Phylogenetic diversity of endophytic *Pestalotiopsis* species in *Pinus armandii* and *Ribes* spp.: evidence from rDNA and β-tubulin gene phylogenies

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The diversity of endophytes associated with bark and needles of *Pinus armandii* and leaves of *Ribes* species was investigated and resulted in 132 isolates being identified as *Pestalotiopsis* species. Isolates were grouped into morphospecies based on morphology and cultural characteristics and named based on morphological characters. To supplement identifications, ribosomal DNA (ITS and 5.8S) and β-tubulin gene sequences from 37 isolates representing diverse morphological and cultural characteristics were analyzed phylogenetically (maximum parsimony and Bayesian criteria). Phylogenies corroborated with morphologies and are largely congruent with previously established taxonomic schemes. Phylogenetic comparison of the two genes reveals that ITS based phylogenies alone were not suitable to infer phylogenetic relationships among *Pestalotiopsis* species as most of the clades did not receive adequate statistical support. β-tubulin phylogenies were better resolved but a combined dataset was found to be more appropriate. Although results are generally concordant with published studies, there seems to be several inconsistencies in grouping taxa at the species level. The phylogenetic relationships of species in relation to culture morphology, as well as to host and tissue association are discussed.

Key Words: β-tubulin gene, conidial characters, cultural characteristics, *Pestalotiopsis*, phylogeny, rDNA gene

Introduction

We carried out a biodiversity survey on the endophytic fungi of *Pinus armandii* and *Ribes* species during 2003-2004 in Southern China (Hu, 2005).

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Pinus armandii is an economically and ecologically important tree in Yunnan Province and other places in China (Anon., 1994), but it is often infected with five-needle blister rust (*Cronartium ribicola* J.C. Fisch.) which can cause serious damage (Ren, 1993; Kinloch, 2003). *Ribes* species are alternative hosts of five-needle blister rust. A large number of studies have shown that many fungi exist as endophytes during part of their life cycle (e.g. Wang *et al*., 2005; Devarajan and Suryanarayanan, 2006; Duong *et al*., 2006; Gonthier *et al*., 2006). We were therefore interested in establishing the endophytic fungi present in both hosts as the information may be important in future studies that deal with the applicability of fungal endophytes as potential disease controlling agents (El-Morsy *et al*., 2006).

In this paper we report endophytic *Pestalotiopsis* strains isolated from bark and needles of *Pinus armandii* and leaves of *Ribes* species. *Pestalotiopsis* was established by Steyaert (1949). It is a complex anamorphic genus, in which the species can be saprobes, plant pathogens or endophytes (Steyaert, 1949; Guba, 1961; Suto and Kobayashi, 1993; Strobel *et al*., 1997, 2000; Rivera and Wright, 2000; Karakaya, 2001; Tagne and Mathur, 2001; Gonthier and Involuta, 2002; Wang *et al*., 2002; Kumar and Hyde, 2004; Sousa *et al*., 2004). Espinosa-Garcia and Langenheim (1990) first reported *Pestalotiopsis funerea* as an ecologically important endophyte from *Sequoia sempervirens*. Several *Pestalotiopsis* species have since been isolated as endophytes (Strobel *et al*., 1997, 2000; Guo, 2002; Worapong *et al*., 2003). Strobel *et al*. (1996) isolated *Pestalotiopsis microspora* as an endophyte from *Taxus wallichiana* and found that this fungus has the ability to produce taxol, which had been reported to be effective against cancer (Qiu *et al*., 1994). The identification of *Pestalotiopsis* species based on morphology is however, complicated because there are few morphological characters available to distinguish taxa at the species level. Associations with hosts have commonly been reported in the literature and many new taxa have been published based on host association (Guba, 1961; Sun and Ge, 1990; Chen and Wei, 1993, 1997; Wei and Chen, 1994; Zhao and Li, 1995; Chen *et al*., 2002, 2003; Wang *et al*., 2002; Wei and Xu, 2004).

Molecular studies have shown that *Pestalotiopsis* is monophyletic (Jeewon *et al*., 2002, 2003a, 2004) and Jeewon *et al*. (2003a) discussed the important characters to group species in this genus. Jeewon *et al*. (2004) also investigated associations between host and *Pestalotiopsis* species and found that host-specificity is not suitable for differentiating taxa to species. Thus, *Pestalotiopsis* species are not host related as in *Anthracoidea* (Hendrichs *et al*., 2005; Guo, 2006).

The objectives of this study were to use morphology and phylogenetic analyses to (1) assess the diversity of endophytic *Pestalotiopsis* species from

bark and needles of *Pinus armandii* and leaves of *Ribes* species, (2) test the reliability of grouping species based on cultural characteristics and (3) find out whether phylogenies from ribosomal DNA (ITS and 5.8S) and β-tubulin gene are consistent with morphological based species identification.

Materials and methods

Isolation and culture

Isolation of endophytes from tissues was carried out by modifying the methods outlined by Kumar and Hyde (2004), Wei and Xu (2004) and Promputtha *et al*. (2005). Isolates were grown on Potato Dextrose Agar (PDA) to mature and identified based on sporulating structures. The 132 strains comprised 20 species of *Pestalotiopsis* based on morphology and cultural characteristics (results not shown). The colony morphology (including colour, growth rate, texture, and mycelial form) of each strain was noted and recorded. Twenty-five conidia from each strain were chosen randomly to measure length and width of conidian and the three median pigmented cells, number and length of apical appendages and basal appendages and to describe the colour and shape of conidia and the characters of apical appendages. Each strain was identified to species based on the keys and descriptions provided by Steyaert (1949), Guba (1961), Sutton (1980) and Nag Raj (1993). The cultures were deposited in China General Microbiological Culture Collection Centre (CGMCC), Institute of Microbiology, Chinese Academy of Sciences, Beijing, China. Based on cultural characters and conidial characters, 37 isolates of *Pestalotiopsis* representing 11 species were selected for this study (Tables 1, 2). All fungi strains used in this experiment were cultured on PDA and incubated at 25ºC for 7 days before DNA extraction.

DNA extraction, polymerase chain reaction (PCR) amplification and sequencing of the ITS rDNA and β-tubulin gene

Total genomic DNA was extracted by following a protocol as outlined by Jeewon *et al*. (2004), Cai *et al*. (2005, 2006) and Photita *et al*. (2005). Polymerase chain reaction (PCR) amplification products were obtained with the use of two pairs of primers, universal primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAG-TCGTAACAAGG-3') (described by White *et al*., 1990) and β-tubulin BT2A (5'-GGTAACCAAATCGGTGCTGCTTTC-3') and BT2B (5'-ACCCTC-AGTGTAGTGACCCTTGGC-3') (Glass and Donaldson, 1995; O'Donnell and

Table 1. Source of strains used in this study.

