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Improved resolution of major clades within *Tuber* and taxonomy of species within the *Tuber gibbosum* complex

Gregory Bonito[1]
Department of Biology, Duke University, Durham, North Carolina 27708–0338

James M. Trappe
Department of Forest Ecosystems and Society, Oregon State University, Corvallis, Oregon 97331–5752

Pat Rawlinson
North American Truffling Society, P.O. Box 296, Corvallis, Oregon 97339

Rytas Vilgalys
Department of Biology, Duke University, Durham, North Carolina 27708–0338

Abstract: *Tuber gibbosum* Harkn., described from northern California, originally was thought to be a single, variable species that fruited from autumn through winter to spring. It has become popular as a culinary truffle in northwestern USA, where it is commercially harvested. Morphological studies suggested it might be a complex that includes at least two species. We conducted morphological and phylogenetic studies of the complex to determine how many species it might contain and how they differed morphologically, geographically and seasonally. We also provide the first LSU phylogeny for the genus *Tuber*. Phylogenetic analyses resolve nine major clades in the genus with high bootstrap support and distinguish the Gibbosum clade from the Aestivum, Excavatum, Macrosporum, Magnatum, Melanosporum, Puberulum, Rufum and Spinoreticulatum clades. Further analyses of ITS and LSU regions revealed four distinct species in the Gibbosum complex. Although morphologically similar the four species differ in spore size and shape and in peridial anatomy. These species share the synapomorphy of having suprapellis hyphae with distinctive, irregular wall swellings at maturity; we have not seen this hyphal type in any other *Tuber* spp. worldwide. The three new species are named and described as *T. bellisporum* Bonito & Trappe, *T. castellanoi* Bonito & Trappe and *T. oregonense* Trappe, Bonito & Rawlinson.

Key words: Ascomycota, hypogeous fungi, ITS, LSU, mycorrhizae, Oregon white truffle, Pezizales, phylogeny, Tuberaceae

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[1] Corresponding author. E-mail: gmb2@duke.edu

INTRODUCTION

In 1899 H.W. Harkness published his seminal paper on California hypogeous fungi, the first serious work on these fungi in North America. *Tuber gibbosum* Harkn. was included among the 48 new species he described. Gilkey (1925) described a related species, *T. giganteum* Gilkey, but later reduced that species to synonymy with *T. gibbosum* (Gilkey 1939). *Tuber gibbosum* appeared to be the most abundant species of the genus in the Pacific Northwest, ranging from the San Francisco Bay area of California north to Vancouver Island, British Columbia. It was thought to fruit from early autumn through winter and into the early summer and to be primarily, if not exclusively, associated with Douglas-fir (*Pseudotsuga menziesii*) at relatively low elevations west of the Cascade Mountains. *Tuber gibbosum* has been hypothesized to be part of the Puberulum clade, based on morphology, because of its light-colored fruit body and alveolate-reticulate spore ornamentation (Wang et al. 2007).

Tuber gibbosum is called the Oregon white truffle because of being white when immature and its aromatic resemblance to *Tuber magnatum* Pico, the Italian white truffle. However several color and aromatic differences have been detected among seasonal collections of *T. gibbosum* and amateur collectors have differentiated two varieties, autumn and spring. Since the 1980s both have been commercially harvested from forests in the Pacific Northwest (Lefevre et al. 2001).

The aim of this study was to use both molecular phylogenetics and morphology to assess species diversity within the *Tuber gibbosum* complex and to place them within the larger context of the genus *Tuber*. We selected a broad geographic and seasonal array of collections for rDNA sequencing and morphological analyses. As a result we present a comprehensive LSU phylogeny for the genus *Tuber* that included representative taxa from North America, Europe and Asia, established a new epitype for *T. gibbosum* and described three other species in the Gibbosum complex.

MATERIALS AND METHODS

Material studied.—We investigated more than 100 collections from the *Tuber gibbosum* complex, representing a broad geographic and seasonal range, from the mycological

collections of the Oregon State University Herbarium (OSC). Fresh specimens of other species found during the study in North America, Italy and China also were studied and accessioned into OSC. Types and paratypes were examined and molecular data from these collections have been included whenever possible. Specimens from the National Fungus Collections (BPI) and the Herbarium of Università di Bologna (BOLO), Italy, also were studied. Collection numbers and geographic origin of collections used in molecular analyses are provided (TABLE I).

Molecular methods.—DNA was extracted by the chloroform extraction technique (Gardes and Bruns 1993). For DNA extraction glebal tissue was ground, dried in cetyl trimethylammonium bromide (CTAB) in sterile sand and large cubic zirconium beads in a mini bead-beater 1–2 min (Biospec Products, Bartlesville, Oklahoma). The internal transcribed spacer region (ITS1, 5.8S and ITS2) and part of the nuclear ribosomal large subunit (LSU) locus were amplified with universal fungal primer sets ITS5– ITS4, 5.8S-LR3 and LROR– LR5 (Vilgalys and Hester 1990, White et al. 1990). The PCR protocol began with an initial denaturation at 94 C (3 min), followed by 35 cycles at 94 C (1 min), 50 C annealing (30 s), and a 72 C extension (1 min), with a final extension at 72 C (7 min). Each 25 μL PCR reaction consisted of 4 μL dNTP (1.25 μM, 2.5 μL PCR buffer, 1 μL BSA, 1.25 μL primer 1 (10 μM), 1.25 μL primer 2 (10 μM), 0.15 μL *Taq* DNA polymerase (0.5 U), 4.8 μL water and 10 μL DNA extract (~ 10 ng/μL). Two microliters of each PCR product were loaded into a 1.0% agarose gel buffered with TAE and stained with 2 μL SYBR Safe (Invitrogen, Carlsbad, California) per 80 mL TAE. Gel electrophoresis products were viewed on a GelDoc XR imager (Bio-Rad Laboratories Inc., Hercules, California). PCR products were cleaned in QIAGEN quick-clean columns and used for PCR sequencing reaction. Sanger sequencing was performed with Big Dye chemistry 3.1 (Applied Biosystems, Foster City, California) with the forward primer ITS5, 5.8S or LROR and the reverse primer ITS4, LR3 or LR5. DNA sequences were determined on an ABI3700 capillary sequencer (Applied Biosystems, Foster City, California).

Phylogenetic analyses.—DNA sequences were manually trimmed and edited with Sequencher 4.0 (Gene Codes Corp., Ann Arbor, Michigan) and sequence-similarity values were calculated. Both ITS and LSU sequences were queried against the NCBI public database GenBank by use of the BLASTN algorithm to verify that sequences were of *Tuber* (Altschul et al. 1990). DNA sequences were aligned in MacClade 4.0 and adjusted by eye (Maddison and Maddison 2002). Ambiguously aligned regions were excluded from the alignment. Phylogenetic analyses first were carried out for genus *Tuber* with LSU data to determine both the phylogeny of the genus and appropriate outgroup taxa for analyzing the *T. gibbosum* group. A second set of phylogenetic analyses then was conducted to assess finer scale structuring within the Gibbosum group and included greater taxon sampling within this group and an additional genetic marker (ITS). Analyses were conducted on both the ITS and LSU alignments, individually and combined. Unweighted maximum parsimony (MP) heuristic searches were calculated by PAUP 4.0b10 with 1000 random addition sequences and 5000 bootstrap replicates (Swofford 2002). Maximum likelihood (ML) searches were conducted with GARLI (Zwickl 2006) and Bayesian inference (BI) with MrBayes (Ronquist and Huelsenbeck 2003). Alignments have been accessioned in TreeBASE (s2482), and the 133 sequences are available on GenBank under accession numbers FJ809749–FJ809882 (TABLE I).

Light microscopy.—Herbarium specimens were hand-sectioned with a razorblade and rehydrated in water or embedded in paraffin, thin-sectioned (± 8 μm thick), stained with safranin-fast green and made into permanent slides, then characterized anatomically by light microscopy. Six morphological characters were assessed from multiple collections of *T. gibbosum* and the three new species described here: (i) peridial structure and thickness as measured in cross section; (ii) glebal trama and sterile vein structure; (iii) ascus shape, size, wall thickness; (iv) length and width of spores (n = 40) in one-, two-, three-, four- and five-spored asci (excluding ornamentation) for at least three collections of each species; (v) spore ornamentation (height, number of alveolae along spore length, size of alveolae); (vi) presence of surface hyphae with irregular wall thickenings. To determine the significance of spore size variation among species analysis of variance (ANOVA) and Tukey's honestly significant difference tests were calculated with R statistical software (http://www.r-project.org). Where two dimensions of structures are reported in the descriptions, length is followed by width. Q refers to the length/width ratio.

Scanning electron microscopy.—Cross sections of dried herbarium specimens were attached to aluminum mounts with double-sticky tape. These were gold-palladium sputter coated at a nominal coating thickness of 15 nm with a Hummer 6.2 system (Anatech Ltd., Springfield, Virginia). Peridial, glebal and spore structures were characterized at 10 kv with a Philips XL30 environmental scanning electron microscope under high vacuum (FEI Co., Hillsboro, Oregon).

RESULTS

Molecular analyses.—Phylogenetic inferences of LSU rDNA with a GTR + G + I model of nucleotide substitution having four substitution classes, 64 ingroup taxa and 537 unambiguously aligned characters (174 parsimony informative characters) support *Tuber* as monophyletic and place the *T. gibbosum* complex as a distinct clade within the genus. The sister group of the Gibbosum clade however was not resolved. Eight additional major clades were resolved in *Tuber*: Aestivum, Excavatum, Magnatum, Spinoreticulatum, Rufum, Melanosporum, Macrosporum and Puberulum (FIG. 1).

