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DNA Barcoding of *Dendrobium moschatum* (Banks) Sw. Specimen from Makawanpur, Central Nepal

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Abstract

The genus *Dendrobium*, with sparse distribution in nature, is one of the largest genera of Orchidaceae. DNA barcoding could be the best option for rapid and accurate identification of the *Dendrobium* species. The objective of the present study is to delineate the *Dendrobium* species using DNA barcoding technology. Here, we used a specimen of *Dendrobium* sp. collected from Brindaban Botanical Garden, Makawanpur (540 m asl) as a test object. We amplified and sequenced three chloroplast loci, *rbcL* (Ribulose-1,5- bisphosphate carboxylase), *matK* (Maturase K) and *psbA-trnH* (intergenic spacer) from the specimen. We retrieved twelve accessions of plastome sequences from NCBI, representing six *Dendrobium* species *(D. candidum*, *D. crepidatum*, *D. chrysanthum*, *D. denneanum*, *D. fimbriatum* and *D. moschatum*) reported in Nepal. Similarly, one accession of plastome of *Bulbophyllum epiphytum* was also retrieved, to be used as an out-group. Respective aligned sequences of *rbcL*, *matK* and *psbA-trnH* were extracted from each accession. Evolutionary analysis was performed following the Maximum Likelihood approach using MEGA X. The result showed that the evolutionary tree generated with combined sequences of all three loci (*rbcL*, *matK* and *psbA-trnH*) was better compared to that generated with sequence of single locus. However, additional markers are required for higher accuracy.

Keywords: Accessions, *Dendrobium*, Evolutionary tree, Molecular markers, Plastome

Introduction

Orchidaceae is the largest vascular plant family, which consists of ca. 736 genera and ca. 28,000 species in the world (Chase et al., 2015; Christenhusz & Byng, 2016). *Dendrobium* is one of the most diverse genera comprising approximately 1200-1500 species in the world (Xu et al., 2015), out of which 30 species have been reported in Nepal (Shrestha et al., 2022). Species of this genus are used for medicinal and horticultural purposes (Chinese Pharmacopoeia Editorial Committee [CPEC], 2010; Feng et al., 2015; Xu et al., 2015). More than 20 species of *Dendrobium* with its different parts are traded under different names in Nepal (Pyakurel et al., 2019; Shrestha et al., 2010; Subedi et al., 2013). Due to overexploitation and habitat destruction, *Dendrobium* species demand immense efforts for their conservation. Consequently, all the species of this genus have been enlisted in CITES appendices I and II (Convention on International Trade in Endangered Species of Wild Fauna and Flora [CITES], 2023).

The vast diversity of *Dendrobium* species combined with a high degree of morphological resemblances among them, makes it problematic to identify the species with morphological studies alone (Adams, 2011; Xu et al., 2015). Precise as well as rapid identification of the species is crucial for conservation and management of *Dendrobium*, and DNA barcoding could be an effective solution towards that end. DNA barcoding is the molecular technology that involves sequencing specific and standard regions of DNA for species identification (Asahina et al., CBOL plant working group, 2009; 2010; Feng et al., 2015). This technology can also be used for the identification of botanical origins of crude drugs (Asahina et al., 2010).

In this study, we attempted to identify best performing molecular markers to delineate the *Dendrobium* species. There is ambiguity in selecting molecular markers for species differentiation in *Dendrobium*, as findings of different researches are not consistent. Lahaye et al. (2008) proposed *matK* as universal DNA barcode for flowering plants including orchids. Asahina et al. (2010) found *matK* effective to

distinguish *Dendrobium* species. Liu et al. (2019) and Nguyen et al. (2020) proposed ITS (Internal Transcribed Spacer) as barcode for *Dendrobium*. Xu et al. (2015) recommended combined sequence of *matK* and *ITS* as the core barcode for *Dendrobium*. Here, we isolated and sequenced three chloroplast loci viz. *rbcL*, *matK* and *psbA-trnH* as possible DNA barcodes for *Dendrobium* species.

