Molecular and morphological description of *Pestalotiopsis hainanensis* sp. nov., a new endophyte from a tropical region of China

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During a survey of the diversity of *Pestalotiopsis* species in Hainan Province, a tropical region of China, a new endophytic fungus *Pestalotiopsis hainanensis* was isolated from the stem of *Podocarpus macrophyllus* at Xinglong Tropical Botanical Garden. The new species is morphologically distinguished from similar species such as *P. karstenii* in having unbranched and short apical appendages, from *P. heteroconis* in the absence of basal appendages, and from *P. westerdijkii* in median cell colour and absence of basal appendage. Furthermore, this new species has a large conidium length/width ratio. Phylogenetic analyses based on ITS regions (ITS1, 5.8S and ITS2) and beta-tubulin 2 gene (*tub2*) indicate that *P. hainanensis* is phylogenetically distinct from *P. karstenii*, *P. heterocornis* and *P. westerdijkii*.

Key words: Beta-tubulin; endophyte; new species; phylogeny; rDNA

Introduction

Pestalotiopsis Steyaert is the anamorph of *Pestalosphaeria* Barr belonging to the family *Amphisphaeriaceae* (Barr, 1975; Sutton, 1980). The conidia of *Pestalotiopsis* are usually fusiform, 5-celled, with three brown to fuliginous median cells and hyaline end cells, and with two or more apical appendages arising from the apical cell. At present, inter-specific delineation of this genus is based on morphology of the conidia (Guba, 1961; Nag Raj, 1993), conidiogenesis (Sutton, 1980) and teleomorph association (Barr, 1975, 1990; Zhu *et al*., 1991; Metz *et al*., 2000).

Approximately 220 species of *Pestalotiopsis* have been described (http://www.indexfungorum.org/Names/Names.asp), and many of them have morphological characters that overlap in many aspects. Many of them were named as a new species only according to its occurrence on new host plants (Venkatasubbaiah *et al*., 1991; Pal and Purkayastha, 1992; Chen *et al*., 2002,

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2003). The taxonomic affinities of *Pestalotiopsis* species have been confused and equivocal. This has made it necessary to evaluate the traditional taxonomy of the genus by molecular phylogenetic analysis.

The morphological characters having phylogenetic significance have been demonstrated and discussed by Jeewon *et al*. (2003) and Wei *et al*. (2005). Molecular studies indicated that *Pestalotiopsis* species isolated from same hosts are not necessarily related (Jeewon *et al*., 2004; Wei, 2004). It was proposed that when a new *Pestalotiopsis* species is described, morphological characters should be taken into account rather than host association and molecular phylogenetic information is also necessary to prove that the taxon is unique from other known species (Jeewon *et al*., 2004; Wei and Xu, 2004).

Pestalotiopsis species are an important group of endophytic fungi (Okane *et al*., 1998; Suryanarayanan *et al*., 1998; Cannon and Simmons, 2002; Toofanee and Dulymamode, 2002; Wei and Xu, 2003, 2004; Kumar and Hyde, 2004; Photita *et al*., 2004; Wang *et al*., 2005; Gonthier *et al*., 2006). At present at least 23 *Pestalotiopsis* species have been reported as endophytes, some of which produce secondary metabolites with a great potential for anti-microbial and anti-tumor medicinal application (Espinosa-Garcia and Langenheim, 1990; Strobel *et al*., 1996, 1997, 2000; Brown *et al*., 1998; Fröhlich *et al*., 2000; Li *et al*., 2001; Guo, 2002; Wei and Xu, 2003; Worapong *et al*., 2003; Bettucci *et al*., 2004; Kumar *et al*., 2004; Wang and Guo, 2004; Wei and Xu, 2004). However, many endophytic *Pestalotiopsis* species have never been identified due to the complication and difficulty in using existing morphological characters (Okane *et al*., 1998; Suryanarayanan *et al*., 1998, 2000; Toofanee and Dulymamode, 2002).

