

## New North American truffles (*Tuber* spp.) and their ectomycorrhizal associations

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**Abstract:** Recent surveys of belowground fungal biodiversity in México and USA have revealed many undescribed truffle species, including many in the genus *Tuber*. Here we describe seven new species: *Tuber beyerlei*, *T. castilloi*, *T. guevarai*, *T. lauryi*, *T. mexiusanum*, *T. miquihuanense* and *T. walkeri*. Phylogenetic analyses place these species within the Maculatum group, an understudied clade of small truffles with little apparent economic value. These species are among the more taxonomically challenging in the genus. We collected *Tuber castilloi*, *T. mexiusanum* and *T. guevarai* as fruit bodies and ectomycorrhizae on *Quercus* spp. in forests of eastern México. *Tuber mexiusanum* has a particularly broad geographic range, being collected in eastern USA under *Populus deltoides* and in Minnesota and Iowa in mixed hardwood forests. *T. walkeri* is described from the upper midwestern USA, and *T. lauryi* and *T. beyerlei* occur in the western USA.

**Key words:** Ascomycota, ectomycorrhizae, hypogeous fungi, Pezizales, Pezizomycetes, phylogeny

### INTRODUCTION

Truffle species in the genus *Tuber* have considerable ecological and economic importance (Mello et al. 2006). They form mycorrhizal associations with both gymnosperm and angiosperm woody plants as well as some orchids, including *Epipactis*, and are involved in helping to acquire nutrition for their hosts (Wurzburger et al. 2001, Bidartondo et al. 2004, Walker et al. 2005). Truffles contribute to the diet of mammals, insects and slugs, which in turn aid in spore dispersal (McGraw et al. 2002, Hochberg et al. 2003, Maser et al. 2008). Humans also consume and market truffles, and a few *Tuber* species have been brought into cultivation (e.g. *T. melanosporum*, *T. aestivum*, *T. borchii*) and are now the basis of a multimillion dollar industry worldwide (Palenzona et al. 1972, Mello et al. 2006, Paolocci et al. 2006).

To date, 38 species of *Tuber* have been described from North America (TABLE I). Of these, 27 are described from the United States and 11 from México (Trappe and Guzmán 1971, Cázares et al. 1992, Trappe et al. 1996, Trappe and Cázares 2006, Guevara et al. 2008). No new *Tuber* species have been described from Canada thus far, although several species occur there. Historically European names have been applied to many North American *Tuber* spp. (Harkness 1899, Gilkey 1954, Trappe and Cázares 2000), but the validity remains questionable in many cases (Bonito and Trappe unpubl). Recent molecular studies of *Tuber* spp. in USA and northern and central México indicated that most North American *Tuber* species are diverse and genetically distinct from their European counterparts (Guevara et al. 2008, Bonito et al. 2010). For instance, Bonito et al. (2010) identified 12 putatively undescribed *Tuber* species that could be ascribed to the Maculatum clade, seven of which were represented only by mycorrhizae or soil clone sequence data.

Here we describe seven new *Tuber* species that belong phylogenetically within the Maculatum clade, a group of small truffles that has challenged taxonomists for centuries. *Tuber castilloi*, *T. miquihuanense* and *T. guevarai* are described from eastern México, *T. mexiusanum* is described from México to eastern and upper midwestern USA, *T. beyerlei* from Oregon and Idaho, *T. lauryi* from western Oregon and *T. walkeri* from upper midwestern USA. These new species are differentiated from related taxa by morphology, biogeography and variation in the internal transcribed spacer (ITS) region of rDNA.

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TABLE I. Thirty-eight *Tuber* species described from North America. Species belonging to the Maculatum clade are indicated with an asterisk

<i>T. anniae</i> Colgan & Trappe <sup>a, p</sup>
<i>T. argenteum</i> Gilkey <sup>b, h, p</sup>
<i>T. bellisporum</i> Bonito & Trappe <sup>c</sup>
<i>T. besseyi</i> Gilkey <sup>d, p</sup>
* <i>T. beyerlei</i> Bonito, Trappe & Guevara <sup>c</sup>
<i>T. californicum</i> Harkness <sup>f, p</sup>
<i>T. canaliculatum</i> Gilkey <sup>g, p</sup>
<i>T. candidum</i> Harkness <sup>f, o, p</sup>
<i>T. castellanoi</i> Bonito & Trappe <sup>c, p</sup>
* <i>T. castilloi</i> Guevara, Bonito & Trappe <sup>c</sup>
<i>T. citrinum</i> Harkness <sup>f, p</sup>
<i>T. gardneri</i> Gilkey <sup>h, p</sup>
<i>T. gibbosum</i> Harkness = ( <i>T. giganteum</i> Gilkey) <sup>f, p</sup>
* <i>T. guevarai</i> Bonito & Trappe <sup>c</sup>
<i>T. guzmanii</i> Trappe & Cázares <sup>i, p</sup>
<i>T. harknessii</i> Gilkey <sup>h, p</sup>
<i>T. irradians</i> Gilkey <sup>h, p</sup>
* <i>T. lauryi</i> Bonito, Trappe & Guevara <sup>c</sup>
<i>T. levissimum</i> Gilkey <sup>b, p</sup>
* <i>T. linsdalei</i> Gilkey <sup>h, p</sup>
<i>T. longisporum</i> Gilkey <sup>j, p</sup>
<i>T. luomai ad int.</i> Trappe <sup>k, p</sup>
<i>T. lyonii</i> Butters = ( <i>T. texense</i> ) <sup>l, p, s</sup>
* <i>T. mexicanum</i> Guevara, Bonito & Cázares <sup>c</sup>
* <i>T. miquihuanense</i> Guevara, Bonito & Cázares <sup>c</sup>
<i>T. monticola</i> Harkness <sup>f</sup>
<i>T. oregonense</i> Trappe & Bonito <sup>c</sup>
<i>T. pacificum</i> Trappe & Castellano <sup>o</sup>
<i>T. phleboderma</i> (Gilkey) Trappe comb. nov. <sup>k</sup>
<i>T. quercicola</i> Frank, Southworth, & Trappe <sup>o</sup>
<i>T. regimontanum</i> Guevara, Bonito, et Rodriguez <sup>q</sup>
<i>T. separans</i> Gilkey <sup>b, s</sup>
* <i>T. shearii</i> Harkness <sup>g</sup>
<i>T. sphaerosporum</i> Gilkey <sup>h, p</sup>
<i>T. spinoreticulatum</i> Uecker & Burdsall <sup>r, p</sup>
<i>T. unicolor</i> Gilkey <sup>g</sup>
* <i>T. walkerii</i> Healy, Bonito & Guevara <sup>c</sup>
* <i>T. whetstonense</i> Frank, Southworth, & Trappe <sup>o</sup>

<sup>a</sup> *Mycotaxon* 64:438 (1997).<sup>b</sup> *University of Calif. Publ. Bot.* 6:318 (1916).<sup>c</sup> *Mycologia*, 102, 2010.<sup>d</sup> *Ore. St. Monog., Bot.* 1:40 (1939).<sup>e</sup> This paper.<sup>f</sup> *Proc. Calif. Acad. Sci., Ser. 3, Bot.* 1:274 (1899).<sup>g</sup> *Mycologia* 12:99 (1920).<sup>h</sup> *Index of Fungi*.<sup>i</sup> *Int. Jour. Med. Mush.* 8:279 (2006).<sup>j</sup> *Mycologia* 17:251 (1925).<sup>k</sup> In preparation.<sup>l</sup> *Mycotaxon* 60:365 (1996).<sup>m</sup> *Monogr. Tuberc.* (Milano): 45 (1831).<sup>n</sup> *Mycotaxon* 75:176 (2000).<sup>o</sup> *Mycotaxon* 95:229 (2006).<sup>p</sup> *Act. Bot. Yunn.* 56:39 (2009).<sup>q</sup> *Rev. Mex. Micol.* 26: (2008).

TABLE I. Continued

<sup>r</sup> *Mycologia* 69:626 (1977).<sup>s</sup> *Mycologia* 84:345 (1992).<sup>t</sup> *Mycologia* 63:320 (1971).

Further, an ectomycorrhizal association with angiosperms was confirmed by molecular techniques for four of these new species.

## MATERIALS AND METHODS

*Sample collection.*—Over the past decade we studied more than 100 *Tuber* ascomata from northeastern México and various locations in the United States (SUPPLEMENTARY TABLE I, TABLE II). Specific localities have been itemized under *Specimens examined*. Specimens were preserved following recommendations of Castellano et al. (1989), and when possible duplicate splits have been deposited in the herbaria José Castillo Tovar (ITCV), Oregon State University (OSC), Duke University (DUK), Iowa State University (ISC) and University of Minnesota (MIN). Previously accessioned herbarium specimens of *Tuber*, including type collections from OSC and ITCV, also were examined during this study (SUPPLEMENTARY TABLE I).

*Morphological observations.*—Morphological data were obtained by the methods of Castellano et al. (1989), Gilkey (1939) and Pegler et al. (1993). Examined characters included ascoma size, surface texture and color, peridial structure, spore length and width (excluding ornamentation), length/width ratio (Q), shape, wall thickness, number of reticular meshes along and across spore axes, height of reticular walls, color, ascus size, shape, wall thickness and number of spores/ascus. Hand-cut sections were mounted in 5% KOH and Melzer's reagent for light microscopy. Spore measurements of *Tuber* spp. in KOH compared to those in water showed no KOH effect (J. Trappe unpubl). Microscopic structures were measured and photographed under a compound microscope and stereomicroscope. For scanning electron microscopy, specimens were fixed in 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, postfixed in 1% OsO<sub>4</sub>, then dehydrated to absolute ethanol, critical point dried, mounted on sticky tape on a stub, and sputter-coated with gold and palladium. Spores were digitally imaged with 10 kV in a JEOL 5800LV SEM in the Microscopy and Nanoimaging Facility at Iowa State University.

