Phylogenetic analysis of endophytic *Pestalotiopsis* species from ethnopharmaceutically important medicinal trees

Tejesvi[1,2], M.V., Tamhankar, S.A.[3], Kini, K.R.[1], Rao. V.S.[3] and Prakash, H.S.[1*]

[1]Department of Biotechnology, University of Mysore, Manasagangotri, Mysore-570 006, Karnataka, India.
[2]Department of Biology, PO Box 3000, FIN-90014 University of Oulu, Finland
[3]Agharkar Research Institute, Agharkar road, Pune, Maharashtra, India

Tejesvi, M.V., Tamhankar, S.A., Kini, K.R., Rao, V.S. and Prakash, H.S. (2009). Phylogenetic analysis of endophytic *Pestalotiopsis* species from ethnopharmaceutically important medicinal trees. Fungal Diversity 38: 167-183.

The phylogenetic diversity in 30 endophytic *Pestalotiopsis* strains and two *Bartalinia robillardoides* strains isolated from the medicinal plants *Azadirachta indica*, *Holarrhena antidysenterica*, *Terminalia arjuna* and *T. chebula* were analyzed using internal transcribed spacer (ITS) restriction fragment length polymorphism (ITS-RFLP) and sequence analysis of the ITS region of ribosomal DNA (rDNA). The amplified rDNA fragment length ranged from 548-607 bp. rDNA ITS sequence analysis provided greater resolution for distinguishing isolates of *Pestalotiopsis* than ITS-RFLP analysis with five restriction enzymes Alu I, Hae III, Ava II, Hpa II and Taq I. The ITS-RFLP profiles were highly distinctive for *P. virgatula* and *P. theae* and also exhibited a high level of intraspecific polymorphism in strains of *P. microspora*. Endophytic and pathogenic *Pestalotiopsis* species clustered into distinct groups based on analysis of ITS sequences. The clusters grouped irrespective of the host or parts of the trees or locations (Mysore, Srirangapatna, Nanjungud, Gopalswamy hills and Sullia) from which they were isolated. Further studies on *Pestaliotiopsis* endophytes and the naming of species are suggested.

Key words: genetic diversity, ITS sequences, ITS-RFLP, polymorphism, pathogens, rDNA.

Article Information
Received 5 May 2009
Accepted 16 July 2009
Published online 1 October 2009
*Corresponding author: Tejesvi, M.V.; e-mail: hsp@appbot.uni-mysore.ac.in; mvtejesvi@gmail.com

Introduction

Pestalotiopsis species are anamorphic members of the family *Amphisphaeriaceae* (Kang *et al*., 1999) and there are 230 species listed in *Index Fungorum* (http://www.index fungorum.org/Names/Names.asp) and they are usually found in tropical and subtropical plants throughout the world (Jeewon *et al*., 2004; Tejesvi *et al*., 2007, 2008; Ding *et al*., 2009; Liu *et al*., 2009). *Pestalotiopsis* species are widespread plant pathogens (Zhang *et al*., 2003) or saprotrophs on bark and decaying plant material (Agarwal and Chauhan, 1988) or degraders of plant material (Osono and Takeda, 1999) and also saprobes of decaying wild fruits (Tang *et al*., 2003). *Pestalotiopsis theae* has been identified as pathogenic on tea, *Camellia sinensis* (Koh *et al*., 2001). *Pestalotiopsis* species appears to be genetically diverse as almost every isolate has distinct biochemical and phenotypic traits (Li *et al*., 1996). *Pestalotiopsis* species have gained much attention and importance in recent years as they produce many important secondary metabolites (Strobel, 2002; Harper *et al*., 2003; Kumar *et al*., 2004).

Endophytes are microorganisms that colonize and cause unapparent asymptomatic infections in healthy plant tissues (Wilson, 1995; Hyde and Soytong, 2008) and temperate and tropical plants and even lichens may harbor numerous endophytic species (Kumar and Hyde, 2004; Gonthier *et al*., 2006; Li *et al*., 2007; Sánchez Márquez *et al*., 2007, 2008; Rungjindamai *et al*., 2008; Tao *et al*., 2008). Endophyte diversity in woody plants is little understood and estimations of their diversity have been based on the ‘‘morphospecies’’ concept (Lacap *et al*., 2003) or morphological similarity to known species (e.g. Ganley *et al*., 2004; Wei *et al*., 2007a; Oses *et al*., 2008). The

majority of the endophytes reported are from leaves and twigs (e.g. Fröhlich *et al*., 2000; Photita *et al*., 2001; Huang *et al*., 2008), but there are very few reports pertaining to the isolation and diversity of endophytes from the inner bark and roots of the tropical plants (Tejesvi *et al*., 2007; Oses *et al*., 2008).

Fungal species have been separated and described traditionally on the basis of morphology, cultural characteristics including pigment production, presence of microconidia and chlamydospores and new species are still described on such basis (Rheeder *et al*., 1996; Leslie *et al*., 2005; Zhao *et al*., 2007). Morphology has limitations as fungal characters can change depending upon host and environment (Egger, 1995). The number of morphological markers available is also generally low, which makes delimitation from related species difficult (Magnani *et al*., 2005). In recent years, rapid developments in molecular techniques and multidisciplinary approaches have had a great impact on fungal taxonomy (Tan and Niessen, 2003; Phillips *et al*., 2007; Shenoy *et al*., 2007a; Zhu *et al*., 2008; Thongkantha *et al*., 2009).

A more precise assessment of diversity and identification of fungi can be achieved using the polymerase chain reaction (PCR) and restriction fragment length polymorphisms (RFLP) of the internal transcribed spacer (ITS) of the fungal nuclear ribosomal DNA repeat (Egger, 1995). The PCR-RFLP technique with specific oligonucleotide primers ITS 1 & ITS 4 (White *et al*., 1990) and restricting with different endonucleases, has been successfully used to analyze regions of ribosomal DNA of various groups of fungi (Gomes *et al*., 1999; Glen *et al*., 2001; Cai *et al*., 2006; Shenoy *et al*., 2007a; Hyde and Soytong, 2008) and can be considered as a useful tool. Sequence analysis of the ITS region of nuclear ribosomal DNA has been widely used for molecular identification and phylogenetic diversity studies of fungi. Their have also been some studies on the phylogeny and diversity of endophytes isolated from inner bark and roots of medicinal plants using molecular methods, especially in the identification of morpho-species (Lacap *et al*., 2003; Promputtha *et al*., 2005). Earlier studies from our laboratory have shown that *Pestalotiopsis* is one of the most dominant genera in *Azadirachta indica*, *Holarrhena antidysenterica*, *Terminalia arjuna* and *T. chebula* (Mahesh *et al*., 2005; Tejesvi *et al*., 2005, 2006, 2007). The aim of the present study was to isolate *Pestalotiopsis* species from these trees, to assess (a) the phylogenetic diversity of the isolated species and attempt to identify them, (b) analyze the endophytic and pathogenic *Pestalotiopsis* species.

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, Srirangapatna, Gopalaswamy hills, Sullia and Mysore regions of South India (Table 1). Two to three bark samples of 2 × 4 inches were collected from the trunk regions, about 1.5-2 m above ground level. Four to five roots and twigs were collected from the respective host plants. Each sample after collection was tagged and placed in a separate polythene bag, brought to the laboratory and processed within 24 hours of collection.

Isolation, identification and preservation of endophytes

Samples were cleaned thoroughly in running tap water for 5 minutes before processing. Small bark, twig and root parts were surface-disinfected by sequential washes in 70% (v/v) ethanol (1 min) and 3.5% (v/v) NaOCl (3 min), followed by rinsing with sterile water and allowed to surface dry under sterile conditions. Two hundred segments of bark, twigs and roots from each plant were collected from different locations and 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 and were incubated in a light chamber for two weeks at 12 h 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 a stereo-binocular microscope (Leica, Germany) and transferred onto potato dextrose agar (PDA) medium. Fungal spore formation was facilitated by

Table 1. Details of tree species and sampling sites employed in the study.

Plant name	Family	Plant code	Ethnobotanical use*	Site of collection	Location	Vegetation type
Terminalia arjuna W. & A.	Combretaceae	TA	Cardiotonic, febrifuge, antidysenteric	Nanjangud (Nan) Srirangapatna (Sri) Mysore (Mys)	12° 07’N & 76° 44’E 12° 25’N & 76° 40’E 11° 45’N & 75° 57’E	Riparian Dry deciduous
Azadirachta indica A. Juss	Meliaceae	AZ	Skin troubles, rheumatism	Mysore (Mys)	11° 45’N & 75° 57’E	Dry deciduous
Holarrhena antidysenterica Wall. Ex. DC	Apocynaceae	HA	Heart disease, diarrhoea	HD Kote (HDK) Kharapur (KA) Sullia (SU)	11° 25’N & 76° 40’E 11° 25’N & 76° 40’E 12° 71’N & 75° 68’E	Deciduous
Terminalia chebula Retz.	Combretaceae	TC	Diarrhoea, skin diseases	Gopalaswamy Hills (GH)	11° 57’N & 75° 12’E	Dry deciduous

*Source: Yoganarasimhan (1996).

inoculating cultures on sterilized banana leaf pieces amended with the PDA medium. Identification of species was achieved based on the morphological and taxonomic keys provided by Steyaert (1949), Guba (1961), Sutton (1980) and Nag Raj (1993). Isolates of *Pestalotiopsis* were maintained in cryovials on PDA layered with 15% glycerol (v/v) at -80ºC at Department of Biotechnology, University of Mysore, Mysore, India.