As the diversity of host plants is great, there should be abundant endophytic *Pestalotiopsis* species in nature, especially in tropical regions. Hainan Province is the only area located in the tropical zone with high plant diversity and a favorable environmental condition for *Pestalotiopsis*. There are more than 20 *Pestalotiopsis* species recorded in Hainan province as plant pathogens (Chen and Wei, 1993, 1997; Chen *et al*., 2002; Wei and Chen, 1994). However, no endophytic *Pestalotiopsis* species has been reported from this area. As a part work of Flora Fungorum Sinicorum on *Pestalotiopsis* and allied genera, *Pestalotiopsis* species have been isolated and identified since 2004. Among the 43 species identified (unpublished data), a new endophytic fungus is described here as *Pestalotiopsis hainanensis* based on morphological characters and molecular phylogenetic analysis of the ITS regions (ITS1, 5.8S, ITS2) and beta-tubulin 2 gene (*tub2*).

Materials and methods

Strain isolation and culture

Healthy branches of *Podocarpus macrophyllus*, about 10 cm long and 1 cm diam., were collected from Xinglong Tropical Botanical Garden, Hainan Province of China in April 2004. The leaves and twigs were separated from branches and washed with running tap water, then sterilized with 75% ethanol (60 seconds), 1.3% NaClO (5 minutes) and 75% ethanol (30 seconds) (Wei and Xu, 2004). Samples were washed three times with sterilized water, then cut into pieces 1 cm long and placed on potato dextrose agar medium. The tissues were incubated at 25ºC for 3-20 days and checked regularly. Pure fungal cultures were obtained by single spore isolations following the methods outlined by Lacap *et al*. (2003) and Promputtha *et al*. (2005).

Hyphal tips from the colony margin were removed on new Petri-dishes with potato dextrose agar medium. When the colony grew to 2 cm diam., autoclaved segments of carnation leaf (*Dianthus caryophyllus* L.) were added aseptically on the colony to promote sporulation (Fisher *et al*., 1982; Strobel *et al*., 1996) and fruiting body morphology was observed under a light microscope.

DNA extraction, PCR amplification and DNA sequencing

The fungus was grown on potato dextrose agar for 7 days at 25ºC. The mycelia were harvested from the plates and total genomic DNA was extracted according to the methods of Wang *et al*. (2005).

The ITS region of rDNA were amplified using primers ITS4 and ITS5 (White *et al*., 1990). Part of the *tub2* gene was amplified using primers bt2a and bt2b (Glass and Donaldson, 1995). PCR was performed in a 25 µL reaction containing 100 ng genomic DNA, 10 × PCR reaction buffer including 1.5 µM $MgCL_2$, 0.4 µM each primer, 200 µM of each deoxyribonucleotide triphosphate and 1.25 unit *Taq* polymerase. The thermal cycling program was as follows: 3 min initial denaturation at 95ºC, followed by 34 cycles of 40 s denaturation at 94ºC, 60 s annealing at 50ºC for ITS primers and at 55ºC for *tub2* primers, 1 min extension at 72ºC, and a final 10 min extension at 72ºC. A negative control using water instead of template DNA was included in the amplification process. Four microliters of PCR products from each PCR reaction were examined by electrophoresis at 75 V (4 V cm^{-1}) for 2 h in a 0.8% (W/V) agarose gel in 1 × TAE buffer (40 mM Tris, 1 mM EDTA, pH 8.0) and visualized with UV light after staining with ethidium bromide (0.5 µg mL^{-1}).

PCR products were purified using the PCR Purification Kit (Go3S) according to the manufacturer's protocol. Purified PCR products were directly sequenced in the ABI PRISM 377 DNA sequencer (Applied Biosystems). Both DNA strands were sequenced with primers mentioned above.

