Vietnam Journal of Biotechnology **20**(2): 279-287, 2022

INSIGHT INTO *PAPHIOPEDILUM X DALATENSE* AVER. (ORCHIDACEAE) ORIGIN BASED ON MORPHOLOGICAL AND MOLECULAR MARKERS

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Received: 04.11.2021
Accepted: 24.01.2022

SUMMARY

Paphiopedilum callosum, *Paphiopedilum villosum* and *Paphiopedilum × dalatense* are endangered species and are currently listed as Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2019) Appendix I species. Thus, the international trade for these naturally occurring species is forbidden. However, the *in vitro* propagated materials and their inter-species hybrids forced by breeders can be traded for commercial purposes. *P. × dalatense* is an endemic slipper orchid species of Vietnam with high aesthetic value. Currently, the natural reserves of this species have been greatly reduced by overexploitation. In this study, *P. × dalatense* was demonstrated to be the reversible hybrid species between *P. callosum* and *P. villosum* based on both morphological and molecular markers. *P. × dalatense* possesses intermediate morphological characteristics between *P. callosum* and *P. villosum* which could be recorded in leaf, in flower and its components such as lip, petal, and dorsal sepal. There were 23 sites in ITS1-5.8S rRNA-ITS2 region sequence of *P. × dalatense* which possess the nucleotide characteristic of both *P. callosum* and *P. villosum*. There were two out of three *P. × dalatense* investigated samples possessed partial *mat*K gene which was identical to the corresponding sequence in *P. villosum* and the last sample possessed identical partial *mat*K gene to *P. callosum*. Proving *P. × dalatense* to be the reversible hybrid species between *P. callosum* and *P. villosum* is the essentially scientific base for commercial breeding of *P. × dalatense* by artificial hybridization which contributes to avoid overexploiting the natural *P. × dalatense* resources.

Keyword: ITS1-5.8S rRNA-ITS2, hybrid species, *Paphiopedilum × dalatense,* partial *mat*K gene, reversible

INTRODUCTION

Paphiopedilum genus includes about 75 species which distribute in tropical Asia, from South India and East Himalaya to Philippines, New Guinea and the Solomon Islands. There are about 22 to 26 naturally original and hybrid *Paphiopedilum* species distributed in Vietnam. With flower's special structure - the lip's shape looks like the shoe, slipper orchid species and their hybrids have been infatuated, collected and cultivated in many countries around the world.

Natural slipper orchid species usually distribute in the high mountain regions with the altitude above sea level of 800 - 1500 m, however there are several species distribute the lower altitude such as *P. concolor*. Slipper orchids have

been found in cool, shady, high humidity habitats, i.e. temperature from 18 to 24ºC, light intensity from 30 to 40% sunlight, humidity about 80% is the suitable condition for growth and development of *Paphiopedilum* species. Almost slipper orchid species naturally grow in the craggy terrains such as upright cliffs in mountainous regions or ledges near waterfalls or under the canopies of tall trees (Averyanov *et al.*, 2003).

Paphiopedilum callosum (Rchb. f.) Stein has Vietnamese name is "Van hai" meaning veined slipper orchid and characterized by clear vein in the dorsal sepal. This is a natural slipper orchid species which has scattered distribution in different areas in Thailand, Cambodia, Lao and Vietnam. In Vietnam *P. callosum* naturally grows in evergreen, dense primary forest with broadleaf trees, seasonal rain climate. This species usually grows on leaf humus belonging to grass layer in the forest like *Cymbidium* species. However, occasionally this species can be found on rocks covered with moss (Averyanov *et al.*, 2003). Nowadays, it's extremely rare to meet *P. callosum* in nature in Vietnam because this species grows in the low altitude places, easy to be exploited resulting in a depletion of the species reserves.

