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Kalapuya brunnea gen. & sp. nov. and its relationship to the other sequestrate genera in Morchellaceae

Matthew J. Trappe[1]
James M. Trappe
Department of Forest Ecosystems and Society, Oregon State University, Corvallis, Oregon 97331-5752

Gregory M. Bonito
Department of Biology, Duke University, Durham, North Carolina 27708

Abstract: *Kalapuya* is described as a new, monotypic truffle genus in the Morchellaceae known only from the Pacific northwestern United States. Its relationship to other hypogeous genera within Morchellaceae is explored by phylogenetic analysis of the ribosomal LSU and EF1α protein coding region. The type species, *K. brunnea*, occurs in Douglas-fir forests up to about 50 y old on the west slope of the Cascade Range in Oregon and in the Coastal Ranges of Oregon and northern California. It has a roughened, warty, reddish brown to brown peridium, a solid whitish gleba that develops grayish brown mottling as the spores mature, and produces a cheesy-garlicky odor at maturity. Its smooth, ellipsoid spores resemble those of *Morchella* spp. but are much larger. The four hypogeous genera known in the Morchellaceae, *Kalapuya, Fischerula*, *Imaia* and *Leucangium*, are distinct from the epigeous genera *Morchella* and *Verpa*, but it is uncertain whether they resulted from a single transition to a hypogeous fruiting habit or from multiple independent transitions. *Kalapuya*, locally known as the Oregon brown truffle, has been commercially harvested for culinary use.

Key words: Ascomycota, Douglas-fir, EF1α, *Fischerula*, hypogeous fungus, *Leucangium*, LSU rDNA, Morchellaceae, Pezizales, taxonomy, truffle

INTRODUCTION

A brown to reddish brown ascotruffle collected in northwestern Oregon for several years has been locally known as the ''Oregon Brown Truffle''. Because it resembles the Oregon Black Truffle (*Leucangium carthusianum* [Tul. & C. Tul.] Paol.) in size, texture, glebal appearance and habitat, it had been assumed to be an undescribed *Leucangium* sp. However its spore shape differs from that of *L. carthusianum* and molecular analyses also distinguish it from *Leucangium* and other known genera. Here we describe this genus and its only known species, *Kalapuya brunnea*, and discuss its relationship with other genera within the Morchellaceae.

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[1] Corresponding author. E-mail: trappem@gmail.com

MATERIALS AND METHODS

Sections were prepared for light microscopy by hand and mounted in dH_2O, Melzer's reagent and cotton blue as well as by microtoming of paraffin-embedded specimens and staining the thin sections in safranin-fast green. All microscopic measurements were made in dH_2O mounts at 400× or 1000× with a Zeiss GSL research microscope. Melzer's reagent was used to test for amyloid reactions and cotton blue for cyanescent reactions.

Glebal tissue samples were sequenced at the Institute for Genome Sciences and Policy at Duke University. Clean fungal tissue was removed from within sporocarps, placed in microcentrifuge tubes and ground with micropestles. DNA was extracted with 24:1 chloroform:isoamyl alcohol and PCR amplified with the primer sets 897R-NS24 (SSU), ITS5–LR5 (ITS and LSU), RPB2_5F-RPB2_7R (RPB2) and 1577F-2218R (EF1α). Information on primers can be found at http://www.aftol.org/primers.php. PCR products were viewed on 1% agarose gels stained with SYBR safe (Invitrogen, Carlsbad, California). Successful amplicons were cleaned with the enzymes exonuclease I and alkaline phosphotase (New England Biolabs, Ipswich, Massachusetts). Bidirectional sequencing was performed with the above primers and the Big Dye Sequencing Kit 3.1 (Applied Biosystems, Foster City, California) on an ABI3730 capillary sequencer (Applied Biosystems). Sequences were edited with Sequencher 4.1 (Gene Codes Inc., Ann Arbor, Michigan) and aligned to reference sequences with MacClade 4.0 software (Maddison and Maddison 2002). Phylogenetic analyses were conducted with parsimony and maximum likelihood optimization criteria in PAUP* 4.0b10 (Swofford 2002) and by Bayesian inference with MrBayes (Ronquist and Huelsenbeck 2003) under a GTR + G + I model of nucleotide substitution. Although not included in our phylogenetic analyses, ITS, SSU and RPB2 sequences were generated for the taxon described here. All sequences produced in this study have been accessioned by GenBank; those used in our phylogenetic analysis (ITS, LSU, SSU, RPB2, EF1α) are summarized (TABLE I) with GenBank accession numbers. In developing the LSU phylogram, along with six collections of the new taxon, we included *Fischerula subcaulis* Trappe 1975, *Imaia gigantea* (Imai 1933) Trappe & Kovács, two species of *Leucangium*, two species of *Verpa*, three species of *Morchella* and taxa in Discinaceae and Tuberaceae as outgroups. We included taxa within the Helvellaceae and Tuberaceae as outgroups for the EF1α phylogeny.

