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Bretziella, a new genus to accommodate the oak wilt fungus, *Ceratocystis fagacearum* (Microascales, Ascomycota)

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Abstract

Recent reclassification of the Ceratocystidaceae (Microascales) based on multi-gene phylogenetic inference has shown that the oak wilt fungus *Ceratocystis fagacearum* does not reside in any of the four genera in which it has previously been treated. In this study, we resolve typification problems for the fungus, confirm the synonymy of *Chalara quercina* (the first name applied to the fungus) and *Endoconidiophora fagacearum* (the name applied when the sexual state was discovered). Furthermore, the generic placement of the species was determined based on DNA sequences from authenticated isolates. The original specimens studied in both protologues and living isolates from the same host trees and geographical area were examined and shown to represent the same species. A lectotype was designated for *Chalara quercina* and *Endoconidiophora fagacearum* and an epitype linked to a living ex-epitype isolate was designated. Phylogenetic analyses confirmed that the species resides in a well-supported monophyletic lineage in the Ceratocystidaceae, distinct from all other genera in the family. The new genus *Bretziella* is described to accommodate the oak wilt fungus.

Keywords

Quercus, Ceratocystidaceae, Microascales, heterothallic

Introduction

Oak wilt is a serious disease of many *Quercus* spp. in the Midwestern and Eastern United States, as well as Texas (Juzwik et al. 2011). The disease was first described in the 1940's (Henry 1944, Bretz 1953) and sporadic, localized outbreaks occur frequently in the established range, although the disease is viewed by many as a manageable (Juzwik et al. 2011, Horie et al. 2013). However, with a growing global awareness of invasive alien species and their potential to cause destructive epidemics (Brasier 2008, Wingfield et al. 2015), oak wilt is considered one of several significant diseases that threaten the health of *Quercus* spp. worldwide (Gibbs 1981, 2003, Brasier 2001).

Oak wilt is caused by a fungus in the genus *Ceratocystis*, which is widely known as *Ceratocystis fagacearum* (Juzwik et al. 2008, 2011, Harrington 2009). The genus was originally described to accommodate the sweet potato pathogen, *C. fimbriata* (Halsted 1890). Since that time many morphologically similar species were described in or transferred to this genus, resulting in an aggregate genus incorporating more than 70 species a century later (Upadhyay 1981). DNA sequence analyses revealed that *Ceratocystis sensu* Upadhyay included two phylogenetically distinct groups (Hausner et al. 1993, Spatafora and Blackwell 1994). Several subsequent studies confirmed that the one group, including the type species of *Ophiostoma*, previously treated as *C. pilifera*, resides in the Ophiostomataceae (Ophiostomatales, Sordariomycetidae). The second group, including *C. fimbriata*, resides in the Ceratocystidaceae (Microascales, Hypocreomycetidae) (Réblová et al. 2011, De Beer et al. 2013a).

Generic boundaries within the Ceratocystidaceae were recently reconsidered based on DNA sequence data for three gene regions in 70 species (De Beer et al. 2014). Phylogenetic analyses showed that the family includes at least seven well-supported monophyletic lineages accepted as distinct genera, as well as four minor, unresolved lineages. De Beer et al. (2014) thus redefined *Ceratocystis s. str.* and *Ambrosiella*, re-instated and emended descriptions for *Chalaropsis*, *Endoconidiophora*, and *Thielaviopsis*, and described two new genera, *Davidsoniella* and *Huntiella*. The unresolved lineages included *Thielaviopsis basicola*, *Ceratocystis adiposa*, and *Ambrosiella ferruginea*. In a subsequent study, Mayers et al. (2015) re-instated the genus *Phialophoropsis* to accommodate *A. ferruginea* and *A. trypodendri*, and described an additional genus, *Meredithiella*.

The fourth unresolved lineage in the study of De Beer et al. (2014) included the single taxon, *Ceratocystis fagacearum*. The asexual state of the fungus was described first as *Chalara quercina* (Henry 1944). Bretz (1951) and Hepting (1951, 1952) soon discovered that the fungus was heterothallic and that the sexual state could be induced in culture by crossing isolates of opposite mating type. Bretz (1952) proceeded to describe the sexual state as *Endoconidiophora fagacearum*. However, Bretz was not aware that in the previous year, Bakshi (1951) reduced *Endoconidiophora* (Münch 1907) to synonymy with *Ceratocystis*, a treatment that soon gained wide acceptance (Moreau 1952, Moreau and Moreau 1952, Hunt 1956). In his monograph of *Ceratocystis*, Hunt (1956) transferred *E. fagacearum* to that genus.

During the course of the six decades following the Hunt (1956) monograph, the oak wilt fungus was treated as *Ceratocystis fagacearum*, with its asexual (anamorph) name, *Chalara quercina* as heterotypic synonym (Nag Raj and Kendrick 1975, Upadhyay 1981, Seifert et al. 1993, De Beer et al. 2013b). Following the dual nomenclature system, Paulin-Mahady et al. (2002) suggested that the asexual state of *C. fagacearum* should be treated as *Thielaviopsis quercina*. This was because the type species of the genus *Chalara*, *Chalara fusidioides*, was clearly different from the taxa related to *Ceratocystis* and was suggested to belong to the Leotiales.

De Beer et al. (2014) restricted *Ceratocystis* to species previously treated in the *C. fimbriata* complex. *Endoconidiophora* was confined to species previously treated in the *C. coerulescens* complex, with *E. coerulescens* as the type species. Based on the phylogenies presented by Mbenoun et al. (2014) and De Beer et al. (2014), *Thielaviopsis* with *T. ethacetica* as the type species, included species previously treated in the *C. paradoxa* complex. Consequently, none of the four genera (*Ceratocystis*, *Endoconidiophora*, *Thielaviopsis* or *Chalara*) are available to accommodate *C. fagacearum*, which resides in a lineage distinct from these genera (De Beer et al. 2014). Because the isolate representing *C. fagacearum* was not from a type specimen, De Beer et al. (2014) concluded a generic placement of the species could not be considered prior to resolving the typification of *E. fagacearum* and *Ch. quercina*. These authors also suggested that sequences of additional isolates should be included in such a study.

The aim of this study was firstly to consider the appropriate generic placement of the oak wilt fungus in the Ceratocystidaceae based on phylogenetic analyses of the three gene regions used by De Beer et al. (2014), and including additional isolates of the fungus. Secondly, all available materials used in the protologues of the two species were obtained to address unresolved typification issues. The synonymy of *Ch. quercina* and *E. fagacearum* and priority of the basionyms was also resolved against the backdrop of contemporary nomenclatural practices (McNeill et al. 2012, 2015).

Materials and methods

Herbarium specimens and isolates

Herbarium specimens labelled as *Chalara quercina* from the study of Henry (1944), and *Endoconidiophora fagacearum* from the study of Bretz (1952), were obtained respectively from the National Fungus Collections (BPI) (U.S. Department of Agriculture, Beltsville, Maryland) and the Forest Service (FP) (Center for Forest Mycology Research, Madison, Wisconsin). Each specimen included dried cultures and notes. In addition, four isolates of *Ceratocystis fagacearum* that were isolated from diseased oak trees in the USA, available from the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (University of Pretoria, Pretoria, South Africa), were included in the study (Table 1). The epitype for *C. fagacearum* was deposited in BPI.

Table 1. Isolates of *Bretziella fagacearum*[†] used in this study.

Culture numbers[‡]	Host	Locality	GenBank accession number		
			60S	LSU	MCM7
CMW 2039 = CBS 130770	*Quercus* sp.	Minnesota	=KM495518	=KM495341	=KM495430
CMW 2656[EP] = CBS 138363	*Quercus rubra*	Iowa	KM495518[§]	KM495341	KM495430
CMW 2658	*Quercus* sp.	Iowa	=KM495518	=KM495341	=KM495430
CMW 38759 = CBS 129241	*Quercus* sp.	Iowa	=KM495518	=KM495341	=KM495430

[†] Information on other species and isolates included in this study and their GenBank accession numbers are available in De Beer et al. (2014).

[‡] CMW = Culture collection of the Forestry and Agricultural Biotechnology Institute, University of Pretoria, Pretoria, South Africa, CBS = Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands.

[§] Where DNA sequences of different isolates were identical, we only deposited one sequence representing each haplotype in GenBank. Identical sequences obtained from other isolates are indicated with '='

[EP] = Ex-epitype.

PCR, DNA sequencing and phylogenetic analyses

Three gene regions, the nuclear ribosomal DNA large subunit (LSU), the 60S ribosomal protein RPL10 (60S), and mini-chromosome maintenance complex component 7 (MCM7), were amplified and sequenced for all four living isolates. These gene regions were the same as those selected and used by De Beer et al. (2014) to define generic boundaries in the Ceratocystidaceae. In addition to these, sequences were determined of the ribosomal internal transcribed spacer region (ITS) and translation elongation factor 1-α (TEF1α), respectively the universal DNA barcode (Schoch et al. 2012) and secondary barcode (Stielow et al. 2015) for fungi, for isolate CBS 138363 = CMW 2656. Total genomic DNA was extracted with PrepMan® Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, California) following the protocols used by Duong et al. (2012). Primers, PCR and PCR sequencing protocols used were the same as those described by De Beer et al. (2014).

Representative species of the dominant genera in the Ceratocystidaceae were included in the phylogenetic analyses. The recently described *Meredithiella* was not included because appropriate sequence data were not available for this taxon. Species of *Knoxdaviesia* and *Graphium* were included as outgroups. Datasets for each of the three gene regions were compiled and aligned separately with the online version of MAFFT v. 7 (Katoh and Standley 2013) and concatenated into a single dataset for subsequent analyses. Maximum likelihood (ML) and Bayesian inference (BI) were carried out on the concatenated dataset. ML analysis was conducted using raxmlGUI v. 1.3.1 (Silvestro and Michalak 2012). Ten runs of a maximum likelihood search with the GTR+G model were performed, followed by 1000 bootstrap searches. BI analysis was conducted using MrBayes v. 3.2 (Ronquist et al. 2012). Ten parallel runs with the GTR+G model were performed for 5 million generations. Trees were sampled every 100th generation. The first 25 % of the tree samples were discarded as burn-in, and Bayesian posterior probabilities were computed from the remaining trees.

Morphology

Morphological characters of sexual and asexual structures taken from the herbarium specimens and living isolates were compared with each other and with the original descriptions (Henry 1944, Bretz 1951, 1952). For morphological studies, isolates were grown on 2 % yeast malt agar (YMA). In an attempt to obtain sexual structures, the four isolates were crossed with each other in all possible combinations on 2 % water agar in the presence of sterilized oak twigs. The plates were incubated at room temperature under near UV light.

Microscopic structures taken from herbarium specimens were mounted and studied in 10 % KOH, and those from living cultures were mounted in water, later replaced with 85 % lactic acid in which they were then studied. Up to 50 measurements were made for each characteristic structure where possible. Microscopic structures were studied with a Nikon SMZ18 stereoscope and a Nikon Eclipse Ni compound microscope. Images were captured using a Nikon DS-Ri2 camera. Measurements were made using the Nikon Imaging Software (NIS) Elements (v. 4.3).

Results

Phylogenetic analyses

DNA sequences obtained for the LSU, 60S, and MCM7 regions of the four living isolates were used for phylogenetic analyses. These sequences, as well as the ITS and TEF1α sequences for CBS 138363 = CMW 2656 (ex-epitype, see below), have been deposited in the RefSeq Targeted Loci (RTL) database in NCBI GenBank (Schoch et al. 2014).

A total of 39 isolates representing 35 species were included in the phylogenetic analyses. Alignment of the 60S dataset resulted in ambiguously aligned regions and long gaps that were a result of the inconsistency in the presence/absence of introns and highly variable intron sequences. Gap-containing positions from the 60S dataset were thus excluded from further analyses. After removing all gap positions, the 60S dataset consisted of 314 characters with 105 variable characters. The LSU dataset consisted of 875 characters with 173 variable characters. The MCM7 dataset consisted of 628 characters with 321 variable characters. The ML and BI analyses of the concatenated dataset of all three gene regions resulted in trees with almost identical topology. Monophyletic clades representing all genera included in the analyses could be identified and these clades were strongly supported in both ML and BI analyses.

The four *C. fagacearum* isolates included in this study formed a well-supported monophyletic clade (Figure 1) that was most closely related to, but distinct from, *Phialophoropsis*. The only difference observed between the BI and ML trees was the positioning of *Thielaviopsis* in relation to other genera. In the ML tree, *Thielaviopsis* formed a sister clade to those of *Endoconidiophora* and *Davidsoniella*, but with no support. This was in contrast to the BI tree, where *Thielaviopsis* formed a clade basal to those of *Ceratocystis s. str.*, *Chalaropsis*, *Endoconidiophora* and *Davidsoniella* with high posterior probability values.

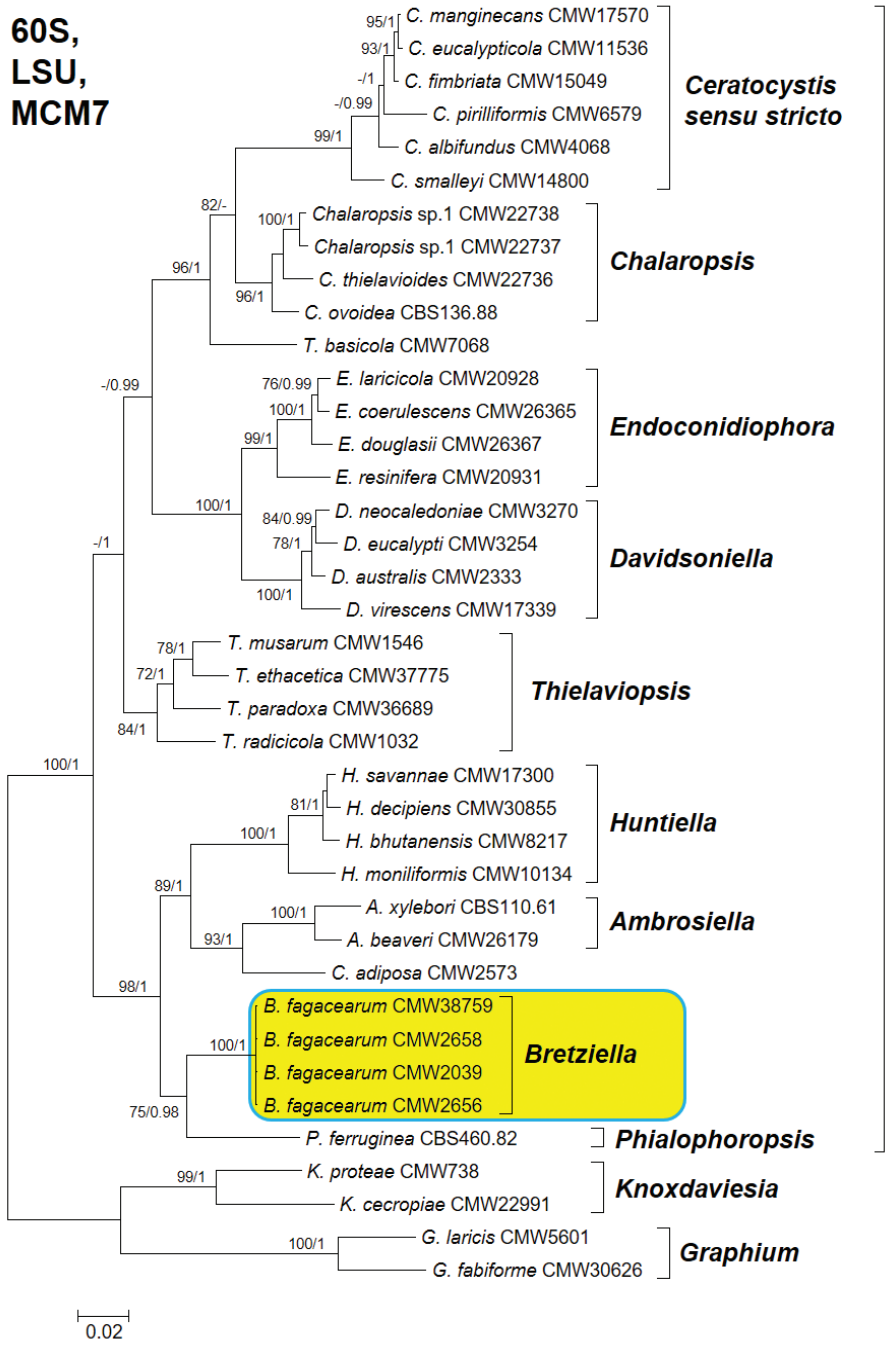

Figure 1. Bayesian phylogram derived from the analyses of the concatenated dataset (60S, LSU, MCM7). Maximum likelihood bootstrap values (≥ 70 %, 1000 replicates) and Bayesian posterior probabilities values (≥ 0.95) are indicated at nodes. "-" indicated no phylogenetic support or the support values are below 70% for ML and 0.95 for BI.

Morphology

The herbarium specimen of *Chalara quercina* (BPI 595712) from study of Henry (1944) consisted of a dried culture with dark brown to grey clumps of aerial hyphae present. Only asexual structures were obtained from this specimen (Figure 2A, E, F, K).

Description: Conidiophores cylindrical tapering towards the apex, single, upright, straight or slightly curved, occasionally branched, pale to dark brown, becoming paler to the apex, 3–9 septate, up to 140 µm long including conidiogenous cells, 3–5 µm wide at the base. *Conidiogenous cells* cylindrical, tapering towards the apex, slightly pigmented to hyaline, 20–32 µm long, 2.5–3.5 µm wide at the base, 2–3 µm wide near the apex. *Conidia* endogenous, hyaline, rectangular shaped, 4–8.5 × 2–3 µm, produced in chains. *Aleurioconidia* not observed.

The herbarium specimen of *Endoconidiophora fagacearum* (FP 97476) from the study of Bretz (1952) consisted of a few broken pieces of dried agar covered with a thick, grey to dark brown mycelial mat. A few ascomatal necks were observed with their bases completely embedded in the mycelial mats, which also contained asexual structures (Figure 2B, C, D, G, H, L).

Description: *Ostiolar hyphae* observed in a single ascomatal neck hyaline, divergent. *Ascospores* recovered from broken ascoma hyaline, ellipsoidal, occasionally curved, 4.5–9.5 × 2–3.5 µm, embedded in gelatinous sheath. *Conidiophores* cylindrical tapering towards the apex, single, upright, straight or slightly curved, occasionally branched, pale to dark brown, becoming paler towards the apex, 2–6 septate, up to 100 µm long including conidiogenous cells, 3.5–5 µm wide at the base (these measurements reflect a limited number of intact conidiophores due to the brittle condition of the specimen). *Conidiogenous cells* cylindrical, tapering towards the apex, slightly pigmented to hyaline, 19–35 µm long, 2.5–3.5 µm at the base, 2–3.5 µm near the apex. *Conidia* endogeneous, hyaline, rectangular-shaped, 3–6.5 × 2–3 µm, produced in chains. *Aleurioconidia* not observed.

Laboratory crosses between the living isolates (Table 1) treated to date as *Ceratocystis fagacearum*, did not yield sexual structures and produced only asexual structures (Figure 2I, J, M).

Description: On 2 % YMA with oak sticks mycelia fluffy, pale to dark grey. Spore-bearing structures hidden in mycelial mat. *Conidiophores* cylindrical, tapering towards the apex, single, upright, straight or slightly curved, occasionally branched or reduced to conidiogenous cells, pale to dark brown, becoming paler towards the apex, 3–9 septate, up to 155 µm long including conidiogenous cells, 3–5 µm wide at the base, often constricted at septum. *Conidiogenous cells* cylindrical tapering towards the apex, slightly pigmented to hyaline, 25–35 µm long, 2.5–4.5 µm wide at the base, 2.5–3.5 µm wide near the apex. *Conidia* endogenous, rectangular shaped, hyaline, 3.5–9 × 1.5–3.5 (avg. 5.9 × 2.5 µm), produced in chains. *Aleurioconidia* not observed.

Features of the conidiophores were almost identical between the two herbarium specimens and the living isolates (Figure 2E–J). Conidial dimensions, however, showed some variability between the original descriptions and our observations. Henry (1944)

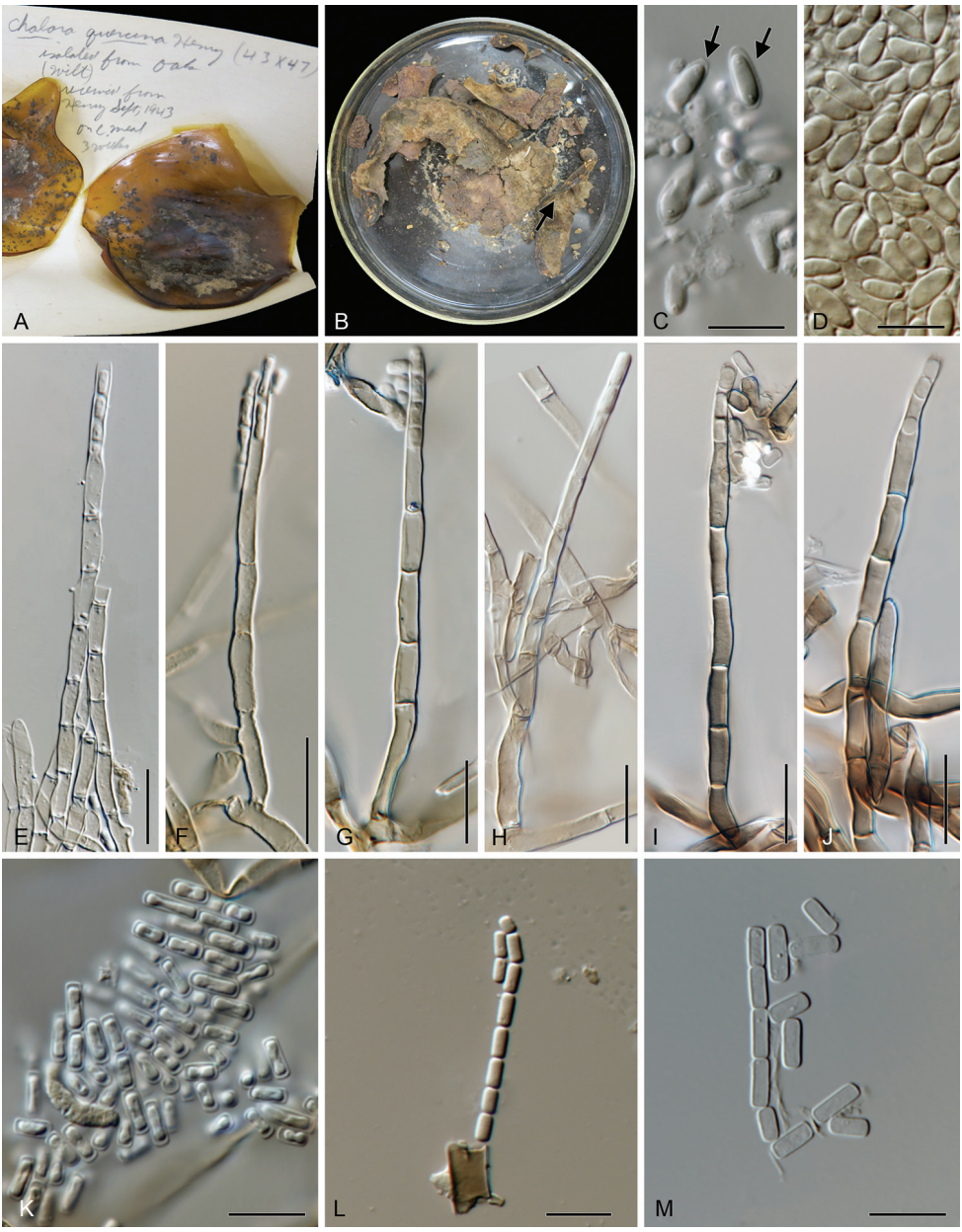

Figure 2. Morphological features of herbarium specimens and a living isolate of the oak wilt fungus. **A, E, F, K** *Chalara quercina* (BPI 595712, Lectotype) **B, C, D, G, H, L** *Endoconidiophora fagacearum* (FP 97476, Lectotype) **I, J, M** Living isolate treated as *Ceratocystis fagacearum* (CMW 2656 = CBS 138363, ex-epitype) **A, B** Dried cultures (arrow in **B** indicates the piece where ascomata were found) **C, D** Ascospores with sheaths (arrows) **E–J** Conidiophores **K–M** Conidia. Scale bars: **C–J** = 20 μm, **K–M** = 10 μm.

described conidia in the range of 4–22 × 2–4.5 µm, whereas in this study the size range of conidia from his specimen (BPI 595712) was 4–8.5 × 2–3 µm. Bretz's (1952) description of conidia reflected a mixture of endogenous conidia and aleurioconidia (see Mbenoun et al. 2014), described as 'thick-walled, olivaceous to brown, polymorphic spores, 3.5–5.5 µm wide to 5 to 20 µm long, formed endogenously and intercalarily in hyphae, which may also produce hyaline endoconidia'. We observed only hyaline, endogenous conidia in the range of 3–6.5 × 2–3 µm from the Bretz specimen (FP 97476). This concurs with the description of Henry (1944), and observations based on the holotype specimen of Bretz (now lost, see below) by Nag Raj and Kendrick (1975) and Upadhyay (1981). It is also consistent with more recent observations that aleuriocondia do not occur in this species (Paulin-Mahady et al. 2002, Harrington 2009). The conidial dimensions taken from the living isolate (CMW 2656) were in the range of 3.5–9 × 1.5–3.5 µm, and corresponded with those on both herbarium specimens.

Culture characteristics of the fresh isolates were similar to those of the Bretz specimen (FP 97476), forming fluffy, thick mycelial mats containing the sexual structures (Figure 2A, B). The morphology of the dried culture of Henry (BPI 595712) differed from the other two specimens. However, the original description (Henry 1944) reads as follows: 'mycelial mat fluffy, 1–3 mm high, white, becoming gray to olive-green with occasional patches of tan', and is consistent with the morphology of the cultures examined in this study as well as that for the Bretz specimen.

Only a few broken ascomata were removed from the Bretz specimen (FP 97476) for this study. The shape of the ascomata was similar to those described by Bretz (1952). Diverging ostiolar hyphae were observed on the specimen and corresponded to Bretz's description of 'a cluster or fringe of hyaline filaments' that terminated in the 'long, black beaks'. The ascospores were 4.5–9.5 µm long and 2–3.5 µm wide, consistent with those reported by Bretz (1952) that were 5–10 × 2–3 µm. Bretz (1952) described the ascospores as 'elliptical and slightly curved', but did not specifically mention a sheath; a feature also not mentioned by Hunt (1956) when he provided the new combination for *Endoconidiophora fagacearum* in *Ceratocystis*. However, Upadhyay (1981) described ascospores from the lost holotype of Bretz (see below) as 'elongate ellipsoid or elongate orange section shaped in side view, cylindrical to elliptical in face view, end view not seen, surrounded by a uniform hyaline gelatinous sheath, 5–11 × 2.5–3.5 µm including sheath'. Our observations of the ascospores (Figure 2C, D) included the presence of sheaths surrounding the ascospores, consistent with the description of Upadhyay (1981).

Taxonomy and nomenclature

Morphological comparisons with herbarium specimens representing *Chalara quercina* and *Endoconidiophora fagacearum*, confirmed that the four living isolates included in this study represented the same taxon. Unresolved typification and nomenclatural issues relating to this taxon are considered below. Phylogenetic analyses including

DNA sequences showed that the four isolates grouped in a well-supported clade in the Ceratocystidaceae (Figure 1), distinct from all other genera recently defined by De Beer et al. (2014) and Mayers et al. (2015). The lineage clearly represents an undescribed, at present monotypic genus in the *Ceratocystidaceae*, described as follows:

***Bretziella* Z.W.deBeer, Marinc., T.A.Duong & M.J.Wingf., gen. nov.**
MycoBank MB822520

Etymology. Named after Theodore W. Bretz who first discovered and described the sexual state of the type species of this genus (Bretz 1951, 1952).

Diagnosis. The genus is distinguished from all other genera of the *Ceratocystidaceae* based on the mycelial mats that it forms on infected oak trees. These mats form pressure cushions or pads that push the bark away from the underlying sapwood. This causes cracks in the bark, exposing the mats to fungal-feeding arthropod vectors, primarily nitidulid beetles.

Type species. *Bretziella fagacearum* (Bretz) Z.W.deBeer, Marinc., T.A.Duong & M.J.Wingf.

Description. *Ascomatal bases* black, globose, with undifferentiated ornamental hyphae, often embedded in mycelial mat. *Ascomatal necks* elongated, black at base, lighter at apex. *Ostiolar hyphae* present. *Asci* dehiscent. *Ascospores* one-celled, hyaline, ellipsoidal, occasionally curved, embedded in hyaline sheath. *Conidiophores* arise laterally from vegetative hyphae, occasionally branched. *Conidiogenous cells* phialidic, cylindrical, pale to dark brown. *Conidia* unicellular, cylindrical with flattened ends, hyaline, borne in chains of varying length. *Aleurioconidia* not present.

Ecology and distribution. The only known species in the genus causes vascular wilt on various oak species in North America.

***Bretziella fagacearum* (Bretz) Z.W.deBeer, Marinc., T.A.Duong & M.J.Wingf., comb. nov.**
MycoBank MB822521
Figures 2, 3

Bas.: *Endoconidiophora fagacearum* Bretz, Phytopathology 42: 436. 1952; *Ceratocystis fagacearum* (Bretz) Hunt, Lloydia 19: 21. 1956.

