ISOLATING MICROSATELLITE FROM *Amorphophallus variabilis* AND ITS APPLICATION FOR POPULATION STUDY IN DRAMAGA CONSERVATION FOREST, INDONESIA

EDI SANTOSA[1*], CHUN LAN LIAN[2], YOKO MINE[3], KEN TAKAHATA[3] and NOBUO SUGIYAMA[4]

[1]*Department of Agronomy, Faculty of Agriculture, Institut Pertanian Bogor, Bogor 16680, Indonesia*
[2]*Asian Natural Environmental Science Center, University of Tokyo, Tokyo 188-0002, Japan*
[3]*Faculty of Agriculture, Tokyo University of Agriculture, Kanagawa 243-0034, Japan*
[4]*Graduate School of Agricultural and Life Sciences, University of Tokyo, Tokyo 113-8657, Japan; Present address: Faculty of Agriculture, Tokyo University of Agriculture, Kanagawa 243-0034, Japan*

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ABSTRACT

Amorphophallus variabilis Blume, a member of Araceae, is a fleshy perennial tuber crop endemic in Java Island, Indonesia. The plant produces white edible corm; and it was used as food during famine time before 1960s. Rapid ecological changes and land fragmentations in Java in recent times threaten populations of *A. variabilis*. Here, compound microsatellite markers were developed in order to develop conservation strategies in the populations. Twelve primers pairs produced high polymorphism ranging from 5 to 22 alleles per locus. The observed and expected heterozygosities ranged from 0.191 to 0.851 and 0.380 to 0.943, respectively. This high allelic diversity indicates that these markers are suitable for the study on population genetic structure. Cross-amplification on related and non-related species was performed. Application of the markers on populations from Dramaga Conservation Forest revealed high allelic richness, high diversity within and among populations. Genetic distance among populations increased with an increase of geographic distance. Present study suggested that, it is important to study population of *A. variabilis* in Java in order to understand the population genetic structure and develop effective *in situ* conservation programs.

Keywords: Araceae, genetic population, SSR, tuber crop, white iles-iles

INTRODUCTION

Amorphophallus variabilis Blume, synonym *Brachyspatha variabilis* (Blume) Schott, an Araceae, is a diploid endemic perennial tuber crop native to Java. Its distribution in Indonesia is exclusively in Java, Kangean and Madura Islands (Jansen *et al.* 1996; Yuzammi 2000). The plant naturally grows in less disturbed land under partial trees shading and at gap of forest trees at low altitude up to 700 to 900 m above sea level (Jansen *et al.* 1996; Sugiyama & Santosa 2008). *A. variabilis* is locally called white *iles-iles* (Safii 1981; Wiyani 1988) reflecting the white color of the tuber. It is easily distinguished morphologically from *Amorphophallus muelleri* Blume (Sugiyama & Santosa 2008) known as yellow *iles-iles*.

* Corresponding author: edisang@gmail.com

The plant produces a single underground corm with several cormlets; corms and cormlets exhibit dormancy during dry season (Jansen *et al.* 1996). The corm was utilized as staple food in Java particularly during famine situation before 1960s. Corm of *A. variabilis* contains high glucomannan (ca. 35% on a dry weight basis) (Ohtsuki 1968; Safii 1981; Wiyani 1988). Glucomannan is used as an important material in beverage, food and pharmaceutical industries (Jansen *et al.* 1996; Alonso-Sande *et al.* 2009).

Botanically, a large genetic diversity has been observed within *A. variabilis* plants, including inflorescence (Santosa *et al.* 2004) and leaf morphologies (Sugiyama & Santosa 2008). According to Yuzammi (2000), the petioles vary from pure green to dark brown with different color and pattern of spots. Therefore, *A. variabilis*

germplasm could be used as breeding materials (Sugiyama & Santosa 2008). According to Zhang *et al.* (1998), several *Amorphophallus* species could be hybridized.

The monoecious unisexual inflorescence produces unpleasant odor during anthesis (Kite & Hetterschieid 1997) to attract pollinators. Red bright mature berries develop after cross-pollination by Nitidulidae insects (Santosa *et al.* 2004). Seeds are dispersed long distance by birds and clonally propagules i.e. cormlets were dispersed nearby mother plants (Sugiyama & Santosa 2008). Thus, in an undisturbed population, both ramets and genets co-exist.

Recently, the sliced corms of *A. variabilis* are used as feeds for swine (Santosa *et al.* 2004; Sugiyama & Santosa 2008). People collect the corms without considering the conservation. On the other hand, land fragmentations and agricultural intensifications in agroforestry system for cash crops (Santosa *et al.* 2005) disturbed the populations. Consequently, the populations of *A. variabilis* in Java are in danger. High exploitation has been known causing population decline and loss of genetic diversity in many species (Leimena *et al.* 2007; Nuryanto & Susanto 2010; Santosa *et al.* 2010).

