ORIGINAL ARTICLE

DNA Barcoding of Endemic and Endangered Orchids of India: A Molecular Method of Species Identification

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ABSTRACT

Background: Orchids are economically important, particularly in horticulture, floristry, pharmaceutical, and fragrance industries. India is a paradise for 1256 orchids, of which 31% (388 species) are endemic. Although Indian orchids are known in floristry, there is still a lot of room to use their bioactive compounds to promote their commercialization. Easy and accurate identification is first step towards conservation and commercial cultivation of endemic and endangered orchid species. This requirement can be accomplished using DNA barcoding technique. **Materials and Methods:** A total of 62 samples belonging to 35 species, 7 genera were collected for DNA barcoding studies. Evolutionary divergences and barcoding gap were calculated using MEGA-X software to screen the most suitable barcode region among the *ITS, matK, rbcL,* and *trnH-psbA* loci. BLAST analysis was used to identify barcoding locus presenting maximum species resolution. Phylogenetic analyses were performed to test harmony between barcoding and taxonomy. **Results:** We have generated 133 barcoding sequences, out of which, 46 sequences were found unique and new to GenBank database. Evolutionary divergence analysis showed the best result for *ITS*, where intra-specific and inter-specific divergence ranged between 0.0000–0.0300 and 0.0322–0.3765 correspondingly. It indicated clear barcoding gap, which was sufficient to robustly infer identities for taxa. BLAST-based evaluation concluded that largest number of barcode sequences (94.64%) could be identified using *ITS* locus followed by *rbcL* (78.69%) and *matK* (51.61%). In addition, the optimal phylogenetic tree was established using the *ITS* locus sequences, which complemented the orchid taxonomy. **Conclusion:** This study recommends *ITS* as best single locus barcoding region for identifying the orchids of India.

Key words: Internal transcribed spacer, interspecific divergence, maturaseK, phylogenetic analysis, ribulose bisphosphate carboxylase large chain

SUMMARY

- Orchids have been evoked worldwide eminence in recent years, owing to its wide range of long lasting flowers and medicinal properties. India is enriched with various species of orchids which require detail scientific exploration. DNA barcoding and molecular phylogenetic techniques allowed rapid and accurate species identification which is being used extensively for species identification and characterization of flora and fauna. *ITS, matK, rbcL,* and *trnH-psbA,* are the common genomic regions used for DNA barcoding of plants.

Our study considered different endangered and endemic species of *Aerides, Bulbophyllum, Coelogyne, Cottonia, Dendrobium, Paphilopedium,* and *Trias* genera consisting of 62 specific samples from 35 orchid species of 7 genera. Our study based on distance, BLAST and tree-building methods suggested that *ITS* is the best barcoding region to be considered as the barcode for these samples in comparison to the other regions studied. Interestingly, we were able to identify 20, 12, and 14 sequences of *ITS, matK,* and *rbcL* respectively, which were unique and new for GenBank database; and taxonomic lineages of 10 endemic Western Ghats species, which were unrecognized to NCBI database.

Morphologically similar orchids   Amplification and sequencing of ITS loci   Phylogenetic analysis and generation of barcoding library
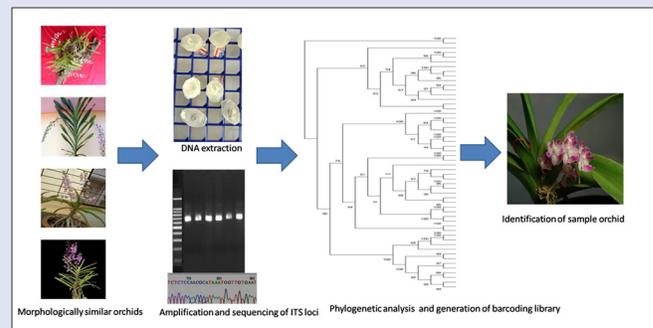
Abbreviations used: *accD*: Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta gene; *atpF-atpH*: ATP synthase subunit b-delta gene; EtBr: Ethidium bromide; *matK*: Maturase K gene; *ndhJ*: NAD(P)H-quinone oxidoreductase gene; nrDNA: Nuclear ribosomal DNA; *rbcL*: Ribulose bisphosphate carboxylase large subunit gene; *rpoB*: Beta subunit of RNA polymerase gene; *rpoC1*: DNA-directed RNA polymerase subunit beta gene.

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INTRODUCTION

Orchids have wide varieties and are spread all over the world. They are well-known for their beautiful, long-lasting flowers, and traditional medicinal values.[1,2] A recent survey of orchids found that species concentration is highest in northeastern India, and endemism of orchids is largest in Western Ghats.[3] Although orchids are rich in many useful bioactive compounds and can be important members of commercial herbal medicines, cosmetics, flavors, and fragrances market, they are always been ignorant by traders, researchers, and ecologist because of the lack of easy identification methods.[4] Moreover, deforestation as well as illegal over-harvesting of orchids for their horticultural and floricultural values caused serious threat to orchid survival conditions such as

specific pollination methods, climatic conditions, recent speciation, and improper distribution of symbionts.[5] Correct identification of

endangered, critically endangered and vulnerable species is necessary for planning and management of *in situ* and *ex situ* conservation methods in India which may lead to commercialization of orchids.[6] This requirement has led to the development of a method for quick and accurate identification of species using DNA barcode technology.[7] This technique relies on (i) developing on-line digital barcode library from the reliable sample vouchers of taxonomically identified species and (ii) comparing unknown samples of delimiting species to the library for their identification and molecular characterization.[7,8] This method is also effective during scarcity of plant DNA samples, where minute amount of dry, damaged, immature, or processed sample is enough to provide appreciable outcome. Information gathered from DNA barcodes can be used beyond taxonomic studies and will have far-reaching implications across many fields of biology, including herbal drug producers, flavor and fragrance industry, ecology, evolutionary biology, conservation biology, and non-professional users such as customs officers and forensic specialists to identify morphologically similar species and their herbal products.[9] Hence, the implementation of DNA barcoding methods for discrimination and identification is a need of hour to accelerate conservation and commercialization of endemic and endangered orchids of India.

Ample experiments lead to the identification of specific genes and genomic regions that could serve as the standard DNA barcode for plants, animals, or insects. Consortium for the Barcoding of Life working group proposed plastid genes: Partial ribulose bisphosphate carboxylase large chain (*rbcL*) and maturase K (*matK*) as universal barcode for land plants.[10] In addition to these markers, conserved plastid genes: *accD, ndhJ, rpoB, rpoC1,* and *ycf5*; plastid intergenic spacer regions: *trnH-psbA, atpF-atpH and psbK-psbI*; and nuclear ribosomal internal transcribed spacer regions: *ITS* (*ITS*1+5.8S+*ITS*2), *ITS1,* and *ITS2* were also found as promising DNA barcoding regions for the identification of different plant families.[11-13] Although there are 1256 orchids found in India, very few reports are available on barcoding of Indian orchids.[12,14,15] In the present study, the potential of four candidate barcodes *rbcL, matK, psbA-trnH,* and *ITS* for the identification of 35 species belonging to seven orchid genera was analyzed. Subsequently, phylogenetic mapping was conducted to find its harmony with taxonomy.

