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Tuber luomae, a new spiny-spored truffle species from the Pacific Northwest, USA

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Abstract: *Tuber luomae,* a new truffle species known only from the Pacific Northwest, USA, is distinguished by spiny, non-reticulate spores and a two-layered peridium — the outermost layer (pellis) consists of inflated, globose to subpolygonal cells and the inner (subpellis) of narrow hyphae. ITS sequence analyses show that it has phylogenetic affinity to other *Tuber* species in the Rufum clade. The only other members of the Rufum clade with a strongly developed peridiopellis of large, inflated cells are the southern European *T. malacodermum* and *T. pustulatum* and the northern Mexican *T. theleascum*. We find it interesting that this peridial structure that is uncommon in the Rufum clade has been found in geographically disjunct species.

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INTRODUCTION

In 1980 Dr. Dan Luoma collected six *Tuber* ascomata on Orcas Island of the San Juan Islands, located in the straits separating coastal northwestern Washington (USA) from southern Vancouver Island (Canada). Microscopic examination revealed that although this collection has spiny spores and macroscopic characteristics that place it within the Rufum clade (Bonito *et al*. 2010), it is distinguished by a pellis of subglobose to globose or subpolyhedral cells up to 35 µm broad. Three other *Tuber* collections with the same morphological features have been discovered. Two of the collections were in northwestern Oregon, consisting of one ascoma in Benton County in 1962 and two ascomata in Clackamas County in 1995; one collection in southwestern Oregon consisted of one ascoma in Jackson County in 2012. We here describe this new species, *Tuber luomae*, and use ITS rDNA sequence data to place it within the Rufum clade of the genus *Tuber*.

MATERIALS AND METHODS

Specimens

Spiny-spored *Tuber* species, collected by raking (Weber *et al*. 1997) during the past four decades, were examined in this study. When possible, data on color and morphology of fresh ascomata were recorded and the specimens photographed prior to air-drying. Sections of the type specimen of *Tuber luomae* were paraffin-embedded, sectioned by microtome, and stained with Safranin O/ Fast-green (Johansen 1940). The other specimens were rehydrated in water and characterized anatomically by light microscopy. Spore measurements exclude the surface ornamentation of spines.

Phylogenetic analysis

Collections examined in this study and analyzed by molecular methods are presented in Table 1 along with their collection information and GenBank accession numbers (the Benton County collection had been preserved in an ethanol-formalin solution that had dried; reliable sequences were not obtainable).

Two procedures were used for molecular analysis. In the first, prior to DNA extraction, a small piece of previously unexposed glebal tissue was removed from the holotype (OSC 148707) collection with a sterile razor blade and pulverized with the aid of sterile sand, cubic zirconia beads and a Mini-Beadbeater (Biospec Products, Bartlesville, OK). DNA was extracted with 24:1 chloroform:isoamyl alcohol and precipitated with isopropanol. The internal transcribed spacer region (ITS1, 5.8S nrDNA, and ITS2) and part of the nuclear ribosomal large subunit (LSU) locus were amplified with the universal fungal primer sets ITS5 – ITS4 and LROR – LR5 (Vilgalys & Hester 1990, White *et al*. 1990, Bertini *et al*. 1999). The PCR cocktail and protocol followed that of Healy *et al*. (2009). PCR products were stained with 1× SYBR Safe (Invitrogen, Carlsbad, CA) and visualized on a 1.0 % agarose gel buffered with TAE buffer. Gel electrophoresis products were viewed on a GelDoc XR imager (Bio-Rad Laboratories, Inc., Hercules, CA). Qiagen Quick-Clean columns were used to clean PCR products prior to sequencing.

A second set of molecular methods was applied to the paratype specimens from Clackamas (OSC 148706) and Jackson (OSC

Table 1. Collections examined in this study and analyzed by molecular methods along with their collection information and GenBank accession numbers (the Benton County collection had been preserved in an ethanol-formalin solution that had dried; reliable sequences were not obtainable).

