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Molecular identification of three *Habenaria* species from Binh Chau-Phuoc Buu Nature Reserve, Vietnam

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Abstract

The present provides molecular data for three *Hebenaria* species such as *Habenaria diphylla* (Nimmo) Dalzell, *H. khasiana* Hook.f. and *H. rostellifera* Rchb.f. collected from Binh Chau-Phuoc Buu Nature Reserve, Vietnam for the first time. Along with other DNA sequences from GenBank database, the phylogenetic trees for *Habenaria* species from Vietnam have been established.

Keywords

Habenaria diphylla, *Habenaria khasiana*, *Habenaria rostellifera*, ITS, *trn*L-F regions, phylogeny

Introduction

Habenaria Willd. is one of the large genera of the family Orchidaceae Juss., including about 876 species that grow in tropical and subtropical regions (1). The Orchids of Vietnam illustrated survey recorded 35 species for the flora of Vietnam (2). Recently, two new species, *H. austrosinensis* Tang & F.T. Wang and *H. diphylla* (Nimmo) Dalzell**,** have been described for the flora of Vietnam (1, 3). Therefore, the total number of the known species of *Habenaria* in Vietnam was 37.

Binh Chau–Phuoc Buu Nature Reserve is the only remaining coastal primary dipterocarp forest of Vietnam located in Xuyen Moc District, Ba Ria-Vung Tau Province, Vietnam. According to the report of Management Board of Binh Chau-Phuoc Buu Nature Reserve, Binh Chau-Phuoc Buu Forest has a diverse system of flora which includes 142 families and 796 plant species. In 2019 and 2020, our field works in Binh Chau-Phuoc Buu Nature Reserve disclosed the presence of three *Habenaria* species, including *H. diphylla* (Nimmo) Dalzell, *H. khasiana* Hook.f. and *H. rostellifera* Rchb.f. Our previous work has recorded *H. diphylla* for the flora of Vietnam (1). Herein, the ITS and trnL-F sequences of three *Habenaria* species collected from Binh Chau-Phuoc Buu Nature Reserve were successfully amplified and sequenced. Notably, the ITS and trnL-F regions of *H. diphylla* were reported for the first time.

Materials and Methods

Plant samples collection

Specimens of *H. diphylla*, *H. khasiana* and *H. rostellifera* were collected in Binh Chau-Phuoc Buu Nature Reserve, Bung Rieng Ward, Xuyen Moc District, Ba Ria-Vung Tau Province, Vietnam, on 30th August 2019 (Fig. 1). All vouchered specimens were encoded as collected Le VS 221, Le VS 226, Le VS 229 and deposited at Herbarium of Binh Chau-Phuoc Buu Nature Reserve. Furthermore, ITS and *trn*L-F sequences of 18 species of *Habenaria* genus and

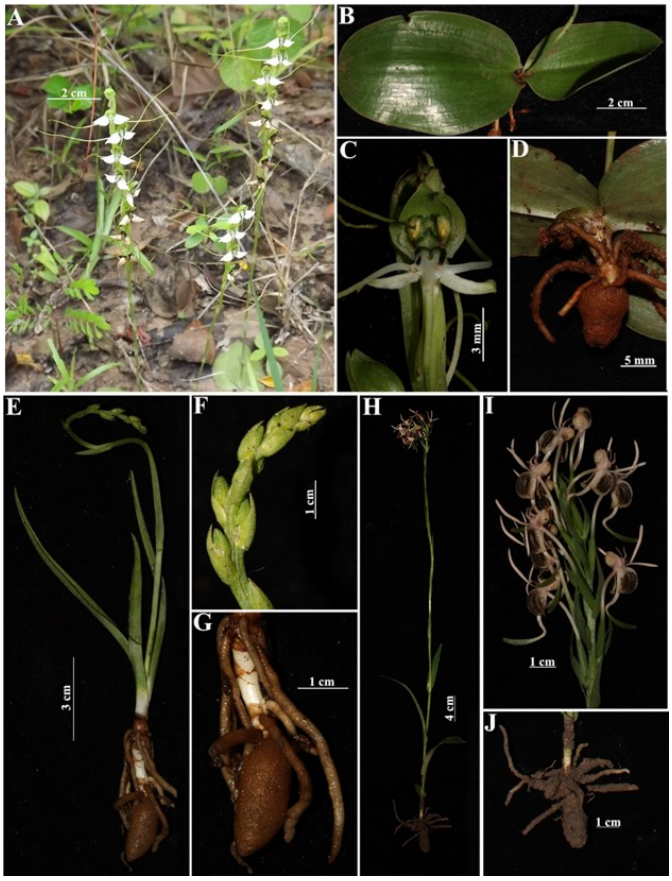

Fig. 1. A-D: *Habenaria diphylla*, E-G: *H. khasiana* and H-J: *H. rostellifera*

Platanthera chlorantha from the Genbank database were used in this study for the establishment of the phylogenetic tree (Table 1).

