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Morphology and DNA marker for distinguishing *Paphiopedilum hangianum* and *Paphiopedilum emersonii* from Vietnam

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ABSTRACT

Genus *Paphiopedilum* has species having lovely flowers which are incredibly attractive to everyone. Their ornamental and commercial value caused over-collection and illegal poaching and trade. Due to these reasons, nowadays, the Venus slipper orchids are facing to deplete in nature. Therefore, it is important to consider these species conservation. Mainly, it is necessary to prioritize the identification and phylogenetic analysis methods of the genus *Paphiopedilum* which includes many species with similar morphological characteristics. Consequently, it isn't easy to distinguish the identical species of this genus when the plants are young or not yet fully flowering. Therefore, this study aimed to distinguish two *Paphiopedilum* species, i.e. *P. hangianum* and *P. emersonii*, which have similar morphological characteristics, through comparative morphological analysis and differences in DNA barcoding sequences. To solve the problem associated with species identifications, a morphological comparison table was created with the four DNA sequence markers *matK*, *rbcL*, *rpoC1* and *trnH-psbA*. The results of the morphological analysis showed that *P. hangianum* and *P. emersonii* are significantly different from each other in the flower's characteristics. While the difference in leaf morphology of both selected species is found very little, it is also distinguishable upon careful comparison. Moreover, the DNA barcoding indicator gave accurate and rapid distinctions between the two species, even when the plants are young or without flowers. Furthermore, this DNA barcoding can establish an evolutionary

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relationship between the two selected species and the other species of the genus Paphiopedilum. The results of this study also suggested that the indicator trnH-psbA is a suitable marker for distinguishing these two species and can be applied for the phylogenetic analysis of the genus *Paphiopedilum* in Vietnam.

1 Introduction

Genus *Paphiopedilum* Pfitzer (Vennu's slipper) belongs to the family Orchidaceae, which can be easily recognized by its unique flower structure with a deformed, sac-like central petal called a com lips. Venus's Slipper orchids are a small but prominent branch of the Orchid family, representing one of the most specialized lines of insect-pollinated flowering plants. Genus *Paphiopedilum* is native to southeast Asia (Myanmar, Thailand), northern India, southern China and New Guinea, with more than 80 species distributed worldwide (Braem et al. 1998; Braem et al. 1999; Cribb 1998, Koopowitz 2008). Vietnam has the world's most considerable diversity of *Paphiopedilum* genera. Averyanov et al. (2004) listed 22 species of the *Paphiopedilum* genus, including four natural hybrids of Vietnam.

Based on the initial morphology, the genus *Paphiopedilum* has been divided into three subgenera *Parvisepalum*, *Brachypetalum*, and *Paphiopedilum.* Subgenus *Paphiopedilum* is a heterogeneous group with the highest number of species. Meanwhile, the subgenus *Paphiopedilum* is divided into five sections: *Paphiopedilum*, *Barbata*, *Pardalopetalum*, *Cochlopetalum* and *Coryopetalum* (Averyanov et al. 2004). Later, the species *P. canhii* was discovered and added to a section *Pygmaea* under subgenus *Paphiopedilum* due to the interbreeding characteristics between subgenus (Gorniak et al. 2014) or even a new subgenus *Megastaminodium* to contain this species (Braem and Gruss 2012).

Two species of genus *Paphiopedilum*, i.e., *P. hangianum* (Perner and Gruss 1999) and *P. emersonii* (Koop and Cribb 1986), are endemic to Vietnam and distributed only in some provincial mountainous areas such as Thai Nguyen, Bac Kan, Tuyen Quang, and Ha Giang (Averyanov et al. 2004). These species give the most beautiful flowers and are very popular not only in Vietnam but also in many countries around the world. Morphologically these two species could not be distinguished without flowers (Averyanov et al. 2004; Dang et al. 2017; Vu et al. 2019).

Traditional methods based on morphological characteristics are not found suitable for the identification of these two species before flowering; therefore, a combination of traditional and modern molecular markers-based techniques like DNA barcodes can be an alternative to this problem (Xu et al. 2014; Vu et al. 2019; Bui et al. 2022, Cahyaningsih et al. 2022, Worthy et al. 2022). DNA barcoding molecular identification method provides accurate and reliable data to identify target species with the help of selected molecular markers by passing a specific DNA region. Further, the selection of appropriate marker gene sequences will increase the efficiency of species identification. The nuclear marker *ITS* is the most widely used molecular marker (Hollingsworth et al. 2009; Singh et al. 2012; Wu et al. 2012; Yukawa et al. 2013; Xu et al. 2015; Veldman et al. 2018; Tran et al. 2018). However, the chloroplast genome in plants has many features relevant for DNA markers, either in coding sequences (such as *rbcL* and *matK*) or intergenic regions (such as trnH-psbA). The molecular marker trnH-psbA has been widely used for identification purposes in previous studies, with species resolution up to 100% across a wide range of 72 plant genera (Kress and Erickson 2007) or over a small range of species within a genus (Parveen et al. 2012; Bolson et al. 2015). In addition, the combination of multiple loci of the *trnH-psbA*, *rpoB*, *rpoC1*, *rbcL*, and *matK* has been recommended for taxonomy and phylogenetic studies (Gorniak et al. 2014; Vu et al. 2019).

Furthermore, for the genus *Paphiopedilum*, the first barcode-based species identification study was conducted in India by Parveen et al. (2012). This study obtained excellent results with five barcodes gene *rpoB*, *rpoC1*, *rbcL*, *matK*, and ITS. Further, Gorniak et al. (2014) combined morphological characteristics, cytological analysis, and DNA barcode gene markers to identify species *P. canhii*, which was previously controversial in the taxonomic system because of its characteristics of interference between different genera. Additionally, studies on DNA barcoding with several markers such as *ITS*, *LEAFY*, *ACO*, *matK*, *trnL*, *rpoB*, *rpoC1*, and *trnH-psbA* to identify genus *Paphiopedilum* were conducted, especially in Vietnam (Trung et al. 2013; Vu et al. 2020).

Therefore, this study aims to observe, analyze and describe morphological characteristics of the stem, roots, leaves, and flowers to identify two orchid species, i.e., *P. hangianum* and *P. emersonii*. Our objectives are also to provide new insight into the molecular classification of these two species through the chloroplast and nuclear DNA barcode sequences and further to distinguish species *P. hangianum* from *P. emersonii* by using DNA barcode combined with morphological characterization.

2 Materials and Methods

2.1 Collection of plant materials

A total of 5 individuals of each species, i.e., *P. hangianum* and *P. emersonii*, were collected by a field trip in 2017 from four northern

provinces, i.e., Thai Nguyen, Bac Kan, Ha Giang and Tuyen Quang of Viet Nam (Table 1; Figure 1) and cultivated under the greenhouse of the Faculty of Biotechnology, Thai Nguyen University of Sciences, Viet Nam. So far, we have obtained three flower samples. The young leaves of each species were collected into Fancol tubes and preserved at a temperature of 4^{o}C for DNA extraction.

