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Characterization of Indonesian pigmented rice (*Oryza sativa*) based on morphology and Single Nucleotide Polymorphisms

NUR SITI KURNIASIH[1], RATNA SUSANDARINI[1], FEBRI ADI SUSANTO[2], TRI RINI NURINGTYAS[1,2], GLYN JENKINS[3], YEKTI ASIH PURWESTRI[1,2,♥]

[1]Departement of Tropical Biology, Faculty of Biology, Universitas Gadjah Mada. Jl. Teknika Selatan, Sekip Utara, Sleman 55281, Yogyakarta, Indonesia. Tel./fax.: +62-274-580398, ♥email: yekti@ugm.ac.id
[2]Research Center for Biotechnology, Universitas Gadjah Mada. Jl. Teknika Utara, Depok, Sleman 55281, Yogyakarta, Indonesia
[3]Institute of Biological, Environmental and Rural Sciences, Aberystwyth University. Aberystwyth, United Kingdom

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Abstract. *Kurniasih NS, Susandarini R, Susanto FA, Nuringtyas TR, Jenkins G, Purwestri YA. 2019. Characterization of Indonesian pigmented rice (*Oryza sativa*) based on morphology and Single Nucleotide Polymorphisms. Biodiversitas 20: 1208-1214.* Indonesia has many cultivars of pigmented rice, but many variants have not been characterized using morphological characters and molecular markers. SNPs (Single Nucleotide polymorphisms) have been used in previous studies to identify the Indica and Japonica subspecies. Characterization of wheter a line belongs to the Indica or Japonica subspecies is useful information for rice breeders, especially to generate line exhibiting the strong hybrid vigor. Morphological characters are used to determine the relationship between cultivars using cluster analysis. The SNP markers were amplified by PCR, sequenced and compared with sequences in the GenBank. Based on morphological characters, ten cultivars divide into two clusters. SNPs distinguish Indica and Japonica subspecies, and show that Hitam Lampung, Aek Sibundong, Melik, Hitam Toraja, Merah Kalimantan, and Merah Sumbawa belong to the Indica subspecies while Cempo Ireng and Pare Eja belong to Japonica. Abang Segreng and Hitam Toraja could not be clearly assigned to either the Indica or Japonica subspecies.

Keywords: Morphological and molecular characterization, *Oryza sativa*, pigmented rice, SNP markers

INTRODUCTION

Rice (*Oryza sativa* L.) is the staple food for more than half the world's population (Thippeswamy et al. 2014). Based on the place of origin, rice is divided into 3 different subspecies or varietal groups, namely Sinica (Japonica), Javanica and Indica (Le heron et al. 2016). Based on the geographical distribution, plant and grain morphology, hybrid sterility and serological reaction, rice is divided into Indica and Japonica subspesies (Chang and Bardenas 1965). According to Thomson et al. (2007), 68% of 330 rice accessions in Indonesia are Indica subspecies and 32% are Japonica. The Indica subspecies has longer seeds, high stature, soft plant tissue, a large number of tillers, is fragile and has non-sticky textured seed, and it is responsive to temperature. The Japonica subspecies has rounded seeds, medium stature, small seedlings, and sticky textured seeds (Le heron et al. 2016; Tripathi et al. 2016).

Indonesia has many regions growing various pigmented rice landraces, each of which has a local name. Most of the rice cultivated by local communities is from local seeds that have not been characterized and certified. This is potentially confusing since one cultivar may have more than one name, and many local variants may actually be identical. In addition, the variation in phenotype of pigmented rice can cause problems in classification, especially with respect to distinguish both Indica and Japonica subspecies.

Characterization of pigmented rice can be performed using morphological characters as well as molecular markers which provide useful objective information for plant breeding efforts, especially for rice improvement (Supriyanti et al. 2015). Hybridization of Indica and Japonica subspecies will generate the strong hybrid vigor, which has increased rice yields such as increased biomass, size, and growth rate (Lu et al. 2009; Feng et al. 2015; Guo et al. 2016). According to Utami et al. (2009), the differences and similarities in morpho-physiological characters can be used for identification and are easier and cheaper than molecular markers (Sivarajan 1984). However, molecular markers can be used to characterize genotypes quickly and precisely.

