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Tuber qujingense and *T. songlu*, two new species from Yunnan, China

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Shanping Wan and Jianwei Liu have contributed equally to this work.

Abstract

Tuber qujingense and *T. songlu* are described as new species based on both phylogenetic and morphological analyses. Phylogenetically, these two species are clearly distinct from described species of *Tuber*. Morphologically, these two species have whitish smooth ascomata, pubescent and prosenchymatous peridium and 1–4 spored asci; however, *T. qujingense* is characterized by its fusiform ascospores and taller alveolar walls (4.5–11 µm) while *T. songlu* has larger ellipsoid ascospores and lower alveolar walls (1–7 µm).

Keywords: White truffles, phylogeny, taxonomy

Introduction

Tuber P. Micheli ex F.H. Wigg. (1780: 109) (Ascomycota, Pezizales) is one of the most quintessential genera of hypogeous fungi, which has important edible and economic values (Mello *et al*. 2006; Jeandroz *et al*. 2008; Trappe *et al*. 2009; Bonito *et al*. 2013). Furthermore, *Tuber* species play a key role in forest ecosystems, including disturbed forests, where they are often common ectomycorrhizal symbionts. *Tuber* speciation and function in ecosystems are tightly linked to their ectomycorrhizal (ECM) ecology and putative co-diversification with major plant families including Pinaceae (pines), Fagaceae (oak/beech), Myrtaceae (eucalypts), and Salicaceae (willows/poplar) and to adaptations for animal dispersal in the Northern and Southern Hemispheres (Kauserud *et al*. 2010; Zambonelli *et al*. 2016).

Recent taxonomic and molecular studies on *Tuber* in China have revealed several new species (García-Montero *et al*. 2010; Fan *et al*. 2016a, b; Wan *et al*. 2016, 2017a, b). During our survey of hypogeous fungi in Yunnan, China, two whitish truffles were found under pure coniferous forest in Huize County, Qujing City, Yunnan Province. According to observation and analysis, we found two specimens that showed clear differences compared with other *Tuber* species. The results of the molecular and morphological studies confirmed that the two collections are two undescribed species which we present in this paper with descriptions, illustrations and molecular work and name *T. qujingense* and *T. songlu.*

Materials and methods

Morphological studies

Fresh samples were collected from forests of *Pinus armandii* Franch. in Yunnan, China. Macromorphological description

was based on fresh ascomata, and microscopic examinations were later conducted from dry material following the methods of Yang and Zhang (2003). Hand-cut sections were mounted in 5% (w/v) KOH and examined under a light microscope (Leica DM2500, Leica Microsystems, Wetzlar, Germany). For evaluation of the range of spore size, 40 ascospores were measured from the specimen. Ascospores are given as length × width (l × w), and the extreme values are in parentheses; the abbreviation 'Q' represents the range of ratio of spore length to spore width calculated for each spore. For scanning electron microscopy (SEM), spores were scraped from the dried gleba onto double-sided tape, and this was mounted directly on an SEM stub, coated with gold-palladium, and examined and photographed using a JSM-5600LV SEM (JEOL, Tokyo, Japan). The specimens are deposited at the Herbarium of Cryptogams, Kunming Institute of Botany, Chinese Academy of Sciences (HKAS).

