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Shifts in dominant tree species modulate phyllosphere microbial diversity and function in successional forests

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Abstract

Background Phyllosphere microbiome plays a crucial role in maintaining plant fitness. However, its response to changes in dominant tree species during forest succession still remains poorly understood.

Methods In this study, microbial isolation and high-throughput sequencing techniques were used to analyze the community structure and diversity of phyllosphere microbes in pure *Pinus massoniana* forests, mixed *P. massoniana* and *Liquidambar formosana* forests, and pure *L. formosana* forests.

Results The results showed that the isolation rates of key plant fungal pathogens varied significantly in phyllosphere across forest types. In pure pine forest, *Fusarium* was most prevalent in pine needles, while in the mixed forest, *Alternaria* was dominant. For *Liquidambar* leaves, *Phyllosticta* dominated in pure forests, while *Colletotrichum* was prevalent in the mixed forests. Alpha diversity analysis revealed that higher microbial richness and diversity in the mixed forest compared to the pure forest. The bacterial community structure in *Liquidambar* leaves differed between the pure forest and the mixed forest. Co-occurrence networks confirmed more complex and stable microbial compositions and interactions in the mixed forest. Bacterial communities in pine needles exhibited higher functional capacity for methanotrophy and nitrogen fixation in the mixed forests.

Conclusions The results demonstrate that the mixed forests foster greater microbial diversity, complexity, and functional potential in the phyllosphere compared to the pure forests, highlighting the importance of forest composition in shaping phyllosphere microbial communities.

Keywords Phyllosphere microbe, Forest succession, Microbial community composition, Plant pathogens

Introduction

The phyllosphere refers to the aerial parts of living plants, including leaves, stems, buds, flowers, and fruits [52]. Microorganisms such as bacteria and fungi are able to colonize this environment despite its challenging conditions, such as nutrient scarcity, antibacterial substances released by leaves, and significant fluctuations in temperature and humidity [47]. Phyllosphere microbial communities are shaped by both biotic and abiotic factors. Studies indicate that plant species [41], leaf characteristics [28, 33], seasonal variations [59, 61, 62], and

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geographic location [32] significantly affect these communities. In the phyllosphere, diverse microbial interactions occur, including bacteria and fungi, as well as between these microorganisms and their host plants. These relationships range from parasitism and commensalism to mutualism. The host plant provides essential nutrient to phyllosphere microbes [47], which in turn, contributes to plant health by fixing nitrogen, promoting growth, suppressing pathogens, and enhancing disease resistance [6, 36].

Recent studies have underscored the critical role phyllosphere microbial. For instance, Innerebner et al. [20] found that *Sphingomonas* strains could inhibit the plant pathogen *Pseudomonas syringae* by competing directly for glucose, fructose, and sucrose. However, research in this field remains limited. Many studies focus on the effects of individual tree species or specific factors on phyllosphere microbial communities, often neglecting the broader influence of different forest types. A deeper understanding these interactions is crucial for elucidating the complex relationships between tree species, phyllosphere microbes, and forest ecosystems.

Traditional microbial isolation and culture methods have historically dominated phyllosphere microbial studies, yet they come with significant limitations. Most microbes in natural environments are unculturable, with only a small fraction successfully isolated and grown in pure culture. Research indicates that only 0.1 to 3% of environmental bacteria are considered culturable [3]. The development of high-throughput sequencing (HTS) technology has revolutionized the analysis of phyllosphere microbial communities, enabling researchers to assess community structure and function with increased resolution and efficiency. Unlike culture-based methods, HTS can detect a diverse array of microorganisms without the need for cultivation, providing extensive genomic information [17]. However, HTS also has limitations: it cannot differentiate between active and inactive microorganisms, and sequencing biases hinder the study of microbial ecological functions [34]. Additionally, the identification of potential biocontrol agents still relies on traditional culturing methods [26]. While traditional methods are essential for investigating the physiological characteristics and functions of specific microorganisms, HTS allows for rapid and comprehensive profiling of microbial community structure and potential functions. Integrating these approaches is crucial for advancing our understanding of phyllosphere microbial communities and their ecological roles.

In this study, we investigate the changes of phyllosphere fungal and bacterial communities across different forest types. We aim to explore the response of phyllosphere microbes to changes in tree species in the pure and mixed forests during forest succession. The primary hypothesis posits that dominant tree species might be the main driver shaping the phyllosphere microbiome.

Methods

Study site and sampling

The study was conducted at Linggu Temple in Zijin Mountain, Nanjing, China (32°16′15″ N, 118°48′00″ E), located within the subtropical and warm temperate zones. The annual average sunshine duration is 1,628.8 h, and the average temperature is 19.6°C, with winter temperatures reaching −3.7°C and summer temperatures reaching 38.8°C. The annual average precipitation is 1,530.1 mm, ranging from 1,091 to 2,371.4 mm [51].

Since the 1930s, Zijin Mountain has been entirely covered by *Pinus thunbergii* Parl. and *P. massoniana* Lamb. However, in 1982, the area began to experience pine wilt disease, caused by the pinewood nematode (*Bursaphelenchus xylophilus*), leading to the natural growth of *Liquidambar formosana* Hance. and *Quercus acutissima* Carruth, which gradually replaced the declining pine trees. The establishment of broad-leaved forests, along with competition for nutrient, has further exacerbated the decline of the pine forests. Currently, the predominant forest type in the scenic area consists of the mixed forests (comprising both pine and broad-leaved species), followed by pure pine forests and pure broad-leaved forests [44]. For this study, three forest types were selected: a pure *P. massoniana* forest, a mixed forest (*P. massoniana* + *L. formosana*), and a pure *L. formosana* forest. The intermediate mixed forest is defined by the equal proportion (1:1) between *P. thunbergii* and *L. formosana* trees [40] (Figure S1). Five trees were sampled from both pure forest and mixed forests for *P. massoniana* and *L. formosana*, respectively, resulting in a total of 20 trees. Each selected tree was spaced more than 50 m apart. Needle/leaf samples were collected from three directions of each tree into sterile bags in October 2022 and were delivered to the laboratory and stored at 4 °C and −20 °C for further analysis.

Isolation and identification of bacteria and fungi

Needle/leaf tissue was cut into small pieces (5 mm × 5 mm), resulting in a total of 30 small pieces. The surface of the leaf tissue was disinfected in a laminar flow hood using 75% alcohol for 30 s. Subsequently, the leaf tissue was immersed in 1% NaClO for 90 s, followed by three rinses with sterile water, and placed on potato dextrose agar (PDA) and Luria–Bertani (LB) plates for the isolation of fungi and bacteria, respectively. The plates were incubated in the dark at 25°C for three days. Pure cultures were obtained by transferring mycelial edges and colonies onto fresh PDA and LB plates. Preliminary

classification of the isolates was conducted based on morphological characteristics.

