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Assessment of the DNA Barcodes Characteristic of *Phalaenopsis deliciosa* based on *matK, rbcL,* and ITS

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ABSTRACT. Indonesia has high biodiversity for plant species, including orchids with medicinal potential such as *Phalaenopsis deliciosa*. Generally, morphological characters, especially in flowers are used for orchids identification. However, when the plants are not in the flowering period, the identification becomes difficult. Therefore an alternative method, such as molecular identification (DNA barcoding) needs to be applied for the best solution. This research aims to identify and compare three markers (*matK, rbcL*, ITS) for their function as potential barcodes for *Phalaenopsis deliciosa*. This study was conducted by DNA amplification using three different markers set. The data were analyzed using Bioedit, BLAST, and ClustalX. The result found that the identity level of *matK, rbcL,* and ITS to other orchids species was 99-98%, 98%, and 94-96%, respectively. Furthermore, *matK* and ITS showed high specificity for *Phalaenopsis deliciosa*, and are therefore recommended as the best molecular identification marker of genus *Phalaenopsis*.

Keywords: DNA barcoding; molecular identification; orchid; *Phalaenopsis deliciosa*

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INTRODUCTION

Indonesia is a country with high biodiversity. The government declared through PERMENHUT No. 35 of 2007 (KLHK, 2007) that one of the potential biodiversity to be developed are ornamental plants, particularly orchids and those with medicinal properties (Hani *et al*., 2014; Wahyudiningsih & Nion, 2017; Utami & Hariyanto, 2019). Orchids belong to *Orchidaceae* family and has the most species-rich angiosperm when compared to other flowering plants group (Givnish *et al*., 2016; Fay, 2018; Tsiftsis *et al*., 2019). Furthermore, Indonesia has about 5000 species of orchids, about 1500 species have been identified (Schuiteman *et al*., 2010; Semiarti, 2012), and reports that some have the potential to be used as raw material for medicines (Hani *et al*., 2014; Teoh, 2016; Perwitasari *et al*., 2020). Some contain chemicals that are useful for therapeutic, such as glycerides, alkaloids, and others. When these plants are handled appropriately, they are useful as herbal medicines and have the potential to be used as a substitute for synthetic drugs. Herbal medicines are acceptably safe for health and have a dramatically fewer side effects (Taheri *et al*., 2011; Ekor, 2014; Moreira *et al*., 2014; Yao *et al*., 2016).

In general, morphological characteristics are used when identifying orchids and other plants. However, this approach sometimes meet obstacle since the vegetative characters of orchids are almost identical. Therefore, the existence of flowers as generative organs for identification is indispensable (Su *et al*., 2013; Feng *et al*., 2015; Dirks-Mulder *et al*., 2017). For precision and also to support the conventional approach, the molecular markers through DNA barcoding used short fragments to identify the specimens is an alternative method (Jinbo *et al*., 2011; Li *et al*., 2011; Kim *et al*., 2014). DNA barcoding use marker selection that distinguish specific differences between species (Takamiya *et al*., 2011; Saddhe & Kumar, 2018). The CBOL Plant Working Group (2009) suggests the use of two plastid genes, namely *maturase-K* (*matK*) and *ribulose-1.5-bisphosphate carboxylase* (*rbcL*) as the standard barcodes for plant DNA, and one potential nuclear ribosome DNA, namely the Internal Transcribed Spacer (ITS) region (Feng *et al*., 2015). Previously, the potential barcodes for *Paphiopedilum* and *Thrixspermum*

orchids were investigated using in silico approaches (Rohimah *et al*., 2018; Sindiya *et al*., 2018). Therefore, this research aims to identify and compare three markers (*matK*, *rbcL*, ITS) for their function as potential barcodes for *Phalaenopsis deliciosa*. The core barcode for *Phalaenopsis deliciosa* thus can evaluate the tropical biodiversity especially in Indonesia.

