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Tuber eburneum and *Tuber mujicii*: New pine-associated *Tuber* species from eastern North America

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ABSTRACT

The ectomycorrhizal truffle genus *Tuber* is widespread and diverse. Recent sampling of ascomata, ectomycorrhizal root tips, and environmental sequences has resulted in the identification of many *Tuber* species that cannot be assigned to described species and require formal description. Using morphological and molecular phylogenetic analysis, we describe two North American *Tuber* species associated with pines (*Pinus* spp.). *Tuber eburneum*, sp. nov., is an early-diverging taxon in the Melanosporum clade that differs substantially from all other taxa in that clade due to its light-colored peridium and gleba, lack of peridial warts, and peridial hairs that are ornamented with small, irregular protrusions. *Tuber mujicii*, sp. nov., is a whitish truffle species in the Puberulum clade. Although *T. mujicii* is morphologically similar to many related taxa, it can be distinguished by a combination of characters, including peridium color, spore size, number of ascospores per ascus, and number of reticulations across the spore surface.

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Eastern North America; ectomycorrhizal fungi; Pezizales; truffle; Tuberaceae; 2 new taxa

INTRODUCTION

The genus *Tuber* comprises an estimated 180–230 species of truffle fungi that are distributed across the Northern Hemisphere (Bonito et al. 2010). Although the truffle form has evolved independently in several major lineages of fungi, species in the genus *Tuber* are notable for their ecological and economic importance. *Tuber* species are common ectomycorrhizal (ECM) symbionts with a number of woody gymnosperm and angiosperm host plants as well as some orchids (Bonito et al. 2010; Jacquemyn et al. 2016; Lancellotti et al. 2014). Several *Tuber* species are also cultivated for commercial harvest as culinary delicacies due to their intense aromas and flavors. By forming nutrient-dense and highly aromatic ascomata belowground, *Tuber* truffles have evolved as a food source for many animal species (Ori et al. 2018; Trappe et al. 2009).

Despite the widespread interest in the ecology and cultivation of *Tuber*, many species remain undescribed. Using a global sample of *Tuber* sequences, Bonito et al. (2010) constructed a phylogenetic analysis of all major lineages of *Tuber*, which included many undescribed taxa that were assigned temporary numeric species designations (phylotypes). The main objective of the current study is to document and formally describe two of these undescribed *Tuber* species that are associated with North American species of *Pinus: Tuber* sp. 13, an early-diverging taxon in the Melanosporum clade, and *Tuber* sp. 17 in the Puberulum clade (Bonito et al. 2010).

MATERIALS AND METHODS

Microscopy.— Specimens were sectioned by hand with a razor blade and mounted in 3% KOH or deionized water for observation of microscopic features. In addition, one specimen of *Tuber* sp. 13 (RH73) was chemically fixed and a portion was embedded in resin following the protocol of Curry and Kimbrough (1983). Resin-embedded material was cut into sections that were 2 µm thick using glass knives. These sections were mounted on slides, stained with toluidine blue O (Sigma-Aldrich, St. Louis, Missouri), and imaged with a Zeiss Axiocam HR3 system (Thornwood, New York). A portion of the chemically fixed material was processed for scanning electron microscopy (SEM) following the protocol of Healy (2003). For each mature specimen, spores were measured at 1000× magnification. Because of the wide variation in the dimensions of spores that grow in asci with different numbers of spores, we measured and tabulated the dimensions of spores from 1-spored, 2-spored, 3-spored, 4-spored, 5-spored, and 6-spored asci separately. We measured

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spore length and width (excluding ornamentation), Q-ratio (length/width), ornamentation height, and ascus length, width, and shape. Standard deviations (as ±) are provided for measurements with mean values reported. For *Tuber* sp. 17, which has alveolate-reticulate spores, we measured the number of reticulate meshes per spore length and width. We also measured peridium thickness, shape, color, and arrangement and peridium cell length and width following Healy et al. (2016). Color notations follow Ridgway (1912). Measurements were made with a Zeiss Axio Imager A2 (Oberkochen, Germany) compound microscope. Voucher specimens were dried and accessioned in the Fungal Herbarium of the Florida Museum of Natural History at the University of Florida (FLAS).

Molecular methods.—Genomic DNA was extracted from dried specimens with a standard cetyltrimethylammonium bromide (CTAB) extraction protocol (Gardes and Bruns 1993) and from fresh specimens using an alkaline extraction buffer following the methods of Vandepol et al. (2020). For specimens without existing GenBank sequence accessions, the nuclear rDNA internal transcribed spacer (ITS) region ITS1-5.8S-ITS2 and nuclear large subunit (28S) rDNA were polymerase chain reaction (PCR)-amplified using the standard primer pairs ITS1f-ITS4 (Gardes and Bruns 1993; White et al. 1990) and LROR-LR5 (Hopple and Vilgalys 1994) following the protocols of Gardes and Bruns (1993). PCR amplification of the translation elongation factor 1-alpha (*tef1*) and the second largest subunit of RNA polymerase II (*rpb2*) was performed using the Tuberaceae-specific primers *ef1α*-Tub-for, *ef1α*-Tub-rev, and *rpb2*-Tub-rev (Bonito et al. 2013) and the fungal-specific primer *fRPB2*-5F (Liu et al. 1999), using PCR protocols described in Bonito et al. (2013). PCR products were visualized on 1.5% agarose gels, enzymatically cleaned with exonuclease 1 and alkaline phosphatase (Glenn and Schable 2005), and Sanger sequenced bidirectionally with the same primers by Eurofins Genomics (Louisville, Kentucky).

Phylogenetic analysis.—Reference ITS, 28S, *tef1*, and *rpb2* sequences were chosen to represent the closest relatives of each proposed new species. For both species, more reference ITS sequences were available than for other loci. *Tuber* sp. 13 was previously identified as a basal lineage in the Melanosporum clade, so reference sequences were chosen for all described species in the Melanosporum clade and several species from its sister lineage, the Rufum clade (Bonito et al. 2013). *Tuber magnatum* Picco was chosen as an outgroup based on its position in the multilocus phylogenetic analysis of *Tuber* by Bonito et al. (2013).

Sequences were chosen for *Tuber* sp. 17 based on BLAST search similarity to sequences of *Tuber* sp. 17 and published phylogenetic analyses of related taxa in the Puberulum clade (Guevara-Guerrero et al. 2015; Healy et al. 2013; Kinoshita et al. 2018, 2011; Páez et al. 2018). Sequences from more than 30 new collections of Puberulum clade *Tuber* specimens from the eastern USA were generated for this study and included in the phylogenetic analyses. *Tuber gibbosum* Harkn. was selected as an outgroup based on its position in the multilocus phylogenetic analysis of *Tuber* by Bonito et al. (2013). A table of all sequences used in both phylogenetic analyses is included in SUPPLEMENTARY INFORMATION 1.