Paphiopedilum villosum (Lindl.) Stein has a Vietnamese name is "Hai vang" meaning yellow slipper orchid and characterized by large and attractive flowers. This species has a wide and varied distribution from Eastern North India, North Myanmar, Western South Yunnan to North Thailand. This species is relatively popular in the locality of Da Lat, especially in Lang Bian mountain and is the only slipper orchid species possesses mainly epiphyte, it is rarely found in the rocks, but at the inaccessible rock tops of steep cliffs in gorges along alpine streams (Averyanov *et al.*, 2003). Currently, *P. villosum* is recorded in the Red list of the plant in Vietnam as EN B1+ 2b, c, e (Ban, 2007).

Paphiopedilum × *dalatense* Aver. was described in 2001 by Averyanov and this author himself considered this species was the hybrid between *P. villosum* and *P. callosum* due to *P.* × *dalatense* possesses the intermediate characteristics in morphology between the two hypothetically parental species. This species is characterized by a relatively large flower with an olive-green vein in dorsal sepal and small black-purple flat warts along upper margin toward the base of petals, these make *P.* × *dalatense* its own unique beauty. In nature, *P.* × *dalatense* could be found at evergreen primary forests in Don Duong district, Lamdong province (Averyanov, 2001). Nowadays, this species become rare to meet in nature due to overexploitation and the narrowing of the habitats. Demonstration *P.* × *dalatense* was the hybrid species between *P. villosum* and *P. callosum* based on morphological and molecular proofs will support to commercial breeding *P.* × *dalatense* by artificial hybridization. However, up to the present time, there has been no in-depth study to determine the genetic relationship between the three species of *P. callosum*, *P. villosum* and *P.* × *dalatense* found although the artificial hybrids between still possess similar morphological characteristics to *P.* × *dalatense*.

In this study, the intermediate characteristics in morphology between *P. callosum* and *P. villosum* expressed in *P.* × *dalatense* are shown together with the comparison DNA data of ITS1-5.8S rRNA-ITS2 nuclear region and chloroplast partial *mat*K of three investigated species to make an insight into the origin of *P.* × *dalatense*.

MATERIALS AND METHODS

Materials

Materials for morphological investigation are all of adult individuals of three studied species (*P. callosum*, *P. villosum* and *P.* × *dalatense*) which were collected from their natural habitats in Don Duong district, Lam Dong province, Vietnam from 1999 to 2019 and have been cultivated to keep and to conserve in Tay Nguyen Institute of Scientific Research. These individuals were previously classified and identified by Nong Van Duy. In addition, the dry

specimens in VTN botanical museum also used in the research.

In study on molecular characteristics, leaf of three alive above adult individuals per each studied species were sampled for analysis. Samples belong to *P. callosum* were coded as A1-A3, *P. villosum* as B1-B3, and *P.* × *dalatense* as C1-C3, respectively.

Recording and comparing morphological characteristics

The throughout method in the process of recording morphological characteristics of surveyed species and determining the intermediate characteristics between *P. villosum* and *P. callosum* species expressed in *P.* × *dalatense* is the comparative morphological method in accordance with described by Thin (2007), Klein and Klein (1970).

The differences among three studied species were investigated in vegetative organs, i.e. quantity, shape, size, color of leaves and reproductive organs, i.e. shape, size, distribution of basal color and vein color and warts of flower and its components such as dorsal sepal, petal, lip. Based on that, indicating which morphological characteristics in *P.* × *dalatense* were the intermediate characteristics between *P. villosum* and *P. callosum*.

Identification hybrid species based on partial DNA sequence of ITS1-5.8S rRNA-ITS2 and *mat*K gene

DNA extraction

Total genomic DNA was extracted using CTAB protocol I (Weising *et al*., 2005) with a modification of adding 10% SDS to the extraction buffer, which was then dissolved in water for subsequent use.

DNA isolation and amplification by PCR

PCRs were performed in 50 µL reactions containing 25 µL My Red HS Taq mix (Bioline), 2.5 µL 10 pmol/µL forward primer, 2.5 µL 10 pmol/µL reverse primer and approximately 50 ng DNA templates. The reactions were performed in a Mastercycler ® nexus thermocycler (Eppendorf, Germany) with the following thermal programs: (i) For ITS1-5.8S rRNA-ITS2 region: initial denaturation at 94°C for 7 min, 35 cycles of 94°C for 30 s, 51°C for 1 min, 72°C for 1 min 30 s; final extension at 72°C for 10 min; (ii) For partial *mat*K gene: initial denaturation at 94°C for 5 min; 36 cycles of 94°C for 45 s, 51°C for 45 s, 72°C for 1 min 30 s; final extension at 72°C for 15 min.

