Effects of Robinia pseudoacacia-Fraxinus Mixed Planting on Soil Bacterial Community Structure and Diversity in the Yellow River Delta (Post-print)

Authors: Huang Yali, Tian Qi, Qin Guanghua, An Ran, Ma Fengyun, Jing Ruyan, Chen Bojie

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Abstract

To investigate the effects of Robinia pseudoacacia-Fraxinus mixed plantations on soil bacterial community structure and diversity in the Yellow River Delta, high-throughput sequencing technology was employed to analyze and compare the soil bacterial community structure and diversity among Robinia pseudoacacia-Fraxinus mixed plantations, Robinia pseudoacacia pure stands, and Fraxinus pure stands. The results showed that: A total of 36 bacterial phyla were identified across the mixed plantation and the two pure stands. Acidobacteria, Proteobacteria, and Actinobacteria (with relative abundance >10%) were the dominant bacterial groups shared in the soils of Robinia pseudoacacia-Fraxinus mixed plantations and both pure stands; Nitrospirae was the dominant bacterial group in Robinia pseudoacacia pure stand soils. The relative abundance of each bacterial phylum differed significantly among different plantation soils. Mixed plantations altered soil bacterial community structure and enhanced bacterial diversity. The soil bacterial species number, Chao1 index, and Shannon index in Robinia pseudoacacia-Fraxinus mixed plantations were 1934.5, 2629.1, and 9.1, respectively, which were significantly higher than those in the two pure stands. Correlation analysis indicated that soil water content was significantly positively correlated with Actinobacteria; pH was highly significantly positively correlated with Gemmatimonadetes and significantly negatively correlated with Acidobacteria. Bacterial diversity was significantly positively correlated with soil water content and significantly negatively correlated with available potassium and organic matter content. The study demonstrates that soil bacterial community structure in Robinia pseudoacacia-Fraxinus mixed plantations differs from that in the two pure stands, with significant differences in diversity, and that Robinia pseudoacacia-Fraxinus mixing alters bacterial community structure and

increases bacterial diversity.

Full Text

Preamble

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Effects of a Mixed Plantation of *Robinia pseudoacacia* and *Fraxinus velutina* on Soil Bacterial Community Structure and Diversity in the Yellow River Delta

Huang Yali[1], Tian Qi[1], Qin Guanghua[2], An Ran[1], Ma Fengyun[1]*, Jing Ruyan[1], Chen Bojie[1]

[1]College of Forestry, Shandong Agricultural University; Laboratory of Ecology and Environment, Shandong Agricultural University
[2]Shandong Academy of Forestry, Jinan 250014, China

Abstract: To investigate the effects of a mixed plantation of *Robinia pseudoacacia* and *Fraxinus velutina* on soil bacterial community structure and diversity in the Yellow River Delta, high-throughput sequencing technology was used to analyze and compare the soil bacterial community structure and diversity in mixed and pure plantations of these species. The results showed that a total of 36 bacterial phyla were detected across the mixed plantation and both pure stands. Acidobacteria, Proteobacteria, and Actinobacteria were the dominant bacterial taxa shared among the two pure forests and the mixed forest, each with a relative abundance greater than 10%. Nitrospirae was the dominant bacterial taxon specifically in the *R. pseudoacacia* pure stand. The relative abundance of bacteria in the mixed plantation of *R. pseudoacacia* and *F. velutina* differed significantly from those in the pure plantations. Among the three plantation types, the mixed forest exhibited the highest observed species number (1934.5), Chao1 index (2629.1), and Shannon index (9.1). There was a significant positive correlation (0.995) between soil water content and Actinobacteria abundance, a very significant positive correlation (0.999) between soil pH and Gemmatimonadetes abundance, and a very significant negative correlation (-0.909) between soil pH and Acidobacteria abundance. Bacterial diversity showed a very significant positive correlation with soil water content and a very significant negative correlation with soil available potassium. The results demonstrated that the mixed plantation of *R. pseudoacacia* and *F. velutina* altered the soil bacterial community structure and increased bacterial diversity compared with the two pure stands.

