GENETIC RELATIONSHIP OF SEVERAL MORPHOLOGICAL AND MOLECULAR CHARACTERISTICS OF *Phalaenopsis amabilis* (L.) Blume ORCHIDS FROM THE MERATUS MOUNTAINS OF SOUTH KALIMANTAN, INDONESIA

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Received: 16 January 2021, Revised: 24 January 2022, Accepted: 15 April 2022

GENETIC RELATIONSHIP OF SEVERAL MORPHOLOGICAL AND MOLECULAR CHARACTERISTICS OF *Phalaenopsis amabilis* (L.) Blume ORCHIDS FROM THE MERATUS MOUNTAINS OF SOUTH KALIMANTAN, INDONESIA. *Phalaenopsis amabilis* (L.) Blume orchid is one of the most popular orchid species in the world. However, this ornamental plant is threatened in its natural habitat, the Meratus Mountains of South Kalimantan, Indonesia. This study determines and analyzes the genetic relationship among several morphological characteristics of *P. amabilis* from this region combined with molecular (RAPD) markers. A total of ten orchid samples comprised of nine moth orchids (*P. amabilis*) and one species (*P. cornu-cervi*) as an outgroup, as well as ten RAPD primers were used in this study. Based on the morphological markers, the moth orchids have a moderate level of genetic diversity, indicated by Shannon's index value of 0.5. In contrast to molecular markers, this germplasm shows high genetic polymorphism, shown by the polymorphism degree of 100% for all primers used. The cluster analysis shows that this germplasm can be divided into two clusters for morphological and five for molecular markers. Following these markers, the grouping of moth orchids was nearly corresponding to their origin. Thus, this information could be useful as a reference for orchid conservation and breeding programs in the future.

Keywords: Breeding and conservation, genetic polymorphism, orchid, *Phalaenopsis*

KEKERABATAN GENETIK ANTARA BEBERAPA KARAKTER MORFOLOGI DAN MOLEKULER DARI ANGGREK Phalaenopsis amabilis *(L.) Blume ASAL PEGUNUNGAN MERATUS, KALIMANTAN SELATAN, INDONESIA.* Phalaenopsis amabilis *(L.) Blume merupakan salah satu jenis anggrek terpopuler di dunia. Namun tanaman hias ini telah terancam di salah satu habitat aslinya, yaitu Pegunungan Meratus, Kalimantan Selatan, Indonesia. Tujuan penelitian ini adalah untuk menentukan dan menganalisis kekerabatan genetik dari beberapa karakter morfologi dari* P. amabilis *dari wilayah tersebut dan menggabungkannya dengan penanda molekuler (RAPD). Sebanyak sepuluh sampel anggrek, terdiri atas sembilan anggrek bulan (*P. amabilis*) dan satu spesies outgroup (*P. cornu-cervi*), serta sepuluh primer RAPD telah digunakan dalam penelitian ini. Berdasarkan penanda morfologi, anggrek ini memiliki tingkat keragaman genetik sedang, ditunjukkan dengan nilai indeks Shannon sebesar 0,5. Berbeda dengan penanda molekuler, plasma nutfah ini menunjukkan variasi genetik yang tinggi, ditunjukkan dengan derajat polimorfisme sebesar 100% untuk semua primer yang digunakan. Hasil analisis klaster menunjukkan bahwa plasma nutfah ini terbagi menjadi dua kelompok utama untuk penanda morfologi dan lima kelompok untuk penanda molekuler. Berdasarkan kedua penanda, pengelompokan plasma nutfah ini relatif berkaitan dengan wilayah asalnya. Dengan demikian, informasi ini diharapkan dapat digunakan sebagai acuan untuk program konservasi dan pemuliaan anggrek pada masa mendatang.*

Kata kunci: Pemuliaan dan konservasi, variasi genetik, anggrek, Phalaenopsis

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 doi:10.20886/ijfr.2022.9.1.63-72

