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MOLECULAR CHARACTERIZATION OF MYCORRHIZAL FUNGI ISOLATED FROM THE ROOTS OF *CYMBIDIUM SPECIES*

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ABSTRACT

Orchidaceae is the largest flowering plant family which consists of nearly 25000 species. Symbiotic association is found in orchids association with the endophytic fungi in their roots. It is important to find the fungi which play an important role in orchid germination and seedling development. In the present study, the fungi from the roots of *Cymbidium* species are isolated. DNA isolation of the cultured fungi was done. The isolated genomic DNA was amplified using internal transcribed spacer region and LSU region. The new DNA sequences were identified at genus and species level using BLAST software tool (NCBI, USA) and deposited in NCBI. The DNA sequences that shown maximum similarity to query sequences were selected and analysed using MEGA 6.0 (Molecular Evolutionary Genetics Analysis) for phylogenetic analysis. The phylogenetic tree was generated based on Neighbour joining method.

KEYWORDS: ITS, LSU, *Cymbidium bicolor*, *Cymbidium aloifolium*, molecular identification and *Ceratobasidium species*.

INTRODUCTION

The Orchidaceae is the largest and most diverse plant families, distributed worldwide (Dressler *et al*., 1993). Orchidaceae represents the largest flowering plant family with more than 27,135 accepted species (The plant list, 2010). Symbiotic mycorrhizal fungi association is seen in all members of the family (Rasmussen, 1995). Mycorrhizal association is known to be important to orchids because they depend on the presence of suitable fungal partners for

seed germination and seedling development (Rasmussen *et al*., 2002). Orchid mycorrhizas are morphologically different from other mycorrhizas and involve a phylogenetically distinct group of soil fungi. The mycorrhizal association is ubiquitous but very important symbiosis in nature, which plays an essential role in the maintenance of most terrestrial ecosystems (Smith and Read, 2008). Therefore, a complete understanding of the mycorrhizal fungi of the many threatened orchid species is required for conservation action plans.

Molecular taxonomic identification of the endophytic fungi of orchid species has now revealed, the diversity of orchid associates is much complex (Tondello *et al*., 2012). The *Rhizoctonia*-like fungi includes members of the *Ceratobasidiaceae*, *Sebacinales* and *Tulasnellaceae* (Yukawa *et al*., 2009). DNA barcoding is a novel technique which enables species identification and taxonomic classification using a short and standard DNA sequence (Huang *et al*., 2013). ITS of the nuclear DNA is one of the most popular loci in systematic and phylogenetic studies. About 400-800bp of ITS, makes it easier for sequencing and provide sufficient discrimination power among the species (Dentinger *et al*., 2011).

The aim of the present investigation is to collect the *Cymbidium aloifolium* and *Cymbidium bicolor* root samples, isolate and culture the associated fungi from it. The cultured fungi were identified using sequencing and phylogenetic analysis.

MATERIALS AND METHODS

Collection of plant samples

The plant species belong to the *Cymbidium aloifolium (L.) Sw.* and *Cymbidium bicolor (Lindl.)* roots were from different locations of Eastern ghats. Samples from each accession will be randomly cut off with an ethanol-disinfected sickle and placed separately in sterile polythene bags to avoid moisture loss. The collected species were taken care not to damage the roots for the culture of orchid mycorrhizae and the materials are transported to laboratory within 12h and stored at 4 ° C until isolation procedures were completed.

Culture of endophytic fungi

The collected samples are washed thoroughly with sterile distilled water and air dried before they are processed. The root were surface sterilized by immersing them sequentially in 70% ethanol for 3min and 0.5% Sodium hypochlorite for 1min and rinsed thoroughly with sterile distilled water. The excess water is dried under laminar airflow chamber. Then, with a sterile scalpel, outer tissues are removed and the inner tissues of 0.5cm size are carefully dissected

and placed on petriplates containing Potato Dextrose Agar. The media are supplemented with streptomycin sulphate (100mg/L) to suppress bacterial growth. The plates are then incubated at 25±2 ° C until fungal growth appeared. The plant segments are observed once a day for the growth of endophytic fungi. Hyphal tips growing out the plated segments were immediately transferred into PDA slant and maintained at 4°C (Athipunyakom *et al*., 2004).

Molecular characterization of fungal isolates

Identification of fungal endophytes had been done using DNA sequencing. Fungal endophytes are cultivated on PDA Broth (Himedia) by placing agar blocks of actively growing pure culture (3mm in diameter) in 250ml Erlenmeyer flasks containing 100ml of the medium. The flasks were incubated at 25±2 ° C for 3 weeks with periodical shaking at 70 rpm. After the incubation period, only the cultures actively growing in PDA Broth were taken out and filtered through sterile cheese cloth to remove the mycelial mats. A modified fungi DNA extraction method was used to isolate DNA from fungi (Aamir *et al*., 2015). The sequencing reaction was performed with ABI big dye cycle sequencing terminator reactions (Applied Bio systems) at Eurofins Genomics, Bangalore.