MATERIALS AND METHODS

Sampling of orchids and herbarium preparation

Orchid samples were collected from different geographical regions of India: Agasthyamalai (Tamil Nadu); Gurukula Botanical Sanctuary (Kerala); Mullayanagiri, Kemmannugundi, Medikeri (Karnataka); Kalsubai (Maharashtra); forest region of Assam; forest of Nagaland; and from Botanic Garden of Meise (BGM), Meise, Belgium. GPS locations of collection points in India are shown in Figure 1. Belgium samples were originally collected from India and were conserved *ex situ* in BGM, Belgium. Many individuals of the same species were collected from different locations to find intra-specific distances among geologically far species. Field samples were identified by Dr. K Sashidhar, President of "The Orchid Society of Karnataka" (TOSKAR), based on the reproductive or vegetative characters available at the time of collection.

Herbarium specimen of orchid samples were prepared and submitted for preservation to the Department of Microbiology and Biotechnology of the Bangalore University, Bengaluru, India. Accessions numbers of remaining six plants were obtained from the BGM, Belgium.

DNA extraction

Healthy fresh leaves of all the samples collected from different locations were cut into small pieces and dried in shade for 1 day. All leaf pieces were desiccated in labeled silica gel dark bottles for 7–10 days prior to the DNA isolation. Genomic DNA of field samples was isolated using CTAB methodology.[16] CTAB buffer was modified by adding 2% soluble PVP to remove phenolic compounds from plant leaves. GenElute, Plant Genomic DNA Miniprep Kit (Sigma) was used for rapid isolation of high-quality DNA from dried leaf samples collected from Belgium. The manufacturer's protocol was followed to pursue the DNA isolation. The isolated DNA obtained from any of the method was checked for quality by electrophoresis (0.8% TAE agarose gels containing Ethidium bromide at 7V/cm constant voltage) and visualized by a UV transilluminator. The DNA quantity was also checked using spectrophotometer. DNA samples having 260/280 ratio more than 1.6 were considered for amplification.

Amplification and sequencing of selected loci

Four major barcoding loci for plants–*ITS, matK, rbcL,* and *trnH-psbA* were amplified for all the orchids studied using known universal primers listed in Table 1.

Polymerase chain reaction (PCR) amplification of targeted DNA regions was performed using Applied Biosystems GeneAmp PCR machine. PCR conditions and PCR reactions are explained in Table 1. All the PCR reagents were acquired from Invitrogen, Thermo Fisher Scientific Corporation and primers were synthesized from Sigma-Aldrich Corporation. PCR products were visualized using 1% agarose gels stained with ethidium bromide (0.5 mg/mL) in a Bio-Rad Gel imaging system. Amplified DNA was purified using GenElute™ PCR Clean-Up Kit. The cleaned PCR products were stored at −20°C and were sequenced thereafter. Sequencing was done using DNA Analyzer: 3730 × l by Applied Biosystems using Sanger method.

Polymerase chain reaction data analysis

Amplification success was computed by taking percentage ratio of amplified products and DNA samples used for PCR, whereas sequencing success rate was calculated by the percentage ratio of the number of high quality sequences and the total number of PCR product used for sequencing. The obtained DNA sequences were aligned using ClustalX 2.1 and gaps were filled based on necessity.[17] These sequences were submitted to GenBank database through Banklt-NCBI-NIH.

Determination of candidate barcode sequences
method

The sequences from each candidate loci were aligned using Clustal X2 software.[17] A global multiple sequence alignment method was used for the *ITS, rbcL,* and *matK* sequences. Genetic distances were calculated to quantify sequence divergence among the individuals using Kimura two parameter (K2P) models in Molecular Evolutionary Genetics Analysis-version X (MEGA-X), computer software (developed by Pennsylvania State University, Pennsylvania).[18] The pairwise distances were calculated using 1000 bootstrap replication for all the barcodes. Pairwise deletion option was chosen to treat the gaps and missing data. Individual locus wise K2P distance matrix was generated by aligning DNA sequences of particular locus for all the species. Two species were considered as distinct, if their inter-specific distance was more than the maximum intra-specific distance. Individuals of same species were considered different variety if there was intra-specific distance. The differ ence between the greatest intra-specific distance and the smallest inter-specific distance, i.e., "Barcoding Gap" was also determined. Candidate barcode sequence was identified based on the barcoding gap where there was no overlap between the intra- and inter-specific distances.

BLASTn method

Percentage identity of our sequences with GenBank nucleotide database was determined using megablast option of BLASTn program (Basic
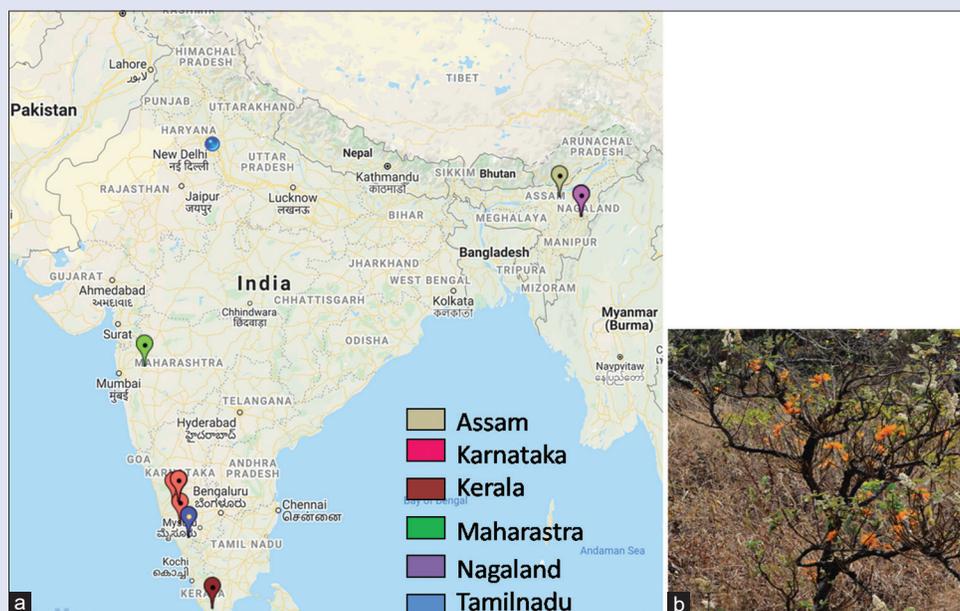
Figure 1: Geographical mapping of orchids at hotspots in India. (a) Different colors denote collection sites based on the recorded GPS. (b) *Dendrobium jerdonianum* at its natural habitat

