Fun with the discomycetes: revisiting collections of Korf's anamorphic *Pezizales* and Thaxter's New England truffles leads to a connection between forms and the description of two new truffle species: *Pachyphlodes pfisteri* and *P. nemoralis*

Rosanne HEALY
Caroline HOBART
Genevieve E. TOCCI
Lilla BÓNA
Zsolt MERÉNYI
Aurelia PAZ CONDE
Matthew E. SMITH

Ascomycete.org, 7 (6) : 357-366.
Novembre 2015
Mise en ligne le 30/11/2015

Summary: *Pachyphlodes* is a pleiomorphic pezizalean truffle genus, originally described from Europe. Color is an important diagnostic character for the species in this genus, and is naturally, if unfortunately, obscured in dried herbarium specimens. Therefore, some notable experts have understandably misidentified some of the species in herbaria. This study was part of a larger effort to use molecular phylogenetic analyses followed by morphological study to sort the relationships and species limits within *Pachyphlodes*. One clade of *Pachyphlodes* that we refer to here as the /nemoralis clade was previously unrecognized for its unique spore ornamentation of coalesced spine tips present in all species in this clade studied to date. A North American species was misidentified as *P. melanoxanthus*, while a European species was misidentified as *P. citrinus*, or *P. ligericus*. Here we describe these two species in the /nemoralis clade as *P. pfisteri* from North America and *P. nemoralis* from Europe. We also link a mitosporic form to *P. pfisteri*, and revisit the concept of the mitosporic form called *Glischroderma*. We dedicate this paper to Richard P. Korf, who's work on pezizalean anamorphs inspired this study.
Keywords: Ascomycota, *Pezizales*, *Pachyphlodes nemoralis*, *pfisteri*, *Pachyphloeus*, spore mats, *Glischroderma,* phylogeny.

Introduction

In 1994 Dr. Richard P. Korf gave a marvelous talk and paper titled "Fifty years of fun with the discomycetes and what's left to do" (Korf, 1994). This talk featured a visible mat of vegetative hyphae that is formed in either a discrete or indeterminate patch and that produces spores through mitosis (here after referred to as a "spore mat"). Korf and his students collected these spore mats on soil in the woods around Ithaca, New York. He recognized the affiliation of these spore mats with the *Pezizales* and he tentatively identified them as *Glischroderma* Fuckel, a poorly known form genus erected for a spore mat described from Germany (Fuckel, 1870: 34). His talk discussed the morphological ambiguity of this species (i.e. does it have a peridium?), its identity, and what was known about spore mats in other species that are affiliated with the *Pezizales*. He gave a piece of a spore mat to Dr. Keith N. Egger for DNA sequencing and the 28S rDNA sequence was phylogenetically affiliated with *Scabropezia* and *Pachyphlodes* (Norman & Egger, 1999). Here we revisit the morphology and phylogenetic affinity of Korf's spore mat collection and show that the sequences of this anamorphic form match the ascocarp of a new North American truffle species, *Pachyphlodes pfisteri* sp. nov. We also describe a European relative from the same molecular phylogenetic clade *Pachyphlodes nemoralis* sp. nov.

Material and Methods

Material studied

We studied fresh European and North American collections of spore mats and ascomata as well as herbarium collections borrowed from Cornell University (CUP), Harvard University (FH), Oregon State University (OSC), the Swedish Museum of Natural History (S), and the Real Jardín Botánico (MA) herbaria as well as specimens kindly provided by Dr. James M. Trappe. All measurements are based on material rehydrated in water (spores) or 5% KOH (to inflate asci and peridia). Reactions were recorded in 5% KOH and Melzer's solution. For each species description, average measurements were calculated based on 50 mature meiospores or mitospores and 20 asci. Material was observed in Melzer's solution to assess the amyloid reaction of asci. For study of the peridium in the spore mat of *Pachyphlodes pfisteri*, a fresh spore mat was fixed and embedded in resin following Curry & Kimbrough (1983) and then 20 serial sections 3 µm thick were cut with glass knives, mounted on slides and stained with Toluidine Blue O. For scanning electron microscopy (SEM), dried material was revived in 3% KOH and then gradually dehydrated to absolute ethanol followed by critical point drying (CPD). CPD pieces of gleba were placed on carbon tape on aluminum stubs, sputter coated with gold/palladium, and viewed with 10 kV on a scanning electron microscope in the University Imaging Center at the University of Minnesota, St. Paul. Voucher specimens are deposited in the Farlow Herbarium of Harvard University (FH), Royal Botanic Garden at Kew (K), University of Lille (LIP), and University of Florida Fungal Herbarium (FLAS).

Molecular methods

We attempted ITS and 28S rDNA sequencing for all described species of *Pachyphlodes*. Of the twelve described species and two varieties of *Pachyphlodes*, sequences were available or newly obtained for all except *P. saccardoi* (Mattir.) Doweld, and *P. melanoxanthus* subsp. *xanthocarnosus* (Soehner) Doweld. Sequences and morphological study of *P. lateritius* (Fogel & States) Doweld and *P. macrosporus* (Calonge) Doweld 2013 revealed that they do not belong to *Pachyphlodes sensu stricto* so they are not included in these analyses. *P. lateritius* is a *Geopora* and *P. macrosporus* is a *Hydnotrya*. Specimens of *P. saccardoi* and *P. melanoxanthus* subsp. *xanthocarnosus* were available for morphological study but were too old to obtain sequences. We judged from their morphologies and the morphology and sequences of *P. ligericus* (Tul. & C. Tul. ex Berk.) Zobel and *P. austro-oregonensis* (J.L. Frank & Trappe) Doweld that these species would not be pertinent to the species we are describing here, so they are not included in our phylogenetic analyses or discussion. Fresh or dried ascomata and mitosporic spore mats were placed in CTAB extraction buffer and DNA was extracted using a standard chloroform method (Gardes & Bruns, 1993). ITS and 28S rDNA was PCR-amplified with forward primers ITS1F, ITS5 and reverse primers ITS4, LR5, and LR3 (Gardes & Bruns, 1993; Vilgalys & Hester, 1990; White *et al.*, 1990). Amplicons were cleaned and sequenced bidirectionally with the same primers as for PCR. Sanger sequencing was performed at the University of Florida Interdisciplinary Center for Biotechnology Research (Gainesville, FL), and at the Beckman Coulter Genomics Laboratory (Danvers, MA). New sequences are depo-

sited in the National Center for Biotechnology Information under accession numbers KT899960-KT899976, KU170603- KU170604, and KU171101-KU171105.

Phylogenetic analysis

Sequences were edited and assembled in Geneious Pro v 5.6.7 (Drummond *et al.*, 2012). For both ITS and 28S, highly similar sequences were found through BLAST searches in NCBI. Sequence sets for ITS and 28S were aligned separately in MAFFT v. 6.822 (Katoh & Toh, 2010), and manually optimized in SeAl v. 2.0a11 (Rambaut, 2007). Phylogenetic analyses included RAxML and Bayesian analyses. Priors for posterior probability were selected using the Aikake Information criterion with the software package jModeltest (Posada, 2008). The model chosen for analyses of the ITS alignment was TPM1uf+I, and the 28S alignment was GTR+I+G. Bayesian analyses were run in MrBayes v. 3.2.3 (Huelsenbeck & Ronquist, 2001) for 20,000,000 generations in two parallel runs, with trees sampled every 1000th generation, and the first 25% of sampled trees discarded as burn in. Adequacy of mixing of the chains was checked in Tracer (Rambaut & Drummond, 2007). Maximum likelihood analyses were conducted using RAxML-HPC2 v 8.1.11 (Stamatakis, 2014) with a GTR + gamma model of nucleotide substitution. 1000 bootstrap iterations were performed with rapid bootstrapping. MAFFT, RAxML, and MrBayes analyses were carried out on XSEDE on the CIPRES Science Gateway v. 3.3 (Miller *et al.*, 2010). The datasets included 41 taxa and 641 sites for the ITS alignment, and 30 taxa and 816 sites for the 28S alignment.

