Mycol Progress (2015) 14: 83
DOI 10.1007/s11557-015-1107-2

ORIGINAL ARTICLE

A new whitish truffle, *Tuber thailandicum* from northern Thailand and its ectomycorrhizal association

Nakarin Suwannarach[1] · **Jaturong Kumla**[1] · **Saisamorn Lumyong**[1]

Received: 19 February 2015 / Revised: 21 August 2015 / Accepted: 24 August 2015 / Published online: 8 September 2015

Abstract A new species of whitish truffle, *Tuber thailandicum*, is described based on collections from northern Thailand. This species is characterized by whitish ascomata with dark brown gleba and subglobose spores with an alveolate reticulum. *Tuber thailandicum* is similar to *T. castilloi*, but differs in the thicker peridium and wider spores in one-spored asci. Molecular analysis of the internal transcribed spacer region and large subunit of ribosomal DNA also supports that *T. thailandicum* is clearly different from previously described whitish truffle species. It grows in mycorrhizal association with *Betula alnoides*, and the morphology and anatomy of mycorrhizae are described. Moreover, the identification of mycorrhizal status was confirmed by molecular methods.

Keyword *Ascomycota* · Hypogeous fungi · Taxonomy · Phylogeny · Ectomycorrhizal symbiosis

Introduction

Truffles (*Tuber* spp.) are edible fungi and belong to the order *Pezizales*, family *Tuberaceae*. Generally, truffle species produce hypogeous ascocarps and are the most expensive fungi in the world (Hall et al. 2007; Stobbe et al. 2012, 2013). Périgord black truffle (*T. melanosporum* Vittad.), Italian white truffle (*T. magnatum* Pico) and summer truffle (*T. aestivum* Vittad.) are a highly prized food in European countries (Hall et al. 2007; Bonito et al. 2010a; Stobbe et al. 2013). Oregon whitish truffles (*T. gibbosum* Harkn. and *T. oregonense* Trapp, Bonito & P. Rawl.) are commercially harvested in North America (Bonito et al. 2010b). *Tuber indicum* Cooke & Massee is one of the renowned commercial black truffles in China (García-Montero et al. 2010; Mortimer et al. 2012). Chinese truffles have been exported to Australia, Europe, Japan and America since the 1980s, and the popularity of truffles worldwide has increased (Rey 2001; Hall et al. 2003; Mortimer et al. 2012). Truffles form ectomycorrhizal symbiosis on a wide range of shrubs and trees including members of the families *Betulaceae*, *Cistaceae*, *Corylaceae*, *Fagaceae* and *Pinaceae* (Weden et al. 2004; Hall et al. 2007; Trappe et al. 2009; Bonito et al. 2011). Currently, more than half of the harvested truffle yield is produced in orchards (Hall et al. 2003). Traditional identification of *Tuber* species is based on morphological characteristics. There are 296 *Tuber* records in Index Fungorum (http://www.indexfungorum.org/ Names/Names.asp). This index might include many synonyms and misidentifications; therefore, it is difficult to estimate worldwide *Tuber* species diversity. Morphological variations associated with environmental conditions and developmental stage may make this manner of identification difficult. Closely related truffles are difficult to distinguish visually (Chen and Liu 2007; Kinoshita et al. 2011). Although Kirk et al. (2008) noted 86 *Tuber* species worldwide, this figure remains uncertain because of the limitations regarding information from Asia. Recently, molecular taxonomical analyses have provided powerful tools in the identification of *Tuber* species (Chen and Liu 2007; Karkouri et al. 2007; Bonito et al. 2010a, b; Kinoshita et al. 2011; Guevara et al. 2013).

Section Editor: Franz Oberwinkler

✉ Saisamorn Lumyong
saisamorn.l@cmu.ac.th

Nakarin Suwannarach
suwan_461@hotmail.com

Jaturong Kumla
Jaturong_yai@hotmail.com

[1] Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand

Truffles have been researched in Asia for many decades (Trappe 1976; Liu 1985). *Tuber indicum* Cooke & Massee, found in India, was the first Asian truffle to be discovered (Cooke and Massee 1892). In the 1980s, four new *Tuber* species were reported, *T. liaotongense* Wang, *T. taiyuanense* Liu, *T. tianshanense* Tao and *T. xizangense* Xu from China (Liu and Liu 1994; Tao 1988; Wang et al. 1998; Xu 1999; Chen and Gong 2000). *Tuber huidongense* Wang and *T. zhongdianense* He, Hai, Li & Y. Wang were reported from China in 2002 and 2004, respectively (Wang and He 2002; He et al. 2004). In 2005, *T. umbilicatum* Chen & P.G. Liu and *T. furfuraceum* Hu & Y.I. Wang were reported from China and Taiwan, respectively (Chen et al. 2005; Hu and Wang 2005). Since 2010, more than ten known new species of *Tuber* were reported from China (García-Montero et al. 2010; Fan et al. 2011a, 2011b, 2012a, 2012b, 2012c, 2013; 2014; Deng et al. 2013; Su et al. 2013; Li et al. 2014). In addition, Kinoshita et al. (2011) reported that *Tuber* biodiversity in Asia is considered to be high and not well documented. However, no *Tuber* species have been reported from Thailand. During an investigation of ectomycorrhizal fungi associated with tree species in northern Thailand, we found an interesting species of *Tuber* under *Betula alnoides* Buch.-Ham. ex G. Don. (Betulaceae), which has white ascomata. It resembled several known whitish truffles, such as *T. castilloi* Guevara, Bonito & Trappe (Guevara et al. 2013) and *T. oregonense* Trappe, Bonito & P. Rawl. (Bonito et al. 2010b) from North America, *T. magnatum* and *T. borchii* Vittad. from Europe (Riousset et al. 2001) and *T. latisporum* Juan Chen & P.G. Liu from China (Chen and Liu 2007). However, detailed morphological observation and phylogenetic analysis of internal transcribed spacer (ITS) and large subunit (LSU) regions of ribosomal DNA revealed that it is a new species, which we describe in this present paper. Moreover, the ectomycorrhizal association with *B. alnoides* was confirmed and described by molecular techniques.

Material and methods

Sample collection

Ascomata of *Tuber thailandicum* were collected from Huay Kok Ma village (18°40′30″N, 98°54′25″E, elevation 1240 m), Doi Suthep-Pui National Park, Chiang Mai Province, Thailand in May 2014. Soil type in the field was sandy clay loam (52.5 % sand, 19.1 % silt and 28.4 % clay) with a pH of 4.5. Ascomata were wrapped in aluminum foil or kept in plastic specimen boxes until they were transported back to the laboratory, and photographs were taken within 24 h. The specimens were dried at 40–45 °C and deposited at the Research Laboratory for Excellence in Sustainable Development of Biological Resources, Faculty of Science, Chiang Mai University, Thailand (SDBR-CMU).

Morphological studies

Macromorphological data were derived from fresh specimens. Color names and codes follow Kornerup and Wanscher (1967). The micromorphological data were derived from dried specimens rehydrated in 95 % ethanol followed by distilled water, 5 % potassium hydroxide (KOH) or Melzer's reagent. Size data of anatomical features are based on at least 50 measurements of each structure under a light microscope (Olympus CX51, Japan). For spore statistics, Q is the ratio of spore length divided by spore width and **Q** is the average Q of all specimens±standard deviation. For scanning electron microscopy (SEM), ascospores were scraped from the dried specimen onto double-sided tape, which was mounted directly on an SEM stub, coated with gold, examined and photographed with a JEOL JSM-5910 LV SEM (JEOL, Japan).