Keywords: Illumina MiSeq; Yellow River Delta; mixed forest; soil bacterial; community structure; bacterial diversity

Introduction

Soil microorganisms constitute a vital component of forest ecosystems, serving as important participants in material transformation and nutrient cycling processes in soil. They play crucial roles in soil structure formation, degradation of harmful substances, and improvement of plant nutrient availability. Among soil microbial groups, bacteria represent the most abundant and widely distributed taxa, performing essential functions in promoting the decomposition of organic residues and transformation of potential soil nutrients, thereby enhancing soil fertility. Soil bacterial community diversity serves as an important indicator of soil quality. In forest ecosystems, investigating soil bacterial community structure and diversity facilitates understanding of the interrelationships among soil, plants, and bacteria.

Traditional methods for studying soil bacterial community structure and diversity, such as dilution plating and Biolog techniques, involve cumbersome procedures, long experimental durations, and low detection efficiency, making it difficult to comprehensively understand bacterial community characteristics. High-throughput sequencing technology, also known as next-generation sequencing, offers significant advantages over conventional methods, including high sequencing throughput, high accuracy, and the ability to more comprehensively reflect environmental bacterial community structures. With continuous development of high-throughput sequencing technology, research on soil microbial flora has become increasingly sophisticated, enabling more efficient and comprehensive understanding of soil microorganisms.

The Yellow River Delta region features a unique ecosystem type, representing a typical saline-alkali area with fragile ecological conditions that severely constrain local economic development. To improve the ecological environment and promote economic growth, large-scale artificial forests have been established in the Yellow River Delta since the 1980s. Salt-tolerant tree species such as *Robinia pseudoacacia* and *Fraxinus velutina* are commonly selected for afforestation in this region. While numerous studies have investigated carbon storage, afforestation techniques, and management models for artificial forests in this area, research on soil microbial communities remains limited. This study employs Illumina MiSeq high-throughput sequencing technology to analyze and compare soil microbial community structure and diversity among pure *R. pseudoacacia* stands, pure *F. velutina* stands, and mixed *R. pseudoacacia*-*F. velutina* plantations in the Yellow River Delta. The objective is to elucidate the effects of mixed planting on soil bacterial communities in artificial forests, providing a scientific basis for cultivation management and soil fertility maintenance.

1 Study Area and Sample Plot Description

The study site is located in Hekou District, Dongying City, Shandong Province (118°53 27 -118°55 41 E, 37°59 14 -37°88 23 N). The region experiences a warm temperate semi-humid monsoon climate with an average annual temperature of 12.94°C, average annual frost-free period of 234 days, and average annual precipitation of 690.6 mm. Precipitation distribution is uneven throughout the year, with summer rainfall accounting for 69.25% of the annual total. The average annual sunshine duration is approximately 2728.5 hours, and average relative humidity is 69.2%. The soil texture is heavy clay, with surface salt content ranging from 0.4% to 3.0%. The main soil types are coastal saline soil and fluvo-aquic soil. Primary afforestation species include *Robinia pseudoacacia*, *Fraxinus velutina*, *Ulmus pumila*, *Ailanthus altissima*, *Populus* spp., and *Sophora japonica*. The understory vegetation consists mainly of *Cynodon dactylon*, *Erigeron acer*, and *Chenopodium album*.

This study selected three types of artificial forest sample plots: pure *R. pseudoacacia* stands, pure *F. velutina* stands, and mixed *R. pseudoacacia*-*F. velutina* stands. Stand characteristics are presented in .

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2 Sample Collection and Processing

2.1 Sampling Method

Sampling plots of 20 m × 20 m were established in each stand type. Within each plot, five sampling points were arranged in a quincunx pattern. Surface weeds and litter were removed, and soil samples were collected from the 5–20 cm soil layer. Soil samples from the same plot were thoroughly mixed, visible roots were removed, and the composite sample was used for analysis. A total of nine soil samples were collected (three replicates per stand type). Each sample was divided into two portions: one portion was placed in sterilized sealed plastic bags, stored in liquid nitrogen for DNA extraction; the other portion was passed through a 0.20 mm sieve for physicochemical property analysis.

