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Calongea, a new genus of truffles in the Pezizaceae (Pezizales)

by

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Abstract

Healy, R.A., Bonito, G. & Trappe, J.M. 2009. Calongea, a new genus of truffles in the Pezizaceae (Pezizales). *Anales Jard. Bot. Madrid* 66S1: 25-32.

Phylogenetic analysis of the ITS and LSU rDNA of *Pachyphloeus* species from Europe and North America revealed a new truffle genus. These molecular analyses plus sequences downloaded from a BLAST search in GenBank indicated that *Pachyphloeus prieguensis* is within the Pezizaceae but well outside of the genus *Pachyphloeus*. Morphological differences in the peridial and glebal hyphae and spores distinguish this genus from *Pachyphloeus*. We here propose the monotypic new genus *Calongea*, with the type species *C. prieguensis* comb. nov., in honor of Prof. Francisco de Diego Calonge, who has long studied the truffle fungi of Spain and participated in describing the type species of *Calongea*.

Keywords: *Pachyphloeus*, ITS, LSU, Ascomycota, molecular analysis, morphology, phylogeny.

Resumen

Healy, R.A., Bonito, G. & Trappe, J.M. 2009. Calongea, un nuevo género de trufas en las Pezizaceae (Pezizales). *Anales Jard. Bot. Madrid* 66S1: 25-32 (en inglés).

El análisis filogenético del ITS y LSU rDNA de especies europeas y norteamericanas de *Pachyphloeus* revelan un género nuevo de trufa. Los datos moleculares de este estudio, además de las secuencias obtenidas de una búsqueda BLAST en GenBank, indican que *Pachyphloeus prieguensis* es un miembro de la familia Pezizaceae pero no está relacionado con ninguna otra especie hipogea o epigea de dicha familia. Encontramos diferencias morfológicas en las hifas del peridio y gleba así como en las esporas que morfológicamente distinguen éste de *Pachyphloeus*. Proponemos el nuevo género monotípico *Calongea*, con *C. prieguensis* comb. nov. como la especie tipo, en honor a uno de los descubridores originales, Prof. Francisco de Diego Calonge.

Palabras clave: *Pachyphloeus*, ITS, LSU, Ascomycota, datos moleculares, morfología, filogenia.

Introduction

Pachyphloeus prieguensis Mor.-Arr., J. Gómez & Calonge was described in 1996 from deciduous woodlands with calcareous soil in Spain (Moreno-Arroyo & al., 1996). Some of its distinctive features include pallid, pink-tinged veins and labyrinthiform, mostly hollow canals within a tuberous, minutely verrucose, foul-smelling, dark reddish-brown to blackish-brown ascocarp. One challenge to identifying or describing truffles is that their relationships are confused by convergent morphologies. In their adaptation to a sequestrate habit, they gain features such as odors adapted for spore dispersal through mycophagy and lose features such as apothecia and forcible spore discharge adapted for aerial spore dispersal. Consequently, these sporocarps often resemble others in their ecological niche more than they resemble their most closely related epigeous relatives (Trappe & Claridge, 2005; Læssøe & Hansen, 2007).

During a phylogenetic study of *Pachyphloeus* species with use of ITS and LSU rDNA, *P. prieguensis* sequences consistently fell outside of the genus when analyzed with maximum parsimony and maximum likelihood optimization criteria. In addition, the ITS and LSU sequences of *P. prieguensis* were not very similar to any accessions held in Genbank's DNA sequence database (82% and 93% similarity, respectively). These revelations prompted us to re-examine the morphology of *P. prieguensis*. In so doing, we

found differences in the peridial structure, gleba and spore ornamentation that distinguish this taxon from *Pachyphloeus*.

Materials and methods

Dried pieces of ascocarps were rehydrated in 3% KOH and either viewed with bright field microscopy or dehydrated in an ethanol series to 100%, critical point dried in a DCP-1 Denton Critical Point Drying Apparatus (Denton Vacuum Inc., Cherry Hill, NJ), mounted on aluminum stubs with double-sided tape, silver painted around the specimen edges, sputter-coated for 120 sec with Au/Pd, and visualized with 15 kV in a JEOL 5800LV SEM. All images were digitally captured. Spore measurements were taken from 50 randomly selected spores, under 100× oil immersion.

