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Research Article

Genetic Relationship of 22 Pigmented Rice from Central Java and East Java Based on Simple Sequence Repeat (SSR)

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ABSTRACT

Pigmented rice contains distinct bioactive compounds compared to regular white rice. The study aimed to determine the genetic diversity of 22 pigmented local rice varieties originating from East Java and Central Java Province, Indonesia. This research used a PCR-based molecular marker approach utilizing plant pigment marker genes, namely Simple Sequence Repeat (SSR). The research commenced with planting and maintaining the rice plants, DNA isolation according to the cetyltrimethylammonium bromide (CTAB) method, DNA amplification, and PCR visualization, followed by phylogenetic tree construction using PAST 4.0 software. Based on the allele structure analysis, four main clusters of pigmented rice were identified. The identified polymorphisms and clustering patterns provide valuable insights for future plant evolution, breeding, and conservation research. In addition, genetic differentiation suggests a high level within the population. Based on allele structure analysis, black rice shares genetic characteristics with other rice colours, including red, brown, and white. In conclusion, black rice shows the most complex allele structure for colour-related genes among all rice colours.

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INTRODUCTION

Rice belongs to Poaceae family and is one the most important cereal crops in the world. White rice is one of the rice that largely consumed by people around the world. Futhermore, pigmented rice including red, brown, and black rice are also member of rice which safety to consume. The colours in pigmented rice are from anthocyanin and proanthocyanidin (Bhat et al. 2020). The location of the pigments among cultivars is generally found in the pericarp or rice bran. High anthocyanin content is indicated by a darker rice bran colour. Meanwhile, the red colour in pericarps is caused by the presence of proanthocyanidins. Black rice from Java contains higher level of anthocyanin than red rice. The pigment in rice come from phenylalanine protein which a precursor in the synthesis of secondary metabolite compounds in plants, including flavonoid and anthocyanin (Fatchiyah et al. 2020). Similarly, Sari et al. (2021) reported that phenylalanine content in black rice is higher than in white rice, where it acts as precursor protein on expression of anthocyanin.

People have become aware of healthy lifestyles by changing old habits to consume healthier, including pigmented rice. In these recent years, research related to pigmented rice has gained increasing interest due to its high levels of bioactive compounds (Dwiwibangga et al. 2022; Fatchiyah et al. 2023; Fatchiyah et al. 2020). Pigmented rice, especially Indonesian local rice, is rich in bioactive compounds that differ from regular white rice. One of the main compounds found in pigmented rice is anthocyanin, which has strong antioxidant properties and has been associated with health benefits such as antidiabetes (Dwiwibangga et al. 2022) and has anti-inflammation activity anticancer and anti hypertension (Wijayanti et al. 2021; Fatchiyah et al. 2023). Additionally, pigmented rice has antioxidant activity from its phenolic compounds such as ferulic acid and p-coumaric acid. (Samyor et al. 2017). Other phytochemicals, such as flavonoids and carotenoids, and high fiber content are also characteristic of pigmented rice. Important vitamins and minerals, such as B-complex, vitamin E, magnesium, and selenium, can also be found in pigmented rice (Mbanjo et al. 2020). The bioactive compounds in pigmented rice can vary depending on the type of rice and environmental factors. These bioactive compounds provide various health benefits for consumers.

The results of previous studies showed that based on the morpho-agronomic analysis resulted that there were 22 clusters of pigmented rice. Two major clusters emerged from the grouping according to their morpho-agronomic characteristics. Different types of rice pigments are present in 20 out of the 22 rice groups in cluster 1. Meanwhile, Wojaloka and IR Ngawi Hitam make up cluster 2. Both of these varieties are categorized as black rice (Husnah et al. 2024).

Improving the genetic quality of plants requires genetic diversity to produce superior plants (quality, quantity, color, and resistance to pests and diseases) (Manzo-Sánchez et al. 2021). Indonesia's local pigmented rice has extensive diversity and has been studied for its potential in plant development and genetic identification. Research on pigmented rice includes studies on morphological variation in six local pigmented rice varieties from Malang Regency (Shinta et al. 2014), genetic diversity and clustering in North and East Kalimantan (Hamidah et al. 2024), and anthocyanin variation in four local black rice varieties (Basith et al. 2023).

Understanding this genetic diversity is crucial for plant breeding efforts to develop pigmented rice varieties with superior color, enhanced nutritional quality, and increased resistance to environmental stress. Simple Sequence Repeats (SSRs) or microsatellites are short, tandemly repeated DNA sequences which repeat several times in the genome. SSRs play a key role in genetic polymorphism. SSR polymorphism are widely used to assess genetic diversity, with the number of different SSR alleles in a population provides insight into genetic variability. SSRs are more effective because they are polymorphic, abundant, and well-distributed in the genome (Sarif et al. 2020). A previous study by Sari et al. (2021) used SSRs to determine the genomic, proteomic, and anthocyanin nutritional value of three cultivar of Indonesian local black rice. Analysis of genetic variation in marker genes can provide insights into the genetic diversity responsible for color in pigmented rice. The genetic variety underpinning colour in pigmented rice can be understood by analyzing genetic variation in marker genes using SSR. Thus, the aim of this research study is to explore the genetic diversity of 22 East Java and Central Java pigmented rice.