Table 1. List of fungi with their host, habitat and accession number used in this study

Taxon	Isolates	Host	Habitat	GenBank accession number	
				ITS	β-tubulin
Pestalotiopsis aquatica	PSHI2002Endo321	*Podocarpus macrophyllus* (Thunb.) D. Don.	Endophytic	AY687303	DQ333571
P. clavispora	PSHI2002Endo389	*Camellia sinensis* O. Ktze	Endophytic	AY682929	DQ333572
P. conigena	PSHI2002Endo309	*C. nitidissima* Chi	Endophytic	AY687301	DQ333573
P. crassiuscula	PSHI2002Endo356	*P. macrophyllus*	Endophytic	AY687868	DQ333574
P. disseminata	PSH2000I-066	*P. imbricatus* Bl.	Pathogenic	AY687870	DQ333575
P. gracilis	HKUCC8320	*Scaevola hainanensis* Hance	/	AF409962	/
P. hainanensis	PSHI2004Endo166	*P. macrophyllus*	Endophytic	DQ334863	DQ137861
P. heterocornis 1	PSHI2002Endo391	*P. macrophyllus*	Endophytic	AY681491	DQ137865
P. heterocornis 2	PSHI2002Endo408	*C. sasanqua* Thunb.	Endophytic	AY681492	DQ137866
P. heterocornis 3	PSHI2002Endo303	*C. japonica* L.	Endophytic	AY687874	DQ137867
P. jesteri	/	*Fragraea bodenii* Wernh.	Endophytic	AF377282	/
P. karstenii 1	PSHI2001Path201	*C. japonica*.	Pathogenic	AY681472	DQ137858
P. karstenii 2	PSHI2002Endo353	*C. japonica*	Endophytic	AY681474	DQ137859
P. karstenii 3	PSHI2002Endo402	*C. sasanqua*	Endophytic	AY681476	DQ137860
P. kunmingensis	PSHI2002Endo766	*P. macrophyllus*	Endophytic	AY373376	DQ333576
P. lawsoniae	PSH2000I-057	*Pinus massoniana* Lamb.	Pathogenic	AY687871	DQ333577
P. mangifolia	PSHI2002Endo672	*C. sasanqua*	Endophytic	AY687306	DQ333578
P. microspora	PSHI2002Endo747	*C. sinensis*	Endophytic	AY681484	DQ333579
P. neglecta	PSHI2002Endo401	*P. nagi* (Thunb.) Zoll & Mor.	Endophytic	AY682932	DQ141530
P. olivacea	PSHI2002Endo696	*C. sasanqua*	Endophytic	AY687883	DQ333580
P. paeoniae	PSHI2002Endo8801	*Taxus yunnanensis* Cheng & L.K. Fu.	Endophytic	AY687311	DQ333581
P. paeoniicola	PSHI2002Endo3502	*P. nagi*	Endophytic	AY687310	DQ333582

Table 1 continued. List of fungi with their host, habitat and accession number used in this study

Taxon	Isolates	Host	Habitat	GenBank accession number	
				ITS	β-tubulin
P. photiniae	PSHI2002Endo403	*C. sasanqua*	Endophytic	AY682942	DQ333583
P. rhododendri	BRIP 25628	*Antidesma ghaesembilla* Gaertn.	/	AF409986	/
P. subcuticularis	PSHI2002Endo882	*T. yunnanensis*	Endophytic	AY687878	DQ333584
P. theae	PSHI2001path205	*C. sinensis*	Endophytic	AY681479	DQ137870
P. versicolor	PSHI2004Endo124	*Tamarindus indica* L.	Endophytic	DQ334862	DQ333585
P. westerdijkii	PSHI2004Endo98	*Allamanda cathartica* L.	Endophytic	DQ137856	DQ137862
Seiridium cardinale	ICMP 7323	*Cupressocyparis leylandii* Dallim.	Pathogenic	AF409995	/
S. ceratosporum	PHSI2001Pathcw07	*Vitis vinifera* L.	Pathogenic	AY687314	DQ137857
S. cardinale	CMW2133	*Cupressus sempervirens* L.	Pathogenic	/	AF320504

Note: /, no data.

