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Synergy between CBD/Nagoya Protocol and ITPGRFA on access and benefit-sharing on plant genetic

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Abstract. Utilization of plant genetic resources (PGR) is a prerequisite for benefit-sharing. However, scientists cannot fully utilize PGR because access to their PGR has been governed by Material Transfer Agreement (MTA) on Mutually Agreed Terms (MAT). For example, plant breeders face difficulties when they hybridize PGR accessed under CBD/Nagoya Protocol (CBD/NP) with PGR received from the Multilateral System (MLS) under the International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA). The difficulties of plant breeders could be overcome through developing a Breeding Model combining bilateral and multilateral approaches, with particular focus on transaction of PGR from *in situ* to *ex situ*. The bilateral element in the Breeding Model is expected to be acceptable by providing countries since the benefit will be shared back to them. It will also make the MLS more attractive to users since it will introduce new PGR from *in situ*.

Keywords: ABS, breeding model, CBD, ITPGRFA, Nagoya protocol.

1. Introduction

Although Access and Benefit-Sharing (ABS) of PGR has been a hot topic of international debate for more than quarter of a century, few people have analyzed it comprehensively, from the perspective of both the Nagoya Protocol (NP) of the Convention on Biological Diversity (CBD) and the International Treaty on Plant Genetic Resources for Food Agriculture (ITPGRFA). Breeders who utilize PGR may raise questions on how to share the benefits arising from the hybridization of PGR governed by different ABS conditions. Contrary to this, if two or more PGR are crossed, where all of which had been accessed under the Standard Material Transfer Agreement (SMTA) conditions, then only one payment is required [1].

Even for crop species listed in Annex 1 to ITPGRFA, not all the PGR are exchanged using the SMTA, because Contracting Parties are obliged “to include all the PGR listed in Annex 1 and that are under the management and control of the Contracting Parties and in the public domain” (Article 11.2 of the ITPGRFA). PGR held in private sector or university are not necessarily in the Multilateral System (MLS); SMTA does not automatically apply to these PGR [2].

To improve the MLS, we should combine the two different rules, i.e. bilateral rules under CBD/NP and multilateral rules under ITPGRFA. We can merge the two rules into a Breeding Model utilizing Article 13.2 (h) of the ITPGRFA, which stipulates that “access to PGR for food and agriculture found in *in situ* conditions will be provided according to national legislation or, in the absence of such

legislation, in accordance with such standards as may be set by the Governing Body". To make it attractive to all stakeholders, incentives to utilize the Breeding Model should be carefully designed.

2. Question from the seed industry

In Japan, some seed companies have raised question on ABS in crop breeding. "When I incorporate two PGR into a new variety, one from CBD/NP world and the other from ITPGRFA world, should I make two cumulative benefit-sharing payments for the new variety?" (Figure 1).

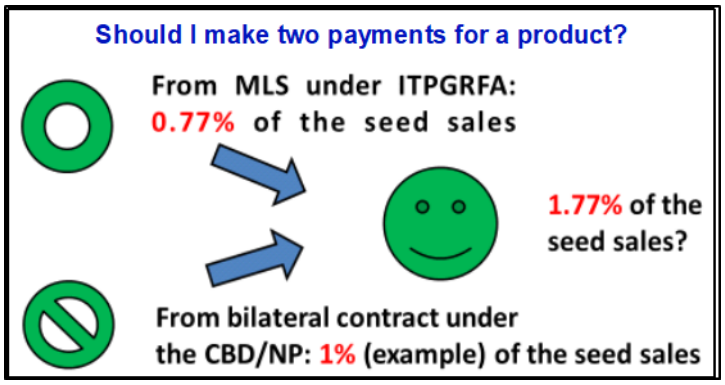

Figure 1. A question from seed industry.

To avoid such a complication, the MLS of ABS of ITPGRFA was carefully designed so that hybridization among PGR accessed through the system will not create problem in the benefit-sharing, since (a) no need to negotiate Mutually Agreed Terms (MAT) to access to PGR (as in NP), since SMTA is used for all transfers, and (b) no cumulative benefit-sharing payments as stipulated in Para 2 of the Annex 2 to the SMTA "Where a Product contains a Plant Genetic Resource for Food and Agriculture accessed from the Multilateral System under two or more material transfer agreements based on the Standard Material Transfer Agreement only one payment shall be required under paragraph 1 above".

The author considers that few people participating in the respective governing body sessions have ever imagined such a question because legally speaking, CBD/NP and ITPGRFA are independent. But in a real-world of PGR exchange, plant breeders cannot say that these two instruments are independent.

3. Comparative analysis of the ABS rules in CBD/NP and ITPGRFA

To understand why Japanese seed industry has raised such a question, we need to briefly analyze the two ABS instruments, bearing in mind the pros and cons of each instrument. The CBD/NP is designed on the principle of "the sovereign rights of States over their natural resources" (Article 15, para 1 of CBD). Therefore, acquisition of PGR is subject to Prior Informed Consent (PIC) of providing country and on MAT (Article 15, para 5 and 4 of CBD). This means that the ABS rules under CBD/NP are based on "bilateralism" [3]. The pros of the rule are flexibility in setting ABS conditions (through MAT negotiation), and the benefits are definitely returned to the providing country. The cons are time-consuming negotiations and accumulation of benefit-sharing payments if more than one PGR are involved because all the MTA for the PGR are equally valid.

Contrary to this, ITPGRFA is designed on "multilateralism" [4]. The pros are easy and uniform ABS conditions and avoidance of cumulative payments as long as the PGR in the MLS are hybridized to each other. The con is the fact that the benefits do not necessarily return to the providing country because the MLS shares the benefits through the Benefit-Sharing Fund.

4. Synergetic implementation of the ABS instruments

To answer the questions raised by the seed industry and help them to fully benefit from the ABS instruments, the author introduces a new Breeding Model combining the pros of the two instruments. The idea is to fully utilize the Article 13.2 (h) of the ITPGRFA, which stipulates that "access to PGR found in *in situ* will be provided according to national legislation, or in the absence of such legislation, in accordance with such standards as may be set by the Governing Body of the ITPGRFA." The author believes that Article 13.2 (h) is the point of contact between the two instruments, which enables the synergetic implementation of CBD/NP and ITPGRFA [5].

The skeleton of the Breeding Model is as follows:

(a) Provider and user jointly explore and evaluate PGR found in *in situ* conditions of the providing country, and both start plant breeding program for developing new variety of plants for their respective needs. Within a certain period of time (for example, five years), materials collected in the joint exploration will be used exclusively by the provider and user.
(b) After that period of time, the materials collected in the above joint exploration will be released into the MLS of the ITPGRFA.
(c) Then, the MLS will be more attractive to users since the available gene-pool for the MLS is enriched with new and unique materials.
(d) At the time of transfer to the MLS, materials already under development by the provider and the user are allowed to enjoy special treatment. Such materials already under development are regarded as if they were derived from the MLS (Figure 2).

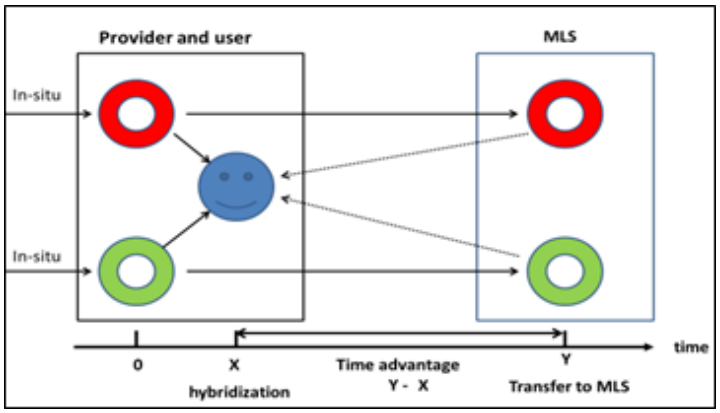

Figure 2. Time advantage of the proposed breeding model.

The incentives for the provider and user to employ this model are:

(a) The benefits derived from the utilization of the materials will go back to the provider.
(b) The provider and user enjoy the time advantage in developing new variety of plants. They have already started plant breeding programs (Y-X) years earlier than any other competitors who will receive the materials from the MLS (after year Y at the earliest).

The Breeding Model also enriches genetic diversity in the MLS because many samples in the MLS are duplicated materials from other *ex situ* collection. MLS will become more attractive to users because its contents will be enriched through the introduction of fresh materials originating from *in situ* conditions.

5. Concluding remarks

Synergetic implementation of CBD/NP and ITPGRFA should be strengthened to assist the exchange of PGR in everyday business of plant breeders. The proposed Breeding Model is an idea aiming at breaking through the present situation, combining the pros of these instruments, as well as bridging *in situ* and *ex situ*. It also enriches MLS under ITPGRFA through the introduction of new materials collected from *in situ*.

6. Acknowledgement
The idea of this Breeding Model is a fruit of discussions by a small Ad-Hoc Group, ABS Japan. The author expresses his gratitude to all the members of the Group: Mr. Kamogawa, T., Mr. Kondo, T., Dr. Morioka, H. and Prof. Okuno, K. The content of this article was first presented at the Workshop for Nagoya Protocol and Plant Treaty National Focal Points in South and Southeast Asia held from 27 to 30, March 2017 at IRRI.

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Genome editing for potato (*Solanum tuberosum* L.)–current status and future prospects

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Abstract. Genome editing has revolutionized crop improvement. For a heterozygous tetraploid and vegetatively propagated crop such as cultivated potato, *Solanum tuberosum* ssp. *tuberosum* L. (2*n* = 4*x* = 48), gene editing presents tremendous opportunities for trait improvement. In potato, traits such as improving cold storage and processing, herbicide tolerance, self-compatibility and modified starch quality have been targeted utilizing CRISPR/Cas9 and TALENs reagents in diploid and tetraploid clones. In our research program, we have used gene editing to modify herbicide resistance, overcome self-incompatibility in diploid potatoes, and assess off-target effect of the gene editing reagents. The potential to generate transgene-free plants via genome editing coupled with a recently streamlined regulatory route by the US Department of Agriculture for plants engineered by this technology, crops with genomic resources, and established genetic transformation and regeneration procedures such as potato are in-line to benefit from the technology's full potential. This paper summarizes the developments in genome editing platforms and delivery mechanisms applicable to various crop species with a focus on potato, a global food security crop, providing insight into current advances, challenges and future prospects of utilizing genome editing for trait improvement.

Keywords: genome editing, potato, CRISPR/Cas9, TALEN.

Mass genome sequencing of crops and wild relatives to accelerate crop breeding: the digital rice genebank

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Abstract. The advent of next generation sequencing, and more recently third generation sequencing, has enabled researchers to begin interrogating the genomic information of thousands of accessions of conserved genetic resources as well as cultivated varieties. Here, we describe recent results from the 3,000 Rice Genomes Project (3K RGP) and summarize a few of the projects for other crop species. The 3K RGP has served as the catalyst to create a digital rice genebank with the intent to sequence many more of the conserved accessions held in the International Rice Genebank Collection in the coming years. We are progressing with generating high quality reference builds for the 15 subpopulations defined by the 3K RG analyses. These along with reference builds of the wild relatives will allow access to the unique genomic regions specific to particular cultivated types and wild relatives. We have also begun efforts to sequence a further 10,000 accessions of rice in collaboration with partners in China. Yet, in-depth sequence data and initial comparative bioinformatic analyses are not enough to promote efficient use. Hence, high throughput phenomic screening in multiple environments, development of novel genetic populations, computational genetics and modeling will be necessary to understand the link between genotype to phenotype and its environmental control. The combination of these approaches underpinned by computational analyses will allow identification of novel genes and alleles that can be deployed into elite varieties for sustainable crop improvement.

Keywords: genetic resources, high throughput phenomics, next generation sequencing, rice.

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Integrating the intangible traditional forms of farming knowledge and practices of the Alur people of North-Western Uganda into the IP laws of Uganda

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Abstract. Documentation of traditional knowledge about traditional forms of farming knowledge and practices is essential to prevent its erosion over time, to enable its accessibility to subsequent generations of the same community, as well as other communities to attempt value-addition and possible benefit-sharing among various stakeholders, and finally to link innovation, investment and enterprise. Therefore, in the absence of documentation, potential investors and entrepreneurs would have to bear a very high transaction cost in order to seek information about potentially viable and useful IP produced by local communities and individual innovators. The transaction cost for a community to scout potential partners for value addition would even be higher. In such a situation, a single TK documentation could be beneficial. Accordingly, Integrating Intangible Traditional Farming Knowledge and practices in the IP laws of Uganda for global opportunities is a project intended to identify, collect, organize, register or record traditional forms of farming knowledge and practices (TK), as a means to dynamically maintain, manage, use, disseminate and or protect TK (positively or defensively). It is intended to form part of a comprehensive, thought-through the process of TK documentation and, in effect to act the documented knowledge would be shared only following the directions of the provider of knowledge. Unless authorized by the provider of knowledge, it will not be shared with anyone for any purpose and will be kept in the register as a confidential entry. However, broad categories of the knowledge or practices will be shared, so that interested seekers of this knowledge can be put in touch with providers.

Keywords: traditional forms, Alur people, IP laws.

1. Introduction

Integrating intangible traditional farming knowledge and practices in the intellectual property (IP) laws of Uganda for global opportunities is a project that was successfully implemented with support from SIDA and PRV by W Gilbert through Okoro Coffee Growers Cooperative Union Ltd. [1]. The project's focus was among others intended to identify, collect, organize, register or record traditional forms of farming knowledge and practices (TK), as a means to dynamically maintain, manage, use, disseminate and/or protect traditional knowledge either positively or defensively. This is intended to form part of a comprehensive, thought-through the process of TK documentation.

Okoro Coffee Growers Cooperative Union Ltd. is a farmer-based cooperative union whose mandate among others is to empower and promote the interest of smallholder farmers of West Nile region and safeguard the norms, values, beliefs, indigenous knowledge and practices, and also

collaborate with actors and institutions that promote culture and strengthen Uganda's diverse cultural identities to uphold the existing heritage for community empowerment [2].

This change project was implemented in Arua, Nebbi and Zombo Districts in the North-Western Uganda and lie between latitudes 2030' and 2045' N and between longitudes 30045' and 31010' E. The northern boundary of the districts is marked by Arua District. River Nile and Lake Albert highlight the eastern border. Bulisia District lies to the south-east while the Democratic Republic of Congo DRC marks the west and south-west limits. These boundaries are primarily political. The two districts occupy a total area of 3,288 km^2 of which 83.19% is arable land, 2.91% game reserve, 6.4% swamps and open water and 7.5% forest reserve.

The pilot project identified various intangible traditional farming knowledge and practices from the sampled districts and among these include practices need urgent safeguarding (Koya) to prevent them from complete disappearance. The objective of the project is to identify, collect, organize, register or record traditional forms of TK, and to create new IP rights through scientific validation of the traditional forms of TK and collaborative research and development.

2. Methodology

2.1. Community consultation–inception of the project

The consultations involved two aspects, namely the involvement of different stakeholders at all levels and identification of elements. Stakeholders consulted included community members, Okoro Coffee Growers Cooperative Union's Bod (as the host), District Commercial Office of Zombo, Nebbi and Arua Districts, kerAlur (Traditional kingdom), Nyaravur Cultural Troops, Jang'Okoro Cultural Troops, Nyapea Cultural Groups. NGO/CSOs such as life concern a human right based local organization, human rights crew, Zombo District Farmers Association (an umbrella organization for the farmers in the district), Zombo District Farm Institute (agricultural research institute), and some selected elders from the District of Arua, Nebbi and Zombo.

During the inception of the project, focus was put on tangible and intangible elements of the TK potential threats to their enactment and the transmission, availability of associated tangible elements and resources, concerned organizations (NGOs and others) and expected results, to ensure coherent and integrated results-based management (RBM) to frame the documentation process.

The consultations included the community consent to participate in the project, identification of elements, and culture bearers consent to provide prior information. This also necessitated the community to identify practices that needed urgent safeguarding by listing them.

2.2. Identification of the community data collectors

The community members identified the community data collectors. The community data collectors included both men and women, youth and elderly who were able to communicate in local language and from the community and sub-counties of operation.

2.3. Training of the community data collectors

The project trained six community data collectors. The training took two days during January 2018 and was flagged off to the field to collect information from the people familiar with the cultures and farming practices of the Alur. The fieldwork was carried out in the community for a period of two months (February–March 2018). The participatory approach was promoted by the project. The data collectors were divided into groups of two per district. This was to allow for the collection of data effectively. Each team was to identify an element of farming practice and interview the cultural bearers, until all the information was exhausted, then transcribes the data for the day.

2.4. Data collection strategy

Both primary and secondary data were collected and analysed as part of the documentation process. Different instruments were designed to cater for the different categories of stakeholders that were interviewed. These included; the board and management tool, farmers' tool, local NGO tool and the

focus group discussion tool. The tools were aligned with the project objectives and indicators measured. The tools were pretested to ensure that they can capture relevant and valid information.

In consultation with OCGCU, the project used both random and purposive strategies to select participants from the community during the project documentation. The sampling was done at the zonal level to make sure that all zones located in the three districts targeted are represented in the project implementation. There are seven zones include Zone A-Errusi, Zone B-Paidha, Zone C-Pakadha, Zone D-Nyapea, Zone E-Zeu, Zone F-Warr and Zone G-Kango. From each zone, two producer societies were selected making a total of 14. From each primary society, six farmers were selected making a total of 84 farmers.

The project planned to interview key informants who were purposively selected as follows, Okoro Coffee Cooperative Union board members, agricultural researchers, development practitioners and policymakers, district production officers (Nebbi, Arua, Zombo, farmers, cultural groups and religious leaders).

Focus group discussions were held with beneficiaries of the project to gain a better understanding of general community and institutional perspective on the means to dynamically maintain, manage, use, disseminate and or protect TK (positively or defensively). One FGD was held in each of the three selected zones making a total of 3 FGDs. A gendered approach was taken into consideration during the selection of members for the group discussions i.e. men, women and the youths. Notes were taken during the discussions. The discussion was guided by an interview guide.

Key project design and implementation activities were reviewed during the documentation process in order to properly understand the context and situation of the TK to date. Findings from the document review further informed the methodology and enabled refinement of the project framework by filling information gaps and helping to identify further data collection needs. Some of the documents that were reviewed include but not limited [3].

Whether TK is secret and confidential, sacred, individually or communally held, orally transmitted, documented and systematized in some form, codified, already partially documented or subject to customary restrictions for use or disclosure. The content or expression of TK, whether TK is technical knowledge or know-how, embodied in a tangible product or related to TCEs, TK and biological resources/genetic resources; whether specific biological or genetic resources be collected as part of the documentation, or are resources imbued with distinctive characteristic developed through traditional methods of selection, breeding or processing. How extended is the TK use and dissemination, whether the TK is non-disclosed or disclosed to the general public (made publicly available) or to individuals that do not belong to the community like researchers, students alternatively or known by a community individual or leader or elder, the community as a whole, a group, an indigenous nation, other social actors; whether TK commercialised or traded in some form.

Applicable national and international legislation, focussing on what specific laws and regulations are applicable for traditional farming knowledge and practices, which can provide specific IP advice in this regards. Other relevant legal regimes and instruments like customary laws and traditions, community protocols, biodiversity laws and access and benefit-sharing legislation.

2.5. Data analysis and interpretation

The project team compiled and analysed all collected data on progress towards meeting the project targets, results achieved, and gaps reported. For qualitative data, the initial step was to read through the FGD transcripts several times while making notes in the transcript. All investigators participated in this process. Disagreements or issues needing further clarity were resolved through discussions and triangulation of data source. Qualitative content analysis technique was used. This technique refers to what the text talks about with relationship aspects and involves in-depth interpretation of underlying meanings of the text. Data was therefore condensed, i.e. shortened without losing quality. Open coding was done and codes grouped into categories and then themes identified.

Qualitative data from crucial informant Interview and in-depth FGD interviews were analyzed manually where variables run through the interviews, the data was quantified, whenever possible, all data were triangulated.

3. Results and discussion

3.1. Farming practice–Koya (the practice of communal)

One outstanding traditional farming knowledge (Koya) identified and documented. This practice of communal work among the Alur community of Nebbi and Zombo Districts was a measure of affirmative action to help chiefs, vulnerable and needy persons to dig a plot, build a house in order to provide social support and show solidarity to them by the community members. The practice manifests itself under the domain of social practice, rituals and festive events.

The practice of communal work was a collective effort by able and energetic persons usually mobilized or hired as volunteers to dig a plot or build a house for a clan chief or a vulnerable member within the clan. The chief clan elder who mobilizes people of communal work is ritually prepared by taking him for throat cleaning at dawn to a secret spot of the river bank. The major categories of those who are vulnerable are women, widows, the aged, the disabled, widower who are characterized by low status, limited access to ownership of assets, low participation in decision making and inadequate social wellbeing and security. It is an approach focused on empowering the disadvantaged to gain greater access to and control over resources. A crucial step in this process is to stimulate people to organize themselves efficiently and functionally so that they can gain control over their situation using the strength of their numbers.

The person organizing communal work would prepare what he/she had to appreciate the communal workers and inform the community mobilizer. The mobilizer would come and assess the quantity, fix the day for the work and inform the workers to go for the work on the appointed day. The communal workers would then come for the work on the agreed day and date. They would work wholeheartedly then go for a reception organized for them either on that day of the work or any other appointed day.

Communal work was mainly organized through the guidance of the elders who provided leadership during work because of their experience. Working together to provide service empowered the disadvantaged socially and economically. People worked for others in expectation of a return in food or drinks. The central idea was to assist people and to help others. Young children were made to work and were given food with fish because goat meat was worked for by the elders. The Alur women worked together in harvests of millet, simsim, groundnuts, sorghum and on the other hand men worked on digging fields, construction works and hunting.

Insensitivity to the disadvantaged persons, deteriorating relations between the people and the traditional chiefs, the younger have generation lost for respect for and loyalty to traditional chiefs, the allegiance paid to traditional chiefs has shifted to property owners whose decisions are more respected, insensitivity to the vulnerable people, food insecurity among communities, inadequate labour force due to rural-urban migration by the younger generation, selfishness and discrimination against the vulnerable, the introduction of white-collar jobs for the youth, poor attitude towards helping the needy and above all, laziness, idleness among the youth and inadequate skilled workforce [4].

Most younger generation now segregate against the vulnerable people in communities, poverty and disparities within the communities, low level of sensitization of the masses, contrary foreign cultural practices and influence, insufficient appreciation of cultural values, norms, practices and lack of cohesion, social isolation between cultural institutions, the vulnerable and the younger generation leading to generation gap since most young people spend most of their times in school, inadequate collaboration and coordination among communities, food insecurity among the vulnerable people to reward the labours, low household income, apathy and low attitude of the younger generation, poverty which limits access to the service, lack of maintenance of cultural norms and values, weak institutional arrangements on the side of cultural institutions, poverty and disparities within communities, people are becoming more egoistic on self-centred, differences in education and in social status make the

society more heterogeneous thus limiting promotion of common interests, needs, priorities and, although education is seen as a way of development, it can alienate young people from their family and culture and loss of respect by the younger generation for traditional chiefs besides, their failure to pay allegiance to them has shifted property ownership and decision making and finally, disrespect by the youth are among the factors hindering the transmission of the element [5].

3.2. Availability of associated tangible elements and resources

Land on which communal work is done is owned by the traditional chiefs and the vulnerable persons. The materials used for construction such as poles, reeds, mud, grass and strings have become scarce due to population pressure. Animals such as goats and birds such as chicken are available. Foodstuff such as maize, cassava and millet for making local brews and for consumption are available.

The land is a permanent asset. Materials used for construction such as poles, reeds, mud, grass, strings, etc. are readily available and can easily be obtained. The animals such as goats and birds such as chicken can be reared. Foodstuff such as maize, cassava and millet can be planted and be grown or produced. The skills for doing work can be trained and transmitted. Positive traditional practices are supported by healthy family life can be learnt. Attitude and behaviour of people towards communal work can still be acquired.

3.3. Project outcomes

The outcomes of the project are the preservation of traditional knowledge for community-oriented objectives, such as education, awareness, cultural preservation and for developing more systematic future research projects.

To date, the data generation and compilation, characterization of materials underlying the farming practices (tangible and intangible elements), community consultation, and document review was done so far. However, a new IP right for integrating intangible traditional farming knowledge and practices through scientific validation, and collaborative research and development of TK was not done yet.

3.4. Recommendation

- Existing human resources to do communal work.
- Support from NGO's to advocate for the preservation of culture.
- All the chiefdoms among the Alur community need to support positive traditional practices in support of the practice.
- Local government to set aside some fund for further documentation.
- Politicians and NGOs to get involved in formulating policies concerning support to the vulnerable members of communities, e.g. income-generating activities and construction of structures to give them social support and protection.
- Need to promote community participation in their cultural norms.
- The private sector should steer community participation in the promotion of the practice through the creation of favourable climate.

4. Concluding remarks

Traditional knowledge may be produced by individuals, by groups of individuals or local or indigenous communities. Some of this knowledge may be kept confidential to the originators and their descendants and may be accessed only with restrictions; some may be disseminated locally, but may, nonetheless, be restricted in scope or terms of accessibility; some of this knowledge may be shared widely within a community and with outsiders, so that the knowledge becomes public domain. Integrating intangible traditional farming knowledge and practices in the IP laws of Uganda for global opportunities, being a pilot project was majorly guided by document review from Ministry of Gender Labour and Social Development. There are quite several practices identified, but one elaborated is outstanding and being practised across the three districts understudies.

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Intellectual property right and farmer protection in accessing genetic resources

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Abstract. Intellectual Property Rights (IPR) in the field of agriculture are regulated under the Patent Law, the Plant Breeder's Right Act and the Plant Cultivation System Act. The decision Number 99/PUU-X/2012 of the Constitution Court granted the right of farmers to access genetic resources (GR). The study aims to assert an application relevance of the law on the events in concreto complying with the provisions of law or contract. This is normative law research. This study revealed that if the Plant Breeder's Right Act and the Patent Act are linked to access to GR, they are not adequately regulated of how fair its benefit-sharing from the use of genetic resources, especially to local communities or farmer which had traditional knowledge (TK). The benefits of IPR are, a form of legal protection against the TK of the population over local varieties, it can utilize GR, and as conservation. IPR has progressively been incorporated into agriculture, namely Law Number 4/2006 concerning the Ratification of the International Treaty on Plant Genetic Resources for Food and Agriculture (PGRFA) and Law Number 11/2013 on the approval of the Nagoya Protocol on access to GR and the fair and equitable benefit-sharing arising from their utilization to the Convention on Biological Diversity. Based on this, it is essential to consider equal and balancing protection between breeder rights and farmer rights.

Keywords: farmer protection, genetic resource, intellectual property right.

1. Introduction

In the last decade, Access and Benefit-Sharing (ABS) issues had increasingly become part of the international and national policy and legal agendas related to biodiversity in general and genetic resources (GR) in particular. Approximately 18 countries have developed long-distance access and ABS, which are well summarized and covered by regulations on biodiversity. Some countries that have developed national laws include the Philippines, Brazil, Peru, India, Ethiopia and others. Indonesia is still in the process of discussing the Genetic Resources Bill, which provides Access and Benefit-Sharing arising from the utilization of GR.

In Indonesia, the National Law on GR has not yet been regulated, however Article 33 paragraph 3 of the Indonesia Constitution which said that any natural resources, including GR, are under the authority of the Government and should be used as much as possible for the people welfare, can be the base for any law and regulation on GR. Other law related to GR management are Law Number 32/2009 on Environmental Protection and Management, Law Number 12/1992 on Plant Cultivation Systems, Law Number 29/2000 on Plant Variety Protection and Law Number 4/2006 on the

Ratification of the International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA). Law Number 1/2014 which amendmended Law Number 41/1999 on Forestry, Law Number 5/1990 on Conservation Natural Resources and Ecosystems, Law Number 41/2014 on Amendment to Law Number 18/2001 on Animal Livestock and Health and Law Number 45/2009 on the Amendment to Law Number 31/2004 on Fisheries.

National laws on ABS arising from the utilization of GR, are still sectorial or partial in nature, such as Law Number 12/2012 on Plant Cultivation Systems, Law Number 5/1994 on Ratification of the United Nations Convention on Biological Diversity (CBD), Law Number 29/2000 on Plant Variety Protection, Law Number 21/2004 on the Ratification of the Cartagena Protocol on Biodiversity, Law Number 4/2006 on Ratification of ITPGRFA and Law Number 11 of 2013 on the Adoption of the Nagoya Protocol of the United Nations CBD. So far, those laws have yet been able to mine the economic potential of GR and prevent its misappropriation or biopiracy.

Developed countries that have technology in the field of biotechnology are very dependent on GR from developing countries that do not have sufficient ability, both in terms of human resources, technology and funds for the development of research and technology. Research and development in the biotechnology field, which is at risk, is inseparable from the lack of financial support or investment. It is technological progress need rewards [1] and incentives [2] from the findings of the technology.

One form of reward given to the invention must at least be protected with intellectual property rights (IPR) such as patent or plant variety protection. Commercialization of technology products will generate income that can be used to fund future research and technology development. Pharmaceutical companies closely related to biotechnology are based their research and development of pharmaceutical products from GR, coming from developing countries.

The process of accessing GR is often done by biopiracy. Biopiracy is essentially the act of taking valuable resources from a country's traditional knowledge (TK) that is packaged and redeveloped without giving contribution to the holders of TK's. Some people view biopiracy as the act of stealing valuable resources for the commercial gain of developed countries and multinational corporations [3]. To those with this view, biopiracy represents a disingenuous repackaging of TK to secure monopoly rents for the biopirate excluding the original innovator from a claim to these rents. The fact that the potential returns from investing in modern biotechnology accrue only to those who hold IPR biased in favours of contemporary biotechnology and against TK raises significant inequity concerns.

While the definition of bioprospecting is an umbrella term describing the discovery of new and useful biological samples and mechanisms, typically in less-developed countries, either with or without the help of indigenous knowledge, and with or without compensation. In this way, bioprospecting includes biopiracy and the search for previously unknown compounds in organisms that have never been used in traditional medicine [4]. TK, the questions of how IPR can protect benefit farmers in accessing GR should be answered. Therefore, this study aims to ascertain whether the application of the law on the events complies with the provisions of law or contract.

2. Materials and methods

The study is carried out by using normative legal research. Normative legal research examines the implementation of the provisions of positive law and contracts factually on any particular legal events. According to Mertokusumo [5], the target of the research is legal norms (rules) to gain: das sollen through research librarianship, as well as events or behaviour in the sense of the fact or the das sein through field research. On the other hand, Soekanto Soerjono [6] argues that legal research includes "normative legal research" that consists of examination of the role of law, the principle of synchronization of law, legal history, comparative law, as well as "empirical legal research" which consist of identification and effectiveness of the regulation.

A qualitative method and juridical analysis are conducted in this study. A descriptive research specification which provides data or image, may be detailed about the people, circumstances and other facts, with the author's descriptive methods can only compare certain phenomena. The specification is

used with the goal of obtaining an overview of farmer protection in accessing the global resource. Data collection was performed through interviews and selecction in the form of documents which is sourced from literature, legislation, official documents and other documents. The respective documents could be research results, scientific articles/papers, scholarly journals and scientific papers. This evaluation can generate an overview of the implementation of the IPR and farmer protection in accessing GR.

3. Results and discussion

Patents are one type of IPR that are most closely related to the use of GR. Provisions in the patent system related to the use of GR are as follow: Patents granted for each invention, both products and processes, in all fields of technology throughout the invention are new, have inventive steps and can be applied in the industry (TRIPs Article 27 [1] and Law Number 14/2001 on Patent). Microorganisms found in nature or the results of genetic engineering are patentable (see TRIPs Article 27 [3]).

The distributive justice approach is a balance in seeing the excess of IPR protection, especially in the commercialization, access and utilization of GR carried out through biopiracy. The principle of distributive justice is the concept of justice [7] which is closely related to the issue of human dignity, commons good and human rights. The basic question of this distributive justice approach is: what exactly constitutes a "fair", "just" or "equitable" distribution? In the context of using GR, is biopiracy a good thing? Is the utilization and commercialization of the product taken without permission, without the contribution can be justified? How should ABS (www.cbd.int/abs) be carried out (equitable distribution)? This approach brings a different perspective, especially from the angle of protection of the interests of indigenous people, who are holders, guardians of GR that have been utilized without proper contributions and rewards. Differences [8] in the protection of the interests of the private sector (pharmaceutical companies) and the interests of the state as well as in the use of GR also have a major influence on how the problem of using these GR is solved.

Some countries have formulated disclosure requirements in the patent law, steps which are called transparency, which aims to ensure the proposal from the patented of GR. These countries include:

1) Belgia, Patent Law; Project: Law Number 2005-04-28/33: *Loi modifiant la loi du 28 mars 1984 sur les brevets d invention, en ce qui corcerne la brevetabilite des inventions biotechnologiques.*
2) Bolivia; Supreme Decree Number 24676, Article 2, Final Provisions VII-Seventh.
3) Brazil; Provisional Measure Number 2.186-16 (23 August 2001).
4) China; Patent Law Amendment (2008), Article 5(2), 26(5).
5) Costa Rica; Biodiversity Law 7788, Article 80; Rules on Access (2003) Art. 25.
6) Denmark; Act 412, 31 May 2000 Amending Danish Patent Act, paragraph 3; Danis Penal Code 163.
7) Egypt; Egyptian Law Number 82 of 2002 on the Protection of Intelectual Property Rights, Art. 13.
8) New Zealand; Patent Bill 2009 and section 17 Patent Act (1953).
9) Norwegia; Patent Law Amendment 2004, section 8b.
10) Panama; Executive Decree Number 25 (28 April 2009) Art. 19.
11) Portugal; Biodiversity Law (10 August 2002) Ar. 4c.
12) Romania; Patent Law 64/1991, rule 14.1.c) source shall be indicated.
13) South Africa; Patent Law Amendment (7 December 2005).
14) Swiss; Amendment of Patent Law of 22 June 2007, RO 2008 2551, Art. 49 a.
15) Thailand; Act on Protection and Promotion of Traditional Thai Medicine Intelligence B.E. 2542.
16) Venezuela; Biodiversity Law 2009.

Ratification of the Nagoya Protocol through Law Number 11/2013 by the Indonesian Parliament is one of the opportunities for Indonesia to obtain benefit-sharing arising from of GR. ABS provision is a means offered by the Nagoya Protocol to protect biodiversity, including for Indonesia. The application of ABS provisions in Indonesia can prevent biopiracy. The Nagoya Protocol recognizes state sovereignty in protecting their GR. These GR are not freely traded, but in accessing must meet the

provisions set out in the protocol, namely based on Prior Informed consent and Mutually Agreed Terms (MAT), as well as the involvement of indigenous/traditional communities and for preventing theft of biodiversity (Table 1).

Table 1. Differences between law and policy about GR on sovereignty rights.

State	Right of sovereignty			
	Accessing	Benefit-sharing	Farmer right	Traditional Knowledge
Brazil	Depend on outsider variety	Good access mechanism; must permit from the government; benefit-sharing is not transparent; joining in Multilateral System (MLS)	No specific regulation	Provide protection through national legislation and regional cooperation
USA	*Ex situ* collection majority	Good access mechanism; submit profit sharing to the parties	No specific regulation	No regulation
Germany	Can meet their own needs but use more modern varieties	There is no further mechanism at the national level; access on PGR controlled privately is done through contractual relations; join the MLS	No specific regulation	No regulation
China	Very rich in PGR, but many of them are not monitored by state management programs, although there are investments	There are bilateral mechanisms, but have not ratified ITPGRFA so that they are not joined into the MLS	No specific regulation	No regulation
Indonesia	Abundant PGR, but many of them are not monitored by state management programs, although there are investments	The access mechanism is still in the process; profit-sharing is still unclear	No specific regulation	No regulation

The effectiveness of the application of ABS provision sharing is not enough by only ratifying the Nagoya Protocol, but should be further detailed with regulations at the national level. Then, in the policy in the field of IPR regulation, the government has also prepared a draft amendment to the Patent Act with a draft amendment to patents, specifically in article 25 of the change to the Law states that "if the invention relates to and, or originates from GR and or TK, it must be clearly and correctly stated the origin of GR and/or TK in the description". By applying the Sources of Origin Country principle, eating is an aspect of dampening the onset of biopiracy. Thus, biopiracy is a form of bioprospecting, where both actions are characterized by two main characteristics: first, the absence of permission to access, and the absence of compensation/rewards that can be given to GR holders related to TK.

Government policy by giving authority to local communities in managing biodiversity is the right choice to avoid community conflict. This authority is a form of appreciation for the contribution of the comunity for conserving biodiversity. Management must also be in line with the mandate in Article 33

of the Constitution and the fifth principle of Pancasila. Conceptually, regulation of the existence of Nagoya Protocol has provided a way for Indonesia to protect abundant biodiversity through ABS.

The international regulation through the Nagoya Protocol stipulates specific guidelines and conditions to access to genetic or biological resources. For example, access should be subject to prior informed consent of the party or country providing the resources (state of origin of the resources or a party that has acquired the resources following the CBD). Further, the so-called TK plays a vital role in local communities through constant development and exchange. Access to this knowledge is also subject to similar procedures, but with the participation of the holders of such knowledge. Hence, the benefits from the utilization of biological resources are supposed to be shared in a fair and equitable way through MAT, including non-monetary gain. For example, the French Centre for Agricultural Research for Development (CIRAD) promotes advantages, such as research collaboration, training, transfer of technology, co-publication, co-ownership of results, and hence the regulatory mechanisms on ABS may represent a useful tool to stimulate research cooperation. A MAT can be signed without reference to the ABS provision in the CBD or the Nagoya Protocol. However, as more than 30 countries are parties to these treaties, there are several advantages of reffering to the Nagoya Protocol ABS-components in the MTA, there is a higher degree of security as the legal basis for the MTA improvements. Therefore, legal sanctions may be avoided and the capacity to obtain a Certificate of Origin to publish increases. IPR may also be easier to agree, and the ethical basis of the MTA will be ensured.

There are many reasons why MTAs are growing its importance. The informal exchange among farmers built upon TK is less typical today than it was before. Knowledge creation and innovation increasingly take place in the context of systematic research and research cooperation across countries. In this process transfer of biological or genetic materials is not only common, but also grows in volume and importance in line with the increasing importance of the bio-economy and attention given to biodiversity, climate change, food security and other challenges. The frontier between basic and commercially oriented research is becoming blurred, and the global organization of, e.g. drug development induces complex divisions of labour in clinical trials with implications for the transfer of GR. One of the purposes of these international treaties is to create conditions to facilitate access to GR. However, today MTAs cannot be understood without the context of international treaties and national regulations. They may amount to restrictions impeding research and development. This is a continuous balancing act. Therefore, these are necessary to ensure that developing countries, typically being the providers of materials and biodiversity, can benefit from the exchange and transfer of biological materials.

4. Conclusions

Patents can be used as economic support and utilization of GR. However, the license also opens up opportunities for abuse from its use. Legal protection for the usage of GR and TK is to provide a foundation for their recognition TK with easy-access permits and fair distribution of the benefits TK. Equitable benefit-sharing is then implemented in MAT. The rights of farmers include the right to store, use and exchange and sell seeds, protection of TK, participation in making decisions and fair ABS. The utilization and access of GR and TK involve the participation of indigenous peoples in granting permits. TK Fair distribution of benefits is based on contractual relations, of which, the role of government as a legal subject of international agreements becomes crucial to guarantee rights indigenous peoples and farmers in terms of the use of GR and TK.

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Strengthening seed delivery system for enhanced adoption of improved sorghum varieties among smallholder farmers in Malawi, Mozambique and Zambia

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Abstract. Many improved sorghum varieties and hybrids have been developed by public research institutions in Malawi, Mozambique and Zambia. However, the seeds are not readily available to small-scale farmers in remote rural areas. The non-availability of improved seed is due to various reasons, including low demand for certified seed and therefore, low profitability. Sorghum has been unattractive to large seed companies, and hence is not taking up production and distribution of these improved sorghum varieties to farming communities leading to low adoption. As a result, a seed system strengthening project was initiated to enhance seed delivery in remote rural areas that cannot access seeds easily. The project is in the first year stage and is being implemented in fourteen districts of Malawi, Mozambique and Zambia. The project activities include early generation seed production of selected sorghum varieties, the establishment of promotional demonstration fields, building the capacity of seed producers and farmers in seed production through training and creating a platform for players in the sorghum seed value chain. The specific objectives are: (i) to improve the availability of sufficient quantities of early generation seed to seed producers, (ii) to strengthen the capacity of sorghum seed value chain players involved in the promotion of released sorghum varieties, and (iii) to promote the use of sorghum certified seed by small scale farmers. The research outputs so far achieved are 250 farmers have been trained in seed production, 10 tons of basic seed of selected varieties have been produced and 144 sites have been established.

Keywords: food security, Malawi, Mozambique, sorghum varieties, Zambia.

1. Introduction

Sorghum (*Sorghum bicolor*) is one of the most essential traditional cereal crops of the hotter and drier regions of the tropics and subtropics [1]. This plant species is the second most important cereal crop in sub-Saharan Africa [2]. Global sorghum is the second most important cereal after maize with 22% of the total area of cereals [3]. The demand for sorghum has been increasing as reflected in the trend for the increasing area under sorghum production in Africa over the last fifty years [3].

Unfortunately, crop production and productivity has not kept pace with increasing demand due to several limitations: i) insufficient availability of improved seed, ii) lack of information dissemination, iii) few alternative end uses, iv) poor marketing strategies, v) poor grain quality, and vi) lack of fertilizer use as observed by Mbulwe [4]. Macauley [3] also observed that crop productivity had not kept pace with this increasing demand due to both a lag in crop improvement efforts in the crop and the extreme environmental conditions and the low agricultural input under which this crop is grown. Thus it is immediately evident that crop improvement efforts combined with improved agronomic practices, are a must for this crop in Africa, especially because of the reducing arability of land. Interventions of the Bill and Melinda Gates Foundation-supported HOPE project (harnessing opportunities for productivity enhancements) for sorghum and millets (http://hope.icrisat.org) that started in 2009, have demonstrated that yield gains from as low as 17 to as high as 141 per cent for sorghum is possible through the use of improved varieties and associated improved agronomic practices (http://hope.icrisat.org).

Additional support is required to enable the strengthening of the crop improvement process, seed production and delivery systems for improved varieties [3,5]. Due to its excellent adaptation to semi-arid and arid climates, the proportion of total grain production represented by the sorghum in semi-arid countries of Africa is very high. In 2011, Zambia accounted for 12% of the regional sorghum production that stood at 213.336 tons [6], which was lower than that produced by Malawi and Mozambique.

In Zambia, sorghum ranks third in terms of importance as staple cereal food crop after maize and rice [7] and contributes highly to national food security. In Zambia, the major sorghum producing areas are southern and western provinces and to a lesser extent eastern, central, northwestern and Luapula provinces [7]. In Mozambique, sorghum is a major cereal grain with a cultivated area of 27 million of hectares, and it is considered as a food security crop in most of the provinces in the country, especially in regions where rainfall is a limiting factor for maize and rice production.

Since the early 1980s, a total of 16 improved sorghum varieties have been developed [8] and released by Zambia Agriculture Research Institute (ZARI), but the seed of these varieties is not readily available at farmer level. Past public investments in sorghum research in Malawi, Mozambique and Zambia resulted in the release of a number of sorghum varieties with superior agronomic performances. However, the seed is not available to small-scale farmers in remote rural areas.

The formal seed sector through seed companies is expected to be the channels for production and distribution of these improved sorghum varieties to farmers. However, this sector has been reluctant to engage in seed production and marketing of sorghum, primarily because of its low profitability and erratic seed demand. This constraint has highly contributed to the low uptake of the improved sorghum varieties. In the 2000s, there has been a renewed effort to improve seed accessibility with focus on supporting private sector (small and medium enterprises) and also the establishment of seed business-friendly regulations across the region such as harmonized seed regulations [9]. Despite these efforts as observed by Mbulwe [4], seed companies that were tasked to be conduits of government released varieties have failed to deliver citing low demand and the high cost of distributing the seed in far-flung areas. This has been cited as one of the many contributing factors to the low adoption of these varieties. Most seed companies have continued to focus more on profitable crops such as maize.

In Zambia, most smallholder farmers involved in sorghum production source their seeds mainly from informal channels such as farm-saved seeds leading to low productivity and production of the crop. When small seed dealers are capacitated and linked to readily available sources of improved crop varieties, these channels could play a significant role in the increased adoption of varieties of less attractive crops such as sorghum. These small seed companies could play a catalytic role in the adoption of these crop varieties. The small seed dealers are highly decentralized, and therefore, more appropriate channel for the diffusion of improved varieties to small scale farmers is needed. Interventions, such as capacity building of decentralized seed dealers through training, improve linkages to sources of improved sorghum varieties, provision of adequate quantity and quality of foundation and certified seeds, provision of market information on improved varieties to the

surrounding farmers and beyond, will improve accessibility of improved adoption of improved sorghum varieties and increase sorghum production.

The adoption of the released improved varieties in sorghum growing areas of Mozambique has been minimal and mostly unknown because of the inadequacies of the seed system. Lack of seed and poor distribution of improved sorghum varieties has been pointed as the main constraints for the farmers to increase their production and productivity. As a result, farmers continue to use their local varieties which have low productivity. In Malawi, the seed system for sorghum is challenged by lack of a mechanism in place to increase the production of certified seed of improved sorghum varieties to be taken by farmers. Also there is a limited partnership in the sorghum value chain to enable and motivate farmers' access certified seed of improved varieties.

Several sorghum farmers in Malawi have continued to use local varieties, although there are some improved sorghum varieties such as Pilira1, Pilira 2 and PN3, which have been developed and released by the breeding program. In part, this has been due to unavailability of basic seed for the production of certified seed and farmers' lack of knowledge of the existence of improved sorghum varieties. Kasinthula Research has been multiplying seed of these improved varieties to make seed available to farmers. In order to enhance the adoption of improved sorghum varieties in sorghum growing areas, the demonstration plots of improved sorghum varieties were established on lead farmers' fields. Establishment of field demonstration plots of improved sorghum varieties aimed to popularize the varieties to farmers around the sorghum growing area. The demonstration plots to be managed by the farmers with the supervision of the extension and research staff will serve to promote improved sorghum varieties among farmers.

The overall goal of the project is to increase sorghum production through the promotion of accessibility to and availability of adequate quantities of seed of improved sorghum varieties by small scale farmers. Specifically, the project aims at: (i) improving availability of sufficient quantities of early generation seed (basic seed) of improved sorghum varieties for supply to seed producers; (ii) strengthening the capacity of sorghum seed value chain players, including small seed companies, agro-dealers, and farmer cooperatives involved in the promotion of improved sorghum varieties; (iii) promoting the use of improved sorghum varieties and certified seed by small scale farmers.

2. Materials and methods

2.1. Improving the availability of sufficient quantities of early generation seed to seed producers

The primary seed was produced by the free seed producers and distributed to small and emerging seed producers on a cost-recovery basis with the supervision of the breeders and seed certification authority. The primary seed was then made available to seed producers, for the production of certified seed. This activity was done through formal seed sector involving emerging seed companies and informal seed sector through community-based organizations with supervision from extension and seed certification authority staff in order to ensure and assure adherence to recommended seed quality production standards.

2.2. Strengthening the capacity of sorghum seed value chain players involved in the promotion of improved sorghum varieties

Relevant training sessions have been organized at a smallholder farmer level in aspects of the quality of seed production, processing and storage (Figure 2). This activity is expected to entail training and supervision of farmers to ensure adherence to quality standards. All participating farmers' cooperatives were provided with small scale seed processing equipment such as seed graders, blowers, and seed packaging to be used at the farm level. The agro-dealers were also trained and linked to the seed producers.

2.3. Promoting the use of sorghum certified seed by small scale farmers

In order to enhance the adoption of improved sorghum varieties in sorghum growing areas, the demonstration plots of improved sorghum varieties have been established on lead farmers' fields. The

demonstration plots were managed by the farmers with the supervision of the extension and research staff. The demonstration plots served as a means of promoting improved sorghum varieties among farmers. They are also channels for communicating other agronomic practices, such as the use of herbicides, time of planting, spacing, and weed management. At an appropriate period, field days were organized in order for farmers to share experiences and knowledge gained through the project activities on the farm.

3. Results and discussion

3.1. Increased availability of sorghum basic seed

Through the project interventions the availability of, and accessibility to a certified seed of improved sorghum varieties to smallholder farmers are enhanced. So far, ten tons have been produced per participating country (Figure 1). The availability of primary and certified seed will be increased to 20 tones for each country by 2019.

Figure 1. Sorghum foundation seed multiplication field.

3.2. Enhanced capacity of farmers groups and local seed dealers through specialized skills and knowledge

A total of 6 farmer groups, each comprising 20 farmers and at least ten seed dealers per project site has received training in seed production, processing and marketing. Through the project interventions, especially capacity enhancement activities, local and decentralized seed dealership will be improved in the project sites (Figure 2).

Figure 2. Farmers and extension staff are receiving seed production training.

3.3. Increased number of decentralized seed dealers in the seed delivery of improved sorghum varieties

The project has managed to create awareness among seed dealers about the opportunities that exist in sorghum trade. Most dealers have accepted to include sorghum on their shelves. The project is promoting the model where the foundation and certified seed of improved variety are produced and marketed within the same area. This model enhances the accessibility of the seed. Through the support of the project, the number of seed dealers in the project sites is projected to be increased by 10 per project site.

3.4. Increased number of small scale farmers using improved sorghum varieties

Through promotional activities such as field demonstrations of improved sorghum varieties and increased participation of decentralized seed dealers and awareness creation on the benefits of using improved sorghum varieties and certified seed, more improved sorghum varieties will be availed to the local farmers. For each project site, a total of 250 farmers are estimated to be using improved sorghum varieties by year 3 of the project. The number of farmers showing interest in sorghum production has sharply increased due to market opportunities highlighted through the sorghum value chain players' platform as well as companies in the brewing industry showing interest to buy sorghum grain.

3.5. Increased number of improved sorghum varieties promoted in the project areas

Currently, in year two, on average three improved varieties are being used by farmers in each site. Most farmers have lost their local varieties, hence they have switched to improved ones in order for them to continue growing sorghum. Through field promotional demonstrations of improved sorghum varieties, the number of varieties being utilized by small scale farmers will be increased by more than three varieties in each site.

4. Conclusions

The interventions of the project have already started showing positive results in the increased amounts of foundation seed being produced, a number of farmers adopting improved sorghum seed, agro-dealers marketing sorghum seed, and farmers and staff undergoing to training. These results clearly show that the intended project objectives will be achieved.

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Community-based access and benefit-sharing platform and its role in biodiversity, culture and intellectual property rights

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Abstract. The world is experiencing a paradigm shift towards new technical uses of genetic resources (GR), which is likely to have a significant impact on communities across the world. These new technologies comprise genome sequencing, gene editing, computational biology, nanotechnology among others, yet the technological innovation driving this shift is occurring only among a few elite, high-tech innovation actors, high-end universities, companies and research institutions. Kenya is a member of the World Trade Organizations (WTO) Agreement on Trade-Related Aspects of Intellectual Property Rights–TRIPS and party to the Convention on Biological Diversity (CBD) and its Cartagena and Nagoya Protocols. This survey interrogated constitutional provisions concerning Biodiversity, Culture and Intellectual (BCI) property rights, with a view of promoting community-based Access and Benefit-Sharing (ABS) platform. It highlights provisions of Articles 11, 40.5 and 69 of the constitution and interrogates the Protection of Traditional Knowledge and Cultural Expressions Act of 2016 that aims at ensuring compliance with these constitutional provision and aims at building trust between users and providers of traditional knowledge, associated GR and traditional cultural expressions and creating a *sui generis* mechanism for the protection of traditional knowledge and cultural expressions. In this survey, GR are defined as microorganisms, plants and animal materials, including indigenous seeds, genetic plant varieties and traditional animal breeds.

Keywords: biodiversity, intellectual property right, genetic resources.

1. Introduction

The world is experiencing a paradigm shift towards new technical uses of genetic resources (GR), which is likely to have a massive impact on communities across the world. These new technologies comprise genome sequencing, gene editing, computational biology, nanotechnology among others, yet the technological innovation driving this shift is occurring only among a few elite, high-tech innovation actors, high-end universities, companies and research institutions.

However, farmers and local communities are being left behind entirely, pausing a risk, since there is no capacity and language to understand the sophisticated technology and articulate their needs concerning the scientific, legal and policy developments caused by the shift. Major challenges are:

1) Translating the advanced technological progress to make it accessible and understandable to farmers and local communities.
2) Identifying and analyzing the needs of farmers and local communities concerning the new technologies and transformations in the use of GR.

3) Articulating the needs of farmers about the new GR technologies in the advanced technical language of the high-end innovation actors.
4) Relaying those needs to the high-end innovation actors and relevant policy-making institutions.
5) The shift from genetic material to data as critical inputs for GR-based innovation is accompanied by a movement from Access and Benefit-Sharing (ABS) into the IP system as the primary regulatory frameworks. Kenya is a member of the World Trade Organizations (WTO) Agreement on Trade-Related aspects of Intellectual Property Rights [1]–TRIPS and party to the Convention on Biological Diversity (CBD) and its Cartagena and Nagoya Protocols [2].

The CBD of 1992 comprises the international legal framework for access to GR, traditional knowledge (TK) and benefit-sharing (ABS). The relevant provisions on ABS are concretized in and made internationally binding through Nagoya protocol to the Convention. This has fundamentally changed the terrain of working with GR in research and development, and national laws have to be reviewed to ensure compliance with its obligations.

Articles 11, 40.5 and 69 of the constitution and the Protection of Traditional Knowledge and Cultural Expressions Act [3] aim at ensuring compliance with these obligations and build trust between users and providers of TK, associated GR and traditional cultural expressions (TCEs). The Traditional Knowledge and Cultural Expressions Act of 2016 aims at creating a *sui generis* mechanism for the protection of TK and cultural expressions. An exciting aspect of this act is that it defines GR as microorganism, plant and animal material, including indigenous seeds, genetic plant varieties and traditional animal breeds.

Numerous challenges are still standing in the way before this can be achieved. This survey examined some of the outstanding issues identified based on a pilot case study of selected counties and national regulatory institutions and interrogated how they are coping with resulting challenges. Could disclosure of the source of origin of GR and associated TK in Intellectual Property Rights (IPR) applications help in enforcing ABS requirement at the community level.

To find the actual picture in the counties, and capacity of relevant institutions to enforce this requirement, this survey was carried out in three pilot counties of Kajiado, Busia and Vihiga with the aims of:

1) Finding the level of preparedness by the county governments to document TK and TCEs and establish county repositories as required by Section 4 of the Protection of Traditional Knowledge and Cultural Expressions Act of 2016.
2) Determining the existence of repositories and guidelines for documentation and access to TK, associated GR and TCEs as required by Sections 10, 18, 19, 20 and 25 of the Protection of Traditional Knowledge and Cultural Expressions Act of 2016.
3) Establish the existence of guidelines for examining biological inventions and suitability of the patent law to enforce the disclosure requirement.
4) Developing short- and long-term recommendations for implementation of the Protection of Traditional Knowledge and Cultural Expressions Act of 2016, and promotion of Community-based ABS Platforms (CABS Platform).

2. Policy framework

Protection of Traditional Knowledge and Cultural Expressions Act of 2016 is an Act of parliament that provides a framework for the protection and promotion of TK and TCEs. It gives effect to the Constitution of Kenya 2010 [4], article number 11, 40.5 and 69 of the Constitution of Kenya and recognizes GR, culture and protection of IPR.

Article 11 describes culture as the foundation of the nation and as the cumulative civilization of the people and the nation of Kenya. It calls for the state, through an act of parliament, to recognize ownership and the role of communities in the conservation and promotion of their scientific and indigenous technologies, and to provide for the protection of IPR of the people of Kenya. Nagoya Protocol, on the other hand, provides a transparent legal framework for the effective implementation

of the third objective of the CBD. It applies to GR covered by CBD and to the benefits arising from its utilization. It also covers TK and TCE with GR held by indigenous and local communities.

The erosion of our cultures and the loss of Kenya's heritable (genetic) resources underscore the need for policies and legislation to conserve and regulate access to GR, protect TK and practices and facilitation of the equitable sharing of benefits arising from the use of such resources. This pilot project interrogated the implication of enforcing the disclosure of origin of biological resources and, or associated traditional knowledge in patent and utility model applications. It is believed that this framework with the disclosure requirement of the country of origin can facilitate contracts and licensing agreements for bioprospecting between communities, research and development institutions and other development partners.

In phase I, the survey attempted to establish the level of awareness by counties on Constitutional Provisions in Articles 11, 40.5 and 69, and determine existence of guidelines on documentation and access to TK, associated GR and TCEs in the county TK and TCEs as required by Sections 10, 18, 19, 20 and 25 of the Protection of Traditional Knowledge and Cultural Expressions Act of 2016.

In phase II, the TK and GR unit of Kenya Industrial Property Institute interrogated the suitability of IP regime in ensuring Returns on Community-based Assets (ROCBA), comprising Patents/Utility Models and breeder's rights, by requiring disclosure of the source of origin of TK and associated GR.

Phase I

Phase I activities attempted to establish the level of awareness by counties about Constitutional Provisions in Articles 11, 40.5 and 69, and determine existence of guidelines on documentation and access to TK, associated GR and TCEs as required by Sections 10, 18, 19, 20 and 25 of the Protection of Traditional Knowledge and Cultural Expressions Act of 2016.

Phase II

Phase II interrogated the suitability of patent and utility model application procedures with the aim of intending to establish its suitability in ensuring ROCBA, by requiring disclosure of the source of origin of TK and associated GR in their application.

3. Finding of the survey in the pilot counties

The following ten questions were put to respondents in the pilot counties of Kajiado, Busia and Vihiga. Discussed herewith are the findings:

1) *Does your county have designated county and sub-county culture, agriculture and livestock officers?*

   All the three counties have designated county and sub-county culture, agriculture and livestock officers.

2) *Does your county have a Biodiversity, Culture and Intellectual (BCI) property policy?*

   It was also established that the County Government of Busia has a draft of BCI property rights policy that was developed in the 2016/2017 financial year. However, the draft is yet to be debated at the county assembly. It is anticipated that once the draft is debated, it shall be enforced to ensure that communities in Busia County will enjoy returns on their biodiversity, culture and intellectual property assets.

   It was established that county governments of Kajiado and Vihiga do not have a BCI property rights policy. However, there are community-based activities in the counties under the intangible cultural heritage and natural products initiative that could culminate in the development of a county BCI property rights policy.

   This county BCI property rights policies aim at providing guidelines on documentation and access to TK, associated GR and TCEs of communities in the counties. The policies are expected to provide guidelines on enforcement of Annex 1 of Nagoya Protocol, in the context of granting of Prior Informed Consent (PIC) and drafting of mutually agreed terms on accessed TK, associated GR, and TCEs.

3) *Are you aware of the Constitutional Provisions in Articles 11, 40.5 and 69?*

All respondents in the pilot counties answered that they are aware of Constitutional Provisions in articles 11, 40.5 and 69, whose substantive issues related to biodiversity, culture and intellectual property rights.

4) *Are you aware of "The Protection of Traditional Knowledge and Cultural Expressions Act of 2016"?*

It was established that the three counties are not aware of The Protection of Traditional Knowledge and Cultural Expressions Act of 2016, an Act of parliament that aims at creating a *sui generis* mechanism for the protection of TK and cultural expressions in Kenya and gives effect to enforcement of Articles 11, 40 and 69 (1) (c) of the constitution. This lack of awareness is at institutional and individual levels.

Section 4 of The Protection of Traditional Knowledge and Cultural Expressions Act of 2016, sets out responsibilities of county governments that include initial registration, preservation and conservation, facilitation of access and sharing of information and allocation of financial resources for TK and TCEs.

5) *How is the county government planning to enforce provisions of Section 4?*

It was established that all the three counties plan to enforce provisions of Section 4 through the county implementation activities in the Integrated Plan (CIP) of 2018–2022. This plan has aspects of documenting intangible cultural heritage and the generation, protection and commercialization of community-based natural products.

It has envisaged that the CIPs and the county BCI policies will provide a framework and guidelines on documentation and development of sub-county repositories, for the purpose to collect and compile information including documentation, storage, preservation and conservation, protection and promotion and facilitation of collaboration on access to, or sharing of information and data relating to TK, associated GR and TCEs.

Under Section 10 of the Act, communities are empowered to promote their TK, associated GR and TCEs, and to control its use and share in benefit from its commercial exploitation, where communities are to adopt their own rules and procedures for authorization of use by third parties.

6) *How is the county government planning to enforce provisions of Section 10?*

It was established that the three counties plan to enforce provisions of Section 10 through the CIP of 2018–2022. An emerging issue from Kajiado county respondents was concerning the absence of an intellectual property protection regime for animal breeds (gap identified in IP protection), [see Use of Livestock Resources for Food Security in Light of Climate Change policy brief of April 2017–breeding program and infrastructure–the case of Red Maasai sheep in Kenya. This was mainly about the definition of GR in the Traditional Knowledge and Cultural Expressions Act of 2016, which is defined as a microorganism, plant and animal material, including indigenous seeds, genetic plant varieties and traditional animal breeds.

It was the opinion of respondents from Kajiado County that animal breeders deserve an animal breeder's rights regime, just like plant breeders enjoy plant varieties protection and all benefits that are derived from obligations under Art. 9 of the FAOs ITPGRFA. They argue that, just like the plant breeders have plant breeder's rights, animal breeders also need an animal breeder's rights regime.

This argument is in line with Article 1(3) of the Paris Convention for protection of industrial property, which states that, industrial property shall apply not only to industry and commerce proper, but likewise to agricultural, and extractive industries, and all manufactured or natural products, for example, wines, grain, tobacco leaf, fruit, cattle, minerals, mineral waters, beer, flowers and flour.

7) *Protection against unlawful acts under Section 18 of the Act is modelled along with the conventional Intellectual Property Rights by conferring positive protection subject to exceptions and limitations under Section 19. How is the county government planning to enforce provisions of Sections 18 and 19?*

It was established that the county governments, in consultation with the national government, shall set up mechanisms to prevent misappropriation, misuse or unlawful access and exploitation of TK, associated GR and cultural expressions, where any user shall be required to obtain prior informed consent from the community holding the knowledge and use of the knowledge and associated GR shall be compatible with fair practice, relevant customary laws, protocols and practices and acknowledges the moral rights of communities, to ensure that communities realize returns on their assets.

8) *One of the main challenges faced in Kenya is the use of TK to create works that are protected under existing Intellectual Property Rights. This is addressed by Section 20(1), which requires written authorization of the use of such works for commercial purposes. How is the county government planning to enforce provisions of Section 20(1)?*

The three-county governments indicated that they shall confer all the rights as stipulated in the Traditional Knowledge and Cultural Expressions Act of 2016, including the monetary and non-monetary rights (shared IPRs) as anticipated by NEMAs Environment and co-ordination (conservation of biological diversity and resources, access to GR and benefit-sharing) regulations of 2006 and Annex 1 of Nagoya Protocol.

9) *Section 25 of the Act cover provisions on the authorization of use of TK and cultural expressions, or notification of agreements. How is the county government planning to enforce provisions of Section 25?*

The county governments of Kajiado, Busia and Vihiga indicated that they should collaborate with the national IPR offices (Kenya Industrial Property Institute, Kenya Copyrights Board) to ensure that TK, associated GR and TCEs are protected against all acts of misappropriation, misuse, unlawful access or exploitation.

It is expected that the draft county culture, biodiversity and intellectual property rights policies (BCI) shall ensure those holders of TK, associated GR or cultural expressions shall grant access, authorizations, assignments or licenses in respect of protected TK or cultural expressions in writing and copy submitted to the cabinet secretary and the respective county executive committee member in charge of matters relating to TK and culture.

The Traditional Knowledge and Cultural Expressions Act of 2016 seeks to regulate the use of TK and cultural expressions and ensure that the communities or individuals holding the knowledge can control their access and use, benefit from commercial exploitation, protect against misuse and misappropriation and promote its use and preservation.

10) *How is the county government planning to enforce these provisions?*

In response to question 10 with Table 1 on cultural industries, it was established that all the listed cultural expressions 1–16 are found in the pilot counties of Kajiado, Busia and Vihiga, and that they shall be documented.

It is also envisaged that several products that have a particular appellation to any geographical location in the pilot counties shall be documented and appropriate community collective marks, geographical indications and appellations of origin registered.

The cultural industries listed in Table 1, such as architecture, music, art, visual arts and performances, to a certain extent involves the use of TK and TCEs. Songs, dances and culinary art of preparation, cooking and presentation of traditional food, is the main attraction of such cultural days and events.

In Busia County, it was established that all the listed cultural expressions 1–16 in Table 1 are found and shall be documented. The central communities in the county are Bakhayo, Banyala, Marachi, Samia, Teso and Luo. Marachi community has traditional mats that could qualify for a community collective mark that can be registered if the community forms and registers an association.

Table 1. Relates to question 10.

Creative economic sub-sector	The protection of intellectual property
Architecture	Copyright
Advertising	Copyright
The fine arts antiquities	Copyright and traditional cultural expressions
Craft	Copyright, trademark and design industry
Design interior	Copyright and design industry
Design visual communication	Plant and design industry
Fashion	Copyright and design industry
Film, animation, and video	Copyright
Photography	Copyright
Application and games developer	Copyright
Music	Copyright
Performing arts	Copyright
Publishing	Copyright
Television and radio	Copyright
Culinary	Trademark and Geographical Indications (an indication of origin)

It was the opinion of the Busia county cultural officer that cultural products like Marachi baskets that could constitute have a unique appellation due to TK of the Marachi community and be protected by a community collective mark. The Marachi community has the potential for developing a collective mark for its products.

It was found that almost every community in the pilot counties had a unique product that can qualify for registration of community collective marks. This could leverage on the one village one product (OVOP) initiative by the Ministry of Industry Trade and Cooperatives, which aims at branding at least one product from each community in each county.

In Vihiga County, it was established that all the listed cultural expressions 1–16 in Table 1 are found. The County came out most active in this category, where it has an elaborate, annual cultural festivities calendar/week that features cultural expressions from Maragoli community on 26 December, Banyore community on 27 December, Tiriki community on 28 December, and Teriki/Nandi community on 30 December every year.

Analysis of findings from the three pilot counties of Kajiado, Busia and Vigiga

1) All respondents in the pilot counties of Kajiado, Busia and Vihiga were aware of the Constitutional Provisions of Articles 11, 40.5 and 69.
2) All respondents in the pilot counties of Kajiado, Busia and Vihiga lacked awareness of the existence of The Protection of Traditional Knowledge and Cultural Expressions Act of 2016, which is an Act of Parliament for actualizing Constitutional Provisions in Articles 11, 40.5 and 69.

As per the definition of GR provided for in The Protection of Traditional Knowledge and Cultural Expressions Act of 2016, there is lack of an appropriate regime for the protection of Animal Breeders Rights, which was identified as a gap.

4. Finding for phase II

4.1. Kenya Copyrights Board

Kenya Copyrights Board was interrogated on issues related to the development of implementing regulations for the Traditional Knowledge and Cultural Expressions Act of 2016, and the establishment of the National Repository as required by Section 5.

Section 5 of the Traditional Knowledge and Cultural Expressions Act of 2016 requires the national government to:

1) Establish and maintain a repository at the Kenya Copyright Board.
2) Promote and conserve TK and cultural expressions of communities in Kenya.
3) Protect traditional knowledge and cultural expressions from misuse and misappropriation.
4) The facilitation of access to information and the sharing of information and data relating to TK and cultural expressions.

What is the Kenya Copyrights Board doing to ensure that the Protection of Traditional Knowledge and Cultural Expressions Act of 2016 is enforced?

Findings:

It was established that Kenya Copyright Board, the custodian of the Act, is developing draft implementation guidelines and regulations for the Traditional Knowledge and Cultural Expressions Act of 2016 and shall involve stakeholders. It is hoped that the guidelines and implementing regulations shall help National and County Governments to meet their obligations as required by Sections 4 and 5.

4.2. Kenya Industrial Property Institute

The survey sought to know how the institute proposes to enforce the disclosure requirement in patents and Utility model applications to ensure ABS on accessed and commercialized TK and associated GR. It also sought to establish how biological inventions, including biotechnological inventions, are examined.

The manager patent was requested to respond to the following questions, the County Governments of Kajiado, Busia and Vihiga indicated that they should collaborate with the national IPR offices (Kenya Industrial Property Institute [KIPI], Kenya Copyrights Board and Kenya Plant Health Inspectorate Service) to ensure that TK and associated GR and TCEs are protected against all acts of misappropriation, misuse, unlawful access, or exploitation.

1) What is KIPI doing to ensure that TK and associated GR of communities is not misappropriated, misused, unlawful accessed or exploited through patents and utility models?
2) Are there guidelines for examination of biological inventions, including biotechnology?

4.3. Analysis of Kecobo and KIPI

It was established that the Industrial Property Act (IPA) has provision for patent and utility model protection for microorganisms or other self-replicable material, products of GR and herbal products and nutritional formulations that give new effects.

The response from the manager patents was that a proposal had been made to the State law office to amend IPA to call for disclosure of the source of TK and associated GR in patent and utility model applications to ensure that communities benefited from their accessed TK and associated GR. Once the State law passes the amendments, KIPI shall have it enforced to ensure that communities realize returns on their assets.

It was further established that there are no guidelines on the examination of biological inventions, including biotechnology applications. It was found that the absence of the guidelines has caused a lack of clarity in the execution of biological-based inventions, including biotechnology.

Biological inventions are examined based on provisions of Section 29 of IPA 2001 and Regulation 11 of Implementing Regulations of 2002. It was found that neither of the designated depository institutions (KALRO and Kenya Medical Research Institute [KEMRI]) has guidelines on depositing and maintaining microorganisms for patent purposes.

A case to note is KE/P/2013/001960, [PCT/JP2012/062935] that relates to and involves the use of a microorganism which is considered not to be available to the public. According to the requirements of Section 29 of the IPA [5], the applicant was required to deposit the culture of the microorganism with Kenya Agricultural Research Institute (KARI) or KEMRI. Although the nine claims have been examined substantively and found to be novel, inventive and industrially applicable, a patent cannot be granted because the depository requirement has not been met, hence, the application is considered as insufficiently disclosed.

Implications of this lack of clarity are that biotechnology applications are technically prohibited, which is contrary to the spirit of Article 16 of the CBD on sustainable application and adaptation of biotechnology.

Copyrights Board, who are custodians of The Protection of Traditional Knowledge and Cultural Expressions Act of 2016 should, in collaboration with Council of Governors, create awareness on obligations of all the 47 counties on matters related to biodiversity, culture and intellectual property rights.

To guarantee Returns on Community Based Assets (ROCBA), the disclosure of origin of biological resources and or associated traditional knowledge in intellectual property applications is required. This proposal for amendment has been submitted to the State Law Office. It stipulates that, where the subject matter of an IP application concerns, is derived from or developed with biological resources and, or associated TK of communities or individuals, the applicants should disclose the community or individual in the community providing the resources and, or associated TK and from whom they were obtained.

The applicants are also required to provide information including evidence of compliance with the Constitutional Provisions of Articles 11 and 69 among other legal requirements in the providing county for prior informed consent for access and fair and equitable benefit-sharing of benefits arising from the commercial or other utilization of such resources and, or associated TK.

As per the definition of GR provided in The Protection of Traditional Knowledge and Cultural Expressions Act of 2016 and lack of an appropriate regime for the protection of animal breeders rights, it is recommended that the identified gap be filled with and appropriate intellectual property regime.

Animal husbandry, a branch of agriculture concerned with animals that are raised for meat, fibre, milk, eggs, or other products and that include day-to-day care, selective breeding and the raising of livestock, was a significant concern of Kajiado County.

It can be observed that the Kyrgyz Republic has a law on the legal protection of selection achievement and governs economic and moral relations arising out of the creation [discovery, development], legal protection and use of selection achievements for which patents have been granted in the Kyrgyz Republic. Czech Republic has a law on the legal protection of new varieties of plants and breeds of animals and protects new plant and animal varieties [6,7].

These two regimes could provide possible options for addressing this gap to the selection and protection of traditional animal breeds. An animal breeder's regime shall ensure exclusive rights for the breeders and communities. This can help in recouping the investment in research and development of traditional animal breeds and grant opportunities for licensing. Communities will also increase their negotiation power and create a positive image for both researchers and communities. It is anticipated that this may prove useful for raising funds, finding business and add the market value of the traditional animal breeds.

Option one:

An animal breeder regime could be developed based on the Kyrgyz Republic Law on the legal protection of selection achievement of May 26,1998 governs economic and moral relations arising out of the creation [discovery, development], legal protection and use of selection achievements for which patents have been granted in the Kyrgyz Republic.

Option two:
An animal breeders regime could be developed based on the Czech Law on the legal protection of new varieties of plants and breeds of animals protects new plant and animal varieties, [See Kyrgyz republic law on the legal protection of selection achievements and Czech law on the legal protection of new varieties of plants and breeds of animals].

5. Concluding remarks
It is highly recommended that Kenya develops an IPR regime for traditional animal breeds based on any of these two options. As per the absence of guidelines for examination of biological inventions, it is recommended that they are developed by KIPI.

The Biological Diversity Act, 2002 (starting now referred to as BD Act of India) provides a mechanism for access to the GR and benefit-sharing accrued from there. To facilitate this access and benefit-sharing and in order to prevent any unauthorized use of the biological resources of India, in 2005 suitable amendments were made in Section 10 of the Patents Act, 1970, wherein disclosure of the source and geographical origin of the biological material was made mandatory in an application for patent when the said material is used in an invention. Beside a declaration by the applicant regarding the required permission from the competent authority was inserted in Form 1 of the Patents Rules, 2003.

It is recommended that KIPI develops such guidelines for clarity and streamline examination of biological-based inventions. On the other hand, since KEMRI and KALRO lack capacity to handle deposited microorganisms for patent prosecution, Kenya should ascent to the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for Patent Procedure. Lack of guidelines for examination of biological inventions, including biotechnology, was identified in the examination procedures of KIPI.

A look at case KE/P/2013/001960, [PCT/JP2012/062935] demonstrates effects of this lack of clarity on where to deposit microorganisms for patent examination purposes, that has technically prohibited KIPI from substantively examining and granting a patent to the applicant.

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PhilRice Genebank: recent developments in managing and sharing the Philippine rice germplasm

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Abstract. The Genetic Resources Division (GRD) of PhilRice collects and conserves rice genetic resources to ensure the future generations of available seeds needed to build better rice plants in facing climate change and growing population. At present, GRD maintains the national collection of rice genetic resources with 7,129 accessions. To effectively manage the germplasm collection, the search for, development of, and implementation of the best conservation strategies and innovation in technology have been the utmost priority of the GRD. Thus, georeference data such as latitude, longitude and elevation of germplasm origin during collecting mission were recorded using a handheld global positioning system (GPS) receiver. The e-Seedfile software was developed to provide virtual access of the reference collection for regenerated germplasm seed verification and valid type confirmation for new and old germplasm collection. Barcoding, on the other hand, facilitated accurate inventory of seed stocks, making the distribution and regeneration of germplasm more efficient. Moreover, paperless data collection using android application was implemented for immediate data validation and accurate data downloading from tablets to workstations, making it an ideal tool for germplasm characterization. Furthermore, the current database system was upgraded and adjusted to adopt the use of digital object identifier (DOI) through registration to the global information system (GLIS) on Plant Genetic Resources for Food and Agriculture (PGRFA). The DOI allows the use of material to be tracked, thus meeting the legal obligations of the SMTA and monitor the impact of genebank collections in utilization in research and breeding programs. These innovative technologies are of great importance to expand the toolbox for the management and conservation of the germplasm collection that will help enhance the long-term conservation of rice diversity and easy access to germplasm and germplasm-related information.

Keywords: barcoding, characterization, DOI, e-seedfile, GPS, rice.

1. Introduction

The Philippines lies in one of the eight centres of crop origin and diversity recognized by the great Russian conservationist Nikolai Vavilov [1]. Philippines hotspot is also identified as one of the world's biologically most productive countries [2]. It is an archipelago characterized by a wide

variation in climate, eco-geography, and farming systems, which sustain and promote an extensive diversity of rice.

Rice has a high phenotypic diversity results from a long history of domestication, driven by human demographic expansion, and by sympatry of the cultivated rice ecotypes with their wild relatives [3]. The abounding diversity of rice in the Philippines is a national heritage that must be highly valued. These rice genetic resources possess a vast wealth of desirable genes that serve as one of the most important raw material in meeting the current and future needs of rice improvement as well as production programs. These are the building blocks for the development of new varieties to address sustainable development and meet the needs of the continuously growing population. Their efficient conservation and use are critical to safeguarding food and nutrition security, now and in the future.

Maintaining biodiversity for food and agriculture is a national responsibility and cooperative efforts are needed to halt genetic erosion. Its preservation is of paramount importance as these germplasms are the repository of useful genes for plant scientists to solve future problems. However, despite being ecologically rich, Philippines ranks among the top countries with the largest number of threatened species with extinction [4]. The threat of losing these valuable materials makes conservation efforts increasingly more urgent and essential. Thus, the need to conserve essential rice germplasms has been addressed by PhilRice with the establishment of the genebank.

Genebank enabled the conservation of plant genetic materials *ex situ* to ensure seed availability and survival. PhilRice established a rice genebank that will address the conservation of these rice genetic resources for the future generation. Germplasm conservation activities involved manual processing of dried materials for permanent packaging and medium-term storage. Processing included manual sorting to remove all inert and damaged materials as well as separate mixtures. Identities will be cross-referenced against the original seed and panicle files. A properly managed and well characterized germplasm collection will attract an intensified utilization by breeders, leading to more diverse and better-designed varieties which are the critical technology in high productivity rice farming. As more information is available about the germplasm, the more comprehensive selection and diversity of materials can be made available for use in the breeding program and for future generations to appreciate.

Plant genetic resource conservation merits far greater attention than it is now receiving. Recent years have seen increasing efforts to improve both *in situ* and *ex situ* conservation methods, which in theory would foster dynamic conservation of plant species and populations [5,6]. Rice genetic resources conservation is crucial to ensure germplasm sources for further crop breeding [7]. Innovations in technology are expected to expand the toolbox for the management and conservation of the collection that will help enhance the long-term preservation of biodiversity and the easy access of germplasm and germplasm-related information [8]. Thus, to effectively manage the germplasm collection, the search for, development of, and implementation of the best conservation strategies and innovation in technology have been the utmost priority of the genebanks.

2. Review of related literature

In the effort to preserve biodiversity, there has been a concerted world effort to explore, collect, conserve and document the genetic diversity of this important germplasm before they are lost forever. Genebanks around the world play a vital role in the conservation, availability and use of a wide range of plant genetic resources, with the overall aim of long-term conservation. They help bridge the past and the future by ensuring the continued availability of genetic resources for research and improved seed delivery for a sustainable and resilient agricultural system. Well managed genebanks safeguard genetic diversity and make the materials available as a source of variation for plant breeding and selection [9]. Genebanks as a concrete example of *ex situ* conservation ensure that stored materials are readily accessible, can be well characterized and documented and are relatively safe from external threat [10]. This method of conservation cannot, though, provide the opportunity for a wild relative to continue the evolutionary process that a species undergoes in its natural environment. However, it

keeps germplasm safe when plants are destroyed in their natural habitat and they have the advantage, for the user, of making material from widely scattered localities available in one place ready for use.

Conserving and increasing the sustainable use of plant genetic resources is necessary for achieving food security and addressing nutritional requirements of present and future generations. Sustainable conservation of these plant genetic resources depends on the effective and efficient management of genebanks through the application of standards and procedures that ensure the continued survival and availability of plant genetic resources. Therefore, it is vital to conserve the diversity of plant genetic resources so that it is available to the global community [9].

In order to increase the efficiency and effectiveness of germplasm conservation efforts to discover, conserve, and use new qualities in plant genetic resources. Technological advances and developments on germplasm repositories and seed banks must be generated to foster innovation platforms [11]. Availability of detailed documentation of passport, phenotypic, and genetic data increases the value of all genebank accessions. Inclusion of georeferenced sources, habitats, and sampling data in collection databases facilitate interpretation of genetic data for genebank accessions [12]. Furthermore, genebanks often conserve multiple samples that have the same cultivar name because they are genetically distinct. Regardless of the cultivar name, genebanks attempt to maintain the genetic composition of accessions unchanged from sample to sample and from generation to generation. Where the sample originally received is genetically heterogeneous, the genebank may choose to split it into homogeneous groups to be conserved as independent accessions, or keep it as a heterogeneous population for conservation as a single accession [13]. While accession identifiers are assigned to enable each accession to be identified uniquely, and rigorous quality standards are followed to ensure that samples of the accession remain true to type [9]. These advancements and innovations will contribute in the generation of a complete and detailed picture of global rice diversity in the future, thus, definitely play a vital role in the conservation, management and utilization of rice genetic resources.

One hundred and nine non-redundant accessions were selected from the available KKN in LNG as a whole collection model (Table 1). In order to construct our whole collection model genetic data set, a random individual for each non-redundant accession was selected and associated with 24 highly informative genomes spread SSR data. Molecular characterization procedures have been published elsewhere [14].

2.1. Assembly of rice germplasm

Conservation of plant genetic resources, guarantees the ability of seeds to survive in storage which has been successfully applied by genebanks worldwide for the preservation and exchange of crop genetic resources threatened by genetic vulnerability [15]. Exploration and collection comprise a problematic and challenging phase of genetic conservation. A systematic approach to germplasm collection program is necessary to ensure that the maximum range of diversity of germplasm will be collected in a cost and time efficient manner. As a rule, germplasm collecting is undertaken for two purposes for conservation and utilization. Rice improvement programs rely on the vast gene pool represented in genebanks for the source of genes and novel alleles needed to build better rice.

PhilRice through Genetic Resources Division (GRD) collects and conserve rice genetic resources. Collecting activities prioritized the underrepresented provinces and tribal area and stored at PhilRice's genebank. To expand existing collections, field collections in priority locations all over the country are performed and donations from individuals, organizations and institutions are actively encouraged. Georeference data such as latitude, longitude and elevation of germplasm origin during collecting mission were recorded using a handheld global positioning system (GPS) receiver. To date, PhilRice Genebank currently holds 16,233 collections and 7,129 of which are assigned as accessions, identifying them as unique among the registered collections (Table 1).

Table 1. Current status of materials conserved inside PhilRice Genebank.

Biological status	Accessions	Collections	Total
Philippine TRVs	3,943	2,955	6,898
Breeding/research materials	1,615	3,313	4,928
Improved cultivars	631	739	1,370
Unspecified germplasm	494	1,024	1,518
Foreign TRVs	430	787	1,217
Wild rice	16	28	44
Farmers' lines		*258*	258
Total	7,129	8,869	16,233

2.2. Germplasm conservation

Conservation of rice germplasm is a continuous process. The PhilRice Genebank follows the international standards on various processes which includes registration of incoming (new) germplasm collections, preparation of seed files (for seed verification/identity), seed cleaning, viability test (germination rate), slow drying of seeds to achieve 6% seed moisture content (MC) and packaging in standard foil packets for storage in medium-term (active collections) and duplicated in long-term (base collection) storage facilities of PhilRice Genebank.

Regeneration of genebank collections is necessary due to decreasing seed viability as well as diminishing amount of seeds over time through active distribution. Seed multiplication on the other hand, is the best way to revitalize stocks to maintain the genetic integrity of germplasm collection. To keep these valuable materials alive, regeneration has to be undergone to maintain their viability and genetic integrity.

As part of the PhilRice Genebank continuous improvement, germplasm conservation procedures and facilities are still being improved. The facilities of the genebank ensure the long-term preservation of this critical rice diversity. In 2017, PhilRice constructs the facility for genetic resources conservation and management of rice germplasm and acquire the needed equipment for its full operation (Figure 1). Construction of genetic resources building the facility will house upright freezers (-20°C) as storage facilities for *ex situ* germplasm conservation of rice genetic resources. Laboratories for seed health and quality testing, and molecular laboratory for DNA fingerprinting and storage of DNA bank of all rice germplasm collections will also include in the new facility. It is located in PhilRice CES, Maligaya, Munoz, Nueva Ecija. This facility in PhilRice CES was selected since it is the central repository or rice germplasm in the Philippines.

Figure 1. The newly constructed PhilRice Genebank facility.

2.3. Germplasm inventory

Managing genebanks involves repeatedly identifying the samples, or accessions, to track them and to update the information describing them after various processes like pathogen testing, cleaning, multiplication, characterization, evaluation, seed storage and inventory, and distribution. This process is both time-consuming and prone to human error if it is done using conventional hand registration with paper records [16]. To ensure that the conserved germplasm are same as the original collection, seed identity was verified through cross-checking with available seed files, planting plans and panicle files. Comparison between the seed lot and the seed file was done to verify the identity of the seed lot and the status of the seed quality (i.e. mix, mismatch, infected, etc.) were also noted.

To minimize labelling and handling mistakes during seed production process, a computer-generated barcode labels (accession number, lot number and cropping season) for field tags and seed production process. Barcoded labels printed on polyester paper using thermal printer have been tested for readability and durability under field and screen house conditions for an expanded application [17]. Furthermore, color coded vacuum sealed foil was used for the precise inventory of seed stocks, making the more efficient inventory, conservation, distribution and regeneration of germplasm. Blue code for wild rice, red for germplasm with restricted access, yellow for base collections and white for active collections (Figure 2).

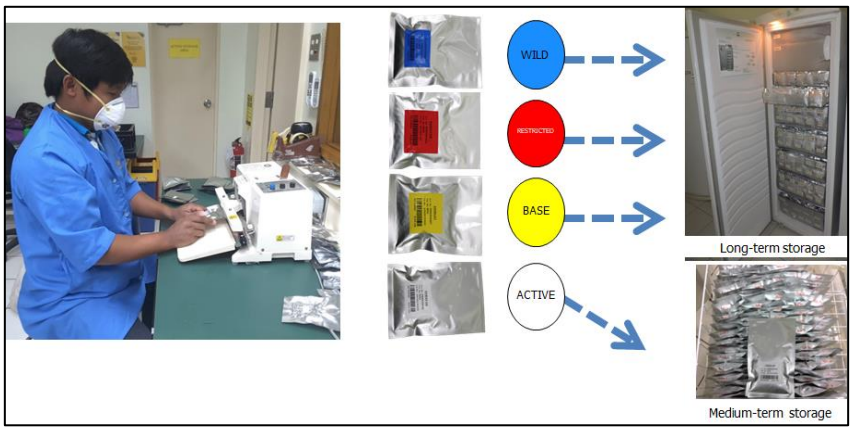

Figure 2. Color-coded classification of foil packets.

The e-Seedfile software was developed to provide virtual access to the reference collection for seed verification of the regenerated germplasm and valid type confirmation of the new and old germplasm collection. While panicle file was also initiated to provide a virtual reference to the accession.

2.4. Germplasm characterization

Assessment of agro-morphological diversity among rice germplasm is an essential endeavour in any genetic resources management and crop improvement. Discovery of desirable traits from traditional rice varieties (TRVs) and incorporating these traits in rice breeding efforts would greatly benefit rice farmers to mitigate the effect and better manage rice production in changing environmental conditions. This study provides information on the morphological characterization of TRVs used to establish each accession's genetic identity, to identifying varieties with desirable traits for direct utilization and potential donors for crop improvement and assess the extent of genetic diversity of the collections.

Many software packages are available for assessing phenotypic diversity parameters that increased the efficiency of germplasm curators and, plant breeders to speed up the crop improvement [18]. Recently, paperless data collection using an android-based application called FieldLabV2.9 developed by IRRI is being implemented for immediate data validation and accurate data downloading from tablet PC to the central database of GRD. Efficient paperless data collection using android technology

results in immediate data validation and accurate data downloading from tablets to workstations, making it an ideal tool in germplasm characterization [17].

2.5. Germplasm distribution and information management

The complex processes of managing rice germplasm collection at PhilRice Genebank are supported by its in-house documentation system called Germplasm Management System (GEMS). This serves as a central repository of integrated rice germplasm information that provides links to the different genebank operations, from registration, characterization, evaluation and seed inventory to seed distribution to end-users thus increase the efficiency in doing its decision-support that helps genebank managers.

There are also datasheets, record books and forms since it is not always possible to record/upload data directly into the system. These datasheets can be used for the chain of procedures performed by different genebank staff in their respective tasks. These are also correctly filed, bound and archived for future references and back-up files. Recently, GEMS was upgraded into advanced version 'GEMS v2.0' that involves redesigning of the system architecture and adding new functions in synchronization with the current documentation needs of the Genebank. All germplasm data uploaded in the database were also checked and validated.

One of the constraints in the utilization of germplasm by breeders, researchers and farmers is the access. At present, the development of its web-based version is being done for broader dissemination of information to breeders, researchers and other stakeholders.

2.6. Germplasm exchange

Genebank collections around the world hold the raw genetic materials needed to breed new and better plant type to feed the growing population. Many holders of these plant genetic resources have their documentation and management system. However, there is no standardized or shared method for assigning unique identifiers relative to the accessions. The digital object identifiers (DOIs) has been implemented as an agreed method for the assignation of global identifiers to standardize the method of providing accessions' permanent unique identifiers. The use of DOI allows the materials to be tracked as these genetic resources are being shared, duplicated, and used among institutions. Through DOI, the impact of genebank collections in utilization in research and breeding programs are monitored, including its conformance to the legal obligations stated in the SMTA. Recently, a multicountry construction of a test platform for the development and allocation of unique identifiers of rice germplasm was implemented in Asia. This initiative was organized by the Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD) in collaboration with the secretariat of the International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA) and the International Rice Research Institute (IRRI), in participation of genebank curators from Bhutan, India, Indonesia, Malaysia, Philippines and Zambia.

2.7. PhilRice germplasms with DOI

There are variations in characters observed in 101 registered cultivars (Table 2). Maturity days ranged from 99 to 193 DAS, where in Taal 2 was found to be early maturing while Panesopek was late maturing. Plant height had an average of 151.50 cm. The 100 seed weight ranged from 1.3 g to 3.3 g, where in Iniba-bain and Gundang were observed to have the heaviest grain weight (3.3 g). The rice collections that exhibited the longest panicle (>35 cm) is Bandera.

In terms of qualitative traits, some of the traditional Philippine varieties has glabrous leaves (Table 3). Glabrous leaves rice was usually fed to ruminants. For the DOI registered germplasm, Sinatyar and Taal 2 have glabrous leaves. On the other hand, extreme lodging resistance were observed on Ligantong, Bimmotiti and Kintuman (Calipago).

Table 2. Descriptive statistics of the 101 registered cultivars based on 12 quantitative traits.

Descriptor	Mean	SE	Sd	Min	Max
Maturity	141.24	± 2.0	20.6	99	193
Grain width	2.63	± 0.1	0.5	2.00	4.00
Grain length	8.13	± 0.1	1.0	5.00	10.00
Culm diameter	4.86	± 0.1	0.6	4.00	6.00
Culm length	128.80	± 3.4	23.6	67.00	162.00
Culm number	14	± 0.4	2.6	10	20
Grain weight	2.45	± 0.1	0.4	1.3	3.3
Leaf blade length	63.08	± 2.0	13.8	37	99
Leaf blade width	1.44	± 0.0	0.3	0.8	2
Ligule length	20	± 0.6	4.4	10	30
Panicle length	25.28	± 0.4	3.2	18	35
Plant height	151.50	± 4.4	30.8	88	187

Table 3. Varieties with desirable qualitative attributes.

Descriptor	Descriptor state	Name of cultivar
Leaf pubescence	Glabrous	Sinatyar, Taal 2
Culm lodging resistance	Very strong	Ligantong, Bimmotiti, Kintuman (Calipago)

Some of the registered varieties are resistant to a specific disease. Reactions to the blast were resistant in Hinamog, Nagpili, Bandera, Casungsong, Caboyo, Sologanon, Binolawan, Paliod, Korasisi and Kalisokasaya. Meanwhile, Doctor, Guinabioka and Kintuman (Calipago) showed resistance to bacterial leaf blight. On the otherhand, Kalagnon was resistant to sheath blight. There are several available germplasm accessions that could offer resistance against major rice diseases, which could be considered as option for selection of parent materials for rice breeders to produce rice lines/varieties that would perform well against problematic rice diseases. It is highly recommended to continue exploring the potential of available rice lines/varieties against major rice diseases that would further expand the option for breeders in selecting parent materials and also to monitor the stability of resistance of the earlier tested accessions.

3. Concluding remarks and future perspective

There are significant constraints in the genebank operations that undeniably affect its capacity to manage rice germplasm. The present storage conditions in PhilRice are far from ideal. During germplasm storage, unstable power supply and cold room malfunction adversely affect the quality and viability of stored seeds. Desired temperature and corresponding relative humidity levels are unattained regarding international genebank standards. Continual upgrading and expansion of laboratory and storage facilities will be pursued to enhance the capacity of the genebank to store high-quality seeds for long period. To maximize storage life, maintain genetic integrity and ensure availability of high-quality seed and information of the collection, PhilRice Genebank continuously upgrading its facilities and optimizing its protocols.

An essential aspect of genebank management is to secure duplicates of germplasm for safety back-up to mitigate the risk of its partial or total loss caused by natural or human-made catastrophes. Although a portion of PhilRice Genebank accession were duplicated in IRRI Genebank, there is a need

to send (if not all), at least the core collections to ensure materials are safely duplicated in other locations so that if a collection suffers a loss, germplasm can be restored from duplicate sets.

Lots of things are yet to be done, increasing inter-disciplinary approaches to facilitate fast, reliable and accurate conservation and management. PhilRice Genebank is also on the move to explore increased collaboration with various institutions in the field of bioinformatics, DNA/RNA sequencing, and protein profiling. Innovations in technology are expected to expand the toolbox for the management and conservation of the collection that will help enhance the long-term preservation of biodiversity and the easy access of germplasm and germplasm-related information. These advancements and innovations will contribute in the generation of a complete and detailed picture of global rice diversity in the future, thus, definitely play a vital role in the conservation, management and utilization of rice genetic resources.

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Digital object identifier (DOI) application for rice germplasm collection at Yogyakarta AIAT

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Abstract. In 2014, local rice exploration survey identified 76 local rice accessions from Yogyakarta. These local rice collections have been stored in Yogyakarta AIAT cooler facilities. Yogyakarta AIAT has assigned the digital object identifier (DOI) to 55 of its local rice collections. The assignation of DOI will be useful for the local rice collection for their availability for transfer with the Standard Material Transfer Agreement (SMTA) in the Multilateral System (MLS) of Access and Benefit-Sharing (ABS) of the International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA).

Keywords: digital object identifier (DOI), information, local rice, Yogyakarta.

1. Introduction

Daerah Istimewa Yogyakarta (DIY) is one of the provinces in Java Island in Indonesia. DIY Province is bordered with the Indonesian ocean to the south, and several regencies of Central Java Province such as Klaten to the northeast, Wonogiri to the southeast, Purworejo to the west, and Magelang to the northwest. It lies between 7°33'−8°12' South Latitude and 110°00'−110°50' East Longitude of Greenwich and cover an area of 3,185.80 km² or 0.17% of Indonesia (1,860,359.67 km²). The province is divided into five districts, namely Sleman, Bantul, Kulon Progo, Gunungkidul and Yogyakarta City.

According to typology, the DIY Province consists of volcanic plateau, karst material to coastal sand dune from 0−2,910 m above sea level (m asl). The volcanic plateau is located in Sleman, where Mount Merapi is located, and some are in Gunungkidul that has a plateau with the primary material of karst or sediment. Some of the lowland areas are in Sleman, Bantul, Yogyakarta City and Kulon Progo. Coastal sandy and mangrove areas are in Kulon Progo [1]. Diversity of land typology in this province causes variations in genetic resources, in particular, food crops such as rice.

Rice cultivations in Sleman, Bantul and Kulon Progo are mostly irrigated rice cultivation, while in Gunungkidul, which has dry land characteristics, is rainfed rice cultivation. Agriculture areas in DIY according to BPS [1] were 53,553 ha. Rice production in 2014 was 945,136 tons; it increased 2.78% from the previous year [2].

Farmers in DIY usually cultivate paddy new superior varieties, such as IR 64, Ciherang, Situ Bagendit, Pepe and Inpari (Inbred Irrigated Fields Rice). However, with DIY land typology, there may be some farmers cultivate local rice variety. Local rice is a type of rice plant existed for a long time and is still being cultivated until today in certain regions [3]. Local rice is one of the potential genetic

resources that are cultivated for specific food consumption reason. Local rice varieties majority have specific characters, such as unique taste, grain form and grain colour, resistance to a specific pest, disease or abiotic stresses on their specific location. These genetic advantages can be extracted in developing new rice cultivars [4].

In 2014, Yogyakarta Assessment Institute for Agricultural Technology (AIAT) which is one of the technical implementing units of Indonesian Agency for Agricultural Research and Development (IAARD), has a mandate for exploring DIY genetic resource potentials especially for local rice food crops [5]. The local rice exploration activity identified that DIY has around 76 accessions. The local rice exploration also identified local origin in this region [6,7]. The collected local rice accessions seeds are being stored in Yogyakarta AIAT.

The local rice seed collection is stored in a closed plastic container, labelled, and then put in the cooler. Their seed rejuvenation was tested both on farmers and in a limited form like pots. Rejuvenation purpose is to determine the level of diversity and save genetic diversity [8]. Several local white rice accessions in the collection have been characterized [9,10]. Their study revealed that DIY local white rice had a high genetic diversity based on its morphological characters.

Assessment on five local black rice accessions (Melik, Pari Ireng, Cempo Ireng, Jlitheng and Bantul black rice) showed that their plant height and number of productive tiller characters had a wide genetic diversity [11]. On the other hand, the length and width of grain, number of filled grains per panicle and days of maturing characters had narrow genetic diversity.

Genetic diversity is not only seen through its morphological character, but can also be indicated by the biochemical characters. Biochemical character is carried out to find out the potential advantages of each local paddy of DIY. The genetic diversity of several local black rice cultivars based on rice colour parameters and total anthocyanin content had been identified [12]. Genetic factors provide a more significant role than environmental factors in determining the diversity of colour parameters and the total anthocyanin content.

DIY local rice showed a high level of genetic diversity. To save genetic material collections, conservation efforts are needed. Conservation is divided into three groups, namely *in situ* conservation, which is an effort to maintain collections in their original habitat, carried out by conservationists or participatory farmers; *ex situ* conservation, an effort to maintain collections from the place of origin until stored in gene banks; on-farm conservation, which is to maintain collections by rejuvenating (updating) collections made by breeders in government and private research institutions [13].

Ex situ conservation does not stop at the activity of storing collections in gene bank. However, it includes documented information processing activities related to specific identities that contain information on genetic diversity and specific potential of collections. Documented information related to the systematic genetic diversity of collections will make it easier for breeders to obtain sources of genetic diversity appropriately and used it in a specific improvement character.

In 2016, the Food and Agriculture Organization (FAO) collaborated with Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD), IAARD has signed a Letter of Agreement (LoA) for implementing a multicountry construction with a focus on the codevelopment and transfer of technology information related to genetic resources of food crops, especially local rice accessions. The objectives of the agreement are to bridge the gap between the information requirements of gene bank curators, rice breeder, and more targeted upstream biological researchers, and to support applied germplasm curation and forward-looking rice breeding programs and strategic rice research.

The specific agreement is to identify local rice genetic resource material into digital object identifier (DOI) which is a method for the assignation of global identifiers as the permanent and unambiguous method for the identification of rice accessions. This agreement also developed a platform to establish an automatized system to system connections to add value to the material being transferred within and from Multilateral System (MLS), and meeting both scientific need and legal obligations of the Standard Material Transfer Agreement (SMTA).

The purpose of this study was to provide a support for the agreement between FAO-ICABIOGRAD through registering DIY local rice, which has been collected by the Yogyakarta AIAT, on the application of a digital identification operating system through ICABIOGRAD registration system.

