

Genotypic Analysis of Two Local Swamp Rice Based on Microsatellite Markers

(Analisis Genotipe Dua Padi Rawa Lokal Berdasarkan Markah Mikrosatelit)

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ABSTRAK

Argo merupakan padi rawa lokal asal Kalimantan yang banyak dibudidayakan oleh petani lokal. Pada kegiatan eksplorasi di Kalimantan ditemukan dua tipe padi rawa lokal tersebut yang fenotipenya berbeda. Oleh sebab itu, perlu dilakukan verifikasi genotipe untuk menentukan perbedaan kedua genotipe padi rawa lokal tersebut. Teknologi sidik jari DNA menggunakan markah SSR dapat membantu verifikasi genotipe secara cepat dan tidak dipengaruhi oleh faktor lingkungan. Tujuan penelitian ini ialah mengetahui tingkat kemiripan Argo-1 dan Argo-2 dengan genotipe padi rawa dan padi irigasi lainnya menggunakan markah mikrosatelit. Penelitian dilaksanakan di Laboratorium DNA, Balai Besar Penelitian Tanaman Padi. Sebanyak delapan varietas unggul dan lokal digunakan sebagai bahan genetik beserta dua padi lokal Argo (Argo-1 dan Argo-2). Genotipe padi dianalisis dengan menggunakan delapan markah mikrosatelit. Hasil analisis molekuler menunjukkan bahwa kedua tipe Argo menunjukkan pita DNA yang berbeda berdasarkan penanda RM228, tetapi secara genetik mereka terkait erat. Jarak genetik antara kedua kultivar Argo adalah 0,143. Argo-1 dan Argo-2 berada pada klaster yang sama dengan Inpara 4 dan Siam KDK, namun berada pada kelompok yang berbeda dengan varietas padi irigasi seperti Mekongga dan Inpari 9. Berdasarkan penelitian ini, Argo-1 dan Argo-2 tidak dianjurkan untuk disilangkan dengan Inpara 4 dan Siam KDK karena keragaman genetik yang terbentuk akan sempit.

Kata kunci: Padi rawa, markah SSR, jarak genetik, varietas lokal.

ABSTRACT

Argo is a local swamp rice from Kalimantan which is widely cultivated by local farmers. Exploration activities in Kalimantan found two types of local rice named Argo but demonstrated different phenotypes. Therefore, genotypic verification is needed to determine the differences between the two Argo rice genotypes. DNA fingerprint could help genotype verification rapidly and the result is not influenced by environmental factors. The aims of this study were to determine the level of similarity of Argo-1 and Argo-2 with swamp rice and other irrigated rice genotypes using microsatellite markers. A total of eight improved and local varieties were used as genetic material with the two local rice Argo (Argo-1 and Argo-2). The rice genotypes were analyzed by using eight microsatellite markers. The results of the molecular analysis showed that the two types of Argo showed different DNA bands based on the RM228 marker but genetically they were closely related. The genetic distance between the two Argo cultivars is 0.143. Argo-1 and Argo-2 were located in the same cluster as Inpara 4 and Siam KDK, but they were in the different groups from those of irrigated rice varieties such as Mekongga and Inpari 9. Based on this research, Argo-1 and Argo-2 are not recommended to be crossed with Inpara 4 and Siam KDK because the genetic diversity formed will be narrow.

Keywords: Swamp rice, SSR markers, genetic distance, local variety.

INTRODUCTION

Harvested area of rice (*Oryza sativa* L.) crops in Java Island has decreased from 52.0% in 1980 to 46.3% in 2014 (Pasandaran and Suherman 2015). Therefore, it is necessary to expand paddy fields outside Java which are dominated by suboptimal land. One of the suboptimal lands is swampland. Rice productivity in swamp ecosystems is still low (2 t/ha) compared to the national average of rice productivity of 5.2 t/ha (Statistics Indonesia 2020). Complex stresses in swamps include uncontrollable water conditions, Fe poisoning, aluminum poisoning, salinity, and blast disease. Therefore, efforts to develop tolerant varieties and exploration of swamp rice cultivars were carried out to obtain local superior varieties to be used in a breeding program to increase rice productivity in the swamps.

