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ORIGINAL ARTICLE

Improving the backbone tree for the genus *Pestalotiopsis*; addition of *P. steyaertii* and *P. magna* sp. nov.

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Abstract A novel, saprobic *Pestalotiopsis* species isolated from the decaying leaves of *Pteridium* sp. collected in France is described as *Pestalotiopsis magna*. The novelty of the species is confirmed based on phenotypic analyses of conidial characters and phylogenetic analyses of sequence data. *Pestalotiopsis magna* can also be distinguished from similar and related species by its larger conidia. Phylogenetic species recognition, based on combined, multilocus alignment of the internal transcribed spacer (ITS), partial β-tubulin, and partial translation elongation factor 1-alpha (*tef1*), strongly supported the monophyly of *P. magna* with relation to other versicolorous species. The ex-type culture of *P. steyaertii* was also sequenced and placed in the backbone tree for *Pestalotiopsis*.

Keywords New species · Phylogeny · Saprobe

Introduction

Pestalotiopsis Steyaert (1949) is an appendage-bearing, conidial, asexual fungus (coelomycetes) in the family *Amphisphaeriaceae* (Barr 1975, 1990; Kang et al. 1998), and is common in tropical and temperate ecosystems (Bate-Smith and Metcalfe 1957). Species of *Pestalotiopsis* cause a variety of diseases in plants (Maharachchikumbura et al. 2013a, b, c; Zhang et al. 2012a, b) and are also often isolated as endophytes (Xu et al. 2010; Maharachchikumbura et al. 2012; Debbab et al. 2013). They are not highly host-specific, and their taxa may have the ability to infect a range of hosts (Hopkins and McQuilken 2000). Due to their ability to switch life-modes, many endophytic and pathogenic *Pestalotiopsis* species persist as saprobes (Hu et al. 2007; Maharachchikumbura et al. 2012) and have been isolated from dead leaves, bark and twigs (Guba 1961; Maharachchikumbura et al. 2012). Several species have been recovered from soil, polluted stream water, wood, paper, fabrics, and wool (Guba 1961).

Pestalotiopsis consists of around 250 species, most of which were named according to their host associations (Guba 1961; Steyaert 1949; Kohlmeyer and Kohlmeyer 2001). However, molecular data has shown that the genus needs revision (Maharachchikumbura et al. 2011, 2012; Zhang et al. 2013), and many of the traditional species may be spurious. This calls for critical re-examination of the genus, using both morphological studies and a multigene phylogeny based on ex-type and ex-epitype cultures (Maharachchikumbura et al. 2012, 2013a).

The current paper aims to provide a complete morphological and molecular characterization of *P. magna*, a new *Pestalotiopsis* species isolated as a saprobe from dead fern leaves in France. We also re-examined and sequenced an ex-type culture of *P. steyaertii* Mordue, and provide here a description and sequence data for this species, thereby strengthening the backbone tree for *Pestalotiopsis* at the species level.

Materials and methods

Isolation and identification

Decaying Bracken (*Pteridium* sp.) leaves were collected from Rimont village, France in August 2011. The isolation of *P.*

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magna followed the methods used by Maharachchikumbura et al. (2012). The ex-type culture of *P. steyaertii* (IMI 192475) was obtained from the Centre for Agricultural Bioscience International (CABI), and cultured on potato dextrose agar and autoclaved pine needles on synthetic nutrient-poor agar (PNA) (Crous et al. 2006) at room temperature (25 °C). The morphology of fungal colonies was recorded according to the method used by Maharachchikumbura et al (2012). Fungal mycelium and spores were observed under the light microscope and photographed. All microscopic measurements were measured with Tarosoft image framework (v. 0.9.0.7), and with 30 conidial, and 15 conidioma and conidiogenous cell measurements. The fungal strains that were used for this study are listed in Table 1.