Selection of isolates and preparation of single-spore cultures

Thirty strains of *Pestalotiopsis* and two strains of *Bartalinia robillardoides* were selected for this study based on their morphological characters, occurring on different hosts and five different locations (Table 1). Nineteen isolates of *P. microspora* were isolated from inner bark, twigs and roots of *T. arjuna* growing in Mysore, Srirangapatna and Nanjangud regions of Karnataka, seven isolates from *T. chebula* from Gopalaswamy hills, three isolates from *A. indica* from Mysore, and one isoate from *H. antidysenterica* growing in Sullia region. Two isolates of *Bartalinia robillardoides* isolated from *A. indica* were used as outgroup for comparison. For each strain, conidia were isolated, and single spore cultures were grown on PDA at 25 ^{o}C for 7-10 days.

DNA extraction and amplification

For cultivation of fungi, three mycelial plugs taken from actively growing colony margins using a 5-mm diameter cork borer were inoculated in a 100 ml Erlenmeyer flasks containing 50 ml potato dextrose broth. The isolates were grown in still culture at 25°C in the dark. The fungi were grown for 7 to 10 days, harvested and stored at -80°C until DNA extraction. DNA was extracted from 0.5 to 1.0 g of mycelium according to the modified method of Saghai-Maroof *et al*. (1984). DNA concentration was estimated by measuring absorbance at 260 and 280 nm using a spectrophotometer. Target regions of the rDNA ITS (ITS1, 5.8S, ITS2) were amplified symmetrically using primers ITS 1 and ITS 4 (White *et al*., 1990). Amplifications were performed in a total reaction volume of 25 µl containing 200 µM of each dNTP, 2.5 mM $MgCl_2$, 5 pM of each primer, 1.5 units *Taq* DNA polymerase and 50 ng of template DNA. PCR amplifications were performed in a thermal cycler (Biometra, 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 min. PCR amplification products were electrophoretically separated on 1.5% (w/v) agarose gels at 100 V for 2 hours in 1x TBE buffer (89 mM Tris, 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. A 100 bp size marker was used as reference (Bangalore Genei, India).

Selection of restriction enzyme

Restriction enzymes were selected based on the restriction sites present in the ITS region of *Pestalotiopsis* spp. sequence data of representative strains from the National Center for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov/BLAST/>). These sequences were obtained and analyzed for restriction sites using Gene Runner software available for free (http://www.genelink.com/tools/gl-downloads.asp). Restriction enzymes, *Alu* I, *Hae* III, *Ava* II, *Hpa* II and *Taq* I (Sigma Aldrich, India) were selected for the study.

Restriction digestion

The RFLP analysis was carried out by precipitating the PCR product with 100 mM NaCl and 2.5 volumes of absolute ethanol. Samples were kept at -20°C for 2 h and centrifuged at 10,000 rpm for 15 min. The pellet was washed with 70% (v/v) ethanol, resuspended in 10 µl of water and digested with different restriction enzymes at 37°C. The DNA fragments were size-fractionated with 2.5% (w/v) agarose gel electrophoresis, stained with ethidium bromide (0.5 µg/ml) and photographed under UV light.

ITS-RFLP data analysis

The ITS-RFLP data were recorded by scoring all DNA bands and were compiled in a binary matrix in which 1 indicated presence and 2 absence of a fragment. The data were converted to distance matrices based on Nei (1978) unbiased minimum distance. The

distance matrices were then used to construct a dendrogram by the unweighted pair-group method with arithmetic mean (UPGMA) using Tools for Population Genetic Analysis (TFPGA v1.3) (Miller, 1997).

Sequence assembly and alignment

Amplification products obtained from PCR reactions with unlabeled ITS primers (ITS1 and ITS4) were used for sequencing. Sequencing reactions were carried out using Big Dye Terminator Cycle Sequencing kit v3.1 (Applied Biosystems, Foster City, CA, USA), as per the manufacturer's instructions. Extension products were then purified using an ethanol/EDTA precipitation protocol and analyzed on an ABI 3100 *Avant* Genetic analyzer (Applied Biosystems, Foster City, CA, USA) as recommended by the manufacturer. DNA sequences obtained for each strain from each forward (ITS1) and reverse (ITS4) primer were inspected individually for quality. Both strands of the DNA were then assembled to produce a consensus sequence for each strain using Gene Runner software. All sequences were aligned using Clustal X with default settings (Thomson *et al*., 1997).

Phylogenetic analyses

Phylogenetic analysis was performed under weighted parsimony, with heuristic search, using PAUP v4.0b10 (Swofford, 2003). The search used the stepwise addition option and was repeated 10 times from different starting points with tree-bisection-reconnection (TBR) branch swapping. All characters were equally weighted and alignment gaps were treated as missing data or fifth base. Confidence in specific clades from the resulting topology was tested by bootstrap analysis with 1,000 replicates. Tree scores including consistency index (CI), retention index (RI), rescaled consistency (RC) index and homoplasy index (HI) were also calculated for all the trees. Kishino-Hasegawa tests (Kishino and Hasegawa, 1989) and Templeton tests (Templeton, 1983), as implemented in PAUP 4.0. Trees were viewed in Treeview (Page, 1996). *Bartalinia robillardoides* was used as the outgroup (Jeewon *et al*., 2002).

Besides the morphological characterization and ITS-RFLPs, ITS sequence analysis was also done for molecular authentication of species of *Pestalotiopsis*. Sequence data analysis was carried out by a stepwise approach. First, BLAST database searches were performed with full-length ITS fragments as queries to reveal relationships to published sequences.

Results

ITS-RFLP

The ITS region of 30 *Pestalotiopsis* strains and two *B*. *robillardoides* isolates showed extensive length polymorphisms (Fig. 1a). The length of the amplified rDNA fragment ranged from 550 to 600 bp. Based on the polymorphisms of the ITS region, the 32 isolates could be assigned into two groups, 20 *Pestalotiopsis* isolates irrespective of host or location formed the first group with a length of 550 bp (approx) and the other ten along with two strains of *B*. *robillardoides* formed a second group having a length of 600 bp (approx). The PCR products were cleaved by the restriction enzymes *Alu* I (Fig. 1b), *Hae* III, *Ava* II, *Hpa* II and *Taq* I to detect a wider range of polymorphisms. The ITS regions of most of the strains had restriction sites for each of the endonucleases tested. The restriction patterns obtained with the restriction enzymes, except with TaqI were not strain specific; several strains had the same pattern. Cleavage with *Taq* I showed additional polymorphism, however characterization of individual strains was still not possible. Restriction fragments obtained with all the endonucleases tested were used to determine genetic distances among the strains. UPGMA-analysis revealed four major clusters (Fig. 2). The first cluster contained the two strains of *B*. *robillardoides*. The second cluster contained 10 isolates of *P*. *microspora*, three isolates of *P*. *virgatula*, two isolates of *P*. *clavispora* and two *Pestalotiopsis* species (sp. 2 and 3). The third cluster contained three isolates of *P*. *theae* which could be differentiated. The fourth cluster contained seven *P*. *microspora* species and two *Pestalotiopsis* species (sp. 1 and 4). No intraspecific variation was detected in *B*. *robillardoides* and *P*. *virgatula* whereas some variations were observed among *P*. *theae* isolates, belonging to the third cluster. In contrast, isolates of the other

