SPECIFICITY OF MYCORRHIZAL FUNGI ISOLATED FROM THE ROOTS OF *VANDA TESTACEA* FROM FIVE DIFFERENT REGIONS USING DNA BARCODING

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ABSTRACT: Orchidaceae is one of the longest families of flowering plants which consist of nearly 25000 species. Vanda testacea is an epiphytic perennial which is occurring from the Indian subcontinent to Indochina at the elevations of 500 to 2000 meters. Mycorrhizal association of orchids is essential because for its seed germination and seedling development. The present study is carried out to identify the specific mycorrhizal fungi from the roots of Vanda testacea from five different locations using DNA barcoding. Two loci used for this purpose were Internal Transcribed Spacer region and Large Subunit region. The new DNA sequences were identified 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. It shows the similarity in the mycorrhizal fungi identified from five different locations of Vanda testacea.

KEYWORDS: Vanda testacea, Mycorrhizal Specificity, Phylogeny, Tulasnella species, DNA barcoding

INTRODUCTION

Orchidaceae is the one the longest families in flowering plants in the world. It consists of nearly 25000 species. Orchids have three main growth habitat; soil dwelling (terrestrial), on other plants (epiphytic) and on rock surfaces (lithophytic) (Smith and read, 1997). *Vanda testacea* is one among the species which is epiphytic in nature mainly known for its medicinal properties. Almost all plant parts (roots, leaves, flowers) in powder form or as an extract are used as herbal medicines to cure rheumatism, bronchitis, nervous disorders, piles, and inflammations as well as a potential anti-cancerous drug (Chauhan,1990). Orchid mycorrhizas are morphologically different from other mycorrhizas and involve a phylogenetically distinct group of soil fungi. 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 taxonomic method that uses a short genetic marker in an organism's DNA to identify it as belonging to a particular species. 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 present study involves the collection of *Vanda testacea* root samples from five different regions, isolation of mycorrhizal fungi from its roots and find its specificity using DNA barcoding by two different loci (ITS and LSU).

MATERIALS AND METHODS

Collection of plant samples

The plant species *Vanda testacea* roots were collected from five different locations Iduki, Kodaikannal, Kolli hills, Shevaroy hills and Sirumalai. Samples 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 were 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 3minutes and 0.5% Sodium hypochlorite for 1minute 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, Oat meal Agar and Corn Meal 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 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.

RESULTS AND DISCUSION

Fungal Isolation from the roots of *Vanda testacea*

Almost all the fungal hyphae were isolated from the dark incubated plates, rather than the light dark resime plate. Plate 1 shows the mycorrhizal isolates of fungi species grown on PDA, OMA and CMA were white and lack spores. Mycelium was floccase in early stages of growth, but as culture aged, mycelia became increasingly apparent to the agar surface often into large clumps. Nearly, 10 mycorrhizal endopyhtes and 13 non- mycorrhizal fungi were isolated from the roots of *Vanda testacea*. Mycorrhizae associated with orchids were basically endophytic in nature. The fungi which form mycorrhizal associations belong to the group are *Armillaria*, *Ceratobasidium*, *Erythromyces*, *Moniliopsis*, *Mycena*, *Russulaceae*, *Serendipita*, *Thanatephorus* and *Tulasnella*. The study of Tan *et al.*, 1999 showed that there is important role of the fungi on seed germination of *Spathoglottis plicata*. However, Hayakawa *et al.*, 1999 showed different results, there was no significant effect of the isolated fungus on in vitro seed germination.