Species	Collection ID[1]	Origin	GenBank Accesion No.
Tuber candidum	SOC 727	USA: Oregon	AY830856
Tuber ferrugineum	n/a	n/a	AF132506
Tuber huidongense	IFS Y. Wang 89924	China	DQ478632
Tuber indicum	Tind-my02	China	DQ329365
Tuber liaotongense	Tsp-hr02	China	DQ478645
Tuber luomae	*JT6003	USA: Washington	FJ809887
	RB12-139	USA: Oregon	MH142475
	JT17457	USA: Oregon	MH142474
Tuber lyonii	JT5665	USA: Texas	FJ809883
Tuber melanosporum	1015	Israel	AF167096
Tuber melosporum	AH31737	Spain	JX402095
Tuber nitidum	BM105	Spain	FJ809885
Tuber pustulatum	JT32319	Spain	FJ809889
	AQUI 9725	Spain	MK211278
Tuber quercicola	SOC 733	USA: Oregon	AY918957
Tuber regimontanum	*ITC909	Mexico	EU375838
Tuber rufum	BOLO1506-1	Italy	AY112894
Tuber rufum var. lucidum	AZ2097	Italy	FJ809888
Tuber spinoreticulatum	*Uecker188	USA: Maryland	FJ748913
Tuber taiyuanense	IFS Y. Wang 610	China	DQ478637
Tuber theleascum	AQUI 9729	Mexico	MK211283
Tuber wenchuanense	FL-2013d strain HMAS 60239	China	JX267044

[1] Asterisk (*) denotes type collection.

151373) counties; the Sigma Extract-N-Amp™ (Sigma-Aldrich, St. Louis, MO) protocol was followed by use of 15 µL of extract solution and 30 µL of dilution solution. PCR was performed with the universal primer set ITS1-F – ITS4 (White *et al*. 1990, Gardes & Bruns 1993) or the reverse primer ITS4Tuber, which was designed to improve specificity for *Tuber* (CTC GAC TCG TAG AAG ACA CT, Bonito unpublished). PCR products were stained with ethidium bromide after gel electrophoresis. PCR products were cleaned with ExoSAP-IT® (Affymetrix, Santa Clara, CA) prior to sequencing.

Sanger sequencing was performed in both directions using a BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) with ITS1-F, ITS5 or LROR (forward) and ITS4, ITS4Tuber or LR5 (reverse). DNA sequences were determined on an ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Foster City, CA). Forward and reverse sequences were assembled and Sequencher v. 4.0 (Gene Codes, Ann Arbor, MI) was used to manually edit sequences and ambiguous regions at the ends were removed. Both ITS and LSU sequences were queried against the NCBI public database GenBank with the BLASTN algorithm for comparison with other sequences and to verify that the sequences belonged to *Tuber*.

DNA sequences were aligned manually for phylogenetic analysis with MacClade v. 4.0 (Maddison & Maddison 2002) and ambiguously aligned regions were excluded. Phylogenetic inference was conducted on the ITS alignment with RAxML computed through the CIPRES web portal (www.phylo.org) with a GTR model nucleotide substitution, and 1 000 bootstrap iterations. Outgroup taxa belonging to the Melanosporum clade were used because they are known to comprise the phylogenetic sister group to the Rufum clade within the genus *Tuber* (Bonito *et al*. 2010, 2013). Sequences produced with these procedures were deposited in GenBank under accession numbers FJ809887, MH142474 and MH142475, as was an LSU sequence not used in the alignment (FJ809812). Sequence alignments were deposited at TreeBASE under the accession number 22992.

RESULTS

Taxonomy

The description of *Tuber luomae* is based on the holotype. The three paratypes were carefully examined and did not differ in any significant details.

Tuber luomae Trappe, Eberhart, Piña Páez & Bonito, ***sp. nov.*** MycoBank MB807402. Fig. 1.

Etymology: In honor of distinguished mycologist Dr. Dan Luoma, collector of the holotype, for his many contributions to mycology.

Typus: **USA**, Washington, San Juan Co., Orcas Island, Camp Orkila, elev. 20 m, 31 Oct. 1980, *D. Luoma,* Trappe 6003 (**holotype** OSC 148707; ITS GenBank FJ809887, LSU GenBank FJ809812).

