Description and identification of *Ostryopsis davidiana* ectomycorrhizae in Inner Mongolia mountain forest of China

QING-ZHI YAO[1]
WEI YAN[2]
HUI-YING ZHAO[1]
JIE WEI[2]
[1] Life Science College
[2] Forestry College
Inner Mongolia Agriculture University
Huhhot, 010018, P. R. China
Email: yaoqingzhi@imau.edu.cn

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Abstract: The ectomycorrhizal (ECM) fungal composition and anatomical structures of root samples of the shrub *Ostryopsis davidiana* were examined. The root samples were collected from two plots in the Daqing Mountain and Han Mountain around Hohhot, Inner Mongolia of China. Basing on morpho-anatomical features of the samples, we have got totally 12 ECM morphotypes. Twelve fungal taxa were identified via sequencing of the internal transcribed spacer region of their nuclear rDNA. Nine species are *Basidiomycotina*, incl. *Thelephoraceae* (*Tomentella*), *Cortinariaceae* (*Inocybe* and *Cortinarius*), *Tremellaceae* (*Sebacina*), *Russulaceae* (*Lactarius*), and *Tricholomataceae* (*Tricholoma*), three *Ascomycotina*, incl. *Elaphomycetaceae* (*Cenococcum*), *Tuberaceae* (*Tuber*), and *Pyronemataceae* (*Wilcoxina*). *Cenococcum geophilum* was the dominant species in *O. davidiana.* The three *Tomentella* and the two *Inocybe* ECMF of *O. davidiana* are very common in Inner Mongolia.

Zusammenfassung: Die Pilzdiversität der Ektomykorrhiza (ECM) und deren anatomische Strukturen von Wurzelproben des Strauches *Ostryopsis davidiana* wurden untersucht. Die Wurzelproben wurden aus zwei Untersuchungsflächen im Daqing Berg und Han Berg nahe Hohhot, Innere Mongolei, China, gesammelt. Basierend auf morpho-anatomischen Merkmalen wurden 12 ECM-Morphotypen charakterisiert. Zwölf Pilz-Taxa wurden durch Sequenzierung der internen transkribierten Spacer-Region der nuklearen rDNA identifiziert. Neun Arten sind Basidiomycotina, inkl. *Thelephoraceae* (*Tomentella*), *Cortinariaceae* (*Inocybe* und *Cortinarius*), *Tremellaceae* (*Sebacina*), *Russulaceae* (*Lactarius*) und *Tricholomataceae* (*Tricholoma*), drei *Ascomycotina*, inkl. *Elaphomycetaceae* (*Cenococcum*), *Tuberaceae* (*Tuber*) und *Pyronemataceae* (*Wilcoxina*). *Cenococcum geophilum* war die dominierende Art in *O. davidiana*. Die drei *Tomentella* und die beiden *Inocybe* ECMF von *O. davidiana* sind in der Inneren Mongolei häufig.

Ectomycorrhizal (ECM) fungi play an important role in nutrient transportation, interspecific interactions, and maintenance of biodiversity in ecosystems (SIMARD & al. 1997). Knowledge of ECM fungal diversity has traditionally relied on fruit-body surveys. Fruit-bodies are ephemeral sexual structures that often require specific detection methods such as raking of soil and turning over dead wood. Moreover, many ECM fungal lineages have never been found producing fruit-bodies (TEDERSOO & al. 2010).

The absence of fruit-bodies and short-term superficial search efforts has resulted in the discrepant view of above- and below-ground fungal diversity and community structure (GARDES & BRUNS 1996, but see SMITH & al. 2007).

