# Genetic diversity and antifungal activity of species of *Pestalotiopsis* isolated as endophytes from medicinal plants

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Tejesvi, M.V., Kini, K.R., Prakash, H.S., Ven Subbiah and Shetty, H.S. (2007). Genetic diversity and antifungal activity of species of *Pestalotiopsis* isolated as endophytes from medicinal plants. Fungal Diversity 24: 37-54.

The genetic diversity of fungal endophytes in root, bark and twigs of four medicinally important plants, *Azadirachta indica*, *Holarrhena antidysenterica*, *Terminalia arjuna* and *T. chebula* were examined. Thirty isolates of *Pestalotiopsis* and two isolates of *Bartalinia robillardoides* were genotypically compared by RAPD techniques and 241 reproducible polymorphic bands were obtained using 23 random primers. The data was subjected to unweighted pair-group (UPGMA) cluster analysis. The isolates grouped into four main clusters and subgroups, group I contained 12 isolates, group II contained 3 isolates of *P. virgatula*, group III contained 10 isolates including *P. microspora*, *B. robillardoides*, *P. theae* and *Pestalotiopsis* spp., group IV contained five isolates of *P. microspora* and finally one *Pestalotiopsis* spp. did not fall into any group. The ethyl acetate extracts of isolates from *Terminalia arjuna* showed greater antifungal activity than those from other medicinal trees against six test organisms *viz*., *Alternaria carthami*, *Fusarium oxysporum*, *Fusarium verticilloides Macrophomina phaseolina*, *Phoma sorghina* and *Sclerotinia sclerotiorum*, with the zone of inhibition ranging from 4 to 25 mm in diameter. The results indicate that RAPD can be employed for detecting genetic diversity of *Pestalotiopsis* species from medicinal plants and for pre-selection of these isolates for bioactive screening programme.

**Key words:** *Azadirachta indica*, *Holarrhena antidysenterica*, *Terminalia arjuna*, *Terminalia chebula*, RAPD, secondary metabolites

## Introduction

Fungal endophytes live within their host plants without causing any apparent disease symptoms (Bacon *et al*., 2000; Promputtha *et al*., 2005; Wang *et al*., 2005). The majority of plant species examined to date harbour

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endophytic fungi within asymptomatic aerial tissues, such that endophytes represent a ubiquitous, yet cryptic, component of terrestrial plant communities (Arnold *et al*., 2003; Gonthier *et al*., 2005). Studies to date have focused primarily on the quantification of isolates obtained from surface-disinfested fragments of plant species. Frequently different fungi can be obtained in a single isolation from a twig or inner bark (Carroll, 1995; Gonthier *et al*., 2005). Endophyte diversity in woody plants is poorly understood and estimation of their diversity has been based on the morphological species concept (Ganley *et al*., 2004; Promputtha *et al*., 2005; Duong *et al*., 2006).

*Pestalotiopsis* is an anamorphic genus of the family *Amphisphaeriaceae* (Kang *et al*., 1999; Jeewon *et al*., 2003) and are commonly present in tropical and subtropical species of plants. Although this organism is a widespread saprobe on bark and decaying plant material, it also occurs as endophytes from a wide variety of plant families around the world (Metz *et al*., 2000; Harper *et al*., 2003; Strobel *et al*., 2003; Gonthier *et al*., 2005; Devarajan and Suryanarayanan, 2006). The geographical distribution and ubiquitous occurrence of *Pestalotiopsis* suggest an important role for this genus in the forest ecosystem. This prevalence may suggest an important role for these fungi in forest ecosystems, and they are dubbed as `the *E. coli* of the temperate and tropical rainforest systems' (Strobel, 1998). *Pestalotiopsis* species have gained much attention in recent years as they have been found to produce many important secondary metabolites (Strobel, 2002). The ambuic acid, pestacin and isopestacin isolated from *Pestalotiopsis* species are found to possess antifungal activity against *Pythium ultimum* (Li *et al*., 2001; Harper *et al*., 2003; Strobel *et al*., 2002). Further more, jesterone and hydroxyl-jesterone isolated from *P. jesteri* from a medicinal tree *Terminalia morobensis* is having anti-oomycetic activity (Li and Strobel, 2001).

Several biochemical methods exist to detect genetic diversity in fungi including the use of isozymes (Micales *et al*., 1986), restriction fragment length polymorphisms (RFLP) and other molecular markers (Karp *et al*., 1996). Randomly amplified polymorphic DNA (RAPD) has been used increasingly for genetic variation studies (Williams *et al*., 1993). Although the technique has been criticized for several undesirable features such as non co-dominant inheritance, anonymous nature (Backeljau *et al*., 1995), lack of positional homology (Van de Zande and Bijlsma, 1995) and especially inconsistent reproducibility (Staub *et al*., 1996), RAPD data can detect genetic diversity between related species (Harvey and Botha, 1996) and within species (Van Oppen *et al*., 1996). Because of its ease of use and low cost, the technique has profoundly affected our ability to explore genetic variation in organisms.

