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Research Article

MOLECULAR ANALYSIS OF GENTIC DIVERSITY OF ENDOPHYTIC MYROTHECIUM SPP. BY RAPD AND ISSR MARKERS

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ABSTRACT

India has immense variety of plants with endophytic organisms within them without any risk of life. In order to study genetic relatedness in ten isolates of *Myrothecium* species which is a fungus plant pathogen obtained as an endophyte from bark and twigs from *Garcinia* spp. were studied with RAPD and ISSR markers. The DNA markers are designed as a potential tool for mapping genetic divergence between individuals or within related species. Hence, in this research, we concentrate on applications, the advantages and limitations of RAPD, and ISSR molecular markers for genome fingerprinting of endophyte genotypes, to characterize genetic diversity and phylogenetic/evolutionary relationships between them. Five RAPD primers, OPM-01 to OPM-05 generated 193 polymorphic bands consisting of 72 markers. Three ISSR primers v*iz.*, ISSR-01, ISSR-02 and ISSR-07 generated 93 polymorphic bands with a marker score of 41. The dendrogram produced using the unweighted pair group method with arithmetic means (UPGMA) and cluster analysis made using Nei's genetic distance resulted in the formation of isolates into four main groups and revealed a rather close phylogenetic relationship among some isolates whereas isolates M-2 and M-5 showed surprisingly large genetic variation and evolutionary divergence from other isolates.

Keywords: Genetic Diversity, RAPD, ISSR, Endophytes, *Myrothecium* verrucaria*, Garcinia*

INTRODUCTION

The term endophyte is derived from two Greek words *endo* "within" and *phyton* "plant" that denotes a broad spectrum of microorganisms which reside inside any part of the plant body with varying life spans and life styles [1]. Bacon and White [2] gave an inclusive and widely accepted definition of endophytes "Microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects". Although symptomless nature of endophyte colonization propose that they generally exist with mutualistic symbiotic relationship with the host plant, the observed biodiversity of endophytes reveals that in some rare cases they can exert aggressive saprophytic and parasitic interactions [3].There is a hypothesis suggesting that many fungal endophytes producing different types of secondary metabolites with bioactive potential properties which can modify host genome by horizontal gene transfer (HGT) in which fungal genes coding for those metabolites gets transferred to the host genome. As a result of HGT, plants become able to secrete many valuable compounds, in fact medicinal properties of many plants has been directly attributed to the HGT taking place between fungal endophyte and the host plant [4]. *Myrothecium sp.* is a fungus frequently found throughout the world and also it is a predominant endophyte often isolated from plants. It is a highly potent cellulose decomposer; it has been formulated into a pesticide for the control of nematodes and weeds, Suryanarayanan *et al*. [5] examined that endophyte status of dry tropical forests of Southern India representing 24 hosts (17 families). They adopted a sampling method that simulated conditions of the humid, tropical Panamanian forest. They revealed that the host specificity is crucial to extrapolate global fungal biodiversity as it is dependent on the fungus-plant ratios. Traditionally, fungal species have been classified on the basis of morphology and cultural characteristics like pigment production, presence of microconidia and chlamydo spores [6, 7]. However, the number of these markers available is generally low, which makes classification difficult, as also the identification of related species [8]. PCR-based molecular marker techniques have been employed in the detection of endophytic fungi in previous studies. These conventional PCR- based molecular markers can identify endophytic fungi specifically, but it cannot be used to quantify endophytic fungal biomass within plant tissues. *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 bioactives and could be further exploited to foster the identity of the novel molecule [9].

