Genetic diversity of elephant foot yam (Amorphophallus paeoniifolius) and relatives from the Meratus Mountains of South Kalimantan, Indonesia

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Research Article

Genetic Diversity of Elephant Foot Yam (*Amorphophallus paeoniifolius*) and Two Other Relatives from the Meratus Mountains of South Kalimantan, Indonesia

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ABSTRACT
Elephant foot yam *(Amorphophallus paeoniifolius)* is a tuber crop with high economic value, so it is very prospective to be developed. This study aimed to characterize and determine the genetic diversity and relationship of *A. paeoniifolius* and two other relatives from the Meratus Mountains of South Kalimantan, Indonesia, using the *rbc*L marker. Eight samples of *A. paeoniifolius* and three other ones (outgroups), two of *A. muelleri* and one of *A. borneensis,* were used in the study. The genetic diversity was determined using the nucleotide diversity index (π), whereas the phylogenetic relationships were reconstructed using the Maximum Likelihood (ML) and Neighbor-Joining (NJ) methods. The results show that this germplasm has a high diversity at an inter-species level of 0.95% and a low at intra-species (0.33%). The phylogenetic analyses revealed that *Amorphophallus* from this region separated into different clades, three for NJ and one for ML. In this case, *A. paeoniifolius* var. *sylvestris* from Bati-Bati, Tanah Laut is closely related to *A. paeoniifolius* var. *hortensis* from Marajai, Balangan. In conclusion, although *Amorphophallus* from the Meratus Mountains of South Kalimantan, Indonesia, shows a high diversity at an inter-species level, the phylogenetic analyses revealed a unique relationship. This finding is expected to be a reference in supporting efforts to conserve, cultivate, and utilize sustainable *Amorphophallus*, globally and locally, particularly for the Dayak Meratus community of the South Kalimantan, Indonesia.

INTRODUCTION

The Meratus Mountains, which extend from the Hulu Sungai Tengah (HST) to the Tanah Laut (Tala) regencies of the South Kalimantan, Indonesia, is part of the world's mega-diversity that reserves abundant genetic resources. *Amorphophallus* is one of the local genetic resources of this region that have been underutilized by the local people, especially the Dayaks. For a long time, they only used this plant as the second food source after rice and sometimes as an offering food (*sesaji*) in the ritual ceremony for land clearing (King et al. 2017).

Nowadays, *Amorphophallus* is a type of tuber crop with high economic value in the global market, so the productivity of this cultivated plant should be increased to fulfil market demands (Mekkerdchoo et al. 2016). The tuber of this plant is a source of glucomannan (a type of carbohydrate) and other substances, which can be used for the food and health industry (Mekkerdchoo et al. 2016). Even today, the tuber has been exported to other countries, especially Japan, with relatively large volumes, around 3,000 tons/year. However, its export needs are often not met (Poerba et al. 2009). Hence, the opportunities for its cultivation are still very wide opened. In other words, various efforts, like preserving, maintaining, and developing this local crop, are very urgent to be conducted.

Genetic characterization is one of the essential tasks in supporting those programs, both the preservation, cultivation, and utilization of this plant. In brief, this activity is the one key in conservation and breeding programs (Malhotra et al. 2018). In general, conservation aims to ensure the continuing survival of species, habitats, and biological communities and interactions between species or species with their ecosystems. Besides, breeding can be used directly to preserve and utilize several genes with essential agronomic traits for future purposes (Acquaah 2017). In the Meratus Mountains of South Kalimantan, Indonesia, the characterization of these plants has been limited to only use morphological markers. Meanwhile these markers have certain limitations, some of which were also greatly influenced by environmental factors. In addition, the morphological characters are very inefficient and time-consuming due to the long period of the generative/flowering phase (Sunaryo 2015).

