Pestalotiopsis species on ornamental plants in Yunnan Province, China

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Pestalotiopsis species were obtained from diseased leaves of ornamental plants collected in Yunnan Province, China. Morphological comparison and phylogenetic analysis of combined sequence data of the internal transcribed spacer (ITS), partial β-tubulin and partial translation elongation factor 1-alpha (*ef1*) showed that the isolates comprised seven species of *Pestalotiopsis*. Three species, *Pestalotiopsis ericacearum, P. gaultheriae* and *P. rhododendri,* are new to science and described herein.

Keywords: leaf blight, new species, pathogen, phylogeny.

Pestalotiopsis, an appendage-bearing conidial asexual form in the family Amphisphaeriaceae (Barr 1975, 1990, Kang *et al*. 1998, 1999), is widely distributed throughout tropical and temperate ecosystems (Maharachchikumbura *et al*. 2011). It is an important plant pathogenic genus (Yasuda *et al*. 2003; Das *et al*. 2010; Maharachchikumbura *et al*. 2011, 2013 a) with about 250 species, traditionally named according to their host associations (Guba 1961, Steyaert 1949, Venkatasubbaiah *et al*. 1991, Kohlmeyer & Volkmann-Kohlmeyer 2001). Many of these names are likely, however, to be erroneous as molecular data has shown that the genus needs revision (Cai *et al*. 2011; KoKo *et al*. 2011; Maharachchikumbura *et al*. 2011, 2012). *Pestalotiopsis* species have been also often isolated as endophytes (Liu *et al*. 2006; Hu *et al*. 2007; Wei *et al*. 2007; Watanabe *et al*. 2010; Botella & Diez 2011; Rocha *et al*. 2011, Debbab *et al*. 2011, 2012; Maharachchikumbura *et al*. 2012), or occur as saprobes (Wu *et al*. 1982, Agarwal & Chauhan 1988, Yanna *et al*. 2002, Hu *et*

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al. 2007, Liu *et al*. 2008, Maharachchikumbura *et al*. 2012). Endophytic species have been shown to produce a wide range of chemically novel diverse metabolites (Xu *et al*. 2010; Maharachchikumbura *et al*. 2011; Debbab *et al*. 2011, 2012; Maharachchikumbura *et al*. 2013 b).

We surveyed diseases of ornamental plants in Kunming Botanical Gardens and surrounding areas in Yunnan Province, China. We constantly observed *Pestalotiopsis* and related taxa associated with disease symptoms of ornamental plants. The aim of the current paper is to provide molecular characteristics of *Pestalotiopsis* species associated with ornamental plants, and three new species are also introduced.

Materials and methods

Sample collection and isolation

Fresh specimens of *Pestalotiopsis* species were obtained from leaf spots on living leaves of ornamental plants in Yunnan Province, China. To induce sporulation, diseased leaves were placed in sterilized Petri dishes with moistened sterile filter papers for 1 to 2 days after collection. Acervuli were rehydrated in water for examination and sectioning. Specimens were examined under a Leica MZ16A stereo microscope and observations and microphotographs were made under the light microscope (Nikon Ei800 and Leica DM3000); for some hyaline structures differential interference contrast microscopy was used. Hand sections (4–10 µm thick) were made with a sharp razor blade and sections were transferred to a drop of water, a drop of lactic acid or a drop of cotton blue for examination and photography. The morphology of fungal colonies was recorded following the method used by Maharachchikumbura *et al*. (2012). A single conidium culture technique was used to obtain pure colonies of the fungi following the method used by Chomnunti *et al*. (2011).

DNA extraction and PCR conditions

Biospin Fungus Genomic DNA Extraction Kit (Produced Bioer Technology Co., Hubei, China) was used to extract total genomic DNA directly from the acervuli. The ITS and 5.8S region of rDNA molecule was amplified using primer pairs ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') (White *et al*. 1990), β-tubulin gene region was amplified with primer pairs BT2A (5'-GGTAACCAAATCGGTGCTGCTTTC-3') and BT2B (5'- ACCCTCAGTGTAGTGACCCTTGGC-3') (Glass & Donaldson 1995, O'Donnell & Cigelnik 1997) and *ef1* was amplified using the primer pairs EF1-526F (5'-GTCGTYGTYATYGGHCAYGT-3') and EF1-1567R (5'-ACHGTRCCRATACCACCRATCTT-3') (Rehner 2001). PCR was performed with the 25 µl reaction system consisting of 19.5 µl of double distilled water, 2.5 µl of 10× Taq buffer with $MgCl_2$, 0.5 µl of dNTP (10 mM each), 0.5 µl of each primer (10 µM), 0.25 µl Taq DNA polymerase (5 U/µl), 1.0 µl of DNA template. The thermal cycling program followed the method used by Maharachchikumbura *et al*. (2012, 2013 a).

