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Notes on *Tuber huidongense* (*Tuberaceae*, *Ascomycota*), an endemic species from China

Xiao-Juan Deng [1,3], Juan Chen [1,2], Fu-Qiang Yu [1] & Pei-Gui Liu [1*]

pgliu@mail.kib.ac.cn

[1] *Key Laboratory of the Biodiversity and Biogeography, Kunming Institute of Botany Chinese Academy of Sciences, Kunming 650204, P. R. China*

[2] *Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences Beijing 100193, P. R. China*

[3] *Graduate School of Chinese Academy of Sciences Beijing 100039, P. R. China*

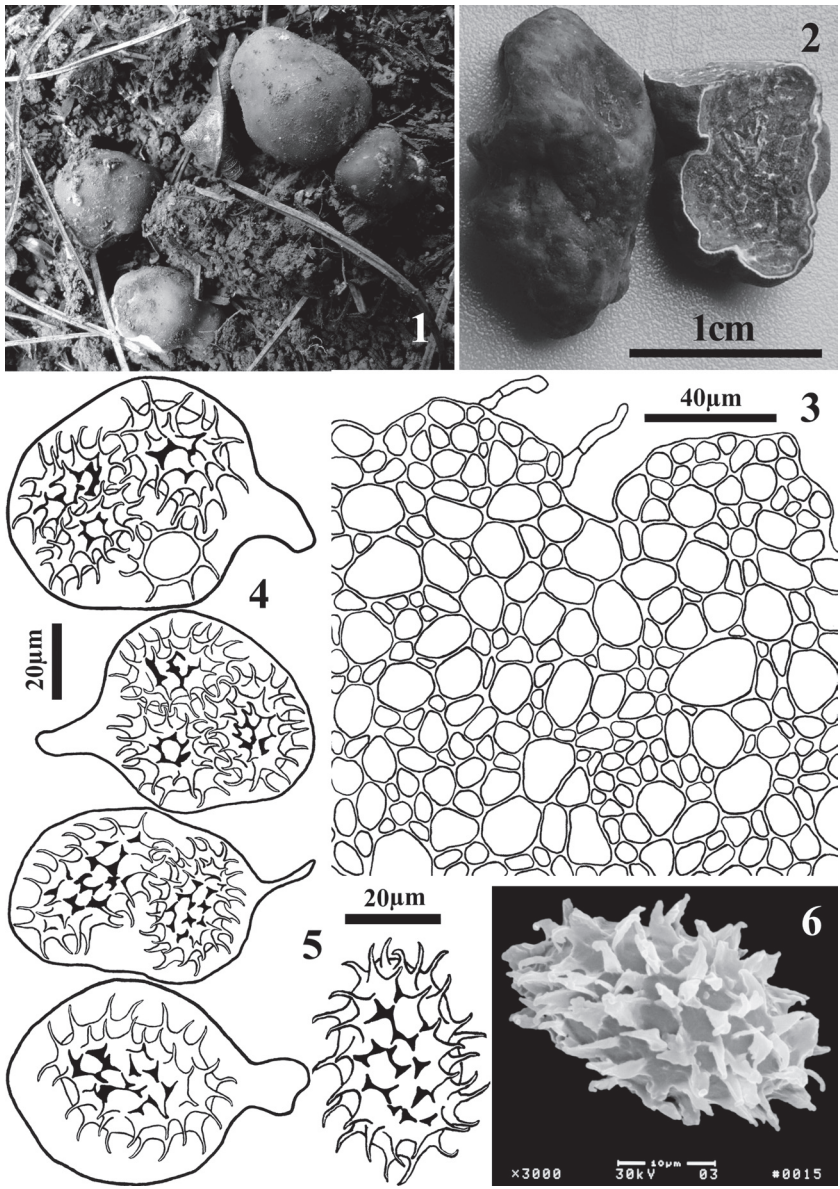
Abstract — Based on material collected from Yunnan and Sichuan, China, *Tuber huidongense* is re-described and illustrated in detail. A key to distinguish *T. huidongense* and its allied species found in China is provided. *T. furfuraceum* is synonymized with *T. huidongense*. Molecular phylogenetic analysis using ITS-rDNA sequences demonstrated that *T. huidongense* is a well-supported species in the *T. rufum*-clade. Morphological characters of *Tuber huidongense* × *Pinus armandii* mycorrhizae are also illustrated and described for the first time.

