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Full Length Article

Evolution of the Soil Bacterial Community Structure during the Development of *Pinus massoniana* Plantations in Subtropical China

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Abstract

Soil bacterial communities can form links between forest trees and soils, but the effect of stand age on the soil bacterial communities and diversity is still unclear. The technique of IlluminaMiSeq high-throughput sequencing was used to detect abundance and diversity of the 16S rRNA gene of bacteria to evaluate the evolution of the soil bacterial communities and diversity changes over time (13, 25, 38 and 58 years old) of *Pinus massoniana* Lamb. plantations in subtropical China. The results showed that the succession of *P. massoniana* plantations significantly influenced the structure and diversity of the bacterial communities. The Shannon and Simpson diversity indexes of soil bacteria were the highest in 25 year-old *P. massoniana* plantations, whereas the diversity index reached the lowest value in the 38-year-old *P. massoniana* plantations. The Chao1 and Ace indexes were the highest in 58 year old *P. massoniana* plantations. At the phylum level, 35 phyla were obtained in all treatments, among which *Acidobacteria* (32.82%), *Proteobacteria* (29.75%), *Actinobacteria* (11.07%) and *Chloroflexi* (10.52%) were the predominant phyla. The dominant genera were *Acidothermus*, *Candidatus_Solibacter*, *Variibacter*, *Bradyrhizobium* and *Acidibacter* (relative abundance larger than 2%). Hierarchical clustering and principal component analysis (PCA) revealed that stand age significantly affected the soil microbial community structure and as a result, the soil microbial community structures in the 13- and 25 year old plantations differed sharply from those in the 38- and 58 year old plantations. The soil bacterial communities, both in terms of structure and diversity, were significantly correlated with soil pH, total nitrogen, and ammonium nitrogen ($P < 0.01$). In conclusion, stand age greatly altered the diversity and structure of the bacterial communities, and soil pH and ammonium nitrogen were the potential environmental factors associated with the bacterial community variations.

Keywords: *Pinus massoniana*; Stand age; Bacterial community structures; 16S rRNA sequencing

Introduction

Soil microbes are the key constituent of forest-soil feedback in the forest ecological system (Fisk and Fahey, 2001). They promote the energy flow and circulation of materials of the ecosystem, affect the forest growth and composition of phytocoenosium (Merilä *et al*., 2010) and participate in numerous ecological processes of the soil ecosystem such as nutrient cycling, decomposition of organic matter, etc. (Hart *et al*., 2005). At present, to avoid overexploitation of natural forests and to meet the human demands for timber, fuel materials and other forest products, planted forests have rapidly expanded throughout the world (Wen *et al*., 2015). The areas of planted forests have currently reached 264 million hectares, accounting for 7% of total forest area in the world (Hunter, 2001; Chu *et al*., 2018). However, with the rapid development of large-scale, continuous and single planted forests, such as eucalyptus (Wu *et al*., 2014), slash pine (Wu *et al*., 2015), larch (Liu *et al*., 1998), cedar (Xia *et al*., 2015) and so on, the excessive consumption of nutrients leads to a series of problems including the decline of land fertility, poor productivity, serious plant disease and insect pests, poor ecosystem stability and so on. As a result, many scholars and forest growers have focused on the maintenance of sustainable planted forests. Considering the sensitivity of soil microbes to changes in the soil environmental and nutrient availability, the change of microflora is often used as one of the important early warning and sensitive indexes for evaluating soil quality changes caused by natural or man-made interference (Stone *et al*., 2015; Zhang *et al*., 2016). The interaction between above- and below-ground parts of a planted forest ecosystem has been a heated research topic. More attention has been paid to the effects of varieties of trees on soil microbial communities (Marschner *et al*., 2004; Berg and Smalla, 2010; Oh *et al*., 2012), but there has been limited research on the stand age. However, there are great differences in circulation of

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materials and energy flow for the forest ecosystems at different stand ages. Different intensities of plant-soil interactions may lead to differences in forest soil environment and biological composition (Hackl *et al*., 2004). In the process of planted forest growth, the structure of the soil microbial community can be directly affected by the difference between litter and root exudates (Moore-Kucera and Dick, 2008) and the composition of the soil microbial community can be indirectly affected by influencing soil physicochemical properties, such as soil pH (Högberg *et al*., 2007), temperature (Cavaglieri *et al*., 2009), soil moisture (Brockett *et al*., 2012), and nutrient content and availability (Lucas-Borja *et al*., 2016). It can be concluded that stand age has a significant impact on the structure and diversity of soil microbial communities (Jia *et al*., 2005; Chatterjee *et al*., 2009). Therefore, it is crucial to study the trend of the soil microbial community structure in the process of planted forest growth for understanding the nutrient requirement of a planted forest, soil environmental status and quality, which is conducive to improved operation and management of planted forests.

