Soil Bacterial Network Characteristics in Pinus sylvestris var. mongolica Plantations in the Hulunbuir Sandy Land (Postprint)

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Abstract

To elucidate the interrelationships among soil bacteria in Mongolian pine (Pinus sylvestris var. mongolica) plantations in the Hulunbuir Sandy Land, this study employed molecular ecological network analysis to compare soil bacterial communities across different soil layers (0-10 cm and 10-20 cm), using Mongolian pine plantations of different stand ages (25, 34, and 43 years) as study subjects and sandy grassland as a control. The results showed that: (1) From 25 to 43 years, the total number of edges in the soil bacterial network increased, while the average path length decreased. With increasing soil depth from 0-10 cm to 10-20 cm, the total number of edges in the plantation soil bacterial network decreased, and the average path length increased. Compared with sandy grassland, the total number of edges in the plantation soil bacterial network was lower. (2) The keystone taxa in the 25-year plantation belonged to Acidimicrobiales, RB41, and MB-A2-108; those in the 34-year plantation belonged to Gaiellales; those in the 43-year plantation belonged to Gaiellales, RB41, Subgroup_7, Subgroup_6, and DA101_{{{soil}}_{{group}}}; and those in the grassland belonged to Latescibacteria. (3) Total nitrogen, ammonium nitrogen, microbial biomass carbon content, and urease activity had significant positive effects on some bacteria with high betweenness centrality in the soil bacterial network ($P<0.05$); invertase and catalase activities, soil water content, and available phosphorus content had significant negative effects on some bacteria with high betweenness centrality in the soil bacterial network ($P<0.05$); soil organic matter had both significant positive and negative effects on some bacteria with high betweenness centrality in the soil bacterial network ($P<0.05$). From 25 to 43 years in Mongolian pine plantations, the soil bacterial network became increasingly complex and compact; with soil depth from 0-10 cm to 10-20 cm, network complexity and compactness decreased; compared with grassland, the complexity of the plantation soil bacterial network was lower. The

43-year plantation had the greatest number of keystone taxon types in the soil bacterial network. Additionally, the soil bacterial network was most strongly influenced by soil organic matter. The research results contribute to a deeper understanding of soil bacterial communities in Mongolian pine plantations in the Hulunbuir Sandy Land and provide scientific and technological support for the sustainable management of these plantations.

Full Text

Abstract

To elucidate the interrelationships among soil bacteria in *Pinus sylvestris* var. *mongolica* plantations of the Hulunbuir Desert, we selected plantations of different stand ages (25, 34, and 43 years) in the Hulunbuir Desert as research objects, using sandy grassland as a control. Molecular ecological network analysis was employed to compare and analyze soil bacterial communities across different soil layers (0–10 cm and 10–20 cm). The results showed that as stand age increased from 25 to 43 years, the total number of edges in the plantation soil bacterial network increased while the average path length decreased. Compared with sandy grassland, the plantation soil bacterial network had fewer total edges. As soil depth increased from 0–10 cm to 10–20 cm, the total number of network edges increased while the average path length decreased. The keystone bacterial taxa in the 25-year plantation belonged to Acidimicrobiales, RB41, and MB-A2-108; those in the 34-year plantation belonged to Gaiellales; those in the 43-year plantation belonged to Gaiellales, RB41, Subgroup_7, Subgroup_6, and DA101_{{{soil}}_{{group}}}; and those in the grassland belonged to Latescibacteria. Total nitrogen, ammonia nitrogen, microbial carbon content, and urease activity showed significant positive correlations with some bacteria exhibiting high betweenness centrality in the soil bacterial network ($P < 0.05$). Invertase and catalase activities, soil water content, and available phosphorus content showed significant negative correlations with some bacteria exhibiting high betweenness centrality ($P < 0.05$). Soil organic matter exhibited both significant positive and negative effects on bacteria with high betweenness centrality ($P < 0.05$). From 25 to 43 years, the soil bacterial network in *P. sylvestris* plantations became increasingly complex and compact. From 0–10 cm to 10–20 cm soil depth, network complexity and compactness decreased. Compared with grassland, the soil bacterial network in plantations was less complex. The 43-year plantation had the greatest number of keystone bacterial taxa. Additionally, soil organic matter exerted the greatest influence on the soil bacterial network. These findings contribute to a deeper understanding of soil bacterial communities in *P. sylvestris* plantations in the Hulunbuir Desert and provide scientific and technological support for the sustainable management of these plantations.