Description: *Ascomata* when dried 20–25 × 25–30 mm, irregular, the surface even or with prominent wrinkles. *Peridium* light orange brown, minutely verrucose with a reddish-brown pellis over a pallid subpellis. *Gleba* with light brown fertile tissue

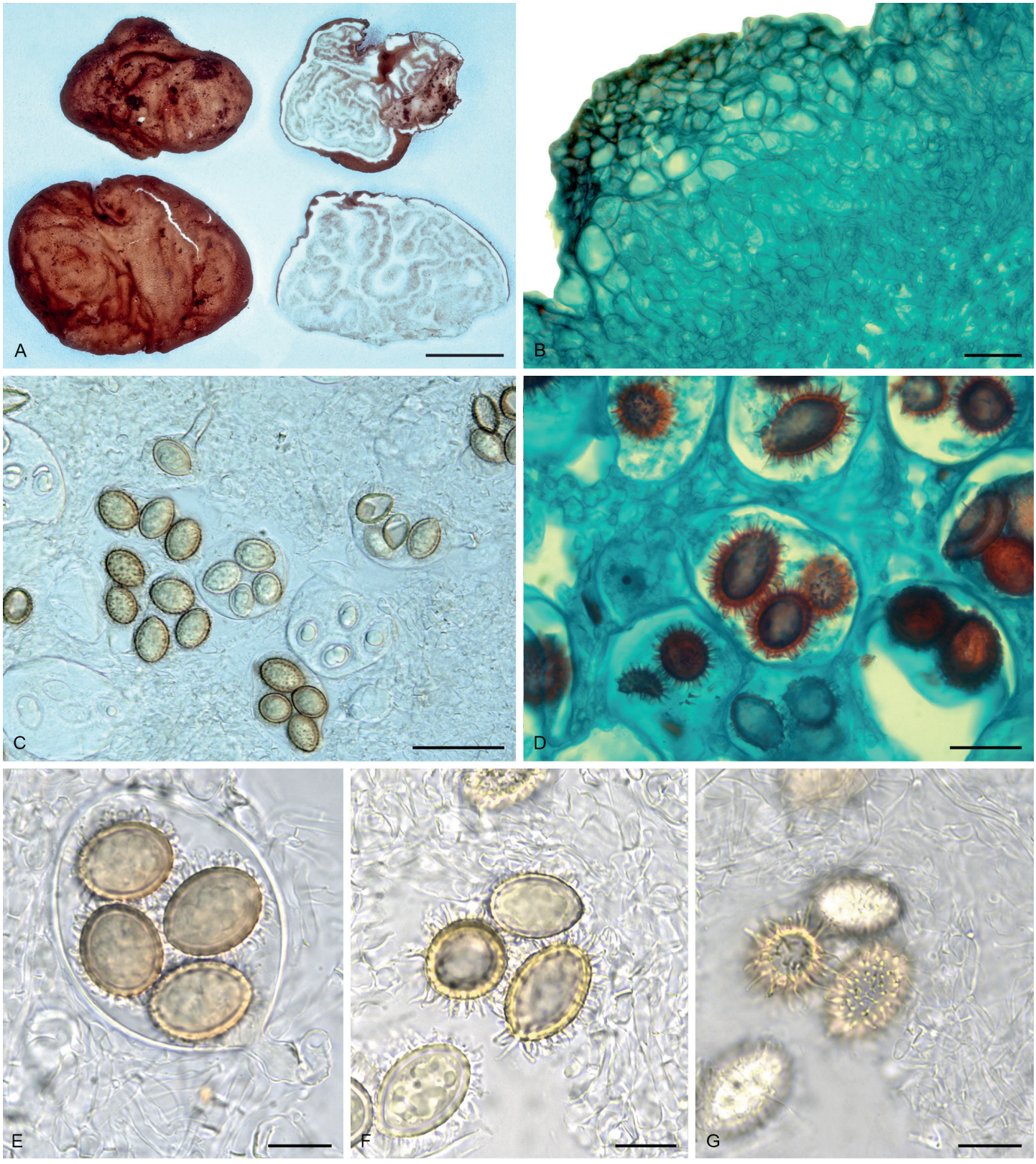

Fig. 1. Morphological characters of *Tuber luomae* (Holotype, OSC 148707). **A.** Ascocarp, surface and cross-section view. **B.** Outer peridium in cross section composed of inflated cells with inner peridium abruptly differentiated as interwoven hyphae, stained with Safranin O/ Fast-green. **C.** One- to four-spored asci. **D.** Spiny spores stained with Safranin O/ Fast-green. **E.** Four-spored ascus with distinctive basal hyphal stem. **F.** Spore ornamentation, highlighting the spines. **G.** Spores at two focal depths illustrating the spines and lack of connecting reticulum. Scale bars: A = 10 mm; B, D = 20 µm; C = 40 µm; E–G = 15 µm.