Swamp rice is generally the local paddy that has a good adaptation in the swampy ecosystem. Many swampy landraces are found in Kalimantan, the largest number of swampy land areas in Indonesia (Djaenudin 2008). In the previous rice exploration activities, it was found 40 accessions of swampy landraces in South Kalimantan (Wahdah et al. 2015), 44 accessions in North Kalimantan (Nurhasanah and Sunaryo 2015), and 104 accessions in West Kalimantan (Hendra et al. 2009).

Argo is a unique landrace rice belonging to the *indica* subspecies, which shows phenotypic superiority over other swampy landraces. It has a similar time of maturity with that of high yielding varieties and is classified as an early maturity. Argo has a slender grain shape and light brown grain color. There are two types of Argo, i.e. Argo-1 and Argo-2, grouped based on their discovery sites. Both landraces have been cultivated widely in the swampy areas of West Kalimantan. We hypothesized that both of the Argo local rice are different with each other, but they have some similarity with that of newly developed swampy rice varieties. Genotypic analysis based on molecular marker for the landraces, therefore, is needed to verify their genetic identity.

Genotypic characterization is one activity on germplasm management, and it was common to apply based on molecular methods. By this activity, we can explore the genetic diversity and genetic relationships among genotypes. Genotypic analysis using molecular markers is very useful for obtaining information on germplasm collection genetic diversity. Genetic diversity information among the selected parents in a breeding program is the basis

for successful development of new rice varieties (Risliawati et al. 2016; Sumarno and Zuraida 2016; Silitonga 2017).

Microsatellites, also termed as simple sequence repeat (SSR) markers, are one of the molecular tools for characterizing rice genotypes. Microsatellite analysis using thousands of validated molecular markers is a proven effective method for characterizing rice genotypes (Temnykh et al. 2000; McCouch et al. 2002; Zhang et al. 2007). Microsatellite markers have good power in distinguishing varieties and populations in plant varieties (Moeljopawiro 2010). Microsatellite analysis is an alternative molecular analysis which has advantages such as quickly characterizing genotypes, identifying genetic distances between genotypes or cultivars, and verifying similarities with superior varieties and others. Such molecular characterization can provide information quickly without being affected by environmental conditions. The aims of this study were to determine the level of similarity of Argo-1 and Argo-2 with swamp rice and other irrigated rice genotypes using microsatellite markers.

MATERIALS AND METHODS

Plant Materials

Genetic materials were two types of Argo cultivars (Kalimantan landraces), seven improved varieties, and one local variety originated from Kalimantan that are hypothesized to be similar to those of Argo-1 and Argo-2 (Table 1).

Genomic DNA Isolation

Genomic DNA was isolated from young leaves. Twenty seeds per genotype were germinated and stored in a germinator, then the seedlings were planted in a screen house. Leaf samples were harvested at the age of 21 days after sowing (DAS). DNA isolation was carried out using 5 young leaves or equivalent of 5 mg. DNA isolation was carried out using CTAB method (Murray and Thompson 1980). The DNA pellet resulted from the isolation was dissolved in 200 ml ddH₂O.

DNA quantity and quality tests were carried out using a NanoDrop Spectrophotometers (Thermo Fisher Scientific, USA). A total of 2 μ l of DNA samples were used for this analysis. Minimum quantity and quality required >50 ng/ μ l and around 1.8–2.0 (in 260/280 column), respectively. Isolated DNA was also run in a 0.8% agarose gel to determine their intactness.

PCR Amplification, Gel Electrophoresis, and Data Scoring

The PCR reaction was done using eight microsatellite markers (Table 2). The SSR markers were selected based on their polymorphism.

The volume of the PCR cocktail used was 25 μ l, consisting of 50 ng of DNA sample, 0.25 M of forward and reverse primers, 100 mM of each dNTP, 1 \times PCR buffer of 20 mM Tris pH 8.3, 50 mM KCl buffer, 1.5 mM MgCl₂, 0.01% gelatin, and 0.5 units of KAPA Taq DNA polymerase. The PCR reaction profile used was 95°C for 5 min, followed by 35 cycles of 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min, then final extension at 72°C for 5 min.

