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Diversity and composition of ectomycorrhizal community on seedling roots: the role of host preference and soil origin

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Abstract As the main source of inocula, ectomycorrhizal (ECM) fungal propagules are critical for root colonization and seedling survival in deforested areas. It is essential to know factors that may affect the diversity and composition of ECM fungal community on roots of seedlings planted in deforest areas during reforestation. We quantitatively evaluated the effect of host plant and soil origin on ECM fungal propagule community structure established on roots of *Castanopsis fargesii*, *Lithocarpus harlandii*, *Pinus armandii*, and *Pinus massoniana* growing in soils from local natural forests and from sites deforested by clear-cut logging in the 1950s and 1960s. ECM root tips were sampled in April, July, and October of 2006, and ECM fungal communities were determined using ECM root morphotyping, internal transcribed spacer (ITS)-RFLP, and ITS sequencing. A total of 36 ECM fungal species were observed in our study, and species richness varied with host species and soil origin. Decreased colonization rates were found in all host species except for *L. harlandii*, and reduced species richness was found in all host species except for *P. armandii* in soil from the deforested site, which implied the great changes in ECM fungal community composition. Our results showed that 33.3% variance in ECM fungal community composition could be explained by host plant species and 4.6% by soil origin. Results of indicator species analysis demonstrated that 14 out of 19 common ECM fungal species showed significant preference to host plant species, suggesting that the host preference of ECM fungi was one of the most important mechanisms in structuring ECM fungal community. Accordingly, the host plant species should be taken into account in the reforestation of deforested areas due to the strong and commonly existed host preference of ECM fungi.

Keywords Ectomycorrhizal fungal community · Host preference · Indicator species analysis · Fungal propagule · Redundancy analysis

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Introduction

Ectomycorrhizal (ECM) association is a kind of fungus–plant symbiosis in which the ECM fungus facilitates the uptake of nutrients and water to the host plant, and in return, the plant provides photosynthates as energy source to the fungi (Smith and Read 2008). ECM fungi play critical roles in the establishment, survival, and fitness of ECM plants, and would thus positively affect the early establishment of vegetation as well as the long-term dynamics of plant communities (Horton et al. 1999; Dickie and Reich 2005; Nara 2006).

Many ectomycorrhizal trees are dominant species in natural forests and are commonly used in reforestation in disturbed areas, and the diversity and composition of ECM fungal propagules would be critical for seedling establishment. Forest logging reduces or even cuts off the carbon input into the roots, and will hence change the structure of ECM fungal community (Jones et al. 2003). The ECM fungal community may include symbiotic structures in plant roots as well as fungal propagules in the soil. Since the number of active ectomycorrhiza on the dying roots would drop to zero within two to several years after clear-cut logging (Hagerman et al. 1999), fungal propagules (typically of spore and sclerotia) would be the main source of inoculum in post-disturbance sites especially where reforestation has been delayed for years. While some studies have shown significant changes of ECM fungal communities after clear-cutting (reviewed by Jones et al. 2003), it is difficult to address if such changes in ECM fungi communities were caused by shifts in the propagule community or removal of ectomycorrhizal roots only. Seedling bioassay using field-collected soils as growth media could determine the ECM propagule community in the deforested or forest areas (Taylor and Bruns 1999; Brundrett and Abbott 1994; Jones et al. 1997; Izzo et al. 2006), and would therefore be helpful in evaluating the impact of soil origin on diversity and structure of ECM community on seedling roots.

Compatibility between fungi and host is important for successful settlement of seedlings. Mycorrhizal fungal species may differ in host spectrums, from narrow (typically genus limited) to broad (across families and orders) (Molina et al. 1992). Since exclusive host specificity is difficult to prove, host preference, which is defined as the significantly biased occurrence of a mycorrhizal fungal species on a particular host plant, is used in field studies (Ishida et al. 2007; Tedersoo et al. 2008). It has been documented that host preference was a determinant factor in structuring the ECM fungal community in different forest ecosystems all over the world (e.g., Kennedy et al. 2003; Richard et al. 2005; Ishida et al. 2007; Tedersoo et al. 2008, 2009; Morris et al. 2009). These results, however, are mainly based on data from mature forests where ECM communities on plant roots may be affected by both mycorrhizal species composition on the roots of neighbor plants and fungal propagules in the soil (Ishida et al. 2007; Kennedy et al. 2003; Tedersoo et al. 2008). While some earlier results from seedling bioassays have indicated that different hosts may selectively associate with different cluster of ECM fungi in the propagule community (Massicotte et al. 1999), the limitation in identification methods and lack of statistical analysis make it difficult to show the "actual" structure of the propagule community of ECM fungi.

Evergreen broad-leaved forests, characterized by a diversity of ECM host plants, are widely distributed in subtropical China. However, a large portion of these subtropical forests were destroyed by clear-cut logging in the 1950s and 1960s (Wang et al. 2007). Great efforts for the reforestation and ecological restoration in these disturbed areas have recently been of great concern by local communities. Considering the fact that many dominant plant species in the subtropical forests of China are obligatory ECM hosts, the successful establishment of symbiotic associations between plant roots and ECM fungi should be critical for seedling survival during forest restoration. It is therefore essential to know the structure of ECM fungal propagule communities both in deforested sites and local forests as well as factors that may affect the diversity and composition of ECM fungal communities on roots of planted seedlings.

Two broad-leaved trees (*Castanopsis fargesii* Franch. and *Lithocarpus harlandii* Rehder.) and two pine trees (*Pinus armandii* Franch. and *Pinus massoniana* Lamb.) were grown in soils from local natural forest stand and from sites deforested by clear-cut logging in the 1950s and 1960s. Species richness and community composition of ECM fungi in seedling roots were determined during the growing season in 2006. We use indicator species analysis to determine and statistically test the preferences of the fungi among the alternative hosts, and multivariate analysis of variance (MANOVA, computed by redundancy analysis) to model the variance of ECM fungal community composition explained by the experimental factors. Our objective was to evaluate the role of host and soil origin in determining the ECM fungal community structure established on seedling roots.

