

# Molecular and morphological data confirmed the presence of the rare species *Mattirolomyces terfezioides* in China



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## ABSTRACT

Although the species *Mattirolomyces terfezioides* ( $\equiv$  *Terfezia terfezioides*) has been recorded from China several times but it is really rare taxon with important ecological and economic value, the conspecificity with European material has never been tested by molecular data. We re-examined three specimens labelled as *T. terfezioides*, one as *T. leonis* and one as *Terfezia* sp. in the herbarium HMAS and obtained five ITS and three LSU sequences. Our morphological observation and DNA sequences show that one specimen (HMAS 83766) labelled as *M. terfezioides* turns out to be *Choiromyces* sp. and the other four are *M. terfezioides*. The ITS and (or) LSU sequences of the Chinese samples are identical with or with 99% similarity to those from the European samples, which fully confirms the presence of *M. terfezioides* in China. The species is currently known from northern China (Hebei Province, Beijing and Shanxi Province). This study shows that *M. terfezioides* has a Euroasia distribution other than European endemism and such distribution might be explained by the co-occurrence with the potential host tree *Robinia pseudoacacia*.

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## 1. Introduction

*Mattirolomyces terfezioides* (Mattir.) E. Fisch., the type species of *Mattirolomyces* E. Fisch. (Pezizaceae, Pezizales), is one of the truffle species repeatedly documented in taxonomic and phylogenetic literatures (Kagan-Zur et al., 2014). It was originally described from Northern Italy by Mattiolo (1887) in the genus *Choiromyces* Vittad. Fischer (1938) erected a monotypic genus *Mattirolomyces* using *Choiromyces terfezioides* Mattir. as the type. Trappe (1971) transferred *Mattirolomyces* to *Terfezia* (Tul. and C. Tul.) Tul. and C. Tul. based on the overlapping characters between some species of *Terfezia* and *Mattirolomyces*. Molecular phylogenetic analyses, however, supported *Mattirolomyces* to be a separate genus from *Terfezia* within the same family, Pezizaceae (Hansen et al., 2001; Læssøe and Hansen, 2007; Percudani et al., 1999), thus making the name *M. terfezioides* has been fixed since then. Unlike *Terfezia* species (desert truffle), which are mostly found in arid to semi-arid sandy environments in Mediterranean region and form mycorrhizae with herbaceous species of Cistaceae (Díez et al., 2002),

*M. terfezioides* is often found under artificially planted trees [e.g. *Robinia pseudoacacia* L., *Diospyros kaki* Thunb. and *Prunus avium* (L.) L.] in southern and central Europe and its mycorrhizal status is not clearly answered up to now.

Among the five known species of *Mattirolomyces*, *M. terfezioides* and *Mattirolomyces spinosus* (Harkn.) Kovács et al. have been recorded from China. *M. spinosus* is only listed by Tai (1979), whereas *M. terfezioides* is one of the mostly documented true truffles in the country (Alsheikh, 1994; Liu, 1991; Liu and Guo, 1984; Liu and Tao, 1989; Liu et al., 2002; Zhang, 1990) and enumerated as one of the Chinese edible fungi (Dai et al., 2010). These records, however, has never been tested with DNA sequence data. Alsheikh (1994) observed a specimen collected from Beijing (HMAS 32656). The identity of this specimen, however, was left as an open issue by Kovács and Trappe (2014) when they said “it (*Mattirolomyces*) includes five species ... from four continents (or five, if we consider the Beijing urban collection of *M. terfezioides* as well...”. Moreover, Kovács and Trappe (2014) found that most of the Chinese desert truffles are misidentified. In such scenario, as well as based on a assumption that truffles normally have a less efficient dispersal ability (Trappe and Claridge, 2005) that will result in relatively narrow distribution (Bonito et al., 2010), it is natural to question if the Chinese specimens labelled as *M. terfezioides* could be conspecific with authentic (European) *M. terfezioides*. Aiming to

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answer this question, we re-examined five historical specimens (possibly) related with *M. terzeioides* in HMAS and amplified the ITS and LSU regions for them. The results are reported herein.