As much as 6 μ l PCR product of each rice genotype was electrophoresed in 8% polyacrylamide gel, run at 50 V for 90 min. A 100 bp DNA ladder was used as a DNA size ladder. PCR products were stained using ethidium bromide and DNA bands were visualized using the GelDoc Go Imaging System (Bio-Rad, USA) under UV light. PCR products were then scored by giving a score of 1 for the presence of an SSR band and 0 for a genotype that has no SSR band.

The PIC values were estimated using the following formula (Botstein et al. 1980):

$$\widehat{PIC}_l = 1 - \sum_{u=1}^k \tilde{P}_{lu}^2 - \sum_{u=1}^{k-1} \sum_{v=u+1}^k 2\tilde{P}_{lu}^2 \tilde{P}_{lv}^2$$

where P_{lu} = population frequency of an allele A_u (or lu p, to indicate the l^{th} locus), and a genotype $u v A_u A_v$ has a population frequency P_{uv} (or P_{luv}).

Genetic distance (D_A) was estimated using Nei and Takezaki (1983) method:

$$D_A = 1 - \frac{1}{m} \sum_{j=1}^m \sum_{i=1}^{a_j} \sqrt{p_{ij} q_{ij}}$$

where p_{ij} and q_{ij} are the frequencies of i^{th} allele at the j^{th} locus in populations X and Y respectively, while a_j is the number of alleles at the j^{th} locus, and m is the number of loci examined.

Phylogenetic tree was constructed from a distance matrix of unweighted pair-group method using arithmetic average (UPGMA) that all automatically analyzed using PowerMarker software.

RESULTS AND DISCUSSION

SSR Marker Polymorphism

The number of alleles observed in this study ranged from 1 to 5 alleles per SSR locus. The value of the greatest gene diversity was demonstrated by RM3571 with a total gene diversity of 0.730 (Table 3). This is supported by the PIC value, where RM3571 has the highest PIC value of 0.680 (Table 3), and therefore, the most informative marker for genetic characterization identified to differentiate among the tested rice genotypes. The mean PIC value of all

Table 1. Two types of Argo and eight improved varieties were used in this study.

Genotype	Origin/pedigree	Biological status	Agroecosystem	Germplasm collection
Argo-1	West Kalimantan	Landrace	Rainfed/swampy	New collection of ICRR
Argo-2	West Kalimantan	Landrace	Swampy	New collection of ICRR
Banyuasin	Cisadane/Kelara	Improved variety	Swampy	ICRR
Inpara 4	IR05F101	Improved variety	Swampy	ICRR
Inpari 9	IR65469-161-2-2-2-3-2-2/IR61979-138-1-3-2-2	Improved variety	Irrigated	ICRR
IR48	Improved variety	Introduction	Irrigated	ICRR
Margasari	Siam Unus/Cisokan	Improved variety	Swampy	ICRR
Mekongga	A2790/2 *IR64	Improved variety	Irrigated	ICRR
Mendawak	Mahsuri/Kelara	Improved variety	Swampy	ICRR
Siam KDK	South Kalimantan	Landrace	Swampy	ICRR

ICRR = Indonesian Center for Rice Research.

Table 2. SSR markers used in this study together with their characteristics.

