ARAŞTIRMA MAKALESİ	RESEARCH ARTICLE

Genetic Diversity and Relationship of Native *Phalaenopsis* Orchids: A Case Study of Indonesian Archipelago

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Abstract

The native *Phalaenopsis* is valuable germplasm for future orchid breeding programs and for its conservation because it provides many beneficial traits or genes. This study aimed to determine and analyze the molecular diversity and phylogeny of Indonesian native *Phalaenopsis* by a DNA barcoding (*mat*K) marker. A total of 19 samples of *Phalaenopsis* orchids were used in this study. All leaf samples of orchid were extracted and purified using a commercial DNA isolation kit from Geneaid Biotech Ltd., Taiwan (GP100). The DNAs were then amplified by specific *mat*K primers: Forward (5'-CGTACAGTACTTTTGTGTTTACGAG-3') and Reverse (5'-ACCCAGTCCATCTGGAAATCTTGGTTC-3'). The DNA targets or products (*mat*K) were purified and sequenced by the Sanger-bidirectional method at 1st Base Ltd., Malaysia. Before further analysis, the *mat*K sequences of *Phalaenopsis* were edited, reconstructed, and aligned with the assistance of Clustal W in the MEGA 11 software. Its genetic diversity was determined using the nucleotide diversity index ($\pi\%$), and the phylogenetic analysis was performed using the maximum likelihood (ML) method with a statistical bootstrap. The phylogenetic relationship was also assessed using principal component analysis (PCA). Based on this marker, the native *Phalaenopsis* has a high genetic diversity ($\pi\% = 1.70$). In addition, the phylogenetic analysis revealed that this germplasm was separated into seven clades, where *P. pantherina* has the closest relation to *P. cornu-cervi* and *P. gigantea*. Conversely, the highest genetic distance was to *P. amabilis* from South Kalimantan and to *P. celebensis* from Sulawesi, at a coefficient divergence of 0.084. Our findings provide an essential foundation for supporting future orchid breeding practices, including conservation, on local and global scales.

Keywords: Breeding program, Genetic diversity, Moth orchid, Ornamental plant, Phylogenetic relationship.

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1. Introduction

Phalaenopsis, commonly known as a moth or moon orchid, is the most famous orchid genus in the world, with a relatively high number of species (Hsu et al., 2018). Globally, this orchid comprises about 66 native species and is distributed mainly in the Asiatic regions (Hinsley et al., 2018), such as India, Sri Lanka, Taiwan, Philippines, and Indonesia (Deng et al., 2015). In Indonesia, more than 20 species of *Phalaenopsis* are present in seven large islands, including Sumatra, Java, Borneo (Kalimantan), Celebes (Sulawesi), Timor (Nusa Tenggara), Moluccas (Maluku), and Papua (Rahayu et al., 2015). In this case, Borneo is the third largest island with a high diversity of orchids worldwide (Siregar, 2008). Besi et al. (2021) estimated that 2500-3000 species (equivalent to 10% of the world's orchids) are present on this island.

In general, native orchids are unique germplasm. They can grow in their habitat without human assistance. Besides, they genetically stored many valuable genes or traits for conservation and breeding programs (Li et al., 2021). However, existing of these orchids in their customary habitat is challenged, even threatened by natural disturbance and human intervention (Mursyidin et al., 2021a). Ecologically, orchids grow in specific habitats, so they are often present in small populations and a narrow-pattern distribution. Deforestation, habitat degradation, overexploitation, and illegal trading are human factors that contribute to reducing this orchid population in nature (Zahara and Win, 2019). Consequently, the preservation, breeding, and analysis of the genetic diversity of *Phalaenopsis* orchids are of great importance.

Conventional methods utilizing morphological markers have been used for years to analyze the genetic diversity of orchids (Kwon et al., 2017). However, these methods are nevertheless time-consuming, and morphological markers are affected by environmental conditions (Nadeem et al., 2018). The genetic diversity of *Phalaenopsis* has also been studied using several molecular markers, e.g., Random Amplified Polymorphic DNA (RAPD) (Niknejad et al., 2009) and Simple Sequence Repeat (SSR) (Tsai et al., 2015). Again, these markers also have drawbacks, such as being highly subjective and producing less precise analytical results (Lee et al., 2017).

