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Full Length Research Paper

Relationship between microbial community and soil properties during natural succession of forest land

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Recently, there has been increasing research focus on microorganism community structure in secondary forest succession. This study aimed at analyzing the community diversity of bacteria and fungi in the forest in six different ecological regions of Changbai Mountains in Jilin Province of China, by terminal restriction fragment length polymorphisms (T-RFLP). Results show that the fungal and bacterial diversity differed greatly between forest succession and plants species. Species richness and the Shannon's diversity index of fungi and bacteria in broad mid-age forest and broad-leaved Korean pine mixed maturity forest were higher than that of other forest successions in the six regions. The results of principal component analysis (PCA) and canonical correspondence analysis (CCA) indicate that the stages of forest successions had no strict specificity with bacterial community diversity but strict specificity with fungal community, while environmental condition had great influence on fungal community diversity and soil nutrient, and pH was a primary influencing factor. These results suggest that the environmental conditions exhibit greater influence on the community diversity of fungi than bacteria. And soil nutrient and pH were more indicative of the change of community diversity of fungi and bacteria.

Key words: Terminal restriction fragment length polymorphisms (T-RFLP), forest succession, Northeast China.

INTRODUCTION

Changbai Mountains of Northeast China has special topographical and geological features. Soil degradation is a major threat to sustainable use of soil ecosystem because it decreases actual and potential level of vegetation cover, partly due to the climatic conditions, industrial pollution and human destruction. It is important to monitor the microbial populations and soil properties after forest succession because microorganisms will drive biogeochemical cycles that control functioning of the ecosystems. For example, fungi play an important role in forest restoration because of symbiosis with plant root; they can facilitate mineral absorption by the host plant, stabilize and improve soil structure, affect the population structure and preserve species diversity (Bothe et al., 2010). Despite the fact that soil fungi are key drivers of carbon and nutrient cycling in forest ecosystems (Wilson and Agnew, 1992), there is little information regarding the effect of forest conversation on soil fungal communities.

In forest ecosystems, dominant plants often cause changes in soil properties, which lead to complex local interactions between vegetation soil (Wilson and Agnew, 1992). The effects of forest conversion on general soil microbial characteristics and biomass have received some attention (Koch and Makeschin, 2004). Indeed, the mycelia of soil-borne decomposer and symbiotic mycorrhizal fungal in forest soils are often substantial (Anderson and Cairney, 2007) and fungi may comprise over a third of the microbial biomass in many forests (Högberg and Högberg, 2002). Couple with the fact that fungal hyphae are not readily decomposed, living and

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Table 1. The description of the study sites in the Changbai Mountains, Northeast China.

Plot	Latitude	Longitude	Altitude	Species
Broad mid-young forest (SF1)	042 21'09.7"N	127 56'28.8"E	912	*Betula platyphylla, Larix gmelinii*, *Fraxinus mandshurica*
Broad mid-age forest (SF2)	042 12'21.3"N	127 46'23.4"E	940	*Betula platyphylla, Populus davidiana, Tilia amurensis, Abies nephrolepis*
Broad old forest (SF3)	042 24'03.1"N	128 05'54.1"E	763	*Betula platyphylla, Populus davidiana*
Broad maturity forest (SF4)	042 21'28.9"N	127 59'00.7"E	835	*Betula platyphylla, Populus davidiana*
Broad-leaved Korean pine mixed maturity forest (SF5)	042 24'05.2"N	128 05'31.6"E	766	*Pinus koraiensis*, *Populus davidiana*
Broad-leaved Korean pine mixed old forest (SF6)	042 21'04.6"N	127 59'15.8"E	833	*Pinus koraiensis*

dead fungal mycelia are likely to represent a significant component of soil carbon sink (Zak et al., 2000). Understanding the soil fungal community response is thus of importance in determining how forest conversion influences soil carbon dynamics.

The soil microbial population is essential for maintaining terrestrial ecosystems because it is responsible for their involvement in organic matter dynamics and regulating nutrient cycling. Moreover, it also responds to small changes that may occur in the soil ecological stress or restoration processes (Kalembasa and Jenkinson, 1973). Thus, microbial communities are key to soil quality and functioning. This has attracted many researchers to assess the degradation processes and restoration strategies by making use of relevant parameters of the soil microbial community. It is well known that a fallow period could increase organic carbon that acts as available substrate for microbial growth, thus, natural succession may potentially have positive effects on soil quality and microbial community.

