Gene 495 (2012) 10–15

Contents lists available at SciVerse ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Assessment of phylogenetic inter-relationships in the genus *Cymbidium* (Orchidaceae) based on internal transcribed spacer region of rDNA

Santosh Kumar Sharma [a], Jeremy Dkhar [b], Suman Kumaria [b], Pramod Tandon [b], Satyawada Rama Rao [a,*]

[a] Department of Biotechnology and Bioinformatics, North-Eastern Hill University, Shillong (Meghalaya), India
[b] Centre for Advanced Studies in Botany, North-Eastern Hill University, Shillong (Meghalaya), India

ARTICLE INFO

Article history:
Accepted 22 December 2011
Available online 5 January 2012

Keywords:
Cymbidium
DNA barcode
ITS
Phylogeny
rDNA

ABSTRACT

Sequence data obtained from nrITS region were used to assess phylogenetic inter-relationships and infrageneric classification of ten *Cymbidium* species collected from north-east India. The final aligned data matrix of combined ITS 1, 5.8S and ITS 2 yielded 684 characters. The ITS 1 and ITS 2 regions showed variable sequence lengths and G + C content (%). The 5.8S region was found to be more conserved (98.71%) followed by ITS 1 (86.12%) and ITS 2 (69.40%). ITS 2 recorded highest percentage of parsimony informative sites (7.46%), high sequence divergence with indels (24.63%), high number of transitions and transversions. ITS sequence data determined the phylogeny of Asiatic Cymbidiums with high bootstrap values. All three proposed subgenera could be distinguished clearly by all four (MP, ML, NJ, and BI) phylogenetic methods. This study validates the utility of ITS rDNA region as a reliable indicator of phylogenetic relationships, especially ITS 2 as probable DNA barcode at higher levels and can serve as an additional approach for identification of broader range of plant taxa especially orchids.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Sequence data including nrDNA, *matK* gene and *rbcl* gene have been found to be useful for elucidating phylogenetic relationships and taxonomic studies in various plant species (Suh et al., 1993; Baldwin et al., 1995; Ainouche and Bayer, 1997; Goel et al., 2002; Van den Berg et al., 2002, 2005; Singh et al., 2007; Dkhar et al., 2010). Several fragments of the chloroplast genome (*trnH-psbA*, *matK*, *rpoC*, *rpoB*, and *rbcL*) in combinations of two or three regions have also been used as candidate regions with highest potential, however, more representative samples should be examined to choose the best candidate in such approach (Shneer, 2009). After comparison of candidate DNA barcodes viz., *psbA-trnH*, *trnH-psbA*, *matK*, *rbcL*, *rpoC1*, *ycf5*, and ITS), it was authentically concluded that ITS sequences (especially ITS 2) of nuclear rRNA genes may be used as potential DNA barcode in highly variable plants for establishment of molecular phylogenetic relationships at species level (Shneer, 2009; Chen et al., 2010). Several studies have shown that these spacer regions can be used for establishment of phylogeny among closely related taxa, identification of species or strains (Hillis and Dixon, 1991) including *Cymbidium* (Cameron et al., 1999; Van den Berg et al., 2002). Phylogeny can also reveal intricacies of the evolutionary process, leading to the development of hypothesis with respect to morphological adaptation, physiological changes or biogeography (Lazaro and Aguinagalde, 1996). Several studies in Orchidaceae have used DNA sequence data to resolve species phylogenetic relationships and infrageneric classification (Bateman et al., 1997; Cox et al., 1997; Ryan et al., 2000; Van den Berg et al., 2002, 2005; Whitten et al., 2000; Williams et al., 2001). Preliminary analysis of phylogenetic relationship in *Cymbidium* based on sequence data from internal transcribed spacers (ITS) of nuclear ribosomal DNA (Zhang et al., 2002) and plastid *matK* (Van den Berg et al., 2002) has already been reported. Cameron et al. (1999), indicated placement of *Cymbidium* among Maxillarieae/Cymbidieae on the basis of *rbcl* DNA sequences as both of these are paraphyletic. Subsequent phylogenetic relationships based on nrITS and plastid *matK* of *Cymbidium* (Van den Berg et al., 2002) affirmed that two or three subgenera can potentially be defined within the genus with some sectional delimitation and suggested a South-East Asian origin for the *Cymbidium*. Most of these studies have found relatively little genetic variation within the genera and consequently phylogenetic reconstruction at that level was difficult.

In the present investigation, data obtained from the nrITS sequences were used to assess phylogenetic relationships and infrageneric classification of Cymbidiums collected from north-east India for the first time. The nrITS sequence data was used to collect, collate, comprehend, compare and analyze the underlying genetic mechanism leading to speciation and subsequent evolution in the genus *Cymbidium*.

Abbreviations: FISH, fluorescent in situ hybridization; PCR, polymerase chain reaction; kb, kilobase(s) or 1000 bp; nrITS, nuclear ribosomal internal transcribed spacer; rDNA, ribosomal DNA; SPAR, single primer amplification reaction; UPGMA, unweighted pair-group method with arithmetic averages; NJ, neighbor joining; MP, maximum parsimony; ML, maximum likelihood.

* Corresponding author. Tel.: +91 364 2722404; fax: +91 364 2550076.
E-mail addresses: srrao22@yahoo.com, srrao@mail.com (S.R. Rao).

