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DNA BARCODING OF SOME INDIAN *COELOGYNE* (EPIDENDROIDEAE, ORCHIDACEAE)

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Abstract

Sub-tribe Coelogyninae (Epidendroideae, Orchidaceae) comprises 16 genera; study of one of these genera, *i.e., Coelogyne* Lindl. reveals sectional relationships with *Pholidota*. Present study was undertaken to develop DNA barcodes of sub-tribe Coelogyninae using *rbcL* (Rubisco large sub unit). The *rbcL* from chloroplast genome tested for as effective barcode. The inter-specific divergence values and species discrimination rates were calculated by Kimura 2 parameter (K2P) using MEGA 4.0. The *rbcL* with average inter-specific divergence values yielded 72.72% species resolution, thus, could distinguish all the species of *Coelogyne* and *Pholidota*. Invariably, the genus *Pholidota* showed close affinity with *Coelogyne*, supporting thereby the inclusion of genus *Pholidota* in the sub-tribe Coelogyninae under tribe Coelogyneae.

Introduction

THE ORCHIDACEAE is one of the largest families of flowering plants. It comprises about 779 genera and 22,500 species (Mabberley, 2008). In India, with 1,229 species spreading over 184 genera, it represents second largest flowering plant family and contributes about 10% of Indian Flora (Kumar and Manilal, 1994). The orchid genus *Coelogyne* Lindl. is distributed throughout South East Asia, Sumatra and Himalayas (Butzin, 1992); with most of the species growing as epiphytes, some species grow as lithophytes or even terrestrial plants (Comber, 1990). In India, most of the orchid habitats are dwindling in state due to many anthropogenic activities, such as habitat destruction and fragmentation, landscape development, river valley projects and other infrastructural developments. Except for a few publications in recent past (Chaudhary *et al.,* 2012; Khasim and Ramesh, 2010; Parab and Krishnan, 2008; Ramudu *et al.,* 2012), no extensive work has been done on molecular characterization of Indian orchids. In view of above background, the molecular characterization of some Indian orchids belonging to sub-family Epidendroideae was analyzed, in the present investigation.

Materials and Methods

For molecular studies, plant materials (one population each) belonging to the tribe Coelogyninae, Sub-tribe Coelogyneae, subfamily Epidendroideae of family Orchidaceae, were collected from different geographical locations of Southern India *i.e.*, Andhra Pradesh, Tamil Nadu and Kerala (Table 1; Fig. 1). Some species were also procured from Tropical Botanical Garden and Research Institute (TBGRI), Pallode (Kerala) and National Orchidarium, Yercaud (Tamil Nadu).

Isolation of Genomic DNA

The genomic DNA was isolated using CTAB (N-acetyl-N,N,N-trimethyl-ammonium bromide) technique (Doyle and Doyle, 1987). Leaf tissue (100 mg) grounded to powder to which was added 600 ml of cold extraction buffer (3% CTAB, 1.42 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0, 2% polyvinylepyrrolidone, 5 mM ascorbic acid); the tissue was further homogenized for 3 min. The entire homogenization process was performed in liquid nitrogen. These homogenized samples were then treated at 65° C for 15 min. and then extracted once with chloroform-isoamyl alcohol (24:1, v/v) to obtain clear supernatant. This supernatant was centrifuged at 12,000 rpm for 5 min. This supernatant containing plant genomic DNA was transferred to fresh test-tube to which added a one-fifth volume of 5% CTAB solution in 0.7 M NaCl, and extracted again with chloroform-isoamyl alcohol. The DNA was precipitated from the supernatant by the addition of two volumes of cold absolute ethanol, incubated at −80°C for 15 min. and the DNA centrifuged at 12,000 rpm for 20 min at 4°C. After rinsing the DNA pellet in cold 70% ethanol, the DNA was dried under vacuum. The dried DNA was resuspended in 100 μl of distilled water. DNA concentration was determined by measuring the absorbance at 260 nm. The DNA quality was assessed by using 1% agarose gel electrophoresis.

