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Full Length Article

Morpho-Anatomical Characterization and Phylogenetic Analysis of Five *Tomentella* Ectomycorrhizae from Leigong Mountain, Guizhou

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Abstract

Mycorrhizal specimens were collected from *Castanopsis fargesii*, *Betula luminifera*, *Pinus massoniana* and *Quercus acutissima* in the Leigong Mountain Nature Reserve and five brown ectomycorrhizal morphotypes were identified from the samples. An analysis of morphological and anatomical characteristics indicated that all five morphotypes had the morphological characteristics of *Tomentella* ectomycorrhizae. rDNA ITS segment phylogenetic analysis indicated that these five morphotypes consisted of ectomycorrhizae formed by *Tomentella* fungi. Phylogenetic analysis of rDNA ITS showed that four of them were *Tomentella ellisii*, *T. fuscocinerea*, *T. bryophila* and *T. stuposa*. The results of this study fill gaps in research on ectomycorrhizal fungi of the genus *Tomentella* in the Leigong Mountain Nature Reserve. © 2019 Friends Science Publishers

Keywords: *Tomentella*; Ectomycorrhizae; Morphological characteristics; Phylogenetic analysis

Introduction

Tomentella Pers. ex Pat belongs to the family Thelephoraceae, order Thelephorales, class Agaricomycetes, phylum Basidiomycota. *Tomentella* fungi are ectomycorrhizal on trees in the families Pinaceae, Fagaceae, Betulaceae and Salicaceae, as well as on some herbaceous and shrubby plants (Bidartondo and Bruns, 2001; Selosse *et al*., 2002; Mccormick *et al.*, 2004; Julou *et al*., 2005). *Tomentella* ectomycorrhizae range from brown to dark brown and have polygonal outer mantle layer cells, emanating hyphae, cystidium or rhizomorphs and often have clamp connections (Nylander, 2004; Jakucs *et al*., 2005; Walker *et al.*, 2005; Agerer, 2006; Jakucs and Erös-Honti, 2008). *Tomentella* is very common and plays a dominant role in the coniferous and deciduous broadleaf forests comprising north temperate, temperate, and tropical forest ecosystems (Huelsenbeck and Ronquist, 2005; Smith *et al.*, 2009; Wei and Agerer, 2010).

Much of domestic research on ectomycorrhizae has focused on the genus *Tomentella* and nearly 20 ectomycorrhizal morphotypes have been described in detail. Molecular identification was conducted in this study by sending approximately 3,000 fruiting body and mycorrhizal sequences from fungi of the genus *Tomentella* to the GenBank database. While domestic research on *Tomentella* ectomycorrhizae got off to a late start, six species of *Tomentella* mycorrhizal fungi were discovered and described on *Pinus yunnanensis* seedlings in 2011 (Xie and Liu, 2011). *Tomentella* mycorrhizal fungi were discovered through ectomycorrhizal morphotype and molecular identification research on *Picea crassifolia* (Fan *et al*., 2011). Five types of *Tomentella* ectomycorrhizae were reported on the morphology and structural characteristics of *P. tabuliformis* in 2013 (Wang and Guo, 2013). *Tomentella* ectomycorrhizae were discovered in key tree species with regional distributions in Inner Mongolia, including *Larix gmelinii*, *P. tabuliformis*, *Betula platyphylla*, *Picea mongolica*, *Populus tremula* and *P. sylvestris* (Wei *et al.*, 2015, 2017). This genus of fungi is widespread and plentiful and that a need existed for in-depth research on the ectomycorrhizae formed by *Tomentella* fungi.

Leigong Mountain Nature Reserve is located in Leishan County, Qiandongnan Miao and Dong Autonomous Prefecture, which is in the southeastern part of Guizhou Province; the reserve encompasses the watershed between the Zhujiang and Yangtze River systems. Because of the Reserve's geographical location and favorable natural conditions as well as the region not having been glaciated during the Quaternary, it constitutes a refuge for many ancient relic species and is rich in biological resources.

