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

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Abstract

The development of hybrid varieties should be supported by the availability of high quality seeds. Genetic purity is one of the quality criteria required for successful seed production of maize hybrid. In producing hybrid seeds, it is frequently contaminated by crossed pollen from another variety or the occurrence of selfing. The objectives of this study were 1) to get microsatellite markers (SSR) specific for male and female parents, 2) to get a percentage of the genetic purity of maize hybrid seeds cv. Bima-3 and Bima-4. The experiments were conducted at field station at University Farm Cikabayan Bogor Agricultural University, and in the molecular laboratory at Indonesian Center for Agricultural Biotechnology and Genetic Resources Bogor, from April until December 2011. Maize hybrid varieties and their parental lines seed used in this research were obtained from Indonesian Cereal Research Institute (ICERI) Maros, South Sulawesi. Five SSR markers selected for parental lines were phi109275, phi96100, phi374118, phi328175, and phi072.

The assessment of genetic purity of two hybrid varieties namely cv. Bima-3 and Bima-4, used specific markers from previous experiment. Fourty samples of individual plants from each maize hybrid variety were tested. From five markers tested, three markers namely phi96100, phi328175 and phi072 produced polymorphic bands and capable to distinguish parental line of two maize hybrids. Microsatellite marker phi96100 was specifically used for testing genetic purity of cv. Bima-4 and phi072 for cv. Bima-3. While phi328175 was specific marker for both maize hybrids. The genetic purity test of cv. Bima-3 and Bima-4 indicated that both varieties had purity levels of 97.5% and 80%, respectivelly. This study showed that SSR markers were more reliable for assessing genetic purity as compared to morphological marker. The results of study are expected to be useful in the verification of genetic purity of maize hybrid seeds accuretly.

Key words : parental lines, polymorphic bands, quality of maize hybrid seed

Introduction

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

The use of hybrid seeds in Indonesia in 2010 amounted to 54% of the maize acreage, and in 2014 was projected to reach 75% (Direktorat Jenderal Tanaman Pangan 2010). The use of hybrid varieties need to be supported with the provision of high quality of seeds. Seed quality includes physical, physiological, genetic quality (Sadjad 1993), and pathology quality (Ilyas 2012). Genetic quality control of seed is very important especially in hybrid seed.

Planting hybrid seeds that genetic quality are not true will decrease in productivity. In this regard it is necessary to develop techniques to identify and test the purity of hybrid and parental lines thus genetic quality can be maintained. With the release of various hybrid varieties, causing difficulties in overcoming the genetic purity, because it is visually difficult to distinguish between varieties of one another. So far, the methods used to test the purity of hybrid is through observations plants in the field (grow out test), but this requires time and substantial resources (Komori and Nitta 2004). In addition, the estimates of genetic purity based on morphological characters are sometimes difficult, because these characters are very influenced by the environment. Technique is still widely used for the protection of plant varieties in Indonesia.

Indonesian's national standards for the purity of maize hybrid seeds are based on grow out test by comparing seeds and plants in the same stage in identical environmental conditions (BSN 2003).With the development of molecular biology, identification of varieties can be done with the help of molecular markers, either by DNA or protein. Molecular marker is an effective tool because it can detect genetic variation and is not influenced by the environment. DNA markers in addition to unlimited in number, are also not affected by environmental and developmental phases of the plant (Tanksley and McCouch 1997).

Microsatellite marker or SSR (Simple Sequence Repeats) can be used to identify and verify varieties of plants (Nunome *et al*. 2003). Application of SSR techniques for fingerprinting of plant species was first reported by Akkaya *et al*. (1992) *in* Shah *et al*. (2009). SSR markers are very useful for various applications in crop improvement because it has a high polymorphism and easy handling (Stachel *et al*., 2000). 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), in soybean (Santoso 2006), and in tomato hybrids (Bredemeijer *et al*. 2002; Liu *et al*. 2007). The objectives of this study were 1) to obtain microsatellite markers specific for male and female parents of maize hybrid, and 2) to get a percentage of the genetic purity of maize hybrid seeds cv. *Bima-3* and *Bima-4*.