Materials and Methods

Plant material and DNA extraction

We collected leaf of *Dendrobium* sp. from the Brindaban Botanical Garden, Makawanpur (Alt. 540 m, Lat. 27.49283°N and Long. 85.04564°E) and dried it in silica gel. The sample code was assigned as BT-23. Total genomic DNA was isolated using CTAB method (Keb-Llanes et al., 2002). Voucher specimens were also collected and stored at KATH (specimen no. M1_22/11/2022).

PCR amplification and sequencing

Three plastid markers *rbcL* (Ribulose-1,5-bisphosphate carboxylase), *matK* (Maturase K) and *psbA-trnH* (the intergenic spacer between the gene coding protein D1, a polypeptide of the photosystem II reaction center (psbA) and gene coding histidine accepting tRNA (trnH)) were amplified (Figure 1) and sequenced using primers listed in Table 1. One or few nucleotides had to be substituted in the primer described previously to maintain the melting temperature and GC content between primer pairs and also to minimize the possibility of self and cross primer dimer formation. The PCR conditions were 35 cycles of denaturation at 94°C for 30 Sec., annealing at 54°C for 30 Sec. and extension at 72°C for 1 min. The PCR conditions were same for all three primer pairs.

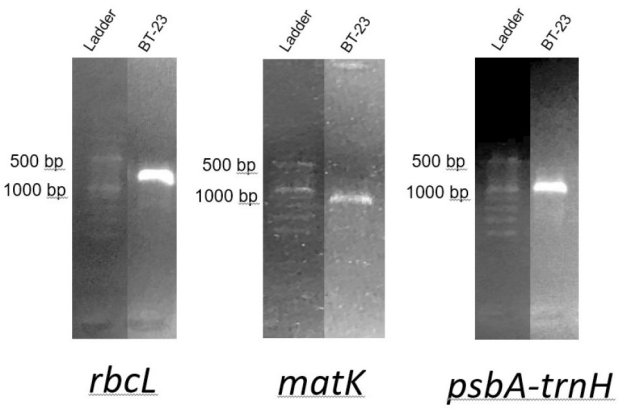

Figure 1: PCR amplification of *rbcL*, *matK* and *psbA-trnH* from BT-23

The sequencing was carried out in ABI310 Genetic Analyzer. The newly generated sequences of *rbcL* and *matK* were registered at the NCBI and the assigned NCBI accessions are presented in Table 2. Also, a very short sequence read was obtained for *psbA-trnH*, which was not shared with the NCBI, but has been given in Appendix I.

Table 2: GenBank accessions generated in the study

Species	Locus	GenBank accession
Dendrobium moschatum (Banks) Sw.	*rbcL*	OQ187817
	matK	OQ144654

Sequence downloads and data analysis

We retrieved twelve accessions of plastome sequences from the NCBI, representing six *Dendrobium* species reported from Nepal. Similarly, one accession of plastome of *Bulbophyllum epiphytum* was also retrieved (Table 3). Respective aligned sequences of *rbcL*, *matK* and *psbA-trnH* were extracted from each accession manually using SnapGene viewer tool.

Table 1: Primers used in the study

Locus	Primer name	Sequence (5'⟶3')	Remarks
rbcL	rbcL-F	ATGTCACCACAAACAGAGACTAAAG	Modified from Kress et al., 2009
	rbcL-R	GTAAAATCAAGTCCACCACG	
matK	matK-F	CCATCCATCTAGAAATCTTGGTTC	Modified from Yu et al., 2011
	matK-R	GCTGTAATAATGAGAAATATTTCTGC	
psbA-trnH	psbA	GTTATGCATGAACGTAATGCTC	Modified from Sang et al., 1997
	trnH	CGCGCATGGTGGATTCACAATC	Modified from Tate et al., 2003