Because phylogenies resulting from ITS and LSU datasets were internally congruent these datasets were combined for finer scale analyses of genetic diversity within the Gibbosum group (FIG. 2). The final data

TABLE I. Collection information for specimens and sequences used in this study for molecular analyses

Species Name	Voucher	Location	GenBank number	
			ITS	LSU
Choiromyces alveolatus	JT22830	Oregon, USA		AF435826
Choiromyces meandriformis	RH691	Iowa, USA		FJ809794
Choiromyces meandriformis	GB285	Oregon, USA		FJ809795
Dingleya verrucosa	JT12617	New Zealand		U42686
Reddellomyces donkii	JT13292	California		U42687
Tuber aestivum	JT23265	Serbia		FJ809843
Tuber aestivum	SG14	Gotland, Sweden		EU424155
Tuber aestivum	JT30500	Gotland, Sweden		FJ809844
Tuber cf. *anniae*	JT22986	Washington, USA	FJ809851	FJ809804
Tuber bellisporum PARATYPE	JT11679	Oregon, USA	FJ809855	FJ809826
Tuber bellisporum HOLOTYPE	JT7270	Oregon, USA	FJ809856	FJ809827
Tuber bellisporum PARATYPE	JT6060	Oregon, USA	FJ809857	FJ809828
Tuber borchii	Y86	Austria		AY515305
Tuber borchii	GB32	Emilia-Romagna, Italy	FJ809852	FJ809799
Tuber brumale	GB52	Emilia-Romagna, Italy		FJ809817
Tuber brumale	GB53	Emilia-Romagna, Italy		FJ809818
Tuber californicum		California, USA		AF127120
Tuber canaliculatum	JT17440	West Virginia, USA		FJ809841
Tuber canaliculatum	JT12448	Quebec, Canada		FJ809842
Tuber candidum	SRC625	California, USA		DQ974807
Tuber castellanoi PARATYPE	JT4568	California, USA	FJ809858	FJ809829
Tuber castellanoi PARATYPE	JT19924	California, USA	FJ809859	FJ809830
Tuber castellanoi HOLOTYPE	JT28069	California, USA	FJ809860	FJ809831
Tuber dryophilum	GB66	Emilia-Romagna, Italy		FJ809800
Tuber dryophilum	GB63	Emilia-Romagna, Italy		FJ809801
Tuber excavatum	JT8539	Italy		FJ809824
Tuber excavatum	BM100	Spain		FJ809825
Tuber ferrugineum	MA2721	unknown		FJ809809
Tuber gennadii	BM667	Spain		FJ809849
Tuber gibbosum	JT12477	Oregon, USA	FJ809861	FJ809832
Tuber gibbosum	JT26632	Washington, USA	FJ809862	FJ809862
Tuber gibbosum EPITYPE	JT6555	California, USA	FJ809863	FJ809833
Tuber gibbosum	JT27987	Oregon, USA	FJ809864	FJ809834
Tuber gibbosum	JT30681	Oregon, USA	FJ809866	FJ809866
Tuber gibbosum	J11493	Oregon, USA	FJ809867	FJ809867
Tuber gibbosum	JT30580	Oregon, USA	FJ809868	FJ809868
Tuber gibbosum	JT22789	Oregon, USA	FJ809869	FJ809869
Tuber gibbosum	JT29402	Oregon, USA	FJ809870	FJ809870
Tuber gibbosum	JT12396	Oregon, USA	FJ809865	FJ809865
Tuber guzmanii ISOTYPE	JT3474	Morelos, Mexico		FJ809803
Tuber indicum group A	JT15417	Sichuan, China		FJ809820
Tuber indicum group A	KUN29360	China		FJ809821
Tuber indicum group B	GB339	China		FJ809822
Tuber indicum group B	GB237	China		FJ809840
Tuber irradians	JT19426	Oregon, USA	FJ809850	FJ809796
Tuber irradians	JT7696	Oregon, USA		FJ809797
Tuber levissimum	HS2025	unknown		FJ809798
Tuber liaotongense	OSC87602	China		FJ809813
Tuber luomai	JT17457	Oregon, USA		FJ809812
Tuber lyonii	GB112	Georgia, USA	EU394704	EU394704
Tuber lyonii	GB119	Virginia, USA		FJ809808
Tuber macrosporum	JT13362	Emilia-Romagna, Italy		FJ809838
Tuber macrosporum	JT19458	Emilia-Romagna, Italy		FJ809839
Tuber maculatum	TL5974	Denmark		AJ969627

TABLE I. Continued

Species Name	Voucher	Location	GenBank number	
			ITS	LSU
Tuber magnatum	JT19369	unknown		FJ809847
Tuber magnatum	JT19460	Emilia-Romagna, Italy		FJ809848
Tuber melanosporum				AF435821
Tuber melanosporum	GB255	Spain		FJ809819
Tuber cf. *nitidum*	BM105	Spain		FJ809807
Tuber oligospermum	Y94A	Austria		AY515306
Tuber oregonense PARATYPE	JT27985	Oregon, USA	FJ809871	FJ809871
Tuber oregonense PARATYPE	JT28266	Oregon, USA	FJ809872	FJ809872
Tuber oregonense PARATYPE	JT27173	Oregon, USA	FJ809873	FJ809873
Tuber oregonense HOLOTYPE	GB284	Oregon, USA	FJ809874	FJ809835
Tuber oregonense PARATYPE	JT12387	Oregon, USA	FJ809875	FJ809875
Tuber oregonense PARATYPE	JT22669	Oregon, USA	FJ809876	FJ809876
Tuber oregonense PARATYPE	JT22691	Washington, USA	FJ809877	FJ809877
Tuber oregonense PARATYPE	JT27945	Oregon, USA	FJ809878	FJ809836
Tuber oregonense PARATYPE	JT8767	Oregon, USA	FJ809879	FJ809837
Tuber oregonense	GB287	Oregon, USA	FJ809880	FJ809880
Tuber oregonense PARATYPE	JT15112	Oregon, USA	FJ809881	FJ809881
Tuber oregonense PARATYPE	JT28263	Oregon, USA	FJ809882	FJ809882
Tuber panniferum	BM70	Spain		FJ809845
Tuber panniferum	JT12835	Cordoba, Spain		FJ809846
Tuber pseudoexcavatum	CJ408	Yunnan, China		FJ809816
Tuber puberulum	TL3857	Denmark		AJ969625
Tuber regimontanum HOLOTYPE	ITCV909	Nuevo Leon, Mexico	EU375838	EU375838
Tuber rufum	GB211	Italy		FJ809810
Tuber rufum	GB213	Italy		FJ809811
Tuber cf. *shearii*	JT9933	Michigan, USA		FJ809802
Tuber sphaerosporum	JT12487	Oregon, USA	FJ809853	FJ809805
Tuber sphaerosporum	JT19772	Oregon, USA	FJ809854	FJ809806
Tuber spinoreticulatum	RH158	Iowa, USA		FJ809814
Tuber spinoreticulatum ISOTYPE	Uecker188	Maryland, USA		FJ809815

matrix included 1256 unambiguously aligned characters (201 parsimony informative), 28 ingroup sequences and five outgroup taxa. A GTR + G model with four substitution classes was best fit for this dataset. Trees resulting from BI and MP were congruent, but ML analyses consistently placed *T. oregonense* on a long internal node (see FIG. 1). Nonetheless four species (*T. bellisporum*, *T. castellanoi*, *T. gibbosum* and *T. oregonense*) were resolved within the Gibbosum complex with high statistical support under BI, ML and MP criteria. No more than 1% intraspecific ITS variation was detected within collections of these four species. The ITS sequences of *Tuber castellanoi* and *T. bellisporum* had the highest pairwise similarity (94%), while the ITS of *T. oregonense* was the least similar (89% similar to other species in the complex).

Morphological characters.—Species in the Gibbosum clade can easily be distinguished from *Tuber* species in other clades by the peculiar wall thickenings on hyphal tips emerging from the peridial surface at maturity (FIG. 3D). Among other hypogeous Ascomycota a similar formation has been found only on *Choiromyces alveolatus* (Harkn.) Trappe (Trappe 1975). *Tuber bellisporum*, *T. castellanoi*, *T. gibbosum* and *T. oregonense* each are supported by a suite of morphological characters but display considerable intraspecific morphological variation. The consistency of the molecular data let us determine characters useful for identification of mature specimens by morphology.