Molecular studies

Genomic DNA of dried specimens (1–10 mg) was extracted using the fungal Genomic DNA Extraction Mini Kit (FAVORGEN, Taiwan). The internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) was amplified by PCR using ITS1F and ITS4 primers (White et al. 1990) under the following thermal conditions: 95 °C for 2 min, 30 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min, and 72 °C for 10 min on a GeneAmp 9700 thermal cycler (Applied Biosystems, USA). In addition, the large subunit (LSU) region of rDNA was also amplified with LROR and LRO5 primers (Vilgalys and Hester 1990) under the following thermal conditions: 94 °C for 2 min, 30 cycles of 95 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min, and 72 °C for 10 min. Negative controls lacking fungal DNA were run for each experiment to check for any contamination of the reagents. PCR products were checked on 1 % agarose gels stained with ethidium bromide under UV light and purified using NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel, Germany). The purified PCR products were directly sequenced. Sequencing reactions were preformed and the sequences were automatically determined in the genetic analyzer at the 1ST Base Company (Malaysia) using the same PCR primers mentioned above. Sequences were used to query GenBank via BLAST (http://blast.ddbj.nig.ac.jp/top-e.html).

For the phylogenetic analysis, a multiple sequence alignment was carried out using the alignment subroutines in Clustal X (Thompson et al. 1997), and the aligned ITS and LSU sequences deposited at TreeBASE under the numbers 16701 and 16704, respectively. A phylogenetic tree was constructed using the PAUP beta 10 software version 4.0 (Swofford 2002). In maximum parsimony analysis, all characters were equally weighted and gaps were treated as missing data. Heuristic searches with 100 random-addition sequence

replicates and tree-bisection reconnection branch swapping were performed. Bootstrap analysis was conducted with 1, 000 replicates using the same settings as above (Felsenstein 1985). The parsimony tree scores, including tree length and consistency, retention, rescaled consistency, and homoplasy indices (CI, RI, RC, and HI), were calculated. Bayensian phylogenetic analyses were carried out using the Metropolis-coupled Markov chain Monte Carlo (MCMCMC) method with MrBayes v3.2 (Ronquist et al. 2012), under a GRT+I+G model. Markov chains were run for 1,000,000 generations, with six chains and random starting trees. The chains were sampled every 100 generations. Among these, the first 2000 trees were discarded as the burn-in phase of each analysis and the resulting trees were used to calculate Bayesian posterior probabilities.

Characterization of *Tuber thailandicum* ectomycorrhizas

Morphological analysis

Ten ectomycorrhizal root samples were collected in the field point where the ascocarps were found, and separated from soil samples under the stereomicroscope (Olympus TL3, Japan). Ectomycorrhizal morphotypes were characterized based on anatomical and morphological characters following Agerer (1986, 1991, 2006). For anatomical characterization, sections of root tips mounted in 3 % KOH or 1 % Congo Red were observed under a light microscope.

Molecular analysis

The ectomycorrhizal roots having the macro-morphological characteristics of *Tuber* sp. were then analyzed molecularly (Rauscher et al. 1996; Kovács and Jakucs 2006; Boutahir et al. 2013). The ectomycorhizal roots were washed three times with sterilized water. Genomic DNA was extracted from single mycorrhizal root tips as described by Paolocci et al. (1999), using the Nucleospin Plant II kit (Macherey-Nagel, Germany) according to the manufacturer's protocols. The ITS regions of the rDNA of the fungal symbiont were amplified by polymerase chain reaction with ITS1F and ITS4 primers following the thermal conditions described above. PCR products were checked, purified and sequenced. For phylogenetic analysis of the sequences, the same settings as above were used.

For plant identification, the ITS regions of rDNA were amplified from total genomic DNA extracted from ectomycorrhizal colonized root using the primers ITS4 and ITSLeu (Baum et al. 1998) under the following thermal conditions: 94 °C for 3 min, 35 cycles of 91 °C for 1 min, 55 °C for 2 min, 72 °C for 1 min, and 72 °C for 10 min. PCR products were checked, purified and sequenced. Sequences were used to query GenBank via BLAST.

Results

Taxonomy

Tuber thailandicum N. Suwannarach, J. Kumla & S. Lumyong, sp. nov. Figure 1

MycoBank No.: **MB811502**

Diagnosis: Differs from *T. castilloi* due to its thicker peridium and wider spores in one-spored asci.

Holotypus: Thailand, Chiang Mai Province, Muang Distric, Doi Suthep-Pui National Park, Huay Kok Ma village, 18°40′30″N, 98°54′25″E, elevation 1240 m, in a tropical deciduous forest, dominated by *Betula alnoides* and *Castanopsis* spp., 20 May 2014, Nakarin Suwannarach and Jaturong Kumla, SDBR-CMU-MTUF001 (holotypus), GenBank sequence KP196329.

Etymology: *thailandicum* referring to Thailand, where the new species was found.

Ascomata hypogeous, globose to subglobose or irregularly lobed, 2−3.5 cm in diameter, white (2A1) to pale yellow (4A3) when fresh, gradually becoming pale yellow (3A3) to brown (6D8) when dry (Fig. 1a, b). Odor slightly aromatic when mature. Peridium surface minutely verrucose, mostly 150−255 μm thick, composed of two layers (Fig. 1c). Outer cortical layer 75−112.5 μm thick, pseudoparenchymatous, composed of subglobose to subangular, pale yellow or hyaline cells 6−20×4−10.5 μm (Fig. 1d). The outer most cells giving rise the hyphae-like hairs, the hairs 20−63.5 μm long, septate, tapered, usually acute at the apex (Fig. 1e). Inner layer 70−170 μm thick, of intricately interwoven, hyaline hyphae 2−5 μm in diameter, the walls thin to somewhat thickened. Gleba solid, white when young, becoming dark brown at maturity, marbled with distinct, white and meandering veins. Asci globose to subglobose or ellipsoid, hyaline, with thin or slightly thickened walls, 37.5−80×50−87.5 μm, sessile with a short stalk 3−4 μm in diameter, one-spored to four-spored (Fig. 1f). Ascospores subglobose, sometimes globose or broadly ellipsoid, hyaline when young, becoming yellowish brown to brown at maturity, excluding their alveolate-recticulate ornamentation, in one-spored asci 40−65×40−62 μm, in two-spored asci 25−48×20−50 μm, in three-spored asci 22−33×20−29 μm, in four-spored asci 20−28×15−25 μm, Q=1.00−1.28, **Q**=1.09±0.08, ornamented with a regular alveolate reticulum, 3−5 μm high, constituted of mostly hexagonal meshes 10−17.5×7.5−12.5 μm and 3−4 μm across the spore width (Fig. 1g, h).