Sequences were aligned with MUSCLE 3.8.425 (Edgar 2004) using default settings in Geneious 2020.2.4 (Auckland, New Zealand) and checked manually. Unedited alignments were deposited in the Open Science Framework repository, available at https://osf.io/jpyu7/?view_only=1e407246b85d4145a031053b9d6f0a3e. Ambiguously aligned regions were removed from all alignments with Gblocks (Talavera and Castresana 2007) using the least stringent settings for all alignments. After removal of ambiguous regions, an additional alignment of concatenated ITS, 28S, *tef1*, and *rpb2* sequences was created for all specimens for which sequences of at least three of the four loci were available. For the analysis of *Tuber* sp. 13, the following alignments were used: an alignment of 42 ITS sequences of 557 characters (277 were parsimony-informative); an alignment of 34 28S sequences of 854 characters (139 were parsimony-informative); an alignment of 15 *tef1* sequences of 820 characters (77 were parsimony-informative); an alignment of 16 *rpb2* sequences of 733 characters (66 were parsimony-informative); and a concatenated alignment of 19 ITS+28S+*tef1*+*rpb2* sequences (where at least 3 of 4 loci are present) of 2987 characters (500 were parsimony-informative). For the analysis of *Tuber* sp. 17, the following alignments were used: an alignment of 93 ITS sequences of 602 characters (230 were parsimony-informative); an alignment of 33 28S sequences of 874 characters (83 were parsimony-informative); an alignment of 44 *tef1* sequences of 833 characters (109 were parsimony-informative); an alignment of 20 *rpb2* sequences of 785 characters (59 were parsimony-informative); and a concatenated alignment of 46 ITS+28S+*tef1*+*rpb2* sequences (where at least 3 of 4 loci are present) of 3108 characters (393 were parsimony-informative).

Appropriate models of nucleotide substitution for the alignments were selected with PartitionFinder2 (Lanfear et al. 2017) using the "greedy" algorithm (Lanfear et al.

2012). The following models were selected for the analyses of *Tuber* sp. 13: GTR+I+G for ITS and 28S, SYM +G for *tef1*, and K80+I for *rpb2*. For *Tuber* sp. 17, GTR +I+G was selected for ITS, 28S, and *tef1*, and K80+I was selected for *rpb2*. These models were also used for each corresponding partition in the phylogenetic analyses of the concatenated alignments.

Phylogenetic analyses of both species were conducted with maximum likelihood (ML) and Bayesian methods. Initial phylogenetic analyses were conducted separately for each locus, and after determining that there were no supported conflicts in any of the tree topologies, an additional analysis was conducted with a concatenated alignment. ML phylogenetic tree search used RAxML 2.0.0 (Edler et al. 2021) with 1000 bootstrap replicates to evaluate support for nodes. Bayesian analysis was conducted with MrBayes 3.2.2 run on the CIPRES Science Gateway (Miller et al. 2010). Parallel runs of four Markov chain Monte Carlo chains running for 20 million generations were conducted, with trees sampled every 1000 generations and the first 25% of generations discarded as burn-in. Stationarity was evaluated based on the standard deviation of split frequency (less than 0.01), and mixing behavior of the chains were checked in Tracer 1.7.1 (Rambaut et al. 2018). Trees were visualized and rooted in FigTree 1.4.4 (Rambaut 2018). No supported conflicts were found between ML and Bayesian consensus tree topologies for any of the analyses. Nodes were considered supported when ML bootstrap values were ≥70% and Bayesian posterior probability values were ≥0.95.

RESULTS

Phylogenetic analysis.— Both ML and Bayesian phylogenetic analyses show that *Tuber eburneum*, sp. nov. (FIG. 1a, b), and *T. mujicii*, sp. nov. (FIG. 2a, b), are distinct from other *Tuber* species. *Tuber eburneum*, sp. nov., is supported as an early-diverging taxon in the Melanosporum clade, forming a monophyletic group with 100% bootstrap support and a posterior probability of 1 in both ITS and concatenated phylogenies (FIG. 1a, b). There were no supported conflicts in the 28S, *tef1*, or *rpb2* phylogenies (SUPPLEMENTARY INFORMATION 2). This placement in the Melanosporum clade is also supported morphologically by the unique spiny spores, which resemble those found in other species in the Melanosporum clade.

Tuber mujicii, sp. nov., is supported as a distinct taxon in the Puberulum clade, forming a monophyletic group with 97% bootstrap support and a posterior probability of 1 in the ITS phylogeny and 100% bootstrap support and a posterior probability of 1 in the concatenated phylogeny (FIG. 2a, b). There were some supported conflicts among the 28S, *tef1*, and *rpb2* phylogenies, but only among undescribed species (e.g., *Tuber* sp. BL-5 is polyphyletic in the *tef1* phylogeny) or minor differences in resolution of deeper nodes of the trees (SUPPLEMENTARY INFORMATION 3). None of these conflicts challenged the monophylly or relative placement of *T. mujicii* with respect to its sister clades. The clade of species sister to *Tuber mujicii* includes two undescribed species from eastern North America (FIG. 2b) and another species delimited in Bonito et al. (2010) as *Tuber* sp. 16 from eastern North America and Europe, which is only represented in the ITS phylogeny (FIG. 2a).

TAXONOMY

Tuber eburneum Lemmond, Healy & M.E. Sm., sp. nov. FIG. 3
MycoBank MB839047

Typifications: USA. IOWA: Story County, McFarland Park, erumpent in soil under pine needle duff that is 0.5 to 1.5 cm thick, under *Pinus strobus* (white pine) in a small grove of planted trees, 8 Sep 1997, coll. *R. Healy RH73* (FLAS-F-67910) (**holotype**). GenBank: ITS = GQ221451; 28S = JQ925713.

Diagnosis: Tuber eburneum differs from other species in the Melanosporum clade by its light-colored (vinaceous-buff) peridium, the lack of verrucae on the peridium, a pale ochraceous-buff rather than brown-black gleba at maturity, honey-yellow rather than dark brown ascospores, the presence of beaded peridial hairs, and by DNA sequences. Other features of the spores of *T. eburneum*, such as their variable shape (subglobose to ellipsoid) and the ornamentation of spines that are not connected at the base, further help to separate this species from other members of the Melanosporum clade.

Etymology: Referring to the ivory (white with yellowish tints) color of the ascoma.

Ascoma globose to ovoid-ellipsoid, up to 2.1 × 1.9 × 1.8 cm, knobby, white to light buff, with cracked areas on the exposed surface, smooth (not warted). Gleba solid, white when young, white to light buff at maturity, marbled with white veins of sterile tissue. Odor faint and pleasant, but stronger after enclosure in a container, reminiscent of baked potato; taste bitter, raphanoid.

Peridium 70–210 µm thick, composed of three layers: an ectal excipulum up to 40 µm thick but occasionally absent from some parts of the peridium, composed of irregular, cream-buff cells 5–24 × 3–10 µm with walls occasionally thickened up to 1 µm, and interspersed with hair-like cells that project from the ascoma surface (described below); medullary excipulum 40–100 µm thick