Table 3: Plastome sequences retrieved from NCBI

S.N.	Species	GenBank accession
1.	*Bulbophyllum epiphytum*	MN737573.1:82380-83051
2.	*Dendrobium candidum*	KY887994.154604-55141
3.	*Dendrobium candidum*	MZ129032.155309-55844
4.	*Dendrobium chrysanthum*	LC490682.11944-2847
5.	*Dendrobium chrysanthum*	LC490683.11945-2848
6.	*Dendrobium chrysanthum*	LC490684.11945-2848
7.	*Dendrobium crepidatum*	LC490404.11958-2861
8.	*Dendrobium crepidatum*	LC490675.11958-2861
9.	*Dendrobium crepidatum*	LC193509.11939-2842
10.	*Dendrobium denneanum*	LC192955.154366-54901
11.	*Dendrobium fimbriatum*	LC193521.11941-2844
12.	*Dendrobium moschatum*	OM161978.12057-2960
13.	*Dendrobium moschatum*	NC 061572.1

Phylogenetic analysis was performed following the Maximum Likelihood approach and Kimura 2 Parameter (K2P) model with 1000 bootstrapping replications using Molecular Evolutionary Genetics Analysis (MEGA X) tool. The sequence of *Bulbophyllum epiphytum* was used as an out-group to root the tree.

Results and Discussion

matK is better marker than rbcL marker

Phylogenetic analysis using *rbcL* sequences showed rather poor species discrimination power.

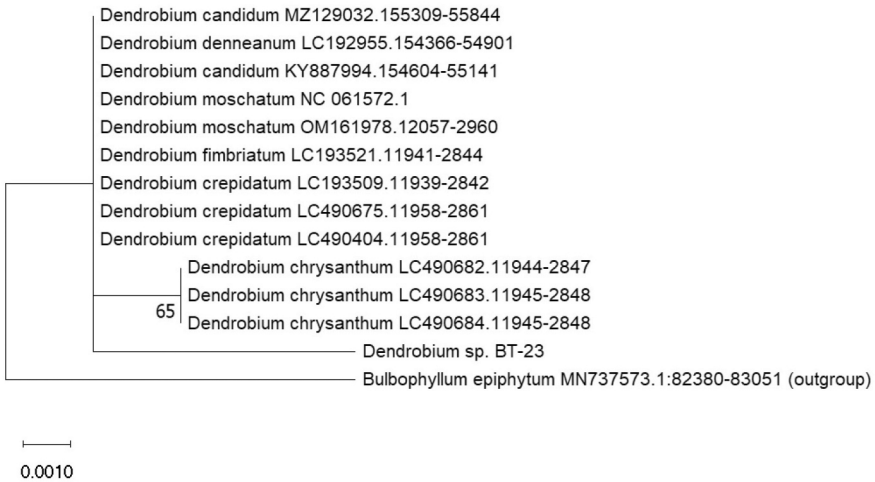

Figure 2: Maximum Likelihood tree generated using *rbcL* sequences based on the K2P model. The number on the branch represent bootstrapping support after 1000 bootstrap replications test. Scientific names are followed by respective GenBank accession numbers. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were a total of 538 positions in the final dataset. Evolutionary analyses were conducted in MEGA X