I. INTRODUCTION

The Meratus Mountains of South Kalimantan Province, Indonesia, is one of the diversity centers of native orchids in the world. In this region, hundreds of native orchids germplasms are currently threatened by natural and human impacts, making it difficult to find in their customary habitat (Muslimah, Rachmawaty, Hoesain, Ninsyh, & Yulianto, 2011). Even though some of them have been categorized as endangered species by CITES (Committee of the International Trade of Endangered Species). One orchid species that is very difficult to find in their customary habitat is the moth orchid (*Phalaenopsis amabilis* (L.) Blume). The local people of this region recognized three forms of the orchids, namely the 'Pelaihari' from the Birah Bajuin Mountain, Tanah Laut Regency, the 'Meratus' from the Hulu Sungai Selatan Regency, and the 'Halong' from the Balangan Regency of South Kalimantan (Rusmayadi, Sumardi, Sudjatmiko, & Kuswidyosusanti, 2017). For some orchid collectors, these three forms are very difficult to distinguish morphologically. Hence, further verification using molecular markers is very necessary.

The moth orchid of 'Pelaihari' is one of the most famous orchids in the world (Muslimah et al., 2011). This moth orchid is generally recognized based on the flower characteristics, both shape, size, motif, and length of the flowering period (Tsai, Chou, Wang, Ko, Chiang, & Chiang, 2015). In general, the flower of the 'Pelaihari' orchid is white, decorated with a yellow-brown spot motif on the labellum, and has flowers with florets reaching more than 50 units on each stalk. The orchid stalk could reach 80 cm, the length of the flowering period is up to 6 months (Muslimah et al., 2011). Consequently, it is not surprising that this orchid is the most searched and is very popular with collectors and breeders around the world. In 2009, this moth orchid was even designated as one of Indonesian's national flowers or "Puspa Pesona" (in Indonesian terms) making it a national superior variety or the best cross-parent material for the breeding program (Muslimah et al., 2011).

In Indonesia, the moth orchids is spread across several large islands, such as Java, Sumatra, Sulawesi, Kalimantan, and Papua (Fatimah & Sukma, 2011). However, the existence of the moth orchids in Meratus Mountain of the South Kalimantan Province is more worrying than the others, as mentioned earlier. Thus, the conservation and breeding programs of the orchid are very urgent to implement. The Indonesian Orchid Association of South Kalimantan branch has carried out some activities to conserve and preserve this germplasm. While these efforts have not been optimally carried out, some activities showed less satisfactory results (Muslimah et al., 2011). The limited information about orchid genetics is one of the obstacles to these two activities.

In general, the conservation and breeding tasks involve several principal activities, one of which is identifying and characterizing germplasm with a comprehensive study (van de Wouw, van Hintum, Kik, van Treuren, & Visser, 2010). According to Pellens and Grandcolas (2016), critical and accurate information about germplasm sources is urgently needed to support these programs. Hence, the objective of our study was to determine and analyze the genetic relationship among several morphological and molecular characteristics of this orchid, particularly by RAPD/Random Amplified Polymorphic DNA markers. While these markers have certain limitations, a combination of these two markers may represent a comprehensive feature of the genetic diversity of this germplasm (Rocha et al., 2020). Until now, many researchers have been using these markers for assessing the genetic diversity of various germplasms, like orchids (Khoddamzadeh, Sinniah, Kadir, & Kadzimin, 2010). In Indonesia, RAPD has been used for analyzing the genetic diversity of orchids but is still very limited. Moreover, the orchid samples are the hybrids, not from the natural areas, as employed by Sulistianingsih and Purwantoro (2012). Thus, the results of our study are

valuable in supporting orchid conservation and breeding programs in the future.

