

Phylogenetic positions of three *Amorphophallus* species natively growing in the Meratus Mountains, South Kalimantan, Indonesia

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Abstract. Mursyidin DH, Hernanda MA. 2021. Phylogenetic positions of three *Amorphophallus* species natively growing in the Meratus Mountains, South Kalimantan, Indonesia. *Biodiversitas* 22: 2821-2828. Information on genetic diversity and relationships (phylogenetic position) of germplasm is essential for conservation and breeding programs in the future. Here, we focused on determining the genetic diversity and phylogenetic positions of three native *Amorphophallus* species from the Meratus Mountains, South Kalimantan, Indonesia, using the *rbcL* marker. The results show that this germplasm has a medium level of genetic diversity (0.63). The phylogenetic analyses (NJ, ML, and MP) revealed that *Amorphophallus* from the region has a unique or specific relationship position. In this case, *A. muelleri* with an accession number of MT818204 and MT818205 are grouping and have close relatedness with *A. muelleri* previously deposited in GenBank (AF497087.1). Three samples of *A. paeoniifolius* (MT818202, MT818203, and MT818206) have also clustered with the same species (DQ012500.1; AF497091.1). An interesting result was shown by *A. borneensis* (MT818211) that demonstrated a close relationship with *A. tinekeae* (DQ012505.1) in NJ and ML analyses, not with a similar species (DQ012484.1) in MP analysis. Further, a bootstrap analysis on an earlier analysis supported the separation. Thus, this information is valuable in supporting the conservation and breeding programs of this germplasm, both locally and globally.

Keywords: Conservation, Genetic diversity, Phylogeny, Plant breeding, Wild species.

Abbreviations: NJ: Neighbor Joining; ML: Maximum Likelihood; MP: Maximum Parsimony.

INTRODUCTION

Amorphophallus, belonging to the Araceae family, is a large genus with over 200 species reported (Claudel et al. 2017). This genus is distributed mainly in Old World tropical forests, including Asia, Oceania, and Africa (Mekkerdchoo et al. 2016), from close to the coastal line to an altitude up to 900 m a.s.l. and adapts to low light intensities (Santosa et al. 2017). *Amorphophallus* exhibits a wide range of agro-ecological adaptation to dry and moist soils. It is abundant under trees shading home gardens, mixed gardens, secondary forests, and agroforestry, as well as open fields (Grob et al. 2002; Santosa et al. 2017). Because of its high abundance, around 70%, Southeast Asia estimated to be the center of diversity of this genus (Claudel et al. 2017).

For a long time ago, several *Amorphophallus* species have been used as a traditional medicine in several Asian countries, particularly in China and India (Dey et al. 2012, Gao et al. 2017a). Recently, this germplasm has attracted an economic interest, both for food resources and pharmaceutical products worldwide (Mekkerdchoo et al. 2016). *Amorphophallus paeoniifolius*, known as elephant foot yam or *A. campanulatus* (syn.), and *A. muelleri* are the two *Amorphophallus* examples with these economic values, particularly for commercial glucomannan and other substances, like ceramide (Mekkerdchoo et al. 2016, Zhong et al. 2018).

In general, *Amorphophallus* shows a high variation morphologically, so that it is very difficult to distinguish each other, especially at intrageneric level (Claudel et al. 2017; Gholave et al. 2017). Nowadays, various DNA markers can be useful to characterize germplasm with close genetic relationships (Terentieva et al. 2020). Of these, chloroplast DNA (cpDNA) or DNA barcode is an excellent marker to study the genetic diversity and relationships of plants because of its conserved gene order, maternal inheritance, and low mutation rate (Gao et al. 2017b). In this study, we have used the *rbcL* region of cpDNA to determine the genetic relationship or phylogenetic position of three wild *Amorphophallus* species from the Meratus Mountains of South Kalimantan, Indonesia.

Indeed, although *rbcL* provides low resolution compared with other cpDNA markers, particularly *matK*, it does offer several advantages, including its presence across the plant kingdom, unambiguous alignment, high primer universality, and high sequence quality (Dong et al. 2014). Furthermore, this barcode region is easy to amplify, sequence, and align in most terrestrial plants and provides a useful backbone to the barcode dataset, despite it having only modest discriminatory power (Hollingsworth et al. 2011). Finally, *rbcL* generates high-quality sequence output with easily retrievable across phylogenetically divergent lineages, and it performs well in discrimination tests in combination with other loci (CBOL 2009).