Materials And Methods

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

Selection of specific molecular markers of male and female parents

Seeds of maize hybrid varieties and their parental lines used in this research were obtained from Indonesian Cereals Research Institute (ICERI) Maros, South Sulawesi. Maize inbred (female/male parents) used were parental lines to produce hybrid *cv. Bima-3* (*Nei9008/MR-14)* and *Bima-4 (G180/MR-14)*. Specific markers selected for parental lines were *phi109275, phi96100, phi374118, phi328175*, and *phi072*.Twenty seeds for each of the parental lines planted in a plastic box with soil media. Samples of young leaves which have perfectly open at 15 days after planting (DAP), were taken from 20 individual seedlings for DNA extraction. DNA isolation, amplification and visualization using the procedure described by George *et al*. (2004), with slight modified.

Sample leaves were cut into small pieces and mixed, put into mortar and added liquid nitrogen to be easily crushed. The DNA (pellet) was tested for its quality, quantity, and subsequently used for PCR reaction. For each PCR reaction 1.5 µl of DNA were used, and 3 µl buffer (5x), 3 µl Enhancer (5x), 0.3 µl dNTP mix (10 µM), 1.5 µl DNA markers (5 µM), 0.15 µl TAG DNA polymerase, 5.55 µl ddH2O were added, then one drop of mineral oil. The following PCR program was used: a denaturation step at 94^{o}C for 2 min, 29 cycles of 30 seconds at 94^{o}C, 1 min at 56^{o}C and 1 min at 72^{o}C.

Finally additional extension was performed for 5 minutes at 72°C. PCR products were placed in each of the weels and added with 4 µl loading dye. The process of electrophoresis using Polyacrylamide Gel Electrophoresis (PAGE) 6% with a constant flow of 100 volts for 70 minutes or until the bromphenol blue reached the bottom of the plate. Furthermore, the gel was separated from the glass plate and immediately immersed in a solution of ethidium bromide while shaking for 10 minutes, and continued immersion in water 15 minutes. Bands of DNA were detected using Bio-Rad Laboratories Segrate Milan Italy. Data were collected for specific bands formed from any parental lines tested.

Genetic Purity Test of Maize Hybrid Seed

Field experiments were carried out in the field station at the University Farm Cikabayan, Bogor Agricultural University. The genetic purity of seeds was tested at moleculer laboratory at Indonesian Center for Agricultural Biotechnology and Genetic Resources Bogor. The assessment of genetic purity of two hybrid varieties namely *cv. Bima-3* and *Bima-4*, used specific markers from previous experiment. The seeds were planted in the field with planting space of 75 cm x 20 cm. Fertilization was done twice i.e. the first fertilization: Urea 100 $kgha^{-1}$ + SP-36 200 $kgha^{-1}$ + KCl 75 kg ha^{-1} were given 7 days after planting (DAP), and the second fertilization: Urea 200 kg ha^{-1} + KCl 25 kg ha^{-1} given 30 DAP. Plant maintenance was done intensively.

Forty samples of plants of each hybrid varieties randomly determined, young leaf samples were tested for genetic purity by using SSR markers. DNA isolation was performed using a mini-prep method as discribed by Doyle and Doyle (1990). The percentage of hybrid genetic purity were calculated based on DNA banding pattern appeared on the individual plant samples, with the following formula:

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

where: TS (total sample) = number of samples/individual plants tested; NH (non-hybrid) = number of samples/individual plants having the same banding pattern with female or male parents, and samples without bands.

Morphological observation (grow out test) were performed on the same sample. Purity test was done by observing morphological characters based on the description of each hybrid varieties especially color of anther and color of cob hair.

Results And Discussion

The identification of specific molecular markers of parental lines

From five markers tested, three markers of them namely *phi96100, phi328175* and *phi072* produced polymorphic bands and were capable to distinguish parental line of two maize hybrid. Microsatellite marker *phi96100* was specific used for testing genetic purity of *cv. Bima*-4 and *phi072* for *cv. Bima-3*. Pabendon (2005) reported that these SSR markers have a high degree of poymorphism. While *phi328175* was specific markers to both hybrids maize (Figure 1). The *phi96100, phi072,* and *phi328175* markers considered to be use in testing the genetic purity of *cv. Bima-3* and *Bima -4*.