Materials and methods

Site descriptions

The study site (30°45′–31°22′ N, 103°25′–103°47′ E, ~780 m above sea level) is located in Dujiangyan city, Sichuan province, Southwest China, with a mean annual precipitation of 1,244 mm and temperature of 15.2°C. The main type of vegetation is subtropical evergreen broad-leaved forests, and tree growth resumes in March, peaks in June to August, and slows down in November (Liang et al. 2007).

Soil collection and experimental design

Soils were collected from either a natural forest site or a deforest site. The natural forest site, about 80 to 90 years old, is located within the Banruosi Permanent Forest

Research Plot of the Chinese Academy of Sciences established in August 2000, in Dujiangyan city, Southwest China. Within this 6.4 ha forest stand, ECM plants include nine species from four genera (*Quercus*, *Lithocarpus*, *Cyclobalanopsis*, and *Castanopsis*) of Fagaceae and a subtropical conifer (*P. massoniana*). Previous study has shown that the major ECM fungi in the natural forest are members of Amanitaceae, Russulaceae, and Boletaceae (Liang et al. 2007). The deforested site, about 2 km away from the natural forest site, had been a natural forest stand before clear-cutting in the late 1950s. Abandoned until the 1970s, this site was modified into terraced plantations of orange, apple, gingko, and crops, then fallowed since the 1990s (Du et al. 2008). A variety of non-ECM plants such as *Saccharum arundinaceum*, *Dicranopteris dichotoma*, *Rhus chinensis*, *Aralia chinensis*, *Ilex chinensis*, and *Rubus* spp. were common species in the deforested site.

Soils (20 cm in depth) were collected from three locations within the natural forest or the deforested site after removal of litter and ground cover plants in late April of 2005. The fresh soil used for growth media was sieved (2 cm) to remove stones and debris. Soil samples from the three locations within each site were mixed, and used as growth media for seedling growth. The site for seedling cultivation was formerly an agricultural land for rice before our experiment. The site was about 0.7 ha, and about 0.5 km away from the natural forest site and 2 km from the deforested site.

Four plant species were used in our experiment, of which *C. fargesii* and *L. harlandii* are late-successional broad-leaved species, and *P. armandii* and *P. massoniana* are pioneering conifers. Seeds were surface sterilized with 70% alcohol for 3 min and then soaked in 2% (*v*/*v*) sodium hypochlorite solution for 10 min for *P. armandii* and *P. massoniana*, and 20 min for *C. fargesii* and *L. harlandii* according to Bécard and Piché (1992). Sterilized seeds were rinsed using distilled water and transferred to pre-moistened filter paper in Petri dishes (18 cm diameter) to germinate at 18–24°C for 3 weeks. The germinated seeds were transferred into a new Petri dish and stored at 6 to 8°C in refrigerator before planted into the pots. For each host plant species, six pre-germinated seeds were sowed and four growing seedlings were kept in one plastic growth pot (28 cm diameter and 32 cm height) containing the soils mentioned above. Five blocks were set up to provide replications in an open area located between the two soil sampling sites. Within each block, 24 treatments (2 soil origins × 4 plant species × 3 harvest dates) were arranged in a random split-plot design. There were three pots for each treatment resulted in a total of 360 pots (24 treatments × 3 pots × 5 blocks) from the five blocks. Sixty pots of autoclaved control (4 plant species × 5 blocks × 3 harvest dates) were also used in the experiment which contained autoclaved soil (deforested/forest=1:1, 121°C for 2.5 h). No irrigation was performed since rainfall was plentiful for plant growth during the experiment period (from May 2005 to October 2006). Fifteen pots were sampled for each tree species per treatment and five pots were sampled for the autoclaved soil control at each harvest date, and roots of two seedlings per pot were sampled and kept at 4°C until ECM morphotyping.

Morphotyping of ectomycorrhiza

Root tips of two seedlings per pot were pooled up to give a sample of 100 root tips for morphotyping according to the online DEEMY key (http://www.deemy.de). Distinctive characteristics including color, luster, branching, texture, emanating hyphae, fungal hyphae mat, and rhizomorphs were recorded with a digital camera fixed to a dissecting microscope (SZH 10, Olympus, Japan). Root tip numbers from each morphotype were recorded, and ambiguous characters were rechecked after further molecular identification (see below). Twenty root tips of each morphotype from every pot were preserved in a plastic centrifuge tube with 650 ml cetyl trimethyl ammonium bromide (CTAB) buffer (100 mM Tris, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% CTAB, and 0.2% β-mercaptoethanol) at −20°C before DNA extraction.

DNA extraction, amplification, and sequencing

Three to five root tips per morphotype from each sample in the CTAB buffer were transferred to a plastic centrifuge tube (1.5 ml) containing 20 μl CTAB buffer, and then crushed by a screw drill, finally, an additional 630 μl CTAB buffer was added and gently shaken. The subsequent incubation, extraction, centrifugation, and purification procedure followed the modified method of Gardes and Bruns (1993). The internal transcribed spacer (ITS1, 5.8S, ITS2) region of ribosomal DNA was amplified by polymerase chain reaction (PCR) using the primers ITS1F and ITS4 (Gardes and Bruns 1993). The PCRs mixture (25 μl) included 1 μl template DNA, 0.2 mM deoxyribonucleotides, 2.5 μl 10× PCR buffer (Takara, Otsu, Japan; MgCl2 included), 0.2 μM each primer, 0.5 mg ml^{-1} bovine serum albumin, and 1.5 U Taq DNA polymerase (Takara, Otsu, Japan). Samples were amplified using a PTC-200 thermal cycler (Gene Amp, Waltham, MA, USA). The PCR protocol was as follows: initial denaturation of 94°C for 5 min followed by 30 cycles of 40 s (94°C), 40 s (55°C), 40 s (72°C), followed by a final extension of 72°C for 10 min. PCR products of ITS region were characterized by restriction enzyme digestion (AluI, DpnII, HinfIand HaeIII, New England Biolabs Inc. Beverly, MA, USA). Five microliters of restriction fragment length polymorphism

(RFLP) products were examined by electrophoresis at 70 V for 2.5 h in a 1.8% (*w*/*v*) agarose gel in 0.5× TBE buffer. Gels were stained with ethidium bromide (0.5 mg/ml) and photographed under UV light. Each unique RFLP type was directly sequenced or cloned according to the PROMEGA manual (Promega Inc., Madison, WI, USA) before sequencing. DNA was sequenced with a 3730 DNA Capillary Sequencer (Applied Biosystems, USA) by the Sunbiotech Co. Ltd. (Beijing, China).