This study took *Tomentella* ectomycorrhizae discovered at the Leigong Mountain Nature Reserve as its research subject, describing the morphological characteristics, making molecular identification and constituting the first report on fungi in the genus *Tomentella* from Leigong Mountain Nature Reserve.

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Materials and Methods

There were four different plant communities (evergreen broadleaf forest composed of *Castanopsis eyrei* and *C. fargesii*; deciduous broadleaf forest composed of *B. luminifera, P. adenopoda* and *Platycarya strobilacea*; warm coniferous forest composed of *P. massoniana* and *Taiwania flousiana;* and scrub composed of *Quercus acutissima* and *Q. fabri*) in Leigong Mountain Nature Reserve. After randomly selecting adult trees in sampling areas, we collected soil samples containing small roots around the trees, packaged the samples, and preserved them for further processing by freezing at 20°C at different times during June–August of 2015 and 2016.

Sample processing: The samples were soaked in water, washed in running water, and roots were cut into sections. Mycorrhizae were picked off the root sections under a stereomicroscope and sorted on the basis of their morphological characteristics. After each type of mycorrhizae had been divided into portions, one portion was preserved in Formaldehyde–acetic acid-ethanol fixative (FAA) solution for use in morphological examination and description of characteristics, and another portion was preserved in 2% cetyl trimethylammonium bromide (CTAB) solution for use in molecular identification.

Morphological examination: A stereomicroscope (Olympus SZX10, Japan) was used to examine the macroscopic morphological characteristics of mycorrhizae: branching type, branch tip shape, color, surface characteristics, and emanating hyphae. The fungal mantle was scraped off unbranched tips and prepared on temporary slides, which were used to observe structural characteristics, including the inner and outer mycelium tissue types in the mantle, arrangement of mycelium, emanating hyphae characteristics, and rhizomorph type.

Molecular Identification

DNA Extraction and PCR Amplification

DNA extraction: Each mycorrhizal type was identified by morphology and structural characteristics and one or two root tips were selected from each group for the extraction of DNA. The TaKaRa MiniBEST Plant Genomic DNA Extraction Kit was used for extraction.

PCR amplification: The PCR reaction system (50 μL) consisted of template (2 μL), ITS1f (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCTTATTGATATGC) primers (2 μL each) and 2× Master Mix (25 μL), with the remaining volume made up of ddH_2O. The PCR amplification reaction was performed in an Eppendorf Mastercycler PCR instrument, with amplification procedures consisting of initial denaturation at 94°C for 5 min; denaturation at 94°C for 40 s, annealing at 54°C for 60 s and extension at 72°C for 60 s for 35 cycles and flattening at 72°C for 10 min. The edited morphological rDNA ITS sequences were subjected to comparative analysis at GenBank (NCBI) and the taxonomic relations of the morphological fungi were determined on the basis of sequence similarity.

DNA extraction was performed using the TaKaRa MiniBEST Plant Genomic DNA Extraction Kit and the ITS segment amplification primers were synthesized by the Shanghai Bioengineering Company. The PCR amplification system consisted of 10x buffer (2.5 μL), dNTPs (2 μL), primers (0.5 μL each), enzymatic test reagent (0.25 μL), DNA template (2 μL) and double-distilled water to make up a volume of 25 μL. PCR amplification reaction thermal cycling parameters consisted of pre-denaturation at 94°C for 5 min; denaturation at 94°C for 30 s, annealing at 54°C for 45 s and extension at 72°C for 1 min for 35 cycles and extension at 72°C for 5 min.

Sequencing: The purified PCR product was sequenced by GenScript. The obtained sequences were edited using BioEdit and compared in UNITE (BLAST), which yielded preliminary species classification.