TABLE I. Collection information for specimen and sequences generated in this study

Species name	Voucher	Location	GenBank numbers				
			ITS[a]	LSU[a]	SSU	RPB2	EF1α
Balsamia sp.	MES84	Yolo Co., CA, USA	—	—	—	—	GU596458
Balsamia sp.	SRC868	Yuba Co., CA, USA	—	—	—	—	GU596459
Dingleya sp.	JT27686	NSW, Australia	—	—	—	—	GU596461
Dingleya sp.	JT27860	Victoria, Australia	—	—	—	—	GU596462
Fischerula subcaulis (H)	OSC131366	Tillamook Co., OR, USA	—	—	—	—	GU596467
Helvella lacunosa	MES218	Riverside Co., CA, USA	—	—	—	—	GU596456
Helvella lacunosa	MSNorCal1	Yuba Co., CA, USA	—	—	—	—	GU596457
Imaia gigantea	JT17444	Haywood Co., NC, USA	—	—	—	—	GU596468
Kalapuya brunnea	GB309	Benton Co.,OR, USA	GQ119349	GQ119349	GU596476	—	—
Kalapuya brunnea	GB310	Benton Co., OR, USA	GQ119350	GQ119350	—	GU596477	—
Kalapuya brunnea	JT30484	Benton Co., OR, USA	GQ119351	GQ119351	—	GU596473	—
Kalapuya brunnea	JT17311	Linn Co., OR, USA	GQ119352	GQ119352	—	—	—
Kalapuya brunnea	JT30506	Linn Co., OR, USA	GQ119353	GQ119353	—	GU596474	—
Kalapuya brunnea (H)	JT32730	Benton Co., OR, USA	GQ119354	GQ119354	—	GU596475	—
Leucangium carthusianum	GB311	Benton Co. OR, USA	GQ119355	GQ119355	—	—	—
Leucangium carthusianum	JT23195	Polk Co., OR, USA	GQ119356	GQ119356	—	—	—
Leucangium carthusianum	JT27191	Mason Co., WA, USA	GQ119357	GQ119357	—	—	GU596471
Leucangium carthusianum	M1248	Italy	GQ379719	GQ379720	—	—	GU596472
Leucangium sp.	JT17223	OR, USA	—	—	—	—	GU596469
Leucangium sp.	JT17201	Polk Co., OR, USA	GQ119358	GQ119358	—	—	—
Leucangium sp.	JT22831	Polk Co., OR, USA	GQ119359	GQ119359	—	—	—
Leucangium sp.	JT22764	Tillamook Co., OR, USA	GQ119360	GQ119360	—	—	—
Leucangium sp.	JT23195	Polk Co., OR, USA	—	—	—	—	GU596470
Reddellomyces sp.	AWC4985	Victoria, Australia	—	—	—	—	GU596460
Tuber gibbosum	JT30580	Clackamas Co., OR, USA	—	—	—	—	GU596463
Tuber melanosporum	GB200	Italy	—	—	—	—	GU596464

(H) = *Holotype*.

[a] In most cases ITS and LSU were submitted as a single sequence, thus they share a single accession number.

RESULTS

Phylogenetic analysis of LSU rDNA and EF1α genes both indicate the new taxon is nested with Morchellaceae and related to but distinct from other known hypogeous genera in this family (e.g. *Fischerula*, *Imaia* and *Leucangium*) and epigeous genera (*Morchella*, *Verpa*, *Disciotis*) (FIG. 1). However the relationships among genera within Morchellaceae remain unresolved (FIG. 2). Morphological characters also clearly distinguish this species from others examined. We accordingly designate a new genus, *Kalapuya*, to accommodate this new species.