Table 2. Morphological characteristics of *Pestalotiopsis hainanensis* compared with similar *Pestalotiopsis* species

***Pestalotiopsis* species**	**Conidium**			**Apical appendage**				**Basal appendage**	**Habit**
	Size (μm)	Length/ width ratio	Median cell	Number	Position	Length (μm)	Tip		
hainanensis	19-22 × 5-6	3.9	Brown to olivaceous	1-3	Apical	1-10	Unknobbed unbranched	Absent	Endophyte
karstenii	15.6-31.2 × 4.6-6.1	3.5	Brown to olivaceous	1-3	Apical	5.4-28.2	Unknobbed branched	Absent	Endophyte, pathogen
heterocornis	18-26 × 5-7	3.7	Brown to olivaceous	1-3	Apical, subapical	13.8-18.8	Unknobbed unbranched	Unbranched	Endophyte
westerdijkii	18.9-23.4 × 6.4-7.7	3.2	Umber to fuliginous	1-3	Apical	2.6-13	Unknobbed unbranched	Unbranched	Endophyte

Phylogenetic analysis

Totally 28 *Pestalotiopsis* strains belonging to 24 species were used for phylogenetic analysis of ITS region and *tub2* gene sequences (Table 1). The sequences were aligned with Clustal X software (Thompson *et al*., 1997) and the results were adjusted manually where necessary to maximize alignment. The alignment data were subsequently used for maximum-parsimony (MP) analysis, in which searches for most parsimonious trees were conducted with the heuristic search algorithm with tree-bisection-reconnection (TBR) branch swapping in PAUP* 4.0b1a (Swofford, 1998). For each search, 1000 replicates of random stepwise sequence addition were performed and 100 trees were saved per replicate. Gaps were treated as missing data. Homologous sequence positions were treated as a discrete character with four possible unordered states (A, G, C, or T), and equally weighted parsimony (with a transition:tranversion ratio of 1:1) was included in the parsimony analysis. Optimal trees were identified using heuristic searches based on 1000 random addition replicates retaining clades compatible with the 50% majority-rule in the bootstrap consensus tree.

Results

Morphology

The new species *Pestalotiopsis hainanensis* isolated from *Podocarpus macrophyllus* are similar to *Pestalotiopsis karstenii*, *P. heterocornis* and *P. westerdijkii* (Table 2). However, *P. hainanensis* has a large conidium length/width ratio and is distinguished from *P. karstenii* in the unbranched, short apical appendages (1-10 vs 5.4-28 μm), from *P. heterocornis* in the absence of an unbranched basal appendage and from *P. westerdijkii* in the different median cell colour (brown to olivaceous vs umber to fuliginous) and absence of basal appendage.

Molecular phylogenetics

The ITS dataset of 30 taxa resulted in a data matrix of 553 sites. Maximum-parsimony analysis yielded six most parsimonious trees with tree length (TL) 188 steps, consistency index (CI) 0.8085, retention index (RI) 0.9347, rescaled consistency index (RC) 0.7557 and homoplasy index (HI) 0.1915. The strict consensus tree was shown in Fig. 2, and *P. hainanensis* did not cluster together with any references.

The *tub2* dataset of 27 taxa resulted in a data matrix of 453 sites. Maximum-parsimony analysis yielded 56 most parsimonious trees with a tree length (TL) of 553 steps. The CI, RI, RC and HI were 0.7993, 0.8827,

0.7055 and 0.2007, respectively. The strict consensus tree was shown in Fig. 3, and *P. hainanensis* did not cluster together with any references.

The results of the ITS region sequence similarity comparisons showed that *P. hainanensis* had similarities with three *P. karstenii* strains (97.4%) and with three *P. heterocornis* strains (96.1-96.6%). It is interesting that the three *P. karstenii* strains have identical 5.8S gene and ITS sequences. *Pestalotiopsis karstenii* 1, as a pathogen, was isolated from leaf and stem of *Camellia japonica* and *P. karstenii* 2 was isolated from *C. japonica* leaf and *P. karstenii* 3 was isolated from stem of *C. sasanqua* as an endophyte (Wei and Xu, 2003).

The results of the *tub2* gene sequence similarity comparisons showed that *P. hainanensis* had similarities with three *P. karstenii* strains (92.3%-93.8%) and with three *P. heterocornis* strains (94.1%).