0378-1119/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.gene.2011.12.052

2. Materials and methods

2.1. Plant sampling

Cymbidiums viz. *Cymbidium aloifolium*, *C. elegans*, *C. eburneum*, *C. mastersii*, *C. gigenteum* (syn. *C. iridiodes*), and *C. cyperifolium* collected mainly from Sikkim and Meghalaya province of North-eastern region of India were analyzed using nrITS sequence data. Plant samples of *C. lowianum* were obtained from Dr. U. C. Pradhan, Chairman, Orchid Specialist Group, Government of India. Plant samples of *C. hookerianum*, and *C. devonianum* were obtained from Orchid Research Centre, Government of Arunachal Pradesh, Tipi, Arunachal Pradesh, India. The plant samples of *C. tigrinum* were obtained from authorized nurseries viz. Green Light nursery, Upper Shillong, Meghalaya and International nursery, Kalimpong, West Bengal after necessary identification by orchid experts. The collected plants were grown in greenhouse of Plant Biotechnology Laboratories, Department of Botany as well as Department of Biotechnology and Bioinformatics of North-Eastern Hill University, Shillong. For each species, a minimum of three individuals and more than one population were analyzed.

2.2. DNA extraction, amplification reaction and sequencing

Genomic DNA of *Cymbidium* species was extracted following Sharma et al. (2011). The PCR primers ITS 4 and ITS 5 of White et al. (1990) were used to amplify the ITS region (ITS 1, 5.8S, and ITS 2) utilizing same primers for sequencing. The amplification program consisted of one cycle of initial denaturation at 94 °C for 4 min followed by 30 cycles of 94 °C for 1 min, 55 °C for 3 min and 72 °C for 1 min with final amplification of 72 °C for 7 min. DNA amplification was performed in a thermal cycler system (Gene Amp® 2700 Applied Biosystems). Amplified PCR products were purified using QIAquick gel extraction kit (QIAGEN, Germany) and sequenced at Xcelris Scientific Pvt. Ltd., India.

2.3. Sequence alignment and indel coding

The boundaries of the ITS region for all ten species of *Cymbidium* were determined by comparing published sequences (Van den Berg et al., 2002). *Cyrtopodium andersonii* was selected as out group and sequences obtained were subjected to multiple sequence alignment using Clustal X program (Thompson et al., 1997) with default settings. Clustal X generated alignments were further re-aligned manually. Gaps were included into analysis and coded automatically in a binary matrix using SeqState v.1.21 (Müller, 2005) applying the simple indel coding strategy (Simmons and Ochoterena, 2000).

2.4. Phylogenetic analysis

The sequence characteristics of the ITS region were calculated using MEGA version 4 (Tamura et al., 2007). Neighbor joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) methods were used to analyze the aligned sequence data matrix. All trees were constructed using Phylip (Felsenstein, 2004). Bootstrap analysis was carried out with 999 random seed and 1000 replicates to examine the relative level of support for individual clades on the cladograms of each search (Felsenstein, 1985). For NJ based method, the distance matrix was estimated following the Kimura 2-parameter model (Kimura, 1980). Rate variation among sites for ITS data matrix was estimated using Diverge 2.0 (Gu, 2001).

The Bayesian inference (BI) of phylogeny was also conducted using MRBAYES v.3.1.2 (Ronquist and Huelsenbeck, 2003). BI analysis was performed for 1,000,000 generations applying the default settings (MCMC, two runs with four chains each, heating temperature 0.2, saving one tree every 100 generations). The best model of molecular evolution for the ITS data matrix was determined using jModelTest 0.1 (Posada, 2008). The GTR model of molecular evolution with gamma-distributed rate variation across sites was assigned to the ITS data. The binary (restriction site) model was applied to the indel partition. All trees were viewed with the program Tree View 1.5 (Page, 1996).

3. Results

ITS sequences of ten *Cymbidium* species have been submitted to GenBank databases (www.ncbi.nlm.nih.gov) and can be accessed under accession numbers as referred in Table 1.

3.1. Sequence length and base composition of ten Cymbidium species

The ITS 1 and ITS 2 regions of ten species of *Cymbidium* presently investigated showed variable sequence lengths and G + C content (%). The sequence lengths of ITS 1 for all the ten species ranged from 239 to 244 bp while ITS 2 sequence lengths ranged from 254 to 265 bp (Table 2). All the ten Cymbidiums revealed sequence length of 155 bp for 5.8S region. The G + C content (%) of ITS 1 was found to be slightly higher as compared to ITS 2 region and average G + C content of 70.37% and 69.49% were recorded for ITS 1 and ITS 2, respectively.

3.2. Sequence alignment

For determining sequence statistics among *Cymbidium* species, 245 and 268 characters were aligned for ITS 1 and ITS 2 respectively. The addition of *Cyrtopodium andersonii* (selected as out-group) resulted in an aligned length of 248 and 281 characters for ITS 1 and ITS 2 respectively. The final aligned data matrix of the combination of ITS 1, 5.8S and ITS 2 yielded 684 characters and was used for resolving the phylogenetic relationship among ten Cymbidiums from north-east India (Fig. S1). The multiple sequence alignments for phylogenetic tree reconstruction are available on request from authors.