Kalapuya M. Trappe, Trappe, & Bonito, gen. nov.
MycoBank MB513040, GenBank GQ119354

Ascomata hypogaea, stereothecia, subglobosa. Peridium porphyreum vel brunneum, verrucosum, verrucis fissuris tenuibus separatis. Gleba solida, albida, contextu fertili griseo-brunneo maculato. Asci ellipsoidei vel globosi. Sporae ellipsoideae, laeves, juventute hyalinae, maturitate succineae.

TYPE SPECIES: *Kalapuya brunnea* M. Trappe, Trappe, & Bonito.

Etymology. *Kalapuya*, after the native American tribe whose ancestral lands encompassed the range of this genus, from the western foothills of the Cascade Range to Coastal Ranges.

Kalapuya brunnea M. Trappe, Trappe, & Bonito, sp. nov.
MycoBank MB 513040, GenBank GQ119354

Ascomata hypogaea, stereothecia, subglobosa vel globosa, 12–80 × 10–45 mm. Peridium porphyreum vel brunneum, verrucosum, verrucis fissuris tenuibus separatis. Gleba solida, albida, contextu fertili griseo-brunneo maculato. Asci globosi, 70–90 × 65–90 μm, pariete 1–3 μm crassis, 6–8 sporis. Sporae ellipsoideae, 32–43 × 25–33 μm, laeves, juventute hyalinae, maturitate succineae.

Macrocharacters. ASCOMATA (FIG. 3) hypogeous stereothecia, subglobose to lobed and furrowed, 12–60(–80) × 10–45 mm, with a subcartilagenous, dendroid, basal attachment that easily breaks off when specimens are removed from soil. PERIDIUM (FIG. 4)

