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Tuber pseudoexcavatum versus *T. pseudohimalayense* — new data on the molecular taxonomy and mycorrhizae of Chinese truffles

José L. Manjón[1], Luis G. García-Montero[2*], Pablo Alvarado[1], Gabriel Moreno[1] & Gabriella Di Massimo[3]

josel.manjon@uah.es **luisgonzaga.garcia@upm.es*

[1]*Dept. Biología Vegetal, Facultad de Biología, Universidad de Alcalá 28871 Alcalá de Henares (Madrid), Spain*

[2]*Dept. Forest Engineering, U.D. Operaciones Básicas, E.T.S.I. Montes Technical University of Madrid (UPM) Ciudad Universitaria s/n, Madrid 28040, Spain*

[3]*Dept. di Biologia Vegetale, Universitá degli studi di Perugia Borgo XX Giugno 74, 06121 Perugia, Italy*

Abstract — The study of Chinese *Tuber* species available in European markets began 14 years ago. *T. pseudohimalayense* was proposed as a new species but has been questioned. We evaluated the validity of *T. pseudohimalayense* by comparing the molecular genetics and ectomycorrhizal morphology of *T. pseudohimalayense*, *T. pseudoexcavatum,* and *T. indicum*. As a result of these studies, we propose *T. pseudoexcavatum* to represent a synonym of *T. pseudohimalayense.*

Key words — *Tuberaceae*, hypogeous fungus

Introduction

Several Chinese *Tuber* species have become commercially available in European markets in recent decades. *Tuber pseudohimalayense* G. Moreno et al. (1997) and *T. pseudoexcavatum* Y. Wang et al. (1998) were found in Spanish markets and proposed as new species.

Tuber pseudoexcavatum has been regarded as common, as confirmed by molecular phylogenic and taxonomic studies (Riousset et al. 2001, Zhang et al. 2005, Wang et al. 2006b). *Tuber pseudohimalayense* appeared to be much rarer. Di Massimo et al. (1998) found *T. pseudohimalayense* in Chinese truffle shipments in Italian truffle markets, and Zhang et al. (2005) and Wang et al. (2006a) cited it from China.

Wang & Hall (2001), who reported that *T. pseudohimalayense* closely resembles *T. sinense* K. Tao & B. Liu, proposed using molecular tools to clarify the taxonomy of this and other truffles from southwestern China. Zhang et al. (2005) synonymized *T. pseudohimalayense* and *T. sinense* with *T. indicum* Cooke & Massee based on morphological studies and rDNA ITS sequence analyses.

Wang et al. (2006a) studied the genetics and phylogeography of Chinese *Tuber* species. On the basis of the ITS and β-tubulin gene sequences they concluded that differences in the taxonomic characters of *T. indicum* Cooke & Massee, *T. sinense*, *T. pseudohimalayense,* and *T. himalayense* B.C. Zhang & Minter represented normal variations within a single species, *T. indicum*.

However, Zhang et al. (2005) and Wang et al. (2006a) were unable to study the *T. pseudohimalayense* holotype collection in the University of Alcalá Herbarium (AH 18331), which was unwilling to risk its loan to another country as the type consists of only one small piece of an ascoma (Moreno et al. 1997).

Comandini & Pacioni (1997) and Zambonelli et al. (1997) synthesized *T. indicum* ectomycorrhizae. In the University of Alcalá (Spain), *T. pseudohimalayense* and *T. pseudoexcavatum* ectomycorrhizae were also synthesized using spores from ascomata of the type collections of both species (holotype of *T. pseudohimalayense* and an isotype of *T. pseudoexcavatum* AH 18387). We found that *T. pseudohimalayense* ectomycorrhizae had a morphological affinity with *T. indicum* ectomycorrhizae; however, they even more resembled *T. pseudoexcavatum* ectomycorrhizae (Manjón et al. 1998, García-Montero et al. 2008).

We here report results of genetic studies of ascomata from types of *T. pseudohimalayense* and *T. pseudoexcavatum*, plus samples of *T. indicum* and describe the morphology of *T. pseudohimalayense* ectomycorrhizae in comparison with those of *T. indicum* and *T. pseudoexcavatum*. Our aim was to assess the validity of *T. pseudohimalayense* and increase our knowledge of the genetics of Chinese *Tuber* taxa.