Key words — truffle, taxonomy, morphology

Introduction

Taxonomic study of the genus *Tuber* in China began with the description of *T. taiyuanense* B. Liu in the 1980s (Liu 1985). Over the last two decades a total of thirteen new species were described from China (Zhang & Minter 1988, Tao & Liu 1989, Wang & Li 1991, Hu 1992, Moreno et al. 1997, Wang et al. 1998, Xu 1999, Wang & He 2002, He et al. 2004, Chen et al. 2005, Hu & Wang 2005, Chen & Liu 2007). Among them, *T. huidongense*, *T. liaotongense* Y. Wang, *T. taiyuanense*, *T. umbilicatum* Juan Chen & P.G. Liu, and *T. furfuraceum* are five taxa that resemble each other. The phylogenetic work by Wang et al. (2007) indicated that *T. huidongense*, *T. taiyuanense,* and *T. liaotongense* clustered in the *T. rufum* group. However, morphological characters of *T. huidongense* were not fully documented and its relation with other species, such as *T. furfuraceum,*

* Corresponding author



Figs. 1–6: *Tuber huidongense*. 1. Ascomata in natural habitat; 2. Dried ascomata; 3. Pseudoparenchymatous tissue of outer layer of peridium with outer peridial hyphae; 4. Asci and ascospores; 5. Line drawing of an ascospore; 6. SEM photomicrograph of an ascospore.

remained unclear. Additional *Tuber huidongense* specimens collected from Yunnan and Sichuan Province, China, made it possible to examine it in depth and reveal the morphological variation among individuals. Mycorrhizae, which were traced to ascomata of *T. huidongense* in a forest of *Pinus armandii* Franch., were confirmed by ITS sequence comparison and their morphological characters are reported herein.

Materials and methods

Macroscopic characters are described from fresh or dried materials, while microscopic characters are based on the dried material. Sections of tissue were made with a razor blade and mounted in 5% KOH. Line drawings were made with a Nikon E400 microscope and the aid of a drawing tube. Scanning electron microscopy (SEM) followed Chen et al. (2005). Statistical analysis of spore measurements followed Yang (2000). Specimens examined were deposited in the Herbarium of Cryptogams, Kunming Institute of Botany, Chinese Academy of Sciences (KUN-HKAS) and Herbarium of Forestry Department, National Taiwan University, Taipei, Taiwan, China (NTUF).

Samples of mycorrhizae were collected beneath an ascomata of collection KUN-HKAS 55305 in Xiangyun County (Central Yunnan, China). The procedure for preparing samples of mycorrhizae for observation followed Agerer (1987–2006). Samples of mycorrhizae were examined with a Leica S8APO stereoscope. Cross-sections of mycorrhizae were made with a Leica CM1100 freezing microtome. The macro- and microscopic characters of mycorrhizae are described and illustrated according to Agerer (1987–2006).