Nowadays, sequencing-based DNA markers, such as DNA barcoding or chloroplast DNA markers, are commonly used in determining the genetic diversity and relationship of orchids (Jheng et al., 2012). While these markers have a few disadvantages, including relatively high costs, they are faster and more accurate in determining the genetic diversity of germplasm (Singh et al., 2017). Among markers present, the Consortium for the Barcode of Life has recommended *maturase* K (*mat*K) as one of the DNA barcoding markers for these objectives, i.e., determining the genetic diversity and phylogenetic relationship of germplasm (CBOL Plant Working Group, 2009).

Conceptually, *mat*K is a functional gene in the chloroplast genome (cpDNA) that shows a high mutation rate and, therefore, is known as a fast-evolving gene (Barthet et al., 2020). Kar et al. (2015) reported that this gene has a substitution rate three times higher than the *rbc*L, another similar gene in cpDNA. This gene also displays varying numbers and sizes of indel (insertions-deletions) mutation. As a result, it provides a high phylogenetic signal for resolving genetic relationships among plants at all taxonomic units (Kar et al., 2015). In other words, *mat*K provides many excessive variability sequences that can be aligned to determine evolutionary relationships of germplasm from species to higher taxonomic levels (Kar et al., 2015).

The objectives of our study are to determine and analyze the molecular diversity and phylogeny of the Indonesian native *Phalaenopsis* using the *mat*K marker. Our study may provide results to support future orchid breeding programs and its conservation, locally and globally, particularly for *Phalaenopsis*.

2. Materials and Methods

2.1. Plant materials

We have collected and used 19 samples of orchids from several parts of Indonesia (*Figure 1*). Samples were categorized into 15 *Phalaenopsis* species and two *Paraphalaenopsis* (used as an outgroup). Detailed information on the collected samples were presented in *Table 1*.

Figure 1. Map of Indonesia showing locations of 19 native Phalaenopsis samples collected for this study.

Table 1. Sampling location (province origin), common name, and length of matK sequence of native Phalaenopsis used in this study.

Sampling location (Province Origin)	Species	Common name	Length of *mat*K (bp)
South Kalimantan	*P. amabilis*	Moon or moth orchids	861
	P. cornu-cervi	Deer antlered *Phalaenopsis*	918
	P. deliciosa	Delicate *Phalaenopsis*	815
	P. difformis	Dark brown *Phalaenopsis*	977
	P. modesta	Modest *Phalaenopsis*	826
	P. pantherina	Panther-like *Phalaenopsis*	803
	P. sumatrana	Sumatran *Phalaenopsis*	891
	P. zebrina	Zebra-like *Phalaenopsis*	832
East Kalimantan	*P. bellina*	Beautiful *Phalaenopsis*	911
	P. gigantea	Giant *Phalaenopsis*	931
	P. lamelligera	Deer antlered *Phalaenopsis*	876
	P. modesta	Modest *Phalaenopsis*	878
	Para. labukensis	Mouse tail orchid	839
West Kalimantan	*P. bellina*	Beautiful *Phalaenopsis*	835
	P. corningiana	Corning's *Phalaenopsis*	811
	Para. serpentilingua	Mouse tail orchid	819
Mentawai, Sumatra	*P. violacea*	Violet moth orchid	795[a]
Sulawesi	*P. celebensis*	Celebes *Phalaenopsis*	1045[b]
Maluku	*P. amboinensis*	Amboin Island *Phalaenopsis*	898

Remarks: [a] the shortest; [b] the longest

2.2. DNA isolation and purification

All samples of orchid leaves were extracted and purified using a DNA isolation kit, commercially from Geneaid Biotech Ltd., Taiwan (GP100). In this stage, 50 g of leaf samples were crushed by mortar and prepared according to the manufacturer's instructions until pure DNA was obtained.

