JOURNAL OF PURE AND APPLIED MICROBIOLOGY, APRIL 2014. Vol. **8**(2), p. 1197-1208

Dynamics of Ectomycorrhizal Fungal Communities in a Chronosequence of *Pinus tabulaeformis* Plantations

Hong-Xia Yu[1], Chun-Yan Wang[1], Hui Chen and Ming Tang*

College of Forestry, Northwest A&F University, Yangling, Shaanxi 712100, China.

(Received: 25 October 2013; accepted: 06 January 2014)

In the semi-arid Loess Plateau of northwestern China, the vegetation deterioration and soil degradation are very severe, and pines are large-scale planted for reforestation. In this study, the dynamics of ectomycorrhizal (EM) fungal communities in a chronosequence of 3, 7, 12, 25 and 55 year-old *Pinus tabulaeformis* plantations were investigated by denatured gradient gel electrophoresis (DGGE) based on fungal internal transcribed spacers of rDNA. A total of 21 DGGE bandtypes were successfully retrieved and sequenced after molecular cloning. The results indicated that they belonged to Ascomycota and Basidiomycota, accounting for 23.81% and 76.19% of the total, respectively. The dominant genus was *Russula*, followed by *Suillus*, and then *Cortinarius*, *Tuber* and *Phialophora*. The DGGE profiles of EM fungal communities changed with seasons as well as plantation ages, and redundancy analysis indicated that shifts in EM fungal community composition affected by soil chemical properties was smaller among plantation ages than among seasons.

Key words: Dynamics, Ectomycorrhizal fungi, Community, Denaturing gradient gel electrophoresis, *Pinus tabulaeformis*, Loess Plateau.

The Loess Plateau is the site of research focus in China because of its severe vegetation deterioration and soil degradation[1–3]. The present lost area in this region is about 450 000 km^2[1], accounting for 72% of the total area (624 000 km^2)[3]. Many restoration projects have already been performed to improve the ecological environment in the region over the past decades. Artificial vegetation was the most important patternadopted in vegetation restoration and reconstruction. As a kind of the most predominant pioneer tree species for artificial reforestation, Chinese pine (*Pinus tabulaeformis* Carr.) was widely planted in the Loess Plateau of northwest China due to its high stress tolerance for cold, drought and poor soil[4, 5].

* To whom all correspondence should be addressed.
Tel/Fax: +86 (0)29 87080157
E-mail: tangm@nwsuaf.edu.cn

Ectomycorrhizal (EM) fungi are essential belowground components for many important ecologically and economically trees across the globe, especially for pines[6]. Pines are perceived as obligate ectomycorrhiza trees, and they do not develop normally without EM mutualistic symbiosis[7, 8]. EM is a form of symbiotic association that occurs between plant roots and certain soil fungi[9]. They facilitate water and nutrients supply required for the survival and growth of plants, and improve plant resistance to pathogens and environmental stress[9]. Zhang *et al*.[10] studied that inoculation with EM fungi could not only improve the growth rate and biomass, but also increase resistance to damping-off caused by *Rhizoctonia solani* in *P. tabulaeformis* seedlings.

Since EM had been detailedly drawn and described in the 1880s, about 6 000 fungi and 20 000–25 000 plants involved in EM symbiotic associations have already been reported[11-13]. In China approximately 500 fungi belonged to 40

families and 80 genera were reported[14]. Among them, 3/4 associated with coniferous trees, including more than 1/3 with pines[14]. EM fungi in soil were exposed to an extremely complex environment and their communities were susceptible to several factors, such as soil properties[15,16], seasonality[17,18], planting ages[19, 20], host plant performance (e.g., growth and phenology)[10, 21] and community[7,22]. The diversity, structure and composition of EM fungi communities in pines have been extensively studied with *P. thunbergii*[23], *P. sylvestris*[24], *P. halepensis*[25], *P. radiate*[26], *P. banksiana*[27], *P. muricata*[19], *P. densiflora*[28] and *P. contorta*[16]. However, little information is known about the EM fungal community in *P. tabulaeformis*, especially in the Loess Plateau of China. Moreover, the variation of EM fungi with planting years and shifting with seasons in the actual processes of restoration are also poorly understood in this region.

The main purpose of the study is to characterize belowground EM fungal communities of *P. tabulaeformis* in relation to planting years and seasonality in plantations of the Loess Plateau. To our knowledge, this study is the first investigation of EM fungal communities of *P. tabulaeformis* and relationships between the recovery of EM fungal communities and reforestation in the Loess Plateau of northwestern China. The seemly identical EM morphotypes may be colonized by two or more different fungal species, so that it is possible to sort different EMs into one type, resulting in an underestimation of the actual EM community composition[23, 29, 30]. The use of molecular profiling would reduce this kind of bias and precisely asalyze the real status of EM fungal communities in *P. tabulaeformis* plantations. In this study, nested polymerase chain reaction (PCR) and denatured gradient gel electrophoresis (DGGE) techniques were used to investigate EM fungal communities in a chronosequence of *P. tabulaeformis* plantations at three different sampling seasons to address: (1) EM fungal diversity and community composition in the *P. tabulaeformis* plantations in the Loess Plateau of China; (2) The seasonal dynamics of EM fungal community; (3) The relationship between EM fungal community and reforestation age.

MATERIALS AND METHODS

Study site

The study site is located in the Lianjiabian forest of the northern Ziwuling region, Heshui County, Gansu Province, China (108°10'–109°18'E, 35°03'–36°37'N). It is a typical hilly and gully region in the Loess Plateau, with an altitude above sea level of 1211–1453 m and a relative height of 200–400 m. This region belongs to mid-temperate continental monsoon climate with an annual average temperature of 7.4 °C and annual average precipitation of 587.6 mm. The soil type is calcareous cinnamon soil and the mainly vegetation type is the deciduous broadleaf forest. The *P. tabulaeformis* plantation forests occupies approximately 53 000 ha, covering 81% of the total area of plantation forests in this region[4].

