Pestalotiopsis camelliae, a new species associated with grey blight of *Camellia japonica* in China

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We have been surveying diseases of ornamental plants in Yunnan Province, China and discovered a previously undescribed species of *Pestalotiopsis* causing grey blight disease on leaves of *Camellia japonica*. The associated causal agent of the disease is introduced as a new species, *Pestalotiopsis camelliae*, in this paper. The taxon can clearly be distinguished from all known species of *Pestalotiopsis* by its morphology and DNA (combined ITS, β–tubulin and *tef1* gene regions) phylogeny. Its most similar relative, *P. furcata* has conidia with more apical appendages (5–9) than in *P. camelliae* (3–6) and the appendages of *P. furcata* consistently divide into branches, that is rare in *P. camelliae*.

Keywords: asexual taxon, leaf spot, *Pestalotiopsis furcata* phylogeny, sp. nov.

During a survey of diseases of ornamental plants in Yunnan Province, China, we constantly observed grey blight of *Camellia japonica* L. (Japanese camellia) and primary identification found a *Pestalotiopsis* species associated with the disease symptom. *Pestalotiopsis* Steyaert is an appendage–bearing conidial asexual form (coelomycetes) in the family Amphisphaeriaceae (Barr 1975, 1990). Species of *Pestalotiopsis* are common in tropical and temperate ecosystems (Bate–Smith & Metcalfe 1957) and may cause plant disease (Das *et al*. 2010, Ko Ko *et al*. 2011, Zhang *et al*. 2012). They are also often isolated as endophytes (Wei *et al*. 2007, Xu *et al*. 2010), or occur as saprobes (Wu *et al*. 1982, Yanna *et al*. 2002).

The taxonomic status of species within the genus is confused and species identification using only molecular or morphological data is problem-

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atic (Hu *et al.* 2007, Maharachchikumbura *et al.* 2011, 2012 a, b). Therefore, distinct morphological characters as well as molecular data are needed to distinguish species in the genus (Maharachchikumbura *et al.* 2011). The aim of the current paper is to describe the distinct morphological and molecular characters of the new *Pestalotiopsis* species associated with *C. japonica* grey blight.

Materials and methods

Sample collection, isolation and identification

Fresh specimens of *Pestalotiopsis* sp. were obtained from leaf spots on living leaves of *Camellia japonica* in Shuangbai, Chuxiong, Yunnan Province, China. To induce sporulation, diseased leaves were placed in sterilized Petri dishes with moistened sterile filter paper. A single conidium culture technique was used to obtain pure colonies of the taxon following the method used by Chomnunti *et al.* (2011). Fungal mycelium and spores were observed under a light microscope (Nikon Ei800 and Leica DM3000) and photographed.

DNA extraction and PCR condition

Biospin Fungus Genomic DNA Extraction Kit (Produced Bioer Technology Co., Ltd. Hubei, China) was used to extract total genomic DNA from fresh fungal mycelia (500 mg) scraped from the margin of a colony on a PDA plate incubated at 25 °C for 7 to 10 days. The ITS and 5.8S region of rDNA molecule was amplified using primer pairs ITS4 and ITS5 (White *et al.* 1990), β–tubulin gene region was amplified with primer pairs BT2A and BT2B (Glass & Donaldson 1995, O'Donnell & Cigelnik 1997) and *tef1* was amplified using the primer pairs EF1–526F and EF1–1567R (Rehner 2001). PCR was performed under conditions used by Maharachchikumbura *et al.* (2012 a).

Phylogenetic analysis

Sequences were optimized manually to allow maximum alignment and maximum sequence similarity as detailed in Liu *et al.* (2011). 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 10000, 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 *tef*1 sequences of 18 isolates of *Pestalotiopsis*, with a *Seiridium* sp. as the outgroup (Tab. 1). The aligned data matrix consisted of 2006 characters (ITS = 543, β–tubulin = 460, *tef1* = 1003); 1454 characters were constant, 163 variable characters were parsimony-uninformative and 389 characters were parsimony-informative. The Kishino-Hasegawa (KH) test showed that the two trees generated from parsimonious analysis were not significantly different and one tree was selected as the best tree (length = 872 steps, CI = 0.826, RI = 0.916, HI = 0.174, RC = 0.756). In the phylogram the *Pestalotiopsis* strains separated into three major clades, named A, B and C, with high bootstrap support (Fig. 1). Clade A comprised species having pale brown or olivaceous concolorous median conidial cells. Clade B comprised species having versicolorous median conidial cells and Clade C species with dark concolorous median conidial cells, with knobbed apical appendages. We constructed phylogenetic trees separately for each gene region (results not shown) and the resolution increased from ITS, β-tubulin to *tef*1, respectively, but the best resolution was obtained when all three genes were combined.