*DNA sequencing and phylogenetic analyses.*—Molecular protocols follow those of Guevara et al. (2008). Briefly, DNA was extracted from sporocarps and mycorrhizae by the chloroform extraction technique using CTAB 2× buffer, and the ITS region was amplified with the primer set ITS1f-ITS4 (White et al. 1990, Gardes and Bruns 1993). The 28S large subunit rDNA was amplified with the primer set LROR and LR5 (Vilgalys and Hester 1990). PCR products were cleaned enzymatically with antarctic phosphatase and endonuclease (New England Biolabs, Ipswich, Massachusetts). Sanger sequencing was performed by Big Dye chemistry 3.1 (Applied Biosystems, Foster City, California)

TABLE II. Comparative morphology of the *Tuber* species described in this study

Species, holotype collection number	Peridium surface	Peridium color and thickness	Pellis and cell size	Subpellis	Dermatocystidia	Spore size without reticulation	Spore shape	Number spore/asci	Geography of the holotype
<i>T. beyerlei</i> Trappe 32597 (OSU)	Finely verrucose	Light brown 200–350 µm	Pseudo. 7–30 µm	Interwoven	Present	20–47 × 19–40 µm	Subglobose to broadly ellipsoid	1–3(–4)	Oregon, USA
<i>T. castilloi</i> ECG 149 (ITCV)	Verrucose	Cream 80–150 µm	Pseudo. 5–38 µm	Interwoven	Present	27–63 × 20–40 µm	Broadly ellipsoid	1–4	NL. Mx.
<i>T. guevarai</i> GG180 (ITCV)	Finely verrucose	Cream to light brown 160–220 µm	Pseudo. 4–30 µm	Interwoven	Present	18–55 × 16–42 µm	Subglobose to broadly ellipsoid	1–4(–5)	Tam. Mx.
<i>T. lauryi</i> Trappe 19425 (OSC)	Smooth to finely verrucose	Light brown 300–1000 µm	Pseudo 4–24 µm	Interwoven	Present	22–50 × 20–41 µm	Subglobose to broadly ellipsoid	1–2(–3)	Oregon, USA
<i>T. mexiusanum</i> GG 181 (ITCV)	Verrucose	Cream to light brown 130–350 µm	Pseudo 7–38 µm	Interwoven	Present	15–50 × 12–36 µm	Subglobose to broadly ellipsoid	1–4(–5)	Tam. Mx.
<i>T. miquihuanense</i> GG885 (ITCV)	Flat to ± pyramidal or angular verrucae	Light to reddish brown 110–360 µm	Pseudo 5–24 µm	Interwoven	Absent	20–50 × 20–39 µm	Subglobose to broadly ellipsoid	1–4(–5)	Tam. Mx.
<i>T. walker</i> RH521 (OSC)	Smooth to finely verrucose	Ocher brown 150–600 µm	Pseudo 4–38 µm	Interwoven	Present	18–53 × 15–45 µm	Globose to ellipsoid	1–4(–5)	Iowa, USA

with the forward primer ITS5 or LROR and reverse primers ITS4 or LR5. DNA sequences were determined on an ABI 3700 capillary sequencer (Applied Biosystems, Foster City California). DNA sequences were viewed and manually edited in Sequencher 4.0 (Gene Codes Corp., Ann Arbor, Michigan). Sequences were queried against GenBank with the BLASTN algorithm to verify that sequences belonged to *Tuber*. Sequences were aligned with MUSCLE (Edgar 2004). Alignments were manually checked and ambiguous regions were excluded in Mesquite 2.5 (Maddison and Maddison 2009).

Phylogenetic analyses were conducted with maximum likelihood (ML) in PAUP\* (Swofford 2002) and Bayesian inference (BI) with MrBayes (Huelsenbeck and Ronquist 2001). The best fit nucleotide substitution model was based on the Akaike information criterion and was implemented in PAUP\* 4d106 (Swofford 2002). ML bootstrap support based on 1000 replicates was assessed with RAxML (Stamatakis et al. 2008) and BI analyses were run through the CIPRES Web portal (<http://www.phylo.org/>). BI was based on parallel runs of 20 000 000 generations with four chains, sampling every 1000 generations.

Intraspecific and interspecific ITS variation was determined by aligning sequences from the species of interest to their closest sister taxa in MUSCLE (Edgar 2004). The alignments were edited manually, but no regions were excluded. Uncorrected *P* values resulting from these ITS alignments were calculated in PAUP\* 4d106 (Swofford 2002). Sequences produced in this study are deposited in GenBank under accession numbers JF419239–JF419315.

*Ectomycorrhiza* surveys.—Ectomycorrhizae of Mexican *Quercus*-dominated forests were surveyed in Victoria (El Madroño, Las Mulas), Miquihua (La Joya), Gómez Farías (San José), Tamaulipas, Monterrey (Chipinque, La Estanzuela, El Barro), Santiago (El Manzano), Higuera (Sierra de Picachos) and Nuevo León, México, in Jul 2008. Woody species in these stands included: *Quercus canbyi*, *Q. polymorpha*, *Q. rugosa*, *Q. rysophylla*, *Q. laceyi*, *Q. greggii*, *Q. miquihuanense*, *Q. clivicola*, *Q. virginiana*, *Q. sartorii*, *Arbutus xalapensis*, *Prunus* sp. and *Juglans* sp.

To sample ectomycorrhizae in the field, roots from *Quercus* were traced from the base of the trunk with a hand garden cultivator until attached clusters of fine roots were visible. Root clusters were sampled from 5–10 trees per site for a total of 70 trees. In some cases, truffles were unearthed, collected and sequenced. Mycorrhizae were soaked 1 h and washed under tap water in a 1 mm sieve on the day of sampling. Eight to 10 mycorrhizal tips were selected per tree, aiming to maximize morphological diversity and not favor any particular morphology. Root tips were preserved individually in CTAB 2× until DNA extraction. A total of 630 individual root tips from all sites were preserved. *Tuber* sequences detected and belonging to the Maculatum clade are reported below.

*Tuber* ectomycorrhizae on *Populus deltoides* were produced in growth chambers in the Duke phytotron. We used field soils collected from *Populus*-dominated forests along the Caney Fork River, Tennessee, as inoculum (Gottel et al. 2011). Cuttings of *Populus* genotype *deltoides* D124 were

surface sterilized by soaking in 3% hydrogen peroxide 10 min. After thorough rinsing, cuttings were soaked in water 3 d and planted in a 50% mixture of field soils and sterile sand. The plants were grown under 870  $\mu\text{mol photons/m}^2 \text{ s}$  of light, with dew point temp of 13 C and relative humidity of 60% (day) and 85% (night). After 5 mo, plants were harvested and their roots washed and inspected for ectomycorrhizae. Single ectomycorrhizal tips were collected for DNA extraction and sequenced (as above). Sequences recovered belonging to *Tuber mexicanum* are reported below.

## RESULTS

*Morphological analyses.*—Truffle species studied here ranged from cream to reddish brown, and all had a pseudoparenchymous pellis and a subpellis of interwoven hyphae. All species except *Tuber miquihuanense* had dermatocystidia on the peridial surface. Ascospores of all species had alveolate reticulation and ranged from globose to ellipsoid as in *T. walkeri* to broadly ellipsoid as in *T. castilloi*. *Tuber castilloi* had the largest spores, *T. beyerlei* the smallest.

*Molecular analyses.*—Because incompletely overlapping sets of taxa were represented in the ITS and LSU datasets, these two loci were analyzed separately. The ITS data matrix consisted of 81 taxa and 430 included characters: 155 parsimony informative, 255 constant and 20 parsimony non-informative. The HKY85+G model of nucleotide substitution was most appropriate for our ITS dataset and a GTR+G+I model for the LSU dataset. The LSU data matrix included 42 taxa and 541 characters: 105 parsimony informative, 414 constant, 22 parsimony non-informative. The ITS and LSU phylogenies are congruent at every supported node, and the seven species described here are supported by high bootstrap values for both loci (FIGS. 1–2). The Maculatum clade consists of two subclades with species distributed widely across North America and Europe (FIGS. 1–2). Sequences analyzed here indicate that species in this clade predominantly form mycorrhizal associations with species of angiosperm hosts belonging to *Quercus*, *Carya*, *Salix*, *Populus*, *Notholithocarpus* and *Epipactis*, with two exceptions being *T. beyerlei* and *T. lauryi*, which have been collected in pure *Pseudotsuga menziesii* forests.

*Ectomycorrhiza field survey in México.*—The genus *Tuber* was found in ectomycorrhizal tissue from 24 oaks and at every oak site we sampled in México. Grouping these ITS sequences at 97% similarity resulted in nine distinct operational taxonomic units (OTUs). Four *Tuber* OTU's that were detected on *Quercus* roots were in the Maculatum clade. ITS sequences of three of these OTUs were quite similar

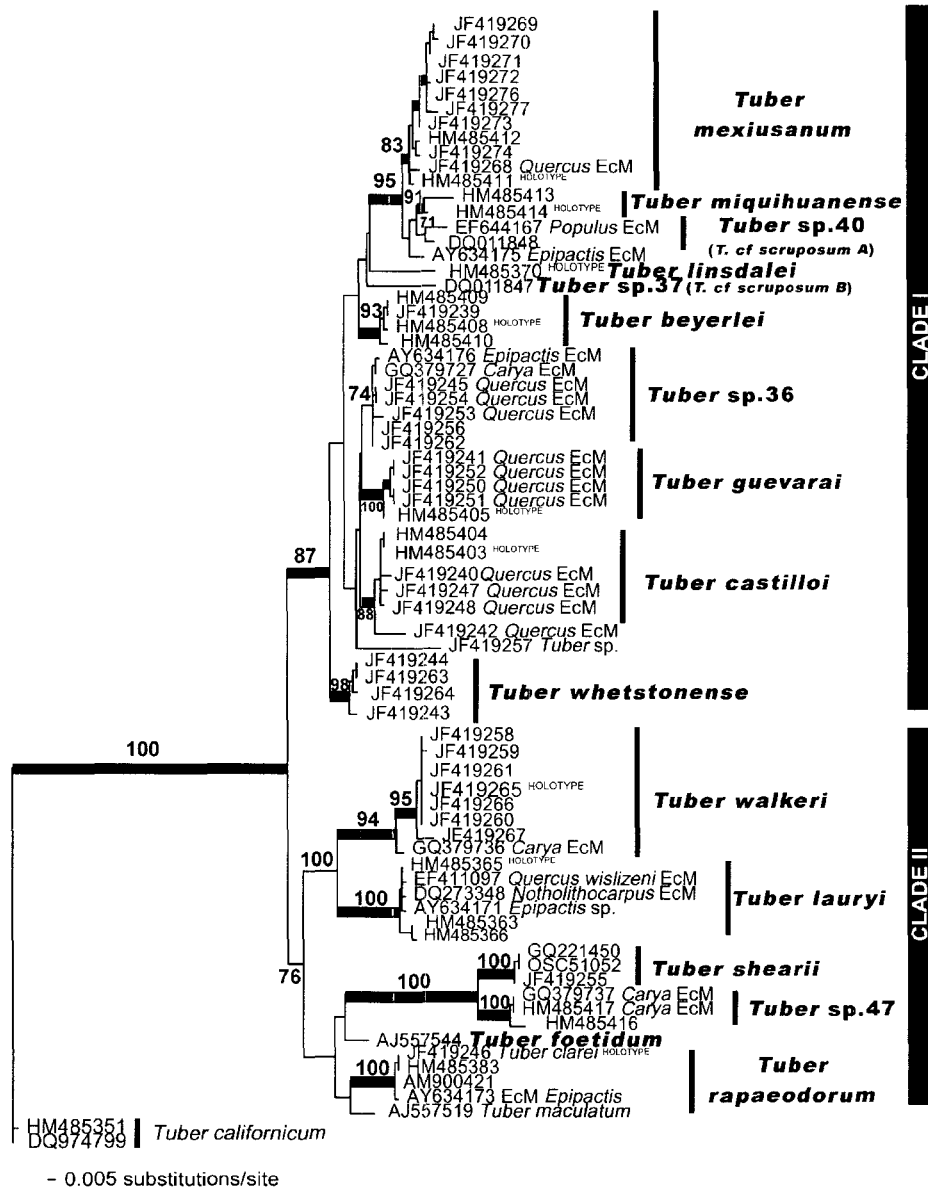


FIG. 1. Most likely tree for the Maculatum clade based on ITS nuclear rDNA with phylogenetic placement of the species described here. Maximum likelihood (ML) bootstrap values are on top of the branches and posterior probabilities based on Bayesian inference (BI) are below branches. Values of > 70 for ML and 99 or above for BI are considered significant. An asterisk was used to denote support values of 100 when space was limited. The seven species describe here are resolved with significant support. Species are labeled with their GenBank accession number. *Tuber californicum* was chosen as outgroup based on previous analyses of white truffles in the Maculatum and Puberulum groups.

to sequences from ascomata of *T. guevarai*, *T. castilloi* and *T. mexiusanum* respectively. *Tuber guevarai* was detected in 15 ectomycorrhizae and four sites, *Tuber castilloi* in 12 ectomycorrhizae from five sites and *Tuber mexiusanum* at only one site (El Madroño, Tamaulipas). Although ectomycorrhizae of *Tuber miquihuanense* were not detected, an ascoma (JT32616) of this species was collected.

