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GENETIC DIVERSITY OF *ALPINIA MALACCENSIS* (BURM.F.) ROSCOE (ZINGIBERACEAE) IN JAVA ISLAND, INDONESIA

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SUMMARY

Alpinia malaccensis (Burm.f.) Roscoe is a rare plant in Java, Indonesia, that produces essential oils. Its sustained decline causes great concern for future uses. Therefore, the study of its diversity in its natural habitat to predict future survival needs serious focus. The present study aims to determine the genetic diversity of *A. malaccensis* in Java based on ISSR markers. The sampling of a total of 11 populations yielded 33 accessions, which underwent amplifying using 12 ISSR primers. Using the molecular data analyzed their relationships, population structure, and genetic diversity. The UPGMA cluster, structure, and principal component analysis (PCA) demonstrated that the accessions divide in three groups, a result that correlates with their geography in Java. Group I consisted of var. *malaccensis* populations, while groups II and III comprised the var. *nobilis* populations, which proved truer among populations of var. *nobilis*. The genetic diversity category of *A. malaccensis* showed moderate based on Nei's genetic diversity (*h* = 0.2892) and Shannon's information index (I = 0.4438). The coefficient of genetic differentiation (G_{ST}) and molecular variance among the populations displayed higher within the individual population, which revealed a significant genetic variance among the 11 populations of *A. malaccensis*. The latest results confirm that ISSR markers can help distinguish the genotypes of *A. malaccensis*.

Keywords: Alpinia, gingers, genetic variation, population structure, molecular marker

Key findings: The results indicate that a genetic variation of *A. malaccensis* can further serve the species' conservation, characterization, and usefulness for future breeding purposes. In addition, this data will form the backbone of a taxonomic database.

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INTRODUCTION

Alpinia malaccensis (Burm.f.) Roscoe belongs to the family Zingiberaceae. Smith (1990) placed it under the subgenus *Alpinia*, section *Alpinia*, and subsection *Catimbium*. This species spread widely from India, through Indochina to Malesia. In the Malesian region, *Alpinia malaccensis* scattered in the Malay Peninsula, Sumatra, Java, and the Moluccas

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(Newman *et al*., 2004; Nurainas and Junaidi, 2007). In different regions of Indonesia, this species has many vernacular names including, 'langkuas malaka,' 'makui malaka' (Malay), 'laja gowah,' 'raja gowah' (Sunda), 'kamijara' (Java), and 'lawasa malacca' (Ambon) (Heyne, 1987).

Alpinia malaccensis served many uses, i.e., as traditional medicine, as a spice in cooking, and as a natural preservative ingredient in the cosmetic industry. Its rhizomes are usually harvested directly from the forest. In India, its rhizome serves as a vegetable and ointment. In the Moluccas (Indonesia), the rhizome provides a chewing spice (*kinang*) eaten with betel nuts (*Areca catechu* L.) to have a melodious and strong voice. Water infused with seeds and adding a little salt makes an anti-nausea treatment. This infusion also helped reduce fever by bathing the sick (Sujianto and Nurhayati, 2019). In Thailand, the rhizome as medicine cures stomach aches, cough, food poisoning, and tuberculosis (Phumthum and Balslev, 2020). Essential oils containing methyl cinnamate as the main active ingredient result in a distinctive aroma of the plant parts (Norazah *et al*., 2005). Each plant organ has essential oils with different levels of activity against microbes, but the oil from the leaf contains the most active inhibitors (Jusoh *et al*., 2020). The rhizome contains a lot of terpenoids, phenolic, and aromatic hydrocarbons (Sirat *et al*., 2011). Terpenoid compounds themselves have antimicrobial, antifungal, antiviral, antiparasitic, antihyperglycemic, antiallergenic, anti-inflammatory, antispasmodic, immunomodulatory, and chemotherapeutic properties (Anggraito *et al*., 2018).

In *A. malaccensis*, the active compound in each population has different qualities with varying quantities (Shen *et al*., 2021). In addition, the quality of compounds may degrade and alter the compound type, especially in the next generation. This results from genetic malformation continuously taking part in changing the metabolic pathways of these compounds (Baulcombe and Dean, 2014; Lu *et al*., 2020). The use of *A. malaccensis* in the production of essential oil reveals a growing industry in Indonesia. Unfortunately, the direct harvest of its rhizomes from the forest without cultivation moves fast (Sujianto and Nurhayati, 2019). With the decrease in forest area and the increasing demand for industrial markets, the said species decreases in its natural habitat. In China, a similar problem occurred for the medicinally useful ginger species *Curcuma wenyujin,* in which the concentration of the secondary metabolites decreases with the number of generations, but at the same time, the demand for secondary metabolites in these plants increases. The problem happened because pharmaceutical industries used this species for manufacturing blood thinners and pain relievers (Zheng *et al*., 2015; Lu *et al*., 2020; Li *et al*., 2021). Thus, knowledge about the genetic diversity of *A. malaccensis* requires immediate action to secure sustainable crop plants with high yields and good quality in the future.