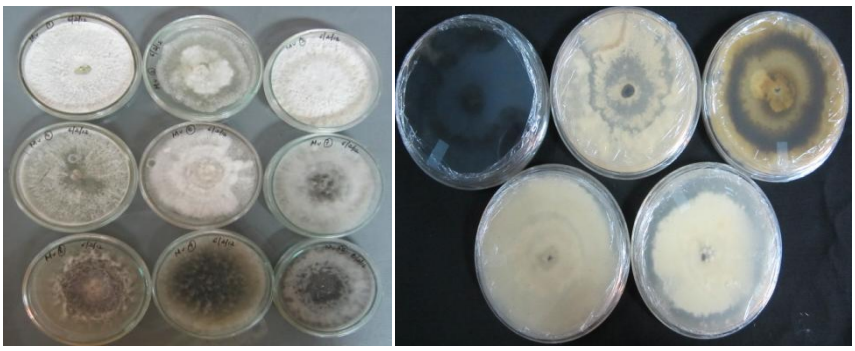
There are some limitations for the detection and identification of endophytic fungi directly from within plant tissues using molecular techniques. Firstly, as only sparse hyphae may exist within the plant tissues, some fungal DNA may be lost during the DNA extraction process, thus only a slight fraction of fungal DNA is included in the total DNA extracted from plant tissues. Secondly, there are inhibitors that may interfere with the PCR amplification in the DNA solution. Thirdly, the universal primers may not perfectly match with some fungal template DNA. In addition, it is important to take into consideration that surface sterilization may not have denatured the DNA of endophytes, although sodium hypochlorite is relatively

effective for this purpose. Therefore, it is likely to be troublesome to amplify all endophytic fungal DNA fragments from the total DNA samples. Another limitation is the limited number of sequences, i.e., less than 1%, of the estimated 1.5 million fungal species presented in NCBI Gene Bank and EMBL database, although there is daily increase in fungal DNA sequences in public databases [10]. This technique can be is used to investigate the genotypic diversity in populations of endophytic fungi and the genetic variation can be observed in the form of amplified polymorphic bands [11]. ISSR technique, which was originally used to measure genetic diversity of plants and animals [12] has been applied in studies of fungi and are well established and have been successfully applied to assess the population genetic diversity and fungal communities in natural environment [13, 14, 15] Thus, ISSR technique combines most of the benefits of RAPD and microsatellite analyzes, and is ideal for studies of genetic variation of endophytic fungal populations. In this investigation attempts were made to probe the genetic variation among the isolate of *Myrothecium* spp. obtained from bark and twigs of *Garcinia* spp. regarding to genome conservation activities and to compare the advantages and limitations of RAPD and ISSR molecular markers for phylogenetic analysis of fungal endophytes presence in herbal plant.

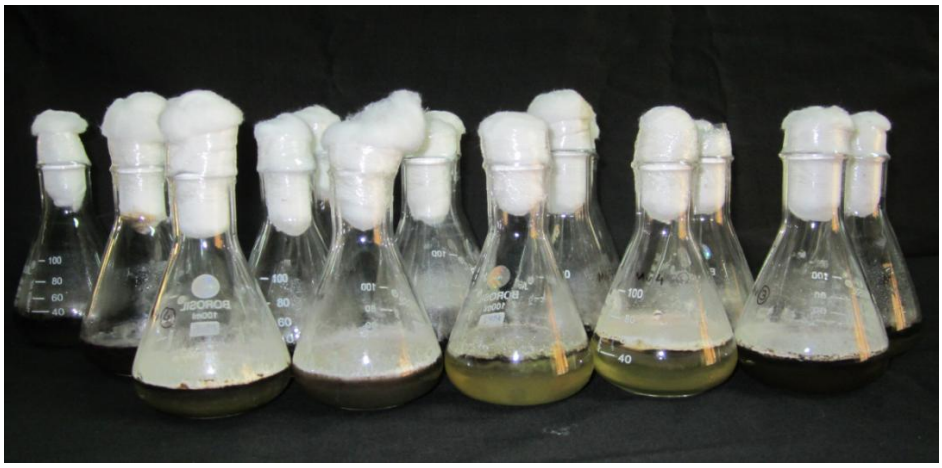
MATERIALS AND METHODS

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)

Figure 1: (a) and (b): Culture morphology of *Myrothecium* sp. on PDA; (c) *Myrothecium* culture in Potato dextrose broth.

DNA isolation:

DNA was extracted from 0.5 to 1.0 g of fresh mycelium according to the method of Saghai-Maroof *et al*. [16]. Mycelia were removed from the PDB by filtration and ground using sterile pestle and mortar with liquid nitrogen until dry powder was obtained.