Species (strain)	Collection date	Collection site	Host/tissue	GenBank accession number	
				ITS	β-tubulin
P. algeriensis (K9DY)	21/04/2004	Dongchuan	*Pinus armandii* bark	EF055186	EF055223
P. carveri (G5HA)	27/04/2003	Tiantaishan	*Pinus armandii* bark	EF055187	EF055224
P. caudata (K14DW)	21/04/2004	Dongchuan	*Pinus armandii* bark	EF055188	EF055225
P. cocculi (C11A)	29/10/2003	Dongchuan	*Pinus armandii* bark	EF055189	EF055226
P. cocculi (DFFW)	20/04/2003	Dongchuan	*Pinus armandii* bark	EF055190	EF055227
P. cocculi (DH12DY)	20/04/2003	Dongchuan	*Pinus armandii* bark	EF055191	EF055228
P. cocculi (EY2AR)	21/08/2003	Dali	*Ribes* spp. leaf	EF055192	EF055229
P. cocculi (PS11BY)	16/04/2004	Dali	*Pinus armandii* bark	EF055193	EF055230
P. cocculi (YC4D)	27/10/2003	Dali	*Pinus armandii* bark	EF055194	EF055231
P. cocculi (YY1C)	27/10/2003	Dali	*Pinus armandii* needle	EF055195	EF055232
P. disseminata (EC3A)	21/08/2003	Dali	*Pinus armandii* bark	EF055196	EF055233
P. funerea (ML4DY)	16/08/2003	Qiaojia	*Ribes* spp. leaf	EF055197	EF055234
P. heterocornis (PN3DW)	16/04/2004	Dali	*Pinus armandii* bark	EF055198	EF055235
P. lespedezae (C17AW)	29/10/2003	Dongchuan	*Pinus armandii* bark	EF055199	EF055236
P. lespedezae (EC12A)	21/08/2003	Dali	*Pinus armandii* bark	EF055200	EF055237
P. lespedezae (ML2A)	16/08/2003	Qiaojia	*Ribes* spp. leaf	EF055201	EF055238
P. lespedezae (PN3AY)	16/04/2004	Dali	*Pinus armandii* bark	EF055202	EF055239
P. lespedezae (PN3DH)	16/04/2004	Dali	*Pinus armandii* bark	EF055203	EF055240
P. lespedezae (SY16AW)	22/10/2003	Qiaojia	*Pinus armandii* needle	EF055204	EF055241
P. lespedezae (SY16E)	22/10/2003	Qiaojia	*Pinus armandii* needle	EF055205	EF055242
P. lespedezae (YY12A)	27/10/2003	Dali	*Pinus armandii* needle	EF055206	EF055243
P. neglecta (EP2DY)	21/08/2003	Dali	*Pinus armandii* bark	EF055207	EF055244
P. neglecta (EY6D)	21/08/2003	Dali	*Ribes* spp. leaf	EF055208	EF055245
P. neglecta (K9AW)	21/04/2004	Dongchuan	*Pinus armandii* bark	EF055209	EF055246
P. neglecta (Q13DW)	05/04/2004	Qiaojia	*Pinus armandii* bark	EF055210	EF055247
P. neglecta (SY19C)	22/10/2003	Qiaojia	*Pinus armandii* needle	EF055211	EF055248
P. neglecta (YY1A)	27/10/2003	Dali	*Pinus armandii* needle	EF055212	EF055249
P. neglecta (YY8A)	27/10/2003	Dali	*Pinus armandii* needle	EF055213	EF055250
P. olivacea (MS002)	12/04/2003	Qiaojia	*Pinus armandii* bark	EF055214	EF055251
P. olivacea (SY17A)	22/10/2003	Qiaojia	*Pinus armandii* needle	EF055215	EF055252
P. olivacea (YY6A)	27/10/2003	Dali	*Pinus armandii* needle	EF055216	EF055253
P. olivacea (YY8C)	27/10/2003	Dali	*Pinus armandii* needle	EF055217	EF055254
P. vismiae (J15DW)	05/04/2004	Qiaojia	*Pinus armandii* bark	EF055218	EF055255
P. vismiae (PC9C)	16/04/2004	Dali	*Pinus armandii* bark	EF055219	EF055256
P. vismiae (PC11A)	16/04/2004	Dali	*Pinus armandii* bark	EF055220	EF055257
P. vismiae (PN3CW)	16/04/2004	Dali	*Pinus armandii* bark	EF055221	EF055258
P. vismiae (Q15DY)	05/04/2004	Qiaojia	*Pinus armandii* bark	EF055222	EF055259

Notes: Dongchuan --- Er Er Er Forest Farm, Dongchuan, Yunnan;
Dali --- Luopingshan Forest Farm, Dali, Yunnan;
Qiaojia --- Mashu Forest Farm, Qiaojia, Yunnan;
Tiantaishan --- Tiantaishan Forest Farm, Qionglai, Sichuan.

Table 2. Conidial morphology of the strains used for this study

Species (Strain)	Conidia length (µm)	Conidia width (µm)	Conidia length /width ratio	Colour of middle cells	Middle cells length (µm)	Length of apical appendages (µm)	Length of basal appendages (µm)
P. algeriensis (K9DY)	20-22.5	5-7.5	2.3-2.6	almost concolorous, olivaceous, the lowest one slightly lighter	12.5-15	5-12.5	3.8-5
P. carveri (G5HA)	17.5-25	7.5-8.8	2.6-3	almost concolorous, olivaceous, the lowest one slightly lighter	12.5-15	10-27.5	2.5-5
P. caudata (K14DW)	27.5-35	6.3-7.5	4-4.7	almost concolorous, brown, the lowest one slightly lighter	17.5-20	10-17.5	5-8.8
P. cocculi (C11A)	22.5-30	6.3-7.5	3.8-5.3	almost concolorous, olivaceous, the lowest one lighter	15-16.3	10-17.5	3.8-6.3
P. cocculi (DFFW)	20-27.5	6.3-7.5	3.8-5.3	almost concolorous, olivaceous, the lowest one lighter	13.8-17.5	5-16.3	2.5-5
P. cocculi (DH12DY)	22.5-27.5	5-7.5	3.6-5	almost concolorous, olivaceous, the lowest one lighter	15-17.5	8.8-15	2.5-8.8
P. cocculi (EY2AR)	20-25	5-6.3	3.4-4	almost concolorous, pale brown, the lowest one lighter	12.5-16.3	5-17.5	5-10
P. cocculi (PS11BY)	22.5-27.5	5-6.3	4.5-6	almost concolorous, pale brown, the lowest one slightly lighter	15-17.5	7.5-17.5	2.5-7.5
P. cocculi (YC4D)	20-22.5	7.5-8.8	2.6-3	almost concolorous, pale brown, the middle one darkest, the lowest one lightest	12.5-15	15-27.5	5-15
P. cocculi (YY1C)	22.5-25	5-6.3	3.6-5	almost concolorous, olivaceous, the lowest one lighter	12.5-17.5	10-32.5	5-11.3
P. disseminata (EC3A)	22.5-27.5	6.3-7.5	3-4	almost concolorous, pale brown, the lowest one lighter	15-17.5	12.5-20	3.8-5
P. funerea (ML4DY)	32.5-35	7.5-8.8	3.7-4.6	almost concolorous, pale brown	20-25	7.5-20	10-15
P. heterocornis (PN3DW)	22.5-25	6.3-7.5	3-4	almost concolorous, pale brown, the lowest one lighter	12.5-15	12.5-30	2.5-6.3
P. lespedezae (C17AW)	20-27.5	7.5-8.8	2.7-3.7	almost concolorous, pale brown, the middle one darkest, the lowest one lightest	13.8-17.5	15-22.5	2.5-7.5
P. lespedezae (EC12A)	20-25	7.5-8.8	2.3-3.2	almost concolorous, pale brown, the lowest one lighter	12.5-15	10-15	5-6.3
P. lespedezae (ML2A)	21.3-25	7.5-8.8	2.4-3.3	almost concolorous, pale brown, the lowest one lighter	13.8-17.5	12.5-17.5	3.8-8.8
P. lespedezae (PN3AY)	20-22.5	7.5-8.8	3-3.5	almost concolorous, pale brown, the middle one darkest, the lowest one lightest	12.5-10	12.5-22.5	5-7.5
P. lespedezae (PN3DH)	20-25	6.3-7.5	2.6-4	almost concolorous, pale brown, the middle one darkest, the lowest one lightest	13.8-15	10-25	5-7.5
P. lespedezae (SY16AW)	20-22.5	7.5-8.8	2.5-3	almost concolorous, pale brown, the lowest one lighter	12.5-15	15-27.5	2.5-5
P. lespedezae (SY16E)	20-25	7.5-10	2-3.3	almost concolorous, brown, the lowest one lighter	15-17.5	10-22.5	2.5-10