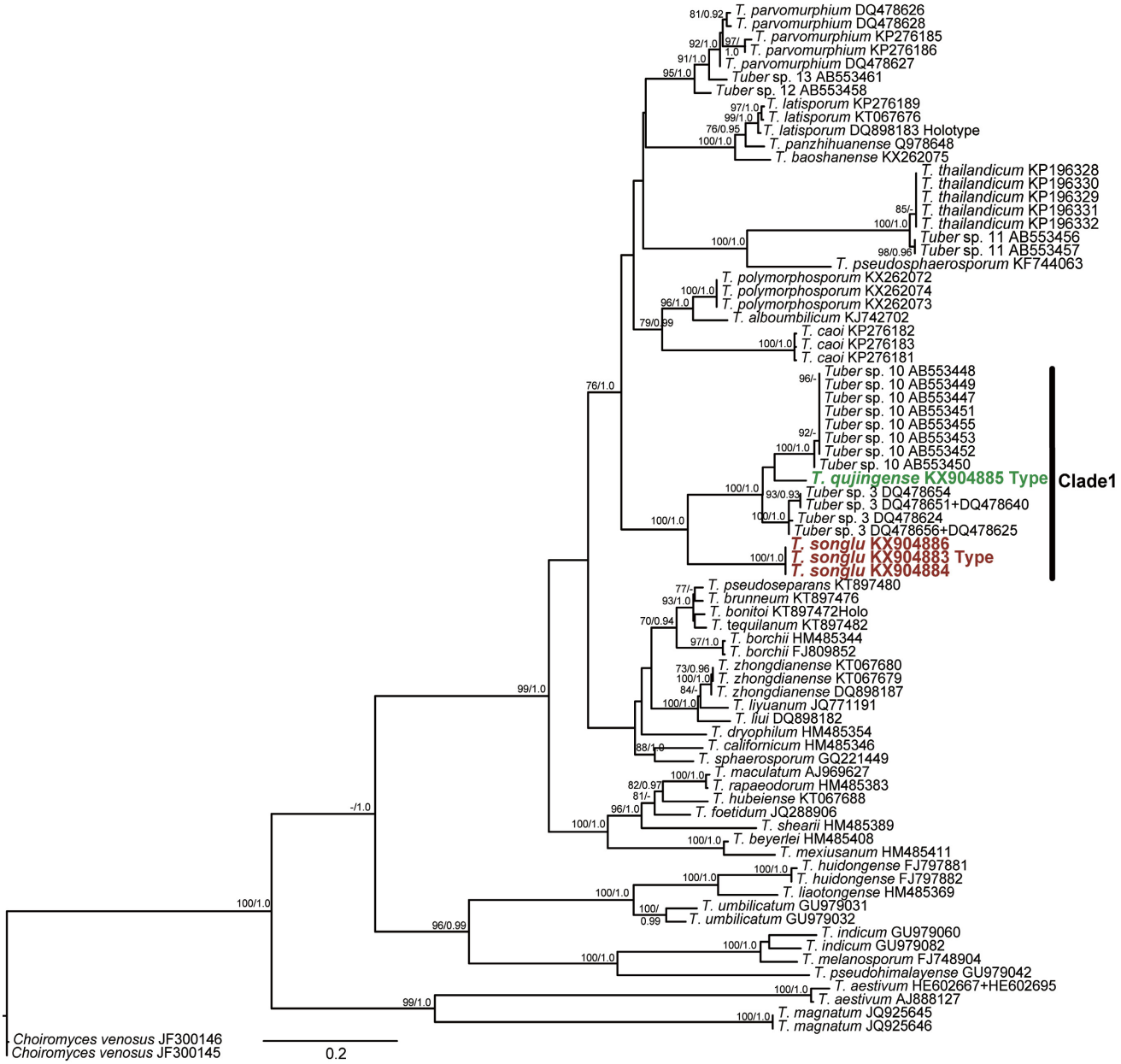

FIGURE 1. RAxML tree based on ITS sequences of *T. qujingense*, *T. songlu* and related species. Bootstrap (BS) values derived from Maximum Likelihood (ML) analysis (≥ 70%) and Posterior Probabilities (PPs) from Bayesian Inference (≥ 0.90) are shown above or beneath the branches at nodes. New sequences are in colored bold font.