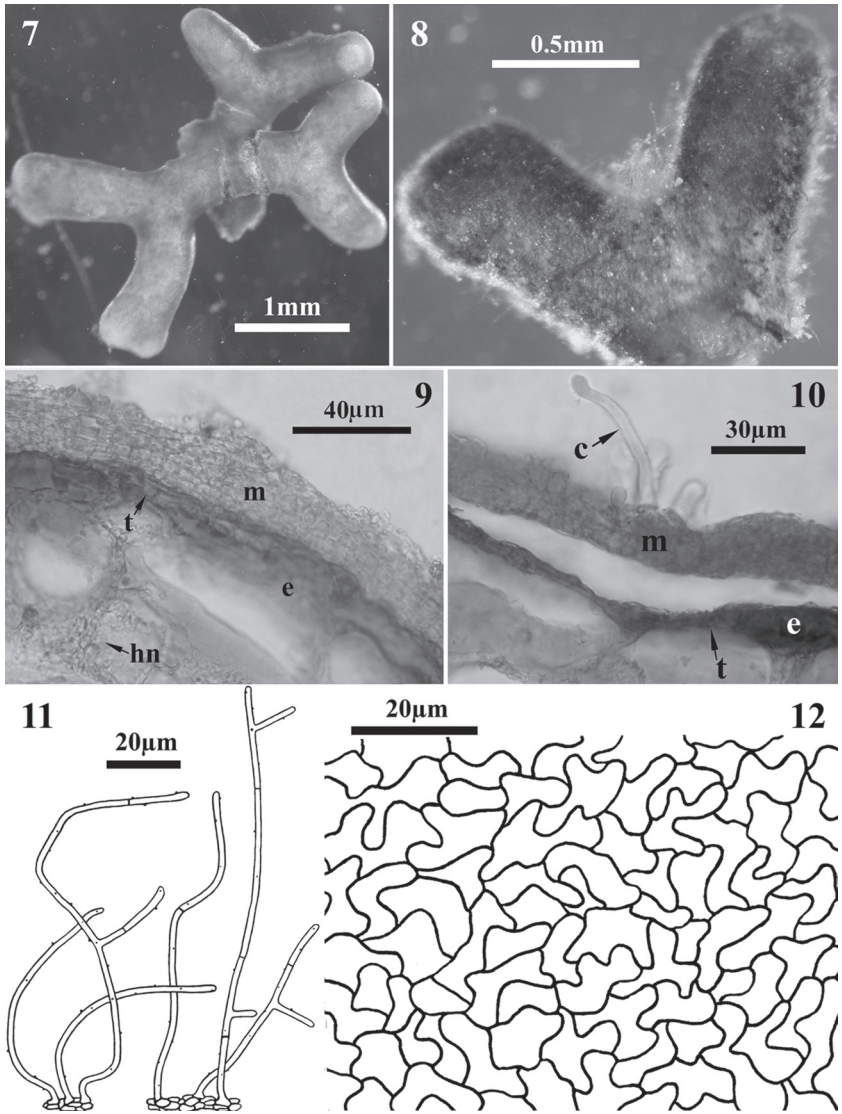
DNA was extracted from samples using modified CTAB (Doyle 1987). The primers ITS5 (White 1990) and ITS4LNG (Paolocci et al. 1999) were used to amplify the ITS region of the DNA from ascomata and mycorrhizae. The primers Nad3-1 and Nad3-2 (Soranzo 1999) were used to amplify the mitochondrion gene of host plant in mycorrhizae. PCR reaction solution and cycling parameters in Chen & Liu (2007) were used with necessary modification. PCR reaction was performed on a Takara TP100 thermal cycler. Amplification products were electrophoresed on a 1% agarose gel, and purified with Sangon's purification kit. Sequencing was performed with a BigDye® Terminator v3.1 Cycle Sequencing Kit on an ABI 3730XL automatic sequencer. Software and methods used in sequence alignment and phylogenetic analysis followed Chen & Liu (2007).

Results

Morphology of ascomata — Figs. 1–6

Tuber huidongense Y. Wang, Mycotaxon 83: 191 (2002).

= *Tuber furfuraceum* H.T. Hu & Y. Wang, Mycotaxon 93: 155 (2005).



Figs. 7–12: Mycorrhizae formed by *T. huidongense* on *P. armandii*. 7. Bifurcate mycorrhizal roots; 8. Mycorrhizal root tips with emanating hyphae; 9, 10. Cross section of mycorrhizae, cystidia (c), epidermis (e), hn (Hartig net), mantle (m), tannins (t); 11. Emanating hyphae; 12. Pseudoparenchymatic structure of outer mantle layer.

Ascomata subglobose, irregularly globose, ellipsoid, furrowed slightly or irregularly, 0.5–2.5 cm in diam; surface yellow-brown to red-brown when fresh, brown when dried, under stereoscope verrucose or slightly furfuraceous, pubescent or not, pubescence more visible when fresh. Gleba whitish when young, brown to nearly black when mature, marbled with pale to light brown veins (Figs. 1, 2).

