New Species of Whitish Truffle, Tuber Lithocarpii Sp. Nov., Belonging to the Puberulum Clade, Discovered Under Lithocarpus Konishii in Taiwan

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Abstract

Background

Diversity of *Tuber* species in Taiwan is still poorly known, with only *T. formosanum* from *Melanosporum* clade, *T. furfuraceum* from *Rufum* clade and *T. elevatireticulatum* from *Puberulum* clade were recorded in recent 30 years. During our survey of hypogeous fungi in Taiwan, another whitish truffle was collected from roots of *Lithocarpus konishii* along a forest road in southern Taiwan in January 2018.

Results

The whitish truffle is herein described as a new species *Tuber lithocarpii*, which is appeared to differ from most closely related taxon by its small ascomata size, reddish-brown gleba color, small and globose ascospores size with large pentagonal or irregular marbled reticulated ornamentation. Phylogenetic analysis revealed that *T. lithocarpii* formed a monophyletic clade within *Puberulum* clade and placed sister to the Thai whitish truffle *T. thailandicum* with strong branching support.

Conclusions

Tuber lithocarpii, which is appeared to differ from the previous recorded species based on morphological and phylogenetic evidences. This is an important and encouraging discovery in Taiwan as to facilitate cognition of Asian truffles, the following development of biotechnological applications and truffle cultivation of native species.

Background

Index Fungorum lists out 327 species, subspecies and varieties of genus *Tuber* over the world. However, diversity of *Tuber* species in Taiwan is still poorly known, not to mention other 'truffles' such as *Elaphomyces* and *Hydnотrya* species. Only three species of *Tuber* were found in Taiwan in 1987, 2005 and 2017 respectively, which were *T. formosanum* Hu from *Melanosporum* clade (invalidly described in 1992 due to the lack of designated holotype and later re-typification in 2013) (Hu, 1992; Qiao et al., 2013), *T. furfuraceum* H.T. Hu & Y. Wang from *Rufum* clade (Hu & Wang, 2005) and *T. elevatireticulatum* from *Puberulum* clade (Lin et al., 2018). Our research of Taiwan native hypogeous fungi has been carried out since 2015, fortunately another species of *Tuber* was collected from roots of *Lithocarpus konishii* (Hayata) Hayata in southern Taiwan and appeared to be distinguishable from other previous recorded species based on morphological and phylogenetic evidences.

Methods

Morphological and molecular analysis methods followed methodology described by Lin et al. (2018). Ascocarp was collected with three-pronged garden cultivators and the pH value of adjacent soil were measured by JENCO 6010M pH meter following protocol of the manufacturer. Soil attaches on ascocarp

were cleaned with dry toothbrush, and then cut into halves for observing gleba color or color change under air exposure. Sections of fresh tissue were made with a razor blade by hand, then mounted in 0.1% (w/v) cotton blue in lacto-phenol for describing morphological characteristics. Ascospore dimensions, with the ornamentation excluded, were based on at least 200 randomly selected ascospores by eyepiece. The abbreviation Q represents the range of ratio of spore length to spore width calculated for each spore, and Qm with $\pm$ standard deviation is the mean of the Q value. Holotype was deposited at Herbarium of Taiwan Forestry Research Institute, Taipei, Taiwan (Index Herbarium: TAIF).

DNA was extracted using the TANBead[®] fungal Nucleic Acid Extraction Kit and TANBead[®] Nucleic Acid Extractor (Taiwan Advanced Nanotech Inc., Taipei) following the protocol of the manufacturer. DNA was then amplified and sequenced the internal transcribed spacer (ITS) with universal primer ITS4/ ITS5 (White et al., 1990). PCR was performed in 25 μ l reactions containing 2.5 μ l DNA template, 1 μ l primer each, 8 μ l ddH₂O and 12.5 μ l 2 $\times$ Taq Master Mix (Genomics Bioscience and Technology CO., Ltd., New Taipei City, Taiwan). PCR were run as an initial denaturation at 94°C for 3 min, then at 94°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 30 s by 30 cycles and a final extension at 72°C for 5 min on a multigene thermal cycler (Labnet International, Inc.). The PCR products were sent to Seeing Bioscience Co., Ltd. for purification and sequencing by Sanger Sequencing Method (ABI 3730).

