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Three new species of *Entoloma* subgenus *Pouzarella* from China based on morphological and molecular data

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Abstract

In the present paper, three additional species of *Entoloma* subgenus *Pouzarella* viz. *E. erectoides, E. griseocarpum* and *E. rubropilosum* are described from China. *E. rubropilosum* is a typical species in section *Pouzarella*; *E. griseocarpum* and *E. erectoides* are members of sect. *Dysthales*. The taxa are further confirmed by ITS, RPB2, LSU and mtSSU analyses and phylogenetic relationships with other *Entoloma* subgen. *Pouzarella* species are also discussed. ITS sequence analysis showed that the sizes of the entire ITS region and ITS1 are remarkably divergent, while the ITS2 is conserved in length within *Entoloma* subgen. *Pouzarella*. Molecular analyses, based on the combined dataset, demonstrated that species diversity of subgen. *Pouzarella* in China is much higher than previously thought, in the present study twenty phylogenetic species from China are taken into consideration. On the other hand, morphological and molecular analyses suggested that classification of *Entoloma* subgen. *Pouzarella* probably has to be fundamentally re-adjusted based on additional data.

Keywords

Entolomataceae, systematics, taxonomy, multi-gene analyses

Introduction

Pouzarella Mazzer is a distinctive group of entolomatoid species that was accepted as a genus by some researchers (Horak 2008; Largent 1994; Mazzer 1976). Others consider it as a subgenus of *Entoloma* P. Kumm. (Noordeloos 1979, 1992, 2004; Noordeloos

and Gates 2012). Recent molecular phylogenetic studies (Co-David et al. 2009; Baroni and Matheny 2011; He et al. 2013; Largent and Bergemann 2015) based on multiloci showed that *Pouzarella* actually represent a distinct monophyletic group separated from the other entolomatoid groups. In addition, taxa of *Pouzarella* are easily recognised both by macro- and micromorphological characters. However, here we still treat it as a subgneus of *Entoloma* s.l. because accepting *Pouzarella* as a separate genus would make *Entoloma* s.l. paraphyletic. Taxonomical revision of other well supported, distinct clades of *Entoloma* s.l. is therefore needed before a formal decision on the generic status of *Pouzarella* can be made. When such a revision is achieved, we predict, that *Pouzarella* will be a well-defined genus based on morphological and molecular evidence. In the field, species of *Entoloma* subgen. *Pouzarella* are often overlooked due to their small and dull-coloured basidiomes. However, the inconspicuous species are widespread and have been reported from tropical to temperate regions. So far, more than seventy species have been described worldwide (Mazzer 1976; Horak 1980, 1983, 2008; Desjardin and Baroni 1991; Noordeloos 1992, 2004; Noordeloos et al. 1992; Manimohan et al. 1995; Baroni and Ortiz 2002; Manimohan et al. 2006; Karstedt et al. 2007; Baroni et al. 2008; Largent et al. 2011; Baroni et al. 2012; Noordeloos and Gates 2012; He et al. 2013; Largent and Bergemann 2015; Raj and Manimohan 2017).

Basidiomes of members in subgen. *Pouzarella* are easy to recognise. However, many species have in common small basidiome size and greyish colours and, therefore, it is difficult to distinguish them to species by morphological characters only. Accordingly, both morphological and molecular data are needed to refine the species concept and understand the diversity of these small agarics in *Entoloma* subgen. *Pouzarella*.

In previous studies, seven species of *Entoloma* subgen. *Pouzarella* were reported to occur in China (Ying 1995; He et al. 2013). However, we believe that subgen. *Pouzarella* remains poorly understood in this region, rich in many diverse ecological habitats. In continuation of previous surveys, further field work was carried out in southwest and northeast China. More than 50 samples matching the concept of *Entoloma* subgen. *Pouzarella* were collected and many turned out to be different from the locally already known species. As a first step, three distinctive new taxa are described in the present study whereas the other specimens were shelved for the moment because of scarcity of material. To further confirm the three new taxa and infer the affinities amongst representative species of *Entoloma* subgen. *Pouzarella*, phylogenetic analysis was carried out based on the combination of ITS, RPB2, mtSSU and nLSU sequences.

Materials and methods

Morphological descriptions

Fresh basidiomes were photographed in the field and described macroscopically. Colour notations follow Kornerup and Wanscher (1978). Microscopic examination was done using a Leica DM5000B microscope. Basidiospores, basidia and pileipellis were

mounted and measured in 5% potassium hydroxide (KOH) and/or 1% Congo Red. Pigmentation of the micro-structures was observed in distilled water. Measurements of the basidiospores excluded hilar appendix (apiculus) and at least 30 basidiospores of each specimen were measured. Q represents the length to width ratio of a basidiospore in profile view; ***Q*** represents the average Q of all basidiospores and is given ± standard deviation; *x* represents the means of basidiospore length and width ± standard deviation. All cited collections, including the holotypes, are deposited at the Mycological Herbarium of Soil and Fertilizer Institute, Sichuan Academy of Agricultural Sciences (SAAS), Chengdu, China and the Herbarium ZT of ETH Zurich, Switzerland.

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted with Biospin Fungus Genomic DNA Extraction Kit following the manufacturer's instructions. PCR amplification was performed using DreamTaq™ Green PCR Master qMix (2×), Fermentas. The primers for RPB2 amplification were rpb2-6F and rpb2-7R, rpb2-i6f and rpb2-i7r (Matheny 2005; Co-David et al. 2009). ITS regions were amplified with ITS5 and ITS4; LR0R and LR5, MS1 and MS2 were used for nLSU and mtSSU amplification, respectively (White et al. 1990; http://www.biology.duke.edu/fungi/mycolab/primers.htm).

Sequence alignment and phylogenetic analyses

Sequences used in phylogenetic analyses are listed in Table 1 and aligned in muscle 3.6 (Edgar 2004). The aligned sequences were manually modified where necessary in Mega 6.0 (Tamura et al. 2013). Phylogenetic analyses were based on the combined ITS, nLSU, RPB2 and mtSSU sequences. For the ITS region, only ITS1 and ITS2 were kept for further analyses. Conflicts between the ITS, nLSU, RPB2 and mtSSU datasets were evaluated by comparing the topologies resulting from the phylogenetic analysis of the single gene. As no conflict was detected amongst the well supported clades of the different trees, sequences of the four genes were combined for further analyses.

Maximum likelihood analysis – ML analysis was carried out by the web RAxML Version 8 (http://www.phylo.org/sub_sections/portal/) under the GTRGAMMAI model with 1000 bootstrap replicates (Stamatakis 2014). "Find best tree using maximum likelihood search" option was selected when analysis was undertaken.

Maximum parsimony analysis – MP analysis was performed using PAUP* version 4.0b10 (Swofford 2003). All characters were treated as unordered and of equal weight. Gaps were treated as missing data. Bootstrap values (BS) were obtained from 1000 replicates.

Bayesian analysis – Bayesian analysis was performed using MrBayes 3.2.6 (Ronquist and Huelsenbeck 2003). The best substitution models for each marker were selected using the Akaike Information Criterion (AIC) in jModelTest 2.1.7 (Darriba

Table 1. A list of taxa, specimens and GenBank accession numbers of sequences used in this study.

Taxa	Collection No.	Origin	GenBank accessions				Remarks
			ITS	LSU	RPB2	mtSSU	
Entoloma albostrigosum	DL Largent 9641	Australia: Queensland	–	HQ876535	HQ876513	HQ876557	GenBank ID: *Pouzarella albostrigosa*
	DL Largent 9663	Australia: Queensland	–	HQ876536	HQ876514	HQ876558	GenBank ID: *P. albostrigosa*
E. araneosum	ME Noordeloos 200314	China: Jilin	KC710056	GQ289153	GQ289225	GQ289293	
E. barringtonense	DL Largent 9901 (Holotype)	Australia: Queensland	–	HQ876524	HQ876543	HQ876546	GenBank ID: *P. parvula*
E. changchunense	HMJAU 3886 (Holotype)	China: Jilin	–	JQ993095	–	JQ993061	
E. crassicystidiatum	GDGM 28821	China: Guangdong	KC678997	JQ291567	JQ993085	JQ993058	
	GDGM 27357 (Holotype)	China: Guangdong	KC678996	JQ291569	JQ993083	JQ993056	
E. debile	DLL 9784	Australia: Queensland	–	HQ876528	HQ876506	HQ876550	GenBank ID: *P. debilis*
E. dindenense	DL Largent 9623	Australia: Queensland	–	HQ876527	HQ876505	HQ876549	GenBank ID: *P. fusca*
E. erectoides	SAAS 1232 (Holotype)	China: Sichuan	**MH020746**	**KU534255**	**KU534496**	–	
	SAAS 945	China: Sichuan	**MH020769**	**KU534239**	**KU534498**	–	
	SAAS 1361	China: Jilin	**MH020755**	–	**KU534484**	–	
E. farinosum	DL Largent 9934 (Holotype)	Australia: Queensland	–	HQ876516	HQ876495	HQ876538	GenBank ID: *P. farinosa*
	DL Largent 9900	Australia: Queensland	–	HQ876515	HQ876494	HQ876537	GenBank ID: *P. farinosa*
E. furfuraceum	GDGM 28818 (Holotype)	China: Jinlin	JX975293	JQ993094	JQ993084	JQ993062	
	SAAS 104	China: Jilin	–	KU534240	–	–	
E. griseocarpum	SAAS 1230	China: Tibet	**MH020753**	**KU534253**	**KU534500**	**KU534438**	
	SAAS 1328 (Holotype)	China: Tibet	**MH020766**	**KU534256**	**KU534501**	**KU534455**	
	SAAS 951	China: Sichuan	**MH020770**	**KU534242**	**KU534499**	**KU534457**	
E. lageniforme	DL Largent 9895	Australia: Queensland	–	HQ876523	HQ876502	HQ876545	GenBank ID: *P. lageniformis*
E. lasium	DL Largent 9662	Australia: Queensland	–	HQ876529	HQ876507	HQ876551	GenBank ID: *P. lasia*
	DL Largent 9670	Australia: Queensland	–	HQ876530	HQ876508	HQ876552	GenBank ID: *P. lasia*
	DL Largent 9807	Australia: Queensland	–	HQ876533	HQ876511	HQ876555	GenBank ID: *P. lasia*
	DL Largent 9811	Australia: Queensland	–	HQ876534	HQ876512	HQ876556	GenBank ID: *P. lasia*
	DL Largent 9729	Australia: Queensland	–	HQ876531	HQ876509	HQ876553	GenBank ID: *P. lasia*
	DL Largent 9778	Australia: Queensland	–	HQ876532	HQ876510	HQ876554	GenBank ID: *P. lasia*
E. nodosporum	TENN:068582	USA: Tennessee	KY744163	MF797654	–	–	GenBank ID: *P. nodospora*
E. pamiae	DL Largent 9794	Australia: Queensland	–	HQ876517	HQ876496	HQ876539	GenBank ID: *P. pamiae*
	DL Largent 9834	Australia: Queensland	–	HQ876519	HQ876498	HQ876541	GenBank ID: *P. pamiae*
	DL Largent 9808	Australia: Queensland	–	HQ876518	HQ876497	HQ876540	GenBank ID: *P. pamiae*
E. perbloxamii	MEN 2004071 (Holotype)	Australia: Tasmania	KC710117	GQ289178	GQ289249	GQ289318	
E. pilocystidiatum	DL Largent 9848	Australia: Queensland	–	HQ876520	HQ876499	HQ876542	GenBank ID: *P. pilocystidiata*

Taxa	Collection No.	Origin	GenBank accessions				Remarks
			ITS	LSU	RPB2	mtSSU	
E. pilocystidiatum	DL Largent 9932 (Holotype)	Australia: Queensland	–	HQ876521	HQ876500	HQ876543	GenBank ID: *P. pilocystidiata*
	DL Largent 9949	Australia: Queensland	–	HQ876522	HQ876501	HQ876544	GenBank ID: *P. pilocystidiata*
E. prunuloides	MEN 200340	Slovakia	KC710073	GQ289184	GQ289255	GQ289324	
E. rubropilosum	SAAS 406 (Holotype)	China: Sichuan	**MH020761**	**KU534218**	**KU534488**	**KU534439**	
	SAAS 1112	China: Tibet	**MH020767**	**KU534252**	**KU534502**	**KU534454**	
E. setiforme	DL Largent 9809 (Holotype)	Australia: Queensland	–	HQ876525	HQ876503	HQ876547	GenBank ID: *P. setiformis*
	DL Largent 9810	Australia: Queensland	–	HQ876526	HQ876504	HQ876548	GenBank ID: *P. setiformis*
E. silvanum	K(M) 191739 (Holotype)	India: Kerala	KY643747	KY643724	–	–	
E. sp. 1	SAAS 894	China: Sichuan	**MH020765**	**KU534245**	**KU534491**	**KU534447**	
E. sp. 2	SAAS 1088	China: Jilin	**MH020749**	**KU534246**	–	**KU534441**	
	SAAS 1210	China: Jilin	**MH020752**	**KU534248**	–	**KU534449**	
E. sp. 3	SAAS 249	China: Sichuan	**MH020759**	**KU534243**	–	–	
E. sp. 4	SAAS 1209	China: Jilin	**MH020751**	–	**KU534492**	**KU534448**	
	SAAS 291	China: Jilin	**MH020760**	–	**KU534486**	**KU534444**	
E. sp. 5	SAAS 1360	China: Jilin	**MH020754**	**KU534249**	–	**KU534456**	
E. sp. 6	SAAS 1464	China: Sichuan	**MH020756**	**KU534258**	**KU534493**	**KU534450**	
E. sp. 7	SAAS 100	China: Sichuan	**MH020747**	–	–	**KU534442**	
E. sp. 8	080301	China: Sichuan	**MH020745**	**KU534254**	–	–	
	SAAS 102	China: Sichuan	**MH020748**	–	**KU534489**	**KU534443**	
E. sp. 9	SAAS 1527	China: Shaanxi	**MH020758**	**KU534251**	**KU534495**	**KU534452**	
E. sp. 10	SAAS 529	China: Sichuan	**MH020763**	**KU534244**	**KU534497**	**KU534446**	
	SAAS 772	China: Sichuan	**MH020764**	**KU534241**	**KU534487**	**KU534458**	
E. sp. 11	SAAS 526	China: Shaanxi	**MH020762**	**KU534257**	**KU534490**	**KU534445**	
E. strigosissimum	152	Italy	JF908004	–	–	–	
E. subaraneosum	GDGM 28823 (Holotype)	China: Jilin	JQ320113	JQ410329	–	–	
	KA 12-1534	South Korea	KJ523135	–	–	–	
E. tenuissimum	GDGM 28813	China: Jilin	JX975295	JQ993097	JQ993086	JQ993059	
	GDGM 28814 (Holotype)	China: Heilongjiang	JX975294	JQ993096	JQ993087	JQ993060	
E. violaceovillosum	P. Manomohan 645 (Holotype)	India: Kerala	–	GQ289205	GQ289273	GQ289345	
E. yunnanense	GDGM 28815	China: Yunnan	JQ320108	JQ320128	–	JQ993057	

Sequences in bold and marked with "KU" and "MH" are newly generated in this study. Sequences marked with "GQ" were from Co-David et al. (2009). Sequences marked with "HQ" were from Largent et al. (2011). KC710056, KC710073 and KC710117 were from Morgado et al. (2013). KC678996, KC678997 and sequences marked with "JQ" and "JX" were from He et al. (2013). KY643747 and KY643724 were from Raj and Manimohan (2017). JF908004 was from Osmundson et al. (2013). KJ523135 was from Kim et al. (2015). KY744163 and MF797654 are unpublished.

et al. 2012). GTR+I+G model was selected for nLSU, GTR+G for mtSSU and ITS and SYM+G for RPB2. Two runs of six Markov chains were run from random starting trees for 6 million generations and sampled every 100 generations. Every time the diagnostics were calculated, 25% of the samples from the beginning of the chain were discarded. Runs were stopped after the average standard deviation was below 0.01. Bayesian posterior probabilities (BPP) were determined after calculating a 75% majority rule consensus tree.

Taxonomy

Entoloma erectoides **Xiao L. He & E. Horak, sp. nov.**
MycoBank No: MB828692
Figs 1a, b, 2

Diagnosis. *E. erectoides* is distinguished by the greyish brown pileus covered with silvery fibrils, large basidiospores (13.5–17.5 × 8–9.5 μm) and presence of ovoid to subutriform cheilocystidia.

Type. CHINA. SICHUAN PROV.: Yajiang County, Gexigou National Nature Reserve, 29°33'N, 100°50'E, elevation ca. 2980 m, August 2014, He XL (SAAS 1232, holotype; ZT 14180, isotype).

Etymology. *Erectoides*, refers to the suberect to erect fibrils on the pileus.

Description. Pileus 5–15 mm broad, bluntly conic, convex or campanulate, dry, slightly hygrophanous, greyish-brown to brown (5C2–5D2), densely covered with suberect fibrils or minutely fibrillose squamules; fibrils silvery greyish, striate from entire margin to near centre. Lamellae sinuate, ventricose, distant, up to 3.5 mm wide, moderately thick, with two tiers of lamellulae, dark grey to brownish-grey, with entire and concolorous edge. Stipe 40–60 × 1–2.5 mm, central, cylindrical, equal, dry, concolorous with pileus, densely covered with grey to greyish fibrils, hollow, surface dry, with a pale yellow brownish to pale brownish strigose base. Context thin, concolorous with pileus. Odour and taste not distinctive.

Basidiospores (13–) 13.5–17.5 × 8–9.5 (–10.5) μm (x = 15.5 ± 0.5 × 8.8 ± 0.3 μm), Q = 1.50–1.94 (***Q*** = 1.72 ± 0.03), heterodiametric, strongly angled in profile and face views with 6–9 facets, appearing nodulose, pale yellow brownish, thick-walled. Basidia 39–48 × 13–18 μm, subclavate or clavate, 4-spored. Aborted basidia scattered in the hymenium, often filled with dark brown amorphous cytoplasmic pigment. Lamellar trama dark brown, composed of parallel, cylindrical, heavily encrusted and thin-walled cells, 6–15 μm wide. Lamellar edge sterile. Cheilocystidia 33–90 × 12–33 μm, broadly ovoid to utriform (32–65 × 22–30 μm) or lageniform (70–90 × 16–20 μm), with pale brownish, intracellular pigment, slightly thick-walled. Pileipellis a trichoderm composed of clustered and suberect hyphae, walls externally encrusted with

Figure 1. Basidiomata of the new species. **a, b** *E. erectoides* (SAAS 1361, SAAS 1232) **c, d** *E. griseocarpum* (SAAS 951, SAAS 1328) **e, f** *E. rubropilosum* (SAAS 406, SAAS 1112).

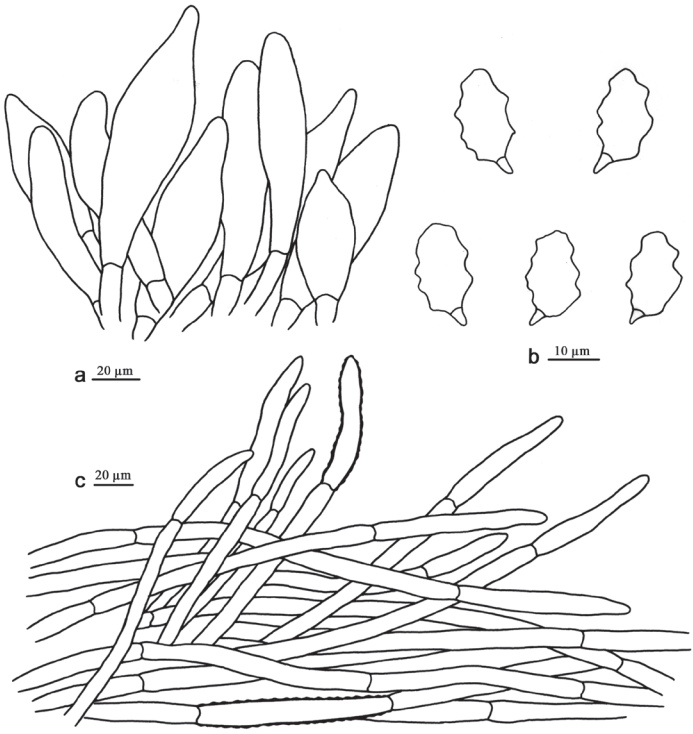

Figure 2. Microscopic structures of *E. erectoides* (drawn from the holotype). **a** Cheilocystidia **b** Basidiospores **c** Pileipellis.

brown pigment; terminal cells 30–50 (–90) × 8–15 µm, cylindrical to slightly fusoid; subpellis composed of cylindrical, encrusted hyphae, up to 20 µm broad. Stipitipellis composed of thin-walled and pale yellowish-brown encrusted hyphae; terminal cells 40–80 × 9–15 µm, cylindrical to slender fusoid, walls encrusted with pale yellow-brown pigment. Oleiferous hyphae absent. Clamp connections absent.

Habitat. Scattered or gregarious on soil and amongst leaf litter in broadleaf forest dominated by *Quercus* or on soil amongst decaying leaves of *Betula*, *Pandus* and *Abies*.

Additional collections examined. CHINA. SICHUAN PROVINCE: CHINA: SICHUAN PROV. Yajiang County, Gexigou National Nature Reserve, 29°33'N, 100°50'E, elevation ca. 2980 m, 24 July 2013, He X.L. (SAAS 945). JILIN PROV.: Antu County, Changbai Mountains, 42°10'N, 127°55'E, elevation ca. 750 m, 25 August 2014, He X.L. (SAAS 1361).

Comments. Morphologically, *Entoloma erectoides* is a member of section *Dysthales*. In literature, a few species in section *Dysthales* are described having silvery fibrils or squamules on pileus and stipe. Accordingly, *E. erectoides* can be confused with the Argentinean *E. calileguense* Blanco-Dios (as *Pouzarella variabilis* T.J. Baroni, Albertó, Niveiro & B.E. Lechner in Baroni et al. 2012). Both species have silvery greyish-brown erect fibrils or squamules on the pileus and stipe. However, the latter species is easily separated by the much larger basidiospores (16–23.5 × 10–12 µm, Baroni et

al. 2012). *E. farinosum* (Largent & Skye Moore) Noordel. & G.M. Gates, reported from Australia, differs by globose or nearly napiform cheilocystidia (Largent et al. 2011). In addition, this taxon is separated from *E. erectoides* by molecular evidence. *E. tenuissimum* T.H. Li & Xiao-Lan He, also recorded from China, is distinguished by the smaller and slimmer basidiomes and taxonomically is also distinctly different based on molecular analysis (He et al. 2013). *E. argenteolanatum* (T.J. Baroni, Perd.-Sánch. & S.A. Cantrell) Noordel. & Co-David was found on decaying leaves of tropical trees and shrubs in the Dominican Republic and is characterised by denser and longer silvery fibrils and the place of discovery in the Caribbean on the island of Hispaniola (Baroni et al. 2008). The other grey-brown species with silvery fibrils in section *Versatile* could be distinguished by the innately fibrillose pileus and stipe and colourless hymenial cystidia.

Entoloma griseocarpum **Xiao L. He & E. Horak, sp. nov.**
MycoBank No: MB828701
Figs 1c, d, 3

Diagnosis. *E. griseocarpum* is characterised by the greyish-brown pileus, large basidiospores (12.5–15.5 × 7.5–9 μm) and broadly clavate, ovoid to lageniform cheilocystidia.

Type. CHINA. TIBET: Linzhi, Lulang, 29°94'N, 94°79'E, elevation ca. 3800 m, 18 September 2014, He X.L. (SAAS 1328, holotype).

Etymology. *griseocarpum*, refers to the greyish-brown coloured basidiomes.

Description. Pileus 5–20 mm broad, hemispherical, convex, bluntly conic to broadly campanulate, dry, not hygrophanous, greyish-brown to brown (4D3–4E3), densely covered by suberect hispid or minutely squamulose overall, denser in centre; fibrils dark grey, pale grey brownish or concolorous with pileal surface (4D2–4D3), striate from entire margin to near centre. Lamellae sinuate with short decurrent tooth, ventricose, distant, moderately thick, up to 3 mm broad, with two tiers of lamellulae, dark grey to brownish-grey, with entire and concolorous edges. Stipe 20–50 × 0.7–1.5 mm, cylindrical, equal, dry, concolorous with pileus, densely covered with pale yellow brownish flocculose hairs, hollow, with a dirty yellowish to pale yellow brownish strigose base. Context thin, concolorous with pileus. Odour and taste not distinctive.

Basidiospores 12.5–15.5 (–17) × (6.5–) 7.5–9 (–9.5) μm (x = 13.8 ± 0.3 × 8.3 ± 0.3 μm), Q = 1.60–1.94 (***Q*** = 1.71 ± 0.02), heterodiametric, strongly angled in profile and face view with 6–10 facets, appearing nodulose, pale yellow brownish, thick-walled. Basidia 35–55 × 11–13 (–15) μm, subclavate to clavate, 4-spored. Aborted basidia scattered in the hymenium, filled with dark brown amorphous cytoplasmic pigment. Lamellar trama dark brown, composed of parallel, cylindrical, heavily encrusted and thin-walled elements. Lamellar edges sterile. Cheilocystidia 23–50 × (10–) 12 20 μm, broadly clavate, ovoid to lageniform; with brownish, intracellular pigment, slightly thick-walled. Pileipellis a trichoderm composed of yellow brown, suberect and multiseptate hyphae, walls heavily encrusted with brown

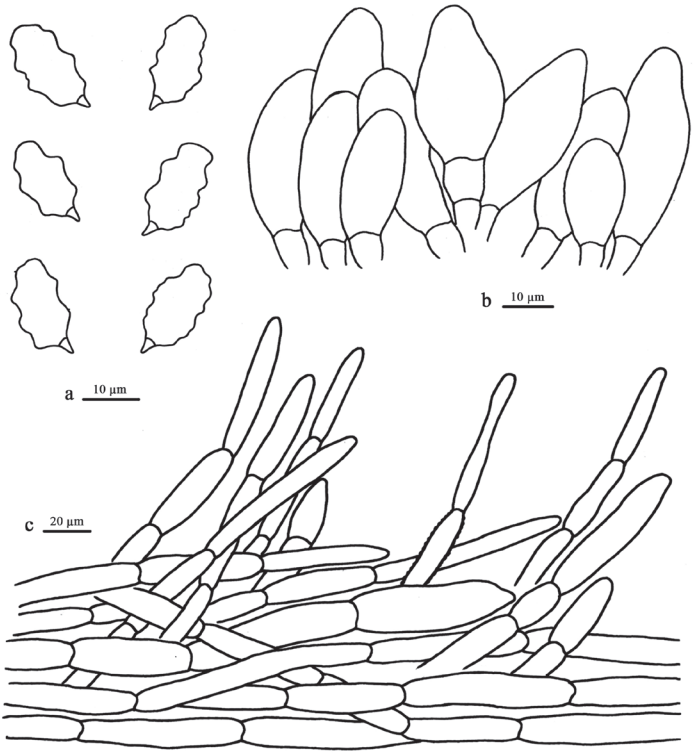

Figure 3. Microscopic structures of *E. griseocarpum* (drawn from the holotype). **a** Basidiospores **b** Cheilocystidia **c** Pileipellis.

pigment; terminal cells 35–105 × 8–27 μm, cylindrical, subclavate or bullet-shaped, thin to moderately thick-walled; subpellis composed of cylindrical encrusted hyphae, up to 25 μm diam. Stipitipellis composed of yellow-brown encrusted hyphae; terminal cells 40–80 × 4–10 μm, slender cylindrical with obtuse apex, thin-walled, sparsely encrusted with pale yellowish-brown pigment. Oleiferous hyphae absent. Clamp connections absent.

Habitat. Scattered on soil amongst decaying litter in mixed conifer-broadleaf forest dominated by *Quercus*, *Betula*, *Rhododendron* and *Abies*.

Additional collections examined. CHINA. TIBET: Linzhi, Lulang, 29°94'N, 94°79'E, elevation ca. 3800 m, 18 September 2014, He X.L. (SAAS 1230, SAAS 1657, SAAS 1751, SAAS 1871). SICHUAN PROV.: Jiuzhaigou, 33°28'N, 103°59'E, elevation ca. 3000 m, 20 July 2013, He X.L. (SAAS 951).

Comments. The greyish-brown pileus covered by suberect hispid or minutely squamulose, the brown external encrustations on pileipellis and stipitipellis and the cylindrical terminal cells of pileipellis and stipitipellis indicate *E. griseocarpum* belongs to the sect. *Dysthales*. It is very similar to *E. albostrigosum* (Largent & Abell-Davis) Blanco-Dios and *E. lasium* (Berk. & Broome) Noordel. & Co-David (Largent et al. 2011). However, *E. albostrigosum* is distinguished by the white strigose base and

E. lasium differs by the smaller basidiospores (8.9–14.5 × 5.1–8.7 µm, Largent et al. 2011). In addition, the two species are distant from *E. griseocarpum* following phylogenetic analysis. *E. puertoricense* Blanco-Dios (as *P. caribaea* T.J. Baroni & B. Ortiz in Baroni and Ortiz 2002) resembles *E. griseocarpum* by the brownish-grey coloured basidiomes but is separated by its broader basidiospores (12.5–16.5 × 8.3–11.3 µm, Q = 1.26–1.65, Baroni and Ortiz 2002). Moreover, *E. puertoricense* was discovered in a tropical habitat in Puerto Rico (Baroni and Ortiz 2002). The similar *E. japonicum* (Hongo) Hongo, described from Japan, is also reminiscent of *E. griseocarpum* in the brownish pileus but is distinguished by the much larger basidiospores (15–18.5 × 9–10.5 µm, Hongo 1959). The well-known *E. dysthales* (Peck) Sacc. also differs by the larger basidiospores (14–20 × 7.5–10 µm, Mazzer 1976). *E. fulvolanatum* (Berk. & Broome) Blanco-Dios from Sri Lanka is not only separated by its type locality but also by the narrower basidiospores measuring 12–16 × 7–8 µm (Mazzer 1976). Two species in subgen. *Pouzarella*, recently described from geographically neighbouring India, viz. *E. peechiense* K. N. A. Raj & Manim. and *E. silvanum* K. N. A. Raj & Manim., have somewhat similar basidiomes as compared to *E. griseocarpum*; however, their ITS and LSU sequences are distinctly different (Raj and Manimohan 2017). The third Indian species *E. lomapadum* Manim., Joseph & Leelav. is readily recognised by the much smaller basidiospores measuring 11–13 × 6–9 µm (Manimohan et al. 1995). There were four other species in subgen. *Pouzarella* which showed some similarities to *E. griseocarpum*. *E. fibrillosipes* (Murrill) Noordel. & Co-David is distinguished by the much larger basidiospores (17–22 × 7.5–10 µm, Mazzer 1976). *E. subdeceptivum* Courtec. and *E. rotula* (Romagn.) Noordel. & Co-David are lignicolous (Mazzer 1976). *E. homomorphum* (Romagn.) Singer differs by the larger basidiospores (15–19 × 9–11.5 µm, Mazzer 1976).

Entoloma rubropilosum **Xiao L. He & E. Horak, sp. nov.**
MycoBank No: MB828699
Figs 1e, f, 4

Diagnosis. *E. rubropilosum* is distinct due to its reddish-brown coloured pileus and stipe, large basidiospores (13–17 × 7.5–9.5 µm), broadly clavate cheilocystidia, distinctive thick-walled setiform caulocystidia and terminal cells of the pileipellis hyphae.

Type. CHINA: SICHUAN PROV.: Yajiang County, Gexigou National Nature Reserve, 29°33'N, 100°50'E, elevation ca. 2950 m, 24 July 2013, He X.L. (SAAS 406, holotype).

Etymology. *Rubropilosum*, refers to the reddish coloured fibrils on the pileus.

Description. Pileus 7–20 mm broad, conical-convex, truncate conical to broadly campanulate, dark reddish-brown (8D2–8D3) at first, becoming greyish-orange to pale beige brownish (5B2–5C2), dry, slightly hygrophanous, densely covered by reddish-brown erect or suberect squamules and fibrils; fibrils much denser at disc, margin not striate or very slightly striate only. Lamellae adnate to sinuate, ventricose, up to 2.5 mm wide, relatively

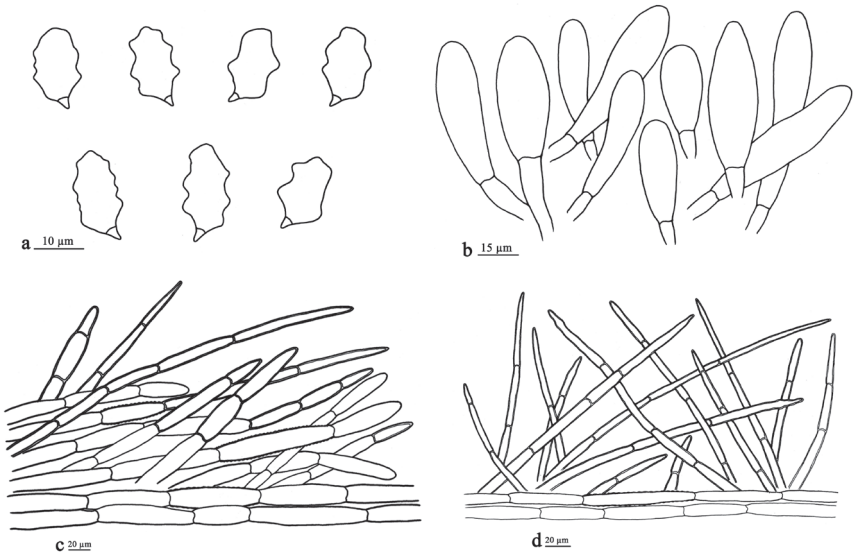

Figure 4. Microscopic structures of *E. rubropilosum* (drawn from the holotype). **a** Basidiospores **b** Cheilocystidia **c** Pileipellis **d** Stipitipellis.

thick, with two tiers of lamellulae, brownish-pink when mature, with concolorous and entire edges. Stipe 40–73 × 0.8–2 mm, central, cylindrical, hollow, densely covered with rust reddish hairs or fibrils, very dark brown strigose at base. Odour and taste not distinctive.

Basidiospores (12.5–) 13–17 × 7.5–9.5 µm (x = 15.2 ± 0.5 × 8.5 ± 0.3 µm), Q = 1.53–1.98 ($\boldsymbol{Q}$ = 1.76 ± 0.02), heterodiametrical, with 6–8 facets in profile and face views, sometimes multi-angled to nodulose, pale brownish, thick-walled. Basidia (32–) 38–45 (–50) × 12–16 µm, clavate, 4-spored. Aborted basidia inconspicuous. Lamellar edges sterile. Cheilocystidia 25–50 × 12–18 µm, broadly clavate, with faintly pale brownish, intracellular pigment, slightly thick-walled. Pleurocystidia absent. Pileipellis a trichoderm composed of brown hyphae; terminal cells 23–110 × 6–18 µm (diameter was measured at the base), slender setiform, gradually tapering towards subacute apex, sometimes subfusoid to somewhat bullet-shaped, thick-walled, with intraparietal and intracellular brown pigment; subpellis composed of cylindrical, relatively thin-walled hyphae, encrusted with yellow-brown pigment. Stipitipellis composed of loosely entangled, rather slender hyphae; terminal cells 45–120 × 5–11 µm (diameter was measured at the base), distinctly setiform with obtuse or subacute apex, thick-walled, with intraparietal and intracellular brown pigment. Oleiferous hyphae absent. Clamp connections absent.

Habitat. Scattered on soil amongst decaying litter in broadleaf forest dominated by *Quercus* or in mixed forest with *Quercus*, *Betula*, *Rhododendron* and *Abies*, also on soil in bamboo forest.

Additional collections examined. CHINA. SICHUAN PROV.: Yajiang County, Gexigou National Nature Reserve, 29°33'N, 100°50'E, elevation ca. 2950 m, 24 July 2013, He X.L. (SAAS 765); 24 July 2013, He X.L. (SAAS 706); 3 August 2014, He X.L. (SAAS

1488, SAAS 1112, ZT 14179). TIBET: Linzhi, Lulang, 29°94'N, 94°79'E, elevation ca. 3800 m, 18 September 2014, He X.L. (SAAS 1618, SAAS 1087); Linzhi, Kadinggou, 29°50'N, 93°26'E, elevation ca. 2950 m, 24 September 2014, He X.L. (SAAS 1456).

Comments. The setiform terminal cells of pileipellis and stipitipellis place *E. rubropilosum* in sect. *Pouzarella*. It is readily recognised in the field. A few species of *Entoloma* subgen. *Pouzarella* with reddish-brown fibrils or squamules have been reported in literature (Mazzer 1976; Baroni et al. 2008). *E. ferreri* (T.J. Baroni, Perd.-Sánch. & S.A. Cantrell) Noordel. & Co-David is distinguished by dark blackish stains on the pileus caused from handling and non-setiform pileocystidia and caulocystidia (Baroni et al. 2008). *E. strigosissimum* (Rea) Noordel. is separated by the larger basidiospores [15–19 (23) × 8.5–10.5 (11.5) μm, Mazzer 1976]. *E. squamifolium* (Murrill) Singer might be confused with *E. rubropilosum* due to the ferruginous hairs on the stipe (Mazzer 1976). However, *E. rubropilosum* can be distinguished by the setiform pileocystidia and caulocystidia. Furthermore, type of *E. squamifolium* was collected in a tropical location. The recently described *E. wayanadense* K. N. A. Raj & Manim. from India, also discovered in a tropical area, is similar to *E. rubropilosum* in its greyish-orange pileus with long hairs and the setiform terminal cells of pileipellis, but differs by the absence of cheilocystidia. In addition, the partial ITS sequence (419 bp, KY 643748) of *E. wayanadense* is quite different from that of *E. rubropilosum* (Raj and Manimohan 2017).

Key to the species of *Entoloma* subgen. *Pouzarella* described from China

1	Pileus reddish-brown or greenish-brown	**2**
–	Pileus greyish-brown	**3**
2	Pileus covered with reddish-brown suberect fibrils	***E. rubropilosum***
–	Pileus greenish-brown with reddish tinge, zonate	***E. changchunense***
3	Pileus covered with appressed or suberect silvery fibrils	**4**
–	Pileus squamulose or covered with suberect brownish fibrils	**5**
4	Pileus covered with appressed silvery fibrils	***E. subaraneosum***
–	Pileus covered with suberect silvery fibrils	**6**
5	Pileus fibrillo-squamulose or squamulose-tomentose, growing in tropical forest	***E. crassicystidiatum***
–	Pileus covered with erect or suberect fibrils	**7**
6	Pileus pale brownish with pinkish tinge, basidiospores larger, average (16.8 ± 0.5) × (10.8 ± 0.3) μm	***E. tenuissimum***
–	Pileus greyish-brown, basidiospores smaller, average (15.5 ± 0.5) × (8.8 ± 0.3) μm	***E. erectoides***
7	Average spore length less than 13 μm	***E. furfuraceum***
–	Average spore length more than 13 μm	**8**
8	Pileus greyish-brown, striate from entire margin to near centre	***E. griseocarpum***
–	Pileus peach brown, not striate	***E. yunnanense***

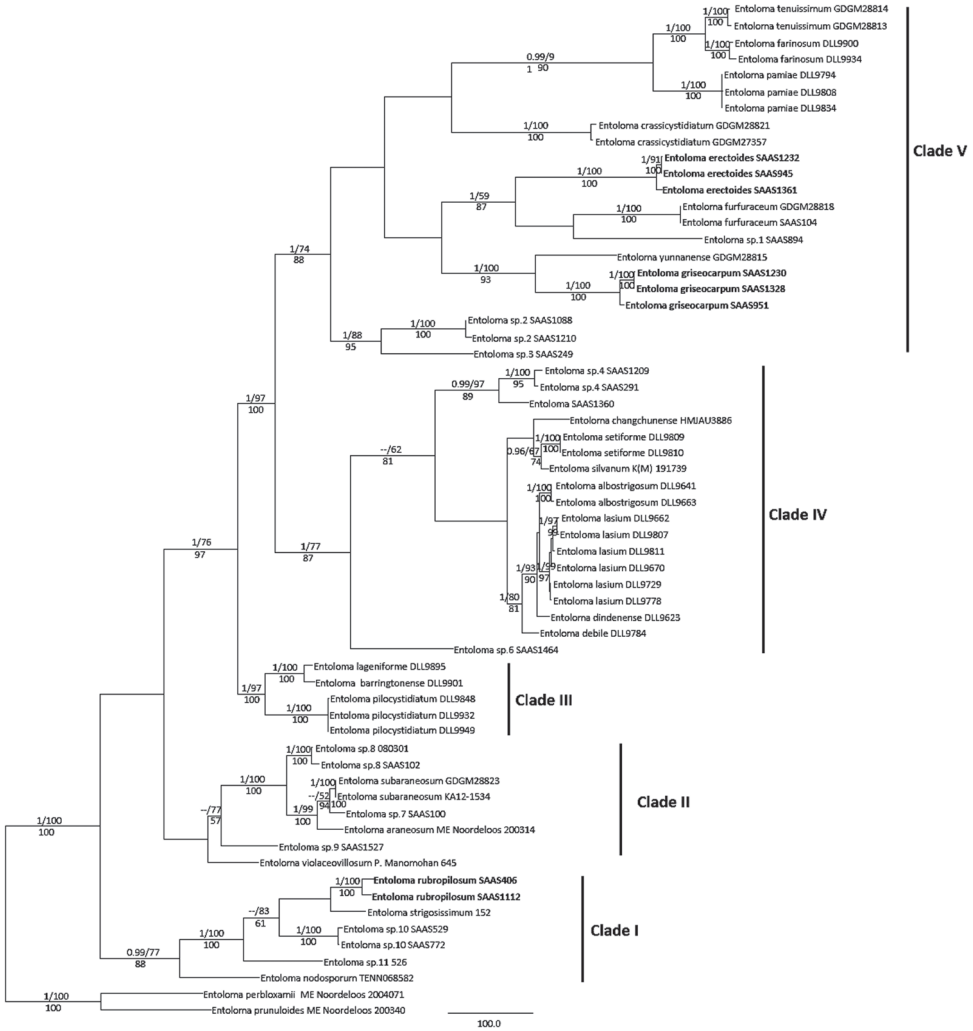

Figure 5. Phylogenetic relationships of *Entoloma* subgen. *Pouzarella* species inferred from the combined ITS, LSU, mtSSU and RPB2 dataset (new species are in bold). Bayesian posterior probability values (BPP > 0.90) and MP BS support values (> 50%) are indicated above branches as BPP/BS; RAxML BS support values (> 50%) are listed below branches.

Molecular analysis

A total 76 sequences were generated in this study and they were deposited in GenBank. The combined dataset in the molecular analyses is composed of 62 specimens and 2925 aligned sites. MP, ML and Bayesian analyses produced almost the same topologies except for the unsupported branches and the MP tree is shown (Fig. 5).

For the ITS sequences used in the analyses, both the size of the entire ITS1-5.8-ITS2 region as well as for ITS1 and ITS2 were separately compared. It is remarkable

that the sizes of the entire ITS region and ITS1 were significantly divergent. In general, the total length of ITS sequences in subgen. *Pouzarella* ranged from 591 bp to 1086 bp. ITS1 was highly variable in length, while the length of ITS2 is relatively conserved within subgen. *Pouzarella*. ITS1 and ITS2 spacer varied from 229 to 690 bp and from 202 to 255 bp, respectively. However, it is noteworthy that two groups of the whole ITS region and ITS1 spacer could be partitioned in length. One group was varying from 591 bp to 709 bp and the other from 967 bp to 1086 bp. For the 5.8S region, 159–162 bp were yielded. Despite 5.8S is highly conserved, 4 indels and 11 nucleotide substitutes were found in this region. Regarding RPB2 sequences, their length was considerably conserved.

The three new species in this study were placed in different clades, showing they are quite different from each other. *E. rubropilosum* in relatively close to *E. strigosissimum* in the analyses, but similarity of their ITS sequences is only 84%. *E. griseocarpum* is grouped with *E. yunnanense* J.Z. Ying, but more than 150 different bases were observed in their ITS sequences. *E. erectoides* and *E. furfuraceum* nested in the same clade; however, more than 100 different bases were detected amongst their ITS sequences.

Results and discussion

In the present study, three new species of *Entoloma* subgen. *Pouzarella* viz. *E. griseocarpum*, *E. erectoides* and *E. rubropilosum*, are reported from southwest China. The description is based on morphological and molecular characters. Together with the seven afore-mentioned species, ten taxa of *Entoloma* subgen. *Pouzarella* are now recorded for China. Five of those have been discovered in the northeast of China, four in southwest China and only one was reported from southern China (Ying 1995; He et al. 2013).