Peridium 150–300 µm thick (excluding verrucae, which are 10–50 µm tall), composed of two layers: outer layer 80–150 µm thick, pseudoparenchymatous, composed of subglobose or subangular yellow- brown cells, 4–30 × 4–21 µm, cell walls 2–3 µm thick; inner layer 90–150 µm thick, composed of intricately interwoven, thin-walled hyphae 1–5 µm diam; outer peridial hyphae pale yellow-brown, cylindrical, sometimes with inflated ends, 15–30 µm long, 3–5 µm in diam, not septate or with one septum, originating from superficial cells (Fig. 3). Asci 42–70 × 35–55 µm excluding stalk, ellipsoid or subglobose, stalk measuring 8–25 × 6–14 µm, 1–4(–5) spored (Fig. 4). Ascospores ellipsoid to narrowly ellipsoid, spiny-reticulate, yellowish brown at maturity; in 1-spored asci 25–44(–45) × (19–)20–28 µm, 2-spored asci 25–40 × (16–)18–24(–28) µm, 3-spored asci (25–)27–35(–38) × (17–)18–22(–29) µm, 4-spored asci (22–)25–38(–40) × 16–21(–27) µm; Q = (1.10–)1.25–1.80(–2.24), **Q** = 1.50 ± 0.18 (200/10/5); spines 4–6(–7) µm high, some curving at apex; meshes 4–9 × 3–8 µm, some incomplete, 3–5 across the spore width and 4–6 along the spore length; walls 2 µm thick (Fig. 5, 6).

Habitat and distribution: under *P. armandii*-dominated forests mixed with *Betula alnoides* Buch.-Ham. ex D. Don, *Myrsine africana* L., and *Cotoneaster franchetii* Bois, or under *Cyclobalanopsis glauca* Oerst.

Specimens examined: CHINA. SICHUAN PROVINCE: Huidong Co., Jiangzhou (E102°47', N26°56'), Minde village, in *P. armandii* forest, alt. 2070 m, 26 Nov. 1989, Y. Wang 89923 (IFS89923-Holotype); Huidong Co. (E102°58', N 26°63'), wild edible mushroom market, 3 Nov. 2006, J. Chen 410 (KUN-HKAS 52008), J. Chen 419 (KUN-HKAS 52015); Huili Co. (E102°24', N26°66'), wild edible mushroom market, 6 Nov. 2006, J. Chen 420 (KUN-HKAS 52016). YUNNAN PROVINCE: Kunming City, Shuanglong, Jiulongwan, Erdanshishan (E102°48.394', N25°09.928'), in *P. armandii* and *Betula alnoides* mixed forest, alt. 2260 m, 10 Nov. 2007, X. J. Deng JD-03 (KUN-HKAS 55304); Xiangyun Co., 320 national highway, toll station (E100°32.043', N25°26.943'), in mixed forest dominated by *P. armandii*, *Myrsine africana*, and *Cotoneaster franchetii*, alt. 1950 m, 2 Dec. 2007, X. J. Deng XY-03 (KUN-HKAS 55305). TAIWAN: Nan-tou Co., under *Cyclobalanopsis glauca*, alt. 1200m, Dec. 2002, H. T. Hu 0201(Holotype of *T. furfuraceum*,in NTUF).

Morphology of mycorrhizae of *T. huidongense* × *P. armandii* Figs. 7–12

Mycorrhizal system mostly bifurcate, rarely unramified, 1–3.5 mm long, with 1–3 orders of ramifications; short-distance exploration type. Main axes 0.4–0.5 mm, slightly bent. Unramified ends straight, dark brown in older

parts, brownish in young parts and often whitish to pale brown at the apex, cylindrical and with a rounded apex, 0.5–1 mm long and 0.4–0.6 mm in diam; cortical cells not visible; mantle not transparent, with locally darker patches. Emanating hyphae hyaline, loosely woolly, sometimes scattered and distributed unequally. KOH 5%, cotton-blue, and Melzer's reagent reactions not distinctive. Rhizomorphs lacking. Sclerotia lacking.

