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Effects of Microbial Communities on Elevational Gradient Adaptation Strategies of *Pinus yunnanensis* Franch. and *Pinus densata* Mast. in a Mixed Zone

Dejin Mu [1,2], Junrong Tang [1,2], Nianhui Cai [1,2], Shi Chen [1,2], Yingnian He [1,2], Zijun Deng [2], Yi Yang [2], Dan Yang [2], Yulan Xu [1,2] and Lin Chen [1,2,*]

[1] Key Laboratory of National Forestry and Grassland Administration on Biodiversity Conservation in Southwest China, Southwest Forestry University, Kunming 650224, China
[2] College of Forestry, Southwest Forestry University, Kunming 650224, China
* Correspondence: linchen@swfu.edu.cn

Abstract: *Pinus densata* Mast. is considered a homoploid hybrid species that originated from the putative parent species *Pinus tabuliformis* Carr. and *Pinus yunnanensis* Franch., but the mechanism of the adaptive differentiation of *P. densata* and its parents in native habitats has not been reported. Therefore, the overlapping distribution areas between *P. densata* and *P. yunnanensis* in the heart of the Hengduan Mountains were chosen. The adaptive differentiation mechanism of the homoploid hybrids and their parents with respect to the elevational gradient was studied based on the morphological features and the different strategies of recruiting endophytic microbial communities from the rhizosphere soil. The results showed that (1) the height and diameter at breast height were the greatest at 2600 m and 2900 m, and from 2700 m to 2900 m, three-needle pines (*P. yunnanensis*-like type) transitioned into two-needle pines (*P. densata*-like type). (2) The recruitment of rhizosphere microbial communities was driven by the C, N, P and pH values which showed significant elevation features. (3) There was a significant difference in the recruitment strategies of endophytes between the *P. yunnanensis*-like type and *P. densata*-like type. *Pinus densata* mainly reduced the recruitment of Mucoromycota (fungi) and increased the recruitment of Proteobacteria (bacteria), which may be related to environmental adaptability, quorum sensing and the metabolism of auxiliary factors and vitamins at high elevations. (4) The root endophytic microbiome was enriched in the rare groups from the rhizosphere soil microbial pool. The results of this study provide new insights and new ideas for environmental adaptability and differentiation in homoploid hybrid speciation.

Keywords: adaptation strategy; elevational gradient; homoploid hybrid speciation; *Pinus densata*; *Pinus yunnanensis*

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1. Introduction

Mountain ecosystems are excellent systems to study microbial communities because many climate and soil variables change within a relatively small distance [1]. Elevational gradients usually alter soil properties, including pH, organic matter content, water content, total nitrogen, phosphorus, potassium, available nitrogen, phosphorus and potassium, which differentiate niches [2,3]. Soil microbial elevational diversity patterns have been extensively studied in mountain ecosystems [4]. Soil pH is considered to have the strongest direct impact on the diversity and community structure of soil microorganisms [5–8]. On a relatively large geographical scale, soil characteristics, soil quality, season, climate, elevation and other factors change the soil microbial pool, thus affecting the recruitment of root endophytes [9,10]. However, the elevational gradients of mountain ecosystems, rapidly alter both biotic and abiotic environments, are an ideal system in which to examine how plant hosts recruit microbial symbionts with changes in environmental conditions.