Therefore, in order to develop effective program on genetic conservation of *A. variabilis*, it is important to evaluate population size using reliable genetic markers, such as microsatellite. Microsatellite or simple sequence repeat (SSR) is widely used to develop conservation program in many species (Abdul-Muneer 2014; Nachimuthu *et al.* 2015). In this study, we developed microsatellite of *A. variabilis* using compound isolation method for the first time.

Many methods have been developed for microsatellite isolations e.g. based on sequence data (Lian *et al.* 2001; Fatimah & Sukma 2011), cloning, enrichment library and dual-suppression methods (Lian & Hogetsu 2002; Lian *et al.* 2006). In the present study, compound microsatellites are isolated according to procedure of Lian *et al.* (2006). The objective of this study was to develop microsatellite markers from *A. variabilis* and its application for genetic population study.

MATERIALS AND METHODS

Plant Material

Plant materials were collected separately for microsatellite development and population study i.e. from Bogor Botanical Garden (BBG) and Dramaga Conservation Forest (DCF), respectively with permission from the authorities. According to Yuzammi (2000), Bogor area including the sampling sites, is the center of *A. variabilis* diversity.

To develop microsatellite markers, 2 - 5 g fresh and healthy leaves of *A. variabilis* were collected during rainy season in 2005 at the BBG, Bogor, West Java Province, Indonesia. The leaves were cleaned using moist tissue paper and put into a plastic bag containing silica gel, then stored at -30 $^{\circ}$C until being used in genomic DNA extraction. Microsatellite development was conducted in the Laboratory of Forestry, Asian Natural Environmental Science Center, University of Tokyo, Japan from 2009 to 2010.

For population study, *A. variabilis* accessions were sampled and characterized in the DCF (244 m asl), Bogor, West Java Province, Indonesia from 2010 to 2011. DCF is managed by the Indonesia Ministry of Environment and Forestry. Around 40 of the 60 ha DCF area is dedicated for conservation forest, locally known as the CIFOR forest (CIFOR = Center for International Forestry Research). DCF is located about 8 km northwest of Bogor Botanical Garden, 1 km west of Situ Gede Lake and 1 km north of Cisadane River. Three populations were selected for this study, i.e. A (-6.5509798, 106.7497507,17z), B (-6.5519391,106.7494932,17z) and C (-6.5548596,106.7507271,17z) (Fig.1). In each population, plants were sampled from an area of about 0.5 to 1 ha.

All plants having pseudo stems thicker than 2 cm at 10 cm above soil surface and were spaced more than 1 m apart were collected. Plants spaced less than 1 m apart were considered as a ramet (similar genet) and not sampled. Petiole colors of accessions were characterized (Table 1). Initially, 35, 25 and 38 samples were collected, and after optimizing the DNA results a set of 11, 21 and 15

