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Impact of tree species on barley rhizosphere-associated fungi in an agroforestry ecosystem as revealed by 18S rDNA PCR-DGGE

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Abstract Agroforestry systems have been considered a form of sustainable land use. Woody species in agroforestry systems can improve soil physicochemical properties by supplying leaf or stem litter. However, little is known about fungal community structure and diversity in agroforestry systems. In the present study, the culture-independent 18S rDNA-based polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) method was used to investigate fungal community structure in rhizosphere and bulk soil in *Populus euramevicana*-barley and *Taxodium distichum*-barley agroforestry systems. DGGE profiling and cluster analysis revealed that the fungal community structure in the rhizosphere was more complex than that of bulk soil. Our results also indicated that the rhizosphere fungal community in barley was less affected by *T. distichum* than by *P. euramevicana*. In addition, an increase in the relative abundance of certain rhizosphere fungal populations was detected in this agroforestry system. Sequencing of prominent DGGE bands revealed an increase in the rhizosphere of a fungal species belonging to the genera *Chaetomium*, which includes potential biocontrol agents. A rare cellulolytic fungus, *Acremonium alcalophilum*, was found in the bulk soil from *P. euramevicana* and barley grown under *P. euramevicana*. Taken together, our findings may provide new insights into agroforestry practices.

Keywords PCR-DGGE · Fungal community · Rhizosphere · Agroforestry

Introduction

The rhizosphere, a soil region adjacent to and influenced by plant roots, is characterized by intense microbial activity (Hinsinger et al. 2009; McNear 2013). Plant rhizospheres can provide abundant nutrients and a relatively stable environment, thus forming an attractive habitat for microorganisms (Saito et al. 2007). The host plant is thought to shape soil microbial community structure through root exudates, including both organic and inorganic compounds (de Ridder-Duine et al. 2005; Haichar et al. 2008). The resident microorganisms in the rhizosphere, particularly fungi, in turn exert profound effects on plant growth. Fungi encompass a wide

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variety of organisms that play important and diverse roles in soil ecological functions. Fungi are involved in processes ranging from the decomposition of organic matter to the provision of nutrients and water to plants and cycling of minerals (Hoshino and Matsumoto 2007; Finlay 2008). Some fungi with antagonistic activities benefit their plant hosts by protecting them from plant pathogens (Gomes et al. 2003; Berg et al. 2005), whereas other fungi are well-known pathogenic species that cause a variety of plant diseases (Thorn 1997).

Information about the diversity, population dynamics and community structure of rhizosphere fungi is critical to achieve a better understanding of their ecosystem functioning. Conventional culture-based methods once played an important role in the evaluation of fungal communities. However, many fungi are refractory to laboratory culture, and some of these culture methods are time-consuming and laborious, thus limiting their usefulness for fungal community analysis (Saito et al. 2007; Gomes et al. 2003). To overcome these limitations, a number of PCR-based culture-independent molecular techniques have been developed to assess fungal diversity in the environment. These methods include denaturing gradient gel electrophoresis (DGGE) (van Elsas et al. 2000; Vainio and Hantula 2000), terminal restriction fragment length polymorphism (T-RFLP) (Edel-Hermann et al. 2004), and single-strand conformation polymorphism (SSCP) (Zachow et al. 2009). Among these, PCR-DGGE has been widely used in the analysis of microbial diversity, for both bacteria and fungi. Previous studies that used this method have provided vital information regarding the diversity and population dynamics of soil and rhizosphere fungi (Hoshino and Matsumoto 2007; Gomes et al. 2003; Broeckling et al. 2008; Manici and Caputo 2010).

Agroforestry is a land use system that incorporates trees to grow in farming systems in association with crops or livestock, simultaneously or sequentially (Reynolds et al. 2007; Fanish and Priya 2013). Agroforestry practices have been considered sustainable, functionally biodiverse and environmentally friendly land use alternatives for treeless agroecosystems. These systems can offer land-users multiple opportunities, including enhancing farm productivity and improving soil fertility, and can also provide protective functions, such as nutrient cycling, microclimate improvement, and carbon sequestration (Fanish and Priya 2013; Udawatta et al. 2009). According to the components present, agroforestry systems are classified as agri-silviculture systems, which are composed of trees with crops, agri-horticulture systems, which combine fruit trees with crops, silvipastoral systems, which mix trees and animals, or agri-silvipastoral systems, which combine trees with both crops and animals (Fanish and Priya 2013).

Trees in agroforestry systems can improve soil quality and soil physical properties, thus potentially influencing the composition and function of the soil microbial community. However, studies on the diversity and population dynamics of microbial communities in agroforestry systems are rare. At present, only a few works on this topic have been reported. Using fatty acid methyl ester (FAME) analysis, Vallejo et al. (2012) found that the structure and composition of microbial communities in silvopastoral systems shifted in different land management systems. In another study, arbuscular mycorrhizal (AM) fungal diversity was assessed using spore morphology, which showed that spore density was greater in tree and crop rhizospheres than in the soils, and it was higher in tree roots than crop roots (Pande and Tarafdar 2004). The microbial population dynamics of soil under traditional agroforestry systems were also examined using the traditional culture-dependent technique (Tangjang et al. 2009). Seasonal changes in bacterial and fungal population were found. For bacteria the highest population was during spring and it was autumn for fungi. The important role of plant species in shaping the rhizosphere-associated fungal community structure remains unclear. To address these issues, this study investigated the structure and composition of fungal communities in the rhizosphere and bulk soils in an agroforestry system (trees with crops) using the 18S rDNA PCR-DGGE molecular approach.

Materials and methods

Site description

The study site is located in the Sheyang forestry farm, Sheyang, Jiangsu Province, P.R. China (33°33′30″–37′30″N, 120°24′35″–30′35″E). The climate of the study area is northern subtropical maritime monsoon,

with a mean annual temperature of 14.5 °C. The average monthly temperature ranges from 8.0 °C (January) to 27.1 °C (June). The mean annual precipitation in this area is 1069.0 mm, and the annual evaporation is 1407.4 mm. The level of underground water varies between 1.5 and 2.0 m. The soil type is predominantly silt loam, with a pH of approximately 8.0. The field study was permitted by Sheyang forestry farm, Jiangsu Province, and the authors declared that the field studies did not involve endangered or protected species.

Agroforestry system design

The agroforestry system used in this study is of the agri-silviculture type, which was set up in November 2010. We randomly designed three agroforestry treatments: two containing barley (*Hordeum vulgare* L.) as well as *Populus euramevicana* or *Taxodium distichum* (L.) Rich and a barley-only control. The three treatments were next to each other and have similar soil characteristics and cropping histories. The variety of barley planted in these plots was *H. distichum*. The trees used in this system were planted independently and tree species were planted with 4 × 6 and 3 × 4 m spacing, respectively. Trees were four years old when sampling. The experimental design is presented in Fig. 1.