2.3. DNA quantification, amplification, and sequencing

The concentration and purity of the extracted DNA samples were determined using a UV-VIS spectrophotometry method. By specific primers of *mat*K (Le et al., 2020), i.e., Forward (5'-CGTACAGTACTTTTGTGTTTACGAG-3') and Reverse (5'-ACCCAGTCCATCTGGAAATCTTGGTTC-3'), the DNAs were amplified as following these conditions (Mursyidin et al., 2022): Initial denaturation was carried out at 94ºC for 5 min and denaturation at 94ºC for 30 sec. This was followed by 30 sec of alignment at the annealing temperature (48ºC); 45 sec of alignment at 72ºC for extension, and 7 min of alignment at 72ºC for final extension (72ºC). The reaction was repeated for 35 cycles and employed in a total volume of 25 µL, i.e., DNA template (2 µL; 20 ng), *mat*K-F/R primers (1 µL; 0.2 µmol), and 22 µL of PCR mix (MyTaq HS Red Mix, Bioline, UK). The separation of the DNA target (product) was conducted with electrophoresis of agarose gel (2%) in a 1X TBE buffer solution and GelRed DNA stain (Biotium Inc., USA) and visualized with a UV transilluminator. Finally, the purification and sequencing of DNA products was done by the Sanger-bidirectional method at 1st Base Ltd., Malaysia.

2.4. Data analysis

The analysis was initiated by manual reconstruction and editing of the *mat*K consensus sequence of *Phalaenopsis*, with the assistance of ClustalW in the MEGA 11 software (Tamura et al., 2021). After completion, all sequences were aligned using similar programs (Tamura et al., 2021) and in MultAlin (Mitchell, 1993). The purpose of multiple alignments was to observe the conserved region and some mutational events, such as substitution (transition-transversion) and indels (insertion-deletion) therein. Several variables were also analyzed, including GC content, polymorphic region (variable site; parsimony-informative; singleton site), transition/transversion ratio (Ti/Tv), including genetic diversity (π%). The latest (genetic diversity) was determined using the Nei and Li (1979) criteria: low (0.1 - 0.4); moderate (0.5 - 0.7), and height (0.8 - 2.0). The final analysis was the phylogenetic tree reconstruction carried out using the maximum likelihood (ML) method with a statistical bootstrap, a Kimura-2 parameter of substitution model, and a Nearest-Neighbor-Interchange (NNI) interference approach (Kimura, 1980; Tamura et al., 2021). The phylogenetic relationship was also confirmed using principal component analysis or PCA (Bahar et al., 2019; Mursyidin and Khairullah, 2020).

3. Results and Discussion

Determination of genetic diversity and the phylogenetic relationship of native orchids is beneficial in supporting future orchid plant conservation and breeding programs. According to Yusop et al. (2022), the native orchid is a germplasm with high genetic diversity for use in these activities, mainly as the parental cross. This study's native orchids of *Phalaenopsis* showed high genetic diversity (π%, 1.70) based on *mat*K markers (*Table 2*). The high genetic diversity is strongly correlated with the mutations that occur in it, i.e., substitutions (transitions and transversions) and indels (insertion-deletions) (*Figure 2*).

Table 2. Molecular characteristics of matK of Indonesian native Phalaenopsis

Parameter	*mat*K
Sequence length observed (bp)	795-1045
GC content (%)	31.42
Nucleotide diversity (π%)	1.70
Singleton site/SNP (bp)	126
Transition/transversion bias value (*R*)	0.70
Polymorphic sites (bp)	175

According to Suriani et al. (2021), variations in the order of gene sequence in cpDNA, including the *mat*K gene, are due to a single nucleotide mutation (SNP) present in the region. In this study, SNP recorded 126 incidents (*Table 2*). The number is almost the same as the report of Chen and Shiau, (2015) on the *mat*K sequence of *Anoectochilus*.

Molecularly, *mat*K is one of the chloroplast genes involved in photosynthesis. According to Barthet et al. (2020), this gene exhibits a relatively high mutation, which is beneficial for genetic diversity and relationship studies. Kar et al. (2015) reported that *mat*K has a substitution rate three times higher than other chloroplast genes,

