ISSN (print) 0093-4666 ISSN (online) 2154-8889

MYCOTAXON

http://dx.doi.org/10.5248/124.155

Volume 124, pp. 155–163 *April–June 2013*

A reassessment of excavated *Tuber* species from China based on morphology and ITS rDNA sequence data

Li Fan[1*], Jin-Zhong Cao[2] & Yu Li[2]

[1] *College of Life Science, Capital Normal University, Xisanhuanbeilu 105, Haidian, Beijing 100048, China*
[2] *Institute of Mycology, Jilin Agricultural University, Changchun 130118, China*
Correspondence to *: *fanli@mail.cnu.edu.cn*

Abstract — A composite study was made of the morphology and phylogeny of Chinese *Tuber* species that produce ascomata with a cavity at the base (excavated ascomata). One new species, *T. neoexcavatum,* was found and is here described. It is characterized by brown ascomata with distinctly verrucose surface and prosenchymatous peridium. The presence of *T. excavatum* in China is still in doubt. A key is provided for all excavated species of *Tuber.*

Key words — *Ascomycota*, molecular analysis, taxonomy, truffle

Introduction

Species of *Tuber* that produce distinct cavities at the base of ascomata are often referred to as 'excavated species.' The ascomal surface within the cavity of such species differs considerably from the surface covering the outer ascomata (Cao 2010). For example, *T. pseudohimalayense* (= *T. pseudoexcavatum*) produces larger pyramidal warts inside the cavities but smaller ones outside the cavities, even forming a distinct area with the same large pyramidal warts in some abnormal ascomata that lack cavities. The only exception is *T. mesentericum* Vittad., in which pyramidal warts completely cover the ascomal surface (Riousset et al. 2001).

Occasionally other *Tuber* species (e.g., *T. nitidum* Vittad. and *T. taiyuanense* B. Liu in the Rufum group; *T. liyuanum* L. Fan & J.Z. Cao in the Puberulum group) produce an umbilicus or a distinct pit at the base of an ascoma, giving them the appearance of an excavated species. However, these *Tuber* species could never be mistaken for excavated species, because there is no clear difference between the cavity interior and exterior inside and a cavity is not consistently present within all ascomata in the collection.

Tuber species with true cavities were first described by Vittadini in 1831 (*T. excavatum* and *T. mesentericum*) and Quélet in 1880 (*T. fulgens*). Two Chinese species — *T. pseudoexcavatum* (Wang et al. 1998) and *T. sinoexcavatum* (Fan et al. 2011) — also produce ascomata with cavities. During intensive investigation of the Chinese *Tuber* species with excavated ascomata, the authors found a new excavated species. Here we describe the new species, discuss all three Chinese excavated species, and reassess their taxonomic status.

Materials & methods

Morphological studies

Fresh specimens of the new species described here were collected from Baoshan City, Yunnan Province, P.R. China and deposited in BJTC (Herbarium Biology Department, Capital Normal University in Beijing). The dried specimens were deposited in BJTC and HKAS (Herbarium of Cryptogams, Kunming Institute of Botany, Chinese Academy of Sciences). Macro- and microscopic characters were described from fresh specimens and rehydrated specimens. Sections were made with a razor blade and mounted in 3% KOH for observation. Specimens were dried for archival purposes, and permanent slides were prepared by staining in Melzer's reagent, rinsing in water and mounting in polyvinyl lactic glycerol. For scanning electron microscopy (SEM), ascospores were scraped from fresh or dried gleba onto doubled–sided tape mounted directly on an SEM stub, coated with gold–palladium, and examined and photographed with a Hitachi S–4800 SEM.

Molecular methods

Samples from herbarium material were crushed by shaking for 3 min at 30 Hz (Mixer Mill MM 301, Retsch, Haan, Germany) in a 1.5 ml tube together with one 3 mm diameter tungsten carbide ball. Total genomic DNA was then extracted using the PeqLabE.Z.N.A._Fungal DNA kit following the manufacturer's protocol. The ITS regions were 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, and 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. The other sequence data of ITS rDNA included in this study were downloaded from GenBank. GenBank numbers are shown in 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 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.