Phylogenetic analysis

Raw sequence data were optimized following the method used by Maharachchikumbura *et al*. (2012). A maximum parsimony analysis (MP) was performed using PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (Swofford 2002). Ambiguously aligned regions were excluded and gaps were treated as missing data. Trees were inferred using the heuristic search option with TBR branch swapping and 1000 random sequence additions. Maxtrees were set up to 5000, branches of zero length were collapsed and all multiple parsimonious trees were saved. Tree length [TL], consistency index [CI], retention index [RI], rescaled consistency index [RC], homoplasy index [HI], and log likelihood [-ln L] (HKY model) were calculated for trees generated under different optimality criteria. The robustness of the most parsimonious trees was evaluated by 100 bootstrap replications resulting from maximum parsimony analysis, each with ten replicates of random stepwise addition of taxa (Felsenstein 1985). The Kishino–Hasegawa tests (Kishino & Hasegawa 1989) were performed in order to determine whether the trees inferred under different optimality criteria were significantly different. Trees were viewed in Treeview (Page 1996).

Results

A phylogenetic tree was constructed using combined ITS, β-tubulin and *ef1* sequences of 30 isolates of *Pestalotiopsis,* with *Seiridium* sp. as the outgroup taxon (Tab. 1). The aligned data matrix consisted of 2068 characters; 1452 characters were constant, 217 variable characters were parsimony-uninformative and 399 characters were parsimony-informative. The Kishino-Hasegawa (KH) test showed that first tree generated from parsimonious analysis was the best tree (length= 1324 steps, CI= 0.647, RI= 0.831, HI= 0.353, RC= 0.538).

In our study we found many species of *Pestalotiopsis* associated with diseases in ornamental plants and various disease symptoms including *Pestalotiopsis karstenii* on leaf spots on living leaves of *Camellia reticulata* (Fig. 1 a), *P. clavata* on leaf spots on living leaves of *Rhododendron delavayi* (Fig. 1 b), *P. clavispora* on leaf spots on living leaves of *Camellia japonica* ((Fig. 1 c) and *P. trachicarpicola* on leaf spots on living leaves of *Podocarpus macrophyllus* (Fig. 1 d). Our identifications are based on morphology and three gene combined molecular data (Fig. 2).

Taxonomy

Pestalotiopsis ericacearum Y. M. Zhang, Maharachch. & K. D. Hyde, **sp. nov.** – Fig. 3

MycoBank no.: MB 803235

Etymology. – The specific epithet is based on the host family *Ericaceae*, from which the fungus was isolated.

Tab. 1. *Pestalotiopsis* isolates used in this study.

Taxon	Isolates	GenBank Accession Number		
		ITS	β-tubulin	*ef1*
P. adusta (Ellis & Everh.) Steyaert	ICMP 6088	JX399006	JX399037	JX399070
P. asiatica Maharachch. & K. D. Hyde	MFLUCC 12-0286	JX398983	JX399018	JX399049
P. camelliae Y. M. Zhang, Maharachch. & K. D. Hyde	MFLUCC 12-0277	JX399010	JX399041	JX399074
P. chinensis Maharachch. & K. D. Hyde	MFLUCC 12-0273	JX398995	-	-
P. chrysea Maharachch. & K. D. Hyde	MFLUCC 12-0261	JX398985	JX399020	JX399051
P. clavata Maharachch. & K. D. Hyde	MFLUCC 12-0268	JX398990	JX399025	JX399056
P. clavata	IFRDCC 2394			
P. clavispora	MFLUCC 12-0281	JX398979	JX399014	JX399045
P. clavispora	IFRDCC 2391			
P. diversiseta Maharachch. & K. D. Hyde	MFLUCC 12-0287	JX399009	JX399040	JX399073
P. ellipsospora Maharachch. & K. D. Hyde	MFLUCC 12-0283	JX399016	JX399016	JX399047
P. ericacearum Y. M. Zhang, Maharachch. & K. D. Hyde	IFRDCC 2439			
P. foedans (Sacc. & Ellis) Steyaert	CGMCC 3.9123	JX398987	JX399022	JX399053
P. furcata Maharachch. & K. D. Hyde	MFLUCC 12-0054	JQ683724	JQ683708	JQ683740
P. gaultheriae Y. M. Zhang, Maharachch. & K. D. Hyde	IFRD 411-014			
P. inflexa Maharachch. & K. D. Hyde	MFLUCC 12-0270	JX399008	JX399039	JX399072
P. intermedia Maharachch. & K. D. Hyde	MFLUCC 12-0259	JX398993	JX399028	JX399059
P. jesteri Strobel, J. Yi Li, E. J. Ford & W. M. Hess	MFLUCC 12-0279	JX399012	JX399043	JX399076
P. karstenii (Sacc. & P. Syd.) Steyaert	IFRDC OP13			
P. linearis Maharachch. & K. D. Hyde	MFLUCC 12-0271	JX398992	JX399027	JX399058
P. rhododendri Y. M. Zhang, Maharachch. & K. D. Hyde	IFRDCC 2399			
P. rosea Maharachch. & K. D. Hyde	MFLUCC12-0258	JX399005	JX399036	JX399069

