NOTE / NOTE

Ectomycorrhizal community composition of *Pinus tabulaeformis* assessed by ITS-RFLP and ITS sequences

Qin Wang and Liang-Dong Guo

Abstract: Ectomycorrhizal (ECM) fungal composition was examined in a *Pinus tabulaeformis* Carr. forest. A total of 28 root samples of *P. tabulaeformis* were collected in June and September. Thirty-five ECM morphotypes were identified according to ECM morphological characters, and 26 ECM fungi were identified based on the analyses of ITS-RFLP and ITS sequences. *Tomentella*, *Sebacina*, and *Tuber* were common genera, and Atheliaceae sp., *Lactarius deliciosus*, *Tomentella ferruginea*, and *Tomentella* sp. 3 were dominant species. Of these ECM fungi, 13 were found in June, 19 in September, and 6 during both sampling times. Atheliaceae sp. and *T. ferruginea* were the dominant fungi both in June and September. *Lactarius deliciosus* was dominant in June, but rare in September. *Tomentella* sp. 3 was dominant in September but rare in June.

Key words: ectomycorrhizal fungi, diversity, molecular identification, pine.

Résumé : Les auteurs ont examiné la composition de la mycoflore ectomycorhizienne (ECM) dans une forêt de *Pinus tubulaeformis* Carr. Ils ont récolté un total de 28 échantillons racinaires du *P. tubulaeformis*, en juin et septembre. Ils ont identifié 35 morphotypes d'ECM selon les caractères morphologiques des ECM, et ils ont pu identifier 26 espèces de champignons ECM, en se basant sur les séquences ITS-RFLP et ITS. Les genres les plus communs appartiennent aux *Tomentella*, *Sebacina* et *Tuber*; des espèces d'Atheliaceae et les *Lactarius deliciosus*, *Tomentella ferruginea*, et *Tomentellea* sp. 3 constituent des espèces dominantes. Parmi ces espèces d' ECM, 13 ont été retrouvées en juin, 19 en septembre et six aux deux périodes d'échantillonnage, en juin et en septembre. L'espèce d'Atheliaceae et le *T. ferruginea* constituent les espèces dominantes à la fois en juin et en septembre. Le *L. deliciosus* domine en juin, mais devient rare en septembre, alors que le *Tomentella* sp. 3 domine en septembre, mais rare en juin.

Mots-clés : champignon ectomycorhiziens, diversité, identification moléculaire, pin.

[Traduit par la Rédaction]

Introduction

Ectomycorrhizas are symbiotic structures formed between soil fungi and plant roots. The ectomycorrhizal (ECM) fungi exchange soil-derived nutrients for carbohydrates from the host plants and are beneficial to host species in conferring resistance to abiotic or biotic stresses (Smith and Read 2008). ECM plants are dominant or common species in many natural forests. ECM fungi therefore play important ecological roles in nutrient transfer, inter-or intra-specific interactions, and the maintenance of biodiversity in ecosystems (Simard et al. 1997). Understanding the diversity of ECM fungal communities in relation to their hosts is key to understanding the ecology and function of fungus–plant associations in natural ecosystems.

Investigations of ECM fungal communities based only on aboveground fruit bodies cannot provide a complete picture of ECM communities in natural ecosystems (Gardes and Bruns 1996; Grogan et al. 2000). This is because although fruit-bodies are necessarily associated with ectomycorrhizas, a fungus forming ectomycorrhizas may not always form sporocarps, which are affected by biotic and abiotic factors in natural ecosystems (Horton and Bruns 2001). Furthermore, identification of belowground ECM fungi according