II. MATERIAL AND METHOD

A. Plant Materials

A total of ten orchids samples with several morphological characteristics (Table 1), comprising nine moth orchids (*P. amabilis*) species and one of the deer-antlered orchid *P. cornu-cervi* (as an outgroup) were collected randomly from three locations of the Meratus Mountains, including three regencies of South Kalimantan, Indonesia, namely Tanah Laut (Tala), Balangan and Hulu Sungai Selatan (HSS) (Figure 1). Specifically, for the hybrid orchid outgroup samples were obtained from the Food, Agriculture, and Fisheries Service Agency of Banjarmasin City and an orchid collector in Banjarbaru regency, South Kalimantan. For morphological analysis, samples were directly observed at the sampling locations. For molecular analysis, orchid leaves were sampled and put into a plastic bag containing silica gel.

B. Morphological Observation

The morphological observations were carried out directly at the sampling locations on six morphological characters, those were: resilience of flowers, thickness of the petals, length of the flower stalk, number of blossoms

Table 1. List of orchid samples employed in the study

Local Name	Code	Scientific Name	Origin (Regency)
Anggrek bulan 'Pelaihari'	PA-01	*P. amabilis*	Tanah Laut
Anggrek bulan 'Halong'	PA-02	*P. amabilis*	Balangan
Anggrek bulan 'Halong'	PA-03	*P. amabilis*	Balangan
Anggrek bulan 'Halong'	PA-04	*P. amabilis*	Balangan
Anggrek bulan 'Halong'	PA-05	*P. amabilis*	Balangan
Anggrek bulan 'Meratus'	PA-06	*P. amabilis*	Hulu Sungai Selatan
Anggrek bulan 'Halong'	PA-07	*P. amabilis*	Balangan
Anggrek bulan[a]	PA-08	*P. amabilis*	Tanah Laut
Anggrek bulan[a]	PA-09	*P. amabilis*	Tanah Laut
Anggrek bulan gergaji[b]	PC	*P. cornu-cervi*	Tanah Laut

Remarks: ahybrid, as comparison ban out group

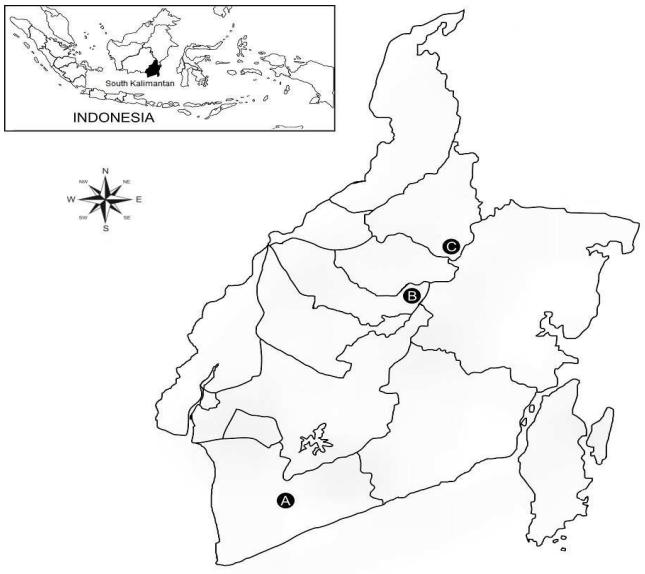

Figure 1. A map of South Kalimantan showing the three sampling locations (along the Meratus Mountains), where the moth orchids were collected: The regency of Tanah Laut (A), Hulu Sungai Selatan (B), and Balangan (C)

per stalk, and visibility of veins on leaves, as well as leaf shapes.

C. Molecular Analysis

Molecular analysis of the orchid was conducted in the Laboratory of Genetics and Molecular Biology, Faculty of Mathematics and Natural Sciences, Universitas Lambung Mangkurat, Indonesia. The activities started with the extraction of DNA by the DNAzol kit protocol following manufacturer's procedure. The DNA was then quantified and measured by UV-Vis spectrophotometer at 260 nm wavelengths. Amplification of this genetic material was conducted using 10 of the 22 selected RAPD primers (Table 2) (Sulistianingsih & Purwantoro, 2012) with a total volume of 20 µl, consisting of 17 µl master mix PCR (containing deionized water; PCR buffer; dNTPs; $MgCI_2$, Taq DNA polymerase), 1.5 ng/µL of each primer (100 picomoles) and 1.5 ng/µL of genomic/template DNA.