Genetic variation analysis below the genus level often uses molecular markers to identify genetic changes due to environmental and genetic differences. No reports on genetic diversity in *A. malaccensis* existed, and only a few studies of its close relatives and species took place. Four molecular markers have successfully been used to profile the genetic variation in *Alpinia* spp., namely Amplified Fragment Length Polymorphism (AFLP), Sequence Repeat Amplified Polymorphism (SRAP), Random Amplified Polymorphic DNA (RAPD), and Inter Simple Sequence Repeats (ISSR). The AFLP markers efficiently analyzed the genetic diversity in *A. galanga* (Khunpiban *et al*., 2010), *A. oxyphylla* (Wang *et al*., 2012; Pan *et al*., 2021), and *A. officinarum* (Yang *et al*., 2011). Among populations of Indonesian 'galangal,' the SRAP markers showed low genetic diversity (Maulidah *et al*., 2019).

A study reported the ISSR markers profiling of *Alpinia galanga* accessions from India revealed its genetic diversity with a low level of population differentiation (GST = 0.2139) (Rajasekharan *et al*., 2016). Based on RAPD and ISSR markers, Basak *et al*. (2018) found a narrow range of genetic distance among the three studied populations of species *A. nigra*. Compared with RAPD, the ISSR marker was able to produce two unique bands for *A. galanga*, but the ISSR marker was found better in discriminating *A. galanga* accessions (Parida and Nayak, 2019). Several advantages of using the ISSR marker in assessing genetic diversity include simple, rapid, reproducible, and inexpensive (Wang *et al*., 2012).

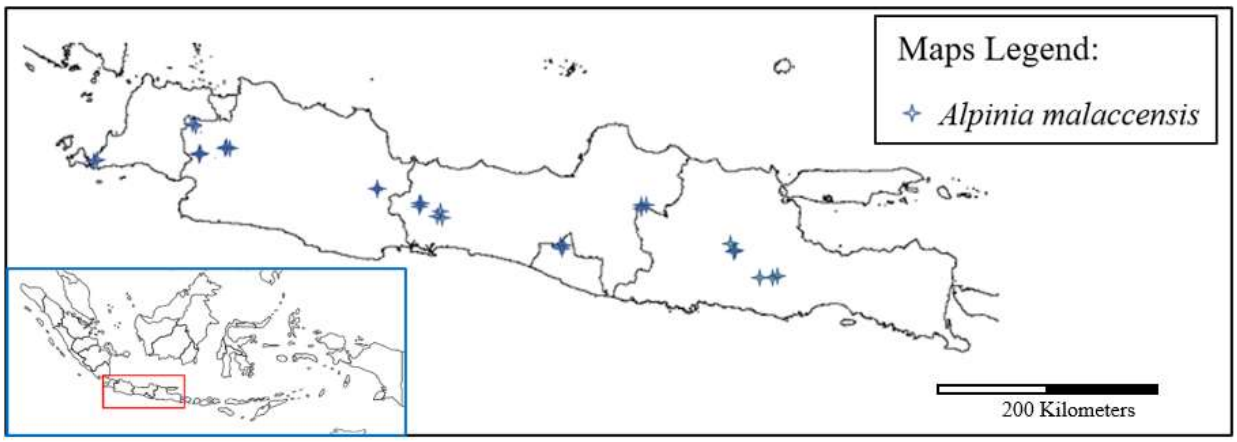

Figure 1. Sampling sites of *A. malaccensis* in Java, Indonesia.

Simple sequence repeats are distributed throughout the genome (Godwin *et al*., 1997; Gemmill and Grierson, 2021). Therefore, the ISSR marker showed the potential of discriminatory power for assessing clonal plant species (Velasco-Ramirez *et al*., 2014) and suitability for analyzing the genetic variation of *A. malaccensis*. In various species, the genetic variation data can be used for their breeding (Zheng *et al*., 2015) and conservation (Wang *et al*., 2012) in the future. Moreover, this data support taxonomic studies. The study determined the genetic diversity in various populations of *A. malaccensis* in Java, Indonesia, using ISSR markers, a first-ever undertaking to provide genetic diversity data for *A.malaccensis*.