Table 1: List of amplification criteria and primers used for amplification of the candidate DNA barcodes loci

Barcoding Locus	Amplification criteria	Amplification details
ITS	PCR Reaction System	20 μL: 2.0 μL 10× PCR Buffer, 2.0 μL MgCl2 (25mM), forward and reverse primers 1μL (10 μM) each, 2 μL dNTPs (2.5mM), 1 μL DMSO, 2 μL BSA (1mg/ml), 2 μL DNA and 0.2 μL Taq Pol.
	PCR Reaction Process	Procedure 1: 95°C, 4 min; 35× (94°C, 45s; 59°C, 1 min; 72°C, 1 min); 72°C, 7 min; hold at 4°C.
		Procedure 2: 94°C, 1 min; 32× (94°C, 30s; 50°C, 1min; 72°C, 45s); 72°C, 5 min; hold at 4°C.
	Primers	ITS-17SE-f: 5'-ACGAATTCATGGTCCGGTGAAGTGTTCG-3'[i]
		ITS-26SE-r: 5'-TAGAATTCCCCGGTTCGCTCGCCGTTAC-3'[i]
		ITS5-f: 5'-GGAAGTAAAAGTCGTAACAAGG-3' [ii]
		ITS4-r: 5'-TCCTCCGCTTATTGATATGC-3'[ii]
matK	PCR Reaction System	20 μL: 2.0 μL 10× PCR Buffer, 2.0 μL MgCl2 (25mM), forward and reverse primers 1μL (10 μM) each, 2 μL dNTPs (2.5 mM), 1 μL DMSO, 2 μL DNA and 0.2 μL Taq Pol.
	PCR Reaction Process	Procedure 1: 94°C, 4 min; 32× (94°C, 30 s; 52°C, 50 s; 72°C, 50 s); 72°C, 7 min; hold at 4°C.
		Procedure 2: 94°C, 4 min; 32× (94°C, 1 min; 48°C, 40s; 72°C, 50 s); 72°C, 7 min; hold at 4°C.
	Primers	matK-1RKIM-f: 5'-ACCCAGTCCATCTGGAAATCTTGG-3' [10]
		matK-3FKIM-r: 5'-CGTACAGTACTTTTGTGTTTACGA-3'[10]
		matK-2.1f: 5'-CCTATCCATCTGGAAATCTTAG -3' [iii]
		matK-5r: 5'-GTTCTAGCACAAGAAAGTCG-3' [iii]
rbcL	PCR Reaction System	20 μL: 2.0 μL 10×PCR Buffer, 2.0 μL MgCl2 (25mM), forward and reverse primers 1 μL (10 μM) each, 2 μL dNTPs (2.5mM), 2 μL DNA and 0.2 μL Taq Pol.
	PCR Reaction Process	Procedure 1: 95°C, 4 min; 35× (94°C, 30s; 55°C, 1 min; 72°C, 1 min); 72°C, 7 min; hold at 4°C.
		Procedure 2: 94°C, 1 min; 35× (94°C, 30s; 52°C, 1min; 72°C, 1 min); 72°C, 7 min; hold at 4°C.
	Primers	rbcL1f: 5'-ATGTCACCACAAACAGAGACTAAAGC-3'[iv]
		rbcL724r: 5'-TCGCATGTACCTGCAGTAGC-3'[v]
		rbcLa-f: 5'-ATGTCACCACAAACAGAGACTAAAGC-3'[vi]
		rbcLa-r: 5'-GTAAAATCAAGTCCACCCCG-3' [vii]

Contd...

Table 1: Contd...

Barcoding Locus	Amplification criteria	Amplification details
trnH-psbA	PCR Reaction System	20 µL: 2.0 µL 10× PCR Buffer, 2.0 µL MgCl2 (25mM), forward and reverse primers 1µL (10 µM) each, 2 µL dNTPs (2.5 mM), 2 µL DNA and 0.2 µL Taq pol.
	PCR Reaction Process	Procedure 1: 94°C, 4 min; 35× (94°C, 30 s; 50°C, 40 s; 72°C, 1 min); 72°C, 7 min; hold at 4°C.
	Primers	psbA3'f: 5'-GTTATGCATGAACGTAATGCTC-3'[viii]
		trnHf-05r: 5'-CGCGCATGGTGGATTCACAATCC-3' [viii]

i) Edwards D, Horn A, Taylor D, Savolainen V, Hawkins JA. Taxon. DNA barcoding of a large Genus, *Aspalathus* L. (Facaceae). Taxon 2008;57:1317-27, ii) White TJ, Bruns TD, Lee SB, Taylor JW. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand GH, Sninsky JJ, White TJ, editors. PCR Protocols: A Guide to Methods and Applications. San Diego: Academic Press; 1990. p. 315-22, iii) Ford CS, Ayres KL, Toomey N, Haider N, Stahl VAJ, Kelly LJ, *et al.* Selection of candidate coding DNA barcoding regions for use on land plants. Bot J Linnean Soc 2009;159:1-11, iv) Fay MF, Cameron KM, Prance GT, Lledo MD, Chase MW. Familial relationships of rhabdodendron (Rhabdodendraceae): Plastid *rbcL* sequences indicate a caryophyllid placement. Kew Bull 1997;52:923-32, v) Olmstead RG, Michaels HJ, Scott KM, Palmer JD. Monophyly of the asteridae and identification of their major lineages inferred from DNA sequences of *rbcL*. Ann Mo Bot Gard 1992;79:249-65, vi) Levin RA, Wagner WL, Hoch PC, Nepokroeff M, Pires JC, Zimmer EA, *et al.* Family-level relationships of onagraceae based on chloroplast rbcL and ndhF data. Am J Bot 2003;90:107-15, vii) Kress WJ, Erickson DL, Jones FA, Swenson NG, Perez R, Sanjur O, Bermingham E. Plant DNA barcodes and a community phylogeny of a tropical forest dynamic plot in Panama. Proc Natl Acad Sci USA 2009;106:18621-6, viii) Hamilton MB. Four primer pairs for the amplification of chloroplast intergenic regions with intraspecific variation. Mol Ecol 1999;8:513-25. *ITS: Internal Transcribed Spacer; rbcL*: Ribulose bisphosphate carboxylase large chain, *matK*: Maturase K, PCR: Polymerase chain reaction

Local Alignment Search Tool for nucleotides). Our sequences were taken as query sequence and BLASTn was run to find similarity with reference databases. The top hit with 100% query coverage and E value-0.00, was compared with query sequences to find percent similarity. Query sequences were claimed as barcoding sequences in two cases (i) when query and best match sequences were conspecific individual, i.e., individuals of same species, (ii) in case query species nucleotide data were not available on NCBI database and query sequence was best matched with congeneric species (other species of the same genus). If identified sequences were found 100% identical to the individual of same species, it intended our barcoding sequences are known to the NCBI database. In case of proximity (98.90%–99.99%) of the generated sequences with individuals of same species available in database, our sequences were considered as new barcodes and total number of individuals showing intra-specific differences was noted manually.