In the phylogenetic analyses, 35 taxa in subgen. *Pouzarella* were included and twenty phylogenetic species from China were recovered, suggesting a high species diversity in this geographical region. Five distinct clades (Clades I–V) were observed and the three new species are phylogenetically separated from each other. Based on morphological characters, *Pouzarella* (as a genus or subgenus of *Entoloma* s.l.) was divided into three sections (*Dysthales*, *Pouzarella* and *Versatiles*, Mazzer 1976; Noordeloos 1992). In our phylogenetic tree, Clade I corresponds to sect. *Pouzarella* while the taxa belonging to Clade II are accommodated in sect. *Versatile*. Except for several species of uncertain position, most members of Clade III, Clade IV and Clade V belong to the traditional sect. *Dysthales* morphologically. *E. rubropilosum* is nested in Clade I, which also includes *E. nodosporum* (G.F. Atk.) Noordel.and *E. strigosissimum*, as well as two still unknown species (*Entoloma* sp. 10 and *Entoloma* sp. 11) collected in China that morphologically fit the concept of sect. *Pouzarella*. These four species possess setiform pileocystidia and caulocystidia, somewhat reddish-brown or reddish fibrils on stipe and pileus. *E. erectoides* and *E. griseocarpum* are placed in Clade V.

The length of the ITS sequence of the species nested in Clade I, Clade II and Clade IV is relatively short, ranging from 591 bp to 709 bp. Available ITS sequences of *Entolo-*

ma subgen. *Pouzarella* species in Clade V are recorded from 967 bp to 1086 bp. Unfortunately, no ITS sequences are available for comparison of the taxa belonging to Clade III. Eventual combination, referring both to morphological and molecular evidence, may in the future fundamentally change the classification of *Entoloma* subgen. *Pouzarella*.

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RESEARCH ARTICLE

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Neoprotoparmelia gen. nov. and *Maronina* (Lecanorales, Protoparmelioideae): species description and generic delimitation using DNA barcodes and phenotypical characters

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Abstract

Multilocus phylogenetic studies revealed a high level of cryptic diversity within the lichen-forming fungal genus *Maronina* (Protoparmelioideae, Parmeliaceae). Coalescent-based species delimitation suggested that most of the cryptic molecular lineages warranted recognition as separate species. Here we study the morphology and chemistry of these taxa and formally describe eight new species based on phenotypical and molecular characters. Further, we evaluate the use of ITS rDNA as a DNA barcode for identifying species in this genus. For the first time, we obtained an ITS sequence of *Maronina australiensis*, the type species of the genus and showed that it is phylogenetically not closely related to species currently placed in *Maronina* or *Protoparmelia*. We assembled a dataset of 66 ITS sequences to assess the interspecies genetic distances amongst the twelve *Maronina* species using ITS as DNA barcode. We found that *Maronina* and *Protoparmelia* form a supported monophyletic group whereas *M. australiensis* is sister to both. We

therefore propose a new genus *Neoprotoparmelia* to accommodate the tropical-subtropical species within Protoparmelioideae, with *Neoprotoparmelia corallifera* as the type, *N. amerisidiata*, *N. australisidiata*, *N. brasilisidiata*, *N. capensis*, *N. crassa*, *N. pauli*, *N. plurisporibadia* and *N. siamisidiata* as new species and *N. capitata*, *N. isidiata*, *N. multifera*, *N. orientalis* and *N. pulchra* as new proposed combinations. We provide a key to *Neoprotoparmelia* and confirm the use of ITS for accurately identifying species in this group.

Keywords
ITS, lichenised fungi, BPP, new genus, new species, Parmeliaceae, taxonomy

Introduction

The taxonomic status of the genus *Maronina* and its phylogenetic relationships have been a matter of debate. *Maronina* was formally described in 1990 (Hafellner and Rogers 1990) for two species with multispored asci, the type species *Maronina australiensis* and *M. multifera*. The authors suggested a close relationship of *Maronina* and *Protoparmelia* based on ascus characters and considered *Maronina* a multispored derivative of *Protoparmelia*. Later, molecular data confirmed the phylogenetic relationship between *Maronina* with *Protoparmelia* and *Maronina* was merged with *Protoparmelia* (Papong et al. 2011). Recently, Kraichak et al. (2017) suggested the use of a temporal banding approach for a consistent grouping of taxa at higher taxonomic levels, i.e. at family and genus level, for lichen-forming fungi. This approach identifies a divergence time of ~102–112 Ma for families and 29–33 Ma for genera (Kraichak et al. 2017). Based on this approach, the genus *Maronina* has been resurrected (*Maronina-Protoparmelia* split ~70 Ma; Divakar et al. 2017, Kraichak et al. 2017, Singh et al. 2018) and both genera, *Protoparmelia* and *Maronina*, have been placed together in the subfamily Protoparmelioideae (Parmeliaceae). Currently, the genus *Protoparmelia* comprises arctic, boreal, temperate and Mediterranean species, whereas the genus *Maronina* comprises subtropical and tropical species.

Presently *Maronina* includes 11 species (Aptroot 2002, Aptroot et al. 1997a, 1997b, 2007, 2013, Barbero et al. 2006, Elix 2007, 2009, Hafellner and Rogers 1990, Kantvilas and Elix 2007, 2010, Lendemer and Lumbsch 2008, Papong et al. 2011). Molecular data are available for six species (*Maronina capitata*, *M. corallifera*, *M. multifera*, *M. orientalis*, *M. isidiata* and *M. pulchra*). A recent study aimed at molecular identification of species in *Maronina* and *Protoparmelia*, based on a multilocus dataset and species delimitation analysis, included these six species and two putatively novel species (Singh et al. 2015). Molecular analysis confirmed the presence of the above-mentioned species in *Maronina* and suggested seven additional species: *M. isidiata* A, *M. isidiata* B (Brazil), *M. isidiata* C (Thailand), *M. isidiata* D (Australia), *M. isidiata* E (Australia), *M.* ZA (South Africa) and *M.* KE (Kenya). These candidate species were strongly supported by species delimitation approaches BP&P and speDeSTEM (Singh et al. 2015), but not formally proposed at the time. In the present study, we describe the seven novel *Maronina* species *sensu* Singh et al. (2015) and a further new saxicolous species from Brazil based on phenotypical and molecular evidence. In addition, we include the type specimen *M. australiensis*, which has not been sequenced before.

Materials and methods

We included 66 ITS rDNA sequences of *Protoparmelia* and *Maronina* in this study. Out of these, 61 ITS sequences are from Singh et al. (2015) and five sequences are new, representing two additional specimens of *M. capitata*, two sequences of a new taxon *M. plurisporibadia* and a sequence of the type species of the genus *Maronina*, *M. australiensis* (Table 1).

Molecular methods

For DNA extraction, amplification and sequencing, we followed the protocols from Singh et al. (2015). We used the *Protoparmelia* specific primers (Suppl. material 1:Table S1) and Ex Taq polymerase (Takara Bio Europe, France) for the PCRs. Generating an ITS sequence from the 32-year-old *M. australiensis* sample required a PCR cloning approach. The amplified products were cloned into the pJET1.2 / blunt cloning vector using the Thermo Scientific CloneJET PCR cloning kit and transformed into *E. coli* XL1-Blue cells (for details see: https://www.chem-agilent.com/pdf/strata/200249.pdf). The cloned PCR products were analysed using the "colony PCR". For the PCR reactions and sequencing, we used the pJET1.2 Forward Sequencing Primer and the pJET1.2 Reverse Sequencing Primer. We performed a BLAST search using the *M. australiensis* ITS sequence to infer the phylogenetic affinities of *M. australiensis*.

Phylogenetic analyses

We aligned the sequences using MAFFT v5 with Geneious version 5.6.5 (Katoh et al. 2005, Drummond et al. 2011). To infer the phylogenetic position of *M. australiensis* within Protoparmelioideae, we produced an alignment using the ITS sequences of *M. australiensis*, *Protoparmelia* and *Maronina* species. Using this alignment, we generated a maximum likelihood tree using the ITS sequences from *Protoparmelia* (9 species, 20 sequences) and *Maronina* (13 species including the type species, 44 sequences; Fig. 1), with GTR + G as the substitution model. This dataset contains overall 66 sequences, including 2 sequences of the outgroup (Gypsoplacaceae). The maximum likelihood search was performed using the RAxML-HPC BlackBox v8.1.11 on the Cipres Scientific gateway (Miller et al. 2010, Stamatakis 2014).

Analysis of sequence variation in the ITS barcode marker

To infer intra- and interspecific ITS sequence variation within and amongst putative lineages of *Neoprotoparmelia* (*Maronina* s.l.), we calculated pairwise distances amongst *Neoprotoparmelia* species (*Maronina* s.l. species, 43 sequences from 12 species, excluding

Table 1. Specimens used in this study. New sequences are indicated in bold.

Species	Sample ID as in BOLD database	Voucher	Accession number ITS rDNA
Gypsoplaca macrophylla	NA	USA, Rosentreter 15995 (F)	KF650781
Gypsoplaca sp.	NA	USA, Spribille 38752 (GZU)	MK046745
Maronina australiensis	NA	Australia, Hafellner 17823 & Rogers, holotype (GZU)	MK046744
Neoprotoparmelia australisidiata	IS120074	Australia, Kantvilas 228/10, HO 559228 (HO)	KP822275
	IS120075	Australia, Kantvilas 289/07, HO 545660 (HO)	KP822276
N. brasilisidiata	IS140153	Brazil, Cáceres & Aptroot ISE 21684, holotype (ISE)	KP822271
	IS140154	Brazil, Cáceres & Aptroot ISE 13673 (ABL)	KP822272
	IS140192	Brazil, Cáceres & Aptroot 21648 (ISE)	KY066262
N. capensis	ZA120814	South Africa, Crespo, Divakar, Hawksworth, Amo & Lumbsch 14c, MAF-Lich. 19627, isotype (MAF)	KP822302
	ZA120815	South Africa, Crespo, Divakar, Hawksworth, Amo & Lumbsch 39a, MAF-Lich. 19625, isotype (MAF)	KP822303
	ZA120816	South Africa, Crespo, Divakar, Hawksworth, Amo & Lumbsch 44e, MAF-Lich. 19628 isotype, (MAF)	KP822304
	ZA120817	South Africa, Crespo, Divakar, Hawksworth, Amo & Lumbsch 63f, MAF-Lich. 19584 holotype (MAF)	KY066279
N. capitata	CAJF821184	USA, Lendemer 9044 (NY)	JF821184
	CA140194	Brazil, Cáceres & Aptroot ISE 22138 (ISE)	MK046746
	CA140195	Brazil, Cáceres & Aptroot ISE 22207 (ISE)	MK046747
N. corallifera	CO120073	Thailand, Papong & Konhin 6601pp, 554585 (HO)	KY066260
	CO120744	Thailand, Papong 7100 (MSUT)	KY066261
	CO120302	Thailand, Papong 6483 (MSUT)	KP822264
N. crassa	IS120052	Australia, Elix 38202, CANB 800762 (CANB)	KY066265
	IS120053	Australia, Elix 38207, CANB 800763 (CANB)	KY066266
	IS120056	Australia, Elix 39795, CANB 783253 (CANB)	KP822273
	IS120057	Australia, Elix 39804, CANB 783259 (CANB)	KY066264
	IS120058	Australia, Elix 39805, CANB 783260 holotype (CANB)	KP822274
N. multifera	MU140152a	Brazil, Cáceres & Aptroot ISE 13667 (ABL)	KP822291
	MU140152b	Brazil, Cáceres & Aptroot Ise 13667 (ABL)	KP822292
	MU140198	Brazil, Cáceres & Aptroot, ISE 9559 (ISE)	KY066270
	MU140201	Brazil, Cáceres & Aptroot ISE 22119 (ISE)	KY066271
N. orientalis	OR120077	Thailand, Papong 6612, HO-554582 (HO)	KY066274
	OR120296	Thailand, Papong 6922 (MSUT)	KP822295
	OR120298	Thailand, Papong 7033 (MSUT)	KP822296
	OR120301	Thailand, Papong 6487 (MSUT)	KP822297
	ORJF821182	Thailand, Papong 6922 (MSUT)	JF821182
N. pauli	Ke1	Kenya, Kirika & Lumbsch 3821-1 holotype (EA)	KP822279
	Ke2	Kenya, Kirika & Lumbsch 3821-2 isotype (F)	KP822280
N. plurisporibadia	140189	Brazil, Cáceres & Aptroot ISE 22130 holotype (ABL)	MK046748
	140190	Brazil, Cáceres & Aptroot ISE 22161 (ABL)	MK046749
N. pulchra	PU120061	Australia, Elix 37379, CANB 803643 (CANB)	KY066277
	PU120062	Australia, Elix 38452, CANB 769060, (CANB)	KY066276
	PU120063	Australia, Elix 39560, CANB 789446 (CANB)	KP822298
	PU120064	Australia, Elix 37097, CANB 800711 (CANB)	KP822299
	PU120066	Australia, Elix 39787, CANB 781897 (CANB)	KP822300
	PU120067	Australia, Elix 39791, CANB 783250 (CANB)	KY066275
	PU120068	Australia, Elix 39798, CANB 783256 (CANB)	KY066278
	PU120069	Australia, Elix 39806, CANB 783261 (CANB)	KP822301
N. siamisidiata	130029	Thailand, P. & B. v.d. Boom 46872 (Hb. v.d. Boom)	KP822277
	130030	Thailand, P. & B. v.d. Boom 46947 (Hb. v. d. Boom)	KP822278

Species	Sample ID as in BOLD database	Voucher	Accession number ITS rDNA
Protoparmelia badia A	NA	Austria, Muggia & Hafellner 68478 (GZU)	KF562191
P. badia A	NA	Slovenia, Hafellner 71474 (GZU)	KP822209
P. badia B1	NA	Italy, Dal Grande & Singh FR 68881 (FR)	KP822251
	NA	Italy, Dal Grande & Singh FR 68882 (FR)	KP822252
	NA	Spain, v. d. Boom 46079 (Hb. v. d. Boom)	KP822242
P. badia C	NA	Spain, Crespo, Rico, Ruibal & Boluda, MAF-Lich. 19437 (MAF)	KP822260
	NA	Spain, Crespo, Rico, Ruibal & Boluda, MAF-Lich. 19438 (MAF)	KP822261
P. hypotremella	NA	Canada, Lendemer 14431B (NY)	KP822268
	NA	Canada, Lendemer 14563 (NY)	KP822269
P. memnonia	NA	Norway, Haugan 9612 (O)	KF562194
	NA	Norway, Holien 13370 (TRH)	KP822282
P. montagnei A	NA	Turkey, Crespo, Divakar, Lumbsch & Candan, MAF-Lich. 19465 (MAF)	KP822283
	NA	Turkey, Crespo, Divakar, Lumbsch & Candan, MAF-Lich. 19469 (MAF)	KP822286
P. montagnei C	NA	Spain, Crespo, Rico & Ruibal MAF-Lich. 19427 (MAF)	KP822288
	NA	Spain, Crespo, Rico & Ruibal MAF-Lich. 19428 (MAF)	KP822289
	NA	Spain, Crespo, Cubas, Núñez & Divakar, MAF-Lich. 19462 (MAF)	KY066267
	NA	Turkey, Divakar, Crespo, Candan & Lumbsch, MAF-Lich. 19467, (MAF)	KP822287
P. ochrococca	NA	USA, McCune 31673 (OSU)	KP822293
P. oleagina	NA	Norway, Johnsen, L-92691 (BG)	KY066273
	NA	Norway, Tønsberg 41328, L-92554 (BG)	KY066272

M. australiensis and the outgroup). Pairwise distances between different haplotypes were reported as the number of nucleotide substitutions per site (s/s). Average genetic distance was calculated on the BOLD workbench (Barcode of Life Data Systems, BOLD; Ratnasingham and Hebert 2007). The ITS distance was inferred based on pairwise comparisons of all sequences. ITS sequences from the candidate species circumscribed in Singh et al. (2015) and the newly generated sequences, including the voucher information, were submitted to the BOLD database, under the project name '*Neoprotoparmelia* species description'.

Morphological and chemical methods

For the samples *Maronina isidiata* A, *M. isidiata* B, *M. isidiata* C, *M. isidiata* D, *M. isidiata* E and *M. plurisporibadia* (in Singh et al. 2015), morphological examination was performed with an Olympus SZX7 and pictures were taken with Nikon Coolpix 995. Hand-made sections of ascomata and thallus were studied in water, 5% KOH (K) and/or Lugol's reagent (1% I_2) after pre-treatment with KOH (IKI). Microscopic photographs were prepared using an Olympus BX50 with Nomarski interference contrast and Nikon Coolpix 995.

For the samples *Maronina* ZA and *M.* KE, morphological examination was performed under a Nikon SMZ-1500 stereomicroscope and Nikon Eclipse-80i microscope, with bright field and DIC. Photographs were taken with a Nikon DS-Ri2 coupled to the microscope and stereomicroscope. Observations and measurements of ascospores and conidia were made in water. When possible, for each species, at least 30 spores and conidia from different specimens were measured and length width (l:b) were calculated. In the description of the new species, n (number of spores and conidia measured) are given in parentheses. Spot tests (K, C, I and Pd) and thin-layer chromatography (TLC) were carried out following Orange et al. (2010). We used TLC solvent system C (200 ml toluene / 30 ml acetic acid), with concentrated acetone extracts at 50 °C spotted on to silica gel 60 F254 aluminium sheets (Merck, Darmstadt).

Results and discussion

In the ML phylogenetic tree of Protoparmelioideae, both *Protoparmelia* and *Maronina* s.l. form supported monophyletic clades (Fig. 1). *Protoparmelia* and *Maronina* s.l. are supported as sister groups, whereas *Maronina australiensis* is sister to the *Protoparmelia-Maronina* s.l. clade. This suggests that *Maronina*, as currently circumscribed, is polyphyletic. The heterogeneous nature of *Maronina* has already been indicated by Kantvilas and Elix (2007), based on ascomatal characters. Originally, Hafellner and Rogers (1990) described two species in *Maronina*, namely *M. australiensis* from Australia and *M. multifera* from South America. Later, Kantvilas and Elix (2007) described another species, *M. hesperia*, from Australia and pointed out that *M. multifera* differs chemically and morphologically from *M. australiensis*. *Maronina australiensis* and *M. hesperia* contain depsides instead of depsidones as found in *M. multifera* and paraphyses in *M. australiensis* and *M. hesperia* are slender and mostly simple, whereas those in *M. multifera* are branched and anastomosing. The authors suggested *Maronina* s.str. to be a strictly Australian genus, comprising *M. australiensis* and *M. hesperia* only. In the present study, we support this hypothesis, based on molecular evidence, which confirms that *M. australiensis* and *M. multifera* are not closely related. Instead, the morphological and chemical properties of *M. multifera* are very similar to the other *Maronina* s.l. species, e.g. presence of depsidones and branched paraphyses. *Maronina multifera* forms a well-supported monophyletic clade with other *Maronina*. s.l. taxa (Fig. 1 this study and Singh et al. 2015, 2017 based on a 6-locus phylogeny). Based on molecular and phenotypical evidence, we thus propose to restrict the genus *Maronina* s.str. to *M. australiensis*, the type species of the genus and *M. hesperia*. In its restricted circumscription, the genus *Maronina* is currently only known from Australia. To accommodate the *Maronina* s.l. taxa, sister of *Protoparmelia*, we propose the new genus *Neoprotoparmelia* with *N. corallifera*, as the type species. The following species are here recognised in *Neoprotoparmelia*: *N. capitata*, *N. isidiata*, *N. multifera*, *N. orientalis* and *N. pulchra* and

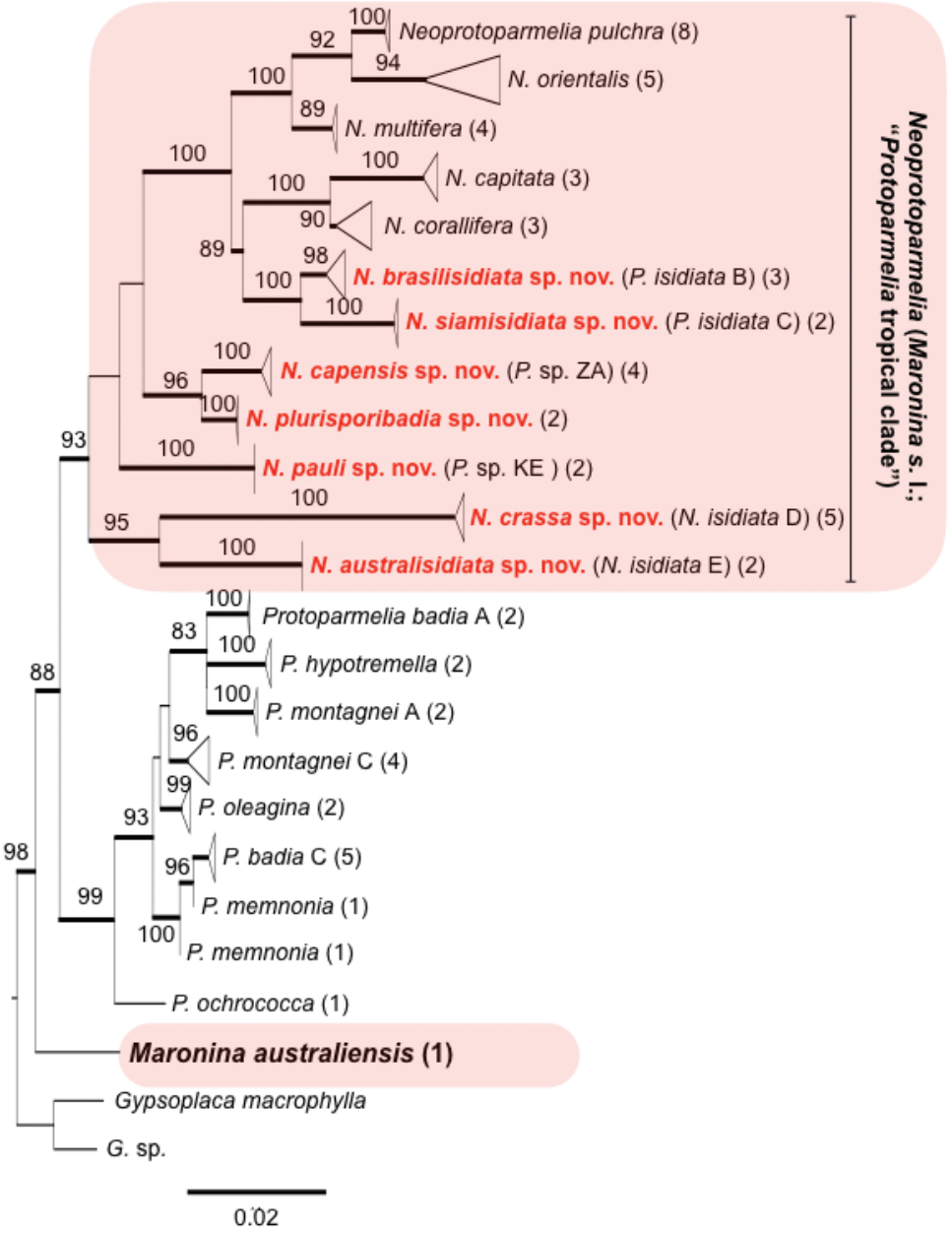

Figure 1. Phylogeny of Protoparmelioideae based on maximum likelihood analysis of ITS rDNA sequences of *Protoparmelia* and *Neoprotoparmelia* (*Maronina* s.l.). Numbers above branches indicate bootstrap support. Terminal clades were collapsed at the species level for clarity of presentation. The length of the triangle corresponds to branch lengths. Numbers in parentheses indicate number of specimens included in collapsed clade. Voucher information of each specimen in a clade is given in Table 1; New *Neoprotoparmelia* species are marked in red and bold.

eight new described species as *N. amerisidiata*, *N. australisidiata*, *N. brasilisidiata*, *N. capensis*, *N. crassa*, *N. pauli*, *N. plurisporibadia* and *N. siamisidiata*. All *Neoprotoparmelia* species are well supported in the ML tree inferred from the ITS sequences (Fig. 1). The genus occurs throughout the tropics.

The presently available data do not allow us to infer the exact phylogenetic position of *Maronina* s.str. (*M. australiensis* and *M. hesperia*). The first 30 BLAST hits of the *M. australiensis* ITS fragment suggest close affinity of *M. australiensis* to *Lecanora* species.

Distance summary

The mean intra- and inter-specific divergence was 0.56% (SE = 0.01) and 19.94 (SE = 0.01), respectively (Table 2). Our results thus show that, within species, divergence was much lower than inter-species divergence for all *Neoprotoparmelia* species (Table 2, Fig. 2). The maximum sequence divergence amongst individuals of a species was, in all cases, lower than the minimum interspecies sequence divergence, which supports the barcode-based taxonomic assignments of *Neoprotoparmelia* species (Table 2, Fig. 2). The maximum intraspecific genetic variation did not overlap with the nearest neighbour and a barcode gap was present amongst all neighbouring species (Fig. 2). Hence, we conclude that ITS is a suitable barcode marker to identify *Neoprotoparmelia* species.

Table 2. Genetic distances amongst *Neoprotoparmelia* species.

Species	Mean Intra-species distance	Max Intra-species distance	Nearest Neighbour	Distance to NN
The mean, maximum intra-specific distances and distance to the nearest neighbour				
Neoprotoparmelia capitata	0.43	0.69	*N. corallifera*	3.66
N. corallifera	1.39	1.92	*N. capitata*	3.66
N. brasilisidiata	1.97	2.95	*N. siamisidiata*	7.49
N. siamisidiata	0.54	0.54	*N. brasilisidiata*	7.49
N. crassa	0.23	0.71	*N. corallifera*	16.65
N. australisidiata	0.0	0.0	*N. corallifera*	18.45
N. pauli	0.0	0.0	*N. plurisporibadia*	13.09
N. multifera	0.25	0.64	*N. corallifera*	7.12
N. orientalis	1.75	3.57	*N. pulchra*	5.95
N. pulchra	0.32	0.72	*N. orientalis*	5.95
N. capensis	0.58	1.61	*N. plurisporibadia*	9.02
N. plurisporibadia	0.16	0.16	*N. capensis*	9.02
Intra-species and inter-species genetic distances				
Category	Minimum distance (%)	Mean distance (%)	Maximum distance (%)	SE distance
Intraspecific	0.00	0.56	3.57	0.01
Interspecific	3.66	19.94	30.34	0.01

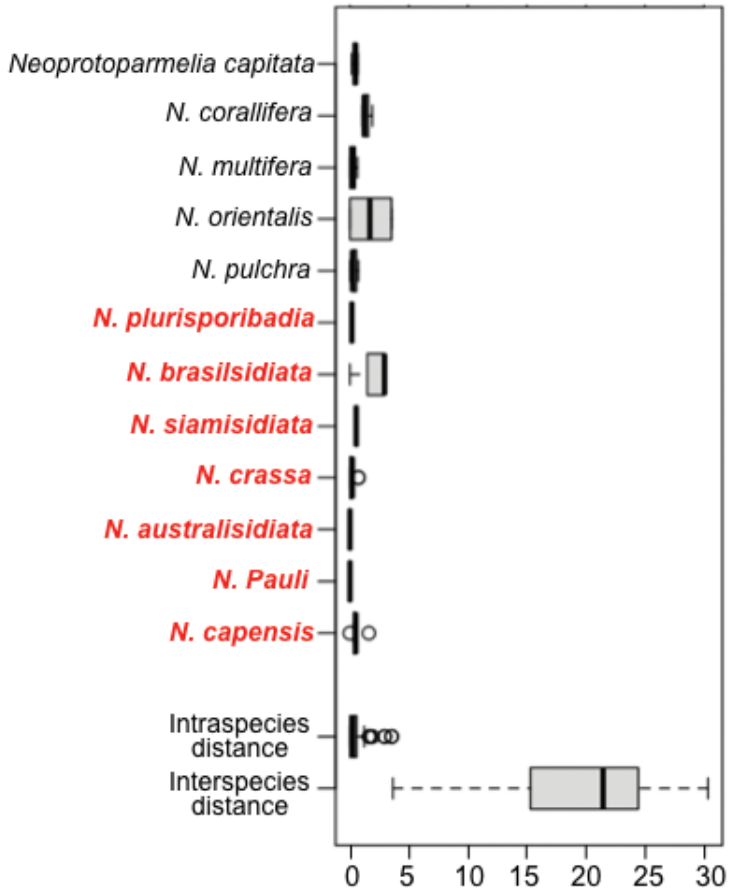

Figure 2. Boxplot showing the intra- and interspecific genetic distances of *Neoprotoparmelia* species and the overall intraspecific distances from all species and pairwise interspecific distances. New species described in this study are marked in red.

Taxonomic conclusions

Maronina **Hafellner & R. W. Rogers, Biblioth. Lichenol. 38: 100. 1990**
MycoBank no.: MB25517
Figure 3

Type species. *Maronina australiensis* Hafellner & R. W. Rogers. Type. AUSTRALIA (Fig. 3). Queensland, Tandora about 25 km ENE of Maryborough, sea level, 25°27'S, 152°52'E, mangroves, on *Rhizophora stylosa*, 23 August 1986, J. Hafellner 17823 & R. W. Rogers (holotype GZU).

Based on molecular and phenotypical evidence, we propose *Maronina* s.str. to be a strictly Australian genus, comprising *M. australiensis* and *M. hesperia* Kantvilas & Elix

Figure 3. *Maronina australiensis* (type species of *Maronina*), holotype Hafellner 17823 & Rogers (GZU). Scale bar: 1 mm.

only, as was suggested by Kantvilas and Elix (2007)) and Kantvilas et al. (2010). The genus *Maronina* contains depsides instead of depsidones as found in *Neoprotoparmelia*. Paraphyses in *Maronina* are slender and mostly simple, whereas those in *Neoprotoparmelia* are branched and anastomosing.

Neoprotoparmelia **Garima Singh, Lumbsch & I. Schmitt, gen. nov.**
MycoBank no.: MB826940
Figures 4–13

Type species. *Neoprotoparmelia corallifera* (Kantvilas & Papong) Garima Singh, Lumbsch & I. Schmitt

Etymology. Derived from the Greek *neos* (=new) and its close relationship to *Protoparmelia*.

Diagnosis. Thallus crustose. Apothecia lecanorine, broadly adnate to sessile; thalline margin distinct. Proper excipulum cupulate, hyaline. Asci 8- to multispored, clavate, variations of the *Lecanora*-type (Hafellner 1984, Kantvilas and Elix 2007, Kantvilas et al. 2010). *Paraphyses* sparingly branched and anastomosing; apices clavate and brown-pigmented. Ascospores ellipsoid to fusiform to elongate, non-halonate. Pycnidia immersed, globose. Conidia bacilliform.

Chemistry. *Neoprotoparmelia* species mainly produce depsidones of the alectoronic acid chemosyndrome.

Distribution and ecology. The taxa of this genus occur in open habitats, mostly on bark, with only a few species growing on siliceous rock. This genus has a Pantropical distribution and is currently known from Australia, Brazil, Kenya, Papua New Guinea, South Africa, Thailand and south-eastern USA.

Remarks. The new genus is morphologically similar to *Maronina* but can be distinguished by containing depsidones instead of depsides as found in *Maronina* and branched paraphyses. The genus is morphologically similar to *Protoparmelia* but was recognised as "tropical *Protoparmelia* clade" in Singh et al. (2015). The asci are essentially variations of the *Lecanora*-type sensu Hafellner (1984), and mainly coincides with those well studied by Kantvilas and Elix (2007) and Kantvilas et al. (2010). A detailed illustration of the ascus of *N. pulchra* is given in Aptroot et al. (1997a: 148, fig. 101a); it is similar to the ascus illustrated of *Protoparmelia badia* by Hafellner (1984: 393, fig.40).

Neoprotoparmelia amerisidiata **Garima Singh & Aptroot, sp. nov.**
MycoBank no.: MB827474
Figure 4

Type. USA. Georgia, McIntosh Co., Sapelo Island, Sapelo Island Wildlife Management Area, 31°26'00"N, 81°22'10"W, on bark of *Quercus*, 16 December 2009, J. Lendemer 20995 (holotype: NY).

Diagnosis. Similar to *Neoprotoparmelia brasilisidiata*, but differing by the thicker, 0.07–0.11 mm wide, isidia.

Etymology. Named after its distribution in North America and the presence of isidia.

Description. Thallus up to ca. 0.05 mm thick, shiny, pale olive-green to olive-grey, continuous, delimiting marginal prothallus line (brown, thin or absent). Isidia always numerous, initially widely dispersed or somewhat clustered, eventually covering much of the thallus, up to 1.5 mm long, persistently 0.07–0.11 mm wide over their whole length, cylindrical, usually irregularly repeatedly branched and somewhat nodulose, glossy, pale olive-green to olive-grey, tips distinctly brown and dull. Apothecia and pycnidia not observed.

Chemistry. Spot tests: medulla of thallus and isidia UV++ greenish-white, C–, P–, K–, KC+ pink. TLC: alectoronic acid (major), dehydroalectoronic acid (minor or trace) and β-alectoronic acid (trace).

Distribution and ecology. On tree bark in forest. Known only from the south-eastern USA (North Carolina, Alabama, Georgia, Mississippi and Florida).

Reference sequences. (specimen: Lendemer 20995, holotype: NY). KY012827 (mtSSU), KY066301(nuLSU).

Remarks. This species comprises the specimens recovered within '*P. isidiata* A' in '*Protoparmelia* tropical clade' in Singh et al. (2015). It is morphologically most similar to *N. brasilisidiata* which only differs by the generally thinner isidia. Some specimens have been reported before as *Protoparmelia isidiata* (Lendemer and Lumbsch 2008).

Additional specimens examined. USA. Florida, Gilchrist Co., Waccasassa Flats, 5 December 1993, R.C. Harris 31685, 31755 (NY), R.C. Harris 31685 (NY); USA. Georgia, McIntosh Co., Sapelo Island, Sapelo Island Wildlife Management Area, 15 December 2009, J. Lendemer 20745, 20727 (NY).

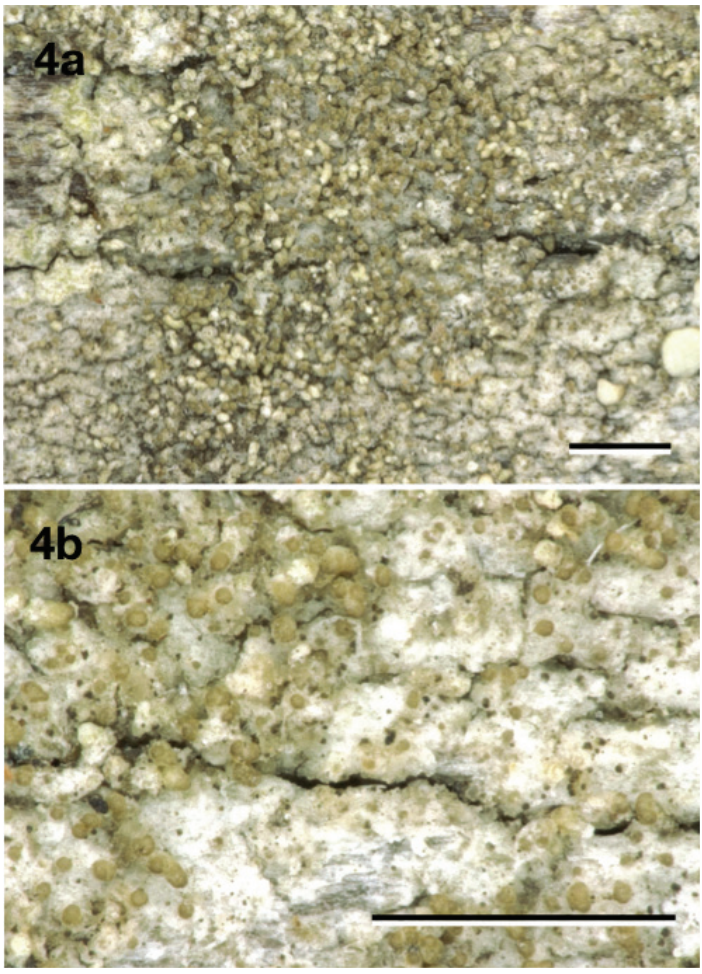

Figure 4. *Neoprotoparmelia amerisidiata*, holotype Lendemer 20995 (NY). Scale bar: 1 mm.

Neoprotoparmelia australisidiata **Garima Singh & Aptroot, sp. nov.**
MycoBank no.: MB826943
Figure 5

Type. AUSTRALIA. Northern Territory, 2 km N of Emerald Springs, 13°37'23"S, 131°36'40"E, on *Erythrophloeum chlorostachys*; 22 September 2007, G. Kantvilas 289/07 (holotype: HO 545660).

Diagnosis. Similar to *Neoprotoparmelia isidiata*, but differing by the larger number of isidia per thallus areole.

Etymology. Named after Australia and the presence of isidia.

Description. Thallus consisting of almost contiguous, flat to convex areoles with irregular shape, of up to ca. 0.1 mm thick and 0.7 mm wide, somewhat shiny, pale brown to dark brown or pale olive-green to olive-grey, marginal prothallus black, thin

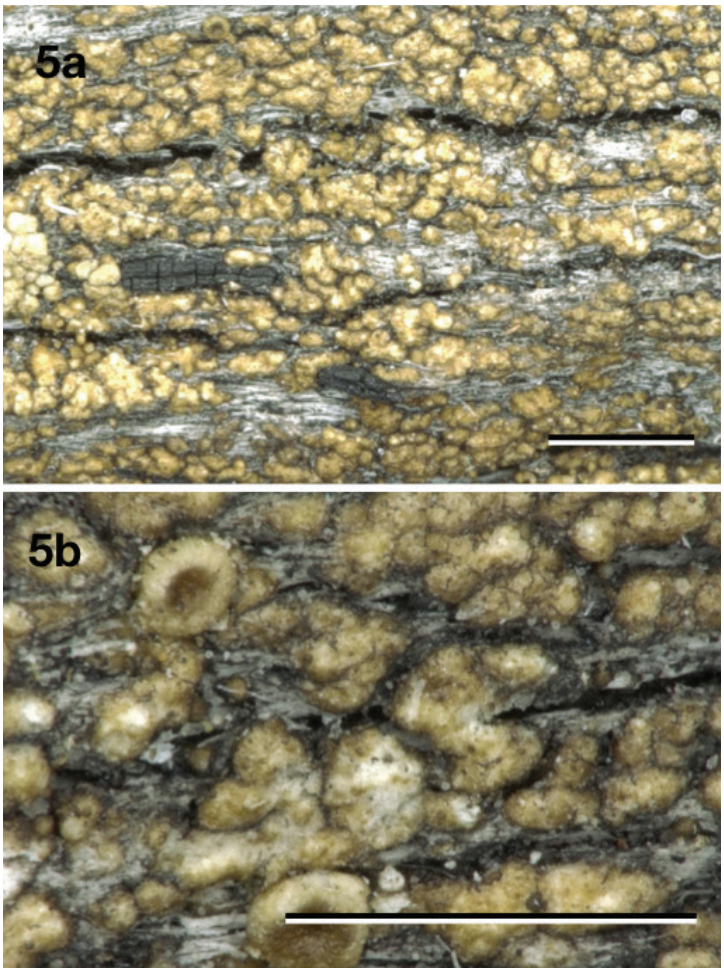

Figure 5. *Neoprotoparmelia australisidiata*, holotype Kantvilas 289/07 (HO 545660). Scale bar: 1 mm.

or absent. Isidia usually in groups on almost each thallus areole, up to 0.9 mm long, persistently 0.07–0.1 mm wide over their whole length, cylindrical, usually rather irregularly once or more rarely repeatedly branched and somewhat nodulose, somewhat shiny, pale to dark brown or pale olive-green to olive-grey, of thallus colour, tips not darkened or somewhat brown. Apothecia (only young ones observed) sessile, round, 0.4–0.6 mm diam., disc concave to flat, smooth, glossy, orange brown. Margin glossy, ca. 0.05 mm wide, glossy brown at the outside, slightly higher than the disc. Hymenium hyaline, not inspersed with oil droplets, up to 50 µm high; epihymenium fuscous brown, pigment in K becoming soluble and paler; hypothecium hyaline, up to 90 µm thick including subhymenium; excipulum hyaline throughout, with a 5–12 µm thick layer of cortex, without crystals, with algae, extending below the hypothecium (cupulate). Paraphyses branched, ca. 2.5 µm wide, not thickened at the tips. Mature asci and ascospores not observed. Pycnidia not observed.

Chemistry. Spot tests: medulla of thallus and isidia C–, P–, K–, KC+ pink, UV+ greenish-white. TLC: alectoronic acid (major), dehydroalectoronic acid (minor or trace) and β–alectoronic acid (trace).

Distribution and ecology. On wood or bark of trees in open or closed forests. Known only from Australia (Northern Territory & New South Wales).

Reference sequences. (specimen: Kantvilas 289/07, holotype: HO 545660). KP822276 (ITS), KP822466 (mtSSU), KP823523 (*TSR1*).

Remarks. This species comprises the specimens recovered within '*P. isidiata* E' in '*Protoparmelia* tropical clade' in Singh et al. (2015) and referred to as *Maronina* in Divakar et al. (2017) and Singh et al. (2018). Coalescent-based species delimitation inferred from the six-locus dataset supports these taxa as distinct lineage from the other isidiate samples collected from the geographically distant populations. This species is morphologically very similar to *Neoprotoparmelia isidiata*, but has larger and contiguous thallus areoles, usually bearing more isidia. Members of this species may differ considerably in colour and the abundance and maximum length of the isidia.

Additional specimens examined. AUSTRALIA. New South Wales, Maxwells Flora Reserve, S of Eden, 195 m alt., 26 October 2010, G. Kantvilas 228/10 (HO 559228).

Neoprotoparmelia brasilisidiata **Garima Singh, M. Cáceres & Aptroot, sp. nov.**
MycoBank no.: MB826944
Figure 6

Type. BRAZIL. Sergipe, Parque Nacional Serra de Itabaiana, 10°44'57"S, 37°20'20"W, ca. 200 m alt., on bark of tree, 10 May 2014, M. Cáceres & A. Aptroot 21684 (holotype: ISE, isotype: ABL).

Diagnosis. Very similar to *Neoprotoparmelia amerisidiata*, but differing by having thinner, 0.04–0.08 mm wide, isidia.

Etymology. Named after the country of discovery, Brazil and the presence of isidia.

Description. Thallus up to ca. 0.05 mm thick, shiny, pale olive-green to olive-grey, continuous, marginal prothallus brown, thin or absent. Isidia always numerous, initially widely dispersed or somewhat clustered, eventually covering much of the thallus, up to 1.5 mm long, persistently 0.04–0.08 mm wide over their whole length, cylindrical, usually rather irregularly repeatedly branched and somewhat nodulose, glossy, pale olive-green to olive-grey, tips distinctly brown and dull. Apothecia sessile, round or usually with wavy outline, 0.6–1.3 mm diam., disc flat, smooth, dull, dark brown. Margin dull, ca. 0.15 mm wide, of thallus colour, not or only slightly higher than the disc. Hymenium hyaline, not inspersed with oil droplets, up to 80 µm high; epihymenium olive-brown, pigment in K becoming soluble and paler; hypothecium hyaline, up to 75 µm thick including subhymenium; excipulum hyaline throughout, with a 7–15 µm thick layer of cortex without crystals, with algae, extending below the hypothecium (cupulate). Paraphyses branched, ca. 2.0 µm wide, not thickened at the

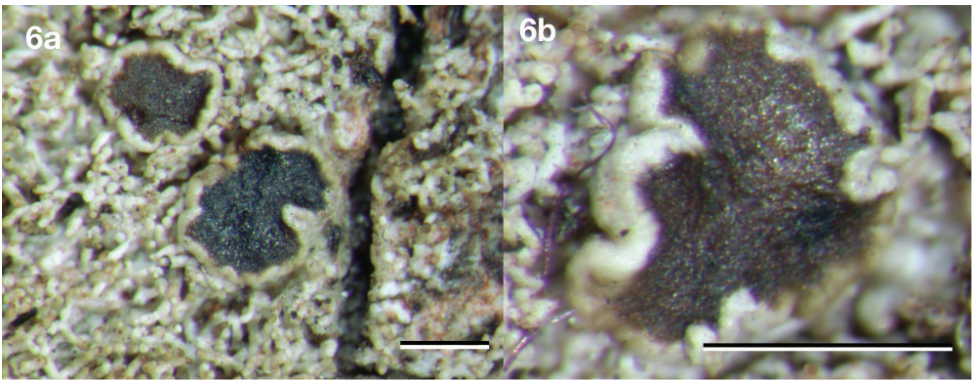

Figure 6. *Neoprotoparmelia brasilisidiata*, holotype Cáceres & Aproot 21684 (ISE). Scale bar: 1 mm.

tips. Asci 8-spored, cylindrico-clavate, up to 55 × 13 µm. Ascospores hyaline, simple, narrowly ellipsoid, not constricted, 9–11 × 2–3 µm, without appendages. Pycnidia not observed.

