

Delimitation of *Tuber pseudohimalayense* and *T. pseudoexcavatum* based on morphological and molecular data

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Abstract – The taxonomic relationship between *Tuber pseudohimalayense* and other Chinese black truffles including *T. indicum*, *T. himalayense*, *T. sinense*, *T. pseudoexcavatum* has been controversial. Several studies have treated *T. pseudohimalayense* as a synonym of *T. indicum*. Morphological and molecular analyses of the isotype of *T. pseudohimalayense* in this study suggest that *T. pseudohimalayense* and *T. pseudoexcavatum* are a single species, distinct from *T. indicum*. Although the name *T. pseudoexcavatum* is much more widely used in the recent truffle literature than *T. pseudohimalayense*, the latter has priority over *T. pseudoexcavatum* based on the International Code of Botanical Nomenclature (ICBN). This study documents additional morphological variation among individuals of *T. pseudohimalayense*, a commercial truffle species and endemic to the Himalayan region based on a large number of Chinese materials.

Truffles / Taxonomy / ITS / LSU / morphological variation

INTRODUCTION

With more than 63 species worldwide, *Tuber* F.H.Wigg. is one of the largest genera of hypogeous fungi in the *Pezizales* (*Ascomycota*) in the northern hemisphere, mainly distributed in Mediterranean Europe and North Africa, North America and South and East Asia (Kirk *et al.* 2001; Ceruti *et al.*, 2003). *Tuber* species form ectomycorrhizae on a wide range of trees and shrub species such as *Quercus*, *Corylus* and *Pinus* and produce edible fruitbodies, known as truffles. The Asian black truffles, *T. indicum* Cooke & Masee, *T. sinense* K. Tao & B. Liu, *T. pseudohimalayense* G. Moreno, Manjón, J. Díez & García-Mont, and *T. pseudoexcavatum* Y. Wang, G. Moreno, Rioussset, Manjón & G. Rioussset have been recorded from China (Tao *et al.*, 1989; Zang *et al.*, 1992; Moreno *et al.*, 1997; Wang *et al.*, 1998). These species are characterized by dark-brown to black ascocarps with conspicuous warts and spiny or spiny-reticulate ascospores, which makes it difficult to distinguish these from each other.

Tuber pseudohimalayense was described in 1997 from a Chinese specimen which had been exported to Spain market (Moreno *et al.*, 1997). This species has peculiar spinose-reticulate ascospores, asci with 1-7 spores and black-brown ascocarps covered with pyramidal warts. Morphologically it is very similar to *T. himalayense*, a species originally described from India, northwestern Himalayan region, but it differs from the latter in its regular and complete spinose-reticulate ornamentation of the spores. In contrast, spores of *T. himalayense* are ornamented predominantly with spines or irregular reticulum (Zhang & Minter, 1988). A suite of studies have treated *T. pseudohimalayense* as a synonym of *T. indicum* (Mabru *et al.*, 2004; Zhang *et al.*, 2005; Wang *et al.*, 2006a), but this interpretation was based on specimens of *T. indicum*, which had been annotated as *T. pseudohimalayense* (Paolocci *et al.*, 1997; Roux *et al.*, 1999).

Tuber pseudoexcavatum was described as a new commercial species in 1998 based on some specimens collected from Sichuan province, southwestern China (Wang *et al.*, 1998). Ascocarps which are subglobose, deeply excavated, with a pyramidal warted surface, and ascospores with spinose-reticulate ornamentation were considered to be its diagnostic characters.

At the start of this study, we hypothesized that *T. pseudoexcavatum* and *T. pseudohimalayense* would be a same species because of the morphological very similar original descriptions for both species. To clarify the taxonomic relationship between *T. pseudohimalayense*, *T. indicum* and *T. pseudoexcavatum*, the holotype of *T. indicum*[K(M)39493], the isotypes of *T. himalayense*[K(M)32236] and *T. pseudohimalayense*(AH18331) and many specimens of *T. indicum* and *T. pseudoexcavatum* from China were examined morphologically and their internal transcribed spacer (nrITS) region and ribosomal large subunit rDNA (LSU) were sequenced and analyzed. Although Manjón *et al.* (2009) suggested that the two species mentioned above are conspecific based on evidence from mtLSU sequence analyses, the morphological variation among individuals of *T. pseudoexcavatum* was not documented. In this respect, the present study can be considered a complement to Manjón *et al.* (2009).