This reaction was performed by using Thermal Cycler PCR (Techne, TC3000G, USA) with a cycling condition (Mursyidin & Daryono, 2016): initial denaturation at 94°C for 5 min; denaturation at 94°C for 30 secs, annealing at 37°C for 30 secs, and extension to 72°C for 1.5 min (these stages were repeated for 45 cycles); and a final extension at 72°C for 7 min. The amplified DNA was separated by 1.5% of agarose gel electrophoresis and 1X TBE buffer (pH 8.0) as a supporting medium. These samples were stained by nucleic acid gel stains (GelRed, Biotium, USA) and observed with the DNA ladder (100 bp, Vivantis). Observation of DNA fragment of each primer which was generated conducted by UV transilluminator and digital camera (Nikon Coolpix L610).

Table 2. Selected RAPD primers employed in the study

Primers	Sequences (5'-3')	GC content (%)
OPA-02	TGCCGAGCTG	70
OPA-04	AATCGGGCTG	60
OPB-01	GTTTCGCTCC	60
OPB-06	TGCTCTGCCC	70
OPB-07	GGTGACGCAG	70
OPS-12	CTGGGTGAGT	60
OPA-09	GGGTAACGCC	70
OPA-10	GTGATCGCAG	60
OPB-05	GATGACCGCC	70
OPB-10	CTGCTGGGAC	70

Source: Sulistianingsih and Purwantoro (2012)

D. Data Analysis

Data were analyzed both for morphological and molecular markers. Data analysis was started with scoring and standardizing the obtained morphological dataset. Shannon-Weaver diversity index (H') was used to determine the phenotypic diversity of this germplasm. Diversity indices were calculated based on phenotypic frequency using the standardized Shannon-Weaver Diversity index formula (Rabara, Ferrer, Diaz, Newingham, Ma, & Romero, 2014). Multivariate statistical analyses of characterization data were conducted using cluster analysis. This analysis was done using the MVSP ver. 3.1 software (Kovach, 2007). The distance matrix was generated using the Euclidean Distance Coefficients and used as input for clustering using the unweighted pair group of arithmetic means (UPGMA) method.

For molecular data, each DNA fragment that develops at a particular rate of electrophoresis gel was measured by using a linear regression equation and considered as a single locus. Hence, the same DNA fragments of some individual plants were interpreted as one homologous locus. The locus was then converted into binary matrix data by scoring the value of one (1) if there is a DNA fragment and zero (0) if there is no DNA fragment. The binary matrix data was then derived into a genetic distance matrix. To calculate the genetic distance of the genotype pairs found in different individuals, the Dice coefficient was applied. Based on the value of genetic similarity then the clustering analysis was conducted. The clustering analysis and reconstruction of a phylogenetic relationship of this germplasm were performed using the UPGMA with the assistance of the NTSys ver. 2.2 (Rohlf, 2009). A bootstrap analysis was also

performed to evaluate the internal nodes on the dendrogram.

III. RESULTS AND DISCUSSION

A. Morphological Characteristics

Although the three forms of orchids from the Meratus Mountains appear to be the same (relatively difficult to distinguish) morphologically (Figure 2), the results of further observations of some of these characters show quite significant differences (Table 3). For example, based on leaf shape, the three forms of orchids could be distinguished because the leaf shapes are different, namely *ovate* for 'Pelaihari', *elliptic* for 'Meratus', and *lanceolate* for 'Halong' (Table 3). Other differences appear in the leaf veins and thickness of petals, whereas the 'Pelaihari' orchids has visible leaf veins and thin petals (Table 3). Based on Table 3, it is also seen that 'Pelaihari' orchids have different characteristics compared to the other two orchids found in the Meratus Mountains, namely a long stalk of the flower with 9-17 blooms, a long resilience of the flower blooms (4-6 months), and a yellow-brown spot at the labellum, as well as a V-shape at the end of the labellum. The complete information on the differences in morphological characters of the orchids was presented in Table 3.