One ITS-rDNA sequences were obtained from the only holotype ascocarp sample of *T. lithocarpii* and were submitted to GenBank through DDBJ with Accession Numbers LC609779 (Tateno et al, 2002). Other whitish *Tuber* sequences were obtained from GenBank database for phylogenetic analysis (Table 1), with blackish truffle *Tuber melanosporum* as the outgroup. Sequences were aligned using MAFFT 7 (Kato and Standley 2013) with default settings, and poorly aligned sites were identified using Gblocks 0.91b (Castresana 2000) with gaps allowed in conserved blocks and with all other parameters left as default values. Ambiguous sites were excluded from phylogenetic analyses. Maximum likelihood (ML) analyses were conducted with MEGA 6.0 (Tamura et al. 2013) using K2P model. Bootstrap analyses were conducted with 1000 replications (Felsenstein 1985). Bayesian phylogenetic analyses were conducted with MrBayes 3.2.6 (Ronquist et al. 2012), for evaluating the effect of different phylogenetic approach. K2P model was used and MCMC chains were run for 1,000,000 generations, sampling every 100th tree. Among these, the first 20% trees were discarded as burn-in phase and the remaining trees were used to calculate Bayesian posterior probabilities. The consensus tree was viewed with FigTree 1.4.3 (Rambaut 2014). Pairwise comparison were conducted with MEGA 6.0 to calculate the similarities between pairs of selected sequences.

Table 1
–Details of the whitish *Tuber* ITS sequences used in phylogenetic study

Taxa		Voucher no.	Origin	GenBank Accession no.	References
1.	<i>T. alboumbilicum</i>	# YAAS L2324	China	KJ742702	Li et al. (2014)
2.	<i>T. anniae</i>	# JT13209	USA	HM485338	Bonito et al. (2010a)
3.	<i>T. asa</i>	M1828	Italy	HM485341	Bonito et al. (2010a)
4.	<i>T. bellisporum</i>	JT7270	USA	FJ809856	Bonito et al. (2010b)
5.	<i>T. borchii</i>	Tar042	Italy	KT165326	Belfiori et al. (2016)
6.	<i>T. californicum</i>	JT22590	USA	HM485351	Bonito et al. (2010a)
7.	<i>T. castellanoi</i>	JT19924	USA	FJ809859	Bonito et al. (2010b)
8.	<i>T. cistophilum</i>	AH39275	Spain	JN392231	PMID:22314593
9.	<i>T. dryophilum</i>	GB37	Italy	HM485354	Bonito et al. (2013)
10.	<i>T. elevatireticulatum</i>	# XTAM3	Taiwan	MF540618	Lin et al. (2018)
11.	<i>T. flavidosporum</i>	# K213	Japan	AB553446	Kinoshita et al. (2016)
12.	<i>T. gibbosum</i>	JT19424	USA	HM485362	Bonito et al. (2010a)
13.	<i>T. huizeanum</i>	# BJTC FAN186	China	JQ910651	Fan et al. (2013a)
14.	<i>T. incognitum</i>	OSC150066		GQ221447	/
15.	<i>T. japonicum</i>	# N88	Japan	AB553444	Kinoshita et al. (2016)
16.	<i>T. latisporum</i>	# HKAS 44315	China	DQ898183	Chen and Liu (2007)
17.	<i>T. lauryi</i>	JT19425	USA	HM485365	Guevara et al. (2013)
18.	<i>T. lijiangense</i>	# HKAS52005	China	KF805727	Fan and Yue (2013)
19.	<i>T. lithocarpii</i>	# DNW1	Taiwan	LC609779	This study
20.	<i>T. liui</i>	HKAS 48269	China	DQ898182	Chen and Liu (2007)
21.	<i>T. liyuanum</i>	# BJTC FAN162	China	JQ771191	Fan and Cao (2013)

Holotype.

* New species described in this study are **bold** as indication.