3.3. Sequence divergence among Cymbidiums

The 5.8S region has been found to be more conserved as evidenced from the number of conserved sites (153 out of 155, 98.71%), followed by ITS 1 (86.12%) and ITS 2 (69.40%). On the contrary, relatively a higher sequence divergence was recorded for ITS 2 (Table 2). Percentage of sequence divergence based on substitution and substitution plus indels was 10.61% and 12.65% for ITS 1; 21.64% and 24.63% for ITS 2 and 1.29% for 5.8S region respectively. The number of synapomorphic sites for ITS 1 was 7 (2.86%) whereas it was 13 (4.85%) for ITS 2. No synapomorphic site was recorded in case of 5.8S region. Autapomorphic sites are more in number for ITS 1 (26, 8.70%) than ITS 2 (15, 5.58%). ITS 2 recorded highest percentage (7.46%) of parsimony informative sites. Both regions recorded frequent transitions rather than transversions. The number of indels for ITS 1 and ITS 2 was 5 and 8 respectively. The high number of total transitions and transversions were recorded in ITS 2 (Table 2).

Table 1
Taxonomic status of *Cymbidium* species investigated and their GenBank accession numbers obtained for sequence data related to complete ITS region.

Species	Subgenus	Section	GenBank accession no.
C. devonianum	*Cymbidium*	Biggibarium	JF729005
C. aloifolium		Cymbidium	JF729014
C. lowianum	*Cyperorchis*	Iridorchis	JF729007
C. hookerianum			JF729010
C. giganteum			JF729013
C. eburneum		Eburnea	JF729012
C. mastersii			JF729006
C. tigrinum		Parishiella	JF729008
C. elegans		Cyperorchis	JF729009
C. cyperifolium	*Jensoa*	Maxillarianthe	JF729011

Table 2
Sequence data analysis of ITS 1, 5.8S and ITS 2 regions in 10 species of *Cymbidium*.

Taxa	Length (nt)	G + C (%)	Aligned length (nt)	Sequence divergence (%)		Number of							Ti	Tv
				Substitution	Substitution + indels	Deletions/ indels	VS (%)	CS (%)	SS (%)	AS (%)	PIS (%)	MSS (%)		
ITS 1														
Cymbidium														
C. devonianum	242	71.9	245	10.61	12.65	1/5	26 (10.61)	211 (86.12)	7 (2.86)	18 (7.35)	7 (2.86)	1 (0.41)	19	6
C. mastersii	241	69.3				2/5								
C. lowianum	241	71.4				2/5								
C. tigrinum	244	68.4				1/5								
C. elegans	240	69.6				3/5								
C. hookerianum	241	70.5				2/5								
C. cyperifolium	241	69.7				2/5								
C. eburneum	241	71.4				2/5								
C. giganteum	240	70.0				3/5								
C. aloifolium	239	71.5				3/5								
Cymbidium* + *Cyrtopodium			248	22.18	26.21	–/10	55 (22.18)	151 (60.89)	6 (2.42)	46 (18.55)	10 (4.03)	3 (1.21)	37	15
5.8S														
***Cymbidium* spp.**	155	60.6–61.3	155	1.29	1.29	0	2 (1.29)	153 (98.71)	0	2 (1.29)	0	0	0	2
Cymbidium* + *Cyrtopodium			155	11.61	11.61	0	18 (11.61)	137 (88.39)	0	18 (11.61)	0	0	13	5
ITS 2														
Cymbidium														
C. devonianum	256	69.1	268	21.64	24.63	3/8	58 (21.64)	186 (69.40)	13 (4.85)	34 (12.69)	20 (7.46)	11 (4.10)	28	19
C. mastersii	256	69.1				4/8								
C. lowianum	265	71.7				2/8								
C. tigrinum	265	70.6				2/8								
C. elegans	256	69.1				4/8								
C. hookerianum	258	68.9				2/8								
C. cyperifolium	257	68.9				2/8								
C. eburneum	257	68.5				3/8								
C. giganteum	257	69.3				3/8								
C. aloifolium	254	69.7				3/8								
Cymbidium* + *Cyrtopodium			281	33.45	38.08	–/13	94 (33.45)	148 (52.67)	19 (6.76)	60 (21.35)	28 (9.96)	15 (5.34)	54	25

Abbreviations: Exact G + C content (%) cannot be accounted for due to the presence of ambiguity codes in sequence data; VS: variable sites; CS: conserved sites; SS: synapomorphic sites; AS: Autapomorphic sites; PIS: parsimony informative sites; MSS: multiple substitution sites; Ti: transitions; Tv: transversions.

3.4. Phylogenetic relationship among Cymbidium species

The NJ, MP, ML and BI methods were used to assess the phylogenetic relationship of the genus *Cymbidium* based on the combined nucleotide sequence data of ITS 1, 5.8S and ITS 2 (Fig. 1). A clear relationship among subgenera is observed in all the trees generated through four phylogenetic methods. Barring BI analysis, all other phylogenetic methods support the placement of subg. *Cymbidium* at the base of the tree followed by a clade comprising subg. *Cyperorchis* and subg. *Jensoa*. The association between the two clades was indicated with varying support. MP and NJ trees resolved members of subg. *Cymbidium* as first clade comprising of two species viz. *C. devonianum* and *C. aloifolium* and collapsed both species into a single group (Figs. 1A and B). All the four trees clearly resolved close relationship of two members of subg. *Cyperorchis* i.e. *C. elegans* (section: Cyperorchis) and *C. mastersii* (section: Eburnea) with support of high bootstrap values which further corroborates earlier reports (Van den Berg et al., 2002; 2005). Interestingly, the position of *C. cyperifolium* (Subg. *Jensoa*, section Maxillarianthe) clade is contradictory as all four methods favor different placement within the phylogenetic tree. Excluding BI analysis based tree, all three methods (MP, NJ, and ML) based trees (Figs. 1A–D), clearly resolved relationship among major clades as well as association between subgenera.