DNA was extracted from selected representative strains from each fungal and bacterial group using the cetyltrimethylammonium bromide (CTAB) extraction method. The fungal internal transcribed spacer region (ITS) was amplified by polymerase chain reaction (PCR) with the forward primer ITS1 (5'-TCCGTAGGTGAACCTGCG C 3') and the reverse primer ITS4 (5'TCCTCCGCTTAT TGATATGC 3') set [37]. The PCR reaction mixture comprised a total volume of 50μl, consisting of 25μl 2×Premix Taq, 2μl Primer-F (10μM), 2μl Primer-R (10μM), 1ng template DNA, and 20μl ddH_2O. The PCR cycling conditions were as follows: initial denaturation at 94 °C, for 10 min, followed by 37 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s, elongation at 72 °C for 90 s, and a final extension at 72 °C for 10 min.

For bacterial 16SrDNA amplification, PCR was performed with the forward primer 27F (5'-AGAGTTTGA TCCTGGCTCAG 3') and the reverse primer 1492R (5-'GGTTACCTTGTTACGACTT 3') set [55]. The PCR reaction mixture contained a total of 50μl, consisting of 25μl 2×Premix Taq, 2μl Primer-F (10μM), 2μl Primer-R (10μM), 1ng template DNA, and 20μl ddH_2O. The PCR cycling conditions included initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, elongation at 72 °C for 45 s, and a final extension at 72 °C for 10 min.

PCR products were assessed with 1% agarose gel electrophoresis, purified, and subsequently sequenced by Nanjing Sipujin Co., Ltd. (Nanjing, China). The obtained sequences were aligned and BLAST on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to further determine the strain taxonomy.

The sequences of all fungal and bacterial isolates were deposited in the NCBI GenBank under accession numbers provided in Table S1&2.

Calculate the isolation rate (IR) of phyllosphere fungi and bacteria using the following formula:

IR = (Number of isolates of a specific strain obtained from leaf samples / Total number of isolates obtained from leaf samples) × 100%

DNA extraction and high-throughput sequencing

The genomic DNA was isolated from each needle/leaf sample using a Plant Genomic DNA Kit (Tiangen Biotech Company, Beijing, China) following the description of the manual. DNA concentration was measured by a Nanodrop ND-1000 spectrometer (NanoDrop Technologies, Wilmington, DE, USA). The fungal ITS2 was amplified by PCR with the forward primer ITS3F (5'-GCATCG ATGAAGAACGCAGC 3') and reverse primer ITS4R (5'-TCCTCCGCTTATTGATATGC 3') [24]. Each PCR reaction consisted of triplicate 50 μl mixture containing 25μl 2×Premix Taq, 2μl Primer-F (10μM), 2μl Primer-R (10μM), 50ng Template DNA, and nuclease-free water. A PCR control was included with sterile water rather than DNA to monitor potential contamination. The PCR cycling conditions were as follows: 3 min of initial denaturation at 95 °C, followed by 33 cycles of 20 s denaturation at 95 °C, 20 s annealing at 56 °C, 30 s elongation at 72 °C, and a final extension at 72 °C for 5 min.

The bacterial 16S rDNA V5-V7 region was amplified by PCR with the forward primer 799F (5'-AACMGGA TTAGATACCCKG 3') and reverse primer 1193R (5'-ACGTCATCCCCACCTTCC 3') [39]. The PCR reactions mixture also totaled 50 μl, consistING of 25 μl 2×Premix Taq, 2.5 μl Primer-F (10 μM), 2.5 μl Primer-R (10 μM), 50 ng template DNA, and nuclease-free water. A PCR control was included with sterile water rather than DNA. The PCR cycling conditions were: 5 min of initial denaturation at 94 °C, followed by 31 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s, elongation at 72 °C for 30 s, and a final extension at 72 °C for 8 min.

The PCR products were assessed with 1% agarose gel electrophoresis (Figure S2), followed by purification and sequencing by Magigene (Guangdong Magigene Biotechnology Co., Ltd. Guangzhou, China) on the Illumina NovaSeq 6000 platform. The raw sequencing reads were deposited in the NCBI Sequence Read Archive (SRA) database under the Accession Number: PRJNA1170359.

Data processing and statistical analysis

After demultiplexing, sequences were quality-filtered with fastp (v0.19.6) [12] and merged with FLASH (v1.2.11). High-quality sequences were denoised using DADA2 [9], a plugin in the Qiime2 (v2020.2) [8] pipeline, achieving single nucleotide resolution based on error profiles within samples. DADA2-denoised sequences are referred to as amplicon sequence variants (ASVs). To minimize the effects of sequencing depth on alpha and beta diversity measures, the number of sequences per sample was rarefied to 20,000, resulting in an average Good' s coverage of 97.90%. Taxonomic assignment of ASVs was performed using the Naïve Bayes consensus taxonomy classifier in Qiime2 with the SILVA 16S rRNA database (v138) for bacteria and the UNITE database (v8) for fungi. Sequences annotated as chloroplast, mitochondrial, or plant-derived were bioinformatically removed to ensure that only bacterial or fungal sequences remained for further analysis. Metagenomic function was predicted using FAPROTAX and FUNGuild for bacteria and fungi, respectively.

Alpha diversity indices, including species richness (Sobs), species diversity (Shannon), and species evenness (Shannon eveness), were calculated with normalized

ASV data using Mothur v1.30.1 [42]. One-way analysis of variance (ANOVA) was employed to examine significant differences in alpha diversity and dominant taxa across different forest types, with post-hoc testing conducted using the Tukey Honest Significant Difference (TukeyHSD) method. ANOVA was performed using SPSS version 26 (IBM, Armonk, NY, USA), with significance set at $P<0.05$. Principal Coordinates Analysis (PCoA), Mantel test, and permutational multivariate analysis of variance (PERMANOVA) analyses, and data visualizations were performed using R version 4.3.0 (R Foundation for Statistical Computing), primarily with the following packages: vegan v.2.6.4, ggplot2 v.3.4.3, ggpubr v.0.6.0, ggsignif v.0.6.4, and ggcor v.0.9.8.1. PCoA and PERMANOVA, based on Bray–Curtis similarities after 999 permutations, were used for fungal and bacterial community structure analysis. Community diversity, along with the relative abundance of fungal and bacterial phyla and genera, was visualized using GraphPad Prism version 8.0.0 for Windows (GraphPad Software, Boston, Massachusetts, USA, www.graphpad.com). A co-occurrence network for fungal/bacterial genera with relative abundance greater than 0.01% and present in at least three samples was constructed based on Spearman correlation coefficients ($r>0.7$) and significance ($P<0.05$). A heatmap was generated on the bioinformatics platform (https://www.bioinformatics.com.cn/) for functional predictions.

Results

Composition of isolated fungi and bacteria in phyllosphere

The isolated fungi were preliminarily classified into 35 groups based on colony morphology (color, texture, and edge features) and growth rate (Figure S3). Following ITS-rDNA sequence identification, 28 fungal species in total were obtained (Table S1).