This study aimed at investigating the relationship between microbial community and soil properties on selected sample sites during the natural succession of forest in Changbai Mountains. The results obtained will provide a theoretical basis for the application of eco-environment restoration.

MATERIALS AND METHODS

Field site

The study sites were selected in Baihe County of Jilin Province on the Changbai Mountains, Northeastern China, located at 42°24'N, 128°5'E (Table 1). Characterized by alpine landforms with deep deposits of dark brown forest soil, the area has a semi-humid climate with an annual mean temperature of 3.5°C and precipitation of 700 mm. The soil, developed from volcanic ash, is classified as Eutric cambisol (FAO classification) with high organic matter content in surface layer. All the soils investigated were developed and covered with vegetation for different periods of time. The soil is sandy loam in 3 to 8 cm and gravelly sand in 8 to 24 cm depth. The soil bulk density is 0.35 gcm^{-3} in 0 to 10 cm and 0.68 gcm^{-3} in 10 to 20 cm layer. The dominant tree species are *Pinus koraiensis*, *Fraxinus mandschurica*, *Acer mono*, and *Tilia amurensis*.

The study sites were randomly selected, including a broad mid-young forest, broad mid-age forest, broad maturity forest, broad old forest, broad broad-leaved Korean pine, broad broad-leaved Korean pine mixed old forest. The description of each site is shown in Table 1.

Soil sampling and analysis

Soil samples were collected on October 2011. Five soil samples at each site were randomly collected from topsoil at a depth of 0 to 5 cm, and then mixed together as one sample. Soil cores were immediately transported to the laboratory and stored at -20°C for 4 days prior to sieving. The five soil cores from each sample point were then mixed together to form a single soil sample. After carefully removing the surface organic materials and fine roots, each mixed soil sample was divided into two portions. One portion was air-dried for the analysis of soil chemical properties. The other was sieved with a 2-mm screener and the total DNA extracted using the UltraClean™ 12888 soil DNA kit (Mo Bio Laboratories Inc., Solana Beach, CA, USA). DNA was eluted in 50 µL Tris-EDTA (pH 8.0) and store at -80°C. The description of each site is shown in Table 2.

ITS rDNA gene amplification

For analyses of the fungi community, we used primers ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCTTATTGATATGC) to amplify the internal transcribed spacer region (ITS) of the nuclear rDNA gene cluster. For the terminal restriction fragment length polymorphisms (T-RFLP) analysis, the ITS1F primer 5' in each pair was labeled with the fluorescent dye 5-FAM (synthesized by Shanghai Majorbio biotech Inc). Polymerase chain reaction (PCR) was carried out in 50 µL reaction volumes using 2 µL of purified DNA (10~20 ng), 0.5 mM of

Table 2. Soil physical and chemical properties at the different stages of secondary forest succession.

Plot	pH	Organic carbon (g/kg)	Total nitrogen (g/kg)	Available P (mg/kg)
SF1	5.6	130.75 ± 2.02	11.39 ± 0.16	3.34 ± 0.61
SF2	5.3	150.84 ± 1.41	10.75 ± 0.19	4.28 ± 0.22
SF3	5.3	120.40 ± 0.77	9.87 ± 0.07	4.62 ± 0.38
SF4	5.8	130.75 ± 1.22	10.85 ± 0.08	3.53 ± 0.48
SF5	5.5	140.43 ± 1.07	11.21 ± 0.07	2.79 ± 0.84
SF6	5.4	110.49 ± 1.07	9.33 ± 0.09	3.92 ± 0.56

primers ITS1F and ITS4, 1.5 mM MgCl, 0.2 mM dNTP, and 2.5 U Taq DNA polymerase (TAKARA, Japan) on an ABI 9700 Thermal cycler. An initial denaturation step of 5 min at 94°C was followed by amplification for 34 cycles at the following conditions: 60 s at 94°C, 60 s at 51°C, 60s at 72°C. A final 8 min extension at 72°C completed the protocol.