2. Materials and methods

2.1. Steps for registering genetic material

Genetic material application steps included:

1) Installing Oracle virtual box.
2) Installing Ubuntu.
3) Installing Php My Admin (Bitnami).
4) Registration (registration) of genetic material.

Before preparing to install the application, the descriptor must prepare a data description of local rice accordingly [14]. The data consisted of information about:

1) Sample_id: A string that identifies the Plant Genetic Resources for Food and Agriculture (PGRFA) that is being registered. It is a unique identifier for the PGRFA in the provider's management. Yogyakarta AIAT as a technical implementation unit had this ID number by registered materials to ICABIOGRAD.
2) Date: Date in which PGRFA became part of the collection. Date fragments (yyyy-mm and yyyy) are also accepted.
3) Hold_wiews code: FAO/WIEWS code of the breeding institution. We use ICABIOGRAD code number wiews.
4) Hold_PID: Uncomplicated SMTA PID of the breeding institution or individual if available. ICABIOGRAD would give the PID code number to Yogyakarta AIAT.
5) Hold_name: Surname and name for individuals or organization name where the PGRFA material is maintained. In this part, ICABIOGRAD will maintain Yogyakarta AIAT's material.
6) Hold_address: Address of the providing institution or individual, multiple lines are accepted. It will be Yogyakarta AIAT.
7) Hold_country: ISO-3166 alpha-3 country code (https://en.wikipedia.org/wiki/ISO_3166-1_alpha-3) of the providing institution or individual.
8) Method: Method through which the PGRFA has been acquired. Mandatory. See Table 1 for the codes accepted by this element.
9) Genus: The taxon of the genus for the PGRFA. At least one between <genus> and <cropname> must be provided.
10) Species: Authority for the scientific name.
11) Spauth: Authority for the subtaxon at the most detailed level provided.
12) Subtaxa: Any additional intraspecific taxon such as subspecies, variety, form, group and so on.
13) Bio_status/Biostatus: Biological status of the PGRFA. It can be a local name. See Table 2 below.
14) Mls_status: Code that identifies the status of the PGRFA about the MLS. See Table 3 below.
15) Coll_sid: Code number that identifies the materials came from.
16) Coll_site: Description of where the PGRFA was collected.
17) Coll_source: Code of the nature of the location where the PGRFA was collected. See Table 4 below.
18) Coll_lat: Latitude where the PGRFA was collected in either dd°mm'ss"X (where X is N or S) format or ddd.xxxxx (up to 5 decimals, preceded by a minus sign for S) format. No spaces are allowed.
19) Coll_lon: Longitude where the PGRFA was collected in either dd°mm'ss"X (where X is E or W) format or ddd.xxxxx (up to 5 decimals, preceded by minus sign for W) format. No spaces are allowed.
20) Coll_elevation: Elevation of collecting site in m asl.

21) Coll_date: Date on which the PGRFA was collected. Date fragments are also accepted when an only a year or year and month are known.
22) Ancestry: Pedigree or other description of the ancestry of the PGRFA and how it was bred. Please note that this column is a list of identifiers assigned to the PGRFA locally in your or somebody else collection (e.g. the accession number for gene banks).

Table 1. Code for a method of collection of PGRFA.

Code	Description
Acqu	Acquisition
Ihcp	In-house copy
Ihva	In-house variant
nodi	Novel distinct PGRFA
obna	Observation–Natural
Obin	Observation–Inherited

PGRFA = Plant Genetic Resources for Food and Agriculture.

Table 2. Code for the biological status of PGRFA.

Code	Description	Code	Description
100	Wild	414	Inbred line (parent of hybrid cultivar)
110	Natural	415	Segregation population
120	Semi-natural/Wild	416	Clonal selection
130	Semi-natural/Sown	420	Genetic stock
200	Weedy	421	Mutant
300	Traditional cultivar/landrace	422	Cytogenetic stocks
400	Breeding/research material	423	Other genetic stocks
410	Breeder's line	500	Advanced or improved cultivar
411	Synthetic population	600	GMO
412	Hybrid	999	Other
413	Founder stock/base population		

PGRFA = Plant Genetic Resources for Food and Agriculture.

Table 3. Code for identification of the status of PGRFA about the MLS.

Code	Description
0	No available under MLS
1	Available under MLS
11	The PGRFA belongs to a crop listed in Annex I and is under the management and control of a Contracting Party to the Treaty and declared to be in the public domain
12	The sample is in a collection subject to an agreement concluded under Article 15 of the Treaty
13	The holder received the sample with SMTA
14	The holder has voluntarily placed the PGRFA into the MLS
15	The PGRFA is derived from, and distinct from, material previously received from the MLS is still under development and not yet ready for commercialization, and may be made available at the discretion of the developer

PGRFA = Plant Genetic Resources for Food and Agriculture, MLS = Multilateral System, SMTA = Standard Material Transfer Agreement.

Table 4. Source/code for description.

No.	Description	No.	Description
10	Wild habitat	25	Pasture
11	Forest or woodland	26	Farm store
12	Shrubland	27	Threshing floor
13	Grassland	28	Park
14	Desert or tundra	30	Market or shop
15	Aquatic habitat	40	Institute, experimental station, research organization, gene bank
20	Farm or cultivated habitat	50	Seed company
21	Field	60	Weedy, disturbed or ruderal habitat
22	Orchard	61	Roadside
23	Backyard, kitchen or home garden (urban, peri-urban, or rural)	62	Field margin
24	Fallow land	99	Other

All data must be input on Microsoft excel (.xls) program and upload in excel CSV (comma delimited; .csv) format.

3. Results and discussion

In 2014, Yogyakarta AIAT had successfully explored, invented, and carried out activities on 76 Yogyakarta local rice. By the end of 2014, based on the identification and characterization activity, there was 55 accessions of Yogyakarta local rice that were ready to be registered in the DOI. The results of the registration of the digital numbering have been archieved in the ICABIOGRAD registration system.

The 55 accessions of Yogyakarta local rice have a variety of features, such as rarity or can only be found in a specific location in DIY. Other features included specific pericarp colour (white, red, or black), specific aroma, the potential value such as high production and some other accessions had potential on drought-resistant rice accessions. There was specific accession on irrigated rice, upland rice, and sticky rice. Unfortunately, in this DOI system there is no column that contains a character or specific characteristics of local rice.

Information related to Yogyakarta local rice digital numbering can be accessed through https://ssl.fao.org/glis/. Yogyakarta local rice that have been registered through the ICABIOGRAD digital numbering system is listed in numbers 05020-30654 up to numbers 05020-30654. It is believed that in the future, DOIs will become the global standard for public identification of PGRFA, which facilitate linkage between the material and diverse sources of information associated with the material. DOIs platform will give many beneficial opportunities. This platform could establish automatized system-to-system connections to add value to the material being transferred within and from MLS, thus meeting both scientific needs and legal obligations of the SMTA. More specific beneficial is a simple reliable mechanism to identify accessions that are duplicated across gene bank [14]. Importantly, this system could bridge the gap between the information requirements of gene bank curators, rice breeders, and more targeted upstream biological researchers, and support the applied germplasm curation and forward-looking rice breeding programs and strategic rice research.

According to the DOIs beneficial mechanism as a gene bank, they will directly have benefit from the adoption of better tools and methodologies for the documentation of PGRFA information related to rice accessions. Rice plant breeders and users of the multilateral system could get facilitated from a globally access of associated information beyond the passport data. For research institutions, they will get additional information on the availability of accessions that may help the decision-making process

on regeneration, conservation, and improvement of specific characteristic needed in developing a new rice cultivar, thus will have a positive impact on national plant breeding programs.

4. Conclusions

Yogyakarta AIAT has registered 55 local rice through ICABIOGRAD registration system. Registration of local rice genetic resources is needed as one of the efforts to complete information that meets the needs of digital communication between gene bank curators, rice breeders and other agricultural researchers who have interest in accessing genetic resources that can be used to regenerate, conserve, and also improve specific characteristics that is needed in developing a new rice cultivar.

5. Acknowledgement

The author would like to thank ICABIOGRAD 'DOI Project Management Team' (Dr. M. Sabran, Dr. Puji Lestari, Dr. Nurul Hidayatun and Dr. Hakim Kurniawan) for giving us a chance for participating in the local rice management DOI project and writing scientific papers related to DOI of Yogyakarta local rice.

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Decision support system based on database system of genetic resources for Central Kalimantan local crops to develop *ex situ* and *in situ* conservation

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Abstract. The results of exploration and inventory of local crop genetic resources from Central Kalimantan have been compiled into a genetic resource database system. The data and other information that have been integrated into a database system can be further developed systematically to become a decision support system so that it can be utilized as a useful tool in decision making. This system can be built by combining data, models and analytical tools in an application system with a user-friendly interface. This system can be utilized in the preparation of genetic resources development planning, such as for the determination of location for *in situ* and *ex situ* conservation areas in Central Kalimantan. The objective of this study was to develop such system, through the application of MS Access database as the Database Management System (DBMS) for storage (repository) and accessing database system applications that have been prepared interactively. The System is systematically designed to include geographic information system and based on the spatial analysis which can provide guidance and recommendation for zoning areas based on the suitability of plant commodities, both inside and outside of their natural habitat environment.

Keywords: system, support, decision, genetic resources, Central Kalimantan.

1. Introduction

Central Kalimantan province is located around the equator line with a total area of approximately 15 million hectares. It has vast natural biodiversity containing local plant genetic resources. In 2014, it was reported that there were approximately 937 species of genetic resources comprising food crops, horticulture, medicinal crops and fisheries/livestock [1]. They have been widely used and managed by local communities with indigenous knowledge through traditional farming system. The existence of this plant genetic resource is closely linked to the culture of those local communities. It should be therefore be conserved in order to preserve Indonesia's biodiversity and cultural richness [2,3].

Local crop genetic resources can be further managed and developed to support sustainable life in the future. As an important part of biodiversity, it is an asset for all sectors and contributes to sustainable development, food security and better nutrition [4]. The importance of plant genetic resources for humankind has been well recognized in the recent decades and many would argue that diversity is essential for allowing sustainable development of various human activities [5]. The need of

conservation, utilization, monitoring and management of plant genetic resources for food and agriculture has been promoted since the '90s [6,7].

The initial stage of the effort to manage genetic resources involves inventory and data collection of local plant genetic resources. The information of plant genetic resources can then be used for long term preservation of agricultural crop genetic biodiversity in addition to enriching agro-biodiversity with new genetic resources to serve the needs of agricultural research [8]. This study was conducted in order to develop a database system as an integral part of a Decision Support System (DSS). Data compilation based on the result of exploration and inventory of biodiversity was incorporated into the system. The PC-based application called MS Access was utilized to develop a database system complete with its DSS. It is expected to generate valuable information for genetic resource management and planning, especially for determining zonation areas for conservation and for the development of those areas.

2. Materials and methods

This study was conducted using data that had been collected and compiled from exploration and inventory of plant genetic resources. Information regarding local crops was obtained through field surveys in several locations using a purposive sampling approach throughout the whole areas of Central Kalimantan, which covers 15,451,287 hectares (Figure 1).

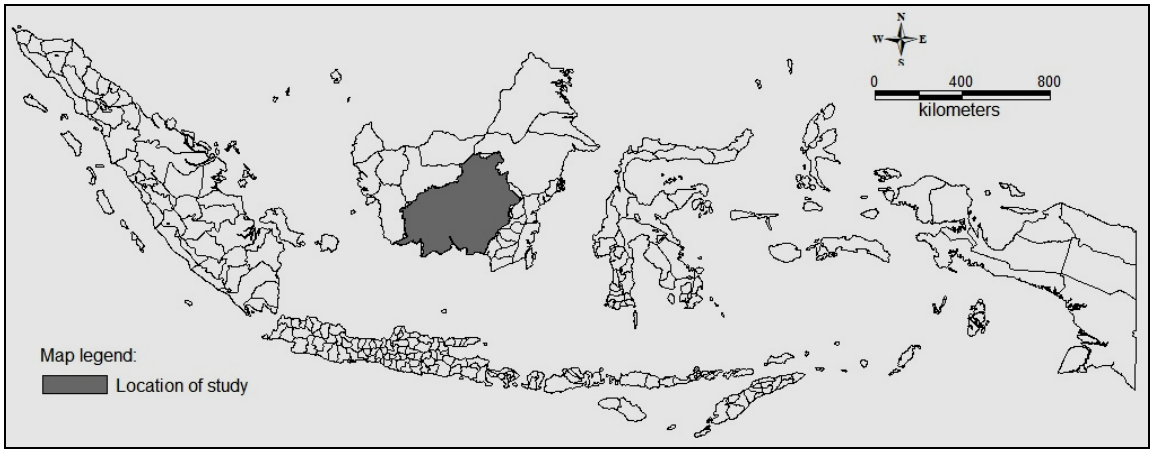

Figure 1. Situation map of Indonesia showing the location of the study.

The PC-based MS Access application was employed in this study to create a database system which would be further developed to include DSS. This application is used due to its effectiveness for Database Management System (DBMS) [9]. The simple DSS was constructed as a planning database, where its logical data structure can be used to assist the decision makers for planning and management [10].

The Geographic Information System (GIS) application was also incorporated in order to supply the database with spatial data, as well as facilitating, improving, storing, displaying, future utilization and updating data [11–15]. As a part of GIS, the base map of the land system at reconnaissance scale was used in order to identify land suitability of each local crop according to its original habitat data. The use of land system information as part of spatial secondary data with its recurring pattern principle was used to determine other suitable areas for *in situ* and *ex situ* development based on the similarities of landform, soil and vegetation with the original habitat and having a relatively uniform climate [16–18]. The general procedure of this study is described in Figure 2.

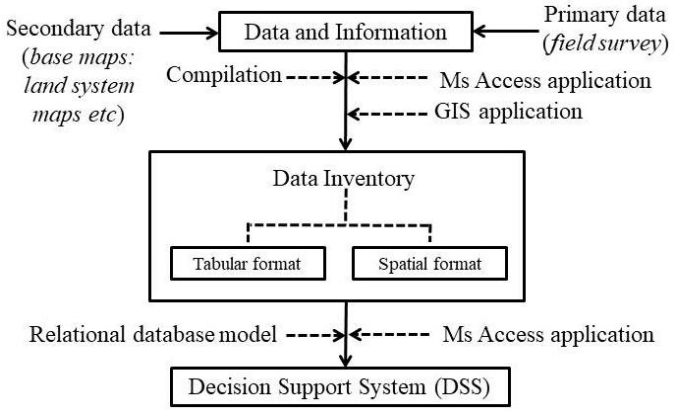

Figure 2. Flowchart of general procedure in this study.

3. Results and discussion

3.1. The status of plant genetic resources in Central Kalimantan

Central Kalimantan province is located between 00°46'58''S–03°33'43''S latitudes and 110°42'48''E–115°50'39''E longitudes. It consists primarily of drylands and wetlands typology with various agroecosystem within each typology [16,17]. The drylands, also known as upland areas, are located in central to northern part with an elevation range of 100 to 500 meters above sea level. The wetlands are found in the Southern part at an elevation below 100 meters. Most of the upland areas are highly weathered, acidic, infertile and have poorly buffered soils [20], while the southern part is dominated with lowland areas consisting of swamp and peatland. In general, with appropriate land management, the arable land has low to high potential for cultivation of wetland rice and moderate potential for dryland food crops and perennial crops [21]. This potential is further enhanced by climate condition within the tropics, which allows cultivation to be expanded based on habitat suitability.

Exploration has identified approximately 937 species of food crops, horticulture, medicinal crops and fisheries/livestock, and most of them have been cultivated traditionally with indigenous knowlege [1,22]. Geographically, they are scattered at many locations in the whole Central Kalimantan Province, as shown in Figure 3.

3.2. Developing a database management system

Relevant information regarding the plant genetic resources and inventory data were organized into a database system within MS Access environment. The PC-based application program called MS Access was used in this study because of its simplicity for developing database management [23].

Using a relational database model, all data was initially classified according to the group of crop, i.e. food crops, fruit crops, ornamental crops and medicinal crops. Species classification was then determined in each group, followed by addition of relevant information to provide useful information regarding general description, original habitat, spatial distribution, recommendation for *ex situ* and *in situ* conservation and propagation area, cultivation techniques and benefits value (for medicinal crops).

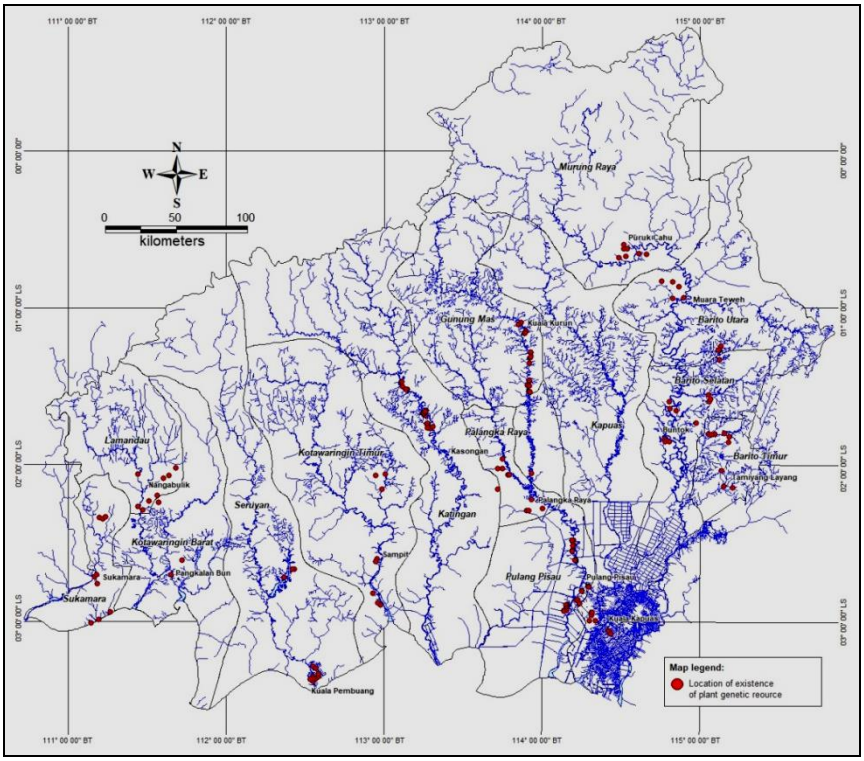

Figure 3. The distribution of identified plant genetic resources from explorations in Central Kalimantan.

The decision-making part of DSS was constructed and built based on its logical data structure so that managers will find it easy to use. Several screen-captures representing parts of DSS are shown in Figure 4.

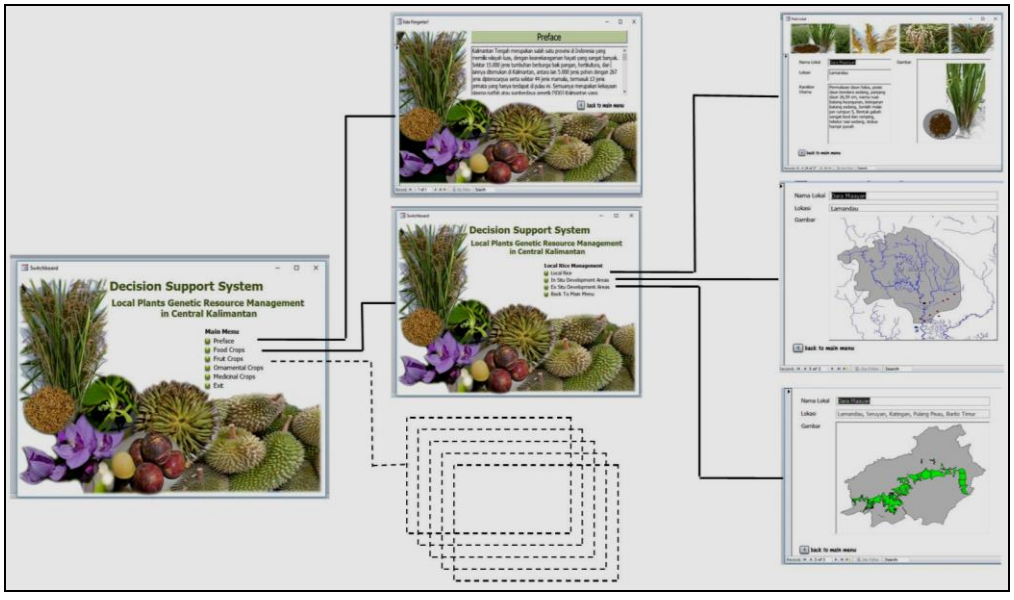

Figure 4. Several windows view representing part of DSS for genetic resources management in Central Kalimantan.

4. Conclusions

In order to support preservation and management of local crops, relevant information of plant genetic resources should be organized into a database system that can be further developed to become a decision support system. It can assist the planning process of preservation and management of local crops through the determination of development areas for *ex situ* and *in situ* conservation.

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MARDI rice genebank: important roles in data management and data sharing

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Abstract. Malaysia has agreed to adopt the International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA) and acceded to the Treaty in 2004. Under Article 10 of the Treaty, the contracting parties should allow easy access of the genetic resources under Annex 1 Crop through Multilateral System, and Benefit-Sharing raised from the utilization of the resources. To implement the Treaty, MARDI has actively involved in the implementation through the exchange of seeds particularly rice accessions using Standard Materials Transfer Agreement (SMTA) and in data sharing for easy access of rice genetic resources. MARDI has started to involve in adoption and implementation of Digital Object Identifiers (DOIs) and also contributed to the Genesys database though a project named 'Building Genesys Catalog of Phenotypic Datasets of Malaysian Rice Germplasm'. In Malaysia, rice genetic resources are conserved at the National Rice Genebank in Penang. The genebank was established in 1989 with the primary purpose to collect and conserve rice genetic resources (*Oryza* spp.) in Malaysia. Currently, the genebank has conserved a total of 13,020 accessions of rice germplasms consisted of landraces collected throughout the country, introduced varieties, and breeding materials. The rice germplasms are an important source of genetic materials for the development of new rice varieties. To date, 48 rice varieties were developed and introduced to the farmers and local communities. Those varieties possessed consequential and unique traits such as high yield and resistant to pest and diseases, to make them valuable to farmers and local communities. Those varieties are registered and freely accessed in Global Information System (GLIS) website for DOI implementation. Besides, a total of 22 phenotypic datasets including datasets for morphological traits, pest and diseases (brown planthopper, blast and bacterial leaf blight), and quality and speciality traits, are prepared and published in the Genesys portal (database). Data sharing is essential to enhance the breeding program in the country and sharing of valuable genetic resources with other countries for the future development of new varieties for food security and to promote sustainable agriculture.

Keywords: Malaysia, MARDI, DOI, rice genetic resources, ITPGRFA.

1. Introduction

Malaysia is located between 2° and 7° north of the equator and longitudes 100° and 119° east. This South-East Asian sovereign covers an area of about 329,758 km^2, consisting of Peninsular Malaysia, and the states of Sabah and Sarawak. Malaysia has been recognized as one of the world's twelve mega

diversity developing countries in the world. It harbours at more than 170,000 species [1] with more than 15,000 species of flowering plants [2]. The plant species are presented by more than 2,500 tree species, 3,000 species of orchids, 500 species of ferns, 60 species of grasses and bamboos, and many others [2].

Malaysia implemented the National Policy on Biological Diversity in 1998 and ratified the Convention on Biological Diversity in 1994. Ten years later, Malaysia agreed to adopt the International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA) and acceded to the Treaty in 2004. Under Article 10 of the Treaty, the contracting parties should allow easy access of the genetic resources under Annex 1 Crop through Multilateral System and Benefit-Sharing raised from the utilization of the resources. Consequently, MARDI has actively involved in the Treaty through the exchange of rice genetic resources by using the Standard Material Transfer Agreement (SMTA) and also in data sharing for easy access to the genetic resources. Recently, MARDI has involved in adoption and implementation of Digital Object Identifiers (DOIs) for rice germplasm and also contributed to Genesys portal (database) through a project named 'Building Genesys Catalog of Phenotypic Datasets of Malaysian Rice Germplasm' funded by Global Crop Diversity Trust (Crop Trust).

In Malaysia, rice genetic resources are conserved in the National Rice Genebank located at MARDI Seberang Perai, Pulau Pinang. The genebank was established in 1989 with the primary purpose to collect and conserve the rice genetic resources (*Oryza* spp.) in Malaysia. Currently, the genebank has conserved a total of 13,020 accessions of rice germplasm consisted of landraces collected throughout the country, introduced varieties, and breeding materials. These rice germplasms are important as genetic resources for research and development of new rice varieties in Malaysia. To date, 48 rice varieties were successfully developed by MARDI Rice Breeders and were introduced to the farmers and local communities. However, only 45 MARDI varieties were selected for DOI registration. These varieties possessed consequential and unique traits such as high-yielding, aromatic and colored, and resistant to pest and diseases, thus making them valuable to the farmers and local communities.

This paper will discuss on the establishment of data management in MARDI and the contribution of MARDI in data-sharing mechanism to support easy access of rice genetic resources established under the International Treaty on Plant Genetic Resources for Food and Agriculture (ITPGRFA or the 'Treaty').

2. Data management in MARDI

To support the efforts for sustainable use, development, and conservation of plant genetic resources in the country, MARDI has developed a database information system known as MARDI Agrobiodiversity Information System (AgrobIS) (Figure 1). The information system was established to conserve data on collection and conservation of genetic resources related to agrobiodiversity components, namely plants, arthropods and microbes [3]. It is a web-based application and capable of handling specific characters of each agrobiodiversity component [3]. The system is an innovative technology for handling agrobiodiversity information using licensed free software, MySQL for database development and PHP for the web interface.

For rice germplasm, the information system consists of five categories: (1) Passport data–basic information of the accession such as accession number, variety name, original country, collector name, acquisition date and seed source; (2) Characteristics–consists of morphological characterization data on quantitative and qualitative traits. It is based on Rice Descriptor List developed by IRRI and Biodiversity International; (3) Evaluation–consists of screening data for biotic (drought, salinity) and abiotic stresses (brown planthopper, foliar blast and bacterial leaf blight); (4) Quality traits–consists of data on speciality traits of grain such as amylose content, gelatinization temperature, seed coat color, endosperm type and scent (aroma); (5) Seed Management–consists of information on management of the collection in genebank, i.e. specific location in medium and long term storage, duplicated area, regeneration details, seed quantity and result of germination test.

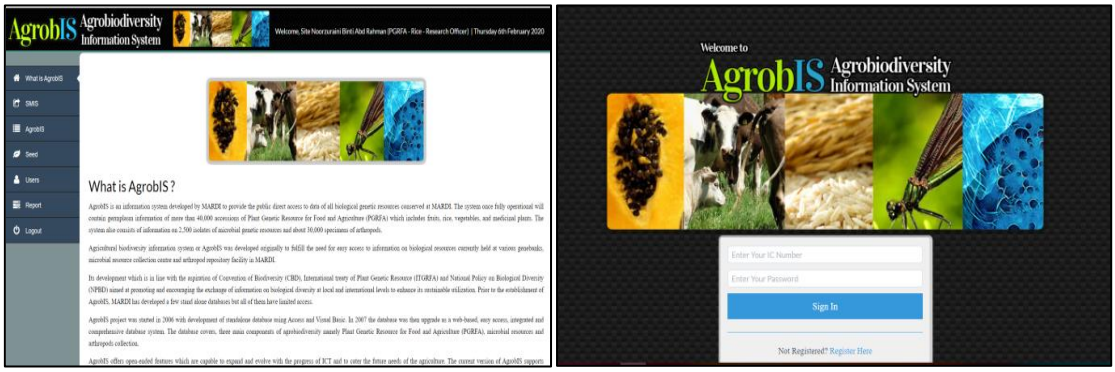


Figure 1. The overview of the Agrobiodiversity Information System (AgrobIS).

3. Building Genesys catalog of phenotypic datasets of Malaysian rice germplasm

MARDI has involved in 'Building Genesys Catalog of Phenotypic Datasets for Malaysian Rice Germplasm' as part of a larger Crop Trust project named 'A Genesys Catalog of Phenotypic Datasets Linked to Genebank Accessions'. The project started in 2017 and will end in August 2018. The objective of the project is to contribute to the building of metadata-based catalogue of accession-related phenotypic datasets on Genesys. In this project, 22 datasets of rice germplasm based on selected planting season were successfully published on Genesys (Figure 2). The datasets covered 624 rice accessions of landraces and introduced varieties for morphological characterization data (9 datasets), bacterial leaf blight (10 datasets), brown planthopper (1 dataset), foliar blast (1 dataset) and rice specialty traits (1 dataset).

The datasets for morphological characterization provide a broad spectrum of variation of morphological traits among the accessions (Table 1) which are fundamental in order to provide information for breeding program [4]. The highest coefficient of variation observed in culm number (33.11) followed with ligule length (31.98). Besides that, there are several accessions showed resistant for certain diseases. The bacterial leaf blight dataset observed 53 accessionsare resistant and 84 accessions are moderately resistant (Figure 3). Meanwhile, the foliar blast dataset observed nine accessions are highly resistant and 14 accessions are resistant to foliar blast disease (Figure 4). The rice specialty traits observed 25 accessions with low amylose content, 10 accessions with intermediate amylose content and five accessions with high amylose content (Figure 4). In Malaysia, most of the people preferred rice varieties with low to intermediate amylose content. The rice is less sticky and soft texture.

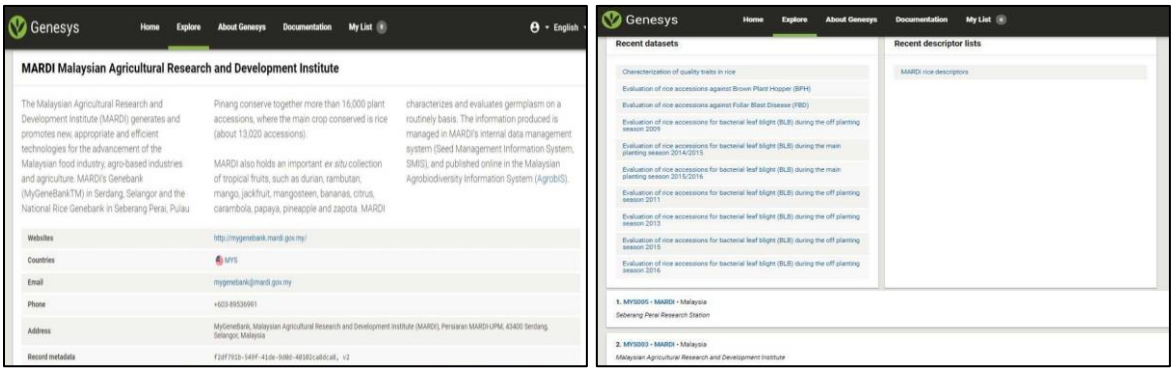


Figure 2. List of data on characterization and evaluation of Malaysian rice germplasm in the Genesys Catalog.

Table 1. The statistical analysis of selected rice germplasm in the Genesys Catalog.

Variable	N	Mean	Max	Min	Std dev	Std error	Variance	Coefficient of variation
Flag leaf length (cm)	153	34.87	73.6	15.0	9.06	0.73	82.06	25.98
Flag leaf width (mm)	153	12.83	21.8	6.2	2.62	0.21	6.84	20.38
Leaf length (cm)	143	44.65	82.6	16.2	9.95	0.83	98.95	22.28
Leaf width (mm)	143	10.81	18.8	6.0	2.21	0.18	4.88	20.44
Ligule length (mm)	142	16.27	38.7	5.2	5.20	0.44	27.08	31.98
Culm length (cm)	148	79.80	144.6	32.4	23.48	1.93	551.44	29.43
Culm number	148	14.29	32.0	5.0	4.73	0.39	22.37	33.11
Panicle length (cm)	149	24.05	34.8	9.8	3.77	0.31	14.22	15.68
Maturity	68	131.24	172.0	105.0	17.20	2.09	296.00	13.11

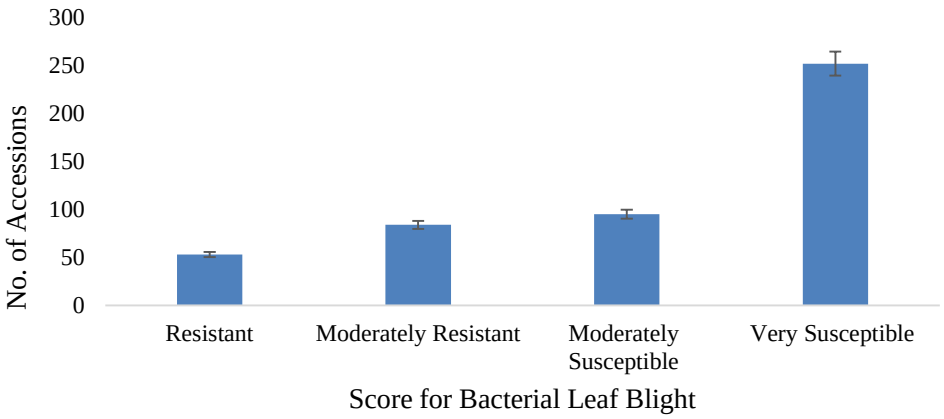


Figure 3. The score of bacterial leaf blight among the selected rice germplasm which are available in the Genesys Catalog.

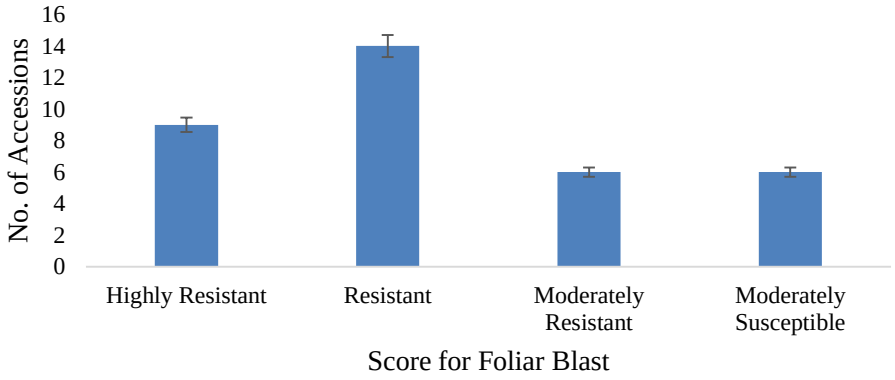


Figure 4. The score of foliar blast disease among the selected rice germplasms which are available in the Genesys Catalog.

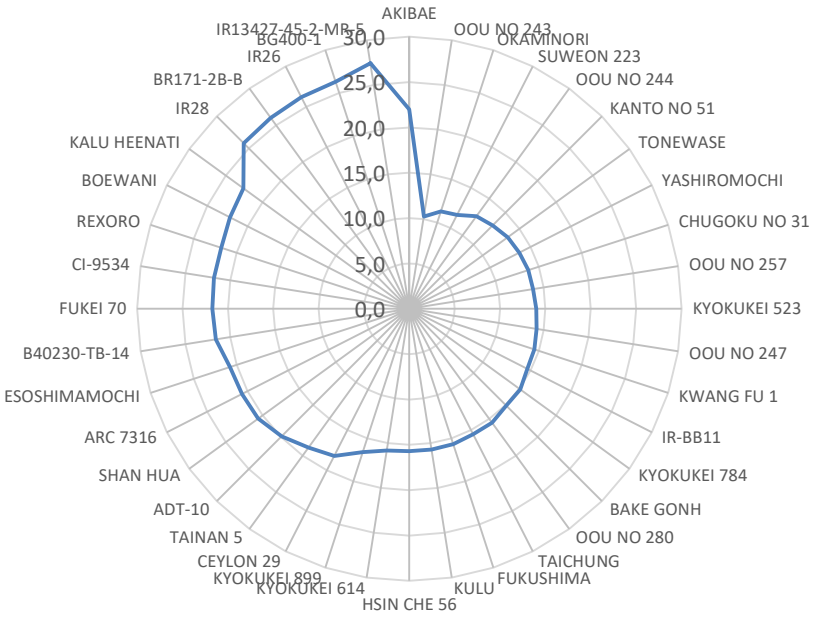


Figure 5. Data on amylose content which are available in the Genesys Catalog.

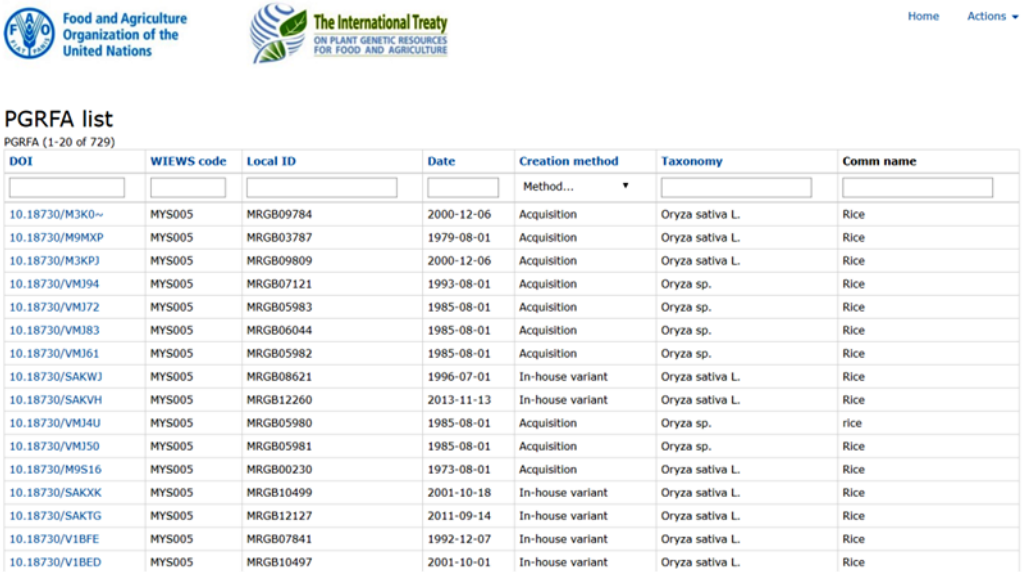
4. DOI implementation on rice germplasm in MARDI

MARDI was invited to be involved in the Project "W3B-PR-29-Indonesia: Multicountry Construction of a Test Platform for the Development and Allocation of Unique Identifiers to Rice Germplasm, linking the MLS information infrastructure and the DivSeed repository". A total of 708 accessions of Malaysian rice germplasms had been registered for DOIs (Figure 6). The accessions include 45 accessions of MARDI varieties, 591 accessions of Malaysian landraces and 72 accessions of introduced varieties from International Rice Research Institute (IRRI).

The released varieties consisted of the first variety released in 1964 until 2017. In Malaysia, the breeding program was started by the Department of Agriculture (DOA) in year 1960s. The first variety named Malinja was released in 1964 for double cropping planting season [5]. Malinja was derived from the cross between Siam 29 (landraces with photoperiod sensitivity) and Pebifun (introduced variety with non-photoperiod sensitivity). The variety has maturity days about 150 days from sowing. Mahsuri was the second released variety which was also popular during that period, but it had two serious weaknesses, i.e. susceptible to blast disease and lodging problem. Improvement was made to the variety through hybridization. Remadja and Sigadis, two varieties from Indonesia, were used as blast resistant donor varieties. Meanwhile, a few introduced varieties such as IR8, IR119 and IR2070 were used for the improvement of plant type and lodging problem in Mahsuri [5]. The last variety released in 1970s was Bahagia. The strain was selected from segregating IF5 (F4) population brought from IRRI. After four years of selection, the variety was released in 1968 [5]. Bahagia had good physical grain quality, medium height and relatively good yield.

The varietal improvement program in Malaysia has begun from the introduction of exotic elite breeding lines and varieties started with IR8. IR8 was introduced in 1966 for tropical irrigated lowlands. It was the first semi-dwarf and high yielding rice variety [6,7]. The variety increased yield potential of irrigated rice from 6 to 10 ton per ha in the tropics. In MARDI, the variety was used extensively in crosses either to improve the variety itself or to increase the yielding potential of other varieties [5]. Between 1968 and 1969, IR8 had been used in 53 crosses either with landraces, released varieties, varieties with resistance to pests and diseases or varieties with good grain quality. From

1972 to 1978, all the MARDI released varieties derived from these crosses namely were Murni, Masria, Jaya, Sri Malaysia I, Sri Malaysia II and Pulut Malaysia I.


Food and Agriculture Organization of the United Nations
The International Treaty ON PLANT GENETIC RESOURCES FOR FOOD AND AGRICULTURE
Home Actions

PGRFA list

PGRFA (1-20 of 729)

DOI	WIEWS code	Local ID	Date	Creation method	Taxonomy	Comm name
				Method...		
10.18730/M3K0~	MYS005	MRGB09784	2000-12-06	Acquisition	Oryza sativa L.	Rice
10.18730/M9MXP	MYS005	MRGB03787	1979-08-01	Acquisition	Oryza sativa L.	Rice
10.18730/M3KPJ	MYS005	MRGB09809	2000-12-06	Acquisition	Oryza sativa L.	Rice
10.18730/VMJ94	MYS005	MRGB07121	1993-08-01	Acquisition	Oryza sp.	Rice
10.18730/VMJ72	MYS005	MRGB05983	1985-08-01	Acquisition	Oryza sp.	Rice
10.18730/VMJ83	MYS005	MRGB06044	1985-08-01	Acquisition	Oryza sp.	Rice
10.18730/VMJ61	MYS005	MRGB05982	1985-08-01	Acquisition	Oryza sp.	Rice
10.18730/SAKWJ	MYS005	MRGB08621	1996-07-01	In-house variant	Oryza sativa L.	Rice
10.18730/SAKVH	MYS005	MRGB12260	2013-11-13	In-house variant	Oryza sativa L.	Rice
10.18730/VMJ4U	MYS005	MRGB05980	1985-08-01	Acquisition	Oryza sp.	rice
10.18730/VMJ50	MYS005	MRGB05981	1985-08-01	Acquisition	Oryza sp.	Rice
10.18730/M9S16	MYS005	MRGB00230	1973-08-01	Acquisition	Oryza sativa L.	Rice
10.18730/SAKXK	MYS005	MRGB10499	2001-10-18	In-house variant	Oryza sativa L.	Rice
10.18730/SAKTG	MYS005	MRGB12127	2011-09-14	In-house variant	Oryza sativa L.	Rice
10.18730/V1BFE	MYS005	MRGB07841	1992-12-07	In-house variant	Oryza sativa L.	Rice
10.18730/V1BED	MYS005	MRGB10497	2001-10-01	In-house variant	Oryza sativa L.	Rice

Figure 6. List of Malaysia rice germplasm registered for DOI in Global Information System (GLIS).

Starting in 1978, the breeding program established new objectives based on current problems occurred in rice plantation. The objectives were targeted to solve the problems on pests and diseases, problem soils, drought and semi-deep water, rainfed and regional specificity. The introduced varieties used to achieve these objectives were from India, Bangladesh, Thailand, Indonesia, Philippines (including IRRI), Japan, Korea, China and others. Many landraces were also used as donors, especially for blast resistance, rice varieties for rainfed areas, and to broaden the genetic base. The local semidwarf mutants derived from mutation technique were also used as donors of dwarf genes.

Till date, MARDI has released three white glutinous rice varieties, one black glutinous rice variety, two Clearfield rice varieties that carry the resistant gene to imidazolinone to prevent weedy rice problems, four quality rice varieties, one aerobic rice variety and the remaining were ordinary rice. The ordinary rice usually consists of high yielding traits such as many tillers, long panicle, heavy grain weight and also resistant to pests and diseases.

Figure 7. The upland and rainfed rice cultivation in Malaysia.

Besides, selected valuable rice germplasm conserved in MARDI Rice Genebank were also successfully registered for DOIs, especially the Malaysian landraces. The landraces were collected throughout the country and is maintained and conserved in seeds genebank [1]. The landraces are important as a source of new genes [1] and essential for the development of superior recombinants in crop improvement and development of new rice variety [1,8]. The landraces are divided into two groups based on planting practices namely upland (Figure 1) and rainfed lowland (Figure 2).

Upland rice can be found in some area in Peninsular Malaysia but commonly found in Sabah and Sarawak. It is usually cultivated for home consumption by rural people in the states [9]. Landraces are mostly photoperiod sensitive, lengthy maturation, tall plant types, susceptible to lodging and less responsive to fertilizer. In MARDI, landraces have been successfully used in pre-breeding to generate desirable genotypes for new rice varieties. There are several outstanding landraces used in rice improvements such as Radin Goi, Pongsu Seribu 2, Mayang Ebos, Siam 29, Pulut Sutera, Tangkai Rotan, Engkatek and Secupak. All the registered accessions for DOI are stated under Annex 1 crop. They can be accessed by the contracting parties of the Treaty for research and crop improvement to achieve global food security and promoting sustainable agriculture in each country.

5. Challenges handling the toolkit DB for DOI registration

The DOI Toolkit DB was developed using Linux and the system ran using Java application. The setup of the Toolkit required large storage capacity of the computer system up to 8,000 MB or 4 GB. Thus, only with the large storage capacity of the system may ensure the success of the installation process. The Toolkit also requires the users to be familiarized with the SQL for running the trigger for registration. The trainees during the workshop were mostly the genetic resource persons who faced problems in handling the SQL for running the triggers. Further improvement of the system with the most effortless installation process and friendly use of DOI Toolkit for registration may be required for future and global use.

6. Conclusions

The involvement and contribution of MARDI in both projects showed the commitment of MARDI for data sharing mechanism established by the Treaty. The contribution will enhance rice breeding program and sharing valuable information on rice genetic resources with other countries for future research as well as for rice varietal development to achieve global food security and for promoting sustainable agriculture locally and globally.

7. References

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Identification of soybean (*Glycine max* [L.] Merr.) mutants and improved varieties having diverse drought tolerance character using SSR marker

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Abstract. Global climate change has a huge impact on the agricultural world. The water scarcity that happened in some areas can lead to decreased food production, including soybean in Indonesia. Indonesia has a number of soybean genotypes with diverse drought tolerance which have been obtained using various approaches and could be one of the alternatives in responding to the problem. This study aimed to identify soybean mutant genotypes generated from *in vitro* selections and improved varieties using Simple Sequence Repeat (SSR) markers. A total of 10 mutant lines and 20 improved varieties from Indonesia were genotyped using 10 SSR markers adopted from literatures and ten newly designed SSR markers. The research was conducted in ICABIOGRAD molecular biology laboratory from November 2017 to February 2018. The amplicons were scored as binary data and analyzed using NTSYS and PowerMarker softwares. The new SSR markers were designed based on the variants searched from the IAARD genome database (www.genom.litbang.pertanian.go.id) and showed unambiguous amplicons. The results showed that adopted SSR markers were able to detect more alleles, a higher range of major allele frequency, gene diversity, heterozygosity and Polymorphism Information Content (PIC) compared to the newly designed markers. Phylogenetic analysis showed that all of the soybean mutants were grouped in the same cluster with the parent (Sindoro). This molecular marker-based information of soybean mutants along with the improved varieties in this study could be useful for assisting breeding strategy in screening parental lines to develop drought tolerant soybean varieties in the future.

Keywords: soybean, mutant, improved varieties, drought tolerance, SSR.

1. Introduction

Soybean is one of important crops with a nutritional role as a source of protein. Unfortunately, soybean production in Indonesia is only able to cover 30% of national consumption while the rest is fulfilled from imports [1]. The opportunity to increase national soybean production through increasing productivity and expanding the planting area gives a hope that soybean self-sufficiency can still be achieved in the future.

Water availability is one of the critical factors that influence soybean productivity. According to Taufiq and Sundari [2], excess water will cause flooding and aeration stress while lack of water will

cause drought stress. When soybean plants experience drought stress, the production will decrease dramatically, so that the risk of harvest loss is getting bigger [1].

The types of soybean varieties have an influence in determining the amount of yield losses arising from drought stress. The use of drought tolerant soybean varieties can help reduce the level of damages caused by drought stress [3]. Development of drought tolerant soybean varieties can be pursued through cross-breeding, purification of local varieties, introduction of varieties, induced mutation and production of transgenic plants. Induced mutation, both physically and chemically, aims to increase the genetic diversity in plants through changes in the composition of genetic materials, therefore, selection process can be more targeted according to the desired character [4,5]. According to Herison et al. [6], induced mutations are expected to produce mutants with potential characters that are better than their original individuals, and the enhanced mutants can be released as new improved varieties in the future.

The mutants need to be characterized to determine how far the genetic changes occur. The changes that occur due to mutations can be observed through changes in morphology, anatomy and at the level of DNA [7]. So far, the phenotype is the easiest way to characterize the plants, but this method has a considerable disadvantage that is still influenced by environmental factors and it is difficult to distinguish accessions with close genetic relationship [8]. On the contrary, the characterization using molecular markers has the advantage of being more accurate because it is not influenced by environmental factors, the time required is faster and it is even able to discriminate genetic differences between mutants and the wild type/parent cultivars [9,10]. Simple Sequence Repeat (SSR) is one of the molecular markers widely applied in analyzing plant genetic diversity, the study of phylogeny, and marker assisted selections [11]. These markers are short tandem DNA sequences that are 1−6 base pairs long and are widely distributed in the plant genome area [12]. The advantages of these markers are that they are codominant, have high level polymorphism and reproducibility, and the Polymerase Chain Reaction (PCR) based application is easy [13,14]. The objective of this study was to identify soybean mutant genotypes from *in vitro* selection and improved varieties that have diverse drought tolerance character using SSR markers. The molecular information from this study could be useful for assisting breeding strategy in screening parental lines to develop drought tolerant soybean varieties in the future.

2. Materials and methods

2.1. Genetic materials

The mutant lines were derived from Sindoro variety irradiated using several dossages of gamma ray. The mutants were selected *in vitro* using media containing polyethylene glycol (PEG). The plantlets then were acclimatized and planted in ICABIOGRAD greenhouse in Bogor and the leaves were harvested for DNA extraction. Meanwhile, the DNA from twenty national varieties were extracted directly from the seeds. Molecular analysis was conducted from November 2017 to February 2018 at Molecular Biology Laboratory, ICABIOGRAD.

2.2. Genomic DNA extraction

DNA was extracted using the Doyle and Doyle [17] method with some modifications. A total of 0.5 grams of leaf pieces or one soybean seed were ground in a mortar containing 500 µl extraction buffer (100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA pH 8.0, 2% (w/v) cetyltrimethylammonium bromide (CTAB), 2% (w/v) polyvinylpyrrolidone (PVP) and 0.38% (w/v) sodium disulphite. The mixture was then put in a 2 ml microtube, followed by the addition of more extraction buffer until the volume reached 1 ml. Subsequently, two µl of β-mercaptoethanol were added per sample followed by incubation at 65°C for 15 minutes. Next, 800 µl of chloroform:isoamyl alcohol solution (24:1) was added to each sample and was then centrifuged at a speed of 12,000 rpm for 10 minutes at 20°C. The supernatant was transferred to the new microtube. Furthermore, 3 M sodium acetate pH 5.2 was added at 1/10 of supernatant volume and followed by the addition of cold isopropanol at one supernatant volume. The mixture was incubated at -20°C for one hour. After that, the mixture was centrifuged at a

speed of 12,000 rpm for 10 minutes at 20°C. The DNA pellet was then washed using 70% ethanol and dried using DNA Speedvac Concentrator (Thermo Scientific, USA). The dry pellet was dissolved in 100 µl TE solution (10 mM Tris pH 8.0 and 1 mM EDTA) and diluted to 10 ng/µl for a good amplification in PCR.

Table 1. The list of soybean genotypes used in this study.

Genotype	Information	Drought resistance status
M#1 to M#10	Mutant	Tolerant
Baluran	Improved varieties	Susceptible
Jaya Wijaya	Improved varieties	Moderate
Bromo	Improved varieties	Moderate
Krakatau	Improved varieties	Moderate
Tidar	Improved varieties	Moderate
Dering 1	Improved varieties	Tolerant
Leuser	Improved varieties	Moderate
Orba	Improved varieties	Moderate
Anjasmoro	Improved varieties	Susceptible
Slamet	Improved varieties	Moderate
Grobogan	Improved varieties	Moderate
Sindoro	Improved varieties	Susceptible
Kaba	Improved varieties	Moderate
Tanggamus	Improved varieties	Moderate
Wilis	Improved varieties	Susceptible
Cikuray	Improved varieties	Moderate
Galunggung	Improved varieties	Susceptible
Dieng	Improved varieties	Tolerant
Kawi	Improved varieties	Moderate
Lumut	Improved varieties	Tolerant

2.3. Development of SSR primers

Ten primers adopted from Cregan et al. [19] were used in this study (Table 2). We also used ten newly designed SSR primers that were obtained by filtering of variants based on the alignment of five soybean genotypes including Tambora, Grobogan, B3293, Malabar and Davros with reference sequence from Williams 82 variety [20]. From the total sequences, 100% putative SSR primers were retrieved and their motives ranged from di-nucleotide to hexa-nucleotide motives. The chosen sequences with SSR motifs were of good quality for designing SSR primer using BatchPrimer3 program [18]. The sequences of these newly designed primers have been uploaded in IAARD genome database (www.genom.litbang.pertanian.go.id).

2.4. DNA amplification

Each sample was amplified in a total reaction of 10 µl containing 10 ng DNA, 2× MyTaq HS (Bioline, UK) at up to 5 µl, 0.5 µl forward and 0.5 µl reverse primers with a concentration of 10 µM, and sterile $_{dd}H_2O$. Amplification was carried out using 20 pairs of SSR markers derived from previously

mentioned reference and the newly developed primers (Table 2). PCR reaction was carried out in T1 Thermocycler (Biometra, Germany) machine with the following PCR profile: initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 52°C for 1 min and extension at 72°C for 1 min. The PCR reaction was ended with the final extension step at 60°C for 15 min. The PCR results were run on 8% polyacrylamide gel containing 1× TBE buffer with a voltage of 90 V for 2 hours. The polyacrylamide gel was then stained using ethidium bromide and visualized under UV light using a UV Transilluminator (Bio-Rad, USA).

Table 2. The list of SSR markers used in this study.

Marker	Sequence (5'– 3')	Chromosome	References
Satt009	F: CCAACTTGAAATTACTAGAGAAAT R: CTTACTAGCGTATTAACCCTTG	3	[19]
Satt030	F: AAAAAGTGAACCAAGCC R: TCTTAAATCTTATGTTGATGC	13	[19]
Satt147	F: CCATCCCTTCCTCCAAATAGAT R: CTTCCACACCCTAGTTTAGTGACAA	1	[19]
Satt308	F: GCGTTAAGGTTGGCAGGGTGGAAGTG R: GCGCAGCTTTATACAAAAATCAACAA	7	[19]
Satt197	F: CACTGCTTTTTCCCCTCTCT R: AAGATACCCCCAACATTATTTGTAA	11	[19]
Satt191	F: CGCGATCATGTCTCTG R: GGGAGTTGGTGTTTTCTTGTG	18	[19]
Satt463	F: TTGGATCTCATATTCAAACTTTCAAG R: CTGCAAATTTGATGCACATGTGTCTA	7	[19]
Satt431	F: GCGTGGCACCCTTGATAAATAA R: GCGCACGAAAGTTTTTCTGTAACA	16	[19]
Satt045	F: TGGTTTCTACTTTCTATAATTATTT R: ATGCCTCTCCCTCCT	15	[19]
Satt294	F: GCGGGTCAAATGCAAATTATTTTT R: GCGCTCAGTGTGAAAGTTGTTTCTAT	4	[19]
SoySSR1.1	F: GACATTGGAGGACATGTAGAC R: GTCTTTACTGCAATGCAACTC	1	Newly designed
SoySSR2.1	F: TAACCTGCAAATGGTCAACTA R: TGAGAAGATCACAACGGATAC	2	Newly designed
SoySSR3.1	F: TAAATTTGGATCAGATGCTGT R: GTCCAATCAAGAAAACAGTTG	3	Newly designed
SoySSR4.1	F: CTGTTGAAGTGTAATCGTTAAAA R: TTGCCGTTGATATAATCCTTA	4	Newly designed
SoySSR5.1	F: ATGAAGCCCAGAGAGTACAAC R: CCCAAGTCTGAACATTACTCA	5	Newly designed
SoySSR6.1	F: AACCACCTTGGTATTTCACTT R: TAACTGCCCAGAATTAGTTGA	6	Newly designed
SoySSR7.1	F: CAACAACCATGTCACTATACG R: AGGATTTCTTTGGAGATTGG	7	Newly designed
SoySSR8.1	F: CTCAACAACAACAACAAC R: GAATGTGTCATGCAAATACAA	8	Newly designed
SoySSR9.1	F: GGAAGATTGATTCAAAAGTCA R: CGAGAAAGTGATTGTGAGAA	9	Newly designed
SoySSR10.1	F: ATCAAACCCGAACTTATTCTT R: GGTCCCTAAGAAAGGAGATTA	10	Newly designed

2.5. SSR data analysis

Amplicons of each primer on all individual sample were scored as binary data and SSR allelic size was determined using GelAnalyzer software [20]. The binary data then were analyzed using the

Unweighted Pair-Group Method with Arithmetic-Sequential Agglomerative Hierarchical and Nested (UPGMA-SAHN) program on NTSYS version 2.1 [21]. The genetic similarity value between soybean genotypes was calculated based on the Simple Matching (SM) coefficient using SIMQUAL subprograms. Furthermore, the scoring alleles were also analyzed using PowerMarker version 3.25 [19] to determine the major allele frequency values, genetic diversity, heterozygosity and Polymorphic Information Content (PIC) produced by the markers.

3. Results and discussion

3.1. Markers polymorphism analysis

The polymorphism analysis showed that the newly designed soybean markers have a lower number of alleles than the adopted markers from [16] (Table 3 and 4). In addition, the newly designed markers also have lower major allele frequency and genetic diversity values than the adopted markers (Figure 1). Among all markers being used, nine adopted markers had heterozygous alleles in the soybean genotype with the range of 0.55 to 1. On the other hand, there were only two newly designed markers which had heterozygous alleles, such as SoySSR5.1 and SoySSR10.1. There were nine adopted markers with PIC>0.7, which according to the criteria of Hildebrand et al. [24], are an informative marker. In contrast, there were only four newly designed markers with PIC>0.7.

Table 3. Polymorphisms statistics of adopted SSR markers.

Marker	Allele number	Allele size range (bp)	Major allele frequency	Gen diversity (He)	Heterozigosity (Ho)	PIC
Satt009	8	144–219	0.42	0.75	0.80	0.71
Satt030	9	169–215	0.28	0.81	0.93	0.79
Satt147	10	184–235	0.20	0.86	0.97	0.84
Satt308	9	126–201	0.18	0.86	0.87	0.84
Satt197	13	149–240	0.33	0.80	0.90	0.78
Satt191	12	185–268	0.18	0.87	1.00	0.86
Satt463	8	123–161	0.40	0.78	0.55	0.76
Satt431	11	174–253	0.18	0.86	0.60	0.85
Satt045	8	148–182	0.33	0.77	0.77	0.74
Satt294	3	237–246	0.60	0.55	0	0.48
Sum	91					
Average	9.1		0.31	0.79	0.74	0.77

The adopted markers from Cregan et al. [19] were designed based on the mapping of 606 SSR loci on three mapping populations: USDA/Iowa State *G. max* × *G. soja* F_2 population, the University of Utah Minsoy × Noir 1 recombinant inbred population, and the University of Nebraska Clark × Harosoy F_2 population. They have been used in several previous studies as reported by Yani [25], Safina [26] and Tasma et al. [27] and demonstrated high polymorphism level. On the contrary, the newly designed markers were designed by randomly selecting several of the SSR loci identified from the five soybean genotypes which may share high similarity in SSR. In addition, these newly designed primers had never been used in previous studies to confirm their polymorphism in Indonesian soybean genotypes. Therefore, in the future it is necessary to design more new markers using other SSR loci with higher level of polymorphisms.

Table 4. Polymorphisms statistics of newly designed SSR markers.

Marker	Allele number	Allele size range (bp)	Major allele frequency	Gen diversity (He)	Heterozigosity (Ho)	PIC
SoySSR1.1	6	253–274	0.33	0.76	0	0.72
SoySSR2.1	3	237–249	0.43	0.61	0	0.52
SoySSR3.1	4	239–255	0.37	0.73	0	0.68
SoySSR4.1	3	219–229	0.50	0.60	0	0.52
SoySSR5.1	3	152–181	0.50	0.62	1.00	0.54
SoySSR6.1	5	253–272	0.27	0.78	0	0.74
SoySSR7.1	8	244–280	0.30	0.81	0	0.78
SoySSR8.1	11	259–323	0.17	0.88	0	0.87
SoySSR9.1	5	199–225	0.34	0.74	0	0.69
SoySSR10.1	4	242–282	0.58	0.57	0.07	0.51
Sum	52					
Average	5.2		0.38	0.71	0.11	0.66

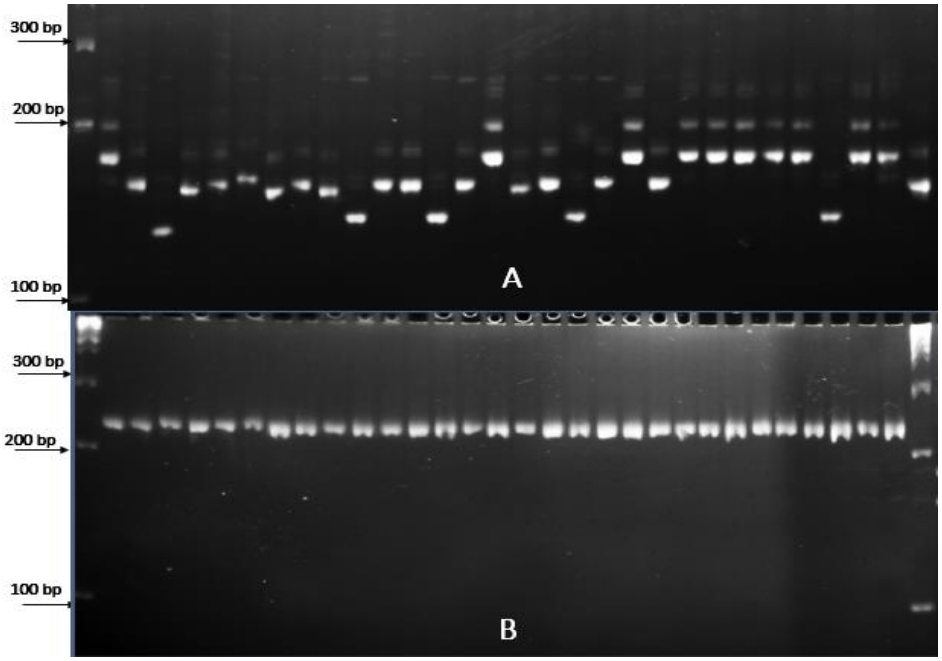

Figure 1. The electrophoresis results of soybean samples that were amplified using SSR markers. A = Satt 308, B = SoySSR4.1.

3.2. Phylogenetic analysis and the potential development of mutant lines

The phylogenetic analysis showed that the thirty soybean genotypes separated into two main clusters at the genetic similarity coefficient of 0.71 (Figure 2). The first cluster is divided into two subclusters, namely subcluster IA and IB. The subcluster IA consisted of 14 genotypes and subcluster IB consisted of 13 genotypes. Meanwhile, the second cluster consisted of the remaining three genotypes, namely Leuser, Anjasmoro and Galunggung.

All mutant lines were grouped in subcluster IA together with the native variety Sindoro, except mutant line M#1 that was grouped in subcluster IB. Overall, the classification of soybean genotypes in this study was not based on the character of drought tolerance resistance, but it was more based on

their phylogenetic relationship. The results of the genetic similarity matrix showed that there were two genotypes (M#4 and M#5) with very close relationship with genetic similarity values of 88.1%. In addition, there were two genotypes (Anjasmoro and Dieng varieties) with the farthest relationship with genetic similarity value of 62.7% (data not shown).

After several tests in the greenhouse, the drought tolerant mutant lines used in this study have the potential to be developed either to be released as new improved varieties or used as parents in cross-breeding (data not shown). Among the ten mutant lines, there were three lines that still had the highest genetic similarity to Sindoro variety, namely M#2, M#3 and M#6 with a value of 79% (data not shown). We assumed that the high value of genetic similarity to the parental variety probably indicates that the mutation process has not been entirely successful in changing the composition of genetic materials in those lines. According to Widiastuti et al. [7], each individual plant has a different response to radioactivity, and certain radioactivity doses could induce mutations strongly in certain individuals but not necessarily capable of inducing mutations in other individuals. In addition, mutations often happen randomly and which part of the chromosome will be mutated could not be predicted. According to Walling et al. [28], soybeans have a total of 20 pairs of chromosomes, while the SSR markers used in this study did not fully represent all chromosomes in soybeans.

M#1 mutant line had the farthest genetic distance from the parent, Sindoro. In the dendrogram, this line was grouped in subcluster IB, separated from Sindoro in subcluster IA (Figure 2). The M#1 grouped with Dering 1 variety with the genetic similarity of 87.4%. Dering 1 is a national soybean variety with drought tolerant character during the reproductive phase, and this variety is still tolerant until the water content reaches 30% [29]. In the future, the M#1 line could be a potential new drought tolerant soybean variety after multiple trials in the fields with drought phase and genetic stability tests.

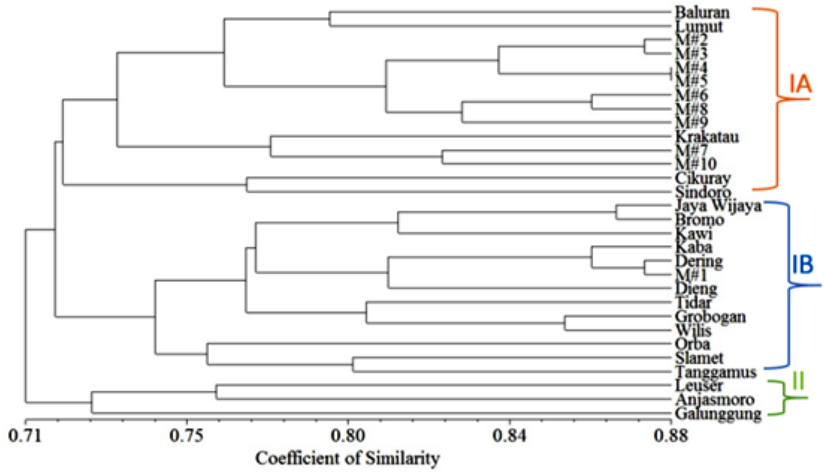

Figure 2. Dendrogram of 30 soybean genotypes based on UPGMA-SAHN program in NTSYS.

Besides being potentially released as an improved variety, the mutant lines used in this study also could be used as parental lines in crossing activities. By using Dering 1 variety as a donor character of drought resistance, the M#9 line which has a genetic distance of 37% to Dering 1 has the greatest potential to be crossed. According to Izzah and Reflinur [30], the genotypes with large genetic distance can be used as parental candidates for cross breeding to produce progeny with added value or heterosis effect from each parent. In contrast, the genotypes with close genetic distance should not be used as parental in cross-breeding to prevent the occurrence of inbreeding depression. Inbreeding depression, the opposite of heterosis, is the decreased progeny vigour due to the increased homozygosity level as a result of crosses between two individuals with close genetic relationship [29]. Inbreeding depression in plants will cause the plants to be stressed, which is identified by the decrease of plant height, less vigor, sensitive to pest and disease attack, decrease of fruit number and increased

fruit abortion, and the appearance of various unwanted characters due to the combination of recessive alleles [31]. Overall, this study results suggested the possibility to select parental lines either of mutant lines or improved varieties for heterotic crosses in breeding program using molecular markers.

4. Conclusions

Molecular identification of the soybean genotypes based on twenty SSR markers separated thirty genotypes into two clusters at genetic similarity of 0.71. All of the mutant lines were grouped in the first cluster, together with the parental variety Sindoro. From the 10 mutant lines, only M#1 showed a close genetic relationship with Dering1 variety. This mutant has a potential to be developed as a new variety with drought tolerance character. Nine markers adopted from previous studies and four newly developed markers had PIC>0.7, indicating as informative markers which could be applied to distinguish soybean varieties/germplasm in the future.

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Yield stability and agronomical performance of inland swamp rice lines developed using molecular breeding

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Abstract. Yield stability of rice varieties in diverse locations is an important aspect in a varietal recommendation to ensure better economic benefits for farmers. Multi-location trials in different locations or seasons will identify consistently high-performing varieties. This study aimed to test the yield stability of selected promising lines previously developed by means of Marker-assisted Breeding. Field trials were conducted in three different types of swampland locations: Lampung, South Kalimantan and Riau. The experiments were carried out in a randomized complete block design with three replications. A total of 15 selected rice lines were evaluated. Seedlings were transplanted to 2 m × 5 m plots with 20 cm × 20 cm planting distance. Yield stability was estimated using various analyses that measure coefficient of variability (Cvi), coefficient of regression (bi), deviation parameter (δ2), coefficient determination (Ri2) and AMMI biplot. Combined analysis of variance showed that line effect (G), environmental effect (E), and G × E interaction were statistically significant different. Using CVi25% and bi<1 threshold followed by AMMI biplot analyses, four lines (line number 1, 4, 6 and 11) were classified as having broad stability. Five lines (line number 5, 12, 13, 14 and 15) showed less yield stability but had better adaptation in tidal swamp environment. The other two lines (line number 3 and 7) showed a greater yield in the coastal swamp area, suggesting their potential as adapted lines suitable to this swampy environment.

Keywords: inland swamp rice lines, molecular breeding, yield stability.

1. Introduction

Indonesia has vast arable lands, especially outside of Java Island, which could play major roles in supporting food crops production. There are around 33.4 million hectares of swampy areas distributed in Sumatra, Kalimantan, Papua and Sulawesi Islands. However, only 1.3 million hectares have been reclaimed by the government and they are mostly located in Sumatra and Kalimantan. Swampy areas also have various rice production constraints, including flash flooding, stagnant flooding, acid sulfate soils, pests, diseases and weeds. Droughts also often occur in the upper portion of swamps. Swamplands are rich in acidic soils that contain a high concentration of iron that can cause iron toxicity. In Indonesia, 2.27 million ha of rice are cultivated in peatlands, tidal swampy lands, red-yellow podzolic soils, permanently flooded lowlands, poor drainage lowlands and new wetland fields, which are characterized by high iron content [1,2]. These type of lands spread throughout the Indonesian archipelago [1,2].

One of the approaches to get promising lines is testing the performance of selected lines in widely different agro-ecological conditions to evaluate their adaptability. Selected rice lines which show low variation in diverse conditions are likely to have broad adaptability. On the other hand, lines with varied responses to diverse environmental conditions may have specific adaptation to specific environments, which can be optimized to obtain maximum yield potential and yield component [3].

Genotypes tested in different locations and different years may have significant fluctuations in yield due to variations in soil fertility, unpredictable rainfall and the presence of other biotic and abiotic stresses. Differential responses of genotypes to different environmental conditions are termed as Genotype by Environment Interaction (GEI). It reduces progress in plant breeding programs since it obfuscates the association between phenotype and genotype value [4,5]. However, GEI can be exploited by selecting superior genotypes for each specific target environment, or avoided by selecting widely adapted and stable genotypes across a wide range of environments [6].

GEI can be quantified using several procedures that are based on evaluation of genotypes under multiple environments. These methods are classified into univariate and multivariate stability statistics [7]. The most widely used ones are univariate methods, which are based on regression of the mean value of each genotype on the environmental index or marginal means of environments [8,9]. Multivariate analysis is an alternative and complementary method for evaluating genotype stability. Additive Main Effects and Multiplicative Interaction (AMMI) model is also widely used for studying GEI [10].

To support national rice production and food security, extensification strategy at sub-optimal marginal land (swampland) is one of the approaches that can be attempted. Tolerance to Fe toxicity and adaptability in swampy environment is one of the keys to successful expansion to such land to increase rice production. Through molecular breeding approach, ICABIOGRAD has developed promising rice lines for swampy area. These lines were selected using molecular markers based on targeted genes which are known to contribute to Fe toxicity tolerance, such as *IRT1* (iron regulation transporter) gene that plays a role in strategy I of the Fe tolerance mechanisms [11–13], *NAS* (nicotianamine synthase) gene, which is active in long-distance transport of iron [12,14] and *FRO* (Fe^{3+} chelate reductase) gene, which converts insoluble Fe^{3+} to soluble Fe^{2+} [12,15,16].

The phenotypic stability of a variety is influenced by its ability to respond to diverse environments. Thus, it is important to perform yield stability test of rice lines to select the ones which have the most stable yield in different locations. The objective of this study was to determine the stability of yield traits of promising inland swamp rice lines across three different locations and planting seasons. The output of this research is selected rice lines with wide adaptation and/or specific adaptation to swampy environment.

2. Materials and methods

2.1. Planting materials, experimental design and test locations

A total of 15 selected rice lines and two check varieties (Table 1) were evaluated from 2015 to 2017 at three locations consisting of three environments: swampy coastal land (Taman Bogo, Lampung), type

B tidal swampy land (KP Belandean, South Kalimantan) and type C tidal swampy land (KP Siak, Riau).

Table 1. List of genotypes used in the study.

Lines	Pedigree	Code
B14301E-KA-17-a	Kao Daok Mali-105-9/B13143-8-MR-3-KA-14//Inpara 5	B1-17a
B14308E-KA-38	Setail/Inpara 2//Code	B2-38
B14315E-KA-1	B11844-MR-29-7-1/Inpara 3//Cisantana	B3-1
B14316E-KA-4	B11844-MR-29-7-1/Inpara 5//Code	B4-4
B14339E-KA-14	IR42/Ciherang	B5-14
B14354E-KA-4	Banyuasin/Ketankutuk	B6-4
B14357E-KA-4-b	Siakraya/B13132-7-MR-1-KA-6	B7-4-b
B13925E-KA-1-a	Swarna Sub-1/Mekongga	B8-1-a
B13926E-KA-29-a	Swarna Sub-1/Ciherang	B9-29-a
B13957E-KA-50	Batanghari/Conde	B10-50
B13983E-KA-44	Inpari 9/Swarna Sub1	B11-44
B13988E-KA-40	Cimelati/Inpara 3//Inpari 9/FR13A	B12-40
IR70213-10-CPA-2-UBN-B-1-1-3	Mekongga/Inpara 3//Mekongga/Inpara 3	I13-3
B13522E-KA-5-B	Kebo/BR11 Sub-1	B14-5-B
B13545E-KA-1-B	Ciherang/Swarna Sub-1//Ciherang///Inpara 3	B-15-1-B
IR64	Sensitive Control	IR64
Mahsuri	Tolerant Control	Mhs

The locations where the trials were conducted differ in soil type, altitude, temperature and rainfall received per annum (Table 2). At each location, the trial was laid out in a randomized complete block design (RCBD) with three replications. Each plot had six rows that were 5 m long with 0.2 m spacing between rows. Fertilizer (urea and DAP) was applied as per the recommendation for each respective location. All of the DAP was applied at planting time while one-third of urea was applied at planting, one-third at tillering and the remaining one-third at panicle initiation. A seed rate of 60 kg/ha was used, and seeds were directly drilled in a row. Plantings were done in the main rainy season following the optimal dates in each respective location.

Table 2. Description of the experimental locations.

Location	Elevation (m asl)	Latitude	Longitude	Annual rainfall (mm)	Average temperature (°C)	Swampy type
Taman Bogo, Lampung	30	$50°58^{1}01^{11}$ S	$105°30^{1}44^{11}$ E	2,143	26.9	Coastal
KP Siak, Riau	10	$100°03^{1}$ N	104° 0° E	2,000	25.9	Tidal, type C
KP Belandean, South Kalimantan	31	$4°10^{1}14^{11}$ S	$57°19^{1}13^{11}$ E	1,796.9	27	Tidal, type B

2.2. Data collection and statistical analysis

Data were collected for the following traits: days to heading, days to maturity, panicle length, plant height, filled grains/panicle, fertile tillers/plant, grain yield and 1,000-seed weight. Grain yield (t/ha)

was estimated based on extrapolation at 14% moisture level on the basis of four central harvestable rows. The grain yield and other agronomic parameters were subjected to analysis of variance using the SAS version 8.1 software. The stability of grain yield data was also analyzed using several methods described by Finlay and Wilkinson [8], Eberhart and Russell [9], Francis and Kannenberg [17] as well as AMMI analysis.

3. Results and discussion

3.1. Soil chemical compositions of the field experiments

The results of soil analysis (Table 3) showed that among the three locations, the swampland in South Kalimantan had the highest soil acidity (pH 3.8). The other areas in Lampung and Riau had lower soil acidity: pH 4.0 and 4.6, respectively. Taman Bogo swampland (Lampung) also suffered from iron toxicity. As a comparison, normal upland soil in a nearby area had pH 5.4.

Table 3. Soil chemical status in three locations of swampy lowland area at dry season (DS) and wet season (WS) in 2015–2017.

Description	Taman Bogo, Lampung 2015 (DS and WS)		KP Belandean, South Kalimantan 2016 (WS)	KP Siak, Riau 2017 (DS)
	Normal site	Iron toxicity site	Iron toxicity site	Iron toxicity site
pH (H_2O)	5.4	4.0	3.8	4.6
C organic (%)	1.14	1.1	5.1	2.3
N total (%)	0.09	0.09	0.25	0.13
C/N	12.7	11	-	17
P_2PO_5 Bray 1 (ppm)	10.5	6.8	12.8	12.47
K_2O Morgan (ppm)	30	27.7	8.0	77.67
Exchangeable base (me/100 g)				
P	0.32	0.31	-	0.22
K	0.02	0.02	0.034	0.57
Ca	0.03	0.01	1.50	0.05
Mg	0.04	0.02	1.37	0.28
Na	0.01	0.01	0.62	0.03
Fe (ppm)	765	2,030	631	334.38
Pyrite as total Fe & S (%)	0.01	0.02	4.37	0.02
Texture (%)				
Clay	29	18	69	-
Silt	33	38	31	-
Sand	39	44	0	-

3.2. Analysis of variance and agronomic performance

The variance analysis for plant height, fertile tillers/plant, days to flowering, number of filled grain and grain yield (t/ha) showed significant differences ($P \leq 0.01$) attributed to the effects of genotypes and locations (Table 4). The G × E interaction effect was also significant ($P \leq 0.01$) in all characters except for plant height. Significant effect of G × E interaction indicates that the influence of location on traits observed on each genotype in different environments is different. In particular, the number of fertile tillers per plant, days to flowering, number of filled grain and the average of grain yield (t/ha, converted from 4 m × 5 m plots) were the characters that were significantly influenced by different environments ($P \leq 5\%$).

Table 4. ANOVA for plant height, fertile tillers/plant, days to flowering, number of filled grain, and grain yield of 15 genotypes of promising rice lines evaluated at three locations of swampland inTaman Bogo (Lampung), KP Belandean (South Kalimantan) and KP Siak (Riau) in 2015–2017.

Source	df	*F* value	df	*F* value	df	*F* value	df	*F* value	df	*F* value
L	2	181.44**	2	12.27**	2	224.5**	2	101.9**	2	1.021**
Rep*L	6	0.78ns	6	11.6**	6	1.39ns	6	2.41*	6	1.05ns
G	16	3.49**	16	1.65**	16	4.68**	16	1.62**	16	1.18**
G*L	32	5.68	32	1.90**	32	2.89**	32	1.93**	32	3.80**
cv		8.04%		10.42%		4.23%		13.51%		14.70%

L = location, Rep = replicate, G = genotype.

The yield performance of the tested lines varied from 4.16 (B6-4 line) to 5.7 (B12-40 line) t/ha. There are six lines with the average grain yield of more than 5 t/ha (Table 5).

Table 5. Combined observations of trait performance of 15 genotypes of promising rice lines evaluated at three locations of swampland in 2015–2017[a].

Genotype	PH[b]	FT/P[c]	DtF[d]	FG[e]	AvrGY (t/ha)[f]
B1-17a	103.22a-e	13.89a	89.78bc	91.23ab	4.33ab
B2-38	97.86b-e	11.93ab	83.33cd	101.64a	4.22bc
B3-1	108.58abc	12.93ab	81.89d	85.18ab	4.64ac
B4-4	99.02a-e	15.24a	89.11bc	83.53ab	5.40abc
B5-14	88.16de	9.98b	83.11cd	99.23a	5.51abc
B6-4	97.96b-e	12.6ab	88.44bcd	85.93ab	4.16bc
B7-4-b	94.13b-e	12.42ab	88.22bcd	89.89ab	5.07abc
B8-1-a	94.42b-e	12.47ab	93.44b	105.33a	4.72ac
B9-29-a	108.27abc	14.22a	93.56b	105.06a	4.44ab
B10-50	109.52abc	14.91a	91.06b	94.88a	4.52ac
B11-44	101.9a-e	12.38ab	93.89b	83.84ab	4.59ac
B12-40	106.26a-d	11.93ab	88.67bcd	109.66a	5.7abc
I13-3	111.14ab	14.36a	94.67ab	100.32a	5.32abc
B14-5-B	108.77abc	11.6ab	95.11ab	84.02ab	4.87ac
B-15-1-B	92.26cde	13.16ab	90.89b	87.96ab	5.07abc

[a] Location and planting seasons (PS): Taman Bogo (Lampung) at PS II 2015 until PS I 2016, KP Belandean (South Kalimantan) at PS II 2016–2017 and KP Siak (Riau) at PS II 2016–2017.
[b] PH = plant height.
[c] FT/P = number of fertile tillers per plant.
[d] DtF = days to flowering.
[e] FG = number of filled grain.
[f] AvrGY = average grain yield (t/ha, converted from 4 m × 5 m plots).

3.3. Stability analysis of grain yield performance

Stability parameter tests from the three locations were presented in Table 6. Analyses were done based on Lin et al. [18] concept, who stated that a genotype is classified as stable if: (1) its variance between environments is small; (2) its response to the environment is proportional to the mean of all tested

genotype's responses; (3) the residual mean square from the regression model for environmental index is small. The parameters were quantified using various approaches described in the following sub-sub-sections.

3.3.1. Finlay and Wilkinson analysis. In this method, stability was analyzed based on the regression coefficient (bi) between the mean of a genotype compared to the general mean of all genotypes tested in all environments. This method can explain the stability and adaptability of each genotype. Finlay and Wilkinson [8] used the bi values to classify stability standards into three groups: (1) if bi>1, stability is lower than the mean; (2) if bi = 1, stability is the same with the mean; (3) if bi<1, stability is higher than the mean.

3.3.2. Francis and Kannenberg analysis. Stability analysis based on this method used the diversity coefficient (CVi) of each tested genotype as a standard [17]. The smaller the value of CVi coefficient, the more stable the genotype. The value of CVi can be divided into four groups: (1) low (CVi<25%), (2) slightly low (CVi = 25–50%), (3) slightly high (CVi = 50–70%) and (4) high (CVi = 75–100%). Based on those categories, there were eight lines that had CVi<25%, namely: B1-17-a, B2-38, B3-1, B4-4, B6-4, B7-4-b, B10-50, and B11-44.

Table 6. Yield stability parameters of promising swampland rice lines.

Genotype	Taman Bogo, Lampung	KP Belandean, South Kalimantan	KP Siak, Riau	Yi[a]	CVi[b]	bi[c]	δ2[d]	Ri[e]
B1-17-a	3.89	3.89	5.20	4.32	17.52 (S)	0.60 (S)	0.209	0.535
B2-38	3.87	4.20	4.60	4.22	8.62 (S)	0.32 (S)	0.037 (S)	0.999 (S)
B3-1	4.81	3.71	5.40	4.64	18.52 (S)	0.31 (S)	0.033	0.150
B4-4	4.59	5.62	6.00	5.4	13.54 (S)	0.61 (S)	0.224	0.665
B5-14	3.73	6.07	6.73	5.51	28.63	1.30	0.341	0.289
B6-4	3.81	3.59	5.07	4.15	19.2 (S)	0.58 (S)	0.189	0.406
B7-4-b	4.80	5.22	5.20	5.07	4.68 (S)	0.17 (S)	0.012 (S)	0.796 (S)
B8-1-a	2.48	4.68	7.00	4.72	47.88	2.00	0.003 (S)	0.841 (S)
B9-29-a	2.91	4.09	6.33	4.44	39.17	1.53	0.097	0.722
B10-50	3.31	5.46	4.80	4.52	24.36 (S)	0.61 (S)	0.224	0.117
B11-44	3.95	4.02	5.80	4.59	22.83 (S)	0.85 (S)	0.622	0.424
B12-40	2.99	6.71	7.40	5.7	41.62	1.90	0.007	0.064
I13-3	3.65	5.44	6.87	5.32	30.31	1.42	0.190	0.704
B14-5-B	3.05	5.04	6.53	4.87	35.86	1.53	0.097	0.639
B15-1-B	3.37	5.30	6.53	5.07	31.45	1.39	0.223	0.579

[a] Yi = yield predicted based on Finlay-Wilkinson stability.
[b] CVi = genetic diversity coefficient [17].
[c] S = yield is stable (<25%).
[d] bi = genotype regression coefficient [8], scored as S if bi<1.
[e] δ2 = deviation parameters.
[f] Ri = determination coefficient, scored as S if δ2 small and R1 approaching 1.

3.3.3. Eberhart and Russell analysis. In this method, the analysis was based on the deviation from regression of the average of each genotype on different environment index [9]. A genotype is stable if the residual of mean squares from their line regression is small. Stability parameters were determined

based on the value of the deviation (δ_2) and the coefficients determination (R1) of each genotype. The stability of a genotype is verified if it has small δ_2 value and high R_1 value (approaching 1).

3.3.4. Stability analysis using AMMI model. Combined analysis of variance for grain yield (GY) showed that the effects of genotype, environments and their interaction (G × E) were significant (Table 7). Significant effect of G × E interactions could be further analyzed with biplot analysis to figure out the interaction patterns between genotypes and environments using AMMI model.

Table 7. Combined ANOVA of grain yield in three environments.

Source	Df	Sum Sq	Mean Sq	*F* value	Pr (>*F*)
Environment	2	129.93	64.96	12.28	0.008
Rep(Env)	6	31.74561563	5.291	3.006	0.0098
Genotype	16	41.46792582	2.592	1.473	0.00126
G × E	32	71.00774979	2.22	1.261	0.00194
PC1	17	48.290547	2.841	1.61	0.0076
PC2	15	22.717203	1.514	0.86	0.0061
Residuals	96	168.96	1.760		

AMMI method is a popular extension of ANOVA for studying G × E interaction [10]. This method extracts genotype and environment main effects and uses interaction of principal components to explain patterns in G × E or residual matrix, which provides a multiplicative model [19]. The AMMI model is a two-factor data analysis with genotype as the main factor and environments as the additive factor, while the effect of G × E interaction are modeled as a bilinear model [20].

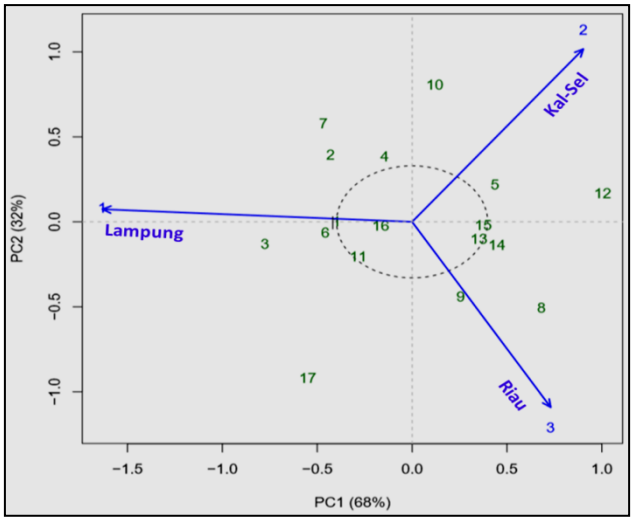

Figure 1. AMMI biplot for grain yield (kg/ha) of 15 upland rice genotypes (G) and three environments (E) using genotypic and environmental scores. 1 = Taman Bogo, Lampung (2015), 2 = KP Belandean, South Kalimantan (2016), 3 = KP Siak, Riau (2017).

3.3.5. AMMI biplot stability. The best way to visualize the interaction patterns between genotypes and environments is provided in the biplot analysis of G × E [21]. This can be used to predict the possible existence of different environment which will enable the genotype to grow well [22]. Genotype and environment additive main effect against their respective first multiplicative term PC1 dan PC2 was charted in AMMI biplot (Figure 1).

The AMMI biplot in Figure 1 explained which genotypes were stable on all three environments tested or flourished in specific locations. Mattjik and Sumertajaya [20] stated that if a genotype is stable, it will be plotted close to the zero point (0.0), which means that it shows general adaptation to all environments used in the test. Genotypes plotted far from the zero point and closer to a specific location line means that it has a better adaptation to that specific location [23]. Based on those criteria, genotypes number 1, 4, 6 and 11 were indicated as the stable genotypes, while genotypes number 5, 12, 13, 14 and 15 were optimum in tidal swamp environments. Genotype number 3 and 7 were shown to be optimum in coastal swamp area.

Yield stability concepts proposed by Francis and Kannenberg [17] and Eberhart and Russel [9] are statistical analyses of yield stability, but can only indicate if one genotype is classified as a stable genotype or not. On the other hand, the concept from Finlay and Wilkinson [8] was a dynamic method that can also show the adaptability pattern of a genotype. By using AMMI method, the G × E pattern which affects yield stability can be visualized using biplot analysis.

4. Conclusions

Yield stability of rice is important in a varietal recommendation to indicate whether the variety could be planted in a broad range of environments or not. The G × E interaction is a key factor in yield and yield component performance. G × E interaction is an important source of rice yield variation and its visualization in a biplot is a powerful tool for depicting the response of genotypes in different environments. Five lines (line number 5, 12, 13, 14 and 15) showed less yield stability, but optimum growth in a tidal swamp environment. Two lines (line number 3 and 7) showed optimum yield in the coastal swamp area.

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6. Authors contribution

DWU: main contributor, designed the experiment, analyzed and interpreted data, and wrote the manuscript. IR: analyzed the data. IK, PS and S: conducted the field experiment.

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Application of induced mutation technique to improve genetic variability of Indonesian traditional rice varieties

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Abstract. The development of rice genotype is vital to guarantee food security to cope with global climate changes and increasing population growth. Biodiversity is spread throughout the Indonesian archipelago. Many Indonesian local varieties are resistant to biotic and abiotic stresses that may be useful for rice breeding program. They are well-adapted to specific environment and have good aroma and eating quality, but have some weaknesses, such as late maturity, susceptible to lodging, unresponsive to fertilizer and low yield. Induced mutation breeding is useful for increasing genetic variability to develop genotypes with several interesting agronomical characters and yield. Center for Isotope and Radiation Application, National Nuclear Energy Agency (CIRA-NNEA) has been conducting the induced mutation technique for genetic improvement of rice. Pandan Putri, an early maturing mutant variety that was derived from irradiated Pandan Wangi variety from Cianjur area, is one of successful improvements of traditional rice varieties using mutation technique. The other success stories are described in this paper. It is concluded that nuclear technology can be applied for induced mutation breeding to improve several agronomical traits.

Keywords: nuclear technology, induced mutation, traditional rice, genetic improvement.

1. Introduction

Rice (*Oryza sativa* L.) is the primary food crop for most of Asian countries. In Indonesia, rice plays an important role in providing food and nutritional security and eradicating poverty. To achieve stable growth in rice production in keeping with the increasing population, a strong effort is required to boost productivity, break yield barriers and provide safety against fluctuations in climatic conditions. Securing the availability of rice production and ensuring sustainable community food needs in Indonesia can be done through the cultivation of modern and traditional rice varieties. Modern rice varieties are obtained through the stages of rice breeding activities which are then released nationally. Most of the traditional rice varieties existed in Indonesia belongs to the *tropical japonica* sub-species (known as *javanica*) [1]. Until now, most of these traditional rice varieties continue to be cultivated in several regions in Indonesia.

Based on the number of existing traditional rice germplasm, it is estimated that only 10–15% of these varieties are continuously planted by farmers. In the future, this number is likely to decline if there is no systematic effort to preserve local rice varieties [2]. Extensive genetic diversity in traditional rice is a genetic potential that controls several important traits. Therefore, traditional

varieties are very useful in rice breeding activities to obtain superior traits and to expand the genetic background of superior varieties to be produced [3].

Traditional rice varieties have been reported to have resistance to various environmental stresses including resistance to pest and diseases and are adapted to specific locations. Therefore, they have been cultivated for generations by most farmers, although it has some morphological characters such as relatively long growth duration [4]. Traditional rice that still survives to date is a cultivar produced by natural selection for decades or even hundreds of years, so that it generally has good characteristics preferred by the community such as delicious rice taste and resistance to abiotic stresses [5].

Genetic erosion of traditional rice germplasm in Indonesia began after the green revolution program implemented by the government at the end of the 1960s. The introduction of high-yielding varieties which tends to have shorter harvesting age triggerred mass extinction of traditional varieties because many farmers turned to these superior varieties [6]. The yield of traditional varieties was far lower than modern varieties, and thus they were no longer planted by farmers and started to extinct. To anticipate their extinction whilst preserving their positive characteristics and increasing its economic value, breeding activities need to be carried out to improve their genetic without changing other positive properties.

Cross-breeding (or recombinant breeding), which is based on hybridization of different genotypes followed by trait selection, has become a common practice in plant breeding. Further advancements in plant breeding for the induction of genetic alterations through physical and chemical mutagen laid the foundation of another type of plant breeding known as mutation breeding. The variation so created is further amplified by recombination of alleles on homologous chromosomes and their independent assortment at meiosis [7]. The most noticeable effects of irradiation on the transmission of the genetic materials are its inhibiting action on meiosis. Spontaneous and induced mutations are the primary source of all genetic variations existing in any organism, including plants [8].

Induced mutations with mutagenic agents have been used to create genetic variations from which desired mutants can be selected. Mutation breeding has become a very productive breeding tool that offers the possibility of inducing desired attributes that are not found in nature or that have been lost during domestication. Mutation breeding involves the development of new varieties by generating and utilizing genetic variability through mutagenesis [7]. The possibility of increasing the genetic variability of rice varieties by means of ionizing radiation had more consideration from plant breeder point of view [9]. This paper summarizes the development and achievement of mutation induction techniques in improving the genetic background of Indonesian traditional rice.

2. Morpho-agronomic characteristics of Indonesian traditional rice varieties

Indonesia, known as an agrarian country, is located around the equator. Its tropical climate with only two seasons has made this country rich in biodiversity including traditional rice germplasm. Abundant rice genetic resources are found in Indonesia. Almost every region has more than one traditional rice varieties that have been cultivated for generations. For example, Kewal variety from Banten, Barak Cenana from Tabanan (Bali), Jembar from West Java, Pandan Wangi from Cianjur (West Java), Rojolele from Klaten (Central Java), Kuriak Kusuik from West Sumatra, Siam Datu from South Kalimantan, etc.

The exisiting traditional rice varieties are cultivated by the farmer's community and controlled by the government. Therefore, traditional varieties are more adaptable to climate change than introduced varieties. Approximately, 3,800 local rice germplasms are registered by Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development [10]. "Fur" or "Gundil" is an example of Indonesian rice variety known by the peasant community and widely cultivated in Java, Lombok, Bali, Sumbawa, and other several remote areas.

Generally, local farmers cultivate the traditional rice varieties in unfavorable growing environments, such as dry land in hilly areas, acidic dry land, swampy land, "lebak" or lowlands that are often flooded, tidal swamps that tend to be saline and other marginal lands. Therefore, it is possible to make the traditional rice varieties more adapted and tolerant to biotic and abiotic stresses with good

taste according to people's preferences. Several traditional rice varieties have been identified as having resistance to biotic stresses, such as rice gall midge, brown planthopper, bacterial leaf blight, orange leaf disease, leaf blast, neck blast, *Rice stripe virus* and rice tungro disease, and abiotic stresses, such as drought, Al toxicity, Fe toxicity, salinity, low temperature and shading [3].

Majority of Indonesian traditional rice have long panicles, low tiller number, round seeds which make them hard to fall, wide leaves, intermediates amylose content and photoperiod insensitive [1]. Rice belonging to the *javanica* group has usually long hair and dense grain (*sericeous*), and a tail/hair at the end of the grain [6].

Each traditional variety adapts well to the area where the plant originates, with the taste of rice according to the preference of the local community and has a specific aroma. Other characteristics are strong and deep rooting, but not responsive to fertilizer application. Resistance to plant pests and diseases is present in many traditional rice and wild rice [11]. A list of characterized and identified 456 traditional rice accessions from Indonesia with resistance to pests and plant diseases have been published [3].

The traditional rice are commonly late maturity and tall, which makes them susceptible to lodging and produce low tiller number. In general, traditional varieties have also low yield potential and less ideal plant architecture. Traditional rice is commonly cultivated in suboptimal land such as dry land of ex-forests in an effortless way with no chemical inputs for fertilizers and pesticides.

Farmers usually prepare seeds for the next planting season in a traditional way by relying on their own crops. Thus, the quality of the seeds, especially the level of purity, is very low, which affects production. Due to low purity of seed, the appearance of traditional rice varieties in the field in terms of plant height, harvesting days, grain shape and color, seem to be diverse [12]. Efforts have been made by the regional government to improve the seed purity of traditional rice and release them as superior varieties. Until 2012, a total of ten local rice varieties have been purified and released in Indonesia (Table 1).

Table 1. Local varieties that have been purified and released by regional government in 2004–2018 [12].

Traditional varieties	Province	Year of release
Rojolele	Central Java	2003
Pandan Wangi	West Java	2004
Anak Daro	West Sumatra	2007
Kuriek Kusuik	West Sumatra	2009
Junjung	West Sumatra	2009
Caredek Merah	West Sumatra	2010
Lampai Kuning	West Sumatra	2014
Siam Mutiara	South Kalimantan	2008
Siam Saba	South Kalimantan	2008
Cekow	Riau	2012
Karya	Riau	2012

The regional government's aims for the release of traditional rice varieties are (1) to obtain the legality that traditional varieties deserve to be regional superior varieties with specific site, (2) to obtain legality for efforts to produce certified seeds, (3) to obtain equal rights in the use of qualified seeds, and (4) to increase the benefits and economic values of local variety seeds for the community and local government.

3. Induced crop mutagenesis

Crop mutation breeding based on mutation techniques approach has been used for more than 50 years, and more than 1,000 cultivars of 44 crop species have been released [13]. Mutagenesis is a powerful tool that has been used to create genetic materials for plant breeding. The primary aim in mutation breeding is to develop and improve well adapted plant varieties by modifying one or two major traits to increase their productivity or quality through inducing physical or chemical mutagens in seeds and other planting materials [14].

Among different type of physical mutagen, gamma rays are widely employed for mutation studies because they have shorter wave length and possess more energy per photon than x-rays, and penetrate deeply into the tissue. Gamma rays have been a popular method to improve the qualitative and quantitative characters for many crops [9]. Most of the gamma ray irradiation doses were acute. High doses of radiation caused higher sterility of M_1 plants, but in some cases, low doses of irradiation stimulated the growth and yield of M_1 plants [15]. Irradiating seeds with suitable doses of gamma rays produces physiological or genetical changes in plant tissue which may affect the yield of plant [16].

Increasing the genetic variability of rice varieties through ionizing radiation is more preferred by plant breeder to start plant mutation breeding. Until now, mutation breeding technique has become a very productive breeding tool that offers the possibility of inducing desired attributes that are not found in nature, or that have been lost during domestication. There are two major outcomes resulted from mutation breeding: improved varieties that are directly used for commercial cultivation and new genetic stocks with improved characters or with better combining ability of traits.

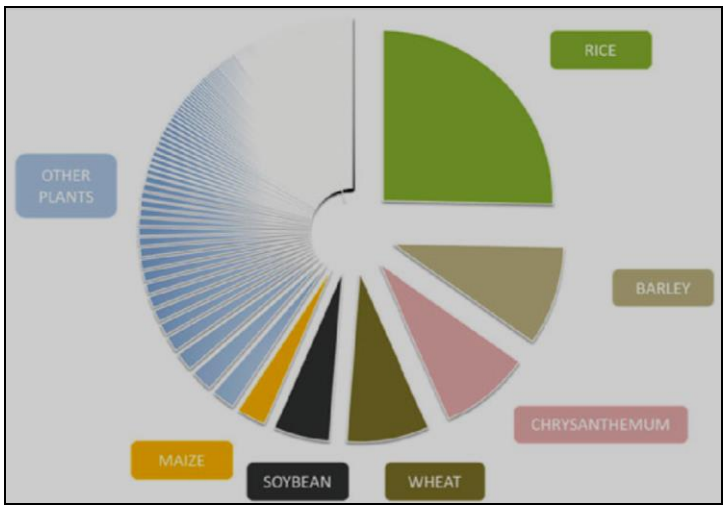

Figure 1. Pie chart representing officially registered mutant crop varieties. The Mutant Variety Database contains 3,222 entries out of which 2,456 are seed propagated and 367 are vegetatively propagated plants [17].