The anatomy of ECM has been studied over 120 years since GIBELLI (1883) and FRANK (1885). But it lasted almost a century until the structure of ECM was recognized as being essential for studies on fungal relationships (GODBOUT & FORTIN 1985). Despite over 100 years of investigation on the subject, the number of species described using morph-anatomical features is relatively small (about 343 species, DE ROMAN & al. 2005). ECMs that are morpho-anatomically well studied are presently restricted to only some fungal groups. An affiliation of non-identified, but comprehensively described ECM to higher hierarchical levels is sometimes impossible due to the lack of distinctly useful features. Thus, more detailed descriptions of ECM are needed. Species-by-species analyses, linking the molecular identification approach of ECM fungi to a certain tree species are quite numerous in Europe, but rare in China. Only in recent years, the ECM morpho-anatomical features of *Castanopsis fargesii* (WANG & al. 2011), *Quercus liaotungensis* (WANG & al. 2012), *Pinus tabulaeformis* (WANG & GUO 2013, WEI & AGERER 2010, 2011), *Picea crassifolia* and *Betula platyphylla* (FAN & YAN 2013) were described.

Ostryopsis (*Betulaceae*) is a small genus endemic to China (CHEN & al. 1999), and only two species, *O. davidiana* DECNE and *O. nobilis* BALF. f. et W. W. SM. are recognized (CHEN 1994). The former is mainly distributed in N China and the latter is limited to the SW China. These two species provide a good model system to explore the diversification pattern of plants at the species level. In addition, both of them play an important role in restoring the local ecosystem as pioneering tree species when naturally or artificially destroyed (CHEN 1994). Morpho-anatomical descriptions of ECM on *O. davidiana* are limited, and no other anatomical types on *O. davidiana* have been described up to now in details. The key to these ECM anatomical types could provide basic knowledge for determination of ECM anatomical types on *O. davidiana* and be useful for other kinds of studies on *O. davidiana*, e.g. for investigating ECM fungal diversity and richness, species composition, for selecting suitable inocula for reforestation, or for estimating the dynamics of ECM fungi composition in a long term or under climate changes.

Materials and methods

Site description: The Daqing Mountain (40° 37′ to 40° 57′ N; 110° 45′ to 111° 32′ E) locates along the northern edge of the North China plate. It extends about 200 km from east to west. It is the prominent part of the Yinshan Mountains, which is an important water source for agricultural irrigations in the south-middle area of Inner Mongolia, named TuMoChuan plain.

The Han Mountain (The Saihanwula National Nature Reserve in China) (43° 59' to 44° 27' N; 118° 18' to 118° 55' E) lies in Balinyou County, Inner Mongolia Autonomous Region. Its conservation subjects include forests, grasslands, wet-land ecosystems and some rare species. The geography of this area is middle-elevation Mountains, in which the highest altitude is 1997m s. m. on Wulan Mountain. The location of this reserve is at the boundary between grassland and forest between south Asian broad-leaved and Taiga forests. The biodiversity is fairly rich with characters species and endemics. There are 665 plant species in 318 genera and 85 families, 37 mammal species in 14 families and 6 orders, 148 bird species in 46 families and 17 orders known. In September 2001 it was recognized by UNESCO (United Nations Educational, Scientific and Cultural Organization) as a biosphere reserve.

Tab. 1. Molecular identification of ectomycorrhizal fungi on root tips of *Ostryopsis davidiana* based on ITS sequences.

Type	Closest blast match (GenBank accession No.)	Query/reference ITS length (similarity %)	ECM fungus	Sites
1	*Cenococcum geophilum* (DQ179119)	969/999(97 %)	*Cenococcum geophilum*	Daqing and Han
2	*Wilcoxina rehmii* (DQ069001)	525/529(99 %)	*Wilcoxina rehmii*	Daqing
3	*Tuber liaotongese* (GU979037)	500/504(99 %)	*Tuber liaotongese*	Han
4	*Sebacina* sp. (AF440651)	603/622(97 %)	*Sebacina* sp.	Daqing and Han
5	Uncultured *Tricholomataceae* (AF377210)	656/670(98 %)	*Tricholoma* sp.	Daqing
6	*Lactarius pubescens* (AY606953)	639/676(99 %)	*Lactarius pubescens*	Daqing and Han
7	*Cortinarius coerulescentium* (DQ083781)	577/620(93 %)	*Cortinarius* sp.	Daqing and Han
8	*Inocybe splendens* (FN550911)	656/663(99 %)	*Inocybe splendens*	Han
9	*Inocybe fuscidula* var. *fuscidula* (HQ604476)	675/697(97 %)	*Inocybe fuscidula* var. *fuscidula*	Daqing and Han
10	*Tomentella ramosissima* (TRU83480)	649/668(97 %)	*Tomentella ramosissima*	Daqing and Han
11	*Tomentella fuscocinerea* (emb.FN594853)	575/586(98 %)	*Tomentella fuscocinerea*	Daqing and Han
12	Uncultured *Tomentella* (EU726321)	661/671(99 %)	*Tomentella* sp. 2	Daqing