Amplification of rbcl and Sequencing

The universal primers downloaded from Kew website

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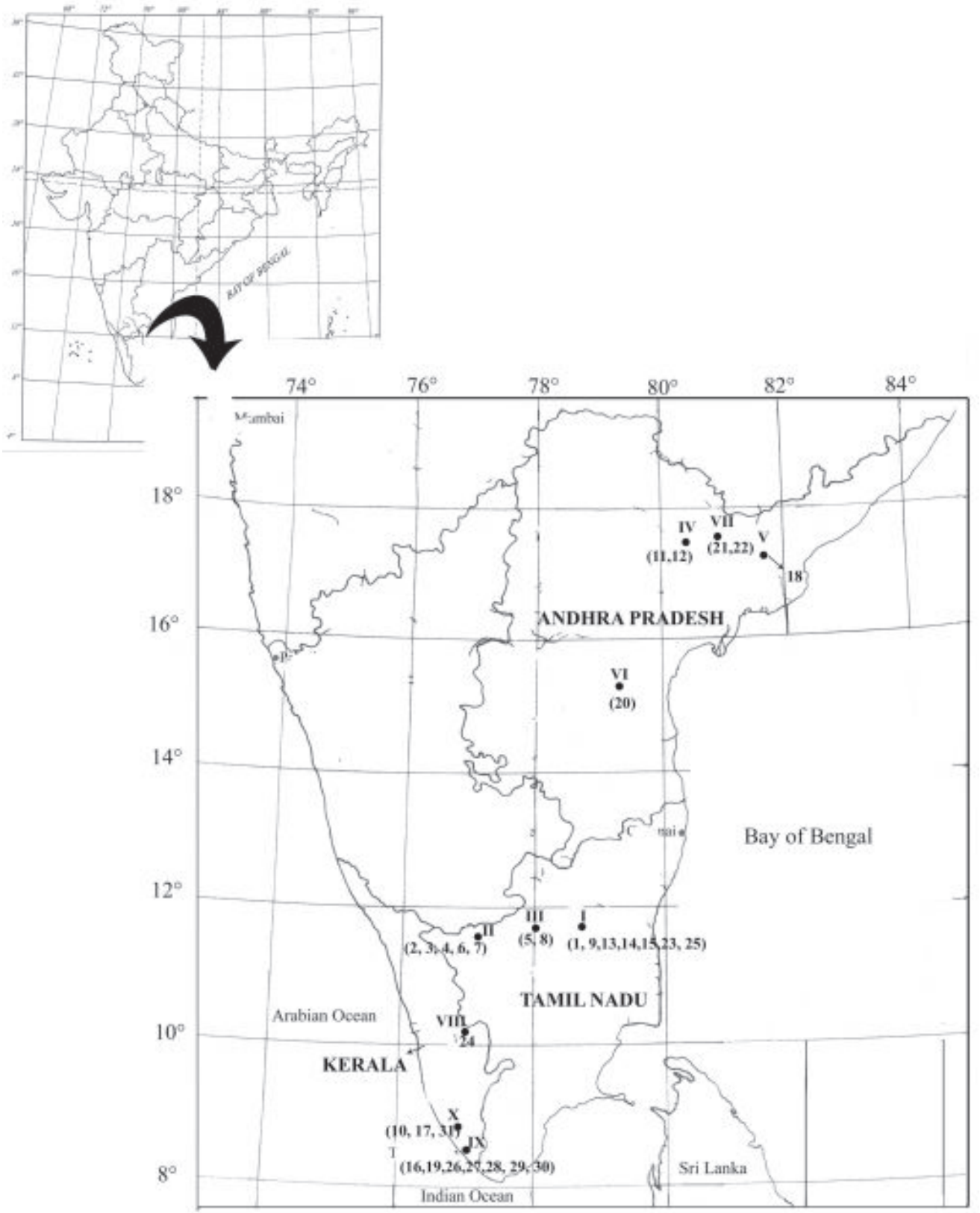


I. Shevroy Hills, Yercaud (TN); II. Dodabetta, Ooty (TN); III. National Orchidarium, Yercaud (TN); IV. Paderu (AP); V. Chinthapalli (AP); VI. Giddaluru (AP); VII. Lthugadda (AP); VIII. Karumancode (KE); IX. TBGRI, Pallode (KE); X. Pallode (KE).)

