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A molecular and morphological re-examination of the generic limits of truffles in the tarzetta-geopyxis lineage – *Densocarpa*, *Hydnocystis*, and *Paurocotylis*

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ABSTRACT

Truffle species within the /tarzetta-geopyxis lineage share smooth, globose, hyaline spores, but differ in the amount of convolution of hymenia in ascomata. The relationships among truffle species in this lineage have historically been confused. Phylogenetic analyses of the ITS and 28S nuclear ribosomal DNA from recently collected members of the /tarzetta-geopyxis lineage from Asia, Austral Asia, North America, and South America prompted a reinvestigation of species and generic limits in the truffle genera *Hydnocystis*, *Paurocotylis*, and *Stephensia*. Our analyses support emendations of *Hydnocystis* and *Paurocotylis*, abandonment of *Stephensia* and the resurrection of the genus *Densocarpa*. Nomenclatural changes include the transfer of *Stephensia bombycina* to *Hydnocystis*, the transfer of *Hydnocystis singeri* and *Stephensia bynumii* to *Paurocotylis*, the reinstatement of *Densocarpa* for *Stephensia shanori* and transfer of *Stephensia crocea* to *Densocarpa*. This is the first detection of the genus *Paurocotylis* in the Americas. We describe three new species, *Hydnocystis transitoria* from North America, *Paurocotylis patagonica* from South America, and *Paurocotylis watlingii* from Australia. Our work highlights the unexplored diversity, morphological plasticity, and remaining taxonomic problems among truffles in the /tarzetta-geopyxis lineage.

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Introduction

The /tarzetta-geopyxis lineage, as defined by Tedersoo et al. (2006), includes the truffle genera *Paurocotylis* and *Stephensia*, and the cup fungus genera *Geopyxis* and *Tarzetta* (Tedersoo et al. 2006). This lineage was considered part of the *Pyronemataceae* until recently. Phylogenetic analyses based on multiple genetic loci of 93 species across 40 genera did not support a monophyletic *Pyronemataceae* when the earliest diverging lineages were included. One of four lineages recommended for exclusion from the *Pyronemataceae s. str.* was /tarzetta-geopyxis (Hansen et al. 2013). Conflicting results from analyses of these four lineages suggest more data are necessary to place them into a new or existing family (Hansen et al. 2013). Recent delimitation of the *Geopyxis* species in the /tarzetta-geopyxis lineage left the phylogenetic placement of the truffle genera unresolved (Wang et al. 2016). Discoveries of new truffle species in /tarzetta-geopyxis from Asia, Austral Asia, North America, and South America (Healy et al. 2013) prompted us to re-examine the generic limits and ecology of truffles in this lineage. In particular, we re-examined the genera *Hydnocystis, Stephensia*, and *Paurocotylis* which phylogenetic analyses show are included in the /tarzetta-geopyxis lineage (Wang et al. 2016). The genus *Densocarpa*, a monotypic genus originally described for *Densocarpa shanorii*, is currently considered a synonym of *Stephensia shanorii*. *Densocarpa* is reinstated here to accommodate species in the clade with *D. shanorii*.

Background on truffle genera in the /tarzetta-geopyxis lineage

The genus *Hydnocystis* was described by L. R. and C. Tulasne from ascomata in sandy forests of Europe and Russia (Tulasne & Tulasne 1844). Species of *Hydnocystis* are macroscopically distinguished by an enclosed, finely puberulent to tomentose ascoma that has a single opening leading to a non-convoluted, hollow cavity lined by a white hymenium in a palisade of paraphyses and cylindrical asci with colorless, smooth, uniseriate spores (Tulasne & Tulasne 1844). Burdsall (1968) revised the genus and noted some additional characteristics, the most important of which were eguttulate, globose spores (originally described as ellipsoid by Tulasne & Tulasne 1844) and elongated paraphyses forming an epithecium over the asci.

Over time, 12 species were placed in *Hydnocystis* but most have been transferred to other genera. Gilkey (1916) synonymized *Hydnocystis compacta* Harkness with *Genea intermedia* Gilkey due to its similarly ornamented spores. This species was subsequently transferred to the monotypic genus *Gilkeya* M.E. Sm. & Trappe as *Gilkeya compacta* (Harkn.) M.E. Sm. & Trappe, based on molecular phylogenetic evidence (Smith et al. 2006). Burdsall (1968) revised the genus and considered *Hydnocystis* monotypic, including only the type species *Hydnocystis piligera*. He excluded the other species based on glebal and spore characters. Most excluded species either lacked a true epithecium (*Hydnocystis convoluta* McAlpine, *Hydnocystis singeri* Gilkey), had amyloid asci (*H. convoluta*), had ornamented spores (*Hydnocystis echinospora* Rodway, *Hydnocystis thwaitesii* Berk. & Br.), or had ellipsoid to subglobose spores (*Hydnocystis arenaria* Tul. & C. Tul., *Hydnocystis beccarii* Mattir., *Hydnocystis californica* Gilkey, *Hydnocystis clausa* (Tul. & C. Tul.) Ceruti). Burdsall did not suggest an alternate genus for *H. singeri* (Burdsall 1968). After Burdsall's revision, *Hydnocystis japonica* (Kobayasi) Trappe (=*Protogenea japonica* Kobayasi) was added to the genus. Trappe (1975) transferred *P. japonica* to *Hydnocystis* because of its morphological similarities with *H. piligera*. *Hydnocystis japonica* is found among leaf litter in various types of forests and has the main characters of the genus. This species is most frequently under a non-ectomycorrhizal conifer, *Cryptomeria japonica* (Thunb. ex L.f.) D. Don.

At present, *H. piligera* and *H. japonica* are the only undisputed species in *Hydnocystis*. We recently discovered an undescribed North American species of *Hydnocystis*, including its anamorphic state, which stimulated our work on the /tarzetta-geopyxis lineage. From our analyses we infer that *Stephensia bombycina* (Vittad.) Tul. & C. Tul., the type species of *Stephensia*, is nested phylogenetically within the genus *Hydnocystis*. Like *Hydnocystis*, *S. bombycina* also produces an anamorph (Fontana & Giovannetti 1987). Mitospores of *S. bombycina* germinated and grew in culture (Fontana & Giovannetti 1987). Thus, the spores and spore-bearing structures of this state are conidia and conidiophores, respectively.

The genus *Stephensia* (Vittad.) Tul. & C. Tul. was previously shown to be paraphyletic (Læssøe & Hansen 2007; Perry et al. 2007; Alvarado et al. 2011; Wang et al. 2016), though no formal nomenclatural changes have been proposed. With the revelation during our analyses that the type species of *Stephensia* is a *Hydnocystis*, we attempted to place the remaining *Stephensia* species in the most plausible genera for which there is molecular evidence. *Stephensia* currently comprises eight species, including *S. bombycina, Stephensia bynumii* Trappe, Bushnell, & Castellano, *Stephensia colomboi* De Vito, *Stephensia crocea* Quél., *Stephensia peyronelii* Mattirolo, *Stephensia sumatrana* Boedijn, *Stephensia shanorii* (Gilkey) Gilkey and *S. varia* Rodway. *Stephensia shanorii* was first described in the monotypic genus *Densocarpa* (Gilkey 1954). After noticing the similarities between *Densocarpa shanorii* and *S. crocea*, Gilkey abandoned her new genus and transferred *D. shanorii* to *Stephensia* (Gilkey 1961). However, she noted dissimilarities between *S. shanorii* and the type species, *S. bombycina* (Gilkey 1961). Here we resurrect *Densocarpa* to accommodate both *S. shanorii* and *S. crocea*. The morphological and phylogenetic affiliations of the other species previously treated as *Stephensia* are discussed below.

The genus *Paurocotylis* Berk., with *Paurocotylis pila* Berk. as the sole member, has been fully and accurately described through the collective efforts of successive mycologists who studied fresh collections at various stages of maturity. Initially the affiliation of this species was uncertain. Berkeley (1855) noted that asci could be present, and that it might be similar to *S. bombycina*. He also mentioned a superficial similarity to *Arachnion* (Lycoperdales). The type specimen was a single, over-mature ascoma in which most of the asci are collapsed in a cavity with a mass of loose spores and glebal hyphae. Berkeley's erroneous interpretation that this taxon was potentially a basidiomycete was followed by Saccardo, thereby

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causing confusion for later researchers (Dennis 1975). Patouillard (1903) reinterpreted the type, observed asci, and recognized similarities with *Hydnocystis,* such as the hollow ascoma, excipulum of pseudoparenchyma, and smooth, globose spores. Dennis (1975) studied development in fresh, immature specimens from Great Britain where the species had been introduced. He observed that *P. pila* is initially a solid ascoma with a thin peridium. The asci develop among thin, branched hyphae, and are cylindrical, with 6–8 uniseriate, smooth, globose spores with a single large guttule. The gleba becomes hollow due to evanescent asci that leave powdery spores to partially fill the space in the gleba. Pegler *et al.* (1993) added that the peridium is thick and composed of isodiametric cells that are smaller towards the surface. They observed a poorly developed hymenium with no paraphyses. *Paurocotylis pila* is typically bright red, fruits on the soil surface, and is thought to be saprobic and bird-dispersed (Castellano *et al.* 2004; Læssøe & Hansen 2007; Trappe & Claridge 2015).

Ecologically, the /tarzetta-geopyxis lineage has a mixture of saprobic and biotrophic habits. *Tarzetta* is unequivocally ectomycorrhizal (EcM) (Tedersoo *et al.* 2010) but various trophic strategies have been assigned to taxa in *Densocarpa*, *Geopyxis, Hydnocystis, Paurocotylis*, and *Stephensia*. Wang *et al.* (2016) showed with multigene analyses that *Geopyxis* is paraphyletic, occurring in at least five well-supported clades, and some taxa live as plant or lichen endophytes. Other authors have implicated some *Geopyxis* species as biotrophic (Egger 1986; Egger & Paden 1986b,Vrålstad *et al.* 1998), weakly to moderately pathogenic (Egger 1986; Egger & Paden 1986a), saprobic (Petersen 1970; Egger 1986; Hansen *et al.* 2013), or a combination of trophic strategies (Egger 1986; Vrålstad *et al.* 1998; Hansen *et al.* 2013; Tedersoo *et al.* 2013). *Hydnocystis* species have been considered EcM (Molina *et al.* 1992; Rinaldi *et al.* 2008) or non-ectomycorrhizal (Tedersoo *et al.* 2010). *Stephensia* species have been considered EcM (Molina *et al.* 1992; Rinaldi *et al.* 2008), mycorrhizal with orchids (Bidartondo *et al.* 2004; Wang *et al.* 2016), or non-mycorrhizal (Hutchison 1989; Tedersoo *et al.* 2010). *Paurocotylis* species have been considered EcM (Brundrett *et al.* 1996; Rinaldi *et al.* 2008) or saprobic (Dennis 1975; Castellano *et al.* 2004).

The goals of this work were to: 1) phylogenetically place newly discovered truffle taxa from Austral Asia, North America, and South America in the /tarzetta-geopyxis lineage (*Densocarpa*, *Hydnocystis*, *Paurocotylis*, and *Stephensia*); 2) clarify the taxonomic status of *Stephensia* and assign species of *Stephensia* to appropriate genera based on phylogenetic and morphological evidence; 3) describe three new species (*Hydnocystis transitoria, Paurocotylis patagonica, Paurocotylis watlingii*), designate an epitype for *H. japonica,* and transfer *H. singeri* to *Paurocotylis*; 4) infer the most likely ecological strategies (endophytic, mycorrhizal, or saprobic) of truffle taxa in the /tarzetta-geopyxis lineage; and 5) identify unifying morphological characters for the genera within the /tarzetta-geopyxis lineage.

Materials and methods

DNA extraction, amplification, and sequencing

We obtained 46 new sequences and downloaded 90 additional nuclear rDNA sequences from NCBI to analyze phylogenetic relationships in the /tarzetta-geopyxis lineage. We used sequences from the internal transcribed spacer regions (ITS1–5.8S–ITS2, referred to as ITS) to aid in species delimitation (Schoch *et al.* 2012), and the 28S rDNA (LSU) to resolve relationships within the /tarzetta-geopyxis lineage. DNA was isolated from unexposed mature glebal tissue of fresh or dried ascomata. Extraction followed a standard chloroform method. The ITS and 5′ end of the 28S region of nuclear rDNA were amplified with ITS5 and LR5 (Vilgalys & Hester 1990; Bertini *et al.* 1999). We used NS7/ITS2 (White *et al.* 1990) to obtain sequences of *Stephensia shanorii*. PCR products were visualized on a 1 % agarose gel, and excess primer and nucleotides were removed with exonuclease 1 and shrimp alkaline phosphatase (U.S.B. Corporation, Cleveland, OH, USA). Amplicons were sequenced bidirectionally on an ABI PRISM 3700 DNA Analyzer in the Biomedical Genomics Laboratory at the University of Minnesota and the Interdisciplinary Center for Biological Research at the University of Florida using NS7, ITS1f, ITS4, ITS5, LR5, and LR3. Sequences were manually edited and assembled with Sequencher v. 5.1 (Gene Codes Inc., Ann Arbor, MI, USA). New sequences were deposited in GenBank under the numbers JX424575, KT361822–KT361850, KT428713–KT428714, KY321428–KY321441 (Table 1). Alignments and trees are deposited in TreeBase (http://purl.org/phylo/treebase/phylows/study/TB2:S19360).

Phylogenetic analyses

For alignment, all sequences were trimmed in SeAl v 2.0a11 (Rambaut 2007) before the 'CATTA' motif at the end of the 18S gene and before the 'ACCTCNNATCAGGTAGGGAT' motif of the 28S. When numerous hits of highly similar (>97 % identity) endophyte ITS sequences were returned from BLAST searches, sequences were chosen to represent host and geographic diversity. Taxa in the 28S data set included new sequences from this study and sequences representative of major lineages in the Pyronemataceae based on Hansen *et al.* (2013). All sequences were aligned with MAFFT v 7.187 (Katoh & Toh 2010), and manually adjusted in Se–Al. For the ITS alignment, ambiguous regions were excluded in Gblocks under the least stringent settings (Castresana 2000). Phylogenetic inference support included maximum likelihood (ML) bootstrapping and Bayesian posterior probabilities (BPP) from Bayesian inference (BI) analyses. ML was performed with RAxML 8.1.24 (Stamatakis 2014) using a GTR + Gamma model of nucleotide substitution. Rapid bootstrapping was implemented with 1000 replicates. The optimal model of evolution and priors for BI analyses were determined with jModelTest 0.1.1 (Posada 2008) using the Akaike information criterion for each partition. The ITS model was SYM+I+G; the 28S model was TIM3+I+G. Analyses were run for 20 000 000 generations, parameters were sampled every 1000th generation, and the first 25 % was discarded as burn-in. Stationarity, mixing, and estimated sample sizes were checked using Tracer v1.4 (Rambaut & Drummond 2007). MAFFT, ML, and BI analyses were implemented in XSEDE on the CIPRES Science Gateway v. 3.3 (Miller *et al.* 2010). Posterior probabilities were taken out to two decimal points (1/100th). The 28S rDNA alignment had 798 positions for 74 taxa, with *Orbilia auricolor* serving as

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Table 1 – Species sequenced for this study, their phylogenetic identities and most recently accepted name, collection or herbarium number, geographic origin, collector and year, and GenBank accession numbers.