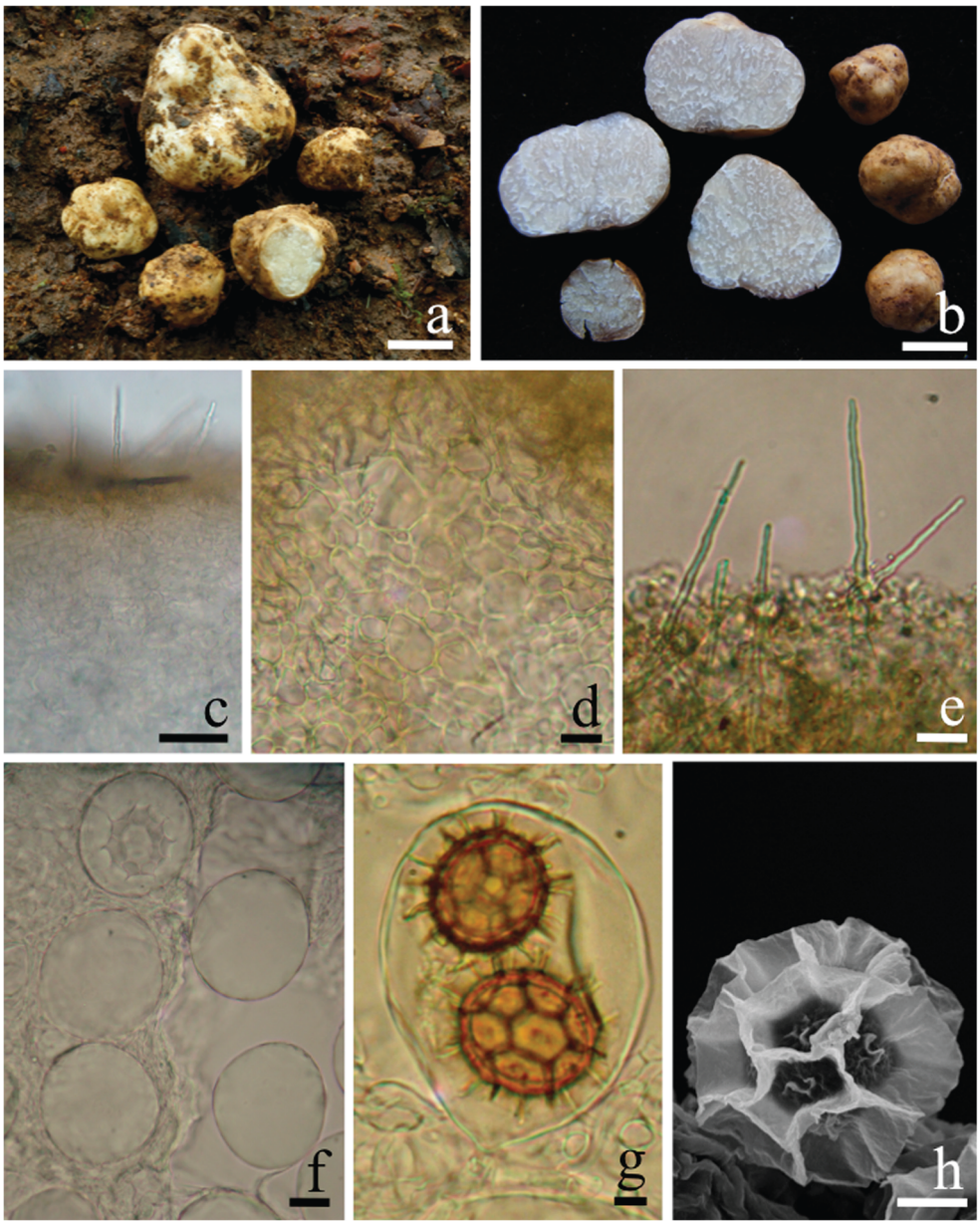

Fig. 1 *Tuber thailandicum*. (**a**) and (**b**), Ascomata. (**c**). A cross section of peridium. (**d**). Pseudoparenchymatous tissue of the outer layer of peridium. (**e**). The hyhae-like hairs on the surface of a peridium. (**f**) and (**g**). Asci and ascospores as observed under a compound microscope. (**h**). Ascospore as observed with a scanning electron microscope. *Scale bar* a and b=1 cm, c= 50 μm, d and e=10 μm, f= 25 μm, a and h=10 μm

Ecology and Distribution: Hypogeous, solitary or in groups in calcareous soils under *B. alnoides*, fruiting during the rainy season. Known only from Thailand.

Other Material Examined: THAILAND: Huay Kok Ma village, 20 May 2014, Nakarin Suwannarach (SDBR-CMU-MTUF001, SDBR-CMU-MTUF002), 5 June 2014, Jaturong Kumla and Santhiti Vadthanarat (SDBR-CMU-MTUF003).

Note: *Tuber thailandicum* resembles *T. castilloi* but *T. castilloi* has a smaller ascomata size (1.1−2.5 cm in diameter), thinner peridium (80−150 μm), and narrower spores in one-spored asci (40−65×40−62 μm). *Tuber thailandicum* and *T. castilloi* spores are subglobose to broadly ellipsoid, while those of *T. castilloi* are mostly broadly ellipsoid (Guevara et al. 2013). *Tuber castilloi* forms mycorrhizal associations with *Quercus* spp. in North America.

Phylogenetic analysis

The ITS sequences from ascomata of *T. thailandicum* SDBR-CMU-MTUF001, SDBR-CMU-MTUF002 and SDBR-CMU-MTUF003 were deposited at GenBank under accession numbers KP196328, KP196329 and KP196330, respectively. In addition, the LSU sequences of *T. thailandicum* SDBR-CMU-MTUF001, SDBR-CMU-MTUF002 and SDBR-CMU-MTUF003 were deposited under accession numbers KP196333, KP196334 and KP196335, respectively. The

Tuber sequences for phylogenetic analysis were obtained in this study and from GenBank database (Table 1). *Tuber magnatum* was used as the outgroup. The aligned dataset of 55 sequences of the ITS consisted of 773 characters, of which 221 characters were constant, 131 variable characters were parsimony uninformative, and 431 characters were parsimony informative. Heuristic searches resulted in 22 equally parsimonious trees with a length of 1779 steps, CI=0.567, RI=0.816, RC=0.462 and HI=0.473. While the aligned data set of 46 sequences of LSU consisted of 733 characters of which 421 characters were constant, 137 variable characters were parsimony uninformative, and 175 characters were parsimony informative. Heuristic searches resulted in ten equally parsimonious trees with a length of 546 steps, CI=0.703, RI=0.861, RC=0.606 and HI=0.404. The phylogenetic dendrograms of ITS and LSU sequences are shown in Figs. 2 and 3. Both phylograms indicated that *T. thailandicum* clearly differed from other whitish truffle species and formed a monophyletic clade with a bootstrap support of 100 %. Based on the ITS analysis, the whitish truffles were separated into three main clades. *Tuber thailandicum* stands within the Gibbosum clade together with *T. bellisporum*, *T. castellanoi*, *T. gibbosum* and *T. pseudospaerosporum*, and forms a sister taxon to *T. pseudospaerosporum* with 98 % bootstrap support. The Maculatum clade comprises ten whitish truffle species (*T. castilloi*, *T. foetidum*, *T. guevarai*, *T. lauryi*, *T. maculatum*, *T. mexiusanum*, *T. miquihuanense*, *T. pseudomagnatum*, *T. shearii* and *T. walkeri*). The remaining clade includes 16 species, *T. alboumbilicum*, *T. borchii*, *T. dryophilum*, *T. latisporum*, *T. liui*, *T. liyuanum*, *T. microspaerosporum*, *T. oligospermum*, *T. panzhihuanense*, *T. puberulum*, *T. sinosphaerosporum*, *T. sinopuberulum*, *T. spherosporum*, *T. sphaerospermum*, *T. vesicoperidium* and *T. zhongdianense*. From the LSU region analysis, the number of operational taxonomic units used in phylogenetic analysis were fewer than in the ITS analysis because of limited availability of LSU sequence data in the database. Moreover, the ITS region is a more precise marker for the identification and species-level determination of *Tuber* than the LSU region (Chen and Liu 2007; Bonito et al. 2010a). Therefore, this study used only the ITS analysis to group the *Tuber* species corresponding to Bonito et al. (2010a).

Characterization of *Tuber thailandicum* ectomycorrhizas

Two ITS sequences of the mycorrhizae were obtained. Phylogenetic analysis confirmed that the fungal symbiont of the selected mycorrhizas was in the same clade as *T. thailandicum* fruiting bodies (Fig. 2). These sequences were deposited at GenBank under accession numbers KP196331 and KP196332, respectively and both sequences had 100 % similarity with *T. thailandicum*. Therefore, the fungal symbiont was *T. thailandicum*. For plant identification, the ITS sequence from ectomycorrhizal roots in field, containing 592 bp, was deposited in GenBank under accession number KP713780 and closely resembled the ITS sequence AY763114 and AY761101 from *B. alnoides* (Li and Shoup 2005), with a similarity of 99 % by BLAST. Therefore, the mycorrhizal root formed by the colonization of *T. thailandicum* was *B. alnoides*.