Prior existence of our sequences in the database with 100% identity to other species/genus and lesser similarity with alike species individuals does not allow our sequences to be used as barcodes. These sequences were considered incorrect. Identification was stated ambiguous when query sequence was found common (100% identical) among more than one species of that genus. Barcoding locus differentiating maximum species based on evolutionary distance and showing maximum number of barcoding sequences based on the percentage identity analysis was considered as preferred barcode candidate gene for the accounted orchids.

Phylogenetic analysis

Phylogenetic trees were constructed implementing discrete character method-Maximum Likelihood (ML) with 100 bootstraps available in MEGA X version.[18] ML method applies complex evolutionary model and is known to be reliable to infer phylogenetic analysis. In ML method-based exercises, Kimura two-parameter (K2P) model were used. Complete deletion option was chosen to treat the gaps and missing data. Barcoding sequences identified using similarity analysis of BLASTn method were used to construct phylogenetic tree. Species identification was considered successful only when all conspecific and congeneric individuals formed a single clade supported by bootstrap $P > 50$ in the ML tree. Obtained results of the phylogenetic analysis were compared with existing taxonomic classification[19] to confirm complementary character of barcoding and taxonomy.

RESULTS

Sampling of orchids

A total of 62 samples belonging to 35 different orchid species were collected, out of which, 23 species were endemic to Western Ghats of India and 12 were endemic to Northeast India. Vouchers for 56 specimens were deposited in the Bangalore University herbarium and accession numbers were collected. Accession numbers of 6 vouchers were collected from BGM, Belgium. Species name, voucher number, accessions numbers, geographical distribution, conservation status based on Convention on International Trade in Endangered Species of Wild Fauna and Flora, specific date of collection and place of collection of orchid samples were recorded and represented in Table 2.

Amplification and sequencing success of barcoding loci

The amplification success of four loci, namely, *ITS, matK, rbcL*, and *trnH-psbA* were noted as 97%, 100%, 100%, and 41%, respectively. These outcomes were further condensed to 93%, 100%, 98%, and 25%, respectively, after sequencing of the PCR products using the same primers. As *trnH-psbA* locus showed very low success rate for amplification and sequencing of orchids, we did not consider that for species resolution and identification studies.

Chromatograms generated by automated DNA sequencers were further interpreted and analyzed to remove the outcome of the improper and heterozygous (double) peaks of sequences. In total, we obtained 178 sequences of *ITS, matK*, and *rbcL* loci from the 62 samples, representing 35 species of 7 genera. These sequences accession number are mentioned in Table 3. Among 35 species studied, taxonomic lineages of 10 endemic Western Ghats species were noted "unrecognized" to NCBI database. Thus, this study make scientific populace familiar with barcoding sequences of 10 unrecognized species of Western Ghats, India, namely *Bulbophyllum acutiflorum, Bulbophyllum fimbriatum, Bulbophyllum fuscopurpureum, Bulbophyllum mysorense, Bulbophyllum tremulum, Coelogyne mossiae, Coelogyne odoratissima, Dendrobium panduratum, Trias stocksii*, and *Trias bonaccordensis*.

Table 2: Details of orchid species collection

Species Name	Voucher number	Species accession number	Geographical Distribution	Status according to CITES	Place of collection	Date of collection
A. crispa	DS001	BUH1001	WG	VU	Medikeri	September 06, 2016
	DS002	BUH1002			Kemmannugundi	November 06, 2016
A. maculosa	DS003	BUH1003	WG	EN	Kemmannugundi	November 06, 2016
	DS004	BUH1004			Gurukula Botanical Sanctuary	October 15, 2016
A. odorata	DS005	BUH1005	NE	VU	Assam Forest	September 25, 2016
	DS006	BUH1006			Nagaland Forest	September 20, 2016
A. multiflora	DS007	BUH1007	NE	D	Nagaland Forest	September 20, 2016
	DS008	XX-o-BR-19960050			BGM, Belgium	October 25, 2016
A. ringens	DS009	BUH1008	WG	D	Medikeri	September 06, 2016
	DS010	BUH1009			Gurukula Botanical Sanctuary	October 15, 2016
A. rosea	DS011	BUH1010	NE	EN	Assam Forest	September 25, 2016
	DS012	XX-O-BR-20160268			BGM, Belgium	October 25, 2016
B. acutiflorum	DS013	BUH1011	WG	EN	Medikeri	September 06, 2016
B. fimbriatum	DS014	BUH1012	WG	EN	Medikeri	September 06, 2016
	DS015	BUH1013			Mullayanagiri	March 10, 2018
B. fuscopurpureum	DS016	BUH1014	WG	EN	Medikeri	September 06, 2016
	DS017	BUH1015			Mullayanagiri	March 10, 2018
B. mysorense	DS018	BUH1016	WG	EN	Medikeri	September 06, 2016
	DS019	BUH1017			Mullayanagiri	March10, 2018
B. tremulum	DS020	BUH1018	WG	EN	Medikeri	September 06, 2016
	DS021	BUH1019			Mullayanagiri	September 10, 2018
C. peduncularis	DS022	BUH1020	WG	VU	Kemmannugundi	November 06, 2016
C. breviscapa	DS023	BUH1021	WG	VU	Medikeri	September 06, 2016
C. cristata	DS024	BUH1022	NE	CR	Assam Forest	September 25, 2016
	DS025	XX-o-BR-19073822			BGM, Belgium	October 25, 2016
C. flaccida	DS026	BUH1024	NE	VU	Assam Forest	September 25, 2016
C. mossiae	DS027	XX-o-BR-19073820	WG	CR	BGM, Belgium	October 25, 2016
C. nitida	DS028	BUH1025	NE	VU	Nagaland Forest	September 20, 2016
	DS029	BUH1026			Assam Forest	September 25, 2016
C. nervosa	DS030	BUH1027	WG	EN	Kemmannugundi	September 06, 2016
	DS031	BUH1028			Gurukula Botanical Sanctuary	October 15, 2016
	DS032	BUH1029			Mullayanagiri	March 10, 2018
C. odoratissima	DS033	BUH1030	WG	VU	Gurukula Botanical Sanctuary	October 15, 2016
C. ovalis	DS034	BUH1031	NE	VU	Nagaland Forest	September 20, 2016
	DS035	XX-o-BR-190740170		VU	BGM, Belgium	October 25, 2016
C. graminifolia	DS036	BUH1032	NE	VU	Kemmannugundi	November 06, 2016
C. pandurata	DS037	XX-o-BR-190700243	NE	VU	BGM, belgium	October 25, 2016
	DS038	BUH1033			Nagaland forest	September 20, 2016
D. anceps	DS039	BUH1032	NE	EN	Nagaland forest	September 20, 2016
D. aphyllum	DS040	BUH1033	NE	VU	Nagaland Forest	September 20, 2016
D. aqueum	DS041	BUH1034	WG	VU	Agasthyamalai	August 04, 2017
	DS042	BUH1035			Mullayanagiri	March 10, 2018
D. barbatulum	DS043	BUH1036	WG	VU	Kemmannugundi	November 06, 2016
	DS044	BUH1037			Agasthyamalai	August 04, 2017
D. jerdonianum	DS045	BUH1038	WG	EN	Mullayanagiri	March 10, 2018
	DS046	BUH1039			Kemmannugundi	November 06, 2016
	DS047	BUH1040			Kalsubai	March 19, 2018
D. nanum	DS048	BUH1041	WG	EN	Agasthyamalai	August 04, 2017
	DS049	BUH1042			Mullayanagiri	March 10, 2018
D. nobile	DS050	BUH1043	NE	D	Assam forest	September 25, 2016
	DS051	BUH1044			Nagaland forest	September 20, 2016
D. ovatum	DS052	BUH1045	WG	VU	Agasthyamalai	August 04, 2017
	DS053	BUH1046			Mullayanagiri	March 10, 2018
D. panduratum	DS054	BUH1047	WG	VU	Agasthyamalai	August 04, 2017
	DS055	BUH1048			Mullayanagiri	March 10, 2018
P. druryi	DS056	BUH1049	WG	CR	Agasthyamalai	August 04, 2017
	DS057	BUH1050			Mullayanagiri	March 10, 2018
T. stocksii	DS058	BUH1051	WG	CR	Kalsubai	March 10, 2018
	DS059	BUH1052			Agasthyamalai	August 04, 2017
	DS060	BUH1053			Mullayanagiri	March 10, 2018
T. bonaccordensis	DS061	BUH1053	WG	VU	Agasthyamalai	August 04, 2017