On the other hand, determination of phylogenetic relationships among wild *Amorphophallus* species is very urgent to undertaken, mainly for germplasm conservation, evaluation, and utilization in future breeding programs (Gao et al. 2017b). According to Gao et al. (2017a), wild relatives of domesticated plants provide important gene reservoirs for improving commercial cultivars. In recent years, handful of phylogenetic studies have been undertaken on some *Amorphophallus* species using chloroplast DNA (cpDNA) regions (Gao et al. 2017b; Sedayu et al. 2010). However, the phylogenetic studies of *Amorphophallus* species in Indonesia, particularly South Kalimantan, have not been well performed, except in Java (Nikmah et al. 2016, Wahyudi et al. 2016), Bali, and Lombok (Kurniawan et al. 2011).

Thus, the objectives of our study were to (i) estimate the genetic diversity of the *Amorphophallus* native to the Meratus Mountains, South Kalimantan, Indonesia by *rbcL* cpDNA marker, (ii) determine the genetic relationships or phylogenetic position of this germplasm from the region with other *Amorphophallus* species included in GenBank database, and (iii) provide some recommendations on the conservation and utilization of this germplasm for local, regional, and national government, particularly in Indonesia.

MATERIALS AND METHODS

Plant materials

We have used a total of 80 samples of *Amorphophallus*, comprises six samples (including three species, namely *A. paeoniifolius*, *A. muelleri*, and *A. borneensis*) collected directly from the Meratus Mountains, South Kalimantan, Indonesia (Table 1), and 74 other species, obtained randomly from GenBank database (Table 2).

DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from collected leaf samples using the plant genomic DNA extraction kit (Geneaid Biotech Ltd., Taiwan) following a manufacturer's protocol. The DNAs were quantified using a UV-VIS spectrophotometer (NanoVue, GE Healthcare, UK) and standardized on 1.0% (w/v) agarose gels. The *rbcL* gene was amplified and sequenced with primers *rbcL*-F (5'-ATGTCACCACAAACAGAGACTAAAGC-3') and *rbcL*-R (5'-GTAAAATCAAGT CCACCRCG-3') from Gholave et al. (2017). Amplifications were conducted using the SimpliAMP Thermocycler PCR (Applied Biosystem, USA). A 25 μ L reaction mix, composed of 22 μ L PCR mix (MyTaq HS Red Mix, Biorline, UK), 2.0 μ L (10 μ M) primer forward and reverse, and 1 μ L (10 \times diluted containing ca. 10 ng DNA) template. The amplified DNA fragments were then separated by 2% agarose gel with a 1X TBE buffer solution, stained with a DNA dye (FluoroVue, SMOBiO Technology, Taiwan), and observed by UV transilluminator. The successful amplified DNA was then sent to 1st Base Ltd., Malaysia, for purification and sequencing bidirectionally using the Sanger method.

All sequences were deposited in GenBank with accession numbers shown in Table 1.

Genetic diversity and phylogenetic analyses

Sequences were analyzed, edited and assembled using the MEGA-X software (Kumar et al. 2018). The genetic diversity was determined using the nucleotide diversity index (π) method (Nei and Li 1979). Meanwhile, the level of genetic diversity was referred to Nei (1987) category, where 0.1 to 0.4 is low, 0.5 to 0.7 is the medium, and 0.8-2.00 is high. For phylogenetic analysis, multiple sequence alignments of sequences were performed with Clustal Omega (Sievers & Higgins 2018) and edited manually using a similar software used previously to obtain an unambiguous sequence alignment. Interspecific genetic divergences were calculated using Kimura 2-Parameter (K2) distances in MEGA-X following the instruction of the CBOL for distance calculations (Kumar et al. 2018). Three phylogenetic analyses, namely Maximum Likelihood (ML), Maximum Parsimony (MP), and Neighbor-Joining (NJ) were also carried out in MEGA-X. The bootstrap analysis with 1000 replicates was applied for evaluated the topological robustness of the phylogram (Lemey et al. 2009).

