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Molecular Detection of Endophytic, *Myrothecium* spp. by ITS-Sequencing Technique

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Abstract: *Endophytic fungi are microorganisms with immense production secondary metabolites having biological activity features, which exist within plants without any hazard for their living. Ten isolates of endophytic Myrothecium verrucaria species, recovered from bark and twigs of Garcinia spp. in order to detection and identification of this fungus plant, and to analyze their genome fingerprints with help of ITS-sequencing. ITS region is one of the most famous nuclear DNA sequences for genetic inference at the generic and genus levels in fungus. Hence, the target regions of rDNA, ITS 1, ITS 2 regions and 5.8S gene were amplified using primers ITS 1 and ITS 4. In this research, the phylogenetic evolutionary relationship of the desired endophyte were evaluated from the sequences of the ITS region. The amplification and subsequent sequencing of highly conserved ITS region showed genetic similarity among 10 isolates of Myrothecium spp (M), and based on the polymorphisms of the ITS region, the 10 isolates gave a length of ~ 550 bp amplicon.*

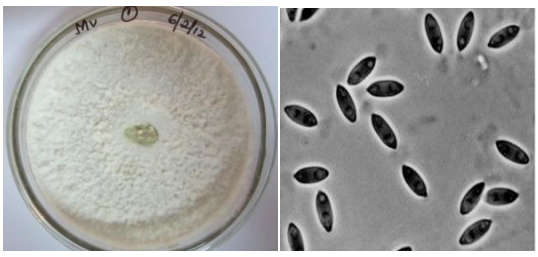
Keywords: *Phylogenetic relationship, ITS sequencing, Endophytes, Myrothecium verrucaria, Garcinia, polymorphism*

1. INTRODUCTION

India has immense variety of plants with endophytic organisms within them without any risk of life [1]. Endophytes, found ubiquitous in all plant species in the world, contribute to their host plants by producing plenty of substances that provide protection and ultimately survival value to the plant. Many researchers have proven that endophyte is a new and potential source of novel natural products for exploitation in modern medicine, agriculture and industry [2].

Myrothecium sp. is a fungus commonly found throughout the world and also it is a predominant endophyte often isolated from plants. It is a highly potent cellulose decomposer, and has been formulated into a pesticide for the control of nematodes and weeds Suryanarayanan *et al*. [3]. Liu *et al.,* [4]have isolated some antifungal metabolites like roridin A, verrucarin A, 8β-acetoxy-roridin H, cerevisterol, N-phenyl-β-amino-naphthalene (macro cyclic trichothecenes) from.

Myrothecium sp. residing in white croaker *Argyrosomus argentatus*. Amagata *et al.,* [5] have elucidated the structures and cytotoxic properties of trichoverroids and their macrolide analogues produced by saltwater culture of *Myrothecium verrucaria*. These metabolites have huge potential in treating patients suffering from secondary fungal infections in case of immunocompromised diseases like AIDS and in case of patients being treated with immunosuppressors.



(a) **(b)**

Fig1.*Culture plate (a) and conidial morphology of endophytic Myrothecium sps. isolated from Garcinia spp. (b)*

Garcinia species finds application in traditional medicine to treat various infections. Fungal endophytes of medicinal plants occupy a unique habitat, highly diverse and are important sources of natural metabolites of pharmaceutical importance. Some endophytes isolated from *Garcinia* plants are a potential source for bioactive and could be further exploited to foster the identity of the novel molecule [6].

Molecular sequencing techniques coupled with PCR have recently been strongly utilized in the discovery and identification of endophytic fungi based on phylogenetic analysis and sequence similarity analogy. Molecular fingerprinting techniques are powerful tools in the detection of population genetic composition and diversity of endophytic fungi. Further development of these markers to give clearance analysis of endophytes in plants will considerably enhance their value, and will permit the sensitive detection of endophyte incidence in plant populations.