Sequence analyses

Sequences with identity less than 97% in base pairs across the ITS region were considered as sequences of different species. These sequences were first compared with the sequences of identified sporocarps in a local database in Bioedit 7.1, and then with sequences deposited in the UNITE (Koljalg et al. 2005) and the GenBank database. The maximum likelihood method was used to infer the phylogenetic placement of fungal sequences in reference to known sequences from the UNITE and GenBank with PhyML 3.0.1 using GTR + I model (Guindon and Gascuel 2003).

Statistical analyses

ECM fungal identity of each RFLP type was determined by ITS sequencing. Species richness per pot was defined as the number of species in a given growth pot. Colonization rate was expressed as the percentage of root tips colonized. Relative abundance is the number of ECM root tips of a fungal species divided by the total number of ECM root tips for all species in a host plant species (or soil origin). Frequency is the number of samples of an ECM fungal species that occurred divided by the total number of samples in a host plant species (or soil origin). Relative frequency is the frequency of individual species divided by the sum of frequencies for all fungal species. Important value is the mean of relative frequency and relative abundance of fungi. We used species accumulation curves to assess whether we have adequately sampled the community or not, and to compare the ECM fungal richness among host plant species and soil origins. Calculations of species accumulation curves were implemented by function specaccum() available in the vegan package using the "random" method. This method can be used to find the mean accumulated species and its standard deviation from random permutations of the data, or subsampling without replacement (Gotelli and Colwell 2001). A two-way repeated measure ANOVA was used to test the effect of host, soil origin, and their interaction on ECM fungal species richness in each growth pot.

A three-way or two-way MANOVA by redundancy analysis (RDA) was used to test the effect of soil origin, host species, harvest date, and their interactions on ECM fungal community composition using the averaged abundance data table. The MANOVA was computed by the multivariate method of RDA, as proposed by Legendre and Anderson (1999). This method provides permutation tests for the main effect and interaction terms and the form of the test is more appropriate than parametric tests for community composition data, if the data are not normally distributed. In the MANOVA model, each main factor (host species and soil origin) was coded using Helmert contrasts. Since these factors were crossed and the design was balanced, the Helmert variables representing the main factors were orthogonal to each other. Their interaction, which was represented by variables that were the products of the variables coding for the main factors, were also orthogonal to the main factors. To test the effect of the main factors and their interaction on fungal community composition, Helmert variables corresponding to the factor or interaction of interest were used as the explanatory matrix, while the remaining factors and interactions in the Helmert matrix were used as covariables. The fungal species abundance data were Hellinger-transformed before each RDA test, since this transformation is appropriate for community composition response data and gave the best results in most cases for our data. That transformation consists in dividing each abundance value by the sum of the fungus abundances in a row to remove the effect of the total number of fungus isolates per row, and taking the square root of the result (Legendre and Gallagher 2001). The statistical significance of each main factor and interactions were assessed with 9999 permutations by the function rdaTest() in the rdaTest package for R (Legendre and Durand 2010).

Indicator species analysis was carried out to assess the host and soil substrate preference of ECM fungus (De Cáceres and Legendre 2009; De Cáceres et al. 2010). Indicator species analysis was conducted for the ECM fungal species that had relative abundances >1% on any host tree species. Among the available indices, we used the point-biserial correlation coefficient (r_{pb}), which is available in the R-language function multipatt() of the indicspecies package. The r_{pb} index is the Pearson correlation computed between a quantitative variable of species abundance data and a binary variable indicating whether the site belongs to a site group combination under study, or not. This index is defined as:

$$r_{pb} = \left(N \times a_p - a \times N_p\right) / \sqrt{\left(N \times 1^2 - a^2\right) \times \left(N \times N_p - N_p^2\right)}$$

where N is the total number of samples; N_p, the number of sites belonging to the target group; a_p, the sum of the

abundance values of the species within the target group; *a*, the sum of the abundance values of the species over all samples; and *l*, the norm of the vector abundances of the species (see De Cáceres and Legendre 2009, for details). A higher r_{pb} value denotes a stronger association strength between fungus and hosts. A permutation test is used to determine if a species is statistically significantly associated with a site group under the null hypothesis of no relationship. Under this null hypothesis, the observation of a species at a site belonging to the target site group is by chance only. Three independent tests had been run for the preference of ECM fungal species: to hosts, soil origins, and seasons.

All statistical tests were performed in R 2.11.0 (R Development Core Team 2009) with package vegan 1.17-2 (Oksanen et al 2010), rdaTest 1.6 (Legendre and Durand 2010), and indicspecies (De Cáceres and Legendre 2009; De Cáceres et al. 2010; see http://sites.google.com/site/miqueldecaceres/).

Results

Colonization rates of ECM fungi

The two-way repeated measure ANOVA showed that hosts significantly affected ECM colonization rates ($P<0.001$, Fig. 1a). The difference of ECM colonization rates between soils varied with host species. Colonization rates were significantly higher in forest soil than in the deforested soil for *C. fargesii*, *P. armandii*, and *P. massoniana* species, but similar colonization rate was found in these two soils for *L. harlandii* (Fig. 1a).