Table 1 Codes, coordinates and addresses of samples collection in 4 provinces of Northern Vietnam

Species	Codes	Coordinates	Receiving place/coordinate	Characteristic
P. ermesonii	HH01	Lat: 21.75729 Long: 105.8877	Mo Ba, Tan Long, Dong Hy Thai Nguyen –Viet Nam	have flower
	HH02	Lat: 22.52493 Long: 105.6295	Cao Tan, Pac Nam, Bac Kan – Viet Nam	adulthood
	HH03	Lat: 22.52493 Long: 105.6400	Cao Tan, Pac Nam, Bac Kan – Viet Nam	have flower
	HH04	Lat: 21.75729 Long: 105.8999	Lan Quan, Tan long, Dong Hy, Thai nguyen – Viet Nam	have flower
	HH05	Lat: 21.76717 Long: 105.96411	Khuon Muc, Cuc Duong, Dong Hy, Thai Nguyen – Viet Nam	nurseling
P. hangianum	HA01	Lat: 21.9666667 Long:106.05	Ban Buoc, Phuc Yen, Lam Binh, Tuyen Quang, Vietnam	nurseling
	HA02	Lat: 21.96666 Long: 106.045	Ban Buoc, Phuc Yen, Lam Binh, Tuyen Quang, Vietnam	have flower
	HA03	Lat: 21.9711 Long: 106.02	Ban Buoc, Phuc Yen, Lam Binh, Tuyen Quang, Vietnam	have flower
	HA04	Lat: 22.6935 Long: 105.0908	Linh Ho, Kim Thach, Vị Xuyên, Ha Giang, Vietnam	have flower
	HA05	Lat: 22.6826 Long: 105.2493	Thuong Tan, Bac Me, Ha Giang, Vietnam	adulthood

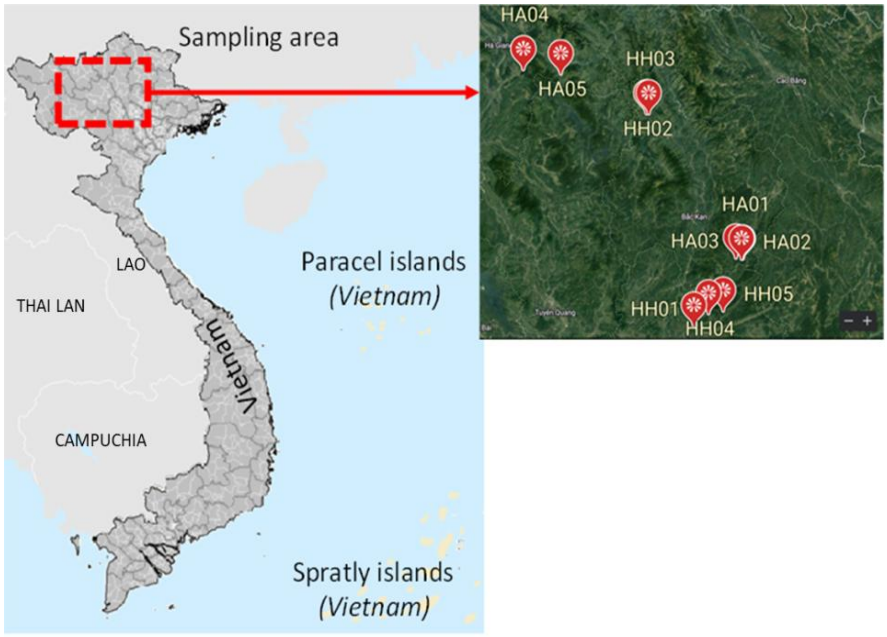

Figure 1 Sampling map in some provinces of Northern Vietnam

2.2 Morphological identification

The plant materials were directly observed, and the characteristics of each plant part were described in detail to compare with existing documents and identification keys (Koopowitz and Cribb 1986; Perner and Gruss 1999; Averyanov et al. 2004, Koopowitz 2008).

2.3 DNA extraction, amplification

Total DNA was extracted using the modified CTAB method to match the experimental conditions in Vietnam (Collins and Symons 1992). Leaf samples were ground in liquid nitrogen, supplemented with separation buffer and incubated at 65^{o}C for two hours. A solution of chloroform: isoamyl alcohol (24:1) was added to the tube containing the separation buffer and the sample in a 1:1 volumetric ratio to separate the DNA. The sample was centrifuged at 13000 Rpm/g for 10 min, the supernatant was transferred to a new tube, and isopropanol (1:1 vv sample: isopropanol) was added to precipitate the DNA. Specific primer pairs were used to multiply the gene fragments. Primers used in PCR for the amplification: *matK*, trnH-psbA, *rpoC1*, *rbcL* (Tate and Simpson, 2003, Parveen et al. 2012). The detailed sequences of the used primers are shown in Table 2. The PCR reaction components and heat cycle multiplying genes are shown in Table 3 and 4.

2.4 Sequencing, alignment, and phylogenetic analyses

PCR products were purified for sequencing on an ABI PRISM 3100 Avant Genetic Analyzer automatic nucleotide sequencer. Sequences were blasted in NCBI and processed with Snapgene and BioEdit software, and gene sequence-based taxonomy was built with MEGA X software. DNA sequences were imported to MEGA

Table 2 Primer sequences for the DNA barcode gene

Primers	Nucleotide sequence	Annealing temperature	Expected sizes	Reference
trnH-psbA	F,5'-GTTATGCATGAACGTAATTGCTC-3'	52	600 bp	Tate and Simpson (2003)
	R,5'-CGCGCATGGTGGATTCACAATCC-3'			
matK	F,5'-CGATCTATTCATTCAATATTTC-3'	52	900 bp	Parveen et al. (2012)
	R,5'-TCTAGCACACGAAAGTCGAAGT-3'			
rpoC1	F,5'-GTGGATACACTTCTTGATAATGG-3'	52	600 bp	Parveen et al. (2012)
	R,5'-CCATAAGCATATCTTGAGTTGG-3'			
rbcL	F,5'-ATGTCACCACAAACAGAAAC-3'	52	750 bp	Parveen et al. (2012)
	R,5'-TCGCATGTACCTGCAGTAGC-3'			

Table 3 PCR reaction mixture volume and concentrations for all barcodes

Components	Barcode locus			
	rbcL	*MatK*	*rpoC1*	*trnH-psbA*
PCR Master Mix (2X)	12.5µL (×1)	12.5 µL (×1)	12.5 µL (×1)	12.5 µL (×1)
Forward & reverse primers	1 µL (10µM)	1 µL (10 µM)	1 µL (10µM)	1 µL (10µM)
Distilled water	4.5	4.5	4.5	4.5
DNA (50 µg/µl)	1 µL	1 µL	1 µL	1 µL

Table 4 PCR cycling profile for each barcode locus

Components	Barcode locus			
	rbcL	*MatK*	*rpoC1*	*trnH-psbA*
Initial denaturation	94°C, 5 min	94°C, 5 min	94°C, 5 min	94°C, 5 min
Denaturation	94°C, 45s	94°C, 45s	94°C, 45s	94°C, 45s
Annealing	54°C, 30s	52°C, 30s	54°C, 30s	52°C, 30s
Extension	72°C, 50s	72°C, 40s	72°C, 60s	72°C, 50s
Final extension	72°C, 7 min	72°C, 7 min	72°C, 7 min	72°C, 7 min

X for alignment with sequences from the GenBank database with the addition of the outgroup species *P.hangianum* and *P.emersonii*. Maximum Likelihood analyses with 1000 bootstrap replications and the Tamura-Nei model (1993) of sequence evolution were used to construct a phylogenetic tree (Tamura and Nei 1993).