Molecular analyses were done with DNA extracted from previously unexposed portions of mature gleba taken from the inside of air-dried ascocarps. DNA was extracted with 24:1 chloroform:isoamyl alcohol and precipitated in isopropanol. Both the internal transcribed spacer region (ITS1, 5.8S, and ITS2) and three divergent domains (D1, D2, D3) of the ribosomal large subunit (LSU) locus were amplified by use of the universal fungal primer set ITS5 - LR5 (Bertini & al., 1999; Vilgalys & Hester, 1990). PCR conditions and the handling of PCR products were as described in Healy & al. (2009). Sanger sequencing was performed on an ABI3700 (Applied Biosystems, Foster City, CA) using Big Dye chemistry version 3.1 (Applied Biosystems, Foster City, CA) with the forward primer ITS5 and reverse primer LR5. DNA sequences were assembled and manually edited with Sequencher 4.0 (Gene Codes, Ann Arbor, MI) and queried against GenBank (http:// blast.ncbi.nlm.nih.gov/Blast.cgi) to compare with other sequences and to verify that sequences were of the target group. Sequences from top blast hits (LSU) were combined with our data and were aligned manually using Mesquite 4.0 (Maddison & Maddison, 2002). Ambiguously aligned regions were excluded from the analyses. Phylogenetic analyses were conducted by the maximum parsimony and maximum likelihood methods. Parsimony analyses were carried out by a heuristic search in PAUP 4.0b10 with 1000 random addition sequences and 5000 bootstrap replicates (Swofford, 2001). Two independent maximum likelihood analyses based on a general-time-reversible 6-parameter model of evolution were run using the software program GARLI and included 100 bootstrap replications (Zwickl, 2006). The truffle species *Genea harknessii* from within the Pyronemataceae was chosen as an outgroup based on previous studies focused on the systematics of the Pezizomycetes (Hansen & Pfister, 2006). Sequences for *Calongea prieguensis* produced in this study were deposited in GenBank under accession numbers FJ228462 and FJ228463. Herbarium abbreviations are according to the Index Herbariorum (http://sweetgum.nybg.org/ih/herbarium_list.php).

Results of molecular analyses

BLAST results of *C. prieguensis* sequences of the LSU (Table 1) and ITS/5.8S (Table 2) rDNA sequences had highest similarity (93% for LSU; 82% for ITS) to Genbank accessions of species in the *Peziza depressa-Ruhlandiella* clade of Læssøe & Hansen, 2007.

Phylogenetic relationships of 27 ingroup Pezizaceae taxa based on maximum likelihood analyses of LSU rDNA are depicted in Figure 1. This most likely tree resulted from analyses that included 993 characters, of which 255 were parsimony-informative, 71 were variable but parsimony-uninformative (parsimony analysis) and 667 were constant. Bootstrap values for significantly supported nodes (>70 %) are labeled with parsimony bootstrap on top and likelihood bootstrap values below.

Table 1. Top BLAST results for *Calongea prieguensis* LSU rDNA.

Taxonomic name	Maximum coverage	Maximum identity	Alignment scorea	Genbank ccession	E-value
Peziza ellipsospora	97%	93%	1299	AF335139	0.00
Peziza domiciliana	97%	92%	1297	AF335137	0.00
Peziza badiofusca	97%	92%	1297	AF335132	0.00
Peziza limnaea	97%	92%	1291	AF335147	0.00
Peziza whitei	97%	92%	1286	AF335168	0.00
Ruhlandiella berolinensis	88%	93%	1179	AF335175	0.00

Table 2. Top BLAST results for *Calongea prieguensis* ITS/5.8S rDNA.