Morphological Taxonomy

Specimens were sampled and processed using conventional methods guided by the Royal Botanic Gardens, Kew (4). Species identification was performed by comparison of morphological vegetative and reproductive characteristics of published description of *Habenaria* genus (5, 6).

Total genomic DNA extraction and PCR amplification

Total genomic DNA was extracted from fresh leaves of *H. diphylla*, *H. khasiana* and *H. rostellifera* using Gene Jet Plant Genomic DNA Purification Mini Kit (Thermo Fisher Scientific, USA) according to the manufacture's instruction. Amplification of the ITS and *trn*L-F regions was performed on Mastercycler machine (Eppendorf, Germany) using the primers (7, 8) procedures, including ITS primers (forward: 5'TCCGTAGGTGAACCTGCGG3', reverse: 5'TCCTCCGCTTATTGATATGC3'), and *trn*L-F primers (forward: 5'CGAAATCGGTAGACGCTACG3', reverse: 5'ATTTGAACTGGTGACACGAG3'). The PCR mixture was the 25 µl reaction mixture containing 12.5 µl Go-Taq green master mix (Promega, USA), 1.25 µl of each forward and reverse primers (10 µM), 9.5 µl nuclease-free deionized water and 0.5 µl DNA template (25 µg/ml). The PCR program consisted of 3 min at 95 °C; 35 cycles of 1 min at 94 °C, 1 min at 55 °C, 2 min at 72 °C); and a final extension at 72 °C for 10 min. The PCR products were purified and sequenced using ABI 3130 XL Sequencer.

Sequencing data analysis

The ITS and *trn*L-F sequences of other species of *Habenaria* genus were queried and *Platanthera chlorantha* was obtained from the Genbank database. DNA sequences generated from this study along with those download Genbank were assembled and aligned using the ClustalW (9) to recognize the homology between sequences. The sequence datasets were analyzed with phylogenetic method (maximum parsimony) using PAUP*4.0a146 (10) with *Platanthera chlorantha* as the outgroup species (11). Bootstrap values of 50% or higher were performed to obtain cluster supports. The percent identity values between the sequences were calculated using the alignment tool from NCBI.

Table 1. Sequences of eighteen *Habenaria* species and *Platanthera chlorantha* from GenBank database used in this study

Scientific name	Accession number (ITS/*trnL-F*)	Scientific name	Accession number (ITS/*trnL-F*)
H. pantlingiana	MF944309/MF945323	*H. rhodocheila*	KY966607/MF945375
H. petelotii	MF944311/MF945377	*H. malintana*	MF944306/MF945381
H. stenopetala	MF944324/MF945265	*H. praetermissa*	MF944313/-
H. limprichtii	MF944301/MF945209	*H. dentata*	KY966605/KR350362
H. medioflexa	MT500668/MF945235	*H. lucida*	MT500671/MT507762
H. myriotricha	KY966606/-	*H. acuifera*	MF944278/MF945225
H. commelinifolia	MF944288/MF945252	*H. linguella*	MF944303/MF945210
H. reflexa	MF944314/MF945388	*H. rostrata*	MT500676/MT507767
H. ciliolaris	MF944287/MF945384	*H. tonkinensis*	MF944326/MF945228
		P. chlorantha	MT179753/MF945194

Results and Discussion

The PCR products of *trn*L-F and ITS sequences of *H. diphylla*, *H. khasiana* and *H. rostellifera* were presented as the clear bands which had similar sizes of predicted bands (7, 8) (Fig. 2). The ITS and *trn*L-F sequences of three studied species after editing had the sizes about 532 and 980 bps

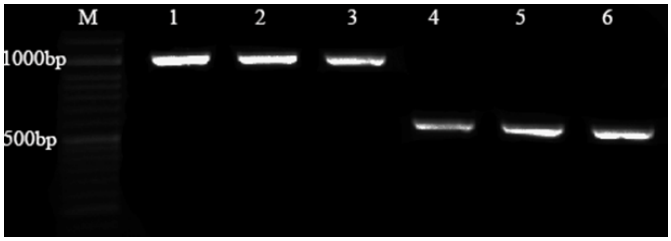

Fig. 2. PCR amplification result of *trn*L-F (1-3) and ITS regions (4-6) of *H. diphylla*, *H. khasiana* and *H. rostellifera*. M: ladder.

respectively. The ITS and *trn*L-F sequences of *H. diphylla*, *H. khasiana* and *H. rostellifera* were registered in NCBI database. The accession numbers of ITS/*trn*L-F regions were MT305990/MT303814, MT305991/MT303815 and MT305992/MT303816 respectively.