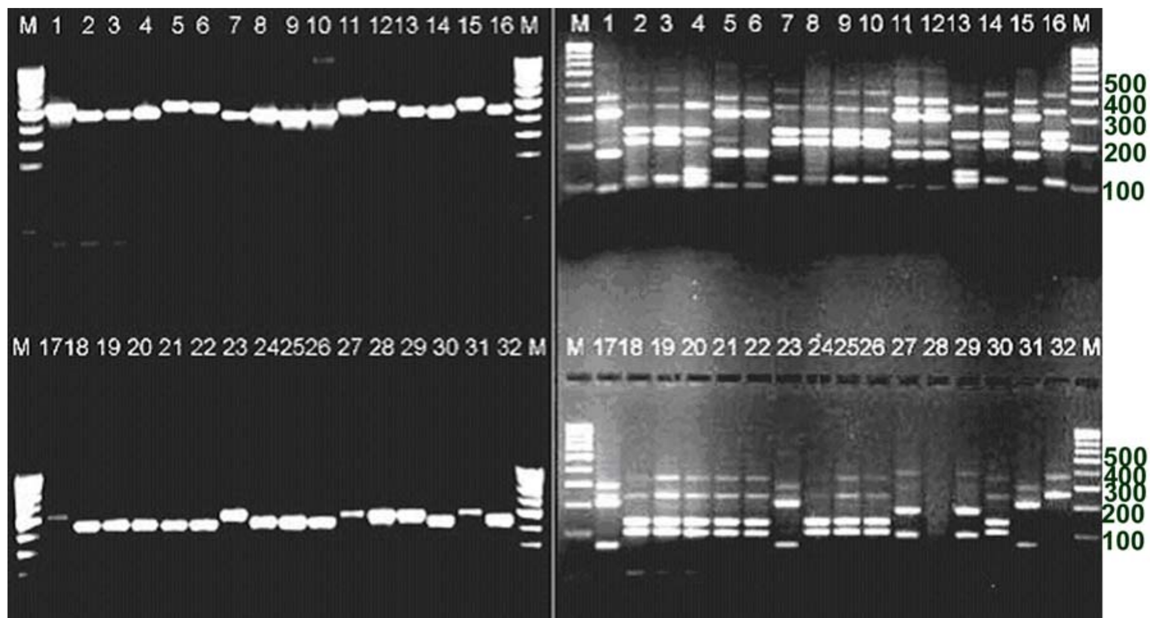

Fig. 1. Internal transcribed spacer region of *Pestalotiopsis* and *Bartalinia* (Lane 27 & 29) isolates amplified with primers ITS1 and ITS4 (A) and digested with *Alu* I (B). M, marker 100 bp ladder. Lane numbers 1 to 32 correspond to the isolates in Table 2.

Pestalotiopsis species were dispersed all over the dendrogram.

ITS sequence and phylogeny

The amplified products of the ITS regions were sequenced and analyzed. Sequenced strains with the accession numbers including their original sampling site, initial morphological characterization and the results of the BLAST searches are listed in Table 2. Phylogenetic studies revealed considerable variations among the isolates collected from four individual trees of different locations. All the sequences were aligned using Clustal X with default setting. Base frequencies across taxa (mean frequencies: A=0.25272, C=0.220 04, G=0.21883, T=0.30841) as determined by the $\chi2$ test implemented in PAUP v4.0b10 were homogeneous. Of 619 characters, 450 were constant, 19 were parsimony uninformative, and 150 were parsimony informative. The most parsimony rooted trees (total length = 475 steps; CI = 0.853; RI = 0.970) is shown in Fig. 3. Similar results were obtained using neighbor joining analyses (results not shown). Most of the clades were supported by high bootstrap values (> 60%) except two with 54% and 57% bootstrap support.

The phylogram of *Pestalotiopsis* species had five clades (Fig. 3), in which isolates of *P. microspora* are distributed across the phylogram irrespective of the trees from which they were isolated. Clade I contained *P. microspora* isolates from TA, TC and AZ. Two isolates of *P. clavispora* and *Pestalotiopsis* species (sp. 2 and 3), three isolates of *P. virgatula* and three isolates of *P. theae* are grouped in the clade II, III and IV, respectively. Clade V contained seven *P. microspora* and two unidentified *Pestalotiopsis* species (sp. 1 and 4). Two *B. robillardoides* isolates were used as outgroup.

Comparisons of the endophytic and pathogenic Pestalotiopsis species

A further phylogenetic analysis based on ITS sequences was conducted to compare endophytic *Pestalotiopsis* species sequenced in this study with pathogenic *Pestalotiopsis* species in GenBank to find out their relationships and to authenticate the endophytic species. The pathogenic *Pestalotiopsis* sequences retrieved from GenBank are listed in Table 3. Analysis of the data set with weighted parsimony analysis using gaps as missing state, yielded 54 most parsimonious trees (Fig.4). Of the 571 characters, 413 were constant, 38 were parsimony uninformative, and 120 were parsimony informative. The most parsimony tree of the 54 most parsimonious unrooted trees (total length = 860 steps; CI = 0.726; RI = 0.942) from analyses of this data set is shown in Fig. 4. Base frequencies were as follows: A=0.25407, C=0.21691, G=0.21769, T=0.31133.

Table 2. Database typing of endophytic fungi from four medicinal plants *viz., Terminalia arjuna* (TA), *Terminalia chebula (TC)*, *Azadirachta indica* (AI) and *Holarrhena antidysenterica* (HA).

Sl. No.	Fungal isolate	Accession number	Locations	Morphological characterization	Source	Closest match in the BLAST	Incl. score	Accession numbers	(%)
	Terminalia arjuna								
1	TA-4	**AY924262**	Mysore	*Pestalotiopsis* sp.1	Bark	*Pestalotiopsis neglecta*	1199	**DQ000992**	**100**
2	TA-6	**AY924263**	Srirangapatna	*Pestalotiopsis clavispora*	Root	*Pestalotiopsis clavispora*	993	**AY682929**	**98**
3	TA-8	**AY924264**	Srirangapatna	*Pestalotiopsis clavispora*	Root	*Pestalotiopsis clavispora*	993	**AY682929**	**98**
4	TA-37	**AY924265**	Mysore	*Pestalotiopsis theae*	Bark	*Pestalotiopsis theae*	991	**AY681481**	**98**
5	TA-38	**AY924266**	Mysore	*Pestalotiopsis microspora*	Bark	*Pestalotiopsis microspora*	1162	**AF377288**	**99**
6	TA-57	**AY924267**	Mysore	*Pestalotiopsis microspora*	Twig	*Pestalotiopsis microspora*	1170	**AF377288**	**99**
7	TA-59	**AY924268**	Srirangapatna	*Pestalotiopsis* sp.2	Root	*Pestalotiopsis clavispora*	993	**AY682929**	**99**
8	TA-60	**DQ402052**	Srirangapatna	*Pestalotiopsis* sp.3	Root	*Pestalotiopsis clavispora*	892	**AY682929**	**98**
9	TA-63	**AY924270**	Nanjangud	*Pestalotiopsis microspora*	Twig	*Pestalotiopsis microspora*	1021	**AF377292**	**98**
10	TA-70	**AY924271**	Srirangapatna	*Pestalotiopsis microspora*	Bark	*Pestalotiopsis microspora*	1013	**AF377292**	**98**
11	TA-73	**AY924272**	Nanjangud	*Pestalotiopsis* sp.4	Twig	*Pestalotiopsis neglecta*	1096	**DQ000992**	**98**
12	TA-76	**AY924273**	Mysore	*Pestalotiopsis microspora*	Bark	*Pestalotiopsis microspora*	1154	**AF377296**	**99**
13	TA-100	**AY924274**	Nanjangud	*Pestalotiopsis theae*	Twig	*Pestalotiopsis theae*	1037	**AY681479**	**99**
14	TA-102	**AY924275**	Srirangapatna	*Pestalotiopsis microspora*	Root	*Pestalotiopsis microspora*	1013	**AF377292**	**99**
15	TA-103	**AY924276**	Srirangapatna	*Pestalotiopsis microspora*	Bark	*Pestalotiopsis microspora*	1146	**AF377296**	**98**
16	TA-112	**AY924277**	Nanjangud	*Pestalotiopsis microspora*	Bark	*Pestalotiopsis microspora*	1021	**AF377292**	**99**
17	TA-118	**AY924278**	Mysore	*Pestalotiopsis microspora*	Bark	*Pestalotiopsis microspora*	1043	**AF377288**	**98**
18	TA-122	**AY924279**	Nanjangud	*Pestalotiopsis microspora*	Bark	*Pestalotiopsis microspora*	1007	**AF377292**	**97**
19	TA-126	**AY924280**	Nanjangud	*Pestalotiopsis microspora*	Bark	*Pestalotiopsis microspora*	1021	**AF377292**	**98**
	Terminalia chebula								
20	TC-315	**AY924281**	Gopalaswamy hills	*Pestalotiopsis virgatula*	Bark	*Pestalotiopsis virgatula*	997	**AY687880**	**98**
21	TC-319	**AY924282**	Gopalaswamy hills	*Pestalotiopsis virgatula*	Bark	*Pestalotiopsis virgatula*	997	**AY687880**	**98**
22	TC-320	**AY924283**	Gopalaswamy hills	*Pestalotiopsis virgatula*	Bark	*Pestalotiopsis virgatula*	997	**AY687880**	**98**
23	TC-324	**AY924284**	Gopalaswamy hills	*Pestalotiopsis microspora*	Bark	*Pestalotiopsis microspora*	1114	**AY377288**	**98**
24	TC-326	**AY924285**	Gopalaswamy hills	*Pestalotiopsis microspora*	Bark	*Pestalotiopsis microspora*	1021	**AY377292**	**98**
25	TC-9	**AY924286**	Gopalaswamy hills	*Pestalotiopsis microspora*	Bark	*Pestalotiopsis microspora*	1021	**AY377292**	**98**
26	TC-13	**AY924287**	Gopalaswamy hills	*Pestalotiopsis microspora*	Bark	*Pestalotiopsis microspora*	1021	**AY377292**	**98**
	Azadirachta indica								
27	AZ-32	**AY924288**	Mysore	*Bartalinia robillardoides*	Bark	*Bartalinia robillardoides*	981	**AF405301**	**96**
28	AZ-35	**AY924289**	Mysore	*Pestalotiopsis microspora*	Bark	*Pestalotiopsis microspora*	991	**AF377292**	**97**
29	AZ-46	**AY924290**	Mysore	*Bartalinia robillardoides*	Bark	*Bartalinia robillardoides*	975	**AF405301**	**96**
30	AZ-71	**AY924291**	Nanjangud	*Pestalotiopsis microspora*	Bark	*Pestalotiopsis microspora*	995	**AF377292**	**97**

Table 2. (continued) Database typing of endophytic fungi from four medicinal plants *viz., Terminalia arjuna* (TA), *Terminalia chebula (TC)*, *Azadirachta indica* (AI) and *Holarrhena antidysenterica* (HA).