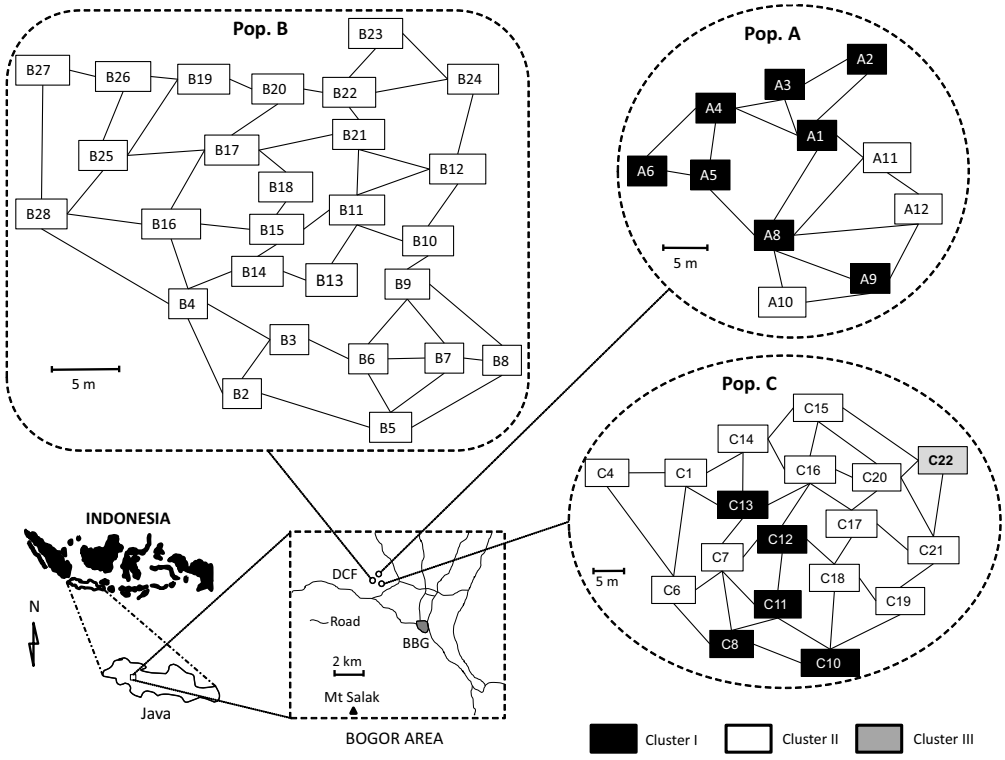

Figure 1 Site and accession positions within populations in Dramaga Conservation Forest (DCF), Bogor, Indonesia
(Note: Lines represent distance and visibility between two accessions; accessions without lines represent blockage by dense trees; color represents cluster membership; bar represents 5 meter)

accessions were used for further analysis from A, B and C populations, respectively. The other samples were excluded from analysis because they failed to amplify, or produced multiple and unclear bands.

Microsatellite Development

A modified cetytrimethyl ammonium bromide (CTAB) method was adopted for conducting genomic DNA extraction (Zhou *et al.* 1999). DNA from 1 g dry leaves was dissolved in a final volume of 200 µL water and stored in -30 °C until use. Isolation of codominant compound microsatellite markers was performed according to the method of Lian *et al.* (2006). In brief, the *Hae* III blunt-end restriction enzyme was used to digest DNA sample. The digested DNA was then ligated to an adaptor using a DNA Ligation Kit (TaKaRa Shuzo, Japan). Fragments flanked by a microsatellite region at one end were amplified from the constructed DNA library using SSR primers, $(AC)_6(AG)_5$ or $(TC)_6(AC)_5$ and an adaptor primer, AP2 (5´–CTATAGGGCACGCGT GGT–3´). The PCR products were subcloned using a pT7 Blue Perfectly Blunt Cloning Kit (Novagen, USA) according to the manufacturer's instructions. The positive clones were amplified using the U19 and M13 reverse primers, and then sequenced using a Thermo Sequenase Pre-mixed Cycle Sequencing Kit (Amersham Biosciences) plus the T7 or U19 primer labeled with Texas Red (Sigma-Aldrich) on SQ-5500E sequencer (Hitachi, Tokyo). A primer (IP1) was designed from the sequenced region flanking to the SSR. The IP1 and corresponding microsatellite primers were used as the marker.

In addition to microsatellites developed from *A. variabilis*, twenty microsatellite markers which were developed from *A. paeoniifolius* by Santosa *et al.* (2007) and stored in GenBank were evaluated for its suitability toward *A. variabilis* genotyping. In order to extend usefulness of the microsatellite markers, the developed markers from *A. variabilis* were evaluated on several Araceae members and other root crops species.

PCR Amplification and Detection

PCR reaction was carried out in a reaction mixture (10 µL) containing 5 - 10 ng DNA, 0.2 mM of each dNTP, 1 PCR buffer (Mg^{2+} free,

Table 1 Characterization of *A. variabilis* accessions obtained from Dramaga Conservation Forest, Bogor, Indonesia

Population	Code	Petiole color	Population	Code	Petiole color
A	A1	Pure green	B	B20	Brown with small white spot
A	A2	Pure green	B	B21	Brown with large white and green spots
A	A3	Brown with white spot	B	B22	Brown with large light green spot
A	A4	Green with brown spot	B	B23	Brown with white spot
A	A5	Green with white spot	B	B25	Green with large white spot
A	A6	Green with brown spot	B	B26	Dark brown-black with white spot
A	A8	Pure green	B	B27	Brown with small white spot
A	A9	Brown with black spot	B	B28	Brown with small dark brown spot
A	A10	Brown with dark brown spot	C	C1	Pure green
A	A11	Brown with black spot	C	C4	Brown with white spot
A	A12	Dark green with brown spot	C	C6	Green with grey spot
B	B2	Grey with brown spot	C	C7	Light green with brown spot
B	B3	Brown with green spot	C	C8	Brown with pink spot
B	B4	Grey with green spot	C	C10	Pure green
B	B5	Brown with green spot	C	C11	Light green with grey spot
B	B6	Brown with white spot	C	C12	Pure green
B	B7	Green with small white spot	C	C13	Brown with white and black spot
B	B8	Light green with black and white spot	C	C14	Brown with light brown spot
B	B9	Light green	C	C16	Brown
B	B10	Brown with brown and black spot	C	C17	Dark brown with pink spot
B	B11	Pure green	C	C20	Pure green
B	B14	Light brown with white spot	C	C21	Dark brown with pink and black spot
B	B16	Dark green with white spot	C	C22	Light green with black spot
B	B18	Brown with black spot			