This study aimed to characterize and determine the genetic diversity and relationship of *Amorphophallus paeoniifolius* synonym to *A. campanulatus* (elephant foot yam) and two other relatives from the Meratus Mountains of South Kalimantan, Indonesia, using the *rbc*L marker. Conceptually, this marker its own advantages, mainly being the ability to distinguish germplasm with its very close genetic relationship (Wattoo et al. 2016). Besides, this marker is more accurate and reliable than others and has generated unbiased or unambiguous data (Lee et al. 2017; Singh et al. 2017). Thus, the results of our study are expected to be a reference in supporting efforts to conserve, cultivate, and utilize sustainable *Amorphophallus*, globally and locally, particularly for the Dayak Meratus community of the South Kalimantan, Indonesia.

MATERIALS AND METHODS

Plant materials

A total of eleven samples of *Amorphophallus* comprise of eight for *A. paeoniifolius* and three others, including two species as the outgroup, namely *A. muelleri* and *A. borneensis*, were used in this study (Table 1). All plant materials were collected by a purposive sampling method from seven locations of South Kalimantan, Indonesia (Figure 1).

Molecular characterization

Molecular characterization was carried out in the Laboratory of Genetics and Molecular Biology, Faculty of Mathematics and Natural Sciences, University

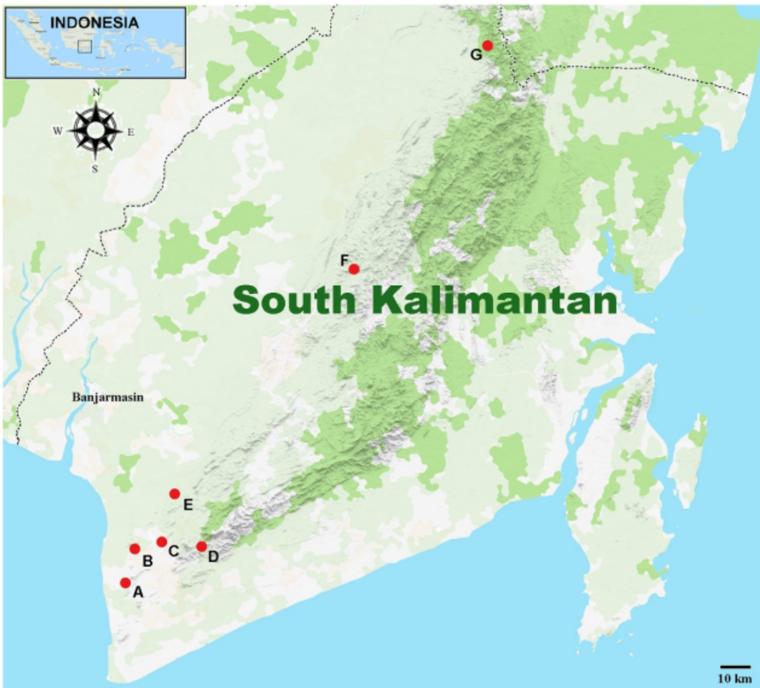

Figure 1. Map of South Kalimantan, Indonesia, showing seven sampling locations where the *Amorphophallus* were collected: A. Telaga, Takisung, Tanah Laut; B. Ranggang, Takisung, Tanah Laut; C. Angsau, Pelaihari, Tanah Laut; D. Sungai Bakar, Bajuin, Tanah Laut; E. Kait-Kait, Bati-Bati, Tanah Laut; F. Batung, Piani, Tapin; G. Marajai, Halong, Balangan.

Table 1. List of *Amorphophallus* samples used in this study and their origin.