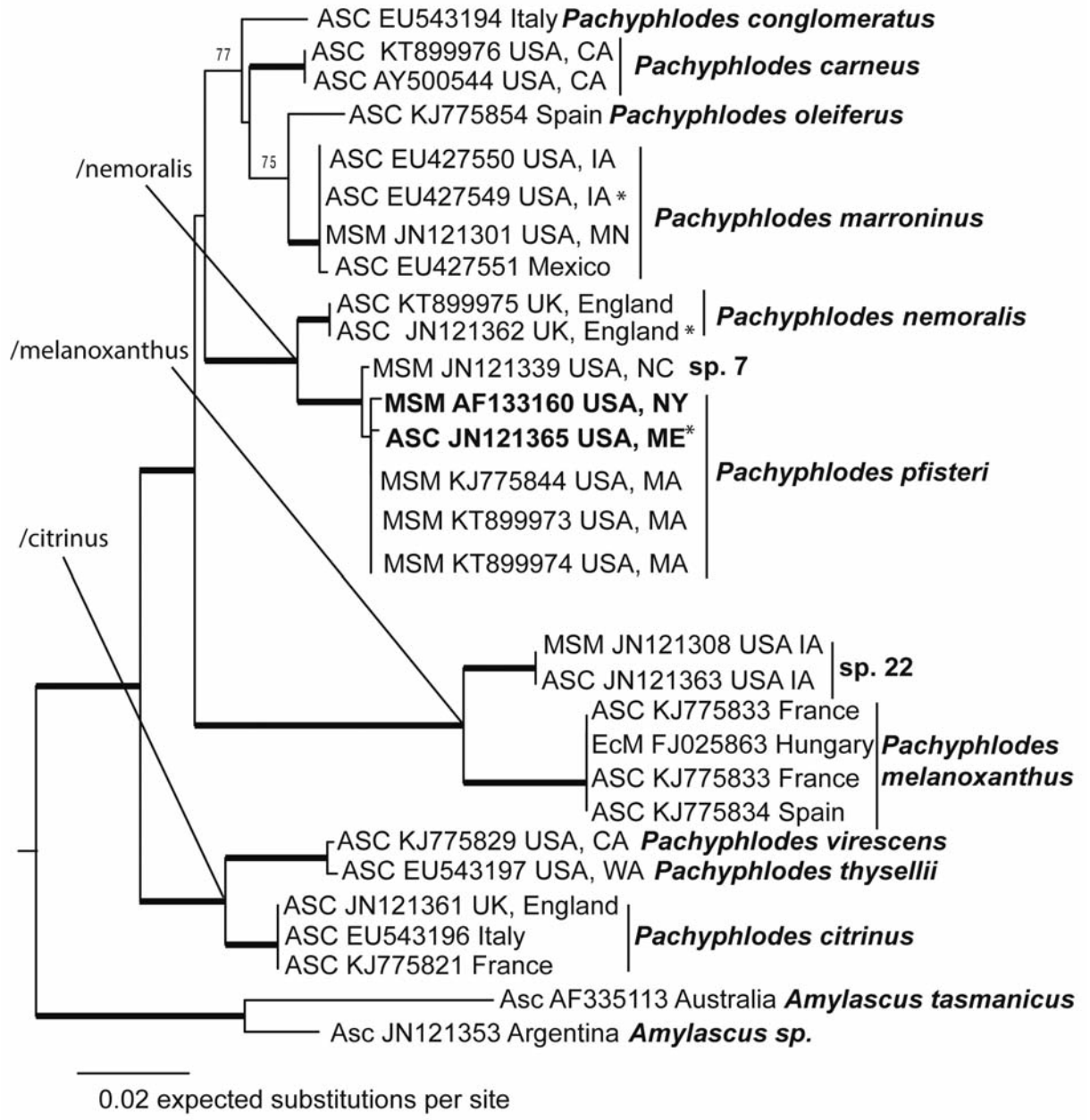

Plate 1 – Maximum likelihood phylogenetic tree based on 28S rDNA sequences of described *Pachyphlodes* species and undescribed species in the /nemoralis clade. The phylogeny was rooted with two species of *Amylascus*. Thickened branches indicate >80% ML bootstrap support and posterior probability values greater than 0.95 for Bayesian analysis. Sequences from ascomata (ASC), mitospore mats (MSM) and ectomycorrhizal root tips (EcM) are indicated along with GenBank accession numbers and locations of origin. Sequences in bold are K. Egger's sequences of R.P. Korf's spore mat (Norman & Egger, 1999) and our sequence of the ascoma of *P. pfisteri* MES-306. Asterisks denote sequences from holotype specimens.

Results

Of the species analyzed, three monophyletic clades were inferred from RAxML and Bayesian phylogenetic analyses of the 28S locus of *Pachyphlodes*: the /citrinus clade (including *P. citrinus* (Berk. & Broome) Doweld, *P. thysellii* (W. Colgan & Trappe) Doweld, and *P. virescens* (Gilkey) Doweld); the /melanoxanthus clade (including *P. melanoxanthus* and an undescribed species from North America referred to as "species 22"); and the /nemoralis clade, including only undescribed species (Pl. 1). The affiliations of *P. marroninus* (Healy, Bonito & Guevara) Doweld, *P. carneus* (Harkn.) Doweld, *P. conglomeratus* (Berk. & Broome) Doweld, and *P. oleiferus* J. Cabero & J. Pérez are unresolved within *Pachyphlodes* based on this limited set of taxa and the 28S locus. The 28S sequence from Korf's spore mat from