MATERIALS AND METHODS

Plant material and procedure

Collection of 33 accessions of *A. malaccensis* took place from 11 locations in three regions in Java, Indonesia: 1) Western Java - Ujung Kulon National Park, Banten; Mt. Halimun Salak National Park; Jasinga, Bogor; Dramaga campus of IPB University, Bogor; Mt. Ciremai, Kuningan. 2) Central Java - Mt. Slamet; Banyumas; Mt. Lawu; Kaliurang, Yogyakarta), and 3) Eastern Java - Mt. Semeru and Tahura Raden Soerjo (Figure 1). At each location, gathering three accessions took place from the population. Each accession was taken from a different clump than the other accessions. As much as 100 g of young and healthy leaves of each accession underwent collection, and labeling, then, placed into a Ziplock plastic bag with silica gel.

DNA extraction and PCR amplification

Molecular analysis of genetic diversity consists of three stages: DNA extraction, DNA amplification via polymerase chain reaction (PCR) technique, and electrophoresis. Extraction of *A. malaccensis* genomic DNA used the Plant Genomic DNA Extraction Miniprep System (Viogene, Taipei, Taiwan). The subsequent DNA amplification used 12 ISSR primers (Table 1). The total volume of each PCR reaction was 20 µl consisting of 10 µl Thermo Scientific™ DreamTaq Green PCR Master Mix (2X) (Thermo Fisher Scientific, Sweden), 5 µl ddH2O, 3 µl primer (0.5 µM), and 2 µl DNA (100 ng). The amplification underwent 35 cycles. Each cycle consisted of a pre-denaturation stage at 94ºC for 4 min, denaturation at 94ºC for 50 sec, annealing at 46ºC – 55ºC for 1 min, elongation at 72ºC for 1 min, and the final elongation stage at 72ºC for 5 min. Amplicons have undergone electrophoresis on 1% agarose gel at 100 volts for 45 min with a standard 1 kb DNA ladder (Thermo Scientific, USA). Electrophoresis results observations used a UV–transilluminator instrument (WiseDoc, Daihan Ltd, South Korea).

Data analysis

The DNA fragments and bands resulting from electrophoresis assessment as binary data, classified the presence of band scored as 1, and the absence of band scored as 0. Molecular data arrangement into a binary matrix to calculate the similarity coefficients used the simple matching (SM) index, genetic diversity, population structure, and genetic relationship of *A. malaccensis*. Estimating the value of

Table 1. ISSR primer names, number of observation bands, number of polymorphic band, and polymorphism percentage of *A. malaccensis.*

Primer name	Sequence (5'-3')	Annealing temperature (ºC)	Number of observation bands	Number of Polymorphic bands	Polymorphism (%)
G7	$(AG)_8C$	46.0	6	6	100
H1	$(GGGGT)_3$	53.0	6	6	100
H2	$(GA)_9T$	53.0	7	7	100
H6	$(GT)_8T$	48.0	6	6	100
H7	$(AC)_8Y$	48.0	8	7	87.50
H10	$(AG)_8TC$	53.0	7	7	100
H11	$(AG)_8YA$	55.0	16	13	81.25
H20	$(AC)_8CYT$	55.0	3	3	100
I	$(CA)_8YC$	55.0	4	4	100
M	$(AC)_8YG$	50.0	5	5	100
P9	$(AG)_8T$	46.0	11	10	90.91
Q2	$(AG)_8TC$	53.0	4	4	100
Total		-	83	78	
Average		-	6.92	6.50	96.64

Notes: Y: Pirimidin (C, T), R: Purin (A, G)

molecular variance (AMOVA), the significance of genetic differences, and pair-wise population differentiation (PhiPT) used the GenAlex 6.5 (Peakall and Smouse, 2012). The genetic diversity indices when populations are in Hardy-Weinberg equilibrium consist of: the number of alleles (Na), the effective number of alleles (Ne), Shannon's information index (I), genetic diversity (h), percentage of polymorphic loci (PPL), total genetic diversity (H_T), genetic diversity within populations (H_S), coefficient of genetic differentiation (G_{ST}), gene flow among populations (Nm), and Nei's genetic distance among populations, calculated using the Population Genetic Analysis software, version 1.32 (PopGene32) (Yeh and Boyle, 1997).

Population structure and genetic relationship calculations used the principal component analysis (PCA), and to generate a dendrogram of *A. malaccensis* used the Unweighted Pair Group Method with Arithmetic Average (UPGMA) in NTSys software version 2.1.1a (Rohlf, 1998). The calculation of population structure based on Bayesian cluster analysis employed STRUCTURE 2.3.4 (Pritchard *et al*., 2000). Initially, conducting a simulation run of the STRUCTURE assumed the number of genetic clusters/subpopulations (K) of 1 to 10 with 20 iterations for each K. The Monte Carlo Markov Chain was run for 100,000 steps, following a burn-in period of 10,000 steps under the admixture model, the correlated allele frequencies model, and no prior population information. Computing the optimum number of K used the K method of Evanno *et al*. (2005) using STRUCTURE HARVESTER (Earl and Vonholdt, 2012).