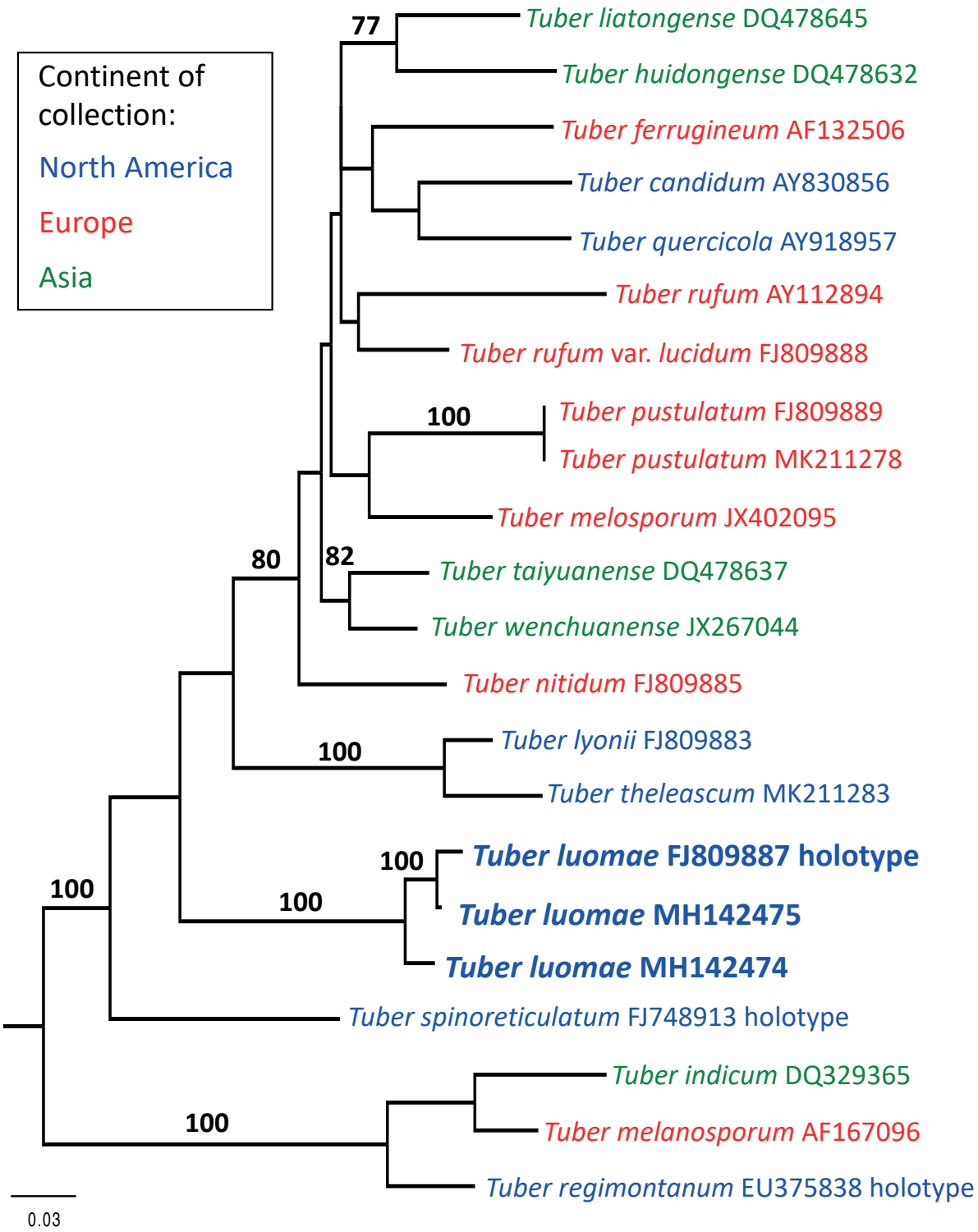

Fig. 2. Phylogenetic reconstruction of the *Tuber rufum* clade based on maximum likelihood analysis of the ITS rDNA region with 16 ingroup and 3 outgroup taxa (*T. melanosporum*, *T. indicum*, and *T. regimontanum*). The analysis was based on a GTR model of nucleotide substitutions and resulted in a most likely tree with a log likelihood score of –ln 4198.629. Scale bar = 0.03 substitutions per site.

marbled with prominent white veins. *Odor* in the field mildly acrid. *Peridium* ± 500 µm thick, the outermost layer (pellis) ± 150 µm thick with crowded flattened warts 55–180 µm tall and 100–375 µm broad, of ellipsoid to subglobose or subpolyhedral cells 6–30(–38) µm broad, those of the outer ± 50 µm of the ascomata surfaces with yellow to yellowish brown walls 1–3 µm thick imparting an orange brown color to the tissue, below which the inflated cells are hyaline and walls mostly < 1 µm thick; subpellis ± 350 µm thick, gradually to abruptly differentiated from the pellis as interwoven, hyaline, thin-walled hyphae 2–4(–8) µm broad. *Asci* hyaline, globose to ellipsoid or pyriform, 65–80 × 40–60 µm excluding the basal hyphal stem, 10–38 × 7–9 µm; walls thin (< 1 µm) in youth, with age thickened to 2 µm. *Spores* 1–5/ascus, ellipsoid to broadly