Molecular phylogeny

DNA extraction, PCR amplification, and DNA sequencing

Total genomic DNA was extracted from fresh fungal mycelia (500 mg), scraped from the margin of a colony on a PDA plate incubated at 25 °C for 7–10 days (Guo et al. 2000). The ITS, β-tubulin and *tef1* genes were amplified using primer pairs ITS4/ITS5 (White et al. 1990), BT2A/BT2B (Glass and Donaldson 1995; O'Donnell and Cigelnik 1997), and EF1-526F or EF728F/EF1-1567R or EF2 (Carbone and Kohn 1999; O'Donnell et al. 1998; Rehner 2001). Polymerase chain reaction (PCR) was performed with the 25-μl reaction system consisting of 19.5 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 of Taq DNA polymerase (5 U/μl), and 1.0 μl of DNA template. The thermal cycling program followed Maharachchikumbura et al (2012).

Phylogenetic analysis

Sequences were optimized manually to allow maximum alignment and maximum sequence similarity, as detailed in Maharachchikumbura et al (2012) (Table 1). A maximum likelihood analyses was performed with an Apple-Mac computer using user-friendly, graphical, front-end software, raxmlGUI version 1.3 (Silvestro and Michalak 2011). The optimal ML tree search was conducted with 100 separate runs, using the default algorithm of the program from a random starting tree for each run. The final tree was selected among suboptimal trees from each run by comparing likelihood scores under the GTRGAMMA substitution model.

In addition, Bayesian Analyses (BA) were performed using MrBAYES 3.1.2 (Huelsenbeck and Ronquist 2001). Suitable models were first selected using models of nucleotide substitution for each gene, as determined using MrModeltest (Nylander 2004), and included for each gene partition. The GTR+I+G model was selected for ITS and the HKY+I+G for β-tubulin and *tef1*, and these were incorporated into the analysis. The analyses of four Markov Chain Monte Carlo (MCMC) chains were run from random trees for 100,000,000 generations and sampled every 1,000 generations. The temperature value was lowered to 0.15, burn-in was set to 0.25, and the run was automatically stopped as soon as the average standard deviation of split frequencies reached below 0.01. The resulting trees were printed with FigTree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/). Sequences generated in this study were deposited at GenBank, while the alignments and trees were deposited in TreeBASE (www.treebase.org/treebase/index.html).

Results

Phylogenetic analysis

The combined dataset of ITS, β-tubulin, and *tef1* contained 45 strains representing 29 taxa of *Pestalotiopsis* with *Seiridium* sp. as the outgroup, and consisted of 1,633 total characters, including gaps. Bayesian analysis resulted in a tree with largely the same topology and clades as the RAxML tree. The *P. steyaertii* strain formed a distinct, well-supported clade separate from the currently recognized species in the versicolorous clade. The new species, *P. magna*, clustered as an outlying species in the versicolorous clade with high branch-length support (Fig. 1).

Taxonomy

Pestalotiopsis magna Maharach. & K.D. Hyde, sp. nov. Fig. 2a–j.

MycoBank: MB 805405

Etymology: The specific epithet is based on the larger size of the conidia compared to most species in the versicolour clade, and the Latin word for large is *magnus*.

Holotype: MFLU 13-0594

Description

Saprobic on decaying leaves. Sexual state: unknown. Asexual state: *conidiomata* 200–400 μm diam, pycnidial, globose, brown, semi-immersed on PDA releasing black conidia in a slimy, globose, glistening mass. *Conidiophores* indistinct. *Conidiogenous cells* discrete to lageniform, hyaline, smooth and thin-walled, 3–8×2–6 μm, proliferating 1–2 times percurrently, collarette present and not flared. *Conidia* (40)42–46(47)×(9)9.5–12 μm (mean ± SD=44.1±1.4×11.0±0.6 μm), fusiform to clavate, straight to slightly curved, 4-septate; basal cell obconic with a truncate base, hyaline or sometimes pale brown, thin- and smooth-walled, 8.5–9 μm long; three median cells (30)31–33.5(34) μm long (mean ± SD=31.8±1.4 μm), brown, septa and periclinal walls darker than rest of the cell, versicolored,