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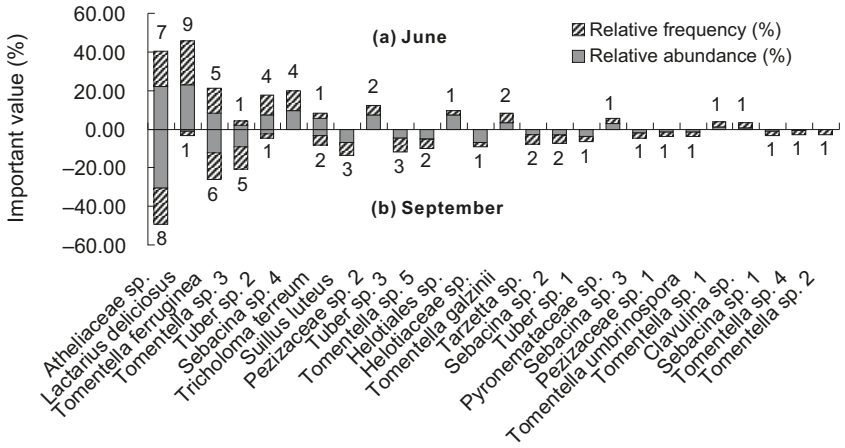
Table 1. Identification of ectomycorrhizal fungi on root tips based on BLASTN against the National Center for Biotechnology Information database.

Morphotype	ECM fungus	GenBank accession No.	Closest blast match reference taxa (GenBank accession No.)	ITS length (bp) and similarity (%)*
ECM1	*Lactarius deliciosus*	GQ985409	*Lactarius deliciosus* (EU423920)	647/647 (100)
ECM2	*Tricholoma terreum*	GQ985410	*Tricholoma terreum* (AY573540)	611/612 (99)
ECM3	*Suillus luteus*	GQ985411	*Suillus luteus* (AY898620)	604/604 (100)
ECM4	Atheliaceae sp.	GQ985412	Atheliaceae sp. (EU649086)	506/505 (95.5)
ECM5	*Clavulina* sp.	GQ985413	*Clavulina cf. rugosa* (DQ974712)	610/615 (95.1)
ECM6	*Tomentella ferruginea*	GQ985414	*Tomentella ferruginea* (AF272909)	536/537 (98.3)
ECM7	*Tomentella umbrinospora*	GQ985415	*Tomentella umbrinospora* (AF272920)	547/547 (97.1)
ECM8	*Tomentella galzinii*	GQ985416	*Tomentella galzinii* (AJ421255)	541/541 (97.2)
ECM9	*Tomentella* sp. 1	GQ985417	*Tomentella viridula* (AF272914)	541/540 (96.5)
ECM10	*Tomentella* sp. 2	GQ985418	*Tomentella fuscocinerea* (DQ974776)	578/580 (92.9)
ECM11	*Tomentella* sp. 3	GQ985419	*Tomentella* sp. (EF218826)	578/578 (95.8)
ECM12	*Tomentella* sp. 4	GQ985420	*Tomentella* sp. (EU668275)	576/575 (94.6)
ECM13	*Tomentella* sp. 5	GQ985421	*Tomentella* sp. (EU668206)	578/578 (98.1)
ECM14	*Sebacina* sp. 1	GQ985422	*Sebcina* sp. (AF440651)	533/531 (96.6)
ECM15	*Sebacina* sp. 2	GQ985423	*Sebcina* sp. (AF465191)	536/536 (95.9)
ECM16	*Sebacina* sp. 3	GQ985424	*Sebcina* sp. (DQ974768)	532/529 (97.4)
ECM17	*Sebacina* sp. 4	GQ985425	*Sebcina epigaea* (AF490397)	531/511 (91.6)
ECM18	Helotiaceae sp.	GQ985426	Helotiaceae sp. (EF619696)	463/465 (90.7)
ECM19	Helotiales sp.	GQ985427	Helotiales sp. (DQ182427)	464/464 (99.8)
ECM20	Pezizaceae sp. 1	GQ985428	Pezizaceae sp. (DQ974749)	565/612 (90.2)
ECM21	Pezizaceae sp. 2	GQ985429	Pezizaceae sp. (DQ974687)	556/555 (95.7)
ECM22	Pyronemataceae sp.	GQ985430	Pyronemataceae sp. (EU726302)	475/477 (95.6)
ECM23	*Tarzetta* sp.	GQ985431	*Tarzetta* sp. (EU326167)	528/488 (72.7)
ECM24	*Tuber* sp. 1	GQ985432	*Tuber* sp. (EU326690)	523/575 (87.2)
ECM25	*Tuber* sp. 2	GQ985433	*Tuber furfuraceum* (FJ176920)	503 /521 (85.5)
ECM26	*Tuber* sp. 3	GQ985434	*Tuber* sp. (DQ898183)	559/554 (80.6)