Sampling procedure

Five *P. tabulaeformis* plantations, established for 3, 7, 12, 25, and 55 years, respectively, were chosen for this study. Samples were collected in May, August and November 2010. At each sampling season, five sampling plots were selected at the study site. From each sampling plot, four *P. tabulaeformis* individual were randomly selected with 20 m intervals between each other. Whereafter, rhizosphere soil (transect depth of 5–20 cm) was sampled according to the methods described by Kidd *et al.*,[31]. Fine roots of each *P. tabulaeformis* individual were collected by tracing four laterals from the taproot to avoid contamination of other plant roots. Samples in sealed bags were put into ice box and taken back to the laboratory for next analysis.

Soil properties analysis

Root-free soil samples collected from the same plantation forest were thoroughly mixed and sieved through a 2 mm sieve for the analysis of soil chemical properties. The organic matter content was measured according to the method of Yeomans and Bremner[32]. Soil available phosphorus content was assayed by the method described by Olsen *et al.*,[33]. Nitrate and ammonium nitrogen concentrations were determined by a Bran andandLuebbe AA3 continuous flow analytical system (Norderstedt, Germany) after being extracted in 1 mol/L KCl solution[34].

EM colonization

The grid-line intersect method was used to measure EM colonization[35]. Roots were washed gently over sieve to remove debris and adhering soil with tap water, and spread out evenly in a 9 cm (diameter) a Petri dish after being cut into 1 cm long sections. For each sample, a total root length of 320 cm was used (80 randomly selected root segments at a time and repeated four times). EM colonization was assessed under a stereomicroscope (Olympus SZX7) and calculated as the percentage (%) of the number of colonized root-grid intersections divided by the total number of examined root-grid intersections.

DNA extraction

Equal numbers of EM root tips from each plot were sampled randomly (52 per tree; 208 per plot; 1040 per season; 3120 in total)[36, 37]. EM root tips were washed three to five times with sterile distilled water and stored in 2 mL microcentrifuge tubes at -20 °C before the extraction of nucleic acids. Total DNA was extracted using a modified cetyltrimethyl ammonium bromide method as developed by Gardes and Bruns[38].

Nested PCR

The extracted DNA from EM roots was used as template in nested PCR. The primer pairs ITS1-F [38] and ITS4[39], ITS1-F with a GC-clamp (40 bases) adhered to the 52 end and ITS2[39] were chosen for the first and second round of PCR reactions, respectively. All PCR amplifications were carried out in a 50 µL reaction volume, containing 2.5 µL template, 10 mM Tris-HCl (pH 8.3), 5 0 mM KCl, 2.5 mM $MgCl_2$, 0.25 mM of each dNTP, 0.2 µM of each primer and 1.25 unitsTaq DNA polymerase. The same PCR cycling parameters were performed for both amplification rounds in nested PCR with a S1000 thermal cycler (Bio-Rad, USA) as follows: 94 °C for 5 min, then 35 cycles of 95 °C for 45s, 55 °C for 45s and 72 °C for 45s, and a final extension of 72 °C for 10 min. PCR products were analyzed by 1% (w/v) agarose gel electrophoresis, stained with ethidium bromide (EB) and visualized under UV light. Obtained PCR products were stored at -20 °C for subsequent DGGE analysis.

DGGE analysis

PCR products were subjected to DGGE by using the DCode universal mutation detection system (Bio-Rad, Hercules, CA, USA)[40,41] to analyze the EM fungal community. To minimize potential variations of between-gel, comparisons were confined to lanes within a single gel. Gel contained 8% (w/v) polyacrylamide (40% solution, Acrylamide/bis-acrylamide = 37.5:1, w/w). Vertical denaturing gradient was prepared for 30% (12.6 g urea, 12 % (v/v) formamide) to 60% (25.2 g urea, 24 % (v/v) formamide). To integrate the nested PCR products into the gel as soon as possible, DGGE was primarily run at 200 V for 8 min, and then performed at 70 V for 13 h in 1× TAE buffer at a constant temperature of 58 °C. After being stained with EB, gel was visualized under UV light, and then photographed by Gel Doc imaging system (Bio-Rad, Hercules, CA, USA).

Cloning and sequencing

All of the detected bands were excised from the DGGE gel under UV light, and then were mashed and incubated in 30µL sterile ddH_2O at 4 °C overnight. PCR products were purified using TIANgel Midi Purification Kit (Tiangen Biotech CO., LTD, Beijing, China). Purified PCR products from each isolated target band were ligated to the pGEM-T Easy vector (Promega, Madison, WI, USA) and transformed into *Escherichia coli* DH5á competent cells by following the manufacture's protocol. Positive clones were screening from the transformed cells by using blue-white spot procedures according to Gao *et al.*,[42]. Clone inserts were checked by PCR amplification (PCR reaction system and conditions as described above) using primers of ITS1-F and ITS2. The clones which tested positive were sent to TianyiHuiyuan Bioscience and Technology Inc. (Beijing, China) for sequencing.

Sequence analysis

To prove the identity of EM fungal phylotypes, obtained sequences were analyzed by basic local alignment search tool (BLAST) for nucleotide blast and compared with sequences deposited in GenBank. Based on sequence homology, closest matches sequences were used for taxonomic classification and divided into three categories: species level identity > 97%, genus level identity 95-97% and family level identity < 95%[43]. The best representatives of each individual DGGE bandtypes were deposited in GenBank database under accession numbers KF032594–KF032614. Each molecular operational taxonomic unit was regarded as one EM species

defined by Tedersoo *et al.*,[13]. Sequences were edited and trimmed manually using BioEdit software (version 7.0.9.0) and aligned by Clustal X 1.81. Finally, the neighbor-joining tree was constructed by using MEGA version 5.05 with the Kimura two-parameter model[44].

Data analysis

Statistical data were subjected to one-way analysis of variance (ANOVA) ($P< 0.05$) using SAS version 8.1 (SAS Institute Inc., Cary, NC). The test of significance was performed by applying Duncan's test ($P< 0.05$). Figures were done with SigmaPlot 10.0 software (Systat Software Inc., Germany).