Table 1 Isolates used in this study

Taxa	Isolates	GenBank Accession Number		
		ITS	β-tubulin	*tef1*
P. adusta (Ellis & Everh.) Steyaert	ICMP 6088	JX399006	JX399037	JX399070
P. adusta	MFLUCC10-146	JX399007	JX399038	JX399071
P. anacardiacearum Y.M. Zhang, Maharachch. & K.D. Hyde	IFRDCC 2397	KC247154	KC247155	KC247156
P. asiatica Maharachch. & K.D. Hyde	MFLUCC 12-0286	JX398983	JX399018	JX399049
P. camelliae Y.M. Zhang, Maharachch. & K.D. Hyde	MFLUCC 12-0277	JX399010	JX399041	JX399074
P. camelliae	MFLUCC 12-0278	JX399011	JX399042	JX399075
P. chinensis Maharachch. & K.D. Hyde	MFLUCC 12-0273	JX398995	–	–
P. chrysea Maharachch. & K.D. Hyde	MFLUCC 12-0261	JX398985	JX399020	JX399051
P. chrysea	MFLUCC 12-0262	JX398986	JX399021	JX399052
P. clavata Maharachch. & K.D. Hyde	MFLUCC 12-0268	JX398990	JX399025	JX399056
P. clavispora (G.F. Atk.) Steyaert	IFRDCC 2391	KC537808	KC537822	KC537815
P. clavispora	MFLUCC 12-0280	JX398978	JX399013	JX399044
P. clavispora	MFLUCC 12-0281	JX398979	JX399014	JX399045
P. diversiseta Maharachch. & K.D. Hyde	MFLUCC 12-0287	JX399009	JX399040	JX399073
P. ellipsospora Maharachch. & K.D. Hyde	MFLUCC 12-0283	JX399016	JX399016	JX399047
P. ellipsospora	MFLUCC 12-0284	JX399015	JX399015	JX399046
P. ericacearum Y.M. Zhang, Maharachch. & K.D. Hyde	IFRDCC 2439	KC537807	KC537821	KC537814
P. foedans (Sacc. & Ellis) Steyaert	CGMCC 3.9178	JX398989	JX399024	JX399055
P. foedans	CGMCC 3.9123	JX398987	JX399022	JX399053
P. foedans	CGMCC 3.9202	JX398988	JX399023	JX399054
P. furcata Maharachch. & K.D. Hyde	MFLUCC 12-0054	JQ683724	JQ683708	JQ683740
P. gaultheria Y.M. Zhang, Maharachch. & K.D. Hyde	IFRD 411-014	KC537805	KC537819	KC537812
P. inflexa Maharachch. & K.D. Hyde	MFLUCC 12-0270	JX399008	JX399039	JX399072
P. karstenii (Sacc. & P. Syd.) Steyaert	IFRDCC OP13	KC537806	KC537820	KC537813
P. intermedia Maharachch. & K.D. Hyde	MFLUCC 12-0259	JX398993	JX399028	JX399059
P. linearis Maharachch. & K.D. Hyde	MFLUCC 12-0271	JX398992	JX399027	JX399058
P. magna Maharachch. & K.D. Hyde	MFLUCC 12-652	KF582795	KF582793	KF582791
P. rhododendri Y.M. Zhang, Maharachch. & K.D. Hyde	IFRDCC 2399	KC537804	KC537818	KC537811
P. rosea Maharachch. & K.D. Hyde	MFLUCC12-0258	JX399005	JX399036	JX399069
P. samarangensis Maharachch. & K.D. Hyde	MFLUCC 12-0233	JQ968609	JQ968610	JQ968611
P. saprophyta Maharachch. & K.D. Hyde	MFLUCC 12-0282	JX398982	JX399017	JX399048
P. steyaertii	IMI 192475	KF582796	KF582794	KF582792
P. theae (Sawada) Steyaert	MFLUCC12-0055	JQ683727	JQ683711	JQ683743
P. theae	SC011	JQ683726	JQ683710	JQ683742
P. trachicarpicola Y.M. Zhang & K.D. Hyde	MFLUCC 12-0263	JX399000	JX399031	JX399064
P. trachicarpicola	MFLUCC 12-0264	JX399004	JX399035	JX399068
P. trachicarpicola	MFLUCC 12-0265	JX399003	JX399034	JX399067
P. trachicarpicola	MFLUCC 12-0266	JX399002	JX399033	JX399066
P. trachicarpicola	MFLUCC 12-0267	JX399001	JX399032	JX399065
P. trachicarpicola	IFRDCC 2403	KC537809	KC537823	KC537816
P. trachicarpicola	OP068	JQ845947	JQ845945	JQ845946
P. umberspora Maharachch. & K.D. Hyde	MFLUCC 12-0285	JX398984	JX399019	JX399050
P. unicolor Maharachch. & K.D. Hyde	MFLUCC 12-0275	JX398998	JX399029	JX399063
P. unicolor	MFLUCC 12-0276	JX398999	JX399030	–
P. verruculosa Maharachch. & K.D. Hyde	MFLUCC 12-0274	JX398996	–	JX399061
Seiridium sp.	SD096	JQ683725	JQ683709	JQ683741

