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Tuber in China: *T. sinopuberulum* and *T. vesicoperidium* spp. nov.

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Abstract — Two new species are described and illustrated. *Tuber sinopuberulum* is recognized by its glabrous peridium and globose to subglobose ascospores. *Tuber vesicoperidium* is distinguished by its peridial structure of large, thick-walled swollen cells. The two new species are supported by molecular studies.

Key words — *Ascomycota*, *Tuberaceae*, truffle

Introduction

The first species of *Tuber* P. Micheli ex F.H. Wigg. to be described in China was *T. taiyuanense* B. Liu in 1985 (Liu 1985, Cao et al. 2011). Of the additional 28 species since described, 19 appear endemic to China (Table 1). The other nine species (*T. aestivum* Vittad., *T. borchii* Vittad., *T. californicum* Harkn., *T. excavatum* Vittad., *T. indicum* Cooke & Massee, *T. lyonii* F.K. Butters, *T. maculatum* Vittad., *T. puberulum* Berk. & Broome, *T. rufum* Picco) have been recorded from India, Europe, or North America (Song 2005; Cao 2010). In recent years interest in Chinese *Tuber* species has accelerated partly because some are edible and are being marketed both at home and abroad. Marketplaces where locals sell their wild mushrooms have proven to be excellent places to find new species. Here we describe two new *Tuber* species, which we discovered in a Kunming (Yunnan Province, China) mushroom market where locals, including the Yi people (Wikipedia 2011), offer wild mushrooms for sale.

Materials & methods

Morphological studies

Truffles were collected from the mushroom market in Kunming, China on 20 December 2010. Specimens were deposited in BJTC (Herbarium of Biology Department, Capital Normal University). Fresh specimens were described both macro-

Table 1. *Tuber* species described from China since 1985

Species	Reference
T. sinense K. Tao & B. Liu	Tao et al. 1989
T. liaotongense Y. Wang	Wang, 1990
T. gigantosporum Y. Wang & Z.P. Li	Wang & Li 1991
T. formosanum H.T. Hu	Hu 1992 (from Taiwan)
T. pseudohimalayense G. Moreno et al.	Moreno et al. 1997
T. pseudoexcavatum Y. Wang et al.	Wang et al. 1998
T. liui A.S. Xu	Xu 1999
T. xizangense A.S. Xu	Xu 1999
T. huidongense Y. Wang	Wang & He 2002
T. zhongdianense X.Y. He et al.	He et al. 2004
T. furfuraceum H.T. Hu & Y. Wang	Hu & Wang 2005 (from Taiwan)
T. umbilicatum Juan Chen & P.G. Liu	Chen et al. 2006
T. latisporum Juan Chen & P.G. Liu	Chen et al. 2007
T. lijiangense L. Fan & J.Z. Cao	Fan et al. 2011
T. sinoexcavatum L. Fan & Yu Li	Fan et al. 2011
T. polyspermum L. Fan & C.L. Hou	Fan et al. 2012a
T. sinoalbidum L. Fan & J.Z. Cao	Fan et al. 2012a
T. microspermum L. Fan & J.Z. Cao	Fan et al. 2012b
T. microspiculatum L. Fan & Yu Li	Fan et al. 2012b

and microscopically. Razor-blade sections were mounted in 3% KOH or stained with Melzer's reagent, rinsed, and mounted in polyvinyl lactic glycerol to make permanent slides for archiving with dried specimens. For scanning electron microscopy (SEM), ascospores were scraped from the dried gleba onto double-sided tape mounted directly on an SEM stub, coated with gold-palladium, and examined and photographed with a HITACHI S-4800 SEM.