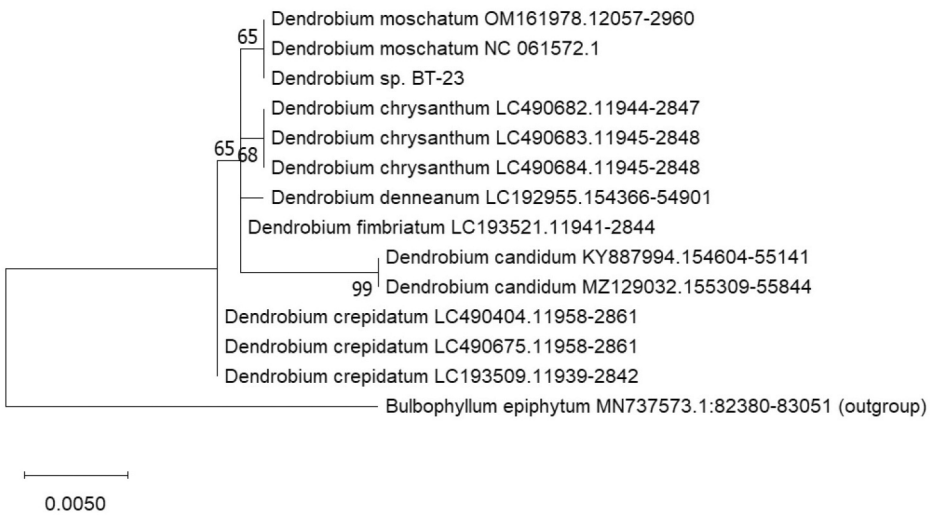

Figure 3: Maximum Likelihood tree generated using *matK* sequences based on the K2P model. The number on the branches represent bootstrapping support after 1000 bootstrap replications test. Scientific names are followed by respective GenBank accession numbers. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were a total of 904 positions in the final dataset. Evolutionary analyses were conducted in MEGA X

This marker was able to delineate *D. chrysanthum* only. Most of the species under the study fall under the same clade (Figure 2). Interestingly, the tree generated with *matK* sequence performed better (Figure 3) which is consistent with previous reports (Asahina et al., 2010; Lahaye et al., 2008).

Tree generated with combined rbcL, matK and psbA-trnH is better

The tree generated with combined sequences of *rbcL*, *matK* and *psbA-trnH* was better than that generated with single locus *matK*. Specifically, for *D. chrysanthum*, the bootstrapping support value significantly increased from 68 to 94 (Figure 3 and 4). Moreover, our test object BT-23 could be the *D. moschatum* as it clumped at the clade of *D. moschatum*, but the specimen seems somewhat different compared with two other accessions of *D. moschatum* (Figure 4).

rbcL, matK and psbA-trnH markers are still not sufficient enough for Dendrobium species discrimination

Based on the evaluation of recoverability, sequence quality and species discrimination level, Consortium for the Barcode of Life's (CBOL) plant working group (2009) recommended the combination of *rbcL* and *matK* as a plant barcode. Contrastingly, our results show that the tree generated with *rbcL*, *matK* and *psbA-trnH* combined sequences are better but still not sufficient enough, as bootstrapping support values are less than 50 for some branches (Figure 4), suggesting the necessity of more markers for precise delineation of *Dendrobium* species.

Li et al. (2021) suggested *ndhF* and *ycf1* in along with *matK* as barcodes for Orchids, *ndhF* and *ycf1* could be the potential loci to be added for analysis. For medicinal orchids, Raskoti and Ale (2021) found ITS and ITS+*matK* as the most efficient single and multi-loci barcodes respectively. Several reports also recommended ITS as barcode for *Dendrobium* (Liu et al., 2019; Nguyen et al., 2020), but Feng et al. (2015) stressed that ITS2 region is not a sufficient enough barcode to identify *Dendrobium* species. Also, Xu et al. (2015) recommended *matK* + ITS as core barcode for *Dendrobium*, but one should be very careful while combining chloroplast and nuclear DNA sequences. It may represent an oversimplified version of genetic history as chloroplast and nuclear DNA may reflect distinctly different evolutionary histories (Wei et al., 2014).