The isolation rate of *Fusarium* in pine needles was significantly higher in the pure pine forest compared to the mixed forest ($P<0.05$). In the *Liquidambar* leaves, the isolation rate of *Alternaria, Diaporthe,* and *Colletotrichum* decreased in the pure *Liquidambar* forest compared to the mixed forest, while *Phyllosticta* was increased in abundance (Fig. 1a).

The isolated bacteria were preliminarily classified into 14 groups based on colony morphology (shape, size, color, transparency, edge features, and surface texture), and growth patterns (Figure S3). Following 16S-rDNA sequence identification, 9 bacterial species in total were obtained (Table S2). The isolation rate of *Serratia* decreased in the pure pine forest compared to the mixed forest, while *Alcaligenes* showed an increase in abundance. In *Liquidambar* leaves, the isolation rate of *Serratia* was higher in the pure *Liquidambar* forest compared to the mixed forest, whereas *Bacillus* exhibited a decrease in abundance. (Fig. 1b).

Phyllosphere microbial community diversity

The α-diversity of fungal and bacterial communities varied with forest type. The fungal species richness in pine needles in the mixed forest (PMF) was significantly higher than that in the pure pine forest (PPF) ($P<0.05$) (Fig. 2a). In the mixed forest, the species richness, diversity, and evenness of pine needles were significantly higher than those of *Liquidambar* leaves ($P<0.05$) (Fig. 2a-c). For bacteria, the species richness and diversity in pine needles in the mixed forest (PMF) were also significantly higher than in the pure pine forest (PPF) ($P<0.05$) (Fig. 2d & 2e). Similarly, in the mixed forest, the bacterial species richness, diversity, and evenness of pine needles were significantly higher than those of *Liquidambar* leaves ($P<0.05$) (Fig. 2d-f). However, the bacterial α-diversity of *Liquidambar* leaves did not differ between the pure *Liquidambar* forest (LPF) and mixed forest (LMF) (Fig. 2d-f).

Phyllosphere microbial community composition

The Venn analysis showed that the unique fungal ASVs in pine needles and *Liquidambar* leaves accounted for 23.7%, 33.2%, 13.4%, and 16.3% across pure pine forest, mixed pine and *Liquidambar* forest, and pure *Liquidambar* forest, respectively (Fig. 3a), while shared ASVs accounted for 3.2%. The unique bacterial ASVs in pine needles and *Liquidambar* leaves accounted for 22.6%, 29.1%, 21.9%, and 18.7% across the same forest types (Fig. 3b), with the shared ASVs accounting for 2.0%.

The results of PCoA and PERMANOVA indicated significant differences in the fungal community structure in the pine needle between the pure pine forest (PPF) and mixed forest (PMF), as well as in the *Liquidambar* leaves between the pure *Liquidambar* forest (LPF) and mixed forest (LMF) ($P<0.05$) (Fig. 4a, Table S3). In contrast, the bacterial community structure in the *Liquidambar* leaves varied between the pure *Liquidambar* forest (LPF) and mixed forest (LMF), while no significant difference was observed in the bacterial community structure in the pine needles between the pure pine forest and mixed forest ($P<0.05$) (Fig. 4b, Table S3).

The obtained fungal sequences were classified into four phyla, 456 genera. The dominant phylum was Ascomycota (91.06%), followed by Basidiomycota (5.65%), Mucoromycota (0.012%) (Fig. 5a). The relative abundance of Ascomycota and Basidiomycota varied among different forest types. Specifically, the abundance of Ascomycota in pine needles in the mixed forest (PMF) was significantly higher than in the pure pine forest (PPF) ($P<0.05$) (Fig. 5e), whereas the relative

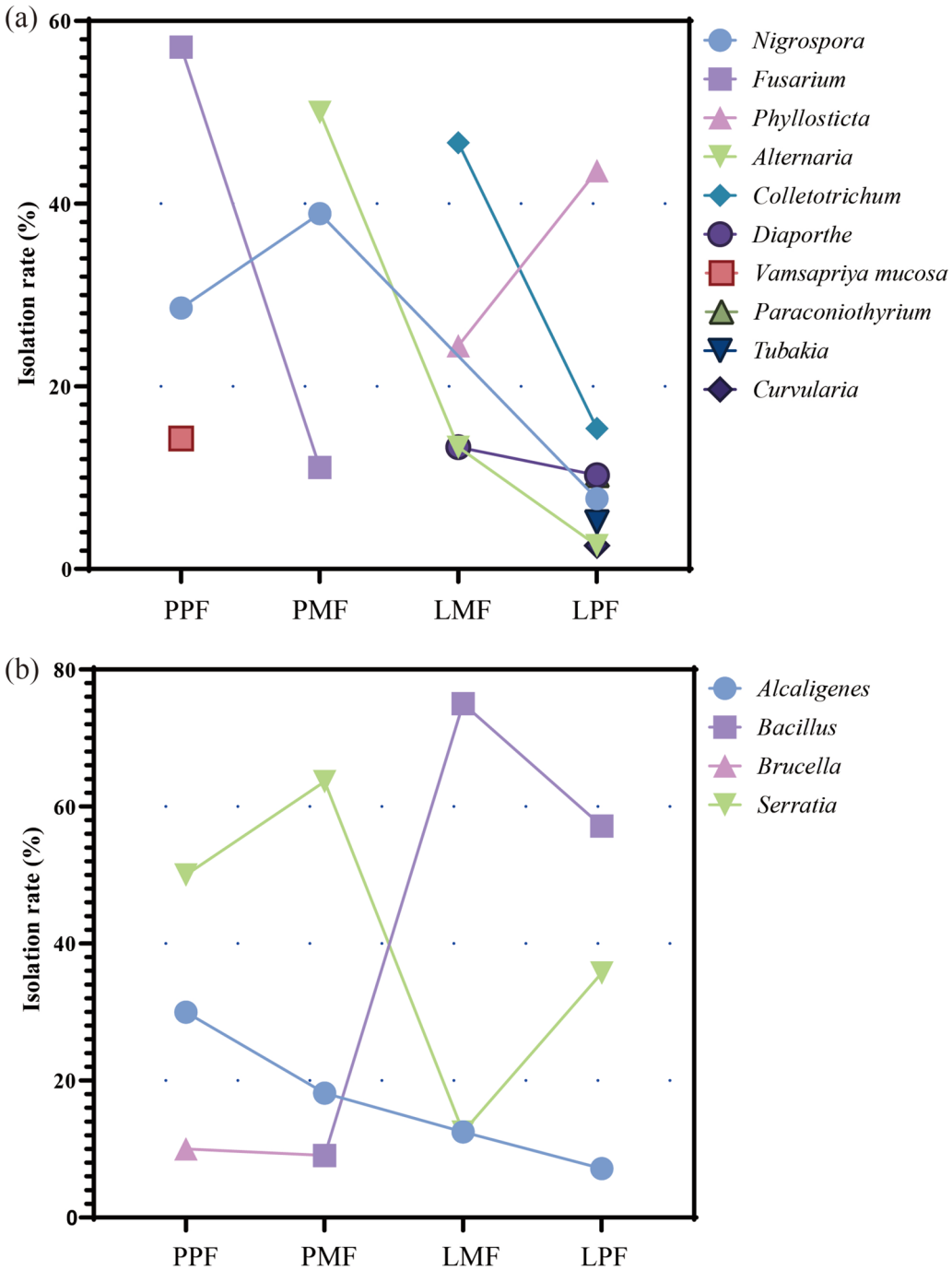

Fig. 1 The isolation rates of fungal (a) and bacterial (b) genera from pine needles and *Liquidambar* leaves during forest succession. PPF: Pine needles in the pure pine forest, PMF: Pine needles in the mixed forest, LMF: *Liquidambar* leaves in the mixed forest, LPF: *Liquidambar* leaves in the pure *Liquidambar* forest