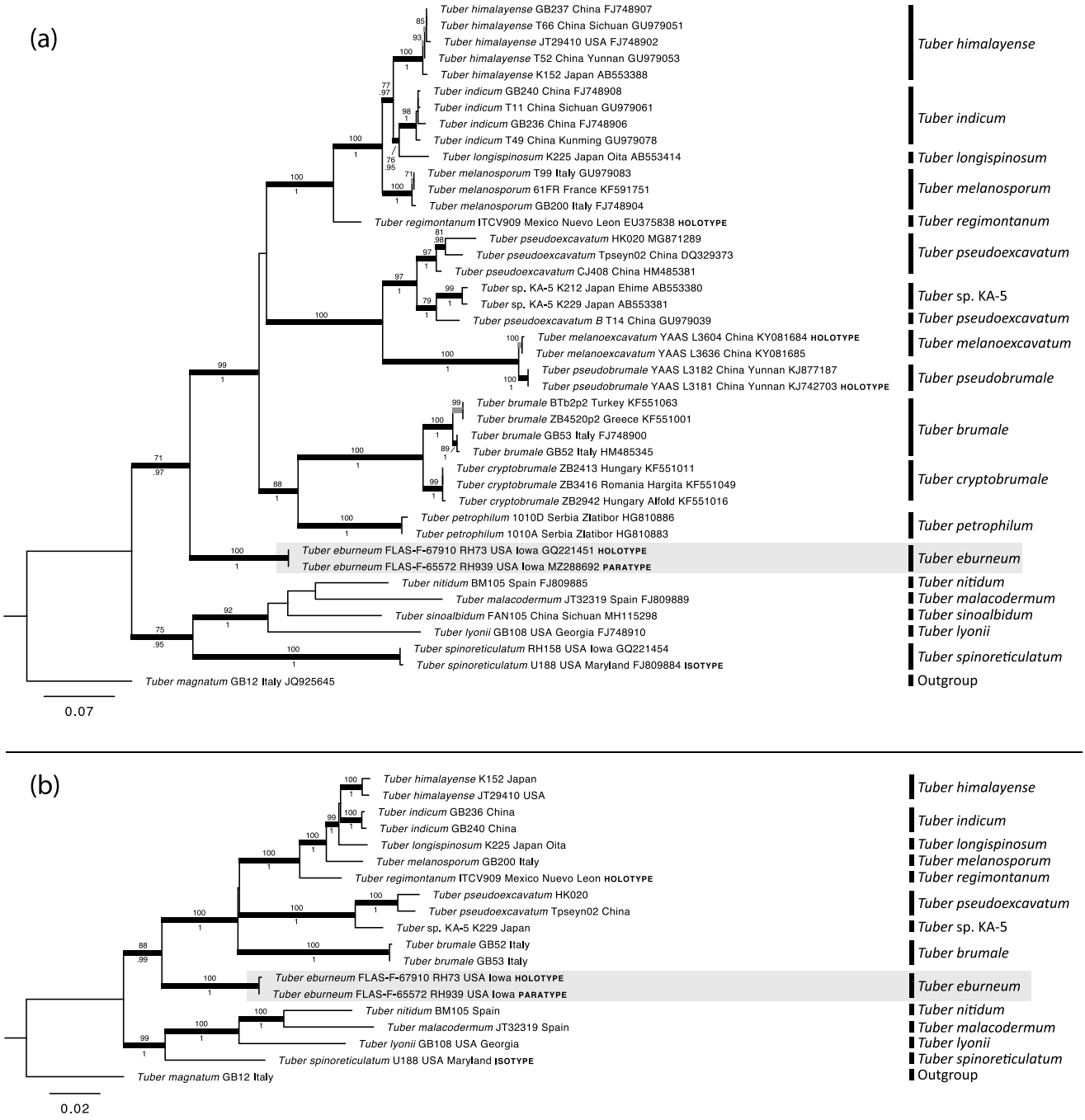

Figure 1. Maximum likelihood phylogenies based on (a) ITS rDNA and (b) concatenated ITS+28S+*tef1*+*rpb2* sequences showing placement of *Tuber eburneum*, sp. nov., in the Melanosporum clade. Branches are considered supported when bootstrap values are ≥70% or Bayesian posterior probability values are ≥95%. Supported values are placed above (bootstrap) or below (posterior probability) each branch. Branches that are supported by both ML bootstrap support and Bayesian posterior probability are indicated with thickened black branches, whereas branches that are supported by only one method are colored gray.

with inflated cells of textura angularis, on average 14 × 7 µm but some inflated and elongated up to 26 × 10 µm; excipulum of interwoven hyphae forming a textura intricata of irregular to elongated cells, elongated cells up to 23 µm in length and 2–3 µm wide at septa. Peridial hairs scattered to dense on ascoma surface, often in tangled clusters, projecting 12–55(–130) µm from surface, although bases of individual hairs are often entangled and difficult to determine. Peridial hairs 2–9 µm wide, infrequently septate, often beaded in appearance due to swellings beneath the surface of the hyphal wall (FIG. 3). Trama of textura intricata with interwoven hyphae 2–3(–5)