Sl. No.	Fungal isolate	Accession number	Locations	Morphological characterization	Source	Closest match in the BLAST	Incl. score	Accession numbers	(%)
31	AZ-75	**AY924292**	Nanjangud	*Pestalotiopsis microspora*	Bark	*Pestalotiopsis microspora*	1132	**AF377288**	**99**
Holarrhena antidysenterica									
32	HA-33	**AY924293**	**Sullia**	*Pestalotiopsis theae*	Root	*Pestalotiopsis theae*	1045	**AY681481**	**99**

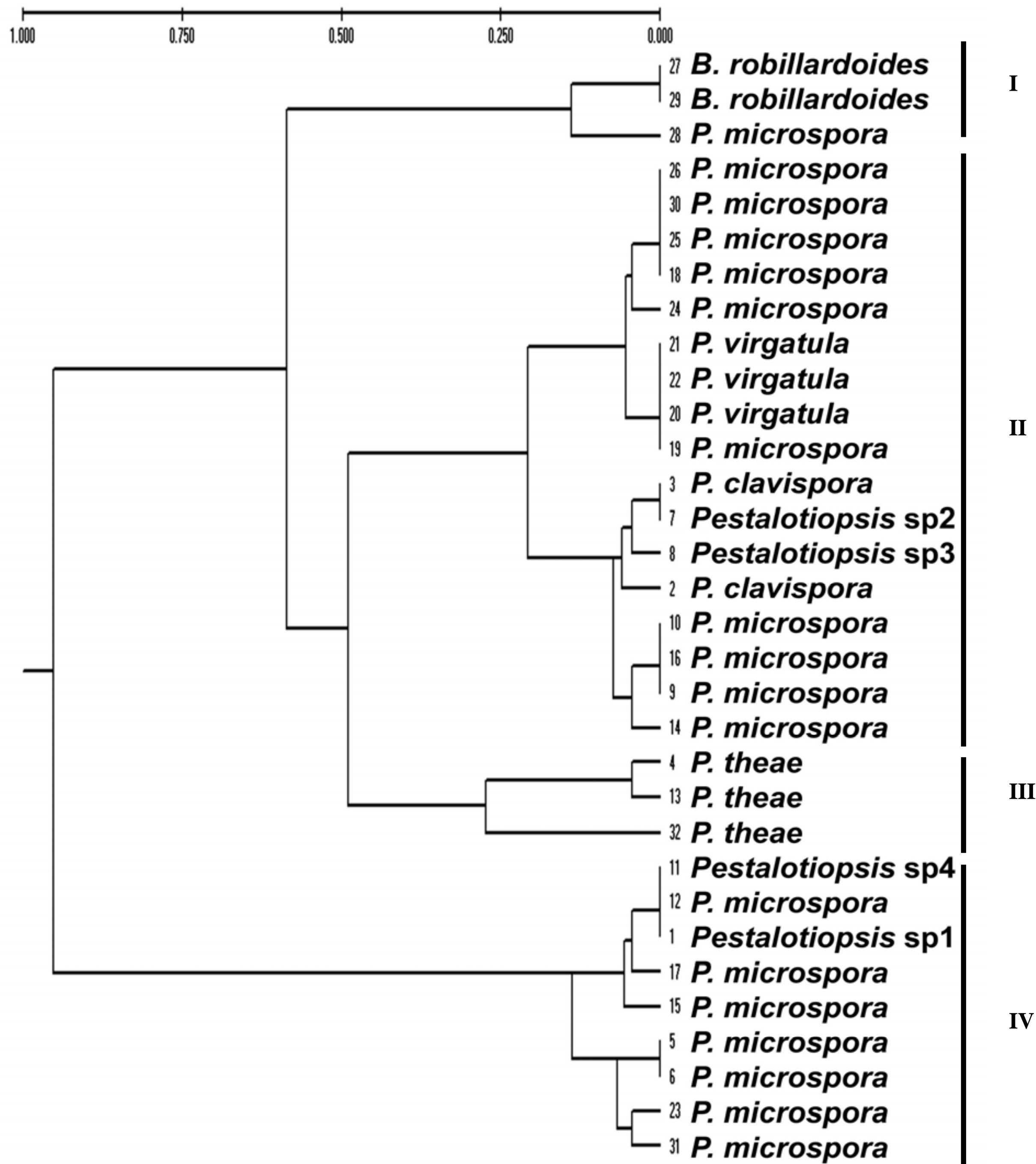

Fig. 2. Dendrogram based on UPGMA-cluster-analysis of the restriction patterns (*Alu* I, *Hae* III, *Ava* II, *Hpa* II and *Taq* I) of the ITS regions of the rDNA of 30 *Pestalotiopsis* and two *Bartalinia* isolates.

Interestingly most of the endophytic species formed separate clades except for four of them i.e. Sp. 1 and 4 of *Pestalotiopsis* and TA-76 and TA-103 of *P. microspora*. The fungal endophytes were distributed throughout the tree topology and were present in six of the seven clades (Fig. 4). The endophytic and pathogenic isolates of *P. theae* were differentiated phylogenetically and their grouping was supported with high bootstrap confidence of 98%. Most of the pathogenic *Pestalotiopsis* spp. were not closely related to endophytic isolates of *Pestalotiopsis*; they formed separate clade i.e., *P. virgatula* (**AY687879**) *P. photiniae* (**AY682938**) *P. photiniae* (**AY682937**), *P. photiniae* (**AY682936**), *P. versicolor* (**AY687300**), *P. clavispora* (**AY682927**) and *P. photiniae* (**AY682939**).

Table 3. The list of pathogenic *Pestalotiopsis* spp. sequences retrieved from GenBank.

Fungal species	Host	Source / pathogen	Locations	Accession Number
P. photiniae	*Camellia japonica*	Pathogen on leaves	Zhejiang Province, China	**AY682936**
P. karstenii	*Camellia japonica*	Pathogen on leaves	Zhejiang Province, China	**AY681472**
P. photiniae	*Camellia sinensis*	Pathogen on leaves	Zhejiang Province, China	**AY682938**
P. photiniae	*Camellia sinensis*	Pathogen on leaves	Zhejiang Province, China	**AY682937**
P. theae	*Camellia sinensis*	Pathogen on leaves	Zhejiang Province, China	**AY681479**
P. theae	*Camellia sinensis*	Pathogen on leaves	Yunnan Province, China	**AY681478**
P. theae	*Camellia sinensis*	Pathogen on leaves	Zhejiang Province, China	**AY681477**
P. clavispora	*Caryota urens*	Pathogen on leaves	Guangxi Province, China	**AY682927**
Pestalotiopsis sp.	*Gardenia jasminoides*	Diseased leaf	Hubei Province, China	**AY688467**
P. uvicola	*Mahonia acanthifolia*	Pathogen on leaves	Zhejiang, China	**AY687297**
Pestalotia lawsoniae	*Pinus massoniana*	Pathogen on leaves	Guangxi Province, China	**AY687871**
P. photiniae	*Pinus massoniana*	Pathogen on leaves	Guangxi Province, China	**AY682939**
P. adusta	*Podocarpus macrophyllus*	Pathogen on leaves	Zhejiang, China	**AY687298**
P. cryptomeriae	*Podocarpus nerrifolius*	Pathogen on leaves	Zhejiang, China	**AY687299**
P. disseminata	*Podocarpus imbricatus*	Pathogen on leaves	Guangxi Province, China	**AY687870**
P. virgatula	*Podocarpus macrophyllus*	Pathogen on leaves	Zhejiang Province, China	**AY687879**
P. oxyanthi	*Podocarpus macrophyllus*	Pathogen on leaves	Zhejiang Province, China	**AY687875**
P. heterocornis	*Podocarpus macrophyllus*	Pathogen on leaves	Zhejiang Province, China	**AY687873**
P. disseminata	*Podocarpus macrophyllus*	Pathogen on leaves	Guangxi Province, China	**AY687869**
P. versicolor	*Podocarpus nagi*	Pathogen on leaves	Zhejiang, China	**AY687300**

Discussion

Endophytic *Pestalotiopsis* species have been isolated from many tree species throughout the world (Hu *et al*., 2007; Arnold, 2007; Ding *et al*., 2009; Liu *et al*., 2009). Cannon and Simmons (2002) studied species diversity of endophytic fungi in 12 plant species belonging to nine families in the Iwokrama Forest Reserve, Guyana, and their results showed that different *Pestalotiopsis* species were associated with different plants. In the present study the extent of diversity in endophytic *Pestalotiopsis* species was similar among the seven sites and this indicates that the distribution of endophytic *Pestalotiopsis* species is ubiquitous and is not greatly affected by geographical variation. However, the number of *Pestalotiopsis* species isolated from *Holarrhena antidysenterica* and *Azadirachta indica* was lower than from *Terminalia arjuna* and *Terminalia chebula*. This is mainly because *H. antidysenterica* and *A. indica* was collected from only two regions as compared to the other trees.