Species	Local Name	Origin (Village/District/ Regency)	Geographic Coordinate	Elevation (m-asl)
A. paeoniifolius var. *sylvestris*	*Bagang*	Sungai Bakar (upper), Bajuin, Tanah Laut	3°77'95°S; 114°85'56°E	93
A. paeoniifolius var. *sylvestris*	*Bagang*	Sungai Bakar (lower), Bajuin, Tanah Laut	3°77'53°S; 114°85'45°E	64
A. paeoniifolius var. *sylvestris*	*Bagang*	Kait-Kait, Bati-Bati, Tanah Laut	3°58'42°S; 114°81'86°E	41
A. paeoniifolius var. *sylvestris*	*Bagang*	Ranggang, Takisung, Tanah Laut	3°83'40°S; 114°69'29°E	17
A. paeoniifolius var. *hortensis*	*Suweg*	Batung, Piani, Tapin	2°93'72°S; 115°40'90°E	230
A. paeoniifolius var. *hortensis*	*Suweg*	Telaga, Takisung, Tanah Laut	3°83'40°S; 144°72'27°E	24
A. paeoniifolius var. *hortensis*	*Suweg*	Marajai, Halong, Balangan	2°37'29°S; 115 ° 70'20 °E	53
A. paeoniifolius var. *hortensis*	*Suweg*	Angsau, Pelaihari, Tanah Laut	3°79'55°S; 114°78'47°E	24
A. muelleri	*Porang*	Sungai Bakar (upper), Bajuin, Tanah Laut	3°78'86°S; 114°85'74°E	197
A. muelleri	*Porang*	Sungai Bakar (lower), Bajuin, Tanah Laut	3°77'40°S; 114°85'43°E	60
A. borneensis	*Maya*	Batung, Piani, Tapin	2°93'73°S; 115°41'01°E	242

of Lambung Mangkurat (ULM) and Laboratory of Molecular Biology, Agricultural Quarantine Agency (Class I) Banjarmasin, South Kalimantan. The activity began with DNA isolation from the leaves of all *Amorphophallus* samples of Meratus Mountain, South Kalimantan, which was successfully collected, using the DNAZol@Direct protocol (Molecular Research Center Inc., USA). DNA genomes were then quantified by UV-VIS spectrophotometry (NanoVue, GE Healthcare, UK). The DNAs were then amplified using the *rbc*L marker (Table 2) and a PCR machine (SimpliAMP, Applied Biosystem, USA).

The total volume of PCR reactions used in the study was 25 µL, consisting of 2 µL of 20 ng genomic DNA (templates), 1 µL of 0.2 µmol for each primer, and 22 µL of PCR mix (MyTaq HS Red Mix, Bioline, UK). The PCR reaction was carried out following the instructions of Mursyidin et al. (2021), with the following conditions: (1) initial denaturation, 94°C for 5 min; (2) denaturation, 94°C for 30 sec; (3) annealing, 48°C for 30 sec; (4) extension, 72°C for 45 sec; and (5) final extension, 72°C for 7 min.

The amplified DNAs were separated by gel electrophoresis with 2% agarose and a 1X TBE buffer solution. After electrophoresis, the gel was stained with GelRed (Biotium, USA). Furthermore, DNA fragments in the gel were observed on UV transilluminator light and documented using a digital camera. DNA fragments that were amplified then sent to 1st Base Ltd., Malaysia, for purification and sequencing bidirectionally using the Sanger method.

Data analysis

The partial sequences of *rbc*L of *A. paeoniifolius* and two other relatives (outgroup), were edited, assembled, and analyzed using the software of MEGA-X (Kumar et al. 2018). The gapped regions in the alignment were excluded from subsequent analysis unless some positions included nucleotide diversity (Mursyidin et al. 2018). The genetic diversity was determined using the nucleotide diversity index (π) with three-level categories, i.e., low (0.1 - 0.4), medium (0.5 - 0.7), and high (0.8-2.00) (Nei & Li 1979; Nei 1987). For phylogenetic analysis, multiple sequence alignments of sequences were performed with Clustal X ver. 2.0 (Larkin et al. 2007). The phylogenetic relationships were reconstructed using the Maximum Likelihood (ML) and Neighbor -Joining (NJ) methods on the program of MEGA-X (Kumar et al. 2018). In these analyses, the bootstrap method with 1,000 replicates and the substitution model of Kimura 2-Parameter was applied to reconstruct and evaluate the phylogenetic trees (Felsenstein 1985; Kumar et al. 2018).