RAPD analysis:

Genetic diversity among isolates of *Myrothecium* spp. from *Garcinia* spp. was assessed using RAPD. Five primers v*iz*., M-01, M-02, M-03, M-04 and M-05 (Operon Technologies Inc., USA), were used for reproducible banding patterns. Polymerase chain reaction (PCR) amplifications for RAPD analysis were performed in a total reaction volume of 25 µl containing 1X PCR buffer, 10 mM of each dNTP, 10 pM of each primer, 1.0 units *Taq*DNA polymerase (Fermantas, Vilnius, Lithuania) and 50 ng of template DNA. RAPD PCR amplifications were performed in a thermal cycler (Eppendorf, Germany) with an initial denaturing step of 94 ºC for 3 min, followed by 45 amplification cycles of 94 ºC for 1 min, 36 ºC for 1 min, and 72 °C for 2 min and a final extension step of 72 °C for 10 min. PCR amplification products were electrophoretically separated on 1.4% Agarose gels at 100V for 2 hours in 1X TBE buffer (89 mMTris, 89 mM Boric acid, and 2 mM EDTA), stained with ethidium bromide, visualized under 300 nm UV light and photographed using a Molecular Imager, imaging system (Gel-Doc XR$^+$, BIORAD, USA). A 1 kb size marker was used as reference (Genei Bangalore, India).

RAPD data from the amplifications were recorded by scoring polymorphic DNA bands and were compiled in a binary matrix in which 1 indicated presence of band and 0 the absence of band. The data were converted to distance matrices based on Nei (1978) unbiased minimum distance. The Distance matrices were

then used to construct a dendrogram (1000 bootstrapping) by the unweighted pair-group method with arithmetic mean (UPGMA) using Tools for Population Genetic Analyses (TFPGA Ver 1.3) [17].

ISSR analysis:

Genetic diversity among endophytic isolates of *Myrothecium* spp. from *Garcinia* spp. was assessed using ISSR. Three primers v*iz*., ISSR-01, ISSR-02 and ISSR-07 [18], used for reproducible banding patterns were synthesized from Sigma-Aldrich (USA). Polymerase chain reaction (PCR) amplifications for RAPD analysis were performed in a total reaction volume of 25 µl containing 1X PCR buffer, 10 mM of each dNTP, 10 pM of each primer, 1.0 units *Taq*DNA polymerase (Fermantas, Vilnius, Lithuania) and 50 ng of template DNA. RAPD PCR amplifications were performed in a thermal cycler (Eppendorf, Germany) with an initial denaturing step of 94 ºC for 3 min, followed by 45 amplification cycles of 94 ºC for 1 min, 43 ºC for 1 min, and 72 °C for 2 min and a final extension step of 72 °C for 10 min. PCR amplification products were electrophoretically separated on 1.0% Agarose gels at 100V for 2 hours in 1X TBE buffer (89 mMTris, 89 mM Boric acid, and 2 mM EDTA), stained with ethidium bromide, visualized under 300 nm UV light and photographed using a Molecular Imager, imaging system (Gel-Doc XR+, BIORAD, USA). A 1kb size marker was used as reference (Bangalore Genei, India).

Amplified ISSR products were analyzed by scoring polymorphic DNA bands and were compiled in a binary matrix in which 1 indicated presence of band and 0 the absence of band. The data were converted to distance matrices based on Nei (1978) unbiased minimum distance. The distance matrices were then used to construct a dendrogram (1000 bootstrapping) by the unweighted pair-group method with arithmetic mean (UPGMA) using Tools for Population Genetic Analyses (TFPGA Ver 1.3) [17].

RESULTS AND DISCUSSION

DNA Isolation:

The DNA isolated from different isolates of *Myrothecium sp.*, was confirmed by running the extracts on gel and concentration of DNA was estimated using a nanodrop.

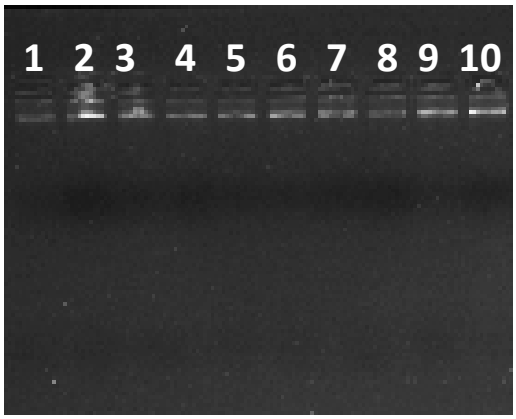
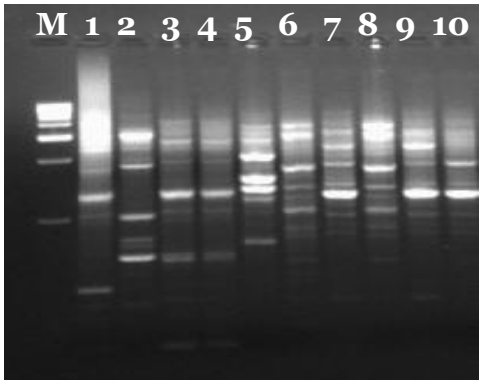