3 Results

3.1 Morphological characteristics of two *Paphiopedilum* species

Botanical characteristics (stems, roots, flowers, etc.) of the collected orchid samples were recorded by direct observation and the results are presented in Table 5 and Figures 2 & 3. The results of the morphological analysis showed that both *P. hangianum* and *P. emersonii* were very similar in shape and size in the absence of flowers. The leaves size, color and shape are very similar without much difference. Here are a few subtle differences that can help differentiate these two species even without flowers. Both *P. hangianum* and *P. emersonii* stems have stacked leaves; however, the leaves of *P. hangianum* are closely overlapping to form a regular V, and the leaves extend straight up. In contrast, *P. emersonii* leaves are somewhat looser; the leaf neck is wide when holding the plant straight in hand, and the leaves tend to hang horizontally. The *P. hangianum* leaves are dark green, while *P. emersonii* leaves are slightly lighter and more glossy. The *P. hangianum* leaf margins are somewhat wavy, making the leaves slightly warped above and below. However, these distinct characteristics were only observed when the plants matured and grew under the same conditions. These distinguishing characteristics are not apparent with young trees or trees collected from the forest. Flowers are the main identifying

Table 5 Diagnostic morphological characters of *P. hangianum* and its closest *P. emersonii*

Characters	*P. hangianum*	*P. emersonii*
Stem		
Height	Less than 10 cm	Less than 10 cm
Arrangement of Leaves	Two rows, overlapping close,	Two rows, overlapping loose,
Leaves		
Number per plant	6 to 8	6 to 8
Apex	Obtuse	Obtuse
Shape	Oblong	Oblong
Length (cm)	More than 20cm	More than 20cm
Width (cm)	Medium 3 to 6 cm	Medium 3 to 6 cm
Upper surface colour	Green, Light mixed (near uniform)	Light green, Light mixed (near uniform)
Lower surface colour	Green	Green
Inflorescence and flower		
Peduncle length	More than 20 cm	More than 20 cm
Floral bract	Green colour and broadly lanceolate	Green, white colour and broadly lanceolate
Flower width in front view	8-9 cm in average, horizontal egg shape	7-8 cm in average, circle shape
Dorsal sepal	6-6,5×4-4,2cm, dorsal sepal with oval shape, convex curvature, entire margin and yellow dominant	4-4, 5× 2, 8-3cm, dorsal sepal with oblong oval shape, light undulate curvature, undulate margin and white dominant
Synsepalum	5, 5-6× 5-5,5cm, synsepalum with sub-orbicular shape, obtuse apex and yellow dominant. Synsepalumyellow pattern inside	5-5, 2 × 4-4, 2cm, synsepalum with sub-orbicular shape, obtuse apex and yellow dominant. Synsepalum white with a yellow line on synsepalum length, absent pattern inside
Petal	7-7, 5×4, 2-4, 5cm, Petal with oval with slightly round tip shape, obtuse apex	5-5, 2×4-4,2cm, Petal with sub-orbicular shape, obtuse apex
Lip	4-4, 2×2-2.2 cm, pale yellow, glossy, the lower part is. The inside has many small dots about 1mm in size, reddish brown.	3, 2-3.5 ×1, 6-2 cm, the outer surface is not flat but rough, with two lobes. The inner surface has many prominent dark brown dots.
Staminode	1.2 × 0.8 cm, carried dark yellow anther with a brown border, about 2 mm in size. The stigma is large, glossy yellow, oval to ovoid-elliptical, about 6mm in size.	1.5× 0.8 cm, carried pale yellow anthers, about 2 mm in size. The stigma is large, glossy yellow, oval to ovoid-elliptic, about 6 mm in size.

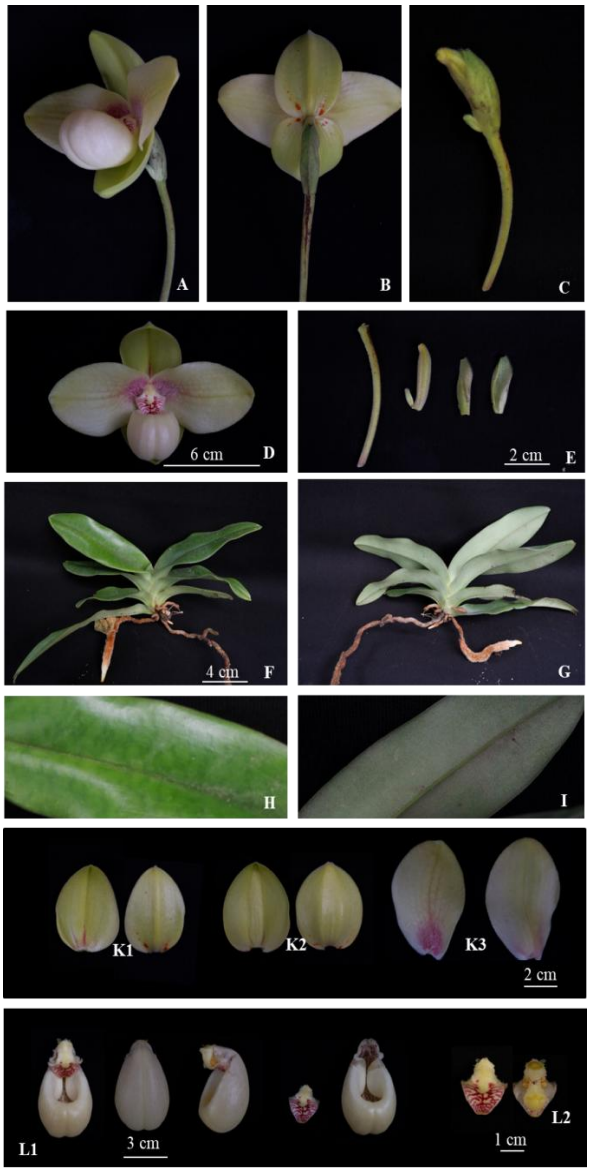

Figure 2 Morphology of stems, leaves and flower structure of *P. hangianum* A-D. Flower; E. Peduncle, ovary and Floral bract; F-I. Sterm and leaves; K1. Dorsal sepal; K2. Synsepalum; K3. Petal; L1. Lip; L2. Staminode