Table 1. Sources of sequences for molecular analysis

Species	Voucher specimen	Geographical origin	ITS
Terfezia boudieri Chatin	mot08	Algeria	AF276673
	DDtb1	Tunisia	GU474809
Tuber aestivum Vittad.	E1	Italy	AF516788
	E17	Italy	AY226042
T. borchii Vittad.	GB62	Italy	HM485342
	GB45	Italy	HM485344
T. excavatum Vittad.	Texc-eu02	France	DQ329362
	BM100	Spain	FJ748899
?T. excavatum	HKAS 52006	China	GQ217540
T. fulgens Quél.	M2435	Italy	HM485358
	SFI: TUBFUL/041008B	Slovenia	FN433150
T. magnatum Picco	Tm4	Italy	AJ586308
	—	Italy	EU807975
T. melanosporum Vittad.	A59	France	AF106878
	Tm13	France	AF132501
T. mesentericum	TmsW095-W029 (UPS)	Sweden	AJ888047
	TmsW088-W047 (UPS)	Sweden	AJ888044
T. neoexcavatum	**BJTC FAN184 (Holotype)**	**China**	**JX458715**
T. pseudoexcavatum	Tpse-yn05	China	DQ329374
T. pseudohimalayense	BJTC FAN120	China	JX458716
T. rufum Picco	Morphotype 5	Italy	AY940646
	1506	Italy	AY112894
T. sinoexcavatum	BJTC FAN130 (Holotype)	China	JX458717
	BJTC FAN166	Chinan	JX458718

TBR branch swapping was employed. The Bayesian analysis was performed with MrBayes 3.1.2 (Huelsenbeck et al. 2001; Ronquist & Huelsenbeck 2003) with two sets of four chains (one cold and three heated) and the stoprule option in effect, halting the analyses at an average standard deviation of split frequencies of 0.01. The sample frequency was set to 100, and the first 25% trees were removed as burn-in. Bayesian posterior probabilities (PP) were obtained from the 50% majority rule consensus of the remaining trees. Two sequences derived from *Terfezia boudieri* were used as outgroup.

Results

Molecular phylogenetics

480 of 688 characters were found to be parsimony-informative. The maximum parsimony analysis of sequences resulted in one most parsimonious tree (Fig. 1) with a length (TL) = 1422 steps, consistency index (CI) = 0.6582, retention index (RI) = 0.8002, homoplasy index (HI) = 0.3418, and rescaled consistency index (RC) = 0.5267.

The phylogenetic analysis (Fig. 1) reveals that the *Tuber* species with excavated ascomata are scattered in different clades with strong support. The new species *T. neoexcavatum* clusters in a clade with other three other

