Soil fungal communities of montane natural secondary forest types in China

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Distinctive plant communities may provide specific physical and chemical properties with soils by specific litters and root exudates to exert effects on soil microorganisms. Past logging activities in the Qinling Mountains induced diverse natural secondary forest types (NSFTs). How these recovered NSFTs regulate patterns of soil microbial communities remain limited. In the study, we used terminal-restriction fragment length polymorphism (T-RFLP) to precisely determine forest type-specific soil fungal diversity and composition in five NSFTs. Our results indicated that NSFTs had significant impacts on the soil fungal communities. The most diverse fungal species were found in the Armand pine (*Pinus armandi*) and Chinese pine (*Pinus tabulaeformis*) forest soils, followed by sharptooth oak (*Quercus aliena* var. *acuteserrata*) and Chinese pine-sharptooth oak forest soils, the wilson spruce (*Picea wilsonii*) forests had the lowest soil fungal diversity. The analyses of community composition suggested that the fungal communities of Armand pine forest soils were similar to those of Chinese pine forest soils, while other communities prominently differed from each other. Stepwise multiple regression analysis revealed that soil silt, clay, pH, and ammonium nitrogen had intimate linkages with soil fungal diversity. Furthermore, the patterns of soil fungal communites were strongly governed by the specific soil environments of the tested NSFTs, as described by canonical correspondence analysis (CCA). Finally, our study showed that soil fungal communities may be mediated by NSFTs via specific soil edaphic status. Hence, such a comparable study may provide fundamental information for fungal diversity and community structure of natural forests and assist with better prediction and understanding how soil fungal composition and function alter with forest type transformation.

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Introduction

Fungi are essential members of soil microbiota that with other microbial components participate in matter cycling of forest ecosystems by gradually degrading and transforming plant organic matters into simple inorganic forms available for organisms (Blackwood *et al.*, 2007; Hoorman, 2011; Phillips *et al.*, 2013). Soil fungi seem to be inclined to acid habitats (Blagodatskaya and Anderson, 1998; Bååth and Anderson, 2003), Grantina *et al.* (2011) also demonstrated that fungal species richness and quantity in acid forest soils (pH = 4.46–5.30) are apparently higher than in arable soils (pH = 6.96–7.69). Studies on fungal community structure and diversity in forest soils not only significantly improve fungal resources, but facilitate better understanding of distribution rules in fungal communities in forest soils.

As growth substrates for forest vegetation, soils directly involve in a variety of ecological processes in forests (van Breemen *et al.*, 1997). In turn, forest vegetation provide soils with characteristics through ecological relevance by exerting significant and long-lasting effects on the formation of soil physical and chemical environments on which soil microbes rely (Kleb and Wilson, 1997; Finzi *et al.*, 1998; Ludley *et al.*, 2008, 2009; Aponte *et al.*, 2010). Therefore, composition and diversity of biotic communities that inhabit forest soils also may change with forest vegetation composition and soil biotopes (O'Malley, 2007; Christ *et al.*, 2011). Hence, forest types may select soil microbes (Konopka, 2011). Nevertheless, we have a poor understanding of this selection mechanism among different forest types. For instance, whether soil fungal communities have similarity between NSFTs dominated by related canopy species, between monospecific and mixed forests, and between coniferous and broadleaved forests is not fully understood.

The Qinling Mountains serve as a critical boundary for climate and vegetation, dividing China into North China and South China. The climate of North China is semi-arid and characterized by warm temperate deciduous broad-leaved forests, while the climate of South China is humid and characterized by subtropical evergreen and deciduous broad-leaved forests (Liu *et al.*, 2009). With its unique status as being the only east-west oriented mountain range (1,600 km) across China' mainland, the Qinling Mountains are a key region of land biodiversity of global importance by the Chinese government. Thus, studies on the mountain forest

ecosystems of the Qinling Mountains are essential for ecological research. Unfortunately, the forests in the Qinling Mountains suffered from extensive logging in the 60s~70s of last century, thereby leaving few primitive forests. The Huoditang forest region on the south-facing slope of the Qinling Mountains experienced final cutting that consequently resulted in the regeneration of diverse NSFTs, 95% of the region is covered by recovered natural secondary forests.

Previous studies have revealed that the final cutting resulted in variations in landscape pattern of different NSFTs (Lei *et al.*, 1996b), community dynamics (Lei *et al.*, 1996c), nutrition cycles (Zhang *et al.*, 1996) and soil properties (Dang *et al.*, 1996a, 1996b; Lei *et al.*, 1996a) in the Qinling Mountains. Of these recovered NSFTs from the Huoditang forest region, soil microbial-associating information remains unknown. In the present study, we applied T-RFLP to quantify the diversity and composition of soil fungal communities of five NSFTs and related the detected differences to measured soil edaphic properties. Our objective was to use these NSFTs to examine how soil fungi specifically respond to vegetation shift. In order to precisely judge the result reliability, three restriction endonucleases were applied to digest PCR amplicons.

Materials and Methods

Study sites

In the study, we examined the differences in soil fungal communities across five main NSFTs from the Huoditang forest region (2037 hectares) on the south-facing slope of the Qinling Mountains (Cheng *et al.*, 2013). The altitude of Huoditang ranges from 800 a.s.l. to 2500 a.s.l. with geographic coordinates of N33°18′–33°28′ in latitude and E108°21′– 108°39′ in longitude. The annual average temperature and precipitation for this region with a 170 day frost-free period is 8°C to 10°C and 900 mm to 1,200 mm, respectively.

The abrupt and broken topography consists mainly of granite and gneiss with a prevailing soil type of mountain brown forest soil. The mean slope is 35° and the mean soil depth is 45 cm.

Soil sampling

In September 2013, soils were sampled from five NSFTs from the Huoditang forest region of the Qinling Mountains. The NSFTs CP, SO, AP, and WS are dominated by Chinese pine (*Pinus tabulaeformis*), sharptooth oak (*Quercus aliena* var. *acuteserrata*), Armand pine (*Pinus armandi*), and wilson spruce (*Picea wilsonii*), respectively, whereas CPSO is occupied by mixed forests of Chinese pine and sharptooth oak. The sharptooth oak forests are the zonal climatic climax communities and the Chinese and Armand pines are the early successional species. Without massive anthropogenic or natural disturbances, the forest communities which are dominated by pines will be subjected to invasion of oaks to form mixed forests, eventually, transforming to communities dominated by oaks. In the study, except for wilson spruce forests (WS) at a higher altitude (> 2000 a.s.l.), the other four target NSFTs intensively distribute within a narrow altitude range (1400 a.s.l.–1700 a.s.l.). Selected NSFTs are approximately 55±5 years and are representatives of the typical forest types across most parts of North China. These NSFTs all grow in acid soil identified as mountain brown forest soil that usually develops on acid parent materials, with a relatively consistent soil development history. By all accounts, these sampling sites were close to each other and had a similar slope degree and elevation to support a relatively homogeneous precipitation, temperature and soil type. Overall, the Huoditang forest region offers a desired study area for elucidating the differential effects of forest vegetation on soil microbial communities.

