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Research Article

Tuber Hudongense: A Critical Asian Truffle to Seek in Europe

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Abstract

Species belong to the genus Tuber (Pezizales, Ascomycota) are ectomycorrhizal (ECM) fungi that produce hypogeous fruiting establishing a symbiotic interaction with plant roots, mainly oaks and hazelnuts. Truffles are known as the gastronomically prized species, with a high market value, due to the expensive delicacies. Although these fungi are distributed worldwide, it has never been discovered in DPR Korea. In this study, we described about the first discovery of *Tuber huidongense* in DPR Korea. Based on the discovery of specimens of *Tuber huidongense* in DPR Korea which represents the first harvest of a true truffle in the country, a phylogenetic investigation was carried out to clarify the existence or otherwise of a complex of species around this truffle, hypothesized by various authors. A genetic analysis of all the ITS sequences available in public database was performed and it appeared very probable that two other species of Tuber (*T. furfuraceum* and *T. lannaense*) described in Far East Asia are later synonyms with *T. huidongense*. Therefore, their synonymization is proposed and the taxonomy of *T. huidongense* is clarified. From a geographical point of view, the species so far seems concentrated in South and East Asia. Its possible presence in Europe (Czech Republic) is considered doubtful to be confirmed.

Keywords

Phylogenesis, Taxonomy, Synonymy, Asian Truffles

1. Introduction

True truffles are the underground fruiting bodies (ascoma pl. ascomata) of species belonging to the genus *Tuber* (Ascomycota, Pezizales) which live in mycorrhizal or endophytic symbiosis with the roots of many plants, both shrubs and herbaceous plants [1]. As for all hypogeous fungi, i.e. fungi with underground fruiting bodies, the distribution of their spores is ensured by various animals (arthropods: mainly insects, molluscs, mammals but also birds and turtles) which identify the ripe ascomata thanks to the odors emanating, feed on them and with their feces they spread them over more or less vast territories [2]. Some species of *Tuber* which have been highly appreciated since ancient times have ascomata of considerable size and important effects on health and the humor and also provide extraordinarily attractive smell and

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taste to humans, becoming an icon of luxury foods [3].

The species of the genus Tuber which naturally occurred only in the northern hemisphere, are now present in forest, parks, gardens and in the specific truffle orchards of almost all continents except Antarctica, spread by several means. Especially, development of an indirect cultivation system made with planting of seedlings that their roots are inoculated with spores or mycelium of economically valuable truffles also hastened the wide spread of truffle species around the world [4, 5].

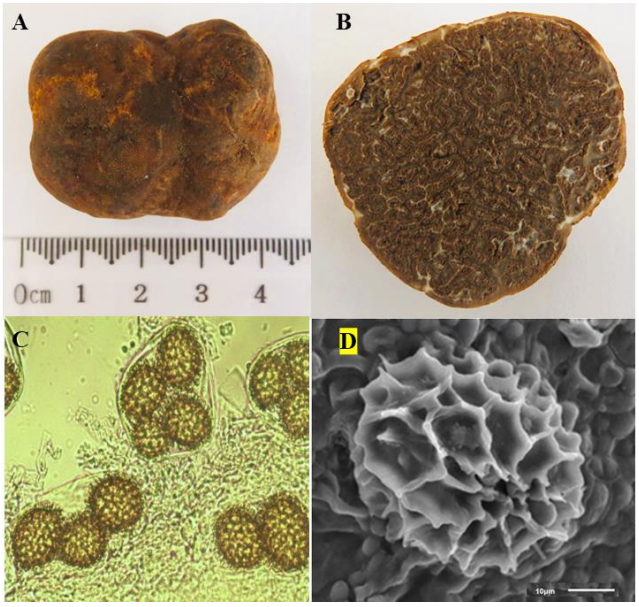

Figure 1. *Tuber huidongense Y. Wang. A) ascoma of T. huidonge; B) gleba; C) spores at optical microscope; D) spore at SEM.*