ECM community established on the four hosts

A total of 35,300 root tips from 353 pots were examined and 30,068 of which were ectomycorrhizal. Three to 11 ECM morphotypes were found from each pot and subjected to DNA extraction and PCR. And totally 51 unique ITS-RFLP types were obtained from 1,240 PCR products. These unique ITS-RFLP types were identified as 36 ECM fungal taxa according to ITS sequences. Of these fungi, 25 were obtained from *C. fargesii*, 20 from *L. harlandii*, 10 from *P. armandii*, and 29 from *P. massoniana*. Results of fungus–host association strength test by indicator species analysis revealed that 14 out of 19 common ECM fungal taxa, accounting for 90.8% of total number of ECM root tips, showed significant host preference (Table 1). Within the 14 ECM fungi, Thelephoraceae sp.1 significantly preferred *C. fargesii*, *Thelephora* sp., *Tomentella* sp.3, *Hymenogaster tener*, *Scleroderma* sp., Thelephoraceae sp.2, Thelephoraceae sp.6, and Thelephoraceae sp.4 to *L. harlandii*, and *Suillus* cf. *placidus* to *P. massoniana*. In addition, Tricholomataceae sp. significantly preferred both *C. fargesii* and *L. harlandii*, *Cenococcum geophilum* to both *C. fargesii* and *P. armandii*, Pezizales sp. to both *C. fargesii* and *P. massoniana*, and Tuberaceae sp.2 and *Wilcoxina mikolae* to both *P. armandii* and *P. massoniana* (Table 1).

The host plant significantly affected ECM fungal species richness (repeated measure ANOVA, $P<0.01$). Species richness was significantly lower in *P. armandii* than in the other three hosts in the forest soil in April and July. In October, the difference in species richness among hosts was more pronounced in the deforested soil (Fig. 1b). The three-way MANOVA by RDA on ECM fungal community composition indicated that the main effect of host plant was significant ($P<0.001$), and a large portion of variance (33.3%) was explained by host (Table 2). However, due to the significant effect of the interaction among host and the other two factors (soil origin and harvest date), we conducted all possible one-way MANOVA to clarify this effect, and found the host effect was so strong that it was consistently significant within each of the two soil origins throughout the three harvest dates (Table 3). The variance in ECM fungal community composition explained by the host plant ranged from 14.6% to 35.7% in different soils and sampling dates ($P=0.001$; Table 3).

Accumulation curves of ECM fungal taxa showed that *C. fargesii*, *L. harlandii*, and *P. massoniana* captured higher proportion of ECM fungi species than *P. armandii* in both soils (Fig. 2a–d). Accumulated numbers of ECM fungal species were similar between the two soils for *C. fargesii* (Fig. 2a), and obviously lower in the deforested soil than in the natural forest soil for *L. harlandii*, *P. massoniana*, and *P. armandii* (Fig. 2b–d).

ECM community from natural forest and deforested sites

Within the 36 ECM fungal taxa identified from the two soil origins, 32 taxa were from the forest soil and 28 species from the deforested soil (Fig. 3). Of these taxa, 24 were shared between the two soil origins. The results of fungi–soil origin association strength test by indicator species analysis revealed that Thelephoraceae sp.1, *C. geophilum*, Tricholomataceae sp., *H. tener*, and Tuberaceae sp.2 significantly preferred the forest soil. In contrast, *W. mikolae*, *Thelephora* sp., *Tomentella* sp.4, and *Thelephora* sp. significantly preferred the deforested soil (Fig. 3). When autoclaved control was considered in the indicator species analysis, most soil-preferred ectomycorrhizal fungus (EMF) species were similar except that three species (*H. tener*, *Thelephora* sp., and *Tomentella* sp.4) with marginal significance ($P>0.01$) were excluded (Table 6). EMF species showing significant preference to

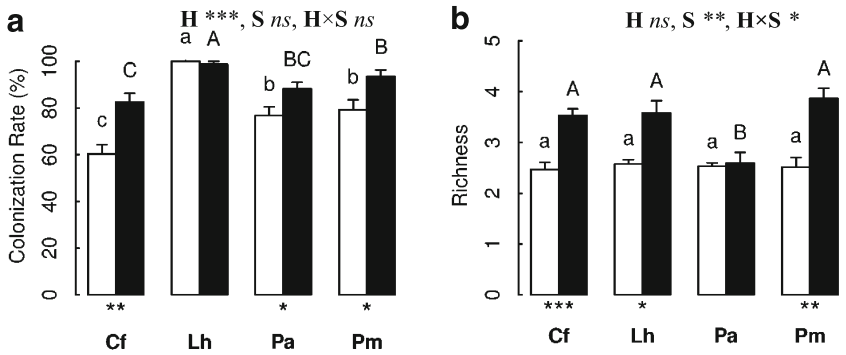

Fig. 1 Colonization rates (**a**) and species richness (**b**) of ECM fungi by host and soil origin. Multiple comparisons of group means among hosts were carried out within each soil origin with LSD by Kruskal–Wallis tests. *White and black bars without shared letters* denote significant differences among host plants within the degrade soil (*lowercase*) and the forest soil (*uppercase*), respectively. Effects of soil origin on ECM fungi richness and colonization rate for a given host within each harvest date was tested by the two-tailed *t* test. Significant effects of soil origin were marked with *asterisks below the rectangle* of each host (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$). Results of two-way repeated measure ANOVA showed the effects of host (*H*), soil origin (*S*), and their interactions ($H \times S$) (*ns*, $P > 0.05$; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$). Abbreviations Cf, Lh, Pa, and Pm correspond to hosts *C. fargesii*, *L. harlandii*, *P. armandii*, and *P. massoniana*, respectively

autoclaved control (Parmeliaceae sp.1 and *Laccaria amethystina*) and the combination of deforest and forest soils (Thelephoraceae sp.2 and Thelephoraceae sp.6) may indicate the composition difference between aerial dispersed spores reaching the study site and EMF propagules in soils of both sites.