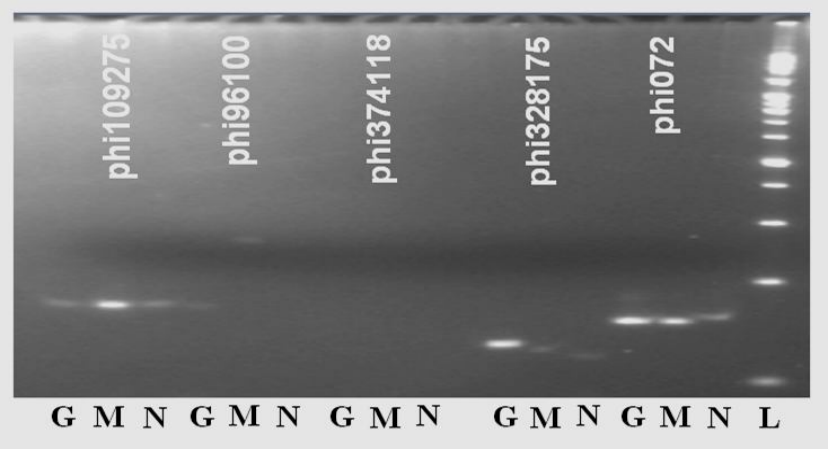

Figure 1. Visualization of specific marker for parental of maize hybrid (G = G180; M = MR-14, N = Nei 9008; L = DNA ladder)

Genetic Purity Test of Hybrid Seeds Maize

Of the 40 samples plants *cv. Bima-4* identified using *phi96100* marker, showed that seven samples (number 4, 6, 8, 9, 19, 31, 39) which similar to the male parent bands (MR-14), and one sample (number 12) which similar female parents bands (G180). Overall the total sample contained 20% of the *cv. Bima-4* seeds that were not genetically pure (Figure 2).

The result of identification showed that there were banding pattern similar to the male parent, it seemed that mixing occurs during harvesting seed or processing activities, while the presence of the same banding pattern with female parent indicated that selfing occured in the production process due to inaccuracies in detasseling.

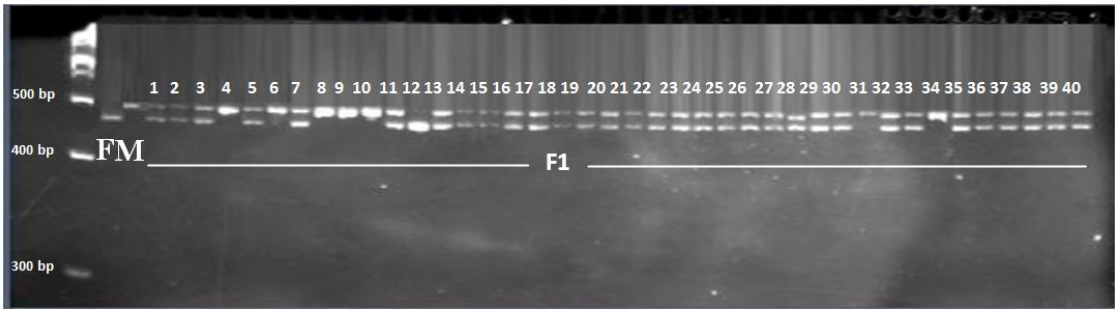

Figure 2. Visualization of DNA banding pattern using SSRs markers *phi96100* through vertical electrophoresis 6% PAGE. F= female parent, M= male parent. No. 1, 2, 3, ... 40 is *cv. Bima-4*

Observations from grow out test, off type was marked with red purplish anthers and reddish cob hair, while individual plants that fit the description either anther or hair cob color were cream. As compared to the grow out test observations, SSR markers can identify more seed contamination (Table 1). Individual plant number 31 in *cv. Bima-4* detected no hybrid, both with SSR markers and grow out test. Incase of plant sample number 39, it was not a hybrid based on grow out test, but it was a hybrid based on SSR. This shows that SSR markers are more accurate in identifying contamination of maize hybrid seeds as compare to the grow out test. Tanksley and McCouch (1997), reported that in addition to that DNA markers are unlimited in number, they are also not affected by the environment and the developmental phases of the plant as morphological markers.