4. Discussion

4.1. Sequence characteristics and molecular analysis

Recently, several molecular phylogenetic studies of Orchidaceae (Cameron et al., 1999, *rbcL*; Freudenstein and Chase, 2001, *nad1b–c* intron; Cameron and Chase, 2000, 18S; Cameron, 2004, *psaB*; Freudenstein et al., 2004, *rbcL* and *matK*) have been carried out. Although extensive samplings of lineages are representatives of the whole family, these studies were mostly based on single target DNA sequence. Besides, studies combining two or three single target DNA have also been published for some tribes, subtribes, or subfamilies (Whitten et al., 2000: Cymbidieae; Pridgeon et al., 2001: Pleurothallidinae; Gravendeel et al., 2001: *Coelogyne* and related genera; Kores et al., 2000: Orchidoideae; Salazar et al., 2003: Cranichideae). Undoubtedly, the greatest contribution in resolving clades was made by the dataset with ITS which can be explained by its greater number of variable sites and their higher rate of sequence divergence including substitutions. The nuclear ribosomal DNA Internal Transcribed Spacers (ITS) seem to be a useful source of information for understanding phylogenetic relationships within genus *Cymbidium*. The levels of variation differed noticeably among the different DNA regions. The two ITS regions, ITS 1 and ITS 2, exhibited greatest number of variable sites with the fastest rate of change (Table 2). These two regions have several folds of variable sites in relation to the total number of sites as compared to 5.8S region. The levels of genetic variation found in *Cymbidium* are somewhat higher than other epiphytic orchids viz. *Cattleya* (Ryan et al., 2000) and *Sophronitis* (Van den Berg et al., 2002). Such variations are apparent in comparatively high number of well supported groups in the ITS phylogeny.

The present findings reveal that ITS 1 and ITS 2 regions of *Cymbidium* have high sequence divergence and G + C content (%). As shown in Table 2, the 5.8S rDNA regions are highly conserved. Most of the variable sites were obtained from ITS 1 and ITS 2 which supports the findings of Lau et al. (2001) on the ITS 2 region among 17 *Dendrobium* species. Although ITS 2 is longer than ITS 1 in sequence length (Table 2), the G + C content in ITS 1 (68.4–71.9%) is relatively higher than that of ITS 2 (68.5–71.7%). Such observations confirm the results of Takaiwa et al. (1985) who reported higher G + C contents in monocotyledon. This is in contradiction to earlier identified angiosperms nrDNA ITS 2 sequences is GC rich (Hershkovitz and Zimmer, 1996).