Despite the broad application of RAPD based genetic markers for analysis of genetic diversity of fungal endophytes in a wide range of natural habitats, little information is available on the species diversity of endophytes. In particular there is very little information about fungal endophytes associated with tropical forest trees (Promputtha *et al*., 2005; Duong *et al*., 2006). RAPD analysis have been successfully used to identify strains (Pryor and Gilbertson, 2000; Jana *et al*., 2003), characterize races (Malvick and Grau, 2001) and to analyze virulence variability related to genetic polymorphisms (Kolmer and Liu, 2000) in phytopathogenic fungi. It has also been used in the study of inter- and intra-specific variability among populations from different (Pazoutova *et al*., 2000) and from the same geographic regions (Walker *et al*., 2001; Ma *et al*., 2001).

The four medicinally important plants, *Azadirachta indica*, *Holarrhena antidysenterica*, *Terminalia arjuna* and *T. chebula*, which have been reported to have anti-bacterial, antifungal, anti-inflammatory, anti-mutagenic, antioxidant, antipyretic, cardiotonic and diuretic properties, were selected for the study (Kaur *et al*., 1997; Bharani *et al*., 2001; Gupta *et al*., 2001). Earlier studies from our laboratory have reported *Pestalotiopsis* spp. as one of the most dominant species from the selected medicinal trees (Mahesh *et al*., 2005; Tejesvi *et al*., 2005). The purpose of the current study was to investigate genetic variation among *Pestalotiopsis* species isolated from medicinal plants and screen the secondary metabolites from them for antifungal activity.

## Materials and methods

### *Collection of plant material*

Inner bark, roots and twigs of *Azadirachta indica* (AI), *Holarrhena antidysenterica* (HA), *Terminalia arjuna* (TA) and *T. chebula* (TC), were collected from the riverbanks of Nanjungud (12º 07'N 76º 44'E), Srirangapatna (12º 25'N 76º 40'E) Gopalaswamy hills (11º 40'N 76º 32 E), Sullia (12º 77'N 75º.58E) and Mysore (12º 18'N 76º 42'E) districts of southern India. Bark samples were collected from trunk regions, about 1.5-2 m above ground level. Four to five roots and twigs were also collected from the respective host plants. Each sample was tagged and placed in separate polythene bags, brought back to the lab and processed within 24 hours of collection.

***Isolation, identification and preservation of endophytes***

Samples were washed thoroughly in running tap water for five minutes before processing. Small bark, twig and root segments were surface disinfested by sequential washes in 70% (v/v) ethanol (1 min) and 3.5% (v/v) NaOCl (2 min), rinsed with sterile water and allowed to surface dry under sterile condition (Schulz *et al*., 1993). Two hundred segments of bark, twigs and roots from each plant collected at different locations were plated on water agar (15g/L) (WA) medium supplemented with streptomycin (Himedia, 100 mg $l^{-1}$). Ten to 15 segments were placed on each plate. Plates were incubated in a light chamber for two weeks at 12 hours light/dark cycles at 23ºC (Suryanarayanan, 1992). After incubation for 15 to 30 days, individual fungal colonies were picked from the edge of an advancing colony with a sterile fine tipped needle under stereo-binocular microscope and transferred onto potato dextrose agar (PDA). Fungal spore formation was encouraged by inoculating cultures on sterilized banana leaf pieces amended with the PDA medium. Species identification was based on the morphological and taxonomic keys provided by Guba (1961) and Steyaert (1949). Isolates of *Pestalotiopsis* were maintained in cryovials on PDA layered with 15% glycerol (v/v) at -80ºC at Department of Applied Botany and Biotechnology, University of Mysore, Mysore, India.

***DNA extraction***

DNA was extracted from 0.5 to 1.0 g of fresh mycelium according to the modified method of Saghai-Maroof *et al*., (1984). A portion of the genomic DNA was diluted to 50 ng/µl for use in PCR and stored at –20ºC or –80ºC.

***RAPD Analysis***

Genetic diversity among isolates of *Pestalotiopsis* from *Azadirachta indica* (AI), *Holarrhena antidysenterica* (HA), *Terminalia arjuna* (TA) and *T. chebula* (TC) (Table 1) was assessed using RAPD. Twenty-three primers v*iz*., A-03, A-11, A-13 and B 01-20 (Operon technologies inc., USA), were used for reproducible banding patterns in this study. Primers were selected based on a preliminary study. Polymerase chain reaction (PCR) amplifications for RAPD analysis were performed in a total reaction volume of 25 µl containing 1X PCR buffer, 2 mM of each dNTP, 2.5 mM $MgCl_2$, 5 pM of each primer, 1.5 units *Taq* DNA polymerase (Bangalore Genie, India) and 50 ng of template DNA. RAPD PCR amplifications were performed in a thermal cycler (Biometra) with an initial denaturing step of 94ºC for 3 min, followed by 45 amplification