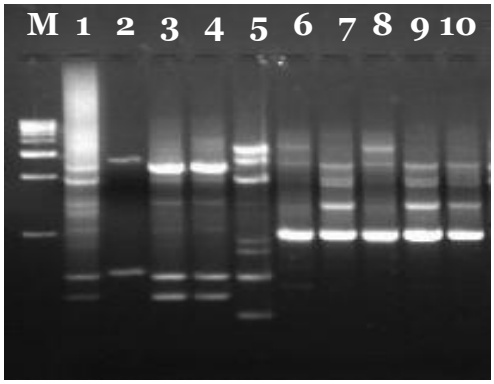
Figure 2: Extracted DNA of *Myrothecium* spp. observed on 1% Agarose gel

RAPD:

RAPD profiles were generated for 10 isolates of *Myrothecium* spp. using five primers (Table 1). A total of 193 polymorphic bands were scored consisting of 72 markers. Each amplification was repeated twice to confirm the reproducibility and only reproducible bands were considered for scoring and analysis. Gels showing typical amplification products are shown in Fig 3.



(a)



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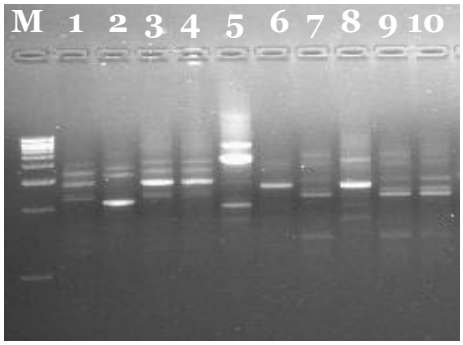

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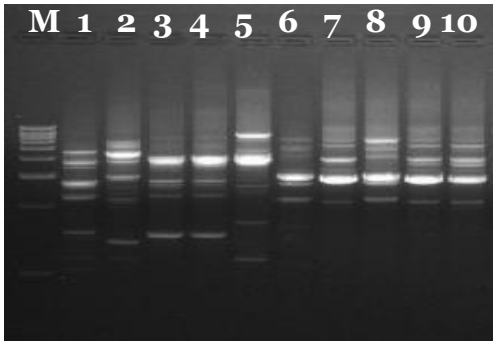

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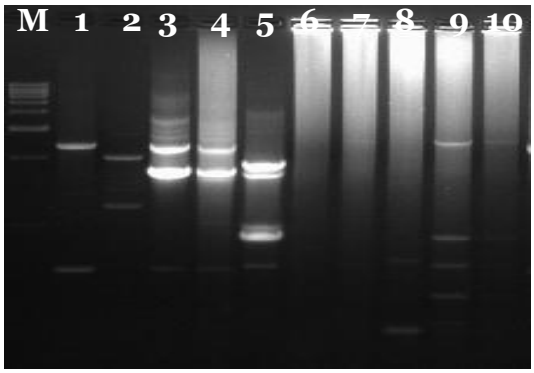

(e)

Figure 3: Agarose gels showing RAPD products of 10 isolates (1-10) of *Myrothecium* spp. obtained by amplifying 50 ng of DNA using the Primers OPM-01 (a), OPM-02 (b), OPM-03 (c), OPM-04 (d) & OPM-05 (e). M = 1Kb marker**.**

Sl No.	Primer	No. Of Polymorphic bands
1.	OPM-01	38
2.	OPM-02	36
3.	OPM-03	29
4.	OPM-04	66
5.	OPM-05	24

Table 1: List of RAPD primers used and number of polymorphic bands obtained.