Contd...

Table 2: Contd...

Species Name	Voucher number	Species accession number	Geographical Distribution	Status according to CITES	Place of collection	Date of collection
D. herbaceum	DS062	BUH1054	WG	VU	Mullayanagiri	March 10, 2018

WG: Western Ghats; NE: Northeast; CR: Critically Endangered; D: Decline; EN: Endangered; VU: Vulnerable; *A. crispa: Aerides crispa; A. maculosa: Aerides maculosa; P. druryi: Paphilopedium druryi; D. panduratum: Dendrobium panduratum; T. bonaccordensis: Trias bonaccordensis; d. herbaceum: Dendrobium herbaceum; A. odorata: Aerides odorata; D. ovatum: Dendrobium ovatum; T. stocksii: Trias stocksii; D. nobile: Dendrobium nobile; D. barbatulum: Dendrobium barbatulum; D. jerdonianum: Dendrobium jerdonianum; D. nanum: Dendrobium nanum; D. anceps: Dendrobium anceps; A. multiflora: Aerides multiflora; A. ringens: Aerides ringens; B. acutiflorum: Bulbophyllum acutiflorum; B. fimbriatum: Bulbophyllum fimbriatum; A. rosea: Aerides rosea; B. fuscopurpureum: Bulbophyllum fuscopurpureum; D. aphyllum: Dendrobium aphyllum; P. druryi: Paphiopedilum druryi; B. mysorense: Bulbophyllum mysorense; B. tremulum: Bulbophyllum tremulum; C. nitida: Coelogyne nitida; C. peduncularis: Cottonia peduncularis; D. aqueum: Dendrobium aqueum; C. breviscapa: Coelogyne breviscapa; C. cristata: Coelogyne cristata; C. flaccida: Coelogyne flaccida; C. mossiae: Coelogyne mossiae; C. nervosa: Coelogyne nervosa; C. odoratissima: Coelogyne odoratissima; C. graminifolia: Coelogyne graminifolia; C. pandurata: Coelogyne pandurata; C. ovalis: Coelogyne ovalis*
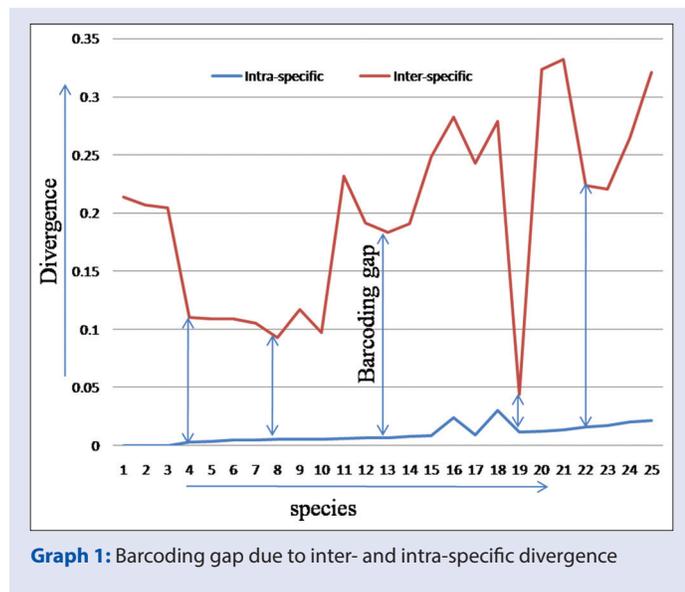
Graph 1: Barcoding gap due to inter- and intra-specific divergence

Determination of candidate barcode sequences
Genetic distance method

The analysis of evolutionary divergence between sequences of all the species using K2P model of distance matrix method showed *ITS* had the much higher inter-specific divergence (0.0322–0.3765) compared to *matK* and *rbcL* (0.0000–0.0802 and 0.0000–0.1294, respectively). The intra-specific divergence was also noted highest for *ITS* (0.0000–0.0300) followed by *rbcL* and *matK* (0.0000–0.0072 and 0.0000–0.0042, respectively). Obvious barcoding gap was found in *ITS* and the overlap between inter-specific and intra-specific variation was noted in *matK* and *rbcL* [Graph 1]. Hence, genetic distance method concluded that sequences generated using *ITS* locus can be considered as the potent DNA barcode for orchids considered in the present study.

BLAST analysis

BLAST-based similarity analysis inferred 53, 32, and 48 barcodes for *ITS, matK,* and *rbcL* respectively. The maximum number of query sequences (94.64%) could be identified as barcoding sequences using *ITS* locus followed by *rbcL* (78.69%) and *matK* (51.61%). Incorrect identification rate was noted 3.57%, 38.71%, and 16.39% for the barcoding candidate genes *ITS, matK,* and *rbcL,* respectively. In this study, 1.76%, 4.91%, and 8.06% sequences of *ITS, rbcL,* and *matK,* respectively, were found ambiguous based on megablast analysis of nucleotides. In case of *ITS* locus, the sequence of *D. jerdonianum* (DS045) was found incorrect; and *A. rosea* (DS012) was found ambiguous based on the similarity analysis [Table 4].