Phylogenetic analysis: The Bayesian method was used to perform phylogenetic analysis of the base sequence of the DNA ITS segment of the identified ectomycorrhizal fungi. After similar sequences had been located in UNITE and NCBI, mafft and Cluster were used to edit the sequences. After performing AIC calculations in MrModeltest v2.3 (Nylander, 2004), we confirmed that the optimal model suitable for analysis was SYM+I+G, set the number of running generations as 1.5 million in Bayesian analysis and ran the program until the average standard deviation was under 0.01 (Huelsenbeck and Ronquist, 2005). Other parameter settings were retained as the defaults. Sump and Sumt commands were subsequently run in MrBayes, the prior 25% of generations were discarded and phylogenetic tree summation and posterior probability (PP) calculations were performed.

Results

Different Mycorrhizal Morphotypes and Anatomical Characteristics

Morphotype 1: The mycorrhiza system has monopodial branching has a pyramidal form, 0–2 grade and is hydrophilic. The terminal branch tip was inflated, rod-shaped and reddish-brown when young and brown when mature; the surface characteristics included a dense wooly coating, a small number of long emanating hyphae and no cystidium (Fig. 1A).

The outer mantle layer consisted of pseudoparenchymatous tissue, the mycelial cells were triangular to polygonal, the mantle was L-type (Fig. 1B), the mycelial cells were 7.4–13.5 × 4.7–10 μm in size and the cell walls were 0.3–0.5 μm thick. The inner layer consisted of plectenchyma (Fig. 1C) and the hyphae were

colorless and had a diameter of 2–4 μm. The emanating hyphae were light brown, few in number, with clamp connections and a diameter of 3 μm and the cell walls were 0.5 μm thick and had a smooth surface (Fig. 1D). The rhizomorphs were undifferentiated, the hyphae had a diameter of 3 μm and the cell walls were 0.5 μm thick (Fig. 1E).

This morphotype was isolated from the root system of *C. fargesii* trees in the evergreen broadleaf forest in Leigong Mountain Nature Reserve.

Morphotype 2: The mycorrhizal system was monopodial, branchless and hydrophilic. The tips were rod-shaped, inflated, brown and surface characteristics included a dense wooly coating, large numbers of emanating hyphae and no cystidium (Fig. 2A).

The outer mantle layer consisted of pseudoparenchymatous tissue, the mycelial cells were triangular to polygonal in shape and the mantle was L-type (Fig. 2B). The mycelial cells were 10−14 × 6−9.6 μm in size. The inner mantle layer consisted of plectenchyma and the hyphae were transparent and colorless (Fig. 2C) and had a diameter of 2−4 μm. Numerous emanating hyphae were present, with a diameter of 3−3.5 μm, a smooth surface and

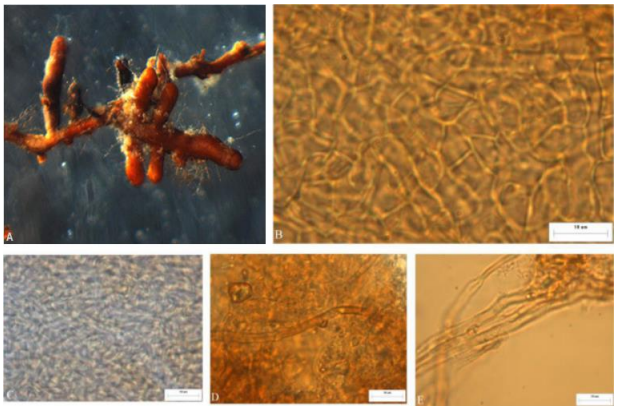

Fig. 1: Morpho-anatomical structural characteristics of morphotype 1

A: External morphological features; **B**: outer mantle layer; **C**: inner mantle layer; **D**: emanating hyphae; **E**: rhizomorph