TAXONOMY

*Tuber beyerlei* Trappe, Bonito & Guevara, sp. nov.

FIG. 3a–d

Mycobank MB564391, GenBank JF419286,

*Diagnosis:* Ascomata 5–20 mm broad, subglobose to globose or irregular, orange brown, light brown when dried. Peridium granulose to irregularly roughened

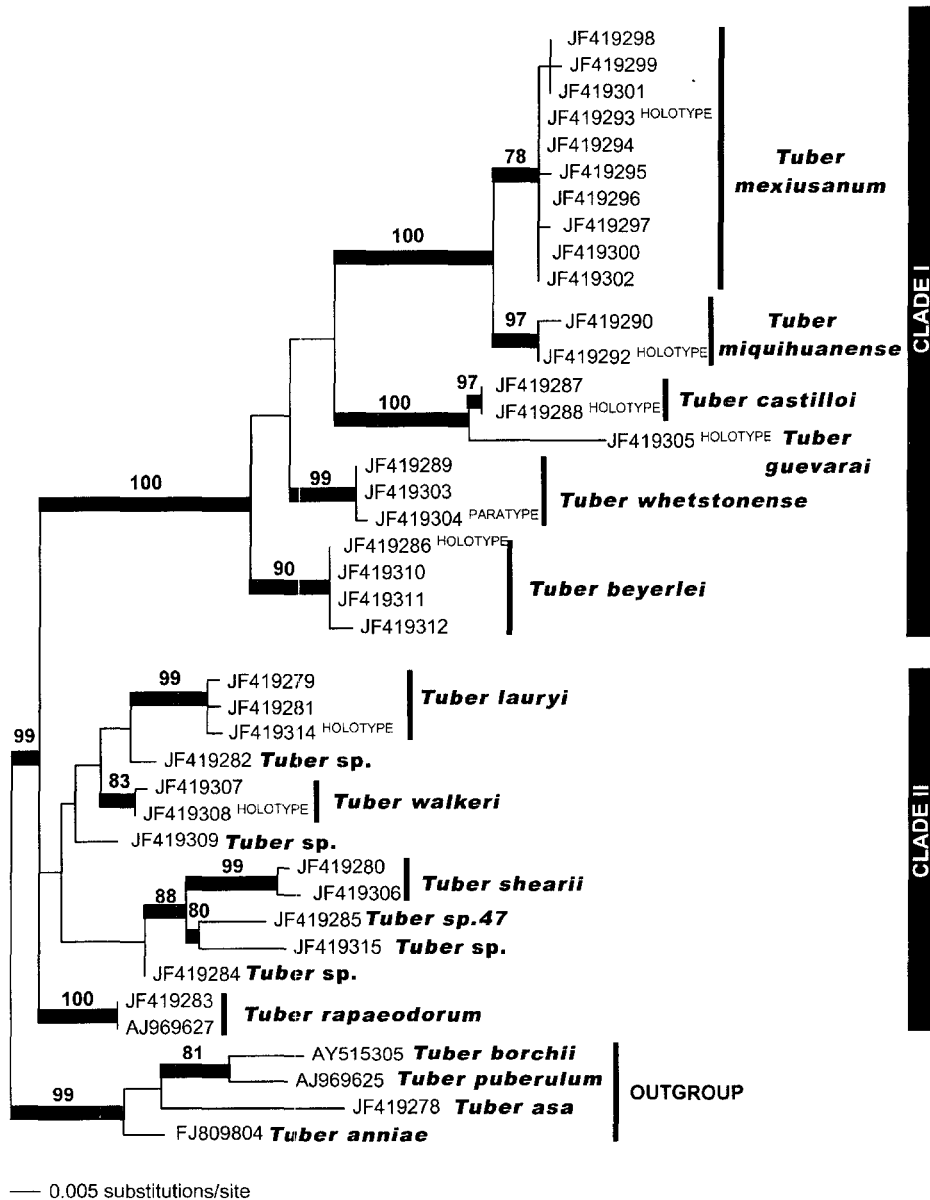


FIG. 2. Most likely tree for the Maculatum clade based on LSU nuclear rDNA with phylogenetic placement of the species described here. Maximum likelihood (ML) bootstrap values are on top of the branches. Values of > 70 for ML are considered significant. The seven species describe here are resolved with significant support. Species are labeled with their GenBank accession numbers. *Tuber borchii*, *T. puberulum*, *T. asa* and *T. anniae*, species in the Puberulum clade, were chosen as outgroup taxa. Those species whose taxonomic affiliation is uncertain were labeled *Tuber* sp.

with 8–10 flat to rounded warts per mm. Gleba solid, light brown marbled with white veins. Odor garlicky. Flavor not recorded. Peridium 200–350  $\mu$ m thick. Spores in one-spored asci 37–47  $\times$  32–40  $\mu$ m; *HOLOTYPE*. USA. OREGON. Clackamas County. Milwaukie, 5807 SE Cedar, 5 Nov 2004, *Adrian Beyerle 2149*, *Trappe 32597* (OSC 130875).

*Etymology*: In honor of Adrian Beyerle, avid truffle collector, long-term member of the North American Truffling Society and discoverer of the holotype.

Ascomata 5–20 mm broad, subglobose to globose or irregular, orange brown, light brown when dried. Peridium granulose to irregularly roughened with 8–10 flat to rounded warts per mm. Gleba solid, light brown marbled with white veins. Odor garlicky. Flavor not recorded.

Peridium 200–350  $\mu$ m thick; pellis a pseudoparenchyma 100–290  $\mu$ m thick, the cells 7–30  $\mu$ m broad, versiform to angular or isodiametric, grouped to form warts, yellowish to reddish brown in KOH, the walls 2–



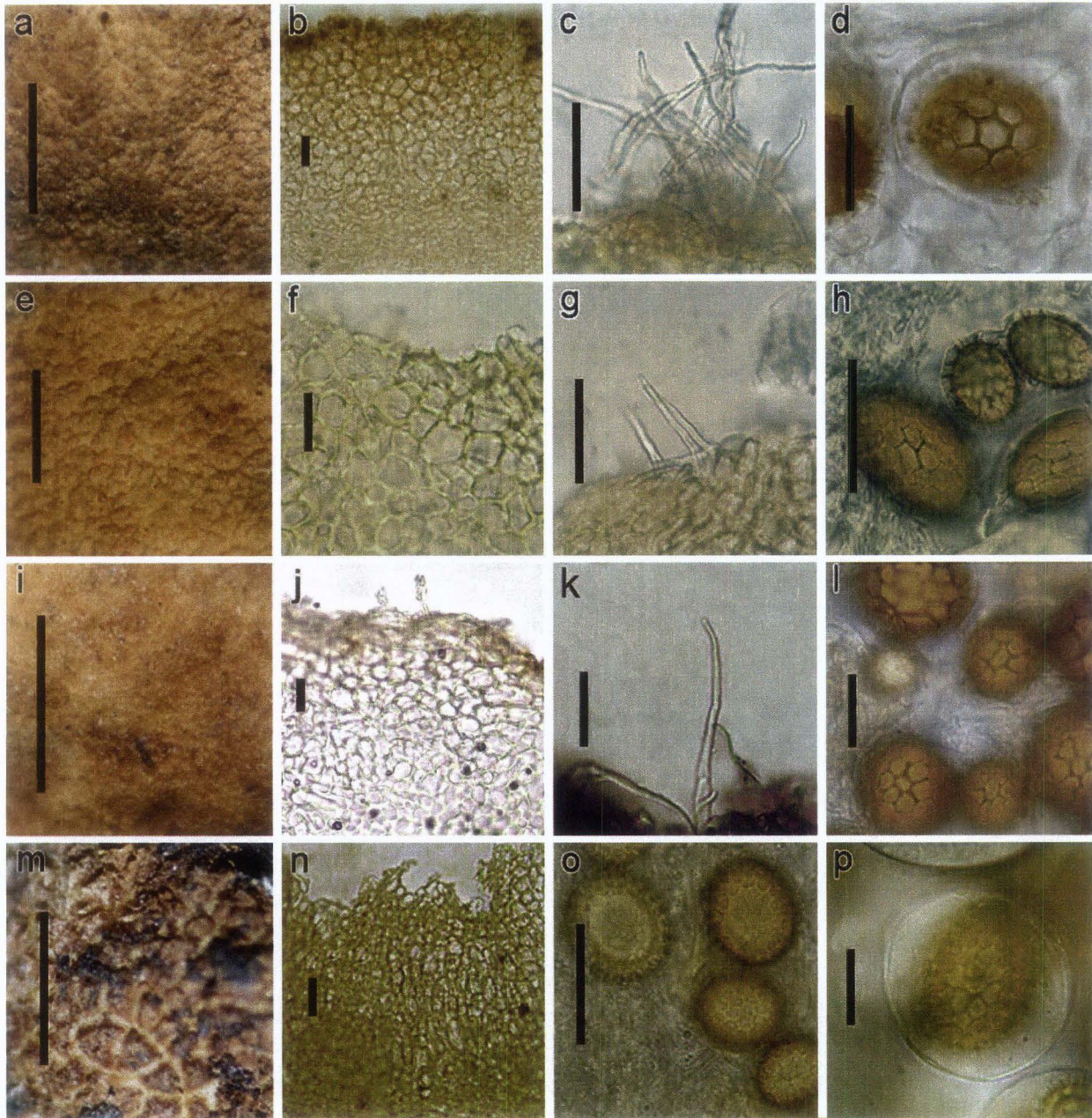


FIG. 3. a–d. *Tuber beyerlei*. a. Peridium (bar = 1 mm). b. Cross section of peridium of a pseudoparenchymatous pellis (bar = 20  $\mu$ m). c. Dermatocystidia (bar = 20  $\mu$ m). d. Spore in an ascus 100 $\times$  (bar = 20  $\mu$ m). e–h. *T. castilloi*. e. Peridium (bar = 1 mm). f. Cross section of peridium of the pseudoparenchyma pellis (bar = 20  $\mu$ m). g. Dermatocystidia (bar = 50  $\mu$ m). h. Ascospores 100 $\times$  (bar = 50  $\mu$ m). i–l. *T. lauryi*. i. Peridium (bar = 1 mm). j. Cross section of peridium of pseudoparenchymatous cells in the pellis and a subpellis with interwoven hyphae (bar = 20  $\mu$ m). k. Dermatocystidia (bar = 20  $\mu$ m). l. Spore in an ascus 100 $\times$  (bar = 20  $\mu$ m). m–p. *T. miquihuanense*. m. Peridium (bar = 1 mm). n. Cross section of peridium of the pseudoparenchymatous pellis (bar = 20  $\mu$ m). o, p. Ascospores 100 $\times$  (bars = 30  $\mu$ m, 20  $\mu$ m).