Sampling and morpho-anatomical characterization:
Surveys of the sampling sites were conducted to collect morphotypes and molecularly identify the ECM fungal species in *Ostryopsis davidiana* plantations in Inner Mongolia Mountain Forest. The sites selected for sampling included The Daqing Mountain and The Han Mountain. Roots at each site were sampled in July 2011 and 2012. In total, 60 soil cores, 30 from each sampling site, were collected. From each *O. davidiana* tree, four soil cores of 15 cm^3 were taken 10–15cm away from the stem, wrapped in polythene bags, tagged, placed in ice boxes and transported to the laboratory for analysis.
Fresh roots were washed free of soil and fresh fine roots of *O. davidiana* were picked out from each soil sample. Although the roots of weeds and other plants were minimised during field sampling, care was taken to distinguish *O. davidiana* roots from the roots of other plants. Remaining soil particles were removed under stereomicroscope. The isolated morphotypes were kept in distilled water for morpho-anatomical characterisation and in 2 % cetyl trimethylammonium bromide (CTAB) in Eppendorf tubes for molecular characterisation and stored at –20 °C. Characterisation of morphotypes followed AGERER (1991, 2006).

DNA extraction, PCR analysis: According to GARDES & BRUNS (1993) DNA of the ECM was extracted from the root samples. The ITS region (ITS1, 5.8S, ITS2) of rDNA from each ECM morphotype was amplified by PCR through using the primer pairs ITS1-F/ITS-4. The final 50 µl reaction mixture contained 1 µl template DNA, 1× PCR buffer, 2.0 mM $MgCl_2$, 0.2 mM each dNTP, 15 pmol of each primer, and 2.5 U Taq polymerase (TransGen Biotech, Beijing, China). The amplification was programmed for a denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation for 40 s at

94 °C, annealing for 50 s at 50 °C, extension for 1 min at 72 °C, and a final 10 min extension at 72 °C. A negative control using sterile Milli-Q water instead of template DNA was included in the amplification process. ITS-PCR products were size-fractionated on 2 % agarose gels. ITS-PCR band sizes were estimated by comparing to a standard 100-bp molecular weight ladder. Molecular identifications were repeated at least one time for each morphotype sample.

DNA sequencing and identification: Following the instruction provided by the manufacturer, the PCR products were purified by using the PCR production purification kit. One representative PCR product was sequenced by using an ABI Prism 3700 Genetic Analyzer (Applied Biosystems, USA). In order to check the most likely chimera breakpoints, all ITS sequences were analyzed by the Chimera Check program of the RDP version 2.7 (MAIDAK & al. 1999). A value of 97 % ITS region identity was used as a DNA barcoding threshold for ECM fungal taxa (TEDERSOO & al. 2007). The ITS sequences generated in this study were used as query sequences to search for similar sequences in NCBI and UNITE (KÕLJALG & al. 2005, 2013) to provide at least tentative identification for the ECM fungi. ITS sequences of OTUs with high similarity (>96 %) to vouchered specimens were assigned to a genus. If the ITS sequences of OTUs showed poor similarity (<96 %) with reference sequences in NCBI and UNITE, a genus or family name was assigned to the OTU based on a combination of similarity and phylogeny (SOUTHWORTH & al. 2009).

Results

ECM fungal community composition:

Twelve ECM morpho-types were obtained from fine roots of *O. davidiana*, from two sites. Identical ITS sequences were obtained from three to five samples of each morphotype. Twelve ECM fungi were identified from the 12 morpho-types based on the analyses of ITS sequences (Tab. 1). Three are Ascomycetes and nine Basidiomycetes. Among the Basidiomycetes, six were identified on species level; three on genus level. For the Ascomycetes, three were identified on species level.