As orchid samples were collected from different geographical locations, intra-specific distance was noted among the conspecific individuals. Intra-specific variations were shown by individuals of 72.72% species studied based on *ITS*. Whereas *rbcL, matK* barcoding loci-based evaluation could find intra-specific variations in 45.83% and 12.5% conspecific individuals, respectively. The sequences showing intra-specific variations were considered as barcode of said species variety. These variations were ranged between 0.01%–1.01% for all three loci [Supplementary Table S1]. In other case, among congeneric species showing inter-specific variation, percentage similarity of nucleotides ranged between 92.00%–98.00% for *ITS*, 98.00%–99.50% for *matK,* and 97.00%–99.50% for *rbcL*. Hence, BLAST-based similarity analysis found *ITS* locus is comparatively potent and precise to identify congeneric and conspecific individuals, whereas percentage of ambiguous or incorrect sequences was quite higher for *matK* and *rbcL*.

Phylogenetic matrices and species resolution

ML-based tree of *ITS* showed higher bootstrap values and species of each genus were clustered on different branches and nodes as monophyletic taxon and then clustered with genus of other clades. These could be correlated with orchid classification. *Aerides* and *Cottonia* genus, which belong to *Aeridenae* subtribe were clustered together. Bulbophyllinae and Dendrobiinae subtribe of Dendrobieae tribe were clustered next to each other. Statistically, all the operational taxonomic units were perfectly bifurcated from their respective nodes with a bootstrap *P* > 50 for most of the subtrees. It confirmed that *ITS* is having high resolution power for molecular classification of orchids. Samples collected from BGM (DS025, DS035, and DS037) were clustered with individuals of the same species collected from India. *C. nervosa* individuals made monophyletic group with bootstrap value 89. *C. pandurata* individuals displayed coalescent stochasticity with branch support value 100 [Figure 2]. Thus, *ITS* locus-based ML phylogenetic tree can be used to identify unknown samples of studied species for molecular classification and identification.

A low bootstrap value (<50) was shown by ML subtrees of *Bulbophyllum, Dendrobium, and Coelogyne* constructed using *matK* locus. Which made this locus unfit for species identification [Figure 3]. Mixed population of *Paphilopedium* (subfamily-*Cypripedioideae*) and *Bulbophyllum* (subfamily-*Epidendroideae*) as well as *Aerides* (Tribe-Vandeae) and *Trias* (Tribe-Dendrobieae) was displayed on evolutionary tree of *rbcL*. Furthermore, lower bootstrap values confirmed that *rbcL* cannot discriminate and identify species according to the taxonomic classification of orchids [Figure 4]. Thus, in this study, *ITS* region showed perfect universality and identification of orchids at congeneric and conspecific level using distance, blast, and tree-Building methods.

Table 3: Accession number assigned by GenBank for *Internal Transcribed Spacer*, maturase K, and Ribulose bisphosphate carboxylase large chain sequences of the listed orchid species and voucher numbers

Species Name	Voucher number	Accession Numbers		
		ITS	matK	rbcL
A. crispa	DS001	MK120006	MK084915	MK125046
	DS002	MK120346	MK084916	MK125047
A. maculosa	DS003	MK120347	MK084917	MK125048
	DS004	MK120348	MK084918	MK125049
A. odorata	DS005	MK120349	MK084919	MK125050
	DS006	MK120350	MK084920	MK125051
A. multiflora	DS007	MK120351	MK084921	MK125052
	DS008	MK120352	MK084922	MK125053
A. ringens	DS009	MK120353	MK084923	MK125054
	DS010	MK120354	MK084924	MK125055
A. rosea	DS011	MK120355	MK084925	MK125056
	DS012	MK120356	MK084926	MK125057
B. acutiflorum*	DS013	MK144519	MK084927	MK155283
B. fimbriatum*	DS014	MK169283	MK084928	MK155284
	DS015	MK169284	MK084929	MK155285
B. fuscopurpureum*	DS016	MK169285	MK084930	MK155286
	DS017	MK169286	MK084931	MK155287
B. mysorense*	DS018	MK169287	MK084932	MK155288
	DS019	MK169288	MK084933	MK155289
B. tremulum*	DS020	MK169289	MK084934	MK155290
	DS021	MK169290	MK084935	MK155291
C. peduncularis	DS022	MK169291	MK084936	MK155292
C. breviscapa	DS023	MK169292	MK089394	MK155293
C. cristata	DS024	MK169293	MK089395	MK155294
	DS025	MK169294	MK089396	MK155295
C. flaccid	DS026	MK169295	MK089397	MK155296
C. mossiae*	DS027	-	MK089398	MK155297
C. nitida	DS028	MK169296	MK089399	MK155298
	DS029	MK169297	MK089400	MK155299
C. nervosa	DS030	MK169298	MK089401	MK155300
	DS031	MK169299	MK089402	MK155301
	DS032	MK169300	MK089403	MK155302
C. odoratissima*	DS033	MK169301	MK089404	MK155303
C. ovalis	DS034	MK169302	MK089405	MK155304
	DS035	MK169303	MK089406	MK155305
C. graminifolia	DS036	MK169304	MK089407	MK155306
C. pandurata	DS037	-	MK089408	MK155307
	DS038	MK169305	MK089409	MK155308
D. anceps	DS039	MK169306	MK089410	MK159238
D. aphyllum	DS040	MK169307	MK089411	MK159239
D. aqueum	DS041	MK169308	MK089412	MK159240
	DS042	MK169309	MK089413	MK159241
D. barbatulum	DS043	MK169310	MK089414	MK159242
	DS044	MK169311	MK089415	MK159243
D. jerdonianum	DS045	MK169312	MK089416	MK159244
	DS046	MK169313	MK089417	MK159245
	DS047	MK169314	MK089418	MK159246
D. nanum	DS048	MK169315	MK089419	MK159247
	DS049	MK169316	MK089420	MK159248
D. nobile	DS050	MK169317	MK089421	MK159249
	DS051	MK169318	MK089422	MK159250
D. ovatum	DS052	MK169319	MK089423	MK159251
	DS053	MK169320	MK089424	MK159252
D. panduratum*	DS054	MK169321	MK089425	MK159253
	DS055	MK169322	MK089426	MK159254
P. druryi	DS056	MK169323	MK089427	MK159255
	DS057	MK169324	MK089428	MK159256
T. stocksii*	DS058	-	MK089429	MK159257
	DS059	-	MK089430	MK159258
	DS060	-	MK089431	MK159259

Contd...

Table 3: Contd...