Over 3,220 crop varieties that have been released through induced mutation technique are being grown in different countries of the world. The majority (80%) of this crop varieties are seed propagated, with almost half of it (48%) are cereals (Figure 1, [17]). Among cereals, rice is the top rank compared to the other crops, whereby 443 rice cultivars have been developed by mutation breeding using EMS, fast neutron and gamma irradiation [18].

Many rice mutants have been developed with new genetic variation and improvement in plant characteristics, both in quantitative and qualitative traits. Numerous studies have reported that many mutant genes controlling important traits like plant height, tiller number and panicle length have been cloned and characterized at the molecular level [19–22]. Babaei et al. [23] also reported that rice mutants have been useful for genetic and physiological assessments of yield-limiting factors.

Dwarfism is one of the important agronomic traits that play a part in increasing rice yield. As many as 80 dwarf mutants of rice have been reported including six high-tillering dwarfs [24]. The following characteristics in rice mutants were subsequently developed: early maturity, endosperm quality, elongated uppermost internode, genetic male sterile, improved nutritional quality because of low phytic acid, giant-embryo mutants of potential interest to the rice oil industry, and adapted Basmati and Jasmine germplasm [25]. In China, the most widely grown rice cultivar, Zhefu 802, induced from Simei 2 by gamma rays, has a relatively short growing period (105–108 d), high yield potential, wide adaptability, high resistance to rice blast and cold tolerance [26].

Basically, plant mutation breeding is an accelerated breeding method which generally takes 7–9 years as opposed to 10–15 years of conventional breeding to produce new cultivar in annual crop. This is because mutation breeding improved an already preferred cultivar for a certain trait. Once mutants have been identified in a population, they can be deployed directly and indirectly in breeding programs [27].

4. Mutation breeding in Indonesian traditional rice varieties

In Indonesia, plant mutation breeding technique was applied in rice since early 1960's by Center for Isotope and Radiation Application, National Nuclear Energy Agency (CIRA-NNEA), the only institute that is engaged in rice mutation breeding in Indonesia. Significant achievements were made from the early stages. In 1982, Atomita 1, the first mutant rice variety in Indonesia, was officially released. This mutant variety was produced from seed irradiation treatment of Pelita 1/1 with gamma rays at a dose of 0.2 kGy. Screening for biotic stress was started in M_2 generation and followed by selections of mutants with desirable morphological and agronomical traits in advanced generations. Atomita 1 has contributed more than 10% of the total rice varieties after 1982. Its contribution has been increasing until these years (Table 2).

Mutation breeding based on nuclear technique has the following advantages in rice improvement in Indonesia: (1) almost all characters in rice could be improved as long as their variations exist in nature, (2) both allelic and non-allelic mutations to those already known in the germplasm collection could be induced, and (3) mutation techniques are useful, particularly for the improvement of traditional varieties for specific traits that could not be improved using cross-breeding [28].

The mutation breeding can effectively change a few traits without changing other characteristics that have been preferred. This method is useful for the improvement of local rice varieties that are already popular in certain areas because of preferred taste of rice by the local community and good adaptability in the area, but are very late in maturity and have low and unstable yielding ability. In addition, high plant architecture makes it unable to stand down and hard to fall especially before harvest. Susceptibility to lodging can reduce the yield both in quantity and quality. Attempts to improve these defects through cross-breeding have failed due to the inability to keep the desired quality preference [28].

Pandan Putri variety is an example of a successful improvement of traditional rice varieties using mutation breeding. This variety is the improvement of Pandan Wangi, a local rice variety, through seed radiation with gamma rays at a dose of 0.2 kGy. Pandan Putri is 45 days earlier but with appearance and taste of rice that are not different from the wild type, Pandan Wangi. The improvement activities of local rice varieties with mutation breeding are growing in line with the rampant efforts of the regional government to purify and release local rice varieties to support regional food self-sufficiency programs by utilizing local wisdom.

Table 2. Genetic improvement in rice mutant varieties developed using mutation breeding in Indonesia (1982–2016).

Name	Year	Parent and treatment	Improved character(s)	Status
Atomita 1	1982	Pelita I/1; 0.2 kGy	Resistant to brown planthopper (BPH) biotype 1 and green leafhopper (GLH); early maturity	Released
Atomita 2	1983	Pelita I/1; 0.2 kGy	Resistant to BPH biotype 1; tolerant to saline soil; early maturity	Released
Atomita 3	1990	Mutant line No. 627/103/PsJ; 0.2 kGy	Resistant to BPH biotype1 and biotype 2; early maturity	Released
Atomita 4	1991	Cisadane; 0.2 kGy	Early maturity; tolerant to high Fe soil (poor soil drainage)	Released
Situgintung	1992	Seratus Malam*; 0.2 kGy	Resistant to BPH biotype 1; intermediate resistant to BPH biotype 2	Released
Cilosari	1996	Mutant line of Seratus Malam (SM 268/PsJ) × IR36	Tolerant to bacterial leaf blight (BLB)	Released
Meraoke	2001	F_1 seeds (Atomita 4 × IR64); 0.2 kGy	Slender seed; tolerant to BLB strain IV; early maturity	Released
Woyla	2001	F_1 seeds (Atomita 2 × IR64); 0.2 kGy	Slender seed; tolerant to BLB strain IV; early maturity	Released
Kahayan	2003	F_1 seeds (Atomita 4 × IR64); 0.2 kGy	Tolerant to BLB strain IV	Released
Winongo	2003	F_1 seeds (Atomita 3 × IR64); 0.2 kGy	Big slender seed; tolerant to BLB srain IV	Released
Diah Suci	2003	F_1 seeds (Cilosari × IR74); 0.2 kGy	Slender seed; tolerant to BLB strain IV	Released
Mayang	2004	F_1 seeds (Cilosari × IR74); 0.2 kGy	Big and slender seed; tolerant to BLB strain IV	Released
Yuwono	2004	IR64; 0.1 kGy	Tolerant to BLB strain IV	Released
Mira-1	2006	Cisantana; 0.2 kGy	Resistant to BPH biotype 1 and 2	Released
Bestari	2008	Cisantana; 0.2 kGy	High yield; resistant to BPH biotype 1 and 2; intermediate resistant to BPH biotype 3	Released
Pandan Putri	2010	PW 1-PsJ; 0.2 kGy	Early maturity; aromatic; semi-dwarf	Released
Inpari Sidenuk	2011	Diah Suci; 0.2 kGy	High yield; slender seed; early maturity	Released
Mugibat	2012	Cimelati; 0.2 kGy	Tolerant to blast fungus race 133; intermediate tolerant to blast fungus race 033 and race 173; intermediate resistant to BPH biotype 1, 2 and 3; long grain and slender seed	Released
Sulutan Unsrat 1	2012	Super Win; 0.2 kGy	High yield; high protein content; intermediate resistant to BPH biotype 1 and 2; tolerant to BLB strain III	Released
Sulutan Unsrat 2	2012	Super Win; 0.2 kGy	High yield; intermediate resistant to BPH biotype 1 and 2; tolerant to BLB strain III	Released
Mustaban	2016	Kewal; 0.2 kGy	High yield; slender seed; early maturity; semi-dwarf	Released
Mustajab	2018	Jembar; 0.2 kGy	Plant architecture; semi-dwarf; high yield; resistant to BLB strain III; medium resistant to BPH biotype 1	Released

Collaboration among CIRA-NNEA, Sam Ratulangi University and the North Sulawesi Regional Government, has resulted in the release of Sulutan Unsrat 1 and Sulutan Unsrat 2 varieties. The two varieties were around 25 days earlier than Superwin's original varieties [12]. At present, collaborations are also being carried out by CIRA-NNEA with several regional governments and higher education in Indonesia (Table 3). Through these collaborations, local rice varieties will be genetically improved by means of induced mutation to obtain varieties with comparable maturity and height with modern varieties, and yet have the original taste and aroma. Mutant varieties with such desirable characteristics will benefit farmers because of shorter growth period, better yield quality and quantity, and relatively higher selling price. At the same time, these mutants can preserve the preferred characteristics of the traditional rice varieties which are almost extinct due to difficulties in competing with modern varieties. CIRA-NNEA is also collaborating with local government of Kota Baru, Paser, East Kalimantan and Sijunjung, West Sumatra to improve local rice varieties through mutation breeding.

Table 3. Radiation treatment of 10 traditional rice varieties done by CIRA-NNEA.

Traditional rice variety	Origin	Growth duration (months)	Plant architecture	Radiation treatment
Simera, Karang Dukuh	Jambi	>4	High	0.2 kGy
Siam Datu	South Kalimantan	>6	High	0.2 kGy
Pandan Wangi	Cianjur, West Java	>6	High	0.2 kGy
Superwin	North Sulawesi	>6	High	0.2 kGy
Dayang Rindu	Musi Rawas	>4	High	0.2 kGy
Payo	Kerinci	>6	High	0.2 kGy
Rojolele	Klaten, Central Java	>4	High	0.2 kGy
Beak Sembalun	NTB	>6	High	0.2 kGy
Barak Cenana	Tabanan, Bali	>6	High	0.2 kGy
Palalawank	Landak, West Kalimantan	>6	High	0.3 kGy

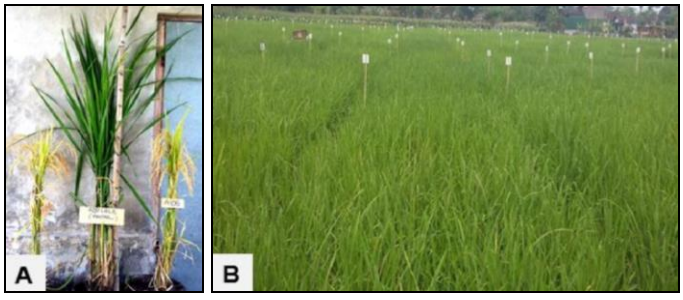

Figure 2. Early and semi-dwarf selected mutant plants (A), early homogeneous and semi-dwarf M_4 lines (B) originating from Rojolele ionized-seed irradiation at a dose of 200 Gy.

5. Conclusions

Mutation breeding has been proved as an effective means to genetically improve Indonesian local rice varieties. This method is useful to change the desired traits without changing preferred traits already present in the rice genetic materials, such as good adaptability in specific regions, flavor and aroma.

Some of Indonesian local rice varieties, such as Pandan Wangi from Cianjur and Superwin from North Sulawesi, have been successfully improved through mutations breeding.

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Diversity analysis in soybean (*Glycine max* [L.] Merrill) mutant lines grown in saline soil using agronomic traits and RAPD markers

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Abstract. Soybean is one of the strategic food crops in Indonesia, but its production is far below the demand due to inadequate area for soybean cultivation. Shifting soybean cultivation to marginal land such as saline soil has been suggested as a realistic solution to increase soybean production. Development of soybean variety tolerant to salinity is the key step to support the cultivation of soybean in saline soil. The objective of this research was to evaluate the genetic diversity in mutant soybean lines generated using gamma rays based on agronomic traits and RAPD markers. A total of 200 irradiated seeds of cultivar Detam-3 were planted in saline soil with electrical conductivity of 1.2–4.3 dS/m. Agronomic traits were evaluated on plants until harvesting time. Genetic analysis using two RAPD markers (OPAA-02 and OPAA-14) was done on 11 plants of each radiation treatment. The results showed that 54 plants survived in saline soil. High level of variation based on agronomic traits was observed in these plants. RAPD analysis revealed 60% and 83.3% polymorphism among 11 plants for OPAA-02 and OPAA-14 markers, respectively.

Keywords: Detam-3, gamma rays, OPAA-02, OPAA-14, saline soil, soybean.

1. Introduction

Soybean (*Glycine max* L. Merr.) is one of the main crops widely planted in many countries. Soybean is used as food, feed and raw materials of industrial product [1]. It has a relatively high protein content (35–46%) [2]. However, national soybean production is far below the demand. Domestic production was 963.18 thousand tons in 2015, while domestic consumption reached 1.56 million tons [3]. The domestic consumption increased by 1.73% each year [4]. On the other hand, the area for soybean cultivation has decreased. The harvested area in 2009 was 722,291 ha, but decreased to 550,793 ha in 2013, and increased to 614,095 ha in 2015 [5]. However, domestic soybean production is unable to meet the soybean demand. Harsono [6] estimated that 2 million ha of cultivation area is needed to fulfil the demand in 2020.

Salinity becomes a global challenge in agricultural production [7]. Up to 80% of plant yield can be lost because of drought and salinity [8]. Development of salinity tolerant variety is the key step to support the cultivation of soybean in saline soil. The first step in a breeding program is creating genetic variation through hybridization [9], exploration [10] and mutation [11]. Mutation breeding has been widely used for inducing variation. Two types of mutation are known, i.e. chemical mutation

using mutagen EMS [12] and physical mutation using gamma rays [13]. Mutation breeding is considered effective to improve traits and efficient to screen new traits [14]. RAPD technique is one alternative for identification plant genetic diversity [15].

This study aimed to evaluate the diversity of gamma rays-mutated soybeans lines based on agronomic traits and RAPD markers. This research is a part of a breeding program to obtain adaptive black soybean mutant plants with better productivity compared to its parents in saline soil.

2. Materials and methods

This research was conducted at saline soil in Dresi Wetan Village, Kaliori Sub-district, Rembang District, Central Java Province and Central Laboratory of Diponegoro University, Semarang City, Central Java Province, from December 2017 to July 2018. Soybean cultivar Detam-3 was irradiated with gamma rays at 0 (control), 160, 208, 258, 304, 352, 400, 448, 496, 544 and 592 Gy at the Center for Isotopes and Radiation Application, National Nuclear Energy Agency (CIRA-NNEA). Two-hundred seeds were planted in saline soil with electrical conductivity (EC) of 1.2–4.3 dS/m. The mutant population was named BSMG (for Black Soybean Mutant Gamma).

Agronomic traits were assessed based on the number of leaves, plant height, number of pods, average seed per pod, seed weight per plant, weight of 1 seed and 100 seeds. Analysis of agronomic traits was done by analysis of variance (ANOVA) at 5% significance level, followed by Dunnett's test to compare each treatment to control plant (Detam-3).

Molecular variations were revealed using Random Amplified Polymorphic DNA (RAPD) markers with two primers (OPAA-02 and OPAA-14) which were previously used as DNA markers for soybean genotypes grown under salt stress [16,17]. Leaf samples were collected from 3-week-old plants of each gamma-rays dose. DNA extraction was carried out using Plant Genomic DNA Kit (Tiangen). DNA amplification was performed by PCR following a modified method from Khan et al. (2013). PCR-mix was composed of 22 µl Master Mix (12.5 µl AmpliTaq Gold 360, 1 µl 360 GC Enhancer, 8.5 µl $_{dd}H_2O$), 1 µl primer (working solution 25 µM) and 2 µl DNA template to make a total volume of 25 µl per sample. The amplification reaction was carried out in a Thermal Cycler (Labnet, MultiGene OptiMAX). The first cycle consisted of denaturation of template DNA at 95°C for 10 min, followed by primer annealing at 37°C for 30 sec and primer extension at 72°C for 2 min. For the next 43 cycles, the period of denaturation was reduced to 30 sec while annealing and extension time remained the same as in the first cycle. The last stage of PCR amplification was final extension at 72°C for 8 min. PCR products were separated on a 1.5% agarose gel and DNA fragments were visualized using GelDoc. Bands were scored as present (1) or absent (0) for each primer. The data was arranged into a binary data matrix as discrete variables. This matrix was subjected to Unweighted Pair-Group Method for Arithmetic Average Analysis (UPGMA) to generate a dendrogram using average linkage procedure. All these computations were carried out using NTSYSpc software.

3. Results and discussion

3.1. Agro-morphological characteristics

The leaf number of BSMG-304, BSMG-400 and BSMG-592 was significantly different from Detam-3. The plant height of BSMG-160, BSMG-208, BSMG-304 and BSMG-544 was significantly different from Detam-3 (Table 1). Under optimum condition the plant height of Detam-3 was approximately 56.9 cm [18]. In our study, the growth of this variety at saline soil was strongly inhibited (14.33 cm), which may be caused by the effect of salinity stress. The decrease in plant height of mutant lines may also be caused by both salinity stress and gamma radiation that can create a mutation in plant, such as dwarfism. Other studies reported that the decrease in plant height was due to the effect of salinity stresses on plant growth [19] or the effect of gamma radiation [8].

Table 1. Morphological characteristics of soybean mutant lines and its wild type (Detam-3) grown in saline soil.

Genotype	Number of crops	Number of leaves	Plant height (cm)
BSMG-160	7	5.33±1.16	8.67±0.76*
BSMG-208	6	13.33±1.53	8.50±3.12*
BSMG-256	3	8.67±0.58	14.00±2.00
BSMG-304	8	24.00±18.25*	7.33±1.89*
BSMG-352	3	10.67±1.15	9.33±2.31
BSMG-400	5	28.00±10.15*	9.83±2.75
BSMG-448	3	13.00±3.46	11.50±2.78
BSMG-496	3	9.33±3.51	12.17±4.75
BSMG-544	0	0.00±0.00	0.00±0.00*
BSMG-592	4	19.00±6.08*	18.33±7.50
Detam-3	12	6.00±5.20	14.33±2.08
CV (%)		46.74	28.88

*Significantly different from the control (Detam-3) based on Dunnett's test at P≤0.05.
CV = coefficient of variation.

Plants affected by salinity stress show delayed growth responses such as decrease of plant height, leaf area, dried apical buds and even plant death [20]. In other crops such as bean, salinity stress in high concentration of NaCl decreases plant height, number of leaves and leaf area [21]. These decreasing parameters are due to the negative effects of ions Na^+ and Cl^- on the rate of photosynthesis, changes in enzyme activity, and also decreased levels of carbohydrates and growth hormones which can cause growth inhibition [21]. Gamma radiation in soybeans can affect the length, width and density of stomata that influence the process of respiration and photosynthesis [13].

3.2. Yield components

The number of seeds per pod for BSMG-256, BSMG-352 and BSMG-544 and seed weight per plant for BSMG-208 were significantly different from control (P>0.05) (Table 2). Under optimum condition, variety Detam-3 produces approximately 51 pods per plant [18]. The same variety produced on average of 4 pods per plant or 96% less (range from 2.00 to 6.67) under salinity stress condition in our study. In the previous study, treatment of variety Dering-1 to three levels of salinity stress, i.e. 3, 6 and 9 dS/m, led to the decreasing number of pods per plant by 32.7, 68.03and 98.10%, respectively [20].

The weight of 1 seed and 100 seeds of BSMG-544 and BSMG-592 were significantly different from the control (Table 2). Under optimum condition, the weight of 100 seeds of Detam-3 is approximately 11.8 g [18], but under salinity stress condition in our study, its weight decreased to 7.95 g. On the contrary, the weight of 100 seeds of BSMG-592 in saline soil increased to 12.67 g, which is categorized as medium-size seed according to Adie and Krisnawati [22] and Putra et al. [23]. Our result showed that gamma radiation with the dose of 592 Gy gave a positive response on the weight of 100 seeds.

Table 2. Yield components of soybean mutant lines and control grown in saline soil.

Genotype	Number of pods	Average of seeds/pod	Seeds weight/plant	Weight of 1 seed	Weight of 100 seeds
BSMG-160	2.00±1.73	1.75±0.43	0.17±1.07	0.06±0.01	5.77±1.53
BSMG-208	6.67±2.89	1.67±0.61	1.15±0.54*	0.11±0.03	10.88±3.49
BSMG-256	6.00±3.46	1.88±0.13*	0.67±0.36	0.06±0.03	6.30±3.32
BSMG-304	4.33±1.15	1.53±0.46	0.58±0.26	0.10±0.05	9.61±4.60
BSMG-352	2.00±0.00	1.83±0.29*	0.30±0.02	0.08±0.01	8.27±1.11
BSMG-400	5.00±1.00	1.39±0.18	0.73±0.10	0.11±0.03	10.88±2.83
BSMG-448	4.67±3.79	1.59±0.36	0.79±0.74	0.10±0.02	10.29±2.09
BSMG-496	4.00±2.00	1.50±0.50	0.40±0.18	0.07±0.02	7.38±2.17
BSMG-544	0.00±0.00	0.00±0.00*	0.00±0.00	0.00±0.00*	0.00±0.00*
BSMG-592	3.33±3.21	1.16±0.29	0.43±0.31	0.13±0.02*	12.67±1.77*
Detam-3	4.00±1.00	1.30±0.26	0.43±0.19	0.08±0.01	7.95±1.42
CV (%)	47.96	20.79	53.28	25.21	25.21

*Significantly different from the control (Detam-3) based on Dunnett's test at P≤0.05.
CV = coefficient of variation.

3.3. Molecular analysis

Based on RAPD analysis, 60% polymorphism level (3 of 5 fragments) and 83.3% polymorphism level (5 of 6 fragments) were found for OPAA-02 and OPAA-14, respectively (Figure 1 and Table 3).

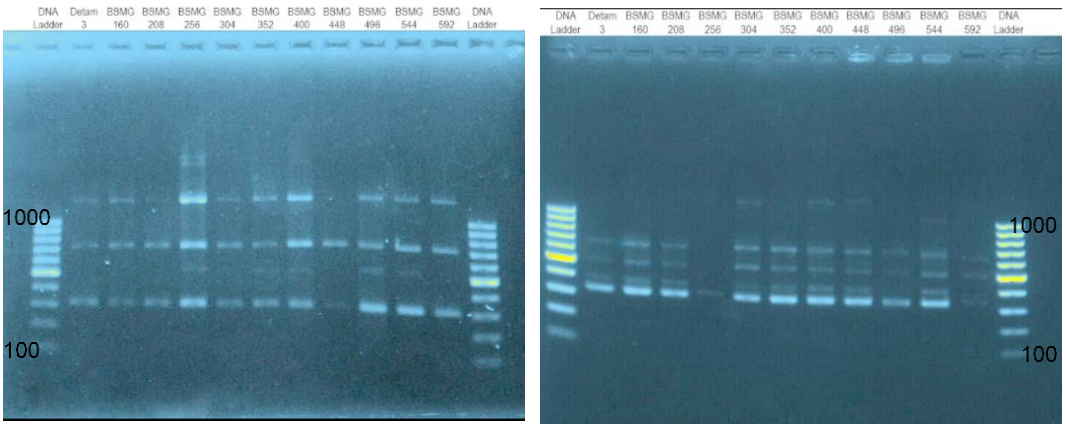

Figure 1. RAPD analysis of several soybean mutant samples derived from gamma-irradiated Detam-3 variety using OPAA-02 and OPAA-14 primers.

Table 3. Overview of DNA fragments amplified in soybean mutant lines derived from gamma-irradiated Detam-3 variety using OPAA-02 and OPAA-14 primers.

Primer	Sequence (5' to 3')	Fragment size (bp)	Total no. of fragment	No. of polymorphic fragment	Polymorphism (%)
OPAA-02	GAGACCAGAC	300–2220	5	3	60
OPAA-14	AACGGGCCAA	300–1210	6	5	83.3

OPAA-02 primer produced 2,220 bp fragment (in BSMG-256) and 520 bp fragment (in BSMG-256, BSMG-352, BSMG-400, BSMG-496 and BSMG-544), which did not appear in control (Detam-3). OPAA-14 primer generated 1,210 bp fragment (in BSMG-304, BSMG-400 and BSMG-448), and 850 bp fragment (in BSMG-544). A band with a size of 1,340 bp present in the control plant was missing in BSMG-448 for OPAA-02 primer. Missing bands with the size of 700, 520 and 370 bp were also observed in BSMG-256 for OPAA-14 primer. The presence and absence of different fragments may indicate the occurrence of genetic mutations in the irradiated seeds (Table 4).

Juwarno and Samiyarsih [24] reported the genetic differences between control plot and 80 mM NaCl plot for three soybean cultivars (Mahameru, Slamet and Dam) subjected to salt stress condition. Genetic instability on soybean subjected to salinity stress was manifested in RAPD profiles as the decrease or the increase in band intensity, disappearance of bands and appearance of new bands as compared to the controls [17].

Table 4. Presence of RAPD bands in black soybean mutant gamma (BSMG) lines derived from gamma-irradiated Detam-3 variety.

Primer name	Fragment size (bp)	Detam-3	BSMG lines									
			160	208	256	304	352	400	448	496	544	592
OPAA-02	2,220				+							
	1,340	+	+	+	+	+	+	+		+	+	+
	740	+	+	+	+	+	+	+	+	+	+	+
	520				+		+	+		+	+	
	300	+	+	+	+	+	+	+	+	+	+	+
OPAA-14	1,210					+		+	+			
	850										+	
	700	+	+	+		+	+	+	+	+	+	+
	520	+	+	+		+	+	+	+		+	+
	370		+	+		+	+	+	+	+	+	
	300	+	+	+	+	+	+	+	+	+	+	+

RAPD analysis of the mutant lines revealed four groups of soybean genotypes (Figure 2). The first cluster comprised of the wild type and mutant line BSMG-592, which showed 100% similarity. The second cluster included BSMG-160, BSMG-208, BSMG-304, BSMG-400 and BSMG-448 at 74% similarity. Two of the mutants (BSMG-304 and BSMG-400) occupied the same branch node. The third cluster included three mutant lines (BSMG-352, BSMG-496 and BSMG-544) with 70% similarity. The highest genetic distance was observed between BSMG-256 and control (Detam-3), which showed 46% similarity.

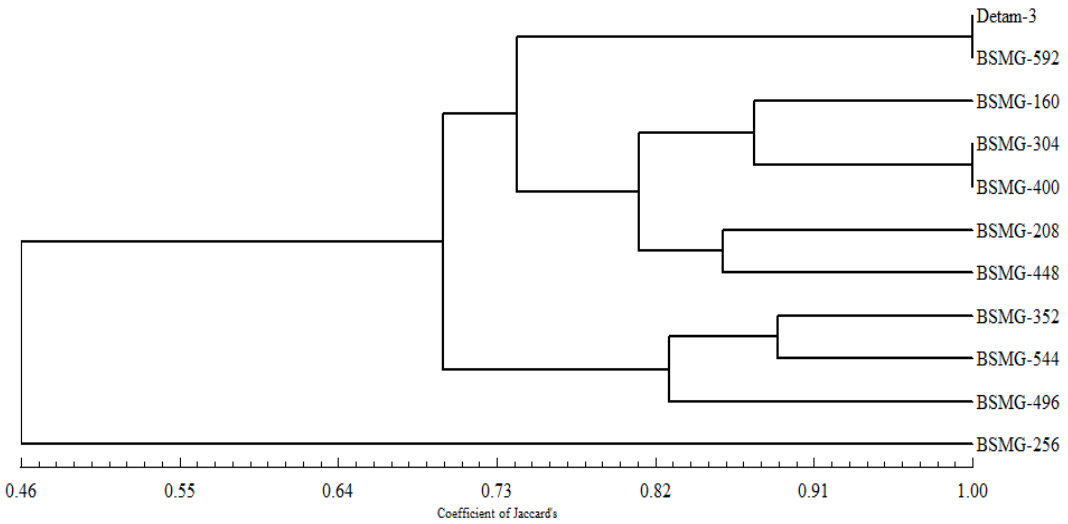

Figure 2. Dendrogram derived from UPGMA clustering analysis based on genetic difference in soybean mutant lines generated from gamma-irradiated of Detam-3 variety.

4. Conclusions

Mutant BSMG-256 had significantly higher weight of 100 seeds than the wild type Detam-3 under salinity stress condition. RAPD analysis using OPAA-02 and OPAA-14 primers showed 60% and 83.3% polymorphism, respectively, among the mutant lines. The highest genetic distance was observed between BSMG-256 and the wild type (46% similarity).

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Agronomic performance of soybean mutant lines based on preliminary and advance yield at dryland area

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Abstract. Since 2013, researchers at ICABIOGRAD have established a development program for soybean varieties with bigger seeds, medium maturity and high productivity through cross-pollination combined with mutation by γ irradiation. In 2016, about 36 homozygous M_6 lines with high productivity and excellent agronomic characteristics were selected for subsequent preliminary and advanced yield test. The research aimed to study the agronomic performance of mutant lines (M_6 and M_7) and to select the best lines that will be used for adaptation testing at different locations and environments. The study was conducted at the Village of Gunung Menyan, Pamijahan Sub-district, Bogor during the first and second planting season of 2017. The experiment was arranged in a randomized block design with three replications. During the first planting season of 2017, a preliminary yield testing was conducted on 36 M_6 lines along with 4 check varieties. For the second season, 20 lines were selected along with 4 check varieties to be tested for advanced yield testing at the same location and method. Advanced yield trial showed that nine mutant soybean lines produced higher yields with excellent agronomic performance, which were 15−26% higher than Panderman's check varieties and 27−31% higher than the Anjasmoro check variety. The nine selected soybean line were SSD-C-M7-342-10, SSD-C-M7-350-18, SSD-D-M7-372-14, SSD-E-M7-387-1, Bulk-C-M7-458, Bulk-C-M7-493-1, Bulk-C-M7-493-20, Bulk-C-M7-499 and SSD-E-M7-404-18. These selected lines will be further assayed for adaptation at different locations and environments in the following year.

Keywords: soybean, mutant lines, yield test, high productivity.

1. Introduction

Soybean is the main food commodity after rice which needs more attention [1]. National production of soybean in Indonesia during 2015 was around 2.33 million tons, which was still far below national consumption, so that import of 1.37 million tons of soybean is still needed. To reduce soybean import, the Indonesian government has made various efforts to increase production, such as expanding the area of soybean plants and increasing productivity and quality in accordance with consumer preferences.

Imported soybeans generally have the large seed (>15 g/100 seeds). Therefore, they are prevalent in tofu and “tempe” industry. However, until now the number of soybean varieties with large seeds that have been released in Indonesia is still limited. Since 2013, ICABIOGRAD has been developing large-

seeded soybean varieties by increasing genetic diversity through cross-pollination combined with mutation by γ ray irradiation [2].

The development of superior soybean varieties is a dynamic and continuous activity, depending on consumer preferences. The breeding procedures typically start with increasing the genetic diversity through various methods such as crossing, mutation by physical or chemical induction, and genetic transformation, followed by selection using various methods (such as bulk, pedigree and SSD method), preliminary and advanced yield test and multilocation yield test [3–7].

The use of irradiation techniques in plant breeding is well-established and does not need a long time to produce results. Gamma irradiation can penetrate seeds and alter the structure and number of chromosome pairs in plant seeds, which in turn causes changes the characteristics of the plants and their offspring. This phenomenon has been used to improve qualitative and quantitative plant traits, such as pest resistance, drought resistance, and early maturity [8–11] Increasing genetic diversity of soybean genetic resources by crossing potential parents (with early maturity and high yielding potential for example) has also been carried out in ICABIOGRAD.

The combination of cross-pollination and irradiation mutations provided more opportunity for success. Since 2009, ICABIOGRAD has been creating improved soybean varieties through mutations [12]. Currently, M_6 mutant lines with early maturity of <75−80 days, higher yield (15−33 g/plant or 15−59% higher than check varieties, and 25−93 pods/plant or 23−59% more than check varieties) have been obtained. These genetic materials need to be evaluated further in preliminary and advanced yield trial.

Preliminary and advanced yield trials are the stages to select the best promising lines before adaptation test at different locations (multilocation test). The best lines that are selected at the multilocation trial will have the opportunities to be released as newly improved varieties.

2. Materials and methods

2.1. Preliminary yield trial

The yield test was conducted during the first season of 2017, at the Village of Gunung Menyan, Pamijahan Sub-district, Bogor. The genetic material used here were 36 M_6 soybean selected lines that originated from irradiated homozygote lines (F10:G.10428 × Panderman) along with 4 check varieties (Mo:F10 G.10428 × Panderman, G.10428, Panderman and Anjasmoro). The experiment was designed in a randomized block design with three replications. Soil tillage was conducted optimally, and a total of 500 g limes (dolomite) with 1 t/ha of manure were applied a week before planting. The genotypes were planted in a plot of 2.4 m × 3 m, with 2 plants/hole. Fertilizers used were 50 kg of urea, 250 kg SP36 and 100 kg/ha KCl were applied 10 days after planted. Pest, diseases and weeds control were carried out as needed. Data were collected for uniformity of plant in the same lines, days to harvesting, seed yield and other agronomic characters.

2.2. Advanced yield trial

The experiment was conducted during the second planting season of 2017 at Pamijahan, Bogor District. The genetic materials used here were 20 M_7-selected lines from preliminary yield testing along with 4 check varieties. The experiment was designed in randomized block design with three replications. Soil tillage was conducted optimally, and a total of 500 g limes (dolomite) with 1 t/ha of manure were applied a week before planting. The genotypes were planted in a plot of 3 m × 4 m, with 2 plants/hole. Fertilizers used were 50 kg of urea, 250 kg SP36 and 100 kg/ha KCl were applied 10 days after planted. Pest, diseases and weeds control were carried out as needed. Data were collected for the flowering date, maturity date, seed yield per plant and seed yield per ha.

3. Results and discussion

3.1. Preliminary yield trial

The yield and other agronomic characters of the genotypes in the preliminary trial at Pamijahan during the first planting season of 2017 are shown in Table 1. There were two lines (SSD-C-M6-338-6 and

SSD-C-M6-342-10) that produced higher yield and better agronomic characters, such as pods number, compared to the four check varieties. Nine mutant lines produced higher yields (36−61% higher than Panderman, or 3−21% higher than Anjasmoro), these lines were: SSD-C-M6-338-6, SSD-C-M6- 342-10, SSD-C-M7-352-20, SSD-D-M6-372-14, SSD-E-M6-387-1, Bulk-C-M6-467, Bulk-C-M6-493, Bulk-D-M6-507 and Bulk-E-M6-567. These lines, along with several other lines, would be included in adaptation tests at various locations and seasons in the following year. The higher yield of SSD-C-M6-342-10 and SSD-C-M6-342-10 lines are mainly contributed by the higher number of pods/plant. Based on previous observation of Asadi et al. [4], the number of pods/plant has a direct effect on seed yield, and more pods will contribute to higher yield.

3.2. Advanced yield trial

The results of the advanced yield trial in Pamijahan during the second planting season of 2017 was shown in Table 3. There were nine soybean lines that produced yield 15−26% higher than Panderman and 20−31% higher than Anjasmoro (Table 3 and 4). These lines mature at 88−91 days, with the height of 41−50 cm, and their seed size ranged from 24−26 g/100 seeds. The seed size of the selected lines was much larger than the two check varieties (Panderman and Anjasmoro) (Table 3). The nine soybean selected lines were SSD-C-M7-342-10, SSD-C-M7-350-18, SSD-D-M7-372-14, SSD-E-M7-387-1, Bulk-C-M7-458, Bulk-C-M7-493-1, Bulk-C-M7-493-20, Bulk-C-M7-499 and SSD-E-M7-404-18. Among the nine selected lines, SSD-C-M7-350-18 and Bulk-M7-E-557 lines produced seed yield above 2.5 t/ha, which were 26% higher than Panderman, and 31% higher than Anjasmoro. The nine selected lines need to be tested in multilocations and seasons, and 1−3 of the best lines (with the highest potential and yield) will have the opportunity to be released as newly improved varieties.

Total seed yield is influenced by other agronomic characters, such as maturity date, plant height, number of branches, number of pods and the number of fertile nodes, as well as the size of seeds (100-seed weight). Correlation analysis between agronomic characters (Table 4) in advanced yield trial detected a significant positive correlation between seed weight (100-seed weight) and maturity date. On the other hand, seed weight had significant negative correlation with the number of pods. Seed yield was negatively correlated with the number of pods/plant, but had a significant positive correlation with seed weight (100-seed weight). The strong correlation means that 100-seed weight can be used as one of the determining characters in soybean selection for high productivity trait. However, to find out the direct or indirect effects between agronomic characters on seed yield, it is necessary to do path analysis between the agronomic characters [4,13,14].

Table 1. Yield and agronomic characters of M_6 soybean lines under preliminary yield trial at Pamijahan, Bogor (2017).

Mutant lines	DF	DM	PH	NB	NP	Yield (t/ha)
SSD-C-M6-338-6	**38**	**95**	**53.1**	**2**	**26**	**2.586**
SSD-C-M6 -342-10	**37**	**93**	**57.4**	**2**	**25**	**2.636**
SSD-C-M6 -350-18	37	93	49.5	1	22	2.072
SSD-C-M6-351-19	37	93	47.9	2	20	1.934
SSD-C-M6-352-20	**38**	**93**	**53.3**	**1**	**23**	**2.277**
SSD-C-M6-353-21	37	93	51.1	2	24	2.212
SSD-C-M6 -358-26	37	93	44.3	2	28	1.935
SSD-D-M6-360-2	37	93	50.2	2	22	2.152
SSD-D-M6-362-4	38	91	50.2	2	21	2.026
SSD-D-M6-372-14	**38**	**92**	**49.0**	**2**	**26**	**2.262**
SSD-D-M6-373-15	38	94	39.5	2	27	1.681
SSD-D-M6-377-19	37	91	44.4	3	25	1.791
SSD-E-M6-387-1	**37**	**93**	**50.6**	**2**	**22**	**2.363**
SSD-E-M6-411-25	38	93	49.6	3	28	1.829
SSD-E-M6-418-32	38	91	47.0	4	29	1.426
Bulk-C-M6-458	38	91	42.2	2	26	2.033
Bulk-C-M6-465	37	93	48.7	2	24	2.122
Bulk-C-M6-467	**37**	**93**	**50.7**	**2**	**25**	**2.248**
Bulk-C-M6-470	37	93	53.3	2	26	2.115
Bulk-C-M6-476	37	94	52.8	2	25	2.020
Bulk-C-M6-493	**38**	**95**	**53.6**	**2**	**26**	**2.269**
Bulk-C-M6-497	38	91	50.7	3	27	2.198
Bulk-C-M6-499	38	95	46.4	2	30	2.178
Bulk-C-M6-501	37	93	44.4	2	21	2.010
Bulk-D-M6-503	37	93	44.8	2	26	2.127
Bulk-D-M6-504	38	92	47.9	2	25	1.868
Bulk-D-M6-506	39	97	49.5	2	25	1.869
Bulk-D-M6-507	**37**	**93**	**51.7**	**2**	**23**	**2.238**
Bulk-D-M6-508	38	92	48.5	2	25	1.960
Bulk-D-M6-534	37	92	50.7	2	23	2.134
Bulk-D-M6-537	37	93	49.1	3	24	2.036
Bulk-E-M6-557	38	93	37.2	3	36	2.192
Bulk-E-M6-561	38	93	48.9	2	27	1.804
Bulk-E-M6-562	38	93	51.9	2	23	1.983
Bulk-E-M6-567	**38**	**93**	**46.1**	**3**	**29**	**2.339**
Bulk-E-M6-580	38	93	50.2	2	22	2.114
Mo (F10G.10428 × Panderman)	36	93	53.0	2	23	2.470
G.10428	37	93	47.8	1	14	2.418
Panderman	37	98	52.8	2	37	1.640
Anjasmoro	42	93	65.3	2	29	2.182
Mean	38	93	49.4	2	25	2.094
LSD 0.05 (%)	1	1	5.4	1	9	0.410

DF = days to flowering (days), DM = days to maturity (days), PH = plant height (cm), NB = number of branch/plant, PN = number of pod/plant.

Table 2. Yield and agronomic characters of M_7 selected mutant lines in advanced yield trial at Pamijahan, Bogor (2017).

Genotypes	DM	PH	NB	NP	NN	100 SW	Yield (t/ha)
SSD-C-M7-338-6	88	45.4	1	24	7	23.74	1.707
SSD-C-M7-342-10	**89**	**47.7**	**1**	**23**	**6**	**24.89**	**2.334**
SSD-C-M7-350-18	**91**	**43.1**	**2**	**17**	**5**	**25.99**	**2.536**
SSD-C-M7-353-21	89.	50.3	2	16	5	23.87	2.298
SSD-D-M7-360-2	89	42.4	2	26	7	21.37	2.231
SSD-D-M7-372-14	**88**	**48.9**	**2**	**24**	**7**	**22.94**	**2.482**
SSD-E-M7-387-1	**91**	**50.1**	**1**	**21**	**7**	**25.56**	**2.460**
Bulk-C-M7-458	**89**	**48.0**	**1**	**23**	**7**	**24.79**	**2.425**
Bulk-C-M7-465	89	46.8	1	26	7	24.49	2.279
Bulk-C-M7-493-1	**90**	**47.6**	**1**	**27**	**7**	**24.37**	**2.346**
Bulk-C-M7-497	87	47.2	1	23	7	24.20	2.089
Bulk-D-M7-503	90	46.9	2	24	7	23.83	2.077
Bulk-D-M7-507	89	50.0	2	23	7	24.26	2.259
Bulk-D-M7-534	89	50.1	2	25	7	22.75	2.057
Bulk-C-M7-493-20	**89**	**40.7**	**2**	**29**	**6**	**23.76**	**2.525**
SSD-D-M7-374-16	89	46.5	1	20	7	25.32	2.130
Bulk-C-M7-499	**88**	**46.6**	**1**	**22**	**7**	**24.45**	**2.320**
SSD-C-M7-358-26	88	38.6	1	26	7	20.81	1.976
Bulk-C-M7-478	88	46.4	2	26	7	22.42	1.920
SSD-E-M7-404-18	**88**	**44.5**	**1**	**21**	**6**	**24.54**	**2.451**
Mo:(G10428 × Pand)-10-1	93	44.4	2	28	7	28.41	2.070
G.10428	91	32.6	1	18	5	34.08	1.534
Panderman	91	44.1	1	32	8	18.34	2.015
Anjasmoro	87	55.6	1	34	7	16.48	1.925
Mean	89	46.0	1.48	24.10	6.63	23.99	2.185
LSD 0.05	2	5.2	NS	8	NS	2.14	0.424

DM = days to maturity (days), PH = plant height (cm), NB = number of branch/plant, NP = number of pod/plant, NN = number of fertile nod, 100 SW = seed weight (g/100 seeds).

Table 3. Yield and agronomic characters of M_7 selected mutant lines in advanced yield trial at Pamijahan, Bogor (2017).

	DM (X1)	PH (X2)	NB (X3)	NP (X4)	NN (X5)	100 SW (X6)
PH	0.143^{ns}					
NB	0.192^{ns}	0.042^{ns}				
NP	-0.247^{ns}	-0.337^{ns}	0.010^{ns}			
NN	-0.284^{ns}	0.124^{ns}	-0.284^{ns}	**0.588****		
100 SW	**0.464***	0.398^{ns}	-0.319^{ns}	**-0.546***	-0.327^{ns}	

DM = days to maturity (days), PH = plant height (cm), NB = number of branch/plant, NP = number of pod/plant, NN = number of fertile nod, 100 SW = seed weight (g/100 seeds).

4. Conclusions

In preliminary yield trial, nine mutant lines produced higher yield compared to check varieties. Of those nine lines, two M_6 lines produced the best yield and better agronomic characters. Those two lines were SSD-C-M6-338-6 and SSD-C-M6-342-10. In advanced yield trial, 9 of 20 selected M6-mutant lines produced higher yields, 15−26% higher than Panderman and 20−31% higher than Anjasmoro), and better agronomic characters. Those nine lines were SSD-C-M7-342-10, SSD-C-M7-350-18, SSD-D-M7-372-14, SSD-E-M7-387-1, Bulk-C- M7-458, Bulk-C-M7-493-1, Bulk-C-M7-493-20, Bulk-C-M7-499 and SSD-E-M7-404-18. The nine selected soybean lines need to be tested in multilocation and seasons in order to select one or more of the best selected promising lines (high potential and yield, and other excellent agronomic characters) will have opportunity to be released as newly improved varieties.

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Related wild species for breeding of tomato resistant to early blight disease (*Alternaria solani*)

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Abstract. Tomato (*Solanum lycopersicum* L.) is susceptible to many fungal diseases, including early blight of foliage caused by the necrotroph fungus *Alternaria solani*. Frequent application of fungicide is the major component to keep the disease low. Resistant tomato cultivar is the most desirable as it can reduce the cost of disease control significantly. So far, sources of resistance to early blight can only be found in wild relative species of tomato, and few of them have been used in traditional breeding. Unfortunately, tomato lines bred from wild donor parent still exhibit poor horticultural performances like low yield, and late maturity, and indeterminate plant habit, which hinders the release of these lines directly as cultivars. The quantitative expression and polygenic control of the early blight resistance trait, as well as the influence of plant developmental stages and environmental conditions, complicate phenotypic selection in traditional breeding. Genotypic selection by using closely linked-markers to the resistance loci is thus preferred, but mapping of early blight resistance QTL in interspecific crosses of tomato have not obtained markers which are useful for marker-assisted breeding. This review presents sources of early blight resistance in wild species of tomato and efforts in dissecting early blight resistance QTL via linkage analysis with molecular markers. Strategies to obtain closely-linked markers and genomics-assisted breeding to facilitate the introgression of useful resistance genes to cultivated tomato are discussed.

Keywords: tomato, early blight, wild related species, marker-assisted breeding.

1. Introduction

The cultivated tomato, *Solanum lycopersicum* L. section *Lycopersicon* (formerly *Lycopersicon esculentum* Mill.) [1] is susceptible to over 200 diseases [2]. Fungal diseases are the most important threat to tomato productivity and can increase production costs by 30% due to application of fungicides alone [3]. Early blight (EB) caused by the necrotroph fungus *Alternaria solani* Ellis and G. Martin, is the most frequent and widely distributed foliar diseases of tomato especially in areas with high rainfall and humidity [4]. About 15–20 fungicidal sprays must be applied per season to achieve reasonable control of EB [5]. Therefore, improvement of cultivars with increased fungal resistance is still the primary goal of private and public tomato breeding program [6].

EB, as the name implies, is strongly associated with tomato with early maturing type. Older senescing leaves and plants at fruiting stage or with heavy fruit load are more susceptible to the disease [4]. Consequently, early-maturing cultivars are more susceptible to EB than medium- or late-

maturing cultivars because leaves of early-maturing cultivars tend to senesce earlier in the growing season [7]. Currently, no early maturing tomatoes have adequate EB resistance under field epidemics and therefore breeders are still developing highly resistant cultivar with desirable horticultural performances [8].

Sources of EB resistance are not known in cultivated tomato but can be found in its wild relative species. However, their utilization in the development of resistant tomato cultivar have been restricted by incompatibility barrier, the quantitative expression of EB which makes difficult the selection of the best and promising progenies, and negative linkage drag introgressed from the donor parent [9, 10, 11]. This review presents sources of resistance in wild related species of tomato to *A. solani*, progress in their utilization in EB resistance breeding, mapping of the EB resistance genes, and strategies to facilitate and accelerate the transfer of resistance to cultivated tomato.

2. Wild related species of tomato

Wild tomatoes are native to western South America and distributed along the coast and in the Andes from Ecuador through Peru and to northern Chile, and in the Galapagos Islands [12]. Wild tomato species grow in a variety of habitats, from coastal regions to high altitude (over 3,300 m) of mountain regions, near river and creeks, as well as in extreme dry habitats [13]. Taxonomic classification based on morphological data combined with molecular data of chloroplast DNA (cpDNA) restriction fragment length polymorphisms (RFLPs), nuclear microsatellites, isozymes, internal transcribed spacers of nuclear ribosomal DNA (ITS; multiple copy), the single-copy nuclear encoded granule-bound starch synthase gene (GBSS or waxy gene), and amplified fragment length polymorphisms (AFLP) recognized 12 wild related species of tomato [14].

3. Early blight resistance in wild related species of tomato

Extensive screening program in the temperate and tropical areas performed in the field or under controlled environment in glasshouse identified six wild tomato species to have high or useful resistance to *A. solani* (Table 1). These are *S. arcanum* (syn. *L peruvianum*), *S. chilense* (syn. *L. chilense*), *S. habrochaites* (syn. *L. hirsutum*), *S. neorickii* (syn. *L. parviflorum*), *S. peruvianum* (syn. *L. peruvianum*) and *S. pimpinellifolium* (syn. *L. pimpinellifolium*) (Table 1). *S. lycopersicoides* was also resistant to this fungus in a laboratory test studying host-pant interaction of fungal necrotroph [15]. The resistance of *S. pimpinellifolium*, the most closely related species to cultivated tomato, to *A. solani* is usually less than the other wild species.

Resistance to *A. solani* is characterized by low percentage of disease severity (in the case of natural or artificial infection in field tests and artificial spray inoculation in glasshouse tests) and small lesion size of less than 1 cm^2 (for droplet inoculation of detached leaves in laboratory or intact leaves on whole plant in glasshouse; Figure 1). Detailed observations on resistant accessions showed that apart from small in size, the frequency of necrotic lesions on resistant species was also lower compared to that on cultivated tomato or susceptible accessions [16].

Fungal growth and sporulation in the lesion of strong resistance source were also limited [15]. Intra-variation of EB resistance occurred within the same species. This was especially observed in *S. habrochaites*, *S. peruvianum* and *S. pimpinellifolium* where different accessions showed a range of disease responses from resistant to highly susceptible [16,17].

Table 1. Wild related species of tomato with resistance to *Alternaria solani*, the fungal agent of early blight disease.

Species and accession	Test method and environment	Disease severity*	Reference
Solanum arcanum LA2157	Droplet inoculation on leaves; glasshouse	1.4 mm^2	[16]
S. chilense G1.1556	Droplet inoculation on leaves; glasshouse	6.7 mm^2	[16]
S. chilense LA3111	Droplet inoculation on leaves; laboratory	Hypersensitive response lesion	[17]
S. habrochaites			
PI126445	Spray inoculation; field	N/A	[18]
PI390513, PI390514, PI390516, PI390658, PI390660, PI390662, PI390663	Spray inoculation; field	N/A	[19]
B6013	Natural infection; field	10.6–10.8%	[20]
LA2100, LA2124, LA2204	Spray inoculation; glasshouse	2.0–2.9 (on a disease scale of 1–9)	[21]
LA2099, LA1777, PI126445, PI390662	Spray inoculation; glasshouse	11.6–21.2%	[22]
S. lycopersicoides LA2951	Droplet inoculation on detached leaflets; laboratory	2 mm^2	[15]
S. neorickii G1.1601	Droplet inoculation on leaves; glasshouse	6.68 mm^2	[16]
S. peruvianum			
LA2192, LA1365, LA1910, LA1983, PI270435, PI365951, PI390665, PE33, PI390671	Spray inoculation; glasshouse	2.0–3.9 (on a disease rating scale of 1–9)	[21]
PE33, PE44, PI390665	Droplet inoculation on leaves; glasshouse	1.46–6.23 mm^2	[16]
S. pimpinellifolium			
PI212408, PI251320, PI365912, PI365928, PI390519, PI303662	Spray inoculation; field	N/A	[19]
EC-65992,EC-65993, EC-85617, EC-96522, EC-121453	Natural infection; field	N/A	[23]
A1921	Natural infection; field	9.0–11.7%	[20]
L4394 (IHR1939)	Spray inoculation; field	38.0%	[24]

*Measured quantitatively based on necrotic lesion size (length × width) in mm^2, percentage of cumulative disease index or defoliation, or percentage of leaf area infected on a diseases rating scale, or measured qualitatively for the presence of small hypersensitive lesion. N/A = data not available.

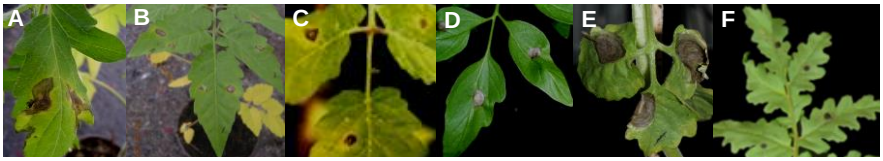

Figure 1. Variation in early blight necrotic lesion sizes among cultivated tomato *Solanum lycopersicum* (A) and its wild relative species *S. arcanum* LA2157 (B), *S. habrochaites* LA2650 (C), *S. neorickii* G1.1601 (D), *S. pennelii* (E), and *S. lycopersicoides* LA2951 obtained after droplet inoculation of *Alternaria solani* spores. Panel A to E were personal documentation, whereas panel F was taken from Smith *et al*. [15].

4. Introgression and marker-assisted breeding for early blight resistance

Introgression breeding often carries negative linkage drag which can persist within a genome despite repeated backcrossing, especially if recombination is suppressed [11]. This phenomenon also occurs in tomato breeding for resistance to EB using wild donor parent. So far, only *S. habrochaites* PI126445 that has been used in the development of resistant tomato and result in several moderately resistant lines [25–28]. EB resistance in these lines is strongly associated with late maturity, low yielding ability, and indeterminate growth habit [22]. Plants with indeterminate/semi-determinate growth habit continue producing younger leaves which are less susceptible to the fungus. Consequently, they appear healthier than determinate plants while they may not possess genetic resistance [9].

Genetic analyses, either classical or via linkage analyses with molecular markers, concluded that EB resistance is under complex genetic control. EB resistance is quantitatively expressed and controlled by additive and non-additive interaction effects of multiple genes and highly influenced by physiological maturity and environmental factors [5,7,24,28,29,30,31,32]. The heritability estimate of EB resistance is low to moderate (0.26–0.72) [5,29,32,34].

The complex and modest heritability of EB inheritance together with the aforementioned confounding factors to EB resistance expression have slowed the breeding process, which relies on phenotypic selection. To speed up breeding process, genotypic selection using closely-linked markers to EB resistance loci is needed. Unfortunately, progress in mapping quantitative trait locus (QTL) with effects on EB resistance in three interspecific crosses, i.e. *S. lycopersicum* NC84173 × *S. habrochaites* PI126445 [26], *S. lycopersicum* cv. Solentos × *S. arcanum* LA2157 [25] and *S. lycopersicum* NCEBR1 × *S. pimpinellifolium* LA2093 [30], has not identified closely-linked markers to EB resistance. Five to 14 QTLs which encompassed a range of marker intervals (1.8 to 73.0 cM) were identified in 7 of 12 tomato chromosomes with a rather low individual effect (3.0% to 25.9%; Table 2). Few QTLs were contributed from the susceptible parent [26]. Some QTLs were species specific, but some were common or in overlapped positions despite identified in different genetic background and environment, indicating their authenticity on EB resistance and deserve further genetic dissection [26].

Within such large intervals, markers are loosely linked with EB resistance and thus are not applicable for marker-assisted breeding because of crossovers between markers and the EB resistance QTLs [2]. Fine mapping to locate the QTLs precisely must be attempted by development of a series of near-isogenic lines (NILs) and sub-NILs consisting of plants each with a different single homozygous introgression containing one target QTL [9]. Marker-assisted selection is applied to speed up the return to recurrent parent type by screening individuals for the presence of the target locus in each generation of backcross and the absence of extraneous donor DNA throughout the rest of the genome. Fine mapping is not only essential for validation the actual effect of individual QTL, but also necessary for reducing the linkage drag associated with introgressed QTL, and to determine whether QTL effects on EB resistance are caused by several tightly linked genes or by one gene with pleiotropic effects [2,4].

Table 2. Quantitative trait loci (QTLs) associated with resistance to *Alternaria solani*, the fungal pathogen of early blight disease of tomato (*Solanum lycopersicum*).

Marker type	Chr[a]	LOD score	PVE[b] or R^2 value	Interval (cM)	Reference
Parent; size and type of linkage mapping population; and type of population for QTL analysis					
S. lycopersicum NC84173 × *S. habrochaites* PI126445; 145 BC_1 plants; 145 BC_1 plants and BC_1S_1 families[c, d]					
RFLPs (141) and RGAs (23)	1	3.5–7.0	7.5–21.9%	73.0	[33]
	2	2.8–2.9	15.3–15.9%	39.6	
	3	2.9	9.1%	13.1	
	5	2.4–2.6	7.3–7.9%	24.2	
	5	2.4–3.7	7.3–11.3%	27.8	
	8	3.0–3.7	9.0–10.3%	16.3	
	8	5.2–5.4	14.3–21.0%	53.8	
	9	2.8–8.2	7.5–25.9%	51.2	
	9	3.7–5.1	10.1–16.2%	34.7	
	10	4.1–6.8	10.1–20.2%	48.0	
	11	3.2–3.8	11.5–13.2%	15.8	
	11	3.0–3.2	7.1–9.9%	22.2	
	12	2.5–3.1	8.3–10.3%	26.3	
	12	4.1	12.9%	26.7	
S. lycopersicum cv. Solentos × *S. arcanum* LA2157; 172–6 F_2 plants; 175 F_2 plants and 156 F_3 families[e]					
31 SSRs, SNPs, and 344 AFLPs	1	4.1	6.8%	31.0	[31]
	2	4.2–5.6	7.2–10.3%	42.0	
	2	3.4– 9.0[f]	7.6–16.2%	18.0	
	5	4.0–6.1	8.1–10.5%	36.0–41.0	
	6	3.7–6.3	8.2–10.8%	21.0–36.0	
	7	7.5–8.3[f]	13.3–16.0%	30.0–33.0	
	9	4.8–5.2	8.2–9.2%	31.0	
	9	4.6–8.7	8.6–15.5%	22.0–23.0	
S. lycopersicum NCEBR1 × *S. pimpinellifolium* LA2093; 172 RILs (F_7); 172 F_7 and 4128 RILs for each F_8 to F_{10} generation[c]					
RFLPs, ESTs, CAPSs, and SSRs (total 294 markers)	2	3.0–3.6	3%	3.5–11.1	[35]
	2	2.5–3.6	8%	13.9–17.2	
	5	3.9–7.1[f]	11–18%	5.7–12.1	
	6	3.7–4.9	16%	2.5–14.2	
	9	3.0–5.1[f]	7–14%	1.8–9.0	

[a] Chr = chromosome.
[b] PVE = phenotypic variation explained.
[c] Identified by linkage analysis of marker with percent defoliation in the field using simple interval mapping (SIM) and composite interval mapping (CIM) approaches.
[d] Selective genotyping was applied.
[e] Identified by linkage analysis of marker with lesion size and percentage of small lesions in glasshouse and disease scores in the field using multiple-QTL-model mapping (MQM) procedure.
[f] Resistance alleles were contributed from the susceptible parent.

Robust marker like single nucleotide polymorphisms (SNPs) which detect minor variation will be useful not only for selection of a resistant genotype at the early seedling stage, but also for dissection of complex quantitative resistance into individual genes, and understanding the genetic basis of correlation between EB resistance and negative horticultural traits [32]. A large number of SNPs obtained from the next generation sequencing (NGS) projects and high throughput genotyping platforms is now available [33,34]. The recent development in genomic research can also aid in QTL dissection. For example the combination of high-throughput transcriptome analyses with a permanent population such as introgressed lines (ILs) [35]. ILs with a differential performance for the trait of interest are comparatively analyzed for transcriptional regulation. Single differential genes identified by microarray analysis are validated using real-time qPCR and then validated for its functionality through mutagenesis or transformation. Closely linked marker can then be used in genomic-assisted breeding [35].

5. Concluding remarks

Seven related wild species of tomato possess medium to strong EB resistance, which is not present in the cultivated tomato. Development of EB resistant tomato lines using wild donor species is still hampered by complex genetic control of resistance and negative linkage drag introgressed from wild donor parent, like late maturity, indeterminism, and reduced yield. Progress in genetic mapping of EB resistance QTL studies has not obtained closely-linked marker to EB resistance genes which can be used in marker-assisted breeding. Development of NILs, sub-NILs and permanent population ILs is necessary to fine-mapping the QTL position, to estimate the actual individual effect of each gene, and to break the linkage between the early blight resistance gene and negative horticultural traits if these traits are caused by several tightly linked genes. The application of recent development in genomic research such as large number of SNPs discovered from the NGS technology and high-throughput transcriptome analyses combined with ILs can aid precise identification of EB resistance genes which are useful for introgression breeding.

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Point mutation of ITS-nrDNA sequences as specific markers of three durian species: *Durio zibethinus*, *D. kutejensis* and *D. lowianus*

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Abstract. Molecular markers are considered more efficient tools than morphological markers for species identification. This research aimed to get specific molecular markers among three durian species *Durio zibhetinus*, *D. kutejensis* and *D. lowianus* using ITS-nrDNA sequences. A number of 139, 7 and 1 ITS sequences of *D. zibethinus*, *D. kutejensis* and *D. lowianus*, respectively, were used in this experiment. Each group of sequences was then aligned to make one consensus sequence. In order to identify the point mutations, the aligned sequences were cut with restriction enzyme *in silico* using Genious ver. 7 software. The simulations found that each consensus sequences has different point mutation forming different restriction site. Consensus sequences of *D. zibethinus* has *Eco*R1 site on base number 280, *D. kutejensis* has *Ama*871 site on base number 394 and *D. lowianus* has *Aco*1 site on base number 135. These signals could be used as specific markers for the three durian species.

Keywords: *Durio*, ITS-nrDNA, point mutation, species markers.

1. Introduction

Indonesia is the centre of origin and distribution of the genus *Durio*, and is found to have high diversity of durian genetic resources [1,2]. Every natural durian production area consists of very high genotype variation; each single tree is a different genotype [3]. Complex germplasm variation, therefore, could create conflict in determining and naming a durian type among community groups. Different naming occurred in the same genotypes. Although, high genetic variation is an essntial resource to variety improvement, either through selection [4] or breeding programs [5].

Phylogenetic analysis is one of the important steps whether to select parent trees or determine breeding strategies. This analysis could determine accessions which genetically contain wild traits that we need, or which accessions have already been developed. Phylogenetic studies are also useful in helping rationalize the number of accessions in conservation activities [6–8]. In this way, it could facilitate an efficient collection management through maintain fewer accession but still contain high genetic diversity.

Phylogeny among organisms is now generally studied based on DNA sequencing polymorphisms from sustainable genes that function as bar codes such as Internal Transcribed Spacers (ITS) [9]. The

rate of mutation in this gene is suitable for phylogenetic study in angiosperms and eukaryotes at low taxonomic levels [9]. ITS-nrDNA has also been used for studies of various plants at the intra-species level [10–12]. The massive use of this gene for phylogenetic studies is due to its advantages among others, including a high number of repetitions in the core genome, fairly rapid evolution through gene crossing and conversion, short size (<700 nt), and conserved sequence areas on both sides which leads to easy amplification [9].

Besides being used for phylogenetic analysis, in this article ITS gene demonstrated to be suitable markers for determination of three durian species, *D. zibethinus*, *D. kutejensis* and *D. lowianus*. It could specifically determine the species based on the differences in Single Nucleotide Polymorphism (SNP) in the ITS sequence which is related to areas that contain point mutation forming restriction enzymes sites.

2. Materials and methods

Materials used in this study were leaf samples from 7 accessions of *D. ketejensis* and 139 accessions of *D. zibethinus*. Isolation and amplification of durian genomic DNA were performed using genomic DNA isolation kits for plants (Geneaid™). Other materials include liquid nitrogen, PVP-40, β-mercaptoethanol, absolute ethanol, isopropanol, TAE buffer, agarose gel, PCR mix reagent (KAPA 2G), deionized water, 1 Kb DNA ladder, loading dye, DMSO, ITS5 primer (5'-TAG AGA AAG GAG AAG TCA TAA CAA-3') and ITS4 (5'-CCC GCC TGA CCT GGG GTC GC 3').

Isolation of durian genomic DNA was carried out following the protocol of the Geneaid™ isolation kit. Modifications were made during three isolation steps: first, by adding PVP-40 as much as 10% of the sample weight; second, 1% β-mercaptoethanol of the supernatant volume was added before incubating in a water bath; third, the incubation period at 65°C for 10 minutes was extended to 180 minutes.

Amplification of the target area of ITS-nrDNA using 50 ul PCR mix was prepared in a 0.2-ml PCR tube consisting of 1× Master Mix KAPA, 20 ng DNA template, 4% DMSO, and 20 µM primers. PCR mix was then amplified in PCR machine type Thermal Cycler 2720 (Applied Biosystem), with pre-denaturation reaction at 94°C for 3 minutes, followed by 25 cycles consisting of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and elongation at 72°C for 60 seconds. Then, PCR was ended with final elongation at 72°C for 5 minutes. The amplification results were confirmed using electrophoresis method. Samples resulting in positive PCR were then directly sequenced using services of a third party (Macrogen Inc., South Korea).

The total of 7 ITS sequences from *D. ketejensis* and 139 from *D. zibethinus* were each aligned to produce one consensus sequence each. The two consensus sequences were then compared with the D. *lowianus* sequence downloaded from NCBI Genebank. The alignment results were then digested *in silico* using restriction enzymes listed in Genious ver. 7 software to determine the position of the mutation points that can be used as PCR-RFLP specific markers.

3. Results and discussion

A product of about 700 nucleotides was obtained from PCR amplification of durian genomic DNA samples using the ITS primers. This product is in accordance with what was conveyed by [9]. The alignment and sequence analysis of the 3 closely related species *D. kutejensis*, *D. zibethinus* and *D. lowianus* indicate that there were different bases in the consensus sequences of the three species which are point mutations. Thirteen point mutations were obtained from *D. kutejensis* sequences. Their positions are bases number 23, 41, 51, 57, 105, 106, 114, 127, 162, 197, 394, 395 and 509. One point mutation was obtained from *D. zibethinus* sequences, which is base number 283. Meanwhile, three point mutations were obtained from *D. lowianus* sequences, which are bases number 52, 135 and 160 (Figure 1).

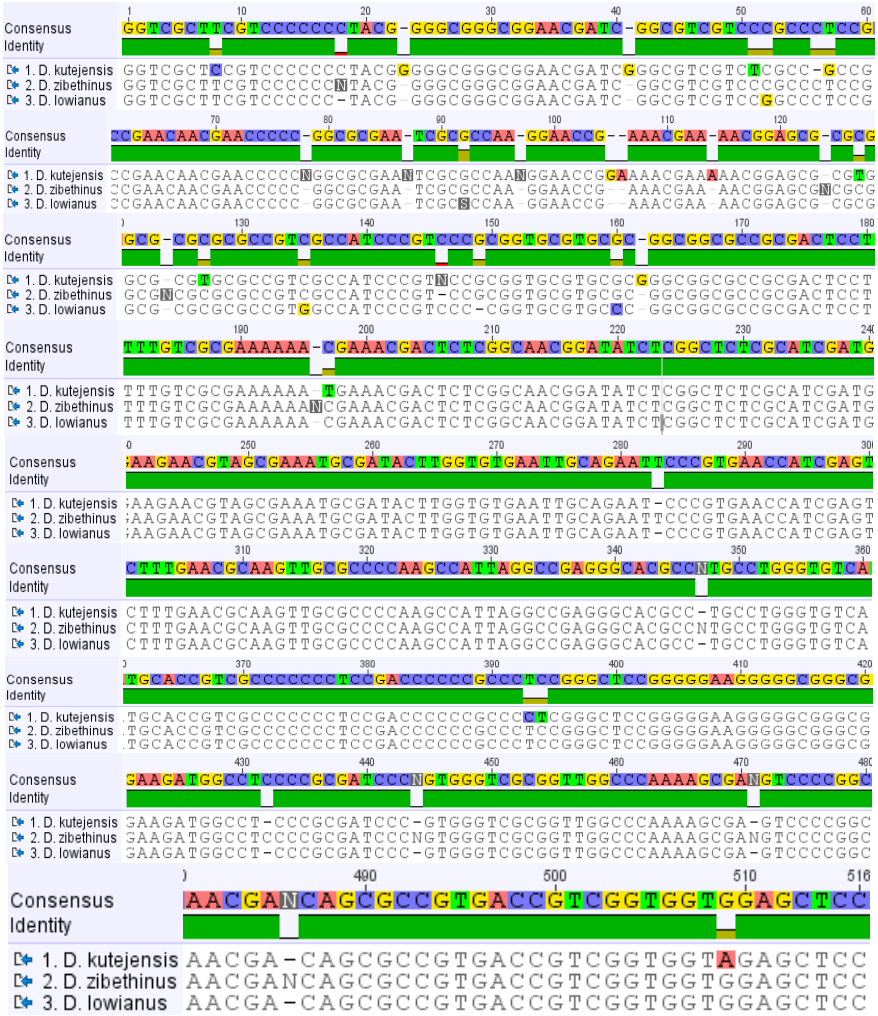

Figure 1. Alignment of ITS-nrDNA sequences of three durian spesies, *D. kutejensis*, *D. zibethinus* and *D. lowianus*, to show the point mutation sites.

In silico analysis amongst three consensus sequences showed the presence of specific restriction sites on each ITS sequences. These restriction sites were the same as one of the point mutations found in the consensus sequence of the three species. Consensus sequences of *D. kutejensis* has *Ama*871 restriction site at base number 394, consensus sequences of *D. zibethinus* has *Eco*R1 restriction site at base number 280, and consensus sequences of *D. lowianus* has *Aco*1 restriction site at base number 135 (Figure 2). The different restriction site found in each consensus sequences showed that ITS-nrDNA sequences have different species signal which could be used as specific markers to differentiate three durian species, *D. zibethinus*, *D. kutejensis* and *D. lowianus*. This finding also showed the evidence that *D. lowianus* stands as different species, whom some researcher consider it to be only as a variation of *D. zibethinus* [1].

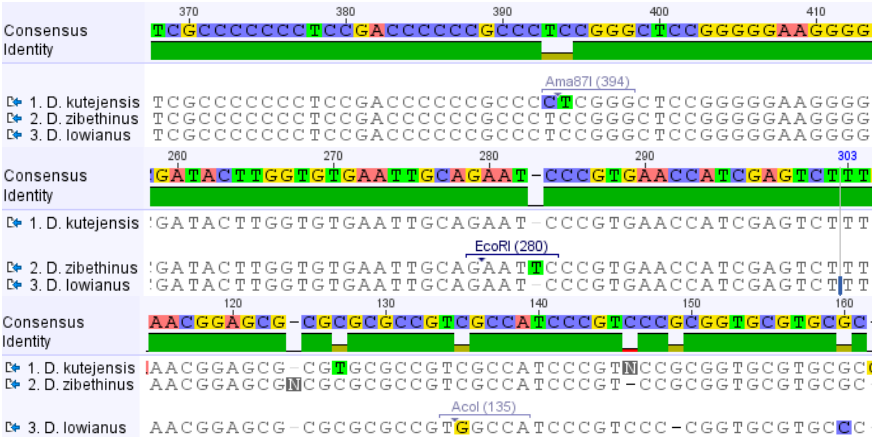

Figure 2. *In silico* analysis of point mutations on ITS-nrDNA sequences among three durian spesies, *D. kutejensis*, *D. zibethinus* and *D. lowianus*, to show enzyme restriction sites as species signals.

The difference of each restriction sites of these sequences can be used as species markers which the detection can be done simply by using PCR-RFLP technique with their respective enzymes without going through sequencing. In this way, the ITS-nrDNA sequences of *D. kutejensis* will be cut into two fragments using the *Ama*871 restriction enzyme, the ITS-nrDNA of *D. zibethinus* will be cut into two fragments using the *Eco*R1 restriction enzyme and the ITS-nrDNA of *D. lowianus* will be cut into two fragments using the *Aco*1 restriction enzyme.

4. Conclusions

Among the three related species *D. kutejensis*, *D. zibethinus* and *D. lowianus*, each of their ITS-nrDNA sequence has a specific restriction site that forms specific signal and could be used as species markers. Further determination for the three species could be simply done by using PCR-RFLP technique.

5. Acknowledgement

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Simulation of QTL by sequencing for agronomic quantitative trait loci detection in small to medium population size in soybean

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Abstract. Quantitative Trait Loci by sequencing (QTL-seq) is a QTL detection method that utilizes the principles of Bulked Segregants Analysis. It detects alleles with extreme frequencies in whole-genome sequence data from two bulked populations with contrasting phenotypes. This approach is less laborious than QTL detection using linkage mapping, and the result had been shown to be comparable in the same mapping population. However, since the genomes of the two bulked populations are completely sequenced, it can facilitate further characterization of the QTL segment and the genes underlying the QTLs. In this study, QTL-seq was simulated using high-density SNP genotyping data from a recombinant inbred population consisting of 188 individuals. The genomes of both parents had been sequenced, and the SNP genotyping identified 2,207 SNP markers that were polymorphic and segregating in the population. Since the markers are dense enough and well distributed across the genome, they can be used to represent the alleles that can be obtained from whole genome resequencing of bulked individuals. The availability of genotype data for each individual in the mapping population also enabled the detection of QTL via linkage mapping. Using data generated from both approaches, various simulations were conducted to compare the results that could be obtained under ideal conditions, as well as less ideal ones such as when the QTL effects are small, the presence of skewed phenotype distribution, and a small number of bulked samples.

Keywords: SNP genotyping, quantitative trait locus detection, QTL-seq, whole-genome resequencing.

1. Introduction

Detection of chromosomal segments containing genes that regulate traits of interest in crops can be a lengthy and costly endeavour. Typical forward genetics approach relies on genetic mapping of the traits in a segregating population, where the location of the gene is inferred from recombination frequency between the trait and DNA markers distributed throughout the genome [1]. Depending on the choice of methods and technology, a significant amount of time, labour, and funding are required to prepare a mapping population, scoring the trait and genotype the DNA markers on each individual, as well as the processing and analyzing the data to generate the map.

Takagi et al. [2] proposed Quantitative Trait Loci by sequencing (QTL-seq) as an alternative mapping method to shorten the time, reduce workload, and reduce cost. It is a variant of Bulked Segregant Analysis (BSA) method [3], where individuals with the highest and lowest phenotypic scores are combined into two bulks, and genotyped using generation sequencing. The allele frequencies of Single Nucleotide Polymorphisms (SNPs) identified from the sequencing data are then compared between the two bulks. SNPs located near the causal gene should show contrasting frequencies in the two bulks, and the authors termed this contrast as SNP index.

The use of whole-genome sequencing simplifies the genotyping process, since screening for polymorphic markers is no longer required. All sequence variations within the population can also be identified from the sequence data [4,5], which will assist in candidate gene identification further down the line. The number of genotyped individuals is also dramatically reduced to just three: the two bulks and one of the parents. This also reduces the wet lab work required to extract the DNA, since each bulk can be treated as a single sample during DNA extraction.

However, some disadvantages can also arise. Since the bulk can only be identified after phenotyping, the total time for phenotyping and genotyping may be longer compared to traditional QTL analysis, where individuals can be genotyped soon after the plants germinate. Sequencing and bioinformatics analysis can also take around one month, so in some cases, this approach could be more time-consuming than regular QTL mapping. Genotyping must also be repeated for multiple traits, as different phenotypes will likely produce the different high and low bulk composition. This approach also needs high depth sequencing [5], which can be expensive for species with large genome size [6]. The resulting analysis also cannot estimate R2 value, as well as the effect of heterozygous alleles.

Despite those disadvantages, this method can still represent a significant saving in some situations and species. Consequently, we attempted to test the applicability of this method in our soybean breeding program under ideal and non optimal conditions. Several non ideal scenarios were tested, such as small sampling size, weak QTL effects and skewed distribution. The results were then compared with regular QTL analysis on a recombinant inbred lines population.

2. Materials and methods

The mapping population consisted of 188 recombinant inbred soybean lines derived from a cross between Tambora and B3293. The phenotype data for this simulation was taken from a single location at Cibalagung, Indonesia. Genotyping was performed using Illumina Soy SNP 6K on the Illumina iScan platform. QTL analysis was first performed using Windows QTL Cartographer [8] to identify strong and weak QTL for comparison with QTL-seq.

QTL-seq simulation was done by first identifying individuals with the highest and lowest phenotypic scores for plant height, seed weight and flowering time traits, to be bulked for SNP index calculation. The bulk size was set at 20 and 10 individuals to see the effect of bulk size on QTL detection. Marker data from each individual were then combined to form a pooled genotype data in each bulk. The delta SNP index (dSNP index) was calculated as the value of the frequency of a major allele in the 'high' bulk minus the frequency of that allele in the 'low' bulk.

dSNP index = (major allele frequency in high bulk)–(allele frequency in low bulk). As an example, a SNP has A and C alleles in the mapping population. The number of A allele in the high bulk of 20 individuals was 32, so its allele frequency is 32/(20×2) or 0.8. In the low bulk, there were only 7 A allele, so the frequency is 7/40 or 0.175. The dSNP index for that marker thus equals to 0.8–0.175 or 0.625. The resulting values for each marker were then plotted as a scatter plot using qqman package in R [8]. For easy comparison, the LOD scores from the same traits from composite interval mapping (CIM) analysis in Windows QTL Cartographer were also plotted in similarly.

3. Results and discussion

The SoySNP6K contains 5,236 SNPs markers selected from Song et al. (2013), which could be assayed concurrently for each individual's DNA. Among those SNP markers, 2,207 were polymorphic in the mapping population, and it should be dense enough to represent most segments that underwent

recombination in the mapping population. Such a high marker density should be able to simulate the pattern generated by even denser marker generating technology like next generation sequencing (NGS).

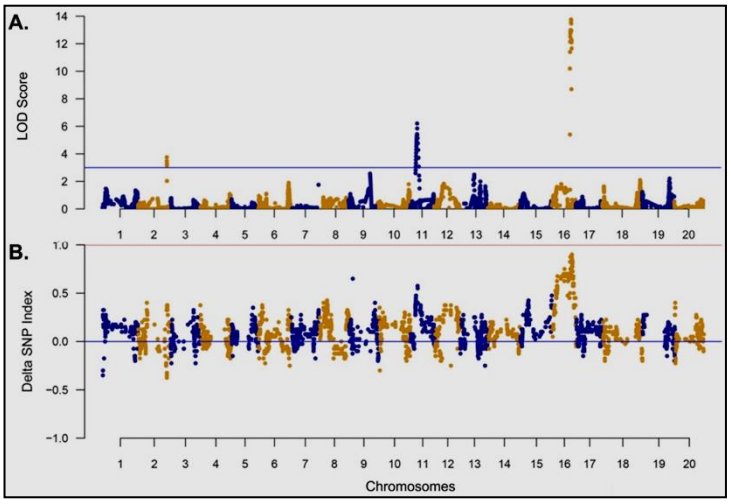

Figure 1. Manhattan plot of QTL detection using CIM in Windows QTL Cartographer (A) and QTL-seq (B) for plant height.

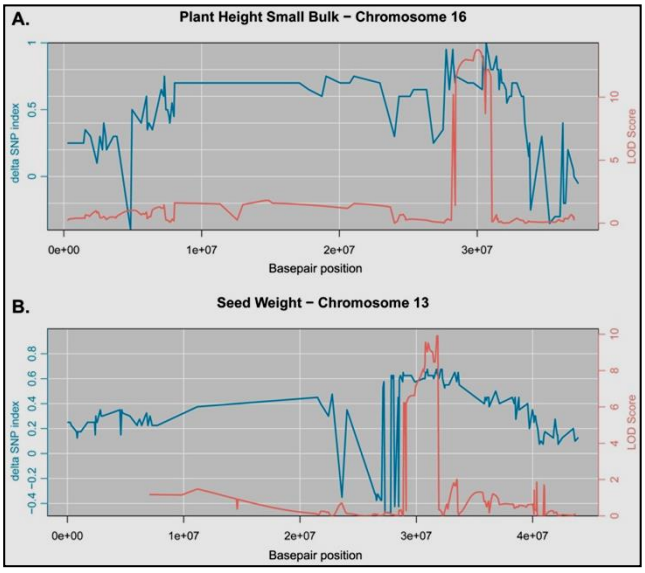

Figure 2. Line plot of LOD scores from Windows QTL Cartographer (blue) and dSNP index from QTL-seq (red) in chromosomes where a strong QTL was detected for plant height (A) and seed weight (B).

For strong QTLs, such as one found in chromosome 16 for plant height (LOD score >13), the locus also showed up as the highest peak in QTL-seq (Figure 1). Likewise, a major QTL for seed weight was detected in chromosome 13 (LOD score >10). Closer inspection of chromosome 16 revealed that the base interval identified by both QTL-seq and regular QTL analysis was mostly similar, with the highest peaks from QTL-seq flanked the same interval that had the highest LOD score in composite interval mapping. However, the shape of the peak from each method was very different (Figure 2).

Composite interval mapping was able to estimate the location of the QTL even when there is no marker data for that segment, an ability that is absent in QTL-seq analysis. For the strong QTL for seed weight in chromosome 13, the peak shapes from each method were also different, but as in plant height, the highest dSNP index values and the highest LOD scores were found to flank largely similar chromosome intervals (Figure 3).

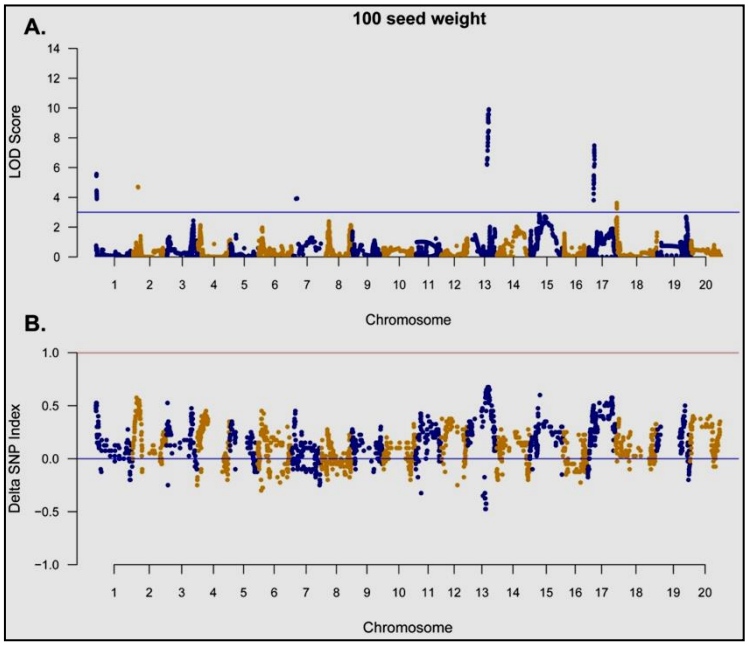

Figure 3. Manhattan plot of QTL detection using CIM in Windows QTL Cartographer (A) and QTL-seq (B) for seed weight.

For weaker QTLs found in plant height and seed weight mapping (LOD score <8), the loci were not always reliably detected using QTL-seq. The second highest QTL for plant height in chromosome 11 was also detected in QTL-seq, but the highest dSNP index in this chromosome was lower than one found in chromosome 9 (Figure 1B), where no significant QTL were detected using composite interval mapping.

The second most significant QTL for seed weight in chromosome 17 was not detected in QTL-seq, as another locus in the same chromosome and several loci in other chromosomes had a higher dSNP index than this QTL (Figure 3B). QTL-seq thus performed less reliably for less dominant QTLs in this mapping population.

Another factor that can influence QTL detection in QTL-seq is the size of the bulk. We observed an increase of dSNP index values when the bulk size was reduced from 20 to 10 at the major QTL for plant height at chromosome 16, although the QTL interval also became enlarged compared to CIM result (Figure 4A and 4B). Logically, the use of smaller bulk size will eliminate individuals with average phenotype scores [11], resulting in the elimination of weak QTL alleles and larger differences of SNP index between the bulk at the expense of decreased sensitivity toward weak-effect QTL. The potential downside is that smaller bulk size will also make the analysis more sensitive to phenotyping error, as individuals that are phenotyped incorrectly will subsequently represent a larger percentage of the bulk. Bulk size reduction appeared not to diminish the ability of QTL-seq to detect weaker QTL, as illustrated by the weaker QTL for plant height at chromosome 11. As in the major QTL for this trait, the dSNP index was higher than when bulk size was set at 20, but the peak value has shifted away from the peak LOD score detected by CIM (Figure 4C and 4D). In this case, we conclude that

although smaller bulk size can improve the contrast between bulks, it can reduce the accuracy of the detection of QTL region by several hundred thousand base pairs.

The last non ideal condition that we tested was skewed phenotypes. In CIM, it is possible to transform the phenotype data of a skewed population to make it resemble a normal distribution [11], and CIM will use the transformed data to find the QTL location. In QTL-seq, phenotype data are only used to select the individuals, so unless the transformation reshuffle the order of the highest to lowest individuals in the phenotype table, the same individuals will still be selected for the highest and lowest bulk respectively, which will negate the benefit of phenotype data transformation.

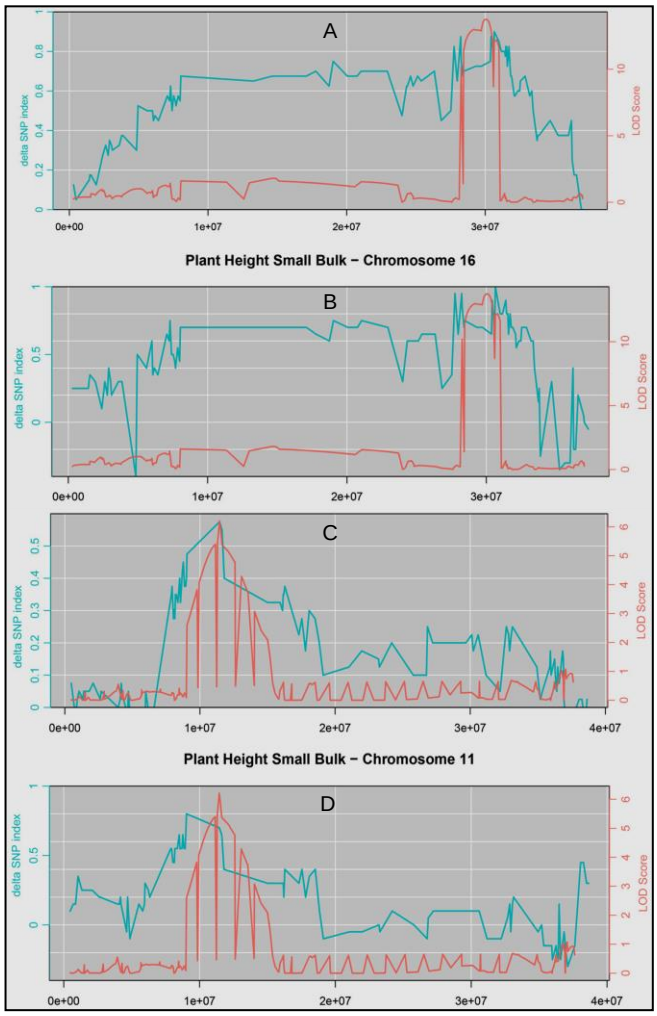

Figure 4. Line plots of LOD scores from Windows QTL Cartographer (blue) and dSNP index from QTL-seq (red). For a strong QTL, a bulk size of 20 individuals (A) and 10 individuals (B) detected similar interval as QTL. For a weaker QTL, the larger bulk size (C) is more consistent with CIM results than the smaller bulk size (D).

The trait that exhibited skewed distribution was flowering time, where the population skewed toward early flowering and a small number of individuals flowered late (Figure 5A). The bulk of early flowering individuals thus could potentially contain a very diverse genetic composition, as the earliest flowering time contained a large percentage of the population, resulting in low contrast between the

late and early flowering bulks. As expected, under such condition QTL-seq did not produce outstanding dSNP index values in loci that were detected by CIM as the location of flowering time QTL in chromosome 11 and chromosome 16 (Figure 5B and 5C).

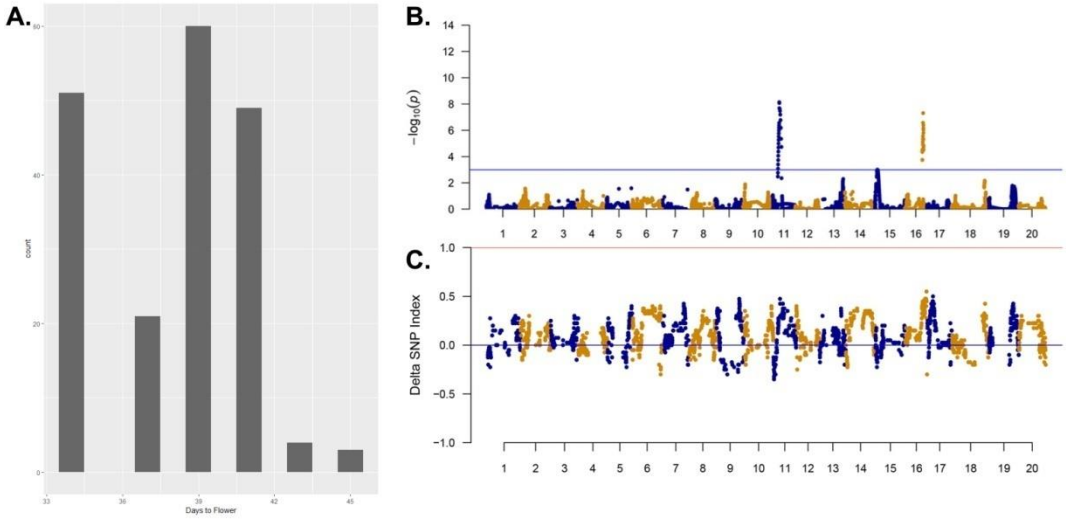

Figure 5. Histogram of flowering time trait in the population (A) and Manhattan plots of QTL detection using CIM in Windows QTL Cartographer (B), and QTL-Seq (C) for flowering time.

4. Conclusions

QTL-seq could reliably detect strong QTL, but less sensitive for detecting weaker effect QTLs. Charts from dSNP index values had different pattern from LOD score charts from linkage-based QTL analysis, but the highest peaks from both methods flanked the same loci, although the QTL interval may shift when the size of the bulk is small and the QTL is relatively weak. QTL-seq was also found to be sensitive to skewed trait distribution. Nevertheless, QTL-seq could be a more economical and less laborious alternative QTL detection method for less complex traits.

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Studies on heritability and genetic variability for grain physical properties in Malaysian rice germplasm

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Abstract. Genetic based information of different traits plays important role in varietal improvement of rice. Twenty rice accessions (*Oryza sativa* L.) obtained from National Rice Gene Bank were evaluated during main season 2015/2016 to estimate heritability, genetic variability and genetic advance for seven grain physical properties. Among the traits, milled grain length/width ratio, milled grain length, milled grain length after cooking and grain length exhibited high estimates of phenotypic coefficient of variation (PCV), and genotypic coefficient of variation (GCV). Highest broad sense heritability and genetic advance was obtained for grain length (98.66% and 62.25%), milled grain length (98.95% and 67.31%), milled grain length/width ratio (98.50% and 80.41%) and milled grain length after cooking (98.93% and 65.44%) which suggest these traits are most probably controlled by additive gene action and hence they can be fixed by selection. However, kernel elongation ratio showed lowest value of broad sense heritability and genetic advance (11.76% and 1.38%, respectively) and may suggest non-additive gene action in their inheritance and selection of this trait may difficult due to high environmental influences. Therefore, improvement of high quality rice with kernel elongation ability may require molecular marker application as it is highly affected by environment for precise selection.

Keywords: germplasm, heritability, genetic advance, genotypic coefficient of variation (GCV), phenotypic coefficient of variation (PCV).

1. Introduction

Grain quality assessment is one of major component in rice breeding program as it determines consumer acceptance for the particular rice variety. Plant breeders typically concentrated on development of high rice variety. However, good grain quality provides better prospects of high economic value for local consumption or for export. Grain physical properties such as uniform shape and general appearance are major factors defining market value [1,2]. Therefore, parental selection from germplasm collection should incorporate wide range of genetic variability for desirable traits. Other than that, it is important to understand the extent of variation that is due to heritable component

and can be used as guidance in planning effective breeding program, as certain quality related traits is influenced by environment.

Genetic parameters, such as genotypic coefficient of variation and phenotypic coefficient of variation are very useful in determining the magnitude of variability that exists in the germplasm. Furthermore, combination of broad-sense heritability and genetic advance estimation allow rice breeder to determine the influence of environment on the expression of genetic factors and repeatability of trait throughout selection process [3]. Kernel elongation trait of indica rice was found to be controlled by genotype, major gene effects and environment interaction using genetic models in two environments [4]. This study was conducted to determine the genetic variability, heritability and genetic advance of grain physical properties in Malaysian germplasm as an early investigation for high quality rice varietal development.

2. Materials and methods

Twenty accessions of rice were evaluated in a field trial in research plot of MARDI Seberang Perai during main season 2015/2016. The seeds were obtained from National Rice Gene Bank, MARDI Seberang Perai (Table 1) and were transplanted in 3 m × 1 m plots with 25 cm spacing in a randomized complete block design (RCBD) with three replications for each accession. Three plants from each accession were selected randomly from each replication for data collection of the following characters which are grain length (mm), grain width (mm), milled grain length (mm), milled grain width (mm), milled grain length/width ratio, milled grain length after cooking (mm) and kernel elongation ratio.

Table 1. List of rice germplasm used in this study.

Acc. number	Rice accession	Sub-species	Origin
MRGB06725	Fawng Nan	*Indica*	IRRI
MRGB00698	Khao Pra Guad 55-2-133	*Indica*	Thailand
MRGB00653	Kapuri	*Indica*	Suriname
MRGB01727	SML 242	*Indica*	Suriname
MRGB10778	Ceysvoni	*Indica*	Suriname
MRGB01846	Washabo	*Indica*	Suriname
MRGB01726	SML 81B-25	*Indica*	Suriname
MRGB01873	ARC7110	*Indica*	India
MRGB09874	Padi Mosilou	*Indica*	East Malaysia (Sabah)
MRGB03532	Rambai Kersik	*Indica*	East Malaysia (Sarawak)
MRGB03337	Karok	*Indica*	East Malaysia (Sarawak)
MRGB05031	Padi Lalang	*Indica*	Peninsular Malaysia
MRGB05022	Lembut Terong	*Indica*	Peninsular Malaysia
MRGB01154	Burong 1	*Indica*	Peninsular Malaysia
MRGB00694	Ketitir	*Indica*	Peninsular Malaysia
MRGB00174	Banyak Anak 192	*Indica*	Peninsular Malaysia
MRGB00490	H 8	*Indica*	Sri Lanka
MRGB00280	Ceylon 8	*Indica*	Sri Lanka
MRGB00533	Hsin Chu 56	*Japonica*	Taiwan
MRGB00348	Cho Ko To	*Japonica*	China

The milled grain length/width ratio was calculated by dividing average length by the average of width of rice kernel. Based on the value, rice grains were classified into short bold (SB), long bold (LB), short slender (SS), medium slender (MS) and long slender (LS) [5]. Furthermore, kernel elongation ratio was calculated by dividing the average length of cooked kernel by the average length of the raw rice [6].

The data was analyzed by analysis of variance (ANOVA) using SAS software version 9.1 (SAS Institute 1998). Moreover, genetic parameters, such as genotypic (σ^2g) and phenotypic (σ^2p) variances, genotypic (GCV) and phenotypic (PCV) coefficients of variation, broad-sense heritability (h^2) and genetic advance (GA), were calculated using mean square values from individual and combined ANOVA tables [7] as the following formula:

$$\sigma^2p = \sigma^2g + \sigma^2e \qquad \sigma^2g = \frac{(M2–M1)}{r}$$

where: r = number of replications, σ^2e= error mean squares, M1, M2 = error and genotype mean squares.

$$GCV = \frac{\sqrt{\sigma^2g} \times 100}{\text{Grand mean}}$$

$$PCV = \frac{\sqrt{\sigma^2p} \times 100}{\text{Grand mean}}$$

$$h^2 = \frac{\sigma^2g}{\sigma^2p}$$

$$GA = K \frac{\sigma^2g}{\sqrt{\sigma^2p}}$$

where: σ^2g = estimated genetic variance, σ^2e = pooled error variance, r = number of replications, K = selection differential and it was 2.06 at selection intensity of 5%.

3. Results and discussion

3.1. Analysis of variance

ANOVA revealed highly significant differences among the accessions for the traits indicating the presence of significant amount of variability among the traits studied except for the kernel elongation ratio (Table 2). Similar results were also reported by Allam et al. [8], Nirmaladevi et al. [9], Gampala et al. [10] and Kumar et al. [11] in their experiments. Mean performance for all traits is presented in Table 3.

Table 2. Analysis of variance (ANOVA) for grain quality traits.

Source of variation	Accession	Replication	Error
df	19 ^{ns}	2 ^{ns}	38 ^{ns}
Grain length	24.46**	0.47*	0.11 ^{ns}
Grain width	0.36**	0.01 ^{ns}	0.01 ^{ns}
Milled grain length	14.16**	0.06 ^{ns}	0.05 ^{ns}
Milled grain width	0.22**	0.002 ^{ns}	0.01 ^{ns}
Ratio MGL/MGW	3.95**	0.02 ^{ns}	0.03 ^{ns}
Grain length after cooking	13.92**	0.13 ^{ns}	0.06 ^{ns}
Kernel elongation ratio	0.004 ^{ns}	0.003 ^{ns}	0.003 ^{ns}

MGL = milled grain length, MGW = milled grain width, ns = no significant differences.
*Significant difference at 5% level, **Significant difference at 1% level in F-test.

Table 3. Mean performance for physical grain properties of tested accessions.

Accession	Grain length (mm)	Grain width (mm)	Milled grain length (mm)	Milled grain width (mm)	Ratio MGL/ MGW	Grain length after cooking (mm)	Kernel elongation ratio
Fawng Nan	11.69±0.22	2.26±0.05	8.42±0.11	2.06±0.04	4.09±0.09	8.64±0.19	1.03±0.03
Khao Pra Quad	11.18±0.31	2.46±0.06	8.01±0.07	2.23±0.06	3.59±0.11	7.93±0.15	0.99±0.02
Kapuri	12.37±0.11	2.50±0.02	8.93±0.12	2.24±0.03	3.99±0.07	8.89±0.25	1.00±0.04
SML 242	11.81±0.37	2.37±0.09	8.84±0.10	2.13±0.04	4.16±0.12	8.83±0.12	1.00±0.01
Cho Ko To	13.28±0.39	2.63±0.09	8.90±0.24	2.34±0.06	3.81±0.08	9.23±0.13	1.04±0.04
Lalang	12.05±0.16	2.43±0.06	8.49±0.13	2.22±0.05	3.83±0.13	8.19±0.25	0.97±0.04
Ceysvoni	12.74±0.22	2.39±0.01	9.12±0.21	2.06±0.03	4.43±0.15	9.67±0.13	1.06±0.01
Padi Mosilou	12.03±0.27	2.45±0.08	8.18±0.14	2.29±0.15	3.59±0.19	8.11±0.10	0.99±0.03
Washabo	11.97±0.23	2.46±0.06	8.91±0.29	2.26±0.03	3.94±0.12	9.21±0.15	1.04±0.03
SML 81B-25	11.88±0.27	2.28±0.06	9.09±0.06	2.03±0.03	4.47±0.06	9.11±0.05	1.00±0.01
Ceylon 8	6.61±0.19	2.66±0.02	4.70±0.10	2.46±0.01	1.91±0.04	4.85±0.21	1.03±0.02
ARC7110	7.07±0.06	2.14±0.05	4.81±0.08	1.90±0.03	2.53±0.08	4.89±0.12	1.02±0.03
Rambai Kersik	6.30±0.04	2.92±0.03	4.30±0.04	2.59±0.04	1.66±0.04	4.41±0.04	1.0±0.01
Karok	6.73±0.08	3.63±0.03	4.79±0.12	3.08±0.04	1.55±0.03	4.83±0.18	1.01±0.02
Lembut Terong	6.13±0.05	2.74±0.09	4.20±0.05	2.42±0.01	1.73±0.03	4.35±0.03	1.04±0.02
Burung (1)	5.91±0.08	2.78±0.14	4.17±0.04	2.42±0.05	1.73±0.06	4.12±0.09	0.99±0.02
Ketitir	6.76±0.05	2.61±0.03	4.55±0.06	2.44±0.01	1.86±0.03	4.85±0.11	1.07±0.01
Banyak Anak 192	7.18±0.16	2.59±0.05	5.06±0.08	2.39±0.05	2.12±0.07	5.07±0.12	1.00±0.03
H8	6.47±0.23	2.82±0.11	3.93±0.12	2.55±0.11	1.54±0.03	4.34±0.14	1.11±0.07
Hsin Chu 56	7.00±0.13	3.21±0.03	4.64±0.08	2.78±0.12	1.67±0.06	5.08±0.04	1.09±0.03
Mean	9.36	2.62	6.60	2.34	2.91	6.73	1.02
LSD ($\alpha = 0.05$)	0.56	0.19	0.37	0.18	0.26	0.40	0.09

MGL = milled grain length, MGW = milled grain width.