Taxon	Isolates	GenBank Accession Number		
		ITS	β-tubulin	*ef1*
P. samarangensis Maharachch. & K. D. Hyde	MFLUCC 12-0233	JQ968609	JQ968610	JQ968611
P. saprophyta Maharachch. & K. D. Hyde	MFLUCC 12-0282	JX398982	JX399017	JX399048
P. theae	MFLUCC12-0055	JQ683727	JQ683711	JQ683743
P. trachicarpicola Y. M. Zhang & K. D. Hyde	OP068	JQ845947	JQ845945	JQ845946
P. trachicarpicola	IFRDCC 2403			
P. umberspora Maharachch. & K. D. Hyde	MFLUCC 12-0285	JX398984	JX399019	JX399050
P. unicolor	MFLUCC 12-0276	JX398999	JX399030	-
P. verruculosa Maharachch. & K. D. Hyde	MFLUCC 12-0274	JX398996	-	JX399061
Seiridium sp.	SD096	JQ683725	JQ683709	JQ683741

Description. – Associated with leaf blotch on living leaves of *Rhododendron arboretum* subsp. *delavayi*; initially black and rounded and later expanding to form a brown blotch. Sexual state not observed. Asexual state: *Acervuli* (200)250–500(600) µm in diam., black, epidermal to subepidermal in origin, separate or confluent, dehiscence irregular. Conidiophores reduced to conidiogenous cells. Conidiogenous cells hyaline, branched or unbranched at the base or above, cylindrical, lageniform or claviform. Conidia (15)16–20(21) × 5–9 µm (av. = 18 × 6.5 µm), fusiform, straight to slightly curved, 4-septate; basal cell conical, hyaline, thin and smooth-walled, 2–3 µm long (av. = 2.6 µm); with three median cells, doliiform to cylindrical, constricted at the septa, concolorous, olivaceous, septa and periclinal walls darker than the rest of the cell, together (9)10–14(15) µm long (av. = 12 µm); second cell from base 4–6 µm (av. = 5 µm); third cell 4.5–6.5 µm (av. = 5.8 µm); fourth cell 4–6 µm (av. = 5 µm); apical cell hyaline, cylindrical, 4–6 µm long (av. = 5 µm); with 3–4 tubular apical appendages (mostly 3), arising from the apex of the apical cell, knobbed at the end, (19)20–43(45) µm long (av. = 32 µm), unequal in length; basal appendage present 2–9 µm (av. = 4 µm).

Holotype. – CHINA, Yunnan Province, Chuxiong, Zixishan, on leaf spots on living leaves of *Rhododendron delavayi*, February 2011, *leg.* Y. Zhang OP023, holotype IFRD 410-008.

Notes. – *Pestalotiopsis ericacearum* is a morphologically characteristic species and its distinctness is supported by molecular phylogeny (Fig. 1, Tab. 2). It has relatively short conidia (16–20 µm) compared to other species that have knobbed apical appendages; *P. kunmingensis* (33.8–46.8 µm), *P. pallidotheae* (21.5–30.7 µm), *P. theae* (22–32 µm) and *P. tecomicola* (23–31.5 µm). In the phylogram (Fig. 2) it is clearly distinguished from *P. jesteri* and *P. karstenii* with high bootstrap support. No cultures were obtained for this species and thus DNA was directly extracted from the acervuli.