MATERIALS AND METHODS

Materials

The pigmented rice originated from East Java and Central Java (Table 1). The samples waere provided by the Study Center of Smart Molecule of Natural Genetics Resources (SMONAGENES), Brawijaya University. In a 20-litre polybag (Green Planterbag) with four replicates, all twenty-two pigmentedrice were planted. There was a 3:1 (ww^{-1}) ratio of soil to compost in the soil sample utilized for the research. Fertilizer with the NPK ratio of 16:16:16 was administered one week following planting.

Table 1. The list of local pigmented rice from Central Java and East Java.

No	Pigmented-rice	Colour of Pigment	Origin	
			Central Java	East Java
1	Wojaloka	B		√
2	Jeliteng	B	√	
3	IR Ngawi Hitam	B		√
4	Melik	B		√
5	Mentik Ngawi	R	√	
6	Arumba	R		√
7	Blambangan A2	R		√
8	Blambangan A3	R		√
9	N790 merah	R	√	
10	Inpari 24	R		√
11	Inpari 32	R		√
12	IR Ngawi Merah	R		√
13	Berlian	W	√	
14	N790	W		√
15	Sigunca 02	W		√
16	Sertani 13	W		√
17	Sigupai	Br		√
18	HMS 700	Br		√
19	Rojolele	W		√
20	Mr 308	W		√
21	Siam-siam	W		√
22	Kabir 07	W		√

Note: B, black rice; R, red rice; W, white rice; Br, brown rice.

Methods

DNA isolation and amplification

DNA was isolated using the Cetyltrimethylammonium bromide (CTAB) method from the leaves of 22 pigmented rice seedlings at 18-days old (Fatchiyah et al. 2011). DNA concentration was quantified using NanoDrop spectrophotometer (ND-1000, NanoDrop Inc., USA). The quality of DNA was confirmed gel electrophoresis using 0.8 % of agarose gel in 1x TBE buffer (pH 8.3) and then run at 100 Volt for 30 minutes. The results were then visualised in a UV transilluminator. In this study, the genomic variability of pigmented rice was assesed using polymerase chain reaction (PCR) using three simple sequence repeat (SSR) genes, namely SSR 202, SSR 224, and SSR 318 (Chen et al. 2017; Sari et al. 2021) where SSR202, SSR 224, and SSR 318 showed the most sensitive marker on the polymorphism of pigmented rice (Sari et al. 2021). The nucleotide base sequence for primer forward and reverse is presented in Table 2. The PCR procedure followed the manufacturer's protocol by Promega (catalogue number M712). The PCR programme was designed for 35 cycle with denaturation stage at 95 °C for 30 s, the annealing stage was 55 °C for 30 s, and extension stage at 72 °C for 45 s. The SSR profile was analysed using 5 % non-denaturating polyacrylamide gels with ethidium bromide stain. The visualization was performed using a UV transilluminator (BioRad, Cat. No. 161-0433).

Data analysis

The results of PCR amplification with SSR markers were scored based on DNA band fragments at each locus. After scoring based on DNA bands, the Polymorphic Information Content (PIC) was calculated. PIC values was categorised into three, as follows: (PIC>0.5) is included in the high category, (0.25<PIC<0.5) is included in the medium category, and (PIC<0.25) is low (Serrote et al. 2020). The next step was to conduct genetic diversity analysis using GenAlEx 6.5 to observe heterozygosity values (H), number of alleles (Na), number of effective alleles per locus (Ne), and Shannon's information index (I). The allele analysis for the structure of the population was performed via STRUCTURE software (Pritchard et al. 2000). Genetic diversity analysis was also conducted using Analysis of Molecular Variant (AMOVA) to obtain diversity between populations and within populations with estimated variants and PhiPT values or population differentiation obtained from GenAlEx version 6.501(Peakall & Smouse 2012). The run parameters was used namely 24 individuals, 27 loci, 4 populations assumed, 100000 burn-in period, 100000 reps with k=1-10, iterations=10x, The scoring results were also carried out to analyze the relationship with PAST 4.0 using the Jaccard similarity index on the UPGMA algorithm.

RESULTS

Characteristics of SSR 202; SSR 224; and SSR 318 markers

The number of rice analysed using SSR markers totalled to 22 pigmented rice varieties. The SSR markers used in this polymorphism analysis are three markers, namely SSR-202, SSR-224, and SSR-318. Based on the three markers used, the values of Na, Ne, I, and PIC were obtained (Table 3). The number of alleles at each locus was calculated by observing at the bands that appear at each locus. Overall measuring fragments, the shortest measuring

Table 2. Nucleotide base sequences of SSR primers used in the study (Chen et al. 2017).

Marker Code	Primer Forward (5' ⟶ 3')	Primer Reverse (5' ⟶ 3')	Chromosome number	Motif	PCR Product (bp)
SSR202	5'-CAGATTGGAGATGAAGTCCTCC-3'	5'-CCAGCAAGCATGTCAATGTA-3'	11	(CT)30	150
SSR224	5'-ATCGATCGATCTTCACGAGG-3'	5'-TGCTATAAAAGGCATTCGGG-3'	11	(AAG)8; (AG) 13	157
SSR318	5'-GTACGGAAAACATGGTAGGAAG-3'	5'-TCGAGGGAAGGATCTGGTC-3'	2	(GT)15	150

range starts at 150 bp. SSR-318 had the largest range of DNA fragment sizes with a range of 150-1000 bp. Meanwhile, SSR-224 exhibited the smallest range, between 150-750 bp.