Material and methods

Material examined: *Tuber pseudohimalayense* (holotype, AH 18331) probably imported from China, January 1995; *Tuber pseudoexcavatum* (isotype, AH 18387) imported from China from pine forests in Yunnan, January 1995; *Tuber indicum* (AH 18329), probably imported from China, January 1995; *Tuber indicum* holotype (K 39493) and *Tuber himalayense* isotype (K 32236) from the Royal Botanic Gardens, Kew; and samples of *Tuber sinense* (personal collection provided by Y. Wang, with immature ascospores).

Molecular methods: Total DNA from *T. pseudohimalayense* and its putative closest relatives among Chinese *Tuber* species (Table 1) was extracted by means of

Table 1. Samples included in molecular comparisons

Species	Herbarium Code	Type collection	ITS GenBank#	28S LSU GenBank#	mtLSU GenBank#
T. pseudohimalayense	AH 18331	Holotypus		FJ233104	FJ792795
T. pseudoexcavatum	AH 18387	Isotypus		FJ233103	FJ792794
T. indicum	AH 18329	–	FJ233102	FJ233102	

the MasterPure™ DNA Purification Kit (Epicentre Biotechnologies, Madison, US) following the manufacturer's instructions. An additional piece of *T. pseudohimalayense* was included to sonicate spores for two periods of 60s in a Cell Disruptor B15 sonifier (Branson). The sample was checked periodically under the microscope to insure spore rupture. After extraction, 1.5 µl resuspended DNA was added to a 50µl PCR mixture with the following concentrations: 1u EcoTaq DNA polymerase with 1× EcoTaq Buffer (Ecogene), $MgCl_2$ 2mM, DNTPs 0.2 mM each. 28S LSU primers U2 (5' – GAC TCC TTG GTC CGT GTT – 3', Sandhu et al. 1995) and LR1 (5' – GCA TAT CAA TAA GCG GAG GA – 3', Van Tuinen et al. 1998), and mitochondrial large ribosomal subunit (mtLSU) primers ML3 (5' – GCT GGT TTT CTA CGA AAC ATA TTT AAG – 3', White et al., 1990) and ML4 (5' – GAG GAT AAT TTG CCG AGT TCC – 3', White et al., 1990) were added at 0.5µM each. PCR reactions were performed under a program consisting of a hot start at 95 °C for 5 min, five cycles at 94 °C, 50 °C and 72 °C (45, 30 and 45 sec, respectively), followed by 30 cycles at 94 °C, 54 °C and 72 °C (45, 30 and 45 sec, respectively) and a final 72 °C step for 10 min. *T. indicum* was amplified once, while *T. pseudoexcavatum* and *T. pseudohimalayense* were amplified twice. PCR products were checked in a 1% agarose gel prior to purification in Sephadex G-50 superfine columns and sequenced in an ABI 3130 sequencer with BigDye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, US) with the same amplification primers as in the sequencing reaction. The sequences were then loaded in MEGA software and visually compared to chromatograms to check for peak reading failures. Sequences were entered into GenBank under the codes specified in Table 1.

Microscopy: For light microscopy, the hand-sectioned samples were mounted in Hoyer's medium. For scan electron microscopy (SEM), the material was rehydrated in concentrated ammonium hydroxide (28–30%) for 30 min, dehydrated in aqueous ethanol (70%) for 30 min, fixed for 2 hr in pure ethylene glycol dimethyl ether (= 1,2-dimethoxymethane) and finally immersed in pure acetone for at least 2 hr, followed by critical point drying and spattering with gold-palladium. This technique uses very little material. The micrographs were taken at the University of Alcalá with a Zeiss DSM–950 SEM. Spore were measured under the oil immersion objective of a light microscope including the ornamentation (Moreno et al. 1997).

Descriptions of ectomycorrhizae: We synthesized *T. pseudohimalayense* ectomycorrhizae with *Quercus ilex* subsp. *ballota* (Desf.) Samp. using spores from the type by applying Bencivenga's (1982) method as modified by Manjón & García-Montero (1996). Plants were grown under controlled environmental conditions in a glasshouse

in the Juan Carlos I Royal Botanical Garden at the University of Alcalá (Madrid, Spain). Mycorrhization of each plant was expressed as the number of *Tuber* mycorrhizae over the total number of non-mycorrhizal tips (Bencivenga et al. 1987). The ectomycorrhiza color was described following the Munsell standard soil color charts (Munsell 1976). Ectomycorrhizae were identified with a stereoscopic microscope (photo-Leica WildMZ8) and a light microscope (photo-Leica LeitzDMRB) as recommended by Agerer (1987–2002), Zambonelli et al. (1993), and Granetti (1995). Mycorrhizae characters of related species were taken from the literature (Table 3).