3.2. Phenotypic and genotypic coefficient of variation

The estimates of PCV, GCV, h^2 and expected GA values as percent of mean are presented in Table 4. Based on the result, PCV values were higher than their corresponding GCV for all traits demonstrates that the variations expressed were not solely due to genotype, but also influenced by environment. However, the value difference between PCV and GCV for most of the traits studied is low which might be less influenced by the environment [11]. This findings is in line with other study that showed small gap of PCV and GCV value of grain properties on selected rice varieties/accessions [12–15].

3.3. Heritability and genetic advance

Heritability is important to determine trait response to phenotypic selection. Improvement of quantitative trait in plants requires reliable estimation of heritability for effective breeding program [16]. Moreover, h^2 is the relative magnitude of phenotypic and genotypic variance of the traits that gives an idea of the total variation accounted to genotypic effect [17]. High h^2 were observed in most of traits studied viz. grain length (98.66), grain width (91.67), milled grain length (98.95), milled grain width (87.50), milled grain length/width ratio (98.50) and milled grain length after cooking (98.93).

This finding is in line with other studies [8–11]. However, heritability value of kernel elongation ratio in this study is low (11.76) which might be contributed by the selection of materials with no notable accessions with kernel elongation trait. Most of other studies reported high heritability value

of this trait viz. 98% [8], 86% [3] and 91.88% [10]. Nevertheless, Devi et al. [15] reported moderate heritability value for kernel elongation ratio (59%) with low genetic advance (7.5%). Although the high heritability value shows the effectiveness of selection based on phenotypic performance, it does not demonstrate any indication to the amount of genetic progress for selecting the superior individuals which is possible by using genetic advance estimation [3].

Table 4. Variance components and heritability values for grain quality traits.

Parameters	σ^2e	σ^2g	σ^2p	h^2	PCV	GCV	GA	GA%
Grain length	0.11	8.11	8.22	98.66	30.63	30.43	5.83	62.25
Grain width	0.01	0.11	0.12	91.67	13.22	12.66	0.65	24.97
Milled grain length	0.05	4.70	4.75	98.95	33.02	32.85	4.44	67.31
Milled grain width	0.01	0.07	0.08	87.50	12.04	11.26	0.51	21.69
Ratio MGL/MGW	0.02	1.31	1.33	98.50	39.63	39.33	2.34	80.41
Grain length after cooking	0.05	4.62	4.67	98.93	32.11	31.94	4.40	65.44
Kernel elongation	0.003	0.0004	0.0034	11.76	5.72	1.96	0.01	1.38

MGL = milled grain length, MGW = milled grain width, σ^2e = error variance, σ^2g = genotypic variance, σ^2p = phenotypic variance, PCV = phenotypic coefficient of variation, GCV = genotypic coefficient of variation, h^2 (%) = broad sense heritability, GA = genetic advance, GA% = genetic advance as percent of mean.

High heritability with high genetic advance is demonstrated by grain length (62.25), milled grain length (67.31), milled grain length/width ratio (80.41) and milled grain length after cooking (65.44) that might indicate additive gene action. Furthermore, high heritability with low genetic advance is demonstrated by grain width (24.97), milled grain width (21.69). Kernel elongation ratio had low heritability with low genetic advance (1.38) that might suggest the traits is highly influenced by the environment and phenotypic selection may not be effective in that particular condition. The estimation of genetic advance is more useful as a selection tool when considered jointly with heritability estimates [19].

4. Conclusions

There is high variability of grain physical properties existed among the accessions studied. The genotypic and phenotypic variances may provide information for high grain quality breeding program. The highest heritability with the highest genetic advance value is demonstrated by milled grain length/width ratio trait that might give useful information as rice grain with long and slender properties as the main feature for development of high quality rice for local market. Moreover, more materials from germplasm need to be explored in order to exploit its desirable trait in breeding program and can be combined with application of marker-assisted selection (MAS) for low heritability traits.

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Functional analysis of an appressorium-spesific gene from *Colletotrichum gloeosporioides*

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Abstract. A novel gene (*CAS2*) specifically expressed during appressorium formation was isolated from *Colletotrichum gleosporioides* using Differential Display RT-PCR. *CAS2* comprises 368 deduced amino acid residues and is 50% identical to a hypothetical protein from *Chaetomium globosum*. ProtFun 2.2 server analysis predicted that Cas2 functions as a transport and binding protein. Based on putative transmembrane domain prediction software (HMMTOP), Cas2 protein is composed of five alpha-helical transmembrane domains with a very short external N-terminus tail and long internal C-terminus. ExPASy ScanProsite analysis showed the presence of integrin beta chain cysteine-rich domain, N-myristoylation site, EGF-like domain, 2Fe-2S ferredoxins, iron-sulfur binding region, VWFC domain, fungal hydrophobins signature, membrane lipoprotein lipid attachment site and Janus-faced atracotoxin (J-ACTX) family signature in CAS2 protein. Mutants with deleted *CAS2* were not significantly different in terms of vegetative growth, conidiation, and appressoria production compared to wild type. However, the *cas2* mutant produced multipolar germination, a feature which distinguishes it from wild type strain. Interestingly, the mutant is non-virulent to mango fruits, indicating that *CAS2* may encode proteins that function as novel virulence factors in fungal pathogens.

Keywords: appressorium formation, *Colletotrichum gloeosporioides*, spesific gene, gene disruption, pathogenicity.

1. Introduction

Anthracnose disease, caused by *Colletotrichum gloeosporioides* or *Glomerella cingulata*, is very common and destructive on numerous crop and ornamental plants worldwide. This fungal pathogen is one of the best-studied species among hemibiotrophic fungi for elucidating various aspects of the host-pathogen interaction with its host. The pathogenicity of *C. gloeosporioides* depends on cellular morphogenesis event. Beginning with conidial attachment onto host surfaces, appropriate physicals and chemicals from host plant induced the conidia to germinate. Subsequently, the tip of the germ tube becomes attached to the surface and begins to swell to form a dome-shaped, highly melanized infection cell, the appressorium [1]. Next, a penetration peg emerges from a small area, adhering appressorium against the host surface [2]. The fungus then uses enormous turgor pressure generated in

the appressorium to drive the penetration peg through underlying plant surface [3]. This morphogenesis is a complex process from initiation to maturation, and involves the expression of a number of genes. Identification and characterization of genes that are active during conidial-appressorium morphogenesis is important to understand the molecular mechanisms of fungal differentiation and pathogenesis, and to develop new control methods that are rationally designed with specific targets in mind.

Several genes have been identified in *C. gloeosporioides* that are specifically expressed during appressorium formation er genes involved in the process that have not been discovered [5]. The first appressorial genes identified, *in/24* and *in/26*, were isolated from the rust fungus *U. appendiculatus* by differential screening of a genomic library [6]. *in/24* is expressed when appressoria begin to mature and its expression is maintained throughout maturation. Likewise, *in/26* is upregulated during appressorial maturation, although it is constitutively expressed at low levels in non-differentiated cells. The functions of these genes are unknown. Using the same approach, two appressorium-specific genes (*Mi/23* and *Mi/29*) were identified from *M. grisea* [7] and their functions are also unknown. An additional *M. grisea* gene, *MPG1*, was isolated by differential cDNA cloning and is abundantly expressed during appressorial differentiation and early plant infection [8] during conidiation and in mycelial cultures starved for nutrient, but the importance of this gene was demonstrated by showing that *mpgl* mutants were impaired in appressorium formation. The protein encoded by *MPG1* is a hydrophobin and in addition to its role in spore and appressorium adhesion, it may also act as a developmental sensor for appressorium formation [8].

In addition to *MPG1*, a *PTH11* gene from *M. grisea* was predicted to encode an appressorial transmembrane protein. *PTH11* was identified by Restriction Enzyme Mediated Integration (REMI) mutation [9], and *pth11* mutants failed to form appressoria on inductive surfaces and have decreased pathogenicity. However, these mutants were responsive to exogenous cAMP, which helps in forming functional appressoria and restoring pathogenicity. A PTH11-GFP fusion protein was found to be localized at the cell membrane. Based on these results, it was suggested the PTH11 protein plays a role in activating appressorium signaling as a receptor for inductive surface cues.

Differential display was used to isolate a novel appressorium-specific genes (*CgCAS2*). The sequence of the gene was used to characterize and predict the features and function of the resulting protein. A gene knockout experiment was also performed to observe the gene's function in appressoria formation and pathogenesis.

2. Materials and methods

2.1. Fungal and culture conditions

C. gloeosporioides PeuB was obtained from the stock culture collection of School of Biosciences and Biotechnology, Universiti Kebangsaan Malaysia. The fungal cultures were grown by frequent subculturing on potato dextrose broth (PDA: Difco, USA). Conidia, germinating conidia, appressoria and mycelia were cultivated and harvested [4].

2.2. Genomic DNA and RNA isolation

Total DNA of *C. gloeopsorioides* was isolated from mycelia using the method described by Hamer et al. [1]. Total RNA of conidia, germinating conidia and mycelia were extracted using TRI REAGENT® solution (Molecular Research Center, USA), while RNA from the appressoria was extracted using TRIZOL® solution in combination with mechanical cell disruption by glass beads [4] of the DNA and RNA was tested using agarose gel electrophoresis. Both DNA and RNA were stored at -20°C until further usage.

2.3. Clonning and sequencing of CgCAS2

Isolation of genes active at the appressoria developmental stage (*CgCAS2*) is based on a differential display of mRNA by reverse transcription polymerase chain reaction (PCR) using arbitrary primers. A conidia suspension at 10^6 conidia/ml was induced and incubated for 7 hours to form appressoria on a

glass petri dish (15 cm in diameter) waxed with rubber leaves and papaya fruit wax. Total RNA from appressoria and mycelia was isolated by a modified method used by Redzuan [4] trary ACP primers were used to perform independent reverse transcription PCR reactions by employing a method of GeneFishing™ (SeeGene, South Korea). After separation on 2% agarose gel, the PCR products showing differential expression in appressoria (compared to mycelia) were cloned into pCR2.1-TOPO vectors using TOPO-TA Cloning Kit (Invitrogen, USA). DNA Walking Kit (SeeGene, South Korea) was then used to obtain the full-length sequence of *CgCAS2*.

Three target specific primers (TSPs, Table 1) were designed from the newly-obtained *CgCAS2* sequence using a DNAWalking SpeedUp™ Premix Kit (Seegene, South Korea). Nested PCR was performed by using the DNA Walking Annealing Control Primers (DW-ACP) provided in the Kit and the three TSPs. Each of the DW-ACPs contained a specific ACP primer-binding site at its 3'-end (5'-AGGTC, 5'-TGGTC, 5'-GGGTC and 5'-CGGTC). The amplification contained 100 ng of *C. gloeosporioides* genomic DNA, 4 µl of 2.5 µM DW-ACP (one of DW-ACP 1, 2, 3, and 4), 1 µl of 20 µM TSP, 1, 25 µl of 2× SeeAmp™ ACP™ Master Mix II, and sufficient distilled water to make up a 50 µl reaction. In the second PCR, four PCR reactions were set up, each of which contained 3 µl of the purified PCR product, 1 µl of the 10 µM DW-ACPN provided in the kit, 1 µl of 20 µM TSP 2, 10 µl of 2× SeeAmp™ ACP™ Master Mix II, and 5 µl of distilled water to make up a 20 µl reaction. In the third PCR, four PCR reactions were prepared, each of which contained 1 µl of the second PCR products, 1 µl of the 10 µM universal primer provided in the kit, 1 µl of 10 µM TSP 3, 10 µl of 2× SeeAmp™ ACP™ Master Mix II and 7 µl of distilled water to make up a 20 µl reaction. All of the PCRs were performed on a PCR Thermal Cycler. The extracted PCR products were cloned into the pGEM®-T Easy Vector System (Promega) and sent to a commercial DNA sequencing service (First Base, Malaysia) for nucleotide sequence determination. After the upstream *CgCAS2* sequence was cloned and sequenced, two primers (Hpw-F and Hpw-R) were used to obtain the whole *CgCAS2* gene.

Table 1. List of oligonucleotide primers used in this study.

Name	Sequence	Usage
HpTsp1	GGTGACGACAATGATTTCT	PCR CgCAS2 ORF
HpTsp2	CCCAGTCCCACTTGTTGT	PCR CgCAS2 ORF
HpTsp3	TGTCACCCAGTTATTTGCT	PCR CgCAS2 ORF
Hpw-F	CCGAGGCATAAACCAGGGACGAG	PCR CgCAS2 ORF
Hpw-R	TGATCCCGTTGGTCTTTGCCTTG	PCR CgCAS2 ORF
TrpC-F	CCATGTCAACAAGAATAAAACGC	PCR integration gene replacement vector

2.4. Transformation-mediated gene replacement

Preparation of sphaeroplasts and transformation of *C. gloeosporioides* were performed according to methods described by Rodriguez and Redman [11] gromycin transformants were selected on regeneration medium containing hygromycin B (300 µg/ml) (Sigma, USA). Before transformation, pN1389-PKAC was linearized with *Kpn*1 restriction endonuclease and precipitated with ethanol. Subsequently, 20 µg of DNA was transfected into *C. gloeosporioides* sphaeroplasts.

2.5. Genomic DNA and RNA blot analyses

DNA digestion, agarose gel fractionation, labeling of probes and hybridization were performed according to the kit manufacturer's instruction and standard methods [12] 2.5 kb fragment of *CgPKAC* DNA probe was labeled with [α-^{32}P] dCTP using Ready To Go™ DNA Labeling kit (-dCTP) (Amersham, USA). Hybridization was carried out with hybridization buffer (1 M $Na_2HPO_4.2H_2O$, 1 M NaH_2PO_4, 0.5 M EDTA, 0.1% [w/v] SDS) at 65°C for 4 hrs for pre-hybridization and hybridized

overnight after the labeled-probes were added. The membrane was washed at 65°C with 2× SSC for 10 min followed by 2× SSC and 0.1% SDS, 1× SSC and 0.1% SDS, and 0.5× SSC and 0.1% SDS until the radioactivity signal was low. The washed blots were exposed to Fujifilm for various times at -80°C.

2.6. Appressorium induction on hydrophobic hard surface

Induction of appressorium was tested on a glass slide coated with rubber wax. A total of 50 µl of wax (in chloroform) was spread on glass slide with cotton bud. Subsequently, 25 µl of conidia suspension containing 10^5 conidia/ml were applied on the glass slides. Appressorium formation was observed every hour for 8 hours.

2.7. Virulence assay

Test for pathogenicity was performed as described by Kim et al. [8]. Mature green mangos were infected with conidia of *C. gloeosporioides*. Two modes of inoculation were applied in the pathogenicity test: inoculation on unwounded and wounded mango fruits. Before inoculation, fruits were surface sterilized with 70% ethanol and left to dry at room temperature. A total of 0.5 ml of conidial suspensions at 2×10^4 conidia/ml was applied to the surface of unwounded fruits by spraying the inoculum with a spray gun (Preval, USA), while wounded fruits were inoculated with 20 µl of condial suspension. Mangoes were arranged in moistened plastic trays and incubated at 30°C for two weeks to observe the disease symptoms. Number of lesions was observed daily.

3. Results and discussion

3.1. Sequence analysis of the CgCAS2 gene

A total of 2,150 bp of DNA sequence, which includes the *CgCAS2* ORF, 900 bp of promoter region, and 39 bp of 3'-end regulatory region, was obtained (Figure 1). The *CgCAS2* encodes a protein with 368 amino acids. A GTCGATGTTG sequence at nucleotide position 901 to 903, complying with the Kozak's rule, was found at the start region of the ORF (Figure 1). Comparison between the sequence of the gene and its cDNA sequence revealed a 1,214 bp ORF, which is interrupted by two introns at nucleotide positions 718 to 771 and 1,003 to 1,058, respectively. The intron/exon splice junction (GTA[Y/A]GT/[A/C]AG) of the two introns are typical of splice site sequences in other *C. gloeosporioides* genes and fit the consensus sequences found in other filamentous fungi. The second intron has the internal splicing sequence GCTAACPr necessary for lariat formation in filamentous fungi [14].

Analysis of the 900 bp upstream sequence of the coding region indicates that the 5' flanking region of the *CgCAS2* contains several potential regulatory elements (Figure 1). TATA box is absent in the *CgCAS2* promoter. However, a TATA-like sequence was detected at position -66 bp upstream of ATG. Genes from filamentous fungi often lack classical regulatory sequence of the 5' and 3' non-coding regions of other eukaryotes, and some filamentous fungi promoters do not contain any TATA boxes [14] II CCAAT signal was identified at -311 bp upstream of the start codon. Other putative regulatory elements identified at the upstream sequence are the GAGA factor binding site at -266, -466 and -668, factor NF-E1/NF-E1a/NF-E1b/NF-E1c binding site (YTATCW) at -422 and transcription factor NF-Y/CTF/CBF binding sites (ATTGG) at -261 and -758.

Within the 3'-untranslated region, a putative polyadenylation sequence (5'-AAATAA-3') is detected at the position 1,244–1,249 downstream from the ATG (Figure 1). During processing of pre-mRNA to mRNA, 5-AAAUAA-3' motif is required for proper RNA cleavage and subsequent polyadenylation. The spacing between the *CgCAS2* AAATAA element at position 1,245 and poly (A) tail is 19 bp in length, indicating that this element is most likely recognized during RNA processing [15] ORF encodes a protein with 368 amino acids.

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   -900    ggcccgacgtcgcatgctcccggccgccatggcggccgcgggaattcgatttcacagaag
   -840    tatgccaagcgaggggggggtctagacgatcgtgcagacatcttcaacgactggttttc
   -780    aataggcgtcaatgagctgcaattggaatggtgatttgggttcgtctcgtgtcaaacaga
   -720    agcttcgcctacccaagccagtgggagggaaatgtgggcaaatagatagcagagagcaag
   -660    ctagcaatacggtacagaatttcgggcgaccttcttgcgtctgctgtgcaatcaaacaaa
   -600    gcggtttgacctcaggaaacagaaggtgtcgcccaaaatgtcactgacatgtctgtccgc
   -540    agaggcacgggcttgggggaaaatgtgtcaaacccaactaccgaccgtcctgaccgaggc
   -480    ataaaccagggacgagagctgcgtcaagaaaccttcgcactcgtttacagtcgcatacta
   -420    tcatagatctgcgtggcatctgagcgatcgcatcgtcccgtttgggttagagcgccgtct
   -360    ccagccgcgcacaacgctgaatggtcccctcatttgatgtgcagcgaaccaatgcacgga
   -300    tgctgcaacttcatgctctggcacatcctcggtgagagattggccagttcgatctcgtgg
   -240    cgcggtttgggaacctcggctcagcttcccggaatatggtttgcaggggttggagttgc
   -180    ttctgctcctggttgaaacgtgatcgtttcgactacaagattgaagtagccccgatgat
   -120    acttggaacatcaatgggaccacgaaaatcatccgactattttcgtttctgtatactgat