MATERIAL AND METHODS

Morphological examination. – Macromorphological descriptions were based on fresh and dry specimens. Micromorphological data and photographs were recorded from the dried specimens after sectioning and mounting in 5% KOH solution for study using a ZEISS Axio Imager AI compound microscope. Scanning electron microscopy (SEM) was performed as explained in Chen & Liu (2007).

Taxon sampling, DNA extraction and PCR amplification – Thirty nine specimens of *T. pseudoexcavatum* were collected from southwestern China (Yunnan and Sichuan provinces). In addition, the isotypes of *T. pseudohimalayense* (AH18331), *T. himalayense* [K(M)32236] and the holotype of *T. indicum* [K(M)39493] were compared and analyzed. Forty *Tuber* samples were selected for molecular analyses. Vouchers and Genbank accession numbers are listed in Table 1. Genomic DNA was extracted from dried or fresh material with the E.Z.N.A. Fungal DNA kit (Omega Bio-Tek, Doraville, Georgia) according to the manufacturer's protocol. PCR amplification and sequence were followed

Table 1. Origin of specimens and accession numbers of sequences from GenBank in this study

<i>Taxon</i>	<i>Voucher specimens Number and code</i>	<i>Locality</i>	<i>Genbank# ITS</i>	<i>Genbank# 28S (LSU)</i>
<i>T. indicum</i> *	T60	Chuxiong, Yunnan, China	GU979059	GU979134
<i>T. indicum</i>		Unknown	AF106882	
<i>T. indicum</i> *	T52	Gongshan, Yunnan, China	GU979053	GU979116
<i>T. indicum</i> *	T15	Kunming market, China	GU979068	
<i>T. indicum</i> *	T10	Paizhuhua, Sichuan, China	GU979050	GU979112
<i>T. indicum</i> *	T66	Paizhuhua, Sichuan, China	GU979051	GU979113
<i>T. indicum</i> *	T113	Huidong, Sichuan, China	GU979081	GU979137
<i>T. indicum</i> *	T115	Yongsheng, Yunnan, China	GU979082	GU979138
<i>T. sinense</i> *	T11	Huidong, Sichuan, China	GU979061	GU979117
<i>T. indicum</i> *	T49	Kunming, China	GU979078	GU979119
<i>T. indicum</i> *	T81	Yimen, Yunnan, China	GU979067	GU979127
<i>T. indicum</i> *	T16	Haikou, Yunnan, China	GU979069	GU979122
<i>T. indicum</i>	Ti20	Unknown	U89362	
<i>T. indicum</i>			AY514308	
<i>T. melanosporum</i>		Unknown		AF435821
<i>T. melanosporum</i> *	T99	Italy	GU979083	GU979139
<i>T. melanosporum</i>	A59	France	AF106878	
<i>T. melanosporum</i>	Tm13	Vaucluse, France	AF132501	
<i>T. melanosporum</i>		Unknown	U89359	
<i>T. pseudohimalayense</i> *	AH18331, T41	Import from China		GU979110
<i>T. pseudoexcavatum</i> *	T14	Huidong, Sichuan, China	GU979039	GU979102
<i>T. pseudoexcavatum</i> *	T29	Huidong, Sichuan, China	GU979040	GU979103
<i>T. pseudoexcavatum</i> *	T80	Yimen, Yunnan, China	GU979046	GU979107
<i>T. pseudoexcavatum</i> *	T33	Huidong, Sichuan, China		GU979108
<i>T. pseudoexcavatum</i> *	T114	Huidong, Sichuan, China	GU979042	GU979109
<i>T. pseudoexcavatum</i> *	T13	Huidong, Sichuan, China	GU979041	GU979104
<i>T. pseudoexcavatum</i> *	T31	Baoshan, Yunnan, China	GU979045	GU979106
<i>T. pseudoexcavatum</i> *	T32	Kunming, Yunnan, China	GU979044	
<i>T. pseudoexcavatum</i> *	T18	Kunming, Yunnan, China	GU979043	GU979105
<i>T. pseudoexcavatum</i>	HKAS 39504	Chuxiong, Yunnan, China	AY514310	
<i>T. brumale</i>		Marche, Italy	AF106880	
<i>T. brumale</i>		Unknown	AF132504	
<i>T. umbilicatum</i> *	CJ409A, T104	Huidong, Sichuan, China	FJ797879	GU979096
<i>T. huidongense</i> *	CJ410A, T105	Huidong, Sichuan, China	FJ797882	GU979094
<i>T. huidongense</i> *	CJ419, T110	Huidong, Sichuan, China	FJ797881	GU979093
<i>T. huidongense</i> *	CJ243, T5	Chuxiong, Yunnan, China	GU979035	GU979091
<i>T. huidongense</i> *	CJ420, T107	Huili, Sichuan, China	FJ797883	GU979099
<i>T. furfuraceum</i> *	T4	Taiwan	GU979034	GU979090
(= <i>T. huidongense</i>)				
<i>T. excavatum</i> *	CJ411A T106	Huidong, Sichuan, China	GQ217540	
<i>T. excavatum</i>	Trappe 19457	Unknown		DQ191677