ISSR:

ISSR profiles were generated for 10 isolates of *Myrothecium* spp. using 3 primers (Table 2). A total of 93 polymorphic bands were scored with a marker score of41.Each amplification was repeated twice to confirm the reproducibility and only reproducible bands were considered for scoring and analysis. Gels showing typical amplification products are shown in Fig 4.

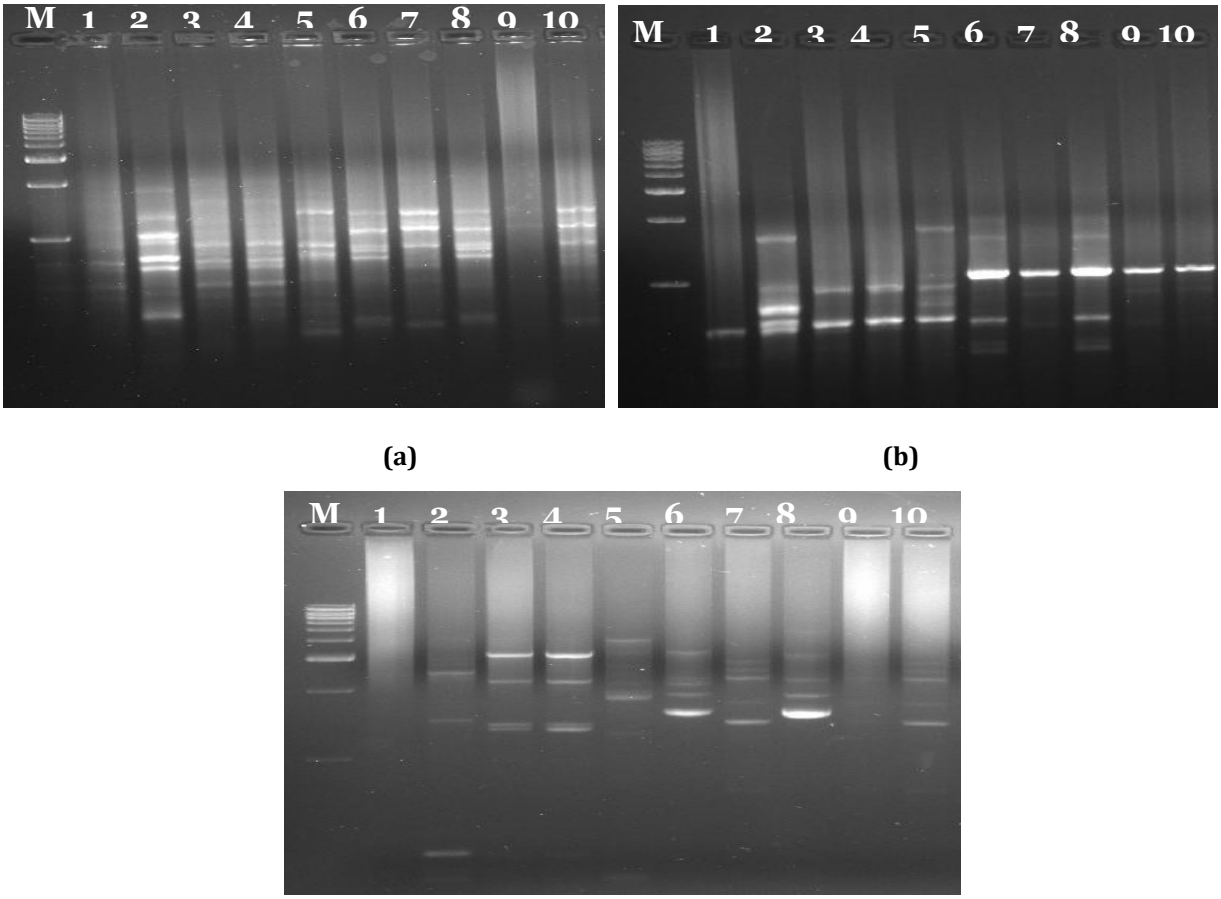

Figure 4: Agarose gel showing ISSR products of *Myrothecium* isolates (Lane 1-10) obtained by amplifying 50 ng of DNA using the Primers ISSR-01 (a), ISSR-02 (b) AND ISSR-07 (c). M = 1Kb marker.