TYPES: USA. Dry culture resulting from a cross between two isolates, locations unknown, from *Quercus* sp., 26 Feb 1952, T.Bretz (Lectotype designated here: FP 97476, MycoBank typification number: MBT 378423). USA. Iowa, on *Quercus rubra*, 1991, S.Seegmueller (Epitype designated here: BPI 893238, MycoBank typification number: MBT 378424; ex-epitype culture CBS 138363 = CMW 2656). Representative sequences from epitype: 60S = KM495518, LSU = KM495341, MCM7 = KM495430, ITS = KU042044, TEF1α = KU042043. See Notes 1, 2 and 3 below.

= *Chalara quercina* Henry, Phytopathology 34: 633. 1944; *Thielaviopsis quercina* (Henry) A.E.Paulin, T.C.Harr. & McNew, Mycologia 94: 70. 2002.
TYPE: USA. Dry culture, Wisconsin, Madison, on *Quercus* sp., Sept. 1943, B.Henry (Lectotype designated here: BPI 595712, MycoBank typification number: MBT 378425). See Note 4 below.

Descriptions. Henry (1944, pp. 631–635, Figure 1); Bretz (1951, p. 298, Figure 1); Bretz (1952, p. 436–437, Figure 1); Stessel and Zuckerman (1953, pp. 65–67, Figure 1); Hunt (1956, p. 21); Nag Raj and Kendrick (1975, pp. 94, 131, figure 32A); Upadhyay (1981, p. 66).

Note 1. Based on the one fungus one name principles adopted in the Melbourne Code (Hawksworth 2011, McNeill et al. 2012), the older basionym of the oak wilt pathogen, *Chalara quercina* (Henry 1944), has nomenclatural priority over *Endoconidiophora fagacearum,* the name Bretz (1952) assigned to the sexual state of the fungus. However, since Hunt (1956) treated the fungus as *Ceratocystis fagacearum*, the latter name were given preference under the dual nomenclature system in all major taxonomic works on the genus to date (Griffin 1968, De Hoog 1974, Nag Raj and Kendrick 1975, Upadhyay 1981, Seifert et al. 1993, Paulin-Mahady et al. 2002, Harrington 2009, De Beer et al. 2013b, 2014, Wingfield et al. 2013, Mayers et al. 2015). During the course of the past approximately 60 years, the name *Ceratocystis fagacearum* has also been adopted by plant pathologists and mycologists working on all aspects of the important disease known as oak wilt and the biology of the fungus (e.g. Shigo 1958, Cobb et al. 1965, Peplinksi and Merrill 1974, Gibbs and French 1980, Juzwik and French 1983, Appel et al. 1990, Kile 1993, Gibbs 2003, Juzwik et al. 2008, 2011). A search on 26 August 2017 for *C. fagacearum* in Google Scholar and Google respectively yielded 1940 and 119000 hits, while the name *Ch. quercina* yielded only 431 and 3330 hits respectively. This provides strong evidence that *C. fagacearum* is the more 'widely used' name (see Hawksworth 2012).

In the present study, we have shown that the oak wilt fungus does not belong in *Ceratocystis s. str.*, *Endoconidiophora*, *Thielaviopsis* or any of the other genera currently accepted in the Ceratocystidaceae (De Beer et al. 2014, Mayers et al. 2015). We have consequently suggested that it is treated in a novel genus for which we have provided the name *Bretziella*. Based on the widespread use of the name *C. fagacearum*, we submitted a formal proposal that its basionym, *Endoconidiophora fagacearum*, is conserved against *Chalara quercina* (= *Thielaviopsis quercina*), to enable the new combination, *Bretziella fagacearum*, proposed above.

Note 2. In the protologue of *E. fagacearum*, Bretz (1952) specified the location of the holotype as 'Type, For. Path. 97476, deposited in the Mycological Collections of the Bureau of Plant Industry, Soils and Agricultural Engineering'. In subsequent studies, the holotype specimen was referred to as 'BPI-FP 97476' (Hunt 1956, Nag Raj and Kendrick 1975, Upadhyay 1981). BPI has confirmed to us that this specimen had been lost. Fortunately, another specimen with the same number (FP 97476) as the one used in the protologue, was recently discovered in the Centre for Forest Mycology

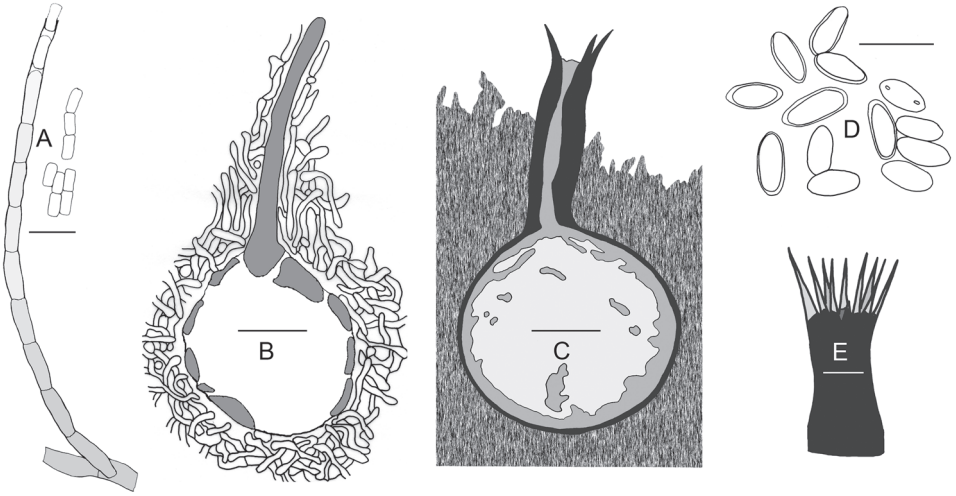

Figure 3. Line drawings of the oak wilt fungus. These illustrations are based on previously published line drawings and observations of the herbarium specimens (BPI 595712, FP 97476) in the present study. **A** Conidiophore and conidia in 10 % KOH (BPI 595712) **B** Ascomatal primordium re-drawn from Wilson (1956) **C** Median, histological section through ascoma embedded in the mycelial mat, re-drawn from Bretz (1952) **D** Ascospores in 10 % KOH (FP 97476) **E** Ostiolar hyphae (FP 97476). Scale bars: **A, D** = 10 μm, **E** = 50 μm, **B, C** = 100 μm.

Research Herbarium USDA-FS-NRS (FP) and was made available for this study. This specimen included a note by T. Bretz dated 26 Feb. 1952, marked as 'type'. It is thus clear that this specimen represents an isotype of *E. fagacearum*. Based on Art. 9.12 (McNeill et al. 2012), we designate FP 97476 as lectotype for *E. fagacearum*.

Note 3. The lectotypes designated here for *Ch. quercina* and *E. fagacearum* both consist of dried specimens for which DNA sequence data are not available. However, based on careful microscopic comparisons between these two specimens and a living isolate from Iowa (Figure 2), we have concluded that the specimens and isolate all represent the same species. Although Bretz (1951, 1952) did not specify the host and location of the (now) lectotype of *E. fagacearum*, he stated that ascomata were obtained from multiple crosses between isolates from several *Quercus* spp. and Chinese chestnut (*Castanea mollissima*) occurring in Missouri, Arkansas, Ohio, Michigan, Pennsylvania, West Virginia, Kentucky, Tennessee, North Carolina, and Virginia. The specimen of Henry (1944) came from an unnamed *Quercus* sp. in Wisconsin, but he also included isolates from several *Quercus* spp. in Illinois, Iowa, and Minnesota in his study. Thus, although our living isolates do not come from the same host species and location as the lectotypes, they originate from the same host genus and geographical area (Midwest and Eastern States) from where isolates have been included in the studies of Henry (1944) and Bretz (1951, 1952). Based on the morphology, host, and origin, we have designated a dried culture of one of our isolates as epitype for *E. fagacearum* to enable the inclusion of the oak wilt fungus in DNA based studies.

Note 4. Henry (1944) lodged the original specimens of *Chalara quercina* in two collections but did not designate either as the holotype. One of these specimens (BPI 595712 = FP 94260) was included in the present study and is designated here as lectotype.

Discussion

The oak wilt fungus is an economically important pathogen in the USA, with the potential to become a serious, alien invasive if it was ever introduced into other countries having oak forests. It is listed as a quarantine organism by the European and Mediterranean Plant Protection Organization (EPPO) and the European Union (EU) (http://www.q-bank.eu/). Making a change to the name of a species having this level of importance must clearly be done responsibly and with care (Crous et al. 2015). Once the Ceratocystidaceae had been revised by De Beer et al. (2014) it became inevitable that *C. fagacearum* would require taxonomic revision, but it was felt that additional data were required to support a name change. In this study, we have shown, based on robust phylogenetic data, that the oak wilt fungus clearly requires a new genus in the Ceratocystidaceae, distinct from all four of the genera (*Ceratocystis*, *Endoconidiophora*, *Chalara* and *Thielaviopsis*) in which it has previously been treated. The alternative of retaining this important pathogen in *Ceratocystis* would be confusing to plant pathologists (Wingfield et al. 2012), phylogenetically incorrect and inconsistent with its unique biology.

In addition to phylogenetic data, the unusual biology of the oak wilt fungus supports the description of the new genus, *Bretziella*, to accommodate this species. After infection of healthy trees through wounds or root grafts, the fungus forms pressure pads under the bark that lead to cracks in the bark, exposing mats of mycelium and fruiting structures, attractive to fungus-feeding arthropods such as nitidulid beetles that then act as vectors of the fungus (Juzwik and French 1983, Harrington 2009, Juzwik et al. 2011). These insects move to fresh wounds on trees perpetuating the infection cycle. There are no other species in the Ceratocystidaceae that share this unique biology.

The choice of an epithet for the new species name in *Bretziella* was problematic. If we were to follow the Melbourne Code strictly, the unknown basionym of the asexual morph, *Ch. quercina*, would have priority over *E. fagacearum*, the basionym for *C. fagacearum* and the name that has been widely used. A formal proposal has thus been submitted to conserve the better known basionym against one that would be unfamiliar to most plant pathologists and mycologists. In this way, it is possible to ensure that even though the species has to be treated in a new genus, the epithet will remain familiar to those working with the fungus.

Subsequent to careful morphological comparisons, two lectotypes and an epitype have bene designated for the two basionyms, *Chalara quercina* and *Endoconidium fagacearum*. These procedures ensure that the basionyms are now permanently linked to specimens. Sequences obtained from the epitype have been deposited in the RefSeq Targeted Loci (RTL) database in NCBI GenBank to enable accurate and reliable

identifications when BLAST searches are conducted (Schoch et al. 2014). In addition, a draft genome sequence for the ex-epitype culture has already been generated and is publicly available (Wingfield et al. 2016). The typifications together with the formal proposal will serve to stabilize the nomenclature of the oak wilt fungus. It is also hoped that they will prevent a need for further name changes for *B. fagacearum* in the future.

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RESEARCH ARTICLE

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Monocillium gamsii sp. nov. and *Monocillium bulbillosum*: two nematode-associated fungi parasitising the eggs of *Heterodera filipjevi*

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Abstract

Monocillium gamsii **sp. nov.** (Ascomycota, Hypocreales, Niessliaceae) isolated from eggs of the cereal cyst nematode *Heterodera filipjevi* is described and illustrated based on morphological and molecular phylogenetic evidence. The new taxon discovered in wheat fields in Turkey destructively parasitises nematode eggs. The infected eggs were readily colonised by the fungus, which produced microsclerotia. The fungus could be grown on artificial media and the parasitism of *M. gamsii* towards *H. filipjevi* was reproducible in vitro. Hyphae penetrating the nematode eggs entirely colonised the embryo, developed into multicellular chlamydospore and dictyochlamydospore-like structures eventually forming microsclerotia. Molecular and morphological differences and similarities between *M. gamsii* and its phylogenetically related species are discussed. *Monocillium bulbillosum* was found to be closely related to the new species. The pathogenicity of *M. bulbillosum* against *H. filipjevi* was also assayed in vitro because of its sister group relationship to *M. gamsii* revealing that this species was also capable of colonising eggs of *H. filipjevi*.

Keywords

Egg-parasitic fungi, Niessliaceae, new species, plant parasitic nematodes, taxonomy, molecular phylogeny, ITS, LSU, *rpb1*, *tef*

Introduction

Various fungi have been reported as natural enemies of plant parasitic nematodes (PPN) (Nordbring-Hertz et al. 2011; Siddiqui and Mahmood 1996; Stirling 2014). A group of these fungi infect females and egg contents of endoparasitic nematodes such as cyst nematodes (Kerry 1988; Rodríguez-Kábana and Morgan-Jones 1988), which are biotrophic plant pathogens establishing a long-term parasitic interaction with their host plants. The unique sedentary life style of this group of PPN render them especially vulnerable of being colonised by their natural enemies (Lopez-Llorca et al. 2008). Cyst nematodes are globally distributed and were the first group of PPN reported to be parasitised by fungi (Kühn 1877), which spurred investigations to find additional nematode-antagonistic fungi ever since [(Tribe 1977) and references therein]. Most egg-parasitic fungi belong to the ascomyceteous Hypocreales, e.g. *Pochonia chlamydosporia* (Goddard) Zare & W. Gams, *Metapochonia rubescens* (Zare, W. Gams & López-Llorca) Kepler, S.A. Rehner & Humber, *Lecanicillium lecanii* (Zimm.) Zare & W. Gams, *Metarhizium* spp., *Purpureocillium lilacinum* (Thom) Luangsa-ard, Houbraken, Hywel-Jones & Samson, and *Trichoderma* spp. (Kerry and Hirsch 2011; Khan et al. 2006; Szabó et al. 2012; Zhang et al. 2014). In contrast, none of the second important group of nematode-antagonistic Ascomycota, the Orbiliomycetes (Baral et al. 2017) has been reported to parasitise nematode cysts and eggs.

Grant and Elliott (1984) reported *Monocillium* sp. parasitising the cysts of the soybean cyst nematode *Heterodera glycines*. This is so far the only report on *Monocillium* antagonising a plant parasitic nematode. The genus *Monocillium* Saksena, 1955 was emended and placed in the Niessliaceae by Gams (1971), and *Monocillium* spp. were regarded as the asexual morphs of the hypocrealean genus *Niesslia* Auersw., 1869. However, the types of both genera have not yet been connected conclusively by elucidation of the life cycle or by molecular data, hence we hesitate to regard these genera as synonymous and treat them as separate taxonomic entities for the time being. The genus *Monocillium* currently comprises eighteen species (http://www.mycobank.org/quicksearch.aspx) and is defined by showing acremonium-like morphology, but is characterised by unbranched conidiophores with phialides having thickened walls in the lower part. The known species were isolated from soil, plant materials such as dead leaves and wood, but also from other fungi, and building material (such as wall paper). Among all *Monocillium* species described so far (Barron 1961; Gams 1971; Gams and Turhan 1996; Girlanda and Luppi-Mosca 1997; Ramaley 2001) *M. curvisetosum* W. Gams & Turhan is the only species which was originally isolated from aphids as an unusual host for this genus. However its potential parasitic association with its host has not yet been reported.

Egg-parasitic fungi attacking cyst nematodes have repeatedly been isolated from all agricultural soils in various geographic regions (Chen and Chen 2002; Dababat et al. 2015).

Experimental wheat fields of the International Maize and Wheat Improvement Centre (CIMMYT) in Turkey, where a significant reduction in population size of the cereal cyst nematode *Heterodera filipjevi* had been observed between two consecutive years (unpublished data), were sampled to isolate and study fungal candidates that could be causally involved in this drop of the nematode population size.

Here we report a so-far undescribed hypocrealean species which destructively parasitised the eggs of *H. filipjevi*. The antagonistic interaction of this fungus with the nematode eggs was studied based on in vitro tests. We also report the antagonistic potential of *M. bulbillosum* as the most closely related species to the herein described fungus, towards the eggs of *H. filipjevi*.

Materials and methods

Sample collection and materials examined

Cysts of *H. filipjevi* were collected from experimental wheat fields of CIMMYT in the Central Anatolian Plateau of Turkey in 2013. The fields located in Yozgat (39°08'N, 34°10'E; altitude 985 m.a.s.l) and Haymana (39°26'N, 39°29'E, altitude 1260 m.a.s.l) were naturally nematode infested. The samples including soil and roots were collected at random from the rhizosphere of wheat plants at the end of the growing season. Cysts were extracted from the collected samples using the modified flotation decanting method (Coyne et al. 2007). From the extracted suspensions, cysts were manually collected under a dissecting microscope and stored in 1.5 ml microtubes at 4 °C either in dry condition or in sterile tap water until further use. For taxonomic and phylogenetic inferences, additional fungal strains were obtained from the Westerdijk Fungal Biodiversity Institute (formerly CBS-KNAW, Utrecht, Netherlands).

Cultural studies

Fungal isolation from eggs of *Heterodera filipjevi*

The field-collected cysts of *H. filipjevi* were scrutinised by using a dissecting microscope to separate symptomatic cysts showing defined discolourations or bearing discernible hyphae, from healthy-looking (i.e. homogeneously brown) or empty cysts. Symptomatic cysts were selected, surface-sterilised in 5% sodium hypochlorite (NaOCl), and dissected to collect their egg contents. Only the nematode eggs showing symptoms of fungal infection were processed for fungal isolation and culture-dependent species identification. A portion of the fungal infected eggs were additionally used for culture-independent identification. The methods applied here, have been described in greater detail in Ashrafi et al. (2017).

Growth rate studies

Growth rates were determined at various temperatures from 15 to 35 °C at 5 °C intervals in the dark or in ambient conditions by placing agar disks (5 mm diam.), excised from the margin of a young potato dextrose agar (PDA) culture onto five replicate plates of PDA, cornmeal agar (CMA), oatmeal agar (OA; 30 g oatmeal, 18 g agar-agar, 1L deionised water), synthetic nutrient-poor agar (SNA; Nirenberg (1976)), and malt extract agar (MEA). The colony diameter was measured weekly for a 3 week period. Colour changes of fungal structures formed in culture were checked using 3% potassium hydroxide (KOH) watery solution.

Pathogenicity tests against *H. filipjevi*

The antagonistic potential of the below described species and *M. bulbillosum*, respectively, was assessed towards *H. filipjevi* in vitro as previously described (Ashrafi et al. 2017). Briefly, healthy cysts and eggs were surface-sterilised and placed either on or at the margin of the growing mycelium of one-month-old PDA or 2% water agar (WA) cultures of the two fungal species. To document the process of colonisation of eggs of *H. filipjevi* by the new fungal species, a slide culture technique was also performed using PDA 1/3 strength (compare Ashrafi et al. (2017)).

Microscopy

Nematode eggs and fungal structures were examined and photographed by a Zeiss Axioskop 2 plus compound microscope and an Olympus SZX 12 stereo microscope equipped with a Jenoptik ProgRes® digital camera. Images were recorded using CapturePro 2.8 software (Jenoptic, Jena, Germany). Nematode eggs colonised by fungi, and fungal structures were mounted in water or lactic acid and photographed. Cysts were photographed in water in a square cavity dish (40×40×16 mm). To illustrate different stages of fungal development and fungal colonisation of nematode eggs, slide cultures were prepared (Gams et al. 1998) and then photographed. Nomarski Differential Interference Contrast (DIC) optic was used for observation and measurements. All measurements were taken in water, and are given as x1–x2 (x3 ± SD), with x1 = minimum value observed, x2 = maximum value observed, x3 = average, and standard deviation (SD), followed by the number of measurements (n).

Scanning electron microscopy was performed on a Quanta 250 scanning electron microscope (FEI Deutschland GmbH, Frankfurt, Germany). Fungal structures of interest were obtained from a one-month-old OA culture grown at 23 °C in the dark and directly analysed using environmental scanning electron microscopy (ESEM). For the experiment, pressures between 410 and 490 Pa at 4 °C were employed. For cooling the sample chamber was equipped with a Peltier stage. Fungal mycelia with abundant

conidia were placed on non-conductive double-sided adhesive discs on a flat specimen stub and positioned on the Peltier stage for cooling. Images were taken at acceleration voltage of 12.5 kV. Scanning speed was 60 µsec. For imaging of beam sensitive fungal structures, the scanning modus was changed to 3 µsec with 20-fold line integration. Images were adjusted in brightness and contrast using Adobe Photoshop software CS 5.1.

Molecular phylogenetic studies

DNA extraction, PCR amplification and DNA sequencing

Fungal genomic DNA was isolated from mycelia grown on PDA using a modified CTAB method, and from individual nematode eggs infected by fungi using the Qiagen DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) as reported in Ashrafi et al. (2017).

For each specimen, four nuclear loci were amplified: The internal transcribed spacers including the 5.8S rDNA gene (ITS) using the primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990); the 5' end of the ribosomal large subunit (LSU) DNA with the primers LROR (Rehner and Samuels 1994) and LR5 (Vilgalys and Hester 1990); partial RNA polymerase II largest subunit 1 (*rpb1*) using the primers cRPB1af and RPB1cr (Castlebury et al. 2004); and partial translation-elongation factor 1-α (TEF) using the primers EF1-983f and EF1-2218r (Castlebury et al. 2004). All PCR reactions were performed as described previously (Ashrafi et al. 2017) with the following thermal programmes: 95 °C (2 min) for initial denaturation followed by 40 cycles of denaturation at 95 °C (30 s), annealing at 52 °C (ITS), 51 °C (LSU), 54 °C (*rpb1*), and 60 °C (TEF) (40 s), extension at 72 °C (1 min for ITS, LSU and *rpb1*, and 1 min and 20 sec for TEF), and a final extension at 72 °C (10 min). Amplicons were purified using the DNA Clean & Concentrator™-5 kit (Zymo Research Corp., Irvine, California, USA) and sequenced by Eurofins Genomics GmbH, (Ebersberg, Germany) with the same primers as used for PCR amplification. Obtained sequences were assembled, edited and trimmed with Sequencher 5.4.1 (Gene Codes Corporation, Ann Arbor, Michigan, USA) and deposited in GenBank under the following accession numbers: MF681481–MF681514. The sequences generated were compared to sequences available in GenBank using a BLASTn search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al. 1990).

DNA sequence alignment and phylogenetic inference

The newly generated sequences together with closely related sequences selected as revealed by BLASTn searches were used for phylogenetic analyses (Table 1). The sequences were aligned using the online version of Mafft v.7 (Katoh and Standley 2013). All sequences were aligned using the iterative refinement methods: Sequences of the *rpb1* and TEF gene regions were aligned using the algorithms implemented in L–INS–i, while LSU and ITS were aligned applying the Q–INS–i algorithm. Only

Table 1. Isolates and accession numbers used in the phylogenetic analyses.

Species	Isolate number	Host / substrate	Locality	GenBank accession numbers				Reference
				ITS	LSU	*rpb1*	*tef*	
Bionectria byssicola	CBS 914.97–GML2665	*Alchornea* branches- leaf litter	Uganda, Brazil	AF358252	GQ506011	GQ506040	KX184977	(Hirooka et al. 2010; Moreira et al. 2016; Schroers 2001)
Hyaloseta nolinae	CBS109837	*Nolina micrantha*, leaf litter	USA, New Mexico	KM231846	KM231726	KM232279	–	(Lombard et al. 2015)
Ijuhya vitellina	DSM104494	*Heterodera filipjevi*, egg	Turkey	KY607535	KY607549	KY607576	–	(Ashrafi et al. 2017)
Monocillium bulbillosum	CBS344.70	mouldy wallpaper	Germany	MF681488	MF681501	MF681513	MF681507	This study
Monocillium gamsii	DSM105458	*Heterodera filipjevi*, egg	Turkey	MF681485	MF681496	MF681512	MF681506	This study
Monocillium gamsii	DSM105459	*Heterodera filipjevi*, egg	Turkey	MF681483	MF681493	MF681511	MF681505	This study
Monocillium gamsii	DSM105460	*Heterodera filipjevi*, egg	Turkey	MF681482	MF681492	MF681510	MF681504	This study
Monocillium gamsii	DSM105461	*Heterodera filipjevi*, egg	Turkey	MF681481	MF681490	MF681509	MF681503	This study
Monocillium ligusticum	CBS684.95	ectomycorrhizae of *Pinus halapensis*	Italy	MF681489	MF681502	MF681514	MF681508	This study
Nisslia exilis	CBS357.70	*Picea abies*, bark	Germany	–	AY489718	AY489645	AY489613	(Castlebury et al. 2004)
Nisslia exilis	CBS560.74	*Pinus sylvestris*, decayed needle	England	–	AY489720	AY489647	AY489614	(Castlebury et al. 2004)

the start and end of the alignments were trimmed manually in Se-Al v2.0 (Rambaut 1996). The following phylogenetic analyses were applied: a Bayesian method of phylogenetic inference using Metropolis Coupled Monte Carlo Markov chains (MC^3) as implemented in the computer program MrBayes v3.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). We used MrModeltest v2.2 (Nylander 2004) to determine the best fitting DNA substitution model for the Bayesian approach. Both the hierarchical likelihood ratio test (hLRT) and the Akaike Information Criterion (AIC) selected the general time reversible model of DNA substitution with gamma distributed substitution rates and invariate sites (GTR+I+G) as the best fitting model for all individual data sets and was implemented for the analyses accordingly. For the Bayesian analyses four incrementally heated simultaneous Monte Carlo Markov chains were run with 2.000.000 generations using random starting trees and flat *prior* distributions. Trees were sampled every 500 generations resulting in a total of 4001 sampled trees. A 50% majority rule consensus tree was computed only from trees of the plateau, and if, additionally, the split frequencies were below 0.01. Thus, 501 trees were discarded as "burnin". Maximum likelihood (ML) analyses were performed using RAxML 7.2.8 (Silvestro and Michalak 2012; Stamatakis 2014) implemented in Geneious 8.1.2 applying the general time-reversible (GTR) substitution model with gamma model of rate heterogeneity and 1000 replicates of rapid bootstrapping. Neighbor-joining (NJ) analyses (Saitou and Nei 1987) was

done in PAUP 4.0b10 in the batch file mode (Swofford 2002) applying the Kimura two-parameter model of DNA substitution (Kimura 1980) with a transition/transversion ratio of 2.0 to compute genetic distances. Support for internal nodes was estimated by 1000 bootstrap replicates (Felsenstein 1985). Two members of Bionectriaceae, *Bionectria byssicola* (Berk. & Broome) Schroers & Samuels and *Ijuhya vitellina* Ashrafi, W. Maier & Schroers, were selected as outgroup to root the trees. The phylogenetic trees were visualised using FigTree v. 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree).

Results

Sample collection and fungal isolation

Among the field-collected samples, a high proportion of cysts was found containing blackish bodies resembling microsclerotia-like structures upon microscopy (Fig. 1A). By dissecting the infected cysts, microsclerotia-like black bodies were found to be colonising the individual nematode eggs (Fig. 1B, C). In some infected eggs the developing juveniles were found to be entirely destroyed exhibiting an olivaceous brownish appearance (Fig. 1D, E). Eggs were colonised by one or occasionally two microsclerotia. When cultured on PDA, hyphae grew out of the microsclerotia of the infected eggs (Fig. 1F), and formed colonies at first white creamy, later becoming blackish dotted centrally with a general dark appearance due to the dense pigmentation (Fig. 1G).

Sequence comparison and phylogenetic inference

The DNA sequences of four different gene regions obtained from the examined specimens of the here described nematode-parasitic fungus were either identical (in TEF and RPB1), or nearly identical (1 base pair (bp) substitution in LSU, and up to 2 bp substitutions in ITS). The most similar DNA sequences found in GenBank using BLASTn searches belonged to *Hyaloseta nolinae*, the sexual morph of *Monocillium nolinae*, and shared similarities of 96% in the ITS region, 99% in the LSU, and 89% in *rpb1*. A similar BLASTn search in MycoBank showed identities of the ITS sequence of 96.6% with *M. bulbillosum*, 93.9% with *H. nolinae* and 92.7% with *Niesslia exosporioides*, and of the LSU sequence of 99.5% with *H. nolinae*, and 99% with both *M. bulbillosum* and *Niesslia exosporioides* suggesting a close relationship with the representatives of the Niessliaceae. Fungal DNA could also be directly isolated and sequenced from individual eggs displaying the typical symptoms of fungal infection. These DNA sequences were identical to the sequences retrieved from pure cultures supporting the conspecificity of the symptom-causing structures within the egg with the isolated pure cultures derived from the eggs.