* Sequences obtained in our lab.

by Chen & Liu (2007). The ITS and LSU sequences were amplified with primers ITS4/ITS5 and LR5/LROR, respectively (White *et al.*, 1990; Moncalvo *et al.*, 2000).

Sequence alignments and phylogenetic analyses – Sequences were edited with SeqMan (DNASTAR Package). Nucleotide sequences were initially aligned with Clustal X 1.83 (Thompson *et al.*, 1997) and adjusted manually in BioEdit Version 5.0.9 (Hall, 1999). Phylogenetic analyses were performed with PAUP version 4.0b10 (Swofford, 2002). LSU gene tree were inferred by Maximum parsimony (MP) method using heuristic search and tree bisection reconnection (TBR) branch swapping with 1000 search replicates. Bootstrap tests were performed using 500 replicates. Bayesian analyses were implemented on combined 5.8S-ITS2 and LSU sequences in MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). A initially run for 2,000,000 generations with four simultaneous Markov chain Monte Caro (MCMC) chains was done to estimate how many generations were required for likelihood scores to reach stationary. Bayesian PP was determined by computing majority rule consensus trees by use of the set of trees that reached stationary phase. A probability of 95% was considered significant.

RESULTS

Morphological analyses

The isotypes of *T. pseudohimalayense* and *T. pseudoexcavatum* shared completely similar morphological features of peridium, asci and ascospores (Fig. 2, Table 2). Their ascocarps' surface were both covered with minutely pyramidal warts and the peridium outer layer was pseudoparenchymatous and composed of subglobose to ellipsoid cells 10-30 μm diam. Asci usually contained 1-7 (8) spores in each ascus and spores are ornamented with spines connected by low ridges to form an regularly alveolate reticulum. In contrast, the peridium of *T. indicum* and *T. himalayense* was composed of polygonal, subglobose or elongated cells, usually 10-20 μm diam and the spore ornamentation was mostly composed of separate spines or an irregular reticulum.