Table 3. Genetic diversity analysis based on SSR markers in pigmented rice located in East Java and Central Java.

Marker	Na	Ne	I	PIC
SSR-202	10	1.710	0.586	0.402
SSR-224	11	1.514	0.498	0.323
SSR-318	6	1.198	0.295	0.162

Na, observed number of alleles; Ne, effective number of alleles ; I, Shannon's information index; PIC, polymorphism information content.

The amplification results revealed 10 alleles for SSR-202, 11 for SSR-224, and 6 for SSR-318. The Ne values were 1.710 for SSR-202, 1.514 for SSR -224, and 1.198 for SSR-318. Shannon's information index (I) informed about the index quantifies the genetic variability or heterogeneity within a population. The value of I in SSR-202, SSR-224, and SSR-318 were 0.586, 0.498, and 0.295, respectively. The PIC values ranged from 0.162 to 0.426 where SSR-318 has the lowest PIC value and SSR-202 has the highest PIC value. These results suggest that SSR-202 may be more effective in distinguishing individuals within a population and serve as a reliable genetic marker in breeding or phylogenetic studies.

Marker gene methods are used in molecular identification to determine genetic diversity and evolutionary relationships between organisms by variations in genetic sequences. The goal is to elucidate the evolutionary history and diversification of organisms, thus paving the way for advanced research in population genetics and phylogenetics. DNA amplification generates DNA band patterns that exhibit varying degrees of polymorphism and monomorphism. The similarity between these patterns suggests closer evolutionary relationships. Figure 1 illustrates the results of DNA amplification based on specific marker genes.

Genetic diversity of pigmented rice from Central and East Java using SSR marker

The rice analysed by SSR markers included 22 pigmented rice, divided into several groups (Table 4). Three SSR markers, SSR-202, SSR-224, and SSR-318, were utilized in this polymorphism investigation. The results showed values for Na, Ne, I, and He (Table 4). The Na value in this study was obtained in the range of 1.059-1.647. The highest Na value was found in the Black and Red Rice groups (1.647), while the lowest was in the parental group (1.059); among the tested rice accessions, Brown Rice had the lowest Na value (1.176). Based on Shannon's diversity index, a value range of 0.367-0.571 was obtained with an average of 0.481. The largest Shannon's diversity index value was owned by the accession of Black and Red Rice (0.571) Based on the three SSR markers used, the overall heterozygosity value was in line with the values of Na, Ne, and I. The highest heterozygosity value was observed in Black and Red Rice (0.412).

Analysis of molecular variance (AMOVA)

Analysis of molecular variance (AMOVA) of rice genotypes based on their color was carried out by analyzing the distribution of genetic diversity among and within the population. The results of the AMOVA analysis showed that the diversity among populations was 22 %, while diversity within population was 78 % (Table 5).

The AMOVA analysis revealed that genetic differentiation in Indone-

Table 4. Genetic diversity of pigmented rice originated from Central Java and East java using SSR markers (202, 224, and 318).

Pigment colour	Na	Ne	I	He
Black Rice	1.647	1.647	0.571	0.412
Red Rice	1.647	1.647	0.571	0.412
Brown Rice	1.176	1.176	0.408	0.294
White Rice	1.412	1.412	0.489	0.353
Mean	1.388	1.388	0.481	0.347