Taxonomic name	Maximum coverage	Maximum identity	Alignment scorea	Genbank ccession	E-value
Peziza depressa	95%	82%	424	EU819535	3e-115
Terfezia "trappei"	95%	81%	412	AF276676	6e-112
Terfezia leptoderma	95%	81%	409	AF396864	8e-111
Terfezia olbiensis	95%	81%	407	AF387657	3e-110
Terfezia claveryi	100%	81%	401	AF276671	1e-108
Tirmania nivea	94%	81%	399	AF276667	5e-108

Taxonomic treatment

Calongea Healy, Bonito & Trappe, **gen. nov.** (Figs. 2-5)

A Pachyphloeo differt exipulo duplici; stratum exteriore pilis brevibus cellulis apicalibus versiformibus, hi in canalibus cavis similita praesentes; stratum interiore hyphis filamentis et cellulis inflatis; canalibusque cavitatibus glebae epithecio peridioideo inclusis; sporae spinis variabiliter dispositis, frequenter curvis, sine perisporis; et ordinatione DNA ITS *et* LSU. *Species typicus: Calongea prieguensis.*

Differing from *Pachyphloeus* by its combination of a 2-layered peridium: outer layer of thick-walled cells giving rise to hairs with versiform tips; inner layer a mix of filamentous hyphae and inflated cells; a peridium-like epithecium with versiform hairs lining the canals and cavities; spores with spines of varying length, width and spacing, the spine bases connected by low ridges to form an irregular reticulum; and ITS and LSU DNA not congruent with other genera in the Pezizaceae.

Etymology: We dedicate this genus to Prof. Francisco de Diego Calonge, whose mycological career encompassed study of many groups, including truffle fungi, and who provided to J. Trappe hospitality, good fellowship, good stories, lessons on Spanish fungi, history, customs, culture, and outstanding food and wine, both in Madrid and on memorable field trips.

Calongea prieguensis (Mor.-Arr., J. Gómez & Calonge) Healy, Bonito & Trappe, **comb. nov.**

Basionym: *Pachyphloeus prieguensis* Mor.-Arr., J. Gómez & Calonge, Bol. Soc. Micol. Madrid 21: 87-88. 1996

Type: SPAIN. Córdoba, Priego, 4 April 1992 (MA).

Illustrations: Moreno-Arroyo & al., Figs. 1-6 (1996); Montecchi & Sarasini, 210-211 (2000); Moreno-Arroyo & al., 158-160 (2005).

GenBank Accession Numbers FJ228463, FJ 228462.

Ascomata 1-3 cm broad, subglobose to irregular. *Peridium* reddish brown in youth, by maturity blackish brown, nearly smooth or minutely verrucose. *Gleba* cream colored in youth, by maturity yellow to yellowish pink or light vinaceous, the context thick and containing elongated, labyrinthiform canals and chambers pallid in youth, by maturity pinkish to brown. *Odor* strong, most unpleasant, sweet-foetid; rehydrated herbarium specimens smell like rotting cardboard.

Peridium of two layers: outer layer 83-165 µm thick including the minute warts formed by mounded cells; cells in face view smoky-brown with golden-brown walls up to 7.4 µm thick, isodiametric to subrectangular, length × width ca 40-41 × 13 µm to 50 × 41 µm, interspersed with occasional large cells up to 100 × 78 µm. Surface cells between the warts giving rise to unbranched, hyaline to smoky-brown hairs up to 60 µm long, with one to several septa and walls 0.5-1.3 µm thick; apical cells of the hairs globose to capitate, cylindrical or ventricose, 10-50 µm long and 10-50 µm broad at the widest point (Fig. 2). The inner peridial layer is of hyaline, filamentous, interwoven cells up to 8.3 µm broad and with walls 0.5 µm thick interspersed with inflated cells up to 20 µm broad with walls up to 3.3 µm thick; this layer grades into the interwoven hyphae of the gleba. The peridium and outer glebal vein tissue together are 50-149 µm thick.

Gleba marbled with hollow canals lined with peridial-like tissue, subtended by veins of fertile tissue. The peridial-like tissue is composed of textura angularis, with large, thick-walled cells and superficial thinner-walled, septate hairs 60 to 100 µm long (Figs. 3, 4), varying from filiform and ± 8 µm broad at the septum with apical cells inflated up to 28 µm, and with the diversity of apical cells as on the peridial hairs.