Chemistry. Spot tests: medulla of thallus and isidia UV+ greenish white, C–, P–, K–, KC+ pink. TLC: alectoronic acid (major), dehydroalectoronic acid (minor or trace) and β-alectoronic acid (trace). Gyrophoric acid has also been reported (Kalb 2004).

Distribution and ecology. On tree bark in parks, open areas, Cerrado and Atlantic rain forests. Neotropical - known from Costa Rica, El Salvador and Brazil, where it is widespread and known from the following states: Sergipe, Matto Grosso, Rio de Janeiro, São Paulo, Maranhão, Tocantins, Minas Geraes and Rio Grande do Sul.

Reference sequences. (specimen: Aptroot 21684, holotype: ISE). KY012831 (mtSSU), KY066305 (nuLSU).

Remarks. This species comprises specimens recovered within '*P. isidiata* B' in '*Protoparmelia* tropical clade' in Singh et al. (2015). It is similar to *N. amerisidiata*, but, however, differs in having slightly thinner isidia. It is a common species on exposed bark in the neotropics and can easily be recognised in the field, from other isidiate crusts even when sterile, due to the strong UV-reaction visible with a portable UV-torch and thus can be distinguished from other isidiate crusts, even when sterile.

Additional specimens examined. BRAZIL. Rio Grande do Sul, Viamão, near Parque Itapua, ca. 100 m alt.; 26 September 2014, M. Cáceres & A. Aptroot 22137 (ABL, ISE); Maranhão, Bananal, 20 km S of Imperatriz, ca. 140 m alt.; 20 October 2016, M. Cáceres & A. Aptroot 28776 (ABL, ISE). Tocantins, near Itaguatins, ca. 150 m alt.; 22 October 2016, M. Cáceres & A. Aptroot 28809 (ABL, ISE). COSTA RICA. Guanacaste, 15 km SSE of Nicoya, ca. 850 m alt.; 22 March 2004, H. Sipman 52086 (B), A. Aptroot 60835, 60836 & 60840 (INB). SAN SALVADOR. Ahuachapán, Parque Nacional El Imposible, ca. 800 m alt.; December 1998, R. Welz 89, 140 & 438 (B).

Neoprotoparmelia capensis **V. J. Rico, A. Crespo & Garima Singh, sp. nov.**
MycoBank no.: MB826945
Figure 7

Type. SOUTH AFRICA. Western Cape prov., between Papendorp and Strandfontein, near Vailkay bridge, 31°41'34"S, 18°13'59"E, ca. 32 m alt., 4 February 2005, A. Crespo, P.K. Divakar, D.L. Hawksworth, G. Amo & T.H. Lumbsch 63f (holotype: MAF–Lich. 19584; isotypes: MAF-Lich. 19624, 19625, 19626 and 19628).

Diagnosis. Morphologically similar to the northern hemispheric *Protoparmelia montagnei* (Fr.) Poelt & Nimis, but mainly differing from it by the presence of alectoronic acid as major secondary metabolite in the medulla. The two species, *P. montagnei* and *N. capensis*, are also genetically not closely-related and belong to different genera.

Etymology. The specific epithet refers to its occurrence in Cape Province of South Africa.

Description. Thallus saxicolous, crustose, up to 8 cm wide, thin and areolate (in younger parts, up to 1 mm thick) to mainly thick and areolate, warted or subsquamulose (up to 2.2 mm thick), irregular or orbicular; surface light grey, pale to strong brown, with whitish mottled-fissured areas (by a locally strong mucilaginous epicortex), dull; delimited, or not, by a blackish hypothalline line. Areoles irregular, polygonal to rounded, up to 2 mm in diam., mainly slightly convex to irregular or flat, surface smooth to irregular, cracked or warted, marginal areoles sometimes lobe-like. Apothecia frequent, 1 to several per areolae, zeorine to lecanorine, immersed and nearly urceolate when young to adnate or sessile and constricted at the base when adult, rounded to irregular, up to 2 mm in diam.; disc brown to brown-black, dull, concave to flat or sometimes convex; thalline exciple persistent or excluded with age, concolorous with thallus to whitish (by a strong mucilaginous epicortex); proper exciple cupulate, up to 70–155 µm thick, coherent, hyphae mainly periclinal with strong mucilaginous walls, margins reduced in young apothecia. Hymenium hyaline to yellowish, coherent, 60–75 µm tall, in the margins somewhat fan-like (together with proper exciple) and exceeding the thalline exciple in adult apothecia; epihymenium light brown to brown, up to 15 µm tall, with few irregular granules; hypothecium and subhymenium hyaline to slightly yellowish, 25–70 µm thick. Paraphyses coherent in water, branched and anastomosed, apices somewhat thickened and mainly surrounded by a brown mucilaginous hood (up to 10 µm wide). Asci clavate, 42–70 × 12–20 µm, 8–spored, amyloid tholus (excluding the axial mass) and surrounding mucilage, *Lecanora*–type (cf. also *Maronina*–type, Kantvilas et al. 2010). Ascospores hyaline, simple, 9–13(–14) × 3.5–5.5(–6) µm (n = 40), fusiform to elongate (l:b = 1.8–2.9), with rounded apices or sometimes slightly apiculate in one end, some with apical hyaline setae. Pycnidia frequent, immersed, globose to oblong, wall hyaline, ostiole tissue with brown to black pigmented walls. Conidia simple, hyaline, 7–17 × 1–1.5 µm (n = 20), bacilliform, straight.

Chemistry. Spot tests: medulla K– or ± unclean yellowish, C–, KC+ unclean rose-red, I–, P–, UV++ greenish-white. TLC: atranorin (traces), α–alectoronic acid (major),

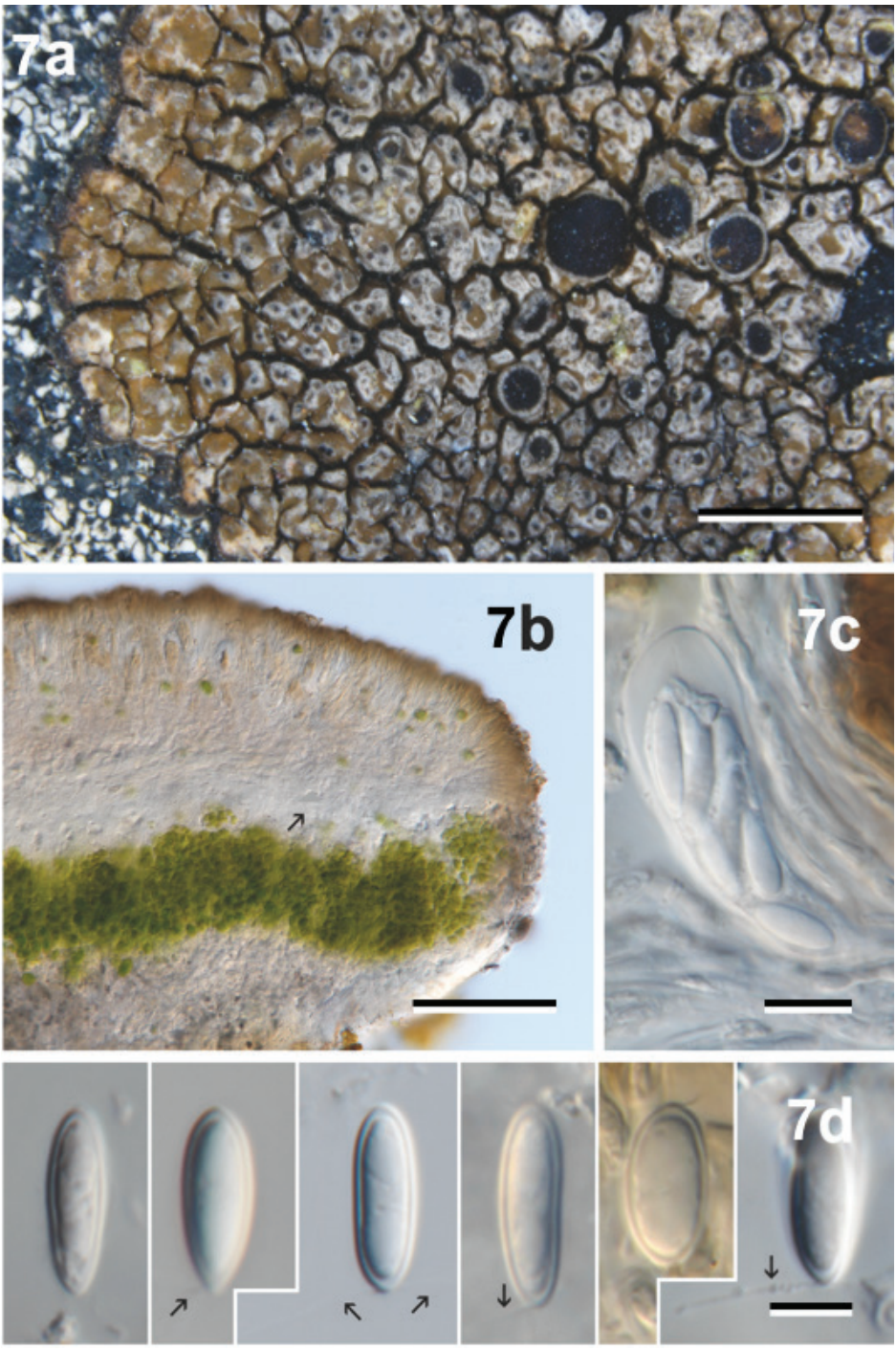

Figure 7. *Neoprotoparmelia capensis*, holotype Crespo, Divakar, Hawksworth, Amo & Lumbsch 63f (MAF-Lich. 19584). **a** Habit **b** Section through centre of apothecium, showing cupular proper exciple (arrow) **c** Ascus **d** Spores, showing setae (arrow). Scale bars: 2 mm (**a**), 100 µm (**b**), 10 µm (**c**), 5 µm (**d**).

unidentified substance (major or traces, closed to norstictic acid, Rf class 4), ± β–alectoronic (traces) and traces of related substances.

Distribution and ecology. Only known from the type locality in the arid northwest of the Cape Region (South Africa), rich in succulent plants (succulent Karoo biomes, cf. Mucina and Rutherford 2006), growing on exposed sandstones next to the Atlantic coast.

Reference sequences. (specimen: Crespo, Divakar, Hawksworth, Amo & Lumbsch 63f, holotype: MAF–Lich. 19584)**.** KY066279 (ITS), KP822500 (mtSSU), KP796385 (nuLSU), KP822184 (RPB1), KP823556 (TSR1).

Remarks. This comprises the specimens recovered within '*P.* sp. ZA' in '*Protoparmelia* tropical clade' in Singh et al. (2015). *Neoprotoparmelia capensis* is morphologically similar to the *Protoparmelia montagnei* complex, in the sister genus *Protoparmelia*, but differs from the latter in its chemistry and distribution. The major secondary metabolite found in *N. capensis* is alectoronic acid whereas, in *P. montagnei*, it is lobaric and/or gyrophoric acids or fatty acids. *Protoparmelia montagnei* is distributed in Eurasia on acid rocks, with mainly a broad Mediterranean distribution, from Turkey to The Canary Islands and from Ireland to Morocco (Coppins and Chambers 2009, Barbero et al. 2006). In contrast, *N. capensis* grows on sandstone in the Cape Region. Molecular data also clearly supports *N. capensis* and *P. montagnei* as distantly related, evolutionary independently lineages (Singh et al. 2015). Details on the morphology and chemistry of the similar *P. montagnei* species complex can be found in Coppins and Chambers (2009) and Barbero et al. (2006). The grey to brown thalli, 8-spored asci, α–collatolic acid absence, distribution and/or molecular data, supports *N. capensis* as an evolutionary independent lineage from the other two saxicolous *Neoprotoparmelia* species here described.

The analysed material of *Neoprotoparmelia capensis* was rich in lichenicolous ascomycetes, some of which make its characterisation confusing. Portions of the studied specimens serve as host to species of *Phacographa* and *Sphaerellothecium* similar to those living on taxa of the *Protoparmelia badia* complex (Hafellner 2009 and Triebel 1989, respectively), causing visible symptoms. A *Phoma*–type fungus, with hyaline pycnidia and conidia, frequently infected the hymenium of *N. capensis*. Moreover, in some adult apothecia of *N. capensis*, an endohymenial *Arthonia* species develops its asci, together with those of the host. The latter two taxa lacked visible symptoms on the host. These four lichenicolous fungi are currently under further investigation and the results will be published in a subsequent study.

Neoprotoparmelia capitata (Lendemer) Garima Singh, Lumbsch & I. Schmitt, comb. nov.

MycoBank no.: MB827475

Basionym. *Protoparmelia capitata* Lendemer, Lichenologist 40: 332. 2008.

Synonym. *Maronina capitata* (Lendemer) Divakar, A. Crespo & Lumbsch in Divakar et al., Fungal Diversity 84: 114. 2017.

***Neoprotoparmelia corallifera* (Kantvilas & Papong) Garima Singh, Lumbsch & I. Schmitt, comb. nov.**
MycoBank no.: MB827476
Figure 8

Basionym. *Maronina orientalis* var. *corallifera* Kantvilas & Papong in Kantvilas et al., Lichenologist 42: 557. 2010.

Synonyms. *Protoparmelia corallifera* (Kantvilas & Papong) Kantvilas, Papong & Lumbsch in Papong et al., Lichenologist 43: 561–567. 2011. *Maronina corallifera* (Kantvilas & Papong) Divakar, A. Crespo & Lumbsch in Divakar et al., Fungal Diversity 84: 114. 2017.

Type. Thailand, Phu Pha Kham, Muk Dahan Province, Nhong Sung District, 16°46'N, 104°43'E, in dry dipterocarp forest, 310 m altitude, 21 June 2009, K. Papong & W. Konhin 6603 p.p.

Figure 8. *Neoprotoparmelia corallifera* (type species *Neoprotoparmelia*), sample Papong 7100. Scale bar: 1 mm.

***Neoprotoparmelia crassa* Garima Singh & Aptroot, sp. nov.**
MycoBank no.: MB827477
Figure 9

Type. AUSTRALIA. Australian Capital Territory, Solar Village, J.A. Elix 39805 (holotype: CANB 783260).

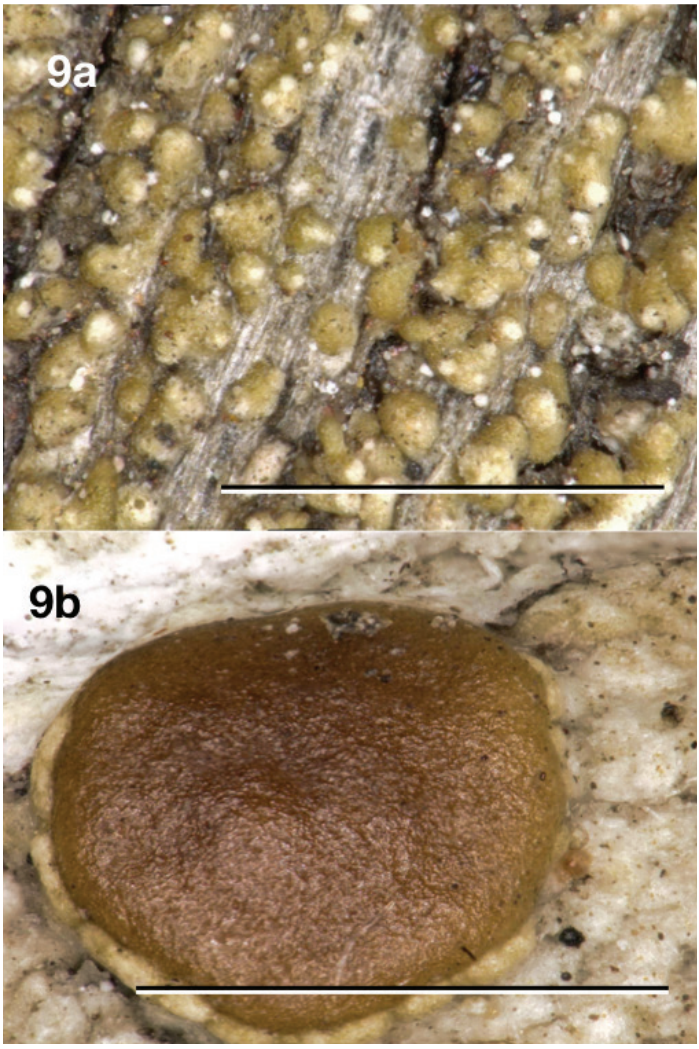

Figure 9. *Neoprotoparmelia crassa* Elix39818. Scale bar: 1 mm.

Diagnosis. Similar to *Neoprotoparmelia isidiata*, but differs from it in having shorter isidia and a thicker thallus.

Etymology. Derived from *crassus* (Lat. = fat) indicating that the thallus is thicker than that of the other isidiate species.

Description. Thallus consisting of contiguous to centrally fusing, flat to rather convex areoles with irregular shape, of up to ca. 0.1 mm thick and 0.3 mm wide, somewhat shiny, pale brown to dark brown, marginal prothallus absent. Isidia covering most of the thallus except the outer margins, globose to ellipsoid, up to 0.15 mm long, persistently 0.07–0.1 mm wide, unbranched, of thallus colour, tips not darkened or somewhat brown. Apothecia and pycnidia not observed.

Chemistry. Spot tests: medulla of thallus and isidia UV+ greenish white, C–, P–, K–, KC+ pink. TLC: alectoronic acid.

Distribution and ecology. On wood or bark of trees in open or closed forests. Known only from Australia (Australian Capital Territory and Northern Territory).

Reference sequences. (specimen: Elix 39805, holotype: CANB 783260). KP822464 (mtSSU), KP822274 (ITS), KP796345 (nuLSU), KP822145 (RPB1), KP822359 (MCM7), KP823521 (TSR1).

Remarks. This comprises the specimens recovered within '*P. isidiata* D' in '*Protoparmelia* tropical clade' in Singh et al. (2015). Similar to *Neoprotoparmelia isidiata* but differing in having a thicker thallus and shorter isidia.

Additional sequenced specimens examined. AUSTRALIA. Same as type, J. A. Elix 39795 (CANB); Northern Territory, Melville Island, H. Streimann 42469 (B, CANB).

***Neoprotoparmelia isidiata* (Diederich, Aptroot & Sérus.) Garima Singh, Lumbsch & I. Schmitt, comb. nov.**
MycoBank no.: MB827478
Figure 10

Basionym. *Protoparmelia isidiata* Diederich, Aptroot & Sérus. in Aptroot et al., Biblioth. Lichenol. 64: 146. 1997.

Synonym. *Maronina isidiata* (Diederich, Aptroot & Sérus.) Divakar, A. Crespo & Lumbsch in Divakar et al., Fungal Diversity 84: 114 (2017).

Type. PAPUA NEW GUINEA. Simbu, Mount Wilhelm, near lake Piunde, 5°47'S, 145°03'E, ca. 3600 m alt.; 5–8 August 1992, A. Aptroot 31494 (holotype: BR).

Description. Thallus consisting of isolated convex areoles of up to ca. 0.1 mm thick and 0.2 mm wide, somewhat shiny, pale brown to dark brown or mottled whitish-grey, on a fully immersed hyaline hypothallus, marginal prothallus black, thin or absent. Isidia usually solitary on almost each thallus areole, up to 0.5 mm long, persistently 0.07–0.1 mm wide over their whole length, cylindrical, usually rather irregularly once or more rarely repeatedly branched and somewhat nodulose, glossy, pale to dark brown, tips dark brown to almost black. Apothecia sessile, initially round, older ones usually with wavy outline, 0.6–3.5 mm diam., disc flat, smooth, glossy, dark brown to orange brown. Margin glossy, ca. 0.25 mm wide, glossy brown at the outside, not or only slightly higher than the disc. Hymenium hyaline, not inspersed with oil droplets, up to 70 µm high; epihymenium fuscous brown, pigment in K becoming soluble and paler; hypothecium hyaline, up to 120 µm thick including subhymenium; excipulum hyaline throughout, with a 20–30 µm thick layer of cortex, without crystals, with algae, extending below the hypothecium (cupulate). Paraphyses branched, ca. 2.5 µm wide, not thickened at the tips. Asci cylindrico-clavate, up to 35 × 9 µm, with 8 mostly biseriate ascospores. Ascospores hyaline, simple, narrowly ellipsoid, not constricted, (9–)11–13(–17) × 2–3 µm, without appendages. Pycnidia not observed.

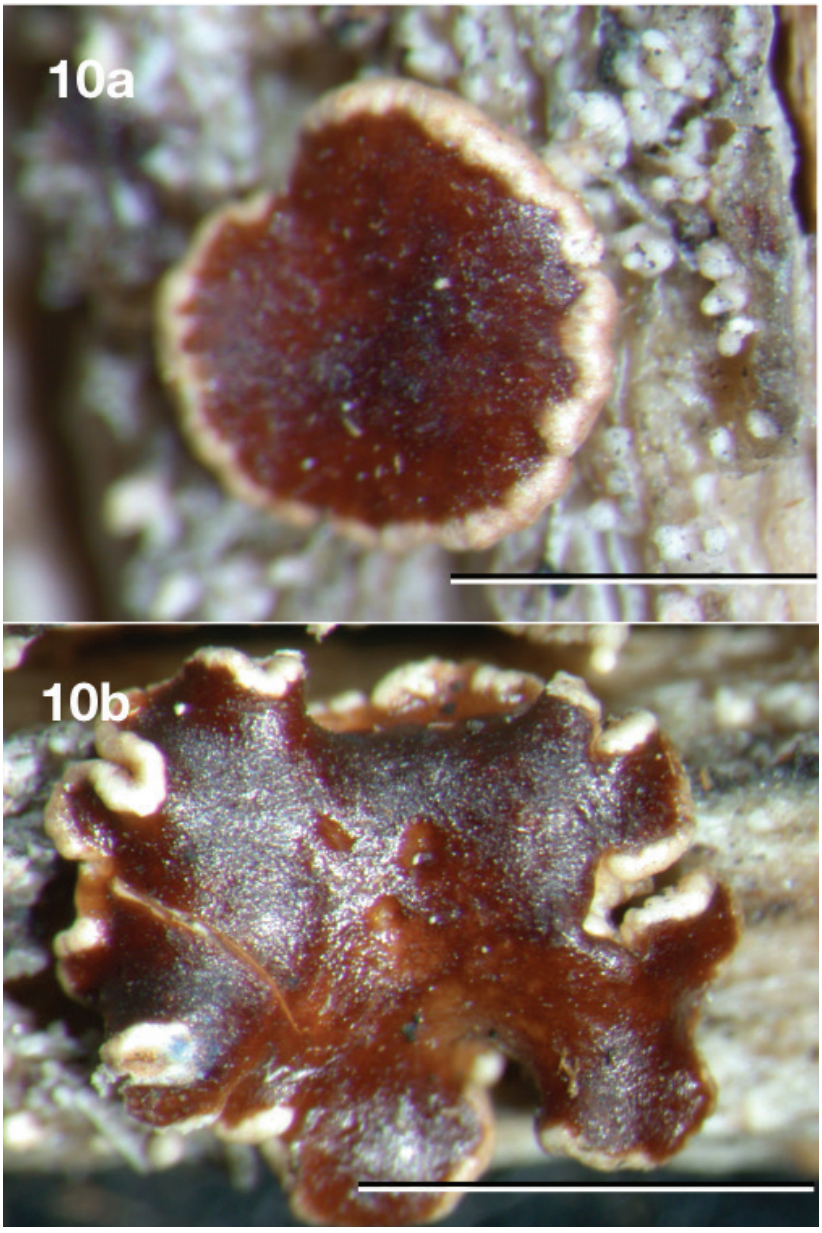

Figure 10. *Neoprotoparmelia isidiata*, holotype Aptroot 31494 (BR). Scale bar: 1 mm.

Chemistry. Spot tests: medulla of thallus and isidia UV++ greenish-white, C–, P–, K–, KC+ pink. TLC: alectoronic acid (major), dehydroalectoronic acid (minor or trace) and β-alectoronic acid (trace).

Distribution and ecology. On bark of trees in forests. Known from Papua New Guinea only.

Remarks. This species differs from the other species by having a thallus consisting of tiny areoles, generally bearing just one isidium each and by large apothecia.

Additional specimens examined. PAPUA NEW GUINEA. Simbu, Mount Wilhelm, near lake Piunde, ca. 3600 m alt.; 5–8 August 1992, A. Aptroot 32711 (BR); P. Diederich 10359 (Hb. Diederich); March 1987, A. Aptroot 18353 (BR).

Neoprotoparmelia multifera **(Nyl.) Garima Singh, Lumbsch & I. Schmitt, comb. nov.**
MycoBank no.: MB827479

Basionym. *Lecanora multifera* Nyl., Acta Soc. Sci. Fenn. 7: 445. 1863.

Synonyms. *Maronea multifera* (Nyl.) Vain., Acta Soc. Fauna Flora Fenn. 7: 100. 1890. *Maronina multifera* (Nyl.) Hafellner & R.W. Rogers, Biblioth. Lichenol. 38: 106. 1990. *Protoparmelia multifera* (Nyl.) Kantvilas, Papong & Lumbsch in Papong et al., Lichenologist 43: 566. 2011.

Neoprotoparmelia orientalis **(Kantvilas & Papong) Garima Singh, Lumbsch & I. Schmitt, comb. nov.**
MycoBank no.: MB827480

Basionym. *Maronina orientalis* Kantvilas & Papong in Kantvilas et al., Lichenologist 42: 557. 2010.

Synonym. *Protoparmelia orientalis* (Kantvilas & Papong) Kantvilas, Papong & Lumbsch in Papong et al., Lichenologist 43: 566. 2011.

Neoprotoparmelia pauli **V. J. Rico, Lumbsch & Garima Singh, sp. nov.**
MycoBank no.: MB827481
Figure 11

Type. KENYA. Eastern Prov., Mwingi Co., Nuu Hill, 01°02'S, 38°20'E, ca. 1000 m alt., inselberg with dry woodland dominated by *Terminalia*, *Combretum* and *Acacia*, on sandstone, 12 March 2014, P.M. Kirika & H.T. Lumbsch 3821 (holotype: EA, isotype: F).

Diagnosis. Similar to *Neoprotoparmelia capensis* but differs from it by having a reduced, olive tinged thallus and smaller apothecia. Moreover, the major secondary metabolite produced by *Neoprotoparmelia pauli* is α–collatolic acid, absent in *N. capensis*.

Etymology. The new species is named after our colleague, the Kenyan lichenologist, Paul M. Kirika, who was one of the collectors of the type material.

Description. Thallus saxicolous, crustose, up to 3 cm wide, rimose to areolate, thin (up to 0.8 mm thick); surface dark brown, olive-brown to light olive-brown, sometimes with whitish mottled-fissured areas (by a locally strong mucilaginous epicortex), dull to slightly shiny; blackish hypothalline line blackish or absent. Areoles

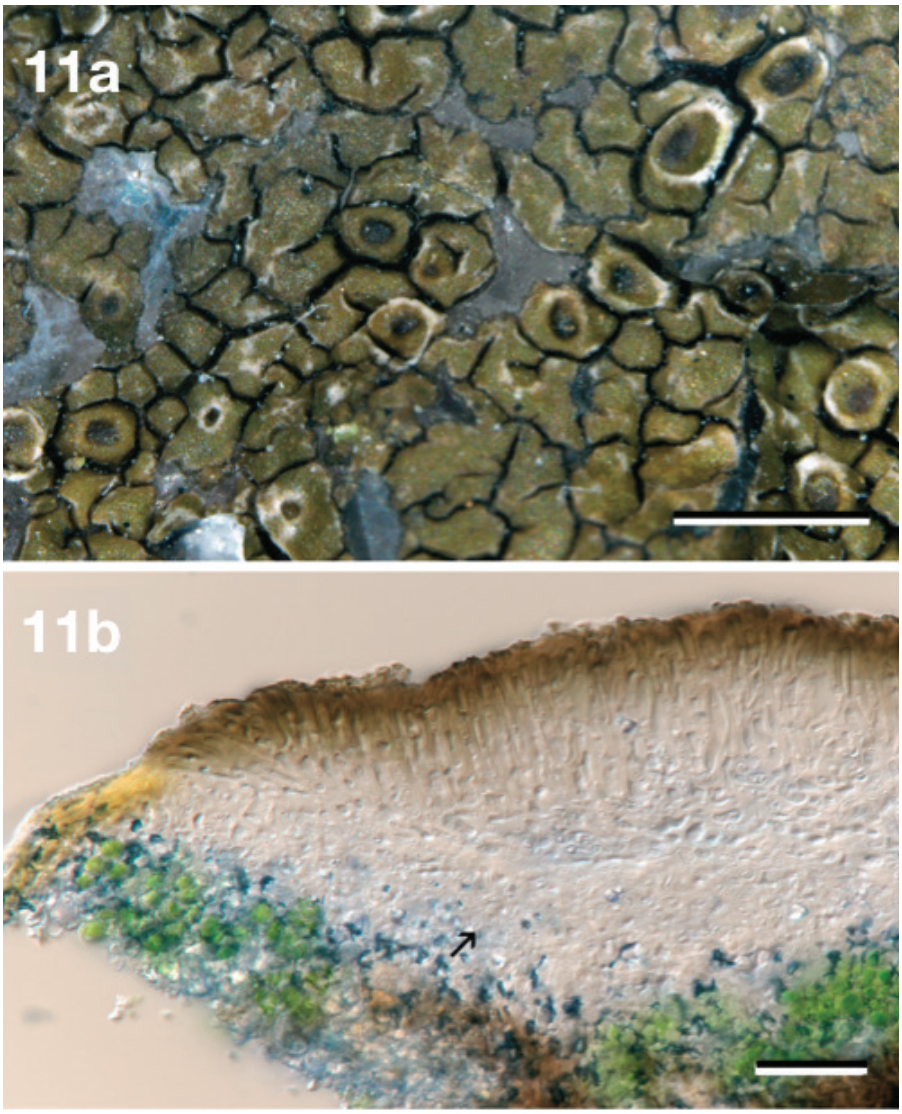

Figure 11. *Neoprotoparmelia pauli*, holotype Kirika & Lumbsch 3821 (EA) **a** Habit **b** Centre of apothecia section, showing cupular proper exciple (arrow). Scale bars: 1 mm (**a**), 20 µm (**b**).

irregular, polygonal to rounded, up to 0.75(–1.2) mm in diam., flat to slightly convex, surface mainly smooth, marginal areoles sometimes lobe-like. Apothecia frequent, 1 per areolae, zeorine to lecanorine, mainly immersed and nearly urceolate or adnate, rounded, up to 0.4 mm in diam.; disc brown to brown-black, dull, concave to flat; thalline exciple persistent, concolorous with thallus to whitish (by a strong mucilaginous epicortex); proper exciple cupulate, up to 35 µm thick, coherent, hyphae mainly periclinal with strong mucilaginous walls. Hymenium hyaline, coherent, 35–60 µm tall; epihymenium light brown to brown, up to 15 µm tall, with few irregular granules;

hypothecium and subhymenium hyaline, 15–35 µm thick. Paraphyses coherent in water, branched and anastomosed, apices somewhat thickened and mainly surrounded by a brown mucilaginous hood (up to 7.5 µm wide). Asci clavate, 50 ×16 µm, 8–spored, amyloid tholus (excluding the axial mass) and surrounding mucilage, *Lecanora*–type (cf. also *Maronina*–type, Kantvilas et al. 2010). Ascospores hyaline, simple, 10–12.5 × 4–5 µm (n = 8), fusiform to elongate (l:b = 2–2.75), with rounded apices or sometimes slightly apiculate in one end, some with apical hyaline setae. Pycnidia immersed, globose to oblong, wall hyaline, ostiole tissue with brown pigmented walls. Conidia simple, hyaline, (9–)10–17 × 1–1.5 µm (n = 20), bacilliform, straight.

Chemistry. Spot tests: medulla K– or ± unclean yellowish, C–, KC–, I–, P–, UV+ greenish-white. TLC: atranorin (minor or traces), α–collatolic acid (major or minor), α–alectoronic acid (minor), unidentified substance (major or traces, closed to norstictic acid, Rf class 4), ± β–alectoronic (traces) and traces of related substances.

Distribution and ecology. Only known from the type locality in Kenya, covered with upland dry forest ecosystems (Wass 1995), growing on exposed sandstones.

Reference sequences. (specimen: Kirika & Lumbsch 3821, holotype: EA). KP822469 (mtSSU), KP822279 (ITS), KP796348 (nuLSU), KP822148 (RPB1), KP823526 (TSR1).

Remarks. Consists of specimens recovered within '*P.* sp. KE' in '*Protoparmelia* tropical clade' in Singh et al. (2015), supported as an evolutionary independent lineage based on the coalescent-based species delimitation analysis. The thalli of the type material were poorly developed, immature apothecia and only a few mature spores were found. This hindered us in providing detailed morphological features (especially ascomatal) and thus future collections may slightly change the morphological description. Its olive-brown thalli, 8-spored asci, α–collatolic acid presence, distribution and/or molecular data supports it as an evolutionary independent lineage from the other two saxicolous *Neoprotoparmelia* species.

***Neoprotoparmelia plurisporibadia* Garima Singh, M. Cáceres & Aptroot, sp. nov.**
Mycobank no.: MB827482
Figure 12

Type. BRAZIL. Rio Grande do Sul, Viamão, near Parque Itapua, 30°05'S, 51°00'W, on granite, ca. 100 m alt.; 26 September 2014, M. Cáceres & A. Aptroot 22130 (holotype: ABL; isotype: ISE).

Diagnosis. Differing from the morphologically similar *Protoparmelia badia* (Ach.) M. Choisy by the presence of multispored asci and different chemistry and distribution.

Etymology. Named after *pluri* = many, spores and *badia* = dark brown.

Description. Thallus consisting of areoles with wavy border of up to ca. 1.3 mm thick and 2.0 mm wide (but mostly much smaller) that are tightly packed together and occasionally become almost lobe-like, somewhat shiny, pale brown to dark brown, marginal prothallus black, thin or absent. Isidia absent. Apothecia immersed in areoles

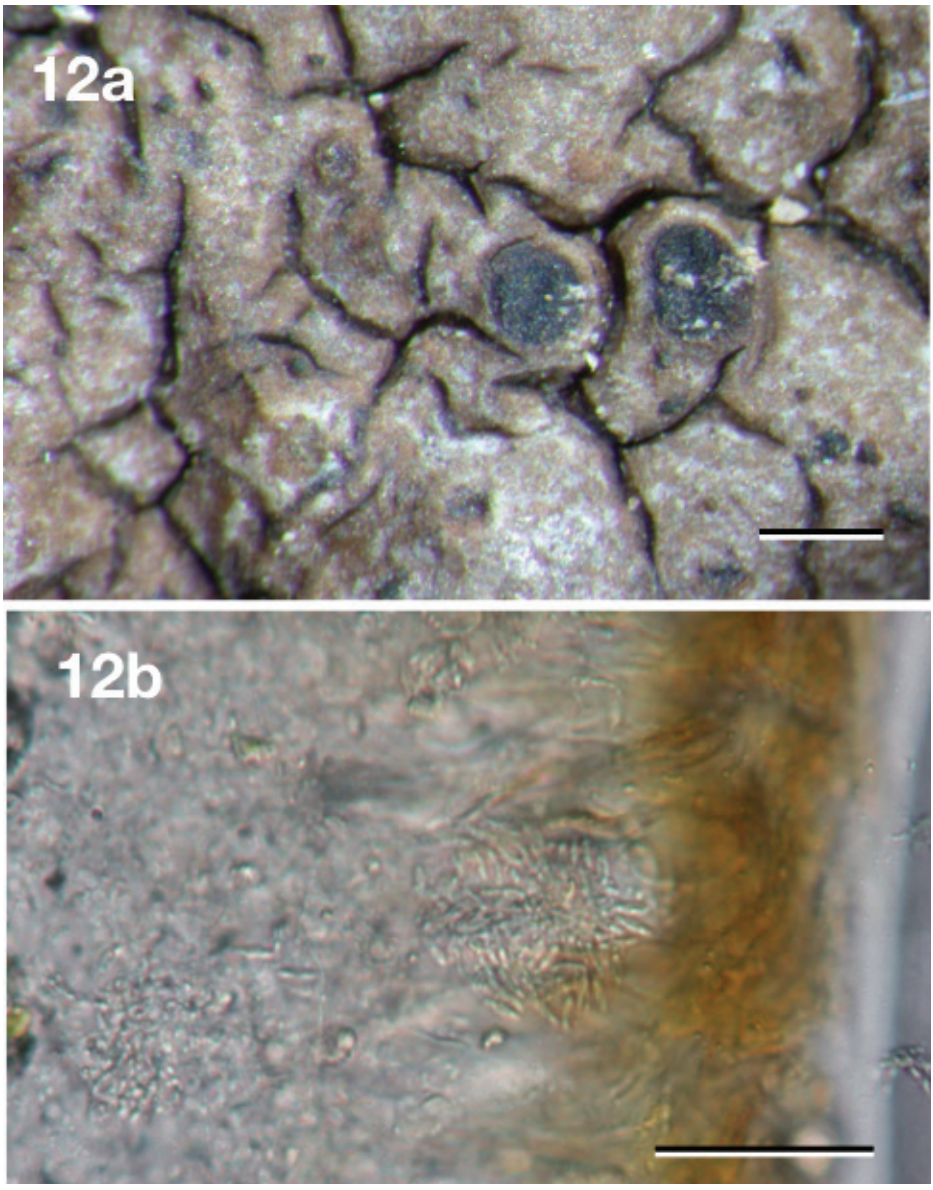

Figure 12. *Neoprotoparmelia plurisporibadia*, holotype Cáceres & Aproot 22130 (ABL). **a** Habitus **b** ascus with ascospores. Scale bars: 1 mm (**a**), 10 micron (**b**).

to erumpent, usually up to one per areole, initially round, later usually compressed and with wavy elongated shape, 0.4–1.3 mm diam., disc concave to flat, smooth, glossy, dark brown. Margin dull, ca. 0.3 mm wide, indistinguishable from the thallus, not or only slightly higher than the disc. Hymenium hyaline, not inspersed with oil droplets, up to 100 µm high; epihymenium fuscous brown, pigment in KOH becoming soluble and paler; hypothecium hyaline, not distinguishable from the thallus medulla and thus extending to over 1 mm; excipulum hyaline throughout, with a 10–15 µm thick layer

of pseudocortex without crystals, with algae, not extending below the hypothecium. Paraphyses simple to somewhat branched, ca. 2.5 µm wide, not thickened at the tips. Asci cylindrico-clavate, blue, up to 95 × 15 µm, with ca. 50 ascospores. Ascospores hyaline, simple or occasionally with a pseudoseptum, narrowly ellipsoid, not constricted, 7.0–8.0 × 2.5–3.5 µm, wall ca. 0.5 µm thick, without appendages. Pycnidia abundant, immersed, dark brown; surrounding areole usually slightly raised. Conidia hyaline, linear to slightly clavate, 5–7.5 × 0.9–1.1 µm.

Chemistry. Spot tests: medulla of thallus UV+ greenish-white, C–, P–, K–, KC+ pink. TLC: alectoronic acid.

Distribution and ecology. On granite in open low mountain area. Known only from Brazil (Rio Grande do Sul).

Reference sequences. M. Cáceres & A. Aptroot 22130, MK046748.

Remarks. Somewhat similar to *Protoparmelia badia*, from which it differs markedly by the multispored ascus and production of alectoronic acid instead of lobaric acid, as occurs in *P. badia*. It can also be distinguished from the other two saxicolous *Neoprotoparmelia* species, *N. pauli*, and *N. capensis*, by distribution and by the presence of approximately 50-spored asci in contrast to the 8-spored asci present in the latter.

***Neoprotoparmelia pulchra* (Diederich, Aptroot & Sérus.) Garima Singh, Lumbsch & I. Schmitt, comb. nov.**
MycoBank no.: MB827483

Basionym. *Protoparmelia pulchra* Diederich, Aptroot & Sérus. in Aptroot et al., Biblioth. Lichenol. 64: 147. 1997.

TYPE: on the S shore of L. Piunde, Pindaunde Valley, Mt Wilhelm, Simbu Province, Papua New Guinea, 05°47'S, 145°43'E, alt. 3600 m, subalpine forest remnants on W slope of valley, 6 Aug. 1992, H. Sipman 35638; holo: B.

Synonym. *Maronina pulchra* (Diederich, Aptroot & Sérus.) Divakar, A. Crespo & Lumbsch in Divakar et al., Fungal Diversity 84: 114. 2017.

***Neoprotoparmelia siamisidiata* Garima Singh & Aptroot, sp. nov.**
MycoBank no.: MB827684
Figure 13

Type. THAILAND. Chiang Mai, Doi Suthep–Ou National Park, Medicinal Garden 18°48'17"N, 98°54'43"E, ca. 1100 m alt., on bark of *Cinchona pubescens*, 13 October 2002, H.J.M. Sipman 48520 (holotype: B).

Diagnosis. Similar to *Neoprotoparmelia brasilisidiata*, but mainly differs from it by the presence of 16–spored asci.

Etymology. Named after the place of discovery, Siam (Thailand) and the presence of isidia.

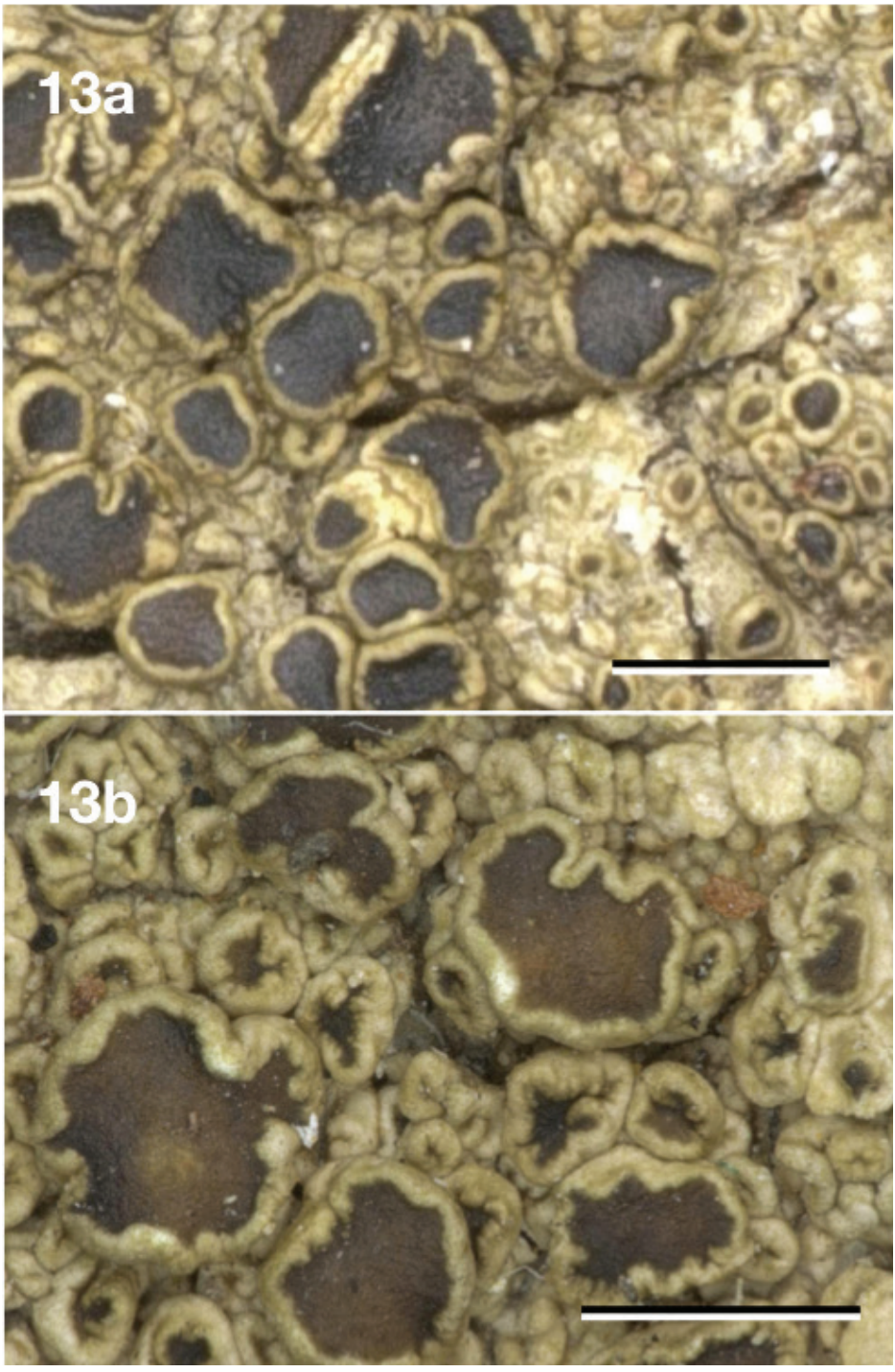

Figure 13. *Neoprotoparmelia siamisidiata*, *v.d.* Boom 46872 (Hb. v.d. Boom). Scale bar: 1 mm.