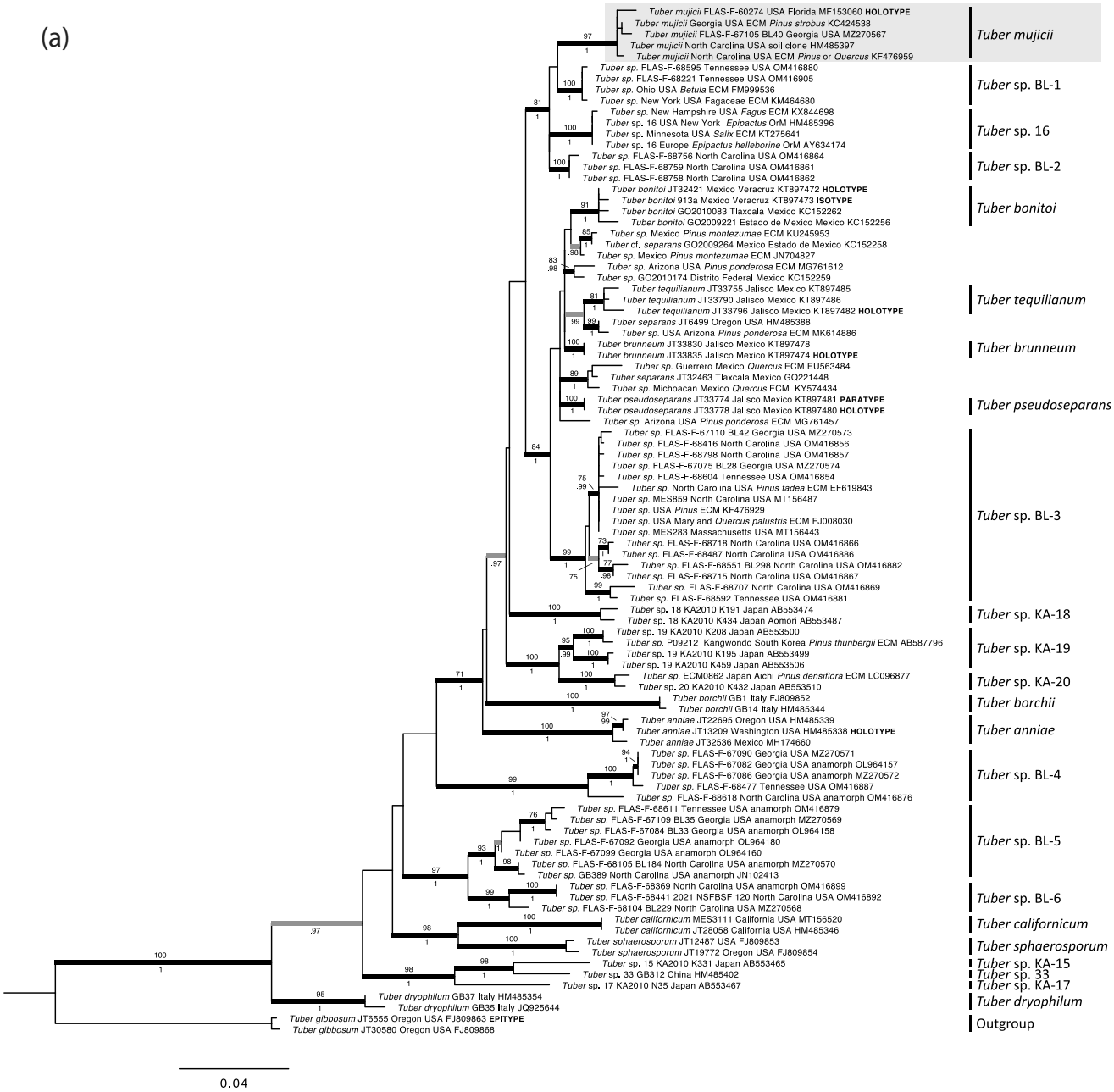

Figure 2. Maximum likelihood phylogenies based on (a) ITS rDNA and (b) concatenated ITS+28S+*tef1*+*rpb2* sequences showing placement of *Tuber mujicii*, sp. nov., in the Puberulum clade. Branches are considered supported when bootstrap values are ≥70% or Bayesian posterior probability values are ≥95%. Supported values are placed above (bootstrap) or below (posterior probability) each branch. Branches that are supported by both ML bootstrap support and Bayesian posterior probability are indicated with thickened black branches, whereas branches that are supported by only one method are colored gray.

μm wide at septa, interspersed with larger hyphae up to 9 μm wide. Asci 44–83(–117) × 32–73 μm, globose to subglobose, hyaline. Short pedicels occasionally observed. Ascospores honey-yellow at maturity, subglobose to ellipsoid with occasional ellipsoid spores having notably tapered ends, spores 1–5 (rarely 6) spores per ascus but 4-spored are most common. Spores in 1-spored asci (29–) 36–47 36–47 × 21–28 μm, Q = 1.4–1.8 (mean 1.6 ± 0.12); in 2-spored asci 21–44 × 20–26 μm, Q = 1–1.8 (mean 1.4 ± 0.28); in 3-spored asci 21–39 × 19–25 μm, Q = 1.0–1.7 (mean 1.3 ± 0.22); in 4-spored asci 20–34 × 18–25 μm, Q = 1–1.7 (mean 1.3 ± 0.19); in 5-spored asci 20–36 × 17–22 μm, Q = 1–1.8 (mean 1.4 ± 0.21). Only one ascus with six spores was observed (in the paratype RH939); spores 19–29 × 15–19 μm, Q = 1.2–1.6 (mean 1.6 ± 0.16). Spores spiny with straight to curved spines, spines 1–5 μm tall and

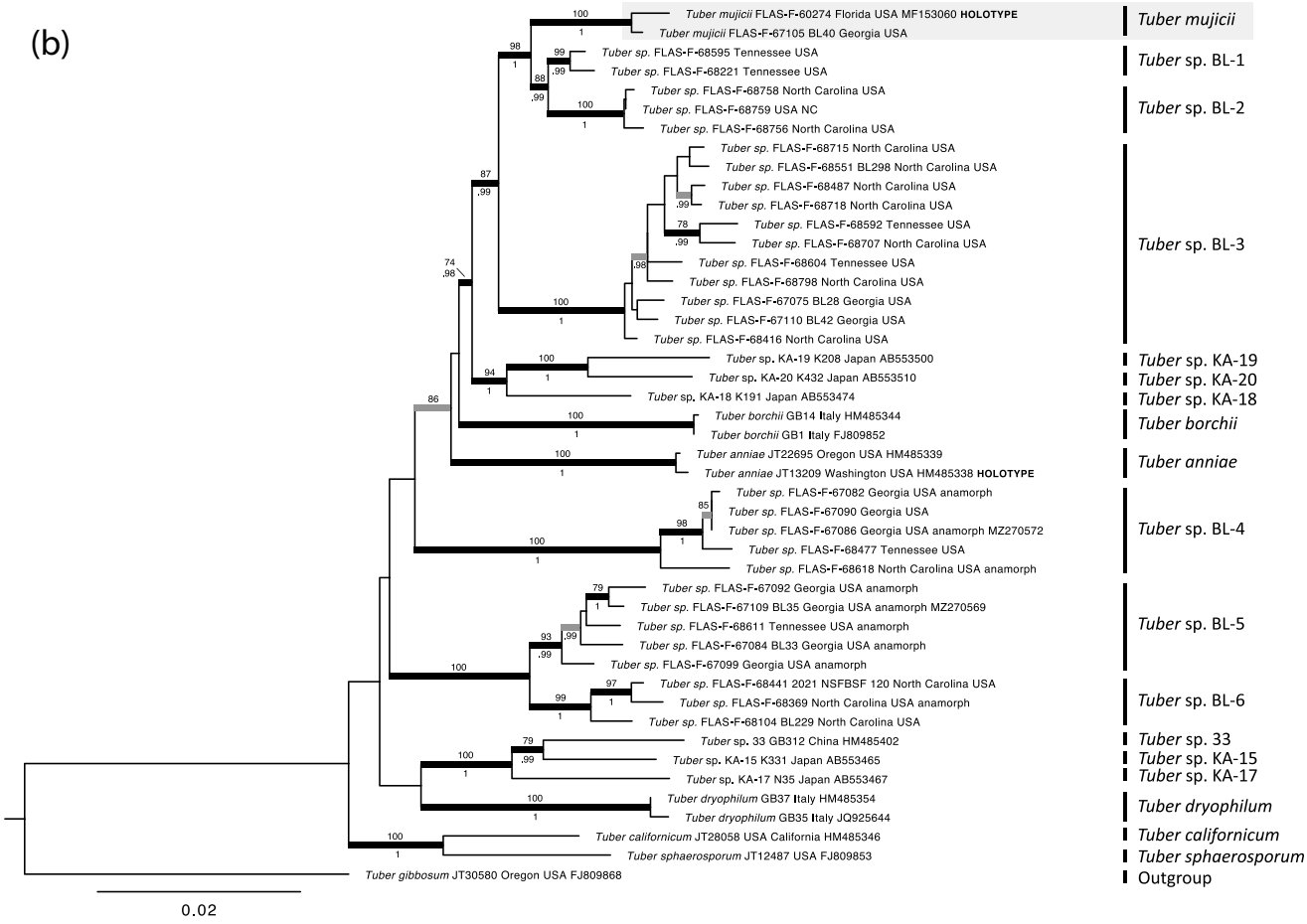

Figure 2. (Continued).

less than 0.5 µm wide. Spines crowded but free from one another and not connected at their bases (FIG. 3).

Ecology and distribution: Associated with Pinaceae, similar to a few other species in the Melanosporum clade (e.g., *T. brumale* Vittad., *T. melanosporum* Vittad., *T. pseudoexcavatum* Y. Wang, G. Moreno, Riousset, Manjón & G. Riousset, *T. himalayense* B.C. Zhang & Minter) but unlike the other North American species belonging to the same clade, *T. regimontanum* Guevara, Bonito & Rodríguez, which was collected in a *Quercus polymorpha* forest.

Additional specimen examined: USA. IOWA: Story County, McFarland Park, hypogeous in soil under pine litter under *Pinus strobus* (white pine) in the same small grove of planted trees as the holotype, 3 Jul 2009, coll. *R. Healy RH939* (FLAS-F-65572) (**paratype**). GenBank: ITS = MZ288692; 28S = MZ288691; *tef1* = JX022610; *rpb2* = JQ954523.