Molecular results support that *P. hainanensis* is a new species which is distinguished from *P. karstenii*, *P. heterocornis* and other *Pestalotiopsis* species.

Taxonomy

Pestalotiopsis hainanensis A.R. Liu, T. Xu & L.D. Guo, **sp. nov.** (Fig. 1)

Fungus in foliis *Dianthi caryophylli* cretus, acervulus discretus vel irregularis in ambitu, plerumque 50.7-221 µm (medio 102.9 µm) in diametro. *Cellulae conidiogenae* discretae vel integratae, lageniformes, ampuliformes vel subcylindraceae, hyalinae, laeves, 8.2-15.5 × 2-3.5 µm (medio 11 × 2.8 µm); *Conidia* 5-cellularia, fusiformia, recta ad subcurvata, 19-22 × 5-6 µm (medio 20.2 × 5.2 µm), subconstricta ad septa; cellulae medianae tres, subcylindraceae, crassitunicatae, laeves, versicolores vel subconcolores, simul 13-15 µm (medio 13.6 µm) longae [cellula secunda a basi brunnea, 3.2-4.5 µm (medio 3.9 µm); cellula tertia olivacea, 3.2-4.4 µm (medio 3.7 µm); cellula quarta brunnea vel olivacea, 2.9-5 µm (medio 3.7 µm)]; cellulae hyalinae exteriores parvae, triangulares, setula apicalis una, raro duae vel tres, brevis, 1-10 µm (medio 3.9 µm) longa; pedicellus vulgo absens; Ratione conidii long./lat. = 3.9:1.

Colonies on potato dextrose agar white, cottony, margin nearly round; acervuli developed in mycelia and gave rise to black spore mass, punctate, discrete, and developed on the carnation leaves on potato dextrose agar, scattered, irregular 50.7-221 µm ($\bar{x}$ = 102.9 µm) in diam. *Conidiogenous cells* integrated, lageniform to ampulliform or subcylindrical, colourless, smooth-walled, 8.2-15.5 × 2-3.5 µm ($\bar{x}$ = 11 × 2.8 µm); *Conidia* fusiform, erect or slight curving, 5-celled, 19-22 × 5-6 µm ($\bar{x}$ = 20.2 × 5.2 µm), slight constricted at septa; intermediate coloured cells subcylindrical, thick-walled, smooth, usually approximate concolorous, together 13-15 µm ($\bar{x}$ = 13.6 µm) long; second cell from the base pale brown, 3.2-4.5 µm ($\bar{x}$ = 3.9 µm); third cell olivaceous, 3.2-4.4 µm ($\bar{x}$ = 3.7 µm) and fourth cell pale brown to olivaceous, 2.9-5 µm ($\bar{x}$ = 3.7 µm); exterior hyaline cells small, trigonal, bearing 1 setula, rarely 2 or 3 setulae, short, 1-10 µm ($\bar{x}$ = 3.9 µm) long; basal appendage absent; mean conidium length/width ratio = 3.9:1.