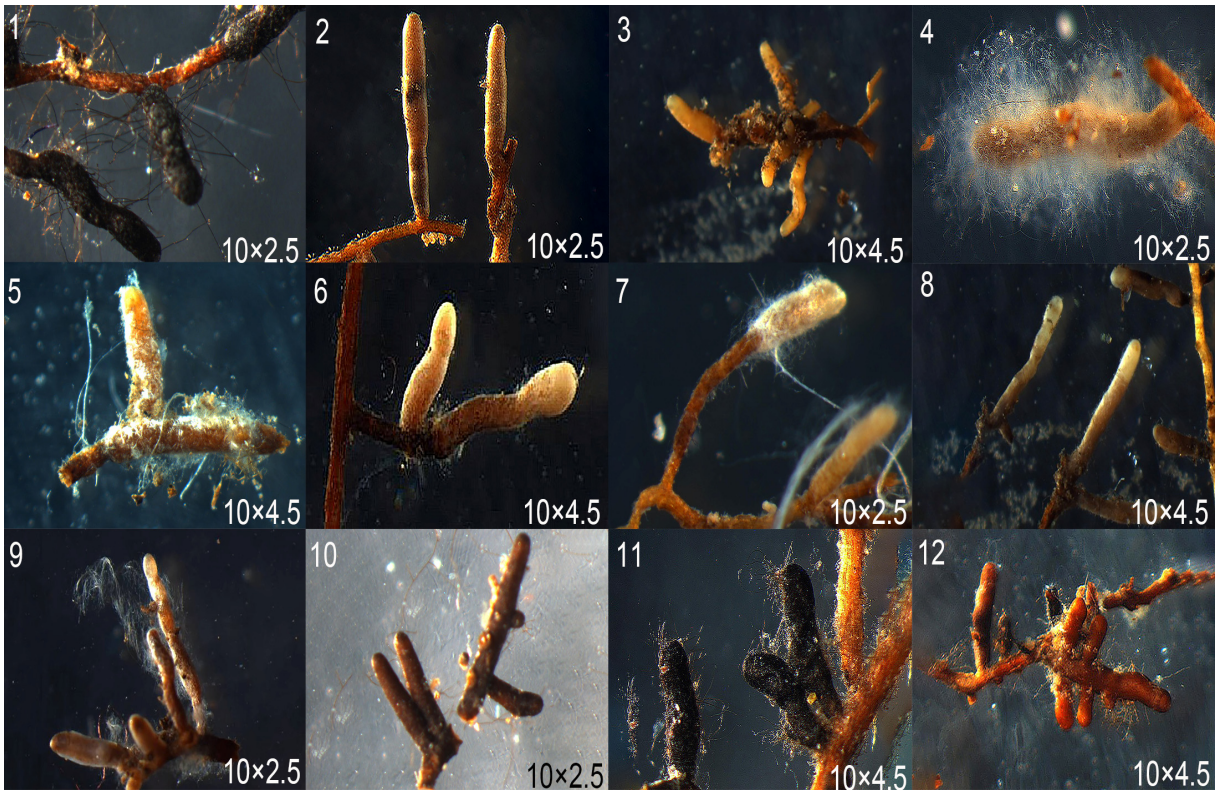
Characters of the ECM morphological–anatomical structure:

Cenococcum geophilum – Morphotype 1 (Figs. 1, 13):
Hydrophilic, monopodial-pinnate or unramified, black, 4–6 mm long, 0.4–0.6 mm diam., unramified ends inflated, with abundant dark rigid emanating hyphae, no rhizomorphs, with abundant globular deep black sclerotia (0.5–1.0 mm diam.), with rigid mycelium radially projecting from the surface, not smooth, shiny. Mantle in plan view: plectenchymatous, hyphae star-like arranged and tightly glued together (Type G), membranaceously brownish, cell wall thick (4.0–6.0 µm), with simple septa.