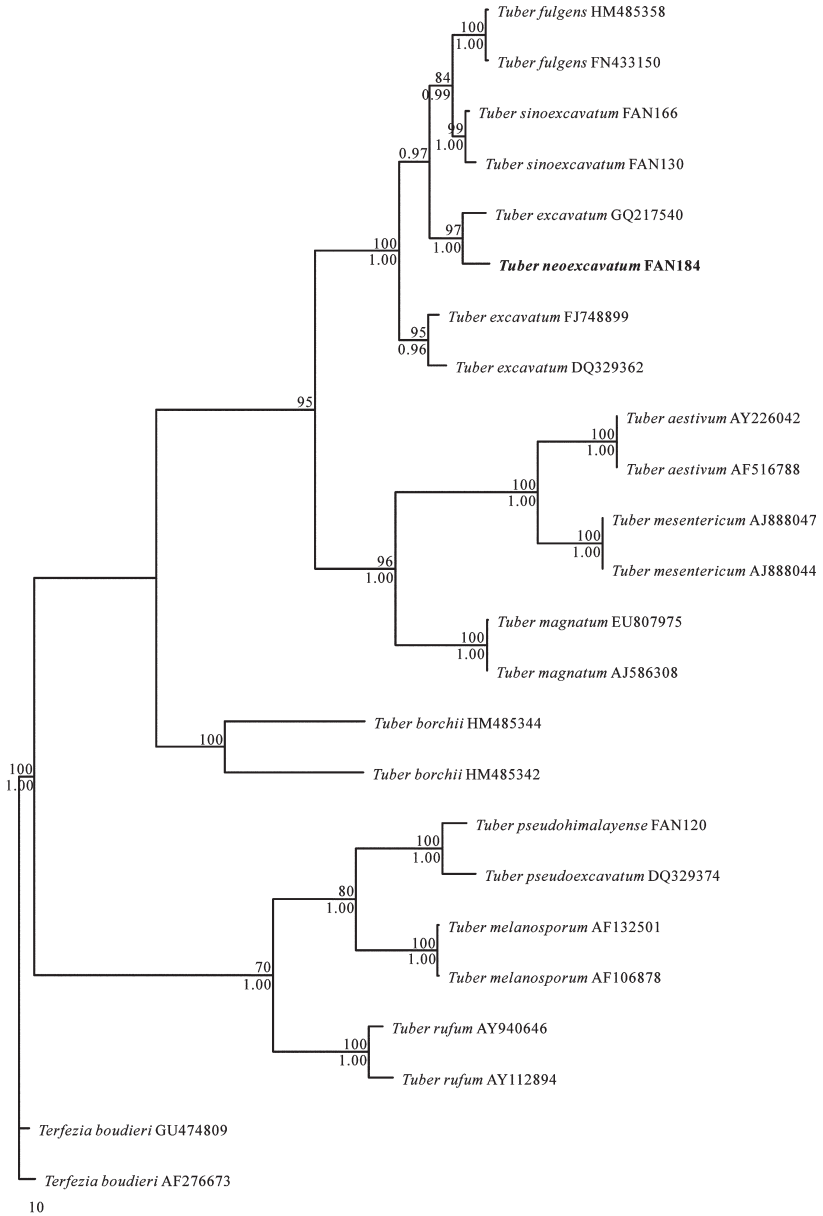

Fig. 1. Phylogeny derived from maximum parsimony analysis of the ITS rDNA sequences of some *Tuber* species with real excavated ascomata, using *Terfezia boudieri* as outgroup. Bootstrap values of more than 70% from 1000 replications are shown above the respective branches. Bayesian posterior probabilities are estimated and clades with PP>0.95 (95%) are marked under the branches.

excavated species: *T. excavatum*, *T. fulgens*, and *T. sinoexcavatum*. Furthermore, four strongly supported subclades (each represented by two sequences) correlate exactly with individual taxa as defined using morphological methods, indicating that the four excavated species are closely related but distinct. The other two excavated species are in two separate clades: *T. pseudohimalayense* grouping with *T. melanosporum* and *T. mesentericum* with *T. aestivum*.

Taxonomy

Tuber neoexcavatum L. Fan &Yu Li, **sp. nov.** Fig. 2

MycoBank MB 801128

Differs from other *Tuber* species by its brown ascoma with a distinctly verrucose surface and prosenchymatous peridium.

Type: China. Yunnan Province, Baoshan City, in soil under forest of *P. yunnanensis* Franch., 28 Dec. 2011, Jin-Zhong Cao 511 (**Holotype**, BJTC FAN184).

Etymology: *neoexcavatum* (Lat.), referring to the similarity to *Tuber excavatum*.

Ascomata hypogenous, subglobose, 3–4 cm in diam., with typical basal cavities (excavated ascomata), covered with distinctly verrucose warts on the surface, yellow brown to brown with shadow of olive green; interior cavity surface covered with many fine, irregular scales and large warts, pale white to yellow-white when fresh. Odor and flavour not recorded. Peridium 250–350 μm thick including the 100–150 μm high verrucose warts, prosenchymatous, composed of interwoven hyphae, light brown and slightly thick-walled towards the outside, 3–6 μm in diam., hyaline and thin-walled towards the inside, 2.5–5 μm in diam. Gleba yellow brown to brown, with the shadow of olive green at maturity, veins white to cream white, more or less radiate from the cavity. Asci subglobose or long oval, hyaline, thin walled, 75–100 × 62.5–80 μm, with a short stalk, 2–4-spored. Ascospores elliptic or long elliptic, occasionally broadly elliptic, hyaline at first, light brown or brown at maturity, reticulate ornamentation on the surface, 30–47.5 × 25–30 μm excluding the ornamentation, meshes 5–6 μm deep, 2–4 across the spore width.

