



## A new species of *Pestalotiopsis* from leaf spots of *Licuala grandis* from Hainan, China

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### Abstract

A new species, *Pestalotiopsis licualacola*, was isolated from grey leafspots of *Licuala grandis* (ruffled fan palm). It is morphologically distinct in having relatively small, greyish brown conidia ( $16\text{--}20 \times 3\text{--}5 \mu\text{m}$ ), and 1–3 short apical appendages without knobs. Phylogenetic analysis based on combination of ITS,  $\beta$ -tubulin and *tef1* gene sequence data clearly distinguishes *P. licualacola* from other species in this genus, with ex-type sequence data in GenBank. Based on morphology and molecular phylogeny we describe it as a new species.

**Key words:** coelomycetes, phylogeny, taxonomy

### Introduction

The ruffled fan palm (*Licuala grandis*) is a dainty palm with a height of approximately 2 m that originates in South East Asia, and is a rainforest understory palm ([www.nationaltropicals.com.au/](http://www.nationaltropicals.com.au/)). During investigations of *Pestalotiopsis* in southern China, we have obtained many isolates of *Pestalotiopsis* spp. from diseased leaves of diverse plants, which we are using for novel compound screening as the genus is biochemically highly creative (Xu *et al.* 2010, Aly *et al.* 2011, Debbab *et al.* 2011, 2012). Among them was an undescribed species isolated from necrotic leaf spots from *L. grandis*. Morphological details are described and a comparison made with related species. Molecular characteristics based on the DNA sequences of three gene loci (ITS,  $\beta$ -tubulin and *tef1*) were also determined.

### Materials & Methods

#### *Morphological and cultural studies*

Diseased leaves of ruffled fan palm were collected from Xinglong County, Hainan Province. Leaf samples were placed in clean paper bags and symptoms were recorded. A single conidium culture technique was preformed to obtain pure colonies of the fungi following the method outlined in Chomnunti *et al.* (2011). The colonies were transferred to 2% potato-dextrose agar (PDA) medium and incubated at room temperature (25°C). Sporulation was induced using sterilized carnation leaves, which were aseptically placed on the surface of the medium with growing mycelium. The morphology of fungal colonies was recorded following the method of Hu *et al.* (2007). Fungal mycelium and spores were observed under the light microscope

(Nikon 80i) and photographed. Methods of examination, photography and isolation followed Boonmee *et al.* (2011). Dried and ex-type cultures of this species are deposited in the Plant Pathology Herbarium of Guizhou University (HGUP).

#### *DNA sequencing and alignment*

Total genomic DNA was extracted from fresh cultures using a modified protocol of Doyle & Doyle (1987) and Lee & Taylor (1990). The ITS and 5.8S region of rDNA molecule was amplified using primer pairs ITS4 and ITS5 (White *et al.* 1990);  $\beta$ -tubulin gene region was amplified with primer pairs BT2A and BT2B (Glass & Donaldson 1995, O'Donnell & Cigelnik 1997); and *tefl* was amplified using the primer pairs EF1-526F and EF1-1567R (Rehner 2001). PCR was performed with the 25  $\mu$ L reaction system consisting of 19.75  $\mu$ L of double distilled water, 2.5  $\mu$ L of 10 $\times$  Taq buffer with MgCl<sub>2</sub>, 0.5  $\mu$ L of dNTP (10 mM each), 0.5  $\mu$ L of each primer (10  $\mu$ M), 0.25  $\mu$ L Taq DNA polymerase (5 U/ $\mu$ l), 1.0  $\mu$ L of DNA template. The thermal cycling program followed Maharachchikumbura *et al.* (2012). The DNA sequences of HGUP4057 in ITS, beta-tubulin and *tefl* regions generated in this study were submitted to GenBank (KC436006, KC481683 and KC481684).