**Table 1.** Endophytic fungi, *Pestalotiopsis* and *Bartalinia* isolated from different locations in southern India.

| Sl.No. | Species | Isolate code name | Source | Locations |
|---|---|---|---|---|
| | ***Terminalia arjuna*** | | | |
| 1 | *Pestalotiopsis* sp1 | TA-4 | Bark | Mysore |
| 2 | *Pestalotiopsis clavispora* | TA-6 | Root | Srirangapatna |
| 3 | *Pestalotiopsis clavispora* | TA-8 | Root | Srirangapatna |
| 4 | *Pestalotiopsis theae* | TA-37 | Bark | Mysore |
| 5 | *Pestalotiopsis microspora* | TA-38 | Bark | Mysore |
| 6 | *Pestalotiopsis microspora* | TA-57 | Twig | Mysore |
| 7 | *Pestalotiopsis* sp2 | TA-59 | Root | Srirangapatna |
| 8 | *Pestalotiopsis* sp3 | TA-60 | Root | Srirangapatna |
| 9 | *Pestalotiopsis microspora* | TA-63 | Twig | Nanjangud |
| 10 | *Pestalotiopsis microspora* | TA-70 | Bark | Srirangapatna |
| 11 | *Pestalotiopsis* sp4 | TA-73 | Twig | Nanjangud |
| 12 | *Pestalotiopsis microspora* | TA-76 | Bark | Mysore |
| 13 | *Pestalotiopsis theae* | TA-100 | Twig | Nanjangud |
| 14 | *Pestalotiopsis microspora* | TA-102 | Root | Srirangapatna |
| 15 | *Pestalotiopsis microspora* | TA-103 | Bark | Srirangapatna |
| 16 | *Pestalotiopsis microspora* | TA-112 | Bark | Nanjangud |
| 17 | *Pestalotiopsis microspora* | TA-118 | Bark | Mysore |
| 18 | *Pestalotiopsis microspora* | TA-122 | Bark | Nanjangud |
| 19 | *Pestalotiopsis microspora* | TA-126 | Bark | Nanjangud |
| | ***Terminalia chebula*** | | | |
| 20 | *Pestalotiopsis virgatula* | TC-315 | Bark | Gopalaswamy hills |
| 21 | *Pestalotiopsis virgatula* | TC-319 | Bark | Gopalaswamy hills |
| 22 | *Pestalotiopsis virgatula* | TC-320 | Bark | Gopalaswamy hills |
| 23 | *Pestalotiopsis microspora* | TC-324 | Bark | Gopalaswamy hills |
| 24 | *Pestalotiopsis microspora* | TC-326 | Bark | Gopalaswamy hills |
| 25 | *Pestalotiopsis microspora* | TC-9 | Bark | Gopalaswamy hills |
| 26 | *Pestalotiopsis microspora* | TC-13 | Bark | Gopalaswamy hills |
| | ***Azadirachta indica*** | | | |
| 27 | *Bartalinia robillardoides* | AZ-32 | Bark | Mysore |
| 28 | *Pestalotiopsis microspora* | AZ-35 | Bark | Mysore |
| 29 | *Bartalinia robillardoides* | AZ-46 | Bark | Mysore |
| 30 | *Pestalotiopsis microspora* | AZ-71 | Bark | Nanjangud |
| 31 | *Pestalotiopsis microspora* | AZ-75 | Bark | Nanjangud |
| | ***Holarrhena antidysenterica*** | | | |
| 32 | *Pestalotiopsis theae* | HA-33 | Root | Sullia |

cycles of 94ºC for 1 minute, 36ºC for 1 minute, and 72°C for 2 minutes and a final extension step of 72°C for 10 minutes. PCR amplification products were electrophoretically separated on 1.4% Agarose gels at 100V for 2 hours in 1X TBE buffer (89 mM Tris, 89 mM Boric acid, and 2 mM EDTA), stained with ethidium bromide, visualized under 300 nm UV light and photographed.

RAPD data from the amplifications were recorded by scoring polymorphic DNA bands and were compiled in a binary matrix in which 1 indicated presence and 2 absence. 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) (Miller, 1997).

### *Antifungal activity*

#### Preparation of fungal extract

*Pestalotiopsis* species was grown in 500 ml volume contained in 1 L Erlenmeyer flasks for 21 days at 23ºC under static conditions with potato dextrose medium (Himedia, India). Fungal mycelia were separated from the culture filtrate by passing through four layers of cheesecloth. The filtrate was extracted with equal volumes of ethyl acetate and ultimately dried by flash evaporation. Six fungal species obtained from the Culture Collection Centre, Dept. of Applied Botany and Biotechnology, University of Mysore, India, were used as the antimicrobial test strains: *Alternaria carthami*, *Fusarium oxysporum*, *F. verticilloides Macrophomina phaseolina*, *Phoma sorghina* and *Sclerotinia sclerotiorum*. These fungi were maintained on PDA at 28°C. The agar disk diffusion method was used to test antimicrobial activity of fungal extract using potato dextrose agar medium (Lemriss *et al*., 2003).