For a long time, true truffles were considered the prerogative of Europe, where they have been a sought-after food since ancient times and where the first studies took place, but for more than a century they have also been known from Asia and North-Central America, and in recent years these last two areas have been considered central in the study of the biodiversity of the genus. In Asia, the first indigenous truffle, *Tuber indicum* [6], was described from India. Among the specimens of this collection deposited in the Kew Herbarium, subsequently, Zhang & Minter (1988) [7] distinguished *T. himalayense*. Some other species of *Tuber* have occasionally been added to the check-list of Asian mushrooms and the new reports have become increasingly frequent, on a morphological basis between the last decade of the 20th century and the first years of the new millennium. A great boost to knowledge in this field has been provided by genetic analyses, and the results place the eastern area of the Asian continent as one of the three main hotspots of biodiversity for the genus *Tuber*. However, in this great influx of new data, some taxonomy issues have arisen. One of these concerns *Tuber huidongense* Y. Wang [8], described based on morphology but with the type never officially characterized at the molecular level. Around this species, belonging to the clade Rufum, other taxa have been described subsequently, leading to the possibility of referring to a Huidongense complex [9]. What was the first finding of a true truffle in the DPR Korea, identified as *Tuber huidongense* gave rise to this taxonomic and biogeographical clarification on this species. Our aim was to characterize at the molecular level and to clarity taxonomically the true truffle first found in the DPR Korea.

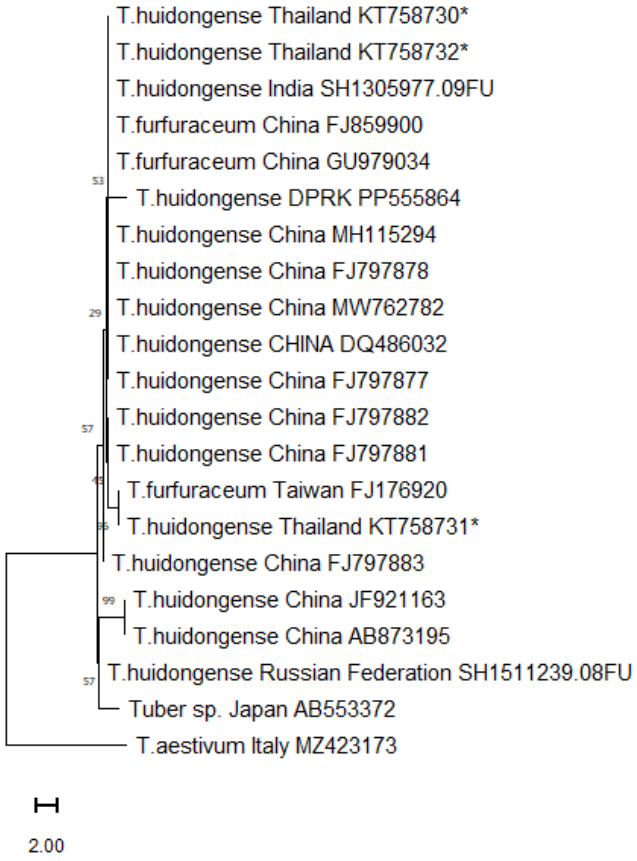

Figure 2. *Maximum likelihood tree obtained from the alignment of ITS nuclear rDNA region sequences. Maximum likelihood phylogenetic analysis was inferred from the ITS nrDNA sequences of Tuber huidongense and similar species (T. furfuraceum and T. lannaense) retrieved from GenBank and UNITE were included in Table 1. Type sequences underlined (KT758730 T. lannaense FJ797882 still listed as T. huidongense in GenBank). The asterisked sequences from Thailand now belong to T. lannaense. Tuber aestivum was included as an outgroup.*

2. Materials and Methods

2.1. Specimens

The truffles were found in a northwest larch, *Larix kaempferi* Lambert 1824 [=*Larix leptolepis* (Sieb et Zucc.) Gord.] forest (38 ° 53 ℕ, 126 ° 32 ℇ, altitude 330m) on September 30th, 2022. Specimens are preserved in the Central

Institute of Mushroom (DPR Korea). Its identification took place on the morphological and molecular levels.

2.2. Morphological Analyses

Microscopic characters of spores, asci, and peridium were examined on hand-made sections or squash preparations obtained from a specimen voucher, using fluorescence microscope (Olympus, CX43, Japan), camera (Cannon, China) and SEM (Jeol, JSM-6610A, Japan). Sample was rehydrated for 10 min in 20% KOH, rinsed with sterile water, and then soaked with 3% KOH, following the procedure described by Leonardi et al. (2019) [10].