Site characteristics are showed in Table 1. For each NSFT, three 20 m × 20 m plots were established. For an explicit research purpose, we did not sample deeper soils because most of the microbial biomass distributed in the surface

Table 1. Environmental characteristics of five NSFTs

Forest type	Plot	Longitude	Latitude	Altitude (m)	Azimuth (°)	Slope (°)	CD[a] (%)	ATH[b] (m)	ADBH[c] (cm)
AP	1	108°32′57″	33°32′80″	1410	34	288	70	12.18	15.58
	2	108°33′13″	33°33′45″	1460	32	198	60	12.57	14.13
	3	108°33′15″	33°33′47″	1483	27	245	70	13.91	18.50
CP	1	108°32′60″	33°32′83″	1420	22	261	80	16.42	15.69
	2	108°32′93″	33°33′27″	1460	27	216	80	14.63	14.04
	3	108°32′94″	33°33′36″	1485	26	243	70	15.44	12.93
CPSO	1	108°26′40″	33°26′13″	1580	34	216	70	10.69	10.56
	2	108°26′92″	33°26′92″	1595	32	235	60	11.33	10.37
	3	108°26′22″	33°26′02″	1512	36	221	70	12.35	14.28
SO	1	108°26′70″	33°25′97″	1717	32	318	60	10.48	13.35
	2	108°27′17″	33°25′92″	1641	34	240	50	10.56	13.73
	3	108°27′14″	33°25′94″	1620	26	277	70	10.45	15.27
WS	1	108°28′42″	33°27′58″	2001	15	74	60	12.23	19.14
	2	108°28′71″	33°27′67″	2079	12	73	50	15.61	20.79
	3	108°28′94″	33°27′85″	2046	16	84	50	14.37	18.52

[a] CD, crown density
[b] ATH, average tree height
[c] ADBH, average diameter at breast height

layer (Babujia *et al.*, 2010; Liu *et al.*, 2012). All soil samples were taken from the surface mineral layer (0–10 cm) after litter removal. In each plot, thirty soil cores were collected using a soil corer (3 cm in diameter), and were pooled into one composite sample. Soil samples were placed in plastic bags and transferred to the laboratory using a cooling box. Afterward, soils were sieved (2 mm mesh) and then stored at 4°C until microbial analysis.

Soil physical and chemical analysis

Both physical and chemical parameters of the samples, including sand, silt, clay, bulk density (BD), porosity, pH, total organic carbon (TOC), ratio of carbon and nitrogen (C/N), total nitrogen (TN), ammonium nitrogen (AN), nitrate nitrogen (NN), total phosphorus (TP), and available phosphorus (AP) were analyzed following the methods described by Liu *et al.* (1996). Measurements for these soil parameters were conducted based on Forestry Standards "Observation Methodology for Long-term Forest Ecosystem Research" of People's Republic of China.

Microbial analysis

Soil fungal DNA extraction : Soil samples were subjected to pretreatment to remove soil contaminants, followed by a fast glass beading DNA extraction procedure. Briefly, soil samples (0.5 g) were mixed with 1.5 ml of TNP + Trition X-100 + Skim Milk (100 mM Tris, 100 mM $Na_4P_2O_7$, 1% PVP, 100 mM NaCl, 0.05% Triton X-100, and 4% skim milk, pH = 10.0), followed by vortexing for 3 min. Mixtures were incubated at 55°C for 5 min and centrifuged at $14,800 \times g$ for 3 min, afterwards the supernatants were discarded. The prewashing cycle was performed 3 times, in which samples were mixed with 0.7 ml of 0.5 M calcium chloride and centrifuged at $14,800 \times g$ for 3 min. The samples were subjected to a fast procedure for cell lysis by glass beading. One ml of DNA extraction buffer (100 mM Tris-HCl, 100 mM sodium phosphate, 1.5 M NaCl, 1% CTAB, pH = 8.0), acid-washed glass beads (< 0.1 mm, 0.4–0.6 mm, and 0.8–1.0 mm, 0.25 g each type) and 200 μl of 20% SDS were added. Mixtures were shaken dramatically in a RETSCH MM 400 Mixer Mill for 3×30 sec at 30 Hz and followed by centrifugation at $8,000 \times g$ for 15 min. Recovered supernatants containing soil fungal DNA were successively extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, v/v/v) and chloroform-isoamyl alcohol (24:1, v/v), respectively. The resulting aqueous phase was recovered by centrifugation at $6,500 \times g$ at 4°C for 10 min and precipitated with 0.6 volume of cold isopropanol and 0.1 volume of 3 M NaAc (pH = 5.2) at room temperature for 2 h. DNA pellets were obtained by centrifugation at $14,800 \times g$ for 20 min at 4°C, washed with cold 70% ethanol twice, and resuspended in TE buffer (pH = 8.0), to give a final volume of 100 μl.

Absorbances of recovered DNA at 230 nm, 260 nm, and 280 nm were determined by using a NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific). An aliquot (5 μl) of crude DNA was analyzed using 0.8% w/v agarose gel and run in 1× TBE at 5 V/cm for 1 h. The gel was stained in ethidium bromide (0.5 mg/ml) and photographed with the Gel Doc XR + System (Bio-Rad).

Fungal ITS PCR amplification : For molecular assessment for soil fungal communities of the five NSFTs, the fungal-specific primer set: ITS1F (5′-CTT GGT CAT TTA GAG GAA GTA A-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) (White *et al.*, 1990; Gardes and Bruns, 1993) were used. ITS1F primer 5′ was labeled with the fluorescent dye 6-FAM. Each PCR reaction mixture (50 μl) contained: 25 μl of 2× Taq MasterMix (Cowin), 2 μl of each primer (10 μmol/L), 1 μl of BSA (0.4 μg/μl), 2 μl of template DNA (20 ng), and 18 μl of ddH_2O. PCR was performed in a MyCycler™ Thermal Cycler (Bio-Rad) by using an initial denaturation step of 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min with a final extension at 72°C for 7 min.