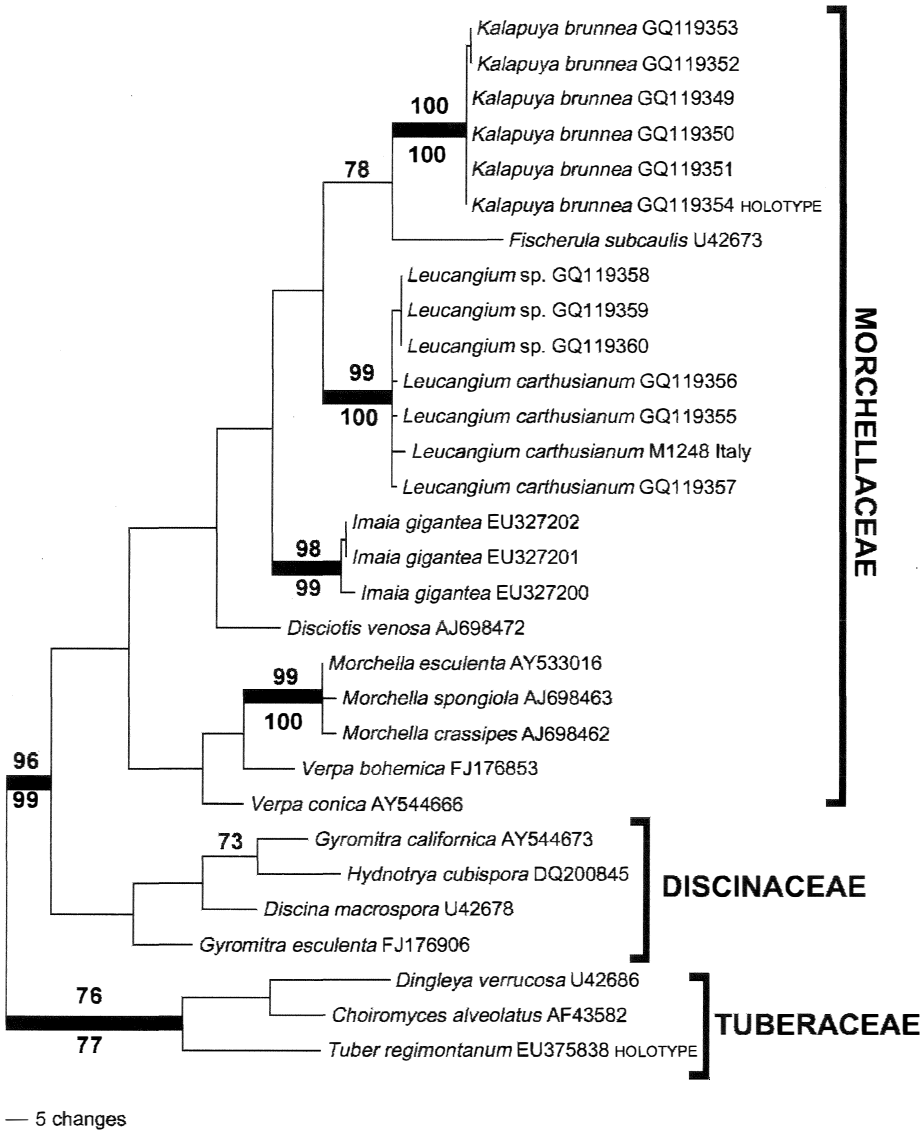


FIG. 1. One of 137 most parsimonious trees. The analysis is based on 787 included characters of the 28S large subunit rDNA, 638 which are constant and 108 that are parsimony informative. Nodes with significant bootstrap support are thickened. Maximum parsimony (MP) bootstrap values based on 1000 replicates are shown above the nodes, and maximum likelihood (ML) bootstrap values based on 1000 replicates are shown below nodes. Taxon names are followed by GenBank accession numbers.

light yellowish brown to orange-brown, reddish brown or brown, often with darker patches or becoming blackish on the upper surface in age, rough to granular, up to 2 mm thick, unevenly covered with patches of flat to rounded warts 0.5–3 mm broad, the larger warts often in turn beset with minute warts, polygonal in face view and separated by narrow fissures, in age often rimose-areolate. GLEBA (FIG. 5) solid, firm, whitish to yellowish gray, with grayish brown mottling of fertile pockets surrounded by sterile, undifferentiated veins. FLAVOR and ODOR mildly garlicky-cheesy, the odor reminiscent of mature Camembert.

Microcharacters. ASCOSPORES (FIG. 6) ellipsoid, 32–43 × 25–38 µm (Q = 1.09–1.68), surface smooth, containing a large, central guttule with crowded tiny droplets inside the spore at its ends and on the sides of the central guttule; spore walls 1–3 µm thick, hyaline in youth becoming amber to pale olive by maturity, nonreactive in Meltzer's reagent, stongly staining in cotton blue. ASCI (FIG. 6) 6–8-spored, ellipsoid to globose or irregular, nonamyloid, 70–110 × 60–