Table 2. Primers were used in this study.

Primer	Position	Sequence (5'-3')	Annealing (°C)	Target (bp)
*rbc*L	*Forward*	ATGTCACCACAAACAGAGACTAAAGC	48	600
	Reverse	GTAAAATCAAGTCCACCRCG		

Source: Gholave et al. (2017).

RESULTS AND DISCUSSION

Results

The partial sequences of the *rbc*L region of *A. paeoniifolius* and two other species originating from the Meratus Mountains of South Kalimantan, Indonesia, were successfully sequenced and aligned. The results show that this germplasm has a different length of *rbc*L, ranging between 542 and 607 bp (Table 3). At the inter-species level, *A. paeoniifolius* has the *rbc*L ranging between 574 and 605 bp. The polymorphic sites and the rate of the substitutional matrix were shown in detail in Table 4 and 5, respectively. Following Table 3 & 4, the partial sequences of *rbc*L of *Amorphophallus* have 24 polymorphic loci, i.e., nine parsimony-informative and 15 singleton sites. Furthermore, these sequences have shown different substitutional rates, 57.51 for transitional mutation and 26.49 for transversional ones (Table 3). Regarding genetic diversity, *A. paeoniifolius* has lower genetic diversity (0.33%) than at the intra-species level (0.95%) (Table 3).

Table 3. Characteristics of the *rbc*L sequences of *A. paeoniifolius* and two other species (outgroup) from the Meratus Mountains of South Kalimantan, Indonesia, including its genetic (nucleotide) diversity.

Parameter	Intra-species	Inter-species
Range of sequence length (bp)	574-605	542-607
Number of polymorphic sites (*S*)	9	24
Substitution-transition rates (%)	33.32	57.51
Substitution-transversion rates (%)	66.68	26.49
Transition/transversion bias value (*R*)	0.50	1.35
GC content (%)	41.68	41.88
Maximum likelihood value (*In*L)	-912.08	-1021.29
Nucleotide diversity (p%)	0.33	0.95

The phylogenetic analyses showed that *Amorphophallus* from the Meratus Mountains of South Kalimantan, Indonesia, was separated into different clades, three for Neighbor-Joining (NJ) and one for Maximum Likelihood (ML) (Figure 2). In this case, *A. paeoniifolius* is generally far separated from two other *Amorphophallus* species (outgroup), namely *A. muelleri* and *A. borneensis*.

The genetic distance analysis (Table 6) revealed that *A. paeoniifolius* var. *hortensis* from Pelaihari, Tanah Laut has a close relationship with similar germplasm from Takisung, Tanah Laut region. Similarly, *A. paeoniifolius* var. *sylvestris* from Takisung, Tanah Laut is also closely related to *A. paeoniifolius* var. *hortensis* from Pelaihari and Takisung, Tanah Laut. In contrast, a far relative relationship showed by *A. paeoniifolius* var. *sylvestris* from Bati-Bati, Tanah Laut with *A. paeoniifolius* var. *hortensis* from Marajai,

Table 4. Polymorphic sites of the *rbc*L sequences of *A. paeoniifolius* and two other species from the Meratus Mountain of South Kalimantan, Indonesia.