The two-way repeated measure ANOVA showed that ECM fungal species richness was significantly affected by soil origin. Species richness in the forest soil was significantly higher than that in the deforested soil for all the hosts and harvest dates except for *P. armandii* in April and July (Fig. 1b). The results of three-way MANOVA by

Table 1 Indicator species analysis showing host preference of ECM fungi species which had relative abundances >1% on any host species

Fungus	*C. fargesii*	*L. harlandii*	*P. armandii*	*P. massoniana*	r_{pb}	*P* values
Wilcoxina mikolae	0.006/0.024	0.001/0.011	**0.562/0.789**	**0.332/0.622**	0.643	*0.0001*
Thelephoraceae sp.1	**0.304/0.470**	0.086/0.256	0.150/0.300	0.154/0.378	0.204	*0.0006*
Cenococcum geophilum	0.107/0.578	0.083/0.533	**0.198/0.700**	0.052/0.378	0.291	*0.0001*
Tomentella sp.3	0.095/0.229	**0.168/0.389**	0.008/0.022	0.077/0.211	0.288	*0.0001*
Tricholomataceae sp.	**0.175/0.349**	**0.107/0.300**	–	0.002/0.011	0.387	*0.0001*
Thelephoraceae sp.6	0.013/0.036	**0.132/0.333**	0.007/0.022	0.036/0.089	0.360	*0.0001*
Thelephoraceae sp.4	0.023/0.048	**0.091/0.211**	–	**0.054/0.178**	0.226	*0.0003*
Scleroderma sp.	0.010/0.036	**0.106/0.278**	–	–	0.416	*0.0001*
Thelephoraceae sp.2	0.036/0.084	**0.062/0.189**	–	0.002/0.011	0.246	*0.0001*
Suillus cf. *placidus*	0.002/0.012	–	–	**0.091/0.200**	0.368	*0.0001*
Pezizales sp.	**0.041/0.072**	–	–	**0.042/0.133**	0.205	*0.0009*
Clavulina sp.	0.043/0.096	0.013/0.056	–	0.025/0.078	0.130	0.0647
Hymenogaster tener	0.021/0.048	**0.052/0.156**	–	0.007/0.022	0.231	*0.0001*
Tomentella sp.4	0.041/0.060	0.024/0.056	–	0.013/0.056	0.109	0.1827
Tuberaceae sp.2	–	–	**0.043/0.133**	**0.035/0.1**	0.232	*0.0005*
Thelephora sp.	0.001/0.012	**0.052/0.133**	0.012/0.044	–	0.263	*0.0001*
Sebacinales sp.	0.026/0.048	–	0.011/0.056	0.017/0.033	0.093	0.3265
Cortinariaceae sp.	0.030/0.060	0.004/0.011	–	0.003/0.011	0.157	0.0138
Sebacinaceae sp.1	–	–	0.004/0.011	0.017/0.056	0.155	0.0170

The figures in the table are frequency/relative abundance values of ECM fungi in corresponding hosts. The r_{pb} value in the table is showing for the fungus–hosts (bold) which have the highest $r_{pb,}$ in comparison with the remaining fungus–hosts combinations. Significant *P* values after Holm correction were shown in italics. Fungi corresponding to one or more than one bold cell entry and a significant *P* value denoted that it significantly preferred one or more than one host species

Table 2 Three-way MANOVA by RDA showing the effects of soil origin, host species, harvest date, and their interactions on ECM fungal community composition

	df	R^2	*F* values	*P* values
Host (H)	3	0.333	27.19	<0.001
Soil origin (S)	1	0.046	11.35	<0.001
Harvest date (D)	2	0.032	3.86	<0.001
H × S	3	0.062	5.08	<0.001
H × D	6	0.066	2.68	<0.001
S × D	2	0.016	1.92	0.002
H ×S × D	6	0.054	2.19	<0.001

RDA on ECM fungal community composition indicated that the two-way and three-way interactions among soil origins, hosts, and harvest dates were significant ($P<0.01$). So the effect of soil origin depended on both host and harvest date (Table 2). Two-way MANOVA analyses by RDA showed that the soil origin significantly affected the ECM community composition and the effect varied with host plant species (Table 4). Variance in ECM community composition explained by soil origin was highest in *P. armandii* (51.9%, $P=0.0001$), and lowest in *C. fargesii* (7.2%, $P=0.0061$). The interaction between soil origin and harvest date explained less than 2% variance in ECM community composition (Table 2).

ECM community composition in the three harvest date

Of the 36 ECM fungal taxa, 29, 31, and 25 taxa were detected in April, July, and October, respectively. According to results from indicator species analysis, *H. tener* and Pezizales sp. significantly preferred to occur in the earlier two harvest dates, and *Scleroderma* sp., *Suillus* cf. *placidus*, and Thelephoraceae sp.4 were significantly more abundant in the latest harvest date October. Tricholomataceae sp. preferred April and October while *Tomentella* sp.1 preferred July (Fig. 3).

Results of the three-way MANOVA by RDA on ECM fungal community composition indicated that although the main effect of harvest date was significant ($P=0.0001$), the variance in ECM fungal community composition explained by harvest date was low (3.2%), and the effect of harvest date depended much on the host (host × harvest date, 6.6%, $P=0.0001$; Table 2). Two-way MANOVA by RDA indicated that the ECM community composition was significantly affected by harvest date in *C. fargesii* (15.6%, $P<0.001$), *L. harlandii* (16.5%, $P<0.001$), and *P. massoniana* (16.9%, $P<0.001$), rather than in *P. armandii* (2.1%, $P=0.834$) (Table 4).