The ITS-RFLP analysis showed intra-specific and interspecific variation among *Pestalotiopsis* isolates. The amplification products of the ITS region of 30 isolates of *Pestalotiopsis* spp. and two isolates of *B. robillardoides* isolated from different locations of South India, ranged from 550 to 600 bp, which is comparable with the sizes of other *Pestalotiopsis* spp. Despite the length polymerphism observed using ITS primers for many of the isolates, only two length mutants were observed, making the differentiation of species impossible. With ITS-RFLP, it was possible to assign the isolates into four main clusters. However, unequivocal species identification was possible only with *P. theae*. *Pestalotiopsis* species differentiation may not be possible with ITS-RFLP; enzymes other than the ones used in this study can be considered to conclude further about the technique. Different *Pestalotiopsis* species should be studied to confirm suitability of ITS-RFLP for its identification. Hence, ITS sequencing and homology searches were carried out to confirm morphological identification and phylogenetic analysis. By using ITS-RFLP (*Nde*II, *Hinf*I and *Rsa*I) Pandey *et al*. (2003) observed no polymorphism among 17 isolates of *Phyllosticta* from different hosts and suggested sequencing of ITS region of *Phyllosticta* isolates to detect variation among them.

Fungal identification is more reliable when classical and molecular approaches are combined (Hyde and Soytong, 2007, 2008; Than *et al*., 2008). In the present study four *Pestalotiopsis* species (sp. 1-4) were assigned species names following blast searches in GenBank. *Pestalotiopsis* sp. 1 and sp. 4 were

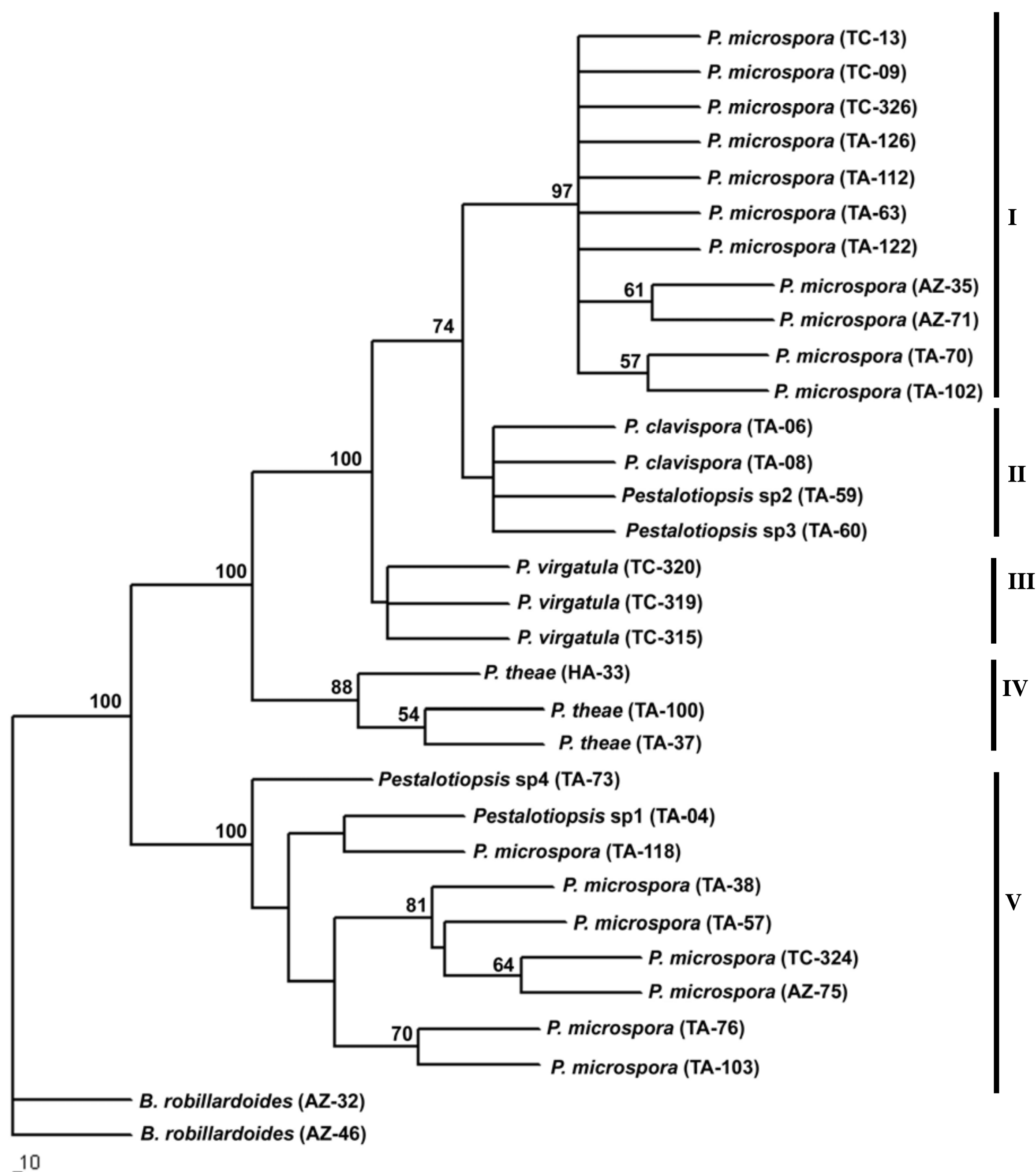

Fig. 3. Phylogenetic analysis of ITS and 5.8S rDNA sequences of endophytic *Pestalotiopsis* spp. The tree shown is the strict consensus of three weighted equally most parsimonious trees and was derived by phylogenetic analysis of 30 *Pestalotiopsis* isolates from different origins. Maximum parsimony bootstrap values (>50%) are shown above the lines. *Bartalinia robillardoides* was used as an outgroup.

close matches with *P. cryptomeriae* and *P. disseminata*, and *Pestalotiopsis* sp. 2 and sp. 3 with *P. microspora* and *P. clavispora* respect-tively (Fig. 4). The problem with this approach however, is that GenBank sequences cannot be verified as they are not usually linked to type material (Hyde and Soytong, 2007, 2008; Hyde and Zhang, 2008). Thus these names may be wrong. Thus, identification based on culture alone, as with endophyte studies, is often misleading (Nag Raj, 1981, 1993). In *Pestalotiopsis* spp. 1-4 identification, should consider morphology and ITS sequence similarity; it is not correct to use phylogenetic analysis as the only basis.

Sequence data from rDNA ITS / 5.8S rRNA has been extensively used to examine relationships among fungi, particularly at the