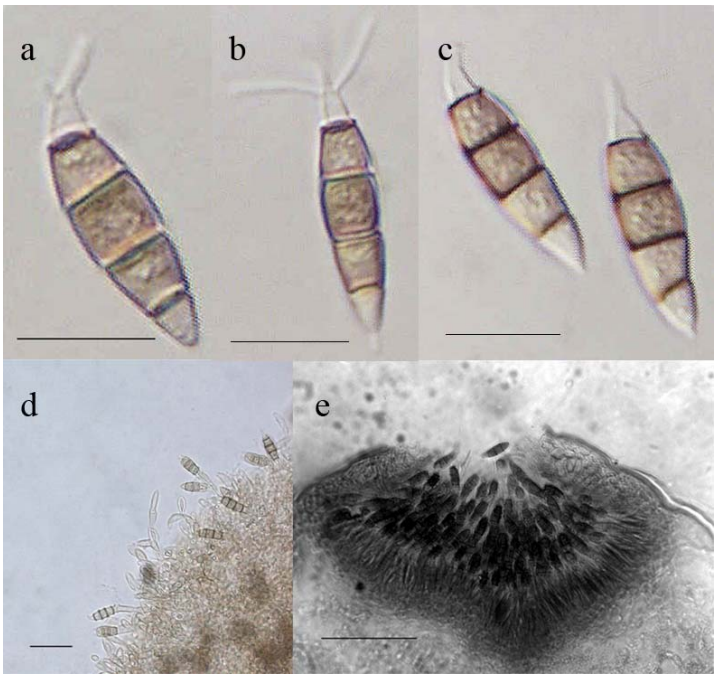

Fig. 1. *Pestalotiopsis hainanensis*. **a, b, c.** Conidia on autoclaved segment of carnation leaf on potato dextrose agar. **d.** Conidiogenous cells on potato dextrose agar. **e.** Acervulus on autoclaved segment of carnation leaf on potato dextrose agar. Bars: a, b, c = 10 µm, d = 20 µm, and e = 50 µm.

Habitat/Distribution: Known to inhabit living stem of *Podocarpus macrophyllus*, Hainan, China.

Holotype : China, Hainan, Xinlong, endophyte of *P. macrophyllus,* 1 May 2004, A.R. Liu, specimen of dried culture stored in the Herbarium Mycologicum Academiae Sinicae (HMAS); extype living culture in the China General Microbiological Culture Collection Center (CGMCC).

Discussion

From the result of phylogenetic analyses *Pestalotiopsis* species can be divided into two groups (X and Y) corresponding to their morphological characters. In group X the median colourous cells are umber to fuliginous, but in group Y median colourous cells are brown to olivaceous. Although *P. hainanensis* and *P. westerdijkii* are similar in