The PCR product was purified using a Tiangen PCR purification kit (Tiangen Biotech, P.R. China). Each purified fluorescent product was digested separately with 2.5 µL (2.5 Units) of the restriction enzymes Alu I in the manufacturer's buffer (total reaction volume 10 µL) for 4 h at 37°C. Digestion products were scanned by the ABI3730 and the results analyzed by Peak Scanner Software V1.0

16S PCR amplification

For T-RFLP analysis, bacterial 16S regions were amplified using 27F (AGAGTTTGATCMTGGCTCAG) labeled with fluorescent dyes 5-FAM and 1492R (GGTTACCTTGTTACGACTT) primers. Amplifications were conducted in 25 µL volumes containing 100 ng DNA, 1.5 µL of primers (10 mM/L), 12.5 µL PCR-Power Mix (TAKARA, Japan) 10 1.0 µL of a bovine serum albumin solution(10 mg ml^{-1}). PCR amplification was performed in an ABI 9700 Thermal cycler with an initial denaturation step of 94°C for 5 min, followed by 34 cycles of 94°C for 30 s, 62 for 1 min, 72°C for 2 min and a final extension step of 72°C for 10 min. Each PCR reaction was run with a negative control containing no DNA. All PCR products were electrophoresed in 1.5% (w/v), stained with ethidium bromide and visualized under ultraviolet (UV) light. The PCR products were purified using the PCR Clean-up Kit (Tiangen Biotech, China) following the manufacturer's instructions, and digested using 5 units Alu I for 4 h at 37°C and 20 min at 65°C, respectively.

In order to determine the sizes of terminal restriction fragment, ca 2.0 µL of each digest, 0.5 µL GeneScan 500 ROX (Applied Biosystems) and 12 µL Hi-Di Formamide (Applied Biosystems) were denatured at 95°C for 5 min and cooled on ice. Sequencing was done on a 50-cm column using POP4 polymer on an ABI PRISM 3130 × 1 genetic sequencer (Applied Biosystems) and the results were analyzed by Peak Scanner Software V1.0.

Statistical analysis

To assess the impact of natural succession after forest on soil chemical properties, and the size and composition of the microbial community, one-way analysis of variance (ANOVA) was used to compare means and least significant difference (LSD) was used to compare the variance. When significance was observed at the $P< 0.05$ level,

Duncan post-hoc test was used to carry out multiple comparisons. All regression analysis was performed using SPSS version 13.0 for Windows. Canonical correspondence analysis (CCA) was calculated by Canoco for windows 4.5.

RESULTS

Soil organic carbon (C) and total nitrogen (N)

Organic C exhibited the highest (150.84 g/kg) and lowest (110.49 g/kg) amount in the soil under SF1 and under SF6, respectively (Table 2). During the secondary forest succession, organic C in the soil increased quickly, and tended to be the highest under SF3. Thereafter, it was decreased and readily became nearly constant. In contrast, a different trend was observed for total N in soil; it decreased from SF1 to SF6, and readily became nearly constant. Overall, organic C and total N in the soil were ranked in the following order: young forest > various successions sites > Korean pine mixed old forest. Moreover, there was a significant correlation between organic C and total N ($y = 0.317 + 0.054x$, $r^2 = 0.802$, $p < 0.0001$, $n = 20$) during the natural succession.

Diversity of fungi and bacteria

The T-RFLP fingerprinting of fungi was performed using the restriction enzyme Alu I. According to the number of T-RFs and the relative peak heights, the Shannon diversity index, Margalef index and Evenness index, species richness of the six regions were calculated (Table 3). The fungal community diversity indices from SF1 to SF6 revealed that fungal diversity differed greatly between regions. The total Shannon index and Margalef index were highest at the broad mid-age forest (SF2), lowest in the broad old forest (SF4), and moderate at the sites of various succession stages, whereas Richness and Evenness had no clear trend with the Shannon index (Table 3).

Also, the T-RFLP fingerprints of bacteria were performed using the restriction enzyme ALU I. The species richness, Shannon diversity index and Evenness index of the nine samples of bacteria were calculated (Table 4). The diversity of bacteria was lower than that of fungi. The bacterial community diversity indices from SF1 to SF6 revealed that bacterial diversity differed greatly between regions. The species richness and Shannon diversity index of SF5 were significantly higher than in the SF1 and SF3, while the difference of between the SF2

Table 3. Diversity indices of fungus in six regions on the Changbai Mountains.

Plot	Shannon	Margalef	Evenness	Richness
SF1	3.40^{ab}	2.35^{ab}	0.97^{b}	33.00^{ab}
SF2	3.62^{b}	2.91^{b}	0.97^{b}	41.33^{b}
SF3	3.60^{a}	2.78^{a}	0.95^{a}	27.00^{a}
SF4	3.13^{b}	1.96^{b}	0.98^{b}	39.67^{b}
SF5	3.44^{b}	2.50^{ab}	0.97^{ab}	35.67^{ab}
SF6	3.47^{b}	2.63^{b}	0.96^{ab}	37.00^{b}

Values with the same letter(s) were not significant different at p = 0.05 by Duncan's post-hoc test.