Comments: Tuber eburneum was previously identified by Bonito et al. (2010) as *Tuber* sp. 13. *Tuber eburneum* differs from other taxa in the Melanosporum clade in several respects. The ascoma of this species has a light-colored peridium that lacks verrucae. This is in stark contrast to all other described species in the Melanosporum clade, which have dark-colored peridia that are covered by conspicuous warts without hairs. *Tuber eburneum* also has a white to light buff gleba at maturity rather than the typical brown-black gleba at maturity that is more common for other species in the Melanosporum clade. The mature ascospores of *T. eburneum* are honey-yellow rather than darker shades of brown seen in other species in this clade. The presence of beaded peridial hairs in *Tuber eburneum* is also a unique identifying characteristic not seen in any other species in the Melanosporum clade.

Microscopically, *T. eburneum* differs conspicuously from its closest relatives in the Melanosporum clade, including *T. brumale, T. cryptobrumale* Merényi, Varga & Bratek, and *T. pseudoexcavatum* (Merényi et al. 2017; Wang et al. 2006). *Tuber eburneum* has only up to 5 (rarely 6) ascospores per ascus instead of the 1–8 spores per ascus seen in *T. pseudoexcavatum* and *T. brumale* and 1–7 seen in *T. cryptobrumale*. Spores of *T. eburneum* have spines that are not connected at the base, which is similar to the spore ornamentation seen in *T. brumale* and *T. cryptobrumale*

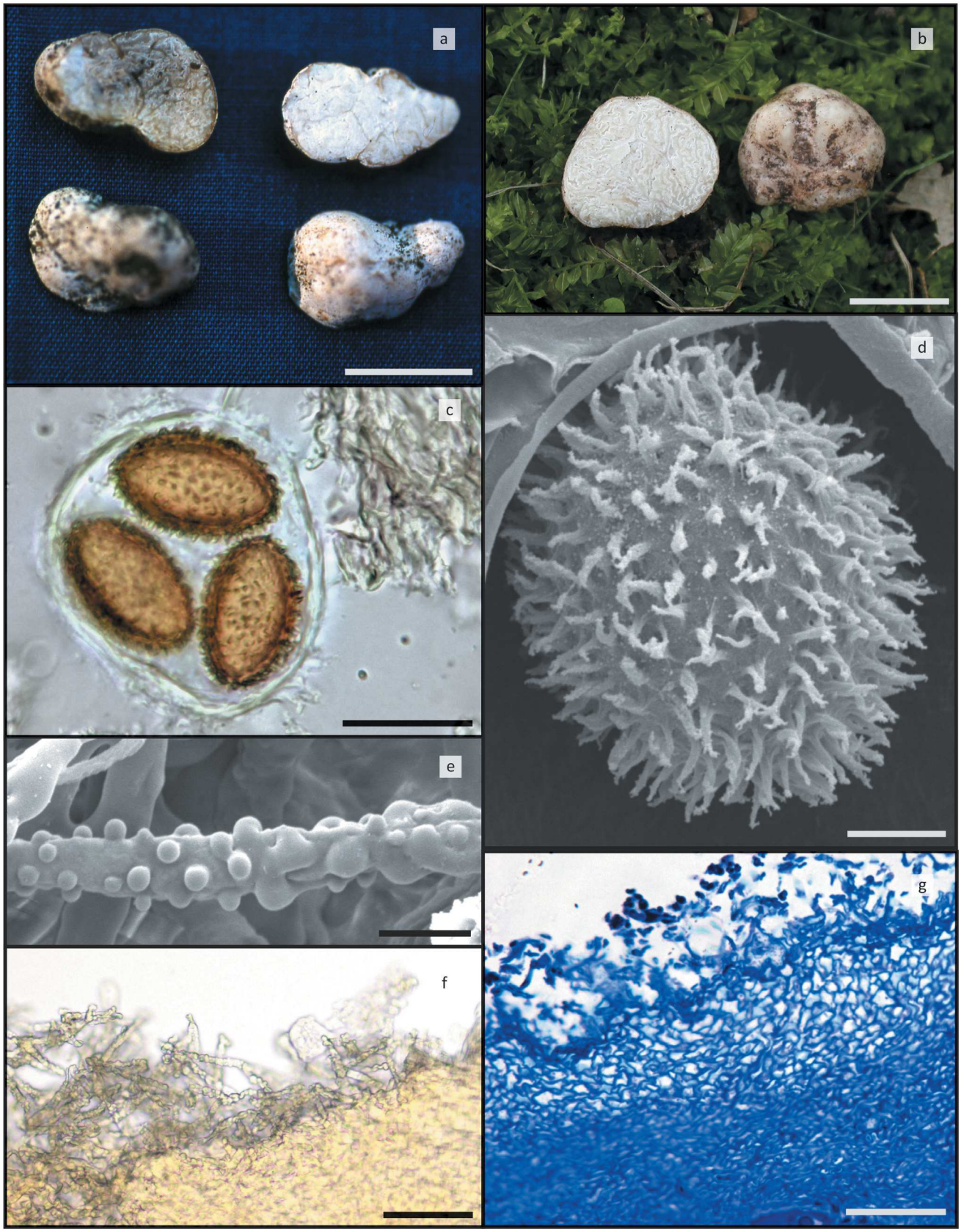

Figure 3. *Tuber eburneum*, sp. nov. a. Ascoma RH73 (FLAS-F-67910) (**holotype**). b. Ascoma RH939 (FLAS-F-65572) (**paratype**). c. Ascospores in ascus. d. SEM of ascospore showing spiny ornamentation. e. SEM of ornamented peridial hairs. f. Hair-like projections on peridium surface. g. Resin section stained in toluidine blue O, showing peridium in cross-section. Bars: a = 1 cm; b = 1 cm; c = 30 µm; d, e = 5 µm; f, g = 50 µm.

but different from those in *T. pseudoexcavatum* where the ornaments are connected at the spore surface and form a spiny-reticulate ornamentation. Connection between spines is a variable character in other Melanosporum clade species, and given the limited number of collections of *T. eburneum*, it is possible that this character is more variable than we have observed. The spore shape is more variable in *T. eburneum* than in *T. brumale* and *T. cryptobrumale*; some spores in *T. eburneum* range from nearly globose to elongate-ellipsoid (Q = 1–1.8), compared with more uniformly ellipsoid spores seen in *T. brumale* (Q = 1.4–1.8) and *T. cryptobrumale* (Q = 1.5–1.7). Geographically, *T. eburneum* is so far only the second species in the Melanosporum clade to be described from North America. The other North American species in the Melanosporum clade, *T. regimontanum*, resembles most other taxa in the clade, with a dark peridium covered by pyramidal warts and a gleba that becomes brown-black upon maturity.

Tuber mujicii Lemmond, Healy & M.E. Sm., sp. nov. FIG. 4
MycoBank MB839190

Typification: USA. FLORIDA: Putnam County, Ordway-Swisher Biological Station, near west entrance, Unit 4-A, in mixed forest beneath *Pinus palustris*, 25 Jan 2017, coll. *Alija Mujic AM-FL17-001* (FLAS-F-60274). GenBank: ITS = MF153060; 28S = MZ270564; *tef1* = OL989331; *rpb2* = OL989366.