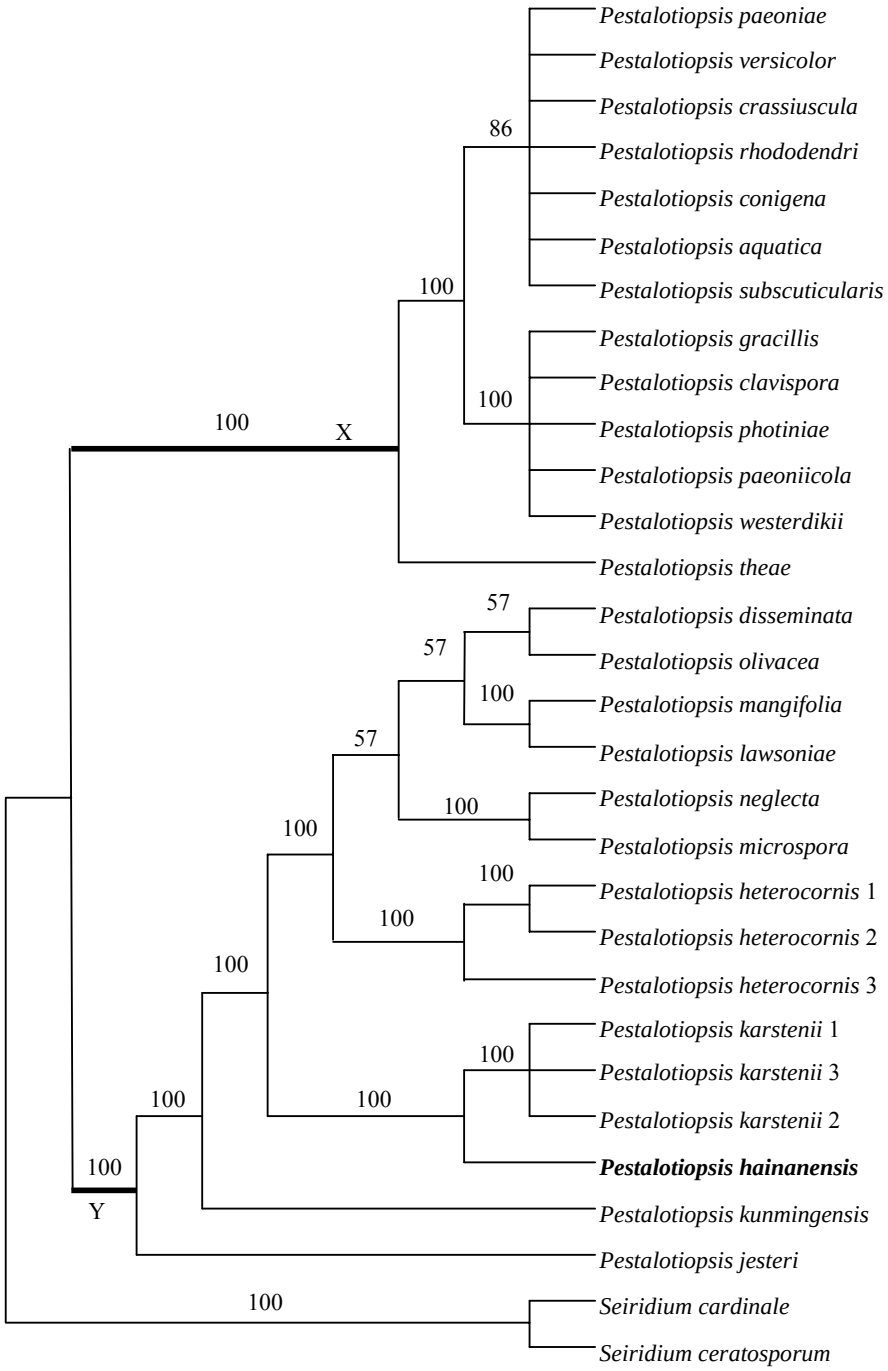

Fig. 2. Strict consensus tree of 6 equally parsimonious trees generated from the ITS region (ITS1, 5.8S and ITS2) sequences of 30 strains showing the relationship of *Pestalotiopsis hainanensis* with reference taxa. The tree rooted with *Seiridium cardinale* and *S. ceratosporum* (TL = 188, CI = 0.8085, RI = 0.9347, RC = 07557, and HI = 0.1915). Bootstrap values greater than or equal to 50% are shown at branches.