Terminal-restriction fragment length polymorphism (T-RFLP) : It should be noted that since tested forests (such as CP, SO, and CPSO) were in close proximity, plots established at the forest edges may have been affected ineluctably by the adjacent NSFTs. On the other hand, the plots located at different slope positions within a given forest may differ in soil physical and chemical conditions. To some extent these factors might have caused a decrease in community homogeneity between three replicate plots of one NSFT, thus increasing community similarity between NSFTs. To eliminate geographical disturbances of different soils for analysis of fungal communities (Hunt *et al.*, 2004; Schwarzenbach *et al.*, 2007), PCR amplicons from three replicate plots in each NSFT were pooled into one composite sample. The PCR amplicons were purified with TIANgel Midi Purification Kit (TIANGEN) according to the manufacturer's protocol. Subsequently, purified amplicons were subjected to restriction endonuclease digestion. Briefly, PCR products were separately digested with *Hha*I (GCG^C, Fermentas), *Msp*I (C^CGG, NEB), and *Rsa*I (GT^AC, NEB) at 37°C for 7 h to produce terminal restriction fragments (TRFs). Digestion reactions (20 μl) contained 4 μl of PCR products (0.8–1.0 μg), 1 μl of 10× buffer, 1 μl of endonuclease (10 U) and 14 μl of ddH_2O. Recovered digestions were precipitated with 2 volumes of cold ethanol by centrifugation at $16,000 \times g$ for 15 min at 4°C for desalting. DNA pellets were then washed with 70% cold ethanol twice and resuspended in 20 μl sterilized ultrapure water.

In preparation for capillary electrophoresis, 2 μl of digestion products were mixed with 12 μl of formamide and 0.5 μl of GeneScan ROX1000 size standard (Applied Biosystems). Mixtures were denatured at 95°C for 4 min and then placed on ice for 5 min. Capillary electrophoresis was performed on an ABI 3730*xl* Genetic Analyzer (Applied Biosystems). Detection of fluorescently labeled 5′-terminal restriction fragments were analyzed by GeneScan 3.7 (Applied Biosystems).

Data analysis

Peaks of TRFs representative for different operational taxonomic units (OTUs) with a height of less than 50 fluorescence units were excluded. The TRFs with less than 50 bp in length were removed, too. An upper fragment length threshold of 710 bp was applied to recover sequences with OTU restriction sites. For quality control, the raw data was compiled and uploaded to T-RFLP analysis EXpedited (T-REX)

Table 2. Soil edaphic factors of five NSFTs[a]

Soil edaphic factor	AP	CP	CPSO	SO	WS	*P*
BD (g/cm^3)	0.93±0.16a	1.07±0.11a	0.81±0.05a	0.82±0.08a	0.94±0.08a	0.060
Porosity (%)	64.69±6.11a	59.87±3.93a	69.52±1.61a	68.95±2.89a	64.54±2.87a	0.062
Silt (%)	2.67±0.20a	1.07±0.10b	4.24±0.24c	3.73±0.13d	2.68±0.28a	<0.01
Clay (%)	65.56±2.15a	32.49±2.48b	80.41±2.28c	71.29±1.65e	54.34±3.85d	<0.01
Sand (%)	31.76±2.18a	66.44±2.58b	15.35±2.39c	24.98±1.77e	42.97±3.64d	<0.01
pH (H$_2$O)	5.89±0.09bc	5.67±0.03cd	5.43±0.10ad	5.23±0.15a	6.08±0.23b	<0.01
TOC (g/kg)	35.14±1.58a	29.86±0.78d	25.96±0.81bc	23.96±1.21b	28.36±3.08cd	<0.01
TN (g/kg)	2.14±0.57abc	1.70±0.09ab	2.08±0.04c	1.34±0.10a	2.35±0.11c	<0.05
C/N	14.17±0.62a	17.61±1.41b	12.49±0.16a	17.92±1.34b	12.10±1.67a	<0.01
NN (mg/kg)	8.99±0.91a	7.07±0.96a	1.62±0.06b	1.77±0.33b	6.26±0.10a	<0.05
AN (mg/kg)	13.55±0.15a	14.91±0.30b	21.30±0.03c	18.87±0.47d	20.50±0.62cd	<0.01
TP (g/kg)	0.48±0.02a	0.61±0.02b	0.39±0.02c	0.26±0.02d	0.47±0.03a	<0.01
AP (mg/kg)	6.81±0.13a	37.55±0.82b	5.29±0.15c	5.21±0.38c	5.21±0.43c	<0.05

[a] Values represent the means of three replicates ± standard deviation, different lowercase letters are significantly different according to LSD's post-hoc test or Dunn's post-hoc test, n=3.

which is a free web-based tool to aid in the analysis of T-RFLP data (Culman *et al.*, 2009). Noise filtering was performed for identifying true peaks based on a standard deviation multiplier set to 1. TRFs were then aligned by selecting a clustering threshold of 1 bp and then imported to MS Excel 2007. Percentages of each TRF peak area of the total peak area of each sample were calculated and TRFs of peak areas over a 1% threshold were further analyzed (Bennett *et al.*, 2008). The normalized peak area was defined as relative abundance of each reserved OTU. The OTUs with a relative abundance over 10% were defined as the dominant species. Hence, the experiment was assigned as 5 soils × 3 restriction endonucleases × 5 replicates, but not all identified OTUs present in the 5 replicate profiles of each enzyme; however, they were present in one or two replicates. Despite the rare abundance, these OTUs were included in the data statistics. It should

be noted that because of the presence heterogeneity of some specific OTUs among replicate profiles, their relative abundance was suggestively below 1% when averaged. All identified OTUs and their average abundance were represented in heatmaps. Based on presence/absence and abundance of OTUs, the Shannon-Wiener index (H) and Evenness index (E) were calculated, respectively. The Richness index (S) was expressed by the identified OTU numbers. Data of soils and fungal diversity between NSFTs were compared using one-way ANOVA with LSD's post-hoc test. When equal variance was not assumed, the non-parametric Kruskal-Wallis test with Dunn's post-hoc test was used. The results were showed in the form of mean ± SD with significant differences detected at the 0.05 level. To determine whether NSFTs exerted effects on soil fungal communities, one-way analysis of similarities (ANOSIM) with 999 permutations was used