Table 2 continued. Conidial morphology of the strains used for this study

Species (Strain)	Conidia length (µm)	Conidia width (µm)	Conidia length /width ratio	Colour of middle cells	Middle cells length (µm)	Length of apical appendages (µm)	Length of basal appendages (µm)
P. lespedezae (YY12A)	20-25	7.5-8.8	3.1-3.7	almost concolorous, pale brown, the lowest one lighter	12.5-15	15-22.5	3.75-7.5
P. neglecta (EP2DY)	22.5-25	7.5-8.8	3-3.3	almost concolorous, brown, the lowest one lighter	12.5-15	5-15	3.8-5
P. neglecta (EY6D)	20-25	6.3-7.5	2.6-4	almost concolorous, brown, the lowest one lighter	12.5-15	10-17.5	2.5-7.5
P. neglecta (K9AW)	22.5-25	5-7.5	3-4.5	almost concolorous, brown, the lowest one lighter	12.5-15	11.3-25	5-6.3
P. neglecta (Q13DW)	20-25	7.5-8.8	2.7-3.3	almost concolorous, brown, the lowest one lighter	12.5-15	10-17.5	5-7.5
P. neglecta (SY19C)	20-22.5	7.5-8.8	3.4-3.7	almost concolorous, pale brown, the middle one darkest, the lowest one lightest	12.5-15	7.5-20	5-7.5
P. neglecta (YY1A)	20-25	6.3-7.5	3-4.3	almost concolorous, pale brown, the middle one darkest, the lowest one lightest	15-16.3	15-22.5	2.5-7.5
P. neglecta (YY8A)	20-25	6.3-7.5	3.6-4.8	almost concolorous, olivaceous, the lowest one lighter	12.5-17.5	10-25	2.5-5
P. olivacea (MS002)	20-25	6.3-7.5	3.6-4.8	almost concolorous, olivaceous, the lowest one lighter	12.5-17.5	101-20	3.8-7.5
P. olivacea (SY17A)	20-25	6.3-7.5	2.7-4	almost concolorous, pale brown, the lowest one lighter	12.5-15	15-27.5	2.5-5
P. olivacea (YY6A)	22.5-25	6.3-7.5	3.7-5.3	almost concolorous, olivaceous, the lowest one lighter	15-17.5	20-37.5	3.75-10
P. olivacea (YY8C)	22.5-25	6.3-7.5	3-4	almost concolorous, pale brown, the lowest one lighter	15-17.5	15-45	5-7.5
P. vismiae (J15DW)	15-22.5	7.5-8.8	2-3	almost concolorous, pale brown, the lowest one lighter	10-15	10-15	2.5-5
P. vismiae (PC9C)	20-25	6.3-8	2-2.2	almost concolorous, pale brown, the lowest one lighter	12.5-15	12.5-20	2.5-5
P. vismiae (PC11A)	20-22.5	6.3-7.5	2.7-3.5	almost concolorous, pale brown, the middle one darkest, the lowest one lightest	12.5-15	12.5-20	2.5-6.3
P. vismiae (PN3CW)	22.5-27.5	6.3-8.8	3-3.4	almost concolorous, pale brown, the lowest one lighter	12.5-17.5	7.5-17.5	3.8-7.5
P. vismiae (Q15DY)	20-22.5	7.5-8.8	3.3-4	almost concolorous, pale brown, the lowest one lighter	10-15	10-15	2.5-5

Notes: 1) The conidial shape of most strains are fusiform, fusoid, straight or curved, so they were not mentioned in the table;
2) Most strains have 2-3 apical appendages and 1 basal appendage, so they were not mentioned in the table.

Cigelnik, 1997), respectively. The amplification reaction included 5-10ng of fungal genomic DNA (3 μL) as template, 5 μL of 10× Mg free PCR buffer, 3 μL of 25 mM $MgCl_2$, 4 μL of 2.5 mM deoxyribonucleotide triphosphate (dNTPs), 1.5 μL of each 10 μM primers (ITS4 and ITS5, or BT2A and BT2B), 31.7 μL sterile water and 0.3 μL of 2.5 units of Taq DNA Polymerase (Promega, Madison, Wisconsin) (total volume of 50 μL). The thermal cycling parameters outlined by Cai *et al*. (2005, 2006) were used. This included an initial denaturation of 95ºC for 3 minutes, followed by 30 cycles consisting of denaturation at 95ºC for 1 minute, annealing at 52ºC for 55 seconds, and extension of 72ºC for 1 minute. A final extension at 72ºC for 10 minutes was added at the end of the thermal cycling. Amplified products were visualised on 1% agarose gel to check for product size and purity. PCR products were purified by using GFX^{TM} PCR DNA and Gel Band Purification Kit under the guide of the manufacturer's protocol (Amersham Biosciences). Purified PCR products were directly sequenced with the above mentioned primers in an automated sequencer (ABI 3730 sequence analyser).

Phylogenetic analyses

Nucleotide sequences generated from the 5.8S gene and the internal transcribed spacers (ITS), and partial β-tubulin gene were aligned using CLUSTALX (1.83) (Thompson *et al*., 1997). With the use of BioEdit (Hall, 1999), manual adjustments were made by inserting gaps to improve the alignments. Phylogenetic analyses based on maximum parsimony (MP) were performed for single gene dataset (ITS & β-tubulin) as well as for a combination of the two genes in PAUP v.4.0b10 (Swofford, 2002). Ambiguously aligned regions were excluded. Based on previous phylogenetic studies, *Seiridium cardinale* (GenBank Accession No: AF409995 (ITS), AF320503 (β-tubulin)) was used as outgroup (Barnes *et al*., 2001; Jeewon *et al*., 2002, 2003a, 2004).

Datasets were initially analyzed using weighted and unweighted parsimony. All characters were unordered. The heuristic search option was used, ignoring invariant and uninformative characters. Random addition of sequences with tree bisection-reconnection (TBR) branch swapping was performed. MulTrees option was in effect, and zero-length branches were collapsed. Weighted parsimony analyses were conducted in which changes among transitions, transversions, and gaps were subjected to a symmetric stepmatrix generated using STMatrix ver.2.2 (François Lutzoni and Stefan Zoller, Biology Department, Duke University) as described by Miller and Huhndorf (2004). Tree scores, including tree length (TL), consistency index

(CI), retention index (RI), rescaled consistency index (RC) and homoplasy index (HI) were also calculated for all the trees generated under different parameters.

MrModeltest 2.2 (Nylander, 2004) was used to get the best-fit model of substitution for maximum likelihood (ML) analyses and MrBayes analyses. ML analyses were carried out using heuristic search, with addition sequence set to "asis" and TBR branch swapping algorithm.

Branch support and stability for all maximum parsimony and neighbour joining analyses were assessed by bootstrap analysis (Felsenstein, 1985). Bootstrap analysis was carried out based on 1000 resampled data sets analyzed with random addition of taxa. Bayesian posterior probabilities were calculated using MrBayes v3.0. (Huelsenbeck and Ronquist, 2001). The implemented model was the same as that for ML analysis. One million generations were run for four chains and sampled every 100th generations resulting in 10,000 trees. The first 1,000 trees were discarded because they represented the burn-in phase of the analysis and the remaining 9,000 trees were used to calculate the posterior probabilities in a consensus tree. Trees were viewed in Treeview (Page, 1996).