Note: Large spot = spot width $\geq$ 10mm
Small spot = spot width $\leq$2.5mm
Otherwise mentioned = spot size was medium
Spot was measured from 10 cm above soil surface to middle petiole length

Applied Biosystems), 2.5 mM $MgCl_2$, 0.25 U of Ampli *Taq* Gold (Applied Biosystems, USA), 0.5 µM of each IP1 primer and the corresponding SSR primer (labeled with Texas Red). The PCR thermal cycler (Applied Biosystems) were used with cycling profile: 9 minutes at 94 ºC, followed by 40 cycles of 30 seconds at 94 ºC, 30 seconds at the locus-specific annealing temperature (Table 2) and 1 minute at 72 ºC, and finally a 5 minutes extension at 72 ºC. The PCR products were electrophoresed on a 6% polyacrylamide gel using an SQ-5500E sequencer, and then analyzed with FRAGLYS ver. 3 software (Hitachi, Tokyo).

Data Analysis

Characteristics of microsatellite markers developed in the present study were tested on *A. variabilis* across all populations (n = 47). Two bands from an individual were considered as different alleles if the differences in molecular weight were bigger than 3 base pair (bp) for the microsatellite containing trinucleotide repeat and 2 bp for the microsatellite containing dinucleotide repeat; these bp different are matter of technical procedure not related to nucleiotide repeat motif (Santosa 2017). The numbers of alleles, observed (H_O) and expected heterozygosities (H_E) and polymorphic information content (PIC) were calculated using CERVUS ver. 3.0.3 software (Marshall *et al.* 1998). Hardy-Weinberg equilibrium (HWE) and linkage disequilibria between loci were tested using GENEPOP version 4.0 software on the web (Rousset 2008). Presence of null allele was estimated using CERVUS ver. 3.0.3 software (Marshall *et al.* 1998). Number of migration (Nm) was estimated using GENALEX software.

Analysis of molecular variation (AMOVA) within (F_{IS}) and among population (F_{ST}) was determined from A, B and C populations separately using GENALEX software in 999 permutations. Cluster analysis was performed using NTSYST Spc 2.11p (Exeter Software, Setauket USA). Presence of allele at each locus was coded in binary form 1 (presence) or 0 (absence). Individual across populations was clustered in UPGMA dendrogram using Jaccard similarity coefficient.

RESULTS AND DISCUSSION

Polymorphism Test

From twenty-two microsatellite loci isolated from *A. variabilis*, twelve loci were polymorphic and codominant. Polymorphic information content (PIC) ranged from 0.351 to 0.932 (Table 2). The observed (H_O) and expected heterozygosities (H_E) ranged from 0.191 to 0.851 and from 0.380 to 0.946, respectively. The full length of amplified region from *A. variabilis* microsatellite had been deposited at GenBank for public access (www.ncbi.nlm.nih.gov/Genbank).

Two of 12 loci isolated from *A. variabilis*, i.e. Avar01 and Avar07, could deviate from HWE proportion (p 0.001) by having an excess of heterozygosities (Table 2). Linkage disequilibria analysis indicated that the loci had high linkages ($p \leq 0.001$), i.e. between Avar02 and Avar04, Avar04 and Avar08, Avar07 and Avar10, and Avar07 and Ampa10. Excluding loci Avar04 and Avar07, the other 10 loci could be used in population genetic study. According to Li *et al.* (2009), a set of 10 - 15 or more loci was desirable for genetic population study. Furthermore, two microsatellite primers from *A. paeoniifolius,* i.e. Ampa10 and Ampa15, produced clear bands in *A. variabilis* accessions. Therefore, in total, fourteen polymorphic microsatellite loci could be used for *A. variabilis* genotyping.

This study is the first work on the development of polymorphic codominant microsatellite markers in *A. variabilis*. Microsatellite markers developed in the present study could be used to identify the polymorphism based on the standards proposed by Lian *et al.* (2006), i.e. clearness of bands, common annealing temperature and different allele sizes. In the present study, annealing temperature was set at 58 and 62 °C. Allele sizes among several loci had large differences in base pair size (Table 2). Therefore, loci Avar01 and Avar09, Avar05 and Avar06, and Avar03 and Avar10 could be mixed together in PCR reaction, to speed up PCR preparation and allele analysis.

