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Genetic diversity of *Amorphophallus titanum* in Bengkulu, Indonesia based on RAPD markers

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Abstract. *Arianto W, Zuhud EAM, Hikmat A, Sunarminto T, Siregar IZ. 2018.* Genetic diversity of *Amorphophallus titanum* in Bengkulu, Indonesia based on RAPD markers. *Biodiversitas 19: 1783-1790.* Titan Arum [*Amorphophallus titanum* (Becc.) Becc. Ex Arcang], a plant species belonging to the family of Araceae is known for its gigantic floral size and elicited rotten fragrance when the flower bloom. Since it remains only found in Sumatran island, many authors categorized the plant as endemic species. The population of the species in the natural habitat has significantly declined because of the conversion of forest land mainly into plantations or other land uses. Considering the importance of conservation attempts to *A. titanum*, a sufficient data on genetic diversity of the species is necessary. The research was aimed to determine the genetic diversity within and among populations of *A. titanum* in some area of protected forests in Bengkulu Province, comprising the population of Palak Siring, Tebat Monok, and Air Selimang. RAPD genetic DNA fingerprinting approach was used to assess the genetic diversity of *A. titanum* using 13 preselected DNA primer: OPA 11, OPA 19, OPC 04, OPN 14, OPN 19, OPU 03, OPU 06, OPU 07, OPB 17, OPC 07, OPO 04, OPU03-1, OPNI 18E. The result revealed that the method has successfully produced several DNA fragments with varied length ranging from 250 bp to 2000 bp with 4-16 variation in polymorphic bands. Based on RAPD marker analysis, the population of Air Selimang was considered as a potential center of diversity of *A. titanum* because of the others two populations had a lower genetic diversity. In general, the genetic diversity among populations was lower than within population. The cluster analysis of the genetic similarity of 22 individuals of the three populations resulted in the separation into two main groups with the first group consisting of 17 individuals (Population Air Selimang and Tebat Monok) and the second group of 5 individuals (Palak Siring population).

Keywords: *Amorphophallus titanum*, genetic diversity, Random Amplified Polymorphic DNA

INTRODUCTION

Bunga bangkai, the local name of Titan Arum (*Amorphophallus titanum* (Becc.) Becc. Ex Arcang) is an important plant species belonging to the family of Araceae. The plant is known by its gigantic floral morphology characterized by a large sheathing bract, a spathe, wrapped the basal portion of the spadix (a racemose inflorescence having a lot of small flowers seating in a fleshy stem axis). The spadix reaches its vertical axis up to 1,6 m - 3 m the reason why it is considered as the plant with the tallest flower in the world (Barthlott and Lobin 1998; Arianto et al. 1999; Giardano 1999). Since 138 years after first discovered, the plant has significantly attracted many researchers in greenhouses or botanical gardens almost all over the world to study many aspects of the plant biology. The studies include plant morphology and anatomy, vegetative (spathe) and generative (spadix) growth and development (Gandawijaja et al. 1983; Barthlott and Lobin 1998; Hejnowicz and Barthlott 2005; Sholihin and Purwantoro 2005; Lobin et al. 2007; Claudel et al. 2012; Purwanto and Latifah 2013), thermogenesis (Barthlott 2009), floral odor analysis (Fujioka at al. 2012), germination (Latifah and Purwantoro 2015), micro-propagation (Irawati 2011), and estimation of genetic diversity in some populations (Poerban and Yuzammi 2008). The Plant have become symbols or flag species in many botanical gardens around the world in an attempt to attract as many visitors to the botanical gardens (Latifah and Purwantoro 2015).

Naturally, *A. titanum* is widespread over the Sumatra rainforest as understory growth in the calcareous soil below the forest canopy. However, the plants also occasionally found in open area, secondary forest, river bank, and in the edge of the road (Hidayat and Yuzammi 2008). Since it remains only found in Sumatran island, many authors categorized the plant as an endemic species (Barthlott and Lobin 1998; Hidayat and Yuzammi 2008). *A. titanum* has three successive phases, i.e., vegetative, dormant, and generative phase. The vegetative phase is an active green photosynthetic stage indicated by the emergence of a single leaf that grows for 6-12 months initiated in early raining season. The vegetative phase has responsibility for producing photosynthate and stored the sugar for

developing tuber. The underground tuber can reach 100 kg in weight. Following the detachment of the leaf, the dormant phase is beginning, and it is entirely underground tuber for 1-4 years before flowering. The generative phase or flower emergence is accidental and cannot be predicted (Bown 1988; Hetterscheid and Ittenbach 1996; Graham and Hadiah 2004).