*ITS length of ECM fungi / reference taxa (% similarity)

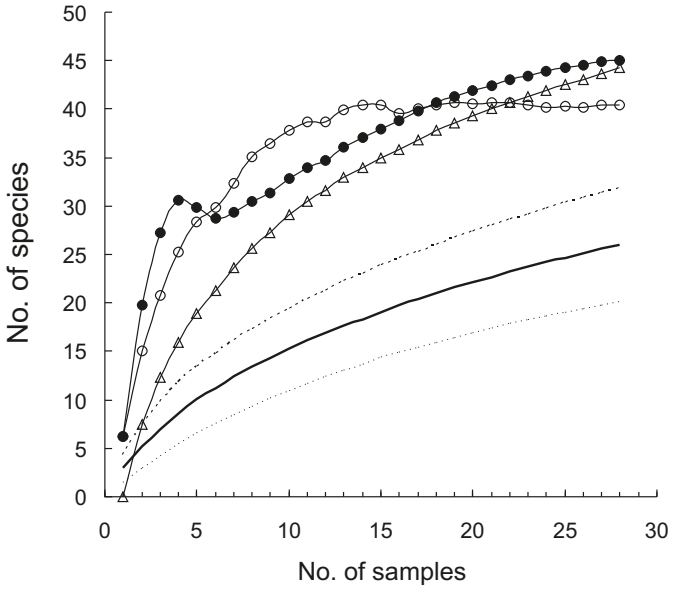
Fig. 1. Ectomycorrhizal fungus community structure observed in *Pinus tabulaeformis*. Numbers above the bars represent the number of samples in which the type was observed. Species data are ranked by importance value. (*a*) Sample collected in June, (*b*) sample collected in September.



to ECM morphological characteristics, is time-consuming and usually not sufficient for species recognition (Gardes and Bruns 1993; Bruns et al. 1998). Therefore, this has long represented a considerable limitation to our understanding of ECM fungal diversity and functioning in natural ecosystems (Debaud et al. 1999; Kretzer et al. 2004; Riviere et al. 2007).

Advances in molecular techniques have allowed consistent identification of ECM fungi, and has facilitated research in natural ecosystems (Smit et al. 2003; Peter et al. 2008). PCR-RFLP combined with sequencing has particularly proven to be an easy and feasible method to provide rapid and accurate identification of fungal taxa from ECM roots, and has greatly increased our understanding of the diversity and

Fig. 2. Minimum species richness estimates and species accumulation curves of ectomycorrhizal fungi on Chinese pine with increasing samples. Line, species accumulation curve; broken lines, 95% confidence interval of the species accumulation curve; open circles, Chao2 curve; filled circles, ICE curve; open triangles, Jackknife2 (n = 1000).



composition of belowground ECM fungal communities in forest ecosystems (Horton and Bruns 2001; Nouhra et al. 2005; Peter et al. 2008).

Chinese pine, *Pinus tabulaeformis* Carr. occurs naturally in 12 provinces in northern China (31°N–44°N, 101°30′E–124°45′E) and is one of the most widely distributed *Pinus* species in China (Xu 1990). Chinese pine is also an important forestation tree species for timber production, and water and soil conservation, with the characteristics of drought resistance and the ability to grow in poor soil (Xu 1990). It therefore plays an essential role in the development and stability of forest resources and ecological function. There have been many studies of ECM fungal communities in conifer-dominated ecosystems (Peter et al. 2001; Palfner et al. 2005; Iwański and Rudawska 2007). However, little is known about ECM fungal communities of *P. tabulaeformis*.

The basic aim of the present study was to determine the belowground ECM fungal community composition of *P. tabulaeformis* and to obtain a quantitative estimate of the relative contributions of each ECM type to community structure. The root system of *P. tabulaeformis* was collected from Dongling Mountain, the Beijing Forest Ecosystem Research Station of the Chinese Academy of Sciences, and the ECM fungi were identified by a combination of stereomicroscope-based morphotyping and molecular techniques.