The Qinghai–Tibet Plateau (QTP) *sensu lato* (*sl*) comprises the plateau, the Himalayas and the Hengduan Mountains, is known as the 'Roof of the World', has the richest species diversity in the Northern Hemisphere and exhibits a high level of endemism [11]. Conifers, which constitute more than 39% of the world's forests, are prominent components of the QTP *sl* [12]. It remains unknown why the mountainous areas at middle latitudes contain the most coniferous species [13]. The QTP *sl* is an important site for species diversity and speciation of conifers. Alpine *Pinus densata* (Pinaceae) is one of the prominent species on the QTP *sl*, with a high drought stress tolerance and adaptation to high elevation habitats [11]. *Pinus densata* Mast. is considered a type species of homoploid hybrid speciation (HHS) that originated from the putative parent species *Pinus tabuliformis* Carr. and *Pinus yunnanensis* Franch. [14]. Evidence from molecular genetics, for example, chloroplast DNA polymorphism [15], allozyme [16–18], simultaneous DNA probes of the 18S rRNA gene and the 5S rRNA gene [19], the paternally inherited chloroplast rbcL gene and maternally inherited mitochondrial nad1 fragments [20,21], demonstrates that *P. densata* is a natural homoploid hybrid and that ecological selection may have played a role in its speciation. Approximately 55 million years ago, the Tibetan Plateau supposedly rose as the Indian continent smashed into Eurasia [22], and *P. densata* were separated from their parents. As the QTP *sl* rose, the hybrid was mainly distributed at high elevations on the Tibetan Plateau, mainly in the Himalayas, and extended to the Hengduan Mountains. Its two parents are currently mainly distributed in low-elevation Yunnan Province and northern China, with distinct niches [11]. At present, *P. densata* and its parents *P. tabuliformis* and *P. yunnanensis* are unlikely to coexist in a small geographical area, and in the overlapping areas it is difficult to find even *P. densata* and one of its parents [23]. There may be a small number of coexisting communities of *P. densata* and *P. yunnanensis* in the heart of the Hengduan Mountains, Yunnan Province [23]. A report showed weak genetic barriers among *P. densata* and its parental species, as revealed by crossing experiments [24]. Therefore, the differences between the microbial, chemical and morphological characteristics of *P. densata* and one of its parents in a mountainous system will be helpful in revealing the niche differentiation of different species of pines from the perspective of microenvironment adaptability. Additionally, the identification and isolation of beneficial microorganisms will be beneficial to enhance the drought and cold resistance of forest seedlings.

The microbial communities of plants that promote growth [25], nutrient uptake [26], stress tolerance [27] and resistance to pathogens [28] play many ecological roles. However, only a few studies have focused on plant microbial community assembly along elevational gradients. For example, in Baima Snow Mountain (28°35′–27°24′ N, 98°55′–99°24′ E, Deqin County, China), the heterogeneity of the richness and community composition of ectomycorrhizal fungi was stronger among different elevational zones than across different seasons [29]. Additionally, Zi et al., (2020) reported that multiple soil factors, especially the content of soil ammonium nitrogen (NH_4^+-N), were the key factors that shaped the bacterial community of ancient wild tea plant roots along elevational gradients [30]. A *Pinus densiflora* forest study showed that elevation negatively affected the species richness of root-inhabiting fungi but did not influence that of soil-inhabiting fungi [31]. However, the recruitment strategy of the symbiotic microbial communities and the functions of the microorganisms in niche differentiation associated with elevational gradients on the scale of a mountain have not been reported.

Here, we can infer that *P. densata* and *P. yunnanensis* can coexist in relatively narrow ranges and that the pines in this area exhibit the composite features of *P. densata* and *P. yunnanensis*. This mixed zone of *P. densata* and *P. yunnanensis* provides a good sample plot for the study of niche differentiation of *P. densata* and *P. yunnanensis* in microhabitats. Therefore, we analyzed the rhizosphere and endophytic microbiomes of *P. densata* and *P. yunnanensis* using next-generation sequencing and measured the morphological features of pines. We hypothesized that (H1) pine trees recruit specific microorganisms from rhizosphere soil as endophytes. Endophytes are related to immunity and metabolism, and rhizosphere microbes are related to the interaction between microbes and the host.

(H2) *P. densata* has both parental characteristics and developed typical characteristics of high elevation adaptation. *P. densata* grows well at high elevations, and its needles show the characteristics of water holding and drought resistance to adapt to high elevations. *P. densata* roots recruit a series of drought-resistant, enhanced metabolism and disease-resistant microorganisms to enhance their adaptability to high elevations.