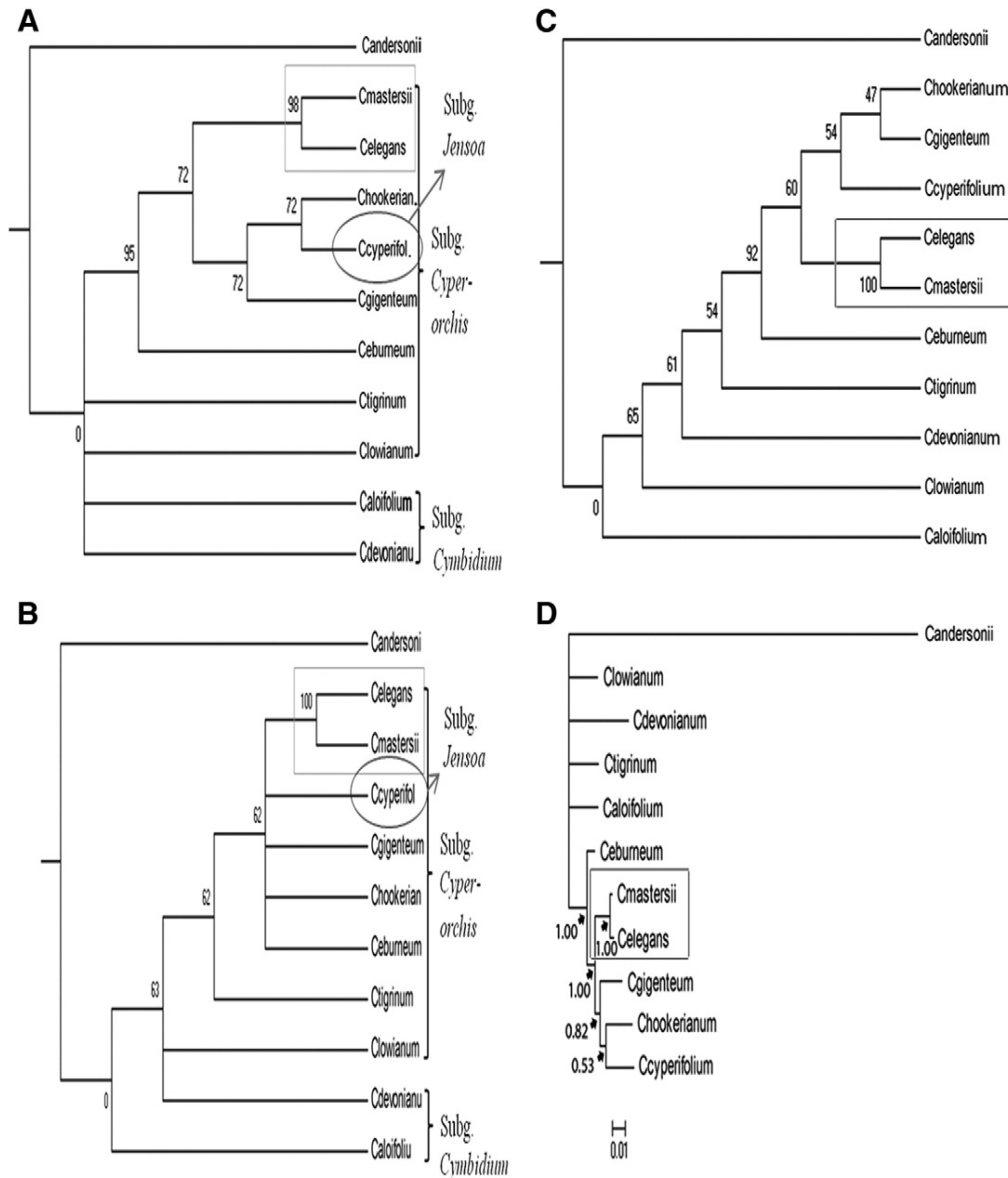

Fig. 1. 50% majority-rule consensus phylogenetic trees for inferring relationships among Indian representatives of the genus *Cymbidium* obtained using four phylogenetic methods viz. (A) maximum parsimony (MP), (B) neighbor joining (NJ), (C) maximum likelihood (ML) and (D) Bayesian inference (BI) of the concatenated ITS 1 and ITS 2 sequence data. BI tree is consensus of 15,000 trees obtained from four runs of Bayesian analysis. Bootstrap values and posterior probabilities are given at the nodes. Branch lengths reflect number of changes per site.

Sequences with more than 50% G + C content (presumed functional ITS paralogs) reported in *Cycas* by Xiao et al. (2010), recorded 64.4% and 65.2% G + C content for ITS 1 and ITS 2 respectively. A case of higher level of sequence divergence in ITS 2 was also observed in present studies which may be due to the fact that substitution and substitution plus indels is relatively much higher compared to ITS 1. ITS 2 of the nuclear rDNA cistron is a widely used phylogenetic marker. Earlier, it was specifically used for low-level inter-specific phylogenetic analyses. However, during last few years, its applications as a marker has been enormously increased for species identification in environmental samples (phylochips) (Engelmann et al., 2009) as a target molecule for barcoding (Ben-David et al., 2007; Moniz and Kaczmarska, 2010; Chen et al., 2010) and for distinguishing species (Muller et al., 2007). Furthermore, it is recently concluded that the rate of successful identification with the ITS 2 was 92.7% at the species level in various plants (Chen et al., 2010).

4.2. Phylogenetic relationship

ITS sequence data clearly resolved the phylogeny of the genus *Cymbidium* with high bootstrap values and all the three proposed subgenera could be distinguished clearly (Du Pay and Cribb, 1988). However some intricacies at sectional level were observed as reported by Van den Berg et al. (2002). The position of *C. mastersii* (subg. *Cyperorchis*, section Eburnea) and *C. elegans* (subg. *Cyperorchis*, section Cyperorchis) is the hallmark feature of the investigation which clustered together in all the methods of analysis. The other member of section Eburnea i.e. *C. eburneum* showed positional differences in the study but did not alter the resolution of the relationship within *C.* subg. *Cyperorchis*. Such differences were also recorded by Van den Berg et al. (2002) too for *C. eburneum* who also opined that this taxon requires additional information which may confirm that *C. mastersii* is sister taxon of *C. eburneum*. Species of subg. *Cyperorchis*

(section Iridorchis) revealed distinguished pattern for *C. hookerianum* and *C. gigenteum*, which followed the general pattern of congruence within section excluding *C. lowianum*. The most plausible explanation for this contrast may be due to several events of hybridization and possible introgression in the group (Van den Berg et al., 2002), which would explain not only the conflicting positions of *C. eburneum* but also a more diffuse and subtler pattern of incongruence persisting in the members of section Iridorchis. Sharma et al. (2010a) also recorded the karyo-morphological variation in these disputed species viz. *C. eburneum*, *C. hookerianum* and *C. mastersii* and proposed high degree of stability in the genus *Cymbidium.* However, the availability of more data is required to determine its exact position within this subgenus and its polyphyletic nature which is lacking for genus *Cymbidium*.