Forward and reverse primers for ITS1-5.8S rRNA-ITS2 region isolation and amplification were ITS 5P (5'- GGA AGG AGA AGT CGT AAC AAG G -3') and ITS 8P (5'- CAC GCT TCT CCA GAC TAC A -3'), respectively. This primer pair was used in the previous study by Moller and Cronk (1997). Forward and reverse primers for partial *mat*K gene isolation and amplification were *mat*K F (5'- CGA TCT ATT CAT TCA ATA TTT C -3') and *mat*K R (5'- GTT CTA GCA CAA GAA AGT CG -3'), respectively. This primer pair was used in the previous study by Zuo *et al*. (2011)

Electrophoresis, purification and sequencing

The PCR products were detected by electrophoresis on 1.0 % agarose gel electrophoresis and then were purified using AccuPrep ® Gel purification Kits (Bioneer, Korea). DNA sequencing was performed using ABI 3730 sequencers (Phu Sa Biochem Ltd., Vietnam)

DNA sequence data analysis

DNA sequence data were processed by Bioedit 5.6.0 (Hall, 1999) and Chromas 2.6.6 (Goodstadt, Ponting, 2001). Mega 6 software was used to align the corresponding sequences (Tamura *et al*., 2013).

From the scientific basis of morphological and molecular data, identify *P.* × *dalatense* is a hybrid between *P. villosum* and *P. callosum*, and indicate which species plays the maternal or paternal role (if any).

RESULTS AND DISCUSSION

The origin of *P.* × *dalatense* based on morphological characteristics

The morphological data recorded for three investigated species (*P. callosum*, *P. villosum* and *P.* × *dalatense*) in this study were not much different from previous studies (Averyanov, 2001; Averyanov *et al*., 2003), but also indicated detail characteristics which were not clearly described by previous authors. These characteristics reflected the intermediate nature between *P. callosum* and *P. villosum* in *P.* × *dalatense* (Figure 1.a), as follows:

In leaf morphology, *P. callosum* were narrowly elliptic, oblong-elliptic or obovate, tridenticulate at the acute apex, ciliate at the base, tessellated pale and dark above, sometimes purple at a base on the lower surface (Figure 1.b1). In *P. villosum* had leaves linear ligulate, acuminate to acute at unequally bilobate apex mid-green on the undersurface, purple-spotted at the base, basal margins ciliate (Figure 1.b2). Meanwhile, leaves of *P.* × *dalatense* were narrowly or oblong-elliptic acute at apex, green/pale-green finely but indistinctly tessellated above, with fine purple-violet speckled toward the base on the lower surface (Figure 1.b3).

Dorsal sepals of *P. callosum* were white flushed with purple in the lower half, veined with purple and green (Figure 1.d1). *P. villosum* had dorsal sepal green with a white margin and central glossy deep maroon areas (Figure 1.d2). *P.* × *dalatense* had dorsal sepal white at the base with lower half flushed pink, pink-brown, purple-brown, purple, or olive-green, veined with olive-green, brown or purple (Figure 1.d3).

Petals of *P. callosum* was white to yellow-green with a purple apical third spotted with maroon on upper margin and sometimes in basal half. Petals sometimes reflexed, sub-sigmoid, ligulate, obtuse or rounded at apex, maroon-ciliate (Figure 1.e1). *P. villosum* has petals glossy reddish-brown with a central maroon stripe. Petals incurved, obovate-spathulate, retuse-emarginate, rounded at apex, glossy, ciliate, purple-villose at the base (Figure 1.e2). *P.* × *dalatense* petals were white to a dull pink, yellowish-green and pale brownish-green with pink, brownish-purple or purple stripes, sometimes few spotted with small black-purple flat warts along upper margin toward the base. Petals reflexed, sub-sigmoidal, ligulate, obtuse or broad apiculate at apex, with black-purple cilia (Figure 1.e3).