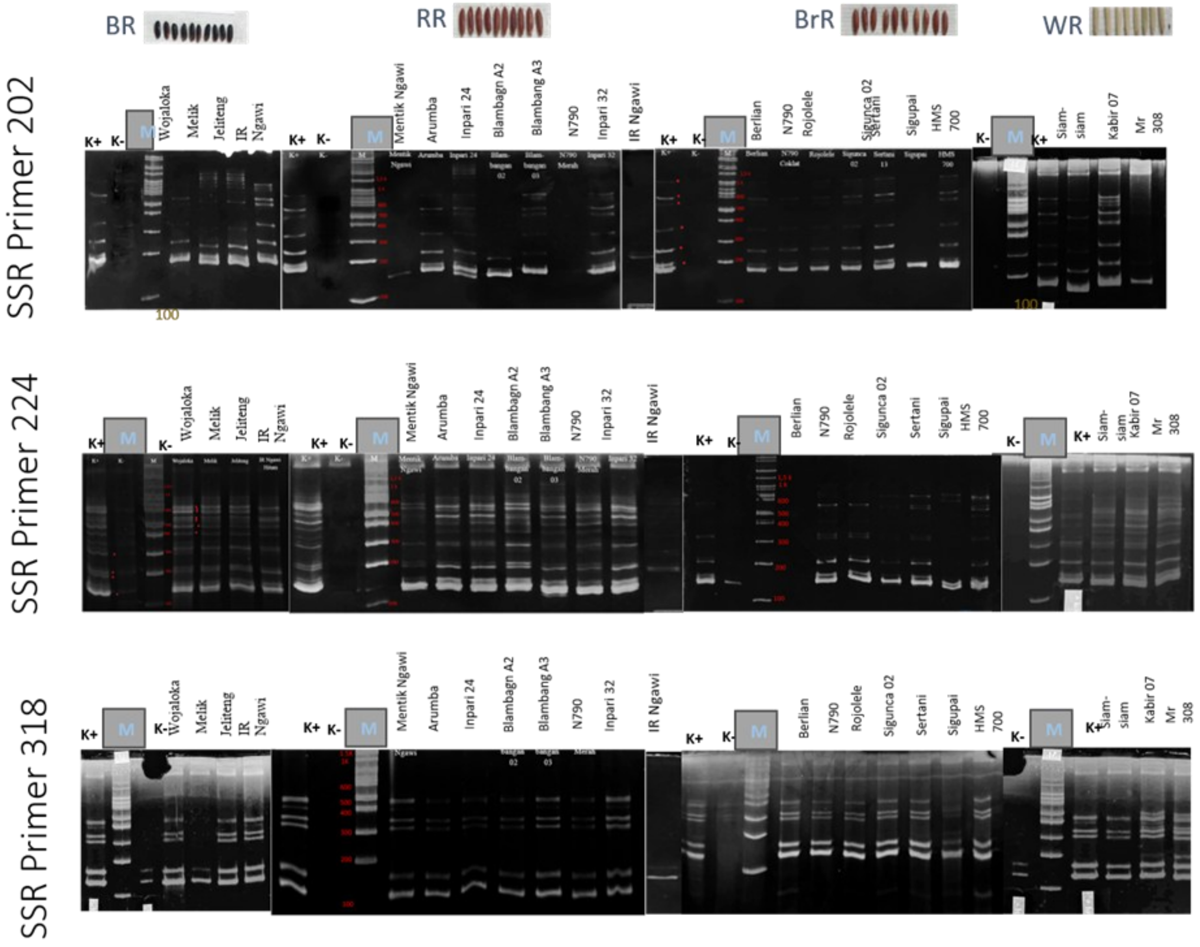


Figure 1. Amplification of 22 local pigmented rice based on SSR marker. SSR 202;SSR 224; SSR 318.

sian pigmented rice varied between and within populations (Table 5). The genetic diversity among the population was low in this study at 22 %. Meanwhile, the value of genetic differentiation in the population showed a higher value of 78 %. This difference in the value of genetic differentiation needs to be supported by the value of PhiPT. In addition, genetic differentiation within the population is highly valued due to the distribution of seeds from pigmented rice originated from Central Java and East Java. The PhiPT value in this study was obtained with a significance of 0.222 with a significance ($P<0.01$) indicating genetic divergences among the population. Overall, local Indonesian pigmented rice exhibits high genetic differentiation.

Genetic relationship of local pigmented rice from East Java and Central Java based on phylogenetic tree

A phylogenetic tree constructed from molecular data aims to identify evolutionary relationships among organisms. In the phylogenetic tree, the closeness of relationships between organisms is reflected in the spatial distance between branches representing those organisms. This closeness is based on

Table 5. Genetic diversity of pigmented rice from Central and East Java using AMOVA.

Source of Variance	df	SS	MS	Est. Var.	%	PhiPT	Prob.
Among Population	4	8955089	2238772	279113	22	0.222	<0.01
Within Population	19	18561994	976947	976947	78		
Total	23	27517083	3215719	1256060	100		

the similarity of genetic sequences, where organisms with more similar sequences are typically placed closer together in the tree. Conversely, organisms with greater genetic differences are positioned farther apart. By molecular data, phylogenetic trees aid in understanding organismal evolution and provide a basis for classification and further research on evolutionary divergence. A phylogenetic tree based on marker genes synthesizes anthocyanin pigments in pigmented rice (Figure 2).