The loci developed from *A. variabilis* cross-amplified in other species (Table 3), indicating that the loci could provide a useful tool for genotyping in other species. Indeed, microsatellite primers are well known for its species-specific (Lian & Hogetsu 2002; Csencsics *et al.* 2010), however, cross-amplifications sometimes exist (Santosa *et al.* 2007; Fatimah & Sukma 2011). Successful cross-amplification is probably due to the presence of homologous loci among them. Santosa *et al.* (2007) have reported that microsatellites isolated by dual-suppression from *A. paeoniifolius* amplified in related species, while Fatimah and Sukma (2011) reported that microsatellite markers obtained from sequence data could amplify across *Phalaenopsis* genus. However, amplification PCR products did not mean produce polymorphic allele in present study. It needs further investigation on the usefulness of the developed loci for genotyping other *A. variabilis* species.

Population and Genetic Diversity of DCF

The average numbers of alleles per locus was 6.5 for 14 loci. The numbers of alleles per locus varied from 3 to 11 in population A, from 3 to 16 in population B and from 3 to 14 in population C (Table 4). Locus Ampa15 produced the smallest number of alleles, whereas locus Ampa10 produced the largest number of alleles across DCF populations. Several accessions failed to amplify, i.e. locus Avar02 for A12 and C20 accessions, locus Avar04 for C6 and C10 accessions, locus Avar06 for B9 and B11 accessions, locus Avar11 for B23 and C17 accessions, locus Ampa10 for C13 accession and locus Ampa15 for A8 and C7 accessions. These accessions probably had null allele for particular loci. Point mutation in the primer annealing sites may lead to the occurrence of null alleles causing the primer failed to amplify (Lian *et al.* 2006).

Table 2 Characteristics of twelve microsatellite markers isolated from *A. variabilis*

Locus	GenBank[z]	Repeat motif	Primer sequence 5' to 3'	T_a (°C)	NA	Allele (bp)	H_O	H_E	PIC
Avar01*	MF527250	$(CT)_6(GT)_7$	F: CTTGTTCGGACCACCTTCTTGACAATC R: (AC)7(AG)3	62	5	120-130	0.489	0.548	0.492
Avar02	MF527251	$(CT)_{12}(GT)_7$	F: CTCAAAATCGAATCTTCTCCATTTTAC R: (AC)7(AG)3	62	14	124-154	0.333	0.877	0.854
Avar03	MF527252	$(CT)_8..(CTT)_{11}..(CT)_5(GT)_7$	F: CACTCTTCCACACTCCCCCTGTTACAC R: (AC)7(AG)3	62	5	128-140	0.213	0.380	0.351
Avar04	MF527253	$(CT)_{13}....(CT)_4(GT)_7$	F: CTTCCCTATGCAGGTGAGTC R: (AC)7(AG)3	58	8	113-155	0.333	0.427	0.396
Avar05	MF527254	$(GT)_5(GA)_7$	F: GTGAAGGAGGTGGGCGTTTTG R: (TC)7(AC)3	58	5	177-187	0.468	0.520	0.460
Avar06	MF527255	$(GT)_{10}(GA)_7$	F: CTAACGACTAAGGACTTAAGC R: (TC)7(AC)3	58	22	63-141	0.511	0.943	0.928
Avar07*	MF527256	$(GT)_{10}(GA)_7$	F: CGCTGATGTACTTGTTGACATTG R: (TC)7(AC)3	58	11	139-171	0.723	0.761	0.719
Avar08	MF527257	$(GT)_8(GA)_7$	F: CATGGTCCATGGGTTTAGCTTTGC R: (TC)7(AC)3	62	20	166-236	0.745	0.933	0.918
Avar09	MF527258	$(GT)_5(GA)_7$	F: GGTGTACGACTAGAGTTTTGTCG R: (TC)7(AC)3	62	7	175-183	0.426	0.676	0.620
Avar10	MF527259	$(GT)_8(GA)_7$	F: GATGTCATTCTCCGCCACCGAGTAG R: (TC)7(AC)3	62	10	196-228	0.851	0.769	0.726
Avar11	MF527260	$(GT)_6(GA)_7$	F: GATACTGCTATACCGAGTGTCCTATG R: (TC)7(AC)3	62	7	153-185	0.200	0.655	0.607
Avar12	MF527261	$(TC)_8G(CT)_2(GT)_5(CT)_5(GT)_7$	F: GGAACACTAGCGAGTACAATGTATC R: (AC)7(AG)3	62	5	135-149	0.191	0.786	0.741

Note: [z] = GenBank repository code of primers and fragments sequence
* = significant deviation from Hardy–Weinberg Equilibrium ($p < 0.01$)
T_a = annealing temperature
NA = number of allele
H_O = observed heterozygosity
H_E = expected heterozygosity
PIC = Polymorphic Information Content
Each reverse primer (R) was tailed with an additional 19 nucleotide (U19) to the 5' end

Table 3 Cross-species amplification of twelve microsatellite primers developed from *A. variabilis*