The mycorrhizae are simple or monopodial-pinnate, straight, cylindrical or sometimes club-shaped and always with rounded ends, 2.0–5.0 mm in length and 0.3–0.6 mm in diameter (Fig. 4a, b). Rhizomorphs were not observed. The color varied with different developmental stages; white when young then becoming pale grey, and dark grey when older. Two kinds of peritrophic elements, emanating hyphae and cystidia, were observed on *T. thailandicum* mycorrhizae. The emanating hyphae are hyaline, 2.0–3.0 μm in diameter, thin-walled, septate, ramified and tortuous (Fig. 4c). They bear abundant crystals and generally grow from a network on the surface of the mycorrhizae. The cystidia are hyaline, needle-shaped, septate, smooth, straight or slightly bent, 40–60 μm in length, 3.0–5.0 μm in diameter and have rounded tips (Fig. 4d). The cystidia grow radially from the surface of mantle and are arranged in a restricted area, often at the apex of the mycorrhizae. Mycorrhizae with cystidia are very rare and sometimes may lack these structures. The outer and inner mantle layers formed a pseudoparenchymatous mantle consisting of angular cells, 4.5–10.0 μm in diameter. The mantle type of hypha was designated as L type after Agerer's (2006) categorization (Fig. 4e). A cross section of the mycorrhizal roots showed a 25–30 μm thick mantle arranged in four to six cell layers, with the Hartig net only reaching the epidermal layer and apparently only the first row of the cortical cells (Fig. 4f).

Discussion

The present study is the first report to describe a new whitish truffle from Thailand and its ectomycorrhizal association. *Tuber thailandicum* is characterized by its white ascomata and subglobose spores with an alveolate reticulum; thus, it belongs to the gibbosum group. The morphological characteristics clearly separate the new species, *T. thailandicum* from other whitish truffle. It has a two-layered peridium that easily separated it from *T. cistophilum*, *T. glabrum*, *T. gennadii*, *T. lacunosum*, *T. maculatum*, *T. oligospermum*, *T. panzhihuanense*, *T. sphaerospermum* and *T. vesicoperidium*, which have a one-layered peridium (Bulman et al. 2010; Alvarado et al. 2012; Fan and Cao 2012c, 2014, Fan et al. 2014; Deng et al. 2013). The presence of hyphae-like hairs on peridial surface of *T. thailandicum* distinguishes it from *T. alboumbilicum*, *T. bellisporum*, *T. castellanoi*, *T. gibbosum*, *T. microspiculatum*, *T. miquihuanense*, *T. oregonense*,

Table 1 Details of the *Tuber* sequences used in this study

Taxon	Voucher	Location	GenBank number	
			ITS	LSU
T. alboumbilicum	YAAS L2324	Sichuan, China	KJ742702	
T. bellisporum	JT7270	Oregon, USA	FJ809856	FJ809827
T. bellisporum	JT6060	Oregon, USA		FJ809828
T. borchii	B-1481	Nagyegyháza, Hungary	AJ557542	
T. borchii	ZB1529	Hungary	JF261384	
T. borchii	CI.-38	Hungary	AJ557541	
T. borchii	AH39139	Madrid, Spain		JN392291
T. borchii	GB32	Italy		FJ809852
T. borchii	GB14	Italy		JQ925682
T. castellanoi	JT19924	California, USA	FJ809859	FJ809830
T. castellanoi	JT28069	California, USA	FJ809860	FJ809831
T. castellanoi	JT4568	California, USA		FJ809829
T. castelloi	ITCV149	Mexico	HM485403	JF419288
T. castelloi	ITCV142	Mexico	HM485404	JF419287
T. dryophilum		Italy	AF003917	
T. dryophilum	GB37	Italy		JQ925688
T. dryophilum	GB35	Italy		JQ925687
T. dryophilum	GB66	Italy		FJ809800
T. foetidum	ZB2452	Garé, Hungary	AJ557543	JF261361
T. foetidum	ZB2489	Szigetújfalu, Hungary	AJ557544	JF261362
T. gibbosum	JT30580	Oregon, USA	FJ809868	
T. gibbosum	JT29402	Oregon, USA	FJ809870	
T. gibbosum	JT6555	California, USA		FJ809833
T. gibbosum	JT27987	Oregon, USA		FJ809834
T. gibbosum	AFTOL-ID1344	Oregon, USA		FJ176877
T. guevarai	ITCV180	Mexico	HM485405	
T. guevarai	4B	Mexico	JF419251	
T. latisporum	HKAS44315	Yunnan, China	DQ898183	
T. latisporum	HKAS30838B	Yunnan, China	DQ898185	
T. lauryi	JT19425	Oregon, USA	HM485365	JF419314
T. lauryi	JT5880	Oregon, USA		JF419279
T. liui	HKAS48269	Xizang, China	DQ898182	
T. liyuanum	FAN162	Yunnan, China	JQ771191	
T. liyuanum	FAN187	Yunnan, China	JQ771193	
T. maculatum	FHS-399	Serbia	FM205644	
T. maculatum	ZB2656	Hungary		JF261366
T. maculatum	TL5974	Denmark		AJ969627
T. magnatum	GB13	Italy	JQ925646	
T. magnatum	T100_mag	Italy	GU979030	
T. magnatum	JT19369	Unknown		FJ809847
T. magnatum	JT19460	Emilia-Romagna, Italy		FJ809848
T. mexiusanum	ITCV181	Mexico	HM485411	
T. mexiusanum	ITCV3785	Mexico		JF419294
T. mexiusanum	GB391	USA		JF419295
T. microsphaerosporum	BJTC FAN152	China	KF805726	
T. miquihuanense	ITCV885	Mexico	HM485414	JF419292
T. miquihuanense	ITCV4026	Mexico		JF419290

Table 1 (continued)

Taxon	Voucher	Location	GenBank number	
			ITS	LSU
T. oregonense	JT15112	Oregon, USA	FJ809881	
T. oregonense	JT28263	Oregon, USA	FJ809882	
T. oregonense	JT27945	Oregon, USA		FJ809836
T. oregonense	JT8767	Oregon, USA		FJ809837
T. oligospermum	AH37867	Italy	JN392259	JN392322
T. oligospermum	AH39338	France	JN392266	JN392319
T. oligospermum	AH39281	Spain		JN392315
T. panzhihuanense	HKAS72015	Sichuan, China	JQ978648	
T. pseudomaganatum	BJTC FAN163	Yunnan, China	JQ771192	
T.pseudosphaerosporum	BJTC FAN260	Yunnan, China	KF744062	
T.pseudosphaerosporum	BJTC FAN250	Yunnan, China	KF744063	
T. puberulum	FHS-389	Serbia	FM205642	
T. puberulum	TL3857	Denmark		AJ969625
T. puberulum	TL11885	Denmark		AJ969626
T. shaerospermum	AH39197	Cáceres, Spain	JN392242	JN392307
T. shaerospermum	AH37798	Temara, Morocco	JN392245	JN392304
T. shaerosporum	OSC75864	Oregon, USA	HM485390	
T. shaerosporum	JT19772	Oregon, USA	FJ809854	
T. shearii	OSC51052	North Carolina, USA	HM485389	JF419280
T. sinopuberulum	BJTC FAN157	Yunnan, China	JQ690073	JQ690070
T. sinosphaerosporum	BJTC FAN135	Yunnan, China	JX092086	
T. sinosphaerosporum	BJTC FAN136	Yunnan, China	JX092087	
T. thailandicum	SDBR-CMU-MTUF001	Chiang Mai, Thailand	KP196328	KP196333
T. thailandicum	SDBR-CMU-MTUF002	Chiang Mai, Thailand	KP196329	KP196334
T. thailandicum	SDBR-CMU-MTUF003	Chiang Mai, Thailand	KP196330	KP196335
T. thailandicum	CMU-MYMTUF01	Chiang Mai, Thailand	KP196331	
T. thailandicum	CMU-MYMTUF02	Chiang Mai, Thailand	KP196332	
T. walkeri	RH569	USA	JF419261	
T. walkeri	RH754	USA	JF419265	
T. walkeri	RH794	USA		JF419307
T. walkeri	RH521	USA		JF419308
T. vesicoperidium	BJTC FAN155	Yunnan, China	JQ690071	JQ690068
T. vesicoperidium	BJTC FAN156	Yunnan, China	JQ690072	JQ690069
T. zhongdianense	HKAS45388B	Yunnan, China	DQ898186	