To generate fewer compound variables to characterize the soil types, the soil chemical parameters were processed by principal component analysis (PCA). Ordination of different sites according to their soil chemical properties was subjected to the first two principal components (PCs) including most of the explanatory variable information of the original set of data. The loading scores of the first two PCs were then performed for person's correlation coefficient to identify relationships between these two PCs and EM fungal variables on the new dependent variables.

DGGE images were digitalized and analyzed using Quantity One software 4.6.2 (Bio-Rad, Hercules, CA, USA). Presence or absence of the bands in each lane of the DGGE gel was converted to a binary matrix. After that, the data were subjected firstly to detrended correspondence analysis (DCA) to decide response model (linear or unimodal) of ordination. The result showed that the max length gradient was 1.94 for EM fungi species data, thus redundancy analysis (RDA) was chosen for inferring relationships between the EM fungal community and environmental variables. RDA was performed using Canoco version 4.5 (Centre for biometry, Wageningen, the Netherlands), focusing the scale of inter-species correlations. Monte Carlo permutation test with 499 replicates was permuted using cyclic shifts. Based on the data of the number and intensity of bands in DGGE profiles, species richness (S), Simpson index (D), Shannon-Weiner index (H) and Evenness index (E_h) were calculated in accordance with the formula below[45,46]:

$$H = -\sum_{i=1}^{S} (Ni / N) \ln (Ni / N)$$

$$E_h = H / \ln S$$

$$D = \sum_{i=1}^{S} (Ni / N)^2$$

Where Ni was the peak density of the ith band, N was the sum of the peak density of all bands in a lane, S was the total band number in a lane. The diversity indices (S, H, E_h and D) of fungi and bacteria were used for the correlation analysis with SAS version 8.1 (SAS Institute Inc., Cary, NC).

RESULTS

Ordination of edaphic characters and colonization of EM fungi

The ordination explained the variation of soil chemical parameters among samples: 67.27% accounted for the variation of the first principal component (PC1) and 20.46% for the second principal component (PC2) (Fig. 1). The loadings of the axes showed that PC1 reflected the organic matter, nitrate nitrogen, ammonium nitrogen and available nitrogen status of the soil, while PC2 reflected the available phosphorus. EM fungal colonization was significantly positively correlated with PC1 ($r = 0.64$, $P< 0.01$) (Table 1), suggesting that as soil organic matter, nitrate nitrogen, ammonium nitrogen and available nitrogen increase, EM fungal colonization increases. EM fungal colonization was not correlated with PC2 ($r = -0.28$, $P> 0.05$) (Table 1), indicating that the influence of soils available phosphorus on EM fungal colonization was not significant in this study (Fig. 2).

EM fungal colonization in the roots of *P. tabulaeformis* was intense, varying from 71% to 97.75% in all samples. Combining all sites or all months together, the highest EM fungal colonization presented in the 12-year site (mean = 95.75%) and May (mean = 92.15%), respectively (Fig. 2). EM fungal colonization showed a negative correlation with sampling time ($r = -0.51$, $P< 0.05$) (Table 1), while the correlation between EM fungal colonization and sampling site had no significant positive correlation ($r = 0.35$, $P> 0.05$) (Table 1).

Seasonal variation in EM fungal colonization was present in all sites, and there was a declining trend from spring to autumn (Fig. 2).

EM fungal DGGE profiles and diversity

Differences in DGGE profiles of EM fungal communities were obviously observed. Firstly, the diversity and composition of the EM fungal community in the same month varied within different sites. From the perspective of the growth phases of trees, several EM fungal bandtypes of DGGE profiles were more common in plantations above 12-year than younger ones. The difference caused by the growth phase was verified within three different sampling seasons. Secondly, the diversity and composition of the EM fungal community in the same site varied within three different seasons. Whereas several bands in the DGGE profiles were common from May to November, several bands were unique at three different seasons. Furthermore, the signal intensities of DNA bandtypes were variable, from strong to weak.

The EM fungal diversity indices were calculated based on the signal intensity and the number of bands in DGGE profiles (Table 2). The species richness ranged from 7 to 19, 6 to 24 and 11 to 16 in May, August and November, respectively (Table 2), and showed a positive correlation with plant age ($r = 0.59$, $P < 0.05$) (Table 1). The Shannon-Wiener index varied among samples from May to November, ca. 2.73–3.97, 2.53–4.28, 3.29–3.89, respectively (Table 2), exhibited a positive correlation with plant age ($r = 0.52$, $P < 0.05$) (Table 1), but was no significant correlation with seasons ($r = 0.23$, $P > 0.05$) (Table 1). Nevertheless, the Evenness index and the Simpsom's index showed non-significant correlation both with plant age and with month ($P > 0.05$) (Table 1), ranged from 0.92–

Table 1. Correlations among EM fungal variables, plant age, seasonand two PCA extracted components of soil parameters (PC1 and PC2)

	EM colonization (%)	Shannon-Wiener indexes (*H*)	Species richness (*S*)	Evenness index (E_h)	Simpson's index (*D*)
Plant age	0.35	0.52*	0.59*	-0.47	0.46
Season	-0.51*	0.23	0.12	0.38	0.29
PC1	0.64**	0.45	0.53*	-0.56*	0.38
PC2	-0.28	-0.04	-0.02	0.17	-0.07

*Correlation is significant at the 0.05 level. **Correlation is significant at the 0.01 level.

Table 2. Shannon-Wiener index (*H*), Richness (*S*), and Evenness (E_h), Simpson index (*D*) of EM fungi at different sampling sites andseasons

Season	Site (years)	Shannon-Wiener indexes (*H*)	Species richness (*S*)	Evenness index (E_h)	Simpson's index (*D*)
May	3	3.17	10	0.95	0.88
	7	2.73	7	0.97	0.84
	12	3.57	14	0.94	0.90
	25	3.53	14	0.93	0.90
	55	3.97	19	0.93	0.92
August	3	2.52	6	0.98	0.82
	7	4.21	21	0.96	0.94
	12	3.31	12	0.92	0.88
	25	3.93	17	0.96	0.93
	55	4.27	24	0.93	0.94
October	3	3.76	15	0.96	0.92
	7	3.29	11	0.95	0.89
	12	3.81	15	0.98	0.92
	25	3.89	16	0.97	0.93
	55	3.60	14	0.95	0.91

0.98 and 0.82–0.94, respectively (Table 2), and both showed the lowest value in the 3-year site at August. The first principal component had a significant influence on EM fungal species richness ($r = 0.53$, $P < 0.05$) (Table 1) and Evenness index ($r = -0.56$, $P < 0.05$) (Table 1), meaning that as soil organic matter, nitrate nitrogen, ammonium nitrogen and available nitrogen increase, the species richness of EM fungi increases while Evenness index decreases.