Taxonomy

Pestalotiopsis camelliae Y. M. Zhang, S. S. N. Maharachchikumbura & K. D. Hyde, **sp. nov.** – Figs. 2–3.
MycoBank no.: MB 800980

T e l e o m o r p h . – Unknown.

D e s c r i p t i o n . – Associated with grey blight on leaves of *Camellia japonica*, initially producing small, rounded, yellow-green spots on the leaves, spots becoming brown to grey, with concentric rings and producing black, scattered acervuli (Fig. 1). Acervuli grey to black, epidermal to subepidermal, separate or confluent, dehiscence irregular, 100–220 µm wide, 76 – 150 µm high, unilocular, glabrous; wall tissue (stroma and parietal cells) only a few cells thick, cell walls thick, outermost layer colourless, inner layers pale brown to brown, encrusted (Fig. 1). Conidiophores reduced to conidiogenous cells. Conidiogenous cells discrete, lageniform, smooth, thin-walled, colourless. Conidia 27–33 × 7.5–9.3 µm (28.5 × 8.5 µm), fusoid, straight to slightly curved, 4–septate, basal cell obconic, colourless, thin- and smooth-walled, 4–7 µm long (5.4 µm), with three median cells, doliiform to subcylindrical, with thick verruculose walls, slightly constricted at the septa, concolorous, olivaceous, septa and periclinal walls darker than the rest of the cell, wall rugose, together 19–22 µm long (20 µm) (second cell from base 5.6–

Tab. 1. Details of *Pestalotiopsis* isolates used in the phylogenetic study.

Taxon	Isolates	Host/Location	GenBank accession number			Reference for sequence
			ITS	β-tubulin	tef1	
P. cf. *algeriensis* (Sacc. & Berl.) W. Wu	SD077	*Camellia sinensis*/China	JQ683718	JQ683702	JQ683734	Maharachchikumbura *et al.* 2012 b
P. cf. *disseminata* (Thüm.) Steyaert	SD034	*C. sinensis*/China	JQ683716	JQ683700	JQ683732	Maharachchikumbura *et al.* 2012 b
P. cf. *menezesiana* (Bres. & Torrend) Bissett	SG064	*C. sinensis*/China	JQ683719	JQ683703	JQ683735	Maharachchikumbura *et al.* 2012 b
P. cf. *menezesiana*	SD072	*C. sinensis*/China	JQ683713	JQ683697	JQ683729	Maharachchikumbura *et al.* 2012 b
P. cf. *microspora* (Speg.) G. C. Zhao & N. Li	SD056	*C. sinensis*/China	JQ683722	JQ683706	JQ683738	Maharachchikumbura *et al.* 2012 b
P. cf. *versicolor* (Speg.) Steyaert	SG100	*C. sinensis*/China	JQ683712	JQ683696	JQ683728	Maharachchikumbura *et al.* 2012 b
P. cf. *versicolor*	SD047	*C. sinensis*/China	JQ683715	JQ683699	JQ683731	Maharachchikumbura *et al.* 2012 b
P. cf. *versicolor*	SD091	*C. sinensis*/China	JQ683714	JQ683698	JQ683730	Maharachchikumbura *et al.* 2012 b
P. camelliae Y. M. Zhang, Maharachchikumbura & K. D. Hyde	OP111	*C. japonica*/China	JX399010	JX399041	JX399074	Maharachchikumbura *et al.* 2012 a
P. camelliae	OP131	*C. japonica*/China	JX399011	JX399042	JX399075	Maharachchikumbura *et al.* 2012 a
P. cf. *virgatula* (Kleb.) Steyaert	SD004	*C. sinensis*/China	JQ683723	JQ683707	JQ683739	Maharachchikumbura *et al.* 2012 b
P. furcata Maharachchikumbura & K. D. Hyde	MFLUCC12-0054/SS017	*C. sinensis*/Thailand	JQ683724	JQ683708	JQ683740	Maharachchikumbura *et al.* 2012 b
P. theae (Sawada) Steyaert	MFLUCC12-0055/SC027	*C. sinensis*/Thailand	JQ683727	JQ683711	JQ683743	Maharachchikumbura *et al.* 2012 b
P. theae	SC011	*C. sinensis*/Thailand	JQ683726	JQ683710	JQ683742	Maharachchikumbura *et al.* 2012 b
P. trachicarpicola Y. M. Zhang & K. D. Hyde	OP068	*Trachycarpus fortune*/China	JQ845947 JQ683720	JQ845945	JQ845946	Zhang *et al.* 2012
Pestalotiopsis sp.	SD012	*C. sinensis*/China	JQ683713	JQ683704	JQ683736	Maharachchikumbura *et al.* 2012 b
Pestalotiopsis sp.	SD072	*C. sinensis*/China	JQ683725	JQ683697	JQ683729	Maharachchikumbura *et al.* 2012 b
Seiridium sp.	SD096	*C. reticulata*/China		JQ683709	JQ683741	Maharachchikumbura *et al.* 2012 b

