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ORIGINAL ARTICLE

Pestalotiopsis yunnanensis sp. nov., an endophyte from *Podocarpus macrophyllus* (Podocarpaceae) based on morphology and ITS sequence data

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Abstract *Pestalotiopsis* is a common and important plant-associated pathogen and endophyte with wide geographical and host distribution. In an investigation of endophytic *Pestalotiopsis* species associated with *Podocarpaceae* in China, a new species *Pestalotiopsis yunnanensis* was isolated from *Podocarpus macrophyllus* in Kunming, southwestern China. This new species produced pycnidium-like conidiamata in culture, distinct from its morphologically similar species, *P. funereoides*, *P. funerea* and *P. thujae*, which produce acervuli in manual media and hosts. *P. yunnanensis* also possesses a greater conidium length/width ratio, and longer apical and basal appendages as its distinguishing morphology characters. Phylogenetic analysis of internal transcribed spacer (ITS) sequences showed that *P. yunnanensis* is a member of *Pestalotiopsis*, and is distinct from morphologically similar *P. funereoides*, *P. funerea*, and *P. thujae*, as well as other *Pestalotiopsis* species. A dichotomous key to 26 *Pestalotiopsis* species occurring on *Podocarpus* plants is also presented.

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Introduction

The genus *Pestalotiopsis* Steyaert was established by Steyaert (1949), following a taxonomic amendment of the genus *Pestalotia* De Not., and about 162 species have been accepted (Kirk et al. 2008). *Pestalotiopsis* is a monophyletic genus and forms anamorphs of *Pestalosphaeria* Barr (Amphisphaeriaceae) (Barr 1975; Jeewon et al. 2003; Kirk et al. 2008). This genus is characterized by relatively fusiform conidia, with 3-colored median cells, 2 colorless end cells, and two or more apical appendages (Steyaert 1949). The inter-specific delineation of *Pestalotiopsis* is based on the morphology of the conidia (Guba 1961; Nag Raj 1993), conidiogeneses (Sutton 1980), and teleomorph association (Barr 1975, 1990; Zhu et al. 1991; Metz et al. 2000). However, the taxonomic affinities of some *Pestalotiopsis* species have been confused and ambiguous, as the morphological characters overlap in many aspects (Maharachchikumbura et al. 2011). Recently, molecular techniques have been successfully employed in species identification and phylogenetic analysis (Jeewon et al. 2003; Wei et al. 2005, 2007; Tejesvi et al. 2009; Liu et al. 2010; Maharachchikumbura et al. 2012).

In an investigation of endophytic *Pestalotiopsis* species associated with *Podocarpaceae* in China, a particular endophytic *Pestalotiopsis* strain from *Podocarpus macrophyllus* (Thunb.) Sweet was isolated and is described here as a new species, based on morphological characters and ITS sequence analysis.

Materials and methods

Sample collection and isolation

This study was carried out in the Kunming Botanic Garden, Chinese Academy of Sciences, in Yunnan Province of southwestern China (25°01′N, 102°41′E). The site is located in the highlands occuring 1,990 m above sea level. The annual mean temperature is 17.4 °C, and the annual mean rainfall is 1,012 mm.

Healthy twigs of *P. macrophyllus* (*Podocarpaceae*) were collected from the Kunming Botanic Garden of Chinese Academy on November 11, 2002. The samples were placed in plastic bags, labeled, and taken to the laboratory. Samples were stored at 10 °C and processed within 4 days of collection. The samples were washed with running tap water and were then surface-sterilized according to the protocol of Guo et al. (2000). After surface sterilization, twigs were cut into ca. 0.5-cm segments and transferred to PDA plates. Plates were incubated at 25 °C for 3–20 days and checked regularly. Endophytic *Pestalotiopsis* strains were grown on autoclaved segments of carnation leaf (*Dianthus caryophyllus* L.) for sporulation (Wei and Xu 2004). All morphological observations and measurements were made in sterile water, with a mean of 30 measurements for each character using a compound microscope (Nikon E600; Japan). *Pestalotiopsis* cultures were grown on malt extract agar (MEA, 2 %) for 1 week and used for the molecular study.