Table 3. Soil fungal diversity of five NSFTs[a]

Diversity index		Enzyme	AP	CP	CPSO	SO	WS	*P*
Average diversity	Richness S	*Hha*I	26.60±1.34a	27.60±0.89a	22.80±0.45b	23.80±0.84b	20.00±0.00c	<0.01
		*Msp*I	30.60±0.89a	21.40±0.55b	18.60±0.55c	18.40±0.55c	18.00±1.00c	<0.01
		*Rsa*I	28.80±1.64a	25.00±1.41b	16.40±1.34c	20.20±0.84e	13.40±1.14d	<0.01
	Shannon H	*Hha*I	3.08±0.05a	3.04±0.03a	2.76±0.02b	2.89±0.04d	2.58±0.02c	<0.01
		*Msp*I	3.26±0.03a	2.89±0.03b	2.49±0.04c	2.47±0.05cd	2.38±0.03d	<0.01
		*Rsa*I	3.15±0.07a	3.00±0.08b	2.28±0.09c	2.57±0.07e	2.07±0.08d	<0.01
	Evenness E	*Hha*I	0.94±0.00a	0.92±0.00b	0.88±0.00c	0.91±0.00b	0.86±0.00d	<0.01
		*Msp*I	0.95±0.01a	0.94±0.01b	0.85±0.01c	0.85±0.01c	0.83±0.01d	<0.01
		*Rsa*I	0.94±0.01a	0.93±0.01a	0.82±0.01b	0.86±0.01d	0.80±0.01c	<0.01
Total diversity	Richness S	*Hha*I	31.00	31.00	25.00	26.00	22.00	
		*Msp*I	40.00	29.00	23.00	24.00	27.00	
		*Rsa*I	37.00	41.00	24.00	29.00	18.00	
	Shannon H	*Hha*I	3.13	3.08	2.80	2.91	2.60	
		*Msp*I	3.36	3.03	2.60	2.54	2.60	
		*Rsa*I	3.31	3.38	2.40	2.75	2.30	
	Evenness E	*Hha*I	0.91	0.90	0.90	0.89	0.90	
		*Msp*I	0.92	0.91	0.80	0.80	0.80	
		*Rsa*I	0.92	0.91	0.70	0.82	0.80	

[a] Values represent the means of five replicates ± standard deviation, different lowercase letters are significantly different according to LSD's post-hoc test or Dunn's post-hoc test, n=5.

to test significant differences between NSFTs based on Bray-Curtis and Euclidean distance matrices. Recovered TRF profiles from distinct NSFTs were clustered with Bray-Curtis distance and Group average (Blackwood *et al.*, 2007). Non-metric multidimensional scaling (NMDS) was carried with Euclidean distance. To detect relationships between diversity indices and environmental factors, stepwise multiple regression analysis was performed. Canonical correspondence analysis (CCA) was used to identify the effects of soil factors on the patterns of soil fungal communities.

Results

Soil physical and chemical properties

Except for soil porosity (P = 0.062, one-way ANOVA) and bulk density (P = 0.060, one-way ANOVA), all P values below the 0.05 significant threshold in the one-way ANOVA or Kruskal-Wallis test, indicated the NSFTs had significant effects on soil properties (Table 2). Both CPSO and SO soils had higher silt and clay contents than other three coniferous soils. The SO soils had the highest C/N and the lowest values for six of nine chemical factors. WS soils had the highest pH and more abundant TN. The coniferous soils (AP, CP, and WS) had higher concentrations of TOC and NN than other two. Specifically, NN contents were approximately 4–5 times higher in coniferous soils than in CPSO and SO soils, yet the CPSO soils had the highest level of AN. Additionally, CP soils were rich in P concentration (TP and AP), even the AP concentrations were 5~6-fold higher than other forest soils.

Soil fungal diversity

Enzyme-specific TRF profiles were produced to estimate diversity of soil fungal communities which inhabited the studied NSFTs. As depicted in Table 3, all diversity indices (Richness S, Shannon H, and Evenness E) significantly differed among the forest soils (P < 0.05). On the basis of TRF profiles, these indices indicated a generally consistent diversity tendency, namely, more diverse fungal species distributed in the two pine soils (AP and CP), regardless of average or total diversity statistics. Additionally, except for the Richness and Shannon of *Msp*I, and the Evenness of *Hha*I

and *Rsa*I based on the total diversity, the spruce forest soils held fungal communities with the least diversity indices.

Soil fungal taxonomic patterns

Venn diagrams exhibited patterns in unique and shared fungal OTUs, based on total OTU statistics (Fig. 1). The OTU number pattern was intensively dependent on applied enzymes. The maximal unique OTUs were found in the CPSO soils for *Hha*I (9, 13.43% of the total), AP soils for *Msp*I (16, 19.28% of the total) and *Rsa*I (12, 14.81% of the total), respectively. Besides, the TRF profiles indicated the OTUs shared by all soils were 2 (2.99%) for *Hha*I, 1 (1.20%) for *Msp*I and 3 (3.70%) for *Rsa*I, respectively. The OTUs shared by 2–4 soils were 34 (50.75%) for *Hha*I, 30 (36.14%) for *Msp*I and 38 (46.91%) for *Rsa*I.

Soil fungal taxonomic abundance

Fungal OTU abundance of forest soils were shown in Fig. 2, which also specifically varied with the enzymes. In *Hha*I profiles, the abundance of 6 OTUs was higher than 10%, OTU 130 (14.29%), 347 (14.16%), and 405 (10.82%) were dominant species in WS soils, OTU 349 (11.08%), 368 (12.10%) in CPSO soils and OTU 368 (11.13%) in SO soils. In addition, the abundance of OTU 354 in CP soils was up to 14.64%. In *Msp*I profiles, 3 of 5 dominant OTUs (> 10% in abundance) were OTU 204 (13.96%), 646 (10.73%), and 648 (13.29%) found in CPSO soils. OTU 646 also shared a high abundance (18.64%) in SO soils. Besides, OTU 124 (16.23%) and 651 (11.22%) were the dominants of WS soils. For *Rsa*I profiles, there were 4 OTUs with a high abundance to be identified, OTU 70 (13.19%) and 648 (20.29%) in CPSO soils, OTU 614 (22.74%) and 650 (13.80%) in WS soils. OTU 70 (16.15%) was also dominant in SO soils. Certainly, there were OTUs which had an abundance ranging from 5% to 10% to occur across the five forest soils, accounting for 21.92% of the total identified OTU number for *Hha*I, 10.84% for *Msp*I and 16.05% for *Rsa*I.