In brief, morphological observations reveal the unique feature of the three forms of moth orchids found in the Meratus Mountains. The unique feature is characterized by the resilience of flowers bloom and the number of blossoms per stalk, as well as the shape of the observed leaves (Table 3). According to van

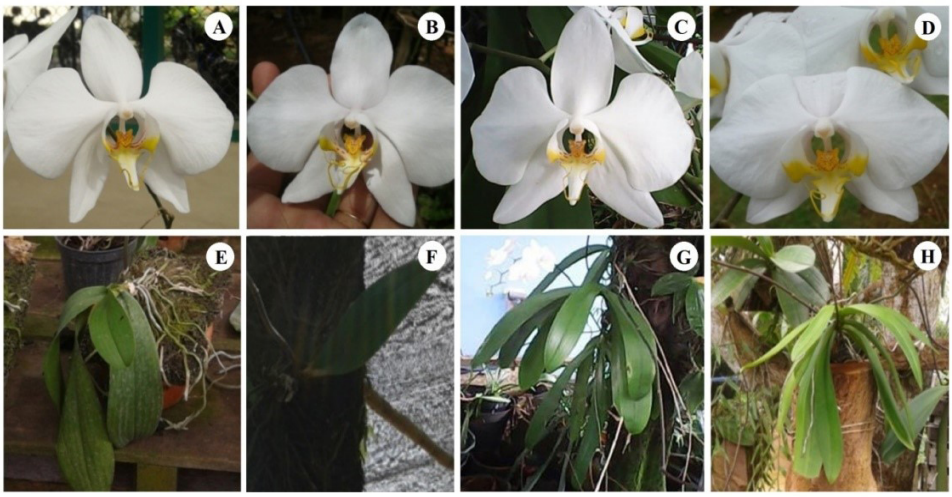

Figure 2. The morphological features of the moth orchid flowers (A-D) and its plant habitus (E-H).

Remarks: A = *P. amabilis* 'Halong'; B = *P. amabilis* 'Meratus'; C = *P. amabilis* 'Pelaihari'; D = *P. amabilis* hybrid, as a comparison)

Table 3. The morphological differentiation of the moth orchids endemically from the Meratus Mountains, South Kalimantan, including the hybrid (a comparison)

Traits observed	*P. amabilis 'Halong'*	*P. amabilis 'Meratus'*	*P. amabilis 'Pelaihari'*	*P. amabilis 'Hybrid'*
The resilience of flowers bloom	± 4-5 months	±2 months	± 4-6 months	< 2 months
The thickness of the petals	Thick	Thick	Thin	Thick
Length of the flower stalk	± 1.5 m	± 1.0 m	± 1.5 m	± 1.0 m
Number of blossoms per stalk	5-15 unit	5-10 unit	9-17 unit	5-10 unit
Visibility of veins on leaves	Invisible	Invisible	Visible	Invisible
Leaf shape	Lanceolate	Elliptic	Ovate	Lanceolate

Huylenbroeck (2018), the uniqueness of this germplasm is also based on the triangular shape of the labellum and the color sharpness of the flowers. Interestingly, based on this study, the 'Pelaihari' shows distinct flower formation; the number of florets in one stalk, and the length of the flowering period is more striking than the other two orchids found in the region. So, it is not surprising that in 2009 this orchid had obtained formal legality from the Indonesian minister of agriculture as a superior national cultivar (Muslimah et al., 2011).

According to Anumalla, Roychowdhury, Geda, Mazid, and Rathoure (2015), although morphological marker has a weakness because it is strongly influenced by environmental factors, breeders are using this marker to evaluate germplasm in the early stages of plant growth and development. In other words, morphological analysis is still used in determining the genetic diversity of germplasm. In genetics, the morphological marker or phenotype is the results of the expression of genotype which is influenced by environmental factors. Thus, further verification of the germplasm studied using more stable molecular markers on environmental factors is very important. Furthermore, a combination of the two markers (morphological and molecular) is expected to produce a more comprehensive feature of the genetic diversity of germplasm (Rocha et al., 2020).