T. pseudomagnatum, *T. pseudosphaerosporum*, *T. sinoalbidum* and *T. sinopuberulum* (Guevara et al. 2013). Moreover, the spore shape of *T. thailandicum* differs from the broadly ellipsoidal, ellipsoid and long-ellipsoid spores of *T. bellisporum*, *T. borchii*, *T. dryophilum*, *T. foetidum*, *T. gibbosum*, *T. glabrum*, *T. latisporum*, *T. liui*, *T. liyuanum*, *T. maculatum*, *T. oregonense*, *T. pseudomagnatum*, *T. rapaeodorum*, *T. sinoalbidum* and *T. zhongdianense* (Xu 1999; He et al. 2004; Chen and Liu, 2007; Bonito et al. 2010a; Bulman et al. 2010; Fan and Cao 2012b; Fan et al. 2014). *Tuber microsphaerosporum* and *T. sinosphaerosporum* are distinguished from *T. thailandicum* by their reticulated globose spores (Fan and Yue 2013; Fan et al. 2012a). *Tuber shearii* has spores similar to *T. thailandicum*, but can be easily differentiated by its smaller reticulum size (9−10×5−6 μm) (Murill 1920). *Tuber thailandicum* shares some morphological characteristics with five North American truffles, *T. castilloi*, *T. guevaria*, *T. lauryi*, *T. mexiusanum* and *T. walkeri*, including a two-layered peridium with hyphae-like hair on its surface and subglobose, sometime globose or broadly ellipsoid reticulated spores (Table 2). However, the peridial thickness in *T. thailandicum* (150−225 μm) was

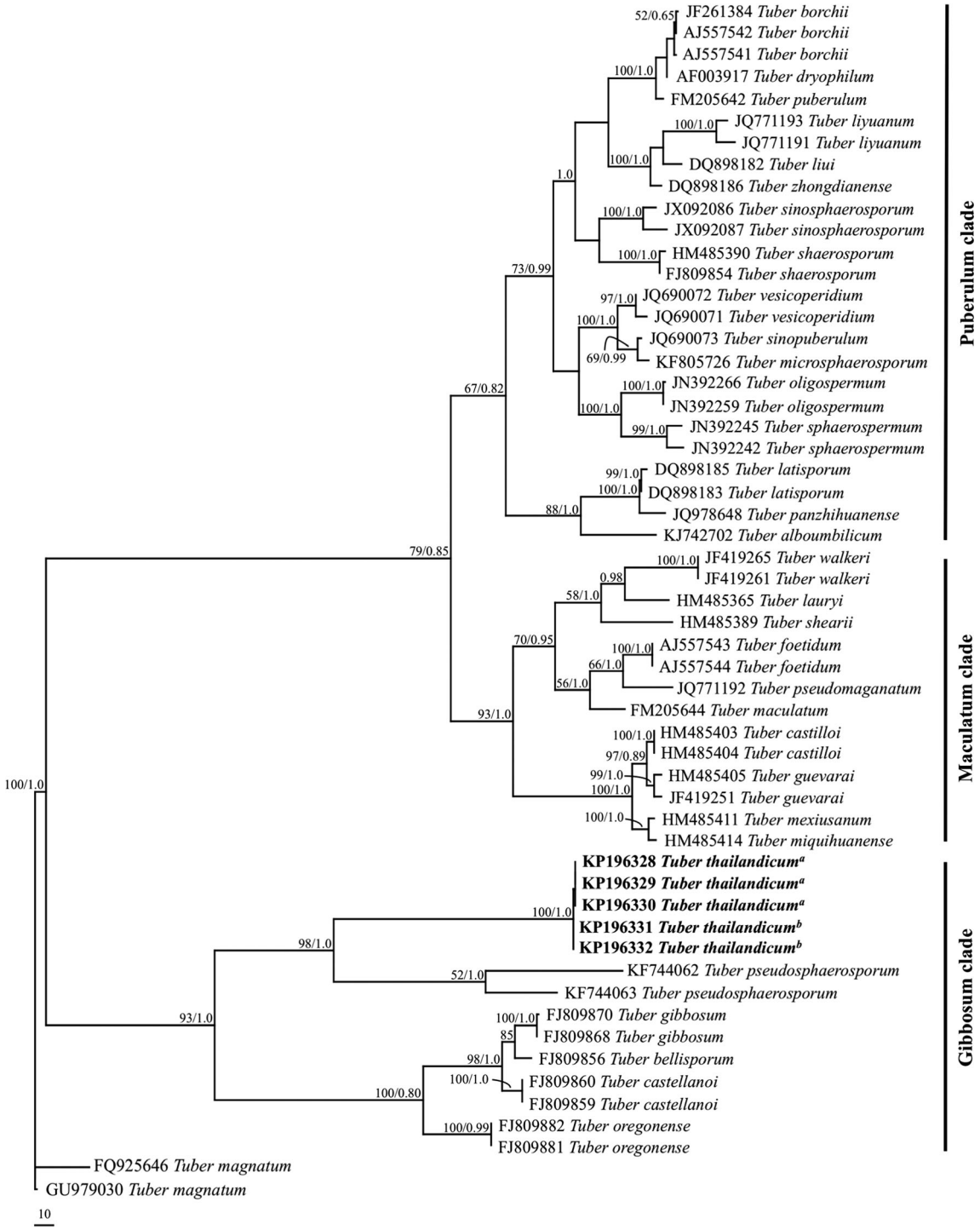

Fig. 2 One of 22 maximum parsimonious trees inferred from a heuristic search of the ITS region of rDNA of 55 sequences. *Tuber magnatum* was used as the outgroup. Numbers above branches identify the bootstrap statistics percentages (left) and Bayesian posterior probabilities (right). Branches with bootstrap values≥50 % are shown at each branch and the bar represents ten shown substitutions per nucleotide position. The sequences from this study are in bold. [a] Sequence from ascomata and [b] Sequence from mycorrhizae

different from *T. castilloi* (80−150 μm) and *T. lauryi* (300 −1000 μm) (Guevara et al. 2013). The smaller ascomata size and 1−5 spores in asci of *T. guevaria*, *T. mexiusanum* and *T. walkeri* distinguished them from the new species reported here (Guevara et al. 2013). Interestingly, our ITS and LSU sequence analyses clearly separate *T. thailandicum* from the other whitish truffles. Based on the ITS sequence analysis, *Tuber thailandicum* forms a sister taxon to *T. pseudosphaerosporum* known from southwestern China with 87 % similarity, while *T. pseudosphaerosporum* has