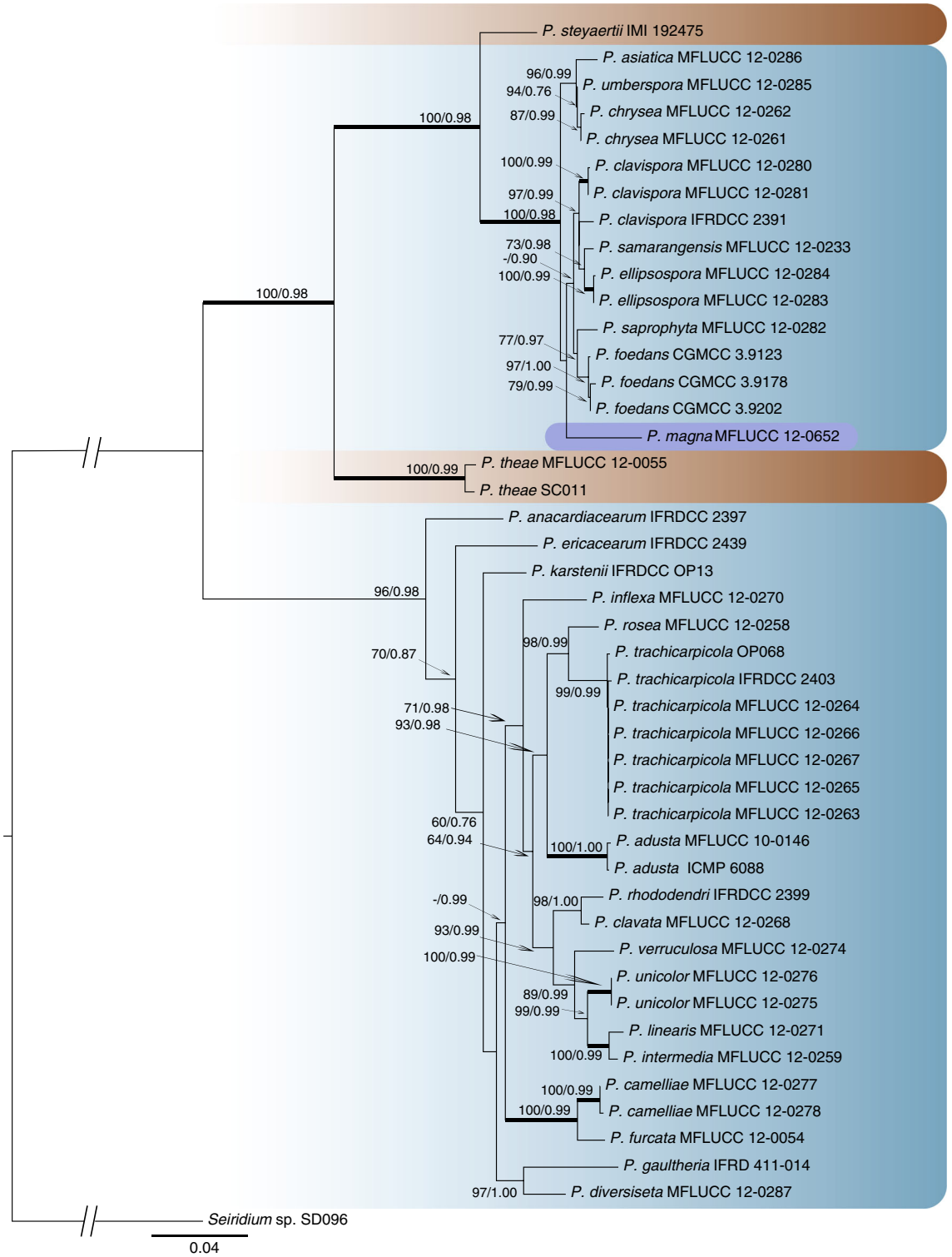

Fig. 1 Maximum likelihood (ML) majority rule consensus tree for the analyzed *Pestalotiopsis* isolates. RAxML bootstrap support values (ML) and Bayesian posterior probabilities (PP) are given at the nodes (ML/PP). Thickened lines indicate a ML of 100. The tree was rooted to *Seiridium* sp.

wall rugose; second cell from base pale brown, 9.5–11.5 μm long; third cell brown, 9.5–11 μm long; fourth cell brown, 10.5–12 μm long; apical cell 5–8 μm long, hyaline, conic to acute; with 2–4 tubular appendages on apical cell, inserted at different loci but in a crest at the apex of the apical cell, unbranched, flexuous, (10)16–26(30) μm long (mean ± SD=23.2±4.2 μm); single basal appendage, tubular, unbranched, centric, 11–15 μm long.