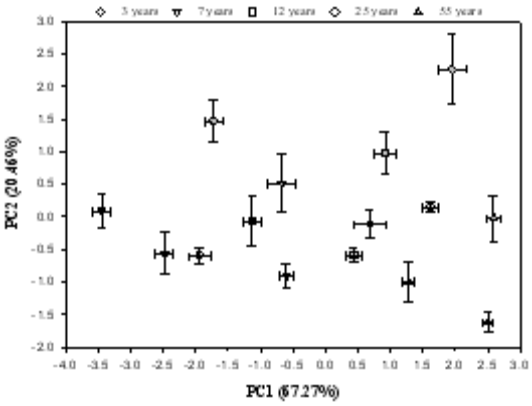

Fig. 1. PCA of soil chemical properties (including soil organic matter, available phosphorus, nitrate nitrogen, ammonium nitrogen, and available nitrogen) marked by sampling site and time. The points represent the means of PC1 and PC2 scores at each sampling site and time. Bars represent the SEs. Open symbols, closed gray symbols and closed black symbols represent May, August and November, respectively

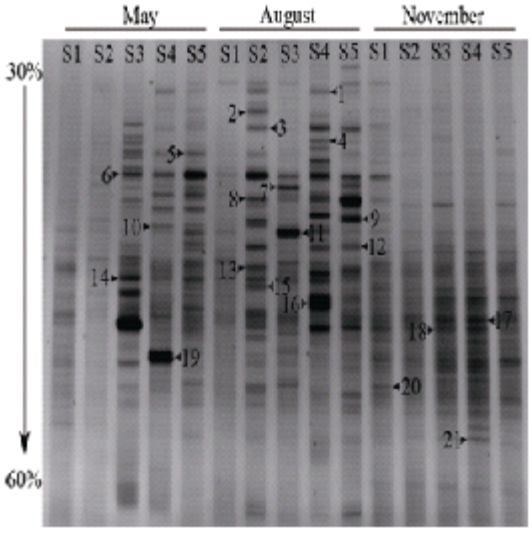

Fig. 3. DGGE pattern of nested PCR-amplified ITS rDNA fragments of EM fungi in the roots of *P. tabulaeformis*. Lanes labeled as S1–S5 represent the samples from 3, 7, 12, 25 and 55 year-old *P. tabulaeformis* plantations at three sampling seasons, respectively. The linear denaturant gradient is from 30% to 60%

Phylogenetic analysis

Twenty one detected bands of the recovering rDNA genes were successfully cloned and sequenced from the DGGE gel (Fig. 4), ranging

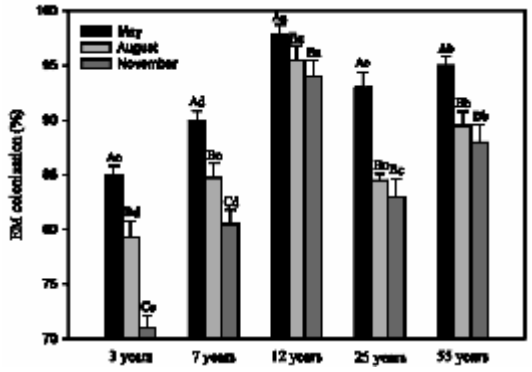

Fig. 2. EM colonization in five investigated *P. tabulaeformis* palntations at three sampling seasons. Bars represent SEs. Different capital letters and lowercase letters above bars indicate significant difference between sampling season and sites, respectively

from 201 to 306 bp. Less than half of them (48%) had a high similarity (> 97%) with fungal ITS sequences in the nucleotide sequence database, so that the identification of obtained sequences to species level was hampered. To provide more information for the identification and phylogenetic status of these sequences, one to four closest matches for each sequence were downloaded and used for phylogenetic analysis (Fig. 4). The results showed that all the obtained sequences belonged to either Ascomycota or Basidiomycota. The respective contributions of Ascomycota and Basidiomycota to the EM fungal community differed in terms of taxa composition and four different indicators of biodiversity (including EM fungal S, D, H and E_h), depending on the plantation age level at three sampling time points. Five obtained sequences of DGGE bandtypes 5–8 and 11 were classified into Ascomycota and closed to the genus *Tuber*, *Phialophora* and *Cenococcum*. The remaining sixteen obtained sequences were grouped in Basidiomycota. Most of basidiomycete fungi belonged to Russulales, Boletales and

Agaricales in the Agaricomycetes, which accounted for 93.75% and 71.43% of basidiomycete fungi and all of the sequenced fungi, respectively. The most common family was the Russulaceae, followed by the Suillaceae and the Cortinariaceae. The dominant genus was *Russula*, followed by *Suillus* and then *Cortinarius*, *Tuber* and *Phialophora*.

