Molecular, morphological, and biochemical identification of sembada merah and sembada hitam rice (*Oryza sativa* L)

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Abstract. Sembada Merah and Sembada Hitam rice were Sleman Yogyakarta Local Genetic Resources which have potential as a functional food. This study aimed to determine the molecular, morphological, and biochemical character of Sembada Hitam and Sembada Merah rice. Molecular identification using RM 220, RM 224 and RM 252 microsatellite markers was carried out in Genetics and Breeding Laboratory of Agriculture Faculty of UGM Yogyakarta. Morphological identification including plant, stem, leaf, flower, panicle and grain character was carried out at AIAT Yogyakarta, meanwhile biochemical identification (amylose and anthocyanin (cyanidin-3-glucoside) content) was conducted in Indonesian Center for Rice Research. Those two rice varieties were identified with Segreng Handayani as a check. The results showed that Sembada Hitam and Sembada Merah rice differ of molecularly, morphologically, and biochemically so that Sembada Hitam and Sembada Merah rice are separate varieties.

1. Introduction

One indicator of the realization of food security is the achievement of rice self-sufficiency. Rice that is consumed as staple food by most people is white rice. On the other hand, there are other types of rice, pigmented rice (red rice and black rice). Red rice and black rice are types of local germplasm of Special Region of Yogyakarta which are still cultivated by some farmers. Gunungkidul district is an area in Yogyakarta known as a producer of red rice, namely Mandel Handayani and Segreng Handayani. These two rice varieties were released by the government as local superior varieties in 2009 under number 2227 / Kpts / SR.120 / 5/2009 (Mandel Handayani) and number 2226 / Kpts / SR.120 / 5/2009 (Segreng Handayani). Red rice is not only produced by Gunungkidul Regency, but Sleman Regency also has a type of pigmented rice namely red rice and black rice known as Sembada Merah and Sembada Hitam.

Red rice and black rice have long been known to be very beneficial for health. [1] reported that colored rice has potential as a source of antioxidants and as a source of functional food. In Japan, red rice is popular as a functional food because of its high content of polyphenols and anthocyanins[2]. Anthocyanin pigments are effective in reducing cholesterol levels in the human body [3]. Anthocyanin has been recognized as a functional health food ingredient because it has antioxidant activity (Satue-Gracia et al. 1997; Nam et al. 2006; Philpot et al. 2006) cit. [4]. In addition, black rice has higher protein, vitamin, and mineral content than white rice[5]. These advantages can provide added value to red rice and black rice so the selling price is higher than white rice. With the increasing of people’s
living standard awareness of health importance, red rice and black rice are popular as functional food. Functional food is food that naturally or through certain processes contains one or more compounds that are considered to have physiological functions for health [6].

Many types of red rice and black rice exist in Yogyakarta region. Exploration results from Assessment Institute for Agricultural Technology (AIAT) Yogyakarta in 2014, Yogyakarta has 15 types of red rice and 16 types of black rice[7]. Therefore, this study aimed to find out the molecular, morphological, and biochemical character of Sembada merah and Sembada hitam rice from Sleman Yogyakarta. In accordance with [8] that evaluation of genetic diversity can be done using morphological, biochemical, and molecular markers.

Molecular markers for many programs will be expensive to use, but it is essential to emphasize the importance of phenotype and selection[9]. Molecular selection with emphasis on breeding values requires precise phenotypic data that supports breeding values. Morphological characters are basic characters in plant classification. Through morphological characters, we can distinguish an individual from others more easily and objectively [10]. Characterization at the morphological level is needed primarily to identify phenotypes and their changes related to their ecotype [11].

On the other hand, [12] stated that marker technology can be used to answer questions relating to genetic diversity, classification, and phylogeny associated with germplasm management, and being a tool for breeding and selection through gene markers. Germplasm identification of plants using molecular markers can provide fast, effective, and accurate results.

2. Materials and Methods

The experiment was conducted from January 2016 to August 2016. The materials used in this study were Sembada Merah rice seeds, Sembada Hitam rice seeds, and Segreng Handayani (as a check variety). For molecular identification, the comparisons used were brown rice Mandel Handayani and Segreng Handayani. Chemicals for molecular identification were DNA analysis chemicals, microsatellite markers attached to the color properties of RM 220, RM 224, and RM 252, PCR kits, and materials for PCR electrophoresis. This study consisted of 3 stages, namely molecular identification (in Genetics and Breeding Lab of UGM Faculty of Agriculture), morphological identification (at Yogyakarta AIAT) and biochemical identification (at Center for Rice Research).

2.1. Molecular Identification

Molecular identification was begun with planting Sembada Merah, Sembada Hitam, Mandel Handayani and Segreng Handayani. Molecular identification used two comparisons of red rice namely Segreng Handayani and Mandel Handayani. Molecular identification consisted of 3 stages: isolation of genomic DNA using CTAB method (Doyle and Doyle, 1990); DNA obtained was used as a template for DNA amplification (PCR reaction); and visualisation of DNA amplification results (PCR) using Metaphore Gel Electrophoresis (MAGE) method.