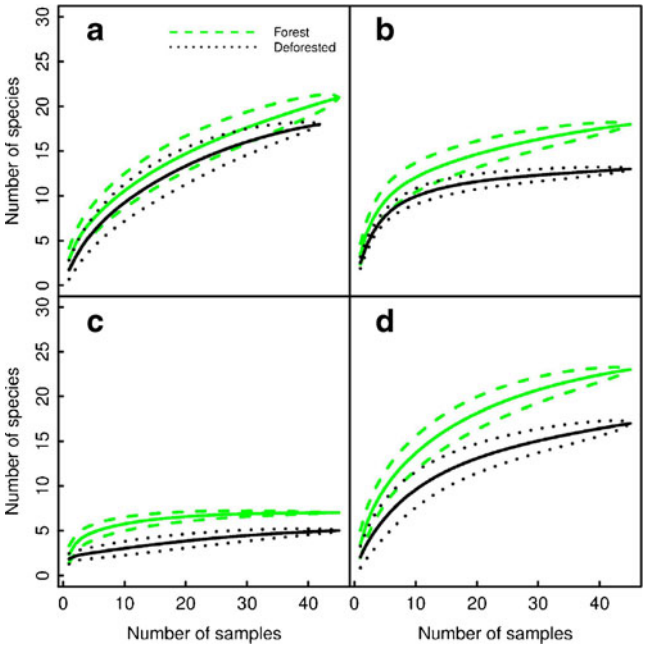

Fig. 2 ECM fungal species accumulation curves of *C. fargesii* (**a**), *L. harlandii* (**b**), *P. armandii* (**c**), and *P. massoniana* (**d**), grown in soil from the deforested and forest sites. The final value of each species accumulation curve equals to the total number of observed species in a given host and site. The confidence intervals were estimated by Bootstrap method

Discussion

Colonization rate of ECM fungi

We found that the ECM colonization rate on the root of three host plants was significantly lower in the deforested soil than that in the forest soil, but it still could provide a high root colonization rate. Several possible reasons could explain the ECM colonization in the deforested soil: (1) ECM fungal propagules could be in a continuous input into the deforested site from adjacent forests (a secondary forest about 300 m away or the natural forest 2 km away) by wind or small mammals

Table 3 One-way MANOVA by RDA to show the effects of host effect on ECM fungal community composition within each harvest date for a given soil origin

	df	April			July			October		
		R^2	*F*	*P*	R^2	*F*	*P*	R^2	*F*	*P*
Deforested site	3	0.357	11.94	0.001	0.293	9.14	0.001	0.338	11.05	0.001
Forest site	3	0.148	4.41	0.001	0.146	4.36	0.001	0.213	6.31	0.001

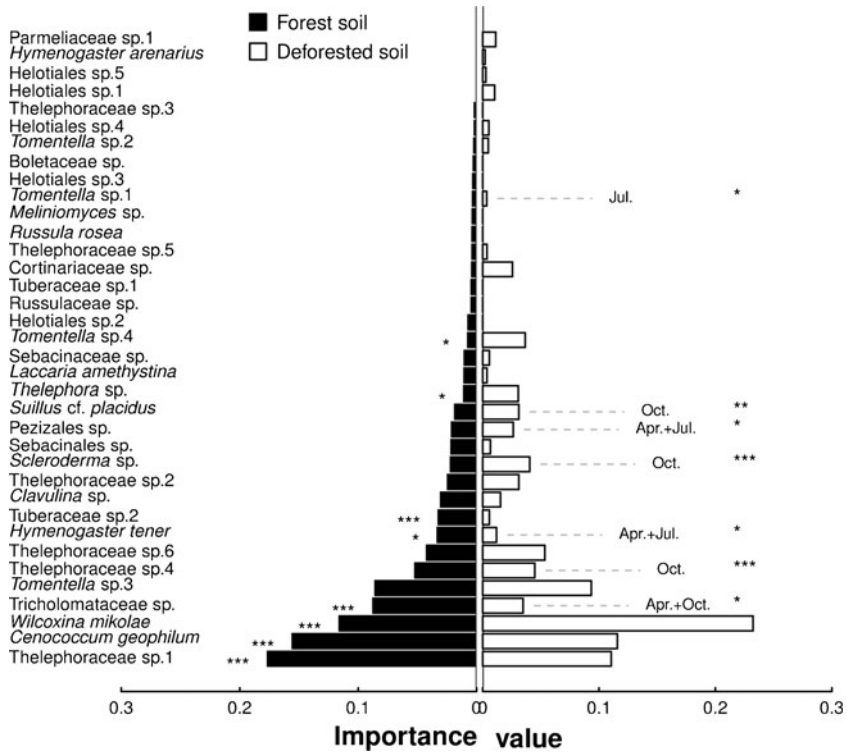

Fig. 3 Important values of ECM fungal species of the natural forest and deforested sites based on pooled samples of all hosts and harvest dates. *Stars* (*left*) and *letters* (*right*) marked on each *rectangular bar* denote a significant site and harvest date preference for the corresponding fungi (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$)

after clearing. Increasing evidences have shown that ECM fungal propagule could be dispersed through these agents over distance (Cazares and Trappe 1994; Frank et al. 2006, 2009; Lilleskov and Bruns 2005; Peay et al. 2010); (2) while the density of ECM fungal propagules may decline in the deforested site, some persistent structures (e.g., some kinds of spores, sclerotia, etc.) may keep viable in these areas; (3) EMF spores from the natural forest near the site for seedling cultivation could also cause colonization of seedling roots.

In accordance with Brundrett and Abbott (1994), we also found that the evaluation of ECM fungal inoculum potential in the deforested site would depend on host plant species used in this experiment. For example, about 60% ECM fungal colonization rate was achieved in *C. fargesii*, while almost 100% in *L. harlandii* in the deforested soil. For these reasons, we propose that several hosts, instead of a single host, should be used to give a prudence evaluation on the ECM inoculum potential in the deforested areas of subtropical China where several ECM hosts co-dominated in the local evergreen broad-leaved forest.