MATERIALS AND METHODS

The samples were obtained from Kebon Agung orchids nursery located in Gebang district, Jember. They were morphologically identified using several identification books such as Orchids of Papua New Guinea (Millar, 1978), Lowland Orchids of Papua New Guinea (O'Byrne, 1994), Key to the genera of Orchidaceae of New Guinea (Schuiteman, 1995), and Flora Malesiana: Orchids of New Guinea (Schuiteman *et al*., 2010). For DNA extraction, the procedures were based on the standard protocol of genomic isolation kit (GeneAll Exgene™ Korea). Briefly, 0.1 g leaf sample was mixed with extraction buffer containing RNAse and crushed using mortar and pestle until homogenized. The mixture was centrifuged and the supernatant was recovered, which was then processed through filter column and dissolved with elution buffer in a total volume of 50 µL. The genomic DNA was then stored until used as a template in PCR amplification.

Subsequently, DNA amplification was performed using three different primer sets: 1) *matK*_F (5′-CTTCTGGAGTCTTTCTTGAGC-3′) and *matK*_R (5′-CCCAATACAGTACAAAA TTGAGC-3′)(Khew & Chia, 2011; Azofeifa-Bolaños *et al*., 2017), 2) *rbcL*_F (5′-ATGTCACCACAAACAGAGACTAAAGC-3′) and *rbcL*_R (5′-GTAAA ATCAAGTCCACCRCG-3′)(Costion *et al*., 2011), and 3) ITS_F (5′-GGCTCTCGCATCGATGAAGA-3′) and ITS_R (5′TAGAATTCCCCGGTTCGCTCGCCGTTAC-3′)(Sun *et al*., 1994; Martins *et al*., 2014). The total volume for PCR was 20 µL, consisting of DNA template, primer set, and PCR premix (AccuPower® PCR PreMix Bioneer Korea). The amplification reaction was performed in three steps include pre-denaturation at 95°C for 5 min, 30 cycles of denaturation at 95°C for 30s, annealing at 53°C for 30 s, and extension at 72°C for 1 min 20s, and the final extension at 72°C for 5 min. After PCR, the product was loaded on 1.25% agarose gel electrophoresis containing EtBr and visualized under UV transilluminator.

The PCR product was then processed for sequencing using Macrogen Service Korea. Subsequently, the data were analyzed using several bioinformatics software such as Bioedit, BLAST, and ClustalX. The phylogenetic tree was constructed using MEGA5.05 (Zhai *et al*., 2013).

RESULTS AND DISCUSSION

Based on morphological characteristics, *Phalaenopsis deliciosa* has shown several characters such as growing epiphyte with a very short stem, monopodial, oblong-lanceolate leaves with undulate margins, and the flowers consist of two lateral sepals, one dorsal sepal, two petals and one labellum (Fig. 1). Furthermore, the flowers bloom sequentially to all directions. These characters are consistent with previous research on the morphology of *Phalaenopsis deliciosa* (Teoh, 2016).

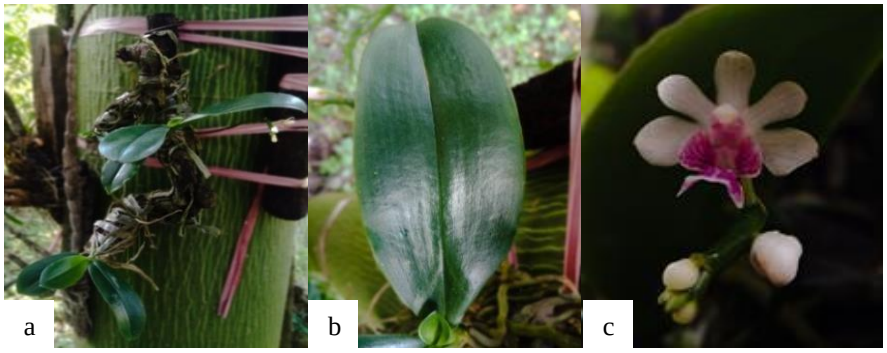

Fig. 1. The morphology of *Phalaenopsis deliciosa* in Kebon Agung orchids nursery: a. short stem; b. oblong-lanceolate leaves; c. flowers