Species	Most recent accepted name	Basionym	Collection/herbarium no.	Geographic origin	Year/collector	Genbank no. ITS	Genbank no. 28S
Densocarpa crocea	*Stephensia crocea*	*Stephensia crocea*	MA-56988	Spain	2001/E. Rubio	KT361829	KT361843
Densocarpa shanorii	*Stephensia shanorii*	*Densocarpa shanorii*	RH328	USA	1999/Sibylla Brown	KT361830	KT361842
Densocarpa shanorii	*Stephensia shanorii*	*Densocarpa shanorii*	RH136	USA	1998/R. Healy	KT361850	–
Densocarpa shanorii	*Stephensia shanorii*	*Densocarpa shanorii*	FLAS-F-59805	USA	2016/K. Gilberg	KY321434	–
Densocarpa sp.	*Stephensia shanorii?*	*Densocarpa*	OSC-150060, Trappe 3319 RH1862	Mexico	1972/J. Trappe	KY321433	KY321429
Hydnocystis bombycina	*Stephensia bombycina*	*Genea bombycina*	OSC-61969, RH955	Serbia	1992/Z. Marjanovic	KT361826	KT361837
Hydnocystis bombycina	*Stephensia bombycina*	*Genea bombycina*	OSC-32395, Trappe 3268, RH1861	Mexico	1972/J. Trappe	KY321435	KY321428
Hydnocystis japonica	*Hydnocystis japonica*	*Protogenea japonica*	KPM-NC-0008613	Japan	1999/Yousuke Degawa	KT361825	KT361835
Hydnocystis japonica	*Hydnocystis japonica*	*Protogenea japonica*	KPM-NC-0018030	Japan	2011/T. Orihara	KT428713	KT428714
Hydnocystis japonica	*Hydnocystis japonica*	*Protogenea japonica*	KPM-NC-24873	Japan	2015/T. Orihara	KY321436	–
Hydnocystis japonica	*Hydnocystis japonica*	*Protogenea japonica*	KPM-NC-24892	Japan	2015/T. Orihara	KY321437	–
Hydnocystis piligera	*Hydnocystis piligera*	*Hydnocystis piligera*	MA-9982	Liechtenstein	No date/Medici. G. Caro	KT361822	KT361838
Hydnocystis piligera	*Hydnocystis piligera*	*Hydnocystis piligera*	MA-29812	Spain	1990/MA Perez	KT361823	–
Hydnocystis transitorius	sp. nov.	*Hydnocystis*	RHAM488	USA	2011/R. Healy	KT361828	–
Hydnocystis transitorius	sp. nov.	*Hydnocystis*	RH950	USA	2009/L. Kumar	KT361827	JX424575
Hydnocystis sp.	*Hydnocystis piligera*	*Hydnocystis piligera*	FH-00290161	China	1998/D. Hibbett, Z. Wang	KT361824	KT361836
Jafnea semitosta	*Jafnea semitosta*	*Peziza semitosta*	ISC-443551	USA	2007/R. Healy	–	KT361849
Paurocotylis bynumii	*Stephensia bynumii*	*Stephensia bynumii*	OSC-58840, JT9004	USA	1986/D. Wheeler	KY321439	KY321430
Paurocotylis patagonica	sp. nov.	*Paurocotylis*	CORD-C00004222	Argentina	2009/E. Nouhra	KT361833	KT361841
Paurocotylis patagonica	sp. nov.	*Paurocotylis*	FLAS-F-59804	Chile	2016/P. Sandoval-Leiva	KY321440	KY321431
Paurocotylis pila	*Paurocotylis pila*	*Paurocotylis pila*	FLAS-F-59803 EB263, RH1859	Australia	2016/G. Gates	KY321441	KY321432
Paurocotylis pila	*Paurocotylis pila*	*Paurocotylis pila*	KPM-NC-23032	New Zealand	2013/P. White	KY321438	KP191791
Paurocotylis singeri	*Hydnocystis singeri*	*Hydnocystis singeri*	CORD-C00004224	Argentina	2008/Robledo	KT361832	KT361840
Paurocotylis watlingii	sp. nov.	*Paurocotylis*	Watling-11725	Australia	1974/R. Watling	KT361831	KT361839
Tarzetta sp.	*Tarzetta*	*Peziza*	DHP-CH30	Chile	DH Pfister	–	KT361846
Tarzetta sp.	*Tarzetta*	*Peziza*	RH1646	USA	2014/R. Healy	–	KT361844
Tarzetta sp.	*Tarzetta*	*Peziza*	RH1525D	USA	2007/R. Healy	–	KT361847
Tarzetta sp.	*Tarzetta*	*Peziza*	RH1526D	USA	2008/R. Healy	–	KT361845
Tarzetta sp.	*Tarzetta*	*Peziza*	DHP-08-654	USA	2008/D.H. Pfister	–	KT361848

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the outgroup. After excluding ambiguous regions of the alignment, the ITS rDNA alignment had 464 positions for 77 taxa and was midpoint rooted.

Specimen morphology and microscopy

Macroscopic characteristics were described from field notes of fresh or dried material. Micro-morphological characteristics and measurements were detailed from razor-excised pieces of air-dried ascomata rehydrated in water or 5 % KOH. Upon rehydration, excised pieces were squash mounted in water, cotton blue, or Melzer's Reagent and viewed with brightfield and differential interference contrast (DIC) microscopy. To determine mean spore diameter spore measurements were taken from 50 randomly selected spores under the highest power of light microscope magnification in water mounts. Color nomenclature follows Kelly & Judd (1955). Fresh material of *Hydnocystis transitoria* was fixed in 2 % paraformaldehyde and 2 % glutaraldehyde in 0.1 M sodium cacodylate buffer, dehydrated in a gradual ethanol series, exchanged with acetone, and infiltrated with a standard formulation of Spurr's Resin (Spurr 1969), and the resin polymerized for 30 h at 70 °C. Pieces were sectioned 3 μm thick, stained with toluidine blue O, and mounted with xylol and Permount. For scanning electron microscopy (SEM), small blocks of glebal tissue were fixed as described above, dehydrated in a graded ethanol series to 100 % ethanol, critical point dried, attached to aluminum stubs, sputter coated with 1/1 gold/palladium and viewed under 10 KV in a JEOL SEM in the Microscopy and NanoImaging Facility at Iowa State University.

Definitions of morphological terms

The term epithecium refers to paraphyses that are elongated well beyond the asci, that may be branched or unbranched, and may or may not be fused (Kirk *et al.* 2008). If paraphyses are interwoven and fused, the tissue is referred to as pseudoparenchyma, as in species of *Genea*. If the paraphyses are not fused, the tissue is referred to as prosenchyma, as in *Hydnocystis piligera* Tul. & C. Tul. (Kirk *et al.* 2008). In *Hydnocystis piligera*, the epithecium of prosenchyma forms a distinct layer that is visible with a hand lens.

Results

DNA sequences and phylogenetic analyses

The ML and BI analyses of the 28S alignment confirmed a monophyletic /tarzetta-geopyxis lineage (Fig 1). *Stephensia* was paraphyletic, with *Stephensia bombycina* inferred within *Hydnocystis*, *Stephensia bynumii* affiliated with *Paurocotylis*, and *Stephensia shanorii* and *Stephensia crocea* inferred together in a clade distant from other genera. Since *Densocarpa* is a valid name for *S. shanorii*, we resurrect its use for this species and its closest relative *S. crocea* as well as an undescribed species from Mexico. *Hydnocystis* was also paraphyletic, with *Hydnocystis singeri* most closely affiliated with *Paurocotylis pila*. Based on our results three phylogenetically distinct truffle genera are supported: *Densocarpa, Hydnocystis*, and *Paurocotylis*. Among the three inferred truffle lineages, *Densocarpa* and *Hydnocystis* had strong bootstrap and posterior probability support, while *Paurocotylis* was weakly supported. *Tarzetta* and *Densocarpa* were inferred as sister to the rest of the /tarzetta-geopyxis lineage. Final ML optimization in RAxML yielded a best tree with log-likelihood of −5787.185458 for the 28S alignment.

Analyses of the ITS region agreed with the placement of truffle species within the /tarzetta-geopyxis lineage as inferred by the LSU analyses. *Stephensia bombycina*, the type species of *Stephensia*, was inferred as a *Hydnocystis*, *S. bynumii* was inferred as a *Paurocotylis*, *S. shanorii*, *S. crocea* were inferred together in a distinct lineage (*Densocarpa*), and *H. singeri* was inferred as a *Paurocotylis*. There was strong support based on ITS for *Densocarpa, Hydnocystis*, and *Paurocotylis* (Fig 2). Final ML optimization in RAxML yielded a best tree with log-likelihood of −3321.778401 for the ITS alignment. In Figs 1 and 2 taxon names have been changed to reflect nomenclatural revisions presented here. Table 1 gives a synopsis of the current and previously accepted names for each taxon.

Direct comparison of ITS sequences in the 830 bp alignment showed 99 % sequence similarity among the nine *Hydnocystis piligera* sequences. ITS sequence similarities between *H. piligera* and other species were 96 % with *Hydnocystis japonica*, 95 % with an undescribed species from China (FH 00290161), and less than 91 % with *S. bombycina* (Serbia and Mexico) and with *Hydnocystis transitoria* sp. nov. from the USA. There were 26 bp differences (5 %) between *H. japonica* (507 bp) and the undescribed *Hydnocystis* from China (505 bp) (FH-00290161). RAxML and BI phylogenetic analyses of both ITS and 28S strongly support a monophyletic clade of *H. piligera*, *H. japonica*, *S. bombycina*, *H. transitoria* sp. nov., and *Hydnocystis* sp. nov. from China (FH-00290161) (Figs 1 and 2).

As surmised by Burdsall (1968), the southern South American species *H. singeri* was not closely related to the core group of *Hydnocystis*. Instead, our analyses show that it was most closely related to *P. pila* from New Zealand. Most taxa in the *Paurocotylis* clade shared a notable insert in the beginning of ITS1. The insert in the *P. pila* sequences (175 bp long of an 873 position alignment) were 93–100 % similar (0–11 bp difference and one ambiguity) to each other and were 83–85 % similar to the insert of *Paurocotylis patagonica* sp. nov. (178 bp long), differing in 27–31 nucleotide positions for each *P. pila* sequence (Supplemental Fig S1). The insert in the *H. singeri* sequence (145 bp) was only 33 % similar to *P. pila*, and 36 % similar to *P. patagonica* sp. nov. from Argentina. The insert in the sequence from *Paurocotylis watlingii* from Australia was relatively short (39 bp), but matched 54 % with *H. singeri* across 55 positions. *Stephensia bynumii* was missing the insert. The ITS1 insert in *Paurocotylis* species was not similar to the insert discovered in some species of *Geopyxis* (*Geopyxis alpine, Geopyxis deceptive, Geopyxis majalis*, and *Geopyxis rehmii*) by Wang *et al.* (2016) (Supplemental Fig S1).

Geopyxis was inferred as paraphyletic, supporting previous analyses (Perry *et al.* 2007; Tedersoo *et al.* 2013; Wang *et al.* 2016). Endophytic sequences from angiosperms,

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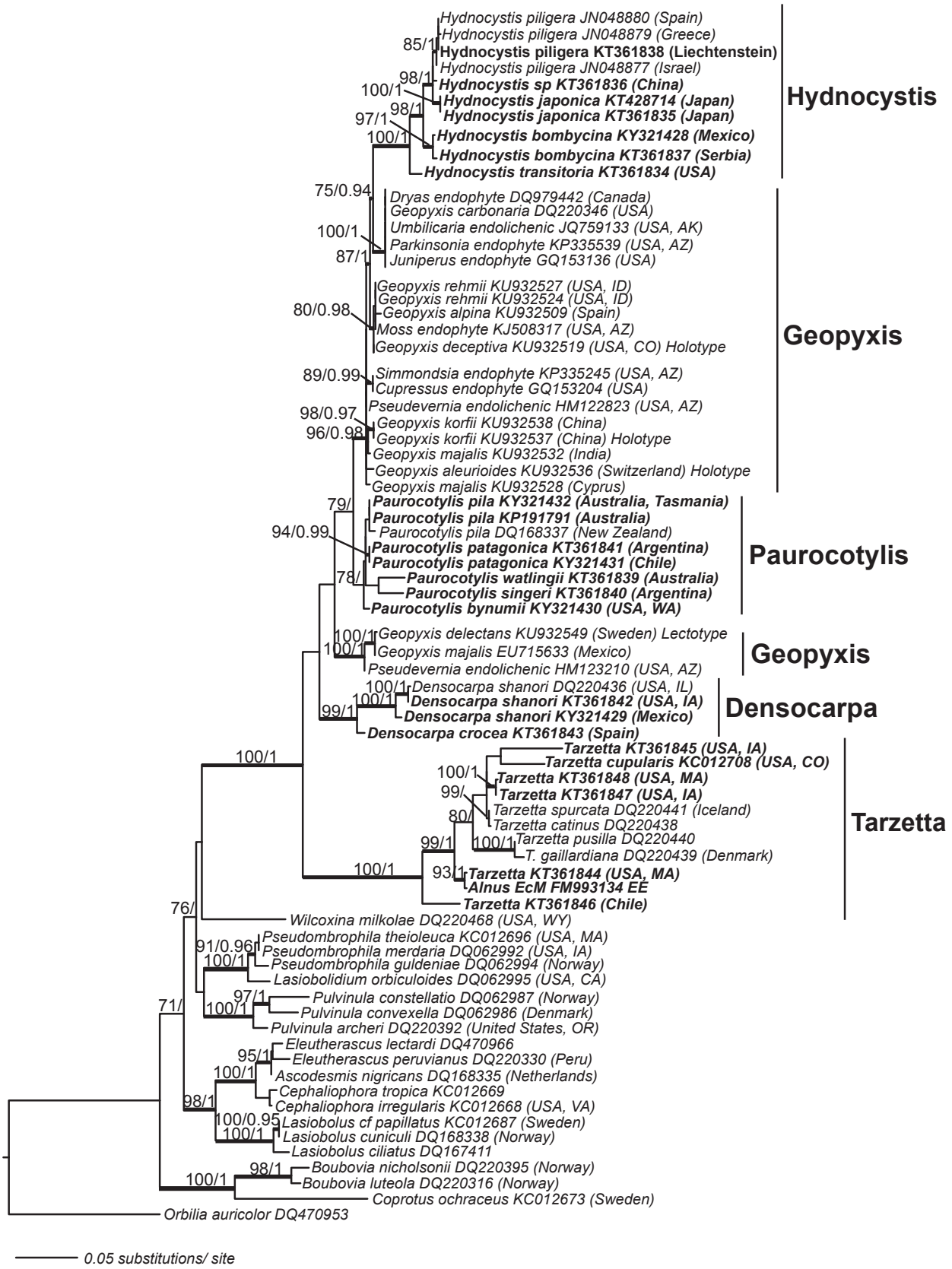

Fig 1 – Phylogenetic tree based on maximum likelihood (ML) analysis of 28S rDNA, with node support from 1000 bootstrap iterations and Bayesian posterior probabilities (BPP). BPP ≥0.95/Bootstrap values ≥70 % are indicated at branch nodes. Thickened branches indicate support from both methods of phylogenetic analyses. Bolded taxa are newly generated sequences. Non-bolded sequences are from GenBank, 'EcM' stands for 'ectomycorrhizal'. Taxon names presented here reflect nomenclatural revisions introduced in the taxonomy section of this paper.