Description. Thallus consisting of slightly convex areoles of up to ca. 0.1 mm thick and 0.3 mm wide which are mostly coalescent to form a rimose thallus, somewhat shiny, pale brown to dark brown or mottled whitish-grey, on a fully immersed dark hypothallus, marginal prothallus black, thin or absent. Isidia always numerous, initially widely dispersed or somewhat clustered, eventually covering much of the thallus, up to 1.5 mm long, persistently 0.05–0.07 mm wide over their whole length, cylindrical, usually rather irregularly once or repeatedly branched and somewhat nodulose, glossy, pale to dark brown, tips generally dark brown. Apothecia sessile, initially round, older ones usually with wavy boundaries, 0.6–1.5 mm diam., disc flat, smooth, glossy, dark brown to orange brown. Margin glossy, ca. 0.25 mm wide, glossy brown at the outside, not or only slightly higher than the disc. Hymenium hyaline, not inspersed with oil droplets, up to 90 µm high; epihymenium fuscous brown, pigment in KOH becoming soluble and paler; hypothecium hyaline, up to 120 µm thick including subhymenium; excipulum hyaline throughout, with a 20–30 µm thick layer of cortex, without crystals, with algae, extending below the hypothecium (cupulate). Paraphyses branched, ca. 2.5 µm wide, not thickened at the tips. Asci cylindrico-clavate, blue, up to 35 × 9 µm, with 16 mostly biseriate ascospores. Ascospores hyaline, simple, broadly ellipsoid, not constricted, 9–11 × 6.5–8 µm, without appendages. Pycnidia not observed.

Chemistry. Spot tests: medulla of thallus and isidia UV+ greenish-white, C–, P–, K–, KC+ pink. TLC: alectoronic acid (major), dehydroalectoronic acid (minor or trace) and β-alectoronic acid (trace).

Distribution and ecology. On tree bark in a Park. Known only from Thailand (Chiang Mai).

Remarks. This comprises the specimens recovered within '*P. isidiata* C' in '*Protoparmelia* tropical clade' in Singh et al. (2015). It is similar to the other four isidiate *Neoprotoparmelia* species but can be distinguished from them by the presence of 16-spored asci. For additional specimens from Thailand, see Aptroot et al. (2007, as *Protoparmelia isidiata*). It can be distinguished from *Neoprotoparmelia corallifera* only by presence of 8-spored asci (Aproot et al. 1997a) and by using molecular data.

Key to *Neoprotoparmelia*

1	Thallus sorediate or isidiate	**2**
–	Thallus lacking soredia or isidia	**9**
2	Thallus sorediate, known from USA and Brazil	***Neoprotoparmelia capitata***
–	Thallus isidiate	**3**
3	Isidia globose to ellipsoid, covering the thallus except margins, Australia	***N. crassa***
–	Isidia otherwise	**4**
4	Isidia up to 1.5 mm tall	**5**
–	Isidia less than 1.5 mm tall	**7**
5	Asci 16-spored, Thailand	***N. siamisidiata***
–	Asci 8-spored	**6**

6 Isidia persistently 0.07–0.11 mm wide over their whole length, SE of the USA..... ***N. amerisidiata***
– Isidia thinner and less regular, South and Central tropical America..... ***N. brasilisidiata***
7 Asci 32–50-spored, Thailand ..... ***N. corallifera***
– Asci 8-spored, Australia or Papua New Guinea **8**
8 Usually several isidia on one thallus areole, Australia..... ***N. australisidiata***
– Each thallus areole with only one isidium, Papua New Guinea ....***N. isidiata***
9 Thallus epiphytic **10**
– Thallus saxicolous **12**
10 Asci 8-spored, Papua New Guinea ..... ***N. pulchra***
– Asci multispored..... **11**
11 Asci 32-spored, South America ..... ***N. multifera***
– Asci 32–50-spored, Thailand ..... ***N. orientalis***
12 Asci multispored, Brazil ..... ***N. plurisporibadia***
– Asci 8-spored **13**
13 Thallus grey to brown, main substance alectoronic acid, South Africa ...***N. capensis***
– Thallus olive, main substance α-collatolic acid, Kenya..... ***N. pauli***

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Supplementary material 1

Table S1: List of primers used in this study

Authors: Garima Singh, André Aptroot, Víctor J. Rico, Jürgen Otte, Pradeep K. Divakar, Ana Crespo, Marcela Eugenia da Silva Cáceres, H. Thorsten Lumbsch, Imke Schmitt

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RESEARCH ARTICLE

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Bacidia albogranulosa (Ramalinaceae, lichenized Ascomycota), a new sorediate lichen from European old-growth forests

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Abstract

A sterile sorediate member of the genus *Bacidia* s.str., *B. albogranulosa*, is described here as a new species. It is characterised by its very thin, pale grey thallus, white, farinose to granular soredia, the production of atranorin and the absence of ascomata and pycnidia. It grows on slightly acidic to subneutral bark of broad-leaved trees in old-growth forests in the Czech Republic, Poland, Ukraine and Russia (European part of the Caucasus). The new species is well characterised by its morphology, secondary chemistry and molecular (nrITS, mtSSU) traits. It is closely related to other atranorin-containing species in the genus, *Bacidia diffracta*, *B. polychroa* and *B. suffusa*.

Keywords

Atranorin, sterile lichens, subneutral bark

Introduction

Bacidia De Not. (Ramalinaceae, lichenised Ascomycota) is a genus of lichenised fungi with crustose thalli, a chlorococcoid photobiont, lecideine or biatorine apothecia and multiseptate oblong to acicular ascospores (Ekman 1996). Many of the species do not produce any lichen substances detectable by TLC, but one or more pigments in the apothecial tissues are known (Ekman 1996, Coppins and Aptroot 2009, Wirth et al. 2013). Names of five acetone-insoluble pigments are derived from *Bacidia* s.str., i.e. Arceutina-yellow, Laurocerasi-brown, Polychroa-brown, Rubella-orange and Schweinitzii-red (Ekman 1996, Meyer and Printzen 2000). The genus *Bacidia* includes approximately 230 species wordwide (Lücking et al. 2016). However, many species named '*Bacidia*' belong to other genera or even other families, so the diversity of *Bacidia*, in its strict sense, is estimated to be 60–90 species (Ekman 1996, 2001, Coppins and Aptroot 2009).

During field research in old-growth forests in Europe, we repeatedly collected a sterile sorediate crust, preliminarly assigned to the genus *Lecanora* Ach. due to the production of atranorin. Surprisingly, molecular data placed the species into *Bacidia* s.str. The currently known members of *Bacidia* s.str., except for *B. sorediata* Lendemer & R. C. Harris (Lendemer et al. 2016), do not produce isidia or soredia, but the thallus of some species consists of granules that very likely have a function as vegetative propagules (Ekman 1996). The new species is related to *B. diffracta* S. Ekman, *B. polychroa* (Th. Fr.) Körb., *B. rubella* (Hoffm.) A. Massal. and *B. suffusa* (Fr.) A. Schneid., which also produce atranorin as the main secondary metabolite (Culberson and Culberson 1969, Ekman 1996). Based on morphological, chemical and molecular characters, we describe this very distinct taxon as new to science.

Material and methods

Sampling, morphology and chemistry

Collected specimens are deposited in KTC, PRA, UGDA and the personal herbarium of J. Malíček. Microscopic descriptions are based on hand-cut sections mounted in water. Lichen secondary metabolites were identified using thin layer chromatography (TLC) in A, B' and C solvents (Orange et al. 2010). Figures were acquired by the stereomicroscope Olympus SZX 12 with the cooled colour digital camera Olympus DP 70 (resolution 12.5 Mpx) in the software QuickPHOTO MICRO 3.0 (Promicra), using an extended depth of field module Deep Focus.

DNA extraction, PCR amplification and sequencing

The Invisorb Spin Plant Mini Kit (Invitek) and CTAB protocol (Cubero et al. 1999) were used for DNA extractions. The fungal ITS rDNA (henceforth ITS) and mitochondrial SSU (mtSSU) were amplified with the following primers: ITS1F (Gardes

Table 1. GenBank accession numbers and voucher information of specimens used in this study. New sequences are indicated in bold.

Taxon	Source – Specimen	ITS	mtSSU
Bacidia albogranulosa 1	Czech Republic, Lanžhot, J. Vondrák 11888 (PRA)	**MK158342**	**MK158332**
Bacidia albogranulosa 2	Czech Republic, Lanžhot, J. Vondrák 11889 (PRA)	**MK158341**	**MK158333**
Bacidia albogranulosa 3	Czech Republic, Šumava Mts, J. Vondrák 17113 (PRA)	**MK158339**	**MK158334**
Bacidia albogranulosa 4	Russia, Caucasus, J. Malíček 9622 (hb. J. Malíček)	**MK158340**	**MK158335**
Bacidia albogranulosa 5	Czech Republic, Moravský kras, J. Malíček 8013 (hb. J. Malíček)	–	**MK158336**
Bacidia albogranulosa 6	Ukraine, Otok, J. Vondrák 12235 (PRA)	–	**MK158337**
Bacidia albogranulosa 7	Czech Republic, Český les Mts, J. Vondrák 12865 (PRA)	–	**MK158338**
Bacidia arceutina	Switzerland, van den Boom 41117 (hb. van den Boom)	–	JQ796829
Bacidia diffracta	Wetmore 26401 (MIN)	AF282090	–
Bacidia ekmaniana 1	USA, Delaware, Lendemer 33783 (NY)	–	KX151745
Bacidia ekmaniana 2	USA, North Carolina, Lendemer 30488A (NY)	–	KX151746
Bacidia fraxinea	Sweden, Johansson 1620 (BG)	AF282088	–
Bacidia polychroa	Knutsson 91–215 (hb. Knutsson)	AF282089	–
Bacidia rosella	Sweden, Ekman 3117 (BG)	AF282086	AY300877
Bacidia rubella 1	Poland, Pojezierze Ilawskie, M. Kukwa 4598 (DUKE)	MG461695	DQ986808
Bacidia rubella 2	Ukraine, Otok, J. Vondrák 12200 (PRA)	**MK158343**	**MK158331**
Bacidia rubella 3	Switzerland, van den Boom 41103 (hb. van den Boom)	JQ796852	JQ796830
Bacidia rubella 4	Sweden, Ekman 3021 (BG)	AF282087	–
Bacidia schweinitzii 1	USA, North Carolina, Lendemer 30548 (NY)	KX151761	KX151749
Bacidia schweinitzii 2	USA, North Carolina, Tripp 2614 (NY)	KX151762	KX151750
Bacidia schweinitzii 3	USA, North Carolina, Lendemer 29364 (NY)	KX151763	KX151751
Bacidia schweinitzii 4	USA, North Carolina, Lendemer 31238 (NY)	KX151764	KX151752
Bacidia schweinitzii 5	USA, Maryland, Lendemer 31855 (NY)	KX151765	KX151753
Bacidia schweinitzii 6	USA, Tennessee, F. Lutzoni (DUKE)	DQ782850	DQ972998
Bacidia sipmanii	Tenerife, Sérusiaux s.n. (hb. Sérusiaux)	JQ796853	JQ796832
Bacidia sorediata 1	USA, Maryland, Lendemer 33869 (NY)	KX151773	KX151760
Bacidia sorediata 2	USA, North Carolina, Lendemer 35031 (NY)	KX151769	KX151756
Bacidia sorediata 3	USA, Delaware, Lendemer 33702 (NY)	KX151767	KX151754
Bacidia sorediata 4	USA, Delaware, Lendemer 33787 (NY)	KX151772	KX151759
Bacidia sorediata 5	USA, North Carolina, Lendemer 35386 (NY)	KX151770	KX151757
Bacidia sorediata 6	USA, Virginia, Lendemer 31692 (NY)	KX151768	KX151755
Bacidia sorediata 7	USA, Virginia, Lendemer 31527 (NY)	KX151771	KX151758
Bacidia suffusa	Wetmore 74771 (MIN)	AF282091	–
Bacidina arnoldiana s.lat.	Poland, Pojezierze Ilawskie, M. Kukwa 4593 (DUKE)	HQ650650	DQ986810
Toninia sedifolia	Canada, Quebec, F. Lutzoni & J. Miadlikowska (DUKE)	HQ650689	DQ972987

and Bruns 1993) and ITS4 (White et al. 1990), mrSSU1, mr SSU2R and mrSSU3R (Zoller et al. 1999). PCR reactions of nrITS and mtSSU were prepared for a 20 µl final volume containing 14 µl double-distilled water, 4 µl MyTaq polymerase reaction buffer, 0.2 µl MyTaq DNA polymerase, 0.4 µl of each of the 25 mM primers and 1 µl of the sample. Amplifications of both loci consisted of an initial 1 min denaturation at 95 °C, followed by 35 cycles of 1 min at 95 °C, 1 min at 56 °C, 1 min at 72 °C and a final extension of 7 min at 72 °C. The PCR products were visualised on a 0.8% agarose gel and cleaned with GenElute PCR Clean-Up Kit (Sigma), according to the manufacturer's protocols. In total, 5 new ITS and 8 mtSSU sequences were generated (Table 1). Two short mtSSU sequences, containing ca. 400 positions, were exluded from the final analysis.

Sequence alignment and phylogenetic analysis

The newly produced sequences were edited in BioEdit 7.2.5 (Hall 1999). The final analyses included the newly generated sequences, the most similar *Bacidia* sequences (identity > 90%) according to a BLASTN search (Altschul et al. 1990) in the GenBank database and sequences of chemically and morphologically similar species (*B. schweinitzii* (Fr. ex Tuck.) A. Schneid., *B. sorediata*) to demonstrate their distant position in the tree. *Bacidina arnoldiana* s.lat. and *Toninia sedifolia* (Scop.) Timdal were selected as an outgroup. The ITS and mtSSU regions were aligned separately using MAFFT 7 (Katoh and Standley 2013) with L-INS-i method (Katoh et al. 2005). Ambiguous positions were excluded from the analysis using Gblocks 0.91b (Castresana 2000), with a less stringent selection, on the Phylogeny.fr server (Dereeper et al. 2008). The final ITS alignment contained 443 positions and 29 sequences; the mtSSU alignment had 730 positions and 28 sequences. Gaps were coded in SeqState by simple coding (Simmons and Ochoterena 2000).

We concatenated the alignments and inferred a phylogeny using MrBayes 3.2.6 (Huelsenbeck and Ronquist 2001; Ronquist et al. 2012). Results of MrModeltest 2.0 (Nylander 2004) suggested the general time reversible model, including gamma-distributed rates across sites modelled with four discrete categories and a proportion of invariant sites (GTR+G+I), as the best substitution model for both regions. Each analysis was performed with two runs, each with four MCMC chains (temperature 0.05). Trees were sampled every 500th generation. Analyses were stopped when the average standard deviation of the split frequencies between the simultaneous runs was below 0.01. To eliminate trees sampled before reaching apparent stationarity, the first 25% of entries were discarded as burn-in and the rest were used to compute a majority-rule consensus tree with Bayesian posterior probabilities for the branches.

A maximum likelihood analysis was performed using RAxML-HPC v. 8.2.10 (Stamatakis 2014) with the GTR+G+I model on the CIPRES Science Gateway (Miller et al. 2010). Non-parametric bootstrap analysis was performed with 1000 bootstrap replicates. The maximum likelihood consensus tree is not shown, but bootstrap values are indicated at branches in the Bayesian tree (Fig. 2).

Results and discussion

Taxonomy

Bacidia albogranulosa **Malíček, Palice, Vondrák & Kukwa, sp. nov.**
Mycobank: MB828612
Fig. 1

Type. CZECH REPUBLIC. Dolnomoravský úval lowland: Břeclav, Lanžhot, protected area Cahnov, 150 m alt., 48°39'22"N, 16°56'25"E, on bark of *Acer campestre*, 1 Apr 2014, J.Vondrák (holotype: PRA-Vondrák 11888).

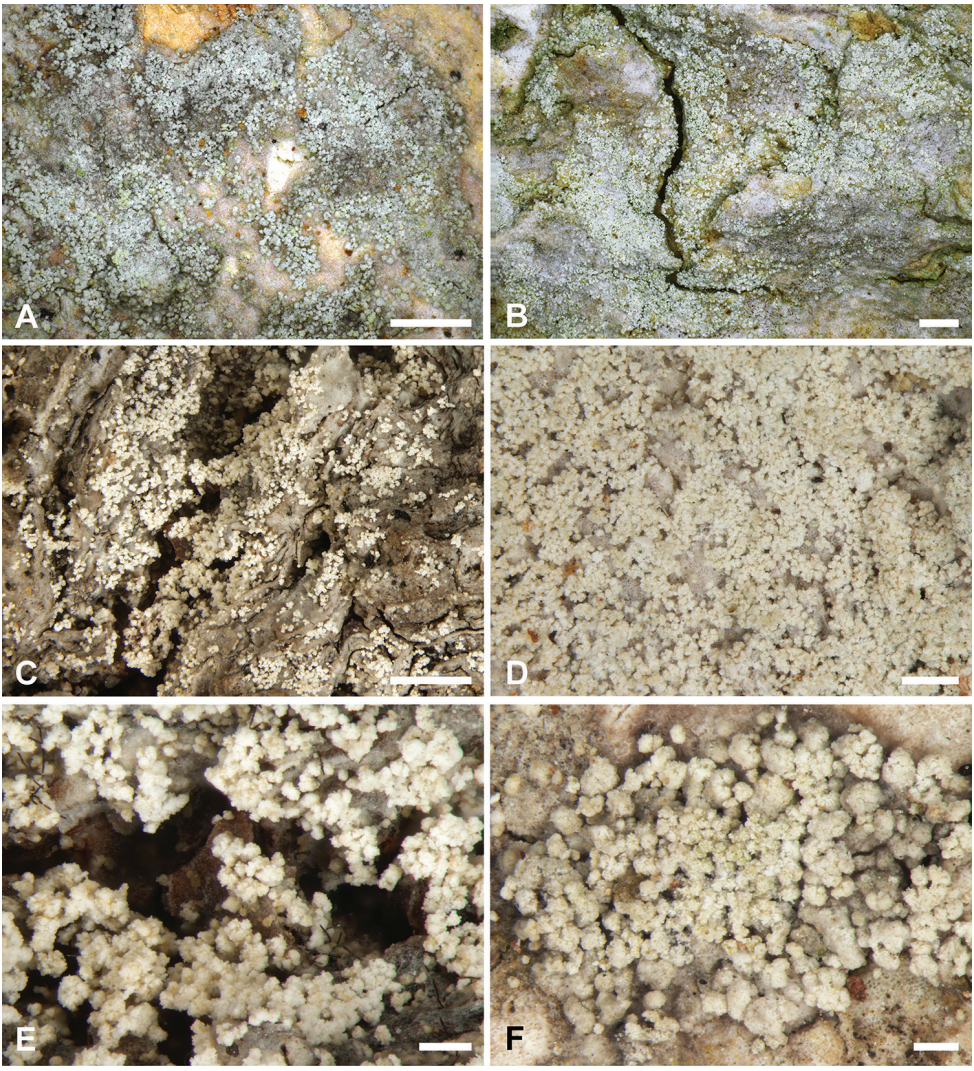

Figure 1. Morphology of *Bacidia albogranulosa*. **A** Holotype (PRA/Vondrák 11888) **B** Common phenotype (Malíček 10802) **C** Typical growth form on old beech trees (Malíček 8166) **D** Phenotype with abundant soredia forming a seemingly leprose thallus (Malíček 8013) **E** Detail of soredia (Malíček 8166) **F** Soredia arising from granules (PRA/Vondrák 11888). Scale bars: 1 mm (**A–C**), 0.5 mm (**D**), 0.2 mm (**E, F**). Photos by J. Malíček (**A, B**) and J. Machač (**C–F**).

Diagnosis. The species is characterised by a grey-white hypothallus or very thin thallus covered by groups of white, farinose to granular soredia or by being completely sorediate. Ascomata and pycnidia are unknown. Atranorin is the only secondary metabolite. The species occurs in old-growth forests on bark of broad-leaved trees with high bark pH (> 5).

Etymology. The name refers to the white rough (granular) soredia that are often present.

Description. The thallus consists of a hypothallus (i.e. without photobiont cells) or, in some parts, a lichenised and thinly episubstratal thallus (up to 100 µm high), which is smooth or partially areolate, pustulate or granular, grey-white to grey, sorediate. A prothallus is absent or very thin and white. Soredia are not produced in clearly delimited soralia, but dispersed in groups or forming a more or less continuous layer, white or, when fresh, yellowish-white, farinose to granular, simple, (25–)35–65 µm in diam., or in consoredia up to 125 µm in diam. Soredia are enclosed by a colourless, more or less compact "wall" without projecting hyphae. The photobiont is trebouxioid, and 5–16 µm in diameter. Ascomata and pycnidia are unknown.

Chemistry. Atranorin detected by TLC (n=20). Numerous tiny crystals of atranorin visible in water mounts of soredia and thallus in polarised light. Spot reactions: K+ yellow, Pd+ yellowish, C–, KC–, soredia UV+ dull orange, thallus UV– or dull yellowish-white (in 365 nm).

Distribution and ecology. The new species is reported from the Czech Republic, Poland, Russia (European part of the Caucasus) and Ukraine. It has already been published under a provisional name, *Bacidia albogranulosa* ined. from the Czech Republic (Vondrák et al. 2016) and the Ukrainian Carpathians (Malíček et al. 2018, Vondrák et al. 2018).

Bacidia albogranulosa grows abundantly in old-growth floodplain and scree forests in the Czech Republic and old-growth ash or hornbeam dominated broad-leaved forests in Poland. It rarely occurs in old-growth beech (Ukraine) and mixed forests (Russia). It has usually been found on a dry and coarse bark of broad-leaved trees with a relatively high bark pH (approximately > 5). The most frequent phorophytes are *Acer campestre* (n=5), *A. platanoides* (11) and *Fagus orientalis*/*sylvatica* (4; overmature or dying trees due to a fungal infection). A few specimens were recorded also on *Fraxinus angustifolia* (2), *F. excelsior* (2), *Carpinus orientalis* (1), *Euonymus europaeus* (1) and *Quercus* sp. (2). The species prefers rather shaded trunks and places not directly exposed to rain, similar to many *Lepraria* species (Saag et al. 2009).

Alyxoria varia (Pers.) Ertz & Tehler, *Bacidia rubella* and the non-lichenised fungus *Dendrothele acerina* (Pers.) P.A. Lemke (on *Acer* spp.) are the most commonly recorded, co-occurring species. In the Czech Republic, the new species was repeatedly found on weathered bark with the red-listed *Gyalecta flotowii* Körb. or *G. ulmi* (Sw.) Zahlbr. It co-occurred also with *Acrocordia gemmata* (Ach.) A. Massal., *Arthothelium spectabile* A. Massal., *Bacidia fraxinea* Lönnr., *B. incompta* (Borrer) Anzi, *Caloplaca flavocitrina* (Nyl.) H. Olivier, *Gyalecta truncigena* (Ach.) Hepp, *Hazslinszkya gibberulosa* (Ach.) Körb., *Inoderma byssaceum* (Weigel) Gray, *Lecania croatica* (Zahlbr.) Kotlov, *Lepraria finkii* (B. de Lesd.) R. C. Harris, *L. vouauxii* (Hue) R. C. Harris, *Opegrapha vermicellifera* (J. Kunze) J. R. Laundon and *Pyrenula nitidella* (Flörke ex Schaer.) Müll. Arg.

Phylogeny. The new species is strongly supported as a distinct clade in the ITS and mtSSU phylogeny (Fig. 2) and belongs to *Bacidia* s.str. sensu Ekman (2001). According to the ITS data, it is closely related to *Bacidia diffracta*, *B. suffusa* and *B. polychroa*. These four species form a well supported group, characterised by the presence

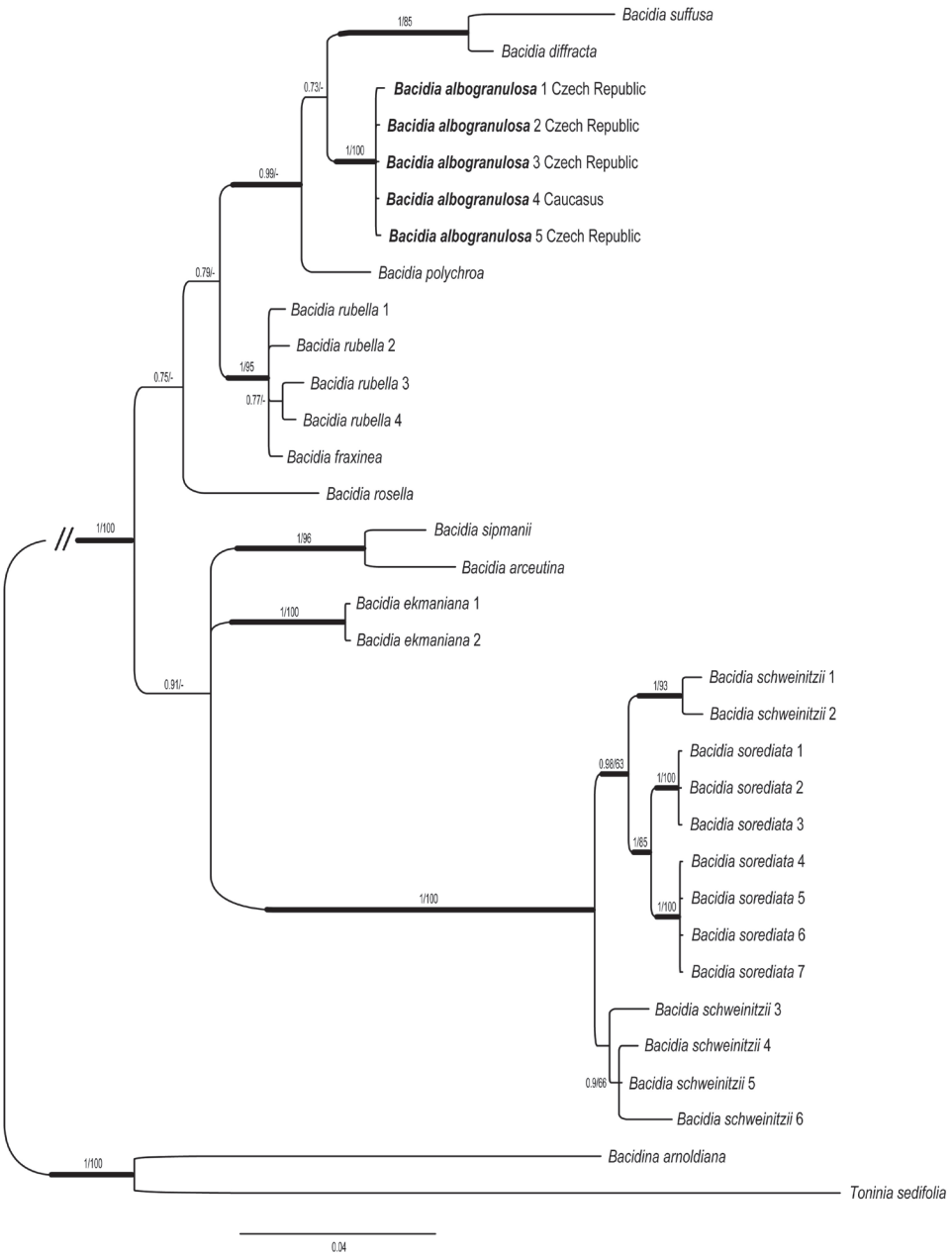

Figure 2. Phylogeny of selected members of *Bacidia* s.str. This is a Bayesian phylogenetic reconstruction based on nrITS and mtSSU sequences. The new species, *Bacidia albogranulosa*, is indicated in bold. Branches with > 0.95 Bayesian posterior probability values are indicated by thicker lines. Bayesian posterior probabilities (first value) and maximum likelihood bootstrap percentages (second value) are indicated.

of the pigments Laurocerasi-brown and Polychroa-brown in the apothecia. *Bacidia albogranulosa* is also related to *B. rubella*, a species it frequently co-ocurrs with. The only sorediate member of *Bacidia* s.str., the North American *B. sorediata*, seems not to be closely related to the new species, based on the ITS and mtSSU sequence data (Fig. 2).

Notes. Although apothecia and pycnidia are unknown, *B. albogranulosa* can be recognised in the field by its white-grey hypothallus or very thin thallus covered by groups of white to yellowish-white soredia that often extend across the entire thallus. Ecologically, the species prefers trees with rough and slightly acidic or subneutral bark in old-growth forests.

Bacidia albogranulosa may macroscopically resemble some *Lepraria* species or poorly developed *Phlyctis argena* (Ach.) Flot., but it clearly differs by having a non-continuous, locally developed thallus, composed of dispersed granular aggregates that disintegrate into soralia at an early stage and by the lack of a fibrous prothallus. Additionally, atranorin alone is not known from any described *Lepraria* species (Saag et al. 2009). Similarly, sorediate European *Lecanora* species contain other substances in addition to atranorin, such as aliphatic acids, depsides/depsidones or terpenoids and usually form thicker thalli or at least a distinct fibrous hypothallus (Malíček et al. 2017). A slightly similar appearance is typical for a few other *Lecanora* species (e.g. *L. compallens* Herk & Aptroot, *L. stanislai* Guzow-Krzem., Łubek, Malíček & Kukwa), producing usnic acid and zeorin and forming a yellowish-greenish to greenish-grey sorediate thallus (Guzow-Krzemińska et al. 2017).

Initial stages of the new species may resemble sterile thalli of *Caloplaca substerilis* Vondrák, Palice & van den Boom. This taxon lacks atranorin and tends to form thin areolate-squamulose, almost evanescent thalli with occassional sulcate or marginal soralia (Vondrák et al. 2013). The closely related species *B. diffracta* produces a similar, finely granular grey thallus and contains atranorin in addition to traces of zeorin. Nevertheless, this species is richly fertile, has larger thalline granules (40–100 µm diam.) and is so far only known from eastern North America (Ekman 1996). The only presently known sorediate member of *Bacidia* s.str., *B. sorediata*, differs in having a better developed, grey-green to dark green thallus, diffuse, rarely confluent soralia and fine soredia. It occurs only in south-eastern North America (Lendemer et al. 2016) and it is not phylogenetically closely related to *B. albogranulosa* (Fig. 2).

Additional specimens examined. CZECH REPUBLIC. Western Bohemia: Český les Mts, Bělá nad Radbůzou, Smolov, protected area Pleš, old-growth mixed forest on scree on E slope, 49°33'02"N, 12°38'21"E, 740–840 m alt., on *Acer platanoides*, 6 August 2014, J.Vondrák 12865 (PRA). Southern Bohemia: Šumava Mts, Volary, Mt Stožec – Medvědice, a mountain scree deciduous old-growth forest at NNE-facing slope, 48°52'49.5"N, 13°50'03"E, on dry bark of *Acer platanoides*, 935 m alt., 7 Aug 2014, Z.Palice 17827, Jul.Palicová & K.Palicová (PRA), ibid.: at NE-facing slope, 48.8802°N, 13.8385°E, 900 m alt., on bark of *Acer platanoides*, 17 Oct 2016, J.Vondrák 17113 & Z.Palice 24362 (PRA). Šumava Mts, Lenora, Mt Zátoňská hora, semi-natural scree deciduous forest at SW-facing slope, just below the top, 48°56'41"N, 13°49'48"E, on bark of *Acer platanoides*, 1022 m alt., 27 June 2018, J.Malíček &

Z.Palice 25133 (PRA). Novohradské hory Mts, Pohorská Ves, nature reserve Žofínský prales, N part of the reserve, old-growth forest at N-NW-facing slope, 48°40'10"N, 14°42'30"E, on bark of *Fagus*, 765–770 m alt., 18 Aug 2016, Z.Palice 22220 (PRA). Central Bohemia: Křivoklátsko Protected Landscape Area, Skryje, Týřov National Nature Reserve, mixed deciduous forest with shady rocky outcrops in valley of Úpořský potok brook S of Vápenný vrch Hill (424 m), 49°58'09"N, 13°47'43"E, 270 m alt., on bark of *Acer platanoides*, 11 Aug 2018, J.Malíček 11990 (herb. Malíček). Southern Moravia: distr. Břeclav, Lanžhot, Ranšpurk National Nature Reserve, ca. 48°40'41"N, 16°56'49"E, floodplain old-growth forest, alt. 150 m, on bark of *Acer campestre*, 10 Oct 2013, J.Malíček 6214 & J.Vondrák (herb. Malíček). Cahnov-Soutok National Nature Reserve, old-growth flood-plained forest 7.5 km SSW of Lanžhot, 48°39'23"N, 16°56'24"E, 150 m alt., on bark of *Acer campestre* and *Fraxinus angustifolia*, 1–3 Apr 2014, J.Malíček 6793, 6832, 6863, M.Kukwa 12409, 12434, 12504, 12514, 12515, 12526, Z.Palice 17686 & J.Vondrák 11889, 12051, 12057, (herb. Malíček, PRA, UGDA). Distr. Blansko, Moravský kras Protect. Landscape Area, Vilémovice, Vývěry Punkvy National Nature Reserve, oak-dominated woodlands on SE-facing slope in surrounding of Blansek castle ruin, 49°22'15"N, 16°43'24"E, alt. 425 m, on bark of *Acer campestre*, 17 Apr 2015, J.Malíček 8013 & V.Lenzová (herb. Malíček).

POLAND. Równina Bielska: Białowieża Primeval Forest, Białowieski National Park, N part of forest section no 286, 52°45'07"N, 23°52'40"E, *Tilio-Carpinetum*, on *Acer platanoides*, May 2014, M.Kukwa 12592 (UGDA); ibid.: forest section no 256, *Tilio-Carpinetum*, on *Acer platanoides*, May 2014, M.Kukwa 12755 (UGDA); ibid.: *Circaeo-Alnetum*, on *Acer platanoides* and bark of fallen *Fraxinus excelsior*, Aug 2014, M.Kukwa 13135a, 13176 & A.Łubek (KTC, UGDA); ibid.: *Tilio-Carpinetum*, on *Acer platanoides*, August and October 2014, M.Kukwa 13292, 14394 & A.Łubek (KTC, UGDA); ibid.: *Tilio-Carpinetum*, on *Acer platanoides*, Aug 2015, M.Kukwa 17195, 17584, 17404 & A.Łubek (KTC, UGDA); ibid.: *Circaeo-Alnetum*, on bark of log (*Fraxinus excelsior*), 24 Aug 2015, M.Kukwa 17446 & A. Łubek (KTC, UGDA).

RUSSIA. Caucasus Mts: Caucasian Biosphere Reserve, Guzeripl', old-growth deciduous mixed forest (*Quercus robur*, *Alnus glutinosa*, *Acer campestre* etc.) at right bank of Belaya River, 0.4 km WSW of margin of village, 43°59'20"N, 40°07'30"E, 700 m alt., on bark of *Acer campestre*, *Carpinus orientalis*, *Fraxinus* and *Quercus robur*, 8–9 June 2016, J.Malíček 9622, 10491, Z.Palice 21600, 21690, 22395, 22622, 22715, 23063, J.Vondrák 14956 & G.Urbanavichus (herb. Malíček, PRA). Guzeripl', a forested crest between Belaya and Molchepa rivers, just ca. 1 km SSE of the village, well-lit mixed forest at N-wards descending crest, 43°59'12"N, 40°08'30"E, on bark of old *Quercus*, 935 m alt., 7 June 2016, Z.Palice 22672, 22964 & J.Vondrák 15532 (PRA). Guzeripl', mixed primeval forest (*Abies nordmanniana*, *Acer trautvetteri*, *Fagus orientalis* etc.) on a ridge and W-facing slope 3.5 km S of village, 43°57'53"N, 40°07'50"E, 1470 m alt., on bark of *Acer platanoides* and *Fagus orientalis*, 14 June 2016, J.Malíček 10802, Z.Palice 22624, 22924, J.Vondrák 15291 & G.Urbanavichus (herb. Malíček, PRA).

UKRAINE. Zakarpattia Oblast Province: Berehovo, Nyzhni Remety: lood-plain forest "Otok" 2.5 km SW of village, close to Mala Borzhava River, 48°14'12"N,

22°48'25"E, 120 m alt., on bark of *Acer campestre*, 23 Oct 2013, J.Malíček 6463 & J.Vondrák (herb. Malíček). Ibid.: "Otok", ca. 4 km SW of village, 48°14'00"N, 22°48'20"E, 190 m alt., on bark of *Acer campestre*, *Euonymus europaeus* and *Fraxinus angustifolia*, 3 June 2014, J.Šoun & J.Vondrák 12235, 12206, 12237 (PRA). Khust, Velyka Uhol'ka, old-growth beech predominated forest in valley of Velika Uhol'ka River, ca. 0.7 km NNE of last houses in village, 48°15'02"N, 23°41'47"E, 500 m alt., on bark of old *Fagus sylvatica*, 13 May 2015, J.Malíček 8166 & Z.Palice 19366 (herb. Malíček, PRA); ibid.: old-growth hornbeam-beech forest, 48°14'43"N, 23°41'39"E, on bark of old *Fagus sylvatica*, 460 m alt., 19 May 2015, Z.Palice 19392 (PRA).

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Three new species of soil-inhabiting *Trichoderma* from southwest China

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Abstract

Fungi in the genus *Trichoderma* are widely distributed in China, including in Yunnan province. In this study, we report three new soil-inhabiting species in *Trichoderma*, named as *T. kunmingense*, *T. speciosum* and *T. zeloharzianum*. Their colony and mycelial morphology, including features of asexual states, were described. For each species, their DNA sequences were obtained from three loci, the internal transcribed spacer (ITS) regions of the ribosomal DNA, the translation elongation factor 1-α encoding gene (tef1) and the gene encoding the second largest nuclear RNA polymerase subunit (rpb2). Our analyses indicated that the three new species showed consistent divergence amongst each other and from other known and closely related species. Amongst the three, *T. speciosum* and *T. kunmingense* belong to the Viride Clade. Specifically, *T. speciosum* is related to three species – *T. hispanicum*, *T. samuelsii* and *T. junci* and is characterised by tree-like conidiophores, generally paired branches, curved terminal branches, spindly to fusiform phialides and subglobose to globose conidia. In contrast, *T. kunmingense* morphologically resembles *T. asperellum* and *T. yunnanense* and is distinguished by its pyramidal conidiophores, ampulliform to tapered phialides, discrete branches and ovoidal, occasionally ellipsoid, smooth-walled conidia. The third new species, *T. zeloharzianum*, is a new member of the Harzianum Clade and is closely associated with *T. harzianum*, *T. lixii* and *T. simmonsii* but distinguished from them by having smaller, subglobose to globose, thin-walled conidia.

Keywords

Rhizospheric fungi, diversity, Hypocreales, taxonomy

* These two authors contributed equally to this work.

Introduction

The genus *Trichoderma* Pers. (Ascomycota, Sordariomycete, Hypocreales, teleomorph *Hypocrea* Fr.) is cosmopolitan, often existing as saprophytes in a diversity of ecosystems, such as agricultural fields, prairies, forests and salt marshes (Gond et al. 2007, Verma et al. 2007, Gazis and Chaverri 2010). Though rarely, they are also found in deserts and freshwater ecosystems. In some woody plants, they are the most abundant endophytes. In addition, a few species of *Trichoderma* are effective in attacking or inhibiting other fungi through their secondary metabolites and these fungi have been exploited as potential biocontrol agents against plant pathogens (Degenkolb et al. 2008, Cheng et al. 2012, Lopes et al. 2012, Mukherjee et al. 2013). A few *Trichoderma* species are crop pathogens and can produce toxins to spoil food. For example, *T. aggressivum* can cause significant crop loss to mushroom production (Oda et al. 2009, Schuster and Schmoll 2010, Kim et al. 2012, 2013).

As multilocus molecular phylogeny enables rapid and accurate identification of *Trichoderma* species, a significant number of *Trichoderma* species have been recently reported based on molecular phylogenetic evidence. Following the guidelines of the International Code of Nomenclature (ICN) for algae, fungi and plants (Melbourne Code, Art. 14.13), 254 names of *Trichoderma* species and two names of varieties in *Trichoderma* were accepted in 2015 (Bissett et al. 2015). Since then, 71 new *Trichoderma* species have been reported. Amongst these 71 species, 15 were described based on cultures from ascospores and the remaining 56 were based on asexual morphs in nature (Chen and Zhuang 2016, 2017a, b, c, Qin and Zhuang 2016a, b, c, d, 2017, Zhu et al. 2017a, b, du Plessis et al. 2018). Most of the new species were isolated from soil (Chen and Zhuang 2016, 2017a, b, c, du Plessis et al. 2018), rotten twigs, stems or barks (Qin and Zhuang 2016a, b, c, d, 2017, Zhu et al. 2017a, b). Several were found associated with the attine ants (Montoya et al. 2016) and on the surface of *Hypoxylon anthochroum* stroma (Sun et al. 2016).

China has an enormous fungal diversity. Amongst the 71 new *Trichoderma* species reported since 2015, 43 were from China (Chen and Zhuang 2017a,b,c, Qin and Zhuang 2017, Zhu et al. 2017a, b). Of these 43 species, 33 were from the soil of different regions (Chen and Zhuang 2017a, b, c), which shows that soil has a high *Trichoderma* diversity. In our survey of *Trichoderma* from soil, 180 *Trichoderma* strains were collected in southwest China and preserved in the Laboratory for Conservation and Utilization of Bioresources, Yunnan University (YMF) and China General Microbiological Culture Collection Center (CGMCC). Three new species were identified based on morphological features and DNA sequence data at three loci: the genes encoding RNA polymerase II subunit (rpb2) and translation elongation factor 1-α gene (tef1) and the internal transcribed spacer (ITS) regions of the nuclear ribosomal RNA gene cluster. Based on the DNA sequence information, we revealed their phylogenetic positions as belonging to the Viride Clade (two species) and the Harzianum Clade (one species).

Materials and methods

Isolates of strains

Soil samples were collected from Luliang and Kunming in Yunnan Province, southwest China. All the samples were stored at 4 °C before use. *Trichoderma* strains were obtained by serial dilutions (1,000 to 1,000,000 fold) and spread on to the surface of Rose Bengal agar with antibiotics (40 mg streptomycin, 30 mg ampicillin per litre) added in a 9-cm-diam. Petri dish, followed by incubation under 25 °C for 5 days. Representative colonies were picked up with a sterilised needle and transferred to new plates containing potato dextrose agar (PDA, Zhang et al. 2013). All putative strains of *Trichoderma* were permanently kept in the Herbarium of the Laboratory for Conservation and Utilization of Bio-resources, Yunnan University, Kunming, Yunnan, P.R. China (YMF). In addition, the holotype strains have been deposited in the China General Microbiological Culture Collection Center (CGMCC).

Morphology characterisation and growth observation

For morphological studies, we used three different media: cornmeal dextrose agar CMD (40 g cornmeal, 2% (w/v) dextrose, 2% (w/v) agar), PDA and synthetic low nutrient agar (SNA). Each strain was first cultured on a PDA plate for 3 days and a small agar piece of 0.5 cm diam. with mycelium was then transferred respectively to new CMD, PDA and SNA plates. Strains were incubated in 9 cm diam. Petri dishes at 25 °C with a 12 h natural light and 12 h darkness interval (Sutton 1980). Colony diameters were all measured after 3 days for morphological descriptions, diameters at 25 °C and 35 °C and the times when mycelia entirely covered the surface of plate were also recorded. For microscopic morphology, photographs were taken with an Olympus BX51 microscope connected to a DP controller digital camera.