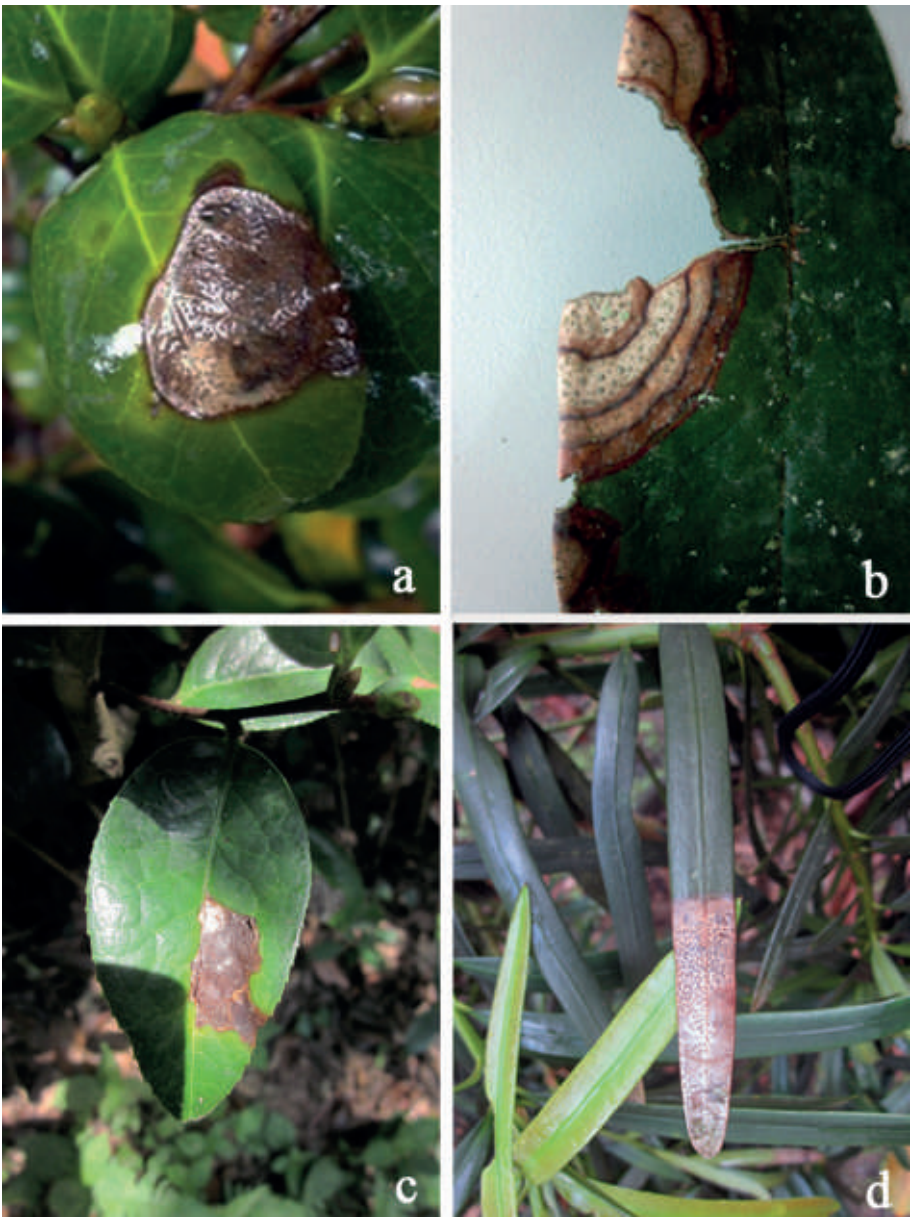

Fig. 1. a P*estalotiopsis karstenii* on leaf spots on living leaves of *Camellia reticulata*. **b** *P. clavata* on leaf spots on living leaves of *Rhododendron delavayi*. **c** *P. clavispora* on leaf spots on living leaves of *Camellia japonica*. **d** P. *trachicarpicola* on leaf spots on living leaves of *Podocarpus macrophyllus*.

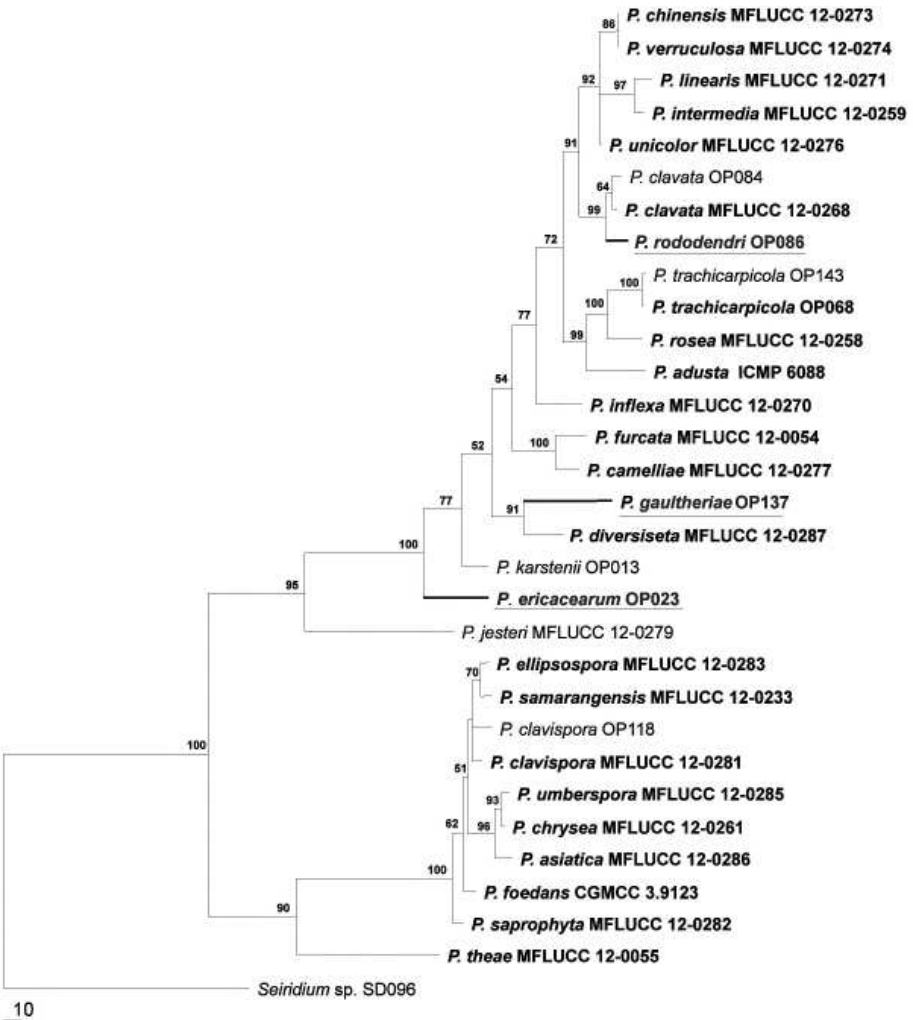

Fig. 2. Maximum parsimony phylogram generated from combined three genes (ITS, β-tubulin and *ef1*) analysis of species of *Pestalotiopsis*. Data were analyzed with random addition sequence, unweighted parsimony and treating gaps as missing data. *Seiridium* spp. placed as outgroup and type and ex-type sequences are in bold. New species are in bold and underlined.