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gymnosperms, lichens, and mosses nested within disparate *Geopyxis* clades (Fig 2). All of the vascular plant endophytes were isolated from above ground tissues, indicating that *Geopyxis* is not EcM. The only sequences from EcM root tips were resolved within *Tarzetta* (Fig 2).

Taxonomy

Here we describe the morphology of truffles in the genera *Hydnocystis*, *Paurocotylis, and Densocarpa*. We describe three new species, make five new combinations, designate one epitype, and amend the descriptions of two species. We provide descriptions of all described truffle species in the /tarzetta-geopyxis lineage in order to effectively compare genera and species.

Hydnocystis Tul. & C. Tul. 1844. *Nuovo Giornale Botanico Italiano* **2**: 59.
Type species: ***Hydnocystis piligera*** Tul. and Tul. 1844. *Nuovo Giornale Botanico Italiano* **2**: 59–60.
Etymology: Latin 'Hydnon', Tuber; 'cystis', bladder.
Description: *Ascoma* a ptycothecium, as defined by Weber *et al.* (1997), globose to ovoid, lobed or folded, with either a simple hymenium lining a hollow cavity or infolded with canals and a small cavity, connected to the exterior by a pore or larger opening, whitish to brown.
Excipulum of pseudoparenchyma; if excipular hairs are present, they are granule-encrusted.
Hymenium a palisade of paraphyses and asci.
Paraphyses narrow, septate, often longer than asci, forming epithecium.
Asci inamyloid, cylindrical, indehiscent, spores arranged uniseriately.
Ascospores globose, smooth, hyaline or nearly so.
Comments: We amend the description of the genus *Hydnocystis* with regards to the degree of ascoma infolding and the granules that encrust the excipular hyphae.

Hydnocystis piligera Tul. and Tul. 1844 *Nuovo Giornale Botanico Italiano* **2**: 59–60.
Isotype. **France**, Var, Hyéres under terebinth (*Pistacia terebinthus*), Dec. 1844, Tul. and Tul. Antibes (CUP 48960).
Etymology: Latin, 'pili', the hair, possibly referring to the hairy ascomata of this species.
Description: *Ascoma* a ptycothecium subglobose to slightly flattened, sometimes lobed, 0.5–4 cm diam., tomentose, pale brown to yellow brown, usually with one large cavity lined by cottony hyphae, gleba white, odor fruity (Montecchi & Sarasini 2000).
Excipulum 500 μm, hairs 4–6 μm diam., septate, granule-encrusted, brown, outer excipulum composed of pseudoparenchyma with cells 10–30 μm diam., outer cells with brown walls. Inner excipulum with smaller cells, hyaline.
Hymenium a palisade of asci and paraphyses that form a thick epithecium.
Paraphyses narrow, 3–5 μm diam., septate, elongated considerably beyond the asci.
Asci cylindrical, 250–300 μm × 30–50 μm.
Ascospores globose, smooth, hyaline, not guttulate, 28–35 μm diam.
Anamorph unknown.
Distribution and habitat: On soil surface or shallowly hypogeous. In many habitats under Pinaceae or Angiosperms. Widespread in Europe, Russia, and Israel.
Specimens examined: **Austria**, Feldkirch, sn date, (CUP-48863); **France**, Var, Hyéres, under terebinth (*Pistacia terebinthus*) Dec. 1844, Tul. and Tul. (Isotype CUP-48960), Antibes, sn date, Torrault (CUP-48975); **Lichtenstein**, Cecinamare, Terremi sabbiosi, under *Pinus, Picea, Eucalyptus*, sn date, Medici. G. Caro (MA-9982); **Spain**, Mallorca, Banyalbufar, Coll de Sa Bastida, 20 December 1990, MA Perez (MA-29812); **Russia**, 6 August 1906, F. Bucholtz B311 (CUP-48971, FH-00290167); Bonifa, 18 June 1903, R. Maire (CUP-48973); Kemmern, 10 August 1901, 8 July 1903, F. Bucholtz b311 (CUP-48970, CUP-48972, FH-00290163, FH-00290165).
Comments: There is little to no ITS variability among specimens of *H. piligera*. The sequences in GenBank, most of which are from Alvarado *et al.* (2011), matched the sequences obtained for this study. Morphology is the same as described for the type specimen and the more recently studied specimens treated by Agnello (2011), Alvarado *et al.* (2011), Burdsall (1968), and Montecchi & Sarasini (2000).

Hydnocystis bombycina (Vittad.) Healy and M. E. Sm., **comb. nov**.
MycoBank MB814818
Basionym: *Genea bombycina* Vittad. *Monographia Tuberaceaarum*. (Milano): 29 (1831).
Synonym: *Stephensia bombycina* (Vittad.) Tul. & C. Tul *Comptes rendus hebdomadaires des séances de l'Académie des Sciences* **21**: 1433 (1845).
Type: Location unknown. Not examined.
Etymology: Latin, 'silky', referring to the silky ascomata. Vittadini named this species within the genus *Genea,* and most *Genea* have peridia with rough, warted surfaces and obvious peridial hairs. It is possible that Vittadini considered *H. bombycina* to be 'silky' and smooth in comparison.
Description: *Ascoma* a ptycothecium, subglobose, depressed, cracked, up to 2 cm diam., tomentose, light brown to yellow brown, drying reddish brown (Pegler *et al.* 1993), with a single cavity in the gleba that is connected to the exterior through a pore. Gleba white, marbled with white to cream-colored veins or open canals where the hymenium is folded. Odor of H_2S.
Excipulum 800 μm, hairs 2–6 μm diam., septate, granule-encrusted, brown with brown walls, outer excipulum composed of somewhat parallel hyphae that radiate to surface, continuing beyond surface as hairs, layer 100 μm thick, outer cell walls brown. Inner excipular layer 700 μm thick, of somewhat parallel hyphae (Hawker 1954).
Hymenium a palisade of paraphyses and asci.
Paraphyses 2–3 μm diam., uninflated, septate, often elongated beyond ascus tips to grow into cavity between opposing hymenia that result from infolds.
Asci cylindrical 180–270 × 20–30 μm, tapering to elongated narrow stalk, with up to eight uniseriately (typically) to irregularly biseriately arranged ascospores (Hawker 1954), unreactive in Melzer's solution.
Ascospores globose, smooth, hyaline, 19–22 μm diam. (original description), but reported to vary from 16 to 34 μm

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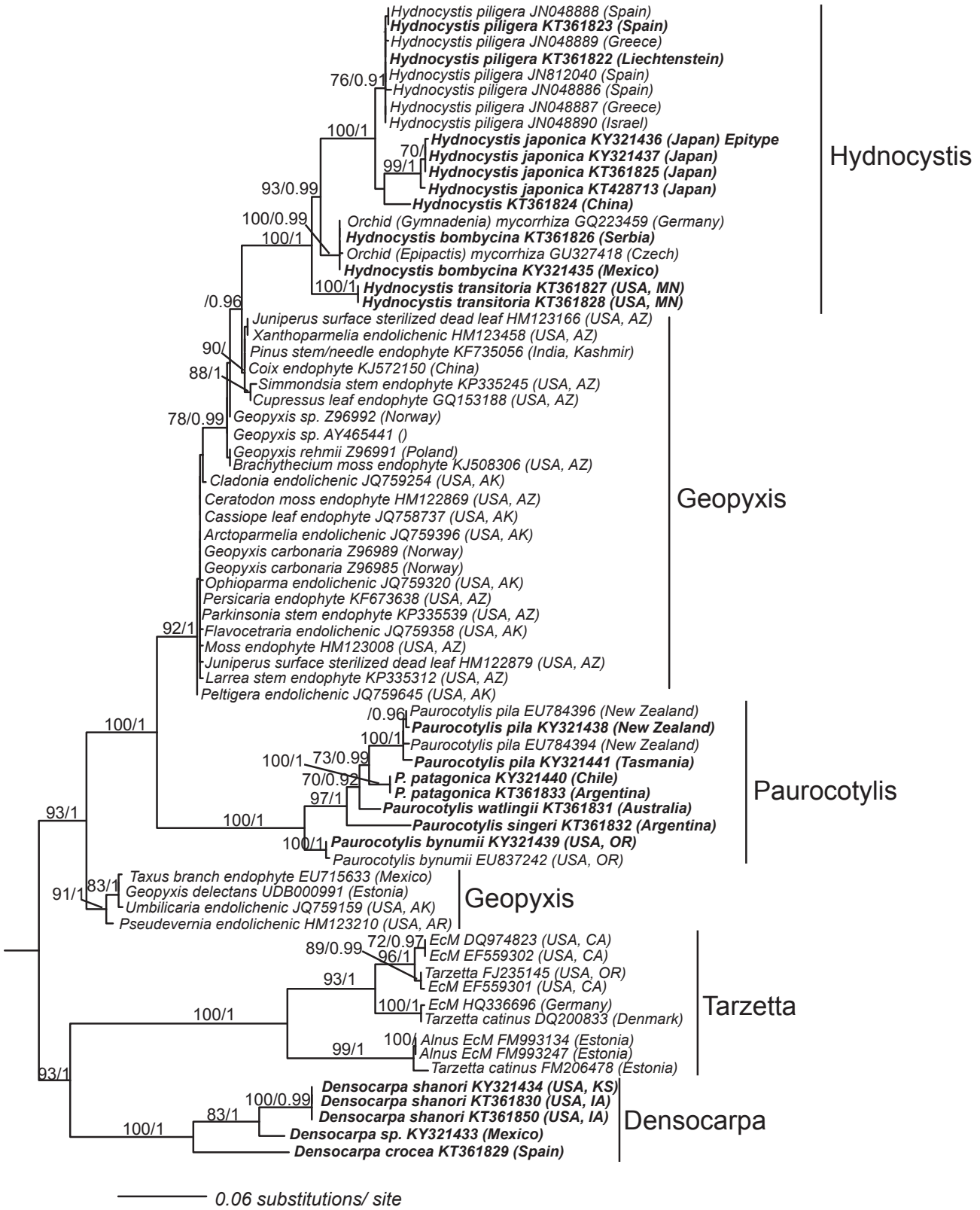

Fig 2 – Phylogenetic tree based on maximum likelihood (ML) analysis of ITS rDNA, with node support from 1000 bootstrap iterations and Bayesian posterior probabilities (BPP). BPP ≥0.95/Bootstrap values ≥70 % are indicated at branch nodes. Thickened branches indicate support from both methods of phylogenetic analyses. Bolded taxa are newly generated sequences. Non-bolded sequences are from GenBank, 'EcM' stands for 'ectomycorrhizal'. Taxon names presented here reflect nomenclatural revisions introduced in the taxonomy section of this paper.

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diam. (spore measurements of various authors summarized in Gyosheva *et al.* 2012). Contents of mature spores cyanophylous in cotton blue, walls light blue.

Anamorph produced from ascospores on malt agar. Buff colored. Conidiophores sympodial, septate, repeatedly dichotomously branched, encrusted with granules, with one to three terminal conidia produced, leaving scars at the location of their release. Conidia globose 6.5–8 μm diam. to ovoid 7.8–9.7 × 5.8–6.5 μm, warted (Fontana & Giovannetti 1987).

Distribution and habitat: On or under the soil surface or in woodland litter in forests dominated by conifers or angiosperms. Europe, Israel, Mexico. Attempts to synthesize mycorrhizae with *Salix* and *Quercus* were unsuccessful and *H. bombycina* was easy to culture (Fontana & Giovanetti 1980). Terrestrial orchid roots have yielded ITS sequences that match *H. bombycina* (Fig 2) (Wang *et al.* 2016).

Specimens examined: **United Kingdom**, England, Somerset, Burrington Coombe, sn date, L. Hawker, Hawker 777 (OSC-34416); **Germany**, Eimersdorf/Saar, 15 August 1968, G. Gross 189 (OSC-131641); **Italy**, Firenze, Mattirolo (OSC-34458); **Serbia**, Grisevac Svrljig, 21 October 1992, Z. Marjanovic, Z. Marjanovic-20, Trappe 23260, (OSC-61969); **Mexico**, Mexico City, Colonia Cuauhtémoc, Shirley Courts Hotel on Sullivan St., under *Pinus*, *Cupressus*, other plants, 14 June 1972, J. Trappe, Trappe 3268, (OSC-34395, FH-284692).

Comments: *Hydnocystis bombycina* was described by Vittadini (1831) as *Genea bombycina* from a specimen collected in Italy, and then redescribed as the type species for *Stephensia* by Tulasne & Tulasne (1851). Hawker (1954) added microscopic details in her re-analysis of this species. The specimen from Serbia that was sequenced for our phylogenetic analysis matches the description for *H. bombycina*, which is morphologically similar to other *Hydnocystis* species but varies in a pronounced infolding of the hymenium. Characteristics of *H. bombycina* and other *Hydnocystis* species include the ptycothecial form, ascomatal opening, peridium with encrusted hyphae, cylindrical, stipitate asci in a palisade, slender paraphyses that overgrow the asci, globose spores that are hyaline, inconspicuously guttulate, and uniseriately arranged, and white to cream-colored gleba. Paraphyses of *H. bombycina* are not fused.

The anamorph of *H. bombycina* is similar to the anamorph of *H. transitoria*. Anamorphs of both species are sympodial, have warted, globose to ovoid conidia, septate, branched, warted conidiophores, and conidial scars at the location of conidial release. The anamorph of *H. bombycina* differs from that of *H. transitoria* in producing only terminal conidia. Differences may be due to the growth regimens; the *H. bombycina* anamorph was produced in pure culture whereas the *H. transitoria* anamorph developed in its natural habitat. There were no attempts to culture *H. transitoria*.

Similarity among the 28S sequences and the ITS sequences of the Serbian and Mexican collections were within 99 %. Both are well supported within the *Hydnocystis* clade.

Since the type species of *Stephensia* is a *Hydnocystis* species, the genus *Stephensia* is a later synonym. *Hydnocystis* (described in 1844) has priority over the name *Stephensia* (described in 1845). *Hydnocystis bombycina* was previously recognized as having a close relationship with *H. piligera* (Alvarado *et al.* 2011) and the paraphyly of *Stephensia* was also recognized previously (Læssøe & Hansen 2007; Perry *et al.* 2007; Alvarado *et al.* 2011; Wang *et al.* 2016).

Hydnocystis transitoria L. Kumar, Healy & M.E. Sm., **sp. nov**.

MycoBank: MB814213

Fig 3

Diagnosis: *Hydnocystis transitoria* is a North American species distinguished from other *Hydnocystis* species by its distinct ITS sequence, nearly smooth ascoma, its thick, well-developed epithecium of parallel and interwoven paraphyses tips, and its envelopment of asci within paraphyses that are interwoven and sometimes fused. Among *Hydnocystis* species with a simple cavity, it is distinguished by much smaller spores.