Results

Molecular analysis: Amplified partial 28S LSU and mtLSU sequences from the manually extracted samples of *Tuber pseudoexcavatum* and *T. pseudohimalayense* and the sonicated sample of *T. pseudohimalayense* are identical to each other but different from *T. indicum*. Further validation by means of rDNA ITS amplification with primers ITS5 (5' – GGA AGT AAA AGT CGT AAC AAG G– 3', White et al., 1990) and ITS4 (5' –TCC TCC GCT TAT TGA TAT GC– 3', White et al., 1990) was unsuccessful for all samples except *T. indicum*, probably due to sample age or deterioration. Comparison of the *T. indicum* ITS sequence to public databases by means of nucleotide BLAST search in NCBI web service (http://www.ncbi.nlm.nih.gov/) showed almost complete identity (99%) with the other *T. indicum* sequences. These indicate *T. pseudoexcavatum* and *T. pseudohimalayense* belong to a single taxon, unrelated to *T. indicum* (Figs. 1–2).

Morphology: Because the molecular analysis indicated that *T. pseudohimalayense* and *T. pseudoexcavatum* represented the same species, we re-examined their macro and microscopic characters. The holotype fragment of *T. pseudohimalayense* has a brown peridium and a slight excavation similar to that of the *T. pseudoexcavatum* ascoma, characters that were overlooked when *T. pseudohimalayense* was originally described. The *T. pseudohimalayense* holotype shows 1–7 spored asci and 1–8 spored asci occur in the species as a whole. Peridium and spores of the two species do not differ (Table 2; Figs. 3–4).

Ectomycorrhizae: The root tips of *Q. ilex* subsp. *ballota* seedlings inoculated with *T. pseudohimalayense* averaged 50% mycorrhization (standard deviation ± 17). No ectomycorrhizae formed by other fungi were detected. *Tuber pseudohimalayense* ectomycorrhizae (Fig. 5) were concentrated in the proximal and median part of the root system. Therefore, the techniques for inoculating and producing mycorrhized plants, as well as the substrates used, gave good results for this truffle species. Macro- and micromorphology of ectomycorrhizae of *T. melanosporum* Vittad. and four Chinese *Tuber* spp. are compared in Tables 3–4.

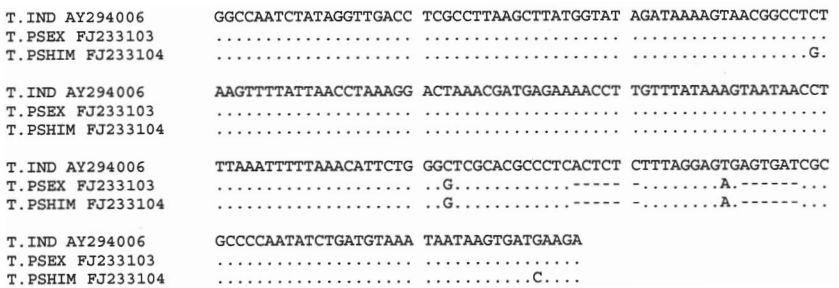

Fig 1. Partial alignment of the mitochondrial large ribosomal subunit (mtLSU) of the studied samples. Conserved bases (·), deleted bases (-).