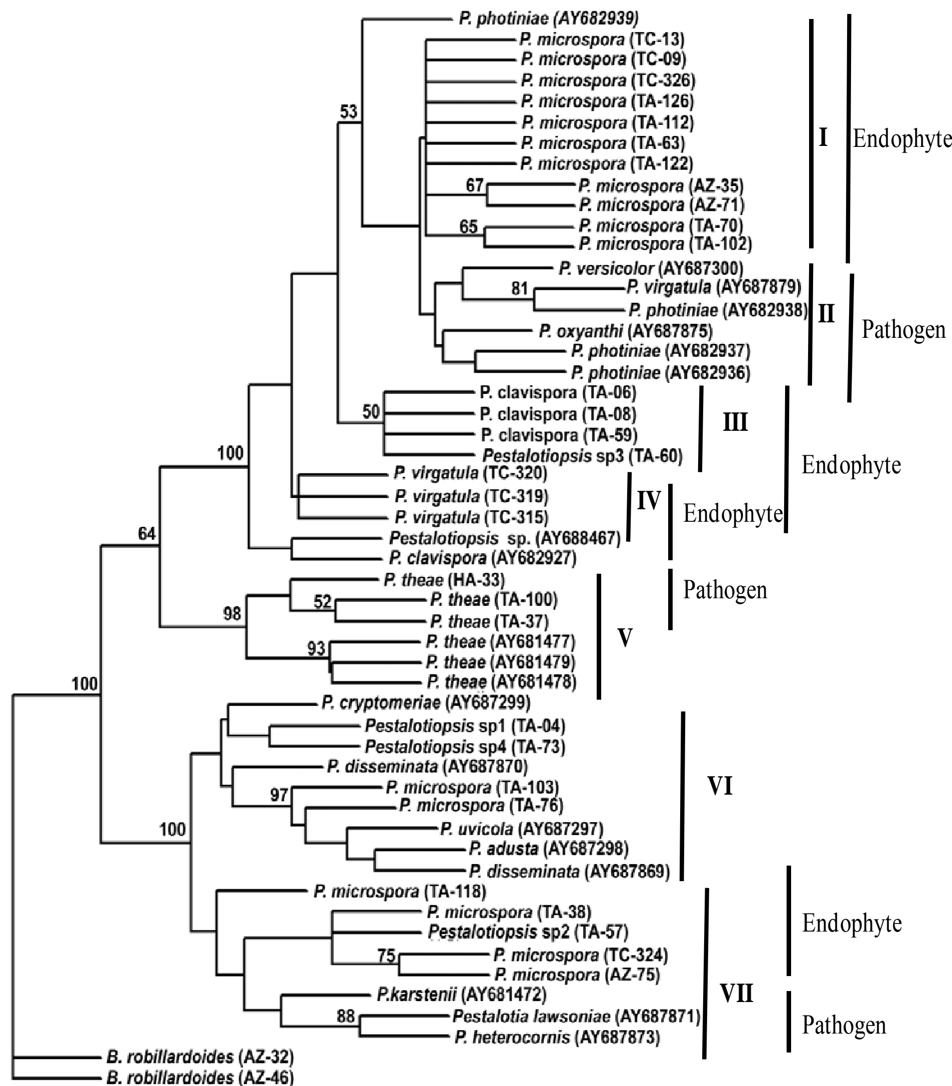

Fig. 4. Phylogenetic analysis of ITS and 5.8S rDNA sequences of *Pestalotiopsis* spp. The tree shown is the strict consensus of 54 equally most parsimonious trees and was derived by PAUP analysis of 52 isolates, of which 30 were *Pestalotiopsis* spp. and two *B. robillardoides* isolates were from medicinal plants (all from this study) and 20 were pathogenic *Pestalotiopsis* spp. (sequences from Genbank). Pathogenic strains taken from GenBank and EMBL databases are indicated with accession numbers in round parentheses. The maximum parsimony bootstrap values (>50%) are shown above the lines. *Bartalinia robillardoides* was used as an outgroup.

species level (Baayen *et al*., 2002; Jeewon *et al*. 2003, 2004; Than *et al*., 2008). Hence, molecular techniques are considered useful in the identification and classification of endophytes (Guo *et al*., 2000; Promputtha *et al*., 2005, 2007; Wang *et al*., 2005). ITS data can provide a more comprehensive assessment of non-sporulating fungal species diversity associated with woody roots, twigs and bark, compared to morphology. PCR and DNA sequencing combined with morphological verification can provide accurate identifications of fungal diversity by validating taxonomic identification. A molecular study of fungal endophytes of woody roots identified 27 genera associated with Douglas-fir [*Pseudotsuga menziesii*] and Ponderosa pine (*Pinus ponderosa*) (Hoff, 2002). Two predominant groups were observed, the first corresponding to an ascomycetous genus of heat-resistant fungi (*Byssochlamys*) that is active in bioprotection, and the second a zygomycetous genus (*Umbelopsis*) that is a common soil fungus not previously detected in living roots of these tree species. This approach

has limitations: the researcher cannot be certain that a fungus whose sequence has been entered in the database has been accurately identified (Hyde and Soytong, 2007; 2008), and the low representation of fungi from some substrates in GenBank.

Wei *et al*. (2007b) analysed the ITS regions of endophytic and pathogenic strains of *P. karstenii*, *P. lawsoniae* and *P. theae* and clearly differentiated these strains. Brown *et al*. (1998) studied endophytic fungal communities of the *Musa acuminata* species complex and found that *Colletotrichum gloeosporioides*, *C. musae* and *Phyllosticta musicola*, which are pathogens and can also be endophytes. *Deightoniella torulosa*, an endophyte isolated from wild banana (*Musa acuminata*) caused leaf spots *in vitro* on banana leaves (Photita *et al*., 2004), while the ITS sequences demonstrated that the pathogenic *Colletotrichum* isolated from banana were different from the endophytic isolates (Photita *et al*., 2005). Many pathogenic *Pestalotiopsis* species were confirmed as endophytes in earlier studies (Wei and Xu, 2003a,b; Wei *et al*., 2005). Therefore *Pestalotiopsis* species may have endophytic as well as pathogenic life stages.

Jeewon *et al*. (2002, 2003, 2004) used both morphological (conidial morphology) and molecular criteria (ITS sequence data) to classify *Pestalotia* and *Pestaliotiopsis* species. *Pestalotiopsis* species had considerable diversity in morphology and the isolates grouped together based on similarities in conidial morphology. Similar results were observed by phylogenetic analysis of *P. theae*, *P. virgatula* and *P. clavispora* in this study. ITS sequence analysis of endophytic and pathogenic *Pestalotiopsis* strains resulted in pathogenic strains forming a separate clade from the endophytic strains. Interestingly, three isolates of endophytic and pathogenic *P. theae* also grouped in the separate clades, but this may also be due to geographical isolation (Fig. 4). The pathogenic strains of *P. theae* were isolated from diseased leaves of *Camellia sinensis* in China, whereas the endophytic *P. theae* strains were isolated from *Terminalia arjuna* and *Holarrhena antidysenterica*. ITS sequence analysis of pathogenic strains of *Guignardia citricarpa* and endophytic *Guignardia* strains indicated that the pathogenic and endophytic strains belonged to distinct species (Baayen *et al*., 2002), with *P. capitalensis* occurring as an endophyte in a wide range of hosts.

Endophytic *Pestalotiopsis* species isolated from medicinal plants are genetically more diverse than previously observed based on morphological characters. The present results also support the findings of Jeewon *et al*. (2004) which conclude that *Pestalotiopsis* species can be determined based on morphological characters rather than host–specificity or location. *Pestalotiopsis* species identification based on ITS-RFLP has limited application as it cannot be used to definitively identify species, for example large intraspecific variation was detected in *P. microspora*. ITS sequence data helped us to assign species putative names to some *Pestalotiopsis* isolates and detect phylogenetic diversity among species. Endophytic and pathogenic isolates of *P. theae* could also be differentiated using phylogenetic analysis of the ITS region.

This study provides data on endophytic *Pestalotiopsis* species on medicinal trees however, we recognize that there are inadequacies with the techniques used and further work is needed. *Pestalotiopsis* is a genus in serious need of taxonomic study. However, future studies require that the type material is reexamined and described with full descriptions and then identical fresh collections with living ex-types can be designated (Hyde and Soytong, 2007). In this way a stable basis for species names can be initiated. The present system of putting names to fresh collections based on comparison with existing monographs (e.g. Guba, 1961) cannot work, as descriptions are brief and based mostly on host and thus identifications will be subjective. Similarly, names of species in GenBank are likely to be erroneous. This has already been initiated in genera such as *Colletotrichum* and *Mycosphaerella* (Crous *et al*., 2007; Shenoy *et al*., 2007b; Than *et al*., 2008).

Pestalotiopsis species are of considerable interest to researchers and pharmacists due to their ability to synthesize a wide range of economically important bioactive molecules (Strobel, 2002; Tomita, 2003; Ding *et al*., 2009; Liu *et al*., 2009). Hence we are currently pursuing fermentation studies on these endophytes to produce a wide array of secondary

metabolites which can be screened against therapeutic targets. This study has used traditional isolation techniques because we required strains for bioprospecting, however it would be interesting to use DGGE, and random cloning analysis to establish the presence of endophytes using total DNA extraction (e.g. Duong *et al*., 2006; Seena *et al*., 2008).

Acknowledgement

The authors thank The Department of Biotechnology (DBT), India, for providing financial support. Tejesvi MV is grateful to the Indian Council of Medical Research (ICMR) for the award of a Senior Research Fellowship.

References

Agarwal, A.K. and Chauhan, S. (1988). A new species of the genus *Pestalotiopsis* from Indian Soil. Indian Phytopathology 41: 625-627.

Arnold, A.E. (2007). Understanding the diversity of foliar endophytic fungi: progress, challenges, and frontiers. Fungal Biology Reviews 21: 51-66.

Baayen, R.P., Bonants, P.J.M., Verkley, G., Carroll, G.C., van der Aa, H.A., de Weerdt, M., van Brouwershaven, I.R., Schutte, G.C., Maccheroni, W. Jr., Glienke de Blanco, C. and Azevedo, J.L. (2002). Nonpathogenic isolates of the citrus black spot fungus, *Guignardia citricarpa*, identified as a cosmopolitan endophyte of woody plants, *G. mangiferae* (*Phyllosticta capitalensis*). Phytopathology 92: 464-477.

Brown, K.B., Hyde, K.D. and Guest, D.I. (1998). Preliminary studies on endophytic fungal communities of *Musa acuminata* species complex in Hong Kong and Australia. Fungal Diversity 1: 27-51.

Cai, L., Jeewon, R. and Hyde, K.D. (2006). Phylogenetic investigations of Sordariaceae based on multiple gene sequences and morphology. Mycological Research 110: 137-150.

Cannon, P.F. and Simmons, C.M. (2002). Diversity and host preference of leaf endophytic fungi in the Iwokrama Forest Reserve, Guyana. Mycologia 94: 210-220.