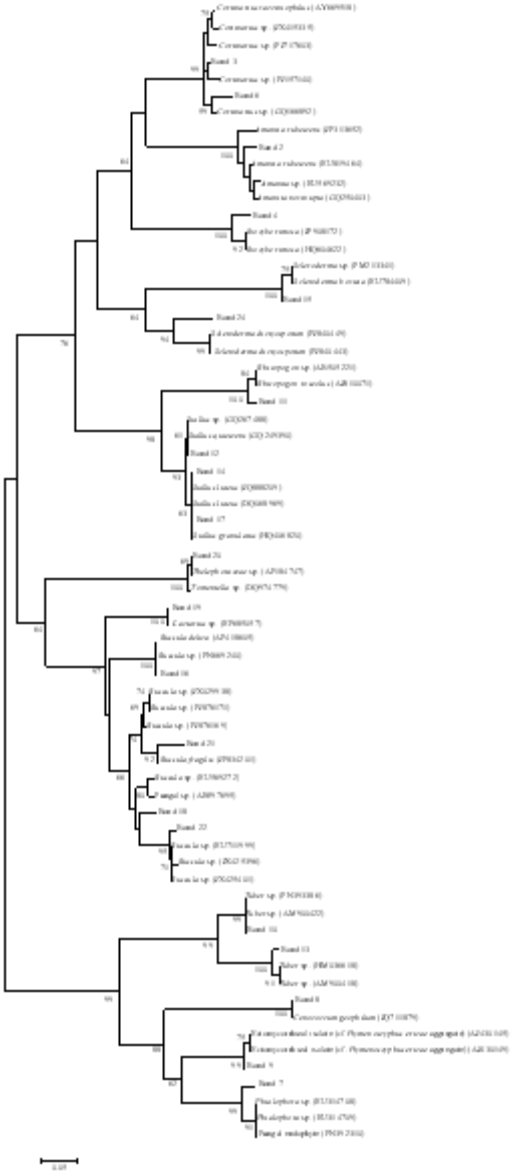

Fig. 4. Neighbor-joining phylogenetic tree of identified EM fungi in the roots of *P. tabulaeformis* based on their ITS rDNA sequences. Bootstrap analysis was performed using 1,000 replicates. Bootstrap values > 50% were shown

RDA of the EM fungal community

RDA analysis was used to assess relationships between the soil environmental variables and EM fungal community (Fig. 5). The result showed that the first two axes of RDA explained 25.7% and 7.9% of the microbiological variances with high species-environment correlation of 0.83 and 0.90, respectively. In Fig. 5, lines with arrows denoted soil environmental variables, the direction of arrows denoted positive or negative relationships between soil environmental variables and axis in different quadrants, the length of lines denoted relationships between fungi species and soil environmental variables. The majority of species were represented in the right part of RDA ordination diagram, and all of the soil chemical properties were also represented in the right part, indicating that the majority of species were positively affected by soil chemical properties. Nevertheless, the RDA result also indicated that the sole influence of certain soil factor to the EM fungal community was difficult to be estimated in the present study. The RDA triplot also revealed that samples from each

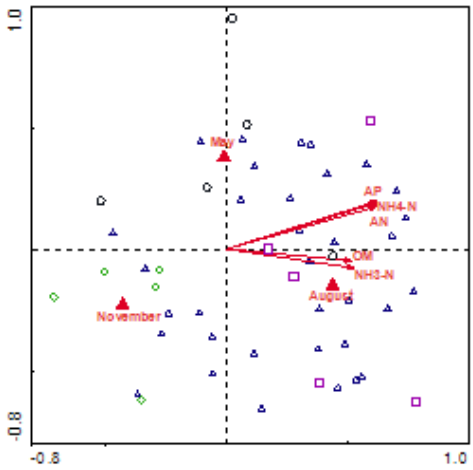

Fig. 5. RDA depicting relationships between EM fungi in a chronosequence of *P. tabulaeformis* plantations and the soil chemical variables at three sampling seasons. The proxy variables and nominal variables are represented by solid lines with filled arrows and closed triangles, respectively. Open triangles indicate EM fungal species. Open circles, square and rhombus represent sampling sites in May, August and November, respectively. Abbreviations are listed as follows: OM, organic matter; NO_3^-N, nitrate nitrogen; NH_4^-N, ammonium nitrogen; AN,

sampling season separated in ordination space among EM fungal communities, however, the total variation in the species composition of the EM fungal community among field sites was much smaller with respect to the time of plantation age.

DISCUSSION

The number of detected fungal species depended on the quantity of used EM roots and the efficiency of DNA extraction and amplification. Moreover, the EM fungal community is very complex and composed of numerous populations, resulting in a smear of DGGE bandtypes, so that it's difficult to identify each and every individual[41]. Totally, 21 fungal taxa were obtained from the five investigated *P. tabulaeformis* plantations at three seasons (Fig. 4), this number was less than those detected form *P. tabulaeformis*(26 taxa;[47]), *P. strobes* (53 taxa; [17]), and *P. contorta* (81 taxa;[16]) in natural ecosystems and *P. resinosa* (39 taxa;[48]) in plantations,but more than those from *P. muricata* (20 taxa;[49]) in natural ecosystemsand *P. halepensis* (12 taxa;[25]) and exotic *P. radiate* in plantations(19 taxa; [26]).

The obtained 16 basidiomycete and 5 ascomycetemycobionts from *P. tabulaeformis* plantations have been recorded as EM partners in pines, such as *P. thunbergii*[23], *P. halepensis*[25], *P. radiate*[26], *P. banksiana*[27], *P. muricata*[19], *P. contorta*[16], *P. densiflora*[28] and *P. tabulaeformis*[47]. Of all the detected fungi in this study, DGGE bandtype 6 had relatively strong signal intensities in most sites and was identified as *Cenococcum geophilum* with a high similarity of 99%. Obase *et al*.[23] revealed that *C. geophilum* was one of the most common EM fungi across the world. *Cenococcum* abundantly colonized in forest ecosystems[23] and were considered to be well adapted to disturbance, probably because of their sclerotia[50, 51]. Russulaceae and *Russula* were the most common family and genus colonized in the roots of *P. tabulaeformis*, respectively. Russuloid species were reported to be the most frequent and dominant EM fungi in forests[52]. Kjoller[53] studied on the disproportionate abundance between EM roots and their associated mycelia in soil, and revealed that russuloid species were more dominant in roots. This may be one possible reason why they were detected frequently in the present study.

Seasonal fluctuations, as a common phenomenon, have been observed in the communities of arbuscular mycorrhizal fungi[54], soil fungi[55], phyllosphere fungi[56], as well as EM fungi in coniferous trees[17] or other types of trees[18]. From the DGGE profile in this study, it was seen that the diversity, structure and composition of the EM fungal communities in *P. tabulaeformis* plantations changed through seasons (Fig. 3).