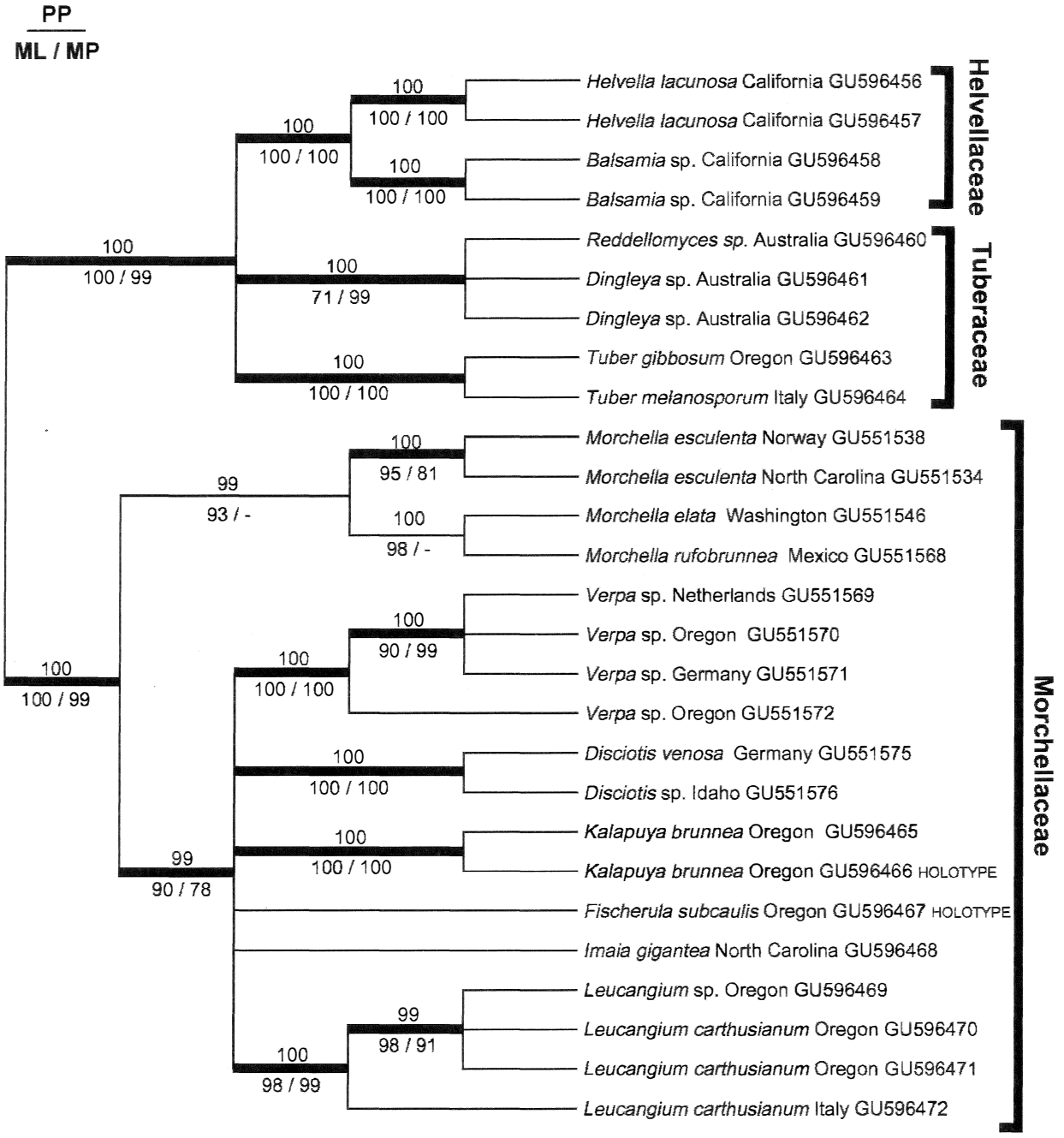
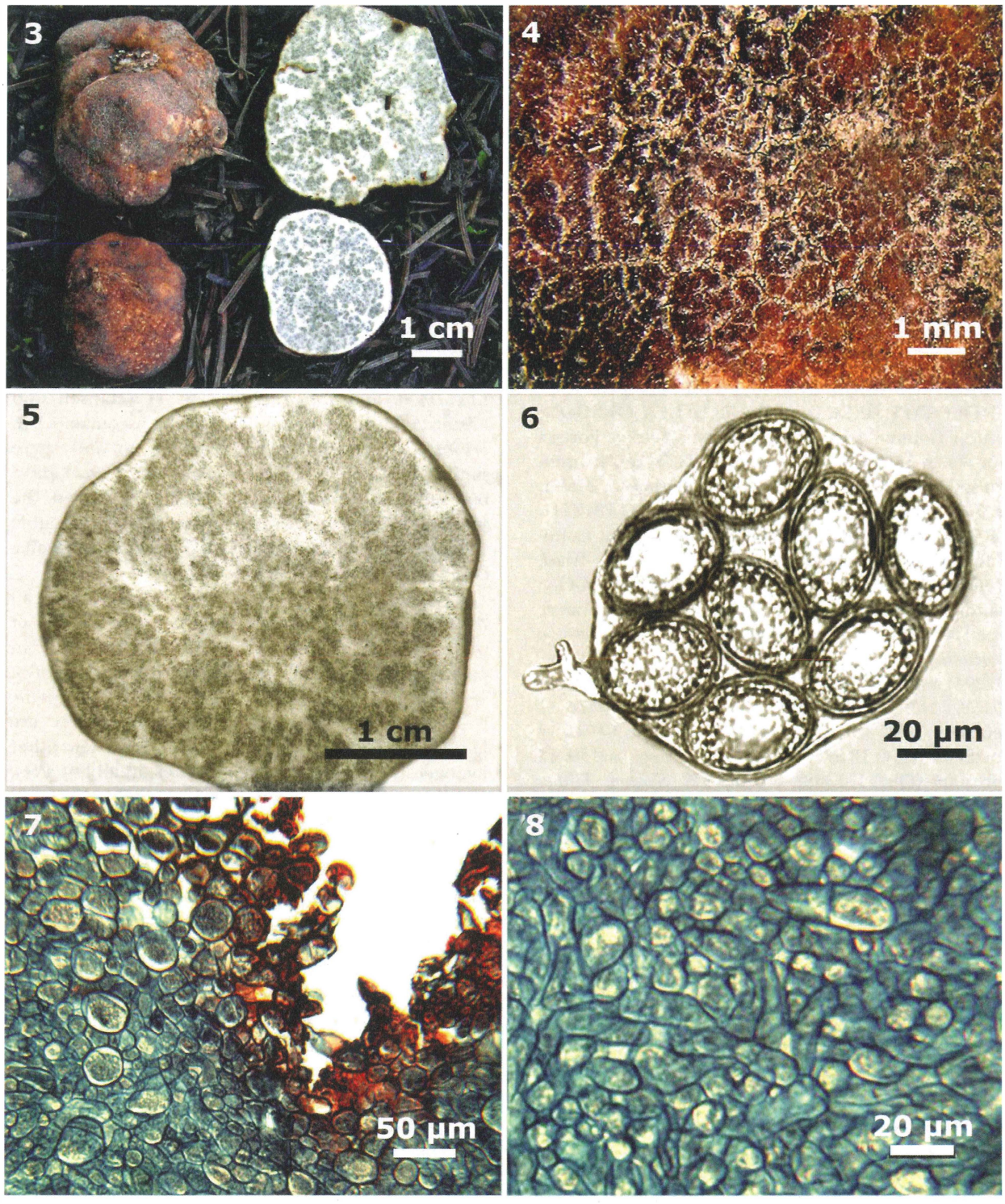