Moreover, morphological features (size, shape and ornamentation of spores) varied significantly among collections of *T. pseudoexcavatum*. For instance, the spores of some specimens were mainly ellipsoid and the Q (length/width ratio) = (1.05–)1.2–1.56(–1.67), Q (average of all ascospores sampled along with standard deviation) = 1.40 ± 0.12 (KUN-HKAS44324a, 44325a, 44338, 49744, 49747, 47618, 47619, 37469, 39504, 42399), other specimens have broad ellipsoid spores [Q=(1.05–)1.1–1.44(–1.5), Q=1.26 \pm 0.10] (KUN-HKAS47617, 47616, 44991, 41313, 44346, 44343, 52016) and a few specimens have subfusiform ascospores ornamented incompletely with a reticulum or even spines (KUN-HKAS 44324a,44325)(Fig. 1 m-o).

Molecular analysis

We were unsuccessful in extracting DNA from the *T. indicum* holotype and *T. himalayense* isotype because the samples were probably too old.

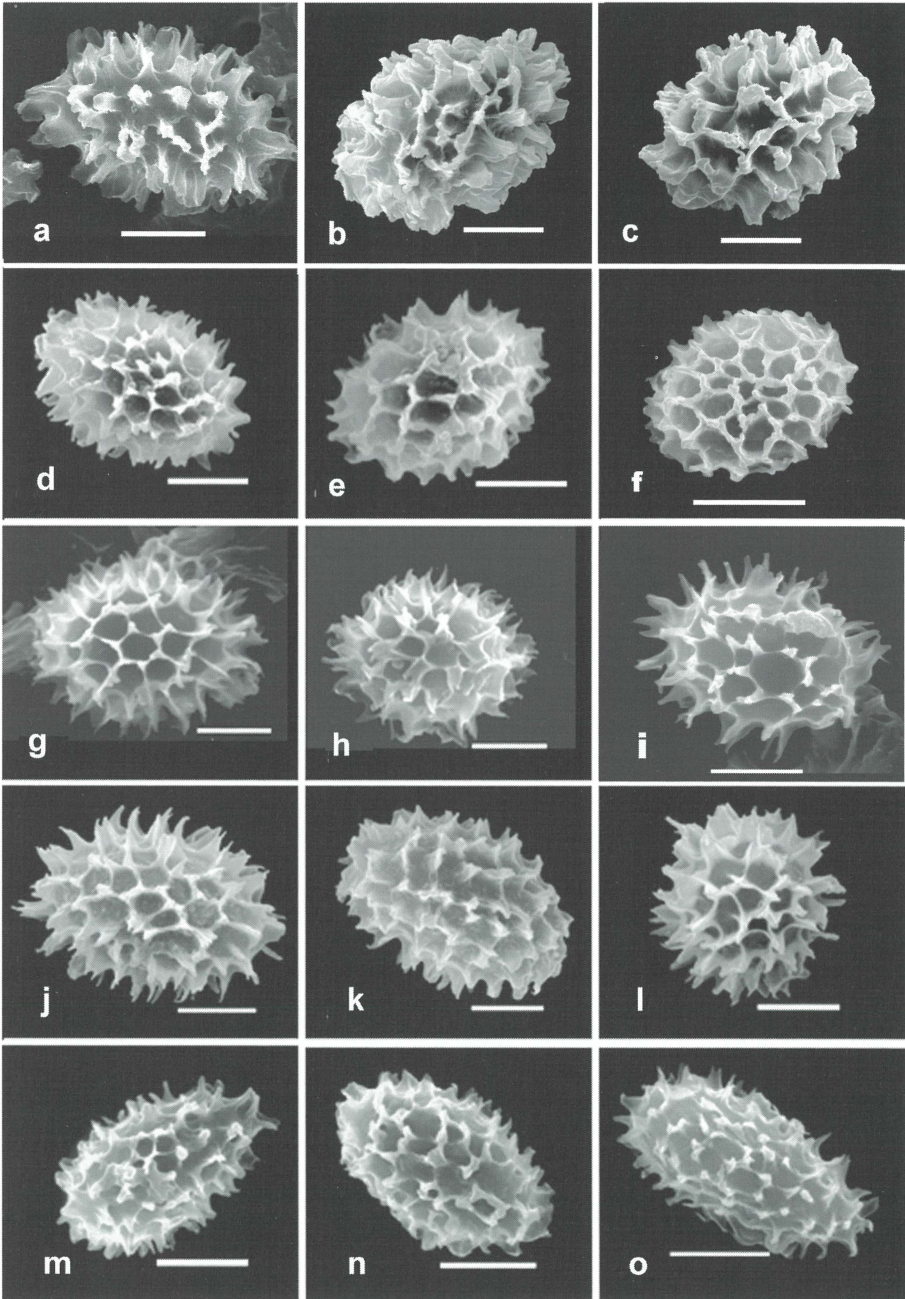


Fig. 1. SEM of *Tuber* species ascospores, showing the detail of ornamentation. **a.** *T. indicum* **Holotype** K(M)39493; **b-c.** *T. himalayense* **Isotype** K(M)32236; **d-f.** *T. pseudohimalayense* **Isotype** AH18331. **g-i.** *T. pseudoexcavatum* KUN-HKAS47617; **j-l.** *T. pseudoexcavatum* KUN-HKAS52011; **m-o.** *T. pseudoexcavatum* KUN-HKAS44325b; Scale bar = 10 μ m.