We also assessed the combinability ot the two datasets by adopting the method used by Miller and Huhndorf (2005) which simply compares highly supported clades (bootstrap support values ≥70% and Bayesian poserior probabilites ≥95% among trees generated by different datasets) to detect conflict. If there is no conflict among the highly supported clades in the trees generated from single gene datasets, this implies that the genes share similar phylogenetic histories and phylogenetic resolution and support can be increased by combining the datasets.

Results

Diversity of Pestalotiopsis from hosts

The 132 strains of *Pestalotiopsis* from bark and needles of *Pinus armandii* and leaves of *Ribes* species were identified into 20 species based on the morphological characteristics of conidia. Specifically, 111 strains (19 species) were isolated from bark of *Pinus armandii*, 12 strains (5 species) from needles of *Pinus armandii* and 11 strains (7 species) from leaves of *Ribes* species. Bark had the highest species diversity and all species isolated from needles occurred on bark. With the exception of *Pestalotiopsis monochaetioides*, all species isolated from leaves of *Ribes* species also occurred on bark of *Pinus armandii*.

Cultural and morphological characters

Colony morphology of isolates of *Pestalotiopsis* species was variable (Table 3). Even under the same growth conditions, differences were observed in colour, growth rate and texture among the same species. This was particularly noticeable after the same strain was subcultured a few times. For example, when *P. lespedezae* (C17AW) was first isolated, the colony was pink and cottony, covered the Petri dish (10 cm in diameter) within four days and sporulated with abundant fruit bodies. After two subcultures, the colony became white, there was a sharp decline in the growth rate (the diameter was less than 4 cm after 10 days) and fruiting bodies were only sparse.

A great number of abnormal conidia appeared following repeated subculture. The lengths of the apical and basal appendages also varied. When *P. funerea* (ML4DY) was first isolated, the average length of apical appendages was 12.5-22.5 µm. However after being subcultured twice, the apical appendages were 5-15 µm long. There were no distinguishable differences in other conidial characteristics (conidial length, median cells length, conidial width and colour of median cells) among the different generations.

Phylogenetic results

ITS phylogenies

ITS1 and ITS2 regions, including the 5.8S rDNA gene, of the studied strains were 593 bp. Weighted and unweighted maximum parsimony, treating gaps as missing data or newstate, resulted in only one tree with identical topology (results not shown).

Based on MrModeltest 2.2, model K80+G was chosen to be most appropriate for ML analyses and MrBayes analyses. ML analysis resulted in a tree that was topologically similar to that from MP analyses (results not shown). Generally, all strains were grouped into 5 distinct clades, but there were confident statistical supports for only two clades.

Table 3. Colony morphology of the strains used for this study.

Species (Strain)	Edge	Top colour	Reverse	Colony textura	Elevation	Colony growth rate (mm/d)
P. algeriensis (K9DY)	fimbriate	pink and pale brown	dark brown; zonate	fluffy	raised	12
P. carveri (G5HA)	fimbriate	dirty pink, with some brown parts	orange	fluffy	raised	13
P. caudata (K14DW)	fimbriate	pink	orange; unclear zonate	cottony	flat	13
P. cocculi (C11A)	entire	yellow	light yellow; unclear zonate	fluffy	raised	14
P. cocculi (DFFW)	fimbriate	white	light orange	cottony	flat	13
P. cocculi (DH12DY)	fimbriate	pink	light orange; unclear zonate	cottony	flat	12
P. cocculi (EY2AR)	fimbriate	white	light orange; unclear zonate	cottony	flat	12
P. cocculi (PS11BY)	entire	dirty white, with some brown parts	light orange; unclear zonate	cottony	flat	14
P. cocculi (YC4D)	fimbriate	white	orange; zonate	cottony	flat	7
P. cocculi (YY1C)	fimbriate	light brown	dark brown	cottony	flat	13
P. disseminata (EC3A)	fimbriate	light brown	light brown			10
P. funerea (ML4DY)		white				10
P. heterocornis (PN3DW)	fimbriate	white	light orange	cottony	flat	13
P. lespedezae (C17AW)	entire	pink	pink	fluffy	raised	14
P. lespedezae (EC12A)	fimbriate	pink	light orange	cottony	flat	12
P. lespedezae (ML2A)	fimbriate	white	light orange	cottony	flat	10
P. lespedezae (PN3AY)	fimbriate	pink	orange	cottony	flat	13
P. lespedezae (PN3DH)	fimbriate	pink, with some brown parts	orange	fluffy	raised	13
P. lespedezae (SY16AW)	entire	white	light brown; zonate	cottony	flat	14
P. lespedezae (SY16E)	fimbriate	white	orange	cottony	flat	12
P. lespedezae (YY12A)	fimbriate	white	orange	cottony	flat	8
P. neglecta (EP2DY)	entire	pink	light orange	cottony	flat	14
P. neglecta (EY6D)	fimbriate	dirty white	light brown; zonate	cottony	flat	13
P. neglecta (K9AW)	fimbriate	white and pale brown	brown; zonate	fluffy	raised	12
P. neglecta (Q13DW)	fimbriate	white	light orange	cottony	flat	10
P. neglecta (SY19C)	fimbriate	light pink	pink	cottony	flat	13
P. neglecta (YY1A)	fimbriate	white	light orange	cottony	flat	13
P. neglecta (YY8A)	fimbriate	white	orange	cottony	flat	12

Table 3 continued. Colony morphology of the strains used for this study.

Species (Strain)	Edge	Top colour	Reverse	Colony textura	Elevation	Colony growth rate (mm/d)
P. olivacea (MS002)	fimbriate	White	white	cottony	flat	8
P. olivacea (SY17A)	fimbriate	dirty white	light orange	cottony	flat	13
P. olivacea (YY6A)	fimbriate	white	light orange	cottony	flat	7
P. olivacea (YY8C)	entire	white	light orange	fluffy	raised	14
P. vismiae (J15DW)	entire	white	light orange; unclear zonate	fluffy	raised	14
P. vismiae (PC9C)	fimbriate	white	white	cottony	flat	9
P. vismiae (PC11A)	undulated	white	white	cottony	flat	13
P. vismiae (PN3CW)	undulated	dirty white	orange	cottony	flat	10
P. vismiae (Q15DY)	entire	dirty white	light orange; unclear zonate	fluffy	raised	14

Note: 1) The mycelia of all strains are aerial, so they were not mentioned in the table;

β-tubulin phylogenies

The β-tubulin gene dataset comprised 456 bp. Weighted parsimony treating gaps as missing data generated one tree (results not shown) which was identical in topology to those obtained from unweighted parsimony.

With the use of MrModeltest 2.2, model HKY+G was chosen for ML searches and MrBayes analyses. Transition weighted three times over transversion, and other parameters were as follows: shape parameter of 0.2161 and - ln likelihood = 374.2062. Estimated base frequencies were as follows: A = 0.2116, C = 0.3181, G = 0.2266 and T = 0.2437. When transition–transversion ratio and shape parameter were estimated, one tree was obtained, which was also identical in topology to that from weighted parsimony (results not shown).

All strains were grouped into 6 distinct clades, essentially similar to those in ITS phylogenies. All of the clades received strong statistical support except one clade characterized by DH12DY, K14DW and ML4DY (no bootstrap support in MP but with 76% Bayesian posterior probabilities). However, the phylogenetic positions of some strains were inconsistent in ITS phylogenies and β-tubulin phylogenies.