DNA extraction, PCR amplification and sequencing

For each strain, genomic DNA was extracted from mycelium growing on PDA harvested after 3 days of growth, following the method of Wang and Zhuang (2004). For the amplifications of ITS, rpb2 and tef1 gene fragments, three different primer pairs were used: ITS4 and ITS5 for ITS (White et al. 1990), EF1-728F (Carbone and Kohn 1999) and TEF1LLErev (Jaklitsch et al. 2005) for tef1 and tRPB2-5F and tRPB2-7R for rpb2 (Chen and Zhuang 2016). Each 25 µl PCR reaction consisted of 12.5 µl T5 Super PCR Mix (containing Taq polymerase, dNTP and Mg2+, Beijing TsingKe Biotech Co., Ltd., Beijing), 1.25 µl of forward primer (10 µM), 1.25 µl of reverse primer (10 µM), 1µl DNA template, 5 µl of PCR buffer and 4.5 µl sterile

water. PCR reactions were run in an Eppendorf Mastercycler following the protocols described by Zhuang and Chen (2016). PCR products were purified with the PCR product purification kit (Biocolor BioScience & Technology Co., Shanghai, China), and sequencing was carried out in both directions on an ABI 3730 XL DNA sequencer (Applied Biosystems, Foster City, California) with primers used during PCR amplification. GenBank accession numbers of sequences generated in this study are provided in Table 1.

Phylogenetic analyses

Preliminary BLAST searches with tef1, rpb2 and ITS gene sequences of the new isolates against NCBI and UNITE databases identified species closely related to our three isolates. Based on this information, we downloaded tef1, rpb2 and ITS sequences of 40 strains, representing 25 species. To show the phylogenetic position of *T. zeloharzianum*, 11 of the 14 species belonging to the *T. harzianum* complex were included. The remaining three species in this complex were not included because their rpb2 sequences are not available in NCBI.

Three alignment files were generated, one for each gene and converted to NEXUS files with ClustalX 1.83 (Thompson et al. 1997) to identify the phylogenetic positions of these species. The three alignments were then combined with BioEdit 7.1.9.0 (Hall 1999). The phylogenetic analyses included 1008 characters for rpb2, 1233 characters for tef1 and 590 characters for ITS. All characters were weighted equally and gaps were treated as missing characters.

Maximum Likelihood (ML) analysis was computed by RAxML (Stamatakis 2006) with the PHY files generated with ClustalX 1.83 (Thompson et al. 1997), using the GTR-GAMMA model. Maximum likelihood bootstrap proportions (MLBP) were computed with 1000 replicates. Bayesian Inference (BI) analysis was conducted with MrBayes v3.2.2 (Ronquist and Huelsenbeck 2003). The Akaike information criterion (AIC) implemented in jModelTest 2.0 (Posada and Darriba 2008) was used to select the best fit models after likelihood score calculations were done. The base tree for likelihood calculations was ML-optimised. HKY+I+G was estimated as the best-fit model under the output strategy of AIC, Metropolis-coupled Markov chain Monte Carlo (MCMCMC) searches were run for 2000000 generations, sampling every 500th generation. Two independent analyses with four chains each (one cold and three heated) were run until the average standard deviation of the split frequencies dropped below 0.01. The initial 25% of the generations of MCMC sampling were discarded as burn-in. The refinement of the phylogenetic tree was used for estimating Bayesian inference posterior probability (BIPP) values. The Tree was viewed in FigTree v1.4 (Rambaut 2012), values of Maximum likelihood bootstrap proportions (MLBP) greater than 70% and Bayesian inference posterior probabilities (BIPP) greater than 90% at the nodes are shown along branches.

Table 1. Species, strains and their corresponding GenBank accession numbers of sequences used for phylogenetic analyses.

Name	Strain	GenBank accession number		
		ITS	rpb2	tef1
Trichoderma afarasin P. Chaverri & Branco-Rocha	Dis 314F	FJ442259	FJ442778	FJ463400
T. afroharzianum P. Chaverri, F.B. Rocha & Druzhinina	GJS 04-186	FJ442265	FJ442691	FJ463301
T. asperelloides Samuels	GJS 04-187	JN133553	JN133560	JN133571
	GJS 04-116	GU198301	GU248411	GU248412
	GJS 08-87	–	GU198272	GU198241
T. asperellum Samuels, Lieckf. & Nirenberg	GJS 90-7	EU330956	EU338337	EU338333
	GJS 01-294	EU856297	FJ150788	EU856323
	GJS 06-294	GU198307	GU198266	GU198235
	CGMCC 6422	KF425754	KF425755	KF425756
	GJS 05-328	GU198318	EU248614	EU248627
T. atrobrunneum F.B. Rocha, P. Chaverri & Jaklitsch	GJS 04-67	FJ442273	FJ442724	FJ463360
T. atroviride P. Karst	DAOM 222144	AF456916	FJ442754	AF456889
T. gamsii Samuels & Druzhinina	GJS 04-09	DQ315459	JN133561	DQ307541
T. guizhouense Q.R. Li, McKenzie & Yong Wang	S628	–	KJ665273	KJ665511
T. harzianum Rifai	T55	KX632511	KX632568	KX632625
	T18	KX632492	KX632549	KX632606
	T2	FJ884174	KX632534	KX632591
	CBS 226.95	AY605713	AF545549	AF348101
	T11	KX632600	KX632543	KX632486
T. hispanicum Jaklitsch & Voglmayr	S453	JN715595	JN715600	JN715659
T. inhamatum Veerkamp & W. Gams	CBS 273.78	FJ442680	FJ442725	AF348099
T. junci Jaklitsch	CBS 120926	FJ860761	FJ860540	FJ860641
T. kunmingense Y. Zhang	YMF 1.02659	**KJ742800**	**KJ742801**	**KJ742802**
T. lentiforme P. Chaverri, Samuels & F.B. Rocha	Dis 218E	FJ442220	FJ442793	FJ463310
T. lieckfeldtiae Samuels	GJS 00-14	DQ109528	EU883562	EU856326
T. lixii P. Chaverri	GJS 97-96	AF443920	KJ665290	AF443938
T. pleuroti S.H. Yu & M.S. Park	CBS 124387	HM142363	HM142372	HM142382
T. pleuroticola S.H. Yu & M.S. Park	CBS 124383	HM142362	HM142371	HM142381
T. pyramidale Jaklitsch & P. Chaverri	S73	–	KJ665334	KJ665699
T. rifaii F.B. Rocha, P. Chaverri & Samuels	Dis 337F	FJ442621	FJ442720	FJ463321
T. samuelsii Jaklitsch & Voglmayr	S5	JN715596	JN715599	JN715651
T. simmonsii P. Chaverri, F.B. Rocha, Samuels & Jaklitsch	S7	–	KJ665337	KJ665719
T. speciosum Z.F. Yu & X. Du	YMF 1.00205	**MH113929**	**MH155270**	**MH183184**
T. theobromicola Samuels & H.C. Evans	Dis 85f	DQ109525	FJ007374	EU856321
T. valdunense Jaklitsch	CBS 120923	FJ860863	FJ860605	FJ860717
T. viride Pers	CBS 119325	DQ677655	EU711362	DQ672615
T. yunnanense Z.F. Yu & K.Q. Zhang	CBS 121219	GU198302	GU198274	GU198243
T. zeloharzianum Z.F. Yu & X. Du	YMF 1.00268	**MH113932**	**MH158996**	**MH183181**
Nectria eustromatica Jaklitsch & Voglmayr	CBS 125578	HM534897	HM534887	HM534876

Results

Sequence analyses

The final alignments and the trees obtained have been deposited in TreeBASE (TreeBASE accession number: 23172). Phylogenetic positions of the new species were ascertained by analyses of the combined tef1, rpb2 and ITS dataset containing 2831 characters, of which 487 characters were constant, 2344 were variable.

In our analyses, sequences from 41 strains including 21 strains of the Harzianum Clade, 19 strains of the Viride Clade and an outgroup taxa, *Nectria eustromatica* were used to construct the phylogenetic tree. Of the three new species, *T. speciosum* and *T. kunmingense* belonged to the Viride Clade, whereas *T. zeloharzianum* were located in the Harzianum Clade. These two clades formed a monophyletic group, which is generally consistent with what was found in a previous study (Jaklitsch and Voglmayr 2015). The three new species each clustered with different species to form well-supported clades. *T. speciosum* was closely related with *T. samuelsii* Jaklitsch & Voglmayr, *T. hispanicum* (Jaklitsch & Voglmayr) Jaklitsch & Voglmayr and *T. junci* Jaklitsch. This clade had high statistics support (BIPP/MLBP = 100%/85%). *T. kunmingense* fell within a clade formed by strains of *T. asperellum* Samuels, Lieckf. & Nirenberg, but there was a distinct genetic distance between *T. kunmingense* and strains of *T. asperellum*. Similarly, *T. zeloharzianum* was phylogenetically distinct but associated with *T. harzianum* Rifai, *T. lixii* (Pat.) P. Chaverri and *T. simmonsii*. Jaklitsch & Voglmayr.

Taxonomy

***Trichoderma speciosum* Z.F. Yu & X. Du, sp. nov.**
MycoBank MB825469
Figure 2

Etymology. Latin, *speciosum* refers to showy and splendid colony on PDA.

Diagnosis. Characterised by tree-like conidiophores, branches paired or in whorls of 3–4, spindly to fusiform phialides (5.0–10.0 × 2.0–3.0 μm), subglobose to globose conidia (3.7–4.9 × 3.1–3.8 μm). Differs from *T. hispanicum* by paired branches, whorled and thinner phialides, subglobose to globose conidia. Differs from *T. samuelsii* by paired and compact branches, subglobose to globose conidia and the character of pustules on SNA. Differs from *T. junci* by whorled, smaller phialides and subglobose to globose conidia.

Type. CHINA. From soil of tobacco rhizosphere, Luliang, Yunnan Province, 24°57'22"N, 103°46'30"E, 1800 m alt., Jul 2007, Z.F. Yu (YMF 1.00205, holotype), Ex-type culture CGMCC 3.19079.

Description. Mycelium covers plate after 72 h at 25 °C and 30 °C on CMD, no growth at 35 °C. Colony homogenous, pale yellowing, not zonate, outline circular.

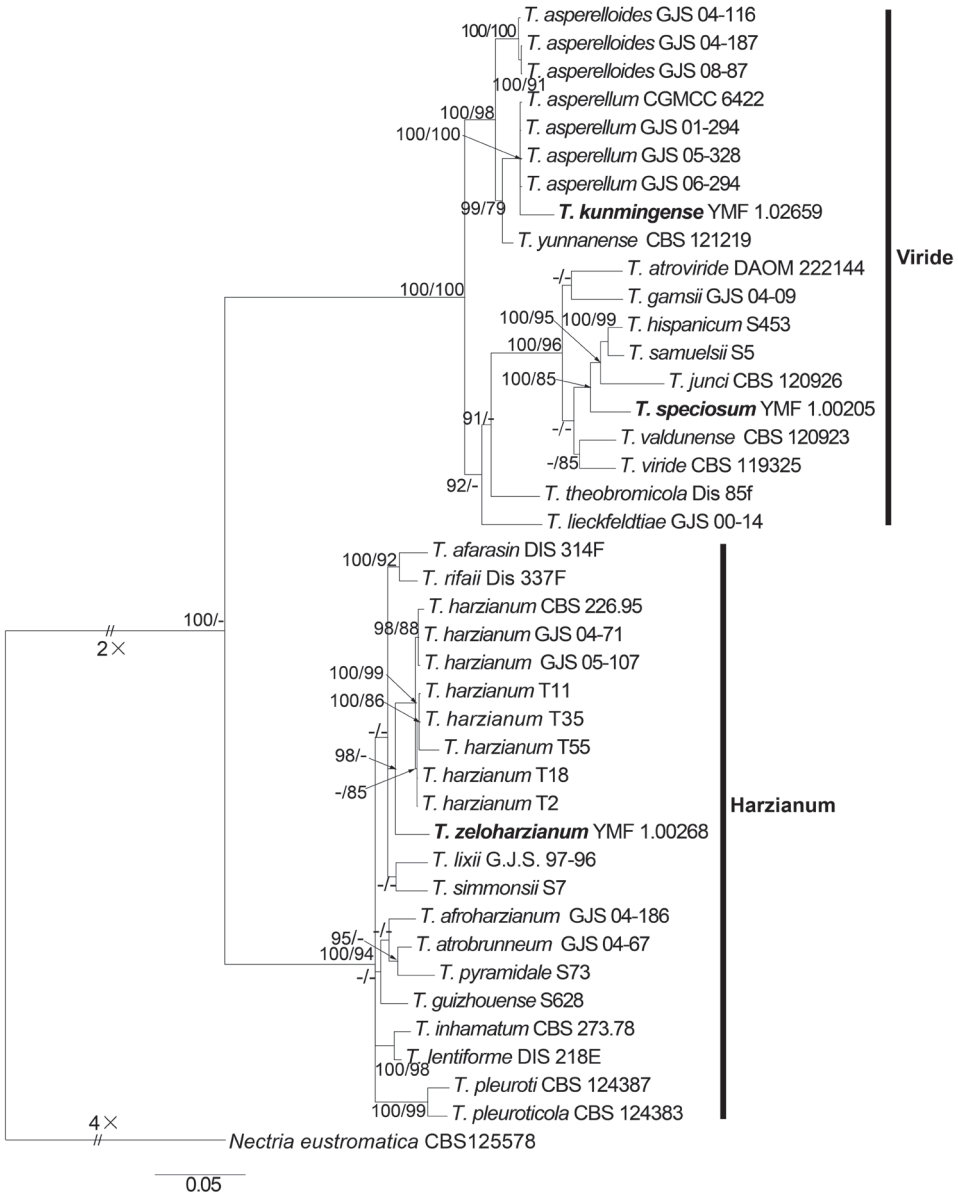

Figure 1. Phylogenetic tree based on Bayesian analysis of the combined tef1, rpb2 and ITS sequences. *Nectria eustromatica* is used as the outgroup. Bayesian posterior probabilities greater than 0.90 are given at the nodes (left). Maximum likelihood bootstrap values greater than 70% are given at the nodes (right). The scale bar shows the expected changes per site. New species proposed are in boldface.

Aerial hyphae sparse, relatively abundant at margin, distinctly radial, arachnoid. Conidial production noted after 4 days.

On PDA, mycelium covers the plate after 72 h at 25 °C and 30 °C, no growth at 35 °C. Colony circular, typically zonate, yellow-green colony homogeneous distrib-

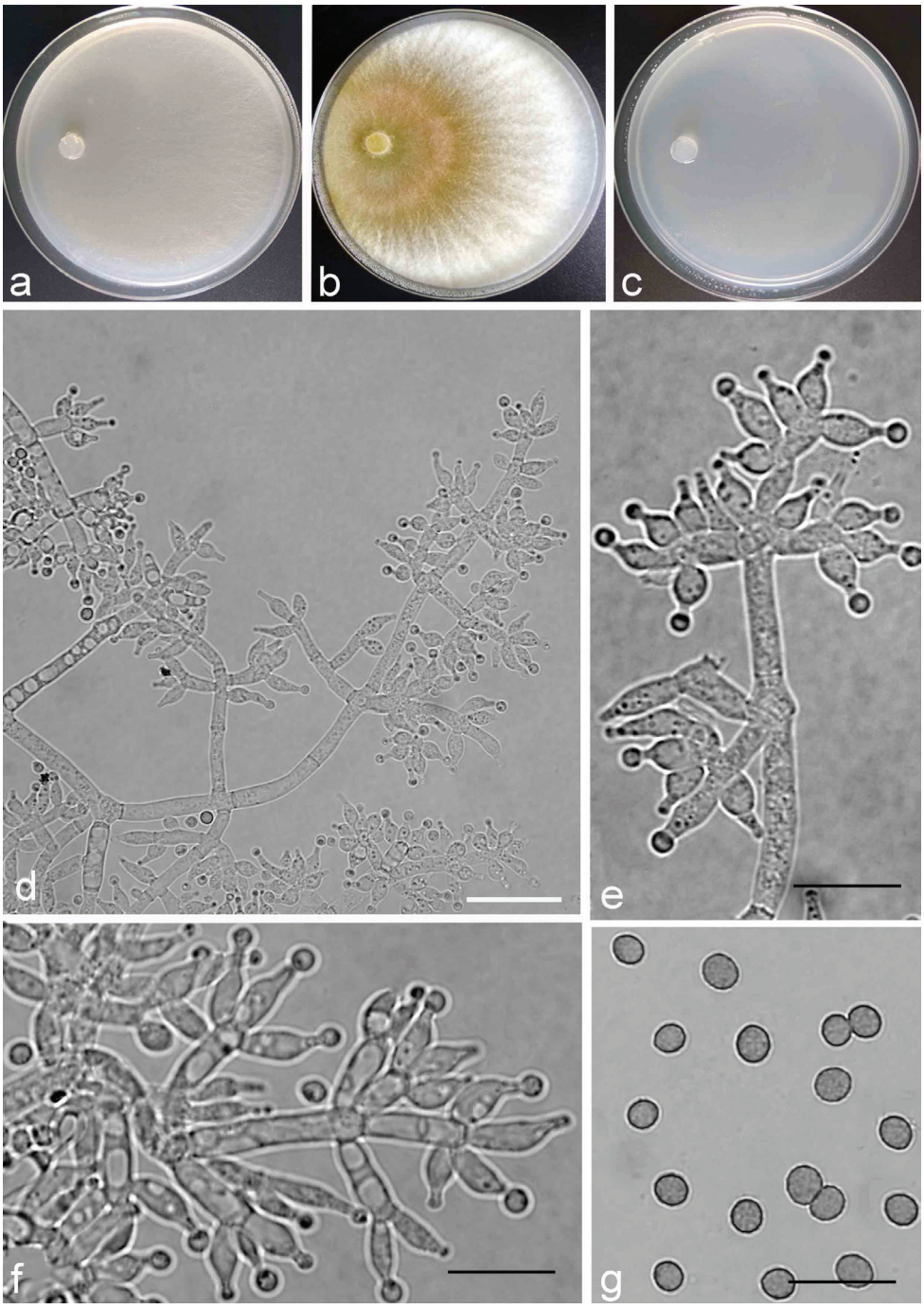

Figure 2. Cultures and anamorph of *Trichoderma speciosum*. **a–c** Cultures (**a** on CMD, 3 days **b** on PDA, 3 days **c** on SNA, 3 days) at 25 °C **d–f** Conidiophores and phialides (SNA, 4 d) **g** Conidia (SNA, 20 d); Scale bars: 10 μm (**d–g**).

uted around the point of inoculation, forming a coarse circle. Whitish aerial hyphae distributed on the agar surface in external zone, hairy, dense and radial. Conidial production noted after 3 days.

On SNA after 72 h, colony radius 37–38 mm at 25 °C, mycelium covers the plate after 120 h, 56–59 mm at 30 °C after 72 h, no growth at 35 °C. Colony hyaline, thin, fan-shaped with indistinct outline. Aerial hyphae scarcely degenerating. Conidial production noted after 5 days, minute white pustules formed around central part of the colony, turning green after 6 days. Conidiophores tree-like, comprising a main axis with second branches, base 3.0–4.0 µm wide, second branches paired or in whorls of 3, sometimes second branches branched again, the distance between neighbouring second branches is (12.0–) 15.0–29.0 (–30.0) µm, main axis and branches terminating in whorls of up to five phialides. Conidiogenous cells phialides lageniform or ampulliform, arising singly or in 2–4; 5.0–10.0 × 2.0–3.0(–3.5) µm, length/width ratio 1.7–3.6 (–4.2), non-equilateral when curved. Conidia ovoid to short ellipsoidal, verrucose (3.6–)3.7–4.9(–5.0) × (3.0–)3.1–3.8(–4.2) µm, length/width ratio (1.0)1.1–1.4(–1.5).

Habitat and distribution. In soil from tobacco rhizosphere in part of cultivated land of south-western China.

Teleomorph. Not known

Remarks. *Trichoderma speciosum* is phylogenetically most closed related to three species – *T. hispanicum*, *T. samuelsii* and *T. junci* (Jaklitsch et al. 2012; Jaklitsch 2011). The three species were isolated from ascospores and only *T. speciosum* was isolated from the anamorph. However, *T. speciosum* differs from these three species in having verrucose, subglobose to globose conida, while conidia of *T. hispanicum* and *T. samuelsii* are oblong and smooth and those of *T. junci* are ovoid to ellipsoidal with length/width ratio 1.3–1.8(–2.2).

In addition, side branches of *T. hispanicum* are often unpaired, phialides often singly, whereas branches of *T. speciosum* are generally paired or in whorls of 3–5. For *T. samuelsii*, branches are sparser and phialides with l/w of (1.7–)2.5–4.6(–7.1) are more slender than those of *T. speciosum*. Phialides of *T. junci* are also more slender than those of *T. speciosum*, which are narrowly lageniform.

***Trichoderma kunmingense* Z.F.Yu & J.Y.Li, sp. nov.**
MycoBank MB808878
Figure 3

Etymology. Latin, *kunmingense*, refers to the site in which this species was found.

Diagnosis. Characterised by pyramidal fashion conidiophores, ampulliform to tapered phialides (6.0–9.0 × 2.5–4.5 µm), discrete branches and ovoid, occasionally ellipsoid, smooth-walled conidia (3.4–4.4 × 2.7–3.4 µm). Differs from *T. asperellum* by slightly shorter and sometimes more whorled phialides, mostly obovoid conidia.

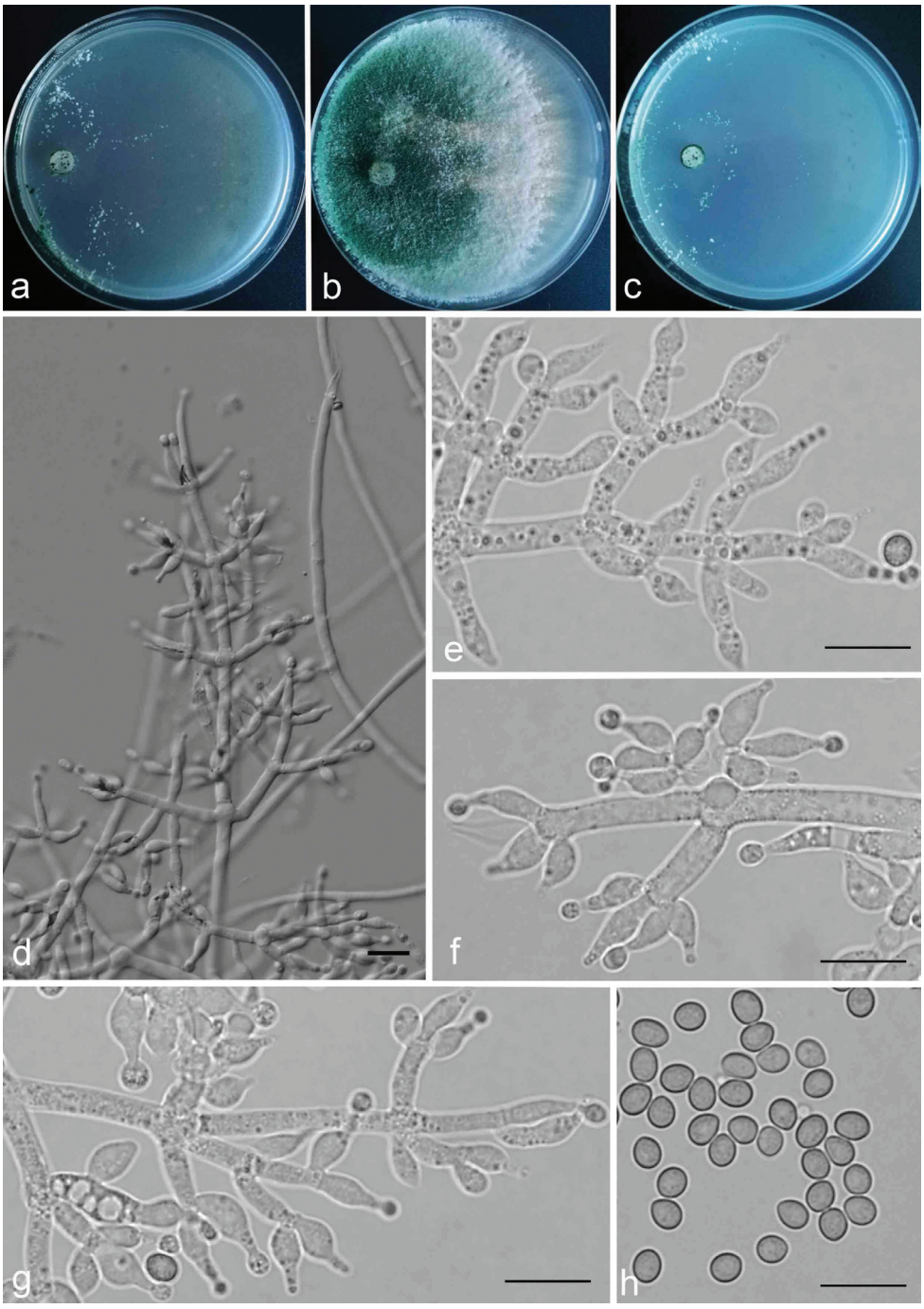

Figure 3. Cultures and anamorph of *Trichoderma kunmingense*. **a** on CMD at 30 °C, 3 days **b, c** Cultures (**b** on PDA, 3 days **c** on SNA, 3 days) at 25 °C **d–g** Conidiophores and phialides (SNA, 4 d) **h** Conidia (SNA, 20 d); Scale bars: 10 μm for (**d–h**).

Differs from *T. yunnanense* by sparser branches and more whorled, smaller phialides and conidia.

Type. CHINA. Kunming, Yunnan, 24°52'28"N, 102°49'34"E. 1929 m alt, in soil, Aug 2007, Y. Zhang (YMF 1.02659, holotype), Ex-type culture CBS 125635.

Description. Colony on CMD after 72 h radius 35–50 mm, mycelium covering the plate after 96 h at 25 °C, 55–59 mm at 30 °C and 41–46 mm at 35 °C after 72 h. Colony hyaline, margin distinctly noted. Aerial hyphae are indistinctly observed, radiate and sparse, white pustule formed from inner zone, asymmetrical to pulvinate, loosely arranged. Conidial production noted after 48 h. No diffusing pigment produced.

Mycelium covers plate after 72 h at 25 °C and 35 °C on PDA and radius 52–56 mm at 30 °C. Colony layered distinctly, margin conspicuous and radial. Aerial hyphae, hairy to floccose, dense internal zone, but relative sparse on margin, abundantly and flat in a large green disc around the inoculums, turning green after 24 h of conidiation.

Colony on SNA after 72 h radius 48–50 mm, mycelium covering the plate after 96 h at 25 °C, 53–56 mm at 35 °C and covering the plate at 30 °C after 72 h. Colony and pustules are similar to that on CMD, colony hyaline and smooth, the shape of pustules more regular, sometimes hemispherical, loosely distributed around the point of inoculation. Conidiophores well defined, branching 2–3 times in a pyramidal fashion, with the longest branches verticillate on the discrete main axis, the base 2.2–3.9(–4.4) µm wide, branched toward the tip, the distance between neighbouring second branches are 11.0–38.5 µm. Phialides arising generally 1–3 times repetition on each branches or in whorls of 3–5, ampulliform to tapered, slightly constricted at the base, often straight or less sinuous or curved toward apex of conidiophore, mostly (5.0–) 6.0–9.0(–10.0) × 2.5–4.5 µm, length/width ratio (1.3–)1.4–3.4(–3.6). Conidia obovoid, sometimes ellipsoidal, smooth-walled, both ends broadly rounded or at the base slightly narrower, 3.4–4.4 × 2.7–3.4 µm, length/width ratio (1.1–)1.2–1.6, pale green when viewed singly, usually greenish in mass.

Specimen examined. PR China, Kunming, Yunnan Province, 24°52'N, 102°49'E, elev. 1929 m, isolated from soil samples, Aug. 2007, by Y. Zhang (Holotype, YMF 1.02659; ex-type culture, YMF 1.026591, CBS 125635).

Habitat and distribution. In garden soil of Kunming city of southwest China.

Teleomorph. Not known

Remarks. *Trichoderma kunmingense* can be distinguished from *T. asperellum* Samuels, Lieckfeldt and Nirenberg, by having more crowded branches and phialides. *T. asperellum* typically forms whorls of 2–4 phialides, whereas phialides of *T. kunmingense* sometimes attain 5 phialides. Although the phialides are ampulliform in both species, the phialides of *T. asperellum* are slightly longer (type strain: 7.2–11.5 µm) than those of *T. kunmingense*. Moreover, conidia of *T. asperellum* have inconspicuous and small ornamentation, but those of *T. kunmingense* are smooth and conidia are slightly longer (type strain: 3.5–4.5 × 2.7–4.0 µm) (Samuels et al. 1999, Samuels and Ismaiel 2010).

Trichoderma kunmingense and *T. yunnanense* Yu and Zhang are also closely related in the phylogenetic tree, but branches and phialides of *T. yunnanense* are more crowded

than those of *T. kunmingense*. Phialides in *T. yunnanense* arising separately or more often paired with branches, rarely in whorls of 3 (Yu et al. 2007). Conidia of *T. yunnanense* (4.0–5.0 ×3.5–4.0 μm) are also larger than those of *T. kunmingense*.

***Trichoderma zeloharzianum* Z.F. Yu & X. Du, sp. nov.**
MycoBank MB825472
Figure 4

Etymology. Greek *zelo-*, meaning emulation + *harzianum*, referred to *Trichoderma harzianum*

Diagnosis. Characterised by pyramidal conidiophores, verticillate branches, ampulliform to lageniform phialides (5.5–10.0 × 2.5–3.5 μm) and subglobose to globose, thin-walled conidia (2.7–3.1 × 2.4–2.6 μm). Differs from *T. harzianum* by verticillate branches, 3–6 whorled phialides on terminal of each branch and thinner conidia. Differs from *T. lixii* by verticillate and compact branches, more terminal phialides on main axis and smaller conidia. Differs from *T. simmonsii* by verticillate branches and longer conidia.

Type. CHINA. Yunnan: Qujing City, Luliang county, 25°05'25"N, 103°56'42"E, 1800 m alt., in soil, Jul 2007, Z.F. Yu (YMF 1.00268, holotype), Ex-type culture CGMCC 3.19082.

Description. On CMD after 72 h, colony radius 59–62 mm, mycelium covers the plate after 96 h at 25 °C; 43–45 mm at 30 °C and 46–52 mm at 35 °C after 72 h. Colony yellowing, margin distinct. Aerial hyphae fertile and conspicuous, hairy radial, distributed on surface, green conidial production noted after 4 days.

On PDA after 72 h, colony radius 57–58 mm, mycelium covers the plate after 96 h at 25 °C. Covering the plate at 30 °C and 38–42 mm at 35 °C after 72 h. Colony white, margin distinct. Aerial hyphae abundant, hairy to floccose, denser around central disc. Green conidiation noted after 3 days.

On SNA after 72 h, radius 59–65 mm, mycelium covers the plate after 144 h at 25 °C, 64–65 mm at 30 °C and 29–37 mm at 35 °C after 72 h. Aerial hyphae sparsely, slightly radial and conspicuous zonate. Conidiophores well defined, branching 2–3 times in a pyramidal fashion. Branches paired or a whorl of 3–4, the distance between neighbouring second branches is 16.0–39.0 μm, base 3.0–4.0 μm wide, each branch terminating in a whorl of 3–6 phialides, phialides ampulliform to lageniform, often verticillated up to 5 around the main axis near the apex, rarely singly arising, (4.5)5.5–10.0(–11.0) × 2.5–3.5(–4.0) μm, length/width ratio (1.4–)1.8–3.4(–3.6). Conidia smooth on surface, subglobose to globose, sometimes obovoid, (2.6–) 2.7–3.1(–3.2) × (2.3–) 2.4–2.6(–2.7) μm, length/width ratio (1.0–)1.1–1.3(–1.4).

Habitat and distribution. In soil from tobacco rhizosphere in part of cultivated land of south-western China.

Teleomorph. Not known

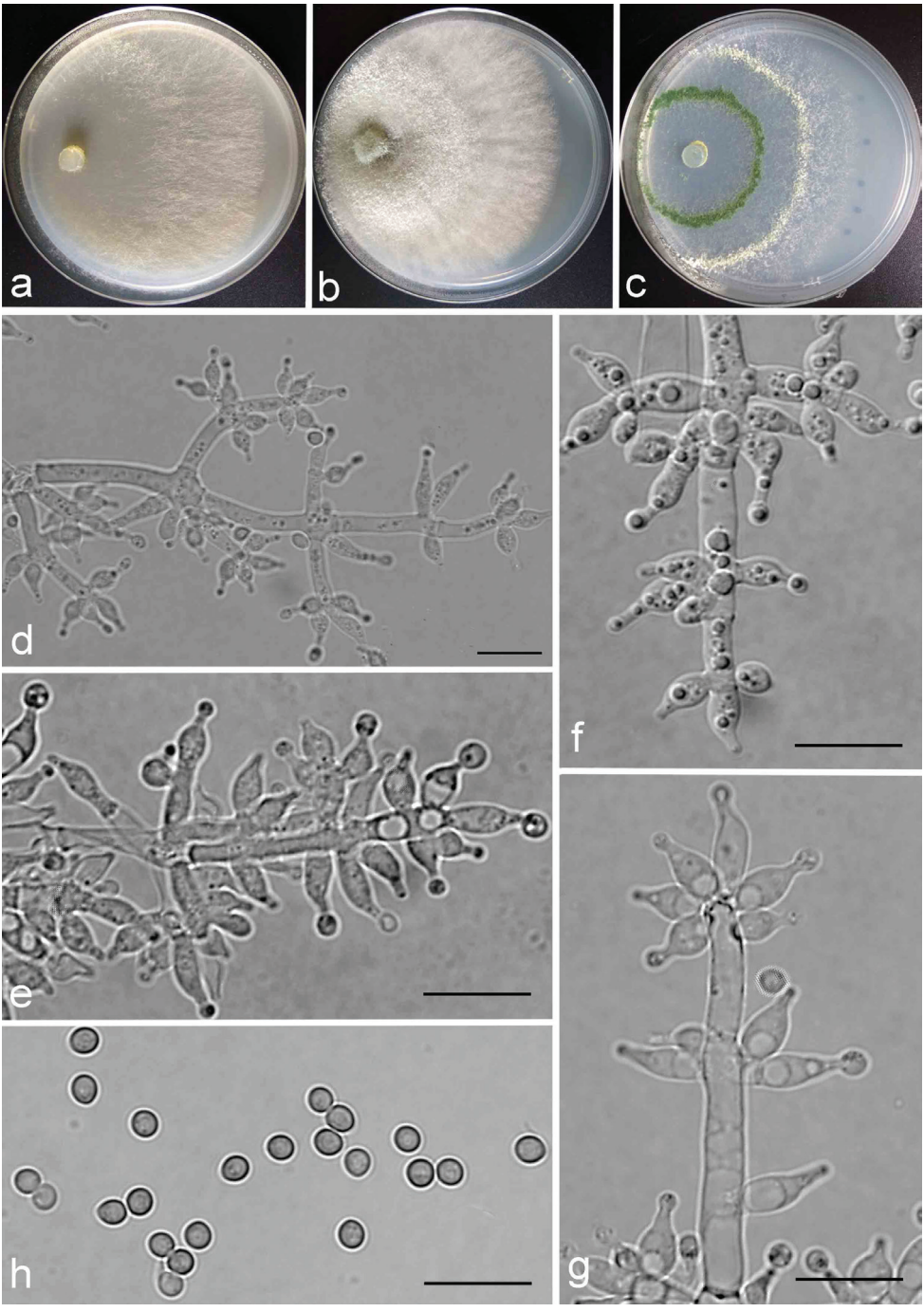

Figure 4. Cultures and anamorph of *Trichoderma zeloharzianum*. **a–c** Cultures (**a** on CMD, 3 days **b** on PDA, 3 days **c** on SNA, 3 days) at 25 °C **d** Conidiophore-like structures (SNA, 4 d) **e–g** Conidiophores and phialides (SNA, 4 d) **h** Conidia (SNA, 20 d); Scale bars: 10 μm for **d–h**.

Remarks. *Trichoderma zeloharzianum* forms a single branch with *T. harzianum* Rifai as sister clade. Morphologically, *T. harzianum* is similar to *T. zeloharzianum* in their shape of conidiophores and phialides, but the branches of *T. harzianum* are opposite of each other and each branch terminating in a whorl of 2–5 phialides (Chaverri et al.2015), while *T. zeloharzianum* is clearly distinguishable by having verticillated branches and 3–6 terminal whorled phialides. In addition, the conidia of *T. harzianum* are generally wider [(2.0–)2.5–3.0 (–3.7) µm] than those of *T. zeloharzianum*.

Trichoderma lixii differs from *T. zeloharzianum* also by having opposing pairs of branches and fewer terminal phialides (2–4) on main axis. Beyond that, closely spaced branches are common in *T. lixii* (Chaverri et al.2015), whereas for *T. zeloharzianum*, neighbouring branches are more compact and the conidia of *T. lixii* are usually larger [(2.5–)3.0–3.5 (–3.7) × (2.2–)2.5–3.2(–3.5) µm] than those of *T. zeloharzianum*.

Trichoderma simmonsii is also distinguished obviously from *T. zeloharzianum*, except their differences about opposing branches (Chaverri et al. 2015), the phialides are more stout and shorter ((4.2–)5.2–6.5 (–9.0) µm) than those of *T. zeloharzianum*. Furthermore, *T. simmonsii* is commonly constricted below the tip to form a narrow neck (Chaverri et al. 2015); however, this character is not found in *T. zeloharzianum*.

Discussion

The application of molecular barcode for fungal taxonomy has led to a re-evaluation of morphology-based taxonomy of *Trichoderma*. A recent study suggested that tef1 introns could provide a high resolution to this genus and is shown to be superior to other phylogenetic markers (Jaklitsch et al. 2012). Rpb2 sequences appeared powerful due to their suitable interspecific variations (Jaklitsch and Voglmayr 2015). ITS sequences are identical or nearly identical for several species of the genus (e.g. those of *T. hispanicum*, *T. koningii*, *T. viridescens* and *T. samuelsii*), therefore this marker alone is not useful for phylogenetic reconstruction or for barcoding of these fungi (Druzhinina et al. 2005, Jaklitsch et al. 2012). Together, due to their universality and reliability for species in the *Trichoderma* genus, these three loci were chosen for this study.

Based on the combined analysis of sequences from three genes, phylogenetic positions of three species were ascertained, amongst which *T. zeloharzianum* belonged to the Harzianum clade. *T. zeloharzianum* has the characteristic of typical *T. harzianum*-like morphology containing pairs or verticils branches, ampulliform to lageniform phialides and globose to subglobose or broadly ovoid conidia (Chaverri et al. 2015). The *T. harzianum* species complex is a cosmopolitan and ubiquitous species, playing important roles in ecology and economy. Chaverri et al. (2015) disentangled this species complex recognising 14 species. In the present study, 11 of the 14 species from the Harzianum Clade were included for analyses. *T. zeloharzianum* is the most closely related to *T. harzianum*, with the latter being more broadly distributed. The sexual and asexual morphs for *T. lixii*–*T. harzianum* have been rejected (Druzhinina et al. 2010,

Atanasova et al. 2013) and Chaverri et al. (2015) and also showed that *T. lixii* and *T. harzianum* are closely related but represent separate species. Here, we found *T. zeloharzianum* is more closely to *T. harzianum* than to *T. lixii*.

Both *T. speciosum* and *T. kunmingense* belong to the Viride Clade. The study of Jaklitsch and Voglmayr (2015) indicated that the structure of the Viride Clade is complex, as there are additional subclades, such as the Hamatum/ Asperellum Clade, the Rogersonii Clade, the Neorufum Clade and several smaller subclades. Samuels et al. (2006) showed that asexual morphs of the Viride Clade often have verrucose conidia. In the present study, *T. kunmingense* with smooth conidia is found phylogenetically related to *T. asperellum* and *T. asperelloides*, two species with verrucose conidia and both belonging to the Asperellum subclade. However, *T. speciosum* with warted conidia could not be assigned to any specific subclade.

Species of the Harzianum and Viride Clades were commonly isolated from soil. However, the number of published soil-inhabiting *Trichoderma* species is limited compared with that on woody substrates. Furthermore, the sexual states of most soil-inhabiting species are unknown (Chen and Zhuang 2016). China is rich in species diversity of the *Trichoderma* genus. Future studies will likely reveal more new taxa in soil, which could provide a better understanding of the relationship between asexual and sexual states of some species in the genus.

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RESEARCH ARTICLE

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Melanospora (Sordariomycetes, Ascomycota) and its relatives

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Abstract

The order Melanosporales comprises a large group of ascomycetes, most of them mycoparasites, characterized by the production of usually ostiolate, translucent ascomata, unitunicate asci, and unicellular, pigmented ascospores with germ pores or germ slits. The most studied taxa are *Melanospora* and *Sphaerodes*, but the boundaries with other morphologically closely related genera are not well resolved. In this study, the taxonomy of *Melanospora* and related taxa have been re-evaluated based on the analysis of nuclear rDNA, actin and elongation factor genes sequences of fresh isolates and numerous type and reference strains. The genus *Melanospora* has been restricted to species with ostiolate ascoma whose neck is composed of intermixed hyphae, and with a phialidic asexual morph. *Microthecium* has been re-established for species of *Melanospora* and *Sphaerodes* without a typical ascomatal neck or, if present, being short and composed of angular cells similar to those of the ascomatal wall, and usually producing bulbils. Three new genera have been proposed: *Dactylidispora*, possessing ascospores with a raised rim surrounding both terminal germ pores; *Echinusitheca*, with densely setose, dark ascomata; and *Pseudomicrothecium*, characterized by ascospores with indistinct germ pores. Dichotomous keys to identify the accepted genera of the Melanosporales, and keys to discriminate among the species of *Melanospora* and *Microthecium*, as well as a brief description of the accepted species of both genera, are also provided.

Keywords

Ceratostomataceae, *Dactylidispora*, *Echinusitheca*, Melanosporales, *Microthecium*, *Pseudomicrothecium*, soil, *Sphaerodes*, 4 new taxa

Introduction

The family Ceratostomataceae (Winter 1887) includes nearly 100 species, often mycoparasitic and characterized by ostiolate and rostrate, or less frequently non-ostiolate, translucent ascomata, unitunicate and evanescent asci, brown or exceptionally hyaline, unicellular ascospores with a germ pore at each end, or less frequently with only one germ pore or a germ slit, and phialidic asexual morphs or bulbils. Currently, that family is included in the Melanosporales (Chaudhary et al. 2006, Zhang et al. 2006, Hibbett et al. 2007, Li et al. 2016, Schultes et al. 2017), although historically it had been placed in Aspergillales (Gaümann 1964), Hypocreales (Alexopoulos 1962, Spatafora and Blackwell 1994a, Rehner and Samuels 1995, Jones and Blackwell 1998, Zhang and Blackwell 2002) and Sphaeriales (Bessey 1950, Dennis 1968). This family comprises 11 sexually reproducing genera, i.e. *Arxiomyces*, *Melanospora*, *Persiciospora*, *Pteridiosperma*, *Pustulipora*, *Rhytidospora*, *Scopinella*, *Setiferotheca*, *Sphaerodes*, *Syspastospora* and *Vittatispora*. *Melanospora*, the largest genus of this family (more than 50 species), was established by Corda (1837) to accommodate *Ceratostoma chionea* and two new species, *Melanospora zamiae* and *Melanospora leucotricha*, with the former chosen later as the type species (Kowalski 1965). *Melanospora* is characterized by usually ostiolate ascomata with a long neck and a translucent, pale yellow to reddish brown ascomatal wall, and mostly smooth-walled, brown, ellipsoidal to citriform, rarely discoid or fusoid ascospores, with a depressed germ pore at each end, occasionally surrounded by a raised rim (Guarro et al. 2012). Related genera are *Microthecium* and *Sphaerodes*. The former was erected by Corda (1842) to distinguish *Mi. zobelii* from *Melanospora* spp. by the presence of non-ostiolate, usually immersed ascomata; and *Sphaerodes* was introduced by Clements (1909) to separate *Melanospora episphaeria* from *Melanospora* spp. by its reticulate ascospores. However, the generic boundaries between *Melanospora* and its relatives remained obscure. Doguet (1955) carried out a revision of *Melanospora*, synonymizing several species and transferring additional species from other genera, mostly from *Sphaeroderma*, which had been proposed by Fuckel (1877) and distinguished from *Melanospora* by the absence of an ascomatal neck. Doguet (1955) considered the production of a neck as a non-stable taxonomic character influenced by the nature of the substrate where the fungus grows, and segregated the genus in several sections on the basis of the morphology of the ascomata (presence or absence of neck, and its size when present) and ascospores (shape and ornamentation). The most comprehensive revision of *Melanospora* and related genera was carried out by Cannon and Hawksworth (1982), based mainly on the structure of the ascospore wall under SEM, resulting in the transfer of species of *Microthecium* to *Melanospora* and to *Sphaerodes*. However, recent molecular studies demonstrated that these two latter genera are polyphyletic (Zhang and Blackwell 2002, Fan et al. 2012, Li et al. 2016, Schultes et al. 2017). Other genera included in the family are: *Arxiomyces*, which produces ovoid to ellipsoidal ascospores with a rounded apex and a truncate base with a large sunken germ pore (Cannon and Hawksworth 1982, 1983); *Persiciospora*, characterized by ascospores with a pitted wall and a faint reticulation (Cannon and Hawksworth 1982); *Pteridiosperma*, with ascospores ornamented with longitudinal wing-like appendages (Krug and Jeng 1979);

Pustulipora, distinguished by its ascospores with a germ pore at each end surrounded by a blistered, rarely cushion-like structure showing an irregular pustulate appearance (Cannon 1982); *Rhytidospora*, characterized by non-ostiolate ascomata with a cephalothecoid ascomatal wall (Krug and Jeng 1979); *Scopinella*, producing brown, cuboid-ellipsoidal ascospores with two prominent longitudinal germ slits (Cannon and Hawksworth 1982); *Setiferotheca*, which produces ascospores similar to those of *Arxiomyces* and ascomata with a crown of dark brown setae surrounding the ostiole (Matsushima 1995); *Syspastospora*, possessing ascomata with a long neck composed of parallel arranged hyphae and cylindrical ascospores with a large terminal slightly sunken germ pore at each end (Cannon and Hawksworth 1982); and *Vittatispora*, which produces ascomata similar to those of *Syspastospora* and citriform ascospores with a longitudinal, thick, hyaline ridge (Chaudhary et al. 2006). Practically all taxonomic studies on these fungi have been based exclusively on the morphological characterization of the reproductive structures of preserved fungarium specimens, since unfortunately due to their mycoparasitism, many of these fungi do not grow in pure culture or do not produce ascomata in absence of their hosts. On the other hand, obtaining reliable nucleotide sequences from members of the Melanosporales is also difficult because of the usually large amount of DNA of their hosts. Based on the study of several freshly-isolated soil-borne fungi and of reference and type strains obtained from various culture collections, we have re-examined the phylogenetic relationships of the most relevant genera of the Ceratostomataceae. Consequently, the genus *Melanospora* has been redefined, *Microthecium* has been re-established, and three new genera have been proposed.