Soil sampling

Five soil sampling treatments were included in this study, which were *P. euramevicana*, *T. distichum*, *P. euramevicana* + barley, *T. distichum* + barley and barley. Soil samples were collected from the bulk and rhizosphere soil 10–15 cm below the ground surface and 50 cm away from the tree trunk. For the collection of rhizosphere soil, excess bulk soil not tightly adhering to the roots was removed by vigorous shaking. There were three replicates for agroforestry treatments (true replicate sites) and three sampling locations were randomly selected in each plot 50 m apart. At each sampling location, five replicates (pseudo-replicates), i.e., five subsamples, were collected within a 2 × 2 m square. All the subsamples were subsequently sieved and pooled to make one composite soil sample. And three composite soil samples for each treatment were collected. The collected soil samples were placed into polyethylene bags and carried back to the laboratory in a portable cool container.

DNA extraction and PCR amplification

Soil DNA was extracted from 0.5 g of fresh soil using the E.Z.N.A.™ Soil DNA Kit (Omega Bio-tek Inc., Norcross, GA, USA). To obtain rhizosphere-associated fungal DNA, 5 g of the roots containing tightly adhering soil were suspended in 50 ml of sterile 0.85% NaCl solution and shaken for 30 min. After that the supernatant was centrifuged for 10 min at 1000 rpm, and the pellet resulted was used for DNA extraction. The extracted DNA was preserved at −80 °C until further use. PCR amplification of the targeted fungal 18S rDNA was carried out in a 50 μL reaction volume following Vainio and Hantula (2000) using universal fungal primers GC-FR1 (5′-AI CCA TTC AAT CGG TAI T-3′) and FF390 (5′-CGA TAA CGA ACG AGA CCT-3′). A 40-bp GC-clamp was added to the 5′ end of the FR1 primer. PCR was performed in an S1000 thermal cycler (Bio-Rad, Hercules, CA) with the following thermocycling program: initial denaturation at 95 °C for 8 min, followed by 30 cycles of 95 °C for 30 s, 50 °C for 45 s and 72 °C for 2 min, with a final extension step at 72 °C for 10 min. The PCR products were electrophoresed in 1.5% (w/v) agarose gels and stained with GelStain (TransGen Biotech, Beijing, China). PCR products were further purified for DGGE analysis using the E.Z.N.A.® Cycle-Pure Kit (Omega Bio-tek Inc., Norcross, GA, USA).

DGGE analysis

DGGE was performed using a DGGE-2001 system (CBS Scientific, USA). PCR products were separated on 8% (w/v) polyacrylamide gels with a 40–60% denaturing gradient (where 100% denaturing was defined as 40% deionized formamide plus 7 M urea). Electrophoresis was run in 1 × TAE buffer at 60 °C. The voltage was set at 20 V for 15 min and then at 100 V for 16 h. The gels were then stained with SYBR Green I at a 1:10,000 dilution (Invitrogen, Eugene, OR, USA) for 30 min. Images were viewed by UV transillumination and scanned using a ChemiDOC XRS instrument (Bio-Rad, USA).

The DGGE banding patterns were analyzed using Quantity One 1-D software (Bio-Rad, USA) following normalization of the gel image. Similarity dendrograms

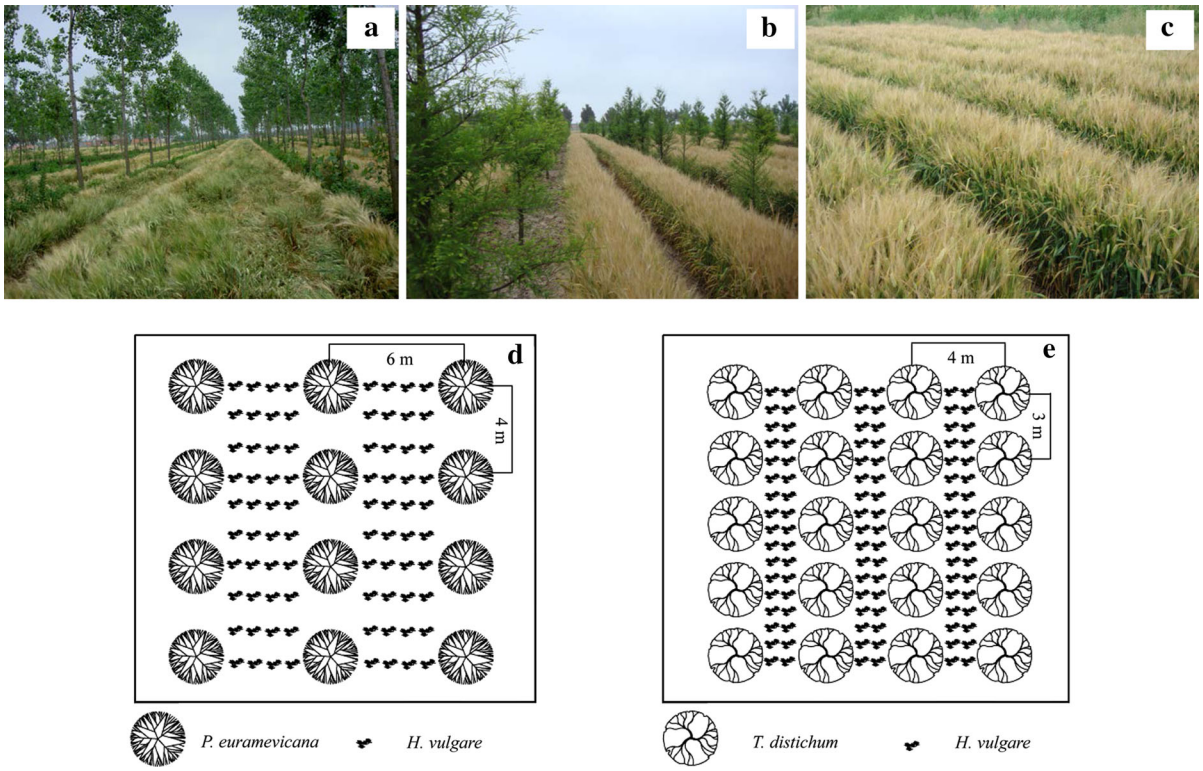

Fig. 1 Experimental design. **a** *Populus euramevicana*- barley agroforestry system, in which *P. euramevicana* trees were planted in rows between alleys of barley; **b** *Taxodium distichum*-barley agri-silviculture system; **c** barley; **d** and **e** illustrations showing the spatial arrangements of trees and crops in the agroforestry system