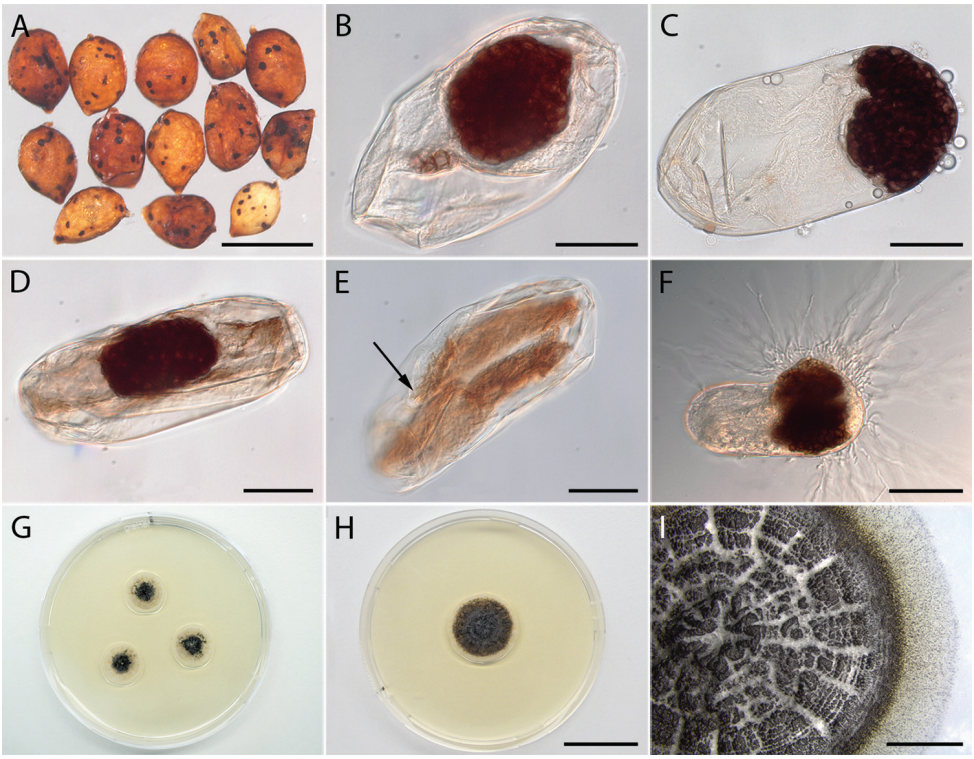

Figure 1. Naturally infested cysts and eggs of *Heterodera filipjevi* with *Monocillium gamsii*, and pure cultures obtained from infected eggs. **A** Field collected symptomatic cysts bearing parasitised eggs. **B–E** Nematode eggs infected by *M. gamsii* **B, D** Nematode eggs containing microsclerotia of *M. gamsii* **E** An embryonated egg containing a second stage juvenile (J2) parasitised by *M. gamsii* (arrow points at nematode's stylet) **F** A nematode egg containing microsclerotia, and hyphae growing out of it **G–H** colony of *M. gamsii* grown on PDA **G** colonies developing from three individually plated infected eggs **H** A 25-d-old culture grown at 25 °C in the dark **I** The surface of a five-month-old culture detailing the sclerotioid masses covering the colony surface. Single microsclerotia can be seen as little black dots at the margin of the culture. Scale bars: 800 µm (**A**); 30 µm (**B–E**); 50 µm (**F**); 2 cm (**H**); 5 mm (**I**).

The final combined ITS, LSU, *rpb1* and *tef* dataset comprised 11 strains representing 7 species with a total alignment length of 2949 bp (603 ITS, 797 LSU, 649 *rpb1*, 900 *tef*). The topologies of the phylogenetic trees were identical using Bayesian inference (Fig. 2), neighbor-joining or maximum likelihood (not shown). The four sequenced strains of the here described nematode egg-colonising fungus were recovered as a highly supported monophyletic group with a close sister group relationship to *M. bulbillosum* and with *H. nolinae* as the next-closest relative. In the second monophyletic clade of Niessliaceae, two strains of the type species of *Niesslia*, *N. exilis*, proved to be paraphyletic with respect to *M. ligusticum* (Fig. 2).

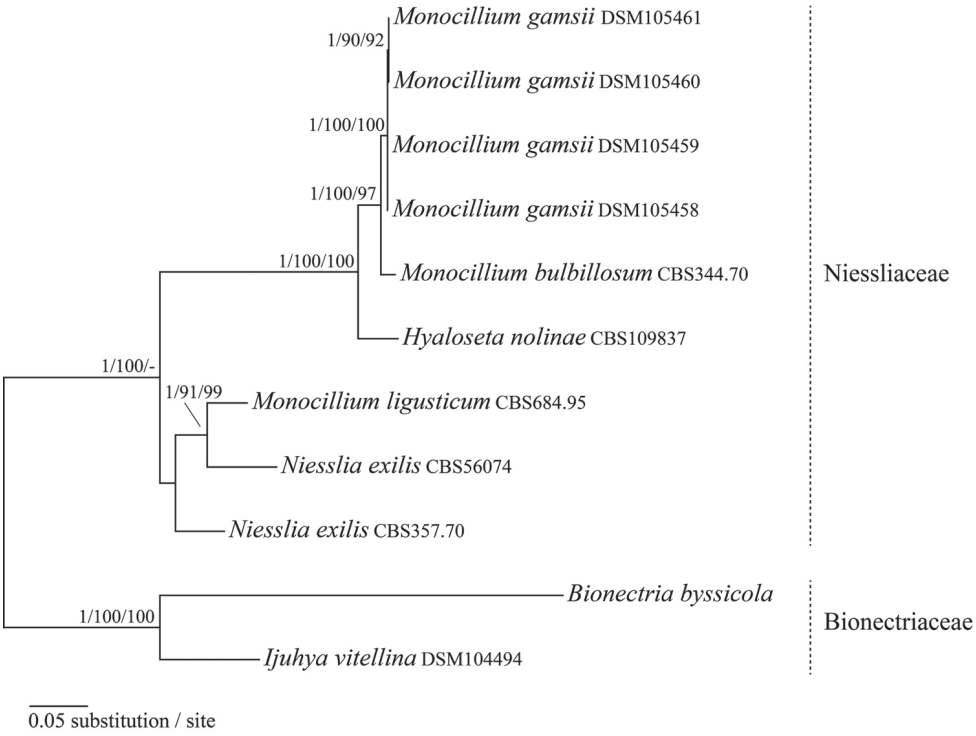

Figure 2. Bayesian inference of phylogenetic relationships using four strains of the here described nematode parasite and all Niessliaceae present in GenBank based on an alignment of ITS, LSU, *rpb1*, and *tef* sequences using GTR+I+G as nucleotide substitution model. Depicted is a 50% majority rule consensus tree derived from 3500 trees from the stationary phase of a Monte Carlo Markov Chain. *A posteriori* probability (BIpp) values greater than 0.95, and bootstrap values of neighbor-joining (NJBT) and maximum likelihood (MLBT) analyses greater than 0.7 are given above branches (BIpp/NJBT/MLBT). Two representatives of the Bionectriaceae, *Bionectria byssicola* and *Ijuhya vitellina*, were used to root the tree.

Taxonomy

***Monocillium gamsii* Ashrafi & W. Maier, sp. nov.**
MycoBank No: MB 823248
Figs 1H, I, 3

Holotype. Turkey, Yozgat, experimental wheat field: dried culture on PDA, originating from an individual egg from a cyst of *Heterodera filipjevi*, isolated by Samad Ashrafi, August 2013, dried culture on PDA, deposited at the herbarium of the Botanic Garden and Botanical Museum Berlin-Dahlem: B700016491.

Ex-holotype strain: DSM 105458, deposited in the open collection of the Leibniz-Institut DSMZ Deutsche Sammlung von Mikroorganismen und Zellkulturen

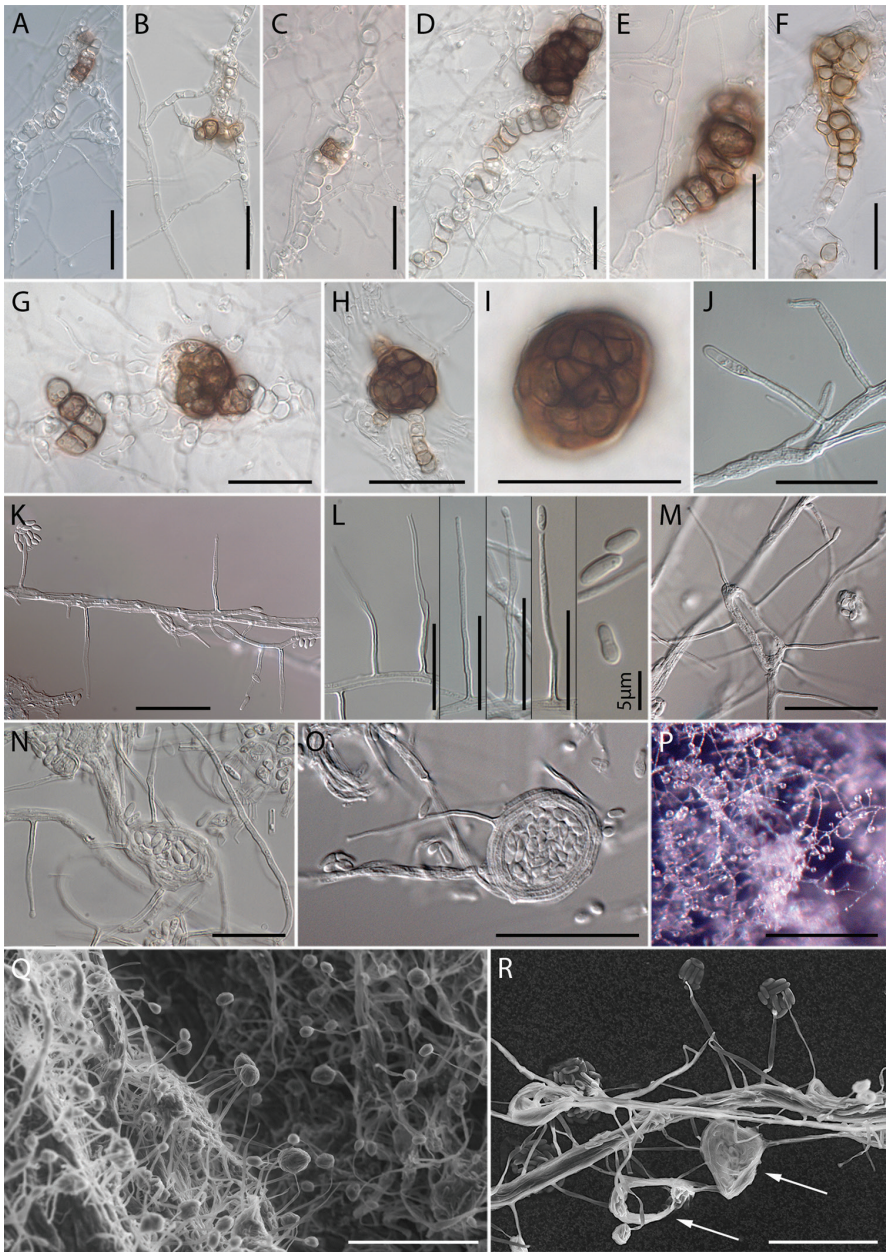

Figure 3. Micrographs of *Monocillium gamsii*. **A–C** Hyphal growth by intercalary development of chlamydospore and dityochlamydospore-like structures filled with guttules **D–H** initiation of microsclerotia by interweaving or coiling of dictyochlamydospores, and growth to full size **I** Highly pigmented sclerotium at maturity displaying a *textura angularis* on surface view **J–N** Setae, phialides and conidia **M–O** Formation of phialides on coiling hyphae **P** Conidial heads, conidia cohering in wet heads **Q, R** SEM: **Q** Phialides from mycelium with conidial heads, arising from hyphae **R** coiling hyphae (arrows), and detail of phialides bearing conidia **A–I** from PDA 1/3 strength **J–P** from PDA; **Q, R** from OA. Scale bars: 30 µm (**A, H, I, K, M, O, R**); 20 µm (**B–G, J, L, N**); 200 µm (**P**), 50 µm (**Q**).

GmbH, GenBank accession numbers: ITS: MF681485; LSU: MF681496; *rpb1*: MF681512; *tef*: MF681506.

Additional material examined. From the same location: DSM 105459 (dried culture on PDA, B700016492), GenBank accession number: MF681483 (ITS), MF681493 (LSU), MF681511 (*rpb1*), MF681505 (*tef*); DSM 105460 (dried culture on PDA, B700016493), GenBank accession number: MF681482 (ITS), MF681492 (LSU), MF681510 (*rpb1*), MF681504 (*tef*); DSM 105461, GenBank accession number: MF681481 (ITS), MF681490 (LSU), MF681509 (*rpb1*), MF681503 (*tef*); and CBS 141176.

Etymology. In honour and memory of Prof Walter Gams for his outstanding works on the genera *Monocillium* and *Niesslia*.

Diagnosis. Naturally occurring infected eggs often accommodating one subglobose, strongly pigmented, dark brownish microsclerotium.

Description. Colonies slow-growing, at 20 °C on PDA reaching 10–12 mm diam. (7d), 19–22 mm (14 d), 25–32 (21 d); optimum temperature for growth 25 °C, 14–16 mm (7 d), 22–25 mm (14 d), 31–34 mm (21d); at 30 °C 10–11 mm (7d), 15–17 (14 d), 22–25 mm (21 d), no growth observed at 35 °C; optimum temperature for growth in other examined cultural media 25 °C, after 21 d reaching 31–32 mm diam. (CMA), 36–40 mm (MEA), 40–50 mm (OA), 32–40 mm (SNA); colonies on PDA finely wrinkled, slightly elevated centrally, first pale creamy, later centrally becoming dotted, greyish-brown to fuscous black due to formation of darkly pigmented microsclerotia, margins and reverse pale creamy. Vegetative hyphae hyaline, thin-walled, forming strands or coils, often with dictyochlamydospore-like structures, occasionally bearing setae with elongate, ellipsoid tips, variable in size. Chlamydospores or dictyochlamydospores mostly developing intercalary, filled with small guttules, gradually pigmented, turning brownish firstly at cell walls, interweaving to form microsclerotia. Cells of microsclerotia angular, pigmented, first pale olivaceous brown filled with guttules, later dark brown, forming a *textura angularis* in surface view. Guttules often absent in mature and strongly melanised sclerotial cells. Microsclerotia later covering the entire colony, developing sclerotioid masses, not changing colour in KOH. Phialides often separated from hyphae by a basal septum, thick-walled in the lower part, the wall thickening distinct at about 1/3 to 1/2 of the total length from the base, thin-walled from ca. midpoint extending to the tip, occasionally slightly inflated in the middle part, gradually tapering to the tip, 21–39 µm (28.7 ± 4.4) in length, 1.0–2.1 µm (1.4 ± 0.2) wide at the base (n = 90), solitary, arising directly from hyphae or hyphal rope, occasionally arising from hyphal coils surrounding several conidia. Conidiogenesis abundant, conidia hydrophilic, adhering in watery droplets, oblong, rarely clavate or ampulliform, one-celled, smooth-walled, 4.1–7.4 × 1.4–2.9 µm (4.9 ± 0.6 × 2.1 ± 0.3) (n = 250). Sexual morph not observed.

Development of *M. gamsii* in nematode eggs in vitro

Monocillium gamsii infected cysts and eggs of *H. filipjevi* in vitro. Initial indications of infection were observed in healthy nematode cysts placed on the fungal colonies

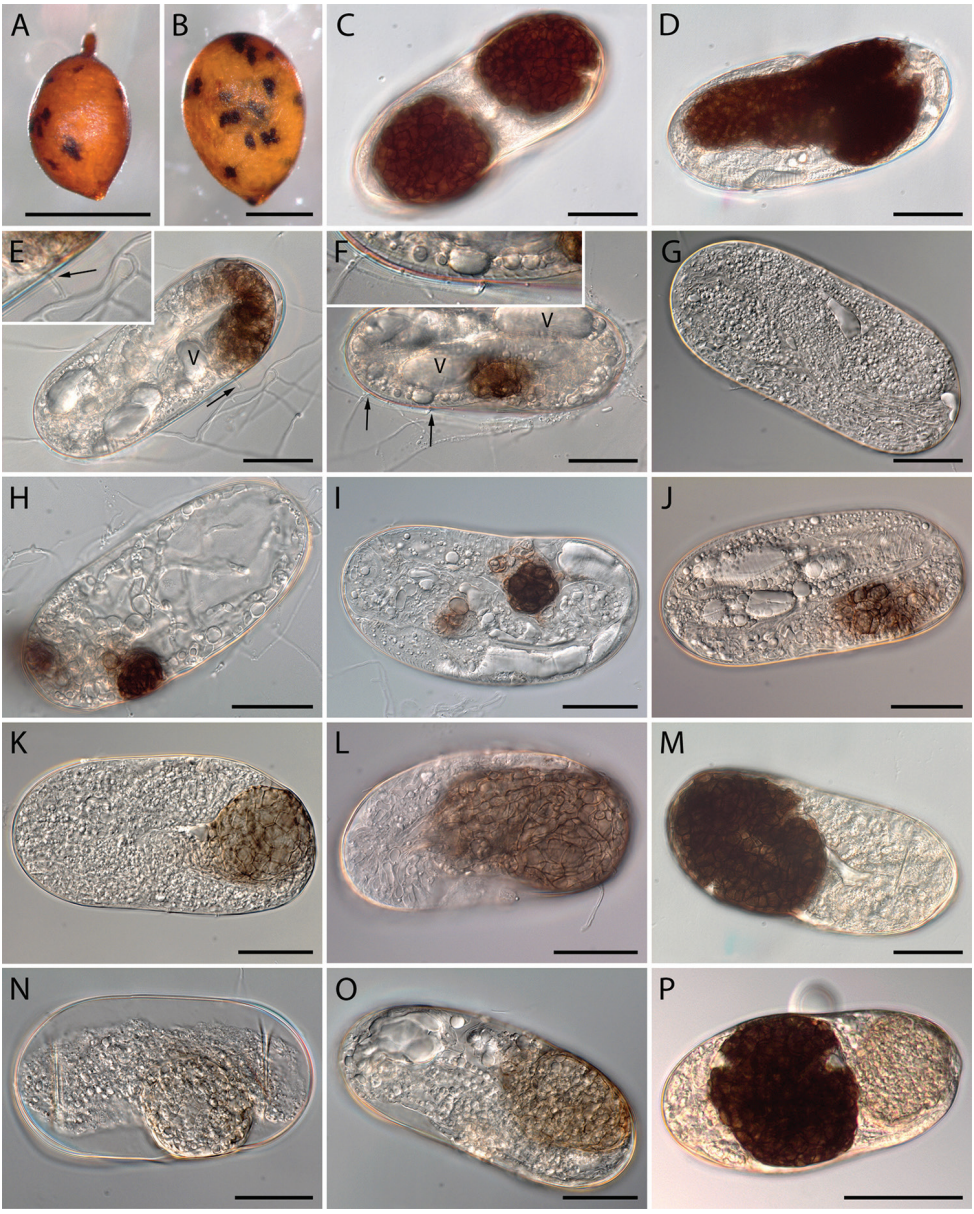

Figure 4. Cysts and eggs of *Heterodera filipjevi* infected by *Monocillium gamsii* exhibiting colonisation in vitro. **A, B** infected cysts rendered black-dotted due to fungal-colonised eggs containing microsclerotia **C, D** Eggs with mature sclerotia, extracted from symptomatic cysts **E, F** Individual hyphae penetrating eggshell (arrows indicate the individual hyphae; V indicates vacuole-like structures) **G–M** Fungal development inside the eggs **G, H** Earlier stages of infection in unembryonated eggs **I, J** Fungal development in the body cavity of developing juveniles where enlarged, thick-walled cells are formed and coalesce to initiate microsclerotia formation **K–M** Microsclerotia developing to full size and pigmentation **N–P** Pigmentation in microsclerotia from pale-olivaceous to darkly brown. Scale bars: 600 µm (**A**); 300 µm (**B**); 30 µm (**C–O**); 50 µm (**P**).

within 2–3 weeks (Fig. 4A, B). The fungus rendered the homogenously brown healthy looking cysts black-dotted, bearing a strong resemblance to the naturally infected cysts collected from fields. By dissecting the symptomatic cysts, nematode eggs were found to be colonised with darkly pigmented spherical to subglobous microsclerotia formed inside the body cavity of the developing juveniles (Fig. 4C). Similar to some naturally infected eggs, sclerotioid masses were also found in some artificially infected samples colonising almost the entire egg (Fig. 4D).

In the slide cultures, fungal infection of eggs was initiated by individual hyphae directly penetrating the eggshell and body cuticle of developing juveniles (Fig. 4E, F). Following penetration, filamentous hyphae entirely colonised the unembryonated eggs (Fig. 4G, H) or the body cavity of the developing juveniles (Fig. 4I, J), enlarged (Fig. 4H–J), occasionally inflated, forming thick-walled, finely pigmented, and guttules-filled cells (Fig. 3J), which eventually coalesced to form discrete microsclerotia with a *textura angularis* appearance (Fig. 4K–M). Infection studies revealed that such microsclerotia could be formed 7–10 d after the incubation of nematode eggs with the fungus.

Pigmentation of microsclerotia occurred during fungal development from hyaline to olivaceous brown and later strongly brownish melanised cells (Fig. 4N–P). Microsclerotia developing inside the artificially infected eggs displayed a *textura angularis* and were indistinguishable from those found in the naturally infected samples. At the early stages of development, microsclerotial cells were often filled with guttules (oil-like bodies), which were not observed in the mature microsclerotia. At the early stages of fungal infection (up to 10 d after inoculation), some vacuole-like structures were observed inside the eggs along the body cavity of developing juveniles (cf. Fig. 4E, F) with a glistening reflexive appearance, which were not observed at later stages of development, or in the field collected samples containing mature sclerotia.

Parasitism of *M. bulbillosum* towards *H. filipjevi*

The antagonistic potential of *M. bulbillosum* was also examined against *H. filipjevi* in vitro. Eggs of *H. filipjevi* were infected by *M. bulbillosum* in the course of 2–4 weeks. The infection symptoms were similar to the symptoms described for *M. gamsii*. *Monocillium bulbillosum* rendered cysts black dotted, containing eggs colonised with microsclerotia. In early stages of infection, eggs were entirely colonised with filamentous hyphae which later developed into microsclerotia with a *textura angularis* on the surface (Fig. 5A–F).

Discussion

The results obtained from comparative morphological characteristics and molecular phylogenetic inference using four gene regions, suggested *M. gamsii* as a new species. Within the genus *Monocillium*, only *M. bulbillosum*, *M. curvisetosum*, *M. indi-*

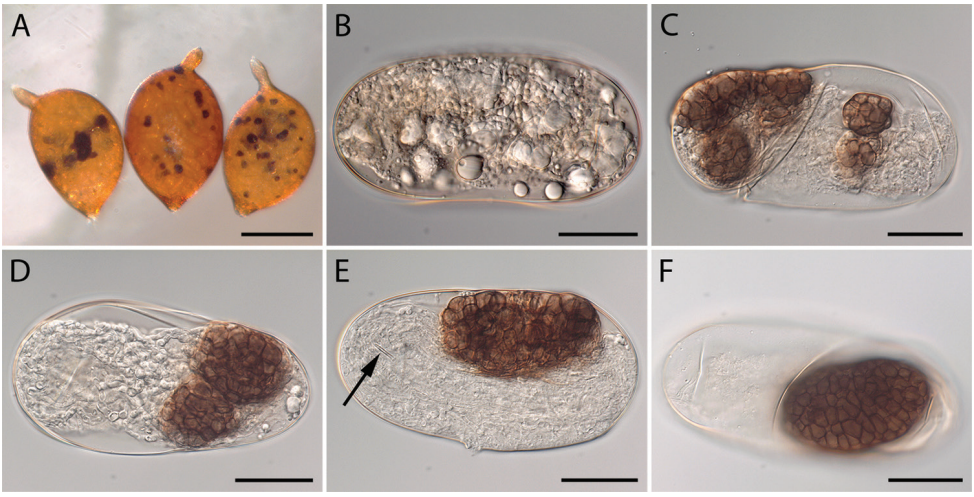

Figure 5. Cysts and eggs of *Heterodera filipjevi* infected by *Monocillium bulbillosum* in vitro. **A** Symptomatic cysts infected by *M. bulbillosum* **B** Infected egg showing early stage of fungal colonisation **C–F** Formation of microsclerotia in nematode eggs (arrow points at nematode's stylet). Scale bars: 300 µm (**A**); 30 µm (**B–F**).

cum and *M. ligusticum* have been reported to form (micro-) sclerotia in culture, as is also the case in *M. gamsii.* In the (micro-) sclerotia forming group, *M. gamsii* can be separated from *M. indicum* by producing conidia cohering in watery heads instead of dry conidia. *Monocillium curvisetosum* produces dry and globose conidia, while *M. gamsii* produces oblong and watery conidia. The new taxon differs from *M. ligusticum* by having much shorter phialides: 21–39 µm vs (35–) 40–70 (–140) µm (Girlanda and Luppi-Mosca 1997). The difference between these two species is also strongly supported by sequence comparison (Fig 2). According to phylogenetic inference, *M. bulbillosum* is very closely related to but separable from *M. gamsii*. Both *M. gamsii* and *M. bulbillosum* form microsclerotia in culture, however *M. bulbillosum* forms mainly bulbillose and individually distinct microsclerotia while these structures in *M. gamsii* are mostly confluent and non-separable. *Monocillium gamsii* grows slightly faster in comparative growth tests on PDA. In addition, *M. gamsii* forms setae, and its conidia are clearly longer than those of *M. bulbillosum* (4.1–7.4 × 1.4–2.9 µm vs 2.9–3.5 × 1.8–2.1 µm). They also differ clearly by the habitat they were originally isolated from. While *M. gamsii* was isolated from the eggs of nematodes in a semiarid region in the Central Anatolian plateau of Turkey, *M. bulbillosum* was isolated only once from wall paper in Kiel Germany (Gams 1971). Interestingly though, *M. bulbillosum* was also able to parasitise eggs of *H. filipjevi* in our in vitro assays and formed microsclerotia in the infected eggs in a similar manner as *M. gamsii*.

Hyaloseta nolinae (asexual morph: *Monocillium nolinae*) was included in this study according to a BLASTn search in GenBank, showing a high sequence similarity with the sequences of *M. gamsii* as query. In the phylogenetic analyses presented here it forms a highly supported monophyletic group with *M. gamsii* and *M. bulbillosum* (Fig. 2). In contrast to *M. gamsii*, *M. nolinae* (the asexual morph of *H. nolinae*) does not form

microsclerotia in culture. *Hyaloseta* Ramaley, 2001 was described as a monotypic genus from Asparagaceae (formerly Agavaceae) in New Mexico developing both conidia and ascomata on the fibrous leaves of its host (Ramaley 2001). According to the limited phylogenetic evidence presented here *M. gamsii* and *M. bulbillosum* could be transferred to the holomorph genus *Hyaloseta*. However, the differential characters used to define *Hyaloseta* in comparison to *Niesslia* are subtle. Therefore, as long as an extensive molecular phylogenetic analysis of all representatives of *Niesslia* and *Monocillium* is pending, it seems less disruptive to place the new species in the 'anamorph genus' *Monocillium*.

It is intriguing that microsclerotia, which represent the main symptoms of fungal infection of nematode cysts and eggs in both *M. bulbillosum* and *M. gamsii* were readily reproduced in fungal pure cultures and were also formed in artificially infected nematode eggs. Apart from the essential role of conidia in fungal reproduction and dispersal, it seems that microsclerotia also play an important part in the developmental cycle of these fungi, at least with respect to those parts of their life cycle that could be assayed in vitro here and during which it interacts with nematodes. *Monocillium gamsii* was found in field-collected dried cysts in the semiarid Central Anatolian Plateau. In nature, fungal sclerotia are generally considered as resting structures by which the fungus may tolerate abiotic stresses like dessication, and can thus survive until favourable conditions return. Support for this hypothesis comes from the observation that we were able to isolate *M. gamsii* from field-collected cysts obtained by culturing the microslerotium-containing infected eggs that had been kept for more than one year in dry conditions either at 4 °C or at room temperature. Furthermore, nematode cysts are protective structures in which nematode eggs can survive for many years in soil in the absence of host plants or in adverse environments. By colonising the cyst contents, i.e. the mucilaginous matrix and the eggs, the egg-colonising fungi for example *M. gamsii* and *M. bulbillosum*, may thus benefit from this "specific" niche where they may have equivalent prolonged-survival conditions.

Our microscopic observations of the in vitro tests revealed that *M. gamsii* is capable of destructively and quickly parasiting the nematode eggs within the cysts first by penetrating the eggshell, followed by profilic formation of microsclerotia. We did not observe formation of any specific infecting structure like in the case of the recently described cyst and egg-parasitic fungus *Ijuhya vitellina* which developed appressoria (Ashrafi et al. 2017). Incubation of cysts on the colony of *M. bulbillosum* demonstrated that this species can also parasitise the nematode eggs in a similar manner, and even form microsclerotia. These observations suggest that both species may be candidates for nematode biocontrol.

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Three new species of *Hydnophlebia* (Polyporales, Basidiomycota) from the Macaronesian Islands

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Abstract

The genus *Hydnophlebia* includes two species of wood-inhabiting fungi, *Hydnophlebia chrysorhizon* and *Hydnophlebia omnivora*. Both are characterized by cream to reddish-orange, resupinate basidiome, with hydnoid hymenophore, margin with strands, a monomitic hyphal system, tubular to ventricose cystidia and elliptical spores. In this paper, a taxonomic study of *Hydnophlebia*, using morphology and molecular analyses of large subunit nuclear ribosomal DNA (LSU) and the internal transcribed spacer nrDNA operon (ITS), is reported. Three new species, *Hydnophlebia canariensis*, *H. gorgonea* and *H. meloi*, from the Macaronesia bioregion (Canary Islands and Cape Verde Archipelago), are described.

Keywords

Agaricomycetes, corticioid fungi, phylogeny, taxonomy, Canary Islands, Cape Verde Archipelago

Introduction

Hydnophlebia was erected by Parmasto (1967) to accommodate *Hydnum chrysorhizon* Torr. A few years later, the type species was transferred to *Phanerochaete* P. Karst. (Budington and Gilbertson 1973), and *Hydnophlebia* was neglected for a long time. Ryvarden et al. (2005) reintroduced it with a brief description taken from the original Latin diagnosis: basidioma resupinate, membranous, reddish-orange, hymenophore hydnoid with aculei, margin with rhizomorphs, hyphal system monomitic, tubular to

ventricose usually few cystidia, and spores ellipsoid, smooth, and thin-walled. According to Hjortstam and Ryvarden (2009), two species should be included in this genus of wood-inhabiting corticoid fungi: *Hydnophlebia chrysorhizon* (Torr.) Parmasto and *Hydnophlebia omnivora* (Shear) Hjortstam & Ryvarden.