Spore length and Q differed significantly among the four species (FIG. 4), in one-, two-, three- and four-spored asci. *T. castellanoi* with Q = 1.0–1.6 and *T. oregonense* with Q = 1.5–2.5(–2.9) differed the most from each other and from *T. bellisporum* (Q = 1.0–2.1) and *T. gibbosum* (Q = 1.1–1.8). Although spores of *T. oregonense* and *T. bellisporum* are similar in width, they differ significantly in length and Q. Conversely spores of *T. gibbosum* and *T. bellisporum*

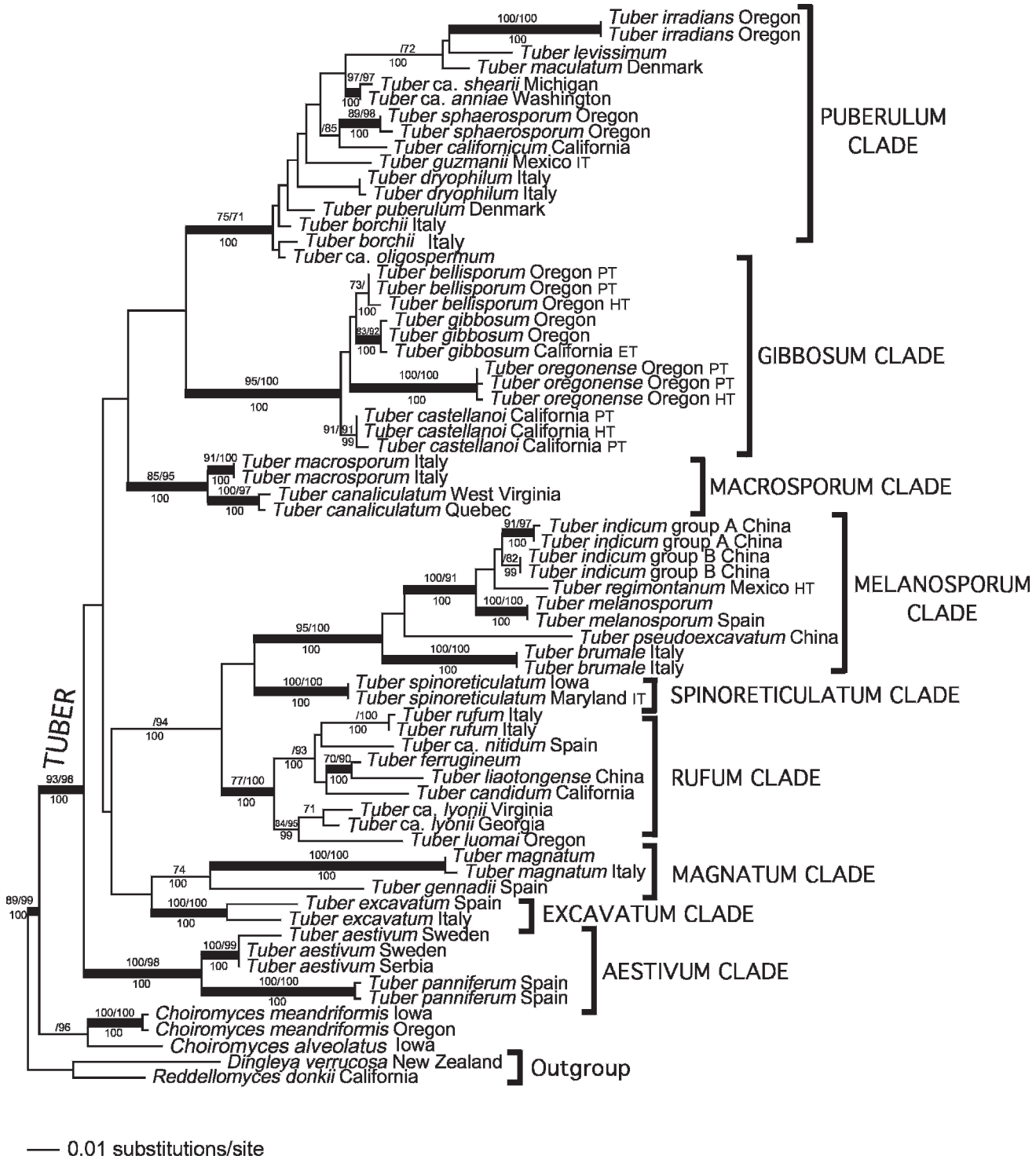

FIG. 1. Most likely tree for genus *Tuber* based on LSU nuclear rDNA showing the phylogenetic placement of the Gibbosum clade within the genus. Maximum parsimony (MP) bootstrap values are shown on top of the branches, followed by maximum likelihood (ML) bootstrap values. Posterior probabilities (BI) are below branches. Thickened branches represent nodes supported with high bootstrap values by all three methods of inference (> 70 for MP and ML, 100 for BI). Species labels include Latin binomials and geographic origin (when known). Nine major *Tuber* clades are resolved and labeled. Southern hemisphere species from other genera in the Tuberaceae were included as outgroups. HT = holotype, ET = epitype, PT = paratype, IT = isotype.

FIG. 2. Phylogeny of the Gibbosum clade based on ITS and LSU nuclear rDNA inferred by Bayesian analysis (BI). Bootstrap values are shown on top of branches with maximum parsimony (MP) followed by maximum likelihood (ML), and posterior probabilities are shown below branches. Thickened branches represent nodes supported with high bootstrap values by all three methods of inference (> 70 for MP and ML, 100 for BI). Species are labeled by their Latin binomial. HT = holotype, ET = epitype, PT = paratype.

may overlap in length and Q but spore widths overlap little (FIG. 4).

Some spores in well matured specimens of each species showed an even ''microreticulum'' when viewed in optical cross section (FIG. 3E, F, G, I, K); Montecchi and Sarasini (2000, p 282) illustrated this phenomenon in a species they identified as *T. gibbosum* and regarded it as a ''double reticulum'', with a small-meshed reticulum nested on the spore surface within the alveolae of the larger reticulum. In our studies however SEM showed no such structures on spore surfaces of any of the four species in the Gibbosum complex (FIG. 3M–P) or on the inner surface of the spore wall (FIG. 3P). *T. castellanoi* has

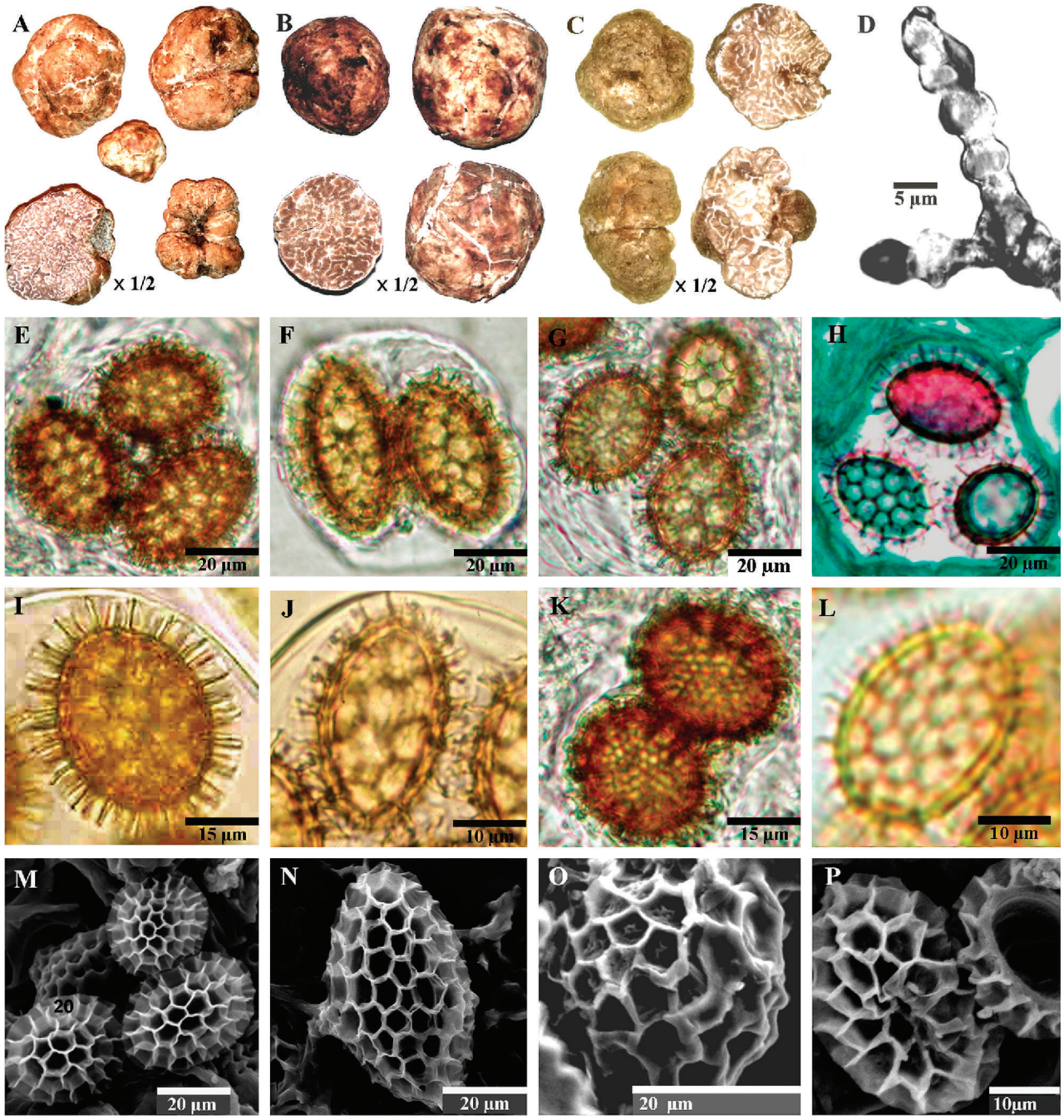

FIG. 3. Morphological characteristics of species in the Tuber gibbosum group. Fresh ascomata: A. *T. gibbosum*; B. *T. oregonense*; C. *T. castellanoi*. D. Beaded peridial hyphae of *T. bellisporum* but characteristic of all four species in this group. Asci: E. *T. gibbosum*; F. *T. oregonense*; G. *T. castellanoi*; H. *T. bellisporum*. Ascospores: I. *T. gibbosum*; J. *T. oregonense*; K. *T. castellanoi*, showing ''microreticulation''; L. *T. bellisporum*. SEM Micrographs: M. *T. gibbosum*; N. *T. oregonense*; O. *T. castellanoi*; P. *T. bellisporum*.

some minute, irregularly raised prominences on the spore surfaces within the alveolae as seen by SEM (FIG. 3O), but these do not resemble the ''microreticulum'' that appears when the light microscope objective is focused on the optical cross section of its spores (FIG. 3K). We are collaborating with an optical engineer, Frank Evans, to discover the origin of the ''microreticulum''.