In China, 33.18% of forests are planted forests, with a total area of 6.9×10^7 ha, which is the largest area of planted forests in the world (Pan *et al*., 2011). *P. massoniana* is characterized by a high adaptability to different environmental conditions, rapid growth and resistance to drought and unfavorable soil conditions. In subtropical China, it is widely used in plantation planting and is one of the most important coniferous species in reforestation projects (Wen *et al*., 2015). *P. massoniana* covers a total area of 12 million hectares, accounting for 7.74% of the total area of trees in China (Meng *et al*., 2014). Most previous studies on *P. massoniana* plantations have focused on soil nutrients, enzyme activity, litter evolution, plant diversity and soil animal functional groups (Wen *et al*., 2014; Zhou *et al*., 2017), reflecting changes in the soil quality of artificial forests. Only a few studies have investigated changes in the soil microbial community in forest plantations, mainly concentrating on soil microbial quantity and microbial biochemical functions, using traditional methods (Cai and Ding, 2013). However, such methods frequently underestimate microbial community diversity and richness and are therefore not suitable for describing any physiological differences. The use of Illumina high-throughput sequencing techniques to study soil microorganisms enables the detailed investigation of the soil microflora and comprehensively analyzes the taxonomy, phylogeny and functional diversity of the microbial community (Quail *et al*., 2008).

In this paper, evolution of the soil bacterial community structure and diversity with the age (13, 25, 38 and 58 years old) of *P. massoniana* plantations in subtropical China was explored using the high-throughput sequencing of the 16S rRNA gene. The main objectives of this study were as follows: (1) determination of the diversity and composition of the soil bacterial communities among *P. massonia* plantations of different ages and (2) investigation into the relationship between physicochemical properties and the soil bacterial communities to illuminate the distribution characteristics of the soil bacterial communities related to soil nutrients.

Materials and Methods

Site Description and Soil Sampling

The study area was located at the Guizhou Province Forestry Academy of Sciences, China (106°44′20″E, 26°29′35″N). The climate is a typical subtropical monsoon climate, with an annual average temperature of 15.2°C, an average annual precipitation of 1,198 mm, an annual average relative humidity of 77% and an average annual number of sunshine hours of 1,412 h; the frost-free period extends to over 278 days. The soil type is classified as yellow soil. We selected *P. massoniana* plantations with similar site conditions and different ages, namely, 13, 25, 38 and 58 years (Table 1). The understory was dominated by the shrubs *Rubus pirifolius* and *Mallotus* spp., while the herb layer mainly consisted of *Imperata cylindrica* and *Dicranopteris pedata*. Three 20 × 20 m plots were established at each site in August 2017. The spacing between plots was at least 50 m. In each sample plot, five soil samples (0 to 10 cm beneath the litter layer) were collected with the help of a 5 cm in diameter drill. The samples were mixed to obtain one composite sample per site, stored in ice boxes, and transported to the laboratory. Stones and course plant debris were removed and the samples were sieved through a 2 mm sieve. One part of each sample was frozen at -80°C for the subsequent analysis of the soil microbial community, while another part was used fresh to determine soil ammonium nitrogen, nitrate nitrogen, and moisture content. The remaining soil was used to determine soil pH, total nitrogen, and organic carbon after air drying.

Soil Physicochemical Analysis

Soil moisture content was determined *via* oven drying at 105 ± 5°C to a constant weight (Shanghai, China). Soil pH (soil to water ratio of 1:2.5) was determined *via* the potentiometry method. Briefly, 10 g of air-dried soil were placed into a beaker with 25 mL of CO_2-free distilled water. The mixture was stirred and after 30 min of standing, the pH was determined (Shanghai, China). Soil total nitrogen content (TN) was determined *via* the Kjeldahl method (Lu, 1999); soil organic carbon content (SOC) was determined by the potassium dichromate oxidation method (Walkley, 1935). Soil ammonium nitrogen (NH_4^+-N) and nitrate nitrogen ($N0_3^-$-N) were measured *via* indophenol blue colorimetry and disulfonic acid colorimetry, respectively (Weatherburn, 1967; Doane and Horwath, 2003).

Soil DNA Extraction and High-throughput Sequencing

Soil DNA extraction was conducted using the E.Z.N.A.®

soil DNA kit (OMEGA, G.A., U.S.A.) according to the manufacturer's instruction, using approximately 0.5 g of fresh soil. The purity and integrity of the extracted DNA were assessed *via* 1% agarose gel electrophoresis and the concentration and purity of the extracted DNA were evaluated with the help of a nucleic acid quantometer (NanoDrop ND-1000). The V3-V4 regions of the bacterial 16S rRNA gene were amplified using a primer set: 338F(5′-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′-GGACTACHVGGGTWT CTAAT-3′) (Wei *et al*., 2018). The volume of the PCR reaction mixture was 50 μL, containing 25 μL Premix Taq DNA polymerase (5 U·μL^{-1}) (TaKaRa, Dalian, China), 4 μL DNA template (1–10 ng), 0.2 μL forward primer (20 ng/μL), 0.2 μL reverse primer (50 ng/μL) and balanced ultrapure water (ddH_2O). The PCR amplification conditions were as follows: predegeneration for 5 min at 95°C, 30 cycles of amplification at 94°C for 40 s, 58°C for 30 s and 72°C for 60 s and extension at 72°C for 5 min. The PCR products were detected by using 2% agarose gel electrophoresis and then recycled using the AxyPrep DNA gel extraction kit (AXYGEN Company). Subsequently, library inspection was carried out by using the Thermo NanoDrop 2000 ultraviolet trace spectrophotometer and 2% agarose gel electrophoresis. High-throughput sequencing for the V3-V4 zone of the 16S rRNA gene was conducted by Illumina Miseq platform (PE300, Shanghai Personal Biotechnology Co., Ltd.). All sequences have been deposited in the NCBI Sequence Read Archive (SRA) database under accession number PRJNA491760.