Marker	Chromosome	Associated gene*	Forward (5'→3')	Reverse (5'→3')	References
RM261	4	<i>Bph12</i>	GGTTGAACCCAAATCTGCA	CTTTGATAGCGGCTTTGTCC	Yang et al. (2002)
RM228	10	Drought	CTGGCCATTAAGTCCTTGG	GCTTGCGGCTCTGCTTAC	Venuprasad et al. (2011)
RM248	7	Root traits	TCCTTGTAAGTCTGGTCCC	GTAGCCTAGCATGGTGCATG	Selvi et al. (2015)
RM20590		<i>Xa7</i>	TTCGATGAGCACCTTTCTTGTC	GCCTCGCGATTCACTTATGC	Utami et al. (2010)
RM3571	8	<i>Hd12</i>	GAGGACCCCGAATCGATC	AACAGGGCCGGTTAAGTAG	Utami et al. (2016)
RM8213	4	<i>Bph17</i>	AGCCCAAGTATACAAAGATG	GCGAGGAGATACCAAGAAAG	Sun et al. (2005)
AMS	2	Palatability	CTTCCAAGGACCCCATCCT	CCCAACATCTCCGTCAGAAT	Lestari et al. (2009)
GBSS1	6	Palatability	CAAATAGCCACCCACACCAC	CTTGACAGATGTTCTTCTGATG	Lestari et al. (2009)

*The marker analyzed is associated with the respective genes described in the table.

markers was 0.413. The average value of PIC in this study is quite informative based on Botstein et al. (1980) because it is still in the range of $0.5 > \text{PIC} > 0.25$ as an indicator of informative markers. The PIC value is required to select the appropriate marker that can distinguish between accessions of particular plant genotypes under study (Terryana et al. 2018).

The RM3571 and GBSS1 markers have high reproducibility of PCR products among the ten rice genotypes tested. The DNA band polymorphisms of the two markers in two Argo genotypes are different

from those of other rice genotypes tested (Figures 1 and 2). Green arrows indicate the DNA bands of Argo-1 and Argo-2 showed different patterns compared to DNA bands of other rice genotypes in South Kalimantan including that of the local adapted rice genotype, Siam KDK (Figures 1 and 2).

RM228 can clearly differentiate Argo-1 and Argo-2. Argo-2 has two DNA bands with 125 bp and 500 bp in sizes, which were absent in Argo-1 (Figure 3). This indicates that SSR marker RM228 can be used as a fingerprint to differentiate the two Argo genotypes.

Table 3. Allele number, gene diversity, and PIC values of eight SSR markers used to analyze rice genotypes in this study.

SSR marker	Allele number detected	Gene diversity	PIC value
RM261	3	0.505	0.442
RM228	4	0.328	0.313
RM248	2	0.420	0.332
RM20590	3	0.465	0.420
RM3571	4	0.730	0.680
RM8213	1	0.000	0.000
AMs	3	0.515	0.460
GBSS1	5	0.700	0.656
Mean	3	0.458	0.413

PIC = polymorphism information content.

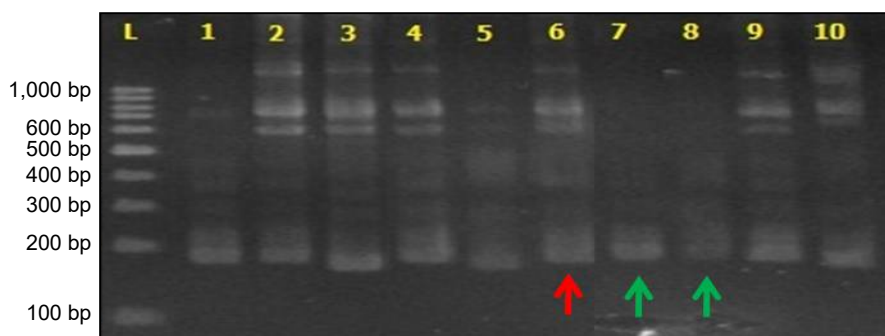


Figure 1. DNA polymorphism of two Argo genotypes and other eight rice genotypes genotyped using RM3571 marker. L = 100 bp DNA ladder, 1 = Mendawak, 2 = Mekongga, 3 = Inpara 4, 4 = Margasari, 5 = IR48, 6 = Siam KDK, 7 = Argo-1, 8 = Argo-2, 9 = Banyuasin, 10 = Inpari 9. Green arrows indicate DNA bands of Argo-1 and Argo-2; red arrow indicates DNA band of Siam KDK, a local swampy rice genotype.