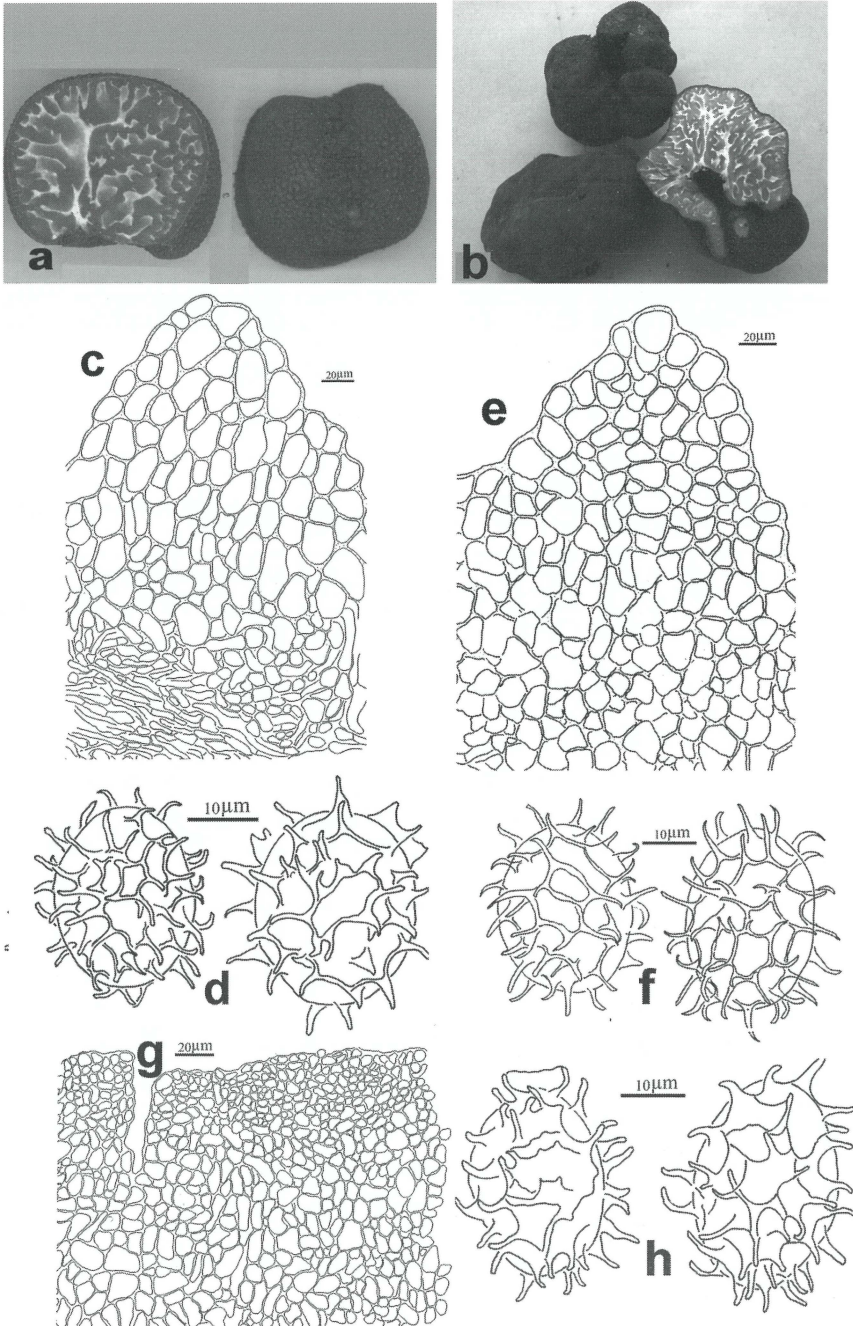


Fig. 2. Morphological characters of *T. pseudoexcavatum* and *T. pseudohimalayense*. **a-b-c-d.** *T. pseudoexcavatum* (**a-c-d.** KUN-HKAS47617; **b.** KUN-HKAS52011). **e-f.** *T. pseudohimalayense* (isotype AH18331). **g-h.** *T. himalayense* isotype [K(M) 32236]. **a-b.** Fresh ascocarps. **c-e-g.** Pseudoparenchymatous tissue of outer layer of peridium. **d-f-h.** Ascospores.

Table 2. Morphological characters of type of *T. indicum* and isotypes of *T. himalayense* and *T. pseudohimalayense*

Fungal taxa	Voucher specimens	Ascomata surface configuration			Peridium	Number of spores of asci	Ascospores		
		Color	Warts	Cavity			Shape	Size (μm)	Ornamentation
<i>T. indicum</i>	K(M)39493 Holotype	Black	Polygonal, 1-2.5 mm wide and slight pointed at the apex.	no	subglobal or elongated cell usually $\leq 20 \mu\text{m}$	(1)3-5(6)	ellipsoidal, $Q = 1.490 \pm 0.11$	(18)20-32(38) \times (15)16-20(26)	Spine, sparse and free, 3-6(7) μm high