#### Preparation of inoculum

The fungal inoculum was prepared from 5 to 15 day old culture grown on PDA medium. The Petri dishes were covered with 10 mL of distilled water and the conidia were scraped using sterile spatula. The spore density of each inoculum was adjusted with spectrophotometer ($\lambda$ = 530 nm) to obtain a final concentration of approximately $10^4$ spores/mL. The antifungal inhibitor Nystatin (Himedia, India) was used as a control. Each disk (Whatman filter paper) containing 50 µg of fungal extract (5 mg/mL) was applied on PDA medium, previously inoculated with test strain. Plates were first kept at 4ºC for at least 2 hours to allow the diffusion of chemicals, and then incubated at 28ºC. Inhibition zones were measured after 48 hours of incubation. Experiments were carried out in two replicates and the means of the inhibition zones were calculated (Table 2).

**Table 2.** Antifungal activity of *Pestalotiopsis* and *Bartalinia* extracts (50 µg/disc) against the fungal strains tested by disc-diffusion assay.

| Sl. No. | Culture number | Fungal isolates | *Fusarium oxysporum* | *Macrophomina phaseolina* | *Alternaria carthami* | *Sclerotinia sclerotiorum* | *Fusarium verticilloides* | *Phoma sorghina* |
|---|---|---|---|---|---|---|---|---|
| | | | Inhibition zone diameter around test disc | | | | | |
| Nystatin* | | | 12 | 10 | 23 | 13 | 18 | 16 |
| Negative control | | | - | - | - | - | - | - |
| 1 | TA-4 | *Pestalotiopsis* sp. 1 | - | 4 | 22 | - | 20 | - |
| 2 | TA-6 | *Pestalotiopsis clavispora* | - | - | 25 | - | - | 6 |
| 3 | TA-8 | *Pestalotiopsis clavispora* | - | - | - | - | - | - |
| 4 | TA-37 | *Pestalotiopsis theae* | - | - | 15 | - | 8 | 6 |
| 5 | TA-38 | *Pestalotiopsis microspora* | - | - | - | - | - | - |
| 6 | TA-57 | *Pestalotiopsis microspora* | - | - | - | - | - | - |
| 7 | TA-59 | *Pestalotiopsis* sp. 2 | - | - | 10 | - | 10 | - |
| 8 | TA-60 | *Pestalotiopsis* sp. 3 | - | - | 12 | - | 13 | - |
| 9 | TA-63 | *Pestalotiopsis microspora* | - | - | 17 | - | 15 | 6 |
| 10 | TA-70 | *Pestalotiopsis microspora* | - | - | 18 | - | 6 | - |
| 11 | TA-73 | *Pestalotiopsis* sp. 4 | - | 5 | 14 | - | 12 | 8 |
| 12 | TA-76 | *Pestalotiopsis microspora* | - | - | - | - | - | - |
| 13 | TA-100 | *Pestalotiopsis theae* | 3 | - | 8 | - | 8 | - |
| 14 | TA-102 | *Pestalotiopsis microspora* | - | - | - | - | - | - |
| 15 | TA-103 | *Pestalotiopsis microspora* | - | 4 | 15 | - | 8 | 14 |
| 16 | TA-112 | *Pestalotiopsis microspora* | - | - | 23 | - | 6 | - |
| 17 | TA-118 | *Pestalotiopsis microspora* | - | - | - | - | - | - |
| 18 | TA-122 | *Pestalotiopsis microspora* | - | - | - | - | 12 | 4 |
| 19 | TA-126 | *Pestalotiopsis microspora* | - | - | - | - | - | 12 |
| 20 | TC-315 | *Pestalotiopsis virgatula* | - | - | - | - | - | - |
| 21 | TC-319 | *Pestalotiopsis virgatula* | 2 | 6 | - | - | 8 | - |
| 22 | TC-320 | *Pestalotiopsis virgatula* | - | - | - | - | - | - |
| 23 | TC-324 | *Pestalotiopsis microspora* | - | - | 6 | - | - | - |
| 24 | TC-326 | *Pestalotiopsis microspora* | - | - | - | 8 | - | - |
| 25 | TC-9 | *Pestalotiopsis microspora* | - | - | - | - | - | - |
| 26 | TC-13 | *Pestalotiopsis microspora* | 6 | - | 4 | - | - | - |
| 27 | AZ-32 | *Bartalinia robillardoides* | - | - | - | - | - | - |
| 28 | AZ-35 | *Pestalotiopsis microspora* | - | 6 | - | - | - | - |
| 29 | AZ-46 | *Bartalinia robillardoides* | - | - | - | - | - | - |
| 30 | AZ-71 | *Pestalotiopsis microspora* | - | - | - | - | 9 | - |
| 31 | AZ-75 | *Pestalotiopsis microspora* | 5 | - | - | - | - | - |
| 32 | HA-33 | *Pestalotiopsis theae* | - | - | - | 6 | - | - |