abundance of basidiomycetes was significantly lower in the mixed forest ($P < 0.05$) (Fig. 5c). At genus level, *Aureobasidium* was the most abundant genus (16.39%), followed by *Trichomerium* (3.85%), *Cladosporium* (2.87%), *Alternaria* (2.79%), *Malassezia* (1.64%), and *Nigrospora* (1.02%) (Fig. 5b). The abundance of *Aureobasidium* and *Trichomerium* in pine needles in the pure pine forest (PPF) was significantly higher compared to the mixed forest (PMF) ($P < 0.05$) (Fig. 5d). In contrast, *Cladosporium*, *Alternaria*, *Nigrospora*

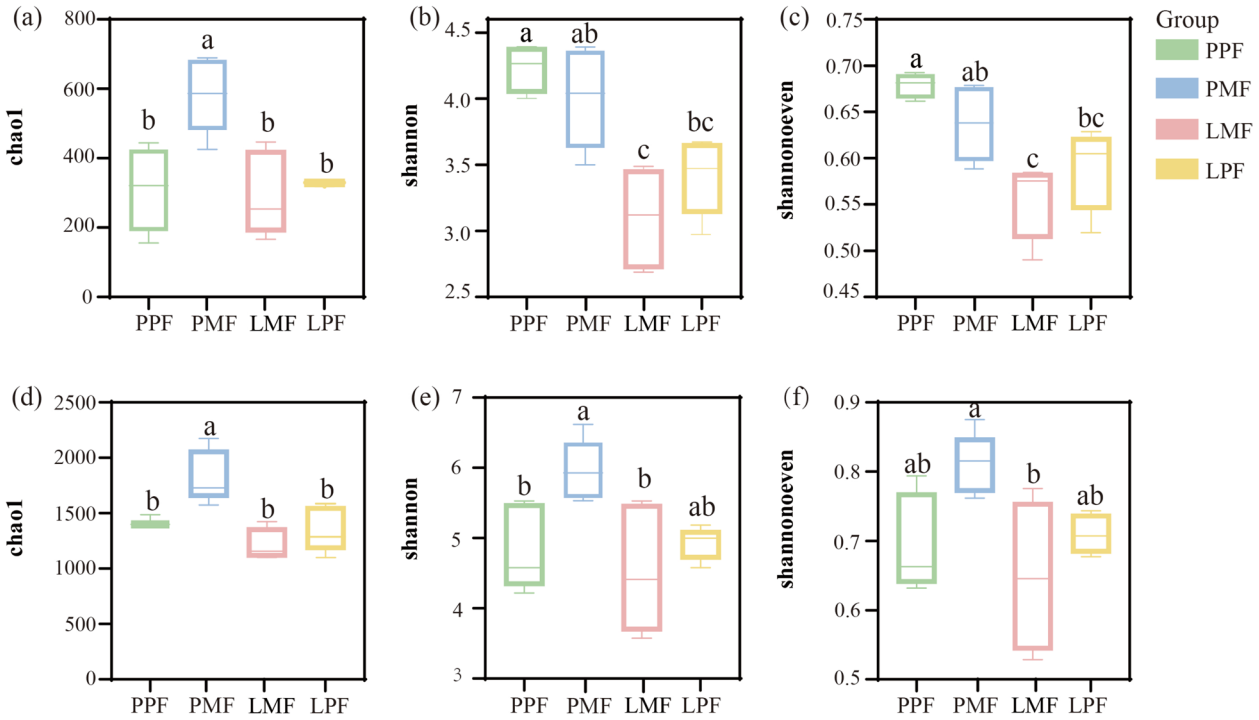

Fig. 2 The *α*-diversity of phyllosphere fungal community (a-c) and bacterial community (d-e) among different forests during forest succession. Different letters indicate significant differences ($P < 0.05$) between forest types. PPF: Pine needles in the pure pine forest, PMF: Pine needles in the mixed forest, LMF: *Liquidambar* leaves in the mixed forest, LPF: *Liquidambar* leaves in the pure *Liquidambar* forest

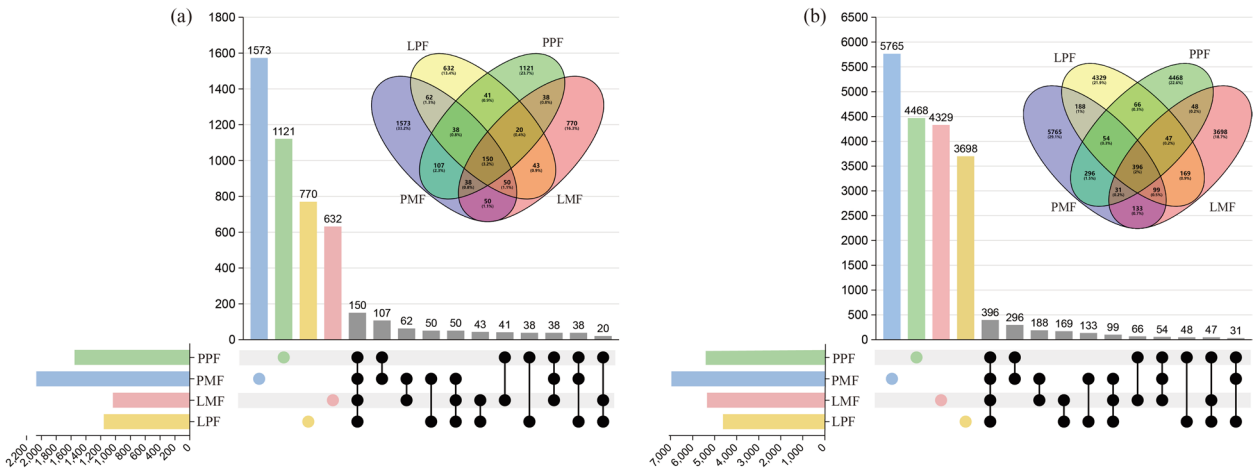

Fig. 3 The Venn diagrams showing the shared and unique ASVs of fungal community (a) and bacterial community (b) among different forests during forest succession. PPF: Pine needles in the pure pine forest, PMF: Pine needles in the mixed forest, LMF: *Liquidambar* leaves in the mixed forest, LPF: *Liquidambar* leaves in the pure *Liquidambar* forest

exhibited the highest abundance in the mixed forest (PMF) ($P < 0.05$) (Fig. 5d).