were generated using the UPGMA (unweighted pair group method with arithmetic averages) clustering method. UPGMA also known as average linkage, uses clustering approach and uncorrected data to create phylogenetic trees. It's a distance based method and assumes constant rates of evolution over different lineages. To evaluate the fungal complexity of each sample, the following three indices of biodiversity were applied: species richness (S), the Shannon–Weaver index (H) and the equitability index (E). S was determined from the number of DGGE bands in each lane. The Shannon index was calculated using the formula $H = -\sum P_i \ln P_i$, where P_i (calculated as $P_i = n_i/N$) is the importance probability of the bands in a lane. In this formula, n_i is the peak height of the i-th band, and N is the sum of all peak heights in the DGGE profile (Yu and Morrison 2004). E was calculated by the function $E = H/\ln S$. Principal component analysis (PCA) was performed with Canoco software, version 4.5 (Microcomputer Power, Ithaca, NY, USA), according to Ogino et al. (2001).

Gene cloning, sequencing and phylogenetic analysis

Dominant bands were excised from DGGE gels and placed in sterile Eppendorf tubes containing E.Z.N.A.™ Poly-Gel DNA Extraction Kit reagent (Omega Bio-tek Inc., Norcross, GA, USA), and DNA was purified from the excised gel fragments following the procedure recommended by the manufacturer. The purified DNA was then re-amplified with the FF390/FR1-GC primer pair using the former PCR protocol. The PCR products were subjected to a second DGGE to check the purity of the excised bands. Single bands that showed electrophoretic mobility identical to the original bands were excised and reamplified with the primers FF390 and FR1 (without the GC clamp). The amplified DNA fragments were ligated into the *pEASY*-T1 Simple Cloning vector (TransGen Biotech, Beijing, China), and the ligation mixes were transformed into *Trans*1-T1 Phage Resistant Chemically competent cells (TransGen Biotech, Beijing, China)

according to the manufacturer's instructions. The positive recombinants were identified by PCR using M13F and M13R primers. Clones with the correct insert were sequenced with T7 sequencing primers using an ABI 377 DNA sequencer (Applied Biosystems, Warrington, UK). DNA sequences were compared with 18S rDNA sequences available in the NCBI blast database (http://www.ncbi.nlm.nih.gov) using BLAST-N.

For phylogenetic analyses, sequences were initially aligned using Clustal X 1.81 software (Thompson et al. 1997), and a phylogenetic tree was constructed using the neighbor-joining method in MEGA version 6 (Tamura et al. 2013). A phylogeny test was performed to estimate the confidence of the tree topology by bootstrap analysis with 1000 replicates.

Statistical analysis

Statistical comparisons were carried out to examine fungal communities in the rhizosphere and bulk soil samples by analysis of 18S rDNA PCR-DGGE data of triplicates among groups of treatments. Comparisons were made by one-way ANOVA based on Dunn's multiple comparison test using SigmaStat 3.5 software. Data were presented as the mean ± SEM. And a P value of <0.05 was considered statistically significant difference between treatments.

Results

Fungal community structure and diversity in the rhizosphere

To characterize the structure of the rhizosphere-associated fungal community, PCR-DGGE analysis was conducted using the universal fungal primer set GC-FR1/FF390. As shown in Fig. 2a, numerous DGGE bands of various intensities were detected, and the DGGE profiles displayed complex banding patterns. Although a few strong dominant bands were present in all of the samples, a large number of well-resolved weak bands were also observed in the profiles, causing the gel lanes to exhibit unique banding patterns. The DGGE profiles were further studied by cluster analysis. Clustering of the banding patterns revealed a range of similarity from 47 to 84% among the samples (Fig. 2b). Two main clusters were formed at the 47% similarity level. The profiles of the rhizosphere soil samples taken from *P. euramevicana* and from barley grown under *P. euramevicana* formed one cluster, which had a similarity of 50–84%. The profiles of the other samples were separated into another cluster, which included the samples taken from *T. distichum*, barley and barley grown under *T. distichum*. The similarity in this group ranged from 49 to 81%. Furthermore, the profile of one replicate of *P. euramevicana* rhizosphere soil was separated into the subcluster formed by rhizosphere soil samples of barley grown under *T. distichum*. In addition, one profile of the rhizosphere soil from barley grown under *P. euramevicana* was included in the group formed by two replicates of *P. euramevicana*.

DGGE fingerprinting of fungal communities in bulk soil

The fungal community in the bulk soil was also analyzed by DGGE. In contrast to the rhizosphere pattern, the DGGE profiles of the bulk soil samples revealed the presence of only two dominant bands accompanied by numerous low-intensity bands in all samples, indicating the dominance of few populations (Fig. 3a). In addition, the DGGE profiles of the soil samples from the field grown with *T. distichum* had fewer bands compared with other samples. The dendrogram generated by UPGMA cluster analysis revealed two distinct clusters with 72–80% and 73–86% similarity (Fig. 3b). The similarity between the two clusters was 61%. The profiles generated from the replicate soil samples of the field grown with *T. distichum* were grouped together, while the other samples belonged to another cluster. In contrast to the rhizosphere samples, the molecular fingerprints of the soil samples showed relatively little variation among replicates of the same treatment.

Fungal diversity

The richness and diversity of fungi in the rhizosphere and bulk soil samples were calculated using several diversity indices, including S, H and E. As shown in Fig. 4a, fungal richness in the rhizosphere and bulk soil of *T. distichum* both tended to be lower than that of other samples, particularly in the bulk soil where there were only 15 DGGE bands, compared with 24–27 bands in the other samples. The Shannon index H for