Holotype designated here: **United States of America**, Minnesota, Fillmore Co., Forestville State Park, hypogeous in mesic *Quercus*-dominated woodland. N43°38′ 28.3″ W92°13′17.3″, 21 July 2009, Leticia Kumar, RH950 (MIN-928483).

Etymology: Latin, 'transitoria', intermediate, referring to our interpretation of this species having paraphyses intermediate between the unfused elongated paraphyses in the prosenchymatic epithecia of *H. piligera* and the pseudoparenchymatic epithecia of more highly modified *Hydnocystis* such as *H. bombycina* comb. nov.

Description: *Ascoma* a ptycothecium, hypogeous, laterally flattened and somewhat rounded in outline, 4–8 mm diam. × 1.5–3 mm high with an opening 1–4 mm diam. (Fig 3A), leading into a simple cavity lined by a regular, unfolded hymenium that is cottony in appearance due to a well-developed epithecium (Fig 3B, arrows). Peridium light brown (ISCC-NBS 74) when fresh, turning darker (ISCC-NBS 78) when dried, covered with sparse well-spaced short granule-encrusted hairs (Fig 3C). The scent of fresh fruiting bodies a mixture of a sour odor (reminiscent of soy sauce) and a sweet odor (reminiscent of maple syrup). When dried, the ascoma had a strong sweet scent of maple syrup or pearly everlasting flowers (*Anaphalis margaritacea*).

Excipulum 500 μm, of pseudoparenchyma, short microscopic excipular hairs, 4–8 μm wide, arising sporadically from the outermost cells (Fig 3D), outer excipulum 100–170 μm, light golden brown (ISCC-NBS 74) with cells up to 30 μm thick, walls 2–3 μm thick. The inner excipulum, 240–300 μm thick, walls 1 μm thick. The excipular layers are of textura angularis (Fig 3E).

Hymenium a palisade of asci and paraphyses.

Paraphyses 2–10 μm diam., parallel to interwoven and sometimes fused to form a hyaline textura intricata (Fig 3F–I) that embeds the asci and extends beyond them to form a well-defined epithecium, 63–197.5 μm thick, composed of parallel to interwoven and sometimes fused hyphae (Fig 3F, I). Subhymenium hyaline with cells up to

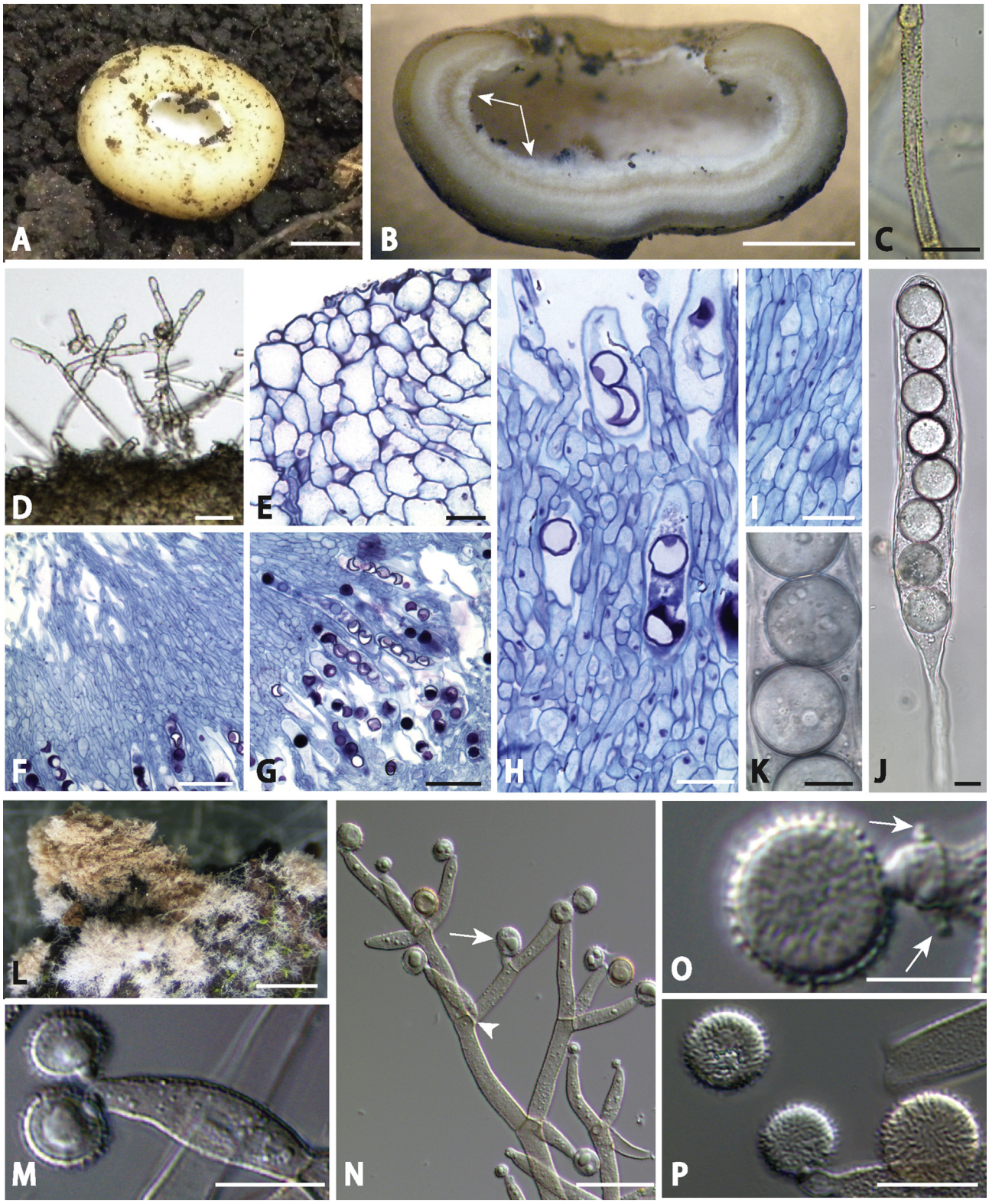

Fig 3 – *Hydnocystis transitoria* (A–P). (Type specimen RH950) (A). Ascoma with opening into cavity; (B). Ascoma cut in half to expose cavity lined with regular palisade of hymenium overtopped by cottony epithecium (arrows); (C–K). Brightfield microscopy; (C). Excipular hypha encrusted with granules; (D). Projecting hyphal tips of the excipulum; (E–H) Resin embedded sections stained in toluidine blue O; (E). Excipulum composed of textura angularis; (F). Epithecium; (G). Hymenium with asci and paraphyses; (H). Higher magnification of asci interspersed with interwoven paraphyses; (I). Epithecium of parallel hyphae mixed with textura intricata; (J). Cylindrical ascus with uniseriate spores, and tapered base; (K). Ascospores; (L–P).

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20 μm diam., of textura intricata. Epithecial cells 2–16 μm wide, with average diam. of 4 μm. Paraphyses tips separate and distinct at the epithecium leading edge.

Asci 185–217 μm long, 16–23.5 μm diam. tapering to their bases (Fig 3J), with obtuse apices, 8-spored, uniserate, J-in Melzer's. Croziers not observed.

Ascospores globose, smooth, containing inconspicuous oil droplets (Fig 3J–K) 18–24.5 μm, with an average size of 21 μm in diam.

Anamorph: (Fig 3L–P). Mycelial spore mat on woody debris, colonies small (~1 cm in diam.), pinkish-brown (Fig 3L). Hyphae, including conidiophores, encrusted with granules (Fig 3M–N), 4–6 μm diam. at the septum, even or swollen up to 6–8 μm between septa. Conidiophores sympodial, terminal, or at the base of septa along the main axis (Fig 3N arrowhead), with one to four branches observed per node, one to two concurrent conidia per apex, and up to five conidiogenous scars per apex (Fig 3O arrows). Branches 2 μm long with conidium almost sessile (Fig 3N arrow) to 32 μm long, tapering at apex (Fig 3N). Mature conidia globose to ovoid (laterally or longitudinally flattened) with closely spaced truncated spines (Fig 3O–P). Size excluding ornamentation: (6.5–) 7–9 (–11) with a median of 8 μm for globose spores or 7–8.5 × 7–9 μm with a median of 8 × 8.5 μm for ovoid spores. Spines 1–1.5 μm long. There was no attempt to culture the anamorph.

Distribution and habitat: Two ascomata were collected in July and the anamorph was collected two years later in October from the same site in Minnesota. This site is on the Paleozoic Plateau, characterized by limestone/dolostone/sandstone and shale bedrock, covered by silty glacial deposits in the uplands and sandy gravel in the river valleys. The area is known for its karst topography (Williams 2009). The truffles were shallowly hypogeous under leaf litter in a hardwood forest of *Quercus rubra*, *Q. alba*, *Carya ovata*, *Tilia americana*, *Ostrya virginiana*, *Acer* sp. and *Celtis occidentalis*. The anamorph was collected on a fallen twig in the same woods. These are the only known collections of *H. transitoria*. This species is presumed to be biotrophic, saprobic, or of a non-ectomycorrhizal habit such as orchid mycorrhizae, since there is no EcM evidence of any *Hydnocystis* from root tips.

Anamorph examined: **United States of America**, Minnesota, Fillmore Co., Forestville State Park, hypogeous in mesic *Quercus*-dominated woodland. 2 October 2011, R. Healy, AM488 (MIN-929321).

Comments: There are key microscopic morphological differences between *H. transitoria* and the two previously described and accepted *Hydnocystis* species, *H. japonica* and *H. piligera*. *Hydnocystis transitoria* has parallel to interwoven paraphyses that encase the asci in textura intricata, the epithecium is composed of parallel to interwoven hyphae, and sparse excipular hairs are present on the ascoma. In contrast, *H. piligera* has separate (non-fused) paraphyses (Tulasne & Tulasne 1851), an epithecium formed by unfused paraphyses, and ascomata that are densely tomentose (Montecchi & Sarasini 2000). Similar to *H. japonica*, the paraphyses of *H. piligera* overgrow the asci to form a prosenchymatic epithecium. The peridium of *H. piligera* is more densely tomentose than the peridium of either *H. japonica* or *H. transitoria*. *Hydnocystis bombycina* comb. nov. differs in its densely tomentose ascoma and highly invaginated hymenium. The spores of *H. transitoria* are smaller than those reported for *H. piligera* and *H. japonica*, but are within the size range reported for *H. bombycina* (de Vries 1985; Gyosheva *et al.* 2012).

Hydnocystis japonica (Kobayasi) Trappe, Mycotaxon 2: 115. 1975.

Mycobank (epitype): MBT374117

Fig 4A–E

Basionym: *Protogenea japonica* Kobayasi, 1964 [1963]. *Transactions of the Mycological Society of Japan* **4**:119–120.

Types: **Japan**, Kagoshima Prefecture, Utizume, collected by H. Indoh, K. Aoshima, Y. Kobayasi on 14 November 1962 (Isotype CUP-JA-003467) annotated as *Hydnocystis japonica* by J.M. Trappe 12 November 1974. Epitype designated here: **Japan**, Kagoshima Pref., Mt. Koba, Minamiosumi-cho, collected by T. Orihara on 12 May 2015 (KPM–NC–0024873).

Etymology: 'japonica', Japan, the country from where this species was described.

Description: *Ascoma* a ptycothecium, laterally flattened, more or less rounded, 8–12 mm diam. × 5–7 mm high with an opening up to 2 mm in diam (Fig 4A). into a large central cavity lined with a regular unfolded to once folded white hymenium that is cottony on the surface due to a well-developed epithecium. The peridium light brown (ISCC-NBS 74), smooth except for a basal tuft of white mycelium.

Excipulum 500–550 μm, projecting hyphal tips ('hairs') with cell walls 1 μm thick, light golden brown (ISCC-NBS 74), with short granule-encrusted, outer excipulum composed of textura angularis with more or less isodiametric cells, outermost cell walls 1–1.5 μm thick (Fig 4B). The subtending layer is of textura intricata, 100–150 μm thick, hyaline.

Hymenium a palisade of paraphyses and asci.

Paraphyses 3–4 μm diam., septate, parallel (Fig 4C), extending beyond the asci, where they appear to adhere at the tips (although in the type description, the tips are free). Disentangled tips of paraphyses are irregularly rough (Fig 4D).

Asci cylindrical, 270–400 × 35–40 μm, tapering to a narrow elongated base (Fig 4E), with three to eight uniseriately arranged spores of varying sizes.

Ascospores globose, smooth, hyaline, not guttulate, 25–32 μm diam., average of 30 μm.

Anamorph unknown.

Distribution and habitat: On soil or shallowly hypogeous, Japan.

Anamorph; (L). Mitospore mat; (M–O). DIC microscopy; (M). Two conidia produced at the tip of a conidiophore; (N). Conidiophore branches at septa (arrowhead), sometimes short (arrow), and conidia at branch tips; (O). Conidia denticles (arrows) and scars; (P). Warted ornaments of conidia and encrusted conidiophores. – Scale bars (A, B, L) = 2 mm, (C, E, H, I, N) = 20 μm, (D) = 25 μm, (F, G) = 100 μm, (J, K, M, P) = 10 μm, (O) = 5 μm.