DNA extraction, amplification and sequencing

Total DNA was extracted from fresh cultures following the protocol of Guo et al. (2000). The internal transcribed spacer (ITS) region of rDNA was amplified using primer pairs ITS5 and ITS4 (White et al. 1990). Amplification was performed in a 50-μl reaction volume which contained PCR buffer [20 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 20 mM Tris–HCl, pH8.4], 200 μM of each deoxyribonucleotide triphosphate, 15 pmols of each primer, c. 100 ng template DNA, and 2.5 U of *Taq* polymerase (Biocolor BioScience & Technology, Shanghai, China). The thermal cycling program was as follows: 3 min initial denaturation at 95 °C, followed by 35 cycles of 40 s denaturation at 94 °C, 50 s annealing at 52 °C, 1 min extension at 72 °C, and a final 10 min extension at 72 °C. A negative control using purified water (Cascada Biowater; Pall, NY, USA) instead of template DNA was included in the amplification process. Four microliters of PCR product from each PCR reaction was examined by electrophoresis at 75 V for 2 h in a 0.8 % (W/V) agarose gel in 1×TAE buffer (0.4 M Tris, 50 mM NaOAc, 10 mM EDTA, pH 7.8) and visualized under UV light after staining with ethidium bromide (0.5 μg ml^{-1}). PCR products were purified using PCR Cleanup Filter Plates (MultiScreen® PCRμ96; Millipore, USA) according to the manufacturer's protocol. Purified PCR products were directly sequenced with primer pairs as mentioned above in an ABI 3730-XL DNA sequencer (Applied Biosystems, USA).

Phylogenetic analysis

The ITS sequences of *P. yunnanensis*, 30 representative *Pestalotiopsis* species, and *Truncatella angustata* (Pers.) S. Hughes (*Amphisphaeriaceae*) as an outgroup, were included in the phylogenetic analyses, to determine the phylogenetic relationship of the new taxon within the genus. All sequences were aligned with Clustal X1.81 (Thompson et al. 1997) and the results were adjusted manually where necessary to maximize alignment. All sites were treated as unordered and unweighted, and gaps were treated as missing in the phylogenetic analyses.

The alignment data was subsequently used for maximum parsimony (MP) analysis, in which search for most parsimonious trees was conducted with the heuristic search algorithm with tree-bisection-reconnection (TBR) branch swapping in PAUP 4.0b10 (Swofford 2002). For each search, 1,000 replicates of random stepwise sequence addition were performed and all trees were saved per replicate. The strength of the internal branches of the trees was tested with bootstrap analyses using 1,000 replications with the same search settings.

Bayesian analysis of the same alignment dataset was conducted in MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001), following the protocol of Sun and Guo (2010). The best-fit evolutionary model was determined for each dataset by comparing different evolutionary models via MrModeltest 2.3 (Nylander et al. 2008). The GTR+G model for ITS datasets was selected by AIC in MrModeltest 2.3, with a random starting tree. The prior probability density is a flat Dirichlet (all values are 1.0) for both Revmatpr and Statefreqpr as default settings. Four simultaneous chains of Markov Chain Monte Carlo were run starting from random trees and sampling every 100 generations. The analysis was halted at 2,000,000 generations, when the calculation reached stationarity. At the end of the analysis, 20,000 trees were generated and 5,000 trees were excluded as the "burn in" when calculating the posterior probabilities. Bayesian posterior probabilities (BPP) were obtained from the 50 % majority rule consensus of the trees kept. If>95 % of the sampled trees were contained in a given clade, it was considered to be significantly supported by the data.