* 10 µg/ ml

## Results

Nineteen isolates of *Pestalotiopsis* species were obtained from inner bark, twigs and roots of *Terminalia arjuna* growing in the Mysore, Srirangapatna and Nanjangud regions of Karnataka. Seven isolates were obtained from *Terminalia chebula* from Gopalaswamy hills. Three isolates of *Pestalotiopsis* and two isolates of *Bartalinia robillardoides* were isolated from *Azadirachta indica* from Mysore and one isolate of *Pestalotiopsis theae* from *Holarrhena antidysenterica* growing in Sullia region. RAPD profiles were generated for 30 isolates of *Pestalotiopsis* spp. and two isolates of *B. robillardoides* using 23 primers. A total of 241 polymorphic bands were scored, with an average of 10.4 bands per primer. Each amplification was repeated twice to confirm the reproducibility and only reproducible bands were considered for scoring and analysis. Portions of gels showing typical amplification products are shown in Fig. 1.

Cluster analysis with UPGMA using genetic distances was performed to generate a dendrogram (Figs 2, 3) illustrating the overall genetic relationships within the species studied. In the present study, there were four main groups (Fig. 2). Group I contained 12 isolates, group II contained 3 isolates of *P. virgatula*, group III contained ten isolates i.e. *P. microspora*, *Bartalinia robillardoides*, *Pestalotiopsis theae* and *Pestalotiopsis* spp., group IV contained five isolates of *P. microspora*. One *Pestalotiopsis* spp. did not fall into any group. RAPD analyses (Fig. 1) from morphologically similar and dissimilar taxa from the same and different hosts were analyzed in order to establish whether the *Pestalotiopsis* species clustered together. The isolates from *Azadirachta indica* were distant from each other and they clustered with isolates of *Terminalia arjuna* and *T. chebula* independent of the host from which they were isolated. In addition, the two endophytic isolates *Bartalinia robillardoides* from *Azadirachta indica* appeared to be similar and grouped together. Isolates of *Pestalotiopsis theae*, isolated from the bark of *Terminalia arjuna* at Mysore appeared in a separate group. Another interesting example is the relationship of five *Pestalotiopsis microspora* (6, 5, 17, 23 and 31) isolates of group IV, which were isolated from *Terminalia arjuna*, *T. chebula* and *Azadirachta indica* respectively falling into one group.

Eighteen isolates of *Pestalotiopsis microspora* collected from different host and locations were also analyzed for genetic diversity. *Pestalotiopsis microspora* isolates did not show any similarity and they could not be grouped into more specific clusters.

The antifungal activities of the ethyl acetate extracts obtained by *Pestalotiopsis* spp. fermentation were quantitatively assessed by the presence

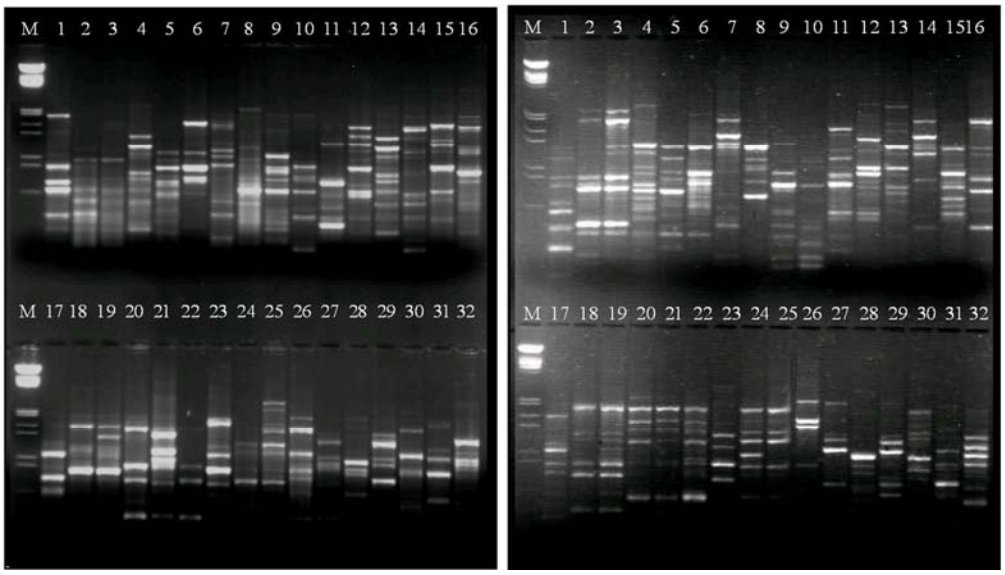


**Fig. 1**. Agarose gel showing RAPD products of *Pestalotiopsis* and *Bartalinia* isolates obtained by amplifying 50 ng of DNA using the Primer OPB-05 (Fig 1a) and OPB-17 (Fig. 1b). Lane numbers 1 to 32 correspond to the strain numbers in Table 1.

or absence of inhibition zones and zone diameters (Table 2). Results showed that the extracts from 20 (62.5%) isolates inhibited the growth of most of the pathogenic fungi. Higher inhibition was observed for *Alternaria carthami*, *Fusarium verticilloides and Phoma sorghina*. Maximal inhibition zones for the fungi sensitive to the ethyl acetate extract of *Pestalotiopsis* were in the range of 4–25 mm (Table 2). Isolates 4, 9, 11 and 15 showed significant inhibition zones against *Alternaria carthami*, *Fusarium verticilloides* and *Phoma sorghina*. Minimum inhibition zones were observed for isolates obtained from *Terminalia chebula*, *Azadirachta indica*, and *Holarrhena antidysenterica* isolated from different locations for all the six fungi tested (Table 2).

