

High Resolution Microsatellite Marker Analysis of Some Rice Landraces Using Metaphor Agarose Gel Electrophoresis

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Abstract

Microsatellite markers or simple sequences repeats are DNA - based molecular techniques that are used to see the different among accessions and inbred lines. There are three methods to analysis the results of the polymerase chain reaction of microsatellite markers namely polyacrylamide gel electrophoresis (PAGE), capillary electroforesis, and Metaphor Agarose Gel Electroforesis (MAGE), and the Use of MAGE assessed more easily and economically the polymorphic pattern of DNA markers. This study aimed to obtain fast, effective and efficient in term of easy and cheap technique to identify microsatellite markers of some black rice cultivars and F₂ populations from crosses between black with white rice. The results showed that MAGE successfully separated clearly SSRs alleles with different sizes of less than 25 bp .

Keywords: microsatellite markers, Metaphor Agarose Gel Electrophoresis (MAGE), and rice cultivar .

Introduction

Black rice became popular and began to be consumed as a functional food naturally or through certain processes, contained one or more compounds that were considered to have beneficial physiological functions to health. One of compounds present in black rice was anthocyanin (Yawadio *et al.*, 2007). Black rice has potential as a viable source of antioxidants.

There was color diversity of black rice from dark purple to light purple. Therefore the identification of superiority was required so that it could be used as breeding material. Pabendon *et al.* (2003) suggested that knowledge about germplasm diversity and kindship among breeding materials were very important to plan crosses to produce hybrid lines. Lines that were made from genetically diverse sources could

produce better hybrids compared with close kindship.

The evaluation of genetic diversity can be done with morphological, biochemical and molecular markers (Solouki *et al.*, 2008). The use of molecular markers as a genetic marker is a tool which is able to look at genetic differences between inbred lines. Molecular markers hold two important roles in plant breeding programs, namely as a determinant of fingerprint and marker in the selection link with certain desirable phenotypic characteristics (McCouch and Tanksley, 1991). Pabendon *et al.*, (2011) said that the marker technology can be used to answer questions related to genetic diversity, classification, and phylogeny related to germplasm management and tool of breeding and selection through gene markers. Several technologies based on DNA marker that are used widely were RFLP (Restriction Fragment Length Polymorphism), AFLP (Amplified Fragment Length Polymorphism), SSR (Simple Sequence repeats), and SNP (single nucleotide polymorphism) (Asif *et al.*, 2008).

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Among numbers of molecular markers available, microsatellites or Simple Sequence Repeats (SSRs) have become frequent used marker system. Microsatellite markers consisted of 1-6 base pairs motif of DNA structure, repeated five times or more in a row sequentially (Vigouroux *et al.*, 2002). Such markers spread and covered the whole genome, especially in eukaryotic organisms. This marker has been used in various studies, including genetic diversity for the identification of plant variety, and selection process of plant breeding material as called of Marker Assisted Selection (MAS). According to Azrai (2005), selection with DNA markers based on genetic traits were stable and not affected the environment.

The simplicity of SSRs in the amplification and detection of DNA fragments, and high levels of generated polymorphism making this method was an ideal to be used in large sample numbers. SSR could be harvested using PCR (Polymerase Chain Reaction) technique. PCR used only small amounts of DNA with a small DNA segment amplification, approximately 100-300 bp (base pairs) of the genome. Moreover, SSRs could be applied without damaging the plant material because only a few was used in DNA extraction or may use other parts such as seeds or pollen (Senior *et al.*, 1996). SSR technology was widely used because it was relatively practice, accurate, and produced high level of polymorphism, and even allowed the observation of multiple markers simultaneously (multiplex), and spread evenly throughout the genome (Susanto *et al.*, 2009).

Another advantage of using microsatellite was codominant nature, meaning that it could distinguish between homozygous and heterozygous (Powell *et al.*, 1996). Several considerations of using microsatellite in genetic studies included (1) SSR markers were abundant and evenly dispersed in the genome, diversity was very high (many alleles within loci), codominant and location of the genome could be known; (2) reproducibility

and accuracy were very high; (3) these markers were very accurate for distinguishing the genotype, seed purity evaluation, mapping, and genotyping of selection for the desired characteristic; and population genetic studies and analysis of genetic diversity. Microsatellite markers has been widely used to identify the genetic diversity of several kinds of plant germplasm, such as Sorghum (Bucheyeki *et al.*, 2009), maize (Pabendon *et al.*, 2007), red local rice (Utami *et al.*, 2009) and wheat (Zhang *et al.*, 2005).