Diagnosis: Tuber mujicii can be distinguished from other North American *Tuber* species in the Puberulum lineage by a combination of characters, including clay-colored to tawny-olive peridium, the lack of dermatocystidia, the 1–2 (rarely 3) ascospores per ascus, the generally subglobose as opposed to ellipsoid spores, the number of reticulations across the spore surface, and the ITS rDNA sequences.

Etymology: In honor of mycologist and truffle biologist Alija Mujic, collector of the holotype specimen.

Ascoma globose-ovoid and clay-colored to tawny olive, surface smooth to minutely verrucose, with occasional fissures on the peridium surface (FIG. 4). Fresh ascomata approximately 1.5 × 1.7 × 1.7 cm. Gleba solid and marbled drab with white veins of sterile tissue that emanate from multiple points along the interior of the excipulum. Aroma earthy, pleasant, and mild; flavor not recorded. Peridium 220–510(–700) µm thick, composed of two distinct but intergrading layers. Ectal excipulum a thin layer (30–140 µm) of variably shaped cells forming a structura globosa-angularis, most cells isodiametric to angular and (3–)5–10 µm in diameter, interspersed with larger elongate cells up to 22 × 7 µm. Walls of outermost cells lightly pigmented cinnamon-buff, becoming hyaline toward medullary excipulum. Medullary excipulum intergrading with ectal excipulum, 180–375(–610) µm, composed of loosely packed hyaline hyphae forming a textura intricata, cells 3–5 µm wide at the septa. Tramal hyphae hyaline, interwoven hyphae forming a textura intricata, cells 3–7 µm wide at the septa. Asci globose to ellipsoid and mature asci astipitate and with 1–2(–3) spores per ascus, 1-spored asci 78–119 × 44–88 µm, 2-spored asci 78–112 × 44–76 µm, 3-spored asci uncommon and 82–95 × 65–93 µm. Spores cinnamon-buff to clay color, ellipsoid to subglobose, with reticulate alveolate ornamentation, alveolar walls 1–5 µm tall. Ascospores in 1-spored asci 44–63 × 36–58 µm, Q = 1.0–1.5 (mean 1.2 ± 0.1); ascospores in 2-spored asci 32–58 × 28–46 µm, Q = 1.0–1.4 (mean 1.1 ± 0.1); ascospores in 3-spored asci 30–44 × 27–42 µm, Q = 1–1.2 (mean 1.2 ± 0.1). Spore ornamentation alveolate with 5–7 alveolar meshes along the length and 3–7 across the width of spores in 1-spored asci, 2–7 per spore length and 2–6 per spore width in 2-spored asci, and 3–7 per spore length and 3–6 per spore width in 3-spored asci.

Ecology and distribution: Tuber mujicii has been identified primarily in association with pines (*Pinus* spp.) from sites across the southeastern USA, although association with *Quercus* and other angiosperm hosts cannot be ruled out. Records include an ascocarp collection from Florida in a pine-dominated forest as well as a root tip sequence from *P. strobus* seedlings in Georgia (KC424538) (Cowden and Peterson 2013) and an additional unidentified root tip sequence putatively from *Pinus* seedlings from an unpublished study in North Carolina (KF476959). An immature ascocarp was collected in Georgia in a mixed forest habitat with low pine density.

Comments: Tuber mujicii was previously identified by Bonito et al. (2010) as *Tuber* sp. 17. *Tuber mujicii* generally conforms to the morphological characteristics of truffles in the Puberulum lineage as summarized by Lancellotti et al. (2016), particularly the small ascomata size and smooth texture, pseudoparenchymatous excipulum (peridium), globose and astipitate asci, and alveolate-reticulate ascospores. However, the reddish-brown ascomata color, the lack of dermatocystidia, the 1–2 (rarely 3) spores per ascus, and the generally subglobose spores collectively help to differentiate this species from other North American taxa in the Puberulum lineage.

Tuber mujicii can be distinguished from other phylogenetically affiliated North American *Tuber* species by a number of morphological features. The clay-colored to

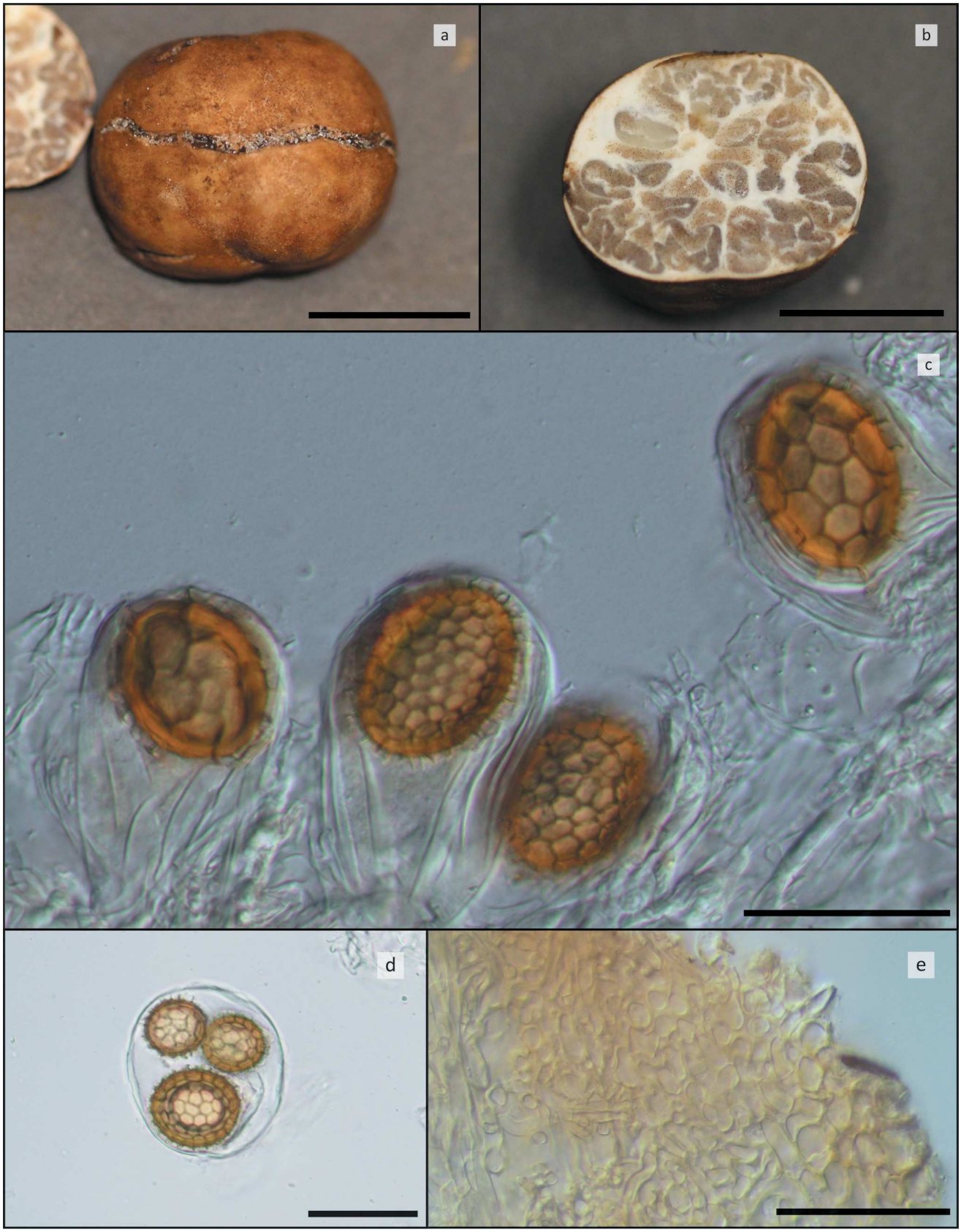

Figure 4. *Tuber mujicii*, sp. nov. a. Ascoma. b. Cross-section of fresh ascoma. c. Ascospores in asci, note variability of reticulations. d. Ascospores in ascus. e. Ectal excipulum of rounded to angular and occasional elongate cells. All images of FLAS-F-60274 (**holotype**). Bars: a, b = 1 cm; c–e = 50 µm.