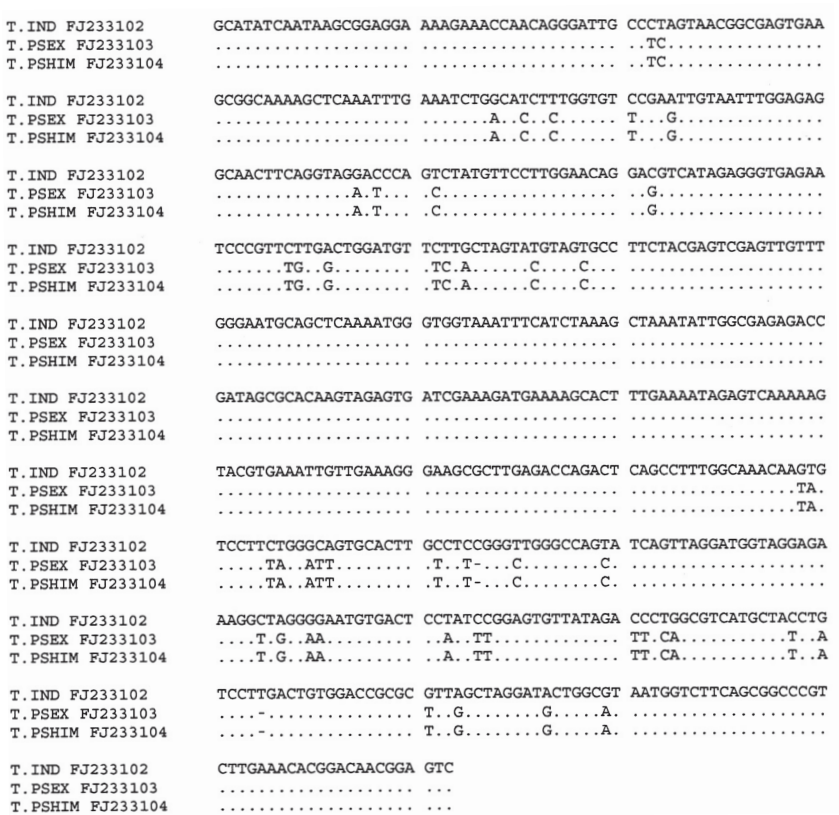

Fig. 2. Partial alignment of the genomic large ribosomal subunit (nrLSU 28S) of the studied samples. Conserved bases (·), deleted bases (-).

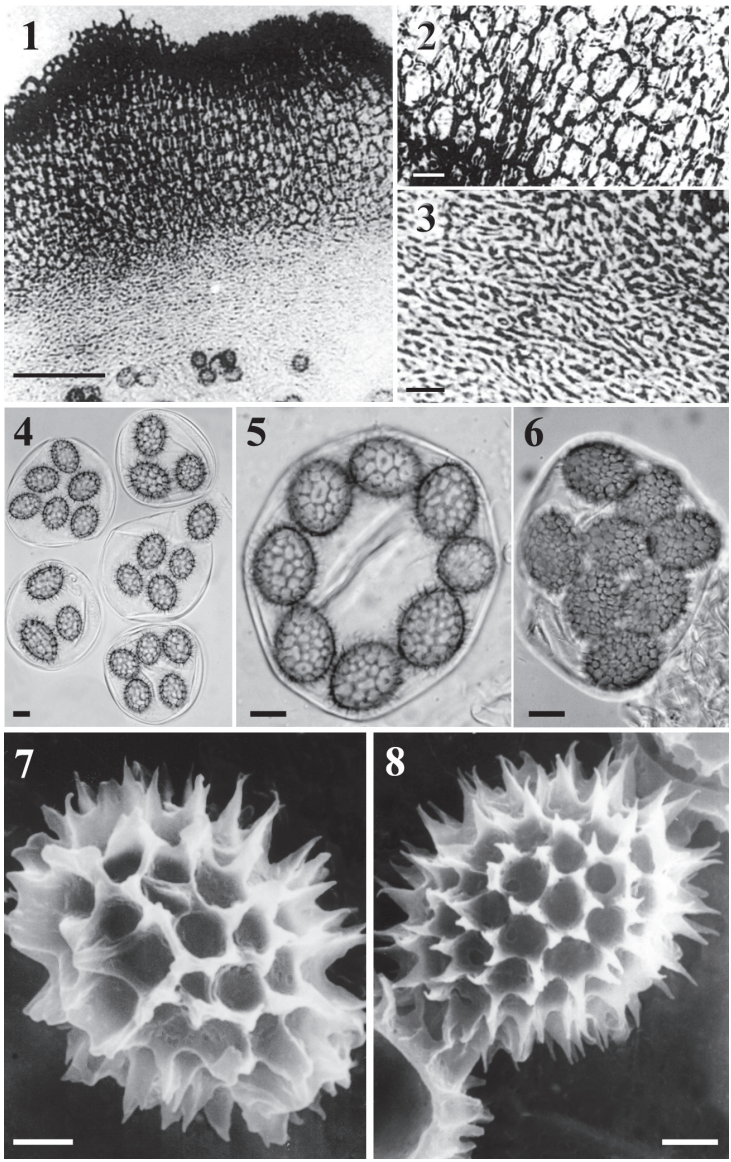

Fig. 3. *Tuber pseudoexcavatum* (Wang et al. 1998). 1) Detail of the external covering of the peridium. 2) Thick-walled globose cells of the external covering of the peridium. 3) Plectenchymal cells of the internal covering of the peridium. 4–6) Globose ascus with 3 to 8 ascospores, where the spiny-reticular ornamentation is apparent. 7–8) Thorny reticulations of spores, under the S.E.M.