Wilcoxina rehmii – Morphotype 2 (Figs. 2, 14):
Hydrophilic, monopodial, club-shaped, unramified ends not inflated, the tip of the mycorrhizal constriction beaded, brownish, up to 9 mm long, 0.2–0.3 mm diam-, mantle matt, smooth, non-transparent, sparse white short emanating hyphae, no rhizomorph. Mantle in plan view: Plectenchymatous, without pattern (type E). Emanating hyphae with simple septa, smooth.

Tuber liaotongense – Morphotype 3 (Figs. 3, 15):
Hydrophilic, monopodial-pinnate or pyramidal, yellowish to brown, 11–13 mm long, 0.7–0.9 mm diam., unramified ends uninflated with sparse short emanating hyphae, mantle smooth, transparent, with soil particles. Mantle in plan view: pseudoparen-

chymatous with epidermoid cells (Type Q); thin mycelia cell wall, smooth margin, hyphae septate, no shrinking, and no clamp connections.



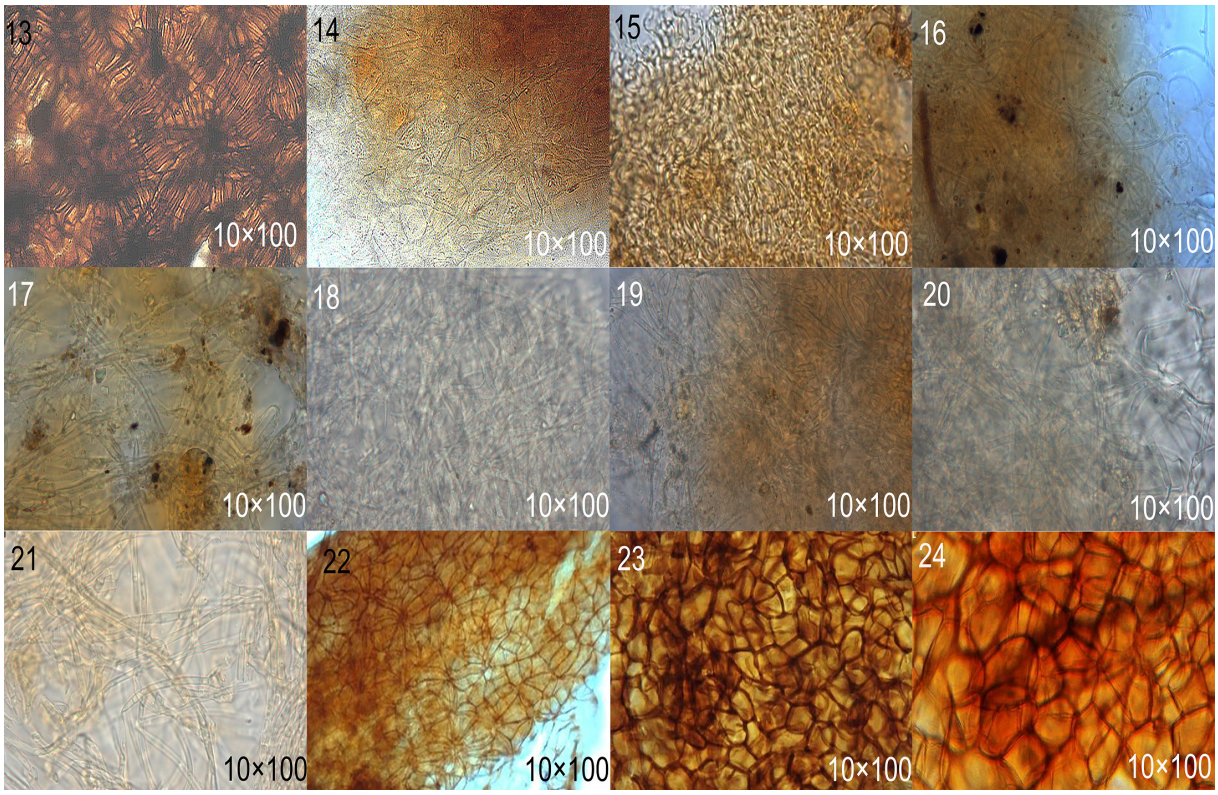
Figs. 1–12. Ectomycorrhizae on roots of *Ostryopsis davidiana*. – Fig. 1. *Cenococcum geophilum*. – Fig. 2. *Wilcoxina rehmii*. – Fig. 3. *Tuber liaotongense*. – Fig. 4. *Sebacina* endomycorrhiza. – Fig. 5. Uncultured *Tricholomataceae*. – Fig. 6. *Lactarius pubescens*. – Fig. 7. *Cortinarius coerulescentium*. – Fig. 8. *Inocybe splendens*. – Fig. 9. *Inocybe fuscidula var. fuscidula*. – Fig. 10. *Tomentella ramosissima*. – Fig. 11. *Tomentella fuscocinerea*. – Fig. 12. Uncultured *Tomentella*. – Bars: Figs. 1, 2, 4–8, 10–11 = 0.1 mm; Figs. 3, 9 = 1 mm; Fig.12= 0.5 mm.