The phylogenetic trees among 3 studied species and other *Habenaria* plants from Vietnam were established and the results were presented in Fig. 3. The data showed that the classification of species of *Habenaria* foliage and general flower shape (2, 5, 6). The arrangement of *H. rostellifera* and *H. rostrata* analyzed by molecular markers is similar to that used by morphological analysis. Accordingly, 2 species were grouped together in both ITS and *trn*L-F phylogenetic trees with bootstrap values of 100% and 98% respectively (Fig. 3A and B). By using the BLAST tool in NCBI, there are differences of two molecular markers (ITS and *trn*L-F*)* of the studied species to those of closely related species in GenBank, however. As a results, the pairwise alignment of ITS region between *H. rostellifera* and *H. rostrata* showed that the entire aligned length of this region of two species was 589 bp. Two sequences had 588 homologous positions and 1 non-homologous position. Meanwhile, the entire aligned length of *trn*L-F region of *H. rostellifera* and *H. rostrata* was 764 bp in which there had 761 homologous positions and 3 non-homologous positions.

H. diphylla was firstly described as a new species in India (12). This species was also found in Nepal, Bhutan, Bangladesh, China, Myanmar, Thailand, Philippines (5, 6, 13). Recently, *H. diphylla* was recorded for the flora of Vietnam, whose distribution were identified in Binh Chau-Phuoc Buu Nature Reserve, Vietnam (1). The ITS and *trn*L-F regions of *H. diphylla* was firstly shown by the present