1     ATGTTGTTGCCCAATGGGGGAGAAATCATACCCAACCCGTTTCTATCAATACCCATTGCC
       M  L  L  P  N  G  G  E  I  I  P  N  P  F  L  S  I  P  I  A
61    GCCGGGCTAGCAGTGGCCGCAGCGCAAGGTCACGGAGCAATTAGGATTTCCGGAGTTAAA
       A  G  L  A  V  A  A  A  Q  G  H  G  A  I  R  I  S  G  V  K
121   CCCGAAAATGTTCGCAGACTAGCCTACATCGCTGCGCTCGCTAGTTTTGTTCTCTCGACG
       P  E  N  V  R  R  L  A  Y  I  A  A  L  A  S  F  V  L  S  T
181   ACGGAATATCTCAACAAATGGTCAGCAAATAACTGGGTGACAGACAACAAGTGGGACTGG
      E  Y  L  N   K  W   S  A  N   N  W  V  T  D   N  K  W  D  W
241   GATCGAGAAATCATTGTCGTCACCGGCGGCAGCAGCGGCATCGGCGCAAGCATCATCAAG
      D  R  E  I  I  V  V  T  G  G   S  S  G   I  G   A  S   I  I  K
301   CACATCTTCGCAAGAAACCCCAAAGCGACCATTGTAGTGGTTGACTTGGCACCGTTATCA
       H  I  F  A  R  N  P  K  A  T  I  V  V  V  D  L  A  P  L  S
361   TGGGAACCACCCAAGGGCTCCAAGCTTCACTACTTCAAGTGTGACCTGACCGACACGGCG
       W  E  P  P  K  G  S  K  L  H  Y  F  K  C  D  L  T  D  T  A
421   GCACTGAAGACGCTTTGCACTCTCATTCGAACTCAGGTTGGGGATCCTACGGTTCTCATC
       A  L  K  T  L  C  T  L  I  R  T  Q  V  G  D  P  T  V  L  I
481   AATAATGCCGGCATTGCGCGGGGTGCAACAATTATGGAAGGCTCATATGCCGACATTGAG
       N  N  A  G  I  A  R  G  A  T  I  M  E  G  S  Y  A  D  I  E
541   CTCACCGTGAAGACAAATCTCATTGCGCCCTTCCTGTTGACGAAGGAGTTCCTGCCGTAT
       L  T  V  K  T  N  L  I  A  P  F  L  L  T  K  E  F  L  P  Y
601   ATGGTTCGCAGGAATCATGGACATATCGTCAACATCGGGTCGATGAGTTCGGTGGTCCCA
       M  V  R  R  N  H  G  H  I  V  N  I  G  S  M  S  S  V  V  P
661   CCCGTCAGAATCGCAGATTATTCTGCAACTAAAGCAGGACTAACTGCCATGCATGAGgtc
       P  V  R  I  A  D  Y  S  A  T  K  A  G  L  T  A  M  H  E
721   agtctactggtgaccacgccccaaagccgacgcttgactgacatagtacagTCTTTGCAA
                                                          S  L  Q
781   CTCGAGTTGAAGTACATCCACAAAGCACTGAAAGTTCGACAAACGCTTGGAATCTTCGGC
       L  E  L  K  Y  I  H  K  A  L  K  V  R  Q  T  L  G  I  F  G
841   TTCATCAGGACGCCTCTTGTTCCGTTCAACCCCGGACAGCCACATTTCGTTATGCCACTG
       F  I  R  T  P  L  V  P  F  N  P  G  Q  P  H  F  V  M  P  L
901   CTTCATGTCGATACTGTTGGTGAGGCAATTGTTAATGGACTTTACAGCGGATACGGCGGG
       L  H  V  D  T  V  G  E  A  I  V  N  G  L  Y  S  G  Y  G  G
961   ACCATTTACCTTCCTAGAATCATGTCTTTGGTGACTGCACTCgtaagttgtaaaattatc
       T  I  Y  L  P  R  I  M  S  L  V  T  A  L
1021  ccaaaaacaattagcatggggctaacagaatcaaacagAGGGCAGGGCCGGAATGGATA
                                            R  A  G  P  E  W  I
1081  TGGCGCCTAGCGCGAGAGACAACCGCCAGTGCAAAGGATATCCCTTACACCCCCCGCCAG
       W  R  L  A  R  E  T  T  A  S  A  K  D  I  P  Y  T  P  R  Q
1141  AAGATTAATGACTTGACGGGCACGTTTGACTTGGAAGAGGCTAGCAAGGCAAAGACCAAC
       K  I  N  D  L  T  G  T  F  D  L  E  E  A  S  K  A  K  T  N
1201  GGGATCAAGAATGAGCTTTAATCATCGAAACATTTCATCAATGTAAATAAGTATATTTTG
       G  I  K  N  E  L  -
```

Figure 1. Nucleotide sequence of CgCAS2 fragment showing the deduced amino acid residues and the two intron regions within the ORF. The deduced amino acids (368 residues) are indicated with abbreviations and shown below the ORF. Intron sequences are shown in lower case red letters and underlined. The potential CAAT box, TATA box, GAGA factor, factor NF-E1/NF-E1a/NF-E1b/NF-E1c binding site (YTATCW), transcription factor NF-Y/CTF/CBF binding sites (ATTGG) and polyadenylation (ATAATAA) are underlined and marked in blue letters.

The predicted CgCAS2 protein has a theoretical molecular mass of 41.7 kDa and a calculated isoelectric point of 9.4. PSORT (http://psort.nibb.ac.jp) analysis showed that there is a 65.2%

possibility that this protein is located in the cytoplasm, 17.4% in the mitochondria, 13% in the nucleus and 4.3% in the endoplasmic reticulum. Analysis of the N-terminal amino acid sequence using SignalP software predicted the presence of a signal sequence that is 24 amino acids long.

Similarity search against known proteins showed that the deduced amino acid sequence of CgCAS2 shares significant homology with some hypothetical proteins from other fungi, and the highest hits were with a hypothetical protein from *C. globosum* (CHGG09887) with 50% identity, hypothetical protein from *A. niger* (An14g01270) with 46% identity and hypothetical protein from *M. grisea* (MGG01604) with 40% identity. CgCAS2 is rich in Ala (9.8%) and Leu (10.1%).

3.2. Disruption of CgCAS2

Gene disruption was performed to test for the possible involvement of *CgCAS2* in appressorium morphogenesis. To construct a gene replacement vector, a 2.3 kb hygromycin resistance (*hph*) gene cassette was inserted into *Hin*dIII site of a cloned 1.8 kb *CgCAS2* fragment in pGEMCAS2 to generate the final construct, pGEMCAS2-hph (Figure 2A). Linear and circular versions of pGEMCAS2-hph were transfected into the sphaeroplasts of *C. gloeosporioides* wild type strain PeuB. Schematics of the homologous integration is shown in Figure 2B.

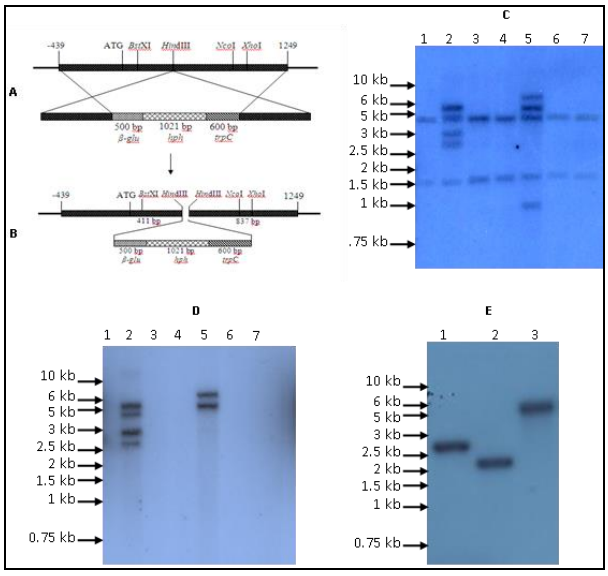

Figure 2. Schematic presentation of the strategy used for *CgCAS2* gene disruption in *C. gloeosporioides*. (A) Restriction map of the *CgCAS2* locus. (B) Partial map of the pGEMCAS2-hph replacement vector. DNA blot analysis of *CgCAS2* gene replacement in transformant *Cgcas2-x1* (lane 1), *Cgcas2-x2* (lane 2), *Cgcas2-x3* (lane 3), *Cgcas2-x15* (lane 4), *Cgcas2-c1* (lane 5), *Cgcas2-c2* (lane 6) and *C. gloeosporioides* wild type strain PeuB (lane 7). Genomic DNA was digested with *Xho*1 and probed with 1.8 kb of *CgCAS2*. (C) 1.1 kb of *hph* fragments. (D) The band in *Cgcas2-x2* and *Cgcas2-c1* samples showed different patterns of DNA fragments compared to the wild type strain when hybridized with 1.8 kb of *CgCAS2* probe. Probing with 1.1 kb of *hph* confirmed that both mutants carried the hygromcin resistant gene cassette in the mutant genome. To confirm that targeted integration has taken place in *Cgcas2-x2* and *Cgcas2-c1*, genomic DNA was digested with *Kpn*I and probed with 1.1 kb of *CgCAS2* gene. (E) A single band was detected when genomic DNA was digested with *Kpn*I and probed with 1.8 kb of *CgCAS2 gene*. In the *Cgcas2-c1* mutant, an increase in 2.3 kb (hygromycin cassette) was observed when compared to the wild type. Lane 1: wild type, lane2: *Cgcas2-x2*, lane3: *Cgcas2-c1*.

A total of 35 hygromycin-resistant transformants were isolated by single spore isolation and subcultured on PDA plate containing 300 g/ml hygromycin. All transformants were screened using PCR with HpF-F and HpF-R primers, which are complementary to the native *CgCAS2* DNA fragment, as well as with TrpC-F and HpF-R primers. TrpC-F primer was designed based on TrpC terminator sequence in the hygromycin resistance gene cassette. In two transformants, *Cgcas2-x2* and *Cgcas2-c1*, HpF-F and HpF-R primers did not produce the expected ~1.7 kb PCR fragment, indicating that there is an insertion of *hph* DNA fragment into the *CgCAS2* locus. TrpC-F and HpF-R primers amplified a ~1.5 kb amplicon in *Cgcas2-x2* and *Cgcas2-c1*, but not in the wild type strain that do not have *hph* gene cassette insertion (Figure 2B).

Cloning and sequencing of that fragment confirmed that homologous integration at the *CgCAS2* locus took place in the *Cgcas2-c1* mutant only. The disruption of *CgCAS2* in *Cgcas2-c1* mutant was also confirmed by Southern blot analysis (Figure 2C, 2D and 2E). In *Cgcas2-c1*, three extra bands

with the size of ~1 kb, ~6 kb and ~7 kb were observed (Figure 2C). Hybridisation with the hygromycin phosphotransferase (*hph*) gene showed that *Cgcas2-c1* produced bands with different sizes, whereas no signals were observed for the wild type (Figure 2D). To further clarify if gene replacement had occurred within *CgCAS2* locus, the genomic DNAs were digested with *Kpn*I, which has no restriction sites in wild type *CgCAS2*. When it was hybridized with the 1.7 kb fragment of *CgCAS2*, only *Cgcas2-c1* had a ~6 kb fragment, in contrast to the ~2.7 kb fragments seen in the wild type strain (Figure 2E). To test the expression of the *CgCAS2* gene by the mutant, total RNA extracted from appressoria of the wild type and *Cgcas2-c1* mutant were subjected to Northern blot analysis using the *CgCAS2* cDNA as a probe. The results confirmed the absence of *CgCAS2* transcripts in the appressoria of *Cgcas2-c1* mutants, whereas a *CgCAS2* transcript was detected in the wild type (Figure 3).

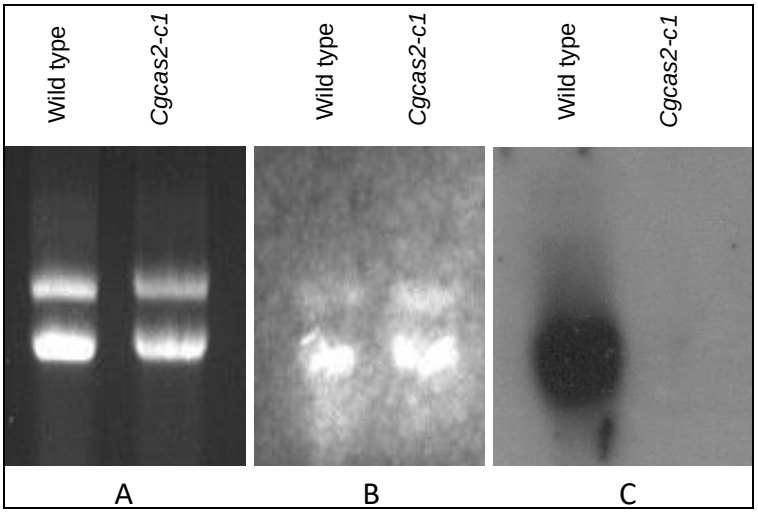

Figure 3. RNA blot analysis of total RNA obtained from appressoria of the wild type and the *Cgcas2* mutant of *C. gloeosporioides*. (A) The total RNA was extracted from 7-hour old appressoria induced with rubber leave wax on Petri dish. RNA was electrophoresed. (B) Blotted onto nitrocellulose membrane. (C) Hybridized with a α-^{32}P-dCTP labeled 1.7 kb fragment of *CgCAS2* gene.

3.3. The effect of CgCAS2 disruption on C. gloeosporioides morphogenesis

The *Cgcas2* mutant strains had the typical grayish color and colony morphology similar to the wild type strain when grown on PDA. The growth rate of *Cgcas2* mutants, which was measured on PDA Petri dish cultures, is the same as that of the wild type after incubation at ambient temperature for one week. The *Cgcas2* mutant produced vegetative hyphae and abundant aerial mycelia. No obvious differences in conidial morphology was observed between the wild type and the *Cgcas2* mutant, but the amount of conidia production was slightly different (data not shown). This indicates that *CgCAS2* is essential for conidiation in *C. gloeosporioides*.

The effects of *CgCAS2* deletion mutant on germination and appressorium formation were assayed on hard surface glass slide coated with rubber leaf wax. Conidia produced by *Cgcas2* mutants were able to germinate and form appressoria. These mutant appressoria were melanised properly and had regular shapes. However, the percentage of germ tubes and appressoria formation was significantly reduced in the *Cgcas2* mutants compared to the wild type strain (Figure 4). In addition, the *Cgcas2* mutant conidia produced multipolar germination, in contrast with unipolar germination found in wild type conidia. However, appressoria differentiation only occurred at the tip of one of the germ tubes in both mutant and wild type. The remaining germ tubes in the mutant were unable to differentiate to

form appressoria. The *Cgcas2* mutant also produced longer germ tubes before forming appressoria, while the wild type conidia produced sessile appressoria (Figure 5).

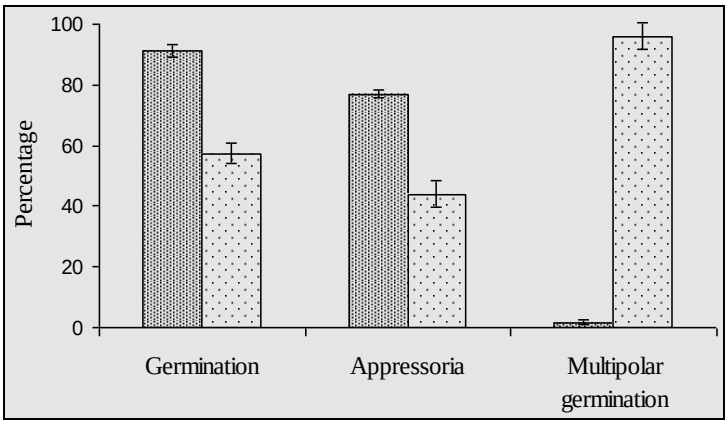

Figure 4. Percentage of germination, appressorium formation and multipolar germination of the wild type (▨) and the *Cgcas2* mutant (▭) conidia of *C. gloeosporioides* on hydrophobic hard surface glass slide coated with rubber leaf wax. The mean values of the same coloured bars inscribed with a common letter are not significantly different base on statistical analysis (P<0.01).

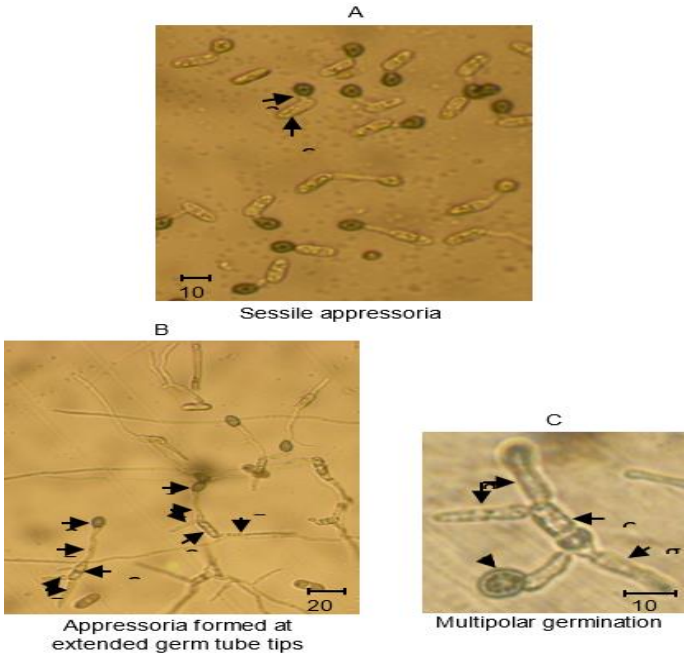

Figure 5. Light microscope observation of sessile appressorium formation in the wild type (A) and appressorium formation at extended germ tube tips of *Cgcas2* mutant (B) of *C. gloeosporioides*. Multipolar germination (B and C) of *Cgcas2* mutant on the hard surface of hydrophobic glass slides coated with rubber leave wax. The image was captured with an Olympus phase contrast microscope (200× magnification for A and B; 400× magnification for C) and a Nikon digital camera. a = appressorium, c = conidium, g = germ tube.

3.4. CgCAS2 is required for C. gloeosporioides pathogenicity

To determine the role of *CgCAS2* in pathogenesis, conidia of *Cgcas2* deletion mutants were inoculated onto mango fruits. Two methods of inoculation, i.e. direct inoculation onto wounded fruits and spray inoculation onto unwounded fruits, were employed to test for pathogenesis. In wounded fruits, the wild type strain induced typical brown lesions on fruits 3 days after inoculation (DAI) and developed typical necrotic, sunken anthracnose symptoms 7 DAI. In contrast, small brownish lesions were observed 3 DAI with the *Cgcas2* mutant, which did not develop into typical anthracnose symptoms seen in the wild type. Anthracnose disease severity was measured by lesion diameters and the *Cgcas2* mutant induced significantly smaller lesions than the wild type strain (Figure 6 & 7). When conidia were inoculated on unwounded fruits, initial symptoms by the wild type strain appeared 4 DAI and severe sunken lesion symptoms were observed 9 and 10 DAI. However, smaller brown lesions were observed on unwounded fruits sprayed with *Cgcas2* mutant conidia 6 DAI. Disease severity (based on the number of lesions) was nearly 3-fold lower in *Cgcas2* mutant compared to the wild type strain. In addition, lesions induced by *Cgcas2* mutant did not further develop into typical anthracnose symptoms. These results indicate that *CAS2* has an important role in pathogenesis of *C. gloeosporioides*.

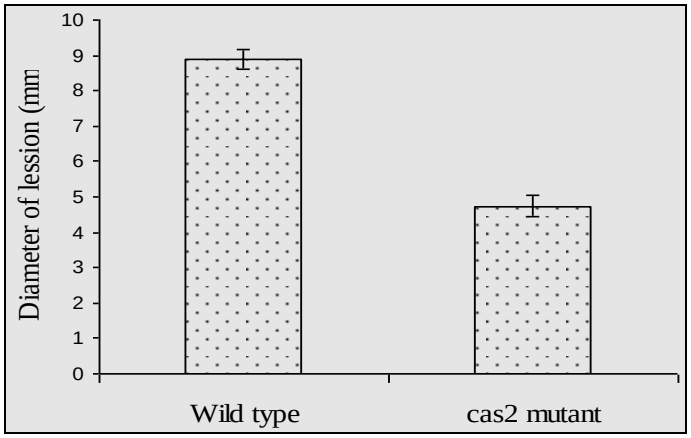

Figure 6. Disease severity of mango inoculated with the wild type and the *Cgcas2* mutant of *C. gloeosporioides*.

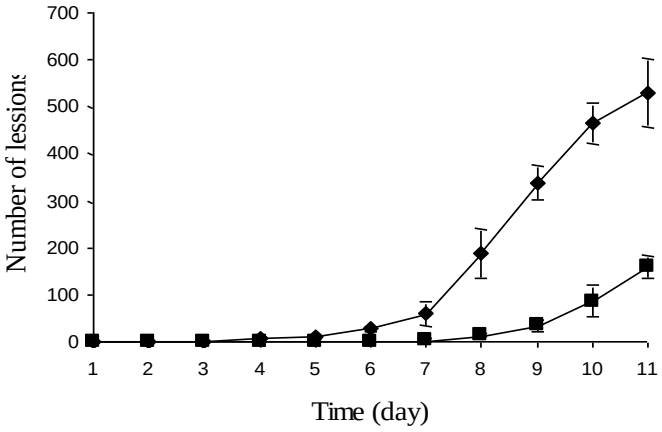

Figure 7. Disease severity of mango inoculated with the wild type (♦) and the *Cgcas2* mutant (■) *C. gloeosporioides*.

CgCAS2 is present as a single copy gene in *C. gloeosporioides* genome and is uniquely expressed in the appressoria [4]. No *CgCAS2* transcripts were detected in other growth stages including conidia, germ tubes and mycelia. Comparison of CgCAS2 protein sequence with known proteins from other organisms showed that it has similarities with hypothetical proteins from several fungal species including *C. globosum, A. niger* and *M. grisea*. *CgCAS2* sequence contains putative casein kinase II phosphorylation site, glycosaminoglycan attachment site, protein kinase C phosphorylation site and short-chain dehydrogenases/reductases. The presence of the putative kinase-dependent phosphorylation motifs in CgCAS2 and the importance of kinase signaling in *C. gloeosporioides* infection pathway signify a possible role of this protein in plant infection process [16], a putative epidermal growth factor (EGF)-like domain signature, which is a receptor for soluble growth factors like ECM. Furthermore, the HMM analysis predicted that *CgCAS2* is composed of five alpha-helical transmembrane domains with the N-terminus located outside of the membrane and the C-terminus inside of the membrane. This prediction strongly suggests that *CgCAS2* could be a cell receptor in the *C. gloeosporioides* appressorium.

In higher eukaryotes, integrins use bidirectional signaling to integrate the intracellular and extracellular environments [17]. For incoming signals, ligand binding activates intracellular signaling pathways. For outgoing signals, signals received by other receptors of neuronal cells activate intracellular signaling pathways that impinge on integrin cytoplasmic domains, and make the extracellular domain competent for ligand binding on a time-scale of less than 1 second [18] family of small integral membrane proteins, shows close association with integrins [19] were first identified in mammals and play a major role in cell shape remodelling including migration, cell-to-cell contact, and motility/invasion during diverse biological processes, such as parasite entry, sperm-egg fusion, B-cell/T-cell contact, neuron out-growth, and metastasis [15] st tetraspanin gene identified was in *M. grisea* and named as *PLS1* [21] mologous to *PLS1* have been identified in other ascomycetes [16-17] are reduced in virulence [23]. In this study, *Cgcas2* mutant was also significantly reduced in virulence and did not produce typical anthracnose symptoms, but instead produced small brown lesions that are low in abundance. A simple hypothesis can be proposed, whereby mutant conidia consume more energy to produce multidirectional germination than unipolar germination, which in turn lower the mechanical force and reduce available enzymes for penetration.

Such mechanism was observed in *Cgpkac* mutant, which has reduced pathogenicity towards unwounded fruits due to the failure of appressoria to penetrate intact fruit skins, as cAMP-PKA signaling pathway is involved in the degradation of lipid bodies to generate turgor pressure. However, the effect of the deletion in the *Cgcas2* mutant with regards to the reduction in pathogenicity in wounded fruits is not well understood. It is postulated that the deletion of the *CgCAS2* resulted in high levels of phosphodiesterase and low levels of cAMP, which causes the inactivation of cAMP-PKA signaling pathway. In addition, the cAMP-PKA pathway regulates appressoria related cell wall degrading enzymes. Inactivation of cAMP-PKA pathway by deletion of *CgCAS2* may have resulted in the mutant being unable to produce cell wall degrading enzyme. More work is needed to determine whether CgCAS2 is involved in the cAMP signal cascade that regulates parasitic-saprophytic switching mode. To resolve this, it is important to detect the intracellular cAMP level and phosphodiesterase activity in *Cgcas2* mutant compared to the wild type.

4. Conclusions

CgCAS2 may be a cell receptor in the *C. gloeosporioides* appressorium involved bidirectional signaling to integrate the intracellular and extracellular environments. *CgCAS2* plays an important role in the coordination of cellular processes required for pathogenic and saprophytic development in *C. gloeosporioides* appressoria.

5. Acknowledgement

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Genetic improvement of Aceh local rice variety Sikuneng to produce green super rice lines adaptive to abiotic stresses

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Abstract. The high genetic diversity of rice landraces of Aceh has great potential to contribute to the world's food security program in the future, especially in adapting to climate changes and environmental degradation. The objective of this research was to develop a new superior rice line adaptive to abiotic stress and maintains high productivity despite limited agricultural inputs. An Aceh's local variety, Sikuneng, was artificially crossed with an isogenic line IRBB27 and the F_1 plants were self-pollinated for two generations. The F_3 lines were cultivated in drought stress and high salinity conditions with the application of aerobic rice system and flash irrigation. It was discovered that the F_3 lines showed significantly improved plant height, number of panicles per plant, panicle length, grain weight per plant and grain yield potential per hectare. The plant height of the F_3 lines varied from 98 to 192 cm, whereas the plant height of Sikuneng was 172 cm. The number of panicles of F_3 lines ranged from 10.3 to 43.5, whereas Sikuneng had only 8.7 panicles. The panicle length of F_3 lines ranged from 23.9 to 47.3 cm and Sikuneng had only 28.1 cm of panicle length. The grain weight per plant of F_3 lines varied from 69.8 to 196.7 g, in contrast to the grain weight of Sikuneng which was 64.5 g. The weight of 1,000 grains of F_3 lines ranged from 21.0 to 34.9 g, whereas that of Sikuneng was 26.9 g. While the yield potential of Sikuneng was 7.6 t/ha, the yield potential of the F_3 lines varied from 7.2 to 13.9 t/ha. The highest yield potential was shown by Skn-68-1 line. Further evaluation and selection process need to be conducted in the next generations until the improved characters of the new lines are stable.

Keywords: Aceh, IRBB27, genetic improvement, green super rice, Sikuneng, abiotic stress.

1. Introduction

Among the impacts of climate change, drought is a serious threat, especially in lowland rain-fed areas where 75% of world rice supply is produced [1]. Extreme weather has led to increased frequency of abiotic stresses in plants, such as droughts, floods and heatwaves [2,3]. These extreme conditions are the major threats to food security, particularly in Asia, including Indonesia. More than 4 billion people live in Asia, where 90% of rice was produced and consumed as a staple food [4,5]. Therefore, the task of achieving and maintaining food security will be a big responsibility for Indonesia. The problem is further complicated by high population growth, environmental degradation, water limitation, and land degradation. Therefore, it is urgent to develop new rice varieties that are able to adapt to global climate change and can maintain high productivity under extreme environmental conditions despite water or fertilizer shortages [6,7]. A suitable solution is to develop Green Super Rice (GSR) through a plant breeding program by crossing adaptive local varieties to superior varieties. The GSR rice is

developed through innovative introgression breeding strategies that utilize heterosis or codominance principles to improve rice varieties for sub-optimal or marginal land. GSR is a rice cultivar that can maintain high productivity despite less input (water, fertilizer and pesticide). Marcaida et al. [8] and Yorobe et al. [9] suggested that GSR cultivars in IRRI are able to overcome drought and soil fertility shortages or other extreme conditions.

Recently, a local superior variety in Aceh, Sikuneng, has been successfully crossed with the isogenic line IRBB27 obtained from IRRI. Sikuneng has a number of advantages including high yield potential and adaptive to extreme conditions, but it matures in 4.5 months and tall, which makes it susceptible to lodging. A previous study showed that local rice varieties from Aceh have several advantages, such as resistant to salinity and drought stresses [10], have higher yield potential and tolerant to high temperatures [10]. IRBB27 has high productivity, resistant to diseases, early maturing (3.5 months) and has the genes that make the architecture of the plant to be shorter with compact stems. Some of the F_2 generations derived from the cross between Sikuneng and IRBB27 showed improvement in several traits compared to the two parents, i.e. their yield potential is above 12 t/ha and mature at 100–110 days. The objective of this research was to produce a GSR rice line with high yield potential and able to adapt under low fertilizing and water-saving inputs, resistant to pest and diseases, and tolerant to drought stress and high temperature, by exploiting the heterosis aspect of crop breeding.

2. Materials and methods

This study was carried out in an experimental farm of Syiah Kuala University, Aceh, Indonesia from March to September 2017. The cultivated land for selection activities was a sub-optimal rice field in Darussalam, Banda Aceh, Indonesia. The genetic materials tested were 100 F_2 rice lines derived from a cross between a local superior rice variety, Sikuneng, and IRBB27 which was introduced from IRRI through Temasek Life Science, Singapore. F_3 seeds were harvested from the F_2 population in the Seed Science and Technology Laboratory, Syiah Kuala University. Fifty F_3 seeds from each F_2 lines were planted on the same location in 600 m^2 dry saline land situated 4 km from a beach at an altitude of 3 m above sea level. Seedlings were planted in dry, non-puddled and non-saturated soils which received intermittent irrigation which was supplied by aerobic system using saline water with a salinity level of about 8–12 mmhos/cm.

The pedigree selection method was carried out by recording genealogies of selected progeny from generation to generation. For each cross, one panicle was planted in two rows following a wide hill spacing system called 2:1 *Jajar Legowo*. Fifteen-day-old seedlings were transplanted with a spacing of 25 cm between rows and 25 cm between hills, with one plant per hill. Selection was conducted visually (qualitatively) based on desired criteria, especially for plant height, number of panicles, grain weight, panicle length and yield potential. Selection was done on the population as well as individuals to obtain plants with desired characters.

3. Results and discussion

The F_3 lines derived from a cross between Sikuneng and IRBB27 showed wide variation in the yield components, including the number of panicles, panicle length, grain weight per plant, weight per panicle or yield potential (Table 1). Generally, the F_3 rice lines produced higher number of panicles than the two parents (Figure 1). a similar phenomenon can also be found in the length of the panicle (Figure 2A). It was found that the panicle length of Skn 9-4 F_3 rice lines was significantly higher than that of their parents. The grain weight per plant was also significantly higher than that of the parents (Figure 2B). The number of panicles of Skn 44-5 F_3 rice lines was significantly increased compared to that of their parents. The same result was discovered on the yield of the F_3 rice lines. Yield potentials of F_3 rice lines were generally higher than that of both parents. These chracters were the main selection criteria, aside from the semi dwarf phenotype. This indicates that the two parental lines contributed favorable alleles for yield component traits.

Table 1. Yield components of F_3 lines derived from a cross between Sikuneng and IRBB27.

Genotype	Panicle number	Length of panicle (g)	Grain weight per plant (g)	Grain weight per panicle (g)	Yield potential (t/ha)
Sikuneng	8.7	28.1	64.5	8.1	7.6
IRBB27	17.2	25.8	72.7	4.2	8.5
Inpari 30	20.4	27.2	74.1	3.7	8.3
Skn 47-1	24.9	32.3	149.4	6.2	10.6
Skn 68-1	27.5	36.8	196.7	8.3	13.9
Skn 44-5	39.2	35.4	162.3	4.2	11.0
Skn 45-4	17.7	26.2	69.8	4.1	7.2
Skn 44-4	29.4	31.1	109.9	3.8	11.4
Skn 111-16	22.5	23.9	84.4	3.8	8.8
Skn 43-2	43.5	30.7	181.7	4.2	13.0
Skn 110-8	35.4	33.8	171.9	4.9	10.0
Skn 29-2	22.8	31.5	193.4	8.9	13.7
Skn 109-1	242	36.2	142.6	5.9	10.1
Skn 109-9	26.5	38.1	156.5	6.0	12.4
Skn 9-4	10.3	47.3	73.6	7.3	7.7
Skn 110-1	21.6	28.6	102.8	4.9	10.7
Skn 110-3	18.2	26.7	71.7	4.0	7.5

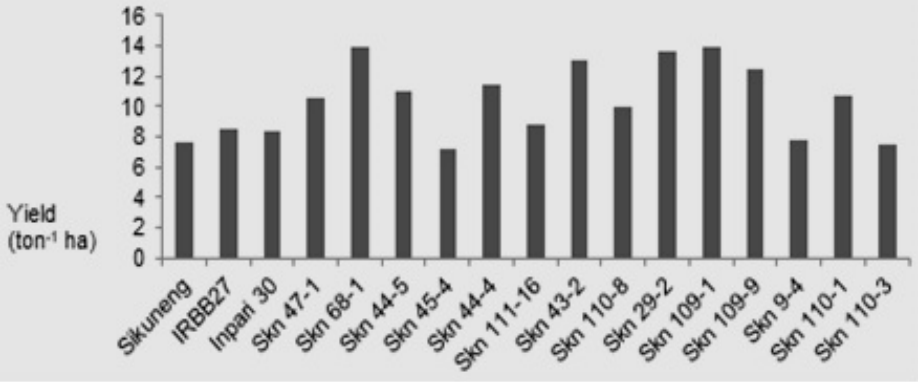

Figure 1. Yield potential of the best selected F_3 lines derived from a cross between Sikuneng and IRBB27.

The F_3 lines Skn-68-1 and Skn-29-2 showed the highest grain weight per plant, i.e. 196.7 and 193.4 g, respectively (Table 1). However, selection needs to be continued in the next generations to fix the superior alleles in the selected progeny. The highest yield was found on Skn-68-1 and Skn-29-2, which reached 13.9 and 13.7 t/ha, respectively (Figure1). This indicates that the F_3 lines from the cross between Sikuneng and IRBB27 have great potentials to produce GSR. Development of GSR is an important strategy to increase rice production in China compared to the existing varieties obtained through conventional rice breeding or hybrid rice [12]. After nearly a decade of cultivation, super rice

accounts for more than 60% of the total area planted with rice and has contributed about two billion dollars for China's national economy [13]. It was also discovered that the F_3 rice lines grew well in the aerobic system that was applied by cultivating the rice plants in dry, non-puddled and non-saturated soils with supplemental intermittent irrigation (Figure 3).

Figure 2. F_3 rice lines and their two parents (P1 Sikuneng and P2 IRBB27). (A) Panicle length of Skn 9-4 line was significantly longer than their parents. (B) Panicles number of Skn 44-5 line was significantly higher than their parents.

Figure 3. The F_3 rice line progenies of Sikuneng × IRBB27 grew well in dry, non-puddled and non-saturated soils with supplemental intermittent irrigation system.

Development of new rice varieties adapted to global climate change is highly needed in order to maintain high productivity under extreme environmental conditions despite fertilizer or water shortages [6,7]. A possible solution for this is by developing GSR through a plant breeding program using highly adaptive local varieties, which are crossed with newly available superior lines. Selection can then be performed to obtain GSR cultivars with high productivity and efficient in fertilizer and water input [14].

4. Conclusions

The F_3 lines derived from a cross between Sikuneng and IRBB27 produced higher panicle number, panicle length and grain weight per panicle than those of both parents. F_3 lines Skn-68-1 and Skn-29-2 showed the highest grain weight per plant, i.e. 196.7 and 193.4 g, respectively.

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Agronomic performance of several doubled-haploid lines derived from anther culture of black rice × white rice

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Abstract. Black rice is a well-known type of rice in Indonesia, but its availability is still limited. So far, only one black rice variety that has been released by rice breeders. Recently, public demand for black rice increases due to its health benefits. Therefore, the development of new variety of black rice is needed. Anther culture is often used to accelerate the process in obtaining homozygous lines in the form of doubled-haploid (DH) plants for rice breeding. This research aimed to evaluate the agronomic performance of several DH lines derived from anther culture. The experiment was conducted at Sukamandi, West Java, during the dry season of 2017. Plant materials used in this study were 13 DH lines derived from anther culture of F_1s and five improved varieties as control. The F_1s were obtained from crosses between local black rice Melik with two white rice varieties, Inpari 13 or Fatmawati. The experiment was conducted in a randomized complete block design with three replications. Observation was conducted on plant height, number of productive tiller, days to flower, days to harvest, panicle length, number of grain per panicle, number of filled and empty grain number per panicle, yield, and pericarp color. The results showed that plant height, number of tillers, yield and yield components of DH lines were significantly different compared to some of the control varieties. Ten DH lines were similar in yield compared to Inpari 13, but five of them produced higher yield than Inpari 13. Days to flower of DH lines and control varieties were similar (75–89 days). The pericarp color of DH lines varies from dark purple to reddish-brown (maroon).

Keywords: agronomic characters, doubled-haploid (DH) lines, anther culture, black rice.

1. Introduction

Black rice has more benefit compared to white rice, because it contains nutrients such as anthocyanin, various vitamins and minerals, including iron, vitamin A and B [1–3]. The pericarp layer of black rice accumulates anthocyanin, which is known as an antioxidant beneficial for human health. Black rice has a pericarp, aleurone, and endosperm with red, blue, or purple with deep red-blue-purple color, which indicate anthocyanin content [4]. Some antioxidants in black rice are reported to be soluble in fat, while anthocyanins dissolve in water and can be spread in the body [5]. It was reported that some

health benefits from black rice due to its association with a reduced risk of heart disease, cancer and memory improvement [4,6].

Black rice can be planted on various lands, not only in lowlands but also in highlands. Anthocyanin content of black rice increases with the increasing altitude and the presence of a favorable environment [7]. Limited quantity of black rice is currently available in the market,but the price is quite expensive, up to 2–3 times of white rice price. Meanwhile, the demand for black rice tends to increase because of the better public awareness of the health benefit of black rice.

A number of local black rice exists in several rice areas in Indonesia. Local black rice cultivars such as Cibeusi was found in West Java; Aen Metan and Hare Kwa were found in East Nusa Tenggara; while Melik, Cempo Ireng, Jeliteng, Wulung and Sirampog were found in Central Java [2]. All these local varieties were tall (>150 cm) and have a low yielding ability [2,3,8], but can be used as the gene source(s) in rice breeding programs. Selection of doubled-haploid (DH) derived from anther culture can shorten the duration of the breeding process because the agronomic character of the DH lines is stable from generation to generation [9]. Maeda et al. [10] reported three loci on chromosomes 1, 3 and 4 which contained genes that control color (black/purple) in rice by assessing them with RM8129, RM15191 and RM2441 SSR markers, respectively. Two genes that control purple pericarp were found in chromosome 4 (*b* genes) and chromosome 1 (*a* genes), while those controlling black pigmentation in pericarp was located in a locus on chromosome 3.

This research aimed to evaluate agronomic performance of several DH lines derived from anther culture of black rice × white rice to obtain black rice lines that has good agronomic characters, such as dwarf (90–115 cm), high yield (>5 t/ha) and early maturing (90–120 days after sowing) which are equivalent to improved variety.

2. Materials and methods

The research was carried out at Sukamandi Experimental Station in dry season (DS) 2017. Fourth generation of doubled-haploid (DH4) lines were tested. These lines were obtained from anther culture of F_1s derived from the cross between the local black rice Melik with two improved varieties of white rice (Inpari 13 and Fatmawati), i.e. Melik/Inpari 13^2, Melik^2/Inpari 13, Melik/Fatmawati^2 and Melik^2/Fatmawati. A total of 13DH4 lines and five improved varieties (Inpari24, Aek Sibundong, Inpari 32, Inpari 13 and Fatmawati) as control were planted in a randomized complete block design (RCBD) with three replications. Each line was planted in a plot size of 2 × 5 m^2 at seedling stage of ±21 days after sowing (DAS) and plant spacing of 25 cm × 25 cm. Fertilizer used were 300 kg urea/ha, 100 kg TSP/ha and 100 kg KCl/ha. Observation was conducted on plant height, days to flower, days to harvest, number of productive tiller, yield per plot, panicle length, number of grain per panicle, number of filled grain, number of empty grain, weight of 1,000 grains and the color of pericarp.

3. Results and discussion

Variance analysis showed that there was a significant difference between doubled haploid (DH) lines and control varieties in all traits observed (Table 1).The yield of ten DH black rice lines were not significantly different from that of the control varieties, i.e. Inpari 13, Fatmawati, Inpari 24, Aek Sibundong and Inpari 32 varieties (Table 2). Unfortunately, the local black rice Melik was not included in this research due to its late-maturing trait which is more than 150 days [11].

Five DH lines derived from Melik/Inpari 13 had higher yield (7–7.3 t/ha) than the parent, Inpari 13 (6.9 t/ha). There were also two DH lines derived from Melik/Fatmawati^2 that had a yield of 6.9–7.2 t/ha, while Fatmawati only yielded 6.8 t/ha. Out of the five improved varieties tested, Inpari 24 (brown rice) had the highest yield (7.3 t/ha). In this research, seven DH lines were selected, i.e. YD1-61-1-1, YD1-71-1-1, YD1-51-2-1, YD1-51-2-2, YD1-48-1-2, YD6-1-1-1 and YD6-1-1-2, based on their higher or equivalent yields compared to the parental varieties and Aek Sibundong variety (7–7.2 t/ha). These lines can be tested further in advanced yield trials atseveral locations.

Table 1. Analysis of variance of agronomic characters of DH lines at Sukamandi in dry season of 2017.

Traits	CV (%)	Mean square	F value	Significance level
Plant height	8.9	292.04	16.94	**
Number of panicle	13.8	14.79	3.18	*
Days to flower	4.0	52.18	60.96	**
Panicle length	7.6	12.53	7.21	**
Number of grain per panicle	17.2	2914.90	4.52	**
Number of filled grain per panicle	18.3	2084.08	3.83	**
Number of empty grain per panicle	31.6	190.87	2.90	**
1,000 grain weight	4.5	4.00	3.05	*
Yield (t/ha)	11.7	2.06	1.94*	*

**Significantly different at α = 1%, *Significantly different at α = 5%.

Table 2. Yield of DH lines at Sukamandi Experimental Station in dry season of 2017.

Line no.	Genotype	Yield (t/ha)	Line no.	Genotype	Yield (t/ha)
1	YD1-61-1-1	7.13	10	YD5-37-1-2	6.4
2	YD1-71-1-1	7.13	11	YD5-37-1-3	6.17
3	YD1-51-2-1	7.13	12	YD6-1-1-1	7.17
4	YD1-51-2-2	7.13	13	YD6-1-1-2	6.87
5	YD1-51-2-3	6.07		Inpari 13	6.9
6	YD1-48-1-2	7.00		Fatmawati	6.8
7	YD2-29-2-1	4.80*		Inpari 24	7.3
8	YD5-10-1-2	5.33*		Aek Sibundong	7.05
9	YD5-37-1-1	5.16*		Inpari 32	6.3
	LSD 5%				1.7
	LSD 1%				2.3
	CV (%)				11.7

1–6 = lines derived from the crossof Melik/Inpari13^2, 7 = Melik^2/Inpari13, 8–11 = Melik/Fatmawati^2, 12–13 = Melik^2/Fatmawati.
*Significantly different at α = 5%.

The days to flower of DH lines ranged from 75 to 84 days, while the days to harvest ranged from 115 to 125 days (Table 3), which were similar to the control varieties Inpari 13 and Fatmawati. However, these lines had earlier harvesting time (30 days faster) when compared to Melik, which are harvested at 150 DAS [11]. For flowering time, there were three lines with late flowering time, i.e. YD1-61-1-1, YD1-71-1-1 and YD2-29-2-1, at 84, 83 and 90 DAS, respectively. The difference in flowering time to Inpari 13 and Fatmawati was 3–10 days. Flowering and harvesting time are positively correlated with the character of grain weight per plant [12]. In other words, higher grain weight per plant typically requires longer flowering and harvesting time. Therefore, it is difficult to obtain an early maturing variety with high yield. However, this theory contradicted with the results of this research. DH line YD2-29-2-1 showed late maturity trait (125 days) but had low yielding ability (4.8 t/ha), as shown by the low number of filled grains and productive tillers. Susanto et al. [13] also

reported similar results, where longer harvesting days was not followed by yield increase. However, the DH lines in this research were still categorized as early-maturing rice varieties (105−124 DAS) according to the Indonesian Center for Rice Research [13] and they produced higher yield when compared to one of the parents, the local black rice Melik.

Six DH lines, i.e. YD1-61-1-1, YD1-71-1-1, YD1-51-2-1, YD1-51-2-2, YD1-51-2-3 and YD1-48-1-2 had 16-17 productive tillers per plant (Table 3). These lines were not significantly different from Inpari 13 and Fatmawati as well as the other three control varieties. The number of productive tiller is an important agronomic trait in rice plant[14]. The number of productive tiller per plant is positively correlated with the character of grain weight per plant, and hence high yield on rice is supported by the number of productive tillers per plant [12].

Table 3. The average value of agronomical traits of DH lines observed at Sukamandi Experimental Station in dry season of 2017.

Genotype	DTF (DAS)	DTH (DAS)	HT (cm)	NPT (tiller/hill)	NTG (tiller/hill)	NFG	NEG	PL (cm)	W1000 (g)
YD1-61-1-1	84**	119*	113.2	17	150.6	130.8	19.8	27.44	26.3
YD1-71-1-1	83*	118*	110.6	16.8	159.9	135.5	24.4	26.41**	26.7
YD1-51-2-1	80	115	106	15.9	153.9	139	14.9*	26.22**	25.3
YD1-51-2-2	80	115	106.8	17.2	161.32	140.21	21.11	26.72**	24.3*
YD1-51-2-3	80	115	102.8**	18.9	149.9	127.56	22.3	25.78**	25
YD1-48-1-2	81	116	96.6*	16.7	160.34	142.23	18.11	26.47**	25.3
YD2-29-2-1	90**	125 **	130.8**	12.6*	143	124.33*	18.7	23.11**	26
YD5-10-1-2	80	115	93.6*	17.2	118.2**	97.00**	21.2	26.20**	23.7*
YD5-37-1-1	75	110**	105.4	14.4	167.8	134.67	33.1	27.21**	25.3
YD5-37-1-2	79	114	107.2	13.7	188.3	148.44	39.9**	26.04**	25.7
YD5-37-1-3	77	112*	115.3	13.4*	204	170.69	33.3	28.3	25.3
YD6-1-1-1	80	115	126.8**	14.3	171.6	157.78	13.8	26.34**	26.3
YD6-1-1-2	79	114	127.0**	16.2	163.8	135.89	27.9	26.77**	25.3
Inpari 13	80	115	111.9	15.6	206.6	190.44	16.1	30.36	26.3
Fatmawati	80	115	117.2	13.7	246.9	216.44	30.4	32.06	28
Inpari 24	81	116	106.7	19.2	145.6	134.67	10.9	27.57	27.3
Aek Sibundong	80	115	108.4	19.8	165.7	148.11	17.6	26.79	27.7
Inpari 32	85	120	109.7	18.3	159.2	145.56	13.7	23.48	27.3
CV %	4	2.8	8.9	13.8	17.2	18.3	36.1	7.6	4.5
LSD 5%	2.7	2.7	6.9	6,193	40.7	66.95	23.3	2.18	3.11
LSD 1%	3.6	3.6	9.2	8,311	54.9	89.71	31.2	2.93	4.18

DTF = days to flower, DAS = days after sowing, DTH = days to harvest, HT = plant height, NPT = number of productive tiller, NTG = number of grain per panicle, NFG = number of filled grain per panicle, NEG = number of empty grain per panicle, PL = panicle length, W1000 = weight of 1000 grains.
**Significantly different at α = 1%, *Significantly different at α = 5%.

Plant height, days to flower, number of productive tiller, panicle length and 1,000 grains weight had the coefficient of variance (CV) of less than 10%, while the number of grain per panicle, number of filled grains per panicle and yield had CV that ranged from 17−30%. The number of empty grain per panicle had the highest CV, i.e. 36% (Table 1). This means that generally, DH lines derived from

Melik/Inpari 13 or Melik/Fatmawati had similar characteristics in terms of plant height, productive tillers number, panicle length and grain size.

The plant height of two DH lines, namely YD1-48-1-2 and YD5-10-1-2, were shorter (94-97 cm) than Inpari 13, whereas three lines, namely YD2-29-2-1, YD6-1-1-1 and YD6-1-1-2, were taller (127–131 cm) than Inpari 13. Inpari 13 and Fatmawati had 112 cm and 117 cm height, respectively. Plants with high postures tend to experience lodging in the grain filling and maturity phases. Higher posture plants also tend to produce smaller numbers of tillers, while shorter plants have a large number of tillers. Consistent with that, our results showed that YD1-48-1-2 and YD5-10-1-2 lines with the plant height of 94–97 cm, had 17 tillers, while YD2-29-2-1 line with a plant height of 131 cm had 13 tillers (Table 3). The height of rice plants is influenced by genetic factors. The plant height of irrigated rice is usually lower (<130 cm) than upland or rainfed rice. Local rice usually has higher plant height (150 cm) and it correlates with large culm character.

Seed size is related to the weight of 1,000 grains. DH lines tested in this research had an average 1,000 grains weight of 26 g (Table 3). The highest weight of 1,000 grains was recorded in Fatmawati (28 g) and Inpari 13 (26.3 g), while the lowest was observed in YD5-10-1-2 (23.7 g). The grain size of DH lines was not significantly different from that of Inpari 13 and Fatmawati, except for YD1-51-2-2 (24.3 g) and YD5-10-1-2 lines that were significantly different from Fatmawati.

The number of empty grain per panicle did not affect the overall yield of DH lines tested. The average number of empty grains was 22 per panicle and the highest number was 39.9 empty grains per panicle. In the control varieties, Fatmawati produced 30 empty grains per panicles, while Inpari 13 produced 16 empty grains per panicles. Fatmawati and Inpari 13 apparently had a high number of empty grain per panicle. However, the percentage of empty grain was only 12 and 8% for Fatmawati and Inpari 13, respectively. Fatmawati often have a high number of empty grain (>30%) and also less total number of panicle. Thus, the number of filled grain per panicle could be less as well. Besides the number of productive tillers, the yield of rice also depends on the number of grains per panicle.

The diversity of grain color of the DH lines was only observed from the color of rice pericarp and its anthocyanin levels were not measured. The grain color in the progeny of Melik/Inpari 13^2 was reddish-purple, while darker color grain was expressed in the progeny of Melik/Fatmawati (Figure 1). According to the Annual Activity Report in 2017, the anthocyanin level of Melik variety was 300 ppm when tested by Yogyakarta AIAT, but it was more than twofold level (780.6 ppm) when measured by ICABIOGRAD.

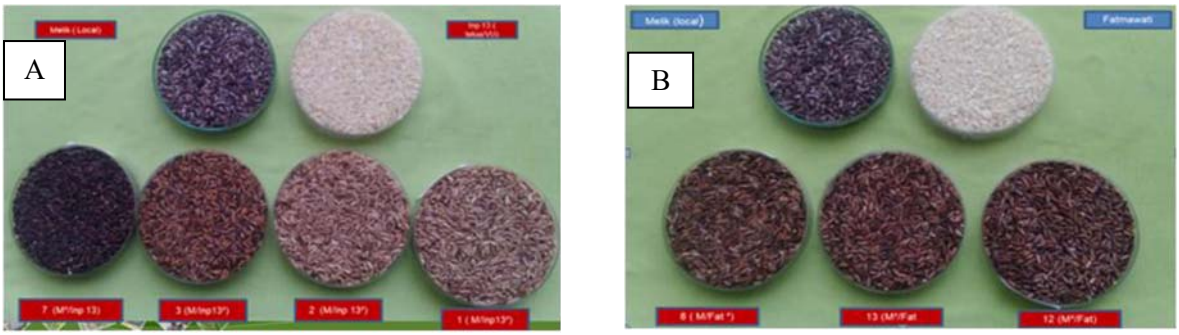

Figure 1. Diversity of rice color in DH lines progeny of Melik/Inpari 13^2 (A) and Melik/Fatmawati^2 (B).

4. Conclusions

Ten DH lines (YD1-61-1-1, YD1-71-1-1, YD1-51-2-1, YD1-51-2-2, YD1-51-2-3, YD1-48-1-2, YD5-37-1-2, YD5-37-1-3, YD6-1-1-1 and YD6-1-1-2) showed an early maturity trait (115 days), which was not significantly different to that of Fatmawati and Inpari 13. These lines can be categorized into two groups, one which had reddish-purple grain color (YD1-61-1-1, YD1-71-1-1, YD1-51-2-1, YD1-51-2-2, YD1-51-2-3 and YD1-48-1-2), and the other which had reddish-black grain color (YD5-37-1-

2, YD5-37-1-3, YD6-1-1-1 and YD6-1-1-2). The yield range of the first group, which derived from Melik/Inpari 13), was 6.1 to 7.13 t/ha. The second group, which was derived from Melik/Fatmawati[2], had yield in the range of 6.4 to 7.2 t/ha. Advanced yield trials in several locations need to be conducted to evaluate the agronomical performance and anthocyanin content, as well as their resistance to major pests and diseases in rice.

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In silico identification of three putative *SWEET* genes in *Metroxylon sagu*

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Abstract. Recent studies have identified Sugars Will Eventually be Exported Transporters (SWEET), a novel type of sugar transporters in diverse plant species. This gene family selectively transports different kinds of sugar substrates, including sucrose, fructose and glucose. In this paper, three *Metroxylon sagu SWEET* genes (*MsSWEET-X*, *MsSWEET-Y* and *MsSWEET-Z*), predicted to be involved in starch accumulation, were identified from the NCBI EST database. A comparative analysis was carried out against *Arabidopsis thaliana* TAIR and *Elaeis guineensis* NCBI genome databases resulting in amino acid residues similarity of three *MsSWEET* genes of 21.32 to 76.25 %. One full-length coding sequence (CDS) of 229 amino acids from *MsSWEET-X* has been annotated as opposed to the partial CDSs from the other two. Three types of putative protein domains (Calreticulin, Glycosyl hydrolases and Triose-phosphate transporter) were predicted for *MsSWEET-X*, *MsSWEET-Y* and *MsSWEET-Z*, respectively. Multiple Alignment sing Fast Fourier Transform (MAFFT) has identified three conserved amino acid motifs (Motif-A, Motif-B and Motif-C) among three compared species. Phylogenetic analysis using Maximum-Likelihood Estimation has revealed two genes *AtCRT3* and *MsSWEET-X* at the upstream of initial tree branches (0.17 and 0.12 length) showing their early evolutionary orthology. By contrast, *MsSWEET-Y* gene was predicted to be the latest homolog of *SWEET16* and *SWEET17* undergoing speciation events from both *Arabidopsis* and oil palm. Taken together, these results showed that even though the oil palm and sago palm shared the common ancestry of monocotyledonous family, their *SWEET* genes were divergent. The gene *MsSWEET-X* was highly close to its homolog in *Arabidopsis*.

Keywords: *in silico*, MAFFT, maximum-likelihood estimation, *Metroxylon sagu*, phylogenetic analysis, *SWEET*.

1. Introduction

The Sugars Will Eventually be Exported Transporters (SWEET), a novel type of sugar transporters have been identified in diverse plant species, including thale cress, tomato, wheat, rubber tree and oil palm [1–4]. Various copy numbers were found ranging from 15 to 29 *SWEET* genes for each plant species. This gene family selectively transports different kinds of sugar substrates, including sucrose, fructose and glucose across the cell membrane regulating physiological aspects in plant species [2,5]. SWEET proteins were generally encoded by seven helices transmembrane domains consisting of a typical tandem repeat of three transmembrane domains, *SWEET*-type TMD helices [5,6].

Considered as a potential staple crop due to its high starch content, sago palm (*Metroxylon sagu*) incorporates the mechanism of sugar transport resulting in starch storage that has not yet been elucidated. The *M. sagu SWEET* gene family is potentially one of the gene families which have an indispensable role in the process of sugar storage. In addition, the genome information for this species is equally limited. An Expressed Sequence Tags (ESTs) library from the leaves of sago palm was published [7]. The database showed that the majority of 372 transcripts was potentially contributed in primary metabolism such as sugar biosynthesis and storage as well as stress tolerance.

This paper aimed to *in silico* identify and characterize the *M. sagu* SWEET gene family by performing comparative analysis from ESTs database against *A. thaliana* and *E. guineensis* genomic databases. Multiple sequence comparisons using MAFFT and BLAST using public platform Galaxy were respectively carried out to identify orthologous sequences between those three species. Phylogenetic analysis was constructed using PhyML to group evolutionary relationship of *SWEET* genes. TMHMM analysis was performed to predict the capability of transmembrane activity for SWEET protein.

2. Materials and methods

2.1. In silico comparative analysis of the SWEET gene family in sago palm, thale cress and oil palm

The *M. sagu* ESTs with accession numbers of JK731189-JK731342 and JK731189-JK731600 were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/). The ESTs consist of 372 tentative unique genes (TUGs) sequences [7]. The ESTs were annotated and stored in the Southern France Galaxy bioinformatics platform (http://galaxy.-southgreen.fr/galaxy/). Thirty-three nucleotide sequences and protein residues of *Arabidopsis thaliana SWEET* (*AtSWEET*) gene family were downloaded from PLAZA 4.0 Dicots Database (https://bioinformatics.psb.ugent.be/ plaza/versions/plaza_v4_-dicots/). In addition, nineteen nucleotide sequences and protein residues of *Elaeis guineensis SWEET* (*EgSWEET*) gene family were downloaded from PLAZA 4.0 Monocots Database (https://bioinformatics.psb.ugent.be/plaza/-versions/plaza_v4_monocots/).

An NCBI MEGA-BLAST + tblastn of *AtSWEETs* and *EgSWEETs* against the *M. sagu* ESTs were carried out at the Galaxy platform using the modified method of Piyatrakul et al. [8] and Putranto et al. [9]. The expectation value cutoff was set to 0.001 using the scoring matrix of BLOSUM62. The results of tblastn were sorted to ensure no duplicated genes noted. The selection was based on the parameters consisting of: (1) hits sharing >50% of sequence similarity with minimum 150 bp length, (2) one unique gene for each location in the EST, (3) one EST hosting more than one gene in the different location, and (4) low E-value.

2.2. Manual annotation and conserved domain analysis of M. sagu ESTs

Manual annotations of *M. sagu* genes were carried out on selected EST encoding potential SWEET domains using Geneious v5.3.6 (Biomatters Ltd, USA). The annotation included the non-translated region (5'- and 3'-UTR), coding sequences (CDS) and protein or domain motifs [10,11]. The putative CDS region for each *M. sagu SWEET* genes was verified using BLASTn in the NCBI (https:// blast.ncbi.nlm.nih.gov/Blast.cgi). The analysis of the conserved domain in the CDS region followed the protocol of Putranto et al. [9]. Each EST containing *SWEET* transcript was screened using the NCBI Conserved Domain Database Search (CDD) (www. ncbi.nlm.nih.gov/-Structure/cdd/cdd.shtml). Multiple sequence alignments between MsSWEET proteins were carried out using Fast Fourier Transform (MAFFT).

2.3. Phylogenetic analysis of MsSWEET against two species models and transmembrane domain analysis

Phylogenetic analysis was performed to determine the orthology and paralogy of a putative *MsSWEET* gene family. The comparative analysis was done on MsSWEET protein residues against 23 AtSWEET and 19 EgSWEET full-length protein residues. The phylogenetic tree was assembled using the BioNJ in the PhyML online software (http://www.atgc-montpellier.fr/phyml) [12]. Model of amino acid substitution used WAG. Bootstrap for the consensus tree was made as many as 6,506 replicates.

The transmembrane domain encoded by MsSWEET proteins was predicted using online server analysis TMHMM V2.0 (www.cbs.dtu.dk/services/TMHMM/). The method used a hidden Markov model (HMM) approach to model various regions of a model protein [13].

3. Results and discussion

3.1. In silico comparative analysis of MsSWEET against AtSWEET and EgSWEET gene families

The identification of several members of *MsSWEET* genes has been successfully performed using *in silico* comparative analysis on the EST database of Wee and Roslan [7] against two databases of model species. Three putative transcripts encoding SWEET proteins (MsSWEET-X, MsSWEET-Y and MsSWEET-Z) were identified from the EST accession numbers JK31566.1, JK31234.1 and JK731407.1, respectively (Table 1). The length of the transcripts ranged from 999 to 1071 bp. The sequence's similarity of *MsSWEET* genes against *AtSWEET* and *EgSWEET* genes were ranged from 21.32 to 76.25% covering 232 to 921 amino acid residues. One full-length coding sequence (CDS) of 229 amino acids from *MsSWEET-X* has been annotated as opposed to the partial CDS from the other two. The partial protein sequences were 57 and 136 amino acid residues. Three types of putative protein domains (Calreticulin, Glycosyl hydrolases and Triose-phosphate transporter) were predicted for *MsSWEET-X*, *MsSWEET-Y* and *MsSWEET-Z*, respectively. The length of the amino acid domains were 141, 57 and 129 bp.

Table 1. Identification of three putative MsSWEET genes using the *in silico* comparative analysis.

Gene name	Wee's ESTs			Galaxy tblastn				Protein sequence		Domain protein		
	Seq ID	Length (bp)	Species	Ref ID	Similarity (%)	Length (aa)	E-value	CDS	Length (aa)	Name	Accession	Length (aa)
MsSWEET-X	JKT731566.1	1,071	*Ath*	AT1G08450.1	76.25	424	1.66E-84	Full-length	229	Calreticulin	cl02828	141
MsSWEET-Y	JKT731234.1	999	*Ath*	AT5G63840.1	47.368	921	8.75E-12	Partial	57	GH31	cl25582	57
MsSWEET-Z	JKT731407.1	1,055	*Egu*	XP_010922652.1	21.324	232	0.05	Partial	136	TPT	cl26744	129

3.2. Phylogenetic analysis of MsSWEET with AtSWEET and EgSWEET protein residues

The phylogenetic analysis of MsSWEET with AtSWEET and EgSWEET protein residues were carried out. Distinctive evolutionary developments of three putative MsSWEET proteins have been identified (Figure 1). The evolutionary relationship starts with AtCRT3 and MsSWEET-X at the upstream of the *SWEET* gene family with 0.17 and 0.12 value of genetic distance. Furthermore, the MsSWEET-Z had a close orthologous relationship with AtVAL1 with a branch value of 0.11. In general, the *AtSWEET* and *EgSWEET* gene family shared close homolog structures in which both of these families were found in the massive cluster. The protein MsSWEET-Y was relatively close with AtSWEET16 (a branch value of 0.38) and AtSWEET17 (a branch value of 0.56). Regarding the phylogenetic tree structure, of the three compared species, the protein MsSWEET-Y was the latest developed in the *SWEET* gene family resulted from the previous speciation of *A. thaliana*. This result confirmed a potential distinctive function of MsSWEET-Y compared with the other MsSWEET proteins.

The matching of *M. sagu*, *A. thaliana* and *E. guineensis SWEET* genes were carried out using tblastn of SouthGreen Galaxy. The identification of protein domain was carried out using the NCBI CDD.

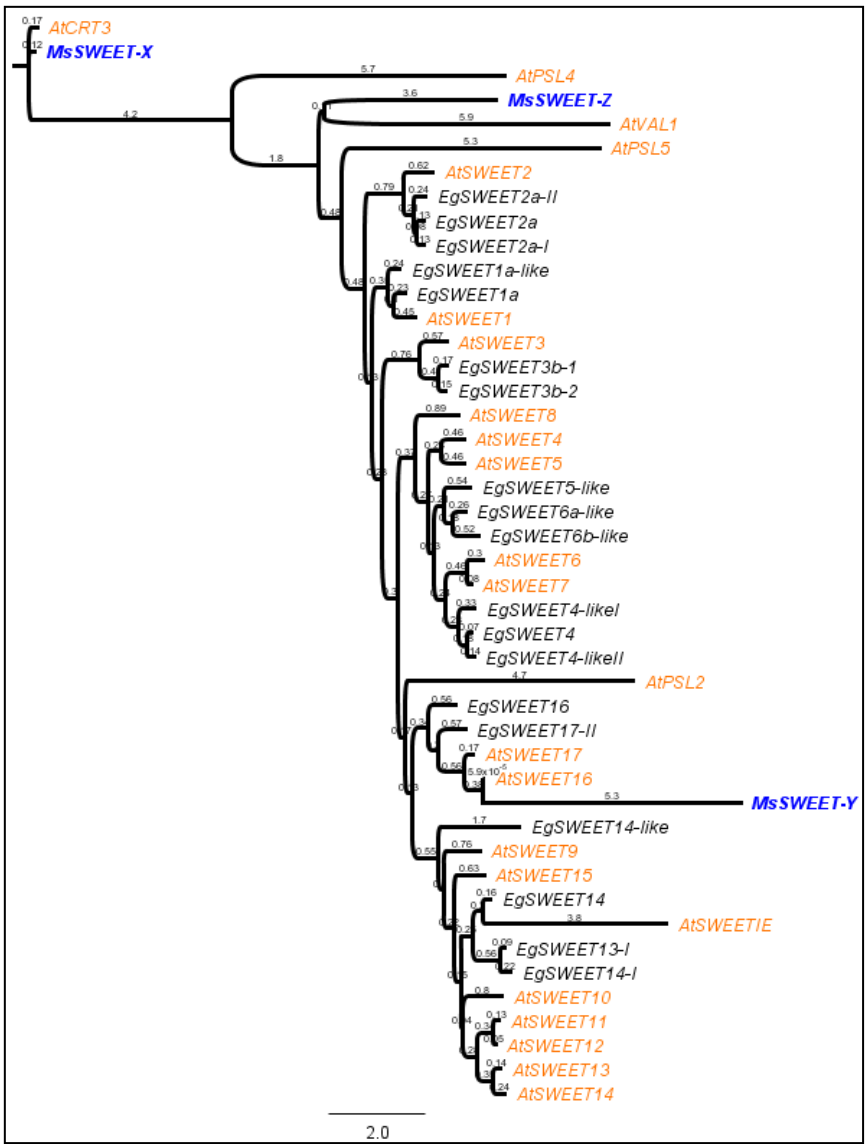

Figure 1. Phylogenetic analysis of 3 MsSWEET against 23 AtSWEET and 19 EgSWEET protein residues. The evolutionary history was inferred using the BioNJ method of PhyML online software. Bootstrap for the consensus tree was made as many as 6,506 repetitions. The bar showed an evolutionary distance of 2.0.

3.3. Conserved motifs and transmembrane domain of putative MsSWEET genes

The multiple sequence alignment using MAFFT has identified three differentiated groups (A, B and C) among the three compared species (Figure 2). Motif-A included 62 amino acid residues dominated by leucine (L) amino acid. Motif-B and Motif-C covered 37 and 27 amino acid residues, respectively. Each of *MsSWEET* gene identified in this work harboured unique sequences differed from *A. thaliana* and *E. guineensis*. In addition, the prediction of the transmembrane domain using TMHMM V2.0 in

three MsSWEET proteins showed that only MsSWEET-Z encodes four predicted transmembrane domains. The locations of predicted transmembrane domains are in 5th–27th, 42nd–61st, 74th–96th and 100th–121st amino acid positions of MsSWEET-Z protein (Figure 3).

MsSWEET-X showed 76.25% orthology to AtCRT3 (AT1G08450.1) encoding calreticulin protein. In *Arabidopsis*, AtCRT3 demonstrated to be involved in the response against the bacterial Pathogen-Associated Molecular Pattern (PAMP) [14]. This result hypothesized a potentially wide range function of the *SWEET* gene family in plants. As for, MsSWEET-X, it has a potentially similar function with AtCRT3.

MsSWEET-Z was identified with a low percentage of similarity (21.32%) to *E. guineensis* protein XP_010922652.1 due to its partial state of the sequence. The comparison to the EST database has its limit as this kind of library was prone to a sequence gap due to the random cloning of expressed CDS. However, reference studies showed that this protein refers to predicted bidirectional sugar transporter SWEET2a and is responsible in sugar transporter. The phylogenetic tree showed a close relationship between *EgSWEET2a* and *AtSWEET2*. The gene *AtSWEET2* encoded a SWEET2 protein mediating both low-affinity uptake and efflux of sugar across the plasma membrane in *Arabidopsis*. The partial similarities of *MsSWEET-Z* to *EgSWEET2a* was confirmed by four transmembrane domains detected using TMHMM V2.0. The transmembrane domain was generally found with helical structure and is responsible for regulating the membrane across activity. This result confirmed the predicted function of *MsSWEET-Z* as one of the key genes responsible for sugar transporter in sago palm.

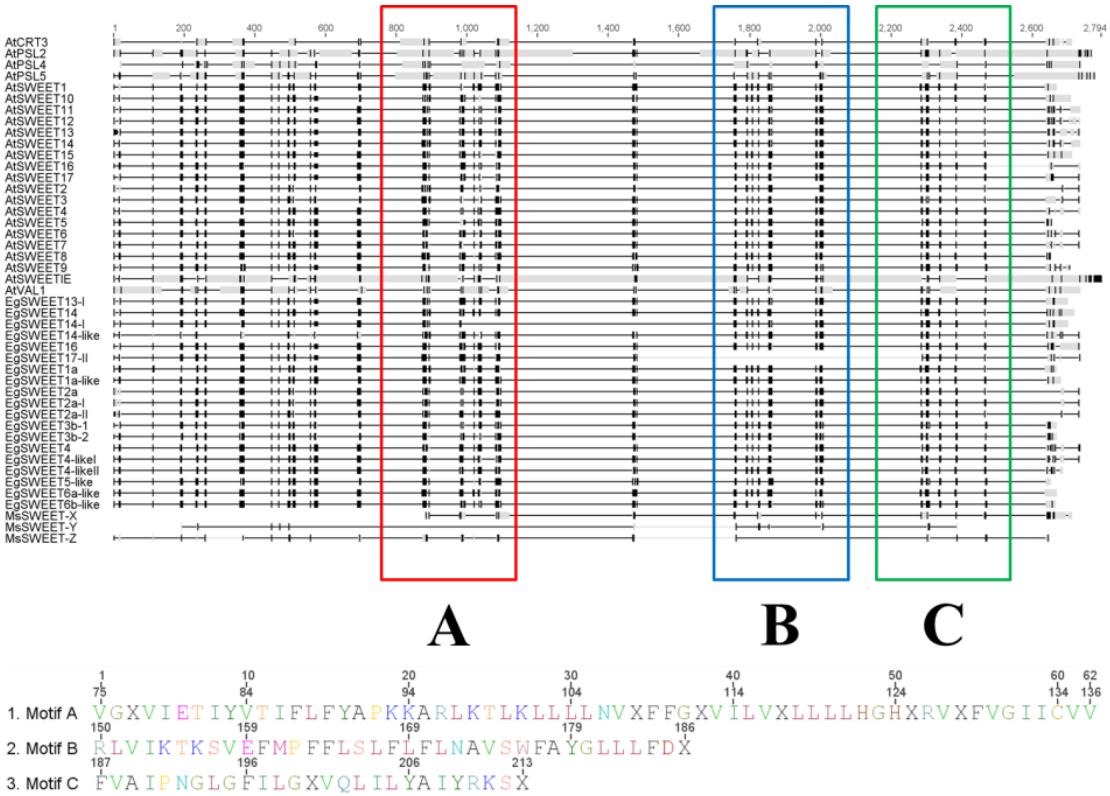

Figure 2. The multiple sequence alignment using MAFFT of the *SWEET* gene family of *M. sagu*, *A. thaliana* and *E. guineensis*. The protein motifs (A, B and C) were identified. The MAFFT was performed on the Galaxy platform with the parameters: matrix BLOSUM 62, maximum numbers of iterations 1,000 and distance method 6 mers.

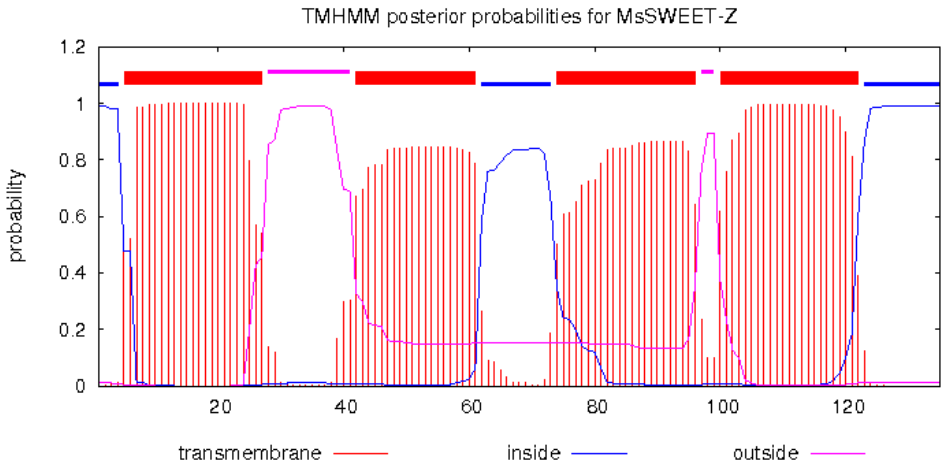

Figure 3. The predicted transmembrane domain encoded by MsSWEET-Z. The analysis was performed using online server TMHMM V2.0.

4. Conclusions

The *in silico* comparative sequence analysis has successfully identified three putative *MsSWEET* genes (*MsSWEET-X*, *MsSWEET-Y* and *MsSWEET-Z*). This work correspondingly showed that even though the oil palm and sago palm shared the common ancestry of the monocotyledonous family, their *SWEET* genes were divergent. The gene *MsSWEET-X* was highly close to its homolog in *Arabidopsis* (*AtCRT3* gene) as opposed to the gene *MsSWEET-Z* predicted to be the real transmembrane protein. Future research should focus on the gene expression of each *MsSWEET* gene in accordance with the sago palm with high producing starch.

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Targeted mutation of *GA20ox-2* gene using CRISPR/Cas9 system generated semi-dwarf phenotype in rice

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Abstract. Recently, the engineered CRISPR/Cas9 system has been applied to rapidly and efficiently modify the targeted gene(s) in a wide variety of plants. Recent studies of successful targeted mutagenesis using the CRISPR/Cas9 system with a single gRNA expression in rice plants have been reported. *GA20ox-2* is a gene encoding an oxidase enzyme involved in the biosynthesis of gibberellin and linked to *sd1* locus. A previous study revealed that mutation of this gene resulted in shorter stature of rice plant due to defects in the gibberellin's signalling pathway. Here, we studied targeted mutation of *OsGA20ox-2* gene in rice using the CRISPR/Cas9 system with the expression of two gRNAs. In this study, we introduced a single plasmid vector of CRISPR/Cas9 system harboring dual gRNAs to modify *OsGA20ox-2* gene in a rice model cv. Kitaake via *Agrobacterium*-mediated transformation. Targeted mutagenesis of *OsGA20ox-2* gene using CRISPR/Cas9 generated nine mutated rice lines with a mutation frequency of 90%. Most mutated lines (50%) had mutations in both *OsGA20ox-2* gRNA. They resulted in homo-diallelic mutation type with 44 bp deletion, while three lines were heterozygous, one line was homo-diallelic with 2 bp insertion, and one line had no mutation. The K15 mutated rice line was identified as a homozygous two-nucleotide insertion and had the semi-dwarf phenotype, demonstrating that *OsGA20ox-2* gene had been disrupted.

Keywords: rice (*Oryza sativa* L.), CRISPR/Cas9 system, targeted mutagenesis, semi-dwarf.

1. Introduction

Rice improvement is essential to meet the increasing food demand due to rapid growth in population. Extreme weather events and the availability of natural genetic resources are other issues that have endangered the global food security [1]. Therefore, improving yield, biotic and abiotic tolerance is crucial to increase and stabilize the productivity of rice crop. Conventional breeding, such as hybridization and mutation techniques, have significantly contributed to increasing rice productivity. Molecular approaches, including marker-assisted backcrossing (MABC) and genetic engineering (GE) technology, have also played an important role in overcoming the limitations of conventional breeding and enhancing crop productivity. However, these technologies still have some disadvantages, such as

being time-consuming and labor-intensive. In the case of GE approach, the complex regulatory process followed by time-consuming and costly safety analysis, as well as the public acceptance of GE crops, are a great challenge [2]. Hence, a directed, rapid, and low-cost method is essential for developing high-yielding, biotic or abiotic stress resistant rice varieties. Targeted editing technology of key functional genes promises to be a powerful tool in accelerating varietal improvement [3].

Genome editing system can be applied to modify gene(s) rapidly in a precise and predictable manner. The technology has a great potential to accelerate crop basic research and improvement program. A more recently developed genome editing system is clustered regularly interspaced short palindromic repeat (CRISPR)/Cas (CRISPR-associated) based on RNA-guided engineered nucleases and a guide RNA complex. The most widely used system is the type II CRISPR/Cas9 employing a Cas9 endonuclease from *Streptococcus pyogenes* [4]. The system allows the creation of double-stranded breaks (DSBs), which can lead to gene mutations due to non-homologous end-joining (NHEJ) repair or gene replacement or correction as a result of homologous recombination-based repair (HR). In most cases, NHEJ causes random insertions or deletion (Indels), which can result in frameshift mutations if they occur in the coding region of the gene, effectively creating a gene knockout. The CRISPR/Cas9 system has been successfully used for efficient genome editing and applied to gene modification in plants, such as rice [5–8], wheat [6,9], maize [10], potato [11], tomato [12] and sweet orange [13].

GA20ox-2 is a gene encoding an oxidase enzyme involved in the biosynthesis of gibberellin, a plant growth hormone, and it is tightly linked to the *sd1* locus [14]. This gene was isolated from rice using degenerate primers based on the conserved domain of the *GA20ox* gene in rice (*OsGA20ox-1*) [15] and *Arabidopsis* (*GA5*) [16] and located on the chromosome 1 [14]. *OsGA20ox-2* is strongly expressed in leaf blades, stems, and unopened flowers. Increased expression of the gene in those tissues, especially in the leaf blade and stem, resulted in a semi-dwarf phenotype in the enzyme-defective *sd1* mutants, as indicated by shorter leaves and stems [17]. Further information explained that *sd1* mutants still allow the flowers to develop and be fertilized normally although the mutants experienced a loss of function of *GA20ox-2* gene. Because of the normal flower formation and fertilization, the yield of *sd1* mutant plants would be stable and not affected by the reduced plant height.

The aim of this research was to study targeted gene mutation of *OsGA20ox-2* using CRISPR/Cas9 system to generate semi-dwarf phenotype in rice. Our recent work has demonstrated that we have successfully introduced the single plasmid vector of the CRISPR/Cas9 system harboring dual gRNAs to modify *OsGA20ox-2* gene in a rice model cv. Kitaake via *Agrobacterium*-mediated transformation. Here, we showed the CRISPR/Cas9 had induced mutations in the targeted endogenous plant gene *GA20ox-2* and they were detectable in T_0 rice plant. The K15 mutated rice line was identified as a homozygous two-nucleotide insertion and had the semi-dwarf phenotype, demonstrating that *OsGA20ox-2* gene had been disrupted.

2. Materials and methods

2.1. Plant material and plasmid

The rice variety Kitaake (*Oryza sativa* L. spp. *japonica*) was used as material for *Agrobacterium*-mediated transformation as previously described. The plant transformation vector was based on pBY02-Cas9 (kindly provided by Dr. Bing Yang), a binary vector for *Agrobacterium*-mediated rice transformation containing a maize *ubiquitin* 1 promoter driving the rice codon-optimized *Cas9* gene and a CaMV 35S promoter driving *hpt*II gene expression for hygromycin resistance.

2.2. Construction of guide RNA of OsGA20ox-2 gene

Construction of guide RNA gene was performed using an intermediate vector pENTR-gRNA that can express two different gRNAs [18]. The pENTR-gRNA vector containing two cloning sites, the first cloning site is facilitated with 2×*Btg*ZI downstream of one rice U6 promoter and another cloning site with 2×*Bsa*I downstream of the second rice U6 promoter. CRISPR/Cas9 construct was designed in a binary T-DNA vector for co-expression of *Cas9* gene and guide RNA. *OsGA20ox-2* gene-specific

gRNAs sequences (gRNA1-GA20ox-2 and gRNA2-GA20ox-2) were cloned into entry vector pENTR-gRNA. Two complementary oligonucleotides (24 nucleotides) from each gRNA that target a specific genomic locus of *OsGA20ox-2* gene were annealed to generate a double-stranded DNA oligonucleotide (dsOligo). Both guide RNAs of *OsGA20ox-2* gene were then inserted into pENTR-gRNA through the 2×*Btg*ZI and 2×*Bsa*I cloning sites, respectively. The gRNA cassette containing two gRNA of *OsGA20ox-2* was finally combined with pBY02-Cas9 by using the Gateway LR Clonase (Thermo Fisher Scientific, USA). The CRISPR/Cas9 construct containing the *Cas9* gene and guide RNA was transformed into *A. tumefaciens* strain LBA4404, and the transformation vector was then used for rice transformation.

2.3. Rice in vitro culture and transformation

Mature *japonica* cv. Kitaake rice seed was dehusked, surface sterilized and placed onto callus induction medium (4 g/l NG salt, 300 mg/l casamino acid, 2.8 g/l L-proline, 30 g/l sucrose and 4 g/l Gelrite, pH 5.8) containing 2 mg/l 2,4-D. The callus tissue derived from the mature embryo is then used as the starting material for transformation. *Agrobacterium*-mediated rice transformation steps followed the protocol as previously described [19]. The transgenic rice lines were grown in a greenhouse with standard maintenance as recommended. Mature seeds were collected from T_0 plants and used for further genotyping analysis.

2.4. Genotyping transgenic rice plant

Total genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method from the transgenic rice plants [20]. The genomic DNA was used as a template to amplify the specific region surrounding the CRISPR/Cas9 target sites of *OsGA20ox-2* gene using a pair of specific primer, i.e. OsGA20ox-2-F (5'-TCATGTCTGTCCAGTGGCAAC-3') and OsGA20ox-2-R (5'-CACCATCGTTTTAATTACCCCATT-3'). The PCR fragments were directly sequenced to identify the pattern of mutations by sending the fragment to a Lab Service at PT Genetika Science. Sequence data were analyzed by alignment to the target sequence of transgenic line compared to one of the wild type plants using the BLAST program.

3. Results and discussion

Two guide RNAs have been designed from exon 1 of *OsGA20ox-2*. To enhance mutagenesis success in the targeted exon of *OsGA20ox-2* gene, two closely located target sites in *OsGA20ox-2* gene were selected for gRNA construction. The position of the gRNAs of *OsGA20ox-2* is shown in Figure 1. The gRNA1-GA20ox-2 was designed in sense orientation and contained the start codon ATG in order to enhance the chances of mutation of the target gene. Modification of ATG start codon will make the gene unable to start the transcription process, and therefore, the gene will malfunction.

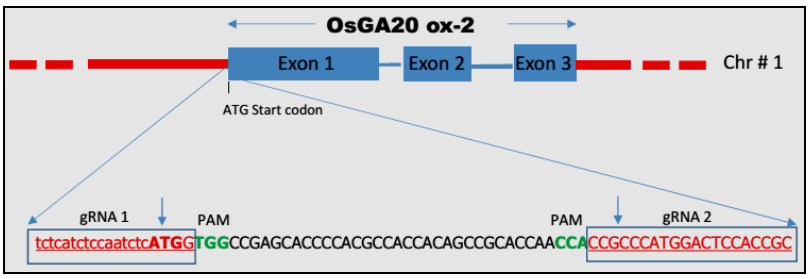

Figure 1. Position of dual gRNAs of *OsGA20ox-2* gene in chromosome 1. Both gRNAs are located in exon 1 of the gene. The gRNA1 was designed in sense orientation containing start codon ATG, while the gRNA2 was in antisense orientation with 44 bp distance from gRNA1.

The intermediate vector pENTR-gRNA, which has two different rice U6 small nuclear RNA gene promoter (PU6-1 and PU6-2), was used to express the dual gRNAs of *OsGA20ox-2*. The gRNA1-GA20ox-2 was cloned at 2×*Btg*ZI sites from the first gRNA scaffold in a tail-to-tail orientation downstream of PU6-1. Meanwhile, the gRNA2-GA20ox-2 was inserted at 2×*Bsa*I cloning sites that were part of the second gRNA scaffold in a tail-to-tail orientation downstream of PU6-2. Two sequential steps of cloning enabled the insertion of the custom two gRNAs into *Btg*ZI dan *Bsa*I restriction enzyme sites in the pENTR vector to produce the intermediate construct pENTR-gRNA-OsGA20ox-2 (Figure 2).

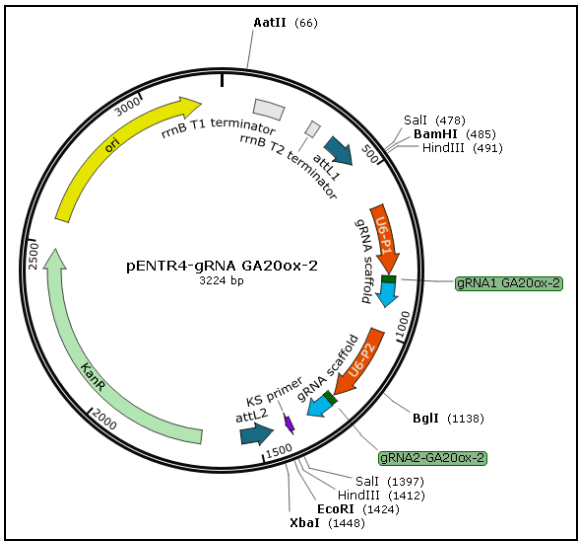

Figure 2. The intermediate vector pENTR-gRNA-OsGA20ox-2 carrying dual guide RNA of *OsGA20ox-2* gene, i.e. gRNA1-GA20ox-2 and gRNA2-GA20ox-2.

To evaluate the efficacy of the CRISPR/Cas9 system in inducing double-strand breaks (DSB) and initiating target gene mutation in rice, we selected *OsGA20ox-2* gene as the target. This gene encodes an oxidase enzyme involved in the biosynthesis of gibberellin. Rice has only one copy of the *OsGA20ox-2* gene and if this gene is knocked out, it would result in a semi-dwarf phenotype indicated by shorter leaves and stems. The standard protocol of *Agrobacterium*-based rice transformation [19] (Figure 3) was used to introduce the CRISPR/Cas9-gRNA-GA20ox-2 to generate 10 independent rice lines.

Following the genetic transformation, T_0 independent lines that showed resistance to hygromycin were identified. To detect mutations in the targeted sequence regions in the T_0 plants, the DNA of ten independent T_0 rice lines were amplified using specific primers for the specific region surrounding the CRISPR/Cas9 target site of *OsGA20ox-2*. PCR amplification showed that all ten plants produced DNA fragment or amplicon with a size of 768 bp (Figure 4). The amplicon from each plant was then subjected to DNA sequencing analysis to identify mutagenesis of the target mutation sites.

Based on DNA sequencing analysis, it was found that there were various combinations of mutations in the representative rice lines as illustrated in Figure 5. Nine mutated rice lines were identified from the ten independent transgenic lines, which means that the mutagenesis frequency was 90%.

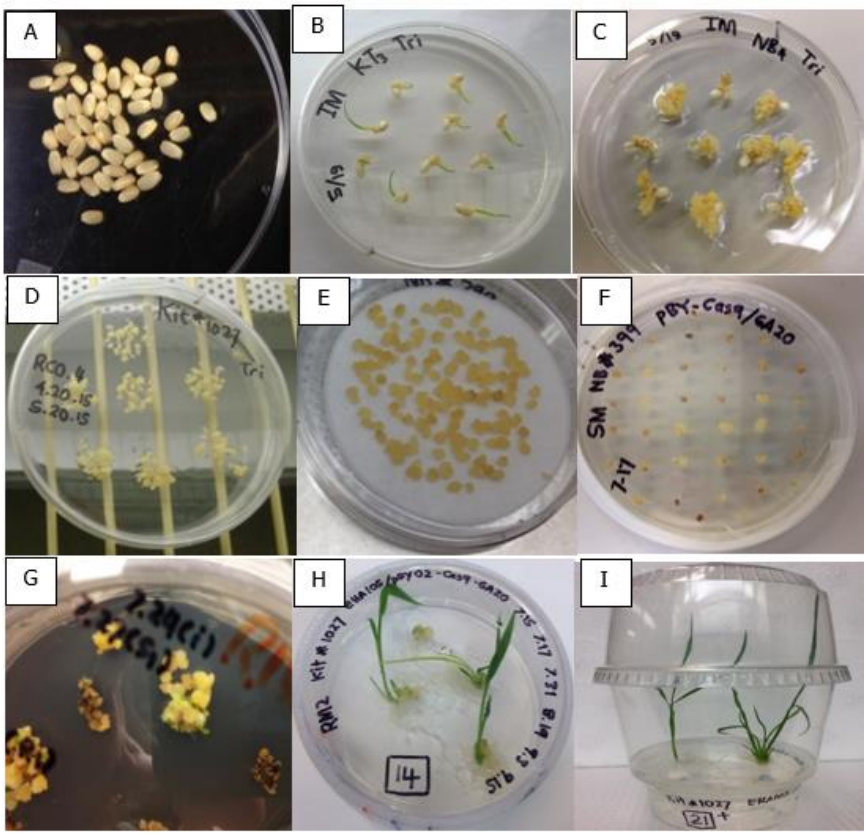

Figure 3. *Agrobacterium*-based rice transformation using mature embryos of rice cv. Kitaake. (A–D) Callus induction steps to produce embryogenic calli. (E) Co-cultivation the calli with *A. tumefaciens* suspension. (F) Selection of resistant callus in hygromycin-containing medium (50 mg/l). (G) Selected callus formed green spots. (H) Regenerated calli. (I) Plantlet in rooting medium.

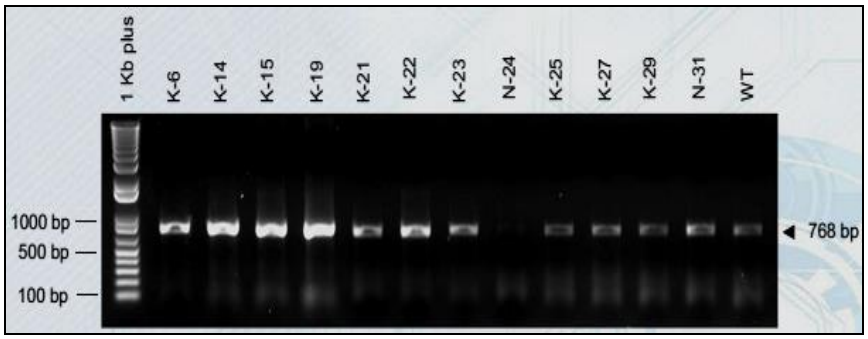

Figure 4. PCR amplification of the specific region in *OsGA20ox-2* gene using a pair of specific primers flanking the mutation target site.

Lines	Nucleotide	Mutation type
WT	AATCTCATGGTGGCCGAGCACCCCACGCCACCACAGCCGCACCAACCACCGCCCATGGAC	
K-6	AATCT---GGCGGCCGAACACCCCCCGCCGCCACGGCCGCGCCAACCCCCGAGCCAGGGAC	nd, heterozygous
	AATCTCA----------CCCATGGAC	
K-14	AATCTCA----------CCCATGGAC	- 44, homo-diallelic
K-15	AATCTCAaTGGTGGCCGAGCACCCCACGCCACCACAGCCGCACCAACCACCGaCCCATGG	+ 2, homo-diallelic
K-19	AATCTCA----------CCCATGGAC	-44, homo-diallelic
K-21	AATCTCATGGTGGCCGAGCACCCCACGCCACCACAGCCGCACCAACCACCGCCCATGGAC	No mutation
K-22	AATCTCA----------CCCATGGAC	-44, homo-diallelic
K-23	TCTCTCG----------CCCGTGTC----------TGGCC	nd, heterozygous
K-25	AATCTCA----------CCCATGGAC	-44, homo-diallelic
K-27	AATCTCAATGGTGGACGAGCACCCCACGCCAGCACAGGCGCACCAACCACCGCGCCCGCG	nd, heterozygous
	AATCTCA----------CCCATGGAC	
K-29	AATCTCA----------CCCATGGAC	-44, homo-diallelic

Figure 5. CRISPR/Cas9-gRNA-induced mutations at the target sites of *OsGA20ox-2* from 10 transgenic lines. Various mutations combinations were found in selected T_0 rice plants. Nucleotide in red represent the gRNA1 and gRNA2 and green underlined nucleotides indicate PAM. Dashes for deletion and blue lowercase letter for insertion.

Half of the mutated lines had alterations in both OsGA20ox-2 gRNA and resulted in homo-diallelic mutation type with 44 nt deletion, while 3 lines were heterozygous, one line was homo-diallelic with 2 nt insertion, and one line had no mutation (Figure 5). These results indicated that CRISPR/Cas9 technology could generate transgenic rice lines with mutated gene of interest easily with high efficiency in the T_0 generation. The high efficiency of targeted mutagenesis in this study is possibly due to the unique characteristics of the CRISPR/Cas9 system. The specificity of CRISPR/Cas9 is not affected by DNA methylation of target gene sequences [21]. The CRISPR/Cas9 technology is advantageous for gene modification in plants with high GC content in their genome such as rice. Hsu et al. (2014) proposed that the high mutation rate was also affected by the GC content of the target gene. We observed that *OsGA20ox-2* gene has 56.9% GC content, which might lead to the high mutagenesis frequency of *OsGA20ox-2* gene.

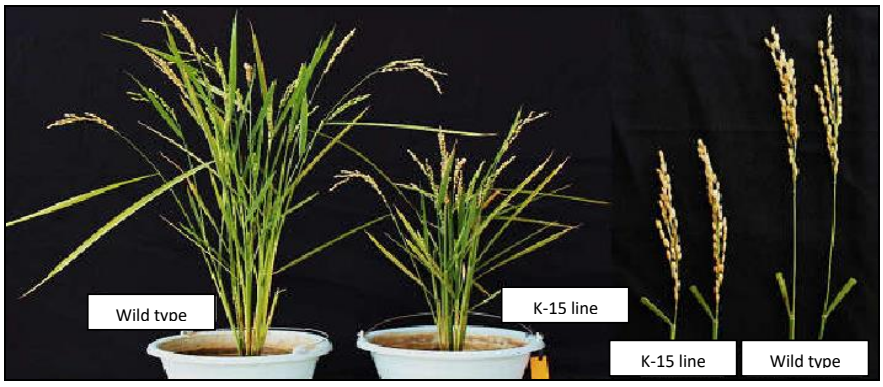

Figure 6. Comparison of CRISPR/Cas9 mutant line K-15 and wild type cv. Kitaake in plant height and panicle length characters.

The results also revealed that targeted mutagenesis of *OsGA20ox-2* with dual gRNA can be generated with high efficiency. Dual gRNA construct could efficiently generate targeted mutagenesis since all mutated lines experienced mutation in targeted sites for the dual of gRNA-GA20ox-2. In this experiment, we adopted a strategy to enhance the efficiency of mutagenesis for genome editing. We designed and constructed two gRNAs targeting *OsGA20ox-2* gene to increase the success rate or

improve the possibility that at least one gRNA will be active for mutagenesis [10]. Another important utility of 2-gRNAs-for-1-gene approach is to enable large deletion mutations in the targeted gene.

In our experiment, homozygous mutations were found in the T_0 plants, accounting for 60% of all T_0 plants (Table 1). It was also observed that half of the homozygous mutations were deletion and all deletions were 44 bp in length (Figure 5). The deletion was caused by the cleavage of double-stranded DNA by Cas9 at a position three base pairs upstream of the PAM sequence from each of gRNA. Homozygous insertion mutation occurred in one plant (K-15) with 2 bp insertion. This result revealed the tendency that the targets showing higher mutation rates were more likely to have homozygous mutations at T_0 [22].

Table 1. Agronomic characters observed on CRISPR/Cas9-T_1 mutant rice lines.

Line	Indel (T_0)	Plant height (cm)	Number of tiller	Exsertion	Leaf flag length (cm)	Length panicle (cm)	Number of fertile seed	Number of sterile seed
WT	Control	82.48	10.50	19.93	36.61	12.74	39.96	4.14
K6.1	Hetero-diallelic	67.94	5.00	20.39	27.04	13.14	46.78	4.22
K14.1 +	44 bp deletion	81.45	9.40	21.55	28.59	12.59	46.10	4.23
K15 +	2 bp insertion	54.31	8.27	14.60	21.79	11.48	32.88	3.75
K19.1 +	44 bp deletion	72.89	8.44	18.36	32.85	12.13	39.04	5.53
K21 +	No mutation	76.40	8.00	21.94	30.5	13.35	48.13	4.27
K23	Hetero-diallelic	73.77	7.58	20.26	29.77	13.26	45.90	3.62
K25.1	44 bp deletion	66.41	5.88	19.77	25.90	12.13	36.24	4.57
K27.1 +	Hetero-diallelic	65.03	6.50	17.02	23.99	11.31	28.00	5.67
K29	44 bp deletion	82.07	8.79	16.09	30.33	15.50	28.66	19.78
N24.2	No mutation	75.72	14.08	15.29	28.57	15.23	37.23	13.8
N31.1	44 bp deletion	69.86	8.85	16.09	30.33	15.50	28.66	19.78

WT = wild type.

To evaluate their phenotypic characters, nine T_1 mutant lines were planted in a greenhouse. Observations indicated that the mutant lines had agronomic characters that were almost similar with the wild type. However, there was one line that showed significant differences from the wild type, i.e. mutant line K-15, especially for plant height and flag leaf length characters (Table 1, Figure 6). It proved that knocking out *OsGA20ox-2* resulted in a semi-dwarf phenotype that was indicated by shorter leaves and stems, which is similar to results found by another group [17]. Based on this result, we summarized that CRISPR/Cas9 system was highly efficient for genome editing in rice. Homozygous mutant alleles were readily found in T_0 plants. Similar result was also obtained by Zhang et al. [22]. This finding confirms CRISPR/Cas9 system as a technology for inducing rapid and accurate modifications in plant genome, thus reducing the breeding time when compared with conventional breeding or genetic engineering. This study also proves that the CRISPR/Cas9 system is a powerful tool in rice improvement through targeted gene editing.

4. Conclusions

Targeted mutagenesis of *OsGA20ox-2* gene using CRISPR/Cas9 generated nine mutated rice lines with a mutation frequency of 90%. Half of the mutated lines had alterations in both OsGA20ox-2 gRNAs and resulted in homo-diallelic mutation type with 44 bp deletion, while 3 lines were heterozygous, one line was homo-diallelic with 2 bp insertion, and one line had no mutation. The K15

mutated rice line was identified as a homozygous two-nucleotide insertion and had the semi-dwarf phenotype.

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CRISPR/Cas9 system for disruption of biochemical pathway for sterol synthesis in *Artemisia annua* L.

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Abstract. WHO recommends artemisinin-based combination therapy for curing malaria which is still a health problem in Indonesia. *Artemisia annua* L. is the primary source for artemisinin comes from subtropical China and India. Some introduction materials are grown in Indonesia, but they produce low concentration of artemisinin. Artemisinin synthesis uses the isoprenoid pathway, in which farnesyl diphospate (FDP) serves as the main precursor. FDP is the main precursor for sterol synthesis as well. The gene controls sterol biosynthesis is *Squalene synthase* (*SQS*). So then *SQS* is considered a competitive gene for artemisinin biosynthesis. CRISPR/cas9 is the most advanced system for genome editing, and can be used to induce a targeted-mutation. The aim of this research was to elevate Artemisinin content through disruption of the biochemical pathway of sterol synthesis using CRISPR/Cas9. Materials and methods used were as follows. *Artemisia* CRISPR/Cas9 seeds were processed and grown in selection medium and then soil. Seventy-seven *Artemisia* kanamycin resistant lines were produced. Fifty-two (of 77) lines were confirmed to have T-DNA by PCR with SQS-Fn and SQS-Rn primers, produced about 710 bp DNA fragment. By using SQS-Fs and SQS-Rs primers, those produced shorter DNA fragments (about 470 bp). A high-resolution electrophoresis QIAxcel was applied to identify for any nucleotide difference occured in PCR product of lines. Results showed that 44 lines (84.61%) have varied sizes with one to three nucleotides differences compared to control (plasmid, 468 bp). By assuming this analysis was right, it may be said that mutations has occured in *SQS* gene of *Artemisia* lines due to the CRISPR/Cas9.

Keywords: *Artemisia annua* L., artemisin, *squalene synthase* (*SQS*) gene, CRISPR/Cas9, targeted-mutation.

1. Introduction

As a world health problem, Malaria caused by *Plasmodium palciparum* has been treated by so many cures found for centuries. For a long time, quinine has been used for treating malaria, but in the 1960s *P. palciparum* started to show the signs of resistance against quinine-derived drugs [1]. In 2001, The World Health Organization (WHO) recommends Artemisinin Based Combination Therapies (ACTs) as the most effective way to treat malaria [2]. To date, *A. annua* is still the main commercial source of

artemisinin. Besides its antimalarial function, artemisinin has also been reported to have antiviral, anticancer and antischistosomal, resulting in its high demand by the pharmaceutical industry. Unfortunately, the supply of artemisinin is significantly restricted by the low content of artemisinin (0.01–0.1% leaf DW) in *A. annua* [3]. A promising approach to enhance the content of artemisinin and consequently to reduce the price of artemisinin is to use plant metabolic engineering to obtain a higher content of artemisinin in transgenic plants. It is possible now to regulate the biosynthesis of artemisinin in a direct way because several genes that are critical for synthesizing artemisinin have been cloned [3]. The synthesis of artemisinin belongs to isoprenoid pathway. In this pathway, farnesyl diphospate (FDP) serves as the main precursor for synthesis both sterol and sesquiterpen, such as artemisinin. The gene control biosynthesis of sterol is *Squalene synthase* (*SQS*) while artemisinin controlled by amorhpha-4, 11-diene synthase (ADS). *SQS* is considered as a competitive enzyme for artemisinin biosynthesis [4] (Figure 1).

Therefore, to increase Artemisinin content of *Artemisia*, the expression of *SQS* gene must be inhibited. In order to inactivate this gene, a clustered regularly interspaced palindromic repeats/ CRISPR-associated protein Cas9 (CRISPR/Cas9) which is the most advanced system for genome editing can be used to induce a targeted-mutation. CRISPR/Cas9 is revolutionizing genome editing technology with minimal off-targets in the present era. Genome editing using CRISPR/Cas9 utilizes a 20-bp guide RNA (gRNA) sequences that uses base pairing to direct Cas9 nuclease to target site and generates double-strand breaks (DSB). Following DSB is DNA repairing mechanism. During DNA repairing process, mutations are often introduced [5]. Besides gene deletions, CRISPR/Cas9 is useful for inserting specific DNA fragment into target sites and specifically altering the transcriptional activity of genes by fusing transcriptional activation or repression domains to an inactivated Cas9 [6,7]. The objective of this research was to elevate artemisinin concentration through disruption of the biochemical pathway of sterol synthesis using the CRISPR/Cas9 system. This is the first report about attempts to apply CRISPR/Cas9 system in *Artemisia*. We produced CRISPR/Cas9 vectors to targeting of *SQS* gene and about 44 *Artemisia* CRISPR/Cas9 lines indicated for some mutations.

2. Materials and methods

2.1. Seeds preparation

Seeds were harvested from 10 individual stalks each of four plants. One plant was either transformed with *Agrobacterium tumefaciens* containing of pHEE401 or pKSE401 plasmid. Both plasmids were transformed by *A. tumefasiens* strain AGL1 and GV3101. Seeds were sterilized using 10% of sodium hypochlorite, 70% of ethanol and 1% of plant tissue culture (PSM). Sterilized seeds were cultured in Murashige and Skoog (MS) medium with additional about 50 mg/l hygromycin (antibiotic) as selectable marker. While for seeds from plants which were transformed with pKSE40, 50 mg/l kanamycin was applied. Seeds of the wild type of *Artemisia* were grown on similar media without antibiotic. All samples were placed in the cool growth room (24–28°C) for seeds germination.

2.2. Acclimatization

Seedling of *Artemisia* were transplanted into the soil medium in polybags (diameter of 30 cm) and maintained in the plastic house at Balithi, Cipanas. Plants were grown in high land altitude about 1,000 m above sea level which is an optimum condition for *A. annua* to grow appropriately. Three months after planting, observation on plant morphology was conducted. Observation was included the shape of leave and stem, plant height and number of branches.

2.3. DNA preparation

DNA was isolated from young fresh leaves using a CTAB method (according to Agrawal et al. [8]) with some modification. Leaves were crashed in 2 ml eppendorf tube using chop-stick and with addition of liquid nitrogen. Extracted DNA was dissolved with 50 µl TE buffer and 1 µl RNase (10 mg/ml) was added to remove RNA. After that, the DNA solution was checked quantitatively using

Nanodrop spectrophotometer and qualitatively using 1% agarose gel electrophoresis and visualized using Chemidoc.

2.4. PCR for detection

DNA that diluted with nucleus free water (NFW) into 10 ng/µl of DNA was used as a template for PCR. The total reaction of PCR 10 µl consisted of 1.9 µl NFW, 5.0 µl 2× 2G Ready Mix KAPA, 0.6 µl $MgCl_2$ 25 mM, 0.5 µl DMSO, each 0.5 µl forward and reverse primers (10 nM/µl) and 1 µl DNA (10 ng/µl). Two pair of primers was used for identification of *Artemisia* CRISPR/Cas9 lines (Table 1). PCR products were checked by electrophoresis and by high resolution electrophoresis QIAxel (Qiagen) in order to obtain the approximate size of DNA amplicon of samples.

Table 1. Two pair of primer used for identification of *Artemisia* CRISPR/Cas9 lines.

Primers	Sequences
AaSQS-Fn	5'-TTTCTGAAGGTGGTACAAAGG-3'
AaSQS-Rn	5'-TTCGCCTGAAGAATGGAAGAG-3'
AaSQS-Fs	5'-GTGGTACAAAGGAATACAAAGTTCTC-3'
AaSQS-Rs	5'-CTCTTTACATATAAATTTTGCCATCC-3'

3. Results and discussion

3.1. Seed preparation and planting

Since two CRISPR/Cas9 plasmid vectors were inserted into the plant by two strains of *Agrobacterium*, GV3101 (coded by G) and AGL1 (coded by A), then we have four populations. They were labelled as G0 and A0 when containing of pHEE401 (with hygromicin resistant gene, *hpt*II) and transformed by Agrobacterium GV3101 and AGL1, respectively. Such similar populations, plants were labelled G2 and A2 since they contained pKSE401 (with kanamycin resistant gene selectable marker, *nptII*). Sterilized seeds for each seed batch were screened or selected using either hygromicin (50 mg/l) or kanamycin (50 mg/l) antibiotic in MS medium. During selection processes, several populations showed growth (Figure 1A). The surviving plants were considered as a transformed plant (transformant). It may indicate that the plasmid has been integrated into the plant genome. Nevertheless, some dying plants were found in medium MS and it may inhibit the transformed plants by secreting inhibitors or preventing transport of essential nutrients [9]. Then, the transformed plants were moved to new MS medium (Figure 1B). When plants grow bigger, they were immediately transplanted into the soil medium within polybag in the glass house for acclimatization (Figure 1C).

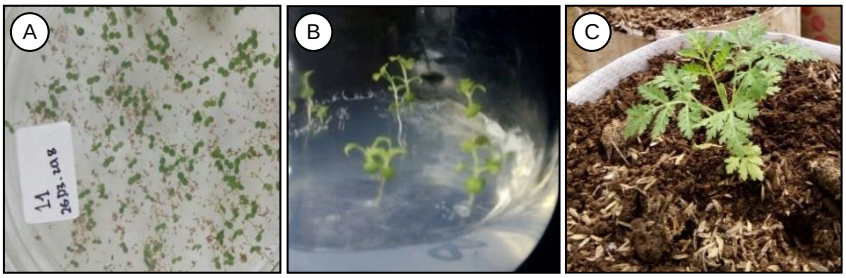

Figure 1. Preparation of *Artemisia* CRISPR/Cas9 plant. (A) Seeds selection in MS medium containing of antibiotic. (B) Transformant plants in new MS medium containing of antibiotic. (C) Tranformants plant transplanted into soil in polybag (diameter 30 cm).

3.2. Acclimatization

Until the acclimatization period finished, there were 77 survival plants/lines consisting of 7 lines of A0, 29 of G0, 40 plants/lines of G2, and 1 non-transgenic plant. So it is assumed that 36 lines contained T-DNA of pHEE401 and 40 lines contained T-DNA of pSKE401. Plants phenotype seemed slightly various and some variation in leaf arrangement are shown in Figure 2 (A, B and C). The main color of the stem and leaf are dark green but there are a few light-green leaves. The height of the plants reached 2.5 meters. Five of 77 plants (6.5%) have more than one main axis (symphodial) at the bottom part of plants, but most of the plants are monopodial. Since *A. annua* has been spreaded around the countries, it is possible that the phenotype of *A. annua* in each country is different. The diversity of phenotype may affect the content of artemisinin.

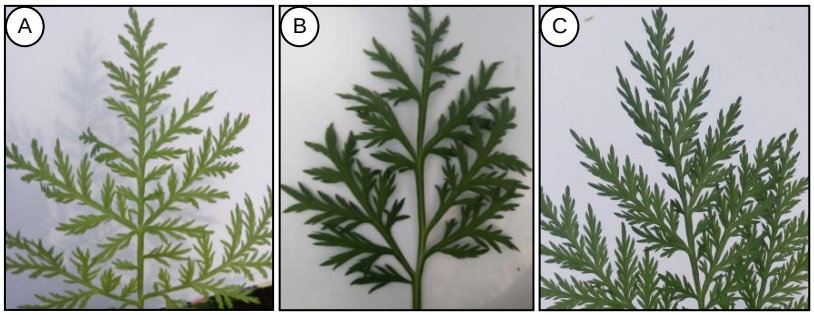

Figure 2. The representative of variation of *A. annua* leaves. (A) Distant-light green. (B) Distant-dark green. (C) Compact-dark green.

3.3. DNA Preparation

DNA isolation method for *A. annua* was a critical point for its result and will influence the molecular analysis result as well. Using liquid nitrogen, CTAB buffer and purifying agent successfully generated good extracted DNA. The quantity of extracted DNA was checked by spectrophotometer, and DNA concentration ranged from 78.8 up to 6,225.2 ng/µl. Both the quality and the quantity of extracted DNA were appropriate for molecular analysis.

3.4. PCR Detection

Based on the electrophoresis of amplified DNA (PCR product), results showed (Figure 4A, 4B and 4C) that 52 lines produced about 700 bp of DNA fragment. These mean T-DNA of CRISPR/Cas9 plasmid had been inserted into the *Artemisia* plant genome. In contrast, 25 lines did not produce such amplification of 700 bp DNA fragment (about 32.47%) and this should be investigated further, since plants were germinated and grown on MS selection medium after sterilization.

It has been reported that Cas9 protein endonuclease will do DNA double strands cutting at 3 to 4 bp after the protospacer adjusting motif (PAM) site, directed by guideRNA and DNA repair process often makes mistakes. It gives possibilities for deletion and or insertion to occur. Based on those, gRNA that was designed using E-CRISP based upon SQS cDNA gene sequence (GenBank: AF405310.1), after found complement in the genome will direct Cas9 protein endonuclease to cut the DNA with precision. Then it is expected to induce some mutation [5].

Conventional electrophoresis equipment was not able to show such differences (in size of DNA fragment produced by PCR) (Figure 3A, 3B and 3C) induced by mutation. Because of that, we tried to use a high-resolution electrophoresis (HRE) QIAxcel (Qiagen) to analysis PCR product of *Artemisia* CRISPR/Cas9 lines before to do sequencing. The equipment was designed to analyses DNA fragment with accuracy down to 3 to 5 bp (protocol). It, however, is only possible applied for DNA with size of smaller than 500 bp. Another set of PCR was made using another pair of primer (SQS-Fs and SQS-Rs) (Figure 4A). By using this equipment, amplicons can be measured and visualized as shown in Figure

4B. For example, sample number 12 has a size of 465 bp (Figure 5C). From these analyses, we managed to get data that showed differences down to 1 to 3 nucleotides compared to the plasmid.

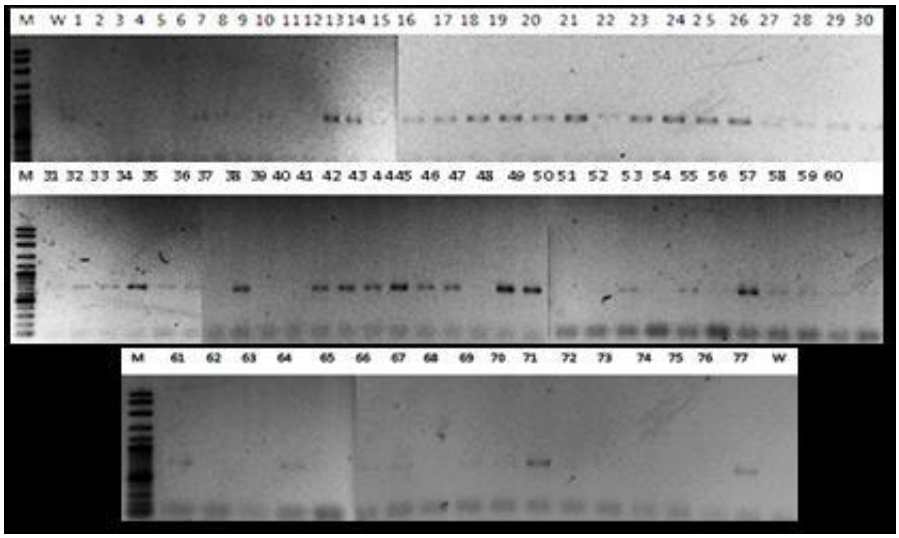

Figure 3. Electrophoregram of PCR product produced by *Artemisia* CRISPR/Cas9 plants. A = samples 1–30, B = samples 31–60, C = samples 61–77.

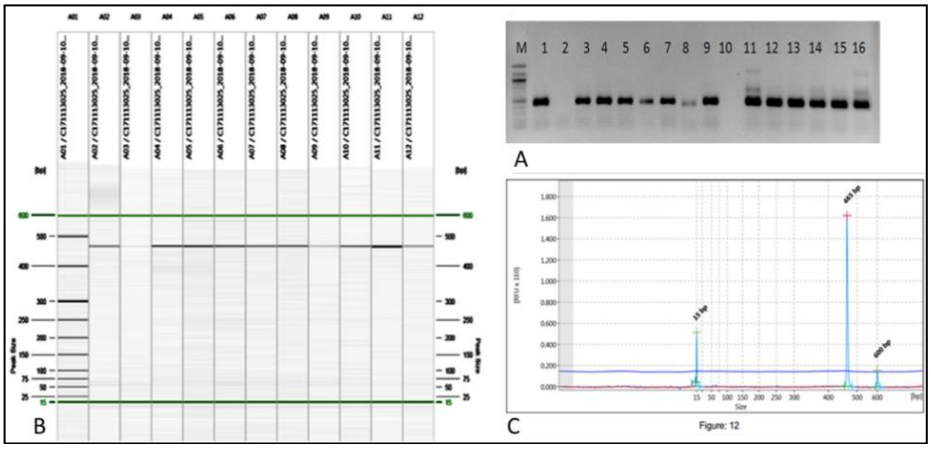

Figure 4. Electrophoregram of PCR product of *Artemisia* CRISPR/Cas9 using two types of electrophoresis. (A) Conventional. (B) High resolution electrophoresis (QIAxcel). (C) Graphyc shows the size of PCR product of line number 12.

From PCR product analysis using HRE QIAxcel, we obtained 44 of 52 *Artemisia* CRISPR/Cas9 lines had changes in their size compared to fragment resulted by the plasmid. Changes encountered were 84.62% (average) has small number deletion between 1 to 3 nucleotides (Figure 5). These may indicate that about 84.62% of lines were mutated. While another 15.38% of lines were not mutated, because they have similar size of DNA amplification (amplicon) as the control plasmid (468 bp). Of that 84.62% of mutated lines, about 69.23% had 1 nucleotide deletion, 11.54% had 2 nucleotides deletion and only about 3.83% of lines had 3 nucleotides deletion.

These experiments results may indicate that designed gRNA for *SQS* gene (target) can direct Cas9 protein endonuclease to do DNA breaks. For sizes confirmation, sequencing of PCR products are

needed. Then the next step is to align sequences of CRISPR/Cas9 lines compared to the *SQS* coding sequence (Genebank: AF405310.1) thath may lead to conclusion whether any protein changes and or an early stop codon has occured. Concentration of artemisinin of these 52 lines is being analyses. Both sequence and artemisinin information data may be sought for its corellation.

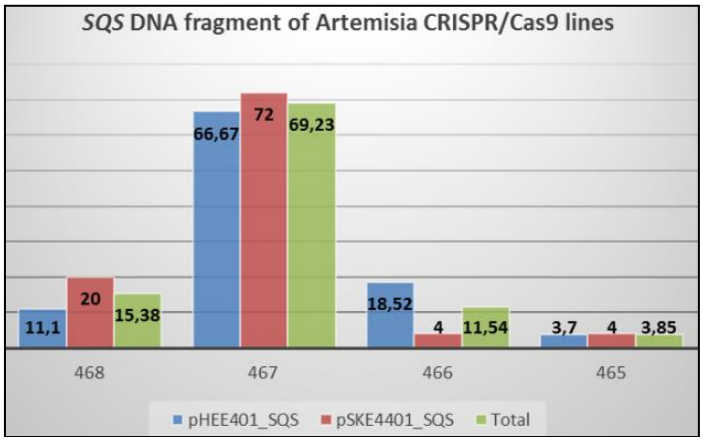

Figure 5. Percentages of CRISPR/Cas9 lines with nucleotide reductions (1 up to 3) induced by pHEE_SQS, pSKE401_SQS plasmids.

4. Conclusions

This is the first report about application of the CRISPR/Cas9 system onto *Artemisia*. Experiments carried out were successfull in generating of 52 *Artemisia* CRISPR/Cas9 lines and induced nucleotides changes in size of *SQS* gene of 44 *Artemisia* CRISPR/Cas9 lines (84.67%). Analysis of PCR products using high-resolution electrophoresis QIAxcel abled to show nucleotide differences among PCR products of lines, varied from 1 to 3 nucleotides. DNA sequencing and artemisinin content analysis are being carried out for confirmation. These would be able to find out whether nucleotide deletions affect the DNA sequence shifted, consequently changed the protein or *SQS* gene transcriptional activity. Information data on artemisinin concentration of these 44 lines will give a further confirmation about mutation that induced by CRISPR/Cas9 system, which can disrupt sterol synthesis and consequently increased the artemisinin content.

5. Acknowledgement

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Improvement of Inpari 30 and Situ Bagendit rice varieties for tolerance to drought through spike-stalk injection method

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Abstract. Extreme climate change requires rice varieties adaptable to drought condition. Adaptation will play an important role in ensuring the sustainability of food security. This research aimed to improve drought tolerance of Inpari 30 and Situ Bagendit varieties through Spike-Stalk Injection Method (SIM). DNAs from several plant species, such as rice (cv. Cabacu), grasses (*Echinochloa crusgalli/E. colona*, elephant grass/*Pennisetum purpureum, Bothriochloa pertusa*, *Cenchrus echinatus*, *Sorghum nitidum*, *Ischamemum timorensis* and *Guinea grass*), sugarcane, wild rice (*Oryza nivara*), maize and sorghum were injected to tillers of cultivar Inpari 30 and Situ Bagendit. The first set of M_1 Situ Bagendit-SIM and M_1 Inpari 30-SIM seeds were treated with 20% of PEG 8000 solution for 10 days. Some of the well germinated seeds were planted in pots and maintained untill harvest. The selected M_2 Situ Bagendit-SIM and M_2 Inpari 30-SIM and the second set of M_1 Situ Bagendit-SIM and M_1 Inpari 30-SIM seeds were planted at Muara Experimental Station, Bogor (West Java). The result of PEG 8000 assay showed that M_1 Situ Bagendit-SIM-elephant grass, sugarcane and *O. nivara* had significantly longer radicle length and higher fresh weight compared to Situ Bagendit, while M_1 Inpari 30-SIM-sugarcane, sorghum and elephant grass had significantly longer radicle and plumule length, and higher radicle weight compared to Inpari 30. Field trial showed that the mutant lines of Situ Bagendit-SIM performed better than those of Inpari 30-SIM. The grain weight of M_1 Situ Bagendit-SIM-Cabacu, *jajagoan* grass, maize, sugarcane, *O. nivara* and *B. pertusa*, and M_2 Situ Bagendit-SIM-*O. nivara* and elephant grass were higher than that of Situ Bagendit. The grain weight of M_1 Inpari 30-SIM-*jajagoan* grass was also higher than that of Inpari 30. Therefore, SIM could be an alternative way to develop genetic variation of rice plant.

Keywords: DNA injection, field trial, grasses, mutation, PEG 8000.

1. Introduction

Drought affects rice plants in various aspects. Plant responses during drought stress at the cellular and molecular levels include cell volume reduction, decreasing leaf area, increasing root-to-shoot ratio and

stomatal sensitivity [1]. These responses are also accompanied by a reduction in photosynthesis rate; an increase in the accumulation of dissolved osmotic compounds, such as proline, betaine sugar and alcohol sugar; changes in enzyme activity and gene expression; and production of long and thick roots and formation of cuticle layers in leaves [2].

The lack of water in the root area causes slow cell division activity in the root meristem area, facilitating to reduce the dry weight of the root besides decreasing the potential of water [3].Therefore, drought-tolerant rice varieties are expected to overcome this problem and able to produce grains, even though under water limitation.

Spike-Stalk Injection Methods (SIM) is one of methods for developing new rice lines done by Chinese National Hybrid Rice Research researchers [4], which was inspired by the injection method into rye [5]. This method has been successfully applied on rice by injecting grass DNA (*Echinochloa crusgalli*) to rice stems when the plants are in early booting stage [4]. DNA is expected to be carried through the water and nutrients flow (through xylem) into the rice genome during meiosis. SIM can overcome the barriers of inter-specific hybridization of cultivated rice and non-AA genome wild rice during chromosomes pairing. This method has been used to generate new rice germplasm by injecting DNAs from a variety of plant species such as wild rice, maize, sorghum, *E. crusgalli* and *Pannicum maximum* [4], and resulted in several hybrid rice varieties [6].

Inpari 30, also known as Ciherang-*Sub1* rice variety, is an irrigated rice with submergence tolerant [7]. It is necessary to combine submergence tolerant properties with drought tolerance in rainfed rice field. Situ Bagendit is an upland rice which was released in 2003. This variety is well adapted in irrigated and upland rice field. The performance of Inpari 30 and Situ Bagendit rice varieties is preferred by farmers [8]. This study aimed to improve Inpari 30 and Situ Bagendit rice varieties by increasing their drought tolerance through SIM.

2. Materials and methods

This research was conducted in a glasshouse of Indonesian Center for Agricultural Biotechnology and Genetic Resources Reserch and Development (ICABIOGRAD) and in a field at Muara Experimental Station (250 m asl), Bogor, from January 2016 to July 2017. Two rice varieties, Inpari 30 and Situ Bagendit, were grown in pots in the glasshouse until panicle formation. Genomic DNAs from several plant sources (Table 1) were isolated following the method of Doyle and Doyle [9]. DNA concentration was adjusted to 450 ng/µl in 1× SSC solution. A total of 50 µl of DNA solution was injected gradually to the second node from the bottom of the panicle following the method of Zhao et al. [4]. Injected panicles were maintained until seed maturity.

2.1. PEG 8000 drought tolerance phenotypic selection

The first set of M_1 seeds were derived from Inpari 30 and Situ Bagendit which were injected with sorghum, elephant grass, wild rice (*O. nivara*), *(E. crusgalli*/*E. colona)*, sugarcane and Cabacu rice (hereafter named as M_1 Inpari 30-SIM or M_1 Situ Bagendit followed by the name of DNA source). Seeds of these mutants were surface sterilized with 70% ethanol solution for 5 minutes. The seeds were then washed three times with sterilized distilled water. Germination assays were performed by evenly distributing the seeds in 10-cm-diameter sterilized petri dishes. Each dish was moistened with 10 ml distilled water and germinated for 3 days.

Germinating seeds of similar size or those with 2 mm of plumule and radicle length were selected. The seeds were transferred to 10 cm-diameter sterilized Petri dishes and immersed for 10 days in 20 ml distilled water containing 20% of polyethylene glycol (PEG) 8000 solution [10], which gave osmotic potential of -5,11 bar or -0,511 Mpa [11]. The experiment was designed in a completely randomized design (CRD) with three replications. The length of plumule and radicle, as well as fresh weight, was measured from 10 germinating mutant seeds of each line selected randomly. The M_1 seedlings from the PEG treatment were planted to potted soil and maintained until the M_2 Inpari 30 and M_2 Situ Bagendit seeds set.

Table 1. List of plant species used as DNA injection sources.

Source	Species/cultivar/common name/accession number	Origin
Rice	*Oryza sativa* cv. Cabacu	Brazil
Wild rice	*O. nivara* 103840 (Reg. 05012-00001)	Philippines (ICABIOGRAD collection)
Maize	Lamuru (Reg. 05002-03696)	Maros District, South Sulawesi Province (ICABIOGRAD collection)
Grass	*Jajagoan* grass/*Echinochloa crusgalli* (= *E. colona*)	Bogor, West Java Province
Grass	Elephant grass/*Pennisetum purpureum*	Bogor, West Java Province
Sugarcane	-	Bogor, West Java Province
Sorghum	Mutiara Kulonprogo L70 (Reg. 05005-00074)	Kulon Progo District, Yogyakarta Province (ICABIOGRAD collection)
Grass	*Bothricochloa pertusa*	Kupang, East Nusa Tenggara Province
Grass	*Cenchrus echinatus*	Kupang, East Nusa Tenggara Province
Grass	*Sorghum nitidum*	Kupang, East Nusa Tenggara Province
Grass	*Ischamemum timorensis*	Kupang, East Nusa Tenggara Province
Grass	Guinea Grass	Kupang, East Nusa Tenggara Province
Grass	Molato Grass	Kupang, East Nusa Tenggara Province

2.2. Field test

M_1 and M_2 seeds from both varieties were sown in 3 m × 6 m plots at Muara Experimental Station, Bogor (250 m asl), without replication. These populations consisting of 13 lines of M_1 Inpari 30-SIM, 13 lines of M_1 Situ Bagendit-SIM, 7 lines of M_2 Inpari 30-SIM (sown in 35 plots) and 7 lines of M_2 Situ Bagendit-SIM (sown in 35 plots). Both wild types (Inpari 30 and Situ Bagendit), IR20 (drought-sensitive check variety) and Cabacu (drought-tolerant check variety) were also sown. The agronomic characters of these mutants were compared to those of the wild types.

3. Results and discussion

3.1. PEG 8000 screening

The initial screening for drought-tolerance of M_1 Inpari 30-SIM and M_1 Situ Bagendit-SIM used 20% of PEG 8000 solution (w/v). After 10 days of incubation in the solution, longer radicle and/or plumule and higher radicle fresh weight than those on wild types were generally observed on SIM mutant lines (Table 2 and Table 3, Figure 1).

The average percentage of germinated seeds from PEG 8000 selection of M_1 Inpari 30-SIM was 15.07% with the highest rate was shown by M_1 Inpari 30-SIM-*O. nivara* (24.1%) and the lowest was by M_1 Inpari 30-SIM-elephant grass (8.6%). The average percentage of germinated seeds from PEG selection of M_1 Situ Bagendit-SIM was 17.37% with the highest rate was in M_1 Situ Bagendit-SIM-sorghum (41.1%) and the lowest was in M_1 Situ Bagendit-SIM-*O. nivara* (7,3%) (data not shown).

Radicle length of M_1 Inpari 30-SIM and M_1 Situ Bagendit-SIM showed significant difference compared to its wild types (Inpari 30 and Situ Bagendit). Radicle length of M_1 Inpari 30-SIM-sugarcane, -sorghum, -elephant grass, -Cabacu and -*E. crusgalli* were longer than that of Inpari 30 (Table 2). Similarly, radicle length of M_1 Situ Bagendit-SIM-elephant grass, -sugarcane and -*O. nivara* were longer than that of its wild type (Table 3).

The plumule length of M_1 Inpari 30-SIM-sugarcane and M_1 Inpari 30-SIM-*E. crusgalli* significantly differed and exceeded that of the other M_1 Inpari 30-SIM and its wild types (Table 2). On

the contrary, the plumule length of M_1 Situ Bagendit-SIM and the wild type were not significantly different, except for M_1 Situ Bagendit-SIM-*O. nivara* (Table 3).

The fresh weight of germinated seeds of M_1 Inpari 30-SIM and M_1 Situ Bagendit-SIM showed significant difference compared to their wild types. The fresh weight of M_1 Inpari 30-SIM-sorghum, -SIM-sugarcane, -*E. crusgalli*, -Cabacu, and -*O. nivara* were higher than that of Inpari 30 (Table 2). Likewise, the fresh weight of M_1 Situ Bagendit-*O. nivara*, -elephant grass, -sugarcane, -Cabacu, -*E. crusgalli* and -maize were higher than those of its wild types (Table 3).

Table 2. Plumule and radicle length of M_1 Inpari 30-SIM and M_1 Situ Bagendit-SIM mutants after treatment with 20% of PEG 8000 solution for 10 days.

No.	Rice variety/mutant	Plumule length (cm)[a]	Radicule length (cm)	Germinated seed fresh weight (g)
1	Cabacu	0.94±0.12 ab	4.71±0.61 b	0.041±0.01 ab
2	Inpari 30	1.18±0.64 bc	3.16±0.92 a	0.031±0.01 d
3	Inpari 30-SIM-*E. crusgalli*	1.87±1.03 a	3.41±0.98 cd	0.040±0.01 ab
4	Inpari 30-SIM-maize	1.09±0.32 bc	0.33±0.30 f	0.031±0.00 d
5	Inpari 30-SIM-elephant grass	1.38±0.52 b	3.67±0.68 c	0.035±0.01 cd
6	Inpari 30-SIM-sorghum	1.32±0.58 bc	4.59±0.70 b	0.043±0.01 a
7	Inpari 30-SIM-sugarcane	2.18±0.71 a	6.04±0.80 b	0.042±0.01 ab
8	Inpari 30-SIM-Cabacu	1.21±0.50 bc	3.42±0.85 cd	0.039±0.01 abc
9	Inpari 30-SIM-*O. nivara*	1.20±0.41 bc	1.55±0.37 e	0.037±0.01 bc

[a] Values followed by the same letter within one column are not significantly different at 5% level according to DMRT.

Table 3. Plumule and radicle length of M_1 Situ Bagendit-SIM and M_1 Situ Bagendit-SIM mutants after treatment with 20% of PEG 8000 solution for 10 days.

No.	Rice variety/mutant	Plumule length (cm)[a]	Radicle length (cm)	Germinated seed fresh weight (g)
1	Cabacu	1.45±0.12 b	3.16±0.61 b	0.042±0.007 a
2	Situ Bagendit	0.84±0.22 b	0.32±0.20 d	0.031±0.009 d
3	Situ Bagendit-SIM-*E. crusgalli*	0.88±0.33 b	1.22±0.53 c	0.036±0.006 bc
4	Situ Bagendit-SIM-maize	0.75±0.15 b	0.97±0.66 c	0.035±0.006 bc
5	Situ Bagendit-SIM-elephant grass	0.86±0.32 b	4.62±0.77 a	0.039±0.004 ab
6	Situ Bagendit-SIM-sorghum	0.95±0.36 b	3.27±0.76 b	0.033±0.007 cd
7	Situ Bagendit-SIM-sugarcane	0.83±0.31 b	4.14±0.76 a	0.037±0.006 bc
8	Situ Bagendit-SIM-Cabacu	0.93±0.30 b	1.03±0.65 c	0.036±0.005 bc
9	Situ Bagendit-SIM-*O. nivara*	1.45±0.74 a	4.51±1.45 a	0.042±0.007 a

[a] Values followed by the same letter within one column are not significantly different at 5% level according to DMRT.

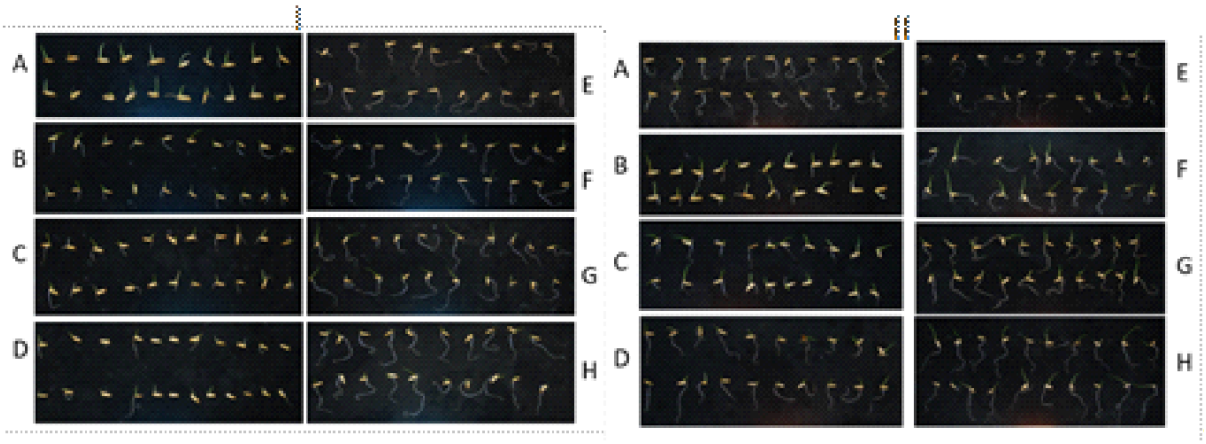

Figure 1. The performance of radicle and plumule of M_1 Inpari 30-Spike-Stalk Injection Method (SIM) mutants and M_1 Situ Bagendit-SIM mutants after treated with 20% of PEG 8000 solution for 10 days.
I. A = Situ Bagendit (wild type), B–H = mutants derived from Situ Bagendit injected with DNA from rice cv. Cabacu, *jajagoan* grass (*E. crusgalli*), maize, sugarcane, sorghum, *O. nivara* and elephant grass, respectively.
II. A = Inpari 30 wild type, B–H = mutants derived from Inpari 30 injected with DNA from maize, *O. nivara*, rice cv. Cabacu, sorghum, *jajagoan* grass, elephant grass and sugarcane, respectively.

3.2. Field test

The selected mutant lines from the first set (M_2 Inpari 30-SIM and M_2 Situ Bagendit-SIM) and the second set (M_1 Inpari 30-SIM and M_1 Situ Bagendit) were planted in the field experimental station. The results of the field test can be seen in Table 4 and 5.

M_1 and M_2 Inpari 30-SIM mutant lines were significantly different from the wild type for all parameters, except for the panicle length. These mutant populations showed shorter plant height and also lower number of filled grain, 100-grain weight and the total grain weight. However, the panicle number of M_1 Inpari 30-SIM-*E. crusgalli*, -SIM-*B. pertusa*, and -SIM-molato grass; and M_2 Inpari 30-SIM-sugarcane, and -SIM-maize mutants were higher than that of Inpari 30. On the contrary, M_1 Inpari 30-SIM-*E. crusgalli* had higher grain weight than the wild type (Table 4).

The agronomic performance of M_1 and M_2 Situ Bagendit-SIM mutant lines were not significantly different to that of the wild type (Table 5). Nevertheless, the number of panicle in M_1 Situ Bagendit-sugarcane and M_2 Situ Bagendit-*O.nivara* were slightly higher than that of Situ Bagendit (Table 5). Additonally, the grain weight of M_1 Situ Bagendit-SIM-Cabacu, -SIM-*E. crusgalli*, -SIM-maize, -SIM-sugarcane, -SIM-*O. nivara*, and -SIM-*B. pertusa* and M_2 Situ Bagendit-SIM-*O. nivara* and -SIM-elephant grass exceeded that of its wild (Table 5).

Results from the PEG treatment and the field test were consistent. In PEG assay, DNA from *E. crusgalli*, sugarcane, maize and *O. nivara* resulted in longer plumule and radicle and higher fresh weight of germinated seeds, which corresponded to longer panicle length and higher grain weight in the field (Table 2–5).

Table 4. Agronomic profile of M_1 and M_2 Inpari 30-SIM mutants grown in Muara Experimental Station, Bogor in 2017.

Rice variety/mutant[a]	Plant height (cm)[b]	Number of panicles	Panicle length (cm)	Number of grains/ panicles	Number of empty grains/ panicles	100-grain weight (g)	Grain weight/ plant (g)
IR20	93.35b-d	14.86de	25.02b	122.70a	33.69d-h	2.80a	18.99c-h
Cabacu	111.31a	10.41e	24.83b	58.38e-j	45.48c-h	2.90a	8.34e-h
Situ Bagendit	89.98c-h	20.33a-d	24.71b	116.93ab	15.05h	2.43ab	37.41a
Inpari 30	93.94bc	16.25c-e	24.55b	100.64a-c	30.90d-h	2.49ab	22.51b-e
M_1 Inpa-SIM-sorghum	89.08c-i	19.60a-d	23.30b	90.00b-e	23.08f-h	2.50ab	32.56a-c
M_1 Inpa-SIM-Cabacu	82.30ij	21.10a-d	21.66b	60.00e-i	44.60c-h	2.29a-d	6.45e-h
M_1 Inpa-SIM-*E. crusgalli*	94.93bc	26.00a	25.33b	96.27a-d	34.27d-h	2.40ab	41.12a
M_1 Inpa-SIM-maize	87.73c-i	17.00b-e	23.86b	83.67c-e	44.07c-h	2.24a-d	12.20e-h
M_1 Inpa-SIM-sugarcane	88.40c-i	20.90a-d	23.14b	80.20c-f	42.15c-h	2.11a-d	22.17b-e
M_1 Inpa-SIM-*O. nivara*	84.80g-j	18.60a-d	23.10b	70.20c-g	37.20d-h	2.36a-c	19.43c-h
M_1 Inpa-SIM-elephant grass	90.60d-g	20.60a-d	22.90b	37.80h-j	62.60c-e	2.33a-c	5.37f-h
M_1 Inpa-SIM-*B. pertusa*	90.60d-g	25.20ab	24.64b	99.60a-c	25.60e-h	2.44ab	36.69ab
M_1 Inpa-SIM-*C. echinatus*	83.00h-j	20.20a-d	24.20b	95.00a-d	27.20e-h	2.34a-c	21.45c-f
M_1 Inpa-SIM-*S. nitidum*	85.87e-j	23.73a-c	23.24b	64.33d-h	31.07d-h	2.43ab	16.46d-h
M_1 Inpa-SIM-*I. timorensis*	80.20j	23.12a-d	22.36b	83.92c-e	21.28gh	2.27a-d	19.06c-h
M_1 Inpa-SIM-Molato grass	86.25d-j	25.75a	22.80b	31.38ij	75.00bc	2.47ab	5.15gh
M_1 Inpa-SIM-Guinea grass	90.80d-g	22.40a-d	26.76b	88.60b-e	46.80c-h	2.55ab	20.63c-g
M_2 Inpa-SIM-sorghum	98.08b	21.04a-d	25.14b	64.48d-h	43.68c-h	2.39ab	14.99d-h
M_2 Inpa-SIM-Cabacu	92.72b-e	20.72a-d	24.11b	45.28g-j	46.48c-h	2.26a-d	10.86e-h
M_2 Inpa-SIM-*E. crusgalli*	85.10f-j	20.90a-d	23.17b	28.60j	60.30c-f	1.09e	3.46h
M_2 Inpa-SIM-maize	92.33d-f	25.33ab	32.80ab	29.87ij	97.33ab	1.45c-e	8.44e-h
M_2 Inpa-SIM-sugarcane	89.04c-i	30.04a-d	23.90b	49.68f-j	98.88ab	1.41de	6.90e-h
M_2 Inpa-SIM-*O. nivara*	85.60e-j	15.35c-e	27.60ab	83.80c-e	112.40a	1.76c-e	12.62e-h
M_2 Inpa-SIM-elephant grass	84.80f-j	18.00a-e	24.45b	65.48d-h	59.04c-g	2.42ab	10.70e-h
CV (%)	5.095	25.64	28.44	26.49	50.05	2.65	52.88

[a] Inpa = Inpari 30.

[b] Values followed by the same letter within one column are not significantly different at 5% level according to DMRT.

Seed germination and seedling development are very important for early establishment of plants under stress condition. It has been reported that seed germination and early seedling growth are potentially the most critical stages for water stress [12] and drought stress impairs the seed germination [13]. Therefore, analysis of germination and seedling growth traits and their response to drought can be useful for selection of rice genotypes tolerant to drought [14].

PEG solution is commonly used in drought tolerance stress experiments because its ethylene content binds water, making it inaccessible to roots and resulting in plant dryness [15]. Either PEG 8000 solution at 20% concentration [10] or PEG 6000 solution at 25% concentration [16] can be used for drought tolerance selection. In this study, we used 20% of PEG 8000 solution as drought determinator to mimic drought stress condition. In the previous study, seed germination was severely affected by 20% of PEG solution [17]. Under water stress, low water potential is a determining factor for inhibiting seed germination [18].