RESULTS

ISSR locus polymorphism detection

In 33 accessions of *A. malaccensis*, obtaining good quality DNA at a concentration of 100-300 ng/µl appears in Table 1. Almost all the accessions had sharp DNA bands. The results of DNA electrophoresis, amplified using the ISSR primers, also showed good outputs. The total produced 83 bands showed all polymorphic, with the average number of bands at 6.92 bands per primer. The percentage of polymorphism in all primers displayed more than 81%. Two primers produced many observational and polymorphic bands (≥10), namely H11 ([AG]8YA) and P9 ([AG]8T).

Analysis of genetic diversity and population structure

The average number of alleles (Na) in all the populations was higher than the effective number of alleles (Ne) (Table 2). The number of alleles (Na) ranged from 1.1449 (RS) to 1.2464 (YG), while the Na value in the total population displayed 1.9855. The effective allele value (Ne) ranged from 1.1043 (JS) to 1.971 (YG), and the Ne value in the total population discloses 1.4876. The genetic diversity of each population ranged from 0.0580 (JS) to 1.1095 (YG), while genetic diversity in the total population was 0.2892 (Table 2). The value of Shannon's information index (I) ranges from 0.0830 (JS) to 0.1568 (YG), while the index value for the total population reveals 0.4438. The percentage of

Table 2. Genetic diversity parameters of *A. malaccensis* in Java, Indonesia.

Populations	Na	Ne	h	I	PPL (%)
JS	1.1304	1.1043	0.0580	0.0830	13.04
SL	1.1884	1.1507	0.0837	0.1199	18.84
CM	1.1739	1.1391	0.0773	0.1107	17.39
LW	1.2319	1.1855	0.1031	0.1476	23.19
UK	1.2319	1.1855	0.1031	0.1476	23.19
IPB	1.1884	1.1507	0.0837	0.1199	18.84
BN	1.2319	1.1855	0.1031	0.1476	23.19
HS	1.2319	1.1855	0.1031	0.1476	23.19
YG	1.2464	1.1971	0.1095	0.1568	24.64
SM	1.2029	1.1623	0.0902	0.1291	20.29
RS	1.1449	1.1159	0.0644	0.0922	14.49
Total	1.9855	1.4876	0.2892	0.4438	98.55
St. Dev.	0.1204	0.3539	0.1663	0.2087	–

Notes: Na: Number of Alleles, Ne: Number of Effective Alleles, h: Genetic Diversity, I: Shannon's Information Index, PPL: Percentage of Polymorphic Loci, UK: Ujung Kulon National Park (Banten), HS: Mt. Halimun Salak National Park, JS: Jasinga, Bogor, IPB: IPB University, Dramaga, Bogor, CM: Mt. Ciremai, Kuningan (West Java), SL: Mt. Slamet, BN: Banyumas, LW: Mt. Lawu (Central Java), YG: Yogyakarta (Yogyakarta), SM: Mt. Semeru, and RS: Tahura Raden Soerjo (East Java).

Table 3. Total genetic diversity of *A. malaccensis* in Java, Indonesia.

Sample numbers	H_T	H_S	G_{ST}	N_M
33	0.2892	0.089	0.6923	0.2223

Notes: H_T: Total genetic diversity, H_S: genetic diversity within populations, $G_{ST:}$ coefficient of genetic differentiation, N_M: gene flow among populations.

Table 4. Nei's genetic distance among populations of *A. malaccensis* in Java, Indonesia.

POP	JS	SL	CM	LW	UK	IPB	BN	HS	YG	SM
SL	0.0582									
CM	0.1583	0.1898								
LW	0.1597	0.2188	0.1554							
UK	0.3180	0.3643	0.1263	0.1659						
IPB	0.3045	0.3191	0.3209	0.1639	0.2769					
BN	0.3580	0.3386	0.2344	0.2807	0.1746	0.1639				
HS	0.3010	0.3209	0.3739	0.2979	0.3751	0.0643	0.1297			
YG	0.4527	0.4557	0.1919	0.1464	0.1295	0.2234	0.1812	0.3787		
SM	0.5511	0.5130	0.2899	0.2560	0.2420	0.3050	0.3042	0.4559	0.1133	
RS	0.4607	0.4576	0.2004	0.2070	0.1597	0.3099	0.2338	0.4530	0.0611	0.0685

Notes: POP: Populations, UK: Ujung Kulon National Park (Banten), HS: Mt. Halimun Salak National Park, JS: Jasinga, Bogor, IPB: IPB University, Dramaga, Bogor, CM: Mt. Ciremai, Kuningan (West Java), SL: Mt. Slamet, BN: Banyumas, LW: Mt. Lawu (Central Java), YG: Yogyakarta (Yogyakarta), SM: Mt. Semeru, and RS: Tahura Raden Soerjo (East Java). Green box (JS-SL): the lowest genetic distance value, blue box (SM-JS): the highest genetic distance value.

polymorphic loci (PPL) in each population tends to be low, ranging from 13.04% (JS) to 24.64% (YG), while the total percentage of polymorphic loci in the total population was very high, reaching a value of 98.55%.