SNPs (Single Nucleotide polymorphisms) are widely used as molecular markers and are based on the differences of one nucleotide [Singh et al. 2013; Vignal et al. 2002). *SNPs* were used to characterize many crops, such as cotton, rice, corn and tomato. Feltus et al. (2004) successfully identified 408,898 candidates of nucleotide polymorphism which can be used as SNPs markers to identify Indica and Japonica subspecies. Based on this research, 5 candidate SNPs are used to distinguish ten pigmented rice accessions into Indica and Japonica subspecies. This study is important and valuable for improvment and production of Indonesian pigmented rice.

MATERIALS AND METHODS

Materials

Ten rice cultivars were obtained from local farms in Indonesia (Table 1). Nipponbare and Pokkali were obtained from Balai Benih Sukamandi, Bogor, Indonesia. Four replicate pots of each genotypes were grown in a green house from November 2017 until June 2018 under defined condition avoiding nutrient deficiency, pest and disease attack. Individuals of each genotype were planted in pots with four replications.

Morphological characterization

Thirty four morphological characters were observed and recorded from vegetative to reproductive growth stages based on Silitonga et al. (2003) and Biodiversity International (2007).

Molecular characterization

DNA extraction

DNA was extracted using the modification of Apitz et al. (2014). One hundred mg leaf tissue was extracted by grinding in 800 µl of extraction buffer containing 2.42 g Tris-HCl pH 8; 0.88 g NaCl; 5 ml EDTA 0.5 M, and 10 % SDS, followed by centrifugation at 13,000 rpm at 4^0C for 1 min. The DNA was precipitated by 700 µL of isopropyl alcohol and was centrifuged for 15 min at 13,000 rpm and 4^0C. DNA was washed with 70% ethanol. DNA was dissolved in 50 µL ddH_2O.

DNA amplification

Maxima Hot Start Green (Thermo Scientific, USA) was used to amplify DNA containing SNPs markers obtained from http: //www.plantgenome.uga.edu/snp. Primers used to amplify DNA containing Indica or Japonica SNPs were designed with Primary 3 software (Table 2). The DNA was sequenced using the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA).

Data analysis

Morphological characters were analyzed using UPGMA cluster analysis to determine the similarity between cultivars, and principal component analysis to determine the significance of the characters in the grouping. Cluster analysis and principal component analysis were performed by the MVSP program (Kovach et al. 2007). Molecular characterization was performed by comparing the sequence of ten cultivars with the rice genome in GenBank.

RESULTS AND DISCUSSION

Morphological characterization

Pigmented rice consists of black, red, and purple variants. It has phenolic compounds, flavonoids, and anthocyanins (Goufo and Trindade 2014). Red and black rice were used in this study. Red rice consist of Aek Sibundong, Abang Segreng, Merah Kalimantan, Pare Eja and Merah Sumbawa. Black rice consist of Hitam Lampung, Melik, Cempo Ireng, Hitam Kalimantan, and Hitam Toraja. Ten cultivar show variation in morphological characters (Table 3; Figure 1). Planting period of ten cultivars varies between 110-215 days, the shortest is Abang Segreng and the longest is Hitam Kalimantan.

UPGMA (Unweighted Pair Group Method using Arithmetic average) cluster analysis was performed using Canberra distance. The cluster analysis of ten pigmented rice cultivars based on morphological character shows that the ten cultivars of rice can be divided into 2 different groups, A and B (Figure 2). The distinguishing characters are the awn, and extension from the lemmas of the rice florets (Figure 3). An awn is a fibrous bristle, formed as an extension of varying lengths from the the midrib (middle nerve) of the lemma. A group does not have an awn and B group has an awn can be short, medium and long.