Keywords: soil microbes; molecular ecological network; stand age; soil depth; *Pinus sylvestris* var. *mongolica*; the Hulunbuir Desert

Introduction

Soil bacteria represent a crucial group within the soil microbiome and constitute an important component of terrestrial ecosystems [?]. Soil bacteria drive material cycling and energy flow in terrestrial ecosystems, playing an irreplaceable role in ecosystem stabilization, regulation, and restoration [?]. Climate, vegetation, and soil properties are generally recognized as key factors influencing the composition and functional dynamics of soil bacterial communities [?]. Furthermore, interspecific relationships among soil bacteria are inseparable from bacterial community structure. Research indicates that soil bacterial communities exhibit vast structural complexity with intricate relationships, including both positive synergistic interactions and negative antagonistic interactions. These complex relationships play crucial roles in shaping bacterial communities and significantly influence the maintenance of ecosystem functions and services [?]. However, due to limitations in traditional methods for identifying and determining soil bacterial interactions, our understanding of these relationships remains incomplete.

Molecular ecological network analysis represents an emerging exploratory data analysis method that visualizes microbial interactions and system stability based on random matrix theory combined with gene sequencing technology. This technique has become an important tool for dissecting microbial interactions and their influencing factors [?]. Studies have revealed that soil microbial communities in northern Chinese forests exhibit more complex interrelationships than those in southern forests [?], and that interactions between soil bacteria and eukaryotes in northern Chinese deserts strengthen with increasing mean annual precipitation [?]. Specific interactions within bacterial networks may either increase or decrease biodiversity [?]. Precisely because of these interactions, bacterial networks can better coordinate various soil bacteria, and when communities are disturbed, they can recruit alternative pathways with different bacterial assemblages to achieve specific functions, thereby providing a buffering effect [?]. Therefore, molecular ecological network analysis serves as an important means to further resolve interactions among soil bacteria and their influencing factors, and represents a crucial approach for revealing the dynamic changes in soil bacterial network structure and their co-variation with ecological processes.

Pinus sylvestris var. *mongolica* is a vital tree species for windbreak and sand fixation in the sandy regions of northern China [?], and interrelationships among soil bacteria significantly impact the growth and health status of *P. sylvestris* trees [?]. The "buffering" function of soil bacterial networks plays an important role in helping *P. sylvestris* forests resist environmental changes [?]. In light of this, this study selected *P. sylvestris* plantations of different stand ages in the Hulunbuir Desert as research objects, using adjacent sandy grassland as a control. Molecular ecological network analysis was employed to reveal the structural characteristics of soil bacterial networks and their influencing factors in *P. sylvestris* plantations, with the aim of deepening our understanding of soil bacterial interrelationships and providing theoretical basis and scientific support for the sustainable management of *P. sylvestris* plantations in the region.

Materials and Methods

Study Area

The study area is located in the Hulunbuir National Forest Park, Inner Mongolia (119°31 -119°43 E, 49°05 -49°13 N), which features a north temperate continental semi-arid climate. The mean annual temperature is -0.3°C, mean annual precipitation is 359.4 mm, mean annual evaporation is 1148.5 mm, and annual sunshine duration is approximately 2559 h with 121 frost-free days. The main soil type is aeolian sandy soil in a dark chestnut soil zone. Dominant tree species include *Pinus sylvestris* var. *mongolica*, *Quercus mongolica*, *Populus tomentosa*, and *Betula platyphylla*. Major herbaceous plants include *Potentilla chinensis*, *Stipa capillata*, *Leymus chinensis*, *Setaria viridis*, and *Sanguisorba officinalis*.