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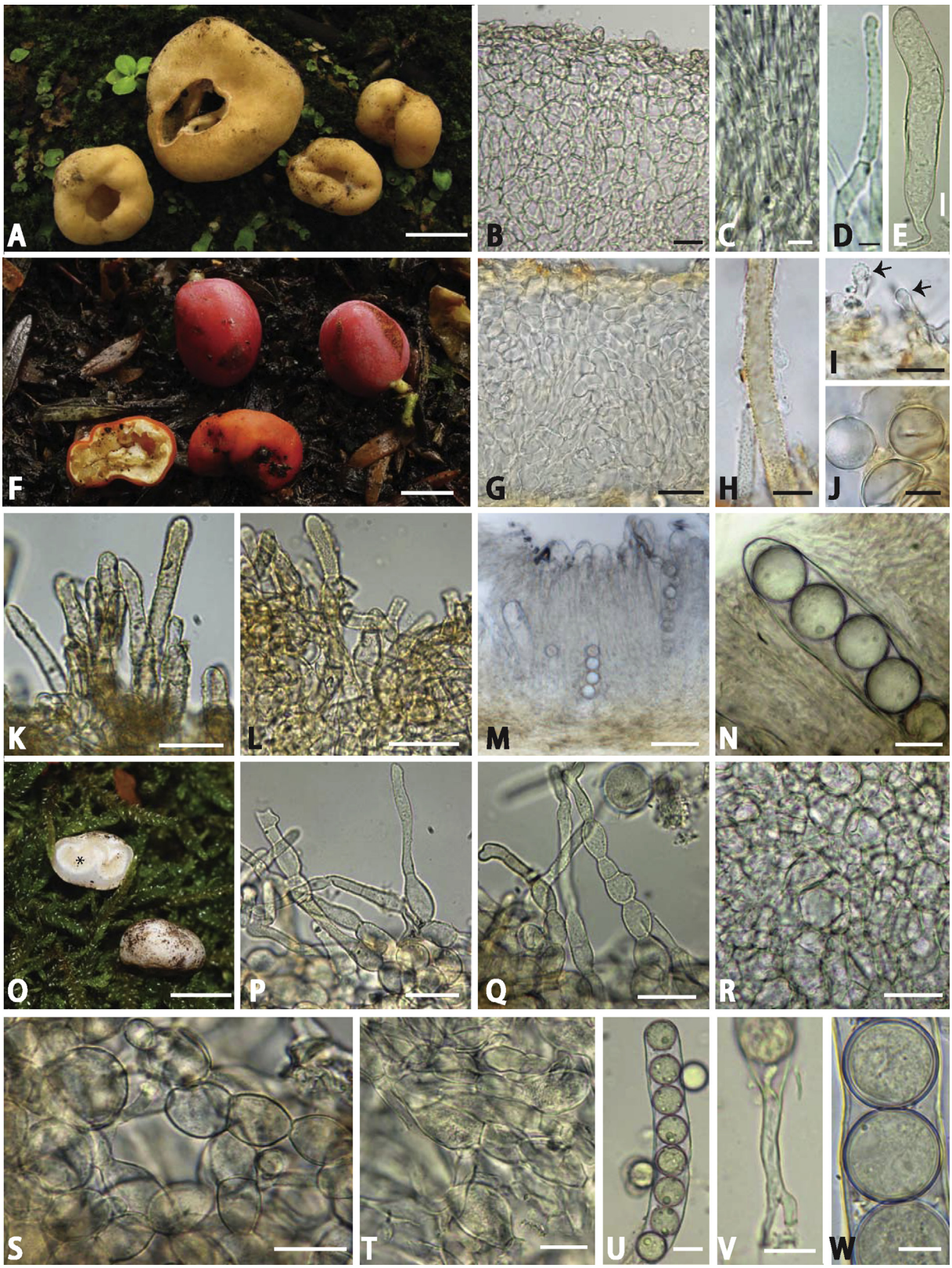

Fig 4 – (A–E) *Hydnocystis japonica*. (A). Ascomata; (B–E). Brightfield microscopy. (B). Excipulum; (C). Paraphyses; (D). Tip of paraphysis; (E). Immature ascus; (F–I) *Paurocotylis pila* (F) Ascoma cut in half (lower) and podocarp fruits (upper); (G–N) Brightfield microscopy. (G). Excipulum; (H). Projecting granular encrusted excipular hypha; (I). Hyphal ends (arrows) of excipular cells; (J). Ascospores; (K–N). *Paurocoylis singeri* (Robledo 1858). (K). Tomentum of encrusted hyphal tips of outer excipular hyphae; (L). Outer excipulum construction of loosely interwoven, unfused hyphae; (M). Regular hymenium of asci and paraphyses shorter than asci; (N). Ascus with ascospores; (O–W). *Paurocotylis patagonica* sp. nov. (Type specimen EN59). (O).

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Specimens examined: **Japan**, Kagoshima Prefecture, Utizume, 14 November 1962, H. Indoh, K. Aoshima, Y. Kobayasi (Isotype CUP-JA-003467 as *Protogenea japonica* annotated as *Hydnocystis japonica* by JM Trappe 12 November 1974); Minamiosumi-cho, Mt. Koba, on ground in deciduous broadleaf forest, 12 May 2015 T. Orihara (Epitype KPM–NC–0024873); Nishinoomote-shi, Anjoh, along Waseda River on ground under *Cryptomeria japonica* 12 August 2015 T. Orihara (KPM-NC0024892); Kanagawa Prefecture, Yugawara-machi, Mt. Tensho, 14 November 2011 T. Orihara (KPM-NC 18030, FLAS F-59178); Hayama-machi, Nangoh-uenoyama Park, 23 October 2011, S. Inoue (KPM-NC 18029, FLAS F-59177); Isehara-shi, Hinata, 17 November 1999 Y. Degawa (KPM-NC 8613); Odawara-shi, Iryuda, K. Maruyama, 25 October 2003, J. Ohta (KPM-NC 11669, FLAS F-59176).

Other specimens examined:

China, Mixed forest of *Picea* and *Abies* with occasional *Cupressus, Juniperus* and *Betula* along stream. 31°41′46″ N, 100°42′19″ E, 3620 m, May 1998, D. Hibbett and Z. Wang wz2209 (FH-00290161, FH-00290162). (*Hydnocystis* sp., originally identified as *H. piligera*).

Comments: Kobayasi (1963) described the ascomata as having an opening but he indicated that some ascomata were closed. This was one of the reasons, along with the nearly smooth peridium and occurrence on the soil surface, that he erected a new genus (*Protogenea*) for this species. Trappe (1975) recognized the key similarities of *P. japonica* with *H. piligera*, along with the variability of the diagnostic criteria, and correctly placed it in *Hydnocystis*. The opening in *H. japonica* is typically larger than the opening in *H. piligera*. Since the type specimen is too old for effective sequencing, we have designated as epitype a recently sequenced Japanese collection (KPM-NC0024873) collected ca. 6 km west of the holotype locality that agrees in every respect with the type description. A specimen collected in China (*Hydnocystis* sp. FH 290161) was originally identified as *H. piligera* but later identified as *H. japonica* by Wang & Pei (2001) based on morphological similarity. With phylogenetic analysis, however, the Chinese specimen apparently represents an undescribed species. This specimen should be studied in greater detail to determine if there are consistent morphological differences.

Paurocotylis Berkeley in Hooker, 1855. Botany of Antarctic Voyage of H.M. Discovery Ships Erebus and Terror, in the years 1839–1843: Flora Novae-Zelandiae, II. London, pp. 188. Type species. Paurocotylis pila Berkeley in Hooker, 1855. Botany of Antarctic Voyage of H.M. Discovery Ships Erebus and Terror, in the years 1839–1843: Flora Novae-Zelandiae, II. London, pp. 188.

Etymology: Greek, 'pauro', few, 'cotylis', a cavity, presumably referring to the cavity that characterizes the type species of this genus.

Description: *Ascoma* a ptycothecium with solid, white gleba that may develop a cavity.

Excipulum: Outer excipulum of interwoven hyphae. Projecting hyphae granule-encrusted.

Hymenium: Paraphyses and asci in a palisade.

Paraphyses thin, branched.

Asci stalked, inamyloid, indehiscent, cylindrical, early deliquescing in some species. *Ascospores* globose, smooth, hyaline, not conspicuously guttulate.

Anamorphs unknown.

Paurocotylis pila Berkeley in Hooker, 1855. Botany of Antarctic Voyage of H.M. Discovery Ships Erebus and Terror, in the years 1839–1843: Flora Novae-Zelandiae, II. London, pp. 188.

Fig 4F–J

Type: **New Zealand**, North Island, Tehawera collected by Colenso on the ground, (K-171066). Not examined.

Etymology: Latin, 'pila', sphere, presumably referring to the globose shape of the ascomata.

Amended description (from Dennis 1975): *Ascoma* a globose ptycothecium bright crimson (v.R 11) drying to a dull rufous (Berkeley 1855), soft, up to three cm, collapsing and becoming wrinkled. Gleba solid, white, and veined in young ascomata, becoming cream-colored with powdery spores surrounding large cavity in mature specimens. Although the peridium of *Paurocotylis pila* appears smooth (Fig 4F–G), there are microscopic granule-encrusted hyphae that project from the excipulum (Fig 4H–I). Odor of ascomata not distinctive.

Excipulum: Outer excipulum 200–300 μm thick, of compactly interwoven hyphae 3–12 μm broad partially inflated to form pseudoparenchyma (Fig 4G); surface and projecting hyphae in outermost layer granule-encrusted (Fig 4H–I); walls 1 μm thick. Inner excipulum colorless, pseudoparenchymatous, composed of subglobose cells 12–40 μm in diam.

Hymenium of paraphyses and asci, but asci are early evanescent.

Paraphyses slender, branched, septate, hyaline, 3 μm diam.

Asci cylindrical with long stalk, 200–300 × 20–25 μm, inamyloid, with 4–6 spores.

Ascospores globose, smooth, non-guttulate, hyaline to yellowish, 17–23 μm in diam. with walls ~1 μm thick (Fig 4J).

Distribution and habitat: Native to New Zealand, Australia, and Tasmania but apparently naturalized in the United Kingdom (Dennis 1975). It is typically found under *Podocarpus* in New Zealand (Trappe & Claridge 2015), and in a gravel pit under *Crataegus, Sambucus, Urtica, Chamaenerioun,* and *Brachythecium* in Nottingham, England.

Specimens examined: **New Zealand**, det. Lloyd, sent to Roland Thaxter 1921, (FH-374294); North Island, Tongariro National Park, Ketetahi Road, under *Podocarpus* sp., 19 May 2013, T. Orihara TO-13-NZ-19-1 KPM-NC 23051); Bay

Ascoma halves showing gleba (upper left) with hymenium surrounding modified epithecium (asterisk); (P–W). Brightfield microscopy. (P). Hyphal ends ('hairs') of outer excipulum; (Q). Moniliform excipular hyphal tips; (R). Inner excipulum of pseudoparenchyma; (S). Outer excipulum with unfused interwoven hyphae; (T). Interwoven hyphae in the gleba, above the asci (modified epithecium); (U). Ascus with ascospores; (V). Forked base of ascus; (W). Ascospores. – Scale bars (A) = 1 cm, (B, E, K, N, S, T, U, V) = 20 μm, (C, H, I, J, W) = 10 μm, (D, F, O, P, Q, R) = 5 μm, (G, L) = 30 μm, (M) = 40 μm.

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of Plenty, Whinray Scenic Reserve, 14 May, 2013, Petra White TO-13-NZ-11-5 (KPM-NC 23032); Kaitoke Regional Park, Swingbridge Trail, on humus in broadleaf and podocarp forest, 7 May 1997, D.J. and E.G. McLaughlin DJM1050 (MIN-863002). **Australia**, Tasmania, Mt. Wellington, Hobart, in wet sclerophyll litter, elevation 300 m; 42° 54′S 147° 15′E, 30 May 2016, G. Gates EB263 (FLAS-F-59803).

Comments: The specimen from Tasmania differs from the New Zealand specimens because the Tasmanian specimen is light brown with encrusted hairs in the excipulum whereas the specimens from New Zealand are red and mostly smooth or with minute hairs. The hairs of the Tasmanian specimen were generally short, in scattered clusters, and extended away from the exciple. However, some were long, repent, forming a loosely interwoven outer layer in some places along the excipulum. They originated from cells that were in the outer layer of the exciple. At the molecular level, the ITS sequence from the Tasmanian specimen was ca. 99 % similar to other *P. pila* collections from New Zealand and Australia. Further investigation using other loci may help to clarify whether the Tasmanian collection represents a new species or a divergent morphology within *P. pila*. It has been suggested that the New Zealand taxon adapted to bird dispersal, as evidenced by its attractive bright red coloration and smooth peridium. The color and shape resemble the podocarp fruits of the bushes that it is often found fruiting under (Trappe & Claridge 2015).

Paurocotylis singeri (Gilkey) Nouhra, Healy & ME Sm., **comb. nov.**

MycoBank: MB814821

Basionym: *Hydnocystis singeri* Gilkey, 1962 [1961]. *Mycologia* **53**: 216.

(Fig 4K–N)

Holotype: **Argentina**, Tucumán, 10 March 1955 R. Singer Gilkey850 (Holotype OSC-25345).

Etymology: 'singeri', In honor of the mycologist Rolf Singer who collected the type specimen.

Description (from Gilkey 1961): *Ascoma* a ptycothecium, subglobose, 1 cm, even to furrowed, white with a simple to partially partitioned cavity, minutely puberulent. Surface hairs originate within the excipulum rather than from surface cells (Burdsall 1968). Gleba white, with one or two cavities. Excipulum composed of pseudoparenchyma.

Hymenium of asci and paraphyses in palisade.

Paraphyses simple to branched, shorter than the asci.

Asci cylindrical, 190–228 μm long, 8-spored.

Ascospores globose, smooth, hyaline, 21 μm diam.

Description of Paurocotylis singeri (CORD-C00004224 Robledo #1858): Ascoma a ptycothecium, white, 15 × 11 × 8 mm when dry, with chambers in the gleba, tomentose with light brown encrusted projecting hyphae (Fig 4K) which are the ends of interwoven, unfused, branching hyphae that can be traced to various levels within the outer excipulum, hyphae 6–8 μm diam., occasionally swollen to 9–10 μm in projecting hyphae.

Excipulum: Outer excipulum of loosely interwoven hyphae (Fig 4L), constricted at the septum and swollen up to 16 μm between septa. Inner excipulum similar but tightly packed, and fused to form pseudoparenchyma.

Hymenium of cylindrical asci with narrow paraphyses that are about 20 μm shorter than the asci (Fig 4M–N). The hymenium is invaginated and lines a convoluted chamber. Subhymenium of interwoven to parallel hyphae, 3–8 μm diam.

Paraphyses 3 μm in diam., septate, even, not inflated at the tips.

Asci 210–268 × 29–36 μm with 2–8 spores per ascus. In asci with fewer than eight spores, degenerated spore remnants are often visible.

Ascospores globose, smooth, inconspicuously guttulate, 21–24 μm diam. with 2 μm thick walls (Fig 4N).

Distribution and habitat: Known only from the Yungas forest region of Argentina.

Specimens examined: **Argentina**, Tucumán, 10 March 1955, R. Singer Gilkey850 (Holotype OSC-25345, Isotype CUP-048961); Jujuy Province, Departmento Ledesma, Parque Nacional Calilegua, Sendero Tataupa, Latitude 23° 44′ 33.3″ south, Longitude 64° 51′ 8″ west, epigeous on soil in montane subtropical Yunga forests, 2 April 2008, Robledo and Rajchenberg Robledo 1858 (CORD-C00004224, FLAS F-59171).

Comments: The Robledo 1858 specimen described above was collected near the type locality and fits the type description in most regards. It differs in that the asci are often less than 8-spored and the spores are 21–24 μm with an average of 22 μm whereas spores for *H. singeri* were described as being 21 μm in diam. or less. Although the spore size and number of spores per ascus differ from the type description, the overall similarities and proximity of collection to the type locality support our identification of this taxon as *P. singeri*. Further collections will be needed to help resolve the morphological concept of this species. Our new collection is clearly similar to the type and both morphological and molecular data indicate that this species is a *Paurocotylis*, not a *Hydnocystis*. This specimen was collected in a type of forest with only one EcM host, the infrequently occurring subcanopy tree *Pisonia zapallo* Griseb. *Paurocotylis* has not been detected on the roots of *Pisonia zapallo*, but few studies have included this host (Geml *et al.* 2014).

Paurocotylis patagonica Nouhra, Healy and M.E. Sm. **sp. nov.**

MycoBank: MB814820

Fig 4O–W

Diagnosis: *Paurocotylis patagonica* is a South American species that can be distinguished from other *Paurocotylis* by its genetic ITS profile, its white ascoma, lack of a distinct simple cavity, its outer excipular and projecting hyphae of granule-encrusted moniliform cells, and its indeterminate paraphyses.

Holotype designated here: **Argentina**, Neuquen Province, Lanin National Park, Ruta a Hua Hum, 12 May 2009, Eduardo Nouhra EN59 (CORD-C00004222).

