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Molecular Identification of Endophytic Fungi isolated from *Rhynchosia beddomei*, An Endemic Medicinal Plant of Tirumala Hills.

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ABSTRACT

The present research reports the isolation of fungal endophytes from the mature leaves of *Rhynchosia beddomei*, an endemic medicinal plant of Tirumala hills. Out of seven endophytic fungi isolated, four were identified phenotypically viz., *Aspergillus flavus*, *Alternaria alterata*, *Pestalotiopsis spp*, *Colletotrichum gleosporiodes*. An another isolate was characterized at molecular level using fungal specific 18S rRNA primers and identified as *Aspergillus japonicus*.

Keywords: Endophytic fungi, *Rhynchosia beddomei*, *Aspergillus japonicus*, Molecular identification, 18S rRNA analysis,

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INTRODUCTION

Endophytes are microorganisms (e.g. fungi and bacteria) that live symbiotically for all or part of their life cycle inside the healthy tissues plant without causing any visible sign of infection [1]. It has also been shown that some fungal endophytes can produce various bioactive compounds which influence the biomass yield and production of bioactive compounds in plants [2,3] and also play an important role in recycling of nutrients in natural ecosystems [4,5]. Therefore, understanding how fungal endophyte communities differ in species composition and tissue or host preference is critical to know worldwide fungal species diversity, the evolutionary context of endophyte-plant associations, and ecosystem functioning [6]. *Rhynchosia beddomei* Baker (Fabaceae, vernacular name - adavivuluva) is a rare and endemic medicinal plant distributed in Seshachalam hills of Eastern Ghats of Andhra Pradesh, India [7]. The leaves of *R. beddomei* were used for wounds, cuts, boils and rheumatic pains [8]. The present investigation was taken up to isolate and characterize endophytic fungi from *Rhynchosia beddomei*.

MATERIALS AND METHODS

Plant Material Collection

Rhynchosia beddomei Plant material was collected from Tirumala Hills of seshachalam range falling under the Eastern Ghats of India. Plant materials was confirmed with taxonomist, Department of Botany, Sri Venkateswara University, Tirupati, and Andhra Pradesh, India. Fungal endophytes were isolated within 24 h after sampling.

Isolation of Endophytic Fungi

For isolation of endophytic fungi, mature leaves of *Rhynchosia beddomei* were collected. Leaves were first washed thoroughly with running tap water and then with liquid detergent Labolene. Thereafter, leaves washed thoroughly with sterile double distilled water. Then leaves were transferred into a laminar air flow (LAF) chamber. In the LAF chamber, leaves were first surface sterilized with 80% ethanol (v/ v) for 30 s followed by thorough washing with sterile double distilled water. Finally, leaves were surface-sterilized with 10% hydrogen peroxide for 5 min and then followed by thorough washing with sterile double distilled water. Such surface-sterilized leaves were then cut into small pieces of 2x2 cm size and were injured with sterile surgical blade prior to its inoculation. Leaf pieces were aseptically transferred to Petri dishes containing agar-agar (2%) solidified Potato Dextrose Agar medium (PDA). Plates were incubated at $24\pm 2^{\circ}\text{C}$ for 10 days. After 10 days, fungal mycelia that grew on the surface of the PDA medium were picked and transferred onto PDA plates. Pure fungal cultures were then identified by 18S rRNA gene amplification and sequencing.

Phenotypic identification of Fungal Endophytes

The endophytic fungal isolates were identified microscopically on the basis of their critical morphological structure such as growth pattern, hyphal features, arrangement of spores and further by staining with lactophenol cotton blue.

Molecular Characterization of Fungal Endophytes

DNA Extraction

The fungal mycelia grown on PDA were harvested from the surface and placed into 1 ml sterilized double distilled water and frozen in liquid nitrogen. The mycelia were mechanically disrupted using mortar and pestle. The extraction and purification of DNA were performed according to the method described by dellaporta [9].

PCR Amplification and Sequencing of 18S rRNA gene

For PCR amplification of gene coding for the small ribosomal subunit (18S rRNA gene) of fungi, the fungal domain specific ITS1 and ITS4 primers were used. Amplification was performed in CG palmcycler. The amplification products were verified by electrophoresis in 0.8% w/v agarose gel. The amplified ITS products were purified using quick gel extraction kit. Sequencing was done at MWGAG Biotech, Bangalore, India. Sequences were compared with consensus sequence data from public databases Genbank (<http://www.ncbi.nlm.nih.gov>) by using the BLAST N sequence match routines. The sequences were aligned using the CLUSTAL X program [10] and phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4.0 [11]. The phylogenetic reconstruction was done using the neighbor joining (NJ) algorithm, with bootstrap values calculated from 1,000 replicate runs, using the software routines included in the MEGA software.