## 2. Materials and methods

### 2.1. Materials

Five specimens under *Terfezia* (where *M. terzeioides* has long been placed) deposited in HMAS were studied. Three of them were labelled as *Terfezia terzeioides*, one as *Terfezia leonis* and one as *Terfezia* sp. This sampling includes a specimen collected from Shanxi Province in October, 1983 (HMAS 76805). Since many specimens have been transferred from the Mycological Herbarium of Shanxi University to HMAS and this specimen meets the date and locality of the specimen cited by Liu and Guo (1984), we believe this specimen presents the voucher that Liu and Guo (1984) used to the report *Terfezia eonis* [later corrected to *T. terzeioides* by Liu and Tao (1989) and Liu (1991)] in China. HMAS 32656, HMAS 60273, HMAS 76805 and HMAS 88581 were described as *T. terzeioides* by Zhang (1990). The specimen (HMAS 32656) labelled as *T. leonis* was cited by Alsheikh (1994) under *M. terzeioides*. This is the only specimen under the name *T. leonis* collected before 1963, and we believe this is the voucher of *T. leonis* in Teng (1963). The specimen labelled as *Terfezia* sp. (HMAS 83766) was cited as a desert truffle record in China by Kovács and Trappe (2014).

### 2.2. Morphological observation

Macroscopic observations are based on dried specimens. Microscopy mainly followed Kovács et al. (2011). Dried ascomata were sectioned with a stainless razor blade. Slides were made by mounting the tissue in 5% or 10% KOH. Micro-morphological features observed included shape and size of ascus, number of ascospore in mature asci, size, shape and surface ornamentation of mature ascospores. Slides were observed under a Leica DM2500 stereoscope and photographed with a Leica DFC450C camera installed in it. Thirty spores that came from different asci or

dissociate outside the asci were measured from mature ascoma. Reactions were tested using Melzer's reagent and Cotton Blue.

### 2.3. DNA extraction, PCR and phylogenetic analyses

Total DNA was extracted from dried gleba with a modified CTAB protocol (Doyle and Doyle, 1987). Since the most samples are rather old (up to 54 years old), an extra purification step was performed for the extracted DNA using GeneClean® II Kit (MP Biomedicals), according to the manufacturer's instructions. The primer pairs ITS5 + ITS4 (or ITS1 + ITS4) and LROR + LR5 were used to amplify the ITS region and part of the 28S respectively (White et al., 1990; R. Vilgalys lab, <http://www.biology.duke.edu/fungi/mycolab/primers.htm>). PCR amplification was performed with Takara® DNA polymerase (Dalian, China) using the following protocol (25 µl reaction mixture): 2.5 µl buffer, 2.5 µl 0.1% BSA, 2 µl 2.5 mM dNTPs, 0.5 µl 10 µM of forward and reverse primers, 0.2 µl 5 U/µl Taq polymerase, 5 µl total DNA solution, and 12 µl ddH<sub>2</sub>O. The following PCR programs were used: 5 min at 94 °C, 38 cycles of 1 min at 94 °C, 1 min at 58 °C and 1 min 30 s at 72 °C, and a final extension of 72 °C for 10 min. For two samples with problem to get the whole ITS region, HMAS 76805 and HMAS 88581, internal primers ITS2 and 5.8SR were used with ITS1 and ITS4 respectively to amplify the ITS-1 and ITS-2 regions separately. Cycling parameters for the two short regions were set as: an initial denaturalization step for 5 min at 94 °C, 38 cycles consisting of 30 s at 94 °C, 30 s at 60 °C, and 50 s at 72 °C, and a final extension at 72 °C for 7 min. The PCR products, pre-judged by gel electrophoresis were purified and sequenced at Sangon Biotech Corporation, Shanghai, China. Sequences were deposited in GenBank with accession numbers in Table 1.