^ Outgroup taxa

Taxa		Voucher no.	Origin	GenBank Accession no.	References
22.	<i>T. maculatum</i>	Mac1	Italy	AF106889	/
23.	<i>T. microsphaerosporum</i>	# BJTCFan152	China	KF805726	Fan and Yue (2013)
24.	<i>T. microverrucosum</i>	# BJTC FAN1422	China	JN870099	Fan et al. (2011)
25.	<i>T. oligospermum</i>	AH37867	Italy	JN392259	Alvarado et al. (2012)
26.	<i>T. oregonense</i>	# DUKE GB284	USA	NR121356	Bonito et al. (2010b)
27.	<i>T. pacificum</i>	OSC62159		EU837241	/
28.	<i>T. panzhihuanense</i>	# DXJ267	China	JQ978648	Deng et al. (2013)
29.	<i>T. polyspermum</i>	LF2011a	China	JF921165	/
30.	<i>T. pseudomagnatum</i>	# BJTC FAN163	China	JQ771192	Fan and Cao (2013)
31.	<i>T. pseudosphaerosporum</i>	BJTCFan250	China	KF744063	Fan and Yue (2013)
32.	<i>T. puberulum</i>	FHS389	Serbia	FM205642	Marjanovic et al. (2010)
33.	<i>T. separans</i>		USA	HM485385	/
34.	<i>T. sphaerosporum</i>	OSC75864	USA	HM485390	/
35.	<i>T. shearii</i>	OSC51052	USA	HM485389	Bonito et al. (2010a)
36.	<i>T. sinopuberulum</i>	# BJTC FAN157	China	JQ690073	Fan et al. (2013b)
37.	<i>T. piceatum</i>		China	MH115318	/
38.	<i>T. sinosphaerosporum</i>	# BJTC FAN135	China	JX092086	Fan et al. (2013c)
39.	<i>T. sphaerospermum</i>	AH39197	Spain	JN392242	/
40.	<i>T. thailandicum</i>	# CMU-MTUF1	Thailand	KP196328	Suwannarach et al. (2015)

Holotype.

* New species described in this study are **bold** as indication.

^ Outgroup taxa

Taxa		Voucher no.	Origin	GenBank Accession no.	References
41.	<i>T. turmericum</i>	# BJTC FAN473	China	KT758837	Fan et al. (2015)
42.	<i>T. vesicoperidium</i>	# BJTC FAN155	China	JQ690071	Fan et al. (2013b)
43.	<i>T. xanthomonosporum</i>	# YAAS L3185	China	KJ162154	Qing et al. (2015)
44.	<i>T. zhongdianense</i>	# wang0299	China	DQ898187	Chen and Liu (2007)
45.	[^] <i>T. melanosporum</i>	MelBc	France	AF300826	/
46.	[^] <i>T. melanosporum</i>	A51	Italy	AF106876	/
# Holotype.					
* New species described in this study are bold as indication.					
[^] Outgroup taxa					

Results

Taxonomy

Tuber lithocarpii **K.F. Wong & H.T. Li & sp. nov.** Figures 1 & 2

Etymology: Referring to the truffle found from roots of *Lithocarpus konishii* (Hayata) Hayata

ASCOCARP hypogeous, solitary, irregular, 15 mm long × 11 mm wide, 1.35 g in fresh weight, solid, cream white to light-brownish, smooth surface. *PERIDIUM* two-layered; inner layer 50–75 µm thick, hyaline, composed of intricately interwoven hyphae; outer layer 112.5–225 µm thick, light brownish, pseudoparenchymatous, composed of globose, subglobose, rod-shaped, polygonal or oval cells, 3.8–22.5 × 2.5–10 µm diam. Hyphae-like hairs arise from outermost cells, hyaline, septate, tapering towards the ends, 25–92.5 µm long, 2.5 µm diam. at the top and 2.5–3.75 µm diam. at the base. *GLEBA* light brown to reddish-brown, marbled with whitish veins, cavities found in gleba. *ASC*/ globose or ellipsoid, 50–112.5 × 37.5–87.5 µm, with stalk, 5 µm long, 5 µm diam., 1–3(–4)-spored. *ASCOSPORES* globose or subglobose, hyaline at first, becoming brown when mature, 20–30 × 20–30 µm (1-spored), 17.5–28 × 17.5–27.5 µm (2-spored), 17.5–28 × 15–27.5 µm (3-spored), 17.5–25 × 17.5–25 µm (4-spored), Q is 1–1.13 and Qm is 1.02 ± 0.05, wall 1.25–5 µm thick, ornamented with pentagonal or irregular reticulations, with meshes varying in size, 5–22.5 × 5–12.5 µm, 2–4(–5) across the ascospore length and 2–4 across the ascospore width.

Specimens examined : TAIWAN, Kaohsiung City, Maolin District, 10K Duona forest road, associated with roots of *Lithocarpus konishii* (Hayata) Hayata, 29 Jan 2018, collected by Chieh-Lung Lin and Yi-Hong Chen, DNW1 (holotype); ITS sequence: LC609779.