Asci irregularly arranged in the fertile veins, oblong to pyriform, fresh measurements according to Moreno-Arroyo & al. (1996) 65-125(140) × 30-50 µm; not blueing (inamyloid) in Melzer's (iodine) solution, even after pre-treatment with 3% KOH, producing eight irregularly biseriate to inordinately arranged spores. Filamentous, interwoven to parallel hyphae among asci 5-8.3

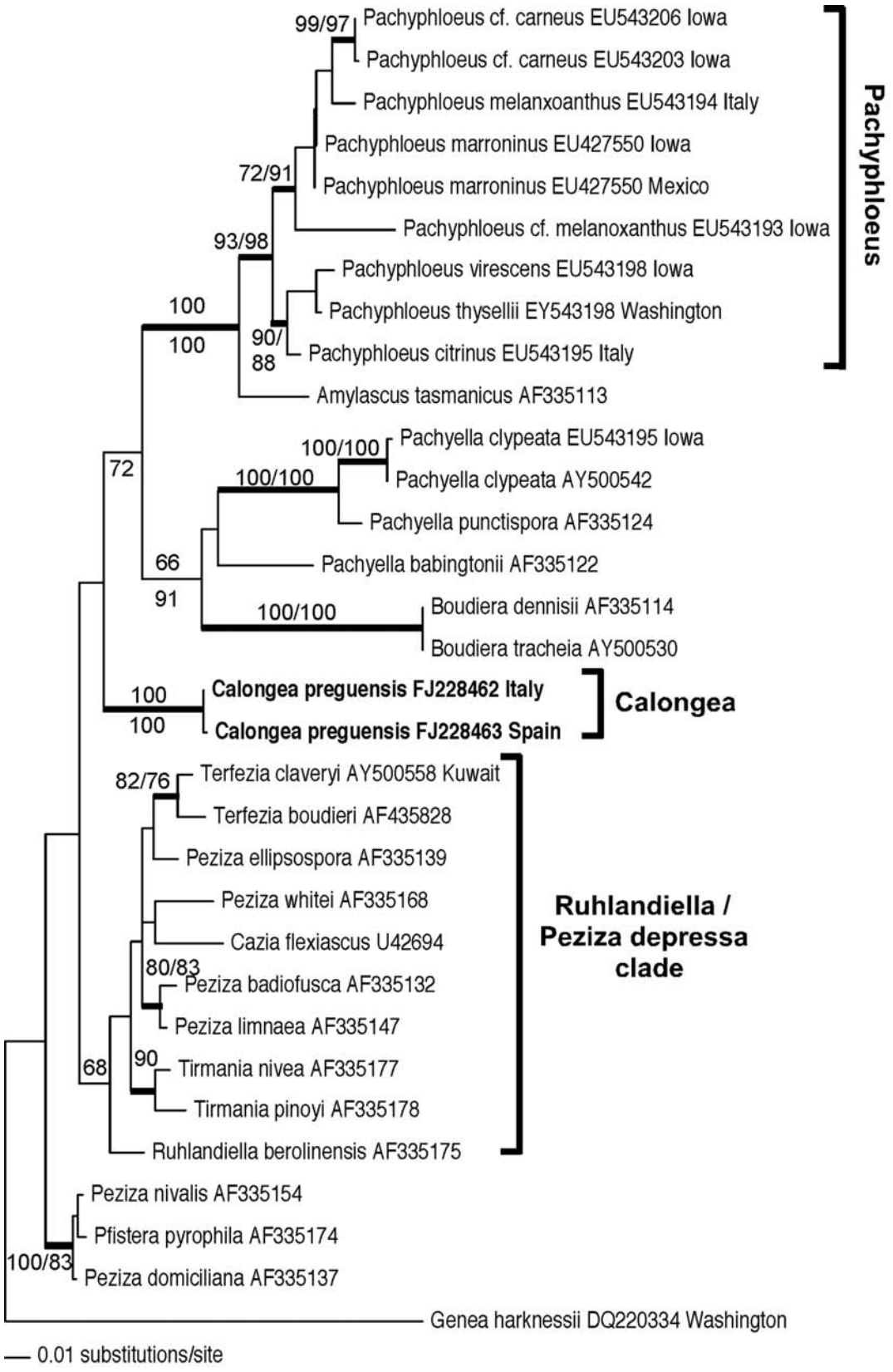

Fig. 1. Most likely tree from maximum likelihood analyses of LSU rDNA with 31 ingroup Pezizaceae. Bootstrap values for significantly supported nodes (>70 %) are thickened with parsimony bootstrap on top and likelihood values below. Taxon labels include the Latin binomial followed by the Genbank accession number and geographic origin (when known). Clades of focus in this paper have been labeled.