Molecular methods

Total DNA was extracted from pieces of dried ascomata with a modified CTAB procedure (Gardes & Bruns 1993). Universal primer pairs ITS1F/ITS4 (Gardes & Bruns 1993, White *et al*. 1990) and LROR/LR5 (Vilgalys & Hester 1990) were used for the amplifications of the internal transcribed spacers 1 and 2 with the 5.8S rDNA (ITS) and the large subunit of the nuclear ribosomal region (nrLSU), respectively.

Polymerase chain reactions (PCR) were performed using the following procedure: 25 μL of PCR reaction solution contained 1 μL DNA, 1 μL (5 μm) of each primer pair, 2.5 μL 10 × buffer (Mg^{2+} plus), 1μL Dntp (1 mM), 0.5 μL BSA (0.1%), 0.5 μL MgCl2, 1 U of Taq DNA polymerase (Takara Tag, Takara Biotechnology, Dalian, China). PCR reactions were run as follows: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 52 °C for 1 min and 72 °C for 1 min. The final reaction was followed by an extension at 72 °C for 10 min. The PCR products were sent to Sangon Biotech Corporation (Shanghai, China) for purifying and sequencing.

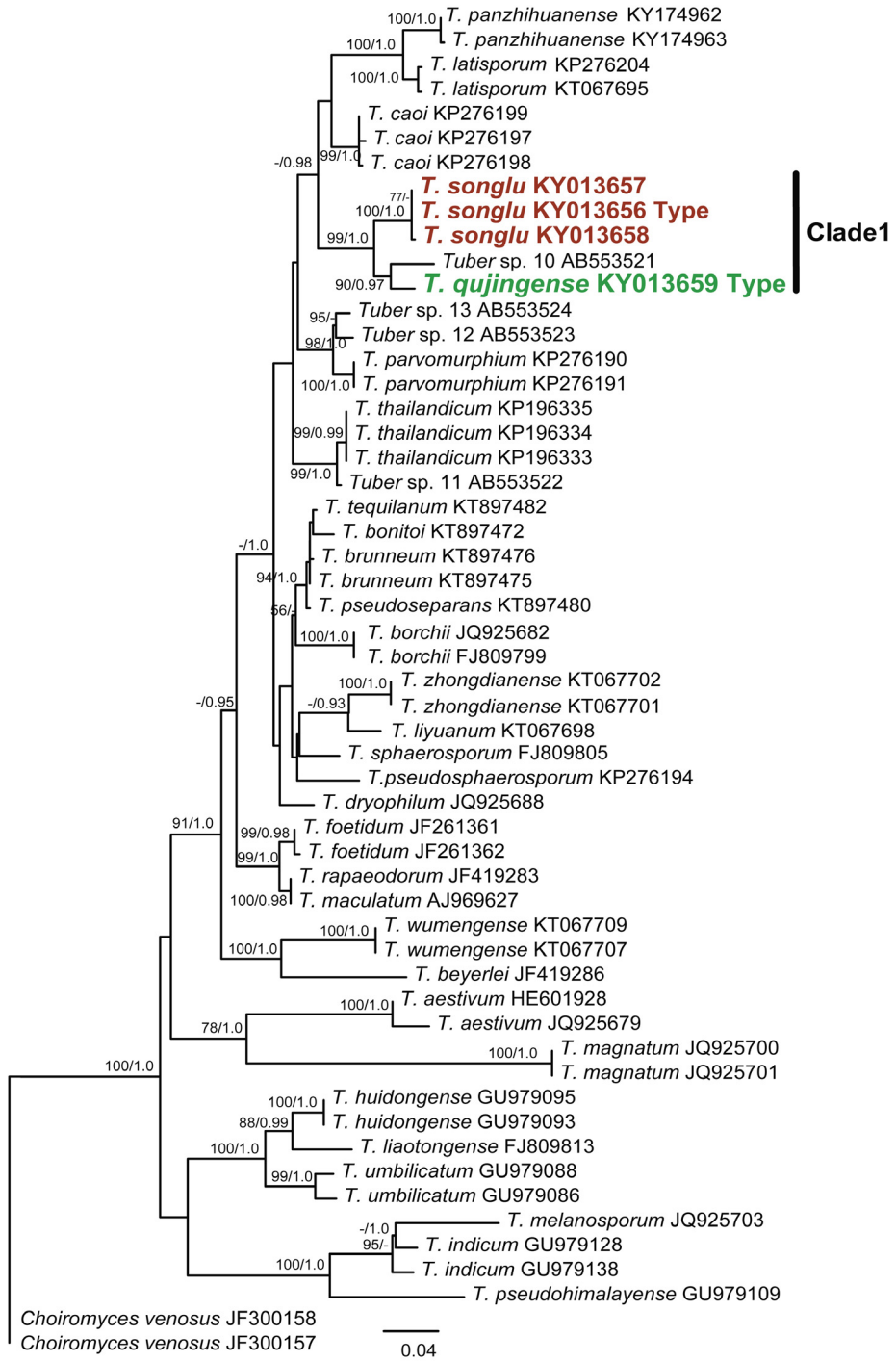

FIGURE 2. RAxML tree based on nrLSU sequences of *T. qujingense*, *T. songlu* and related species. Bootstrap (BS) values derived from Maximum Likelihood (ML) analysis (≥ 70%) and Posterior Probabilities (PPs) from Bayesian Inference (≥ 0.90) are shown above or beneath the branches at nodes. New sequences are in colored bold font.

Phylogenetic analysis

ITS and nrLSU sequences from studied specimens were compiled together with sequences from reference taxa curated in GenBank (http://www. ncbi.nlm.nih.gov/). An ITS and an nrLSU dataset were used to clarify the phylogenetic position of the two new species in this genus. Two sequences derived from *Choiromyces venosus* (Fr.) Th. Fr. were selected and used as outgroups (Figs. 1, 2).