Internal Transcribed Spacer (ITS) is a molecular sequencing technique has been successfully employed for fungal identification and phylogenies based on the sequence analyses of non-coding ITS regions of rDNA.In some studies not only the ITS region but also 18S and 28S rDNA fragments have been employed in the identification of endophytic fungi at various taxonomic levels [7].

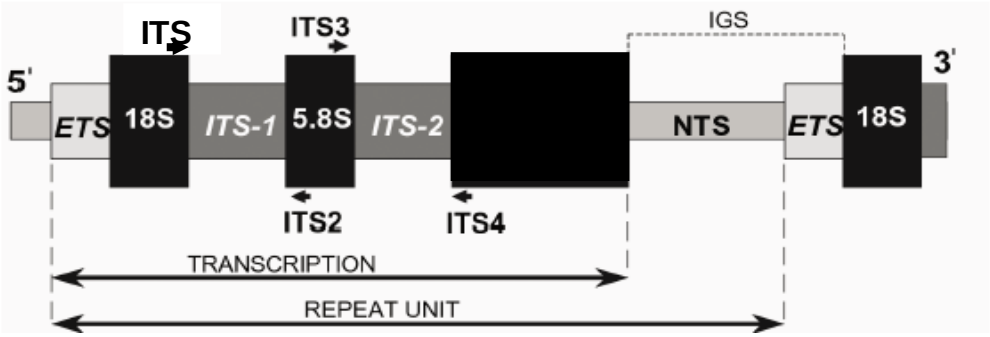


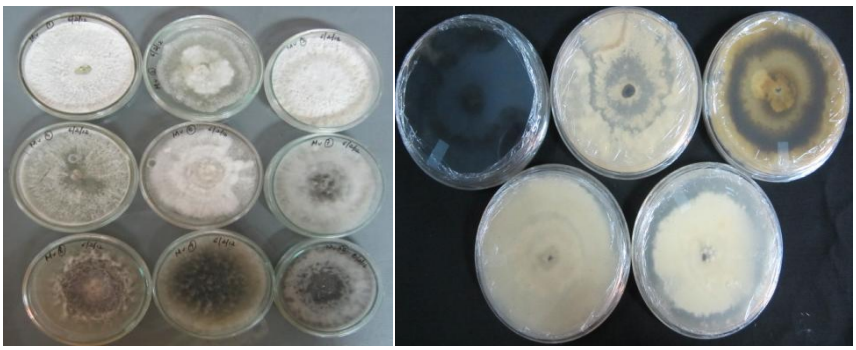
Fig2. *Basic structure of repeat unit of nrDNA. Genes are indicated in black; ETS=external transcribed spacer; IGS=intergenic spacer; ITS=internal transcribed spacer; NTS=non-transcribed spacer; small arrows indicate approximate location of PCR primers used in this study, with primer names. Not drawn to scale.*

In the present research attempts were made tofingerprint the genome diversity of fungal endophytes associated with *Myrothecium* isolated from an herbal plant *Garcinia*, and to generate the ITS sequence data to elucidate the knowledge of phylogenetic evolutionary relationships in selected endophytic fungi.