In addition to the attractive flower character, *Phalaenopsis deliciosa* is used as a herbal ingredient, therefore, it is very important in orchid biodiversity (Ming, 2000; Huda *et al*., 2017). However, there are limited data related to genetic biodiversity for the DNA barcodes. As evidence, only *matK* is available in NCBI's GenBank for *Phalaenopsis deliciosa* (Hidayat *et al*., 2005). Furthermore, the samples were used to complete the existing data and its DNA barcodes were explored using three molecular markers. All primer sets for each marker was tested for amplification reaction through PCR. The results showed that the bands that appeared corresponded to the proposed sizes, which include ± 450 bp, ± 600 bp, and ± 500 bp for *matK*, *rbcL*, and ITS, respectively (Fig. 2).

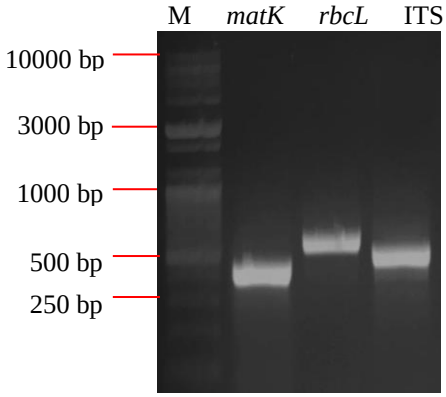

Fig. 2. The amplified PCR product of *Phalaenopsis deliciosa* using *matK*, *rbcL* and ITS primer sets

Table 1. BLAST result of *matK* sequence from *Phalaenopsis deliciosa*

Species	Accession Number	Per. Ident	Query Cover	E-value	Source
Phalaenopsis deliciosa	KY966929.1	99.28%	100%	0.0	China
Phalaenopsis deliciosa	KJ733590.1	99.28%	100%	0.0	China
Phalaenopsis deliciosa	DQ091320.1	99.28%	100%	0.0	USA
Phalaenopsis deliciosa	AB217749.1	99.03%	100%	0.0	Japan
Phalaenopsis pulcherrima	MG459020.1	98.79%	100%	0.0	China
Phalaenopsis pulcherrima	KJ733593.1	98.79%	100%	0.0	China
Phalaenopsis pulcherrima	EF079282.1	98.79%	100%	0.0	Poland
Phalaenopsis chibae	AB217748.1	98.79%	100%	0.0	Japan
Doritis pulcherrima	AB217726.1	98.79%	100%	0.0	Japan
Phalaenopsis zhejiangensis	KJ733594.1	98.55%	100%	0.0	China

These results are consistent with related research that the proposed *matK* used for barcoding is in the range of 450 bp (Khew & Chia, 2011; Azofeifa-Bolaños *et al*., 2017). Relevant results were also illustrated in the amplification of PCR band using the *rbcL* and ITS primer set (Fig. 2), which shows the appropriate range size and are also consistent with the previous reports (Costion *et al*., 2011; Martin *et al*., 2014).

The sequences from the three different primer sets were analyzed with BLAST for alignment, and MEGA5.05 for phylogenetic tree construction. Table 1 summarizes the BLAST results of *matK* sequence from the sample with the existing data in NCBI GenBank.

The result showed that the *matK* sequence from samples have high similarity with those from *Phalaenopsis deliciosa* in China, USA, and Japan with an identity percentage greater than 99%. This indicates that *matK* is able to discriminate *Phalaenopsis deliciosa* down to the species level. The BLAST result is also in mutual agreement with the phylogenetic tree created (Fig. 3).