DNA sequences were assembled in Sequencher 4.1.4 (Gene Codes Corp., Ann Arbor, MI). The obtained sequences were firstly submitted to the Nucleotide Basic Local Alignment Search Tool (BLAST) to find sequences with high homology. For *M. terzeioides* samples, 17 ITS sequences and six LSU sequences with 97–100% similarity were retrieved from GenBank. Duplicate sequences with identical characters were removed if they have the same

**Table 1**  
Specimens used for comparison on DNA sequences and phylogenetic analyses in this study. Sequences generated by this study are in bold.

Species	Voucher	Locality	Collector and date	GenBank No.	
				ITS	LSU
<i>Elderia avenivaga</i>	OSC 111751	Australia	R. Helms, 1891	GQ231733	GQ231734
<i>Elderia avenivaga</i>	OSC 111641	Australia	D. Albrecht, 2000	GQ231736	GQ231737
<i>Mattiolomyces austroafricanus</i>	OSC 58845	South Africa	E. L. Stephens	GQ231752	GQ231753
<i>Mattiolomyces mexicanus</i>	OSC 131669	Mexico	J. Muñoz, 1980.07.08	HQ660378	HQ660379
<i>M. mulpu</i>	OSC 131319	Australia	E. Mantajara, 1983.05.26	GQ231739	GQ231740
<i>M. spinosus</i>	Ellis & Everhart 1782	USA	E. Forges, 1886.11	HQ660381	HQ660382
<i>M. spinosus</i>	CUP 56967	Pakistan	S. Ahmed, 1949.08	HQ660384	HQ660385
<i>M. terzeioides</i> (labelled as <i>T. leonis</i> )	HMAS 32656	China: Beijing	D.L. Guo & H.Z. Li, 1961.09.20	<b>KT963175</b>	<b>KT963180</b>
<i>M. terzeioides</i>	HMAS 60273	China: Hebei Province	Z.J. He & Z.J. Han, 1986	<b>KT963177</b>	<b>KT963179</b>
<i>M. terzeioides</i>	HMAS 76805	China: Shanxi Province	S.X. Guo, 1983.10.17	<b>KT963176</b>	—
<i>M. terzeioides</i> (labelled as <i>Terfezia</i> sp.)	HMAS 88581	China: Shanxi Province	S.X. Guo, 1984.05	<b>KT963178</b>	—
<i>Choiromyces</i> sp. (labelled as <i>M. terzeioides</i> )	HMAS 83766	China: Heilongjiang Province	J.X. Zhuang, 2001	<b>KU531609</b>	<b>KT531618</b>
<i>M. terzeioides</i>	Trappe 4548	France	L. Rioussat, 1974.11.02	GQ231754	—
<i>M. terzeioides</i>	MA 8212	Spain	1984.08.30	GQ422438	—
<i>M. terzeioides</i>	Bratek 1131	Hungary	Z. Bratek, 1996.11.13	AJ272445	—
<i>M. terzeioides</i>	Bratek 1873	Hungary	Z. Bratek, 1998.10.15	AJ305045	—
<i>M. terzeioides</i>	Bratek 2197	Hungary	Z. Bratek, 1991.09.10	AJ272443	—
<i>M. terzeioides</i>	KMG 10125_4	Hungary	G.M. Kovács, 1999.08.30	AJ305169	—
<i>M. terzeioides</i>	Rob 01	Hungary	J. Díez	AF276680	—
<i>M. terzeioides</i>	Rib 02	Hungary	J. Díez	AF276681	—
<i>M. terzeioides</i>	environmental sample	Hungary	—	AJ875015	—
<i>M. terzeioides</i>	environmental sample	Hungary	—	AJ875016	—
<i>M. terzeioides</i>	KMG 10124	Italy	G.M. Kovács, 1995.12.02	AJ305170	—
<i>M. terzeioides</i>	17086	Italy	A. Montecchi, 1989.10.10	JF908728	—
<i>M. terzeioides</i>	KFRI 2829	South Korea	—	KT025693	—