Crous, P.W., Braun, U. and Groenewald, J.Z. (2007). *Mycosphaerella* is polyphyletic. Studies in Mycology 58: 1-32.

Ding, G., Li, Y., Fu, S., Liu, S., Wei, J. and Che, Y. (2009). Ambuic acid and torreyanic acid derivatives from the endolichenic fungus *Pestalotiopsis* sp. Journal of Natural Products 72:182-6.

Duong, L.M., Jeewon, R., Lumyong, S. and Hyde, K.D. (2006). DGGE coupled with ribosomal DNA gene phylogenies reveal uncharacterized fungal phylotypes. Fungal Diversity 23: 121-138.

Egger, K.N. (1995). Molecular analysis of ectomycorrhizal fungal communities. Canadian Journal of Botany 73: 1415-1422.

Fröhlich, J., Hyde, K.D. and Petrini, O. (2000). Endophytic fungi associated with palms. Mycological Research 104: 1202-1212.

Ganley, R.J., Brunsfeld, S.J. and Newcombe, G. (2004). A community of unknown, endophytic fungi in western white pine. Proceedings of National Academy of Sciences 101: 10107- 10112.

Glen, M., Tommerup, I.C., Bougher, N.L. and O'brien, P.A. (2001). Interspecific and intraspecific variation of ectomycorrhizal fungi associated with *Eucalyptus* ecosystems as revealed by ribosomal DNA PCR–RFLP. Mycological Research 105: 843-858.

Gomes, E.A., Barros, E.G., Kasuya, M.C.M. and Araujo, E.F. (1999). Molecular characterization of *Pisolithus* spp. isolates by rDNA PCR-RFLP. Mycorrhiza 8: 197-202.

Gonthier, P., Gennaro, M. and Nicolotti, G. (2006). Effects of water stress on the endophytic mycota of *Quercus robur*. Fungal Diversity 21: 69-80.

Guba, E.F. (1961). Monograph of *Pestalotia* and *Monochaetia*. Harvard University Press. Cambrigde Masssachusetts USA.

Guo, L.D., Hyde, K.D. and Liew, E.C.Y. (2000). Identification of endophytic fungi from *Livistona chinensis* (*Palmae*) using morphological and molecular techniques. New Phytologist 147: 617-630.

Harper, J.K., Ford, E.J., Strobel, G.A., Arif, A., Grant, D.M., Porco, J., Tomer, D.P. and Oneill, K. (2003). Pestacin: a 1, 3-dihydro isobenzofuran from *Pestalotiopsis microspora* possessing anti-oxidant and antimycotic activities. Tetrahedron 59: 2471-2476.

Hoff, J.A. (2002). Fungal diversity in woody roots of east-slope Cascade ponderosa pine (*Pinus ponderosa*) and Douglas-fir (*Pseudotsuga menziesii*). M.Sc. thesis, Washington State University, Pullman, WA.

Hu, H.L., Jeewon, R., Zhou, D.Q., Zhou, T.X. and Hyde, K.D. (2007). Phylogenetic diversity of endophytic *Pestalotiopsis* species in *Pinus armandii* and *Ribes* spp.: evidence from rDNA and β-tubulin gene phylogenies. Fungal Diversity 24: 1-22.

Huang, W.Y., Cai, Y.Z., Hyde, K.D., Corke, H. and Sun, M. (2008). Biodiversity of endophytic fungi associated with 29 traditional Chinese medicinal plants. Fungal Diversity 33: 61-75.

Hyde, K.D. and Soytong, K. (2007). Understanding microfungal diversity - a critique. Cryptogamie Mycologie 28: 281-289.

Hyde, K.D. and Soytong, K. (2008). The fungal endophyte dilemma. Fungal Diversity 33: 163-173.

Hyde, K.D. and Zhang, Y. (2008). Epitypification: should we epitypify? Journal of Zhejiang University-Science B 9: 842-846.

Jeewon, R., Liew, E.C.Y. and Hyde, K.D. (2002). Phylogenetic relationships of *Pestalotiopsis* and allied genera inferred from ribosomal DNA

sequences and morphological characters. Molecular Phylogenetics and Evolution 25: 378-392.

Jeewon, R., Liew, E.C.Y. and Hyde, K.D. (2004). Phylogenetic evaluation of species nomenclature of *Pestalotiopsis* in relation to host association. Fungal Diversity 17: 39-55.

Jeewon, R., Liew, E.C.Y., Simpson, J.A., Hodgkiss, I.J. and Hyde, K.D. (2003). Phylogenetic significance of morphological characters in the taxonomy of *Pestalotiopsis* species. Molecular Phylogenetics and Evolution 27: 372-383.

Kang, J.C., Hyde, K.D. and Kong, R.Y.C. (1999). Studies on Amphisphaeriales: the Amphisphaeriaceae (*sensu stricto*). Mycological Research 103: 53-64.

Kishino, H. and Hasegawa, M. (1989). Evaluation of the maximum likelihood model estimates of the evolutionary tree topologies from sequence data, and the branching order in Homonoidea. Journal of Molecular Evolution 29: 170-179.

Koh, Y.J., Shin, G.H. and Hur, J.S. (2001). Seasonal occurrence and development of gray blight of tea plants in Korea. Plant Pathology Journal 17: 40-44.

Kumar, D.S.S. and Hyde. K.D. (2004). Biodiversity and tissue-recurrence of endophytic fungi from Tripterygium wilfordii. Fungal Diversity 17: 69-90.

Kumar, S.S., Cheung, H.Y., Lau, C.S., Chen, F. and Hyde, K.D. (2004). *In vitro* studies of endophytic fungi from *Tripterygium wilfordii* with anti-proliferative activity on human peripheral blood mononuclear cells. Journal of Ethnopharmacology 94: 295-300.

Lacap, D.C., Hyde, K.D. and Liew, E.C.Y. (2003). An evaluation of the fungal 'morphotype' concept based on ribosomal DNA sequences. Fungal Diversity 12: 53-66.

Leslie, J.F., Zeller, K.A., Lamprecht, S.C., Rheeder, J.P. and Marasas, W.F.O. (2005). Toxicity, pathogenicity, and genetic differentiation of five species of *Fusarium* from sorghum and millet. Phytopathology 95: 275-283.

Li, J.Y., Strobel, G.A., Sidhu, R., Hess, W.M. and Ford, E. (1996). Endophytic taxol producing fungi from Bald Cypress *Taxodium distichum*. Microbiology 142: 2223-2226.

Li, W.C., Zhou, J., Guo, S.Y. and Guo, L.D. (2007). Endophytic fungi associated with lichens in Baihua mountain of Beijing, China. Fungal Diversity 25: 69-80.

Liu, L., Liu, S., Chen, X., Guo, L. and Che, Y. (2009). Pestalofones A-E, bioactive cyclohexanone derivatives from the plant endophytic fungus *Pestalotiopsis fici*. Bioorganic and Medicinal Chemistry 15: 606-13.

Magnani, M., Fernandes, T., Prete, C.E.C., Homechim, M., Ono, U.Y.S., Vilas-Boas, L.A., Sartori, D., Cristina, M., Furlaneto, S. and Fungaro, M.H.P. (2005). Molecular identification of *Aspergillus* spp. isolated from coffee beans. Scientia Agricola 62: 45-49.

Mahesh, B., Tejesvi, M.V., Nalini, M.S., Prakash, H.S., Kini, K.R., Subbiah, V. and Shetty H.S. (2005). Endophytic fungi from *Azadirachta indica* (A. Juss.) inner bark. Current Science 88: 218-219.

Miller, M. (1997). Tools for Population Genetic Analysis (TFPGA) 1.3: A Windows Program for the Analysis of Allozyme and Molecular Population Genetic Data. Computer software distributed by author.

Nag Raj, T.R. (1981). Genera coelomycetum. XIX. *Discosiella*, a lichenized mycobiont. Canadian Journal of Botany 59: 2519-2533.

Nag Raj, T.R. (1993). Coelomycetous Anamorphs with Appendage Bearing Conidia. Mycologue Publications, Waterloo, Ontaria, Canada: 1101.

Nei, M. (1978). Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89: 583-590.

Oses, R., Valenzuela, S., Freer, J., Sanfuentes, E. and Rodriguez, J. (2008). Fungal endophytes in xymlem of healthy Chilean trees and their possible role in early wood decay. Fungal diversity 33: 77-86.

Osono, T. and Takeda, H. (1999). Decomposing ability of interior and surface fungal colonizers of beech leaves with reference to lignin decomposition. European Journal of Soil Biology 35: 51-56.

Page, R.D.M. (1996). TREEVIEW: an application to display phylogenetic trees on personal computers. Computational and Applied Biosciences 12: 357-358.

Pandey, P.K., Reddy, M.S. and Suryanarayanan, T.S. (2003). ITS-RFLP and ITS sequence analysis of a foliar endophytic *Phyllosticta* from different tropical trees. Mycological Research 107: 439-444.

Phillips, A.J.L., Crous, P.W. and Alves, A. (2007). *Diplodia seriata*, the anamorph of "*Botryosphaeria*" *obtusa*. Fungal Diversity 25: 141-155.