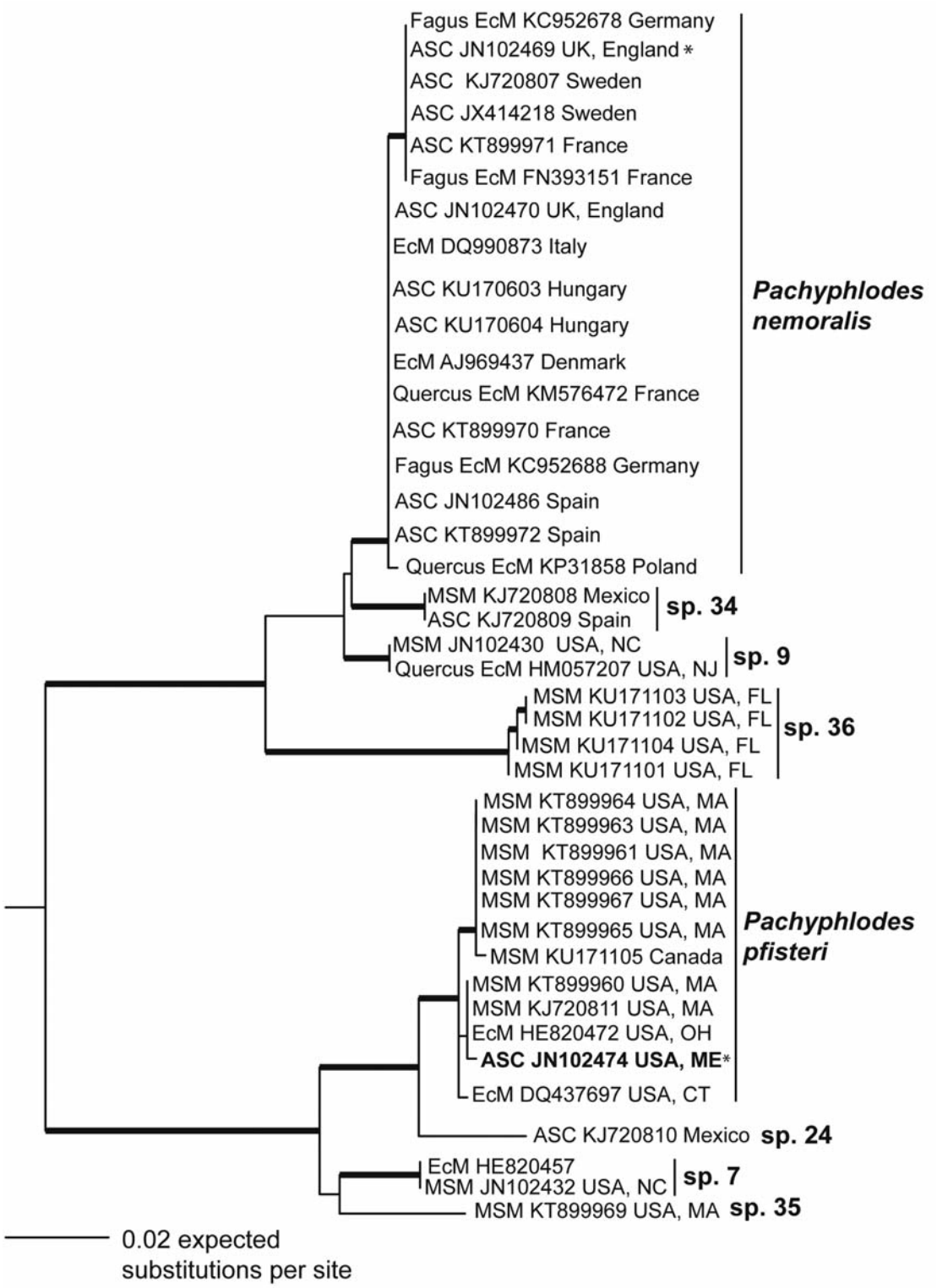

Plate 2 – Midpoint rooted phylogenetic tree based on maximum likelihood analysis of ITS rDNA sequences of taxa in the /nemoralis clade, including *Pachyphlodes nemoralis*, *P. pfisteri*, and undescribed species designated with provisional species hypothesis numbers. Thickened branches denote >70% ML bootstrap support and posterior probability values greater than 0.95 for Bayesian analysis. Sequences from ascomata (ASC), from mitospore mats (MSM) and ectomycorrhizal root tips (EcM) are indicated along with GenBank accession numbers and locations of origin. The sequence in bold is from the ascoma of *P. pfisteri* MES-306. Asterisks denote sequences from holotype specimens.

New York (AF133160) was highly similar to sequences from an ascoma from Maine (JN121365, MES-306) and several spore mats from Massachusetts (Pl. 1).

Phylogenetic analyses of the ITS rDNA resolved eight species within the /nemoralis clade (Pl. 2). Four of these species (sp. 7, sp. 9, sp. 35 and sp. 36) are known only from sequences of spore mats and ectomycorrhizal root tips and are expressed in numerical terms as species hypotheses. These species number designations are intended to stabilize hypothesized species until they can be described (Kõljalg *et al*., 2013) which will permit more effective communication about them (e.g. where undescribed *Pachyphlodes* species are detected on ectomycorrhizal root tips). Sequences of an ascoma from Maine (JN102474) were resolved in the same clade as those of spore mats collected in Massachusetts, New York and Ontario, Canada, as well as ectomycorrhizal root sequences from Connecticut and Ohio. We describe this North American species as *Pachyphlodes pfisteri* sp. nov. Sequences from ascomata collected in England, France, Hungary, Italy, Romania, Spain, and Sweden were resolved in the same clade as ectomycorrhizal root tips from Denmark, France, Germany, Italy, and Poland. We describe this European species as *Pachyphlodes nemoralis* sp. nov.

Descriptions

Pachyphlodes pfisteri Tocci, M.E. Sm. & Healy, *sp. nov.* – Mycobank: MB814777

Genbank reference sequence: JN102474.

Diagnosis: *Pachyphlodes pfisteri* can be distinguished from other species by a combination of the solid yellow gleba, cylindrical excipular warts, brown peridium, inordinate (randomly arranged) asci with a short pedicel embedded within interwoven hyphae of the gleba, asci that typically have eight spores irregularly biseriate to disordered, lack of distinguishable paraphyses, and globose yellowish spores with short, capitate spines that coalesce at the tips to nearly cover the entire spore. Ascospores excluding ornaments range from 13–15 (16) µm diam. with an average of 14.7 µm.

Holotype: MES-306 at the Farlow Herbarium (FH).

Etymology. In honor of Dr. Donald H. Pfister, a lifelong student of the *Pezizales* and a teacher who has inspired us to study and love these fungi as well.

Ascoma irregularly subglobose, 12 × 13 mm, surface with irregularly distributed conical warts, brown with a greenish tinge, gleba light yellow with translucent yellowish sterile veins, base with sparse emanating hyphae (Pl. 3, figs. 1-2).

Excipulum of two layers. Outer excipulum about 140 µm thick, composed of *textura angularis*, with warts up to 120 µm high (Pl. 3, fig. 3), yellow brown in water, orange brown in 5% KOH in outermost cells, pigment soluble in KOH, but only slightly soluble in water, cells up to 34 µm broad, with 1 µm thick walls in outermost cells, interior cells with notably thinner cell walls. Inner excipulum about 70 µm thick, composed of *textura prismatica*, hyaline, thin walled, similar to glebal hyphae. In 5% KOH, many oil-like drops released from the excipulum (Pl. 3, fig. 4). There are cells in the excipulum that appear to have different contents from the majority of cells, but this heterogeneity was only visible when tissue is mounted in KOH (Pl. 3, fig. 5, asterisks). **Paraphyses** not recognizable as such. **Asci** irregularly distributed in the gleba among interwoven hyphae, variable in shape from oval to pyriform with a short pedicel (Pl. 3, fig. 6), 82–126 (152) µm in length, 36–53 (62) µm in width including pedicel, pedicel (8) 10–20 (28) µm long × (5) 8-12 µm diameter widening at the base where it is bifurcate as a remnant of a crozier origin (Pl. 3, fig. 6 arrow). Spore arrangement irregularly biseriate to disordered (Pl. 3, figs. 6-7), typically with 8 spores but some asci with 6 or 7 spores, spore remnants often visible in asci with less than 8 spores. No reaction of asci in Melzer's solution. **Ascospores** (Pl. 3, figs. 6-9) globose, hyaline to light yellow, size range including ornaments 15–18.4 µm with an average of 16.6 µm, spores excluding ornaments range from 13–15 (16) µm with an average of 14.7 µm. Ornamentation on average 1 µm high, consisting of short capitate spines (Pl. 3, figs. 6-8) that coalesce at the tips to produce a nearly solid covering over the spore by maturity (Pl. 3, fig. 9). Similar ascospores were observed on specimens collected by Thaxter in New England in 1901 (Pl. 3, fig. 10).

Mitotic spore mat pale pink to cream white in color (Pl. 4, fig. 1), produced on top of leaf litter under *Quercus*, and one spore mat was collected on the lower trunk of a *Quercus* tree. Spore mass is cream colored. Spore mats dense with hyphae and spores. Surface composed of hyphal tips that aggregate to form dark pink-brown acute tips that are visible with a hand lens. Fresh mats viscous sticky liquid when handled/crushed. Some mats have the dry remains of this liquid, which when desiccated appears thin, translucent, and of brittle consistency like that of dried egg whites. This character appears to be variable, with many mats lacking a brittle "peridium" layer. Spore mat hyphae are branching (Pl. 4, fig. 2), with spores produced blastically through denticles on most internal hyphae (Pl. 4, fig. 3 arrow). Denticles 2 µm long × 1.5 µm wide at the base, tapering to the spore attachment. Hyphae 5–8 µm in diam, commonly 7 µm in diam. Mitotic spores are globose to subglobose and warted (Pl. 4, fig. 4), and average 5.5 µm diam including warts, ranging 5.25–6.25 µm in diam. All parts hyaline when viewed with a light microscope. It is difficult to understand the structure and development of spore mats as the act of sectioning through them crushes them, displacing the various parts. Therefore, a fresh spore mat was fixed, embedded in plastic, serial sectioned, and stained to better understand orientation of the various morphological features. In resin-embedded material that was sectioned in series, photographed and studied, aggregated hyphae (A) were observed to grow from near the base of the spore mat to the top, where they bend over the mat to form a thin covering (C). This covering may be interpreted as a peridium. Plate 4, figures 5-7 are part of a series of sections that together showed aggregated hyphae growing through the spore mat and ultimately bending over the top (indicated by the line drawings and arrow on Pl. 4, figs. 5-6). However, this thin peridium-like structure does not completely cover the entire spore mat. There are lacunae in this seemingly haphazard covering. The aggregating hyphae branch internally (within the spore mat) and produce mitospores along the branches within the mat (Pl. 4, fig. 7).