Results

Taxonomy

Pestalotiopsis yunnanensis J.G. Wei, T. Xu & L.D. Guo, sp. nov. Fig. 1

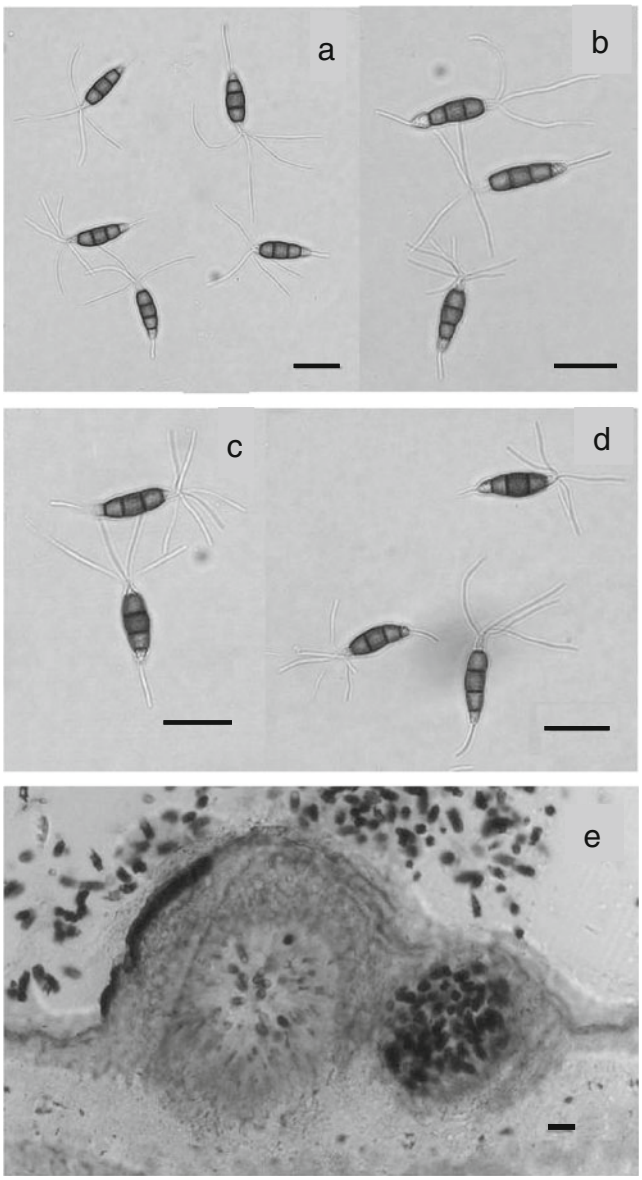

Fig. 1 Morphological characters of *Pestalotiopsis yunnanensis* from culture on carnation leaves. **a**–**d** Conidia with 3–7 long apical appendages and long basal appendage, **e** pycnidioid conidiomata. *Bars* (**a**–**e**) 20 μm

MycoBank No.: MB 800649

Fungus in foliis *Dianthus caryophyllus* cretus. Conidiomatbus pycnidioidebus erumpentibus pustuliformibus uniloculariibus, orbicularibus vel ovalibus ambitu, plerumque 80–180 μm in diametro, aliquando ad 230 μm. Cellulae conidiogenae discretae vel integratae, lageniformes, ampuliformes vel subcylindraceae, hyalinae, 8–17.3×2.5–4.9 μm (medio 12.8×3.3 μm) semel vel bis prolificantes. Conidia fusiformia, quadriseptata, 26–33.8× 6.5–8.2 μm (medio 28.8×7.2 μm); cellulae mediae tres, subcylindraceae vel doliiformes, crassitunicatae, concolores, laeves, 16.3–20.8 μm (medio 18.7 μm) longae [cellula secunda a basi olivacea, 5.5–6.2 μm (medio 5.7 μm); cellula tertia umberina-brunnea, 6.8–7.8 μm (medio 7.4 μm); cellula quarta umbrino-brunnea, 5.9–7.5 μm (medio 6.8 μm)]; cellula apicalis conica, hyalina, laevis, 3.9–6.5 μm (medio 5.1 μm) longa; cellula basalis obconica, laevis, hyalina, 3.9–6.2 μm (medio 5.1 μm) longa; appendices apicales 3–7, plerumque 4–5, tubulares, nonramosae, 19.5–40.3 μm (medio 28.9 μm) longae; appendix basalis centrica, 8.7–22.9 μm (medio 16.4 μm) longa; ratione conidii long./lat. = 4.0 : 1.

Type: CHINA, Yunnan Province, Kunming, Kunming Botanic Garden of Chinese Academy, 25°01′N, 102°41′E, 1,990 m above sea level, healthy twigs of *P. macrophyllus* (*Podocarpaceae*), 11. 11. 2002, Ji–Guang Wei. Holotype (HMAS 96359) was kept in Herbarium Mycologicum Institute Microbiologici Academiae Sinicae. The living culture was deposited in the Department of Plant Protection, College of Agriculture & Biotechnology, Zhejiang University.

Etymology The specific epithet was based on the site where this species was isolated.