Name of Samples	Nucleotide Positions																							
	1	14	34	35	37	43	86	155	164	254	266	270	281	341	470	497	560	571	574	576	587	596	599	607
A. paeoniifolius var. *sylvestris* (lower Bajuin, Tanah Laut)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-
A. paeoniifolius var. *hortensis* (Piani, Tapin)	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	A	T
A. paeoniifolius var. *hortensis* (Pelaihari, Tanah Laut)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	A	A
A. paeoniifolius var. *hortensis* (Takisung, Tanah Laut)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	A	A
A. paeoniifolius var. *sylvestris* (Bati-Bati, Tanah Laut)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	G	T	-	-	-	-
A. paeoniifolius var. *sylvestris* (upper Bajuin, Tanah Laut)	-	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	A	A
A. paeoniifolius var. *sylvestris* (Takisung, Tanah Laut)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	A	A
A. paeoniifolius var. *hortensis* (Marajai, Balangan)	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	T	A	A
A. muelleri (upper Bajuin, Tanah Laut)*	-	.	.	.	.	.	G	.	.	.	T	G	G	C	C	A	T	.	.	.	.	T	A	A
A. muelleri (lower Bajuin, Tanah Laut)*	-	.	.	.	.	.	G	.	.	.	T	G	G	C	C	A	T	.	.	.	.	T	A	A
A. borneensis (Piani, Tapin)*	-	-	A	A	G	C	.	C	G	G	.	C	G	.	C	.	.	T	.	-	-	-	-	-
Consensus	T	A	G	T	T	T	T	T	A	A	C	T	A	T	T	G	C	G	A	G	C	A	T	-

Notes. * Outgroup; Yellow highlight = parsimony informative site; Green highlight = singleton sites.

Balangan, at a distance coefficient of 0.0053 (Table 6). At the inter-species level, *A. paeoniifolius* var. *sylvestris* from Takisung, Tanah Laut, was closely related to *A. muelleri* from lower Bajuin, Tanah Laut (0.0133). A far relative relationship (0.0207) showed by *A. paeoniifolius* var. *sylvestris* (Bati-Bati, Tanah Laut) with *A. muelleri* (lower Bajuin, Tanah Laut).

Table 5. Maximum likelihood estimates of substitution matrix of the *rbc*L sequences of *A. paeoniifolius* and two other species from the Meratus Mountain of South Kalimantan, Indonesia.

From\To	Intra-species				Inter-species			
	A	**T**	**C**	**G**	**A**	**T**	**C**	**G**
A	-	*8.33*	*8.33*	**8.33**	-	*5.31*	*5.31*	**14.38**
T	*8.33*	-	**8.33**	*8.33*	*5.31*	-	**14.38**	*5.31*
C	*8.33*	**8.33**	-	*8.33*	*5.31*	**14.38**	-	*5.31*
G	**8.33**	*8.33*	*8.33*	-	**14.38**	*5.31*	*5.31*	-

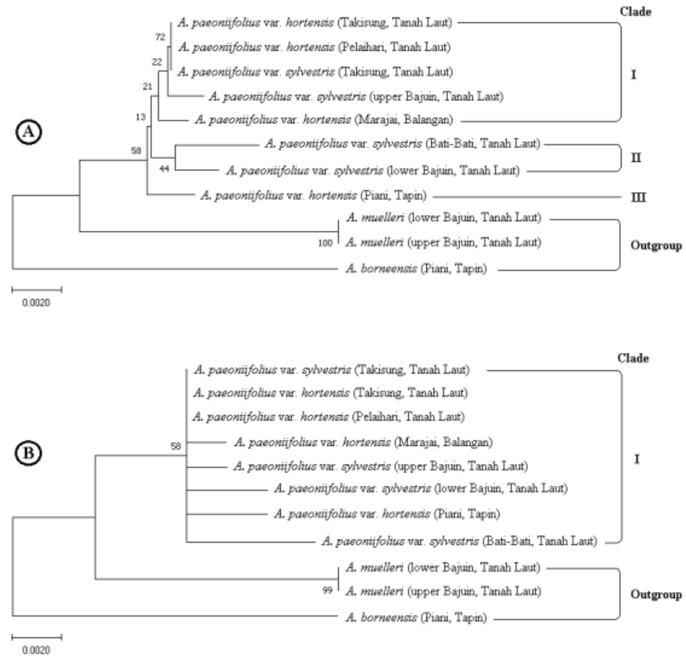

Figure 2. Phylogenetic relationship of elephant foot yam (*A. paeoniifolius*) and two other relatives (outgroup) from the Meratus Mountains of South Kalimantan, Indonesia, based on the NJ (A) and ML (B) methods.