RESULTS AND DISCUSSION

Results

Amorphophallus has a different length of *rbcL* sequences, recorded between 543-1491 bp (Table 3). In this case, the shortest nucleotide (543 bp) is shown by *A. borneensis* from the Meratus Mountains of South Kalimantan, Indonesia, whereas the longest by *A. henryi* (1491 bp). Following Table 3, the *rbcL* sequence of *Amorphophallus* has many polymorphic sites (126 loci) and a level of nucleotide diversity (0.63). Furthermore, this sequence also has several genetic information, such as BIC (8904.75), AIC (7386.04), and lnL values (-3534.79), including Transition/Transversion bias value (2.06) and GC content (42.91%). Table 4 presented several polymorphic sites (nucleotide position), specifically on *Amorphophallus* from the South Kalimantan, Indonesia. Following Table 4, there are 17 loci of polymorphic site, where substitution (particularly transversion) is the most case, and a remaining (smallest part) is deletion.

Phylogenetic analysis using three approaches (NJ, ML, and MP) revealed specific positions of *Amorphophallus* from South Kalimantan (Figures 1-3, red highlight). In general, the three analyses generated a relatively similar phylogenetic tree, where *Amorphophallus* from the region are grouped consistently within other germplasms deposited in GenBank. For example, two samples of *Amorphophallus* species, namely *A. muelleri* from South Kalimantan (MT818204 & MT818205), are grouping and close relatedness with previous *A. muelleri* at GenBank (AF497087.1). Similarly, three samples of *A. paeoniifolius* (MT818202, MT818203, and MT818206) clustered with the same species (DQ012500.1; AF497091.1), also with others, such as *A. pranii*, *A. koratensis*, *A. scaber*, and *A. opertus* (Figures 1-3).

Table 1. List of *Amorphophallus* samples collected from the Meratus Mountains, South Kalimantan, Indonesia.

Species	Origin	Nucleotide length (bp)	Accession number
<i>A. paeoniifolius</i>	Lower Bajuin, Tanah Laut, South Kalimantan	606	MT818202
<i>A. paeoniifolius</i>	Upper Bajuin, Tanah Laut, South Kalimantan	606	MT818203
<i>A. paeoniifolius</i>	Piani, Tapin, South Kalimantan	606	MT818204
<i>A. muelleri</i>	Lower Bajuin, Tanah Laut, South Kalimantan	606	MT818205
<i>A. muelleri</i>	Upper Bajuin, Tanah Laut, South Kalimantan	606	MT818206
<i>A. borneensis</i>	Piani, Tapin, South Kalimantan	543	MT818211

Table 2. List of *Amorphophallus* obtained from GenBank database and its length sequences of the *rbcL*