Material examined / Ascoma: USA. Maine: Washington Co., Steuben, near Eagle Hill Institute off of Hwy 1 under *Quercus* 7-Aug-2009, coll. Genevieve Lewis-Gentry, MES306 (FH, **holotype**); FLAS F-59179 (isotype). York Co., Kittery Pt., Goodwin's Pines, hypogeous under pine needle layer under *Pinus strobus*, Sep-1896, coll. R. Thaxter, det. H.M. Gilkey (FH 00284164). New Hampshire: Carroll Co., Intervale, Sep-1901, coll. R. Thaxter, det. H.M. Gilkey (FH 00284156). Mitospore mats: USA. Massachusetts: Middlesex Co., near Concord, Estabrook Woods on rotting wood, 10-Jul-2014, coll. R. Healy EBW1 (FH). Worcester Co., Petersham, Harvard Forest, Tom's Swamp, on leaf litter under red oak, 4-Sep-2013, coll R. Healy RHAM HF4 (FH), RHAM HF5 (FH), RHAM HF6 (FH), RHAM HF7 (FH), RHAM HF8 (FH), RHAM HFA (FH), RHAM HFB (FH). Worcester Co., Petersham, Harvard Forest, Prospect Hill along French Road on leaf debris under *Quercus rubra* 6-Sep-2013, coll. R. Healy RHAM HF17 (FH). New York: Tomkins Co., Malloryville, Eames Bog, halfway up N. slope, enmeshing *Fagus* husk, 5-Oct-1994, coll. R.P. Korf (CUP-62650). Tomkins Co., Ithaca, Coy Glen on mossy log, 12-Oct-1994, coll. P. Mullin, det. R.P. Korf (CUP-62653). Schuyler Co., Alpine, Hendershot Gulf, Lost Gorge, 14-Sep-1994, coll. Mycology Class, det. R.P. Korf (CUP-62646).

Habitat and distribution: Ascomata shallowly hypogeous, mitospore mats formed above ground on forest litter, logs, and the

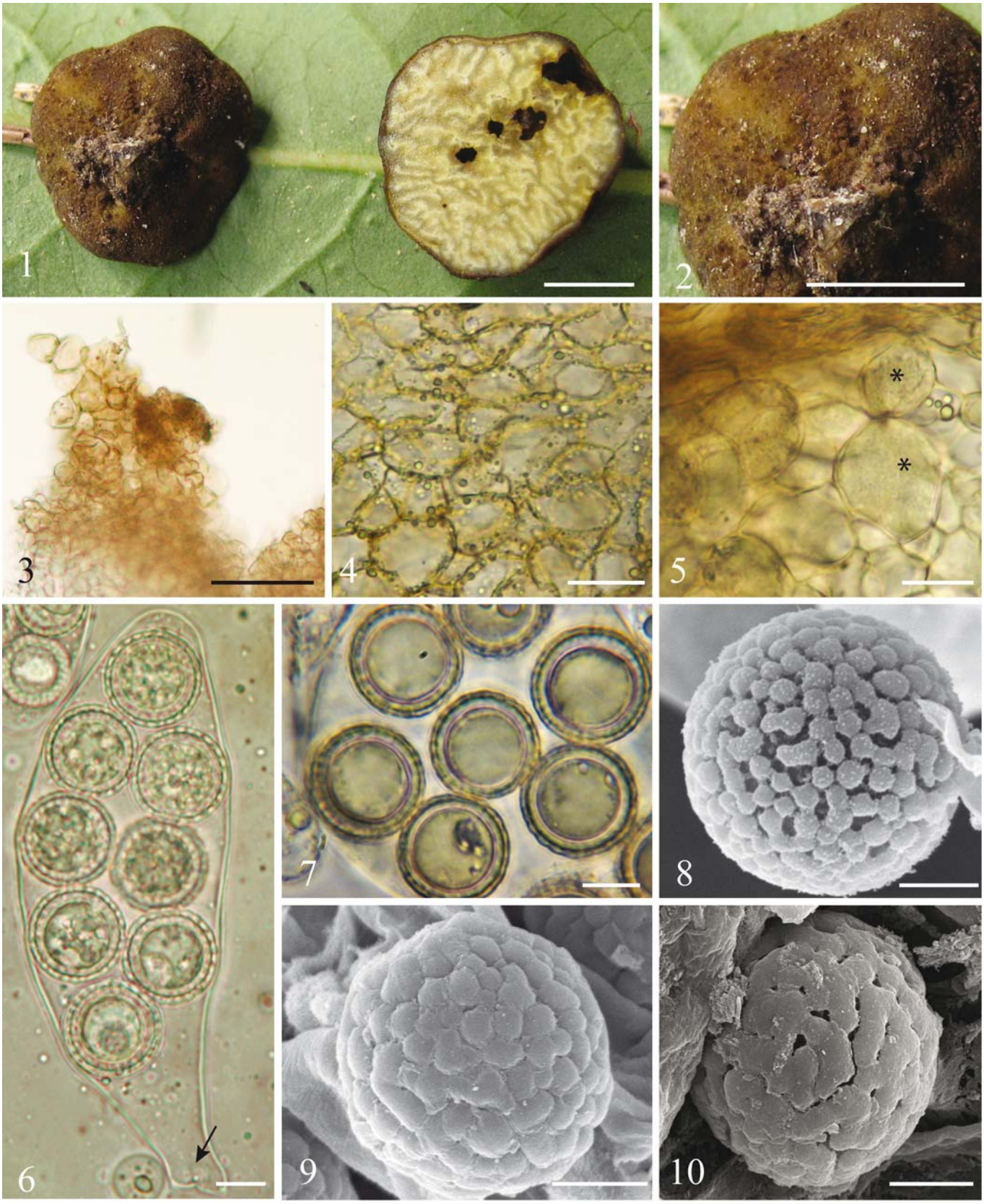

Plate 3 – *Pachyphlodes pfisteri*

Fig. 1-9) Holotype MES 306 (FH, USA: Maine; Isotype FLAS F-59179). 1) *Pachyphlodes pfisteri* ascoma showing exterior base (left) and gleba (right); Bar=5 mm. 2) Conical warts on the peridium; Bar=5 mm. Fig. 3-7) Bright field compound microscopy of *P. pfisteri* features mounted in 5% KOH. Fig. 3) Excipular wart; Bar=25 µm. Fig. 4) Ectal excipulum with oil drops in cells; Bar=30 µm. 5) Ectal excipulum showing cells with heterogeneous content in KOH. One content type indicated by asterisks; Bar=30 µm. 6) Ascus with bifurcate base (arrow); Bar=15 µm. 7) Spores in ascus; Bar=10 µm. 8) SEM of immature meiospore showing beginning of coalescence of spine tips; Bar=3 µm. 9) SEM of mature meiospore showing complete coalescence of spine tips; Bar=5 µm. 10) Mature spore from one of Thaxter's 1901 collections of *P. pfisteri* from New Hampshire (FH 00284156, USA); Bar=25 µm.

lower part of tree trunks. Ectomycorrhizal with trees in mixed or hardwood woodlands and forests in Northeastern North America.