RESULTS

Phenotypic Identification of Fungal Endophytes

Sporulating structures of fungi were considered as diagnostic features for identification of endophytes. Sporulating isolates such *Aspergillus flavus*, *Alternaria alternata*, *Pestalotiopsis spp*, *Colletotrichum gleosporioides* were showed in Figure 1. The colonies of *Aspergillus flavus* an ascomycete, appeared like powdery masses of paleyellow-green color spores. Hyphae are septate, hyaline, growth pattern is usually threaded like and thick mycelia mats were seen. *Alternaria alternata*, an ascomycete, formed whitish brown colony on the growth medium. Conidia are yellow brown or pale brown in color, appeared in a chain consisting 5-8 conidia. *Pestalotiopsis spp*, an endophytic fungus significant in its function i.e. capable of biodegradation of plastics. The colonies are brownish red in color. *Colletotrichum gleosporioides*, a deuteromycete produced one- celled, hyaline, oblong, slightly curved conidia.

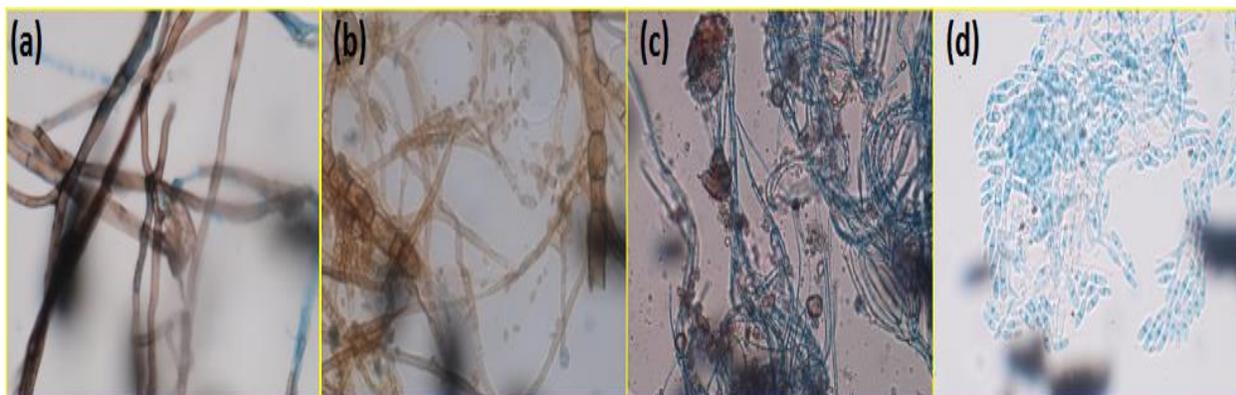
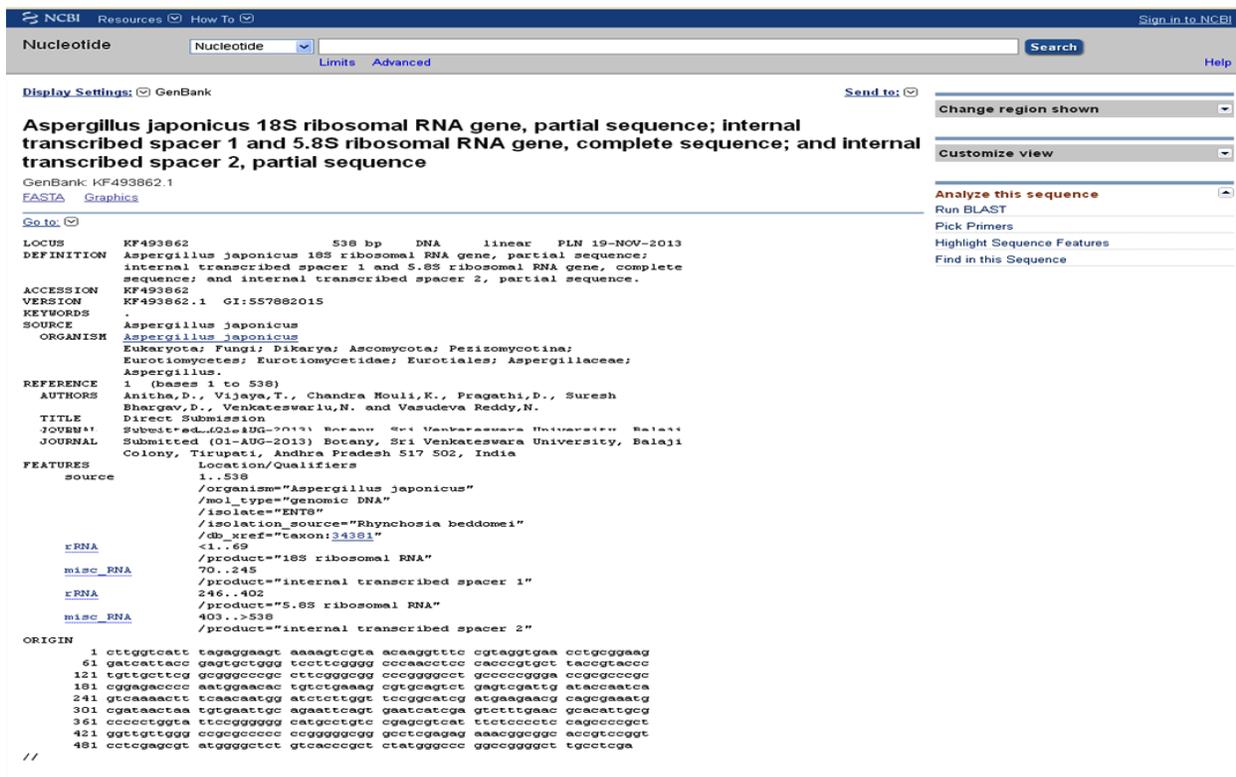