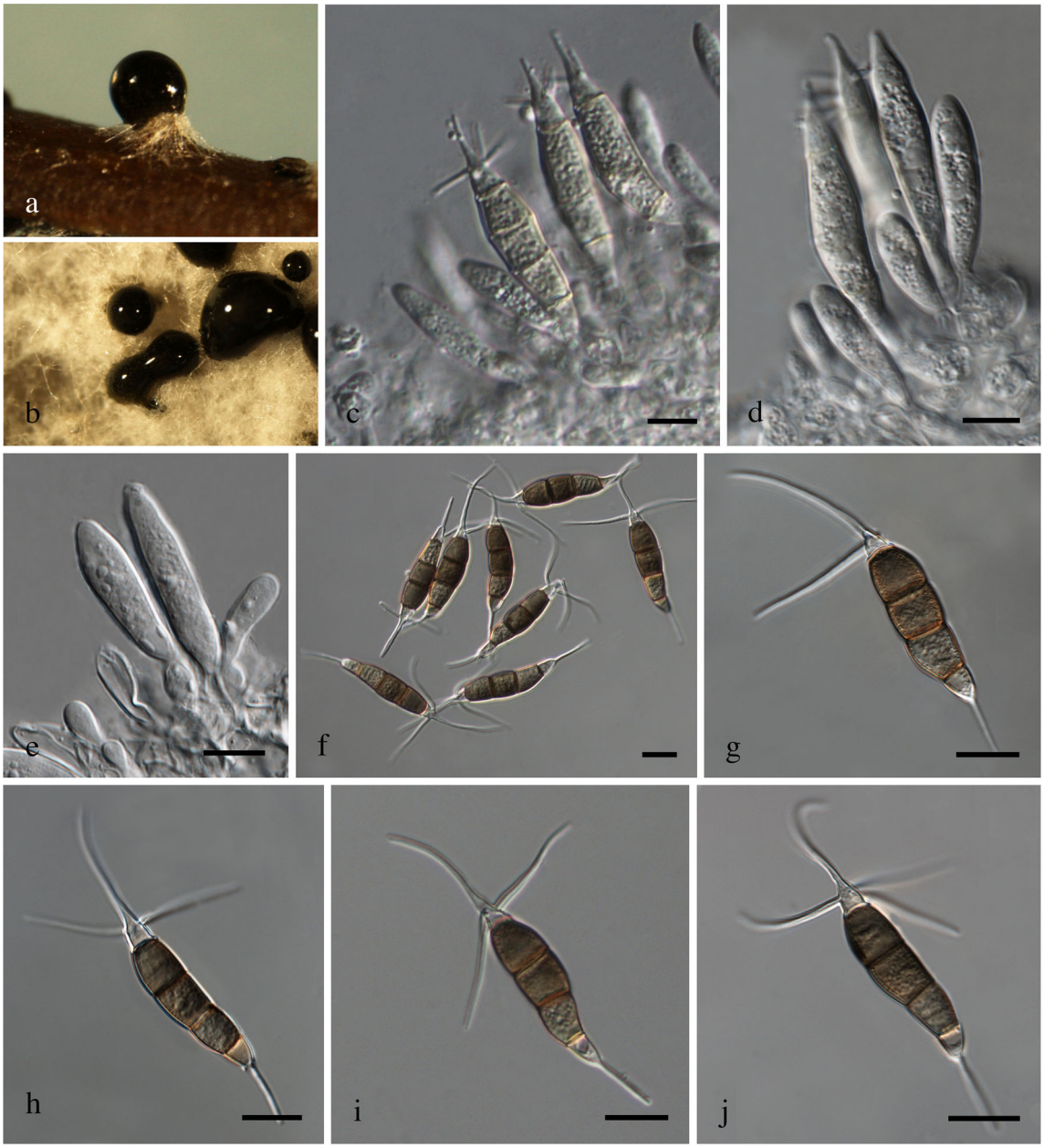

Fig. 2 *Pestalotiopsis magna* (MFLUCC 12-0652). **a** Conidioma on Water Agar with sterile pine needles. **b** Conidiomata on PDA. **c–e** Conidiogenous cells and developing conidia. **f–j** Conidia. Scale bars=10 μm

Colonies fast growing on PDA, attaining 50–70 mm diam after 7 days at 25 °C, edge entire, yellowish white, dense, aerial mycelium on surface, fruiting bodies black; reverse similar in color.

Holotype: France, Ariège, Rimont, on decaying leaves of *Pteridium* sp., Aug 2011, coll. K.D. Hyde, isol. S.S.N. Maharachchikumbura, (MFLU 13-0594 holotype); ex-type living culture = MFLUCC 12-0652.

Notes: *Pestalotiopsis magna* is an outlying species in the versicolorous clade and is distinguished from related species by its larger conidia. The morphologically overlapping species in conidial size are *P. grandis* Dube & Bilgrami (26–48×7–8 μm), *P. hughessii* Steyaert (34–45×7–11 μm), *P. kunmingensis* J.G. Wei & T. Xu (33–47×7.5–10 μm), *P. macrospora* (Ces.) Steyaert (30–45×9–12 μm) and *P. montellicoides* (Doyer) Steyaert (35–48 7.5–10.6 μm) (Steyaert 1949, 1953; Guba 1961; Dube and Bilgrami 1966). However, with the exception of *P. kunmingensis*, the three median cells in all of the above species are concolorous, in contrast to versicolorous in *P. magna*. Molecular data shows that *P. kunmingensis* clusters in the concolorous group (Maharachchikumbura et al. 2012, 2013a) and apical appendages in *P. magna* are not knobbed, like those in *P. kunmingensis*.