Parmasto (1968) included *Hydnophlebia* in tribe Byssomerulieae (Corticiaceae) together with other genera, such as *Byssomerulius* Parmasto, *Chaetoderma* Parmasto, *Crustoderma* Parmasto, and *Phanerochaete*, while Larsson (2007), in his phylogenetic classification for corticioid fungi, included it in Meruliaceae, Polyporales. More recently, Floudas and Hibbett (2015) presented a four gene phylogenetic analysis of phanerochaetoid taxa and confirmed *Phanerochaete* as polyphyletic and *Hydnophlebia* as a genus of its own.

During our survey of corticioid fungi from Macaronesia (Canary Islands and Cape Verde Archipelago), nine hydnoid specimens were initially identified as belonging to the genus *Phanerochaete*. BLAST search of the large subunit of the nrDNA (LSU) sequences showed high similarity with a sequence published in Wu et al. (2010) and identified as *Phanerochaete chrysorhizon* (Torr.) Budington & Gilb. (AF139967). In the analysis by Wu et al. (2010) this sequence was recovered within a clade (clade V) containing i.a. *Phlebia* sensu stricto and a number of taxa with typically odontoid or hydnoid hymenophore, quite far from the *Phanerochaete* core group. BLAST search of the internal transcribed spacers of the nuclear ribosomal gene (ITS) sequences, which gave high similarity to sequences labelled as *Phanerochaete chrysorhizon* (AY219359) and *Phanerochaete omnivora* (Shear) Burdsall & Nakasone (AY219360) published in de Koker et al. (2003). Like later Wu et al. (2010) also de Koker et al. (2003) found that these taxa were not related to the *Phanerochaete* core group.

The aim of this study was to characterize and classify our specimens from Macaronesia, using morphological data and molecular analyses of LSU and ITS regions.

Materials and methods

Sampling, morphological studies and line drawings

Specimens were collected in the Canary Islands and Cape Verde Archipelago (Table 1), and are deposited in the mycological collection (MA-Fungi) of the Real Jardín Botánico herbarium in Madrid, Spain; the initials MD correspond to M. Dueñas, and Tell. to M.T. Telleria. The type specimens of *Hydnum chrysorhizon* (NY!) and *Hydnum omnivorum* Shear (BPI!) were included in the morphological analyses. Colours of dried basidiomata are given according to ISCC-NBS Centroid Color Charts (Kelly and Judd 1976). Dried specimens were also used for light microscope studies and drawings. Measurements and drawings were made from microscopic sections mounted in 3% aqueous KOH and/or Congo Red solution and examined at magnifications up to 1250× using an Olympus BX51 microscope. The length and width of 30 spores and 10 basidia were measured from each sample. Line drawings were made with a Leica DM2500 microscope with the aid of a drawing tube.

DNA isolation and sequencing

Genomic DNA was extracted from eight collections (Table 1) using DNeasy® Plant Mini Kit (QIAGEN, Valencia, CA), following the manufacturer's instructions. Basidiomes were disrupted using Tissue-Lyser II (QIAGEN, Germany) and glass beads. Lysis buffer incubation was overnight at 55 °C.

Total DNA was used for PCR amplification of the D1–D2 region of the large subunit (LSU) and the internal transcribed spacer region (ITS) of the nuclear ribosomal gene. The primers LR0R (Rehner and Samuels 1994) and LR7 (Vilgalys and Hester 1990) were used to amplify the region of the LSU nrDNA; the primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) were used to obtain amplifications of both ITS regions, including the 5.8S of the ribosomal RNA gene cluster and flanking parts of the small subunit (SSU) and large subunit (LSU) nuclear ribosomal genes. Individual reactions to a final volume of 25 µL were carried out using illustra™ PuReTaq™ Ready-To-Go™ PCR Beads (GE Healthcare, Buckingham) with a 10 pmol/µL primer concentration, following the thermal cycling conditions used in Martín and Winka (2000).

Negative controls lacking fungal DNA were run for each experiment to check for contamination. The reactions were run with the following parameters for the LSU nrDNA: initial denaturation at 94 °C for 5 min, then 36 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 1.5 min, with a final extension at 72 °C for 10 min, and 4 °C soak; for the ITS nrDNA: initial denaturation at 95 °C for 5 min, then 5 cycles of denaturation at 95 °C for 30 s, annealing at 54 °C for 30 s, and extension at 72 °C for 1 min, followed by 33 cycles of denaturation at 72 °C for 1 min, annealing at 48 °C for 30 s, and extension at 72 °C, with a final extension at 72 °C for 10 min and 4 °C soak.

PCR products were checked on 2% agarose D1 low EEO (CONDA, Pronadisa™) gels and subsequently purified using the QIAquick Gel PCR Purification (QIAGEN) kit according to the manufacturer's instructions. The purified PCR products were sequenced using the same amplification primers at Macrogen Korea (Seoul, Korea).

Sequencher v. 4.2 (Gene Codes Corporation, Ann Arbor, MI) was used to edit the resulting electropherograms and to assemble contiguous sequences (Table 1 in bold). BLAST searches (Altschul et al. 1997), using the MEGABLAST option were done to compare the sequences obtained against the sequences in the EMBL/GenBank/DDBJ databases (Cochrane et al. 2011, 2016).

Sequence alignment and phylogenetic analyses

The LSU and ITS sequences obtained were aligned separately using Se-Al v. 2.0a11 Carbon (Rambaut 2002) for multiple sequences.

To infer phylogenetic relationships of Macaronesian specimens within Meruliaceae, the LSU sequences were compared with homologous sequences retrieved from the

Table 1. Specimens of *Hydnophlebia* species described as new, and EMBL/GenBank/DDBJ and UNITE accessions included in the LSU and ITS nrDNA analyses. The asterisk (*) after the taxon names denotes type species of the genus. The specimens with uncertain generic placement are listed at the end of the table; in Fig. 1 and 2, the uncertainty is indicated by brackets around the name. Isolates and/or voucher specimens are indicated as they appear in GenBank and UNITE accessions.

Names after our LSU or ITS analyses	Names included in EMBL/GenBank/DDBJ and UNITE	Isolate/Voucher	GenBank/UNITE accessions	
			LSU	ITS
*Abortiporus biennis**	*Abortiporus biennis*	KEW210	AF287842	–
*Cabaladontia queletii**	*Phlebia queletii*	FCUG 722, culture	AF141626	–
*Ceriporia viridans**	*Ceriporia viridans*	FO24398	AJ406518	
*Ceriporiopsis gilvescens**	*Ceriporiopsis gilvescens*	O/Haussknecht98	DQ144618	–
*Climacodon septentrionalis**	*Climacodon septentrionalis*	HHB-13438-sp	AF518610	–
*Crustodontia chrysocreas**	*Phlebia chrysocreas*	FPL-6080	AY293199	–
*Crustodontia chrysocreas**	*Phlebia chrysocreas*	KHL10216 (GB)	AY586695	–
*Cymatoderma elegans**	*Cymatoderma elegans*	Halling9064 (NY)	JN649341	–
Hydnophlebia canariensis	*Hydnophlebia canariensis*	17035Tell., MA-Fungi 86622, Holotype	**KF528103**	**KF483012**
Hydnophlebia canariensis	*Hydnophlebia canariensis*	17038Tell., MA-Fungi 86623	**KF528104**	**KF483013**
Hydnophlebia canariensis	*Hydnophlebia canariensis*	17674Tell., MA-Fungi 86619	**KF528100**	**KF483009**
*Hydnophlebia chrysorhizon**	*Phanerochaete chrysorhizon*	FP-102002-sp (CFMR)	–	AY219359
*Hydnophlebia chrysorhizon**	*Phanerochaete chrysorhizon*	T-484, RGT 871020/12	AF139967	–
*Hydnophlebia chrysorhizon**	*Hydnophlebia chrysorhizon*	T 484	–	KP135335
*Hydnophlebia chrysorhizon**	*Hydnophlebia chrysorhizon*	FP-134985	–	KP135336
*Hydnophlebia chrysorhizon**	*Hydnophlebia chrysorhizon*	HHB-18767	–	KP135337
*Hydnophlebia chrysorhizon**	*Hydnophlebia chrysorhizon*	FD-282	–	KP135338
Hydnophlebia gorgonea	*Hydnophlebia gorgonea*	13327MD, MA-Fungi 86642	**KF528122**	**KF483031**
Hydnophlebia gorgonea	*Hydnophlebia gorgonea*	19110Tell., MA-Fungi 86658	**KF528139**	**KF483048**
Hydnophlebia gorgonea	*Hydnophlebia gorgonea*	19111Tell., MA-Fungi 86659, Holotype	**KF528140**	**KF483049**
Hydnophlebia gorgonea	*Hydnophlebia gorgonea*	19133Tell., MA-Fungi 86664	**KF528145**	**KF483054**
Hydnophlebia meloi	*Hydnophlebia meloi*	19071Tell., MA-Fungi 86654, Holotype	**KF528135**	**KF483044**
Hydnophlebia omnivora	*Phanerochaete omnivora*	HHB-5969-sp	–	AY219360
Hydnophlebia omnivora	*Hydnophlebia omnivora* 2[a]	ME-497	–	KP135332
Hydnophlebia omnivora	*Hydnophlebia omnivora* 2[a]	HHB-6228-sp	–	KP135333
Hydnophlebia sp. 1	*Hydnophlebia omnivora* 1[a]	KKN-112-sp	–	KP135334
Hydnophlebia sp. 2	*Phlebia* sp.	TU108437	–	UDB016816
*Junghuhnia crustacea**	*Junghuhnia crustacea*	X1127, O. Miettinen 13852,1 (H)	JN710554	–
*Junghuhnia crustacea**	*Junghuhnia crustacea*	X262, O. Miettinen 2954,1 (H)	JN710553	–
*Lamelloporus americanus**	*Lamelloporus americanus*	X670, T. Laessoe 10119 (O, H)	JN710567	–
*Lilaceophlebia livida**	*Phlebia livida*	FCUG 2189, culture	AF141624	–
*Merulius tremellosus**	*Phlebia tremellosa*	FPL-4294	AY293200	–
*Merulius tremellosus**	*Phlebia tremellosa*	FCUG 1813, culture	AF141632	–
*Merulius tremellosus**	*Phlebia tremellosa*	F15198 (UBC)	DQ384584[b,c]	DQ384584[b,c]

Names after our LSU or ITS analyses	Names included in EMBL/GenBank/DDBJ and UNITE	Isolate/Voucher	GenBank/UNITE accessions	
			LSU	ITS
*Merulius tremellosus**	*Phlebia tremellosa*	CIRM-BRFM 968	–	GU731568
*Mycoacia fuscoatra**	*Mycoacia fuscoatra*	KHL13275 (GB)	JN649352	JN649352
*Mycoacia fuscoatra**	*Phlebia subserialis*	KUC8041, culture	AY858370	
Mycoacia nothofagi	*Mycoacia nothofagi*	KHL13750	GU480000	–
Mycoacia nothofagi	*Phlebia nothofagi*	AH31887	GQ259416	–
Mycoacia nothofagi	*Phlebia nothofagi*	KHL13750	GU226430	–
*Mycorrhaphium adustum**	*Mycorrhaphium adustum*	KHL12255 (GB)	JN710573	–
*Mycoaciella bispora**	*Mycoaciella bispora*	EL13_99	AY586692	–
Phlebia acerina	*Phlebia acerina*	FCUG 568, culture	AF141615	–
*Phlebia radiata**	*Phlebia radiata*	culture?	AB325676	–
*Phlebia radiata**	*Phlebia radiata*	FCUG2423, culture	AF141627	–
*Phlebia radiata**	*Phlebia radiata*	FPL6140	AF287885	–
*Phlebia radiata**	*Phlebia radiata*	GEL5258	AJ406541	–
*Phlebia radiata**	*Phlebia radiata*	KUC8034, culture	AY858369	–
*Phlebia radiata**	*Phlebia radiata*	TM03_491	EU522844	–
*Phlebia radiata**	*Phlebia radiata*	JLL-15608-sp. (CFMR)	–	AY219366
*Phlebia radiata**	*Phlebia radiata*	ATCC 64658, culture	–	EF491867
Phlebia rufa	*Phlebia rufa*	FCUG 2397	AF141628	–
*Scopuloides hydnoides**	*Scopuloides hydnoides*	KHL11916 (GB)	EU118665[c]	EU118665[c]
*Scopuloides hydnoides**	*Scopuloides hydnoides*	GEL3859	AJ406573	–
*Scopuloides hydnoides**	*Scopuloides hydnoides*	GEL3139	AJ406574	–
*Steccherinum ochraceum**	*Steccherinum ochraceum*	KHL11902 (GB)	JQ031130	–
*Steccherinum ochraceum**	*Steccherinum ochraceum*	Ryberg sn. (GB)	EU118670	–
Specimens "incertae sedis"	*Mycoacia aurea*	GEL5339	AJ406535	–
	Mycoacia aurea	NH14434	AY586691	–
	Phlebia setulosa	PH106520, culture	GU461311	–
	Phlebia setulosa	PH11749, culture	GU461312	–
	Phlebia setulosa	PH5105, culture	GU461313	–
	Phlebia setulosa	AH31879	GQ259417	–
	Phlebia subochracea	FCUG 1161, culture	AF141630	–
	Phlebia subochracea	KGN 162/95 (GB)	EU118656[b]	EU118656[b]
	Phlebia suserialis	FCUG1434, culture	AF141631	–
	Phlebiella griseofulva	GEL4492	AJ406517	–

[a]Names as indicated in Floudas & Hibbett (2015)

[b]Unpublished sequence

[c]These sequences contain part of SSU, complete ITS region (ITS1 + 5.8S + ITS2) and D1-D2 of LSU nrDNA.

EMBL/GenBank/DDBJ databases (Cochrane et al. 2011), mainly from Hibbett et al. (2000), Parmasto and Hallenberg (2000), Thorn et al. (2000), Hibbett and Binder (2002), Langer (2002), Larsson et al. (2004), Binder et al. (2005), Han et al. (2005), Kim et al. (2005), Larsson (2007), Hallenberg et al. (2008), Porter et al. (2008), Moreno et al. (2011), Miettinen et al. (2012), and Sjökvist et al. (2012), Floudas and Hibbett (2015). In order to clearly identify the genus of the Macaronesian specimens, we selected reference sequences from some of the genera included in Meruliaceae by

Larsson (2007), and from genera included by Wu et al. (2010) in his clade V that covers *Phlebia* sensu stricto and several taxa with odontoid or hydnoid hymenophore. Moreover, sequences of type species of different genera listed in MycoBank (Crous et al. 2004, Robert et al. 2013, http://www.mycobank.org) as belonging to Meruliaceae were selected from EMBL/GenBank/DDBJ databases, mainly from references mentioned above (Table 1). Based on Binder et al. (2005) and Floudas and Hibbett (2015), two sequences of *Steccherinum* Gray (residual polypore clade) were included as outgroup. Where ambiguities in the alignment occurred, the alignment generating the fewest potentially informative characters was chosen (Baum et al. 1994). Alignment gaps were marked "–", unresolved nucleotides and unknown sequences were indicated with "N".

A maximum parsimony analysis (MP) was carried out; minimum length Fitch trees were constructed using heuristic searches with tree-bisection-reconnection (TBR) branch swapping, collapsing branches if maximum length was zero and with the MulTrees option on in PAUP*4.0b10 (Swofford 2003), with a default setting to stop the analysis at 100 trees. Gaps were treated as missing data. Nonparametric bootstrap (MP-BS) support (Felsenstein 1985) for each clade, based on 10,000 replicates using the fast-step option, was tested. The consistency index, CI (Kluge and Farris 1969), retention index, RI, and rescaled consistency index, RC (Farris 1989) were obtained. The maximum likelihood (ML) analysis was done in PAUP*Version 4.0b10, with the GTR+I+G model selected by this programme; for assessing branch support, 1000 non-parametric bootstrap replicates (ML-BS) were performed with the fast-step option. A third analysis was done by a Bayesian approach (Larget and Simon 1999, Huelsenbeck et al. 2001) using MrBayes 3.2 (Ronquist et al. 2012) and assuming the general time reversible model (Rodríguez et al. 1990), including estimation of invariant sites and a discrete gamma distribution with six categories (GTR+I+G), as selected by PAUP*Version 4.0b10. Two independent and simultaneous analyses starting from different random trees were run for two million generations with 12 parallel chains, and trees and model scores saved every 100th generation. The initial 1000 trees were discarded as burn-in before calculating the 50% majority-rule consensus tree and the posterior probability (PP) of the nodes, as described in Telleria et al. (2010). A combination of bootstrap proportions and posterior probabilities was used to assess the level of confidence for a specific node (Lutzoni et al. 2004; Wilson et al. 2012). The phylogenetic trees were viewed with FigTree v. 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/) and edited with Adobe Illustrator CS3 v. 11.0.2 (Adobe Systems).

For molecular characterization of the Macaronesian specimens, the ITS sequences were compared with homologous sequences retrieved from the EMBL/GenBank/DDBJ (Cochrane et al. 2011, 2016) and UNITE (Abarenkov et al. 2011, Kõljag et al. 2013, http://unite.ut.ee/cite.php) databases (Table 1), mainly from de Koker et al. (2003), Larsson (2007), Hilden et al. (2008), Wu et al. (2010), Sjökvist et al. (2012), and Floudas and Hibbett (2015).

Based on our previous phylogenetic trees obtained from LSU, two sequences of *Phlebia radiata* Fr. were selected as outgroup (AY219366, EF491867). Alignment gaps were marked "–", unresolved nucleotides and non-sequenced nucleotide positions within the data matrix were indicated with "N". A maximum parsimony analysis (MP)

was carried out under heuristic search, following the same criteria as mentioned above for LSU; maximum likelihood (ML) and Bayesian approaches were also performed, using the GTR+I+G as selected by PAUP*Version 4.0b10 and MrModeltest 2.3. The ML and Bayesian analyses were done with the same programs, and followed the same criteria as mentioned above for LSU.

Alignments and phylogenetic trees have been deposited at TreeBase: http.//purl.org/phylo/tree-base/phylows/study/TB2:S21012

Results

Sixteen new sequences from the Macaronesian specimens were generated in this study (Table 1). The LSU sequence contains the domain D1-D2, and the ITS sequence the regions ITS1, 5.8S nrDNA and ITS2.

LSU analyses

The LSU dataset contains 57 sequences and 908 aligned positions, of which 682 were constant, 82 parsimony uninformative, and 144 parsimony-informative. Maximum parsimony analysis yielded 100 most parsimonious trees (613 steps long, CI = 0.4731, HI = 0.6164, RI = 0.7399) under a heuristic search. Almost identical tree topologies were generated after parsimony and Bayesian analyses. The 50% majority-rule consensus tree from the Bayesian analysis is shown in Fig. 1, with percentage of bootstrap (MP-BS and ML-BS) and posterior probabilities indicated on the branches. The circumscription of clade V from Wu et al. (2010) is indicated in this figure.

All sequences obtained from Macaronesian specimens cluster in a supported clade (MP-BS = 70%, ML-BS = 80 %, PP = 1.0), with sequences from de Koker et al. (2003) and Floudas and Hibbett (2015) under *Hydnophlebia*. Most sequences are distributed over two supported clades, one containing four specimens from São Vicente (MP-BS = 96%, ML-BS = 95 %, PP = 1.0), and the other three specimens from Canary Islands (MP-BS = 93%, ML-BS = 95 %, PP = 0.98). Specimen 19071Tell., from Fogo Island, appears together with sequences KP135334 and KP135218, under *H. omnivora* 1 and *H. omnivora* 2 in Floudas and Hibbett (2015); although this relationship is not well supported (MP-BS = 93%, ML-BS = 95 %, PP = 0.98). The two *H. chrysorhizon* sequences (AF139967 and KP135217 from previous authors) form a fairly well supported clade with the specimens from Saõ Vicente (MP-BS = 61%, ML-BS = 65 %, PP = 1.0).

ITS analyses

The ITS nrDNA dataset contains 26 sequences and 851 aligned positions, of which 575 were constant, 103 parsimony uninformative, and 173 parsimony-informative. After heuristic search, the 100 trees had 447 steps, CI = 0.7136, HI = 0.3798 and RI

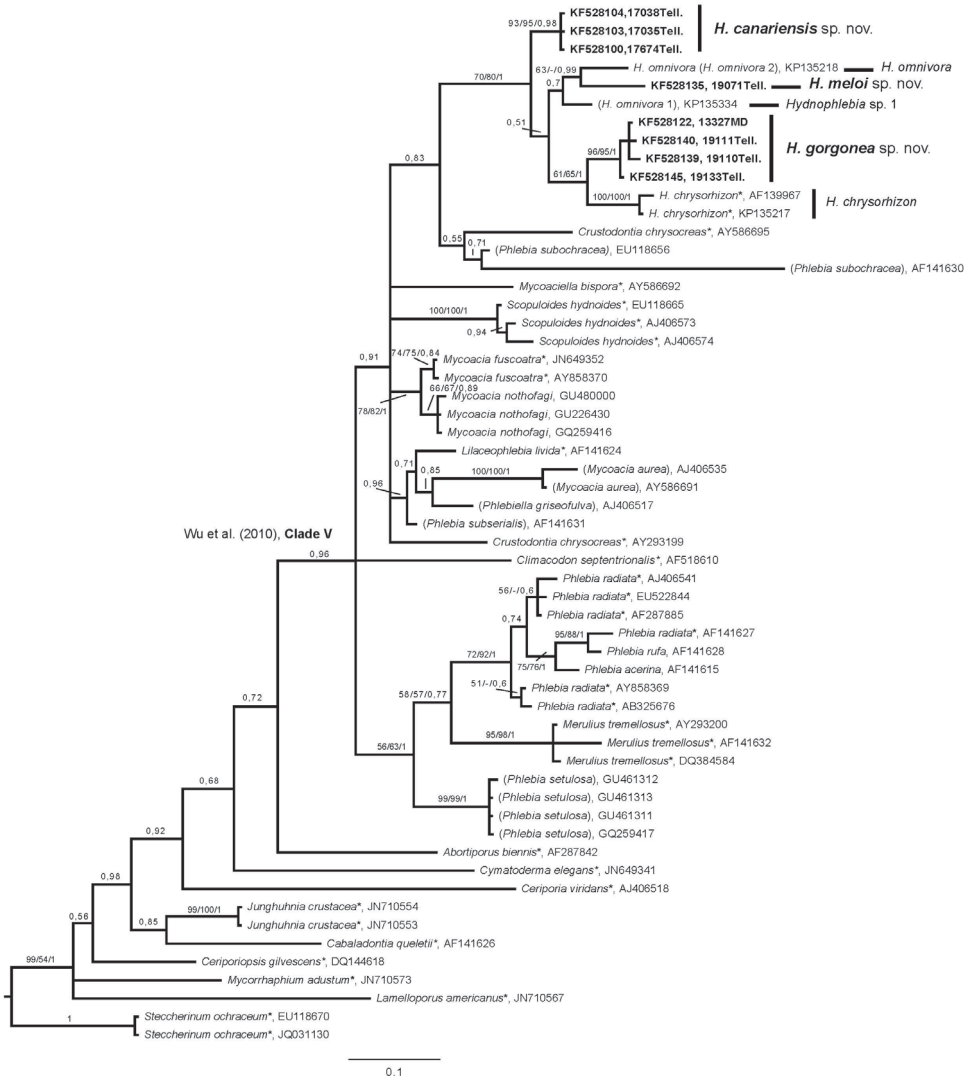

Figure 1. The 50% majority rule Bayesian tree inferred from D1-D2 LSU nrDNA assuming the GTR + I + G model of corticioid fungi included in Table 1. Parsimony bootstrap values (> 50%) maximum likelihood bootstrap values (> 50%) and Bayesian posterior probabilities (> 0.95) are indicated on the branches. Clade V from Wu et al. (2010) is indicated. Taxon name between parentheses indicate specimens with uncertain generic placement. Sequences of the new species described in this paper, *H. canariensis, H. gorgonea* and *H. meloi* are in bold. The asterisk (*) after the taxon names denotes type species of the genus.

= 0.7831. Almost identical tree topologies were generated after parsimony (data not shown), maximum likelihood (data not shown) and Bayesian analyses. The 50% majority-rule consensus tree from the Bayesian analysis is shown in Fig. 2, with percentage of bootstrap (MP-BS and ML-BS), and posterior probabilities indicated on the branches.

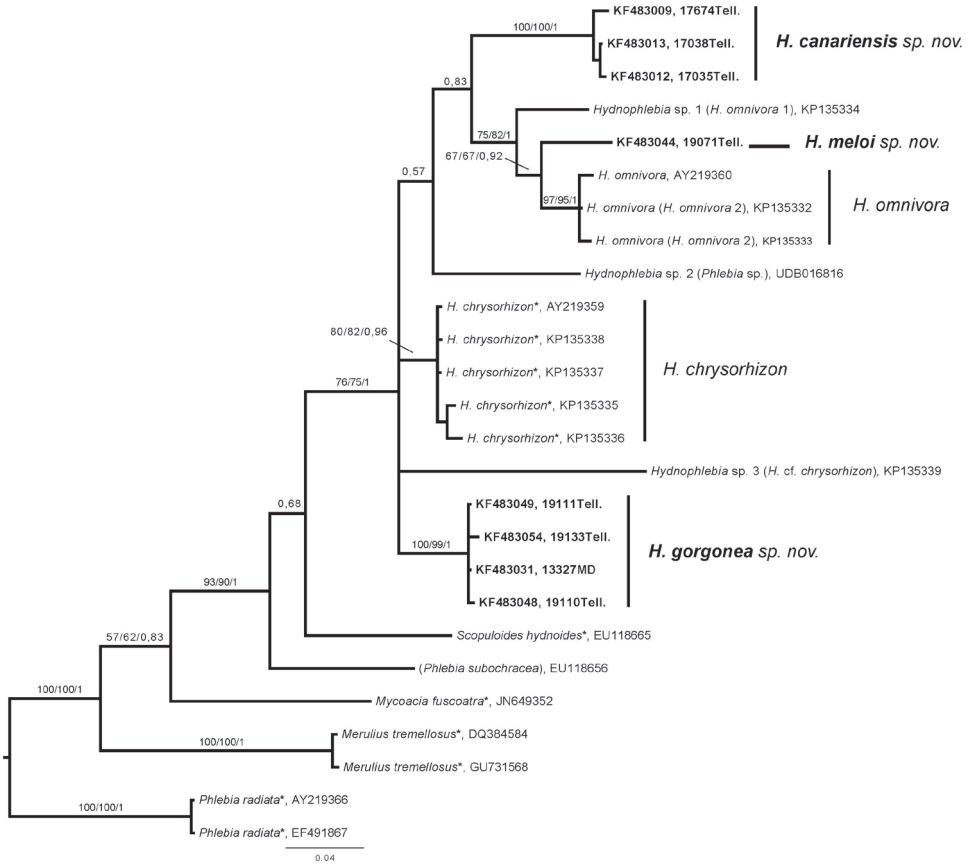

Figure 2. The 50% majority rule Bayesian tree inferred from ITS nrDNA assuming the GTR + I + G model of corticioid fungi included in Table 1. Parsimony bootstrap values (> 50%), maximum likelihood bootstrap values (> 50%) and Bayesian posterior probabilities (> 0.95) are indicated on the branches. Taxon name between parentheses indicate specimens with uncertain generic placement. Sequences of the new species described in this paper, *H. canariensis, H. gorgonea* and *H. meloi* are in bold. The asterisk (*) after the taxon names denotes type species of the genus.

Similar to the LSU analyses, the sequences from Macaronesian specimens form a clade (MP-BS = 76%, ML-BS = 75%, PP = 1.0), together with downloaded sequences of *Hydnophlebia* from the USA (Arizona, Florida, Illinois, New York and Puerto Rico) and Canada, identified in Floudas and Hibbett (2015) as *H. chrysorhizon*, *H.* cf. *chrysorhizon*, *H. omnivora* 1 and *H. omnivora* 2; as well as sequences of *H. chrysorhizon* from Illinois and *H. omnivora* from Arizona published by de Koker et al. (2003) that we consider represent the modern interpretation of these two species. Sequence UDB016816 from Madagascar, in Fig. 2, labelled as *Hydnophlebia* sp. 2, (under *Phlebia* sp. in UNITE database) also clusters in this clade.

The five sequences from Canada and the USA identified as *H. chrysorhizon* grouped in a highly supported clade (MP-BS > 80%, ML-BS > 82%, PP = 1.0). The sequences

UDB016816, labelled *Hydnophlebia* sp. 2 from Madagascar, and KP135339, labelled *Hydnophlebia* sp. 3 (*H.* cf. *chrysorhizon* in Floudas and Hibbett 2015) from Puerto Rico, did not group with other *Hydnophlebia* sequences.

The new sequences generated for this work are distributed over three clades. These clades are here described in the order they occur from top to bottom in Fig. 2.

The first group (MP-BS = 100%, ML-BS = 100%, PP = 1.0) contains sequences of 17035Tell., 17038Tell., and 17674Tell. from El Hierro and Fuerteventura Islands, collected on different substrates.

A second clade (MP-BS > 75%, ML-BS > 82%, PP = 1.0) encloses specimen 19071Tell., from Fogo Island, collected on *Sarcostemma daltonii* Decne (Asclepiadaceae), and the sequence indicated in Fig. 2 as *Hydnophlebia* sp. 1 (*H. omnivora* 1 in Floudas and Hibbett 2015) and three sequences of *Hydnophlebia omnivora* (two of them as *H. omnivora* 2 in Floudas and Hibbett 2015).