Based on the QIIME analysis platform, original FASTQ files were filtered and spliced, and the chimera were removed (Fadrosh *et al*., 2014). After sequence alignment, operational taxonomic units (OTUs) were classified according to the 97% similarity level. The most dominant sequences of each OTU were chosen as the representative sequences, which were classified based on the reference sequences and the classification system provided by RDP (Wang *et al*., 2007). The diversity indexes (Chao1 index, ACE index, Shannon index and Simpson index) were calculated in Mothur (Schloss *et al*., 2013).

Data Analysis

Multiple comparison, variance analysis, and correlation analysis of soil physicochemical properties and bacterial α diversity index were performed using the software package SPSS 21.0 (IBM Corp., N.Y., U.S.A.); R software was employed to carry out hierarchical clustering, a principal component analysis and a redundancy analysis.

Results

Soil Physicochemical Properties

Forest age significantly affected the soil physicochemical properties ($P < 0.05$) (Table 2). Soil pH varied between 4.49 to 5.15, with a maximum value at a forest age of 25 years, which was significantly different from that at other forest ages ($P < 0.05$). With the increase of forest age, soil moisture content showed a decreasing trend, while soil organic carbon first decreased and then increased over time. The maximum value was found at a forest age of 13 years and significantly differed from those found at ages 38 and 58 ($P < 0.01$). With increasing forest age, total nitrogen, ammonium nitrogen, and nitrate nitrogen levels presented a trend of increasing first and then decreasing, following the order 25 years > 13 years > 58 years > 38 years. The C: N ratio was highest at 38 years and lowest at 25 years ($P < 0.01$).

Diversity of Soil Bacterial Communities

The Illumina MiSeq platform was employed to conduct gene sequencing for the V3-V4 zone of the 16S rRNA bacterial gene, resulting in a total of 262,076 effective sequences. The obtained sequence numbers after quality control were 32,928, 34,358, 34,987 and 36,506 for 13, 25, 38 and 58 years stands, respectively (Table 3). Sequences with a similarity level ≥ 97% were clustered into an OTU. Soil from the 25 year old site contained the largest number of OTUs (1,897), while soil from the 13-year-old site had the lowest number (1,423). The sequencing coverage of each soil sample was in the range of 0.9475–0.9802, showing that the determined sequences can fully reflect the bacterial community type and structure of each sampling area. The Chao1 index varied between 1,728 and 2,061, with a maximum at 58 years and a minimum at 13 years. The ACE index varied between 1,762 and 2,291 and increased with the increase of forest age. The variance analysis showed that the forest age significantly affected the Shannon index (8.39 to 9.71; $F = 67.89$; $P < 0.01$) and the Simpson index (0.9905 to 0.9966; $F = 24.65$; $P < 0.01$), both with maximum values at 25 years and minimum values at 38 years. Soil bacterial abundance was the highest at 58 years, while the diversity index was the highest at 25 years.

Based on the results of the correlation analysis, the Chao1 index and the ACE index were not significantly correlated with soil physicochemical indexes (Table 4). However, the Simpson index and the Shannon index were positively related with soil pH, total nitrogen and ammonium nitrogen ($P < 0.01$) and negatively correlated with the carbon nitrogen ratio ($P < 0.05$).

Soil Bacterial Community Comparisons

At the phylum level, a total of 35 taxa were obtained. There are nine phyla whose relative abundances are more than 1%; the nine phyla were *Acidobacteria*, *Proteobacteria*, *Actinobacteria*, *Chloroflexi*, *Planctomycetes*, *Verrucomicrobia*, *Gemmatimonadetes*, *Nitrospirae* and *Firmicutes* (Fig. 1). Among them, *Acidobacteria*,

Table 1: General information about *P. massoniana* plantations of different ages

Age	Elevation	Geographic coordinate	Aspect	Slope (°)	Density /(Tree·hm^{-2})	Mean tree height /m	DBH/cm
13	1 262	106°44′6″E 26°31′29″N	NE	10	1 226	8.1	12.1
25	1 196	106°44′11″E 26°32′27″N	SE	15	925	14.6	19.7
38	1 244	106°44′30″E 26°33′5″N	SE	22	614	20.2	31.4
58	1 249	106°43′44″E 26°32′57″N	SW	18	575	21.5	35.3