2.3. Molecular Analysis

2.3.1. DNA Extraction, Amplification, and Sequencing

Genomic DNA from fungal tissue was extracted using a CTAB method according to the manufacturer's protocol. Final DNA elution was performed in a volume of 100 µl elution buffer. The quality of the isolated material was determined by a UV–VIS NanoDrop One spectrophotometer or by 1% agarose gel electrophoresis. The obtained isolates were stored at −20 ℃ until further analysis steps.

2.3.2. Amplification of DNA

For optimization of target ITS rDNA fragment amplification, we used the primer couple ITS1F (5′- CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC) [11]. For each PCR run performed, a negative control (control of reagent contamination) and a positive control with 100 ng of high-quality genomic DNA of *Pleurotus* sp. were always additionally made. The PCR was run under the following conditions: initial denaturation 95 ℃ for 15 min, denaturation 95 ℃ for 20 s, primer annealing 55 ℃ for 40 s, elongation 72 ℃ for 1 min and final elongation 72 ℃ for 10 min. The number of cycles for the reaction was 35. Each PCR was performed in a volume of 25 µl, with the following composition: 13 µl of 2×PCR master mix (Sangon, China), 1 µl (100 µM) of each primer, 9 µl of ddH_2O, 1 µl of extracted DNA. Amplification products were visualized electrophoretically on a 1.5% agarose gel with the addition of a Green DNA Gel Stain (Sangon, China).

2.3.3. Sequence Alignment and Phylogenetic Analysis

The purified PCR products were directly sequenced by ABI 3500 genetic analyzer (Thermofisher, U.S.A.) using the PCR primers mentioned above. The sequences were assembled and edited and used to query GeneBank via BLAST. For the phylogenetic analysis 21 nucleotide sequences were obtained from GenBank database in this study (Table 1). The multiple sequence alignment was carried out using MAFFT7, using the E-INS-i iterative refinement algorithm [23]. The final data set contained a total of 519 positions. A maximum likelihood (ML) phylogenetic tree was constructed using MEGA11, and bootstrapping algorithm for 1000 replications using the Kimura 2-parameter model with a Gamma distribution of rates among sites [24]. *T. aestivum* epitype sequence was used as outgroup.

Table 1. *Accession numbers from GenBank [12] and UNITE [13] and collection localities of the specimens analysed in this study. The asterisks indicated as a new species with the name of Tuber lannaense.*

Species	Accession number	Origin	Location	Reference
T. huidongense	FJ797882	ascoma	CHINA, Sichuan, Huidong	[14]
T. huidongense	FJ797878	ascoma	CHINA, Yunnan, Kunming	[14]
T. huidongense	FJ797881	ascoma	CHINA, Sichuan, Huidong	[14]
T. huidongense	FJ797883		CHINA, Sichuan, Huili	[14]
T. huidongense	FJ797877		CHINA, Yunnan, Xiangyun	[14]
T. huidongense	DQ486032	ascoma	CHINA, Sichuan, Panzhihua	[8]
T. huidongense	MH115294	ascoma	CHINA, Yunnan, Kunming	[15]
T. huidongense	JF921163	ascoma	CHINA	Fan unpublished
T. huidongense	AB873195	ECM	CHINA, Tangshan, Nanjing, Jiangsu	Zong et al. unpublished
T. huidongense	MW762782	ascoma	CHINA, Yunnan, Huize	Li & Zhao unpublished
T. huidongense	KT758731*	ascoma	THAILAND	[16] as T. lannaense
T. huidongense	KT 758732*	ascoma	THAILAND	[16] as T. lannaense

Species	Accession number	Origin	Location	Reference
T. huidongense	KT758730*	ascoma	THAILAND	[16] as T. lannaense
T. huidongense	SH1511239.08FU	soil	RUSSIAN FEDERATION, Primorsky Krai	[17]
T. huidongense	SH1305977.09FU	soil	INDIA, Dehradun	[18]
T. huidongense	PP555864	ascoma	DPR KOREA,	This study
Tuber sp.3	AB553372	ascoma	JAPAN	[19]
T. furfuraceum	FJ859900	ascoma	CHINA	[14]
T. furfuraceum	GU979034	ascoma	CHINA	[20]
T. furfuraceum	FJ176920	ascoma	TAIWAN	[21]
T. aestivum	MZ423173	ascoma	ITALY	[22]