2.2 Soil Physicochemical Property Analysis

Soil physicochemical properties were measured using conventional methods: soil water content by the ring method; pH by potentiometry (soil:water ratio 1:2.5); soil organic matter (SOM) by potassium dichromate oxidation; available phosphorus (AP) by molybdenum-antimony anti-colorimetry (soil:water ratio 1:5); available nitrogen (AN) by alkali diffusion method; available potassium (AK) by flame photometry; and electrical conductivity by conductometry.

2.3 Soil Microbial DNA Extraction

Soil sample genomic DNA was extracted using a commercial kit. Sample purity and concentration were detected using a UV spectrophotometer. Appropriate amounts of sample were placed in centrifuge tubes and diluted with double-distilled water (ddH O) to 1 ng/ L. The V4 region of the 16S rRNA gene was amplified using primers 515F–806R. PCR products were purified using a gel recovery kit, and equal concentrations of purified products were mixed. Library construction was performed using the NEB Next Ultra DNA Library Prep Kit for Illumina, followed by sequencing on the Illumina MiSeq platform.

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3 Data Processing and Analysis

Raw sequencing data (Raw Data) were obtained from the Illumina MiSeq/HiSeq platform. Barcode sequences were used to demultiplex reads into separate sample datasets (Raw Tags). Low-quality sequences were filtered based on a default quality threshold. Raw Tags were truncated at the first low-quality base, and sequences with consecutive high-quality bases shorter than the default length were removed. The remaining Clean Tags were assembled using FLASH software. Assembled reads were further filtered and compared against the Gold database to detect and remove chimeric sequences, yielding final Effective Tags for analysis.

3.1 Species Annotation and Abundance Calculation

Effective Tags from all samples were clustered using UPARSE software based on sequence similarity. A 97% similarity threshold was used to cluster sequences into operational taxonomic units (OTUs). Representative sequences were selected as those with the highest frequency in each OTU. The GreenGene database was used for species annotation, and abundance of each taxon in each sample was calculated.

3.2 Diversity Analysis

Alpha diversity indices including observed species number, Chao1, Shannon, and Simpson were calculated using QIIME (Version 1.7.0). Rarefaction curves were generated by random sampling. SPSS 22.0 software was used for one-way ANOVA followed by Duncan' s test to detect significant differences among treatments, with $P < 0.05$ indicating significant differences and $P < 0.01$ indicating highly significant differences.

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4 Results

4.1 Sequencing Data Analysis

High-throughput sequencing yielded 54,813, 31,281, and 23,281 raw sequences for the mixed plantation, pure *R. pseudoacacia*, and pure *F. velutina* stands, respectively. After filtering low-quality sequences, 53,607, 30,964, and 23,146 effective sequences were retained. These sequences were clustered into OTUs at 97% similarity. Rarefaction curves showed that the number of observed species increased with sequencing depth but began to plateau, indicating that additional sequencing would contribute minimally to discovering new species. The curves for all three stand types approached saturation, though not completely, suggesting the sequencing data were adequate but additional data might reveal minor new species. The mixed plantation exhibited the highest species richness.

4.2 Bacterial Diversity Analysis

The mixed plantation soil harbored the highest bacterial diversity, with observed species number, Chao1 index, and Shannon index of 1934.5, 2629.1, and 9.1, respectively. These values were significantly higher than those in pure *R. pseudoacacia* stand (81.23%, 68.62%, and 91.46% of mixed stand values, respectively) and pure *F. velutina* stand (91.48%, 96.86%, and 95.84% of mixed stand values, respectively). All diversity indices differed significantly between the mixed plantation and both pure stands ($P < 0.05$). presents the detailed diversity analysis.

[Figure 1: see original paper] shows the rarefaction curve analysis of OTUs for the three stand types: pure *R. pseudoacacia* (HK), pure *F. velutina* (BL), and mixed *R. pseudoacacia-F. velutina* (FBC).

