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Two new species of *Pestalotiopsis* from Southern China

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Abstract

Three *Pestalotiopsis* isolates were obtained from leaves of *Coffea arabica* and *Rhodomyrtus tomentosa*. Among them, two isolates produced versicolorous conidia, and the other produced concolorous conidia. Phylogenetic analysis based on a combination of ITS, β-tubulin and *tef*1 gene sequence data clearly confirms that they belong to two species and distinguishes them from other species in this genus, with ex-type sequence data in GenBank. On the basis of evidence from morphology and molecular phylogeny they are described as new species, *Pestalotiopsis coffeae-arabicae sp. nov.* and *P. rhodomyrtus sp. nov.*

Key words: coelomycetes, phylogeny, taxonomy

Introduction

Species in the genus *Pestalotiopsis* have received much attention in recent years, not only because of their role as plant pathogens, but also as common endophytes, which have been shown to produce a wide range of chemically novel metabolites (Xu *et al*. 2010, Debbab *et al*. 2011, 2012, Maharachchikumbura *et al*. 2011, 2013). We surveyed the *Pestalotiopsis* diversity in southern China. Among them were two undescribed species, described below, which were isolated from *Coffea arabica* and *Rhodomyrtus tomentosa* leaves. Morphological details are provided and a comparison made with related species. Molecular characteristics based on the DNA sequences of three gene loci (ITS, β-tubulin and *tef1*) were also determined.

Materials & Methods

Morphological and cultural studies

Diseased leaves of *Coffea arabica* and healthy leaves of *Rhodomyrtus tomentosa* were collected from Hainan and Guangxi Provinces. Leaf samples were placed in clean paper bags and symptoms were recorded. A single conidium culture technique was performed 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 a

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light microscope (Nikon 80i) and photographed. Methods of examination, photography and isolation followed Boonmee *et al.* (2011). Holotypes and ex-type cultures of the two species are deposited in the Plant Pathology Herbarium of Guizhou University (HGUP) and isotypes are deposited in Mae Fah Luang University (MFLU) Herbarium, Chiang Rai, Thailand.

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), 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 with the 25 µL reaction system consisting of 19.75 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), and 1.0 µL of DNA template. The thermal cycling program followed Maharachchikumbura *et al*. (2012). The DNA sequences of ITS, beta-tubulin and *tef1* regions generated in this study were submitted to GenBank.

TABLE 1. Strains used in phylogenetic analyses and their corresponding GenBank accession numbers.

Species	Sources	GenBank accession		
		ITS	Bt-tubulin	*tef*
P. adusta	ICMP6088	JX399006	JX399037	JX399070
P. asiatica	MFLUCC 12-0286/ NN047638	JX398983	JX399018	JX399049
P. chinensis	MFLUCC 12-0273/ NN047218	JX398995	-	-
P. chrysea	MFLUCC 12-0261/ NN042855	JX398985	JX399020	JX399051
P. clavata	MFLUCC 12-0268/ NN047134	JX398990	JX399025	JX399056
P. clavispora	MFLUCC 12-0281/ NN043133	JX398979	JX399014	JX399045
P. coffeae-arabicae	HGUP4015	KF412647	KF412641	KF412644
P. coffeae-arabicae	HGUP4019	KF412649	KF412643	KF412646-
P. diversiseta	MFLUCC 12-0287/ NN047261	JX399009	JX399040	JX399073
P. ellipsospora	MFLUCC 12-0283	JX398980	JX399016	JX399047
P. foedans	CGMCC 3.9123	JX398987	JX399022	JX399053
P. inflexa	MFLUCC 12-0270/ NN047098	JX399008	JX399039	JX399072
P. intermedia	MFLUCC 12-0259/ NN047642	JX398993	JX399028	JX399059
P. jesteri	MFLUCC 12-0279/ NN042849	JX399012	JX399043	JX399076
P. linearis	MFLUCC 12-0271/NN047190	JX398992	JX399027	JX399058
P. rhodomyrtus	HGUP4230	KF412648	KF412642	KF412645
P. rosea	MFLUCC12-0258/ NN047135	JX399005	JX399036	JX399069
P. saprophyta	MFLUCC 12-0282/ NN047136	JX398982	JX399017	JX399048
P. theae	MFLUCC12-0055	JQ683727	JQ683711	JQ683743
P. theae	SC011	JQ683726	JQ683710	JQ683742
P. trachicarpicola	MFLUCC 12-0263/ NN047072	JX399000	JX399031	JX399064
P. trachicarpicola	MFLUCC 12-0264/ NN047196	JX399004	JX399035	JX399068
P. trachicarpicola	MFLUCC 12-0265/ NN046983	JX399003	JX399034	JX399067
P. trachicarpicola	MFLUCC 12-0266/ NN046978	JX399002	JX399033	JX399066
P. trachicarpicola	MFLUCC 12-0267/ NN047099	JX399001	JX399032	JX399065
P. trachicarpicola	OP068	JQ845947	JQ845945	JQ845946
P. umberspora	MFLUCC 12-0285/ NN042986	JX398984	JX399019	JX399050
P. unicolor	MFLUCC 12-0276/ NN046974	JX398999	JX399030	-
P. verruculosa	MFLUCC 12-0274/ NN047309	JX398996	-	JX399061
Seiridium sp.	SD096	JQ683725	JQ683709	JQ683741