At the moment there were three methods of electrophoresis to analyze the results of the amplification using PCR of SSR, namely the Polyacrylamide Gel Electrophoresis (PAGE), capillary electrophoresis and Metaphor Agarose Gel Electrophoresis (MAGE). PAGE was the most common method and very useful (Cregan and Quigley, 1997). Amplification products in a polyacrylamide gel was usually visualized with radioactive, and fluorescent labels, and even silver staining. However, this technique required visualization of a radioactive activity which was quite expensive and dangerous, and required a longer exposure time. Capillary electrophoresis was used to determine the length of polymorphism microsatellite markers (Marino *et al.*, 1995), but this method required highly sophisticated instruments and expensive labeled primer.

Metaphor Agarose Gel Electrophoresis (MAGE) was another approach to separate alleles of microsatellite markers (Abdurakhmonov *et al.*, 2007). Metaphor agarose delivered twice resolution capability of a product best agarose, metaphor agarose provided clear and good resolution up to 20-800 bp DNA fragments with 2% concentration, approached the polyacrylamide gel resolution.

Based on these conditions, this research was conducted to find a method that was faster, more effective and efficient due to easy use and cheap in price with high-resolution to identify genotypes using microsatellite in some black rice landraces.

Materials and Methods

Plants Materials and DNA Isolation

Eleven landraces of black rice and 2 commercial white cultivars (Table 1) and 30 individual numbers of plant populations of F₂ generation from crosses of black rice of Magelang hairless (S) and Situbagendit white rice (G) were used in this study. DNA rice genome was isolated from plants materials using CTAB (Cetyl-trymethyl ammonium bromide) method (Doyle and Doyle, 1990).

Table 1. Eleven black rice landraces and 2 commercial white cultivars

Black rice landraces	Population
1. Melik	Black rice
2. Jliteng	Black rice
3. Cempo Ireng	Black rice
4. Pari Ireng	Black rice
5. Black rice of NTT	Black rice
6. Blaack rice of Magelang (hairy)	Black rice
7. Black rice of Bantul	Black rice
8. Black rice of Magelang (hairless)	Black rice
9. Black rice of Sragen	Black rice
10. Black rice of Wonosobo	Black rice
11. Black rice of Banjarnegara	Black rice
12. Situbagendit	White rice
13. Inpari 6	White rice

Young leaves of approximately 0.05 g which taken from the nursery were crushed by adding 800 mL CTAB preheated buffer (65 °C) and poured in the micro tube. Tubes were incubated for 60 min at 65 °C with inverted every 10 minutes. A 24 : 1 chloroform isoamyl alcohol solution (CIAA) of 400 mL was added to the tube. The tube was vortexed, and then centrifuged at 12.000 rpm for 15 minutes. The supernatant was taken out and put into a new micro tube. Three M sodium acetate was poured into the tube as much as 1/10 volume of the supernatant. After the addition of cold isopropanol as much as 2/3 the total volume (supernatant + sodium acetate), the tube was inverted properly and stored in the freezer for 24 hours. Furthermore, the tube was centrifuged at 12.000 rpm for 10 minutes. The supernatant was taken and added 500 mL of 70% ethanol and the tube was inverted.

Furthermore, the tube was centrifuged at 12.000 rpm for 5 minutes. The supernatant was discarded and the sediment was dried. Once dried, DNA was dissolved with 50 mL sterile aquabidest, then the DNA was ready to be quantified .

