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DNA Barcoding of Endangered *Paphiopedilum* species (Orchidaceae) of Peninsular Malaysia

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Abstract

In this study, the efficacy of four DNA markers and their combinations (*rbcL*, *matK*, ITS, *trnH-psbA*) as barcode markers were tested across the endangered *Paphiopedilum* species from Peninsular Malaysia. Four species of *Paphiopedilum* were sampled and barcoded. The DNA barcodes reliabilities were evaluated using NCBI BLASTn program, phylogenetic tree via Neighbour-Joining method with 1000 bootstrap replicates in MEGA 6 and barcoding gap assessment. *matK* is the most promising barcode with high sequence quality (100%), high accuracy in BLASTn (100%), clear resolution of species in Neighbour-Joining phylogenetic tree (100%) and a distinct barcoding gap followed by ITS, *trnH-psbA* and *rbcL*. The combination of barcode regions revealed the lack of variation in *rbcL* and *trnH-psbA* but they are still useful for preliminary identification followed up by *matK* for accurate identification.

Key Words: DNA barcodes, *Paphiopedilum,* identification, endangered species, taxonomy

Introduction

Peninsular Malaysia has been blessed with a large number of plant species with high representation of the Orchidaceae family including the genus *Paphiopedilum*. It is a special orchid group and commonly known as lady's slipper orchids because of the pouch-like labellum that is reminiscent of a lady's slipper. *Paphiopedilum* is taxonomically placed under the subfamily Cypripedioideae which is shared with four other genera, all of which have the similar labellum feature (Cribb 2014). Due to its beautiful and unique flowers, slipper orchids have been collected and poached for ornamental purposes but this has caused its number to dwindle in the wild. Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) has placed *Paphiopedilum* in Appendix I which prevents its trade across borders of the country in which it belongs to.

Fewer species occur in the peninsular than in Borneo Malaysia but its appeal is no less alluring. Vegetative characters of the plants that are very much similar to each other prevent correct identification of species that are particularly under threat. As such is the problem, DNA barcoding has been proposed as a simple yet effective means by which identification of the individual species can be done accurately. Each barcode is unique to each species and gives clear-cut identification even between closely related species (Chase & Fay 2009).

The applications of DNA barcoding now has far reaching effects and has been demonstrated to be extremely useful in many cases. Sønstebø *et al.* (2010) reconstructed past vegetation in the Arctic using data obtained through DNA barcoding. Studies that focus on regulation and control of food are able to employ barcoding methods to screen unwanted adulterants in herbal remedies usually included in by unscrupulous manufacturers (Selvaraj *et al.* 2012; Techen *et al.* 2014). In fish sales, retailers and restaurants often deceptively mislabel fish names in order to sell those fishes at a higher price that it actually is. Through DNA barcoding, Wong *et al.* (2008) discovered that twenty five percent of seafood in North America is potentially mislabeled highlighting the greater utility of DNA barcoding in law enforcement especially with food and safety regulations.

The ability of DNA barcoding in taxonomy makes it a very useful tool in identification of plants and slipper orchids in particular. In this study, we tested the efficacy of the core and supplementary barcodes of plant DNA barcoding by single use and also in combination for *Paphiopedilum* in Peninsular Malaysia and thus suggest the ideal barcode for identification.

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Materials and method

Samples collection and identification

Paphiopedilum found in Peninsular Malaysia were collected and studied. Sample collections were performed in two ways; firstly by conducting a field trip and collecting the wild plants using the convenient sampling method and secondly by obtaining plants that are sold in trusted orchid nurseries. Collection in the wild was based on information of distributions found in the literature and from herbariums specimens. A total of 17 specimens were used in this study comprising of 4 species. The species were *Paphiopedilum barbatum* (Lindl.) Pfitzer (1903: 91)*, Paphiopedilum callosum* var. *sublaeve* (Rchb.f) P. J. Cribb (1987: 188)*, Paphiopedilum niveum* (Rchb.f) Stein (1892: 478) and *Paphiopedilum lowii* (Lindl.) Stein (1892: 476). *Paphiopedilum bullenianum* (Rchb.f.) Pfitzer (1894: 40) was not included in the study though it occurs in Peninsular Malaysia. This was due to the lack of fresh specimens for study. For identification, the collected samples were compared to the descriptions in Seidenfaden & Wood (1992) and Cribb (1998). Unidentifiable specimens were tagged with a collection number until barcoding confirms correct identification.

DNA extraction and PCR

DNA was extracted using the CTAB protocol (Doyle & Doyle 1987) with modifications. Extracted DNA was stored in -20°C and used as the template for generating DNA barcodes. The chosen barcode regions *rbcL*, *matK*, ITS and *trnH-psbA* are as recommended by CBOL Plant Working Group *et al.* (2009). Table 1 list out the barcode locus and the primers used for amplification.

TABLE 1. Barcoding locus and primers used in amplification.