Materials and methods

Fungal isolates

The strains included in this study are listed in Table 1. Fresh isolates were obtained from samples following previously described procedures for the activation of dormant ascospores in soil using acetic acid and phenol solutions (Stchigel et al. 2001, García et al. 2003). Ascomata were transferred to 55 mm diam. Petri dishes containing oatmeal agar (OA; oatmeal flakes, 30 g; agar-agar, 20 g; distilled water, 1 L) using a sterile needle, which were then incubated at 15, 25 and 35 °C.

Morphological study

For cultural characterization, isolates were grown for up to 30 d on OA, potato carrot agar (PCA; grated potatoes, 20 g; grated carrot, 20 g; agar-agar, 20 g; L-chloramphenicol, 100 mg; distilled water, 1 L), and potato dextrose agar (PDA; Pronadisa, Madrid, Spain) at 5, 10, 15, 20, 25, 30, 35 and 40 °C. Color notations in parentheses are from Kornerup and Wanscher (1984). Vegetative and reproductive structures were examined under an Olympus BH-1 brightfield microscope by direct mounting in lactic acid

Table 1. Isolates and reference strains of members of Melanosporales included in the combined phylogenetic study.

Taxa	Strain	Source	GenBank accession number			
			LSU	ITS	*act*	*tef1*
Dactylidispora ellipsospora	NBRC 31376^{T}	Forest soil, Papua New Guinea, Buin, Bougainville Island	KP981451	03137601*	KP981545	KP981579
Dactylidispora singaporensis	NBRC 30865^{T}	Soil, Singapore	KP981452	03086502*	KP981546	KP981580
Echinusitheca citrispora	CBS 137837^{T} = FMR 12767^{T}	Forest soil, USA, North Carolina, Great Smoky Mountains National Park, Cataloochee Creek Campground	KP981453	KP981477	KP981547	KP981581
Nectria cinnabarina	CBS 127383	Austria, Niederösterreich, Litschau	HM534894	HM534894	–	HM534873
Melanospora damnosa	CBS 113681	Soil, France, Pont d'Espagne	KP981454	KP981478	KP981543	KP981582
Melanospora kurssanoviana	NBRC 8098	Unknown	KP981455	KP981479	KP981548	KP981583
Melanospora verrucispora	NBRC 31375^{T}	Forest soil, Papua New Guinea, Kebil, Chimb Dist.	KP981456	KP981480	KP981549	KP981584
Melanospora zamiae	NBRC 7902	Unknown	KP981457	00790201*	KP981544	KP981585
Microthecium ciliatum	NBRC 9829	Soil, unknown	KP981458	KP981481	KP981524	KP981586
Microthecium compressum	NBRC 8627	Unkown	KP981459	00862701*	KP981525	KP981587
Microthecium fayodii	FMR 12363	Soil, Tennessee, Great Smoky Mountains National Park, Cosby Creek trail	KP981460	KP981482	KP981526	KP981588
Microthecium fimbriatum	NBRC 8523	Unknown	KP981461	KP981483	KP981527	KP981589
Microthecium fimicola	NBRC 8354	Unknown	KP981462	KP981484	KP981528	KP981590
	FMR 5483	Soil, Australia, Moara	KP981463	KP981485	KP981529	KP981591
	FMR 12370	Soil, Spain, Gran Canaria	KP981464	KP981486	KP981530	KP981592
	FMR 13418	Soil, Spain, Aragon, Los Valles Occidentales	KP981465	KP981487	KP981531	KP981593
Microthecium fusisporum	NBRC 8806	Unknown	KP981466	00880601*	KP981532	KP981594
Microthecium japonicum	FMR 12371	Soil, Spain, Gran Canaria, Pico de Osorio	KP981467	KP981488	KP981533	KP981595
Microthecium levitum	FMR 6218 = CBS 966.97	Soil, Nepal, Bhadgaon	KP981468	KP981489	KP981534	KP981596
	FMR 10098	Soil, Nigeria, Enugu, Nsukka	KP981469	KP981490	KP981535	KP981597
	FMR 13884	Soil, Spain, Catalonia, Vall Fosca	KP981470	KP981491	KP981536	KP981598
Microthecium quadrangulatum	CBS 112763^{T}	Soil, Spain, Asturias, Muniellos Biological Absolute Reserve	KP981471	KP981492	KP981537	KP981599
Microthecium retisporum	NBRC 8366	Soil, Japan	KP981472	00836601*	KP981538	KP981600
Microthecium sepedonioides	FMR 11933	Forest soil, Spain, Aragón, valle de Ordesa	KP981473	KP981493	KP981539	KP981601
Microthecium sp.	FMR 6725 = CBS 102190	Desert soil, Egypt, Sinai	KP981474	KP981494	KP981540	KP981602
Microthecium sp.	FMR 7183 = CBS 108937	Forest soil, New South Wales, Sydney, Blue Mountains	KP981475	KP981495	KP981541	KP981603
Microthecium tenuissimum	CBS 112764^{T}	Soil, Spain, Murcia, Sierra de Espuña, Umbria de Peña Apartada	KY628706	KY628705	–	–

Taxa	Strain	Source	GenBank accession number			
			LSU	ITS	*act*	*tef1*
Microthecium zobelii	NBRC 9442	Decaying carpophore of *Coriolus flabelliformis*	KP981476	00944201*	KP981542	KP981604
Pseudallescheria fusoidea	CBS 106.53^{T}	Soil, Panama, Guipo	EF151316	AY878941	–	–
Pseudomicrothecium subterraneum	BJTC FAN1001^{T}	From *Tuber indicum*, China, Yunnan	JN247804	–	–	–
Vittatispora coorgii	BICC 7817^{T}	Soil, India, Western Ghats, Coorg District, Kakkabe	DQ017375	–	–	–

BICC: Biocon culture collection, Bangalore, India; BJTC: Capital Normal University, Beijing, China; CBS: Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands; FMR: Facultat de Medicina, Reus, Spain; NBRC: Biological Resource Center, Chiba, Japan. ^{T} indicates type strains. * sequences retrieved from NBRC database.

and water of the ascomata and/or microcultures grown on OA and PDA. Pictures were obtained with a Zeiss Axio Imager M1 brightfield microscope. The samples for scanning electron microscopy (SEM) were processed according to Figueras and Guarro (1988), and SEM micrographs were taken at 15 keV with a Jeol JSM 840 microscope.

Molecular study

The DNA of the fungal isolates (Table 1) was extracted and purified directly from the colonies according to the Fast DNA Kit protocol (MP Biomedicals, Solon, Ohio). The amplification of the small subunit (SSU), the D1–D3 domains of the large subunit (LSU) and the internal transcribed spacer region (ITS) of the nuclear rDNA, and the fragments of actin (*act*) and translation elongation factor 1-α (*tef1*) genes were performed according to White et al. (1990) (SSU), Vilgalys and Hester (1990) (LSU), Cano et al. (2004) (ITS), Voigt and Wöstermeyer (2000) (*act*) and Houbraken et al. (2007) (*tef1*). A BigDye Terminator 3.1 cycle sequencing kit (Applied Biosystems Inc., Foster City, California) was used to sequence both strands with a combination of the same primers used in the amplification. PCR products were purified and sequenced at Macrogen Europe (Amsterdam, The Netherlands) with a 3730XL DNA analyzer (Applied Biosystems), and the consensus sequences were obtained using SeqMan (version 7.0.0; DNASTAR, Madison, WI, USA). A phylogenetic study based on the analysis of SSU sequences of the isolates and type and reference strains of the Melanosporales and of some members of the Chaetosphaeriales, Coniochaetales, Coronophorales, Hypocreales, Microascales, Sordariales and Xylariales, using *Thelebolus ellipsoideus* (Thelebolales) as outgroup, was performed to confirm the taxonomic placement of our isolates. A subsequent study, carried out to infer the phylogenetic relationships among members of the Melanosporales, was based on the analysis of a combined data set including the ITS, LSU, *act* and *tef1* sequences of our isolates and of type and reference strains of a large number of the Melanosporales, including *Nectria cinnabarina* and *Pseudallescheria fusoidea* as outgroups. The Maximum-Likelihood (ML) and Bayesian Inference (BI) methods were used in phylogenetic analyses as described by Hernández-Restrepo et al. (2016). Bootstrap support (BS) ≥70 and posterior probability values (PP) ≥0.95 were considered significant. The sequences generated in

this study were deposited in GenBank (Table 1 and Fig. 1) and the alignments used in the phylogenetic analyses were deposited in TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S17079). Sequences retrieved from GenBank and NBRC included in the SSU and combined analyses are shown in Fig. 1 and Table 1, respectively.

Results

The SSU phylogenetic study was based on an alignment of 1023 bp and produced a single ML tree (Fig. 1) inferred from a RAxML analysis. The members of the Melanosporales including our isolates were placed in a highly supported main clade (100 % BS / 0.99 PP), and the isolate CBS 137837, whose morphological features did not match any previously described taxon, occurred as a basal branch clearly separated from the other Melanosporales, which grouped together with a high support (95 % BS / 0.98 PP) and separated into three subclades. The first one (96 % BS / - PP), contained most of the isolates morphologically identified as *Melanospora*, *Persiciospora* and *Sphaerodes*, including the type and reference strains of *Melanospora brevirostris*, *M. fimbriata*, *M. fusispora*, *M. levita*, *M. zobelii*, *Papulaspora sepedonioides*, *Pteridiosperma ciliatum*, *Sphaerodes compressa*, *S. fimicola*, *S. retispora*, *S. quadrangularis* and *S. tenuissima*, without significant genetic variation among them. The second subclade (80 % BS / - PP) comprised the type strains of *Sphaerodes ellipsospora* and *Sphaerodes singaporensis* and a reference strain of *Melanospora kurssanoviana*, which resulted clearly separated from the other two, which grouped with high support (100 % BS / 0.98 PP). In the third subclade (100 % BS / 1 PP) were nested the type species of *Melanospora* (*M. zamiae*), the type strains of *Melanospora verrucispora* and *Sphaerodes mycoparasitica*, and reference strains of *Melanospora damnosa* and *Melanospora tiffanii*.

The lengths of the individual alignments used in the combined data set were 802 bp (LSU), 535 bp (ITS), 727 bp (*act*) and 846 bp (*tef1*), respectively, and the final total alignment was 2910 bp. In the ML tree derived from the RAxML analysis of the combined data set (Fig. 2), the Melanosporales were highly supported (100 % BS / 1 PP) and subdivided into seven lineages. The first clade (89 % BS / 1 PP; Clade *Microthecium*) grouped all our isolates, with the exception of CBS 137837, and type or reference strains of *Melanospora fimbriata*, *M. fusispora*, *M. levita*, *M. zobelii*, *Papulaspora sepedonioides*, *Pteridiosperma ciliatum*, *Sphaerodes compressa*, *S. fimicola*, *S. retispora*, *S. quadrangularis* and *S. tenuissima*. All the fungi belonging to this clade have non-ostiolate ascomata, or when a neck is present, it is short and composed of angular cells similar to those of the ascomatal wall. Also, bulbils (microsclerotial-like asexual propagules) are present in most of these species. In spite of the high morphological variability shown by members of this clade, the loci used in the phylogenetic analysis were not able to separate the species from each other. The second clade (100% BS / 1 PP; Clade *Melanospora*) comprised the type species of *Melanospora*, *M. zamiae*, the type strain of *M. verrucispora* and a reference strain of *M. damnosa*. The members of this clade produce ostiolate ascomata with a long neck composed of hyphae irregularly arranged and ending in a crown of setae. In addition, an asexual morph is commonly present, which is characterized by solitary, sessile, flask-shaped phialides producing from rounded to ellipsoidal conidia. The third lineage comprised only

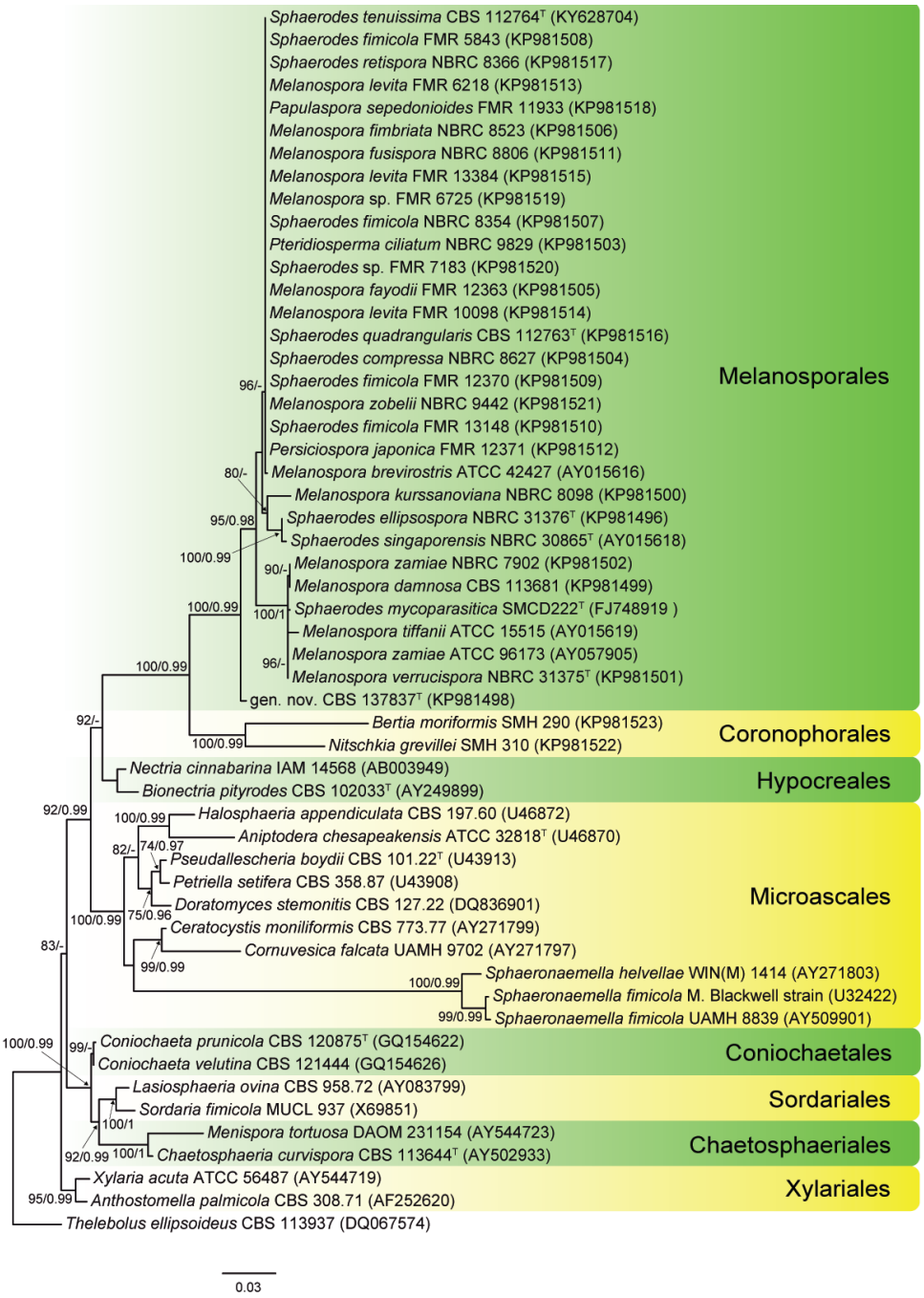

Figure 1. RAxML phylogram obtained from SSU sequences of isolates and type and reference strains included in the Melanosporales, and strains belonging to the orders Chaetosphaeriales, Coniochaetales, Coronophorales, Hypocreales, Microascales, Sordariales and Xylariales. *Thelebolus ellipsoideus* was used as outgroup. RAxML bootstrap support (BS) values above 70 % and Bayesian posterior probability scores above 0.95 are shown at the nodes. Type strains of the different species are indicated with T.

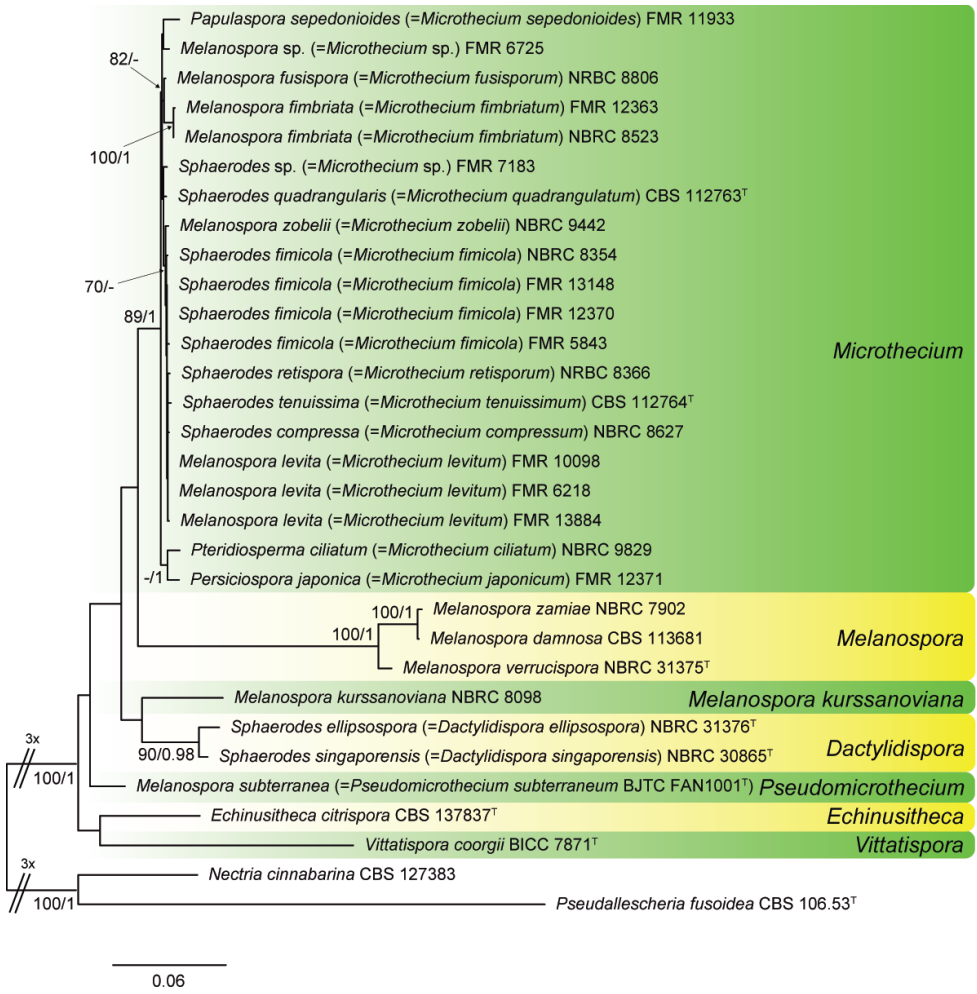

Figure 2. RAxML phylogram obtained from the combined ITS, LSU, *act* and *tef1* sequences of our isolates and type and reference strains of the order Melanosporales. *Nectria cinnabarina* and *Pseudallescheria fusoidea* were used as outgroup. RAxML bootstrap support (BS) values above 70 % and Bayesian posterior probability scores above 0.95 are shown at the nodes. GenBank accession numbers are indicated in Table 1. Type strains of the different species are indicated with T.

a reference strain of *Melanospora kurssanoviana*, which failed to sporulate in pure culture. The fourth clade (90 % BS / 0.98 PP; Clade *Dactylidispora*) was composed of the type strains of *Sphaerodes ellipsospora* and *S. singaporensis*, both characterized by ascospores with a raised rim surrounding the germ pores. Finally, the isolate CBS 137837 and the type strains of *Melanospora subterranea* and *Vittatispora coorgii* formed three independent branches. The isolate CBS 137837 produces globose, non-ostiolate, densely setose, dark ascomata and smooth-walled ascospores with a depressed germ pore at each end, while the other two species of this clade also possess morphological features unique in the Melanosporales, e.g. ascospores with indistinct germ pores in *M. subterranea* and with a longitudinal, thick, hyaline ridge in *V. coorgii*.

Taxonomy

Key to the accepted genera of the Melanosporales producing sexual morphs (adapted from Cannon and Hawksworth 1982)

1 Ascospores with two longitudinal germ slits ..... ***Scopinella***
– Ascospores with germ pores **2**
2 Ascospores with a broad germ pore and a small basal appendage **3**
– Ascospores with a germ pore at each end **4**
3 Ascomata with a crown of dark brown setae surrounding the ostiole ..... ***Setiferotheca***
– Ascomata without setae ..... ***Arxiomyces***
4 Ascospores oblong or cylindric-fusiform, and germ pores crateriform ..... ***Syspastospora***
– Ascospores and germ pores otherwise **5**
5 Ascomata ostiolate; neck long, composed of hyphae **6**
– Ascomata non-ostiolate or ostiolate; neck absent or short, conical, composed of angular cells similar to those of the ascomatal wall **7**
6 Neck composed of irregularly arranged hyphae ..... ***Melanospora***
– Neck composed of parallel arranged hyphae ..... ***Vittatispora***
7 Ascospores with indistinct germ pores ..... ***Pseudomicrothecium***
– Ascospores with conspicuous germ pores **8**
8 Germ pores surrounded by hyaline structures **9**
– Germ pores without such structures **10**
9 Germ pores with a raised rim ..... ***Dactylidispora***
– Germ pores with a blistered, rarely cushion-like structure ..... ***Pustulipora***
10 Ascomatal wall cephalothecoid ..... ***Rhytidospora***
– Ascomatal wall not cephalothecoid **11**
11 Ascomata dark, densely setose ..... ***Echinusitheca***
– Ascomata translucent, glabrous or surrounded by hyphae-like hairs ..... ***Microthecium***

Dactylidispora **Y. Marín, Stchigel, Guarro & Cano, gen. nov.**

MycoBank: MB812079

Type species. *Dactylidispora ellipsospora* (Takada) Y. Marín, Stchigel, Guarro & Cano. Holotype and ex-type strain: NBRC 31376.

Description. *Ascomata* superficial, globose to pyriform, ostiolate or not, yellowish-brown, appearing dark brown when the ascospores are mature, glabrous or setose; *necks* cellular, short, conical, with a crown of setae surrounding the ostiole; *ascomatal wall* membranaceous, of *textura angularis*. *Paraphyses* absent. *Asci* 8-spored, broadly clavate, short-stipitate, without apical structures, evanescent. *Ascospores* one-celled, at first hyaline, becoming brown to dark brown when mature, fusiform or citriform, umbonate and

truncate at the ends, smooth-walled, with one germ pore at each end; *germ pores* depressed, surrounded by a raised rim. *Conidiophores* reduced to conidiogenous cells. *Conidiogenous cells* phialidic, solitary, flask-shaped. *Conidia* hyaline, subglobose to ovoid, smooth-walled.

Etymology. From Greek *δακτυλίδης*–, ring, and from Latin –*spora*, spore, due to the raised rim that surrounds the germ pores of the ascospores.

Notes. The most distinctive characteristic of *Dactylidispora* is the production of smooth-walled ascospores with a germ pore at each end surrounded by a raised rim. *Vittatispora*, proposed as a new genus by Chaudhary et al. (2006), also produces a raised rim surrounding the germ pores. However, both genera can be easily distinguished by the nature of the ascomatal neck, which is composed of angular cells in *Dactylidispora* and of parallel arranged hyphae in *Vittatispora*; and by the presence of a hyaline ridge running the entire vertical length of the ascospore between the germ pores in *Vittatispora*. Moreover, in our phylogenetic study (Fig. 2), *Vittatispora* also constituted a lineage independent from the other members of the Melanosporales. *Pustulipora* is also morphologically similar to *Dactylidispora* being characterized by blistered, rarely cushion-like structures surrounding the germ pore (Cannon 1982). However, unfortunately, *Pustulipora* could not be included into this phylogenetic study since living cultures were not available.

The presence of a raised rim was also described in *Melanospora collipora* (Stchigel et al. 1997), which is here transfered to *Dactylidispora* even though it was not possible to include this species in the phylogenetic study.

Dactylidispora collipora **(Stchigel & Guarro) Y. Marín, Stchigel, Guarro & Cano, comb. nov.**
MycoBank: MB812080

Melanospora collipora Stchigel & Guarro, in Stchigel, Guarro & Figueras, Mycol. Res. 101: 446. 1997. [Basionym]

Notes. This species produces ascomata with a crown of setae around the ostiole, ellipsoidal ascospores, and bulbils.

Dactylidispora ellipsospora **(Takada) Y. Marín, Stchigel, Guarro & Cano, comb. nov.**
MycoBank: MB812081

Microthecium ellipsosporum Takada, in Kobayasi et al., Bull. natn. Sci. Mus., Tokyo 16: 527. 1973. [Basionym]
≡ *Sphaerodes ellipsospora* (Takada) D. García, Stchigel & Guarro, Stud. Mycol. 50: 67. 2004.

Notes. *Dactylidispora ellipsospora* is characterized by non-ostiolate ascomata, fusiform ascospores and absence of asexual morph.

***Dactylidispora singaporensis* (Morinaga, Minoura & Udagawa) Y. Marín, Stchigel, Guarro & Cano, comb. nov.**
MycoBank: MB812082

Melanospora singaporensis Morinaga, Minoura & Udagawa, Trans. Mycol. Soc. Japan 19: 142. 1978. [Basionym]
≡ *Sphaerodes singaporensis* (Morinaga, Minoura & Udagawa) D. García, Stchigel & Guarro, Stud. Mycol. 50: 67. 2004.

Notes. *Dactylidispora singaporensis* is distinguished by its ostiolate ascomata, citriform ascospores, and phialidic asexual morph.

***Echinusitheca* Y. Marín, Stchigel, Dania García, Guarro, A.N. Mill. & Cano, gen. nov.**
MycoBank: MB812084
Fig. 3

Type species. *Echinusitheca citrispora* Y. Marín, Stchigel, Dania García, Guarro, A.N. Mill. & Cano. Holotype and ex-type strain, respectively: CBS H-21596, CBS 137837 = FMR 12767.

Description. *Ascomata* superficial or immersed, solitary to gregarious, globose, non-ostiolate, strongly setose, semi-translucent, pale brown to brown, appearing black when ascospores are mature; *setae* straight, becoming sinuous toward apex, pale

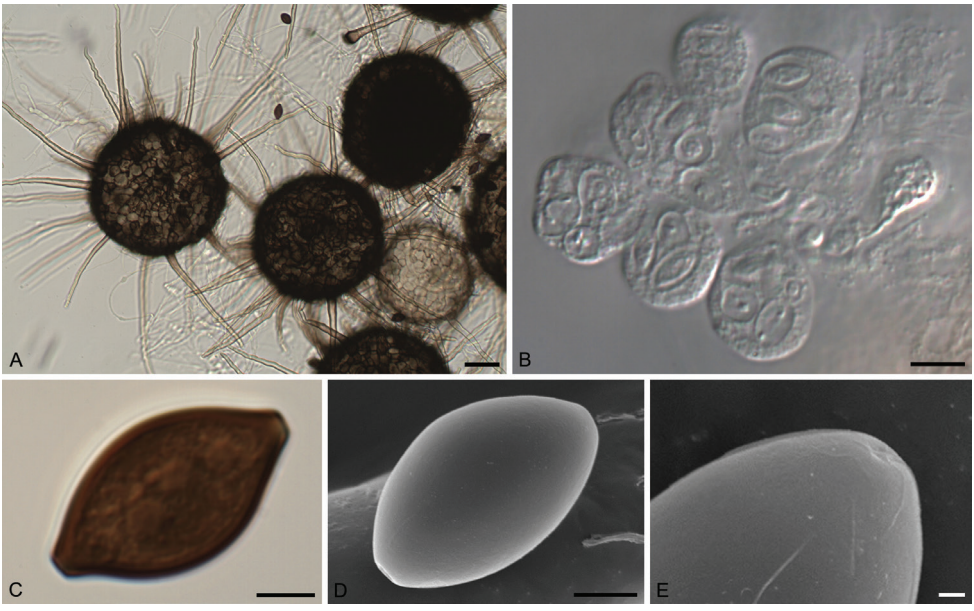

Figure 3. *Echinusitheca citrispora* (CBS 137837^{T}). **A** Ascomata **B** Asci **C, D** Ascospores **E** Depressed germ pore. Scale bars: 50 µm (**A**); 10 µm (**B**); 5 µm (**C, D**); 1 µm (**E**).

brown to brown, non-septate, rarely 1-septate, thick-walled, verrucose to tuberculate, sometimes branched; *ascomatal wall* membranaceous, of *textura angularis* to *textura globulosa*. *Asci* 8-spored, globose to subglobose, non-stipitate, without apical structures. *Ascospores* at first hyaline, becoming brown to dark brown when mature, ellipsoidal, one-celled, smooth-walled, with a depressed germ pore at each end.

Etymology. From Latin *echinus*–, sea urchin, and from Greek –*τείχος*, wall, because of the ascomata resemblance to a sea urchin, due to the abundance of setae.

Notes. This genus is characterized by dark, strongly setose, non-ostiolate ascomata. Apart from *Echinusitheca*, the other genera of the Melanosporales characterized by the production of dark semi-translucent ascomata are *Arxiomyces* and *Scopinella*, but both genera differ from *Echinusitheca* by the production of long ascomatal necks. Moreover, *Scopinella* can be easily distinguished from *Echinusitheca* by its cuboid-ellipsoidal ascospores with two prominent longitudinal germ slits, and *Arxiomyces* by its ellipsoidal ascospores that are rounded at the apex and truncated at the base, and with a broad germ pore that bears a mucilaginous and collapsing appendage.

Echinusitheca citrispora **Y. Marín, Stchigel, Dania García, Guarro, A.N. Mill. & Cano, sp. nov.**
MycoBank: MB812085
Fig. 3

Type. USA, North Carolina, Great Smoky Mountains National Park, Cataloochee Creek Campground (35.1375; -83.4915), forest soil, 15 July 2008, A.N. Miller, M. Calduch and A.M. Stchigel, holotype CBS H-21596, cultures ex-type CBS 137837 = FMR 12767.

Description. Colonies on PDA attaining a diam. of 70–75 mm after 14 d at 35 °C, cottony and granulose due to the presence of a large number of ascomata, white with grey to black dots, depressed at the centre and margins fringed; reverse yellowish-white to pale yellow (4A2 to 4A3) and with olive brown (4F2) dots. Colonies on OA attaining a diam. of 50–60 mm in 14 d at 35 °C, cottony and granulose due to the presence of numerous ascomata, margins arachnoid, white to orange white (5A2) with brownish grey dots (5F2); reverse yellowish-white to golden grey (4A2 to 4C2). Minimum, maximum, and optimum temperature of growth are 20, 40 and 35 °C, respectively. *Mycelium* composed of hyaline to pale yellow, septate, branched, smooth-walled hyphae, 1–3 µm diam. *Ascomata* non-ostiolate, immersed into the mycelium, solitary or gregarious, globose, 130–280 µm diam., setose, semi-translucent, pale brown to brown, appearing black when ascospores are mature; *setae* straight, becoming sinuous toward apex, 20–200 µm long, 5–20 µm wide at base, tapering gradually to a rounded tip of 2–5 µm diam., pale brown to brown, non-septate or rarely 1-septate, thick-walled, verrucose to tuberculate, sometimes branched at apex; *ascomatal wall* membranaceous, 30–40 µm thick, composed of 5–6 layers of flattened cells of 5–30 µm diam. of *textura angularis* to *textura globulosa*. *Asci* 8-spored, globose to subglobose,

20–25 × 15–20 µm, soon evanescent, non-stipitate, without apical structures, irregularly disposed at the centrum. *Ascospores* irregularly arranged in the asci, one-celled, at first hyaline, becoming brown to dark brown when mature, smooth- and thick-walled, ellipsoidal, 20–27 × 10–15 µm, with one germ pore at each end; *germ pores* 0.75–2 µm diam., depressed. *Asexual morph* absent.

Etymology. From Latin *citrum*-, lemon, and *-spora*, spore, referring to the lemon-shaped ascospores.

***Melanospora* Corda, Icon. fung. (Prague) 1: 24. 1837, emend.**
Fig. 4

Type species. *Melanospora zamiae* Corda, Icon. fung. (Prague) 1: 24. 1837. Representative strain: NBRC 7902.

Description. *Ascomata* superficial to immersed, globose to subglobose, ostiolate, yellowish-orange or reddish, tomentose or glabrous, usually with a long neck composed of intermixed hypha, with a crown of rigid, hyaline, septate, smooth- and thick-walled setae; *ascomatal wall* membranaceous, translucent, of *textura angularis*. *Periphyses* present. *Paraphyses* absent. *Asci* 8-spored, clavate, rounded at apex, without apical structures, thin-walled, evanescent. *Ascospores* one-celled, at first hyaline, becoming brown to dark brown when mature, fusiform, ellipsoidal or citriform, smooth-walled, reticulate or verrucose, with a terminal apiculate or depressed germ pore at each end. *Asexual morph* phialidic, hyaline. *Bulbils* uncommon.

Notes. This genus is distinguished by translucent ascomata with a neck composed of intermixed hyphae and with an apical crown of setae, smooth or ornamented ascospores with an apiculate germ pore at each end, and a phialidic asexual morph. The neck of *Melanospora* spp. is morphologically similar to those of *Syspastospora* and *Vittatispora*, which are also composed of hyphae. *Syspastospora* was introduced in 1982 by Cannon and Hawksworth to accommodate *Melanospora parasitica*, with three additional species described later (*S. boninensis*, *S. cladoniae* and *S. tropicalis*). This genus differs from *Melanospora* in the production of cylindrical to barrel-shaped ascospores with a large, slightly sunken germ pore at both ends (ellipsoidal, citriform or fusiform, having much smaller, apiculate or depressed germ pores in *Melanospora*). *Vittatispora* can be distinguished from *Melanospora* by the production of ascospores with a thick, hyaline, longitudinal ridge and a raised rim surrounding the germ pores. Moreover, *Syspastospora* and *Vittatispora* differs from *Melanospora* in the structure of the ascomatal neck, which is composed of hyphae in a parallel arrangement in both genera (interwoven hyphae in *Melanospora*).

Melanospora is now restricted to species with ascoma bearing a neck composed of interwoven hyphae and mostly ending in a crown of setae. This kind of neck differentiates this genus from *Microthecium*, which has a neck composed of angular cells similar to those of the ascomatal wall and possessing a crown of setae surrounding the ostiole rather than disposed at apex of the neck. The only exception is *Melanospora*

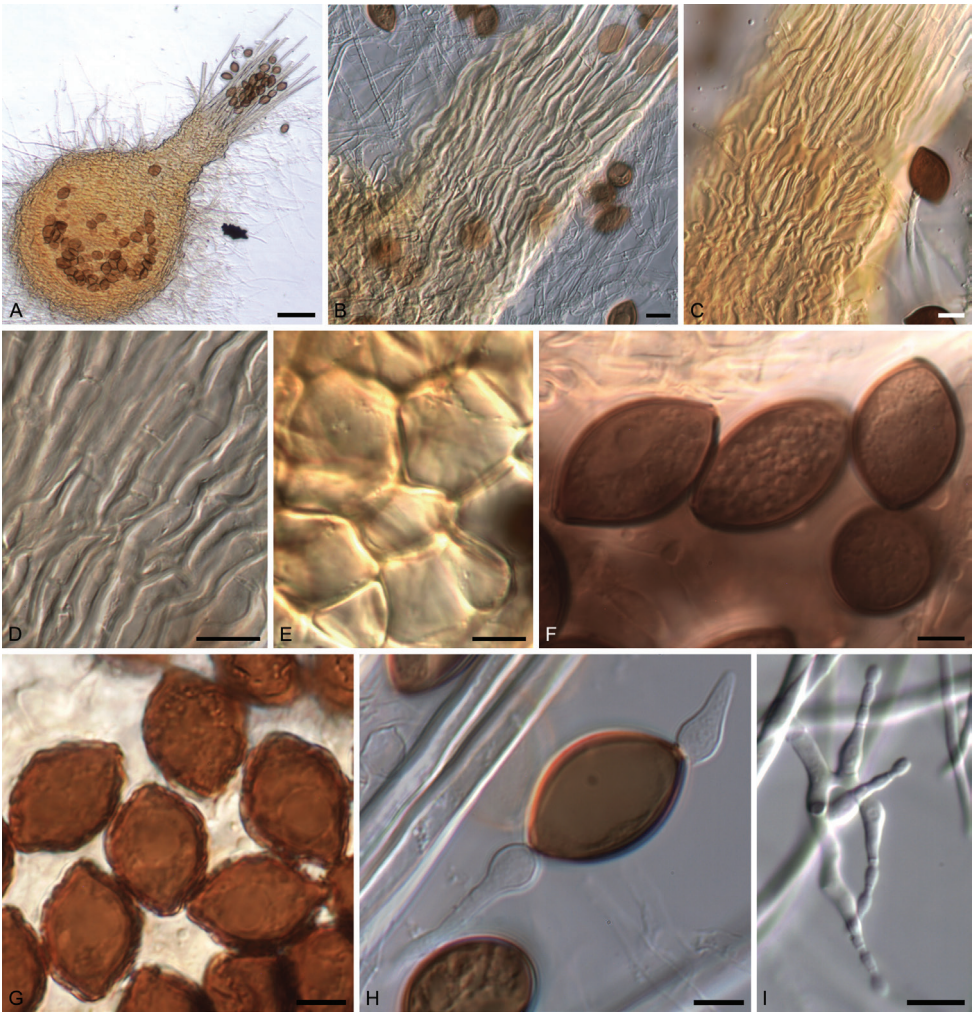

Figure 4. Morphological features of the genus *Melanospora*. *Melanospora damnosa* (CBS 113681). **A** Ascoma **B** Ascomatal neck **D** Detail of hyphal neck **F** Ascospores **H** Ascospore germinating. *Melanospora zamiae* (NBRC 7902) **C** Ascomatal neck **E** Detail of ascomatal wall. *Melanospora verrucispora* (NBRC 31375^{T}) **G** Ascospores **I** Phialidic asexual morph. Scale bars: 50 µm (**A**); 10 µm (**B–E, I**); 5 µm (**F–H**).

mycoparasitica that does not have this sort of neck, being short, cellular and without the crown of setae at the top of this, although this could be due to the fact that it was described and illustrated at an early stage of ascomal development. In a study on the development and cytology of *Melanospora tiffanii*, Kowalski (1965) illustrated early stages of development with the neck appearing similar to that of *M. mycoparasitica*.

Long hyphal necks are produced in *Melanospora arenaria*, *Melanospora caprina*, *Melanospora chionea*, *Melanospora langenaria*, *Melanospora longisetosa* and *Melanospora washingtonensis*; therefore, these have been kept in the emended genus *Melanospora*, although they were not included in the phylogenetic study.

Key to the species of *Melanospora*

1	Ascospores with the surface ornamented	**2**
–	Ascospores smooth-walled	**4**
2	Ascospores irregularly verrucose	***M. verrucispora***
–	Ascospores reticulate	**3**
3	Ascospores coarsely reticulate	***M. mycoparasitica***
–	Ascospores slightly reticulate	***M. tiffanii***
4	Ascospores discoid-ellipsoidal	**5**
–	Ascospores otherwise	**7**
5	Asci 4-spored; ascospores 14–19 × 12–14 × 8–9 µm	***M. longisetosa***
–	Asci 8-spored; ascospores smaller	**6**
6	Neck 250–400 µm long; ascospores 7.5–16 × 6–12 × 4–7 µm	***M. chionea***
–	Neck 150–200(–260) µm long; ascospores 10.5–12(–13.5) × 9–10.5(–12) × 7–9 µm	***M. washingtonensis***
7	Ascomata usually narrower than 100 µm; ascospores citriform to rhomboidal	***M. damnosa***
–	Ascomata usually broader than 100 µm; ascospores ellipsoidal to citriform	**8**
8	Ascomata strongly tomentose; neck 1500–2000 µm long	***M. caprina***
–	Ascomata weakly or not tomentose; neck shorter than 1500 µm	**9**
9	Neck shorter than 250 µm long	***M. zamiae***
–	Neck longer than 800 µm long	**10**
10	Setae longer than 100 µm	***M. arenaria***
–	Setae up to 50 µm long	***M. lagenaria***

Melanospora arenaria **L. Fisch. & Mont., in Montagne, Annls. Sci. Nat., Bot., sér. 4 5: 337. 1856.**

Notes. *Melanospora arenaria* is characterized by ascomata with a long neck and ellipsoidal to citriform, smooth-walled ascospores. It is similar to *Melanospora caprina*, but differs in having less tomentose ascomata with a shorter neck. Also, it is similar to *M. lagenaria*, differing only by the size of the setae at the top of the ascomatal neck. Molecular data is necessary to confirm that both species correspond to different species since the size of the setae could be influenced by the culture media on where these grew.

Melanospora caprina **(Fr.) Sacc., Syll. fung. (Abellini) 2: 462. 1883.**

Sphaeria caprina Fr., Fl. Danic. 11: tab. 1859, fig. 2. 1825. [Basionym]
≡ *Ceratostoma caprinum* (Fr.) Fr., Summa veg. Scand., Section Post. (Stockholm): 396. 1849.

≡ *Cerastoma caprinum* (Fr.) Quél., Mém. Soc. Émul. Montbéliard, Sér. 2 5: 522. 1875.
= *Sphaeria vervecina* Desm., Annls Sci. Nat., Bot., sér. 2 17: 13. 1842.
≡ *Melanospora vervecina* (Desm.) Fuckel, Jb. nassau. Ver. Naturk. 23-24: 126. 1870.
= *Melanospora vervecina f. arundinis* Sacc., Syll. fung. (Abellini) 2: 461. 1883.

Notes. *Melanospora caprina* is distinguished from the other species of the genus by its larger, white, densely tomentose ascomata with a very long neck, and ellipsoidal to citriform, smooth-walled ascospores with slightly apiculate germ pores.

Melanospora chionea (Fr.) Corda, Icon. fung. (Prague) 1: 24. 1837.