Phylogenetic analyses

Combination sequence data obtained from three gene regions (ITS, β-tubulin and *tef1*) 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. 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. DNA sequences from our *Pestalotiopsis* isolates were compared with those that originated from Maharachchikumbura *et al.* (2012), with *Seiridium* sp. (SD096) as outgroup.

Results

Phylogenetic analysis

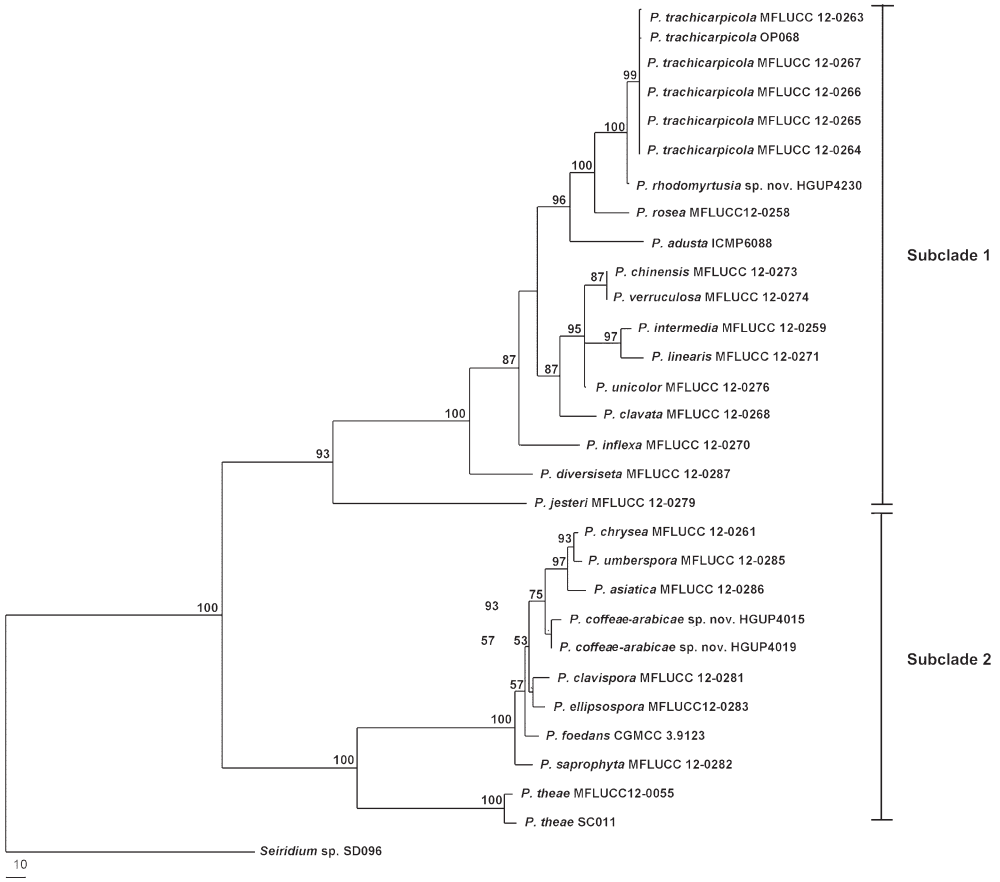

FIGURE 1. Topology showing the most parsimonious tree, inferred from combined ITS, β -tubulin and *tef1* gene regions. Bootstrap values higher than 50% are shown. The tree was rooted with *Seiridium* sp. (SD096).