ellipsoid, ornamented with crowded, discrete hyaline spines 3–4 × 1–1.5 µm. Spores in 1-spored asci (infrequent) 30–40 × 24.5–35 µm (Q = 1.18–1.45), 2-spored 25–33.5 × 21–28 µm (Q = 1.04–1.45), 3-spored 23–30 × 18.5–23 µm (Q = 1.21–1.32), 4-spored 20–26 × 14–21 µm (Q=1.19–1.4),

5-spored (infrequent) 18–26 × 15–21 µm (Q = 1.2–1.31); walls ± 2 µm thick, light yellow brown.

Distribution, habitat, and phenology: Cascade Mountains of far southwest Oregon and north on its western slopes as well as at the eastern slope of the Coast Ranges, thence to Washington's far north in the San Juan Islands; elevations range from 20 to 1 545 m. Habitats range from various mixtures and ages of *Pseudotsuga menziesii, Abies grandis,* and *Alnus rubra* to in one case, a partially harvested old-growth and in another, a pure stand of *Tsuga heterophylla.* The one collection in August was relatively immature, the other three were well matured.

Paratypes: **USA**: Oregon, Benton County, Rock Creek Park (abandoned) off State Hwy. 34, elev. 137 m, in soil, 24 Aug. 1962, *R. Benjamin* No. 38, OSC 158256; Clackamas County, Hwy. 224, SW of Timber Lake, elev. 550 m, in soil, 1 Nov. 1995, *A. Beyerle* B316, OSC 148706 (Trappe 17457); Jackson Co., Conde Creek, elev. 1 545 m, in soil, 4 Nov. 2012, *R. Brock* RB12-139, OSC 151373.