Effect of host plants on ECM fungal community structure

We found host preference was common in the propagule community in our study site, where fungi species with significant host preference occupied 90.8% of total number of ECM root tips. Since rare species could not show significant host preference in statistical analysis, proportion of species with host preference in common species is usually calculated to describe the universality of host preference in ECM fungus communities. Host preference has been found widely existing in mature forests, i.e., 14.5% (eight species out of 55 common species) ECM fungal species showed significant host preference in a Japanese mixed conifer–broadleaf forests (Ishida et al. 2007), 66.7% (18 species out of 27 common species) in a Tasmanian wet sclerophyll forest (Tedersoo et al. 2008),

Table 4 Two-way MANOVA by RDA to show the effects of soil origin, harvest date, and their interactions on ECM fungal community composition within each host

	df	*C. fargesii*			*L. harlandii*			*P. armandii*			*P. massoniana*		
		R^2	F	P	R^2	F	P	R^2	F	P	R^2	F	P
Soil origin (*S*)	1	0.072	2.39	0.006	0.113	5.50	<0.001	0.519	29.19	<0.001	0.157	6.17	<0.001
Harvest date (*D*)	2	0.156	2.58	<0.001	0.165	4.01	<0.001	0.021	0.59	0.834	0.169	3.31	<0.001
$S \times D$	2	0.046	0.75	0.812	0.228	5.53	<0.001	0.033	0.93	0.483	0.062	1.21	0.221

and 66.7% (four species out of six common species) in a neotropical forest in Western Amazonia (Tedersoo et al. 2009). Most studies on ECM fungal host preference were based on data of mycorrhiza investigation from natural forests (Tedersoo et al. 2008; Richard et al. 2005; Ishida et al. 2007; Tedersoo et al. 2009; Morris et al. 2009), and the host preference in the propagule community are seldom concerned. Considering the little overlap between the propagule community in soils and ECM fungus community on host roots in natural forests (Taylor and Bruns 1999), the dominance of ECM fungi with significant host preference in propagule community is poorly known. Our results indicated that among 19 ECM fungal species with >1% relative abundance on any host species, 14 species contributing to 90.8% of the total number of ECM root tips showed significant host preference in the propagule community. This implied that host preference could also be a mechanism in structuring ECM fungal propagule community.

Our results also indicated that the host plant played an important role in determining the composition of ECM fungal propagule community and 33.3% of the variance could be explained by host. By analyzing the data from Massicotte et al. (1999) with partial RDA, we found host explained 30% (using presence/absence data) and 40% (abundance data) variance in ECM propagule community composition. Host effect on ECM fungal community composition was also significant and a considerable portion of variance was explained by hosts in mature forest. For example, the variations explained by host were 13.2% in a mixed conifer–broadleaf forest (Ishida et al. 2007), 10% in a California woodland (Morris et al. 2009), and 19.5% in a neotropical rainforest (Tedersoo et al. 2009). The results from all these studies indicated that host species is one of the drivers in structuring ECM fungi communities.

One interesting point is whether the observed host effect on ECM fungal community composition in our study was mostly contributed by the non-native *P. armandii*, since this plant species could harbor only a very limited number of ECM fungal species. The host effect on ECM fungal community composition is still significant (24%, variance was explained, $P<0.001$) even when the data of *P. armandii* was excluded in the three-way MANOVA by RDA. One-way MANOVA by RDA also confirmed the significant effect of host (7–27% variance was explained, $P<0.001$).

Effect of soil origin on ECM community structure

We also found a significant difference in the ECM fungal propagule composition between the forest and deforested soils, which resulted from the biased occurrences of some dominant or common ECM fungi in the two sites. For example, Thelephoraceae sp.1, *C. geophilum*, and Tricholomataceae sp. were significantly more abundant in the forest soil, while *W. mikolae* significantly more proliferated in the deforested soil. These results were consistent with the occurrence of *C. geophilum* decreased while Pezizalean fungus increased with increasing distance to forest edge (Dickie and Reich 2005). Species of ECM fungi dominant in both soil origins were Thelephoraceae sp.1, *C. geophilum*, *W. mikolae*, Tricholmataceae sp., and *Tomentella* sp.3. These fungi are typical early colonizer with ruderal life strategy, and usually dominant in the post-disturbance ECM fungal propagule community (Baar et al. 1999; Buscardo et al. 2010; Izzo et al. 2006; Taylor and Bruns 1999). The differences of ECM fungus community compositions between two soil origins may be also a result of adaptation of these fungal species to changed soil environments. The removal of host trees with a possible loss of mycorrhizal functioning would cause the changes of soil characteristics, such as soil moisture, soil organic matter, soil nitrogen, and phosphate content. The different response of ECM fungi to these soil parameters would also be probably responsible for the changes of ECM fungus community. Since our site for seedling culture was 500 m away from the natural forest, the natural dispersal of EMF propagules should also be considered. Such dispersals from natural forests could be found at a distance from several meters to over several kilometers, and could cause contamination of seedling bioassay (Dickie et al. 2004; Frank et al. 2006, 2009; Stottlemyer et al. 2008; Peay et al. 2010). Natural dispersal of fungal propagules during seedling cultivation could possibly obscure the contrast of ECM fungal propagule community composition between the nature forest and the deforested site in our study. For a specific-soil-preferred EMF species detected in our study, their preference could thus be more significant if the effects of spore contamination were excluded. Our results of specific-soil-preferred EMF species were much reliable, since they showed significant biased occurrences between the two soils even under a condition of spore contamination (Fig. 3 and Table 6).

Intra-annual variations of ECM fungal community

It has been documented that some fungal species in the ECM fungal community showed significantly biased occurrences in different seasons (e.g., Courty et al. 2008; Koide et al. 2007; Walker et al. 2008). We also found some ECM fungal species preferred to appear in a specific harvest date (Fig. 3), which might be due to the growth and reproductive traits of these fungi or interactions between fungal species. Considering that these species were non-dominant species in the ECM fungal community, the harvest date could explain only a limited

portion of variance (3.2%) of ECM fungal species data (Table 2). Season may be less important in determining ECM fungal community as compared with host identity and soil origin.

In summary, a total of 36 ECM fungi were observed in the present study, and species richness varied with host species and soil origin. The decreased colonization rate and species richness implied the decline of fungal propagule density or the changes in ECM fungal community composition in the deforested site after long-term absence of host plants. Our results showed that 33.3% variance in ECM fungal community composition could be explained by the host plant species and 4.6% by the soil origin. Results of indicator species analysis showed that most common ECM fungal species showed significant preference to the host plant species, suggesting that the host preference of ECM fungi was one of the most important mechanisms in structuring ECM fungal community. Our results have implications both in evaluating viable ECM fungal propagules and selecting a plant species for the reforestation of deforested or degraded areas.