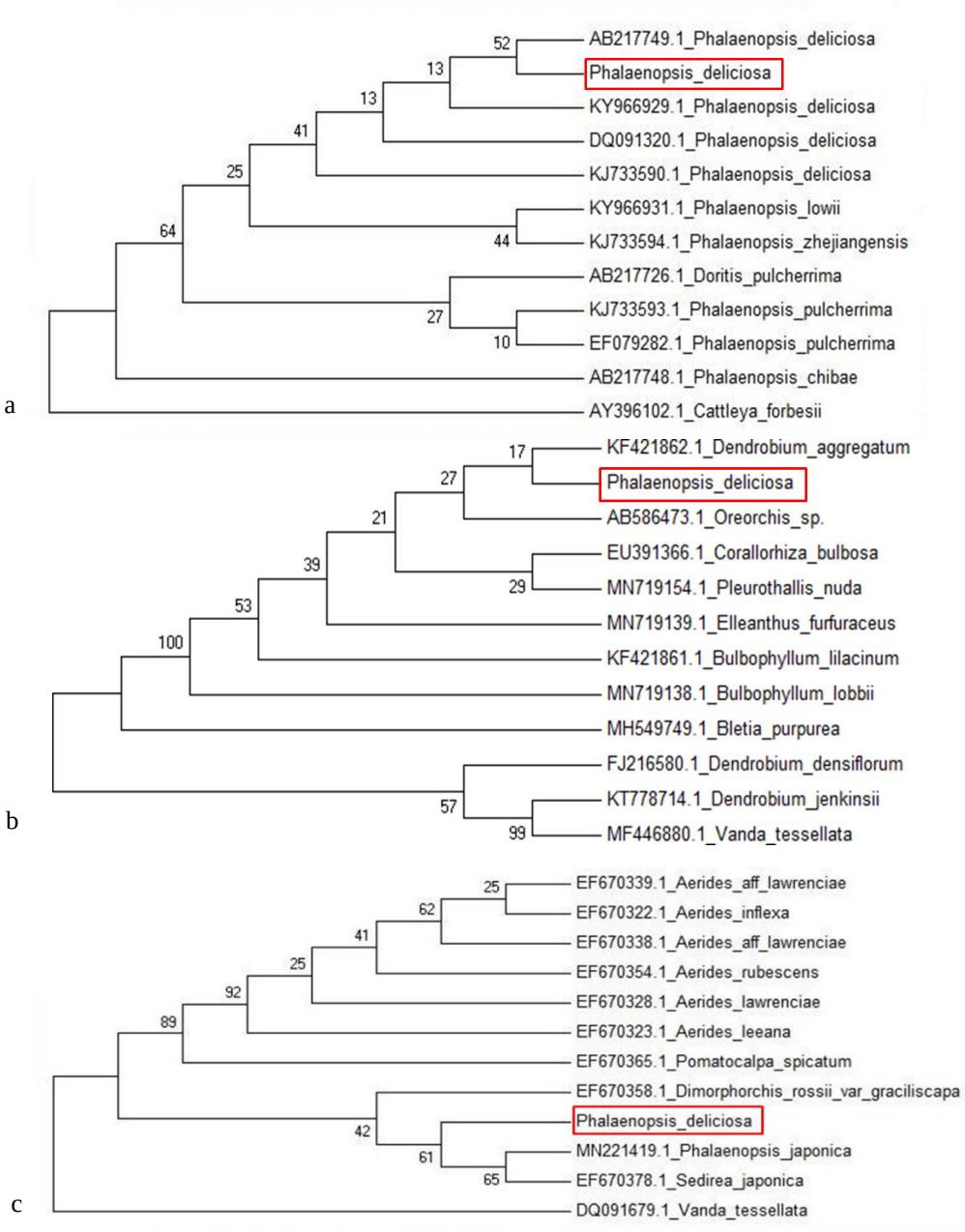

Fig. 3. Phylogenetic tree constructed from: a. *matK* sequences; b. *rbcL* sequences; c. ITS sequences of *Phalaenopsis deliciosa* by MEGA5.05 software

Contrary to *matK*, the sequence of *rbcL* and ITS originating from *Phalaenopsis deliciosa* are not available in NCBI GenBank. Therefore, the sequences originating from this research are newly established. Table 2 and Table 3 illustrate the BLAST results of *rbcL* and ITS sequences, from *Phalaenopsis deliciosa* compared to several orchids species available in GenBank.