Indonesia government designated *A. titanum* as a protected species according to Government Regulation No. 7/1999 (Appendix PP No. 7/1999) and Regulation of Ministry of Environment and Forestry Number 20/ MENLHK/SETJEN/KUM.1/6/2018 concerning in protected species of plants and animals. Based on the 1997 IUCN Red List of Threatened plants, *A. titanum* is classified into Vulnerable (VU). However, in 2002 this species was excluded from the IUCN list because of the lack of available data on population and its presence in nature.

Previous surveys indicated that there was a tendency that the population of *A. titanum* plants has become diminished. The conversion of natural forest for other land used has considered as significant contributor threatening their existence. (Hidayat and Yuzammi 2008). Therefore, if land use changes continue, it will threaten the species existence in nature. Real conservation effort is needed to protect the species in their natural habitat.

Genetic diversity is one aspect of biological diversity that is important for the conservation program (Dyke 2003). Conservation activities require sufficient information of the status of genetic diversity of target species (Heywood and Dullo 2005). Research into genetic diversity of *Amorphophallus* genera has been partially carried out, including *A. paenofiifolius* (Sugiyama et al. 2006), *A. muelleri* (Poerba and Martanti 2008), *A. rivieri* (Hu et al. 2011), *A. variabilis* (Santosa et al. 2012), *A. muelleri* (Wahyudi et al.2013), 35 species of *Amorphophallus* from China and Thailand (Mekkerdchoo et al. 2016), *A. paenofiifolius* (Mandal et al.2016), *A. paenofiifolius* (Santosa et al. 2017).

Research on genetic diversity of *A. titanum* is still relatively limited. A previous report on the genetic diversity of *A. titanum* was published by Poerba and Yuzammi (2008) using 22 accessions of *A. titanum* from West Sumatra and Bengkulu. The study only examines the RAPD profile and genetic dissimilarity analysis, but it was still lacking in discussing genetic diversity measures such as diversities within the population and among populations, genetic distances, and genetic population structures.

One approach that is still being used to determine the genetic diversity of *A. titanum* is Random Amplified Polymorphic DNA (RAPD) markers. The RAPD technique is cost-effective, easy and quick to assay, produces polymorphisms of DNA bands in large quantities, requires no knowledge of the genomic background being analyzed and is easy to obtain the random primers needed to analyze the genomes of all organism types (Tingey et al. 1994; Beebee and Rowe 2008). Although this method has many drawbacks, especially the consistency of its product amplification (Jones et al. 1997), optimizing extraction, well-prepared PCR conditions, and appropriate primer selection would overcome this limitation. The recent research was aimed to determine the genetic diversity of *A. titanum* using a genetic marker of Random Amplified Polymorphic DNA (RAPD).

MATERIALS AND METHODS

Study area

The sampling sites situated on three populations of the plants found in protected forest area in Bengkulu Province, Indonesia consisting of Air Selimang population and Tebat Monok population in Kepahiang District, as well as Palak Siring population in North Bengkulu District as shown in Figure 1. The number of individuals, geographical location, and altitude of the *A. titanum* population were shown in Table 1.

Procedures

Collection of leaflets samples of *A. titanum* was conducted in the 3 populations, namely population of Air Selimang (13 individuals), population of Tebat Monok (4 individuals) and population of Kepala Siring (5 individuals). From each plant, we took 2-3 leaflets, then the leaflets were cut in 2 cm x 2cm and then put in plastic clip bag with silica gel with a volume ratio of 1: 5 (Santoso et al. 2003).

Extraction of DNA

Genomic DNA was extracted using modified CTAB (Cetyl Trimethyl Ammonium Bromide) method referring to Weising et al. (2005) and Aritonang et al. (2007).