Fig. 1. India map with marked places of collection of orchid plant material.

Table 1. List of species taken for molecular studies

S.No.	Species	Place of collection and elevation	Habitat and host tree	Voucher No.
	Sub family Epidendroideae			
	Tribe Coelogyneae			
	Subtribe Coelogyninae			
1	*Coelogyne breviscapa* Lindl.	Shevroy Hills, Yercaud (TN), 1800 m	Epi & *Alnus nepalensis*	ANUH 1001
2	*C. corymbosa* Lindl.	Dodabetta (TN), 2200 m	Epi & *Mangifera indica*	ANUH 1002
3	*C. cristata* Lindl.	Shevroy Hills, Yercaud (TN), 1800 m	Epi & *Terminalia alata*	ANUH 1025
4	*C. flaccida* Lindl.	Dodabetta, Ooty (TN), 2200 m	Epi & *Castanopsis indica*	ANUH 1003
5	*C. nervosa A. Rich.*	Dodabetta (TN), 2100 m	Epi & *Schima wallichii*	ANUH 1004
6	*C. nitida* Lindl.	National Orchidarium, Yercaud, (TN)	_____	ANUH 1005
7	*C. ovalis* Lindl.	Dodabetta, Ooty (TN), 2200 m	Epi & *Terminalia bellirica*	ANUH 1006
8	*C. prolifera* Lindl..	Dodabetta, Ooty (TN), 2200 m	Epi & *Terminalia alata*	ANUH 1007
9	*C. trinervis* Lindl.	TBGRI,Pallode (KE)	_____	ANUH 1026
10	*Pholidota articulata* Lindl.	TBGRI,Pallode (KE)	_____	ANUH 1027
11	*P. pallida* Lindl.	Shevroy Hills, Yercaud (TN), 1800 m	Epi & *Mangifera indica*	ANUH 1009

aligned with chloroplast genome of some orchids and some changes towards 5′ end were taken as new primers for amplification of *rbcL* locus. The new primers were obtained from Helini Biomolecules, Chennai, Tamil Nadu, India (Table 2).

Table 2. Primers used for amplification of *rbcL* DNA barcodes.

S.No.	Locus	Primer name	Primers sequence
1.	*rbcL*	*rbcL_F*	5′ ATGTCACCACAAACAGAGACTAAAGC 3′
		rbcL_R	5′ GAAACGGTCTCTCCAACGCAT 3′

The PCR reaction mixture contained 1 unit of *Pfu* DNA polymerase, 2 μl each of 10X PCR buffer with $MgSO_4$, 2 mM of each of the dNTPs, 10 μM forward and reverse primers and 20-30 ng of template DNA. The final volume was made to 20 μl with autoclaved MQ. For amplification of *rbcL* locus from the chloroplast genome the thermal cycle followed was: one cycle of 5 min at 94°C; 35 cycles of 30 sec each at 94°C, 40 sec at 50°C, 1 min at 72°C with a final extension of 7 min at 72°C. The PCR products were electrophoresed in 1% TAE agarose gel, containing 5 mg/ml EtBr and visualised on a UV trans-illuminator. The PCR products are cleaned by Exo-SAP method**:** The samples that yielded single band of amplicon were cleaned using mixture of two enzymes - exonuclease and shrimp alkaline phosphatase (Exo-SAP method). The enzymes treatment degrades the unused primers left after the amplification and removes the phosphate group from all the left over dNTPs in the reaction mixture. The removal of these two components is necessary as these can cause hindrance in Sanger's di-deoxy sequencing reaction. A total of 2 μl of enzyme mixture, consisting of 0.5 μl of exonuclease I, 1 μl of shrimp alkaline phosphatase and 0.5 μl of MQ was used to clean 8 μl of PCR product making it a total of 10 μl of reaction mixture. The mixture was then incubated at 37°C for 15 min, 85°C for 15 min, and finally held at 4°C in a thermal cycler. The cleaned up PCR products were stored at -20°C. The purified PCR product was subjected to bi-directional Sanger's di-deoxy sequencing (Sanger *et al.*, 1977) using BigDye terminator v3.1 cycle sequencing kit on ABI Prism 3700 DNA Analyzer (Applied Biosystems Inc., USA). The sequencing reaction mixture contained 0.5 μl BigDye v3.1 ready reaction mixture, 3 μl PCR product, 2 μl 5X sequencing buffer, 1 μl either forward or reverse primer (10 μM) and 3.5 μl autoclaved MQ making the final volume of 10 μl. The thermal cycle for sequencing was 30 cycles of 10 sec each at 96°C, 5 sec at 50°C and 4 min at 60°C. The cleaning up of sequencing products was carried out using 1 ml of 0.5M Na_2 EDTA (pH 8.0) diluted with 11 ml of MQ. 15 μl of this solution along with 81 μl of absolute ethanol