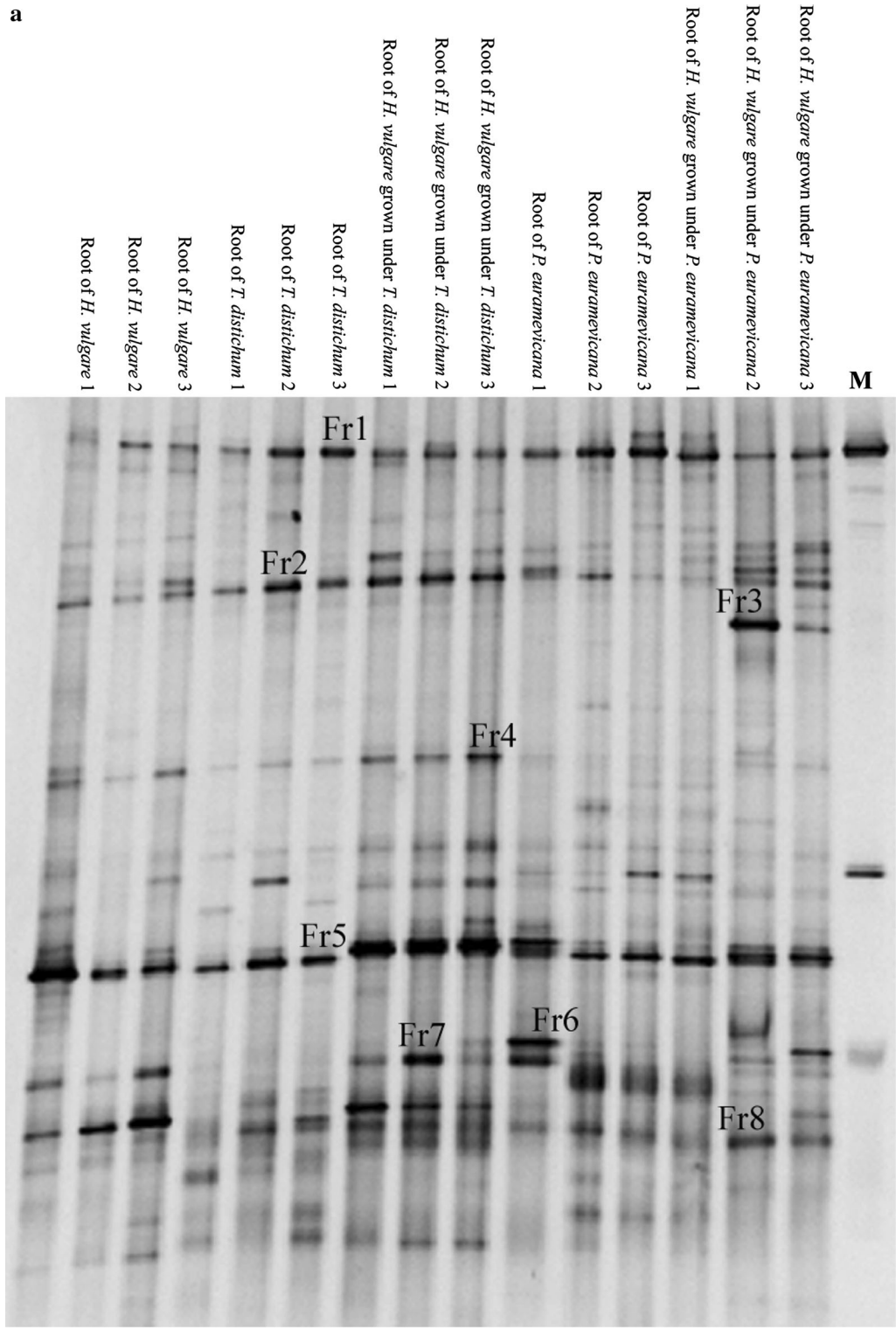

Fig. 2 DGGE banding patterns recovered from rhizosphere samples (**a**) and UPGMA cluster analysis of the banding patterns (**b**). M, DNA marker. Fr1 to Fr8 correspond to bands that were cloned and sequenced

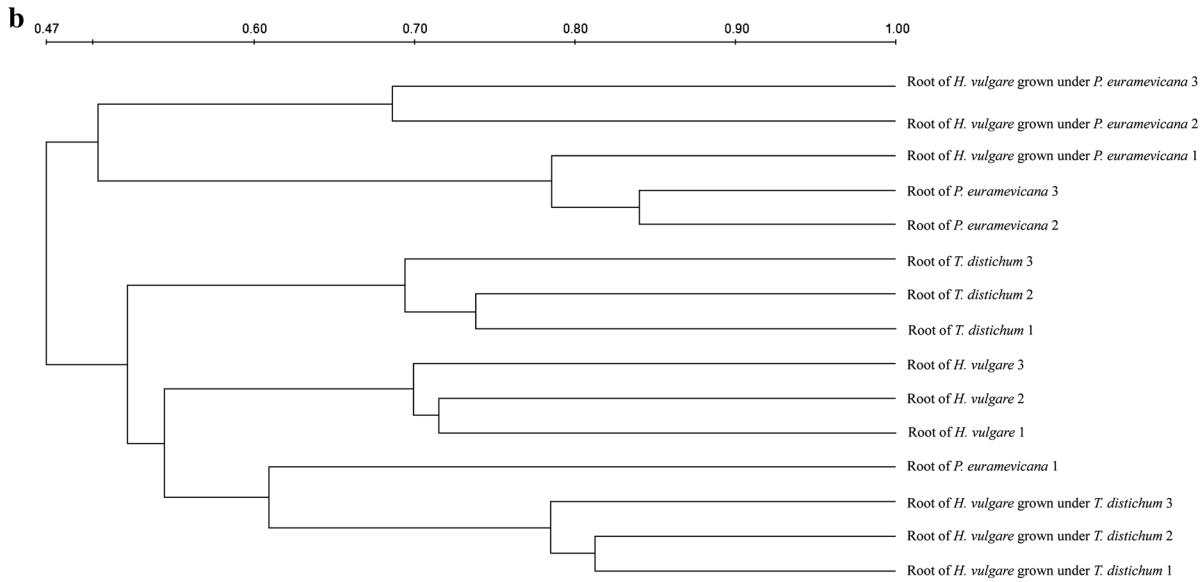

Fig. 2 continued

the rhizosphere soil was almost equivalent among the different treatments, with values between 2.71 and 2.99 (Fig. 4b). In contrast to the rhizosphere soil, the index for the bulk soil of *T. distichum* was significantly lower than that of the other treatments ($P < 0.05$). The equitability index *E* was constant for both rhizosphere soil and bulk soil, and no significant differences were observed (Fig. 4c).

PCA analysis

To further investigate the complex DGGE patterns, principal component analysis were performed (Fig. 5). The PCA plot generated from the DGGE profiles of the rhizosphere samples explained 30.2% (x axis) and 23.5% (y axis) of the variance (Fig. 5a). Three main groupings were observed in the PCA plot. The first group consisted of the samples from the rhizosphere of barley together with the rhizosphere samples of barley grown under *T. distichum*. The second group was composed of the three samples from *T. distichum*, and the third group clustered three rhizosphere samples of barley grown under *P. euramevicana* and two samples from *P. euramevicana*. One replicate of *P. euramevicana* rhizosphere soil was separated from all three groups. When PCA analysis was performed on the DGGE profiles of bulk soil samples, the samples from fields grown with *T. distichum* (not include bulk soil samples from barley grown under *T. distichum*) clustered together in a pattern that could be clearly distinguished from the other samples (Fig. 5b).