Locus	Primer name	Primer sequence	Reference
rbcL	*rbcL*-F	5'- ATGTCACCACAAACAGAAACTAAAGC	Parveen *et al.* (2012)
	rbcL-R	5'-CTTCGGCACAAAATAAGAAACGATCTC	
matK	*matK*-1F	5'- ATCCATATGGAAATCTTGGTTC	Parveen *et al.* (2012)
	matK-1R	5'- GTTCTAGCACACGAAAGTCG	
ITS	ITS1	5'- TCGTAACAAGGTTTCCGTAGGT	Parveen *et al.* (2012)
	ITS2	5'- GTAAGTTTCTTCTCCTCCGCT	
trnH-psbA	*trnH*2	5'- CGCGCATGGTGGATTCACAATCC	Tate & Simpson (2003)
	psbA	5'- GTTATGCATGAACGTAATGCTC	

All amplifications were performed using GoTaq(R) Flexi DNA Polymerase (Promega Corporation) in a 0.2 mL PCR tube for a total reaction volume of 25 µL. The PCR components were listed in Table 2. PCR amplifications were performed in a PCR thermocycler (Eppendorf AG Mastercycler ep gradient) with the appropriate cycling profile (see Table 3). The PCR products were then visualized on an agarose electrophoresis gel stained with nucleic acid stain (EtB"Out" Nucleic Acid Staining Solution, Yeastern Biotech Co. Ltd.). The denaturation, annealing, and extension were repeated for 35 times for *rbcL*, *matK* and ITS and 30 times for *trnH-psbA*.

TABLE 2. PCR reaction mixture volume and concentrations for all barcodes. Total volume is 25µL for each reaction mix.

Components	Barcode locus			
	rbcL	***matK***	**ITS**	***trnH-psbA***
Buffer	5.0 µL (×1)	5.0 µL (×1)	5.0 µL (×1)	5.0 µL (×1)
Magnesium chloride, $MgCl_2$	2.0 µL (2mM)	2.0 µL (2mM)	2.0 µL (2mM)	2.0 µL (2mM)
dNTP	0.5 µL (0.2µM)	0.5 µL (0.2µM)	0.5 µL (0.2µM)	0.5 µL (0.2µM)
Forward & reverse primers	2.25 µL (0.9 µM)	1.25 µL (0.5 µM)	1.5 µL (0.6 µM)	1.25 µL (0.5 µM)
Taq polymerase	0.125 µL (0.025u/ µL)	0.125 µL (0.025u/ µL)	0.125 µL (0.025u/ µL)	0.125 µL (0.025u/ µL)
Distilled water	11.875 µL	13.875 µL	13.515 µL	13.875 µL
DNA	1 µL	1 µL	1 µL	1 µL

TABLE 3. PCR cycling profile for each barcode locus.

Cycling stage	Barcode locus			
	rbcL	***matK***	**ITS**	***trnH-psbA***
Initial denaturation	95.0°C (2 mins)	95.0°C (2 mins)	95.0°C (2 mins)	94.0°C (3 mins)
Denaturation	95.0°C (30s)	95.0°C (30s)	95.0°C (30s)	94.0°C (30s)
Annealing	52.8°C(30s)	50.0°C (30s)	55.0°C (30s)	49.0°C (30s)
Extension	72.0°C (30s)	72.0°C (30s)	72.0°C (30s)	65.0°C (90s)
Final extension	72.0°C (5 mins)	72.0°C (5 mins)	72.0°C (5 mins)	65.0°C (7 mins)

Sequencing, sequence editing and data analyses

Sequencing was outsourced (1st Base Laboratories Sdn. Bhd., Seri Kembangan, Selangor) and performed using Sanger bi-directional sequencing in both forward and reverse directions. Generated forward and reverse sequences for each species of each barcode were assembled into one contiguous sequence and manually edited to remove low quality base calls from both ends of the sequence. Ambiguous bases were labeled as 'N' within the sequence (de Vere *et al.* 2015). The sequence editing processes were performed using ChromasPro. Quality of sequences was obtained from BOLD Systems version 4 online (http://www.boldsystems.org/) by evaluating the number of ambiguous bases present in a sequence whereby a lower percentage of ambiguous bases indicates a high sequence quality and vice versa. Data analyses were divided into three parts, identity of barcode sequences using NCBI BLASTn, phylogenetic trees and the barcoding gap. The edited sequences were used as NCBI BLASTn query to determine species identity and the barcodes with correct species identity were taken into account as barcode accuracy. Prior to phylogenetic tree construction, the barcode sequences were aligned in CLUSTLX (Larkin *et al.* 2007). Phylogenetic trees were constructed using Neighbour-Joining phylogenetic tree with 1000 bootstrap replicates. All trees were constructed in MEGA 6 (Tamura *et al.* 2013). Using the compute pairwise distances in MEGA 6, the genetic pairwise distances for every sample in all barcode loci were calculated and the global barcoding gap was assessed. The combined data set was done by merging the required sequence text files into one sequence respectively. Analysis follows the similar procedure as mentioned above.

