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A novel species of *Pestalotiopsis* causing leaf spots of *Trachycarpus fortunei*

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Abstract – Specimens of a new *Pestalotiopsis* species were obtained from leaves of *Trachycarpus fortunei* from Kunming Botany Garden, Kunming, Yunnan Province, China, where it caused serious leaf blotch and defoliation in the garden. Single ascospore isolates from the teleomorph produced identical colonies with black slimy conidial masses. Morphological characteristics of the conidia produced in culture accorded well with the genus *Pestalotiopsis*. Based on morphological characters and molecular analysis, *Pestalotiopsis trachicarpicola* sp. nov. is introduced and both its asexual and sexual forms are described.

Coelomycetes / new species / holomorph / *Pestalosphaeria*

INTRODUCTION

We are in the process of studying the pathogens of ornamental plants in Yunnan Province. The study involves collecting fresh specimens, isolation, and molecular analysis, and reporting the known and the novel pathogens, so as to strengthen plant quarantine, integrated pest management and diagnosis of fungal diseases of these plants. In this paper we address a species of *Pestalotiopsis* causing serious leaf spotting disease of *Trachycarpus fortunei* (Chinese windmill palm, Arecaceae).

Pestalotiopsis is a confused genus with 234 names (http://www.index fungorum. org/names/names.asp; accession date, 2012.02.25), which is in need of molecular characterization. Maharachchikumbura *et al.* (2011) reviewed the genus and noted there were only four sequenced type or epitype strains available and

showed that names had been widely misapplied to species in GenBank. The sexual stage of *Pestalotiopsis* has been reported for 12 species, as *Pestalosphaeria* (http://www.indexfungorum.org/names/Names.asp) but the morphs have rarely been recorded on the same host plant (Barr 1975; Nag Raj 1985; Hyde, 1996). It is not always clear that two stages are definitely the same biological species, as it is rare to find the sexual stage or *Pestalosphaeria*-morph of *Pestalotiopsis.* However, it is possible to link them through single spore isolation or through molecular evidence.

Only one name can now be applied to any fungus (Taylor 2011) and Maharachchikumbura *et al.* (2011) pointed out that the older *Pestalotiopsis* name should be applied to both morphs and this is followed in this paper. In this study we isolated the sexual morph into pure culture, where it produced a *Pestalotiopsis* asexual morph. Molecular and morphological analysis showed this to be a new species of *Pestalotiopsis*, which is described here as *P. trachicarpicola* Y.M. Zhang & K.D. Hyde. This is a severe pathogen of the Chinese palm *Trachycarpus fortunei*, causing serious leaf spotting leading to defoliation.

MATERIALS AND METHODS

Sampling. Fresh specimens were obtained from leaf spots on living leaves of *Trachycarpus fortunei* in Kunming Botanical Gardens, Yunnan Province, China. Diseased leaves were placed in a moist chamber for 1 to 2 days following collection. Specimens were examined under a Leica MZ16A stereo microscope and fine forceps were used to remove one or two ascomata from the necrotic tissues of the leaf spots, which were mounted in water and lactic acid or stained with cotton blue. 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 were made with a sharp razor blade and thin sections (4-10 μm) were transferred to a drop of water, a drop of lactic acid or a drop of cotton blue for examination and microphotography.

Culturing. Single spore isolation followed the method of Chomnunti *et al.* (2011). Ascomata or asci were removed from the substrate and broken open in 50-100 μL sterilized water on a sterile glass slide in order to provide a spore suspension. An homogenous spore suspension was then transferred with a sterilized pipette, onto the surface of a water agar or PDA plate with antibiotics, and carefully shaken to spread the suspension. The unsealed plate was incubated at 25ºC. Spores were checked within 6 hours and then every 24 hours to establish germination. Once the spores had germinated, a sterilized syringe needle was used to pick up a small piece of agar containing a single germinated spore, which was transferred onto water agar or PDA plates, and incubated at 25ºC until it reached a colony diam. of 1-2 cm. A small piece of mycelium with agar was then transferred to another PDA plate and it was considered that a pure culture had been obtained.