The total genetic diversity (H_T) in *A. malaccensis* showed 0.2892, while the coefficient of genetic differentiation (G_{ST}) revealed 0.6923. It indicated that 69.23% of the total genetic variation of *A. malaccensis* in Java occurs among the population, and the remaining about 30.77% occurs within the population. The level of gene flow among the populations occurs with a fairly small value (0.2223) (Table 3). Nei's genetic distance displayed a correlation with varietal and geographic differences. The highest value of the genetic distance was 0.5511 (SM-JS), and the lowest value was 0.0582 (SL-JS) (Table 4). Molecular variance among the populations indicated higher than within the individual population (Table 5). The AMOVA also showed a significant genetic variance among the populations of *A. malaccensis*, indicated by the PhiPT value (0.568), which was higher than P (rand ≥ data) (0.001). The PhiPT value has been determined based on the formula: Est. Var. between populations / Est. Var. total while obtaining the P (rand ≥ data) used the probability in statistical tests (Blyton and Flanagan, 2006; Ennami *et al*., 2017; Akhtar *et al*., 2021).

Table 5. Analysis of Molecular Variance (AMOVA) of *A. malaccensis* in Java, Indonesia.

Source	df	SS	MS	Est. Var.	Est. Var. (%)	PhiPT	*P*-value
Among Pop	10	227.939	22.794	6.063	57	0.568	0.001
Within Pop	22	101.333	4.606	4.606	43	-	-
Total	32	329.27		10.669	100	-	-

Notes: df: degree of freedom, SS: sum of the square, MS: mean square, Est. Var.: estimated variance, PhiPT: the total proportion of genetic variation (Est. Var. among population: Est. Var. total population), *P*-value: Probability value, Pop: population.

Genetic relationship of *A. malaccensis*

Analyzing the genetic relationships of *A. malaccensis* using UPGMA cluster analysis showed that the 33 accessions divide into three groups at a similarity coefficient of 67%, namely, group I, group II, and group III (Figure 2). Group I comprised two populations of *A. malaccensis* var. *malaccensis*, namely, JS and SL populations. Group II consisted of three populations of *A. malaccensis* var. *nobilis* (Ridl.) I.M. Turner from Western Java Island. Group III consisted of six populations of var. *nobilis* from Central and Eastern Java Islands, Indonesia.

The most likely number of K, identified to be 3, resulted from the STRUCTURE HARVESTER according to the K values (K = 300) (Figure 3). The individual membership proportion (Q) showed higher than or equal to 60% and as many as 100%, 100%, and 66.7%, respectively, of individuals exclusively placed in three distinct clusters (Figure 4). Individuals with a membership proportion of less than 80% included IPB1, BN2, and BN3, with dominant genetic proportions of a genetic cluster B, but also containing the genetic parts of clusters A and B. Other admixture individuals consisted of CM, LW, and UK populations, being the dominant genetic proportion of cluster B but also containing a proportion of cluster A.

The principal component analysis (PCA) on the genetic relationships among the populations of *A. malaccensis* indicated consistency with those revealed by UPGMA and STRUCTURE (Figure 5). Based on the PCA results, the populations also formed three distinctly distributed groups, namely groups I, II, and III. Group I consists of two populations of var. *malaccensis*, namely, JS and SL. Group II consisted of three other populations of var. *nobilis*, namely, IPB, HS, and BN and group III comprised six populations of var. *nobilis*, namely, CM, LW, UK, YG, RS, and SM.