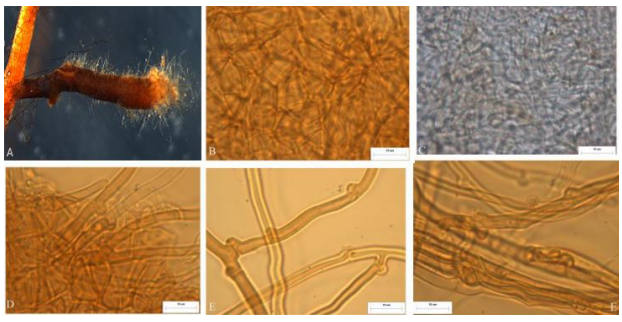

Fig. 2: Morpho-anatomical structural characteristics of morphotype 2

A: External morphological features; **B**: outer mantle layer; **C**: inner mantle layer; **D**: emanating hyphae; **E-F**: rhizomorphs

with clamp connections (Fig. 2D). The rhizomorphs were composed of two types of hyphae, which had different diameters and colors; the fine hyphae were pale yellow, 3−3.5 μm in diameter and had cell walls 0.5 μm thick. The coarse hyphae were deep brown, 4–4.5 μm in diameter and had cell walls 0.8 μm thick. Both types of hyphae had clamp connections and smooth surfaces. (Fig. 2E and F) This morphotype was isolated from the root system of birches in the broadleaf forest in Leigong Mountain Nature Reserve.

Morphotype 3: The mycorrhizal system was monopodial, branchless and hydrophilic. The tips were rod-shaped, inflated, slightly curved and black; surface characteristics included a dense wooly coating, large numbers of emanating hyphae and no cystidium (Fig. 3A).

The outer mantle layer consisted of pseudoparenchymatous tissue; the mycelial cells were arranged like piled stones on the mantle surface, were triangular to polygonal in shape and formed star-shaped arrangements; the mantle was K-type (Fig. 3B) and mycelial cells were 7–12 × 5–8 μm in size. The inner mantle layer consisted of pseudoparenchymatous tissue and the mycelium consisted of pseudoparenchymatous cells (Fig. 3C). The hyphae had a diameter of 2–5 μm. Numerous emanating hyphae were present, light brown and conical in shape, with 1–3 compartments, no clamp connections and a length of 120–150 μm. The mycelial cells of the basal and outer mantle layers were conjoined, with curvature near the base, sharp tips, usually no branching but occasionally branching near the apex, a base 9–10 μm, a central diameter of 3–5 μm and relatively thick cell walls, which reached 1 μm in thickness (Fig. 3D and E).

This morphotype was isolated from the root system of *P. massoniana* in the warm coniferous forest in Leigong Mountain Nature Reserve.

Morphotype 4: The mycorrhizal system was monopodial and rod-shaped, branchless and hydrophilic. The tips were not inflated, slightly contracted, and brownish-red; surface characteristics included a sparse wooly coating, a small number of emanating hyphae, and no cystidium (Fig. 4A).

The outer mantle layer consisted of pseudoparenchymatous tissue, the mycelial cells were horn-shaped, round cells were often present on the mantle surface, the mantle was K-type (Fig. 4B) and the mycelial cells were 12–17 × 8–12 μm. The round cells had thick cell walls (Fig. 4D), which were 0.8–3 μm thick. The inner mantle layer consisted of plectenchyma (Fig. 4C) and the hyphae were colorless and had a diameter of 1.5–3 μm. The emanating hyphae were numerous, light brown to brown, had a diameter of 3–4.5 μm, had cell walls 0.6–0.8 μm thick, had clamp connections and a smooth surface and often had elbow-shaped curves (Fig. 4E and F). This morphotype was isolated from the root system of oaks in scrub in Leigong Mountain Nature Reserve.

Morphotype 5: The mycorrhizal system was monopodial, rod-shaped, branchless and hydrophilic. Tips were not inflated and were brownish-black. Surface characteristics included a dense wooly coating, a small number of short