Additional specimen examined — **China**. **Sichuan Province**, Huidong county, in soil under forest of *P. armandii* and *P. yunnanensis*, 3 Nov. 2006, Juan Chen 411 (HKAS 52006, as *T. excavatum*)

Comments — Ascomata of *Tuber neoexcavatum* resemble those of *T. pseudohimalayense* (= *T. pseudoexcavatum*), including the color and small distinct surface warts, but *T. neoexcavatum* is easily distinguished by its spino-reticulate ornamented ascospores. *Tuber excavatum, T. fulgens,* and *T. sinoexcavatum* also produce similar ascomata, but *T. excavatum* is distinguished by its pale and smooth or finely papillar ascomata and *T. fulgens* and *T. sinoexcavatum* produce globose or subglobose ascospores. The European species, *T. mesentericum,* is also excavated but differs from *T. neoexcavatum* in its blackish ascomata that

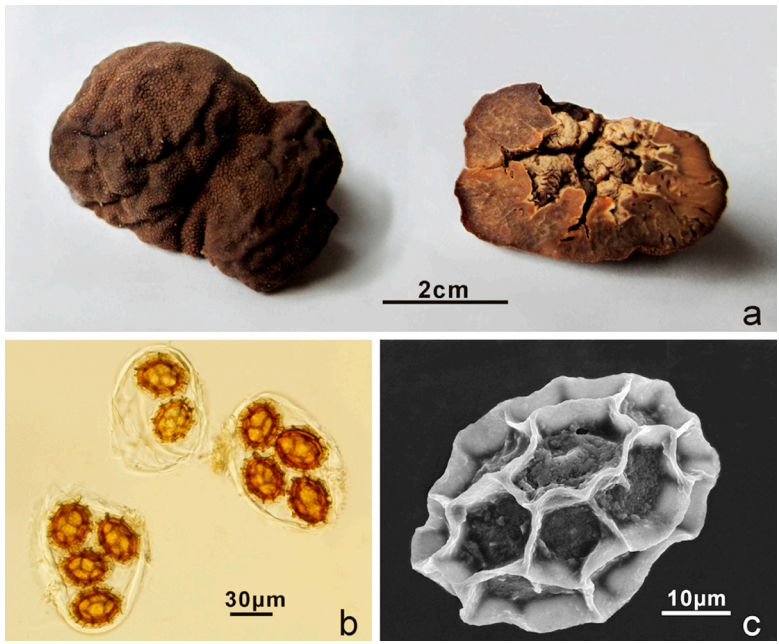

Fig. 2. *Tuber neoexcavatum* (BJTC FAN184, holotype). a Ascomata; b Asci and ascospores observed under light microscope; c Ascospore observed under SEM.

are completely covered with large pyramid warts. In addition, the peridium structure in *T. neoexcavatum* is typically prosenchymatous, which is completely (including the verrucose warts) composed of interwoven hyphae. The ITS rDNA phylogeny (Fig. 1) places *T. neoexcavatum* sequences within an evidently independent clade (BP = 97, PP = 1.00) and clearly supports the new species as independent.

Chen et al. (2008) reported *T. excavatum* in China based on specimen HKAS 52006 collected in 2006 from Sichuan Province (also see Cao 2010). However, our ITS rDNA sequence analysis of HKAS 52006 and other excavated *Tuber* species (Fig. 1) places sequence GQ217540 (from HKAS 52006) in a clade with our *T. neoexcavatum* but not with the *T. excavatum* sequences from France and Spain. In examining HKAS 52006, we could find no ascomal surface and peridial differences between the specimen of Chen et al. (2008) and the type specimen of *T. neoexcavatum*. We therefore redetermine HKAS 52006 as *T. neoexcavatum*.

As all other reports of *T. excavatum* in China are not linked to specimens, there is no firm evidence for *T. excavatum* being present in China. Whether *T. excavatum* occurs in China or not needs further investigation.

Tuber sinoexcavatum L. Fan & Yu Li, Mycotaxon 116: 352 (2011)

Specimens examined — **CHINA**. **Sichuan Province**, Panzhihua City, under the soil of *Pinus* sp., 20 Dec. 2007, De-fu Liu (Holotype, BJTC Fan130); **Yunnan Province**, Huizhe County, in soil under forest, 12 Nov. 2011, Shao-ping Li 005 (BJTC Fan166).

Distribution: China: Sichuan Province; Yunnan Province.

Comments—*Tuber sinoexcavatum* was originally described from one collection (BJTC Fan130), but in 2011 an additional specimen (BJTC Fan166) was found in Yunnan Province, China. The sequence analyses (Fig. 1) support the two specimens as conspecific. It should be pointed out that the ascospores are near regularly globose in the type specimen, but in the new specimen about half the ascospores are subglobose or broadly elliptic. This phenomenon also occurs in *T. latisporum* Juan Chen & P.G. Liu and *T. subglobosum* L. Fan & C.L. Hou (Fan et al. 2013). The *T. latisporum* ascospores are broadly elliptic to subglobose, but also sometimes globose in some ascomata in the same collection, while in some ascomata *T. subglobosum* may occasionally produce a large proportion of broadly elliptic ascospores rather than typical subglobose ones.