No	Species[z]	Family	Origin of the specimen	Loci AVAR											
				01	02	03	04	05	06	07	08	09	10	11	12
1	*Aglaonema pictum* (Roxb.) Kunth.	Araceae	BBG	++	++	-	++	++	+	+	++	+	+	++	++
2	*Alocasia alba* Schott.	Araceae	BBG	++	++	++	++	++	-	+	++	-	+	++	++
3	*Anthurium* spp.	Araceae	BBG	++	++	+	-	++	-	+	++	-	+	++	++
4	*Amorphophallus konjac* K. Koch	Araceae	Tanashi Farm, UT	++	++	+	++	++	++	+	++	+	+	++	++
5	*Amorphophallus muelleri* Blume	Araceae	IPB	++	++	++	-	++	++	+	+	++	+	-	++
6	*Amorphophallus paeoniifolius* (Dennst.) Nicolson	Araceae	Kuningan, Indonesia	++	++	++	++	++	++	++	++	++	++	++	++
7	*Amorphophallus titanum* (Becc.) Becc. Ex. Arcang	Araceae	BBG	++	+	+	-	-	-	+	-	+	+	+	+
8	*Caladium bicolor* (Aiton) Vent.	Araceae	IPB	++	++	+	++	++	++	+	-	-	++	++	++
9	*Colocasia esculenta* (L.) Schott	Araceae	IPB	++	+	+	-	++	++	++	-	++	-	++	+
10	*Dieffenbachia fournieri* N.E. Br.	Araceae	BBG	++	++	+	++	++	-	-	-	-	+	++	++
11	*Dracontium gigas* (Seem.) Engl.	Araceae	BBG	++	++	++	++	++	-	+	-	-	+	-	++
12	*Homalomena pendula* (Blume) Bakh. f.	Araceae	BBG	++	++	++	++	++	-	+	+	++	+	++	++
13	*Ipomoea batatas* (L) Lam.	Convolvulaceae	BBG	++	++	++	++	++	-	+	++	-	+	-	+
14	*Manihot esculenta* Crantz	Euphorbiaceae	BBG	++	-	++	+	-	-	+	+	-	+	-	+
15	*Nelumbo* spp.	Araceae	Tanashi Farm, UT	++	++	+	++	++	++	+	-	-	++	+	+
16	*Schismatoglotis calyptrate* (Roxb.) Zoll. & Moritzi	Araceae	BBG	++	-	++	++	++	+	+	++	++	++	++	++
17	*Spathiphyllum cannaefolium* Schott	Araceae	BBG	++	++	++	+	++	+	+	++	++	-	++	++
18	*Solanum lycopersicum* L.	Solanaceae	Tanashi Farm, UT	++	++	++	-	-	++	+	+	+	+	+	+
19	*Solanum tuberosum* L.	Solanaceae	Cianjur, Indonesia	++	++	++	++	++	-	++	++	++	+	+	+
20	*Tacca* spp.	Araceae	BBG	++	++	++	++	++	+	++	-	-	+	-	+
21	*Xanthosoma sagitifolium* (L.) Schott.	Araceae	BBG	++	++	++	++	++	++	+	++	-	++	++	++
22	*Zamioculcas zamiifolia* Engl.	Araceae	BBG	++	++	++	++	-	++	+	+	-	+	++	++

Note: [z] = One individual per species
BBG = Bogor Botanical Garden
UT = University of Tokyo-Japan
IPB = Institut Pertanian Bogor
'–' = no amplification
'+' = amplification single band
'++' = multiple bands
Amplification band did not always produce microsatellite allele

Table 4 Description for three *Amorphophallus variabilis* populations collected from Dramaga Conservation Forest, Bogor, Indonesia

Population	Number of allele			H_O	H_E	F_{IS}
	Effective	Richness	Specific			
A (n=11)	5.43	3.56	1.14	0.481	0.645	0.240
B (n=21)	7.36	4.58	1.93	0.464	0.667	0.282
C (n=15)	6.71	4.20	1.29	0.465	0.681	0.294

Note: F_{IS} = Fixation Index calculated using formula: $(H_E - H_O) / H_E$ or $1 - (H_O / H_E)$
n = number of sample, evaluated using 10 microsatellite loci from *A. variabilis* and two loci from *A. paeoniifolius*

Number of effective alleles of A, B and C populations were 5.43, 7.36 and 6.71, respectively. B population showed higher allelic richness as compared to A and C populations. More than one specific allele per locus existed in each population. Out of 147 alleles generated by 14 loci across populations, 61 alleles were specific to particular population. A, B and C populations had 16 (26.2%), 27 (44.3%) and 18 (29.5%) specific alleles, respectively. H_O value was the highest in A population, while H_E value was the highest in C population. Fixation indices (F_{IS}) averaged for each population across all loci had high values ranging from 0.240 to 0.294 (Table 4).