Combinations of ITS and β-tubulin

The combined ITS and β-tubulin dataset was 1049 bp. MP heuristic search treating gaps as newstate with unequally weighted parsimony generated eight trees. The consensus tree was used to explain the phylogenetic relationships among the strains (Fig.1) with TL = 384 steps, CI = 0.844, RI = 0.894, RC = 0.754, and HI = 0.156.

Phylogenetic analysis grouped *Pestalotiopsis* strains into 6 clades (Fig.1). Taxa in Clade A constitute a strongly supported monophyletic group (96% bootstrap support and 99% Bayesian posterior probabilities). This clade can be divided into two groups: the first one comprised *Pestalotiopsis lespedezae* strains, *P. neglecta* and *P. olivacea* whereas the second one consists of *P. caudata*, *P. cocculi* and *P. funerea*. This phylogeny is topologically similar to those obtained from single gene analyses

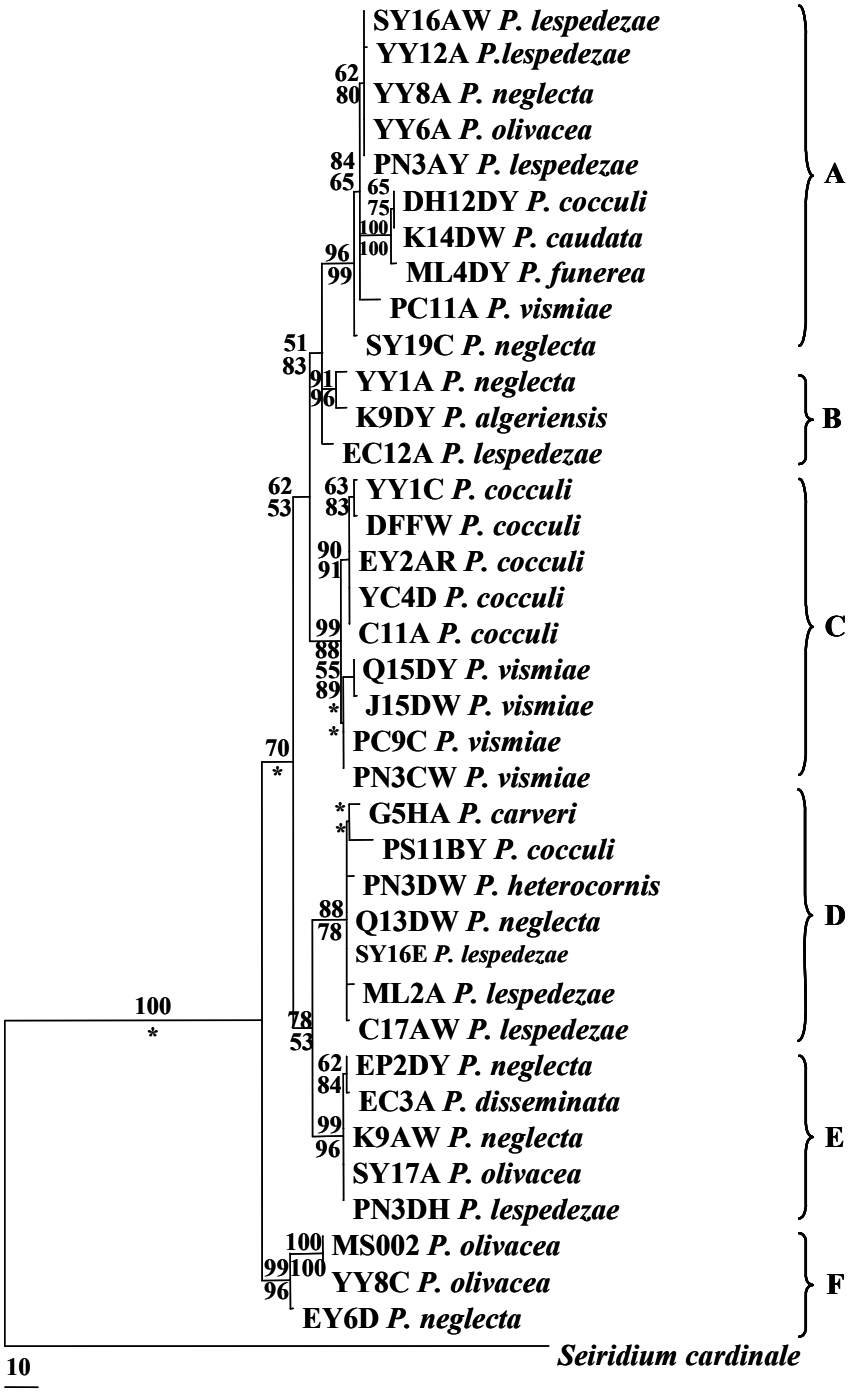
Clade B had 91% and 96% support (bootstrap and Bayesian posterior probabilities respectively). In both, ITS and β-tubulin phylogenies, *P. neglecta* and *P. algeriensis* clustered together but was also found to be related to other strains.

The strains in Clade C clustered together with high bootstrap confidence (99%), but with lower Bayesian posterior probabilities (88%). Most of these strains belong to *Pestalotiopsis cocculi* and *P. vismiae*.

Clade D also received high bootstrap support (88%) but low Bayesian posterior probabilities (78%). This clade included 5 species: *Pestalotiopsis carveri*, *P. cocculi*, *P. heterocornis*, *P. lespedezae* and *P. neglecta*.

Clade E is similar to Clade D, with 99% and 96% support from bootstrap and Bayesian posterior probabilities respectively. This clade had 4 species: *Pestalotiopsis disseminata*, *P. lespedezae*, *P. neglecta* and *P. olivacea*.

Fig. 1. Phylogenetic tree generated from a combination of ITS and partial β-tubulin sequence analysis using PAUP v.4.0.b10 (*Seiridium cardinale* (AF409995 for ITS and AF320503 for β-tubulin) is the outgroup). Numbers above branches indicate that given branches were supported more than 50% from 1000 bootstrap replications. Numbers below branches indicate the given branches were supported more than 50% with Bayesian analysis. * indicates that the given branches were supported less than 50% by bootstrap or Bayesian analysis. Letter A-F represent the different groups possessing different morphological characters.


SY16AW P. lespedezae
YY12A P.lespedezae
YY8A P. neglecta
YY6A P. olivacea
PN3AY P. lespedezae
DH12DY P. cocculi
K14DW P. caudata
ML4DY P. funerea
PC11A P. vismiae
SY19C P. neglecta
YY1A P. neglecta
K9DY P. algeriensis
EC12A P. lespedezae
YY1C P. cocculi
DFFW P. cocculi
EY2AR P. cocculi
YC4D P. cocculi
C11A P. cocculi
Q15DY P. vismiae
J15DW P. vismiae
PC9C P. vismiae
PN3CW P. vismiae
G5HA P. carveri
PS11BY P. cocculi
PN3DW P. heterocornis
Q13DW P. neglecta
SY16E P. lespedezae
ML2A P. lespedezae
C17AW P. lespedezae
EP2DY P. neglecta
EC3A P. disseminata
K9AW P. neglecta
SY17A P. olivacea
PN3DH P. lespedezae
MS002 P. olivacea
YY8C P. olivacea
EY6D P. neglecta
Seiridium cardinale
A
B
C
D
E
F
10

Clade F, which comprises of 2 strains of *Pestalotiopsis olivacea* (MS002 and YY8C) and 1 strain of *P. neglecta* (EY6D), clustered together with high statistical support, 99% from bootstrap analysis and 96% from Bayesian posterior probabilities respectively. It should be pointed out that, however, in ITS analyses, the phylogenetic position of EY6D (*P. neglecta*) was different. It clustered with *P. vismiae*, but the relationship was not supported.