especially *rbc*L, so often called a rapidly evolving gene. In contrast, indels are occasionally found in the gene, demonstrating a conservative pattern of nucleotide replacement (Clegg, 1993). However, indels in some plant families raise many questions about whether the gene can maintain its structure and function in the cell, especially in protein expression (Kar et al., 2015).
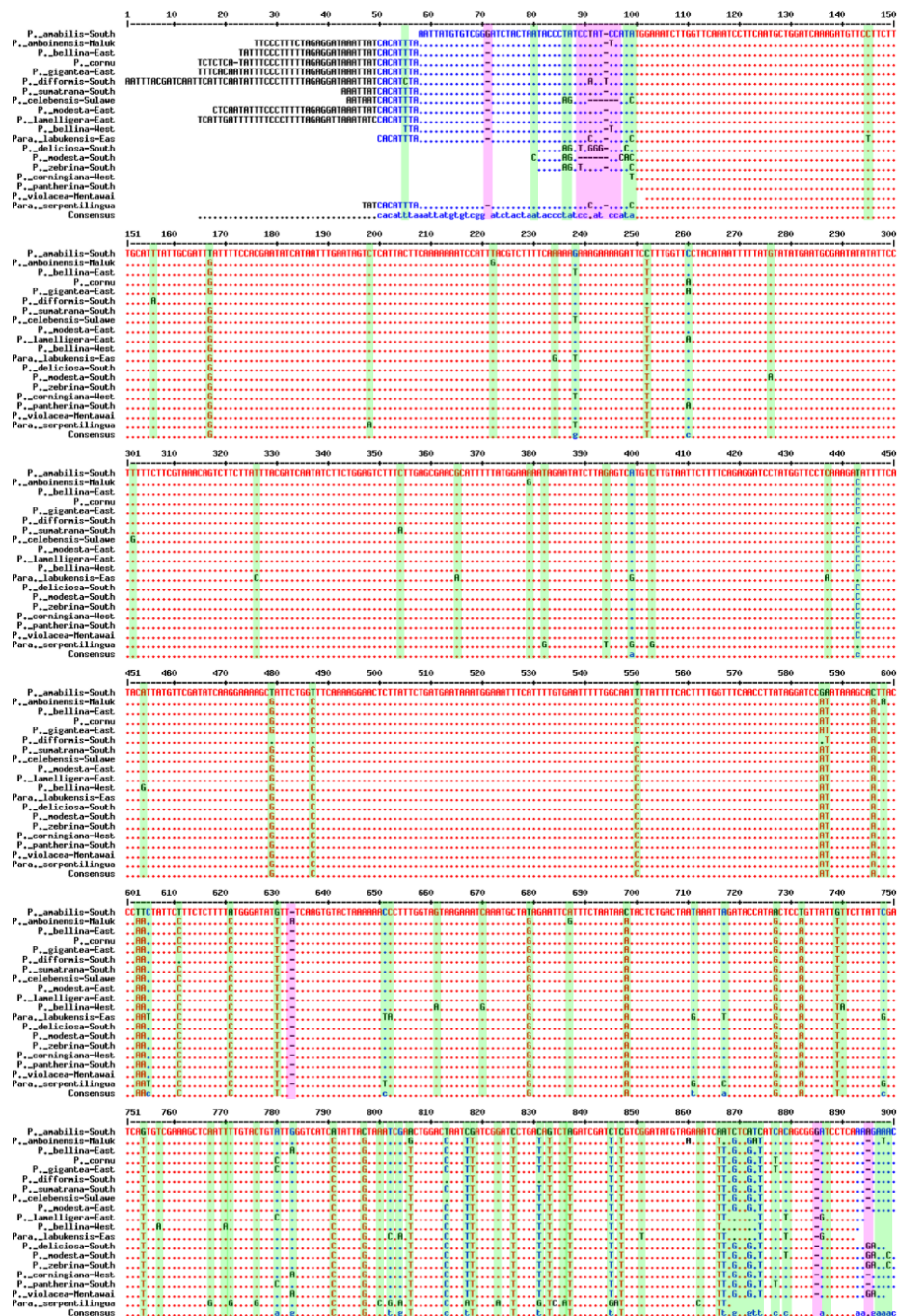

Figure 2. The multiple alignments of matK sequences, showing a barcoding motif of Indonesian native Phalaenopsis. Red marks = a conserved region; green highlight = substitutions; purple highlight = insertions-deletions (INDELs)

According to Chen and Shiau (2015), this mutation causes a difference in the length of the *mat*K sequence. In this study, the partial region of *mat*K of native *Phalaenopsis* ranged from 795 to 1045 bp in length (*Table 2*). Conversely, the complete gene was 1500 bp (Thakar et al., 2016) to 1536 bp in length (Mustafa et al., 2018). Partially, those gene has a different size in some plants, such as *Ficus* (Li et al., 2012), *Lycopersicum* (Căprar et al., 2017), and *Oryza sativa* (Mursyidin et al. 2021b), for about 830 bp–857 bp.