Table 2: Soil physicochemical properties in stands of different ages

Age	pH	Moisture (%)	SOC (g kg^{-1})	TN (g kg^{-1})	NH_4^+-N (mg kg^{-1})	$N0_3^-$N (mg kg^{-1}))	C/N ratio
13	4.80 ± 0.08b	22.36 ± 1.19a	21.22 ± 1.83a	0.85 ± 0.03abc	3.09 ± 0.16b	1.69 ± 0.40b	24.73 ± 1.761ac
25	5.15 ± 0.03a	19.54 ± 1.26b	18.51 ± 1.96b	1 ± 0.14a	4.51 ± 0.04a	2.98 ± 0.10a	18.65 ± 2.61b
38	4.53 ± 0.1c	18.82 ± 1.61bc	15.81 ± 0.37b	0.65 ± 0.11b	1.88 ± 0.16d	2.41 ± 0.15a	24.97 ± 4.19a
58	4.49 ± 0.07c	16.59 ± 0.93c	16 ± 0.68b	0.77 ± 0.1b	2.31 ± 0.37c	2.61 ± 0.47a	20.82 ± 1.92ab

Values shown are means and standard error (n=3). Different letters indicate significant differences between different stand ages ($P < 0.05$)

Table 3: Sequence statistics and diversity index of soil bacterial in stands of different ages

Age	Reads	OTU	Chao1 index	ACE index	Shannon index	Simpson index	Coverage
13	32928 ± 2830b	1423 ± 110b	1727.67 ± 62.93a	1761.60 ± 179.84a	8.56 ± 0.08b	0.9917 ± 0.0004b	0.9689 ± 0.0271a
25	34358 ± 1738a	1897 ± 145a	2039.49 ± 75.58a	2067.3 ± 203.38a	9.71 ± 0.11a	0.9966 ± 0.0005a	0.9802 ± 0.0294a
38	34987 ± 627b	1486 ± 16b	1963.3 ± 86.84a	2088.67 ± 52.82b	8.39 ± 0.03b	0.9905 ± 0.0007b	0.9519 ± 0.0041a
58	36506 ± 715a	1632 ± 37a	2060.85 ± 94.89a	2290.65 ± 80.40a	8.57 ± 0.05b	0.9915 ± 0.0005b	0.9475 ± 0.00471a

Values shown are means and standard errors (n=3). Different letters indicate significant differences between different stand ages ($P < 0.05$)

Table 4: Pearson correlation coefficients between bacterial community α diversity index and soil properties

Item	pH	Moisture	SOC	TN	NH_4^+-N	$N0_3^-$-N	C/N ratio
Chao1 index	0.003	-0.29	-0.461	0.108	0.025	0.305	-0.394
ACE index	-0.188	-0.424	-0.572	-0.018	-0.134	0.317	-0.358
Simpson index	.881**	0.065	0.239	.781**	.889**	0.438	-.632*
Shannon index	.883**	0.063	0.184	.752**	.911**	0.52	-.644*

Note: ** Significant $p < 0.01$; * Significant at $p < 0.05$

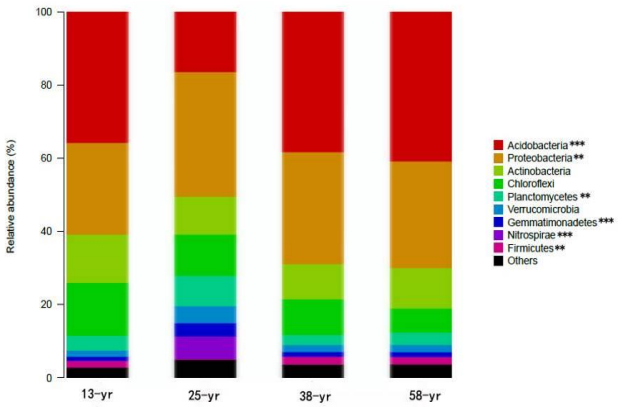

Fig. 1: Relative abundance of the different stand age soil samples at the phylum and genus levels. Significant differences in relative abundance between different treatments were marked with an asterisk. *$P < 0.05$, **$P < 0.01$, *** $P < 0.001$

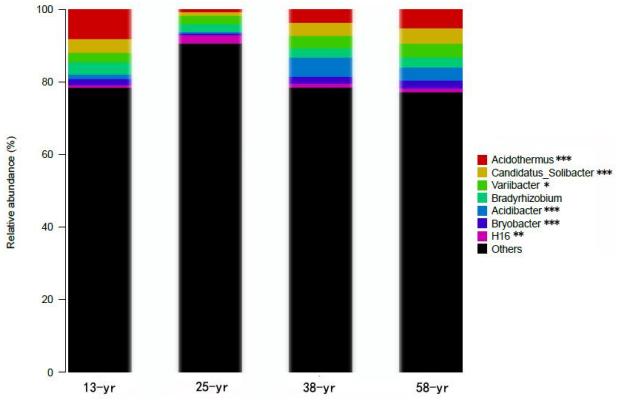

Fig. 2: Relative abundance of the different stand age soil samples at the genus level. Significant differences in relative abundance between different treatments were marked with an asterisk. *$P < 0.05$, **$P < 0.01$, *** $P < 0.001$

Proteobacteria, *Actinobacteria*, and *Chloroflexi* were the dominant taxa, with relative abundances of 16.43–40.81%, 25.06–34.07%, 9.77–13.19% and 9.74–14.50%, respectively. In the 25-year-old site, *Proteobacteria* had the highest relative abundance, accounting for 34.07% of the total number of bacteria.