Sl No.	Primer	No. Of Polymorphic bands
1.	ISSR-1	25
2.	ISSR-2	46
3.	ISSR-7	22

Table 2: List of ISSR primers used and number of polymorphic bands obtained.

RAPD cluster analysis:

Cluster analysis with UPGMA using genetic distances was performed to generate a dendrogram (Fig. 5) illustrating the overall genetic relationships within the species studied. The isolates were divided into four main groups. In general, the isolates from the same species occurred together in the same cluster. The four

main clusters have divided into 5 sub-clusters. The dendrogram suggests that out of 10 isolates 3, 4 and 7, 9 and 6, 8 are closely related since they are present in the same sub-cluster. Isolate 1 along with sub-cluster 3, 4 forms a main cluster and isolate 10 along with sub-cluster 7, 9 forms another main cluster indicating close phylogenetic relationship. The isolates 2 and 5 do not belong to any main cluster/ sub-cluster thus indicating early evolutionary divergence from a common ancestor.

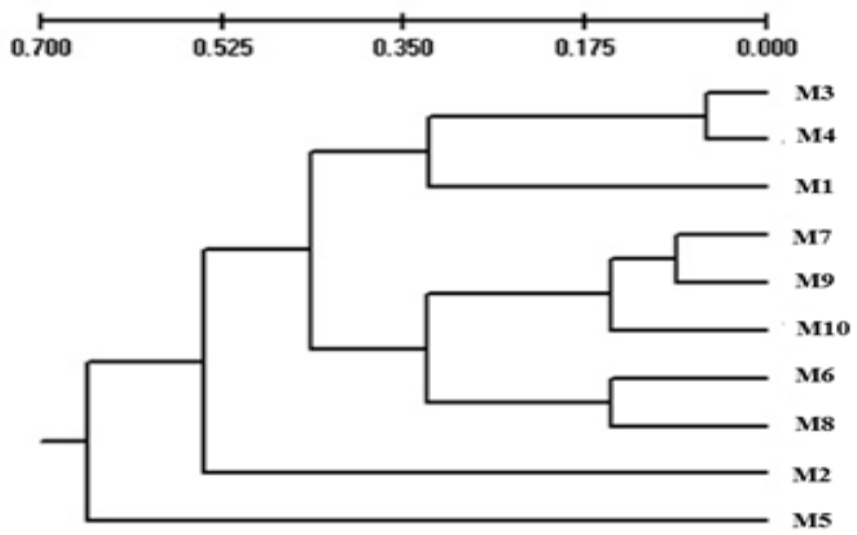

Figure 5: Unweighted pair group method with average (UPGMA) cluster diagram of the relationships between 10 *Myrothecium* isolates. The dendrogram was based on presence or absence of bands in RAPD analysis (Figure 3).

ISSR cluster analysis:

Cluster analysis with UPGMA using genetic distances was performed to generate a dendrogram (Fig. 6) illustrating the overall genetic relationships within the species studied. The isolates were divided into four main groups. In general, the isolates from the same species occurred together in the same cluster. Out of four main clusters only the first cluster further divides to form two sub-clusters. The isolates 7, 10 and 1, 9 form two sub-clusters of the first main cluster thus suggesting genetic similarity among the above mentioned members. The isolates 6 & 8 and 2 & 5 and 3 & 4 form their own main clusters indicating genetic similarity among the members of the clusters and genetic variation among the members of the nearby clusters.