Comments: This species was collected by Roland Thaxter in Maine and New Hampshire in 1896 and 1901. These collections (in the dried state) were identified by Helen Gilkey as *Pachyphlodes melanoxanthus*, but the ascospores and excipular warts are clearly the same as those in the type specimen of *P. pfisteri*. *Pachyphlodes melanoxanthus* has spiny spores with free, acute tips. Some of the spore mats collected by Korf and his students (Pl. 4, fig. 8) were morphologically similar in color and spore morphology (Pl. 4, fig. 9) with those from Harvard Forest collections of *P. nemoralis*. Korf described his collection of CUP62650 as "pinkish white", and "enmeshing *Fagus*

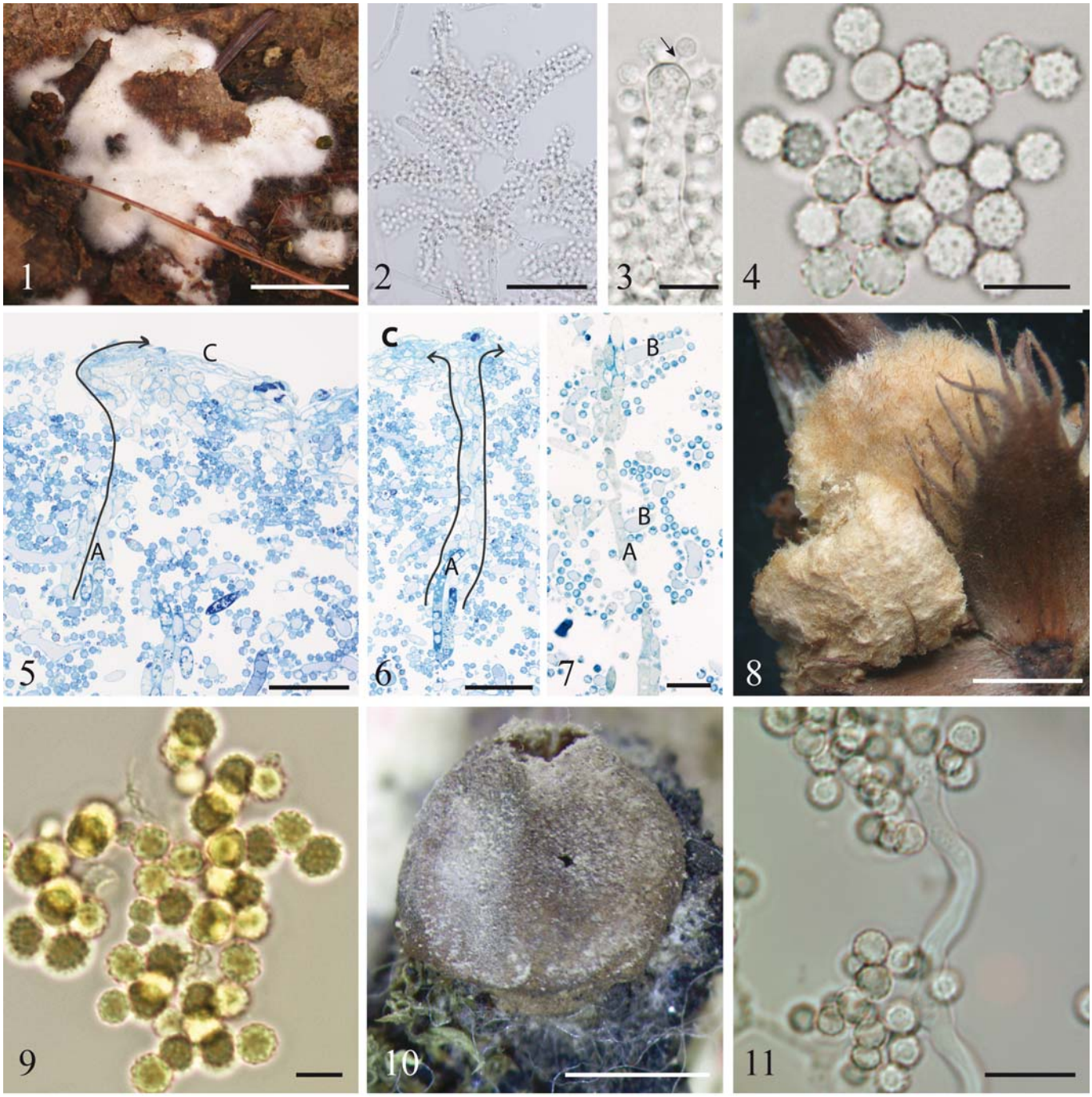

Plate 4 – Spore mats of *Pachyphlodes pfisteri*

1) Spore mat of HF8 on leaf litter; Bar=1 cm. Fig. 2-4) Mitospores of HF 17: 2) Mitospores covering branched hyphae; Bar=25 µm. 3) Mitospores attached to mitospore-bearing hyphae by thin denticle (arrow); Bar=10 µm. 4) Mitospores; Bar=10 µm. Fig. 5-7) *Pachyphlodes pfisteri* EBW1 selected resin sections from a series of sections. 5) Section showing interior of spore mat, with drawn line and arrow indicating the location of the aggregated hyphae (A) growing at a 90˚ angle to the top of the spore mat and bending over the top to interweave with hyphae in the covering (C); Bar=50 µm. 6) Additional section showing aggregated hyphae (A) growing at a 90˚ angle to the top of the mat, and bending at the covering to interweave with the hyphae of the covering (C); Bar = 50 µm. 7) Section of aggregated, non-sporulating hyphae (A) with branches (B) bearing mitospores (arrow); Bar= 25 µm. Fig. 8) Spore mat enmeshing Fagus husk from Korf's collection CUP-62650 (identified as *Glischroderma*, MA, USA); Bar=5 mm. Fig. 9) Mitotic spores mounted in Melzer's solution, from Korf's collection CUP-62653 (identified as *Glischroderma*, MA, USA); Bar=10 µm. Fig. 10) *Glischroderma cinctum* spore mat VH-2676 (UK, England), collected by C. Rea in 1912, from the Höhnel's Herbarium of FH; Bar=2 mm. Fig. 11) *Glischroderma cinctum* mitospores and hypha from C. Rea collection of 1912 (UK, England), from the Swedish Museum of Natural History, S F-139756; Bar=10 µm.

capsule and *Thuja* twig" (notation in specimen packet). It has dried down to a pinkish brown color (Pl. 4, fig. 8), but the description is very similar to *P. pfisteri* spore mats which were likewise pinkish white and collected on forest debris. While there is no ITS sequence available for Korf's collection, the 28S sequence is highly similar to that of *P. pfisteri*. The spore mats of *P. pfisteri* differ from *Glischroderma cinctum* Fuckel described from England. The peridium of *G. cinctum* is robust (Pl. 4, fig. 10) and the spore ornaments much finer (Pl. 4, fig. 11).

Pachyphlodes nemoralis Hobart, Bóna & Conde, *sp. nov.* – MycoBank: MB814778

GenBank reference sequence: JN102469.

Diagnosis: *Pachyphlodes nemoralis* can be distinguished from other species by a combination of brown peridium with angular warts, yellow umbilicate region, solid yellow to greenish yellow or greenish brown gleba, inordinate asci embedded within a gleba of interwoven hyphae, asci with short pedicels, asci with usually eight irregularly biseriate to disordered globose spores ornamented with short capitate spines that coalesce at the tips to nearly cover the entire spore. Ascospores (13.2) 14–15 (16.8) µm diam. excluding ornamentation.

Holotype: Royal Botanic Gardens at Kew 173683 (K).

Etymology: Latin, pertaining to woods and groves, the habitat of this species.