Discussion

Diversity of Pestalotiopsis from Pinus armandii and Ribes species

Of the 132 endophytic strains of *Pestalotiopsis* isolated, 123 strains (19 species) were from *Pinus armandii* and 11 strains (7 species) were from *Ribes* species. *Pinus armandii* and *Ribes* species were found to harbor abundant *Pestalotiopsis* species, illustrating that more than one *Pestalotiopsis* species can dwell in the same host. In Guba's (1961) *Monograph of Monochaetia and Pestalotia*, 8 species from *Pestalotia* and *Monochaetia* were reported from 23 species of *Pinus* (e.g. *P. strobus* and *P. sylvestris*) either as saprobes or pathogens. For example, *Pestalotia conigena*, *P. foedans*, *P. funerea* and *P. stevensonii* were recorded from *Pinus strobus*; *Pestalotia conigena*, *P. funerea*, *P. hartigii* and *P. stevensonii* from *Pinus sylvestris*; *Monochaetia pinicola*, *Pestalotia conigena*, *P. foedans* and *P. funerea* from *P. palustris*. Nag Raj (1993) also reported 6 species of *Pestalotia* from 4 species of *Pinus*, in which 4 species of *Pestalotia* (*P. conigena*, *P. funerea*, *P. macrochaeta* and *P. strobilicola*) are from *Pinus sylvestris*. Although these species have been names as "*Pestalotia*", we reclaim that they belong to the genus *Pestalotiopsis* (Jeewon *et al*., 2002, 2003a). More than one endophytic *Pestalotiopsis* species can be also isolated from one host (Strobel *et al*., 2000; Cannon and Simmons, 2002; Kumar and Hyde, 2004; Lu and Wu, 2004). Four endophytic *Pestalotiopsis* species were obtained when Strobel *et al*. (2000) studied endophytic fungi from *Fragraea bodenii* in Papua New Guinea. Cannon and Simmons (2002) isolated 5 endophytic species of *Pestalotiopsis* from living asymptomatic leaves of 12 tree species from two locations in the Iwokrama Forest Reserve, Guyana. Lu and Wu (2004) isolated 5 endophytic species of *Pestalotiopsis* from tea bushes in Southern Henan Province, China. Similarly, when Kumar and Hyde (2004) studied endophytic fungi from *Tripterygium wilfordii*, 5 species of *Pestalotiopsis* were isolated.

Cultural variation

Dube and Bilgrami (1965) studied cultural variations and their implications on conidial morphology of *Pestalotiopsis darjeelingensis*. They found that there were morphometric differences in the conidial and appendage length between the same species isolated from the same material and those obtained from culture. They reported that the average conidial length was greater in culture, while the length of the apical appendages were shorter. Purohit and Bilgrami (1969) also found considerable variations and abnormalities in conidial morphology under cultural conditions.

In this study we found that when strains were cultured in the same media and under the same environmental conditions, conidial length, conidial width and length of 3 median pigmented cells of the same strain remained similar even after several subcultures. The length of apical and basal appendages, however, was variable, possibly being affected due to growth in the artificial media. We also noticed a remarkable difference in cultural characteristics among morphologically similar strains. When one particular strain was subcultured a few times on freshly made artificial media, cultural morphology such as appendage length changed. This phenomenon is not restricted to *Pestalotiopsis* species, but has been reported in several other studies, especially among pathogenic coelomycetous species, for example *Colletotrichum* (Smith and Black, 1990; Gunnell and Gubler, 1992; Wu and Zhang, 1994 a, b, c; Wu *et al*., 1995; Photita *et al*., 2005). This is important in the taxonomy of *Pestalotiopsis* species and therefore caution is needed when describing species based on cultural characteristics.

Host/tissue relationship

Zhou and Hyde (2001) discussed host-specificity in fungi. They reported that some endophytes, obligate pathogens and mycorrhizal fungi were host-specific. For most saprobic fungi and facultative pathogens, however, host-specificity is not suitable. They used the term host-recurrence to describe the repeated occurrence of saprobic species on a particular host. Yanna *et al*. (2001) studied fungal communities on 7 species of decaying palm fronds. They also reported evidence for host-specificity and host-recurrence on different hosts and frond parts. Species of *Pestalotiopsis* were reported as facultative pathogens, saprobes and endophytes (Steyaert, 1949; Guba, 1961; Suto and Kobayashi, 1993; Strobel *et al*., 1997, 2000; Rivera and Wright, 2000; Karakaya, 2001; Tagne and Mathur, 2001; Gonthier and Mycologici, 2002; Wang *et al*., 2002; Zhang *et al*., 2003; Kumar and Hyde, 2004; Sousa *et al*.,

2004). Based on the definition of host-specificity, this genus is not host-specific and host is not suitable for identification. However, in previous studies of *Pestalotiopsis*, much emphasis has been placed on host-specificity, and many new combinations or species were published based on occurrence on a particular host (Guba, 1961; Chen *et al*., 2002, 2003). Jeewon *et al*. (2004) investigated the phylogenetic relationships among different species of *Pestalotiopsis* on the same and different hosts. They found that isolates from the same host were not necessarily phylogenetically related. On the contrary, a close phylogenetic relationship was apparent between isolates with similar morphological characters. This also indicated that species of *Pestalotiopsis* should not be treated as host-specific, unless proper pathogenicity tests or other tests are performed.

In this study, we also investigated the relationships of strains of endophytic *Pestalotiopsis* from two hosts (*Pinus armandii* and *Ribes* spp.). Phylogenies obtained largely correspond to previously published studies that strains isolated from similar hosts were unrelated. That means endophytic *Pestalotiopsis* are not host-specific. In contrast, a close phylogenetic association is found among strains characterized by similar morphologies. For example, ML4DY, EY2AR, ML2A and EY6D were all isolated from *Ribes* species, but they were distributed in four different clades (Fig. 1).

We also tested the relationships of *Pestalotiopsis* strains from two tissues of *Pinus armandii*. Ten strains were from needles and 23 strains were from bark. Although strains were isolated from the same tissue types, strains with similar morphological characters were found to be more phylogenetically related. That means endophytic *Pestalotiopsis* are not tissue-specific either.

There are 222 epithets of *Pestalotiopsis* in Index Fungorum, http://www.indexfungorum.org/Names/Names.asp. There have been some nomenclatural and taxonomic amendments that some species have been transferred to other genera, such as *Monochaetia*, *Pestalotia* and *Truncatella* or have been treated as synonyms of other species (e.g. *Pestalotiopsis dichaeta* has been treated as synonym of *Pestalotiopsis microspora*). Only 50 species were accepted in *Dictionary of the Fungi* by Kirk *et al*. (2001). Hawksworth (2005) commented on the number of fungal species in relation to biodiversity and cited *Pestalotiopsis* as an example where taxonomists might have either underestimated or overestimated species numbers.

Pestalotiopsis species can be endophytes, pathogens or saprobes and the intimate relationship with the hosts in an endophytic or pathogenic life mode might indicate that taxa have evolved with their host and therefore the genus should be specious. This appears to be the case in genera such as *Mycosphaerella* and its anamorphs (Ayala-Escobar *et al*., 2006). However our

results do not indicate this. The number of species that should be accommodated in *Pestalotiopsis* therefore remains unclear, but is unlikely to be as high as the 222 names in *Index Fungorum*. Further work based on morphological and molecular studies needs to be carried out to determine which species are acceptable in this genus.

Phylogenetic significance of morphologies

Morphological characters are important in identifying *Pestalotiopsis* species (Steyaert, 1949; Guba, 1961; Sutton, 1980; Nag Raj, 1993). Characters used however are few and often overlap. This results in identification problems and difficulties in differentiating species. In genera such as *Colletotrichum*, molecular phylogeny has been helpful in establishing species concepts (Photita *et al*., 2005).