Table 4. Diversity indices of bacteria in six regions on the Changbai Mountains.

Plot	Shannon	Margalef	Evenness	Richness
SF1	2.73^{ab}	1.34^{ab}	0.97	16.67^{a}
SF2	2.91^{ab}	1.59^{ab}	0.97	18.33^{ab}
SF3	2.30^{a}	1.05^{a}	0.97	13.00^{a}
SF4	2.20^{a}	0.92^{a}	0.97	11.33^{a}
SF5	3.22^{b}	2.16^{b}	0.97	27.67^{b}
SF6	2.82^{ab}	1.57^{ab}	0.96	19.67^{ab}

Values with the same letter(s) were not significant different at p = 0.05 by Duncan's post-hoc test.

and SF6 was not significant.

Principal component analysis (PCA)

PCA was performed to analyze the microbial community in top soils. Results of fungus (Figure 1A) showed that the first principal component's contribution rate was 19.94% and eighteen samples were divided into two groups, with samples SF2, SF3 and SF6 in one group and the others in a second group; the second principal component of the contribution rate was 14.18% and the two parts of that samples were further divided into three categories, distributed into three quadrants.

On the other hand, the PCA of bacteria (Figure 1B) showed that the first principal component's contribution rate was 24.28%, and eighteen samples were divided into two parts, with samples SF1 and SF4 in one group, SF3 with one of SF6 in second group, and the rest in another group; the second principal component of the contribution rate was 13.04% and eighteen samples divided into two categories, distributed into three quadrants. These results indicate that nature succession had no specificity with bacterial community diversity, while the fungal community diversity showed difference obviously.

The relationship between microorganism structure of community and environment

CCA was performed to analyze the relationships between environment and microorganism structure community (Figure 2). Figure 2A shows that the size 252.7, 239.5 and 85.1 bp, was positively correlated with N and pH, but negatively correlated with C, C/N and P. The size of 88.9 and 185.5 bp was positively correlated with N, pH, C, C/N, whereas no correlation with P. The size of 252.0, 426.5, and 471.3 bp was positively correlated with P and negatively with C, C/N, N, and pH. However, the size of 380.2 bp located near the original point, has no correlation with the environment.

Figure 2B shows the bacteria's CCA. Seventeen T-RFS were divided into four quadrants. The sizes of 58, 59, 60 and 87 located in the first quadrant, were positively correlated with P, C and C/N, whereas negative with pH and N. The sizes of 51, 89, 76, 75, 342, and 180 were positively correlation with pH and N, but negative with P, C, and C/N. In addition, the sizes of 74, 57, 106, 204, 278 and 294 were positively correlated with pH and N, C, C/N, and negative with P. The size of 50 was positively correlation with C, C/N, P, but no correlation with pH and N. These results indicate that fungal and bacterial diversity in forest succession was mainly influenced by environment.

DISCUSSION

Variation of organic carbon and nitrogen in soil

The vegetation composition can have fundamental effects on soil properties (Rutigliano et al., 2004; Singh et