Species Name	Voucher number	Accession Numbers		
		ITS	matK	rbcL
T. bonaccordensis*	DS061	-	MK089432	MK159260
D. herbaceum	DS062	MK169325	MK089433	-

*These species were first time recognized by NCBI and GenBank database through this study. A. crispa: Aerides crispa; A. maculosa: Aerides maculosa; D. panduratum: Dendrobium panduratum; T. bonaccordensis: Trias bonaccordensis; A. odorata: Aerides odorata; D. ovatum: Dendrobium ovatum; D. haemoglossum: Dendrobium haemoglossum; T. stocksii: Trias stocksii; D. nobile: Dendrobium nobile; P. parishii: Paphiopedilum parishii; D. barbatulum: Dendrobium barbatulum; D. jerdonianum: Dendrobium jerdonianum; D. nanum: Dendrobium nanum; D. anceps: Dendrobium anceps; A. multiflora: Aerides multiflora; A. ringens: Aerides ringens; B. acutiflorum: Bulbophyllum acutiflorum; B. fimbriatum: Bulbophyllum fimbriatum; A. rosea: Aerides rosea; B. fuscopurpureum: Bulbophyllum fuscopurpureum; D. aphyllum: Dendrobium aphyllum; P. druryi: Paphiopedilum druryi; B. mysorense: Bulbophyllum mysorense; C. nitida: Coelogyne nitida; C. peduncularis: Cottonia peduncularis; D. aqueum: Dendrobium aqueum; C. breviscapa: Coelogyne breviscapa; C. cristata: Coelogyne cristata; C. flaccida: Coelogyne flaccida; C. mossiae: Coelogyne mossiae; C. nervosa: Coelogyne nervosa; C. odoratissima: Coelogyne odoratissima; C. graminifolia: Coelogyne graminifolia; C. pandurata: Coelogyne pandurata; C. ovalis: Coelogyne ovalis

DISCUSSION

DNA barcoding has been proposed as a powerful taxonomic tool for species identification. In this study, the core barcodes (matK and rbcL) had better performance in PCR amplification and sequencing when compared with *ITS*. Deprived success of existing *ITS* primers and reduced sequencing success of this regionmight be explained by the incomplete concerted evolution of this nuclear multiple-copy region.[13,20,21] *psbA-trnH* exhibited a low success rate, whereby 75% samples failed to generate high quality bidirectional sequences might be due to the presence of a poly (T) tail at about 100 bp from the *psbA* primer.[22] Previous studies found that Nuclear ribosomal DNA region (*ITS*) evolves rapidly, leading to create genetic distances that can differentiate closely related, congeneric species.[23,24] The inter-specific divergence among different species, accountable for identification and phylogenetic variations in present study might be due to the same reason. Higher intra-specific variation among conspecific species may be explained by the issue of intragenomic diversity in *ITS* due to the presence of sequences in multiple copies in the genome.[25,26] Higher inter-specific diversity and larger barcoding gap is always considered suitable to find DNA barcode.[27] In this study, *ITS* loci were found most suitable for the distinguishing orchids based on higher inter-specific diversity and barcoding gap.

Sequence analysis using BLAST yielded higher species resolution for *ITS* region among all the markers used in the present study. BLAST's higher resolution can be explained by *ITS* greater sensitivity to sequence length, as well as inclusion of indel variation and orthology/paralogy conflation.[22] Earlier reports on *Dendrobium*[9] and medicinal plants of Iran[14] also found BLAST analysis as a proficient method for species identification. *ITS* sequence data are universally used in plant phylogenetic studies despite of its complex and unpredictable evolutionary behavior.[28] Their highly variable noncoding regions may not be useful to study the phylogenetic relationships of high-level taxa, but could be a good source to investigate phylogenetic relationships at lower levels, such as intra-generic levels and intra-specific varieties.[29,30] This might be the reason why individuals of same species and genus were clustered as monophyletic clade in our study. *ITS* sequence-based identification and phylogenetic relationship of orchids have been studied earlier in *Dendrobium*,[20,31] *Habenaria*,[32] and *Paphiopedilum*[33] and was found successful.
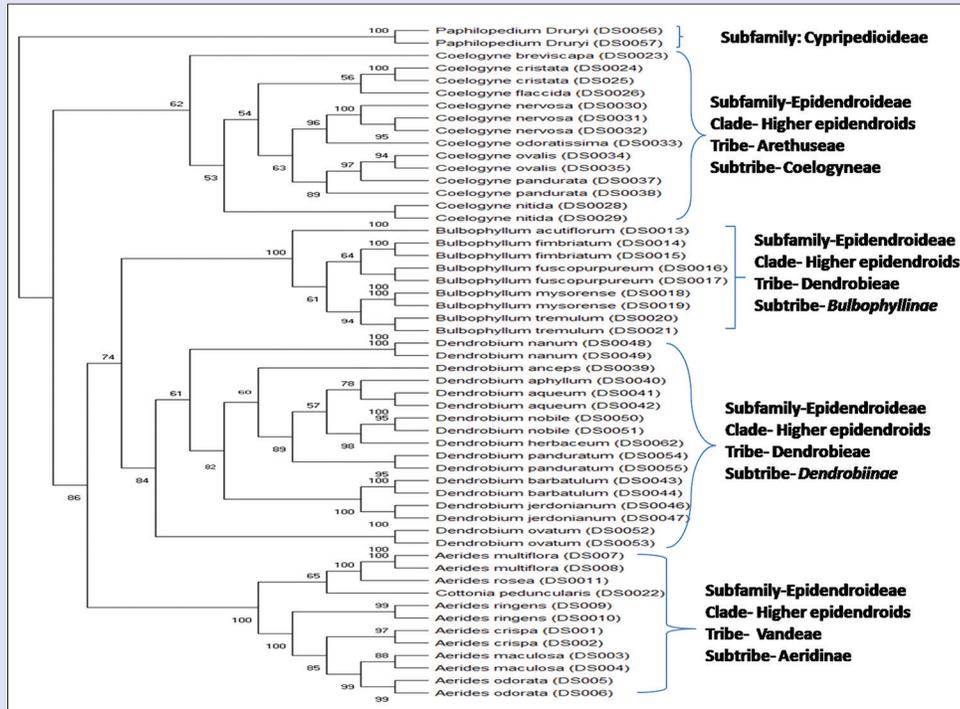
Figure 2: ML tree using ITS DNA barcodes
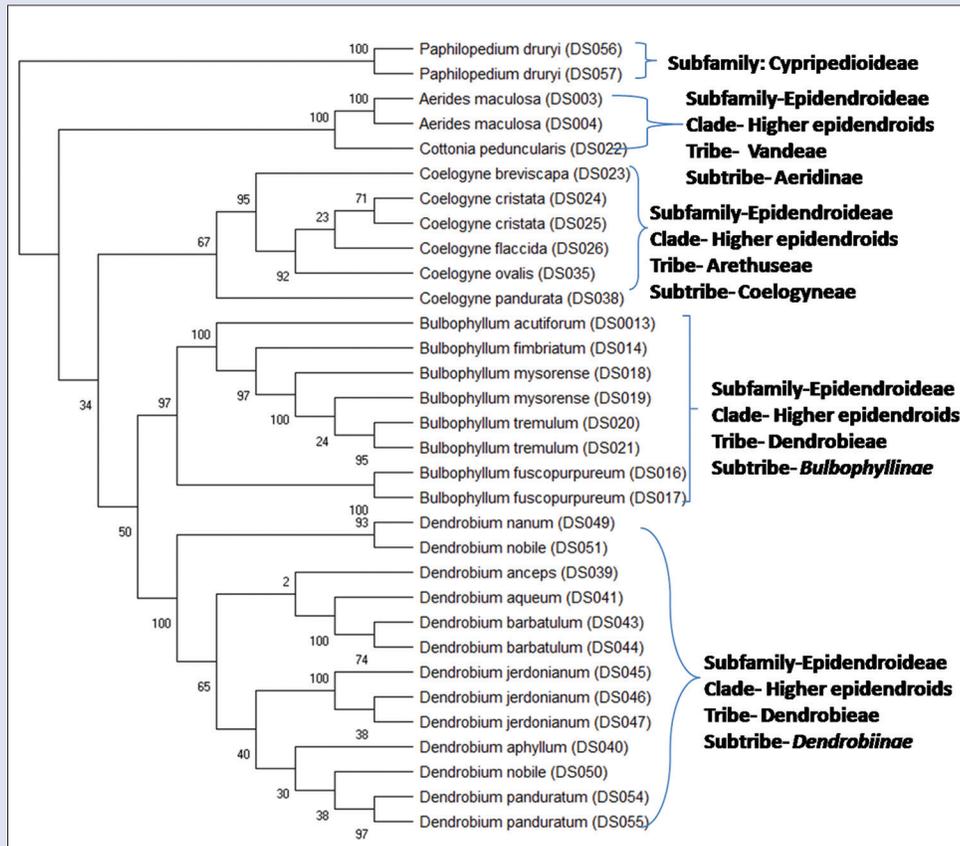
Figure 3: ML tree using matK DNA barcodes