Table 1. Ten rice cultivars and origin

Cultivars	Origin
Red rice	
Abang Segreng	Gunung Kidul, Yogyakarta
Aek Sibundong	Balitbangtan
Merah Kalimantan	South Kalimantan
Merah Sumbawa	Sumbawa, West Nusa Tenggara
Pare Eja	Malino, South Sulawesi
Black rice	
Cempo Ireng	Gunung Kidul, Yogyakarta
Hitam Kalimantan	South Kalimantan
Hitam Lampung	South Lampung, Lampung
Hitam Toraja	Toraja, South Sulawesi
Melik	Bantul, Yogyakarta

Table 2. Primer used to amplify DNA containing the Indica-Japonica SNPs

Primer code	Primer	Sequence	PCR product (bp)	Chromosome number	SNPs location (base number in PCR fragment/ rice genome)	Annealing (^{0}C)
A	Reverse	5’ GTTGGGTCAGGCGAGAAATC 3’	160	12	76/ 2900	54
	Forward	5’ TCACCAACAAGTACAAGCGC 3’				
B	Reverse	5’ ATGATGACAAGGGAGGTGACA 3’	150	9	88/43,237,333	54
	Forward	5’TAACTTCAGGAGCCCTCTAACC 3’				
C	Reverse	5’TTTTCTCCCAATCCGCTGCT 3’	163	1	72/481,481	54
	Forward	5’ATTGGAGTCTTGCCGATGAG 3’				
D	Reverse	5’TGCAGGAGTGACTTCAGGAA 3’	162	8	52/72,058	54
	Forward	5’ ATTGTGGTACTCGGCCTTGA 3’				
E	Reverse	5’AAAGCTCCAGGGACCACATC 3’	162	8	94/83,990	54
	Forward	5’ GGCTGCTTGACACCCTTATG 3’				

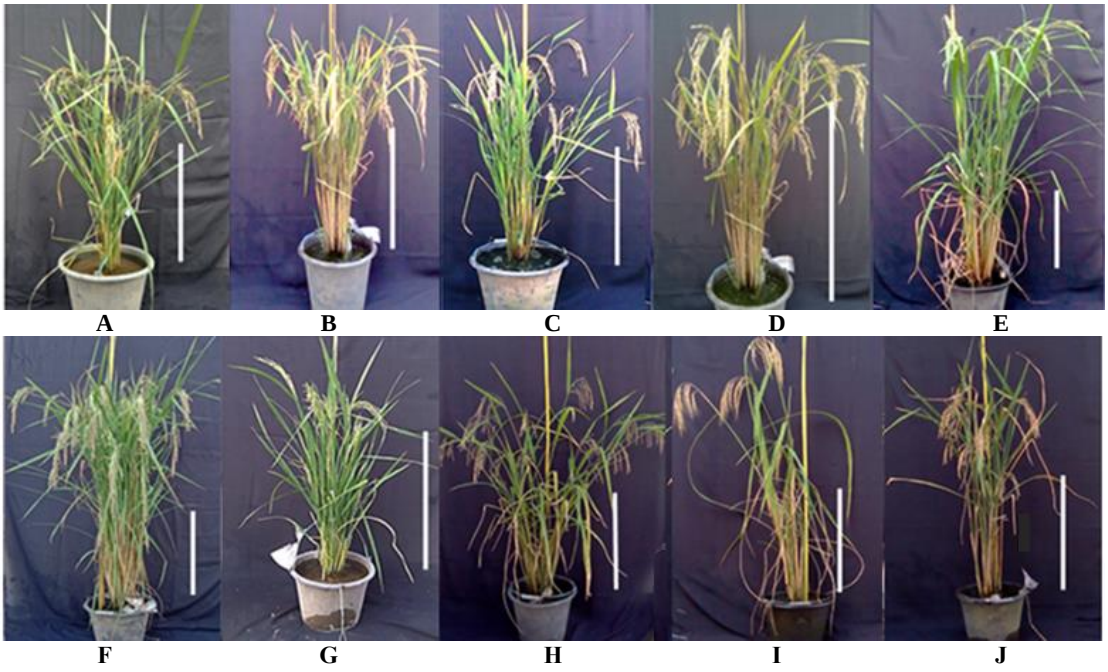

Figure 1. The cultivars of Indonesian pigmented rice used in this study. A. Hitam Lampung; B. Aek Sibundong; C. Abang Segreng; D. Melik; E. Cempo ireng; F. Hitam Kalimantan; G. Merah Kalimantan; H. Hitam Toraja; I. Pare eja; J. Merah Sumbawa. White bar = 50 cm