Test of DNA quality

The DNA quality test was initiated by preparing agarose 1% (0.33 g agarose in 33 mL buffer TAE) being diluted in microwave for 3 minutes. Afterward, GelRed was added as much as 0.5 µL and decanted into gel mold until being viscous (± 10 minutes). In the electrophoresis process, DNA was taken as much as 3 µL. Afterward, 1 µL blue juice was added, mixed and put into gel well. Electrophoresis ran for 20 minutes. After electrophoresis finished, the gel was lifted and the DNA bands were documented under UV transilluminator TPX - 20. LM.

Polymerase Chain Reaction (PCR)

Extraction results DNA were amplified using machine AB Applied Biosystem Veriti TM Thermal Cycler (www.aapliedbiosystem.com). As many as 13 primers (Table 2) from Operon Technology Ltd being used were OPA-11, OPA-19,OPC-04,OPN-14, OPN-19,OPU-03, OPU-06, OPU-07,OPB-17,OPC-07, OPO-04, OPU03-1,OPNI-18E with annealing temperature of 36℃-38℃.

Data analysis

The interpretation of the RAPD profiles used a binary variable based on the presence or absence of amplification products. The value is one if the band present and zero if it absent. The binary data were analyzed using POPGENE 32 version 1.31 software (Yeh and Yang 1999). Ntedits

version 1.07c (Jamshidi and Jamshidi 2011) and NTSys version 2.0 (Rohlf 1997) and Structure version 2.3.4 (Pritchard et al. 2010). The inter and intrapopulation diversity of genetic distance data generated from POPGENE is used for Clustering analysis with Unweighted Pair method. The Group Method with Arithmetic Mean (UPGMA) uses NTsys version 2.0 which will produce a dendrogram. Population structure analysis using STRUCTURE version 2.3.4 software (Pritchard et al. 2010).

Table 1. Number of sample *Amorphophallus titanum*, geographic position, and altitude of the growing site

Population sites	No. of samples	Geographic coordinate		Altitude (m asl.)
		Latitude	Longitude	
Palak Siring	5	3º25'14.05	102º15'48.51	374-406
Tebat Monok	4	3º40'16,98	102º33'26.27	655-661
Air Selimang	13	3º45'43,51	102º37'11,58	773-804