Mantle 15–25 μm thick, pseudoparenchymatic in cross-section, composed of 6–8 layers of hyphal cells; cells 2–10 × 2–8 μm, pale yellow-brown, round to elliptical; slightly discernible with different layers; Outer mantle layers pseudoparenchymatic in structure with interlocking epidermal cells arranged in a puzzle-like pattern in plan view [mantle type M in Agerer (1987–2006)]; cells 10–18 × 5–10 μm, thin-walled. Emanating hyphae cylindrical, thin-walled, bent and septate, 70–150 μm long and 2–3 μm in diam, ends rounded; simple or ramified almost perpendicular and a considerable distance from septum, branches 10–40 μm long; surface warty, warts distributed more or less evenly. Cystidia emanating from outer layer of mantle, cylindrical, thin-walled, 15–45 μm long, 5–8 μm in diam. Tannin cells present, 8–12 × 10–15 μm, red-brown to red, one row. Hartig net composed of 1–2 layers of hyphal cells, two rows thick [Hartig nets type A in Agerer (1987–2006)], cells 1–3 × 2–4 μm.

Phylogenetic analysis

Twenty-four partial internal transcribed spacer ribosomal DNA (ITS-rDNA) sequences of eleven *Tuber* species (*T. candidum* Harkn., *T. excavatum* Vittad., *T. ferrugineum* Vittad., *T. huidongense*, *T. furfuraceum*, *T. liaotongense*, *T. pseudoexcavatum* Y. Wang et al., *T. quercicola* J.L. Frank et al., *T. rufum* Pico, *T. taiyuanense*, and *T. umbilicatum*) were used for analysis (Table 1). *Tuber excavatum* was selected as outgroup. Of the 516 characters analyzed 352 characters were constant, 71 were variable and 93 were parsimony informative. 90 ambiguous characters were excluded from the analyses.

The ITS phylogenetic tree revealed two major well-supported clades. Clade II is composed of *T. pseudoexcavatum*; In clade I *T. liaotongense* (subclade I), *T. huidongense* (subclade II), *T. umbilicatum* (subclade III), and *T. rufum* (subclade IV) formed subclades with high bootstrap support (≥96%) respectively. One sample of *T. taiyuanense* and two samples of *T. umbilicatum* formed a subclade with bootstrap support of 71%. Three samples identified as *T. candidum*, *T. ferrugineum*, and *T. quercicola* were also included in clade I (Fig. 13).

Discussion

When originally described, the ascomata of *T. huidongense* were described as "rough with scattered hairs" (Wang & He 2002). However, our careful observation found that some specimens (KUN-HKAS 52016, KUN-HKAS55304) had hairs

on the surface of ascomata and others not. Those collections proved to be conspecific in our phylogenetic tree (Fig. 13). Based on this, we regard this character as inconsistent in *T. huidongense* and it might be affected either by environmental factors or by mechanical friction. Significant variations were also found to exist between individuals in the shape of spores and to numbers of spores per ascus. For example, the spores of KUN-HKAS 55305 had the lowest **Q** value (**Q** = 1.35) and an extremely high proportion of 1-spored and 2-spored

Table 1: Origin of the fungal sequences.