The relative abundances of *Acidobacteria*, *Proteobacteria*, *Planctomycetes*, *Gemmatimonadetes*, *Nitrospirae* and *Firmicutes* significantly differed among the different forest ages ($P < 0.05$). With the increase in forest age, the relative abundance of *Actinobacteria* first decreased and then increased. After 25 years of afforestation, the relative abundance of Acidobacteria gradually increased and reached the maximum value

(40.81%) at 58 years; the relative abundances of *Proteobacteria*, *Chloroflexi*, *Gemmatimonadetes*, *Planctomycetes* and *Verrucomicrobia* first decreased and then increased. *Nitrospirae* accounted for 0.1, 6.06, 0.08 and 0.1%, respectively, at 13, 25, 38 and 58 years, showing significant variation over time.

At the genus level, we excluded the genera accounting for less than 1% of all genera and classified the remaining genera into a group of "remaining taxa, including *Acidothermus*, *Candidatus_Solibacter*, *Variibacter*, *Bradyrhizobium*, *Acidibacter*, *Bryobacter* and H6 (Fig. 2). Among them, *Acidothermus*, *Candidatus_Solibacter*, *Variibacter* and *Bradyrhizobium* were the dominant species, accounting for 0.79–8.18%, 1.0%–4.18%, 2.29–3.74% and 2.21–3.41%, respectively. The relative abundance of *Acidothermus* significantly differed between different forest ages, similar to replacing it with *Candidatus_Solibacter*, *Variibacter*, *Acidibacter*, *Bryobacter* and H16.

The relative abundances of *Acidothermus*, *Candidatus_Solibacter*, *Bradyrhizobium* and *Bryobacter* followed the order 13 years > 58 years > 38 years > 25 years. *Acidibacter* showed the highest relative abundance at 38 years and the lowest at 25 years. With increasing forest age, the relative abundance of *Variibacter* first decreased and then increased, while that of H6 first increased and then decreased.

Correlation Between Bacterial Community Structure and Soil Physicochemical Properties

Clustering analysis results at the OTU level showed that the soil bacterial community structure differed significantly between different forest ages. The three samples from the 25 years old site were clustered together, at a considerable distance from those of the other sites. This indicates that the soil bacterial community is significantly different at 25 years compared to the other ages. Further, the samples from the 38 years old site were similar to those from the 58-year-old site (Fig. 3a). The principal component analysis further confirmed that there were obvious differences in terms of the soil bacterial community structure at different forest ages. The first and second axes explained 81.43 and 8.29% of the bacterial community structure, respectively and their cumulative contribution rate reached 89.72%. Hence, these two principal components could explain the original variables (Fig. 3b). In terms of PC1 and PC2, the soil bacterial community structure at the forest ages of 13 and 25 years both greatly deviated from those at the ages of 38 years and 58 years, indicating that the soil bacterial community at different forest ages significantly varied with increasing forest age. In addition, the soil bacterial community in the 38 years old forest was similar to that of the 58 years old forest, confirming the higher community similarity of the soil at these two forest ages.

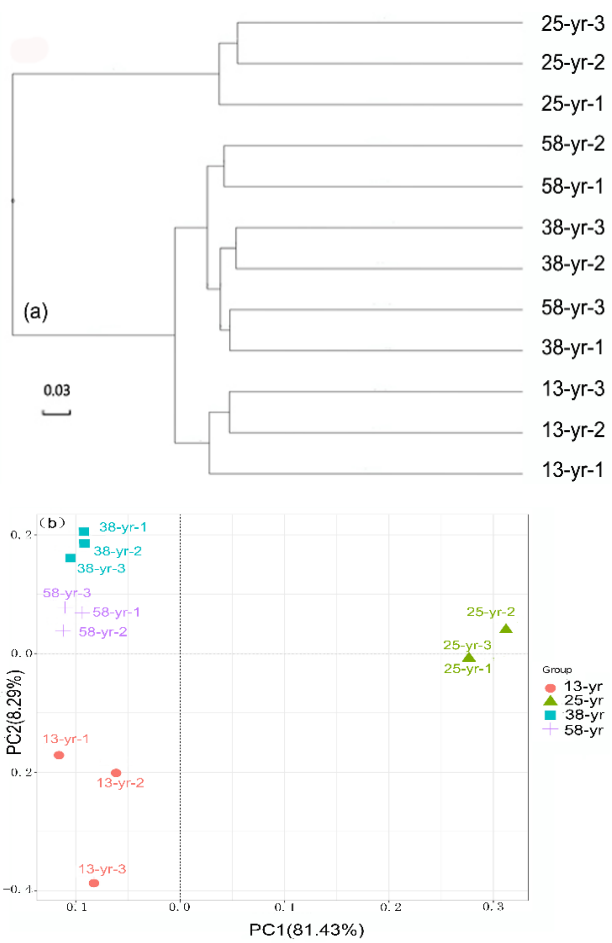

Fig. 3: Hierarchical clustering tree (**a**) and principal component analysis (**b**) of the bacterial community structure in stands of different ages