Note: asl: above sea level

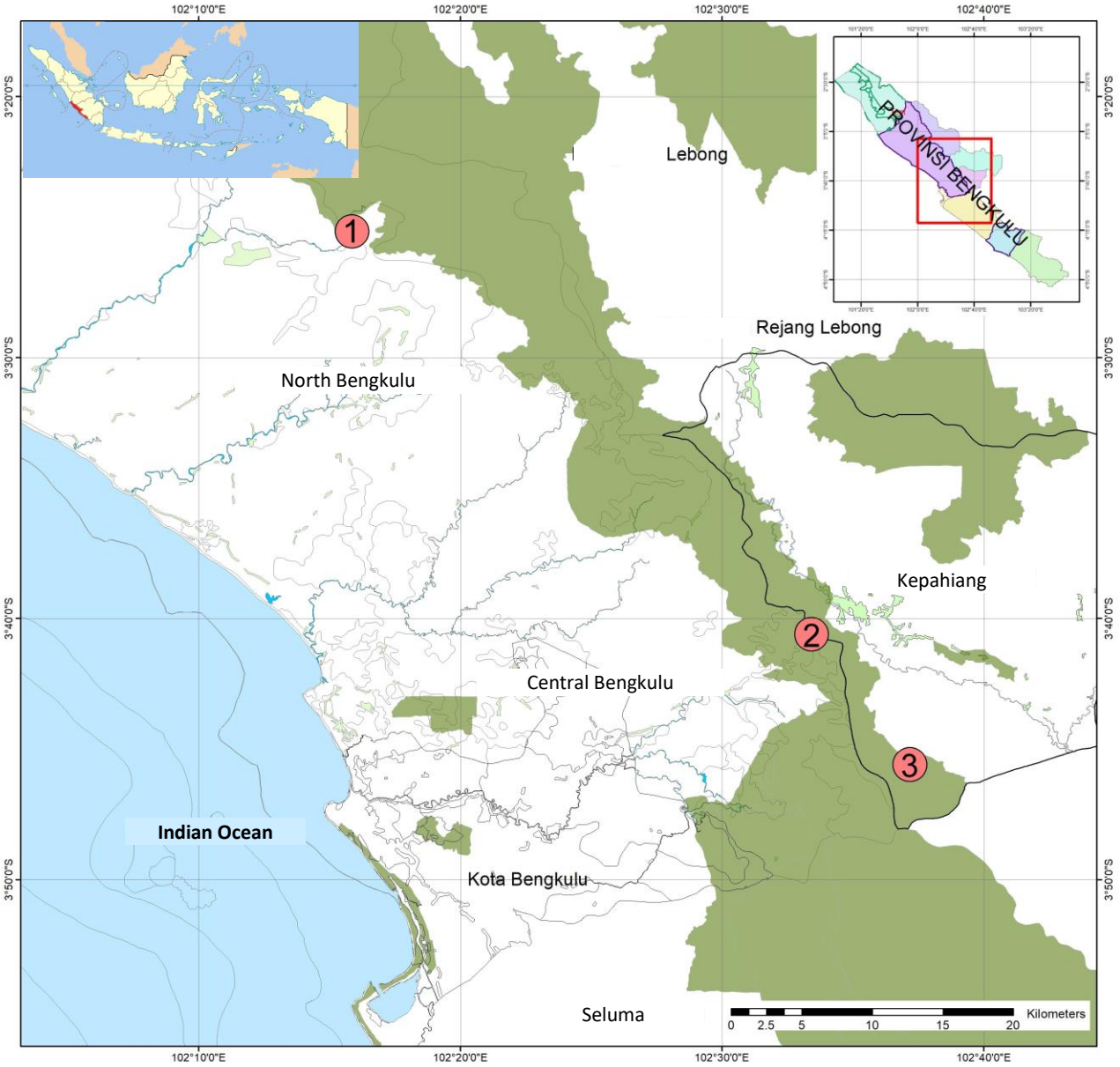

Figure 1. The selected sampling sites of *Amorphophallus titanum* in protected forest area in Bengkulu Province, Indonesia. 1. Palak Siring, 2. Tebat Monok, 3. Air Selimang

RESULTS AND DISCUSSIONS

RAPD profile

Amplification of total DNA genome using 13 RAPD primers in 22 *A. titanum* samples produced clear and reproducible PCR products as presented in Figure 2.

The result revealed that there were 124 DNA fragments with length ranging from 250 bp (base pair) up to 2000 bp with 75-100% polymorphic DNA (Table 3). The appropriate temperature for these 13 primers is 36℃ and 38ºC. The results showed that the RAPD markers used had high levels of polymorphism. On average each primer produces 9.5 bands. The highest number of polymorphic bands (n=16) is found on OPA primer 19, while the lowest band number (n=4) is present in OPU03-1 primer. Based on Poerba and Yuzammi (2008), eight RAPD primers on 25 accessions of *A. titanum*, produced successfully 143 DNA fragments of 100 bp to 1.1 Kb, which has 137 (95.80%) polymorphic bands. Similar results were reported by Poerba and Martanti (2008) in *Amorphophallus muelleri*, 5 RAPDs used had 69.05% polymorphism bands and 30.95% monomorphic bands. Based on research of Mekkerdchoo et al. (2013) on 35 species of *Amorphophallus* spp in China and Thailand obtained 269 bands ranging from 150 to 5000 bp and All amplified fragments were found to have 100% polymorphic bands. In *A. albus* found of a total of 154 bands scored, which ranged from 150bp to 2 kb and averaged 7.3 bands per primer, 32 were polymorphic with 20.8% polymorphism (Hu et al. 2008). In *A. paeoniifolius* using ten microsatellite loci found all loci produced highly polymorphic alleles (Santoso et al. 2003). The existence of a polymorphic gene means that some individuals in the population have heterozygous genes. All levels of genetic variation contributing to the population's ability to adapt to environmental changes (Wise et al. 2002).