Fungal community similarity

On the basis of Bray-Curtis distance, dendrograms were constructed to visually determine if the tested soils had similarity in fungal community structure (Fig. 3). The soil fungal communities from the five NSFTs were divided into 4 groups, the
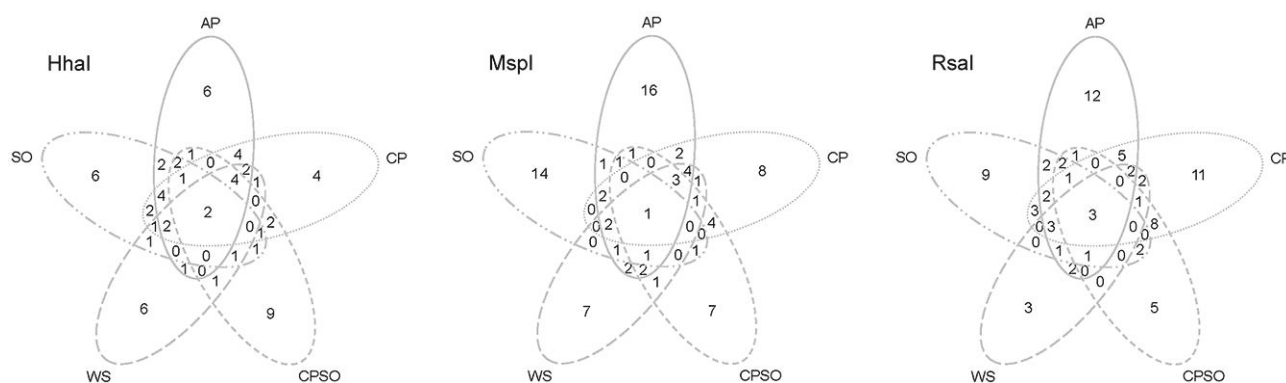

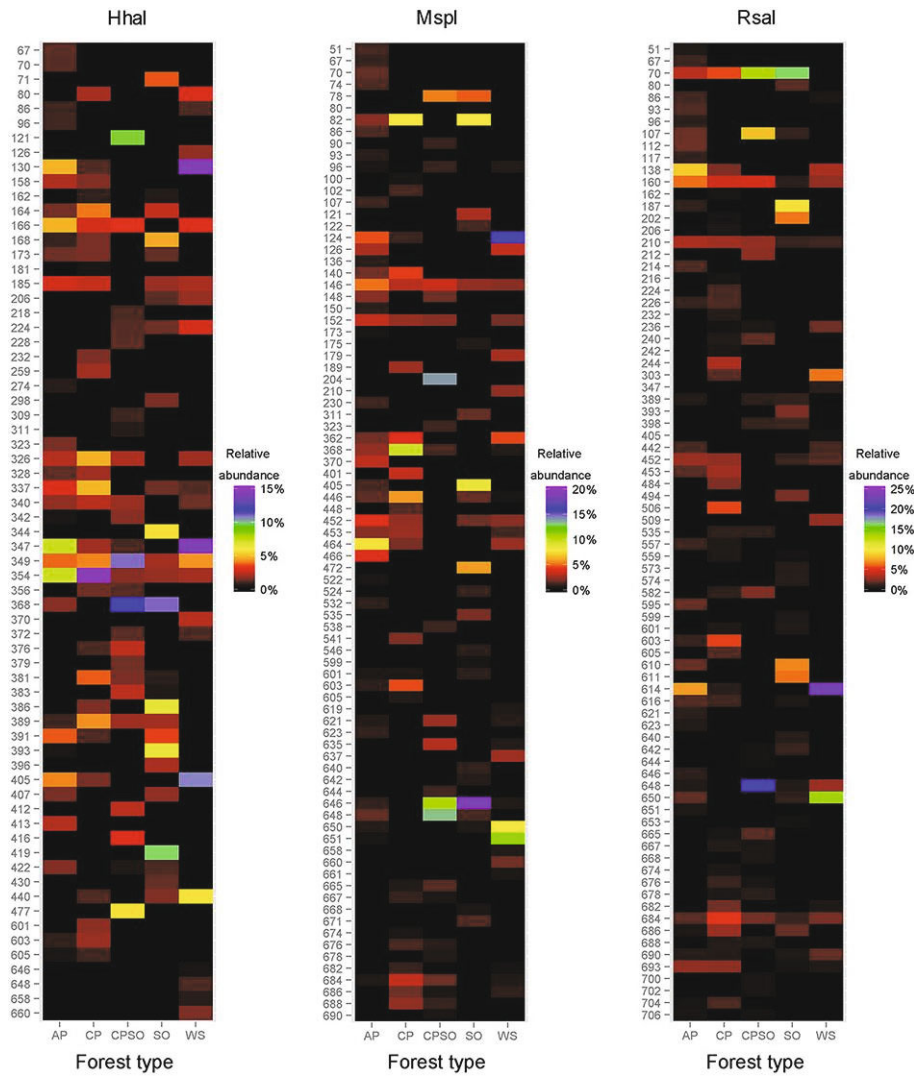**Fig. 1.** Venn diagrams of shared and unique OTUs of five NSFTs.