Etymology: 'patagonica', in reference to Patagonia, the region of South America where this species was discovered.

Description: *Ascoma* a ptycothecium, small, 7 × 4 mm, white, with one hymenium-lined central region stuffed with sterile hyphae that are slightly yellowish in mass (Fig 4O); exterior minutely felty.

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Excipulum: The outer excipulum is 60–120 μm thick, slightly brownish. Emanating hyphae are septate, hyaline, finely encrusted hyphae that originate from various levels within the outer excipulum. The free hyphal tips are 4 μm diam. at septum, many swollen 10–12 μm between septa (Fig 4P–Q). The excipulum is 280–400 μm thick, hyaline, and composed of interwoven hyphae that fuse to form pseudoparenchyma (Fig 4R). The outer excipulum can be teased apart to reveal many unfused interwoven hyphae (Fig 4S). Hyphae constricted to 5 μm at the septum, and swollen to 8–36 μm between septa, appearing beaded or moniliform (Fig 4S), with walls that are about 1 μm thick. Subhymenium is of interwoven hyphae, 5 μm diam. at septum, sometimes swollen 16–40 μm between septa (Fig 4T).
Hymenium a palisade of asci and modified paraphyses.
Paraphyses very slender, not fused, 2–4 μm wide, some straight, others tortuously bent between and beyond asci. The paraphyses are greatly elongated, and stuff the region above the asci where they are parallel to interwoven, hyaline, and are often swollen between the septa. This tissue may be thought of as a modified epithecium.
Asci cylindrical to slightly clavate (Fig 4U), tapered to the forked base (Fig 4V), 24–25 (–28) μm × 100–270 μm, walls 1 μm thick, usually about 170 μm in length and of even width tapering to base, with 4– typically 7–8 uniseriate spores at maturity.
Ascospores globose, smooth, with inconspicuous guttules (Fig 4W), pinkish, (16–)19–26 μm, with an average of 22.6 μm diam., varying in size, with walls two layered 1.6 μm thick (Fig 4W).
Distribution and habitat: Known from mixed forests in the Patagonian region of the Andes Mountains in Argentina and Chile.
Specimens examined: **Argentina**, Neuquen Province, Lanin National Park, Ruta a Hua Hum, Sitio 3 (b1), in organic soil with roble pellin (*Nothofagus obliqua*), Latitude 40° 08′ 56.6″ south, Longitude 71° 36′ 29.8″ west, 12 May 2009, Eduardo Nouhra EN59 (Holotype CORD-C00004222). **Chile**, Puyehue National Park, Anticurra, Sendero La Princesa, in mixed forest, 5 May 2016, Pablo Sandoval Leiva, MES-1666 (SGO, FLAS-F-59804).
Comments: This species shares a long insert near the beginning of the ITS with *P. pila* that has over 80 % similarity in DNA sequence (Supplemental Fig S1). Phylogenetic analyses of the ITS and 28S place these two species closer to each other than to the other species in the clade. *Paurocotylis patagonica* differs from *P. singeri* and *P. bynumii* in having paraphyses that are much longer than the asci, with the ends incorporated into a modified epithecium. *Paurocotylis patagonica* differs from *P. pila* in having ascoma that are white rather than red, having asci that are not evanescent, and having a chamber above the asci that is stuffed by a modified epithecium rather than containing a hollow cavity. *Paurocotylis patagonica* is most similar in morphology to *Paurocotylis watlingii* from Australia (FLAS F-59172), but in *P. patagonica*, the tomentum is less dense and the spores are smaller on average.

Paurocotylis watlingii Healy and M.E. Sm. sp. nov.
MycoBank: MB819377
Fig 5A–E

Diagnosis: *Paurocotylis watlingii* is an Australian species distinguished from other *Paurocotylis* species by its distinct ITS sequence and combination of brown tomentose ascoma, modified epithecium in which paraphyses are indeterminate, lack of glebal cavity, and relatively large spores.
Holotype designated here: **Australia**, Queensland, Lamington National Park, Binna Burra, 27 March 1974, coll. R. Watling, Trappe 11725 (FLAS F-59172).
Etymology: In honor of the collector, the eminent mycologist Roy Watling.
Description: *Ascoma* a ptycothecium, clay-colored with white, unstratified gleba with slightly sinuous streaks, 8 × 8 mm, slightly flattened, slightly subtomentose with granule-encrusted hyphae (Fig 5A).
Excipulum: Outer excipulum composed of brown-walled textura intricata, mostly unfused and can be teased apart (Fig 5B). Outer excipular hyphae emanate from the ascoma to form the tomentum (Fig 5B–C); hyphal ends 6 μm, swollen to 10 μm, or of even diam. Outer excipular hyphae constricted at the septum (5 μm) and inflated between septa to 26 μm (Fig 5B). Inner excipulum similar but hyphae fused and more densely packed together (Fig 5D).
Hymenium a palisade of asci and elongated paraphyses, 3 μm diam., that form a modified epithecium.
Paraphyses are indeterminate in a modified epithecium. Hyphae are tortuously bent. Subhymenium of uninflated, interwoven hyphae.
Asci long, narrow, with eight or fewer uniseriate spores/ascus.
Ascospores smooth, globose, pink-walled, inconspicuously guttulate, 22–28 μm diam., with an average size of 25 μm (Fig 5E).
Distribution and habitat: Only known from a subtropical rainforest in Australia, from a single collection found on the soil surface by a mammal burrow, evidently tossed up to the soil surface.
Comments: The paraphyses and modified epithecium of this species are similar to those of *Paurocotylis patagonica*. However, the ascoma of *Paurocotylis watlingii* (FLAS F-59172) is more densely tomentose, with outer excipular hyphae that are darker brown than in *P. patagonica* and the spores are larger on average. This species is described from a single collection.

Paurocotylis bynumii (Trappe, Bushnell & Castellano) Healy & M.E. Sm., **comb. nov.**
MycoBank: MB814822
Basionym: *Stephensia bynumii* Trappe, Bushnell & Castellano, *Mycotaxon* **64**: 432 (1997) (basionym).
Holotype: **United States of America**, Oregon, Turner, under *Corylus* and *Rhus*, 20 June 1980 O. Bynum Trappe 5832 (OSC-58834).
Etymology: 'bynumii', in honor of O. Bynum, the collector of the type specimen.
Description: *Ascoma* a ptycothecium, subglobose, lobed, folded, 1–2 × 1.5–2 cm, yellowish brown with brown tomentum, gleba white to cream-color with pink-brown to orange brown veins. Odor not distinctive.
Excipulum: Outer excipulum 50–150 μm thick, composed of loosely interwoven hyphae that form moniliform cells, 4–9 μm diam., cells inflated to 10–40 μm. Tomentum

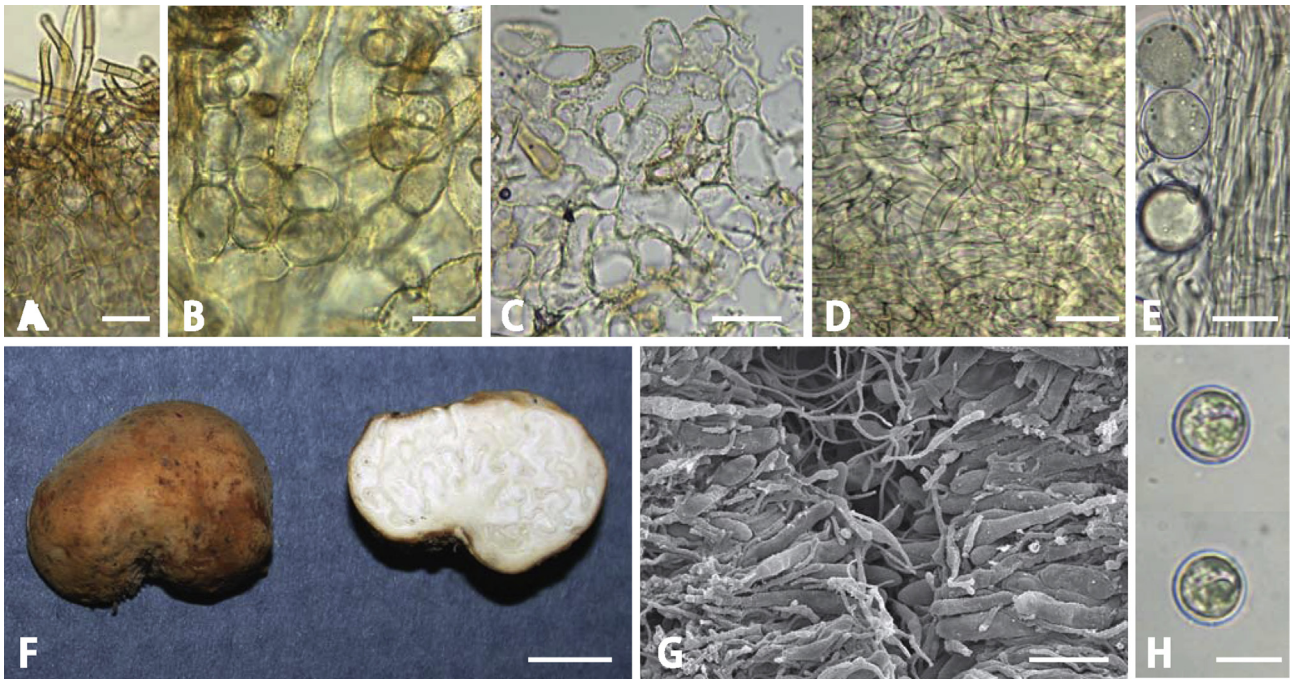

Fig 5 – (A–E). *Paurocotylis watlingii* (FLAS-F-59172) Brightfield microscopy. (A). Excipular tomentum; (B). Outer excipulum of unfused, interwoven, granule-encrusted hyphae, swollen between the septa; (C). Outer excipulum with granule-encrusted hyphae; (D). Excipulum; (E). Hymenium of asci and indeterminate paraphyses; (F–H). *Densocarpa shanorii* (RH1310). (F). Ascoma halves showing gleba (rt); (G). SEM of opposing hymenia of asci and paraphyses that grow into the channel between them; (H). Ascospores. – Scale bars (A, G) = 50 μm, (B, C, D, E) = 20 μm, (F) = 5 mm, (H) = 15 μm.

granule-encrusted, granules hyaline to light yellow. Inner excipulum hyaline, tightly interwoven.

Hymenium a palisade of paraphyses and asci.

Paraphyses narrow 2–3.5 μm diam., septate, same length as asci or slightly longer.

Asci cylindrical, 230–250 × 18–25 μm tapering to long stem and forked base, 6–8 spores arranged uniseriately.

Ascospores globose, smooth, hyaline, 16–21 μm diam.

Distribution and habitat: Only known from Oregon, USA. Hypogeous under Pseudotsuga menziesii, *Corylus cornuta* var. *californica*.

Specimens examined: **United States of America**, Oregon, Marion Co., Turner, Marion Hill Rd, WVNM, under *Corylus* and *Rhus*, 600′ elevation, 20 June 1980, O. Bynum Trappe 5832 (Holotype OSC-58834); 28 June 1980, Trappe 5855 (OSC-58835); under *Tsuga heterophylla*, 10 July 1980, Trappe 5868 (OSC-58836); 26 July 1980, Trappe 5884 (OSC-58837); 2 August 1980, Trappe 5942 (OSC-58838); Junction of County Rds.-Howell Prairie No. 51 and Waconda Rd. No. 6, under maple, 200′ elevation, 9 April 1986, W. Bushnell Trappe 8881 (OSC-58839); Clackamas Co., Beaver Cr., WVCL, under *Pseudotsuga menziesii*, *Alnus rubra* & *Corylus californica*, elevation 500 ft, 15 August 1986, D. Wheeler Trappe 9004 (OSC-58840); Paul Bishop's Tree Farm, 26 July 1987, Trappe 9574 (OSC-58841).

Comments: *Paurocotylis bynumii* is the only member of the genus *Paurocotylis* to have a native distribution in the Northern Hemisphere. It is also the most disjunct, as it occurs in the Northwestern USA, while the others have a Gondwanan distribution. It is sister to the other species in *Paurocotylis*. We place it in *Paurocotylis* as the best current hypothesis, acknowledging that further data may reveal it to be a distinct genus. Morphological features that help to unite this species with other *Paurocotylis* include the brown tomentum of granule-encrusted hyphae, outer excipulum composed of loosely interwoven hyphae that form chains of moniliform cells, and asci that taper to the forked base. The paraphyses are as long as or slightly longer than the asci, and there is no epithecium. In this regard, the morphology is more like that of *P. singeri* than the other *Paurocotylis* species. *Paurocotylis bynumii* is reported as a probable mycorrhizal associate with *Pseudotsuga menziesii* and *Corylus cornuta*, but molecular evidence from root tips is lacking.

Densocarpa Gilkey *N. Amer. Fl.*, Ser. 2 (New York) **1**: 16 1954.

Type species: ***Densocarpa shanorii*** Gilkey [as '*shanori*'], 1954. *N. Amer. Fl.*, Ser. 2 (New York) **1**: 16, 1954[1955]. Mycologia **46**: 786.

Fig 5F–H

Types: **United States of America**, Illinois, Champaign-Urbana, 14 June 1953 L. Shanor Gilkey 764a (Holotype ILL-10894, Isotype OSC-38610).

Synonym: *Stephensia shanorii* (Gilkey) Gilkey (1962) [1961] *Mycologia* **53**(3): 219.

Etymology: 'shanorii', in honor of L. Shanor, the collector of the type specimen.