was added to each well of the PCR plate and mixed thoroughly by inverting the sealed plate. Thereafter, it was kept in dark for 10 min at room temperature and centrifuged at 3220g for 30 min at 18°C. The plate was decanted on a tissue paper and centrifuged in an inverted position at 50g for 1 min. Thereafter, 150 μl of 70% ethanol was added to each well and the plate was centrifuged at 3220g for 10 min at 18°C. The plate was again decanted on a tissue paper and centrifuged in inverted position at 50g for 1 min. The pellets were air-dried for 10 min in dark and 10 μl of the highly deionised formamide to each well was added and the plate was incubated at 42°C for 20 min on a platform shaker. The plate was centrifuged at 700g for 1 min and kept on the plate deck of the sequencer. The plate was locked and linked to the sequencer.

Analysis of DNA Sequence Data

The chromatograms obtained from the sequencer were base-called, using Phred. The sequences with Phred score more than 20 were taken for further analysis. The forward and reverse sequences were trimmed and assembled, using sequencher (Gene Codes Corporation, Ann Arbor, Michigan, USA). Each sequencher project file (.spf) consisted of all the sequences of a single species and its consensus sequence was taken as the representative sequence for that particular species. The sequences were submitted to GenBank, NCBI and accession numbers were obtained for each sequence (Table. 3). The representative sequence of each of the selected species for a particular locus was pasted in a notepad (.txt) file in fasta format and saved to particular location on the hard disk drive. The same notepad file was opened in the Bio Edit version 7.0.9.0 software which is freely available on the internet. The sequences were then aligned using CLUSTAL W, a tool for multiple sequence alignment. After the alignment process is completed, the aligned sequences are opened in a new window. The new file was saved to a location on the hard disk drive as a fasta (.fas) file. The aligned sequences were then retrieved from the fasta file using *Alignment* menu in the toolbar of MEGA version 4.0 software (Tamura *et al.*, 2007). Thereafter, the opened alignment file was closed and a dialog box appeared asking to save the file in MEGA format. After clicking *yes*, the instructions given by the software were followed. After following all instructions, the data was displayed in the MEGA format. The *F7* key was pressed on the keyboard. A table appeared on the screen along with queries about the type of model to be used and the input of nucleotide. After clicking compute, a

Table 3. Gene bank accession numbers of orchid species taken for the molecular studies.

S. No.	Taxa	Gene bank accession number
1	*Acanthephippium bicolor* Lindl.	Submitted to NCBI gene bank
2	*Tainia bicornis* Lindl. Reich.	Submitted to NCBI gene bank
3	*Coelogyne breviscapa* Lindl.	KC 797587
4	*C. corymbosa* Lindl.	Submitted to NCBI gene bank
5	*C. cristata* Lindl.	Submitted to NCBI gene bank
6	*C. flaccida* Lindl.	KC 797588
7	*C. nervosa* A. Rich.	KC 797585
8	*C. nitida* Lindl.	Submitted to NCBI gene bank
9	*C. ovalis* Lindl.	KC 797586
10	*C. prolifera* Lindl.	Submitted to NCBI gene bank
11	*C. trinervis* Lindl.	Submitted to NCBI gene bank
12	*Pholidota articulata* Lindl.	KJ 947345
13	*P. pallida* Lindl.	Submitted to NCBI gene bank
14	*Eria bicolor* Lindl.	Submitted to NCBI gene bank
15	*E. bambusifolia* Lindl.	Submitted to NCBI gene bank
16	*E. polystachya* A. Rich.	KC 7978770
17	*Gastrochilus pulchellus* Lindl.	Submitted to NCBI gene bank