The obtained bacterial sequences were classified into 35 phyla and 674 genera. The dominant phylum was Proteobacteria (53.29%), followed by Actinobacteriota (15.11%), Firmicutes (10.82%), and Bacteroidota (4.11%) (Fig. 6a). The relative abundance of Proteobacteria in the needle/leaf increased gradually with forest succession. Deinococcota in the pine needles in the mixed forest (PMF) showed a significantly higher relative abundance than in the pure forest (PPF) ($P < 0.05$) (Fig. 6c). At genus level, *Massilia* was the most abundant (13.99%), followed

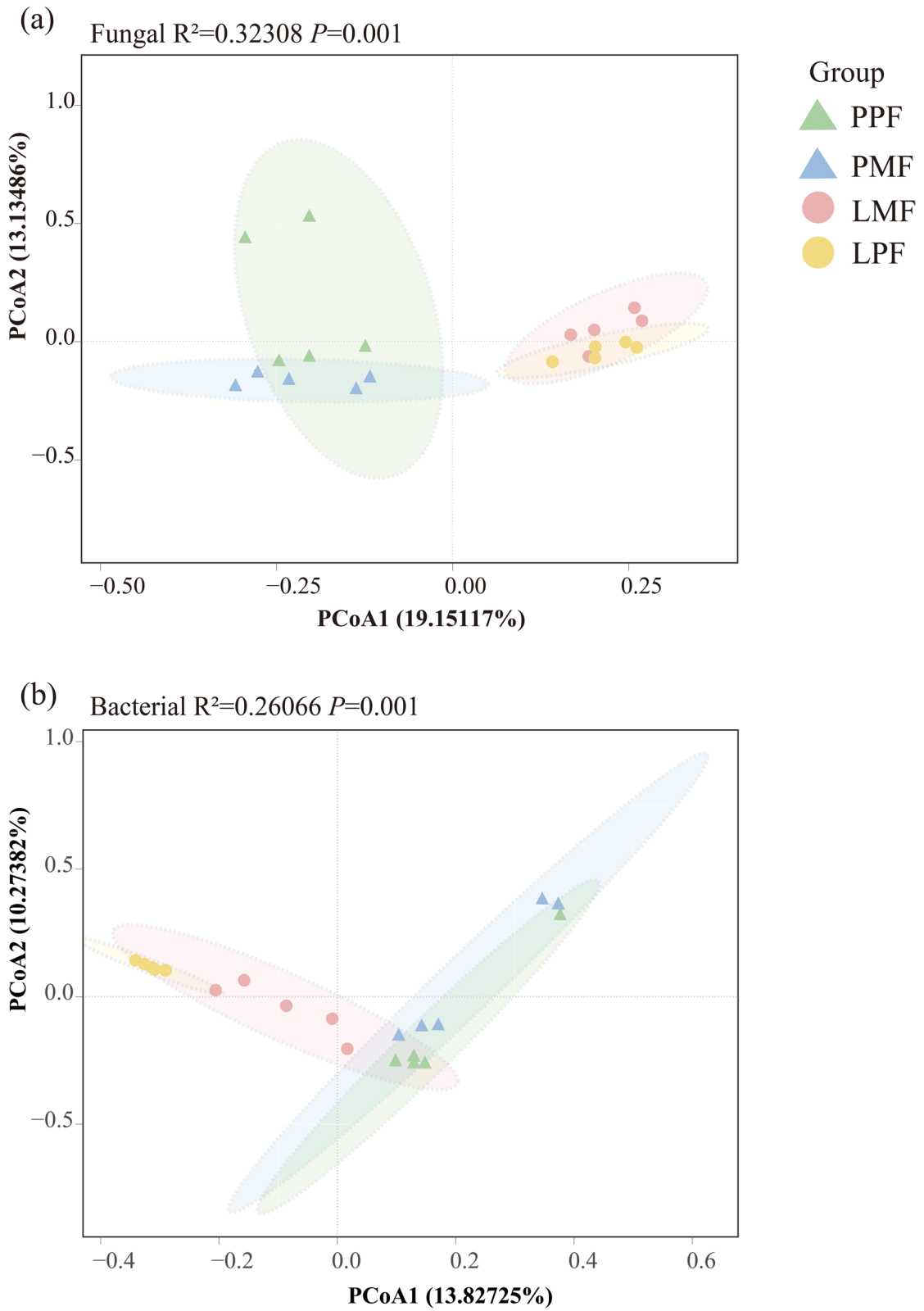

Fig. 4 Principal co-ordinates analysis (PCoA) showing the fungal (a) and bacterial (b) community structure in needle/leaf among different forests during forest succession. PPF: Pine needles in the pure pine forest, PMF: Pine needles in the mixed forest, LMF: *Liquidambar* leaves in the mixed forest, LPF: *Liquidambar* leaves in the pure *Liquidambar* forest.The shape of round (◯) and triangle (▲) representing leaf and needle respectively