Table 3. Variation in morphological characters

Cultivars	Planting period (dap*)	Category of awn length	Anthocyanin coloration of leaf sheath	Intensity of green color of leaf blade	Category of diameter at basal internode	Apiculus color	Category of Panicle length	Category of culm length
Red rice								
Abang Segreng	110	Awnless	Medium	Dark	Thick	Purple	Short	Short
Aek Sibundong	114	Intermediate	Absent	Light	Thin	White	Intermediate	Short
Merah Kalimantan	121	Very short	Absent	Light	Thin	White	Intermediate	Very short-short
Merah Sumbawa	134	Awnless	Absent	Light	Intermediate	White	Intermediate	Short
Pare Eja	127	Very long	Absent	Light	Thick	White	Long	Very short-short
Black rice								
Cempo Ireng	153	Intermediate	Absent	Light	Thick	White	Long	Long
Hitam Kalimantan	215	Very short	Absent	Light	Thin	White	Intermediate	Intermediate to long
Hitam Lampung	116	Intermediate	Absent	Medium	Intermediate	White	Intermediate	Short
Hitam Toraja	128	Awnless	Absent	Light	Intermediate	White	Intermediate	Short-intermediate
Melik	117	Awnless	Absent	Medium	Intermediate	White	Intermediate	Very short-short
Merah Sumbawa	134	Awnless	Absent	Light	Intermediate	White	Intermediate	Short

Note: dap*****: day after planted; category of awn length: very short (<5 mm), intermediate (~15 mm), and very long (>40 mm); category of diameter at basal internode: thin (6-10 mm), intermediate (11-14 mm) and thick (15-18 mm); category of panicle length: short (~15 cm), intermediate (~25 cm), and long (~35 cm); category of culm length: very short-short (51-70 cm), short (71-90 cm), short-intermediate (91-105 cm), intermediate-long (121-140 cm), and long (141-155 cm)

The A group consists of Hitam Toraja, Merah Sumbawa, Melik and Abang Segreng. This group was further divided into 2 subgroups, A1 and A2 based on anthocyanin coloration of the leaf sheath, intensity of the green color of leaf blade, diameter at basal internode of the culm, apiculus color and panicle length. The A1 group contains Hitam Toraja, Merah Sumbawa and Melik which has a medium intensity of green color, intermediate basal internodes, white apiculus and intermediate panicle length. The A2 group contains Abang Segreng which had medium anthocyanin coloration in the leaf sheath, dark intensity of green colour, thick basal internode, purple apiculus, and short panicle length.

The B group was also divided into 2 subgroups, B1 and B2 based upon culm length. The B1 group consists of Hitam Kalimantan, Pare Eja and Cempo Ireng which have intermediate to long culm length (135-146 cm). The B2 group consists of Merah Kalimantan, Aek Sibundong and Hitam Lampung which have very short to short culm length (60-85 cm).

Principal component analysis was performed to determine the dominant characters in characterization. From 34 morphological characters, 6 characters have eigenvalues ≥ 0.25 (Table 4.) The morphological characters were anthocyanin coloration in the leaf sheath, flag leaf length, ligule color, apiculus color, lemma and palea color, and awn length. Leaf length, leaf width, ligule color, collar color, diameter at basal internode of culm panicle, and leaf sheath was significant characters in grouping (Shikari et al. 2009; Lestari et al. 2016).