About the lips, in *P. callosum*, the lip had green, heavily flushed deep maroon and warty on incurved side-lobes (Figure 1.f1). In *P. villosum* the lip had lip have ochre, flushed with pink or reddish and lip tapering to apex (Figure 1.f2). *P.* × *dalatense* had lip pink, yellowish-pink or light yellowish-brown with more deep coloured veins (Figure 1.f3).

Above data showed that *P.* × *dalatense* possessed morphological characteristics which could be considered as intermediaries between *P. villosum* and *P. callosum* in both vegetative and reproductive organs. This is the morphological basis to indicate that *P.* × *dalatense* is a hybrid between the other two species.

The origin of *P.* × *dalatense* based on molecular data

The origin of P. × *dalatense based on ITS1-5.8S rRNA-ITS2 region sequence*

The length of ITS1-5.8S rRNA-ITS2 region sequence in *P. callosum* was 764 bp, in *P. villosum* and *P.* × *dalatense* was 761 bp. Result of careful check the sequences and chromatograms of ITS1-5.8S rRNA-ITS2 region showed that the sequences of same investigated sample induced by sequencing with forward and reverse primers were highly matched; the sequences of the samples belonging to the same species were identical. Comparing ITS1 region among three investigated species indicated that there were 12 nucleotide sites in *P.* × *dalatense* having the specific nucleotides of both *P. callosum* and *P. villosum* (Table 1).