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Appendix

Table 5 Taxonomic affinities of ECM fungi ITS sequences based on BLAST search through GenBank and UNITE databases and inference from phylogenetic analysis

ECM fungus	Accession no.	Closest blast match accession no.	Query/reference ITS length (Similarity %)
Ascomycota			
Tuberaceae sp.1	GQ240939	Uncultured Tuberaceae EU202707	377/408 (92)
Cenococcum geophilum	GQ240926	*Cenococcum geophilum* EU427331	506/517 (97)
Helotiales sp.1	GQ240935	Root-associated fungal sp. EU677762	536/536 (100)
Helotiales sp.2	GQ240931	Ascomycota (Rhododendron type 2) AB089660	520/521 (99)
Helotiales sp.3	GQ240932	Fungal sp.R7 AY699681	520/526 (98)
Helotiales sp.4	GQ240933	Uncultured fungus FN298686	497/532 (93)
Helotiales sp.5	GQ240934	Uncultured Helotiales EU326174	520/526 (98)
Meliniomyces sp.	GQ240929	Uncultured Helotiales DQ273323	534/537 (99)
Parmeliaceae sp.1	GQ240938	*Sphaerosporella brunnea* UDB000994	404/413 (97)
Pezizales sp.	GQ240937	Uncultured fungus GQ205368	543/573 (94)
Tuberaceae sp.2	GQ240940	Tuberaceae FM205701	192/198 (96)
Wilcoxina mikolae	GQ240936	*Wilcoxina mikolae* DQ069000	579/580 (99)
Basidiomycota			
Boletaceae sp1.	GQ240923	Uncultured ECM (*Xerocomus*) EF218744	300/312 (96)
Clavulina sp.	GQ240920	*Clavulina* sp. EU862208	655/677 (96)
Cortinariaceae sp.	GQ240914	Uncultured ECM fungus *Inocybe* EF634110	565/675 (83)
Hymenogaster arenarius	HM358999	*Hymenogaster arenarius* DQ328124	648/653 (99)
Hymenogaster tener	GQ240915	*Hymenogaster tener* AF325633	581/592 (98)
Laccaria amethystina	GQ240913	*Laccaria amethystina* AB211270	668/674 (99)
Scleroderma sp.	GQ240921	Uncultured *Scleroderma* DQ402508	671/677 (99)
Sebacinaceae sp.1	GQ240918	Uncultured *Sebacina* EU910923	611/623 (98)
Sebacinales sp.	GQ240919	Uncultured ectomycorrhiza (Sebacinaceae) EU645626	480/518 (92)
Suillus cf. *placidus*	GQ240922	*Suillus placidus* DQ407257	666/674 (98)
Russula rosea	GQ240916	*Russula rosea* UDB000113	664/677 (98)
Russulaceae sp.	GQ240917	Uncultured *Russula* DQ493564	638/699 (91)
Thelephoraceae sp.1	GQ240904	*Tomentella* UDB003335	626/651 (96)
Thelephoraceae sp.2	GQ240903	Uncultured Thelephoraceae EF619788	600/623 (96)
Thelephoraceae sp.3	GQ240907	Uncultured Thelephoraceae EU498742	621/641 (96)

Table 5 (continued)

ECM fungus	Accession no.	Closest blast match accession no.	Query/reference ITS length (Similarity %)
Thelephoraceae sp.4	GQ240902	Uncultured Thelephoraceae AY748885	608/650 (93)
Thelephoraceae sp.5	GQ240901	Uncultured *Tomentella* FJ210774	607/641 (94)
Thelephoraceae sp.6	GQ240908	*Tomentella* UDB003342	585/617 (94)
Thelephora sp.	GQ240910	*Thelephora terrestris* DQ822828	616/648 (95)
Tomentella sp.1	GQ240905	*Tomentella stuposa* UDB003302	612/629 (97)
Tomentella sp.2	GQ240906	Uncultured *Tomentella* FJ013054	629/648 (97)
Tomentella sp.3	GQ240900	*Tomentella ellisii* DQ068971	596/620 (96)
Tomentella sp.4	GQ240909	Uncultured *Tomentella* FJ013065	629/648 (97)
Tricholomataceae sp.	GQ240911	*Laccaria vinaceoavellanea* AB453023	638/683 (93)

Table 6 Indicator species analysis showing significant preferences of ECM fungi species to soil origin[a]

	Deforested	Forest	Autoclaved CK	r_{pb}	*P* values
Wilcoxina mikolae	**0.451/0.323**	0.287/0.141	**0.417/0.235**	0.175	0.0076
Thelephoraceae sp.1	0.194/0.106	**0.500/0.212**	**0.483/0.194**	0.210	*0.0011*
Cenococcum geophilum	0.383/0.079	**0.708/0.135**	0.367/0.049	0.246	*0.0002*
Tricholomataceae sp.	0.074/0.032	**0.247/0.098**	0.133/0.055	0.162	0.0134
Thelephoraceae sp.6	**0.114/0.069**	**0.129/0.039**	0.033/0.003	0.170	0.0073
Thelephoraceae sp.2	**0.069/0.032**	**0.073/0.021**	–	0.138	0.0347
Tuberaceae sp.2	0.011/0.003	**0.107/0.034**	0.017/0.009	0.171	0.0052
Parmeliaceae sp.1	0.023/0.005	–	**0.067/0.028**	0.186	*0.0013*
Laccaria amethystina	0.006/0.003	0.028/0.005	**0.083/0.027**	0.136	0.0143

Significant preferred soil origins were shown in bold and significant *P* values after Holm correction were shown in italics

[a] Autoclaved CK showed ECM fungal community on plants growing on autoclaved soil (deforested/forest = 1:1)

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