AMOVA analysis showed low F_{ST} values both within and among populations. F_{ST} value across all populations was 0.026, whereas F_{ST} values among A-B, A-C and B-C populations were 0.022 (p = 0.009), 0.034 (p = 0.003) and 0.025 (p = 0.001), respectively, suggesting that each population was slightly conserved. On the other hand, variation among individuals within population was high (64.0%). According to Jansen *et al.* (1996) and Yuzammi (2000), *A. variabilis* has large phenotypic variations in leaf size, petiole and inflorescence color.

The populations exhibited low genetic distance based on Nei (1972), i.e. 0.151 for population A to population B, 0.210 for population A to population C and 0.149 for population B to population C. Individuals across all populations were clustered in three groups (Fig. 2). Group I composed of accessions from A and C populations. Group II composed of accessions from A, B and C populations. Group III contained only one accession from population C, i.e. C22. Population B was exclusively clustered under subgroup II. In each population, with exception for B28, C1, C4, C6 and C7, accessions clustered in the same group were generally located close to each other (Fig. 1). Accessions A10, A11 and A12 from A population were clustered in subgroup II with those from B population. These accessions were geographically close to each other (Fig. 1). This suggests that genetic exchange via pollen occurred between accessions located at the peripheral zone of a population, as stated by Pasquet *et al.* (2008). Sugiyama dan Santosa (2008) stated that Nitidulidae insect becomes important pollinator in *A. variabilis*.

Morphological variations in petiole (Table 1) was unlikely correlated with microsatellite profiles. Accessions with green petiole without spot, i.e. A1, A2, A8, B11, C1, C10, C12 and C20 were clustered into two different groups, i.e. Group I and Group II. This finding was in disagreement with the results of Santosa *et al.* (2012), where flower size, leaf size and the presence of petiole spot were tightly linked with AFLP grouping. The discrepancy between the result of Santosa *et al.* (2012) and this study could be due to the fact that AFLP is a dominant marker. In *A. paeoniifolius*, petiole color, shape and color of spot and petiole roughness are affected by soil fertility (Sugiyama & Santosa 2008). In the previous work, Santosa *et al.* (2004) grouped *A. variabilis* accessions from West Java Province, Indonesia based on inflorescence morphology into four groups. Therefore, it is important to conduct further study to investigate whether petiole and peduncle colors in *A. variabilis* are also affected by soil fertility.