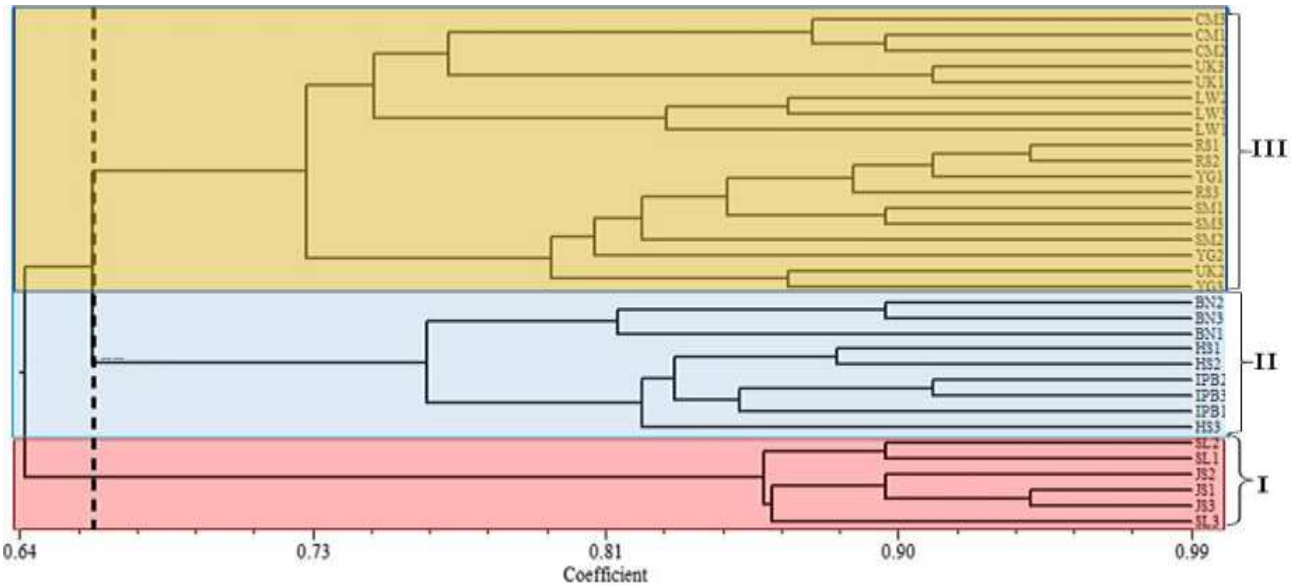

Figure 2. Dendrogram of the genetic relationship of *A. malaccensis* in Java, Indonesia, based on ISSR markers. Group I: red, Group II: blue, and Group III: yellow.

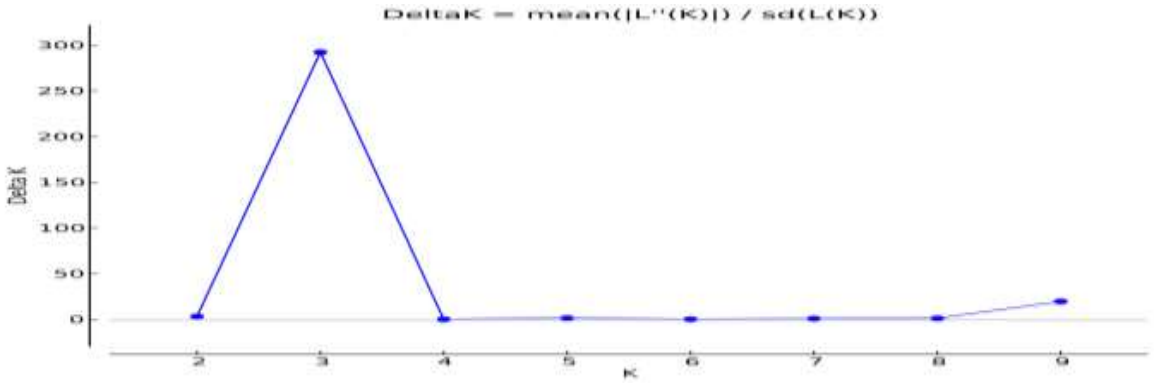

Figure 3. Structure estimation number of clusters for K values ranging from 1 to 10, based on delta K values.

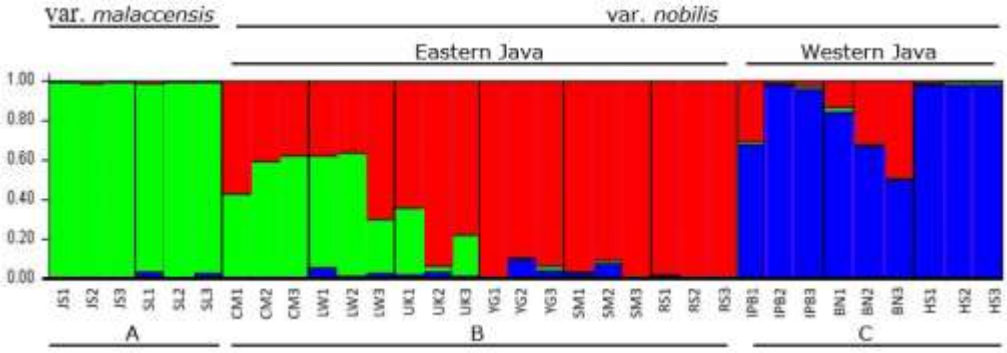

Figure 4. Genetic structure revealed by the Bayesian model-based analysis using STRUCTURE 2.3.4 for *A. malaccensis* populations in Java. Each individual is indicated by a vertical colored bar, and the proportion of the color in each bar represents the probability of membership in the corresponding cluster.