Table 5. Agronomic profile of M_1 and M_2 Situ Bagendit-SIM mutants grown in Muara Experimental Station, Bogor in 2017.

Rice variety/mutant[a]	Plant height (cm)[b]	Number of panicles	Panicle length (cm)	Number of grains/ panicles	Number of empty grains/ panicles	100 grain-weight (g)	Grain weight/plant(g)
IR20	93.35 b-d	14.86 h	25.02 a-f	122.7 a-e	33.69 ab	2.80 ab	18.99 g
Cabacu	111.31 a	10.41 i	24.83 a-g	58.38 g	45.48 a	2.90 a	8.34 h
Inpari30	93.94 b-d	16.25 d-h	24.55 b-g	100.64 ef	30.90 a-d	2.40 a-c	22.51 fg
Situ Bagendit	89.99 b-e	20.33 a-e	24.71 a-g	116.93 a-e	15.05 de	2.43 a-c	37.40 a-e
M_1 Situ-sorghum	93.75 b-d	15.25 gh	25.13 a-f	120.60 a-e	23.15 b-e	2.45 a-c	34.90 a-f
M_1 Situ-Cabacu	100.15 b	17.10 c-h	26.05 a-c	144.55 a	14.75 de	2.56 a-c	46.82 a
M_1 Situ-*Echinocloa crusgalli*	98.60 bc	19.35 a-h	25.50 a-d	137.85 ab	16.20 c-e	2.52 a-c	38.64 a-e
M_1 Situ-maize	95.25 b-e	17.30 c-h	26.53 a	135.15 a-c	15.55 c-e	2.38 a-c	41.04 a-d
M_1 Situ-sugarcane	91.50 b-e	22.30 a	26.50 a	115.45 a-e	16.50 c-e	2.57 a-c	42.31 a-c
M_1 Situ-*Oryza nivara*	87.20 c-e	20.00 a-f	25.00 a-f	115.20 a-e	8.80 e	2.35 a-c	38.37 a-e
M_1 Situ-elephant grass	91.52 b-e	20.96 a-c	24.93 a-g	97.92 ef	45.60 a	2.38 a-c	35.41 a-e
M_1 Situ-*Bothricochloa pertusa*	93.95 b-d	20.85 a-d	25.61 a-d	137.75 ab	31.65 a-c	2.06 c	44.56 ab
M_1 Situ-*Cenchrus echinatus*	87.00 c-e	15.85 e-h	23.04 g	101.10 ef	24.05 b-e	2.14 bc	27.15 e-g
M_1 Situ-*Sorghum nitidum*	88.75 b-c	16.70 c-h	25.08 a-f	101.35 ef	22.70 b-e	2.42 a-c	29.30 d-f
M_1 Situ-*Ischamemum timorensis*	89.47 b-e	15.53 f-h	25.19 a-e	106.93 ef	11.50 e	2.69 a-c	28.36 d-f
M_1 Situ-Molato grass	92.47 b-d	20.07 a-f	24.54 b-g	115.40 a-e	12.80 e	2.31 a-c	38.61 a-e
M_1 Situ-Guinea grass	87.30 c-e	18.57 a-h	24.91 a-g	116.83 a-e	19.87 b-e	2.50 a-c	31.21 c-f
M_2 Situ-sorghum	93.29 b-d	17.77 a-h	25.10 a-f	104.45 d-f	24.50 b-e	2.37 a-c	29.01 d-f
M_2 Situ-Cabacu	86.45 de	17.53 b-h	24.28 c-g	94.98 ef	17.45 b-e	2.54 a-c	32.32 b-f
M_2 Situ-*E. crusgalli*	86.82 c-e	16.87 c-h	23.24 e-g	118.35 a-e	16.80 c-e	2.54 a-c	34.36 a-f
M_2 Situ-maize	82.93 d-f	15.51 f-h	23.71 d-g	98.14 ef	25.05 b-e	2.47 a-c	30.91 d-f
M_2 Situ-sugarcane	79.73 ef	19.55 a-g	23.18 fg	83.51 f	19.90 b-e	2.49 a-c	32.90 b-f
M_2 Situ-*O. nivara*	87.15 c-e	22.02 ab	24.79 a-g	110.12 b-f	18.33 b-e	2.57 a-c	42.91 a-c
M_2 Situ-elephant grass	83.21 d-f	20.18 a-e	24.48 c-g	100.22 e-f	21.47 b-e	2.54 a-c	44.99 ab
CV (%)	9.05	16.87	5.16	17.6	49.05	18.82	24.79

[a] Situ = Situ Bagendit.

[b] Values followed by the same letter within one column are not significantly different at 5% level according to DMRT.

Our results showed that M_1 Inpari 30-SIM-*E. crusgalli* and -sugarcane and M_1 Situ Bagendit-SIM-*O. nivara* and -sugarcane, showed longer radicle and plumule, and also higher fresh weight. These characteristics can help plants to tolerate drought conditions. Long and large root volume will enable plants to adapt in the field with less water. Henry et al. [19] and Comas et al. [20] mentioned that root

architecture, such as diameter, specific root length,and root density, are some important parameters for selecting rice plants for drought tolerance.

The application of SIM has changed the agronomic character in Inpari 30, either for the better or the worse, whereas positive influence of SIM were observed for Situ Bagendit. Zhao et al. [21] mentioned two premises of DNA fragments integrated to rice genome that (1) the expression of gene products in the target rice derived from the inserted DNA fragments retain the intact structures including gene upstream and encoding regions and (2) the regulatory factors of the transcription machinery in rice recognize the upstream sequences of the inserted genes.

In this study, the injected DNA from *E. crusgalli* resulted in higher grain weight in M_1 Inpari 30 and M_1 Situ Bagendit and higher number of panicles in M_1 Inpari 30, but it did not result in significant change in Inpari 30 for other parameters. Rice is a typical C3 plant, whereas *E. crusgalli* is a C4 plant. This weed exhibits fast growth with higher stems, wider and darker green leaves, high rate of photosynthesis, and high efficiency in water and mineral use [22]. DNA fragments transferred from *E. crusgalli* to rice are responsible for exerting the unknown influence on the expression of rice genes [21].

The injected DNA from maize resulted in higher number of panicles in M_1 Inpari 30 and higher number of grain weight in M_1 Situ Bagendit. Li et al. [23] mentioned that the transfer of exogenous corn DNA may induce mutations in rice. If the mutations occur in the functional regions of the genome, this may influence expression of this gene, leading to changes in expression, structure and function of proteins while phenotype variation may also occur in later generations. Ji et al. [24] reported that exogenous corn DNA could change the expression of proteolytic enzymes in mutant rice seedlings, possibly as a result of variation of bases.

The injection of DNA from sugarcane resulted in higher number of panicle and higher grain weight in M_1 Situ Bagendit and higher number of panicles in M_2 Inpari 30. A similar effect was observed for the injection of DNA from wild rice *O. nivara*, where higher grain weight in M_1 and M_2 Situ Bagendit and higher panicle number panicle in M_2 Situ Bagendit were obtained. Zhao et al. [6] reported the development of Yewei B rice from a mutant of V20B through injection DNA of wild rice *O. minuta.* The molecular analysis revealed high DNA polymorphism exists between the mutant and its receptor, indicating that the special DNA fragment from *O. minuta* may be integrated into the genome of the mutant.

The integration of foreign DNA into Inpari 30 and Situ Bagendit genome, respectively, can be proven molecularly. Several class of molecular markers, such as the transferability of rice SSR to Poacea family [25], AFLP [23], as well as SNP markers can be used to detect base differences between wild type and SIM mutants [26,27].

The SIM method has also been analyzed by DNA sequencing [28], SSR, AFLP, RAPD and RFLP markers [6,23,28], differential proteomes [21], whole genome sequencing [29] and restriction-site associated DNA sequencing technology (RADseq) [30].

Results between the PEG treatment corresponded well with the results of the field test for the lines derived from the injection of DNA from *E. crusgalli*, sugarcane, maize and *O. nivara*. This consistency is in agreement with the hypothesis that specific DNA segments might integrate into the genome of the cultivated rice and could be stably passed onto the offsprings [31]. Therefore, SIM can be an effective approach to transform genomic DNA of distantly related species for creating new rice germplasm.

4. Conclusions

Spike-Stalk Injection Method (SIM) on tillers of Inpari 30 and Situ Bagendit rice varieties with DNAs of various plant species have resulted in M_1 mutant seedlings with longer radicle and plumule length and higher fresh weight compared to their wild types. M_1 and M_2 Situ Bagendit-mutants had better agronomic performance than Inpari 30-mutants. Therefore, SIM could be an alternative way to develop genetic variation of rice plant.

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Transformation using RNAi technology for developing potato lines resistance to late blight (*Phytophthora infestans*)

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Abstract. *Phytophthora infestans* (Mont.) de Bary causes late blight, a major disease of potato and tomato that causes production loss at about 10–100% in Indonesia. Breeding for late blight resistance in potato has been carried out in this country to minimize fungicide application. The objective of this study was to generate potato lines resistant to *P. infestans* through RNAi method. A series of studies were conducted, including confirmation of RNAi construct insertion in *Agrobacterium tumefaciens* and PCR analysis, transformation of two potato cultivars using *A. tumefaciens* carrying the RNAi fragment, in addition to selection and molecular analysis of selected plantlet using Polymerase Chain Reaction (PCR). The RNAi plasmid construct was confirmed via PCR analysis using specific primers for *35s* and *Tnos* fragment, and resulted in 500 bp and 250 bp for fragment of *35s* and *Tnos*, respectively. Transformation was performed on 733 Granola's internode explants and 569 Atlantic's internode explants. The transformation process produced 282 explants from Atlantic, while Granola did not produce any transformants. The level of transformation efficiency of Atlantic on selection medium containing hygromycin was 49.61%. Following regeneration step, the 282 selected explants produced 167 plantlets. Based on PCR reaction using specific primers for *hpt* gene, 14 plantlets were PCR positive and contained *hpt* fragment. Overall, *Agrobacterium tumefaciens*-mediated transformation on potato internode explants was successful. Therefore, the selected transformants should be further tested using bioassay for resistance to *P. infestans*.

Keywords: *Agrobacterium tumefaciens*, *Phytophthora infestans*, RNAi, potato.

1. Introduction

Potato (*Solanum tuberosum*) is one of the most important horticultural commodities for staple food. This commodity contains high nutritional content, including carbohydrates, proteins, minerals and amino acids, as well as some important vitamins [1]. In 2016, national potato production in Indonesia was around 1.2 million tons (MT), while national consumption was about 1.0 MT, which was higher than the production. To meet the demand, Indonesia imported around 26,000 ton potatoes. To achieve self-sufficiency in potato production and to become a potato exporter, new Indonesian variety such as Median [2] was developed and released.

The most important problem for potato production is the presence of diseases. The major disease in potatoes is late blight caused by *Phytophthora infestans* (Mont.) De Bary. This pathogen causes damages on many parts of a potato plant, such as leaves, tubers and stems. Late blight was responsible

for the European potato famine in the 19th century, which caused deaths from starvation on more than one million people in Ireland alone [3] and the migration of millions of people [4]. In Indonesia, potato production loss can reach 100% if weather conditions are conducive to the development of *P. infestans*. At present, Indonesian farmers still use several potato varieties that are susceptible to *P. infestans*, such as Granola and Atlantic. Until now, the late blight pathogen was mostly controlled using chemical pesticide. In the United States, more than 15 fungicidal sprays are applied per season, which is considered as a legal practice even though it is harmful to the environment and human health [5]. This is because more than 100 years of concerted breeding efforts across the world to develop resistant potato varieties have not been able to overcome the disease. Conventional breeding techniques that have been used since more than century ago to produce resistant lines found several obstacles, such as limited sources of resistance genes, incompatibility barrier, and insufficient expression levels of the introgressed resistance genes.

Successful development of a resistant potato to control *P. infestans* is considered as a more environmental friendly approach. New techniques have been developed to obtain resistant lines to this pathogen. Research has been initiated to introduce resistance trait from wild relatives to cultivated potato plants, identify molecular markers/QTL associated to resistance, and use genes of interest to transform varieties through cisgenesis. The *RB* gene, which is a major resistance gene from a wild relative potato *S. bulbocastanum* [6], has been mapped, isolated, cloned, and then transformed into Katahdin variety[3].

This genetically-modified potato plant resistant to late blight has been crossed with Indonesian commercial potato variety, Granola and Atlantic. The donor parent was transgenic Katahdin SP951 that carries the *RB* gene modified at the University of Wisconsin, Minnesota, USA, through ABSPII/USAID Project. Some lines among the hybrid progenies had been tested in several potato plantations in West Java and Central Java and showed resistance to late blight [7]. Transcription study of the *RB* gene indicated that in potato lines resistant to late blight the transcript levels were the highest in the foliage and tubers in an age-dependent manner. Expression of *RB* gene was the highest in young tubers and declined as the tubers aged [8].

Since the discovery of RNA interference (RNAi) technology by Romano and Macino [9], this technique is considered as an alternative technique capable of conferring enhanced resistance to fungi and pest [10]. It has been successfully applied to inhibit the growth of rice blast disease by inhibiting the growth and germination of appressoria in *in vitro* experiments [11].

The development of resistant cultivars through RNAi approach is expected to give better results, provide durable resistance, and reduce public concerns about genetically modified products. RNAi technology can be directed to degrade the pathogen's mRNA that enter the host cell or silence endogenous genes of the host cell that enable pathogen infection [12]. Compared to other methods working in protein level, such resistance mechanism in mRNA level is considered as a better approach [13]. Using this method, host-induced gene silencing of fungal genes was obtained in barley infected by powdery mildew *Blumeria graminis*, a biotrophic fungal pathogen[14]. The mechanism of pathogen control by RNAi is not dependent on the production of a foreign protein that could be allergenic or toxic in the host plants, which should make this technology more acceptable than the classic transgenic approaches for disease control [15].

RNAi technology has been considered as a promising approach for pest management, although there are some issues, such as RNAi efficiency, dsRNA degradation, and environmental risk assessments that need to be considered [16]. RNAi study has been investigated on two Kufri Indian Potato varieties (K. Khyati 1037 and K. Khyati 1129), where gene silencing method was used to silence *AVR3a* gene encoding an effector agent in *P. infestans* to infect the host plant. This investigation used siRNA and amiRNA-mediated silencing of *AVR3a* gene and produced moderate resistance against *P. infestans* [17]. The objectives of this study were to conduct *A. tumefaciens*-mediated transformation of potato internode explants and to produce potato plantlets carrying an RNAi fragment.

2. Materials and methods

2.1. Plant materials

Internode explants from 3–4-week-old *in vitro* cultures of Granola and Atlantic were used for transformation. The explants were grown in propagation medium (MS salts) containing $NaH_2PO_4.H_2O$, Myo-inositol and Phytagel Thiamine-HCl, pH 6.0.

2.2. Growing A. tumefaciens LBA4404 containing RNAi construct

A single colony of *A. tumefaciens* LBA4404 containing RNAi construct (Figure 1A; source: Venganza, Inc., Raleigh, NC, USA through ABSPII) inside a T-DNA plasmid pCambia 1300 (Figure 1B) was grown on YEM medium (mannitol [5g/l], yeast extract [0.5g/l], NaCl [0.1 g/l], $MgSO_4.7H_2O$ [0.2g/l], and pH 7.0) containing kanamycin 50 mg/l and shaken for one night at 28°C.

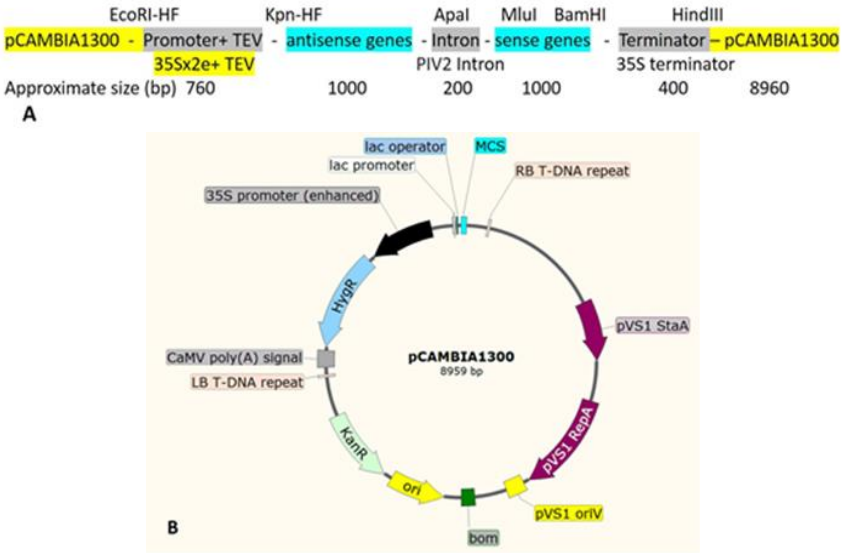

Figure 1. RNAi construct and pCambia1300 plasmid used in this study. **(**A) RNAi construct inside the T-DNA of pCambia1300 plasmid. (B) The map of pCambia1300 plasmid.

2.3. Verification of the plasmid containing RNAi construct

A single colony of *A. tumefaciens* containing RNAi construct was grown in 3–5 ml of liquid YEP medium containing kanamycin antibiotic. The culture was incubated for two nights at room temperature on a shaker set at 200 rpm. The plasmid DNA containing RNAi construct was isolated using alkaline lysis method [18]. The plasmid DNA was used for the next stage of analysis using Polymerase Chain Reaction (PCR) with primers for *hpt*II gene.

2.4. Agrobacterium tumefaciens-mediated transformation on potato internode explant

Transformation was conducted according to the method used by Ziegelhoffer et al. [19]. Pieces of stem segments measuring 0.5 mm long or 0.5 mm square of leaves were placed on a filter paper soaked in liquid MS medium. About 200 explants were immersed in a suspension of *A. tumefaciens* (OD_{600} = 0.6–0.8) for 10–20 minutes. The explants were then transferred to callus induction medium (CIM), covered with aluminum foil and incubated at 22°C for 2–3 days in an incubator. The explants were then transferred to selection induction medium (SIM) containing timentin or carbenicillin and hygromycin. Cultures were incubated for 3 weeks at 18°C with a photoperiod setting of 16 hours. The SIM was replaced every 3 weeks until green shoots appeared. About 1–2 cm of the green shoots were transferred to rooting medium (the medium of propagation) containing hygromycin and incubated at 18°C with photoperiod set at 16 hours and allowed to form strong roots.

2.5. Molecular analysis of the putative transgenic lines

A small piece (0.5 cm^2) of transformed leaf that have already rooted in the rooting medium containing antibiotic was used for plant DNA isolation based on the method used by Fulton [20] and used for PCR analysis. PCR were performed using the specific primers for *hpt*II to detect the presence of the RNAi construct in the transformants following the method of Listanto [21]. Each PCR reaction contained 1× PCR buffer, dNTPs 2.5mM, 2 pmol of forward and reverse primers, 0.2 U *Taq* DNA polymerase and the DNA template. The PCR reaction was started with pre-denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 15 seconds, 61°C for 30 seconds and 72°C for 1 minute, and concluded with a temperature of 72°C for 5 minutes. The results of PCR amplification were separated in 1.5% agarose gel and visualized using Chemidoc.

3. Results and discussion

3.1. Verification of plasmids containing RNAi construct

The verification of plasmids containing RNAi construct was done through plasmid DNA isolation and PCR analysis using primers for 35s and Tnos. The results showed that the plasmid DNA that was transferred into *A. tumefaciens* LBA4404 was still intact, judged from the existence of two fragments, one of which was probably a circular DNA plasmid (Figure 2A). PCR analysis using the primers for *35s* and *Tnos* produced two fragments of promoter 35s and terminator Tnos. The PCR products were 500 bp for 35s fragment and 250 bp for Tnos fragment (Figure 2B). These results confirmed that the RNAi construct was still inside the T-DNA of pCambia1300 plasmid. Thus, the plasmid that contained the RNAi construct was eligible to transform potato explants in order to obtain durable resistance to *P. infestans* in potato. The 35s and Tnos fragments were used to clarify the construct because these fragment are important to control DNA transcription into mRNA that would be used to silence a target gene in *P. infestans*, which is the gene suspected to control the protein expression of elicitin in *P. infestans* [22].

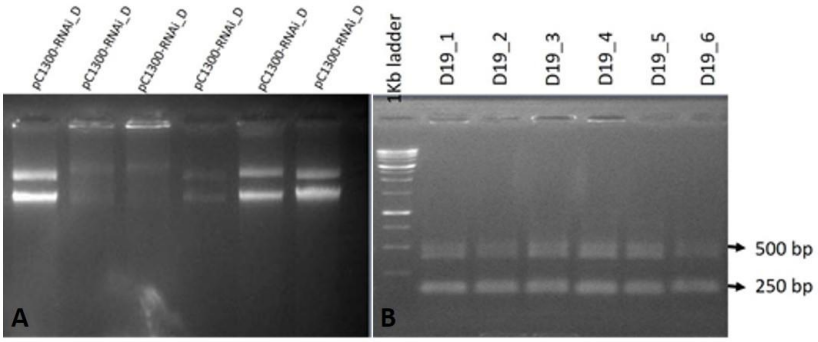

Figure 2.Verification of plasmids containing the RNAi construct using PCR method. (A) DNA plasmid band containing RNAi construct. (B) PCR amplicons of RNAi construct using *35s* and *Tnos* primers.

3.2. RNAi construct transformation

The RNAi inserted into the T-DNA plasmid pCambia1300 was transferred to Granola and Atlantic explants using *Agrobacterium*. The advantage of gene transfer technique using *Agrobacetrium* was that it had specific mechanisms to transfer DNA from its cell into plants using T-DNA. The molecular basis of genetic transformation by *Agrobacterium*in plant cells is DNA transfer from the bacterium followed by integration of a region of a large tumor-inducing (Ti) region into the plant nuclear genome [23]. Based on this mechanism, if a gene of interest is inserted into the T-DNA the expected gene will also be integrated into the target plant genome [24]. The *Agrobacterium*-mediated transformation

using RNAi construct into potato explants produced several plantlets that survived in the selection medium and rooting medium containing hygromycin.

Table 1. Number of selected explants on selection medium containing hygromicin.

Source	Number of explants	Number of surviving explants in selection medium (hygromycin 100)	Number of surviving explants shoots in rooting medium (hygromycin 50)
Atlantic (leaf)	204	1 (0.005%)	1
Granola (leaf)	66	-	-
Granola (internode)	733	-	-
Atlantic (internode)	569	282 (49.61%)	167

Hygromycin was used as a selection agent for the transformants because the pCambia1300 plasmid contains a gene (*hpt*II) for resistance to hygromycin. Transformed explants which still survived in media containing hygromycin indicate that those transformants carried the *hpt*II gene.

Transformation of Granola using 733 internode explants and 66 leaf explants with the RNAi construct did not produce any explants with resistance to hygromycin. However, transformation of Atlantic using 569 internode explants and 204 leaf explants produced 282 internode explants (49.61%) and one leaf explant (0.005%) resistant to hygromycin on selection medium containing 100 mg of hygromycin. The 282 selected explants produced 167 plantlets (shoots), which were transferred to rooting media containing hygromycin (50 mg). The results of RNAi construct insertion to leaf explants and internodes of Atlantic cultivars survived on selection and rooting medium containing hygromycin are shown in Table 1 and Figure 3.

Figure 3. Verification of putative transformed plants containing RNAi construct. (A) Leaf and internode explants of Granola dan Atlantic on selection medium containing hygromycin. (B) Transformed plantlets on rooting medium containing hygromycin.

3.3. Molecular analysis of the putative transformant lines

Molecular analysis was carried out on the surviving plantlets using PCR with specific primers to detect the presence of *hpt*II or the RNAi construct. Thirty-nine of the 168 plantlets were used for PCR analysis using specific primers for *hpt*II gene and 14 plantlets were PCR positive. Transformed plantlets that had DNA fragment of *hpt*II gene are expected to survive on medium containing hygromycin. The presense of *hpt*II gene inside the genome of survived transformants can be used to indicate that the plantlets also contain the RNAi construct. This assumption is based on the structure of the RNAi construct, where *hpt*II gene was inserted inside the T-DNA (Figure 1), so that when the *npt*II or *hpt*II gene is inserted in the plant genome then the RNAi construct should also be in it, or vice versa. According to Gelvin [23], *A. tumefaciens* has the capability to transfer intact T-DNA into plant genomes. Based on the analysis of Bartlett et al. [25], it was demonstrated that the T-DNA inserts

itself into the genome of the target plant started from the right border (RB) and terminated on left border (LB).

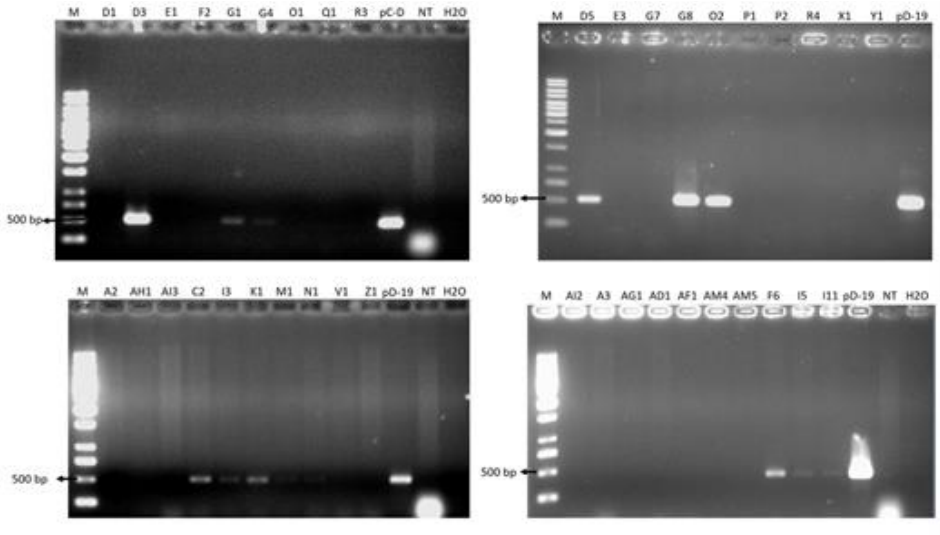

Figure 4. PCR results of transformed plantlets (Atlantic) that contained RNAi using primers for *hpt*II gene. M = 1 Kb DNA ladder, pD-19 = plasmid pCambia1300 containing RNAi contruct, NT = Atlantic non-transformant, H_2O = water.

The results of PCR analysis showed that the 14 transformed plantlets contained the *hpt*II fragment with the size of 500 bp. The existence of *hpt*II gene inside the construct is at the end of T-DNA and close to the LB of pCambia1300 plasmid (Figure 1A). This result also proved that the RNAi construct was also integrated into the genome of transformed plantlets. For further analysis, an experiment should be conducted by employing advanced PCR analysis using specific primers to determine that the transformed plantlets contain the correct construction of RNAi for resistance to *P. infestans.* Whether the resistance is durable or not bioassays to *P. infestans* must be performed in greenhouses or fields.

4. Conclusions

A. tumefaciens-mediated transformation on potato internode explants was simple and successful. The selected transformants should be further tested using bioassay for resistance to *P. infestans.* A total of 14 planlets were PCR-positive for *hpt*II fragment, indicating that RNAi construct for durable resistance to *P. infestans* was successfully integrated into the genome of transformed planlets of Atlantic variety. Further bioassay in greenhouses and in the field should be performed on the plantlets to identify lines with durable resistance to late blight disease.

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Genetic variations of EMS-induced chili peppers (*Capsicum annuum*) cv. Gelora generate geminivirus resistant mutant lines

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Abstract.A mutation breeding program was conducted to improve chili pepper (*Capsicum annuum*) resistance to Geminivirus caused by *Pepper yellow leaf curl virus* (PepYLCV). The disease can cause significant yield losses on chili pepper. This study was conducted to enhance genetic variation in pepper cv. Gelora using ethyl methanesulphonate (EMS) to obtain mutant lines resistant to PepYLCV. Exposure to an EMS solution of 0.5% (v/v) for 30 minutes was used for mutagenizing shoot tips to produce the first population (M_1). After the treatment, shoot tips were cultured on MS medium to regenerate planlets that were subsequently acclimatized to produce M_2 population. A set of 2,955 mutant lines (M_2) were screened in a greenhouse by inoculating PepYLCV isolate Segunung using white flies (*Bemisia tabaci*) as the virus vector. Six weeks after inoculation, the M_2 population was examined for symptoms of Geminivirus and was verified for the presence of PepYLCV infection by PCR using specific primer. The greenhouse assay successfully selected 47 lines (1.6%) of M_2 mutant population that showed no symptoms and no infection to PepYLCV compared to Gelora and Tanjung-2 varieties. Subsequent field test of M_3 mutant lines in Geminivirus endemic area in Lembang, West Bandung, revealed that 15 out of 47 lines showed high resistance to PepYLCV and have good agronomic characters including yield components. A set of primers corresponding to the PepYLCV infection which produced 678 bp in size indicated its good amplification in the susceptible mutant lines, confirming the PepYLCV resistance of 15 selected mutant lines. The resistant mutants were immune to PepYLCV and had potential to be released as improved varieties in the future.

Keywords: *Capsicum annuum*, ethyl methanesulfonate, geminivirus, *Pepper yellow leaf curl virus.*

1. Introduction

Pepper yellow leaf curl virus (PepYLCV) is a pepper-devastating viral pathogen transmitted by whitefly *Bemisia tabaci* [1]. The virus belonging to the family Geminiviridae and genera of Begomovirus have become a serious constraint to chili pepper in tropical and subtropical areas worldwide [2,3]. Chili plants infected with PepYLCV show severe symptoms such as stunting with small, thick, curly leaves and yellowing along leaf margins, which lead to significant fruit loss ranging

from 30 to 100% [4]. First detected on tobacco plants in the East Java in 1932 [5], PepYLCV is now endemic in many provinces of Indonesia and most commonly found in chili pepper and several horticultural crops. Although there is no national data of yield losses of chili caused by Geminivirus infection, Direktorat Perlindungan Tanaman Hortikultura [6] reported that the economic loss of chili due to PepYLCV reached IDR 20 billion in 14 provinces in 2007. In 2009, PepYLCV affected 650 ha of chili plants and caused yield loss up to IDR 16 billion in Kediri, East Java [6]. This disease can spread quickly in correlation with the increase population of *B. tabaci*.

So far, various methods have been used to control PepYLCV distribution, i.e. improvement of cultivation techniques, eradication of infected plants, prevention of vector dispersal and management of vector population using pesticides [4]. However, there are no effective methods to control this disease and its insect vectors. A single whitefly insect can transmit the virus to the plants. Therefore, the use of PepYLCV-resistant plant variety is the most promising method to control this disease. Host resistance plant is mostly preferred besides it is an effective, economical and environmentally friendly method for disease control [7]. Conventional breeding of PepYLCV-resistant plant was conducted through the selection of germplasms to identify possible sources of resistance genes to PepYLCV. As non-center of chili origin, Indonesia has low genetic variability of chili peppers, and it is a great challenge for breeders to collect or to develop a large number of germplasm resources.

Mutations are the primary source of all genetic variations existing in any organism, including plants [8]. Mutation breeding involves the development of new varieties by generating and utilizing genetic variability through chemical, physical and biological mutagenesis [9]. Chemical mutagens are preferably used to induce point mutations [10] and to generate not only loss-of-function, but also gain-of-function phenotypes if the mutation leads to a modified protein activity or affinity [9]. In addition, chemically-induced mutation showed high efficiency in producing individual lines that bear single point missense and non-sense substitutions in hundreds of genes [11]. Among the chemical mutagens, ethyl methanesulfonate (EMS) is considered as an effective one because it can form adducts with nucleotides efficiently, resulting in mispairing among these nucleotides with their complementary bases, and thus, introducing base changes after replication [12].

Previous studies found that EMS is an effective mutagen and can be used to improve desired identifiable characters such as resistance to virus diseases. *Arabidopsis* mutant lines bearing EMS-induced null alleles in the eukaryote initiation factor eIF(iso)4E gene are resistant to infection by several viruses from the genus Potyvirus, including *Turnip mosaic virus* (TuMV), *Lettuce mosaic virus* (LMV) and *Tobacco etch virus* (TEV) [13,14]. Mutagenesis using EMS also caused loss-of-susceptibility due to point mutation of eIF4E gene in *Capsicum annuum* to TMV [15], while in rice caused loss of susceptibility to TMGMV and PMMoV, and *Solanum lycopersicum* to AltMV [16]. EMS-induced mutations in tomato eIF4E were identified by sequencing eIF4E genes from 3,008 M_2 population [17] and by the TILLING of 4,759 M_3 population [18]. Loss of interaction of virus with eIF4E is correlated with a loss of infectivity of the virus, suggesting that the interaction is critical for virus production [19,20].

Induced mutations can rapidly create variability in quantitatively and qualitatively inherited traits in crops. Therefore, the objective of this study was to obtain new germplasm resistant to PepYLCV by the improvement of Gelora chili pepper cultivar.

2. Materials and methods

2.1. EMS mutagenesis

This experiment used Gelora, a red pepper cultivar (*C. annuum* L.) with medium yielding ability but susceptible to PepYLCV. Certified seeds were obtained from PT Sinar Bumi, East Java. The seeds were germinated in 72-hole seedling trays. One hundred shoot tips from 21-day-old seedlings were pre-soaked in disinfectant solution for 1 minute. Shoot tips were treated for 30 seconds at room temperature on a shaker with 0.5% (V/V) of EMS prepared in a 0.1 M phosphate buffer at pH 7.0. Shoot tips were then thoroughly washed three times with sterile distilled water and transferred to vessels containing MS medium supplemented with 2.4-D 3 mg/l and Thidiazuron 0.5 mg/l. Vessels

were kept in a growth chamber at 25±2°C under white light until growing up. Rooting induction was conducted in vessels containing 60 ml of solid MS½N supplemented with NAA 0.5–1.0 mg/l. Subsequently, rooted-plantlets were acclimatized in a screen house at a temperature ranging from 25°C to 30°C. Plantlets of 6 to 8 cm in length were transferred to seed pots (9 cm^2 area) containing a sand and humus mixture (2:1). To maintain humidity, the plantlets were covered with plastic caps and gradually opened during two-week acclimatization. Survival of plants was monitored daily and maintained with recommended cultural practices until fruit harvested. First mutant lines (M_1) seeds were harvested and planted to generate M_2 population.

2.2. Screening for putative resistant mutant lines

A total of 2,955 M_2 of mutant lines were screened to PepYLCV under greenhouse condition at Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development. Gelora, Chiko and Tanjung-2 were used as control varieties. The seeds were germinated in 72-hole seedling trays. The plants were maintained in cages (250 cm × 110 cm × 100 cm) covered with 100-micron mesh cloth. Thirty-day-old plants were inoculated for a period of 48 hours with one or two *B. tabaci* insects which have acquired Begomovirus isolate Segunung for 24 hours. One week after inoculation, plants were transplanted to 9 cm × 15 cm polybags filled with soil and organic matter mixture (12:1).

Disease and agronomic parameters observed were incubation period, symptom types, disease intensity, and also the number and weight of fruits. The disease symptoms were observed during the first harvest period. The disease intensity was scored from 0 to 5 and was used to determine severity level of Begomovirus infection using the formula: DI = Σ(ni × zi)/(N × Z) × 100%, where i = 0–5 score, ni = sum of plant symptoms with score value, zi = value of symptoms score, N = sum of plant, and Z = the highest score of symptoms.

2.3. Establishment of pepper mutant lines in field

The experiment was carried out at Indonesian Vegetables Research Institute, Lembang, West Bandung, under natural infection of PepYLCV. The climate in Lembang is tropical wet and dry, with the average annual temperatures ranges from 17 to 27°C. The rainy season runs from June through October with the average annual rainfall is 1,036.9 mm. A total of 40 mutant lines was arranged in a randomized block design with three replicates. Thirty-day-old seedlings of all mutant lines were individually transplanted in plots covered with polyethylene mulch. The plot size was 1 m × 6 m with 50 cm × 70 cm planting distance. Each plot was comprised of 30 plants per plot. Plants were fertilized with 150 N, P and K with an application rate of 250, 200 and 150 kg/ha, respectively, supplied by a dripping irrigation system. No insecticide was applied to increase successful infestation of *B. tabaci*. Plants were observed for disease intensity, yield components and agronomic traits.

Data were analyzed by means of Statistical Analysis Software (SAS) programme and means were separated by the Least Significant Differences (LSD) test. Genetic variability was calculated based on previous formulas [21] (Table 1).

Table 1. Calculation method of genetic variability.

Source of variant	Degree of freedom	Mean of square	Probability[a]
Replication	[replication(r)-1]	Mean (M3)	$\sigma_e^2 + 21\sigma_u^2$
Mutant lines	[Number of mutant (m)-1]	Mean (M2)	$\sigma_e^2 + 3\sigma_g^2$
Error	(r-1) (m-1)	Mean (M1)	σ_e^2

[a] $\sigma_e^2 = enviroment$; σ_g^2 = genetic variability

$\sigma_g^2 = \frac{M2-M1}{r}$ $\sigma_e^2 = M1$; $\sigma_p^2 = \sigma_g^2 + \sigma_e^2$.

Genotypic coefficients variances (GCV) and phenotypic coefficients variances (PCV) were calculated using the formula:

$$GCV = \frac{\sqrt{\sigma_g^2}}{mean} \times 100\%, \text{ and } PCV = \frac{\sqrt{\sigma_p^2}}{mean} \times 100\%$$

The criteria for GCV and PCV relative is low, medium, and high, if x <10%, 10%< x <20%, and 20%< x, respectively[22].Heritability was calculated using the formula:

$$h_{bs}^2 = \frac{\sigma_g^2}{\sigma_g^2 + \sigma_e^2}$$

Genetic advance was determined as described by Johnson et al. [23]:

$$GA = K(\sigma_p)h^2,$$

where: K= the selection differential (K-2,06 at 5% selection intensity), σ_p = the phenotypic standard deviation of the characters, and h^2 = broad-sense heritability. The genetic advance as percentage of the mean (GAM) was calculated as described by Johnson et al. [23] as follows:

$$GAM = \frac{GA}{mean} \times 100\%,$$

where: GAM = genetic advance as percentage of the mean, and GA = genetic advance.

2.4. Virus detection

The presence of PepYLCV in the samples was detected by PCR analysis using the specific primers for PepLCV (primer PAL1v 1978: 5'-GCATCTGCAGGCCCACATYGTCTTYCCNGT-3' and primer PAR1c 715 5'-GATTTCTGCAGTTDATRTTYTCRTCCATCCA-3') to amplify a 1,600 bp fragment from the intergenic region of PepYLCV component [24]. The procedure of Dellaporta et al. [25] was used for DNA extraction. PCR reactions were performed in a total volume of 50 µl containing 2.5 mM of each dNTP, 1 µl of oligonucleotides (50 ng/µl), 1 µl of *Taq* polymerase (5 U/µl), and 250 ng plant DNA. The PCR conditions were 94°C for 2 min, 50°C for 2 min and 72°C for 2 min, for 35 cycles. The PCR products were analyzed on 0.8% agarose gels.

3. Results and discussion

3.1. Greenhouse screening

A total of 2,955 chili pepper mutant lines were screened for PepYLCV resistance to identify resistant mutant. Results of the greenhouse assay are presented in Figure 1. The data revealed that 82% of 2,955 mutant lines were infected by Begomovirus at three weeks after inoculation (WAI). Until the generative phase, there are only 5% of mutant lines showing no symptom of PepYLCV. Finally, 47 (1.6%) of pepper mutant lines were identified as resistant to PepYLCV because they showed no visual symptoms and contained no particles viruses based on PCR detection. On the contrary, susceptible lines showed systemic symptoms of PepYLCV including stunting with small, thick, curly leaves and yellowing along the leaf margins.

Such resistance responses in different mutant crops against viruses were reported in *C. annuum* mutants to *Tobacco mosaic virus* (TMV) [15], *S. lycopersicum* mutants to TMV [26], rice mutants to *Rice dwarf virus* [27], and *A. thaliana* mutants to TMV [15], *Cucumber mosaic virus* (CMV) [28,29], and *Turnip mosaic virus* [30]. In *A. thaliana*, the screening of large mutagenized populations has led to the identification of a number of mutants in which virus susceptibility is reduced or eliminated. The effect of EMS to *A. thaliana* is inducing mutation on *TOM1*, *TOM2* and *TOM3* genes that are required for efficient TMV [28] and CMV [31] replication in protoplasts. EMS induces C-to-T changes resulting in C/G to T/A substitutions, whereas methyl methanesulfonate produces T/A to G/C transversion and A/T to G/C transitions (1,3,4) [12]. Based on codon usage in *Arabidopsis*, the frequency of EMS-induced stop codon and missense mutations has been calculated to be ~5% and ~65%, respectively [12]. EMS mutagenesis generates randomly distributed mutations throughout the genome [32].

3.2. Field experiment

3.2.1. Selection of chili mutant for resistance lines. Forty-seven selected-chili pepper mutant lines were subsequently tested under natural infestation of whiteflies during the dry season in Lembang, West Bandung, West Java. The disease incidence during the experiment was quite high and evenly spread throughout the crops. Infection symptom of Geminivirus was already visible at three weeks after transplanting, and the disease incidence continued to increase. At 15 weeks after transplanting, the disease incidence on the tested lines was 65.33%, whereas on Gelora, Tanjung-2 and Chiko was 34.67, 42.67 and 32.00%, respectively. These result indicated that the experiment under natural infection of the virus in Lembang was suitable for the identification of chili pepper mutant resistant to PepYLCV. Out of 47 chili mutant lines, 15 mutants were highly resistant to Geminivirus based on visual symptoms and detection by PCR (Table 2). The resistant mutants exhibited 0–5,33% of PepYCLV infection with negative PCR detection. This study showed that mutation breeding is an important aspect in disease management practice. The resulted resistant mutant lines can be further used for selection of suitable genotypes in PepYLCV resistance breeding programme in chili.

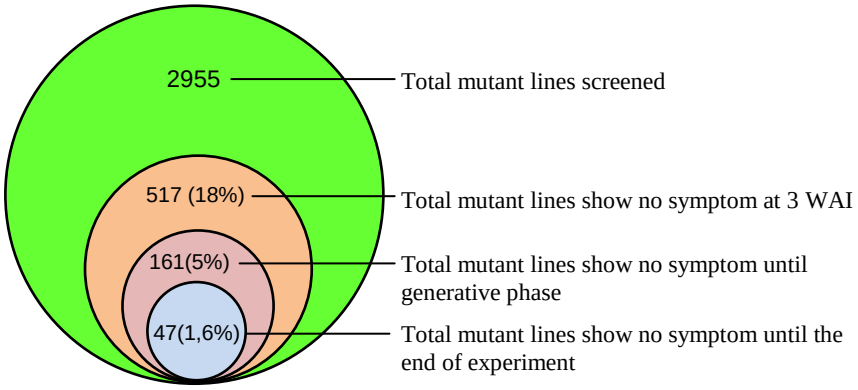

Figure 1. Results of screening of 2,955 chili pepper mutant lines against PepYLCV under greenhouse condition.

3.2.2. Analysis of variance of agronomic and yield component of chili mutant lines. Beside genetic variation in resistance against PepYLCV, chili mutant lines also showed morpho-agronomic variability during the field experiment. The results of variance analysis for all morpho-agronomic characters are shown in Table 3. Significant effects of genotype were observed for all traits under study, except for the number of branch nodes and length of fruit. Genetic variability plays important in crop improvement. The variability that is observed in the basic population is the chance of improvement. The highly significant difference among the mutants for many characters indicated the existence of large genetic variability among the genotypes. These results are in good agreement with some earlier findings of genetic variability of EMS-induced mutagenesis [33–37].

3.2.3. Estimation of chili mutant lines genetic parameters. Table 4 showed estimation of genotypic (Vg) and phenotypic variances (Vp), GCV, PCV and h^2. High Vg and Vp were recorded for plant height (86.01 and 126.48 cm) and harvest period (64.19 and 72.23 days), respectively. The low values of Vg and Vp were observed for the number of branch nodes, weight per fruit, length of fruit, fruit diameter, and length of fruit stalk. In general, the Vps were higher than Vg for all the characters. GCV and PCV for the number of fruits both were 0.00%, whereas for the number of branch nodes were 12.68 and 30.36%, respectively. According to Deshmukh [22], PCV and GCV values greater than 20% are regarded as high and values between 10 and 20% to be medium, whereas values less than 10% are considered to be low. Accordingly, high PCV and GCV were recorded for dichotomous and harvest period, whereas traits with moderate PCV and GCV were height of plant and width of canopy.

The number of branch node had high PCV and medium GCV. Traits having low PCV and GCV were the number of fruits per plant, weight per fruit, length of fruit, diameter of fruit, length of fruit stalk, and weight of seed per plant. High values of PCV and GCV indicated the existence of substantial variability for such characters and selection might be effective based on these characters. A similar finding was reported by earlier researchers for dichotomus and plant height [38]. In this study, the PCV was relatively greater than GCV for all traits; indicating a high contribution of genotypic effect for phenotypic expression of such characters.

Table 2. List of chili mutant lines resistant to PepYLCV under natural infection during the dry season of 2016 in Lembang.

Mutant lines	Disease incidence (%)	PCR	Resistance status	Mutant lines	Disease incidence (%)	PCR	Resistance status
M.25/M3.613.2	45.33	+	S	M3.149.3	20.00	+	S
M.33/M.123.3	5.33	-	R	M3.153.1	1.33	+	S
M.34/M.114.1	14.67	+	S	M3.153.2	8.00	+	S
M.36/M3.114.3	54.67	+	S	M3.167	0.00	-	R
M38/M3.148.2	65.33	+	S	M3.168	0.00	-	R
M.43/M3.139.2	22.67	+	S	M3.176	0.00	-	R
M.44/M3.139.3	4.00	-	R	M3.190	0.00	-	R
M.55/M3.201.2	8.00	+	S	M3.192	0.00	-	R
M.57/M3.801.3	24.00	+	S	M3.200.2	8.00	+	S
M. 58/M3.167.2	30.67	+	S	M3.238.1	9.33	+	S
M.60/M3.167.4	5.33	+	S	M3.238.2	24.00	+	S
M.62/M3.170.3	6.67	+	S	M3.353.1	5.33	-	R
M.63/M3.108.1	0.00	-	R	M3.420.1	26.67	+	S
M.69/M3.200.1	0.00	-	R	M3.517.1	20.00	+	S
M.74/M3.108.3	5.33	+	S	M3.517.2	33.33	+	S
M.76/M3.160.2	4.00	-	R	M3.711.1	20.00	+	S
M.77/M3.160.3	10.67	+	S	M3.711.2	37.33	+	S
M.82/M3.146.1	0.00	-	R	M3.711.3	24.00	+	S
M.84/M3.149.1	0.00	-	R	M3.801.1	12.00	+	S
M.86/M3.123.1	0.00	-	R	M3.801.2	33.33	+	S
M.87/M3.100.2	0.00	-	R	M3.902.1	34.67	+	S
M3.113.3	9.33	+	S	M.902.3	28.00	+	S
M3.122.1	21.33	+	S	Gelora	34.67	+	S
M3.138	6.67	+	S	Chiko	32.00	+	S
M3.148.1	13.33	+	S	Tanjung-2	42.67	+	S

S = susceptible, R = resistant.

Table 3. Mean square of combined analysis for all the characters studied of 47 chili mutant lines of Gelora cultivar and three genotype controls (Gelora, Chiko and Tanjung-2).

Source	Df	Dic	HP	WC	NBN	HvP	NF	WF	LF	FD	LFS	WS
Replication	2	0.904	56.792	0.102	17.846**	31.927	2.69	15.762	50.22	0.006**	0.004	0.058
Mutant	49	113.93**	301.49*	81.63**	0.201	200.60**	1,011.71**	3.11*	58.08	0.10**	0.37**	11.76**
Error	98	1.73	43.469	0.096	0.549	8.047	1.92	0.376	50.53	0.002	0.013	0.016

*Significantly different at 5% level.
**Significantly different at 1% level.
Df = degres of freedom, Dic = dichotomus, HP = height of plant, WC = width of canopy, NBN = number of branch nodes, HvP = harvest period, NF = number of fruits, WF = weight per fruits, LF = length of fruit, FD = fruit diameter, LFS = length of fruit stalk, WS = weight of seed.

Estimates of heritability in broad-sense ranged from 17.9% for the number of branch node to 99.7% for the width of canopy (Table 3). Heritability values greater than 80% were very high, values from 60–79% were moderately high, values from 40–59% were medium and values less than 40% were low [21]. Accordingly, very high heritability was shown by the width of canopy, weight of seed, number of fruit per plant, length of fruit stalk, dichotomous, and harvest period. The higher values of heritability estimations of traits are indicators of a greater proportion of genetic components in relation to the environment [39], and selection for such characters could be fairly easy due to high additive effect [40]. High estimates of broad-sense heritability have also been reported by previous researchers for days to first harvest and fruit length [41], the number of fruit per plant [42] and canopy diameter [43].

Table 4. Genotypic (Vg) and phenotypic (Vp) variances, genotypic (GCV) and phenotypic (PCV) coefficient of variances and broad-sense heritability (h^2) for all traits of Gelora cultivar of *Capsicum annuum*.

Character[a]	Min	Mean	Max	Vg	Vp	GCV (%)	PCV (%)	h^2 (%)	GA	GAM
Dic (cm)	10.55	30.2	32.42	37.40	39.13	20.25	20.71	95.6	12.32	40.7
HP (cm)	34.30	57.2	75.00	86.01	129.48	16.21	19.88	66.4	15.56	27.2
WC (cm)	29.90	33.9	49.87	27.18	27.27	15.35	15.38	99.7	10.73	31.6
NBN	2.40	2.7	3.67	0.12	0.67	12.68	30.36	17.9	0.3	11.1
HvP (days)	24.00	38.8	54.00	64.19	72.23	20.66	21.92	88.9	15.56	40.1
NF	22.33	50.3	71.00	18.35	18.42	0.00	0.00	99.6	8.81	17.5
WF (g)	2.73	4.4	8.47	0.95	4.08	0.01	0.04	23.3	0.97	22.0
LF (cm)	3.86	9.2	11.67	1.59	7.26	0.00	0.02	21.9	1.22	13.3
FD (cm)	0.78	1.1	1.93	0.04	0.09	0.01	0.01	44.4	0.27	24.5
LFS (cm)	2.38	3.0	3.53	0.34	0.35	0.01	0.01	97.1	1.18	39.3
WS (g) per plant	1.13	4.6	7.27	1.98	1.99	0.02	0.02	99.5	2.89	62.8

[a] See Table 3 for explanation of the abbreviations.

The estimated GA values for all characters are presented in Table 4. Due to the different scale of traits, GA was calculated as percentages of GAM. High heritability, along with high GA is an important factor for predicting the resultant effect for selecting the best individuals [44]. Therefore, genetic advance gives an idea of possible improvement of the new population through selections. The genetic gain depends on the amount of genetic variability and magnitude of the masking effect of the environment. Values of genetic advance as a percentage of mean ranged from 11.1 to 62.8. High values of genetic advance as percentage of mean estimates coupled with high estimates of heritability expected in weight of seeds per plant indicate the preponderance of additive gene action for the

expression of these traits [39]. The number of branch node exhibited low heritability and genetic advance, indicating that this trait is controlled by non-additive genetic effects.

4. Conclusions

EMS mutagenesis successfully induced genetic variability in the Gelora cultivar of *C. annuum*. Resistant chili pepper mutant lines against PepYLCV were identified from M_2 generation in a greenhouse screening and verified in a field experiment. The resistant mutant may be used as genetic material to identify the genes controlling resistance against PepYLCV, which is a commercially desirable trait. Chili pepper mutant lines also showed genetic variation in morpho-agronomic and yield component. Some of morpho-agronomic and yield component traits could be used as useful criteria for selection in the chili improvement, due to high variation in genotypic coefficient, heritability estimation, and genetic advance value. Further work is needed to analyze these mutants and determine the genetic reasons underlying the morphological changes in order to genetically improve *C.annuum* cultivars.

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Soil biology characteristics of oil palm land endemic to *Ganoderma* after four years conversion to sugarcane

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Abstract. Basal stem rot disease is the primary disease in oil palm production. One of the control methods to keep the disease severity low is through crop rotation by planting non-host crops for a certain period to reduce the pathogen population. This study aimed to evaluate the biological and molecular characteristics of *Ganoderma* endemic land after converted to sugarcane for four years. Results showed that the total abundance of bacteria in the land ranged from 3.5 × 102–6.1 × 108 CFU/g, whereas phosphates solubilizing bacteria, was in the range of 101–1.0 × 106 CFU/g and N fixing bacteria was 101–2.4 × 104 CFU/g. The genus dominated mycorrhizal fungi from Glomus and Acaulospora with spore population of 1 to 135 spores per 25 g of soil. Another soil biota analyzed was Trichoderma sp. that had population range between 102 to 105 CFU/g. Based on PCR analyses using specific primers Gan2 and Gan3, *Ganoderma* fungi were still detected in selected soil samples and the remaining roots in the field. Nevertheless, the productivity of sugarcane up to 4 ratoons remained high, i.e. more than 90 t/ha.

Keywords: endemic *Ganoderma* land, crop rotation, sugarcane, control technique, biological and molecular characters.

1. Introduction

Basal stem rot (BSR) caused by *Ganoderma* fungi is a significant disease in palm oil cultivation. Various techniques have been done to control the disease, including the use of chemical pesticides, digging holes around infected plants, genetic engineering and application of moderate or tolerant planting material. Nevertheless, there are no effective control methods for severely diseased plants. Alternative methods to break the *Ganoderma* life cycle is through replanting by a special technique [1] and by crop rotation with non-host plants.

Crop rotation can be interpreted as an effort to control pathogen based on the management of inoculum by breaking the life cycle of soil pathogens [2–5]. On the other hand, Partey et al. [6] suggested that crop rotation aims to eradicate inoculum sources which are infested in the land. Furthermore, Zhou et al. [7] suggested that crop rotation can increase crop productivity because of improved soil fertility through the increased level of soil biota population and diversity.

Crop rotation to control *Ganoderma* sp. has not been previously reported. This technique is expected to suppress *Ganoderma*, especially at severe disease level. This research is to study the soil biology characteristics of *Ganoderma* endemic land that have been converted from palm oil to

sugarcane plantation. The data collected can be used to determine the effect of rotation on population and diversity of soil microbes as an instrument not only to control *Ganoderma* sp. but also to restore soil fertility.

2. Materials and methods

2.1. Plant disease history of the land

The experiment was conducted at palm oil plantation at Unit Bekri Plantation, Central Lampung. The land has been planted with oil palm for four generations or approximately 100 years. In that area, the death rate of palm oil has reached more than 30% population or about 70–100 trees per hectare. Production of fresh fruit bunches in productive age crop is also reduced. In endemic block, production has been decreased to ±9,557 kg/ha. Several techniques have been applied to control the disease such as eradication of *Ganoderma* colonized tissue, raising soil bed surrounding basal stem and application of organic materials, such as ash and sludge or application of antagonistic microbes. However, these treatments were not consistently done, leading to the increased of *Ganoderma* disease severity level.

Table 1. Soil microbe population in soil samples taken from 36 blocks of sugarcane cultivation area in ex-oil palm area endemic to *Ganoderma.*

Block no.	Total bacteria (CFU/g)	*Ganoderma* sp. (propagule/g)	*Trichoderma* sp. (propagule/g)	Phosphate solubilizing bacteria (CFU/g)	Nitrogen fixing bacteria (MPN/g)	Lignolytic fungi (propagule/g)	Mycorrhiza (spore/25 g)
02	8.5×10^7	50	1.0×10^5	1.0×10^5	6.1×10^2	2.5×10^2	36
03A	4.0×10^7	10	6.0×10^4	<10	9.4×10^2	1.0×10^2	54
04	4.0×10^7	0	<102	10×10^5	<10	$<10^2$	21
05A	2.6×10^7	20	10×10^4	2×10^5	2.9×10^3	1.5×10^2	19
05C	3.1×10^5	7.3	2.0×10^3	1.8×10^4	7.5×10^3	8.0×10^2	32
05D	3.1×10^6	8.5	<102	1.0×10^4	2.4×10^4	3.0×10^2	22
05F	2.7×10^5	5.5	1.0 x 10^2	1.1×10^4	9.3×10^3	1.5×10^3	13
06A	3.1×10^7	1	1.0 x 10^3	<10	4.6×10^3	1.7×10^2	38
06A.1	3.8×10^8	0	<10	4.5×10^3	3.6×10^2	1.0×10^3	18
06B	3.2×10^6	2	1.5×10^3	<10	1.5×10^3	7.5×10^2	20
06C	3.8×10^8	3	1.5×10^3	<10	2.3×10^3	4.5×10^2	20
07A	1.4×10^7	1	3.5×10^2	7.2×10^3	3.6×10^2	3.0×10^2	4
07B	3.5×10^2	0	$<10^2$	<10	<10	$<10^2$	3
07C	7.0×10^6	6	$<10^2$	1.1×10^3	9.3×10^2	2.5×10^2	7
07D	6.5×10^6	8	$<10^2$	1.6×10^3	<10	1.0×10^2	8
09	6.5×10^7	0	$<10^2$	2.0×10^4	<10	$<10^2$	19
10A	9.2×10^6	10	$<10^2$	2.0×10^4	<10	$<10^2$	21
11A	1.0×10^7	0	0	1.0×10^5	3.6×10^2	$<10^2$	68
12A	1.0×10^7	11	$<10^2$	1.0×10^5	<10	1.5×10^2	18
15A	4.3×10^7	2.5	2.5×10^3	1.2×10^4	<10	4.0×10^2	18
16	3.1×10^7	5.5	2.0×10^3	9.5×10^3	<10	1.0×10^3	1
17	9.5×10^7	1	3.0×10^2	3.0×10^3	<10	1.0×10^2	3
18	5.1×10^8	0	1.5×10^3	6.0×10^3	4.6×10^3	$<10^2$	2
19	6.5×10^6	8	1×10^4	3×10^3	<10	3.0×10^2	1
20	8.0×10^6	10.5	2.5×10^3	1.0×10^2	<10	7.5×10^2	23
36	4.0×10^6	4.5	1.5×10^3	5×10^3	<10	1.5×10^3	23
47A	1.6×10^5	2	$<10^2$	1.8×10^3	<10	1.0×10^2	135
504A	1.1×10^7	2.5	1.5×10^3	1×10^3	3.6×10^2	1.5×10^2	1
545	4.0×10^6	4	1.5×10^3	$<10^2$	4.6×10^3	6.0×10^2	3
585	1.5×10^6	3	1.5×10^3	1.5×10^3	2.9×10^2	3.5×10^2	1
586	1.5×10^7	5.5	1.5×10^3	1.0×10^2	$<10^2$	$<10^2$	31
663	5.5×10^5	0	1.5×10^3	7.0×10^3	7.5×10^2	$<10^2$	9
698	4.3×10^6	0	7.5×10^3	7.5×10^3	$1,1 \times 10^2$	$<10^2$	24
859	1.1×10^6	0	1.5×10^3	$<10^2$	2.1×10^3	$<10^2$	10
981	6.1×10^8	5	3.5×10^2	$<10^2$	3.6×10^2	$<10^2$	2

2.2. Palm oil rotation to sugarcane

Sugarcane varieties planted for crop rotation in *Ganoderma* endemic land in 2012 were PS 881 and KK/BM 9605 (Kidang Kencana). Land preparation was started with intensive soil tillage twice to a depth of 50 cm, soil ploughing twice and ground levelling once. *Ganoderma* biological controlling agent, *Trichoderma* sp. was given once at the beginning of the crop rotation. Sugarcane planting practice was done by the standard method.

After four periods of planting or about four years, composite soil samples and non-decomposed palm oil roots which were still found on land were taken to be analyzed in a laboratory. The total sampling area was 739 ha. About 500 g of soil sample each from 180 sampling points were taken from rhizosphere at a depth of 30–40 cm. These samples represented 32 blocks of sugarcane cultivation area (±699 ha) and 4 blocks of existing palm oil (±40 ha) (Table 1). The soil samples were pooled to make up 36 soil samples. These samples were analyzed for soil biological properties including the abundance of total bacteria, phosphate solubilizing bacteria (PSB), nitrogen-fixing bacteria (NFB), arbuscular mycorrhizal fungi (AMF), *Trichoderma* sp. and *Ganoderma* sp. Also the ligninolytic microbial population was also explored. Bacterial population analysis was done by the standard method using a specific medium. Molecular analysis of *Ganoderma* sp. was performed with Polymerase Chain Reaction (PCR) using Gan2 specific primers [8] and Gan3 [9].

2.3. Analysis of ligninolytic microbial population

Ligninolytic microbe population in soil samples and the remaining palm oil roots that have not decomposed on the land were analyzed using a specific medium containing guaiacol. In this selective agar medium colonies of ligninolytic fungus, including *Ganoderma*, produces brownish halo [10,11]. Soil samples were diluted up to 108, and then 1 ml of each dilution was dispersed on agar media in petri dishes. Tissue samples were directly plated on petri dishes containing the same medium.

2.4. Molecular detection of suspected Ganoderma sp. fungus

DNA isolation was performed on 20 isolates of suspected *Ganoderma* fungus using the ExgeneTM Cell SV DNA Kit, following the protocol in the GeneAll ExgeneTM Protocol Handbook. DNA was amplified using a PCR machine with *Ganoderma* specific primers Gan2 [8] and Gan3 [9]. Amplicons were electrophoresed along with a standard 1 Kb ladder [9].

3. Results and discussion

3.1. Rotation of oil palm to sugarcane

Sugarcane planted in ex-palm oil field endemic to *Ganoderma* showed average growth with high productivity during four planting periods (Table 2). Kidang Kencana variety planted in this area was capable of producing 84.32% of its yield potential, which are 112.5±32.5 t/ha [12]. Therefore, the land is highly supportive of sugarcane growth after crop rotation.

Table 2. The productivity of sugarcane after four years of conversion of oil palm area endemic to *Ganoderma*.

Rotation periods	Average yield (t/ha)
1	101.6
2	99.7
3	91.6
4	93.8
5	87.0

3.2. Analysis of soil biology

Analysis of soil samples showed that the abundance of soil microorganisms varied (Table 3, Figure 2). The data showed that total soil bacteria, PSB, NFB and ligninolytic fungi populations ranged from 102–108 CFU/g, 101–106 CFU/g, 101–104 CFU/g and 102–103 propagules/g soil, respectively. Total soil bacteria population is affected by the type and soil content. Previous research reported a higher population level of total soil bacteria, i.e. 105–106 [13] and 108–109 [14]. Accordingly, the PSB population found in the soil was generally 105–106 CFU/g soil [15]. Marista et al. [16] reported that PSB bacteria found in alluvial soil, peat and red-yellow podzolic (RYP) generally belonged to *Acetobacter*, *Bacillus*, *Flavobacterium*, *Micrococcus*, *Azotobacter*, *Pseudomonas*, *Staphylococcus*, *Escherichia*, and *Paracoccus*.

Table 3. Soil microbe population in soil samples taken from ex-oil palm area endemic to *Ganoderma* after four years conversion to sugarcane.

Analysis	Population range
Bacteria total	3.5×10^2–6.1×10^8 CFU/g
Phosphate solubilizing bacteria	<10–10×10^5 CFU/g
N fixing bacteria	<10–2.4×10^4 CFU/g
Lignolytic fungi	<10^2 –1.5×10^3 propagules per g
Arbuscular mycorrizal fungi	1–135 spore/ 25 g soil
Trichoderma sp.	<10^2–1.0×10^5 CFU per g
Ganoderma sp.	0–50 propagules per g

Varying levels of spore population of mycorrhiza were also observed, ranging from 1 to 135 spores/25 g soil. Meanwhile, the abundance of *Trichoderma* sp. ranged from 102–105 CFU/g soil. The fungus that was taken directly from the soil and plated on *Ganoderma* selective medium (GSM) was confirmed as *Ganoderma* and had a population ranging from 0 to 50 propagules/g soil (Table 3).

Bacteria that grow on nitrogen-free agar medium formed pellicle, a characteristic of NFB. NFB population is relatively low when compared to total bacteria population. While other research reported that NFB function as endophytic microbes such as *Gluconacetobacter diazotrophicus* is found in roots, stems and leaves in a reasonably high population (about 103–107 CFU/g) in various sugarcane plantations in Brazil, Mexico, Cuba and Australia.

The population of AMF spores on soil samples varied, ranging from 1 to 135 spores/25 g soil. This value was lower than the regular rate, which is 35–124 spore/10 g soil [17]. Mycorrhizal species on red-yellow podsolic soil Bekri's crop rotation area was dominated by *Acaulospora* sp. and *Glomus* sp. (Figure 1). AMF form mutualistic symbioses with almost 90% of higher plants; they are capable of improving plant health, soil structure, nutrient uptake and aiding plants to stand dry conditions [18]. Their highest population was observed in soil was 135 spores/25 g soil in sample block 47A, while the lowest was found in block 585 (1 spore/25 g soil sample).

Trichoderma could be found in most of the soil samples, but most of them were in lower a population compared to that reported by Sariah et al. [19], which is 2.1 × 103–2.1 × 104 CFU/g soil. The highest population was obtained from block 02 with population of 105 CFU/g while in block 06 there was no *Trichoderma* found. It seemed that the organic compounds in block 02 were more abundant compared to another block, so that *Trichoderma* best survive.

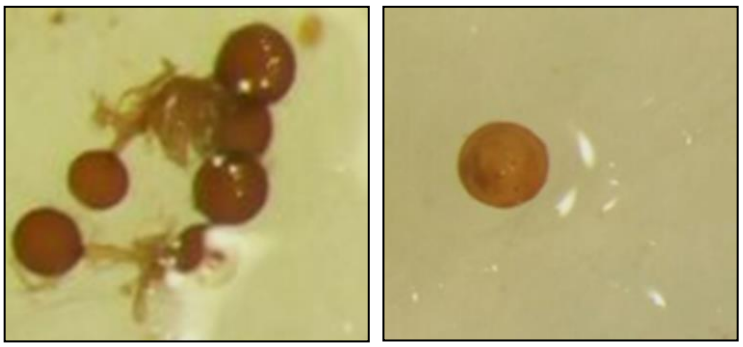

Figure 1. Spores of arbuscular mycorrhizal fungi *Glomus sp.* (left panel) and *Acaulospora sp*. (right panel).

Ligninolytic microbial analysis found ligninolytic microbes in selected soil samples and oil palm root fragments. The rest of the remaining root fragments root can become the food source of ligninolytic microbes which use carbon (C). The range of ligninolytic fungi found was between 102–103 propagules/g soils. *Ganoderma* was simply detected in selective agar medium which contains guaiacol. In this medium, brownish halo appeared surrounding its colony due to the use of guaiacol as the food sources (Figure 2D). There were only 17% of the total soil samples that contained *Ganoderma* of >10–50 propagules/g, 58% had less than ten propagules/g soil, and the remaining 25% did not contain *Ganoderma* propagules.

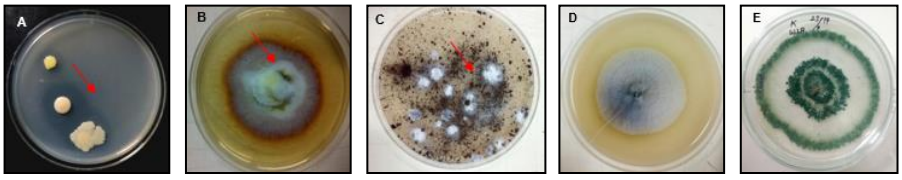

Figure 2. Colonies of fungus isolated from rhizosphere soil samples of sugarcane crops planted in ex-oil palm area endemic to *Ganoderma*. (A) Phosphate solubilizing bacteria. (B) Ligninolytic fungi. (C) Lignin-degrading fungi. (D) Suspected *Ganoderma* on selective agar medium containing guaiacol. (E) *Trichoderma* colony on PDA medium.

3.3. Molecular analysis of suspected Ganoderma fungus

PCR results of DNA isolated from suspected *Ganoderma* grew selective media is shown in Figure 3–5. Based on the molecular identification we found that PCR products of two isolates obtained from soil samples (05b and 545) and one isolate (47) found in the remaining root tissue have the same band size with positive control *Ganoderma* (260 and 454 bp). Meanwhile, isolates 02 and 859 showed negative results on all primers, which indicate that they are not *Ganoderma*. Meanwhile, isolates 05B.1, 17, 19 and 05B.2-3 obtained from non-decomposed oil palm roots tissue showed positive results as *Ganoderma* when identified molecularly. Other fungal isolates suspected to be *Ganoderma* derived from the soil that was positively identified, including isolates 663, 10A, 9, 03A and 36, whereas five other isolates (20, 504A, 12A, 11A and 10A.1) obtained from soil were not identified as *Ganoderma* (Table 4). These results showed that *Ganoderma* could persist in the soil during four years of rotation where they survived well in the remaining oil palm roots.

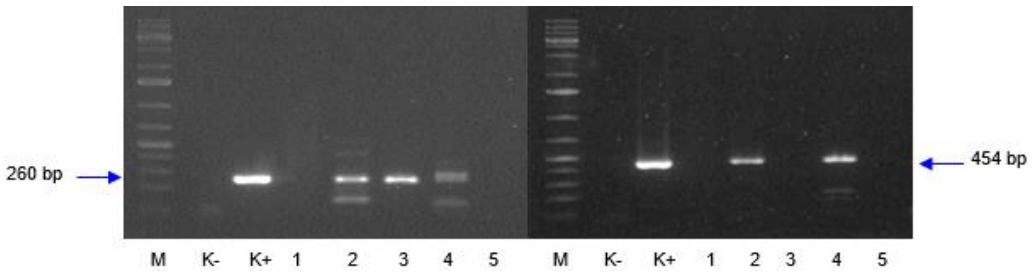

Figure 3. Electrophoregram of PCR results of suspected *Ganoderma* fungus with Gan2 primer (260 bp, left panel) and Gan3 (454 bp, right panel). Negative control (water), positive control (*G. lucidum*), PCR product of isolate 02, isolate 05B, isolate 47, isolate 545 and isolate 859 were loaded in the lanes K-, K+, 1, 2, 3, 4 and 5, respectively.

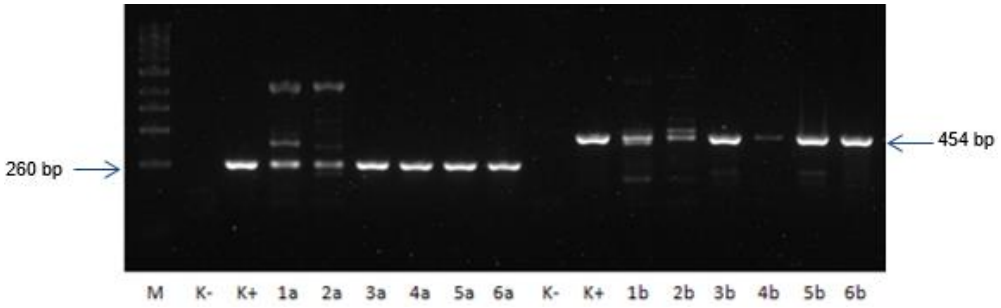

Figure 4. Electrophoregram of PCR results of suspected *Ganoderma* fungus with Gan2 primer (260 bp, left panel) and Gan3 (454 bp, right panel). PCR products of negative control (water), positive control (*G. lucidum*), isolate 663, isolate 09, isolate 019, isolate 017, isolate 05B.1 and isolate 10A were loaded in the lanes K-, K+, 1, 2, 3, 4, 5 and 6, respectively.

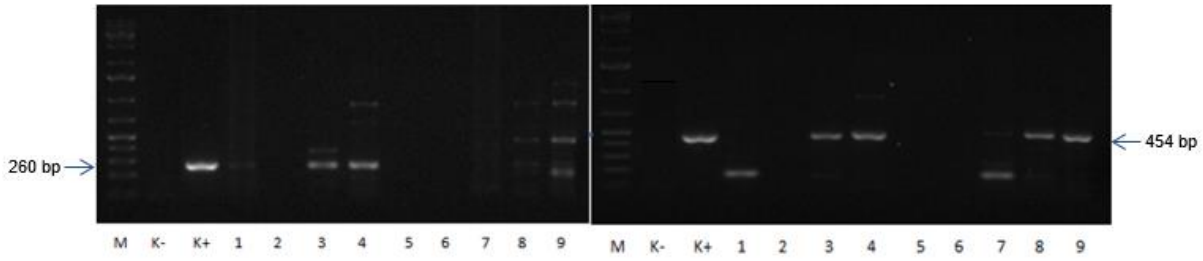

Figure 5. Electrophoregram of PCR results of suspected *Ganoderma* fungus with Gan2 primer (260 bp, left panel) and Gan3 (454 bp, right panel). PCR products of negative control (water), positive control (*G. lucidum*), isolate 12A, isolate 12A.1, isolate 05B.2, isolate 05B.3, isolate 504, isolate 11A, isolate 10A, isolate 03A and isolate 36 were loaded in the lanes K-, K+, 1, 2, 3, 4, 5, 6, 7, 8 and 9, respectively.

Table 4. PCR result of 20 isolates suspected as *Ganoderma* using Gan2 and Gan3 primers.

Isolates code	Origin of isolates	PCR result
03A	Soil	Positive
05B	Soil	Positive
9	Soil	Positive
10A	Soil	Positive
10A.1	Soil	Negative
11A	Soil	Negative
12A	Soil	Negative
20	Soil	Negative
36	Soil	Positive
545	Soil	Positive
859	Soil	Negative
663	Soil	Positive
504A	Soil	Negative
02	Root tissue remains	Negative
05B.1	Root tissue remains	Positive
05B.2	Root tissue remains	Positive
05B.3	Root tissue remains	Positive
17	Root tissue remains	Positive
19	Root tissue remains	Positive
47A	Root tissue remains	Positive

Ganoderma has a saprophytic phase in its life cycle by using C source in organic matter in soil and able to form the resting stage structure, pseudosclerotium [20]. This characteristic enables *Ganoderma* to survive for several years in the soil. Early colonization of oil palm by *Ganoderma* is through saprophytic way rather than pathogenic way [21]. It is recommended to eradicate oil palm organic matter that is contaminated by *Ganoderma* to suppress disease incidence.

4. Conclusions

Sugarcane productivity in *Ganoderma* endemic land after four years of crop rotation or up to ratoon four still high. Soil bacteria, such as phosphate solubilizing bacteria and N fixing bacteria, and soil fungi, such as ligninolytic fungi and arbuscular mycorrhizal fungi, were highly abundant in ex-oil palm land planted with sugarcane. Meanwhile, the population of other soil fungi such as *Trichoderma* sp. was rather low, ranged from 102 to 105 CFU/g soil. The results of the molecular analysis showed that after four years of crop rotation, *Ganoderma* was still found on some soil samples.

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The utilization of local chili variety of Saha Isu as toiletries in West Nusa Tenggara

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Abstract. Chili is commonly used as spice, seasoning, and could help health promoting. Interestingly, a local variety (Saha Isu) from Bima District has been preferred for toiltetries material by indigenous people because of beneficial for headache therapy. The study aimed to know the use of a local chile variety of Saha Isu as toiletries by Bimanese. This study is a qualitative research technique which was done by interview on local people/community and observation of Saha Isu variety in Mpuri Village. These chili have been cultivated from generation to generation decades ago and not distributed yet to other places. In this village, chili was used as shampoo and body scrub material by smoothing and mixing it with grated coconut and a little water. The filtered liquid and remaining dregs were used as shampoo and body scrub, respectively. Total pieces of chili used usually in odd numbers depending on to spiciness level accepted and the availability. In addition to man health, this chili is good for pregnant women and after delivering baby to help control their blood pressure. Based on morphology, Saha Isu has strong spicy-aroma of fruit, dark green young fruit and turned to orange on mature fruit. This preliminary observation need further research on biochemical compound and molecular characterization to enrich the reference about Saha Isu information This traditional knowledge of Saha Isu is important for genetic resources preservation and conservation, and should be a part of activity and responded by institutional participation in Indonesia.

Keywords: Bimanese, toiletries, traditional knowledge, local chili variety, Saha Isu.

1. Introduction

Chili (*Capsicum spp*.) is one of horticultural comodities belonging to berries, genus *Capsicum* (family: Solanaceae). Chili is important on the aspect of food and other aspect, such as for its various benefits of food spicy or seasoning, natural plant color, pharmaceutical ingredient and as sprays for riot control and self-defense. This plant species is well known for its intense organoleptic sensation of hot when consumed. Pungent flavor of chilies is due to a group of closely related alkaloid called capsaicinoids which is only found in the genus *Capsicum*. Several studies have been conducted to examine the effects of chili plants for natural analgesics. Being as medicinal plant, chili is useful to overcome pain, such as rheumatism, thrush, toothache and influenza. Many people also explored chili function for sweat laxative, skin stimulants and increase appetite.

In Indonesia, chili is the second next to nuts and having a high economic value [1]. In one of village called as Mpuri Village, Madapangga Sub-district, Bima District, West Nusa Tenggara Province, chili is used not only for seasoning/spicy on food, but also as an ingredient in toiletries, such as shampoo and body scrub. This traditional knowledge has been known and transferred from generation to generation for more than hundreds years ago. It is noted that a specifyc local chili variety commonly called "Saha Isu", is used its benefit for shampooing. Therefore, this qualitative research is important to be conducted to find out how this genetic resources Saha Isu is closely related to the local wisdom of the local community.

2. Materials and methods

All information and data collected in this study was obtained by interviewed local community and observation of the local chili variety Saha Isu in Mpuri Village, Madapangga Sub-district, Bima District, West Nusa Tenggara (NTB). Interviews were carried out using the snowball sampling method, which used a small number respondents for initial determination and larger sample size as needed. In the beginning, one or two samples of respondents who considerably know well about the issue of Saha Isu to be used as toiletries materials were chosen. If the two previously determined respondents could not provide incomplete information, then other people is considered to be more knowledgeable and can complete the data needed were chosen as. In addition, of morphological characters of the Saha Isu plant was observed in the village.

3. Results and discussion

3.1. Traditional knowledge of Saha Isu in Bima

Most people know that chili is commonly is used as a seasoning/spicy for cooking. In Indonesia, only a few people take advantage of the chili for other purpose related to human health. The indigenous people and local community of Bimanese in West Nusa Tenggara Province has a unique shampooing habit. People in other areas sometimes used natural materials such as coconut milk, olive oil, yoghurt, lemon etc. for toiletries like shampooing. While Bimanese women, particularly housewives, have used chili for shampooing their hair. This is an old habit that has been preserved from generation to generation, suggesting that the value of indigenous/traditional knowledge should be respected.

In an interview with the local people of Mpuri Village, they informed that shampooing using chili was a tradition and at the same time as the medicines for headaches, a vision problems and other aches. They prefer shampooing using this local chili instead of seeing a doctor because natural remedies are believed to be more effective and safe for long use. The tradition of shampooing using chili is maintained by Bimanese woman (pregnant woman, postpartum women, and even any women after long journey). This chili shampooing habit is not only done by women, but also by men.

In addition to chili shampooing, the Bima community is also using it as traditional scrubs from chili pulp with a mixture of various natural spices. This is thought to be influenced by capsaicin content in the Saha Isu. Capsaicin is the substance found in chili which is responsible for its spicy and hot flavor [2]. Moisture content does not play a vital role for the hotness (capsaicin content) of peppers. Capsaicin content of *Capsicum* peppers is dependent of variety and not the moisture or fleshy nature [3]. Capsaicin is effective for treating neuropathic pain and pain associated with conditions such as osteoarthritis, rheumatoid arthritis and psoriasis [4]. Capsaicin has shown potential benefits in managing headaches, including cluster and migrain headaches [5] and to treat the pain [6].

For the first time, people are reluctant using chili shampoo for not rinsing directly. The effect of hot sensation will be overcome by rinsing directly. This shampooing habit is done once every two weeks or every month. Native people reported that this habit become an addiction to them, as when they lessen the application, the disease symptoms such as dizziness and vision problem start appearing. Treatment like this is very favored by women in Bima because besides being natural, it is also less costs for treatment. Based on their experience, after shampooing and applying body scrub with chili, the body feels for more fresh and energetic like it has been injected with vitamins or analgesic

compounds. This condition might be due to the presence of antioxidants and other beneficial compounds in chili.

A number of studies have supported chili to be benefited as pharmaceuticals. Chili extract at a concentration of 0.02–2 mg/ml ethanol contains antioxidant of IC50 0.57 mg/l [7]. Total flavonoid content in *C. annum* (bell pepper), *C. annum* (chili pepper), and *C. frutescens* (chili padi) was compared, of which the highest flavonoid content among these plants were *C. frutescens* with total flavonoids of 0.551 μmol Q/g [8]. Chemical content such as flavonoids are found in extracts of chili which have antioxidant activity against free radicals [9].

3.2. Home-made shampoo and scrub from chili

Chili paste is made by grinding into fine texture and and mixed with coconut milk to make smooth texture. The chili and coconut milk mixture were put in open area overnight in room temperature and ready directly to use for shampooing. Shampooing using chili can be done before or after bathing. Before washing with fresh water, people usually allow it on their hair for next three or four days in order to get sensation of heat and positive effects. The solid waste/pulp from shampoo (chili and coconut) can be used as a body scrub. The chili body scrub is applied by rubbing the pulp on the the desired or symptom area. This is believed to reduce pain. According to Barbano et al. [4], capsaicin effects have been observed on surgical neuropathic pain, postherpetic neuralgia and chronic peripheral polyneuropathy. Bimanese local community experience that the effect of Saha Isu for toiletries is specific for each chili varieties. This information indicates that this local variety of Saha Isu may have specific compounds to allow its uniqueness and needs further exploration of its further benefits as pharmaceuticals.

Chili pepper is also known for rich in ascorbic acid content, which is very essential antioxidant for human nutrition and proper functioning of body [10–11]. Human body can not synthesize vitamin C endogenously, so it is an essential dietary component [12]. Vitamin C is essential in neutralizing free radicals in the body, assimilation of iron, healing of wounds, helps to build skin collagen and defense against bacterial and viral infection [13].

3.3. Morphological characters of chili variety of Saha Isu

In general, the plant of Saha Isu local variety has an appearance similar to the cayenne pepper (Figure 1), but one of the easiest things to distinguish it from other varieties is the fruit color. The ripened fruit is orange, and the more mature the fruit, the more intense the orange color (Figure 2). Based on Shaha et al. [14] study, the color of cayenne pepper was affected the flavonoid content, in which yellow chili has the highest flavonoid and higher antioxidant than green or red one. The morphological characters of Saha Isu is presented in Table 1. This chili is well adapted on 90–120 m above the sea level (m asl). This variety also has been registered to Center for Plant Variety Protection and Agriculture Licencing as a local variety of Bima District, West Nusa Tenggara Province. This local variety become to be recognized as a form of reliable knowledge developed through generations by native peoples in Bima with linked with their lands which have equal status with scientific knowledge.

Saha Isu is cultivated in Mpuri Village and has long been used by Bimanese indigenous people and local community. Surprisingly, the growing area of Saha Isu is limited only in Madapanga Sub-district, as a result its price is very expensive, approximately IDR 2,000.00 per 3 pieces of fruit. This variety is known to be susceptible to pests and diseases, consequently, only a few farmers cultivate it. The main reasons of farmers keep cultivating Saha Isu is in addition to increasing their income and using it for toiletries, and to preserve and conserve of local genetic resources. Thus, this indigenous and traditional knowledge of Saha Isu for toiletries should be managed as traditional resource right. The benefit of their knowledge and tradition must be gained by indigenous Bimanese community.

Table 1. Description of a local chili Saha Isu originating from Bima District.

Character	Description
Plant	
Plant height	30–50 cm
Days to flowering	40 days
Days to harvest	80 days
Stem	
Cross section shape	Cylindrical
Stem diameter	1.25 cm
Stem color	Brownish-green
Leaf	
Leaf shape	Lanset
Leaf color	Dark green
Leaf length	7–11 cm
Leaf width	2–2.5 cm
Flower	
Flower shape	Rotate
Color of petal	Green
Color of crown	White
Color of stigma	Yellowish green
Color of stamen	Yellow
Fruit	
Fruit shape	Hornshaped
Fruit tip shape	Spiky
Fruit length	4.5–7 cm
Fruit diameter	0.8–1.5 cm
Color of young fruit	Dark green
Color of ripened fruit	Orange
Fruit skin thickness	0.9–1.3 mm
Fruit flavor	Spicy
Fruit position	Hanging
Depth of grooves in the locul	Deep
Number of locul	Dominant two
Fruit base curcature	Exist
Depth of identation	Shallow
Capcaisin in placenta	Exist
Weight per fruit	1.14–2.5 g
Seed	
Seed shape	One end rounded, other end tapered
Seed color	Pale yellow
Weight of 1,000-seed	10.4 g
Thickness	1 mm
Peel color	Yellow

Figure 1. Performance of plant of Saha Isu.

Figure 2. Performance of fruit of Saha Isu.

4. Conclusions

The traditional knowledge about Saha Isu used for shampoo and body scrub could not be separated from local wisdom of the Bimanese community. This Saha Isu is a part of the preservation of potential genetic resource of local chili variety which has specific morphological characters. The knowledge of local chili variety utilization to relieve any pain symthomps and blood pressure control has been transferred from generation to generation in Bima.

5. Acknowledgements

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Genetic resources management of local tidal rice in Riau province

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Abstract. There are at least 19 major groups of local tidal rice cultivars distributed in Pelalawan District which were not pure. This study aimed to report the progress of collaboration research among Riau AIAT, Pelalawan District Government and farmers has developed *in situ* and *ex situ* conservation of the local tidal rice cultivars from the year 2007 to 2018. Positive mass selection in Karya population has resulted in KN1-79 line with a yield potential of 6.74 t/ha dry milled grain (DMG), whereas in Cekau population has resulted in C1/KB line with a yield potential of 8.60 t/ha DMG. KN1-79 and C1/KB line have been released as Karya Pelalawan variety and Cekau Pelalawan variety, respectively. Both varieties have been registered by Center of Plant Variety Protection. The drawback of these local cultivars is late in maturity. Karya Pelalawan was crossed to Fatmawati and produced varieties Bono Pelalawan and Mendol Pelalawan which have early maturity character. Cekau 34 was crossed with Cisantana and produced Inpara Pelalawan with new characters such as early maturing, aromatic grain and tolerant to iron toxicity and salinity. Participatory plant breeding has promoted the released varieties to be widely and rapidly distributed as well as easily accepted by farmers. By promoting the ownership of local cultivars to the local government, local cultivars could be as a regional identity. Thus, it has been shown that Pelalawan District Government was actively involved in the development of released cultivars derived from local rice cultivars.

Keywords: genetic resources, genotype, local rice, tidal, participatory plant breeding.

1. Introduction

The decrease in land area for food crops due to land conversion to non agricutural purposes and the low productivity in Riau Province has become a strategic issue nowadays and in the future. Along with the conversion of land functions, local genetic resources of food crops from growing centers are also lost. The Government of Pelalawan District in Riau Province anticipates the decrease in rice production by prioritizing the increase in rice productivity and self-sufficient of rice seeds. Local government efforts to support food self-sufficiency include: (1) establishing rice development areas, (2) purifying and developing local cultivars with high yielding ability, (3) developing local-specific

early maturing lines to increase cropping indexes (CI), (4) developing seed production systems and foster breeders, and (5) establishing food crop management units to conserve genetic resources and produce quality seeds.

About 80,000 ha of tidal rice fields in Riau Province are planted with diverse tidal rice cultivars. Two popular cultivars, Cekau and Karya, which were found in Pelalawan District and released in 2011, are grown once a year. In general, the limitation of once-a-year crops in tidal land is caused by land and climate constraints.

Some of the local varieties have good taste and rice quality according to the local preferences. Local varieties are known to have several important traits for rice improvements such as tolerance to biotic stresses (pests and diseases) and abiotic stresses (flood, drought, Al- and Fe- toxicity, high temperatures and salinity). These traits are only a few positive traits presence in the local varieties. Therefore, the use of local varieties as gene donors in breeding program is highly recommended in order to get superior genes that are local specific and to expand the genetic background of the new developed varieties.

Early maturing and high yielding varieties can change the tidal rice farming system in Riau Province which is dominated by CI 100 to CI 200. One planting season for local cultivars takes 6–7 months, which is expected could be replaced by new early maturing varieties.

To improve the character of local tidal rice and conserve the genetic resources of rice, the Riau Assessment Institute of Agricultural Technology (AIAT) and the Pelalawan Regency Government have carried out the purification of local cultivars and a breeding program. This study aimed to report the progress of research collaboration between Riau AIAT with the local government in the management and development of rice local genetic resources from 2007–2018.

2. Materials and methods

The research was carried out in 2007–2018 in Pelalawan District. A survey was first conducted to record the distribution of local cultivars. All types of rice grown by farmers were collected, observed and purified. Thirty local tidal rice cultivars obtained from the survey were selected and sown in the following experiments.

Seedlings at 21-day-old after sowing were transplanted at a distance of 20 cm × 20 cm with one seedling per planting hole. Basic fertilizers (200 kg/ha urea, 100 kg/ha TSP and 100 kg/ha KCl) were applied at 3 days after transplanting (DAT). The first supplementary fertilizer (50 kg/ha urea and 50 kg/ha KCl) was applied at 30 DAT and the second (50 kg/ha urea) was applied at 60 DAT. Pesticide and herbicide were applied as necessary using the recommended procedures.

Varietal purification was done in the first planting season by positive mass selection method. Panicles from selected rice clumps were advanced in the second planting season by planting one panicle per row. Negative mass selection was applied to these crops. Selected families from these plants were advanced to the next cropping season for seed multiplication.

Two local cultivars (Cekau and Karya), a new plant type variety Fatmawati and a national variety Cisantana were used in cross breeding. Single crosses between Karya × Fatmawati and Cekau × Cisantana were made. The offsprings of these crosses were selected using the pedigree method. One seed of each line was sown per planting hole at a distance of 20 cm × 20 cm. Plants were fertilized with 250 kg/ha urea, 100 kg/ha TSP and 100 kg/ha KCl. The variables observed were plant height, plant age, number of productive tillers, number of filled grains per panicle, number of empty grains per panicle, 1,000-grain weight, yield potential, percentage of broken rice husk composition, percentage of amylose content, resistance to pests and disease and tolerance to iron toxicity and salinity. Resistance to brown planthopper was evaluated based on the Standard Evaluation System (SES) of IRRI [1]. Plant responses to bacterial leaf blight disease were assessed based on the proportion of infected leaf area to the total leaf area according to the SES of IRRI [1]. Analysis of broken rice rendement was done according to SES of IRRI [1].

Evaluation of iron toxicity tolerance was carried out at Taman Bogo Experimental Station, East Lampung, Lampung, from June to September 2016. The land used for the trial was a rice field with

high Fe content (300–400 ppm). Mahsuri and IR64 were planted as tolerant variety and sensitive check, respectively. Seeds were sown until 21-day-old and then transplanted. One seedling of each line or variety was planted in 5-m rows at a planting distance of 20 cm × 20 cm. Fertilizers were applied twice: the first was at planting time with 75 kg/ha of urea, 100 kg/ha of TSP and 100 kg/ha of KCl; the second was at 4 weeks after planting with 75 kg/ha of urea. Iron toxicity tolerance was assessed using the SES of IRRI [1].

Salinity tolerance was evaluated by using hydroponic media (Yoshida solution) containing 120 mM NaCl or Electrical Conductivity (EC) + 11 dS/m. The SES of IRRI [1] was used for evaluation of salinity tolerance. While the process of purification, crossing and testing of lines was carried out in a participatory manner by involving farmers, local government, Riau AIAT and Institution for Seed Certification. Conservation of the genetic resources is carried out both *in situ* and *ex situ*.

3. Results and discussion

3.1. Collection, characterization, and purification of local rice varieties

For decades, new national varieties have not been developed. To date, the dominant cultivars grown by farmers are local variety Cekau and Karya. There were 30 cultivars that have been characterized, of which ten are cultivars grown by farmers until 2018 (Table 1). The remaining 20 cultivars were not found during the survey but were still collected at the Indonesian Center for Rice Research (ICRR) and Riau AIAT. These 30 cultivars can be categorized into 19 main groups.

Table 1. Partial list of local cultivars recorded in rice planting areas in Pelalawan District during a survey in 2006 to 2012.

Name	Age (month)	1,000-grain weight (g)	Yield (t/ha)	Planting area (ha)									
				2006	2007	2008		2009		2010		2011	2012
				S1	S1	S2	S1	S2	S1	S2	S1	S1	S1
Pulau Kijang	5	24.2	2.5	7	1	13	-	-	-	-	-	6	11
Sardani	4	23.3	3.3	2	1	-	-	-	-	-	-	50	60
Pulut Belanda	5	25.7	3.1	5	5	5	-	15	-	15	-	5	4
Pulut Hitam	6	24.5	2.8	13	17	20	-	20	-	20	-	20	30
Lembuk Sawah	4.5	22.0	3.1	30	10	20	-	34	-	26	-	21	14
Pasir	5	26.4	3.0	30	20	20	-	6	-	6	-	11	8
Cekau	5	24.5	3.8	3.5	3.5	3.7	-	4	-	4	-	4	4
Ketek Putih	6	23.3	3.5	20	50	50	-	27	-	33	-	29	25
Karya Rendah	5	20.7	2.7	200	200	200	-	200	-	200	-	100	100
Karya	4.5	23.1	2.8	4	4	4	-	4.5	-	4.5	-	1.5	1.5

S1 = planting season 1, S2 = planting season 2, - = data not available.

New high yielding varieties is favored by farmers in less than ten years after introduction because purified local varieties have gained popularity and shifted many local varieties. According to Oko et al. [2], new varieties should not replace local cultivars because most local cultivars have several advantages. Natural selection has formed local cultivars to have ecological adaptation and thus it may have more advantages than cultivars which are artificially selected by human. Therefore, farmers must be critical in accepting new varieties that may not be holistically comparable.

3.2. Purification of Karya and Cekau cultivars

Tidal rice farming in Riau Province is still dominated by late maturing local varieties which permit only once a year planting season. These local varieties have low to medium productivity and usually comprised of mixed lines. The survey carried out in 2007 had collected 30 types of rice in Pelalawan District. All of these local rice were not pure. The highest impurity was found in Karya and Cekau cultivars with an impurity rate of 78% and 57%, respectively. The level of line mixture in Karya and Cekau were characterized based on the yield and several criteria of phenotypic traits such as stem, flag leaves and tolerance to diseases. Karya cultivar was found to be more heterogenous than Cekau cultivar. It was consisted of 20 different lines, of which 50, 15.4 and 34.6% were worse than, almost equally the same as, and better than Karya in performance, respectively. An equal rate of lines with worse and better performance than Cekau was observed within this population.

Table 2. Agronomic performances of ten lines selected and purified from Karya cultivar.

Genotype	Plant height (cm)	Number of productive tillers	1,000-grain weight (g)	Number of filled grains/ panicles	Number of empty grains/ panicles	Yield (t/ha)
Karya (original population)	167	9	20.45	125	45	3.50
K5	140	11	17.62	255	45	6.18
K5K	148	13	20.42	264	41	7.86
K3A	160	9	22.61	185	15	4.72
KB	166	6	17.80	102	33	1.36
KG	160	9	23.97	201	77	5.42
KM	140	17	21.17	115	14	7.12
KN	170	14	19.46	168	30	5.73
KP	170	13	25.22	156	22	6.42
KR	162	5	19.53	78	33	0.95
KN1	160	13	24.51	169	45	6.74

Impure varieties that have been planted for decades can result in high genetic variability. Significant differences in several characters among cultivars generate high genetic variability [3]. Ten selected genotypes derived from 25 genotypes which are purified from Karya cultivar are presented in Table 2. Five genotypes, namely K5, K5K, KM, KP and KN1 had higher yield potential (>6 t/ha) than the original Karya (3.5 t/ha). Similarly, the selection from Cekau population resulted in three potentially high-yielding genotypes, namely C1/KB, C3/KB and NN1 with a yield potential of 8.60, 7.19 and 8.54 t/ha, respectively (Table 3).

According to Fujino et al. [4], repeated planting of local varieties in unique environmental conditions will produce information on their potential genetic and will be useful as genetic resources in local breeding programs. Selection in diverse local cultivars is based on high yield potential. According to Oladosu et al. [5], differences in the yield character play an important role in the development of rice varieties.

3.3. Crossing of local rice cultivars

One of the obstacles in the rice farming in tidal land is the difficulty in increasing CI due to the narrow planting season, which is largely affected by rainfall and pest infestation. Regular planting season takes place 7 months from August to February (7 months), and hence late maturing local varieties (6–7

months) are planted. One strategy to increase CI is to grow early maturing varieties so that the planting season can be carried out twice within 7 months.

Table 3. Characters of eight lines selected and purified from Cekow cultivar.

Genotype	Height (cm)	Number of productive tillers	1,000-grain weight (g)	Number of filled grains/ panicles	Number of empty grains (panicles)	Yield (t/ha)
Cekau (origin population)	167	10	23.24	198	26	4.20
C/KB	134	11	26.44	100	12	3.64
C1/KB	142	12	31.87	180	35	8.60
C3/KB	149	12	23.99	200	41	7.19
C5/KB	123	7	22.77	76	17	1.51
C6/KB	145	9	29.62	189	32	6.29
C7/KB	141	10	23.98	120	14	3.59
C8/KB	142	6	28.68	114	29	2.46
NN1	167	12	23.03	247	51	8.54

The puposes of the crosses of Pelalawan's local rice cultivars to national released varieties were to shorten the maturity of local rice, increase its productivity without changing too much the good characteristics and the rice taste already preferred by farmers and local consumers. Two crosses were made, i.e. Karya × Fatmawati and Cekau × Cisantana. Fatmawati is a new plant type (NPT) which has a big panicle and high 1,000-grain weight (30.6 g) [6]. According to Khush [7], NPT of rice have compact growth, 8 to 10 productive tillers, large panicles with 200 to 250 filled grains, medium height (semi-dwarf) and upright, have thick leaves with dark green color, deep roots, early maturing and resistant to pests/diseases. In addition, Peng et al. [8] stated that NPT must have few number of tillers but almost every of it are productive, plant height of 90–100 cm, age of 100–130 days, and also thick and strong stems. The cross of Karya × Fatmawati produced Bono Pelalawan and Mendol Pelalawan varieties, whereas the cross of Cekau × Cisantana produced Inpara Pelalawan. Bono Pelalawan variety inherits several characters of Fatmawati such as upright and broad leaves, large panicles, high number of productive tillers (10–12) and big grains (Table 4).

Bono Pelalawan, Inpara Pelalawan and Mendol Pelalawan have been released by the Ministry of Agriculture of Indonesia in 2017. The three varieties represent the majority of consumers' preferences in Indonesia. According to Custodio et al. [9], rice breeding programs must pay attention to farmers' preferences such as tenderness in Southeast Asia and slenderness in South Asia, when considering specific preferences. The results of the crosses showed a gain of advantage in the quality of rice and resistance to salinity stress. These results indicate that breeding practices have created new genetic structures for adaptability to specific environmental conditions and breeding goals [10].

Table 4. Description of three new varieties produced from local genetic resources.

Parameter	Mendol Pelalawan	Inpara Pelalawan	Bono Pelalawan
Origin of cross	Karya/Fatmawati	Cekau 34/Cisantana	Karya/Fatmawati
Plant age (day)	100	105	106
Plant height (cm)	129	116	139
Number of filled grains per panicle	264	280	275
Number of productive tillers	14	12	12
Flag	Somewhat erect	Erect	Erect
Yield potential (t/ha)	8.0	8.20	7.9
Yield average (t/ha)	7.30	7.45	7.3
1,000-grain weight (g)	25.4	27.4	27.6
Rice texture	Glutinuous	Non-glutinuous	Glutinuous
Broken rice husk composition (%)	77.64	79.93	76.56
Amylose content (%)	19.25	25.25	19.22
Other information	Suitable for planting in tidal land types of overflow B and C	Tolerant to Fe (iron) poisoning and salinity	Rather tolerant to Fe (iron) poisoning and salinity

3.4. Participatory plant breeding with locality government and farmers

The low commitment of local government and the change in policies during the period of regional autonomy have constrained the development of food crops in Indonesia. Therefore, local governments must be persuaded to manage their genetic resources and their successful stories in this effort can be a good model for other local governments. Pelalawan Regional Government has given serious attention to the development of food crops and the conservation of plant genetic resources by financing various research activities. These activities included purification of local varieties, development of a new variety through crossing schemes, releasing varieties and conserving genetic resources.

The success of research activities depends on the willingness and readiness of the local government. While most local governments only make use varieties that are provided or produced by national commodity research centers, the Pelalawan District government took more initiatives by developing own varieties and conserving the genetic resources presence in the region. Several strategies were implemented to stimulate the interest of the local government in the use and conservation of their genetic resources, i.e. (1) introducing the superiority and the weakness of their local cultivars, (2) informing the benefits obtained through improving genetic character of local cultivars, (3) informing the opportunities and limiting factors in the rice farming system in the region, (4) informing the research activity progress on regular basis, and (5) increasing their awareness and enthusiasm in the research activities.

Participatory plant breeding can increase the enthusiasm of farmers and local government in breeding process although it can take several years. Through participatory plant breeding, the sense of belonging and sense of responsibility to conserve and developing the lines and varieties they create can be expected. Farmers and Agriculture Service Staff in Pelalawan were actively involved in the selection process of the lines.

Plant breeding programs need to involve a variety of stakeholders in capturing the variety with desired characters in the process of the development of varieties. In participatory plant breeding,

adoption begins during the selection process before the release of varieties. Another difference between participatory breeding programs and conventional breeding is the increase in agrobiodiversity. Biodiversity is higher in the former program because of the rapid changes in the varietal testing locations and the seed accessibility of the new varieties to farmers, which in turn can contribute significantly to food security [11].

Varieties tested in experimental field without involving farmers as partners in evaluating varieties may lead to low adoption rate of varieties. Farmers are the end-users of various varieties produced so that the decisions by farmers when assessing varieties must be considered. By doing so, the selection process is more effective and adoption soon expands [12].

3.5. Fostering seed breeders and development of breeder seeds

The use of high-quality seeds is the initial stage of successful farming. In 2008–2011, one farmer group has been fostered as a seed breeder. Since 2013, the farmer group has developed and distributed candidates of varieties among the members. The breeder seed development program was a collaboration project among breeders, local government and institution for Seed Certification (Table 5). In 2017–2018, there were two active farmer groups that breed local varieties and their essential derivatives with the support from the local government.

Table 5. Breeder seed development programs.

Executor	Activity	Responsibility
Breeder Seed Technical Implementation Unit (UPBS) and seed breeder	Producing nucleus seeds (NS) and breeder seeds (BS); planting BS, foundation seed (FS), stock seed (SS), and extension seed (ES)	Collaborating with the Seed Certification Center and Riau AIAT
Farmers	Planting ES to produce rice	Dealing with rice traders

To support the program of developing high-yielding variety seeds and conserving genetic resources, the Government of Pelalawan District established a Seed Technical Implementation Unit in Mendol Island. Thus, the need for seeds for agriculture on remote islands can be met.

In 2017, the breeder seeds (BS) produced through crossing varieties were planted in 2-ha rice field and in 2018 foundation seeds (FS) were planted in 40 ha rice field. In addition to being reproduced and conserved in their origin (*in situ*), local varieties and new developed varieties were also planted in farmers' rice field in Siak and Meranti Islands District (*ex situ*). *Ex situ* conservation usually involves breeders and cooperative farmers or research partners. Some breeders provide some of their rice fields to be used for growing local rice genetic resources.

Ex situ conservation have been proved to stimulate the adoption of varieties by local farmers. Farmers who are interested in varieties at *ex situ* conservation sites have selected several varieties and developed them independently. The most widely adopted varieties are Inpara Pelalawan, Karya Pelalawan, Cekau Pelalawan and Bono Pelalawan. Zhu et al. [13] stated that *in situ* conservation is an important complement to the *ex situ* conservation of local varieties. Management of mixed cropping (intercropping) of traditional and hybrid rice varieties in China has dramatically increased the cultivation of traditional rice varieties [13].

4. Conclusions

Selection and purification of two local rice varieties Cekau Pelalawan and Karya Pelalawan has resulted in new variety Bono Pelalawan, Mendol Pelalawan and Inpara Pelalawan. Pelalawan District Government has been actively involved in the development of those released cultivars and also collaborated with Riau AIAT in the *in situ* and *ex situ* conservations of local rice genetic resources. Participatory plant breeding program involving local government and farmers has stimulated rapid distribution and adoption of released varieties by farmers. Among the strategies implemented to raise

the interest of local government in funding the research activities were suggesting the local government to obtain the ownership rights of local cultivars and make them as the regional identity, informing the progress of research activities periodically and highlighting the advantages of the research results.

5. Acknowledgement

The authors would like to thank the Pelalawan District Government for funding the research activities.

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Current status of Aceh jernang (*Daemonorops* sp.) and its traditional conservation efforts

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Abstract. Dragon's blood or 'jernang' (in Indonesian language) is referred to the deep and bright red resin obtained from the seeds of rattan palm (*Daemonorops*). Three species of this genus, i.e. *D. draco* (Willd.) Blume, *D. didymophilla* and *D. Micracantha*, are highly value commodity due to their larger fruits and longer fructescences. Dragon's blood has been known in the traditional ancient Chinese medicines as haemostatic agent, antidiarrheal, antiulcer, antimicrobial and anti-inflammatory. Particularly the dracorhodin, a valuable bioactive substance, is closely associated with antitumour and wound healing activity and other industrial need. The distribution of *Daemonorops* is limited to Malaysia, Thailand and western Indonesia, especially in Sumatra. Harvesting *D. draco* seeds has been served as traditional livelihood for some local tribes and farmers in Aceh Province. Resin extracted from Aceh jernang is classified as excellent due to higher content of dracorhodin, but its processing method is still conducted in a very traditional way. The forests in Aceh are suitable for jernang to thrive, but jernang population is decreasing due to deforestation and inappropriate harvesting. Unless conservation efforts are done, jernang might extinct in the near future. Over the past few years, some local farmers have realized the importance of jernang in their livelihoods, and hence they take part in the conservation. This paper dealt with the efforts between a local jernang company, CV Draco Industrial Agribusiness (DIA Group), with Unsyiah in the traditional jernang conservation program.

Keywords: dragon's blood, genetic diversity, non-timber forest product (NTFP).

1. Introduction

Dragon's blood is referred to the deep and bright red resin obtained from the seeds of rattan palm *Daemonorops* as well as from other three distinct plant genera. These genera are (i) *Croton* spp. (Euphorbiaceae), whose dark red sap, known as *Sangre de Drago*, is obtained by cutting the tree trunk. They are mostly grown in Mexico, Venezuela, Ecuador, Peru and Brazil; (ii) *Dracaena* spp., which are mainly distributed in Africa, particularly in Yemen, Somalia and Canary Islands, produce *Dracaena* resin; (iii) *Pterocarpus* spp. or the red sap originated from *Pterocarpusdraco* L., which is mainly found in Jamaica. The name of dragon blood is depicted from an ancient Greek mythology called

'Indian cinnabar' dated back in the first century AD, while other source mentioned from an Indian legend describing a struggle between a hundred-headed dragon and an elephant, which resulted the mixing of the two creatures' blood with a magical substance and is attributed with strong medicinal properties [1]. Dragon's blood was originally produced from *D. cinnabari*, later on from *D. draco*, and recently from *Daemonorops* spp. as some of *Dracaena* sp. like *D. cochincinensis*, which usually inhabits the sunny cliffs with an elevation of 1,300–1,700 m asl is currently in extinction. In China, there are approximately 200,000 of *D. cochincinensis* left in the wild. Thus, since 1987 they have been put in the list as a national endangered species due to over exploitation or excessive collection [2].

Rattan is one of the most economically important non-wood forest products in South East Asia, and mainly used in the construction of diverse furniture as they are well-known for their strength, durability, forming elasticity and lightness. There are 115 species in the genus of rattan palms of *Daemonorops* sp. or 'jernang', although only 10% of them produce the red resin. *Daemonorops draco* (Willd.) Blume, *D. didymophilla*, *D. micracantha*, *D. motleyi*, *D. rubra* and *D. propinqua* are considered as the valuable sources. Particularly, the first three species are the most economically important due to their larger fruits and longer fructescences [1]. In South East Asia, the trees are distributed exclusively in Malaysia, Thailand and Indonesia. In Indonesia, the trees mostly grow well only limited to the western part of Indonesia including in the tropical rain forests of Sumatra, such as Aceh, Jambi, Riau and Bengkulu, and Borneo Islands [3].

In general, its application has been widely known in the traditional ancient chinese medicines for centuries as haemostatic agent, antidiarrhetic, antiulcer, antimicrobial and as anti-inflammatory (Figure 1). Particularly the dracorhodin, a natural biocompound which belongs to the anthocyanin family and distinguished as the valuable pharmacological substance for its antitumour and wound healing activity [2,4]. Besides, it is also applied in artistic applications in most of European churches in the 16th centuries as reverse glass paintings or so called *Hinterglasmalerei*; varnishing, or as red lacquer polish in most of expensive art works of furniture, violin or ceramics. It has also been applied as natural dyeing for clothes by some indigenous tribes of Borneo for its vivid color [5,6].

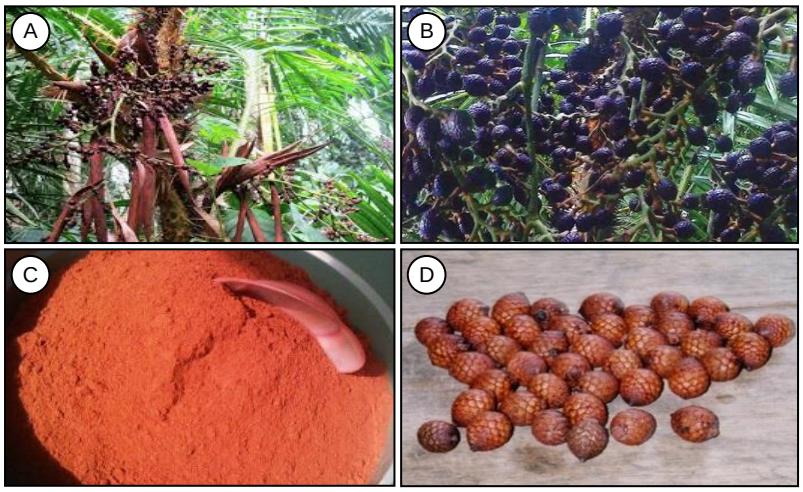

Figure 1. Performance of jernang (*Daemonorops* sp.). (A) Tree of jernang. (B) Jernang fruits at the tree branches. (C) Harvested fruits. (D) Jernang powder or dragon blood as primary raw materials applied in pharmacy and industry (DIA Aceh 2018).

Harvesting the seeds of *D. draco* has been served as traditional livelihood for some local tribes, *Suku Dayak* (in Borneo) and *Suku Kubu*, *Anak Dalam*, *Talang Mamak* and *Melayu Tua* mostly on Jambi Province (in Sumatra), and also regarded as quite profitable additional income for some local farmer. Its economic importance, however, has been realized more and more to a wider community

and not only limited to the local tribes mentioned above. An informal source mentioned that a local farmer could earn over IDR 700 million (local currency) or USD up to 45,000 per year on two hectares land. Such high income could be achieved as the plants do not require much agricultural inputs or special care in general.

The soil composition and physical environment of Aceh forests suit well for jernang to thrive. Therefore, jernang is regarded as one of valuable non-timber forest products (NTFPs) and admitted and classified as 'excellent' due to the extraordinary amount of dracorhodin (personal communication with F. Ismullah in 2018). Nevertheless, its resin extraction processing or methodology is still conducted in a very traditional way. Local farmer or tribes, principally, harvest the seeds in the pristine forest by picking them up with a special 'hook' or cutting down the branches solely instead of the 'whole tree' as they used to conduct it in the past. During the process, fruits are being separated physically, mixed with a special liquid from ethanol based, and then cooked for about one hour. After that, the red dragon's blood resin obtained, is then dried under the sun after the removal of foreign matter, and formed into teardrop-shaped lumps (Figure 2).

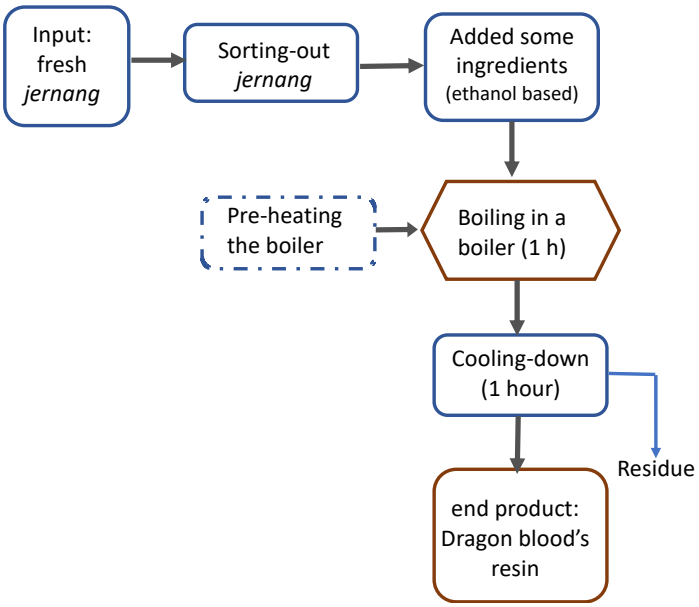

Figure 2. Flow chart of jernang processing (DIA Aceh 2018).

2. The economic importance of jernang

No exact data of exported number of jernang from Aceh has been collected as most of farmer do not consider it as main income activity and many small companies are involved in the supply chain. Nevertheless, it is being estimated from an internet-based source that up to 5,000 kg of raw fruits are harvested monthly. Up to 400 tones of harvested fruits are further traditionally processed per year with a fixed selling price of only IDR 300,000–500,000 (USD 20–34)/kg. In contrast, the price of jernang resin in powder form (Figure 1D) or already processed could reach up to 10 times higher; depending on the quality of dracorhodin the price is within the range IDR 2.5–5.8 million or USD 167–387. For two decades, they are mainly exported to Singapore and China. The prices in the form of red powder could increase sharply if supply is limited. In a lower quality, a fixed price ranging from IDR 550,00−650,000 (USD 37−43) per branch of jernang is also being traded. A branch bearing many jernang fruits are also sold to local companies, which collect the fruits and further process them. One of them is CV Draco Industrial Agribusiness (DIA Group) which was founded and owned in 2014 by the second author (http://djpen.kemendag.go.id/-membership/index.php/frontpage/product_detail/0/2498). This company is located in Ulee Kareng, Aceh Besar and has been exporting up to 125