Of the four species only *T. gibbosum* forms a well developed, epithelial pellis of large, inflated cells; this is usually evident in fairly young specimens (FIG. 5D). *T. castellanoi* forms a relatively weak and variable

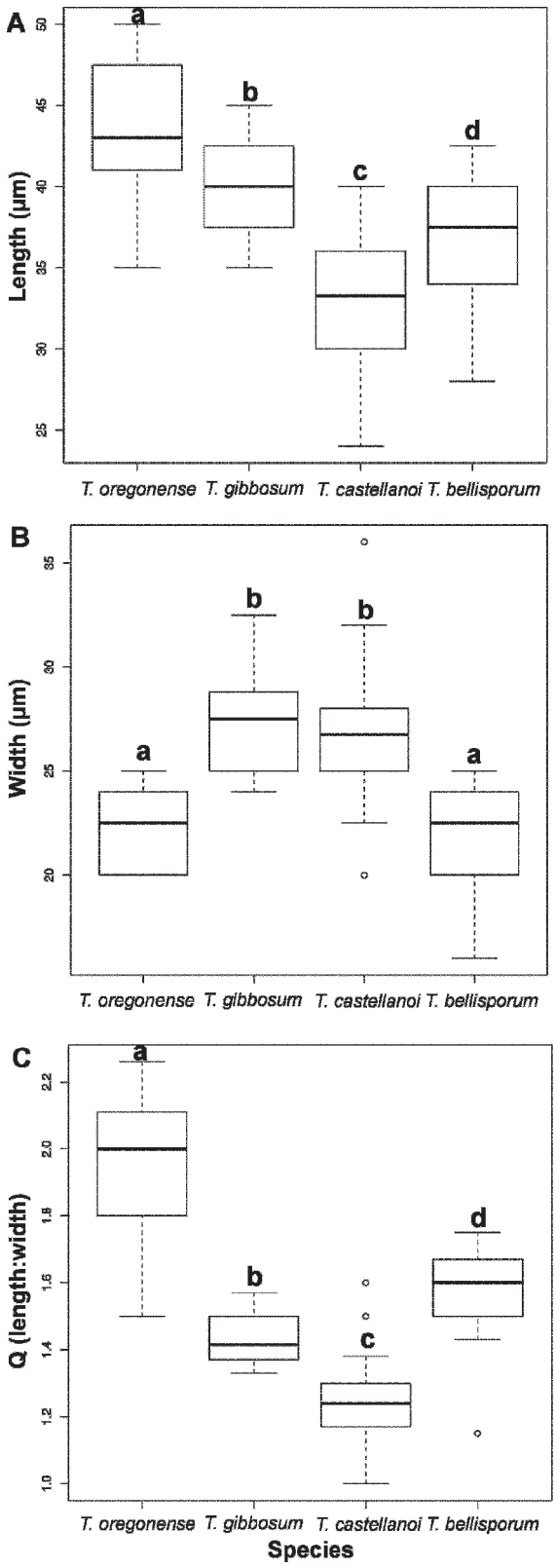

FIG. 4. Boxplots illustrating the distribution in spore length (A), spore width (B) and spore Q (C) for species in the Gibbosum complex. The boxed area represents the interquartile range. Median values are shown by a line within the box. Outlier values are depicted as unfilled dots. Statistically significant differences ($\alpha = 0.05$) based on Tukey's honestly significant difference test are notated by superscript letters above plots; statistical differences occur where letters differ. These data resulted from measurements of spores in two-spored asci, taken from a total of 30 spores for each species (10 spores each from three collections). The same trends are seen for spores from asci containing other numbers of spores (data not shown).

epithelium (FIG. 5C), but its spores are shorter and broader than those of the other three species. *T. bellisporum* spores resemble those of *T. gibbosum*, but individual specimens of the former at most have only clusters of inflated cells interspersed with patches of interwoven hyphae with few inflated cells (FIGS. 4B, 5A). *T. oregonense* has a generally prosenchymentous peridial structure (FIG. 5E) and scattered to frequent, long, subfusoid spores in one- to two-spored asci.

TAXONOMY

Tuber bellisporum Bonito & Trappe sp. nov.
FIGS. 3D, H, L, P; 5A, B
MycoBank MB515121, Genbank FJ809856

Suprapellis peridii maturitate aliquot hyphalibus moniliformibus ob parietes irregulariter incrassatos; sectio transversalis peridii maxime variabilis, ex parte prosenchymata, ex parte pseudoparenchymata, ex parte interposita. Sporae bellae, ellipsoideae vel ovoideae extremis obtusis; ornamento reticulato excludo, in ascis 1-sporis 27–55 × 16–33 µm et Q = (1.0–)1.3–2.0(2.1), in ascis 2-sporis 24–45 × 15–22 µm et Q = (1.2–)1.3–2.1. Holotypus hic designatus: Trappe 7270, Oregon, Douglas County, Steamboat Rock.

Ascomata hypogeous, 4–20(–30) mm broad, subglobose, irregularly lobed and randomly furrowed. *Peridium* white in youth, with age becoming light grayish brown to yellowish brown or orange-brown with ivory to pale brown furrows and patches, 0.15–0.25 mm thick, the surface densely pubescent to scabrous in the patches. *Gleba* solid, at maturity the fertile tissue light to dark brown from the color of the spores, with narrow, white, hypha-stuffed veins that marble the gleba and emerge through the peridium at its furrows. *Odor* in youth fruity-farinaceous, by maturity becoming pungent and ''truffly''.

Spores ellipsoid to ovoid or sometimes globose, light brownish golden at maturity (FIG. 3H, L, P); excluding ornamentation, in one-spored asci 27–55 × 16–32 µm, Q = (1.0–)1.3–2.0(–2.1); in two-spored asci 24–45 µm × 15–22 µm , Q = (1.2)1.3–2.1; in three-spored asci 24–38 µm × 16–25 µm, Q = 1.2–1.9; in four-spored asci 20–36 × 14–22 µm; Q = (1.1–)1.3–1.8(–2.0). Spore wall 2–2.5 µm thick (FIG. 3L).

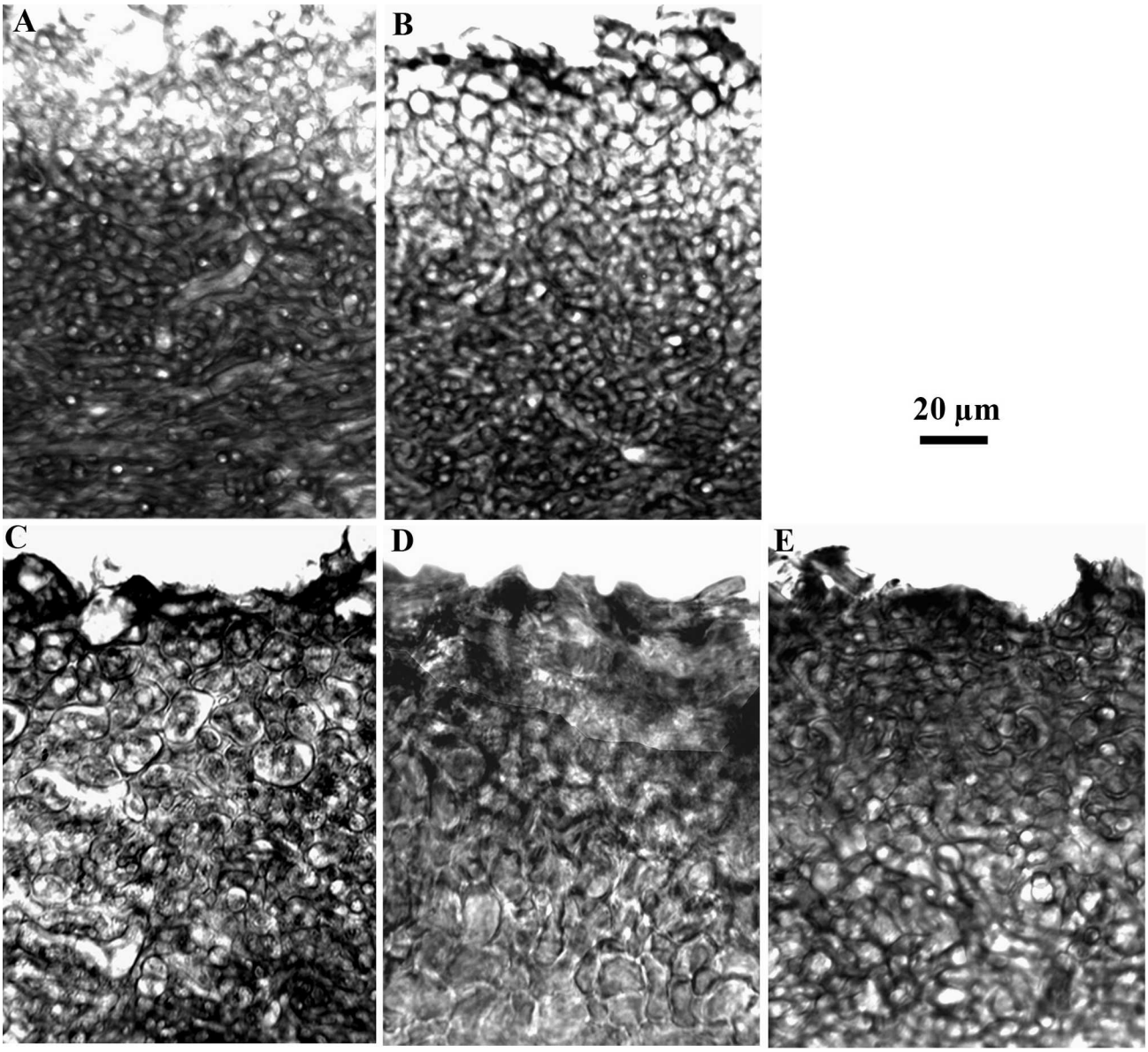

FIG. 5. Cross sections of peridia of *Tuber* species, stained in safranin-fast green. A. *T. bellisporum*, one side of ascoma. B. *T. bellisporum*, other side of same ascoma, illustrating the variation within a single ascocarp. C. *T. castellanoi*. D. *T. gibbosum*. E. *T. oregonense*.