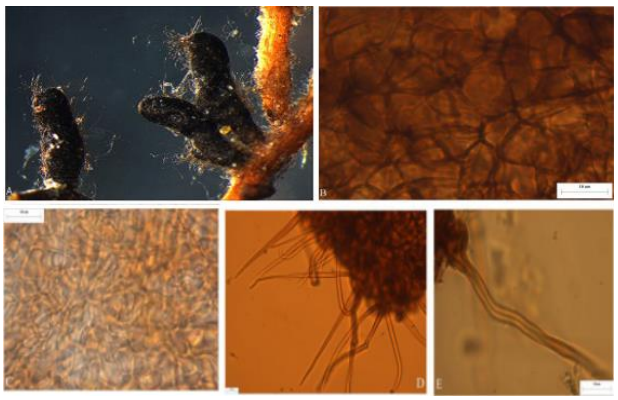

Fig. 3: Morpho-anatomical structural characteristics of morphotype 3
A: External morphological features; **B**: outer mantle layer; **C**: inner mantle layer; **D-E**: emanating hyphae

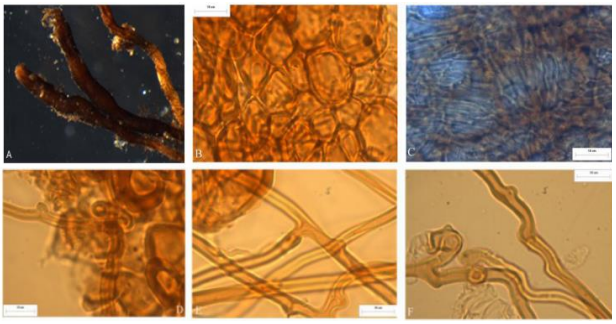

Fig. 4: Morpho-anatomical structural characteristics of morphotype 4
A: External morphological features; **B**: outer mantle layer; **C**: inner mantle layer; **D**: thick-walled round cells; **E-F**: emanating hyphae

emanating hyphae and no cystidium (Fig. 5A).

The outer mantle layer consisted of pseudoparenchymatous tissue, mycelial cells were triangular to polygonal in shape and had a "rose-shaped" arrangement, the mantle type was K-type (Fig. 5B) and the mycelial cells were 11.0–22.0 × 6.0–18.0 μm. The inner mantle layer consisted of plectenchyma, and the hyphae were brown and had a star-shaped arrangement (Fig. 5C). The hyphae had a diameter of 2–4 μm and emanating hyphae were numerous, light brown, with clamp connections and a diameter of 3.5 μm. The cell walls were 0.5 μm thick and had a smooth surface (Fig. 5D). The rhizomorphs were undifferentiated, and the hyphae had a smooth surface and a diameter of 3.5–4 μm

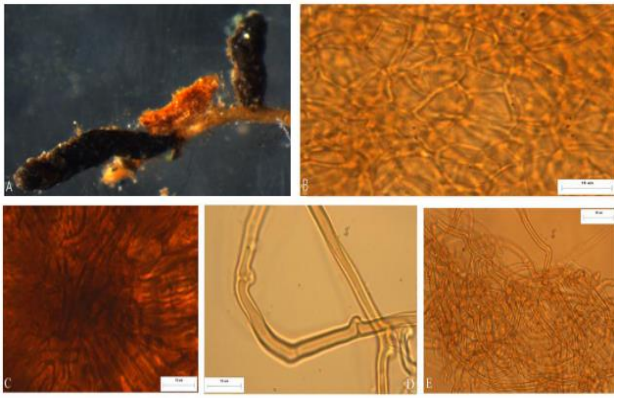

Fig. 5: Morpho-anatomical structural characteristics of morphotype 5
A: External morphological features **B**: outer mantle layer **C**: inner mantle layer **D**: emanating hyphae E: rhizomorph

(Fig. 5E). This morphotype was isolated from the root system of *P. massoniana* in warm coniferous forest in Leigong Mountain Nature Reserve.

Comparison of the Morphological and Structural Characteristics of Five Morphotypes

The five types of ectomycorrhizae collected in this study were all dark brown to black, all had mantles formed by horn-shaped cells and all had emanating hyphae. These morphological characteristics are consistent with the morphological characteristics of *Tomentella* described by Jakucs and Erös-Honti (2008). Table 1 for a comparison of the morpho-anatomical structural characteristics of the five morphotypes.