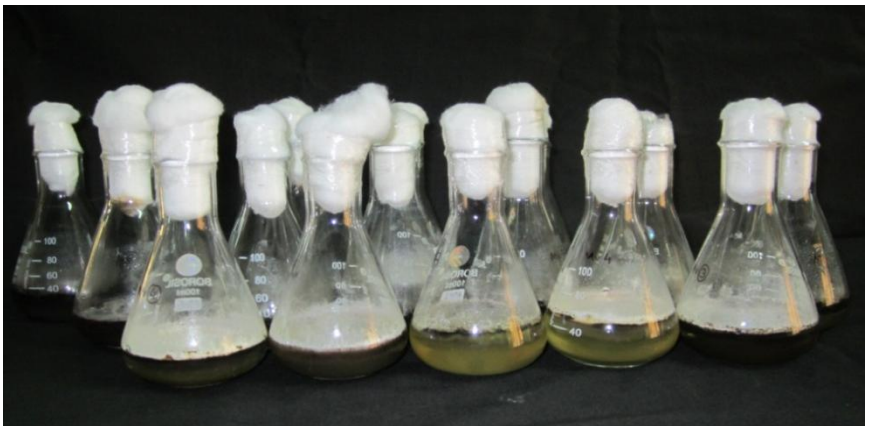
2. MATERIALS AND METHODS

2.1. Fungal Isolates and Culturing Conditions

Ten isolates of *Myrothecium* sp. (*viz.*, M1, M2, M3, M4, M5, M6, M7, M8, M9, and M10) were obtained from bark and twigs from *Garcinia* spp. growing in Western Ghats region of Karnataka. For cultivation of fungi, three mycelia plugs taken from actively growing colony margin using cork borer No. 2 (5-mm diameter) were inoculated into a 100 ml Erlenmeyer flasks containing 50 ml potato dextrose broth (PDB) and grown in still culture at 25°C. The fungi grown for 5 to 7 days were harvested and stored at -80^{0}C until DNA extracted.



(a) **(b)**



(c)

Fig3. *(a) and (b): Culture morphology of Myrothecium sp. on PDA; (c): Myrothecium culture in Potato dextrose broth.*

2.2. DNA Isolation

DNA was extracted from 0.5 to 1.0 g of fresh mycelium according to the method of Saghai-Maroof *et al*., [8]. Mycelia were removed from the PDB by filtration and ground using sterile pestle and mortar with liquid nitrogen until dry powder was obtained.

2.3. PCR Amplification of ITS Region and Electrophoresis

The target regions of the rDNA, ITS 1, ITS 2 regions and 5.8S gene (fig. 2) were amplified using primers ITS 1(5' TCC-GTA-GGT-GAA-CCT-GCG-G 3') and ITS 4 (5' TCC-TCC-GCT-TAT-TGA-TAT-GC 3') [9]. Amplifications were performed in a total reaction volume of 25 µl containing 10 mM of dNTP mix, 10 pM of each primer, 1.0 unit *Taq*DNA polymerase (Fermentas, Vilnius, Lithuania) and 50 ng of template DNA. PCR amplifications were performed in a thermal cycler (Eppendorf, Germany) with an initial denaturing step of 95 °C for 3 min, followed by 35 amplification cycles of 95 °C for 30 sec, 50 °C for 45 sec, and 72 °C for 90 sec and a final extension step of 72 °C for 10 minutes. PCR amplification products were electrophoretically separated on 1.0% (w/v) agarose gels at 100 V for 2 h in 1x TBE buffer (89 mMTris, 89 mM boric acid, and 2 mM EDTA), stained with ethidium bromide (0.5 µg/ml) and visualized under 300 nm UV light and photographed using a Molecular Imager (Gel-Doc XR^+, BIORAD, USA). A 100 bp size marker was used as reference (Bangalore Genei, India).

2.4. Elution of PCR Products

The ~ 550 bp DNA fragment PCR products were checked by electrophoresis on a 1.0% agarose gel, the fragment was eluted and purified using the Nucleo-pore PCR Clean-Up Gel Extraction Kit (Genetix, Molecular Devices, Hampshire) following manufactures instruction:

DNA fragment was excised from agarose gel using sterile blade/ scalpel. For each l00mg of agarose gel 200µl buffer SET was added. For gels containing >2% agarose, double the volume of buffer SET was added. Not more than 400mg of gel was added (or 200mg of >2% gel) per SureExtract spin column. The sample was incubated for 5-10 min at 50^{o}C, pulse vortexed every 2-3 min until the gel slice was completely dissolved. Excess UV exposure damages nucleic acids. The gel slice was weighed and transferred into a clean 1.5ml microcentrifuge tube. The sample was loaded onto SureExtract spin PCR/gel extraction column placed in a 2ml collection tube. Then centrifuged for 1 min at 11,000×g. Discard flow-through and place the column back into collection tube. 700µl of buffer SET3 was added to the SureExtract spin PCR/gel extraction column, Centrifuged for 1 min at 11,000×g. The flow-through was discarded and the column was placed back into the collection tube. Carry-over of chaotropic salt may result in low A_{260}/A_{230} values. The sample was centrifuged for 2 min at 11,000×g to remove buffer SET3 completely. It was made sure the spin column does not come in contact with the flow-through while removing it from the centrifuge and collection tube.