Species	Nucleotide length (bp)	Accession number
<i>A. abyssinicus</i>	1454	AF497060.1
<i>A. amygdaloides</i>	1433	DQ012482.1
<i>A. angolensis</i>	1470	AF497061.1
<i>A. ankarana</i>	1455	AF497062.1
<i>A. baumannii</i>	1482	AF497063.1
<i>A. beccarii</i>	1488	AF497064.1
<i>A. borneensis</i>	1453	DQ012484.1
<i>A. brevispathus</i>	1458	AF497065.1
<i>A. bufo</i>	1381	KT794021.1
<i>A. canaliculatus</i>	1479	AF497066.1
<i>A. cirrifer</i>	1484	AF497067.1
<i>A. coaetaneus</i>	1450	AF497068.1
<i>A. commutatus</i>	1460	AF497069.1
<i>A. corrugatus</i>	1459	AF497070.1
<i>A. dactylifer</i>	1456	DQ012485.1
<i>A. declinatus</i>	1448	DQ012486.1
<i>A. decus-silvae</i>	1454	AF497071.1
<i>A. dracontiodes</i>	1460	AF497072.1
<i>A. eburneus</i>	1459	AF497073.1
<i>A. eichleri</i>	1460	AF497074.1
<i>A. galbra</i>	1458	AF497075.1
<i>A. glossophyllus</i>	1459	DQ012489.1
<i>A. henryi</i>	1491	AF497076.1
<i>A. hewitii</i>	1432	DQ012490.1
<i>A. hirsutus</i>	1482	AF497077.1
<i>A. hirtus</i>	1458	AF497078.1
<i>A. hohenackeri</i>	1430	DQ012491.1
<i>A. hottae</i>	1391	AM905785.1
<i>A. johnsonii</i>	1459	DQ012494.1
<i>A. konjac</i>	1460	AF497080.1
<i>A. konkanensis</i>	1454	DQ012495.1
<i>A. koratensis</i>	1363	KT794052.1
<i>A. krausei</i>	1478	AF497081.1
<i>A. lambii</i>	1473	AF497082.1
<i>A. lanuginosus</i>	1451	DQ012496.1
<i>A. laoticus</i>	1458	DQ012497.1
<i>A. lewallei</i>	1486	AF497083.1
<i>A. longiconnectivus</i>	1454	DQ012498.1
<i>A. longituberosus</i>	1466	AF497084.1
<i>A. margaritifera</i>	1480	AF497085.1
<i>A. maxwellii</i>	1458	AF497086.1
<i>A. mossambicensis</i>	1448	DQ012499.1
<i>A. muelleri</i>	1441	AF497087.1
<i>A. napalensis</i>	1460	AF497088.1
<i>A. napiger</i>	1488	AF497089.1
<i>A. obscurus</i>	1364	KT794063.2
<i>A. ochroleucus</i>	1472	AF497090.1
<i>A. opertus</i>	1381	KT794066.1
<i>A. paeoniifolius</i>	1479	AF497091.1
<i>A. paeoniifolius var. bangkokensis</i>	1439	DQ012500.1
<i>A. palawanensis</i>	1455	AF497092.1
<i>A. pendulus</i>	1459	DQ012501.1
<i>A. pingbianensis</i>	1476	AF497093.1
<i>A. pranii</i>	1381	KT794071.1
<i>A. pusillus</i>	1448	AF497094.1
<i>A. pygmaeus</i>	1460	AF497095.1
<i>A. rhizomatosus</i>	1482	AF497096.1
<i>A. rivieri</i>	1347	AJ005630.1
<i>A. rostratus</i>	1381	KT794077.1
<i>A. sagittarius</i>	1463	AF497097.1
<i>A. salmoneus</i>	1434	DQ012502.1
<i>A. scaber</i>	1381	KT794080.1
<i>A. scutatus</i>	1456	DQ012503.1
<i>A. serrulatus</i>	1381	KT794082.1
<i>A. smithsonianus</i>	1482	AF497098.1
<i>A. sumawongii</i>	1487	AF497099.1
<i>A. symonianus</i>	1472	AF497100.1
<i>A. terrestris</i>	1344	KT794091.1
<i>A. thaiensis</i>	1448	DQ012504.1
<i>A. tinekeae</i>	1427	DQ012505.1
<i>A. titanum</i>	1466	AF497102.1
<i>A. variabilis</i>	1476	AF497103.1
<i>A. yunnanensis</i>	1448	AF497104.1
<i>A. zenkeri</i>	1460	AF497105.1

Table 3. Genetic information for the *rbcL* region of *Amorphophallus* germplasm used in the study¹.

Parameter	<i>rbcL</i>
Range of sequence length (bp)	543-1491
Number of polymorphic sites (<i>S</i>)	126
Bayesian Information Criterion (BIC)	8904.75
Akaike Information Criterion (AICc)	7386.04
Maximum Likelihood Value (lnL)	-3534.79
Transition/Transversion (Ti/Tv) bias value (<i>R</i>)	2.06
GC content (%)	42.91
Nucleotide diversity (π %)	0.63

Note: ¹Based on Kimura 2-parameter model.

Table 4. Polymorphic information for the *rbcL* region of *Amorphophallus* from South Kalimantan, Indonesia.

Nucleotide position	Consensus	Species/GenBank accession number					
		<i>A. paeoniifolius</i> var. <i>Sylvestris</i> (MT818202)	<i>A. paeoniifolius</i> var. <i>sylvestris</i> (MT818203)	<i>A. muelleri</i> (MT818204)	<i>A. muelleri</i> (MT818205)	<i>A. paeoniifolius</i> var. <i>hortensis</i> (MT818206)	<i>A. borneensis</i> (MT818211)
43 ^a	T	C
86 ^a	T	.	.	G	G	.	.
155 ^a	T	C
164 ^a	A	G
191 ^a	C	T	T	T	T	T	T
254 ^a	A	G
266 ^a	C	.	.	T	T	.	.
270 ^{ab}	G	T	T	.	.	T	C
281 ^a	G	A	A	.	.	A	.
341 ^a	T	.	.	C	C	.	.
470 ^a	C	T	T	.	.	T	.
497 ^a	G	.	.	A	A	.	.
560 ^a	C	.	.	T	T	.	.
571 ^a	G	T
596 ^{bc}	T	.	A	.	.	.	-
598 ^{ac}	A	G	G	G	G	.	-
605 ^{ac}	C	A	A	A	A	A	-