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

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

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

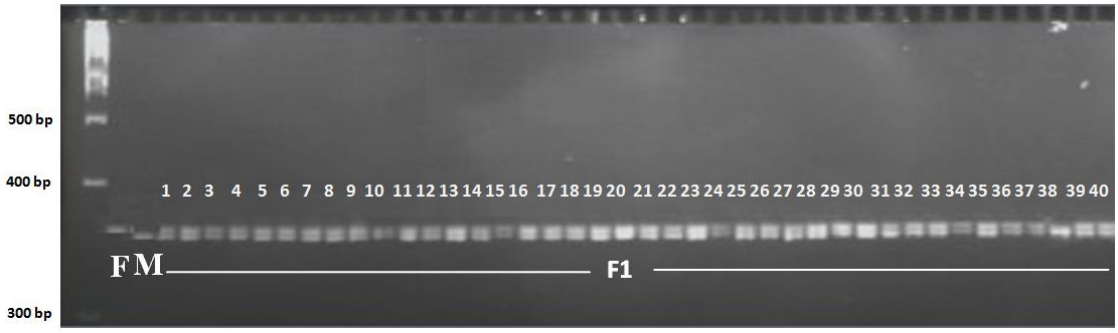

Figure 3. Visualization of DNA banding pattern using SSRs markers *phi96100* through vertical electrophoresis 6% PAGE. F= female parent, M= male parent. No. 1, 2, 3, ... 40 is *cv. Bima-3*

Based on the description of the variety, *cv. Bima-3* has cream color of the anther and of the hair cob. Based on morphological observations of the *cv. Bima-3*, indicated that plant number 28 had anther with purple color, but it fit the description based on SSR markers (Table 2). On the other hand, plant number 38 was identified as non-hybrid base on SSR, but not based on morphological observation. Mulsanti (2011) was found differences in testing genetic purity of rice hybrid between SSR and morphologycal markers.

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

Appearance of plants is controlled by genetic traits and are greatly influenced by environmental factors. Environmental factors such as location and growing season affected the phenotifically appearance of maize plant e.g. grain weight was not maximum. If environmental factors gave strong effect, there was variation in the morphology of the plants. Morphological assessment is subjective and influenced by environmental conditions (Pabendon 2010). Therefore, morphological observation (grow out test) could not be used as the basis for determining the genetic purity of maize plant varieties. Thus, in order to control the purity of maize hybrid varieties and the constituent inbred quickly and accurately the SSR markers could be used.

Conclusion

- Specific SSR markers could be used to identify the genetic purity of maize hybrid the *cv. Bima-3* (phi072, phi328175) and *Bima-4* (phi96100, phi328175).
- Based on SSR markers, the genetic purity of maize hybrid seeds *cv. Bima-3* and *Bima-4 had purity levels of* 97.5% and 80%, respectivelly.
- SSR markers could detect genetic purity of maize hybrid accurately that could not be distinguished by morphological marker.

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References

Akkaya MS, Bhagwat AA, Cregan PB. (1992). Length polymorphism of simple sequence repeats DNA in Soybean. Genetic. 132: 1131-1139

[BSN] Badan Standarisasi Nasional. (2003). Standarisasi Nasional Indonesia "Benih Jagung Hibrida" SNI 01-6944-2003. Jakarta.

Bredemeijer M, Cooke J, Ganal W, Peeters R, Isaac P, Noordijk Y, Rendell S, Jackson J, Roder S, Wendehake K, Dijcks M, Amelaine M, Wickaert V, Bertrand L, Vosman B. (2002). Construction and testing of a microsatellite containing more than 500 tomato varieties. Theor. Appl. Gen. 105: 1019-1026.