Pestalotiopsis steyaertii Mordue, Trans. Br. mycol. Soc. 85(2): 379 (1985)

Description from ex-type culture (Fig. 3a–m)

Description

Saprobic on soil. Sexual state: unknown. Asexual state: *Conidiomata* 300–500 μm diam, pycnidial, globose, brown, semi-immersed on PDA releasing black conidia in a slimy, globose, glistening mass. *Conidiophores* septate at base, branched, colorless, smooth-walled. *Conidiogenous cells* discrete or integrated, short cylindric, hyaline, 5–12×2–4 μm. *Conidia* (25)27–34×7–9.5(10) μm, mean ± SD=30.1±2.2×8.0±0.5 μm, fusiform to clavate, straight to curved, 4-septate; basal cell conical to cylindric, hyaline or pale olivaceous, thin and walled-verruculose, 6–8 μm long; three median cells (16)18–23(25) μm long, mean ± SD=22.1±2.1 μm olivaceous, septa and periclinal walls darker than rest of the cell, versicolored, walled-verruculose; second cell from base pale olivaceous, 6–8 μm long; third cell dark olivaceous, 7–9 μm; fourth cell darker, 6–9 μm; apical cell 6–8 μm long, hyaline or pale olivaceous, conic to hemispherical; apical appendages mostly absent, when present 1–5 tubular appendages on apical cell, inserted at different loci but in a crest at the apex of the apical cell, unbranched, flexuous, (17)20–31(34) μm long, mean ± SD=25.2±3.4 μm; basal appendage mostly absent, when present single, tubular, unbranched, centric, 2–6 μm long.

Colonies fast growing on PDA, attaining 50–60 mm diam after 7 days at 25 °C, edge entire, white, dense aerial mycelium on surface, fruiting bodies black, concentric; reverse similar in color.

Notes: *Pestalotiopsis steyaertii* is a distinct species in terms of its morphology and DNA phylogeny. This species is characterized by its unusual conidial shape. According to Mordue's (1985) observations, most of the isolates of *P. steyaertii* lack apical appendages in conidia. We observed this in the ex-type culture. *P. steyaertii* forms