B. Genetic Diversity

Based on the morphological markers, the moth orchids of the Meratus Mountains have a moderate level of genetic diversity, indicated by Shannon's index value of 0.5 (Table 4). However, two of the morphological traits observed in this study were shown to have a high level of diversity, i.e., the resilience of flowers bloom and the number of blossoms per stalk, with an index of 0.92 each.

Table 4. Genetic diversity (H' index) of the moth orchid based on morphological traits

Morphological traits	H' index
The resilience of flowers bloom	0.92[c]
The thickness of the petals	0.19[a]
Length of the flower stalk	0.19[a]
Number of blossoms per stalk	0.92[c]
Visibility of veins on leaves	0.19[a]
Leaf shape	0.60[b]
Average	0.50[b]

Remarks: [a]low, [b]moderate, [c]high

Table 5. Polymorphism degree of the moth orchids from the Meratus Mountains of South Kalimantan, including their number and size of amplified DNA fragments generated by RAPD markers

Primer	The size range of amplified DNA fragment (bp)	Total of DNA fragment (loci)	Number of polymorphic DNA	Polymorphism (%)
OPA-02	221-1157	24	24	100
OPA-04	312-1698	27	27	100
OPB-01	294-2027	24	24	100
OPB-06	165-1721	32	32	100
OPB-07	171-1501	31	31	100
OPS-12	162-2099	16	16	100
OPA-09	189-527	8	8	100
OPA-10	558-1622	7	7	100
OPB-05	132-319	7	7	100
OPB-10	129-1522	16	16	100
Total/Average		192	192	100

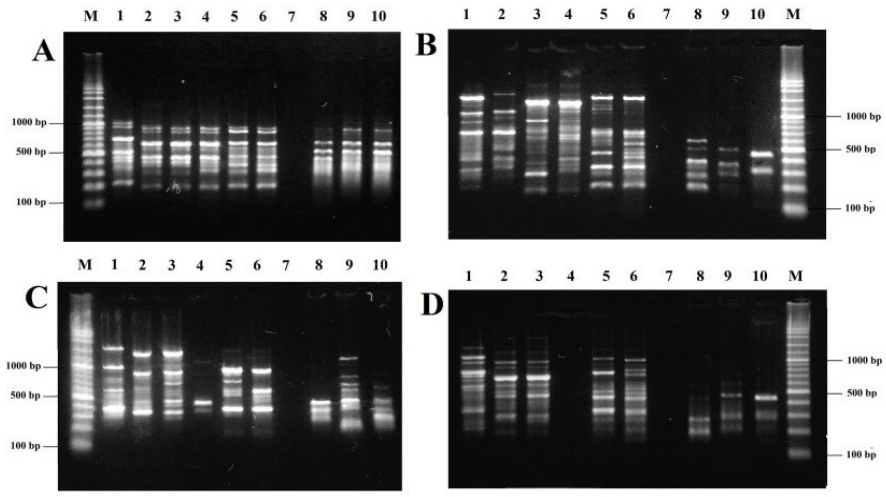

Figure 3. Visualization of amplified DNA fragments generated by four representative RAPD markers used in this study

Remark: A = OPA-02; B = OPB-06; C = OPB-01; D = OPB-07; M = 100 bp DNA ladder (Vivantis); Lane 1 = *P. amabilis* 'Pelaihari'; Lane 2-5, 7 = *P. amabilis* 'Halong'; 6 = *P. amabilis* 'Meratus'; 8-9 = *P. amabili*s hybrid; 10 = *P. cornu-cervi*, an outgroup

In contrast to molecular markers, this germplasm shows high genetic diversity, shown by the polymorphism degree of 100% for all primers used (Table 5). According to Mursyidin and Daryono (2016), genetic diversity can be described using polymorphism, average heterozygosity, and allelic diversity. In RAPD, this genetic diversity or polymorphisms are analyzed by measuring the presence vs. absence of random amplified DNA fragments (Mursyidin and Daryono, 2016).