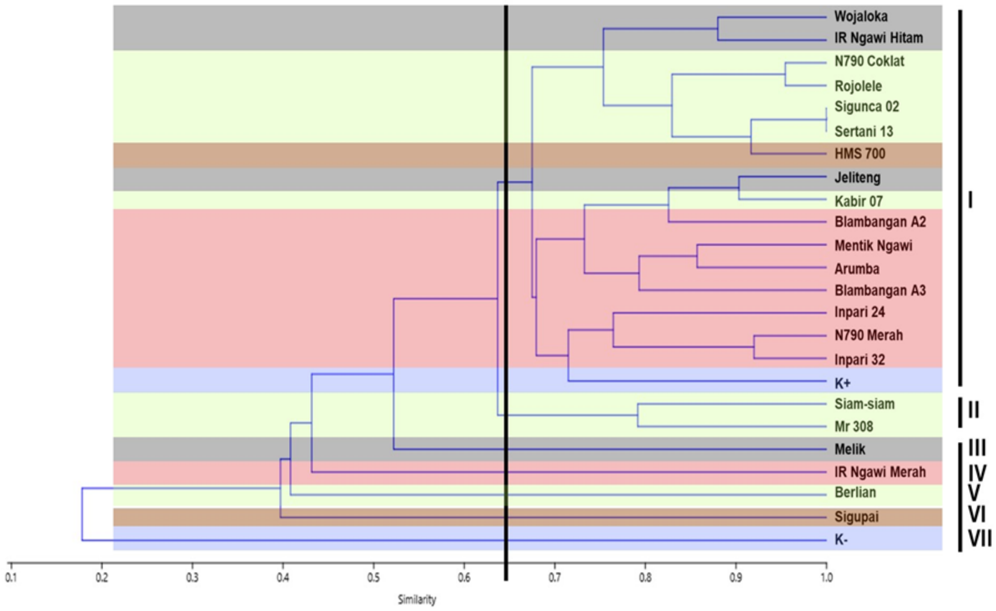
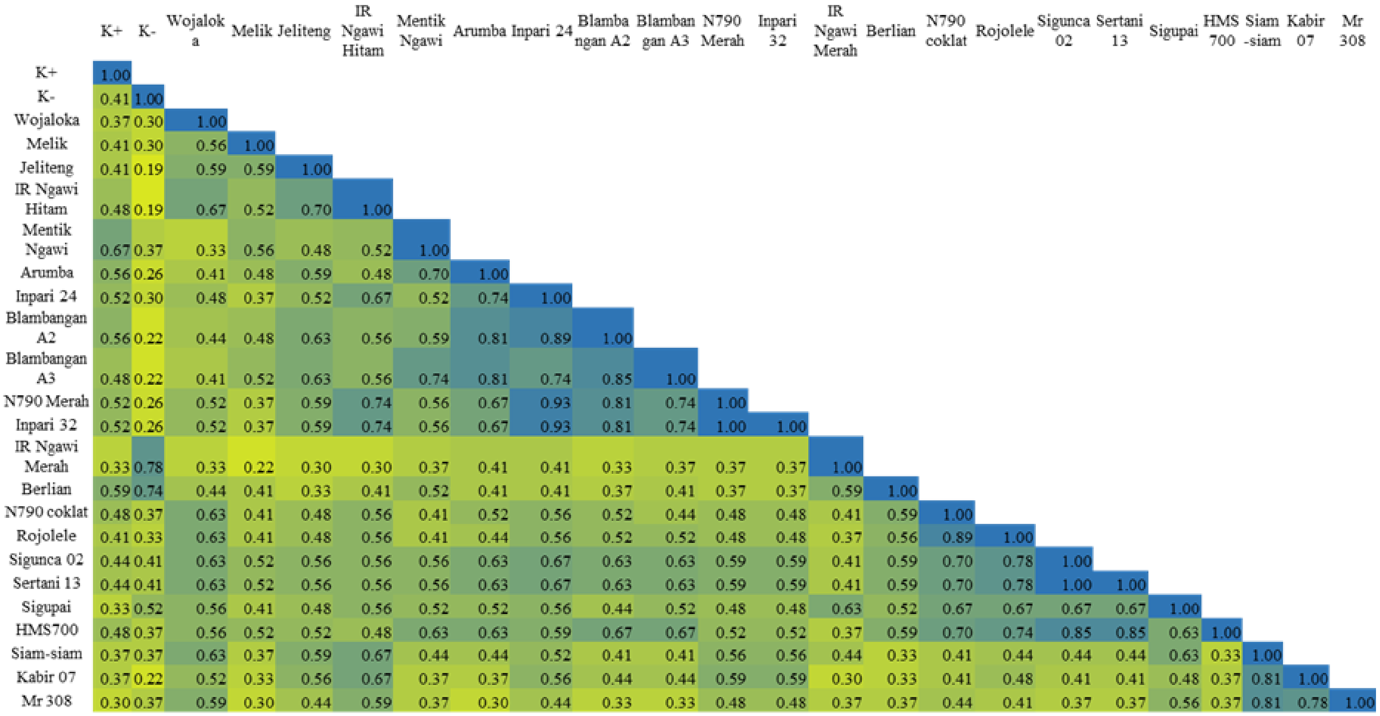


Figure 2. Dendrogram of local pigmented rice generated from UPGMA analysis with PAST 4.0.

Based on the dendrogram results using the UPGMA algorithm and the Jaccard similarity index, 7 clusters were obtained at 0.65 similarity (Figure 2). Cluster I, which had the most accessions, was dominated by Brown, Red, and Black Rice, including the White Rice variety Kabir 07. In cluster I, Jeliteng was more closely related to Kabir 07 and Red Rice's compared to Black Rice's. Cluster II consists of the of White s, namely the Siam-Siam and Mr 308, which are separate from the Kabir 07. Then in order, clusters III-VI were obtained, namely Melik, IR Ngawi Merah, Berlian, and Sigupai. The dendrogram showed that Melik was distinct from other Black Rice, just as IR Ngawi Merah was distinct from other Red Rice. Similarly, Berlian and Sigupai were separated from other Brown Rice varieties.

A similarity index value closer to 1 indicates that the two pigmented rice are more similar, whereas a value lower than 1 and closer to 0 indicates greater dissimilarity. Additionally, the blue color in Figure 3 indicates that the accessions are increasingly similar, while the yellow color indicates a greater dissimilarity, according to the Jaccard index. Figure 3 shows two highly similarpigmented-rice: Inpari 24 with N790 Merah and Inpari 24 with Inpari 32, both of which have a similarity index value of 0.93. This value, being close to 1, suggests high similarity. This is also reflected in the dendrogram, where these pigmented rice are grouped within the same cluster. In contrast, the Melik and IR Ngawi Merah accessions have a much lower similarity index, indicating significant genetic differences between them.