Sample Collection and Processing

In September 2020, we selected three *P. sylvestris* plantations (25, 34, and 43 years old) without management measures and with similar understory vegetation in the Hulunbuir National Forest Park, using nearby sandy grassland as a control. Within each plantation and grassland plot, three 50 m × 50 m sample plots were randomly established. After conducting tree measurements in each plantation plot, three standard trees were randomly selected, and soil samples were collected at the canopy projection area at depths of 0–10 cm and 10–20 cm. In grassland plots, five sampling points were randomly selected for soil collection at the same depths. Approximately 500 g of soil was collected from each sampling point within the same soil layer and thoroughly mixed to form one composite sample, resulting in three replicate samples per treatment. During sampling, soil augers and shovels were sterilized with 75% ethanol to prevent contamination. Concurrently, soil samples were collected using ring knives and aluminum boxes for soil water content determination. All soil samples were stored in an ice box, transported to the laboratory, and transferred to a -80°C freezer for preservation.

Soil Physicochemical Properties Determination

Soil water content (SWC) was determined using the drying method. Remaining soil samples were air-dried and passed through a 2 mm sieve for chemical property analysis. Total nitrogen (TN) and ammonia nitrogen (NH4+-N) were measured using the indophenol blue method. Soil organic matter (SOM) was determined using the potassium dichromate dilution heat method. Available phosphorus (AP) was measured using the molybdenum-antimony anti-colorimetric method. Catalase activity was determined using the potassium permanganate titration method. Urease activity was measured using the sodium hypochlorite colorimetric method. Invertase activity was determined using the Na2S2O3 titration method. Microbial carbon (MBC) was measured using the chloroform fumigation method.

Molecular Identification of Soil Bacteria

Soil bacterial DNA was extracted using the PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, USA). The extracted DNA was subjected to gel electrophoresis detection. The V3-V4 region of the 16S rRNA gene was amplified using primers 338F (5' -ACTCCTACGGGAGGCAGCAG-3') and 806R (5' -GGACTACHVGGGTWTCTAAT-3'). Amplification conditions included an initial denaturation at 95°C for 5 minutes, followed by 27 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 45 seconds, with a final extension at 72°C for 10 minutes. Each soil bacterial sample was amplified in triplicate, and PCR products from the same sample were mixed. After detection via 2% agarose gel electrophoresis, PCR products were purified using the AxyPrepDNA Gel Recovery Kit (Axygen Biosciences, USA), eluted with Tris-HCl buffer, and visualized through 2% agarose electrophoresis. Following library construction, samples were sequenced on the Illumina Miseq platform. Raw sequencing data were processed using Trimmomatic and FLASH software. Based on the Silva Release128/132 database (http://www.arb-silva.de), RDP Classifier (http://rdp.cme.msu.edu/), and Greengenes Release13.5 database (http://greengenes.secondgenome.com/), taxonomic assignment was performed using the usearch algorithm or RDP Classifier. Sequencing data were deposited in the NCBI database under accession number PRJNA875585.

Data Analysis

Bacterial genera with relative abundance greater than 5.00% and present in all samples were selected. Pearson correlation analysis was performed using SPSS 24.0 to calculate correlation coefficients and significance values (r, P). In RStudio, the "igraph" package was used to convert the Pearson correlation coefficient matrix into network files and calculate topological features including total edges, total nodes, average clustering coefficient, average path length, modularity, average connectivity, and betweenness centrality. The within-module connectivity (Zi) and among-module connectivity (Pi) were calculated to determine the modular roles of network nodes [?], including module hubs, connectors, network hubs, and peripherals. Nodes with Zi ≥ 0.62 and Pi ≥ 0.62 were considered keystone species (Table 2), playing important roles both within and among modules. Network files were visualized using Gephi 0.9.2 to generate soil bacterial co-occurrence network diagrams. Each node in the network diagram represents an OTU, and each edge between nodes represents a correlation. To assess the significance of network structure, 100 random networks with the same number of nodes and edges as the co-occurrence network were generated using the "igraph" package, and topological parameters were calculated. The average clustering coefficient of the random networks was compared with that of the bacterial co-occurrence network to determine whether the soil bacterial network exhibited significant cohesion.