Molecular methods

Herbarium samples were crushed by shaking for 3 min at 30 Hz (Mixer Mill MM 301, Retsch, Haan, Germany) in a 1.5 ml tube with one 3 mm diameter tungsten carbide ball. Total genomic DNA was extracted using the PeqLab E.Z.N.A._Fungal DNA kit following the manufacturer's protocol. The ITS region was amplified with PCR using the primers ITS1/ITS4 (White et al. 1990). PCR was performed in 50 µl reactions containing DNA template 2 µl, primer (10 µM) 2 µl each, 2× Master Mix (Tiangen Biotech (Beijing) Co. Ltd.) 25 µl. PCR reactions were run as follows: an initial denaturation at 95 °C for 3 min, followed by 30 cycles at 95 °C for 2 min, 55 °C for 25 s, 72 °C for 2 min, and a final extension at 72 °C for 10 min. The PCR products were sent to Invitrogen Biotechnology Co. Ltd. (Beijing, China) for purifying, sequencing, and editing. Other ITS rDNA sequence data included in this study were downloaded from GenBank (Table 1).

Phylogenetic analyses

DNA sequences were aligned with Clustal X (Thompson et al. 1997). The alignment was manually adjusted with Se-Al v.2.03a (Rambaut 2000). The aligned dataset was analyzed with maximum parsimony (MP) using PAUP*4.0b10 (Swofford 2002). Maximum parsimony analysis was conducted using heuristic searches with 1000 replicates of random-addition sequence, tree bisection reconnection (TBR) branch

Table 2. *Tuber* specimens and sequence numbers used in molecular studies

Species	ITS	LSU
T. sinopuberulum (BJTC FAN157)	JQ690073	JQ690070
T. vesicoperidium (BJTC FAN155)	JQ690071	JQ690068
(BJTC FAN156)	JQ690072	JQ690069
T. borchii	HM485343	FJ809799
	HM485343	
	FJ809852	
T. californicum	DQ974799	AF127120
	HM485351	AF159627
	HM485346	
T. dryophilum Tul. & C. Tul.	HM485353	FJ809800
	HM485354	FJ809801
T. maculatum	EU784428	
	FM205649	
	AJ969627	
	AF106889	
T. oligospermum	FM205506	AY515306
	FM205507	
T. puberulum	AJ557536	
	AJ969625	
T. sphaerosporum Gilkey	FJ809853	FJ809805
	FJ809854	FJ809806
	HM485390	
T. zhongdianense	DQ898186	
	DQ898187	
T. panniferum Tul. & C. Tul.		FJ809845
		FJ809846
T. melanosporum Vittad.	AF132501	GU979139
	AF106878	FJ809819

swapping algorithm. All characters were equally weighted and unordered. Gaps were treated as missing data to minimize homology assumptions. A bootstrap (BS) analysis was performed with 1000 replicates, each with 10 random taxon addition sequences. TBR branch swapping was employed.

Results

Molecular phylogenetics

The maximum parsimony analysis of ITS sequences produced one most parsimonious tree (Fig. 1) with a length (TL) = 613 steps, consistency index (CI) = 0.7276, retention index (RI) = 0.8706, homoplasy index (HI) = 0.2724, and rescaled consistency index (RC) = 0.6335.

The maximum parsimony analysis of LSU sequences produced one most parsi-monious tree (Fig. 2) with a length (TL) = 380 steps, consistency index (CI) = 0.8816, retention index (RI) = 0.8308, homoplasy index (HI) = 0.2724, and rescaled consistency index (RC) = 0.7324.