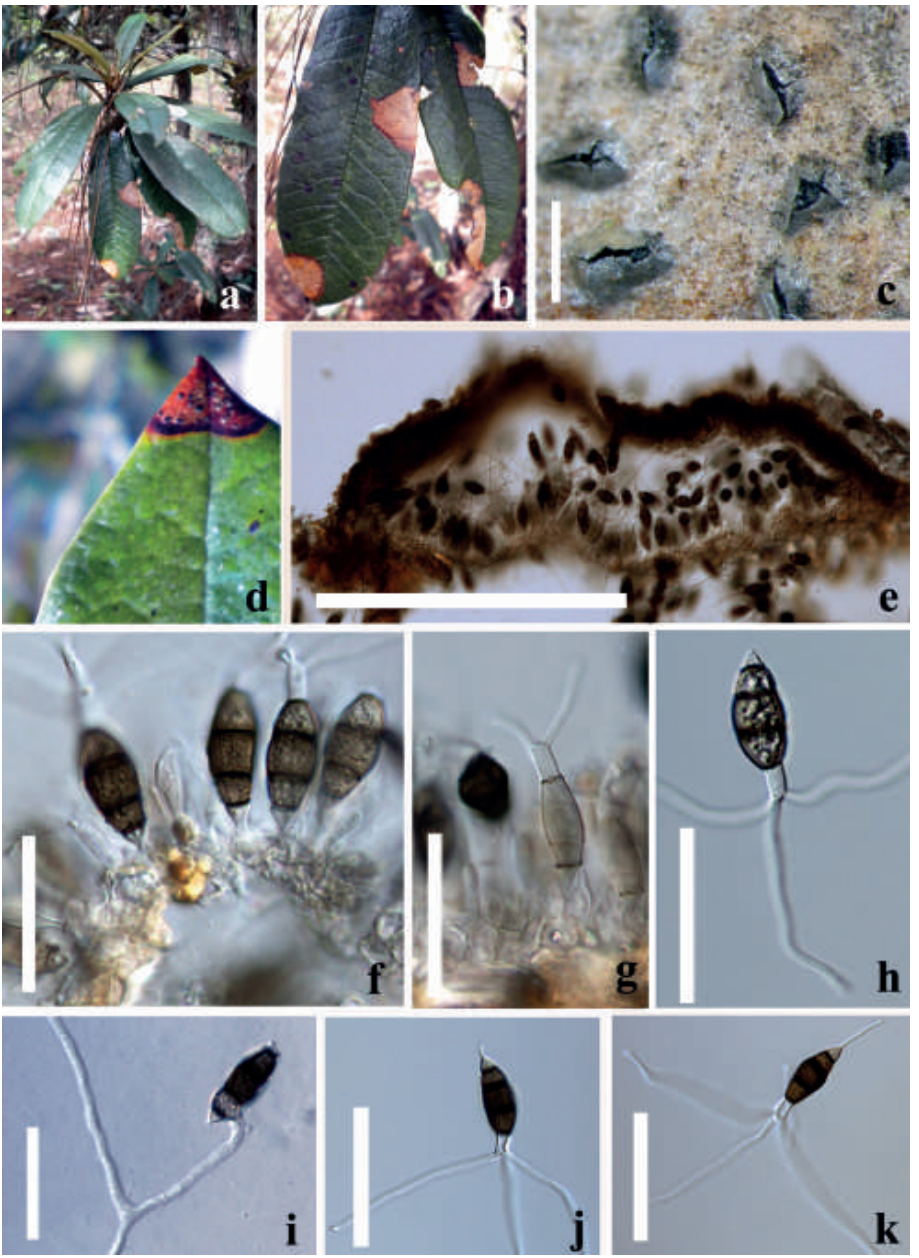

Fig. 3. *Pestalotiopsis ericacearum* (holotype). **a–d** *P. ericacearum* associated with leaf blotch on leaves of *Rhododendron delavayi*. **c** Acervuli, splitting irregularly. **e** Section of acervulus. **f, g** Conidiogenous cells. **h–k** Conidia with knobbed apical appendages. **i** Germination of spore. Bars: c 1000 µm, e 100 µm, f–k 20 µm.

Tab. 2. Synopsis of *Pestalotiopsis ericacearum* and related species.