µm in diameter, but inflated near the borders with peridial-like tissue. The glebal filaments merge with the peridial-like tissue and become indistinguishable from it. Interspersed among the narrow, filamentous hyphae and asci in the fertile veins are large-celled filamentous hyphae with cells swollen up to 58 × 50 µm. These cells have hyaline to brown walls up to 2-5 µm thick, and walls are thickest at the septae whereas cells they connect may be hyaline and thin-walled.

Spores globose, (16)17-19(22) µm (avg. 17.7 µm) including ornamentation, and (13)15-18(19.5) (avg. 15.6 µm) excluding ornamentation. Initially hyaline, at maturity the walls olivaceous brown and inamyloid, the ornamentation of flexuous spines, sometimes curved at the tips, varying in spacing on a given spore, and from 0.5-2.5 µm tall and 0.5-2 µm broad (Fig. 5); a low, irregular reticulum connects the bases of neighboring spines.

Collections examined

SPAIN. **Córdoba:** 15-IV-1990, J. Gómez, *Trappe 12832*, GenBank FJ228463 (OSC). ITALY. **Laconi:** 2-V-1999, *Fantini 1980*, GenBank FJ228462 (OSC).

Collections of Pachyphloeus species examined: ITALY. **Venezia:** Cuneo, Roascio, *Pachyphloeus citrinus* under mixed conifer and broadleaf forest, 12-IX-2000, *M. Machioni* JRWL2197, EU543196 (OSC); *Pachyphloeus melanoxanthus* under *Ostrya* and *Quercus,* I-1999, *Macchioni 1860,* EU543194 (OSC); MEXICO. **Nuevo Leon:** Santiago, El Ranchito, *Pachyphloeus marroninus* under *Quercus polymorpha,* 14-IX-1983, *García 3757,* EU427551 (UNL). UNITED STATES. **Iowa:** Story County, Ames, YMCA Woods, *Pachyphloeus* cf *carneus* under *Quercus macrocarpa,* 9-VIII-2000, *Healy 756,* EU543206 (ISC). Story County, Hickory Grove Park, *Pachyphloeus* cf *melanoxanthus* under *Quercus alba*, *Tilia americana, Carya ovata*, 28-VII-2000, *Healy 735,* EU543193 (ISC); Winneshiek County, Upper Iowa Public Fishing Access, *Pachyphloeus marroninus* under *Quercus alba* and *Q. macrocarpa,* 2-X-1998, *Healy 286,* EU427550 (ISC, OSC); Woodbury County, Stone State Park, *Pachyphloeus* cf *carneus* under *Quercus macrocarpa,* 8-VIII-1999, *Healy 525,* EU543203 (ISC). Woodbury County, Stone State Park, *Pachyphloeus virescens,* 27-VII-2000, *Healy 729,* EU543198 (ISC); **Washington:** Thurston County, Fort Lewis, *Pachyphloeus thysellii* under *Pseudotsuga menziesii,* 18-VIII-1993, *Trappe 13082,* EU543197 (OSC).

Also examined: UNITED STATES. **Iowa:** Webster County, Woodman Hollow State Preserve, *Pachyella clypeata* on log in riparian area in deciduous woods, 18-IX-2005, *Healy,* EU543195 (ISC).