PCR for Microsatellite Amplification

In order to amplify the microsatellite sequence in the genome, PCR technique was used. RM-224 primer with forward: ATCGATCGATCTTCACGAGG; reverse base sequences: TGCTATAAAAGGCATTCGGG was chosen to amplify related microsatellite from total DNA genome of 11 black rice landraces, 2 commercial white rice cultivars and 30 individual plants of F₂ populations from crosses of black rice (Magelang hairless) with Situbangendit white rice cultivar. PCR amplification was performed in a total volume of 10 µl consisted of 5 µl Go Taq Green PCR mix; 2 µl nuclease Freewater; 0.25 µl forward and reverse primers respectively, and 5 µl total DNA genomes. The PCR program was set up for 5 minutes denaturation starters at a temperature of 94 °C, followed by 35 cycles consisted of: 1 minute denaturation at 94 °C, 1 minute annealing process at a temperature of 55 °C, 2 minutes elongation of the primer at 72 °C, the repetition of 2 steps through 4 steps as many as 13 times with touchdown program with a difference temperature of 0.5 °C each cycle and lasted by primer extention at 72 °C for 7 min, and incubation phase at 4 °C.

Gel Electrophoresis

PCR product was analyzed using standard and MetaPhor™ (Lonza) agarose, with different concentrations, i.e. 1 and 2% standard agarose, and 2% metaphor agarose. Each agarose types with different concentrations was dissolved in 1x TBE (Tris-borate-EDTA) buffer separately by heating slowly. After all ingredients were dissolved, 0.01% DNA dye was added. Dissolved agarose was solidified by pouring into a gel tray, stored at 4 °C for 30-60

minutes before use to obtain matrix gel with better resolution. After gel was ready, gel was assembled to electrophoresis tank and soaked with 1 x TBE buffer. PCR results were loaded into the wells and electrophoresed for about 60 minutes at the voltage 80 volts and 400 Amphere. After electrophoresis, SSR products were visualized under UV Transilluminator and photographed.

Result and Discussion

Microsatellites showed high polymorphic, multi-allelic, and abundant in the genome (Kuleung *et al.*, 2006), dispersed throughout the genome (Ijaz and Khan, 2009), and analyzed as DNA fragment in the size of 100 - 350 bp (base pair) (Kelestanie *et al.*, 2013). Microsatellites are usually analyzed on standard agarose or poly-acrylamide gel electrophoresis (Wang *et al.*, 2009). The agarose gel required simple devices, although it did not fit to the DNA segment size of

microsatellite and the result was normally not optimal. In case of poly-acrylamide gel electrophoresis, it was laboratory expensive. An easier, quicker and more reproducible method for electrophoresis and operation would be superior if enough resolution and sensitivity could be achieved. Metaphor system was indeed capable of tracking allelic inheritance (White and Kusunawa, 1997).

Metaphor agarose provided an acceptable resolution separation of 20 - 800 bp fragments that would be fitted to the size of microsatellite given by poly-acrylamide gel resolution, whereas the theoretical resolution capacity was averaged 3.3 bp within the size range of 160 - 307 bps (White and Kusunawa, 1997). Metaphor agarose gel electrophoresis was probably one of easy and economic method to separate allele of microsatellites (Asif *et al.*, 2005).

Figure 1c showed the separation of the two alleles of RM 224 microsatellite markers