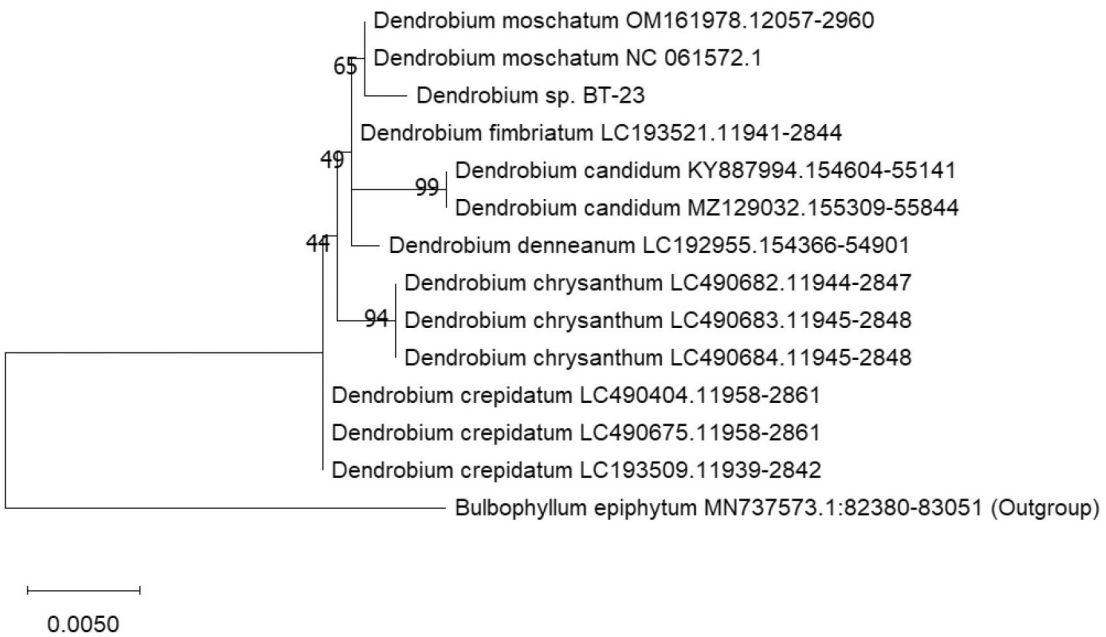

Figure 4: Maximum Likelihood tree generated using *rcbL+matK+psbA-trnH* sequences based on the K2P model. The number on the branches represent bootstrapping support after 1000 bootstrap replications test. Scientific name is followed by respective GenBank accession number. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were a total of 1774 positions in the final dataset. Evolutionary analyses were conducted in MEGA X

Conclusion

Correct identification of species is a prerequisite for species conservation and management. Upon availability of any biological material, DNA barcoding could be the best option for precise and rapid identification. Here, we found that the combination of *rbcL*, *matK* and *psbA-trnH* sequences offered better species discrimination compared to single locus marker, but additional markers are still required for higher accuracy. Some more chloroplast and nuclear markers, for instance, *ndhF*, *ycf1*, ITS, *AS1* etc. need to be tested in order to get a more robust tree of *Dendrobium* species. However, the research provided sufficient indication on the validity of the DNA barcoding approach.

Author Contributions

MSTM designed the research. MSTM, SM, JP and GR performed experiments. MSTM analyzed data and wrote the manuscript. All authors read and approved the final manuscript.

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References

Adams, P. (2011). Systematics of Dendrobiinae (Orchidaceae), with special reference to Australian taxa. *Botanical Journal of the Linnean Society*, *166*, 105-126.

Asahina, H., Shinozaki, J., & Masuda, K. (2010). Identification of medicinal *Dendrobium* species by phylogenetic analyses using *matK* and *rbcL* sequences. *Journal of Natural Medicines, 64*, 133-138.

CBOL Plant Working Group. (2009). A DNA barcode for land plants. *The Proceedings of the National Academy of Sciences USA*, *106*, 12794-12797.

Chase, M. W., Cameron, K. M., Freudenstein, J. V., Pridgeon, A. M., & André, S. (2015). An updated classification of Orchidaceae. *Botanical Journal of the Linnean Society*, *177*, 151-174.

Chinese Pharmacopoeia Editorial Committee. (2010). *Pharmacopoeia of the People's Republic of China. In Theoretical and Applied Genetics*.

Christenhusz, M. J. M., & Byng, J. W. (2016). The number of known plants species in the world and its annual increase. *Phytotaxa*, *261*(3), 201-217.

Convention on International Trade in Endangered Species of Wild Fauna and Flora. (2023). *Appendices I, II & III*.

Felsenstein J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, *39*, 783-791.

Feng, S., Jiang, Y., Wang, S., Jiang, M., Chen, Z., Ying, Q., & Wang, H. (2015). Molecular Identification of *Dendrobium* species (Orchidaceae) based on the DNA barcode ITS2 region and its application for phylogenetic study. *International Joural of Molecular Sciences*, *16*, 21975-21988.