<i>T. himalayense</i>	K(M)33236 Isotype	Black	Polygonal, 1-2.5 mm wide and flattened at the apex	no	subglobal or elongated cell usually $\leq 20 \mu\text{m}$	(1)2-4(5)	ellipsoidal to broadly ellipsoidal, $Q = 1.18 \pm 0.37$	(20)23-37(40) \times (16)17-28(30)	Mostly irregular spinose-reticulate, some spores predominantly spine 4-7 μm high and mesh 7-10 (13) \times 5-9 μm and 3-5 μm high.
<i>T. formosanum</i>	HKAS49707	Black	Polygonal, 1-3 mm, usually pointed at the apex.	no	subglobal or elongated cell usually $\leq 20 \mu\text{m}$	1-4	ellipsoidal, $Q = 1.36 \pm 0.118$	20-36(40) \times 17-28(30)	Mostly free spine and 3-5 μm high, some spores irregular spinose-reticulum
<i>T. pseudohimalayense</i>	AH18331 Isotype	Black	Pyramidal, 0.5-1 mm wide and pointed at the apex	Un-Certain	subglobal cell usually 18-25(30) μm	(1)4-7(8)	ellipsoidal to broadly ellipsoidal, $Q = 1.30 \pm 0.09$	20-30 \times 16-24	Regular spinose-reticulum, mesh 7-11 \times 6-10 μm , and about 1 μm high. Spine usually 4-6(-8) μm high.
<i>T. pseudoexcavatum</i>	HKAS49747	Brown	Pyramidal, 0.5-1.5 mm wide and pointed at the apex	yes	subglobal cell usually 18-25(28) μm	(1)4-7(8)	ellipsoidal to broadly ellipsoidal, $Q = 1.33 \pm 0.11$	17-40 \times 14-32	Regular spinose-reticulum. Mesh 4-12 \times 4-11 μm , and about 1 μm high. Spine usually 4-6(-8) μm high.