In this case, the high *mat*K sequence variation provides a high phylogenetic signal for resolving evolutionary relationships among plant germplasm at all taxonomic levels (Kar et al., 2015). In this context, the high genetic diversity of the native *Phalaenopsis* became a valuable source for the preservation and breeding program of orchid plants. According to Teixeira and Huber (2021), these genetic variations are beneficial in the preservation program, especially in promoting population survival and guaranteeing the adaptive potential of natural populations in the face of rapid environmental change. For plant breeding, genetic diversity is imperative, particularly in developing new cultivars with desired traits (Govindaraj et al., 2015), including parental selection (Aesomnuk et al., 2021) or selecting parents that are genetically distinct (Wu et al., 2021). In other words, determining resources with this high variation will become beneficial for widening the genetic base or gene pool of germplasm (Aesomnuk et al., 2021).

Conceptually, the different levels of genetic diversity are correlated with several factors, such as the breeding system, geographic range and variation, seed dormancy and dispersal mechanism, and natural selection (Huang et al., 2016). Life span and other life-history traits, such as the history of populations, are also affected by the genetic diversity level. Furthermore, environmental factors are often responsible for observed diversity patterns at small spatial scales (Huang et al., 2016; Ozer et al., 2021). However, on a practical level, the improvement of orchid genetic diversity must be continued through hybridization, introgression, mutation, and genetic engineering (Allier et al., 2020).

The phylogenetic analysis, using ML, showed that the native *Phalaenopsis* separates into seven groups or clades (*Figure 3*). Clades II and VI were the largest with 5 and 4 species, respectively. As in the ML, the PCA reveals seven groups (*Figure 4*), albeit with different numbers and compositions of species members. The closest relationship is shown between P. pantherina and P. cornu-cervi from South Kalimantan, as for *P. pantherina* with *P. gigantea* from East Kalimantan (*Figure 5*). Morphologically, although these three orchids have different flower shapes, they have relatively the same primary flower color and are decorated with line or strip motifs on the petals (Lafarge, 2015). In contrast, the farthest relationship by *P. amabilis* from South Kalimantan with *P. celebensis* from Sulawesi, with a divergence coefficient of 0.084 (*Figure 5*). According to Lafarge (2015), morphologically, they have almost the same shape and floral motifs, i.e., flowers with a white-base color and a moth-like shape.
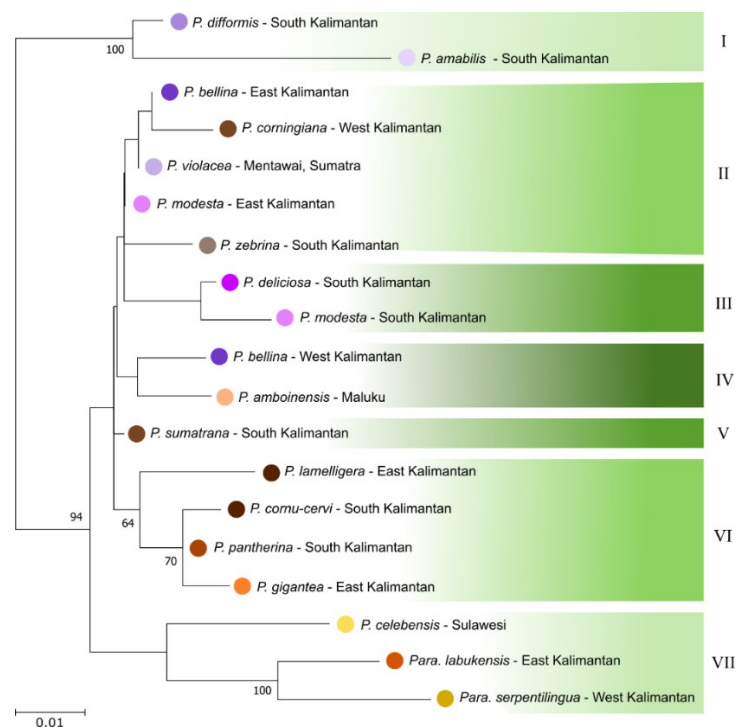