Species	*P. ericacearum*	*P. pallidotheae*[a]	*P. theae*[b]	*P. kunmingensis*[c]	*P. tecomicola*[d]
Conidia size (µm)	16–20 × 5–9	21.5–30.7 × 5.4–7.7	22–32 × 5–8	33.8–46.8 × 7.5–10	23–31.5 × 7.5–8.5
Median cells	concolorous, dark brown	concolorous, pale (light) brown	concolorous, dark brown	concolorous, brown	concolorous, pale brown to brown
Apical appendages:	3	2–4	2–4	2–4	3
Length (µm)	17–40	12.3–39.2	25–50	14.3–52.7	11–16
Tip	knobbed	knobbed	knobbed	knobbed	knobbed
Basal appendages	present	10–20	present	present, branched	present

[a]Watanabe *et al.* (2010); [b]Guba (1961); [c]Wei & Xu (2004); [d]Nag Raj (1993)

Pestalotiopsis gaultheriae Y. M. Zhang, Maharachch. & K. D. Hyde, **sp. nov.** – Fig. 4
MycoBank no.: MB 803236

Etymology. – The specific epithet is based on the host genus *Gaultheria*, from which this fungus was isolated.

Description. – Associated with brown leaf spots on living leaves of *Gaultheria forrestii*. Sexual state not observed. Asexual state: Acervuli 100–310 µm in diam., grey to black, epidermal to subepidermal, separate or confluent, dehiscence irregular. Conidiophores reduced to conidiogenous cells. Conidiogenous cells hyaline, branched or unbranched at the base or above. Conidia fusoid to ellipsoid, straight to slightly curved, 4-septate, (20)23–31(33) × 7–9.5 µm (av. = 26.4 × 8.6 µm), with basal cell obconic, hyaline, thin-walled and verruculose, 3–5 *µm* long (av. = 4.9 µm); with three median cells, doliiform, concolorous when immature, becoming versicolourous when mature, septa and periclinal walls darker than the rest of the cell, wall rugose, together (14)15–20(21) µm long (av. = 17 µm); second cell from base 4–6 µm (av. = 4.8 µm); third cell 5–7 µm (av. = 5.8 µm); fourth cell 4–6 µm (av. = 5 µm); apical cell hyaline, cylindrical 4–5 µm long (av. = 4.5 µm); with 3 tubular appendages, swollen at the tip, arising from the apex of the apical cell, (13)15–50(54) µm long (av. = 35 µm); with basal appendage 2.5–4 µm long, rarely absent.

Holotype. – CHINA, Yunnan Province, Dehong, Mangshi, on leaf spots on living leaves of *Gaultheria forrestii*, September 2011, *leg.* Y. M. Zhang OP 137, holotype IFRD 411-014.

Notes. – *Pestalotiopsis gaultheriae* is a morphologically distinct species, which is also shown in its DNA phylogeny (Fig. 2). Species belongs to the clade comprising *P. gaultheriae* and *P. diversiseta* are concolorous. However, most interestingly, the two upper median cells in *P. gaultheriae* become darker than the lower median cell at maturity. *Pestalotiopsis gaultheriae* has long apical appendages (15–50 µm) when compared with species having similar conidial size, such as *P. diversiseta* (22–30 µm), *P. jesteri* (11–28 µm) and