Molecular studies have shown that *Pestalotiopsis* is monophyletic (Jeewon *et al*., 2002, 2003a, b, 2004). Based on our phylogenies derived from individual and combined ITS and β-tubulin datasets, we also found *Pestalotiopsis* to be monophyletic with high statistical support. *Pestalotiopsis* is easily distinguishable with unique characters. Conidia are usually fusiform, straight or slightly curved, and 3-4-euseptate. The 3 median cells are pigmented (either concolorous or versicolorous). Apical appendages are mostly filiform, occasionally knobbed, one to many (mostly 2-3), branched or unbranched, and arise from the apical cell. Basal appendages are usually present, and arise from the basal cell (Steyaert, 1949). Proper identification at the species level is problematic because of inadequate valid morphological characters and also because many species were named solely based on host.

Identification of *Pestalotiopsis* species has mainly been based on the degree of pigmentation of median cells (either brown concolorous or versicolorous); apical appendage tip morphology (presence or absence of spatula); apical appendage length and spore length and width and basal appendage length (Steyaert, 1949; Jeewon *et al*., 2003a).

In this study, we focused on species that possess median pigmented cells that are concolorous, olivaceous and pale brown and with apical appendages that are not knobbed. The concolorous, olivaceous or pale brown subgroup was separated to 13 subgroups based on the length to width of the conidia (Guba, 1961). Most of our strains are included in subgroup 5 (conidia 18~26 × 5~8 μm) and subgroup 6 (conidia 18~26 × 7~9.5 μm) according to Guba's key. Based on morphometric dimensions, and number of apical appendages as well as conidial shape, we categorized the 37 isolates into 11 species. One major obstacle, however, was to make a proper use of the key provided by Guba

(1961) to identify species. There is so much overlap in morphology that it was difficult to assign a name for each strain or isolate. For instance, the morphological difference among *P. disseminata*, *P. neglecta* and *P. uvicola* is obscure. Similar findings are reported for *P. bicilia*, *P. lawsoniae*, *P. vismiae* and *P. carveri*. Designation of species names appears to be based on personal opinions.

Based on our morphological observations, we concur with the taxonomic arrangement proposed by Jeewon *et al*. (2003a). However, when dealing with a higher number of samples, especially those that possess similar range of conidial length and width, appendage length and similar type of pigmentation, it is very difficult to segregate species. Even our molecular phylogenies generated from two genes appear to be unreliable in justifying whether species groups or subgroups within the section "concolour" is valid or not. It is therefore highly plausible that many species within one clade constitute only 1 species. If this is the case, then there is no doubt that the number of *Pestalotiopsis* species described based on slight morphological differences has largely been overestimated.

Comparison between ITS and β-tubulin gene phylogenies

In this study we selected 37 strains of *Pestalotiopsis* belonging to 11 species based on morphological characters and used DNA sequence analyses to test the validity of our morphologically based identifications. Phylogenetic results obtained show that the ITS gene offered less informative characters than the β-tubulin gene. For ITS analysis, whether we treated gaps as missing data or newstate, the number of informative characters was less than 30 out of 593 (about 5%) and phylogenies could not separate taxa into groups with high statistical support. In β-tubulin analyses, when gaps were treated as missing data, the number of parsimony informative characters was about 11% and when we treated gaps as newstate, more than 15% of characters were parsimony informative. Comparing the two trees, β-tubulin phylogenies were more resolved than ITS based phylogenies. However, neither of the trees from single gene segregated the strains into different groups with high support. On the other hand, a combination of the ITS and β-tubulin datasets offered a better phylogeny to resolve taxonomic questions. As mentioned in other phylogenetic studies, it would be wise to incorporate at least 2 genes so that more accurate phylogenetic interpretation can be inferred from molecular data.

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References

Anon. (1994). Dendrology (Southern Edition). China Forest Press: Beijing, China.

Ayala-Escobar, V., Yáñez-Morales, M.J., Braun, U., Groenewald, J.Z. and Crous, P.W. (2006). *Pseudocercospora opuntiae* sp. nov., the causal organism of cactus leaf spot in Mexico. Fungal Diversity 21: 1-9.

Barnes, I., Roux, J., Wingfield, M.J., Coetzee, M.P.A. and Wingfield, B.D. (2001). Characterization of *Seiridium* spp. associated with cypress canker based on β-tubulin and histone sequences. Plant Disease 85: 317-321.

Cai, L., Jeewon, R. and Hyde, K.D. (2005). Phylogenetic evaluation and taxonomic revision of *Schizothecium* based on ribosomal DNA and protein coding genes. Fungal Diversity 19: 1-21.

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.

Chen, Y.X. and Wei, G. (1993). New combinations of *Pestalotiopsis* from China. Journal of Guangxi Agricultural University 12: 23-35.

Chen, Y.X. and Wei, G. (1997). Continuous notes on congeners of *Pestalotiopsis* in China. Journal of Guangxi Agricultural University 16: 1-9.

Chen, Y.X., Wei G., Chen, W.P., Wang, Z.W. and Lu, Z.H. (2003). Three new species of *Pestalotiopsis* in China. Journal of Guangxi Agricultural and Biological Science 22: 1-4.

Chen, Y.X., Wei, G. and Chen, W.P. (2002). New species of *Pestalotiopsis*. Mycosystema 21: 316-323.

Devarajan, P.T. and Suryanarayanan, T.S. (2006). Evidence for the role of phytophagous insects in dispersal of non-grass fungal endophytes. Fungal Diversity 23: 111-119.

Dube, H.C. and Bilgrami, K.S. (1965). Variations in the conidial morphology of *Pestalotiopsis darjeelingensis* in culture. Current Science 34: 487.

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

El-Morsy, E.M., Dohlob, S.M. and Hyde, K.D. (2006). Diversity of *Alternaria alternata* a common destructive pathogen of *Eichhornia crassipes* in Egypt and its potential use in biological control. Fungal Diversity 23: 139-158.

Espinosa-Garcia, F.J. and Langnheim, J.H. (1990). The endophytic fungal community in leaves of coastal redwood population-diversity and spatial patterns. New Phytologist 116: 89-97.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39: 783-791.

Glass, N.L. and Donaldson, G. (1995). Development of primer sets designed for use with the PCR to amply conserved genes from filamentous ascomycetes. Applied & Environmental Microbiology 61:1323-1330.

Gonthier, P. and Nicolotti, G. (2002). First report of *Pestalotiopsis funerea* on *Cupressocyparis leylandii* in Italy. Plant Disease 86:1402.

Gonthier, P., Massimo, G. 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. Cambridge, Massachusetts USA.

Gunnell, P.S. and Gubler, W.D. (1992). Taxonomy and morphology of *Colletotrichum* species

Guo, L. (2006). Six new species of *Anthracoidea* (Ustilaginales) from China. Fungal Diversity 21: 81-92.

Guo, L.D. (2002). *Pestalotiopsis besseyi*, a new record of endophytic fungi from pine in China. Mycosystema 21: 455-456.

Hall, T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95-98.

Hawksworth, D.L. (2005). Host specificity in *Pestalotiopsis*, in Mycological Research News. Mycological Research 109: 1-6.

Hendrichs, M., Begerow, D., Bauer, R. and Oberwinkler, F. (2005). The genus *Anthracoidea* (*Basidiomycota*, *Ustilaginales*): a molecular phylogenetic approach using LSU rDNA sequences. Mycological Research 109: 31-40.

Hu, H.L. (2005). Study on dendrocola fungi on healthy and infected (by *Cronartium ribicola* Fischer) *Pinus armandii* Franch. MSc. Thesis. Southwest Forestry College, Kunming, Yunnan, China.