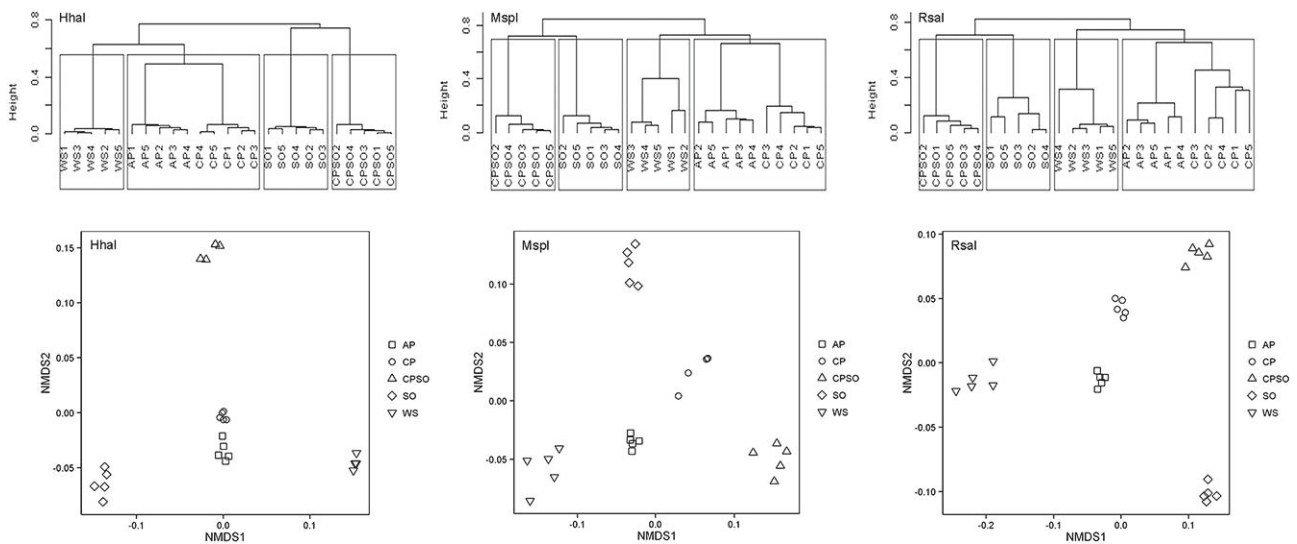**Fig. 2.** Heatmaps of average relative TRF abundance of five NSFTs.

Fig. 3. Dendrograms and NMDS plots of fungal TRF profiles of five NSFTs.

Table 4. ANOSIM of soil fungal communities of different NSFTs

Forest type	Enzyme	Enclidean		Bray-Curtis	
		R	P	R	P
All forests	HhaI	1	<0.001	1	<0.001
	MspI	0.866	<0.001	1	<0.001
	RsaI	0.962	<0.001	1	<0.001
AP, CP, WS vs SO	HhaI	0.710	<0.001	1	<0.001
	MspI	0.603	<0.001	0.994	<0.001
	RsaI	0.562	<0.01	0.865	<0.001
CP, SO vs CPSO	HhaI	1	<0.01	1	<0.01
	MspI	1	<0.01	1	<0.01
	RsaI	1	<0.01	1	<0.01

fungal communities of AP and CP soils were closely clustered into one group, whereas the WS soils served as one group alone. These three fungal communities under coniferous forests were far away from the other two counterparts in CPSO and SO soils. Likewise, the fungal communities from CPSO and SO soils were also separated each other. Based on Euclidean distance, NMDS provided a similar result to the clustering analysis in that AP and CP communities were close, while the fungal communities from other three NSFTs separated from each other and clustered alone (Fig. 3). According to the TRF profiles of the three enzymes, regardless of Bray-Curtis or Euclidean distance, the low *P* and high R values of ANOSIM analysis showed significant differences between NSFTs. This was evident between coniferous forests (AP, CP, and WS) and broad-leaved forests (SO) and between monospecific forests (CP and SO) and mixed forests (CPSO), as suggested by clustering and NMDS analyses (Table 4).

Stepwise multiple regression analysis

Stepwise multiple regression analysis showed that among the five physical factors, silt was an important soil physical factor, which negatively affected soil fungal diversity. On the

contrary, clay showed a remarkably positive linkage with diversity indices (Table 5). For soil chemical factors, soil factors relating to N (TN, NN, AN, and C/N) were significantly correlated with the three diversity indices. In addition, negative relations were found between AN and all indices. Similarly, most of soil fungal diversity indices had a significantly negative association with pH.

Canonical correspondence analysis (CCA)

By analyzing, the Axis 1 explained 33.71% (*Hha*I), 25.04% (*Msp*I), and 24.99% (*Rsa*I) of the total variation, respectively, while the Axis 2 explained 29.73% (*Hha*I), 19.27% (*Msp*I), and 19.09% (*Rsa*I) of the total variation, respectively. The two axes cumulatively explained 63.45% (*Hha*I), 44.31% (*Msp*I), and 44.09% (*Rsa*I) of the total variation, respectively. Generally similar trends may be observed from these plots produced by different three enzymes (Fig. 4). In the plots, all physical and chemical variables were divided into three groups. The soil fungal communities of AP, CP, and WS scattered nearby the Group 1 including 9 soil variables, the patterns of these fungal communities were apparently influenced by these variables, causing separateness of fungal communities of three coniferous forest soils from the other two communities. The fungal communities of CPSO and SO soils were closely related with the Group 2 (AN, Silt, and Clay) and Group 3 (C/N) variables, respectively, resulting in specific fungal species composition. In despite of differences, the similarity in the plots should be noted, this may indicate the result reliability to some extent.

Discussion

Experimental set-up

In the present study, to probe soil fungal communities in recovered NSFTs, microhabitat heterogeneity of sampling

Table 5. Stepwise multiple regressions between fungal diversity and soil edaphic variables[a]

Enzyme	Diversity index		Soil edaphic variables	R^2	P
*Hha*I	Richness S		*Silt(-4.766)	0.4339	<0.05
	Shannon H		*Silt(-0.334) *Clay(0.018)	0.4279	<0.05
	Evenness E		**Silt(-0.053) *Clay(0.003)	0.4636	<0.05
*Msp*I	Richness S		***Silt(-11.654) ***Clay(0.721)	0.6551	<0.01
	Shannon H	Physical variables	***Silt(-0.762) ***Clay(0.048)	0.7161	<0.01
	Evenness E		**Silt(-0.095) *Clay(0.005)	0.6206	<0.01
*Rsa*I	Richness S		**Silt(-11.858) *Clay(0.659)	0.5519	<0.01
	Shannon H		**Silt(-0.810) *Clay(0.043)	0.5561	<0.01
	Evenness E		**Silt(-0.109) *Clay(0.005)	0.5782	<0.01
*Hha*I	Richness S		**pH(-5.354) **TN(8.228) *C/N(0.885) *AN(-0.598) **AP(0.098)	0.9708	<0.001
	Shannon H		***pH(-0.310) **TN(0.281) *NN(-0.022) ***AN(-0.060) *TP(0.246)	0.9883	<0.001
	Evenness E		**pH(-0.031) ***AN(-0.010) *TP(-0.049)	0.9704	<0.001
*Msp*I	Richness S		**pH(-8.167) ***TN(13.247) **AN(-0.955) **TP(-11.006)	0.9857	<0.001
	Shannon H	Chemical variables	**pH(-0.490) **TN(1.025) **AN(-0.072)	0.9836	<0.001
	Evenness E		**TN(0.079) *NN(-0.006) ***AN(-0.017) *AP(0.002)	0.9895	<0.001
*Rsa*I	Richness S		*pH(-5.886) *TN(3.346) ***AN(-1.990)	0.9592	<0.001
	Shannon H		**pH(-0.405) **AN(-0.135)	0.9659	<0.001
	Evenness E		*pH(-0.045) *TN(0.082) ***AN(-0.017) *TP(-0.348) *AP(0.004)	0.9803	<0.001