C. tigrinum (subg. *Cyperorchis*; section Parishiella) has been placed alone without any affinity to any of the members of subg. *Cyperorchis* which may be due to its peculiar morphological and climatic characteristics. It is a rare and endangered *Cymbidium* exclusively growing in cooler and dry climatic regions where temperature rises up to 20 °C. It is smallest member of the genus which does not look like a typical *Cymbidium* when it is not flowering with wide leaves and small, round, clustered pseudo bulbs. Such observations were also recorded by Van den Berg et al. (2002) for *C. dayanum* (section Himantophyllum) which is a morphologically abnormal *Cymbidium*. Such apparent clustering of *C. tigrinum* was also observed while studying the genetic variation using three SPARs viz. RAPD, ISSR and DAMD at DNA level (Sharma et al., 2011).

The clade of *Cymbidium* sect. *Cymbidium* comprising *C. aloifolium* and *C. devonianum* is quite distinct being the base of the trees in the present observation, however, position of *C. devonianum* poses problems since it tends to be closer to subg. *Cyperorchis*. Such observation is in conformity with Van den Berg et al. (2002), who on the basis of ITS data indicated that *C. devonianum* is sister to subg. *Cyperorchis*. Some common morphological characters of subg. *Cyperorchis* and *Cymbidium*, especially in *C. devonianum*, are narrow, acuminate leaf margins and shorter seed types which might be the reasons of affinity and close relationship.

Section *Cymbidium* presents several typical anatomical characters, such as stomata within an elliptical cover and slit-shaped pores and a complete layer of subepidermal schlerenchyma cells (Du Pay and Cribb, 1988). The apparent base of the trees formed by *C. aloifolium* may be due to its several unique features. It is a medicinal, cultivated tropical *Cymbidium* and also a biological indicator of tropical environment (Somashekarappa et al., 1996) with very thick and rigid leaves which resemble Aloes. Such apparent clustering of *C. aloifolium* was also observed while studying the genetic variation using three SPARs at DNA level as well as karyo-morphological studies (Sharma et al., 2010a,b, 2011). Furthermore, physical localization of 45S rRNA genes using FISH technique especially in *C. aloifolium*, and *C. tigrinum* (unpublished data, S2) showed decondensed, dispersed and extended form of hybridization signals (probably transcriptionally active), thus demonstrated the heteromorphism in size, intensities and their appurtenance which might be under epigenetic control. An alternative but not mutually exclusive explanation is that habitat as well as climatic conditions may simply allow a greater fixation rate of chromosomal (and other) variation following from founder effect and effective inbreeding occurring within small and endangered populations occupying novel ecological niches.

Cymbidium subg. *Jensoa* (*C. cyperifolium*) which is the lone member of *Cymbidium* with terrestrial habitat is mostly monophyletic. It derives less support and its clade contradiction favors different placement within the phylogenetic tree. This section also possess some defining anatomical characters in the leaves, such as stomatal cover raised above the surrounding epidermis with a circular shaped pore and papillose epidermal cell surface. However, critical appraisal of some more representatives of this section may better resolve its position within subgenus.

Thus, the ITS region of nrDNA appears to be adequate for resolving infrageneric relationships in this group. This study supports the utility of ITS rDNA region as a reliable indicator of phylogenetic relationships, especially the ITS 2 as one of the probable DNA barcodes at higher levels between and within orchid genera. Future studies should be aimed at sampling the remaining species from different climatic zones and adding more DNA regions to enhance the level of internal support of phylogeny. Other coding DNA regions viz *matK*, *rbcL*, *rpoB*, *rpoC1*, and three non-coding spacers viz *atpF–atpH*, *trnH–psbA*, *psbK–psbI* may be coupled with ITS data to serve as unique DNA barcode for orchids.

Supplementary materials related to this article can be found online at doi: 10.1016/j.gene.2011.12.052.

Acknowledgments

The present work is supported by a grant from University Grants Commission, Government of India, New Delhi, through University with Potential for Excellence (UPE) — Bioscience program. We thank the Department of Biotechnology and Bioinformatics, NEHU, Shillong for providing facilities. Authors are thankful to Dr. P.K. Srivastava, Central Silk Board, Dehradun and Prof. R.C. Verma, Vikram University, Ujjain for their valuable contribution in enhancing the quality of literary presentation of manuscript. Sincere thanks are due to Dr. C.S. Rao, Prof. M.S. Bisht and all members of Plant Biotechnology Laboratories, Department of Botany as well as Department of Biotechnology and Bioinformatics, NEHU, Shillong, for their constant encouragement and help.

References

Ainouche, M.L., Bayer, R.J., 1997. On the origins of the tetraploid *Bromus* species section Bromus (Poaceae): insights from internal transcribed spacer sequences of nuclear ribosomal DNA. Genome 40, 730–743.

Baldwin, B.G., Sanderson, M.J., Porter, J.M., Wojciechowski, M.F., Campbell, C.S., Donoghue, M.J., 1995. The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. Ann. Missouri Bot. Gard. 82, 247–277.

Bateman, R.M., Pridgeon, A.M., Chase, M.W., 1997. Phylogenetics of subtribe Orchidineae (Orchidoideae, Orchidaceae) based on nuclear ITS sequences. 2. Infrageneric relationships and reclassification to achieve monophyly of Orchis sensu stricto. Lindleyana 12, 113–141.

Ben-David, T., Melamed, S., Gerson, U., Morin, S., 2007. ITS 2 sequences as barcodes for identifying and analyzing spider mites (Acari: Tetranychidae). Exp. Appl. Acarol. 41, 169–181.

