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 tef1 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 tef1, 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

Teleomorph. – Unknown.

Description. – 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, $100-220~\mu m$ wide, $76-150~\mu m$ 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 $27-33\times7.5-9.3~\mu m$ ($28.5\times8.5~\mu m$), fusoid, straight to slightly curved, 4–septate, basal cell obconic, colourless, thin- and smoothwalled, 4–7 μm long ($5.4~\mu m$), 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 $19-22~\mu m$ long ($20~\mu m$) (second cell from base 5.6-

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

| - F | Tables | 17 17 0 1/ 100 T | GenBan | GenBank accession number | number | Defendance |
|---|-------------------------|-------------------------------|----------------------|--------------------------|----------------------------|---|
| 1axon - | isolates | пови госапоп | ITS | β-tubulin | tef1 | ควาเลก bas 101 ความสาสพา |
| P. cf. algeriensis (Sacc. & Berl.) W.Wu | SD077 | Camellia sinensis/ China | JQ683718 | JQ683702 | JQ683734 | JQ683734 Maharachchikumbura et al. 2012 b |
| P. cf. disseminata (Thüm.) Steyaert | SD034 | C. sinensis/China | JQ683716 | JQ683700 | JQ683716 JQ683700 JQ683732 | Maharachchikumbura $etal.2012$ b |
| P. cf. menezesiana (Bres. & Torrend) Bissett | SG064 | C. sinensis/China | JQ683719 | JQ683703 | JQ683719 JQ683703 JQ683735 | Maharachchikumbura <i>et al.</i> 2012 b |
| P. cf. menezesiana | SD072 | C. sinensis/China | JQ683713 | JQ683697 JQ683729 | JQ683729 | Maharachchikumbura <i>et al.</i> 2012 b |
| P. cf. microspora (Speg.) G. C. Zhao & N. Li | SD056 | C. sinensis/China | JQ683722 | JQ683706 JQ683738 | JQ683738 | Maharachchikumbura <i>et al.</i> 2012 b |
| P. cf. versicolor (Speg.) Steyaert | SG100 | C. sinensis/China | JQ683712 | JQ683696 JQ683728 | JQ683728 | Maharachchikumbura $etal.2012$ b |
| P. cf. versicolor | SD047 | C. sinensis/China | JQ683715 | JQ683699 JQ683731 | JQ683731 | Maharachchikumbura et al. 2012 b |
| P. cf. versicolor | SD091 | C. sinensis/China | JQ683714 | JQ683698 | JQ683698 JQ683730 | Maharachchikumbura <i>et al.</i> 2012 b |
| P. camelliae Y. M. Zhang, Maharachchikumbura & K. D. Hyde | OP111 | C. japonica/China | JX399010 | JX399041 | JX399010 JX399041 JX399074 | Maharachchikumbura <i>et al.</i> 2012 a |
| P. camelliae | OP131 | C. japonica/China | JX399011 | JX399042 | JX399042 JX399075 | Maharachchikumbura $etal.2012$ a |
| P. cf. virgatula (Kleb.) Steyaert | SD004 | C. sinensis/China | JQ683723 | JQ683707 | JQ683707 JQ683739 | Maharachchikumbura $etal.2012$ b |
| P.furcata Maharachchikumbura & K. D. Hyde | MFLUCC12- 0054/SS017 | C. sinensis/ Thailand | JQ683724 | JQ683708 | JQ683740 | Maharachchikumbura $\operatorname{\it et}$ a l. 2012 b |
| P. theae (Sawada) Steyaert | MFLUCC12- 0055/SC027 | C. sinensis/ Thailand | JQ683727 | JQ683711 | JQ683743 | JQ683727 JQ683711 JQ683743 Maharachchikumbura et al. 2012 b |
| P. theae | SC011 | C. sinensis/ Thailand | JQ683726 | JQ683710 | JQ683742 | J 0683726 J 0683710 J 0683742 Maharachchikumbura $et\ al.\ 2012\ b$ |
| P. trachicarpicola Y. M. Zhang & K. D. Hyde | OP068 | Trachycarpus fortune/China | JQ845947 JQ683720 | JQ845945 JQ845946 | JQ845946 | Zhang et al. 2012 |
| $Pestalotiops is {\rm sp.}$ | SD012 | C. sinensis/China | JQ683713 | JQ683704 | JQ683736 | J Q683704 JQ683736 Maharachchikumbura $etal.2012$ b |
| $Pestalotiops is \ {\rm sp.}$ | SD072 | C. sinensis/China | JQ683725 | JQ683697 | JQ683697 JQ683729 | Maharachchikumbura $etal.2012$ b |
| Seiridium sp. | SD096 | C. reticulata/China | | JQ683709 | JQ683741 | JQ683709 JQ683741 Maharachchikumbura et al. 2012 b |