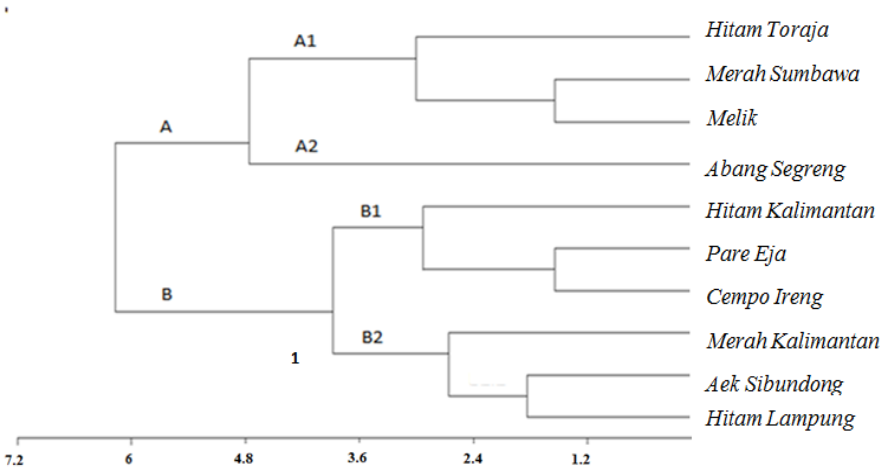

Figure 2. UPGMA dendrogram for morphological characters of 10 rice cultivars. The relationship was based on Canberra distance

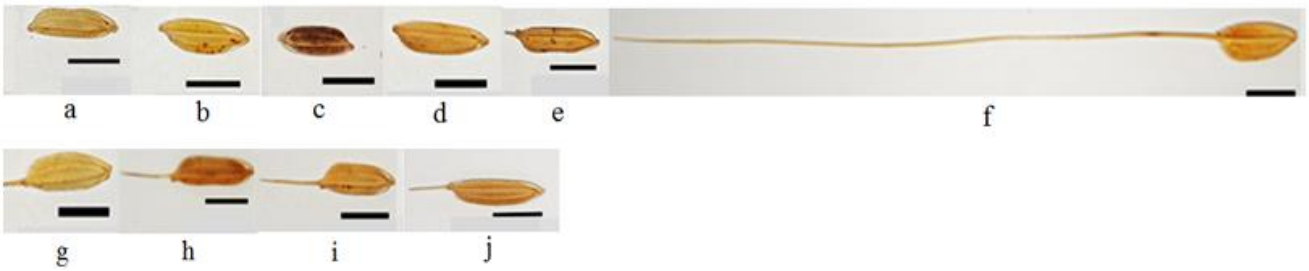

Figure 3. The seeds of ten cultivars of A group, i.e., A. Hitam Toraja; B. Merah Sumbawa; C. Melik, D. Abang Segreng; and B group, i.e., E. Hitam Kalimantan; F. Pare eja; G. Cempo ireng; H. Merah Kalimantan; I. Aek Sibundong; J. Hitam Lampung. Awn is indicated in cultivars E-J. Black bar = 5 mm

Table 4. Eigenvalue of morphological characters in ten rice cultivars

Morphological characters	Axis 1	Axis 2	Morphological characters	Axis 1	Axis 2
Anthocyanin coloration of coleoptile	0.000	0.000	Culm habit	0.140	0.112
Seedling height	0.174	0.037	Culm length	0.195	-0.094
Basal leaf sheath coloration	0.097	0.169	Culm number	0.248	0.009
Anthocyanin coloration in leaf sheath	0.030	**0.391**	Diameter at basal internode	0.223	0.057
Intensity of green color: leaf blade	0.196	0.166	Stigma color	0.102	0.248
Leaf blade attitude	0.102	-0.046	Lemma and palea color	**0.279**	0.069
Leaf blade pubescence	0.157	0.063	Apiculus color	0.106	**0.316**
Auricle color	0.129	0.034	Panicle length	0.229	-0.099
Ligule length	0.240	0.000	Awn color	0.058	-0.135
Ligule character	0.055	0.085	Awn length	0.118	**-0.563**
Ligule Shape	0.202	-0.068	Panicle number per plant	0.187	0.003
Ligule color	0.109	**0.255**	Attitude of panicle	0.107	-0.080
Leaf blade length	0.237	-0.009	Secondary branching of panicle	0.139	0.022
Leaf Blade width	0.230	0.013	Panicle exsertion	0.247	0.003
Flag leaf length	0.179	**-0.314**	Awn color (harvest)	0.063	-0.237
Flag leaf width	0.206	-0.070	Lemma and palea color (harvest)	0.169	0.030
Flag leaf attitude	0.121	-0.038	Seed color	0.245	0.000