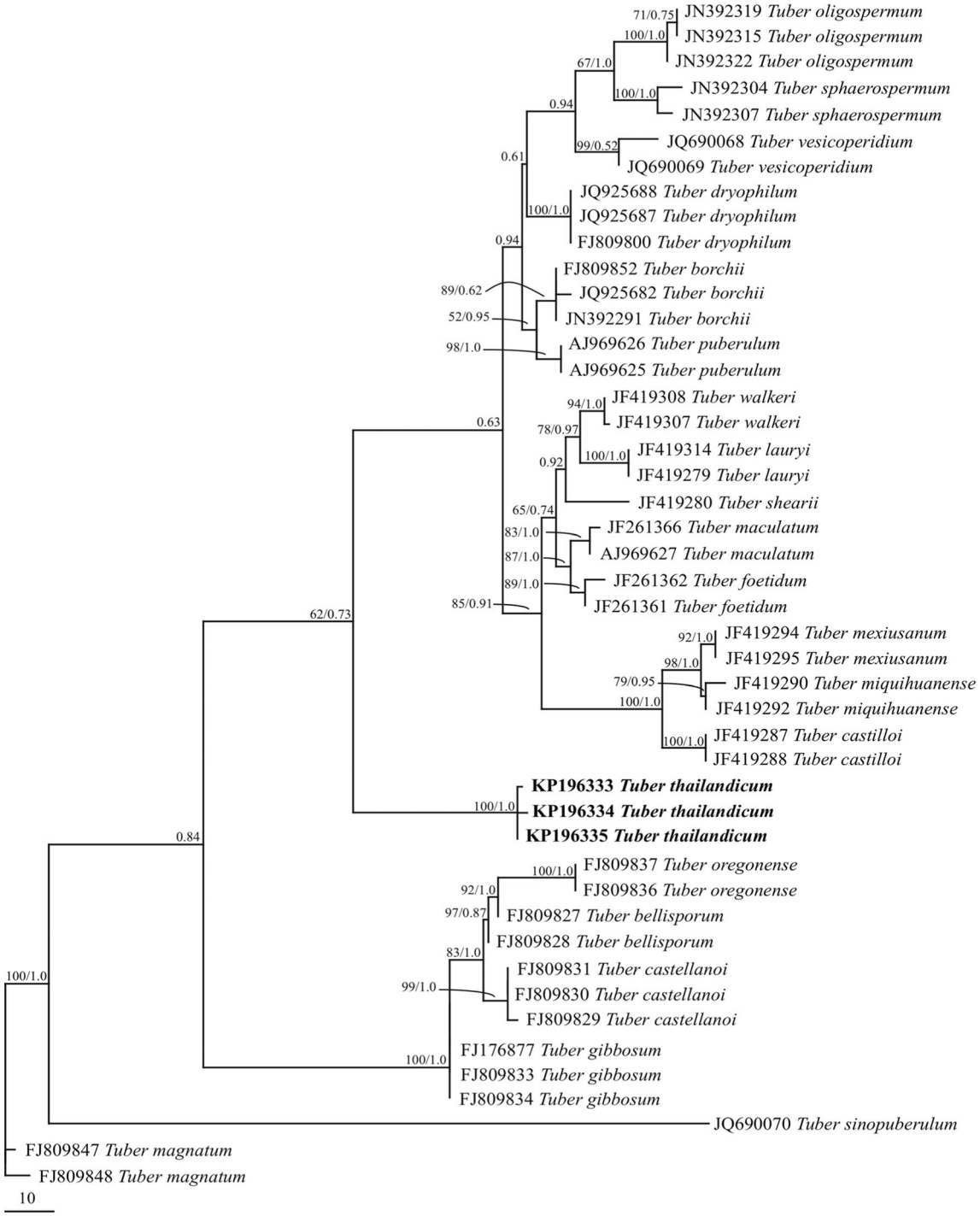

Fig. 3 One of ten maximum parsimonious trees inferred from a heuristic search of the LSU region of rDNA of 46 sequences. *Tuber magnatum* was used as the outgroup. Number above branches identify the bootstrap statistics percentages (left) and Bayesian posterior probabilities (right). Branches with bootstrap values≥50 % are shown at each branch and the bar represents ten shown substitutions per nucleotide position. The sequences from this study are in bold

thicker outer peridium (150−250 μm) and regularly recticuted globose spores (Fan and Yue 2013). Therefore, the combination of morhphological and molecular characteristics strongly supported a new whitish truffle, *T. thailandicum* from Thailand.

Truffles grow in symbiosis with several trees such as beech (*Fagus* spp.), birch (*Butula* spp.), hazel (*Corylus* spp.), oaks (*Quercus* spp.), pine (*Pinus* spp.) and spruce (*Picea* spp.) (Weden et al. 2004; Hall 2007; Trappe et al. 2009; Bonito et al. 2011; Stobbe et al. 2012). The ectomycorrhzae of

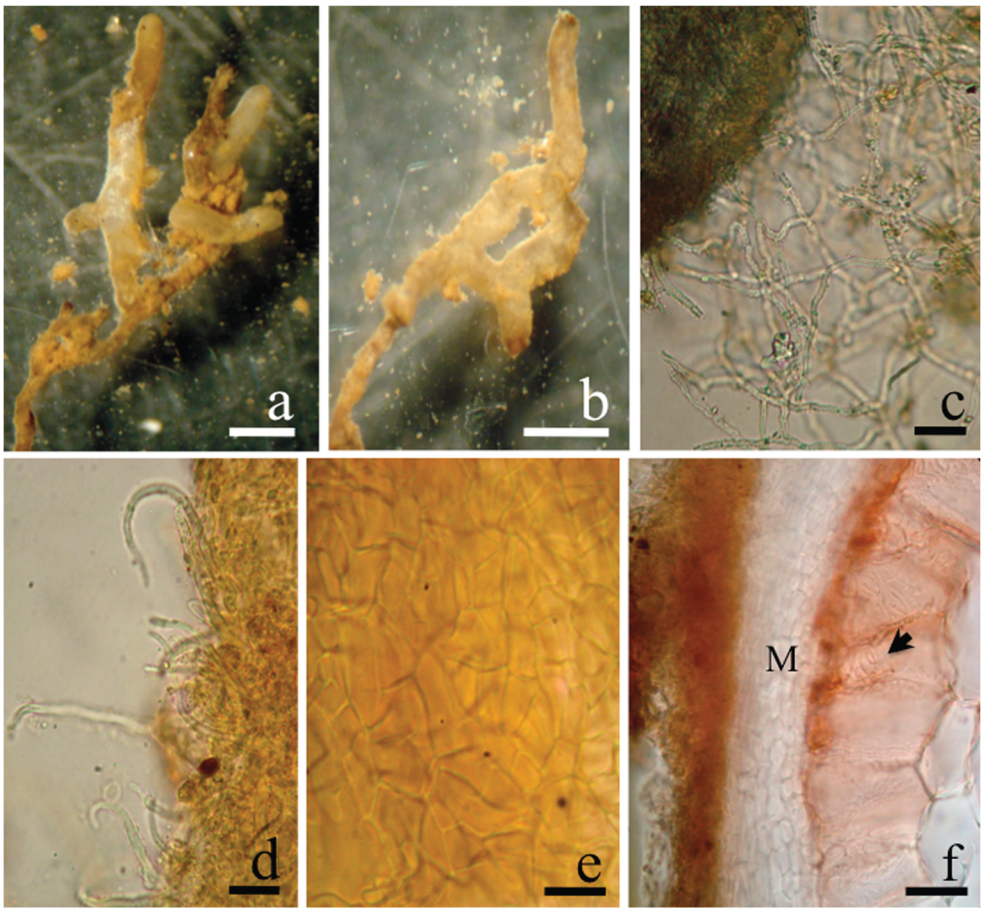

Fig. 4 Morphological and anatomical traits of *T. thailandicum* mycorrhizae with *Betula alnoides*. *a* and *b*. Ectomycorrhizal root tips. *c*. Emanating hyphae with crystals. *d*. Cystidia. *e*. The angular cell of the outer mantle layer. *f*. Cross section of mycorrhizal root tips showing mantle sheath (M) and Hartig net (arrowed). *Scale bars*: a and b=1 mm, c=20 μm, d and e=10 μm, f=25 μm

Table 2 Morphological characters of *Tuber castilloi*, *T. guevaria*, *T. lauryi*, *T. mexiusanum*, *T. thailandicum* and *T. walkeri*

Fugal taxa	Ascomata (cm in diameter)	Peridium		Hair-like structure	Gleba	Number of spore in asci	Ascospore	
		Color/ surface	Thickness (μm)				Shape	Size without reticulation (μm)
T. castilloi[a]	0.1−2.5	Cream/ verrucose	80−150	Present	Light brown	1−4	Subglobose to broadly ellipsoid (mostly broadly ellipsoid)	27−63×0−40
T. guevarai[a]	1.5	Cream to light brown/ finely verrucose	160−220	Present	Brown to dark brown	1−5	Subglobose to broadly ellipsoid	18−55×16−42
T. lauryi[a]	0.6−1.0	Light brown/ smooth to finely verrucose	300−1000	Present	Brown	1−5	Subglobose to broadly ellipsoid	22−50×20−41
T. maxiusanum[a]	1.1−1.4	Cream to light brown/ verrucose	130−350	Present	Brown	1−5	Subglobose to broadly ellipsoid	15−50×12−36
T.thailandicum-[b]	2.0−3.5	White to brown/ verrucose	150−255	Present	Dark brown	1−4	Subglobose to broadly ellipsoid (mostly subglobose)	20−65×15−62
T. walkeri[a]	0.9−1.2	Ocher brown/ smooth to finely verrucose	150−600	Present	Dark brown	1−5	Globose to broadly ellipsoid	18−53×15−45