Calongea prieguensis *and* Pachyphloeus *species compared*

The genus *Pachyphloeus* was described in 1844, typified by *P. melanoxanthus* (Tul.) Tul. & C. Tul., with the following characteristics: 1.) a thick, fleshy peridium with small warts; 2.) a thick-margined orifice stuffed with hyphae, where the interstitial veins come to the surface at the apex of the ascocarp and separate into fissures; 3.) a narrow point of attachment; 4.) sterile veins that marble the gleba, and initially differ in color from the fertile tissue but later the color becomes more uniform overall; and 5.) a well-ordered series of asci with eight globose spores ornamented with spines, irregularly arranged in the ascus, colored at maturity (Tul. & Tul., 1844).

Since the original description of *Pachyphloeus*, it has become evident that another feature that unifies this genus is a single-layered peridium of thick-walled textura angularis (Pegler & al., 1993; Montecchi & Sarasini, 2000; Hansen & al., 2001; Healy, 2002). In contrast, *C. prieguensis* has a duplex peridium composed of a mixture of textura globulosa and textura angularis in the outer layer, subtended by a mixture of interwoven filamentous and inflated hypae. The erecting of a new genus to accommodate *Calongea prieguensis* unifies the type of peridium present on species of *Pachyphloeus*.

Unlike *Pachyphloeus*, the peridium of *C. prieguensis* has short hairs with apical cells of variable shapes and diameters, distinct from their subtending cells. These are also present on the cells lining the hollow (rather then stuffed) canals. The peculiar hairs on the excipulum of *Calongea* have not been reported in *Pachyphloeus*. Some *Pachyphloeus*, such as *P. conglomeratus*, *P. lateritius* and *P. austro-oregonensis* have slender; hyphoid hairs of uniform diameter on the peridium (Berk. & Broome, 1846; Fogel & States, 2002; Frank & al., 2006, respectively), and many species have a basal mycelial tuft. *Calongea* lacks a basal mycelial tuft.

Asci of many *Pachyphloeus* species are arranged in a well ordered to irregular palisade with paraphyses. The canals between opposing hymenia are stuffed with extensions of these paraphyses, and the sterile veins are composed of textura angularis similar to cells of the peridium. In contrast, *Calongea* asci are irregularly arranged in the gleba, and canals are open, not stuffed. In addition, the large-celled, brown-walled filamentous hyphae in the fertile tissue of *Calongea* have not been reported from any species of *Pachyphloeus*. The asci of *P. conglomeratus* are disorganized in the gleba, but unlike *Calongea*, this species has a solid gleba. *Pachyphloeus austro-oregonensis* has empty canals between opposing hymenia, and in places where the excipulum invaginates the gleba (Frank & al., 2006). However, *Calongea* canals are very different, in that they are lined by excipular-like rather than hymenial tissue.

The ornamentation of *Pachyphloeus* spores is of rigid, straight (not curved) short to long, often capitate spines evenly distributed on the spores. Spines on