Colony on PDA pinkish yellow, cottony, fruitbodies ink-like, more or less gregarious, reverse of the culture pinkish. Conidiomata formed on carnation leaves pycnidium-like, pustule-like, unilocular, rounded to oval, 80–180 (230) μm diam. Conidiogenous cells discrete or integrated, lageniform to ampulliform or subcylindrical, colourless, smooth-walled, 8–17.3×2.5–4.9 μm ($\overline{x} = 12.8 \times 3.3\mu m$). Conidia fusiform, 4-septate, 26–33.8×6.5–8.2 μm ($\overline{x} = 28.8 \times 7.2\mu m$); 3 median cells subcylindrical to doliiform, thick-walled, smooth, brown, concolourous, 16.3–20.8 μm ($\overline{x} = 18.7\mu m$) long; in the 3 medium cells, from base the first cell, 5.5–6.2 μm ($\overline{x} = 5.7\mu m$), the second cell 6.8–7.8 μm ($\overline{x} = 7.4\mu m$) and third cell 5.9–7.5 μm ($\overline{x} = 6.8\mu m$); apical cell conic, colourless, smooth-walled, 3.9–6.5 μm ($\overline{x} = 5.1\mu m$) long; basal cell obconical, smooth-walled, colourless, 3.9–6.2 μm ($\overline{x} = 5.1\mu m$) long. Apical appendages tubular, unbranched, 3–7, mostly 4–5, 19.5–40.3 μm ($\overline{x} = 28.9\mu m$) long; basal appendage centric, 8.7–22.9 μm ($\overline{x} = 16.4\mu m$) long. Mean conidium length/width ratio=4: 1.

Habitat/distribution Known to inhabit living twigs of *P. macrophyllus*. China.

Holotype Endophyte of *P. macrophyllus*, strain 789, 11 XI 2002, Kunming, Yunnan.

Phylogenetic analysis

The ITS sequence data from 30 *Pestalotiopsis* species, as well as *T. angustata* used as an outgroup, were included in the phylogenetic analyses. In the alignment of the 31 taxa, the data matrix comprised 537 characters, of which 403 (75 %) characters were constant and 73 (13.6 %) were parsimony informative. The parsimony analysis of the

Fig. 2 Bayesian tree based on ITS sequences. The tree is rooted with *Truncatella angustata*. The numbers at each branch point represents Bayesian posterior probabilities (*left*) and percentage bootstrap support calculated from 1,000 replicates (*right*). *Indicates lack of support for a particular clade. *Bar* indicates 0.1 expected changes per site

alignment data set was subjected to a heuristic search for the most parsimonious trees and a strict consensus tree was obtained from 812 equally most parsimonious trees (CI=0.7828, RI=0.8995, RC=0.7042, HI=0.2172). The same alignment dataset was also performed using MrBayes program, and a similar topology as the strict consensus tree was obtained; thus, a Bayesian tree with posterior probabilities and parsimony bootstrap values at branches was shown in Fig. 2. Phylogenetic analyses indicated that *P. yunnanensis* formed a clade with *P. funerea* (Desm.) Stey., *P. funereoides* Stey., *P. hainanensis* A.R. Liu, T. Xu & L.D. Guo, *P. maculiformans* (Guba & Zeller) Steyaert, and *P. thujae* (Sawada) Y.X. Chen with strong support (BPP=99 %, MP=81 %). Within this clade, *P. yunnanensis* showed a closer relationship with *P. thujae* in the Bayesian analysis (BPP=58 %). *P. yunnanensis* had a 98.3 % (523/532 bp) ITS sequence similarity with *P. thujae*.

Disccussion

Pestalotiopsis yunnanensis is similar to *P. funereoides*, *P. funerea*, and *P. thujae* in morphological characters (Table 1). However, *P. yunnanensis* is able to be distinguished by its pycnidium-like conidiamata in culture as described by Watanabe et al. (1998) from *P. funereoides* and *P. funerea* which produce acervuli in manual media, and *P. thujae* which produces acervuli on its host. *P. yunnanensis* has longer apical and basal appendages than these three species. In addition, the length/width ratio in the conidia of *P.*

Table 1 Morphological characteristics of *Pestalotiopsis yunnanensis* compared with similar *Pestalotiopsis* species

Taxa	Habit	Conidiomata	Conidium (length/width)	Apical appendage			Basal appendage
				Position	Number	Length	
P. yunnanensis	Endophytic	Pycnidium-like	26–33.8×6.5–8.2 μm (4)	Acrogenous	3–7	19.5–40.3 μm	8.7–22.9 μm
P. funereoides (Steyaert 1949)	Pathogenic	Acervular	27–30×7–9 μm (3.56)	Acrogenous	3–6	18–30 μm	5–11 μm
P. funerea (Steyaert 1949)	Pathogenic	Acervular	26–31×8–13 μm (2.71)	Acrogenous	3–6	13–27 μm	2–7 μm
P. thujae (Guba 1961)	Pathogenic	Acervular	25–31×6.5–10 μm (3.53)	Acropleurogenous	3–6	19–22 μm	Short

yunnanensis is greater, with its narrow conidia being distinct from *P. funerea* with wider conidia. *P. yunnanensis* with acrogenous apical appendages is distinguished from *P. thujae* with acropleurogenous apical appendages. Furthermore, the ITS sequence analysis indicated that *P. yunnanensis* is clearly separated from the *P. funerea*, *P. funereoides*, *P. hainanensis*, *P. maculiformans*, and *P. thujae*.