Ascoma sub-globose to oval ptychothecium with umbilicus (Pl. 5, fig. 1, umbilicus indicated by arrow), 2 × 2.4 cm, seal brown (Ridgway, 1912) with surface composed of angular irregular warts (Pl. 5, fig. 2) covered with a citrine dusting when first collected. Emanating mycelium sparse, pink brown. Lower surface in-rolled and warty at edge of umbilicus. Gleba solid, yellow (Pl. 5, fig. 3), sometimes with olive green tinge, browner where translucent, marbled with lighter yellow sterile veins developing from around the inrolled peridium. No perceptible odor.

Excipulum up to 812 µm thick composed of two layers. Ectal excipulum composed of *textura angularis* (Pl. 5, fig. 4), (44) 140–253 (334) µm thick with warts (100) 364–622 (806) µm high composed of cells 17–24 (37) µm broad with 1 µm thick yellow brown walls on the outermost portion, orange-brown in 5% KOH, the pigment slightly soluble in water, and more soluble in 5% KOH. No oil-like droplets or heterogeneous cells evident in KOH. Inner excipulum (120) 152–349 µm thick, composed of *textura prismatica* with thin walls, hyaline (Pl. 5, fig. 5). **Paraphyses** not recognizable. **Asci** irregularly distributed among interwoven hyphae in the gleba, variable in shape but generally pyriform (Pl. 5, fig. 6), (76) 192–126 (167) × (32) 37–60 (70) µm including pedicel, pedicel 30-50 × 16–20 µm that widens to a usually bifurcated base indicative of its origins from a crozier (Pl. 5, fig. 6, arrow), wall 1 µm thick, with 6 to usually 8 spores, with evidence of spore disintegration in asci with fewer than 8 spores, arrangement of spores irregularly biseriate to disordered, walls not reacting in Melzer's reagent. **Ascospores** globose, on average with ornamentation 18 µm diam with a range of 16.8–19 µm, on average excluding ornamentation 14–15 µm diam with a range of 13.2–16.8 µm, ornamentation 2.6 µm high on average, with a range of 1–3.5 µm, spores light yellow (Pl. 5, fig. 7) to occasionally dark brown at maturity, ornamented with capitate spines that coalesce at the tips to form a perisporal covering (Pl. 5, fig. 8); spore walls 1.4–1.8 µm thick on average, with a range of 1–2.2 µm.

No mitosporic form known.

Material examined. United Kingdom: England, Derbyshire, Keddleston Hall, hypogeous by 3 cm under log under old *Fagus sylvatica*, 23-Sep-2008, coll. C. Hobart, K(M)173683, **holotype**; FLAS. F-59181, isotype. West Gloucestershire, Forest of Dean, on soil under leaf litter under *F. sylvatica* and *Quercus* sp., 22-Sep,2009, coll. C. Hobart (K(M)173682; FLAS F-59180). South Yorkshire, Wyming Brook, Sheffield, hypogeous in soil under *Quercus*, *Fagus*, *Betula*, 4-Oct-1995, coll. C. Hobart, det. B. Spooner as *P. citrinus* (K(M)31943). Hungary: Bakony Mountains, Bakonybél, Laposok, 17-Aug-1996, coll. Z. Bratek (ZB1033). Romania: Hargita Mountains, Hargita-fürdői letérő, under *Picea abies*, 3-Oct-1998, coll. B. Pálfy (ZB2281). Spain: Cantabria, Forest of Abiada, on soil under leaf litter *Corylus avellana* and *Fagus sylvatica*, 3-Sep-2011, coll. A. Paz Conde IC03091123 (LIP 0000362; FLAS. F-59183). Cantabria, Villanueva de la Peña, forest of *Quercus petraea*, 13-Jul-2014, coll. P. Chautrand, det. A. Paz Conde IC13071416 (LIP 0000363; FLAS. F-59184). Asturias, Covadonga, at 600 m, under *Corylus avellana*, 15-Aug-2011, coll. F. Garcia, det. E. Rubio as *P. ligericus* (MA-56997). France: Charente-Maritime, Marennes, La Gataudière, 13-Jul-2014, coll. A. Paz Conde, IC14111403, (LIP-0000364; FLAS F-59185). Sweden: Småland, Jönköpings, 24-Sep-1980, coll. R. Carlsson 18, det. L.E. Kers as *P. citrinus* (S F133994). Småland, Jönköpings, under hazel, 2-Oct-1980, coll. R. Carlsson 62, det. L.E. Kers as *P. citrinus* (S F133992). Småland, Jönköpings, under hazel, 4-Oct-1980, R. Carlsson 65, det. L.E. Kers as *P. citrinus* (S F133989). Småland, Jönköpings, under hazel, 4-Oct-1980, coll. R. Carlsson 66a, det. L.E. Kers as *P. citrinus* (S F133993).

Habitat and distribution: Hypogeous or on the soil surface under leaf litter in woodlands where it is ectomycorrhizal with *Quercus* and *Fagus* in Europe.

Comments: *Pachyphlodes nemoralis* has been misidentified as *P. citrinus* or *P. ligericus* in herbaria. For example, in the Real Jardín Botanico de Madrid, MA-56997 was labeled as *P. ligericus*. However, *P. ligericus* is depicted by Tulasne & Tulasne (1851) with spines and no perispore. The spores of MA-56997 have a perispore formed by coalescent spine tips, which is a characteristic of *P. nemoralis* and its close relatives. There has been understandable confusion between *P. citrinus* and *P. nemoralis* (e.g. Pl. 5, fig. 9, spores of SF133993 originally determined as *P. citrinus*), as both have brown warted ascomata with a yellow umbilicus-like region (Pl. 5, fig. 10), and yellow gleba. However, *P. citrinus* spores are not covered by a perispore and the spore ornaments are spines that are longer than in either *P. nemoralis* or *P. pfisteri*. Although the spines of *P. citrinus* are often capitate, they do not coalesce to form a perispore, so the tips are free (Pl. 5, fig. 11). It appears that *P. nemoralis* is restricted to Europe and *P. pfisteri* is restricted to North America. The species in the /nemoralis clade are easily distinguished from *P. melanoxanthus* excipulum and spores. *Pachyphlodes melanoxanthus* has a black ascoma and acute-tipped spiny spores with no perispore (Pl. 5, fig. 12). Since fresh color is a feature that is obscured or lost with drying, a way to distinguish *P. melanoxanthus* from any other described species is to mount the excipulum in 2.5–3% KOH. The color in transmitted light of the ectal excipular cell walls in water is maroon to reddish brown (Pl. 5, fig. 13). In KOH, the color of the cell walls is black (Pl. 5, fig. 14).

Discussion

Pachyphlodes (Zobel, 1854) is a pleomorphic truffle genus in the *Pezizales*. Members of this genus are ectomycorrhizal with *Betulaceae*, *Fagaceae*, and *Tiliaceae* trees in the Northern Hemisphere temperate zone (Frank *et al*., 2006; Erős-Honti & Jakucs, 2009; Healy *et al*., 2013; Tedersoo *et al*., 2009) and their meiospores are dispersed by woodland mammals (Frank *et al*., 2006). The former generic name *Pachyphloeus* Tul. & C. Tul. was illegitimate because that name was previously used for a fossilized *Lepidodendron*-like plant (Göppert, 1836). The name for the *Pachyphloeus* fossil was undoubtedly known to the Tulasne brothers when they described a genus of truffle fungi as *Pachyphloeus* because they included the notation "non Göppert" in their description. Nomenclature was less regulated at that time, however, and the name was accepted until recently.