DNA extraction and PCR condition. Biospin Fungus Genomic DNA Extraction Kit (Produced Bioer Technology Co.) 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 (5'-TCCT CCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAA GG-3') (White *et al.*, 1990), β-tubulin gene region was amplified with primer pairs BT2A (5'-GGTAACCA AATCGGTGCTGCTTTC-3') and BT2B (5' ACCCTC AGTGTAGTGACCCTT GGC-3') (Glass & Donaldson 1995; O'Donnell & Cigelnik 1997) and *tef1* was amplified using the primer pairs EF1-526F (5'-GTCGT YGTYATYGGHCAYGT-3') and EF1-1567R (5'-ACHGTRCCRATACCACC RATCTT-3') (Rehner 2001). PCR was performed by the system under conditions used by Maharachchikumbura *et al.* (2012).

Phylogenetic analysis. Sequences were optimized manually to allow maximum alignment and maximum sequence similarity as described by Maharachchikumbura *et al.* (2012). The aligned dataset was analyzed using PAUP* 4.0b10 (Swofford 2002). *Seiridium* sp. was used as the outgroup, 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 1,000 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 10 replicates of random stemwise 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

As ITS on its own does not sufficiently resolve species distinction in *Pestalotiopsis* (Tempesta *et al.*, 2011), a phylogenetic tree was constructed using a combined ITS, β-tubulin and *tef1* sequence dataset of 16 isolates of *Pestalotiopsis* with *Seiridium* sp. as the outgroup taxon (Tab. 1, Fig. 1). The aligned data matrix consisted of 2019 characters; 1489 characters were constant, 197 variable characters were parsimony-uninformative and 333 characters were parsimony-informative. Kishino-Hasegawa (KH) test showed that the two trees generated from parsimonious analysis were not significantly different (length = 769 steps, CI = 0.848, RI = 0.916, HI = 0.152, RC = 0.777).

In the phylogenetic tree (Fig. 1) our species from *Trachycarpus* forms a well-supported clade with *P.* cf. *algeriensis, P.* cf. *disseminata, P. furcata* and *P.* cf. *microspora.* However, of these four species only *P. furcata*, which is the type strain, can be confidently named. Our new species, *P. trachycarpicola* is very similar to *P.* cf. *disseminata* with 100% bootstrap support and is probably the same species, but this name is likely to be misapplied. We therefore introduce a new species for this *Pestalotiopsis c*ausing leaf spots on *Trachycarpus fortunei.*

Table 1. *Pestalotiopsis* isolates used in this study

Taxon	*Isolates*	*GenBank accession number*		
		ITS	*β-tubulin*	*tef1*
P. cf. algeriensis (Sacc. & Berl.) W.P. Wu	SD077	JQ683718	JQ683702	JQ683734
P. cf. disseminata (Thüm.) Steyaert	SD034	JQ683716	JQ683700	JQ683732
P. cf. menezesiana (Bres. & Torrend) Bissett	SG064	JQ683719	JQ683703	JQ683735
P. cf. menezesiana Bres. & Torrend) Bissett	SD072	JQ683713	JQ683697	JQ683729
P. cf. microspora (Speg.) G.C. Zhao & N. Li	SD056	JQ683722	JQ683706	JQ683738
P. cf. versicolor (Speg.) Steyaert	SG100	JQ683712	JQ683696	JQ683728
P. cf. versicolor (Speg.) Steyaert	SD047	JQ683715	JQ683699	JQ683731
P. cf. versicolor (Speg.) Steyaert	SD091	JQ683714	JQ683698	JQ683730
P. cf. versicolor (Speg.) Steyaert	SD040	JQ683717	JQ683701	JQ683733
P. cf. virgatula (Kleb.) Steyaert	SD004	JQ683723	JQ683707	JQ683739
P. furcata Maharachchikumbura & K.D. Hyde	MFLUCC12-0054 /SS017	JQ683724	JQ683708	JQ683740
P. theae (Sawada) Steyaert	MFLUCC12-0055 /SC027	JQ683727	JQ683711	JQ683743
P. theae (Sawada) Steyaert	SC011	JQ683726	JQ683710	JQ683742
P. trachicarpicola Y. M. Zhang & K.D. Hyde	OP068	JQ845947	JQ845945	JQ683746
Pestalotiopsis sp.	SD012	JQ683720	JQ683704	JQ683736
Pestalotiopsis sp.	SD072	JQ683713	JQ683697	JQ683729
Seiridium sp.	SD096	JQ683725	JQ683709	JQ683741