Results

Sequence quality

Both *rbcL* and *matK* sequences generated 100% high quality sequences while *trnH-psbA* sequences produced 94.12% high quality sequences, the remainder that of medium quality. The barcode that produced the lowest number of high quality sequences is ITS at 89.47% of the sequences. Quality was determined based on the percentage of ambiguous bases found within the sequence. High quality sequences were sequences that exhibited less than 1% ambiguous bases in the overall sequence length. Information on the marker quality is represented in Table 4.

TABLE 4. Barcoding locus and sequence quality statistics (Adapted from BOLD Systems v4 http://www.boldsystems.org)

Locus	High (<1%Ns)	Medium (<2%Ns)	Low (<4%Ns)	Unreliable (>4%Ns)
rbcL	100%	0%	0%	0%
matK	100%	0%	0%	0%
ITS	89.47%	10.53%	0%	0%
trnH-psbA	94.12%	5.88%	0%	0%

BLASTn similarity search

Based on similarity search, *matK* sequences have the highest accuracy at 100%. The accuracy was measured based on the number of correct matches of the query sequences with those in the GenBank database. This is followed by ITS (52.9%), *trnH-psbA* (29.4%) and *rbcL* (5.88%). Thus, *matK* produced barcodes that provide better resolution for species identification than other tested barcodes. All sequences generated were deposited into BOLD data systems under project *Paphiopedilum* of Peninsular Malaysia with GenBank Accession (MG 522876- MG 522926).

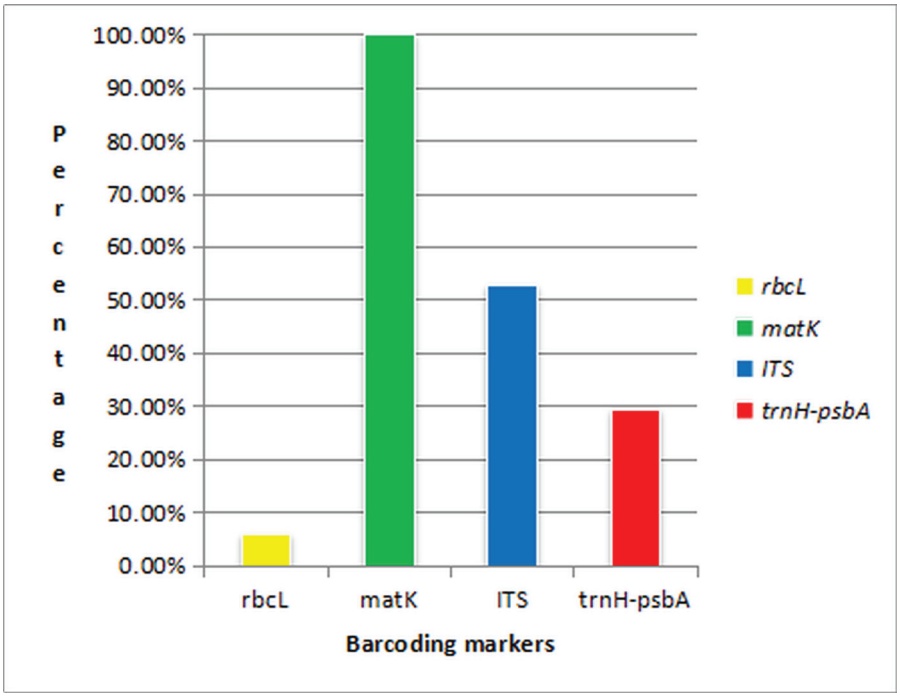

FIGURE 1. BLASTn results of DNA barcodes. Percentage of accuracy was based on the total number of correct matches with the GenBank database.

Phylogenetic trees

All phylogenetic trees were constructed using the Neighbour Joining method and Kimura-2-parameter with 1000 bootstrap replicates. Results obtained from the species clustering showed the effectiveness of the barcodes used. *matK* and ITS (Figure 2 and 3) have the best species resolution as observed by the clustering of conspecific sequences and its congruence with the current taxonomic delimitation of *Paphiopedilum*. *trnH-psbA* and *rbcL* trees show a markedly lower efficiency in distinguishing between the species compared to *matK* and ITS (Figure 4 and 5).

TABLE 5. Species resolution of the individual barcode region and their combinations.

Barcode region	*N* species (*N* accessions)	Species resolution (%)
rbcL	4 (17)	75
matK	4 (17)	100
ITS	4 (17)	100
trnH-psbA	4 (17)	75
rbcL + matK	4 (17)	100
rbcL + ITS	4 (17)	100
rbcL + trnH-psbA	4 (17)	100
matK + ITS	4 (17)	100
matK + trnH-psbA	4 (17)	100
ITS + trnH-psbA	4 (17)	100
rbcL + matK + ITS	4 (17)	100
rbcL + matK + trnH-psbA	4 (17)	100
rbcL + ITS + trnH-psbA	4 (17)	100
matK + ITS + trnH-psbA	4 (17)	100
rbcL + matK + ITS + trnH-psbA	4(17)	100