Figure 1: Sporulating endophytic fungal isolates from *Rhynchosia beddomei* (a) *Aspergillus flavus* (b) *Alternaria alternata* (c) *Pestalotiopsis spp* and (d) *Colletotrichum gleosporioides*

Molecular Characterization of Fungal Endophytes:

Non-sporulating strains were confirmed using 18S rRNA sequencing by using ITS-1 and ITS-4 primers. Based on BLAST search of ribosomal RNA gene sequence, the endophytic fungi were found to be closest homolog of *Aspergillus japonicus* and submitted to the NCBI Genbank with accession no. KF493862 (Figure 2). Phylogenetic tree was constructed based on the closest relationship with consensus sequences and shown in figure 3.



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Aspergillus japonicus 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and 5.8S ribosomal RNA gene, complete sequence; and internal transcribed spacer 2, partial sequence

GenBank KF493862.1
FASTA Graphics

Go to: LOCUS KF493862 538 bp DNA linear PLN 19-NOV-2013
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ACCESSION VERSION KF493862 GI:557882015
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ORGANISM *Aspergillus japonicus*
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REFERENCE 1 (bases 1 to 538)
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Figure 2: Sequence of *Aspergillus japonicus* submitted to NCBI

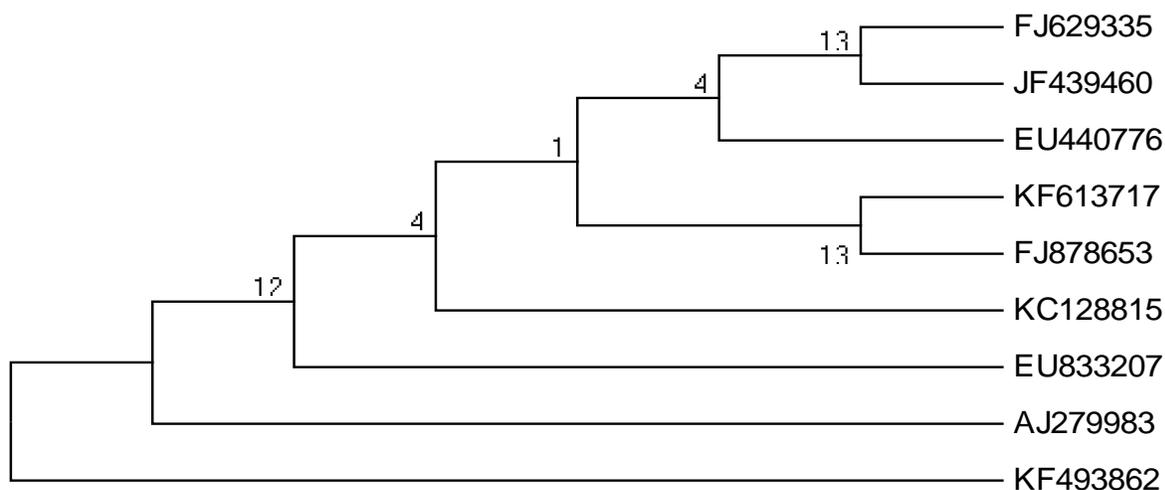


Figure 3: Phylogenetic tree of *Aspergillus japonicus* constructed with MEGA 4 software using the neighbor joining method

DISCUSSION

Endophytic fungi are of highly diverse and intriguing group of microorganisms spend the whole or part of their lifecycle colonizing inter or intracellularly in the healthy tissues of the plant typically causing no apparent symptoms of disease. These are considered as alternative of plant secondary metabolites as they are producing bioactive compounds of pharmaceutical applications such as antibiotics, antioxidants, anticancer and antiviral activities. And hence there is a need for isolation of endophytes and their exploitation at industrial level for producing pharmaceutically important bioactive compounds. In the present study five endophytic fungi from *Rhynchosia beddomei* were isolated and identified. Four isolates were identified based on morphology of the fungal colony, hyphal growth pattern, conidia. Three belongs to ascomycota and one belongs to deuteromycete and suggested the high diversity of ascomycota group fungi. The other isolate was identified based on 18s rRNA sequencing. Due to the high diversity of endophytes, many studies characterized the endophytic fungi using DNA sequences. The preferred nucleotide marker has been ITS because of extensive database in NCBI GenBank. In addition, the ITS region is easy to amplify and, in many cases, provides enough variation to identify species [12, 13]. Many endophytic sequences in NCBI GenBank were remain unidentified. For example, when querying the NCBI nucleotide database using the keyword “endophyte”, almost 50 % were unidentified, labelled as “fungal endophyte”, “uncultured endophytic fungus”, or similar. Many others were identified to family or to genus, but few were identified to species.

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