Note: a: transversion, b: transition, c: deletion

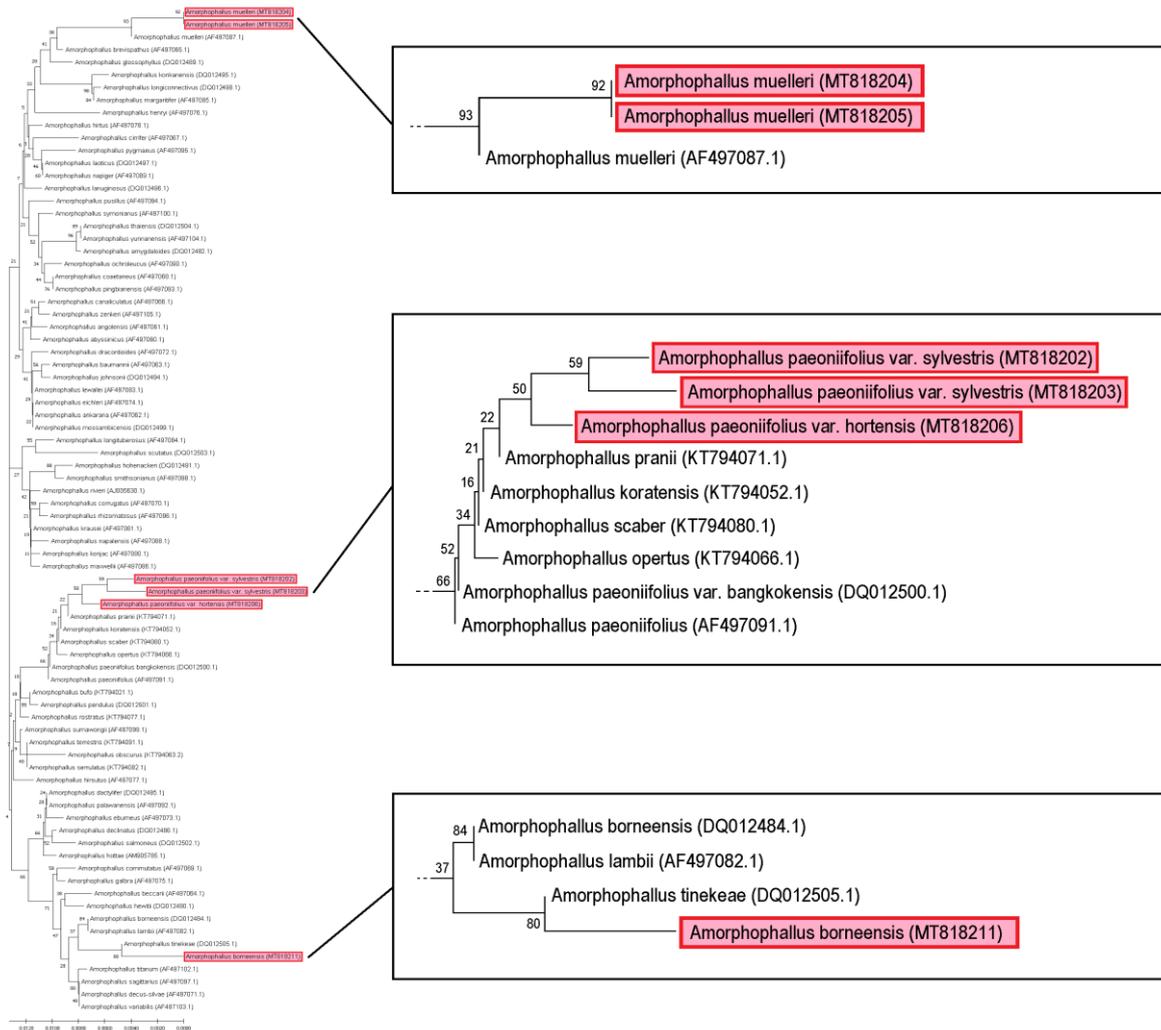


Figure 1. Phylogenetic position of *Amorphophallus* native to the Meratus Mountains of South Kalimantan, Indonesia (red highlight) based on Neighbor-Joining (NJ). The numbers above branches indicate bootstrap values inferred from 1000 replicates