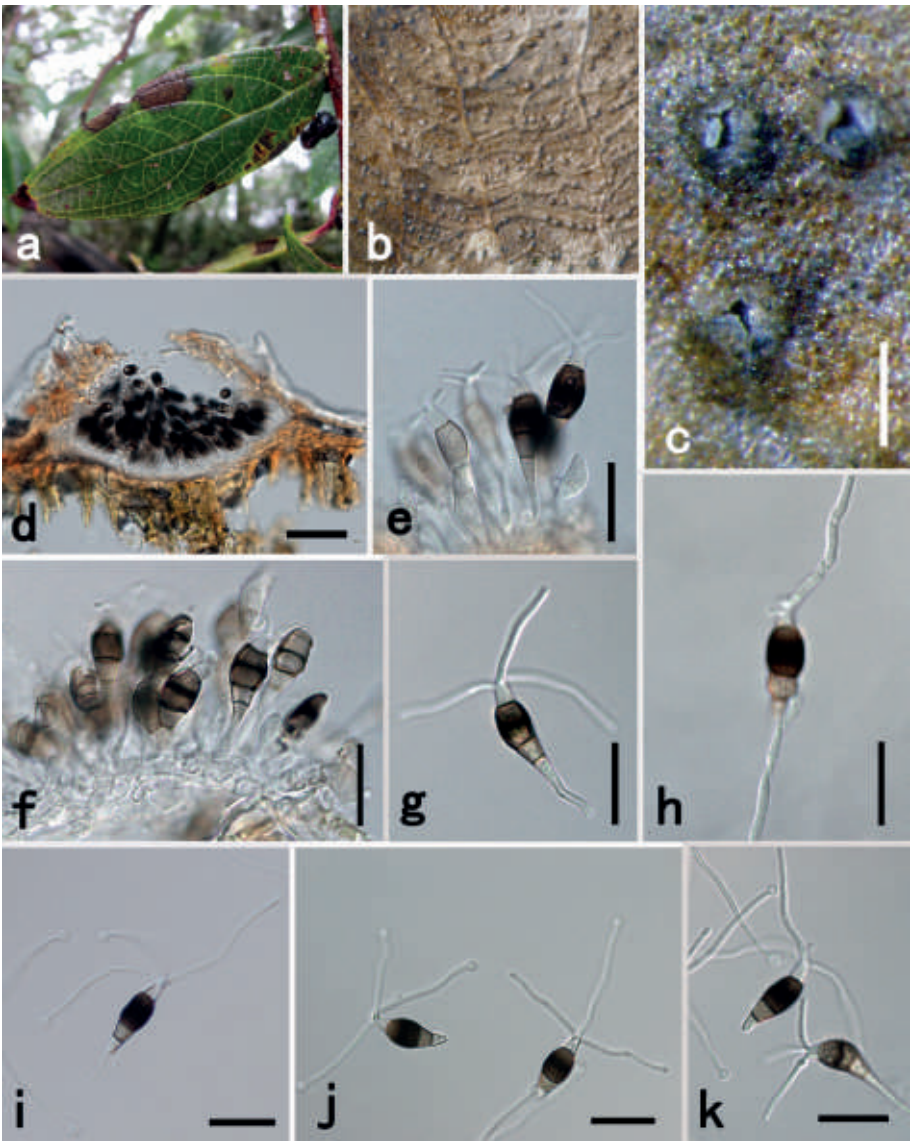

Fig. 4. *Pestalotiopsis gaultheriae* (holotype). **a** *P. gaultheriae* associated with leaf blight on leaves of *Gaultheria forrestii*. **b–c** Acervuli. **d** Section of acervular. **e–f** Conidiogenous cells. **h** Germination of spore. **g–k** Conidia with knobbed apical appendages. Bars: c 200 µm, d 50 µm, e–k 20 µm.

P. pallidotheae (12–40 µm) (Tab. 3). In the phylogenetic tree it forms a sister clade with *P. diversiseta,* however, *P. gaultheriae* is clearly distinguished from *P. diversiseta* by having fewer apical appendages. Furthermore, in *P. diversiseta* apical appendages are sometimes branched and sometimes arise from different parts of the apical cell. No cultures were obtained for this species and thus DNA was directly extracted from the acervuli.

Pestalotiopsis rhododendri Y. M. Zhang, Maharachch. & K. D. Hyde, **sp. nov.** – Fig. 5
MycoBank no.: MB 803237

Etymology. – The specific epithet is based on the host genus *Rhododendron*, from which the fungus was isolated.

Description. – Associated with dead parts of living leaves of *Rhododendron sinogrande*. Sexual state not observed. Asexual state: Acervuli (40)50–190(210) µm in diam., black, epidermal to subepidermal, separate or confluent, dehiscence irregular. Conidiophores indistinct. Conidiogenous cells discrete, simple, short, filiform. Conidia (16)18–27(29) × 5–8 µm (av. = 21 × 7 µm), fusiform, straight to slightly curved, 4-septate; basal cell conical to acute, hyaline, thin and smooth-walled, 3–5 µm long (av. = 4 µm); with three median cells, doliiform to cylindrical, constricted at the septa, concolorous, olivaceous, septa and periclinal walls darker than the rest of the cell, wall rugose, together (12)13–17(19) µm long (av. = 15 µm); second cell from base 4–6 µm (av. = 4.7 µm); third cell 4–6 µm (av. = 4.6 µm); fourth cell 4–6 µm (av. = 4.6 µm); apical cell hyaline, conical, 3–5 µm long (av. = 4 µm); with 3 tubular apical appendages, arising from the apex of the apical cell, knobbed at the end, (5)7–15(18) µm long (av. = 14 µm); basal appendage present 2–6 µm (av. = 4 µm).

Tab. 3. Synopsis of *Pestalotiopsis gaultheriae* and related species.

Species	*P. gaultheriae*	*P. diversiseta*[a]	*P. jesteri*[b]	*P. pallidotheae*[c]	*P. theae*[a]
Conidia size (µm)	23–31 × 7–9.5	27–34×5.5–8	19–23 × 5–7	21.5–30.7 × 5.4–7.7	22–32 × 5–8
Median cells	versicolorous, dark brown	concolorous, olivaceous	concolorous, pale brown to brown	concolorous, pale (light) brown	concolorous, dark brown
Apical appendages:	3	3–5 (sometimes branched)	3–4	2–4	2–4
Length (µm)	15–50	22–30, unequal	11–28	12.3–39.2	25–50
Tip	knobbed	knobbed	knobbed	knobbed	knobbed
Position	apex	top to middle	arising from juncture apical cell	apex	apex
Basal appendages	2.5–4	present	present	present	present

[a]Maharachchikumbura *et al.* (2012 a); [b]Strobel *et al.* (2000); [c]Watanabe *et al.* (2010)