Sequence analysis of DGGE bands

To determine the identities of the dominant fungal populations in the rhizosphere and bulk soil, a total of 19 major bands in the DGGE gel profiles [bands Fr1 to Fr8 in the profile derived from rhizosphere soil (Fig. 2a) and bands Fs1 to Fs11 in the profile derived from bulk soil (Fig. 3a)] were excised and subjected to DNA sequencing. The closest relatives to the excised bands are summarized in Table 1. Eight of the 19 bands excised for sequencing were uncultured environmental fungi. The main band in the DGGE profile derived from rhizosphere soil (Fr5) and the band Fs6 in the profile derived from bulk soil were identical to *Chaetomium* sp. Fr1 and Fs2 were also identical sequences that belonged to an uncultured fungus. The phylogenetic analysis of the remaining ten sequences indicated that they fell into two phyla, ascomycota and zygomycota (Fig. 6). The ascomycota group included seven sequences, and the zygomycota group included three sequences.

Discussion

To date, the culture-independent molecular method PCR-DGGE has not been used to study the structure

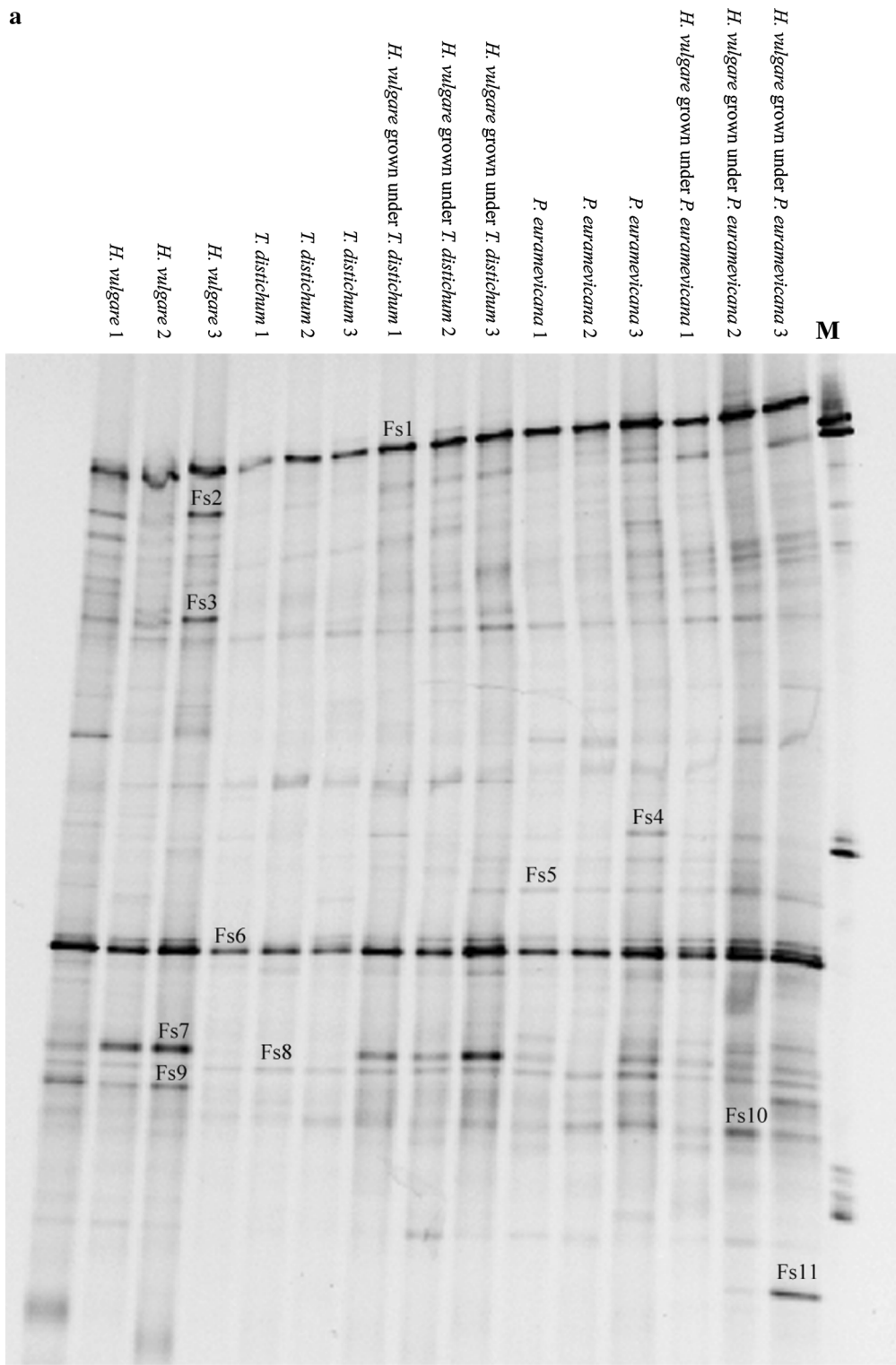

Fig. 3 DGGE profiles of bulk soil samples (**a**) and similarity dendrogram obtained using the UPGMA cluster method (**b**). Lanes M, DNA marker. The dominant bands selected for sequencing are labeled Fs1 to Fs11