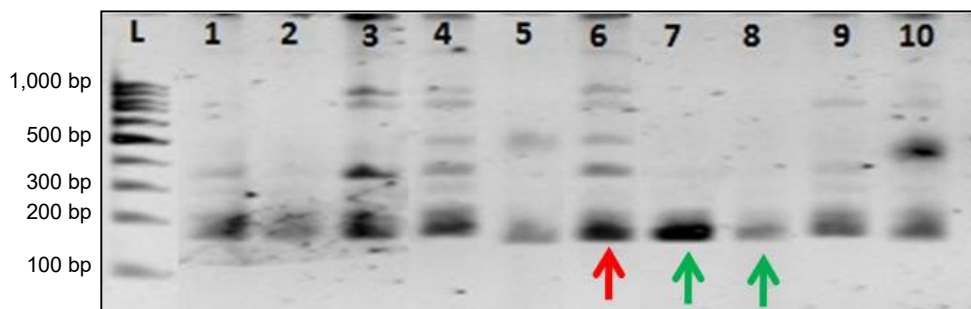


Figure 2. DNA polymorphism of two Argo genotypes and other eight rice genotypes genotyped using GBSS1 marker. L = 100 bp DNA ladder, 1 = Mendawak, 2 = Mekongga, 3 = Inpara 4, 4 = Margasari, 5 = IR48, 6 = Siam KDK, 7 = Argo-1, 8 = Argo-2, 9 = Banyuasin, 10 = Inpari 9. Green arrows indicate DNA bands of Argo-1 and Argo-2; red arrow indicates DNA band of Siam KDK, a local swampy rice genotype.

Genotypic characterization based on microsatellite marker analysis showed that Argo-1 was very closely related to Argo-2. Genotypes Argo-1 and Argo-2 were similar in SSR DNA polymorphism as shown by most of the SSR markers used (Figures 1 and 2). However, the two Argo genotypes still have degree of genetic distance of 0.143 (Table 4).

Based on Figure 2, it is assumed that the clusters formed to follow the similarity of agroecosystems and duration of physiological maturity. Inpara 4 is a late maturity introduced line (135 DAS) and adapted to swampy areas. Siam is a landrace from South Kalimantan. Many types of Siam can be found in the provinces of Kalimantan. Siam is swamp rice and most of the Siamese rice are generally late maturity types (Khairullah et al. 2021). Argo-1 and Argo-2 were also physiologically mature at >130 DAS.

As additional information, Argo-1 and Argo-2 have differences in polymorphic DNA bands in few SSR markers. It was found in the RM228 marker which is a DNA marker for drought tolerance. Argo-2 has two SSR alleles of 125 bp and 500 bp band sizes, respectively, that were absent in Argo-1 (Figure 3). From this data, there are indications of differences in the nature of resistance to drought stress. In the next study, it is necessary to check the resistance properties of these two Argo genotypes to drought stress.

Genetic Diversity among Rice Genotypes Tested

Argo is local rice that is widely grown for generations in Katapang Regency, West Kalimantan, Indonesia. According to the farmers, iron poisoning and blast disease are major problems in swamp areas. It seems that farmers carried out positive selection breeding of Argo local variety based on panicle length, vigor, maturity, and yield. Therefore, the Argo local variety has an early ripening age, around 120 DAS, but still has a tall plant height and is prone to lodging when planted in the rainy season. In the lowlands, this variety also grows well when the water level drops very quickly. It is suspected that Argo is also tolerant to drought stress.

Genetic distance analysis showed that Argo-1 and Argo-2 have a close genetic relationship with genetic distance coefficient between the two Argo genotypes was only 0.143. The genetic similarity level between the two Argo genotypes is 85.7% (Table 4). Argo-1 and Argo-2 have the closest genetic distance with Inpara 4 with a genetic distance coefficient of 0.227 and 0.369, respectively. Argo-1 is also very close with Siam KDK with a genetic distance of 0.286. Argo-1 has a higher genetic distance with Margasari (0.655) and Argo-2 has a higher genetic distance with Inpari 9 (0.584) (Table 4).