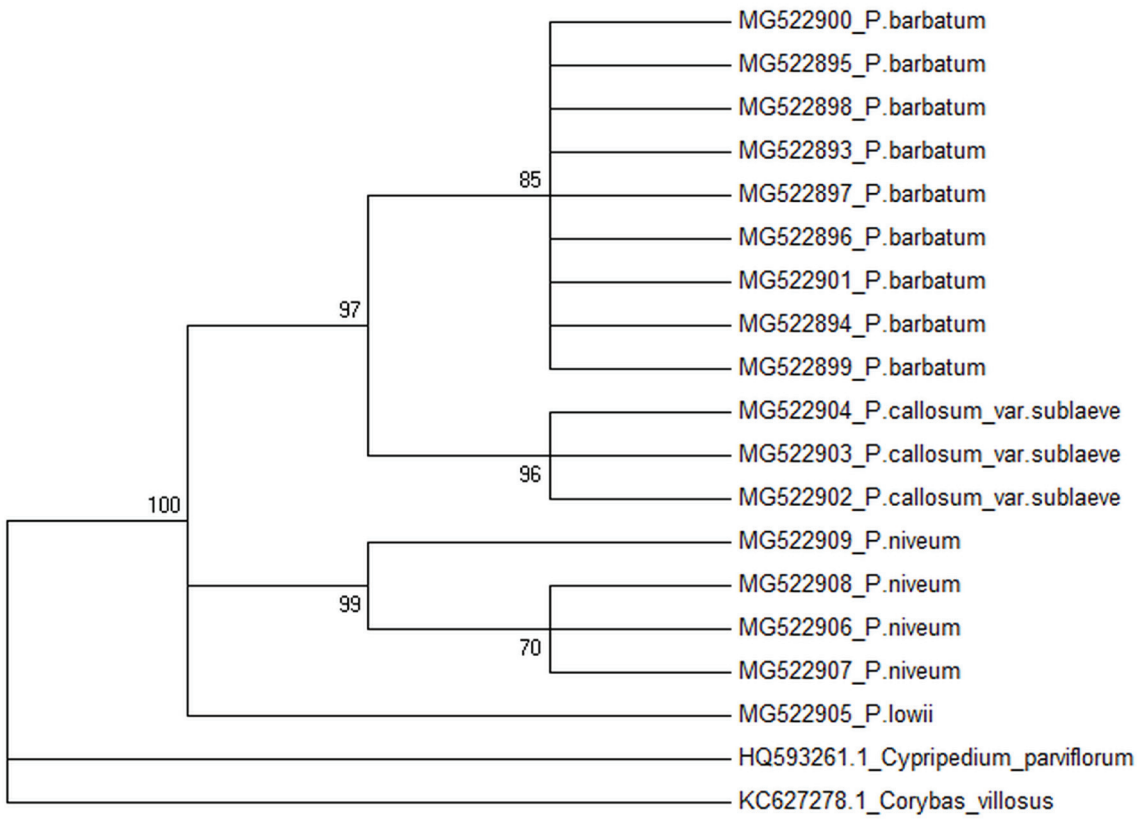

FIGURE 2. NJ bootstrap consensus condensed (cutoff at 50%) phylogenetic tree of *matK* barcodes. Constructed with Kimura-2-parameter and 1000 bootstrap replicates. Outgroups (family Orchidaceae) were obtained from GenBank.