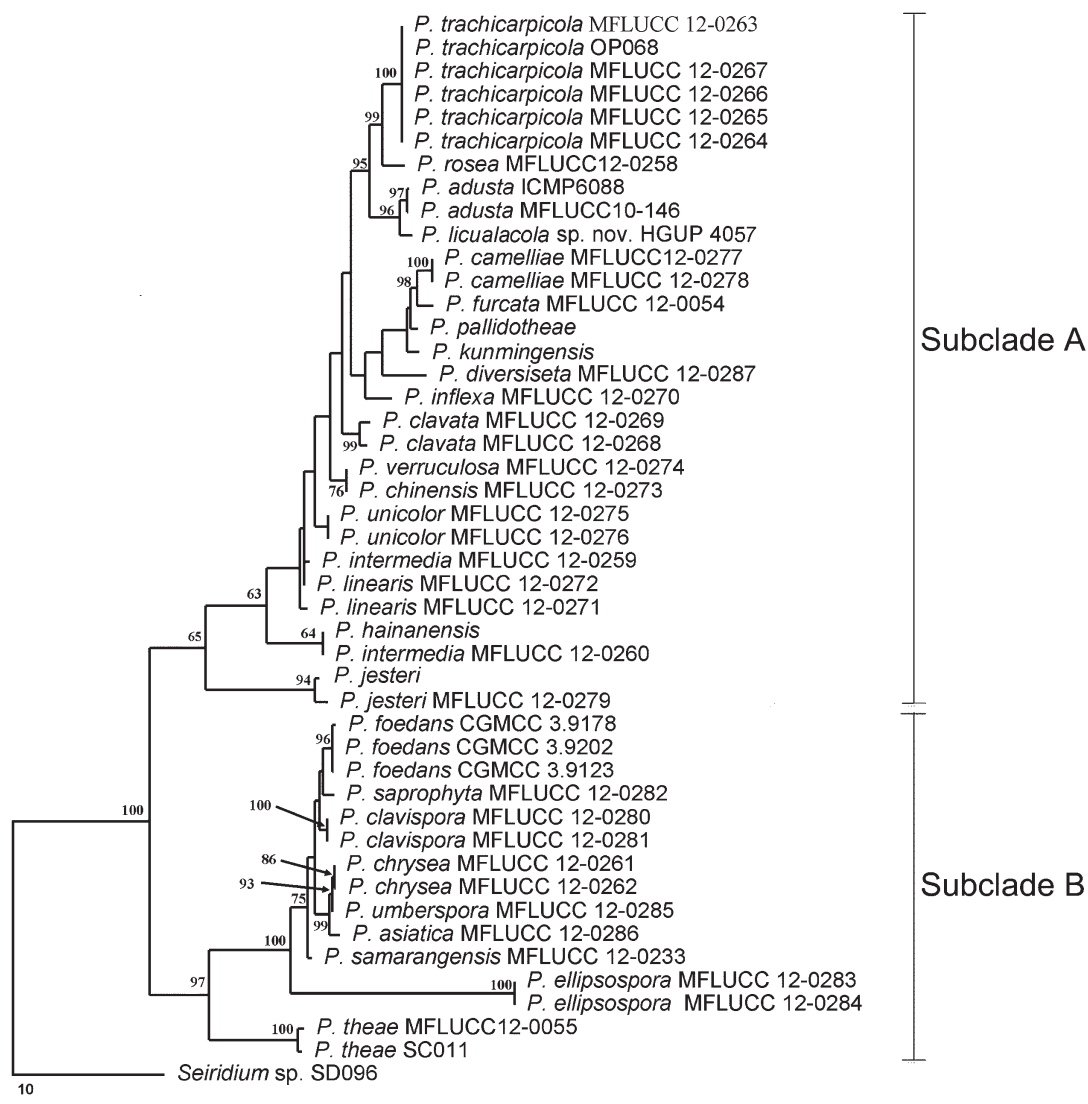
#### *Phylogenetic analyses*

Combination sequence data obtained from three gene regions (ITS,  $\beta$ -tubulin and *tefl*) were aligned using CLUSTALX (v. 1.83) (Thompson *et al.* 1997). The sequences were manually adjusted using BioEdit (Hall 1999), to allow maximum alignment and maximum sequence similarity. Alignments file is available in TreeBASE ([www.treebase.org/treebase-web/home.html](http://www.treebase.org/treebase-web/home.html)) with study ID 13925. 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 1,000 random sequence additions. Maxtrees were set up to 5,000, 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], and homoplasy index [HI] were determined. The robustness of the most parsimonious trees was evaluated by 100 bootstrap replications resulting from maximum parsimony analysis, each with 10 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.