3. Results

3.1. Taxonomy and Phylogenesis

The ITS sequence obtained and the compatibility of its morphological characters allowed the collection to be identified as the one belonging to the Tuber huidongense Y. Wang species (Figure 1, Figure 2). We observed a low intraspecific variability (overall average p-distance = 0.0064) in ITS1-5.8S-ITS2 rDNA region sequences in the different sequences of T. huidongense from South, Southern-East and East Asia. The genetic distance of truffle discovered in DPR Korea with the FJ797882 sequence, considered by us the most reliable to represent the reference barcode of this species, has an average of 0.0232 ±0.0058, in line with the average of all available ITS sequences which is 0.0231 ±0.0082, varying from a minimum of 0.000 for three OTUs [the sequences FJ859900, GU979034 (T. furfuraceum) and KT758730 (T. lannaense)] aligned with FJ176920 (T. furfuraceum) and KT758731 (T. lannaense) respectively to a maximum of 0.03741 (China, MH115294), with those of T. furfuraceum, both from Taiwan (FJ176920) and from the continental China (FJ859900, GU979034) placed at values of 0.02353 and those attributable to T. lannaense have values between 0.025553 and 0.02752. While the genetic distance of the Tuber aestivum outgroup is 0.39526 (average distance 0.40585 ±0.00587).

3.2. Geographical Distribution

Considered an endemic species of China [8, 14] actually from the sequences recovered from public gene banks and from the literature, it presents a hitherto apparently disjointed range that affects many countries of East and South-East Asia, including large islands, and has also been reported as ECM fungi in Central Europe, Czekia [25].

4. Discussion

We showed the specimens of real *Tuber* first found in DPR Korea, which was clarified as *T. huidongense* by the genetic analysis of all the ITS sequences. The type of *Tuber huidongense* (IFS89923) has not been officially sequenced, however, it can be reasonably assumed that the sequence FJ797882 (Table 1), from a specimen collected in the same place of holotype (IFS89923), can represent an authoritative reference [14]. The LSU (KU207731, KU207732, KU207733) and ITS1+ITS2 (KT758730, KT758731, KT758732) sequences first and still recorded as "*Tuber huidongense*" were then used by Suwannarach et al. (2016) [16] to describe the new species *Tuber lannaense* N. Suwannarach & S. Lumyong although blast results with those of *T. huidongense* shows the similarity of 99.81% to 99.19% and 98.80% with *T. furfuraceum*. These data are also confirmed by the phylogenetic tree published [16] for LSU and also for that relating to ITS1+ITS2 where the sequences labeled as *T. lannaense* (KT785730, KT 785731, KT785732) actually belong to "Uncultured bacterium clone OTU1156 16S ribosomal RNA gene" [26]. There was evidently an error of transcription of the actual accession numbers (KT758730, KT758731, KT758732).

The species could also be found in Europe, in fact Grydler et al. (2017) [25] in a survey by sequencing β-tubulin gene from tree roots collected at 322 field sites across the Czech Republic found 8 ectomycorrhiza (OTUs: KX303510, KX303511, KX303533, KX303560, KX303576, KX303578, KX303585, KX303587) with a mean similarity with the GenBank hit of 96.24 ± 0.76%. However, by blasting these Czech sequences with the Asian ones we detected a significantly lower percentage of similarity. Only the harvesting of ascomata of this Czech taxon will be able to clarify its real taxonomy.

5. Taxonomy

Based on both the morphology and genetic similarity of the ITS alone, we propose that the species Tuber huidongense should also include T. furfuraceum and T. lannaense as later synonyms:

Tuber huidongense Y. Wang, Mycotaxon 83: 191 (2002) [8].

= *Tuber furfuraceum* H. T. Hu & Y. Wang, Mycotaxon 93: 155 (2005) [27].

=*Tuber lannaense* N. Suwannarach & S. Lumyong, Mycological Progress 15 (8): 830 (2016) [16].

Therefore the hypothesis of a Huidongense complex may not have any reason to exist, at least from the analysis of the ITS sequences.

Abbreviations

ECM Ectomycorrhizal

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Author Contributions

Su Gyong Jo: Conceptualization, Supervision
Yong Nam Kim: Supervision
Kuk Chol Ri: Methodology
Kwang Myong Kim: Methodology
Kuk Chol Jo: Writing-original Draft Preparation
Il Gwang Yun: Investigation
Jong Hyok Pak: Validation
In Chol Kye: Methodology
Hui Won Kim: Writing-review and Editing
All authors read and approved the final manuscript

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Consent to Publish declaration

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Ethics declaration

Not applicable.

Conflicts of Interest

The authors declare no conflict of interest.

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