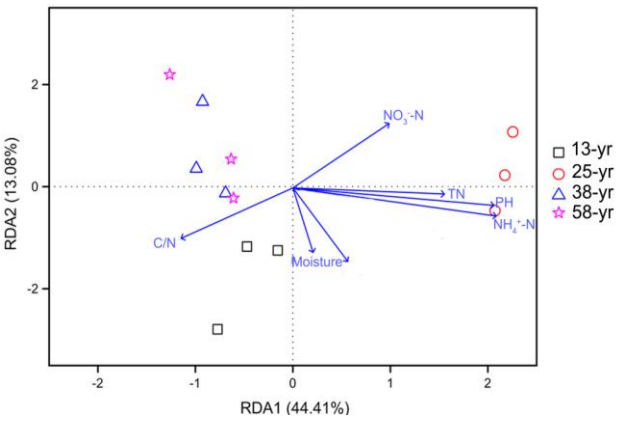

Fig. 4: Redundancy analysis between soil physicochemical properties and the bacterial community structure

To analyze the influences of different soil physicochemical properties on the bacterial community structure, a redundancy analysis was conducted on the bacterial community structure and on soil physicochemical

Table 5: Relationships between soil physicochemical properties and dominant bacterial taxa (RA >1% in all samples)

Abundant taxa	pH	Moisture	SOC	TN	NH_4^+-N	$N0_3^-$-N	C/N ratio
Phylum							
Acidobacteria	-0.919**	-0.217	-0.276	-0.699*	-0.908**	-0.487	0.509
Proteobacteria	0.429	-0.393	-0.214	0.327	0.375	0.644*	-0.476
Actinobacteria	-0.041	0.413	0.54	0.028	-0.011	-0.386	0.283
Chloroflexi	0.384	0.782**	0.474	0.192	0.371	-0.283	0.185
Planctomycetes	0.884**	0.157	0.314	0.768**	0.917**	0.31	-0.542
Verrucomicrobia	0.645*	-0.181	-0.047	0.27	0.625*	0.415	-0.323
Gemmatimonadetes	0.781**	0.089	0.061	0.697*	0.820**	0.625*	-0.658*
Nitrospirae	0.862**	0.046	0.179	0.683*	0.879**	0.608*	-0.578*
Firmicutes	-0.767**	-0.09	-0.166	-0.563	-0.839**	-0.439	0.439
Genus							
Acidothermus	-0.48	0.388	0.261	-0.286	-0.449	-0.656*	0.45
Candidatus_Solibacter	-0.836**	-0.207	-0.144	-0.662*	-0.799**	-0.556	0.558
Variibacter	-0.701*	-0.541	-0.403	-0.643*	-0.749**	-0.018	0.324
Bradyrhizobium	-0.043	-0.007	0.48	-0.165	-0.065	-0.666*	0.483
Acidibacter	-0.831**	-0.434	-0.666*	0-.767**	-0.864**	-0.004	0.348
Bryobacter	-0.871**	-0.268	-0.276	-0.665*	-0.856**	-0.472	0.45
H6	0.690*	-0.172	-0.007	0.533	0.673*	0.533	-0.501

Note: ** Significant $p < 0.01$; * Significant at $p < 0.05$

properties (Fig. 4). The first axis could explain 44.41% of all information, while the second axis explained 13.08%, together explaining 57.49% of the information. In this sense, the first two axes well reflected the relationship between the bacterial community composition and soil environmental factors. Soil pH, total nitrogen content and ammonium nitrogen content all significantly affected the soil bacterial community structure.

Pearson's correlation analysis was conducted on dominant bacterial species and soil physicochemical properties (Table 5). At the phylum level, *Acidobacteria* showed a significantly negative correlation with soil pH, total nitrogen content, and ammonium nitrogen content, while *Proteobacteria* was significantly positively correlated with nitrate nitrogen. *Chloroflexi* showed a significantly positive correlation with soil moisture content and *Planctomycetes* was significantly positively correlated with soil pH, total nitrogen and ammonium nitrogen. *Verrucomicrobia* was significantly correlation with soil pH and ammonium nitrogen, while *Gemmatimonadetes* and *Nitrospirae* were significantly positively correlated with soil pH, total nitrogen, ammonium nitrogen, and nitrate nitrogen and significantly negatively correlated with the carbon nitrogen ratio. *Firmicutes* showed a significantly negative correlation with soil pH and ammonium nitrogen.