Taxon	Code	Voucher	Geographic Origin	ITS1	ITS2
T. liaotongense	L1	/	Fushun, Liaoning, China	DQ478669	DQ478633
T. liaotongense	L2	/	Inner Mongolia, China	DQ478671	DQ478635
T. liaotongense	L3	/	Inner Mongolia, China	DQ478672	DQ478634
T. huidongense	H1	/	Panzhihua, Sichuan, China	DQ486031	DQ486031
T. huidongense	H2	/	Panzhihua, Sichuan, China	DQ486032	DQ486032
T. huidongense	H3	HKAS 52015	Huidong, Sichuan, China	FJ797882	FJ797882
T. huidongense	H4	HKAS 52008	Huidong, Sichuan, China	FJ797881	FJ797881
T. huidongense	H5	HKAS 52016	Huili, Sichuan, China	FJ797883	FJ797883
T. huidongense	H6	HKAS 55305	Xiangyun, Yunnan, China	FJ797877	FJ797877
T. huidongense	H7	HKAS 55304	Kunming, Yunnan, China	FJ797878	FJ797878
T. furfuraceum	H8	NTUF Holotype	Taibei, Taiwan China	FJ859900	FJ859900
T. taiyuanense	T1	HMAS 60234	Xuanhua, Hubei, China	DQ478664	DQ478650
T. umbilicatum	U1	HKAS 52012	Huidong, Sichuan, China	FJ797879	FJ797879
T. umbilicatum	U2	HKAS 52012	Huidong, Sichuan, China	FJ797880	FJ797880
T. rufum	R1	/	Italy	AY112894	AY112894
T. rufum	R2	/	Italy	AY940646	AY940646
T. quercicola	Q1	/	Oregon, USA	AY918957	AY918957
T. candidum	C1	/	Southern Oregon, USA	AY830856	AY830856
T. ferrugineum	F1	/	/	AF132506	AF132506
T. pseudoexcavatum	P1	HKAS 39504	Chuxiong, Yunnan, China	AY514310	AY514310
T. pseudoexcavatum	P2	/	Huili, Sichuan, China	DQ329368	DQ329368
T. pseudoexcavatum	P3	/	Panzhihua, Sichuan, China	DQ329370	DQ329370
T. excavatum	E1	/	Miskolctapolca, Hungary	AJ557545	AJ557545

asci accounting for 40% and 55% respectively, whereas those of KUN-HKAS 52016 had the highest **Q** value (**Q** = 1.63) and a relatively high proportion of 3-spored and 4-spores asci, 35% and 27% respectively (Fig. 14).

At first glance, *T. huidongense* closely resembles *T. furfuraceum*, a species described from Taiwan by Hu & Wang (2005). They were considered different because *T. furfuraceum* had narrower, more ellipsoid spores [**Q** = (1.3–)1.7 (–2.3)] compared to *T. huidongense* (Hu & Wang 2005). However, such variation falls within the range revealed by the specimens of *T. huidongense* (Fig. 14). The conspecificity is also supported by molecular data (Fig. 13). Hence, we conclude that *T. huidongense* and *T. furfuraceum* are synonyms.

The affinity of *T. huidongense* to *T. borchii* Vittad., *T. maculatum* Vittad., and *T. pseudoexcavatum* as supposed by Wang & He (2002) is not yet supported by phylogenetic analysis of Wang et al. (2007), Jeandroz et al. (2008), or our study (Fig. 13). *Tuber huidongense* belongs to the *T. rufum* group, which contains *T. liaotongense*, *T. taiyuanense* from China, *T. rufum* from Europe, and *T. candidum* and *T. quercicola* from North America (Wang et al. 2007, Jeandroz et al. 2008). Our phylogenetic analysis found another member of this group, *T. umbilicatum* (Fig. 13), which was described from China (Chen et al. 2005). Morphologically *T. huidongense* shares a number of characters with this group, such as ascomata with small verrucose peridia and yellowish colored and spiny-reticulate spores. A key to distinguish the taxa from China in this group is provided.

Key to *T. huidongense* and its allied species from China

1. Ascomata with basal cavity ..... *T. umbilicatum*
1. Ascomata without basal cavity 2
2. Ascomata smooth; spores with 5–8 meshes across width ..... *T. taiyuanense*
2. Ascomata verrucose; spores with 3–5 meshes across width 3
3. Spores ellipsoid to narrowly ellipsoid, Q ≥ 1.35; spines 4–6(–7) µm tall, slightly curved at end ..... *T. huidongense*
3. Spores broadly ellipsoid, Q = 1.28; spines 2–4 µm tall, distinctly curved at end ..... *T. liaotongense*

Mycorrhizae of *T. huidongense* × *P. armandii* are characterized by perpendicularly ramified emanating hyphae with warts and cylindrical cystidia, together with a puzzle-like outer mantle layer. Cystidia and warty emanating hyphae also are found on mycorrhizae formed by *T. borchii* and *T. mesentericum* Vittad., however cystidia of the other two mycorrhizae are awl-shaped (Zambonelli et al. 1993, 1995, 1998; Dunabeitia et al. 1996, Rauscher et al. 1996). Roots of *P. armandii* with *T. indicum* Cooke & Massee, mycorrhizae also have puzzle-like mantle layers with epidermal cells and almost perpendicularly ramified emanating hyphae (result unpublished). However, the surfaces of emanating hyphae of the mycorrhizae formed by *T. indicum* are always smooth.