kg monthly or only a quarter of the total demand (500 kg). In order to reduce the supply gap, the company has set up its own nursery for growing seedlings and then sell the young plants to local farmers.

As jernang has become rare, the supply could only reach 10% from the market demand. Moreover, the common extraction method still needs to be enhanced in order to achieve a better quality by reducing the water content and at the same time increasing dracorhodin concentration in the powder (DIA Aceh 2018). Therefore, DIA Group has initiated a collaboration with the Faculty of Agriculture, Unsyiah which serves as a think-tank, provides the technical aspects' supports, e.g. analyzing the powder quality, including the interpretation of results delivered by the chromatography.

3. Conservation efforts

So far, many local tribes and farmers have only search and find jernang in the jungles. However, the population of jernang trees in the jungle has been decreasing due to deforestation or forest land use changes for other purposes. Thus, active harvesting has been not yet fully supported with proper practices because of wrong, general assumptions hold: (a) it is abundantly present, and therefore, people tend to destroy other neighboring plants when searching the plant deep in the forest, (b) it is naturally grown in the 'wild' and cannot be propagated and cultivated, and (c) the individual male trees are often considered as useless and should be completely cut down. This has made female plant difficult to propagate. Consequently, such inappropriate practice would lead to low genetic diversity in *D. draco* [3]. Unless significant conservation efforts being initiated, this plant might face extinction [6]. Over the past five years, many local farmers have initiated to purchase jernang seedlings from local breeder. The demand is relatively high as one farmer might need seedlings to be planted over a land, which is mostly owned by themselves, ranging from 10 up to 30 hectares.

As it was being highlighted before, DIA could only fulfill a quarter from the total demand inclusive from Aceh, which is about 500 kg monthly. In order to fulfill this, DIA has built partnership with some local farmers located in Aceh Jaya, Singkil and Aceh Tengah. The various aspects related with conservation efforts that have been managed are:

i) Provision of small young plants to farmer with a lower price (IDR 30,000 instead of IDR 40,000).
ii) Providing the farmer with a basic know-how in producing seedlings, this includes sharing the basic knowledge of artificial propagation techniques via plant tissue culture mostly from the root parts. The Ministry of Forestry in Jakarta through a program named 'Rumpin' has been initiated providing farmer with free seedlings started from last year.
iii) Active campaign the importance of jernang's conservation linked with farmer's income. Approximately 20 farmers, who previously grew oil palm from Aceh Jaya and Singkil, have earned a higher income from jernang compared to oil palm. They are benefitted from more rapid revenue, that can be earned after 5–7 years waiting; less money spent for purchasing fertilizer and experienced less floods.
iv) Active networking with a higher level such as: (a) lobbying the head of sub-district (*bupati*) of Singkil, for proposing *hutan di luar kawasan* with an area over 50 hectares along the local communities. This semi-protected forests will serve as legitimized conservation or sanctuary for jernang and other valuable wood trees, (b) actively involved in the Forum Group Discussion (FGD) in planning the conservation efforts with the National Ministry of Forestry, and (c) actively joining the discussion with the Ministry of Industry regarding the determination of national product standardization (SNI) of dragon blood's powder ready for exports starting from 2018.

These efforts are conducted with the intention that jernang can be further conserved and regarded as one of the most significant income source for local tribes and farmer.

4. Recommendation to stakeholders

Not all regions in Indonesia are the habitats of jernang. However, the plants are endemic in the seven regions of Aceh, namely Aceh Besar, Aceh Jaya, Southwest and South of Aceh, North Aceh, East Aceh, Aceh Tengah and Singkil (a sub-district located at the border between Aceh and Sibolga in

North Sumatra). Species like *D. draco* and *D. didimophylla* are mainly found specifically in Aceh Jaya and Singkil, respectively. These seven regions, particularly the highland regions of central Aceh in Gayo, are ecogeographically suited well with jernang's plant habits. Usually, the plants are found deeply in the forest in a cluster, and they are not evenly distributed. Over the past five years, the plants have become rare due to forest encroachment and diminishing area of jernang habitats in the forests [7] and these have led to the sharp increase of its price particularly, at the exported destination countries, China and Singapore.

This paper has briefly highlighted how local farmer harvest and conduct the processing method especially in Aceh. Here, we can conclude that most of the methodologies applied are very traditional and have not yet applied high-technology equipment in order to optimize the process as there have been no legal and relevant Standard Operational Procedure (SOP) applied. Moreover, it also mentioned the traditional three party conservation efforts of Aceh jernang, particularly in the provision of seedlings, technical supports by planting and nursery with various farmer groups across Aceh; coordinated and managed by DIA.

In the future, genetic conservation both morphologically or DNA-based marker of local jernang endemic to Aceh forests should be started immediately prior to their extinction. Moreover, an enhanced extraction method by applying advance separation techniques such as spectrophotometric based [8] and ultracentrifugation of harvested fruits, which enables higher yield and purity should be further promoted, especially in the form of competitive grant for local entrepreneur. Further, brand protection in the form of intellectual property right during the patented method should be also secured. Thus, this action should be also supported not only at the community level, but also from multi-stakeholders (higher education, researcher, local authorities and related governmental bodies).

5. Concluding remarks

The information of the current status of Aceh jernang (*Daemonorops* sp.) and their economic importance both to indigenous tribes and local farmer, as well as to local company such as DIA described in this paper will be useful for not only researchers and university academics, but also relevant stake holders. Despite of its positive contribution by the livelihood, unfortunately, this is not accompanied either with an enhanced processing methods in order to achieve higher product quality, noted with higher dracorhodin amount or well-managed conservation efforts for a long term. Some common practices that might worsen the existence of Aceh jernang have been also high-lighted.

6. Acknowledgement

The authors extend their gratitude to jernang farmer community in Aceh Province, who were actively involved with DIA's activity.

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The use of specifically adapted genetic resources for regional economic development

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Abstract. Genetic resources are essential sources for further varietal improvement as well as directly used of their products due to their prime quality product in a particular region but not in other regions. Genetic resources grown for some time in a region probably have been improved their adaptability to the environment. The resourses could be used to develop new varieties adapted to a specific location. If every region has that kind of variety, it will, in turn, improve national productivity of that commodity. Besides, genetic resources having specific adaptability to a specific location and producing a prime quality needed by the consumer may also be useful to boost the economy of that particular region. This kind of products is generally known as the Geographical Indication (GI) products. A GI must identify a product as originating in a given place. Examples of products protected with GI that improve the local economy are: (1) GI Kintamani arabica coffee which before the protection involving 40 groups of farmers (1,750 workers), the price of coffee IDR 25,000.00/kg. After the protection, the farmers' groups become 60 involving 2,640 workers and the price increase to IDR 75,000.00/kg, (2) Muntok white pepper, the price after protection is IDR 170,000.00/kg, whereas the price before protection was IDR 40,000.00/kg. The use of genetic resources in varieties improvement required a tremendous amount of effort and funds, while GI required effort in maintaining quality of products and funds for the promotion of product.

Keywords: genetic resources, plant breeding, geographical indications.

1. Introduction

Genetic resources are essential components for the development of new plant varieties to meet the growing population's demand on their need. However, in the era of globalization, the availability of plant genetic resources is getting scarce due to the fact that the reduction of fertile agriculture land is getting greater than the population increase.

The International Treaty on Plant Genetic Resources for Food and Agriculture (IT PGRFA) is an international legally binding agreement, in harmony with the Convention on Biological Diversity, to guarantee food security through the conservation, exchange and sustainable use of plant genetic resources for food and agriculture (PGRFA), as well as the fair and equitable sharing of benefit arising from its use. The Treaty also recognizes the right of farmers to get protection on their traditional knowledge relevant to plant genetic resources for food and agriculture; participate in sharing benefit-sharing arising from the use of plant genetic resources for food and agriculture; in making decisions, on matters related to the conservation and sustainable use of plant genetic resources for food and agriculture. The Treaty establishes a Multilateral System (MLS) of Access and Benefit-Sharing (ABS)

to facilitate plant genetic resources exchanges and benefit-sharing through a Standard Material Transfer Agreement (SMTA). Besides, there are available genetic resources found in specific location producing products of premium quality resulting in high price can also be used to improve farmers' income due to high demand.

2. Genetic resources

Genetic resources refer to the genetic material of actual or potential value. Genetic material is any material of plant, microbial or other origin containing functional units of heredity. PGRFA are plant genetic materials of actual or potential value for human livelihood, and may include the entire generative and vegetative reproductive material of species with economic and, or social value, especially for the present and future of agriculture, with special emphasis on nutritional plants. It may also be defined as the diversity of material contained in traditional varieties and modern cultivar as well as crop wild relatives and other wild plant spesies that can be used now or in the future for food and agriculture.

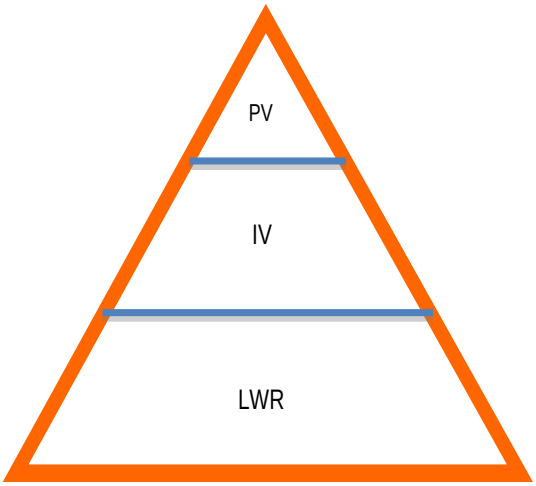

Figure 1. Forms of Plant Genetic Resources: protected varieties, improved varieties, and landraces and wild relatives.

Genetic resources can be grouped into:

1) Protected varieties
   Genetic resources belong to this group consisting new plant varieties eligible for protection. The plant variety must be distinct, i.e. should be distinguishable by at least one essential characteristic from existing or commonly known varieties in any country at the time of filing of the application. It also must be sufficiently uniform in its essential characteristics; which must be stable after repeated propagation.
2) Improved varieties
   Improved varieties or cultivated variety, refers to an assemblage of plants selected for desirable characters that are maintained during propagation. Most cultivars arose in cultivation, but a few are unique selections from the wild. In the International Code of Nomenclature for cultivated plant, cultivar is the most basic catgory.
3) Landraces and wild relatives
   Landraces crop cultivar or animal breed that has been developed through traditional farming practices for many years in aparticular locale without influence from modern agricultural science. A crop wild relative (CWR) is a wild ancestor of the domesticated plant, or another closely related taxon.

3. The use of genetic resources

As shown in Figure 1 three forms of genetic resources that may be used directly or indirectly to develop new plant variety, which, in turn, provide a source of income not only to the developer but also regional economy.

The Convention on Biological Diversity (CBD), which under certain circumstances refers to genetic resources, recognizes the need for equitable sharing of benefits arising from the use of traditional knowledge, innovations and practices relevant to the conservation of biodiversity/genetic resources and the sustainable use of its components.

Access to genetic resources, therefore, should be coupled with equitable benefit-sharing in order to realized the objective of the CBD. Adoption of valid prior informed consent (PIC, Figure 2) procedures in both provider and user countries have a crucial role to play in achieving realization of the CBD's objective of ensuring equity and fairness in benefit-sharing; and in consolidating international ABS governance.

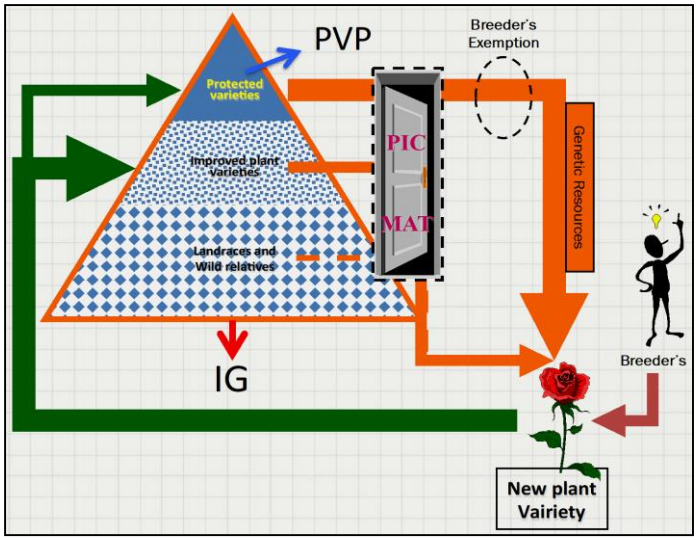

Figure 2. Genetic resources cycles.

PIC is at the very heart of the CBD's compact on ABS. Stakeholders had developed procedures for different purposes (e.g. in MTAs, in patent applications, or the process of product approval for commercialization). Any system should be designed to avoid unnecessary impacts on trade to circumvent any conflicts with World Trade Organization agreements. Any regime has to be developed with the full participation of all stakeholders; only then can it protect the interests of resource providers, in particular concerning traditional knowledge, without being restrictive and preventing desired exchanges of genetic resources.

Under the CBD [1], the concept of Mutually Agreed Terms (MAT) means that a contractual agreement must regulate the access to genetic resources and the sharing of resulting benefits among the parties (the contracting country, as represented by its competent authority, and the party using the genetic resources).

4. Classical plant breeding

The main objective of plant breeding is to improve crop productivity, quality of products (harvested materials), ability to adapt to climate and soil conditions and tolerance or resistance to pests and diseases. Plant breeders use the genetic variations between plants to attain these objectives. Successful adaptation to environmental conditions and success in plant breeding are bounded by the range of the genetic base, as measured by genetic diversity. Genetic variation is needed to address many problems in plant breeding, and is obtained from the biodiversity within the plant genetic resources as shown in

Figure 1 consisted as breeding lines, landraces, primitive forms, wilds and wild relatives, weed races, etc.

In plant breeding, the use of genetic resources is to manipulate plant species in order to create desired genotypes and phenotypes for specific purposes. This manipulation is done, either through controlled pollination, genetic engineering, or both, followed by artificial selection of progeny. Classical plant breeding crosses closely or distantly related individuals to produce new crop varieties or lines with desirable properties. Plants are cross-bred to introduce traits/genes from one variety or line into a new genetic background. For example, rice accession resistant to bacterial leaf blight crossed to high-yielding variety. The goal of the cross being to introduce bacterial leaf blight resistance without losing the high-yield characteristic.

Progeny from the cross would then be backcrossed with the high-yielding parent to ensure that the progeny was most like the high-yielding parent. The progeny from the backcrossing then be tested for yield and bacterial leaf blight resistance and high-yielding resistant plants would be further developed. In crossed pollinated crops, such as corn, plants may also be crossed with themselves to produce inbred lines for breeding. Inbred lines were then used to develop single, double, or three- way cross hybrids.

Plant breeding often, but not always, leads to plant domestication. Plant breeding has been practised for milenia, since the beginning of human civilization. Government institutions and commercial enterprises are alo practicing. International development agencies recognize that crop improvement through plant breeding and development of crops suitable for their environment are vital for ensuring food security.

5. Modern plant breeding

In modern plant breeding or genetic engineering, the DNA in an organism's genome is altered by changing one of the base pair (A-T or C-G). The breeding process may also include the deletion of the whole region of DNA, introduction an additional copy of a gene, or extraction DNA from another organism's genome and combining it with the DNA of that individual. Genetic engineering can be used to enhance or modify the characteristics of an individual organism, e.g. to produce plants that have a higher nutritional value or can tolerate exposure to herbicides. Scientists in a laboratory were able to slow the ripening of tomato by introducing a reverse-orientation copy of an "antisense" gene in a tomato, i.e taking out a gene in the chromosome and putting it back in backwards. This slow ripening tomato increased it shelf life dramatically and was the first genetic engineering crop. In 1994, this genetic engineering tomato was commercialized under the name of FLAVR SAVR tomato and released to the public. However, consumers and retailers in the United States and the United Kingdom resist to Calgene, the company marketing this tomato and tomato paste products, and soon after the tomato project was stopped; since it was no longer profitable.

Studies showed the continued social, environmental and economic benefits of the global adoption of biotechnology in agriculture. The International Service for the Acquisition of Agribiotech Application (ISAAA) [2] report that the global biotech crop area increased in 2017 by 3% or 4.7 million hectares. This increase was primarily due to greater profitability stemming from higher commodity prices, increased market demand both domestically and internationally, and the presence of available seed technologies. As more developing countries increased their biotechnological crop area and continue to allow farmers to adopt biotechnology in food production. With such adoption smallholder farmers see the direct improvements in their crop production; allowing them to provide better lives for themselves and their families. In fact, developing countries now account for 53% of the global biotech area planted.

6. Geographical indication

A sign used on products that have a specific geographical origin and possess qualities or a reputation that are due to that origin is called A Geographical Indication (GI). To function as a GI, a sign must identify a product as originating in a given place, and the qualities, characteristics or reputation of the

product should be essentially due to that place of origin. Since the qualities depend on the geographical place of production, there is a clear link between the product and its original place of production.

Currently, there are various national and international instruments to protect GI from being used by unauthorized parties. Development knowledge base by any society for a period of time owes its origin to the geographical environment and human interactions, the knowledge base becomes the integral part of their economy and tradition. In a globalized society, the knowledges are vulnerable to misuse; hence, the process of preserving the knowledge and heritage is essensial. GIs have intellectual property status since the product gets more value commercially by its mere association with a particular place. GI helps in the identification of a source of a good, which in turn, is related to the quality of good. The laws related to GI apply to a wide variety of goods varying from natural, agricultural to manufactured products. If an area has indicative power then any name related to that area can get legal protection under GI.

In Indonesia, GI have been implemented since the enactment of the Government Regulation Number 51 [3]. One of the most essential requirements in filing for GI protection is that the applicant must submit a book of requirements, under the Law of the Republic of Indonesia Number 20 [4] on Mark and GI refers to Description Document. The description should contain the name of the GI the goods to be protected, the characteristics and qualities of the produced goods, the environmental impact in terms of geography and nature as well as human factors on the characteristics and qualities of goods, the area boundary or area map that is protected by the GI, history and tradition relating to the use of the GI to mark the goods produced by the area, including testimonials from the community on the GI; the production process, processing process and making process which are being applied to enable every producer in that area to produce, process, or make the concerned goods; the methods used for quality testing of the concerned goods; and the labels of the concerned goods showing the GI.

Once the GI registered, the product will be protected indefinitely as long as the specific characteristics and qualities which form the basis of protection remain to exist. GIs can have enormous economic value and are especially useful as marketing tools in emerging markets. So far, there are 65 GI products registered at the Ministry of Law and Human Rights, consisting of fifty-nine domestic GI products, out of which twenty-two products are coffee. Six are foreign products: Champagne, Pisco, Parmigiano Reggiano, Lamphun Brocade, Grana Padano and Tequila.

Farmers' income improvement examples: Kintamani Bali arabica coffee price before GI protection was only IDR 25,000.00/kg, and after the protection become IDR 75,000.00/kg, Muntok white pepper price before GI protection was only IDR 40,000.00/kg, and after the protection become IDR 175,000.00/kg, and Meranti liberica coffee price before GI protection was only IDR 48,000.00/kg, and after the protection become IDR 200,000.00/kg.

Those specially adapted genetic resources having a high reputation in the high producing quality of their products, proof their ability too boost the price through GI. This is not only for the betterment of the farmers, but also for the regional economy.

7. Concluding remarks

Genetic resources is anessential component for varietal improvement in developing high-yielding varieties with the addition of resistance to pest(s), disease(s) or environmental stress. Adapted varieties or landraces capable of producing premium quality products in a specific region known as GI products. Those genetic resources may, eventually, boost the regional economy due to limited growing area with the high demand for the product.

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Geographical Indication
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Genetic diversity of red rice varieties originating from West Java and Banten based on SSR marker related to palatability

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Abstract. West Java and Banten Provinces have diverse local red rice varieties/accessions to support national food security, not only in terms of quantity but also the quality of rice. Good eating quality is closely related to palatability. Palatability is a property that is directly related to the quality of rice feeding, aroma, appearance, taste and texture. This study aimed to analyze the genetic diversity and DNA fingerprint profiles of local red rice accessions from West Java dan Banten using molecular markers related to palatability. A total of 12 red rice accessions and four local red rice accessions from Banten and West Java Provinces were estimated their genetic diversity, respectively. The SSR primers used were Ams (linked with aspartate aminotransferase), GPA (glucosamine-fructose-6-phosphate aminotransferase), GBSS1 (granule-bound starch synthase), CBG (nano cyanogenic β-glucosidase), SS1 (starch synthase), SBE1 (glucosidic linkage of a-polyglucan), RM510 (gel consistency), RM13 (protein content) and RM410 (aromatic). The dendrogram showed two main groups of red rice accessions. The first group consisted of Mayang, Tambleg, Sengkeuhan, Pare Jaketra, Jalawara Hawara, Gadok, Carogol, Beureum Batu, Waren, Segubal, Tampai Beureum and Leger Pondok (similarity reached 80.5%). The second group consists of Kapundung, Cere Beureum and Cireh Hideung with a similarity of 79.5%.

Keywords: Banten, palatability, red rice, SSR, West Java.

1. Introduction

Rice is the main food crop in Indonesia and consumed as staple food. There are various colours of rice depending on the pigment, especially anthocyanin in the pericarp layer, seed coat or aleurons, such as red rice and black rice. The most consumed rice is white one. Red rice contains a high enough vitamin B complex, fibre, essential fatty acids and anthocyanin compounds that are very beneficial to health [1]. Several studies have shown that red rice can be a good source of antioxidants derived from anthocyanin pigments. Islam et al. [2] reported that the red rice population observed in Bangladesh showed differences in coloured pericarp pattern.

Indonesia is known to have a high diversity of rice species and has about 17,000 germplasm accessions [3]. In the 2000s, the number of local rice in the farmers' land had been greatly decreased. Only in certain areas, local rice varieties are still grown by farmers because of the excellent quality and, thus, high selling price. Only a few locations in Banten farmers are still planting local rice

varieties, such as in Cihara, Lebak District. The genetic erosion of rice crops will be more critical if no local rice conservation efforts are made.

Palatability is a property that is directly related to the quality of rice feeding, determined by the fragrance, appearance, taste and texture. The quality of rice flavour is strongly influenced by the physicochemical properties of rice [4]. Some of the genes involved in the synthesis of starch, amino acids, amino sugars and lipids have effects on the physicochemical properties of rice starch which determine the palatability of rice. Therefore, many genetic factors play a role to contribute the rice palatability. Molecular markers have been used to monitor the variation of DNA sequences among varieties. Some molecular markers such as Simple Sequence Repeats (SSR) have been identified to be linked to several genes for essential characters in rice plants [5,6]. Accordingly, we have done research on genetic diversity of West Java and Banten red rice accessions based on palatability SSR markers. The purpose of this study was to analyze the genetic diversity and grouping of accessions of red rice germplasm from the local origin of West Java and Banten based on its DNA fingerprint profile using nine SSR markers linked to the palatability characters.

2. Materials and methods

A total of 16 red rice accessions from Banten dan West Java were used in this study. Red rice accessions from Banten Province are Cere Beureum, Kapundung, Pondok Leger, Tampai Beureum, Segubal, Waren, Beureum Batu, Carogol, Jalahawara, Pare Jaketra, Manikan and Tambleg. Red rice accessions from West Java Province are Cere Hideung, Gadok, Mayang and Sengkuhan. The research was conducted from March to June 2017, in the Greenhouse of Certification Center of Horticulture Plant's Seed at Serang, Banten, and Laboratory of Biotechnology, Faculty of Agriculture, Sultan Ageng Tirtayasa University, Serang, Banten Province.

The research used a descriptive qualitative method including data collection, data analysis and data interpretation. The procedure started with the rice seed seedling for up to 21 days after planting (DAP), and then the DNA was isolated from the young leaves. The DNA isolation used the CTAB method to produce DNA for good PCR amplification. A PCR reaction used nine SSR primers related to rice palatability (Table 1) using standard SSR method and the amplicons were migrated by electrophoresis using 3% agarose gel. Ethidium bromide was used for staining, and then the bands were visualized under UV with Geldoc. The DNA patterns were scanned for the presence of bands and DNA profiles embedded in binary data for "cluster tree analysis". Summary statistics (gene diversity index, PIC and heterozygosity) for all primer was calculated based on the polymoporhic alleles.

Table 1. The SSR primers based on palatability gene.

Primer	Chr	Sequence		Linked gene
		Forward (5′–3′)	Reverse (5′–3′)	
AMs	2	CTTCCAAGGACCCCATCCT	CCCAACATCTCCGTCAGAAT	Aspartate aminotransferase[2]
GPA	11	AATACGCGGCCTTCTCCTAT	TTGATCCGAATGGGTCAAAT	Glucosamine-fructose-6-phosphate aminotransferase[2]
GBSS1	6	CAAATAGCCACCCACACCAC	CTTGCAGATGTTCTTCCTGATG	Granule-bound starch synthase1[1]
CBG	10	AGCTTCCCTAATGGCTTCGT	ATTTGCCAACTTTTGGATGG	Non-cyanogenic β-glucosidase[2]
SS1	6	GATCCGTTTTTGCTGTGCCC	CCTCCTCTCCGCCGATCCTG	Starch synthase 1[1]
SBE1	6	ATTTCTTTGGCCACAGGCGA	CCCAGATTCGGAACAAGAAC	Glucosidic linkage of a-polyglucan[3]
RM510	6	AACCGGATTAGTTTCTCGCC	TGAGGACGACGAGCAGATTC	Gel consistency[4]
RM13	5	TTGGATTGTTTTGCTGGCTCG	GGAACACGGGGTCGGAAGCGAC	Protein content[5]
RM410	9	GCTCAACGTTTCGTTCCTG	GAAGATGCGTAAAGTGAACGG	Aromatic[6]

Chr = chromosome.
[1] [5], [2] [6], [3] [7], [4] [8], [5] [9], [6] [10].

3. Results and discussion

The isolated DNA was diluted to get uniform concentration of about 10–50 ng/µl for PCR. The PCR amplification results were presented in Figure 1.

DNA fingerprint profiles in digital values provided easy identification of rice accessions/varieties. Digital values of DNA patterns based on 9 SSR markers are shown in Table 2. The values showed uniqueness of each accession. Bereum Batu and Waren have the same digital value pattern. The same values were also found between Manikam, Mayang and Tambeleg rice accessions.

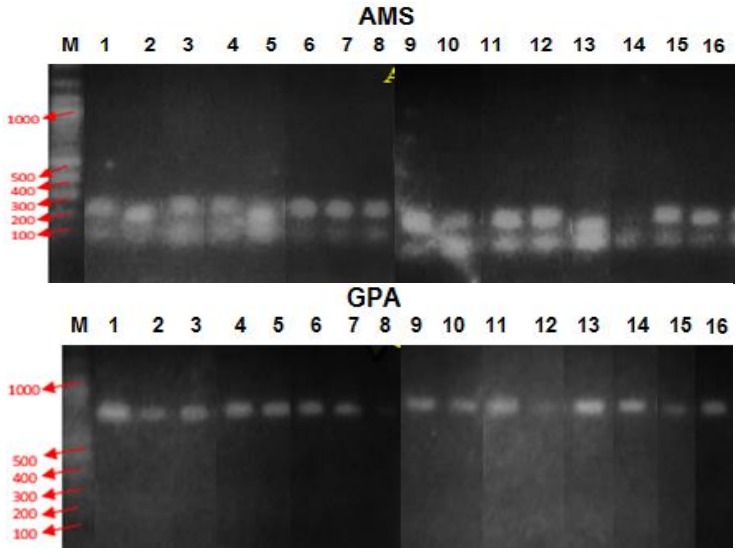

Figure 1. PCR amplification results using molecular markers of AMS and GPA SSR primers based on palatability. M = 100 bp DNA ladder, 1 = Cere Hideung, 2 = Cere Beureum, 3 = Kapundung, 4 = Pondok Leger, 5 = Tampai Beureum, 6 = Segubal, 7 = Waren, 8 = Beureum Batu, 9 = Carogol, 10 = Gadok, 11 = Jalahawara, 12 = Pare Jaketra, 13 = Sengkeuhan, 14 = Tambleg, 15 = Mayang, 16 = Sengkuhan.

The number of alleles, gene diversity, heterozygosity and PIC values of 16 accessions with the 9 SSR is summarized in Table 3. The number of alleles detected was 15–28 alleles, with an average of gene diversity about 0.93. Heterozygosity is obtained from the calculation of gene frequencies at each locus [11]. Based on heterozygosity values, AMs, GBSS1, SS1, SBE1 and RM13 primers are able to distinguish heterozygosity. Conversely, the primers with a heterozygosity value of 0 are assumed to be unable to distinguish heterozygosity (GPA, CBG, RM510 and RM410). The value of heterozygosity close to 0 is low, while the value of heterozygosity close to 1 is high [11].

The polymorphic alleles were analyzed by calculating how many percents of the polymorphic alleles obtained in each SSR primer used. The level of primers informativeness is determined by the calculation of PIC. PIC value provides an estimate of the distinguishing power of a marker by computing not only the number of alleles in one locus, but also the relative frequency of the alleles of an identified population. PIC value becomes the standard to evaluate the genetic markers based on PCR amplified DNA pattern [12,13]. Therefore, PIC value is divided into three classes: highly informative (PIC>0.5), moderately informative (0.25>PIC>0.5) and slightly informative (PIC<0.25). According to Table 3, the average PIC value was 0.93, which means that all of the primers were highly informative. This study used nine primers (AMs, GPA, GBSS1, CBG, SS1, SBE1, RM510, RM13 and RM410), where each primer has been linked to genetically different characters.

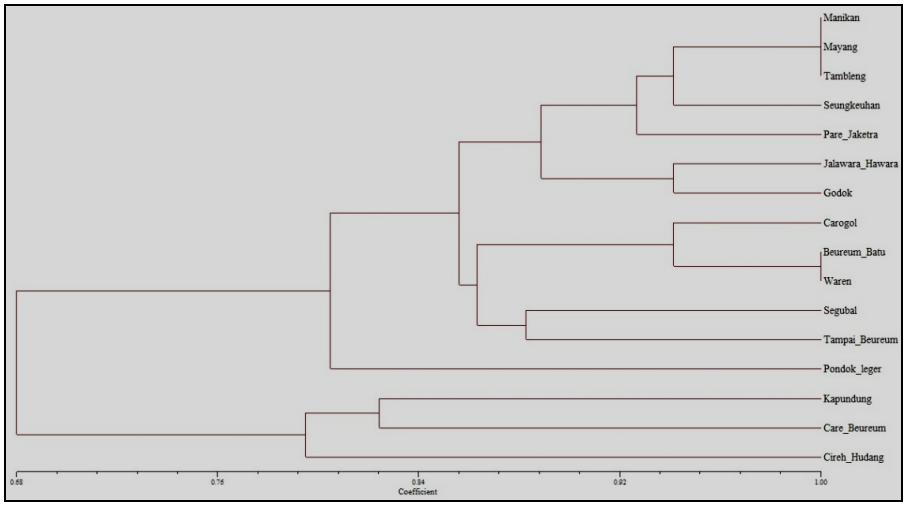

Figure 2. Dendrogram of 16 red rice accessions from West Java and Banten.

Table 2. The digital value of conversion of DNA patterns based on 9 SSR markers.

Accession	Digital values	Accession	Digital values
Manikan	1.011.100.100.101.111	Tambleg	1.011.100.100.101.111
Kapundung	0.111.111.100.101.110	Pondok leger	0.111.100.000.101.111
Carogol	1.011.101.000.101.011	Segubal	1.011.110.000.101.111
Beureum Batu	1.011.101.000.101.111	Tampai Beureum	1.011.100.000.100.111
Care Beureum	0.111.101.000.001.110	Cereh Hideung	0.111.100.101.001.110
Waren	1.011.101.000.101.111	Gadok	1.011.100.000.001.111
Jalawara Hawara	1.011.100.100.001.111	Mayang	1.011.100.100.101.111
Pare Jaketra	1.011.101.100.101.111	Seungkeuhan	1.011.100.110.101.111

Table 3. The number of alleles, gene diversity, heterozigosity and PIC values of 16 accessions with 9 SSR markers in terms of palatability gene.

Primer	Number of alleles	Gene diversity index	Heterozygosity	PIC
AMs	17	0.91	0.07	0.91
GPA	15	0.92	0.00	0.91
GBSSI	19	0.92	0.12	0.92
CBG	19	0.94	0.00	0.94
SSI	28	0.96	0.25	0.96
SBE1	28	0.96	0.03	0.96
RM510	19	0.93	0.00	0.93
RM13	25	0.95	0.31	0.94
RM410	14	0.89	0.00	0.89
Total	184	8.38	0.78	8.36
Average	20.4	0.93	0.09	0.93

AMs markers can predict characters that have a sense of taste and aroma, especially aspartate aminotransferase. Lestari et al. [14] used AMs as DNA markers for eating quality of indica rice in Indonesia. The GPA primer amplified 900 bp fragment which may indicate the presence of glucosamine fructose-6-phosphate aminotransferase. In addition, Utami et al. [15] used GPA primers as DNA markers for physical grain in red rice, indicating its support to red rice research in Indonesia.

The GBSSI primer detected the presence of granule-bound starch synthase genes which function to synthesize amylose *in vivo*. CBG markers are markers for the presence of non-cyanogenic glucosidase located on chromosome number 10. CBG markers can predict the character of the taste and aroma [6].

The SSI primer marked the presence of starch synthase 1 which has a function in the catalytic activity of starch biosynthesis [16]. There are variations in alleles found at the locus associated with starch synthesis, one of which is starch synthase 1 on chromosome number 6 (can be marked by primer SS1) and *Waxy* gene or *Wx* also on chromosome 6 using a GBSS1 primer (granule-bound starch synthase). The existence of the *Wx* gene contributes to amylose levels.

The SBE1 primers are related to the character of the *Waxy* (*Wx*) gene, the soluble starch synthase I (SS1) gene and the starch branching enzyme 1 (SBE1) gene [17]. Primer RM510 can be used to estimate the presence of characters related to the consistency of gel in rice plants [8]. The consistency of the gel will determine the texture of rice after cooking. Rice with soft gel cooking consistency is soft and remains soft even after cooling. Rice with soft gel consistency is preferred by most rice consumers.

The RM13 primer can predict the characters associated with grain protein content in rice which can be seen with a band measuring at 141 bp [9]. The protein content is an indirect indicator of the quality of rice taste. According to Lestari and Koh [6], low protein content and increased stickiness and texture caused high palatability. The physicochemical properties of varied rice starches are genetically affected.

Figure 2 shows a dendrogram consisting of two large groups of red rice. The first group consisted of Mayang, Tambleg, Sengkeuhan, Pare Jaketra, Jalawara Hawara, Gadok, Carogol, Beureum Batu, Waren, Segubal, Tampai Beureum and Pondok Leger. The second group consisted of Kapundung, Cere Beureum and Cireh Hideung. The difference between germplasms in both groups was 32%. Group 1 has a similarity of 80.5%, while the second group has a similarity of 79.5%. Manikam, Mayang and Tambleg accessions are 100% similar. One-hundred percent similarity, which indicates the same palatability characters, was also found between Bereum Batu and Waren. Similarity coefficients are the standard genetic level between accessions. The greater the coefficient of similarity, the more closely the accession is, thus genetically similar. This information is needed in plant breeding activities. However, to date, the physicochemical/palatability properties of various rice accessions are generally still conventionally determined, which requires a large number of rice samples. DNA markers that are developed quickly can be an alternative method for rapid and efficient evaluation, and can predict their potential physicochemical properties.

4. Conclusions

All nine SSR markers related to rice palatability were able to estimate genetic diversity of local red accessions from Banten and West Java. The red rice accessions from these two provinces were highly diverse as reflected by their high gene diversity index and PIC value. All SSR markers proved their high informativeness as indicated by the PIC values. Most of these rice accessions could be distinguished according to the specific DNA fingerprint profiles generated in this study. The red rice accessions belong to two major groups without considering their local origin.

5. Acknowledgement

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Construction of DNA fingerprint for chili pepper varieties using SNAP markers

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Abstract. Establishing the genetic identity of crop varieties has been considered essential for protecting plant breeder and farmer rights, particularly in developing countries like Indonesia. DNA fingerprint using molecular markers is important to give an unambiguous characteristic pattern as a valuable tool for genetic identification. In this study, eight Single Nucleotide Amplified Polymorphism (SNAP) markers were developed and applied to fingerprint 23 varieties of chili pepper. Polymorphism Information Content (PIC) detected in each primer ranged from 0.14 to 0.36 with an average of 0.17. The average of gene diversity was 0.20 among all varieties for total SNAP markers. A phylogenetic tree was subsequently constructed based on their genotypic scores for selected six markers, which separated the 23 varieties into three major groups. The cluster consisted of 2, 5 or 16 varieties. The DNA fingerprints were translated into capital letters representing presence and absence of allele, and they revealed the specific identity of five varieties. A number of varieties possessed the same DNA fingerprint profiles indicating their close genetic distance. Eventhough these SNAP markers were not able to distinguish each variety according to its unique allelic composition, this study could serve as preliminary information to establish genetic fingerprints of chili pepper varieties in Indonesia. Similar studies in the future will benefit from the SNAP found in this study.

Keywords: chili pepper, DNA fingerprint, SNAP.

1. Introduction

Chili pepper belongs to the genus *Capsicum* of the family Solanaceae. It is one of the most important vegetable-spice crops cultivated in tropical regions such as Indonesia. Fruits of chili pepper plants are among the most heavily consumed spices in the world due to their unique colour, taste, pungency, flavour and aroma [1]. Chili pepper is a facultative cross-pollinated crop, and hence, exhibits wide variability for different qualitative and quantitative traits [2,3]. The genus *Capsicum* has a broad genetic diversity, most of these are found growing in the wild and are believed not to have been domesticated. Chili pepper that has been domesticated and cultivated widely in the world comprises five species: *C. annuum*, *C. frutescens*, *C. chinense*, *C. baccatum* and *C. pubescens*. Among these species, *C. annuum* and *C. frutescens* are the main commercial chili pepper traded and cultivated in Indonesia [4].

Genetic identification is critically important in crop plant variety protection. Protection can be granted if the genetic identity of a variety has been proved to be distinct from existing varieties. The uniqueness of a variety is established by tests for distinctiveness. Due to technical limitations, the authentic genetic identification is mainly based on morphological and physiological characters, which are affected by environmental conditions and are often subjective decisions. As a result, different varieties may be difficult to effectively distinguish and arbitrate due to lack of effective species identification methods. Thus, it is an urgent need to establish a set of steady, reliable and easily accessible identification methods for chili pepper varieties to effectively protect their intellectual property rights [5]. Development of molecular marker technology would make it possible to quickly and accurately identify varieties at DNA level, since this technology is not affected by environmental conditions and should be more reproducible and objective [6].

Molecular markers can display the differences in nucleotide sequences which are suitable for DNA fingerprinting of crop varieties [7]. In the last decade, several molecular markers, such as Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Insertions and Deletions (Indel), Inter Simple Sequence Repeats (ISSR) and Simple Sequence Repeats (SSR), were used in chili pepper for developing high density genetic maps [8,9,10], genetic diversity evaluation [11] and are prospective for Marker-assisted Selection [12]. Among all molecular markers, Single Nucleotide Polymorphism (SNP) is the most abundant, robust and feasible for automated high-throughput genotyping [13].

SNP is a single nucleotide DNA variation at specific locations throughout the plant genome. The easiest, most rapid, simplest and allele-specific marker that can be developed utilizing SNP is the Single Nucleotide Amplified Polymorphism (SNAP) marker [14,15]. SNAP marker uses modified allele-specific primers with a mismatched base pair within four bases of the 3'-end in addition to the 3'-end base complementary to the SNP site. The SNAP markers can be developed and applied to construct genetic fingerprint, analyze genetic diversity, kinship and pollen dispersal of target plants [16]. Constructing the DNA fingerprint for chili pepper varieties not only would identify chili pepper species, but also could provide their genetic distance. However, DNA fingerprinting by SNAP markers in chili pepper varieties has not been carried out. In this study, the DNA fingerprints for 23 varieties of chili pepper were constructed by using SNAP markers to provide a reliable scientific basis for the molecular identification and the intellectual property protection of the varieties.

2. Materials and methods

2.1. Plant materials and DNA extraction

A total of 23 chili pepper varieties were used as the plant genetic materials. Detailed information of the chili pepper varieties including name, subspecies, year of release and pedigree is available (Table 1). All chili pepper varieties were grown in a greenhouse until three or four leaves stage of seedling. Genomic DNA extractions from fresh young and healthy leaves were done in Laboratory of Molecular Biology, ICABIOGRAD using cetyltrimethylammonium bromide (CTAB) method [17] with some minor modifications. The quality of extracted DNA was estimated using NanoDrop^{TM} 2000 Spectrophotometer (Thermo Fisher Scientific, USA) and run in 1% (w/v) agarose gel. The samples were visualized under Geldoc-UV Imager (Thermo Fisher Scientific, USA).

2.2. SNAP primer designing

The gene-specific SNAP primers were developed based on previously identified SNP sites (www.genom.litbang.pertanian.go.id) in the genome of chili pepper. The identified SNPs having bi-allelic alternative alleles were selected, and their fragment sequences were adjusted as required for submission for SNAP primer design using the WebSNAPER program (http://ausubellab.mgh.harvard.edu). In WebSNAPER, PCR product with optimum size of 325–375 and absolute size of 300–500 were chosen, while other criteria followed WebSNAPER instruction. After the submission process, optional SNAP primers output with reference and alternate alleles could be seen on display and combination of SNAP primer pairs corresponding to the SNP appeared.

Candidates of SNAP primer pairs with high stability were selected and tested using optimum PCR reaction and program as recommended by WebSNAPER. A pair of primers specific to the corresponding allele with a single band and consistent to the SNP existed in chili pepper varieties could be used as SNAP marker (Table 2).

2.3. DNA amplification

DNA amplification was performed in a T1 Thermocycler (Biometra, Germany). The PCR was performed in 10 µl reaction solution containing 40 ng DNA template, 5 µl Kapa2G Fast Ready Mix (Kapa Biosystems, USA), 0.5 µl each of the forward and reverse primers, and 2 µl sterile $_{dd}H_2O$. PCR conditions for amplification were as follow: pre-denaturation at 94°C for 5 min, 28 or 38 cycles consisting of denaturation at 94°C for 30 s, annealing and extension at 62°C for 1 min, then final extension at 72°C for 10 min and finally stored at 4°C. PCR products for each sample were separated by using 1.5% agarose gel in 1× TAE buffer at 90 V for 90 min to estimate each allele in the SNP site.

2.4. DNA fingerprint based on SNAP and genetic diversity analysis

The molecular data collected from eight SNAP primers were converted into binary format (presence of allele as "1" and absence of allele as "0" representing the reference alleles and alternate alleles, respectively) for analysis with PowerMarker V3.25. Characteristic of the SNAP primer pairs for constructing chili pepper DNA fingerprint were evaluated in the 23 chili pepper varieties in terms of major allele frequency, Nei's gene diversity and Polymorphic Information Content (PIC) using PowerMarker V3.25 software [18]. Molecular Evolutionary Genetics Analysis (MEGA) version 5.0 software [19] was used to develop an Unweighted Pair Group Method of Arithmetic Mean (UPGMA) for evaluating genetic relationships among chili pepper varieties.

Table 1. Detailed information on chili accessions used in this study.

Variety	Species	Year of release	Pedigree
Tanjung-1	*Capsicum annuum*	2001	Natural segregant from Brebes local variety
Tanjung-2	*C. annuum*	2008	Natural segregant from Brebes local variety
Lembang-1	*C. annuum*	2001	Lines selection from Pangalengan local variety
Lingga	*C. annuum*	2011	Lines selection of LV3491
Ciko	*C. annuum*	2011	Lines selection of LV2699
Kencana	*C. annuum*	2011	Lines selection of LV6401
Gelora	*C. annuum*		
Canon	*C. frustecens*	2016	Mass selection from CR017382620115110
AVPP 0207	*C. annuum*		Introduction from AVRDC
Taringe	*C. frustecens*	2008	Mass selection from CR020.0.3.1.2.0
Kresna	*C. frustecens*	2011	
Lembang	*C. frustecens*		Lembang local variety
Landung	*C. annuum*	2011	
Sempurna	*C. annuum*		Natural segregant from Sumatra local variety
Tunduk	*C. frustecens*		
Madun	*C. frustecens*	2013	Lines selection of CR021
Midun	*C. frustecens*		
Andalas	*C. annuum*	2011	Lines selection of CK835
Rama	*C. frustecens*	2011	Lines selection of CR729
Vitra	*C. annuum*		Natural segregant from Sumatra local variety
Tripang	*C. frustecens*		
Prima Agrihorti	*C. frustecens*	2015	Lines selection of R29
Lembang	*C. annuum*		Lembang local variety

Table 2. List of designed and selected Single Nucleotide Amplified Polymorphism (SNAP) primer sets.

Primers	Chr	Primer sequences (5'–3')	Ref	Alt	Ann (°C)	Cycle	Product size (bp)
CaSNAP6_3151	6	F: TTTAATTTTCAAATATCATTGTTCACT TCGAAAACG R: TCCTTCTTAATCACGAAATCAACCCA CTTTCT	A	C	62	38	353
CaSNAP1_0181	1	F: GAAATGCTGAAATAAGTAGCAATAA GAAGCAAAATG R: TTTAAAGCCTTGAGATAAAAGCATAT GTTCTGGAAG	G	A	62	28	375
CaSNAP1_3421	1	F: TATTCAATATTAGGTGAAATGCTCTA GTTGCTCACG R: GGCATTATTCTTAATGCCATTCCACA TAACTAAAAA	C	A	62	28	363
CaSNAP1_5962	1	F: GATCAAATAATGTCATCGGACATGC TCG R: CTGATTTGCGTTTAACTTTGAGAATC CATTTGT	G	A	62	28	373
CaSNAP11_2961	11	F: GAGGCATTGGTGCCTAATCAGGGAT CCTGCTTGTCTGCCCCTCAAAATAGAA R: CCTGCTTGTCTGCCCCTCAAAATAGA A	T	C	62	28	346
CaSNAP11_1679	11	F: TCTGCTGATACCTATTTACCATACTTA TTGAAGACA R: AAAAACATACGGTTACTGATGGCGG ATAGG	A	G	62	28	332
CaSNAP9_4829	2	F: TTTATATTGCCTTACCTATCATTCCTT CACTCTAGC R: TACGCCGAATGGTTGGACTCGCTATA	C	T	62	28	340
CaSNAP9_5132	9	F: AAGTTTGAAATATAGCTTATGCATGC GGGTG R: GAAACTCACCTAAGATATACTATTGA CTCCCCCGAT	G	T	62	38	355

Chr = chromosome, Ref = reference, Alt = alternate, Ann = annealing.

3. Results and discussion

3.1. SNAP markers reliability for DNA fingerprinting construction

All eight SNAP primer pairs could amplify the target sequences in 23 chili pepper varieties (Figure 1). In this study, only reference allele was converted to SNAP, confirming the allele presence and absence depending on the SNP detected in each variety, then translated into nucleotide base scoring profile. Based on the 8 SNAP markers tested on 23 chili pepper varieties, six markers (CaSNAP6_3151, CaSNAP1_0181, CaSNAP11_2961, CaSNAP11_1679, CaSNAP9_4829 and CaSNAP9_5132) revealed polymorphism while 2 SNAP markers (CaSNAP1_3421 and CaSNAP1_5962) were monomorphic, amplifying products for both reference and alternate allele in all the 23 chili pepper varieties. These result indicated that these six markers were suitable for constructing SNAP fingerprint profiles of the 23 chili pepper varieties.

Subsequently, genetic properties for SNAP markers were calculated, including major allele frequency, gene diversity and PIC (Table 3). The average of genes diversity was 0.20 of total genotypes for total SNAP. The usefulness of molecular markers could be measured based on their PIC [20]. PIC is described as the value of a marker for detecting polymorphism in a population and it depends on the number of detectable alleles and distribution of their frequencies. PIC of 6 SNAP

markers used ranged from 0.14 (CaSNAP6_3151 and CaSNAP9_5132) to 0.36 (CaSNAP9_4829). SNAP is co-dominant marker and bi-allelic. However, their PIC is not high as multi-allele microsatellites. As suggested by Guidelines for Molecular Marker Selection and Database Construction, co-dominant markers are favoured as molecular markers for DNA fingerprinting [21].

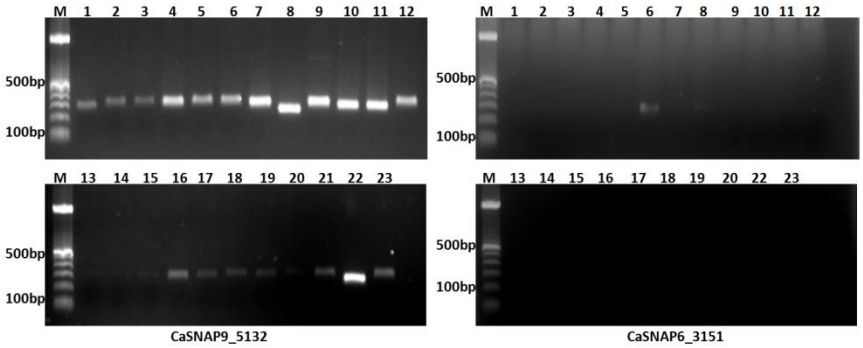


Figure 1. Bands of amplified pattern results after electrophoresis for CaSNAP9_5132 and CaSNAP6_3151, separated by 1.5% agarose gel.

Table 3. Summary of descriptive statistics of 8 SNAP markers in 23 chili pepper varieties.

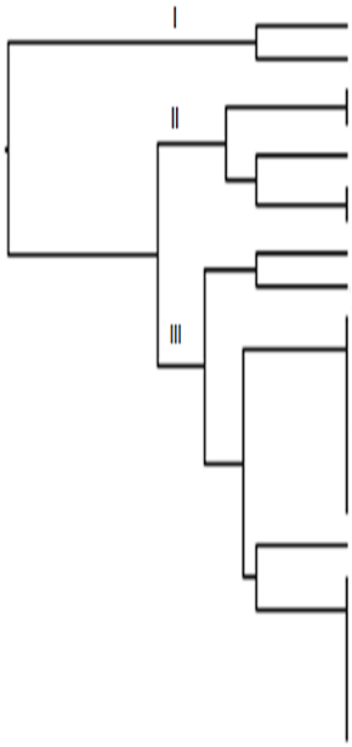
Marker	Major allele frequency	Gene diversity	PIC
CaSNAP6_3151	0.91	0.15	0.14
CaSNAP1_0181	0.86	0.22	0.20
CaSNAP1_3421	1.00	0.00	0.00
CaSNAP1_5962	1.00	0.00	0.00
CaSNAP11_2961	0.86	0.22	0.20
CaSNAP11_1679	0.69	0.42	0.33
CaSNAP9_4829	0.60	0.47	0.36
CaSNAP9_5132	0.91	0.15	0.14
Mean	0.85	0.20	0.17

3.2. DNA fingerprint of chili pepper varieties based on SNAP markers

To identify the genetic diversity between the 23 chili pepper varieties, a phylogenetic tree was subsequently constructed from the six selected SNAP markers based on their genotypic scores using the UPGMA method. UPGMA separated the 23 varieties into three major groups (Figure 2). The first major group consisted of two varieties (Landung and Sempurna), the second major group comprised five varieties (Tripang, Midun, Vitra, Ciko and Tanjung-2) and the remaining varieties (Gelora, Tunduk, Madun, Andalas, Rama, Prima Agrihorti, Lembang [*C. frustecens*], Kresna, Taringe, AVPP-0207, Lingga, Lembang-1, Tanjung-1, Lembang [*C. annuum*], Kencana and Canon) belonged to the third major group. The results of the grouping indicated that the 23 chili pepper varieties of different species could not be distinguished clearly. Some of *C. annuum* species did not separate from *C. frustecens* species.

DNA fingerprinting with molecular markers allows precise, objective and rapid varietal identification. A DNA fingerprinting of 23 chili pepper varieties was constructed with six selected SNAP markers. Based upon the amplicon profile generated by analyzing 23 varieties of chili pepper using six primer pairs, a 6-digit DNA fingerprint for six primer pairs was constructed (Figure 2). For this purpose, the assigned allele for 6 primer pairs was placed from left to right in capital letters. Digits from left to right corresponded to the allele at loci CaSNAP6_3151, CaSNAP1_0181,

CaSNAP11_2961, CaSNAP11_1679, CaSNAP9_4829 and CaSNAP9_5132. For example, fingerprint code for the Tanjung-1 variety was CGTACG, which was from left to right signified scored allele of CaSNAP6_3151, CaSNAP1_0181, CaSNAP11_2961, CaSNAP11_1679, CaSNAP9_4829 and CaSNAP9_5132, respectively.



Variety	Fingerprint Code
Landung	CACGTT
Sempurna	CATGTT
Ciko	CGCGTG
Vitra	CGCGTG
Tanjung-2	CGTGCG
Midun	CGTGTG
Tripang	CGTGTG
Kencana	AGTATG
Canon	AGTACG
Lembang (*C. frustecens*)	CGTACG
Kresna	CGTACG
Taringe	CGTACG
AVPP-0207	CGTACG
Lingga	CGTACG
Tanjung-1	CGTACG
Lembang-1	CGTACG
Lembang (*C. annuum*)	CATATG
Prima Agrihorti	CGTATG
Rama	CGTATG
Andalas	CGTATG
Madun	CGTATG
Gelora	CGTATG
Tunduk	CGTATG

Figure 2. Phylogenetic tree and DNA fingerprint code of 23 chili pepper varieties resulted from UPGMA cluster analysis based on SNAP marker.

The DNA fingerprint code of genotypes would reflect how closely the varieties are related to each other. The six selected SNAP primers were not able to distinguish some of chili pepper varieties (Figure 2.). For instance, seven varieties (Lembang [*C. frustecens*], Kresna, Taringe, AVPP-0207, Lingga, Tanjung-1 and Lembang-1) have the same DNA fingerprint code (CGTACG), and the other six varieties (Prima Agrihorti, Rama, Andalas, Madun, Gelora and Tunduk) also have the same code (CGTATG). Further work is needed to develop the DNA fingerprinting of these chili pepper varieties using SNAP markers.

4. Conclusions

A preliminary DNA fingerprinting database of the 23 chili pepper varieties was built in this study using six SNAP markers, which could be expanded as the number of additional varieties and molecular markers increase. Phylogenetic tree of SNAP markers divided the 23 varieties into three major groups. However, a number of varities possessed the same DNA fingerprint profiles, indicating their close genetic distance. Eventhough these SNAP markers were not able to identify each variety according to its unique code, this study could be useful as preliminary information to establish the genetic identity of chili peppers variety in Indonesia in the future.

5. Acknowledgement
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Development of Laos Khao Kai Noi rice landrace (*Oryza sativa* L.) core collection as a model for rice genetic resources management in the Laos National Genebank

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Abstract. Khao Kai Noi rice is considered as an elite quality landrace in Laos, which has led to its germplasm conservation in the Laos National Genebank. As happens with other germplasm collections, a manageable yet representative sub collection has become an essential element for researchers and breeders to simplify many activities, including those related to crop improvement, phenotype-genotype correlation and determination of diversity hotspots. In this study, 109 accessions were used as a test collection for core collection development to determine the feasibility of collection reduction in a closely related rice group. Three core collections were developed by two established methodologies and evaluated by diversity indexes, allele retention, phylogenetic distribution and geographical location. Based on SSR molecular markers and PowerCore, a reduction to 24 accessions was achieved with the conservation of complete genetic diversity. A K-means based on reduction to 24 accessions rendered slightly lesser results while based on 12 accessions resulted in a 17% diversity loss. These core collections may be useful for genebank management, research and breeding activities in the future. Also, they may as well serve to estimate core collection development behavior in other landraces and cultivars, which is fundamental in genetic resources management and utilization.

Keywords: rice, core collection, Khao Kai Noi, PowerCore, K-means.

1. Introduction

Genebanks play a key role in the conservation, availability and use of a wide range of plant genetic resources for crop improvement in order to provide food and nutrition security. They help to ensure the continued availability of genetic resources that may have become disused in order to aid future research and breeding programs [1]. As collections tend to increase in numbers as time goes by, maintenance costs and management effort increase as well. Also, diversity analysis, phenotype-genotype correlation and low redundancy becomes challenging, which in turn affect the genebank ability to supply users and breeders with the most adequate materials to perform their activities. A suitable solution to this issue is the development of a “core collection” derived from the existing

germplasm collection, which consists of a limited set of accessions, chosen to represent the genetic spectrum in the whole collection in order to facilitate mentioned management, research and supply [2].

This process is generally known as core collection development (CCD) and was first proposed by Frankel and Brown [3], and as stated, it is of high importance as it plays a significant role in the management and use of genetic resources [2]. CCD has been implemented by many genebanks in diverse crops such as rice [4–6], common beans [7], chilis [8], barley [9,10], soybeans [11] and sesame [12]. Although there exist several approaches and different particular objectives, it has become clear that many factors need to be considered for an adequate CCD, where selection criteria and genetic structure play an important role [13], as well as the evaluation procedures that best fit the particular objectives, as some core collections are being created to represent specific sections of germplasm collections [14]. Strategies for CCD of these specific sections need to be further explored and implemented.

The Laos National Genebank (LNG) is located in the Rice Research Center, National Agriculture and Forestry Research Institute (NAFRI). Among its different functions, LNG provides both active and base germplasm collections, which include more than 14,000 rice accessions. This collection includes a group of Khao Kai Noi (KKN), a high quality regional Laos landrace with high importance in local consumption and export value. Currently, about 200 accessions of KKN germplasm are conserved in the LNG, collected in different efforts since 1995 [15−17].

KKN was chosen as the model group for CCD in LNG for two principal reasons. The first one is related to the direct application of the KKN core collection in research of genetic diversity, genetic structure and diversity hotspots determination; which in turn would lead to adequate *in situ*/*ex situ* conservation recommendations, breeding improvement and supply. The second one has to do with CCD in a closely related rice landrace, in order to evaluate the properties of a core collection of such nature, and to be able to extrapolate the CCD methodologies to the entire Laos rice collection, as well as to other important landraces and cultivars in the near future.

PowerCore [18] is a reliable and open access software that has been successfully applied in the development of several subsets from rice collections [19,20], as well as from other crops like chili [8] and Turkish melon [21]. PowerCore implementation provides 100% allelic coverage, yet the target core element number depends on the original collection selecting parameters distribution. A K-means based algorithm [22] may complement our implementation of PowerCore, as it allows determination of a target core element number *a priori*.

In this work, we propose the establishment of core collections from KKN collection in LNB, which could be useful for breeding purposes, identifying diversity hotspots, phenotype-genotype correlation, genebank germplasm management and recommendation for on-farm conservation sites for this important rice landrace, as well as serves as a model for CCD implementation in other landraces and cultivars.

2. Materials and methods

One hundred and nine non-redundant accessions were selected from the available KKN in LNG as a whole collection model (Supplemental Table 1). In order to construct our whole collection model genetic data set, a random individual for each non redundant accession was selected and associated with 24 highly informative genome spread SSR data. Molecular characterization procedures have been published elsewhere [23].

Data was analyzed with PowerCore V1.0 in its default parameters, which provided a core collection number that preserves the totality of allele diversity. This number was used as fixed target in the K-means algorithm for CCD to construct a second core collection with the same number of elements. A third core collection was built with K-means, using half previous fixed target to determine impact on such reduction.

Evaluation of the selected core collections was performed by comparing original collection and selected core collections in terms of: Nei and Shannon-Weaver diversity indexes [18], allelic representation, phylogenetic clustering representation, and, when possible, geographical distribution.

Diversity indexes and allelic representation where calculated with PowerCore. Phylogenetic clustering was determined by an UPGMA dendrogram, which was first constructed by PowerMarker V3.25 software [24] and then visualized by MEGA6 [25], where the representative accessions where tracked. Geographic distribution of those accessions with available geographical reference data were plotted in a Xiengkhouang and Houaphan Laos Provinces Map by ArcGIS (http://doc.arcgis.com/en/arcgis-online/).

3. Results and discussion

To establish representative accessions of KKN as a tool for breeding purposes, diversity hotspots identification, genetic resources management and on-farm conservation sites recommendations for this rice landrace, 109 accessions where analyzed for 24 SSR genome spread molecular markers. The minimum number for complete allelic representation was determined as the 24 accessions selected by PowerCore. Therefore, 24 and 12 accessions where the target for K-means CCD algorithm.

As established above, the PowerCore core collection was able to represent 100% of allele diversity, with a Nei index of 0.48 and a Shannon-Weaver of 0.94. K-means core collections represented 92% of allele diversity, with a Nei index of 0.51 and a Shannon-Weaver of 0.94 when 24 accessions where selected; 83% allelic diversity representation with a Nei index of 0.51 and a Shannon-Weaver of 1.0 when 12 accessions where selected (Figure 1).

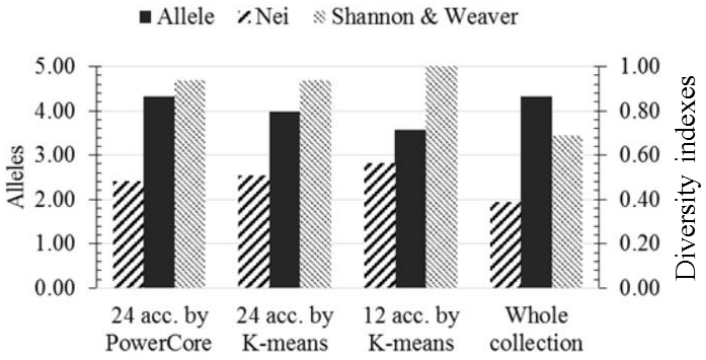

Figure 1. Diversity indexes and allele representation of core accessions of Khao Kai Noi and its whole collection.

Cluster representation among the constructed phylogenetic dendrogram is presented in Figure 2, where constructed core collections with 24 accessions included an accession from each of the 12 major clusters. Constructed core collection with 12 accessions included an accession from 9 of mentioned clusters.

KKN can be classified by its phenotype in 6 groups: “Deng” (red), “Leuang” (yellow), “Hay” (upland), “Khao” (white), “Lai” (striped) and “Lai Dam” (striped and black). Twenty-four accessions included at least a member for each group, while 12 accession core collection failed to represent “Khao” (Supplementary Table 1). The geographical distribution of all core collection accessions were distributed along and covered all provinces where KKN is primarily produced (Figure 3).

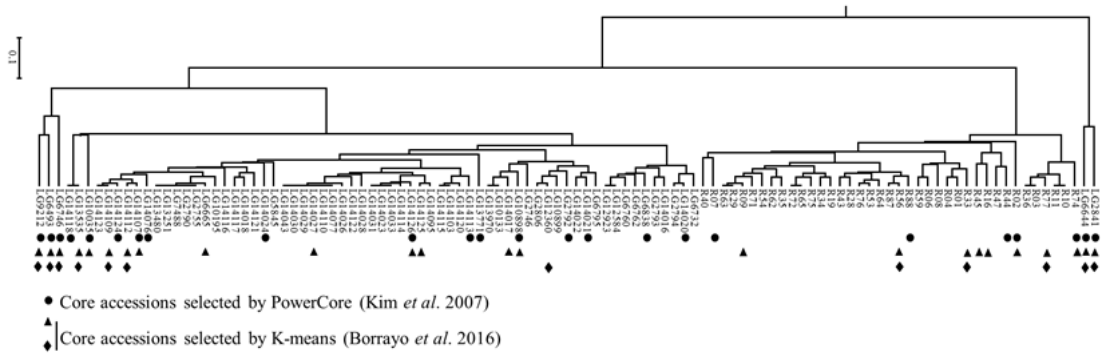

Figure 2. UPGMA dendrogram of 109 accessions of Khao Kai Noi. Marks below each accession indicate its selection as a core collection element.

The core collection consists of a limited set of accessions derived from an existing germplasm collection, chosen to represent the genetic spectrum in the whole collection and should include as much as possible of its genetic diversity [2]. In this study, we aimed to determine the feasibility to create a useful core collection from a closely related rice landrace. A CCD within KKN would prove handful in more than one way, as it can provide priority accessions for *in situ*/*ex situ* conservation, as well as to optimize resources on breeding and research, and provide an important insight of how these CCD methodologies may respond to other landraces or cultivars.

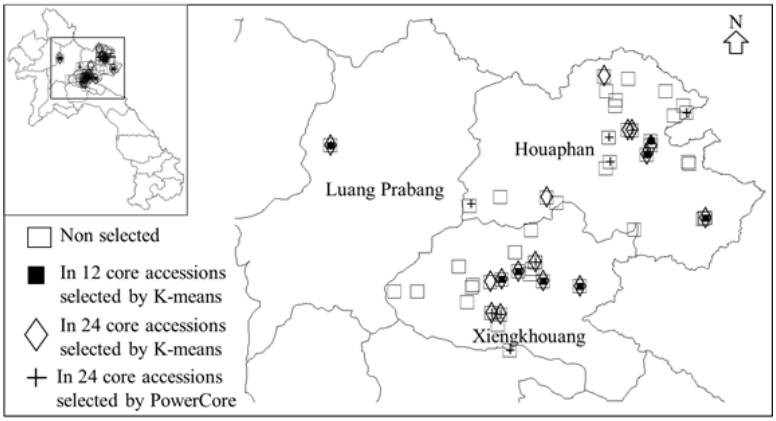

Figure 3. Map of Laos showing the distribution of 62 of 109 accessions of Khao Kai Noi used for collection development. The map was created by using ArcGIS.

The first approach was to determine a core collection that would maintain all possible diversity, which is possible by PowerCore implementation. However, a 22% core collection appear as a high value, distant from the recommended consensus percentage mentioned by Frankel and Brown [3], who indicated that core collections should be reduced to 5% from large and 10% from small collections. This led to the implementation of another methodology that could establish a target value regardless of allele retention percentage. By K-means 24-accession implementation, we wanted to compare methodologies under similar conditions, while 12-accession implementation allow us to determine the behavior under mentioned percentages that have been used in similar CCD [8].

Both PowerCore and K-means 24-accession behaved very similar in terms of diversity indexes, as both clearly reduced redundancy from the original collection. K-means is not able to represent the full allele diversity. This is expected as the selection method for this algorithm is to select a sample closest to centromere of each of the target-number generated groups, which explain why Nei index is higher

in K-means than in PowerCore's selected accessions. In terms of hierarchical clustering and geographic representation, these two core collections had again similar results, proving to be effective methodologies for CCD in the evaluated landrace.

K-means 12-accessions did not comply as effectively as its evaluated counterparts, and a phenotype was not included among the selected elements. However, considering the fact that this core collection had a 50% accession deficit compared to the other ones, it is interesting to note that there was only a 17% allele reduction representation; 2 of the 3 missing cluster representation in the hierarchical clustering were single accession groups; diversity indexes were maximized. It is also important to consider that the main strength of K-means is the inclusion of both agromorphological traits and genetic information, although in this preliminary study only the second one was included. There may be some circumstances where this core collection may prove useful, particularly when priority for conservation or distribution must be provided for budget limitations.

4. Conclusions

In summary, we present three KKN core collections that have their valuable for the landscape's genetic resources management, research and breeding. We believe that the methodologies implemented in this work may be successfully extrapolated to other rice landraces and cultivars, with similar results when selecting a core collection from a highly related original collection.

5. Acknowledgement

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Genetic diversity of local red rice cultivars collections of Yogyakarta AIAT, Indonesia based on morphological character

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Abstract. Identification of local red rice was important to study for genetic diversity. The objective of the study was to identify the morphological characteristics to estime the genetic diversity and heritability in the broad sense of 11 local red rice cultivars. The quantitative data (plant height, leaf length, leaf width, number of productive tillers, panicle length, 1,000-grain weight and number of grain per panicle) were measured by their CVg values for determining their relationships and heritability. The results can be used for parent selection in a breeding program. Results showed that the number of productive tillers, leaf length and the number of grain content per panicle for 11 local red rice from Yogyakarta were efficient and effective characters in selection because the characters have wide of the coefficient of variation genetics and high heritability value.

Keywords: genetic diversity, relationship, morphology, local red rice, Yogyakarta AIAT.

1. Introduction

Red rice, a staple food source known since 2,800 BC, has long been known to be very beneficial to health, as well as staple foods. This red rice could prevent food shortages and nutrition and cure diseases of vitamin A deficiency (farsightedness) and vitamin B. According to Frei [1], rice especially red rice is a major source of carbohydrates, also contains proteins, beta-carotene, antioxidants, and iron. Red rice fibre was relatively easy to be absorbed by intestine than wheat, so it can lighten the intestine burden in performing a peristaltic movement [2] and launching the gastrointestinal system. Thus, conservation of the genetic resources of red rice needs to be addressed and put in high effort, so that the value of benefits can be enjoyed for the community welfare.

The exploration activity of genetic resources team of Yogyakarta AIAT obtained 15 local red rice cultivars which are still cultivated by some farmers in the region of Yogyakarta, Indonesia. Fifteen red rice cultivars have different names depending on the area of origin and farmers who cultivate. Besides, the difference in the name of red rice was thought to be due to the diversity of morphological appearance of the plant to the colour diversity [3].

The genetic resources of local red rice are indispensable in addition to cultivated for consumption required by plant breeders as a parent. Breeders need to use wide genetic diversity for the parent to improve varieties. Utilization of existing genetic resources becomes easier if characterization and

evaluation have been conducted. Characterization of the desired properties is able to estimate the kinship relationship between the cultivars. The phenotype appearance as an expression of genetic diversity can distinguish an individual with other individuals. The study of genetic diversity can be done based on morphological, biochemical and molecular markers [4].

The encoding of morphological properties was heavily influenced by the environment, but morphological properties have been of great benefit in forming several of excellent cultivars since the 1950s. Morphological enrichment can be used to identify multiple germplasm collections. Matching at the morphological level is primarily phenotype recognition and its associated changes in its ecotype [4].

Morphological identification was an easier step at the early stage compared to molecular identification. The morphological markers used were based on simple Mendelian inheritance, such as shape, colour, size and weight. The morphological properties can be used as real clues to specific genes and markers of genes in chromosomes because the properties that affect morphology can be inherited [5]. The phenotype of plants was determined by the genetic and environmental factors [6].

Extensive genetic diversity and high heritability were among the requirements for valid selection [7]. High heritability values indicate that most of the phenotypic diversity caused by genetic diversity, in which selection will gain genetic progress [8]. Based on these matters, this study aimed estimate the genetic diversity and heritability in the broad sense of 11 local red rice cultivars of Yogyakarta AIAT collection based on morphological characters.

2. Materials and methods

2.1. Plant material

The plant materials used in this study were 11 local red rice of Yogyakarta AIAT collection, Indonesia (Table 1). All materials used were planted in plastic pots in a greenhouse using a completely randomized design with three replications. Plant maintenance included watering, fertilizing and pest control were applied if needed.

Table 1. Red rice cultivar of Yogyakarta AIAT collection.

Red rice cultivars	Origin
Merah Pepen	Sleman, Yogyakarta
Sembada Merah	Sleman, Yogyakarta
Saodah Merah	Bantul, Yogyakarta
Mandel	Gunungkidul, Yogyakarta
Cempo Kenanga	Gunungkidul, Yogyakarta
Segreng	Gunungkidul, Yogyakarta
Cempo Jalen	Gunungkidul, Yogyakarta
Mayangan Gundil	Gunungkidul, Yogyakarta
Tangkilan	Gunungkidul, Yogyakarta
Gogo Lembayung	Gunungkidul, Yogyakarta
Andel Merah	Bantul, Yogyakarta

2.2. Observation

A number of morfo-agronomical characters were observed. The parameters included plant height, leaf length, leaf width, number of productive tillers, panicle length, 1,000-grain weight and number of grain per panicle.

2.3. Analysis

Quantitative morphological characters (plant height, leaf length, leaf width, number of productive tillers, panicle length, 1,000-grain weight and number of grain per panicle) were analyzed of variance using completely randomized design (CRD) estimation of genetic variation (Table 2) according to Singh and Chaudhary [9].

Based on results of the analysis the variants, further analysis to determine the genetic diversity shown by the coefficient of genetic diversity (CGD) value was done and calculated according to the Singh and Chaudhary [9] formula.

$$\sigma^2 g = \frac{MSg–MSe}{r}$$

$$CVg = \frac{(\sqrt{\sigma^2 g})}{\overline{X}} \times 100\%$$

According to Moedjiono and Mejaya [10], the CGD that has been obtained can be classified into four criteria, i.e. low diversity (0–25% of the highest CVg), medium diversity (25–50% of highest CVg), high diversity (50–75% of the highest CVg) and very high/very wide (>75% of the highest CVg). Furthermore, the estimated genetic variance of the mean square value of variance analysis follows the Moedjiono and Mejaya [10] formula, which are further used to calculate the value of broad-sense of heritability:

$$H^2 = \frac{\sigma^2_g}{\sigma^2_g + \sigma^2_e / r}$$

σ^2_g = genetic diversity, σ^2_e = EMSe, MSe = mean square of error, r = sum of block.
According to Mc Whirter [11], broad-sense of heritability estimates devided into three categories, low: $H^2<0.20$, medium: $0.20<H^2<0.50$ and high: $H^2>0.50$.

Table 2. Analysis of varian of completely randomized design.

Source	Degree of fredom (Df)	Sum of square (SS)	Mean square (MS)	Expected mean square (EMS)
Genotipe/g	g-1	SSg	MSg	$\sigma^2_{e+} r\sigma^2_g$
Error/e	g(r-1)	SS s	MSe	σ^2_e
Correction total	rg-1			

SSg = sum square of genotype, MSe = mean square of error, σ^2_e = EMSe, σ^2_g = genetic diversity, g = genotipe, r = sum of block.

3. Results and discussion

Phylogenic tree resulted from the clustering of eleven red rice local from Yogyakarta (dendrogram was not shown) showed that there was no duplication between cultivars. Moreover, other characters, such as anatomy, biochemical content, mineral Fe, Zn and other important components need to be observed. There was a correlation between morphological or phenotypic properties and certain biochemical compounds. As reported by Yawadio et al. [13], that pigmented rice has the potential as a source of antioxidants and feasible as a useful food source. Characterization based on morphological markers was usually influenced by macro and microenvironments, and plant life. Therefore, morphological characterization needs to be supported by characterization using molecular markers. According to Dwiatmini et al. [14], molecular markers can give a more accurate picture of the phylogenic tree, because DNA analysis as genetic material is not affected by environmental conditions.

3.1. Genetic variation

Analysis of variance of the coefficient of variation genetic (CVg) of 11 local red rice cultivars was presented in Table 3. The number of productive tillers has the highest value of CVg of 41.83%. According to Moedjiono et al. [10], very high and high CVg values mean of wide or high genetic variability, while those with relatively moderate and low CVg criteria were classified as narrow genetic variability. The number of productive tillers, leaf length and the number of grain content per panicle revealed wide genetic diversity. While characters of leaf width, plant height, panicle length, and 1,000-grain weight have a narrow genetic diversity. A trait that has a very high and high genetic variation value indicates that improvement through selection was possible on this trait.

Table 3. Genetic variation of some morphological properties.

Character	$\bar{x}$	σ^2_g	CVg (%)	Relative value	Criteria of diversity
Leaf width	12.82	5.11	17.63	42.15 (M)	Narrow
Leaf length	49.12	147.84	24.75	59.17 H)	Broad
Number of productive tillers	15.26	40.74	41.83	100.00 (VH)	-
Plant height	129.14	505.41	17.41	41.62 (M)	Narrow
Panicle length	22.75	12.94	15.81	37.80 (M)	Narrow
1,000-grain weight	24.26	3.15	7.32	17.50 (L)	Narrow
Number of grain per panicle	137.12	2042.07	32.96	78.80 (VH)	Broad

CVg = coefficient of variation genetic, σ^2_g = genetic diversity, $\bar{x}$ = average, VH = very high/very broad, H = high/broad, M = medium, L = low.

3.2. Estimating heritability of results and results components

The value of heritability of 11 red rice cultivars was presented in Table 4. Results showed that the value was classified as high, suggesting that the character can be inherited to the next offspring [14]. Selection is an important stage on plant breeding, particularly on characters that contribute to crop adaptation. It will be more effective when the characters have genetic information on estimated heritability, as well as number and type of gene that control the particular traits [15]. Heritability was the most important of a genetic parameter. The heritability probability indicates whether a character was controlled by genetic or environmental factors [14].

Table 4. Heritability of 11 red rice cultivars.

Character	σ^2_g	σ^2_e	R	H^2	Criteria
Leaf width	5.11	0.30	3	0.98	High
Leaf length	147.84	3.77	3	0.99	High
Number of productive tillers	40.74	1.49	3	0.99	High
Culm height	505.41	10.42	3	0.99	High
Panicle length	12.94	3.40	3	0.92	High
1,000-grain weight	3.15	1.67	3	0.85	High
Number of grain per panicle	2,042.07	8.61	3	1.00	High

σ^2_g = genetic diversity, σ^2_e = error mean square, R = replication, H^2 = heritability.

Characters with high heritability values can represent that the effect of genetic factors is more significant on phenotypic appearance than on environmental influences [16]. This was consistent with Welsh [17], which reportes that the overall variation in a population was the result of a combination of

genotype and environmental influences. The heritability value 0 to 1. The value 0 was when all the variations are caused by environmental factors, while the value of 1 when all variations are caused by the genetic factors of the crop varieties. Estimation of heritability value was used as a first step of the selection proses of a segregated population. Consequently, such character can be selected in the early generation and the possibility for further selection addressing on the desired genetic progress.

Concerning to the value of coefficients of genetic diversity, a selection will be efficient and effective when performed on characters with broad genetic diversity coefficient values and high heritability values [18]. Overall, characterization of plant height, stem height, leaf length, number of productive tillers and number of grain per panicle in the local red rice Yogyakarta, Indonesia was an efficient and effective way in selection because the characters have wide of the coefficient of variation genetics and high heritability.

4. Conclusions

The number of productive tillers, leaf length and the number of grain content per panicle on the local red rice Yogyakarta, Indonesia had wide of coefficient of variation genetics and high heritability. Such morphological paramaters in this study was an efficient and effective character included in the selection activity on red rice genetic resources management.

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Tiller profile diversity of upland rice germplasm in ICABIOGRAD gene bank

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Abstract. Tillering ability is an important agronomic trait that determines the yield of rice. Tiller type in rice is categorized as primary, secondary and tertiary tiller which produces panicle at generative phase. Tillering ability as well as the tiller type varies between varieties, especially among germplasms. Our study aimed to identify the tiller profile diversity of upland rice germplasm in ICABIOGRAD Genebank. A total of 100 accessions of local upland rice varieties were planted in a randomized completely block design with two replications under greenhouse condition. The number of tiller from each type was observed weekly from 35 to 63 days after sowing (DAS). The result of the study showed that the number of primary tiller increased slowly from 35 DAS until 63 DAS. After 49 DAS there was no significant addition in secondary tiller number. Generally, the average mean number of tertiary tiller accross the accessions was far below compared to the number of primary and secondary tiller. The highest number of primary tiller, i.e. 8, was expressed by Pae Daye Indolobye (North Sulawesi), Padi Pulut Pute Iteung (East Kalimantan) and K. Puyuk (Central Kalimantan). Up to 22 secondary tillers were formed by Gadabung, a local upland rice variety from Central Kalimantan. Information on tillering ability of local upland variety will benefit rice breeder for selecting appropriate accessions as a gene source for breeding.

Keywords: tiller, accessions, diversity, germplasm, breeding.

1. Introduction

Rice (*Oryza sativa* L.) is one of the most important staple food crops in the world [1]. In tropical countries such as Indonesia, upland rice has significant contribution in supplying staple food for people living in marginal dryland areas [2]. Upland rice cultivation has existed since ancient times in Indonesia. Almost every island in Indonesia are already familiar with upland rice, especially for regions with arid and marginal land. In order to compete, upland rice productivity must be enhanced by the use of new varieties [3].

Tillering and panicle branching are important agronomic traits that affect rice grain yield [4,5]. Characteristics of tillers can be observed starting from the initial growth until the maximum tillering is reached in the filling phase of the seed. Primary tillers grow from the main stems, secondary ones grow from primary, tertiary from secondary tillers, and so on. During the vegetative phase, the maximum number of tiller formed is more than the number of productive tiller in the generative phase

[6]. At generative stage, each tiller type can produce panicle. The panicle that comes out from each type of tiller is called primary, secondary, tertiary and quarternary panicle, respectively [7].

Indonesia is rich in the diversity of rice germplasms. ICABIOGRAD Gene Bank holds a collection of rice germplasm consisting of lowland rice, upland rice, swamp rice and tidal rice. The ability to produce the number of tiller as well as the tiller type varies between varieties, especially among germplasms. Tillering ability of rice is an important parameter for developing new varieties of upland rice. The aim of this study was to identify the tiller profile diversity of upland rice germplasms in ICABIOGRAD Gene Bank.

2. Materials and methods

The experiment used 100 upland rice accessions, of which 10 were planted as lowland rice (Tabel 1). These accessions were originated from 16 provinces in Indonesia. The experiment was done in 2016 under greenhouse condition. Two seeds of each variety were sown in pots (35 cm height and 30 cm in diameter) containing 8 kg of dry soil. Each pot received optimal irrigation treatment and fertilized with 5 g of urea, 2 g of SP36 and 2 g of KCl. Experiments were arranged in a randomized completely block design with two replications. From the beginning of the tiller emergence, each type of tiller was marked with ribbon of different colors. The number of primary, secondary and tertiary tillers were observed weekly starting from 35 days after sowing (DAS) until 63 DAS (figure 1).

Table 1. Upland rice accessions used in characterization of tillering ability.

Code No.	Accession No.	Name	Origin[b]	Code No.	Accession No.	Name	Origin[b]
1	05020-20377	Misik A[a]	1	51	05020-9997	Maisuri	12
2	05020-19976	P. Nyuhu[a]	2	52	05020-20310	Merah	8
3	05020-20497	Umbang Putih[a]	1	53	05020-10272	Pulut Bambo	11
4	05020-15138	Pare Bakato Kaka[a]	3	54	05020-15442	Segajah	6
5	05020-20468	Gerunsai[a]	1	55	05020-20890	Pae Daye Indolobye	7
6	05020-20774	Rebo[a]	3	56	05020-19368	Parab	4
7	05020-20406	Amuntai[a]	1	57	05020-20489	Buntut Kuda	1
8	05020-20417	Pitik	1	58	05020-20924	Ketan Huma	3
9	05020-20991	Rendi Lau	2	59	05020-20426	Korundung	1
10	05020-8555	Munjaw	2	60	05020-20491	Ketan Tomang A	1
11	05020-19974	P. Timai	2	61	05020-20457	Juka	1
12	05020-20465	Merawi	1	62	05020-20731	Ikelo	3
13	05020-5511	Gombal	4	63	05020-20726	Ikiola	3
14	05020-15128	Unknown	3	64	05020-20408	Talun Undang	1
15	05020-19995	Padi Pulut Pute Iteung	2	65	05020-20414	Tokong	1
16	05020-20391	Lengkuas	1	66	05020-4282	Kapas B	1
17	05020-20418	Kahayangan	1	67	05020-20985	Dupa	2
18	05020-20397	Ketan Bahandang	1	68	05020-5205	Papah Aren	9
19	05020-20411	Lemo	1	69	05020-20432	Ketan Saru A	1
20	05020-9200	Ndabulu	5	70	05020-20791	Kemala Water	3
21	05020-15440	Sedang menawan	6	71	05020-19975	P. Pulut Timai	2
22	05020-20450	Talon Jangko	1	72	05020-20502	Humbang	1

Code No.	Accession No.	Name	Origin[b]	Code No.	Accession No.	Name	Origin[b]
23	05020-8294	Pulut Hitam	1	73	05020-20576	Segi	4
24	05020-20466	Talun Sarai	1	74	05020-20422	Sahang	1
25	05020-20581	Pusaka	4	75	05020-20492	Ketan Tomang B	1
26	05020-10479	Paredolo	5	76	05020-20504	Ketan	1
27	05020-12379	Padi Bangkok	4	77	05020-20416	Ketan Baburak	1
28	05020-20427	K. Puyuk	1	78	05020-4170	Si Gupai Kandang	8
29	05020-20018	Sipelang	8	79	05020-20409	Raden Kuning	1
30	05020-20746	Mama Laka	3	80	05020-20775	Muha	3
31	05020-20486	Barito	1	81	05020-20622	Way Rarem	4
32	05020-7950	Surau Parigi	5	82	05020-20467	Ketan Nyaling	1
33	05020-20506	Garu	1	83	05020-9205	Meeto	5
34	05020-5207	Genjah Mayangan	9	84	05020-20768	Nggondo	3
35	05020-20420B	Sangahi	1	85	05020-19980	Padi Saleng	2
36	05020-20500	Umbang Hitam	1	86	05020-20747	Madha Kedhi	3
37	05020-19369	Pare Sintung	4	87	05020-20419	Lanung	1
38	05020-20403	Padi Uwan	1	88	05020-20448	Satum	1
39	05020-20425	Boruk	1	89	05020-20442	Talun Bura	1
40	05020-5505	Segon	4	90	05020-12392	Padi Rabig	4
41	05020-20974	Limar	4	91	05020-19998	P. Turi	2
42	05020-4180	Panada	10	92	05020-20447	Ganahi	1
43	05020-9188	Sakaolutu	5	93	05020-9186	Wulu Mata	5
44	05020-20484	Gunsai	1	94	05020-9550	Ketan Hitam	12
45	05020-20472	Taun Suar	1	95	05020-6345	Markuti[a]	13
46	05020-20405	Siung Basalo	1	96	05020-7545	Si Tambak Padang[a]	8
47	05020-20429	Gadabung	1	97	05020-7390	Sicur[a]	14
48	05020-5247	Sewalan	9	98	05020-21658	Slegreng	15
49	05020-20485	Ti'ung	1	99	05020-21652	Lokal A	15
50	05020-20256	Bho	8	100	05020-INT	Cabacu	16

[a] Also planted as lowland rice in the experiment.
[b] 1 = Central Kalimantan, 2 = East Kalimantan, 3 = East Nusa Tenggara, 4 = West Java, 5 = Southeast Sulawesi, 6 = Lampung, 7 = North Sulawesi, 8 = Nanggroe Aceh Darussalam, 9 = D.I. Yogyakarta, 10 = South Sulawesi, 11 = Riau, 12 = South Sumatra, 13 = East Java, 14 = West Sumatra, 15 = Central Java, 16 = introduction.

3. Results and discussion

A typical primary, secondary and tertiary tiller type of rice is shown in Figure 1. Overall, the number of primary tiller increased slowly from 35 DAS until 63 DAS, whereas the number of secondary tiller increased sharply from 42 DAS to 49 DAS, but increased slightly after 49 DAS (Figure 2). Tertiary tiller started to grow at 49 DAS but its average number accross the accessions was far below the number of primary and secondary tiller.

The ability of plant to form tiller at an early growth stage will largely determines grain yield. All accessions were able to grow primary tiller at 35 DAS in a range of 1 to 10 tillers (Figure 3). The highest number of primary tiller (8) was obtained from three accessions: Pae Daye Indolobye (North Sulawesi), Padi Pulut Pute Iteung (East Kalimantan) and K. Puyuk (Central Kalimantan). Secondary

tillers were already observed at 35 DAS by >50 accessions, with the highest number (3) was expressed by Pae Daye Indolobye.

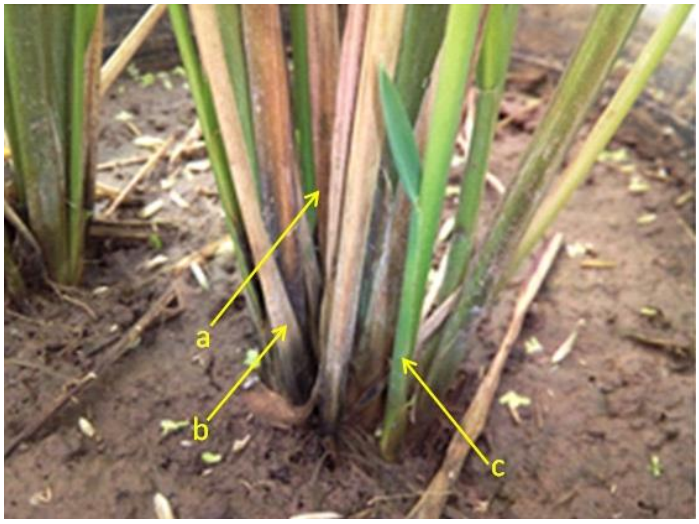

Figure 1. An example of primary (a), secondary (b) and tertiary (c) tiller of rice.

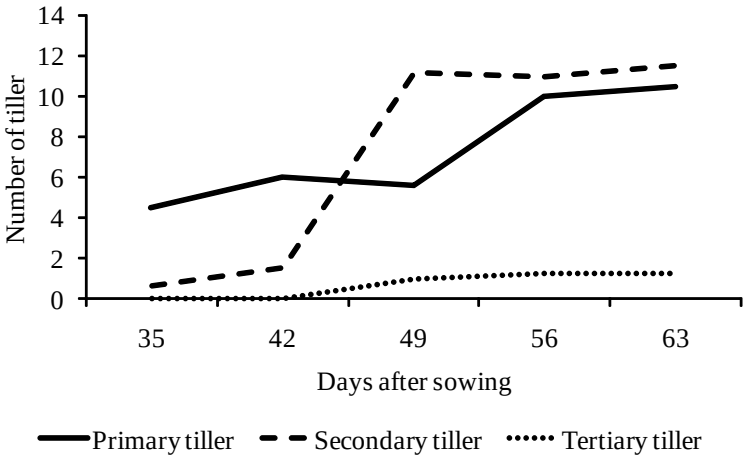

Figure 2. Growth profile of primary, secondary and tertiary tiller of 100 accessions of upland rice.

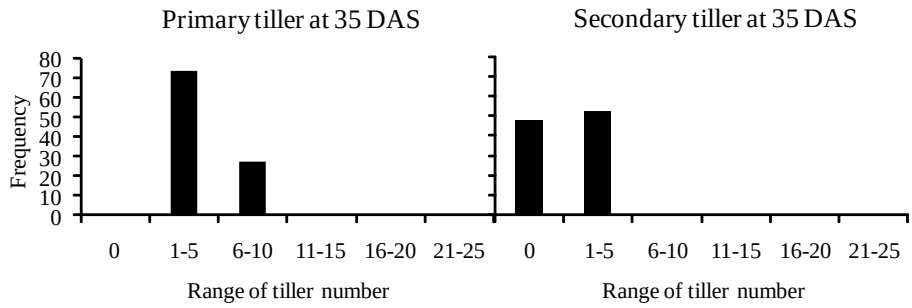

Figure 3. Histogram of primary and secondary tiller number in 100 accessions of upland rice at 35 days after sowing (DAS).

At 63 DAS the number of primary tiller ranged from 6 to 15, whereas a wider range number (1 to 20) of secondary tiller were observed (Figure 4). The majority of accessions (70) were able to grow tertiary tiller in the range of 1 to 6. Gadabung, an accession from Central Kalimantan, produced the highest number of secondary (22) and tertiary tiller (6) at 63 DAS.

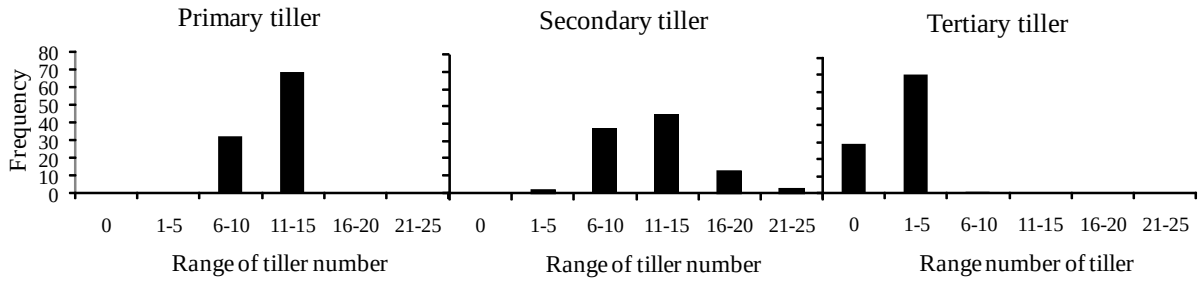

Figure 4. Histogram of primary, secondary and tertiary tiller number in 100 accessions of upland rice at 63 DAS.

Based on the observations from the experiment on 10 accessions which were grown in different soil environments, the tiller number on plants grown as upland rice was higher than those that were grown as lowland rice (Figure 5). From the data, it was found that the number of secondary tiller increased continuously and attained a maximum value at 49 DAS and 63 DAS for upland rice, and at 56 DAS for lowland rice. Generally, the number of tiller increase linerarly at 30 DAS until 70 DAS due to profuse tillering during vegetative growth and then decrease gradually due to plant death and plant aging [8]. The lowest number of panicle bearing tillers were due to mortality of non-effective side tiller [9].

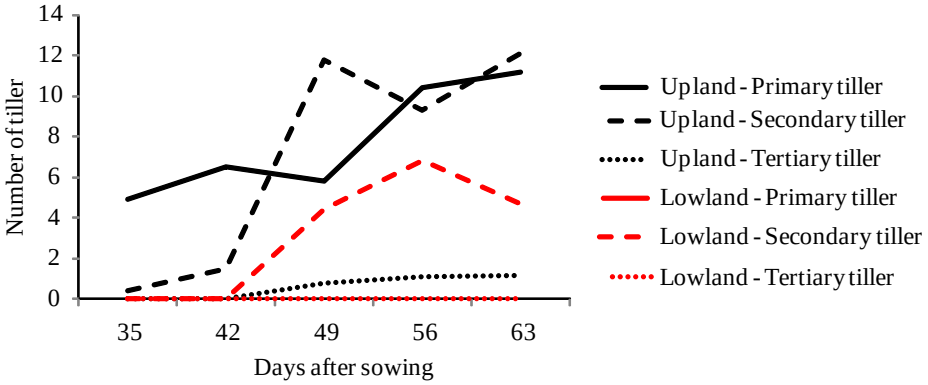

Figure 5. Growth profile of primary, secondary and tertiary tiller of 10 accessions planted as upland and lowland rice.

Differential response in tillering ability was observed among 10 accessions when grown in different soil environment. Under upland environment, Umbang Putih (Central Kalimantan), Rebo (East Nusa Tenggara), P Nyuhu (East Kalimantan) and Misik A (Central Kalimantan) had the highest number of primary tiller (12) during the vegetative phase, whereas under lowland environment, Sicur (West Sumatra) was able to produce 10 primary tiller (Figure 6). For the secondary tiller, the highest number (16) on upland environment was observed on Misik A, whereas that on lowland was expressed by Markuti (East Java).

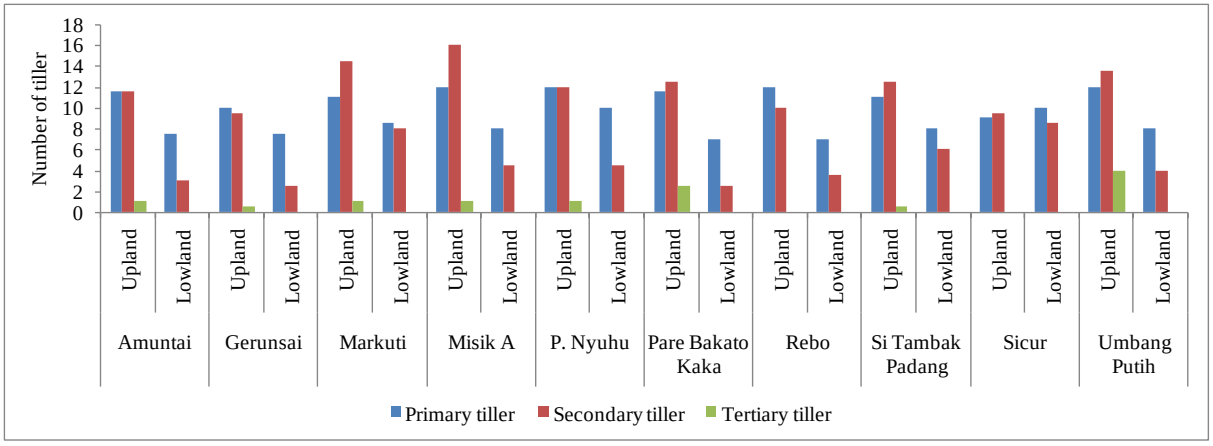

Figure 6. Mean number of primary, secondary and tertiary tiller of 10 rice accessions grown as upland and lowland rice.

During early vegetative growth, rice plant continuously forms new leaves in regular spatial and temporal patterns. The emerging tillers develop from axils of these leaves on the unextended nodes of the main shoot. The early initiated tillers on the main shoot also give rise to secondary tillers and secondary tillers produce tertiary tillers on their stem nodes [10].

Tiller production in rice is largely determined by environmental parameters, which supersede genetic features for expression of complete tillering ability [11]. Tiller development is regulated by a complex network of genetic, hormonal, and environmental factors, making tillering ability a highly plastic trait that allows wild cereals to adapt to different environmental conditions. It is also a major target for manipulation of plant architecture in breeding programs [12]. The formation of tillers plays an important role in determining grain yield, because it is closely related to the number of panicles of land area unit. Small tiller produce very little panicle, whereas too many tillers cause high tiller mortality rate, small panicle size and less optimal tiller, so the consequence is a lack of grain yield [13]. Indeed, reduced tillering has increased productivity in the domestication of maize [14]. In wild type rice plants, a tiller bud is normally formed at each leaf axil, but only those formed on the unelongated basal internodes can grow out into tillers and those formed on the elongated upper internodes become arrested. Secondary tillers are usually formed in wild-type plants, but higher-order tillers, such as tertiary, quaternary and quinternary ones are seldom developed [15].

4. Conclusions

The highest number of primary tiller during the vegetative growth (35 DAS) was expressed by Pae Daye Indolobye, Padi Pulut Pute Iteung and K. Puyuk, local upland rice accessions from North Sulawesi, East Kalimantan, and Central Kalimantan, respectively. Gadabung, an accession from Central Kalimantan had the highest number of secondary tiller (6) at 63 DAS. Information on the tillering ability of local upland rice provided in this study will benefit rice breeder for selecting breeding materials.

5. Acknowledgement

We are grateful to Dr. Chaerani and Dr. Sutoro from ICABIOGRAD for their thorough guidance in the writing process of this manuscript.

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Seed characteristics of local rice accessions from East Barito regency

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Abstract. Central Kalimantan has an abundance of local rice genetic resources scattered in every district. One of the districts with important local rice genetic resources is East Barito. This study aimed to seek unique characters ol local rice varieties in East Barito Regency which could be useful for further genetic improvement. Local rice exploration was conducted during January to February 2018 to obtain local species in the East Barito District. The method of activity is purposive sampling by selecting 4–5 villages within the district based on information of diverse local genetic resources. The data collected consisted of the passport data and the present status of local paddy rice, as well as the character of the seeds to ensure that there was a difference of accession for local paddy genetic resources with the same name. Based on the exploration activity, a total of 27 local rice accessions have been explored from the region, and there were two cultivar local rice accessions that have the same name, i.e. Cantik Manis and Siam Cantik. However, they have different grain shape and other morphological characters.

Keywords: seed, distribution, exploration, character.

1. Introduction

Central Kalimantan with a total area of about 154,000 km^2 has very diverse genetic resources. The genetic resources consist of food, medicinal, ornamental, vegetable, fruits and plantation crops. Based on its agroecosystem, in a dryland with wet climate, many types of fruits, medicinal plants and ornamental plants are found. Whereas in swampland, both tides, lowlands and peat, there are many vegetable crops and other types of food, especially local rice. There were 136 local rice varieties that have been inventoried, and most are found on non-garden land or open lands, such as rice fields and moorland [1].

Rice is widely cultivated throughout the Central Kalimantan and has become the first most crucial food after maize and soybean in terms of cultivation. It is rich in genetic diversity, with hundreds of varieties grown throughout the Central Kalimantan and its economic importance related to agroecological adaptation, household food security, customs, nutritional diversification, income generation and employment. Local rice is genetic resource which has not been paid attention, especially for the effort to purify it, to register and to use it as a source of parents in plant breeding program.

One of the regencies in Central Kalimantan that has quite a lot of genetic resources for rice plants is the East Barito District. Some of these local rice varieties which have unique characters, such as taste quality, have economic value, and are resistant to biotic and abiotic stress [2–6]. This paper presented the results of exploration of cultivars local rice from the East Barito Regency and the identification of the rice grain characters. The objective of this study, therefore, was to seek the unique characters for further rice genetic improvement.

2. Materials and methods

The field surveyed method was used in this study. The field survey conducted in three sub-districts (Patangkep Tutui, Paku and Dusun Tengah) and six villages (Bentot, Jango, Kotam, Tampa, Talohen Hulu and Netampin). The activity was carried out in January–February 2018, by collecting several local rice cultivars grown by local residents. Information about the local names of cultivars was extracted from local farmers through direct interviews or certain people who knew about it, such as village heads and agricultural extension workers. The interview method was done unstructured through simple discussions and conversations. The agronomic character of hull was measured for data collection. All data were analysed by the Analysis of Variance (ANOVA) procedure using SAS software version 9.1. Differences were declared statistically significant when $P < 0.05$. If significant differences were detected, the means were separated by the least significant difference (LSD) at 5% probability level.

3. Results and discussion

The results of exploration conducted in East Barito District indicated that the high genetic diversity of local rice found in this district. A total of 27 local rice cultivars were successfully collected (Table 1). Based on agroecosystem, 9 out of 27 local rice cultivars were collected from irrigated land agroecosystem, while 18 rice cultivars were collected from dryland agroecosystem. Local rice originating from Patangkep Tutui Sub-district was dominated by rice with dryland agroecosystem, and local rice originating from Paku and Central Dusun Sub-districts were dominated by irrigated land paddy. According to the Central Statistics Agency of East Barito Regency (2016), the height of the Patangkep Tutui Sub-district is about 60 m dpl that is higher than the other two districts, Paku Sub-district (15 m dpl) and Central Dusun Sub-district (26 m dpl). The high genetic diversity of paddy rice can be utilized in plant breeding programs to improve the rice variety that has not been used optimally to support the availability of released varieties, so that it can increase the contribution of paddy rice to the national rice production which is still very low, because of its relative low productivity [7].

One of the morphological characters observed in this study was the shape of grain or seeds. This was also conveyed by Lesmana et al. [8] that one of the morphological characteristics used as a differentiator in local rice cultivars is the shape of grain. The shape of grain is also related to the amount of starch content that is different for each cultivar [9].

Grain characters were observed in the exploration, such as color of grain, color of lemma and palea apiculus, color of milled rice, length of grain, weight of grain, thickness of grain, dan weight of 1,000 grains. The mean values of the grain characters evaluated in this study are shown in Table 2. The cultivars showed significant differences for these traits ($P<0.0001$) which suggest the existence of wide variation in the cultivars used in this study. The $LSD_{0.5}$ values indicate the occurrence of real differences among the accessions tested.

The mean of grain length among the cultivars was 0.187±13.452 mm, which ranged from 0.70–1.10 mm. The Dite Intem (2) had the most extended grain length (1.10 mm) followed by Dite Intem (1), Cantik Manis (1) and Cantik Manis (2). They were significantly different from others. Weight of grain significantly varied from 0.17–0.37 mm with a mean value 0.243±32.22 mm. Juntai had a higher value for the weight and thickness of the grain, 0.37 and 0.28 mm, respectively. The mean value of the thickness of the grain among 27 cultivars was 0.189±3.931 mm. Meanwhile, weight of 1,000 grains ranged from 12.00 to 32.00 g with a mean value of 20.537±24.083 g. Dite Intem (1) and Dite Intem (2) had the highest value for the weight of 1,000 grains. Significant variation was observed in the

agromorphological traits, z<0.2 characters of the local rice grain in East Barito Regency tends to be lean, only a few cultivars with rounded grain.

Tabel 1. Local rice cultivars collected from different agroecosystems in East Barito Regency.

Rice cultivar	Agroecosystem	Type of rice	Location (village name)
Tampeko (aromatic)	Upland	Upland rice	Bentot
Lengkong Lehat	Upland	Upland rice	Bentot
Dite Intem (1) (black sticky rice)	Upland	Upland rice	Bentot
Taring Palanuk	Upland	Upland rice	Bentot
Longkong Weat	Upland	Upland rice	Bentot
Hiwau	Upland	Upland rice	Bentot
Dite Intem (2) (black sticky rice)	Upland	Upland rice	Bentot
Juntai	Upland	Upland rice	Jango
Cantik Manis (1)	Upland	Upland rice	Jango
Mayas Putih	Upland	Upland rice	Jango
Lakatan Uban	Upland	Upland rice	Jango
Lampung Gajah	Upland	Upland rice	Jango
Cantik Manis (2)	Upland	Upland rice	Jango
Tamba	Upland	Upland rice	Jango
Tipung	Upland	Upland rice	Jango
Raden Gunung	Upland	Upland rice	Jango
Cantik Manis (3)	Upland	Upland rice	Kotam
Siam Cantik	Irrigated land	Paddy rice	Tampa
Palui	Irrigated land	Paddy rice	Talohen Hulu
Siam Unus	Irrigated land	Paddy rice	Talohen Hulu
Kerdil Jawa	Irrigated land	Paddy rice	Talohen Hulu
Siam Kupang	Irrigated land	Paddy rice	Talohen Hulu
Siam Cantik (1) (harvested using machine)	Irrigated land	Paddy rice	Talohen Hulu
Siam Cantik (2) (manually harvested)	Irrigated land	Paddy rice	Talohen Hulu
Gedagai	Irrigated land	Paddy rice	Talohen Hulu
Lakatan	Irrigated land	Paddy rice	Talohen Hulu
Mainai	Irrigated land	Paddy rice	Netampin

Table 2. Qualitative dan quantitative characters of local rice cultivars in East Barito Regency.

Rice cultivar	Color of grain	Color of awn and steril lemmas	Color of milled rice	Length of grain	Weight of grain	Thick-ness of grain	Weight of 1,000 grains
Tampeko	Dark brown	Dark brown	Clear yellow with scented pandanus	0.80 e	0.25 b	0.21 a	21.50 b
Lengkong Lehat	Yellow straw	Yellow straw	Clear yellow	0.80 e	0.30 b	0.19 b	22.00 b
Dite Intem (1)	Brown	Brown	Black	1.00 a	0.25 b	0.19 b	29.00 a
Taring Palanuk	Yellow straw	Yellow straw	Clear white	0.70 g	0.30 b	0.18 b	19.00 b
Longkong Weat	Yellow straw	Yellow straw	Clear red	0.70 g	0.30 b	0.21 a	21.00 b
Hiwau	Yellow straw	Yellow straw	Clear red	0.80 e	0.20 c	0.19 b	18.00 b
Dite Intem (2)	Dark brown	Dark brown	Black	1.10 a	0.25 b	0.19 b	32.00 a
Juntai	Yellow straw	Yellow straw	Clear white	0.86 d	0.37 a	0.28 a	25.00 a
Cantik Manis (1)	Tawny	Light yellow	Clear white	0.98 b	0.20 c	0.178 b	18.00 b
Mayas Putih	Yellow straw	Yellow straw	Clear white	0.80 e	0.30 b	0.18 b	18.00 b
Lakatan Uban	Dark brown	Yellow straw	Clear yellow	0.90 d	0.25 b	0.20 b	20.50 b
Lampung Gajah	Light yellow	Light yellow	Clear white	0.95 b	0.25 b	0.20 b	23.00 b
Cantik Manis (2)	Brownish-yellow	Light yellow	Clear white	1.00 a	0.17 c	0.19 b	19.00 b
Tamba	Yellow straw	Yellow straw	Clear white	0.90 d	0.20 c	0.19 b	28.50 a
Tipung	Yellow straw	Browning yellow	Clear white	0.80 e	0.30 b	0.17 b	23.50 b
Raden Gunung	Yellow straw	Brown	Clear white	0.90 c	0.30 b	0.20 b	22.00 b
Cantik Manis (3)	Brownish-yellow	Light yellow	Clear white	1.00 a	0.18 c	0.19 b	17.50 b
Siam Cantik	Yellow straw	Light yellow	Clear white	0.80 e	0.25 b	0.17 b	17.00 b
Palui	Yellow straw	Yellow straw	Clear white	0.70 g	0.20 c	0.18 b	15.00 c
Siam Unus	Yellow straw	Yellow straw	Clear white	0.80 e	0.20 c	0.18 b	16.00 c
Kerdil Jawa	Yellow straw	Yellow straw	Clear white	0.70 g	0.20 c	0.17 b	19.00 b
Siam Kupang	Yellow straw	Yellow straw	Clear white	0.75 f	0.20 c	0.18 b	12.00 c
Siam Cantik (1)	Yellow straw	Light yellow	Clear white	0.80 e	0.20 c	0.17 b	14.50 c
Siam Cantik (2)	Yellow straw	Light yellow	Clear white	0.80 e	0.20 c	0.17 b	18.00 b
Gedagai	Yellow straw	Light yellow	Clear white	0.95 b	0.25 b	0.20 b	22.00 b
Lakatan	Brownish-yellow	Light yellow	Milky white	0.70 g	0.30 b	0.19 b	21.50 b
Mainai	Reddish-brown	Yellow straw	Clear white	0.85 d	0.20 c	0.18 b	22.00 b
Mean ± SE				0.187 ±13.452	0.243 ±32.22	0.189 ±3.931	20.537 ±24.083

The yellow straw was dominant of hull color and part of the end of the hull, except for Mainai and Lakatan (Figure 1 and 2). While the polished rice is dominating color for milled rice, except for Lengkong Weat, Hiwau and Lakatan Uban. Desrosiev [10] mentioned that most rice farming carried out in developing countries uses varieties that are appropriate to their environment, and the land to be planted in rice is fully processed and then planted with rice seedlings by means of transplanting.

Figure 1. Mainai and Lakatan hulls.

Figure 2. Dite Intem and Juntai hulls.

The rice produced from each variety is different, some are red, black or purple. The color of rice grain can be given by various things such as red rice seeds that give a reddish color. Anthocyanin color and pigmentation in rice hull apiculus are the factors that affect all aspects of the rice quality in brown rice when harvested, but the compilation of the outer layer of bran that is rich in nutrients is removed, so the rice turns white. Thus, the color of rice grain in addition to being an attraction as well as an indicator of the nutritional content of the rice [11,12].

Figure 3. Local rice cultivars (rounded grains).

Figure 4. Local rice cultivars (long grains).

According to Grubben and Partohardjono [13], differences in grain shape indicate the genetic diversity of the cultivars. Local rice cultivars that have rounded grains (Figure 3) included Lengkong Lehat, Taring Palanuk, Lengkong Weat, Juntai, Mayas Putih, Tipung, Raden Gunung and Lakatan are thought to belong to the *japonica* or *javanica* subspecies. Meanwhile, other cultivars are dominated by long grain shape and slim shape, belonging to the *indica* subspecies (Figure 4). In addition, there were cultivars having the same local name but have different grain shape, such as Dite Intem, Cantik Manis and Siam Cantik. This confusion of naming may occur because farmers often plant more than one cultivar, therefore, it is possible that the seeds are mixed. Taken together, such exploration and characterization of local varieties could be useful for their optimal utilization in tehr future.

4. Conclusions

East Barito was dominated by upland rice with white rice type. The grain characters that use to exploration are color of grain, color of part of the end of grain, color of milled rice, length of grain, weight of grain, thickness of grain dan weight of 1,000 grains. Yellow straw color was dominating grain color. Long grain was the most dominant character found for local rice in this area.

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The problems of *ex situ* genetic conservation at the universities in developing countries: lesson learn from Universitas Gadjah Mada

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Abstract. Agriculture faces enormous challenges for providing sufficient food, feed and fuel raw materials for a growing global population. In the case of food, for instance, global food production must always increase to meet the projection of continuously increase of global food demand. The future challenges of food supply and demand can be addressed by diversification of food sources, introducing high yielding cultivars and improving culture techniques. Food sources can be diversified by collection and evaluation of genetic resources for nutritive values. In contrast, new varieties can be developed through breeding activities that also require genetic resource as genetic material. Genetic resources spread around the world, and to optimally utilize, genetic resource must be explored and conserved both by *in situ* and *ex situ* approaches. The genetic resource exploration through missions requires proper preparation including human resources, logistics and time allocations. Universitas Gadjah Mada (UGM) as a higher education institution has three big university missions, i.e. education, research and community service through student involvement. These three missions through student involvement have been applied to conduct the genetics resource exploration and *ex situ* conservation. The course of genetic resource collection and management has been introduced at different faculties, and because community service at the rural area for two-month times is compulsory for the student, UGM makes use of student to carry out genetic resource exploration and collection. The student must collect the passport data for the genetic resources and send the data to the Agriculture Innovation Center (AIC). In case that seed of genetic material can be found, student must collect also seeds and send to AIC for *ex situ* conservation. Based on UGM experience, *ex situ* conservation, especially seed genebank, faced sustainability problem due to insufficient human and funding resources. UGM integrates some approaches such as crop focusing, networking, student involvement in the characterization and evaluation, and breeding activities to solve such problems.

Keywords: genetic resources, *in situ* and *ex situ* conservation, student involvement.

1. Introduction

Agriculture, as a poineering technology that has a remarkable impact on the planet, has been the foundation of socioeconomic progress. Agriculture will continue to play a significant role in development. Agriculture faces enormous challenges for providing sufficient food, feed and fuel raw