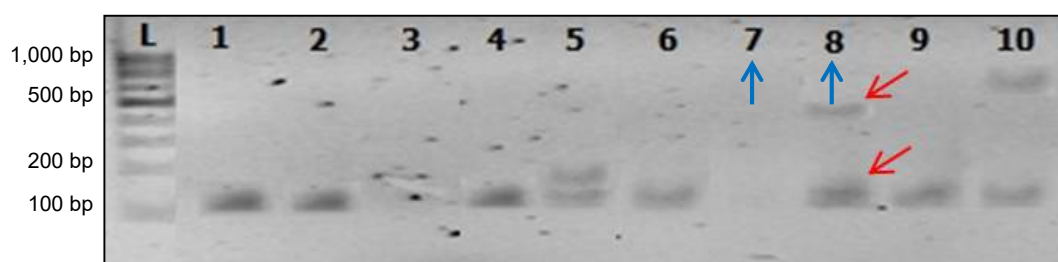


Figure 3. Allele discrimination between Argo-1 and Argo-2 based on genotypic analysis using RM228 marker. L = 100 bp DNA ladder, 1 = Mendawak, 2 = Mekongga, 3 = Inpara 4, 4 = Margasari, 5 = IR48, 6 = Siam KDK, 7 = Argo-1, 8 = Argo-2, 9 = Banyuasin, 10 = Inpari 9. Blue arrows indicate Argo-1 and Argo-2 genotypes, respectively; red arrows indicate two DNA bands that were present in the genome of Argo-2 genotype but was absent in the genome of Argo-1 genotype.

Table 4. Genetic distance comparison of the two Argo rice genotypes with other eight rice genotypes tested in this study.

OUT	Argo-1	Argo-2	Banyuasin	Inpara 4	Inpari 9	IR48	Margasari	Mekongga	Mendawak	Siam KDK
Argo-1	0.000									
Argo-2	0.143	0.000								
Banyuasin	0.613	0.448	0.000							
Inpara 4	0.227	0.369	0.357	0.000						
Inpari 9	0.584	0.547	0.172	0.399	0.000					
IR48	0.512	0.386	0.386	0.399	0.323	0.000				
Margasari	0.655	0.485	0.037	0.399	0.136	0.349	0.000			
Mekongga	0.613	0.448	0.287	0.399	0.386	0.287	0.250	0.000		
Mendawak	0.571	0.412	0.500	0.470	0.573	0.412	0.500	0.287	0.000	
Siam KDK	0.286	0.412	0.438	0.286	0.511	0.573	0.474	0.412	0.563	0.000

Phylogenetic analysis of the ten rice genotypes resulted three main clusters. Clusters 1 and 2 consisted of swamp rice genotypes only. Cluster 3, on the other hand, consisted of irrigated rice genotypes only. Within cluster 1, Argo-1 and Argo-2 were clustered together, therefore they are genetically very similar. In cluster 3, Banyuasin and Margasari were genetically very similar together with IR48 and Mekongga (Figure 4).

Argo-1 and Argo-2 were in one cluster with Inpara 4 and Siam KDK. Inpara 4 is an introduced genotype of International Rice Research Institute (IRRI), Philippines. Inpara 4 is a improved swamp variety that was released in 2010. Siam KDK, on the other hand, is a local swamp rice variety termed as a landrace that has been widely cultivated in Kalimantan. Siam KDK has long been cultivated by the farmers in Kalimantan. Cluster 2 consisted of only one genotype Mendawak. Cluster 3 consisted of five rice genotypes (Inpari 9, Banyuasin, Margasari, IR48, and Mekongga).