2. Materials and Methods

2.1. Site Description and Sample Processing

The samples were collected in Xianggelila City, Yunnan Province, 4 August 2021. August is the most prosperous season for plant growth in Yunnan Province. The sampling sites were located in the heart of the Hengduan Mountains, Xianggelila City, Yunnan Province, China (27°45′44.42″ N, 100°5′38.91″ E). In this study, sampling sites were chosen every 200 m over an elevation gradient, for a total of six representative elevation gradients: 2300 m, 2500 m, 2700 m, 2900 m, 3100 m and 3300 m. According to the flora of China, *P. yunnanensis* and *P. densata* have overlapping areas at 2600 m elevation [32]; therefore, 2600 m was also selected, and a total of seven representative elevations were studied (Supplementary Table S1). Within each elevational band, four plots (20 × 20 m) were randomly selected, separated by at least 100 m from each other (Supplementary Figure S1, Supplementary Table S1). Major plant types were investigated in each plot, and the coverage of major plant components was estimated (Supplementary Table S2). The diameter at breast height (DBH) and tree height of 12 pine trees with DBH greater than 5 cm were randomly selected and recorded in each plot (trees with DBH $\leq$ 5 cm were considered saplings, Supplementary Figure S2). The DBHs were divided into different diameter classes, such as the 6 cm class (5.0 cm $\leq$ DBH $\leq$ 6.9 cm) and the 8 cm class (7.0 cm $\leq$ DBH $\leq$ 8.9 cm).

According to the difficulty of sampling and the planting time, two pines (DBH 6–10 cm) growing for 10–15 years in each plot were randomly selected, and the morphological features of the needles, chemical properties and microbial communities of the rhizosphere soil and roots were then collected. The mature needles of 8 pine trees at each elevation were collected and transported to the laboratory. Fifty clusters of each pine were randomly selected, and the numbers of two- and three-needle clusters, needle length, needle diameter and needle cluster diameter were measured.

For collection of the rhizosphere soil, after removal of the weeds and topsoil in the sampling spots with spades, the rhizosphere soil samples were taken from the root-rich areas in the 10–30 cm depth range from two opposite directions of the pine trees. After removal of loose soil from root surfaces and gravel from root crevices, the soil from root surfaces was gently collected using a 2 mL frozen tube (Supplementary Figure S2). After the rhizosphere soil was collected, the fine roots of the pine trees were cut with scissors sterilized with 75% alcohol and placed in a new 2 mL frozen tube. The 25 g bulk soil samples (10–30 cm) without vegetation were selected and mixed thoroughly from each plot. The bulk soil from four plots within one elevation was then mixed thoroughly. All samples were stored on dry ice. After transportation to the laboratory, the frozen tube with rhizosphere soil was stored at −80 °C. In addition, the fine roots were cleaned and sonicated for 10 min in an ultrasonic bath cleaner (Branson, CT, USA) [33]. The roots were surface disinfected for 5 min in 0.5% NaClO followed by 5 min in 75% CH_3CH_2OH and washed with sterile water. The washed water from the final washing was inoculated on tryptic soy agar (TSA) medium and potato dextrose agar (PDA) medium to verify successful surface sterilization.

2.2. Soil and Root Chemical Properties

In addition, the roots and loose soil around the roots were collected from the 8 pine trees in the abovementioned plots and transported to the laboratory. The soil and roots were dried at 80 °C to a constant weight. The samples were ground to powder by an MM400 ball mill (Retsch, Shanghai, China), and then a 0.2 mm sieve was used to filter the samples. Then, organic carbon, total nitrogen, total phosphorus and soil pH were

measured. Briefly, the organic carbon of the soil and roots was measured by the $K_2Cr_2O_7$-H_2SO_4 volumetric method [34]. Total nitrogen transformed as NH_4^+ was analyzed using a Smartchem200 after digestion by concentrated H_2O_2-H_2SO_4, and the total phosphorus content was also measured by a SmartChem200 automatic chemical analyzer (AMS-Westco, Guidonia, Italy) [35,36]. The pH value of the soil was measured by a pHS-2F pH meter (INESA Scientific Instrument Co., Ltd., Shanghai, China).