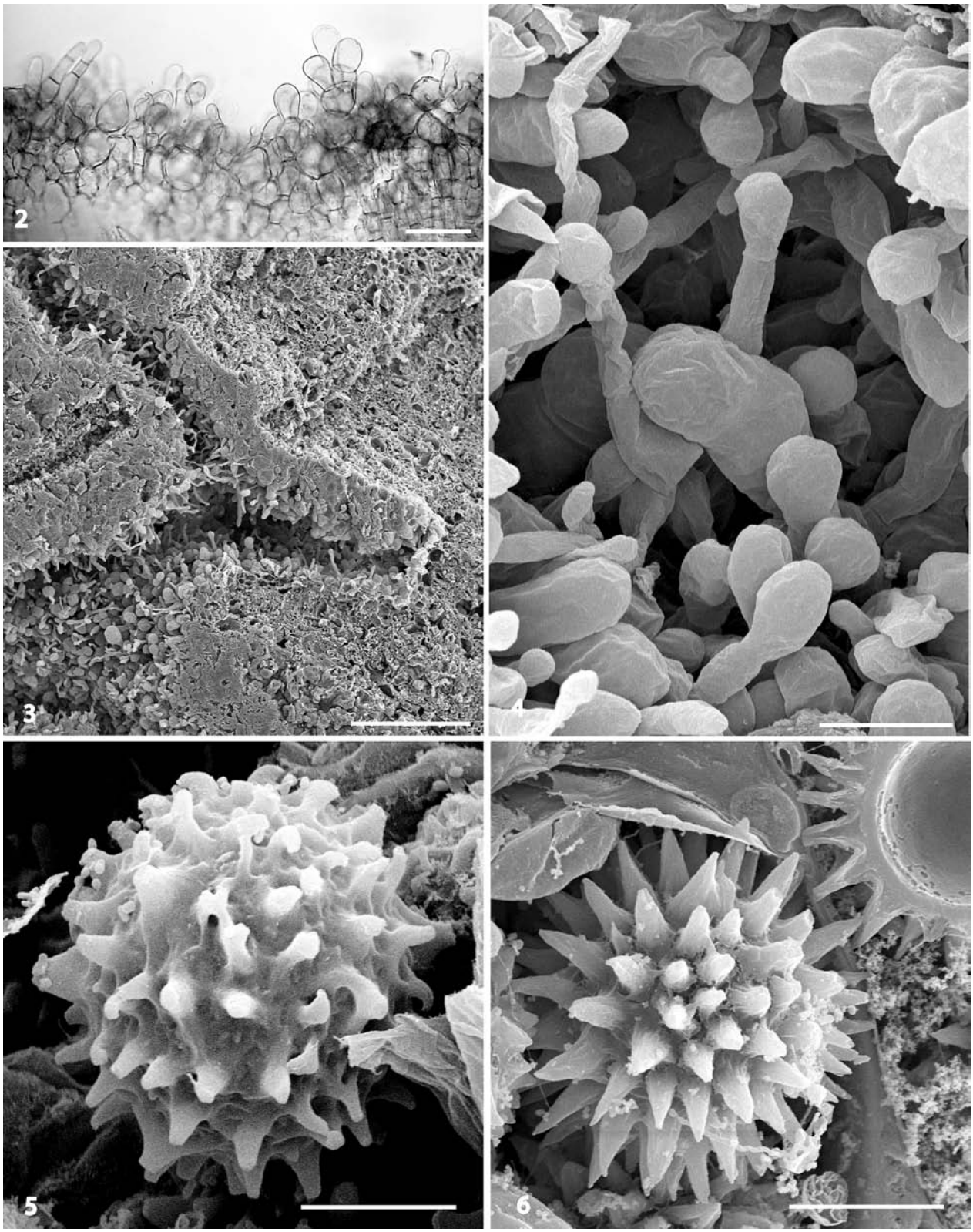

Fig. 2. Bright field microscopy, *Calongea prieguensis* excipular hairs. Scale bar = 50 µm. **Figs. 3-6.** Scanning electron micrographs. **Fig. 3.** Hollow glebal canal lined with hairs in *C. prieguensis*. Scale bar = 250 µm. **Fig. 4.** Higher magnification of Fig. 3 showing details of hairs along the glebal canals. Scale bar = 25 µm. **Fig. 5**. Spore of *C. prieguensis*. Scale bar = 10 µm. **Fig. 6.** Spore of an undescribed, North American *Pachyphloeus* species. Scale bar = 5 µm.

any given spore are uniform in length. Spine development appears to be the same among species of *Pachyphloeus*, until nearly mature, when variations occur. The spine apices may coalesce to partially or fully cover the spores in some species, forming the perisporium (Healy, 2002). The distribution, height, and width of spore spines of *Calongea* are irregular, and the spines themselves are often curved rather than straight. No evidence was seen of a perisporium. *Pachyphloeus* spores most similar to *C. prieguensis* are of an undescribed species from North America that also lacks a perispore, and has straight rather then the more usual capitate spines of other *Pachyphloeus* species (Fig. 6). However, unlike *Calongea*, and in keeping with other *Pachyphloeus*, the spines are rigid, and uniform in length, diameter, and distribution.