Cluster 1 only contains swamp rice genotypes while cluster 3 only contains lowland rice genotypes. Inpara 4 was developed by the Indonesian Agency for Agricultural Research and Development (IAARD) for adaptation in swamp agroecosystems. This superior variety has good adaptation in swampy areas. On the other hand, Siam KDK is a swamp agroecosystem local variety termed as a landrace. Argo-1 and Argo-2 are in one cluster with Inpara 4 and Siam KDK. It is assumed that Argo-1 and Argo-2 are swamp rice genotypes adaptable in swampy agroecosystem. Meanwhile, cluster 3 consists of irrigated rice consisting of Mekongga, Inpari 9, and IR48 varieties. Mekongga is the most popular irrigated rice variety and Inpari 9 is a newly improved variety. Meanwhile,

there are Banyuasin and Margasari varieties in cluster 3. Both varieties are superior varieties that are adaptable in tidal swamp land. Ahadiyat et al. (2014) reported that the genotype cluster in the dendrogram analysis followed the agroecosystem.

Cluster 2 only consisted of one genotype, namely Mendawak. Mendawak is swamp rice that has a lifespan of 120 DAS. On the other hand, Inpari 9, Mekongga, and IR48 are new varieties that have maturity time of <120 DAS. Unlike the three varieties of lowland rice, Margasari and Banyuasin are also in the same cluster. These varieties are types of swamp rice, but can be a cluster of irrigated rice. This is presumably due to similarities based on other characters that have not been revealed yet.

This study also has a future goal to determine the strategy of crossing in the breeding of swampy rice. Based on the results of this study, Argo-1 is not recommended to be crossed with Argo-2, Inpara 4, and Siam KDK because of their close genetic distances. Their crosses will result in progenies with narrow genetic diversity. Based on genetic distance data, it is recommended that Argo-1 should be crossed to Margasari to obtain a wider genetic diversity of the offsprings. Meanwhile, Argo-2 is recommended to be crossed with Inpari 9. The wide genetic diversity will make it easier for breeders to select the best genotypes possible because there will be many superior lines obtain from such crosses. Kristantini et al. (2014) reported that populations developed from crosses between genotypes with high genetic diversity can be used as plant breeding materials to developed superior new rice varieties. Jameela et al. (2014) and Mursyidin and Khairullah (2020) reported that wide genetic diversity enhances the success of selection and cultivar development.

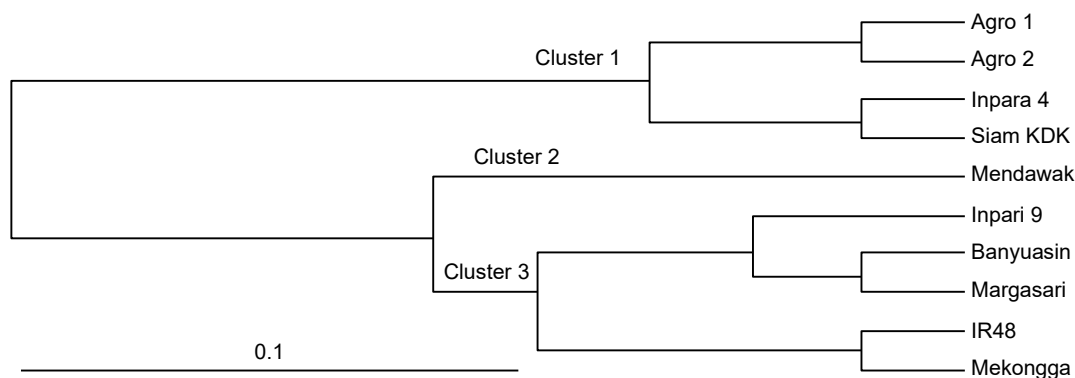


Figure 4. The phylogeny tree of Argo-1 and Argo-2 together with other eight rice genotypes tested based on analysis results using eight SSR markers.

CONCLUSION

The genotypes of two types of swampy landrace were characterized using SSR markers. They are very similar, but still have genetic distance of 0.143. It showed that both genotypes of Argo-1 and Argo-2 may be sister lines. Argo-1 and Argo-2 were in the same cluster as Inpara 4 and Siam KDK, but they clustered in different groups with the irrigated rice genotypes such as Mekongga and Inpari 9. Based on this research, Argo-1 and Argo-2 are not recommended to be crossed with Inpara 4 and Siam KDK because the genetic diversity formed will be narrow.

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AUTHOR CONTRIBUTIONS

All authors equally contributed in this article as the main contributor.

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