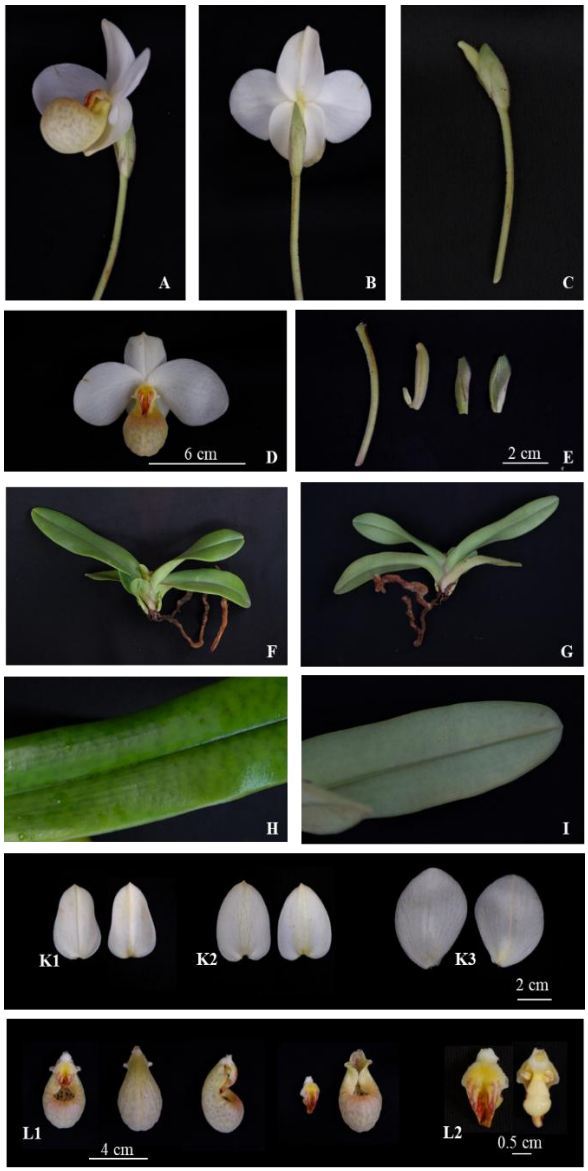

Figure 3 Morphology of stems, leaves and flower structure of *P. emersonii*A-D. Flower; E. Peduncle, ovary and Floral bract; F-I. Stermand leaves; K1. Dorsal sepal; K2. Synsepalum; K3. Petal; L1. Lip; L2. Staminode

feature of plants; although the leaf morphology of the two studied *Paphiopedilum* species are highly similar, their flowers are entirely different. The detailed descriptions of both species' morphological characteristics have been presented in Table 5 and Figures 2 & 3.

3.2 DNA barcode analysis results

Total DNA was extracted from the leaves of the two studied species. The obtained OD value of the isolated DNA was 260/280 which is within the allowable limits (no results expressed). The electrophoresis results of PCR products of *matK*, *rbcL*, *rpoC1* and *trnH-psbA* genes obtained a specific DNA segment; the size is consistent with the theoretical calculation (do not represent the results). PCR products were used for sequencing by the Sanger method on the ABI PRIMS®3100 Avant Genetic Analyzer. The gene sequences were analyzed by the BLAST tool. The tree classification scheme was built by comparing the DNA sequence of the study sample with the common species of the genus *Paphiopedilum* in Vietnam, which was published on GenBank using MegaX software. The maximum Likelihood Method was used for evolutionary analysis. Evolutionary history is based on the Maximum Likelihood method and the Tamura-Nei18 model. The

classification tree based on the most significant likelihood coefficient (-1102.40) is selected and presented.

3.2.1 Differentiation of two *Paphiopedilum* species based on DNA barcode sequence

The *rbcL* sequence obtained from two studied species has a length of 709 nucleotides, which is a highly conserved sequence. Blast results on NCBI coverage (query coverage value) showed 97 - 99% similarities with species of genus *Paphiopedilum* with a similarity coefficient of 99.15% - 99.85%, especially species *P. emersonii* (code NC_053544.1 on GenBank) has 99.86% similarity (99% coverage). Analysis of the *rbcl* sequence similarity of the two studied species with other species of genus *Paphiopedilum* showed that this indicator has a high degree of conservatism, and the selected two species have no genetic difference in the *rbcl* gene. In contrast, other species' differences range from 0.00 - 0.77 (Table 6).

Table 6 Code of gene sequences representing the genus *Paphiopedilum* used for genetic relationship analysis

	GenBank accessionnumber			
	matK	*rpoC1*	*rbcL*	*trnH-psbA*
Section *Paphiopedilum* – Subgenus *Paphiopedilum*				
P.barbigerum		MN153814.1		NC050870.1
P.hirsutissimum		NC050871.1	JN181466.1	NC050671.1
P.gratrixianum	MW284890.1			MW284890.1
P.tranlienianum	KX886262.1	MW794129.1		MW794129.1
P.spicerianum		NC052702.1	MT683624.1	
P.henryum	MK792666.1			
P.helenae	MK792663.1			
P.coccineum	MK792626.1			
Section *Pardalopetalum* Hallier f. & Pfitzer - Subgenus *Paphiopedilum*				
P.dianthum	MF983795.1	MF983795.1	MF983795.1	
P.haynaldianum			AB176547.1	
Section *Barbata* (Kraenzl.) V.A.Albert& Borge Pett. – Subgenus *Paphiopedilum*				
P.purpuratum		NC045279.1	NC045279.1	NC045279.1
P.callosum	KC692133.1			
P.applotonianum				JQ929367.1
Section *Parvisepalum* Aver. & Cribb – Subgenus *Parvisepalum* Karas. & Saito				
P.micranthum	NC045287.1	NC045276.1	NC045278.1	NC045278.1
P.malipoense	MK792675.1			JF796885.1
P.delenatii	NC045278.1	NC041309.1	NC041309.1	NC041309.1
P.armeniacum		LC085347.1	KT388109.1	LC085347.1
P.vietnamense	MK787425.1		JQ182212.1	EF156073.1
Section *Emersonianum* Aver. & Cribb - Subgenus *Parvisepalum* Karas. & Saito				
P.emersonii	MK792646.1			NC053544.1
	MK792647.1			
P.hangianum	KY966590.1			
	MK792652.1			
	MK792653.1			
	MK792656.1			
Subgenus *Brachypetalum* (Hallier) Pfitzer				
P.concolor	JQ929367.1			

The *rpoC1* sequence obtained from 2 studied species has a length of 586 nucleotides with high conservatism. Blast results on NCBI showed that the two studied species were closely related to 27 species of the genus *Paphiopedilum* (coverage reached over 97%), with similarity coefficients from 98.77% - 99.82%. The genetic similarity of the two studied species with other *Paphiopedilum* species in terms of the *rpoC1* sequence is also high. It is impossible to distinguish the two studied species by this indicator (Table 6).