[a] *, *P*<0.05; **, *P*<0.01; ***, *P*<0.001.
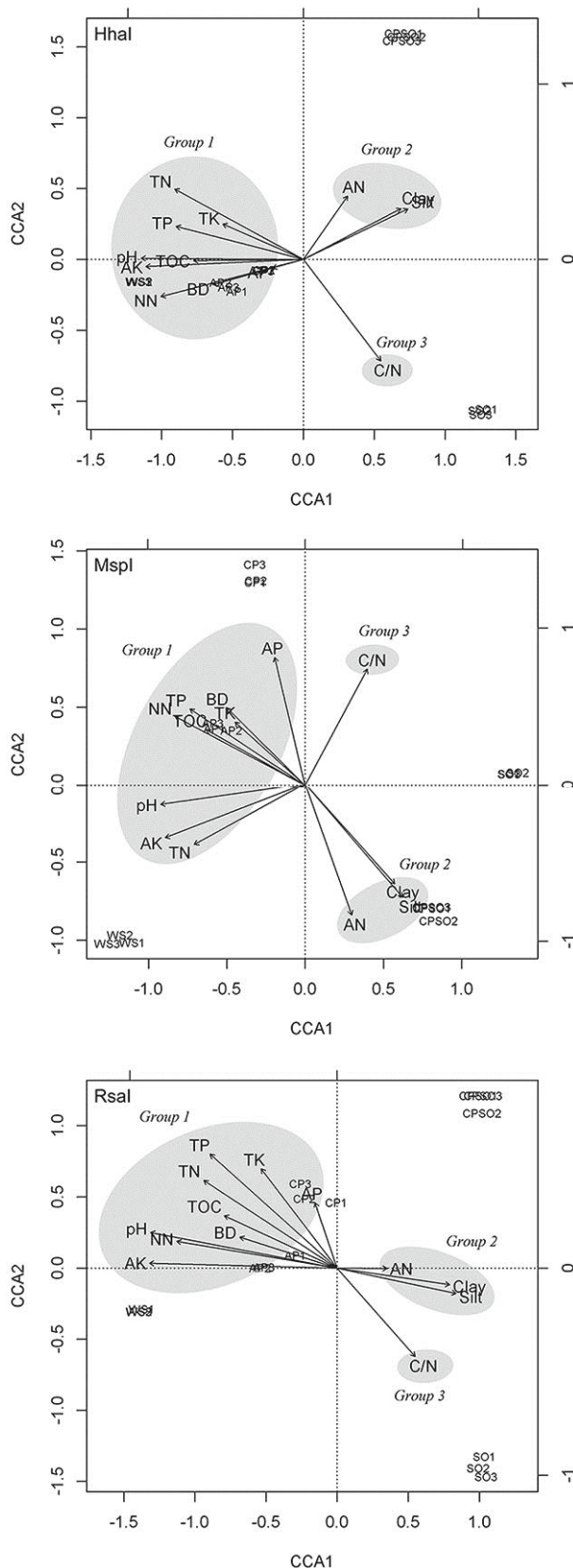
Fig. 4. CCA of the relationships between soil fungal communities and soil edaphic factors.

plots and PCR bias were eliminated. This was achieved by pooling PCR products prior to endonuclease digestion and the use of three restriction enzymes for experimental robustness. Generally consistent results indicated that distinctive NSFTs exerted selective impacts on the fungal communities via forming specific soil environments. Here, the TRF profiles of *Hha*I are taken as an example as follows for convenience in further discussion.

Soil fungal diversity

TRF profiles were indicative of the differences in fungal diversity of the five NSFTs. Stepwise multiple regression analysis was used to detect the linkages between fungal diversity and soil factors of the NSFTs. Among the physical factors, soil silt showed negative relation with fungal diversity, but this particle size only accounted for a tiny fraction (1–4%) across the soils. In contrast, clay was the dominant soil component. Therefore, the impacts of soil texture on fungal diversity should be comprehensively evaluated. For the correlation patterns between diversity indices, silt and clay, we assumed that coarser size particles (silt) are inferior for retainability of soil nutrients and water, whereas finer size particles (clay) have high reactive surface area for absorbing nutrients and holding water (Crowther *et al.*, 2014). Moreover, the finer size particles provide spatial protection for fungi through pore size from other soil organisms (Sessitsch *et al.*, 2001).

Data analysis also revealed that there were several chemical soil factors (pH, TN, C/N, AN, NN, TP, and AP) to closely relate with soil fungal diversity, particularly, soil AN exhibited the strongest linkage. Reports revealing the effects of inorganic nitrogen types on soil fungal diversity are limited. Previous studies have demonstrated that nitrogen deposition enabled the decrease in soil ectomycorrhizal diversity (Lilleskov *et al.*, 2001; Frey *et al.*, 2004) and a loss in non-mycorrhizal diversity also was related to AN addition (Allison *et al.*, 2007). The observed negative relationship between AN and soil fungal diversity in our study was consistent with those patterns. Up to now no relevant literature has been available to give a reasonable interpretation of the negative correlation between fungal diversity and AN. We infer that superfluous AN in the tested soils was in favor of growth of dominant populations and that these populations constrained non-dominant species because of superiority of resource competition to induce reduction of fungal diversity. In our study, pH was also a noticeable chemical factor considering that fungi prefer acid environments. A few studies have demonstrated that soil fungal/bacterial ratio decreased with increase in pH (Bååth and Anderson, 2003; Rousk *et al.*, 2009, 2010). However, Rousk *et al.* (2010) investigated fungal communities across a pH gradient in an arable soil and found that the relative abundance of fungi was unaffected by pH and fungal diversity was only weakly related with pH.