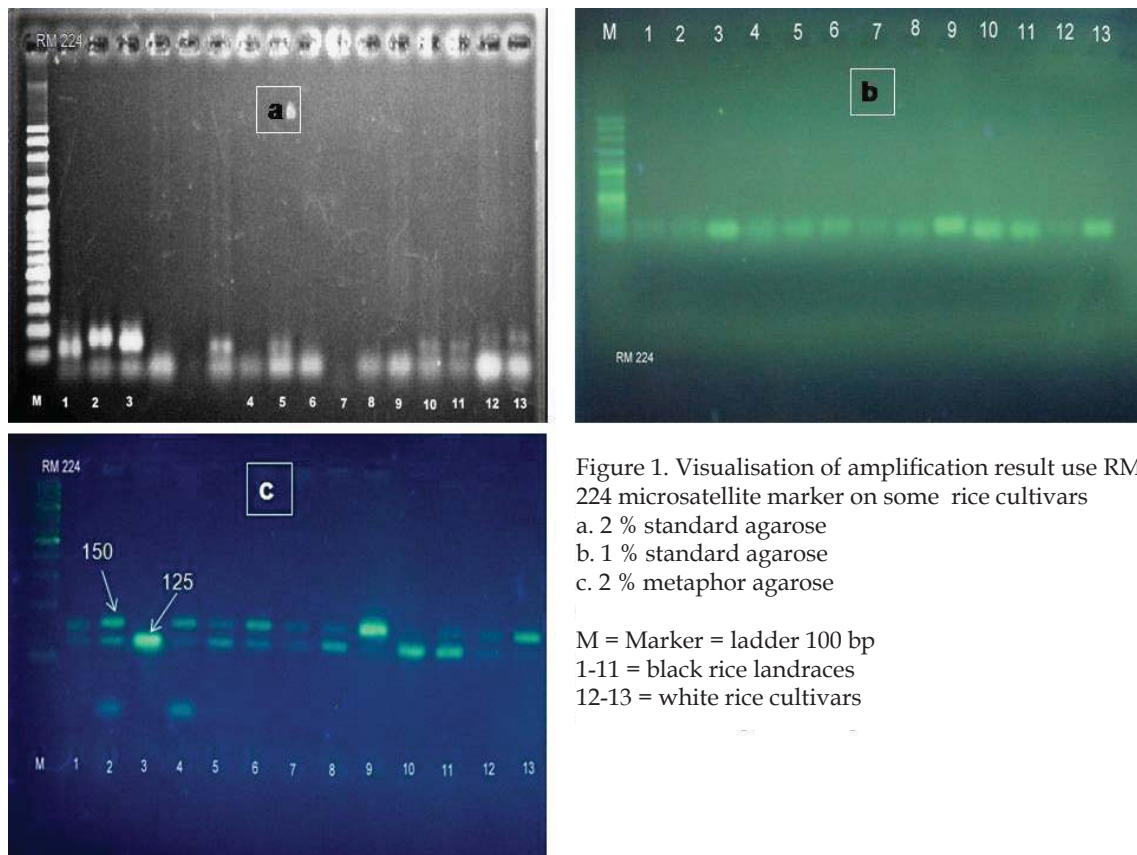


Figure 1. Visualisation of amplification result use RM 224 microsatellite marker on some rice cultivars

- a. 2 % standard agarose
- b. 1 % standard agarose
- c. 2 % metaphor agarose

M = Marker = ladder 100 bp
 1-11 = black rice landraces
 12-13 = white rice cultivars

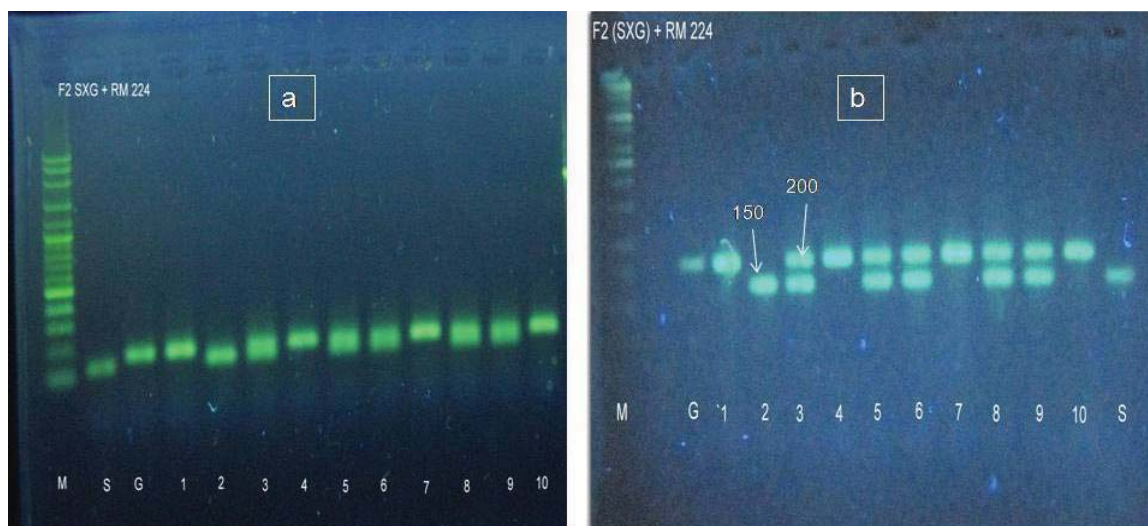


Figure 2. Visualisation of amplification result using RM 224 microsatellite marker on second generation progeny (F_2) from crosses of black rice of Magelang hairless with Situbagendit white rice. a. 1% agarose standard; b. 2% metaphor agarose
 M. Marker= 100 bp ladder
 S = Black rice of Magelang hairless as female parent
 G = Situbagendit white rice as male parent