Table 2. BLAST result of *rbcL* sequence from *Phalaenopsis deliciosa*

Species	Accession Number	Per. Ident	Query Cover	E-value	Source
Bletia purpurea	MH549749.1	98.41%	99%	0.0	USA
Dendrobium aggregatum	KF421862.1	98.24%	100%	0.0	Bangladesh
Corallorhiza bulbosa	EU391366.1	98.40%	99%	0.0	USA
Elleanthus furfuraceus	MN719139.1	98.06%	100%	0.0	Sweden
Bulbophyllum lobbii	MN719138.1	98.06%	100%	0.0	Sweden
Bulbophyllum lilacinum	KF421861.1	98.06%	100%	0.0	Bangladesh
Oreorchis sp.	AB586473.1	98.06%	100%	0.0	Japan
Dendrobium jenkinsii	KT778714.1	98.22%	99%	0.0	India
Dendrobium densiflorum	FJ216580.1	98.39%	98%	0.0	China
Pleurothallis nuda	MN719154.1	98.22%	99%	0.0	Swedia

Furthermore, *rbcL* shows high identity (more than 98%) with several orchids belonging to genus *Bletia, Dendrobium, Corallorhiza, Elleanthus, Bulbophyllum, Oreorchis,* and *Pleurothallis*. Meanwhile, the ITS sequence shows a lower identity (94-96%) with those of several orchids available in the NCBI database. The phylogenetic tree of both *rbcL* and ITS, which shows mutual congruence are shown in Fig. 3b and Fig. 3c.

Table 3. BLAST result of ITS sequence from *Phalaenopsis deliciosa*

Species	Accession Number	Per. Ident	Query Cover	E-value	Source
Phalaenopsis japonica	MN221419.1	96.27%	100%	0.0	South Korea
Sedirea japonica	EF670378.1	96.27%	100%	0.0	Switzerland
Pomatocalpa spicatum	EF670365.1	95.25%	100%	0.0	Switzerland
Dimorphorchis rossii	EF670358.1	95.23%	100%	0.0	Switzerland
Aerides aff. lawrenceae	EF670339.1	95.04%	100%	0.0	Switzerland
Aerides aff. lawrenceae	EF670338.1	95.04%	100%	0.0	Switzerland
Aerides lawrenceae	EF670328.1	95.04%	100%	0.0	Switzerland
Aerides leeana	EF670323.1	95.04%	100%	0.0	Switzerland
Aerides inflexa	EF670322.1	95.04%	100%	0.0	Switzerland
Aerides rubescens	EF670354.1	94.83%	100%	0.0	Switzerland

The molecular markers used are from two different loci, *matK*, and *rbcL* from plastid/chloroplast while ITS is derived from nuclei. This difference led to the strength and weakness of each marker. The study showed that the identity level of *matK*, *rbcL*, and ITS were 99, 98, and 94-96%, respectively. Although all markers demonstrated the ability to discriminate against orchid species, the level of identity/similarity derived from sequence alignment has also become a crucial consideration. Furthermore, one of the best benchmarks of molecular markers in DNA barcoding is the existence of sequence variability, which indicates a high specificity level for identification. Consequently, the *matK* and ITS showed prominent character to be the recommended molecular marker for the identification of *Phalaenopsis deliciosa*.

CONCLUSION

The *matK* and ITS markers showed high specificity for *Phalaenopsis deliciosa.* Therefore, they are recommended as the best marker for the molecular identification of genus *Phalaenopsis.*

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