## **Results**

#### *Phylogenetic analysis*

DNA sequences from our *Pestalotiopsis* isolate were compared with those originated from Maharachchikumbura *et al.* (2012) with *Seiridium* sp. (SD096) as outgroup. The alignment file resulted in a data set comprising 2051 characters including gaps. Of these characters, 1434 were constant and parsimony-uninformative. The 617 parsimony-informative characters included in the parsimonious analyses yielded 18 parsimonious trees (TL = 1514, CI = 0.682, RI = 0.884, RC = 0.603), one of which was selected to represent the topology of the strict consensus tree (Fig. 1). Forty-five *Pestalotiopsis* isolates (27 taxa) formed a large clade with 100% bootstrap support, which was divided into subclades A and B with 65% and 97% bootstrap values, respectively. Our isolate (HGUP4057) clustered in subclade A with *P. trachicarpicola*, *P. rosea* and *P. adusta*, with a high bootstrap value of 95%. HGUP4057 showed a close relationship with two *P. adusta* isolates (ICMP6088 – epitype culture of *P. adusta* and MFLUCC10-146) supported by a strong bootstrap value (96%).



**FIGURE 1.** Topology showing the most parsimonious tree, inferred from combined ITS,  $\beta$ -tubulin and *tef1* gene regions. Bootstrap values smaller than 50% are not shown. The tree was rooted with *Seiridium* sp. (SD096).