Ornamentation an orderly, clean, alveolate reticulum with straight, 5–6-sided alveolae numbering 5–8(–11) along the spore, the corners forming spines 4–5 × 0.5 µm, somewhat broader at the base, the walls straight and as tall as the spines; a ''microreticulum'' appears in some spores when the microscope objective is focused on the optical cross section but not on the spore wall surface or on SEM micrographs of the surface. *Asci* broadly ellipsoid to pyriform in youth; by maturity mostly becoming subglobose to globose, hyaline, thin-walled, 60–77 × 33–58 µm, 1–4(–5)-spored but one-spored asci rare, astipitate at maturity.

Peridiopellis 150–250 µm thick. *Suprapellis* with patches of appressed to loosely arranged, hyaline to yellowish hyphae 3–6(–8) µm broad, the surface pubescence variable, of tangled hyphae and emergent hyphal tips 3–5 µm broad, at maturity some even and smooth, some with granulated surfaces and some moniliform from walls irregularly thickened by hyaline bands 0.5–1 µm thick (FIG. 3D). *Pellis* varying from prosenchymatic to pseudoparenchymatic in patches, of hyaline to subhyaline or yellowish hyphae 3–5 µm broad, near the suprapellis with much thickened and refractive walls, often with scattered to clustered, inflated, plus or minus isodiametric cells

5–15 μm broad (FIG. 5A, B). *Subpellis* variable, 80–175 μm thick, of hyaline to subhyaline, loosely to tightly interwoven, thin-walled hyphae 4–5 μm wide at septa with infrequent to clustered inflated cells up to 20 μm broad. *Gleba* of hyaline, thin-walled, interwoven hyphae 2–7 μm broad with an abundance of cells inflated up to 15 μm.

Distribution, habitat and season. West of the Cascade Mountains from far southwestern Washington south through western Oregon to northwestern California at elevations from near sea level to about 500 m in mesic to xeric habitats; associated with *Pseudotsuga menziesii* in pure stands or in mixture with *Tsuga heterophylla*, other Pinaceae, or *Arbutus menziesii*. Oct–Jun.

Etymology. Latin *belli-* (lovely) and *-sporum* (spored), in reference to the attractive, tidy ornamentation of the spores.

Collections examined. USA. CALIFORNIA: Del Norte County, near Crescent City. Undated, *D.W. Smith*, *Trappe 25868* (PARATYPE OSC 131976). Mendocino County, 12.8 km W of Leggett along California 1. 3 Dec 1978, *J. Graham*, *Trappe 5418* (PARATYPE OSC 131970). Shasta County, Castle Crags State Park. 30 May 1979, *J.M. Trappe 5510* (PARATYPE OSC 131969). OREGON: Douglas County, North Umpqua Road, Steamboat Creek, 300 m. 4 Apr 1963, *W. Simpson*, *Trappe 7270* (HOLOTYPE OSC 131465). Columbia County, W of Scappoose, 105 m, 26 Oct 1971, *R. Oswald*, *Trappe 3033* (PARATYPE OSC 48866). Curry County, 3.2 km S of Natural Bridge 28. Jun 1971, *J.M. Trappe 2760* (PARATYPE OSC 131967). Josephine County, Galice Ranger District. 14 Jan 1990, *M. Amaranthus*, *Trappe 11679* (PARATYPE OSC 49770). Lane County, Garroutte Road 8 km S. of Cottage Grove. 17 Apr 1970 *W.J. Hoopes*, *Trappe 2156* (PARATYPE OSC 131965). Lincoln County, Five Rivers, 85 m. 9 Dec 1985, *E. Grinnell*, *Trappe 8805* (PARATYPE OSC 46755). Linn County, Lebanon, River Drive. 20 Jun 1980, *L. Taylor*, *Trappe 5840* (PARATYPE OSC 131971); Longbow Campground E of Cascadia, 460 m. 6 Dec 1980, *F. Evans*, *Trappe 6060* (PARATYPE OSC 131466). Marion County, Salem, 26 Feb 1970, *V. Hiatt*, *Trappe 2140* (PARATYPE OSC 131964). Tillamook County, Van Duzer Corridor, 215 m. 8 Nov. 1970, *A.H. Smith*, *Trappe 2453* (PARATYPE OSC 131966). Washington County, 1.6 km W of North Plains, 100 m. 31 Oct 1977, *J. M. Trappe 5173* (PARATYPE OSC 48871). WASHINGTON. Skamania County, Underwood, Neull Road., 205 m. 18 May 1998, *P. R. Brehm*, *Trappe 22942* (PARATYPE OSC 139536).

Commentary. Tuber bellisporum resembles *T. gibbosum*, but in addition to genetic differences it differs from *T. gibbosum* by having spores that are on average shorter and narrower. However spore size might not always be useful in differentiating the two because of its high variability within and among specimens. *T. gibbosum* consistently has a pseudoparenchymatic pellis, whereas that of *T. bellisporum* has a mix of pseudoparechyma and plectenchyma. Several peridial cross sections are needed to confirm which peridial type occurs on a single specimen. Both species are easily separated from *T. oregonense* with its frequently long, narrow, subfusoid spores and *T. castellanoi* with its globose to broadly ellipsoid spores. Immature specimens of all four species may have poorly developed peridia with only scattered inflated cells and few or no one- and two-spored asci, so they often cannot be dependably separated at immature stages by morphology alone.

Tuber castellanoi Bonito & Trappe, sp. nov.
FIGS. 3C, G, K, O; 5C
MycoBank MB515122, Genbank FJ809860

Suprapellis peridii maturitate aliquot hyphalibus moniliformibus ob parietes irregulariter incrassatos; sectio transversalis peridii suprapelle prosenchymatica, pelle hypharum inflatarumque subpelle prosenchymatica. Sporae globosae vel late ellipsoideae extremis obtusis; ornamento reticulato excludo, in ascis 1-sporis 33–44 × 24–38 μm et Q = 1.0–1.6, in ascis 2-sporis 24–40 × 20–36 μm et Q = 1.0–1.5. Holotypus hic designatus: Trappe 28069, California, Fresno County, Ross Creek Watershed, Turtle Creek.

Ascomata hypogeous, 7–30(80) mm broad, globose to subglobose to irregularly lobed and randomly furrowed (FIG. 3C). *Peridium* brownish white in youth, with age becoming dull brown with ivory to pale brown lines and patches, 0.1–0.3 mm thick, the surface minutely pubescent, densely in the furrows and more scattered on the exposed lobes. *Gleba* solid, in youth the fertile tissue whitish and marbled with mostly narrow, white, hypha-stuffed veins that emerge here and there through the peridium at its furrows; at maturity the fertile tissue light brown to brown from the color of the spores but the marbling veins remaining white. *Odor* and *flavor* not recorded.

Spores globose to broadly ellipsoid (FIG. 3K), light brownish golden at maturity, in one-spored asci excluding ornamentation 33–44 × 24–38 μm, Q = 1.0–1.6; in two-spored asci 24–40 × 20–36 μm, Q = 1.0–1.5; in three-spored asci 24–36 × 20–30 μm, Q = 1.0–1.4; in four-spored asci 20–35 × 18–28 μm, Q = 1.0–1.4(–1.6). Ornamentation an orderly alveolate reticulum, the alveolae 5–6-sided, (4–)5–7(–8) along spore length, the corners forming spines 2.5–4 × 0.5 μm broad, somewhat broader at the base, the alveolar walls as tall as the spines; a ''microreticulum'' (FIG. 3G, K) appears in some spores when the microscope objective is focused on the optical cross section but not on the spore wall surface or on SEM micrographs of the surface. Spore wall 2.0–2.5 μm. *Asci* globose to broadly ellipsoid or ovoid, sometimes subpolygonal from the pressure of crowded spores, hyaline, thin-walled, 50–80 × 40–60 μm, 1–4(–5)-spored, astipitate at maturity.