At the genus level, *Acidothermus* and *Bradyrhizobium* were significantly negatively correlated with nitrate nitrogen, while *Candidatus_Solibacter* and *Variibacter* showed a significantly negative correlation with soil pH, total nitrogen and ammonium nitrogen. *Acidibacter* and *Bryobacter* were significantly negatively correlated with soil pH, total nitrogen and ammonium nitrogen, while *Acidibacter* showed a significantly negative correlation with organic carbon content. H16 was significantly positively correlated with soil pH and ammonium nitrogen.

Discussion

Soil community structure and community diversity presented different variation trends over time. At all sites, *Acidobacteria*, *Proteobacteria*, *Actinobacteria* and *Chloroflexi* were the dominant phyla (with average relative abundances > 10%), which is similar to the findings of previous studies (Ito *et al*., 2017). This might be associated with the relatively wider ecological amplitude of these phyla. However, despite the similar dominant taxa and the relatively stable soil bacterial community structures during the development of artificial forests, the relative abundances of dominant taxa at different forest ages differed significantly. Hence, although the soil bacterial community structures at different forest ages showed a certain stability, the abundances at the classification level significantly varied. During the development of plantation forests, the differences in canopy structure and aboveground plant community structure affect soil temperature and moisture (Frouz *et al*., 2016; Lucas-Borja *et al.*, 2016), resulting in differences in litter quantities and decomposition rates and, consequently, in physicochemical properties and altered soil microbial community structures. Therefore, it can be concluded that forest age significantly influenced the soil microbial community structure and diversity (Chatterjee *et al*., 2009). *Acidobacteria* was the most abundant bacterial taxa during forest development (except at the 25 years old site), with an average relative abundance of 32.82% (ranging from 16.43–40.81%), which was lower than that of the Dinghu Mountain forest (53.3–67.3%) (Liu *et al*., 2012) but higher than that of the Columbia Forest in Britain (9%) (Axelrood *et al*., 2002). These differences are most likely due to the different vegetation types, climatic conditions, soil types, and soil nutrient patterns (Jangid *et al*., 2011).

The soil microbial community diversity is a key indicator of soil ecological characteristics, reflecting the

level of soil fertility. In this study, there is an "M"-type variation trend instead of a continuously decreasing trend (Fan *et al*., 2013; Wu *et al*., 2015). The values of the Chao1 index and the ACE index were the highest in the 58 years old site, most likely because this site was mature, with abundant undergrowth vegetation and a high species diversity. In addition, plant species composition was complex, resulting in a higher soil bacterial species abundance. The values of the Shannon index and the Simpson index were the highest in the 25 years old site and the lowest in the 38 years old site, indicating that over time, the bacterial diversity in plantation forests decreases. The 25 years old site was still in the initial stage, with a higher competition between forest trees and a higher litter production because of pruning. At the same time, root activity was higher, with a more pronounced production of root exudates, providing carbon and nitrogen for bacterial growth. In contrast, the 38 years old site was already mature, with reduced competition and a lower stand density and litter amount. Hence, larger amounts of nutrients were stored in the organisms and the nutrient return period was long, eventually leading to a lower soil nutrient level and decreased nutrient availability for bacteria. In this study, the Simpson index and the Shannon index were significantly correlated with total nitrogen and ammonium nitrogen, respectively, which might be related to the soil microbial functional groups participating in nitrogen turnover. Nitrogen content in soil is a limiting factor for biological growth (Lebauer and Treseder, 2008) and variations in nitrogen levels result in changes in soil microbial biomass, microbial activity and community structures (Sarathchandra *et al*., 2001). The soil biota directly regulates soil nitrogen supply, and the quantities of ammonifying and denitrifying bacteria in the soil, as well as the corresponding ammonification and nitrification rates, are directly related to the soil nitrogen concentration (Singh and Kashyap, 2007). In view of this, soil microbial functional groups participating in the nitrogen cycle should be investigated on the basis of this study, exploring the interaction between soil microbial functional groups and soil nutrient changes.

Soil nutrient content plays an important role in regulating the microbial community and is the key determinant for survival, species composition, and metabolism of soil microorganisms (Marschner *et al*., 2001). Soil pH can change the soil bacterial community by influencing the bacterial physiological metabolism, changing the competition relationship between microbial communities, or restraining the growth of non-adaptive microorganisms (Lauber *et al*., 2009; Shen *et al*., 2013). In this study, with increasing forest age, the abundance of *Acidobacteria* first decreased and then increased, with soil pH showing the opposite trend. *Acidobacteria* are acidophilic bacteria (Ito *et al*., 2017) and an acidic soil environment is conducive to their metabolic activities, which explains the observed pattern. Chatterjee *et al*. (2009) concluded that soil pH decreased with the increasing forest age of coniferous forests, which is in contrast to the results of our study. There is an increased pH at 13 and 25 years, most likely because *Pinus massoniana* grew more rapidly in these sites, thus required more nutrients, resulting in accelerated nitrogen transformation and increased ammonium and nitrate nitrogen levels. Simultaneously, the root system produced OH^- to maintain the charge balance of the cytomembrane and to buffer the pH value (Wei *et al*., 2017). With the increasing forest age, the soil nutrient content decreased, while the relative abundance of *Acidobacteria* increased, indicating that *Acidobacteria* prefer nutrient-poor environments (Zhao *et al*., 2014). Among *Acidobacteria*, the genera *Acidothermus*, *Candidatus_Solibacter* and *Bryobacter* can use organic matter as a carbon source (Rime *et al*., 2015). According to previous research results (Du *et al*., 2017), high organic carbon contents in the soil are conducive to the growth of *Acidothermus*, *Candidatus_Solibacter* and *Bryobacter*. In this study, however, the soil organic carbon content in the 25 years old site was higher than in the 38- and 58-year-old sites, while the relative abundances of *Acidothermus*, *Candidatus_Solibacter* and *Bryobacter* were lower at 25 years, which may be associated with the soil pH.