tawny-olive ascomata of *T. mujicii* differs from the light-colored *T. pseudoseparans* Guevara, Bonito & Trappe and *T. tequilianum* Guevara, Bonito & Trappe (which are described as white, cream, gray, or light brown), as well as from *T. bonitoi* Guevara & Trappe (yellow with pink dots) and *T. separans* Gilkey (light yellow brown, although Gilkey (1954) describes the color as "earthy-brown") (Guevara-Guerrero et al. 2015). Only *T. brunneum* Guevara, Bonito & Trappe is described as brown, yet not reddish brown, and is similar to *T. mujicii* in having furrows or depressions in the ascoma surface. *Tuber separans* and other related species from North America are all smooth or minutely verrucose on the peridium surface. However, *T. bonitoi, T. brunneum, T. tequilianum*, and *T. pseudoseparans* possess dermatocystidia, whereas *T. mujicii* lacks dermatocystidia.

Tuber mujicii is similar in the number of spores per ascus to *T. separans* and *T. bonitoi*; all three of these species have 1–3 or rarely 4 spores per ascus, whereas the others in this group are described as having up to 4 and rarely 5 spores per ascus. The spore ornamentation found in *T. mujicii* is 1–5 µm high, and this is comparable to what is described from other North American species in the Puberulum lineage as discussed in Guevara-Guerrero et al. (2015). The shape of spores in *T. mujicii*, which ranges from globose to ellipsoid (Q = 1–1.5), is similar to *T. bonitoi* (Q = 1–1.5) and *T. brunneum* (Q = 1–1.5). Other species in this group have spores that are often more broadly ellipsoid, including *T. tequilianum* (Q = 1–1.78) and *T. pseudoseparans* (Q = 1.05–2). Spores of *T. mujicii* are larger than *T. separans, T. bonitoi*, and *T. brunneum*. Additionally, spores in *T. mujicii* have slightly fewer reticulations across the length and width of spores (5–9 across the length and 3–7 across the width) than what has been documented for *T. separans* (3–10 across the length and 7–11 across the width) (Gilkey 1954).

DISCUSSION

Tuber eburneum and *T. mujicii* both associate with Pinaceae hosts, despite belonging to distantly related clades within the genus *Tuber*. Ectomycorrhizal association with Pinaceae hosts is hypothesized to be a derived trait that arose independently in multiple lineages of *Tuber* (Bonito et al. 2013), although the association is observed with varying degrees of specificity. Some species such as *T. gibbosum* are only known to associate with Pinaceae hosts, whereas others such as *T. borchii* can associate with both angiosperm and Pinaceae hosts (Bonito et al. 2013). Both *T. eburneum* and *T. mujicii* have been found in habitats dominated by pines, and a sequence of *T. mujicii* was recovered from an ectomycorrhizal root tip of *Pinus strobus* (FIG. 2). However, we cannot rule out the possibility that one or both species may also form ectomycorrhizal associations with angiosperm hosts. In both cases, more collections and root tip sequences could help establish the host range of these two *Tuber* species.

Tuber eburneum is strikingly different from other species in the Melanosporum lineage, at least in the broad macroscopic features such as aroma, gleba color, and the peridium color and ornamentation. Many *Tuber* species in the Melanosporum lineage are dark in color and have strong odors at maturity, whereas *T. eburneum* is light in color and has an odor described as "faint and pleasant … reminiscent of baked potato." *Tuber eburneum* also has a 157-bp indel in the ITS1 region, which is characteristic of many other species in the Melanosporum lineage (Merényi et al. 2016). However, the lack of other species in this lineage with intermediate characteristics is noteworthy and poses questions about how such drastically different morphological and organoleptic traits came to be present in all other species in the clade.

By contrast, *T. mujicii* reflects the typical characteristics of many other taxa in the Puberulum clade, although both phylogenetic analysis and comparisons of morphological features clearly support species-level differentiation between *T. mujicii* and its closest relatives. Phylogenetic analysis of this species shows that there are many undescribed species closely related to *T. mujicii*, including several known only from collections of mitotic spore mats and ECM root tip sequences. Using a clustering method of 96% ITS sequence similarity (Bonito et al. 2010), we delineate six ITS phylotypes of *Tuber* from eastern North America in our data set that likely correspond to undescribed species (*Tuber* species BL-1 to BL-6; FIG. 2a, b, SUPPLEMENTARY INFORMATION 3). Since the 96% clustering threshold does not separate other described species included in our ITS analysis (e.g., *T. bonitoi* and *T. tequilianum*), this is likely a conservative estimate. These undescribed *Tuber* species are mostly known from recent collections of ascoma and anamorphs from Tennessee, Georgia, and North Carolina. These data indicate that there are many undescribed species of *Tuber* from the eastern USA.

It is also apparent that some specimens identified as *T. separans* belong to phylogenetically distinct groups. Of the two clades containing sequences from putative *T. separans* ascocarps, one is from Oregon in the northwestern USA and the other is from Tlaxcala in central Mexico. Neither clade contains a specimen close to the San Francisco Bay area in California where the type specimen was collected by Harkness and later described in 1916 by Gilkey (Gilkey 1939). Additional collections of *T. separans* from nearby the type locality would be the

best way to clarify the phylogenetic position and geographic distribution of *T. separans*.

Unlike some clades of *Tuber* that are present only on a single continent (e.g., Gibbosum clade in North America or Japonicum clade in Asia), the Melanosporum and Puberulum clades are distributed throughout North America, Europe, and Asia. The phylogenetic position of *T. mujicii* nested among other *Tuber* species from eastern North America adds to the known diversity of *Tuber* in this region, but it does not challenge any assumptions about evolutionary patterns in this group. However, the early-diverging position of *T. eburneum* with sister lineages reported from Eurasia and North America suggests a possible dispersal event from the North American continent to Eurasia and subsequent species radiation in Asia and Europe (Bonito et al. 2013), with at least one subsequent dispersal event back to North America in the *T. regimontanum* lineage. *Tuber himalayense* (reported as *T. indicum*) has been detected in North America but is presumed to be an accidental introduction (Bonito et al. 2011). Alternatively, it is also possible that other inconspicuous, early-diverging members of the Melanosporum lineage from Europe and Asia remain undetected and undescribed.

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DISCLOSURE STATEMENT

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LITERATURE CITED

Bonito GM, Gryganskyi AP, Trappe JM, Vilgalys R. 2010. A global meta-analysis of *Tuber* ITS rDNA sequences: species diversity, host associations and long-distance dispersal. Mol Ecol. 19:4994–5008.