The *matK* sequence obtained from the two studied species has a length of 857 nucleotides. The *matK-HaiHang* sequence blast obtained 100 sequences of species belonging to the Paphiopedilum genus. The highest similarity was 99.87% (MK792656.1 *P. hangianum*), and the lowest was 97.1% (NC_052702.1 *P. spicerianum*). Coverage reached 94 - 99%, the highest similarity was 100% (JQ182193.1 *P. delenatii*), and the lowest was 96.61% (MW528213.1 *P. parishii*). Analysis of the genetic similarity of the two studied species by *matK* indicator showed that the two species have high similarity (the coefficient of difference is only 0.006). The coefficient of difference for other species of the genus *Paphiopedilum* ranges from 0.006 - 1.04.

The *trnH-psbA* sequence obtained from the two studied species has a length of 608 nucleotides. Blast *trnH-psbA-HaiHang* obtained 100 sequences, including 91 sequences of species belonging to the genus *Paphiopedilum* (the remaining nine convergences belong to other genera, including seven species *of Phragmipedium,* one species of *Selenipedium* and one species of *Mixepedium).* Coverage ranged from 70 - 97%, the highest similarity was 99.32% (NC_045278.1. *P. micranthum*), and the lowest was 91.15% (FR851215.1 *M. xerophyticum*).

The result of Blast sequence *trnH-psbA – HaiHuong* is similar to the result of blast *trnH-psbA – HaiHang*; the Blast results were identical to 91 sequences of species belonging to the genus *Paphiopedilum* and nine sequences of species of other genera. Coverage reached 70% - 96%; the highest similarity was 99.83% (NC_053544.1 *P. ermesonii*), and the lowest was 91.29% (FR851215.1 *M. xerophyticum*).

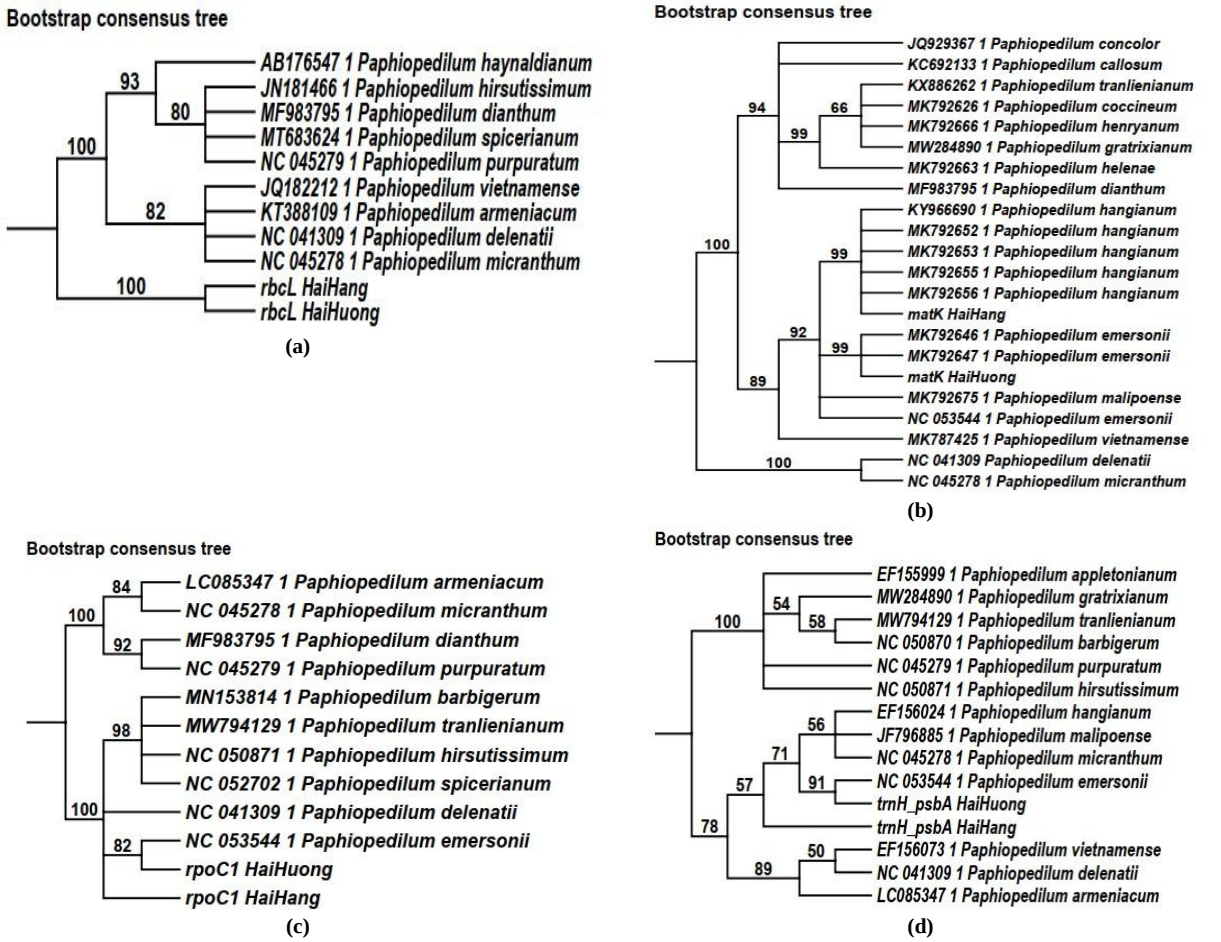

Figure 4 Molecular phylogenetic analysis of the (a) *rbcL*, (b) *matK*, (c) *rpoC1*, and (d) trnH-psbA marker. Bootstrap values are above the nodes of branches. The capital letters and numbers in parentheses are Accession numbers of *Paphiopedilum* species published on GenBank.

3.2.2 Phylogenetic analysis

The classification tree was established based on the *matK*, *rbcL*, *rpoC1*, and *trnH-psbA* markers showing that the two species *P. hangianum* and *P. ermesonii* have a very high degree of closeness. Of the four sequence markers used, only the *trnH-psbA* marker could distinguish these two species. On the tree classification diagram, they are on different branches. The first clade contains only *trnH-psbA-HaiHang* while the second group has the sequence (*trnH-psbA-HaiHuong*, C_053544.1 *P. emersonii*, NC 045278.1 *P. micranthum*, and JF796885.1 *P. malipoense*) with a bootstrap coefficient of 81%. All remaining markers could not distinguish between the two studied species, although *matK* was proposed as the best barcode, with 100% resolution in the two previous *Paphiopedilum* studies (Cahyaningsih et al. 2022; Trung et al. 2013). In addition, *MatK* has been proposed as the standard barcode of many other plant species (Gruss et al. 2018).