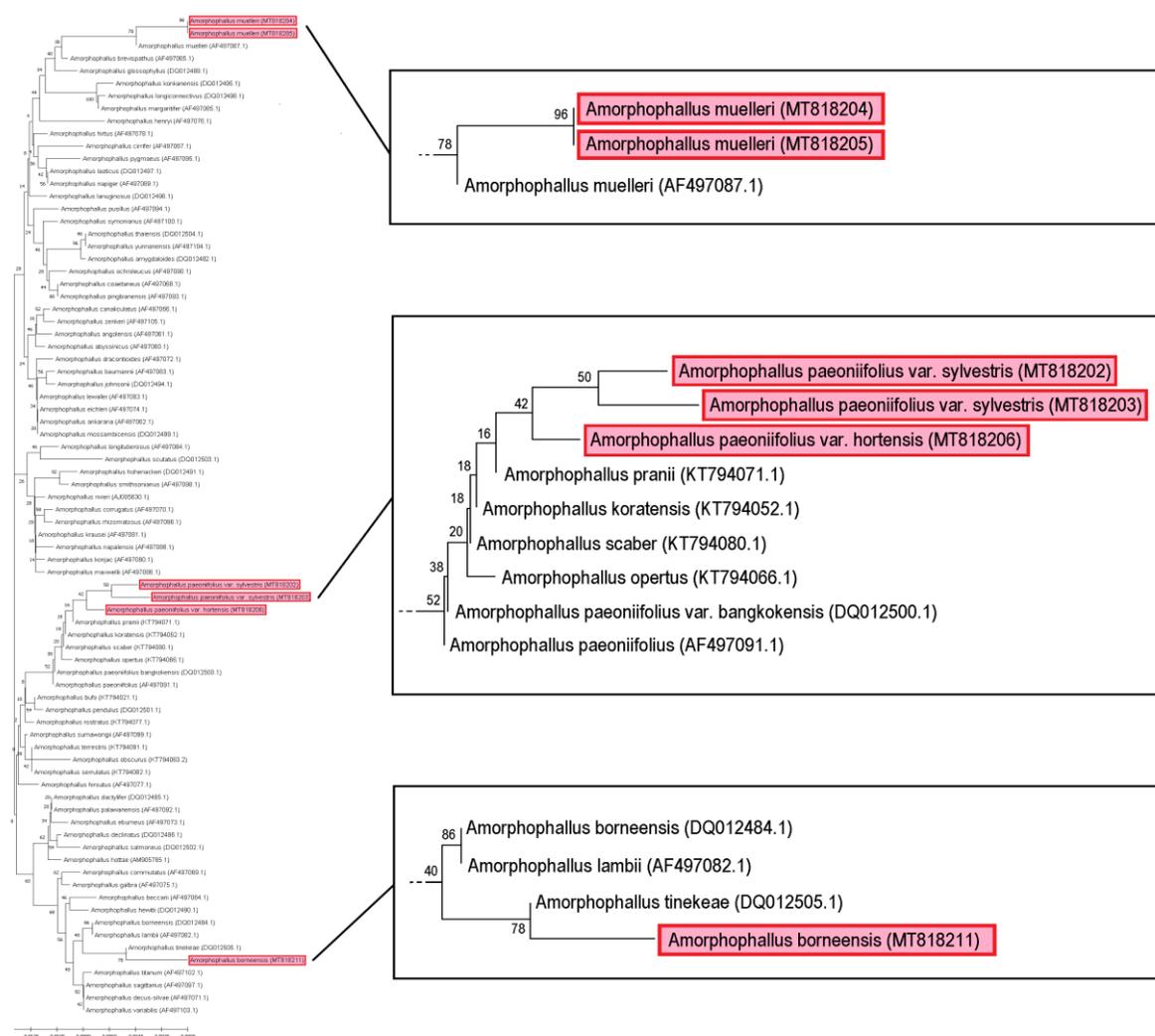


Figure 2. Phylogenetic position of *Amorphophallus* native to the Meratus Mountains of South Kalimantan, Indonesia (red highlight) based on Maximum Likelihood (ML). The numbers above branches indicate bootstrap values inferred from 1000 replicates

Interestingly, *A. borneensis* (MT818211), in this case, although it has a close relationship with *A. tinekeae* (DQ012505.1) in NJ and ML analyses, MP have generated and joined this species with a similar *A. Borneensis* (DQ012484.1). However, a bootstrap analysis showed the lowest value (only 1%) for the last approach (Figure 3), so it is not significant. Moreover, phylogenetic trees generated by NJ and ML have a bootstrap significance of 80% and 78%, respectively (Figure 1 and 2).