Materials and methods

Study site

This study was conducted at *P. tabulaeformis* forest of Dongling mountain, the Beijing Forest Ecosystem Research Station of the Chinese Academy of Sciences, 117 km west of Beijing (39°58′N, 115°26′E). The forest is ca. 30 years old and is located at a mean altitude of 1240 m a.s.l. The mean annual temperature is 2–7 °C and the mean annual precipitation is ca. 500 mm. Other trees in this forest include *Quercus liaotungensis* Koidz. and *Fraxinus chinensis* var. *rhynchophylla* Hemsl. Shrubs in this forest include *Ulmus macrocarpa* Hance, *Amygdalus davidiana* (Carr.) C. de Vos ex Henry, *Armeniaca sibirica* (L.) Lam., *Rhamnus utiliz* Decne., *Lespedeza bicolor* Turcz., *Spiraea trilobata* L., *Leptopus chinensis* (Bunge) Pojark., and *Deutzia parviflora* Bunge. Herbs in this forest include *Carex humilis* Leyss., *Dendranthema lavandulaefolium* var. *seticuspe* (Maxim.) Shih, *Adenophora paniculata* Nannf., *Rabdosia japonica* (Burm. F.) Hara, *Viola variegate* Fisch. ex Link, *Erysimum bungei* (Kitag.) Kitag., *Aleuritopteris argentea* (Gmel.) Fee, *Lathyrus odoratus* Linn., *Spodiopogon sibiricus* Trin., *Deyeuxia arundinacea* (L.) Beauv, and *Thalictrum minus* L. var. *hypoleucum* (Sieb. et Zucc.) Miq. The individuals of *P. tabulaeformis* are more than 90% of the total individuals of trees, and the other trees are less than 10% in this forest.

Ectomycorrhizal root sampling and morphotyping

A sampling site (200 m × 300 m) was established in the *P. tabulaeformis* forest. A total of 28 soil blocks (20 cm × 10 cm, to 20 cm deep) were taken randomly by a shovel from ca. 1 m distance to trunks of *P. tabulaeformis*. Of these, 14 samples were collected on 1 June and 22 September 2007, respectively. Root samples were placed in plastic bags and stored at 4 °C. Roots of *P. tabulaeformis* are brown to red-brown and have a special smell, as well as typical dichotomous and coralloid ectomycorrhizas, which conspicuously distinguish *P. tabulaeformis* roots from the other ECM hosts such as *Q. liaotungensis* in this forest.

After soaking for several hours in tap water, each root sample was then washed free of soil material over a 380 μm sieve in running tap water. Fine roots (<2 mm diam.) were picked manually from the washed sample and were trimmed into ca. 2 cm long sections. The mycorrhizal system was examined under a SMZ-B2 stereomicroscope (Chongqing Optec Optical Instrument Co., Ltd., Chongqing, China). All viable lateral short roots covered by fungal mantle were classified as ectomycorhizae. The presence of a Hartig net was used to confirm ECM status of root tips when questionable. Moribund and dead ECM tips were distinguished from live tips, as they lacked turgidity or were shrunken in appearance and with a dark discoloration of the mantle and cortex. Live ECM root tips were then divided into morphotypes based on general appearance, such as color, luster, size, ramification type, texture, as well as the presence and color of emanating hyphae and rhizomorphs. The tip number of each morphotype in each root sample was counted. For each morphotype, up to three healthy ECM tips were placed in a 1.5 mL microcentrifuge tube and stored at –20 °C for DNA extraction.

DNA extraction and PCR

The DNA extraction protocols followed that of Gardes and Bruns (1993), with minor adjustment. The 5.8S gene and flanking internal transcribed spacer (ITS1 and ITS2) regions of rDNA from each ectomycorrhiza was amplified by PCR using the primer pair ITS1-F/ITS4 (Richard et al. 2005) in a PTC 100™ programable thermal controller (MJ

Research, Waltham, Massachusetts, USA). The final 50 μL reaction mixture contained 1 μL of template DNA, 1× PCR buffer, 2.0 mmol/L $MgCl_2$, 0.2 mmol/L 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.