	K+	K-	Wojaloka	Melik	Jeliteng	IR Ngawi Hitam	Mentik Ngawi	Arumba	Inpari 24	Blambangan A2	Blambangan A3	N790 Merah	Inpari 32	IR Ngawi Merah	Berlian	N790 coklat	Rojolele	Sigunca 02	Sertani 13	Sigupai	HMS 700	Siam -siam	Kabir 07	Mr 308
K+	1.00																							
K-	0.41	1.00																						
Wojaloka	0.37	0.30	1.00																					
Melik	0.41	0.30	0.56	1.00																				
Jeliteng	0.41	0.19	0.59	0.59	1.00																			
IR Ngawi Hitam	0.48	0.19	0.67	0.52	0.70	1.00																		
Mentik Ngawi	0.67	0.37	0.33	0.56	0.48	0.52	1.00																	
Arumba	0.56	0.26	0.41	0.48	0.59	0.48	0.70	1.00																
Inpari 24	0.52	0.30	0.48	0.37	0.52	0.67	0.52	0.74	1.00															
Blambangan A2	0.56	0.22	0.44	0.48	0.63	0.56	0.59	0.81	0.89	1.00														
Blambangan A3	0.48	0.22	0.41	0.52	0.63	0.56	0.74	0.81	0.74	0.85	1.00													
N790 Merah	0.52	0.26	0.52	0.37	0.59	0.74	0.56	0.67	0.93	0.81	0.74	1.00												
Inpari 32	0.52	0.26	0.52	0.37	0.59	0.74	0.56	0.67	0.93	0.81	0.74	1.00	1.00											
IR Ngawi Merah	0.33	0.78	0.33	0.22	0.30	0.30	0.37	0.41	0.41	0.33	0.37	0.37	0.37	1.00										
Berlian	0.59	0.74	0.44	0.41	0.33	0.41	0.52	0.41	0.41	0.37	0.41	0.37	0.37	0.59	1.00									
N790 coklat	0.48	0.37	0.63	0.41	0.48	0.56	0.41	0.52	0.56	0.52	0.44	0.48	0.48	0.41	0.59	1.00								
Rojolele	0.41	0.33	0.63	0.41	0.48	0.56	0.41	0.44	0.56	0.52	0.52	0.48	0.48	0.37	0.56	0.89	1.00							
Sigunca 02	0.44	0.41	0.63	0.52	0.56	0.56	0.56	0.63	0.67	0.63	0.63	0.59	0.59	0.41	0.59	0.70	0.78	1.00						
Sertani 13	0.44	0.41	0.63	0.52	0.56	0.56	0.56	0.63	0.67	0.63	0.63	0.59	0.59	0.41	0.59	0.70	0.78	1.00	1.00					
Sigupai	0.33	0.52	0.56	0.41	0.48	0.56	0.52	0.52	0.56	0.44	0.52	0.48	0.48	0.63	0.52	0.67	0.67	0.67	0.67	1.00				
HMS700	0.48	0.37	0.56	0.52	0.52	0.48	0.63	0.63	0.59	0.67	0.67	0.52	0.52	0.37	0.59	0.70	0.74	0.85	0.85	0.63	1.00			
Siam-siam	0.37	0.37	0.63	0.37	0.59	0.67	0.44	0.44	0.52	0.41	0.41	0.56	0.56	0.44	0.33	0.41	0.44	0.44	0.44	0.63	0.33	1.00		
Kabir 07	0.37	0.22	0.52	0.33	0.56	0.67	0.37	0.37	0.56	0.44	0.44	0.59	0.59	0.30	0.33	0.41	0.48	0.41	0.41	0.48	0.37	0.81	1.00	
Mr 308	0.30	0.37	0.59	0.30	0.44	0.59	0.37	0.30	0.44	0.33	0.33	0.48	0.48	0.37	0.37	0.44	0.41	0.37	0.37	0.56	0.37	0.81	0.78	1.00

Figure 3 illustrates the similarity index values of 22 pigmented rice accessions from East Java and Central Java.

The Jaccard similarity index was used to analyse the genetic relationships among pigmented rice accessions from Central Java and East Javawith highly variable of similarity index value. The dendrogram reveals that rice with the same grain color can still exhibit considerable genetic divergence. For instance, Black Rice accessions are distributed across Jeliteng and Melik. Red Rice generally show close genetic relationships, except for Berlian and Sigupai, which are somewhat different. A similar pattern is observed with IR Ngawi Merah, which is slightly distanced from the other Red Rice. Meanwhile, White Rice, particularly Kabir 07, is separated from other groups such as Siam-Siam and MR 308. Overall, while most rice accessions show close genetic relationships, a few pigmented rice varieties are more distantly related or genetically divergent.