The RDA ordination diagram also clearly showed that the composition of EM fungal community differed among sampling seasons (Fig. 5), suggesting that the seasonality of EM fungal communitymay be driven by the seasonal variation of abiotic environmental factors. Due to the extreme complexity of environment in forest soil, nevertheless, it was difficult to determine the sole influence of one soil factor on the EM fungal community from this study. Furthermore, seasonal changes of EM fungal communities may be relevant to host phenology[21]. In addition, colonization of EM fungi mainly occurred on the fine root tips[9], the lifetime of which usually are as short as one month[18]. This also may be one reason for the highly seasonal variation of EM fungal community.

The EM fungal community varied with the plantation age (Fig. 3), indicating that the diversity and composition of EM fungal community in *P. tabulaeformis* roots changed across a chronosequence of plantations at the same sampling time. It is consistent with previous researches[20,57]. For example, Visser[27] also revealed a distinct sequence of EM fungi in a chronosequence of *P. banksiana* stands located in Canadian boreal forest. Nvertheless, the diversity of EM fungal community in *P. tabulaeformis* plantations did not continuously increase with the plantation age. In the same season, some dominant fungal bandtypes were always present in over 7-year plantations, while some rare fungal bandtypes changed with plantation age. Similar pattern, however, was not found in the 3 and 7-year sites. Therefore, the major change of EM fungal communities in the five investigated *P. tabulaeformis* plantations was only based on the presence or absence of some rare EM fungal phylotypes. It is consistent with previous report that the distribution of some rare EM fungal phylotypes contributed to the species

diversity of EM fungal community[23].

Several reasons may contribute to the changes in diversity, composition and structure of the EM fungal community with plantation age. The first one is the variance of abiotic environment factors. It has been shown that different EM fungal taxa varied in their response to the environmental cue[15, 16], and dominant EM fungi varied as the abiotic environment factors change with stand age[27, 57]. The second one may be the status of host which varied in different phases of plant growth[21, 58].

In addition,shifts of EM fungal communities may also due to the variation in life history characteristics of different EM fungi[13, 20] and interspecific interactions among EM fungi (i.e., competence and coexistence)[59, 60]. Hence, the variation of EM fungal communities with plantation age and season is the result of complex interactions among the edaphic factors, phenology and growth phase of host plants, lifespan of fine root tips, life history characteristics of EM fungi and their interspecific interactions.

CONCLUSIONS

The restorations of vegetation and EM fungal community are very slow in the semi-arid ecosystem of the Loess Plateau, China. EM fungal communities and host plants can interact on the performance and biocoenosis of each other[22, 61], so that the recovery of degraded ecosystem in the Loess Plateau should combine the two together. The evaluation of indigenous mycorrhizal status is only a first step in restoration and rehabilitation of degraded ecosystems by using EM biotechnology[62,63]. Isolation of the dominant and widely distributed EM fungi (e.g. *Russula*) in this region would be crucial for the using of EM biotechnology in future.

ACKNOWLEDGMENTS

This research was supported by the National Natural Science Foundation of China (31270639, 31170567), Special Fund for Forest Scientific Research in the Public Welfare (201404217) and Program for Changjiang Scholars and Innovative Research Team in University of China (IRT1035).

REFERENCES

1. Xin, Z.B., Ran, L.S., Lu, X.X. Soil erosion control and sediment load reduction in the Loess Plateau: policy perspectives. *Int. J. Water Resour. Dev*., 2012; **28**(2): 325–341.
2. Xu, X.Z., Li, M.J., Liu, B., Kuang, S.F., Xu, S.G. Quantifying the effects of conservation practices on soil, water, and nutrients in the Loess Mesa Ravine Region of the Loess Plateau, China. *Environ. manage*., 2012; **49**(5): 1092–1101.
3. Zhang, X.C., Shao, M.A., Li, S.Q., Peng, K.S. A review of soil and water conservation in China. *J. Geogr. Sci*., 2004; **14**(3): 259–274.
4. Yuan, B.C., Yue, D.X.Soil microbial and enzymatic activities across a chronosequence of Chinese pine plantation development on the Loess Plateau of China. *Pedosphere*, 2012; **22**(1): 1–12.
5. Zhang, H.H., Tang, M., Chen, H., Zheng, C.L. Effects of inoculation with ectomycorrhizal fungi on microbial biomass and bacterial functional diversity in the rhizosphere of *Pinus tabulaeformis* seedlings. *Eur. J. Soil Biol*., 2010; **46**(1): 55–61.
6. Teasdale, S.E., Beulke, A.K., Guy, P.L., Orlovich, D.A. Environmental barcoding of the ectomycorrhizal fungal genus *Cortinarius*. *Fungal Divers*., 2012; **58**(1): 299–310.
7. Bahram, M., Kõljalg, U., Kohout, P., Mirshahvaladi, S., Tedersoo, L. Ectomycorrhizal fungi of exotic pine plantations in relation to native host trees in Iran: evidence of host range expansion by local symbionts to distantly related host taxa. *Mycorrhiza*, 2013; **23**(1): 11–19.
8. Cairney, J.W.G. Ectomycorrhizal fungi: the symbiotic route to the root for phosphorus in forest soils. *Plant Soil*, 2011; **344**(1–2): 51–71.
9. Smith, S.E., Read, D.J. (eds): Mycorrhizal symbiosis, 3rd edn. London: Academic Press, 2008.
10. Zhang, R.Q., Tang, M., Chen, H., Tian, Z.Q. Effects of ectomycorrhizal fungi on damping-off and induction of pathogenesis-related proteins in *Pinus tabulaeformis* seedlings inoculated with *Amanita vaginata*. *For. Pathol*., 2011; **41**(4): 262–269.
11. Agerer, R. Fungal relationships and structural identity of their ectomycorrhizae. *Mycol. Prog*., 2006; **5**(2): 67–107.
12. Brundrett, M.C. Mycorrhizal associations and other means of nutrition of vascular plants: understanding the global diversity of host plants

by resolving conflicting information and developing reliable means of diagnosis. *Plant Soil*, 2009; **320**(1–2): 37–77.