Van den Berg, C., Goldman, D.H., Freudenstein, J.V., Pridgeon, A.M., Cameron, K.M., Chase, M.W., 2005. An overview of the phylogenetic relationships within Epidendroideae inferred from multiple DNA regions and recircumscription of Epidendreae and Arethuseae (Orchidaceae). Amer. J. Bot. 92, 613–624.

Cameron, K.M., 2004. Utility of plastid *psaB* gene sequences for investigating intrafamilial relationships within Orchidaceae. Mol. Phylogenet. Evol. 31, 1157–1180.

Cameron, K.M., Chase, M.W., 2000. Nuclear 18S rDNA sequences of Orchidaceae confirm the subfamilial status and circumscription of Vanilloideae. In: Wilson, K.L., Morrison, D.A. (Eds.), Monocots: Systematics and Evolution. CSIRO Publishing, Melbourne, Australia, pp. 457–464.

Cameron, K.M., Chase, M.W., Whitten, W.M., Kores, P.J., Jarrell, D.C., Albert, V.A., Yukawa, T., Hills, H.G., Goldman, D.H., 1999. A phylogenetic analysis of the Orchidaceae: evidence from *rbcL* nucleotide sequences. Amer. J. Bot. 86, 208–224.

Chen, S., Yao, H., Han, J., Liu, C., Song, J., et al., 2010. Validation of the ITS2 Region as a Novel DNA Barcode for Identifying Medicinal Plant Species. PLoS One 5, e8613.

Cox, A.V., Pridgeon, A.M., Albert, V.A., Chase, M.W., 1997. Phylogenetics of the slipper orchids (Cypripediodeae, Orchidaceae): nuclear rDNA ITS sequences. Plant Syst. Evol. 208, 197–223.

Dkhar, J., Kumaria, S., Rao, S.R., Tandon, P., 2010. Molecular phylogenetics and taxonomic reassessment of four Indian representatives of the genus *Nymphaea*. Aquat. Bot. 93, 135–139.

Du Pay, D., Cribb, P., 1988. The Genus *Cymbidium*. Timber Press, Oregon.

Engelmann, J.C., Rahmann, S., Wolf, M., Schultz, J., Fritzilas, E., Kneitz, S., Dandekar, T., Müller, T., 2009. Modelling cross-hybridization on phylogenetic DNA microarrays increases the detection power of closely related species. Mol. Ecol. Res. 9, 83–93.

Felsenstein, J., 2004. PHYLIP (Phylogeny Inference Package) version 3.6. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.

Freudenstein, J.V., Chase, M.W., 2001. Analysis of mitochondrial *nad1b-c* intron sequences in Orchidaceae: utility of length-change characters. Syst. Bot. 26, 643–657.

Freudenstein, J.V., Van den Berg, C., Goldman, D.H., Kores, P.J., Molvray, M., Chase, M.W., 2004. An expanded plastid DNA phylogenetic analysis of Orchidaceae and analysis of jackknife clade support strategy. Amer. J. Bot. 91, 149–157.

Goel, S., Raina, S.N., Ogihara, Y., 2002. Molecular evolution and phylogenetic implications of internal transcribed spacer sequences of nuclear ribosomal DNA in the *Phaseolus–Vigna* complex. Mol. Phylogenet. Evol. 22, 1–19.

Gravendeel, B., Chase, M.W., De-Vogel, E.F., Roos, M.C., Mes, T.H.M., Bachmann, K., 2001. Molecular phylogeny of *Coelogyne* (Epidendroideae, Orchidaceae) based on plastid RLFPs, matK and nuclear ribosomal ITS sequences: evidence for polyphyly. Amer. J. Bot. 88, 1915–1927.

Gu, X., 2001. Maximum-likelihood approach for gene family evolution under functional divergence. Mol. Biol. Evol. 18, 453–464.

Hershkovitz, M.A., Zimmer, E.A., 1996. Conservation patterns in angiosperm ITS 2 sequences. Nucleic Acid Res. 24, 2857–2867.

Hillis, D.M., Dixon, M.T., 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. Q. Rev. Biol. 66, 411–453.

Kimura, M., 1980. A simple method for estimating evolution rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111–120.

Kores, P.J., Weston, P.H., Molvray, M., Chase, M.W., 2000. Phylogenetic relationships within the Diurideae (Orchidaceae): inferences from plastid *matK* DNA sequences. In: Wilson, K.L., D. A. (Eds.), Morrison, Monocots: systematics and evolution. CSIRO Publishing, Melbourne, Australia, pp. 449–456.

Lau, D.T.W., Shaw, P.C., Wang, J., But, P.P.H., 2001. Authentication of medicinal *Dendrobium* species by the internal transcribed spacer of ribosomal DNA. Planta Med. 67, 456–460.

Lazaro, A., Aguinagalde, I., 1996. Phylogenetic relationships between the wild taxa of the *Brassica oleracea* L group ($2n = 18$) using random amplified polymorphic DNA assay. Sci. Hort. 65, 219–227.

Xiao, L.Q., Möller, M., Zhua, H., 2010. High nrDNA ITS polymorphism in the ancient extant seed plant *Cycas*: incomplete concerted evolution and the origin of pseudogenes. Mol. Phylogenet. Evol. 55, 168–177.