The alignment file resulted in a data set comprising 2,020 characters including gaps (ITS: 1–548, β-tubulin: 549–1,015 and *tef1*: 1,016–2,020). Of these characters, 1,646 were constant and parsimony-uninformative. The 374 parsimony-informative characters included in the parsimonious analyses yielded only one parsimonious tree (Fig. 1) (TL = 1040, CI = 0.721, RI = 0.890, RC = 0.642). Twenty-nine *Pestalotiopsis* isolates (22 taxa) formed a strong clade (100% bootstrap support), consisting of two subclades (1 and 2), both with high bootstrap values (subclade 1: 93%, subclade 2: 100%). Our isolate (HGUP4230) was placed in subclade 1, and clustered together with *Pestalotiopsis trachicarpicola* Y.M. Zhang & K.D. Hyde in Zhang *et al.* (2012: 315), *P. rosea* Maharachch. & K.D. Hyde in Maharachchikumbura *et al.* (2012: 118) and *P. adusta* (Ellis & Everh.) Steyaert (1953: 82) supported by a strong bootstrap value (96%). It displayed a very close relationship with *P. trachicarpicola* with 100% bootstrap support. Isolates HGUP4015 and 4019, with only slight sequence differences (data not shown) were assigned to subclade 2 within a sister taxon to the three related species, *P. chrysea* Maharachch. & K.D. Hyde in Maharachchikumbura *et al.* (2012: 107), *P. umberspora* Maharachch. & K.D. Hyde in Maharachchikumbura *et al.* (2012: 121) and *P. asiatica* Maharachch. & K.D. Hyde in Maharachchikumbura *et al.* (2012: 104) with a credible bootstrap value (75%).