materials for a growing global population [1]. Global food production must always increase to meet the projection of continuously increase in food demand. To increase crop production, many agricultural activities are unsustainably carried out due to the overuse or misuse of agrochemicals, irrigation water, fertilizer and other inputs, and the loss of crop rotation. Climate change is a very considerable environmental threat likely to affect the ecosystem and its production potential, and the dynamics of pest and diseases, therefore, it reduces agricultural productivity. The future challenges of food supply and demand can be addressed by diversification of food sources, introducing high yielding cultivars and improving culture techniques. Food sources can be diversified by collection and evaluation of genetic resources for nutritive values. In contrast, new varieties can be developed through breeding activities which also require genetic resource, viz. any genetic material of organism with actual and potential value for human use. Plant breeding should remain a vital component of effective agricultural systems. Plant breeding has contributed to increasing productivity by systematically creating new genotypes with superior adaptation to needs of society, the resource of the production system and the demand of nature in the target environment [2]. Breeding activities successfully improve genetic resource performance with continuously increasing yield potential and adaptation to management practice, because genetic resource provides the building blocks to improve productivity, biotic resistance and abiotic tolerance. Genetic resources spread around the world, and in order to optimally utilize, the genetic resource must be explored and conserved in appropriate ways.

2. Genetic resource conservation at Universitas Gadjah Mada

Genes are the blueprint of organisms and breeding is the art and science of organism improvement which its product can feed the world [3], and the effective use of genetic resource in breeding relies on a thorough understanding of the existing diversity. Genetic diversity plays an essential role in food security, nutrition and sustainable intensive agriculture [4]. Genetic resource as sources of genes can be found everywhere around the world, however, due to deforestation, population growth and climate change, the genetic resource can be eroded. Genetic resource, therefore, must be appropriately conserved. Several types of arguments are used to promote genetic resource conservation, such as species as a valuable resource for humanity, both nowadays and in the future; species play significant role in maintaining a stable environment, the scientific value of species and the opportunity to study and determine ecological processes.

There was a broad consensus on some of the principles of genetic conservation which include (a) high levels of genetic diversity within populations are almost always desirable to ensure that they are genetically sustainable, (b) adaptability is correlated with diversity and should be an essential driver for conservation in response to environmental change, (c) genetic diversity is broadly associated with population size; hence conservation should seek to maintain or create large populations, (d) low levels of genetic diversity are detrimental to populations when they lead to inbreeding depression but can be of particular scientific interest and may indicate ongoing evolution and speciation, (e) gene flow between populations is desirable, but care may be required where small populations have been isolated for an extended period and local adaptation may be swamped, and (f) action to increase landscape permeability for one species may be adverse for another, but what is suitable for most species should take precedence. The concept of genetic resource conservation must capture maximum variation [5].

There are several methods available for preserving crop genetic resources, and they are all necessary for overall, sustainable conservation, however, the standard genetic conservation methods include *in situ* and *ex situ* approaches at local, national and international levels [6].

In situ conservation is a method for preserving crop species, especially crop wild relatives, in nature. *In situ* locations are usually in protected areas, like nature reserves and other places with restricted access. *In situ* methods are necessary for sustainable and useful conservation of essential and potentially critical genetic resources and cultures in which they are embedded. The advantages of *in situ* conservation include preservation of indigenous knowledge; conservation is linked with use and change in the field; allelic richness and genotypic diversity; unique adaption; localized divergence and diversity to meet temporal environmental variation; continuing crop evolutionary process; human

involvement; dispersed sharing of benefits derived from genetic resources. Whereas, the disadvantages are as long as genetic resource remains in the hands of the farmer; it is not directly useful for breeders; farmers can not be trusted to maintain valuable genetic resources; *in situ* conservation is not popular with breeders because of a long and tortuous road that genetic resource must travel between the field and the breeding program; as long as conservation and improvement are directly linked, conservation will be judged by its short-term benefits. Therefore, an ideal area of *in situ* conservation would be peasant farmers (direct land to mouth agriculture), active community, an area of high environmental heterogeneity and ecological complexity, in an area of landrace crop origins or secondary center of crop diversity, and the area should also have the support of agricultural extension services and some market availability for farmers to sell local crops. Although *in situ* conservation represent the most effective way to protect endangered species, it is also evident that not all species can be efficiently preserved at their natural habits [7].

Table 1. Universitas Gadjah Mada field genetic resource conservation.

Field name	Size (ha)	Crop species	Field function
Pagilaran Tea Plantation	1,100	Tea Coffee Quinone	Collection and production
Segayung Cocoa Plantation	160	Cocoa Coconut	Collection and production
Samigaluh Tea and Cocoa Plantation	5	Cocoa	Collection and production
Gunungkidul Wanagama	600	Pinus Eucalyptus	Collection and production
Berbah Agrotechnology Innovation Center	35	Arrow root Breadfruit Cassava Cattle Catfish Corkfish Deer Durian Litchi Orchid Papauw Sauerfruit Sheep Starfruit	Collection
Mangunan Agrotechnology Innovation Center	151	Arrowroot Banana Eucalyptus Ginger Gmelina Glerecidae Jackfruit Pinus Sauerfruit Squamoya Zingiber	Collection

The *ex situ* conservation is the conservation and maintenance of species outside their natural habitat and is used to safeguard population in danger of destruction, replacement and deterioration [5]. *Ex situ* methods are designed to maintain the genetic material in the state in which it is collected, to avoid loss or degeneration of a variety. *Ex situ* methods give rise to one type of diversity because the selection is directed by crop science and commercial/public breeding interests [8]. Approaches to *ex situ* conservation include methods like field genebanks and botanical garden, seed bank and gene libraries.

Field genebank means that accessions in the form of seedlings or clonally propagated species are conserved by cultivating in the open field or pot. Field genebank is the most suitable genetic resource conservation approach for animals and perennial plant which clonally propagated, such as banana, tea and cassava. Field genebank must also be fitted to sexually propagated crops which seeds show recalcitrant characteristic (Table 1). Such conservation can be categorised as static and evolutionary techniques [9]. Static conservation is applied in connection with the relatively intensive breeding program, where identified and characterized genotype are clonally propagated and kept in the clonal archive. In contrast, evolutionary conservation is the opposite to static conservation in the sense that it aims to support genetic changes to the extent that they contribute to continued adaptation. Evolutionary conservation is characterised by plant reproduced by seed in successive generations, and genetic variation between populations from different environments is in general maintained and expected to increase over time [10].

The seed of some species can maintain their initial high germination capacity for many years, and this seed is categorized as orthodox one. Such orthodox seed can be dried and stored at low temperature for a long time. A seedbank is the most conventional for long-term conservation of the genetic resource. Once a seed is kept in refrigeration, it is isolated from the evolutionary process. Seedbank represents the most effective *ex situ* conservation strategy [11]. However, seedbank requires not only essential infrastructure for short and long-term seed storage, but also efficient management of back up regeneration, characterization and evaluation, and data management [12].

At Universitas Gadjah Mada (UGM), the importance of seedbank has been recognised since the university was established. When the Faculty of Agriculture was initiated in 1946, “selection” became one of the sections with the purposes was to conserve and utilize genetic resources around Yogyakarta. To support such purposes, rice seedbank with convenient facilities has been built, and some foreign breeders were invited as a professional expert. The facilities such as coolroom, drying field, screenhouse and seed processing unit could be seen till 1998 at Bulaksumur campus complex of UGM. In 1978, Agriculture Training, Research, and Development Center (ATRDC) was established at Kalitirto, Berbah, Sleman, Yogyakarta, and rice accessions collection was transferred from Bulaksumur to Kalitirto due to better facilities. Old seedbank facilities at Bulaksumur was then used for the winged bean breeding project. The coolroom in Bulaksumur was broken in 1981 and, as a result, winged bean accessions were lost.

In 1998, Bulaksumur campus complex for agriculture was reconstructed; therefore, all seedbank facilities were destroyed. Screenhouse has been moved to Banguntapan, while new coolroom was placed at the new building. New building for the agricultural complex was inaugurated in 2004; however, the coolroom for seedbank was not well performed. The seedbank at ATRDC was also broken almost at the same time. Seed accessions were stored at the refrigerator at different laboratories, and fortunately some accessions still exist until now (Table 2).

In 2015, ATRDC and UGM Mangunan Girirejo Field were merged as Agrotechnology Innovation Center of UGM (AIC-UGM). AIC-UGM was appointed as tropical vegetable genebank through the collaboration between UGM and East West Seed Indonesia (Ewindo). Seedbank of ATRDC will be repaired and enlarged to store some important tropical vegetable seeds such as aubergine, chili pepper, cucumber and yardlong bean, therefore, AIC-UGM will become one of national seedbank focused on the tropical vegetable.

Table 2. Universitas Gadjah Mada seedbank conservation.

Institution name	Crop species	Utilization
Kalitirto Agrotechnology Innovation Center	Aubergine Cucumber Greenbean Hot chili pepper Rice Maize Peas Soybean Tomato Yardlong bean	Collection and some breeding works
Faculty of Agriculture	Rice Maize Peas Tomato Soybean Garlic	Collection and breeding
Faculty of Biology	Rice Maize Strawberry Melon	Collection and breeding

Table 3. Number of province and student for community service in 2014–2018.

Year period	Number of		
	Province	Village	Student
2014	23	280	7,331
2015	27	264	7,077
2016	31	251	6,695
2017	231	231	6,733
2018	221	221	6,279

Source: Community Service Directorate of UGM, 2018 (personal communication).

3. Student involvement in genetic resource

Genetic resource collection can be expensive, and the fund is usually very limited [3]. UGM as a higher education institution has three big university missions, i.e. education, research, and community service, through student involvement. More than 6,000 students with enough knowledge will be sending and stayed at the community both at the urban and rural areas at least two months for community services (Table 3). Several students come from the faculty of agricultural complex, biology, and pharmacy. Such student can conduct the genetic resource exploration and *ex situ* conservation because the course of genetic resource collection and management has been introduced at such different faculties. To make sure that everything will go properly, AIC-UGM in 2018 prepares accession passport and carries out workshop before departure. The student must then collect the

passport data for the genetic resources and send the passport data to AIC-UGM. In the case that seed of genetic material can be found, AIC-UGM also provides seed bags for the student. The student must collect the seeds and send them to AIC for *ex situ* conservation.

4. Problems in *ex situ* genetic resource conservation and their solutions

Based on Fu [13], ten most challenging issues of *ex situ* conservation including insufficient funds, facilities and staff; costly activities requiring laboratories, land, labor, material resources and complex financing for maintaining seed viability; diluted political support; inadequate characterization and evaluation of genetic resource; lack of updated genebank information systems; incomplete diversity coverage; deteriorating genebank support; unbalanced support; lack of professional staff and genebank collapse. Based on UGM experience, *ex situ* conservation, especially genebank, faced sustainability problem due to insufficient human and funding resources. Therefore, UGM integrates some approaches such as crop focusing, networking, student involvement in the characterization and evaluation, and breeding activities to solve such problems.

Crop focusing depends on institution levels at the university. AIC-UGM will focus only to species, which do not become the focus of the faculty or other related institutions in the university. Faculty of agriculture, for instance, has long experience working in tomato and garlic. Therefore, AIC-UGM will work on other vegetable crops such as aubergine, cucumber, hot chili pepper, cosmos, wing bean and yardlong bean. Pagilaran estate plantation grows intensively tea and cocoa tree crops, but fails to work on breeding, nursery and organic fertilizer-based development, AIC-UGM will then intensify on such issues. To speed up the research progress, financial support is given and to improve the research organization. The government also appoints AIC-UGM as a center of excellence for agrotechnology innovation.

The success of breeding programs depends on germplasm [14–16] which can be enriched through an exchange by networking. Networking can be carried out at the national and international levels through correspondences, seminar and symposium, workshop, consortium meeting, publication and collaboration. Correspondence to individual expert and related institutions is an important step to start networking. Although it sometime fails of success and it needs some time, but it must be executed diligently. Seminar and symposium even workshop can be the best way to connect people with similar interest. The attractive theme will encourage many people to participate in the event. After succeeded in correspondence and organizing seminar, symposium and workshop, involvement at the consortium will become a further step to writing useful publication and at the end will produce sustainable collaboration. With focusing on only several crop commodities, networking can be quickly done because AIC-UGM will be easily recognized worldwide based on these commodities. Global collaboration to conserve as much plant diversity as [17] will become an effective and efficient practice for plant genetic resource conservation and management for future generation [18].

A lot of genebanks find difficulties to respond effectively an accession requirement due to inadequate characterization and evaluation [19] which require many human resource or technology investments. The advantage of universities related to human resources is their availability to research because research is compulsory even for bachelor student. In term of the student body, UGM has the most significant number of student in Indonesia and the involvement of the student in the characterization and evaluation of collected accessions will improve the database of every accessions. As a result, accession duplication can be avoided, and the usefulness of the accessions will increase.

Cultivar development through breeding work may be considered as a type of university start-up, which means that university can earn revenue from such activity [20]. With a more complete database of the accessions, breeding works can be conveniently conducted. Breeding duration time probably can be shortened. The economic value of genetic material will be realized. If the wide yielding varieties are frequently developed and released from the collected accessions, and the majority of the growers cultivate these varieties, the funding problems might be solved.

5. Conclusions

UGM has recognized the importance of *ex situ* conservation of genetic resources since it was established. The genetic resource was conserved in *ex situ* collections in the form of fieldbank and seedbank. Seed genebank faced sustainability problem due to insufficient human and funding resources and UGM integrates some approaches such as crop focusing, networking, student involvement in the characterization and evaluation, and breeding activities to solve such problems.

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Diversity of pod weight of Indonesian local groundnut (*Arachis hypogaea* L.) varieties at different harvesting time

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Abstract. Groundnut, a self-pollinated legume, is an important cash crop which has high oil seed content and provides nutritious fodder to livestock. Harvesting time is very critical to the groundnut production, because their maturity determines the pod weight. This study aimed to observe the pod weight of local groundnut varieties of Indonesia at different harvesting time. A total of 50 local varieties groundnut were grown at ICABIOGRAD Experimental Station from May–September 2015. The pods were harvested at 75, 85 and 95 days after planting (DAP) and weighed. The results showed that the accessions reached its physiological maturity at 85 DAP, where the plants reached the highest maturity index (2.28). The average maturity index at 75 and 95 DAP was 1.64 and 1.84, respectively. The pod weightat 75, 85 and 95 DAP ranged from 1.0 to 31.4 g, 4.2 to 35.2 g and 2.0 to 31.6 g, respectively, whereas the seed weight ranged from 1.0 to 20.8 g, 2.2 to 26.4 g and 1.4 to 24 g, respectively. A relatively high seed and hull weight ratio at 85 DAP was found in MLG 1629 Jombang, MLG 7552 Gresik, Hoi Ambon, Lanbau and AH 842Si varieties. The harvesting time should be done at physiological maturity to identify the maturity type among local varieties. It is recommended that the Indonesian local varieties of groundnuts should be harvested at 85 DAP.

Keywords: groudnut, harvesting time, pod weight, seed.

1. Introduction

Groundnut (*Arachis hypogaea* L.) is an essential crop in Indonesia because of its high oil seed content. The crop is grown both as a cash and food crop. Some significant constraints of groundnut production and productivity include the lack of improved varieties, poor cultural practices, insect pests, diseases, weeds, drought, and the non-timely execution of agronomic practices [1,2]. The goal of most groundnut breeding program is to increase yield. Characterization of harvesting time or maturity stage of the crop is an important step in obtaining promising breeding materials.

The time of harvest can contribute significantly on yield and productivity, but most farmers are not aware of groundnut maturity time for harvesting. Generally, local groundnut varieties are harvested at 80–85 days after planting (DAP) for the food industry, whereas the harvesting time is delayed up to 90–100 DAP for industrial seed. Several released variety, such as Domba and Panther, were harvested at 100 DAP, while Badak variety could be harvested at 105 DAP [12].

The impact of late harvesting time are less water content and high percentage of mid-mature kernels [3]. The physiological maturity stage is a recommended measure for determining the harvesting time to obtain high pod yield and kernels quality [4]. Therefore, determination of harvest time is essential as it contributes to yield and productivity. The differences in maturity time were at two levels: between the maturity group of genotypes and between the genotypes within each maturity group [13].

Early harvesting time of groundnut could reduce yield by 15% and economic value by 21% [5]. Harvesting groundnuts too early resulted in immature nuts, low yields, and off-flavors [6] with many immature seeds [3]. Groundnut plant can sustain and develop pods with less than 15% to 20% of flowers produced mature pods. On the other hand, late harvest affects the pod and other yield components. Delaying harvesting time could increase yield, mature kernels, shelling percentage, pod number and pod yield per plant, 100-seed weight, oil and protein contents, and O/L ratio [7–9]. In contrast, delaying harvesting time may also increase yield loss. Premature harvesting reduced yield and kernel quality by 16–25% and delayed harvesting resulted in 30–40% yield losses [4]. Genotype with the highest harvest index is characterized by high yield, performances morphology, weight per pod and number of pod per m^2 [13].

Optimal harvesting time is critical for obtaining high yield of local groundnut varieties as it can reduce yield losses. The late-maturing group tended to have a slightly higher pod weight than the early- and the medium-maturing groups [13]. Information on maturity traits responsible for differences in yield performance among local groundnut genotypes is still lacking. This data can be used to develop appropriate strategies for varietal selection that could improve groundnut yield. Therefore, this study aimed to observe the pod weight performance of Indonesian local groundnut varieties at various harvesting time.

2. Materials and methods

A total of 50 accessions of local groundnut varieties and lines were grown at the Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD) Experimental Station during the dry season of May–September 2015. One seed of each cultivar was sown in 4 rows within 2 m × 3 m plots at a planting distance of 40 cm ×20 cm. The land was disked and harrowed before planting following the procedures for yield trials land preparation. Fertilizers were applied at a dose of 50 kg/ha of urea, 100 kg/ha of P_2O_5 and 75 kg/ha of KCl. Pesticides and fungicides were applied as necessary following the recommended procedures. The standard cultural practices were carried out during the whole growing period [10]. The experiment was well managed in order to avoid drought, nutrient and other stresses. Plants were uprooted at 75, 85 and 95 days after planting (DAP) and observed for yield components, such as pod weight, seed weight, pod number and maturity index. Maturity index is the ratio of seed weight to hull weight.

3. Results and discussion

Harvesting time at physiological maturity resulted in higher groundnut pod and kernel yield compared toharvesting at 75 and 95 DAP, indicating that different harvesting time could affect pod and kernel yield. An early harvesting time can reduce the number of pods per plant which lead to low pod and kernel yields. Pod yield of grondnut varieties directly related to kernel yield [4]. On the other hand, pod maturity can be determined by measuring changes in kernel weight and hull weight during maturity, chlorophyll content, amino acid content and maturity index [3].

In this study, groundnut accessions showed different maturity index at different time of harvest.The maturity index for each accession showed maximum and minimum rates, where the maximum value determines the harvesting time. At 75 DAP, several local varieties had a range of maturity index of 0.50 to 2.78 with an average of 1.6. The other varieties showed a range of maturity index of 0.78 to 3.85 with an average of 2.28 at 85 and 0.35 to 3.16 with an average of 1.84 at 95 DAP (Figure 1 and 2).

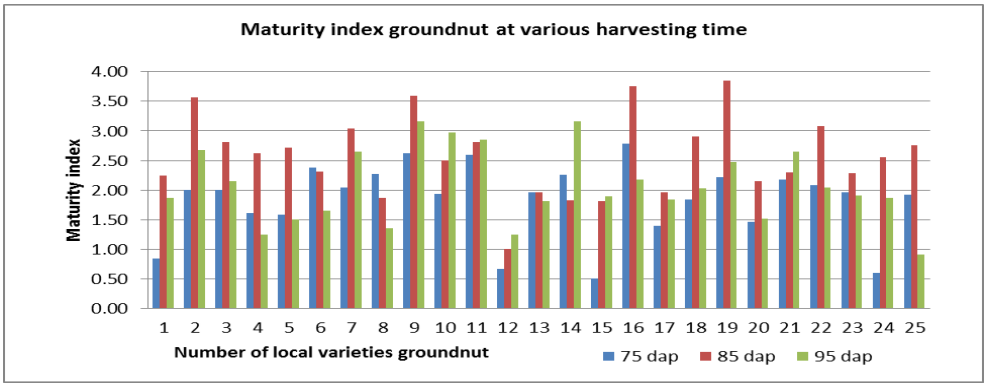

Figure 1. The maturity index of local groundnut varieties number 1–25 at 75, 85 and 95 days after planting (DAP).

Harvesting time determines groundnut pod and kernel yield. Harvesting time at physiological maturity resulted in higher groundnut pod and kernel yield compared to harvesting at early and late physiological maturity. An early harvesting time reduced the number of pods per plant which lead to low pod and kernel yields. On the other hand, delayed harvesting resulted in lower maturity index in some accessions, such as AH 1643 Si (number 10) Banjar, AH 1647 Si (number 11) Karangasem, AH 1656 Si (number 12) Tampaksiring Bali, AH 1681 Si (number 14) Magetan, and lines such as AH 1033 Si (number 47) and AH 1044 Si (number 49) (Figure 1 and 2). All local varieties and lines may show physiological maturity at 80–85 DAP, where they showed 75% mature pod brown and black pod color and maximum seed hull maturity index (2.0–3.4) [3].

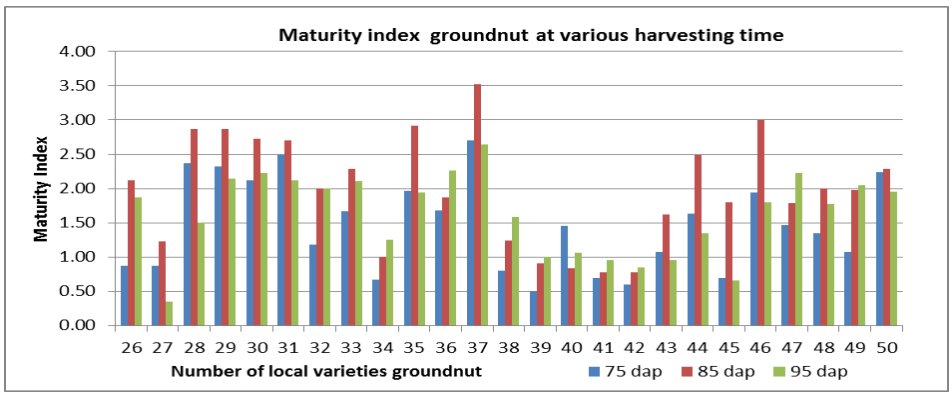

Figure 2. The maturity index of local groundnut varieties number 26–50 at 75, 85 and 95 DAP.

Groundnut is mature when 70–80% of the inner layer of the pods shells turn dark, and the kernels are plump. As reported previously, significant reductions in groundnut yields could occur as a result of premature or delayed harvest [11]. The same result of lower pod yield occurred on other groundnut varieties in Africa when harvested beyond its physiological maturity [4]. Also, harvesting time affect the kernel quality [7,9].

Pod yield of groundnut varieties were positively correlated with kernel yield [4]. The kernel weight harvested at 85 DAP were higher than the kernel that was harvested at 75 and 95 DAP (Table 1). Thus, the kernel weight of local varieties tended to reduce at either early or late harvesting time.

Table 1. The average and range of pod, kernel and hull weight of 50 local groundnut varieties at three harvesting time.

Yield component	75 DAP	85 DAP	95 DAP
Pod weight (g)	14.06 (1.00–3 1.40)	18.15 (4.20–35.20)	18.50 (2.00–31.60)
Kernel weight (g)	9.17 (0.40–20.80)	12.97 (2.20–26.40)	12.32 (1.40–24.00)
Hull weight (g)	4.89 (0.60–10.60)	5.18 (1.60–9.40)	6.18 (0.60–11.40)

DAP = days after planting.

The average kernel weight of local varieties was 9.2, 13.0 and 12.3 g at 75, 85 and 95 DAP, respectively, which suggested lower kernel production at the early harvesting time (Figure 3). However, several accessions produced the highest kernel weight at early harvesting time. The highest kernel weight at 75 DAP was observed on Mlg 7641 Lamongan and Mlg 7548 Tuban lines, whereas at 85 DAP was on Mlg 7579 Probolinggo and AH 1029 Si lines, and at 95 DAP was on Mlg 7579 Probolinggo and AH 1037 Si lines. The highest kernel weight was obtained when pods were fully filled and reached maturity. Early harvested pods were characterized by immature, shrink and small kernels, whereas the late harvested ones sprouted and had high incidence of insect attack on both kernel and pod [4]. Also, the impact of late maturity are less water content, high percentage of mid-mature pods and potential contaminant of *Aspergillus flavus* [3].

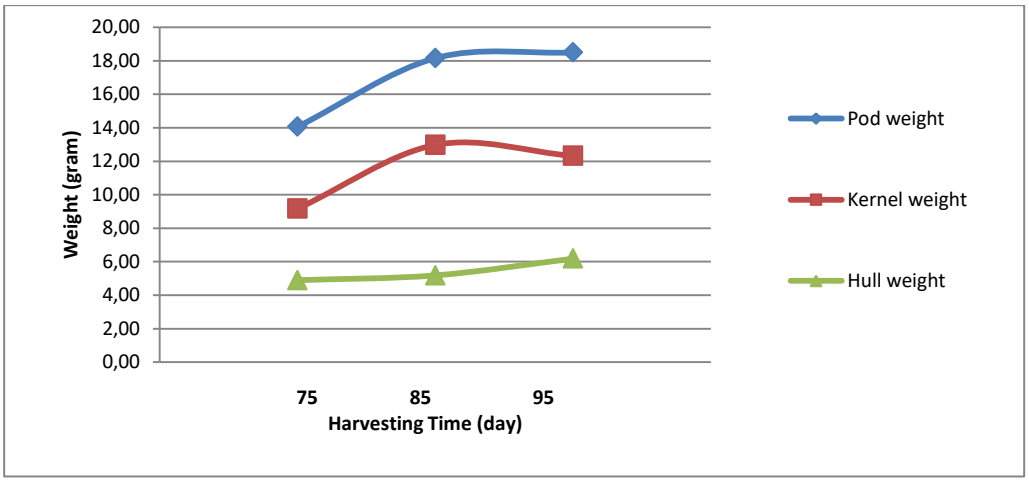

Figure 3. The average pod, kernel and hull weight of 50 local groundnut varieties at three harvesting time.

Harvesting time of groundnut varieties at physiological maturity produced high pod yield. In this study, pod, kernel and hull weight at 85 DAP were higher than those at 75 and 95 DAP (Figure 3). The pod weight harvested at 75, 85 and 95 DAP ranged from 1.0 to 31.4 g, 4.2 to 35.2 g and 2.0 to 31.6 g, respectively. The local varieties Mlg 7641 Lamongan and Mlg 7548 Tuban had high pod weight at 75 DAP, and Mlg 7579 Probolinggo and AH 1029 Si lines at 85 DAP. Mlg 7548 Tuban and Mlg 7579 Probolinggo still showed high pod weight when harvested at 95 DAP. This results affirmed the previous study that high pod weight were mainly recorded at physiological maturity for all local groundnut varieties [4]. The studied groundnut lines have less number of days to 50% flowering and had more number of mature pods at maturity. However, the local varieties with the highest pod weight showed longer period of seed filling duration or still developing pod. Thus, differential pod weight per plant were attributed by genotypes [9].The genotypes with the highest harvest index was those with the highest yield because of the ability to maintain a relatively greater number of pods than the other genotypes [13].

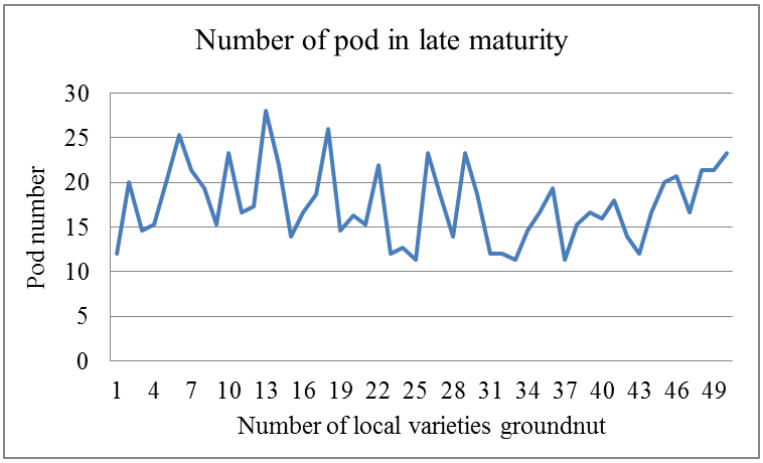

Figure 4. The number of pod of 50 local groundnut varieties at three harvesting time.

The number of pods per m^2 and weight per pod were the two primary components of groundnut yield [13]. The number of pod of local varieties tended to be stable at medium until late harvesting time, i.e. 11 to 28 pods at 95 DAP (Figure 4). The highest pod number was obtained from Mlg 7579 Probolinggo and Kacang Allu (South Sulawesi), whereas the lowest was from Lambuya (North Sulawesi) and line AH 842 Si. Early harvesting time resulted in immature nuts, low yields and off-flavors [6], and consequently reduce the yield, oil content and seeds quality [4]. The effects of pod number per plant on pod yield were influential under different environmental conditions and management. The limitations in assimilate supply during this period, as imposed by drought, reduced flower production, increased flower abortion and pod abscission [13].

The relatively high seed and hull ratio or optimal maturity were found in MLG 1629 Jombang, MLG 7552 Gresik, Hoi Ambon, Lanbau and AH 842 Si, indicating that these varieties had stable maturity index. On the other hand, the local varieties Mlg 7641 Lamongan, Mlg 7548 Tuban, Mlg 7579 Probolinggo, and also the lines AH 1029 Si and AH 1037 Si remained showing high pod weight at maturity time. Therefore, harvesting time groundnut crop is advisable at physiological maturity to identify the different maturity type. For the Indonesia local groundnut varieties the harvesting time will be the best at 85 DAP.

4. Conclusions

The optimaltime to harvest local groundnut varieties of Indonesia was at 85 DAP, as the pod weight, seed weight and maturity index were the highest at this harvesting time. Several varieties with stable seed-hull weight ratio suggested their potential genetic materials for breeding scheme. Different maturity index of the local varieties in this study reflected pod and kernel yield losses in each groundnut variety.

5. Acknowledgement

Authors are highly grateful to researchers of MPGR in ICABIOGRAD, Bogor (Indonesia), who went through entire manuscripts and made positive comments for its improvement, and to express the gratitude to our institution for providing the facilities for this research. Authors are also beholden to technicians for their assistance in the field.

6. Authors contribution

All authors contributed equally to the manuscript. TZ designed, performed the data collection, and drafted the manuscript. NH prepared the plant materials. ND assisted the drafting, revised and finalized the manuscript during publication.

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Animal genetic resources (AnGR) in Mozambique

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Abstract. Mozambique is a country on the eastern coast of Southern Africa, where livestock plays an important role in the agriculture sector, due to its contribution to socio-economic development and poverty reduction. Given the variations in climate, soil fertility, rainfall pattern and altitude among the different areas, the country is divided into ten agroecological regions, each one with its own characteristic production systems and livestock breeds. In the family sector, livestock is mostly composed of indigenous breeds: Landim cattle, goats, pigs and chicken, Angoni and Bovino de Tete cattle breeds and Pafuri goat. In order to restore the quantity and quality of the genetic resources of indigenous breeds and promote their conservation and sustainable use, the country is carrying out several actions and activities, including public awareness campaigns about the importance of indigenous breeds and the need for their conservation, inclusion of AnGR issues in the curriculum of universities and agricultural colleges, characterization of AnGR collection and conservation of semen from native bulls.

Keywords: livestock, family sector, AnGR, breeds.

1. Introduction

Mozambique is a country on the eastern coast of Southern Africa which shares borders with: Tanzania to the north; Malawi and Zambia to the northwest; Zimbabwe to the West, South Africa; Swaziland to the south (Figure 1A). On the East, the section of the Indian Ocean called the Mozambique Channel. It has an area of 801,590 km^2 and about 2,000,000 habitants, distributed in 11 provinces, namely Maputo City, Maputo, Gaza, Inhambane, Sofala, Manica, Tete, Zambézia, Nampula, Cabo Delgado and Niassa. The capital of Mozambique is Maputo and Portuguese is the official language.

The average annual temperature ranges between 20°C and 25°C with two main seasons: rainy/warm season, lasting from November to March, and dry/cool season from April to October [1]. Mean annual rainfall ranges from 350 mm near the Mozambique/Zimbabwe/South Africa border to 2,000 mm in the highlands of Zambezia. In north of the Rio Save, the rainfall is more concentrated with a long dry season, except in parts of the coast. The rainfall in the south of this regionis less concentrated in distribution and winter rains can be encountered in areas closer to the coast [2]. Given the variations among the different areas, the country is divided into ten agroecological regions, each one with its own characteristics of production systems and livestock (Figure 1B).

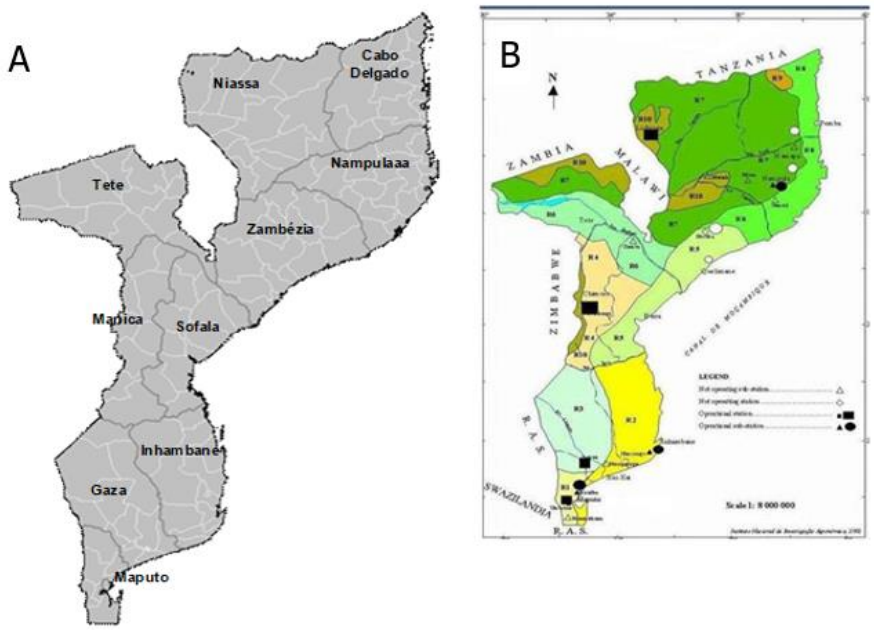

Figure 1. Map of Mozambique (A) and its ten agroecological regions (B).

Livestock plays an important role in the agriculture sector, due to its contribution to the socio-economic development and therefore to poverty reduction. In the family sector, livestock is mostly composed of indigenous breeds of animals, with some degree of mixture with exotic blood. The indigenous breeds are mostly seen as inferior breeds as a result of unsuitable nutritional, sanitary and reproductive management practice [3]. This review paper describes the indigenous breeds in Mozambique, demonstrates their benefits for smallholders' farmers and demonstrates the importance of conserving the Animal Genetic Resources (AnGR) in Mozambique.

2. Characterization of indigenous breeds

The Mozambican breeds have been bred since their origin under natural conditions, with low levels of artificial selection. As a consequence, these breeds have been adapted to indigenous environmental conditions and are resistant to a number of endemic subtropical diseases. However, extensive crossbreeding, replacement with exotic breeds, and social and environmental disasters have placed these indigenous bovines at risk of extinction [4,5]. Most native breeds are named as Landim, which in the South of Mozambique means Indigenous or Local. This name may vary according to the regions and different native languages spoken throughout the country, as in the North (Nampula) native chicken is called Macua. The native breeds of cattle in Mozambique are Landim, Bovino de Tete and Angoni. The Landim breed is based in the southern lowland areas, Tete breed in the Tete province and Angoni in highlands of Tete as shown in the maps (Figure 2) [3].

2.1. Landim cattle

The Landim accounts for more than 70% of the national cattle herd. It is successfully used for meat production, due to its high calving and weaning rates associated with a relatively good dressing percentage, which can reach up to 51–57% with supplementary feeding during the dry season, at 3 to 4 years old. The Landim is small to medium-sized breed and adapts well to harsh environments like drought and forage seasonality. It has low nutrient requirements for maintenance and excellent walking ability in search of grazing and water and tolerant to extreme temperatures (Figure 3A). It is resistant to ticks (*Rhipicephalus* sp.), tick-borne diseases (*Anaplasma* sp. and *Babesia* sp.) and gastrointestinal nematode parasites [6,7].

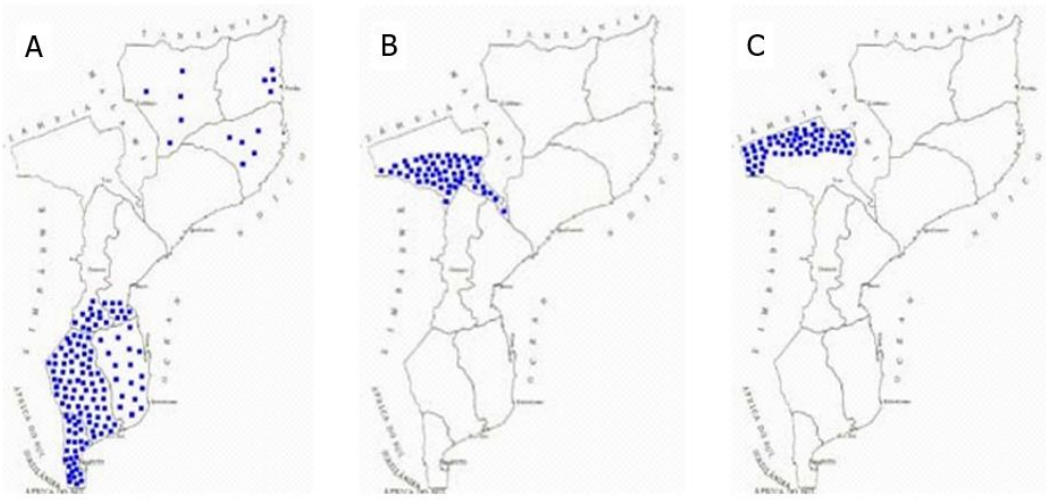

Figure 2. Distribution of native breeds of cattle in Mozambique. (A) Landim. (B) Bovino de Tete. (C) Angoni. Source: FAO.

Figure 3. Native breeds of cattle in Mozambique. (A) Landim. (B) Bovino de Tete. (C) Angoni. The source of photographs A and B are FAO, C is from Vernooij [3].

2.2. Bovino de Tete

Bovino de Tete is morphologically similar to the Landim but smaller in size and with a larger hump and lateral black horns (Figure 3B). Its carcass can yield 85–110 kg depending on the season. Due to its long adaptation to the low rainfall areas and the high temperatures, Bovino de Tete have great ability to survive in a very harsh environment and produce meat as well as draught power. Bovino de Tete represents almost 20% of the national cattle population and is mostly concentrated in the lowland areas of Tete province, in the southern part, in the arid and semi-arid lowland areas of the province [3].

2.3. Angoni

The Angoni cattle are concentrated in the highlands of Tete Province bordering with Zambia and Malawi. It is found in the Angonia plateau, Marávia, Macanga, Fingué and Zumbo, the border with Zambia. It constitutes about 8% of the national cattle herd. The main purpose of this breed is meat production, but it is occasionally used for draught (pulling of carts) and milk production. It is the smallest native cattle breed in size, but the males can weigh up to 730 kg (Figure 3C). Angoni is very resistant to diseases like *Theilleria* in its original environment [4,7].

2.4. Goats and sheep

The Landim goat is the main breed, spread throughout the country with small variations in size and adaptation to harsh conditions (Figure 4A). In Tete province, they look bigger and with a higher fertility rate than in the south of Mozambique. Smallest goats are found in Nampula province and the biggest in Tete regions. The Pafuri goat is bigger and a typical of transhumance system in Gaza

province, where it is milked during the drier periods of the year (Figure 4B). As with other species, the Landim sheep is the most common in the country. The ratio of sheep to goats vary from 1:4 to 1:10 depending on the area, but sheep are mostly used for traditional and religious ceremonies [8].

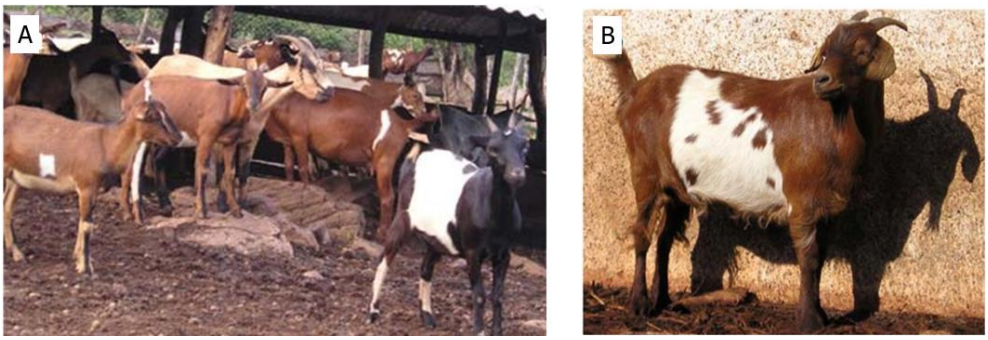

Figure 4. Native goats in Mozambique. (A) Landim. (B) Pafuri. Source: FAO.

3. Management of animal genetic resources (AnGR) in Mozambique

Factors, such as war, shortages of infrastructure, funds and expertise, have contributed to the reduction of productivity of indigenous breeds. Due to these concerns and the need to bridge the huge productivity gaps in developing countries like Mozambique, the production systems is experiencing rapid changes. Indigenous livestock genetic resources, which constitute the largest proportion of livestock in the country, are increasingly being eroded through poorly planned crossbreeding and breed replacements. Their unique genetic attributes, especially those responsible for adaptation, might be lost under such condition.

The country has ratified the Global Plan of Actions (GPA) for the management of AnGR, and several steps have been taken and continued to be monitored to ensure its implementation. It mandated The Institute of Agricultural Research of Mozambique through the Center for Genetic Resources and Assisted Reproduction to identify, develop and test the feasibility of breeding programs and technologies in order to increase the availability of high genetic merit animals, with emphasis on native breeds. They are implemented via the following actions and activities:

a. *Restoration of genetic superiority of indigenous breeds, particularly cattle.* This is achieved by backcrossing existing diluted cows with semen from pure-bred animals.
b. *Crossbreeding and selection studies to develop efficient animals for the production sector.* This is performed on pure native and pure exotic breeds to improve certain traits in the native breeds (dairy and beef) within native breed selection to develop specialized animals, e.g. Landim dairy cows, native beef steers and Landim layers. The Center for Genetic Resources and Assisted Reproduction provides good quality semen, artificial insemination services and the selection of animals of high genetic value for exchange or commercialization between interested breeders.
c. *Revision and update of laws and regulations.* Current laws and regulations dated from the colonial period, therefore there is a need to update to adequately reflect the real current situation of the country. In this context, the animal health regulation has been revised and updated in 2017, which incorporate mainstreaming of AnGR into existing laws and regulations. Most existing laws are weak on issues related to conservation and sustainable use of native breeds, and AnGR focus mainly on raising livestock, improving animal health, access to veterinary services, and identify the strengths and the major shortcomings of sectors that need to be addressed.
d. *Survey and monitoring of AnGR.* The current statistics only cover up to species level and there is no breed information, which makes it difficult for their sustainable use. Therefore, the country is:
    - Putting efforts in capturing information on the breeds of the animals for the inclusion of the information on the coming census.

- Entering data into the Domestic Animal Diversity Information System (DAD-IS) database, a database coordinated by FAO and managed in each country by national coordinators for AnGR.

e. *Performance evaluation of AnGR*. Awareness campaigns are on place to promote animal identification and to implement a national recording scheme to enable identification of superior indigenous breeding stock.
f. *Livestock infrastructures*. Rehabilitation of infrastructures for conservation and management of AnGR, such as sanitary infrastructures like tanks for "carracicid" baths and treatment aisles and productive management infrastructures like booths for the dynamization of breeding programs where the breeders will be based.
g. *Capacity building of Mozambican staff for the management of AnGR*. Through assistance and collaboration with partners, there is a program to strengthen the capacity of veterinary services, increasing their coverage in communities through extensive training of veterinary technicians at various levels and the availability of means of work. There are also programs for the improvement of the cold chains in veterinary services for the conservation and distribution of biological products (vaccines and semen), and also revision and updating of veterinary legislation.
h. *In situ and ex situ conservation of AnGR*. Semen collection of Landim cattle breed and dissemination of information about the importance and superiority of native breeds in the tropics, particularly towards climate changing.

4. Concluding remarks

The genetic diversity of indigenous breeds represents unique resource and opportunity when there is increased demand for livestock products. It is the country's responsibility to make full use of and benefit from it. In the case of Mozambique, awareness of the significance of conservation and sustainable use of AnGR is often limited at the policy level. Such limitations do contribute to the current lack of adequate characterization of indigenous breeds and lower consideration of AnGR in policy decisions. The public sector investment in AnGR development has also declined. Consequently, less attention is paid to breed improvement activities.

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Development of physical color reference for Indonesia paddy collection

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Abstract. One of the objectives of genebank management is to maintain the genetic identity of its collection. In order to maintain the genetic identity, it is important to check new seed against a reference collection. A simple morphological character for determining the reference collection is the color of lemma and palea. Lemma and palea is a pair of bract-like organ in the floret and have similar pattern of pigmentation and therefore can be treated together. The color of lemma and palea determine the seed color. To develop reference collection for grain color characterization of rice germplasm in the Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD) Genebank, 1,000 sample of seeds produced from different year of regeneration are characterized based on the lemma and color categories of the IRRI guidelines for the characterization and evaluation system. Samples of each category were then arranged and photographed. From 11 categories of lemma and palea color described in the guidelines, there are six color categories available from genebank rice collection, whereas five color categories were not found. The six available color categories were straw, golden, brown spot in the straw background, brown line in the straw background, brown, orange-brown and black. Five color categories were not found in the collections i.e. red-purple, purple spots on straw, purple line on straw, purple and white. This material reference could be used for characterization of Indonesian rice. The seeds of the absent categories might need to be aquisitated to the genebank to complete the reference collection.

Keywords: material references, grain color, rice characterization.

1. Introduction

Rice is staple food for Indonesian and most people in South East Asian countries. Indonesia possess high genetic diversity of rice germplasm [1–3]. To ensure the sustainability of rice production in the changing environment, in particular climate changes, Indonesia needs to conserve its rice germplasm *ex situ* as well as on-farm. The National Genebank constructed in 2015 hosted by the Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD), designated as the main genebank to conserve the core collection of rice in Indonesia; although it may be functioned as *ex situ* conservation for other crop as well [4]. The

genebank collects landraces from all regions in Indonesia, as well as improved cultivar and wild relatives. As a new genebank, it has several shorthcoming, e.g. no protocols for sorting the new accession, regeneration and transfer of the materials, duplication of materials, in addition of lack of qualified technician and funding. The number of collections is also limited compared with the capacity of the genebank and the diversity of crops including rice in Indonesia. One important step to overcome those shorthcoming is by improving the genebank management.

The main objectives of genebank management are the preservation of genetic identity and integrity of the germplasm as well as, maintenance of the seed, viability and the promotion of access [5]. Accession maintained in genebank should be remained genetically as similar to the original collected materials as possible. The usual method to check the genetic identity is by checking the morphological traits against descriptor list. Changes in genetic integrity may be caused by genetic drift, unintentional selection and pollen contamination during the regeneration of collection; in addition to mislabelling and seed contamination during the germplasm handling. The greatest change in genetic integrity is the transfer of materials between genebanks. Even a duplicate of a collection might changes its integrity when under the management of different genebank [6]. It is, therefore, important to improve both the handling of accessions within genebank and the protocol for transfer of accessions between genebanks.

Although molecular tools are now available to precisely check and monitor the genetic identity and integrity at the genotype level, morphological characterization can still be used. Characterization facilitates the effort for discriminating phenotypes and allows simple grouping of the accessions [7] Moreover, the characters can be used to facilitate utilization of collections and to detect misidentification and possible errors during genebank operations. Characterization also provides a better insight into the composition of the collection and the coverage of genetic diversity. There are internationally agreed descriptors for characterization, however, characterization is somewhat labour-intensive. Data recording needs to be carried out by trained staff using calibrated and standardized measuring formats, as indicated in the internationally agreed crop descriptor [5].

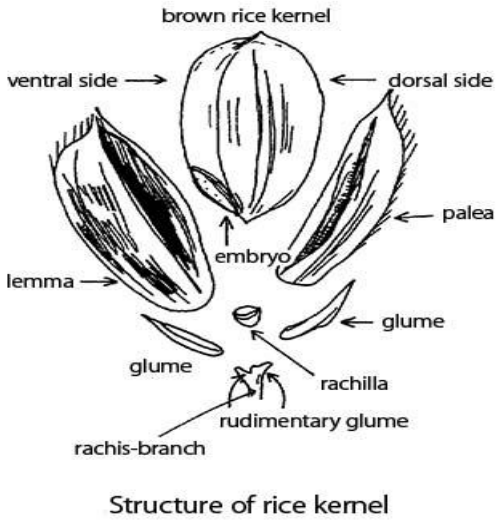

Figure 1. Structure of rice kernel.

Reference collections play an essential role in true-to-type identification [5]. This sample reference can facilitate the identification and characterization process. In early sorting stages of new seeds for the collection, it is important to have reference collection for certain characters that are easy and simple to observe. The lemma and palea color are the simplest morphological character to group rice seed, and therefore, can be used to categorize rice accession at the premilinary sorting of the new

accession. The color is also useful in the eradication of wild type in the cultivated rice, in particular in the farming region where wild type of rice can only be differentiated among purple rice plant or rice plants having some pigmented parts like Madya Pradesh, India. Patil and Sahu [8] suggest the development of varieties with pigmentation to overcome this problem. Lemma and palea are a pair of bract-like organ in the floret (Figure 1) [9]. There is a wide diversity in the distribution, intensity and location of pigment in the lemma and palea. Both lemma and palea show a similar pattern of pigmentation, and therefore, are treated together [10].

The objective of the study is to develop material references for color identification on Indonesia rice germplasm. The availability of physical reference will help curator and technician to characterize and identify rice accession. This will facilitate the characterization program, particularly on the character of rice color.

2. Materials and methods

The color reference was developed based on the rice seed samples of ICABIOGRAD Genebank. A total of 1,000 samples of seed from different years of generation were sampled and characterized. The seed samples were grouped based on the color of lemma and palea in accordance with the IRRI Rice Descriptor [11]. The categories and the availability of each category in the ICABIOGRAD Genebank collection are presented in Table 1. Each seed sample in each category is assigned as sample reference. The samples were photographed and stored in an appropriate pack according to the designation. The photograph was arranged as a paper-printed color reference.

3. Results and discussion

According to the Guidelines for Characterization and Evaluation of Rice [12], there are 35 rice morphological characters, in addition to 12 agronomical characters and 14 characters associated with grain quality. In addition to those characters, there are characters related to symptoms to diseases (23 characters), plant broken due to rodent and birds (2 characters), plant broken caused by insects (11 characters), physical-chemist stresses (4 characters) and characters related to tolerance to deep water such as elongation, submergence tolerance and survival. There are also characters for evaluation of hybrid rice and its progenitors.

There are at least nine characters that are usually used to describe rice seed, i.e. lemma and palea color, the existence of hair in the lemma and palea, the color and the length of sterile lemma, the color of epiculus and the existence of hair on the epiculus and the color and the length of the hair in the epiculus. In the rice seed structure, lemma and palea are the two parts attributing the grain color. Lemma and palea are perianth organs that surround the inner floral organs. Thus, these two organs determine the whole unhusked-grain color [9].

Table 1. Categories of rice seed color and the availability of sample representing each category.

Category	Description	Availability on the collection
0	Straw	v
1	Gold and gold furrows	v
2	Brown spots on straw	v
3	Brown furrows on straw	v
4	Brown (tawny)	v
5	Reddish to light purple	-
6	Purple spots on straw	-
7	Purple furrows on straw	-
8	Purple	-
9	Black	v
10	White	-

As indicated in Table 1, there are 11 categories of the color of lemma and palea, and six categories are available in the ICABIOGRAD Genebank rice collection (Figure 2). The straw color category is the most common category found in the seed collections. The dominance of this color was also found in a study [8] on rice cultivation in Chhattisgarh, India.

Figure 2. Six color categories found in ICABIOGRAD-IAARD sample collection.

No sample found among the seed stock represented the purple group and white color categories. The purple group color categories consisted of four categories, i.e. reddish to light purple, purple spots on straw, purple furrows on straw and purple. The unavailability of those samples is probably due to the lack of genetic materials representing the color variation, or, merely due to the maturity level of the seed materials used in the study.

The purple pigment is controlled by the *Hep* gene [10]. Spikelet color is somewhat different in the flowering to ripening stage. However, no references found regarding the effect of storage duration on the color change of lemma-palea. At ripening, the color of lemma and palea changes into a quite different one [10]. At flowering, anthocyanin distribution in lemma and palea could be categorized as purple tips, purple spread and full purple. The ripening color of lemma-palea acted independently of the color of the early stage. The same color of the lemma-palea in the growing stage might ripen into different color at the ripening stage. Conversely different color of the young probably ripens to the same color at maturity (Figure 3).

Bioversity International [13] determine lemma-palea color differently in early and late observation. There are 15 color categories for early observation, whereas only 11 categories for the late observation. The pre-ripening stage, considered as early observation, is supposed to be conducted after anthesis to hard dough stage. The Bioversity descriptor is subjected to both cultivated rice and wild relatives. Considering that the rice collections consisted of landraces and local varieties, the availability of this descriptor in the collection might open the possibility of expanding the category of this lemma-palea color.

For the *Oryza sativa*, the lemma-palea color is supposed to be characterized at the maturity stage or when the terminal spikelets are ripened [11]. Samples used for color determination in this study were stock samples that were ripen harvested materials and have been stored for years. This level of maturity should provide the final and stable lemma-palea color. Thus, the unavailability of some color categories could be assumed as the lack of materials in the genebank collection. This should be considered for the next acquisition program. Exploring materials with the particular lemma-palea color is needed in order to fill the gap of the genetic variation.

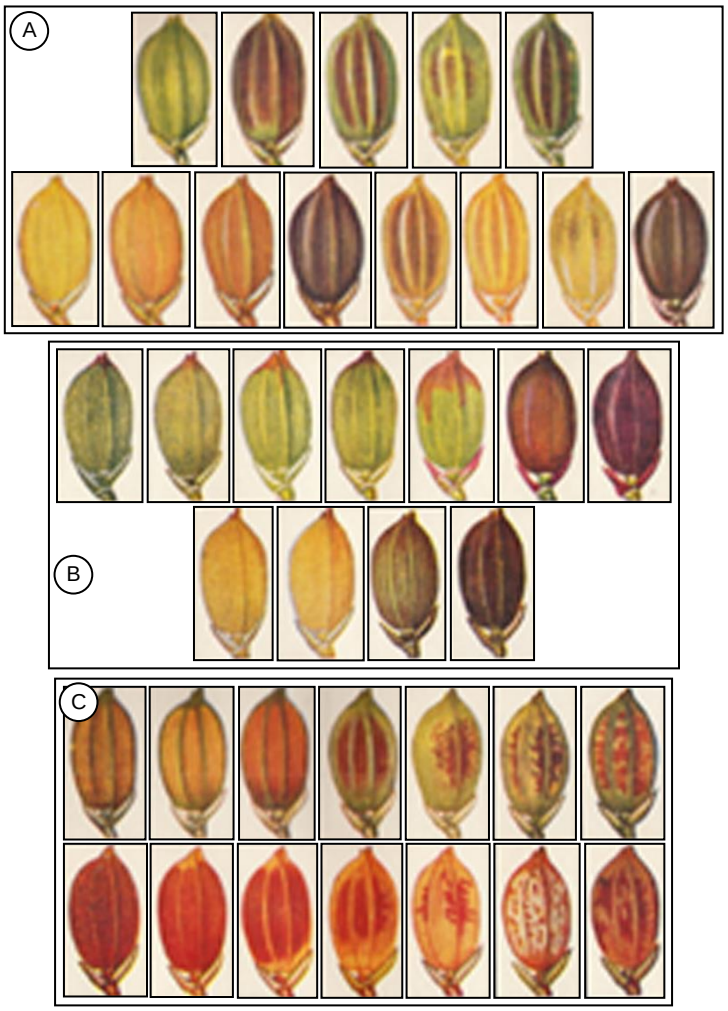

Figure 3. The color change of lemma-palea at flowering and maturity stage [10]. (A) Some color category of flowering changed to broader range of color category at maturity. (B) Some color category of flowering changed to narrower range of color category at maturity. (C) Color category at maturity.

4. Conclusions

There are six color categories found in the stock materials of rice collection in ICABIOGRAD Genebank rice collection. These stock materials can be used as a color reference for characterization of lemma-palea color. Five color categories are not found in the collection. The information about the lack of the five color categories is also useful for targetting the next acquisition plan. The genebank needs to acquire seed samples representing these five colors in order to establish a complete reference. Another consideration is to formulate the need for early-observation of lemma-palea in the pre-ripening stage by using the descriptor by Bioversity international.

In summary, we present three core collections that have their value for the landscape's genetic resources management, research and breeding. We believe that the methodologies implemented in this work may be successfully extrapolated to other rice landraces and cultivars, with similar results when selecting a core collection from a highly related original collection.

5. Authors contribution

NH and MS are the main authors of the manuscript contributed equally in the design and interpreting the observation on the samples. AR contributed in observation and in preparing the sample materials. NAW contributed in adding several paragraphs and commented on the manuscript.

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Screening genetic resources of local accessions of *Capsicum* originated from East Java

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Abstract. Inventory and collection of local chili varieties in production centers in East Java had been carried out in the period 2013–2015 by East Java Assessment Institute for Agricultural Technology (AIAT). A total of 143 accessions of five chili species (*Capsicum annuum* L., *C. frutescens* L., *C. chinense* Jacq., *C. baccatum* L. and *C. pubescens* L) was collected from eight chili production centers in East Java and has been maintained by East Java AIAT. A set of 85 local chili accessions was selected and evaluated for agronomic performance and biochemical compounds. Among the five species, *C. annuum* L. and *C. frutescens* L. dominated the distribution of chili species in East Java. The other three species were still found in some spots of farm households and highlands. *C. pubescens*, locally known as "Bodong" or "Wudel" chili, was found on the high slopes of Mount Semeru, whereas *C.* chinense, known as "Cotoh" chili by the locals, was also found in the highlands of Batu City. Based on agronomic performance there were several accessions with high-yielding potential (≥12 t/ha). Five accessions of *C. frutescens* collected from planting areas in the dryland with dry climate of Blitar not only had high yield potential, but also had high capsaicinoid content, low-fat content, low quercetin and high flavonoids and polyphenols. Phenotypic diversity and geographic origin may be useful as the criteria for selecting a good set of chili accessions.

Keywords: screening, species, accession, chili.

1. Introduction

Local plants with high diversity but have less massive impact directly on welfare are mostly given less attention by policyholders. However, this trend does not apply to chili commodities. The market for high-value food products in developing countries, and unprecedented, can be created by among others, small-scale farmers. They can make the market transition from traditional low-value commodity production to high value. The diversity of plant species and local varieties can be the basis for building new markets, as shown in the case of potato and chocolate varieties [1].

Chili is one of the most important vegetables and spices in the Indonesian food trade. The price fluctuations of chili can cause national economic inflation which compeled policyholders taking steps to develop local varieties of chili. Local chili varieties have adaptive properties under extreme environmental changes, resistance to pests and diseases, and the ability to grow well under low agricultural inputs so they can strive in the yard. Although this commodity can cause national economic inflation, the species and genetic diversity of chili are rarely studied. Therefore, inventory

and collection of local varieties in chili production centers in East Java were carried out in 2013–2015 by East Java AIAT [2]. Parts of the result surveys are described in this paper.

Among 38 recognized *Capsicum* species, five are cultivated, namely *C. annuum* L., *C. frutescens* L., *C. chinense* Jacq., *C. baccatum* L. and *C. pubescens* [3]. Two species, *C. annuum* L. and *C. frutescens* L., were the most cultivated chili in East Java, while the other three were rare but were still found in some farm household spots [2]. Based on the report of Purnomo et al. [15], *C. pubescens* was found in Lumajang which is located in the slopes of Mount Semeru. This species is called "Bodong" or "Wudel" peppers by the locals. *C. chinense*, which was known locally as "Cotoh" chili, was found in the highlands of Junggo, Malang.

From a large collection of chili accessions obtained from the survey, it is necessary to select a set of chili germplasms with promising several uses including product development. Indicators such as phenotypic diversity and geographic origin may be useful as good selection criteria for choosing accessions that are rich in function within a specific timeframe. The selection criteria include botanical classification, geographic origin and biochemical properties of the fruit.

The specific objective of this study was to evaluate the agronomic performance and fruit chemical properties of local chili accessions collected in East Java. The expected outcomes from this study are: (i) accessions with desired characteristics which can be used in the development of local chili varieties in East Java, and (ii) selection criteria using phenotypic diversity and geographic origin of accession.

2. Materials and methods

2.1. Inventory and collection of local chili accessions

Chili production centers in eight regencies (Blitar, Kediri, Malang, Tuban, Lamongan and Mojokerto) in East Java were visited in 2013–2015. Local accessions were collected from diverse topography and altitude (from 150 to 1500 m above sea level, m asl). Genetic materials were collected and maintaned at East Java AIAT.

2.2. Evaluation of agronomic performance and chemical analysis of chili fruit

A total of selected 85 accessions representing five species from eight production centers were evaluated for agronomic performance (Table 1). The evaluation was carried out at Malang Experimental Field, East Java AIAT, from February until September 2016. Seedlings were transplanted on raised beds (3.0 m long × 2.5 m wide × 0.5 m high) at within-row planting distance of 0.6 m and between-bed distance of 1.5 m. Each bed contained 12 plants of each accession. The experimental design used was a randomized block design with two replications. Fruits from selected accessions of *C. annuum* and *C. frutescens* from each production center were subsequently characterized for their chemical compounds. Chemical analysis was carried out in the Food Laboratory of the Food Technology Faculty, Brawijaya University, Malang.

Table 1. Origin and number of local chilli accessions used in agronomic evaluation.

Origin	*Capsicum frutescens*	*C. annuum*	*C. chinense*	*C. baccatum*	*C. pubescens*	Total number
Blitar	5	8	-	-	-	13
Kediri	5	8	-	1	-	14
Lumajang	3	5	-	-	2	10
Malang	3	8	3	1	-	15
Banyuwangi	2	7	-	-	-	9
Tuban	3	5	-	-	-	8
Lamongan	3	6	-	-	-	9
Mojokerto	2	5	-	-	-	7
Total number per species	26	52	3	2	2	85

3. Results and discussion

3.1. Inventory and collection of local chili accessions

The surveys listed 143 chili accessions which were cultivated in diverse topographies and altitudes in East Java. Accessions of *C. annuum* collected from the highlands formed an ellipsoid canopy. Meanwhile, accessions of *C. annuum* taken from the lowlands formed an umbrella canopy. Diversities in the harvesting age within the species were observed among geographic origins, and differences in plant performance were more pronounced among species.

The centers of local chili production in Blitar Regency were drylands with dry climate at an altitude of 150–400 m asl, covering three sub-districts, namely Panggungrejo, Binangun and Wates. The geographic distribution of local chili in Kediri Regency was at lowland and highland up to 1,000 m asl. Air temperature ranged from 23–30°C with an average rainfall of 1,652 mm/day during the rainy season (5–4 months/year). Chili in this region was planted on grey-brown regosol soil in Kepung, Plosoklaten and Puncu Sub-districts. The production center in Lumajang Regency was Pasuruan and Tosari Districts which are located at the slopes of Mount Semeru from 400 to 1,500 m asl in dryland under dry climate with an average rainfall of 1,578 mm per day during the rainy season (5–4 months/year). The chili species in this region is of most interest because the plant has perennial life cycle. The performance of this species is presented in Figure 1.

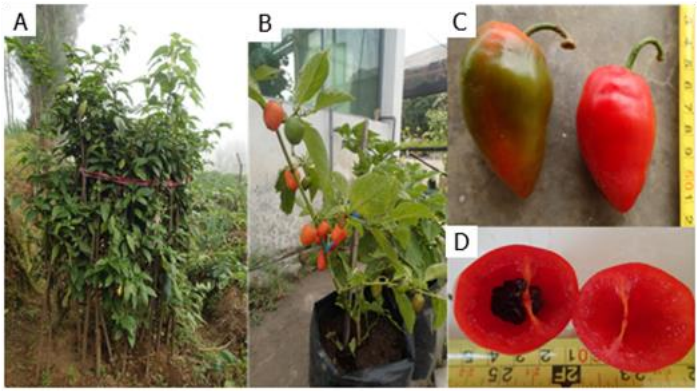

Figure 1. Performance of chili species of *Capsicum pubescens*. (A) Plants in its original habitat in Lumajang Regentcy. (B) Plants in polybags. (C) Fruit. (D) Transverse section of the fruit.

Local chili in Malang Regency is cultivated on terrain topography at 300–1,500 m asl, in Dau, Pujon, Ngantang and Kesembon Sub-districts, in dryland typology with regosol soil under wet climate with an average rainfall of 2,617 mm per day during the rainy season (7–8 months/year). The prodution centers of local chili in Banyuwangi Regency is in the lowlands in some districts such as Wongsorejo, Purwoharjo and Cluring. In Tuban Regency local chili is cultivated in three districts, namely Grabagan, Bancar and Soko; whereas in Lamongan Regency it is planted in two sub-districts, namely Brengkok and Sendangharjo. In Mojokerto Regency local chili is grown in the highlands of Pacet Sub-district.

3.2. Evaluation of agronomic performance and chemical analysis of chili fruit

A set of 85 accessions representing five species from eight chili production centers in East Java was selected for the characterization of the agronomic performance (Table 2). *C. frutescens* produced higher fruit yield compared to the other species when it was planted in lowland area, which is less prospective to be developed as chili production centers in East Java. According to van Zonneveld et al. [4], *C. pubescens* is suitable for highland areas with a wet climate. *C. baccatum* and *C. eximium* are favorite chili species among farmers for their high productivity [5].

Table 2. Agronomic performance of five species of *Capsicum* originated from East Java.

Origin	Species	Number of accessions	Harvesting age (weeks after planting)	Plant height (cm)	Canopy width (cm)	Life cycle	Yield potential (t/ha)
Blitar	*C. frutescens*	5	15–17	133.8±5.6	72.3 ±12.6	A/P	12.5±3.4
	C. annuum	8	13–16	85.3±4.6	86.0±7.5	A	9.2±4.3
Kediri	*C. frutescens*	5	15–17	112.6±7.8	75.3±9.4	A/P	12.8±2.6
	C. annuum	8	13–16	9.7±3.1	86.4±5.2	A	8.6±2.6
	C. baccatum	1	-	69.5	83.4	-	-
Lumajang	*C. frutescens*	3	15–17	138.3±7.6	75.6±5.8	A/P	5.6±1.4
	C. annuum	5	13–16	105.3±9.5	73.4±3.8	A	9.7±4.3
	C.pubescens	2	17–18	218.9±5.1	85.3±4.3	P	5.2±2.1
Malang	*C. frutescens*	3	15–17	138.7±7.8	74.5±3.2	A/P	8.9±3.8
	C. annuum	8	13–16	103.5±6.7	83.7± 4.2	A	10.3±3.1
	C. baccatum	3	15–16	68.9±7.5	85.3±3.2	A	6.4±1.2
	C. chinense	1	12	110.6	43.5	A	3.2
Banyuwangi	*C. frutescens*	2	15–17	119.6±4.3	73.3±26	A/P	9.8±3.2
	C. annuum	7	13–16	87.9±6.3	83.4±5.3	A	7.3±1.8
Tuban	*C. frutescens*	3	15–17	115.4±3.2	75.2±4.1	A/P	12.4±4.5
	C. annuum	5	13–16	83.4±5.5	73.4±3.2	A	7.2±2.2
Lamongan	*C. frutescens*	3	15–17	129.3±4.3	77.5±3.1	A/P	10.4±4.5
	C. annuum	6	13–16	90.1±8,8	83.2±5.5	A	7.3±2.5
Mojokerto	*C. frutescens*	2	15–17	122.7±4.5	73.5±2.8	A/P	5.4±2.1
	C. annuum	5	13–16	92.7±2.2	68.8±5.2	A	9.3±3.4

A = annual, P = perennial, - = no data available.

Fruit chemical analysis revealed that *C. frutescens* accessions from Blitar have a higher level of capcaisinoid, flavonoids and polyphenol compounds, but a lower level of fat content, and quercetin compounds compared to other accessions (Table 3). They also have a higher productivity of about 12.5 t/ha compared to the yield of the other accessions originated from the other production centers (Table 2). The performance of some of these accessions can be seen in Figure 2.

The yield potential of *C. annuum* accessions from Lumajang Regency are comparable to that of *C. fruitescens* accessions from Blitar Regency, but the fruit is not pungent because its capsaicinoid content was as low as 189.6 mg/100 g of ingredients. Mazourek et al. [6] stated that capsaicinoid content affects the spiciness of chili. Spicy flavors are expressed in Scoville Hest Unit Tester units. Spiciness is a unique characteristic of chili caused by a group of alkaloids called capsaicinoids. These alkaloids correlate with antioxidants, polyphenols and fat content in chilli [7,8].

The altitude of planting area may affect chili fruit quality with regard to the levels of capsaicinoids and flavonoids. *C. annuum* and *C. frutescens* accessions from the planting area in the highlands of Lumajang District showed different levels of capsaicinoids and flavonoids contents compared to accessions cultivated in lowland regions (Table 3). Therefore, geographic origin may be used as an indicator for selection criterion of accession with desired character.

Figure 2. Performance of two local accessions of *Capsicum frutescens* originated from Blitar, East Java. (A) BL 1 plants. (B) BL 1 fruit. (C) BL 2 plants. (D) BL 2 fruit.

Table 3. Variation in the biochemical contents of local *Capsicum annuum* and *C. frutescens* accessions originated from eight chili production centers in East Java.

Origin	Species	Number of accessions	Capsaicinoids (mg/100g)	Fat (g/100g)	Flavonoids (mg/100g)	Polyphenols (g/100g)	Quercetin (mg/100g)
Blitar	*C. frutescens*	5	1065.5±142.3	12.3±4.2	11.4±3.4	2.5±0.8	6.6±2.2
	C. annuum	8	312.4±56.4	9.1±3.5	6.1±2.4	1.8±0.3	8.0±2.4
Kediri	*C. frutescens*	5	897.4±123.7	16.3±4.5	9.5±2.3	2.1±0.8	6.7±1.8
	C. annuum	8	304.5±48.7	9.7±2.3	5.9±2.1	1.4±0.2	8.5±2.1
	C. baccatum	1	219.2	7.0	9.8	1.7	9.2
Lumajang	*C. frutescens*	3	575.3±93.8	14.8±5.6	4.2±2,4	1.4±0.6	10.8±2.5
	C. annuum	5	189.6±45.6	9.7±2.4	5.3±3.1	1.4±0.2	12.5±3.4
	C .pubescens	2	213.4±56.8	7.2±4.3	2.8±1.8	1.8±0.3	2.2±07
Malang	*C. frutescens*	3	578.2±89.6	16.7±6.8	6.8±3.2	1.8±0.5	8.2±2.5
	C. annuum	8	198.6±32.5	10.5±3.3	5.5±2.2	1.4±0.4	8.3±2.6
	C. baccatum	3	143.6	7.2	9.9	2.1	10.3
	C. chinense	1	323.1±42.5	7.2±2.7	4.3±2.4	1.9±0.4	4.2±1,1
Banyuwangi	*C. frutescens*	2	886.4±121.5	4.2±1.8	12.5±4.2	2.1±0.8	5.6±1.4
	C. annuum	7	315.8±43.8		5.2±2.3	2.2±0.7	8.4±2.0
Tuban	*C. frutescens*	3	945.3±118.7	5.3±2.1	12.4±3.2	2.3±0.7	5.7±1.8
	C. annuum	5	323.6±45.5	6.8±3.4	5.7±2.2	2.2±0.5	8.3±2.5
Lamongan	*C. frutescens*	3	1005.3±123.4	4.4±1.8	12.1±4.5	2.3±0.6	5.7±1.8
	C. annuum	6	345.7±45.5	6.3±3.1	5.7±2.4	2.1±0.4	7.8±2.0
Mojokerto	*C. frutescens*	2	523.4±92.5	16.7±5.6	5.9±3.3	1.6±0.5	10.4±3.4
	C. annuum	5	198.4±31.3	14.8±4.7	5.0±2.3	1.6±0.3	8.8±3.0

Results of crossing trials and molecular marker analysis in parallel studies (data not shown) revealed that the five domesticated species of *Capsicum* were associated with three major genetic diversity centres of three species in East Java, i.e. *C. annuum*, *C. baccatum* and *C. pubescens*. Based on this finding, it can be stated that East Java has a diverse genetic resource of chili germplasm, which are potential as genetic material for improvement of chili varieties locally. However, *C. annuum* is phylogenetically separated from *C. baccatum* and *C.* pubescens, and thus strong interspecific hybridization barriers exist among them [9].

4. Conclusions

Field evaluation on agronomic performance of 85 chili accessions collected from production centers in East Java revealed great differential characters among the accessions. Five accessions of *C. fruitescens* from Blitar had high levels of capsaicinoids, with low-fat and quercetin contents, but high flavonoid and polyphenols contents. The yield potential of these accessions can reach up to 12 t/ha when planted on drylands under and dry climate. The high level of genetic diversity in the genus *Capsicum* originated from East Java provides genetic materials for plant breeders to develop new high-yielding varieties. Phenotypic diversity and geographic origin of chili accessions may be useful as the criteria for selecting a set of accessions with desirable characters.

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The performance of growth and yield component of soybean varieties in Margodadi village, Ambarawa sub-district, Pringsewu regency, Lampung province, Indonesia

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Abstract. Efforts to increase soybean production require superior varieties which are stable in every agroecology production center. Therefore, it is necessary to perform field evaluation of several new released soybean varieties. The aim of this study was to evaluate the growth and yield potential of soybean varieties developed by Indonesian Legume and Tuber Crops Research Institute (ILETRI) Malang in a field experiment. Four soybean varieties (Devon 1, Dering 1, Gema and Gepak Kuning) were sown in Margodadi Village, Ambarawa Sub-district, Pringsewu Regency, Lampung Province, Indonesia from May to August 2017. Plots were arranged in randomized complete block design with six replicates. The variables observed were crop emergence at 7 DAS sowing, plant height and number of pods at harvest, pest attack on 1-month-old plants and at harvest, and seed yield. The results showed that Gepak Kuning gave the highest yield (2.13 t/ha), which indicated that this variety is suitable to be planted and developed in the location of the study.

Keywords: soybean, varieties, evaluation, agroecosystem.

1. Introduction

One of the successful targets of agricultural development is to achieve self-sufficiency in five basic food crops including soybean. Soybean (*Glycine max*) has long been an important source of protein, fat, mineral and vitamin in the Indonesian diet because it is affordable by most people. In Indonesia, soybean is processed into various food products such as fermented soybean ("tempe" or fermented soybean in blocks, "tauco" or soybean paste and soy sauce), tofu, soybean milk, and other products [1]. At the international level, soybean commodity is repositioned as healthy foodstuff and a highly prospective source of functional food [2].

The demand for soybean increases every year proportionally to population growth [1], while current soybean productivity at the farmers is only 1.3 t/ha [3]. Since the domestic capacity of soybean production could not meet the consumption, the import of soybean is inevitable. Therefore, in efforts to achieve soybean self-sufficiency, support from various aspects, including improved technologies is needed to increase national production. Superior variety is one of the technologies that can contribute significantly to increase the production of soybean. Generally, breeding of superior soybean varieties has been directed to develop cultivars with high potential of seed yield, resistant to pests and diseases, early maturity and seed quality of crops according to consumers wishes [4].

Several improved varieties have been released, mostly by Indonesian Legume and Tuber Crops Research Institute (ILETRI) Malang, which has the mandate to develop new soybean varieties. Among those varieties, Gepak Kuning, Gema, Dering and Devon 1 are soybean with several superior characteristics. Gepak Kuning, released in 2008, is a selection from local varieties with the same name [5]. This variety has high tofu rendement content and can adapt well in paddy field and dryland, both in the rainy season and dry season. The yield potential of Gepak Kuning is 2.86 t/ha. Gema was released on 9 December 2011 [6]. This variety is a selection of breeding lines derived from the cross of an introduced cultivar Shirome to a national variety Wilis [5]. Gema is an early maturing cultivar with harvesting age of less than 75 days and has an average production of 2.47 t/ha. Early maturing type is important for areas with limited rainfall or for cultivation in the second planting time during the dry season when irrigation water decreases [6]. Dering 1 was released in September 2012 [5]. This variety is drought tolerant at the reproductive phase and has a high yield potential up to 2.8 t/ha [7]. In 2015, a new high yielding variety Devon 1 was released [5]. Devon 1 is a selection from the cross of Kawi variety to IAC 100. With the yield potential of 3.09 t/ha and an average of 2.75 t/ha, this variety is expected to be popular among farmers and thus can accelerate the increase of soybean production [3].

Efforts to increase soybean production require superior varieties which can perform well in different agroecological zones. In addition, the selection of a variety is also based on the farmers/users preferences. Therefore, it is necessary to evaluate the growth and yield potential of the soybean varieties above in a field location.

2. Materials and methods

The study was conducted in Margodadi Village, Ambarawa Sub-district, Pringsewu Regency, Lampung Province, Indonesia from May to August 2017. Four varieties (Devon 1, Dering 1, Gema and Gepak Kuning) developed by ILETRI were tested in this study. Two seeds of each variety were sown in 2 m × 2 m plots at 20 cm × 40 cm planting space. The experiments occupied a total area of 2,500 m^2. Plants were fertilized with NPK at a rate of 200 kg/ha and liquid organic fertilizer at a rate of 8 l/ha. NPK was applied near the plant hole at 7 days after sowing (DAS), whereas liquid organic fertilizer was sprayed when plants were 1-month-old. Variables observed were crop emergence at 7 DAS, plant height and pod number at harvest, pest incidence on 1-month-old plants and at harvest, grain weight and yield. Experiments were arranged in a randomized completely block design with six replications for each variety. Mean separation test was done using DMRT at 5% significant level.

3. Results and discussion

3.1. Plant growth and productivity

Among the four varieties, Devon 1 had the lowest growth rate (70%; Figure 1), indicating that seeds of this variety may have declined physiologically. Storing space temperature plays a role in maintaining seed viability during storage, which is influenced by seed moisture content, room temperature and relative humidity. At low temperatures, respiration runs slowly compared to high temperatures. Generally, seeds with low vigor also show poor growth in the field [8,9]. Freshly harvested soybean seeds that are stored for a period of time must have a germination capacity above 85% [9,10]. It has been known that the growth of soybean plants is influenced by the interaction between genetic and environment [11].

Plant height at harvest ranged from 38.58–51.32 cm, which was not significant different from each other (Table 1). These figures were lower than the original description. Agroclimate condition during the growing season may not be conducive for optimal soybean growth because experiments were done at the second planting season but slightly later (May) than the common practice by local farmers (April).

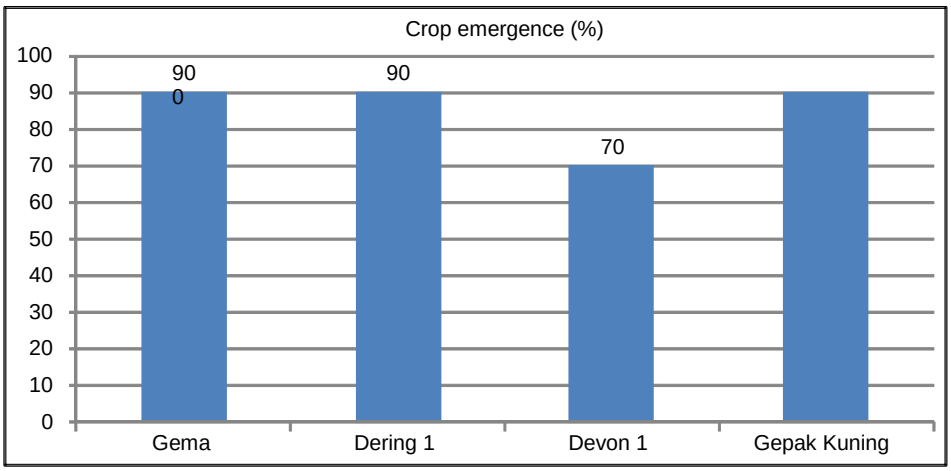

Figure 1. Rate of crop emergence of four soybean varieties at 7 days after sowing in a field in Margodadi Village, Lampung Province, Indonesia from May to August 2017.

The number of pods in soybean plants is strongly influenced by genetic factors [12]. In this study, the highest number of pods was obtained from Gepak Kuning, which was significantly differed from the other three varieties (Table 1). The percentage of empty pods was also the lowest on Gepak Kuning, although it did not differ significantly from the other varieties. The total yield ranged from 1.27 to 2.13 t/ha and was significant different among the four varieties (Table 1). The order of varieties from the highest to the lowest yield was Gepak Kuning, Dering 1, Devon 1 and Gema. The weight of 100 grains of the four varieties was close to the description (Table 1).

Table 1. Growth and yield of four soybean varieties planted in a field in Margodadi Village, Lampung Province, Indonesia from May to August 2017.

Varieties	Plant height (cm)[a]	Number of pod per plant[a]	Empty pods (%)[a]	Yield (t/ha)[a]	Weight of 100 grains (g)[a]
Gema	38.58 a	24.10 b	14.16 ab	1.27 d	11.67 b
Dering 1	47.89 a	32.97 b	10.25 ab	1.87 b	11.00 b
Devon 1	51.32 a	44.20 b	24.42 a	1.47 c	13.67 a
Gepak Kuning	41.80 a	103.80 a	4.92 b	2.13 a	8.00 c

[a] Values followed by the same letter(s) within the same column are not different significantly at 5% level according to DMRT.

Figure 2. Variation in the seed color of Gepak Kuning (A), Dering 1 (B), Devon 1 (C) and Gema (D) soybean varieties released by Indonesian Legumes and Tuber Research Institute (ILETRI).

All those varieties have yellow seed coat color with a varying degree of intensity (Figure 2). The seed coat of Gepak Kuning is greenish yellow, Dering 1 and Devon 1 are yellow, and the seed color of Gema is light yellow [5].

3.2. Leaf and pod damage by insect pests

Insect pests prevailing during the vegetative stage were armyworm (*Spodoptera litura*), whereas at the harvest time were pod borer (*Etiella zinckenella*) and pod sucker (*Riptortus linearis*). Percentage of leaf damage on 1-month-old plants by armyworm was 12 to 18% (Table 2). The highest percentage of leaf damage was observed on Gepak Kuning, but was not statistically significant different from that on Gema and Dering 1. In contrast, the lowest percentage of leaf damage was observed on Devon 1 which did not differ significantly from that on Dering 1. Because the level of leaf damage exceeded the economic threshold (12.5%) [13], insecticide sprays were applied.

Table 2. Percentage of leaf damage by armyworm and pod damage by pod borer and pod sucker on four soybean varieties planted in a field in Margodadi Village, Lampung Province, Indonesia from May to August 2017.

Varieties	Leaf damage by armyworm (%)[a]	Pod damage by pod borer (%)[a]	Pod damage by pod sucker (%)[a]
Gema	16.14 a	6.97 ab	3.73 b
Dering 1	13.08 ab	9.58 a	9.97 a
Devon 1	12.01 b	3.75 b	3.99 b
Gepak Kuning	18.27 a	5.19 b	5.61 b

[a] Values followed by the same letter(s) within the same column are not different significantly at 5% level according to DMRT.

The level of pod damage caused by pod borer and pod sucker reached 3.8 to >9.0%, which were significantly different among varieties (Table 2). Dering 1 was released as pod borer resistant variety [7], but in this study it suffered the most compared to the other varieties. Climatic factors may favor insect prevalence and induced instability of resistance in this variety. Devon 1 was more resistant to three insects, but had low crop growth (Figure 1), high empty pods and low productivity (Table 1).

Although Gepak Kuning was less resistant to insect pests compared to the other varieties, its yield potential was the highest among the four varieties and was close to the description. It is known that variety with high production capacity is due to well adaptability to the environment [14]. Gepak Kuning is a small seed variety, a characteristic that is favored by farmers in the experimental location. Therefore, Gepak Kuning is recommended to be developed in this region.

4. Conclusions

Of the four varieties studied, Gepak Kuning gave the highest yield (2.13 t/ha). Gepak Kuning can be developed in the region of study because it has small seed grain, which is favored by local farmers.

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Inventory and morphological characterization of local upland rice in the highlands of South Sumatra province

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Abstract. South Sumatra has various types of local rice in various agroecosystems that have the potential as plant genetic resources (PGR) of food crops. At present, the existence of the PGR has been diminished due to genetic erosion. Inventory and characterization of local rice genetic resources were carried out in the highlands of South Sumatra during 2014–2017. The study was conducted in Muara Enim, Ogan Komering Ulu, Pagar Alam, and Lahat Regencies. The objective of the study was to inventory and characterize the PGR. The method used was exploration and desk study. Purposive sampling was applied. Morpho-agronomic characterization was carried out *in situ* and *ex situ*. Morphological observations were carried out by visually observing the parts of leaves, stems, panicles and unhulled seeds of the rice accessionsin the location. Morphological characteristics were analyzed descriptively by tabulation. Inventory result showed 17 varieties of local upland rice were spread across four regencies. Local upland rice varieties originating from the regencies with different biophysical characteristics showed differences in morphological characteristics. Among the 17 local varieties, 3 varieties were characterized, namely Setangkai, Barokah and Ayek Keruh. Morpho-agronomic characterization results showed that there was a high diversity of rice accessions collected from different locations in South Sumatra.

Keywords: inventory, characterization, local rice.

1. Introduction

Rice is the staple food for the majority of Indonesian people. According to Indonesian Center for Agricultural Data and Information [1], the average national rice consumption reached 103.18 kg/capita/year. This amount is much higher than the consumption of other foods such as sweet potatoes which only reaches 6.64 kg/capita/year. Efforts to increase rice production must be continued through intensification and extensification to achieve food self-sufficiency. Development of modern biology, such as biotechnology, molecular biology and genetic engineering, is expected to solve the problem of food

availability [2]. However, it relies on the availability of genetic resources. Therefore, local rice varieties have an indirect role in supporting the efforts to meet the food demand.

Local rice varieties are those that have long been adapted to certain areas. This variety has location-specific characteristics for the area. South Sumatra is one of the provinces in Indonesia that hasa high diversity of local rice varieties due to adaptation to the environment where they grow. This diversity supports both the effort to meet the future food demand and the development of genetic resources [3].

Efforts to inventory, identify and conserve local rice varieties are expected to help preserve the biological treasure. Further conservation efforts will eliminate fears of the destruction of local superior rice because there is a tendency for the degradation of local rice varieties, especially in South Sumatra. A survey done by the South Sumatra Assessment Institute for Agricultural Technology (AIAT) from 2003 until 2014 found a decrease in the number of local rice varieties [3]. In one of the highland dryland locations, some local rice varieties previously planted by farmers were already difficult to find. Research needs to be done to get actual data on the existence of local rice varieties. The objectives of this study were: (1) to inventory and map the spread of local rice in highlands of South Sumatra Province, (2) to characterize the morphology and agronomy of each local rice variety, and (3) to collect information on the presence of local rice variety as an initial conservation effort.

2. Materials and methods

The research was carried out during 2014–2017 on the highlands of Muara Enim, Ogan Komering Ulu, Pagar Alam and Lahat Regencies. The method used was exploration and desk study. The samples for characterization were selected by purposive sampling, with five plants were collected for each rice variety. Morpho-agronomic characterization was carried out *in situ* and *ex situ*. Morphological observations were performed by visually observing the parts of leaves, stems, panicles and unhulled seeds of the rice accessions in the location. Morphological characteristics were analyzed descriptively by tabulation.

3. Results and discussion

Diverse geography and agroecosystems in the region of South Sumatra make this province rich in biodiversity and genetic resources. The yield and quality of many local rice varieties in this area are equal to those of improved varieties introduced from outside South Sumatra. The dryland of South Sumatra is distributed on lowland and highland plains. Dry lowland area is divided into flat-wavy, wavy and hilly reliefs. According to Indonesian Center for Soil and Agroclimate Research [4], dryland areas in South Sumatra occupy about 86.4% of the total area of the province. They have great potential in supporting the development of agriculture (food and plantations).

The drylands in the highlands of South Sumatra Province are also suitable for development [5]. Potential dryland areas to be developed are distributed across six regencies, namely Ogan Komering Ulu (OKU), East OKU, South OKU, Muara Enim, Lahat and Musi Rawas [6]. According to Murtilaksono and Anwar [7], dryland areas in South Sumatra are acidic with the following soil characteristics: acidic (pH <5), low organic matter, base saturation <50% (district), high Al, precipitation >2,000 mm/year, low fertility and land productivity that need high input for cultivation. Furthermore, Murtilaksono and Anwar [7] stated that the constraints in the use of acidic dryland are water availability, high soil acidity (low pH), low organic matter and shallow topsoil, very poor in nutrients and rocky soil.

In total 17 local superior rice varieties were found in highland plains of Pagar Alam, Muara Enim and Lahat Regencies, i.e. 10 varieties in Lahat District, 5 varieties in Pagar Alam and 1 variety each in Muara Enim and Ogan Komering Ulu. Several accessions with the same name were found in several villages, although they are morphologically different. Further research is needed to resolve these duplications. The data on the distribution of local rice varieties and the grain characteristics of each variety are presented in Table 1 and Table 2, respectively.

Among the 17 local varieties, 3 varieties were characterized. These were Setangkai found in the village of Pagar Wangi, North Dempo, Pagar Alam at an altitude of 914 m above sea level (m asl); Ayek Keruh in Gunung Lewat, Sukamerindu, Lahat, located at 806 m asl, and in Talang Camai, Selibar, North Pagar Alam at 878 m asl (Figure 1); Barokah in the village of Tumbak Ulas, South Pagar Alam at the altitude of 714 m asl (Figure 2).

Table 1. Distribution of local rice varieties in the highlands of South Sumatra.

Exploration year	Name of variety	Regency	Number
2013	Selebur Rimbe	Muara Enim	1
	Beram, Pulut	Pagar Alam	2
2014	Tambun, Meghun, Gilas Madu, Dayang Rindu, Madu, Abang, Agai Keluang Putih, Henik, Rindik, Merah	Lahat	10
2015	Serubuh Balai	Ogan Komering Ulu	1
2016	Ayek Keruh	Pagar Alam	1
2017	Setangkai, Barokah	Pagar Alam	2
Total			17

In general, those local rice varieties have low productivity, except for Ayek Keruh. Based on a field test in Sukamerindu, Lahat, this accession yielded 8.56 t/ha, which exceeded the yield of Ciherang, an improved national variety (6.4 t/ha). The productivity of Ayek Keruh was also much higher than local rice varieties in Central Java which ranged from 2.2 to 3.8 t/ha. Previously, the highest productivity of local rice varieties was reported from Kalimantan which is 5.18 t/ha. Performance of local variety of Ayek Keruh in Pagar Alam was presented in Figure 1. Ayek Keruh is known to have a high selling value. This early maturing variety (110 days) has a high panicle number per plant (25) and grain number per panicle (>200). This variety is resistant to brown planthopper and blast pathogen. The seed shape is oval and thick (Table 2).

Figure 1. Performance of Ayek Keruh local rice variety at generative stage in Pagar Alam Regency, South Sumatra.

Table 2. Grain characteristicsof local rice varieties in the highlands of South Sumatra.

Name of variety	Regency	Diameter (mm)	Length (mm)	Thickness (mm)	Shape	Lemma and palea (downy/not; hairy/not; color)	Awn (downy/not; hairy/not; color; shape)	100-grain weight (g)
Selebur Rimbe	Sri Tanjung, Muara Enim	2.6	7.9	1.6	Short round	Not downy	Not downy	2.2–2.6
Beram	Pagar Alam	2.9	8.19	1.9	Short round	Not downy	Downy	2.35–2.61
Pulut	Pagar Alam	2.9	8.19	1.9	Short round	Not downy; brownish-yellow	Downy	2.35–2.61
Tambun	Tanjung Telang & Tanah Pilih, Lahat	2.9	8.19	1.9	Short round	Not downy; brownish-yellow	Downy	2.35–2.61
Henik	Tanjung Telang & Payo, Lahat	2.3–2.63	7.5–8.6	1.77–1.85	Oval	Hairless	Downy; yellow end of the grain	1.93–2.09
Dayang Rindu	Tanjung Telang & Tanah Pilih, Lahat	2.63	9.33	2.0	Crescent	Dark	Sharp tip	2.13
Gilas Madu	Gunung Kembang, Lahat	2.97	7.2	1.93	Short round	Hairless	Hairless	2.14
Padi Agai Keluang Putih	Banjar Sari, Lahat	2.7	9.1	1.9	Long oval, curved like a sickle	Hairless	Downy	1.88
Abang	Sugih Waras, Lahat	2.5	9.4	1.8	Hairy; reddish-yellow	Downy	White	2.357
Meghun	Babat Baru, Lahat	3.0	7.47	1.87	Almost round	Hairless	Hairless	1.91
Rindik	Babat Baru, Lahat	2.9	6.47	1.87	Almost round	Hairless	Hairless	1.91
Merah	Jaya Baya, Lahat	2.7	8.5	-	Flat oval	Downy; hairless	Transparent white	2.35
Madu	Babatbaru, Lahat	2.8	7.4	1.9	Thick	Hairless	Hairy; yellow	1.7
Serubuh Balai	Tihang, OKU	2.5	6.9	1.6	Almost round	Hairless	Hairy; yellow	1.602
Ayek Keruh	Talang Camai, Pagar Alam	2.9	7.0	1.9	Oval and thick	Hairless	Hairy; yellow	1.6
Setangkai	Pagarwangi, Pagar Alam	2.5	9.4	1.8	Hairy; reddish-yellow	Hairy; yellow	White	2.357
Barokah	Tumbak Ulas, Pagar Alam	2.6	7.2	1.9	Oval and thick	Hairless; yellowish-brown	Hairy; yellow	1.8

Important characteristics of the three varieties resulting from this characterization activity include adaptability to high altitude up to 700 m asl, relatively higher yield potential compared to other local varieties and extensive adaptatability in local agroecosystems. Other important characters are resistance to pests and diseases, especially brown planthopper and blast. Based on these characteristics, the three varieties are recommended to be developed as adaptive local varieties for the highlands.

Plant performance of the three varieties in the field appeared to be relatively uniform, so seed purification was not needed. This condition was achieved because farmers were accustomed in preparing seeds by themselves by selecting seeds from uniform and healthy plants. By using this method, pure lines were resulted and local farmers claimed that the seed quality they prepare were comparable to the certified seeds.

Figure 2. Performance of Barokah, a local rice variety, at the generative stage in Pagar Alam Regency, South Sumatra.

The massive planting of new improved varieties has a positive impact on national rice production, but has a negative effect on the on-farm conservation of the local rice varieties. Since local rice varieties have developed specific characteristics for adaptation to the local agroecosystems, they need to be protected through on-farm or *ex situ* conservations. Therefore, similar efforts to preserve and develop local varieties must be done to avoid the extinction of useful genetic resources. Inventory and characterization studies that we have initiated are preliminary steps to preserve local rice varieties in the highlands of South Sumatra.

4. Conclusions

Seventeen local rice varieties were identified during the inventory in the highlands of South Sumatra. These local rice varieties were distributed in four regencies in South Sumatra. Ten accessions were found in Lahat, 5 accessions in Pagar Alam and 1 accession each in Muara Enim and Ogan Komering Ulu Districts. Local rice varieties grown in different land typologies with different soil characteristics showed different morphological characteristics. The three varieties are recommended to be developed as adaptive local rice varieties for the highlands, namely Setangkai, Barokah and Ayek Keruh.

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Characterization of 337 exon-based single nucleotide polymorphisms (SNPs) unique to the Indonesian soybean varieties

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Abstract. Genome resequencing of five Indonesian soybean varieties resulted in a total of 2,597,286 single nucleotide polymorphisms (SNPs), 257,598 insertions, and 202,157 deletions. Out of those SNPs, only 95,207 (2.15%) were located in the protein-coding region (exon). The objective of this study was to characterize 337 exon-based SNPs unique to the Indonesian soybean varieties. The study was conducted by taking SNP samples located in the exons using criteria of gene fragments containing the SNPs that were sequenced at least five times within each of the soybean varieties. Out of 95,154 gene-based SNPs detected, only 337 SNPs met the criteria. Each of the soybean varieties was genotyped with the 337 SNP loci, and the genotypic data were scored and analyzed. Results showed that 59 SNPs were common to all five soybean genotypes. A total of 43, 41, 25, 32 and 28 SNPs loci were unique to soybean genotype Davros, Grobogan, Malabar, Tambora and B3293, respectively. These unique SNPs can function as DNA fingerprints for each variety. Out of 59 common SNPs, 24 SNPs were mutations that change the amino acid sequence of the encoded proteins. These genes with amino acid change may have high economic values such as those controlling soybean adaptation in tropical climate, photoperiod insensitivity, disease and insect resistance genes. Expression analyses of the genes with amino acid change showed variation in the expression pattern across different soybean tissues. Functional genomic analysis is necessary to isolate genes useful for breeding purposes.

Keywords: soybean, SNP, genome variation, gene expression, DNA fingerprinting.

1. Introduction

One of the DNA markers recently very popular and which is commonly used, especially in high-throughput marker analysis is single nucleotide polymorphism (SNP) [1,2]. SNP is a DNA variation within the genome that occurs when a single nucleotide changes to another type of nucleotide at the same position. With the rapid development of the genome sequencing technology, SNP markers become more popular due to abundant availability in the genome and can be used in high-throughput genotyping system. SNP marker is biallelic, almost limitless in the genome, easy to be automated and SNP data is easily combined from one laboratory to another making the marker easier to be applied among different laboratories [1–3].

Other types of sequence-based DNA markers include insertion (addition of a base) and deletion (missing of a base) at a specific location within the genome, and both markers are termed as insertion and deletion (indel) [4]. However, the frequency of indel occurrence in the genome is much lower than that of SNPs. SNP and indel are of high-value DNA markers with rapid development in recent years. SNP markers are the basic materials for SNP chip development to be used in a high-throughput genotyping system. The high-throughput genotyping system facilitates gene discovery and quantitative trait loci (QTL) tagging of economically important traits (e.g. yield, seed size, oil content, pest and disease resistances) of agriculturally important crop species such as soybean.

The discovery and detection of superior alleles, genes and QTLs of important traits can be expedited by using high-throughput sequencing and genotyping technologies [5,6]. One of the high-throughput sequencing technology is next generation sequencing (NGS) HiSeq platform that can result in billions of bases (300–600 Gb) at one run of the platform [7] of high-quality sequence due to high sequencing accuracy [6]. A large amount of sequence data resulted by NGS with lower sequencing cost significantly facilitates the improvement of genomic studies of important crop species [8,9]. Supported by modern bioinformatics the important DNA variants useful for breeding can be easily detected to expedite breeding programs.

With the availability of reference genome sequence of various important crop species, the NGS technology becomes very powerful to identify the genomic variation of a crop species through resequencing of various genotypes of the species member to discover superior genes and QTLs together with a large number of DNA markers applicable for plant breeding programs.

Using NGS technology, genotypic characterization of Plant Genetic Resources (PGR) collection can be done in a more comprehensively manner at genome level, and hence the superior gene and QTL discoveries become more efficient, more accurate, and faster. Management of PGR collection in the genebank will be more efficient as all of the materials stored have been identified as genetically distinct.

Resequencing of individual genotypes of a plant species can be easily done with the availability of the reference genome sequence of that particular species. The availability of the reference genome sequence expedites analysis of the resequencing data. Alignment of the resequencing data with those of the reference genome sequences resulted in millions of genomic variations, such as SNPs, indels, and simple sequence repeats (SSRs) [1,10]. These are the main source of DNA markers for breeding purposes [1,6].

The soybean reference genome sequence derived from cultivar Williams 82 has been available since 2010, and the sequence can be accessed by public [11]. The size of soybean genome is about 1.1 Gb predicted to contain 46,430 protein-coding genes. The gene content is about 70% more compared to that of the model plant *Arabidopsis thaliana*. The availability of the soybean reference genome sequence facilitates the identification of the genetic basis of many economically important traits through resequencing studies of many soybean genotypes to support the acceleration of national soybean cultivar development.

The objective of this study was to characterize 337 exon-based SNPs unique to the Indonesian soybean varieties. Characterization included the discovery of exon-based SNPs obtained in all genotypes sequenced (common SNPs) and the ones that existed in only one specific genotype (unique SNPs). The expression pattern of the genes in which the SNPs changed the amino acid sequences was also studied at various soybean tissues.

2. Materials and methods

2.1. Genetic materials

Genetic materials used in this study were five Indonesian soybean varieties, i.e. Grobogan, Tambora, Davros, Malabar and B3293. Grobogan is a superior variety of high productivity with large seed size. Tambora is a superior variety with medium seed size that was introduced from the Philippines. B3293 demonstrated some tolerance to aluminum toxicity and acid soils. Davros is a high productivity soybean variety, and many Indonesian soybean varieties inherit genomes from Davros. Malabar is a

shading-tolerant soybean variety. The selected five varieties are genetically distant based on phylogenetic studies using SSR markers [10,12–14] and are good sources to be used for detecting sequence-based DNA variations such as SNPs and indels.

2.2. DNA isolation, genomic library construction and genome sequencing using NGS Hiseq

Genomic DNAs of the five varieties were isolated using CTAB buffer by following the method of Michiels et al. [15] with a small modification [16]. Genomic DNA libraries were prepared following the method of Tasma et al. [17] and then sequenced in NGS Hiseq platform by using the Illumina reagents, kits, and protocols as previously reported [14,17].

2.3. SNP and indel detection

The alignment of the final sequences derived from five Indonesian genotypes with that of the soybean reference genome sequence Williams 82 [11] was done by using Bowtie2 software [19] followed by SNP identification by using mpileup of Samtools [20]. Effect prediction of SNPs was done using snpEff software [21].

2.4. Exon-based DNA mutation characterization

SNPs identified were then filtered using the following criteria: (i) SNP was located within an exon, (ii) DNA fragment containing the SNP was sequenced at least 5 times (5 times genome coverage) in each of the five soybean genotypes. Among the 95,154 SNPs identified, only 337 SNPs met the criteria. Each soybean genotype (Davros, Grobogan, Malabar, Tambora and B3293) was genotyped with 337 SNP markers, scored and analyzed. The genes containing SNP loci common to all five soybean varieties that changed amino acid composition were further characterized for their expression patterns.

3. Results and discussion

3.1. Common and unique SNPs distributed across five soybean genotypes

Table 1. Common and unique SNPs found in five Indonesian soybean varieties (Davros, Grobogan, Malabar, Tambora and B3293).

Genotype	Number
Unique genic SNP[a]	
Davros	23
Grobogan	41
Malabar	25
Tambora	32
B3293	28
Common genic SNP[b]	59
Total SNPs	208

[a] SNP derived from exon that is unique to a particular soybean genotype analyzed.
[b] SNP derived from exon that belonged to all soybean genotypes analyzed.

Among 95,154 SNPs located within the exon, 337 SNPs were found to have at least five sequence copies in each of the five soybean genotypes, but the identified SNPs were different from the ones in Williams 82 variety. Among the 337 SNPs, 59 SNP loci were found to demonstrate the same

genotypes in all of the five soybean genotypes (i.e. Davros, Grobogan, Malabar, Tambora and B3293), that were different from the ones in the reference genome Williams 82 (Table 1, Figure 1). The 59 SNP loci were called common SNPs.

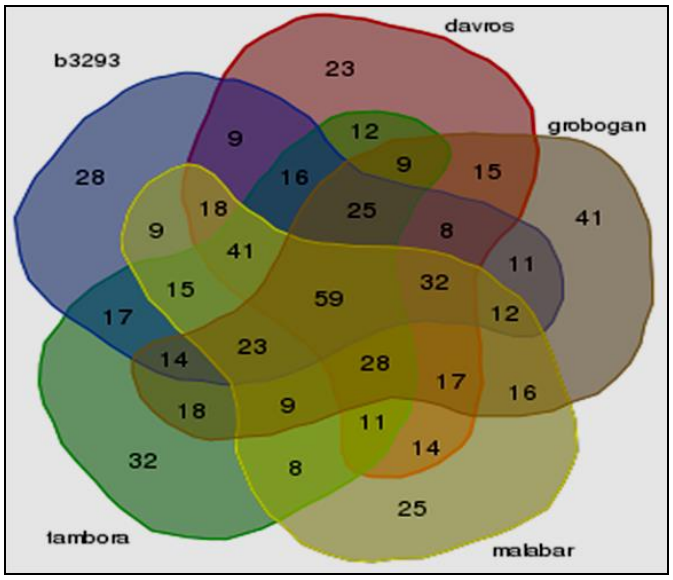

Figure 1. Venn diagram showing the unique and common SNP distributed across the five Indonesian soybean genotypes (Davros, Grobogan, Malabar, Tambora and B3293). Shown in the picture is 59 SNP loci common to all five soybean varieties. Other SNP loci were common to four, three, or two soybean genotypes. The remaining SNP loci were unique to a particular soybean genotype.

Hundreds of unique SNP loci were found only within a particular soybean genotype. A total of 23 SNP loci were observed to be unique in Davros, 41 loci were found to be unique in Grobogan, 25 loci in Malabar, 32 unique loci were discovered in Tambora, and 28 unique SNP loci were found only in the genome of B32933 (Table 2, Figure 2).

These unique SNP loci can be used as fingerprints for that specific genotype and, therefore, they can function as a unique identity of the soybean genotype. Such kind of DNA identity is very important to protect the soybean genotypes from the use by other parties without permission from the germplasm owners. The remaining SNP loci (129 SNPs) were not classified as unique nor common SNPs as they were found in two, three, or four soybean genotypes (Figure 1).

3.2. Exon-based gene mutation characteristics of common SNP loci

Among the 59 SNP loci common to all five soybean varieties but different from the ones in the reference genome Williams 82, a total of 24 SNP loci were SNPs mutations that caused amino acid change of the proteins encoded by the respective genes where the mutated SNPs were located (non-synonymous SNP mutation) (Table 2). Interestingly, among the mutated genes and the proteins they encoded, few might control important phenotypes, such as the ones for tropical adaptation, photoperiod insensitivity, heat/high temperature tolerance, or resistance to tropical soybean diseases and insect pests.

Table 2. Mutated genes that changed their amino acid composition obtained in all five soybean varieties (Davros, Grobogan, Malabar, Tambora and B3293).

Gene designation	Type of protein encoded
Glyma01g42650	Winged-helix DNA-binding transcription factor family protein
Glyma01g44270	RNA-binding (RRM/RBD/RNP motifs) family protein
Glyma03g04810	NB-ARC domain-containing disease resistance protein
Glyma06g40790	Target of AVRB operation1
Glyma06g44770	Glycosyl hydrolase superfamily protein
Glyma07g07530	Receptor-like protein kinase 4
Glyma08g00420	F-box family protein
Glyma08g00420	F-box family protein
Glyma08g01040	DCD (Development and Cell Death) domain protein
Glyma08g01040	DCD (Development and Cell Death) domain protein
Glyma08g24530	Septum site-determining protein (MIND)
Glyma08g26150	0 (the gene product has not been determined)
Glyma09g02860	Myosin heavy chain-related
Glyma11g35320	Phospholipase C 2
Glyma13g26070	Annexin 8
Glyma14g04690	Receptor-like protein 27
Glyma14g04690	Receptor-like protein 27
Glyma16g05600	C2H2 and C2HC zinc fingers superfamily protein
Glyma18g02200	Ethylene insensitive three family protein
Glyma18g46080	Disease resistance protein (TIR-NBS-LRR class) family
Glyma20g06680	Dynamin-related protein 3A
Glyma20g34200	Tetratricopeptide repeat (TPR)-like superfamily protein
Glyma20g34200	Tetratricopeptide repeat (TPR)-like superfamily protein
Glyma20g36630	Ankyrin repeat family protein

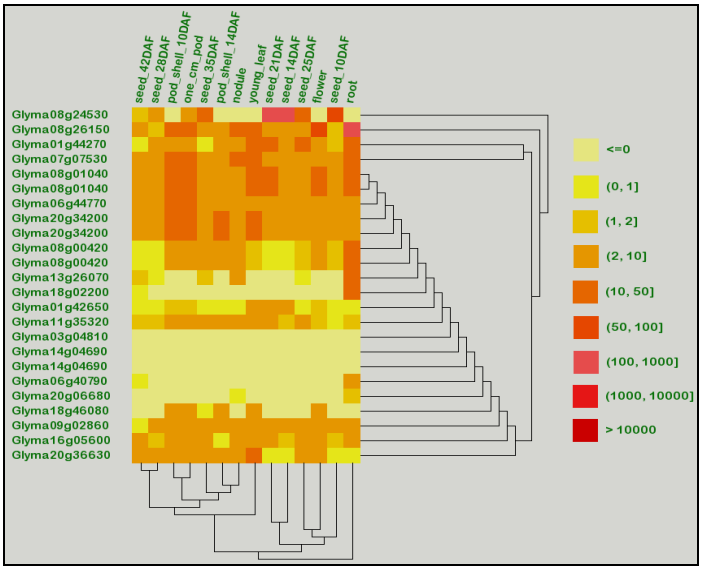

Figure 2. Expression pattern of the 24 mutated genes that were found to be common to all of the five Indonesian soybean varieties expressed in various soybean tissues. DAF = days after flowering.

The twenty-four genes described in Table 2 were differentially expressed across different soybean tissues (Figure 2). Some genes were highly expressed in leaf, root, flower, pod, or seed; while several other genes were very weakly expressed in all tissues (Figure 2). This indicates that most genes identified in this study were tissue-specific.

4. Conclusions

Out of 95,154 SNPs located within the exon, 337 SNPs were found to have at least five sequence copies in each of the five soybean genotypes but were different from the ones in the variety Williams 82. Among the 337 SNPs, 59 SNPs showed capability to differentiate the five soybean varieties compared to the reference variety (Williams 82), and some SNP alleles were also found in only one variety that is potentially used for DNA fingerprinting purposes for that particular variety. Out of 59 common SNPs, 24 were mutations that changed amino acid composition of the encoded proteins. Among the genes with amino acid sequence changes some might be genes of high economic values such as those controlling the soybean adaptation in tropical climate and disease and insect resistance genes. The genes with amino acid change showed variation in the expression pattern across different soybean tissues.

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6. Authors contribution

IMT is the main contributor, responsible for and designed the research, and wrote the manuscripts. DS is member contributor, prepared the genome library and bioinformatics analysis. HR is member contributor, conducted the genome sequencing (NGS) and sequence data analysis.

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