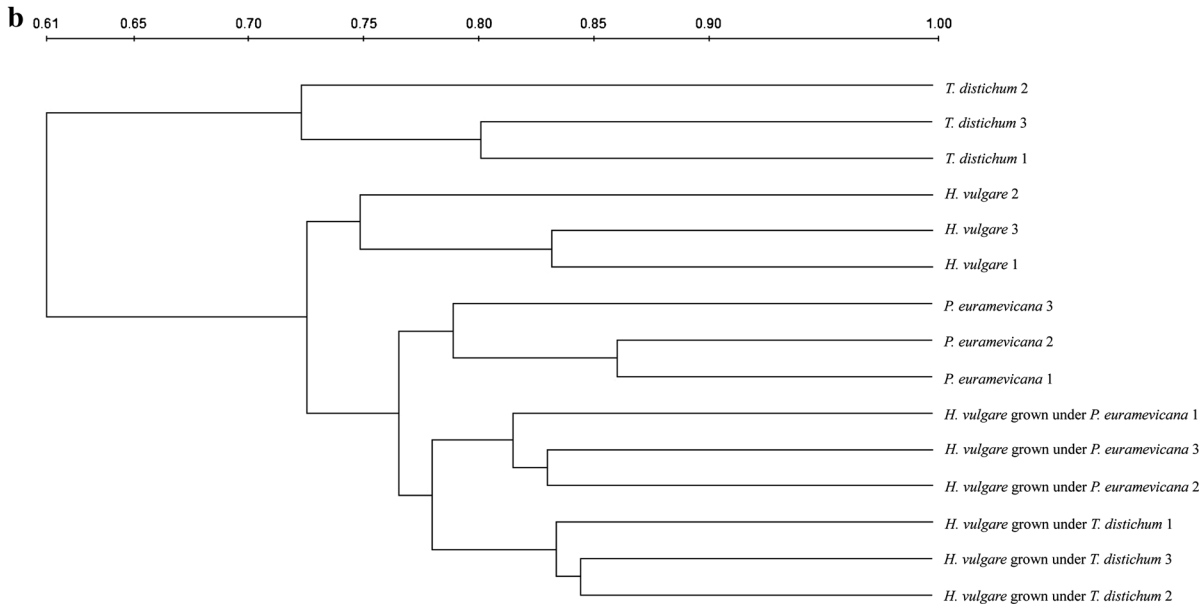

Fig. 3 continued

and composition of the soil microbial community in agroforestry systems. In the present study, we employed 18S rDNA PCR-DGGE to examine the structure and composition of fungal communities in the rhizosphere and bulk soils of *P. euramevicana*-barley and *T. distichum*- barley agri-silviculture systems. Our results indicated that fungal community structure in the rhizosphere was more complex than that of bulk soil. And *P. euramevicana* had much more effect on the rhizosphere fungal community in barley than that of *T. distichum*. It also revealed an increase in the relative abundance of certain rhizospheric fungi in this agroforestry system.

Plant roots have been thought to influence rhizosphere-associated microbial communities in a species-specific manner (Berg and Smalla 2009). Therefore, in this study, we first examined the rhizosphere fungal community by PCR-DGGE analysis. In agreement with findings in bacterial rhizosphere communities (Smalla et al. 2011), we found an increase in the relative abundance of certain fungal populations within the rhizosphere. That is, some bands that were weak in the profile obtained from bulk soil were intensified in the rhizosphere DGGE patterns, e.g., bands Fs2 and Fr1, which DNA sequencing revealed to be derived from the same fungal species. This fungal species was enriched in all roots examined. This rhizosphere effect was also found in the vicinity of maize roots (Gomes et al. 2003). In the DGGE profiles from maize rhizospheres 6 predominant bands were present, while only 3 dominant bands were observed in bulk soil pattern. The enrichment in rhizosphere fungal communities may be attributable to exudates produced by plant roots. These root exudates are rich in ions, enzymes and a wide array of carbon-containing compounds that may play a key role in the enrichment of specific microbial populations in the rhizosphere (Berg and Smalla 2009; Uren 2000). Fewer predominant fungal species were detected in the bulk soil relative to the rhizosphere soil. A similar result was observed by another research group. Broeckling et al. (2008) found that soil fungal biomass declined gradually as plants grew over three generations. They speculated that the depletion of nutrients in the soil over multiple generations might contribute to this decline, and it may represent a transient response toward a new stable community. Our cluster analysis (Fig. 2b) also revealed a relatively high level of similarity between the profiles derived from the roots from the monocultural barley plot and the roots of barley grown under *T. distichum*. This result indicated that *T. distichum* had a lesser impact than *P. euramevicana* on the barley rhizosphere-associated fungal community.

Another finding in our study was that the variability between replicates was higher in samples taken from the roots of *P. euramevicana* than in the other samples (Figs. 2a, b, 5a). It has been suggested that plant health

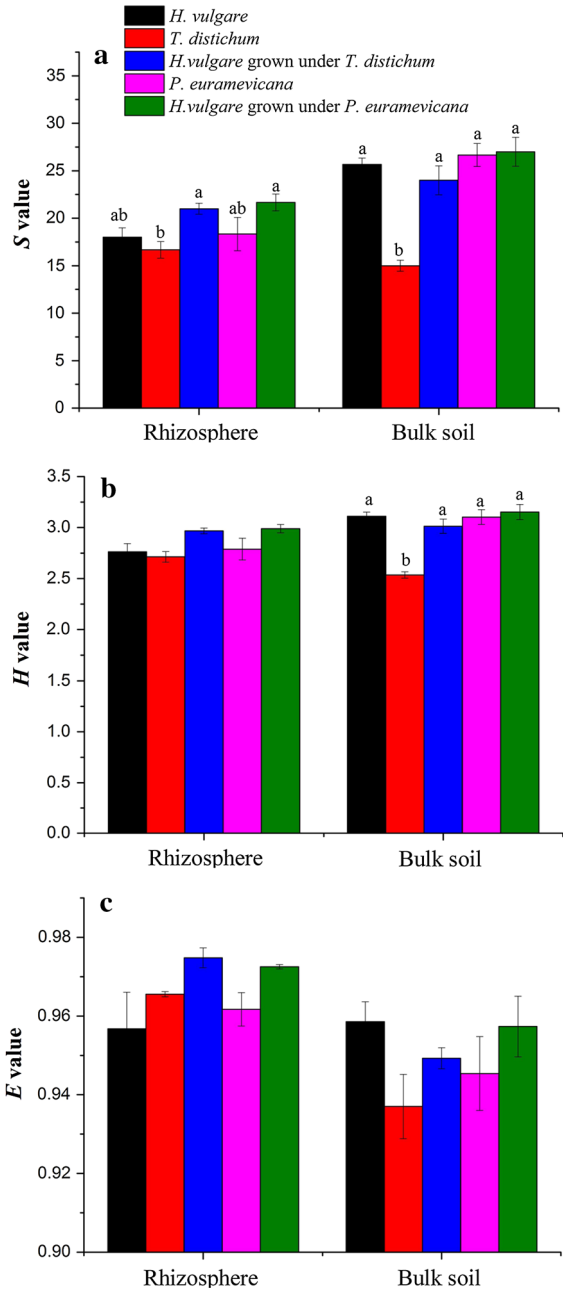

Fig. 4 Diversity analysis of fungi in the rhizosphere and bulk soil. **a** Number of bands in DGGE gels (S), **b** Shannon index (*H*), **c** equitability index (*E*). *Different lowercase letters* in the figure indicate significant differences