NOTE: It was made sure that ethanol contained in buffer SET3 was completely removed. Incubating the columns for 2-5 min at 70^{o}C prior to elution helped in complete removal of ethanol.

2.5. Sequencing of PCR Products

Sequencing of ITS PCR product was carried out after PCR clean up or elution of pure DNA from the ITS gel. The eluted pure product was amplified in a thermal cycler (Applied Biosystems, U.S.A) and subsequently sequenced by an automated sequencer (Applied Biosystems/HITACHI 3500 series) which works on the principle of dideoxynucleotide chain termination method.

Table1. *The quantity of eluted PCR product required for sequencing for different lengths and forms of DNA*

Template PCR product	Quantity
100-200 bp	1-3ng
200-500 bp	3-10ng
500-1000 bp	5-20ng
1000-2000 bp	10-40ng
>2000 bp	20-50ng
Single stranded	25-50ng
Double stranded	150-300ng
Cosmid, BAC	0.5-1ng
Bacterial genomic DNA	2-3ng

Table2. *Reaction mixture and the quantity of the individual components for cycle sequencing PCR*

Reagent	Full Reaction	Half Reaction	Half Reaction
Big dye premix	8 µl	4µl	2µl
Big dye sequence buffer	-	2µl	1µl
Template*	-	-	-
Primers** (10 µM)	1µl	1µl	1µl
Water #			
Total	**20µl**	**20µl**	**10µl**

**Template: add as required for each reaction*

***Primer concentration- 3.2-10 pM*

#Water: add as requires for each reaction

Table3. *PCR cycle sequencing settings for Big dye V3.1*

Initial Denaturation	96º C	1 minute
Denaturation	96º C	10 seconds
Annealing	50ºC	5 seconds
Extension	60º C	4 minutes
Hold at	4°C	

2.6. Ethanol / EDTA Precipitation to Clean up Reactions

5µl of 125 mM EDTA was added to each tube and it was made sure that the EDTA reached the bottom of every tube. This was followed by the addition of 60µl of 100% ethanol to the tubes and all the tubes were finger vortexed and incubated at RT for 15 min. Samples were spun in a microcentrifuge at maximum speed at 4ºC for 20 min and the supernatant was carefully aspirated off. Then 60µl of 70% ethanol was added to the tubes. Samples in the microcentrifuge tubes were spun at maximum speed at 4ºC for 15 minutes and the supernatant was aspirated off. Then the Samples were dried for 15 minutes in a speed vac.

Note: samples were protected from light while drying

2.7. ITS Sequence Analysis

Besides the morphological characterization and ITS-RFLPs, ITS sequence analysis was also done for molecular authentication of species of *Myrothecium*. The analysis of sequence data was carried out by multaligning (using the software Multalin) [10], the available ITS sequences of *Myrothecium* from the National Centre for Biotechnology Information (NCBI) database.

3. RESULTS AND DISCUSSION

3.1. DNA Isolation

The DNA isolated from different isolates of *Myrothecium sp.*, was confirmed by running the extracts on gel and concentration of DNA was quantitatively determined by a Nanodrop method spectrophotometrically (BioRad, California, USA).