(Bars: 1 = 100 µm; 2 = 10 µm; 3 = 20 µm; 4–6 = 10 µm; 7–8 = 5 µm).

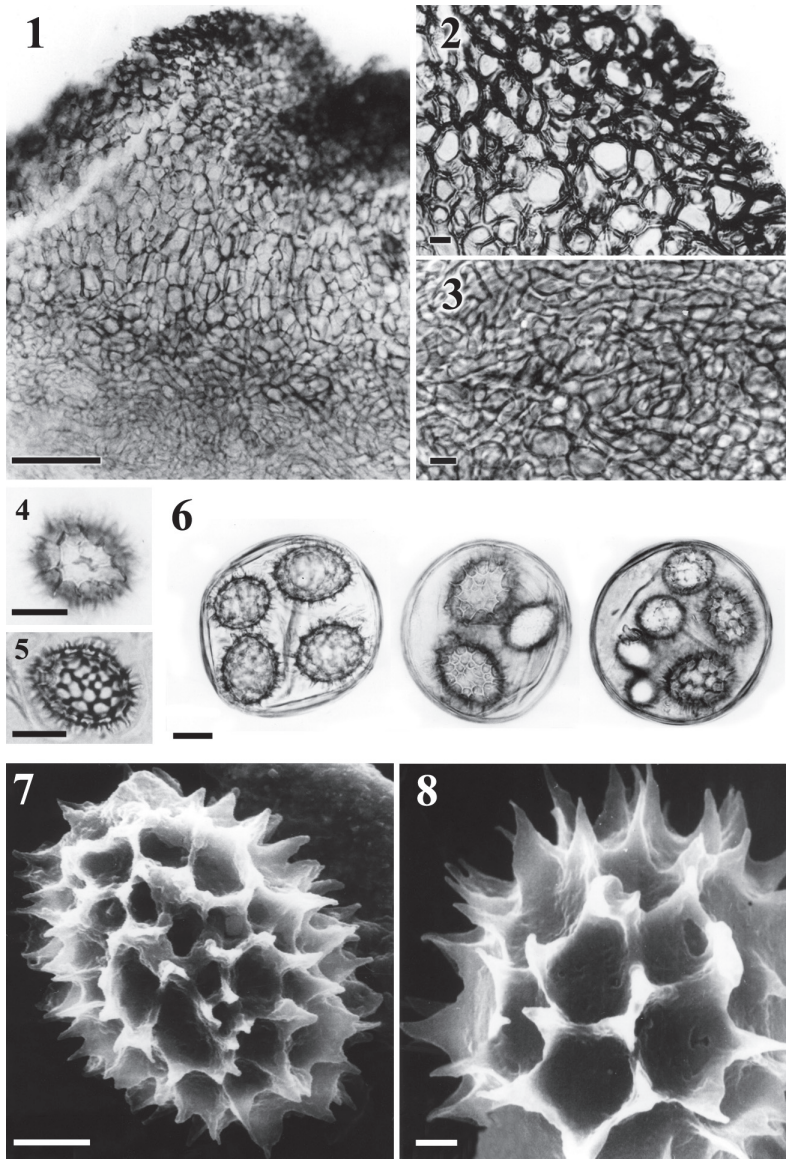

Fig. 4. *Tuber pseudohimalayense* (Moreno et al. 1997). 1) Detail of the external covering of the peridium. 2) Thick-walled globose cells of the external covering of the peridium. 3) Plectenchymal cells of the internal covering of the peridium. 4–5) Ascospores, where the spiny-reticular ornamentation is apparent. 6) Globose ascus with 3 to 7 ascospores. 7–8) Thorny reticulations of spores, under the S.E.M.

(Bars: 9 = 100 µm; 10 = 20 µm; 11–14 = 10 µm; 15 = 5 µm; 16 = 2 µm).

