Seed Genetic Purity Assessment of Hybrid Maize Using Microsatellite Markers (SSR)

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ABSTRACT. The development of hybrid varieties should be supported by the availability of quality seeds. Genetic purity is one of the quality criteria required for successful seed production of hybrid maize. In producing hybrid seeds are often contaminated by crosses pollen from another variety or the occurrence of selfing. The objective of this study was 1) to get microsatellite markers (SSR) specific for male and female parents, 2) to get a percentage of the genetic purity of hybrid maize seeds cv.*Bima-3* and *Bima-4*. The study was conducted at the Laboratory Indonesian Center for Agricultural Biotechnology and Genetic Resources Bogor, from April until December 2011. Parental lines used were to production hybrid *cv. Bima-3* and *Bima-4*. Hybrid maize varieties and their parental lines seed used were derived from Indonesian Cereals Research Institute (BALITSEREAL) Maros and markers SSR selected for parental lines were *phi109275, phi96100, phi374118, phi328175,* and *phi072*. The assessment of genetic purity used two hybrid varieties namely cv.*Bima-3* and *Bima-4*, and used specific markers resulted from identification for both parental hybrid. Fourty samples individual plants of each hybrid maize variety were tested. From 5 markers tested, three of them produced polymorphic bands namely *phi96100, phi328175* and *phi072*. The *phi96100* was specific to parental cv.Bima-4, *phi072* was specific for parental cv.Bima-3. While *phi328175* was specific markers to parental of both hybrids maize were tested. The test of the genetic purity showed that 80% of the cv.Bima-4 and 97.5% cv.Bima-3 hybrid seeds genetically pure. The results of study are expected to be useful in the verification and seed purity test varieties of hybrid maize faster and more accurate.

Keywords: genetic purity, SSR, hybrid maize seed

Introduction

Maize is a commodity priority programmed by the Indonesian government. In recent years, maize production can not meet the nation's needs so that government do import of maize. In order to increase production and productivity of maize, such as the use of hybrid varieties is one of alternative.

The use of hybrid varieties need to be coupled with the provision of high quality seeds. Seed quality involves the physical, physiological, genetic quality (Sadjad 1993), and pathology quality (Ilyas 2010). Planting hybrid seeds that are not genetically pure will decrease in productivity. In this regard it is necessary techniques to identify and test the purity of hybrid and parental thus genetic quality can be maintained. The emergence of hybrid varieties will lead to difficulties in overcoming the genetic purity, since by visual is difficult to distinguish between varieties of one another. The methods used to test the purity of hybrid and parental plants is through observations in the field (grow out test), but this requires time and substantial resources (Komori and Nitta 2004). In addition, the estimated purity morphological characters are sometimes difficult, because these characters are very influenced by the environment. Technique is still widely used for variety protection in Indonesia.

With the development of molecular biology, identification of varieties can be done with the help of molecular markers, either by DNA or protein. Molecular markers is an effective tool for detection by genetic variation, which is not influenced by the environment. In contrast to the morphological and biochemical markers, DNA markers in addition to unlimited in number, also not affected by the environment and or developmental phases of plants (Tanksley and McCouch 1997).

Microsatellite markers or markers SSR (Simple Sequence Repeats) can be used to identify and verify a variety of plants (Nunome *et al*. 2003). A number of other similar studies have been conducted on maize (Pabendon 2005; Senior *et al*. 1998), in rice (Garland *et al*. 1999; Yashitola *et al*. 2002; Mulsanti. 2011), and in tomato hybrids (Liu *et al*. 2007).

The aim of this study was 1) to obtain microsatellite markers specific for male and female parents of hybrid maize, and 2) to get a percentage of the genetic purity of hybrid maize seeds cv. Bima-3 and Bima-4.

Materials and Methods

The experiment was conducted in the laboratory of molecular Indonesian Center for Agricultural Biotechnology and Genetic Resources Bogor and in the filed experiment University Farm Cikabayan, from April until December 2011. The study was conducted in two experimental stages: 1) selection of microsatellite markers specific for male and female parents, and 2) testing the genetic purity of hybrid maize seeds.

Selection of Specific Molecular Markers of Male and Female Parents

Inbred maize (male/female parents) used are parents used to hybrid production cv.Bima-3 and Bima-4, namely Nei9008/MR-14 (cv.Bima-3) and G180/MR-14 (cv.Bima-4). The seeds came from Indonesian Cereals Research Institute (BALITSEREAL) Maros and specific markers selected for parental lines were phi109275, phi96100, phi374118, phi328175, and phi072 (Pabendon 2005).

The seeds planted 20 individuals for each of the parents in a plastic box with soil media. By the time the plant was 15 days after planting (DAP), leaf samples were taken for DNA extraction. DNA isolation, amplification and visualization of DNA banding pattern following the procedure George *et al*. (2004). Part of the plant is taken young leaves that have opened perfectly from 20 individual plants, cut into small pieces and mixed, put into mortal and added liquid nitrogen to be easily crushed.