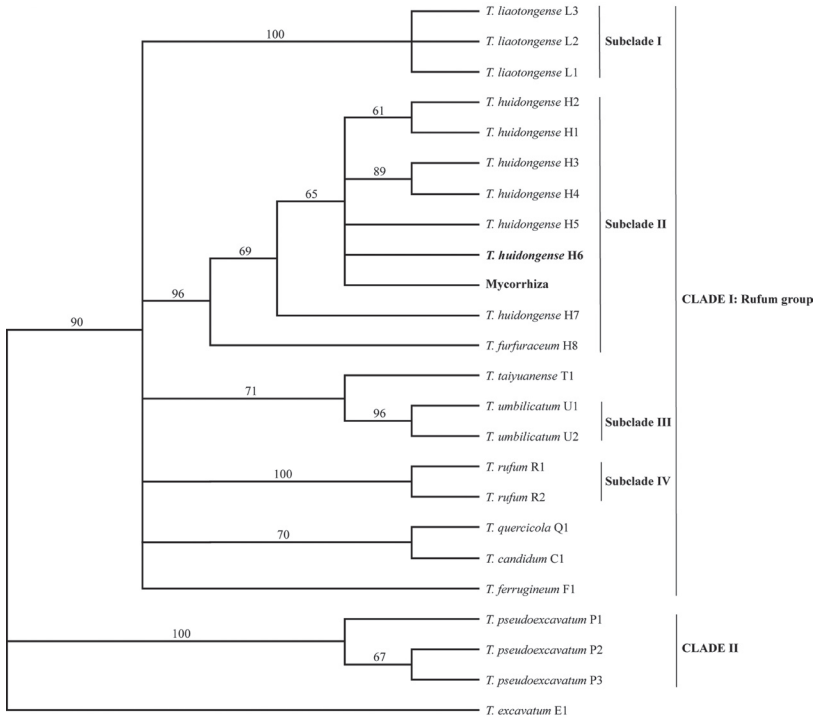


Fig. 13: Maximum parsimony bootstrap consensus tree obtained with ITS1-ITS2 rDNA sequences of *T. huidongense* and related species.

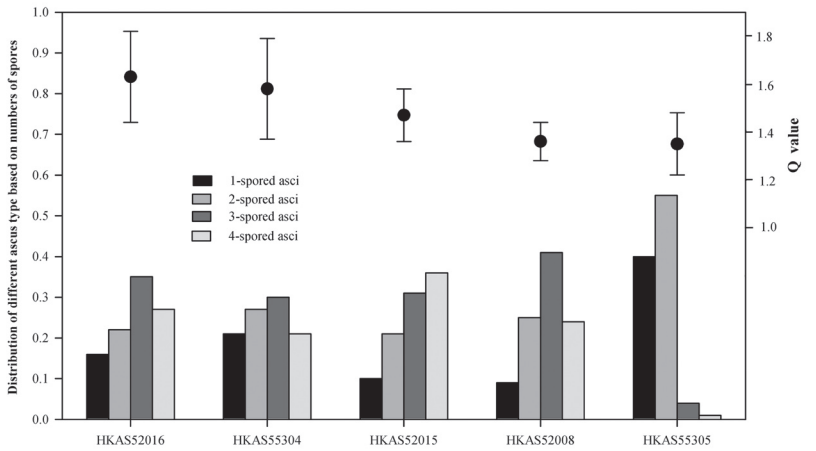


Fig. 14: Q value and distribution of ascus types based on numbers of spores in *T. huidongense*.

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