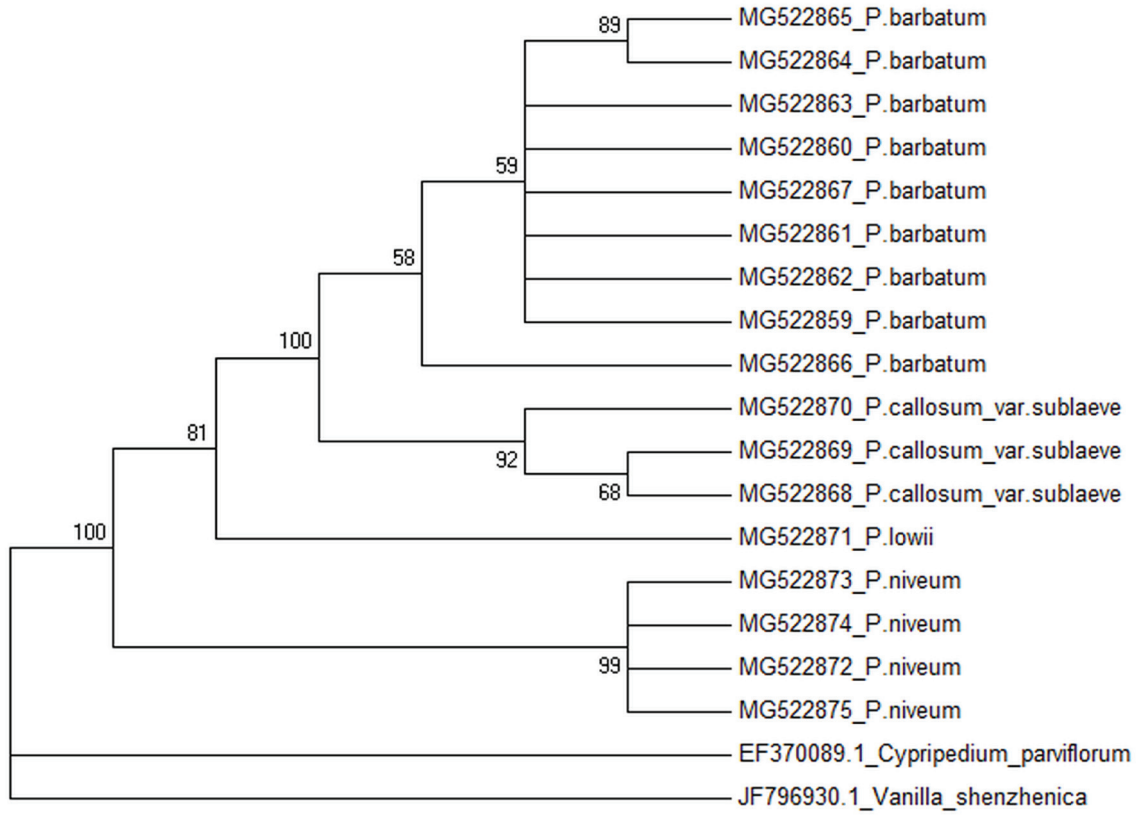

FIGURE 3. NJ bootstrap consensus condensed (cutoff at 50%) phylogenetic tree of ITS barcodes. Constructed with Kimura-2-parameter and 1000 bootstrap replicates. Outgroups (family Orchidaceae) were obtained from GenBank.

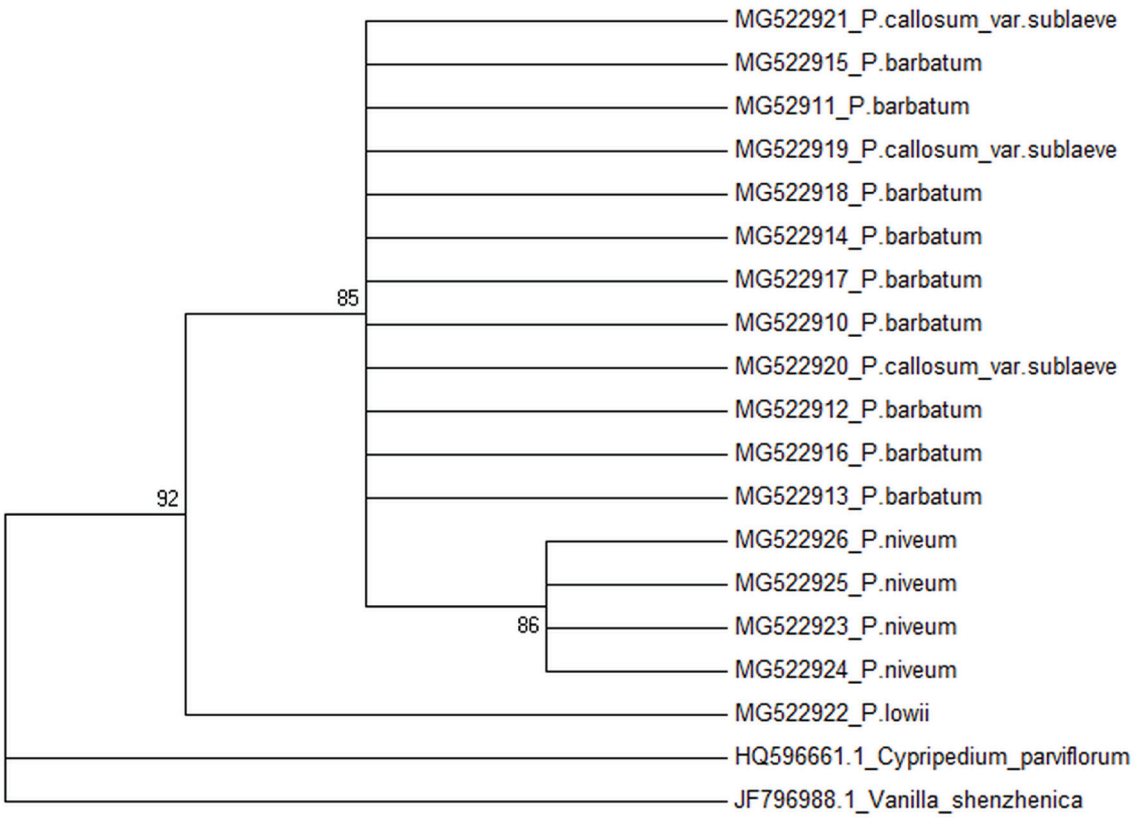

FIGURE 4. NJ bootstrap consensus condensed (cutoff at 50%) phylogenetic tree of *trnH-psbA* barcodes. Constructed with Kimura-2-parameter and 1000 bootstrap replicates. Outgroups (family Orchidaceae) were obtained from GenBank.