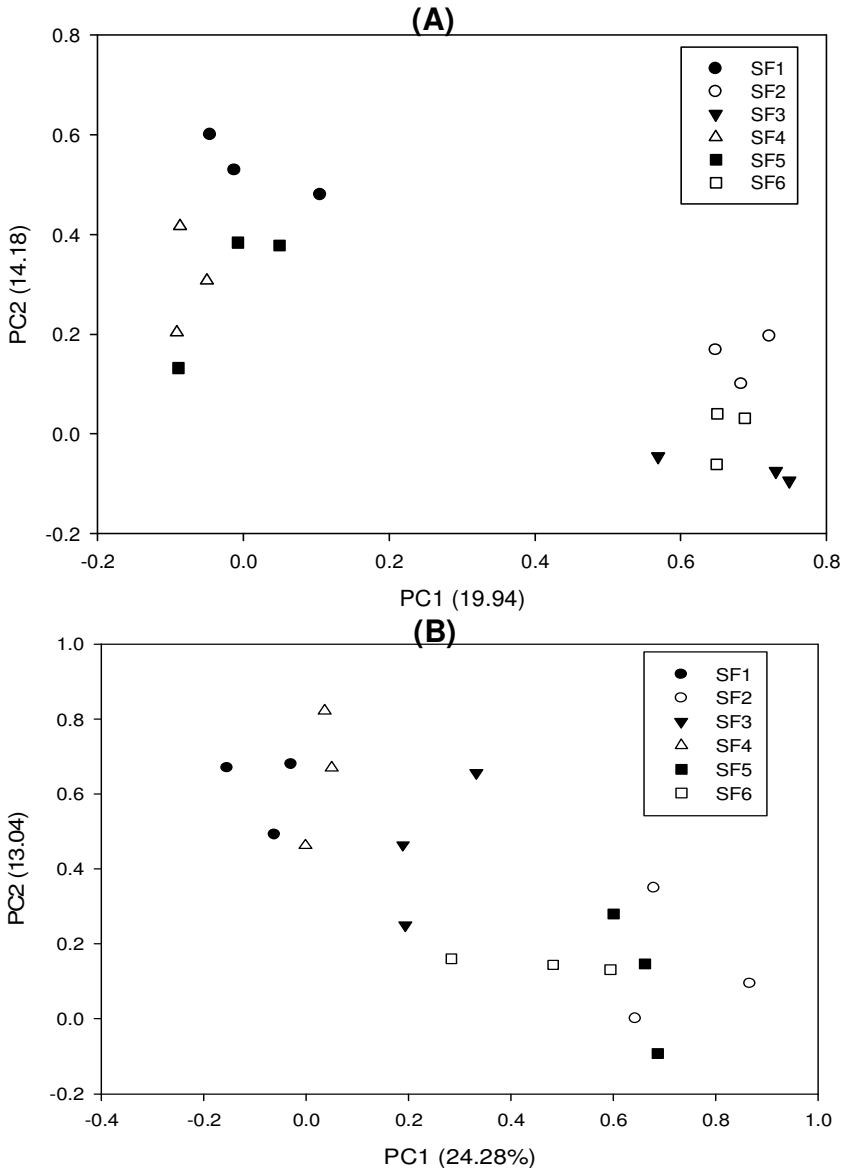

Figure 1. PCA analysis of fungi and bacteria. A, soil fungi; B, soil bacteria. The fungi first principal component's contribution rate was 19.94%, and second principal component of the contribution rate was 14.18%. The bacterial first principal component's contribution rate was 24.28%, and second principal component of the contribution rate was 13.04%.

al., 2004), mainly due to its contribution towards inputting the amount of organic matter into the soil by supplying carbon and energy sources from root exudates and plant remains. In this study, with the age of plantation of forest, the trend changes in the organic C and total N may be related to some factors, which possibly included land-use history, slope position, light and changes in community plant species. Changes in community plant species significantly affected soil organic matter accumulation. For example, annual and biennial plants rapidly grew and covered the land within the broad mid-young forest. This led to severe loss of soil nutrients because of lack of vegetation cover on surface soil and rain leaching. Moreover, pine litter contained a large amount of recalcitrant compounds that resisted decomposition. As a result, there was relatively low soil organic C and total N under SF1 and SF6.

Variation of microbial composition by T-RFLP analysis

The choice of primers and restriction enzymes are crucial in the success of T-RFLP analysis. Only when a variety