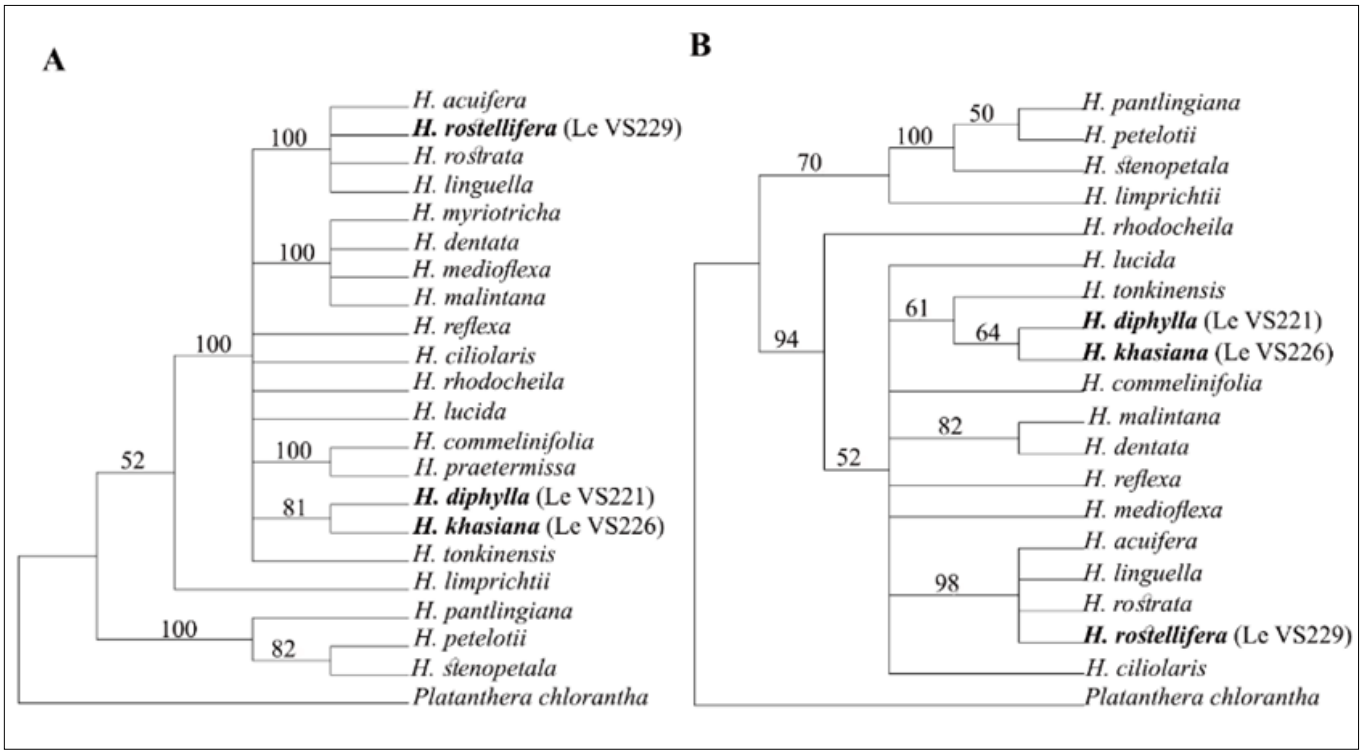

Fig. 3. One of most-parsimonious tree obtained based on the ITS (**A**) and trnL-F (**B**) regions. The bootstrap values of 50% or more are shown above the nodes.

based on ITS sequences was similar with that of *trn*L-F sequences. In Fig. 3, all of three species in this study, including *H. diphylla*, *H. khasiana* and *H. rostellifera* were classified in the group of *Habenaria*, which implied the sequencing data were accurate.

Previous studies provided the information on the distribution of *H. rostellifera* in China, Peninsular Malaysia, Thailand and Vietnam (2, 5, 6). This species is most closely relative to *H. rostrata*. Two species shared many of the same habitat and morphological characteristics, including study. This species was classified in the same group with *H. khasiana* on both ITS and *trn*L-F phylogenetic trees (Fig. 3A and B) with percent identity values of 98% and 97% respectively. Notably, *H. khasiana* belonged to similar morphological group along with *H. tonkinensis* and *H. viridiflora* (5). However, the ITS and *trn*L-F sequences of *H. khasiana* and *H. tonkinensis* had the percent identity values of 96% and 97% respectively**.**

Recent reports have used the molecular data to establish the phylogeny as well as assist classification of *Habe-*

naria species. Accordingly, the 4 *Habenaria* species from Brazil such as *H. reflexicalcar, H. hippocrepica, H. quadriferricola* and *H. espinhacensis* have been described as new species based on morphological and molecular data (14). Similarly, 3 plastids (*matK, trnK* intron and *rps16–trnK*) were used and one nuclear (ITS) regions to establish the phylogenetic relationships and descriptions 1 variety and 4 new *Habenaria* species, including *Habenaria brachydactyla, H. irwiniana, H. minuta, H. pansarinii and H. pansarinii var. minuscula* (15). In addition, the morphological and molecular data have been used to phylogeny and re-taxonomy of two misleading and conflicting *Habenaria* species such as *H. leprieurii* and *H. alpestris.* As a result, one species collected from central-western Brazil, formerly identified as *H. alpestris,* has been described as a new species of which the scientific name was *H. omissa* whereas *H. alpestris* and *H. melanopoda* were conspecific (16). Meanwhile, *H. leprieurii* was classified in a new circumscription for *H. sect. microdactylae* such as *H. leprieurii, H. heptadactyla, H. cruegerii, H. omissa* and *H. cruegeri* var. *flaviflora* (16). Recently, chloroplast *(matK, rbcL)* was used and nuclear (ITS) regions to infer the systematic position of a new *Habenaria* species, *H. sandiegoensis,* from Nepal (17).

Conclusion

In this study, the ITS and *trn*L-F sequences of three *Habenaria* species collected from Binh Chau-Phuoc Buu Nature Reserve were successfully amplified and sequenced. Notably, the ITS and *trn*L-F regions of *H. diphylla* were reported for the first time. Along with other DNA sequences from the GenBank database, the phylogenetic trees for *Habenaria* species from Vietnam have been established.

Authors contributions

This study was designed Hong Thien Van. The studied samples were collected by Van Son Le. All authors performed experiments and handled the research data. Hong Thien Van prepared the manuscript and resolved all the queries of reviewers.

Compliance with ethical standards

Conflict of interest: No conflict of interest was declared by the authors.