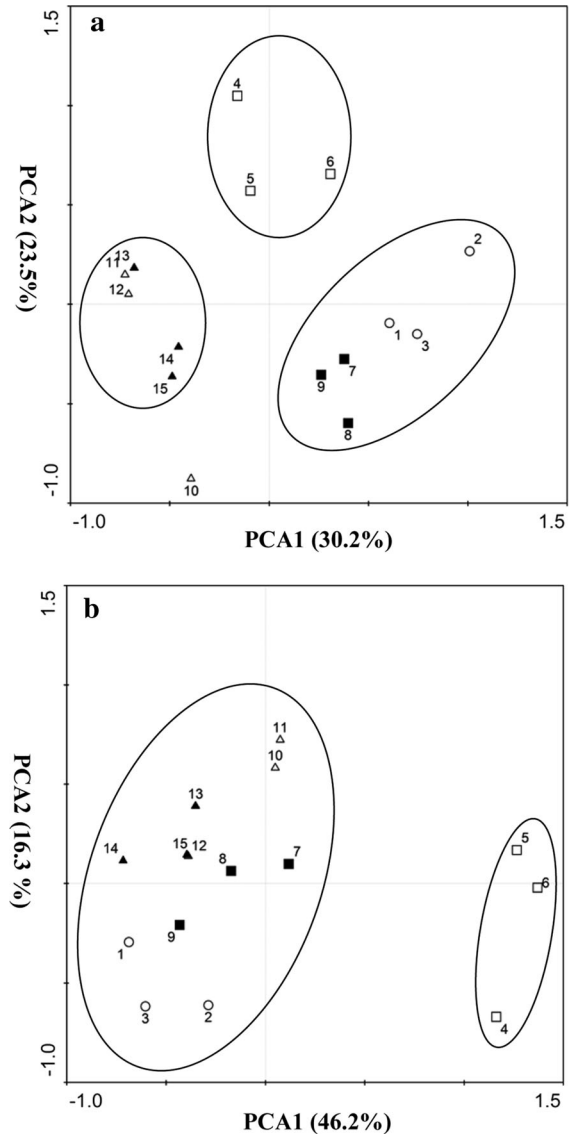

Fig. 5 Principal component analysis of DGGE profiles obtained from rhizosphere (**a**) and bulk soil (**b**). **a** 1, 2 and 3, samples from the roots of the monocultural barley plot; 4, 5 and 6, samples from the roots of *T. distichum*; 7, 8 and 9, samples from the roots of barley grown under *T. distichum*; 10, 11 and 12, samples from the roots of *P. euramevicana*; 13, 14 and 15, samples from the roots of barley grown under *P. euramevicana*; **b** 1, 2 and 3, bulk soil from barley; lanes 4, 5 and 6, bulk soil from *T. distichum*; lanes 7, 8 and 9, bulk soil from barley grown under *T. distichum*; lanes 10, 11 and 12, bulk soil from *P. euramevicana*; lanes 13, 14 and 15, bulk soil from barley grown under *P. euramevicana*

and developmental stage have significant impacts on the structure and composition of microbial communities in the rhizosphere (Gomes et al. 2003; Graner et al. 2003). For example, the fungal community in the rhizosphere of maize growing for 20 days was much different from that of the maize growing for 90 days (Gomes et al. 2003). Root exudates are a driving force

Table 1 Sequencing results of excised bands from DGGE profiles derived from rhizosphere and bulk soil

Bands	Accession No	Closest relative (Accession No)	Identity (%)	Phylum	Order
Fr1	KP781983	Uncultured fungus DGGE gel band SOM06 (KF741858.1)	99	Unknown	Unknown
Fr2	KP781984	*Alternaria alternata* HA4087 (KF962959.1)	99	Ascomycota	Pleosporales
Fr3	KP781985	*Spiromyces minutus* (AF007542.1)	96	Zygomycota	Kickxellales
Fr4	KP781986	*Plectosphaerella sp.* s187 (HQ871886.1)	100	Ascomycota	Glomerellales
Fr5	KP781987	*Chaetomium* sp. CBS 123940 (KC790427.1)	99	Ascomycota	Sordariales
Fr6	KP781988	Uncultured Ceratobasidium DGGE gel band F4 (HM453874.1)	99	Basidiomycota	Cantharellales
Fr7	KP781989	Uncultured soil ascomycete clone s20-71 (AJ515172.1)	99	Ascomycota	Unknown
Fr8	KP781990	Uncultured soil ascomycete clone r20-71 (AJ515164.1)	100	Ascomycota	Unknown
Fs1	KP781991	Uncultured fungus clone T3_IV_3a_20 (EF628892.1)	99	Unknown	Unknown
Fs2	KP781992	Uncultured fungus DGGE gel band SOM06 (KF741858.1)	99	Unknown	Unknown
Fs3	KP781993	Uncultured fungus clone Nikos_52 (HM104572.1)	97	Unknown	Unknown
Fs4	KP781994	*Linderina macrospora* Strain BCRC31802 (JQ004924.1)	100	Zygomycota	Kickxellales
Fs5	KP781995	*Acremonium alcalophilum* Strain CBS 114.92 (JX158486.1)	99	Ascomycota	Glomerellales
Fs6	KP781996	*Chaetomium* sp. CBS 123940 (KC790427.1)	99	Ascomycota	Sordariales
Fs7	KP781997	*Auxarthron umbrinum* UAMH 1874 (AY124499.1)	98	Ascomycota	Onygenales
Fs8	KP781998	*Phoma* sp. MJ76 (HM590661.1)	99	Ascomycota	Pleosporales
Fs9	KP781999	Uncultured fungus DGGE gel band 7 (EU281960.1)	99	Unknown	Unknown
Fs10	KP782000	*Ramicandelaber longisporus* ARSEF 6176 (KC297616.1)	99	Zygomycota	Kickxellales
Fs11	KP782001	*Nannizziopsis barbata* UAMH 11185 (KF466861.1)	98	Ascomycota	Onygenales

in shaping rhizosphere-associated microbial communities (Berg and Smalla 2009). However, the composition of root exudates may vary from plant to plant; thus, the relative abundance and composition of microorganisms in the vicinity of the root may change accordingly (Somers et al. 2004).

Sequencing of the major DGGE bands revealed that the majority of the sequenced bands belonged to two taxa: ascomycota and zygomycota (Table 1), while the rest were uncultured fungi detected as environmental clones. Five of the eight sequences obtained from dominant bands in the rhizosphere samples belonged to ascomycota. Fr2 and Fr5, the two major bands detected in DGGE profiles of rhizosphere soil, had high sequence similarity to *Alternaria alternate* and *Chaetomium* sp., respectively. Previous studies have reported that some species of the genera *Chaetomium* are potential biocontrol agents for a number of soil- and airborne plant pathogens. For example, *Chaetomium globosum* displayed antagonistic activities against a number of plant pathogens, including *Diapothe phaseolorum* f. sp. *Eridionalis*, *Pythium ultimum* (Di Pietro et al. 1992; Dhingra et al. 2003). *Chaetomium spirale* ND35, an endophytic fungus isolated from *Populus tomentosa*, showed potent suppressive activity against several common fruit and forest pathogenic fungi (Gao et al. 2005). Recently, Pontius et al. isolated some chaetoxanthones with antiprotozoal activity from the marine-derived fungus *Chaetomium* sp. (Pontius et al. 2008). *Chaetomium* sp. was found to be enriched in barley roots grown under *T. distichum*.