Description: *Ascoma* a ptycothecium, knobby, folded, sandy in appearance, smooth to slightly verrucose, often cracked,

often grub-infested, sometimes with ants; 0.5–2 cm, light ochraceous buff (Gilkey 1954), light yellowish-pink (ISCC-NBS 28) to light orange brown (ISCC-NBS 57) (Fig 5F), with basal tuft of granule-encrusted hyphae. Gleba pale ochraceous buff (Gilkey 1954), white to cream white with meandering canals (Fig 5F). Odor unpleasant, rafinaceous.
Excipulum 80–180 μm thick. Outer excipulum pale yellowish-brown, of interwoven hyphae 5–10 μm at septum with cells inflated to 15 μm composed of textura intricata that is fused to form small-celled pseudoparenchyma. Excipular hairs sparse, of smooth to encrusted hyphae (granules up to 0.6 μm diam., orange in mounting medium with ethanol), hyaline to yellowish, 5 μm diam. Inner excipular hyphae hyaline, cells less swollen, but otherwise similar to outer excipulum, hyphae 2.5 μm at septum swollen to 5 μm.
Hymenium a palisade of paraphyses and asci, infolded with canals between opposing hymenia.
Paraphyses hyaline, slightly swollen at the tips, 2.5–5 μm diam., some about the same length as asci and others greatly elongated to form a web of hyphae in the canal between opposing hymenia. Elongated paraphyses fuse, and connect opposing hymenia or partially stuff the canals (Fig 5G).
Asci inamyloid, cylindrical, 150–188 μm × 17.5–22.5 μm, walls 1 μm, (3–) 4–8 uniseriate spores, spores in a single ascus often of different sizes, thin-walled smaller spores apparently disintegrating. Ascus tapers, narrowing towards base.
Ascospores smooth, globose, with one large guttule that is irregular in appearance (Fig 5H), (14–) 15.6–17.5 (–20) μm diam. with thick pinkish wall, 1 μm diam.
Anamorph produced in culture from ascospores germinated in 1 % malt extract broth and showed best growth on oatmeal agar (Uecker 1967). Colonized agar becomes brown to gray. Conidiophores verrucose, producing conidia blastically, sympodially, terminally or laterally on long or short branches, singly or in groups of two or more. Conidia warted, brown, globose, 6–8 μm diam., to ovoid, 6–10 × 5–7 μm sometimes with a short stalk (e.g. remnant of the conidiophore). The conidiophores encrusted with dark brown warts. The older hyphae in the colonies becoming brown and granule-encrusted, similar to those of the conidiophores and hyphae at the base of mature ascomata (Uecker 1967).
Distributions and habitat: *Densocarpa shanorii* is known from the Northeast U.S.A., collected in lawns, garden soil, and on wood or woody debris in mixed deciduous and coniferous woods (http://mycoportal.org/portal/index.php, accessed May 2016).
Specimens examined: **United States of America**, Illinois, Champaign Co., Urbana, Pennsylvania St. at Busey, 14 June 1953 L. Shanor Gilkey 764a (Isotype OSC-150061); Illinois St., 1 June 1953 H. Ahles Gilkey 782 (OSC-146659); Brownfield Woods, 1 July 1960 D.D. McLain (OSC-80635); Iowa, Decatur County, near Leon, in bark litter in oak savanna, 12 April 1999, Sibylla Brown RH328 (ISC); Story Co., Ames, Ames High School Woods, on ground in bark litter of elm tree, 29 June 1998, R. Healy 136 (FLAS-F-59175); Story Co., near Ames, McFarland Park, erumpent in soil under *Quercus macrocarpa* and *Tilia americana*, 24 June 2005, R. Healy (FLAS-F-59174); Missouri, St. Louis Co., St. Louis, erumpent in soil in cultivated garden adjacent to natural area, 10 May 2016, Ken Gilberg (FLAS-F-59805). Michigan, Washtenaw Co., Ann Arbor, University of Michigan campus, 17 July 1945 A.H. Smith 34280 (OSC-150059); **Mexico**, Chiapas, ca 5 km S. of Ixtacomitan, Carmino Teapa-Tuxtla, 13 July 1972 Trappe 3319 (OSC-150060).
Comments: The asci in specimens studied here often had more than four mature spores per ascus while the type description states that four or fewer spores per ascus typically reach maturity (Gilkey 1961). However, Uecker (1967) also reported up to eight spores maturing per ascus. The guttules in our *Densocarpa* specimens are not smooth and uniform as in some other Pezizales known to have 'guttulate' spores, and are perhaps of a different composition. Ours are similar to those depicted in Uecker (1967).

Gilkey originally described *Densocarpa* in 1954. Later, however, she observed strong similarities between *D. shanorii* and *Stephensia sumatrana* and decided to transfer *D. shanorii* to the genus *Stephensia* (1961). When she transferred the species Gilkey noted that *D. shanorii* and *S. sumatrana* had more similarities to each other than to *H. bombycina* (referred to in that publication as *Stephensia bombycina*, the type of the genus *Stephensia* – Gilkey 1961). In particular, Gilkey noted the lack of a basal cavity in *D. shanorii* and *S. sumatrana*. Nevertheless, she amended the generic description of *Stephensia* to accommodate *D. shanorii*, adding the character of a notable and foul H_2S odor to her description of *D. shanorii*. Uecker (1967) added more details to Gilkey's description during his cytological study of *D. shanorii*. One of Gilkey's questions regarding differences between *D. shanorii* and *H. bombycina* was answered when Uecker documented that, unlike *H. bombycina*, there is no universal tomentum during any stage of development in *D. shanorii*. However, he noted that the ascomata are completely enclosed throughout all developmental stages and he noted the absence of a basal cavity. He also observed that the asci disintegrate at maturity to release spores into the gleba and he described the spores as having one large or several smaller oil drops.

Densocarpa crocea (Quél.) Healy and M.E. Sm., **comb. nov.**
MycoBank: MB814819
Basionym: Stephensia crocea (Quél) Quél. (1887) Comptes Rendus de ÍAssociation Française pour ÍAvancement des Sciences 15: 489.
Synonym: *Stephensia bombycina* var. *crocea* Quélet (1886) *Enchir. fung.* (Paris): 258.
Type: **Netherlands**, Utrecht, Bunnik, Oud Amelisweerd, 20 October 1984 G. van Immerzeel & G. de Vries (CBS H-18547). Not examined.
Etymology: Latin, 'crocea', saffron-yellow, referring to the orangish yellow color of the peridium.
Description (from De Vries 1985): *Ascoma* a ptycothecium, up to 2 cm diam., yellow, oblong, covered with tomentum, pale orange, darker brown in crevices, gleba whitish to yellow with canals stuffed by elongated paraphyses. Odor raphinoid.
Excipulum 400–700 μm, outer excipulum pale yellow, 40–45 μm thick, composed of pseudoparenchyma, cells 10–20 μm diam., tomentum of granule-encrusted, hyaline

to brown, short hyphae. Inner excipulum of hyaline textura intricata.

Hymenium a palisade of paraphyses and asci.

Paraphyses hyaline to brown, septate, branched, 2.5–5 μm diam., smooth except for the granule-encrusted region elongated beyond the ascus tips.

Asci cylindrical to oblong, tapering to base, 120–200 × 18–23 μm, 4–8 spores.

Ascospores hyaline, globose, smooth, 10–17 (–22) μm diam., wall 1 μm thick.

Anamorph not observed, but cultures were 'easily' obtained on malt extract agar and Sabourad's agar (de Vries 1985).

Specimens examined: **Netherlands**, Noord-Brabant, Drimmelen, in garden near *Betula* and ornamental shrubs, 15 October 1982, Mrs. W. Sommer-Kenniphaas G.A. deVries 1030 & J. Trappe (OSC-41582, as *S. densocarpa*); **Spain**, Asturias, Covadonga, 17 August 2001, E. Rubio (MA-56988).

Comments: If a type specimen exists, its location is unknown. De Vries (1985) described specimens of *S. crocea* as so similar to *S. shanorii* as to be conspecific, as did Fontana & Giovannetti (1987) though no formal transfer was made. Our molecular data supports the placement of *S. crocea* in *Densocarpa*, but suggest *D. crocea* and *D. shanorii* are distinct species. The two species are also geographically separated with *Densocarpa crocea* documented only from Europe and *D. shanorii* known only from North America. A collection tentatively labeled *S. shanorii* from Mexico is molecularly divergent in both ITS and LSU from *S. shanorii* from the USA, suggesting it is a novel species.

Taxa to consider for future analysis of the /tarzetta-geopyxis lineage

No sequence data are currently available for *Berggrenia cyclospora* (Cooke) Sacc. (1889). *Syll. fung.* (Abellini) **8**: 152 but this species has some morphological features in common with the other truffles in the /tarzetta-geopyxis lineage. This pinkish, lobed, terrestrial fungus has globose hyaline spores, no paraphyses, and is known only from New Zealand. Originally described as *Berggrenia aurantiaca* var. *cyclospora* by Cooke (1886), Saccardo (1889) recognized the difference in spore shape (ellipsoid in *B. aurantiaca* and globose in *B. cyclospora*) as worthy of species status and elevated it to *B. cyclospora*. These are the only two recognized species in the genus *Berggrenia*. Lack of paraphyses is a characteristic of *Berggrenia*, but it is not clear from descriptions whether the paraphyses are missing altogether or whether they are modified as indeterminate or interwoven hyphae among the asci. The genus *Berggrenia* is currently considered *incertae sedis* within the Pezizomycotina (Lumbsch & Huhndorf 2007) but the Austral distribution and the globose spores may indicate an affinity with the /tarzetta-geopyxis lineage. Fresh collections and DNA sequences are needed to resolve the phylogenetic affinity of both species in this genus.

Stephensia sumatrana Boedijn, (1939) was described from Sumatra by Boedijn, and later reported by Gilkey (1961) to be more similar to *Densocarpa shanorii* than to *Hydnocystis bombycina*. Illustrations and description by Boedijn (1939) appear similar to *Densocarpa* in the invaginated hymenia lining the canals, and the excipulum composed of pseudoparenchyma. This species is so far known only from Sumatra. Ascomata of this species have a gleba with meandering veins, one small ostiole, a thick excipulum of pseudoparenchyma, an irregular palisade of asci and paraphyses that grow beyond the asci to merge with the hyphae that stuff the canals between opposing hymenia. The asci are cylindrical, with eight or fewer globose, smooth ascospores. The presence of an ostiole in *S. sumatrana* is notably different than other described species in *Densocarpa* but may suggest an affinity with *Paurocotylis* or *Hydnocystis*. Fresh collections and DNA sequences are needed to resolve the phylogenetic affinity of this fungus.

Stephensia colomboi is said to be similar to *H. bombycina* (Læssøe & Hansen 2007). DNA sequences and a morphological comparison with other taxa in the /tarzetta-geopyxis lineage will help to place this species.

Stephensia peyronelii has spores that are not globose, but rather ellipsoid, and may be twice as long as wide (Trappe *et al.* 1997). This species has only known from the type specimen and description. The isotype examined (OSC-131642) was too scanty and old to sample for DNA. A fresh sample would help to place this species. If it belongs in any of the three truffle genera treated here, it will be the only one with ellipsoid rather than globose spores.

Wenyingia sichuanensis Zheng Wang & Pfister (2001), a monotypic species, is described as morphologically similar to *Tarzetta*. Sequence data are needed to determine whether this taxon may also belong to the /tarzetta-geopyxis lineage.

Doubtful and excluded taxa

Additional species placed in *Hydnocystis* include *Hydnocystis arenaria* Tul., *Hydnocystis beccari* Mattir., and *Hydnocystis echinosperma* Rodway. The first two species were placed in *Geopora* by Burdsall (1968) and it was Burdsall's opinion that *H. echinosperma* was a *nomen superfluum* for *Sphaerosoma tasmanica*. Accordingly, we consider these species to belong outside of the /tarzetta-geopyxis lineage.

About half of the legitimately named species originally placed in *Paurocotylis* were later determined to be Glomeromycota. These include *Redeckera fulvum* (Berk. & Broome) C. Walker & A. Schüßsler (synonym: *Paurocotylis fulva* Berk. & Broome 1873), *Glomus fragile* (Berk. & Broome) Trappe & Gerd. (synonym: *Paurocotylis fragilis* Berk. & Broome) and *Glomus macrocarpum* Tul. & Tul. (synonym: *Paurocotylis fulva* var. *zealandica* Cooke).

Placement of the remaining species in the /tarzetta-geopyxis lineage is doubtful because none of these taxa have smooth spores, a defining feature of all known taxa in the /tarzetta-geopyxis lineage. Descriptions of *Paurocotylis echinosperma* Cooke, *Paurocotylis niveus* Rodway and *Paurocotylis prima* Rick clearly include spiny spores. In addition, *P. niveus* is described with globose asci and this feature has not been found in any other taxa in the /tarzetta-geopyxis lineage. No sequences are available for any of these taxa.

Discussion

Phylogenetic analyses

Phylogenetic analyses of the ITS strongly supported three monophyletic truffle genera in the /tarzetta-geopyxis lineage:

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Hydnocystis, *Paurocotylis*, and *Densocarpa*, while inferring a paraphyletic *Geopyxis* as shown by previous work (Hansen *et al.* 2013). *Hydnocystis* and *Densocarpa* were strongly supported by phylogenetic analyses of the 28S region, while *Paurocotylis* was moderately supported by ML only. Unifying morphological characters for the three truffle genera include ascoma with a ptycothecium form, projecting excipular hyphae encrusted with granules, a hymenium of cylindrical asci in a palisade, and smooth, hyaline, globose spores. Anamorphs are known for *Hydnocystis*, *Densocarpa*, and *Geopyxis*, including *Hydnocystis bombycina* (Fontana & Giovannetti 1987) and *Hydnocystis transitoria*, (this study), *Densocarpa shanorii* (Uecker 1967), *Geopyxis majalis* and *Geopyxis carbonaria* (Paden 1972; Vrålstad *et al.* 1998). The anamorphs in the /tarzetta-geopyxis lineage are unified by globose to ovoid warted conidia produced at the tips of branched or simple-septate conidiophores. Species of *Geopyxis*, *H. bombycina*, and *D. shanorii* have all been readily grown in axenic culture and produced sympodial, blastosporic conidia on conidiophores (Uecker 1967; Paden 1972; Fontana & Giovannetti 1987).

Anamorphs may have been described for species in the /tarzetta-geopyxis lineage under other names. Chamuris & Wang (1990, 1998) made a nomenclatural recombination of the anamorph *Rhinotrichum alutaceum* Peck (1881) to their new genus *Stenocephalopsis subalutacea* (Peck) Chamuris & Wang (1998). Peck's species was collected on decayed wood in New York. Chamuris & Wang gained access to fresh collections of the same fungus from plant debris in New York State. Their descriptions and images are strikingly similar to the anamorphs of *H. transitoria* and *H. bombycina*. The similarities include spore mat color, granule-encrusted conidia and conidiophores, denticles remaining on smooth apices after spore release, and branching and conidiation patterns. Chamuris & Wang (1990) also noted that their anamorph was similar to that of *G. majalis* (Paden 1972). Within the taxon *S. subalutacea*, Chamuris & Wang (1990) included spore mats from North America, New Zealand and the United Kingdom. In addition to these localities, Holubová-Jechová (1982) putatively reported the same species (as *Basifimbria subalutacea*) from the Czech Republic, Russia, and Germany. The variability in spore size and ornamentation noted by Holubová-Jechová (1982) may signal that taxa across this wide area are different species. Based on our phylogenetic analyses, it is doubtful that species of truffles in the /tarzetta-geopyxis lineage are found on multiple continents, except in cases where they have been introduced due to anthropogenic activities. It would be interesting to recollect similar spore mats from these localities and to sequence DNA from them to determine if they fall within the /tarzetta-geopyxis lineage. If this is the case, it may help to further eludicate the ecology, and geographic distributions of this group of fungi.

Morphological features that characterize the /tarzetta-geopyxis lineage include ascomata and anamorphs with granule-encrusted hyphae, cylindrical asci, and smooth spores. *Geopyxis* differs from the truffle genera in the clade by the lack of excipular hairs and in the ellipsoid rather than globose spores. Morphological similarities among the epigeous and hypogeous genera include smooth spores, asci that taper at their bases, and similar anamorph characters. In the truffle lineages, further unifying characteristics include globose spores, and the presence of asci and paraphyses in a more or less organized hymenium. Features that help to differentiate among genera in the /tarzetta-geopyxis lineage include excipular structure and spore shape, presence of a well-defined epithecium, and presence of a glebal cavity. The outer excipulum of most *Paurocotylis* is composed of partially to fully unfused interwoven, branched hyphae. *Hydnocystis* can be differentiated by the presence of a cavity, often with an opening connecting the cavity to the exterior, and the presence of a well-defined epithecium in most species. Species of *Densocarpa* have ascomata with open to stuffed canals between folded opposing hymenia but they lack a glebal cavity.