Both datasets were aligned using MAFFT v.7.0 (Katoh & Standley 2013) and then manually edited with BioEdit v.7.0.9 as needed (Hall 1999). The phylogenetic relationships of taxa were inferred using Maximum Likelihood (ML) and Bayesian Inference (BI). ML analysis on the ITS dataset was performed in RAxML v.7.2.6 (Stamatakis 2006) and the GTR + GAMMA substitution model with parameters unlinked. ML bootstrap (BS) replicates (1000) were computed in RAxML with a rapid bootstrap analysis and search for the best-scoring ML tree. Bayesian analysis on the ITS dataset was performed in MrBayes v.3.1.2 (Ronquist & Huelsenbeck 2003) and the GTR + I + G model was selected as the best model under the Akaike Information Criterion (AIC) (Akaike 1974) implemented by MrModeltest v.2.3 (Nylander 2004). Bayesian analysis was carried out using the selected model with four chains sampled every 100 generations and run for a total of 1,000,000 generations. The average standard deviations of split frequencies were less than 0.01 at the end of the run and ESS (effective sampling size) values were > 200. A majority rule consensus tree was built after discarding trees from a 25% burning. Posterior probabilities (PPs) were calculated using the sumt command implemented in MrBayes.

Results

Molecular phylogenetics

ITS and nrLSU sequences were obtained from ascocarps of both *Tuber* species. The final ITS alignment included 79 sequences, which contained 912 aligned sites. Meanwhile the nrLSU alignment included 55 sequences, comprised of 853 aligned sites. The phylogenetic tree based upon the ITS dataset is largely consistent with the nrLSU-based phylogeny.

In both phylogenetic reconstructions, *T. qujingense* and *T. songlu* fell into the same clade but formed different branches of species with strong bootstrap values (Figs, 1, 2, Clade 1). Specifically, both species were resolved in clade 1 that included two undescribed species (*Tuber sp*. 3 and *Tuber sp*. 10) in the phylogenetic tree inferred by ITS sequences; this clade was supported by strong bootstrap values (MLBS/PP = 100/1) (Fig. 1, Clade 1). While in the phylogenetic tree inferred by nrLSU sequences, *T. qujingense* and *T. songlu* were resolved in the clade with *Tuber sp*. 10 but divided into different branches by strong bootstrap values (Fig. 2, Clade 1).