### Taxonomy

***Pestalotiopsis licualacola*** K. Geng, Y. Song, K.D. Hyde & Yong Wang bis, *sp. nov.* (Fig. 2) MycoBank MB 803183

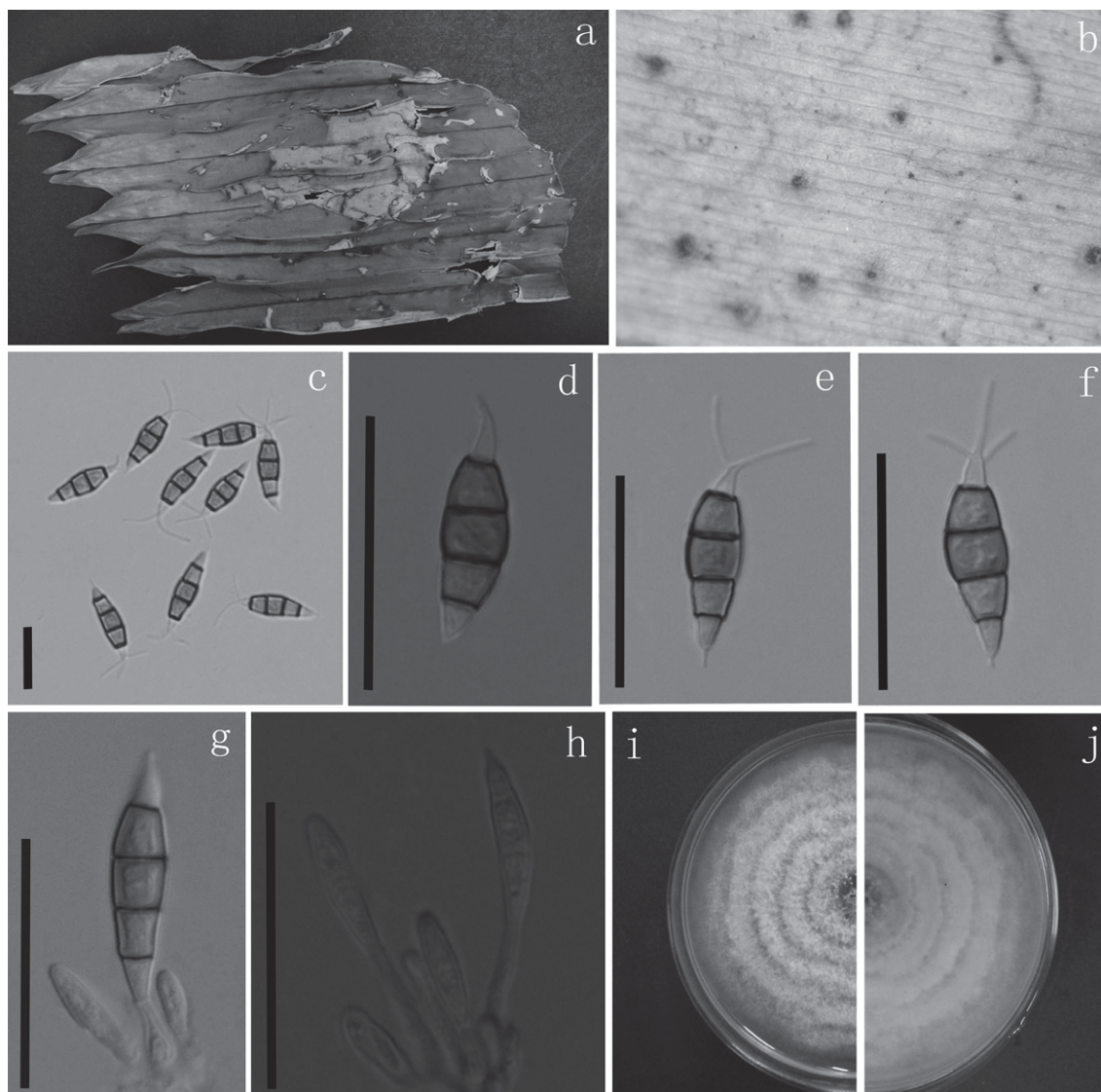
**Type:**—CHINA. Hainan Province: Xinglong County, Tropical Botanical Garden, living leaves of *Licuala grandis*, 8 March 2012, HGUP4057, K. Geng, HGUPd4057, holotype!

Differs from related *Pestalotiopsis* and *Pestalosphaeria* species mainly by its noticeably narrower, fusiform conidia with mostly a single apical appendage.

Colonies on PDA attaining 7 cm diam. after 7 days at 25°C, with edge undulate, whitish, aerial mycelium on surface, fruiting bodies black, concentric; reverse of culture yellow to pale brown. Conidiophores most often indistinct. Conidiogenous cells discrete, hyaline, simple, filiform, 4–10  $\mu$ m long. Conidia 16–20  $\times$  3–5  $\mu$ m ( $\bar{x}$  = 17.4  $\times$  3.9  $\mu$ m), fusiform, straight to slightly curved, 4-septate, smooth, greyish brown; basal cell conical, hyaline, thin-walled, 2–4  $\mu$ m long ( $\bar{x}$  = 2.4  $\mu$ m); with three median cells,

dark brown, concolorous, septa and periclinal walls darker than the rest of the cell, together 9.5–12  $\mu\text{m}$  long ( $\bar{x}$  = 11  $\mu\text{m}$ ); second cell from base 2.7–4.2  $\mu\text{m}$  ( $\bar{x}$  = 3.6  $\mu\text{m}$ ); third cell 2.4–4  $\mu\text{m}$  ( $\bar{x}$  = 3.3  $\mu\text{m}$ ); fourth cell 2.5–3.8  $\mu\text{m}$  ( $\bar{x}$  = 3.2  $\mu\text{m}$ ); apical cell hyaline, conic to subcylindrical, 1.8–3.6  $\mu\text{m}$  ( $\bar{x}$  = 2.4  $\mu\text{m}$ ); with 1–3 tubular apical appendages (mostly 1) without knobs, arising from the apex of the apical cell, 4–9.5  $\mu\text{m}$  long ( $\bar{x}$  = 6.6  $\mu\text{m}$ ); basal appendage filiform, short.