13. Tedersoo, L., May, T.W., Smith, M.E. Ectomycorrhizal lifestyle in fungi: global diversity, distribution, and evolution of phylogenetic lineages. *Mycorrhiza*, 2010; **20**(4): 217–263.
14. He, X.H., Duan, Y.H., Chen, Y.L., Xu, M.G. A 60-year journey of mycorrhizal research in China: past, present and future directions. *Sci. China. Life. Sci*., 2010; **53**(12): 1374–1398.
15. Buee, M., Vairelles, D., Garbaye J. Year-round monitoring of diversity and potential metabolic activity of the ectomycorrhizal community in a beech (*Fagus silvatica*) forest subjected to two thinning regimes. *Mycorrhiza*, 2005; **15**(4): 235–245.
16. Douglas, R.B., Parker, V.T., Cullings, K.W. Belowground ectomycorrhizal community structure of mature lodgepole pine and mixed conifer stands in Yellowstone National Park. *For. Ecol. Manag*., 2005; **208**(1): 303–317.
17. Cowden, C.C., Peterson, C.J. Annual and seasonal dynamics of ectomycorrhizal fungi colonizing white pine (*Pinus strobus*) seedlings following catastrophic windthrow in northern Georgia, USA. *Can. J. For. Res*., 2013; **43**(999): 215–223.
18. Walker, J.F., Miller Jr, O.K., Horton, J.L. Seasonal dynamics of ectomycorrhizal fungus assemblages on oak seedlings in the southeastern Appalachian Mountains. *Mycorrhiza*, 2008; **18**(3): 123–132.
19. Kennedy, P.G., Higgins, L.M., Rogers, R.H., Weber, M.G. Colonization-competition tradeoffs as a mechanism driving successional dynamics in ectomycorrhizal fungal communities. *PloS One*, 2011; **6**(9): e25126.
20. Twieg, B.D., Durall, D.M., Simard, S.W. Ectomycorrhizal fungal succession in mixed temperate forests. *New Phytol*., 2007; **176**(2): 437–447.
21. Dickie, I.A., Moyersoen, B. Towards a global view of ectomycorrhizal ecology. *New. Phytol*., 2008; **180**(2): 263–265.
22. Johnson, D., Ijdo, M., Genney, D.R., Anderson, I.C., Alexander, I.J. How do plants regulate the function, community structure, and diversity of mycorrhizal fungi? *J. Exp. Bot*., 2005; **56**(417): 17514–1760.
23. Obase, K., Lee, J.K., Lee, S.Y., Chun, K.W. Diversity and community structure of ectomycorrhizal fungi in *Pinus thunbergii* coastal forests in the eastern region of Korea. *Mycoscience*, 2011; **52**(6): 383–391.
24. Anderson, I.C., Campbell, C.D., Prosser, J.I. Diversity of fungi in organic soils under a moorland-Scots pine (*Pinus sylvestris* L.) gradient. *Environ. Microbiol*., 2003; **5**(11): 1121–1132.
25. El Karkouri, K., Martin, F., Mousain, D. Diversity of ectomycorrhizal symbionts in a disturbed *Pinus halepensis* plantation in the Mediterranean region. *Ann. For. Sci*., 2004; **61**(7): 705–710.
26. Walbert, K., Ramsfield, T.D., Ridgway, H.J., Jones, E.E. Ectomycorrhizal species associated with *Pinus radiata* in New Zealand including novel associations determined by molecular analysis. *Mycorrhiza*, 2010; **20**(3): 209–215.
27. Visser, S. Ectomycorrhizal fungal succession in jack pine stands following wildfire. *New Phytol*., 1995; **129**(3): 389–401.
28. Ma, D.L., Zang, S.Y., Wan, L.H., Zhang, D.Y. Ectomycorrhizal community structure in chronosequences of *Pinus densiflora* in eastern China. *Afr. J. Microbiol. Res*., 2012; **6**(32): 6204–6209.
29. Horton, T.R., Bruns, T.D. The molecular revolution in ectomycorrhizal ecology: peeking into the black-box. *Mol. Ecol*., 2001; **10**(8): 1855–1871.
30. Taylor, A.F.S. Fungal diversity in ectomycorrhizal communities: sampling effort and species detection. *Plant Soil*, 2002; **244**(1–2): 19–28.
31. Kidd, P.S., Prieto Fernández, A., Monterroso, C., Acea, M.J.Rhizosphere microbial community and hexachlorocyclohexane degradative potential in contrasting plant species. *Plant Soil*, 2008; **302**(1–2): 233–247.
32. Yeomans, J.C., Bremner, J.M. A rapid and precise method for routine determination of organic carbon in soil. *Commun. Soil Sci. Plant*, 1988; **19**(13): 1467–1476.
33. Olsen, S.R., Cole, C.V., Watanabe, F.S., Dean, L.A. (eds): Estimation of available phosphorus in soils by extraction with sodium bicarbonate. Washington DC: USDA Circular 939, U.S. Government Printing Office, 1954; pp 1–19.
34. Li, H., Liang, X.Q., Chen, Y.X., Lian, Y.F., Tian, G.M., Ni, W.Z. Effect of nitrification inhibitor DMPP on nitrogen leaching, nitrifying organisms, and enzyme activities in a rice-oilseed rape cropping system. *J. Environ. Sci*., 2008; **20**(2): 149–155.
35. Brundrett, M., Bougher, N., Dell, B., Grove, T., Malajczuk, N.(eds): Working with mycorrhizas in forestry and agriculture. Canberra: Australian centre for international agricultural research, 1996; pp 179–183.