Tuber sinoexcavatum is morphologically similar to the European species *T. excavatum*, which, however, produces elliptic ascospores. Furthermore, the peridium in *T. sinoexcavatum* is normally covered with fine papillary warts while *T. excavatum* produces smooth or only finely scurfy warts (Pegler 1993, Riousset et al. 2001). Another European species, *T. fulgens,* also produces globose ascospores, but its ascoma is brightly colored and a small hole opens to the outside (Lange 1956, Riousset et al. 2001), unlike the large opening in *T. sinoexcavatum*. Here also the ITS rDNA sequence analyses strongly support three separate species (Fig. 1).

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

= *T. pseudoexcavatum* Y. Wang, G. Moreno, Riousset, Manjón & G. Riousset, Cryptog. Mycol. 19(1–2): 115 (1998)

Specimens examined — **CHINA**. **Sichuan Province**, Huizhe County, in soil under trees, 19 Oct. 2003, Juan Chen 127 (HKAS 44346); 3 Nov. 2006, Juan Chen 412 (HKAS 52010); 5 Nov. 2006, Juan Chen 416 (HKAS 52017); 20 Oct. 2003, Juan Chen 134 (HKAS 44325); 19 Oct. 2003, Juan Chen (HKAS 44346); in soil under *P. armandii*, 15 Dec. 2008, Li Fan 020 (BJTC FAN120); **Yunnan Province**, Kunming City, in soil under forest, 25 Nov. 2002, Zhu-liang Yang 3603 (HKAS 41313); 8 Nov. 2004, Juan Chen 234 (HKAS 47619); 8 Nov. 2002, Juan Chen 287 (HKAS 49744); 9 Mar. 2003, Xing-hua Wang 1477 (HKAS 42399); Jinning County, in soil under trees, 20 Sep. 2006, Juan Chen 427 (HKAS 52032); Yongsheng County, in soil under trees, 29 Oct. 2006, Juan Chen 407 (HKAS 52027); Juan Chen 408 (HKAS 52036).

Distribution: China: Sichuan Province; Yunnan Province.

Comments—Moreno et al. (1997) described *T. pseudohimalayense* for a black truffle with spino–reticulate ornamented ascospores. Although the type

specimen was found in a Spanish truffle market, the authors suspected it was probably imported from China (Moreno et al. 1997). The next year, Wang et al. (1998) published *T. pseudoexcavatum* to accommodate a very common black truffle species in China. It and *T. pseudohimalayense* produce identical ascospores, but *T. pseudohimalayense* is characterized by a distinct cavity at the ascomal base. Manjón et al. (2009) and Chen & Liu (2011) concluded that the two 'species' were definitely conspecific based on their analysis of molecular data. As the earlier published name, *T. pseudohimalayense* has priority over *T. pseudoexcavatum*. We have found it is widely distributed in Yunnan and Sichuan provinces of southwest China during autumn and early winter, and Song (2005) has cited it from Hunan Province. Its spino–reticulate ascospores and 1–8-spored asci separate *P. pseudohimalayense* from all other excavated *Tuber* species.

Discussion

Six total species of *Tuber* with excavated ascomata have been described thus far, including three species from China. As noted above, the European species *T. excavatum* has not yet been confirmed in China.

The ITS rDNA–based phylogeny supports four closely related species — *T. excavatum*, *T. fulgens*, *T. sinoexcavatum, T. neoexcavatum* — within a well-supported clade and shows that *T. pseudohimalayense* is close to *T. melanosporum* and *T. mesentericum* is close to *T. aestivum*. Although *Tuber* species with excavated ascomata appear morphologically similar, however, their rDNA sequence analyses imply that they are definitely polyphyletic.