Sebacina endomycorrhiza – Morphotype 4 (Figs. 4, 16):
Hydrophilic, monopodial-unramified, club-shaped, brown, 3–5 mm long, 0.2–0.4 mm diam., unramified ends inflated, with abundant long villous white emanating hyphae, with soil particles. Mantle in plan view: plectenchymatous (Type D); Emanating hyphae smooth, with simple septa, bifurcation, with slightly inflation at the ramification point.

Uncultured *Tricholomataceae* – Morphotype 5 (Figs. 5, 17):
Hydrophobic, monopodial-ramified, club-shaped, yellowish to brown, 2–3 mm long, 0.3-0.4 mm diam., unramified ends bending and shrinking, mantle shiny, unsmooth, not transparent, with short villous white emanating hyphae, with rhizomorphs, with soil particles. Mantle in plan view: plectenchymatous with ring-like structure (Type A); Emanating hyphae smoothwith simple septa.

Lactarius pubescens – Morphotype 6 (Figs. 6, 18):
Hydrophilic, monopodial-unramification, pinkish, 5–7 mm long, 0.7–1.0 mm diam., unramified ends inflated or not inflated and constricted, short, sparse emanating hyphae, mantle not smooth, not transparent, with rhizomorphs. Mantle in plan view:

plectenchymatous (Type C). Emanating hyphae colourless, smooth, with simple septa. Rhizomorphs uniform.



Figs. 13–24. Plan view of ectomycorrhizal mantle. – Fig. 13. *Cenococcum geophilum*. – Fig. 14. *Wilcoxina rehmii*. – Fig. 15. *Tuber liaotongense*. – Fig. 16. *Sebacina* endomycorrhiza. – Fig. 17. Uncultured *Tricholomataceae*. – Fig. 18. *Lactarius pubescens*. – Fig. 19. *Cortinarius coerulescentium*. – Fig. 20. *Inocybe splendens*. – Fig. 21. *Inocybe fuscidula var. fuscidula*. – Fig. 22. *Tomentella ramosissima*. – Fig. 23. *Tomentella fuscocinerea*. – Fig. 24. Uncultured *Tomentella*. – Bars: 10 µm.

Cortinarius coerulescentium – Morphotype 7 (Figs. 7, 19):
Hydrophobic, monopodial, club-shaped, unramified ends bent, yellowish, 4–6 mm long, 0.1–0.3 mm diam. mantle matt, smooth, non-transparent, white emanating hyphae, with rhizomorphs; Mantle in plan view: plectenchymatous (Type A); Emanating hyphae loosely, uniform in diameter with clamp connections. Rhizomorph undifferentiated, hyphae surface warty.

Inocybe splendens – Morphotype 8 (Figs. 8, 20):
Hydrophilic, monopodial-ramified, rod-like, white to gray, 7–11 mm long, 0.5–0.6 mm diam., unramified ends inflated, mantle shiny, unsmooth, transparent, with short villous emanating hyphae, no rhizomorphs. Mantle in plan view: plectenchymatous (Type C); emanating hyphae smooth with clamp connection.