Peridiopellis 110–250 μm thick. *Suprapellis* a thin layer of appressed, hyaline to yellowish hyphae 2–6 μm broad, the surface pubescence variable, of tangled hyphae and emergent hyphal tips 3–5 μm broad, at maturity some even and smooth, some with granulated surfaces and some moniliform from walls irregularly thickened by hyaline bands 0.5–1(–2) μm thick. *Pellis* a variable pseudoparenchyma of several tiers of subhyaline cells inflated up to 35 μm broad near the suprapellis with much thickened and refractive walls (FIG. 5C). *Subpellis* variable, 90–200 μm thick, of subhyaline, tightly arranged, periclinal, thin-walled hyphae 2–10 μm broad at septa with scattered cells inflated up to 20 μm. *Gleba* of hyaline, thin-walled, interwoven hyphae 2–7 μm broad with scattered cells inflated up to 22 μm.

Distribution, habitat and season. West of the Cascade Mountains from southwestern Washington south through southwestern Oregon to northwestern California at elevations from near sea level to about 915 m, mostly in relatively xeric habitats, associated with *Pseudotsuga menziesii* in pure stands or in mixture with other Pinaceae or *Quercus* spp.; also known from a single locality in the northern Sierra Nevada at ca. 1460 m in a mixed forest of *Abies concolor*, *Pinus lambertiana* and *Pinus ponderosa* but no *Pseudotsuga menziesii*. Feb–Jun, Dec.

Etymology. Possessive in honor of mycologist Michael Castellano (Castellano's *Tuber*) for collecting the holotype and for his contributions to mycology and truffle taxonomy.

Collections examined. USA. CALIFORNIA: Fresno County, Sierra National Forest, Ross Creek Watershed, Turtle Creek, 1460 m. 25 Jun 1997, *M. Castellano, Trappe 28069* (HOLOTYPE OSC 131470) and *B. Oakley, Trappe 19924* (PARATYPE OSC 131471). Humboldt County, Six Rivers National Forest, Cedar Spring Camp, 430 m. 13 May 1976, *J. Trappe 4568* (PARATYPE OSC 131469). Sonoma County, near Santa Rosa, 1997, *D. Arora, Trappe 22662* (PARATYPE OSC 131468). OREGON: Clackamas County, Gladstone, 55 m. 13 Feb 1976, *D.L. Wienecke, Trappe 4540* (PARATYPE OSC 40172). Douglas County, Bureau of Land Management North Bank Habitat Management Area, 2 Dec 1997, *J. Trappe 22295* (PARATYPE OSC 61683). Jackson County, Wellington Butte, 915 m. 20 Apr 1998, *R. Young, Trappe 22799* (PARATYPE OSC 61938). Yamhill County, 5 km E of Newberg, 10 Mar 1977, *R. Koler, Trappe 4948* (PARATYPE OSC 131968). WASHINGTON: Thurston County, Olympia, 6 m. 7 Mar 1986, *M. Haseltine, Trappe 8858* (PARATYPE OSC 46725).

Commentary. In addition to molecular differences from other species in the Gibbosum clade, *T. castellanoi* generally has shorter, globose to broadly ellipsoid spores and a lower Q. It is the only species in the clade recorded from the Sierra Nevada outside the range of *Pseudotsuga*. Usually however it occurs in forests of pure *Pseudotsuga menziesii* or in mixed forests with that species as a component.

Tuber gibbosum Harkn., Proc Calif Acad Sci Ser. 3, 1:273. 1899

= *Tuber giganteum* Gilkey, Mycologia 57:250. 1925.

Genbank FJ809863 FIGS. 3A, E, I, M; 5D

Ascomata 5.0–55 mm broad, the smaller specimens globose to subglobose and randomly furrowed, the larger irregular, lobed and deeply furrowed (FIG 3A). *Peridium* in youth pale olivaceous, soon becoming olive brown to dull brown or orange-brown and often cracking, 0.1–0.2 mm thick, the surface minutely pubescent, densely in the furrows and more scattered on exposed lobes where the pubescence often collapses in age. *Gleba* solid, in youth the fertile tissue whitish and marbled with mostly narrow, white, hypha-stuffed veins that emerge through the peridium at furrows; at maturity the fertile tissue light brown to brown from the color of the spores but the marbling veins remaining white. *Odor* and *flavor* mild in youth, soon becoming strong, pungent and complex, ''truffly''.

Spores ellipsoid to broadly ellipsoid, light brownish golden, in one-spored asci excluding ornamentation 36–60 × 25–37.5 μm, Q = 1.2–1.8; in two-spored asci 28.5–50 × 20–37.5 μm, Q = 1.1–1.8; in three-spored asci 25–50 × 16–32.5 μm, Q = 1.2–1.9; in four-spored asci 22.5–38 × 17–30 μm, Q = 1.1–1.8; in five-spored asci 25–32 × 19–24 μm, Q = 1.30; spore walls 2–5 μm thick; ornamentation an orderly, alveolate reticulum, the alveolae 5–6-sided, (6–)7–10 μm along spore length, the corners forming spines 3–4(–5) × 0.5 μm, much broader at base, the alveolar walls as tall as the spines (FIG. 3E, I, M); a ''microreticulum'' (FIG. 3E, I) appears in some spores when the microscope objective is focused on the optical cross section but not on the spore wall surface or on SEM micrographs of the surface. *Asci* in youth globose to broadly ellipsoid to ovoid or pyriform; at maturity globose to broadly ellipsoid, sometimes cylindrical or misshapen from the pressure of crowded spores within, hyaline, thin-walled in youth, the walls plus or minus 1 μm thick at maturity, 73–100 × (35)65–75 μm, (1–)2–4(–6)-spored, astipitate at maturity.

Peridiopellis 200–300 μm thick. *Suprapellis* with patches of appressed to loosely arranged, hyaline to yellowish hyphae 3–5(–7) μm broad, the surface pubescence variable, of tangled hyphae and emergent hyphal tips 3–5 μm broad, at maturity some even and smooth, some with granulated surfaces and some moniliform from walls irregularly thickened by hyaline bands 0.5–1 μm thick. *Pellis* pseudoparenchymatic, 60–100 μm thick, of several tiers of hyaline to subhyaline or yellowish, isodiametric cells 5–20 μm

broad and some intermingling hyphae 3–5(–10) μm broad, near the suprapellis with much thickened and refractive walls (FIG. 5D). *Subpellis* abruptly differentiated from the pellis, 110–220 μm thick, of hyaline to subhyaline, loosely to tightly interwoven, thin-walled hyphae 2–10 μm broad at septa with scattered to clustered, inflated cells up to 20 μm broad. *Gleba* of hyaline, thin-walled, interwoven hyphae 2–7 μm broad with scattered cells inflated up to 15 μm.

Distribution, habitat and season. West of the Cascade Mountains from southern Vancouver Island, British Columbia, south through western Washington and Oregon to northwestern California's San Francisco Bay below ca. 600 m in pure stands up to ca. 100 y old of *Pseudotsuga menziesii* or *Pseudotsuga* mixed with *Tsuga heterophylla*, *Picea sitchensis*, or *Abies* sp. Often in Christmas tree plantations as young as 5 y; in one such farm the only host species was *Abies procera*. It has been sequenced from *Pinus sabiniana* ectomycorrhizae (Smith et al. 2009). Jan–Jun.

Etymology. Latin *gibbosum* (humped), in reference to the irregular lobes and humps on larger specimens.

Collections examined. CANADA. BRITISH COLUMBIA: Vancouver Island, Royal Oak. 24 Jan 1977, *M. Bell*, *Trappe 4944* (OSC 131484). USA. CALIFORNIA: Marin County, Mill Valley. April. *H.W. Harkness* 162 (HOLOTYPE BPI, ISOTYPE OSC 131473); Point Reyes National Seashore. 4 Apr 1981, *C. Yarwood*, *Trappe 6555* (EPITYPE HERE DESIGNATED OSC 40964). OREGON: Benton County, Paul Dunn Experimental Forest. 28 Feb 2001, *A. Beyerle 1366* (130540). Clackamas County, Beaver Creek, Paul Bishop Tree Farm. 19 Feb 2005, *K. Kittredge*, *Trappe 30580* (OSC). Coos County, Bandon. *H. Gilkey 32* (HOLOTYPE of *Tuber giganteum*, OSC 38614). Douglas County, Rock Creek Fish Hatchery. 15 Apr 1997, *E. Olson*, *Trappe 20407* (OSC). Jackson County, NW head of Humbug Creek. 21 Apr 1998, *J.M. Trappe 22789* (OSC 131486). Josephine County, Myers Valley, Big Pine Campground. 4 Jul 1990, *R. Young & M. Amaranthus*, *Trappe 11493* (OSC 49689). Lane County, 1.6 km S of Mapleton. 19 Mar 1992, *J. Toledo*, *Trappe 12396* (OSC 131482). Polk County, Mill Creek near Buell. 23 Mar 1992, *W. Bushnell*, *Trappe 12477* (OSC 50555). Yamhill County 14 May 2004, *J. Czarnecki*, *Trappe 29402* (OSC 82258). WASHINGTON: Clark County, Vancouver. 23 Feb 1995, *J. Lindgren*, *Trappe 15420* (OSC). King County, Seattle. 7 Apr 2005, *K. Possee*, *Trappe 30681* (OSC 131487). Thurston County, Tenino. 15 Apr 2001, *J. Cosentino*, *Trappe 26632* (OSC 111485).