Proteobacteria frequently participates in the degradation of stable polymers (such as lignin and cellulose), preferentially in the decomposition of plant residues. This phylum includes the heterotrophic nitrogen-fixing bacteria in symbiosis with plants, which can enhance the nitrogen-fixing capacity of the soil (Fazi *et al*., 2005). There is evidence that the abundance of *Proteobacteria* in the soil reflects the nutrient content to some extent (Lin *et al*., 2018) and in a previous study, *Proteobacteria* grew well in a soil environment with a higher pH value (Zhang *et al*., 2014). In this study, during the development of plantation forests, the abundance of *Proteobacteria* in the soil first increased and then decreased, with the highest value in the 25 years old site. This may be associated with the higher soil nutrient content and the higher pH values in this site (Table 3), facilitating the growth of *Proteobacteria*. However, within the *Proteobacteria* phylum, the genera *Variibacter*, *Acidibacter* and *Bradyrhizobium*, which had higher overall relative abundances, had the lowest relative abundances in this site, most likely because different genera respond differently to soil environmental conditions. The genus *Bradyrhizobium* mainly participates in biological nitrogen fixation and accounts for a larger proportion of the nitrogen-fixing bacterial community (Rösch *et al*., 2002). Overall, the relative abundance of *Bradyrhizobium* decreased with increasing forest age, indicating decreased nitrogen levels.

The Gram-positive Actinomycetes are saprophytic bacteria that are able to decompose lignin and cellulose,

playing an important role in humus formation (Buée *et al*., 2010). In the soil, most actinomycetes are beneficial bacteria, and the bioactive substances in their secondary metabolites have antagonistic effects on some pathogenic bacteria (Zeng *et al*., 2013). In our study, the relative abundance of *Actinobacteria* decreased with the increasing forest age, which led to the gradual decrease of their antagonistic effects. This may also be an important factor of soil quality degradation in artificial forests. *Nitrospirae* is associated with soil nitrogen transformation and can oxidize nitrite to nitrate, thus improving the nitrogen use efficiency and promoting the growth of artificial forests. In our study, the relative abundance of *Nitrospirae* had the highest value (6.06%) in the 25 years old site and sharply decreased in sites above the age of 25 years, possibly resulting in a reduced nitrogen use rate and, consequently, decreased soil fertility. *Chloroflexi* use CO_2 as a carbon source *via* photosynthesis, but as they are facultative anaerobic organisms, they do not produce oxygen during photosynthesis (Klatt *et al*., 2013). In our study, the relative abundance of *Chloroflexi* gradually decreased with the increasing forest age, which is probably due to the increasing soil moisture level over time, which led to decreased CO_2 emissions. As a result, the carbon source was not sufficient to sustain the growth of these organisms. *Firmicutes* can secrete large amounts of toxic substances which interfere with the plant nitrogen metabolism and can even lead to plant death (Zeng *et al*., 2013). The relative abundance of *Firmicutes* first decreased and then increased over time; at 25 years, we observed a sharp increase in abundance. This leads us to infer that the slow growth rate and the low productivity over the development of artificial pine forests are related to the presence of *Firmicutes*.

Soil pH, total nitrogen, and ammonium nitrogen were closely correlated with the soil bacterial community. After a reforestation period of 25 years, soil pH, total nitrogen, and ammonium nitrogen gradually decreased, with significant consequences on the soil bacterial community structure.

Conclusion

With the development of forest plantations, the soil quality changes, leading to significant changes in bacterial community structure and diversity. *Acidobacteria*, *Proteobacteria*, *Actinobacteria* and *Chloroflexi* were the dominant taxa in the investigated sites. After a revegetation period of 25 years, the abundances of *Acidobacteria* and *Firmicutes* both increased over time, while those of *Proteobacteria*, *Actinobacteria*, *Chloroflexi*, *Verrucomicrobia*, *Gemmatimonadetes* and *Nitrospirae* decreased, resulting in reduced productivity. Soil pH, total nitrogen, and ammonium nitrogen were the main factors affecting soil bacterial community structure and diversity.

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