

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

K Nugroho\*, M Kosmiatin, A Husni, I M Tasma and P Lestari

Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD), Indonesian Agency for Agricultural Research and Development, Ministry of Agriculture, Jalan Tentara Pelajar No. 3A, Bogor 16111, West Java, Indonesia

\*E-mail: nugrohoxkristianto@gmail.com

**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](http://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                  |
| Willis      | 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](http://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 ddH<sub>2</sub>O. 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<br>R: CTTACTAGCGTATTAACCCCTTG       | 3          | [19]           |
| Satt030    | F: AAAAAGTGAACCAAGCC<br>R: TCTTAAATCTTATGTTGATGC                | 13         | [19]           |
| Satt147    | F: CCATCCCTTCCTCCAAATAGAT<br>R: CTTCCACACCCTAGTTTAGTGACAA       | 1          | [19]           |
| Satt308    | F: GCGTTAAGGTTGGCAGGGTGGAAAGTG<br>R: GCGCAGCTTTATACAAAAATCAACAA | 7          | [19]           |
| Satt197    | F: CACTGCTTTTTCCCTCTCT<br>R: AAGATACCCCAACATTATTTGTAA           | 11         | [19]           |
| Satt191    | F: CGCGATCATGTCTCTG<br>R: GGGAGTTGGTGTTTTCTTG TG                | 18         | [19]           |
| Satt463    | F: TTGGATCTCATATTCAAACCTTCAAG<br>R: CTGCAAATTTGATGCACATGTGTCTA  | 7          | [19]           |
| Satt431    | F: GCGTGGCACCCCTTGATAAATAA<br>R: GCGCACGAAAGTTTTTCTGTAAACA      | 16         | [19]           |
| Satt045    | F: TGGTTTCTACTTTCTATAATTATTT<br>R: ATGCCTCTCCCTCCT              | 15         | [19]           |
| Satt294    | F: GCGGGTCAAATGCAAATTATTTTT<br>R: GCGCTCAGTGTGAAAGTTGTTTCTAT    | 4          | [19]           |
| SoySSR1.1  | F: GACATTGGAGGACATGTAGAC<br>R: GTCTTTACTGCAATGCAACTC            | 1          | Newly designed |
| SoySSR2.1  | F: TAACCTGCAAATGGTCAACTA<br>R: TGAGAAGATCACAAACGGATAC           | 2          | Newly designed |
| SoySSR3.1  | F: TAAATTTGGATCAGATGCTGT<br>R: GTCCAATCAAGAAAACAGTTG            | 3          | Newly designed |
| SoySSR4.1  | F: CTGTTGAAGTGTAATCGTTAAAA<br>R: TTGCCGTTGATATAATCCTTA          | 4          | Newly designed |
| SoySSR5.1  | F: ATGAAGCCCAGAGAGTACAAC<br>R: CCCAAGTCTGAACATTACTCA            | 5          | Newly designed |
| SoySSR6.1  | F: AACCACCTTGGTATTTCACTT<br>R: TAACTGCCCAGAATTAGTTGA            | 6          | Newly designed |
| SoySSR7.1  | F: CAACAACCATGTCATATACG<br>R: AGGATTTCTTTGGAGATTGG              | 7          | Newly designed |
| SoySSR8.1  | F: CTCAACAACAACAACAAC<br>R: GAATGTGTCATGCAAATACAA               | 8          | Newly designed |
| SoySSR9.1  | F: GGAAGATTGATTCAAAAGTCA<br>R: CGAGAAAGTGATTGTGAGAA             | 9          | Newly designed |
| SoySSR10.1 | F: ATCAAACCCGAACCTTATTCTT<br>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.

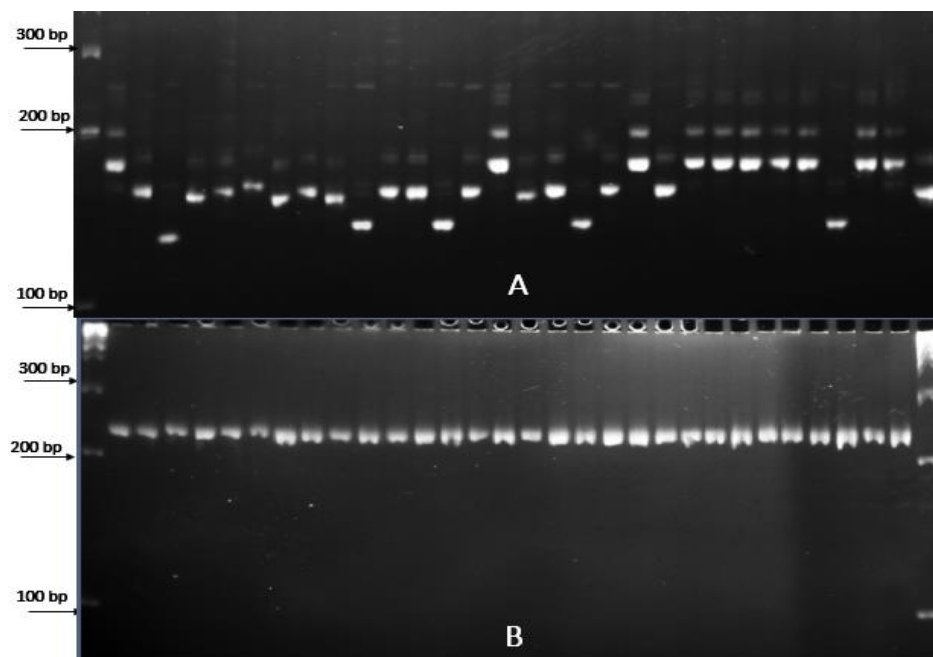
**Table 3.** Polymorphisms statistics of adopted SSR markers.

| Marker  | Allele number | Allele size range (bp) | Major allele frequency | Gen diversity (He) | Heterozygosity (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<sub>2</sub> population, the University of Utah Minsoy × Noir 1 recombinant inbred population, and the University of Nebraska Clark × Harosoy F<sub>2</sub> 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) | Heterozygosity (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

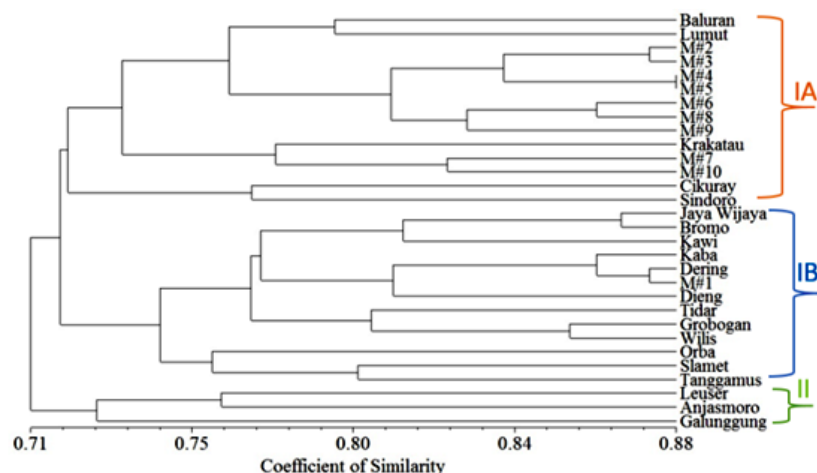
The phylogenetic analysis showed that the thirty soybean genotypes separated into two main clusters at the genetic similarity coefficient of 0.71 (Figure 2). The first cluster is divided into two subclusters, namely subcluster IA and IB. The subcluster IA consisted of 14 genotypes and subcluster IB consisted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