In the bulk soil samples, bands Fs2 and Fs6 appeared to correspond to bands Fr1 and Fr5 detected in rhizosphere soil, but the other bands were not detected in rhizosphere soil. It is worth noting that band Fs5 (99% similarity to *Acremonium alcalophilum*) was present in the bulk soil from *P. euramevicana* and barley grown under *P. euramevicana*. *Acremonium alcalophilum* is an alkalophilic species that can grow in alkaline environments (Nagai et al. 1995). This fungal species is also a rare cellulolytic fungus (Pereira et al. 2013). Thus, the presence of this species in the bulk soil may provide the barley with nutrients through the degradation of cellulose in the soil.

The DGGE method utilized in our study is a culture-independent microbial technique, which has

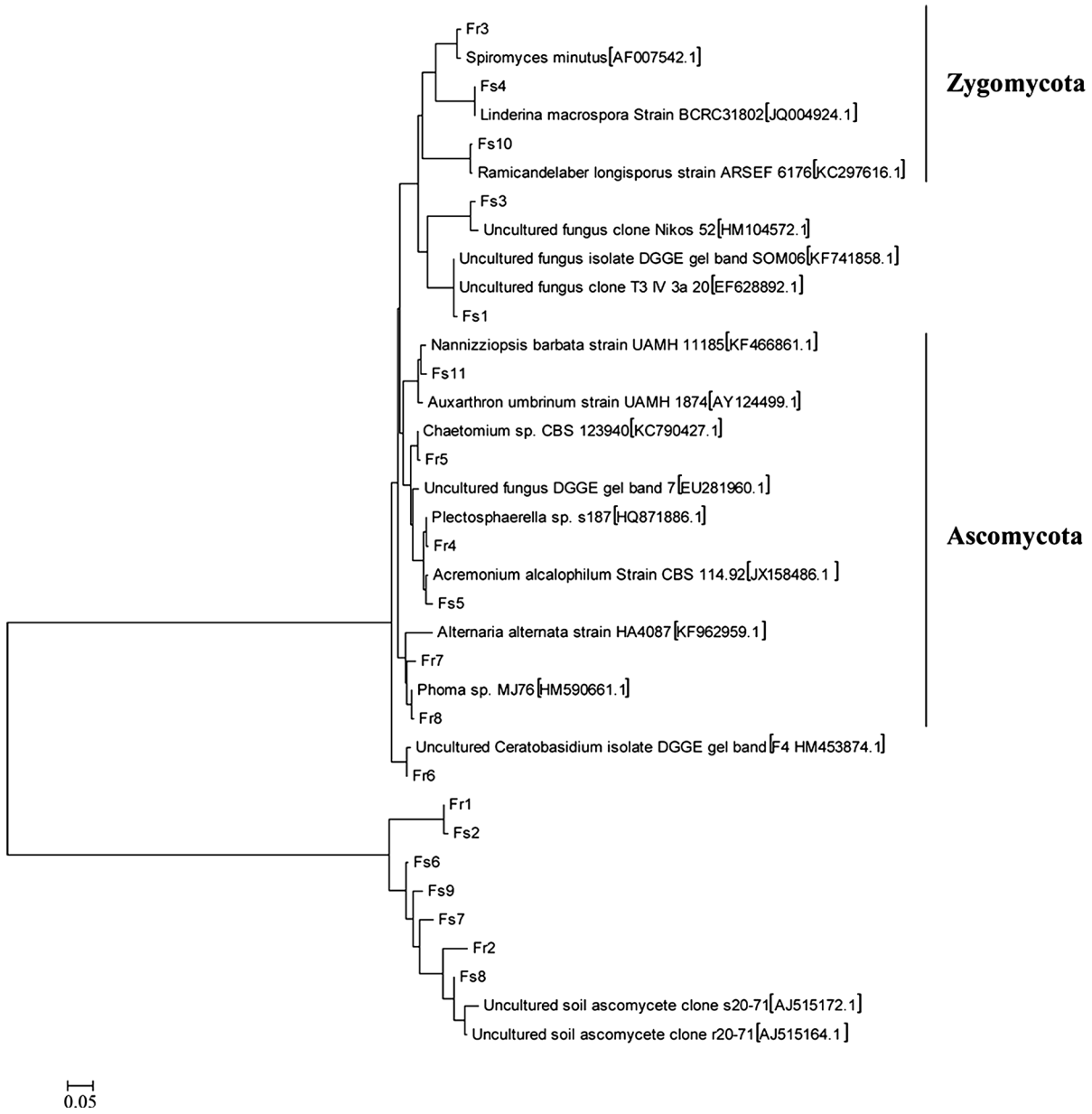

Fig. 6 Neighbor-joining phylogenetic tree for partial sequences of cloned 18S rDNA fragments. The GenBank accession numbers for each sequence are listed in *squared brackets*. The *scale bar* represents 0.05 substitutions per nucleotide position

been widely used in the investigation of environmental microbial communities. This method has many advantages such as it can separate numerous samples on a single gel, thus allowing rapid and simultaneous comparison between samples (O'Callaghan et al. 2006). Additionally, the separated bands on the gel can be excised for subsequent cloning and sequencing, providing phylogenetic information about dominant species in a community. Despite these advantages, there are still limitations for DGGE analysis, including underestimation of minor populations, introduction of DNA contamination during DNA isolation, gel-to-gel variability, etc. (Kowalchuk and Smit 2004). Recently, pyrosequencing, an automated high-throughput sequencing technique was applied to the study of soil microbial communities in a forest ecosystem (Hartmann et al. 2012). In comparison to DGGE, pyrosequencing allows the rapid and accurate

sequencing of nucleotide sequences, which can present high microbial richness coverage and offer an efficient way to target some of the cultivable organisms (Vaz-Moreira et al. 2011; Leite et al. 2012). Therefore, both approaches can be used complementarily for better understanding of microbial communities in agroforestry ecosystem.

Conclusion

Our results showed that tree species may play an important role in shaping the rhizosphere fungal community structure of crop roots in an agroforestry ecosystem. The differences in fungal community could also be caused by other factors that differed between the treatment plots, such as the carbon dynamic in soils stimulated by plant root exudates (Haichar et al. 2008). Moreover, the extent to which soil nutrient availability contributes to fungal communities warrants further investigation and may be helpful in elucidating the link between fungal community structure and soil processes.

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Compliance with ethical standards

Conflict of Interest The authors declare that they have no conflict of interest.

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