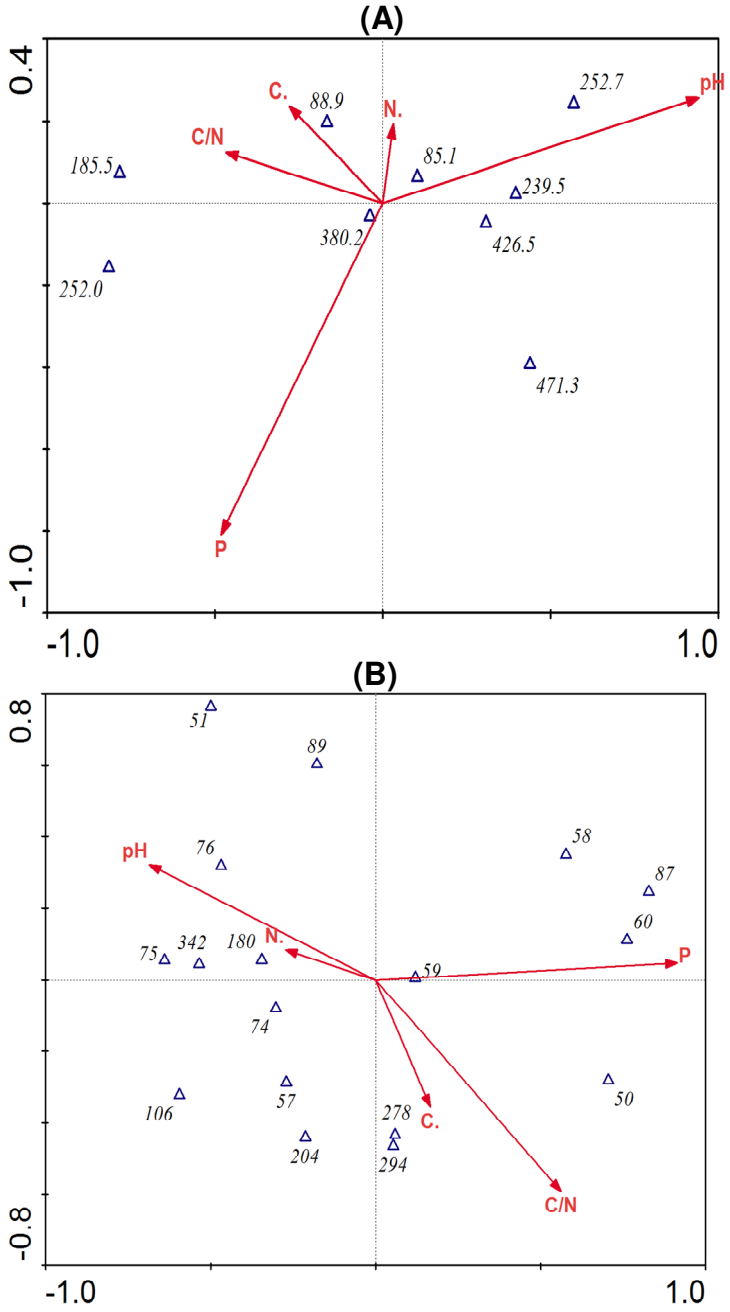

Figure 2. CCA analysis of fungi and bacteria. A, The multi-axial figure of fungal T-RFS and environment conditions; B, the multi-axial figure of Bacterial T-RFS and environmental conditions.

of target microbial fragments are amplified efficiently can T-RFLP analysis truly reflect the microbial diversity of the sample. We used the primers 27F and 1492R to amplify the conserved bacterial V3 fragment, while ITS1F and ITS4 were used to amplify the internal transcribed spacer. The choice of restriction enzymes may affect the ability of T-RFLP to distinguish species. Dunbar et al. (2001) found that fewer terminal restriction fragments (T-RFs) were obtained from double digestion than by combining results from separate single enzyme digests. Therefore, the restriction enzyme Hha I was chosen for microbial analysis.

There were great differences in the fungal and bacterial community diversity between regions. The community diversity indices showed that ecological restoration on the Changbai Mountains was effective. Species richness and the Shannon diversity index of fungi and bacteria in SF5 were higher than that of others in the six regions;

probably the species of SF5 can promote ability of soil aggregate preferably. However, PCA and CCA showed that bacterial community diversity had no strict specificity with the regions in contrast with previous reports (Wallis et al., 2010; Hamman et al., 2007) because the bacterial community diversity depended on not only host plant, but also the environmental conditions such as soil type (Berg and Smalla, 2009), growth stage (Van Overbeek and Van Elsas, 2008) and so on.

Fungal community diversity had strict specificity with the regions as environment condition had great influence on fungal community diversity. This was consistent with the reports of Zhang et al. (2010). The reasons may be that fungi are more sensitive to ambient environmental condition. Zheng et al. (2004) also reported the diversity index of different forest restoration types, and the results were the same with this study. However, Börstler et al. (2006) discovered that the AMF species composition was also related to above-ground plant biodiversity in two differently managed mountain grasslands in Germany. Based on CCA, nutrient and pH were the primary influencing factor. Previous works have also demonstrated that soil properties such as pH and nutrient availability are strong determinants of microbial community structure (Grayston et al., 2004; da Jesus et al., 2010; Ibekwe et al., 2010; Lovieno et al., 2010)

The T-RFLP technique is widely used in microbial ecology (Curlevski et al., 2010). It was first developed by Liu et al. (1997) as a tool for assessing bacterial diversity and comparing the community structure of bacteria in different environmental samples. The technique has the significant advantage of low cost and relative simplicity, permitting sufficient replication to address important ecological questions. Although limited by the fidelity of DNA extraction and quality, it may be the most sensitive molecular method for the study of fungi (Brodie et al., 2002; Singh et al., 2006).

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