Analysis of the taxonomic tree in ability to identify the species grouping found that the markers differed in correspondence with the morphological classification of the identified species. In markers *rbcL* and *rpoC1*, although the two studied species are separated from the other branches according to the morphological classification system, in the branches, there is a mix of species in different sections and subgenus. In the indicator *mat K* and *trnH-psbA*, the two studied species belong to the *Ermersonianum* section and the same clade as *P. malipoense* and *P. micranthum* belong to the *Parvisepalum* section. All remaining branches have species according to the correct section and subgenus as in the traditional taxonomy by morphology (Averyanov et al. 2004). *Matk* and trnH-psbA have good species resolution, which can be used as suitable indicators for the Differentiation and phylogenetic identification of the Venus slippers orchids.

The combination of DNA barcode markers is often used to identify plant species (Rajaram et al. 2019). Guo et al. (2016) recommend the combination of *matK* + atpF-atpH + ITS as a barcode for Venus slippers. However, the combination does not often bring the desired results. In this study, only *matK* and trnH-psbA markers could distinguish the selected two species (Figure 4).

4 Discussion

4.1 Morphological characteristics of two orchid species, *P. hangianum* and *P. emersonii*

Although classifying plants based on morphological characteristics is classical, but necessary and significantly supports the identification. Many plant identification keys have been successfully developed and used to identify various orchid species based on plant morphological structure. Most new species' announcements for the Orchidaceae family are based on plant morphological descriptions, in which flower structure is the most objective criterion (Averyanov et al. 2010; Wang et al. 2017; Gruss et al. 2018, Zheng et al. 2020).

Paphiopedilum is the largest genus and most differentiated and studied in great detail in orchids. Averyanov et al. (2004) established a prominent taxonomic system for the *Paphiopedilum* in Vietnam. This classification system was later developed and used to recognize new species. In this taxonomy, *P. hangianum* and *P. emersonii* are grouped in a section that includes only these two species (Section *Emersonianum* Aver. & Cribb - Subgenus *Parvisepalum* Karas. & Saito). Vu et al. (2020) preliminary classified *Paphiopedilum* into two large groups based on leaf morphology. According to these authors, group 1 includes striated leaves, and Group 2 has leaf species without veins. Group 2 is again divided into group 2A, species with small, long soft leaves, and group 2B, which includes three large and stiff leaves. *P. hangianum* and *P. emersonii* belong to group 2B. After this detailed classification, these authors couldn't distinguish these two species and put them in the same group.

In this study, when directly and meticulously observing the two species over a long period of culture under the same growing conditions, we found that *P.hangianum* and *P. emersonii* have some small distinct features like leaf colour, the arrangement of leaves on the stem, the clarity of the veins on the leaves and the winding of the edges of the leaves, these features can be used to distinguish these two species including. These characteristics have not been described in the previous classification of *Paphiopedilum*. Therefore, these results are new milestones in identifying selected two species. In investigating the ecoregions of the two studied species, we found that, although they share many similarities, they are placed in the same subgenus and section in the taxonomic keys. Still, they are two species with different ecoregions in the wild and rarely encounter them in the same habitat. Similar findings were previously reported by Averyanov et al. (2004).

Although in the genus *Paphiopedilum*, many species are similar in leaf morphology (Averyanov et al. 2004; Vu et al. 2019), each species has distinct characteristics and flowers are used as the primary classification criterion. Venus orchids have a long growth cycle and short flowering time. Therefore, making it difficult to identify by normal morphology, good expertise is needed to distinguish similar species accurately. This makes it difficult to conserve and trade venus orchids, so developing an effective species identification method is necessary, in which a DNA barcode is a potential method. For a long time, using DNA barcodes to classify plants has gradually become a popular tool. Many studies on many plant species use different barcodes and recommend barcodes suitable for them.

Table 7 The distinguishes ability to distinguish two studied species

	MatK	*trnH-psbA*	*RpoC1*	*rbcL*	*trnH-psbA + matK*	*trnH-psbA + rpoC1*	*trnH-psbA + rbcL*	*trnH-psbA + matK + rpoC1*	*trnH-psbA + matK + rbcL*	*rnH-psbA +rbcL+rpoC1*	*matK + trnH-psbA+rpoC1 +rbcL*
Ability to distinguish	-	+	-	-	+	-	-	-	-	-	-

4.2 Insight into the molecular classification of *P. hangianum* and *P. emersonii*

For the genus Orchid, the study of species identification based on barcodes was first conducted by Parveen et al. (2012). Among the eight species of *Paphiopedilum* occurring in India, the study tested five potential barcodes (*rpoB, rpoC1, rbcL, matK*, and nrITS). The results showed that *matK*, with an average interspecies divergence value of 0.9%, yielded a species resolution of 100% of identified species, while ITS only reached 50%, so *matK* was recommended as a barcode to distinguish *Paphiopedilum* species (Table 7). So far, *matK* remains the proposed indicator in studying orchid subspecies. Worthy et al. (2022) also reported the excellent efficacy of the *matK* indicator (compared to the *rbcL* directive) in the barcode study of orchid sub-species and subgenus. In this study, *matK* proved effective in distinguishing two closely related species, *P. hangianum* and *P. emersonii*. Guo et al. (2016) used a database of 107 samples representing 77 *Paphiopedilum* species with eight chloroplast DNA markers and nrITS found; among the single-locus barcodes, nrITS was the most efficient for species identification of the genus (52.27%), while *matK* + atpF-atpH was the most efficient multi-locus combination (28.97%). Moreover, combining matK + atpF-atpH + ITS as a code to identify the genus Paphiopedilum is recommended. Rajaram et al. (2019) used four markers *(rbcL, matK,* ITS, trnH-psbA) to test the 17 samples of 4 endangered *Paphiopedilum* species on the Malixia peninsula. The results found that *matK* is the most potential barcode that has high sequence quality (100%), high accuracy in BLASTn (100%), and precise resolution of species in neighbouring phylogenetic trees (100%), different barcode spacing followed by ITS, are trnH-psbA, and *rbcL*. Multiple indicators and criteria are required for accurate classification in some exceptional cases. For example, in the case of the species *P. canhii*, it was pretty controversial about the taxonomy because of the mixed morphological features among the subgenus. To solve this problem, Górniak et al. (2014) used a combination of morphological data, cytology, and phylogenetic analysis based on DNA barcode (with chloroplast gene markers such as Xdh, matK, trnH-psbA, trnQ-rps16, nuclear genes such as ITS), leaf adaxial epidermal studies, and gynostemium structures were obtained from Scanning Electron Microscopy (SEM) and Light Microscopy (L.M.). Vu et al. (2019) used a DNA barcode to classify 22 species of *Paphiopedilum* in Vietnam. According to the author, trnH - psbA is limited in amplification; ITS is the best single barcode, and the author recommends the *matK*+ITS combination as the most suitable for Vietnam's Venus orchid classification.