3  $\mu$ m thick; dermatocystidia 35–85  $\times$  2–4  $\mu$ m, hyaline, clustered, tapered to the tip, some sinuate, fragile, thin-walled, septate; subpellis 40–90  $\mu$ m thick, of hyaline, periclinal to interwoven hyphae 3–8  $\mu$ m broad at the septa. Gleba of hyaline, interwoven,

thin-walled hyphae, 2–5  $\mu$ m broad at the septa, the cells commonly inflated to 6–15  $\mu$ m broad.

Ascospores subglobose to broadly ellipsoid or sometimes globose, excluding their alveolate-reticulate ornamentation, in one-spored asci 37–47  $\times$  32–



40  $\mu\text{m}$  ( $Q = 1.08\text{--}1.36$ ), two-spored (26–)32–45  $\times$  23–28  $\mu\text{m}$  ( $Q = 1.00\text{--}1.47$ ), three-spored 20–35  $\times$  20–30  $\mu\text{m}$  ( $Q = 1.05\text{--}1.23$ ), and four-spored 23–28  $\times$  19–26  $\mu\text{m}$  ( $Q = 1.07\text{--}1.20$ ), the walls up to 4  $\mu\text{m}$  thick and reddish brown to brown in KOH, reddish brown in Melzer's reagent; reticulum with (4–)6–10 meshes along the spore length and 3–8 across, the alveolar walls 3–5(–7)  $\mu\text{m}$  tall, some spores subalveolate or with irregular reticulation. Asci 60–80  $\times$  50–60  $\mu\text{m}$ , subglobose to broadly ellipsoid, pedicel lacking to prominent, the walls up to 1  $\mu\text{m}$  thick, hyaline in KOH, yellow in Melzer's reagent.

*Distribution, habitat and season:* Oregon, under *Pseudotsuga menziesii*. May and November.

*Specimens examined:* HOLOTYPE. USA. OREGON. Clackamas County. Milwaukie, 5807 SE Cedar, 45 m, 5 Nov 2004, Adrian Beyerle 2149, Trappe 32597 (OSC 130875). PARATYPE. USA. OREGON. Clackamas County. Milwaukie, 5807 SE Cedar, 45 m, 5 Nov 2004, Adrian Beyerle 2401, Trappe 33867 (OSC 130888).

*Comments:* *Tuber beyerlei* differs from *T. levissimum* Gilkey and *T. guevarai* as follows: for *T. beyerlei*, peridium granulate, 200–350  $\mu\text{m}$  thick, spores in one-spored asci 37–47  $\times$  32–40  $\mu\text{m}$ ; for *T. levissimum*, peridium smooth, 420–840  $\mu\text{m}$  thick, spores in one-spored asci 36–58  $\times$  32–52  $\mu\text{m}$  (Gilkey 1939); and for *T. guevarai*, peridium finely papillose with 10–12 papillae per mm to granulate, 160–220  $\mu\text{m}$  thick, spores in one-spored asci 36–55  $\times$  28–42  $\mu\text{m}$ . Our data indicate that most other members belonging to the Maculatum group associate with broadleaf hosts, however the holotype of *T. beyerlei* was found under *Pseudotsuga menziesii*. Ectomycorrhizae belonging *T. beyerlei* have been sequenced recently from truffle orchards in British Columbia, Canada, established with *Corylus* and *Quercus* spp. that had been inoculated with *T. melanosporum* (Shannon Berch pers comm). This report expands the known geographic range for this species and indicates that this species also can form symbioses with broadleaf hosts. The sister species of *T. beyerlei* was not resolved by either ITS or LSU. Both of these rDNA loci place *T. beyerlei* in a clade that also includes *T. mexicanum*, *T. guevarai*, *T. miqihuanense*, *T. castilloi*, *T. whetstonense* and *T. linsdalei*.

***Tuber castilloi*** Guevara, Bonito & Trappe, sp. nov.

FIG. 3e–h

MycoBank MB564391, GenBank JF419288

*Diagnosis:* Ascomata 11–25  $\times$  10–17 mm, subglobose to ovoid, flattened or irregular. Peridium cream yellow to brownish orange or brown, minutely verrucose to papillate with small warts (3–11 per 1 mm), some areas finely roughened, dry, with white furrows. Gleba solid, at maturity light brown marbled with white veins. Odor and flavor not recorded. Dermatozystidia 27–42  $\times$  3–4  $\mu\text{m}$  at

the base. Spores in one-spored asci 27–63  $\times$  21–40  $\mu\text{m}$ . HOLOTYPE. MÉXICO. NUEVO LEÓN. Canyon de Puerto Genovevo, municipality of Santiago, 25 Jun 1985, Cázares 149 (ITCV holotype, OSC 130902 isotype).

*Etymology:* Dedicated to the eminent Mexican mycologist José Castillo Tovar for his many contributions to knowledge of the Mexican mycota.

Ascomata 11–25  $\times$  10–17 mm, subglobose to ovoid, flattened or irregular. Peridium creamy yellow to brownish orange or brown, minutely verrucose to papillose with small papillae (3–11 per mm), some areas finely roughened, dry, with white furrows. Gleba solid, at maturity light brown marbled with white veins. Odor and flavor not recorded.

Peridium 80–150  $\mu\text{m}$  thick; pellis a pseudoparenchyma 50–150  $\mu\text{m}$  wide, cells 5–38  $\mu\text{m}$  broad, versiform to angular or isodiametric, hyaline to reddish brown in KOH, the walls 2  $\mu\text{m}$  thick; dermatocystidia mainly in peridial depressions, single to clustered, 27–42  $\times$  3–4  $\mu\text{m}$  at the base, tapered to the tip, some septate, thick walled, hyaline in KOH; subpellis 50–200  $\mu\text{m}$  thick, delimited from the pellis, of interwoven, hyaline hyphae 3–6  $\mu\text{m}$  broad at septa. Gleba of hyaline, interwoven to periclinal, thin-walled hyphae 3–5  $\mu\text{m}$  broad at the septa.

Ascospores subglobose to broadly ellipsoid, excluding their alveolate-reticulate ornamentation, in one-spored asci 27–63  $\times$  20–40  $\mu\text{m}$  ( $Q = 1.4\text{--}2.3$ ), two-spored 30–50  $\times$  23–34  $\mu\text{m}$  ( $Q = 1.2\text{--}1.9$ ), three-spored 27–40  $\times$  20–32  $\mu\text{m}$  ( $Q = 1.0\text{--}1.8$ ) and four-spored 27–44  $\times$  20–30  $\mu\text{m}$  ( $Q = 1.0\text{--}1.6$ ), the walls 2–3  $\mu\text{m}$  thick and yellowish to reddish brown in KOH, reddish brown in Melzer's reagent; reticulum with 3–10 meshes along the spore length (a few with more than 10) and 3–6 across, the alveolar walls 2–4  $\mu\text{m}$  tall. Asci 50–90  $\times$  50–65  $\mu\text{m}$  including a short pedicel when present, globose to subglobose or ovoid, the wall 1  $\mu\text{m}$  thick, hyaline in KOH, yellowish to brownish in Melzer's reagent.

*Distribution, habitat and season:* México, Nuevo León and Tamaulipas, solitary to gregarious, in forests with *Quercus rugosa*, *Q. rysophylla*, *Q. polymorpha*, *Q. laceyi*, *Arbutus xalapensis*, *Prunus* spp., *Juglans* spp. and *Pinus* spp. at 950 m, June.

*Specimens examined:* HOLOTYPE. MÉXICO. NUEVO LEÓN. Canyon de Puerto Genovevo, municipality of Santiago, 25 Jun 1985, Cázares 149 (ITCV holotype, OSC 130902 isotype). PARATYPES. MÉXICO. NUEVO LEÓN. Canyon de Puerto Genovevo, municipality of Santiago, 25 Jun 1985, E. Cázares 142 (ITCV). TAMAULIPAS. locality Sierra de San Carlos, municipality of San Carlos, 8 Jun 1985, J. García 4739 (ITCV).

*Comments:* *Tuber castilloi* resembles *T. guevarai* in its brown ascomata and peridial structures, but *T. guevarai* has smaller spores in its one-spored asci, 36–55  $\times$  28–42  $\mu\text{m}$ , and 10–12 warts per mm in the



TABLE III. Intraspecific and interspecific ITS variation of *Tuber* species in the Maculatum clade

Species	Sample size (n)	Maximum ITS length (bp) <sup>a</sup>	Intraspecific variation (%)	Interspecific variation (%)
<i>Tuber beyerlei</i>	4	476	< 1.8	> 6.0
<i>Tuber castilloi</i>	4	475	< 1.0	> 2.6
<i>Tuber foetidum</i>	3	580	< 0.5	> 4.9
<i>Tuber guevarai</i>	4	474	< 1.0	> 2.6
<i>Tuber lauryi</i>	10	590	< 1.5	> 6.8
<i>Tuber maculatum</i>	4	588	< 0.1	> 4.6
<i>Tuber mexiusaum</i>	4	473	< 1.5	> 2.1
<i>Tuber miquihuanense</i>	2	472	< 2.2	> 2.6 <sub>a</sub>
<i>Tuber rapaeodorum</i>	42	643	< 1.6	> 4.6
<i>Tuber scruposum</i> A	2	473	< 2.1	> 2.6
<i>Tuber shearii</i>	3	476	< 0.1	> 4.6
<i>Tuber walkeri</i>	7	583	< 2.5	> 6.8
<i>Tuber whetstonense</i>	10	472	< 2.5	> 4.8

<sup>a</sup>Based on the number of base pairs between the end of the 18s SSU motif, CATTAA-, and the beginning 28s LSU motif, -TAGGGATA.

peridium. *Tuber castilloi* also has dermatocystidia as do European species such as *T. puberulum* Berk. & Br., *T. rapaeodorum* Tul. & C. Tul. and *T. borchii* Vittad., but those of *T. castilloi* are 27–42 × 3–4 µm, whereas those of the European species are 50–85 × 6–9 µm (Lange 1956, Pegler et al. 1993). Molecular analyses place *T. castilloi* within the Maculatum clade and sister to *T. guevarai* (FIG. 1). Interspecific variation between these two species is > 2.6%, and intraspecific variation within *T. castilloi* is < 1.0% (TABLE III). Sequence data from ectomycorrhizae confirm that *T. castilloi* forms mycorrhizae with *Quercus* spp.