Characterization of pigmented rice using SNPs

Morphological characters such as seed shape, apiculus, awn length, leaf colour or biochemical assay can be used to characterize Indica and Japonica subspecies (Kovach et al. 2007; Du et al. 2011). However, morphological characters are variable under different environmental conditions. Molecular markers are more stable than morphological characters. SNPs are the predominant polymorphisms in the genome (McNally et al 2009). SNPs as molecular markers were used to identify many crops, such as cotton, rice, corn and tomato (Byers et al. 2012; Chen et al. 2016; Huq et al. 2016).

To assay the effectiveness of the SNPs markers, 5 candidate SNPs were used to distinguish Pokkali and Nipponbare. Nipponbare was assigned to the Japonica group (Matsumoto et al. 2016; Ahn et al. 2016; Huang et al. 2002) and Pokkali was assigned to the Indica group (Lee 2010). B and C primers can be used to amplify DNA containing these SNPs and to distinguish Indica and Japonica subspecies (Figure 4).

As can be seen in figure 5, PCR fragments from primers B and C show that the Hitam Lampung, Aek Sibundong, Melik, Hitam Kalimantan, Merah Kalimantan, and Merah Sumbawa had the same SNPs as Pokkali (Indica subspecies). Cempo Ireng and Pare Eja had the same SNPs as Nipponbare (Japonica subspecies). Hitam Toraja and Abang Segreng share SNPs with Pokkali and Nipponbare. This indicates that the B and C SNP markers did not clearly distinguish between Hitam Toraja and Abang Segreng. Therefore, more SNPs markers are needed to anticipate possible gene recombination.

Comparison of morphological and molecular characterization

Cluster analysis is widely used in plant systematics. The grouping of accessions in this analysis is based on similarities and differences between characteristics (Henderson 2006). Based on morphological and molecular characterization, the color of seed does not determine the groupping of rice. Based on morphological characters, ten cultivars are divided into 2 groups, A and B group. However, based on molecular characterization, ten cultivars are divided into 3 groups, Indica, Japonica and Indica-Japonica (Table 5.). Indica-Japonica subspecies has same nucleotide as Indica and Japonica. Interestingly, morphological analysis show that Pare Eja and Cempo Ireng are included in the Japonica subspecies. The dendogram of the clustering analysis showed that both cultivars are included into the same group (A group) and have a close phylogenetic relationship.

The results of the molecular analysis indicate that Hitam Toraja and Abang Segreng are included in Indica and Japonica subspecies respectively. The dendogram of the clustering analysis of morphological data showed that both cultivars are included in group B. Molecular analysis indicated that Merah Sumbawa, Melik, Hitam Kalimantan, Merah Kalimantan, Aek Sibundong and Hitam Lampung are included in the Indica subspecies. In contrast, the dendogram of the clustering analysis shows that Merah Sumbawa and Melik fall into different groups than the others, indicating that morphological grouping patterns are not always consistent with molecular grouping.