Many previous studies have used DNA barcodes to classify a group or a system of orchids in a country, but no published report has been available to distinguish these two closely related species. In this study, when assessing the species specificity of *P. hangianum* and *P. emersonii* by four single chloroplast makers (*rbcL, rpoC1, matK,* trnH-psbA) and seven maker combinations (trnH-psbA + *matK*, trnH-psbA + *rpoC1*, trnH-psbA + *rbcL*, trnH-psbA + *matK* + *rpoC1*, trnH-psbA + *matK* + *rbcL*, trnH-psbA + *rbcL* + *rpoC1*,*matK* + trnH-psbA+*rpoC1*+*rbcL*) we found trnH-psbA to be the single marker with the best distinction. The trnH-psbA + *matK* complex was the only combination that could distinguish two close species in the Vietnamese *Paphiopedilum* classification system. These are also the two most suitable markers for the Differentiation and phylogenetic determination of the Venus orchid in this study. The analyzed species were grouped according to previous studies' morphological classification. However, using a combination of two markers will be costly in terms of time and cost; therefore, we propose using trnH-psbA as an indicator to differentiate *P. hangianum* and *P. emersonii* and phylogenetic determination *Paphiopedilum* genus of Vietnam.

DNA barcoding is increasingly developing and has many applications; chloroplast genome (super barcodes) brings outstanding applications in taxonomic and phylogenetic research (Liu et al. 2022, Sun et al. 2022). They are overcoming the limitations of previous barcode studies, such as some unanswered phylogenetic questions in *Paphiopedilum*. For example, recent phylogenetic studies indicate widespread reticular evolution within

the genus and that earlier markers cannot address the deep phylogenetic relationship (Tsai et al. 2020). In addition, barcodes have been studied under the name in-silico on the phylogenetic framework associated with the characteristics of *Paphiopedilum* species distributed by a country to determine the species' passport characteristics (Siga et al. 2022). These results will guide future research related to the genus *Paphiopedilum* in Vietnam.

Conclusions

This study identified morphologically and DNA markers to distinguish *P. hangianum* and *P. emersonii* at the flowering and non-flowering stages. Some detailed characteristics of flowers, such as bracts, sepals, synaptic membranes, petals, lips, and stamens, can be used as indicators to distinguish the two species at the flowering stage. Four chloroplast DNA markers such as *rbcL*, *matK*, *rpoC1*, and *trnH-psbA*, were analyzed for the marker as a DNA barcode, and the indicator *trnH-psbA* was proposed as a DNA barcode to distinguish two species of *P. hangianum* and *P. emersonii* at the non-flowering stages. Identifying two similar species, *P. hangianum* and *P. emersonii,* of the genus *Paphiopedilum,* based on morphological characteristics in combination with the DNA barcoding method, solved the identification problems in the absence of flowers or young conditions.

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Conflicts of interest

All authors declare no conflicts of interest.

References

Averyanov, L., Cribb, P., Phan, K.L., & Nguyen, T.H. (2004). Slipper Orchids of Vietnam, Bird Life. Royal Botanic Gardens KEW, World Bank: Ho Chi Minh City, Vietnam 100-120, 308,

Averyanov, V.L., Gruss, O., Chu, X.C., Phan, K.L., Bui, X.D., & Nguyen, T.H. (2010). *Paphiopedilum canhii* - a new species from Northern Vietnam. *Orchids*, *79*(5), 289-290.

Bolson, M., Smidt, E.C., Brotto, M.L., & Silva-Pereira, V. (2015). ITS and trnH-psbA as efficient DNA Barcodes to identify threatened commercial woody angiosperms from southern Brazilian Atlantic rainforests. *PLoS ONE, 10*:e0143049.

Braem, G.J., & Gruss, G.R. (2012). *Paphiopedilum* subgenus *Megastaminodium* Braem & Gruss, a new subgenus to accommodate *Paphiopedilum canhii*. *The 67th Santa Barbara International Orchid Show*, 3-164.

Braem, G.J., Baker, C.O., & Baker, M.L. (1998). *The Genus Paphiopedilum, Natural History and Cultivation*, vol. 1. Botanical Publishers Inc., pp. 182.

Braem, G.J., Baker, C.O., &Baker, M.L. (1999).*The Genus Paphiopedilum, Natural History and Cultivation*, vol. 2. Botanical Publishers Inc., pp. 363.

Bui, T.T.H., Duong, X.A., Nguyen, H.C., Ngo, T.A., Dong, H.G., Ho, M.T., Chu, H.H., Tran, T.T.H., Khuat, H.T., & Tran, D.K. (2022). Morphological characteristics and DNA barcoding in bach hop (*Lilium poilanei* Gagnep) in Vietnam. *Australian Journal of Crop Science,16*(04), 471-478.

Cahyaningsih, R., Compton, L.J., Rahayu, S., Magos Brehm, J., & Maxted, N. (2022). DNA Barcoding Medicinal Plant Species from Indonesia. *Plants, 11*(10), 1375.

Collins, G.G. & Symons, R.H. (1992). Extraction of nuclear DNA from grape vine leaves by modified procedure. *Plant Molecular Biology Reporter, 10*, 233-235

Cribb, P. (1998). The Genus *Paphiopedilum*. 2nd ed. *Natural History Publications (Borneo), Kinabalu & Kew*.

Dang, V.K., Nguyen, T.N., & Vu, H.T. (2017). Screening, designing, pilot evaluation and application of some potentialprimers for molecular discrimination of *Paphiopedilum* species in Vietnam. Vietnam. *Journal of Agriculture and Rural Development in the Tropics and Subtropics, 12*, 113–118.

Gorniak, M., Szlachetko, D.L., Kowalkowska, A.K., Bohdanowicz, J., & Canh, C.X. (2014). Taxonomic placement of *Paphiopedilum canhii* (Cypripedioideae; Orchidaceae) based on cytological, molecular and micromorphological evidence. *Molecular Phylogenetics and Evolution,70*, 429-441.

Gruss, O., Averyanov, V.L., Chu, X.C., & Nguyen, H.T. (2018). A new variety of a natural hybrid of the genus *Paphiopedilum* from Vietnam: *Paphiopedilum* × *aspersum* var. Trantuananhii. *Die Orchidee*, *4*, 52-54.

Guo, Y.Y., Huang, L.Q., Liu, Z.J., & Wang, X.Q. (2016). Promise and Challenge of DNA Barcoding in Venus Slipper (*Paphiopedilum*). *PLoS ONE, 11*(1), e0146880.

Hollingsworth, P.M., Forrest, L.L. & Spouge,J.L. (2009).A DNA barcode for land plant. *Proceedings of the National Academy of Sciences of the United States of America,106*, 12794-12797.