PCR-RFLP analysis

Ten microlitres of each PCR product was combined with 7.5 μL sterile Milli-Q™ water, 0.5 μL restriction endonuclease; either *Hinf*I, *Bsu*RI, *Hha*I, or *Alu*I (MBI Fermentas, Vilnius, Lithuania), 2 μL buffer, and stored overnight at 37 °C. RFLP products were size-fractionated on 2% agarose gels. The gels were stained with ethidium bromide and photographed by AlphaImager™ 2200 (Alpha Innotech Corporation (now Cell Biosciences), Santa Clara, California, USA) under UV light. RFLP band sizes were estimated by comparison with a standard 100 base pair (bp) molecular weight ladder. The fragment length error was ±3% as suggested by Glen et al. (2001). Molecular identifications were repeated at least once for each morphotype sample. Morphotypes with the same RFLP patterns were considered to be formed by the same fungus species.

DNA sequencing and phylogenetic analysis

The PCR products were purified using UNIQ-10 PCR production purification kit (Shanghai Sangon Biological Engineering Technology & Services Co., Ltd, Shanghai, China) according to manufacturer's instruction. One representative PCR product of each RFLP pattern was sequenced using ABI Prism 3700 Genetic Analyzer (Applied Biosystems, Carlsbad, Calif.).

The ITS (ITS1, 5.8S, ITS2) rDNA sequences generated in this study were used as query sequences to search for similar sequences in GenBank and UNITE (Kõljalg et al. 2005) with BLAST program. The ITS sequences of ECM morphotypes sequenced in the present study and reference taxa obtained from GenBank were aligned with ClustalX 1.81 (Thompson et al. 1997) and the results were adjusted manually where necessary to maximize alignment. The alignment data were performed for maximum-parsimony analysis in PAUP* 4.0b1a, using the heuristic search algorithm with tree-bisection–reconnection branch swapping (Swofford 2002). For each search, 1000 replicates of random stepwise sequence addition were performed and 100 trees were saved per replicate. All characters were equally weighted and unordered, and gaps were treated as missing data.

A value of 97% ITS region identity was used as a DNA barcoding threshold. This cut-off level is based on error rates generated by PCR and inter-specific variability within ITS regions as employed in previous studies using ITS sequences for ECM fungal identification from roots and soil (Tedersoo et al. 2008).

Data analysis

Relative frequency is the total number of occurrence of a species divided by the total number of occurrence of all taxa. The relative abundance is the root tip number of a species divided by the total root tip number of all taxa. The relative frequency and relative abundance for each species were then summed for an importance value indicated as a percentage (Horton and Bruns 2001).

A computer program, Estimates version 8, was used to calculate species accumulation (rarefaction) curves and the minimum richness estimators, Chao2, ICE, and Jackknife2 (Colwell 2006).

Results

Ectomycorrhizal fungal community composition

Thirty-five ECM morphotypes were recovered according to ECM morphological characters. A total of 26 unique ITS-RFLP patterns were detected from the ECM morphotypes based on the analyses of four restriction enzymes, i.e., *Hinf*I, *Bsu*RI, *Hha*I, and *Alu*I.

Based on the phylogenetic analyses and sequence similarity comparison of ITS regions (Table 1; see also supplementary data,[2] Figs. S1, S2), a total of 26 ECM fungi including 17 Basidiomycetes and 9 Ascomyceyes were detected. Of these ECM fungi, six were identified to species level. Fourteen ectomycorrhizas were identified to genus level, 5 to family level, and one to order level.

Tomentella, *Sebacina*, and *Tuber* were the most common genera, and represented 36.8%, 15.8%, and 15.8% of the total number of ECM fungus taxa detected in September and 30.8%, 7.7%, and 7.7% of total taxa in June, respectively. *Tomentella* was found in 11 soil samples collected in September and 8 collected in June, *Sebacina* was found in 5 soil samples collected in September and 4 collected in June, and *Tuber* was in 3 soil samples collected in September and 4 collected in June. Atheliaceae sp. (found in 8 samples collected in September, 7 in June), *L. deliciosus* (found in 1 sample collected in September, 9 in June), *T. ferruginea* (found in 6 samples collected in September, 5 in June), and *Tomentella* sp. 3 (found in 5 samples collected in September, 1 in June) were dominant. Importance values for the four species were 49.25%, 3.40%, 26.10%, and 20.9%, respectively, in September, and 40.14%, 45.93%, 21.03%, and 4.35%, respectively, in June (Fig. 1). Twelve ECM fungi were only found in one soil sample. An average of 2.9 ECM fungal taxa were detected from each soil sample with a range of one to six.