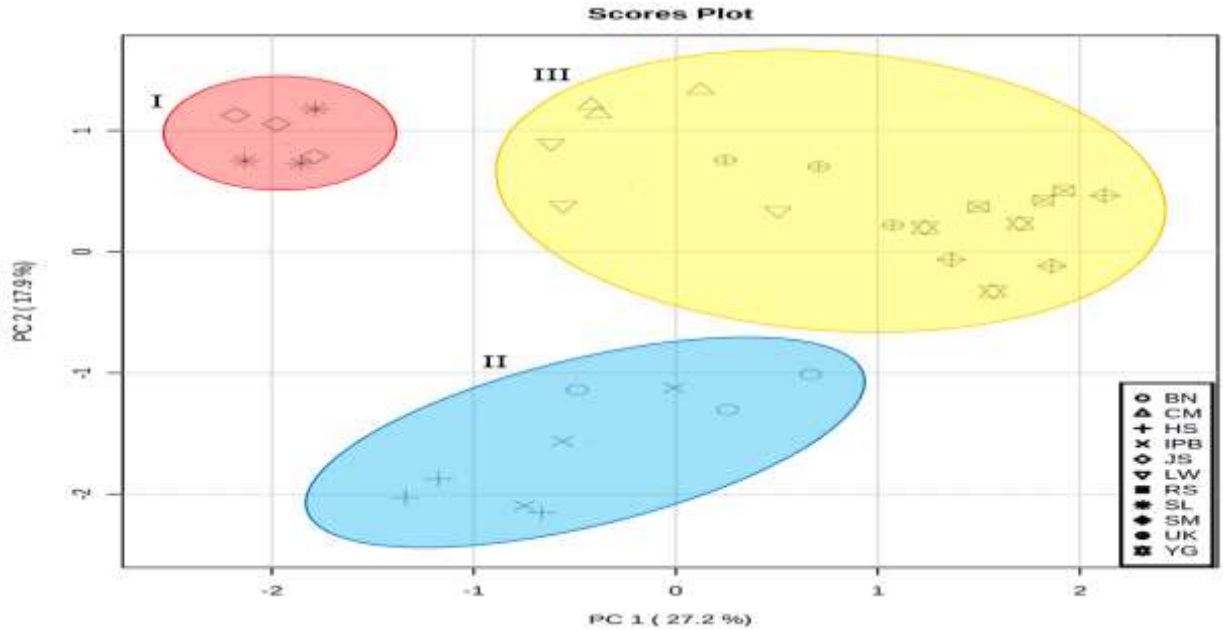

Figure 5. Principal Component Analysis (PCA) of *A. malaccensis* in Java. PC1: Principal Component Axis 1, PC2: Principal Component Axis 2. Group I: red, Group II: blue, Group III: yellow.

DISCUSSION

In a population, the genetic diversity index and the percentage of polymorphic loci reflect genetic diversity (Nei, 1973). The present results showed that the genetic diversity (h) and percentage of polymorphic loci (PPL) of *A. malaccensis* presented low in all the populations. Various factors cause low genetic diversity of a species, such as, a tendency for the species to have vegetative reproduction or self-pollinate, with low mutation rate (Xu *et al*., 2015), or growing in relatively similar habitat conditions, such as, soil type, soil pH, and soil chemical compounds, where the environmental factors are almost the same between the individuals or clumps of a population (Corbett-Detig *et al*., 2015; Aguirre-Liguori *et al*., 2019). Meanwhile, the values of genetic diversity and Shannon information index on the total population exhibited relatively moderate, due probably to the differences in geographical and climatic conditions affecting the genetic diversity among the populations.

Among populations of *A. malaccensis* in Java (H_T), the total genetic diversity showed greater than the genetic diversity within a population (H_S). Based on the AMOVA, the molecular variance among the populations emerged greater than within an individual population. Genetic diversity and molecular variance within a population have a relatively low value related to vegetative reproduction. According to Rajasekharan *et al*. (2016) and Maulidah *et al*. (2019), who investigated *A. galanga* in South India and Indonesia, vegetative reproduction with rhizomes occurs more frequently and faster in the distribution of *A. malaccensis.* Many members of Zingiberaceae have been cultivated, and people usually grow *Alpinia* and other ginger genera using rhizomes because of the opportunity to propagate plants faster this way than from seeds (Cordeiro and Silva, 2003; Rivai *et al*., 2015). The clonal propagation of *Alpinia* results in low genetic variance in the population. The speciation process between *A. malaccensis* population appeared relatively low because the value of N_M was less than 0.25 (N_M = 0.2223), indicating the level of gene flow between the populations was small. The low gene flow can lead to low combinations of two or more gene pools so that populations become more isolated. This has implications for the high genetic diversity among the populations because the genetic exchange between populations was low (Bolnick and Nosil, 2007; Gemmell *et al*., 2018).