Key to *Tuber* species with excavated ascomata

1a. Ascomata blackish; surface with pyramidal warts . . . . . *T. mesentericum*
1b. Ascomata not blackish; surface smooth, verrucose, or with small warts 2
2a. Ascomata distinctly verrucose or with small warts 3
2b. Ascomata smooth or minutely verrucose 4
3a. Ascospores with spino–reticulate ornamentation . . . . . *T. pseudohimalayense*
3b. Ascospores with reticulate ornamentation . . . . . *T. neoexcavatum*
4a. Ascospores mostly elliptic . . . . . *T. excavatum*
4b. Ascospores mostly globose or subglobose. 5
5a. Ascomata golden brown, opening to outside by a small hole . . . . . *T. fulgens*
5b. Ascomata yellow brown to light brown, typically excavated. . . . . *T. sinoexcavatum*

Acknowledgments

We are grateful to Prof. Guozhong Lü and Dr. Ian Hall for reviewing the manuscript. The study was supported by the National Natural Science Foundation of China (No. 31270058, 30770005), the Beijing Natural Science Foundation (No. 5072006). We wish

to extend a special thanks to the Herbarium of Cryptogams, Kunming Institute of Botany, Chinese Academy of Sciences, for providing specimens.

Literature cited

Cao JZ. 2010. The genus *Tuber* in China [Dissertation]. Changchun: Jinlin Agricultural University. 86 p.

Chen J, Liu PG, 2011. Delimitation of *Tuber pseudohimalayense* and *T. pseudoexcavatum* based on morphological and molecular data. Cryptogamie Mycologie 32: 83–93.

Chen J, Wang Y, Liu PG. 2008. Two new records of *Tuber* (*Pezizomycetes*, *Pezizales*) from China. Mycotaxon 104: 65–71.

Fan L, Cao JZ, Liu YY, Li Y. 2011. Two new species of *Tuber* from China. Mycotaxon 116: 349–354. http://dx.doi.org/10.5248/116.349

Fan L, Cao JZ, Hou CL. 2013. *Tuber subglobosum* and *Tuber wenchuanense* — two new species with spino-reticulate ascospores. Mycotaxon 123: 95–101. http://dx.doi.org/10.5248/123.95

Huelsenbeck JP, Ronquist F, Nielsen R,Huelsenbeck JP, Ronquist F, Nielsen R, Bollback JP. 2001. Bayesian inference of phylogeny and its impact on evolutionary biology. Science 294: 2310–2314. http://dx.doi.org/10.1126/science.1065889

Lange M. 1956. Danish hypogeous fungi. Dansk Bot Arkiv 16: 1–48.

Manjón JL, García–Montero LG, Alvarado P, Moreno G, Di Massimo G. 2009. *Tuber pseudoexcavatum* versus *T. pseudohimalayense*—new data on the molecular taxonomy and mycorrhizae of Chinese truffles. Mycotaxon 110: 399–412. http://dx.doi.org/10.5248/110.399

Moreno G, Manjon JL, Diez J, Garcia-Montero LG, Di Massimo G. 1997. *Tuber pseudohimalayense* sp. nov. an Asiatic species commercialized in Spain, similar to the "Perigord" truffle. Mycotaxon 63: 217–224.

Pegler DN, Spooner BM, Young TWK. 1993. British truffles: a revision of British hypogeous fungi. UK: Royal Botanic Gardens, Kew. 224 p.

Rambaut A. 2000. Estimating the rate of molecular evolution: incorporating non-contemporaneous sequences into maximum likelihood phylogenies. Bioinformatics 16: 395–399. http://dx.doi:10.1093/bioinformatics/16.4.395

Riousset L, Riousset G, Chevalier G, Bardet MC. 2001. Truffes d'Europe et de Chine. Paris: Institut National de la Recherche Agronomique. 181 p.

Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574. http://dx.doi.org/10.1093/bioinformatics/btg180

Song MS. 2005. Taxonomic and molecular systematic studies on *Tuber* in China [Dissertation]. Beijing: Graduate School of Chinese Academy of Sciences. 156 p.

Swofford DL. 2002. PAUP* 4.0b10: phylogenetic analysis using parsimony (*and other methods). Sunderland, Massachusetts: Sinauer Associates.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The CLUSTALX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 24: 4876–4882. http://dx.doi:10.1093/nar/25.24.4876

Wang Y, Moreno G, Riousset LJ, Manjón JL, Riousset G, Fourre G, Di Massimo G, Garcia-Montero LG, Diez J. 1998. *Tuber pseudoexcavatum* sp. nov. A new species from China commercialised in Spain, France and Italy with additional comments on Chinese truffles. Cryptogamie Mycol 19: 113–120.

White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. 315–322, in: MA Innis et al. (eds). PCR protocols: a guide to methods and applications. San Diego: Academic Press.