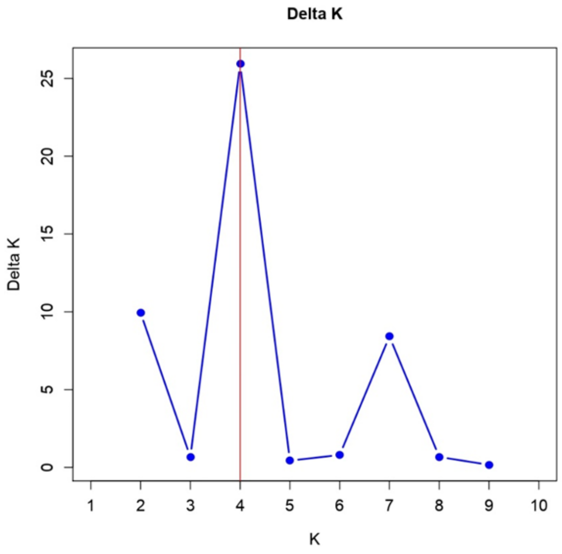


Figure 4. Scatter plot to determine true K value using log probability (ΔK) method; the highest value of ΔK represents the optimum K value (K = 4)

The structure pattern for K = 4 is presented in the bar plot, where the four major clusters (Groups black rice, red rice, brown rice, and white rice) were divided by a straight line (Figure 4). The IR Ngawi Hitam cluster with Hitam Wojaloka is still in the same cluster. Interestingly, the SSR molecular marker analysis in this study aligned with the morphological-agronomic dendrogram results, showing that Hitam Wojaloka and IR Ngawi Hitam are clustered together. Overall, phylogenetic results based on SSR markers were consistent with the phylogenetic characterization of morpho-agronomy of 22 pigmented rice accessions.

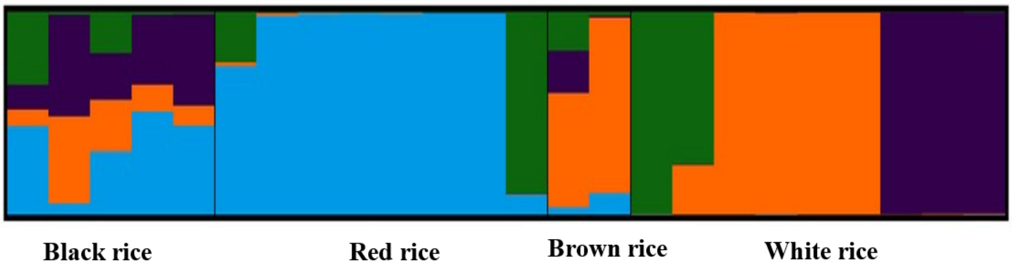


Figure 5. Allele diagram of 22 pigmented rice based on allele structure analysis. The bar plot structure of K = 4 was obtained by STRUCTURE software. The plot shows four major clusters separated by a straight line (Group black rice, red rice, brown rice, and white rice).

Based on the allele structure diagram (Figure 5) shows that from the total of 22 pigmented rice, there were four main clusters. The first cluster is the black rice group, the second is red rice, the third is brown rice, and the fourth is white rice. This indicates that black rice contains a mixture of all the color pigments found in rice. Red rice is a mixture of white and brown rice, but it is considered true red rice. Brown rice, on the other hand, is true brown rice, and it is a mixture of white rice and red rice. However, brown rice, in relation to white rice, is simply white rice with all its alleles intact. In black rice, the alleles are dominated by the color pigments of red, brown, and white rice. Surprisingly, black rice is dominated by a complex allelic structure that encompasses all rice color pigments, as determined by color-related genes. This finding is supported by previous research, which showed that crossing black rice with the local milky white rice from the Ngawi area of East Java produced purple rice, one of the pigmented rice varieties in Indonesia (Wijayanti et al. 2021).

DISCUSSION

Amplification results using three SSR markers in local Indonesian pigmented rice revealed moderate polymorphism detection capabilities for each marker. Rice is a staple food in Indonesia (Kusumardani et al. 2022). SSR marker amplification yielded DNA fragments ranging from 150 to 1000 bp, demonstrating the markers' ability to amplify diverse target sizes. Other studies have reported fewer alleles: 68 from 24 SSR marker loci in 16 Himalayan rice genotypes (Ashraf et al. 2016) and 19 alleles from 18 elite rice from Bangladesh using 4 SSR markers (Mukta et al. 2024). The high allele count observed in this study may result from the genetic diversity among Indonesian pigmented rice accessions. Each population exhibits unique allele frequencies influenced by genotype, geographical distribution, and parental lineage (Budiman et al. 2019).

The PIC value was obtained in the range of 0.426-0.461 or included in the medium category because it has a value of ($0.25<PIC<0.5$) (Serrote et al. 2020). In another study, a value was also obtained that was quite similar to the number of alleles obtained, as many as 110 alleles with a PIC value in the range of 0.26-0.86 in 167 rice accessions from Thailand (Pathaichindachote et al. 2019). McFarlane et al. (2020) also reported that the number of alleles was high due to the large number of markers used. A low number of alleles obtained can be caused by the number of parents and markers used. Based on this study, the value of Shannon's diversity index was obtained with an average of 0.481. When compared to other studies, this value is relatively low. A low Shannon's diversity index suggests that there are only a few alleles present in the population. When the number of alleles is limited, the probability of individual with heterozygosity decreases. Low heterozygosity values indicate that there is a large frequency of homozygous alleles. This mechanism can be caused by inbreeding or self-fertilization (Gao et al. 2019).