Table 6. The genetic distance of *A. paeoniifolius* and two other species (outgroup) from the Meratus Mountains of South Kalimantan, Indonesia.

Code	1	2	3	4	5	6	7	8	9*	10*	11*
1											
2	0.0050										
3	0.0033	0.0033									
4	0.0033	0.0033	0.0000								
5	0.0052	0.0070	0.0052	0.0052							
6	0.0050	0.0033	0.0017	0.0017	0.0070						
7	0.0033	0.0033	0.0000	0.0000	0.0052	0.0017					
8	0.0050	0.0033	0.0017	0.0017	0.0053	0.0033	0.0017				
9*	0.0168	0.0151	0.0134	0.0134	0.0195	0.0151	0.0134	0.0151			
10*	0.0168	0.0151	0.0134	0.0134	0.0195	0.0151	0.0133	0.0151	0.0000		
11*	0.0206	0.0206	0.0206	0.0206	0.0207	0.0206	0.0206	0.0206	0.0263	0.0263	

Notes. * Outgroup; Green highlight = closely related or identic; Yellow highlight = distant related

1 = *A. paeoniifolius* var. *sylvestris* (lower Bajuin, Tanah Laut);
2 = *A. paeoniifolius* var. *hortensis* (Piani, Tapin);
3 = *A. paeoniifolius* var. *hortensis* (Pelaihari, Tanah Laut);
4 = *A. paeoniifolius* var. *hortensis* (Takisung, Tanah Laut);
5 = *A. paeoniifolius* var. *sylvestris* (Bati-Bati, Tanah Laut);
6 = *A. paeoniifolius* var. *sylvestris* (upper Bajuin, Tanah Laut);
7 = *A. paeoniifolius* var. *sylvestris* (Takisung, Tanah Laut);
8 = *A. paeoniifolius* var. *hortensis* (Marajai, Balangan);
9 = *A. muelleri* (upper Bajuin, Tanah Laut);
10 = *A. muelleri* (lower Bajuin, Tanah Laut);
11 = *A. borneensis* (Piani, Tapin).

Discussion

The *rbc*L is a functional gene in the chloroplast genome that is involved mainly in plant photosynthesis and encodes the ribulose-1, 5-bisphosphate carboxylase/oxygenase, or Rubisco (Liu et al. 2012). This gene is in the large single-copy region of the chloroplast genome and shows high homology among different plant germplasm (Dong et al. 2013). Singh and Banerjee (2018) reported that this gene has an intergenic spacer with 600-800 nucleotides. Following CBOL (2009), the *rbc*L gene includes approximately 1,400 nucleotides coding for the large subunit protein, and the length varies slightly among flowering plants (Angiosperm).

In this study, the *rbc*L sequences of *Amorphophallus* were recorded with different lengths, ranging between 542 and 607 bp (Table 3). Specifically, in *A. paeoniifolius* population, the *rbc*L length ranging between 574 and 605 bp. Compared to other studies, *A. paeoniifolius* has a different one, both in partial and complete sequences. For example, in *A. paeoniifolius*, Grob et al. (2002) and Gao et al. (2017) reported the total *rbc*L region of 1,479 bp and 1,391 bp, respectively. In *A. paeoniifolius* var. *campanulatus,* a partial *rbc*L sequence length of 636 bp (Dean et al. 2018). In *A. muelleri*, Grob et al. (2002) reported that this region is around 1,441 bp, while in *A. borneensis*, it is 1,453 bp (Sedayu et al. 2010).