Kimura-2-parameter (K2P) distance matrix for the data was obtained. For constructing neighbour-joining tree, the saved project was opened and 'Phylogeny' was selected on the menu bar and then 'Bootstrap test of phylogeny' option was clicked. The intra- and inter-specific K2P distances were determined, using MEGA 4.0 following the above methodology. The representative sequence for each species was used for determining the inter-specific K2P distances. Species identification success rate for each locus was determined using: (i), the K2P distances arrived at on the basis of analyses of the species utilising MEGA 4.0; (ii), the Neighbour Joining trees constructed with the sequences of the selected loci; and (iii), BLAST method (Ross *et al.*, 2008). The last one involved performing of BLAST searches of representative sequence of each species on NCBI nucleotide database. For the distance based method, species resolution of *rbcL* locus was calculated according to the following formula:

$$\frac{\text{Total number of species} - \text{number of species with zero distance estimate} \times 100}{\text{Total number of species}}$$

For tree-based method, the species resolution was calculated according to the following formula:

$$\frac{\text{Total number of species - number of species which clustered with other species} \times 100}{\text{Total number of species}}$$

In the BLAST method, per cent species discrimination was calculated on the basis of first hit. The first hit with maximum query coverage and identity with the query sequence was considered for the correct sequence identification.

Results

Interspecific K2P Distances and Species Discrimination for rbcL at the Tribe and Subtribe Level

The interspecific variation and species discrimination rates among the congeneric species were also calculated individually for *rbcL* locus for a tribe in subfamily Epidendroideae, *i.e.*, Coelogyneae. The species discrimination rates were calculated both using genetic distance and phylogenetic tree methods. For the later neighbour joining (NJ) trees with thousand bootstrap, replicates were constructed for tested *rbcL* locus. The tribe- wise interspecific variations recorded and per cent species resolutions yielded are given below.

The Tribe Coelogyneae

The tribe Coelogyneae (Epidendroideae) is divided into subtribes namely Thuniinae and Coelogyninae (Dressler, 1993).

Two genera *i.e.*, *Coelogyne* and *Pholidota* of subtribe Coelogyninae were studied. The interspecific K2P distance matrix of 11 *rbcL* sequences obtained from 11 species of Coelogyninae was analysed. Average K2P distance was 0.005 (ranging from 0-0.017); out of 11 species analysed, 7 species had zero distance. The maximum interspecific K2P distance was recorded

Table 4. Interspecific K2P distance among Coelogyneae tribe.

Taxa	*Coelogyne breviscapa*	*C. corymbosa*	*C. cristata*	*C. flaccida*	*C. nervosa*	*C. nitida*	*C .ovalis*	*C. prolifera*	*C. trinervis*	*Pholidota articulata*	*P. pallida*
Coelogyne breviscapa	__										
C. corymbosa	0.004	__									
C. cristata	0.002	0.001	__								
C. flaccida	0.012	0016	0.015	__							
C. nervosa	0.000	0.004	0.002	0.012	__						
C. nitida	0.002	0001	0.000	0.015	0.002	__					
C .ovalis	0.000	0.004	0.002	0.012	0.000	0.002	__				
C. prolifera	0.002	0001	0000	0.015	0.002	0.000	0.002	__			
C. trinervis	0.005	0.004	0.002	0.017	0.005	0.002	0.005	0.002	__		
Pholidota articulata	0.000	0.004	0.002	0.012	0.000	0.002	0.000	0.002	0.005	__	
P. pallida	0.004	0.007	0.006	0.016	0.004	0.006	0.004	0.006	0.009	0.004	__

Table 5. Sectional delineation in the genus *Coelogyne* Lindl.