FIG. 2. Consensus of 10 000 credible trees inferred by Bayesian analysis. The analysis included 27 taxa and 499 characters of EF1α, 146 of which were parsimony informative. Posterior probability (PP) values are shown above the nodes. Maximum likelihood (ML) bootstrap values are shown below the nodes and are followed by maximum parsimony (MP) bootstrap values, based on 1000 bootstrap replicates. Nodes with significant bootstrap support for all methods of inferences are thickened. Taxon names are followed by their geographic origin and GenBank accession numbers.

100 μm, with a stem 10–40 × 6–10 μm and having a forked base; walls hyaline, in youth up to 3 μm thick, thinning to about 1 μm by maturity. ECTAL EXCIPULUM (FIG. 7) with warts up to 150 μm tall of rounded to polyhedral cells 10–28(–50) × 10–25(–50) μm, the walls thin to thickened up to 5 μm near the surface, reddish brown in dH_2O. ENTAL EXCIPULUM (FIG. 8) near the ectal excipulum of rounded, thin-walled cells 10–45 × 10–30 μm mixed with thin-walled hyphae, 5–13 μm broad at septa, hyaline in dH_2O,



FIGS. 3–8. Macro- and microsopic characters of *Kalapuya brunnea*. 3. Ascomata. 4. Peridium, face view. 5. Gleba, cross section. 6. Stipitate ascus with spores. 7. Ectal excipulum, showing a peridial furrow with adjacent warts, stained in safranin-fast green. 8. Ental excipulum, stained in safranin-fast green.

toward the gleba grading to tightly interwoven, hyaline, thin-walled hyphae 5–13 µm broad at septae. GLEBA of loosely interwoven, ascus-bearing, thin-walled, hyaline hyphae 5–13 µm broad at the septae plus scattered inflated cells.

Etymology. Brunnea (Latin): referring to the brown peridium.

Habitat, distribution and season. Hypogeous under Douglas-fir (*Pseudotsuga menziesii*) up to ca. 50 y old in the top 2–10 cm of mineral soil, beneath scant litter layers from near sea level to ca. 500 m in the Cascade Range in Oregon and Coastal Ranges of Oregon and northern California (Humboldt County). Fruiting Oct–Mar, but occasionally as early as Sep and as late as Jul. Its trophic status is unconfirmed but it is thought to be an obligate ectomycorrhizal symbiont with Douglas-fir.

HOLOTYPE HERE DESIGNATED: USA. OREGON, Benton County: 4 km W of Blodgett, Starker Forests Tree Farm (44°36′00.00″N, 123°34′08.70″W), in mineral soil beneath 20 y old *Pseudotsuga menziesii*, 215 m, *M. Trappe, Trappe 32730*, 13 Feb 2009 (OSC 131234).