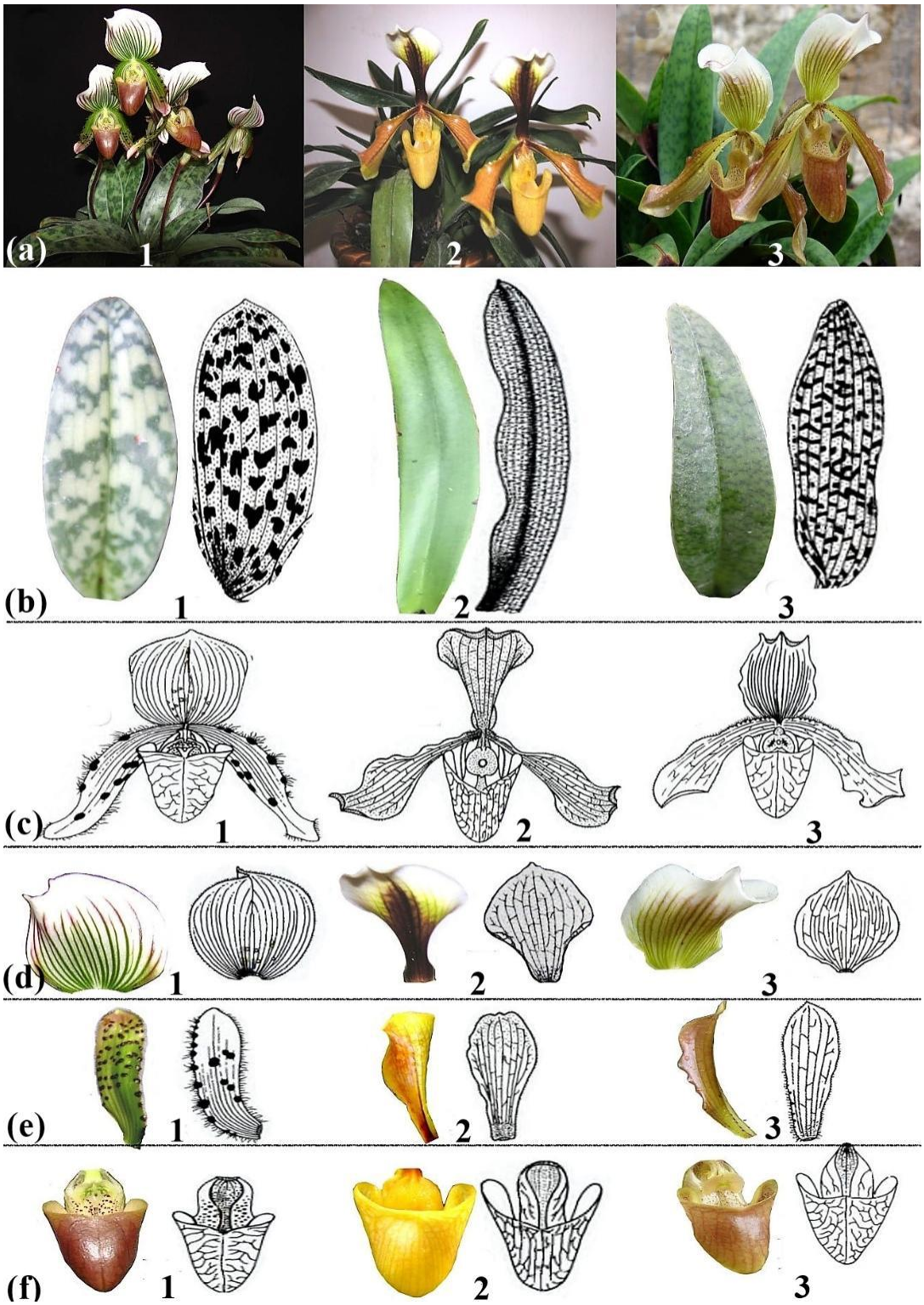

Figure 1. (a) Morphological; (b) Leaf characteristic; (c) Flower characteristic; (d) Dorsal sepal characteristic; (e) Petal characteristic; (f) Lip characteristic; (1) *P. callosum*; (2) *P. villosum* and (3) *P.* × *dalatense*.

Table1. Variation of ITS1 sequence among three investigated species.

Species \ Site	86	117	121	129	165	172	173	184	215	227	236	242	243	248	272
P. callosum	T	G	A	T	T	G	A	T	C	T	G	C	C	C	T
P. villosum	A	A	C	G	C	-	-	A	A	C	C	G	G	T	-
P. × dalatense	W	R	M	K	Y	-	-	W	M	Y	S	S	S	Y	-

Y: C and T; K: G and T; S: C and G; R: A and G; W: A and T; M: C and A.

Table 2. Variation of 5.8S rRNA sequence among three investigated species.

Species \ Site	453	465	510	536	547
P. callosum	C	T	T	A	A
P. villosum	T	C	A	G	T
P. × dalatense	Y	Y	W	R	W

Y: C and T; R: A and G; W: A and T.

Table 3. Variation of ITS2 sequence among three investigated species.

Species \ Site	594	595	603	606	635	637
P. callosum	T	C	A	G	C	G
P. villosum	C	T	G	T	G	A
P. × dalatense	Y	Y	R	K	S	R

Y: C and T; K: G and T; S: C and G; R: A and G

Comparing 5.8S rRNA region among three investigated species indicated that there were 5 nucleotide sites in *P. × dalatense* having the specific nucleotides of both *P. callosum* and *P. villosum* (Table 2).

Comparing ITS2 region among three investigated species indicated that there were 6 nucleotide sites in *P. × dalatense* having the specific nucleotides of both *P. callosum* and *P. villosum* (Table 3).

Thus, in the ITS1-5.8S rRNA-ITS2 region, *P. × dalatense* possessed 23 nucleotide sites which were specific for both *P. callosum* and *P. villosum*. This phenomena was shown clearly in the chromatograms and indicated that *P. × dalatense* received one sister chromatid from *P. villosum* and another from *P. callosum* in certain dyad. Based on sequence data of the ITS1-5.8S rRNA-ITS2, it is recognized that *P. × dalatense* is the naturally hybrid species between *P. callosum* and *P. villosum*.