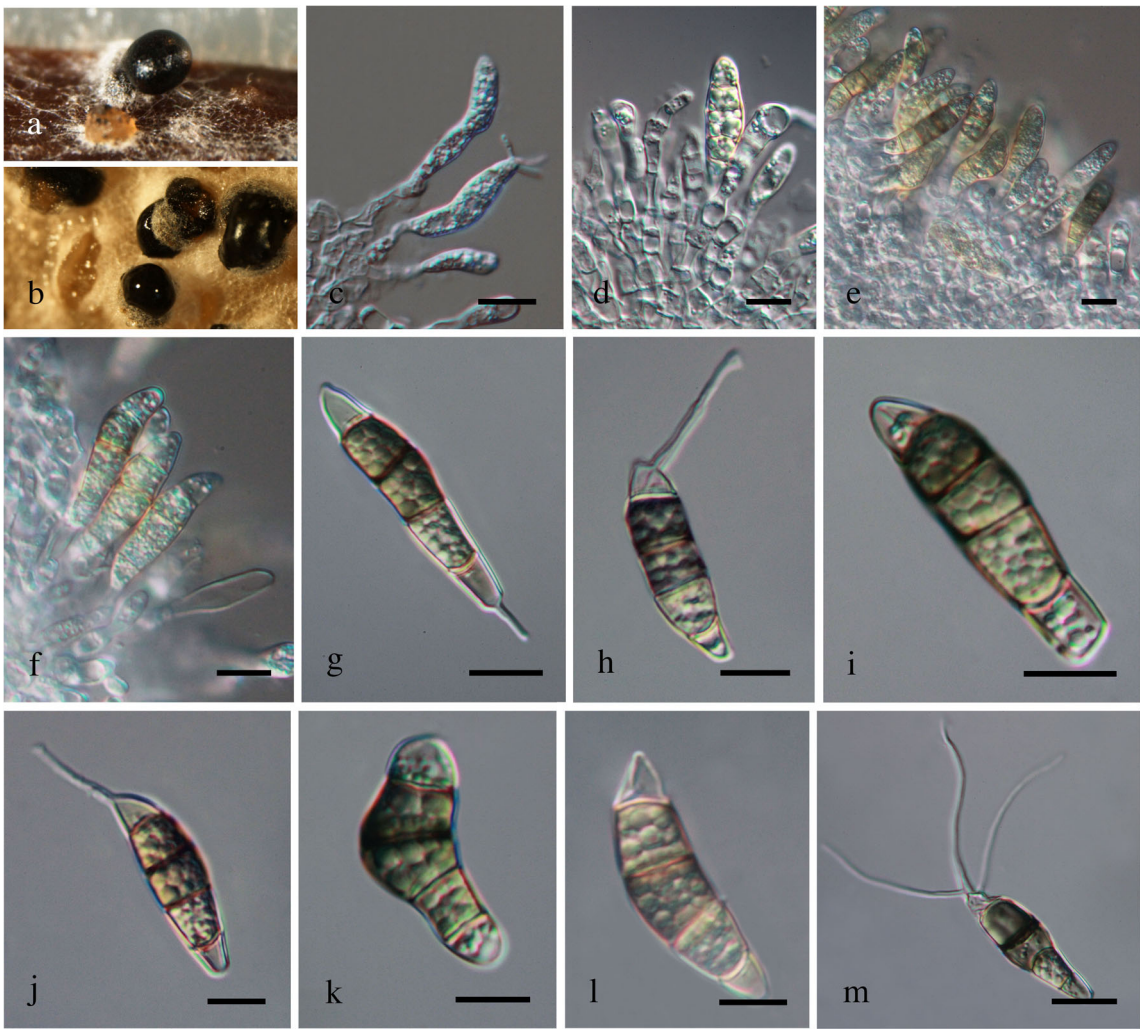

Fig. 3 *Pestalotiopsis steyaertii* IMI 192475. **a** Conidioma on water agar with sterile pine needles. **b** Conidiomata on PDA. **c–f** Conidiogenous cells and developing conidia. **g–m** Conidia. Scale bars=10 μm

a sister group to species having versicolorous median cells and dark concolorous median cells with knobbed apical appendages.

Discussion

Following the discovery of the multimillion-dollar, anti-cancer drug taxol from *P. microspora* (Speg.) G.C. Zhao & N. Li, isolated from *Taxus wallachiana* (Strobel et al. 1996), the importance of the genus *Pestalotiopsis* has increased considerably (Strobel et al. 2002; Xu et al. 2010). Unfortunately, a recent study has shown that taxol production by endophytes is highly unlikely (Heinig et al. 2013). Thus, the trend to look for medicinal metabolite production by endophytes of medicinal plants, which resulted from the Strobel et al. (1996) publication, may have been misguided. There are various reports that *Pestalotiopsis* species produce a diverse array of chemical compounds (Xu et al. 2010), even though the names assigned to the respective taxa lack any taxonomic basis since none of those studies were based on types.

In this study, one new species, *Pestalotiopsis magna* (from the decaying leaves of *Pteridium* sp. from Rimont, Ariège, France), and the ex-type culture of *P. steyaertii* were characterized in terms of morphology and phylogeny. When species are morphologically distinct and molecular evidence shows that they are monophyletic, such species can then be considered as a distinct and valid species in a particular genus (Maharachchikumbura et al. 2011). Both *P. magna* and *P. steyaertii* have distinct morphological characters and separate well in the phylogenetic analysis. *Pestalotiopsis steyaertii* is not a typical member of the genus that is characterized by versicolorous median cells, since it forms unusually-shaped conidia with mostly lacking apical and basal appendages.

Conidial characters are a decisive character in distinguishing *Pestalotiopsis* species; however, host association and geographical location is less informative (Jeewon et al. 2003; Maharachchikumbura et al. 2011). More recently, some new species have been introduced based on morphological and molecular data. Including the two newly-generated, ex-type sequences in the present study, currently 31 *Pestalotiopsis* species have either ex-type or ex-epitype sequences. There are many unusual species in the genus that need re-examination, and we believe that many distinct, well-separated groups will arise from such studies. Through this further research, we can begin to understand the relationships among species in the genus.

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