Taxonomy

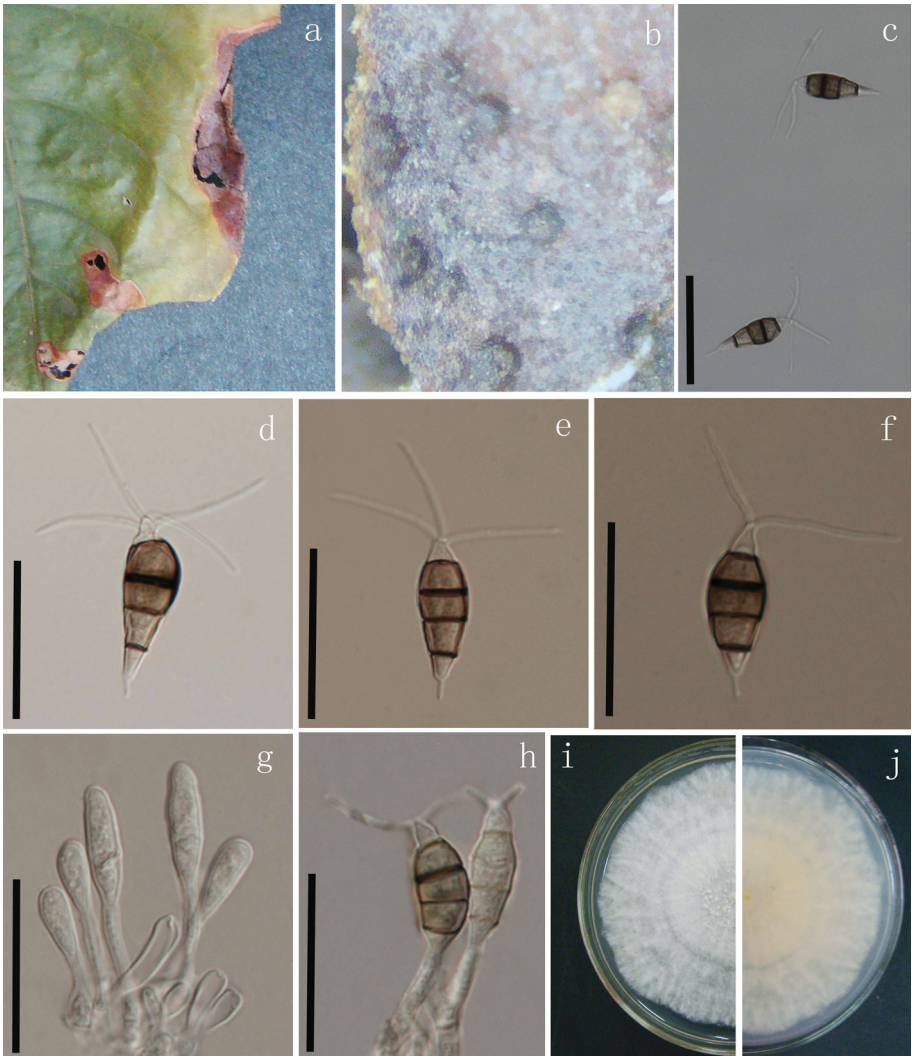

FIGURE 2. *Pestalotiopsis coffeae-arabicae* (holotype). a. Herbarium material—leaves of *Coffea arabica*. b. Conidiomata. c–f. Conidia with versicolorous median cells. g–h. Conidiophores/conidiogenous cells and developing conidia. i–j. Colony on PDA, i. from above, j. from below. Scale bars: c–h=20 µm.

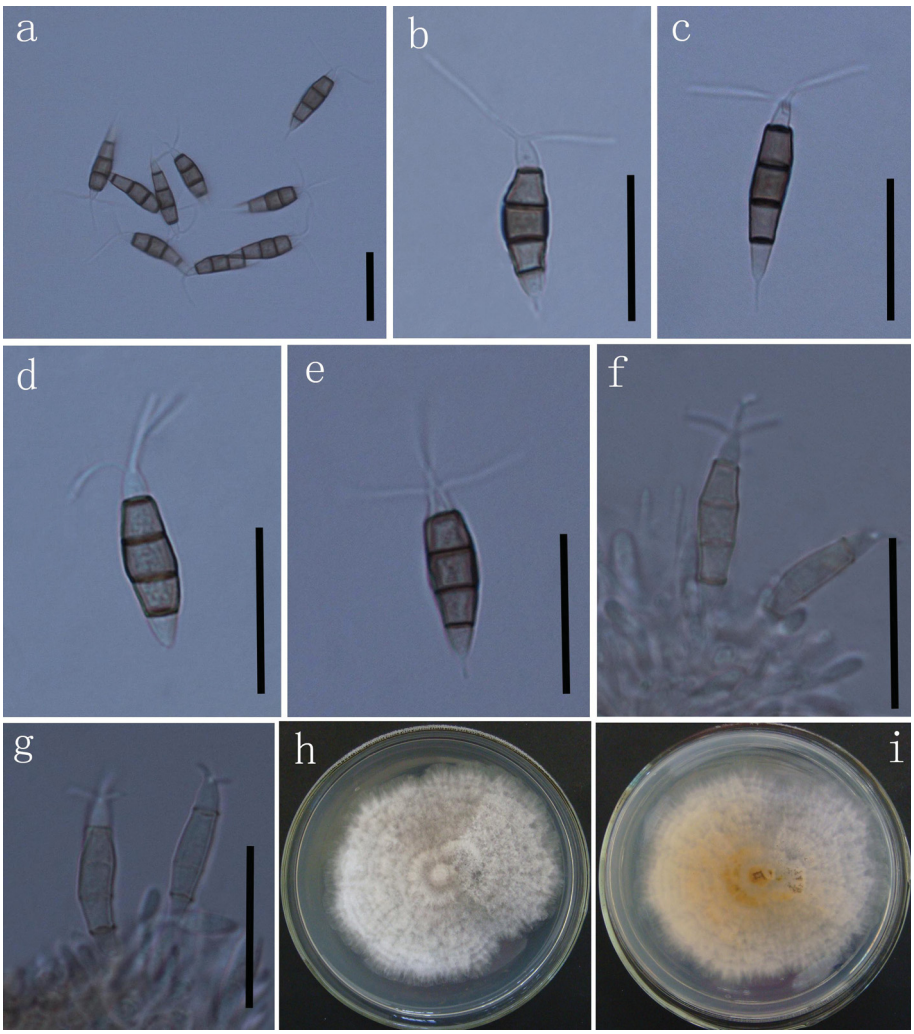

FIGURE 3. *Pestalotiopsis rhodomyrtus* (holotype). a–e. Conidia with concolorous median cells. f–g. Conidiophores/conidiogeous cells and developing conidia. h–i. Colony on PDA, h. from above, i. from below. Scale bars: a–g=20 µm.