Discussion

In this study, *Amorphophallus* has a moderate level of diversity, $p = 0.63$ (Table 3). According to Govindaraj et al. (2015), genetic diversity is one factor in forming a baseline population for natural selection and the evolutionary process. In other words, it has a fundamental role in the future evolutionary trajectory or a prerequisite for future adaptive change of a species. Hence, genetic diversity has profound implications for species conservation and breeding (Lloyd et al. 2016). In conservation practices,

understanding genetic diversity is essential in increasing effectiveness and efficiency, especially for rare species. Because some aspects of conservation biology, such as loss of genetic diversity, only addressed by detailed population genetic studies (Luan et al. 2006).

For plant breeding, genetic diversity becomes more urgent in the context of climate change (Govindaraj et al. 2015). Plant breeders utilize this aspect in plant genetic resources to develop new and improved cultivars with desirable traits, both associated with different biotic and abiotic stress tolerance and farmer-preferred (Swarup et al. 2021). It is also fundamental in generating many essential agricultural phenomena, like heterosis and transgressive segregation. Diverse lines are required for defect correction of commercial varieties and the development of new ones. Thus, identification of various cultivars (if available), creation of diversity (if not exist or limited), and their subsequent utilization are the main objectives of the crop improvement program (Bhandari et al. 2017).