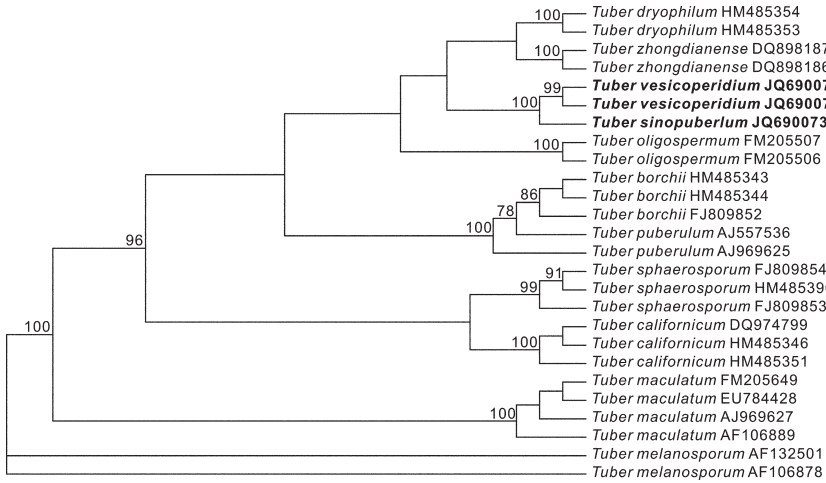

Fig. 1. Phylogeny derived from maximum parsimony analysis of the ITS rDNA sequences of some *Tuber* species with pale ascomata and reticulate ascospores, using *T. melanosporum* as outgroups. Bootstrap values of more than 70% from 1000 replications are shown above the respective branches.

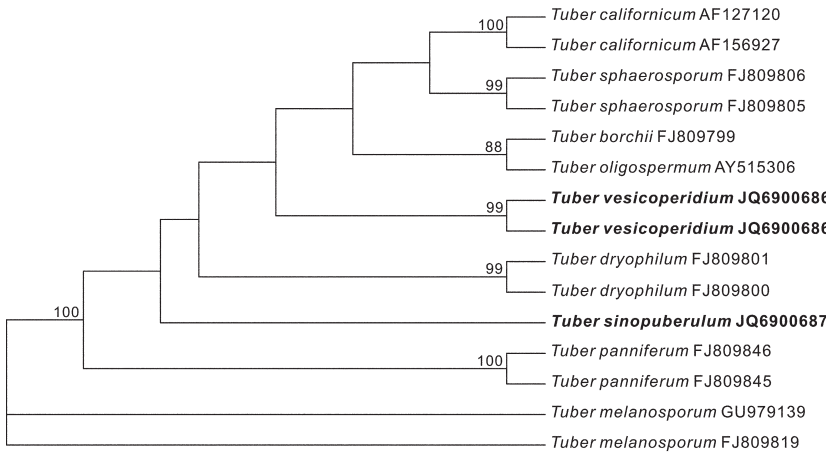

Fig. 2. Phylogeny derived from maximum parsimony analysis of LSU nuclear rDNA sequences of some *Tuber* species with pale ascomata and reticulate ascospores, additionally two sequences of *Tuber panniferum* with spiny ascospores, using *T. melanosporum* as outgroups. Bootstrap values of more than 70% from 1000 replications are shown above the respective branches.

The two new species, *Tuber sinopuberulum* and *T. vesicoperidium,* group into one clade with 100% bootstrap support in the ITS-based phylogenetic analyses (Fig. 1), but in the LSU-based analyses (Fig. 2), they separate into two distinct clades. Two *T. vesicoperidium* sequences group in a clade with strong bootstrap support (99% in both ITS and LSU sequence analyses). *Tuber sinopuberulum* and *T. vesicoperidium* have very similar ascomata and ascospores but very different peridial structures. *Tuber sinopuberulum* typically has a two-layered peridium composed of a pseudoparenchymatous layer and intricate textural layer, whereas *T. vesicoperidium* has a one-layered prosenchymatous peridium with very large and thick-walled swollen cells.

Taxonomy

Tuber sinopuberulum L. Fan & J.Z. Cao, **sp. nov.** Fig. 3

MycoBank MB 564482

Differs from *Tuber puberulum* by the absence of hyphae-like hairs on the peridium surface and by its globose to subglobose ascospores.

Type: China. Yunnan Province, Kunming, from the local mushroom market, 20 Dec. 2010, Jin-zhong Cao 120 (**Holotype**, BJTC FAN157).

Etymology: *sinopuberulum* (Lat.), referring to the similarity to the European species, *Tuber puberulum*.