2.1.1. DNA Isolation. Young leaves (three weeks after transplanting) were taken from each plant population (Sembada Merah, Sembada Hitam, Segreng Handayani and Mandel Handayani) for analysis, 10 samples per population. The genomic DNA isolation from the leaves was carried out using CTAB method [13](with modification). Approximately 0.05 g of leaf sample was homogenized with 800 μL of the heated CTAB buffer (at 65°C) and placed into a microtube. The tube was immersed for 60 minutes at 65°C with inversion every 10 minutes. Subsequently, 400 μL CIAA solution (24/L; v/v) was added and mixed by vortex, then centrifuged at 12,000 rpm for 15 minutes. The supernatant was transferred to the new microtube. Sodium acetate 3 M was added to the tube as much as 1/10 of the supernatant volume. After the addition of cold isopropanol as much as 2/3 of the total volume (supernatant + sodium acetate), the tube was turned back and stored in the freezer for 24 h. Furthermore, the tube was centrifuged at 12,000 rpm for 10 minutes and the supernatant was discarded. 500 μL ethanol 70% was added to the sample and the sample was centrifuged at 12,000 rpm for 5 minutes. The pellet was air
dried and diluted with 50 μL sterile aquabides. The obtained DNA was quantified using a spectrophotometer.

2.1.2. DNA Amplification (PCR). Three microsatellite primers (RM 220, RM 224, and RM 252) related to color properties (Table 1) were used in this study. DNA amplification was performed in 10 μL total volumes with 5 ng/μL DNA concentration in 2.5 μL, 5 μL 2x GoTaq Green PCR mixture; 0.25 μL 10 mM forward primer; and 0.25 μL 10 mM reverse primer. The PCR program was 5-minutes denaturation at 94°C, followed by 35 cycles consisted of denaturation for 1 minutes at 94°C, annealing process for 1 minutes at 55°C, extension process for 2 minutes at 72°C, steps 2–4 were repeated as many as 13 times, with touchdown program (temperature drop regularly) with difference of 0.5°C for each cycle, followed by last extension at 72°C for 7 min. The incubation step was at 4°C for 1 h and the last step was incubated at 10°C.

2.1.3. Visualization of Amplified DNA from PCR. The Metaphore Agarose Gel Electrophoresis (MAGE) method was used to examine the results of DNA amplification. To prepare the 2% gel, 2 g metaphore agarose was added in 100 mL 1x TBE buffer and heated slowly until clear and DNA dye (DNA stain 0.01%) was added to the gel solution. The gel solution was compressed by pouring into the mold, stored at 4°C for 30–60 min before used to obtain a better resolution. After the gel was ready to be used, the PCR results were loaded into the gel well and soaked into a 1X TBE buffer solution into a horizontal electrophoresis device and electrophoresed for about 60 min at 80 V and 400 A. The DNA amplification results were visualized under UV transilluminator and photographed.

2.2. Morphological Identification
Morphological identification by planting the three types of red rice and black rice maintained until harvest. Morphological identification included stem, leaf, flower, panicle and seed characters following the Guidelines for Rice Plant Characterization and Evaluation System [14]. Morphological identification results were made scores and then used for cluster analysis using SAS ver 9.2. so a dendogram was obtained.

2.3. Biochemical Identification
Biochemical identification by analyzing the content of amylose and anthocyanin (cyanidin-3-glucoside) in rice yields of the previous stage (morphological identification stage). Analysis of amylose content followed Cruz and Khush (2000) and analysis of anthocyanin content (cyanidin-3-glucoside) followed Abdel-Aal et al. (2006) with modifications.

3. Results and Discussion

3.1. Molecular Identification
Molecular identification aimed to identify Sembada Merah and Sembada Hitam from Sleman – Yogyakarta genetically, with a check variety of Segreng Handayani and Mandel Handayani. DNA analysis will reveal the genetic differences between red rice and black rice varieties with RM 220, RM 224, and RM 252 microsatellite marker (Figure 1).

Figure 1 shows that by using a primer of RM 220, there was a difference in the size of DNA band from the PCR results between Mandel Handayani and three other varieties (Segreng Handayani, Sembada Merah, and Sembada Hitam), while Segreng Handayani, Sembada Merah and Sembada Hitam had the same size of DNA band. Figure 2, shows that by using the RM 224 marker there was a difference between Sembada Merah with 3 other varieties (Mandel Handayani, Segreng Handayani, and Sembada Hitam), but there was no difference between Mandel Handayani, Segreng Handayani, and Sembada Hitam. Based on PCR results, the use of primers of RM 220 and RM 224 (Figure 1 and Figure 2) had not been able to distinguish the four varieties tested. Therefore, DNA analysis was carried out using different primers namely RM 252.
Figure 1. Result of PCR + RM 220

Figure 2. Result of PCR + RM 224

Figure 3. Result of PCR + RM 252

Note: 08. Mandel Handayani 43. Sembada Merah
15. Segreng Handayani 49. Sembada Hitam

PCR results using primer of RM 252 (Figure 3) showed that there were differences in size of the PCR results from four varieties tested. It can be seen that DNA size of Mandel Handayani was 200 bp, Segreng Handayani was 275 bp, Sembada Merah was 175 bp and Sembada Hitam was 225 bp. Based on these results, using primer of RM 252 can distinguish four varieties of pigmented rice tested (Mandel Handayani, Segreng Handayani, Sembada Merah, and Sembada Hitam).