Commentary. Harkness (1899) recorded the type collection as being under oaks. However we have never found it to be associated with oaks per se. Instead it is primarily associated with *Pseudotsuga menziesii* in pure stands or in association with Fagaceae or other Pinaceae. He was not specific about the type locality, which he listed only as Marin County, Mill Valley, California. Most of that area has since been developed as suburbs, but it has forest and woodland remnants of *Quercus* mixed with *Pseudotsuga menziesii*. While the type collection was ''under oaks,'' roots of *Pseudotsuga* probably grew among the oak roots. Gilkey (1939) synonymized her *Tuber giganteum* with *T. gibbosum*; having examined the holotypes of both species, we agree. *T. gibbosum* is the only member of the Gibbosum clade recorded from Canada.

Tuber oregonense Trappe, Bonito & Rawlinson, sp. nov. FIGS. 3B, F, J, N; 5E

MycoBank MB515123, Genbank FJ809874

Suprapellis peridii maturitate aliquot hyphalibus moniliformibus ob parietes irregulariter incrassatos; sectio transversalis peridii suprapelle prosenchymatica, pelleque subpelle hypharum prosenchymatica cellulis dispersis inflatis. Sporae ellipsoideae vel subfusoideae, extremis obtusis vel subacutis; ornamento reticulato excludo, in ascis 1-sporis 42.5–62.5 × 17.5–30 μm et Q = 1.55–2.5(–2.9), in ascis 2-sporis 32.5–50 × 15–25 μm et Q = 1.5–2.4. Holotypus hic designatus: Bonito GB 284, Oregon, Benton County, Starker Forests Tum Tum Tree Farm near Blodgett.

Ascomata hypogeous, 0.5–5(–7.5) cm broad, the smaller specimens globose to subglobose and randomly furrowed (FIG. 3B), the larger irregular, lobed and deeply furrowed. *Peridium* in youth white, soon developing red to reddish brown or orange brown patches; with age becoming orange-brown to reddish brown overall and often cracking, 0.2–0.4 mm thick, the surface roughened-glabrous to minutely pubescent, densely in the furrows and more scattered on the exposed lobes where the pubescence often collapses in age. *Gleba* solid, in youth the fertile tissue whitish and marbled with mostly narrow, white, hypha-stuffed veins that emerge here and there through the peridium to its surface; at maturity the fertile tissue light brown to brown from the color of the spores but the marbling veins remaining white. *Odor* and *flavor* mild in youth, soon becoming strong, pungent and complex, ''truffly''.

Spores ellipsoid to subfusoid with narrowed ends, light brownish golden, in one-spored asci excluding ornamentation 42.5–62.5 × 17.5–30 μm, Q = 1.55–2.5 (–2.9); in two-spored asci 32.5–50 × 15–25 μm, Q = 1.5–2.4; in three-spored asci 27.5–45 × 15–25 μm, Q = 1.5–2.0 (–2.45); in four-spored asci 25–38.5 × 13–28 μm, Q = 1.4–2.2(2.3); in five-spored asci 28–34 × 22–25 μm, Q = 1.3–1.4; spore walls 2–3 μm thick; ornamentation an orderly alveolate reticulum, the alveolae 5–6-sided, 5–8(–9) along spore length, the corners forming spines (4–)5–7(–8) × 0.5 μm broad, somewhat broader at the base, the alveolar walls uniformly as tall as the spines (FIG. 3F, J, N); a ''microreticulum'' appears in some spores when the microscope objective is focused on the optical cross section but not on the spore wall surface or on SEM

micrographs of the surface. *Asci* in youth globose to broadly ellipsoid to ovoid or pyriform; sometimes the base narrowed stipe-like up to 15 × 7 µm, at maturity globose to broadly ellipsoid or misshapen from the pressure of crowded spores within, hyaline, thin-walled, 60–85 × 65–75 µm, 1–4(–5)-spored, astipitate at maturity,

Peridiopellis 200–300 µm thick plus or minus 80 µm of tightly interwoven hyphae 3–5(–10) µm broad (FIG. 5E), the cells short and with subhyaline walls 0.5–1 µm thick, where the interior veins emerge through the peridium the cells often forming a localized tissue of rounded cells up to 12 µm broad; surface pubescence variable, of tangled hyphae and emergent hyphal tips 2–5 µm broad with thin walls, some even and smooth, some with granulated surfaces and some moniliform from walls irregularly thickened by hyaline bands 0.5–1(–2) µm thick. *Subpellis* abruptly differentiated from the outer layer, 150–220 µm thick, of interwoven, subhyaline, thin-walled hyphae 2–10 µm broad with scattered cells up to 15 µm broad. *Gleba* of hyaline, thin-walled, interwoven hyphae 2–7 µm broad with scattered cells inflated up to 15 µm.

Distribution, habitat and season. West of the Cascade Mountains from southern Puget Sound region of Washington south to southwestern Oregon at elevations from near sea level up to 425 m in pure stands of *Pseudotsuga menziesii* forests up to 100 y old or *Pseudotsuga* mixed with *Tsuga heterophylla*, *Picea sitchensis* or *Alnus* spp.; often in Christmas tree plantations as young as 5 y. Sep–mid-March.

Etymology. Oregon + Latin suffix *–ense* (relating to), in reference to western Oregon being its central region of abundance.

Collections examined. USA. OREGON: Benton County, Starker Forests Tum Tum Tree Farm along Oregon 20 near Blodgett. 3 Feb 2007, *G. Bonito GB 284*, (HOLOTYPE OSC 131409); 3 km S of Peedee, 150 m. 16 Jan 2003, *W. Bushnell 1158*, *Trappe 27985* (PARATYPE OSC 131479). Clackamas County, Beaver Creek, Paul Bishop's Tree Farm, 185 m. 19 Jan 2002, *A.R. Beyerle 1777*, *Trappe 27173* (PARATYPE OSC 131474) and 30 Mar 2002, *A. Beyerle 1790*, *Trappe 28263* (PARATYPE OSC 131478); Beaver Creek Tree Farm, 180 m. 12 Mar 1994, *C. Paapanen*, *Trappe 15112* (PARATYPE OSC 58516. Columbia County, Scappoose, Holiday Road, 305 m. 23 Jan 2001, *A. Beyerle 1343*, *Trappe 27977* (PARATYPE OSC). Curry County, Cape Sebastian Overlook, 220 m. 14 Nov 1985, *G. Menser*, *Trappe 8767* (PARATYPE OSC 46851); Humbug Mountain State Park, 150 m. 18 Mar 1992, *M .Castellano*, *Trappe 12387* (PARATYPE 50523). Douglas County, Melrose, Ray Dorner Ranch, 380 m. 17 Oct 1982, *J. Rawlinson*, *Trappe 7164* (PARATYPE OSC 40976). Josephine County, Sam Brown Campground, 425 m. 1 Mar 1989, *D. Arthur*, *Trappe 11086* (PARATYPE OSC 49220). Polk County, Falls City, Black Rock Road, 245 m. 10 Jan 2003, *A. Beyerle*, *Trappe 28266* (PARATYPE OSC); S. Fork Peedee Creek, Bald Mountain Road. 5 Nov. 1997, *W. Bushnell*, *Trappe 19957* (PARATYPE OSC 60500). Tillamook County, Cape Lookout State Park entrance, 12 m. 26 Oct 2002, *D. Wheeler*, *Trappe 27945* (PARATYPE OSC). Yamhill County, Lafayette, Trappist Monastery, 105 m. 1998, *C. Schneider*, *Trappe 22699* (PARATYPE OSC). Putnam Creek, Luckiamute Tree Farm, 120 m. 11 Jan 1992, *W. Bushnell*, *Trappe 12327* (PARATYPE OSC 50753). WASHINGTON: Clark County, 3.2 km N of View, 260 m. 29 Oct 1994, *D. Wheeler*, *Trappe 15062* (PARATYPE OSC 58230). Grays Harbor County, Montesano, Stewart Tree Farm. 20 Sep 1997, *J. Haseltine*, *Trappe 22691* (PARATYPE OSC 61282); Wynoochee Willie Tree Farm. 19 Nov 1997, *Z. Carter & P. Rawlinson*, *Trappe 19960* (PARATYPE OSC 60515). Lewis County, Cinnabar Road near Mayfield Lake, 205 m. 12 Sep 1992, *N. Wedam*, *Trappe 12640* (PARATYPE OSC 50999).

Commentary. *Tuber oregonense* differs from the other three species of the Gibbosum clade by its long, narrow spores common in one- to four-spored asci and its generally taller spore ornamentation. It is less widely distributed than the others, being recorded only from southwestern Washington and western Oregon. In addition to the molecular differences between the two the largest spores of *T. oregonense* are subfusoid with Q up to 2.9 and a reticulate ornamentation mostly 5–7(–8) µm tall. *Tuber oregonense* fruits primarily in autumn to early winter. *T. gibbosum* in contrast has only ellipsoid spores with Q no larger than 1.45 and a reticulum 3–5(–6) µm tall and fruits from autumn through winter to early summer. Both species are commercially harvested for culinary use. *T. oregonense* is the most commonly collected *Tuber* species in autumn in the Pacific Northwest (Trappe et al. 2009). It often occurs in great numbers in suitable habitats, but in forests the ascomata tend to be at the small end of its size range. The largest specimens have been found in Christmas tree farms or along golf course fairways.