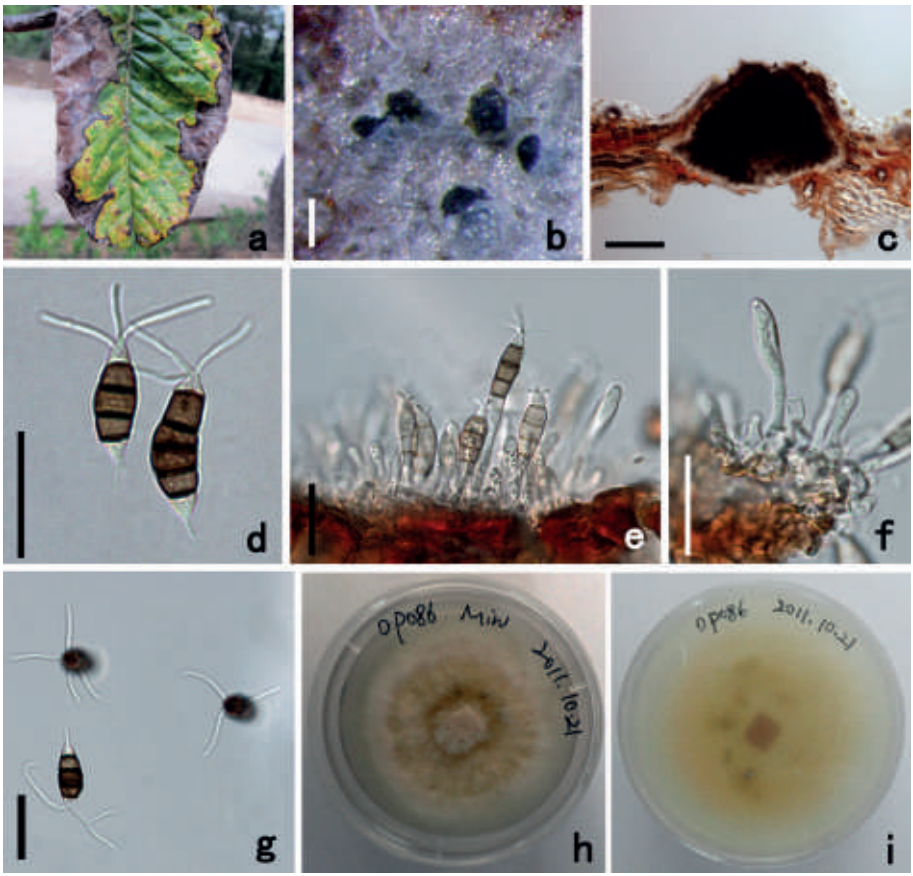

Fig. 5. *Pestalotiopsis rhododendri*. **a** *P. rhododendri* associated with leaf blight on leaves of *Rhododendron sinogrande*. **b** Acervuli, splitting irregularly. **c** Section of acervulus. **e, f** Conidiogenous cells. **d, g** Conidia. **h** *P. rhododendri* colony on PDA from above. **i** *P. rhododendri* colony on PDA from below. Bars: b–c 100 µm, d–g 20 µm.

Colonies on PDA reaching 7 cm diam. after 5 days at 25 °C, with entire edge, whitish and with time change in to pink, with aerial mycelium on surface; fruiting bodies black, gregarious; reverse of culture whitish to pale yellow.

Holotype. – CHINA, Yunnan Province, Chuxiong, Zixishan, on leaf spots on living leaves of *Rhododendron sinogrande*, May 2011, *leg.* Y. Zhang OP086, holotype IFRD 410-018, ex-type culture = IFRDCC 2399.

Notes. – *Pestalotiopsis rhododendri* (18–27 × 5–8 µm) has an overlapping conidial size with *P. clavata* (20–27 × 6.5–8 µm), but it has shorter apical appendages (7–15 *µm*) than *P. clavata* (20–25 *µm*) and it is also separated in DNA phylogeny (Fig. 2).

Discussion

In this phylogenetic study, we include 30 sequenced isolates of *Pestalotiopsis* with seven new strains collected from Yunnan Province, China. Based on morphological and molecular data, we determined that these Yunnan isolates comprise seven species of *Pestalotiopsis* and three are described here as new species.

Previously *P. funerea, P. guepini, P. langloisii, P. macrotricha P. sydowiana P. versicolor* have been reported to be associated with diseases in hardy ornamentals such as *Aralia elata*, *Calluna vulgaris*, *Camellia* sp., *Cupressus* sp., *Erica* sp., *Euonymous fortunei*, *Gardenia* sp., *Rhododendron* sp. and *Taxus* sp. (Pirone 1978, Sutton 1980, Hopkins & McQuilken 2000). Most of these studies indicate that *Pestalotiopsis* are not particularly host-specific and taxa may have the ability to infect a range of hosts (Hopkins & McQuilken 2000, Keith *et al.* 2006; Maharachchikumbura *et al.* 2011).

There are approximately 250 *Pestalotiopsis* names (Index Fungorum 2013). Most species were historically named according to the host from which they were first observed and there is no living type strain for most of these species. Thus, phylogenetic relationships within this large number of species is problematic. However, recently, new species have been introduced based on host occurrence, plus morphological and molecular data (Wei & Xu 2004; Watanabe *et al.* 2010; Strobel *et al.* 2000; Zhang *et al.* 2012; Maharachchikumbura *et al.* 2012, 2013 b), and this will lead to a better understanding of the genus.

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