Studying on the hybrid orchid species in Guatemala, Szlachetko *et al.* (2017) used to investigate ITS1-5.8S rRNA-ITS2 region and gene *XDH* in several *Cypripedium* species including *C. dickinsonianum, C. irapeanum, C. molle* and *C.* × *fred-mulleri* and indicated *C.* × *fred-mulleri* is the naturally hybrid species

between *C. dickinsonianum* và *C. irapeanum* based on *C.* × *fred-mulleri* possesses 4 nucleotide sites in ITS1-5.8S rRNA-ITS2 region and 7 nucleotide sites in *XDH* gene which are specific for both *C. dickinsonianum* and *C. irapeanum*. Comparing to the research of Szlachetko *et al.* (2017), in the current research the number of nucleotide sites specific for both parent species was significantly higher (23 sites comparing to 5 sites).

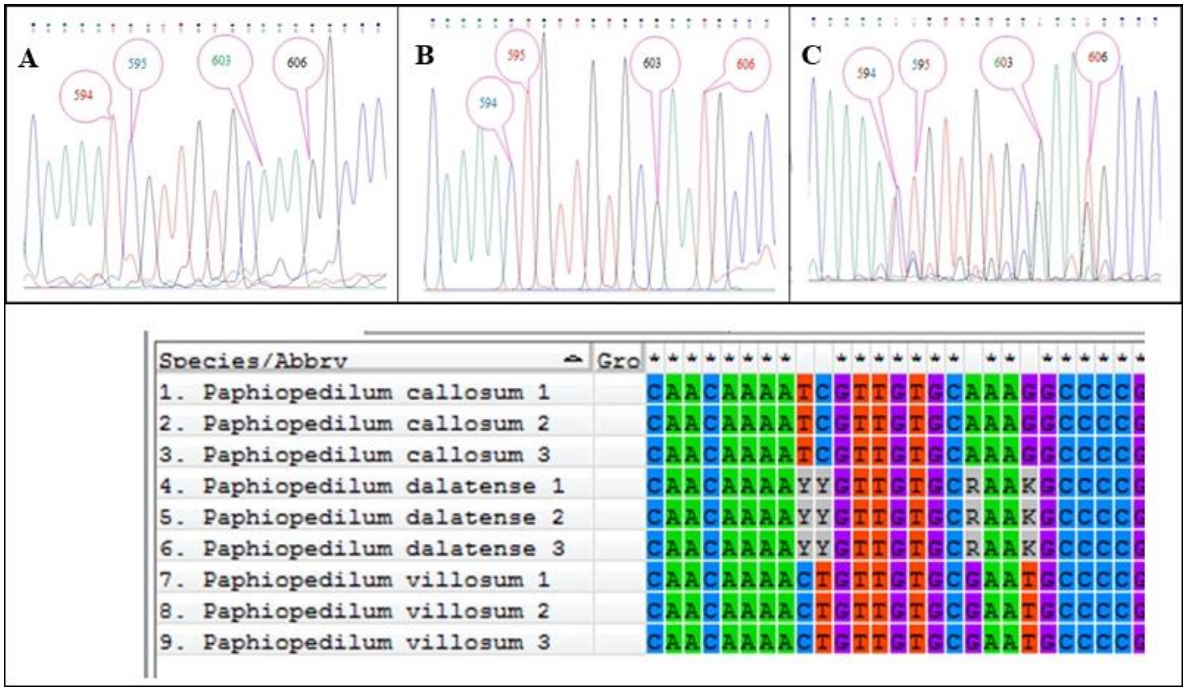

Figure 2. Double peaks at 594, 595, 603, 606 sites in the chromatograms of ITS2 sequence in *P.* × *dalatense* (C) possesses the specific nucleotide of both *P. callosum* (A) and *P. villosum* (B) and result of corresponding alignment.

The origin of P.* × *dalatense based on partial matK gene sequence

The length of partial *mat*K gene sequence were 854 bp for all of investigated species, from 184 site to 1107 site in whole *mat*K gene. The sequences of same investigated sample induced by sequencing with forward and reverse primers were highly matched; the sequences of the samples belonging to the same species were identical in *P. callosum* and *P. villosum* but the samples belonging to *P.* × *dalatense* were different.