Notes: *Tuber lithocarpii* grows in montane area of southern Taiwan with elevation of 1113 m. Only one specimen was found under *L. konishii* tree in a 30-year *Taiwania cryptomerioides* Hayata plantation, at least 8 m apart from the nearest species like *Lithocarpus hancei* (Benth.) Rehder and *Castanopsis indica* (Roxb. ex Lindl.) A.DC. of family Fagaceae. Its surrounding soil is strongly acidic with pH value 4.5 and rich in organic matter content (7 %), but relatively low in potassium oxide, K₂O (81 kg/ha) and calcium oxide, CaO (1551 kg/ha).

Figure 1. *Tuber lithocarpii*. (a) The collection site and host *Lithocarpus konishii*. (b) Reddish-brown gleba with whitish veins. (c)-(d) The mature ascocarp with light-brownish and smooth surface.

Figure 2. Microscopic characteristics of *Tuber lithocarpii*. (a) Peridium and a cross section of pseudo-parenchymatous tissue of peridium. (b)-(c) cavities in peridium. (d) Hyaline and septate hyphae-like hairs arise from outermost cells. (e) Asci with stalk. (f) 3-ascospored asci. (g) 4-ascospored asci.

Phylogenetic analyses

The ITS-rDNA sequences matrix consisted of 46 sequences and 1662 aligned bases, of which 1171 bp were identified as poorly aligned and were excluded by Gblocks. The resultant ITS alignment was 491 bp. The MEGABLAST of National Center for Biotechnology Information (NCBI) queries in GenBank using the ITS sequence of *T. lithocarpii* did not result in fully or nearly complete matches. The top several hits that were identified to species were *T. thailandicum* at ITS (MW470969, query coverage = 98 %, identities = 94.6 %); *T. pseudosphaerosporum* L. Fan (NR153229, query coverage = 99 %, identities = 87.8 %). Pairwise comparisons (Table 2) showed that the similarities with other whitish truffles were in range of 79.6–94.2 %. As Maximum likelihood (ML) and Bayesian analysis yielded similar tree topologies of ITS region, thus the only tree generated from ML analysis is shown in Fig. 3.

Table 2

Pairwise comparisons in similarities (%) of ITS-rDNA sequences obtained from *Tuber lithocarpii* and other closely lineage whitish truffles in *Puberulum* clade.

	<i>T.a</i>	<i>T.li</i>	<i>T.e</i>	<i>T.e</i>	<i>T.la</i>	<i>T.pa</i>	<i>T.ps</i>	<i>T.t</i>
<i>Tuber alboumbilicum</i> (China, KJ742702)								
<i>Tuber lithocarpii</i> (Taiwan, LC609779)	82.6							
<i>Tuber elevatireticulatum</i> (Taiwan, MF540616)	79.8	80.9						
<i>Tuber elevatireticulatum</i> (Taiwan, MF540618)	79.8	80.9	100.0					
<i>Tuber latisporum</i> (China, DQ898183)	86.1	79.6	79.0	79.0				
<i>Tuber panzhihuanense</i> (China, JQ978648)	84.4	79.8	78.4	78.4	97.4			
<i>Tuber pseudosphaerosporum</i> (China, KF744063)	80.8	83.8	79.6	79.6	80.2	79.6		
<i>Tuber thailandicum</i> (Thailand, KP196328)	83.3	94.2	80.7	80.7	82.3	82.5	84.2	
<i>Tuber thailandicum</i> (Thailand, KP196329)	83.5	94.0	80.4	80.4	82.6	82.3	84.3	99.8

Figure 3. Phylogenetic tree of *Tuber lithocarpii* and related whitish truffles based on the ITS-rDNA sequences, with black truffle *T. melanosporum* as the outgroup. Numbers identify the bootstrap values and Bayesian posterior probabilities are indicated near branches as BS/PP. Values of BS and PP below 50% are not indicated. The sequence of new species described in this study is bold as indication.

Discussion

Morphologically, *Tuber lithocarpii* has distinct globose ascospores with large pentagonal or irregular marbled reticulated ornamentation. Comparing with another whitish truffle discovered in Taiwan, *T. elevatireticulatum* have thicker inner layer of peridium (i.e. 810 µm), and more ellipsoid ascospores with larger size (i.e. 50 µm, $Q_m = 1.30 \pm 0.19$) (Lin et al., 2018).