on some black rice landraces and 2 white rice cultivars after electrophoresis at 80 V for about 60 minutes. It appeared that MAGE method using 2% metaphor agarose was clearly able to distinguish two alleles of the RM 224 in the size 125 bp and 150 bp or 25 bp different. Metaphor as also able to differentiate clearly homozygote and heterozygote lines through the production of single and double bands such as column 2 and 3 (figure 1c). These results were not clearly recognized by the use either 1% (Figure 1a) and 2% standard agaroses (figure 1b). The use of either 1% as well as 2% standard agarose (Figure 1a & 1b) did not show good separation as in Figure 1c using 2% metaphor agarose.

Figure 2b showed that Magelang Hairless (S) and Situbagendit (G) parents were able to be distinguished using RM 224 microsatellite markers after electrophoresis at 80 V for about 60 minutes. Similar result was visualized by using 1% standard agarose (figure 2a). The different between standard agarose and metaphor was observed, after they were used to analyze the 10 plants of second generation progeny (F_2) from

crosses of black rice of Magelang hairless with Situbagendit white rice. It revealed that MAGE method using 2% metaphor agarose clearly distinguished two alleles of the RM 224 at the 150 bp and 200 bp fragment sizes. Column 3, 5, 6, 8 and 9 of figure 2a looked thicker which might be a result of coincide 2 bands. In figure 2b, it was clear that the column 3, 5, 6, 8 and 9 produces 2 bands, one band belong to female parent, whereas another band related to male parent so plants at these columns were probably considered as heterozygous lines. In this experiment, metaphor was able to differentiate between homozygous and heterozygous lines as polyacrylamide gel electrophoresis. MAGE was able to visualize the typical characteristics of microsatellite markers as stated by Powell *et al.* (1996) i.e. codominant nature. Powell *et al.* (1996) even stated that there were several considerations for the use of microsatellite markers in genetic studies included (1) such markers were abundant and evenly dispersed in the genome, (2) very high diversity due to many alleles within loci, (3) codominant nature, (4) very high

reproducibility and accuracy; and (5) very accurate for distinguishing the genotype, evaluating seed purity, genetic mapping, and genotyping of desired characteristic, and even studying population genetic and analyzing genetic diversity.

The result of this study indicated that the use of MAGE succeeded in separating the alleles on 25 bp small size different, whereas Asif *et al* (2008) reported the use of microsatellite markers MGHES MAGE-17 for cotton genome could separate DNA alleles in smaller size that was 5 bp. The difference was caused by differences in microsatellite target markers and species.

Polyacrylamide Gel Electrophoresis (PAGE), Metaphor Agarose Gel Electrophoresis (MAGE) and capillary electrophoresis could be used for analysis of microsatellite and all of these methods could provide comparable and reproducible results (Ochsenreither *et al.*, 2006). However, Asif *et al.* (2008) highlighted that MAGE was more effective, while the use of capillary electrophoresis could be done faster and better, but more expensive and required more sophisticated and expertised technology. Mei *et al.* (2004); Bolek *et al.* (2005); and Abdurakhmonov *et al.* (2007) have used the MAGE method to analyze the microsatellite results on cotton crop with similar result. Therefore, the use of MAGE method to analyze microsatellite results could be the right choice because it was more easy and economics. Asif *et al.* (2008) said that in order to achieve better resolution, the new microsatellite survey research for screening of parents in plant breeding, the use of fresh metaphor gel was highly recommended. However, subsequent for screening populations with the same microsatellite, metaphor agarose gel could be reused for five to six times, therefore it became more economics.

Conclusion

Metaphor Agarose Gel Electrophoresis method was effectively and efficiently used to distinguish microsatellite alleles analysis

results on the DNA bands with smaller size different which reached less than 25 bp with a high resolution compared to use of agarose at the same concentration.

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