Table 2. Preselected RAPD Primer used in this study

Primers	Primer Sequence (5'-3')	Length of primer (bp)	T Annealing (°C)	Number of DNA fragments	Polymorphic DNA fragment (%)
OPA 11	CAA TCG CCG T	300-1500	36	8	100
OPA 19	CAA ACG TCG G	250-1500	36	16	100
OPC 04	CCG CAT CTA C	250-2000	36	15	100
OPN 14	TCG TGC GGG T	300-2000	38	11	100
OPN19	GTC CGT ACT G	300-1500	36	9	100
OPU 03	CTA TGC CGA C	350-1650	36	6	100
OPU06	ACC ITT GCG G	300-2000	36	10	100
OPU07	CCT GCT CAT C	300-2000	36	10	100
OPB17	AGG GAA CGA G	200-1850	36	10	100
OPC07	CAC ACT CCA G	300-1900	36	8	100
OPO 04	TCT GGT GAG G	250-1900	36	9	100
OPU03-1	CTA TGC CGA C	400-1900	36	4	(3) 75
OPN18E	AAG GTG AGG TCA	300-2000	38	8	(6) 75
				124	

Table 3. Comparison of primers for RAPD and their amplification products in several studies of *Amorphophallus titanum*

Primer	Sequence base	Fragment length (bp)	Poerba and Yuzammi (2008)		Recent research	
			Total DNA fragment	Polymorphic DNA (%)	Total DNA fragment	Polymorphic DNA (%)
OPA-11	CAA TCG CCG T	300-1500	21	(20) 95.24	8	100
OPA-19	CAA ACG TCG G	250-1500	20	100	16	100
OPC-04	CCG CAT CTA C	250-2000	14	100	15	100
OPN-14	TCG TGC GGG T	300-2000	15	(13) 86.67	11	100
OPN-19	GTC CGT ACT G	300-1500	16	100	9	100
OPU-03	CTA TGC CGA C	350-1650	13	100	6	100
OPU-06	ACC ITT GCG G	300-2000	21	(20) 95.24	10	100
OPU-07	CCT GCT CAT C	300-2000	23	100	10	100
OPB-17	AGG GAA CGA G	200-1850	-	-	10	100
OPC-07	CAC ACT CCA G	300-1900	-	-	8	100
OPO-04	TCT GGT GAG G	250-1900	-	-	9	100
OPU-03-1	CTA TGC CGA C	400-1900	-	-	4	(3) 75
OPN-18E	AAG GTG AGG TCA	300-2000	-	-	8	(6) 75
Total			143	137 (95.80)	124	