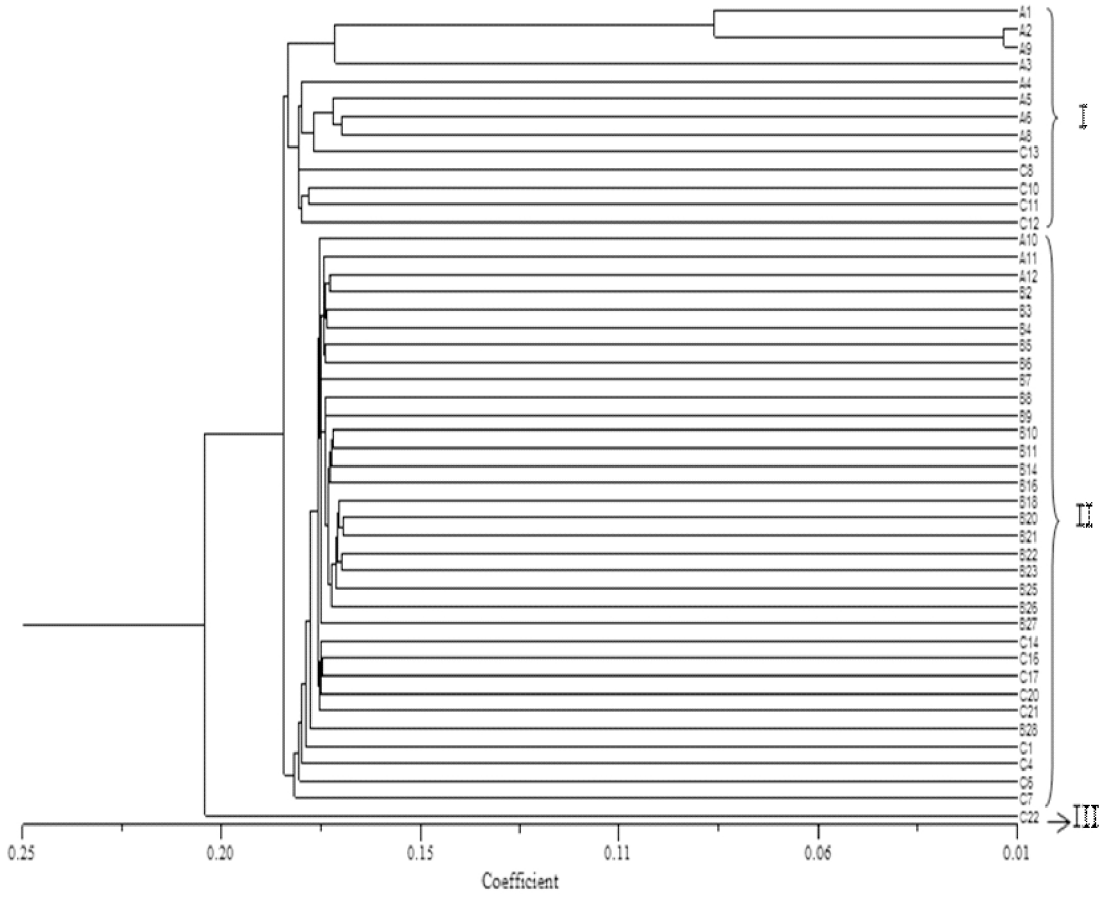

Figure 2 Dendrogram of UPGMA based on Jaccard similarity index of *A. variabilis* constructed using microsatellite data obtained
(Note: A, B, and C codes represent A, B and C populations, respectively)

Conservation Strategy

Three populations evaluated in DCF exhibited unique genetic feature. According to an interview with administrative staff members of DCF, there was no intercropping program, harvesting or any man-made disturbance on the *A. variabilis* populations. Unexpectedly, during field survey, several *A. paeoniifolius* plants were found at DCF. According to local officer, the plants were introduced from Yogyakarta by farm laborers in year 2000s.

Many bushes, weeds and tree seedlings grew densely inside DCF, forming shady condition and blocking more than 75% of sunshine. According to Sugiyama and Santosa (2008), *Amorphophallus* species grow well under shading up to 75% shading level. It was possible that heavy shading disturbed the growth of *A. variabilis*, resulting to low density of the studied-population in DCF. Sugiyama and Santosa (2008) also stated that single mother corms of *A. variabilis* produced 14.3 cormlets per year. Cormlets were detached from mother corm after dormancy release. Two to seven cormlets usually grew close (0 - 13 cm) to mother corms, unlike seedlings from seeds which usually grew apart (> 60 cm) from the mother plant. Considering that mature plant of *A. variabilis* is able to produce 25 - 280 seeds (Sugiyama & Santosa 2008), the population density could increase steadily yearly in undisturbed condition. It is interesting to conduct further study on population dynamic of *A. variabilis* in isolated condition, such as in DCF.

In the present study, several accessions failed to amplify resulting to low number of sampling size in each population (Table 4) that might cause an underestimation of population genetic. According to Li *et al.* (2009), the numbers of effective alleles were determined by the number of sampling size and the numbers of loci used. A set of 40 samples per population is necessary for population study. During DNA extraction, several leaf samples contained large amount of glucomannan. Thus, it is important to improve

DNA extraction method in the near future, to enhance the accuracy of population genetic evaluation.

Genetic distance between A and C populations was significantly larger than those of other population pairs. However, it was still unclear why these populations were distantly separated in terms of genetic profile. Geographically, A and C populations were separated by about 200 meters. The large genetic distance suggested that exchange of genetic materials among A and C populations was restricted. Interestingly, within C population, C22 seemed to be out of group (Fig. 2). This might be caused by the fact that C22 was an introduced seed from other distant populations by birds as primary agent for long-distance seeds dispersal of *A. variabilis* (Sugiyama & Santosa 2008).

The number of estimated genetic exchange or migration among A-B, A-C and B-C populations were 11.23, 7.21 and 9.81, respectively, and were considered as high. Average number of estimated migrant (Nm) based on the number of specific alleles across all populations was high, i.e. 9.39. Smaller degree of genetic differentiation (F_{ST}) among A and B, and B and C populations, indicated populations located closely to each other mostly underwent intense genetic exchange through cross-breeding.

Finding in DCF indicated that the *A. variabilis* existed as a meta-population. Santosa *et al.* (2012) has suggested to conserve single large population in conservation strategy of *A. variabilis*, however, it should be further clarified by evaluating accessions from distant populations across Java Island using codominant microsatellite primers. Many researchers, students and professional workers stated that a large number of *A. variabilis* plants existed in various agroecological condition in West Java and Yogyakarta provinces. Therefore, it is interesting to evaluate *A. variabilis* population from different sites in Java Island to develop conservation strategy for *A. variabilis*.

CONCLUSIONS

Microsatellite primers developed in the present study were applicable for the population study of *A. variabilis*. Three *A. variabilis* populations in Dramaga Conservation Forest exhibited low genetic diversity among populations, but high genetic distance within a population. Increasing number of specific allele with the increasing geographical distances among populations implies the importance to study genetic population from the larger geographical range in Java Island to determine the best conservation strategy for *A. variabilis*.

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