Figure 3. Phylogenetic tree of maximum likelihood (ML), showing a separation of Indonesian native Phalaenopsis into seven clades. The tree was evaluated by bootstrap, indicated by values on internal nodes
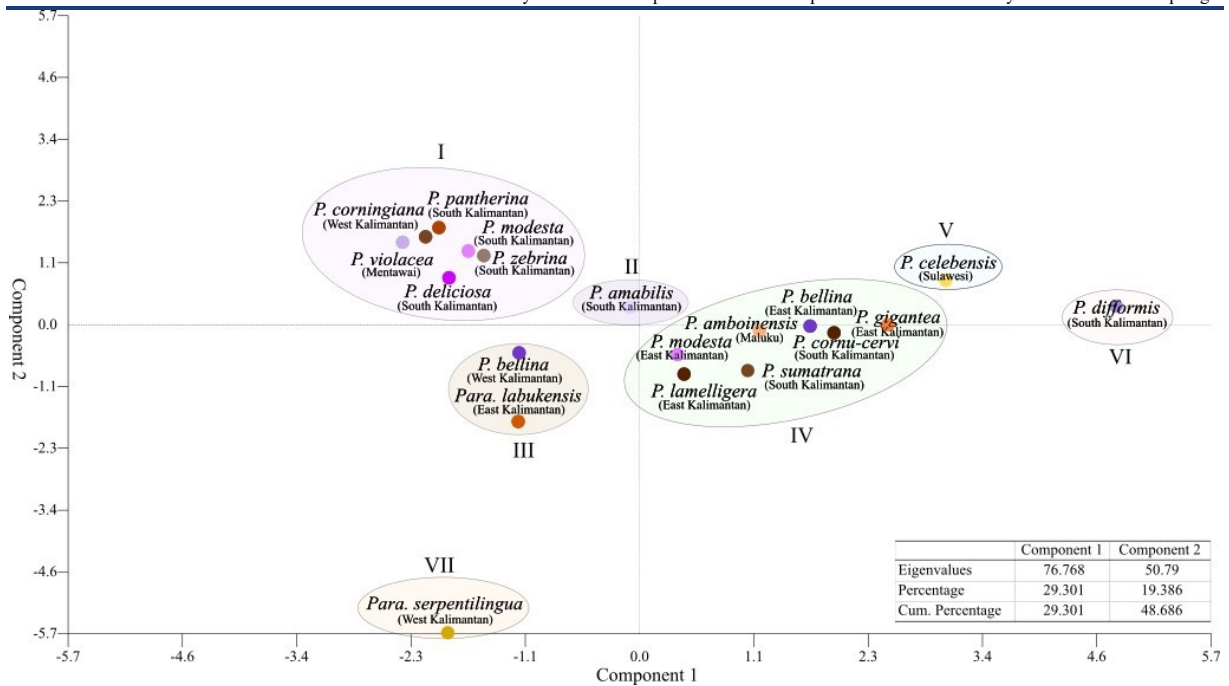
Figure 4. The PCA (principal component analysis) groupes Indonesian native Phalaenopsis into seven clades.
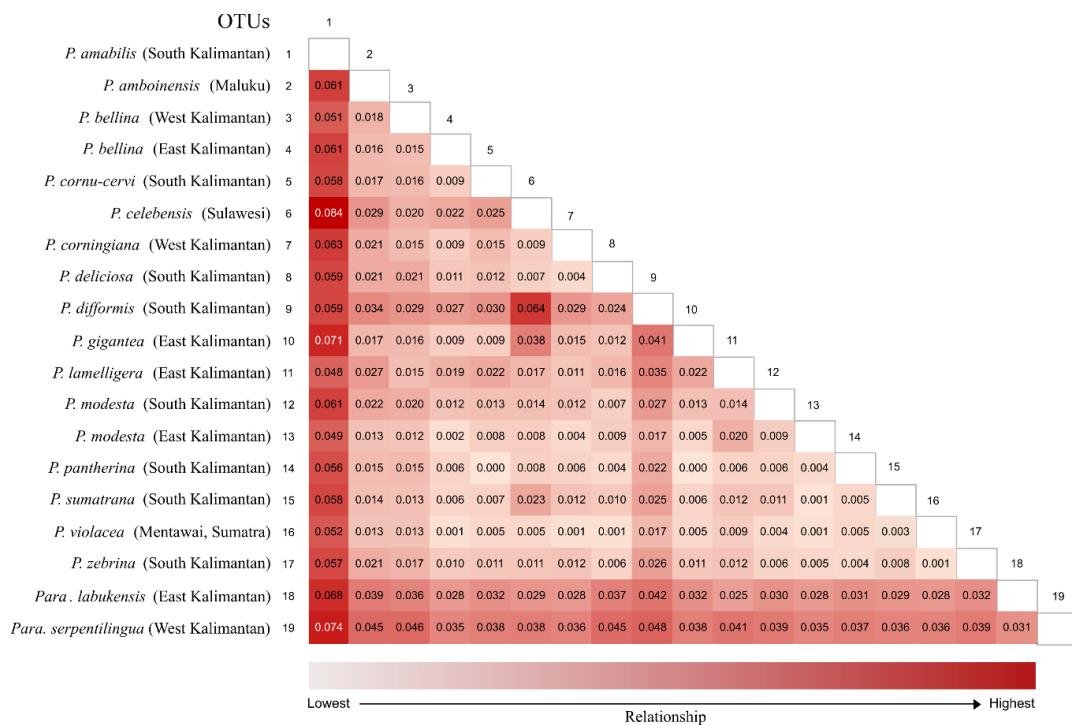