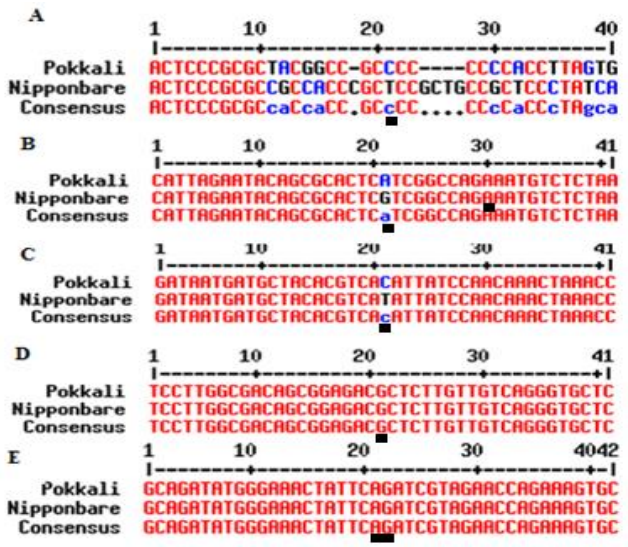

Figure 4. Alignment of Nipponbare and Pokkali sequences using A, B, C, D, and E primers. Black squares indicate the location of SNPs

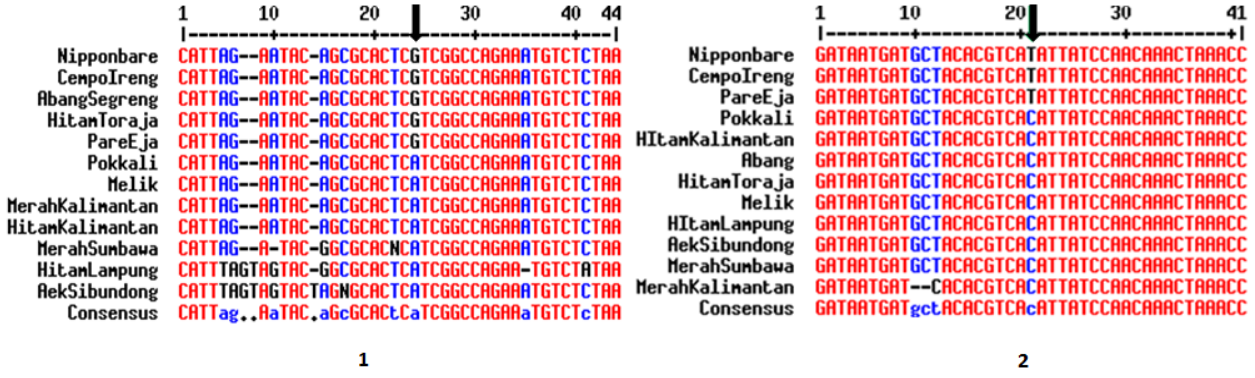

Figure 5. Alignment result of ten cultivars using (1) B and (2) C primers. The black arrows show the location of SNPs

Table 5. Result of morphological and molecular characterization of ten cultivars

Morphological characterization		**Molecular characterization**	
Cultivars	**Group**	**Cultivars**	**Subspecies**
Red rice		**Red rice**	
Abang Segreng	A	Abang Segreng	Indica-Japonica
Merah Kalimantan	B	Merah Kalimantan	Indica
Aek Sibundong	B	Aek Sibundong	Indica
Merah Sumbawa	A	Merah Sumbawa	Indica
Pare Eja	B	Pare Eja	Japonica
Black rice		**Black rice**	
Cempo Ireng	B	Cempo Ireng	Japonica
Hitam Kalimantan	B	Hitam Kalimantan	Indica
Hitam Lampung	B	Hitam Lampung	Indica
Hitam Toraja	A	Hitam Toraja	Indica-Japonica
Melik	A	Melik	Indica

In order to classify various Indonesian rice variants and to eliminate collection duplication, we have started out with five primer pairs to investigate differences between 10 local cultivars. Three of these pairs (A, D and E) were not informative. We suggest using primer pairs B and C as basic tools to set up a comprehensive catalogue of local rice variants in Indonesia. This primer tool-box has to be extended in the future to generate comparable data and to augment the management of pigmented rice germplasm.

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