#### *Tuber guevarai* Bonito & Trappe, sp. nov.

FIG. 4e–h  
Mycobank MB564393, GenBank JF419305

**Diagnosis:** Ascumata 15 × 11 mm, subglobose to ovoid or somewhat flattened, solid, firm. Peridium granulose or with 10–12 papillae per mm, light brown, separable, with a shallow furrow at the base. Gleba solid, marbled, brown to dark brown with white to cream veins. Odor and flavor not distinctive. Dermatocystidia 20–60 × 2–4 µm at the base, scattered, tapered. Spores in one-spored asci 36–55 × 28–42 µm. **HOLOTYPE.** MÉXICO. TAMAULIPAS. San Carlos Municipality, El Diente Mountain, 8 Jun 1985, *G. Guevara 180* (ITCV holotype, OSC isotype).

**Etymology:** Dedicated to the Mexican mycologist and colleague Gonzalo Guevara. In honor of his many contributions to Mexican truffle taxonomy, including the discovery of this species.

Ascoma 15 × 11 mm, subglobose to ovoid or somewhat flattened, solid, firm. Peridium granulose or with 10–12 papillae per mm, light brown,

separable, with a shallow furrow at the base. Gleba solid, brown to dark brown marbled with white to cream veins. Odor and flavor not distinctive.

Peridium 160–220 µm thick; pellis a pseudoparenchyma 60–120 µm thick, cells 4–30 µm broad, angular or isodiametric, reddish brown to dark brown in KOH, walls 2–3 µm thick; dermatocystidia 20–60 × 2–4 µm at the base, scattered, tapered, some septate, thick-walled, hyaline in KOH. Subpellis 80–120 µm thick, grading from the pellis pseudoparenchyma to interwoven, hyaline hyphae 3–4 µm broad near the gleba. Gleba of hyaline, thin-walled, interwoven hyphae 2–6 µm broad at the septae, the cells cylindrical or a few slightly inflated.

Ascospores subglobose to broadly ellipsoid, excluding the alveolate-reticulate ornamentation, in one-spored asci 36–55 × 28–42 µm (Q = 1.2–1.3), two-spored 30–45 × 25–35 µm (Q = 1.1–1.4), three-spored 27–40 × 21–27 µm (Q = 1.1–1.4), four-spored 20–32 × 19–25 µm (Q = 1.0–1.3) and five-spored 18–25 × 16–19 µm (Q = 1.0–1.3), walls 1–2 µm thick, yellowish to reddish brown in mass in KOH and in Melzer's reagent; reticulum with 3–9 alveolar meshes along the spore length, 3–7 across, the walls 3–6 µm tall, some spores showing a slight microreticulum within the alveolar walls. Asci 52–30 µm broad, globose to subglobose with a small pedicel, the walls thick, hyaline in KOH and in Melzer's reagent.

**Distribution, habitat and season:** Known only from the type collection in Tamaulipas, México. Hypogeous, solitary, in a forest with *Quercus canbyi*, *Q. rysophylla*, *Q. clivicola*, *Q. virginiana*, *Q. sartorii* and *Arbutus xalapensis* on soil derived from volcanic rock over cretaceous limestone, June.

**Specimen examined:** **HOLOTYPE.** MÉXICO. TAMAULIPAS. San Carlos Municipality, El Diente Mountain, 8 Jun 1985, *G. Guevara 180* (ITCV holotype, OSC isotype).

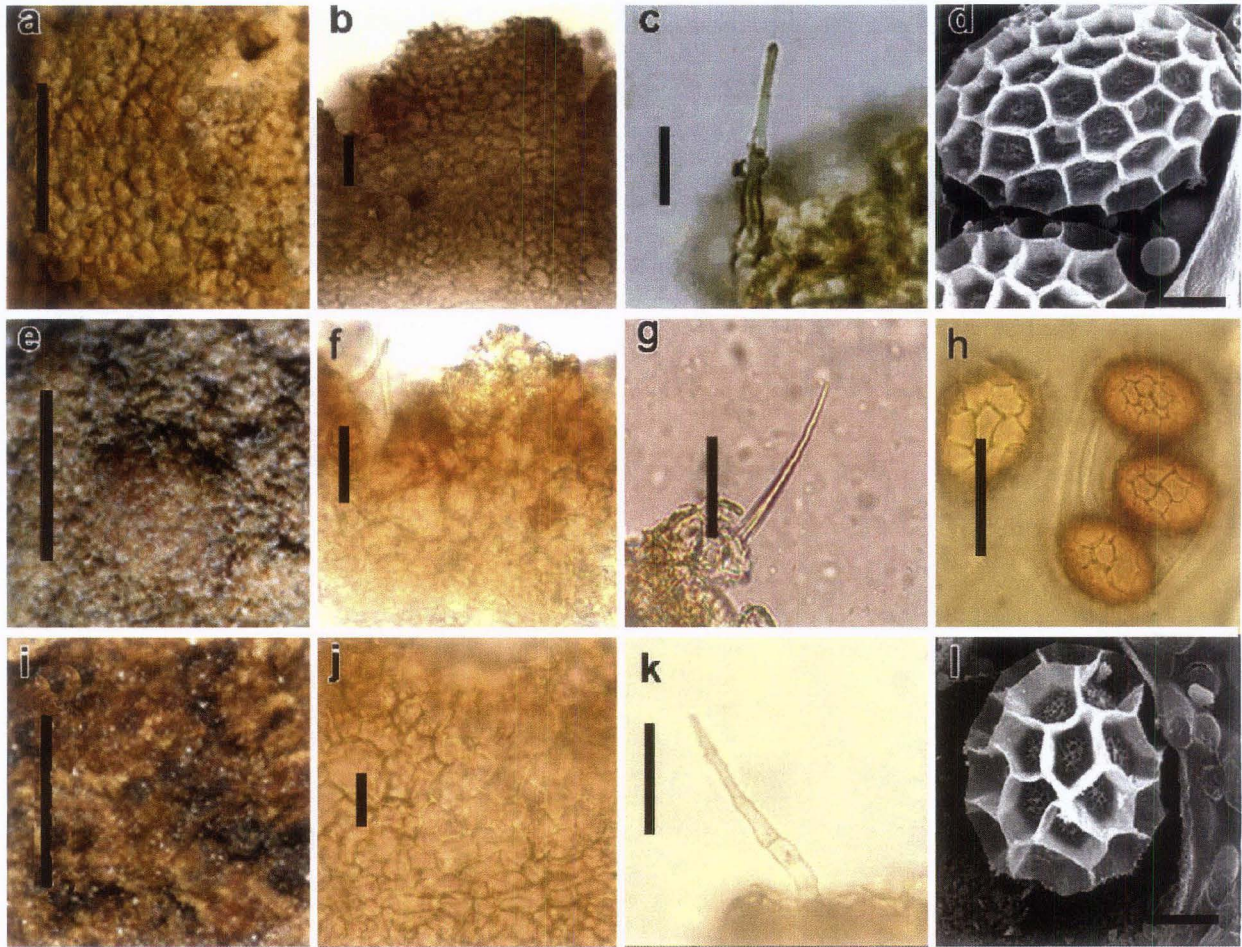


FIG. 4. a–d. *T. mexiusanum*. a. Peridium (bar = 1 mm). b. Cross section of peridium of the pseudoparenchymatous pellis and the subpellis with interwoven hyphae (bar = 50 µm). c. Dermatocystidia (bar = 10 µm). d. SEM micrograph, ascospores with micro-ornamentation (bar = 10 µm). e–h. *T. guevarai*. e. Peridium (bar = 1 mm). f. Cross section of peridium of a pseudoparenchymatous pellis (bar = 10 µm). g. Dermatocystidia (bar = 10 µm). h. Spores in the asci at 100× (bar = 30 µm). i–l. *T. walkeri* peridium (bar = 1 mm). j. Cross section of peridium of the pseudoparenchymatous pellis and the subpellis with interwoven hyphae (bar = 40 µm), k. Dermatocystidia (bar = 20 µm). l. SEM micrograph of the micro-ornamentation of ascospores (bar = 10 µm).

*Comments:* *Tuber guevarai* resembles the European *T. rapaeodorum* Tul. & C. Tul. and *T. borchii* Vittad. in peridial structure, but *T. rapaeodorum*, has scattered dermatocystidia 56–80(–115) µm long and globose spores, and *T. borchii* dermatocystidia are 50–80 µm long (Lange 1956, Pegler et al. 1993, Mello et al. 2000, Halász et al. 2005). The broadly ellipsoid spores of *T. guevarai* distinguish this species from *T. californicum* Harkn. with globose spores. Molecular data place *T. guevarai* within the Maculatum clade and sister to *T. castilloi*. Sequences from ectomycorrhizae confirm its association with *Quercus* (FIG. 1). Interspecific ITS variation between these species is rather low ( $\geq 2.6\%$ ) (TABLE III).

*Tuber lauryi* Trappe, Bonito & Guevara, sp. nov.

FIG. 3i–l

Mycobank MB564394, GenBank HM485365

*Diagnosis:* Ascomata 6–10 × 10–13 mm, globose to subglobose, ellipsoid or irregular. Peridium whitish to brown, smooth but in small patches granulose to finely roughened or with 10–15 rounded warts per mm, up to 1 mm thick. Gleba solid, brown marbled with white to cream veins. Odor and flavor not recorded. Peridium up to 1000 µm thick. Spores in one-spored asci 35–40(–50) × 30–41 µm. *HOLOTYPE*. USA. OREGON. Linn County, Fry Tree Farm, 2 Oct 1996, *J. Trappe 19425* (OSC 130885).

*Etymology:* In honor of Dr Daniel Laury, who found the southernmost collection of the species.

Ascomata 6–10 × 10–13 mm, globose to subglobose, ellipsoid or irregular. Peridium whitish to brown, smooth but in small patches granulose to finely roughened or with 10–15 rounded warts per mm, up to 1 mm thick. Gleba solid, brown marbled with white to cream veins. Odor and flavor not recorded.