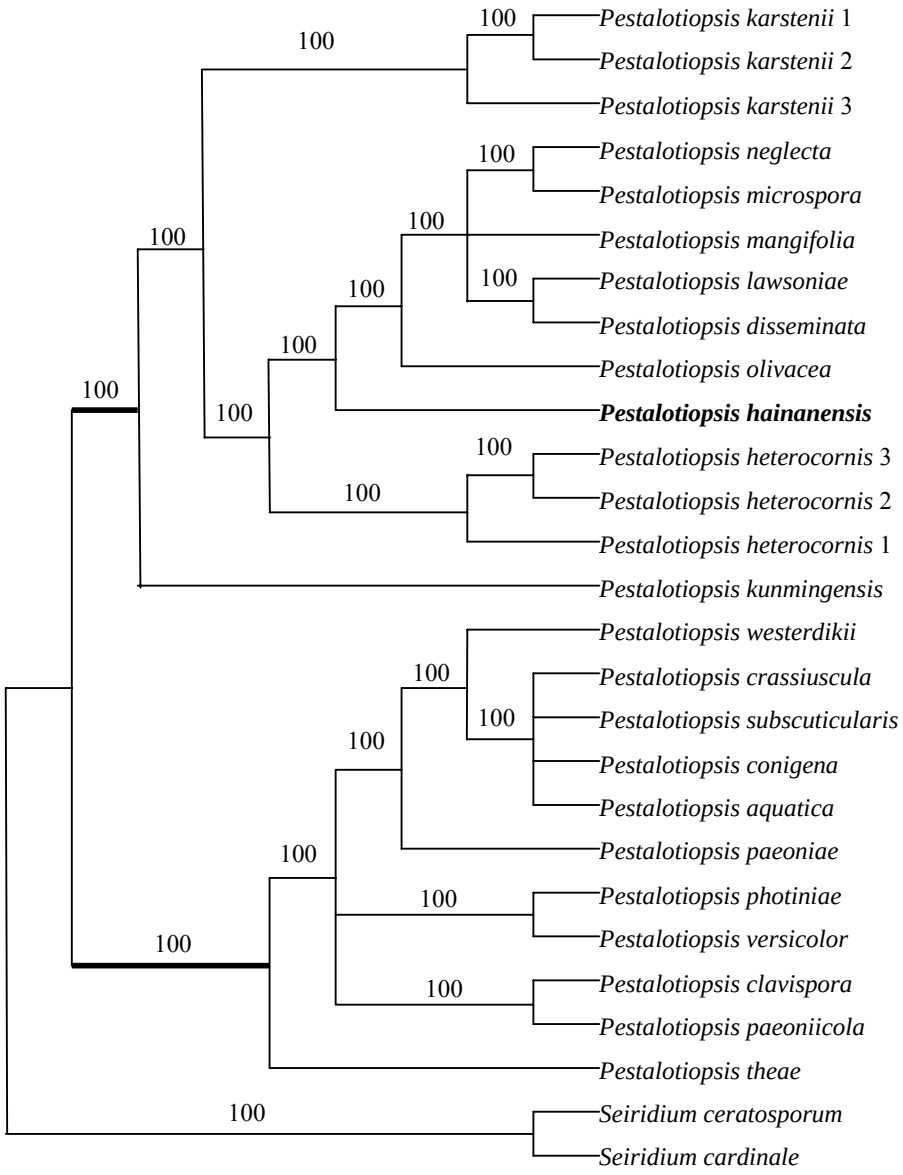

Fig. 3. Strict consensus tree of 56 equally parsimonious trees generated from the beta-tubulin 2 gene (*tub2*) sequences of 27 strains showing the relationship of *Pestalotiopsis hainanensis* with reference taxa. The tree rooted with *Seiridium cardinale* and *S. ceratosporum* (TL = 188, CI = 0.7993, RI = 0.8827, RC = 0.7055, and HI = 0.2007). Bootstrap values greater than or equal to 50% are shown at branches.

some morphological characters, they belong to two different groups on the gene phylogenetic trees.

Griffiths and Swart (1974) recognized that differences in pigmentation of median cells were of taxonomic significance. This corroborated with the results of the Sutton (1961). However, in other studies, pigmentation of the median cells was shown to be unreliable for differentiating certain *Pestalotiopsis* species and argued that colour contrast of median cells is not a dependable character (Purohit and Bilgrami, 1968). Purohit and Bilgrami (1968) suggested that this genus should be studied under uniform conditions. In our study, all the *Pestalotiopsis* strains tested (except for *P. gracilis* (AF409962), *P. jesteri* (AF377282), and *P. rhododendri* (AF409986) sequences from GenBank) were cultured on autoclaved carnation leaves under standard condition. In the present work phylogenetic analyses based on both ITS region and *tub2* gene sequences support pigmentation of median cells as an important taxonomic character in *Pestalotiopsis* (Jeewon *et al*., 2003; Wei *et al*., 2005).

In our previous study on the diversity of the endophytic *Pestalotiopsis* on *Podocarpaceae*, *Theaceae* and *Taxaceae* in southern China, it was demonstrated that each plant hosted more than one endophytic *Pestalotiopsis* species and the species diversity varied among individual host species. For an example, a total of 15 *Pestalotiopsis* species were isolated from *P. macrophyllus*, which were *P. aquatica*, *P. clavispora*, *P. crassiuscula*, *P. heterocornis*, *P. kunmingensis*, *P. menezesiana*, *P. microspora*, *P. neglecta*, *P. olivacea*, *P. oxyanthi*, *P. paeoniae*, *P. photiniae*, *P. rhododendri*, *P. theae* and *P. zonata* (unpublished data). This result also demonstrated that endophytic *Pestalotiopsis* species are not specific to their host plant. However, *P. karstenii* and *P. westerdijkii*, which are similar to the new species, have not been isolated from *P. macrophyllus* in our investigations.

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