The recent study on *A. malaccensis* in Java demonstrated that Nei's genetic distance correlated with geographic and varietal differences among the populations. Species populations from Western Java tend to separate from those in Central and Eastern Java, Indonesia. This was also the case for another species, *Anisodus tanguticus* (Solanaceae), which inhabits a complex topography, and genetic variations occur among the populations of different altitudes (Zheng *et al*., 2008). Meanwhile, Wang *et al*. (2012) and Pan *et al*. (2021), who analyzed genetic variation in *Alpinia oxyphilla* in China, showed that the two parameters above were not correlated with the geographical conditions, but other factors influenced the variation.

Based on the genetic relationship analysis, the resulting grouping pattern of dendrogram, STRUCTURE, and PCA was according to the variety and geographical conditions of *A. malaccensis* populations. Based on differences in varieties, this distinguished Group I from group II and group III. Group I comprised of var. *malaccensis* accessions, while groups II and III consisted of var. *nobilis* accessions. Group II consisted of populations from Western Java, and Group III consisted of six populations from Central and Eastern Java that have relatively drier and higher temperature conditions. So far in this species, the microclimate differences between the western and eastern Java did not cause any character differences between both groups, and even some characters overlap. The amount of rainfall in the western part of Java Island is high at 2200–2400 mm, while the eastern part of Java Island has lower rainfall, around 800–1600 mm (Avia, 2019), and generally, the eastern part of Java Island has a drier climate. This condition also affects the differences in plant species and their behavior in the western and eastern parts of Java Island, Indonesia (Van-Welzen and Raes, 2011).

Geographical roles continued with local adaptation, and the expansion of genotype-adapted clones also occurred in the formation of clusters of 'Bangle' (*Zingiber montanum* (J. Koenig) Link ex A. Dietr.) in several provinces of Indonesia (Ardiyani *et al*., 2021) and *Zingiber zerumbet* (L.) Roscoe ex Sm. in India (Kavitha and Thomas, 2008). Past findings also support the existing results, following the dendrogram based on morphological characters of *A. malaccensis* in West Malesia (Setiawan, 2021). The dendrogram divides into two major groups based on differences in varieties,

namely var. *malaccensis* and var. *nobilis*. Both varieties have different lips, dorsal corolla lobe, and fruit characteristics. The *A. malaccensis* var. *malaccensis* have a yellow line on the edge of the lip with the red line not reaching the edge of the lip, the width of the dorsal corolla lobe narrows at the base, and the fruit is red when ripe. However, *A. malaccensis* var. *nobilis* has many red or purple strips on the edge of the flower lips (Holttum, 1950), the width of the dorsal corolla lobe is the same in all parts, and the fruit is orange when ripe (Setiawan, 2021).

The reproductive system of *A. malaccensis* seemed closely correlated with genetic diversity. The reproductive system of several species from the family Zingiberaceae and Tribe Alpinioideae has a flexibility mechanism on the pistil to avoid self-pollination (Li *et al*., 2002; Ren *et al*., 2007; Takano *et al*., 2013; Su *et al*., 2017; Fernandes *et al*., 2018; Sharma and Kaul, 2020). For example, the pistil of *Alpinia kwangsiensis* divides into two phenotypes, namely, cataflexistyle flowers (position of the stigma above anthers) in the morning until late afternoon and hyperflexistyle flowers (position of the stigma above anthers) in the afternoon to evening. In cataflexistyle flowers, the anthers will release pollen grains in the morning, but fertilization will not occur due to the higher position of the anther so that the pollinator's body parts will not come into contact with the pollen grains (Li *et al*., 2001). Although the flexystyly mechanism causes higher genetic diversity of *Alpinia* and may play a significant role in *Alpinia* speciation, vegetative reproduction of *A. malaccensis* occurs more frequently than sexual reproduction. In addition, the previously described factors, such as, geographical conditions, varietal differences, local adaptation, and the expansion of genotype-adapted clones, are also considered to play a vital role in genetic variation, genetic distance, and genetic relationship.

CONCLUSIONS

The parameters of genetic diversity (h), Shannon information index (I), and the percentage of polymorphic loci (PPL) exhibited low levels for each population, however, in the total population, the values of these three parameters showed moderate. The genetic variation within a population was lower than that among the populations. Genetic diversity among the populations had a high significance value. In *A. malaccensis,* in Java, Nei's genetic distance correlated with geographic distance. Based on the variety and geographical conditions and their UPGMA cluster analysis, STRUCTURE, and PCA, the total population of *A. malaccensis* divides into three groups. Based on ISSR markers, the genetic diversity distinguished two varieties of *A. malaccensis* in Java, i.e., var. *malaccensis* and var. *nobilis*.

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