The minimum richness estimators Chao2, Jackknife2, and ICE predicted that 40, 44, and 45 ECM fungi were associated with Chinese pine, respectively (Fig. 2). Neither accumulation curves nor minimal species richness estimates approached an asymptote with increasing sample size, except for Chao2 (Fig. 2).

Discussion

Ectomycorrhizal fungus species diversity

A total of 26 taxa of ECM fungal species forming a phy-

[2] Supplementary data for this article are available on the journal Web site (http://botany.nrc.ca).

logenetically diverse community were associated with *P. tabulaeformis* in this study. This is in agreement with some previous studies of ECM fungal diversity belowground surveys in conifer stands (Peter et al. 2001). For examples, 25 ECM fungal species were reported in a 100-year-old *Picea abies* (L.) Karst. stand in Sweden (Dahlberg et al. 1997) and in two stands with mature *Abies alba* Mill. situated in the Gran Sasso-Laga National Park (Comandini et al. 1998), respectively. A total of 101 ECM fungal taxa were detected in a mixed-conifer forest with four ECM tree host species *Abies concolor* (Gord et Glend) Lindl., *Abies magnifica* Murr., *Pinus jeffreyi* Murr., and *Pinus lambertiana* Dougl. in the Sierra National Forest, California, USA (Izzo et al. 2005). Furthermore, higher numbers of ECM fungi (34 species) were reported in a *P. abies* field established in 1994 in Central Finland (Korkama et al. 2006), and 123 taxa of ECM fungi were found from root tips of three host species *Eucalyptus regnans* F. Muell, *Nothofagus cunninghamii* (Hook.) Oerst., and *Pomaderris apetala* Labill. in a Tasmanian wet sclerophyll forest (Tedersoo et al. 2008).

We detected low species richness (26 taxa) associated with *P. tabulaeformis* compared with the minimal species richness estimates (40–45 taxa) by Chao2, ICE, and Jackknife2. This is similarly to previous findings (e.g., Tedersoo et al. 2008) of lower species numbers of ECM fungi detected, than were predicted using EstimateS 8. For example, Tedersoo et al. (2007) found that 30 species were associated with native trees in an area of Seychelles, and the minimal species richness estimators Chao2 and Jackknife2 predicted 51.2 and 57.4 species, respectively.

Ectomycorrhizal fungus community composition

There were a few dominant ECM fungi, i.e., Atheliaceae sp., *T. ferruginea*, *L. deliciosus*, and *Tomentella* sp. 3, while the majority (22 fungal taxa) of the ECM fungi was rare in our study. This pattern is an inherent structure for most ECM communities (Taylor 2002).

Species of Atheliaceae have been commonly found to form ectomycorrhizas with conifers in previous studies (Parrent and Vilgalys 2007; Peter et al. 2008). Of these fungi, *Tylospora fibrillosa* (Atheliaceae) was the most dominant species associated with *Picea sitchensis* (Palfner et al. 2005), and *Amphinema byssoides* (Atheliaceae), as a dominant species, formed ectomycorrhizae with conifer seedlings *Pinus contorta* Doug. ex Loud. var. *latifolia* Englem., *Picea glauca* (Moench) Voss, and *Picea mariana* (Mill.) B.S.P. (Gagné et al. 2006). The other ECM fungi found in our study have been reported to form typical ectomycorrhizas with *Pinus* in previous studies (Barroetaveña et al. 2007; Parrent and Vilgalys 2007; Obase et al. 2009). However, *Russula* species, extensively distributed in forest ecosystems as ECM fungi (Horton and Bruns 2001), was not detected in *P. tabulaeformis* forest in our study.

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