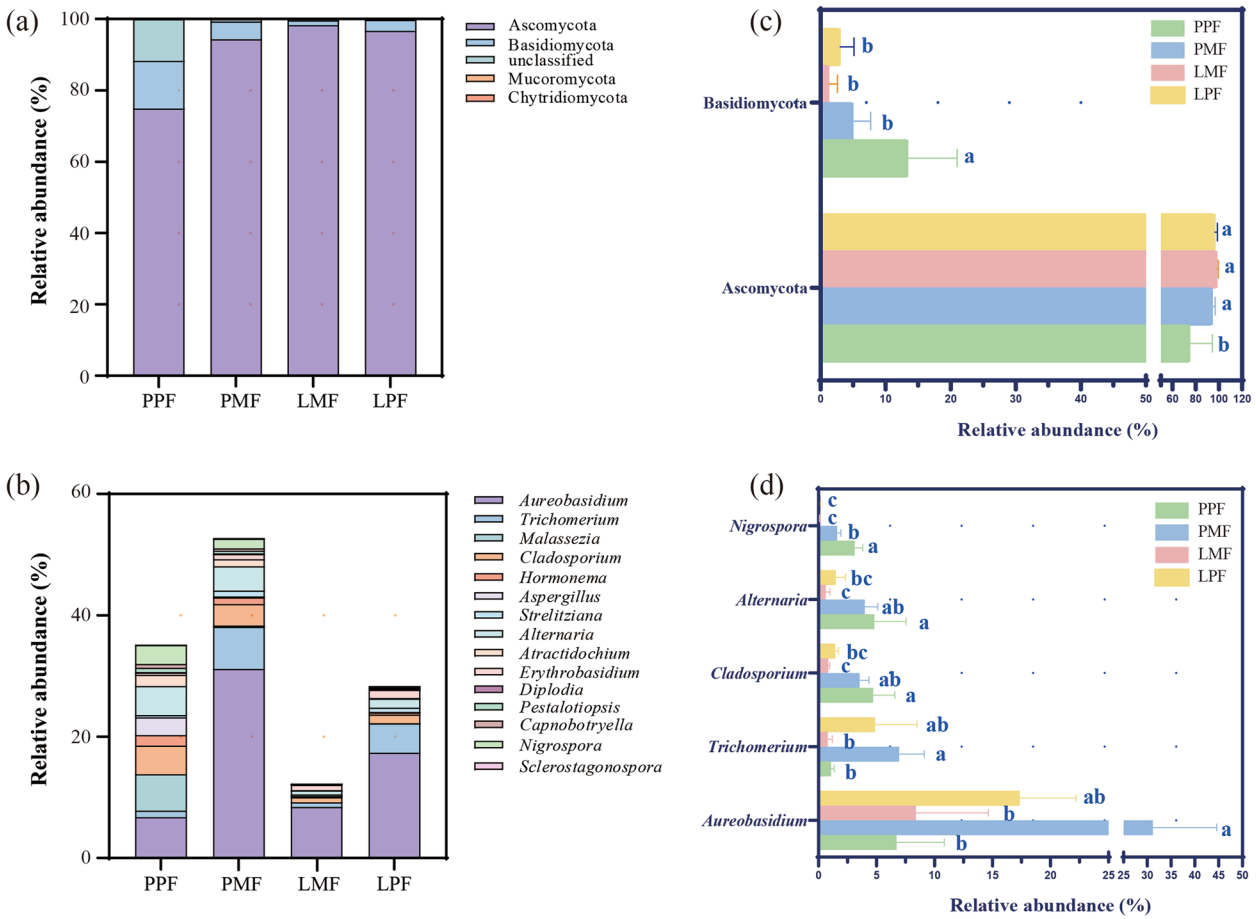

Fig. 5 The relative abundance of dominant fungal phyla in different forests (a), the top 15 most abundant fungal genera (b), and changes in the relative abundance of phyla (c) and genera (d). Different letters indicate significant differences ($P < 0.05$) between different forest types. PPF: Pine needles in the pure pine forest, PMF: Pine needles in the mixed forest, LMF: *Liquidambar* leaves in the mixed forest, LPF: *Liquidambar* leaves in the pure *Liquidambar* forest

by *Cutibacterium* (5.90%), *Methylobacterium-Methylorubrum* (4.26%), and *Staphylococcus* (3.84%) (Fig. 6b). The pine needle in the mixed forest had a higher abundance of *Methylobacterium-Methylorubrum* and *Amnibacterium* compared to the pure forest, while the abundance of *Serratia* in the pine needle in the pure forest was significantly higher than in the mixed forest ($P < 0.05$). Additionally, the abundance of *Massilia* and *Sphingomonas* in the *Liquidambar* leaves in the pure forest (LPF) was significantly higher than in the mixed forest (LMF) ($P < 0.05$) (Fig. 6d).

Phyllosphere microbial community co-occurrence networks

The co-occurrence network analysis revealed that the average degree of the fungal community networks for pine needles and *Liquidambar* leaves in pure pine forest, mixed pine/*Liquidambar* forest, and pure *Liquidambar* forest was 10.872, 13.168, 9.771, and 11.192, respectively. The number of nodes was 78, 113, 70, and 104; the number of edges was 848, 1488, 684, and 1164; and the clustering coefficient was 0.344, 0.613, 0.299, and 0.281 (Fig. 7a-d, Table S4). In the pine needles, the interaction among fungi was higher in the mixed forests, as evidenced by the higher number of nodes and edges, along with increased average degree and clustering coefficient compared to the pure forests. Conversely, this pattern was reversed in the fungal community of *Liquidambar* leaves. For bacterial community co-occurrence network, in both pine needles and *Liquidambar* leaves, the mixed forests exhibited higher node and edge counts, as well as higher average degree and clustering coefficient compared to pure forests, indicating increased bacterial complexity and stability in the mixed forests (Fig. 7e-h, Table S4).