[a] Guevara et al. (2013)

[b] Present study

whitish truffle generally have a pseudoparenchymatous-epidermoid mantle (M type) and needle-shaped cystidia, as have also been detected in *T. borchii* (Rauscher et al. 1996; Benucci et al. 2012; Lancellotti et al. 2014), *T. maculatum* (Zambonelli et al. 1999), *T. oligospermum* (Boutahir et al. 2013), *T. puberulum* (Blaschke 1987), *T. rapaeodorum* (Kovács and Jakucs 2006) mycorrhizae. The shape and size of cystidia and the anatomy of mantle are characteristics used to identify the mycorrhizae of the most economically important *Tuber* species (Kovács and Jakucs 2006; García-Montero et al. 2008; Benucci et al. 2012). In this study, the pseudoparenchymatous, angular outer mantle layer (L type) of *T. thailandicum* mycorrhizae was a important characteristic in distinguishing it from other whitish truffle mycorrhizae. However, previous studies reported that the comparison of younger and older parts of the same mycorrhizal specimen showed variable feature overlap. Variable mantle patterns in the ectomycorrhizae of *T. borchii* were observed and these phenomena were explained as intraspecific and age-related variabilities of the strains (Giomaro et al. 2000). Those observations suggested that the anatomical characteristics of mycorrhizas are not sufficient for identification of the fungal symbiont species (Kovács and Jakucs 2006; Boutahir et al. 2013). Conversely, the molecular analyses have provided powerful tools to identify the genetic differences between fungal symbiont species. Therefore, combining molecular and anatomical approaches is suitable to identify ectomycorrhizae of truffle (Baciarelli-Falini et al. 2006; Kovács and Jakucs 2006; Rubini et al. 2001; Boutahir et al. 2013). In conclusion, our study presents a new whitish truffle, *T. thailandicum* from Thailand. This discovery is important to stimulate the investigations of *Tuber* in Thailand, and will help us understand more on the distribution and ecology of *Tuber* in Asia.

Acknowledgments This work was supported by grants from Chiang Mai University and TRF Research-Team Association Grant (RTA5880006). We are grateful to Prof. Dr. Robert J. McGovern and Mr. Keegan Kennedy for English proof reading.

References

Agerer R (1986) Studies on ectomycorrhizae. II introducing remarks on characterization identification. Mycotaxon 26:473–492

Agerer R (1991) Studies on ectomycorrhizae XXXIV. Mycorrhizae of *Gomphidius glutinosus* and of *G. roseus* with some remarks on Gomphidiaceae (Basidiomycetes). Nova Hedwigia 53:127–170

Agerer R (2006) Fungal relationships and structural identity of their ectomycorrhizae. Mycol Prog 5:67–107. doi:10.1007/s11557-006-0505-x

Alvarado P, Moreno G, Manjón JL (2012) Comparison between *Tuber gennadii* and *T. oligospermum* lineages reveals the existence of the new species *T. cistophilum* (Tuberaceae, Pezizales). Mycologia 104: 894–910. doi:10.3852/ 11-254

Baciarelli-Falini L, Rubini A, Riccioni C, Paolocci F (2006) Morphological and molecular analyses of ectomycorrhizal diversity in a man-made *T. melanosporum* plantation: description of novel truffle-like morphotypes. Mycorrhiza 16:475–484. doi:10.1007/s00572-006-0066-5

Baum DA, Small RL, Wendel JF (1998) Biogeography and floral evolution of Baobabs (*Adansonia*, Bombacaceae) as inferred from multiple data sets. Syst Biol 47:181–207

Benucci GMN, Bonito G, Falini LB, Bencivenga M (2012) Mycorrhization of Pecan trees (*Carya illinoinensis*) with commercial truffle species: *Tuber aestivum* Vittad. and *Tuber borchii* Vittad. Mycorrhiza 22:383–392. doi:10.1007/s00572-011-0413-z

Blaschke H (1987) Vorkommen und charakterisierung der ektomykorrrhizaassoziation *Tuber puberulum* mit *Picea abies*. Z Mykol 53:283–288

Bonito G, Grygansyi AP, Trappe JM, Vilgalys R (2010a) A global meta-analysis of Tuber ITS rDNA sequences: species diversity, host associations and long-distance dispersal. Mol Ecol 19:4994–5008. doi:10.1111/j.1365-294X.2010.04855.x

Bonito G, Trappe JM, Donovan S, Vilgalys R (2011) The Asian black truffle *Tuber indicum* can form ectomycorrhizas with North American host plants and complete its life cycle in non-native soils. Fungal Ecol 4:83–93. doi:10.1016/j.funeco.2010.08.003

Bonito G, Trappe JM, Rawlimson P, Vilgalys R (2010b) Improved resolution of major clades whitish *Tuber* and taxonomy of species within the *Tuber gibbosum* complex. Mycologia 102:1042–1057. doi:10.3852/09-213

Boutahir S, Iotti M, Piattoni F, Zambonelli A (2013) Morphological and molecular characterization of *Tuber oligospermum* mycorrhizas. Afr J Agric Res 8:4081–4087. doi:10.5897/AJAR2013.7354

Bulman SR, Visnovsky SB, Hall IR, Guerin-Laguette A, Wang Y (2010) Molecular and morphological identification of truffle-producing *Tuber* species in New Zealand. Mycol Prog 9:205–214. doi:10.1007/s11557-009-0626-0

Chen J, Liu PG (2007) *Tuber latisporum* sp. nov. and related taxa, based on morphology and DNA sequence data. Mycologia 99:475–481. doi:10.3852/mycologia.99.3.475

Chen J, Liu PG, Wang Y (2005) *Tuber umbilicatum*, a new species from China, with a key to the spinose-reticulate spored *Tuber* species. Mycotaxon 94:1–6

Chen YL, Gong MQ (2000) Truffles *Tuber* spp.: biodiversity and geological distribution. Edible Fungi of China 19:25–26

Cooke MC, Massee G (1892) Himalayan truffles. Grevellea 20:67

Deng XJ, Liu PG, Liu CY, Wang Y (2013) A new white truffle species, *Tuber panzhihuanense* from China. Mycol Prog 12:557–561. doi:10.1007/s11557-012-0862-6

Fan L, Cao JZ (2012) Two new species of white truffle from China. Mycotaxon 121:297–304. doi:10.5248/121.297

Fan L, Cao JZ, Liu YY, Li Y (2011a) Two new species of tuber from China. Mycotaxon 116:349–354. doi:10.5248/116.349

Fan L, Cao JZ, Yu J (2012a) Tuber in China: *T. sinopuberulum* and *T. vesicoperidium* spp. nov. Mycotaxon 121:255–263. doi:10.5248/121.255

Fan L, Cao JZ, Zheng ZH, Li Y (2012b) Tuber in China: *T. microspermum* and *T. microspiculatum* spp. nov. Mycotaxon 199:391–395. doi:10.5248/119.391

Fan L, Feng S, Cao JZ (2014) The phylogenetic position of *Tuber glabrum* sp. nov. and *T. sinomonosporum* nom. nov., two *Paradoxa*-like truffle species from China. Mycol Prog 13:241–246. doi:10.1007/s11557-013-0908-4

Fan L, Hou CL, Cao JZ (2011b) *Tuber sinoalbidum* and *T. polyspermum* – new species from China. Mycotaxon 118:403–410. doi:10.5248/118.403

Fan L, Yue SF (2013) Phylogenetic divergence of three morphologically similar truffles: *Tuber sphaerosporum*, *T. sinosphaerosporum*, and *T. pseudospaerosporum* sp. nov. Mycotaxon 125:283–288. doi:10.5248/125.283