biogeographic origin or of the same material type (azenic culture, environmental samples or ascomata). The 13 sequences left, the four *Mattirolomyces* ITS sequences obtained in this study, and five ITS sequences of *Mattirolomyces austroafricanus*, *Mattirolomyces mexicanus*, *Mattirolomyces mulpu* and *M. spinosus* published by Kovács et al. (2011) were used to conduct the phylogenetic analyses. *Elderia arenivaga*, which is shown to be the closest relative of *Mattirolomyces* by Trappe et al. (2010) and Kovács et al. (2011) was used as outgroup.

Alignments were made using the online version of the multiple sequence alignment program MAFFT v7 (Kato and Toh, 2008), applying the L-INS-I strategy and manually adjusted in BioEdit Version 5.0.9 (Hall, 1999). Maximum Likelihood (ML) and Bayesian Inference (BI) analyses were performed to find the placement of the Chinese samples in the ITS phylogeny of *Mattirolomyces*. ML analysis was conducted in RAxML v7.2.6 (Stamatakis, 2006) and BI in MrBayes v3.2.1 (Ronquist et al., 2012). ML analyses applied the Rapid Bootstrapping algorithm with 1000 replicates, followed by a ML tree search. In the BI analysis, the GTR + I + G model was used and all parameter values, except branch lengths and tree topologies, were set unlinked. The BI analyses were conducted using two

runs with four chains each for  $1 \times 10^7$  generations sampling every 100th tree. A majority rule consensus tree was built after discarding trees from a 25% burning. Trees generated by the two analyses were viewed and then exported as PDF in FigTree v1.3.1.

### 3. Results

#### 3.1. Sequences comparison and molecular phylogenetic analyses

We produced five ITS and three LSU sequences from the five specimens sampled. By BLAST, we found that the ITS and LSU sequence of the specimen HMAS 83766 (KU531609 and KU531618) has 99% similarity with ITS sequence of *Choiromyces* sp. (KP019343) and ten LSU sequences of *Choiromyces* sp. (represented by KP019354, KP019355, KP019356). For the other four samples, we got 17 hits of ITS sequences with 97–100% similarity and six hits of LSU sequences with 98–99% similarity. All the retrieved ITS sequences are labelled as *M. terfezioides* and the six LSU sequences belong to *Mattirolomyces*. Compared with the retrieved ITS sequences, two Chinese samples (HMAS 32656 and HMAS 60273, with complete ITS sequences) have identical ITS



**Fig. 1.** Maximum Likelihood (ML) phylogram of *Mattirolomyces* and based on the ITS region, rooted with *Elderia arenivaga*. ML Bootstrap proportions higher than 70% and posterior probabilities from the Bayesian Inference analysis higher than 0.95 are indicated above and below the branches respectively. Samples are provided with GenBank accessions. Sequences generated in this study are in bold. Samples marked with "\*" are collected under or from the roots of *Robinia pseudoacacia*.

sequences with three Hungary samples (Bratek2197 and two environmental samples with GenBank numbers AJ875015 and AJ875016). We only successfully amplified the ITS-1 and part of the 5.8S regions for HMAS 76805 and HMAS 88581. The two short sequences have one specific change compared with the other sequences of *M. terfezioides*. The two LSU sequences of the Chinese samples (KT963179 from HMAS 60273 and KT963180 from HMAS 32656) have one specific change compared with the only available LSU sequence of *M. terfezioides* from a European sample (Trappe4548). One Chinese sample (HMAS 76805) and three Hungarian samples were collected under or from the root of black locust (*R. pseudoacacia*) (Fig. 1).