Ascomata 2 × 2.5 cm, subglobose or lobed, firm, solid, surface smooth, glabrous, yellow white to light brown at maturity. Odor slight, not pungent. Peridium 250–350 μm thick, two layers; outer layer 80–120 μm thick, pseudoparenchymatous, composed of subangular or subglobose cells 7.5–20 μm in diam., with thin or slightly thickened walls, pale yellowish, no distinct hyphae-like hairs arising from the outermost cells; inner layer composed of intricately interwoven hyphae, hyaline, thin-walled, branch, septate, 3–5 μm in diam. Gleba light brown to brown at maturity, marbled with large and rare, branched white veins continuous with inner peridium. Asci subglobose, ellipsoid or irregular, hyaline, thin-walled, 70–110 × 65–100 μm, sessile or short stalk, 1–5 spored. Ascospores mostly globose or subglobose, a few broad ellipsoid, yellow-brown at maturity, 20–37.5 × 20–32.5 μm excluding ornamentation; ornamentation regularly alveolate reticulum, 2.5–4 μm high, the meshes generally 5–10 across the spore width.

Comments — *Tuber puberulum* from Europe is similar to this new species but can be easily differentiated by the 100–150 μm long hyphal-like hairs densely coating the peridial surface (Lange, 1956; Pegler et al. 1993) and ascospores that are not regularly globose.

The European *Tuber oligospermum* (Tul. & C. Tul.) Trappe and the American *T. californicum* might be confused because of their globose ascospores. However,

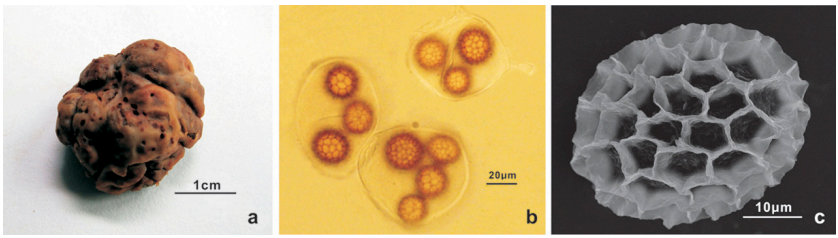

Fig. 3. *Tuber sinopuberulum* (BJTC FAN157, holotype) a. Ascoma. b. Asci and ascospores observed under light microscope. c. Ascospore observed under SEM.

T. oligospermum is distinguished by a one-layered peridium, and *T. californicum* can be separated by its dark colored gleba and puberulent ascomata.

Tuber vesicoperidium (described below) has ascospores similar to *T. sinopuberulum* but can be easily differentiated by its peridial structure characterized by very large and thick-walled swollen cells. The phylogenetic analyses (Figs. 1–2) also show that while *T. sinopuberulum* groups in a clade with *T. vesicoperidium* in the ITS sequence analysis (Fig. 1), they are widely separated in two different clades in the LSU analysis (Fig. 2), suggesting that they are closely related but distinct species.

Tuber vesicoperidium L. Fan, **sp. nov.** Fig. 4

MycoBank MB 564483

Differs from all other *Tuber* species with globose or subglobose ascospores by its one-layered peridium of predominantly large, thick-walled, swollen cells.

Type: China. Yunnan Province, Kunming, from the local mushroom market, 20 Dec 2010, Jin-zhong Cao 118 (**Holotype,** BJTC FAN155).

Etymology: *vesicoperidium* (Lat.), referring to the peridium with larger sized swollen cells.