Fortunately, LSU sequences from the *T. pseudohimalayense* isotype were obtained. Within the LSU region only 3-5 base changes were noted among multiple field collections of *T. pseudoexcavatum* and isotype of *T. pseudohimalayense*.

Forty LSU sequences analyzed in this study included 842 sites, of which 685 characters are constant and 130 are parsimony-informative. The parsimony analysis recovered three major clades (Fig. 3): group *T. huidongense*-*T. umbilicatum* (Clade I), mostly similar to *T. pseudoexcavatum* in spore characters (ascmata yellow-brown, minute papillae and spores spinose-reticulate), group *T. pseudoexcavatum*-*T. pseudohimalayense* (Clade II, ascmata brown, cavity and minutely pyramidal warts and spores spinose-reticulate) and group *T. indicum*-*T. melanosporum* (Clade III, ascmata brown to black, conspicuously warted and spores spiny). The phylogram resulting from the parsimony analysis clustered *T. pseudohimalayense* and *T. pseudoexcavatum* and formed a monophyletic clade with 100 BP support delimiting both taxa from their sister species *T. indicum* and *T. melanosporum* (Fig. 3).

Moreover, Bayesian analyses on combined 5.8S/ ITS2 and LSU data sets recovered the same three major clades as did MP analysis of LSU data. The phylogenetic analysis on combined data set also supported the monophyly of *T. pseudohimalayense* (= *T. pseudoexcavatum*) (Fig. 4). In addition, *T. sinense* appeared to nest within the *T. indicum* group.

The morphological differences between collections of *T. pseudoexcavatum* also were mirrored in ITS sequence length. Two samples (KUN-HKAS 44324a, 44325) displayed only about 650 bp lengths, contrasting with the other samples of *T. pseudoexcavatum* of 860 bp length, due to a 200 bp gap in the ITS1 region.

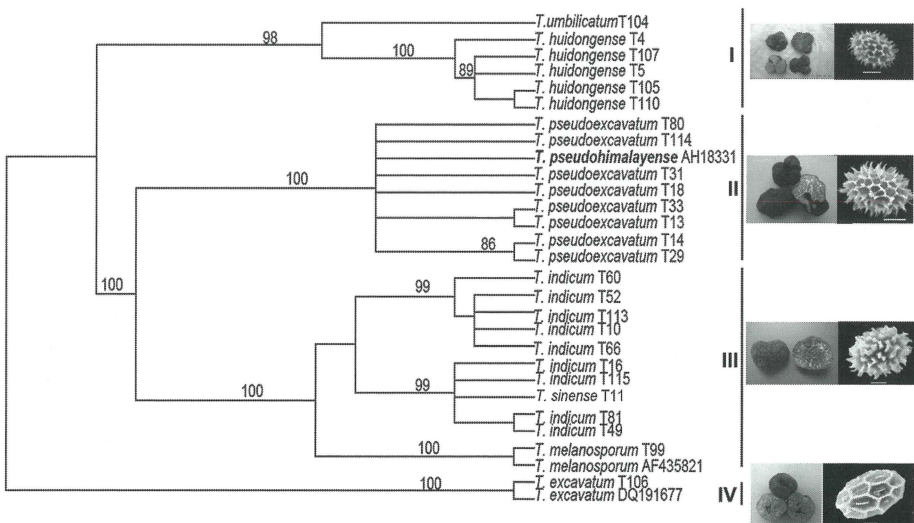


Fig. 3. Strictly consensus tree obtained from maximum parsimony analysis of LSU (total 842 characters, of which 130 are parsimony-informative). Numbers on branches indicate bootstrap values ($\geq 75\%$) from 500 replicates. Bold represents isotype specimens.