Soil fungal community patterns

By comparing TRF profiles, the differences in soil fungal communities of distinct NSFTs were detected. The fungal communities from two pine forest soils (AP and CP) were apparently separated from others, suggesting that they had

compositional distinct, which is in agreement with our expectation based on vegetation and soil edaphic environments (Mitchell *et al.*, 2010). The soils of two pine forests were unique from other soils and comparison among the soil characteristics showed that conspicuous superiority of the most chemical factors was observed in the AP and CP soils. Different soils sharing some fungal OTUs potentially resulted in conformity of community composition, which might be ascribed to similarity of soil physical and chemical properties between the tested soils (Anderson *et al.*, 2012; Carlos *et al.*, 2013). Fungal community dissimilarity might be a result of unique OTU appearance between these soils, as indicated by HhaI TRF profiles, since the fungal OTU 158, 328, 354 and 603 were restricted to AP and CP soils (Fig. 2). These two soils also shared a number of OTUs which were low abundant or absent in other three forest soils, thereby strengthening the similarity of AP and CP communities. Considering that Chinese pine and Armand pine belong to the same genus, they would be expected to have a closer genetic relationship in phylogenesis. Based on the study, it was suggested that NSFTs occupied by different related canopy species may possess more similar microbial communities. This would support other the concept that similar habitats hold similar microbial communities (Green and Bohannan, 2006). Furthermore, we speculate that the rich and diverse nutrients in two pine forest soils could accommodate more fungal species to coexist. In spite of a variety of soil edaphic factors that can influence fungal communities, the CCA was conducive enough to identify the leading factors. As revealed by the CCA, TOC, TN, NN, TP AP, TK, AK, pH, and BD exerted comprehensively effects on patterns of fungal communities of AP and CP soils.

By comparison among the coniferous forests, soil fungal communities of spruce forest (WS) were separated from those of AP and CP forests as depicted by clustering and NMDS analyses. A previous study has reported that WS soils were rich in soil microbial biomass (Cheng *et al.*, 2013). In our study, WS soils had a great amount of soil fungal biomass that belonged to a few fungal OTUs with a high abundance including OTU 130, 347, and 405 in the HhaI profiles, even OTU 614 in the *Rsa*I profiles had an abundance up to 22.74% (Fig. 2). Interestingly, the unique OTU 126, 370, 646, 648, 658, and 660 only occurred in fungal communities of WS soils, this contributed to the discriminations between WS and other soils. Because of the abundance of soil N resources, fungal activities and propagation may not be constrained by N availability in these forest soils (Hoorman and Islam, 2010). On the other hand, higher soil pH was the suspect causing low fungal diversity and unique OTU patterns in WS soils. This can be explained in part since soil fungi likely prefer acidic soils (Blagodatskaya and Anderson, 1998; Bååth and Anderson, 2003; Rousk *et al.*, 2009, 2010; Grantina *et al.*, 2011).

For oak (SO) and pine-oak (CPSO) forest soils, high levels of porosity, silt and clay were found; however, their chemical nutrition was inferior to coniferous forests. In particular, oak forest soils which were merely advantageous in C/N. Such a high C/N might imply that oak forest soils were slightly restricted in N availability (Hoorman and Islam, 2010). As a result, the unique fungal OTU 70, 298, 344, 393, 396,

and 419 appeared in the oak soils (Fig. 2), presumably owing to their superior adaptability to this harsh nutrition environment. For CPSO communities, the initial expectation was that they should be similar to CP or SO communities, or both of them. Yet, our findings revealed that fungal communities in CPSO soils were far differentiated from those in CP and SO soils. Richness in AN, clay, silt and deficit in other chemical nutrients possibly drove the occurrence of unique OTU 121, 218, 228, 309, 311, 342, 412, 416, and 477 in CPSO communities to some extent (Fig. 2). Collectively, soil chemistry differences led to the palpable discriminations between CPSO and CP, SO fungal communities.

Habitats and specialists, generalists

The quantitative analyses on soil fungal community composition suggested that soil fungal distribution is coincident with environmental pattern (Widden, 1987). In our study, most of special species (unique OTUs) did not prevail in the forest soils. Thus, we speculate that fungal species have specific environmental requirements for specific habitats (Pandit *et al.*, 2009; Székely and Langenheder, 2014). Among the sampled local soil sites, chemical sources and sinks differed to form spatial heterogeneity in which a variety of heterogeneous habitats appeared. These fungal specialists are either inactive, dormant or absent in soil habitats mismatching the growth requirements, yet are able to start growing and blossoming when resource conditions are favorable. However, after all, such favorable specific environments are scarce in the soils, causing a lower abundance of these fungal specialists in soils, that is why the PCR products were pooled to remove effects of fungal specialist boost in the individual soil sites (microhabitats with heterogeneity) on overall estimation of fungal communities. On the contrary, fungal generalist species were commonly shared by more soils and evenly distributed in a wide range of different habitats. Fungal generalist species have a strong environment adaptability that is conditioned by dispersal-related mechanisms (Pandit *et al.*, 2009; Cline and Zak, 2014; Székely and Langenheder, 2014). In our study, OTU 166, 185, 326, 337, 340, 347, 349, 354, and 389 in the *Hha*I profiles, OTU 146, 152, 368, 446, 452, 646, and 684 in the *Msp*I profiles and OTU 70, 160, 210, 684, 690, and 693 in the *Rsa*I profiles, all were shared by four or five forest soils.

Conclusion

What should be noted from our study is that similar results for *Hha*I profiles were also identified in the profiles of *Msp*I and *Rsa*I. As the same data of soil edaphic factors were applied in the multivariate analysis, the detected similarity of CCA plots based on different enzymes might mainly derive from the TRF profiles, which can also be approved by diversity statistics, clustering and NMDS. The patterns of soil fungal communities in the CCA plots exhibited a relative consistence with the clustering and NMDS analyzes. In a word, the results in the present study are robust to some extent, objectively reflects the real status of soil fungal communities under the five forest types. Finally, on the basis of three restriction enzymes profiles, we conclude that NSFTs

had significant impact on soil fungal diversity and community structure. Fungal diversity may be mediated by NSFTs via soil texture and N-related factors, while community patterns may be the result of comprehensive impacts of multiple soil factors. The results may further a better understanding of soil fungal communities of different NSFTs and assist with accurate prediction of shifts in soil fungal community composition and function with forest type transformation in the Qinling Mountains.

Acknowledgements

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