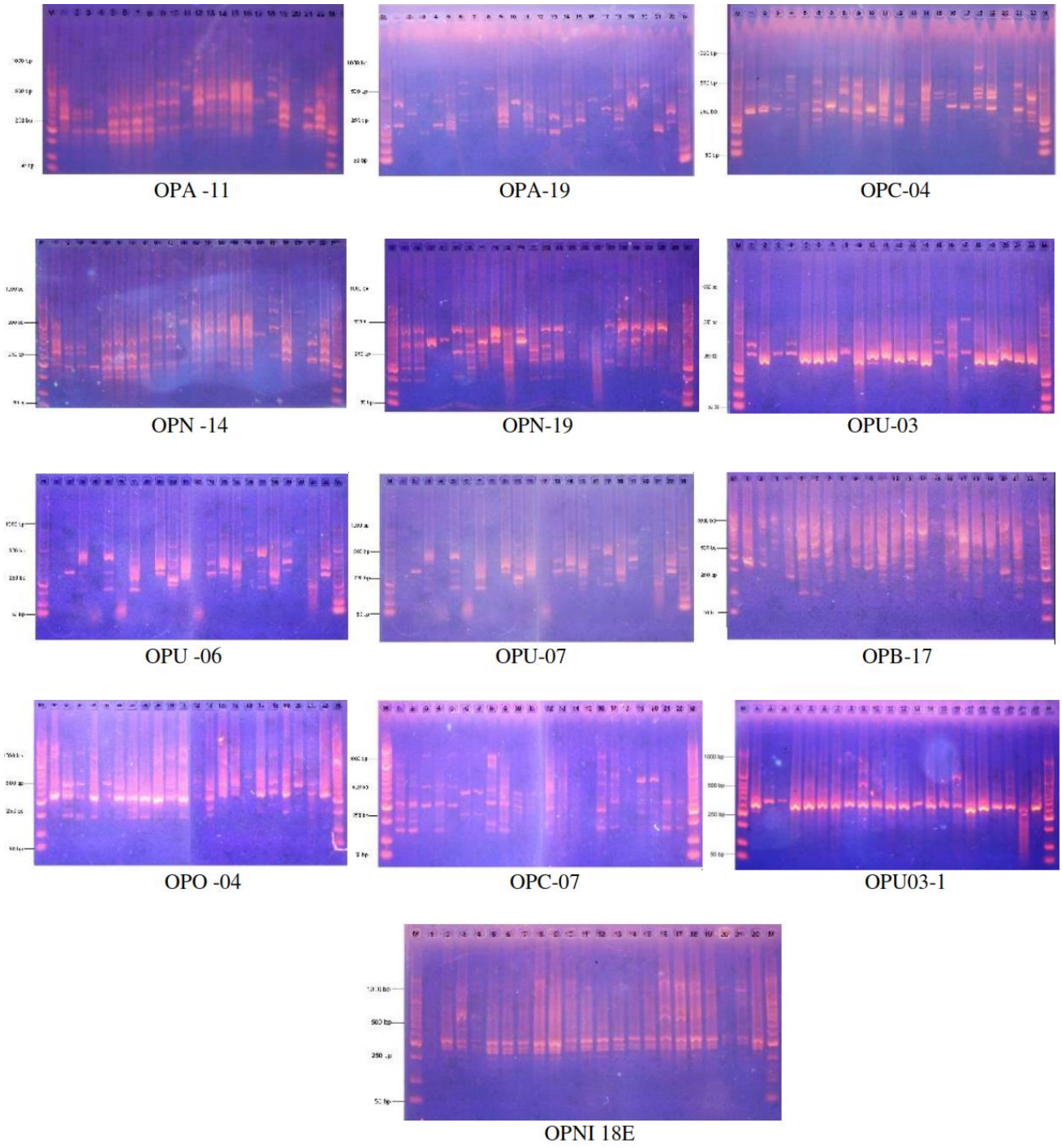

Figure 2. RAPD Amplification product of PCR using 13 primers, line [1-13] Air Selimang, line [14-17] Tebat Monok, line [18-22] Palak Siring. M: Gene Ruler 50 bp DNA Ladder

All preselected thirteen primers has successfully produced 4-16 detectable DNA bands. The highest number of RAPD bands (16 bands) was amplified by primer OPA-19 while the lowest one (4 bands) was resulted by primer OPU03-1 (Table 3). Based on the results of Poerba and Yuzammi's (2008), the number of maximum DNA fragments found in OPU-07 (23 bands), and the minimum number of fragments found in the OPU-03 primer (13 bands). In *A. muelleri* produces 6-11 DNA bands that can be detected and scored, where the maximum number of polymorphic bands 9 is found in primer OPD-04 (Poerba and Martanti 2008). The number and intensity of DNA bands depend on how the primer recognizes its complementary DNA sequence in the DNA of the template used.

Genetic diversity within the population

Genetic diversity of *A. titanum* in Bengkulu varied for each population (Table 4). In Table 4, the population of *A. titanum* in Air Selimang has the highest value for all parameters of genetic diversity (Finkeldey 2005), they were He (0.245), Ne (1.398), PLP (86.29%), Na (1.863) and I (0.381). This condition indicates that the Air Selimang area is probably as one of the centers of *A. titanum* diversity in Bengkulu. A previous report by Poerba and Yuzammi (2008) indicated that value of genetic inequality (dissimilarity) between populations ranges from 0.24-0.52. Genetic diversity in *A. muelleri* ranges from 0.1019 ± 0.1727 to 0.1832 ± 0.2054 (Poerba and Martanti 2008). The amount of genetic diversity in the population is determined by the number of genes that have more than one allele (polymorphic genes).

The lowest genetic diversity was found in the Tebat Monok population with He (0.166), Ne (1,265), PLP (52.42%) and Na (1,524). This is probably due to the Tebat Monok population coming from the same parent. According to Milot et al. 2007, low genetic diversity is predicted to have a negative impact on species viability, and this has become a major concern for conservation.

The high genetic diversity in the Air Selimang population is likely to be influenced by the number of individual per populations that are higher than the other two locations (Palak Siring and Tebat Monok).

Genetic diversity among populations

The total value of genetic diversity in all populations (Ht) (Air Selimang, Tebat Monok, and Palak Siring) is 0.253 with the mean genetic diversity in the population (Hs) is 0.213. The value of genetic diversity between populations (Dst) is 0.040; this value is much lower when compared with the value of Ht and Hs. Genetic differentiation between populations (Gst) is 0.1567 or 15.67%. This means that, in *A. titanum*, a 15.67% differentiation among populations exist. Based on the Gst value, the gene flow level (Nm) is 2,692 (Nm> 1) (Wu et al.2014). These results suggest that gene flow and low differences exist between populations.