36. Korkama, T., Pakkanen, A., Pennanen, T. Ectomycorrhizal community structure varies among Norway spruce (*Picea abies*) clones. *New. Phytol*., 2006; **171**(4): 815–824.
37. Landeweert, R., Leeflang, P., Smit, E., Kuyper, T. Diversity of an ectomycorrhizal fungal community studied by a root tip and total soil DNA approach. *Mycorrhiza*, 2005; **15**(1): 1–6.
38. Gardes, M., Bruns, T.D. ITS primers with enhanced specificity for basidiomycetes-applicationto the identification of mycorrhizae and rusts. *Mol. Ecol*., 1993; **2**(2): 113–118.
39. White, T.J., Bruns, T.D., Lee, S.B., Taylor, J.W.: Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR protocols: a guide to methods and applications* (Innis, M.A., Gelfand, D.H., Sninsky, J.J., White, T.J., eds). New York: Academic press, 1990; pp 315–322.
40. Muyzer, G., Smalla, K. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie Van Leeuwenhoek*, 1998; **73**(1): 127–141.
41. Nakatsu, C.H. Soil microbial community analysis using denaturing gradient gel electrophoresis. *Soil Sci. Soc. Am. J*., 2007; **71**(2): 562.
42. Gao, Z., Li, B.L., Zheng, C.C., Wang, G.Y. Molecular detection of fungal communities in the Hawaiian marine sponges *Suberites zeteki* and *Mycale armata*. *Appl. Environ. Microbiol*., 2008; **74**(19): 6091–6101.
43. Blom, J.M., Vannini, A., Vettraino, A.M., Hale, M.D. Godbold, D.L. Ectomycorrhizal community structure in a healthy and a *Phytophthora*-infected chestnut (*Castanea sativa* Mill.) stand in central Italy. *Mycorrhiza*, 2009; **20**(1): 25–38.
44. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol*., 2011; **28**(10): 2731–2739.
45. Silvestri, G., Santarelli, S., Aquilanti, L., Beccaceci, A., Osimani, A., Tonucci, F., Clementi, F. Investigation of the microbial ecology of Ciauscolo, a traditional Italian salami, by culture-dependent techniques and PCR-DGGE. *Meat Sci*., 2007; 77(3): 413–423.
46. Xu, Z.Y., Tang, M., Chen, H., Ban, Y.H., Zhang, H.H. Microbial community structure in the rhizosphere of *Sophora viciifolia* grown at a lead and zinc mine of northwest China. *Sci. Total Environ*., 2012; **435**:453–464.
47. Wang, Q., Guo, L.D. Ectomycorrhizal community composition of *Pinus tabulaeformis* assessed by ITS-RFLP and ITS sequences. *Botany*, 2010; **88**(6): 590–595.
48. Koide, R.T., Xu, B., Sharda, J., Lekberg, Y., Ostiguy, N. Evidence of species interactions within an ectomycorrhizal fungal community. *New. Phytol*., 2005; **165**(1): 305–316.
49. Gardes, M., Bruns, T.D. Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: above-and below-ground views. *Can. J. Bot*., 1996; **74**(10): 1572–1583.
50. Izzo, A., Nguyen, D.T., Bruns, T.D. Spatial structure and richness of ectomycorrhizal fungi colonizing bioassay seedlings from resistant propagules in a Sierra Nevada forest: comparisons using two hosts that exhibit different seedling establishment patterns. *Mycologia*, 2006; **98**(3): 374–383.
51. Matsuda, Y., Hayakawa, N., Ito, S. Local and microscale distributions of *Cenococcum geophilum* in soils of coastal pine forests. *Fungal Ecol*., 2009; **2**(1): 31–35.
52. Matsuda, Y., Hijii, N. Ectomycorrhizal fungal communities in an *Abies firma* forest, with special reference to ectomycorrhizal associations between seedlings and mature trees. *Can. J. Bot*., 2004; **82**(6): 822–829.
53. Kjøller, R. Disproportionate abundance between ectomycorrhizal root tips and their associated mycelia. *FEMS. Microbiol. Ecol*., 2006; **58**(2): 214–224.
54. Sánchez Castro, I., Ferrol, N., Cornejo, P., Barea, J.M. Temporal dynamics of arbuscular mycorrhizal fungi colonizing roots of representative shrub species in a semi-arid Mediterranean ecosystem. *Mycorrhiza*, 2012; **22**(6): 449–460.
55. Schadt, C.W., Martin, A.P., Lipson, D.A., Schmidt, S.K. Seasonal dynamics of previously unknown fungal lineages in tundra soils. *Science*, 2003; **301**(5638): 1359–1361.
56. Davey, M.L., Heegaard, E., Halvorsen, R., Ohlson, M., Kauserud, H. Seasonal trends in the biomass and structure of bryophyte-associated fungal communities explored by 454 pyrosequencing. *New. Phytol*., 2012; **195**(4): 844–856.
57. Kranabetter, J.M., Friesen, J., Gamiet, S., Kroeger, P. Ectomycorrhizal mushroom distribution by stand age in western hemlock-lodgepole pine forests of northwestern British Columbia. *Can. J. For. Res*., 2005; **35**(7): 1527–1539.
58. Richard, F., Millot, S., Gardes, M., Selosse, M.A. Diversity and specificity of

ectomycorrhizal fungi retrieved from an old-growth Mediterranean forest dominated by *Quercus ilex*. *New*. *Phytol*., 2005; **166**(3): 1011–1023.

59. Kalliokoski, T., Pennanen, T., Nygren, P., Sievänen, R., Helmisaari, H.S. Belowground interspecific competition in mixed boreal forests: fine root and ectomycorrhiza characteristics along stand developmental stage and soil fertility gradients. *Plant Soil*, 2009; **330**(1–2): 73–89.
60. Pickles, B.J., Genney, D.R., Potts, J.M., Lennon, J.J., Anderson, I.C., Alexander, I.J. Spatial and temporal ecology of Scots pine ectomycorrhizas. *New*. *Phytol*., 2010; **186**(3): 755–768.
61. van der Heijden, M.G.A., Klironomos, J.N., Ursic, M., Moutoglis, P., Streitwolf Engel, R., Boller, T., Wiemken, A., Sanders, I.R. Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. *Nature*, 1998; **396**(6706): 69–72.
62. Kropp, B.R., Langlois, C.G. Ectomycorrhizae in reforestation. *Can*. *J*. *For*. *Res*., 1990; **20**(4): 438–451.
63. Marx, D.H., Ruehle, J.L.: Ectomycorrhizae as biological tools in reclamation and revegetation of waste lands. In: *Mycorrhizae for green Asia* (Mahadevan, A., Raman, N., Natarajan, K., eds). Madras: Centre for Advanced Studies in Botany, 1988; pp 336–344.