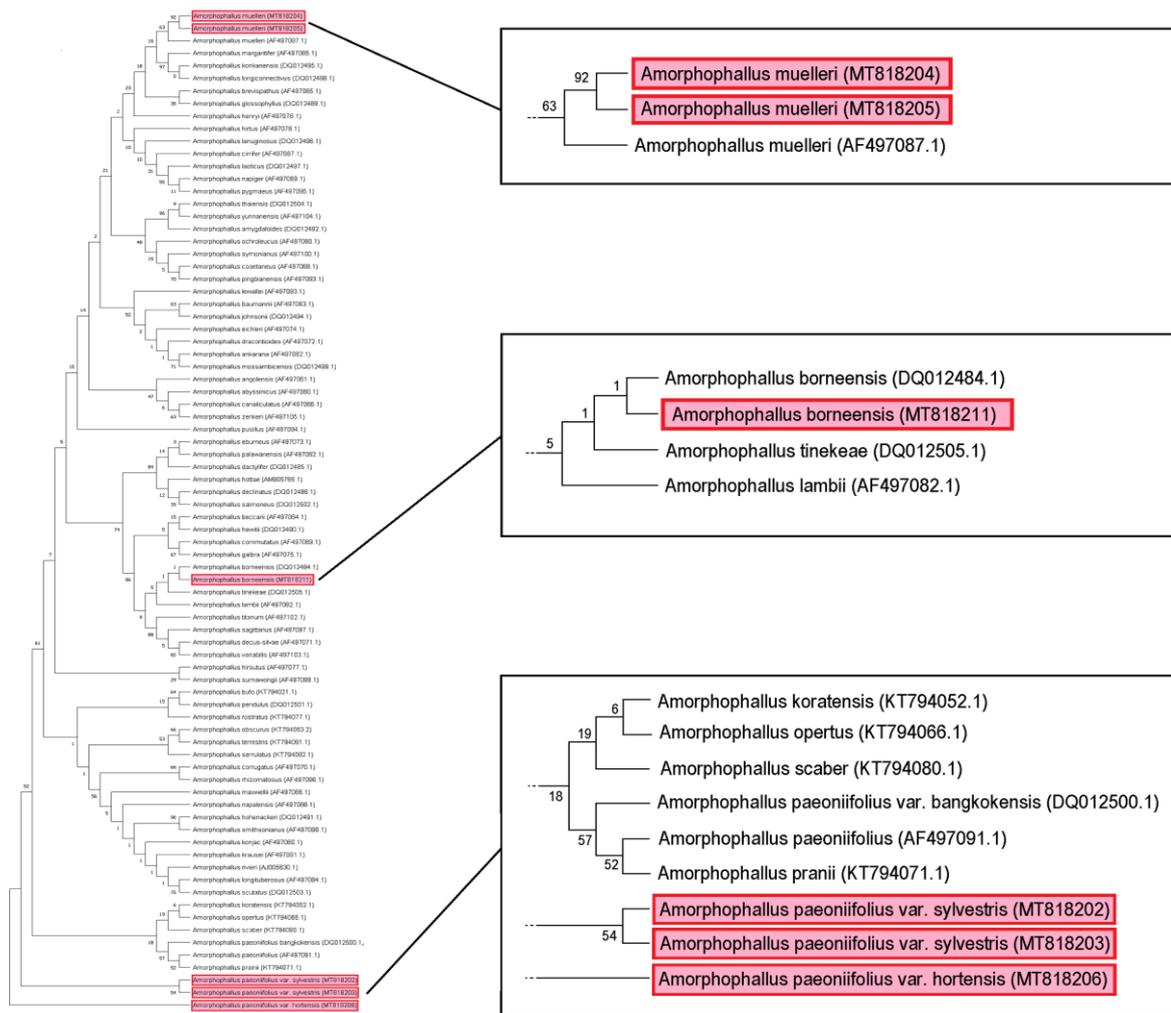


Figure 3. Phylogenetic position of *Amorphophallus* native to the Meratus Mountains of South Kalimantan, Indonesia (red highlight) based on Maximum Parsimony (MP). The numbers above branches indicate bootstrap values inferred from 1000 replicates

Compared to other studies with similar germplasm, there are differences in the value of genetic diversity. *Amorphophallus* in the study has a higher genetic diversity than reported by, for example, Gao et al. (2017a) in wild *A. paeoniifolius* population from Western China ($p = 0.23-0.36$) but lower than similar germplasm in Asia with $p = 0.80$ (Santoso et al. 2017). Referred to Gao et al. (2017a), these differences might be due to the continuous selection had reduced effective population size and increased genetic drift and hitchhiking during domestication. During this process, inbreeding and intensive selection, which narrow the germplasm genetic base, tend to reduce the genetic diversity (Gao et al. 2017a). Further, reduced genetic diversity has been shown to decrease disease resistance and resilience to environmental disturbance and extreme conditions (Lloyd et al. 2016).

According to Lloyd et al. (2016), only as present-day populations need a high genetic diversity to rapidly adapt, future generations will need equally as much genetic diversity to adapt to future changes. Although the genetic diversity is currently high, the rapid rate of evolutionary

change could outpace the rate of adaptation of this species. Consequently, future studies are necessary to understand how the loss of genetic diversity will impact the ability of future generations to continue to cope with environmental change in the population (Lloyd et al. 2016). It is because the low level of diversity may result from a founder effect, genetic isolation, population decline, or natural selection (Gao et al. 2017b).

Molecularly, the level of genetic diversity is affected by polymorphic or mutation events. In this case, the *rbcl* sequence of this germplasm has generated many polymorphic sites (126 loci) with a Transition /Transversion (Ti/Tv) bias value is recorded of 2.06 (Table 3). It means that transition is dominant to transversion and other mutations. Some researchers claim that the transition is more frequently encounter in sequences than transversions, as it can provide easy tolerance from selection pressure (Aloqalaa et al. 2019). In other words, a pattern in which nucleotide transitions are favored several times over transversions is commonly in molecular evolution (Stoltzfus and Norris 2015). It is due to the

biased mutational processes within plant genomes, e.g., cytosine deamination (Lewis et al. 2016). In *Amorphophallus* and others, this case might be a loss of evolutionary potential (Gao et al. 2017a).

Information on the phylogenetic relationship is also urgent and has implications for species conservation and breeding (Flint-García 2013). In this study, *Amorphophallus* revealed complex genetic relationships (Figure 1-3). Specifically, for native germplasm from South Kalimantan, Indonesia, is grouping with similar species in GenBank (Figure 1-3, red highlight). According to Fernández-García (2017), phylogenetic studies can apply in inferring species and their evolutionary history, including helping analyze species delimitation, gene flow, and genetic differentiation. Furthermore, the use of phylogenetic relationship is of current interest given its objective metrics for conservation in the past evolution history, the present genetic status of species, and management for future ones (Fernández-García 2017).

In-plant breeding program, information on this relationship can use in predicting the genetic diversity of the offspring when individuals cross (Acquaah 2012). Conceptually, crossing individuals with distant relationships, the offspring may have high genetic diversity. Conversely, if the individual is a closely related cross, then the genetic is low (Acquaah 2012, Turner-Hissong et al. 2020). Hence, crossing the parent with a close relationship tends to avoid, due to the inbreeding, except in developing a new homozygous or pure-line hybrid (de Los Reyes 2019).

In conclusion, while *Amorphophallus* has a moderate level of genetic diversity, this germplasm revealed complex genetic relationships. Thus, this information is valuable in supporting the conservation and breeding programs of this germplasm, both locally and globally.

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