DISCUSSION

Jeandroz et al. (2008) used 5.8S and ITS2 data to reconstruct the phylogeny and biogeography of *Tuber* and proposed the first phylogenetic classification for the genus. They delimited five major clades (i.e. Aestivum, Excavatum, Rufum, Melanosporum and Puberulum), although Aestivum was not supported with high bootstrap values. Here we provide the first LSU phylogeny for the genus *Tuber* and resolve nine major clades in the genus with high bootstrap support. In contrast to ITS results of Jeandroz et al. (2008), LSU data distinguish the Magnatum and Macrosporum clades as distinct from the Aestivum clade. In addition to resolving the Excavatum, Rufum, Melanosporum and Puberulum clades, LSU data also resolve the Gibbosum and Spinoreticulatum clades.

The Aestivum clade contains the most morphological variation and includes species with large pyramidal warts and alveolate spores as well as *T. panniferum*, which has a tomentose peridium and spiny spores. The Excavatum group includes both *T. excavatum* and *T. fulgens* and is characterized by a basal cavity, a thick and hard peridium and coarsely reticulated spores. The Magnatum clade is represented by *T. magnatum*, which has a pale, glabrous peridium and coarsely reticulated spores. LSU data place *T. gennadii* as sister of *T. magnatum*, although this relationship is not supported statistically by ML bootstrap values (it is supported by MP and BI). *Tuber gennadii* is distinguished by locules that are lined with a hymenium bearing long-stemmed asci. Due to these features Alvarez et al. (1993) placed *Tuber gennadii* in its own genus (*Loculotuber*) within the *Tuberaceae*. These LSU data do not support this taxonomic hypothesis, although from a morphological standpoint *T. gennadii* diverges strikingly from all other *Tuber* spp. The Rufum clade is characterized by a smooth to minutely warted peridium, three-layered ascus walls and spiny spores (Trappe 1969, Trappe et al. 1996). Uecker and Burdsall (1977) placed *T. spinoreticulatum* in the Rufum group because of the structure of its peridium and asci, but LSU data place *T. spinoreticulatum* in its own clade. *Tuber spinoreticulatum* is characterized by a basal cavity, small peridial warts and ascospores ornamented with broad-based spines connected by low walls to form a reticulum. The Melanosporum clade is characterized by large peridial warts and darkly pigmented ascospores ornamented by spines that may connect to form a reticulum. Species in the Macrosporum clade have smooth or minutely warted peridia and globose asci that typically contain fewer than four alveolate-reticulate spores. Species in the Gibbosum clade are distinguished by thick-walled hyphae that emerge from the peridium and often appear moniliform from banded thickenings plus alveolate-reticulate spores. The Puberulum clade is a diverse group of light-colored truffles characterized by a smooth to cracked peridium and globose to elliptical alveolate-reticulate ascospores.

Morphological and molecular data confirm that *Tuber gibbosum* and its three allied species form a group morphologically and genetically distinct from all other *Tuber* species described to date. They are known only from southwestern Canada south through western Washington and western Oregon to the San Francisco Bay area and central Sierra Nevada of California. They all share a synapomorphy unique within genus *Tuber*; their mature peridial suprapellis is beset with scattered to abundant tangled hyphae and hyphal tips with walls irregularly thickened up to 2 μm to produce a beaded appearance. These are not well developed on young specimens but become more evident with maturity. We have examined types or neotypes of nearly all the world's known hypogeous Ascomycota and have not seen these hyphal wall thickenings on any other *Tuber* spp., including those of Europe and Asia. The only other truffle we have found to possess peridial hyphae with such irregularly thickened walls is *Choiromyces alveolatus* (also in the Tuberaceae), which is endemic within the range of the Gibbosum clade but phylogenetically distinct (FIG. 1). The four species within the Gibbosum clade can be separated by a combination of spore characters (FIG. 4). Most other characters either intergrade between the species or are so highly variable even within individual specimens that they have only marginal value in separating the species.

Ceruti et al. (2003) examined a specimen of *T. gibbosum* from California. They did not notice the beaded hyphae on the ascomata surface and synonymized it with the European *Tuber foetidum* Vittad. That species however lacks beaded hyphae (J. Trappe unpubl) and often is associated with deciduous angiosperm trees in Europe (Ceruti et al. 2003).

Montecchi and Sarasini (2000) describe a *Tuber* sp., which they term *Tuber gibbosum*, from *Pseudotsuga* plantations in Europe. However its morphology as described and illustrated by Montecchi and Sarasini (p 281–283) and largely confirmed by us from a dried specimen kindly loaned by Mr Montecchi is in several important respects unlike that of any of the four species we place in the Gibbosum clade: (i) Its peridium is ''pale ochre'' and becomes ''typically cracked in areolae''; the four species of the Gibbosum clade are pallid in youth but soon become mottled with olive brown to orange-brown to dark reddish brown by maturity becoming those colors overall (FIG. 3A–C); they sometimes are cracked but when so not areolate; (ii) its peridium produces ''numerous hairs prominent from the surface, slightly tapered towards the tips.'' Our prolonged search of the Montecchi specimen confirmed that no hyphae with irregular wall thickenings were present. The Gibbosum clade has appressed to tangled surface hyphae, not tapered but at maturity often with irregularly thickened walls (FIG. 3D); (iii) its sterile, broad, white glebal veins are ''arranged towards the base, without touching the external peridium in other areas''; the white sterile glebal veins of the Gibbosum clade tend to be narrow and emerge through furrows randomly distributed over the ascomata as well (FIG. 3A–C); 4) Its asci are ''saccate'' and contain 1–2(–3) spores; asci of the Gibbosum clade are globose to ellipsoid or misshapen by the press of the enclosed spores, which commonly number four and in three of the four

species 5–6. Our attempts at DNA extraction from the Montecchi specimen failed to yield useable DNA. Nonetheless from morphology alone we conclude that this species is not conspecific with any of the four we described in the Gibbosum clade and because it does not have the signature surface hyphae with irregularly thickened walls it probably is not a member of that clade. To our knowledge no similar truffle has been found in Pacific Northwest forests, and we suspect it to be an undescribed species associated with *Pseudotsuga*. When additional specimens become available we plan further DNA extraction attempts to clarify the position of this taxon.

The ranges of all four species in the Gibbosum clade overlap in western Oregon. Inferences on differential species ranges and geographic abundance are speculative at present because they likely reflect abundance of collectors in various parts of the clade range as much or more than real, geographic differences. It does appear that *T. oregonense* is more restricted in range and habitat than the other species, being confined to western Oregon and southwestern Washington in relatively mesic forests, whereas *T. gibbosum* has a greater north-south range and wider habitat adaptation. *T. castellanoi* is the only species known at present from the Sierra Nevada of California, where it also occurs at a considerably higher elevation than do the others; it seems adapted to relatively dry habitats.

That *T. gibbosum* and *T. oregonense* often naturalize and fruit in young *Pseudotsuga menziesii* stands suggests the potential roles that these truffles could have in the forest's ecology and as a renewable resource. Early on these truffles were commercially harvested by raking the forest floor, a practice that disrupted extensive areas of soil where they were abundant (Trappe 1989). The resulting collections often included a large proportion of immature specimens or even other species so the Oregon white truffle gained a reputation of poor quality and consequently low value (Lefevre et al. 2001, Trappe et al. 2009). In recent years increasing numbers of harvesters use trained dogs for truffle hunting in the region. The dogs detect only ripe truffles with higher quality and greater value than those exposed by raking. This practice disturbs the forest floor no more than wild animals do in excavating truffles and might help to improve the reputation and integrity of the developing Pacific Northwest truffle industry.

The phylogeny, novel suprapellis, the limited ranges and the predominant (but not exclusive) association with *Pseudotsuga menziesii* all suggest the Gibbosum clade evolved and speciated in Pacific Northwest USA and adjacent Canada from a common ancestor. However the *Pseudotsuga* species of eastern Asia have not been explored for members of this clade, and they might occur there. Clearly our understanding of *Tuber* phylogeny is enlightened by ITS and LSU rDNA phylogenies, but additional taxa and loci will be needed to test alternative phylogeographic hypotheses and to derive a stable phylogenetic classification system for *Tuber*.

KEY TO TRUFFLES IN THE *TUBER GIBBOSUM* COMPLEX

1. Peridium smooth to roughened or finely pubescent, the surface with scattered to abundant, emergent hyphae having walls with irregularly thickened bands to produce a beaded appearance by maturity. . . . . *Tuber gibbosum* complex. 2.
1. Peridium smooth to warty, roughened, pubescent or tomentose but lacking emergent hyphae having walls with irregularly thickened bands to produce a beaded appearance at maturity. . . . Other *Tuber* spp.
   2. Spores in one- and two-spored asci ellipsoid to subglobose or globose, ≤ 44 µm long excluding the ornamentation; spore length/width ratio (Q) ≤ 1.6. . . . . *T. castellanoi*.
   2. Spores in one- and two-spored asci ellipsoid to subfusoid, many > 44 µm long excluding the ornamentation; Q often > 1.6. . . . . 3.
3. Some spores in one- and two-spored asci subfusoid with tapered ends and Q often ≥ 2.1; spore ornamentation mostly ≥ 5 µm tall. . . . *T. oregonense*.
3. Spores in one- and two-spored asci ellipsoid to broadly ellipsoid with blunt ends and Q ≤ 2.1; spore ornamentation ≤ 5 µm tall. . . . . 4.
   4. Spores of one- and two-spored asci up to 38 µm broad excluding the ornamentation; peridial pellis a well developed pseudoparenchyma with several tiers of inflated cells. . . . . *T. gibbosum*.
   4. Spores of one- and two-spored asci ≤ 33 µm broad excluding the ornamentation; peridial pellis within individual specimens varying from interwoven hyphae to clusters of inflated cells. . . . . *T. bellisporum*.

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