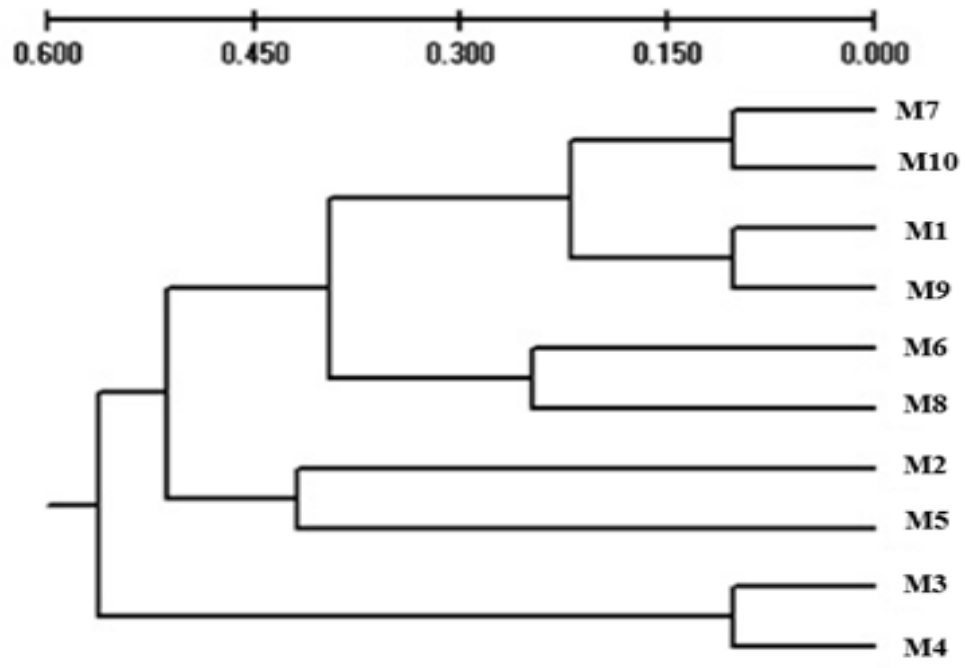

Figure 6: Unweighted pair group method with average (UPGMA) cluster diagram of the relationships between 10 *Myrothecium* isolates (M1 to M10). The dendrogram was based on presence or absence of bands in ISSR analysis (Fig. 4).

Endophytic fungi are one among the most diverse group of microorganisms. Endophytes are present virtually in all plants exerting a mutualistic relationship with the host [2].Extensive colonization of plant tissues by the fungal endophytes creates a "barrier effect", in which local endophytes outcompete and hamper the growth of pathogenic organisms. Fungal endophytes produce many secondary metabolites which in turn prevents the growth of many plant pathogens. They can also supply essential vitamins to plants [19]. *Myrothecium* sp., an endophytic fungus, however also known to exert pathogenic interactions in some plants usually found in close organization with plant tissue makes it an interesting target to study Horizontal Gene Transfer (HGT) between endophytes and the host plant. In this study, *Myrothecium* spp. was isolated from the bark and twigs of plants belonging to *Garcinia* spp. [20]. A more precise determination of diversity and identification of these *Myrothecium* species were obtained by using the genetic markers viz., RAPD and ISSR. RAPD and ISSR have been used increasingly for genetic variation studies [21, 18].In recent years, multidisciplinary approaches have been initiated and the rapid development in molecular biology has a major impact on fungal taxonomy. Construction of phylogenetic trees based on analysis of DNA sequences of relatively small parts of genomes became very popular and provoked many discussions about the diversity among the isolates of *Pestalotiopsis* species using RAPD markers. RAPD and ISSR markers were used for

molecular characterization and genetic analysis of pathogenic strains of *Pestalotiopsis* and the results indicated high morphological and genetic diversity among different isolates [18, 24]. Oroian *et al*., [25] have used RAPD technique for the genetic analysis of *Cyprinuscarpio* spp. RAPD has also been used for molecular characterization of *Fusarium oxysporum* from eggplant [26].

CONCLUSION

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 study we have attempted to characterize and assess the genotype of an endophytic fungus *Myrothecium* spp., which generally are the inhabitants of several plants. Here, in the present study we have isolated this endophyte from *Garcinia* spp.

Advent of new molecular techniques have greatly advanced our understanding of genetic makeup of organisms and molecular marker based techniques like RAPD and ISSR coupled with PCR have become tools of choice for studying the phylogeny, population genetics and hybridization. In this study, both RAPD and ISSR dendrograms revealed the intra-specific genetic diversity and phylogenetic relationships among different isolates of *Myrothecium* sp. This study solidifies the notion that Molecular markers are indispensible part of genetic analysis and it may lead to more research on endophytes, their characterization and assessment of their genetic diversity. Also, understanding the complex host-endophyte interactions like the horizontal gene transfer will eventually lead to judicial exploitation as well as bioprospecting of this rich yet largely unknown natural resource relationship between traditional and molecular taxonomy [22]. Tejesvi*et al.,* [23] reported the genetic.

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