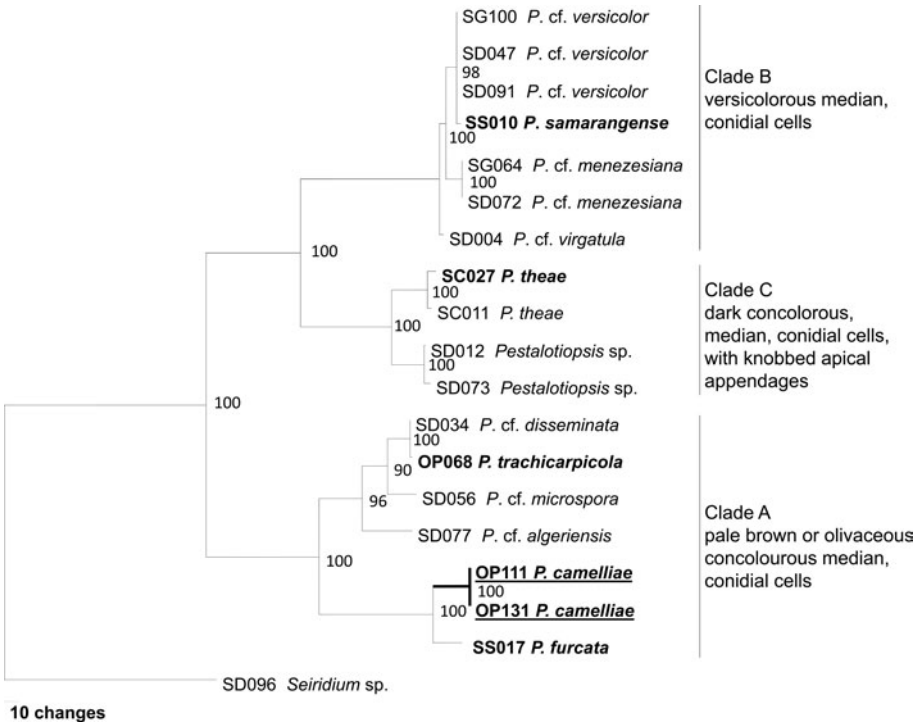

Fig. 1. Maximum parsimony phylogram generated from combine three genes (ITS, β-tubulin and *tef1*) analysis of species of *Pestalotiopsis*. Data were analyzed with random addition sequence, unweighted parsimony and treating gaps as missing data. *Seiridium* sp. was placed as outgroup, ex-type sequences are bold and the new species *P. camelliae* is underlined.

7.4 µm (6.1 µm); third cell 5– 8.3 µm (6.7 µm); fourth cell 6– 8.3 µm (7.2 µm); apical cell colorless, conic to cylindrical 3–6.4 µm long (4.7 µm); 3–6 tubular apical appendages, rarely branched, arising from the upper portion of the apical cell, 13.5–34 µm long (23.8 µm); basal appendages absent.

Colonies relatively fast growing on PDA, reaching 7 cm after 7 days at 25 °C, edge entire, whitish, with dense, aerial mycelium on surface, conidiomata black, gregarious; reverse of culture white.

Etymology. – *camelliae*, in reference to the host genus *Camellia*.

Host plant. – on leaf spots of living leaves of *Camellia japonica*.

Distribution. – so far only known from the type locality Shuangbai, Chuxiong, Yunnan Province, China.

Holotype and further material examined. – CHINA, Yunnan Province, Chuxiong, Shuangbai, on leaf spots of living leaves of *Camellia japonica* L., July 2011, Y. M. Zhang OP111 (IFRD OP111; holotype) – ex-type culture MFLUCC = MFLUCC12-0277; ibid., August 2011, Y. M. Zhang OP131 (IFRD OP131), MFLUCC = MFLUCC 12-0278.