Ceratostoma chioneum Fr., Observ. mycol. (Havniae) 2: 340. 1818. [Basionym]
≡ *Sphaeria chionea* (Fr.) Fr., Syst. mycol. (Lundae) 2: 446. 1823.
≡ *Melanospora chionea* var. *chionea* (Fr.) Corda, Icon. fung. (Prague) 1: 24, tab. 7, fig. 297. 1837.
= *Sphaeria biformis* var. *brachystoma* Pers., Syn. meth. fung. (Göttingen) 1: 60. 1801.
≡ *Melanospora chionea* var. *brachystoma* (Pers.) Sacc., Syll. fung. (Abellini) 2: 461. 1883.
= *Sphaeria leucophaea* Fr., Elench. fung. (Greifswald) 2: 92. 1828.
≡ *Ceratostoma leucophaeum* (Fr.) Fr., Summa veg. Scand., Section Post. (Stockholm): 396. 1849.
≡ *Melanospora chionea* var. *leucophea* (Fr.) Sacc., Syll. fung. (Abellini) 2: 461. 1883.
= *Melanospora antarctica* Speg., Boln Acad. nac. Cienc. Córdoba 11: 233. 1888.

Notes. This species is characterized by white, tomentose ascomata and discoid, smooth-walled ascospores with depressed germ pores.

Melanospora damnosa (Sacc.) Lindau, in Engler & Prantl, Nat. Pflanzenfam., Teil. I (Leipzig) 1: 353. 1897.

Fig. 4A, B, D, F, H

Sphaeroderma damnosum Sacc., Riv. Patol. veg. 4: 64. 1895. [Basionym]

Notes. *Melanospora damnosa* is distinguised by the production of ascomata with a short neck and citriform to rhomboidal, smooth-walled ascospores with a slightly apiculate germ pore at each end.

Melanospora lagenaria (Pers.) Fuckel, Jb. nassau. Ver. Naturk. 23-24: 126. 1870.

Sphaeria lagenaria Pers., Syn. meth. fung. (Göttingen) 1: 58. 1801. [Basionym]
≡ *Ceratostoma lagenaria* (Pers.) Fr. [as 'lagenarium'], Syst. veg., Edn 16: 392. 1827.
≡ *Auerswaldia lagenaria* (Pers.) Rabenh., Hedwigia 1: 116. 1857.

≡ *Cerastoma lagenaria* (Pers.) Quél., Mém. Soc. Émul. Montbéliard, Sér. 2 5: 522. 1875.
≡ *Phaeostoma lagenaria* (Pers.) Munk [as 'lagenarium'], Dansk bot. Ark. 17: 82. 1957.
= *Melanospora lagenaria* var. *tetraspora* Rehm, Hedwigia 30: 259. 1891.

Notes. *Melanospora lagenaria* is similar to *M. caprina,* but the former has less tomentose ascomata with shorter necks ending in a poorly developed crown of setae. This species is also similar to *M. arenaria*. For morphological comparison see Notes of the latter species.

***Melanospora longisetosa* P.F. Cannon & D. Hawksw., J. Linn. Soc., Bot. 84: 130. 1982.**

Notes. This species is characterized by the formation of 4-spored asci and discoid, smooth-walled ascospores.

***Melanospora mycoparasitica* (Vujan.) Y. Marín, Stchigel, Guarro & Cano, comb. nov.**
MycoBank: MB812086

Sphaerodes mycoparasitica Vujan., Mycol. Res. 113: 1173. 2009. [Basionym]

Notes. *Melanospora mycoparasitica* is distinguished by its fusiform, coarsely reticulate ascospores.

***Melanospora tiffanii* Kowalski, Mycologia 57: 279. 1965.**

Notes. This species is distinguished by its fusiform, slightly reticulate ascospores.

***Melanospora verrucispora* Takada, in Kobayasi et al., Bull. natn. Sci. Mus., Tokyo 16: 525. 1973.**
Fig. 4G, I

Notes. This species is characterized by irregularly verrucose ascospores.

***Melanospora washingtonensis* Nitzan, J.D. Rogers & D.A. Johnson, Sydowia 56: 282. 2004.**

Notes. This species is similar to *M. chionea*, but they differ in the length of the neck [150–200(–266) µm in *M. washingtonensis* vs. 250–400 µm in *M. chionea*] and in the size of the ascospores [10.5–12(–13.5) × 9–10.5(–12) × 7–9 µm in *M. washingtonensis* vs. 7.5–16 × 6–12 × 4–7 µm in *M. chionea*], as well as in the presence of a phialidic asexual morph in *M. washingtonensis*.

Melanospora zamiae **Corda., Icon. fung. (Prague) 1: 24. 1837.**
Fig. 4C, E

= *Melanospora leucotricha* Corda, Icon. fung. (Prague) 1: 25. 1837.
= *Melanospora coemansii* Westend., Bull. Acad. R. Sci. Belg., Cl. Sci., sér. 2 2: 579. 1857.
= *Melanospora cirrhata* Berk. in Cooke, Grevillea 16: 102. 1888.
= *Melanospora globosa* Berl., Malpighia 5: 409. 1891.
= *Melanospora pampeana* Speg., Anal. Mus. nac. Hist. nat. B. Aires 6: 287. 1898.
= *Melanospora townei* Griffiths, Bull. Torrey bot. Club 26: 434. 1899.
= *Melanospora rhizophila* Peglion & Sacc., Annls mycol. 11: 16. 1913.
= *Melanospora mattiroloana* Mirande [as 'mattiroliana'], Bull. Soc. mycol. Fr. 32: 72. 1916.
= *Melanospora schmidtii* Sacc., Syll. fung. (Abellini) 24: 650. 1926.
= *Melanospora asclepiadis* Zerova, J. Inst. Bot. Acad. Sci. Ukraine 12: 155. 1937.

Notes. *Melanospora zamiae* is characterized by the production of ellipsoidal to citriform, smooth-walled ascospores with a depressed germ pore at each end. Doguet (1955) described the presence of bulbils; however, later studies did not mention the presence of such sort of propagules (Calviello 1973, Cannon and Hawksworth 1982), which rarely occur in the genus.

Doubtful species

Melanospora aculeata **E.C. Hansen, Vidensk. Meddel. Dansk Naturhist. Foren. Kjøbenhavn 59: 15. 1877.**

Notes. Cultures of this species are not available, but it was originally described as producing small asci (18–21 × 7–8 µm) and ascospores (4–6 × 3–4 µm). This species produced ostiolate ascomata without a neck, typical of *Microthecium*; however, such small ascospores have never been seen in *Microthecium*.

Melanospora endobiotica **Woron., Notul. syst. Inst. cryptog. Horti bot. petropol. 3: 31. 1924.**

Notes. Cultures are not available, and no illustrations were included in the protologue. It was reported as morphologically similar to *Melanospora rhizophila* [now considered a synonym of *Melanospora zamiae* (Doguet 1955)] when it was first described (Woronichin 1924).

Excluded species

***Melanospora arachnophila* Fuckel, Jb. nassau. Ver. Naturk. 23–24: 127. 1870.**

Notes. This species possesses cylindrical asci and hyaline ascospores, features never seen in *Melanospora.* It was previously excluded from *Melanospora* by Doguet (1955).

***Melanospora argadis* Czerepan., Nov. sist. Niz. Rast. 3: 177. 1966.**

Notes. This species shows morphological features never observed in *Melanospora*, e.g. small asci (10–14 × 5–6.5 μm) and olivaceous ascospores (5–5.5 × 3–3.5 μm). The original description is not detailed enough to ascertain its possible taxonomical placement.

***Melanospora exsola* Bat. & H.P. Upadhyay, Atas Inst. Micol. Univ. Recife 2: 331. 1965.**

Notes. This species is excluded from *Melanospora* due to its dark brown, non-translucent, setose ascomata and its small ascospores (4.5–12 × 4–7 μm), which seem to indicate a closer relationship with *Chaetomium*.

***Melanospora gigantea* (Massee & Crossl.) Massee & Crossl., Fungus Flora of Yorkshire (Leeds): 215. 1905.**

Notes. Descriptions of this species and of its basionym, *Sphaeroderma gigantea*, were not found.

***Melanospora lucifuga* (Jungh.) Sacc., Syll. fung. (Abellini) 2: 464. 1883.**

Notes. Cultures are not available, and the original description does not mention asci and ascospores. Therefore, we agree with Doguet (1955) in the exclusion of this fungus from *Melanospora*.

***Melanospora kurssanoviana* (Beliakova) Czerepan., Notul. syst. Sect. cryptog. Inst. bot. Acad. Sci. U.S.S.R. 15: 84. 1962.**

Notes. In our phylogenetic study, *M. kurssanoviana* was placed in an independent lineage far from *Melanospora*. Unfortunately, the only living culture available is sterile.

We did not find any distinctive morphological feature to differentiate this species from other members of the Melanosporales in the original description and in the drawing to introduce it as a new genus.

Melanospora macrospora **P. Karst., Hedwigia 30: 299. 1891.**

Notes. Doguet (1955) excluded this species due to the production of very large cylindrical asci (480–500 × 33–36 µm) and ascospores (42–52 × 28–35 µm), morphological features not observed in any other member of the Melanosporales.

Melanospora octahedrica **Pat., Cat. Rais. Pl. Cellul. Tunisie (Paris): 109. 1897.**

Notes. This species is transferred to *Scopinella* due to the morphology of its ascospores, i.e. octahedral ascospores with two prominent longitudinal germ slits.

Scopinella octahedrica **(Pat.) Y. Marín, Stchigel, Guarro & Cano, comb. nov.**
MycoBank: MB812087

Basionym. *Melanospora octahedrica* Pat., Cat. Rais. Pl. Cellul. Tunisie (Paris): 109. 1897.

Melanospora pascuensis **Stchigel & Guarro, Mycol. Res. 103: 1305. 1999.**

Notes. This species is excluded from *Melanospora* since its neck is cellular or absent, instead it is characterized by a dark ring-like structure around the germ pores of the ascospores (Stchigel et al. 1999). This fungus could represent a new genus since such structure is unique in the Melanosporales, and these kind of structures resulted in being phylogenetically informative, as in the case of *Dactylidispora*, which is distinguished by its ascospores with a raised rim around the germ pores. The type strain of this specimen was contaminated with another fungus and it could not be included in the molecular study.

Melanospora setchellii **(Harkn.) Sacc. & P. Syd., Syll. fung. (Abellini) 16: 564. 1902.**

Notes. This species is excluded from *Melanospora* since it produces cylindrical asci with the ascospores uniseriately disposed, a feature never observed in this genus.

***Melanospora vitrea* (Corda) Sacc., Syll. fung. (Abellini) 2: 463. 1883.**

Sphaeronaema vitreum Corda, Icon. fung. (Prague) 1: 25. 1837. [Basionym]

Notes. Doguet (1955) excluded this species due to its oblong, pale yellow ascospores.

***Microthecium* Corda, Icon. fung. (Prague) 5: 30, 74. 1842, emend.**
Fig. 5

= *Sphaerodes* Clem., Gen. fung. (Minneapolis): 44, 173. 1909.
= *Pteridiosperma* J.C. Krug & Jeng, Mycotaxon 10: 44. 1979.
= *Persiciospora* P.F. Cannon & D. Hawksw., J. Linn. Soc., Bot. 84: 133. 1982.

Type species. *Microthecium zobelii* Corda, Icon. fung. (Prague) 5: 74. 1842. Representative strain: NBRC 9442.

Description. *Ascomata* ostiolate or not, superficial or immersed, globose to subglobose or pyriform, yellowish-orange, orange-brown or reddish, tomentose or glabrous; *necks* short or absent, conical, composed of angular cells similar to those of the ascomatal wall, usually with a crown of hyaline, septate, smooth- and thick-walled setae around the ostiole; *ascomatal wall* membranaceous, translucent, of *textura angularis*. *Periphyses* present. *Paraphyses* absent. *Asci* 8-spored, clavate, rounded at apex, without apical structures, thin-walled, evanescent. *Ascospores* one-celled, at first hyaline, becoming brown to dark brown when mature, ellipsoidal, fusiform, navicular, citriform, plataniform or spindle-shaped, smooth, reticulate, pitted or wrinkled, with a terminal apiculate or depressed germ pore at each end. *Asexual morph* phialidic, hyaline. *Bulbils* usually produced, pale orange to reddish-orange.

Notes. *Microthecium* has translucent ascomata of *textura angularis*, cellular necks short or absent, ascospores smooth-walled or ornamented with a depressed or apiculate germ pore at each end, often producing bulbils and a phialidic asexual morph. *Dactylidispora*, *Pustulipora* and *Pseudomicrothecium* produce ascomata similar to *Microthecium*. However, the two first genera can be distinguished by the presence of a raised rim and blistered structure surrounding the germ pores of the ascospores, respectively, while *Pseudomicrothecium* differs in the production of 2-spored asci and ascospores with indistinct germ pores.

The species *Mi. africanum*, *Mi. beatonii*, *Mi. brevirostratum*, *Mi. episphaerium*, *Mi. foveolatum*, *Mi. geoporae*, *Mi. hypomyces*, *Mi. internum*, *Mi. lenticulare*, *Mi. marchicum*, *Mi. masonii*, *Mi. micropertusum*, *Mi. moureai*, *Mi. nectrioides*, *Mi. pegleri* and *Mi. perplexum* were not included in the phylogenetic study because we could not locate any specimens since the holotypes or living cultures of most of them are not available. However, these species were transferred to *Microthecium* based on their complete and well-illustrated descriptions.

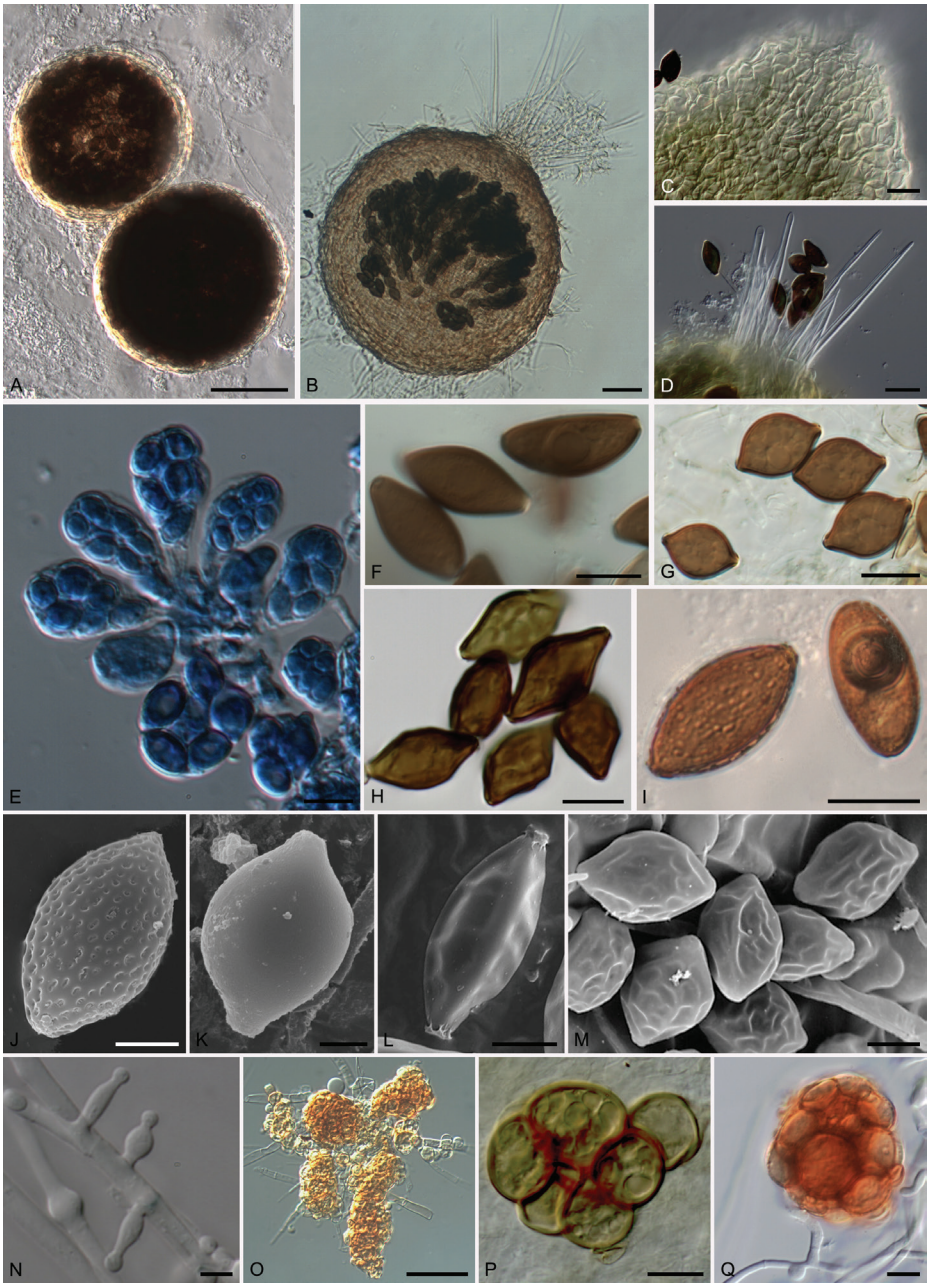

Figure 5. Morphological features of the genus *Microthecium*. *Microthecium levitum* (FMR 10098). **A** Non-ostiolate ascoma **E** Asci **G** Ascospores **K** Ascospore (SEM). *Microthecium fayodii* (FMR 12363). **B** Ostiolate ascomata **F** Ascospores **O** Variable shaped bulbils. *Microthecium fimicola* (FMR 5483). **C** Detail of cellular neck **M** Ascospores (SEM) **P** Bulbil. *Microthecium quadrangulatum* (CBS 112763^{T}). **D** Crown of setae around the ostiole **L** Ascospore SEM. *Microthecium retisporum* (NBRC 8366). **H** Ascospores **N** Asexual morph. *Microthecium japonicum* (FMR 12371) **I** Ascospores **J** Ascospore SEM. *Microthecium sepedonioides* (FMR 11933) **Q** Bulbil. Scale bars: 50 µm (**A, B, O**); 20 µm (**C, D**); 10 µm (**E–I, P, Q**); 5 µm (**J, L–N**); 2.5 µm (**K**).

Key to the species of *Microthecium*

1 Sexual morph absent, only producing bulbils ..... ***Mi. sepedonioides***
– Sexual morph present **2**
2 Ascomata non-ostiolate **3**
– Ascomata ostiolate **13**
3 Ascospores with an ornamented surface **4**
– Ascospores smooth-walled or nearly so **8**
4 Ascospores pitted and with wing-like ridges ..... ***Mi. foveolatum***
– Ascospores coarsely reticulate **5**
5 Asci 4-spored **6**
– Asci 8-spored **7**
6 Ascospores (25–)28–34(–40) × 14–18(–20) µm ..... ***Mi. beatonii***
– Ascospores 22–28 × 12–15 × 9–11 µm ..... ***Mi. perplexum***
7 Ascospores 25–34 × 12–18 µm ..... ***Mi. episphaerium***
– Ascospores 17–20 × 10–12 × 7–9 µm ..... ***Mi. retisporum***
8 Ascomata smaller than 120 µm ..... ***Mi. tenuissimum***
– Ascomata longer than 120 µm **9**
9 Ascospores shorter than 20 µm **10**
– Ascospores longer than 20 µm **11**
10 Ascospores 15–19 × 11–13 × 8–9 µm, with the narrow faces coarsely reticulate and the others smooth ..... ***Mi. compressum***
– Ascospores 10–17 × 8–12 × 9–10 µm, entirely smooth-walled .... ***Mi. levitum***
11 Ascospores fusiform ..... ***Mi. hypomyces***
– Ascospores citriform **12**
12 Ascospores 28–30 × 12–13(–15) µm ..... ***Mi. geoporae***
– Ascospores 18–25 × 8.5–12 × 6–9 µm ..... ***Mi. zobelii***
13 Ascospores with wing-like appendages **14**
– Ascospores otherwise **15**
14 Ascospores wrinkled, (12–)13–18 × (7–)8–10 µm ..... ***Mi. ciliatum***
– Ascospores pitted, (17–)20–22(–24) × 12–14 × 10–12 µm ..... ***Mi. lenticulare***
15 Ascospores ornamentated **16**
– Ascospores smooth-walled **23**
16 Ascospores punctate or punctate-reticulate **17**
– Ascospores reticulate or striate-reticulate **19**
17 Ascospores punctate, ellipsoidal ..... ***Mi. africanum***
– Ascospores punctate-reticulate, ellipsoidal-fusiform **18**
18 Ascospores delicately punctate, asexual morph and bulbils present ..... ***Mi. japonicum***
– Ascospores coarsely punctate, asexual morph and bulbils absent ..... ***Mi. moreaui***
19 Ascospores striate-reticulate **20**
– Ascospores reticulate **21**

20	Ascospores with inconspicuous ridges forming a very coarse reticulum, 18–22(–28) × 9.5–11(–13) × 8–9 µm	***Mi. micropertusum***
–	Ascospores without ridges or reticulum, 26–36 × 13–17 µm	***Mi. masonii***
21	Ascospores with 4–6 prominent longitudinal ribs	***Mi. quadrangulatum***
–	Ascospores without longitudinal ribs	**22**
22	Ascospores spindle-shaped, 19.5–22 × 8.5–11 µm	***Mi. internum***
–	Ascospores citriform to fusiform, 14–20 × 10–17 µm	***Mi. fimicola***
23	Crown of setae absent	***Mi. nectrioides***
–	Crown of setae present around the ostiole	**24**
24	Ascospores citriform	***Mi. marchicum***
–	Ascospores otherwise	**25**
25	Ascospores ellipsoid to citriform, often somewhat plataniform	**26**
–	Ascospores otherwise	**28**
26	Bulbils present	***Mi. fallax***
–	Bulbils absent	**27**
27	Ascospores 21–34 × 11–17 µm	***Mi. brevirostrum***
–	Ascospores 18–22 × 9–11 µm	***Mi. fimbriatum***
28	Ascospores ellipsoid to fusiform	***Mi. fusisporum***
–	Ascospores ellipsoid to navicular	**29**
29	Ascospores (9.5–)11–12(–13) × 4–4.5 µm	***Mi. pegleri***
–	Ascospores longer than 15 µm	**30**
30	Ascospores 16–24 × 8–12 µm	***Mi. fayodii***
–	Ascospores 25–30 × 11–15 µm	***Mi. brevirostratum***

Microthecium africanum **(J.C. Krug) Y. Marín, Stchigel, Guarro & Cano, comb. nov.**
MycoBank: MB812088

Persiciospora africana J.C. Krug, Mycologia 80: 416. 1988. [Basionym]

Notes. *Microthecium africanum* is characterized by ostiolate ascomata and punctate, ellipsoidal ascospores. Two asexual morphs with different conidia have been reported: (i), 1–4(–5)-celled, globose and smooth-walled at first but becoming cylindrical and coarsely verrucose later; (ii), 1–2-celled, large, usually cylindrical and smooth-walled (Krug 1988). However, the type strain was probably not a pure culture because the SSU and LSU sequences match with different species of *Fusarium* and the pictures of the conidia type (i) resemble the chlamydospores produced by several species of this genus.

Microthecium beatonii **D. Hawksw., Trans. Mycol. Soc. Japan 18: 145. 1977.**

≡ *Sphaerodes beatonii* (D. Hawksw.) P.F. Cannon & D. Hawksw., Bot. J. Linn. Soc. 84: 145. 1982.

Notes. This species is characterized by non-ostiolate ascomata, 4-spored asci and very coarsely reticulate, citriform ascospores. These morphological features are also observed in *Microthecium perplexum*, but this species produces ascospores with only a third of the surface coarsely reticulate while the rest remains smooth-walled. *Microthecium episphaerium* and *Mi. retisporum* differ from *Mi. beatonii* in the production of 8-spored asci. Moreover, *Mi. retisporum* produces a phialidic asexual morph and bulbils, which are absent in the other mentioned species, and smaller ascospores (17–20 × 10–12 × 7–9 µm) than in *Mi. beatonii* [28–34(–40) × 14–18(–20) µm], in *Mi. episphaerium* (25–34 × 12–18 µm) and in *Mi. perplexum* (22–28 × 12–15 × 9–11 µm).

***Microthecium brevirostratum* (Moreau) Y. Marín, Stchigel, Guarro & Cano, comb. nov.**
Mycobank: MB812089

Melanospora brevirostrata Moreau, Bull. Trimest. Soc. mycol. Fr. 61: 59. 1945. [Basionym]

Notes. *Microthecium brevirostratum* together with *Mi. fayodii* and *Mi. pegleri* produces ostiolate ascomata, smooth-walled, ellipsoidal to navicular or citriform ascospores and bulbils. *Microthecium brevirostratum* is easily distinguished by ascospores with apiculate germ pores and the presence of a phialidic asexual morph (ascospores show depressed germ pores and lack an asexual morph in other species). *Microthecium fayodii* and *Mi. pegleri* differ in the size of the ascospores, *Mi. pegleri* having the smallest ascospores in *Microthecium* [(9.5–)11–12(–13) × 4–4.5 µm].

***Microthecium brevirostrum* (Fuckel) Y. Marín, Stchigel, Guarro & Cano, comb. nov.**
MycoBank: MB812090

Ceratostoma brevirostre Fuckel, Bot. Ztg. 19: 250. 1861. [Basionym]
≡ *Melanospora brevirostris* (Fuckel) Höhn., Sber. Akad. Wiss. Wien, Math.-naturw. Kl., Abt. 1 123: 94. 1914.
= *Ceratostoma helvellae* Cooke, Grevillea 1: 175. 1873.
≡ *Melanospora helvellae* (Cooke) Sacc., Syll. fung. (Abellini) 2: 462. 1883.
= *Melanospora sphaerodermoides* Grove, J. Bot., Lond. 23: 132. 1885.
= *Melanospora sphaerodermoides* var. *sphaerodermoides* Grove, J. Bot., Lond. 23: 132. 1885.
= *Thielavia soppittii* Crossl., Naturalist, London: 7. 1901.
= *Rosellinia aurea* McAlpine, Fungus Diseases of stone-fruit trees in Australia: 102. 1902.
≡ *Sphaeroderma aureum* (McAlpine) Sacc. & D. Sacc., Syll. fung. (Abellini) 17: 781. 1905.
≡ *Melanospora aurea* (McAlpine) Doguet, Botaniste 39: 124. 1955.
= *Melanospora sphaerodermoides* var. *rubella* Pidopl., Mikrobiol. Zh. 9: 61. 1948.

= *Melanospora camelina* Faurel & Schotter, Revue Mycol., Paris 30: 144. 1965.
= *Melanospora tulasnei* Udagawa & Cain, Can. J. Bot. 47: 1932. 1970.

Notes. *Microthecium brevirostrum*, *Mi. fallax* and *Mi. fimbriatum* produce ostiolate ascomata and ellipsoidal to citriform, often plataniform, smooth-walled ascospores with an apiculate germ pore at each end. *Microthecium fimbriatum* is easily distinguished by its smaller (100–110 µm diam.), reddish ascomata, while *Mi. fallax* differs in the production of bulbils.

Microthecium ciliatum **Udagawa & Takada, Trans. Mycol. Soc. Japan 15: 23. 1974.**

≡ *Pteridiosperma ciliatum* (Udagawa & Y. Takada) J.C. Krug & Jeng, Mycotaxon 10: 45. 1979.

Notes. This species is characterized by non-ostiolate ascomata and ellipsoidal to fusiform ascospores ornamented with wing-like appendages and wrinkles, and the production of a phialidic asexual morph and bulbils. *Microthecium lenticulare* and *Mi. foveolatum* also present ascospores with wing-like appendages, but these are pitted and not wrinkled (as in *Mi. ciliatum*), and neither species produces bulbils. *Microthecium foveolatum* and *Mi. ciliatum* are characterized by non-ostiolate ascomata and the production of a phialidic asexual morph, whereas *Mi. lenticulare* has ostiolate ascomata and lacks an asexual morph.

Microthecium compressum **Udagawa & Cain, Can. J. Bot. 47: 1921. 1970.**

≡ *Sphaerodes compressa* (Udagawa & Cain) P.F. Cannon & D. Hawksw., J. Linn. Soc., Bot. 84: 145. 1982.

Notes. This species is distinguished by the production of non-ostiolate ascomata and citriform, bilaterally flattened ascospores, with the narrow faces coarsely reticulate and the widest faces smooth or nearly so, along with the production of a phialidic asexual morph.

Microthecium episphaerium **(W. Phillips & Plowr.) Höhn., Sber. Akad. Wiss. Wien, Math.-naturw. Kl., Abt. 1 123: 98. 1914.**

Melanospora episphaeria W. Phillips & Plowr., Grevillea 10: 71. 1881. [Basionym]
≡ *Sphaeroderma episphaerium* (W. Phillips & Plowr.) Sacc., Syll. fung. (Abellini) 2: 460. 1883.
≡ *Sphaerodes episphaerium* (W. Phillips & Plowr.) Clem. [as 'episphaericum'], Gen. fung. (Minneapolis): 1−227. 1909.

≡ *Vittadinula episphaeria* (W. Phillips & Plowr.) Clem. & Shear, Gen. fung., Edn 2 (Minneapolis): 281. 1931.
= *Sphaeroderma epimyces* Höhn., Sitzungsberichte der Kaiserlichen Akademie der Wissenschaften Math.-naturw. Klasse Abt. I 116: 103. 1907.
≡ *Melanospora epimyces* (Höhn.) Doguet, Botaniste 39: 125. 1955.

Notes. *Microthecium episphaerium* shows non-ostiolate ascomata and very coarsely reticulate, citriform ascospores. For morphological comparison see Notes of *Mi. beatonii*.

***Microthecium fallax* (Zukal) Y. Marín, Stchigel, Guarro & Cano, comb. nov.**
MycoBank: MB812772

Melanospora fallax Zukal, Sber. Akad. Wiss. Wien, Math.-naturw. Kl., Abt. 1 98: 547. 1889. [Basionym]
= *Melanospora anomala* Hotson, Proc. Amer. Acad. Arts & Sci 48.: 257. 1912.
= Melanospora cervicula Hotson, Proc. Amer. Acad. Arts & Sci. 48: 254. 1912.
= *Melanospora papillata* Hotson, Proc. Amer. Acad. Arts & Sci 48.: 251. 1912.
= *Melanospora phaseoli* Roll-Hansen, Blyttia 6: 73. 1948.

Notes. This species is characterized by ostiolate ascomata, ellipsoidal to citriform, often plataniform, smooth-walled ascospores, and production of bulbils. For morphological comparison see Notes of *Mi. brevirostrum*.

***Microthecium fayodii* (Vuill.) Y. Marín, Stchigel, Guarro & Cano, comb. nov.**
MycoBank: MB812091
Fig. 5B, F, O

Melanospora fayodii Vuill. [as 'fayodi'], Bull. Séanc. Soc. Sci. Nancy, Sér. 2 8: 33. 1887. [Basionym]

Notes. This species is characterized by ostiolate ascomata, ellipsoidal to navicular or citriform, smooth-walled ascospores, and production of bulbils. For morphological comparison see Notes of *Mi. brevirostratum*.

***Microthecium fimbriatum* (Rostr.) Y. Marín, Stchigel, Guarro & Cano, comb. nov.**
MycoBank: MB812092

Sphaeroderma fimbriatum Rostr., Oest. Grönl. Svampe: 25. 1894. [Basionym]
≡ *Melanospora fimbriata* (Rostr.) Petch, Trans. Br. mycol. Soc. 21: 253. 1938.

Notes. *Microthecium fimbriatum* produces ostiolate ascomata, and citriform to plataniform, smooth-walled ascospores with a strongly apiculate and tuberculate germ pore at each end. Although the ascomata was described as small and reddish in the protologue, the strain included in this study (NBRC 8523) shows larger (250–380 µm diam.), orange-brown ascomata. Moreover, our isolate produces bulbils. For morphological comparison see Notes of *Mi. brevirostrum*.

Microthecium fimicola **(E.C. Hansen) Y. Marín, Stchigel, Guarro & Cano, comb. nov.**
MycoBank: MB812093
Fig. 5C, M, P

Melanospora fimicola E.C. Hansen, Vidensk. Meddel. Dansk Naturhist. Foren. Kjøbenhavn 59: 15. 1876. [Basionym]
≡ *Sphaeroderma fimicola* (E.C. Hansen) Sacc., Syll. fung. (Abellini) 2: 460. 1883.
≡ *Sphaerodes fimicola* (E.C. Hansen) P.F. Cannon & D. Hawksw., J. Linn. Soc., Bot. 84: 146. 1982.
= *Melanospora ornata* Zukal, Verh. zool.-bot. Ges. Wien 35: 340. 1886.
≡ *Sphaerodes ornata* (Zukal) Arx, Gen. Fungi Sporul. Cult., Edn 3 (Vaduz): 156. 1981.
= *Sphaeroderma hulseboschii* Oudem., Contrib. Flora Mycol. d. Pays-Bas 11: 23. 1886.
≡ *Melanospora hulseboschii* (Oudem.) Doguet, Botaniste 39: 121. 1955.
= *Melanospora affine* Sacc. & Flageolet, Bull. Soc. Mycol. Fr. 12: 67. 1896.
= *Melanospora manginii* Vincens [as 'mangini'], Bull. Soc. Mycol. Fr. 33: 69. 1917.
≡ *Sphaerodes manginii* (Vincens) Arx, Gen. Fungi Sporul. Cult., Edn 3 (Vaduz): 156. 1981.

Notes. *Microthecium fimicola* is characterized by ostiolate ascomata and coarsely reticulate ascospores with strongly apiculate germ pores at both ends. The other species with ostiolate ascomata and reticulate ascospores are *Mi. internum* and *Mi. quadrangulatum.* The main differences among them are the shape and size of the ascospores, being citriform in *Mi. fimicola*, spindle-shaped in *Mi. internum* and fusiform in *Mi. quadrangulatum*. The production of bulbils has only been observed in our fresh isolates of *Mi. fimicola*, although this was not previously reported.

Microthecium foveolatum **Udagawa & Y. Horie, in Hawksworth & Udagawa, Trans. Mycol. Soc. Japan 18: 149. 1977.**

≡ *Pteridiosperma foveolatum* (Udagawa & Y. Horie) J.C. Krug & Jeng, Mycotaxon 10: 45. 1979.

Notes. This species is easily distinguished by its non-ostiolate ascomata, ellipsoidal to fusiform ascospores ornamented with small pores and thick wing-like ridges usually

longitudinal but often oblique, and production of phialidic asexual morph. For morphological comparison see Notes of *Mi. ciliatum*.

Microthecium fusisporum **(Petch) Y. Marín, Stchigel, Guarro & Cano, comb. nov.**
MycoBank: MB812094

Sphaeroderma fusisporum Petch, Naturalist, London: 58. 1936. [Basionym]
≡ *Melanospora fusispora* (Petch) Doguet, Botaniste 39: 215. 1955.
= *Melanospora fusispora* var. *fusispora* (Petch) Doguet, Botaniste 39: 215. 1955.
= *Melanospora fusispora* var. *parvispora* Matsush., Matsush. Mycol. Mem. 8: 24. 1995.

Notes. *Microthecium fusisporum* is related to *Mi. nectrioides*, both possessing ostiolate ascomata and smooth-walled ascospores. However, *Mi. nectrioides* can be distinguished by the absence of the crown of setae around the ostiole and its citriform ascospores, being fusiform in *Mi. fusisporum*.

Microthecium geoporae **(W. Oberm.) Höhn., Sber. Akad. Wiss. Wien, Math.-naturw. Kl., Abt. 1 123: 98. 1914.**

Guttularia geoporae W. Oberm., Mykol. Zentbl. 3: 9. 1913. [Basionym]

Notes. This species produces non-ostiolate ascomata and citriform, smooth-walled ascospores. Other species previously placed in *Melanospora* characterized by the production of non-ostiolate ascomata and smooth-walled ascospores are *Mi. hypomyces*, *Mi. levitum* and *Mi. zobelii*. *Microthecium hypomyces* is distinguished by its fusiform ascospores (citriform in the other species), and *Mi. levitum* by the presence of bulbils and a phialidic asexual morph. *Microthecium geoporae* and *Mi. zobelii* are distinguished by the size of their ascospores [28–30 × 12–13(–15) µm in *Mi. geoporae* and 18–25 × 8.5–12 × 6–9 µm in *Mi. zobelii*]. *Microthecium tenuissimum* shows similar morphological features to these species but its ascospores are finely reticulate under SEM and its ascomata are smaller (less than 120 µm) than in the other species.

Microthecium hypomyces **(Höhn.) Höhn., Sber. Akad. Wiss. Wien, Math.-naturw. Kl., Abt. 1 123: 50. 1914.**

Sphaeroderma hypomyces Höhn., Sber. Akad. Wiss. Wien, Math.-naturw. Kl., Abt. 1 116: 102. 1907. [Basionym]
≡ *Melanospora hypomyces* (Höhn.) Doguet, Botaniste 39: 215. 1955.

Notes. This species is characterized by non-ostiolate ascomata and fusiform, smooth-walled ascospores. For morphological comparison see Notes of *Mi. geoporae*.

Microthecium internum **(Tehon & G.L. Stout) Y. Marín, Stchigel, Guarro & Cano, comb. nov.**
MycoBank: MB812095

Melanospora interna Tehon & G.L. Stout, Mycologia 21: 181. 1929. [Basionym]

Notes. This species produces ostiolate ascomata and spindle-shaped ascospores with a coarse and irregular reticulum. For morphological comparison see Notes of *Mi. fimicola*.

Microthecium japonicum **(Y. Horie, Udagawa & P.F. Cannon) Y. Marín, Stchigel, Guarro & Cano, comb. nov.**
MycoBank: MB812096
Fig. 5I, J

Persiciospora japonica Y. Horie, Udagawa & P.F. Cannon, Mycotaxon 25: 233. 1986. [Basionym]

Notes. *Microthecium japonicum* is characterized by ostiolate ascomata and ellipsoidal to fusiform, punctate-reticulate ascospores, similar to *Mi. moureai*. However, *Mi. japonicum* produces a phialidic asexual morph and bulbils (absent in *Mi. moureai*) and delicately reticulate ascospores (coarsely reticulate in *Mi. moureai*).

Microthecium lenticulare **(Udagawa & T. Muroi) Y. Marín, Stchigel, Guarro & Cano, comb. nov.**
MycoBank: MB812097

Pteridiosperma lenticulare Udagawa & T. Muroi [as 'lenticularis'], Trans. Mycol. Soc. Japan 22: 20. 1981. [Basionym]

Notes. *Microthecium lenticulare* produces ostiolate ascomata and pitted-walled ascospores with wing-like appendages. For morphological comparison see Notes of *Mi. ciliatum*.

Microthecium levitum **Udagawa & Cain, Can. J. Bot. 47: 1917. 1970.**
Fig. 5A, E, G, K

≡ *Sphaerodes levita* (Udagawa & Cain) D. García, Stchigel & Guarro, Stud. Mycol. 50: 67. 2004.

Notes. This species is characterized by non-ostiolate ascomata, citrifrom and smooth-walled ascospores with umbonate and tuberculate germ pores, presence of

bulbils and phialidic asexual morph. For morphological comparison see Notes of *Mi. geoporae*.

Microthecium marchicum **(Lindau) Y. Marín, Stchigel, Guarro & Cano, comb. nov.**
MycoBank: MB812099

Chaetomium marchicum Lindau, Hedwigia 35: 56. 1896. [Basionym]
≡ *Sphaeroderma marchicum* (Lindau) Sacc. & P. Syd., Syll. fung. (Abellini) 14: 627. 1899.

Notes. *Microthecium marchicum* is characterized by its ostiolate ascomata and citrifrom, smooth-walled ascospores. Its ascospores are similar to those of *Mi. geoporae*, *Mi. hypomyces*, *Mi. levitum* and *Mi. zobelii*, but all of them produce non-ostiolate ascomata.

Microthecium masonii **(Kirschst.) Y. Marín, Stchigel, Guarro & Cano, comb. nov.**
MycoBank: MB812100

Ceratostoma masonii Kirschst., Trans. Br. mycol. Soc. 18: 306. 1934. [Basionym]
≡ *Persiciospora masonii* (Kirschst.) P.F. Cannon & D. Hawksw., J. Linn. Soc., Bot. 84: 135. 1982.

Notes. *Microthecium masonii* is characterized by ostiolate ascomata and ellipsoidal to fusiform, faintly striate-reticulate ascospores. The same type of ascospore ornamentation is also observed in *Mi. micropertusum*, but this latter species is easily distinguished by the presence of inconspicuous ridges forming a very coarse reticulum and a phialidic asexual morph.

Microthecium micropertusum **(Y. Horie, Udagawa & P.F. Cannon) Y. Marín, Stchigel, Guarro & Cano, comb. nov.**
MycoBank: MB812101

Sphaerodes micropertusa Y. Horie, Udagawa & P.F. Cannon, Mycotaxon 25: 236. 1986. [Basionym]

Notes. *Microthecium micropertusum* is distinguished by its ostiolate ascomata, fusiform to citriform or nearly rhombic in outline ascospores with inconspicuous ridges forming a coarse reticulum, and presence of phialidic asexual morph. For morphological comparison see Notes of *Mi. masonii*.

Microthecium moreaui **(P.F. Cannon & D. Hawksw.) Y. Marín, Stchigel, Guarro & Cano, comb. nov.**
MycoBank: MB812102

Persiciospora moreaui P.F. Cannon & D. Hawksw., J. Linn. Soc., Bot. 84: 134. 1982. [Basionym]

Notes. *Microthecium moreaui* is characterized by its ostiolate ascomata, ellipsoidal and pitted-walled ascospores, and production of bulbils. For morphological comparison see Notes of *Mi. japonicum*.

Microthecium nectrioides **(Marchal) Y. Marín, Stchigel, Guarro & Cano, comb. nov.**
MycoBank: MB812103

Sphaeroderma nectrioides Marchal, Bull. Soc. R. Bot. Belg. 23: 25. 1884. [Basionym]
≡ *Melanospora nectrioides* (Marchal) Doguet, Botaniste 39: 121. 1955.
= *Melanospora asparagi* G. Arnaud, Ann. Serv. Epiph. 2: 273. 1915.

Notes. This species produces ostiolate ascomata and citriform, smooth-walled ascospores. For morphological comparison see Notes of *Mi. fusisporum*.

Microthecium pegleri **(D. Hawksw. & A. Henrici) Y. Marín, Stchigel, Guarro & Cano, comb. nov.**
MycoBank: MB812104

Melanospora pegleri D. Hawksw. & A. Henrici, Kew Bull. 54: 795. 1999. [Basionym]

Notes. *Microthecium pegleri* is characterized by ostiolate ascomata, ellipsoidal to plano-convex, smooth-walled ascospores and presence of bulbils. For morphological comparison see Notes of *Mi. brevirostratum*.

Microthecium perplexum **D. Hawksw., Trans. Mycol. Soc. Japan 18: 151. 1977.**

≡ *Sphaerodes perplexa* (D. Hawksw.) P.F. Cannon & D. Hawksw., Bot. J. Linn. Soc. 84: 148. 1982.

Notes. This species produces non-ostiolate ascomata, 4-spored asci and citrifrom ascospores usually with smooth walls, but one third of these are coarsely reticulated. For morphological comparison see Notes of *Mi. beatonii*.

***Microthecium quadrangulatum* (D. García, Stchigel & Guarro) Y. Marín, Stchigel, Guarro & Cano, comb. nov.**
MycoBank: MB812105
Fig. 5D, L

Sphaerodes quadrangularis D. García, Stchigel & Guarro, Stud. Mycol. 50: 64. 2004. [Basionym]

Notes. *Microthecium quadrangulatum* is characterized by ostiolate ascomata and fusiform, reticulate ascospores with strongly apiculate germ pores. For morphological comparison see Notes of *Mi. fimicola*.

***Microthecium retisporum* Udagawa & Cain, Can. J. Bot. 47: 1926. 1970.**
Fig. 5H, N

≡ *Sphaerodes retispora* (Udagawa & Cain) P.F. Cannon & D. Hawksw., J. Linn. Soc., Bot. 84: 149. 1982.
= *Microthecium retisporum* var. *inferius* Udagawa & Cain [as 'inferior'], Can. J. Bot. 47: 1928. 1970.
≡ *Sphaerodes retispora* var. *inferior* (Udagawa & Cain) P.F. Cannon & D. Hawksw., J. Linn. Soc., Bot. 84: 149. 1982.
≡ *Sphaerodes inferior* (Udagawa & Cain) D.W. Li & N.P. Schultes, in Schultes, Murtishi & Li, Fungal Biology 121: 901. 2017.
= *Microthecium retisporum* var. *retisporum* Udagawa & Cain, Can. J. Bot. 47: 1926. 1970.
≡ *Sphaerodes retispora* var. *retispora* (Udagawa & Cain) P.F. Cannon & D. Hawksw., J. Linn. Soc., Bot. 84: 149. 1982.