Table 2. Comparison of fruit body characters in Chinese *Tuber* species

Species	*T. indicum* [1]	*T. himalayense* [1]	*T. pseudo-himalayense* [2]	*T. pseudo-excavatum* [3]
Ascocarp	globose to ellipsoidal	+/- globose	subglobose	subglobose, deeply excavate
Color and surface	black or slightly greyish	black	warts black (+/- pyramidal, wide, flat)	brown to brown-orange, coarsely warted
Peridium (µm)	550–700	700–800	200–500 broad	290–500 thick
Asci (number)	(1–)3–5(–6)	(1–)2–4(–5)	1–7	1–8
Ascospores (µm, including ornamentation)	ellipsoid 30–42 × 23–32	ellipsoidal to globose 28–45 × 23–40	ellipsoidal 18–35 × 16–30	ellipsoidal 29–33 × 21–24
Ornamentation	spines, often slightly hooked	variable, most reticulate, occ. spiny	spines in netted reticulum	spines (5–8 µm tall) in reticulation

[1] Zhang & Minter (1988); [2] Moreno et al. (1997); [3] Wang et al. (1998).

Description of *Tuber pseudohimalayense* ectomycorrhizae

Mycorrhizae in youth slightly club-shaped to almost cylindrical and lacking branches, with age often becoming pinnate branched in a monopodial-pinnate pattern; unbranched ends ≤ 1500 µm × 180 µm, straight; dark brown (7.5 YR 6/6) mycorrhizal tips color; surface smooth with long cystidia especially on the tip; rhizomorphs absent (Fig. 5).

Cystidia sinuous to straight, yellowish; diameter: 2–3.3 µm; septa distance: 20–35 µm; hyphal smooth; often branching near base, with 30% of the cystidia branched in approx. 90º angle.

Outer mantle pseudoparenchymatous, composed of pseudocells with very variable form from irregular polygonal to sinuous cells. Surface extremely irregular with puzzle-like appearance; hyphal pseudocells: (10–)12–30(–32) × (4–)5–12(–13) µm. Inner mantle with a similar irregular and puzzle-like appearance. Total mantle thickness 11-35 µm.

Hartig net present in 2–3 rows of host cortical cells.

Discussion

Ectomycorrhizal fungal diversity is an increasingly complex patchwork (Rinaldi et al. 2008), and *Tuber*, especially in its Chinese range, is not an exception. Moreno et al. (1997) and Di Massimo et al. (1998) proposed *T. pseudohimalayense* as a species characterised microscopically by its peculiar, thick-walled, dark brown ascospores, having an ornamentation of spines with broad basal connections

Table 3. Comparison of ectomycorrhizal morphology in Chinese *Tuber* species observed in international markets

Species	*T. melanosporum* [1]	*T. indicum* [2]	*T. himalayense* [2]	*T. pseudoexcavatum* [3]	*T. pseudohimalayense*
Host	*Quercus pubescens*, *Corylus avellana*, *Pinus sylvestris*	*Quercus pubescens*, *Q. cerris*	*Quercus pubescens*	*Quercus ilex* subsp. *ballota*	*Quercus ilex* subsp. *ballota*
Ramification	simple & clavate with round apex, or monopodial-pinnately branched	simple & monopodial-pinnate or dichotomous branched	mostly unbranched; also branched & monopodial-pinnate	sl.clavate to almost cylindrical & unbranched when very young or branched & monopodial-pinnate	sl. clavate to almost cylindrical & unbranched when very young or branched & monopodial-pinnate
Unramified ends (length)	200–4000 µm long	150–1620 µm long	235–1200 µm long	≤ 2500 µm long	≤ 1500 µm long
(diameter)	2300–450 µm diam	1200–540 µm diam	1500–250 µm diam	≤ 240 µm diam	≤ 180 µm diam
(color)[4]	dark amber to ochre	ochreous-amber 7.5 YR 6/6	ochreous-amber 10 YR 5/8	dark brown 7.5 YR 6/6	dark brown 7.5 YR 6/6
Long cystidia (on smooth surface)	primarily on tip	loose, primarily on tip, less abundant in age	loose, primarily on outer surface, less abundant in age	primarily on tip	primarily on tip

[1] Granetti (1995) and Zambonelli et al. (1993); [2] Zambonelli et al. (1997) and/or Comandini & Pacioni (1997); [3] García-Montero et al. (2008);
[4] Munsell (1976) standard soil color charts.

that form a regular net reticulum composed of variably sized meshes across the whole spore surface. The *T. pseudohimalayense* holotype presents a peridium of 200–500 µm, thinner than that of *T. indicum* (550–700 µm), *T. himalayense* (700–800 µm), and *T. sinense* (550–1300 µm). The cell morphology of the inner and outer layers of the peridium of *T. pseudohimalayense* differs from the other Chinese truffles (see the photographs and descriptions of Manjón et al. 1995; Di Massimo et al. 1996, 1998; Moreno et al. 1997). Finally, *T. pseudohimalayense* holotype asci (1–7 spored) generally contain more spores than *T. indicum*, with 3–5(–6) spored asci, *T. himalayense* with 2–4(–5) spored asci, and *T. sinense* with 1–4 spored asci (Table 2).