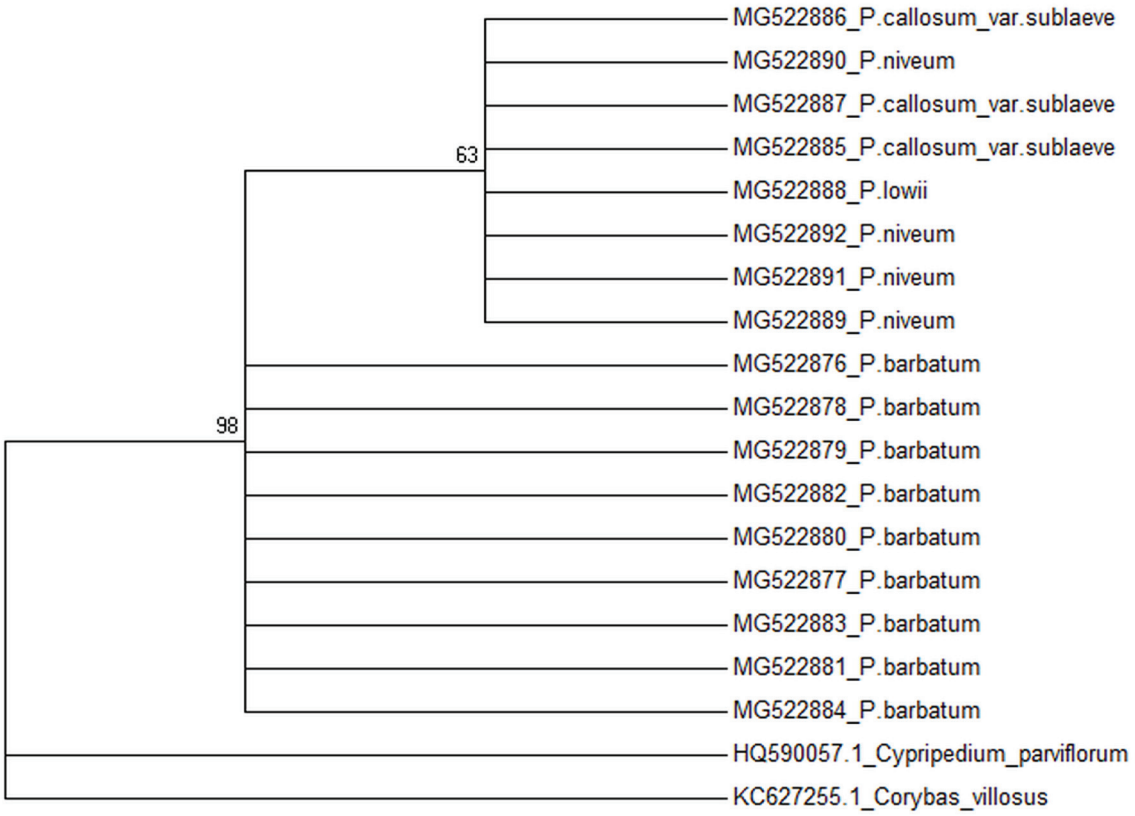

FIGURE 5. NJ bootstrap consensus condensed (cutoff at 50%) phylogenetic tree using *rbcL* barcodes. Constructed with Kimura-2-parameter and 1000 bootstrap replicates. Outgroups (family Orchidaceae) were obtained from GenBank.

Barcoding gap assessment for *matK*, ITS, *trnH-psbA* and *rbcL*

Barcoding gap is present in *matK* between the maximum intraspecific value (0.0037) and minimum interspecific value (0.0049) (Figure 6 (a)), but absent in ITS (overlap at 0.0079), *trnH-psbA* (overlap at 0.0000 and 0.0013) and *rbcL* (overlap at 0.0000, 0.0015, 0.0031 and 0.0046) due to the overlap of genetic pairwise distances of the intraspecific and interspecific distances.

TABLE 6. Presence of barcoding gap of the barcode regions and their combinations.

Barcode region	Barcoding gap
rbcL	Absent
matK	Present
ITS	Absent
trnH-psbA	Absent
rbcL* + *matK	Present
***rbcL* + ITS**	Present
rbcL* + *trnH-psbA	Absent
***matK* + ITS**	Present
matK* + *trnH-psbA	Present
ITS* + *trnH-psbA	Present
***rbcL* + *matK* + ITS**	Present
rbcL* + *matK* + *trnH-psbA	Absent
rbcL* + ITS + *trnH-psbA	Present
matK* + ITS + *trnH-psbA	Present
rbcL* + *matK* + ITS + *trnH-psbA	Absent

Discussions

DNA barcoding is a useful method for species determination but especially in plants; a lot of uncertainties still persist particularly in the use of a specific definite marker as the ultimate barcode. Nonetheless it has been proven to be beneficial due to its ability in identifying plants at any stage of life. Besides observing in detail the characteristics of habit and leaves for example, pollen can be used to accurately identify to genus and even species but melissopalynology often times can only provide identification information up to family (de Vere *et al.* 2012). Animal and fungi barcoding have settled on a suitable marker that presents enough variation to distinguish between species and allow for discovery of new species. Barcoding in plants however, has not decided on a gold standard barcode marker (Chase & Fay 2009).