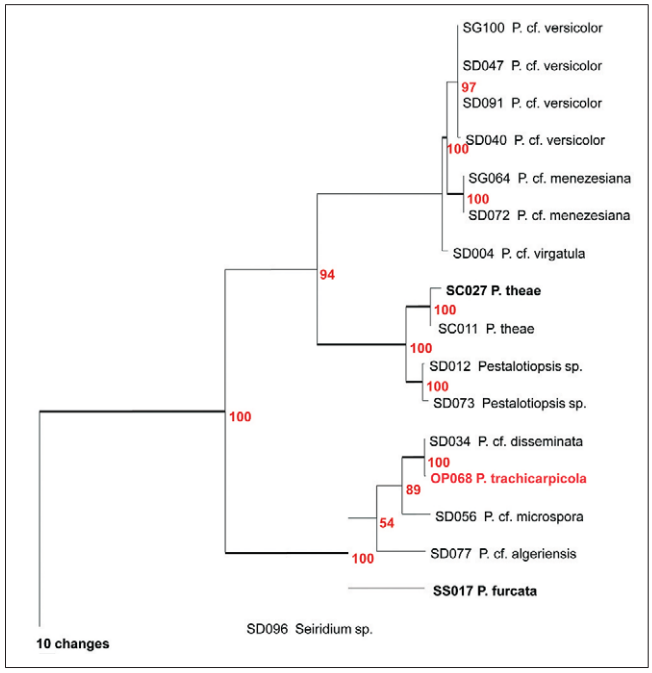

Fig. 1. Maximum parsimony phylogram generated from combined ITS, β-tubulin and *tef1* analysis of *Pestalotiopsis* species. Data were analyzed with random addition sequence, unweighted parsimony and with treating gaps as missing data. Numbers in red give bootstrap values. The outgroup taxon is *Seiridium* sp. and *P. trachicarpicola* and other type or epitype sequences are in bold.

TAXONOMY

Pestalotiopsis trachicarpicola Y. M. Zhang & K.D. Hyde, **sp. nov.** **Figs 2-4**

MycoBank: **MB 564879**.

Etymology: In reference to its occrruence on the host *Trachycarpus.*

Forming leaf spots on *Trachycarpus fortunei* (Fig. 2). ***Ascomata*** 115-215 µm diam × 140-185 µm high ($\bar{x}$ = 177 × 157 µm, n = 10), scattered or gregarious, immersed under slightly raised areas of host epidermis, subglobose to globose, with central black irregular ostioles (Figs 3B, D). ***Peridium*** 20-26 µm wide, comprising 3-5 layers of brown, relatively thick-walled cells of *textura angularis*, inner cells flattened and thin-walled. ***Paraphyses*** 3-5 µm wide, with few septa, base relatively wide and tapering to free ends (Figs 3C, E, F). ***Asci*** 65-76 × 5-14 µm ($\bar{x}$ = 73.6 × 9.3 µm, n = 10), 8-spored, unitunicate, cylindrical, pedicel short, 3.8-5.8 µm long (Figs 3G-H), with a distinct J+, 3-5 µm in diam, amyloid apical ring in ascus apex (Figs 3I). ***Ascospores*** 12-16 × 5-8 µm ($\bar{x}$ = 14.1 × 6.5 µm, n = 30), uniseriate, or 1-seriate in the upper part and 2-seriate at the base, oblong to ellipsoidal or fusiform, smooth or verrucose, pale yellowish brown, 2-3-transversely septate and constricted at the septa, with obconic or semicircular end cells, sometimes the colour of the end cells lighter than cells in the middle cells, cells fairly uniform in size (Figs 3J-L).

Colonies on PDA white, thin, with entire edge, growth determined after 3 days at 25ºC (4.6 cm in 3 days, 1.52 cm/day). After a few weeks, black slimy conidial masses produced on the white colonies, agar changing colour to orange to deep brown (Figs 4E-F). ***Mycelium*** hyaline, sparsely septate, with small guttules, 1.3-6.4 µm diam. ***Conidiophores*** reduced to conidiogenous cells lining the inner wall of the conidiomatal cavity. ***Conidiogenous cells*** discrete, lageniform, smooth, thin-walled, colorless, with 2-4 proliferations. ***Conidia*** fusoid, straight to slightly curved, 4-septate, 19-24.9 × 5.3-6.3 µm ($\bar{x}$ = 22 × 6.0 µm, n = 30), basal cell obconic with truncate base, hyaline, thin- and smooth-walled, 3-5 µm long ($\bar{x}$ = 4 µm), with 3 median cells, doliiform, with thick verruculose walls, constricted at the septa, concolorous, olivaceous, septa and periclinal walls darker than the rest of the cell, together 11.7-13.7 µm long ($\bar{x}$ = 12.4 µm) (second cell from base 3.7-5.7 µm ($\bar{x}$ = 4.7 µm); third cell 3.75-4.7 µm ($\bar{x}$ = 4.2 µm); fourth cell 4-4.7 µm ($\bar{x}$ = 4.4 µm); apical cell hyaline, conic to cylindrical 2.2-4.4 µm long ($\bar{x}$ = 3.2 µm); 3 tubular apical appendages, arising from the upper portion of the apical cell, 9.4-17.8 µm long; basal appendage present, 2.7-5.5 µm long (Figs 4A-D).