Section	Taxa
Ocellatae Pfitzer & Kraenzlin	1. *C. corymbosa*
	2. *C. nitida*
Cristatae Pfitz. ex. Pfitz. & Kranzl.	1. *C. cristata*
	2. *C. nervosa*
Elatae Pfitz. ex. Pfitz. & Kranzl.	*C. stricta*
Fuliginosae Pfitz. & Kranzl	*C. ovalis*
Lentignosae Pfitz. ex. Pfitz. & Kranzl.	*C. breviscapa*
Proliferae sens. str. Lindl.	*C. prolifera*
Flaccidae Lindl.	1. *C. flaccida*
	2. *C. trinervis*

*Arranged according to Subedi *et al.* (2011)

in between *Coelogyne trinervis* and *C. flaccida* (0.017) (Table 4).

Species Resolution

a) Distance based method: The *rbcL* distance matrix of 11 species belonging to subtribe Coelogyninae could not be resolved because of their zero distances with other species and thus resulted in a number of species pairs with distance estimates as zero. Consequently 7 species remain unresolved. Therefore, species resolution based on *rbcL* sequences was 36.36%. The species with zero distance estimates were *Coelogyne breviscapa, C. cristata, C. nervosa, C. nitida, C. ovalis, C. prolifera* and *Pholidota articulata*.

b) Phylogenetic tree building method: The analyses of aligned *rbcL* sequences simplified from 11 species revealed that there were 144 variable sites, parsimony informative 17, singleton sites 29, conserved sites 861 out of total 1127 nucleotide sites. The Neighbour Joining tree with thousand boot straps replicates revealed 3 different clusters comprising 7 unresolved species; thus enabling species resolution of 72.72%. The species clusters were formed (Fig. 2a). These clusters were as follows:

I. *Coelogyne corymbosa, C. nitida, C. prolifera, C. cristata,* and *C. trinervis.*

II. *Pholidota articulata, Coelogyne breviscapa, C. nervosa, C. ovalis, P. pallida.*

C. flaccida— outgroup .

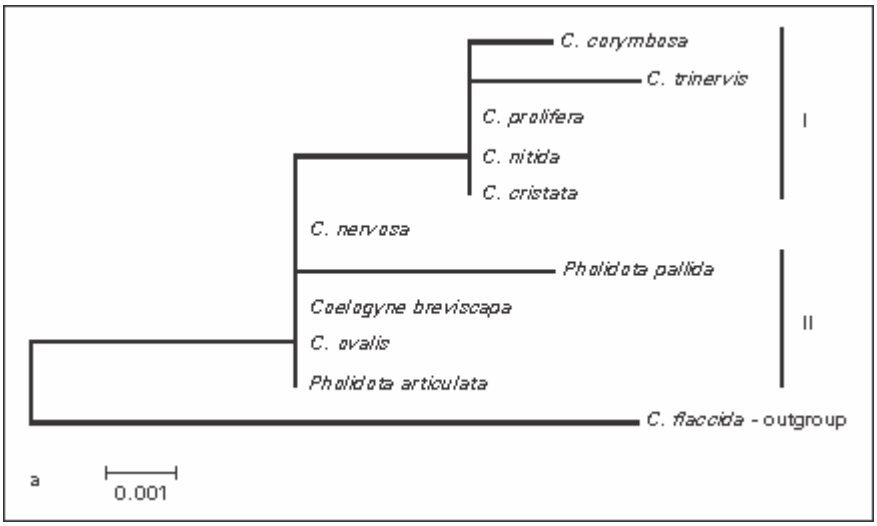
c) BLAST method: The *rbcL* locus of 11 investigated species on BLAST analysis afforded 60.15% species resolution. Species discrimination rate based on this method was 51.29%.

Interspecific K2P Distances and Species Discrimination rates for rbcL at the Generic Level

The interspecific variations and species discrimination rates among the congeneric species were also calculated individually for *rbcL* locus for 3 orchid genera. The species discrimination rates were calculated both using genetic distance and phylogenetic tree methods. For the latter, the Neighbour Joining trees with thousand bootstrap replicates were constructed for tested *rbcL* locus. The genus-wise inter-specific variations recorded and percent species resolutions yielded are detailed below:

Table 6. Average K2P distance among *Coelogyne* species.