In the ITS phylogeny, our four Chinese samples formed a highly supported clade with 11 European samples and one South Korea sample (BI-PP = 1.00, ML-BP = 100%). These European samples are from four countries. There is neither clear genetic nor geographic structure within the clade of *M. terfezioides*. Similar to the results of Kovács et al. (2011), the *M. terfezioides* clade is the earliest divergent clade within *Mattirolomyces*.

### 3.2. Morphological observation

*M. terfezioides* (Mattir.) E. Fisch., In Fischer In Engler A. & Prantl K. Nat. Pfl. Ed.: 39 (1938).

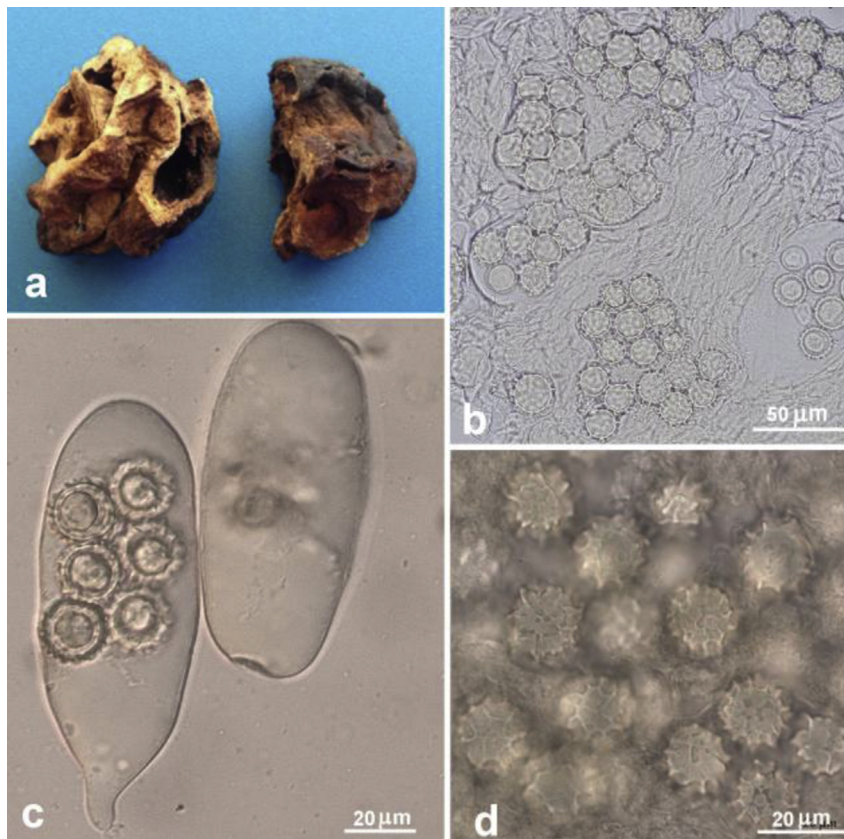
≡ *Choiromyces terfezioides* Mattir., Mém. R. Accad. Sci. Torino, Ser. 2 37: 10 (1887).

≡ *Terfezia terfezioides* (Mattir.) Trappe, Trans. Br. mycol. Soc. 57(1): 91 (1971).

Ascomata (dry specimens, Fig. 2a) hypogeous or subhypogeous, 1.5–3 (–8) cm in diam., subglobose to irregular massy, whitish-yellow to yellow brown, fragile, surface smooth to scabrous, lobed, furrowed or wrinkled. Gleba subsolid, spongy with minute pockets, yellow to yellowish brown, some ascomata with narrow, white to pale yellow veins. Taste and odor sweet when fresh based on record.