When proposing the new Chinese truffle taxa, we did not examine the differences between *T. pseudohimalayense* and *T. pseudoexcavatum* in any further detail, as their ascomata appeared so very different (Table 2). This important omission was shown in the description of *T. pseudoexcavatum*, which reports only that its ascomata differ from *T. sinense*, *T. gigantosporum* Y. Wang & Z.P. Li, *T. indicum*, *T. himalayense,* and *T. pseudohimalayense* in their excavate ascomata and their 8-spored asci. In Table 2 and Figures 3 and 4, we summarise the taxonomical characters of the *T. pseudohimalayense* holotype versus the *T. pseudoexcavatum* holotype (Moreno et al. 1997, Wang et al. 1998). We now can confirm the strong resemblance of the *T. pseudohimalayense* and *T. pseudoexcavatum* holotypes, except that *T. pseudohimalayense* asci range from 1–7 spored vs *T. pseudoexcavatum* that range from 1–8 spored.

Genetic studies on the type collections show that *T. pseudohimalayense* and *T. pseudoexcavatum* represent a single species that is different from *T. indicum*. Our study of voucher collections clearly indicates that *T. pseudohimalayense* was misidentified and provides additional molecular data on the *T. pseudoexcavatum* holotype. 28S LSU and mtLSU sequences were obtained instead of ITS, because the latter failed to be amplify for the *T. pseudohimalayense* holotype, probably due to its poor state of preservation.

Our present proposal of conspecificity of *T. pseudohimalayense* and *T. pseudoexcavatum* is further confirmed by the morphological similarity of their ectomycorrhizae, which are clearly distinguishable from *T. indicum* ectomycorrhizae (Tables 3–4): they are thinner and darker than *T. indicum* ectomycorrhizae, and their outer mantle pseudocells are much larger and more often irregular and sinuous (puzzle form) than those of *T. indicum*.

Accordingly we propose *T. pseudoexcavatum* to be a synonym of the earlier named *T. pseudohimalayense*:

Tuber pseudohimalayense G. Moreno, Manjón, J. Díez & García-Mont., in Moreno et al., Mycotaxon 63: 218 (1997).

= *T. pseudoexcavatum* Y. Wang, G. Moreno, Riousset, Manjón & G. Riousset, in Wang et al., Cryptogamie Mycologie 19: 115 (1998).