Pestalotiopsis coffeae-arabicae Y. Song, K. Geng, K.D. Hyde & Yong Wang bis, *sp. nov.* (Fig. 2) MycoBank MB 804967

Differs from related *Pestalotiopsis* species mainly by its smaller conidia and shorter apical appendages.

Type:—CHINA. Hainan Province: Xinglong County, Tropical Botanical Garden, living leaves of *Coffea arabica*, 8 March 2012, *K. Geng* (HGUP4019, holotype!).

Associated with red-brown leaf spots on living leaves of *Coffea arabica*. Conidiophores usually indistinct. Conidiogenous cells discrete, ampulliform to lageniform, smooth, thin-walled, hyaline, with 2–3 proliferations. Conidia fusoid to ellipsoid, straight to slightly curved, 4-septate, 16–20 × 5–7 µm (n=30, $\bar{x}$ =18.6 × 6.4 µm); basal cell short, conic to obconic, hyaline, thin-walled and verruculose; three median cells 11.8–13.5 µm long (n=30, $\bar{x}$ =12.8 µm), dark brown, septa and periclinal walls darker than the rest of the cell, versicolorous; second cell from base pale brown, 3.5–4.5 µm (n=30, $\bar{x}$ =4.1 µm); third cell darker brown, 3.5–5µm (n=30, $\bar{x}$ =4.1µm); fourth cell darker, 3.7–4.5 µm (n=30, $\bar{x}$ =4 µm); apical cell 2.4–3.1 µm (n=30, $\bar{x}$ =2.7µm), hyaline, obconic to subcylindrical, with 2–4 appendages (mostly 3); apical appendages 11–16 µm long (n=30, $\bar{x}$ =13.5 µm), tubular, arising from the apex of the apical cell; basal appendage, 3–5 µm long (n=30, $\bar{x}$ =3.5 µm), filiform. Colonies on PDA reaching 7 cm diam. after 8 days at 25°C, with undulate edge, whitish, with aerial mycelium on surface; fruiting bodies black, gregarious; reverse of culture pale luteous.

Habitat/ Distribution:—Known to inhabit *Coffea arabica,* Hainan Province, China.

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

Other material examined:—CHINA. Hainan Province: Xinglong County, Tropical Botanical Garden, living leaves of *Coffea arabica*, 8 March 2012, *K. Geng* (HGUP4015!).

Pestalotiopsis rhodomyrtus Y. Song, K. Geng, K.D. Hyde & Yong Wang bis, *sp. nov.* (Fig. 3) MycoBank MB 804968

Differs from *Pestalotiopsis rosea* and *P. adusta* by bigger conidia and its apical appendages, which are shorter than those of *P. trachicarpicola* and *P. rosea*.

Type:—CHINA. Guangxi Province: Liangfeng River National Forest Park, isolated from leaves of *Rhodomyrtus tomentosa*, 2011, *J.G. Wei* (HGUP4230, holotype!).

Conidiophores indistinct. Conidiogenous cells discrete, hyaline, simple, filiform. Conidia 19.7–26.3 × 4.9–6.7 µm (n=30, $\bar{x}$=23.00 × 5.76 µm), fusoid, straight to slightly curved, 4-septate; basal cell conic to obconic, hyaline or pale brown, smooth, thin-walled, 3.6–6.4 µm long (n=30, $\bar{x}$=4.79 µm); three median cells 12.9–16.8 µm long (n=30, $\bar{x}$=15.04 µm), brown, septa and periclinal walls darker than the rest of the cell, concolorous, verruculous; second cell from base 4.3–5.8 µm long (n=30, $\bar{x}$=5.12 µm); third cell from base 3.5–5.7 µm long (n=30, $\bar{x}$=4.73 µm); fourth cell from base 4.3–5.6 µm long (n=30, $\bar{x}$=4.88 µm); apical cell hyaline, obconic to subcylindrical, 2.7–4.1 µm long (n=30, $\bar{x}$=3.56 µm); with 2–3 tubular appendages, arising from the apex of the apical cell, 7.5–14.9 µm long (n=30, $\bar{x}$=10.54 µm), unequal; one basal appendage present, 2.8–4.9 µm long (n=30, $\bar{x}$=3.65 µm), filiform.

Colonies on PDA reaching 7 cm diam. after 8 days at 25°C, with edge crenate, whitish, aerial mycelia on surface, fruiting bodies black, gregarious; reverse of colony pale orange.

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

Habitat/Distribution:—Known to inhabit *Rhodomyrtus tomentosa*, Guangxi Province, China.