Sequences of 13327MD, 19110Tell., 19111Tell., and 19133Tell., all from São Vicente Island on *Prosopis juliflora* (Sw.) DC. (Fabaceae), are distributed in a third clade (MP-BS = 100%, ML-BS = 99%, PP = 1.0).

Taxonomy

Hydnophlebia **Parmasto, Izv. Akad. Nauk Estonsk. SSR, Ser. Biol. 16: 384. 1967**

Type species. *Hydnum chrysorhizon* Torr. in Eaton, Manual Bot.: 309. 1822

Basidioma resupinate, separable, generally reddish orange yellow. Hymenophore hydnoid, with aculei up to 0.5–1.5 mm long, conical to cylindrical. Margin with strands. Hyphal system monomitic with scattered clamps, subicular and strand hyphae thick-walled, colorless to yellowish, encrusted, aculei and subhymenial hyphae thin-walled, also colorless. Cystidia cylindrical to ventricose, colorless, thin-walled, sometimes few. Basidia cylindrical to subclavate, with 4 sterigmata, basal clamp absent. Spores cylindrical, ellipsoid to subglobose, smooth, thin-walled.

Key to species of *Hydnophlebia*

1 Spores narrowly ellipsoid to cylindrical, 4–6 × 2–3 µm (L/W = 1.9). Basidiome orange brown in dry specimens, reddish orange to deep orange in fresh material. Strands very long, yellowish to cream in dry material and reddish orange in fresh specimens, up to 1 mm diam ..... **2. *H. chrysorhizon***

– Spores ellipsoid, broadly ellipsoid or subglobose (L/W ≤ 1.6) **2**

2 Spores subglobose, 4–5.5 × 3–4 µm (L/W = 1.2). Basidiome yellowish white to pale orange-yellow in dry specimens. Margin fimbriate, yellowish white, with strands poorly developed. Cystidia cylindrical, 40–55 × 3–4 µm..... ..... **4. *H. meloi***

– Spores ellipsoid to broadly ellipsoid (L/W ≥ 1.3) **3**

3 Basidiome in small and poorly developed patches, cream-coloured in dry specimens. Clamps present in strand hyphae. Cystidia cylindrical slightly tapered at the apex, 40–70 × 4–5 µm. Spores ellipsoid, 5–6.5 × 3–4 µm (L/W = 1.6) ..... **5. *H. omnivora***

– Basidiome broadly effuse, orange-yellow to brilliant orange-yellow. Clamps absent in strand hyphae **4**

4 Basidiome light orange-yellow in dry specimens. Margin fimbriate, with white, well developed strands. Cystidia of two types, cylindrical with slightly tapered apex and ventricose with subulate apex, 45–55 × 3–5 µm. Spores ellipsoid, 5–7 × 3–4.5 µm (L/W = 1.5) ..... **1. *H. canariensis***

– Basidiome light to brilliant orange-yellow in dry specimens. Margin fimbriate with poorly developed strands. Cystidia cylindrical, sometimes capitate, to ventricose, 45–55 × 4–6 µm. Spores ellipsoid to broadly ellipsoid, 5–7 × 4–4.5 µm (L/W = 1.4) ..... **3. *H. gorgonea***

1. *Hydnophlebia canariensis* Telleria, M. Dueñas & M.P. Martín, sp. nov.
MycoBank MB815729
Figs 3, 4

Diagnosis. This species can be recognized by the orange-yellow basidiome, hydnoid hymenophore with long aculei, up to 1,5 mm, white subiculum, and well-developed white strands. Spores ellipsoid 5–7 × 3–4.5 µm (L/W = 1.5).

Type. Spain. Canary Islands: El Hierro, Frontera, Sabinar de la Dehesa, 27°44'43"N; 18°07'02"W, 610 m alt., on unidentified wood, 26 January 2007, M.T. Telleria, 17035Tell. (holotype: MA-Fungi 86622). LSU sequence KF528103, ITS sequence KF483012.

Etymology. Named after the Canary Islands where the holotype and paratypes were collected.

Description. Basidiome resupinate, effuse, membraneous to ceraceous, yellow (82. v. Y) in fresh specimens and light orange-yellow (70. l. OY) in dry. Hymenophore hydnoid, aculei conical, 0.5–1.5 mm long. Subiculum byssoid, white. Margin fimbriate, white, with well-developed white strands.

Hyphal system monomitic; subicular hyphae 6–8 µm wide, with clamps, thin to thick-walled; strand hyphae 7–11 µm wide, without clamps, thick-walled; aculei hyphae 4–5 µm wide, without clamps, thin-walled and growing perpendicular to the substrate; subhymenial hyphae 3–4 µm wide, without clamps, thin-walled, and loosely interwoven. Cystidia of two types: cylindrical with slightly tapered apex and ventricose with subulate apex, thin-walled, 45–55 × 3–5 µm. Basidia cylindrical to subclavate, 24–28 × 4–6 µm, with 4 sterigmata, basal clamp absent. Spores ellipsoid 5–7 × 3–4.5 µm (L/W = 1.5), thin-walled, colorless, smooth.

Ecology and distribution. On decayed wood and plant debris in arid and semiarid habitats; known only from the Canary Islands.

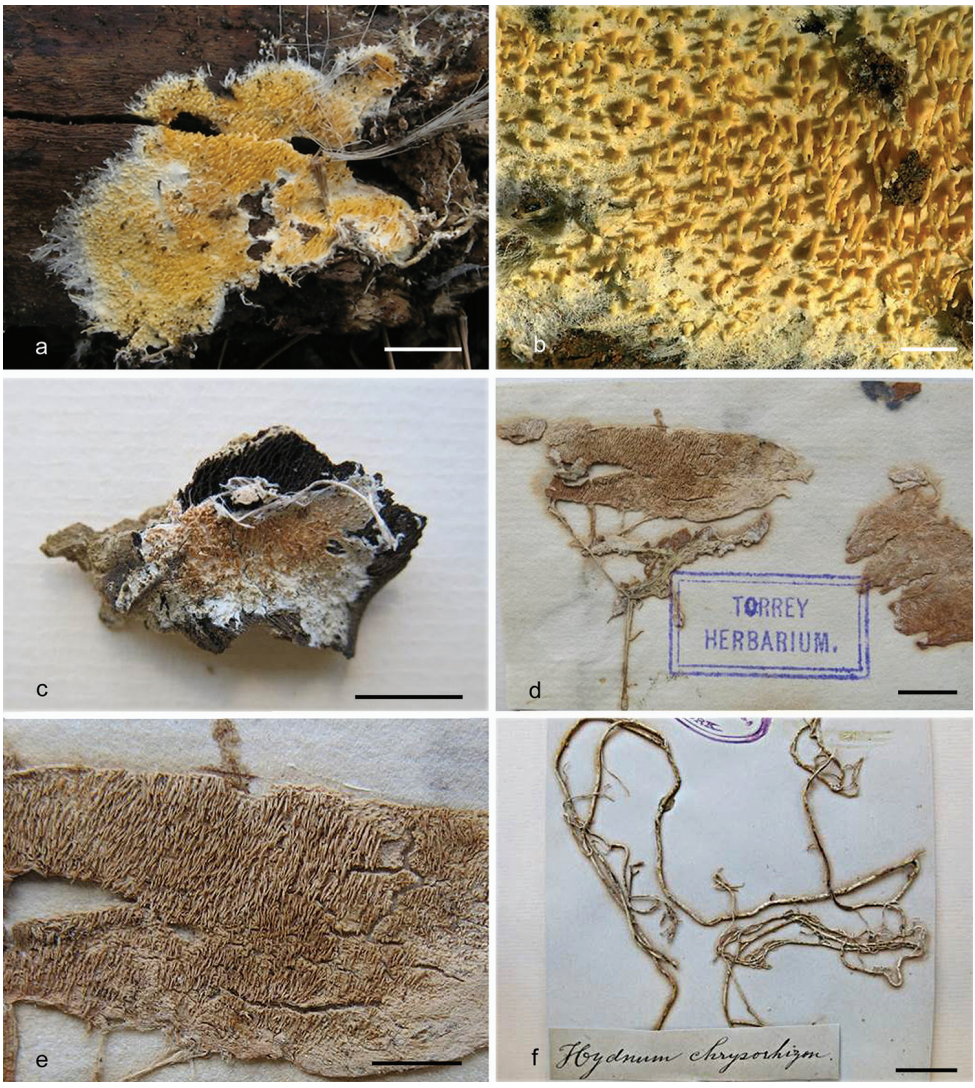

Figure 3. *Hydnophlebia canariensis.* **a**, **b** Collection 17035Tell., MA-Fungi 86622, holotype, basidiome, wet (a) and hymenophore, dry specimen (b) **c** Collection 17038Tell. MA-Fungi 86623, basidiome, dry specimen. *Hydnophlebia chrysorhizon*. Collection NY, lectotype **d** Basidiome, dry specimen **e** Hymenophore, dry specimen **f** Strands, dry specimen. Scale bars: **a, e** = 5 mm; **b** = 1.5 mm; **c, d, f** = 1 cm.

Other specimens examined. Spain. Canary Islands: El Hierro, Frontera, Sabinar de la Dehesa, 27°44'43"N; 18°07'02"W, 610 m alt., on unidentified wood, 26 January 2007, M.T. Telleria, 17038Tell. (MA-Fungi 86623), LSU sequence KF528104, ITS sequence KF483013. Fuerteventura, Pájara, Parque Natural de Jandía, Valle de los Mosquitos, 28°04'36"N; 14°25'23"W, 99 m alt., on *Launaea arborescens*, 05 December 2007, M.T. Telleria, 17674Tell. (MA-Fungi 86619), LSU sequence KF528100, ITS sequence KF483009.

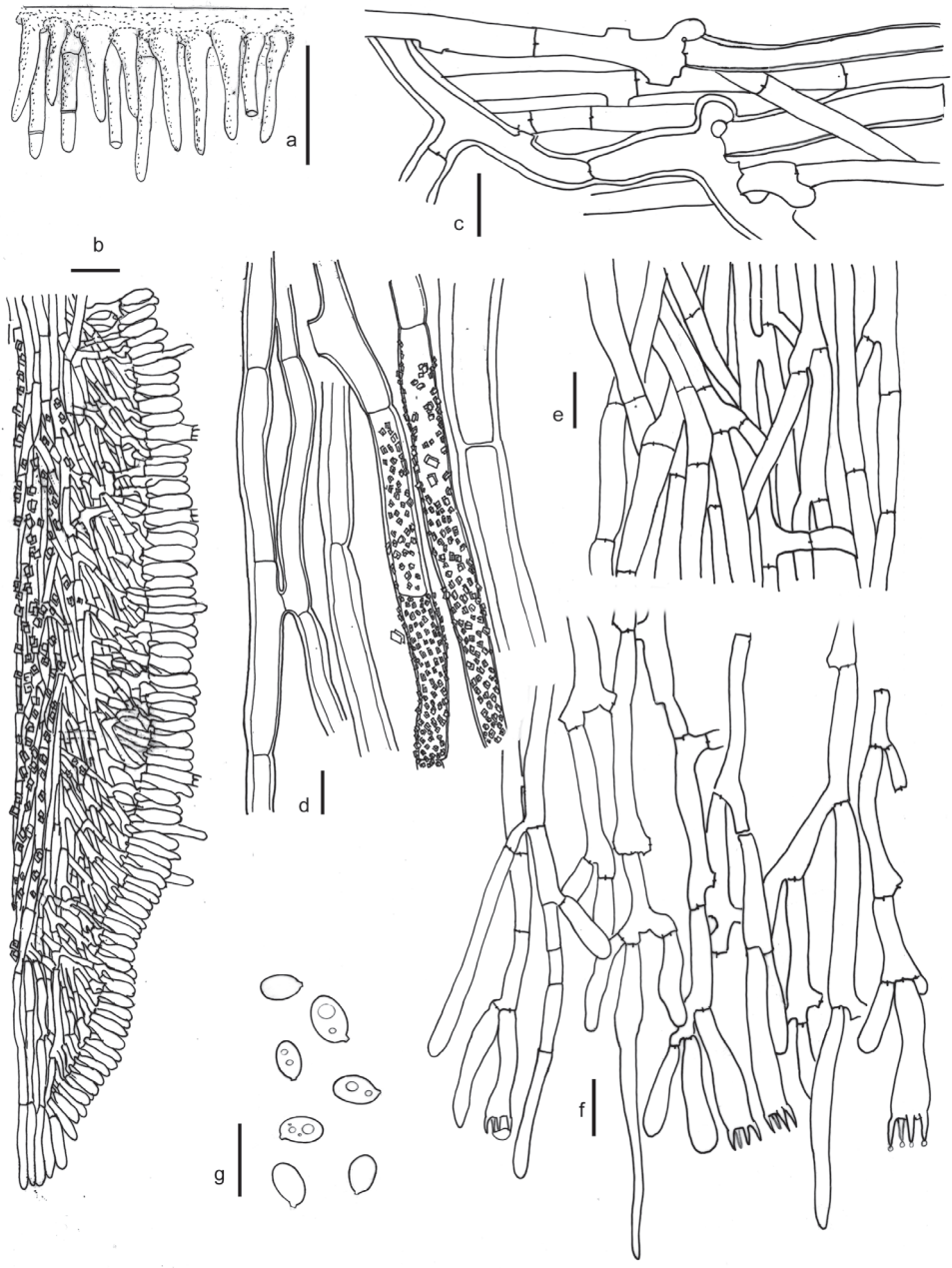

Figure 4. *Hydnophlebia canariensis*. Collection 17035Tell., MA-Fungi 86622, holotype **a** Hymenophore **b** Vertical section through an aculei **c** Subicular hyphae **d** Strand hyphae **e** Aculei hyphae **f** Subhymenial hyphae, cystidia, and basidia **g** Spores. Scale bars: **a** = 1 mm; **b** = 25 µm; **c–g** = 10 µm. Drawing by M. Dueñas.

2. *Hydnophlebia chrysorhizon* (Torr.) Parmasto, Izv. Akad. Nauk Estonsk. SSR, Ser. Biol. 16: 384. 1967

Figs 3, 5

Basionym. *Hydnum chrysorhizon* Torr. in Eaton, Manual Bot.: 309. 1822

Type. USA, *Hydnum chrysorhizon* Torr. in Eaton Man. 3ed. p. 309. 237, C. C., Steward. In herbarium NY! (lectotype, designated by Burdsall 1985).

Description. Basidiome resupinate, effuse, membraneous, easily separable, orange-brown in dry specimens, reddish orange to deep orange in fresh material (Burdsall and Nakasone 1978, Lindsey and Gilbertson 1975, Burdsall 1985, Maekawa 1993). Hymenophore hydnoid, aculei dense, conical to subcylindrical, 1–1.6 mm long. Margin with strands very long and well developed, yellowish to cream in dry specimens, reddish orange in fresh specimens (Burdsall 1985), up to 1 mm diam.

Hyphal system monomitic; subicular hyphae 7–10 µm wide, with clamps, thick-walled, colorless to pale yellow, densely encrusted with colorless crystals and loosely interwoven; strand hyphae 10–17 µm wide, without clamps, thick-walled, colorless, also encrusted; aculei hyphae 4–6 µm wide, with scattered clamps, thin-walled, colorless, and oriented perpendicular to the substrate; subhymenial hyphae 5–7 µm wide, without clamps, thin-walled, colorless, densely interwoven, short-celled. Cystidia not seen, but according to Burdsall (1985) few, cylindrical, thin-walled, hyaline, short, 18–40 × 4.5–6 µm. Basidia clavate, 15–21 × 4–6 µm, with 4 sterigmata, basal clamp absent. Spores narrowly ellipsoid to cylindrical, 4–6 × 2–3 µm (L/W = 1.9), thin-walled, colorless, smooth.

Ecology and distribution. On decayed wood. Described from New York (Eaton 1822), this species has been reported from: Africa: Cameroon, (Roberts 2000), and Seychelles (Hjortstam and Ryvarden 2009); North America: USA, Arizona, Florida, Maryland, Mississippi, New York, North Carolina, South Carolina, Tennesse, Wisconsin (Lindsey and Gilbertson 1975, Burdsall and Nakasone 1978, Burdsall 1985, Nakasone 2012); South America: Argentina, Venezuela, Brazil (Hjortstam and Ryvarden 2007); Meso America: Puerto Rico (Hjortstam and Ryvarden 2009), as well as Saint Vincent and the Grenadines (Nakasone 2012); Asia: Japan (Maekawa 1993).

Other specimens examined. USA. Ohio, Hamilton Co. Sharon Woods County Park, on *Quercus* sticks, 13 October 1973, W.B. & V.G. Cooke 48958. New York, New Dorp, Staten Island, 17 October 1896, col. L.M. Underwood.

Remarks. This species has very long and well-developed strands and, microscopically, it is the only species in the genus with spores narrowly ellipsoid to cylindrical (L/W = 1.9) and scattered clamps in the aculei hyphae.

Based on morphological analyses, Burdsall (1985) considered *Hydnum fragilissimum* Berk. & M.A. Curtis, *Hydnum ischnodes* Berk., and *Hydnum chrysocomum* Underw. as synonyms of *H. chrysorhizon*; and according to Nakasone (2012) *Hydnum schweinitzii* Berk. & M.A. Curtis, *Hydnum chrysodon* Berk. & M.A. Curtis, and *Merulius elliottii* Massee are other synonyms.

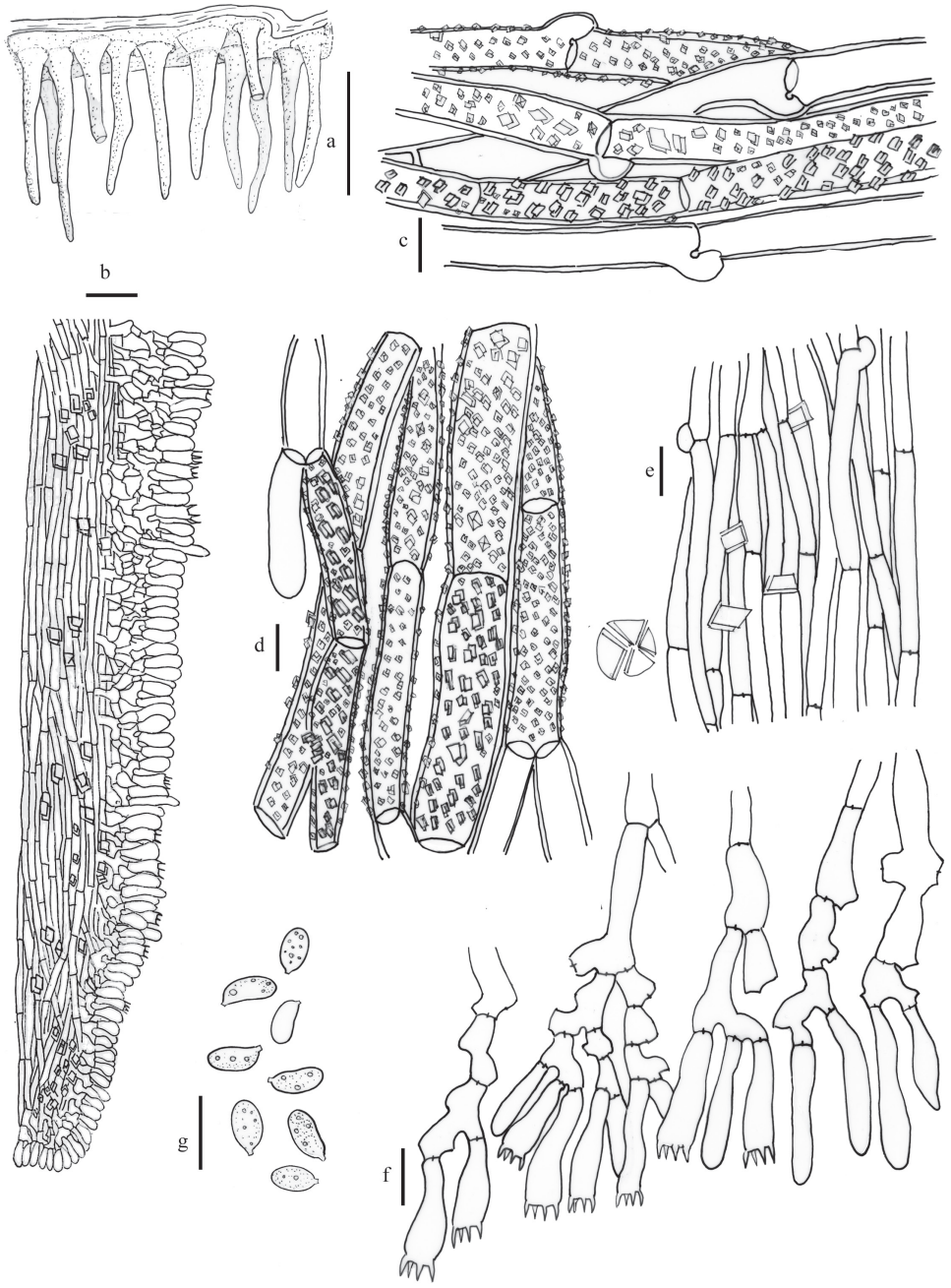

Figure 5. *Hydnophlebia chrysorhizon*. Collection NY, lectotype **a** Hymenophore **b** Vertical section through an aculei **c** Subicular hyphae **d** Strand hyphae **e** Aculei hyphae **f** Subhymenial hyphae and basidia **g** Spores. Scale bars: **a** = 1mm; **b** = 25 μm; **c–g** = 10 μm. Drawing by M. Dueñas.

3. *Hydnophlebia gorgonea* Telleria, M. Dueñas & M.P. Martín, sp. nov.

MycoBank MB815730
Figs 6, 7

Diagnosis. Morphologicaly this species is similar to *Hydnophlebia canariensis*, but can be distinguished by the strands, well developed in *H. canariensis* and poorly so in *H. gorgonea*. Spores ellipsoid to broadly ellipsoid 5–7 × 4–4.5 µm (L/W = 1.4).

Type. Cape Verde. São Vicente: Mindelo, Ribeira da Vinha, 16°51'49"N; 25°00'09"W, 10 m alt., on *Phoenix atlantica*, 26 September 2010, M.T. Telleria, 19111Tell. (holotype: MA-Fungi 86659), LSU sequence KF528140, ITS sequence KF483049.

Etymology. Named after Gorgades, an ancient name for the Cape Verde Islands, Atlantic Ocean.

Description. Basidiome resupinate, effuse, membraneous, easily separable, light orange-yellow (70. l. OY) to brilliant orange-yellow (67. brill. OY). Hymenophore hydnoid, aculei conical, 0.6–1 mm long. Margin fimbriate, white, with poorly developed strands.

Hyphal system monomitic; subicular hyphae 6–8 µm wide, with clamps, thin- to thick-walled, loosely interwoven, hyaline, encrusted with colorless crystals; strand hyphae 12–15 µm wide, without clamps, thick-walled, sometimes gelatinous and also encrusted; aculei hyphae 3.5–4.5 µm wide, without clamps, thin-walled, colorless, growing perpendicular to the substrate; subhymenial hyphae 4.5–8 µm wide, without clamps, thin-walled, colorless, loosely interwoven, and short- to long-celled. Cystidia cylindrical to ventricose, sometimes capitate, thin-walled, 45–55 × 4–6 µm. Basidia cylindrical to subclavate, 22–24 × 6–8 µm, with 4 sterigmata, basal clamp absent. Spores ellipsoid to broadly ellipsoid 5–7 × 4–4.5 µm (L/W = 1.4), thin-walled, colorless, smooth.

Ecology and distribution. This species is known from only two localities of São Vicente Island, Cape Verde Archipelago, on decayed wood of *Phoenix atlantica* and *Prosopis juliflora* in arid habitats.

Other specimens examined. Cape Verde. São Vicente: Mindelo, Ribeira da Vinha, 16°51'49"N 25°00'09"W, 10 m alt., on *Prosopis juliflora,* 26 September 2010, M.T. Telleria, 19110Tell. (MA-Fungi 86658), LSU sequence KF528139, ITS sequence KF483048; M. Dueñas, 13327MD (MA-Fungi 86642), LSU sequence KF528122, ITS sequence KF483031. São Vicente: Ermida, 16°50'26"N; 24°57'23"W, 100 m alt., on *Prosopis juliflora*, 26 September 2010, M.T. Telleria, 19133Tell. (MA-Fungi 86664), LSU sequence KF528145, ITS sequence KF483054.

4. *Hydnophlebia meloi* Telleria, M. Dueñas & M.P. Martín, sp. nov.

MycoBank MB815731
Figs 6, 8

Diagnosis. Similar to *Hydnophlebia omnivora* but differs in having subglobose spores, 4–5.5 × 3–4 µm (L/W = 1.2), instead of ellipsoid, 5–6.5 × 3–4 µm (L/W = 1.6). This is the only species in the genus with subglobose spores.

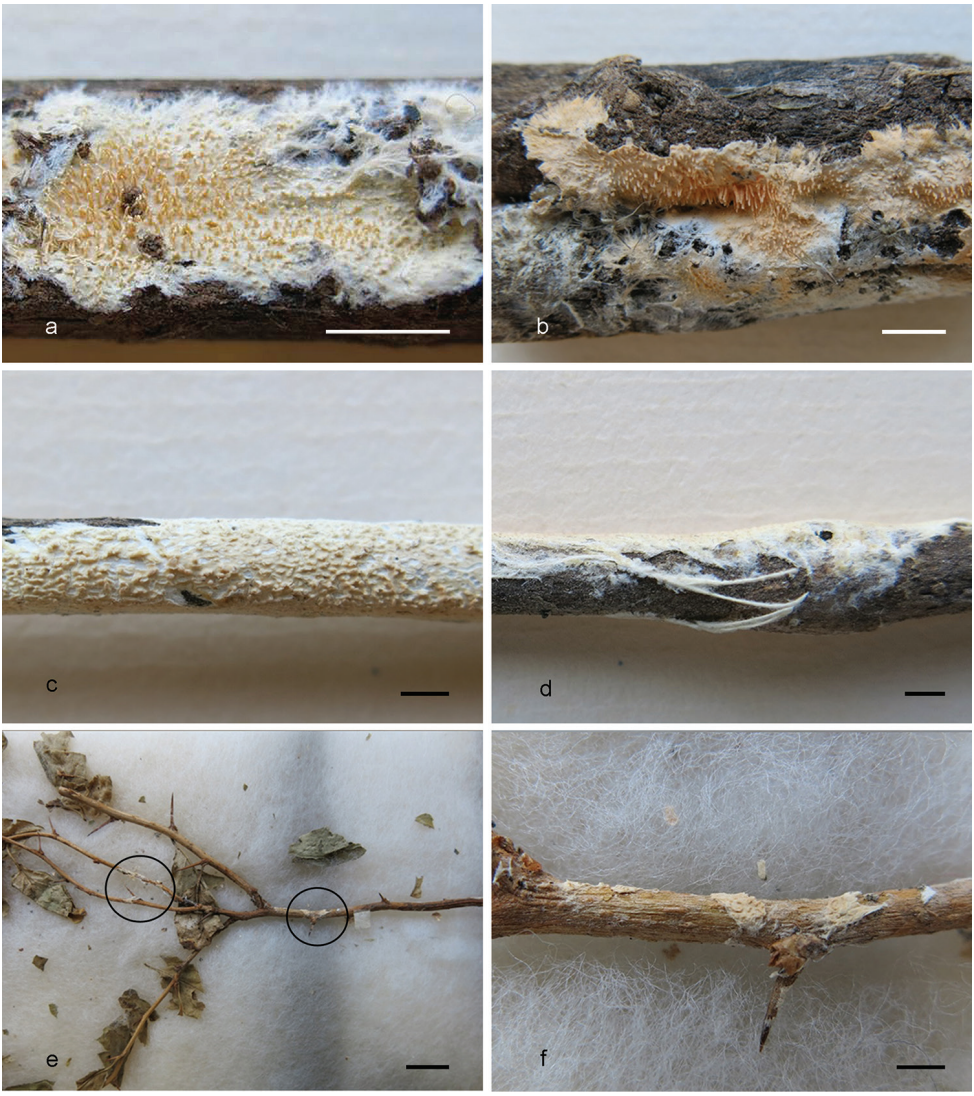

Figure 6. *Hydnophlebia gorgonea.* **a** Collection 19111Tell., MA-Fungi 86659, holotype, basidiome, dry specimen **b** Collection 19133Tell., MA-Fungi 86664, basidiome, dry specimen. *Hydnophlebia meloi* **c**, **d** Collection 19071Tell., MA-Fungi 86654, holotype, basidiome, dry specimen (c), and margin and strands, dry specimen (d). *Hydnophlebia omnivora* **e** Collection 5267 C.R. Shear coll., BPI, holotype **f** Basidiome, dry specimen. Scale bars: **a–b** = 5 mm; **c–d, f** = 2 mm; **e** = 10 mm.

Type. Cape Verde. Fogo: Mosteiros, Miradouro, 15°01'41"N; 24°19'13"W, 283 m alt., on *Sarcostemma daltonii*, 24 September 2010, M.T. Telleria, 19071Tell. (holotype: MA-Fungi 86654), LSU sequence KF528135, ITS sequence KF483044.

Etymology. Named after Ireneia Melo, colleague and friend, Portuguese mycologist from the Botanical Garden of the University of Lisbon.