Peridium 300–1000 µm thick; pellis an irregular pseudoparenchyma 50–150 µm thick, absent in patches, the cells 4–24 µm broad, angular to isodiametric in groups that form warts, in small areas forming chains perpendicular to the peridium, orange to reddish or light brown in KOH, the walls up to 2 µm thick; dermatocystidia scattered, tapered to the tip, sinuate, readily collapsing, thin-walled, hyaline in KOH; subpellis 300–900 µm thick, near the interface with the pellis a dense tissue of periclinal to interwoven, hyaline hyphae 3–5 µm broad at the septa, the cells generally not inflated, the walls up to 1 µm thick. Gleba of hyaline, densely interwoven hyphae 2–5 µm broad at septa, the walls thin or thickened up to 1 µm, only scattered cells somewhat inflated.

Ascospores globose to subglobose or broadly ellipsoid, excluding their alveolate-reticulate ornamentation, in one-spored asci 35–40(–50) × 30–41 µm ( $Q = 1.12$ – $1.33$ ), two-spored 22–38 × 22–35 µm ( $Q = 1.00$ – $1.13$ ), and three-spored 23–28 × 20–28 µm ( $1.0$ – $1.3$ ), the walls up to 6 µm thick, reticulum with 4–10 (–14) alveolar meshes along the spore length, 3–8 across, alveolar walls 2–4 µm tall, some with a micro-reticulation within the alveolae, yellowish to reddish brown or dark brown in KOH, reddish brown in Melzer's reagent. Asci 60–90 × 40–70 µm, broadly ellipsoid, 1–3(–4)-spored, the walls up to 2 µm thick, hyaline in KOH, yellowish in Melzer's reagent.

*Distribution, habitat and season:* Western Oregon. Hypogeous, solitary in mixed forests of *Quercus garrayana* and *Pseudotsuga menziesii* or in pure stands of *Pseudotsuga*. July, October and December.

*Specimens examined:* HOLOTYPE. USA. OREGON. Linn County, Fry Tree Farm, 2 Oct 1996, *J. Trappe 19425* (OSC 130885). PARATYPES. USA. OREGON. Benton County, Lobster Valley, 1.3 miles up Misty Acres Road, 44.311°N, 123°79.3W, 3 Dec 2011, *I. LaVielle, J. Trappe 35446* (OSC) and 3 Dec 2011, *M. Castellano, Trappe 35449* (OSC). Jackson County, Medford, 6 Jul 1997, *D. Laury, Trappe 26987* (OSC 130877). Linn County, 34197 NE Colorado Lake Drive, 26 Oct 2002, *P. Rawlinson, Trappe 28005* (OSC 130878).

*Commentary:* *Tuber lauryi* can be recognized by its usually thick peridium (up to 600 µm even when dried) in combination with its thick-walled asci mostly containing three or fewer spores. It resembles *T. irradians* Gilkey, but the holotype of *T. irradians* has an outer peridium of distinctive radial chains 140–160 µm

long of isodiametric cells, hence the epithet *irradians* (Gilkey 1939), larger spores (36–56 × 28–48 µm) and a peridium no thicker than 380 µm. Phylogenetically *T. lauryi* is close to *T. walkeri* and an unnamed species (FIG. 1) known only from ectomycorrhizae or immature fruit bodies. Sequence data from ectomycorrhizae confirm that *T. lauryi* is mycorrhizal with species of *Quercus*, *Notholithocarpus* and *Epipactis*. However, the two collections from Benton County were found in a pure stand of *Pseudotsuga menziesii*. Bidartondo et al. (2004) also reported a genetically similar *Tuber* sp. associated with orchid (*Epipactis*) roots (FIG. 1 as AY634171) from Berkeley, California, USA.

***Tuber mexiusanum*** Guevara, Bonito & Cázares., sp. nov. FIG. 4a–d  
Mycobank MB564395, GenBank JF419293

*Diagnosis:* Ascomata 8–11 × 11–14 mm, subglobose to lobate, cerebriform or ovoid. Peridium cream-yellow, pale orange to light brown, staining olive green, granulose to finely roughened with 4–7 warts per millimeter, dry, separable, with a white basal furrow. Gleba brown, marbled with white veins. Odor strongly acetone-like. Flavor not recorded. Dermatocystidia scattered or in groups, 22–60 × 1.5–5 µm, tapered. Spores in one-spored asci 20–50 × 16–36 µm. HOLOTYPE. MÉXICO. TAMAULIPAS. San Carlos Municipality, El Diente Mountain, 8 Jun 1985, *G. Guevara 181* (ITCV holotype, OSC-isotype).

*Etymology:* A contraction of México and USA, the geographic range in which this species has been found.

Ascomata 8–11 × 11–14 mm, subglobose to lobate, cerebriform or ovoid. Peridium cream-yellow, pale orange to light brown, staining olive green, granulose to finely roughened with 4–7 warts per millimeter, dry, separable, with a white basal furrow. Gleba solid, brown marbled with white veins, odor strongly acetone-like. Flavor not recorded.

Peridium 130–350 µm thick; pellis a pseudoparenchyma 100–225 µm thick, of angular to isodiametric cells 7–38 µm broad, yellowish to reddish brown in KOH, the walls 2–3 µm thick; dermatocystidia scattered or in groups, 22–60 × 1.5–5 µm at the base, tapered, some septate, thick-walled, hyaline in KOH; subpellis 30–125 µm thick, of interwoven hyphae 3–7 µm broad at the septa, hyaline in KOH. Gleba of hyaline, thin-walled, interwoven to periclinal hyphae 2–5 µm broad at septae.

Ascospores subglobose to broadly ellipsoid or ellipsoid, excluding their alveolate-reticulate ornamentation, in one-spored asci 20–50 × 16–36 µm ( $Q = 1.1$ – $1.7$ ), two-spored 23–30 × 18–26 µm ( $Q = 1.0$ – $1.6$ ), three-spored 21–35 × 12–22 µm ( $Q = 1.0$ – $1.7$ ), four-spored 22–30 × 14–21 µm ( $Q = 1.1$ – $1.7$ ), and



five-spored  $15\text{--}27 \times 12\text{--}20 \mu\text{m}$  ( $Q = 1.1\text{--}1.4$ ), the walls  $1\text{--}2 \mu\text{m}$  thick, yellowish to reddish brown in KOH, reddish brown in Melzer's reagent; reticulum with  $4\text{--}8$  alveolar meshes along the spore length,  $3\text{--}5$  across, the alveolar walls  $2\text{--}7 \mu\text{m}$  tall, some mature spores with irregular reticulation, others with a microreticulation within the alveolar walls. Asci  $40\text{--}80 \times 35\text{--}60 \mu\text{m}$ , globose to subglobose, with or without a rudimentary pedicel, walls  $1 \mu\text{m}$  thick, hyaline in KOH, bluish in trypan blue, yellowish to brownish in Melzer's reagent.

*Distribution, habitat and season:* México, Coahuila and Tamaulipas north to Tennessee, North Carolina, Iowa and Minnesota in USA, hypogeous, solitary to gregarious. In México in oak forests with *Quercus canbyi*, *Q. rysophylla*, *Q. clivicola*, *Q. virginiana*, *Q. sartorii*, *Q. polymorpha*, and *Arbutus xalapensis* on soil derived from volcanic rock over cretaceous limestone or near understory oak within mixed *Abies-Pseudotsuga*-dominated forests. In USA in mesic oak-hickory woods on glacial till or well drained limestone soils in Iowa, oak dominated woods of the driftless area (not recently glaciated) of SE Minnesota and on sandy to silty riparian soils is association with *Populus deltoides* in eastern Tennessee and central North Carolina. Ectomycorrhizae of this species were documented and sequenced (GenBank accession number JN033365) from roots of cuttings grown in Tennessee soils collected along the Caney Fork River under *Populus deltoides*. June through September.

*Specimens examined:* HOLOTYPE. MÉXICO. TAMAULIPAS. San Carlos Municipality, El Diente Mountain, 8 Jun 1985, G. Guevara 181 (ITCV holotype, OSC-isotype). PARATYPES. MÉXICO. COAHUILA. Arteaga municipality, La Siberia, 30 Sep 1983, García 3785 (ITCV, OSC). USA. Iowa, Dubuque County, White Pine Hollow State Preserve, 15 Sep 2000, R. Healy 808 (ISC); Mines of Spain State Park, 16 Sep 2000, R. Healy 817 (ISC). Story County, 10 miles N of Ames, 20 Jul 1997, R. Healy 35 (ISC 435886 as *T. gardneri*). MINNESOTA. Fillmore County, Forestville State Park, 21 Jul 2009, R. Healy 948 and 949 (MIN), and 6 Aug 2009, R. Healy 980 (MIN).

*Comments:* This species closely resembles *T. guevarai* but has wider spores ( $18\text{--}55 \times 16\text{--}42 \mu\text{m}$ ) and  $10\text{--}12$  warts per millimeter in the peridium. *Tuber mexiusanum* also resembles *T. puberulum* Berk & Br., *T. rapaeodorum* Tul. & C. Tul. and *T. borchii* Vitad. from Europe, but these species have bigger dermatocystidia,  $50\text{--}110$ ( $\text{--}115$ ) long (Lange 1956, Pegler et al. 1993, Mello et al. 2000, Halász et al. 2005). Molecular analyses place *T. mexiusanum* within the Maculatum clade and in close relationship to a *Tuber* sp. from roots of an *Epipactis* orchid in Quebec, Canada (FIG. 1 as AY634175 (Bidartondo et al. 2004). Interspecific ITS variation between *T. mexiusanum* and *T. miquihuanense* is low ( $> 2.1\%$ ) (TABLE III).

Sequence data from ectomycorrhizae collected in México confirm that *T. mexiusanum* forms mycorrhizae with *Quercus*. Although collections from Tennessee and North Carolina were still immature, sequence analyses from these collections and from *P. deltoides* ectomycorrhizae confirm their identification. Based on the available data, *Tuber mexiusanum* appears to have the broadest geographic and host range of the species described in this paper.

***Tuber miquihuanense*** Guevara, Bonito & Cázares, sp. nov. FIG. 3m-p

MycoBank MB564391, GenBank HM485414

*Diagnosis:* Ascomata  $30\text{--}20 \text{mm}$ , subglobose, slightly gibbose. Peridium reddish brown to light brown, with a white to cream basal furrow and  $2\text{--}4$  flat to  $\pm$  pyramidal or polygonal warts per millimeter, in some areas granulose to finely roughened or reticulate-pitted, dry, not readily separable. Gleba cream to light brown marbled with white veins. Odor pleasant. Flavor not recorded. Pellis a pseudoparenchyma forming chains perpendicular to the peridial surface. Spores in one-spored asci  $40\text{--}50 \times 30\text{--}39 \mu\text{m}$ . *Holotype:* MÉXICO. TAMAULIPAS. Miquihuana municipality, La Joya, 16 Sep 2006, Guevara 885 (ITCV holotype, OSC 130903 isotype).