Ethical issues: None

References

1. Van HT, Nguyen THV, Le HT, Trinh NN, Huynh NTA, Le VS, Phan TTN, Dang LAT. *Habenaria diphylla* (Nimmo) Dalzell (Orchidaceae), new record for the flora of Vietnam. Plant Science Today. 2021;8(1):185-89. https://doi.org/10.14719/pst.2021.8.1.1014
2. Averyanov L. The orchids of Vietnam illustrated survey part 2 subfamily Orchidoideae. Turczaninowia. 2010;13(2):5-98. https://cyberleninka.ru/article/n/the-orchids-of-vietnam-illustrated-survey-part-2-subfamily-orchidoideae/viewer
3. Ly NS, Tran TT, Do DG, Truong BV. A newly recorded species for Vietnam. Bioscience Discovery. 2018;9(2):222-6. https://biosciencediscovery.com/Vol%207%20No%202/Ly%20Ngoc-Sam93-96.pdf
4. Bridson D, Forman L. The Herbarium Handbook-Third Edition. Royal Botanic Gardens. Royal Botanic Gardens, Kew, UK; 1999.
5. Kurzweil H. The genus *Habenaria* (Orchidaceae) in Thailand. Thai For Bull. (Bot.). 2009: 7-105. https://li01.tci-thaijo.org/index.php/ThaiForestBulletin/article/view/24359
6. Xinqi C, Cribb P. *Habenaria* Willdenow. Flora of China. 2009;25:144-60. http://www.efloras.org/florataxon.aspx?flora_id=2&taxon_id=114408
7. White T, Bruns T, Lee S, Taylor J. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA., Gelfand DH., Sninsky JJ. and White TJ. (eds) PCR Protocols: A Guide to Methods and Applications. 1990. Academic Press, New York. https://www.sciencedirect.com/science/article/pii/B9780123721808500421?via%3Dihub https://doi.org/10.1016/B978-0-12-372180-8.50042-1
8. Taberlet P, Gielly L, Patou G. Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Mol Biol. 1991;17:1105-09. https://link.springer.com/article/10.1007/BF00037152 https://doi.org/10.1007/BF00037152
9. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994;22(22):4673-80. https://doi.org/10.1093/nar/22.22.4673
10. Swofford D. PAUP*. Phylogenetic analysis using parsimony (*and Other Methods). Version 4.0b10. Sinauer Associates, Sunderland, Massachusetts, 2002. http://citeseer.ist.psu.edu/viewdoc/summary?doi=10.1.1.458.6867
11. Jin WT, Schuiteman A, Chase MW, Li JW, Chung SW, Hsu TC. Phylogenetics of subtribe Orchidinae s.l. (Orchidaceae; Orchidoideae) based on seven markers (plastid matK, psaB, rbcL, trnL-F, trnH-psba, and nuclear nrITS, Xdh): Implications for generic delimitation. BMC Plant Biol. 2017;17(1). https://doi.org/10.1186/s12870-017-1160-x.
12. Hooker F. Flora of the Nicobar Islands. Bot. Kew Gard. Misc; 1850.
13. Vinodia S. *Habenaria diphylla* (Nimmo) Dalzell: A new addition to the Orchid flora of Bilaspur district (C.G), Central India. Curr Bot. 2017;8:60-65. https://doi.org/10.19071/cb.2017.v8.321
14. Batista JAN, Vale AA, Carvalho BM, Proite K, Ramalho AJ, Munhoz ACD, Berg C, Bianchetti LB. Four new species in *Habenaria* (Orchidaceae) from the Espinhaço Range, Brazil. Systematic Botany. 2016;41(2):275-92. https://doi.org/10.1600/036364416X691858
15. Batista JAN, Proite K, Bianchetti LB. Descriptions and phylogenetic relationships of four new species and a new name of *Habenaria* (Orchidaceae) from the cerrado and campos rupestres of Brazil. Plant Syst Evol. 2017. https://doi.org/10.1007/s00606-017-1415-x
16. Batista JAN, Reis ADS, Junior JLL, Bianchetti LB. Phylogeny and taxonomy of *Habenaria leprieurii, H. alpestris* and H. sect. *Microdactylae* (Orchidaceae), with descriptions of two new taxa. Phytotaxa. 2018;373(4):241-71. https://doi.org/10.11646/phytotaxa.373.4.1
17. Raskoti BB, Ale R. Molecular phylogeny and morphology reveal a new epiphytic species of *Habenaria* (Orchidaceae; Orchideae; Orchidinae) from Nepal. 2019. PLoS ONE. https://doi.org/10.1371/journal.pone.0223355

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