In this case, the moth orchids of the Meratus Mountains of South Kalimantan, showed a unique profile of DNA fragments generated by RAPD markers (Figure 3). In general, a total of 192 DNA fragments (loci) have been generated by these primers, where each primer resulted in a different number and size of DNA fragments (Table 5). Furthermore, OPA-10 and OPB-05 produced the lowest number of DNA fragments (7 bands), whereas OPB-06 was the highest (32 bands).

The differences in the number and size of DNA fragments in PCR may depend on the attachment site of a DNA primer to the genome of the sample (Clark & Pazdernik, 2013). It means, that each primer has a specific sequence, and the attachment site must be different (Clark & Pazdernik, 2013). These differences are influenced by other factors as well, such as the concentration of $MgCl_2$, DNA template, and DNA polymerase, as well as PCR annealing temperature (Dominigues, 2017; Maddocks & Jenkins, 2017). Siddiqi and Nollet (2019) stated that the presence of polymorphic DNA fragments in a genome reveals the genetic diversity of germplasm. In other words, polymorphic DNA fragments explained the genetic status of germplasm in the population (Chenu, 2015).

C. Genetic Relationships

The cluster analysis shows that this orchid germplasm is divided into different groups, two for morphological (Figure 4) and six for molecular markers (Figure 5). According to Ewens (2013), these differences may be caused by several factors, such as the evolution and adaptation to the local conditions, cross-breeding, population history, speciation, population distribution, and gene flow (Ewens, 2013).

Following the morphological markers, the moth orchid of 'Meratus' was joined by the hybrid in the first cluster, whereas 'Halong' and 'Pelaihari' were in the second. For the molecular

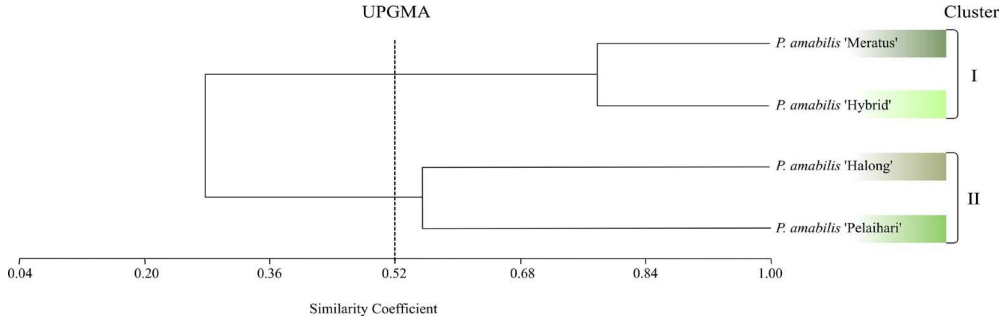

Figure 4. Relationship of the moth orchids from the Meratus Mountains of South Kalimantan, based on morphological characters

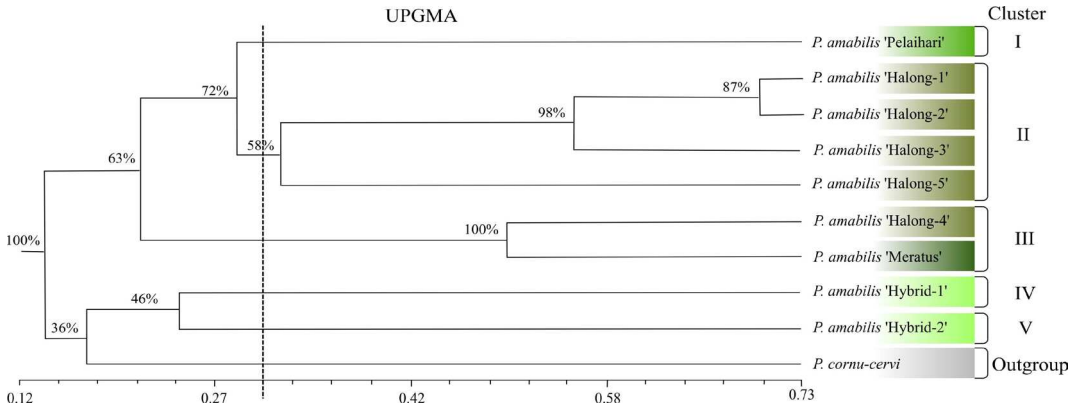

Figure 5. Relationship of the moth orchids from the Meratus Mountains of South Kalimantan, based on RAPD analysis. Note. percentage on nodes were generated by bootstrap 1,000 replicates