Discussion

It is important to clarify relationships amongst *Pestalotiopsis* species as they are not only weak pathogens, but also prolific produces of novel medicinal compounds (Aly *et al*. 2011, Ko Ko *et al*. 2011a, b, Xu *et al*. 2010, Debbab *et al*. 2011, 2012). *Pestalotiopsis coffeae* (Zimm) Y.X. Chen in Chen & Wei (1993: 25), *P. neglecta* (Thüm.) Steyaert (1953: 83; IMI 296896), *P. disseminata* (Thüm.) Steyaert (1949: 319; IMI 281746) and *P. versicolor* (Speg.) Steyaert (1949: 336; IMI 146181) have been reported isolated from *Coffea arabica* (Chen & Wei 1993, http://www.herbimi.info/herbimi/). However, *P. coffeae-arabicae* produced obviously shorter apical and basal appendages (11–16 µm, 3–5 µm) than *P. coffeae* (21.3–25.0 µm, 6.3–8.8 µm) (Ge *et al.* 2009). The conidia of *P. coffeae-arabicae* (16–27.1 × 6.6–7.6 µm) were smaller than those of *P. neglecta* (20–27.1 × 5.9–7.1 µm) and *P. disseminata* (18.5–27.1 × 5.9–7.1 µm), and *P. versicolor* (21.2–28.3 × 7.1–8.3 µm) (Ge *et al.* 2009). In the phylogenetic tree (Fig. 1), *P. coffeae-arabicae* displayed a closer relationship with *P. chrysea*, *P. umberspora* and *P. asiatica*. Their conidial shape and the colour of median cell were also very similar (Maharachchikumbura *et al*. 2012, 2013b). The conidia of *P. coffeae-arabicae* are smaller than those of *P. chrysea*, *P. umberspora* and *P. asiatica* (Maharachchikumbura *et al*. 2012, 2013b). The apical appendages of *P. coffeae-arabicae* are shorter than these three related species. Conidia of *P. rhodomyrtus* are similar in shape to those of *P. trachicarpicola*, *P. rosea* and *P. adusta*. However, the conidia of *P. rhodomyrtus* are bigger than those of *P. rosea* and *P. adusta*. The apical appendages of *P. rhodomyrtus* are shorter than those of *P. trachicarpicola* and *P. rosea*. *Pestalotiopsis rhodomyrtus* possess only one basal appendage, but *P. trachicarpicola* rarely has two, and the length of their basal appendage is different. Phylogenetic analysis also indicated that *P. coffeae-arabicae* and *P. rhodomyrtus* were distinct new species. A detailed morphological comparison of the new species with related species is shown in Table 2.

TABLE 2. Morphological comparison of new taxa and related *Pestalotiopsis* species.

	Conidia			Appendages	
Species	Shape	Size (µm)	Median cells	Apical appendages	Basal appendage
P. rhodomyrtus	fusoid, straight to slightly curved	19.7–26.3 × 4.9–6.7	concolorous	2–3, tubular, 7.5–14.9 µm long	2.8–4.9 µm long, filiform
P. trachicarpicola	fusoid to ellipsoid, broad-clavate, straight to slightly curved	20–25 × 5.5–7.2	concolorous	2–3, tubular, 9–18 µm long	1 rarely 2, 4–8 µm long
P. rosea	fusoid to ellipsoid, straight to slightly curved	17.5–21.8 × 5.7–7	concolorous	1–3, tubular, some branched, 14–22 µm long	2–5.7 µm long, rarely absent
P. adusta	fusiform to ellipsoid, straight to slightly curved	16–20 × 5–7	concolorous	2–3, 6–14 µm long	filiform
P. coffeae-arabicae	fusoid to ellipsoid, straight to slightly curved	16–20 × 5–7	versicolorous	2–4 (mostly 3), tubular, 11–16 µm long	3–5 µm long, filiform
P. chrysea	fusiform, straight to slightly curved	20–24 × 5.5–7	versicolorous	3, tubular, 22–30µm long	3–6 µm long, filiform
P. umberspora	fusiform, straight to slightly curved	19–25 × 6–8	versicolorous	1–3 (mainly 3), tubular, 22–35 µm long	5–7 µm, filiform
P. asiatica	fusiform, straight to slightly curved	20–26 × 5–7	versicolorous	2–4 (mainly 3), tubular, 20–30 µm long	4–8 µm long, filiform

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