Paraphyses absent. Peridium 160–310 μm thick, no clear differentiation from the gleba, composed of inflated hyphae and irregular, hyaline or pale yellowish cells 9–19 μm broad, often collapsing in maturity. Gleba composed of interwoven septate hyphae 2–8 μm broad, with some free hyphal ends. Asci (Fig. 2b and c) randomly arranged in gleba, 10 or (2–) 8-spored, hyaline, globose to ellipsoid, pockety, saccate, cylindrical or clavate, (40–) 55–110 (–130) × (20–) 35–60 (–70) μm, sessile or occasionally sub-stipitate with a short stalk, disintegrating with age, thin-walled, readily separable from glebal hyphae, in youth sometimes the spores clustered in the tip of the ascus, later migrating to the middle, biseriate or irregularly arranged, nonamyloid. Ascospores (Fig. 2b–d) hyaline to pale yellow, globose, (11) 13–19 (–21) μm in diam. excluding the ornamentation (120 spores from four specimens measured); ornamentation of blunt spines connected in an irregular alveolate reticulum 1–4 μm high, mostly have a de Bary bubble and uniguttulate; walls 1–1.5 μm thick, dark yellow to yellowish brown in Melzer's, light blue in Cotton Blue.

Specimens examined: CHINA. Beijing, Luodaozhuang, 1961.9.20, leg. D.L. Guo and H.Z. Li, HMAS 32656; Hebei Province, Wanxian, 1986, leg. Z.J. He and Z.J. Han, HMAS 60273; Shanxi Province,



a. Ascomata; b, c. Asci and ascospores; d. ascospores

Fig. 2. *Mattirolomyces terfezioides*.

Taiyuan, 1983.10.17, leg. S.X. Guo, HMAS 76805 (MHSU 1457); Shanxi Province, Taiyuan, 1984.5, leg. S.X. Guo, HMAS 88581 (MHSU 1458).

#### 4. Discussion

The typical characters of *M. terfezioides* include the whitish to yellowish brown ascomata with subsolid whitish to yellowish gleba with minute pockets asci and globose ascospores with blunt spines connected in an irregular alveolate reticulum 1–4 (–5)  $\mu\text{m}$  high. The four Chinese specimens that were confirmed to be conspecific with European *M. terfezioides* by ITS and LSU data match the morphological descriptions of *T. terfezioides* given by Babos (1981), Király and Bratek (1992), Ławrynowicz et al. (1997), and Alsheikh (1994). Among the other four known species of *Mattirolomyces*, *M. spinosus* is highly similar to *M. terfezioides* (Alsheikh, 1994) and distinguishing the two species has to rely on DNA sequences (Kovács et al., 2011).

Up to now, there are two molecular evidences convincing the presence of *M. terfezioides* in Asia: our data in this study and the GenBank sequences KT025693 from South Korean sample. Alsheikh (1994) cited a specimen from Pakistan under *M. terfezioides* from Pakistan, but this specimen was found to be *M. spinosus* by Kovács et al. (2011) with ITS and LSU sequences. The confirmed conspecificity of the Chinese specimens with European material might be due to shared host. Among our specimens, HMAS 76805 was collected under *R. pseudoacacia*. *M. terfezioides* has been reported to be associated with *R. pseudoacacia* or grow in (mixed) *R. pseudoacacia* forest many times [Bratek et al., 1996; Díez et al., 2002; Montecchi and Lazzari, 1993, and literatures cited by Kovács et al. (2003)]. In our ITS dataset, four samples of *M. terfezioides* are related with *R. pseudoacacia* (Fig. 1). Although Kovács et al. (2003) did not confirm the *M. terfezioides*-*R. pseudoacacia* interaction to be real mycorrhiza, they did find that the root cells of *R. pseudoacacia* could be colonized by the hyphae of *M. terfezioides* or the septate hyphal coils are similar to the endogenous structure formed by *M. terfezioides* (Kovács and Bagi, 2001, Kovács et al., 2007). Given the frequent co-occurrence of *M. terfezioides* with *R. pseudoacacia*, even if they do not form real well-defined mycorrhizae, their internal interaction cannot be excluded. The co-occurrence of *M. terfezioides* with *R. pseudoacacia* in Northern China will add new evidences in understanding the ecological habit and distribution of this edible truffle.

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