## Discussion

Earlier studies from our laboratory had observed that very large numbers of fungal individuals occupy habitats such as tree roots and inner bark (data not shown). The inner bark and twig taken from a single *Terminalia arjuna* tree had yielded 22 genera of fungi that could be readily identified, in addition to few unidentifiable mycelial fungi that could also be cultured (Tejesvi *et al*., 2005). Endophytic fungal diversity has been predicted essentially in the leaves

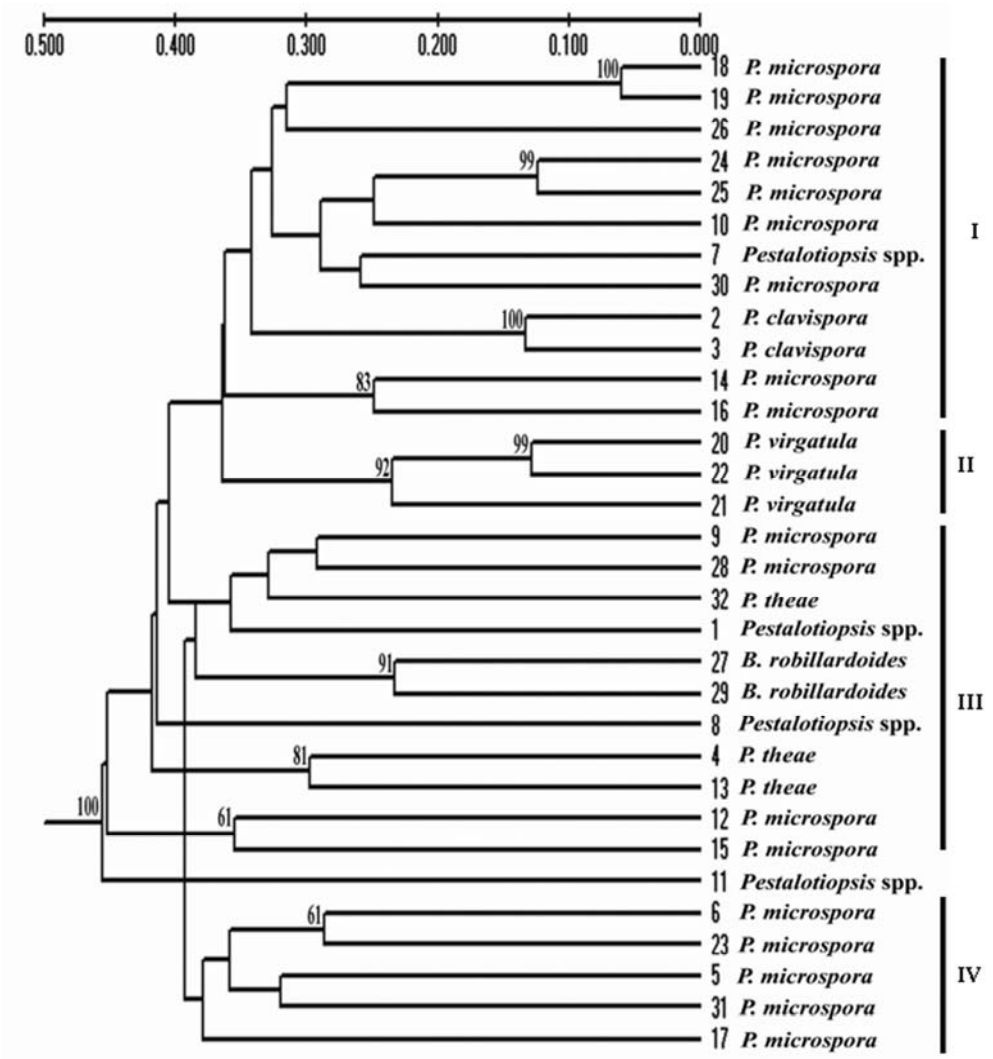


**Fig. 2.** Unweighted pair group method with average (UPGMA) cluster diagram of the relationships between 30 *Pestalotiopsis* and 2 *Bartalinia* isolates. The dendrogram was based on presence or absence of bands (Fig. 1). The internal nodes display more than 50% bootstrapping value using 1000 replicate bootstrapping analysis.