Figure 5. A heatmap, showing the genetic divergence among Indonesian native Phalaenopsis. OTUs = operational taxonomic units

Based on the *rbc*L and *trn*L-F markers, Mursyidin et al. (2021a) reported the close relatedness between *P. amabilis* and *P. celebensis* with a similarity coefficient of 93.90%. On the other hand, based on ITS (internal transcribed spacers) markers, Tsai et al. (2010) reported the close relatedness between *P. amabilis* and *P. sanderiana*; between *P. cornu-cervi* and *P. borneensis*. According to Fatimah and Sukma (2011), the close relatedness of *P. amabilis* to *P. fuscata* is shown by microsatellite markers. Based on the RAPD marker, *P. amabilis* is closely related to *P. hieroglyphica*, whereas *P. cornu-cervi* with *P. mannii* and *P. pantherina* (Niknejad et al., 2009).

According to Acquaah (2015), crossing parents with distant relationships may generate descendants with high diversity. In contrast, crossing elders with close relationships tend to produce descendants with narrow genetic diversity and tend to inbreed (CBOL Plant Working Group, 2009). Briefly, this information is useable in the orchid's breeding and preservation efforts. Furthermore, for the preservation program, our results can be utilized in inferring species and their evolutionary history, including gene flow, genetic differentiation, and species delimitation.

4. Conclusions

Based on the *mat*K region, the native *Phalaenopsis* from Indonesia has a high genetic diversity ($\pi\%=1.70$). The phylogenetic analysis revealed that this germplasm is grouped into seven main clades, where *P. pantherina* has the closest relation to *P. cornu-cervi* and *P. gigantea*. In contrast, the farthest relationship was found to be between *P. amabilis* from South Kalimantan and *P. celebensis* from Sulawesi, with a divergence coefficient of 0.084. Our results provide an essential foundation for supporting future orchid breeding and conservation practices on local and global scales.

Acknowledgment

Ethical Statement

There is no need to obtain permission from the ethics committee for this study.

Conflicts of Interest

We declare that there is no conflict of interest between us as the article authors.

Authorship Contribution Statement

Concept: DHM; Design: DHM.; Data Collection or Processing: MRF; Statistical Analyses: DHM, MRF; Literature Search: MRF; Writing, Review, and Editing: DHM; Read and approved the final manuscript: DHM, MRF.

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