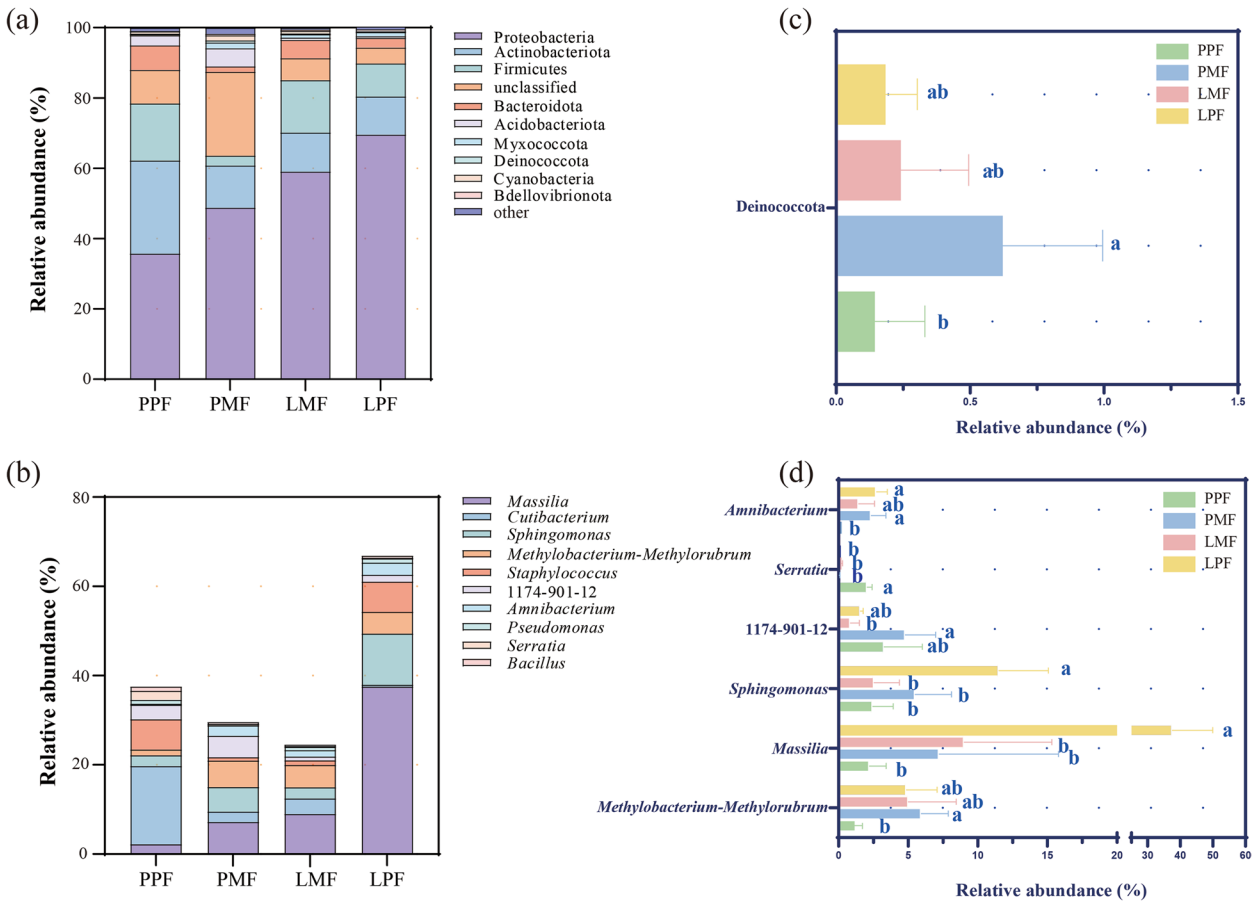

Fig. 6 The relative abundance of dominant bacterial phyla in different forests (a), the top 15 most abundant bacterial genera (b), and changes in the relative abundance of phyla (c) and genera (d). Different letters indicate significant differences ($P < 0.05$) between different forest types. PPF: Pine needles in the pure pine forest, PMF: Pine needles in the mixed forest, LMF: *Liquidambar* leaves in the mixed forest, LPF: *Liquidambar* leaves in the pure *Liquidambar* forest

Phyllosphere microbial community functional prediction

Analysis using FUNGuild classification revealed the Plant Pathogen guild (21.59%) was the most abundant fungal guild, followed by Undefined Saprotroph (21.14%), Animal Pathogen (18.06%), and Epiphyte (13.33%) (Fig. 8a). The abundance of Epiphyte in pine needles was significantly higher in mixed forests than in pure forests ($P < 0.05$) (Figure S4). FAPROTAX prediction identified chemoheterotrophy (24.07%) as the dominant bacterial guild, followed by aerobic chemoheterotrophy (17.85%) and fermentation (5.84%) (Figure S4). Methanotrophy and nitrogen fixation were more abundant in mixed forests, while fermentation, nitrate respiration, nitrate reduction, and nitrogen respiration were lower ($P < 0.05$).

Discussion

We investigated the response of phyllosphere microorganisms to changes in dominant tree species during forest succession. Phyllosphere microorganisms are known to originate from diverse sources, including the surrounding environment and "vertical inheritance" from previous plant generation [56]. This complex origin suggests that microbial community on leaf surfaces is highly dynamic, influenced by both current environmental conditions and historical and successional processes in the forest. Our findings indicate that microbial diversity and species richness in pine needles were significantly impacted by changes in tree species composition. Notably, the fungi and bacterial diversity, as well as species richness, were higher in pine needles in the mixed forests than in pure the pine forests. This increase in microbial diversity is known to enhance the ecosystem functions, stability, and resistance to disturbances [2]. In contrast, we observed no difference in the α-diversity of bacterial and fungal communities on the *Liquidambar* phyllosphere between mixed and pure *Liquidambar* forests. Given that *Liquidambar* is a deciduous tree and pine is a conifer tree, differences in leaf structure and life cycle

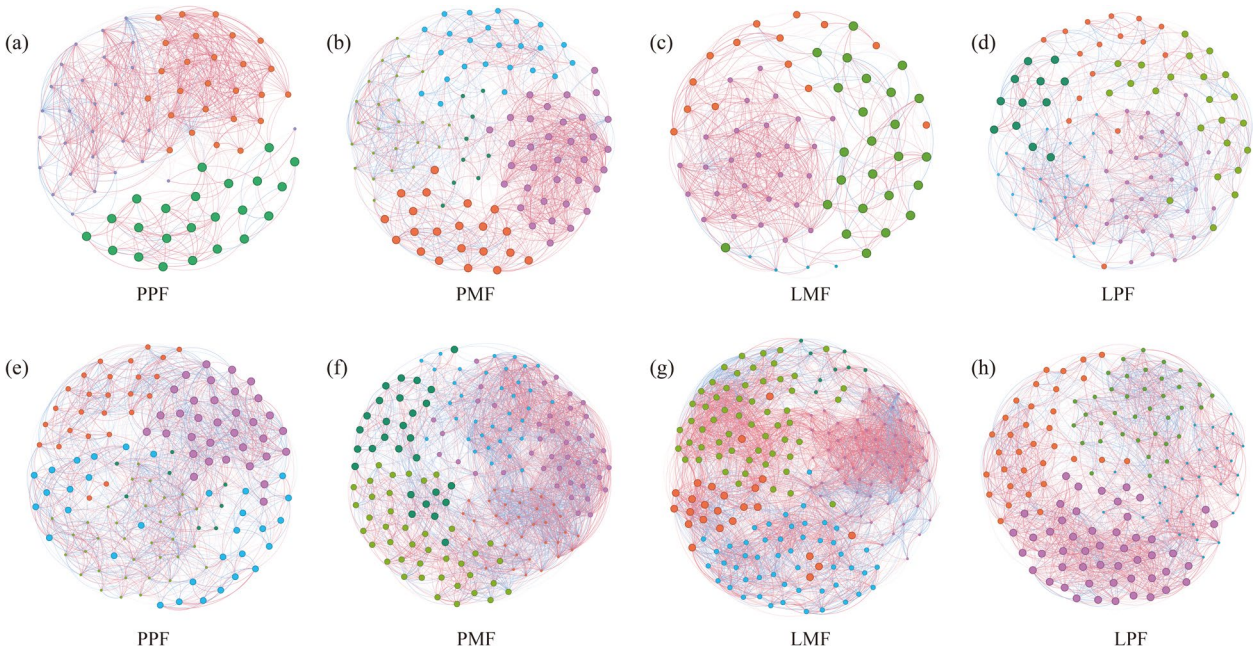

Fig. 7 Co-occurrence networks among fungal community (a-d) and bacterial community (e–h) among different forests during forest succession. Node color indicates species taxa from the same module in each network. Line color indicates positive (pink) and negative (blue) correlation coefficient. PPF: Pine needles in the pure pine forest, PMF: Pine needles in the mixed forest, LMF: *Liquidambar* leaves in the mixed forest, LPF: *Liquidambar* leaves in the pure *Liquidambar* forest

may contribute to the observed disparities in microbial colonization and diversity [11]. In the mixed forests, significant differences in microbial colonization and diversity have been observed between coniferous and broad-leaved trees. The ability of microbes to colonize and establish themselves in the phyllosphere varies not only between tree species but also within different parts of the same leaf [30]. Key factors influencing microbial colonization include leaf surface structure and chemical composition [28]. Research by [4] has shown that leaf traits, such as nutrient content, vary significantly among tree species, potentially leading to microbial distributions that depend on leaf type and forest structure. Consequently, phyllosphere microbial communities in coniferous trees, such as pine, may be more sensitive to changes in dominant tree species during forest succession.