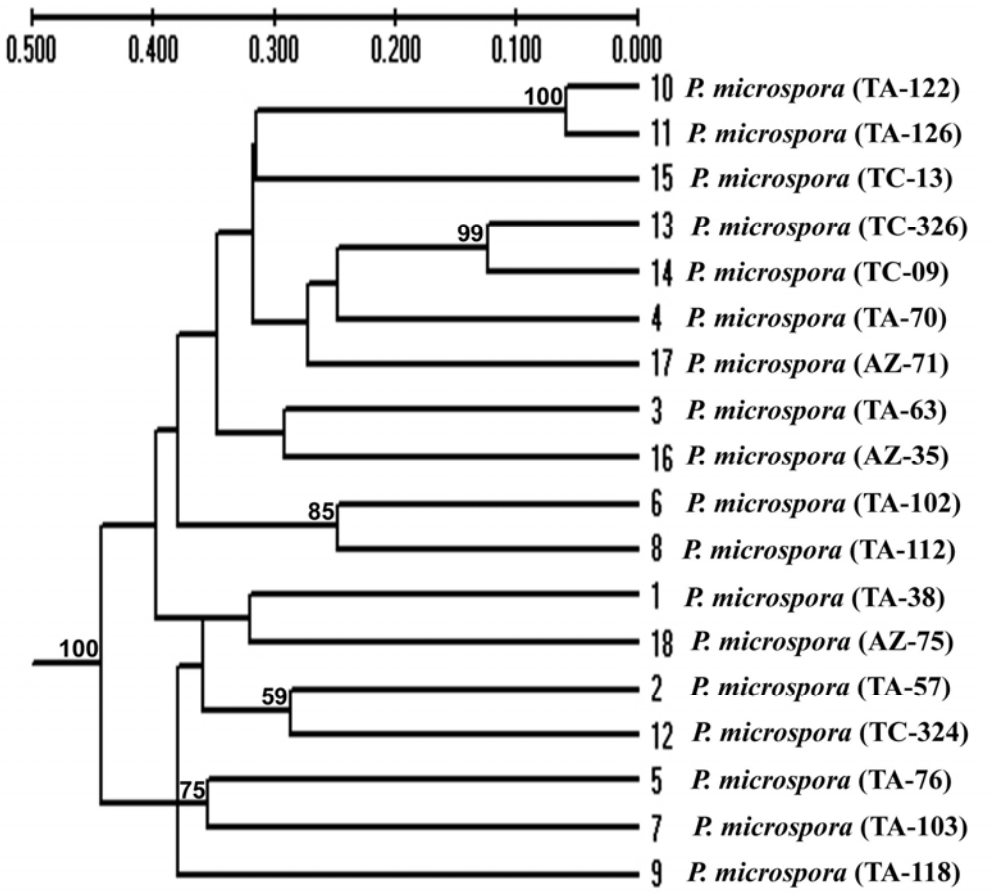


**Fig. 3.** Unweighted pair group method with average (UPGMA) cluster diagram of the relationships between 18 endophytic *Pestalotiopsis microspora* of *T. arjuna*, *A. indica* and *T. chebula* isolated from different locations. The internal nodes display more than 50% bootstrapping value using 1000 replicate bootstrapping analysis.

of many angiosperm taxa of the tropics (Arnold *et al*., 2000; Promputtha *et al*., 2005). The majority of reported endophytes are from leaves and twigs (Cannon and Simmons, 2002; Arnold *et al*., 2003; Suryanarayanan *et al*., 2003; Promputtha *et al*., 2005) and very few reports are available on the isolation and diversity of endophytes from inner bark and roots of the tropical plants (Bills and Polishook 1991; Mahesh *et al*., 2005; Nalini *et al*., 2005 Tejesvi *et al*., 2005). Sixty-nine fungal species were isolated from the bark of a single

*Carpinus caroliniana* tree (Iron wood) by Bills and Polishook (1991) which suggested the enormous extent of fungal diversity in a single plant.

Diversity studies of endophytes from the inner bark and root using molecular techniques is not common. In the present study, RAPD was used to analyze genetic diversity among isolates of endophytic *Pestalotiopsis* species, which were collected from different locations and from different medicinal trees. *Pestalotiopsis microspora* isolated from *Azadirachta indica*, *Terminalia arjuna* and *T. chebula* had greater diversity (Fig. 3). The grouping was not based on host or place of isolation. The genetic differences among the isolates of *Pestalotiopsis microspora* may possibly be due to their isolation from diverse hosts and distinct geographical regions. An analysis of the genetic distance of isolates obtained from four medicinal plants (Table 1) revealed a few key findings: (1) In general, isolates contained within a single plant were genetically diverse (Fig. 3). (2) Isolates did not share any monomorphic markers and this also suggests they are genetically diverse. The results of this study showed a high degree of genetic diversity among the isolates of *P. microspora* (Fig. 3). Wang *et al*. (1997) has described high genetic variability employing RAPD for *Gremmeniella abietina* isolated from one single host tree.

