

DNA Sequencing of *Cymbidium* Bicolor Lindl. Using trnL-F Intergenic Spacer

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ABSTRACT: Molecular markers provide a useful method for genotype characterization and determination of the genetic relationship between varieties. A system based on DNA sequences—which is known as DNA barcoding—will choose one or several standard loci which can be sequenced and compared to differentiate between species. In this study, the trnL-F chloroplast plastid sequences were evaluated for the molecular identification of *Cymbidium bicolor*.

KEYWORDS: trnL-F, *Cymbidium bicolor*, Phylogeny, DNA sequencing and Orchids.

I. INTRODUCTION

Cymbidium orchids are the best known and most widely grown of all orchids. The genus *Cymbidium* of the orchid family Orchidaceae, consisting of 52–55 species and divided into three subgenera (*Cymbidium*, *Cyperorchis* and *Jensoa*), is one of the most well-known and desirable orchids in worldwide horticulture because of its aesthetic appeal and ideal characteristics as a house plant [1]. Because of their ornamental and commercial value, *Cymbidium* orchids have been the subject of taxonomic studies and particularly, species identification [5]. Traditionally, the taxonomy, species and cultivars identification of the genus *Cymbidium* is based on the morphological traits. However, the assessment of those traits is very difficult and morphology is affected by environmental factors. Consequently, some species are very difficult to distinguish and the positions of many species in the evolution and taxonomy of *Cymbidium* are difficult to identify.

Molecular methods, such as molecular marker techniques, molecular phylogenetics and DNA barcoding, provide effective information for taxonomy, species identification and phylogenetics. Choi et al., [2] used RAPD markers to investigate the relationships of *Cymbidium*. Obara-Okeyo and Kakoin [3] identified *Cymbidium* cultivars using RAPD markers. Wang et al., [9] reported the cultivar identification in *C. ensifolium* using ISSR markers. Van den Berg et al., [7] used ITS and matK to elaborate the phylogenetic relationships of *Cymbidium*. Sharma et al., [6] assessed the phylogenetic inter-relationships of *Cymbidium* using ITS. Most of these studies revealed that a limited number of DNA sequences led to relatively little genetic variation within genus *Cymbidium* and therefore phylogenetic resolution and species identification were very difficult. There are many advantages to the chloroplast genome in contrast to the nuclear genome such as haploid, maternal inheritance, single structure, gene content and genome structure high conservation [10]. Parks et al., [4] used chloroplast genomes to increase phylogenetic resolution at low taxonomic levels. The whole cp genomes analyses could contribute to species identification, especially in cultivars identification and population-level studies. Cultivars identification plays an important role in the commercial development of orchids. Phylogenomic analyses based on whole cp genomes will light the way on cultivars identification of *Cymbidium*. These studies had utilized many loci from the plastid genome viz., atpB, rbcL, matK, psaB, trnL-F; one, nad1 intron from the mitochondrial genome, and 26S rDNA and ITS from the nuclear genome. Van den Berg et al., [8] assessed phylogenetic relationships within the *Epidendroid* orchids with emphasis on tribes Epidendreae and Arethuseae using

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combined multiple DNA sequences from nrITS and four plastid regions viz., trnL intron, trnL-F spacer, matK (gene and spacers) and rbcL.

II. MATERIALS AND METHODS

Location and study area

Plant materials were collected from Yercadu, a part of Eastern Ghats, South India is a rich biodiversity hotspot of representing a great aesthetic treasure as well as a grand repository of biological wealth. *Cymbidium bicolor* was collected during February- March 2015 at an altitude of 80-869 m above Mean Sea Level (MSL). The mean temperature during the study period was 21 ± 2 ° C. The leaf sample from the plant was randomly cut off with an ethanol-disinfected sickle and placed separately in sterile polythene bags to avoid moisture loss. The materials were transported to laboratory within 12 h and stored at 4 ° C until isolation procedures were completed.

DNA isolation, PCR amplification and sequencing

DNA was extracted from leaf samples of the plant using Ultra pure genomic DNA preparation Kit (Genei, Bangalore). The chloroplast DNA region of the sample was amplified using trnL-F plastid in 50 µl reaction volumes, each containing 26.6 µl of sterile distilled H₂O, 5 µl of 10X Taq buffer, 2 µl of 25 mM MgCl₂, 1 µl of 10 mM dNTPs, 2.5 µl of trnL-F primer, 0.33 µl of Taq DNA polymerase and 5 µl of extracted genomic DNA. The PCR mixture underwent initial denaturation at 96 ° C for 1 min, denaturation at 96°C for 10 sec, elongation at 72°C for 5 sec and final extension at 72 ° C for 10 min. Direct DNA sequencing was performed using the primer trnL-F on an ABI 3100 automated sequencer following the manufacturer's instructions (Applied Biosystems, Inc.) at Xcelris Labs Limited, Ahmedabad.