The markers chosen for this study represent the most commonly used plant molecular marker with suitable length that will not incur extravagant costs in sequencing and also for its ease in amplification. *rbcL* and *trnH-psbA* both produced high quality sequences as compared to ITS and *matK*. The reason for the difference in the quality of sequences generated lies with the nature of the markers themselves. *rbcL* belongs to the coding region of the plastid genome and is important in the synthesis of the enzyme RuBisCo (Kanevski & Maliga 1994). This particular enzyme is necessary for carbon dioxide fixation for food production in plants. Coding regions tend to resist mutations as they are important for the survival of the plants. Therefore, primers for coding regions are universal and can produce high sequence quality *trnH-psbA* intergenic spacer though not a coding region was still able to produce high quality sequences because the primers used (Tate & Simpson 2003) were designed to start from the end of *psbA* gene (5') to the start of *trnH* gene (3'). The reliability of high quality sequences produced by these barcoding markers seems to be the only advantage it presents in barcoding *Paphiopedilum* of Peninsular Malaysia. The species resolutions provided by both markers were poor as shown by the BLASTn results (Figure 1) and the inability of the Neighbour-Joining trees constructed to differentiate clearly between the 4 species (see Figure 4, 5 and Table 5).

Of the four barcode markers tested, *matK* performed the best. All sequences matched correctly in Genbank database (Figure 1), conspecific sequences were nested together in the same clade in the Neighbour-Joining phylogenetic tree

(see Figure 2) with 100% species resolution (Table 5) and a clear barcoding gap. *matK* sequences were also able to differentiate between the closely related *P. barbatum* and *P. callosum* var. *sublaeve*, therefore supporting the species circumscription by Cribb (1998). The efficacy of *matK* as a barcode for *Paphiopedilum* in India was also supported by the works of (Parveen *et al.* 2012) which prove the ability of *matK* in discrimination even for natural hybrids. Large scale orchid barcoding efforts also promote the use of *matK* as the best barcode for Orchidaceae (Lahaye *et al.* 2007). Sequences of *matK* are able to perform well as DNA barcodes due to its high variation as a result of a higher rate of molecular evolution compared to other coding regions used as barcodes (Hilu *et al.* 2003), notably *rbcL*.

ITS ranks only second best to *matK* based on the results displayed in this study due to its lower quality of sequences produced (Table 4) and the lack of a barcoding gap (Figure 6b). ITS phylogenetic tree (Figure 3) was able to clearly differentiate the species correctly with 100% species resolution; however, the BLASTn result showed lower accuracy for species identification as compared to *matK*. The sequence quality of ITS in this study is also the lowest of the four markers, thus further reducing its qualification as the ideal barcode.

However, ITS have been shown to be effective single barcode relative to other barcode regions when it comes to some genera of Orchidaceae (Singh *et al.* 2012; Xu *et al.* 2015) and even in *Paphiopedilum* (Guo *et al.* 2016). Being a non-coding region, ITS accumulates more variation and thus offers more resolution at lower levels of taxa (e.g. genus, species). There are disadvantages to using ITS, though, with regards to low sequence recovery and the presence of paralogous copies that may occur naturally in the cell (Hollingsworth, 2011).

The barcoding gap is the natural gap that exists between the highest intraspecific value and the lowest interspecific value. This gap is more precisely termed the global barcoding gap (Chapple & Ritchie 2013). The presence of this gap shows that there is a limit to the variation that can occur for all species within a genus and so a threshold of the species limit can then be set. An overlap of this threshold can indicate a cryptic species but most likely shows insufficient variation by the barcode.

The barcoding gap exists in *matK* but not in the other single barcode marker. The barcoding gap exists in all combinations of the barcode regions except for the combination of *rbcL* + *trnH-psbA, rbcL* + *matK* + *trnH-psbA* and *rbcL* + *matK* + ITS + *trnH-psbA*. The *matK* barcoding gap can be used to determine the species boundary in *Paphiopedilum* as there is a clear difference between the lowest interspecific pairwise distance and the highest intraspecific pairwise distance but generalizing this result for setting the species limit for all *Paphiopedilum* is not advisable as this study is localized to species in Peninsular Malaysia. In spite of the geographical constraints presented in this study, barcoding studies according to region is still crucial as it lays the foundation for the universal use of DNA barcoding in plants (Kim *et al.* 2014).

The combination of markers either two, three or four show that additional barcode regions elevates the species resolution due to the added variations (Table 5). Even when combining two of the lowest performing barcodes, *rbcL* and *trnH-psbA*, the resolution increased to 100% but there is a lack of barcoding gap in the earlier noted *rbcL* + *trnH-psbA, rbcL* + *matK* + *trnH-psbA* and *rbcL* + *matK* + ITS + *trnH-psbA*. The variations in the data contributed particularly by *rbcL* and *trnH-psbA* was not sufficient to clearly distinguish the intra- and interspecific variation present in the studied genus. This lack complexity that prevent species discrimination by some chloroplast markers was highlighted by Vu *et al.* (2017) because it represents only the maternal inheritance. The use of a multilocus barcode would face difficulties if one or more from its barcode combination failed to amplify or produce good quality sequences (Li *et al*. 2015).