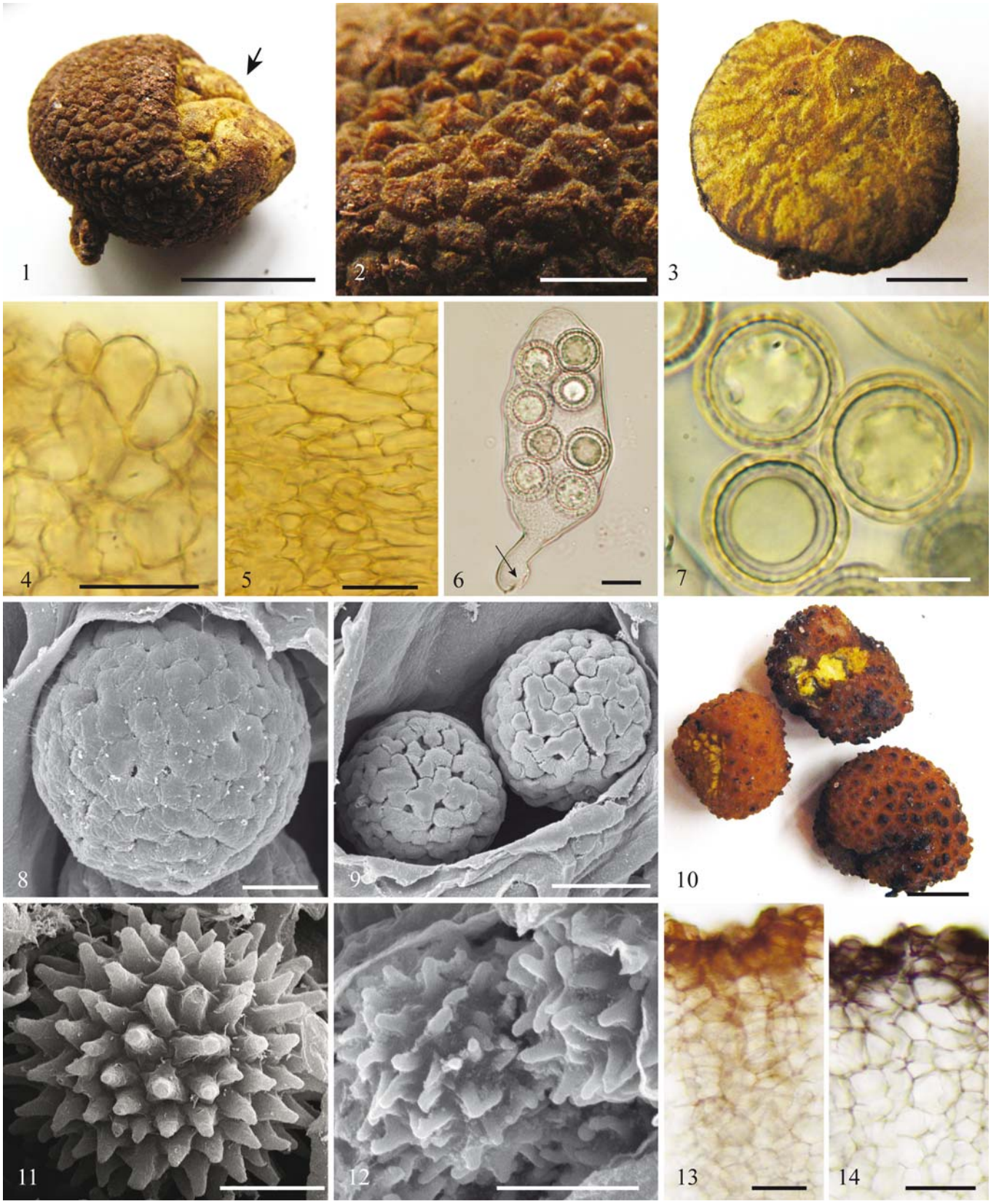

Plate 5 – *Pachyphlodes nemoralis*

Fig. 1-2) Holotype (RBG Kew 173683, United Kingdom) from Kedelston Hall, Derbyshire, England: 1) Yellow umbilicus (arrow) at the apex of the ascoma; Bar=1 cm. 2) Peridium with angular warts; Bar=5 mm. 3) Sectioned ascoma of paratype (FLAS F-59180, United Kingdom) showing yellow gleba; Bar=5 mm. Fig. 4-9) From isotype (FLAS F-59181): 4) Ectal excipulum of *textura angularis*; Bar=30 µm. 5) Inner excipulum of *textura prismatica*; Bar=30 µm. 6) Ascus with bifurcate base (arrow); Bar=15 µm. 7) Ascus with typical light yellow spores; Bar=15 µm. 8) SEM of mature spore covered by coalesced capitate spine tips. Fig. 9. *Pachyphlodes nemoralis* (S F133933, Sweden) SEM of spore; Bar=10 µm. Fig. 10-11) *Pachyphlodes citrinus*: 10) Ascomata (FLAS F-59182, United Kingdom) showing brown angular warts of peridium and yellow umbilicus; Bar=2 mm. 11) SEM of spore of coll. JRWL2197, Italy; Bar=5 µm. Fig. 12-14) *Pachyphlodes melanoxanthus*: 12) SEM of spores of type specimen of *P. melanoxanthus* (RBG Kew 98683, United Kingdom); Bar=10 µm. Fig. 13-14) Bright field microscopy of excipular sections of *P. melanoxanthus* (CaHsk211795). 13) In water; Bar=25 µm. 14) In 3% KOH; Bar=25 µm.

Under the International Code of Nomenclature for algae, fungi and plants (McNeil *et al.*, 2012), the fossil plant name has priority because it was described earlier whereas the truffle name is illegitimate. The next available legitimate name is *Pachyphlodes* Zobel (Corda, 1854), which was given for the species *Pachyphloeus ligericus* Tul. & C. Tul. Zobel (*in* Corda, 1854: 55) erected the genus *Pachyphlodes* to accommodate *P. ligericus*, which he thought differed too greatly from *Pachyphloeus melanoxanthus*, the type species of *Pachyphloeus*, for them to belong to the same genus. The generic name *Pachyphlodes* was not used until recently when all *Pachyphloeus* species were transferred to Pachyphlodes (Doweld, 2013a, 2013b). Unfortunately, after dedicated search there appears to be no fruitbody voucher specimen of the type for *P. ligericus*, which is the type species of the genus *Pachyphlodes*. Therefore, the drawings of two asci, one with immature and one with mature spores (Tulasne & Tulasne, 1851, Tab XIV: V1, V2) should be considered the type specimen, as these were used by Zobel in his description of *P. ligericus* (Art. 9, McNeil *et al.*, 2012).

Korf was the first to suggest that DNA analyses should be used to determine the identity of the spore mats that he and his students collected (Korf, 1994). Twenty years later, the ascomata and spore mats have now been linked as *Pachyphlodes pfisteri*. However, parts of the mystery have yet to be solved. For example, the glutinous peridium of *P. pfisteri* is clearly different from the substantial hyphal peridium that was originally described for the form genus *Glischroderma*. The glutinous substance produced by fresh spore mats, that dries to a brittle layer like desiccated raw egg white, is not always present in *P. pfisteri* spore mats and the hyphae that overlay the top do not completely cover the spore mat. We observed that as the spores mature, they break through the thin weft of hyphae that serves as a rudimentary peridium. As the spores mature, the exterior layer breaks away almost completely. Accordingly, we think that our North American *Pachyphlodes* spore mats are not the same as *Glischroderma sensu stricto* and that the identity of the sexual form of *Glischroderma* remains a mystery. We agree with Korf that *Glischroderma* is a pezizalean spore mat because the spores and spore-bearing hyphae are similar and we have determined that there is a strong phylogenetic signal with the mitospore forms in this order (Healy *et al*., 2013). We invite our other colleagues from Europe to continue Korf's "fun with discomycetes" by re-collecting the spore mats of the enigmatic *Glischroderma* and sequencing them to determine the proper phylogenetic placement and taxonomic identify of this unusual taxon.