To analyze the relationship between bacterial ecological networks and soil factors, the top 30 bacterial taxa with the highest betweenness centrality values in the *P. sylvestris* plantation soil bacterial network were selected to establish network relationships with soil factors. Pearson correlation analysis was performed between betweenness centrality values and soil factors, and correlations with $P < 0.05$ were selected. The "network" package in RStudio was used to visualize the interaction diagram between bacteria and soil factors, which was then exported.

Results

Topological Characteristics of Soil Bacterial Networks

The empirical networks of soil bacterial communities in Hulunbuir Desert *P. sylvestris* plantations exhibited significantly higher modularity, average path length, and average clustering coefficient than random networks, confirming their network characteristics and suitability for studying soil bacterial interactions (Table 3). The topological characteristics of soil bacterial community networks in *P. sylvestris* plantations showed significant differences among stand ages (Table 3). As stand age increased, the total number of network edges and average connectivity increased, while the total number of nodes and average path length decreased. In contrast, the grassland soil bacterial network had more total nodes and edges than the plantations but a shorter average path length. A greater number of total edges indicates increased network complexity [**?**], while a shorter average path length indicates greater network compactness. From 25 to 43 years, the soil bacterial network in *P. sylvestris* plantations became increasingly complex and compact. Compared with plantations, the grassland soil bacterial network was more complex and compact.

The topological characteristics of soil bacterial community networks in *P. sylvestris* plantations also varied significantly between soil layers (Table 3). The surface soil bacterial network (0–10 cm) had higher total edges, average connectivity, and proportion of positive correlations, along with lower average path length, compared with the deeper layer (10–20 cm). Conversely, the grassland surface soil bacterial network had lower total edges, average connectivity, and proportion of positive correlations, along with higher average path length, compared with the deeper layer. As soil depth increased from 0–10 cm to 10–20 cm, the complexity and compactness of the plantation soil bacterial network decreased, while those of the grassland soil bacterial network increased.

Keystone Taxa in Soil Bacterial Networks

A total of 14 connectors were identified across all networks, with no module hubs or network hubs detected. Among these, the plantations contained 13 connectors belonging to Actinobacteria (Acidimicrobiales, MB-A2-108, Gaiellales), Acidobacteria (Blastocatellaceae_{Subgroup}4, Subgroup_6, Subgroup_7), and Verrucomicrobia (DA101_{{{soil}}_{{group}}}). The grassland contained one connector belonging to Latescibacteria (Table 4).

Influencing Factors of Soil Bacterial Networks

Soil physicochemical properties and enzyme activities in Hulunbuir Desert *P. sylvestris* plantations showed strong variability (Table 5). In the surface soil layer, total nitrogen and organic matter content decreased significantly with stand age ($P < 0.05$), microbial carbon content first increased then decreased ($P < 0.05$), and catalase and urease activities first decreased then increased ($P < 0.05$). Grassland soil had significantly higher total nitrogen content but significantly lower urease activity than the plantations ($P < 0.05$). In the deeper soil layer, total nitrogen and microbial carbon content first increased then decreased with stand age ($P < 0.05$), while ammonia nitrogen content decreased significantly ($P < 0.05$). Grassland soil invertase activity was significantly higher than in plantations ($P < 0.05$).