Taxa	*Coelogyne breviscapa*	*C. corymbosa*	*C. cristata*	*C. flaccida*	*C. nervosa*	*C. nitida*	*C .ovalis*	*C. prolifera*	*C. trinervis*
Coelogyne breviscapa	__								
C. corymbosa	0.002	__							
C. cristata	0.000	0.002	__						
C. flaccida	0.022	0.024	0.022	__					
C. nervosa	0.002	0.004	0.002	0.020	__				
C. nitida	0.000	0.002	0.000	0.022	0.002	__			
C. ovalis	0.002	0.004	0.002	0.020	0.000	0.002	__		
C. prolifera	0.002	0004	0.002	0.020	0.000	0.002	0.000	__	
C. trinervis	0.006	0.008	0.006	0.024	0.004	0.006	0.004	0.004	__



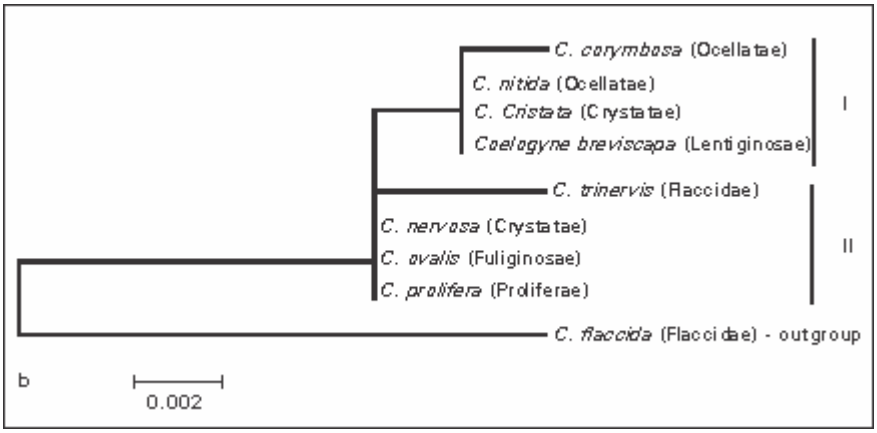


Fig. 2 a-b. a, Dendrogram of tribe Coelogyneae based on phylogenetic tree building method; b, Dendrogram of genus *Coelogyne* based on phylogenetic tree building method.

Coelogyne Lindl.

In genus *Coelogyne*, 9 species have been evaluated in the present investigation. These were *Coelogyne breviscapa*, *C. corymbosa, C. cristata, C. flaccida, C. nervosa, C. nitida, C. ovalis, C. prolifera* and *C. trinervis* (Table 6). The average interspecific K2P distance for *rbcL* was 0.007. The *rbcL* locus had resulted in 3 species pairs with distances estimate zero (Table 5). The species discrimination rate for *rbcL* was 44.44. Out of the 1108 nucleotides site of *rbcL* sequences of nine *Coelogyne* species compared, 276 were variable sites, 11 parsimony informative and 27 singleton sites.

The *rbcL* locus showed species clusters with different number of unresolved species. Thus resulting species resolution was 44.44%. The *rbcL* sequences showed 11 parsimony sites and 276 variable sites.

Pholidota Lindl.

In the genus *Pholidota*, two species, *i.e.*, *P. articulata* and *P. pallida* were studied. Average interspecific distance of *rbcL* locus is 0.002; and the *rbcL* sequences of two species were variable enough to distinguish two species.