A recent effort to delimit the Pezizaceae, on the basis of parsimony analyses of LSU rDNA or on the basis of morphological similarity *(Mycoclelandia* and *Sphaerozone),* included the following fourteen truffle or truffle-like genera in the family: *Amylascus, Cazia, Eremiomyces, Hydnobolites, Hydnotryopsis, Kalaharituber, Mattirolomyces, Mycoclelandia*, *Pachyphloeus*, sequestrate *Peziza (P. whitei* and *P. ellipsospora), Ruhlandiella*, *Sphaerozone, Terfezia* and *Tirmania* (Læssøe & Hansen, 2007). These authors defined ascomycetous truffles as ascocarps produced at or below ground level that lack forcible spore discharge. Here, we refer to genera as "truffle-like" if they fit this definition, but retain forcible spore-discharge (are operculate), and/or lack a peridial covering. Our results, based on both molecular and morphological characters, yielded the discovery of yet another truffle lineage in the Pezizaceae and add to our knowledge of truffle biodiversity. The proposed new genus *Calongea* brings the number of truffles and truffle-like genera in this family to fifteen.

A comparison of Calongea *to other genera in the* Pezizaceae

BLAST results of ITS and LSU sequences from *Calongea prieguensis* have highest affinity to taxa in the *Peziza depressa-Ruhlandiella* clade of the most recent circumscription of the Pezizaceae. Our phylogenetic analyses also place *Calongea* close to (but outside of) this clade. The *Peziza depressa-Ruhlandiella* clade includes apothecial species with asci that are blue over the entire length in iodine solutions (amyloid), rather than at the apex only (Hansen & al., 2001; Hansen & al., 2005; Læssøe & Hansen, 2007). It also includes truffle-like genera that lack a peridium, and instead have an epithecial covering of the hymenium, and weakly amyloid, indehiscent asci *(Ruhlandiella, Sphaerozone* in Dissing and Korf, 1980), truffle-like species that have a distinct peridium, and amyloid, operculate asci *(Peziza ellipsospora, P. whitei* in Trappe, 1979), truffles that have a peridium, and amyloid, indehiscent asci *(Tirmania* in Trappe, 1971), and truffles that have a peridium, with inamyloid, indehiscent asci *(Terfezia* in Trappe, 1971; *Cazia* in Trappe 1989). In these features, *C. prieguensis* is most similar to *Terfezia* and *Cazia*. We agree with previous authors who have observed that operculate and amyloid asci, features traditionally used to delimit the Pezizaceae (Kimbrough, 1970; Korf, 1973), may not be useful for placing the truffles and truffle-like taxa into families (eg. Díez & al., 2002; Læssøe & Hansen, 2007; Trappe, 1979). *Calongea* is an additional truffle in which these delimiting features are lacking, and for which there is morphological and molecular support for placement in the Pezizaceae

Some additional comparisons between *Calongea* and other truffle-like genera in the *Peziza depressa-Ruhlandiella* clade are listed here. *Ruhlandiella* has paraphyses and epithecia that are gelatinous, and the spores are alveolate-reticulate (Dissing & Korf, 2000). The duplex structure of the peridium of *Calongea* is similar to *Peziza*, but *Peziza* spores are ellipsoidal in shape. Like *Calongea*, the peridium of *Cazia* has occasional large cells, but the peridium is glabrous, the gleba is solid, and the spores are ellipsoidal. Among other genera with globose spores, *Terfezia* and *Tirmania* have a similar tissue type in their peridia, but have a solid gleba, rather than hollow canals. Spore ornamentation resembles that of *Terfezia leptoderma* and *T. olbiensis* in variation of spine width, and distribution, and the wartiness along the spore wall surface between spines (see electron micrographs in Montecchi & Sarasini, 2000), which we interpret as incipient spines on *Calongea* spores.

Previous studies based on LSU rDNA have not resolved the higher order relationships among the major clades within the Pezizaceae (Hansen & Pfister, 2006; Læssøe & Hansen, 2007). Our results demonstrate a similar lack of resolution with this locus. Although we can say with confidence that *Calongea* and *Pachyphloeus* are distinct from each other, and that *Calongea* appears close to species in the *Peziza depressa-Ruhlandiella* clade, we have not been able to determine the closest apothecial, truffle, or truffle-like relative of *Calongea.* The use of more genetic markers (e.g. RNA polymerase I & II), the discovery of new taxa, and greater taxon sampling could help resolve the relationships between *Calongea* and other genera within the Pezizaceae.

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