After DNA isolation, followed by testing the quality and quantity of the resulting DNA and PCR reaction. For each PCR reaction used 1.5 µL of DNA and added buffer (5x), Enhancer (5x), 0.2 µL dNTP mix (1 mM), 1.0 µL primer (0.5 mM), 0.1 µL TAG DNA polymerase (5i/il), and 3.2 µL ddH_2O. The solution of each closed with a drop of mineral oil. Amplification process consists of several stages: initial denaturation step at 94°C for 2 minutes, denaturation-1 94°C for 0.5 minutes, 56°C for 1 minute annealing, extension 72°C for 1 minute, 72°C for 5 minutes additional extension. The cycle was repeated 29 times and end up with a cycle elongation at 4°C.

PCR results added 4 µL loading dye to each well, and electrophoresis using PAGE (Polyacrylamide Gel Electrophoresis) 6% with a constant current of 100 volts for 70 minutes or until the bromphenol blue had reached the bottom of the plate. Furthermore, the gel is separated from the glass plate and immediately immersed in a solution of ethidium bromide while shaker for about 10 minutes, and was continued immersion in water for 15 minutes. Bands of DNA detected by the Bio-Rad Laboratories Segrate Milan Italy. Data were collected for specific bands formed at each parents/inbred tested.

Genetic Purity Test of Hybrid Seed Maize

Planting in field experiments carried out in the field station at the University Farm Cikabayan laboratory Research. Testing the genetic purity of seeds laboratory of moleculer at Indonesian Center for Agricultural Biotechnology and Genetic Resources Bogor. Hybrid maize seed varieties tested the genetic purity cv.Bima-3 and Bima-4 came from BALITSEREAL Maros, markers were used to test the genetic purity is polymorphic markers were identified and specific to the parental of hybrid maize from the results of experiment 1.

Planting hybrids in the field at a spacing of 75 cm x 20 cm. The first fertilization dosage: urea 100 kg/ha + SP-36 200 kg/ha + KCl 75 kg/ha was given when the plants are 7-10 day after planting (DAP), and the second fertilization dosage: Urea 200 kg/ha + 25 KCl 75 kg/ha when the plants are 30-35 DAP. Plant maintenance done intensively.

Samples were observed for 40 plants randomly determined for each hybrid variety. Each individual plant leaf samples were taken for genetic purity testing with SSR markers. DNA Isolation by a mini-prep method by Doyle and Doyle (1990). The percentage rate is calculated based on hybrid genetic purity banding pattern that appears on the individual plant samples, with the following formula:

$$\text{Purity hybrid (\%)} = = 1 - \left[\frac{NH}{TS}\right] X\ 100\ \%$$

where: TS (total sample)= number of samples/individual plants were tested
NH (non-hybrid) = number of samples/individual plants having the same banding pattern with female or male parents.

Observation of plant morphological in the field performed on the same sample. Purity test is done by observing morphological characters based on the description of each hybrid varieties tested include: age 50% of male and female flowering (days), plant heights and ear heights, anther and hair ear color, seed types, seed color, the number of rows /ear, and a thousand seeds weight.

Results and Discussion

The Identification of Specific Molecular Markers of Male and Female Parents

From the identification of five markers, there is one marker (*phi109275*) specific for parents identified male and female parents hybrid cv.Bima-4, *phi072* specific markers for parents cv.Bima-3 (MR-14 and Nei9008), and one marker *phi328175* was identified specific to the parental of hybrid cv.Bima-3 and Bima-4 (Figure 1). The *phi96100, phi072*, and *phi328175* markers considered for use in testing the genetic purity of hybrid Bima-3 and Bima-4.

Genetic Purity Test of Hybrid Seed Maize

Of the 40 individual samples *cv.Bima-4* hybrid plants were identified using marker *phi96100*, there were 7 samples of plants (number 4,6,8,9,19,31,39) which is similar to the male parent bands (MR-14), and 1 sample plant (number 12) which is similar to bands female parents (G180). Overall the total sample contained 20% of the *cv.Bima-4* seeds genetically impure (Figure 1). The results showed that there were identification banding pattern similar to the male parent, suggesting that mixing occurs in the process of harvesting or processing activities, while the presence of the same banding pattern with female parent indicate that selfing occurs in the production process due to inaccuracies in doing detaseling.

Figure 1. Visualization of specific marker for parental of hybrid maize.
(G = G180; M = MR-14, N = Nei9008; L = DNA ladder)

Figure 2. Visualization of DNA banding pattern using SSRs markers *phi96100* through vertical electrophoresis 6% PAGE (Polyacrylamide Gel Electrophoresis). No. 1, 2, 3, ... 40 is a hybrid of the *cv.Bima-4* with a range of 500-450 bp.