The SSR primers chosen for this study were very useful in establishing associations with aromatic genes. With 0.03 for heterozygosity and 0.89 for diversity, RM 223 achieved the second-highest level of genetic variation. Conversely, primer RM 9 achieved the highest PIC value of 0.91. The genetic diversity of the 34 rice accessions was categorized into two primary groups: Group A (aromatic), and Group B(non-aromatic) (Susiyanti et al. 2021). The matrix of similarity coefficients, which is based on the fraction of SSR alleles, was also employed in other investigations to determine the degree of relatedness between each cultivar/strain pair. The results indicated that the genotypes used for the study possessed an extraordinary diversity of genetic variation across purple and grey rice. In general, the yield component of rice in each accession studied was influenced by the plant's capacity to adapt to environmental factors during the growth period. The genetic potential of each accession is distinct, which leads to varying levels of variability in growth and yield (Andarini et al. 2022).

Analysis of molecular variance (AMOVA) of rice genotypes based on their color was carried out by analyzing the distribution of genetic diversity between and within the population. The genetic differentiation among populations and within populations can vary. The genetic diversity among the population was 22 % and is categorised as low. Gene flow among pigmented rice from Central and East Java causes low genetic differentiation (Salem & Sallam 2015). Genetic differentiation among populations may result from cross-pollination, allowing random mating between individuals (Zulfahmi et al. 2021). On other hand, genetic diversity within the population is highly valued due to the distribution of seeds from pigmented rice from Central and East Java. The spread of seeds has the potential for gene exchange so it can cause high genetic differentiation (Ahn et al. 2023). Genetic diversity is crucial in crop development due to its benefit on creating resilient, productive, and adaptable agricultural systems. In the field of pigmented rice, genetic diversity improves the nutritional content of regular rice by introducing pigment genes that contain several bioactive compounds, such as anthocyanins (Sari et al. 2021). Further application of genetic diversity is the foundation of innovation in crop development. Genetic diversity provides the raw material to explore new combinations, enabling novel solutions for ensuring food security, improving nutritional quality, and supporting sustainable agriculture.

The PhiPT value in this study was 0.222 ($P<0.01$) indicating genetic divergences among the population. A PhiPT value at range 0.15-0.25 is categorised high genetic differentiation (Wang et al. 2020). High genetic divergence leads to significant genetic variation between populations of the same species, indicating that these populations have become genetically distinct from one another. In this study, high genetic divergence suggests that there has been little or no gene flow between populations due to geographic barriers and ecological isolation.

The kinship relationship of local pigmented rice was calculated based on the number of alleles obtained, which was 29 alleles using the UPGMA algorithm, and a dendrogram was generated to see the overall genetic relationship of 22 pigmented-rice. The results showed that pigmented rice generally grouped according to grain colour: Black, Brown, Red, and White rice. However, from some of these rice groups, separate riceshow that they are closely related to rice based on different grain colors. These results indicate that the morphological character, namely rice grains, can have similarities and genetic differences. Differences between morphological and genetic characteristics may be influenced by environmental factors and post-harvest processing, such as milling (Panwar et al. 2018). Rice grains with special genotypes producing colored rice may lose these characteristics after milling, as the pigments are typically located in the outer bran layer. Once the bran is removed, the remaining grain may appear white or pale. In addition, this milling process is known to have a different color effect on brown rice to become white (Mohidem et al. 2022). In another study conducted on pigmented and aromatic rice from the Western Himalayan region of India, it was found that the same pigmented-riceor type can produce different clusters based on their genetic characteristics (Ashraf et al. 2016).

Based on the UPGMA analysis, the four cluster that found in this research indicated the genetic variation. The investigation of genetic variation is a significant contributor to the enhancement of varieties, which may ultimately result in the achievement of food security. This is because genetic differences enable the creation of recombinants, which are completely necessary for the formation of new varieties (Debsharma et al. 2022). Furthermore, genetic characterisation is critical in identifying and selecting suitable rice cultivars, especially for breeding purposes. The assessment of genetic diversity and the linkages between breeding materials is a significant tool for improving crop improvement (Hamidah et al. 2024).

Based on the allele structure analysis, there are four main clusters of of pigmented rice accessions were identified (Figure 5). In this research, a high number of alleles was obtained due to the of rice accessions originating from different regions, namely Central Java and East Java. Interestingly, black rice appears to be dominated by all rice color pigments randomly by color-related genes. This finding is supported by previous research showing that crossbreeding black and white rice from Ngawi, East Java, produced purple pigmented rice. (Wijayanti et al. 2021).

CONCLUSIONS

This study demonstrates that the SSR markers used for genetic diversity assessment of local pigmented rice from Central and East Java exhibit moderate polymorphism detection, but they effectively amplify specific genetic regions. Additionally, genetic differentiation analysis indicates that most genetic variation (78 %) exists within populations rather than among them, suggesting substantial gene flow between rice accessions from Central and East Java. Allele structure analysis reveals that black rice contains genetic markers associated with red, brown, and white rice, suggesting a complex genetic background influenced by multiple pigmentation-related genes. These findings confirm that black rice possesses a complex allele structure influenced by multiple color-related genes, supporting the hypothesis that its pigmentation results from a combination of genetic factors inherited from other rice varieties. Further research using more marker is highly encouraged to provide more precise genetic analysis.

AUTHOR CONTRIBUTION

Conceptualization, F.F, T.T, Y.AH., and A.S.; validation and data curation F.F., R.N., Y.A.H., and T.T., writing original draft preparation, Y.A.H.; writing, review, and editing, Y.A.H, T.T, and A.S; R.N and F.F are the main authors of this publication. All authors have reviewed and approved the final draft of the manuscript for publication.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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