The structure of phyllosphere microbial communities varied among different forest types. Fungal communities, in particular, exhibited distinct structures depending on forest types. The mixed forests provide a more diverse ecological niche and resources, supporting greater fungal diversity compared to pure forests [53]. These differences in habitats and nutrient availability contribute to unique fungal community composition [50]. However, the bacterial community structure showed no significant differences between the pure pine forests and mixed forests. Previous study has shown that vegetation type, carbon availability and nutrient availability have relatively minor effects on bacterial community structure [16]. This is consistent with Carper et al. [10], who found that local site conditions play a greater role in shaping bacterial community assembly on pine needles than host species identity explaining the relative stability of bacterial communities across different forest types.

In pure pine forests, the relative abundance of Basidiomycota in the fungal community is significantly higher compared to the mixed forests. Basidiomycota may be better adapted to the stable, relatively dry conditions of pine forests, where organic matter decomposition is slow, and nutrient availability is limited [18]. Conversely, Ascomycota is more abundant in the mixed forests, likely due to its ability to rapidly metabolize labile carbon sources [45]. For bacterial community, *Methylobacterium-Methylorubrum* and *Amnibacterium* were more abundant in the mixed forests, indicating that the diversified organic matter inputs in these forests promote the growth of carbon-utilizing bacteria [1, 29]. Methanol, a one-carbon substrate produced during leaf metabolic cycle, serves as a key resource for *Methylobacterium* species, which enhances their fitness during leaf colonization [58]. This indicates that the mixed forests, through the addition of *Liquidambar formosana*, may offer more organic material and moisture, favoring methanol- and carbon-utilizing bacteria [54]. The functional prediction further

supports this, showing significant differences in bacterial functional groups between pine needles in the pure and mixed forests. The mixed forest seems to enhance the abundance of microbes involved in methanotrophy and nitrogen fixation [7], reducing the reliance on anaerobic processes such as fermentation and nitrate respiration [22]. However, these changes were not observed in the pure and mixed *Liquidambar* forests. *Liquidambar* may have already developed tightly integrated microbial communities in pure forests, with efficient carbon and nitrogen resource utilization [25], thus not requiring significant metabolic adjustments in the mixed forests.

Our study also revealed that changes in different dominant tree species during forest succession affected the potential pathogens. In pure pine forests, the most abundant genera- *Alternaria*, *Cladosporium*, and *Nigrospora*, have been reported as potential pathogens [15, 49, 60]. The reduced plant diversity in these forests likely facilitates pathogens' spread [27], leading to a higher abundance of these potential pathogens. Interestingly, *Aureobasidium* was more abundant on the pine needles in the mixed forest, a genus that has attracted attention for its remarkable ability to alleviate plant diseases, making it a promising candidate for biological control applications [21]. Species of *Aureobasidium* are known to produce a variety of bioactive compounds, including hydrolytic enzymes and antifungal metabolites, which can suppress the growth of pathogenic fungi [14]. This suggests that *Aureobasidium* may play a beneficial role in maintaining plant health and ecosystem balance in the mixed forests.

During the isolation and cultivation of phyllosphere microbes, we identified several potential biocontrol agents and pathogens. Many of the fungi isolated belong to genera known to include plant pathogens, such as *Fusarium* [59, 61, 62], *Nigrospora* [43], *Alternaria* [57], *Colletotrichum* [35]. and *Phyllosticta* [59, 61, 62]. These genera are well-documented for their pathogenic potential in various plant species. These pathogens exhibit a quiescent phase, common in Ascomycetes pathogen, where they remain dormant within the host before switching to active phase [38]. High-throughput sequencing results showed that Ascomycetes were more abundant in the mixed forests, least abundant in pure pine forests, and relatively consistent in the *Liquidambar* leaves. This aligns with our isolation and cultivation findings, indicating that forest types influence the isolation rate of potential pathogens. For example, *Fusarium* had a higher isolation rate in pine needles in the pure forests, whereas *Alternaria* was dominant in the mixed forests. On *Liquidambar* leaves, *Colletotrichum* was dominant in the mixed forests, while *Phyllosticta* was more common in the pure forests. These shifts may result from differences in nutrient inputs and environmental conditions, which affect microbial competition and dominance.

Interestingly, we also isolated *Paraconiothyrium*, a potential biocontrol agent [46], from pure *Liquidambar* forests. This genus is recognized for its biocontrol potential and production of antibiotic compounds [48], has shown antagonistic activity against *Colletotrichum* spp. [5]. Notably, the isolation rate of *Colletotrichum* was significantly lower in the pure *Liquidambar* forests, possibly indicating a biocontrol effect by *Paraconiothyrium*. Further research is needed to confirm its role in controlling phyllosphere disease in *Liquidambar* tree. In addition to fungi, we identified several bacterial genera with potential biocontrol properties. *Serratia*, particularly *Serratia marcescens*, was more frequently isolated from pine needles, consistent with high-throughput sequencing data. This genus is known for its insecticidal activity and pathogenicity against *Bursaphelenchus xylophilus* [19]. *Bacillus* spp., widely recognized for their biocontrol abilities [23] and ability to induce systemic resistance in plants [13], were more abundant in the phyllosphere of *Liquidambar* than in pine needles, with the lowest abundance in pure pine forests. However, this finding contradicts the high-throughput sequencing results, potentially due to the thicker leaf cuticle, higher resin, and phenolic compounds in pine needles, which could suppress microbial activity during cultivation.

Conclusions

The introduction of broadleaf trees in pine needle phyllospheres increased microbial diversity and enhanced nutrient-related microorganisms, especially those involved in carbon use and nutrient cycling. In contrast, *Liquidambar* phyllospheres showed no change in microbial diversity between pure and mixed forests, highlighting the differing impacts of tree species on conifer versus broadleaf phyllosphere microbes. Broadleaf introduction also altered pathogen abundance, with *Fusarium* dominating in pure pine forests and *Alternaria* in mixed forests. Similarly, *Colletotrichum* prevailed in mixed forest *Liquidambar* leaves, while *Phyllosticta* was more common in pure forests. High-throughput sequencing revealed lower pathogen abundance and more ASVs in mixed forests, suggesting greater microbial specialization. Co-occurrence network analysis showed more complex, stable microbial interactions in mixed forests, indicating enhanced microbial diversity and function. These findings emphasize that mixed forests promote phyllosphere microbial diversity and resilience, with dominant tree species shaping microbial community structure.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-025-03905-9.

Supplementary Material 1.

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Authors' contributions
Z.-W.G.: Data curation, Formal analysis, Investigation, Visualization, Writing-original draft, Writing review & editing. Y.-L.L.: Data curation, Formal analysis, Z.-N.X. and L.C.: Investigation, Visualization, methodology. N.L. and Y.-C.-Z. L.: Investigation, Validation, methodology. W.-J.M.: Data curation, Methodology, Visualization, Writing-review & editing. H.S. and L.H.: Conceptualization, Funding acquisition, Project administration, Supervision, Writing-review & editing. All authors have read and agreed to the published version of the manuscript.

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Data availability
The datasets generated during the current study are available in the National Center for Biotechnology Information (NCBI) repository under the Accession Number: PRJNA1170359

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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