Ecology of the /tarzetta-geopyxis lineage

It is often assumed that truffles and false truffles are EcM and some authors have erroneously assigned an EcM lifestyle to truffles that are not EcM (e.g. Molina *et al.* 1992; Rinaldi *et al.* 2008). *Geopyxis* was previously considered EcM by some authors (Vrålstad *et al.* 1998; Rinaldi *et al.* 2008). However, evidence suggests that *Geopyxis* and the related truffle genera discussed here do not form EcM symbioses. Lines of evidence include the rapid growth in axenic culture (Vrålstad *et al.* 1998), conidial germination on media (Fontana & Giovannetti 1987), the development of anamorphs in culture, and the development of ascomata among woody litter on the soil surface. Furthermore, except for *Tarzetta*, many of the species in the /tarzetta-geopyxis lineage consistently fruit away from EcM host plants in nature (Dennis 1975; Pegler *et al.* 1993; Trappe & Claridge 2015) and except for *Tarzetta*, there are no credible ITS rDNA sequences from this lineage reported on EcM root tips, even though there have been many molecular studies of roots in areas where *Geopyxis* and allies are regularly collected (Tedersoo *et al.* 2010). In contrast, the anamorphs and ascomata of EcM Pezizales are typically found on bare soil and are regularly recovered from ITS rDNA sequencing of EcM roots (Petersen 1985; Tedersoo *et al.* 2010; Healy *et al.* 2013).

Except for *Tarzetta* species, taxa in the /tarzetta-geopyxis lineage that have been found on roots, have also survived for an extensive period of time after the death of the plant, suggesting they are not obligately mycorrhizal (Vrålstad *et al.* 1998). In addition, some studies have found a positive correlation between burned trees and *Geopyxis* fruiting, a pattern which is atypical for EcM fungi (Greene *et al.* 2010). Other than *Tarzetta*, members of the /tarzetta-geopyxis clade may have biotrophic, saprobic, or mixed trophic modes. Our phylogenetic analyses included many endophyte sequences of angiosperms, gymnosperms, lichens and mosses nested within the /tarzetta-geopyxis lineage. An endophytic lifestyle was previously suggested for *Geopyxis* by Hansen *et al.* (2013) and Tedersoo *et al.* (2013).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funbio.2016.12.004.

REFERENCES

Agnello C, 2011. Ritrovamenti nel Salento di *Hydnocystis piligera* Tul. *Hydnocystis clausa* (Tul. & C. Tul.) Ceruti. *Ascomycete.org* **2**: 9–17.

Alvarado P, Moreno G, Manjon JL, Gelpi C, Kaounas V, Konstantinidis G, Barseghyan GS, Venturella G, 2011. First molecular data on *Delastria rosea, Fischerula macrospora* and *Hydnocystis piligera*. *Boletín de la Sociedad Micológica de Madrid* **35**: 75–81.

Berkeley MJ, 1855. Fungi. In: Hooker JD (ed.), *Botany of Antarctic Voyage of H.M. Discovery Ships Erebus and Terror, in the Years 1839–1843: Flora Novae-Zelandiae, II*. Lovell Reeve, Covent Gardens, London, p. 188.

Bertini L, Amicucci A, Agostini D, Polidori E, Potenza L, Guidi C, Stocchi V, 1999. A new pair of primers designed for amplification of the ITS region in *Tuber* species. *FEMS Microbiology Letters* **173**: 239–245.

Bidartondo MI, Burghardt B, Gebauer G, Bruns TD, Read DJ, 2004. Changing partners in the dark: isotopic and molecular evidence of ectomycorrhizal liaisons between forest orchids and trees. *Proceedings of the Royal Society of London B* **271**: 1799–1806.

Boedijn KB, 1939. Tuberales of the Netherlands Indies. *Bulletin du Jardin botanique de Buitenzorg III* **16**: 236–244.

Brundrett M, Bougher N, Dell B, Grove T, Malajczuk N, 1996. *Working with Mycorrhizas in Forestry and Agriculture*. Australian Centre for International Agricultural Research, Canberra, Australia 374.

Burdsall HH, 1968. A revision of the genus *Hydnocystis* (Tuberales) and of the hypogeous species of Geopora (Pezizales). *Mycologia* **60**: 496–525.

Castellano MA, Trappe JM, Luoma DL, 2004. Sequestrate fungi. In: Mueller GM, Bills GF, Foster MS (eds), *Biodiversity of Fungi Inventory and Monitoring Methods*. Elsevier Academic Press, Burlington, pp. 197–213.

Castresana J, 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Biology and Evolution* **17**: 540–552.

Chamuris GP, Wang CJK, 1990. Stenocephalum, a new genus for *Rhinotrichum subalutaceum* (Hyphomycetes). *Mycologia* **82**: 528–532.

Chamuris GP, Wang CJK, 1998. *Stenocephalopsis*, not *Stenocephalum* as the genus for *Rhinotrichum subalutaceum*. *Mycologia* **90**: 464.

Cooke MC, 1886. Exotic fungi. *Grevillea* **15**: 16.

de Vries GA, 1985. First record of *Stephensia crocea* Quél. in the Netherlands. *Persoonia* **12**: 463–468.

Dennis RWG, 1975. New or interesting British microfungi, III. *Kew Bulletin* **30**: 345–365.

Egger KN, 1986. Substrate hydrolysis patterns of post-fire ascomycetes (Pezizales). *Mycologia* **78**: 771–780.

Egger KN, Paden JW, 1986a. Pathogenicity of postfire ascomycetes (Pezizales) on seeds and germinants of lodgepole pine. *Canadian Journal of Botany* **64**: 2368–2371.

Egger KN, Paden JW, 1986b. Biotrophic associations between lodgepole pine seedlings and postfire ascomycetes (Pezizales) in monoxenic culture. *Canadian Journal of Botany* **64**: 2719–2725.

Fontana A, Giovannetti G, 1980. Osservazioni su *Stephensia bombycina* (Vitt.) Tul. et Tul. in coltura. *Allionia* **24**: 91–98.

Fontana A, Giovannetti G, 1987. The anamorph of Stephensia bombycina. *Mycotaxon* **29**: 37–44.

Geml J, Pastor N, Fernandez L, Pacheco S, Semenova TA, Becerra AG, Wicaksono CY, Nouhra ER, 2014. Large-scale fungal diversity assessment in the Andean Yungas forests reveals strong community turnover among forest types along an altitudinal gradient. *Molecular Ecology* **23**: 2452–2472.

Gilkey HM, 1916. A revision of the tuberales of California. *University of California Publications in Botany* **6**: 288–289 348–349.

Gilkey HM, 1954. Tuberales. *North American Flora, Series 2* **1**: 1–36.

Gilkey HM, 1961. New species and revisions in the order Tuberales. *Mycologia* **53**: 215–220.

Greene DF, Hesketh M, Punden E, 2010. Emergence of morel (*Morchella*) and pixie cup (*Geopyxis carbonaria*) ascocarps in response to the intensity of forest floor combustion during a wildfire. *Mycologia* **102**: 766–773.

Gyosheva M, Konstantinidis G, Assyov B, Kaounas V, 2012. On the occurrence of the truffle-like fungus *Stephensia bombycina* (Ascomycota, Pyronemataceae) in Bulgaria and Greece. *Comptes rendus de l'Académie bulgare des Sciences* **65**: 479–482.

Hansen K, Perry BA, Dranginis AW, Pfister DH, 2013. A phylogeny of the highly diverse cup-fungus family Pyronemataceae (Pezizomycetes, Ascomycota) clarifies relationships and evolution of selected life history traits. *Molecular Phylogenetics and Evolution* **67**: 311–335.

Hawker LE, 1954. British hypogeous fungi. *Philosophical Transactions of the Royal Society of London* **237**: 429–546.

Healy R, Smith ME, Bonito GM, Pfister DH, Ge ZW, Guevara GG, 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**: 1717–1732.

Holubová-Jechová V, 1982. Some notes on *Basifimbria aurea*. *Mycotaxon* **14**: 140–144.

Hutchison L, 1989. Absence of conidia as a morphological character in ectomycorrhizal fungi. *Mycologia* **81**: 587–594.
Katoh K, Toh H, 2010. Parallelization of the MAFFT multiple sequence alignment program. *Bioinformatics* **26**: 1899–1900.
Kelly KA, Judd DB, 1955. *The ISCC-NBS Method of Designating Colors and a Dictionary of Color Names*. United States National Bureau of Standards Circular 553, Washington, D.C 158.
Kirk P, Cannon P, Stalper J, Minter D, 2008. *Dictionary of the Fungi*, 10th edn. CAB International, Oxon, UK.
Kobayasi Y, 1963. On a new species *Protogenea* of the Tuberales. *Transactions of the Mycological Society of Japan* **4**: 119–120.
Læssøe T, Hansen K, 2007. Truffle trouble: what happened to the Tuberales? *Mycological Research* **111**: 1075–1099.
Lumbsch HT, Huhndorf SM, 2007. Outline of Ascomycota. *Myconet* **13**: 1–58.
Miller MA, Pfeiffer W, Schwartz T, 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In: *Gateway Computing Environments Workshop (GCE), 14 Nov 2010, New Orleans*, pp. 1–8.
Molina R, Massicotte H, Trappe J, 1992. Specificity phenomena in mycorrhizal symbiosis: community-ecological consequences and practical implications. In: Allen M (ed.), *Mycorrhizal Functioning: an integrative plant-fungal process*. Chapman and Hall, N.Y.
Montecchi A, Sarasini M, 2000. *Funghi Ipogei D'Europa*. Associazione Micologica, Bresadola, Trento, Italy 178–184.
Paden JW, 1972. Imperfect states and the taxonomy of the Pezizales. *Persoonia* **4**: 405–414 plates 20–22.
Patouillard N, 1903. Note sur le genre *Paurocotylis* Berk. *Bulletin de la Société de Mycologique de France* **19**: 339–341.
Pegler DN, Spooner BM, Young TWK, 1993. *British Truffles. A Revision of British Hypogeous Fungi*. Royal Botanic Gardens, Kew.
Perry BA, Hansen K, Pfister DH, 2007. A phylogenetic overview of the family Pyronemataceae (Ascomycota, Pezizales). *Mycological Research* **111**: 549–571.
Petersen PM, 1970. Danish fireplace fungi, an ecological investigation of fungi on burns. *Dansk Botanisk Arkiv* **27**: 1–97.
Petersen PM, 1985. The ecology of Danish soil inhabiting Pezizales with emphasis on edaphic conditions. *Opera Botanica* **77**: 1–38.
Posada D, 2008. jModelTest: phylogenetic model averaging. *Molecular Biology and Evolution* **25**: 1253–1256.
Quélet L, 1886. *Enchiridion Fungorum in Europa Media et Praesertim in Gallia Vigentium* Doin, Paris 258.
Rambaut A, 2007. *Se-Al: Sequence Alignment Editor* [Computer Program]. http://tree.bio.ed.ac.uk/software/seal/.
Rambaut A, Drummond AJ, 2007. *Tracer v1.4* [Computer Program]. http://beast.bio.ed.ac.uk/Tracer.
Rinaldi AC, Comandini O, Kuyper TW, 2008. Ectomycorrhizal fungal diversity: separating the wheat from the chaff. *Fungal Diversity* **33**: 1–45.
Saccardo PA, 1889. *Sylloge Fungorum*, vol. 8, Padova, Italy.
Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W, 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Science* **109**: 6241–6246.
Smith ME, Trappe JM, Rizzo DM, 2006. *Genea, Genabea* and *Gilkeya* gen. nov.: ascomata and ectomycorrhiza formation in a *Quercus* woodland. *Mycologia* **98**: 699–716.
Spurr AR, 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. *Journal of Ultrastructural Research* **26**: 31–42.
Stamatakis A, 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* **30**: 1312.
Tedersoo L, Hansen K, Perry B, Kjøller R, 2006. Molecular and morphological diversity of pezizalean ectomycorrhiza. *New Phytologist* **170**: 581–596.
Tedersoo L, May T, Smith ME, 2010. Ectomycorrhizal lifestyle in fungi: global diversity, distribution, and evolution of phylogenetic lineages. *Mycorrhiza* **20**: 217–263.
Tedersoo L, Arnold AE, Hansen K, 2013. Novel aspects in the life cycle and biotrophic interactions in Pezizomycetes (Ascomycota, Fungi). *Molecular Ecology* **22**: 1488–1493.
Trappe JM, 1975. Generic synonyms in the Tuberales. *Mycotaxon* **2**: 109–122.
Trappe JM, Bushnell W, Castellano MA, 1997. NATS truffle and truffle-like fungi 6: *Stephensia bynumii* sp. nov. (Ascomycota), with a key to the species of *Stephensia*. *Mycotaxon* **64**: 431–435.
Trappe JM, Claridge AW, 2015. The hidden life of truffles. *Scientific American* **24**: 22–27.
Tulasne LR, Tulasne C, 1844. Fungi nonnulli hypogaei novi v. minus cogniti auct. *Nuovo Giornale Botanico Italiano* **2**: 59–60.
Tulasne LR, Tulasne C, 1851. *Fungi Hypogaei, Histoire et Monographie des Champignons Hypogési*. F. Klincksieck, Paris.
Uecker FA, 1967. *Stephensia shanori*. I. Cytology of the ascus and other observations. *Mycologia* **59**: 819–832.
Vilgalys R, Hester M, 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several species of *Cryptococcus*. *Journal of Bacteriology* **172**: 4238–4246.
Vittadini C, 1831. *Monografia Tuberacearum* Milan 29.
Vrålstad T, Holst-Jensen A, Schumacher T, 1998. The postfire discomycete *Geopyxis carbonaria* (Ascomycota) is a biotrophic root associate with Norway spruce (*Picea abies*) in nature. *Molecular Ecology* **7**: 609–616.
Wang X-H, Huhtinen S, Hansen K, 2016. Multilocus phylogenetic and coalescent-based methods reveal dilemma in generic limits, cryptic species, and a prevalent intercontinental disjunct distribution in Geopyxis (Pyrenomycetaceae s.l., Pezizomycetes). *Mycologia*. http://dx.doi.org/10.3852/16-100 (in press).
Wang Z, Pei K-Q, 2001. Notes on discomycetes in Dongling Mountains (Beijing). *Mycotaxon* **79**: 307–314.
Wang Z, Pfister DH, 2001. *Wenyingia*, a new genus in Pezizales (Otideaceae). *Mycotaxon* **79**: 397–399.
White TM, Bruns T, Lee S, Taylor J, 1990. Amplification and direct sequencing of fungal ribosomal RNA for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds), *PCR Protocols: a guide to methods and applications*. Academic Press, San Diego, pp. 315–321.
Williams MA, 2009. *Land Cover Characteristics in the Karst Region of Southeastern Minnesota* Dissertation. University of Minnesota.
Weber NS, Trappe JM, Denison WC, 1997. Studies on Western American Pezizales. Collecting and describing ascomata-microscopic features. *Mycotaxon* **61**: 153–176.

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