Comparing *T. lithocarpii* with the published Asian whitish truffles, both *T. japonicum* H.Sasaki, A. Kinoshita & Nara and *T. flavidosporum* H.Sasaki, A. Kinoshita & Nara found in Japan, have less ascospores in asci (i.e. 1–3 / 1–2 spores in each asci), larger ascospores size (i.e. 25–50/32–46 µm excluded reticulation) and more meshes across spore width (i.e. 4–6 / 3–4) (Kinoshita et al., 2016). Asian whitish truffle *T. thailandicum* Suwannar., Kumla & Lumyong from Thailand have larger ascomata size (i.e. 2–3.5 cm), darker gleba color (dark brown) and larger ascospores size (i.e. 15–65 µm excluded reticulation), when comparing with *T. lithocarpii* (Suwannarach et al., 2015). On the other hand, *T.*

huizeanum L. Fan & C.L. Hou, *T. latisporum* Juan Chen & P.G. Liu, *T. liui* A.S. Xu and *T. panzhihuanense* X.J. Deng & Y. Wang from China have larger ascomata size (i.e. >1.5 cm) (Chen and Liu, 2007; Deng et al., 2013; Li et al., 2012; Xu, 1999). Apart from the size, *T. panzhihuanense* were distinguishable by only one layer of peridium, darker gleba color (dark grey to black) and larger ellipsoid ascospores (i.e. 28–55×22–45 µm excluded reticulation, Qm = 1.18 ± 0.08). *T. liui* has larger ascospores (i.e. 24–65×40–94 µm excluded reticulation) and more meshes across spore width (i.e. 6–11). *T. huizeanum* do not hyphae-like hairs as *T. lithocarpii*. *T. latisporum* have black gleba and larger ellipsoid ascospores (i.e. 62–93×41–74 µm excluded reticulation, Qm = 1.21 ± 0.09)

Comparing *T. lithocarpii* with other world-wide published whitish truffles, *T. oregonense* Trappe, Bonito & P. Rawl. and *T. lyonii* Butters from the United State have similar morphology, but both of them have red to reddish-brown patches developed on peridium and more ellipsoid ascospores, which is distinguishable from *T. lithocarpii* (Bonito et al., 2010b; Hall et al., 2007). *T. lauryi* Trappe, Bonito & G. Guevara found in the United State have thicker peridium (i.e. 300–1000 µm) and more ellipsoid ascospores. *T. asa* Tul. & C. Tul. from Europe is different from its larger ascomata size (i.e. 2–3.5 cm), whitish gleba color and more number of spores in single asci (1–5) (Agnello & Kaounas, 2011).

Phylogenetic analysis revealed that *T. lithocarpii* formed a monophyletic clade with strong branching support (BS = 1, PP = 1). Bonito et al. (2013) identified 11 main clades within *Tuber*, and *T. lithocarpii* placed under clade 2 within *Puberulum* clade together with *T. latisporum*, *T. panzhihuanense*, *T. alboubilicum*, *T. elevatireticulatum*, *T. pseudosphaerosporum*, *T. thailandicum* with high PP (0.90) support, and formed a sister taxon to the Thai whitish truffles with strong branching support (BS = 1, PP = 1). The phylogenetic tree of ITS showed that *T. lithocarpii* and above mentioned whitish truffle are all come from Asia of Taiwan, Thailand and China might have high evolutionary relationship.

Declarations

Ethics approval and consent to participate : Not applicable

Consent for publication : Not applicable

Availability of data and materials Not applicable

Competing interests

The authors declare no competing interests. All the experiments undertaken in this study comply with the current laws of Taiwan.

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Authors' contributions

CLL, HTL, WWL and KFW collected, recorded and photographed the *Tuber* ascocarp, and all the authors prepared the manuscript. Both authors read and approved the final manuscript.

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Figures

Figure 1

Tuber lithocarpii. (a) The collection site and host *Lithocarpus konishii*. (b) Reddish-brown gleba with whitish veins. (c)-(d) The mature ascocarp with light-brownish and smooth surface.

Figure 2

Microscopic characteristics of *Tuber lithocarpii*. (a) Peridium and a cross section of pseudo-parenchymatous tissue of peridium. (b)-(c) cavities in peridium. (d) Hyaline and septate hyphae-like hairs arise from outermost cells. (e) Asci with stalk. (f) 3-ascospored asci. (g) 4-ascospored asci.

Figure 3

Phylogenetic tree of *Tuber lithocarpii* and related whitish truffles based on the ITS-rDNA sequences, with black truffle *T. melanosporum* as the outgroup. Numbers identify the bootstrap values and Bayesian posterior probabilities are indicated near branches as BS/PP. Values of BS and PP below 50% are not indicated. The sequence of new species described in this study is bold as indication.