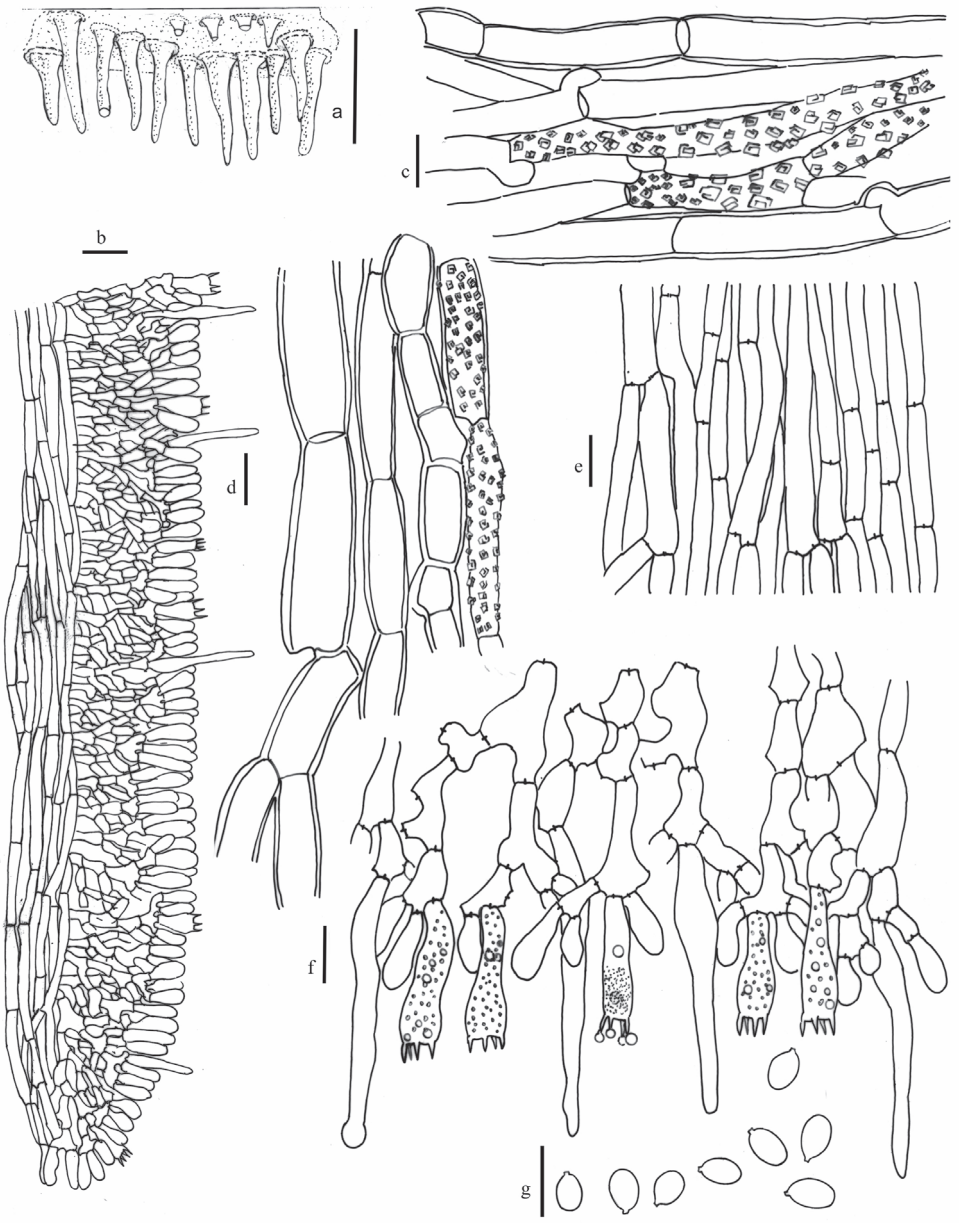

Figure 7. *Hydnophlebia gorgonea.* Collection 19111Tell., MA-Fungi 86659, holotype **a** Hymenophore **b** Vertical section through an aculei **c** Subicular hyphae **d** Strand hyphae **e** Aculei hyphae **f** Subhymenial hyphae, cystidia, and basidia **g** Spores. Scale bars: **a** = 1 mm; **b** = 25 µm; **c–g** = 10 µm. Drawing by M. Dueñas.

Description. Basidiome resupinate, effuse, membraneous to ceraceous, yellowish white (92. y White) to pale orange-yellow (73. p. OY) in dry specimens. Hymenophore hydnoid, aculei conical, 0.5–1 mm long, in dried specimens usually broken. Margin fimbriate, yellowish white, with strands.

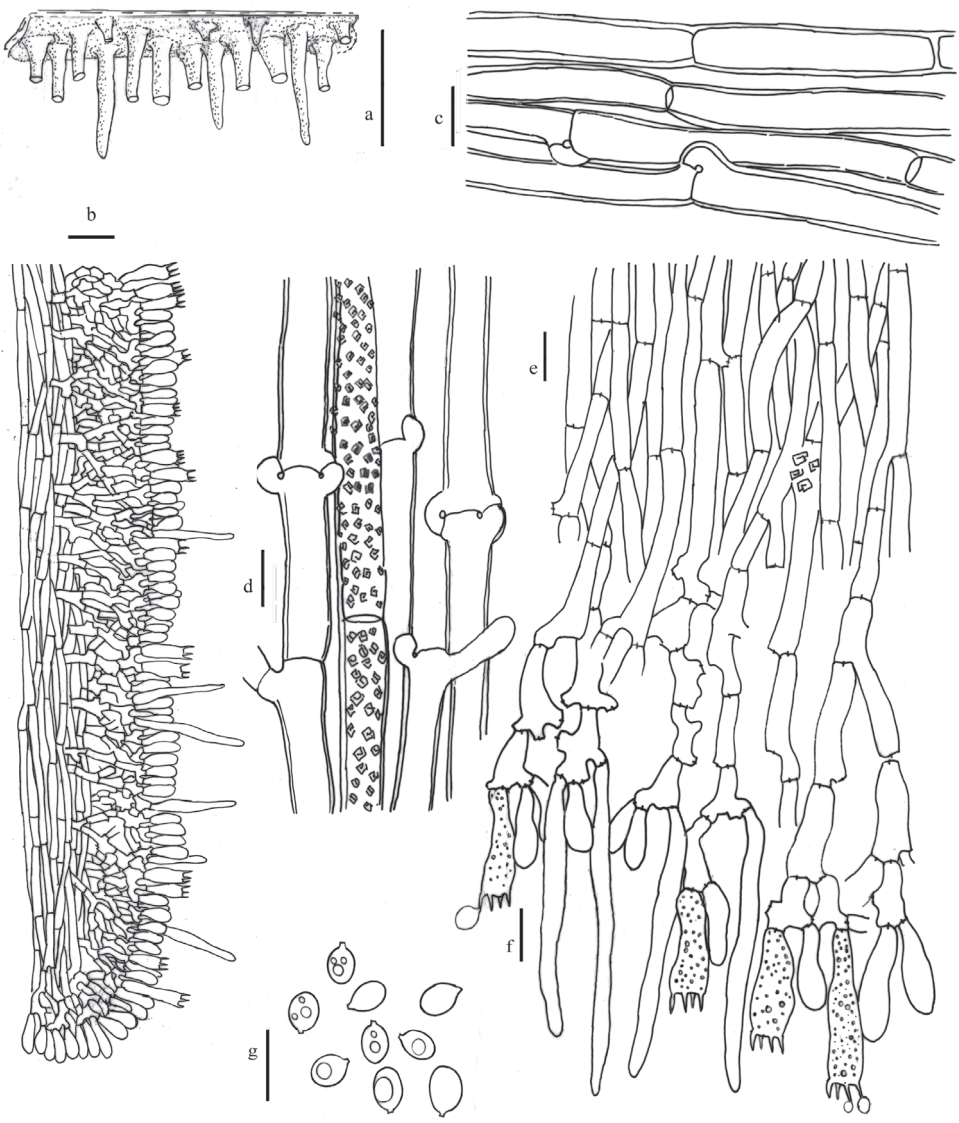

Figure 8. *Hydnophlebia meloi.* Collection 19071Tell., MA-Fungi 86654, holotype **a** Hymenophore **b** Vertical section through an aculei **c** Subicular hyphae **d** Strand hyphae **e** Aculei hyphae **f** Subhymenial hyphae, cystidia, and basidia **g** spores. Scale bars: **a** = 1 mm; **b** = 25 µm; **c–g** = 10 µm. Drawing by M. Dueñas.

Hyphal system monomitic; subicular hyphae 6–7.5 µm wide, with clamps, thick-walled, loosely interwoven; strand hyphae 6–7.5 µm wide, with clamps occasionally double, thick-walled, sometimes encrusted with colorless crystals; aculei hyphae 3.5–4.5 µm wide, without clamps, thin-walled, growing perpendicular to the substrate; sub-

hymenial hyphae 3.5–4.5 µm wide, without clamps, thin-walled, loosely interwoven, short- to long-celled. Cystidia cylindrical, thin-walled, 40–55 × 3–4 µm. Basidia cylindrical to subclavate, 18–26 × 5–7 µm, with 4 sterigmata, basal clamp absent. Spores subglobose 4–5.5 × 3–4 µm (L/W = 1.2), thin-walled, colorless, smooth.

Distribution. Rocky steep slopes, on *Sarcostemma daltonii*, endemic climbing herb of Cape Verde Archipelago. Only known from the type locality in Fogo Island.

Other specimens examined. Cape Verde. Fogo: Mosteiros, Miradouro, 15°01'41"N; 24°19'13"W, 283 m alt., on *Sarcostemma daltonii*, 24 September 2010, M.T. Telleria, 19072Tell. (MA-Fungi 90746).

5. *Hydnophlebia omnivora* (Shear) Hjortstam & Ryvarden, Synopsis Fungorum 26: 10-23. 2009

Figs 6, 9

Basionym. *Hydnum omnivorum* Shear, J. Agric. Res. 30: 476. 1925

Type. USA, C.L.S. Type on Osage Orange [*Macura pomifera*], near Paris, Texas. C.R. Shear coll. Sept. 1903, no. 5267. In herbarium BPI! (holotype).

Description. Basidiome effuse in small and poorly developed patches, cream-coloured in dry specimens. Hymenophore, according to Burdsall (1985), hydnoid, aculei conical to subcylindrical, 0.6–1 mm long; broken or poorly developed in type material. Margin floccose to fibrillose, white, with strands poorly developed.

Hyphal system monomitic; subicular hyphae 8–11 µm wide, with clamps occasionally double, thick-walled, loosely interwoven; strand hyphae 5–9 µm wide, with a few clamps, thick-walled, colorless; aculei hyphae 4–5 µm wide, without clamps, thin-walled, growing perpendicular to the substrate; subhymenial hyphae 5–6 µm wide, without clamps, thin-walled, densely interwoven, short-celled. Cystidia cylindrical, slightly tapered to apex, thin-walled, 40–70 × 4–5 µm. Basidia cylindrical to subclavate, 17–21× 6–7 µm, with 4 sterigmata, basal clamp absent. Spores ellipsoid, 5–6.5 × 3–4 µm (L/W = 1.6), thin-walled, colorless, smooth.

Ecology and distribution. Described from Texas (Shear 1925). According to Burdsall (1985) this species is distributed in the arid regions of southwestern United States, and probably into southern California and northern Mexico. Also reported from Florida (Ginns and Lefebvre 1993) and Uruguay (Martínez and Nakasone 2005).

Remarks. Molecular analyses indicate that this species is related to *H. meloi.* Morphologically they can be distinguised by the shape and size of spores, subglobose 4–5.5 × 3–4 µm in *H. meloi*, and ellipsoid 5–6.5 × 3–4 µm in *H. omnivora*.

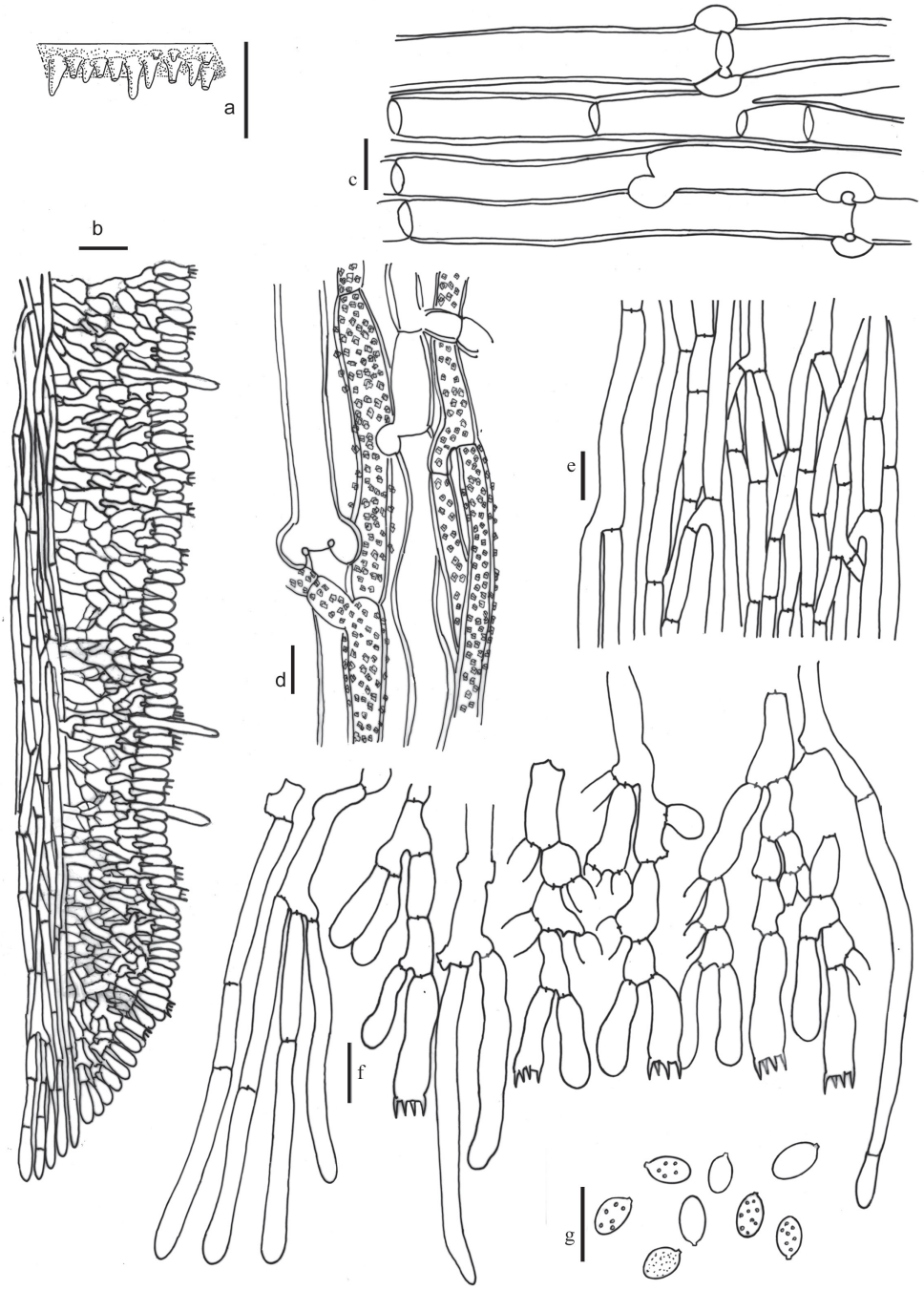

Figure 9. *Hydnophlebia omnivora*. Collection 5267 C.R. Shear coll., BPI, holotype **a** Hymenophore **b** Vertical section through an aculei **c** Subicular hyphae **d** Strand hyphae **e** Aculei hyphae **f** Subhymenial hyphae, cystidia, and basidia **g** Spores. Scale bars: **a** = 1 mm; **b** = 25 µm; **c–g** = 10 µm. Drawing by M. Dueñas.

Discussion

In this study a taxonomic analysis of *Hydnophlebia*, based on morphological and molecular data, is provided. *Hydnophlebia* has been confused with *Phanerochaete* and the two species included, *Hydnophlebia chrysorhizon* and *Hydnophlebia omnivora*, were assigned to the latter genus (Burdsall 1985).

For a long time, *Hydnophlebia* was considered a monospecific genus; however, based on the molecular analyses, both LSU and ITS sequences, as well as a point-by-point comparison of the morphological characters, five species can be discriminated, two already described by other authors (*H. chrysorhizon* and *H. omnivora*), and the three new species from Macaronesia described here (*H. canariensis, H. gorgonea*, and *H. meloi*).

Moreover our results show that three other species could be described, although more collections should be analysed: 1) *Hydnophlebia* sp. 1 under *H. omnivora* 1 in Floudas and Hibbett (2015); 2) *Hydnophlebia* sp. 2 under *Phlebia* sp. from Madagascar in UNITE database; and 3) *Hydnophlebia* sp. 3 under *Hydnophlebia* cf. *chrysorhizon* in Floudas and Hibbett (2015).

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Descolea quercina (Bolbitiaceae), a new species from moist temperate forests in Pakistan

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Abstract

A new species, *Descolea quercina*, is described and illustrated from Northern parts of Khyber Pakhtunkhwa, Pakistan. It is characterized by medium to large basidiomata, squamose to squamose-granulose hygrophanous pileus, and limoniform, verrucose basidiospores with partly concrescent verrucae. Phylogenetic analyses of nuc rDNA region encompassing the internal transcribed spacers 1 and 2 along with 5.8S rDNA (ITS) and nuc 28S rDNA D1-D2 domains (28S) also confirmed it as a new species. A comparison with similar taxa is provided.

Keywords

Basidiomycota, ectomycorrhiza, taxonomy

Introduction

The genus *Descolea* Singer was based on *D. antarctica* Singer, which has agaricoid basidiomata with an annulus, thus resembling *Rozites* or *Pholiotina* spp. (Horak 1971). *Descolea* is currently placed in the *Bolbitiaceae* (Kirk et al. 2008) and is characterized by dry to viscid pileus with or without squamules, central stipe with striated annulus, ochraceous spore deposit, amygdaliform to limoniform, verrucose basidiospores with a smooth apiculus, and a hymeniform pileipellis (Horak 1971). *Descolea* was once

considered to be restricted to the southern hemisphere, however, the known 15 species (Sharma and Kumar 2011) have a wide geographical distribution (Australia, India, Japan, Korea, New Guinae, New Zealand, Pakistan, Siberia, South America) (Horak 1971; Bougher and Malajczuk 1985; Niazi et al. 2007). From Pakistan, only *D. flavoannulata* (Lj.N. Vassiljeva) E. Horak was reported to date. During our macrofungal surveys, we collected a rare and interesting species of *Descolea* from two locations in Northern areas of Khyber Pakhtunkhwa, Pakistan. The species appeared unique and based on discrete morphological characteristics and sequences derived from nuc rDNA region encompassing the internal transcribed spacers 1 and 2 along with 5.8S rDNA (ITS) and nuc 28S rDNA D1-D2 domains (28S), it is described here as new to science.

Materials and methods

Collection and morphological characterization

Collections were made on routine mycological visits to the moist temperate *Quercus* dominated mixed forests of Malam Jabba (Swat district) and Toa valley (Shangla district), Khyber Pakhtunkhwa province, Pakistan. Basidiomata were collected following Lodge et al. (2004) and photographed in their natural habitats. Descriptions of the macro-characters are based on fresh collections and colored photographs. Color codes follow Munsell soil color charts (1975) and are presented in parenthesis after common color names.

Microscopic characters are based on free hand sections from fresh and dried specimens mounted in 5% (w/v) aqueous Potassium Hydroxide (KOH) solution. Measurements of anatomical structures are based on calibrated computer based software "PIXIMÈTRE version 5.9" connected to a compound microscope (BOECO, Model: BM120) and visualized through a microscopic camera (MVV 3000). A total of twenty basidiospores, basidia, cystidia and hyphae were measured from all the collections. For measurements; Q is the range of length/width (L/W) ratio of the total measured basidiospores; Qe is the average L/W ratio of all the measured basidiospores; Me is the average L × W of all the measured basidiospores. Surface of the basidiospores was studied both in 5% KOH solution and scanning electron microscopy (SEM).

DNA extraction

DNA from herbarium specimens was extracted following the procedure mentioned in Peintner et al. (2001). A primer pair ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990) was used to amplify the ITS region and primer pair LR5 and LR0R (Vilgaly's lab http://sites.biology.duke.edu/fungi/mycolab/primers.htm) was used to

amplify the 28S region. Polymerase chain reactions (PCR) were performed in 25 µL volume per reaction. PCR procedure for ITS region consisted of initial 4 minutes denaturation at 94°C, 40 cycles of 1 minute at 94°C, 1 min at 55°C, 1 min at 72°C, and a final extension of 10 minutes at 72°C. PCR procedure for 28S region consisted of initial denaturation at 94°C for 2 minutes, 35 cycles of 94°C for 1 minute, 52°C for 1 minute, 72°C for 1 minute, and final extension at 72°C for 7 minutes. Visualization of PCR products were accomplished using 1% agarose gel added with 3 µL ethidium bromide and a UV illuminator. Sequencing of the amplified products was accomplished through outsourcing (BGI, Beijing Genomic Institute, Hong Kong).

Phylogenetic analyses

The ITS region of the voucher collections MJ-1590, MJ-1590a and AST33 yielded a 725, 732 and 735 bp fragments respectively. Sequences of all the three specimens were used as a reference to BLAST against GenBank. All the query sequences matched 88% with *Descolea phlebophora* E. Horak (HQ533035) and *D. recedens* (Sacc.) Singer (KU523938) from New Zealand. Sequences of other genera, *Descomyces* Bougher & Castellano, *Timgrovea* Bougher & Castellano and *Setchelliogaster* Pouzar, were also downloaded for high similarity with query sequences and used in the subsequent phylogenetic analyses. *Hebeloma fastibile* (Pers.) P. Kumm (AF325643) and *H. circinans* (Quél.) Sacc. (JF908041) were selected as outgroup taxa (Peintner et al. 2001).

The 28S region yielded a 958 bp fragment for MJ-1590 and AST33, while the third collection (MJ-1590a) yielded a noisy sequence which was not included in the final analyses. The query sequences on blast showed 99% similarity with *Descolea recedens* (Sacc.) Singer (HQ827174), *Descolea maculata* Bougher (DQ457664) and *Descolea gunnii* (Berk. ex Massee) E. Horak (AF261523) from USA. Based on high similarity with query sequences, some unknown *Descomyces* species were also included in the phylogenetic analyses. *Hebeloma fastibile* (AY033139) and *H. affine* Smith, Evenson & Mitchel (FJ436324) were used as outgroup taxa.

DNA Sequences were aligned using online webPRANK tool at http://www.ebi.ac.uk/goldman-srv/webprank/ (Löytynoja and Goldman 2010). Maximum likelihood analyses for individual gene regions were performed via CIPRES Science Gateway (Miller et al. 2010) employing RAxML-HPC v.8. Rapid bootstrap analysis/search for best-scoring ML tree was configured for each dataset. For the bootstrapping phase, the GTRCAT model was selected. One thousand rapid bootstrap replicates were run. A bootstrap proportion of ≥ 70% was considered significant. Maximum parsimony (MP) analyses were performed using PAUP* 4.0b (Swofford 2002), with all characters of type unordered and equally weighted. Gaps were treated as missing data. Heuristic searches were performed with 1000 replicates with random taxon addition. MAXTREES was set to 5000 with MulTrees option in effect and TBR branch swapping. All characters were of type 'unord' and equally weighted.

Results

Molecular phylogenetic analyses

The ITS based analysis involved 27 nucleotide sequences. There were a total of 694 characters in the alignment file of which 345 characters were constant, 45 variable characters were parsimony-uninformative while 304 variable characters were parsimony-informative. The tree resulting from the ITS based ML analysis (Fig. 1) was similar to the MP. The distribution of *Descolea* species among different clades is in conformity with Peintner et al. (2001). The sequences from the Pakistani collections (MJ-1590, MJ-1590a and AST33) formed a separate clade with robust bootstrap support (ML 100% and MP 71%), supporting its independent position.

The 28S based analysis involved 17 nucleotide sequences with a total of 941 characters, out of which 867 characters were constant, 16 variable characters were parsimony-uninformative and 58 variable characters were parsimony-informative. The ML phylogram (Fig. 2) was found congruent with MP phylogram (not shown). The sequences from Pakistani collections (MJ-1590 and AST33) formed a separate clade (Fig. 2), with was poorly supported by bootstrap values (ML 71% and MP 73 %), but tree topologies further support its unique position.

Taxonomy

Descolea quercina **J. Khan & A. Naseer, sp. nov.**
MycoBank no: MB820545
Figures 3–5

Type. PAKISTAN. Khyber Pakhtunkhwa Province, Swat district, Malam Jabba valley, 1950 m alt., 25 July 2015, Junaid Khan, MJ-1590, (holotype: SWAT000135).

Diagnosis. Basidiomata medium to large, pileus convex to convex-campanulate with a broad umbo in young stages, light yellowish brown to deep yellowish brown, surface dry, hygrophanous, squamose to squamose-granulose with striate margin; basidiospores limoniform, coarsely verrucose with partly concrescent verrucae.

Description. ***Pileus*** 50–70 mm diameter, convex to convex-campanulate with a broad umbo when young, plane to plano-concave by maturity, light yellowish brown (7.5YR 7/4) to deep yellowish brown (10YR 3/8) with or without olivaceous tinge, surface hygrophanous, squamose to squamose-granulose, scales more or less concentrically arranged, loose, disappearing in age, margin striate; context strong yellowish brown (10YR 5/8), moist, thicker at the center (2–3 mm), color unchanging upon cutting. ***Lamellae*** adnexed, close, light grayish brown (7.5YR 6/4) in young specimens, yellowish brown in mature specimens (7.5YR 7/4), lamellar edge even, lamellulae present, mostly 3 in number, rarely single, often crisped at terminals, some lamellae forking near the stipe. ***Stipe*** 50–70 × 8–12 mm, central,

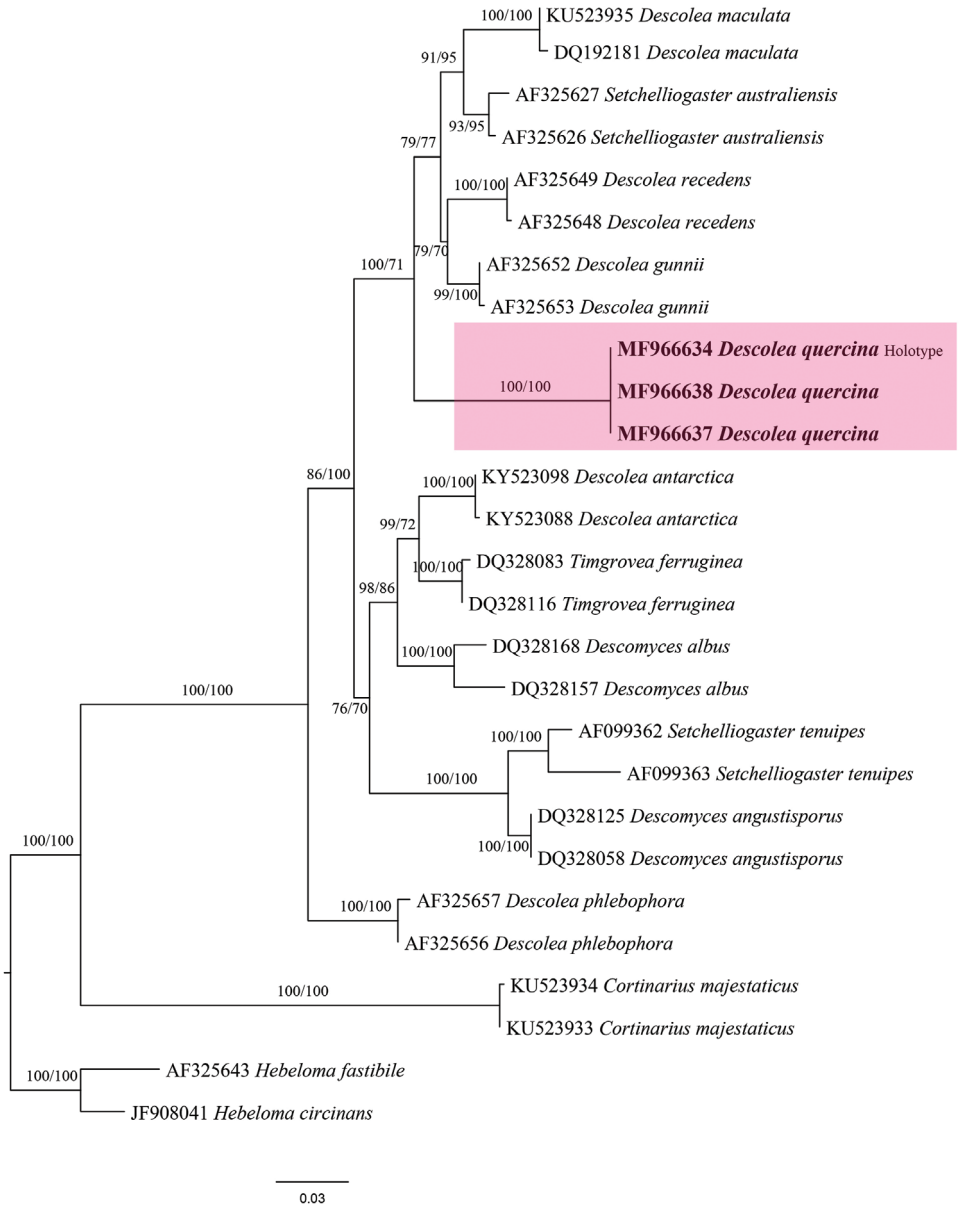

Figure 1. Phylogenetic relationship of *Descolaea quercina* and associated taxa inferred from ITS data. All positions with less than 70% site coverage were eliminated. Maximum likelihood and Maximum parsimony Bootstraps are shown close to the nodes. *Descolea quercina* is represented in boldface

thickening towards base, light yellowish brown (7.5 YR 7/4) to strong yellowish brown (10YR 4/8) and smooth above the ring , yellowish brown (10YR 5/6) and longitudinally fibrillose below the annulus; annulus membranous, concolorous with the lamellae, strongly striate on the upper surface, smooth to slightly scaly

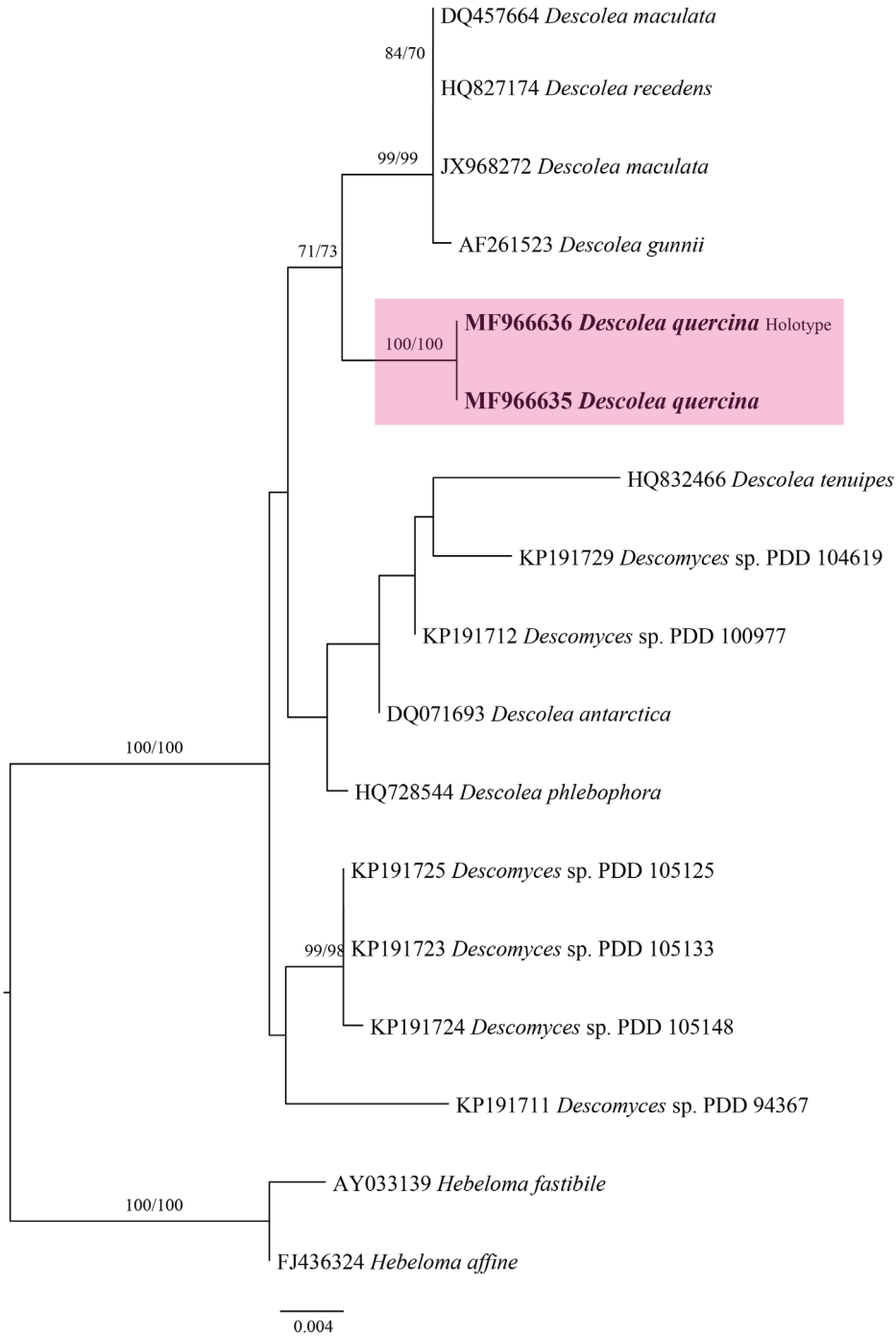

Figure 2. Phylogenetic relationship of *Descolaea quercina* and associated taxa inferred from 28S data. All positions with less than 70% site coverage were eliminated. Maximum likelihood and Maximum parsimony Bootstraps are shown by the nodes. *Descolea quercina* is represented in boldface.