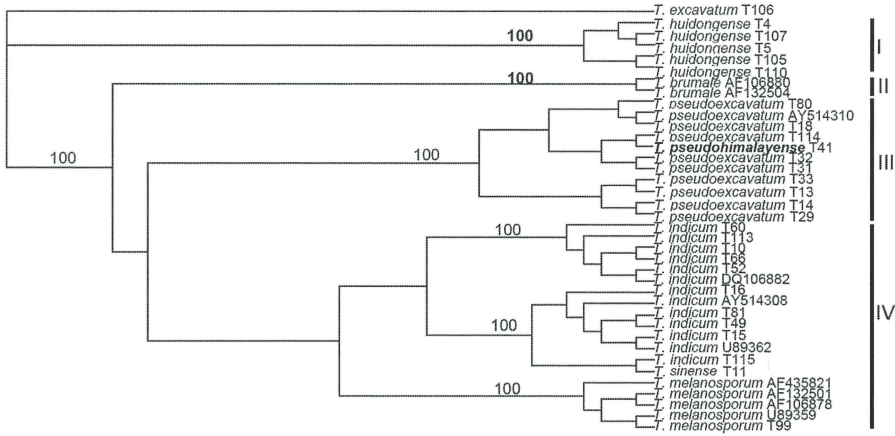


Fig. 4. Phylogenetic relationships of Chinese black truffles derived from Bayesian analysis of combined 5.8S/ ITS2 and LSU sequences (total 1234 characters, of which 330 unique sites). Bayesian posterior probabilities values greater than 95% are indicated above branches.

DISCUSSION

Identification of species with similar morphological characteristics in the genus *Tuber* was always very difficult. A clear delimitation between *T. pseudohimalayense* and other black truffles (*T. indicum*, *T. himalayense* and *T. sinense*) has remained ambiguous owing to the original species description of *T. pseudohimalayense* being based on a single ascocarp (Moreno *et al.*, 1997). Previous studies have treated *T. pseudohimalayense* as a synonym of *T. indicum*, but in fact, morphological features in the original description for *T. pseudohimalayense* are much closer to *T. pseudoexcavatum*.

Our analysis from the isotype of *T. pseudohimalayense* (AH 18331) confirms our initial hypothesis that *T. pseudohimalayense* and *T. pseudoexcavatum* could be conspecific. Morphological characteristics from the isotype specimens of *T. pseudohimalayense* are identical to those of *T. pseudoexcavatum* except for a distinctive cavity. Unfortunately, we failed to obtain the ITS sequences from isotype of *T. pseudohimalayense* due to too small sample and technical reasons (lower quality and quantity of DNA). Therefore, we had to perform the phylogenetic analyses using LSU sequence. The nearly identical LSU sequences (< 5 base changes) for *T. pseudohimalayense* and *T. pseudoexcavatum* suggest they are a single species.

The affinity of *T. pseudohimalayense* (= *T. pseudoexcavatum*) to the Asian black truffle *T. indicum* and the Perigord black truffles *T. melanosporum* and *T. brumale* group was supported in our study. This result agreed with the analysis of Wang *et al.* (2006b) and Jeandroz *et al.* (2008). *T. pseudohimalayense* mostly resembled *T. huidongense* groups (including *T. umbilicatum*, *T. liaotungense* and *T. huidongense* and *T. taiyuanense*) in sporal features and *T. excavatum* in appearance. However, the phylogenetic analyses indicated that *T. pseudohimalayense* is most closely related to *T. brumale*. The latter differs from

T. pseudohimalayense in having a spiny spore ornamentation, rather than a spinose-reticulum.

As a result of the above, we arrive at the following taxonomic decision:

Tuber pseudohimalayense G. Moreno, Manjón, Díez & García-Montero, *Mycotaxon* 63: 217–224, 1997.

= *Tuber pseudoexcavatum* Y. Wang, G. Moreno, L. J. Rioussset, Manjón & G. Rioussset, *Cryptogamie Mycologie* 19(1-2): 113-120, 1998, syn. nov.

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