Taxonomy

Tuber qujingense S. P. Wan, *sp. nov.* (Fig. 3)
MycoBank: MB 839733

Typification: CHINA. Yunnan Province, Huize County (18.103°E, 23.26°N), in humic soil under a pure *Pinus armandii* forest, at about 2400 m, 12 August 2016, *wsp721*, HKAS 95823 (GenBank Acc. No.: ITS = KX904885, LSU = KY013659).

Diagnosis: *Tuber qujingense* differs from related species by its greyish white ascomata, brown snowflake-shaped gleba, prosenchymatous peridium, fusiform ascospores and 1–4 spored asci.

Etymology: Refers to the location of the type collection.

Description: *Ascoma* 2.5 cm in diam, subglobose or irregular, greyish white when fresh, becoming brown when dried. *Peridium* 200–500 µm thick, smooth to pubescent, one layer, prosenchymatous, composed of big, subglobose to subangular cells, (1–) 2–33 (–34.5) × (1–) 1.5–22 (–33) µm, light earthy yellow. *Gleba* solid, brownish purple when mature, marbled with white veins, composed of hyaline, interwoven, thin-walled hyphae, 1.5–6 µm, and cylindrical, inflated hyphae 2.8–50 × 2.8–43 µm. *Dermatocystidia or setae*, straight or bent, obtuse or apiculate at the tip, up to 110 µm long, 6.5 µm in diam, septate, hyaline to whitish. *Asci* (40–) 51–80 × (30–) 31–60 µm, globose to subglobose, pyriform, ellipsoid or irregular, hyaline, sessile or with a short stalk, thin-walled 1–2 µm thick, 1–4 spored. *Ascospores*, fusiform, ellipsoid, sometimes broadly ellipsoid, subglobose, hyaline when young, becoming brown at maturity;

excluding the alveolate-reticulate ornamentation, in 1-spored asci (35–)37–48(–50) × (23–) 25–30(–32) µm (Q = 1.45–1.65), in 2-spored (22–) 28–40(–41) × (14–) 18-27 µm (Q = 1.41–1.7), in 3-spored (20–)3 8–18(–20) × 15–19(–24) µm (Q = 1.23–1.75), and in 4-spored (17–) 20–34(–36) × 14–21(–22) µm (Q = 1.14–1.72); reticulum with 3–10 meshes along the spore length and 3–8 across. The alveolar walls up to 4.5–11 µm tall.

Distribution and habitat: Hypogeous, in soil under pure stand of *P. armandii* in Yunnan Province, China. Known only from China.