marker, the moth orchid of 'Pelaihari' was separated from other samples and formed a single group (Cluster I). The 'Halong' moth orchids were grouped into Cluster II, except for one sample which was grouped to Cluster III, and joined with the 'Meratus'. The hybrids (Clusters IV & V) formed a closely related group to an outgroup.

Following the dendrograms (Figures 4 & 5), the clustering of moth orchid germplasm was near corresponding to their geographic origins. According to Cotrim, Monteiro, Sousa, Pinto, & Fay (2016), the pattern of this relationship may be caused by hybridization and genetic introgression (repeated back-crossing). In this context, hybridization may accelerate speciation via adaptive introgression, or cause near-instantaneous speciation by allopolyploidization (Cotrim et al., 2016).

In brief, these two relationships (dendrograms) also represented a linearization or congruency between morphological and molecular markers. While this congruence needs further verification, some researchers have shown this matter. For example, for forage palm genotype (Rocha et al., 2020), neotropical passerine (Garcia, Barreira, Lavinia, & Tubaro, 2016), and Calochromini (Motyka, Masek, and Bocak, 2017).

Further, a comprehensive analysis of this orchid relationship has been done by several researchers, for example, by Tsai, Chiang, Huang, Chen, & Chou (2010) using the internal transcribed spacer (ITS) sequences. Based on their study, *P. amabilis* is incorporated in the same cluster as *P. sanderiana*, whereas *P. cornu-cervi* is closely related to *P. borneensis*. Based on microsatellite markers, *P. amabilis* is closely

related to *P. fuscata* (Fatimah & Sukma, 2011). Using the RAPD marker, Niknejad, Kadir, Kadzimin, Abdullah, and Sorkheh (2009) reported that P. amabilis has a close relationship with *P. hieroglyphica*, whereas *P. cornu-cervi* with P. mannii and *P. pantherina*. Based on the results of our study, *P. amabi*lis has a distant relationship with *P. cornu-cervi*.

Finally, this information may be valuable for improving the efficiency and effectiveness of plant conservation and breeding programs in the future (Flint-Garcia, 2013; Pellens & Grandcolas, 2016; Singh, 2019). For conservation, information on genetic relationships can apply in inferring species and their evolutionary history, including helping analyze species delimitation, gene flow, and genetic differentiation. In other words, the use of this 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). For plant breeding programs, similar information can be used in predicting the genetic diversity of the offspring when individuals cross (Acquaah, 2012; Turner-Hissong, Mabry, Beissinger, Ross-Ibarra, & Pires, 2020).

IV. CONCLUSION

In this study, morphological and molecular markers provided a unique feature of the genetic relationship of the moth orchids of the Meratus Mountains of South Kalimantan, Indonesia. Based on the cluster (UPGMA) analysis, this germplasm is divided into two (for morphological markers) and five groups (for molecular ones). The results indicate that the grouping of orchid germplasm has quite corresponded to geographic location. While the results provide valuable information to support orchid conservation and breeding in the future, it requires further verification, especially using more accurate molecular markers, such as SNP.

ACKNOWLEDGEMENT

The authors wish to thank the Head of the Food, Agriculture, and Fisheries Service Agency, Banjarmasin City, Indonesia for providing the *P. amabilis* hybrid and *P. cornu-cervi* as a comparison and outgroup, respectively. The author also wishes to thank Rifa'i and Zia for helping with samples collection and molecular analysis in the laboratory.

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