PARATYPES: USA. CALIFORNIA: Humboldt County: Redwood National Park, Lost Man Creek, *B. Wood, Trappe 12658*, 7 Nov 1992 (OSC 131569). OREGON: Benton County: 4 km W of Blodgett, Starker Forests Tree Farm in mineral soil beneath 20 y old *Pseudotsuga menziesii*, 215 m, *G. Bonito GB 309*, 13 Feb 2009 (OSC 132294) and *GB 210* (OSC 132295); *S. Donovan, Trappe 30484*, 6 Nov 2004 (OSC 132389) and *Trappe 30516*, 11 Dec 2004 (OSC 131580); *M. Trappe, Trappe 30494*, 14 Nov 2004 (OSC 131575); *M. Hinds, Trappe 30739*, 12 Apr 2005 (OSC 111388). Monroe, *B. Shelton, Trappe 12328*, 13 Jan 1992 (OSC 131576). Woods Creek, *J. Trappe 1787*, 11 Dec 1968 (OSC 131581), *Trappe 2022*, 18 Oct 1969 (OSC 131582) and *Trappe 2035*, 13 Nov 1971 (OSC 59102). Clackamas County: 305 m, *D. Wheeler, Trappe 11499*, 16 Jul 1990 (OSC 131573). Lane County: Fawn Saddle, 335 m, *F. Evans, Trappe 23442*, 6 Nov 1999 (OSC 131578). Lincoln County: near Harlan, 80 m, *J. Kouni, Trappe 5495*, 10 May 1979 (OSC 131584) and *R. Swartzendruber, Trappe 27983*, 20 Dec 2001 (OSC 131568). Siuslaw National Forest, Alsea Ranger District, CVS Plot 37, *E. Cázares, Trappe 32731*, 18 Sep 2000 (OSC 132390). Siletz watershed ca. 12 miles from U.S. 101, *C. Sousa, Trappe 30525*, 22 Dec 2004 (OSC 131577). Trenholm Saddle Rd W of Alsea, 80 m, *T. Johnson, Trappe 8856*, 1 Mar 1986, (OSC 131588). Linn County: Cedar Creek Road. 5 miles SE of Lebanon, *F. Evans, Trappe 32788*, 7 Feb 2009 (OSC 132391). Wiley Creek, 275 m, *Z. Carter, Trappe 30506*, 26 Nov 1994 (OSC 132390), *F. Morris, Trappe 17473*, 25 Nov 1995 (OSC 131570), *Z. Carter, Trappe 17486*, 6 Dec 1995 (OSC 131571) and *Z. Carter, Trappe 17311*, 8 Jan 1996 (OSC). Near Berlin, 75 m, *R. Hausen, Trappe 7184*, 6 Dec 1982 (OSC 131586), D. Johnson, *Trappe 8825*, 9 Jan 1986 (OSC 131589) and *V. Moore, Trappe 8832*, 10 Jan 1986 (OSC 131587). McDowell Creek Park, 215 m, *R. Hausen, Trappe 7183*, 28 Dec 1982 (OSC 131585). Polk County. Valsetz Lake, 380 m, *D. Wheeler, Trappe 11316*, 13 Oct 1989 (OSC 131574). Tillamook County: Cedar Creek, 490 m, *M. Mahrt, Trappe 22767*, 17 Jun 1996 (OSC 131579). Washington County: Sherwood, 60 m. *R. Dye, Trappe 31753*, 12 Feb 2006 (OSC 112203). Yamhill County: Charles Metsker County Park, 180 m, *S. Ford, Trappe 30503*, 1 Dec 2004 (OSC 131572).

DISCUSSION

Kalapuya brunnea is closely related to three other genera of hypogeous fungi in Morchellaceae, *Fischerula*, *Imaia* and *Leucangium* (FIG. 1) (Hansen and Pfister 2006, Kovács et al. 2008). The ascomata of *K. brunnea* are morphologically similar to and share similar habitats and fruiting seasons with *Leucangium*, but spore morphologies are different. Indeed the spores of *Kalapuya brunnea* more closely resemble those of some *Balsamia* and *Helvella* spp. (Helvellaceae) in shape but are much larger.