Acknowledgements

We thank the following people who generously contributed ascomata or spore mats for this study: Michael Castellano, Gonzalo Guevara, Steve Miller, Brantlee Spakes Richter, Jan Thornhill, James Trappe, and Camille Truong. We gratefully acknowledge the mentorship of Donald Pfister and Zoltán Bratek. We thank the following herbaria and their curators for providing specimens for morphological study: CUP, FH, FLAS, OSC, and S. We thank Gail Celio and the microscopy facility at the University of Minnesota for assistance. Healy received funding from the Arnold Arboretum and Harvard Forest for her DNA work with the spore mats collected at Harvard Forest and Smith received support from the Harvard University Herbaria (HUH). Additional support for Healy and Smith was provided by the Institute for Food and Agricultural Sciences (IFAS) at the University of Florida.

References

Corda A.C.J. 1854. — *Icones Fungorum hucusque cognitorum*. Vol. 6. Prague, Ehrlich, 91 p. + pls. I-XX.

Curry K.J. & Kimbrough J.W. 1983. — Septal structures in apothecial tissues of the *Pezizaceae* (*Pezizales*, ascomycetes). *Mycologia*, 75 (5): 781-794.

Doweld A.B. 2013a. — Nomenclatural novelties. *Index Fungorum*, 31: 1.

Doweld A.B. 2013b. — Nomenclatural novelties. *Index Fungorum*, 32: 1.

Drummond A.J., Ashton B., Buxton S., Cheung M., Cooper A., Duran C., Heled J., Kearse M., Markowitz S., Moir R., Stones-Havas S., Sturrock S., Swidan F., Thierer T. & Wilson A. 2012. — Geneious v5.6.

Erős-Honti Z. & Jakucs E. 2009. — Characterization of beech ectomycorrhizae formed by species of the *Pachyphloeus-Amylascus* lineage. *Mycorrhiza*, 19 (5): 337-45.

Frank J.L., Barry S., Southworth D. 2006. — Mammal mycophagy and dispersal of mycorrhizal inoculum in Oregon white oak woodlands. *Northwest Science*, 80 (4): 264-273.

Fuckel L. 1870 [1869]. — Symbolae mycologicae. Beiträge zur Kenntniss der rheinischen Pilze. *Jahrbücher des Nassauischen Vereins für Naturkunde*, 23-24: 1-459.

Gardes M. & Bruns T.D. 1993. — ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. *Molecular Ecology*, 2 (2): 113-118.

Göppert H.R. 1836. — Die fossilen Farrnkräuter (*Systema filicum fossilium*). *Verhandlungen der kaiserlichen Leopoldinisch-Carolinischen Akademie der Naturforscher*, 17 (suppl.): 1-486 + pls. 1-44.

Healy R., Smith M.E., Bonito G.M., Pfister D.H., Ge Z.W., Guevara G.G., Williams G., Stafford K., Lee T., Hobart C. & Trappe J. 2013. — High diversity and widespread occurrence of mitotic spore mats in ectomycorrhizal *Pezizales*. *Molecular Ecology*, 22 (6): 1717-1732.

Huelsenbeck J.P. & Ronquist F.R. 2001. — Mr. Bayes: Baysian inference of phylogenetic trees. *Biometrics*, 17 (8): 754–755.

Katoh K. & Toh H. 2010. Parallelization of the MAFFT multiple sequence alignment program. *Bioinformatics*, 26 (15): 1899-1900.

Kõljalg U., Nilsson R.H., Abarenkov K., Tedersoo L., Taylor A.F.S., Bahram M., Bates S.T., Bruns T.D., Bengtsson-Palme J., Callaghan T.M., Douglas B., Drenkhan T., Eberhardt U., Dueñas M., Grebenc T., Griffith G.W., Hartmann M., Kirk P.M., Kohout P., Larsson E., Lindahl B.D., Lücking R., Martín M.P., Matheny B., Nguyen N.H., Niskanen T., Oja J., Peay K.G., Peinter U., Peterson M., Põldmaa K., Saag L., Saar I., Schüssler A., Scott J.A., Senés C., Smith M.E., Suija A., Taylor D.L., Telleria M.T., Weiss M. & Larsson K.H. 2013. — Towards a unified paradigm for sequence-based identification of fungi. *Molecular Ecology*, 22 (21): 5271-5277.

Korf R.P. 1994. — Fifty years of fun with the discomycetes and what's left to do. A transcript of the opening lecture of the first Whetzel-Wescott- Dimock lectureship of the Department of Plant Pathology, Cornell University, Ithaca, New York.

McNeill J., Barrie F.R., Buck W.R., Demoulin V., Greuter W., Hawksworth D.L., Herendeen P.S., Knapp S., Marhold K., Prado J., Prud'homme van Reine W.F., Smith G.F., Wirsema J.H. & Turland N.J. 2012. — *International Code of Nomenclature for algae, fungi, and plants (Melbourne Code)*. Regnum Vegetabile 154. Königstein, Koeltz Scientific Books, 208 p.

Miller M.A., Pfeiffer W. & Schwartz T. 2010. — Creating the CIPRES Science Gateway for inference of large phylogenetic trees. Proceedings of the Gateway Computing Environments Workshop (GCE): 14 Nov 2010, New Orleans, 1–8. http://www.ngbw.org/ee/index.php/portal/cite_us.

Norman J.E. & Egger K.N. 1999. — Molecular phylogenetic analysis of *Peziza* and related genera. *Mycologia*, 91 (5): 820-829.

Posada D. 2008. — jModelTest: Phylogenetic Model Averaging. *Molecular Biology and Evolution*, 25 (7): 1253-1256.

Rambaut A. 2007. — Se-Al: Sequence Alignment Editor, http://tree.bio.ed.ac.uk/software/seal/.

Rambaut A. & Drummond A.J. 2007. — Tracer v1.4: http://beast.bio.ed.ac.uk/Tracer

Ridgway R. 1912. — *Color Standards and Color Nomenclature*. Washington, DC. Published by author, 43 p. + 53 pl.

Stamatakis A. 2014. — RAxML Version 8: A tool for Phylogenetic Analysis and Post-Analysis of Large Phylogenies. *Bioinformatics* (2014). doi: 10.1093/bioinformatics/btu033

Tedersoo L., Suvi T., Jairus T., Ostonen I. & Põlme S. 2009. — Revisiting ectomycorrhizal fungi of the genus *Alnus*: differential host specificity, diversity and determinants of the fungal community. *New Phytologist*, 182 (3): 727-735.

Tulasne L.R. & Tulasne C. 1851. — *Fungi Hypogaei. Histoire et monographie des champignons hypogés*. Paris, F. Klincksieck, 222 p. + pls. I-XXI.

Vilgalys R. & Hester M. 1990. — Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of bacteriology*, 172 (8): 4238-4246.

White T.J., Bruns T.D., Lee S. & Taylor J.W. 1990. — Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. *In*: Innis M.A., Gelfand D.H., Sninsky J.J. & White T.J. (eds.). *PCR protocols: a guide to methods and applications*: 315-322. San Diego, Academic Press.

Rosanne Healy
Department of Plant Pathology
University of Florida, Gainesville, FL 32611
USA
rhealy1@ufl.edu

Genevieve E. Tocci
Harvard University Herbaria
22 Divinity Ave., Cambridge, MA 02138
USA
glewis-g@oeb.harvard.edu

Aurelia Paz Conde
Urb. La Llosa, nº 219-F
39509 Villanueva de la Peña, Mazcuerras
Spain
ita-paz@hotmail.com

Matthew E. Smith
Department of Plant Pathology
University of Florida, Gainesville, FL 32611
USA
trufflesmith@ufl.edu

Caroline Hobart
84 Stafford Road
Sheffield, South Yorkshire, S2 2SF
United Kingdom
geastrum@tiscali.co.uk

Lilla Bóna
Department of Plant Physiology and Molecular Plant Biology
Eötvös Loránd University, Pázmány sétány 1/C, 1117 Budapest
Hungary
bonalillaaa@gmail.com

Zsolt Merényi
Department of Plant Physiology and Molecular Plant Biology
Eötvös Loránd University, Pázmány sétány 1/C, 1117 Budapest
Hungary
zmerenyi@gmail.com