DNA sequence assembly and alignment

The forward and the reverse sequence of the *Cymbidium bicolor* were obtained (fig.1). The forward and the reverse sequences were aligned and the consensus was obtained. The sequences were subjected to BLAST and Phylogentic analysis was done (fig.2).

III. RESULTS AND DISCUSSION

Fig. 1: Sequence data: The sequence data obtained by both forward and reverse primer are presented below:

Forward Primer data

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1 AAAACAAATT TCATAGTCCT TTTTATTTAT CCCCTCCTCT TTCGTTTTTT TTTTCCCTGG
61 GGGGAGCTCA TTTGAAGTCA CGGAAATATA TTTCTATCTC CCTTCGCACG GTCCTTTGGC
121 AAAGGGGAGA AAAAAAAAAAT CCTCGTTCCT TTCCCCCCCC CATTCCCTTT TGTTTTGTAT
181 TTTTTTGGAA AATCCAAAAG GAACCTCCAT TTCGAATTAT TTTCCGGGCT GCACCTTTTT
241 GGAATTATTT AAAGTCAAAA TCTTTTTTTT TACTTTTACA AACAAAGAAG GTCTTCTGTT
301 AGAAGATTTA AAAAATTCAT GGCCATTTTT TTAATTGTCC GGCAAAAATG AGGCCGGGGA
361 AAGGGACGGG ACAGCTAAGT TGGGAAAGCA GAGGATGGAA AACCCCGGG CCCCAGAAA
421 AAAAAAAAAA
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Reverse primer data

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1 ACCTACGGCT AGTAACCTCT GTAGCTTCCT GACCTTCCG GTGCATCATC TTAGCAAAT
61 ACTTATATCT ATTTCAATGA AATGAACAGA TCTTCAAAAA TAACACTTTC TTTGTTTGCC
121 ATGTAAGTAA AAAAATTTGG CCTCTAAATA ATTCAATAGC GGAGATTCTT TGAACATACT
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181 TGAACATTTT GATTCTTTAT GTTTTATACC TTGAATACAT AGCATATGAG ATTGGGTTGG
241 AAAAAGATAC TAAGATTTCT ATTCGGATCC CTTTGTGAAA CAACAGATTG CATGAAATGA
301 GAAAAATATT TCAATTTGTT TAAACTGATC TCCACTGATG ATAAAAAAAA AAAACAGGGG
361 AAGGGGAAGA AACAAGAAGG GGGGAAGTAA AGGGGGCTTT TTTTGGGGGA GGGGGGGTCT
421 GGCCCAAAT GGACTTGAAC CAACTAAAAC CA

Figure 1 gives the forward and reverse sequence of the trnL-F intergenic spacer region and it was subjected to BLAST analysis in the NCBI. The similarity of the sequence was obtained and the highest similar sequences were obtained the BLAST. The phylogenetic tree was obtained and the maximum similarity was shown with the *Cymbidium mannii*.

Fig. 2: Phylogenetic tree based on Fast Minimum evolution Method (NCBI)



The phylogenetic tree obtained for the *Cymbidium biclor* based on trnL-F intergenic spacer sequence is presented in figure 2. Thus trnL-F intergenic spacer sequence does not provide enough information for varietal identification. It is suggested to go for other chloroplast DNA markers. However since trnL-F data is highly conserved proves good for similarity searches.

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IV. CONCLUSION

Organelle genome sequencing is becoming a valuable way for improving resolution in phylogenetic studies with the rapid development of biotechnological sequencers, especially at low taxonomic levels. It also could promote population genetic studies and species identification. Closely related species can be distinguished based on information about deletions, insertions, and substitution of nucleotide bases in the trnL-F region. The process and pattern of these events are important for estimating species divergence and are phylogenetically informative. The analysis of cpDNA sequences in the *Cymbidium bicolor* opens up a novel interpretation of relationships among the Orchidaceae species. This allows for more useful taxonomies to be generated.

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