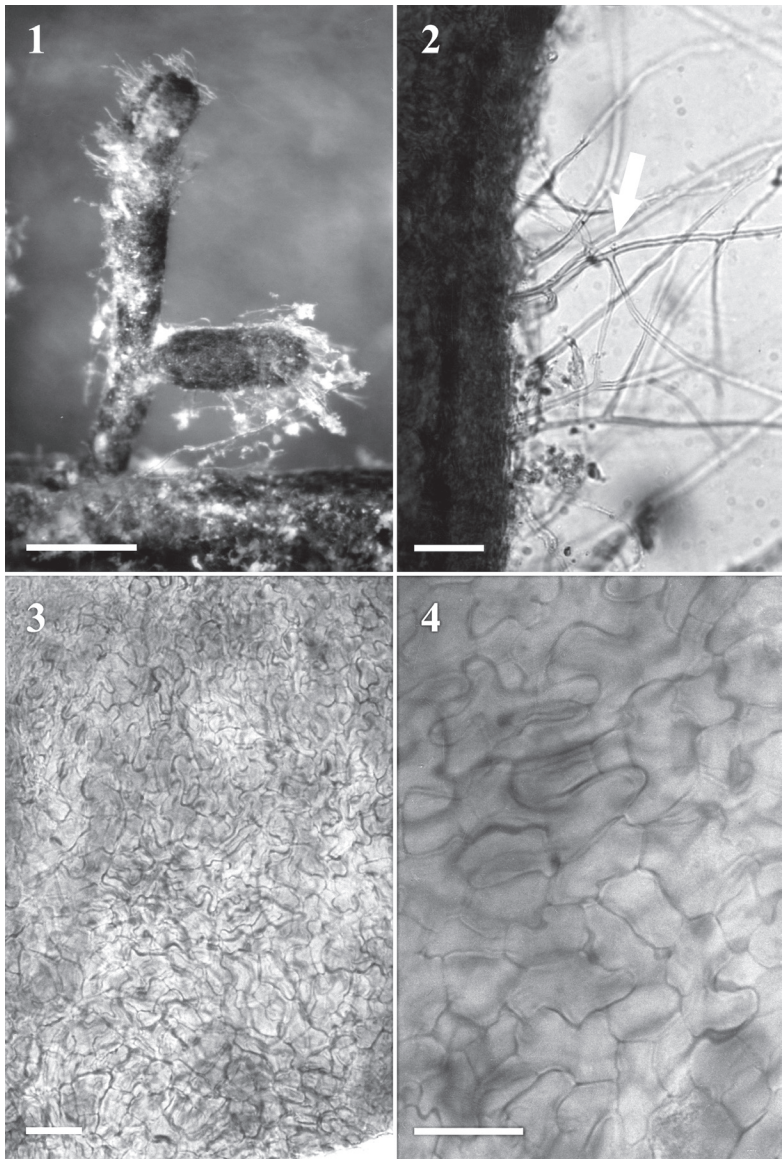

Fig. 5. Ectomycorrhizae of *Tuber pseudohimalayense*. 1) Macroscopic appearance of ectomycorrhiza (50×). 2) Detail of cystidia with right angle-like ramifications (400×). 3) Outer surface of the mantle appearance with a general feature of very irregular puzzle-like shape (100×). 4) Hyphal pseudocells of the outer surface of the mantle with polygon-shaped pseudocells alternating with cells with a sinuous form (1000×)

(Bars: 1 = 500 μm; 2–4 = 10 μm).

Table 4. Comparison of ectomycorrhizal anatomy in Chinese *Tuber* species observed in international markets

Species	*T. melanosporum* [1]	*T. indicum* [2]	*T. himalayense* [2]	*T. pseudoexcavatum* [3]	*T. pseudohimalayense*
Mantle surface pattern (pseudo-parenchymatous)	Puzzle-like: individual cells rounded, with well-defined irregular lobes	Puzzle-like, very heterogeneous, individual pseudocells of two types: rounded & regular and polygonal [mostly smaller & less lobed than in *T. melanosporum*]	Puzzle-like, rather homogeneous; individual pseudocells rectangular, more or less elongated, frequently ramified	Puzzle-like, quite regular, homogeneous; individual pseudocells s-shaped (sinuous)	Puzzle-like, extremely irregular; individual pseudocells of two types: 4–5 sided polygon-shaped and s-shaped (sinuous)
Hyphae (exterior dimensions)					
Length	10.6 (± 2.4) µm (mean)	(8–) 10–16 (–24) µm	(7–) 10–16 (–18) µm	(8–) 10–25 (–26) µm	(10–) 12–30 (–32) µm
Width	4.6 (± 1) µm (mean)	(4–) 5–6 (–10) µm	4–6 µm	(5–) 6–16 (–17) µm	(4–) 5–12 (–13) µm
Cystidia Form	≤ 300 µm long; straight; frequent ~90° and 45° ramifications	≤ 300 µm long; frequent ~90° ramifications	often very long, ≤ 300 µm; ~90° ramifications	very long, sinuous to straight, sectioned; 15% with frequent ~90° ramifications	very long, sinuous to straight, sectioned; 30% with frequent ~90° ramifications
Color	Hyaline	Pale yellow	–	Yellowish	Yellowish
Plinth diam	(2.8–)3.5(–4.4) µm (mean)	2–4(–8) ~90° [tip = 2–3(–6) µm]	2–3 µm	2–4 µm	2–3 µm
Septa distance		25–35 µm	20–40 µm	25–35 µm	20–35 µm

[1] Granetti (1995) and Zambonelli et al. (1993); [2] Zambonelli et al. (1997) and/or Comandini & Pacioni (1997); [3] García-Montero et al. (2008).

Macro and microscopic differences initially observed between the *T. pseudohimalayense* and *T. pseudoexcavatum* holotypes can be explained by gaps in their fruitbody development, maturation stages, and perhaps by differences in the preservation treatments used in the commercial shipments of Chinese truffles. In short, to avoid potential ecological problems in Europe it is essential to know more about the taxonomy, genetics, and morphology of the mycorrhizae of Chinese truffles, so that public agencies can accurately monitor truffle-inoculated seedlings.

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