*Etymology:* For the type locality, Miquihuana municipality, México.

Ascomata  $30\text{--}20 \text{mm}$ , subglobose to slightly gibbose. Peridium reddish brown to light brown, with a white to cream basal furrow and  $2\text{--}4$  flat-polygonal to pyramidal warts per millimeter, in some areas granulose to finely roughened or reticulate-pitted, dry, not readily separable. Gleba cream to light brown marbled with white veins. Odor pleasant. Flavor not recorded.

Peridium  $110\text{--}360 \mu\text{m}$  thick; pellis a pseudoparenchyma  $60\text{--}250 \mu\text{m}$  thick, cells  $5\text{--}24 \mu\text{m}$  broad, angular to isodiametric, some forming chains perpendicular to the peridial surface, yellowish to reddish brown in KOH, thick-walled; subpellis  $50\text{--}112 \mu\text{m}$  thick, of interwoven, hyaline, thin-walled hyphae  $3\text{--}7 \mu\text{m}$  broad at the septa, confluent with the gleba. Gleba of hyaline, thin-walled, interwoven or periclinal hyphae  $2\text{--}7 \mu\text{m}$  broad at septa, cells often slightly inflated.

Ascospores globose to subglobose or broadly ellipsoid, excluding their alveolate-reticulate ornamentation, in one-spored asci  $40\text{--}50 \times 30\text{--}39 \mu\text{m}$  ( $Q = 1.1\text{--}1.4$ ), two-spored  $29\text{--}40 \times 24\text{--}35 \mu\text{m}$  ( $Q = 1.0\text{--}1.2$ ), three-spored  $24\text{--}35 \times 22\text{--}33 \mu\text{m}$  ( $Q = 1.0\text{--}1.4$ ), four-spored  $23\text{--}28 \times 20\text{--}26 \mu\text{m}$  ( $Q = 1.0\text{--}1.2$ ), and five-spored  $20\text{--}28 \times 20\text{--}22 \mu\text{m}$  ( $Q = 1.0\text{--}1.2$ ), the walls  $2 \mu\text{m}$  thick, yellowish to reddish brown in KOH, reddish brown in Melzer's reagent; reticulum with  $4\text{--}$

8 meshes along the spore length, 3–7 across, the alveolar walls 2–5  $\mu\text{m}$  tall, some spores with irregular reticulation. Asci 65–90  $\times$  51–70  $\mu\text{m}$ , globose to subglobose or broadly ellipsoid, with a short pedicel when young, walls 1  $\mu\text{m}$  thick, hyaline in KOH, yellowish to brownish in Melzer's reagent.

*Distribution, habitat and season:* Tamaulipas, México, solitary in pine-oak and mesophyll forests with *Quercus greggii*, *Quercus miquihuanensis*, *Arbutus xalapensis*, *Pinus montezumae* and *P. hartwegii* on calcareous soils 1450–3025 m. July and September.

*Specimens examined:* HOLOTYPE. MÉXICO. TAMAULIPAS. Miquihuauna municipality, La Joya, 3025 m, 16 Sep 2006, Guevara 885 (ITCV holotype, OSC 130903 isotype). PARATYPES. MÉXICO. TAMAULIPAS. Gómez Farías municipality, San José, Rancho El Cielo, 1450 m, 8 Jul 1984, García 4026 (ITCV).

*Comments:* *Tuber miquihuanense* resembles *T. irradians* Gilkey, which also has radially aligned peridial cells but *T. irradians* differs in having ellipsoid spores 40–56  $\times$  36–48  $\mu\text{m}$  and one- to three-spored asci (Gilkey 1939, 1954). Molecular analyses place *T. miquihuanense* as sister taxon with a species from Armenia (Badalyan et al. 2005), annotated as *T. scruposum* Hesse (labeled as *Tuber* sp. 40 in FIG. 1). Although two phylogenetically distinct collections from Armenia have been accessioned in GenBank as *T. scruposum* (labeled A and B in FIG. 1), the Armenian collections have smaller spores (25–40  $\times$  20–25  $\mu\text{m}$ ) than *Tuber miquihuanense* and are mainly associated with *Carpinus betulus*, *Tilia cordata* and *Fagus orientalis* (Badalyan et al. 2005). Interspecific variation between these two species is at the low end of what is observed for other species of *Tuber* (> 2.6%) (TABLE III).

***Tuber walkeri*** Healy, Bonito & Guevara, sp. nov.  
FIG. 4i–l  
Mycobank MB564391, GenBank JF419260

*Diagnosis:* Ascomata up to 9–12  $\times$  8–10 mm, subglobose to ovoid, lobate or irregular. Peridium cream with reddish brown mottled areas, mostly smooth but in some areas slightly verrucose with 8–10 warts per millimeter. Gleba dark brown, marbled with white veins. Odor slightly of fresh coconut. Flavor not recorded. Pellis cells up to 38  $\mu\text{m}$  broad. Spores in one-spored asci 37–53  $\times$  34–45  $\mu\text{m}$ , with a true microreticulation within the alveolar walls. HOLOTYPE. USA. IOWA. Story County, Hickory Grove Park, 6 Aug 1999, R. Healy 521 (ISC).

*Etymology:* In honor of British mycologist Dr Chris Walker for his contributions to knowledge of taxonomy of the Glomeromycota and his collections of Iowa truffles.

Ascomata 9–12  $\times$  8–10 mm, subglobose to ovoid, lobate or irregular. Peridium cream with reddish

brown mottled areas, mostly smooth but in some areas slightly verrucose with 8–10 warts per millimeter. Gleba dark brown, marbled with white veins. Odor slightly of fresh coconut. Flavor not recorded.

Peridium 150–600  $\mu\text{m}$  thick; pellis a pseudoparenchyma 70–300  $\mu\text{m}$  thick, the cells 4–38  $\mu\text{m}$  broad, globose to subglobose or angular, hyaline to yellow or reddish brown in KOH, the walls up to 2.2  $\mu\text{m}$  thick, grading to the subpellis; dermatocystidia 25–70  $\times$  3–6  $\mu\text{m}$  at the base, tapered to the tip, septate, hyaline, scattered or in groups among the warts, the walls 1  $\mu\text{m}$  thick; subpellis 75–300  $\mu\text{m}$  thick, of hyaline hyphae 3–7.5  $\mu\text{m}$  broad at the septa, thin-walled, cells not inflated. Gleba of hyphae similar to those of subpellis.

Ascospores globose to ellipsoid, excluding the alveolate-reticulate ornamentation, in one-spored asci 37–53  $\times$  34–45  $\mu\text{m}$  ( $Q = 1.0$ – $1.5$ ), two-spored 31–45  $\times$  25–38  $\mu\text{m}$  ( $Q = 1.0$ – $1.3$ ), three-spored 29–37  $\times$  23–35  $\mu\text{m}$  ( $Q = 1.0$ – $1.3$ ), four-spored 18–39  $\times$  17–32  $\mu\text{m}$  ( $Q = 1.0$ – $1.2$ ), and five-spored 18–28  $\times$  15–23 ( $Q = 1.0$ – $1.2$ ), yellow to yellow orange in KOH; reticulum with (3–)4–8 alveolar meshes along the spore length, 3–7 across, the alveolar walls 7.5(–10)  $\mu\text{m}$  tall and often reflexed at the margins, with a true microreticulation within the alveolar walls observable with a compound microscope and verified by SEM (FIG. 4l). Asci 49–70 (–80)  $\times$  (45–) 57–75  $\mu\text{m}$ , subglobose to globose, lacking a pedicel, walls 1–2  $\mu\text{m}$  thick, hyaline in KOH.

*Distribution, habitat and season:* Iowa, emergent to shallowly hypogeous, single to clustered in mostly mesic forests of *Quercus alba*, *Q. rubra*, and *Q. macrocarpa* mixed with *Ostrya virginiana*, *Carpinus caroliniana*, *Ulmus* spp. and *Celtis occidentalis* on glacial till as well as well drained soil in a karst landscape August–September.

*Specimens examined:* HOLOTYPE. USA. IOWA. Story County, Hickory Grove Park, 6 Aug 1999, R. Healy 521 (ISC). PARATYPES. USA. IOWA. Dubuque County, White Pine Hollow State Preserve, 20 Aug 1999, R. Healy 544 (ISC); Jasper County, Rock Creek State Park, 1 Aug 1999, R. Healy 513 (ISC); Story County, Ames, YMCA Woods, Aug 9 2000, R. Healy 754 (ISC). Webster County, Woodman Hollow State Preserve, 6 Sep 1999, R. Healy 569 (ISC).

*Comments:* This species is characterized by a microreticulum within the alveolae (FIG. 4l) and pellis cells up to 38  $\mu\text{m}$  broad. It differs from *T. mexicanum* in its nearly smooth, whitish peridium. *Tuber walkeri* can be distinguished from its closest North American relatives, *T. lauryii* and *T. shearii*, by the greater number of spores per ascus (up to five in *T. walkeri*, up to three in the other two) and taller mesh of the spore ornamentation. Phylogenetically *T. walkeri*, *T. lauryii* and *T. shearii* are closely related to *T. rapaeodorum* and *T. foetidum* of Europe (FIG. 1).



## DISCUSSION

Here we described seven new *Tuber* species from North America and placed them phylogenetically within the Maculatum clade of *Tuber*. This brings the number of described *Tuber* species on the continent to 38 (TABLE I). Phylogenetic analyses of ITS and LSU sequences are congruent and distinguish these species from other described species in the Maculatum clade.

Six of the species are associated with angiosperm hosts, including monocot species of *Epipactis* and woody dicot species of *Quercus*, *Populus*, *Salix*, *Carya* and *Notholithocarpus*. The seventh species, *T. beyerlei*, was found under *Pseudotsuga menziesii* in the absence of any other nearby ectomycorrhizal hosts but also has been sequenced from ectomycorrhizae of angiosperms. The European *T. maculatum* and *T. rapaeodorum* are reported to be associated both with ectomycorrhizal deciduous trees and Pinaceae (Halász et al. 2005). Kovács and Jakucs (2005) surveyed *Populus alba* L. *Quercus robur* L. stands in Hungary and detected ectomycorrhizas of four *Tuber* species belonging to the Maculatum clade. Fassi and Fontana (1967, 1969) synthesized mycorrhizae of *Pinus strobus* with *T. maculatum*.

The early reports need to be evaluated with caution, however, because of the difficulty of differentiating similar species in clade Maculatum by morphology alone (White et al. 1990, Mello et al. 2000, Halász et al. 2005). Morphological characters are confounded by variation of edaphic, environmental and genetic factors. In addition, fruit bodies of species in this clade are often small and they are not well studied because they have no economic value as comestibles. In a few cases, phylogenetically distinct species have been accessioned in GenBank under the same name (e.g. *T. maculatum*, *T. foetidum*, *T. scruposum*). These challenges, coupled with the high diversity and existing undescribed species in the clade make it difficult to develop a working morphological key for these pale *Tuber* species at this time.