Discussion

The *rbcL*, a plastid gene, has proven important tool in addressing phylogenetic relationships at various taxonomic levels (Cameron *et al.*, 1999). This gene is located in the large single copy region of the chloroplast genome and encodes the large subunit of ribulose 1,5-biphosphate corboxylase/oxygenase (RUBISCO). It has been sequenced in about 5000 plant species (Sanderson, 2003). Studies on Cypripediodeae by Alberts *et al.* (1994) and Dendrobiinae by Yukawa and Uehara (1996) indicated that the amount of sequence divergence exhibited by *rbcL* is sufficient and suitable for addressing the phylogenetic relationships at generic and species level, within the family Orchidaceae. The DNA bar coding in plants was carried out by Chase *et al.* (2005) and Kress *et al.* (2005). By the recommendations of plant working Group of Consortium for the Barcode of Life (CBOL), *rbcL is* chosen to be tested as DNA barcodes; in the present work, rbcL locus was evaluated according to the standard guidelines in CBOL (http:// www.barcoding.si.edu/ protocols.html) and BOLD [(Barcode of life Datasystems), (Ratnasingam and Herbert, 2007)].

Species Discrimination

Genetic distance, phylogenetic tree method and BLAST analysis methods have been used for evaluating species resolution. The perfect barcoding gap provides in assigning an unknown individual to its respective species correctly (Meyer and Paulay, 2005). In phylogenetic tree method with cluster analysis, the phylogenetic tree is constructed using sequences of the *rbcL* locus based on percent species resolution (China Plant BOL Group, 2011; Lahaye *et al.*, 2008). The NJ tree based analysis for species discrimination also provides a convenient method of viewing the data

as the unresolved species clusters could be identified easily. It also helped in identification of synonymous species *i.e.*, *Pholidota imbricata* and *P. pallida* considered as two different species; later these two names were found to be synonyms in Kew check list (Govaerts *et al.,* 2010). In BLAST method, the barcode sequence of an unknown species individual is BLAST searched for a very similar or identical sequence of the database, containing reference barcodes of correctly identified species and the first hit with maximum score is taken as the species to which the unknown specimen belongs. In the present investigation BLAST species resolution was 55.25. The *rbcL* locus from chloroplast genome exhibited very low and varied species discriminatory powers (50-75%) using all the three methods. The present study is based on previous investigations of *rbcL* locus for reconstructing phylogenies only at family and subfamily level as it had limited application at the species level (Cameron *et al*. 1999; Parveen, 2012; Singh *et al.*, 2012). However among congeneric species, the resolving power was very low.

Interspecific Variability and Phylogenetic Relationships

From all dendrograms constructed based on K2P distances and Bootstrap percentage, it is evident that *Coelogyne flaccida* belongs to *Coelogyne* section *Flaccidae* seems to be a outgroup. However, another species, *i.e.*, *C. trinervis* belonging to same section shows separately in cluster II. These results indicate that *Flaccidae* appears to be polyphyletic. However, Gravendeel and Vogel (2000) observed that *Flaccidae* is monophyletic but with low bootstrap support. It is evident that *C. ovalis* belonging to section *Fuliginosae* shows close affinity with *Proliferae* and *Crystatae*. The present molecular data indicate that *in* to the genus *Coelogyne* is polyphyletic. These results are in accordance with studies of Gravendeel *et al*. (2001, 2005). *Coelogyne* section *Ocellatae* under which *C. corymbosa* and *C. nitida* placed, forms a single cluster (bootstrap percentage of 50 and 24 respectively) with other species of *Crystatae* and *Lentiginosae* (Fig. 2b). The present molecular results indicate that there is clear species discrimination between *C. corymbosa* and *C. nitida.* The term *Ocellatae* refers to the eye-shaped spots present on the lip of the flower; this section is characterized by having relatively few flowers, glabrous ovary and pedicel, and lateral lobes of the lip with distinct colour patches (Subedi *et al.*, 2011). However, Subedi *et al*. (2011) also stated that it is monophyletic based on DNA sequence data. Whereas *Coelogyne* section *Crystatae* under which *C. cristata* and *C. nervosa* were placed, seems to be polyphyletic. However, it needs further study to ascertain the polyphyletic view of entire genus *Coelogyne.*

The present study also showed that there is a clear species discrimination of *Pholidota articulata* and *P. pallida*, but low bootstrap percentage of 24 and 15 respectively. The genus *Pholidota* invariably showed close affinity with *Coelogyne* supporting thereby the inclusion of genus *Pholidota* in the subtribe Coelogyninae of tribe Coelogyneae.

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