Inocybe fuscidula var. *fuscidula* – Morphotype 9 (Figs. 9, 21):
Hydrophilic, monopodial-unramified, club-shaped, yellowish to brown, 5–6 mm long, 1–2 mm diam.; mantle shiny, transparent, with sparse short emanating hyphae. Mantle in plan view: plectenchymatous (Type C); Emanating hyphae smooth, cell wall thin, with clamp connections.

Tomentella ramosissima – Morphotype 10 (Figs. 10, 22): Hydrophilic, monopodial-pinnate or club-shaped, unramified, reddish brown, 7–8 mm long, 0.3–0.4 mm diam., unramified ends not inflated, sparse long thick emanating hyphae, mantle not smooth, not transparent, no rhizomorphs. Mantle in plan view: pseudoparenchymatous with angular cells, forming rosette-like structures (Type K). Emanating hyphae brown, with clamp connections, ramification rectangular.

Tomentella fuscocinerea – Morphotype 11 (Figs. 11, 23): Hydrophilic, monopodial-unramified, club-shaped, unramified-ends inflated and bent, black, 5–6 mm long, 0.6–0.7 mm diam.; mantle shiny, unsmooth, non-transparent, with abundant dark emanating hyphae, no rhizomorphs. Mantle in plan view: pseudoparenchymatous with angular cells, with some roundish cells on the surface (Type K); emanating hyphae thin with clamp connections, brownish.

Uncultured *Tomentella* – Morphotype 12 (Figs. 12, 24): Hydrophilic, monopodial-pinnate or pyramidal, club-shaped, unramified-ends inflated, red to brown, 11–12 mm long, 0.6–0.7 mm diam.; mantle shiny, unsmooth, non-transparent, with sparse long emanating hyphae, with soil particles, no rhizomorphs. Mantle in plan view: pseudoparenchymatous with angular cells (Type K).

Discussion

The main question of this kind of study was how to identify the species of the ECM forming fungus, because insufficiently identified sequences are generally deposited in official sequence databases. Many sequences, especially ITS sequences, are still unidentified to species or just named as uncultured environmental samples. Comparing fruit body DNA from the sample sites was impossible due to the very limited availability of fruit body diversity of the ECM fungi during the sampling period for the ECM. Thus availability of comparable sequences from corresponding fruit bodies in the collection areas is insufficient, because the sampling sites (Daqing Mountain and Sai-HanWuLa Mountain) are arid and semi-arid regions with limited rainfall hindering fructification. Some fungi produce hypogeous fruit bodies and have to be searched for applying sample forks and scratching intensely the upper soil layer (LÆSSØE & HANSEN 2007). This was avoided to leave the study area undisturbed. The fruit bodies of some other fungi, e.g. *Tomentella,* forming only a rather thin and inconspicuous layer on the underside of dead twigs or stems, are easily overlooked. More importantly, the fungal diversity in Inner Mongolia was only scarcely studied during the past time. Therefore, the fruit body identification is not easy for local researchers, leading to a nearly complete lack of such sequences in public databases.

Based on the molecular identification, the ECM morphology and anatomical structures of *O. davidiana* were studied. The results show that the diversity of ECM morphological and anatomical structures is affected by the variations of the ECM fungi species. A much higher ECM diversity of *O. davidiana* was revealed comparing with previous similar investigations. In addition, the existence of the ECMF *Wilcoxina rehmii* is shown for the first time in Inner Mongolia. Most of the *Tuber* species have already been reported from SW China. The three *Tomentella* and two *Inocybe* ECMF on *O. davidiana,* identified in this study, indicate that the *Tomentella* and *Inocybe* spe-

cies are also very common in Inner Mongolia. The *Tuber* ECM occurring on *O. davidiana* may provide more information about the *Tuber* diversity and ecological traits in China. Furthermore, the results may also contribute to the knowledge about the phylogenetic value of the ECM mantle type in taxonomy. The *C. geophilum*, as a generalistic mycobiont, has commonly been found in a variety of forest ecosystems, particularly in drought habitats (PIGOTT 1982, COLEMAN & al. 1989, JANY & al. 2003, PETER & al. 2016).

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