Keb-Llanes, M., Gonzalez, G., Chi-Manzanero, B., & Infante, D. (2002). A rapid and simple method for small-scale DNA extraction in Agavaceae and other tropical plants. *Plant Molecular Biology Reporter, 20*, 299a-299e.

Kimura, M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, *16*, 111-120.

Kress, W. J., Erickson, D. L., Jones, F. A., Swenson, N. G., Perez, R., Sanjur, O., & Bermingham, E. (2009).References Plant DNA barcodes and a community phylogeny of a tropical forest dynamics plot in Panama. *The Proceedings of the National Academy of Sciences USA*, *106*(44), 18621-18626.

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and Evolution*, *35*, 1547-1549.

Lahaye, R., Bank, M. V. D., Bogarin, D., Warner, J., Pupulin, F., Gigot, G., Maurin, O., Duthoit, S., Barraclough, T. G., & Savolainen, V. (2008). DNA barcoding the floras of biodiversity hotspots. *The Proceedings of the National Academy of Sciences USA*, *105*, 2923-2928.

Li, H., Xiao, W., Tong, T., Li, Y., Zhang, M., Lin, X., Zou, X., Wu, Q., & Guo, X. (2021). The specifc DNA barcodes based on chloroplast genes for species identifcation of Orchidaceae plants. *Scientifc Reports*, *11*, 1424.

Liu, H., Fang, C., Zhang, T., Guo, L., & Ye, Q. (2019). Molecular authentication and diferentiation of *Dendrobium* species by rDNA ITS region sequence analysis. *AMB Express*, *9*, 53.

Nguyen, N. H., Vu, H. T., Le, N. D., Nguyen, T. D., Duong, H. X., & Tran, H. D. (2020). Molecular Identification and Evaluation of the Genetic Diversity of *Dendrobium* species collected in southern Vietnam. *Biology*, *9*, 76.

Pyakurel, D., Smith-Hall, C., Bhattarai-Sharma, I., & Ghimire, S. K. (2019). Trade and conservation of Nepalese medicinal plants, fungi, and lichen. *Economic Botany*, XX(X), 1-17.

Raskoti, B. B., & Ale, R. (2021). DNA barcoding of medicinal orchids in Asia. *Scientific Reports, 11*, 23651.

Sang, T., Crawford, D. J., & Stuessy, T. F. (1997). Chloroplast DNA phylogeny, reticulate evolution, and biogeography of Paeonia (Paeoniaceae). *American Journal of Botany*, *84*, 1120-1136.

Shrestha, K. K., Bhandari, P., & Bhattarai, S. (2022). *Plants of Nepal (Gymnosperms and Angiosperms)*. Heritage Publishers & Distributers Pvt. Ltd.

Shrestha, R., Joshi, L., Dhungana, S. K., & Kurmi, P. P. (2010). *Identification manual of Nepalese Dendrobium Orchids*. Department of Plant Resources.

Subedi, A., Kunwar, B., Choi, Y., Dai, Y., Andel, T., Chaudhary, R. P., Boer, H. J., & Gravendeel, B. (2013). Collection and trade of wild-harvested orchids in Nepal. *Journal of Ethnobiology and Ethnomedicine*, *9*, 64.

Tate, J. A., & Simpson, B. B. (2003). Paraphyly of Tarasa (Malvaceae) and diverse origins of the polyploid species. *Systematic Botany, 28*(4), 723-737.

Wang, W., Li, H. L., & Chen, Z. D. (2014). Analysis of plastid and nuclear DNA data in plant phylogenetics evaluation and improvement. *Science China Life Sciences*, *57*(3), 280-286.

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.

Yu, J., Xue, J. H., & Zhou, S. L. (2011). New universal *matK* primers for DNA barcoding angiosperms. *Journal of Systematics and Evolution*, *49* (3), 176-181.

Appendix I: *psbA-trnH* sequence of *Dendrobium moschatum* (Banks) Sw.

CAACAAGATAGCAATCCCCCAATATCTTGTTCTTAGAACAAGATATTGGGGGATTGCTACCTTC
AAAAATTCATATACATACAAAAGTATTATCCATTTATAGATGGAGCT