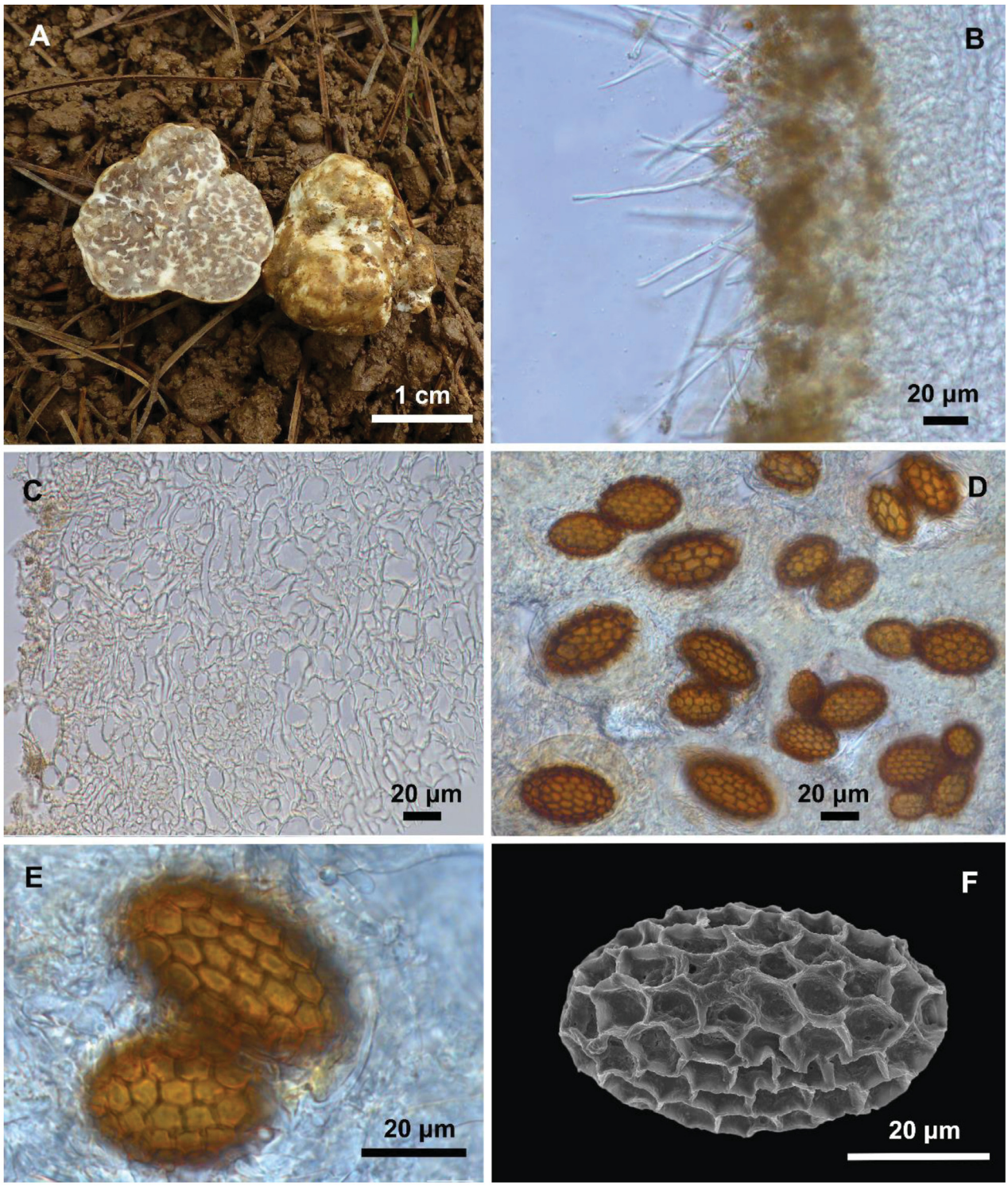

FIGURE 3. *Tuber qujingense* (HKAS 95823, type) **A**. An ascoma and its gleba; **B**. Dermatocystidia; **C**. Peridium section; **D**, **E**. Light micrograph (LM) of ascospores; **F**. SEM photo of an ascospore. Photos by: Shanping Wan.