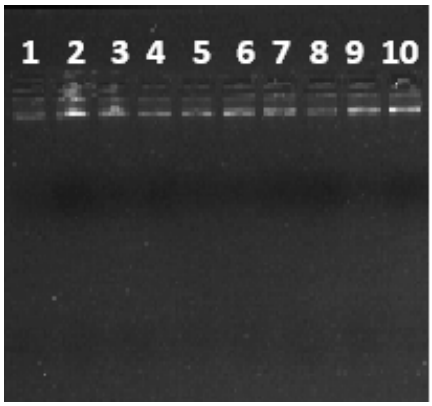


Fig4. *Extracted DNA of Myrothecium spp. observed on 1% Agarose gel*

3.2. ITS

The internal transcribed spacers (ITS) of the rDNA region of 10 *Myrothecium* species showed extensive length polymorphism (Fig. 5). The length of amplified rDNA fragment ranged from 550-600 bp. Based on the polymorphisms of the ITS region, the 10 isolates gave a length of ~ 550 bp amplicon.

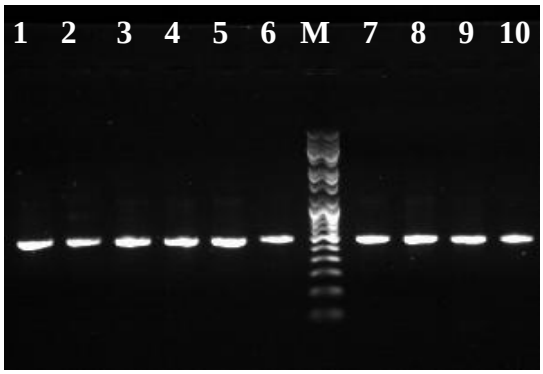


Fig5. *Amplified ITS fragments of Myrothecium spp.(lanes 1-6 & 7-11) visible on 1% Agarose gel; Marker (M): Medium ranged marker.*

3.3. ITS Sequence Analysis

The analysis of sequence data was carried out by multialigning with the available ITS sequences of *Myrothecium* from (Fig 6). NCBI database which showed a homology of 82% with the *Myrothecium* spp. sequenced

A more precise determination of diversity and identification of these *Myrothecium* species were obtained by using the genetic marker viz., ITS. The ITS analysis showed intraspecific and interspecific variation among congeneric species. Because most coding genes are highly conserved, they have been successfully used to assess phylogenetic relationships at higher taxonomic levels. ITS regions benefit from a fast rate of evolution, resulting in greater sequence variation between closely related species. The sequences of these regions therefore generally provide greater resolution at lower taxonomic levels *viz.*, genus and species level. Molecular sequencing techniques such as ITS offer an effective method for the identification of endophytic fungi, particularly for non sporulating isolates, and for the detection of the viable but nonculturable fungi by directly amplified rDNA fragments from plant tissues [11]. Internal Transcribed Spacers (ITS), the non-coding sequences in the genome which gets transcribed along with the genes coding for rRNA and thus are seen in the precursor transcripts of rRNA. The ITS region is now perhaps the most widely sequenced DNA region in fungi [12]. Sequence comparison of the ITS region is widely used in molecular phylogeny because it is easy to amplify even from small quantities of DNA (due to high copy number of rRNA genes). ITS regions are usually highly conserved due to low evolutionary pressure acting on such non-functional sequences. ITS marker has been used for elucidating relationships among congeneric species and closely related genera [13, 14]. In addition to the standard ITS primers used for most studies *viz.*, ITS1 and ITS4 [9], several taxon specific primers have been described that allow selective amplification of fungal sequences [15].