Phylogenetic Analysis

Bayesian analysis (Fig. 6) revealed that the sequences of the five morphotypes were all clustered within the sequences of fungi in the genus *Tomentella*, indicating that all ectomycorrhizae were formed by fungi in the genus *Tomentella*. Morphotype 1 formed a branch with the four species *T. vindula*, *T. galzinii*, *T. subtestacea* and *T. coerufea*. Morphotype 2 formed a branch with *T. ellisii* and had a high level of support (bootstrap 1). Morphotype 3 formed an independent branch with *T. fuscocinerea* (bootstrap 0.94). Morphotype 4 formed an independent branch with *T. bryophila* (bootstrap 1). Morphotype 5 formed an independent branch with *T. stuposa* (bootstrap 1).

Table 1: comparison of the morpho-anatomical structural characteristics of the five morphotypes

Morphotype	External structure			Anatomical structure		
	Color	Surface characteristics	Outer layer	Inner mantle layer	Emanating hyphae	Rhizomorphs
1	Brown	Dense wooly coating	E-type	Plectenchyma	Few, no clamp connections	None
2	Brown	Dense wooly coating	L-type	Plectenchyma	Few, no clamp connections	None
3	Black	Dense wooly coating	Q-type	Pseudoparenchymatous tissue	Many, no clamp connections	None
4	Brownish-red	Sparse wooly coating	M-type	Plectenchyma	Few, with clamp connections	None
5	Brownish black	Dense wooly coating	M-type	Plectenchyma	Few, with clamp connections	None

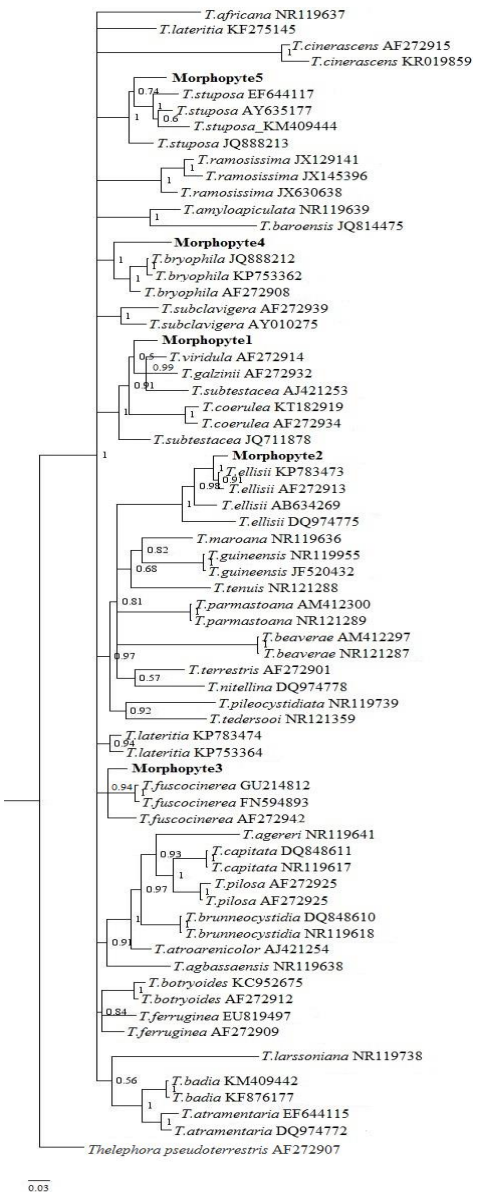

Fig. 6: Bayesian phylogenetic tree based on analysis of rDNA-ITS sequence
Bayesian PPs are indicated at the branch nodes

Consequently, while the species of morphotype 1 could not be identified, the other four morphotypes could all be identified to the species level.

Discussion

This paper identified the five ectomycorrhizal morphotypes obtained from the root systems of *C. fargesii*, *B. luminifera*, *P. massoniana* and *Q. acutissima* in the Leigong Mountain Nature Reserve. A comparison of the morpho-anatomical structural characteristics and phylogenetic analysis of these ectomycorrhizal morphotypes revealed that all were from fungi of the genus *Tomentella* and four morphotypes were identified to the species level.