Figure 3. a–e Basidiomata of *Descolea quercina* sp.nov. **a, b** AST33 **c, d** MJ-1590 (Holotype) **e** Natural habitat (MJ-1590a). Scale bars 12mm for **a, b**; 40 mm for **c–e**

below, margin appendiculate; context fibrous, interior hollow at the center, flesh whitish above the annulus, yellowish brown (10YR 5/6) below, moist. Smell and taste rancid when cut.

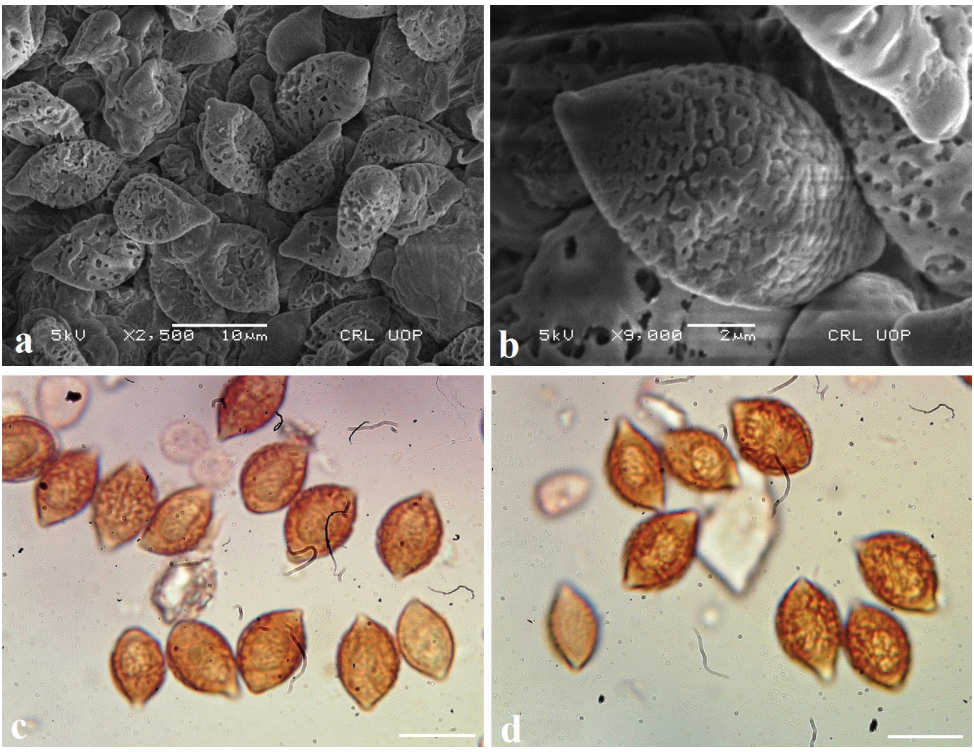

Figure 4. a–d Basidiospores of *Descolea quercina* (MJ-1590) **a**, **b** SEM **c**, **d** in KOH solution. Scale bars 10 µm for **a, c, d**; 2 µm for **b**.

Basidiospores (10–) 11.5–13 (–14) × (6.5–) 6.7–8.6 (–9) µm, Q = 1.4–1.7 (–1.9), Me = 12.0 × 7.9 µm, Qe = 1.5, limoniform, with prominent papilla, coarsely verrucose, verrucae partly concrescent, with prominent smooth apiculus, perispore distinct, without germ-pore, plage smooth, rust brown in KOH. ***Basidia*** 25–40 × 8–12 µm, clavate, tetra-sterigmated, rarely bi sterigmated, sterigmata 3–5 µm long, with clamp connections at the bases. ***Cheilocystidia*** 40–45 × 10–15 µm, broadly clavate to clavate, some with acute apices 4–6 µm long. ***Pleurocystidia*** similar to cheilocystidia, lanceolate to clavate, some with sub-acute to sub-capitate apices, appendix longer (6–8 µm) than with cheilocystidia. ***Pileipellis*** a hymeniform layer, consisting of broadly clavate, clavate to fusiform elements, 20–25 × 10–20 µm, strongly encrusted with golden brown pigment. Hyphae of the universal veil thin walled, cylindrical, 3–6 µm in diameter, strongly encrusted with golden brown pigment, clamp connections present.

Known distribution. PAKISTAN, Khyber Pakhtunkhwa province, Swat district, Malam Jabba valley, Kishawra village. PAKISTAN Khyber Pakhtunkhwa Province, Shangla district, Toa valley.

Ecology. Associated with *Quercus* species. Season July-August

Etymology. The epithet "quercina" refers to association of this taxon with *Quercus* species.

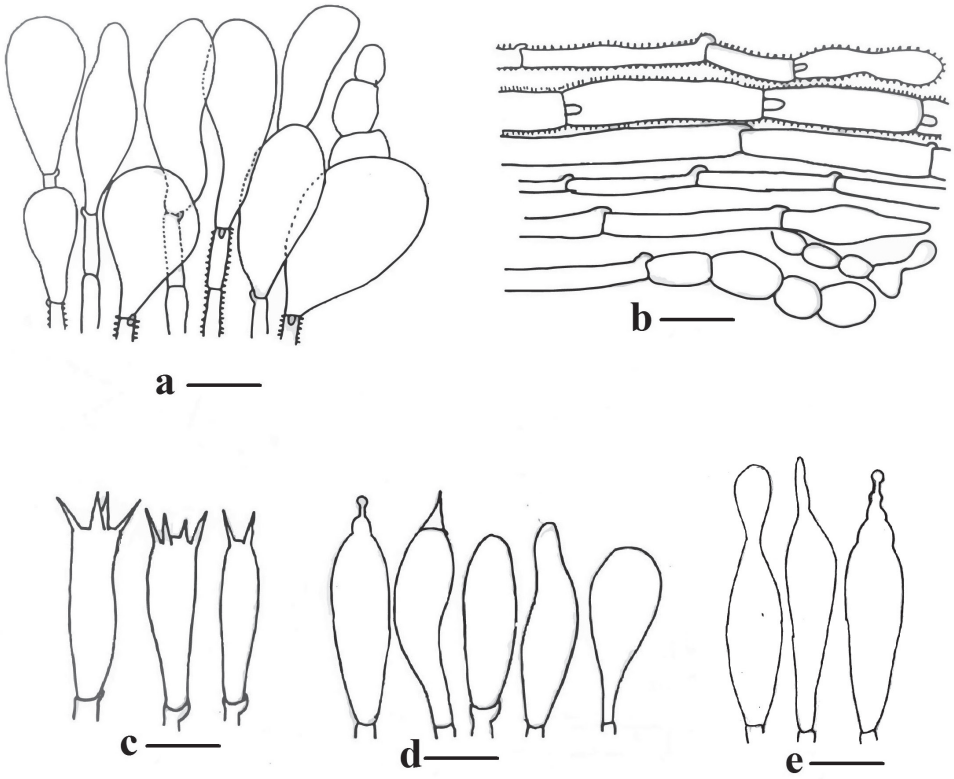

Figure 5. a–e Microscopic structures of *Descolea quercina* (Holotype): **a, b** Pileipellis **c** Basidia **d** Cheilocystidia **e** Pleurocystidia. Scale bars 13 µm for **a, b**; 16 µm for **c–e**.

Conservation status. The species is very rare and is currently reported from two locations in the districts of Shangla and Swat in the northern areas of Khyber Pakhtunkhwa province, Pakistan.

Additional specimens examined. Pakistan, Khyber Pakhtunkhwa province, Shangla district, Toa valley, 2000 m alt., among decomposing litter under *Quercus incana*, 15 July 2015, Arooj Naseer, AST33, (LAH35218). Pakistan, Khyber Pakhtunkhwa province, Swat district, Malam Jabba valley, 1900 m alt., on soil under *Quercus dilatata* Royle, 25 July 2015, Junaid Khan, MJ-1590a, (LAH35219).

Discussion

Descolea quercina is characterized by medium to large basidiomata, with light yellowish brown to deep yellowish brown color, hygrophanous and squamose to squamose-granulose pileus, light brown to yellowish brown stipe with strongly striated annulus and fibrillose base, and limoniform, coarsely verrucose basidiospores with a smooth apiculus covering the partly concrescent verrucae.

Table 1. Morphological comparison of *Descolea quercina* with morphologically similar species.

Characters/ Species	Size of fruiting body	Color	Surface features	Size and shape of basidiospores	Ornamentation	Associated with
D. quercina sp. nov.	Pileus 50–70mm, stipe 50–70 × 8–12 mm	Light yellowish brown to deep yellowish brown with or without olivaceous tinge	Squamose to squamose-granulose, margin striated	11.5–13 × 6.7–8.6 µm, Q = 1.5, limoniform	Coarsely verrucose, verrucae partly concrescent	*Quercus*
D. flavoannulata (Lj.N. Vassiljeva) E. Horak.	Pileus 50–80 mm, stipe 60–100 × 7–10 mm	Melleous ocher to dark brown	Radially wrinkled, sprinkled with concentrically arranged, small, floccose scales	12–16 × 8–9 µm , limoniform	Coarsely verrucose	*Castanopsis*, *Larix*, *Pinus*, *Quercus*
D. gunnii (Berk. ex Massee) E. Horak	Pileus 10–45 mm, stipe 15–60 × 1.5–7 mm	Dark brown to ochraceus	Densely appressed fibrillose-squamulose, striated at the margin,	9.5–12 × 6–7 µm, sub–limoniform	Verrucose with smooth mucro	*Leptospermum*, *Nothofagus*
D. pallida E. Horak	Pileus 10–40 mm, stipe 20–60 × 2–5 mm	Yellowish to reddish-brownish	Distinctly slimy, radially wrinkled, striated at the margin	10–13 × 5–6.5 µm, amygdaliform–limoniform	Isolated minute warts	*Nothofagus*
D. phlebophora E. Horak	Pileus 10–30 mm, stipe 30–70 × 2–6 mm,	Reddish brown to liver brown	Deeply wrinkled at the center and radially veined, striate near the margin, veil remnants absent	8–11.5 × 5–6 µm, amygdaliform	Minutely warted	*Nothofagus*
D. pretiosa E. Horak	Pileus 70–85 mm, stipe 75–80 × 11–13 mm	Fuscous with slight olivaceous tinge to date brown when moist, becoming rich brownish ochraceous with olivaceous tinge	Strongly rugulose, with small, floccose, loose scales	12–14.5 × 7–8 µm, limoniform	Strongly verrucose by isolated warts	*Abies*, *Picea*, *Pinus*, *Taxus*
Cortinarius majestaticus (E. Horak) T.P. Andreson & Orlovich	Pileus 30–70 mm, stipe 40–80 × 8–15 mm	Dark brown with a olive-greenish tinge	Slimy, without squamules, margin striated and there wrinkled	12.5–15 × 7–8 µm, amygdaliform	Strongly warted	*Nothofagus*

Morphologically, *D. quercina* and *D. pretiosa* E. Horak, resemble each other, in particular because of similar sized basidiomata, color, and limoniform basidiospores. However, *D. pretiosa* differs from *D. quercina* by its strongly scaly pileus, somewhat larger basidiospores (12–14.5 × 7–8 µm) with isolated warts, and habitat under conifers (Horak 1971). *Descolea majestatica* E. Horak, because of similar size, color, and strongly warted basidiospores with a plage could also be misidentified as *D. quercina*. However, *D. majestatica* is easily differentiable by its slimy pileus lacking squamules and larger (12.5–15 × 7–8 µm), amygdaliform basidiospores (Horak 1971). Based on these differences and phylogenetic evidence *D. majestatica* recently was transferred to *Cortinarius*, *C. majestaticus* (E. Horak) T.P. Anderson & Orlovich (Anderson and Orlovich 2016). *Descolea flavoannulata* (Lj.N. Vassiljeva) E. Horak, another large-sized taxon already reported from Pakistan (Niazi et al. 2007), resembles *D. quercina* by somewhat similar basidiomata colors and limoniform basidospores, however, it has a radially wrinkled pileus and larger basidiospores (12–16 × 8–9 µm) without plage.

Based on phylogenetic evidence, *D. quercina* is sister to a clade circumscribing *D. maculata*, *D. gunnii* and *D. recedens*. *Descolea maculata* also has a pileus with appressed squamulae, similar colored basidiomata, and basidiospores of almost the same size (10–13 × 6–7.5 µm). But *D. maculata* has a rippled or wrinkled pileus surface and amygdaliform to sublimoniform basidiospores, which are minutely verrucose (Bougher and Malajczuk 1985). Comparison of *D. quercina* with other closely related species is given in Table 1.

Descolea quercina is a striking new species associated with *Quercus* in temperate areas of Pakistan. The ecology and biogeography of this species are particularly significant since most *Descolea* species associated with Fagaceae are native to the Southern hemisphere (New-Zealand, Australia, South America). The only known *Descolea* species associated with *Quercus* or *Castanopsis* and occurring in the Northern hemisphere are now *D. flavoannulata* and *D. quercina*.

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RESEARCH ARTICLE

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A new species and a new record of *Laccaria* (Fungi, Basidiomycota) found in a relict forest of the endangered *Fagus grandifolia* var. *mexicana*

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Abstract

Two species of *Laccaria* discovered in relicts of *Fagus grandifolia* var. *mexicana* forests in eastern Mexico are described based on the macro- and micromorphological features, and their identity supported by molecular analysis of the internal transcribed spacer (ITS) and large subunit (LSU) of the ribosomal RNA gene. The phylogeny obtained here showed that one of the Mexican species is nested in an exclusive clade which in combination with its striking morphological features, infers that it represents a new species, while the other species is placed as a member in the *Laccaria trichodermophora* clade. This is the first report in Mexico of *Laccaria* with *Fagus grandifolia* var. *mexicana* trees, with which the reported species may form ectomycorrhizal association. Descriptions are accompanied with illustrations of macro- and micromorphological characters and a discussion of related taxa are presented.

Keywords

Ectomycorrhizal fungi, ITS, Neotropical fungi, nLSU, Tricholomatales

Introduction

It has long been recognized that *Laccaria* species are important ectomycorrhizal associates of ectotrophic plants worldwide (Mueller 1992). They are known to form interactions, for example with members of the *Pinaceae*, *Dipterocarpaceae*, *Fagaceae*, *Betulaceae*, *Myrtaceae*,

Tiliaceae and *Salicaceae* (Kropp and Mueller 1999, Wilson et al. 2013). Some species as *Laccaria laccata* and *L. bicolor* have been considered host-generalists, and inclusive, have been subject of a lot of *in vitro* experimentation worldwide. However, recent studies developed based on molecular systematics showed that under those names, complexes of species are included (Taylor et al. 2006, Jargeat et al. 2010, Vincenot et al. 2012, Sheedy et al. 2013, Popa et al. 2014). A wide ectomycorrhizal host range has also been attributed to *L. amethystina*, but in this case it has some support for its generalist abilities at the population genetics level by Roy et al. (2008), while consideration for cryptic biological species was discarded, at least among the populations sampled in France.

In the monographic work of *Laccaria* by Mueller (1992), 19 species are recognized from North America, and 40 worldwide. New or potential undescribed species from different regions, based on morphological and molecular characteristics of fructifications, or on DNA identifications of environmental samples, have been discovered recently (Wang et al. 2004, Osmundson et al. 2005, Sheedy et al. 2013, Wilson et al. 2013, 2017, Montoya et al. 2015, Luo et al. 2016, Popa et al. 2014, 2016). Nowadays, MycoBank recognizes 112 records in this group of fungi, and additionally, Wilson et al. (2017) inferred 116 phylogenetic species from 30 countries covering the known geographic range of *Laccaria*. During the advances on the systematics of the group, a small number of morphological (macro- and microscopic) features had been found taxonomically informative (McNabb 1972, Mueller 1992), which may be the cause of false interpretations, leading to conceptual misunderstandings. In fact, since early taxonomic studies on the group, the need to revise the species of *Laccaria* commonly treated under names widely cited in the literature was considered as an important task, due to the existence of different, even undescribed species, confused under apparently well-known ones, such as in the groups of *L. laccata* (Scop.) Cooke and *L. proxima* (Boud.) Pat. (Singer 1967, Mueller and Sundberg 1981, Irving et al. 1985). For example, the study by Sheedy et al. (2013) based on DNA multigene sequences, even noted that cryptic phylogenetic species were not nested as sister taxa. Thus, strict species identifications and achieving phylogenetic inferences with stronger resolution in *Laccaria*, will aid in building a robust data set, dealing with each species ectomycorrhizal host range.

In Mexico, the reports of the diversity of the genus *Laccaria* include about 17 species (Aguirre-Acosta and Pérez-Silva 1978, Bandala et al. 1988, Montoya et al. 1987, 2015, Cifuentes et al. 1990, Pérez-Silva et al. 2006, Garibay-Orijel et al. 2009). The edibility and use of some species as food has been documented (e.g. Montoya et al. 1987, Montoya-Esquivel et al. 2002, 2003, Lampman 2007, Pérez-Moreno et al. 2008) and ectomycorrhizae formed under *in vitro* culture conditions, isolated from native specimens have also been achieved (Santiago-Martínez et al. 2003, Carrasco-Hernández et al. 2010, Galindo-Flores et al. 2015). Molecular studies on most of those records are needed not only to support their identifications but for being included in phylogenetic studies. *Laccaria roseoalbescens* T. J. Baroni, Montoya and Bandala, described as new (Montoya et al. 2015) from the mesophytic forest in Veracruz, was recognized under morphological features and confirmed through phylogenetic DNA sequence analyses and recently incorporated by Luo et al. (2016) in their mo-

lecular phylogeny to confirm the distinction of the new *L. rubroalba* X. Luo, L. Ye, Mortimer & K.D. Hyde from China.

We have under research the fungal community associated to the two southernmost relicts of mesophytic forests dominated by *Fagus grandifolia* var. *mexicana* in the American Continent. This tree species is currently in danger of extinction and in the Red list of Mexican cloud forest trees, inhabiting a narrow range of nearly 145 hm^2 in Mexico (Rodríguez-Ramírez et al. 2013, Montoya et al. 2017). Taking into account its current status, we consider important to document the associated fungal species with particular focus to the ectomycorrhizal forming species. During our study, we found two species of *Laccaria* which after their morpho- and molecular analyses we concluded that with strong support can be recognized, one as *L. trichodermophora* G.M. Mueller and the other, as a distinct undescribed species close to *L. angustilamella* Zhu L., Yang & L. Wang from China. As both are part of the unknown potential mycobionts of this endangered ectotrophic tree species, we were motivated to document them.

Materials and methods

Sampling and morphological study of basidiomes

Random visits were conducted during August–September 2005 and 2007, in two stands of *Fagus grandifolia* var. *mexicana* from Veracruz, Mexico, one in Acatlán Volcano, Acatlán (19°40'43.9"N; 96°51'9.8"W, 1840 m) and the other in Mesa de la Yerba, Acajete (19°33'37.2"N; 97°01'9.8"W, 1900 m). Basidiomes of *Laccaria* growing close to *Fagus* were gathered. Macromorphological characters and color were recorded, alphanumeric color codes in descriptions refer to Kornerup and Wanscher (1967). Measurements and colors of micromorphological structures were recorded in 3% KOH. Basidiospores were studied in Melzer's reagent. Methods to determine spore ranges are those used by Montoya and Bandala (2003), with 45–50 spores measured per collection (length and width of the sporoid excluding the ornamentation) and given as a range with the symbol $\overline{X}$ representing mean values. $\overline{Q}$ represents the basidiospore length/width ratio and is given as range of mean values. Line drawings were made with a drawing tube. The examined specimens studied are deposited in XAL herbarium (acronym from B. Thiers, continuously updated; Index Herbariorum: http://sweetgum.nybg.org/ih/). The SEM images were obtained after critical point drying of pieces of lamellae previously rehydrated in ammonia, fixed in glutaraldehyde and dehydrated in an ethanol series (Bandala and Montoya 2000).

DNA extraction, PCR amplification, and sequencing

Genomic DNA of the Mexican specimen was extracted according to Montoya et al. (2014). PCR was performed to amplify the ITS (Internal Transcribed Spacer) and LSU (Large Subunit) regions of the nuclear rDNA, using primers ITS1F, ITS5/ITS4,

LR0R/LR21, LR7 (Vilgalys and Hester 1990, White et al. 1990, Gardes and Bruns 1993). PCR conditions, as well as procedures for the purification of amplified PCR products, cycle sequencing reactions and their purification were done according to Montoya et al. (2014). Once sequences were assembled and edited, they were deposited at GenBank database (Benson et al. 2017) (Table 1).

Phylogenetic methods

The phylogenetic analysis was performed with the sequences obtained in this study, as well as some retrieved from GenBank (http://www.ncbi.nlm.nih.gov/) derived from the Blast analysis (only those that best match), and complemented with related sequences used by Osmundson et al. (2005), Montoya et al. (2015) and Wilson et al. (2017) (Table 1). For this purpose, we constructed a dataset (ITS+LSU) using PhyDE v.0.9971 (Müller et al. 2010), also with MEGA 6.06 (Tamura et al. 2013) calculated the best evolutionary model and constructed the phylogenetic tree under the method of Maximum Likelihood (ML) with 500 bootstrap replications, and finally with MrBayes v 3.2.6 (Ronquist et al. 2012) constructed the phylogenetic tree (as Montoya et al. 2014) under the method of Bayesian Inference (BI). The phylogenies from ML and BI analyses were displayed using Mega 6.06 and FigTree v1.4.3 (Rambaut 2016) respectively.

Results

A total of 13 new ITS and 28S sequences for *Laccaria* were generated in this study (Table 1 and alignment in TreeBASE S21413). They were obtained from *Laccaria* samples proceeding from the two stands of *Fagus grandifolia* var. *mexicana* in the subtropical cloud forest in central Veracruz (sample AR24 comes from a conifers forest in Veracruz) (Table 1). Only bootstrap values of ≥70% and posterior probabilities (ML/PP) of ≥0.90 were considered and indicated on the tree branches. The phylogeny displayed (Fig. 1) inferred the Mexican samples clustered in two distinct clades. A group clearly related to *Laccaria trichodermophora* and another, in a separate clade, representing an undescribed species.

Taxonomy

Laccaria squarrosa **Bandala, Montoya & Ramos, sp. nov.**
MycoBank: MB823034
Figs 2–5

Holotype. MEXICO, Veracruz State, Mpio. Acatlán, Volcán de Acatlán, Aug 14 2007, DM 63 (XAL). Terrestrial under *Fagus grandifolia* var. *mexicana*.

Table 1. *Laccaria* taxa included in this study: samples, location and GenBank accession number for sequences.