**Etymology:** —In reference to the host, *Licuala grandis*, from which this fungus was first isolated.



**FIGURE 2.** *Pestalotiopsis licualacola* (holotype). a. Herbarium material, leaves of *Licuala grandis*. b. Conidiomata. c–f. Conidia with concolorous median cells. g–h. Conidiophores/conidiogenous cells. i–j. Colony on PDA, i. from above, j. from below. Scale bars: c–h = 20  $\mu\text{m}$ .

## Discussion

It is important to clarify relationships amongst *Pestalotiopsis* species as they are weak pathogens, but also prolific producers of novel medicinal compounds (Xu *et al.* 2010, Aly *et al.* 2011, Ko Ko *et al.* 2011a, b, Debbab *et al.* 2011, 2012). Several *Pestalotiopsis* species are known from palms and perhaps *Pestalospaeria elaeidis* is the most commonly recorded (Hyde 1996). In the phylogenetic tree, *Pestalotiopsis licualacola* clusters with *P. adusta*, *P. licualacola*, *P. rosea* and *P. trachicarpicola*. Only *P. licualacola* produces fusiform conidia, and mostly possesses a single apical appendage. The conidia of *P. licualacola* are

noticeably narrower ( $17.4 \times 3.9 \mu\text{m}$ ) than those of *P. adusta* ( $19 \times 6 \mu\text{m}$ ), *P. rosea* ( $19.2 \times 6.2 \mu\text{m}$ ) and *P. trachicarpicola* ( $23.5 \times 6.5 \mu\text{m}$ ) (Zhang *et al.* 2012, Maharachchikumbura *et al.* 2012). The apical appendages of *P. licualacola* are shorter ( $4\text{--}9.5 \mu\text{m}$ ) than those of *P. adusta* ( $6\text{--}14 \mu\text{m}$ ), *P. rosea* ( $14\text{--}22 \mu\text{m}$ ) and *P. trachicarpicola* ( $9\text{--}18 \mu\text{m}$ ) (Maharachchikumbura *et al.* 2012). In the combined dataset (ITS, beta-tubulin and *tefl* gene regions), *Pestalotiopsis* species clustered in two main subclades (A and B) with high (65% and 97%) bootstrap support (Fig. 1), which supported earlier reports (Jeewon *et al.* 2003, Liu *et al.* 2010, Maharachchikumbura *et al.* 2011, 2012). Phylogenetic analysis indicated that *P. licualacola* is a distinct species.

Hu *et al.* (2007), Liu *et al.* (2010) and Maharachchikumbura *et al.* (2012) suggested that a combined multigene dataset would better resolve the taxonomy of *Pestalotiopsis* and this approach is confirmed here. However, we can only compare our species with those with ex-type sequence data in GenBank. Several other species are known from palms but lack sequence data in GenBank. *Pestalosphaeria elaeidis* ( $27\text{--}34 \times 6\text{--}7 \mu\text{m}$ ), *Pestalotiopsis palmarum* ( $16\text{--}22 \times 5\text{--}7 \mu\text{m}$ ) and *P. macrochaeta* ( $22\text{--}31 \times 8\text{--}10 \mu\text{m}$ ) differ from *P. licualacola* ( $16\text{--}20 \times 3\text{--}5 \mu\text{m}$ ) because they have distinctly longer and wider conidia (Guba 1961, Booth & Robertson 1961, Zhang *et al.* 2003). *Pestalotiopsis phoenicis* has appendages with knobbed ends (Guba 1961, Chen & Wei 1993), while *P. gibberosa* ( $14\text{--}20 \times 6\text{--}8 \mu\text{m}$ ) differs from *P. licualacola* ( $16\text{--}20 \times 3\text{--}5 \mu\text{m}$ ) because it has wider, broadly clavate conidia with darkened central cells (Guba 1961, Chen & Wei 1993), thus confirming the novelty of *P. licualacola*.

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