Data analysis

The new DNA sequences were identified at genus and species level using BLAST software tool (NCBI, USA). The sequences were deposited in the NCBI. The DNA sequences that shown maximum similarity to query sequences were selected and analyzed using MEGA 6.0 (Molecular Evolutionary Genetics Analysis) for phylogenetic analysis. The phylogenetic tree was generated based on Neighbour Joining Method.

RESULT AND DISCUSION

Fungal Isolation from *Cymbidium aloifolium* and *Cymbidium bicolor*

Plate 1 shows the isolates of fungi species grown on PDA medium were white when young but after three weeks ranged from brown to light brown. Mycelium was floccase in early stages of growth, but as culture aged, mycelia became increasingly apparent to the agar surface often into large clumps. Mycorrhiza associated with orchids are basically endophytic and the fungi which form mycorrhizal associations belong to the group *Armillaria, Ceratobasidium, Erythromyces, Moniliopsis, Mycena, Russulaceae, Serendipita, Thanatephorus* and *Tulasnella*.

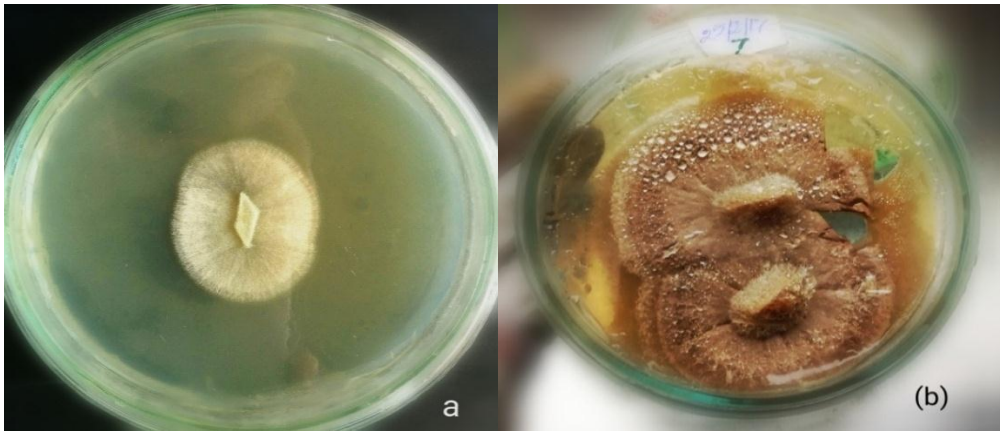

Plate 1: Cultured Mycorrhizal Fungi from the roots of *Cymbidium aloifolium* and *Cymbidium bicolor.*

DNA Isolation, Amplification and Sequencing

DNA isolation of cultured fungi was carried out successfully and purified using Sodium acetate- ethanol precipitant. The isolated genomic DNA was amplified using PCR and the amplicons were obtained approximately at the length of 500 - 800bp for the ITS and LSU region. The amplicons were eluted and sequenced using forward and the reverse primers and the sequence chromatograms were successfully obtained.

Phylogenetic Analysis

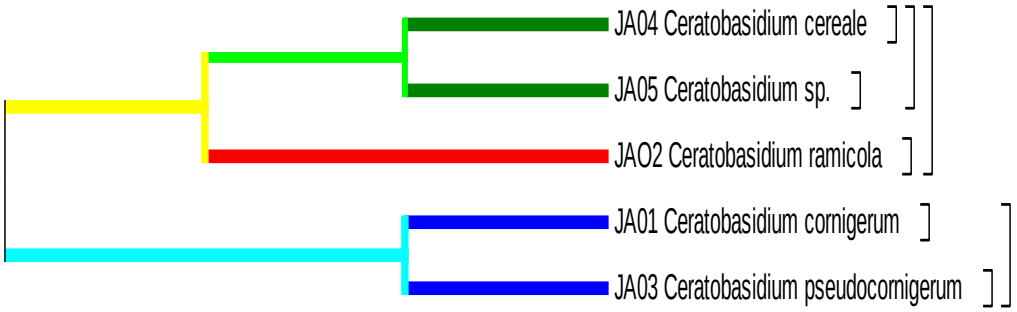

Figure 1: Evolutionary relationships of taxa.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura Nei and Kumar, 2004) and are in the units of the number of base substitutions per site. The analysis involved 5 nucleotide sequences. Codon positions included were

1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 334 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al*., 2013).

Both morphological and molecular studies of orchid mycorrhiza reveal that many terrestrial orchids have extremely specific associations often with fungi from a single teleomorph (Shefferson *et al.,* 2007). Much research needs to be done to unravel the underlying mechanism of mycorrhizal associations in epiphytic orchids which may help to prevent their decline and extinction in nature.

CONCLUSION

Mycorrhizal fungi were successfully isolated from the roots of *Cymbidium aloifolium* and *Cymbidium bicolor* belonging to different accessions from the regions of Eastern Ghats. From the cultured fungi DNA was isolated, amplified and sequenced using ITS region and large subunit region. The phylogenetic tree obtained by MEGA 6.0 suggests that the five fungi isolated from different accession are *Ceratobasidium* species and are closely related. The isolated mycorrhizae from *Cymbidium aloifolium* and *Cymbidium bicolor* which has genus specificity but the species are not specific.

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