The results herein indicate that *Pestalotiopsis* species, despite being isolated from a small number of host and from different regions, possess considerable diversity in morphology and isolates grouped together based on similarities in conidial morphology as elaborated by Jeewon *et al*. (2002, 2003). Our studies also suggest that in the range of 40 km there is abundant diversity of endophytes as it differs in plant diversity, species composition and annual rainfall. Species of *Chaetomium*, *Pestalotiopsis*, *Myrothecium*, *Penicillium* and *Trichoderma* were the most frequently isolated endophytic fungi from the selected medicinal plants (data not shown).

The isolates from *Terminalia chebula* (TC-315, TC319, TC-320, TC-324, and TC-9) grouped together apart from other pairs, but isolates were distinguishable from each other (Fig. 2). RAPD techniques can be employed to detect genetic diversity as well as metabolic variation by checking for different activities. RAPD markers did not differentiate and place the isolates into respective host or locations from which they were isolated. The relationship between species isolated from the same or different hosts do not support phylogenetic analysis and also morphologically similar species form close relationships rather than the isolates of the same host (Jeewon *et al*., 2004). Multiple strains of *Pestalotiopsis* spp. can occur on a single or multiple hosts has been shown in the present and previous studies (Jeewon *et al*., 2003, 2004)

The study on endophytic fungi from medicinal plants has received much attention in recent years as they are believed to be an excellent source of biologically active compounds. In the present study, 32 of the fungal extracts that were tested for antifungal activity against six plant pathogens, many showed inhibitory activities. The isolates which have minimal activity or no activity against the six pathogens tested clustered separately in group IV (5, 6, 17, 23 and 31) and group II (20, 21 and 22). The isolates (18, 19, 10, 7 and 2) showing higher antifungal activity (>10 mm) for one or more fungi grouped with the isolates without antifungal activity (26, 24, 25, 30 and 3) (< 10 or no activity) in the first cluster. Isolates 11, 12 and 15 showing inhibitory activities against two or more fungi clustered separately with a genetic distance of 0.45 from other isolates. Furthermore, isolate 11 does not cluster with any other isolates in the study. Interestingly, *Bartalinia robillardoides* (27 and 29) that did not had any inhibitory effect on any of the fungi, also grouped together in cluster III.

Antimicrobial activities of compounds biosynthesized by the plant endophytes have been reported only by a few groups (Nishioka *et al*., 1997; Huang *et al*., 2001; Li *et al*., 2001; Harper *et al*., 2003; Strobel *et al*., 2001; Strobel *et al*., 2002). Liu *et al*., (2001) has evaluated 39 endophytes for *in vitro* antifungal activity against crop-threatening fungi *Gaeumannomyces graminis* var. *tritici*, *Rhizoctonia cerealis*, *Helminthosporium sativum*, *Fusarium graminearum*, *Gerlachia nivalis* and *Phytophthora capsici*. Out of 39 endophytes investigated, 21 were shown to have *in vitro* antifungal activity to all or a few of the tested phytopathogens.

*Pestalotiopsis* species are of considerable interest to researchers and pharmacists due to their capabilities of synthesizing a wide range of economically important bioactive molecules (Strobel, 2002; Tomita, 2003). It would be wise to screen as many isolates of *Pestalotiopsis* as possible for screening for bioactives if the taxonomic identification is made only to genus level (Jeewon *et al*., 2004). The large variation in *P. microspora* isolated from *Terminalia arjuna* from different regions shown by RAPD-PCR suggests that the species varies genetically (Fig. 3). This also suggests that RAPD-PCR may be useful for genetic variation studies. In addition, it may provide useful keys to select the isolates for further screening based on the RAPD profiles. This method can be employed for (1) differentiating the isolates for screening for bioactives (2) preliminary screening to reduce the cost of fermentation and laborious work of screening for bioactives (3) de-replicating the isolates and (4) selection of more diverse group of fungi with in the genus (isolate 11 in our study). Assessing the potential metabolic diversity of a fungal species cannot be adequately performed by investigating a single representative isolate

because significant metabolic diversity is present among isolates of a single species collected from a single site (Seymour *et al*., 2004). This emphasizes the enormous capacity for secondary metabolic variation that appears to exist in fungal genomes (Galagan *et al*., 2003).

In conclusion, the lack of relationship between *Pestalotiopsis* spp., host and locations was not surprising. Host specificity is poorly defined for this species and is differently expressed under various conditions in the same host (Jeewon *et al*., 2004). Results of this study confirm the presence of inhibitor(s) in the ethyl acetate extract of *Pestalotiopsis* spp. This provides an insight for exploring the antifungal bioactives for better management of fungal pathogens. Furthermore, from this study, the *Pestalotiopsis* spp. (TA-73) can be characterized for novel metabolites based on the RAPD profile.

## Acknowledgments

The authors thank Department of Biotechnology (DBT), India for providing financial support and Sampath Kumara, PhytoMyco Research Pvt. Ltd. Nanjangud, for his help in identification of the plant samples. Mr. Tejesvi is grateful to the Indian Council of Medical Research (ICMR) for the award of a Senior Research Fellowship (SRF), and also to Dr. Nalini and Mr. Mahesh for their generous advice in the laboratory.

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(Received 25 November 2005; accepted 15 October 2006)