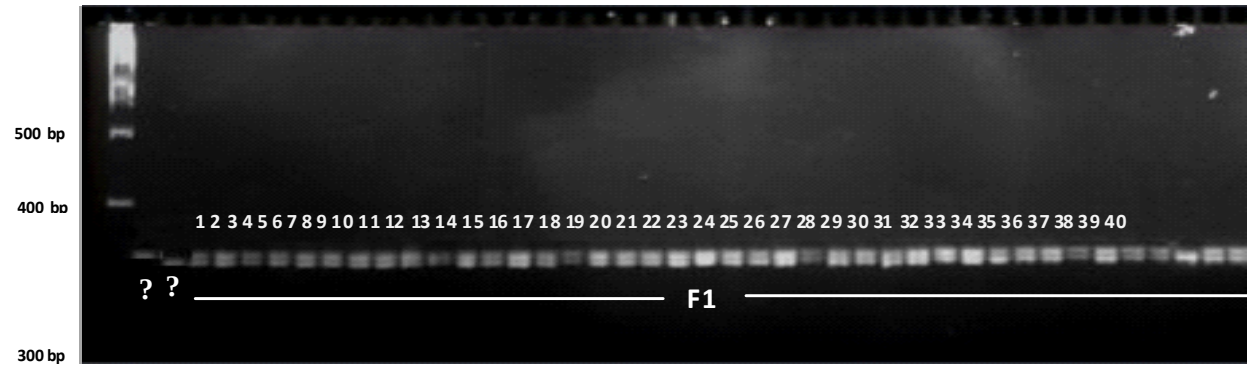

Figure 3. Visualization of DNA banding pattern using SSRs markers *phi072* through vertical electrophoresis 6% PAGE (Polyacrylamide Gel Electrophoresis). No. 1, 2, 3, ... 40 is a hybrid of the *cv.Bima-3* with a range of 400-350 bp

As compared to the morphological observations, SSR markers can identify the more seed contamination (Table 1). This showed that SSR markers are more accurate in identifying seed contamination of hybrid maize as compare to the morphology observations. Tanksley and McCouch (1997), reported that in addition to the DNA markers are not limited in number, are not also affected by the environment and the developmental phases of the plant as morphological markers. The individual plant number 31 was detected as not hybrid maize of *cv.Bima-4*, both either SSR markers or morphological observations. In contrast to the sample plants number 39 based on used grow out test not proved to be a hybrid.

Genetic purity testing of the hybrid *cv.Bima-3* by using *phi072* marker, showed that 97.5% of hybrid seeds produced genetically pure, only 2.5% of the bands have the same pattern with male parent (MR-14) (Figure 3). It is suspected that mixing occurs in the process of harvesting or processing activities in the warehouse.

In hybrid *cv.Bima-3* plant number 28 anther color different between original variety by morphological marker, but not identified in testing with SSR markers (Table 2). While the plant number 38 identified as non-hybrid assay SSR, otherwise not visible on morphological observations. Mulsanti (2011) reported the result of differences in testing genetic purity of hybrid rice between SSR and morphologycal markers.

In general, hybrid morphological characteristics are presented in Table 3. Morphological characters that visually and used as a basis to distinguish the hybrid and non-hybrid maize was the anther color and hair color of cob, while the other characters are relatively uniform in accordance with the description of each hybrid varieties tested. Other morphological characters are influenced

Table 1. The purity of hybrid seeds *cv.Bima-4* based on SSR markers and grow out test.

Purity test	Number of sample	Plant off type (%)	Sample number
SSR	40	20	4,6,8,9,10, 12,31,34
Grow out test	40	5	31,39

Table 2. The purity of hybrid seeds cv.Bima-3 based on SSR markers and grow out test.

Purity test	Number of sample	Plant off type (%)	Sample number
SSR	40	2.5	38
Grow out test	40	2.5	28

Table 3. Hybrid morphological characters cv.Bima-3 and Bima-4 on field testing in Cikabayan.

Morphological characters	cv.Bima-3	cv.Bima-4
Plant heights (cm)	± 174	±156
50% hair of ear (days)	55-61	55-61
50% pollen (days)	54-59	53-59
Steam color	Green slightly purple	Green (100%)
Anther color	Cream (97,5%)	Cream (95%)
Ear hair color	Cream (100%)	Cream (100%)
Ear types	Astigmatism	Astigmatism
Ear heights (cm)	80-110	72-100
Seed types	Semi Pearl	Pearl
The number of rows/cob	12-16	12-14
Seed color	Orange	Orange
Weights of 1,000 seed (g)	280,32	315,29

environment so it is difficult to base the determination of the purity of hybrid seeds.

Appearance of plants is controlled by genetic traits are greatly influenced by environmental factors. If environmental factors exert a more powerful, there will be variations in the morphology of the plant. Morphological assessment is subjective and influenced by environmental conditions. Thus, in order to control the purity of hybrid maize varieties and inbred constituent quickly and accurately the SSR markers could be used.

Conclusion

- There were three specific markers that can be used to identify the purity of hybrid maize the cv.Bima-3 and Bima-4.
- Identify the SSR markers, 80% of the seeds cv.Bima-4 and 97.5% of the seeds cv.Bima-3 in accordance with its constituent inbred or genetically pure. While based on morphological observations, there are 5% of the seeds Milky-4 and 2.5% Bima-3 were identified as mixed crops
- Moleculer marker can detect genetic purity of hybrid maize accurately that can not be distinguished by morphological marker.

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