The cluster analysis of 22 accessions (individuals) of *A. titanum* in three populations (Figure 3) had the similarity coefficient ranged from 0.02 to 0.5. The accession that has the closest similarities with a coefficient value of 0.022 is found in the Palak Siring collection, i.e. PS 5 with PS 2 and PS3. In the coefficient of similarity 0.452, the 22 individual *A. titanum* from three locations were separated into 3 clusters, the C cluster is the accession sampled from Air Selimang (AS1, AS2, AS3, AS7, AS8, AS9, AS10, AS11, AS12, AS13), the D cluster is filled by accession from Tebat Monok (TM1, TM2, TM3, TM3) and The B cluster is an accessions from Palak Siring (PS1, PS2, PS3, PS4 and PS5). In coefficient 0,476 Air Selimang and Tebat Monok joined in a single cluster, they separated with Palak Siring population. This grouping indicates that Air Selimang and Tebat Monok have a close relationship if compared with Palasiring population.

Genetic distance

Table 6 indicates that the population of Air Selimang and Tebat Monok has the closest genetic distance that is 0.0513 if it is compared to the genetic distance between the population of Tebat Monok with Palasiring, i.e., 0.0932 or Air Selimang to Palak Siring, i.e., 0.0886. If we look at the data of geographical distance shows the same pattern with genetic distance. The population of Air Selimang to Tebat Monok has the closest geographical distance, i.e., 12.20 km than the geographical distance between Air Selimang with Palak Siring, i.e., 54.45 km. Based on these results, it can argue that the value of genetic distance and geographical distance are positively correlated. The genetic distance in *A. muelleri* ranges from 0.0255 to 0.3593 (Poerba and Martanti 2008). This result supports the previous statement by Schnabel and Hamrick (1990) and Alpert et al. (1993), the genetic distance correlates with geographical distance.

Table 4. Parameter value of genetic diversity *Amorphophallus titanum* population

Population	PLP (%)	N	Na	Ne	He	I
Air Selimang	86.29	13	1.863	1.398	0.245	0.381
Palak Siring	70.97	5	1.709	1.376	0.229	0.352
Tebat Monok	52.42	4	1.524	1.265	0.166	0.257
Average	69.893	-	1.699	1.346	0.213	0.330

Note: N: Number of the individual. Na: Observed number of Allele Ne: Effective number of the allele, PLP; Percentage Locus Polymorphic, He: expected heterozygosity, I: Shannon's index

Table 6. Genetic distance based on Nei's Unbiased Measures and geographical distance (Km) among *Amorphophallus titanum* population

Population	Air Selimang	Tebat Monok	Palak Siring
Air Selimang	*	$12,20^{D}$	$54,45^{D}$
Tebat Monok	0.0513^{d}	*	$42,20^{D}$
Palak Siring	0.0886^{d}	0.0932^{d}	*

Note: ^{d} is value of genetic distance and $^{D)}$ is value of geographical distance

Table 5. The mean value of genetic diversity based on analysis of Nei (1978) using RAPD marker

Species	Location	Ht	Hs	Gst	Dst	Nm
Amorphophallus titanum	Air Selimang, Palak Siring, and Tebat Monok	0.253	0.213	0.157	0.040	2.692

Note: Ht: value of genetic diversity in all population; Hs: genetic diversity within population; Dst: genetic diversity among population; Gst: genetic differentiation; Nm: Gene flow