Fig. 2. *Pestalotiopsis trachicarpicola* causing serious leaf spotting leading to defoliation of *Trachycarpus fortunei* in Kunming Botanical Garden.

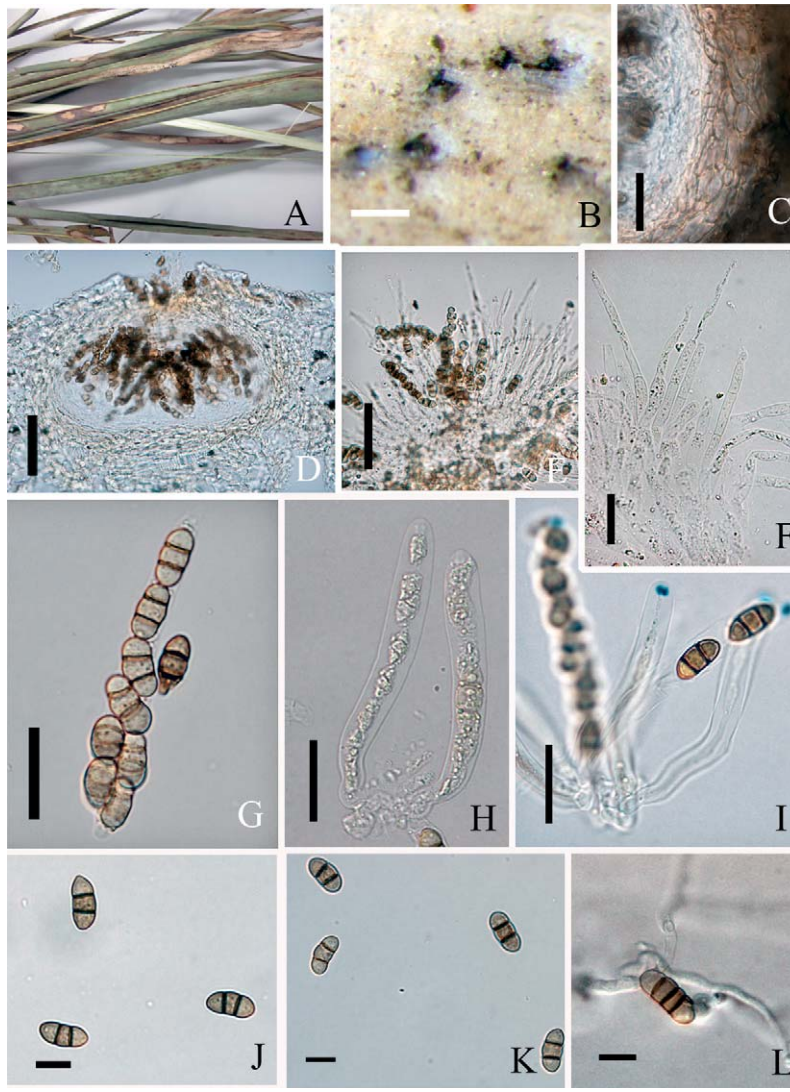

Fig. 3. *Pestalotiopsis trachicarpicola* (holotype). **A. B.** Leaf spot on living leaves. **C.** Peridium with five cell layers. **D.** Section of ascomata. **E.** Asci and paraphyses **F.** Hyaline paraphyses with septum. **G. H.** Mature and immature unitunicate asci. **I.** Asci in Melzer's reagent, note the distinct J+ apical ring. **J. K.** Ascospores. **L.** Ascospore germination. Scale Bars: B = 200 µm, C. F. G. H. I. = 20 µm, D. = 50 µm, J. K. L. = 10 µm.