4.3 Soil Bacterial Community Relative Abundance Analysis

At the phylum level, 36 bacterial phyla were detected across the mixed plantation and two pure stands. The mixed plantation, pure *R. pseudoacacia*, and pure *F. velutina* stands contained 29, 25, and 26 phyla, respectively. Eight phyla had relative abundances exceeding 1% in all stands: Acidobacteria, Proteobacteria, Actinobacteria, Nitrospirae, Chloroflexi, Gemmatimonadetes, Planctomycetes, and Verrucomicrobia.

Acidobacteria, Proteobacteria, and Actinobacteria were dominant taxa shared among all three stands, each with >10% relative abundance. Nitrospirae was the dominant taxon specifically in the *R. pseudoacacia* pure stand. The relative abundances of these phyla differed significantly among stand types. Acidobacteria abundance in the mixed stand (28.72%) was significantly higher than in the *R. pseudoacacia* pure stand but significantly lower than in the *F. velutina* pure stand. Proteobacteria abundance in the mixed stand (24.40%) was significantly higher than in the *F. velutina* pure stand but significantly lower than in the *R. pseudoacacia* pure stand. Actinobacteria abundance in the mixed stand was

significantly higher than in both pure stands. Nitrospirae was only dominant in the *R. pseudoacacia* pure stand, with relative abundance of 11.85%, while its abundance in the mixed stand and *F. velutina* pure stand was only 4.76% and 8.68%, respectively.

The mixed plantation soil contained a unique bacterial phylum, WPS-2, not detected in pure stands. The relative abundances of the eight major phyla in the mixed plantation and pure stands ranged from 1.20% to 7.77%. [Figure 2: see original paper] illustrates the soil bacterial community structure at the phylum level.

4.4 Soil Physicochemical Properties

The mixed plantation soil had the highest water content, with pure *R. pseudoacacia* and pure *F. velutina* soils containing 76.1% and 68.1% of the mixed stand' s water content, respectively. Electrical conductivity was lowest in the mixed plantation soil, with significant differences from both pure stands, while the two pure stands did not differ significantly. Soil pH showed no significant differences among stands and all were alkaline. Available phosphorus content in the mixed plantation was 2.7 mg/kg, significantly higher than in the *F. velutina* pure stand but significantly lower than in the *R. pseudoacacia* pure stand. Available potassium content in the mixed plantation did not differ significantly from the *R. pseudoacacia* pure stand. Available nitrogen content was lowest in the mixed plantation soil. Organic matter content was also lowest in the mixed plantation, with pure *R. pseudoacacia* and pure *F. velutina* soils containing 37.1 g/kg and 33.6 g/kg, respectively—significantly higher than the mixed stand. summarizes the soil physicochemical properties.

4.5 Relationships Between Bacterial Community and Soil Physicochemical Properties

4.5.1 Correlations Between Bacterial Community Structure and Soil Properties Correlation analysis revealed significant relationships between major bacterial phyla and soil properties. Soil pH showed very significant positive correlations with Gemmatimonadetes (0.999) and significant negative correlations with Acidobacteria (-0.909). Available phosphorus showed significant negative correlations with Acidobacteria abundance. Available potassium showed significant positive correlations with Acidobacteria, Proteobacteria, Nitrospirae, and Gemmatimonadetes. Actinobacteria showed significant positive correlations with soil water content and very significant negative correlations with electrical conductivity. presents the correlation coefficients between major bacterial phyla and soil physicochemical properties.

4.5.2 Correlations Between Bacterial Diversity and Soil Properties Bacterial diversity indices showed positive correlations with soil water content and negative correlations with electrical conductivity, available potassium, available nitrogen, and organic matter. The Shannon index exhibited a significant

positive correlation with soil water content (r = 0.957). The Chao1 index showed significant negative correlations with available potassium (r = -0.997) and organic matter content (r = -0.999). details the correlations between bacterial diversity indices and soil properties.