Notes. This species is characterized by non-ostiolate ascomata, reticulate citriform ascospores with apiculate germ pores, a phialidic asexual morph and presence of bulbils. For morphological comparison see Notes of *Mi. beatonii*.

***Microthecium sepedonioides* (Preuss) Y. Marín, Stchigel, Guarro & Cano, comb. nov.**
MycoBank: MB812106
Fig. 5Q

Papulaspora sepedonioides Preuss, Linnaea 24: 112. 1851. [Basionym]

Notes. *Microthecium sepedonioides* only produces bulbils and the sexual morph has never been observed.

***Microthecium tenuissimum* (D. García, Stchigel & Guarro) Y. Marín, Stchigel, Guarro & Cano, comb. nov.**
MycoBank: MB812107

Sphaerodes tenuissima D. García, Stchigel & Guarro, Stud. Mycol. 50: 65. 2004. [Basionym]

Notes. This species is characterized by non-ostiolate ascomata and citriform, ellipsoidal in lateral view, finely reticulate ascospores with strongly apiculate germ pores. For morphological comparison see Notes of *Mi. geoporae*.

***Microthecium zobelii* Corda, Icon. fung. (Prague) 5: 74. 1842.**

≡ *Sphaeria zobelii* (Corda) Tul. & C. Tul., Fungi hypog.: 186. 1851.
≡ *Ceratostoma zobelii* (Corda) Berk., Journal of the Royal Horticultural Society 4: 402. 1860.
≡ *Melanospora zobelii* (Corda) Fuckel, Jb. nassau. Ver. Naturk. 23-24: 127. 1870.
= *Melanospora zobelii* var. *zobelii* (Corda) Fuckel, Jb. nassau. Ver. Naturk. 23-24: 127. 1870.
= *Melanospora coprophila* Zukal, Sber. Akad. Wiss. Wien, Math.-naturw. Kl., Abt. 1 98: 544. 1889.
= *Melanospora marchicum* Lindau, Hedwigia 35: 56. 1896.
= *Melanospora zobelii* var. *minor* Pidopl., Mikrobiol. Zh. 9(2-3): 60. 1948.

Notes. *Microthecium zobelii* produces non-ostiolate ascomata, and citriform, smooth-walled ascospores with slightly apiculate germ pores. For morphological comparison see Notes of *Mi. geoporae*.

□□

***Microthecium ryvardenianum* Aramb. & Gamundí, Agarica 6: 124. 1985.**

Notes. This species is considered as doubtful because it presents morphological features atypical of *Microthecium* (e.g. allantoid ascospores when immature becoming striate when mature).

***Pseudomicrothecium* Y. Marín, Stchigel, Guarro, Cano, gen. nov.**
MycoBank: MB812108

Type species. *Pseudomicrothecium subterraneum* (L. Fan, C.L. Hou, P.F. Cannon & Yong Li) Y. Marín, Stchigel, Guarro & Cano. Holotype and ex-isotype strain: BJTC FAN1001, K[M] 172128.

Description. *Ascomata* non-ostiolate, globose, translucent, pale brown to brown, appearing dark brown when the ascospores are mature, glabrous or setose; *ascomatal wall* membranaceous, of *textura angularis*. *Asci* 2-spored, clavate, short-stipitate, without apical structures, evanescent. *Ascospores* one-celled, at first hyaline, becoming dark brown to blackish when mature, ellipsoidal to citriform, umbonate and truncate at both ends, with a terminal indistinct germ pore at each end. *Asexual morph* absent.

Etymology. The name refers to the morphological resemblance to *Microthecium*.

Notes. The new genus *Pseudomicrothecium* is proposed here to accommodate *Melanospora subterranea* because it constitutes a separate lineage in our phylogenetic study. This genus is characterized by its non-ostiolate ascomata, similar to those of *Microthecium*, 2-spored asci and smooth-walled ascospores with an indistinct germ pore at each end. Asci containing two ascospores have only been observed in some species of *Scopinella* (i.e. *Scopinella gallicola* and *S. sphaerophila*). However, *Scopinella* can be easily distinguished from *Pseudomicrothecium* by the production of ostiolate ascomata with long necks and cuboid-ellipsoidal ascospores with two prominent longitudinal germ slits.

***Pseudomicrothecium subterraneum* (L. Fan, C.L. Hou, P.F. Cannon & Yong Li) Y. Marín, Stchigel, Guarro & Cano, comb. nov.**
MycoBank: MB812109

Basionym. *Melanospora subterranea* L. Fan, C.L. Hou, P.F. Cannon & Yong Li, Mycologia 104: 1434. 2012.

Discussion

We have revised the taxonomy of relevant members of the family Ceratostomataceae based on the analyses of the SSU, LSU, ITS, *act* and *tef1* nucleotide sequences. This study strongly supported the order Melanosporales proposed by Zhang and Blackwell in 2007 (Hibbett et al. 2007). The phylogenetic inference showed seven lineages corresponding to the genera *Dactylidispora*, *Echinusitheca*, *Melanospora*, *Microthecium*, *Pseudomicrothecium* and *Vittatispora*, and to *Melanospora kurssanoviana*. Our results agree with previous studies (Zhang and Blackwell 2002, Fan et al. 2012) which already suggested and demonstrated that the ornamentation of the ascospores under SEM, a feature traditionally used to delimit most of the genera in the Melanosporales, is not useful for estimating phylogenetic relationships among these fungal taxa. Similarly, the morphology of the ascospores is of weak taxonomic value and a poor predictor for the generic delimitation of members of the family Sordariaceae, resulting in the synonymy of two relevant genera, i.e. *Gelasinospora* and *Neurospora* (Dettman et al. 2001, García et al. 2004, Nygren et al. 2011). In our study, two of the largest genera of the Melanosporales, *Melanospora* and *Microthecium*, grouped species with both smooth and ornamented ascospore walls. By contrast, a phylogenetic study of the Lasiosphaeriaceae (Miller and Huhndorf 2005) revealed that the morphology of the ascomatal wall was more

phylogenetically informative than that of the ascospores, with several new genera proposed (i.e. *Immersiella*) or emended (i.e. *Lasiosphaeria*, *Lasiosphaeris* and *Schizothecium*) (Miller and Huhndorf 2004, Cai et al. 2005). Here, the erection of the new genus *Echinusitheca* is a clear example of the relevance of the ascomatal morphology in the taxonomy of these fungi, and in fact this taxon together with *Arxiomyces* and *Scopinella* are the only genera in the Melanosporales that show dark semi-transluscent ascomata. In this context, although *Echinusitheca* has ascospores similar to those of *Melanospora* and *Microthecium*, this genus constitutes one of the lineages phylogenetically most distant within this order.

Another lineage considerably distant from the other members of the Melanosporales is constituted by the clade represented only by the species *Melanospora kurssanoviana*, suggesting that this fungus could represent a new genus. However, this new taxon is at this moment not proposed because its colonies, in spite of attempts to induce sporulation, remain sterile and a detailed morphological study was not possible. The infertility of the cultures is probably due to the fact that an important part of the members of this fungal group show a peculiar habitat developing a certain degree of mycoparasitism and requiring the presence of the host to complete the biologic cycle and develope reproductive structures. The mycoparasitism of *Melanospora*, *Syspastospora* and the species previously placed in *Persiciospora* and *Sphaerodes* has already been demonstrated by numerous authors (Doguet 1955, Calviello 1973, Jordan and Barnett 1978, Harveson and Kimbrough 2000, 2001), and this ability has been exploited in the biocontrol of phytopathogenic fungi (Vujanovic and Goh 2009, Goh and Vujanovic 2010, Kim and Vujanovic 2016, 2017).

The genus *Sphaeronaemella*, which is characterized by pale and translucent ascomata, was thought to be related to *Melanospora* (Cannon and Hawksworth 1982). However, we do not agree with this relationship because it differs from the Melanosporales in the production of hyaline ascospores, as opposed to the pigmented ones in the members of that order. By contrast, our results correlate with those of other authors that demonstrated a closer phylogenetic relationship of this genus with the members of the order Microascales (Spatafora and Blackwell 1994b, Hausner and Reid 2004). In fact, our SSU tree seems to indicate that *Sphaeronaemella* could represent a new family of the Microascales; however, further studies including more taxa and additional genes are needed to more accurately confirm its taxonomic status.

The placement of our isolate of *Persiciospora japonicum* in the *Microthecium* clade once more demonstrated that the ornamentation of the ascospores, which is pitted in *Persiciospora* spp., is of poor taxonomic value, and consequently all the species of *Persiciospora* should be transferred to *Microthecium*. As it was above mentioned, the species of this latter genus show a typical cellular ascomatal neck which is also present in *Persiciospora* and constitutes a common feature in both genera. Surprisingly, in some previous phylogenetic studies, the species of *Persiciospora* were placed in the Hypocreales, closely related to *Nectria* (Zhang and Blackwell 2002, Maharachchikumbura et al. 2015, Schultes et al. 2017). However, this could be probably explained by a possible contamination of the cultures of *Persiciospora* spp. with an hypocrealean host (Fan et al. 2012). The same situation may have occurred with the cultures of *Scopinella* and *Syspastospora*, which led to a possible erroneous classification of both taxa in the

Hypocreales (Zhang and Blackwell 2002, Chaudhary et al. 2006, Fan et al. 2012, Maharachchikumbura et al. 2015, Schultes et al. 2017).

Pteridiosperma ciliatum, a member of the Melanosporales with ascospores ornamented with longitudinal wing-like ridges that anastomose each other to form a well defined reticulum (a distinctive feature of *Pteridiosperma*), was also found in the *Microthecium* clade, proving once again that the ascospore ornamentation is not phylogenetically informative. Consequently, we have synonymyzed the genus *Pteridiosperma* with *Microthecium* since *Pteridiosperma* spp. show non-ostiolate ascomata, or if ostiolate, they show a short neck composed of angular cells, which are typical morphological characteristics of *Microthecium*.

Another genus that our results demonstrated should be synonymized and included in *Microthecium* is *Sphaerodes* because its type species, *S. episphaerium*, shows morphological features (non-ostiolate ascomata) that fit with the current circumscription of that emended genus. Most of the species of *Sphaerodes*, with the exception of *S. ellipsospora* and *S. singaporensis*, which are now located in the new genus *Dactylidispora*, and *S. mycoparasitica*, which is now placed in *Melanospora*, are also transferred to *Microthecium* since these produce non-ostiolate or ostiolate ascomata without a neck, or less frequently with a short neck composed of angular cells similar to the ascomatal ones. Another relevant feature of the genus *Microthecium* is the production of bulbils. These propagules are typical of *Papulaspora*, an anamorphic genus that encompasses more than 40 species. Although it was initially accepted as a genus without a sexual morph (Hotson 1912), its link with species of *Melanospora* and *Chaetomium* has been reported (Roll-Hansen 1948, Zhang et al. 2004). In our phylogenetic study *Papulaspora sepedonioides*, the type species of the genus, was nested in the *Microthecium* clade, and therefore transferred to this genus. The relationship of this species with the Melanosporales had already previously been demonstrated by Davey et al. (2008) and Li et al. (2016). However, it has been demonstrated that *Papulaspora* is a polyphyletic genus, and other species of the genus have been reported as belonging to the classes Leotiomycetes and Sordariomycetes (Ascomycota). Therefore, the other species of *Papulaspora* not linked to the species of *Microthecium* should be transferred to other taxonomic groups. The relationship of some species of *Papulaspora* with the Melanosporales is also suggested by the production of similar phialidic synanamorphs (Van Beyma 1931, Hotson 1942).

The most recent new combination performed in *Sphaerodes*, *S. inferior*, was done to accommodate *S. retispora* var. *inferior* since it was not clustering with *S. retispora* var. *retispora* (Schultes et al. 2017). However, we suspected that the sequences of *S. retispora* var. *retispora* deposited in GenBank were contaminated with the hypocrealean host. In order to corroborate it, we studied such sequenced strain demonstrating that it was effectively contaminated. Therefore, *S. inferior* is here considered a synonym of *Mi. retisporum* since the morphological differences are insufficient to recognize this variety as a different species.

There are important morphological differences among the strains of *Microthecium* that suggest the presence of several additional cryptic species in the genus; however, our phylogenetic study, in spite of having used five loci, was not able to resolve the boundaries among them.

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Gliophorus glutinosus sp. nov. (Hygrophoraceae, Agaricales) from Eastern Himalayan region of India

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Abstract

An interesting species of the genus *Gliophorus* (sect. *Glutinosae*), collected from Sikkim Himalaya in India, is described here as *G. glutinosus* **sp. nov.** after thorough morphological examination and phylogenetic analysis. The species is mainly characterised by its strongly glutinous basidiomata throughout, particularly on the twisted stipe, decurrent lamellae with glutinous edge, gelatinised cheilocystidia, presence of pleuropseudocystidia and absence of clamps in hyphae of the pileipellis. This communication includes detailed morphological description, illustrations, comparison with the allied taxa, nrITS based phylogeny of this novel taxon and a key to the species under *Gliophorus* sect. *Glutinosae*.

Keywords

Agaricomycetes, Basidiomycota, *Gliophorus*, Hygrophoraceae, macrofungi, phylogeny, Sikkim, taxonomy

Introduction

Gliophorus Herink (1958: 80) is a member of tribe Humidicuteae, subfamily Hygrocyboideae of Hygrophoraceae (Agaricomycetes, Basidiomycota) and featured by its glutinous basidiomata, variously coloured but never bright red; sinuate or decurrent lamellae, which are sometimes gelatinised at edge; basidiospores smooth, hyaline, thin-walled, inamyloid, ovoid to ellipsoid; presence or absence of ixocheilocystidia; basidia mostly 4-spored, presence of basal clamp in basidia and basidioles; irregular hymenial trama; pileipellis an ixotrichoderm (Lodge et al.

2014, Singh et al. 2017). This small genus (only 13 members were recorded under this genus in Index Fungorum, http://www.indexfungorum.org/names/Names.asp) encompasses 3 sections: sect. *Gliophorus,* sect. *Glutinosae* (Kühner) Lodge & Padamsee in Lodge et al. (2014: 6) and sect. *Unguinosae* Herink (1959: 81) (Lodge et al. 2014). *Gliophorus* sect. *Glutinosae*, typified by *Gliophorus laetus* (Persoon 1800: 48) Herink (1959: 84), is further characterised by plano-convex pileus that is often indented in centre; green, olive, blue, violet, pink, salmon, yellow, buff, orange or orangish-brown coloured pileus; decurrent lamellae with gelatinised edge; cheilocystidia usually present and embedded in gelatinous matrix (ixocheilocystidia); basidiospores mostly binucleate.

During a macrofungal survey and collection tour to different forested areas of South Sikkim, two of us (DC & KD) came across a very interesting and tiny member of *Gliophorus* sect. *Glutinosae*. After detailed macro- and micromorphological characterisation, coupled with the phylogenetic studies based on the sequence data of nuclear ribosomal internal transcribed spacer (nrITS) region of that species, it was shown to be distinct from any other known species in *Gliophorus* and is proposed here as *G. glutinosus* sp. nov. Detailed morphological description, supporting illustrations and phylogenetic inference is presented here for this novel species.

Material and methods

Morphological study

Macromorphological characters were recorded in the forest and in base-camp from two collections of 13 fresh and dissected young to mature basidiomata. Images of the fresh basidiomata were captured with a Canon Power Shot SX 50 HS. Colour codes and terms are mostly after Methuen Handbook of Colour (Kornerup and Wanscher 1978). Micromorphological characters were observed with a compound microscope (Nikon Eclipse Ni-U). Sections from dried specimens were mounted in a mixture of 5% potassium hydroxide (KOH), 1% Phloxine and 1% Congo red or in distilled water. Micromorphological drawings were prepared with a drawing tube (attached to the Nikon Eclipse Ni) at 1000× magnification. The basidium length excludes sterigmata. Basidiospore measurements were recorded in profile view from 30 basidiospores. Spore measurements and length/width ratios (Q) are recorded here as: minimum–*mean*–maximum. Herbarium codes follow Thiers 2018 (continuously updated).

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from dried herbarium specimens (100 mg) using the XcelGen Fungal gDNA Mini Kit (Xcelris Genomics, Ahmedabad, India). The nuclear ribosomal ITS region was amplified using the primers ITS1F and ITS4 (White et al.

1990). Amplification (with PCR) was performed in a 50 µl reaction mix comprising 2 µl template DNA (10–20 ng), 0.5 U Taq DNA polymerase (Sigma-Aldrich, India), 5 µl 10X Taq DNA polymerase buffer, 1 µl 200 µM of each dNTP (Sigma-Aldrich, India), 1 µl 10 pmol primer and the remaining volume made up by H_2O (Sterile Ultra Pure Water, Sigma-Aldrich). This amplification was done using an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) with the following parameters: 5 min step at 95 °C, followed by 30 cycles of 1 min at 95 °C, 30 s at 55 °C and 1 min at 72 °C and a final 7 min extension step at 72 °C. Products from PCR were then purified with QIAquick PCR Purification Kit (QIAGEN, Germany) and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The sequencing products were run on 3730×l DNA Analyzer (Applied Biosystems, USA). The raw DNA files were edited and combined using FinchTV and ChromasLite v. 2.01. The sequences generated from two collections (DC 17–28 and DC 17–38) were deposited in GenBank (MH392195 and MH392196).

Phylogenetic analysis

Phylogenetic analyses were based on internal transcribed spacer (ITS) nuclear ribosomal DNA sequences data to establish the phylogenetic placement of the new species. Datasets including reference sequences and outgroup were prepared following relevant literature (Ainsworth et al. 2013, Lodge et al. 2014, Singh et al. 2017), BLAST searches (Altschul et al. 1997) and data retrieved from public databases such as GenBank (Clark et al. 2016) and UNITE (Kõljalg et al. 2013). Multiple sequence alignment was performed using MAFFT v.7 (Katoh and Standley 2013). The aligned loci were loaded in PAUP* 4.0b 10 (Swofford 2001) and the best-fit substitution model of nucleotide evolution (GTR+I+G) was carried out in MrModeltest 3.7 (Posada and Crandall 1998). Bayesian inference was computed in MrBayes v.3.2.2 (Ronquist et al. 2012). Bayesian posterior probabilities (BPP) were calculated in two simultaneous runs with the Markov chain Monte Carlo (MCMC) algorithm (Larget and Simon 1999). Markov chains were run for 1000000 generations, saving a tree every 100th generation. Default settings in MrBayes were used for the incremental heating scheme for the chains (3 heated and 1 cold chain), unconstrained branch length [unconstrained: exponential (10.0)] and uninformative topology (uniform) priors. The analysis was allowed to terminate when the average standard deviation of split frequencies was below 0.01. The first 25% of trees was discarded as burn-in (Hall 2004). Simultaneously, with the same dataset, a full search for the best-scoring Maximum likelihood tree was conducted in RAxML (Stamatakis 2006) using the standard search algorithm (ITS1-5.8S-ITS2 data partitioned, 1000 bootstrap replications). The significant threshold was set above 0.95 for Bayesian posterior probability (BPP) and above 70% for Maximum likelihood bootstrap support (MLB). Phylograms (Figs 1, 2), inferred from Maximum likelihood method and Bayesian phylogeny, are presented showing MLB and BPP values, respectively, for the eligible branches.

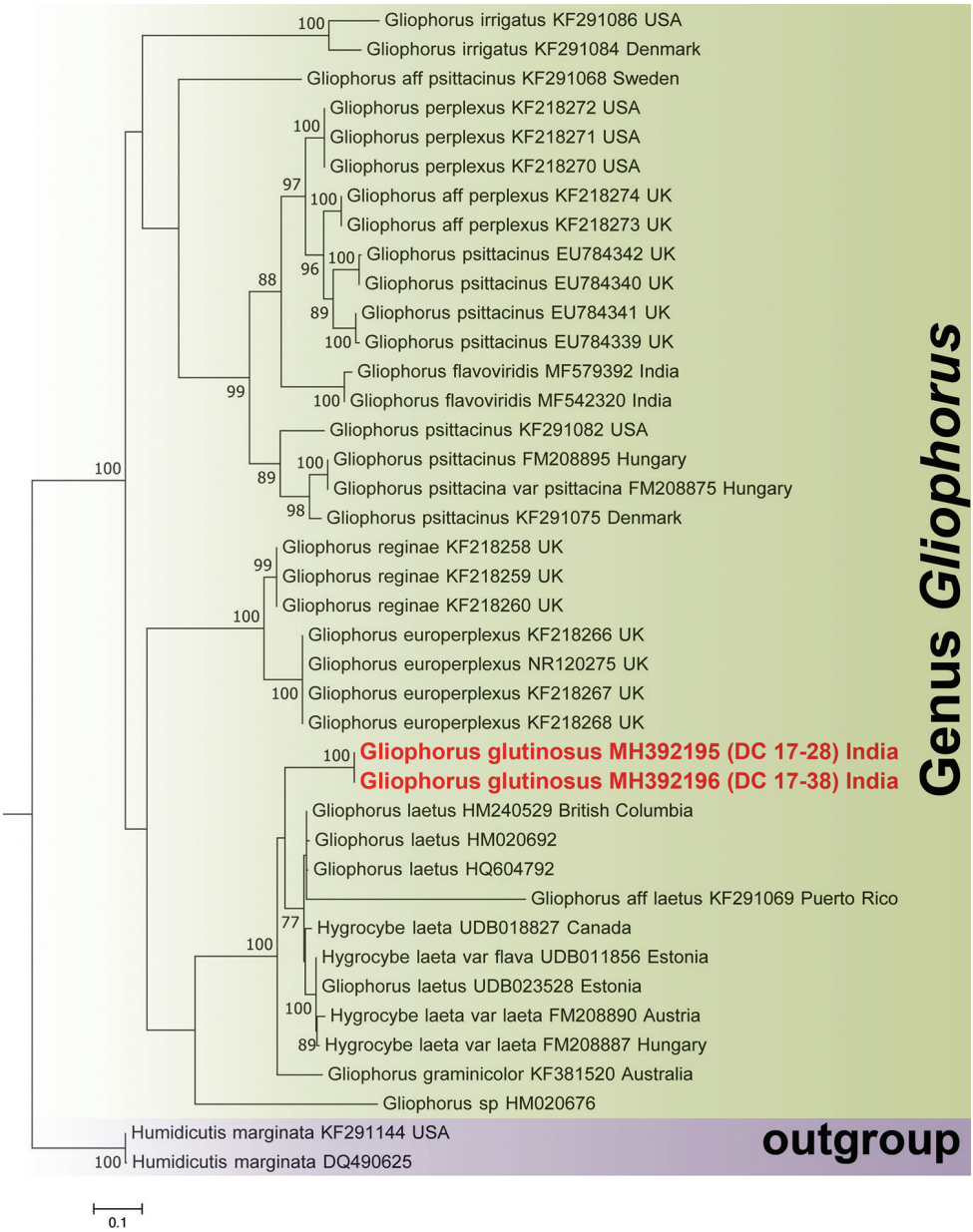

Figure 1. Phylogram resulting from Maximum likelihood analyses of nrITS region. Nodes were annotated with MLB values. MLB values > 70% are shown. Sequences derived from the novel species *Gliophorus glutinosus* (MH392195 and MH392196) are shown in red and bold.

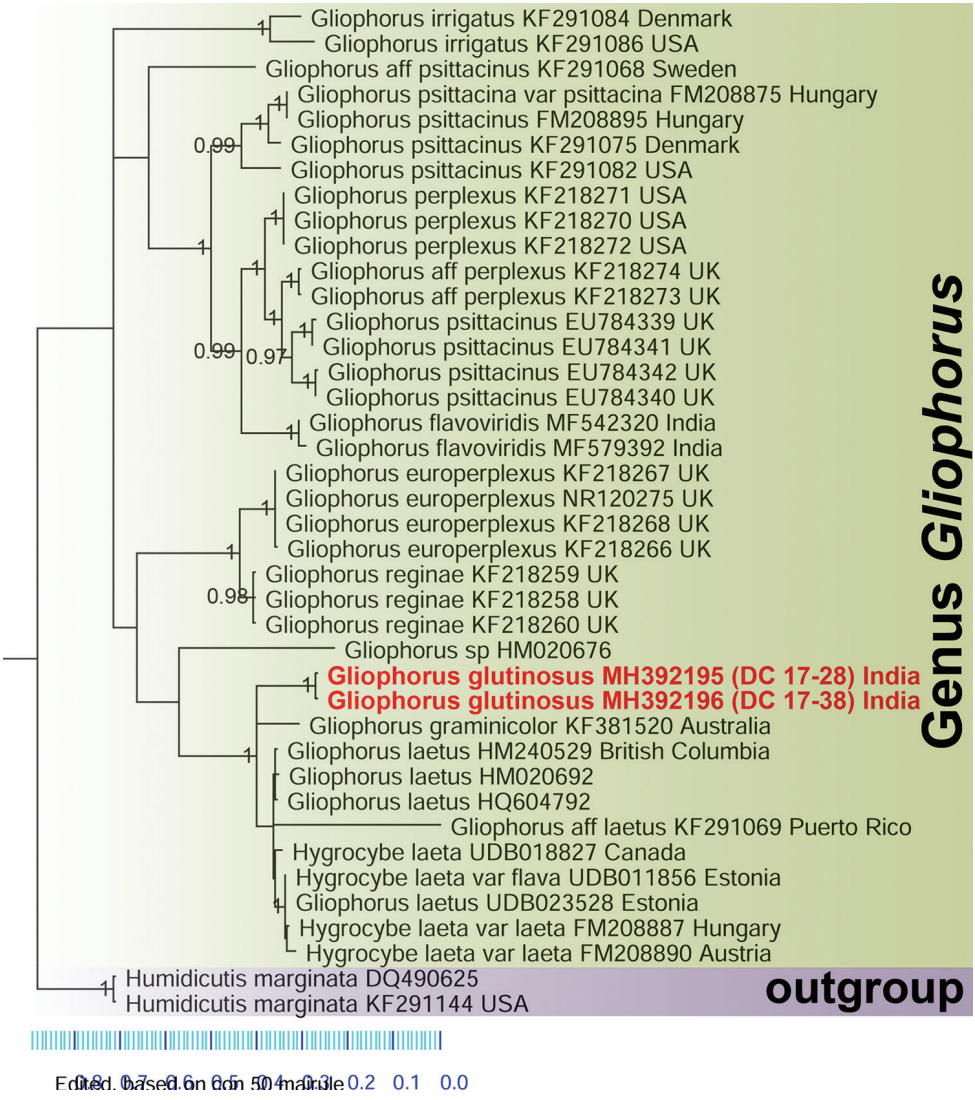

Figure 2. Phylogram resulting from Bayesian phylogeny of nrITS region. Nodes were annotated with BPP values. BPP values > 0.95 are shown. Sequences derived from the novel species *Gliophorus glutinosus* (MH392195 and MH392196) are shown in red and bold.

Results

Phylogeny

The nrITS-sequence dataset consists of 40 sequences. In the Maximum likelihood analysis (Fig. 1), the two Indian collections of the proposed novel species, *Gliophorus glutinosus* (MH392195-DC 17–28 and MH392196-DC 17–38) clustered together (MLB = 100%) as a distinct species and appeared sister to the clade (MLB = 77%) bearing *G. laetus* from Europe, North America, Puerto Rico and Austria (HM240529, HM020692, HQ604792, KF291069, UDB018827, UDB011856, UDB023528, FM208887, FM208890). In turn, *G. laetus* and *G. glutinosus* clustered together as sister (MLB = 100%) to an Australian collection of *G. graminicolor* E. Horak (1973: 176) (KF381520). Similarly, in our Bayesian phylogeny (Fig. 2), the two Indian collections of *Gliophorus glutinosus* clustered together (BPP = 1.00) and appeared sister to the clade bearing *G. laetus* from Europe, North America, Puerto Rico and Austria (HM240529, HM020692, HQ604792, KF291069, UDB018827, UDB011856, UDB023528, FM208887, FM208890). The Australian collection of *G. graminicolor* (KF381520) also appeared as nested (without ancestral information) between the *G. laetus* cluster and *G. glutinosus*.

Taxonomy

Gliophorus glutinosus **K. Das, D. Chakr. & Vizzini, sp. nov.**
Mycobank: MB 825657
Figs 3, 4

Diagnosis. Distinguished from all the allied taxa by its nrITS sequence and possessing a combination of features like typically twisted stipe submerged under thick gluten, sticky pileus, presence of gluten at lamellar edge, decurrent lamellae, indistinct odour, ixocheilocystidia and presence of pleuropseudocystidia and absence of clamps in pileipellis hyphae.

Type. INDIA. Sikkim: South District, Thangse, 1962 m alt., 27°18.496'N, 88°21.519'E, 23 August 2017, *D. Chakraborty* & *K. Das*, *DC 17–28* (Holotype CAL!).

Etymology. The epithet "*glutinosus*" refers to the highly glutinous stipe surface.

Pileus 5–20 mm diam., convex with a shallow central depression at disc when young, becoming plano-convex at maturity; surface highly glutinous, sticky, sulcate-striate, greyish-orange (6C–B5), brownish-orange (5C5), becoming pale orange to orange white (7C7, 6A3–2) with maturity, sometimes whitish to pastel yellow (2A4) at centre; margin crenate; context ≤ 2 mm thick, concolorous with pileus surface. *Lamellae* subdecurrent to decurrent, moderately close to subdistant (11 per 10 mm at pileus margin), viscid, pale orange to orange white (5A3–2); lamellulae in 3 series; edges glutinous, concolorous with face of lamellae, viscid. *Stipe* 10–60 × 2–5 mm, central,

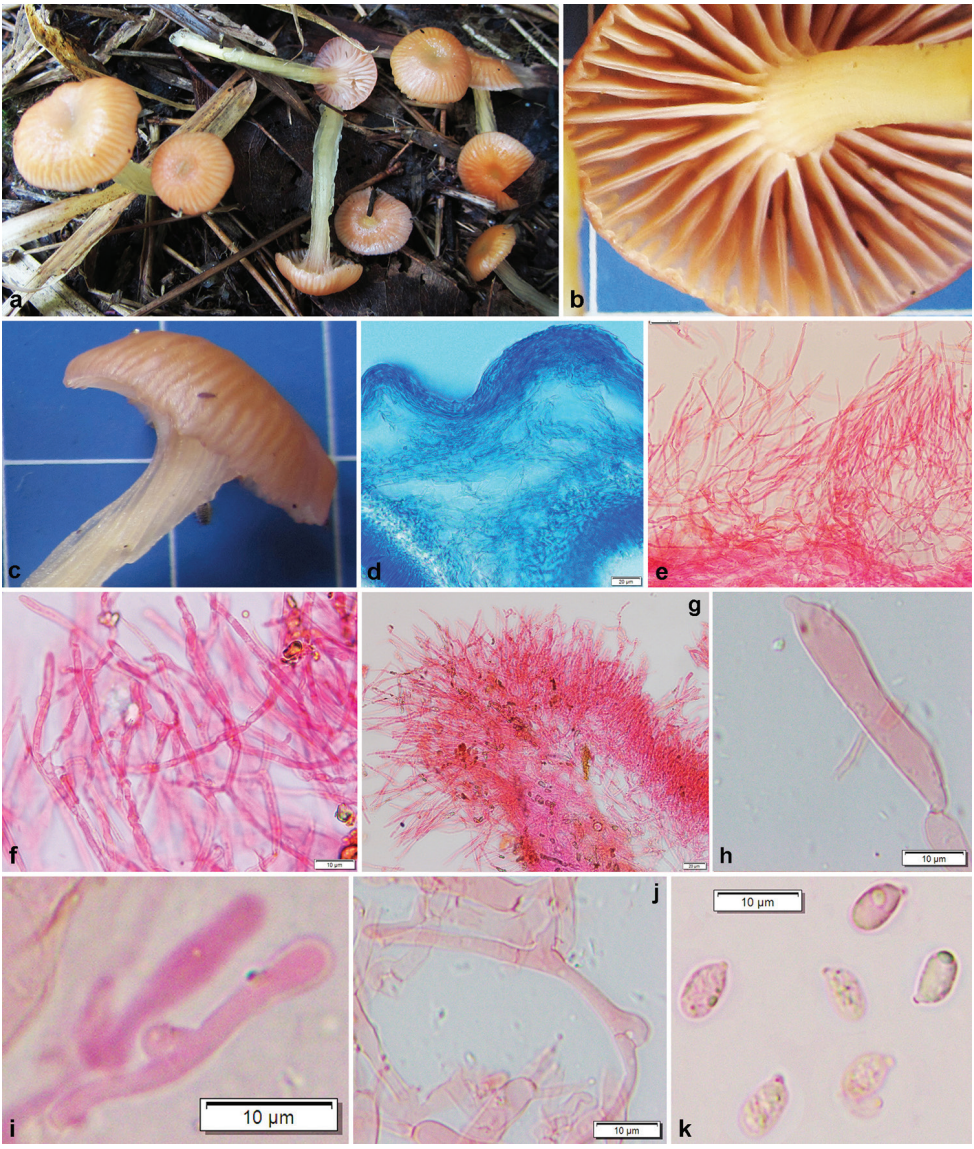

Figure 3. *Gliophorus glutinosus* (from DC 17–28, holotype). **a–c** Fresh basidiomata in field or in base-camp **d** Transverse section through pileipellis showing ixocutis pattern (under cotton blue) **e, f** Hyphal elements in pileipellis (after removal of gluten) **g** Cheilocystidia (after removal of gluten at lamellae edge) **h** Pleuropseudocystidium **i** Basidioles **j** Hyphae in hymenophoral trama **k** Basidiospores. Scale bars: 20 µm (**d, e, g**); 10 µm (**f, h, i, j, k**).

hollow, cylindrical, often gradually broaden towards base, twisted, longitudinally furrowed, submerged under thick sticky gluten (1 mm); surface upper half pale orange (5A3) and pale yellow to light yellow (4A3–4) towards base. *Taste* and *odour* indistinct. *Spore print* not obtained.

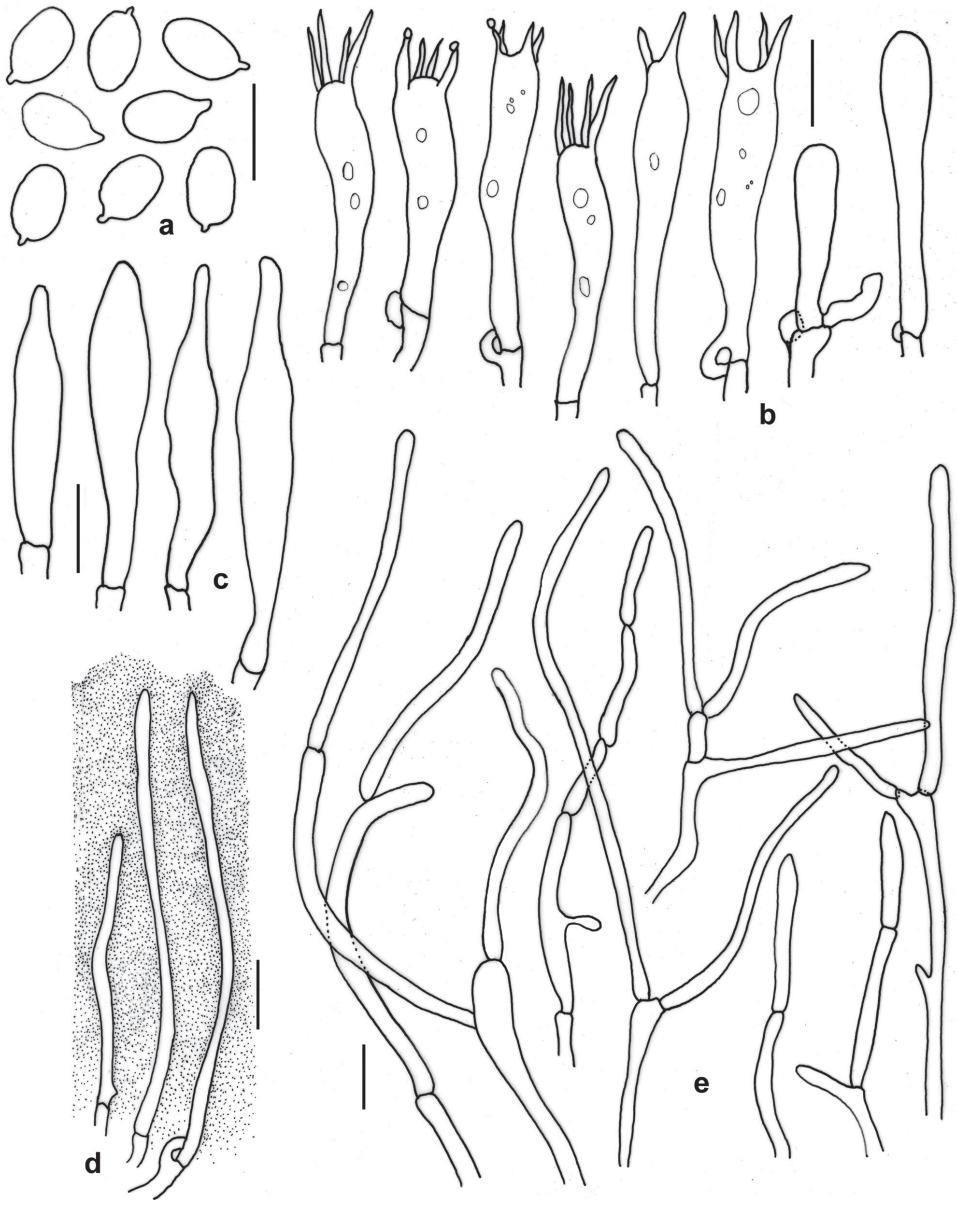

Figure 4. *Gliophorus glutinosus* (from DC 17–28, holotype) **a** Basidiospores **b** Basidia and basidioles **c** Pleuropseudocystidia **d** Ixocheilocystidia **e** Hyphal elements in pileipellis (without gluten and in 5% KOH). Scale bars: 10 µm (**a–e**).

Basidiospores 6–*7*–8 × 3–*4.1*–5 µm (n = 30, Q = 1.5–*1.72*–2.16), elongate-ellipsoid to nearly cylindric, smooth, thin-walled, hyaline, inamyloid, uni- to multiguttulate. *Basidia* 30–38 × 5–7 µm, clavate, thin-walled, with a basal clamp-connection, 2- to 4-spored; sterigmata up to 10 µm long. *Lamellar edge* sterile. *Cheilocystidia* 35–62 × 2–5 µm, slender, occasionally septate, mostly clustered together, gelatinised (embed-

ded in gelatinous matrix). *Pleuropseudocystidia* 31–40 × 5–7 µm, rare, subclavate to appendiculate or fusoid. *Subhymenium* 16–23 µm thick, not gelatinised. *Hymenophoral trama* subregular, consisting of clamped hyphae (3–10 µm diam.), terminal and subterminal cells 17–48 µm long, terminal cells often inflated. *Pileipellis* an ixocutis (when mounted in water or cotton blue), 25–60 µm thick, submerged under thick gluten (seen when mounted with cotton blue), composed of suberect, thin-walled, septate and frequently branched hyphae (observed when mounted in 5% KOH making it free from gluten); terminal elements 15–40 × 2–5 µm, with rounded apex, clamps absent. *Stipitipellis* an ixocutis (when mounted in water or cotton blue) to an ixotrichoderm (when revived in KOH), mostly similar to that of pileipellis.

Habitat/ Distribution. Growing in groups or gregariously on soil amongst leaf-litter of angiospermous plants.

Additional specimen examined. INDIA. Sikkim: South District, Thangse, 1962 m alt., 27°18.496'N, 88°21.519'E, 23 August 2017, *D. Chakraborty* & *K. Das*, DC 17–38 (CAL).

Discussion

The combination of features, such as significantly sticky small basidiomata, distinctively twisted stipe which is completely submerged within a thick (1 mm) transparent layer of gluten, decurrent lamellae with glutinous (sticky) edges, presence of pleuropseudocystidia (sterile elements arising deep in the hymenophoral trama and protruding into the hymenium) and absence of clamps in hyphae of pileipellis, separate *G. glutinosus* from all the known species of *Gliophorus*. Features, such as decurrent lamellae with sticky edges, planoconvex to slightly depressed pileus and presence of ixocheilocystidia, placed the Indian collection under *Gliophorus* sect. *Glutinosae*. In fact, in the phylogenetic analysis (Figs 1–2), the new species forms a strongly supported clade together with *G. laetus*, type species of the sect. *Glutinosae* and with *G. graminicolor*. To our best knowledge, this is the first report of the presence of pleuropseudocystidia in a *Gliophorus* species or, in general, in *Hygrocybe* s.l. (Singer 1986, Boertmann 2010, Lodge et al. 2014). So far, only cheilopseudocystidia have been described as present, albeit rarely, in *Hygrocybe* s.l. (Boertmann 2010, Lodge et al. 2014).

Morphologically, *G. glutinosus* is similar to *G. laetus* [≡*Hygrocybe laeta* (Pers.) P. Kumm. (1871: 112); ≡*Hygrophorus laetus* (Pers.) Fries 1838: 329] but the latter differs by having significantly larger basidiomata (pileus 10–50 mm diam., stipe 30–120 mm long), stipe which is never twisted and less glutinous and showing greyish-lilac tinges at apex; a strongly gelatinised and up to 140 µm thick subhymenium, presence of cuticular clamped hyphae and having an unpleasant odour, described as like burned rubber, burned hair, fish or animal cages (Hesler and Smith 1963, Arnolds 1974, 1990, Boertmann 2010, Bessette et al. 2012). *Gliophorus graminicolor* E. Horak [≡*Hygrocybe graminicolor* (E. Horak) T.W. May & A.E. Wood 1995: 148] from Australia (Tasmania included) and New Zealand is though genetically close to this novel Indian species and can be separated by possessing brown to greenish-brown or grass green coloured

pileus and stipe, less viscid stipe, odour and taste unpleasant, like burnt hair, presence of clamps in pileus hyphae (Horak 1973, 1990, Young and Wood 1997 as *Hygrocybe batesii* A.M. Young (in Young and Wood 1997: 956), Young 1999, 2005, Young and Mills 2002). *Hygrocybe noelokelani* Desjardin & Hemmes (1997: 621), from Hawaii, shows a deep pink, pastel red or pale red pileus, a non-twisted, less viscid stipe, ovoid to broadly ellipsoid, spores (up to 6 µm wide) and presence of large clamp-connections on pileipellis hyphae (Desjardin and Hemmes 1997). *Hygrocybe corallina* Leelav., Manim. & Arnolds (2006: 125), from Kerala, India, has pale red to coral-red basidiomata with bright red lamellae, larger spores [7–10(–11) × 4.5–6.5 µm], clamps observed in all parts of basidioma and the hymenophoral trama regular, made up of medium-sized to long, thin-walled elements, 100–500 × 3–20 µm (Leelavathy et al. 2006).

Key to the species in *Gliophorus* sect. *Glutinosae* worldwide

(* indicated species included in the section are based on morphology alone)

1 Pileipellis as ixocutis **2**
– Pileipellis as ixotrichoderm **6**
2 Pileus whitish to pale argillaceous; lamellae adnate.... ***G. pallidus* E. Horak***
– Pileus dark and/or bright coloured; lamellae decurrent **3**
3 Pileus reddish-brown or lilac pink, liver brown; cheilocystidia absent..... ***G. versicolor* E. Horak***
– Pileus greenish-blue, green or orange-brown; cheilocystidia present **4**
4 Pileus orange-brown, strongly glutinous; stipe twisted, embedded in a thick layer of gluten; basidia 2- to 4-spored ..... ***G. glutinosus* (DC 17–28)**
– Pileus green to greenish-blue, surface moderately glutinous; stipe equal; basidia 4-spored **5**
5 Pileus green; lamellae whitish with greenish tinge; odour burnt-hair like, unpleasant..... ***G. graminicolor***
– Pileus greenish-blue, turning bluish-lilac with age; lamellae lilac blue or pale greenish-blue; odour none ..... ***G. lilacipes* E. Horak***
6 Pileus pinkish-orange, orange brown or pastel red to pale red; lamellae subdecurrent to decurrent **7**
– Pileus green or yellow; lamellae broadly adnate **9**
7 Clamp-connections in pileipellis frequent..... ***G. laetus***
– Clamp-connections in pileipellis very rare..... **8**
8 Pileus orange brown, paler with age; lamellae decurrent, pale yellow; stipe tapering down or broader at middle..... ***H. viscidibrunnea* Bougher & A.M. Young***
– Pileus pink or pastel red or pale red; lamellae subdecurrent, pale pinkish-white; stipe cylindrical ..... ***H. noelokelani****
9 Pileus greenish; lamellae greenish..... ***G. pseudograminicolor* A.M. Young***
– Pileus yolk yellow to lemon yellow; lamellae yellow ..... ***G. chromolimoneus* (G. Stev.) E. Horak***

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