Huelsenbeck, J.P. and Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 17: 754-755.

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. 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. (2003b). Molecular systematics of the Amphisphaeriaceae based on cladistic analyses of partial LSU rDNA gene sequences. Mycological Research 107: 1392-1402.

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

Karakaya, A. (2001). First report of infection of kiwifruit by *Pestalotiopsis* sp. in Turkey. Plant Disease 85: 1028.

Kinloch, B.B.Jr. (2003). White pine blister rust in North American: past and prognosis. Phytopathology 93: 1044-1050.

Kirk P.M., Cannon, P.F., David, J.C. and Stalpers, J.A. (2001). *Ainsworth & Bisby's Dictionary of the Fungi*. 9th Edition. CAB International, UK.

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

Lu, D.S. and Wu, X.Q. (2004). Taxonomic study on the dendrocola of the tea plantations in southern area of Henan Province. Journal of Tea Science 24: 243-248.

Miller, A.N. and Huhndorf, S.M. (2004). A natural classification of *Lasiosphaeria* based on nuclear LSU rDNA sequences. Mycological Research 108: 26-34.

Miller, A.N. and Huhndorf, S.M. (2005). Multi-gene phylogenies indicate ascomal wall morphology is a better predictor of phylogenetic relationships than ascospore morphology in the Sordariales (Ascomycota, Fungi). Molecular Phylogenetics and Evolution 35: 60-75.

Nag Raj, T.R. (1993). *Coelomycetous anamorphs with appendage bearing conidia*. Mycologue Publications, Waterloo, Ontario, Canada.

Nylander, J.A.A. (2004). MrModeltest v2. Program distributed by the author. http://www.ebc.uu.se/systzoo/staff/nylander.html. Evolutionary Biology Centre, Uppsala University.

O'Donnell, K. and Cigelnik, E. (1997). Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. Molecular Phylogenetics and Evolution 7: 103-116.

Page, R.D.M. (1996). Treview: An application to display phylogenetic trees on personal computers. Computer Applications in the Biosciences 12: 357-358.
pathogenic on strawberry. Mycologia 84: 157-165.

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.

Purohit, D.K. and Bilgrami, K.S. (1969). Variations in the conidial morphology of genus *Pestalotiopsis*. Indian Phytopathology 22: 275-279.

Qiu, D.Y., Huang, M.J., Fang, X.H. and Zhu, C. (1994). Isolation of an endophytic fungus associated with *Taxus yunnanensis*. Mycosystema 13: 314-316.

Ren, W. (1993). Yunnan Forest Diseases. Yunnan Science and Technology Press, Kunming, China.

Rivera, M.C. and Wright, E.R. (2000). First report of azalea petal blight caused by *Pestalotiopsis guepinii* in Argentina. Plant Disease 84: 100.

Smith, B.J. and Black, L.L., (1990). Morphological, cultural and pathogenic variation among *Colletotrichum* species isolated from strawberry. Plant Disease 74: 69-76.

Sousa, M.F., Tavares, R.M., Geros, H. and Lino-Neto, T. (2004). First report of *Hakea sericea* leaf infection caused by *Pestalotiopsis funerea* in Portugal. Plant Pathology 53: 535.

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., Hess, W.M., Li, J.Y., Ford, E., Sidhu, R.S. and Summerell, B. (1997). *Pestalotiopsis guepinii*, a taxol-producing endophyte of the Wollemi pine, *Wollemia nobilis*. Australian Journal of Botany 45: 1073-1082.

Strobel, G.A., Li, J.Y, Ford, E., Worapong, J., Gary, I.B. and Hess, W.M. (2000). *Pestalotiopsis jesteri*, sp.nov. an endophyte from *Fragraea badenii* Wernh., a common plant in the southern highlands of Papua New Guinea. Mycotaxon 76: 257-266.

Strobel, G.A., Yang, X.S., Sears, J., Kramer, R., Sidhu, R.S. and Hess, W.M. (1996). Taxol from *Pestalotiopsis microspora*, an endophytic fungus of *Taxus wallichiana*. Microbiology 142: 435-440.

Sun, X.A. and Ge, Q.X. (1990). Ten new combinations of *Pestalotiopsis* from China. Journal of Zhejiang Agriculture University 16(Suppl. 2): 141-150.

Suto, Y. and Kobayashi, T. (1993). Taxonomic studies on the species of *Pestalotiopsis*, parasitic on conifers in Japan. Transactions of the Mycological Society of Japan 34: 323-344.

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

Swofford, D.L. (2002). PAUP*: Phylogenetic Analysis Using Parsimony (*and other methods). Version 4.0b10. Sinauer Associates, Sunderland, Massachusetts USA.

Tagne, A. and Mathur, S.B. (2001). First report of chlorotic spot of maize caused by *Pestalotiopsis neglecta*. Plant Pathology 50: 791.

Thompson, 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 24: 4876-4882.

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.

Wang, Z.W., Chen, W.P., Wei, G. and Chen, Y.X. (2002). Eight new recombination species of *Pestalotiopsis* in China. Journal of Guangxi Agricultural and Biological Science 21: 151-156.

Wei, G. and Chen, Y.X. (1994). Further notes on congeners of *Pestalotiopsis* in China. Journal of Guangxi Agricultural University 13: 115-128.

Wei, J.G. and Xu, T. (2004). *Pestalotiopsis kunmingensis*, sp. nov., an endophyte from *Podocarpus macrophyllus*. Fungal Diversity 15: 247-254.

White, T.J., Bruns, T.L., Lee, S. and Taylor, J.W. (1990). Amplication and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols*: *a Guide to methods and Applications* (eds. M. Innis, D.H. Gelfand, J.J. Sninsky and J.T. White). Academic press, San Diego, USA.

Worapong, J., Inthararaungsorn, S., Strobel, G.A. and Hess, W.M. (2003). A new record of *Pestalotiopsis theae*, existing as an endophyte on *Cinnamomum iners* in Thailand. Mycotaxon 88: 365-372.

Wu, W.P. and Zhang, Z.M. (1994a). Study on taxonomy of genus *Colletotrichum* Cda. I. Classification and nomenclature of the genus. Journal of Hebei Agricultural University 17(2): 24-30.

Wu, W.P. and Zhang, Z.M. (1994b). Study on taxonomy of genus *Colletotrichum* Cda. II. Division of species. Journal of Hebei Agricultural University 17(2): 31-37.

Wu, W.P. and Zhang, Z.M. (1994c). Study on taxonomy of genus *Colletotrichum* Cda. III. Species with falcate conidia in Hebei. Journal of Hebei Agricultural University 17(4): 19-25.

Wu, W.P., Zhang, Z.M. and Li Y.Q. (1995). Study on taxonomy of genus *Colletotrichum* Cda. V. Species with straight conidia in Hebei. Journal of Hebei Agricultural University 18(3): 63-67.

Yanna, Ho, W.H. and Hyde, K.D. (2001). Fungal communities on decaying palm fronds in Australia, Brunei, and Hong Kong. Mycological Research 105: 1458-1471.

Zhang, J.X., Xu, T. and Ge, Q.X. (2003). Notes on *Pestalotiopsis* form Southern China. Mycotaxon 85: 91-99.

Zhao, G.C. and Li, N. (1995). Thirty-four species of *Pestalotiopsis* in Yunnan. Journal of Northeast Forestry University 23: 21-33.

Zhou, D.Q and Hyde, K.D. (2001). Host-specificity, host-exclusivity, and host-recurrence in saprobic fungi. Mycological Research 105: 1449-1457.

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