Following Table 3, there are a different number of polymorphic sites (*S*), mutation rates (especially substitutions), and the number of transition/ transversion bias values (*R*) on the *Amorphophallus*, both at the intra- and inter -species levels. In general, the number of polymorphic sites, substitution-transition rates, and a transition/transversion bias value are relatively higher in the intra-species than the inter-species level, except for substitution-transversion rates (Table 3). However, at the inter-species level, the mutation rates of transitional substitutions are higher than transversion for each nucleotide probability. At the intra-species level, both substitutions (transition and transversion) are equal (Table 5).

According to Stoltzfus and Norris (2015), a transition/transversion bias is described for the difference ratio, which is the effect of a complex function of sequence divergence degree. Generally, transitions are more often found in most sequences than transversions (Aloqalaa et al. 2019). Hence, these phenomenon are common in molecular evolution (Stoltzfus & Norris 2015). However, this underlying phenomenon is not universal, as observed in grasshopper (*Podisma pedestris*) and two types of metazoans, namely *Drosophila* and the Mammalian (Keller et al. 2007).

Conceptually, mutation, both substitution and indel, tends to cause changes in the biochemical properties of protein products (Keller et al. 2007). According to Ripley (2013), the mutation is permanent changes inherited in the genes or nucleotide sequences (genome) of an organism, and it can affect a single nucleotide (point mutation) or some that are close to each other (segmental mutation). This phenomenon is closely related to nucleotide -based evolutionary changes or genetic diversity emerge (Nei 2007). Thus, this event is an initial step in establishing a primary population for natural selection and an integral part of evolution and genetic diversity (Govindaraj et al. 2015).

Regarding genetic diversity, *Amorphophallus* from this region shows a higher diversity in inter-species level (0.95%) than intra-species (0.33%) (Table 3). According to Bhandari et al. (2017), this is a normal phenomenon. The higher the taxonomical hierarchy is, the higher of genetic diversity among different communities of species occurs. However, this is the opposite for the lower taxonomical one. In this context, therefore, genetic diversity is referred to the diversity present within different genotypes of the same species. Bhandari et al. (2017) defined genetic diversity as the variation of heritable characteristics present in a population of the same species.

The phylogenetic analysis revealed that *Amorphophallus* from South Kalimantan formed different clades, three for NJ and one for ML. However, in this case, the *rbc*L region could not resolve this germplasm, particularly *A. paeoniifolius* (intra-specific level) well into two varieties, namely *sylvestris* and *hortensis*. Such cases are also reported by several researchers, e.g., Ude et al. (2019) in yam (*Dioscorea*), Dong et al. (2011) in *Pterygiella*, and Chandrasekara et al. (2021) in *Cinnamomum* accessions. According to Newmaster et al. (2006), this may be corresponding with the lower nucleotide substitution rates of this gene used. Hence, using others or combines two or more DNA barcoding markers are recommended to be conducted.

However, information on genetic diversity and its relationships is valuable in supporting the breeding and conservation programs in the future, particularly for parental selection and the development of novel superior cultivars (Acquaah 2017).

CONCLUSION

Based on the *rbc*L marker, *Amorphophallus* from the Meratus Mountain of South Kalimantan, Indonesia, has high diversity, particularly at an inter-species level and low at intra-species one. The phylogenetic analyses revealed that this germplasm is separated into three main clades, both for NJ and ML, where *A. paeoniifolius* var. *sylvestris* from Bati-Bati, Tanah Laut has closely related to *A. paeoniifolius* var. *hortensis* from Marajai, Balangan. This information may be used as a reference in supporting the conservation and breeding efforts of *Amorphophallus*, both locally and globally.

AUTHORS CONTRIBUTION

DHM and BZ conceived and designed the experiments; MAH did the fieldwork and performed the experiments; DHM and MAH analyzed the data and wrote the manuscript; DHM and BZ reviewed the manuscript internally.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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