Photita, W., Lumyong, S., Lumyong, P. and Hyde, K.D. (2001). Endophytic fungi of wild banana (*Musa acuminata*) at Doi Suthep Pui National Park, Thailand. Mycological Research 105: 1508-1514.

Photita, W., Lumyong, S., Lumyong, P., McKenzie, E.H.C. and Hyde, K.D. (2004). Are some endophytes of *Musa acuminata* latent pathogens? Fungal Diversity 16: 131-140.

Photita, W., Taylor, P.W.J., Ford, R., Hyde, K.D. and Lumyong, S. (2005). Morphological and molecular characterization of *Colletotrichum* species from herbaceous plants in Thailand. Fungal Diversity 18: 117-133.

Promputtha, I., Jeewon, R., Lumyong, S., McKenzie, E.H.C. and Hyde, K.D. (2005). Ribosomal DNA fingerprinting in the identification of non sporulating endophytes from *Magnolia liliifera* (Magnoliaceae). Fungal Diversity 20: 167-186.

Promputtha, I., Lumyong, S., Dhanasekaren, V., McKenzie, E.H.C., Hyde, K.D. and Jeewon, R. (2007). A phylogenetic evaluation of whether

endophytes become saprotrophs at host senescence. Microbial Ecology: 53: 579-590.

Rheeder, J.P., Marasas, W.F.O. and Nelson, P.E. (1996). *Fusarium globosum*, a new species from corn in southern Africa. Mycologia 88: 509-513.

Rungjindamai, N., Pinruan, U., Choeyklin, R., Hattori, T. and Jones, E.B.G. (2008). Molecular characterization of basidiomycetous endophytes isolated from leaves, rachis and petioles of the oil palm, *Elaeis guineensis*, in Thailand. Fungal Diversity 33: 139-161.

Saghai-Maroof, M.A., Soliman, K., Jorgensen, R.A., Allard, R.W. (1984). Ribosomal DNA spacer length polymorphisms in barley: Mendelian inheritance, chromosomal location and population dynamics. Proceedings of National Academy of Sciences 81: 8014-8018.

Sánchez Márquez, S., Bills, G.F. and Zabalgogeazcoa, I. (2007). The endophytic mycobiota of the grass *Dactylis glomerata*. Fungal Diversity 27: 171-195.

Sánchez Márquez, S., Bills, G.F. and Zabalgogeazcoa, I. (2008). Diversity and structure of the fungal endophytic assemblages from two sympatric coastal grasses. Fungal Diversity 33: 87-100.

Seena, S., Wynberg, N. and Bärlocher, F. (2008). Fungal diversity during leaf decomposition in a stream assessed through clone libraries. Fungal Diversity 30: 1-14.

Shenoy, B.D., Jeewon, R. and Hyde, K.D. (2007a). Impact of DNA sequence-data on the taxonomy of anamorphic fungi. Fungal Diversity 26: 1-54.

Shenoy, B.D., Jeewon, R., Lam, W.H., Bhat, D.J., Than, P.P., Taylor, P.W.J. and Hyde, K.D. (2007b). Morpho-molecular characterisation and epitypification of *Colletotrichum capsici* (*Glomerellaceae*, *Sordariomycetes*), the causative agent of anthracnose in chilli. Fungal Diversity 27: 197-211.

Steyaert, R.L. (1949). Contributions à l'étude monographique de *Pestalotia* de Not. Et *Monochaetia* Sacc. (*Truncatella* gen. nov. et *Pestalotiopsis* gen. nov.). Bulletin Jardin Botanique État Bruxelles 19: 285-354.

Strobel, G.A. (2002). Rainforest endophytes and bioactive products. Critical Reviews in Biotechnology 22: 315-333.

Suryanarayanan, T.S. (1992). Light-incubation: a neglected procedure in mycology. The Mycologist 6: 144.

Sutton, B.C. (1980). The Coelomycetes. Commonwealth Mycological Institute, UK.

Swofford, D.L. (2003). PAUP*. Phylogenetic Analysis Using Parsimony (*and other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Tan, M.K. and Niessen, L.M. (2003). Analysis of rDNA ITS sequences to determine genetic relationships among, and provide a basis for simplified diagnosis of *Fusarium* species causing crown rot and head blight of cereals. Mycological Research 107: 811-821.

Tang, A., Hyde, K.D. and Corlett, R.T.C. (2003) Diversity of fungi on wild fruits in Hong Kong. Fungal Diversity 14: 165-185.

Tao, G., Liu, Z.Y., Hyde, K.D. Lui, X.Z. and Yu, Z.N. (2008). Whole rDNA analysis reveals novel and endophytic fungi in *Bletilla ochracea* (*Orchidaceae*). Fungal Diversity 33: 101-122.

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

Tejesvi, M.V., Kini, K.R., Prakash, H.S., Ven Subbiah, Shetty, H.S. (2008). Antioxidant, antihypertensive, and antibacterial properties of endophytic *Pestalotiopsis* species from medicinal plants. Canadian Journal of Microbiology 54:769-80.

Tejesvi, M.V., Mahesh, B., Nalini, M.S., Prakash, H.S., Kini, K.R., Ven Subbiah and Shetty, H.S. (2005). Endophytic fungal assemblages from inner bark and twig of *Terminalia arjuna* W and A. (Combretaceae). World Journal of Microbiology and Biotechnology 21: 1535-1540.

Tejesvi, M.V., Mahesh, B., Nalini, M. S., Prakash, H.S., Kini, K.R., Ven Subbiah, and Shetty, H.S. (2006). Fungal endophyte assemblages from ethnopharmaceutically important medicinal trees. Canadian Journal of Microbiology 52: 427-435.

Templeton, A.R. (1983). Nested clade analysis of phylogenetic data: testing hypotheses about gene flow and population history. Molecular Ecology 7: 381-397.

Than, P.P., Shivas, R.G., Jeewon, R., Pongsupasamit, S., Marney, T.S., Taylor, P.W.J. and Hyde, K.D. (2008). Epitypification and phylogeny of *Colletotrichum acutatum* J.H. Simmonds. Fungal Diversity 28: 97-108.

Thomson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. and Higgins, D.G. (1997). The Clustal-X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Research 25: 4876-4882.

Thongkantha, S., Jeewon, R., Vijaykrishna, D., Lumyong, S., McKenzie, E.H.C. and Hyde, K.D. (2009). Molecular phylogeny of *Magnaporthaceae* (Sordariomycetes) with a new species, *Ophioceras chiangdaoense* from *Dracaena loureiroi* in Thailand. Fungal Diversity 34: 157-173.

Tomita, F. (2003). Endophytes in Southeast Asia and Japan: their taxonomic diversity and potential applications. Fungal Diversity 14: 187-204.

Wang, Y., Guo, L.D. and Hyde, K.D. (2005). Taxonomic placement of sterile morphotypes of endophytic fungi from *Pinus tabulaeformis* (Pinaceae) in northeast China based on rDNA sequences. Fungal Diversity 20: 235-260.

Wei, J.G. and Xu, T. (2003a). Biodiversity of endophytic fungi *Pestalotiopsis*. Biodiversity Science 11: 162-168.

Wei, J.G. and Xu, T. (2003b). *Pestalotiopsis karstenii*, a new record of endophytic fungi from *Camellia sasanqua* in China. Mycosystema 22: 666-668.

Wei, J.G., Xu, T., Guo, L.D. and Pan, X.H. (2005). Endophytic *Pestalotiopsis* species from southern China. Mycosystema 24: 481-493.

Wei, J.G., Xu, T., Guo, L.D., Liu, A.R. Zhang, Y. and Pan, X.H. (2007b). Endophytic *Pestalotiopsis* species associated with plants of Podocarpaceae, Theaceae and Taxaceae in southern China. Fungal Diversity 24: 55-74.

Wei, Y.K., Gao, Y.B., Zhang, X., Su, D., Wang, Y.H., Xu, H., Lin, F., Ren, A.Z., Chen, L. and Nie, L.Y. (2007a). Distribution and diversity of *Epichloë/Neotyphodium* fungal endophytes from different populations of *Achnatherum sibiricum* (Poaceae) in the Inner Mongolia Steppe, China. Fungal Diversity 24: 329-345.

White, T.J., Bruns, T., Lee, S. and Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: A Guide to Methods and Applications*. (eds. M.A. Innis, D.H. Gelfand, J.J. Snisky and J.J. White) Academic Press, San Diego: 315-322.

Wilson, D. (1995). Endophyte - the evolution of a term, and clarification of its use and definition. Oikos 73: 274-276.

Zhang, J.X., Xu, T. and Ge, Q.X. (2003). Notes on *Pestalotiopsis* from Southern China. Mycotaxon 85: 91-92.

Zhao, G.Z., Liu, X.Z. and Wu, W.P. (2007). Helicosporous hyphomycetes from China. Fungal Diversity 26: 313-524.

Zhu, G.S., Yu, Z.N., Gui, Y. and Liu, Z.Y. (2008). A novel technique for isolating orchid mycorrhizal fungi. Fungal Diversity 33: 123-137.