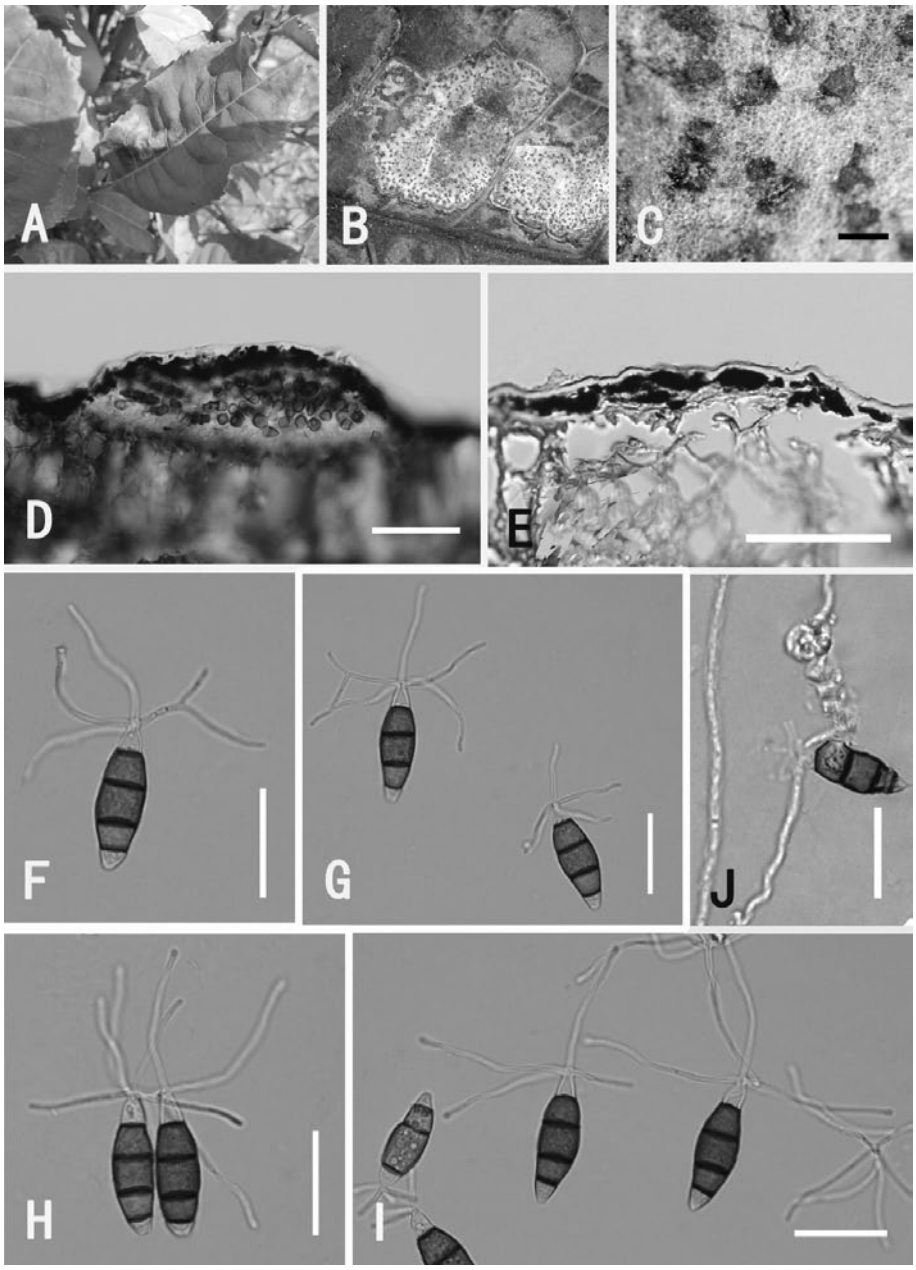

Fig. 2. *Pestalotiopsis camelliae* (holotype) from host. **A** Blight on leaf of *Camellia japonica*. **B, C** Conidiomata, splitting irregularly. **D** Section of conidiomata. **E** Conidiophores/ conidiogenous cells. **F–I** Conidia with 3–6 appendages. **J** Germination of the conidia. Bars: **C** 200 µm; **D, E** 50 µm; **F–J** 20 µm.