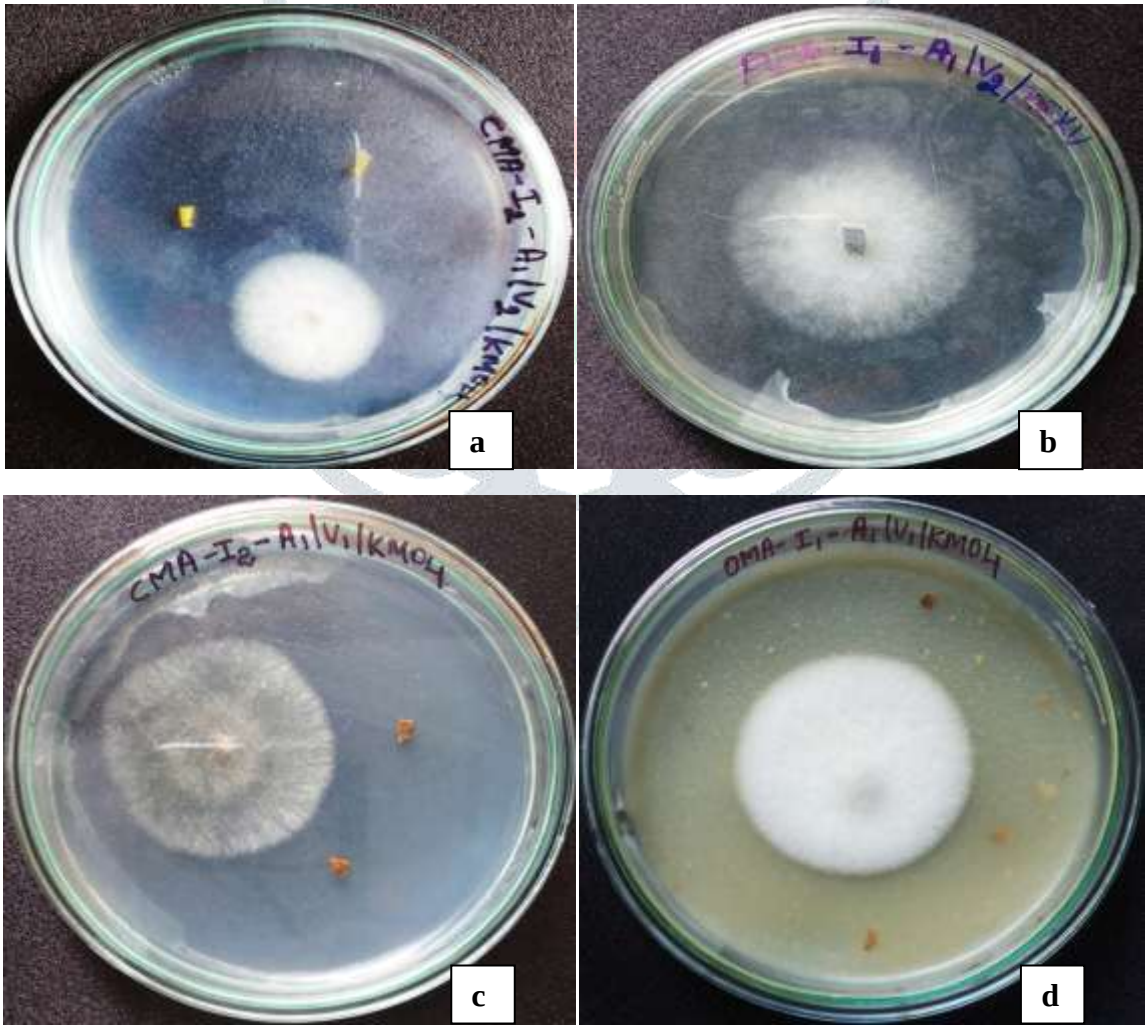

Plate 1: Cultured Mycorrhizal Fungi from the roots of *Vanda testacea* on PDA, OMA and CMA.

DNA Isolation, Amplification and Sequencing

Since the fungus was sterile it could not be identified morphologically and by microscopic observation because it did not produce any spore. 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. The sequences were deposited in the NCBI Gen bank and the accession numbers were obtained as MH468786 and MH479401-MH479405.

Phylogenetic Analysis

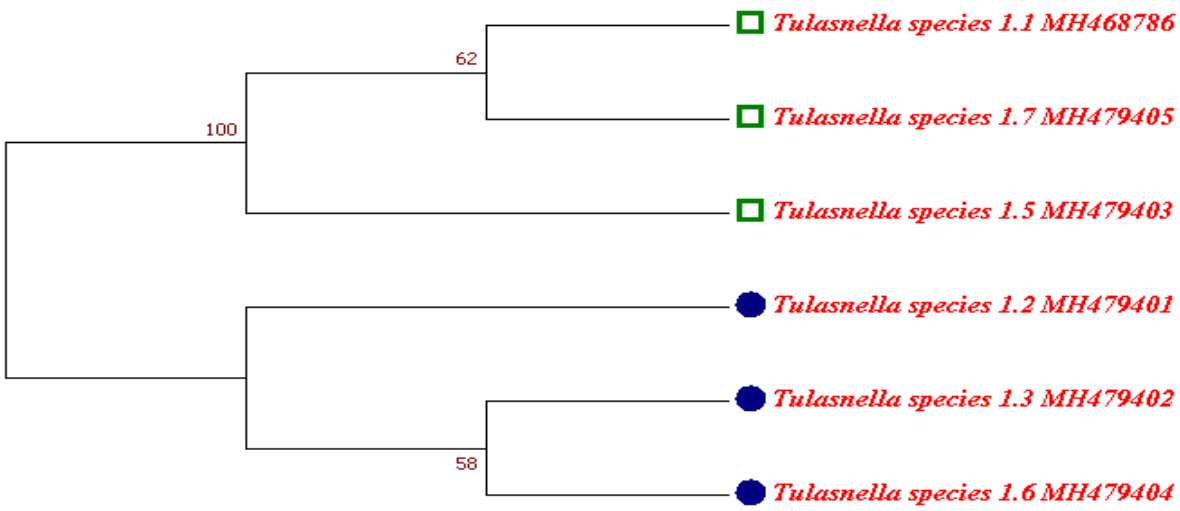

Figure.1 Evolutionary relationships of taxa.

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 2.95755627 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). 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 6 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 361 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). The same type of methodology was also followed by Kasmir *et al*., 2011 for identifying the fungal endophytes. They also reported the ITS region of NCBI data base was compiled and the closest similarities was showed the fungus species. Furthermore, they also reported that, molecular identification had been useful in precisely screening fungal endophytes than relying on microscopic features alone. 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 *Vanda testecea* from five different regions. 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 *Tulasnella species* which are closely related. The isolated mycorrhizae from *Vanda testecae* are specific to it plant and help in the plant growth and seedling development.

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