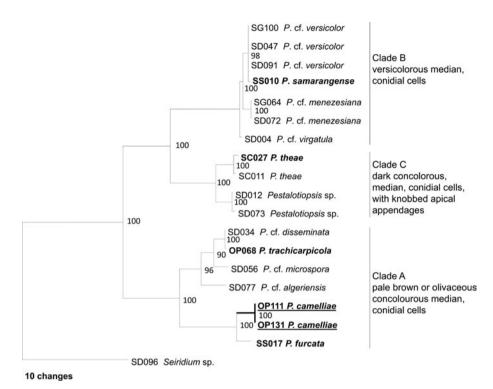


Fig. 1. Maximum parsimony phylogram generated from combine three genes (ITS, β -tubulin and tef1) analysis of species of Pestalotiopsis. Data were analyzed with random addition sequence, unweighted parsimony and treating gaps as missing data. Seiridium sp. was placed as outgroup, ex-type sequences are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

 $7.4~\mu m$ (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 $^{\circ}$ 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.

D i s t r i b u t i o n . – 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.

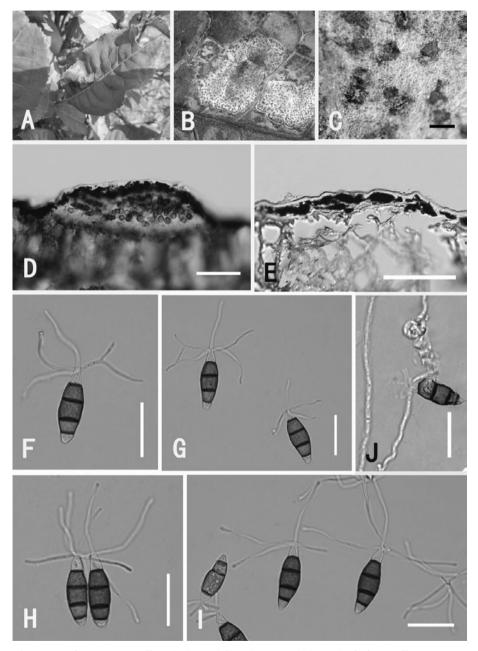


Fig. 2. Pestalotiopsis camelliae (holotype) from host. A Blight on leaf of Camellia japonica. B, C Conidiomata, splitting irregularly. D Section of conidiomata. E Conidiophores/ conidiogenous cells. F–I Conidia with 3–6 appendages. J Germination of the conidia. Bars: C 200 μ m; D, E 50 μ m; F–J 20 μ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

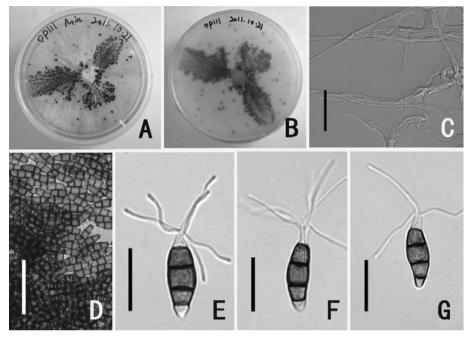


Fig. 3. Pestalotiopsis camelliae (holotype) from culture. A, B Colony on PDA, A from above, B from below. C Hyaline mycelium from colony. D Black slimy mass of conidia. E–G Conidia. Bars: C–G 20 μ m; D 50 μ m.

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 $(26.8–33\times7.5–9.3~\mu\text{m})$, although these overlap in size with those of *P. furcata* $(29–39\times8.5–10.5~\mu\text{m})$. *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* $(27–33\times7.5–9.3~\mu\text{m})$ lack basal appendages when comparing those of morphologically similar species such as *P. leucopogonis* Nag Raj $(27–32\times7.5–9.5~\mu\text{m})$, *P. macrospora* (Ces.) Steyaert $(30–40\times7–9~\mu\text{m})$ and *P. nattrassi* Steyaert $(27–33\times8–9~\mu\text{m})$.

| 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) Median cells | 27–33 × 7.5–9.3 concolorous, olivaceous | 29–39 × 8.5–10.5 concolorous, olivaceous | 27–33 × 8–9 concolorous, brown | $27-32 \times 7.5-9.5$ concolorous, brown | 19–22 × 5–6 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 |

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

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

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