DISCUSSION

It is now evident from recently published molecular studies on *Tuber* and new species descriptions that *Tuber* is even more diverse than previously suspected (Bonito *et al*. 2010, Fan *et al*. 2011, 2012a, b, 2013, Alvarado *et al*. 2012a, Guevara *et al*. 2013, Zambonelli *et al.* 2016). In their assessment of the molecular diversity of *Tuber*, Bonito *et al*. (2010) and Healy *et al*. (2016) reported that the Rufum clade is among the more diverse in the genus and includes numerous cryptic undescribed species.

The Rufum clade is distributed across the Northern Hemisphere with centers of endemism in Europe, Asia, and North America (Bonito *et al*. 2013). While most species in this clade are characterized by spiny spores, some, such as *T. spinoreticulatum* and *T. lyonii*, have spiny spores with low reticulations (Uecker & Burdsall 1977, Trappe *et al*. 1996), whereas *T. liaotongense* has reticulate spores (Cao *et al*. 2011), and *T. melosporum* has smooth spores (Alvarado *et al*. 2012b).

Tuber luomae, known only from four collections in the Pacific Northwest (USA), is characterized by spiny-spores and a thick peridiopellis consisting of a pseudoparenchyma of inflated, subglobose to subpolygonal cells. Until recently, the only other described species in clade Rufum with a strongly pseudoparenchymatous pellis was *T. malacodermum* (Fischer 1923), known only from the holotype from Switzerland. Unlike *T. luomae,* it has a smooth peridiopellis, and the spines on its spores are occasionally to frequently connected by low ridges ('lines') on the spore surface, sometimes forming a partial reticulum. In addition, *T. malacodermum* has smaller asci but longer ascus stems than *T. luomae.* Despite their similar and distinctive peridial anatomy, they are readily distinguished by their spore ornamentation, ascus size, and ascus stem length.

Leonardi *et al*. (2019) examined the *T. malacodermum* holotype, as well as putative *T. malacodermum* ascomata collected in Spain, Corsica, and Mexico. Because morphological characters separated the type specimen from all the other collections, the authors concluded that the holotype is the only known collection of *T. malacodermum*. The type collection was too poorly preserved to yield DNA sequences, but molecular analysis of the newer species placed them into two previously unidentified groups. The specimens from Spain and Corsica were named *T. pustulatum*, whereas the Mexican specimens were named *T. theleascum* (Leonardi *et al.* 2019). Molecular analyses by Leonardi *et al.* (2019) show that *T. luomae* is more closely related to the North American *T. theleascum* than to the European *T. pustulatum* (Fig. 2).

Tuber luomae resembles *T. pustulatum* in the structure of the peridium; both species have a two-layered peridium with an outermost layer composed of globose to angular cells and a subpellis of interwoven hyphae. They differ in the spore ornamentation; spines are shorter in *T. luomae* (up to 4 µm long) in comparison with *T. pustulatum* (up to 7 µm long). Moreover, the spines in *T. pustulatum* are united at the base, forming a reticulum, while the spines in *T. luomae* are discrete (Fig. 1F, G). *Tuber luomae* is also similar to *T. theleascum* in peridial structure, but differs in the thickness of the peridium (*T. theleascum* 160–250 µm, *T. luomae* ± 500 µm). They also differ in spore ornamentation, with spines often interconnected in *T. theleascum* but not connected in *T. luomae*.

These four species (*Tuber luomae, T. malacodermum*, *T. pustulatum* and *T. theleascum*), which have in common a peridiopellis of isodiametric cells rather than the narrow hyphae of all other known members of the clade, are derived from separate lineages. We find it interesting that this peridial structure that is uncommon in the Rufum clade has been found in geographically disjunct species (Fig. 1). *Tuber luomae* has phylogenetic affinity to other North American *Tuber* species in the Rufum clade. In the Pacific Northwest, other Rufum clade species (*T. candidum* and *T. quercicola*) are readily distinguished from *T. luomae* by having a peridiopellis of tightly interwoven hyphae that lack or have only widely scattered inflated cells. *Tuber luomae* can be regarded as rare, considering that hypogeous sporocarps (truffles) have been extensively sought by many dozens of collectors producing thousands of collections over more than a century of searching in western Washington and Oregon.

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Conflict of interest: The authors declare that there is no conflict of interest.

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