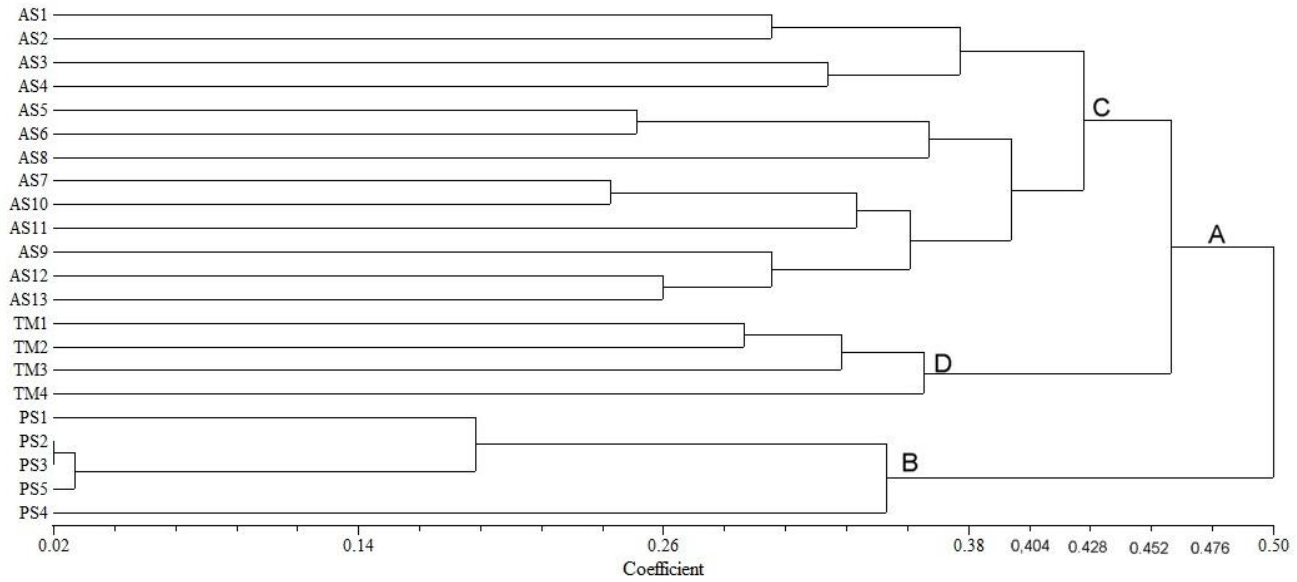

Figure 3. Dendrogram UPGMA of 22 *Amorphophallus titanum* accessions in all location. Note: Accession AS1-AS13: Air Selimang, TM 1-TM4: Tebat Monok, PS1-PS5: Palak Siring

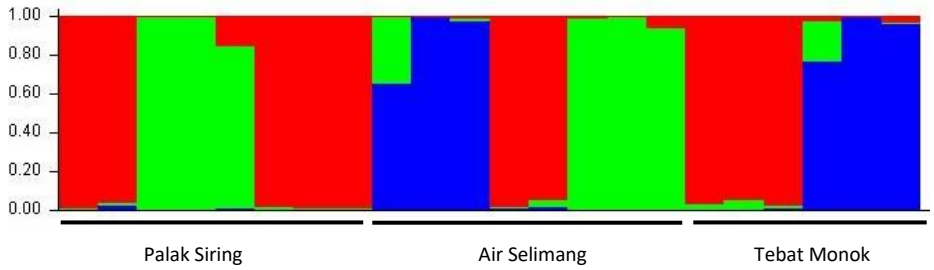

Figure 4. Bayesian clustering Analysis among three populations of *Amorphophallus titanum* using STRUCTURE (K=3)

The structure of population genetics

Structure harvester is used to assess the level of genetic stratification in multi-locus datasets. The result of harvester structure analysis shows that the best dataset number for the three *A. titanum* populations is K = 3 (DeltaK 3 = 29.230). This condition indicates that the three population of *A. titanum* (Air Selimang, Tebat Monok, and Palak Siring) consisting of 22 individuals can be divided into 3 clusters, namely Air Selimang in the first cluster, Tebat Monok in the second cluster, and the remaining third cluster for Palak Siring. The same color pattern in figure 4 illustrates the population has a general genetic structure. The genetic structure of the population is influenced by several factors such as the mating system, genetic drift, population size, seed distribution, gene flow, evolutionary history and natural selection (Hamrick and Godt 1990).

In conclusion, the analysis of genetic diversity of three *A. titanum* populations in Protected Forest Areas in Bengkulu Province revealed that the Air Selimang Population (He = 0.245) was defined as the potential center of genetic diversity of *A. titanum*, because it has the highest diversity value, while the population of Tebat Monok (He = 0.166) has the lowest genetic diversity. The average genetic diversity among populations is lower than the genetic diversity within a population. The results of the clustering analysis of *A. titanum* produced two clusters, where the Palak Siring population is separated from the population of *A. titanum* Tebat Monok and Air Selimang.

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