Table 4: Basic local alignment search tool for nucleotides similarity analysis based barcoding efficiency of candidate loci

Locus	Orchids representing barcoding sequences (%)	Orchids showing incorrect sequence (%)	Orchids showing ambiguous sequence (%)	Orchids representing conspecific sequences (%)
ITS	94.64	3.57	1.76	72.72
matK	51.61	38.71	8.06	12.5
rbcL	78.69	16.39	4.91	45.83

ITS: Internal Transcribed Spacer; *rbcL*: Ribulose bisphosphate carboxylase large chain, *matK*: maturase K
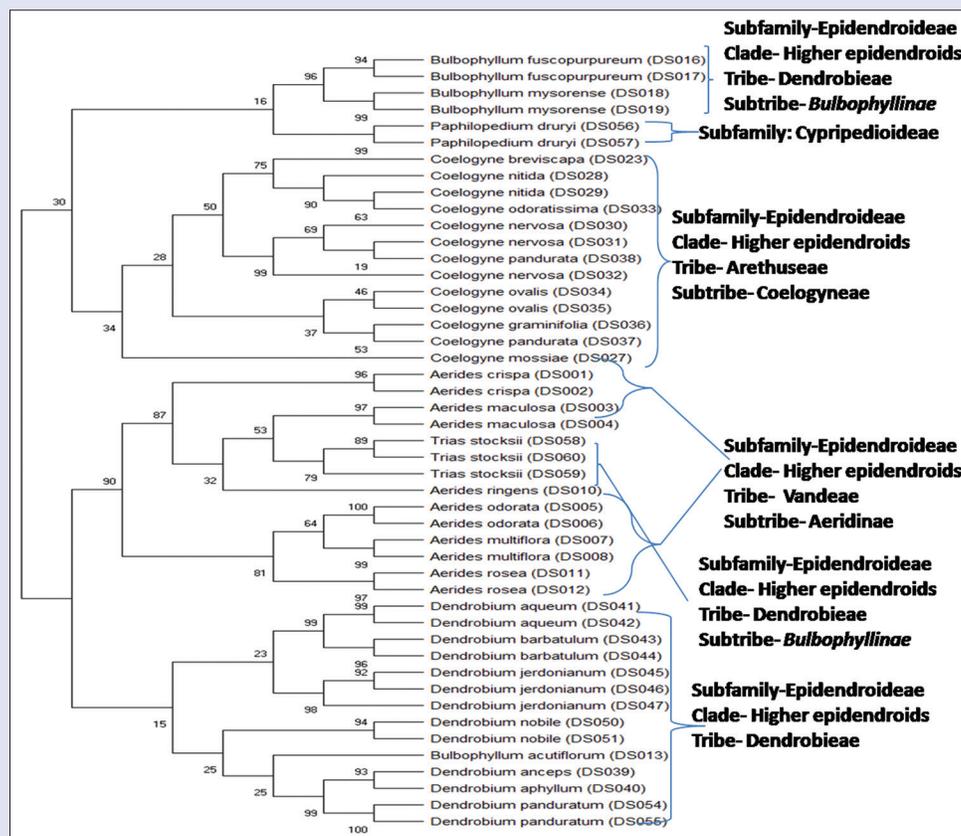
Figure 4: ML tree using rbcL DNA barcodes

China Plant BOL Group proposed that the nuclear ribosomal DNA-*ITS*, or subset of this marker-*ITS*2, should be incorporated alongside rbc*L* + mat*K* into the core barcode for seed plants, particularly for angiosperms.[11] Several recent studies also recommended that *ITS* region in combination with *trnH-psbA*, *matK* and/or *rbcL* are best barcode region for *Schisandraceae* family,[23] *Terminalia*[22] and many orchid species.[12] In contrast, our study found *matK* and *rbcL* loci could not discriminate Indian orchids, might be because we sampled many more closely related species within single genera to assess absolute rather than relative discriminatory power of the tested barcode markers. Hence, the present study outcome based on different methods (distance, BLAST, and tree-building) is strongly supporting earlier report that suggests *ITS* region as most successful barcoding region for *Dendrobium*,[14] *Crawfurdia*,[16] and other medicinal plants like *Saussurea* subg *Amphilaena*.[34]

CONCLUSION

In this study, we worked on 62 samples of 35 endemic and endangered Indian orchids species belongs to 7 genera. This study made scientific populace of NCBI familiar with 10 unrecognized species of Western Ghats, India. We identified 133 barcoding sequences, out of which, 20, 12, and 14 sequences were found unique and new to GenBank for *ITS*, *matK*, and *rbcL*, respectively. Further, our study based on distance, BLAST and tree-building methods suggested that *ITS* is the best region to be considered as single locus barcode for the identification of orchids of India.

Conflicts of interest

There are no conflicts of interest.

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