Material examined: CHINA, Yunnan Province, Kunming, Kunming Botanical Gardens, on leaf spots on living leaves of *Trachycarpus fortunei*, March 2011, K.D. Hyde OP068 (IFRD 9026, holotype), extype living culture IFRDCC 2440; CHINA, Yunnan Province, Kunming, Kunming Botanical Gardens, on leaf spots on living leaves of *Trachycarpus fortunei*, September 2011, Y.M. Zhang OP145 (IFRD 411-019).

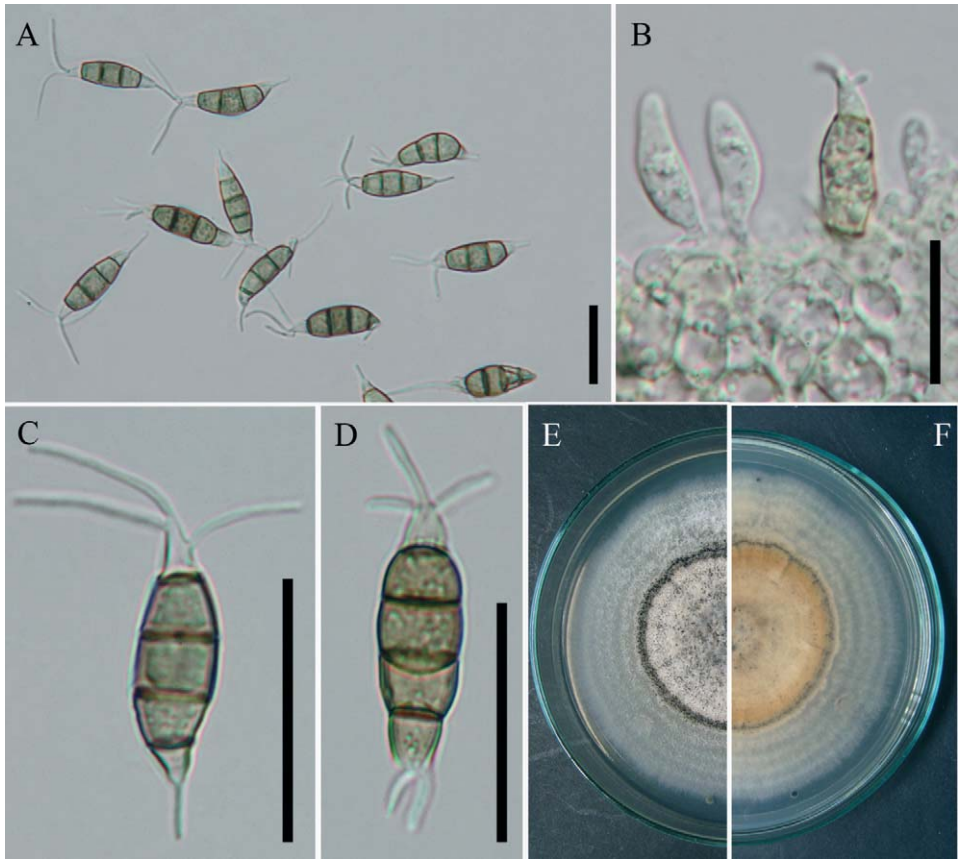

Fig. 4. *Pestalotiopsis trachycarpicola* (holotype). **A.** Colonies producing black slimy masses of conidia. **B**. *Conidiogenous cells* discrete and lageniform **C-D.** Conidia. **E. F.** Colony on PDA, E from above, F from below. Scale Bars: A-D = 20 µm**.**

DISCUSSION

Pestalotiopsis palmarum (Cooke) Steyaert has previously been reported to cause leaf spots on *Cocos nucifera* L. (Guba, 1961), however this taxon differs from *P. trachycarpicola* because in *P. palmarum* the three median cells of the conidia are versicolorous while in *P. trachycarpicola* they are concolorous. The sexual morph of *Pestalotiopsis trachycarpicola* is morphologically most similar to *Pestalosphaeria accidenta* P.L. Zhu, T. Xu & Q.X. Ge (Zhu *et al.*, 1991). *P. accidenta* was recorded on *Rhododendron latoncheae* Franch, and ascomata are 315-420 µm diam, which is considerably larger than those of *P. trachycarpicola (*116-214 µm in diam). The asci and ascospores of both species are similar, but the ends of the ascospores of *P. trachicarpicola* are more rounded and some are verrucose. There are presently 12 species of *Pestalosphaeria* and, following Maharachchikumbura *et al.* (2011), these will all need to be transferred to *Pestalotiopsis*, which is both the oldest and more commonly used name.

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