Tuber songlu S.P. Wan, *sp. nov.* (Fig. 4)
MycoBank: MB 839734

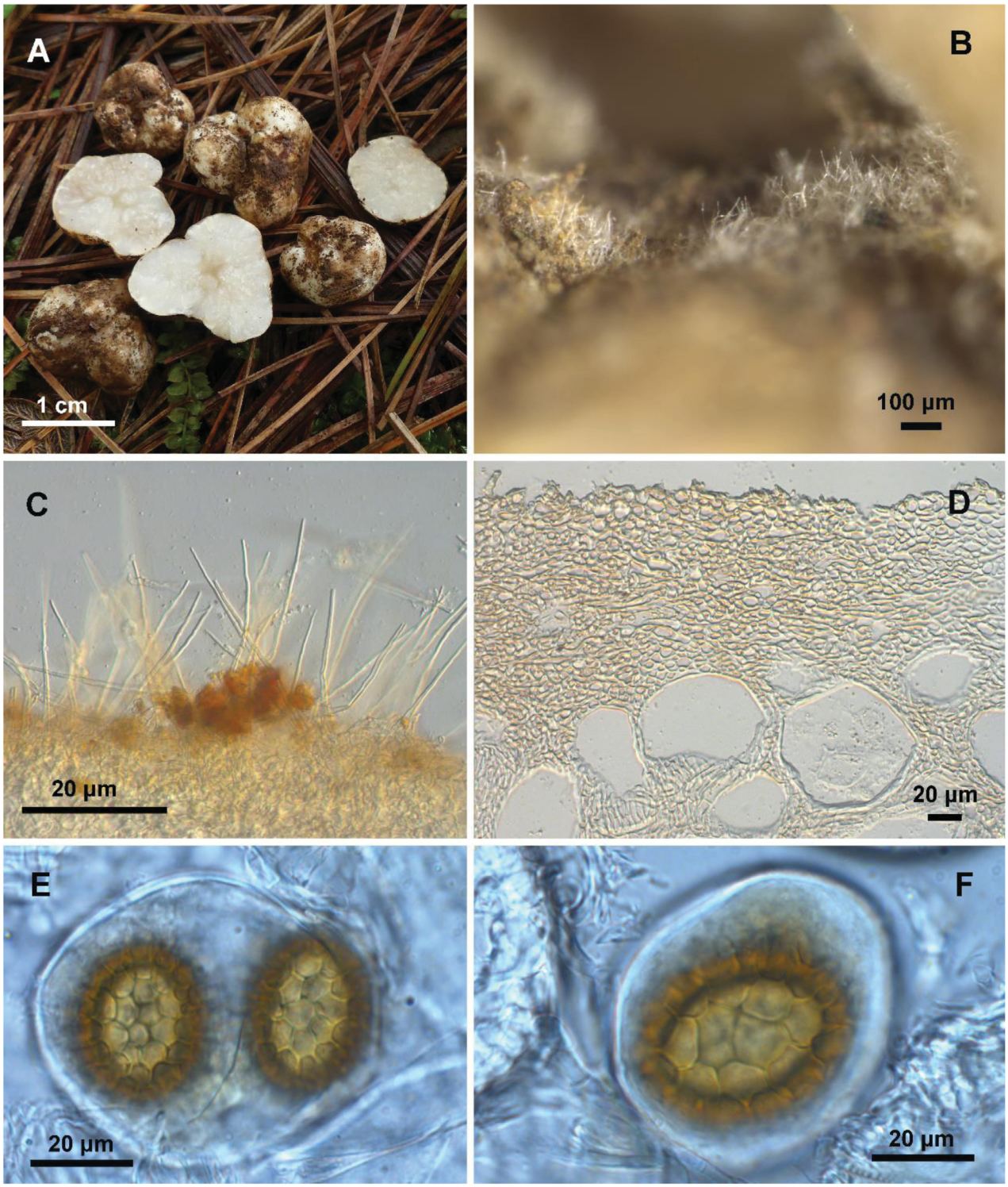

FIGURE 4. *Tuber songlu* (HKAS 95771, type) **A**. Ascomata and glebae; **B**–**C**. Dermatocystidia; **D**. Peridium section; **E**, **F**. Light micrograph (LM) of ascospores. Photos by: Shanping Wan.

Typification: CHINA. Yunnan Province, Huize County (29.103°E, 25.56°N), in humic soil under a pure *Pinus armandii* forest, at about 2415 m, 14 August 2016, *wsp695*, HKAS 95771 (GenBank Acc. No.: ITS = KX904883, LSU = KY013656).