Some bacterial taxa with high betweenness centrality in the Hulunbuir Desert soil bacterial network showed significant correlations with soil water content, organic matter, available phosphorus, ammonia nitrogen, total nitrogen, microbial carbon, catalase, invertase, and urease ($P < 0.05$), with soil organic matter having the greatest influence (Figure 3). Specifically, soil organic matter showed significant positive correlations with *Microvirga* (OTU235) and *Burkholderia-Paraburkholderia* (OTU451), but significant negative correlations with *Gaiella* (OTU29) and Acidobacteriaceae_{Subgroup}1 (OTU544). Soil water content and invertase showed significant negative correlations with Gemmatimonadaceae (OTU145). Ammonia nitrogen showed significant positive correlations with RB41 (OTU256) and Subgroup_6 (OTU194). Available phosphorus showed significant negative correlations with Acidobacteriaceae_{Subgroup}1 (OTU155) and DA101_{{{soil}}_{{group}}} (OTU2222). Catalase showed significant negative correlations with MB-A2-108 (OTU432) and Subgroup_6 (OTU12). Microbial carbon showed significant positive correlations with *Burkholderia-Paraburkholderia* (OTU451) and *Roseiflexus* (OTU773).

[Figure 1: see original paper] [Figure 2: see original paper] [Figure 3: see original paper]

Discussion

Characteristics of Soil Bacterial Network Structure in *P. sylvestris* Plantations

The topological properties of soil bacterial networks in Hulunbuir Desert *P. sylvestris* plantations exhibited dynamic changes with stand age. From 25 to 43 years, although bacterial species richness decreased, the networks became increasingly complex and compact. Previous studies have shown that shorter average path lengths indicate higher efficiency of material cycling, energy flow, and information transfer among species in soil bacterial networks [?], suggesting that when environments experience substantial disturbance, the response of plantation soil bacteria accelerates while their stability decreases [?]. The increasing complexity and frequent interactions among bacteria may represent a strategy to counteract the negative effects of plantation aging.

Compared with *P. sylvestris* plantations, grassland soils harbored more abundant bacterial species with increased interactions, resulting in larger and more complex networks, consistent with previous findings [?]. This difference may arise because *P. sylvestris* roots can secrete metabolites that recruit beneficial bacteria while excluding harmful ones, effectively filtering soil bacteria through host effects [?]. Although the grassland bacterial network was more compact than that of plantations, indicating lower community stability [?], this finding aligns with previous research.

Soil bacterial networks in Hulunbuir Desert *P. sylvestris* plantations displayed distinct topological features across soil depths. With increasing soil depth, bacterial interactions in plantations decreased, average path length increased, and network complexity and compactness declined, consistent with previous studies [?]. This pattern likely reflects the vertical distribution of soil resources and environmental conditions. The 0–10 cm soil layer contains abundant nutrients, larger pores, and higher oxygen content, creating favorable conditions for bacterial growth and communication [?]. Additionally, positive and negative correlations in co-occurrence networks represent synergistic and antagonistic interactions, respectively [?]. Synergistic interactions among surface soil bacteria facilitate survival, reproduction, and cooperative decomposition of litter [?]. Although synergistic interactions in *P. sylvestris* plantations gradually weakened while antagonistic interactions increased with depth from 0–10 cm to 10–20 cm, synergistic interactions remained dominant overall, promoting more efficient litter decomposition and nutrient provision for *P. sylvestris* growth [?].

Ecological Functions of Keystone Taxa in Soil Bacterial Networks

Keystone bacterial taxa differed among stand ages, demonstrating species turnover phenomena. The 43-year plantation contained the greatest diversity of keystone taxa, primarily belonging to Acidobacteria. In addition to Gaiellales and RB41, Subgroup_6, Subgroup_7, and DA101_{{{soil}}*{{group}}} were also identified as keystone taxa. In the mature plantation stage, nutrient return becomes a crucial ecological process, and Acidobacteria taxa play important roles in plant cellulose decomposition [?]. Previous studies have indicated that DA101*{{{soil}}_{{group}}} is a dominant taxon in arid grasslands, and drought stress can lead to its proliferation [?].

Different vegetation types exhibited differences in keystone taxa composition. The grassland keystone taxon belonged to Latescibacteria, which generally adopts a saprophytic lifestyle and prefers anaerobic, nutrient-rich habitats [?]. This suggests that grassland soil oxygen content and porosity may be lower than in plantations. The greater diversity of keystone taxa in plantations indicates higher network stability [?], suggesting that plantation networks are more stable than grassland networks, consistent with our conclusions.