Phylogenetic analysis revealed that while morphotype 1 formed an independent branch with *T. galzinii*, *T. subtestacea*, *T. coerulea* and *T. viridula*, great differences existed in the morpho-anatomical structural characteristics between morphotype 1 and these species. For instance, while *T. galzinii* and *T. subtestacea* both have cystidium with clamp connections (Wei and Agerer, 2010), morphotype 1 lacks this type of cystidium. Xie and Liu (2011) described two types of ectomycorrhizae that are similar to *T. coerulea* and *T. viridula*, and neither have emanating hyphae with clamp connections, but the emanating hyphae of morphotype 1 have clamp connections. Accordingly, determining the relations among the ectomycorrhizal fungi in this branch will require further in-depth research.

Morphotype 2 formed an independent branch with *T. ellisii*, but because no detailed description exists for *T. ellisii*, the morpho-anatomical characteristics of the types could not be compared. *T. ellisii* has been found in Sweden, Italy, France, Hungary, Estonia and the United States and is both an ectomycorrhizal fungi and a mycorrhizal fungi of orchids (Jakucs *et al.*, 2015). Morphotype 3 had the same ectomycorrhizal typology as *T. fuscocinerea* and they both have outer mantle layers consisting of pseudoparenchymatous tissue, in which triangular to polygonal cells form star-shaped arrangements, inner mantle layers consist of pseudoparenchymatous cells and emanating hyphae have no clamp connections and have thick cell walls extending from the cells in the outer mantle layer. *T. fuscocinerea* has been reported in Sweden, Hungary, Denmark and Canada and is both an ectomycorrhizal fungus and a mycorrhizal fungus of orchids (Jakucs *et al.*, 2015). Morphotype 4 formed an independent branch with *T. bryophila*. In terms of morpho-anatomical structural characteristics, both have outer mantle layers composed of horn-shaped cells and round cells in groups on the surface of the outer mantle layer; have emanating hyphae that are extremely numerous, brown, thick-walled and possess clamp connections. *T. bryophila* is found in many parts of Europe, including Denmark, Portugal, Hungary, Austria, Scotland, Norway, Sweden and Finland and Estonia (Jakucs *et al.*, 2015). Xie and Liu (2011) have reported that *T. bryophila* forms ectomycorrhizae with *Pinus yunnanensis* in Yunnan Province, China. Morphotype 5 formed an independent branch with *T. stuposa*; both have outer mantle layers composed of horn-shaped cells, an inner mantle layer composed of hyphae in a star-shaped arrangement, brown emanating hyphae with clamp connections, a smooth surface and undifferentiated rhizomorphs. At present, *T. stuposa* is the species in this genus that has been researched most extensively, and it has been described in detail several times. This species has an extremely broad geographical distribution, is found in Europe and North America, is a major taxon that is

ectomycorrhizal on many species of trees (Jakucs and Erös-Honti, 2008) and plays an important role in forest ecosystems.

International gene libraries (NCBI and UNITE) currently lack any sequence information on the fruiting bodies of *Tomentella* fungi from China, which is likely because the fruiting bodies of *Tomentella* fungi commonly adhere to dead branches, rotten wood and rocks; are difficult to find and are often overlooked. While research on *Tomentella* ectomycorrhizae can provide clues for research on the fruiting bodies of fungi in this genus, research in China on the fruiting bodies of *Tomentella* fungi needs to be strengthened.

Conclusion

Apart from morphotype 1, which could not be identified to the species level and morphotype 2, whose morpho-anatomical structural characteristics could not be verified, the results of phylogenetic analysis of morphotypes 3–5 were supported by the morphotypes' morpho-anatomical structural characteristics. The findings of this study indicate that *T. ellisii*, *T. fuscocinerea*, *T. bryophila* and *T. stuposa* are found in Inner Mongolia and provide information that can be used in future research on the ecological function of *Tomentella* fungi.

Acknowledgements

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