Bonito G, Smith ME, Nowak M, Healy RA, Guevara G, Cázares E, Kinoshita A, Nouhra ER, Domínguez LS, Tedersoo L, et al. 2013. Historical biogeography and diversification of truffles in the Tuberaceae and their newly identified Southern Hemisphere sister lineage. PLOS ONE. 8: e52765.

Bonito GM, Trappe JM, Donovan S, Vilgalys R. 2011. The Asian black truffle *Tuber indicum* can form ectomycorrhizas with North American host plants and complete its life cycle in non-native soils. Fungal Ecol. 4:83–93.

Cowden CC, Peterson CJ. 2013. Annual and seasonal dynamics of ectomycorrhizal fungi colonizing white pine *(Pinus strobus)* seedlings following catastrophic windthrow in northern Georgia, USA. Can J For Res. 43:215–23.

Curry KJ, Kimbrough JW. 1983. Septal structures in apothecial tissues of the Pezizaceae (Pezizales, Ascomycetes). Mycologia. 75:781–94.

Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32:1792–97.

Edler D, Klein J, Antonelli A, Silvestro D. 2021. raxmlGUI 2.0: a graphical interface and toolkit for phylogenetic analyses using RAxML. Methods Ecol Evol. 12:373–77.

Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. Mol Ecol. 2:113–18.

Gilkey HM. 1939. Tuberales of North America. Oregon State Monographs: Studies in Botany. 1:1–63.

Gilkey HM. 1954. Tuberales. North Am Flora. 2:1–29.

Glenn TC, Schable N. 2005. Isolating Microsatellite DNA Loci. Methods Enzymol. 395:202–22.

Guevara-Guerrero G, Bonito G, Cázares-González E, Healy R, Vilgalys R, Trappe J. 2015. Novel *Tuber* spp. (Tuberaceae, Pezizales) in the Puberulum group from Mexico. AscomyceteOrg. 7:367–74.

Healy RA. 2003. *Mattirolomyces tiffanyae*, a new truffle from Iowa, with ultrastructural evidence for its classification in the Pezizaceae. Mycologia. 95:765–72.

Healy RA, Smith ME, Bonito GM, Pfister DH, Ge ZW, Guevara GG, Williams G, Stafford K, Kumar L, Lee T,

et al. 2013. High diversity and widespread occurrence of mitotic spore mats in ectomycorrhizal Pezizales. Mol Ecol. 22:1717–32.

Healy RA, Zurier H, Bonito G, Smith ME, Pfister DH. 2016. Mycorrhizal detection of native and non-native truffles in a historic arboretum and the discovery of a new North American species, *Tuber arnoldianum* sp. nov. Mycorrhiza. 26:781–92.

Hopple JS, Vilgalys R. 1994. Phylogenetic relationships among coprinoid taxa and allies based on data from restriction site mapping of nuclear rDNA. Mycologia. 86:96–107.

Jacquemyn H, Waud M, Lievens B, Brys R. 2016. Differences in mycorrhizal communities between *Epipactis palustris*, *E. helleborine* and its presumed sister species *E. neerlandica*. Ann Bot. 118:105–14.

Kinoshita A, Nara K, Sasaki H, Feng B, Obase K, Yang ZL, Yamanaka T. 2018. Using mating-type loci to improve taxonomy of the *Tuber indicum* complex, and discovery of a new species, *T. longispinosum*. PLoS ONE. 13:e0193745.

Kinoshita A, Sasaki H, Nara K. 2011. Phylogeny and diversity of Japanese truffles (*Tuber* spp.) inferred from sequences of four nuclear loci. Mycologia. 103:779–94.

Lancellotti E, Iotti M, Zambonelli A, Franceschini A. 2014. Characterization of *Tuber borchii* and *Arbutus unedo* mycorrhizas. Mycorrhiza. 24:481–86.

Lancellotti E, Iotti M, Zambonelli A, Franceschini A. 2016. The Puberulum group sensu lato (whitish truffles). In: Zambonelli A, Iotti M, Murat C, editors. True truffle (*Tuber* spp.) in the world: soil ecology, systematics and biochemistry. Switzerland: Springer International Publishing; p. 105–22.

Lanfear R, Calcott B, Ho SYW, Guindon S. 2012. PartitionFinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. Mol Biol Evol. 29:1695–701.

Lanfear R, Frandsen PB, Wright AM, Senfeld T, Calcott B. 2017. PartitionFinder 2: new methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. Mol Biol Evol. 34:772–73.

Liu YJ, Whelen S, Hall BD. 1999. Phylogenetic relationships among ascomycetes: evidence from an RNA polymerase II subunit. Mol Biol Evol. 16(12):1799–808.

Merényi Z, Varga T, Bratek Z. 2016. *Tuber brumale*: the most controversial *Tuber* species. In: Zambonelli A, Iotti M, Murat C, editors. True truffles (*Tuber* spp.) in the world: soil ecology, systematics, and biochemistry. Switzerland: Springer International Publishing; p. 49–68.

Merényi Z, Varga T, Hubai AG, Pitlik P, Erős Á, Trappe JM, Bratek Z. 2017. Challenges in the delimitation of morphologically similar species: a case study of *Tuber brumale* agg. (Ascomycota, Pezizales). Mycol Prog. 16:613–24.

Miller M, Pfeiffer W, Schwartz T. 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In: Proceedings of the Gateway Computing Environments Workshop (GCE), New Orleans, LA. p. 1–8.

Ori F, Trappe J, Leonardi M, Iotti M, Pacioni G. 2018. Crested porcupines (*Hystrix cristata*): mycophagist spore dispersers of the ectomycorrhizal truffle *Tuber aestivum*. Mycorrhiza. 28:561–65.

Páez CP, Bonito GM, Guevara-Guerrero G, Castellano MA, Garibay-Orijel R, Trappe JM, Rámirez RP. 2018. Description and distribution of *Tuber incognitum* sp. nov. and *Tuber anniae* in the Transmexican Volcanic Belt. MycoKeys. 41:17–27.

Rambaut A. 2018. Fig Tree v. 1(4):4.

Rambaut A, Drummond AJ, Xie D, Baele G, Suchard MA. 2018. Posterior summarization in bayesian phylogenetics using tracer 1.7. Syst Biol. 67:901–04.

Ridgway R. 1912. Color standards and color nomenclature. Washington (DC): Published by the author; 43 p, 53 pl.

Talavera G, Castresana J. 2007. Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. Syst Biol. 56:564–77.

Trappe JM, Molina R, Luoma DL, Cázares E, Pilz D, Smith JE, Castellano MA, Miller SL, Trappe MJ. 2009. Diversity, ecology, and conservation of truffle fungi in forests of the Pacific Northwest. USDA Forest Service, Pacific Northwest Research Station. Report No.: PNW-GTR-722.

Vandepol N, Liber J, Desirò A, Na H, Kennedy M, Barry K, Grigoriev IV, Miller AN, O'Donnell K, Stajich JE, et al. 2020. Resolving the Mortierellaceae phylogeny through synthesis of multi-gene phylogenetics and phylogenomics. Fungal Divers. 104:267–89.

Wang Y, Tan ZM, Zhang DC, Murat C, Jeandroz S, Le Tacon F. 2006. Phylogenetic relationships between *Tuber pseudoexcavatum,* a Chinese truffle, and other *Tuber* species based on parsimony and distance analysis of four different gene sequences. FEMS Microbiol Lett. 259:269–81.

White BT, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA Genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ, editors. PCR Protocols: a Guide to Methods and Applications. San Diego. p. 315–22.