Regulatory Effects of Soil Factors on Soil Bacterial Networks

Soil physicochemical properties represent important drivers of soil bacterial communities in the Hulunbuir Desert, primarily regulated by soil organic matter. Vegetation significantly influences soil properties, particularly carbon content [?]. As a crucial component of the soil nutrient pool, organic matter exhibits diverse relationships with different soil bacteria [?] and represents a major factor affecting bacterial community structure [?], consistent with our findings. Both increases and decreases in soil organic matter can stimulate bacterial activity, and bacteria subsequently influence plant nitrogen and phosphorus uptake through various physiological responses, making organic matter supply essential for forest growth [?].

Soil enzyme activities also drive soil bacterial communities in the Hulunbuir Desert. The diversity of regulatory factors suggests that the ecological balance in this region is not easily disrupted [?]. As soil nutrients change in the sandy land, bacterial metabolic pathways diversify. Bacteria secrete various enzymes to degrade these complex nutrients [?]. Urease, catalase, and invertase play indispensable roles in material cycling in the Hulunbuir Desert. Concentrated rainfall during summer and autumn increases urease activity [?], enhancing the activity of bacteria closely associated with urease in the network and enabling more efficient litter decomposition. This decomposition process significantly increases soil catalase activity [?], which decomposes hydrogen peroxide into water and oxygen, thereby mitigating hydrogen peroxide toxicity to plants [?]. Invertase contributes substantially to glucose transformation during litter decomposition [?], and increased enzyme activity facilitates greater network connectivity among soil bacteria participating in material and energy transformation processes in *P. sylvestris* plantations.

Changes in available phosphorus content significantly affect certain bacterial taxa playing important functional roles in the network [?], while ammonia nitrogen and total nitrogen also positively regulate the soil bacterial network because nitrogen and phosphorus are both primary limiting nutrients and important elements affecting bacterial metabolic activity in terrestrial ecosystems [?, ?]. The cycling of carbon, nitrogen, and phosphorus constitutes a vital component of material cycling in *P. sylvestris* forest ecosystems, and changes in these elements can alter interaction types among soil bacteria (e.g., shifts between antagonistic and synergistic interactions), thereby modifying network complexity [?, ?, ?]. Additionally, changes in ammonia nitrogen and organic matter content significantly affect microbial carbon content [?], which reflects soil biological activity and forms the supporting foundation of soil bacterial networks. Total nitrogen, ammonia nitrogen, and available phosphorus showed significant correlations with bacteria belonging to RB41, Subgroup_6, and MB-A2-108 ($P < 0.05$), whose taxonomic levels overlap with those of keystone taxa, suggesting they may perform similar ecological functions. When external disturbances occur, soil bacterial networks can recruit these bacteria to replace keystone taxa and maintain specific ecological functions, thereby achieving buffering effects [**?**].

Conclusion

(1) The soil bacterial network in Hulunbuir Desert *P. sylvestris* plantations became increasingly complex and compact from 25 to 43 years, though bacterial community sensitivity to environmental disturbances increased, with the 43-year plantation being most vulnerable. From 0–10 cm to 10–20 cm soil depth, network complexity and compactness decreased while community sensitivity to environmental disturbances declined. In contrast, the grassland soil bacterial network became more complex and compact with increasing depth, with heightened community sensitivity to disturbances. Compared with grassland, the plantation soil bacterial network exhibited lower complexity.

(2) Keystone bacterial taxa differed among *P. sylvestris* plantations of different stand ages in the Hulunbuir Desert. The 25-year, 34-year, and 43-year plantations contained 3, 1, and 5 different types of keystone taxa, respectively, with Acidobacteria being the most abundant phylum. The grassland contained one keystone taxon belonging to Latescibacteria.

(3) The soil bacterial network in Hulunbuir Desert *P. sylvestris* plantations was driven by soil physicochemical properties and enzymatic activities including soil water content, available phosphorus, organic matter, total nitrogen, ammonia nitrogen, microbial carbon, urease, invertase, and catalase. Soil organic matter exerted the greatest influence on bacteria with high betweenness centrality in the network.

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