Bonito et al. (2010) compared a global sampling of *Tuber* ITS sequences and defined *Tuber* OTUs conservatively as those sequences sharing 96% ITS rDNA similarity. Although suitable for most *Tuber* species, we think this definition may be too stringent for some species in the Maculatum group. For example, *T. miquihuanense*, *T. mexiusanum* and *T. scruposum* (A) are below this threshold as are *T. castilloi* and *T. guevarai*. On the other hand, if we had considered *T. miquihuanense*, *T. mexiusanum* and *T. scruposum* (A) as a single species, it would have the highest rate of intraspecific variation (> 5%) and broadest geographic distribution of any known *Tuber*

species. The collection labeled *T. scruposum* (A) was collected in natural forests in Armenia, which is geographically distant and ecologically distinct from sites in México where *Tuber miquihuanense* is found. The fact that we can find unique genetic, morphological and geographic characters that distinguish these fungi supports our decision to treat them as distinct species. Their ITS sequences still diverge by over 2% and both are most similar to uncultured ectomycorrhizal sequences from their respective continent, criteria we think are important in our decision to delimit them as separate (non-interbreeding) species. Results from LSU rDNA analyses also corroborate the delimitation of these species.

In contrast, when sequences from *Tuber* collections made at great distances apart have little genetic distance (< 1% between isolates), this may be an indication that a recent introduction has occurred (Bonito et al. 2010). Such is likely the case for sequences of the holotype for *T. clareii* from Australia and *T. rapaeodorum* from Europe (FIG. 1). *Tuber clareii*, originally described by Gilkey, was collected from the edge of a golf course in Australia. In all cases on record, *Tuber* collections from Australia are associated with introduced hosts. Based on sequence analysis of the holotype of *T. clareii*, we identify this as the European species *T. rapaeodorum*. *Tuber rapaeodorum* in fact might be among the most cosmopolitan *Tuber* species, and it appears to have dispersed globally (Bonito et al. 2010). On the basis of morphology alone, and from study of the holotypes of *T. clareii*, *T. rapaeodorum* and *T. maculatum*, Trappe and Cázares (2000) had earlier regarded *T. clareii* as a synonym of *T. maculatum*, a conclusion not supported by sequence data.

Jeandroz et al. (2008) estimated divergence times for major nodes within the genus *Tuber*. The node supporting the Maculatum clade was calculated to have diverged approximately 30 000 000 y ago, making this among the most recently diverged group of species in *Tuber*. This recent divergence may explain part of the low interspecific variation detected among North American species and their European counterparts. However, based on this date, the North Atlantic bridge that linked Europe and North America would have separated by the time the Maculatum group is estimated to have radiated (Jeandroz et al. 2008). Asia therefore may seem to be a likely migratory land route between North America and Europe, however, apart from one collection from Nepal, there are no sequences yet of truffles from Asia that belong to the Maculatum clade. However, *Tuber* biodiversity in Asia is considered high and not well documented (Kinoshita et al. 2011). Discovery of the Maculatum lineage in Asia would fill a gap in understanding its biogeography.

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## LITERATURE CITED

- Badalyan RA, Hovsepyan M, Iotti M, Zambonelli A. 2005. On the presence of truffles in Armenia. *Flora Mediterr* 15:683–692.
- 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. *Proc R Soc Lond B Biol Sci* 271:1799–1806, doi:10.1098/rspb.2004.2807
- Bonito G, Gryganskyi A, Trappe J, Vilgalys R. 2010. A global meta-analysis of *Tuber* ITS rDNA sequences: species diversity, host specificity and long-distance dispersal. *Mol Ecol* 19:4994–5008, doi:10.1111/j.1365-294X.2010.04855.x
- Castellano MA, Trappe JM, Maser Z, Maser C. 1989. Key to spores of the genera of hypogeous fungi of north temperate forest with special reference to animal mycophagy. Eureka, California: Mad River Press. 186 p.
- Cázares E, García J, Castillo J, Trappe JM. 1992. Hypogeous fungi from northern México. *Mycologia* 84:341–359, doi:10.2307/3760186
- Edgar RC. 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinform* 5:1–19, doi:10.1186/1471-2105-5-113
- Fassi B, Fontana A. 1967. Sintesi micorrizica tra *Pinus strobus* e *Tuber maculatum* I. Micorrizze e sviluppo dei semenzali nel secondo anno. *Allionia* 13:177–186.
- , ———. 1969. Sintesi micorrizica tra *Pinus strobus* e *Tuber maculatum* II. Sviluppo dei semenzali trapiantati e produzione di ascocarpi. *Allionia* 15:115–120.
- Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for basidiomycetes—application to the identification of mycorrhizae and rusts. *Mol Ecol* 2:113–118, doi:10.1111/j.1365-294X.1993.tb00005.x
- Gilkey HM. 1939. Tuberales of North America. Oregon State monographs. *Stud Bot* 1:1–63.
- . 1954. Tuberales. *North America flora* 2:1–36.
- Gottel NR, Castro HF, Kerley M, Yang ZM, Pelletier DA, Podar M, Karpinetz T, Uberbacher E, Tuskan GA, Vilgalys R, Doktycz MJ, Schadt CW. 2011. Distinct microbial communities within the endosphere and rhizosphere of populus deltoides roots across contrasting soil types. *Appl Environ Microbiol* 77:5934–5944, doi:10.1128/AEM.05255-11
- Guevara G, Bonito G, Cázares E, Rodríguez JA, Vilgalys R. 2008. *Tuber regimontanum*, new species of truffle from México. *Rev Mex Micol* 26:17–20.
- Halász K, Bratek Z, Szeg D, Rudnóy S, Rácz I, Lásztity D, Trappe JM. 2005. Tests of species concepts of the small, white, European group of *Tuber* species based on morphology and rDNA sequences with special reference to *Tuber rapaeodorum*. *Mycol Prog* 4:291–298, doi:10.1007/s11557-006-0132-6
- Harkness HW. 1899. Californian hypogeous fungi. *Proc Calif Acad Sci* 1:241–292.
- Hochberg ME, Bertault G, Poitrineau K, Janssen A. 2003. Olfactory orientation of the truffle beetle, *Leiodes cinnamomea*. *Entomol Exp Appl* 109:147–153, doi:10.1046/j.1570-7458.2003.00099.x
- Huelsenbeck JP, Ronquist F. 2001. MrBayes: Bayesian inference of phylogenetic trees. *Bioinform* 17:754–755, doi:10.1093/bioinformatics/17.8.754
- Jeandroz S, Murat C, Wang YJ, Bonfante P, Le Tacon F. 2008. Molecular phylogeny and historical biogeography of the genus *Tuber*, the ‘true truffles’. *J Biogeogr* 35: 815–829, doi:10.1111/j.1365-2699.2007.01851.x
- Kinoshita A, Sasaki H, Kazuhide N. 2011. Phylogeny and diversity of Japanese truffles (*Tuber* spp.) inferred from sequences of four nuclear loci. *Mycologia* 103:779–794, doi:10.3852/10-138
- Kovács GM, Jakucs E. 2006. Morphological and molecular comparison of white truffle ectomycorrhizae. *Mycorrhiza* 16:567–574, doi:10.1007/s00572-006-0071-8
- Lange M. 1956. Danish hypogeous macromycetes. *Dan Bot Arkiv* 16:1–84.
- Maddison WP, Maddison DR. 2009. Mesquite 2.6: a modular system for evolutionary analysis. Periodical mesquite: a modular system for evolutionary analysis. <http://mesquiteproject.org>.
- Maser C, Claridge AW, Trappe JM. 2008. Trees, truffles and beasts—how forests function. New Brunswick, New Jersey: Rutgers Univ Press. 281 p.
- McGraw R, Duncan N, Cázares E. 2002. Fungi and other items consumed by the blue-gray taildropper slug (*Prophyaon coeruleum*) and the papilose taildropper slug (*Prophyaon dubium*). *Veliger* 45:261–264.
- Mello A, Murat C, Bonfante P. 2006. Truffles: much more than a prized and local fungal delicacy. *FEMS Microbiol Lett* 260:1–8, doi:10.1111/j.1574-6968.2006.00252.x
- , Vizzini A, Longato S, Rollo F, Bonfante P, Trappe JM. 2000. *Tuber borchii* versus *Tuber maculatum*: neotype studies and DNA analyses. *Mycologia* 92:326–331, doi:10.2307/3761569
- Palenzona M, Chevalier G, Fontana A. 1972. Sintesi micorrizica tra i miceli in coltura di *Tuber brumale*, *T. melanosporum*, *T. rufum* e semenzali di conifere e latifoglie. *Allionia* 18:42–52.

- Paolocci F, Rubini A, Riccioni C, Arcioni S. 2006. Reevaluation of the life cycle of *Tuber magnatum*. *Appl Environ Microbiol* 72:2390–2393, doi:10.1128/AEM.72.4.2390-2393.2006
- Pegler DN, Spooner BM, Young TWK. 1993. British truffles, a revision of British hypogeous fungi. London, Kew: Royal Botanic Gardens. 242 p.
- Stamatakis A, Hoover P, Rougemont J. 2008. A rapid bootstrap algorithm for the RAxML Web servers. *Sys Bio* 57:758–771, doi:10.1080/10635150802429642
- Swofford DL. 2002. PAUP\* 4.0: phylogenetic analysis using parsimony (\*and other methods). Sunderland, Massachusetts: Sinauer Associates.
- Trappe JM, Cázares E. 2000. *Tuber maculatum* around the world. *Bull Sem Fédération Assoc Mycol Méditerr n.s.* 18:107–112.
- , ———. 2006. *Tuber guzmanii*, a new truffle from southern México. *Int J Med Mushrooms* 8:279–282, doi:10.1615/IntJMedMushr.v8.i3.90
- , Guzmán G. 1971. Hypogeous fungi from México. *Mycologia* 63:317–332, doi:10.2307/3757764
- , Jumpponen AM, Cázares E. 1996. NATS truffle and truffle-like fungi 5. *Tuber lyonii* (= *T. texense*), with a key to the spiny-spored *Tuber* species groups. *Mycotaxon* 60:365–372.
- Vilgalys R, Hester M. 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *J Bacteriol* 172:4238–4246.
- Walker JF, Miller OK, Horton JL. 2005. Hyperdiversity of ectomycorrhizal fungus assemblages on oak seedlings in mixed forests in the southern Appalachian Mountains. *Mol Ecol* 14:829–838, doi:10.1111/j.1365-294X.2005.02455.x
- 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*. San Diego, California: Academic Press. p 315–321.
- Wurzbarger N, Bidartondo MI, Bledsoe CS. 2001. Characterization of *Pinus* ectomycorrhizae from mixed conifer and pygmy forests using morphotyping and molecular methods. *Can J Bot* 79:1211–1216, doi:10.1139/cjb-79-10-1211