Kalapuya, *Fischerula*, *Imaia* and *Leucangium* all have a similar peridial structure: a tissue of plus or minus isodiametric cells raised in warts overall or in patches. They all produce exceptionally large spores: *Kalapuya*, 32–43 µm; *Fischerula*, 60–100 µm; *Imaia*, 42–62 µm; and *Leucangium*, 60–90 µm. These are larger than those that occur in all but a few other hypogeous genera or, for that matter, in all but a few species of the epigeous *Morchella* and *Verpa* in Morchellaceae. The spores of all these genera otherwise differ strikingly. Those of *Morchella* and *Verpa* are ellipsoid, smooth and lack guttules and cytoplasmic droplets; *Kalapuya*, ellipsoid, smooth, containing a large, central guttule and abundant droplets; *Leucangium*, fusoid-apiculate (similar to spores of *Discina* spp. in Discinaceae), smooth, containing a large guttule but lacking droplets; *Imaia*, globose, enclosed in a thick, mucilaginous sheath permeated with meandering canals and apparently lacking a large guttule or droplets; *Fischerula*, ellipsoid, ornamented with agglutinated spines or conical warts. *Kalapuya* and *Imaia* share a trait not reported for the other two hypogeous members of Morchellaceae: the asci have thickened walls in youth but the walls thin strikingly by maturity (Kovács et al. 2008). Conversely the asci of *Fischerula subcaulis* initiate with thin walls that become thicker and multilayered with maturity (Trappe 1975). Given these morphological divergences, it would be difficult to place these four hypogeous genera in any extant family without molecular evidence and placing them

in the same family as *Morchella* would be highly implausible.

All four genera are limited either in distribution or have strikingly disjunct distributions. *Kalapuya* is known only from northwestern California and western Oregon. *Fischerula* has one species known only from Oregon and Washington and a second species that occurs only in southern Europe (Trappe 1975). *Imaia* has only one species, but it is disjunct with one population in Japan and another in the Appalachian Mountains of eastern USA (Kovács et al. 2008). The only described *Leucangium* sp., *L. carthusianum*, also has two disjunct populations, southern Europe and Pacific northwestern USA (Trappe 1979); other rare, undescribed species are known from a few collections in western USA.

Kalapuya brunnea originally was thought to be a new species of *Leucangium*, due to similarities in size, peridial texture, gleba and habitat. The ectomycorrhizal genus *Leucangium* currently contains only one described species, *L. carthusianum* (Tul. & C. Tul.) Paol. *Leucangium carthusianum* (*Picoa carthusiana* Tul. & C. Tul., syn. *L. opthalmosporum* Quél.) was originally described from a mixed forest of beech and fir in the Chartreuse Mountains of eastern France. It appears to be uncommon in Europe, where its range extends approximately from central Italy to eastern France and southern Germany.

A disjunct population of *Leucangium carthusianum* also occurs in North America, from southwestern British Columbia to Douglas County, Oregon. The North American population appears to associate exclusively with Douglas-fir west of the Cascade crest, most commonly in stands less than 50 y old (Trappe et al. 2007).

A taxonomic history of *Leucangium* is provided in Li (1997). Ultrastructural analysis of *Leucangium carthusianum* by Li (1997) showed similarities with Morchellaceae in septal structure but aspects of ascospore morphology reminiscent of Helvellaceae. Recent molecular work has had difficulty placing *Leucangium* and it has been considered to be part of either the Discinaceae or Morchellaceae lineage (O'Donnell et al. 1997, Læssø and Hansen 2007). A combined analysis of LSU and SSU data by Hansen and Pfister (2006) and more recent analysis of SSU data by Kovacs et al. (2008) support the placement of *Leucangium* in Morchellaceae. Our analyses also support the placement of *Leucangium* within Morchellaceae.

Fischerula was placed in the Helvellaceae by Trappe (1975), but molecular studies by O'Donnell et al. (1997) and Læssø and Hansen (2007) support its placement within the Morchellaceae. Analyses by Hansen and Pfister (2006), Kovacs et al. (2008) and the data presented here confirm that conclusion.

The tribal and geographic names of the Kalapuya have accumulated several orthographic variants over the years. Dr David Lewis, manager of the Cultural Resources Department of the Confederated Tribes of Grand Ronde, confirmed Kalapuya as the spelling now used by the tribe. His inquiries among tribal members did not reveal any knowledge or traditional use of these or other truffles; some members regard most mushrooms as taboo foods.

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