Koopowitz, H. & Cribb, P.J. (1986) Original description of *Paphiopedilum emersonii*. *The Orchid Advocate*, *12*(3): 84-86

Koopowitz, H. (2008). *Tropical Slipper Orchids: Paphiopedilum and Phragmipedium Species and Hybrids*. Portland, Oregon: Timber Press

Kress, W.J., & Erickson, D.L.(2007). A two-locus global DNA barcode for land plants: the coding rbcL gene complements the non-coding trnH-psbA spacer region. *PLoS ONE*, *2*(6)*: e508.*

Liu, H., Ye, H., Zhang, N., Ma, J., Wang, J., Hu, G., Li, M., & Zhao, P. (2022) Comparative Analyses of Chloroplast Genomes Provide Comprehensive Insights into the Adaptive Evolution of *Paphiopedilum* (Orchidaceae). *Horticulturae*, *8*, 391. https://doi.org/10.3390/horticulturae8050391

Parveen, I., Singh, H. K., Raghuvanshi, S., Pradhan, U. C., & Babbar, S. B. (2012). DNA barcoding of endangered Indian *Paphiopedilum* species. *Molecular ecology resources*, *12*(1), 82–90. https://doi.org/10.1111/j.1755-0998.2011.03071.x

Perner, H. & Gruss, O. (1999). Original description of *Paphiopedilum hangia* num. 6.

Rajaram, M.C., Yong, C.S.Y., Gansau, J.A., & Go, R. (2019). DNA Barcoding of Endangered *Paphiopedilum* species (Orchidaceae) of Peninsular Malaysia. *Phytotaxa.*, *387*(2), 94–104

Siga, A., Sarkar. A., Konwar, P., Saikia, J., Saikia, S. P., & Banik. D. (2022). Trait-linked phylogenetic framework of *Paphiopedilum* distributed in India revealed species passport trait to prevent unethical trade through in-silico study. *South African Journal of Botany*,*150*(11):420-430. DOI: 10.1016/j.sajb.2022.07.021

Singh, H.K., Parveen, I., Raghuvanshi, S., & Babbar, S.B. (2012). The loci recommended as universal barcodes for plants on the basis of floristic studies may not work with congeneric species as exemplified by DNA barcoding of *Dendrobium* species. *BMC Research Notes*, *5*, 42.

Sun, Y., Zou, P., Jiang, N., Fang, Y. & Liu, G. (2022) Comparative Analysis of the Complete Chloroplast Genomes of Nine *Paphiopedilum* Species. *Frontiers in Genetics*, *12*, 772415, 1:19. doi:10.3389/fgene.2021.772415

Tamura, K. & Nei M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution*, *10*, 512-526.

Tate, J.A., & Simpson, B.B. (2003). Paraphyly of Tarasa (Malvaceae) and diverse origins of the polyploid species. *Systematic Botany*, *28*,723–737.

Tsai, C.C., Liao, P.C., Ko, Y.Z., Chen, C.H., & Chiang, Y.C. (2020). Phylogeny and Historical Biogeography of *Paphiopedilum* Pfitzer(Orchidaceae) Based on Nuclear and Plastid DNA. *Frontiers in Plant Science*, *11*, 126. doi:10.3389/fpls.2020.00126

Tran, D.D., Khuat, H.T., La, T.N., Nguyen, T.T.T., Pham, B.H., Nguyen, T.K., Tran, H.D., Do, M.T., & Tran, D.K. (2018). Identification of Vietnamese Native *Dendrobium* Species Based on Ribosomal DNA Internal Transcribed Spacer Sequence. *Advanced Studies in Biology*, *10*, 1–12.

Trung, K.H., Khanh, T.D., Ham, L.H., Duong, T.D., & Khoa ,T. (2013). Molecular Phylogeny of the Endangered Vietnamese *Paphiopedilum* Species Based on the Internal Transcribed Spacer of the Nuclear Ribosomal DNA. *Advanced Studies in Biology*, *5*, 337–346

Veldman, S., Kim, S.J., van Andel, T.R., Bello Font, M., Bone, R.E., Bytebier, B., Chuba, D., Gravendeel, B,, Martos, F,, Mpatwa,G., et al. (2018). Trade in Zambian Edible Orchids-DNA Barcoding Reveals the Use of Unexpected Orchid Taxa for Chikanda. *Genes*, *9*, 595.

Vu, H.T., Bui, M.H., Vu, Q.L., Nguyen, T.D., Tran, H., Khuat, H.T., & Le, L. (2019). Identification of Vietnamese *Paphiopedilum* Species Using Vegetative Morphology. *Annual Research & Review in Biology*, *34*(1), 1-14.

Vu, H.T., Vu, Q.L., Nguyen, T.D., Tran, N., Nguyen, T.C., Luu, P.N., Tran, D.D., Nguyen, T.K., & Le, L. (2020). Genetic Diversity and Identification of Vietnamese Paphiopedilum Species Using DNA Sequences. *Biology*, *9*(1) 9, 2020.

Wang, M., Lan, S.-R., & Liu, Z.J. (2017). *Paphiopedilum notatisepalum*, a new species of slipper orchid (Cypripedioideae, Orchidaceae) from China based on morphological and DNA evidence. *Phytotaxa*, *302*(2), 156–164.

Worthy, S. J., Bucalo, K., Perry, E., Reynolds, A., Cruse-Sanders, J., Pérez. A. J. & Burgess, K. S. (2022). Ability of *rbcL* and *matK* DNA barcodes to discriminate between montane forest orchids. *Plant Systematics and Evolution*, *308*: 19. DOI: 10.1007/s00606-022-01809-z

Wu, C.T., Gupta, S.K., Wang, A.Z.M., Lo, S.F., Kuo, C., Ko, Y.J., Chen, C., Hsieh, C.C., & Tsay, H.S. (2012). Internal Transcribed Spacer Sequence Based Identification and Phylogenic Relationship of *Herba Dendrobii*. *Journal of Food and Drug Analysis*, *20*, 143–151.

Xu, Q., Zhang, G.Q., Liu, Z.J., & Luo, Y.B. (2014). Two new species of *Dendrobium* (Orchidaceae: Epidendroideae)from China: Evidence from morphology and DNA. *Phytotaxa*, *174*(3), 129-143.

Xu, S., Li, D., Li, J., Xiang, X., Jin, W., Huang, W., Jin, X., &Huang, L. (2015). Evaluation of the DNA Barcodes in *Dendrobium* (Orchidaceae) from Mainland Asia. *PLoS ONE, 10,* e0115168.

Yukawa, T., Kinoshita1, A., &Tanaka, N. (2013). Molecular Identification Resolves Taxonomic Confusion in Grammatophyllum speciosum Complex (Orchidaceae). *Bulletin of the National Museum of Nature and Science, 39,* 137–145.

Zheng, B. Q., Zou, L.H., Wan, X., & Wang, Y. (2020). *Dendrobium jinghuanum*, a new orchid species from Yunnan, China: evidencefrom both morphology and DNA. *Phytotaxa, 428*(1): 030–042.