Table 4. Variation of partial *mat*K gene among three investigated species.

Site on the investigated partial *mat*K gene	184	636	702	805
Site on the *mat*K gene	437	889	955	1058
P. callosum, 3 investigated samples	G	A	G	A
P. villosum, 3 investigated samples	T	C	T	G
P. × *dalatense,* sample C1	T	C	T	G
P. × *dalatense,* sample C2	T	C	T	G
P. × *dalatense*, sample C3	G	A	G	A

Based on the partial *mat*K gene, the variation among three investigated was shown in Table 4.

Table 4 indicated that C1 and C2 *P.* × *dalatense* samples possessed identical partial *mat*K gene to *P. villosum* samples but C3 *P.* × *dalatense* sample possessed identical partial *mat*K gene to *P. callosum* samples. Accordingly, partial *mat*K gene in *P.* × *dalatense* could be identical to any of in *P. callosum* and *P. villosum*.

In most flowering plants, the hybrid between two parent species or the next generation of the reproductive species possess the plastid genome inherited from maternal species or maternal individuals respectively (Corriveau, Coleman, 1988). In that study, these authors investigated more than 200 angiosperm species belong to various plant families and indicated that about one fourth of total investigated species possessing plastid genome is biparentally inherited, mainly in families such as Strelitziaceae, Plumbaginaceae, Passifloraceae, Onagraceae, Geraniaceae. In all of representatives of Orchidaceae, including the taxa belong to *Paphiopedilum* genus in the study of Corriveau and Coleman (1988), the plastid DNA is maternally inherited.

The genus of *Paphiopedilum* also belongs to the most maternally inherited plastid DNA angiosperm species and this is confirmed again in the study of Chochai *et al*. (2012).

Because the *mat*K gene is located on the chloroplast, its sequence in the hybrid species is identical to species playing maternal role in *Paphiopedilum* species. In case of *P.* × *dalatense* in the current study, partial *mat*K gene in *P.* × *dalatense* could be identical to any of in two parental species. Thus, *P.* × *dalatense* can be proved to be the reversible hybrid species between *P. villosum* and *P. callosum*.

Szlachetko *et al.* (2017) also used to investigate 3'*trn*K-*mat*K sequence of *Cypripedium dickinsonianum, C. irapeanum, C. molle*, *C.* × *fred-mulleri* and indicated *C.* × *fred-mulleri* is the naturally hybrid species between *C. dickinsonianum* (paternal species) and *C. irapeanum* (maternal species) because *C.* × *fred-mulleri* shares the identical 3'*trn*K-*mat*K sequence with *C. irapeanum* while *C. dickinsonianum* differs to *C. irapeanum* and *C.* × *fred-mulleri* in four sites. It was different from the study of Szlachetko *et al.* (2017), in the current study, *P.* × *dalatense* was proved to be the reversible hybrid species.

Together with the information that the samples were collected from Don Duong district, Lam Dong province, where the three investigated species shared the same natural distribution, the morphological and molecular data achieved in this research proved that *P.* × *dalatense* is the reversible hybrid species between *P. callosum* and *P. villosum*.

CONCLUSION

In morphological characteristics of vegetative and reproductive organs, *P.* × *dalatense* species possessed the intermediate characteristic between *P. callosum* and *P. villosum*. Based on DNA sequence data of nuclear ITS1-5.8S rRNA-ITS2 region, *P.* × *dalatense* possessed 23 nucleotide sites which are specific for both *P. villosum* and *P. callosum*. Based on DNA sequence data of plastid partial *mat*K gene, two out of three investigated *P.* × *dalatense* samples were identical to *P. villosum* and the last sample was identical to *P. callosum*. All of these findings on morphological and molecular characteristics proved that *P.* × *dalatense* is the reversible hybrid species between *P. callosum* and *P. villosum*.

Acknowledgements: *The authors gratefully acknowledge Tay Nguyen Institute for Scientific Research, Vietnam Academy of Science and Technology and Tay Nguyen Program 2016 - 2020, project TN18/T08 for their support in completing this study.*

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