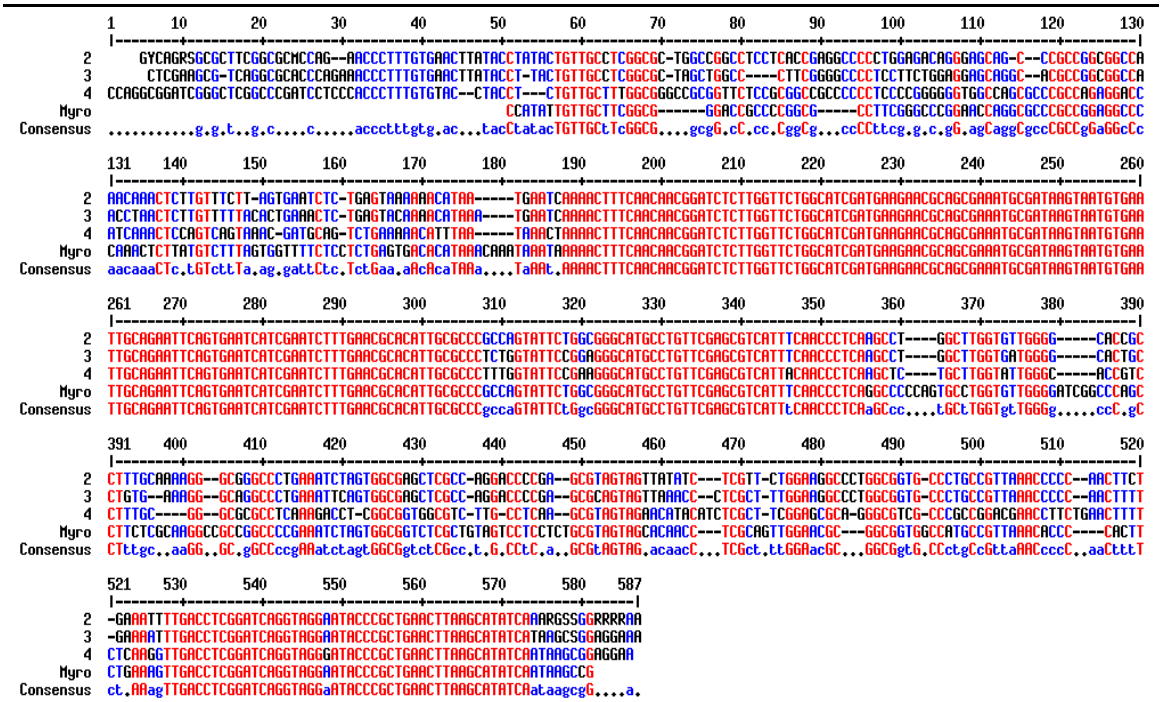


Fig6. *Multiple alignments of ITS sequences with the sequences of Myrothecium in NCBI database*

In the present research, the amplification and subsequent sequencing of highly conserved ITS region showed genetic similarity among 10 isolates of *Myrothecium* spp (M). The amplicon length of 550bp was obtained using two primers ITS-01 and ITS-04 and sequence similarity of 82% with *Myrothecium* spp. revealed during multiple alignments using MultAlign software [10] decisively proved that the isolates belonged to the genus *Myrothecium.* The goal of this study was to characterize the endophytic fungi from plants belonging to the genus *Garcinia* and analyze their genetic diversity using molecular marker *viz.*, ITS. The study was largely successful as we observed considerable intra-specific variation among the isolates of *Myrothecium* spp. The ITS analysis helped us to group the isolates according their genome fingerprints.

4. CONCLUSION

Here, in this investigation we have isolated this endophyte from *Garcinia* spp. The fungal endophytes have garnered huge attention among researchers and pharmaceutical companies in recent years because of their unmatched ability to synthesize novel natural metabolites. However researchers have so far characterized only a few of these organisms mainly from well-known medicinal plants. In this research we have attempted to characterize and assess the genotype of an endophytic fungus *Myrothecium* spp., which generally are the residents of several plants, and give insight to the fungal endophytes colonized with the herbal plant, *Garcinia*.

Development of new molecular techniques have greatly advanced our conception of genetic makeup of microorganisms and molecular-sequencing based techniques like ITS coupled with PCR have become tools of choice for mapping genetic divergence between individuals or within related species, population genetics and hybridization. The present study, the multiple alignment of the resulting sequence with the sequences showed homology with *Myrothecium* sp. This inspection solidifies the notion that Molecular markers are essential part of phylogenetic analysis and it may lead to more research on endophytes, their characterization and assessment of their genetic diversity.

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