Based on the results of DNA analysis using microsatellite markers that were linked to the pigment properties (RM 220 and RM 252), the Sembada Merah and Sembada Hitam varieties were separate varieties and different from the comparative varieties (Mandel Handayani and Segreng Handayani). The two check varieties used were varieties that have been released as superior varieties such as Mandel Handayani and Segreng Handayani. Therefore, Sembada Merah and Sembada Hitam rice were separate varieties that differ from the other two check varieties.

3.2. Morphological Identification

The color of the lower stem of Sembada Merah is purple, Segreng Handayani also has purple stem color, and Sembada Hitam has green stem color. The grain forms of Sembada Merah, Sembada Hitam, and Segreng Handayani are same, they are slim, but they have – different rice color. Sembada Merah has red rice color, Sembada Hitam has black rice color and Segreng Handayani has red rice color on epidermis only (Figure 4). Morphological differences can be seen from the age of plant, stem color, foot culm color, leaf midrib color, auricle color, ligula color, and rice color. Morphological observation data was made scores and then used for cluster analysis to obtain a dendogram (Figure 5).
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Figure 4. Rice color of (a) Sembada Merah, (b) Sembada Hitam, (c) Segreng Handayani

Figure 5. Dendogram based on morphological characters

Figure 5 shows that Sembada Merah and Sembada Hitam were different from the Segreng Handayani as a check variety. Sembada Merah was one group with Segreng Handayani because there were some qualitative characteristics in common, including the rice color (red). However, Sembada Merah and Segreng Handayani have more character differences so they have a great genetic distance. Whereas Sembada Hitam was not in the same group as Sembada Merah or Segreng Handayani. Therefore, Sembada Merah and Sembada Hitam were separate varieties that were different from a check variety, based on plant morphological characteristics.

3.3. Biochemical identification
The results of nutrition analysis from Sembada Merah, Sembada Hitam and Segreng Handayani (for comparison) were presented in Table 1.

Based on Table 1, it appears that cyanidin-3-glucoside was a fairly high anthocyanin from Sembada Merah and Sembada Hitam, this advantage was an added value that was not owned by white rice. Even Segreng Handayani rice as a comparison did not have or did not detect the presence of cyanidin-3-glucoside. Cyanidin-3-glucoside (C3G) was one type of anthocyanin in black rice, another type of anthocyanin was peonidin-3-glucoside (P3G) [15]. Cyanidin-3-glucoside is anthocyanin as an antioxidant which is important for health. Anthocyanin has a function as antioxidants, compounds that can donate electrons to neutralize the damaging properties of free radicals[16]. Free radicals are atoms or
molecules that are very active and unstable because the structure of atoms or molecules has one unpaired electron. To be stable, this atom or molecule needs to get another electron by taking an electron from another molecule. Karainova et al. (1990) and Kamei et al. (1995) cit.[17] stated that anthocyanin has the ability as an anticancer and prevent coronary heart disease by preventing the narrowing of arteries.

Table 1. Results of nutritional analysis of Sembada Merah, Sembada Hitam and Segreng Handayani

| Variable                         | Sembada Merah | Sembada Hitam | Segreng Handayani |
|----------------------------------|---------------|---------------|-------------------|
| Amylosa (%)                      | 20.01         | 5.89          | 18.44             |
| Anthocyanin (cyanidin-3-glucoside µ /100g) | 1.2           | 369.5         | Nd (not detected) |

Furthermore, Frei (2004) cit. [18] stated that red and black rice, in addition to strongly supporting the absorption of particles into the body and convert beta-carotene to vitamin A, are also antioxidant and anti-inflammatory compounds which lead to anticancer in the body. Sembada Hitam and Sembada Merah were different from the check variety (Segreng Handayani) based on anthocyanin content (cyanidin-3-glucoside).

The amylose content of Sembada Merah and Sembada Hitam was different from Segreng Handayani variety as a check variety, so Sembada Merah and Sembada Hitam rice varieties were separate varieties that different from the comparison varieties. Amylose content is an indicator of rice extinction. This study showed that Sembada Hitam and Sembada Merah have different / lower amylose content compared to a check variety (Segreng Handayani). Therefore, Sembada Hitam and Sembada Merah rice have a delicious and fluffier rice flavor. This is indicated by the lower levels of amylose and the higher amylopectin which shows the stickiness with good taste of rice. [19] reported that the smaller the amylose content or the higher the amylopectin level, the more sticky the rice. Whereas [20] stated that amylose content between 20-22% was rice that had a fluffier taste. In line with this opinion [21] stated that rice containing high amylose produced dry rice, whereas rice containing low amylose produced sticky and soft rice.

4. Conclusion
Sembada Hitam and Sembada Merah rice differ of molecularly, morphologically, and biochemically so that Sembada Hitam and Sembada Merah rice are separate varieties.

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