Species diversity of endophytic *Pestalotiopsis* on *P. macrophyllus* is abundant. Wei et al. (2007) reported 15 endophytic *Pestalotiopsis* species isolated from *P. macropyllus* in south China. Whereas Gure et al. (2005) isolated five strains of *Pestalotiopsis* from seeds of *Podocarpus falcatus* (Thunb.) Mirb. in Ethiopia, among which one strain was identified as *P. guepinii* (Desm.) Steyaert based on its morphological characters, one strain was identified as *P. neglecta* (Thüm.) Steyaert based on ITS sequence and three strains were not identified.

There have been more than 20 *Pestalotiopsis* species recorded on species of *Podocarpus* L'Hér. ex Pers. (Guba 1961; Sun and Ge 1990; Zhu et al. 1991; Wei and Chen 1994; Wei and Xu 2004; Wei et al. 2005, 2007; Gure et al. 2005; Liu et al. 2007), and a dichotomous key to 26 *Pestalotiopsis* species occurring on *Podocarpus* plants is presented here:

1 Tip of apical appendages of conidia spathulate
2 Tip of basal appendage of conidia spathulate *P. theae*
2* Tip of basal appendage of conidia not spathulate *P. kunmingensis*
1* Tip of apical appendage of conidia not spathulate
3 The number of apical appendages usually 5
4 Three to six apical appendages and conidiomata acervular *P. funerea*
4* Three to seven apical appendages and conidiomata pycnidioid *P. yunnanensis*
3* Usually 3 apical appendages
5 Median cells of conidia concolorous
6 One to three apical appendages
7 Apical appendages of conidia mostly 1–10 μm and basal appendage absent *P. hainanensis*
7* Apical appendages of conidia 9–22 μm, one attenuated and basal appendage present *P. heterocornis*
6* Usually 3 apical appendages
8 Conidia 18–26×5–8 μm
9 Conidia elliptic
10 Apical appendages 8–23 μm long *P. disseminata*
10* Apical appendages 14–32 μm long *P. olivacea*
9* Conidia narrow
11 Apical appendages 8–23 μm long *P. neglecta*
11* Apical appendages 6–15 μm long
12 Apical appendages usually 10 μm long, basal appendage short *P. podocarpi*
12* Apical appendages usually 3–15 μm long and basal appendage 4–5 μm long *P. microspora*
8* Conidia 22–30 μm×5–8 μm
13 Apical appendages 10–25 μm long *P. mangifolia*
13* Apical appendages 15–38 μm long *P. caroliniana*
5* Median cells of conidia versicolorous
14 Intermediate colored cells contracted, the color of upper two cells umber to brown and the lowest cell yellow brown
15 Conidia 20–30 μm long
16 Apical appendages 16–26 μm long *P. paeoniae*
16* Apical appendages 10–25 μm long *P. oxyanthi*
15* Conidia 18–26 μm long
17 Apical appendages of conidia stout *P. aquatica*
17* Apical appendages of conidia slender
18 Apical appendages 4–22 μm long *P. zahlbruckneriana*
18* Apical appendages 13–32 μm long *P. crassiuscula*
14* Intermediate colored cells strongly contrasted?contracted, upper two cells fuliginous to opaque and the lowest cell light brown
19 Conidia 25–30 μm long *P. rhododendri*
19* Conidia 19–26 μm long
20 Conidia 7–10 μm long
21 Apical appendages filiform *P. paeoniicola*
21* Apical appendages thick
22 Conidia ovate-oblong or pyriform, 19–23 μm×8–10 μm *P. diospyri*
22* Conidia clavate-fusiform, 22–27 μm×7.5–9.5 μm *P. versicolor*
20* Conidia 6–8.5 μm wide
23 The minimum length of apical appendages 9 μm
24 Apical appendages 9–25 μm long *P. zonata*
24* Apical appendages 9–31 μm long *P. menezesiana*
23* The minimum length of apical appendages larger than 9 μm
25 Apical appendages 20–30 μm long *P. cinchonae*
25* The minimum length of apical appendages less than 20 μm
26 Apical appendages 17–31 μm long *P. clavispora*
26* Apical appendages 16–33 μm long *P. photiniae*

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