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

García-Montero LG, Díaz P, Martín-Fernández S, Casermeiro MA (2008) Soil factors that favour the production of *Tuber melanosporum* carpophores over other truffle species: a multivariate statistical approach. Acta Agric Scand B 58:322–329. doi:10.1080/09064710701743286

García-Montero LG, Díaz P, Massimo GD, García-Abril A (2010) A review of research on Chinese *Tuber* species. Mycol Prog 9:315–335. doi:10.1007/s11557-009-0647-8

Giomaro G, Zambonelli A, Sisti D, Cecchini M, Evangelista, Stocchi V (2000) Anatomical and morphological characterization of mycorrhizas of five strains of *Tuber borchii* Vittad. Mycorrhiza 10:107–114. doi:10.1007/s005720000065

Guevara G, Bonito G, Trappe JM, Cázares E, Williams G, Healy RA, Schadt C, Vilgalys G (2013) New north american truffles (*Tuber* spp.) and their ectomycorrhizal associations. Mycologia 105:194–209. doi:10.3852/12-087

Hall IR, Brown GT, Zambonelli A (2007) Taming the truffle, the history, lore and science of the ultimate mushroom. Timber Press, Portland

Hall IR, Yun W, Amicucci A (2003) Cultivation of edible ectomycorrhizal mushrooms. Trends Biotechnol 21:433–438. doi:10.1139/B04-051

He XY, Li HM, Wang Y (2004) *Tuber zhongdianense* sp. nov. from China. Mycotaxon 90:213–216. doi:10.5248/121.255

Hu HT, Wang Y (2005) *Tuber furfuraceum* sp. nov. from Taiwan. Mycotaxon 93:155–157

Karkouri KE, Murat C, Zampieri E, Bonfante P (2007) Identification of internal transcribed spacer sequence motifs in truffles: a first step toward their DNA bar cording. Appl Environ Microbiol 73:5320–5330. doi:10.1128/AEM.00530-07

Kinoshita A, Sasaki H, Nara K (2011) Phylogeny and diversity of Japanese truffles (*Tuber* spp.) inferred from sequences of four nuclear loci. Mycologia 103:779–794. doi:10.3852/10-138

Kirk PM, Cannon PF, Minter DW, Stalpers JA (2008) Ainsworth and Bisby's dictionary of the fungi, 10th edn. Oxon, UK

Kornerup A, Wanscher JH (1967) Methuen handbook of colour, 2nd edn. Eyre Methuen, London

Kovács GM, Jakucs E (2006) Morphological and molecular comparison of white truffle ectomycorrhizae. Mycorrhiza 16:567–574. doi:10.1007/s005720060071-8

Lancellotti E, Iotti M, Zambonelli A, Franceschini A (2014) Characterization of *Tuber borchii* and *Arbutus unedo* mycorrhizas. Mycorrhiza 24:481–486. doi:10.1007/s00572-014-0564-9

Li J, Shoup S (2005) Phylogenetic of *Betula* (Betulaceae) inferred from sequences of nuclear ribosomal DNA. Rhodora 107:69–86. doi:10.3119/04-14.1

Li SH, Zheng LY, Liu CY, Wang Y, Li L, Zhao YC, Zhang XL, Yang M, Xiong HK, Qing Y, Wang L, Zhou DQ (2014) Two new truffles species, *Tuber alboumbilicum* and *Tuber pseudobrumale* from China. Mycol Prog 13:1157–1163. doi:10.1007/s11557-014-1004-0

Liu B (1985) New species and new records of hypogeous fungi from China I. Acta Mycol Sin 4:84–89

Liu B, Liu YH (1994) Study on hypogeous fungi in China. Chinese Fungi 9:157–165

Mortimer PE, Karunarathna SC, Li Q, Gui H, Yang X, Yang X, He J, Ye L, Guo J, Li H, Sysouphanthong P, Zhou D, Xu J, Hyde KD (2012) Prized edible Asian mushrooms: ecology, conservation and sustainability. Fungal Divers 56:31–47. doi:10.1007/s13225-012-0196-3

Murrill WA (1920) Another new truffle. Mycologia 12:157–158

Paolocci F, Rubini A, Granetti B, Arcioni S (1999) Rapid molecular approach for a reliable identification of *Tuber* spp. ectomycorrhizae. FEMS Microbiol Ecol 28:23–30. doi:10.1016/S0168-6496(98)00088-9

Rauscher T, Müller WR, Chevalier G, Agerer R (1996) *Tuber borchii* Vitt. In: Agerer R (ed) Colour atlas of ectomycorrhizae. Einhorn Verlag, Schwäbisch Gmüad, p 113

Rey JF (2001) Creation et origine de la commercialisation de la truffe de Chine en France et dans le monde. In: Corvoisier M, Olivier JM, Chevalier G (eds) Federation Française des Trufficulteurs, Proceedings du Ve Congrès International Science et Culture de la Truffe, Paris, pp 517–518

Riousset G, Riousset L, Chevalier G, Bardet MC (2001) Truffe d'Europe et de Chine. INRA, Paris

Ronquist F, Teslenko M, van der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and modelchoice across a large model space. Syst Biol 61:539–542. doi:10.1093/sysbio/sys029

Rubini A, Paolocci F, Granetti B, Arcioni S (2001) Morphological characterization of molecular-typed *Tuber magnatum* ectomycorrhizae. Mycorrhiza 11:179–185. doi:10.1007/s005720100116

Stobbe U, Büntgen U, Sproll L, Tegel W, Egli S, Fink S (2012) Spatial distribution and ecological variation of re-discovered German truffle habitats. Fungal Ecol 5:591–599. doi:10.1016/j.funeco.2012.02.001

Stobbe U, Egli S, Tegel W, Peter M, Sproll L, Büntgen U (2013) Potential and limitation of Burgundy truffle cultivation. Appl Microbiol Biotechnol 97:5215–5224. doi:10.1007/s00253-013-4956-0

Su KM, Xiong WP, Wang Y, Li SH, Xie R, Baima D (2013) *Tuber bomiense*, a new truffle species from Tibet, China. Mycotaxon 126:127–132. doi:10.5248/126.127

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

Tao K (1988) Taxonomic study on hypogeous fungi of China. The University of Shanxi, Taiyuan, Dissertation

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higguns DG (1997) The CLUSTAL_X windows interface: flexible stratgies for multiple sequene alignment aided by quality analysis tools. Nucleic Acids Res 24:4876–4882

Trappe JM (1976) Notes on Japanese hypogeous ascomycetes. Trans Mycol Soc Jpn 17:209–217

Trappe JM, Molina R, Luoma DL, Cázares E, Pilz D, Smith JE, Castellano MA, Miller SL, Trappe MJ (2009) Diversity, ecology, and conservation of truffle fungi in forests of the pacific northwest. Portland, Oregon

Vilgalys R, Hester M (1990) Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. J Bacteriol 172:4239–4246

Wang Y, He XY (2002) *Tuber huidongense* sp. nov. from China. Mycotaxon 83:191–194

Wang Y, Moreno G, Riousset L, Manjón JL, Riousset G, Fourré G, Di Massimo G, García-Montero LG, Díez J (1998) *Tuber pseudoexcavatum* sp. nov. a Chinese species commercialised in Spain, France and Italy with comments on Chinese truffles. Cryptogam Mycolog 19:113–120

Weden C, Chevalier G, Danell E (2004) *Tuber aestivum* (syn. *T. uncinatum*) biotopes and their history on Gotland, Sweden. Mycol Res 108:304–310. doi:10.1017/S0953756204009256

White TJ, Burns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, Whitish TJ (eds) PCR protocols, a guide to methods and applications. Academic Press, San Diego, pp 315–322

Xu AS (1999) A taxonomic study of the genus *Tuber* in Xizang. Mycosystema 18:361–365

Zambonelli A, Iotti M, Amicucci A, Pisi A (1999) Caratterizzazione anatomo-morfologica delle micorrize di *Tuber maculatum* Vittad. su *Ostrya carpinifolia* Scop. Micol Ital 28:29–35