Ascomata 2–4 cm, subglobose or lobed, a few of slight furrows, firm, solid, surface smooth, glabrous, white to pale yellow, light brown at maturity. Odor slight, not pungent. Peridium 550–650 μm thick, one layer, prosenchymatous, composed of intricately interwoven hyphae, hyaline, thin-walled, branched, septate, 3–7.5 μm in diam., distinctly intermixed with large swollen cells of mostly ellipsoid-shape 50–130 μm in diam. with wall 5–7.5 μm thick, towards the inner side of the peridium, the number of the swollen cells less, integrating with the interwoven hyphae only, more or less parallel to the surface; no distinct hyphae-like hairs arising from the outermost cells. Gleba grey brown to dark brown at maturity, marbled with large and rare, branched, white veins continuous with peridium. Asci subglobose, ellipsoid or irregular, hyaline, thin-walled, 65–95 × 50–70 μm, sessile, 1–5 spored. Ascospores mostly globose, a few subglobose, yellow-brown at maturity, 20–35.5 × 20–32.5 μm in 2–5 spored asci and 37.5–45 × 37.5–42.5 μm in 1-spored asci excluding ornamentation;

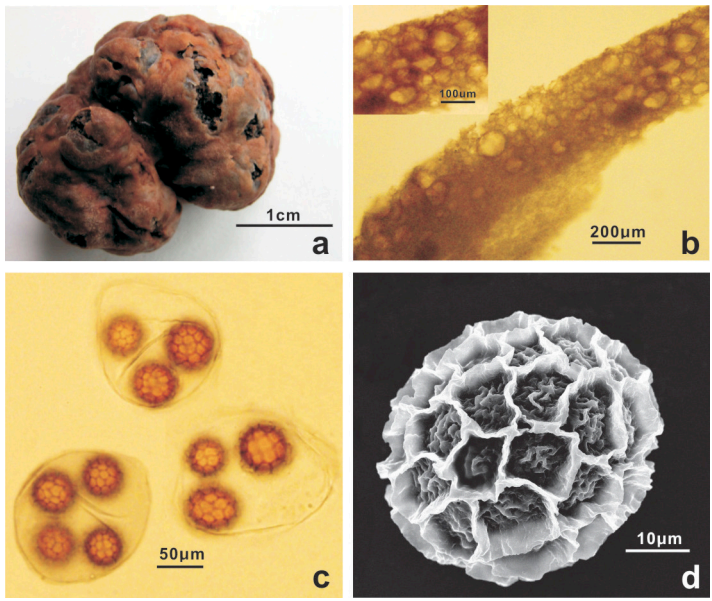

Fig. 4. *Tuber vesicoperidium* (BJTC FAN155, holotype) a. Ascoma. b. Peridium with the large, thick-walled swollen cells. c. Asci and ascospores observed under light microscope. d. Ascospore observed under SEM.

ornamentation regularly alveolate, 2.5–4 μm high, the meshes generally (2–) 3–6(–8) across the spore width.

Additional specimen examined: **China**. **Yunnan Province**, Kunming, from the local mushroom market, 20 Dec 2010, Jin-zhong Cao 119 (BJTC FAN156).

Comments — *Tuber vesicoperidium* is characterized mainly by its one-layered peridium and the large, thick-walled, swollen cells occupying nearly all the peridial tissue, thereby distinguishing it from all other known *Tuber* species with globose or subglobose ascospores. Large swollen cells also occur in some *Tuber* species with a two-layer peridium and spiny-reticulate ascospores, such as *T. borchii*, *T. zhongdianense*, and *T. malacodermum* E. Fisch., but with the swollen cells confined to the outer peridium. Lange (1956), Pegler et al. (1993), and Chen & Liu (2007) note that the shape and wall thickness of the swollen cells do not differ greatly from the surrounding cells and they do not normally exceed 70 μm in diam.

ITS (Fig. 1) and LSU (Fig. 2) sequence analyses group the *T. vesicoperidium* sequences in a clade with strong support (BP 99 for both analyses), supporting *T. vesicoperidium* as a distinct species. In the ITS sequence analysis (Fig. 1), the two *T. vesicoperidium* sequences group together with *T. sinopuberulum* with strong support (BP 100). This close relationship is reflected by their very similar

ascomata and ascospores, but the completely different peridial structures also indicate that the two taxa are morphologically independent.

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