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5 Discussion

5.1 Effects of Tree Species Mixing on Soil Bacterial Communities

Numerous practices and studies have demonstrated that pure plantations exhibit lower biodiversity, stability, and ecological function compared with mixed forests. Consequently, mixed-species plantations are currently recommended for artificial forest cultivation. Mixed forests fundamentally alter vegetation types, and previous research has shown that vegetation type can influence soil bacterial community structure and diversity. This study detected differences in dominant bacterial groups between the mixed *R. pseudoacacia-F. velutina* plantation and pure stands in the 5–20 cm soil layer. While Acidobacteria, Proteobacteria, and Actinobacteria were shared dominant groups across all stands, Nitrospirae was specific to the *R. pseudoacacia* pure stand. Significant differences in phylum-level abundances were observed among stand types.

These findings align with previous studies reporting distinct soil microbial communities among different artificial forest types. The differences in bacterial community structure between mixed and pure stands likely result from variations in tree species composition and configuration, which affect root metabolism, litter decomposition, and soil physicochemical properties. Different tree species create distinct litter composition, decomposition rates, and soil improvement conditions, altering the environment and nutrient availability for soil bacteria. Research has demonstrated significant relationships between microbial functional diversity and plant species diversity, with biodiversity playing important roles in improving soil environment and promoting ecosystem stability.

The mixed plantation enriched tree species composition compared with pure stands, consequently altering soil bacterial communities. Tree species mixing changes vegetation composition at the macroscopic level and modifies soil microbial communities at the microscopic level, which may represent one mechanism underlying the greater stability of mixed forests relative to pure stands. This study focused on the shallow soil layer (5–20 cm), which is influenced by tree roots, microclimate, understory vegetation, and litter. Further research is needed to examine effects of mixed plantations on soil bacterial communities at different soil depths to comprehensively understand their impacts.

5.2 Bacterial Communities and Soil Physicochemical Properties

Soil bacteria are highly sensitive to environmental changes, with soil nutrient content directly affecting bacterial communities. Soil pH exhibits the most sig-

nificant influence on bacterial community structure. Many studies have shown that Gemmatimonadetes thrives in alkaline soils, while Acidobacteria prefers acidic conditions. In acidic soils (pH 4.4–5.8), Gemmatimonadetes exhibits low relative abundance or is undetectable, whereas in alkaline soils (pH 8.01–8.79), its relative abundance reaches 2.5%–7.8%, becoming a dominant group. The alkaline soil environment in the Yellow River Delta coastal saline area likely promotes the growth of alkaliphilic Gemmatimonadetes, resulting in its high relative abundance.

Acidobacteria, known as acidophilic bacteria that grow well in acidic soils, was still dominant in the alkaline soils of the Yellow River Delta but at much lower abundances (25.1%–49.2%) compared with acidic forest soils in other regions (53.3%–67.8%). The alkaline environment likely suppresses some Acidobacteria members while promoting alkaliphilic bacteria, reducing nutrient acquisition for Acidobacteria and consequently lowering its relative abundance.

Soil salinization also strongly affects microbial activity, with higher salt concentrations increasing competitive pressure among bacteria and inhibiting microbial growth. Electrical conductivity serves as a comprehensive indicator of soil salinity. The mixed plantation soil exhibited significantly lower electrical conductivity than pure stands, while simultaneously supporting higher bacterial diversity. These results are consistent with previous research showing that microbial community diversity decreases with increasing salt concentration. High salinity reduces root exudates, decreasing nutrient availability for soil microorganisms and inhibiting their growth and reproduction. In the saline soils of the Yellow River Delta coastal area, promoting mixed plantations can reduce soil salinity and provide favorable conditions for bacterial growth, thereby increasing soil bacterial diversity.

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6 Conclusion

The mixed *R. pseudoacacia-F. velutina* plantation in the Yellow River Delta exhibited higher bacterial abundance than pure stands. Acidobacteria, Proteobacteria, and Actinobacteria were dominant in the mixed plantation and pure *F. velutina* stand, while Nitrospirae was dominant specifically in the *R. pseudoacacia* pure stand. The mixed plantation altered soil bacterial community structure and increased bacterial diversity compared with pure stands. Soil improvement in the mixed plantation changed bacterial community structure and enhanced diversity. This study compared soil bacterial diversity characteristics among stand types within the same season and small spatial scale; further research is needed to investigate temporal and spatial variations in soil microbial communities with stand structure.

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Note: Figure translations are in progress. See original paper for figures.