Taxon	Voucher	Location	GenBank	
			ITS	28S
Cortinarius violaceus	MTS 4854 (WTU)	USA: Washington	DQ486695	DQ457662
L. alba	AWW438	China: Yunnan-Shangrila	JX504094	JX504178
L. alba	F1120750	China	JX504126	JX504206
L. alba	F1121461	China	JX504129	JX504209
L. alba	GMM6131	China: Chang Bai Shan	JX504131	JX504210
L. amethystea	FP-98556	Germany: Vorpommern	DQ499640	–
L. amethystea	TUB 011464	Germany	AF539737	–
L. amethysteo-occidentalis	AWW556	USA: California, Nevada Co.	JX504107	JX504191
L. amethysteo-occidentalis	AWW590	USA: Oregon, Benton Co.	JX504112	JX504195
L. amethystina	ALB183	China: Tibet	JX504092	JX504176
L. amethystina	F1123822	USA: Wisconsin	KU685760	KU685911
L. amethystina	GMM7041	Russia: Caucasus	KU685654	KU685797
L. amethystina	GMM7621	France: Forest comaniale de Ste. Croix	JX504150	JX504224
L. amethystina	LaAM-08-1	–	JGI Genome	JGI Genome
L. angustilamella	BAP226	China: Yunnan	JX504118	JX504201
L. angustilamella	HKAS58714	China: Yunnan, Yongping	JX504168	JX504244
L. aurantia	KUN-F 78557-Type	China: Yunnan	JQ670895	–
L. aurantia	MB-FB-101109	China: Yunnan	JQ681209	–
L. bicolor	AWW539	USA: Illinois	KM067817	KU685763
L. bicolor	AWW537	USA: Illinois, Johnson Co.	JX504105	JX504189
L. major	GMM6012	Costa Rica	KU685758	KU685909
L. major	GMM6019	Costa Rica	KU685757	KU685908
L. nobilis	F1091206	USA: Michigan	KU685636	KU685779
L. ochropurpurea	JMP0038	USA: Wisconsin	EU819479	–
L. ochropurpurea	KH_LA06_016	USA: Louisiana	KU685721	–
L. ochropurpurea	PRL3777	USA: Illinois	KU685732	JX504246
L. ochropurpurea	PRL4777	USA: Illinois	KU685733	KU685883
L. proxima	F1133825	USA: Mississippi	KU685642	KU685786
L. roseoalbescens	LM5042	Mexico: Veracruz	KJ874327	KJ874330
L. roseoalbescens	LM5099-Type	Mexico: Veracruz	KJ874328	KJ874331
L. salmonicolor	GMM7596-Type	China: Tibet	JX504143	JX504218
L. salmonicolor	GMM7602	China: Tibet	JX504145	JX504220
L. sp.	A0561	Japan: Sapporo	JX504082	–
L. sp.	A0573	Japan: Narusawa	KU685617	–
L. sp.	GMM6800	Guatemala	KU685756	KU685907
L. squarrosa[a]	DM121	Mexico: Veracruz	MF669960	MF669967
L. squarrosa[a]	DM63-Type	Mexico: Veracruz	MF669958	MF669965
L. squarrosa[a]	DM93	Mexico: Veracruz	MF669959	MF669966
L. trichodermophora	TENN42523-Type	USA: Texas	DQ149868	–
L. trichodermophora	F1111951	Costa Rica	KU685640	KU685784
L. trichodermophora	GMM7733	USA: Texas, Tyler Co.	JX504157	JX504230
L. trichodermophora	KH_LA06_013	USA: Louisiana	KM067881	KU685872

Taxon	Voucher	Location	GenBank	
			ITS	28S
L. trichodermophora	GMM7735	USA: Texas	KM067872	–
L. trichodermophora	KH-LA06-012	USA: Louisiana	KM067880	–
L. trichodermophora	GMM7734	USA: Texas	KM067871	–
L. trichodermophora	KH-LA06-007	USA: Louisiana	KM067874	–
L. trichodermophora	KH-LA06-008	USA: Louisiana	KM067875	–
L. trichodermophora	tri1125225	USA: Rocky Mountains	DQ149855	–
L. trichodermophora	KH-LA06-010	USA: Louisiana	KM067878	–
L. trichodermophora	KH-LA06-011	USA: Louisiana	KM067879	–
L. trichodermophora	KH-LA06-009	USA: Louisiana	KM067876	–
L. trichodermophora	KH-LA06-009B	USA: Louisiana	KM067877	–
L. trichodermophora	KH-LA06-004	USA: Louisiana	KM067873	–
L. trichodermophora	HC-PNNT-112	Mexico: Mexico State	KT875031	–
L. trichodermophora	GO-2009-266	Mexico: Mexico State	KC152147	–
L. trichodermophora	HC-PNNT-157	Mexico: Mexico State	KT875032	–
L. trichodermophora	GO-2009-305	Mexico: Distrito Federal	KC152149	–
L. trichodermophora	GO-2010-124	Mexico: Veracruz	KC152144	–
L. trichodermophora	EF36	Mexico	KT354980	–
L. trichodermophora	CB08167	Mexico: Mexico State	KT875029	–
L. trichodermophora	GO-2009-228	Mexico: Mexico State	KC152146	–
L. trichodermophora	GO-2010-126	Mexico: Veracruz	KC152145	–
L. trichodermophora	GO-2010-082	Mexico: Tlaxcala	KC152152	–
L. trichodermophora	GO-2009-225	Mexico: Mexico State	KC152143	–
L. trichodermophora	GO-2009-484	Mexico: Tlaxcala	KC152151	–
L. trichodermophora	HC-PNNT-192	Mexico: Mexico State	KT875033	–
L. trichodermophora	GO-2009-210	Mexico: Mexico State	KC152148	–
L. trichodermophora	HC-PNNT-132	Mexico: Mexico State	KT875030	–
L. trichodermophora	GO-2009-314	Mexico: Jalisco	KC152150	–
L. trichodermophora	HC-PNNT-099	Mexico: Mexico State	KT875034	–
L. trichodermophora	GMM7714	USA: Texas	KM067867	–
L. trichodermophora	GMM7712	USA: Texas	KM067866	–
L. trichodermophora	GMM7716	USA: Texas	KM067869	–
L. trichodermophora	HMJAU26938	–	KP128033	–
L. trichodermophora	GMM7703	USA: Texas	KM067865	–
L. trichodermophora	GMM7697	USA: Texas	KM067863	–
L. trichodermophora	GMM7698	USA: Texas	KM067864	–
L. trichodermophora[a]	Montoya 4393	Mexico: Veracruz	MF669961	MF669968
L. trichodermophora[a]	Montoya 4394	Mexico: Veracruz	MF669962	MF669969
L. trichodermophora[a]	AR24	Mexico: Veracruz	MF669964	MF669970
L. trichodermophora[a]	Bandala 4282	Mexico: Veracruz	MF669963	–
Psathyrella rhodospora	MP133 MN	–	DQ267129	AY645058

[a]samples and sequences obtained here.

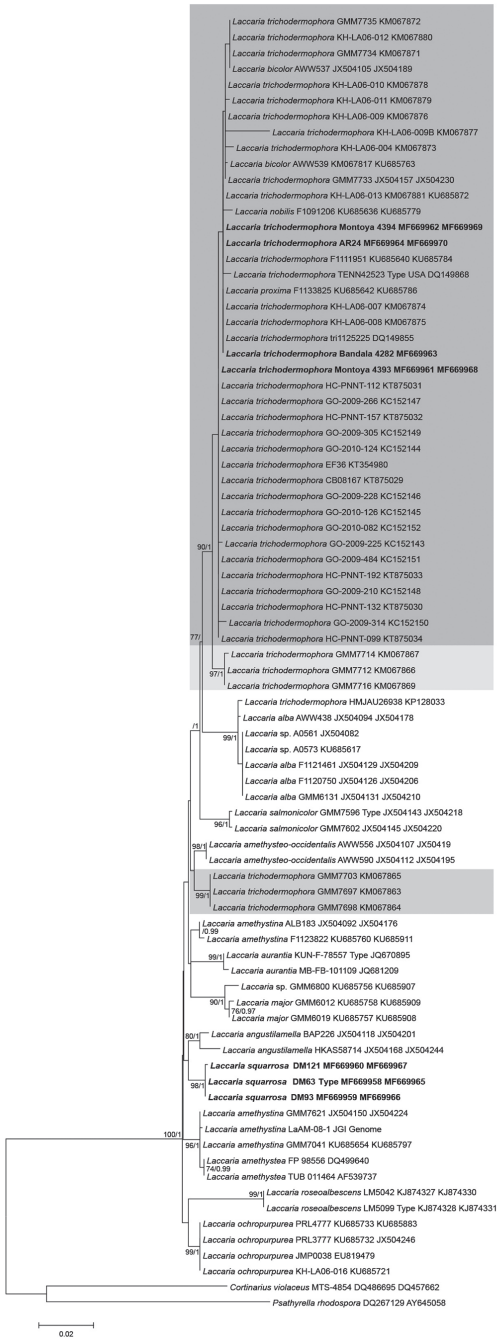

Figure 1. Phylogenetic relationships within *Laccaria* species inferred from the combined ITS and LSU sequence data by maximum likelihood method. Tree with the highest log likelihood (–4163.7219), the percentage of trees in which the associated taxa clustered together (only values ≥ 70% are considered) is shown next to the branches, followed by the posterior probabilities (only values ≥ 0.90 are indicated) obtained after Bayesian inference. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

Figure 2. *Laccaria squarrosa*, basidiomes. **a, b** habit **c, d** pileus surface details **a, c** DM 121 **b** DM 63 (holotype) **d** DM 93. Scale bars: 10 mm.

Diagnosis. *D*iffers from other species by having medium sized basidiomes, with pinkish to pale brownish-orange colors, smooth to finely squarrose surfaces, especially on the stipe, basal mycelium with whitish to pale brownish with pinkish tinges, and globose, echinulate basidiospores, 7–10 (-11.5) × 7–10.5 µm, with the echinulae 0.5–1.4 in length, 0.45–0.9 µm in width at base, subcylindrical to contorted cystidia and pileipellis arranged in a cutis with mounds of intermixed and irregularly projected hyphae.

Gene sequences ex-holotype. MF669958 (ITS), MF669965 (LSU).

Etymology. referring to the characteristic squarrose surfaces of basidiomata.

Pileus 10–82 mm diam convex to plane-convex, at times slightly depressed at center, surface squamulose to squarrose with age, pinkish (6B3–2) with pale yellowish tinges

Figure 3. *Laccaria squarrosa*, lamellae attachment and habit. **a, c** DM 121 **b** DM 63 (holotype) **d** DM 93. Scale bars: 10 mm.

towards the center or brownish-orange (5B6–5) when young; margin recurved, striate, edge thin. *Lamellae* 1–8 mm in length, adnate to subdecurrent, at times slightly undulate, subdistant or distant, pinkish to pale pinkish (6B4), 1–2 lamellulae per lamellae of different sizes. *Stipe* 50–155 × 5–9 mm, cylindrical, widened towards the base, squamulose to squarrose overall, more densely scaly towards the apex and when old, squamules brown, pinkish to ochraceous or ochraceous-orange. *Basal mycelium* pale whitish to brownish (6D6), with pinkish tinges in some areas. KOH negative overall surfaces.

Basidiospores 7–10 (-11.5) × 7–10.5 µm, $\bar{X}$=7.8–10.7 × 7.7–9.48 µm, $\bar{Q}$=1.01–1.12, globose, pale brownish, thin walled, hyaline, inamyloid, echinulate; under SEM the echinulae appear acute, 0.5–1.4 in length, 0.45–0.9 µm in width at base, shorter towards the hylar appendix area, this latter structure (also called the apiculus) consisting of a tube with rounded ending. *Basidia* 35–66 × 10–15 µm, clavate to narrowly clavate, thin walled, mostly tetrasporic, at times tri- or bisporic, sterigmata 10 µm length, some with refringent contents, clamped, hyaline. *Pleurocystidia* 20–38 × 3–6 µm, subcylindrical, contorted, sinuous, hyaline, thin walled. *Cheilocystidia* 14–40 × 2–5 µm, subcylindrical, rarely narrowly utriform, contorted, sinuous, hyaline, thin walled. *Pileipellis* a regular compact cutis, hyphae periclinally oriented, also with projected mounds of intermixed hyphae, which form the pileus scales irregularly projected; hyphae cylindrical, some widened 4–10 µm diam, some septate, hyaline, inamyloid, yellowish in

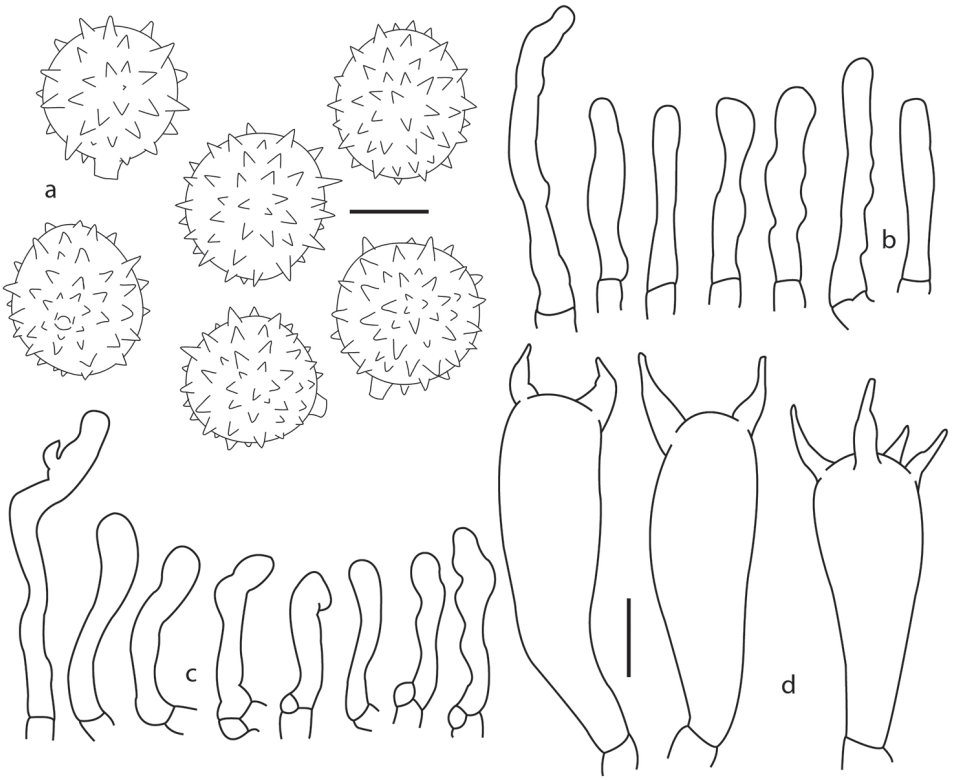

Figure 4. *Laccaria squarrosa*, **a** basidiospores **b** pleurocystidia **c** cheilocystidia **d** basidia **a, c** DM 121 **b** DM 63 (holotype) **d** DM 93. Scale bars: 5 μm (**a**); 10 μm (**b–d**).

mass and somewhat refringent in some parts, thin walled, clamped. *Context hyphae* cylindrical, faintly yellowish in mass, 4–9 (-14) μm diam, thin walled, up to 1 μm diam, hyaline, inamiloyd, septate. *Hymenophoral trama* regular, compact, composed by cylindrical hyphae, pale yellowish in mass, 3–8 μm diam, septate, hyaline and inamyloid, thin walled. *Clamps present*.

Habitat. Terrestrial, solitary, under *Fagus grandifolia* var. *mexicana.*

Additional studied material. MEXICO, Veracruz, Mpio. Acatlán, Volcán de Acatlán, Sep 18 2007, DM 121. Mpio. Acajete, Mesa de la Yerba, Aug 28 2007, DM 93 (all at XAL).

Discussion

In the phylogeny presented here that is based on sequences used in the worldwide survey of *Laccaria* by Wilson et al. (2017) and complemented with some from GenBank (Fig. 1) and sequences of *L. squarrosa*, described here, this new taxon is clearly shown to be phylogenetically isolated from other *Laccaria* species. *Laccaria squarrosa* is dis-

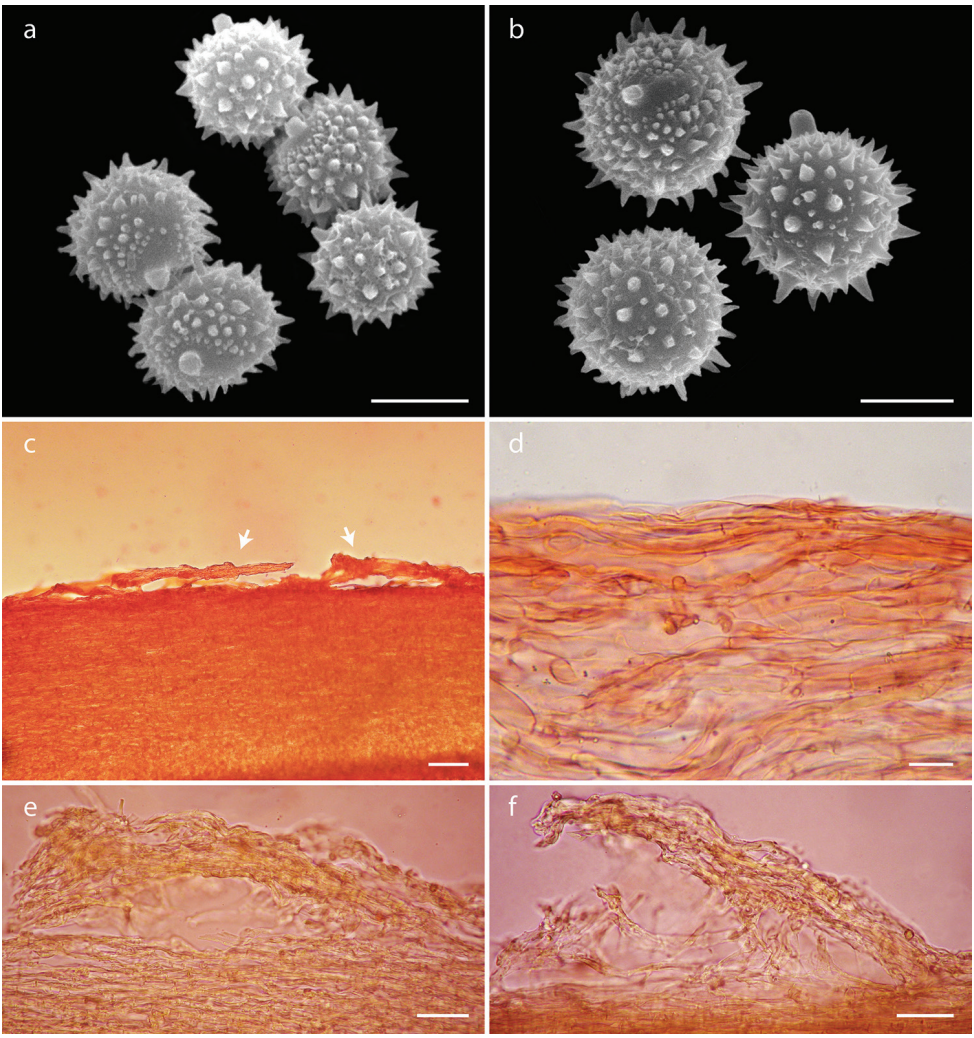

Figure 5. *Laccaria squarrosa*, **a–b** basidiospores under SEM **c–f** details of the pileipellis **c–d** cutis (arrow indicating scales) **e–f** details of the pileipellis scales **c–f** DM 63 (holotype). Scale bars: 5 µm (**a**); 2 µm (**b**); 100 µm (**c**); 20 µm (**d**); 50 µm (**e–f**).

tinct by possessing typical medium sized basidiomes with scaly surfaces, more obvious especially on the stipe and by having the basal stipe mycelium whitish to pale brownish with pinkish tinges. Microscopically it differs by globose, echinulate basidiospores, cylindrical cystidia and pileipellis arranged in a cutis with mounds of intermixed and irregularly projected hyphae. In Fig. 1, *L. squarrosa* is shown to be phylogenetically close to *L. angustilamella* Zhu L., Yang & L. Wang from China. This later species is characterized, however, by having a marasmioid to mycenoid habit, with a short basidiome size (pileus 20–30 mm diam), narrow (2 mm length) and subdistant lamellae,

non-scaly stipe, with more ellipsoid basidiospores (Q up to 1.18) and larger echinulae (2.0–) 2.5–5.0 µm long and up to 2.5 µm wide at base (Wang et al. 2004).

Color features of the basidiomes and whitish mycelia relate *Laccaria squarrosa* to metasection *Laccaria* (Mueller 1992), where it superficially resembles *L. proxima* (Boudier) Patouillard. This later species, however, can be distinguished based on the longitudinally striate stipe, with a fibrillose surface only, ellipsoid basidiospores [9–11.5 × 6.7–8 (-8.8) µm, Q = 1.25–1.35 (-1.4)], having shorter echinulae (0.5–1 µm length), pleurocystidia absence and larger cheilocystidia [19–66.5(-92) × 2–8.5(-16.5) µm] (Mueller 1992). Among the species in the genus, *Laccaria nobilis* A.H. Smith, *L. amethysteo-occidentalis* G.M. Muell., *L. trichodermophora* and *L. ochropurpurea* (Berk.) Peck also produce fibrillose to somewhat scaly pileus surfaces. *Laccaria ochropurpurea* even can have recurved scales on the stipe surface. However, all those taxa clearly differ from *Laccaria squarrosa* by basidiomes and mycelia with violaceous colors, besides other macro and microscopical features (Mueller 1992).

Laccaria trichodermophora G.M. Mueller (Figs 6–7) was previously reported from Mexico (as *L. farinacea* sensu Singer) by Montoya et al. (1987) from conifer forests of Cofre de Perote National Park areas. The collections from *Fagus grandifolia* var. *mexicana* forest here studied, were collected in the locality of Mesa de la Yerba (Veracruz), on Aug 04 2005, Montoya 4393, Montoya 4394; Aug 28 2007, Bandala 4282 (XAL). Excepting by narrower hyphae disposed in the pileipellis mounds and the basidiospores including broadly ellipsoid to ellipsoid shapes, exhibit a similar morphological variation as those described by Mueller (1984, 1992) and other collections reported before from Mexico. A summary of the main morphological features that characterize the studied materials are: *pileus* 15–55 mm diam, fibrillose to fibrillose-minutely scaly, brownish-orange (6C6–C7), light brown or pale pinkish-brown or pale brownish towards the margin (6D6, 7C5–C4), hygrophanous. *Lamellae* 2−6 mm in length, adnate to sinuate, close to subdistant, pinkish or incarnate (6A2−B3). *Stipe* 20–75 × 2–8 mm, cylindrical, attenuated towards the apex, striate, fibrillose and fistulous. hygrophanous, concolorous to pileus but more pinkish-violaceous (13CD2) towards the base. *Basal mycelium* violaceous becoming white. *Basidiospores* 6–9 (-10) × 6–8.5 (-10) µm, $\overline{X}$= 6.9–8.18 × 6.84–7.9 µm, $\overline{Q}$= 1.00–1.05, globose, hyaline, echinulate, under SEM the echinulae appear 0.8–1.13 × 0.6–0.8 µm. *Basidia* 27–65 (-80) × 7–13 µm, clavate, tetra or at times tri-sporic, hyaline, thin walled, clamped. *Cheilocystidia* 12–49 × 2–6 µm cylindrical to narrowly clavate, at times somewhat utriform, hyaline, thin walled, frequently clamped. *Pileipellis* composed of periclinally oriented hypahe 3–10 µm diam, in a more or less cutis arrangement but with frequent mounds of intermixed or erect hyphae, with terminal elements 25–125 × 10–13 µm, cylindrical to clavate other somewhat utriform 20–65 × 5–17 µm. *Clamps present*.

In the phylogeny obtained (Fig. 1), the sampled sequences of this species appear in three clades. One of them, with collections from North America, included the type specimen (DQ149868) and 21 specimens from Mexico. Our collections clustered in this later lineage interestingly with one sample from Costa Rica too. The other two clades are composed of specimens from Texas, one of them sister to the type clade, and

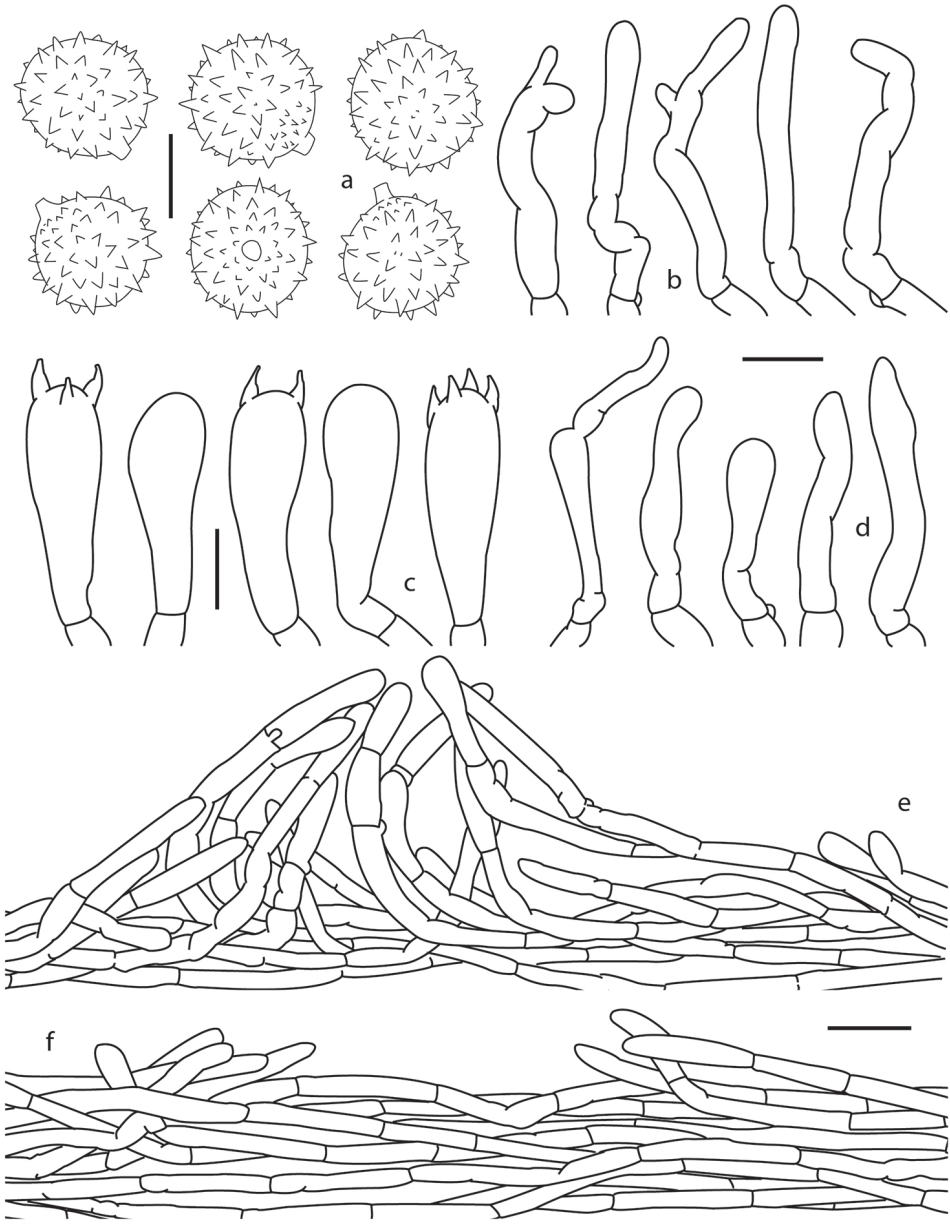

Figure 6. *Laccaria trichodermophora,* **a** basidiospores **b** pleurocystidia **c** basidia **d** pileipellis **e** cheilocystidia **a–e** Montoya 4393. Scale bars: 5 µm (**a**); 10 µm (**b–c, e**); 25 µm (**d**).

the third clearly separated, probably representing an undescribed species. A specimen (KP128033) labeled as *L. trichodermophora* in the GenBank, clustered in *L. alba* group from Asia in our analysis. This sample lacks geographic information and could well be a misidentified collection.

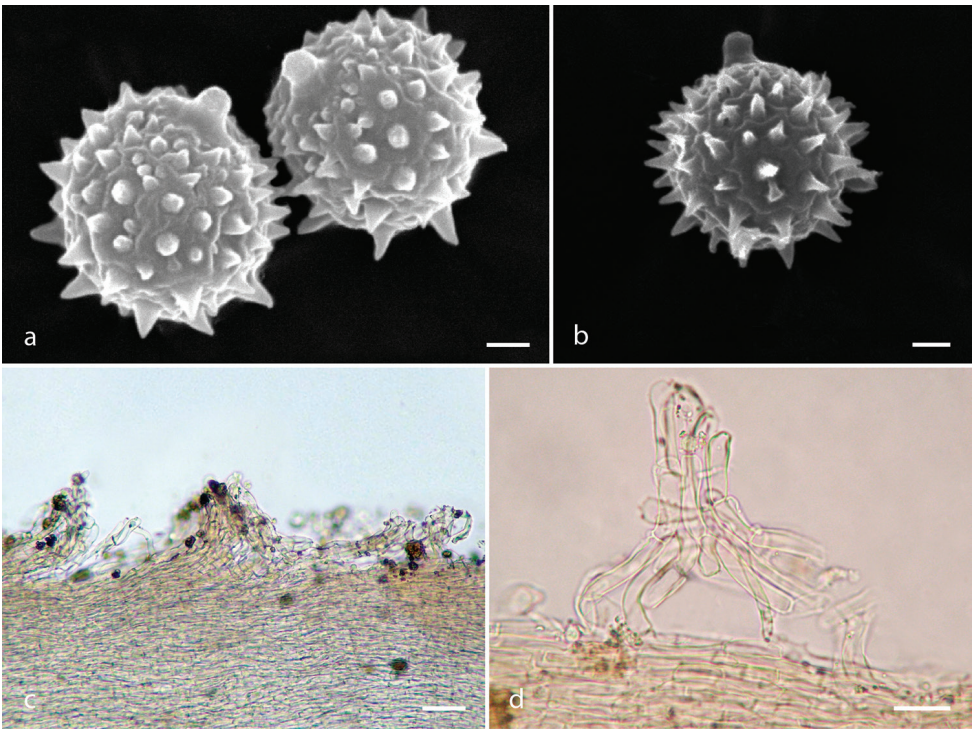

Figure 7. *Laccaria trichodermophora*, **a–b** basidiospores under SEM **c–d** pileipellis **a, c–d** Montoya 4393 **b** Bandala 4282. Scale bars: 1 µm (**a–b**); 50 µm (**c**); 25 µm (**d**).

There are no previous reports of *Laccaria trichodermophora* being associated with *Fagus grandifolia var. mexicana.* This report serves as the first documentation of this association. According to the reports of *L. trichodermophora*, it shows a wide ecological range. Mueller (1992) observed that all collections of this *Laccaria* species from the southeastern United States appeared to be associated with *Pinus*. He also collected it, in Costa Rica, beneath Neotropical species of *Quercus*. In central Mexico, in the states of Tlaxcala and Michoacán, it has been recorded associated to mixed *Pinus-Alnus* and *Pinus-Abies* forests (Montoya et al. 1987, Montoya-Esquivel et al. 2004). In the eastern part of Mexico, in Veracruz, it has been found (as *L. farinacea* sensu Singer) in monodominant *Pinus* and mixed *Pinus-Abies* forests (Montoya et al. 1987). In this later country, it is interesting to note that, basidiomes of this species, specially from conifers, are sold in local markets as edible fungi (Montoya et al. 1987, Montoya-Esquivel et al. 2004). Based on the available ecological information of the samples in the phylogenetic tree (Fig. 1), a wide host range for *L. trichodermophora* type specimen clade can be inferred. Among the potential hosts, it can be recognized as occurring with *Fagus grandifolia*, *Pinus elliottii*, *P. palustris* and *Quercus* sp. in Texas, as well as *P. patula*, other species of Pinaceae and *Quercus* spp. in both US and in Mexico, and the endangered *F. grandifolia* var. *mexicana* as confirmed here. *Abies religiosa* represents

another host also, as proved by data from two sequences (MF669964 and MF669970) (Table 1, Fig. 1) obtained here, from the sample AR24, from an *A. religiosa* forest at Cofre de Perote National Park in Veracruz, Mexico.

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