Direktorat Jendral Tanaman Pangan. (2010). Roadmap swasembada jagung 2010 – 2014. Kementrian Pertanian RI. Jakarta.

Doyle JJ, Doyle JL. (1990). Isolation of plant DNA from fresh tissue. Focus. 12: 13-15

Garland SHL, Lewin L, Abedinia M, Henry R, Blakeney A. (1999). The use of microsatellite polymorphisms for the identification of Australian breeding lines of rice (*Oryza sativa* L.). Euphytica. 108: 53−63.

George MLC, Regalado E, Warburton M, Vasal S, Hoisington D. (2004). Genetic diversity of maize inbred lines in relation to downy mildew. Euphytica. 135: 145−155.

Ilyas S. (2012). Ilmu dan teknologi benih: Teori dan hasil penelitian. IPB Press. 138 hal

Komori, T, Nitta N. (2004). A simple method to control the seed purity of *japonica* hybrid rice varieties using PCRbased markers. Plant Breeding 123: 549−553.

Liu Li-Wang, Y. Wang, Yi-Qin Gong, Tong-Min Zhao, G. Liu, Xiao-Yan Liand Fa-Min Yu. (2007). Assesment of genetic purity of tomato (*Lycopersicum esculentum* L.) hybrid using molekuler markers. J. Sci. Horti. 115: 7–12.

Mulsanti IW. (2011). Identifikasi Dan Evaluasi Kemurnian Genetik Benih Padi Hibrida menggunakan Marka Mikrosatelit. [Thesis]. SPS. IPB. Bogor.

Nunome T, Suwabe K, Iketani H, Hirai M. (2003). Identification and characterization of microsatellites in eggplant. Plant Breeding 122: 256−262.

Pabendon MB, Mejaya MJ, Subandi, Dahlan M. (2005). Sidik jari empat varietas jagung hibrida beserta tetuanya berdasarkan marka mikrosatelit. Zuriat. 16 (2): 192 – 200.

Pabendon MB, Mejaya MJ, Koswara J, Aswidinnoor H. (2010). Korelasi jarak genetik berbasis marka mikrosatelit inbrida jagung dengan bobot biji F1. J. Penelitian Pertanian Tanaman Pangan. 29(1): 11 – 17.

Sadjad S. (1993). Dari benih kepada benih. PT Gramedia Widiasarana Indonesia. Jakarta.

Santoso TJ, Utami DW, Septiningsih EM. (2006). Analisis sidik jari DNA plasma nutfah kedelaimenggunakan markah SSR. J.AgroBio; 2(1):1-7

Senior ML, Murphy JP, Goodman MM, Stabungr CW. (1998). Utility of SSR for determining genetic similarities and relationships in maize using an agarose gel system. Crop Sci. 38: 1088−1098.

Shah Z, Munir I, Ali S, Iqbal A, Mumtaz S, Nwaz R, Swati ZA. (2009). Genetic diversity of Pakistani maize genotypes using chromosome specific simple sequence repeat (SSR) primer sets. African J. of Biotech. 8(3): 375-379.

Stachel M, Lelly T, Grausgruber H, Vollman J. (2000). Application of microstallites in wheat for studying genetic differentiation caused by selection for adaptation and use. Theor. Appl. Genet. 100: 242-248

Tanksley SD, McCouch SR. (1997). Seed banks and molecular maps: Unlocking genetic potential from the wild. Science. 277: 1063 - 1066.

Xin Ye-Yun, Zhang Zhan, Xiong Yi-Ping, Yuan Long-Ping. (2005). Identification and purity test of super hybrid rice with SSR molecular markers. Rice Science. 12(1): 7–12.

Yashitola J, Thirumurugan T, Sundaram RM, Naseerullah MK, Rhamesa MS, Sarma NP, Sonti RV. (2002). Assessment of purity of rice hybrids using microsatellite and STS marker. Crop Sci. 42: 1369−1373.