Using *matK* as the core barcode for identification have been proven in other *Paphiopedilum* studies (Parveen *et al.* 2012; Guo *et al.* 2016), but the resolution power of *matK* vary with other DNA barcoding works done on other plant families (de Vere *et al.* 2012; Parmentier *et al.* 2013; Saarela *et al.* 2013). Thus, to circumvent this issue in plant DNA barcoding the suggestion by Newmaster *et al.* (2006) of having a two-tier identification system would be best. Sequencing a common barcode like *rbcL* would give identification at higher taxonomic levels (e.g. order, family). Once the order or family has been identified, then the recommended specific barcode for the family can then be used to identify the species accurately. Based on the BLASTn results attained in this study, all barcode markers were able to correctly identify to genus and for further identification at the species level we propose that the *matK* barcode be used.

Conclusions

The use of DNA barcoding has great potential especially in identification and taxonomy of plants to clearly elucidate the species and remove confusion on very closely related species. Using the *matK* barcode, accurate identification of species in *Paphiopedilum* can be ascertained and can act as a molecular tag while supporting the morphological data by works of earlier taxonomists.

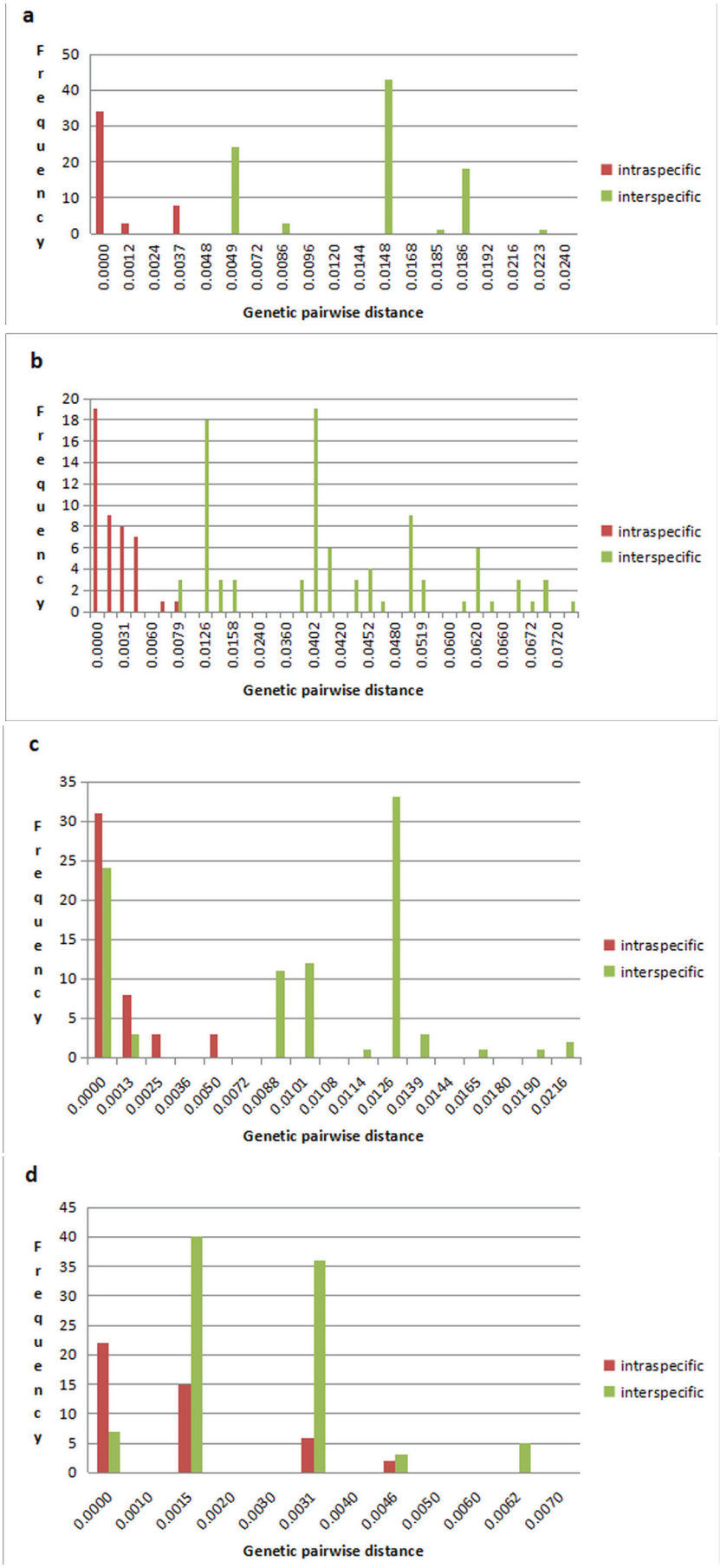

FIGURE 6. Barcoding gap assessment based on the distribution of intraspecific and interspecific distances. No overlap of intraspecific and interspecific distances indicates a barcoding gap. a: *matK* (present), b: ITS (not present), c: *trnH-psbA* (not present), d: *rbcL* (not present).

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