Moniz, M.B., Kaczmarska, I., 2010. Barcoding of diatoms: nuclear encoded ITS revisited. Protist 161, 7–34.

Müller, K., 2005. SeqState: primer design and sequence statistics for phylogenetic DNA datasets. Appl. Bioinformatics 4, 65–69.

Muller, T., Philippi, N., Dandekar, T., Schultz, J., Wolf, M., 2007. Distinguishing species. RNA 13, 1469–1472.

Page, R.D., 1996. TreeView: an application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. 12, 357–358.

Posada, D., 2008. jModelTest: phylogenetic model averaging. Mol. Biol. Evol. 25, 1253–1256.

Pridgeon, A.M., Solano, R., Chase, M.W., 2001. Phylogenetic relationships in *Pleurothallidinae* (Orchidaceae): combined evidence from nuclear and plastid DNA sequences. Amer. J. Bot. 88, 2286–2308.

Ronquist, F., Huelsenbeck, J.P., 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.

Ryan, A., Whitten, W.M., Johnson, M.A.T., Chase, M.W., 2000. A phylogenetic assessment of *Lycaste* and *Anguloa* (Orchidaceae:Maxillarieae). Lindleyana 15, 33–45.

Salazar, G.A., Chase, M.W., Soto Arenas, M.A., Ingrouille, M., 2003. Phylogenetics of Cranichidae with emphasis on Spiranthinae (Orchidaceae, Orchidoideae): evidence from plastid and nuclear DNA sequences. Amer. J. Bot. 90, 777–795.

Sharma, S.K., Kumaria, S., Tandon, P., Rao, S.R., 2011. Single primer amplification reaction (SPAR) reveals inter- and intra-specific natural genetic variation in five species of *Cymbidium* (Orchidaceae). Gene 483, 54–62.

Sharma, S.K., Rajkumari, K., Kumaria, S., Tandon, P., Rao, S.R., 2010a. Karyo-morphological characterization of natural genetic variation in some threatened *Cymbidium* species of Northeast India. Caryologia 63, 99–105.

Sharma, S.K., Rawat, D., Kumar, S., Kumar, A., Kumaria, S., Rao, S.R., 2010b. Single primer amplification reaction (SPAR) reveals intra-specific natural variation in *Prosopis cineraria* (L.) Druce. Trees Struc. Func. 24, 855–864.

Shneer, V.S., 2009. DNA barcoding is a new approach in comparative genomics of plants. Genetica 45, 1436–1448.

Simmons, M.P., Ochoterena, H., 2000. Gaps as characters in sequence-based phylogenetic analyses. Syst. Biol. 49, 369–381.

Singh, A., Devarumath, R.M., Rao, S.R., Singh, V.P., Raina, S.N., 2007. Assessment of genetic diversity, and phylogenetic relationships based on ribosomal DNA repeat unit length variation and Internal Transcribed Spacer (ITS) sequences in chickpea (*Cicer arietinum*) cultivars and its wild species. Genet. Resour. Crop Evol. 54, 810–825.

Somashekarappa, H.M., Narayana, Y., Radhakrishna, A.P., Karunakara, N., Balakrishna, K.M., Siddappa, K., 1996. Bioindicators in the tropical forest of Kaiga environment. J. Environ. Radioactivity 31, 189–198.

Suh, Y., Thien, L.B., Reeve, H.E., Zimmer, E.A., 1993. Molecular evolution and phylogenetic implications of internal sequences of nuclear ribosomal DNA in Winteraceae. Amer. J. Bot. 80, 1042–1055.

Takaiwa, F., Kiyoharu, O., Masahiro, S., 1985. Nucleotide sequence of the 17S–25S spacer region from rice rDNA. Plant Mol. Biol. 4, 355–364.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599.

Thompson, J.D., Gibson, T.J., Plewniak, F., Mongin, F.J., Higgins, D.G., 1997. Clustal X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.

Van den Berg, C., Ryan, A., Cribb, P.J., Chase, M.W., 2002. Molecular Phylogenetics of *Cymbidium* (Orchidaceae:Maxillariae): Sequence data from internal transcribed spacer (ITS) of nuclear ribosomal DNA plastid *matK*. Lindleyana 17, 102–111.

White, T.J., Bruns, T., Lee, S., Taylor, J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H., Sninsky, J.J., White., T.J. (Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, Inc, New York, pp. 315–322.

Whitten, W.M., Williams, N.H., Chase, M.W., 2000. Subtribal and generic relationship of *Maxillarieae* (Orchidaceae) with emphasis on Stanhopeinae: combined molecular evidence. Amer. J. Bot. 87, 1842–1856.

Williams, N.H., Chase, M.W., Fulcher, T., Whitten, W.M., 2001. Molecular systematics of the Oncidiinae based on evidence from four DNA sequence regions: expanded circumscriptions of *Cyrtochilum*, *Erycina, Otoglossum*, and *Trichocentrum* and a new genus (Orchidaceae). Lindleyana 16, 113–139.

Zhang, M.Y., Sun, C.Y., Hao, G., Ye, X.L., Liang, C.Y., Zhu, G.H., 2002. A preliminary analysis of phylogenetic relationships in *Cymbidium* (Orchidaceae) based on nrITS sequence. Acta Bot. Sin. 44, 588–592.