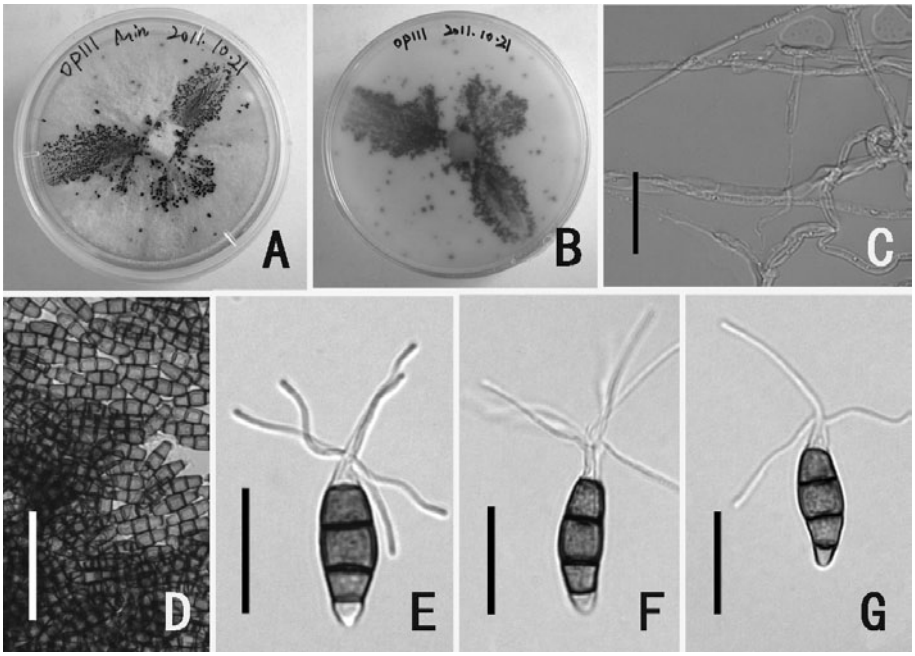

Fig. 3. *Pestalotiopsis camelliae* (holotype) from culture. **A, B** Colony on PDA, **A** from above, **B** from below. **C** Hyaline mycelium from colony. **D** Black slimy mass of conidia. **E**–**G** Conidia. Bars: **C**–**G** 20 µm; **D** 50 µm.

Discussion

Pestalotiopsis contains numerous species and several species are complexes within which species can only be distinguished by using multi-gene sequence data. This is true of many other plant pathogenic genera that have been recently studied (Cai *et al*. 2011, Summerell *et al*. 2011).

Some morphological characters of *Pestalotiopsis camelliae* and related species are synoptically compared in Tab. 2. *Pestalotiopsis camelliae* is similar to *P. furcata* Maharachchikumbura & K. D. Hyde but is a distinct species in terms of its characteristic morphology and DNA phylogeny. It has relatively small conidia (26.8–33 × 7.5–9.3 µm), although these overlap in size with those of *P. furcata* (29–39 × 8.5–10.5 µm). *Pestalotiopsis furcata* has more apical appendages (5–9) than *P. camelliae* (3–6). The apical appendages of *P. furcata* consistently divide into branches while this is a rare character in *P. camelliae*. In addition, *P. camelliae* separates from *P. furcata* with high bootstrap suppport (100%) (Fig. 1). The conidia of *P. camelliae* (27–33 × 7.5–9.3 µm) lack basal appendages when comparing those of morphologically similar species such as *P. leucopogonis* Nag Raj (27–32 × 7.5–9.5 µm), *P. macrospora* (Ces.) Steyaert (30–40 × 7–9 µm) and *P. nattrassi* Steyaert (27–33 × 8–9 µm).

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

Species	*P. camelliae*	*P. furcata*	*P. nattrassi*[b]	*P. leucopogonisc*[c]	*P. hainanensi* A.R. Liu, T. Xu & L.D. Guo[d]
Conidia size (µm)	27–33 × 7.5–9.3	29–39 × 8.5–10.5	27–33 × 8–9	27–32 × 7.5–9.5	19–22 × 5–6
Median cells	concolorous, olivaceous	concolorous, olivaceous	concolorous, brown	concolorous, brown	concolorous, brown to olivaceous
Apical appendages:	3–6	5–9	1–4	7–11	1–3
Length (µm)	13.5–33.7	20–35	25–44	12–19	1–10
Branching	sometimes	branched	no	no	no
Position	apex	apex	apex	3 rows (top, middle and bottom)	apex
Basal appendages	absent	absent	absent or present	absent or present	absent

[a] Maharachchikumbura *et al.* (2012 b), [b] Guba (1961), [c] Nag Raj (1993), [d] Liu (2007)

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