Diagnosis: *Tuber songlu* differs from other species by its whitish ascomata, dense spine-like dermatocystidia, ellipsoid ascospores and 1–4 spored asci.

Etymology: Refers to the local name of the fungus.

Description: *Ascomata* 1–2 cm in diam, globose to subglobose or irregular, white, light reddish brown when fresh, becoming brown when dried. *Peridium* 130–500 µm thick, densely pubescent, one layer, prosenchymatous, composed of subglobose to subangular cells, (1–) 1.5–18(–23) × (1–) 1.5–7(–8.5) µm, hyaline. *Gleba* solid, brown when mature, marbled with brownish yellow veins, composed of hyaline, interwoven, thin-walled cylindrical hyphae, 1.5–8.5 µm broad at the septa, and interwoven, inflated hyphae 2–8.5 µm broad. *Dermatocystidia*, straight or bent, obtuse or apiculate at the tip, up to 230 µm long, 7 µm in diam, multiseptate, hyaline to whitish. *Asci* (63–) 72–96 × 54–75 (–81) µm, globose to subglobose, pyriform, ellipsoid or irregular, hyaline, sessile or with a short stalk, thin-walled 1–2 µm thick, 1–4 spored. *Ascospores*, ellipsoid, sometimes broadly ellipsoid, subglobose, hyaline when young, becoming brown at maturity; excluding their alveolate-reticulate ornamentation, in 1-spored asci (41–) 42.5–51.5 (–56) × (26.5–) 32.5–40 (–41) µm (Q = 1.10–1.70), in 2-spored (22–) 24–42 (–46) × (18–) 19–34 µm (Q = 1.10–1.51), in 3-spored (20–) 26–33 (–35) × 19 (–21)–29 (–30) µm (Q = 1.13–1.24), and in 4-spored (19–) 20–27.5 (–29) × 15–17 (–27) µm (Q = 1.1–1.2); reticulum with 3–8 meshes along the spore length and 3–8 across. The alveolar walls up to 1–7 µm tall.

Distribution and habitat: Hypogeous, in soil under a pure forest of *Pinus armandii* in Yunnan Province, China. Known only from China.

Other material examined: CHINA. Yunnan Province, Province, Huize County, in soil under a pure forest of *P. armandii*, December 2016, *wsp701*, HKAS 95777 (GenBank Acc. No.: ITS = KX904884, LSU = KY013657); *wsp749*, HKAS 95851 (GenBank Acc. No.: ITS = KX904886, LSU = KY013658).

Discussion

Here we use morphology and phylogenetic analyses of the ribosomal ITS and nrLSU regions to delimit *T. qujingense* and *T. songlu* from related species. DNA analyses revealed less than 88.3% ITS sequence similarity between *T. songlu* and *T. qujingense*, *Tuber sp*. 10 and *Tuber sp*. 3. *Tuber qujingense* forms a distinct branch and is related to the Japanese species *Tuber sp*. 10, but they share less than 94.6% ITS sequence similarity with each other. In the genus *Tuber*, species delimitation based on 95% or 96% similarity in ITS has been suggested by previous studies (Bonito *et al*. 2010; Kinoshita *et al*. 2011). Thus, *T. songlu* and *T. qujingense* are significantly different from the known species and are thus phylogenetically distinct.

Morphologically, *T. qujingense* and *T. songlu* share characteristics with the species of Clade 1: whitish, hairy ascomata and reticulate spores (Wang *et al*. 2007; Kinoshita *et al*. 2011; Wan *et al*. 2017a). However, *T. qujingense* and *T. songlu* both have prosenchymatous peridium and 1–4-spored asci while *Tuber sp*. 10 has plectenchyma peridium and 2–3-spored asci (Kinoshita *et al*. 2011). Besides, *T. qujingense* differs in its fusiform ascospores when compared with the larger and ellipsoid ascospores of *T. songlu.* Distinction based on these morphological findings are further supported by phylogenetic analyses (Figs. 1, 2).

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