2.3. DNA Isolation and Sequencing

For high-throughput sequencing, the total DNA of 200 mg of sterilized fine roots was extracted using the CTAB method for each root sample [37], and 0.5 g of soil was extracted with a commercial DNA extraction kit for each rhizosphere soil sample (FastDNA® SPIN Kit for Soil, MpBio, Shanghai, China). DNA quality was monitored by 0.8% agarose gel electrophoresis, and extracted DNA was diluted to a concentration of 1 ng/μL and stored at −20 °C until further processing. The diluted DNA was used as a template for PCR amplification. For bacterial diversity analysis, the V5-V7 variable regions of 16S rRNA genes were amplified with nested PCR using the first and second pair of PCR primers 799F-1392R and 799F-1193R, respectively [38,39]. For fungal diversity analysis, the ITS1 region was amplified with the universal primers ITS1F-ITS2R [40]. Twenty-microliter PCRs were run containing 4 μL 5×FastPfu Buffer, 2 μL 2.5 mM dNTPs, 0.8 μL forward primer (5 μM), 0.8 μL reverse primer (5 μM), 0.4 μL FastPfu Polymerase, 0.2 μL BSA, and 10 ng template DNA, and molecular-grade water was added to 20 μL. The V5-V7 variable regions of the 16S rRNA gene reaction were assessed in two rounds: the first round consisted of 3 min of initial denaturation at 95 °C, 27 cycles of 30 s at 95 °C, 30 s at 55 °C and 45 s at 72 °C, with a final 10 min elongation at 72 °C, and the second round consisted of 3 min of initial denaturation at 95 °C, 13 cycles of 30 s at 95 °C, 30 s at 55 °C and 45 s at 72 °C, with a final 10 min elongation at 72 °C. The ITS1 region of fungi was subjected to 3 min of initial denaturation at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at 55 °C and 45 s at 72 °C, with a final 10 min elongation at 72 °C. Then, library construction and sequencing were performed using the Illumina MiSeq PE250 platform by Shanghai Majorbio Biopharm Technology Co., Ltd. (Shanghai, China). Raw sequences have been deposited in the Sequence Read Archive under Bioproject PRJNA870036.

2.4. Bioinformatic Analysis

Raw sequencing data were in the FASTQ format. Paired-end reads were quality-filtered by Trimmomatic and merged by FLASH software v1.2.11 [41]. We also removed low-quality sequences with average quality scores below 20 using a 50-bp sliding window trimming approach. The merging parameters were 10 bp minimal overlapping, 200 bp maximum overlapping and a 20% maximum mismatch rate. Sequences of each sample were separated according to barcodes (exactly matching), and reads containing ambiguous bases were removed. Then, reads with chimeras were detected and removed. These bioinformatic analyses were conducted using Quantitative Insights Into Microbial Ecology (QIIME v1.9.1) pipeline [42]. Clean reads were subjected to primer sequence removal and clustered to generate OTUs using UPARSE software v11 with a 97% similarity cutoff [43]. The representative read of each OTU was selected using the QIIME package. All representative reads were taxonomically identified against the Silva (Version 138) and Unite (Version 8.0) databases for bacteria and fungi using the RDP classifier (confidence threshold of 70%).

2.5. Data and Statistical Analyses

All reads obtained from 119 samples (8 rhizosphere soils, 8 root samples and one bulk soil sample from one elevation × 7 elevations) were divided into 25,705 bacterial OTUs and 7018 fungal OTUs. We then subsampled the remaining 45,860 bacterial reads and 96,322 fungal reads (minimized read sample) using MOTHUR v1.35.1 “sub.sample”. All data were analyzed according to data descriptions based on subsampled data. The rarefaction curve for each sample was calculated using MOTHUR v1.35.1 “rarefaction.single”

and plotted using GraphPad Prism 9 (GraphPad Software, Inc., San Diego, CA, USA). Raincloud plots were generated according to Allen et al., (2019) using R v3.4.3 to investigate the variations in the morphological features with elevational gradients [44]. Linear regression was performed using the R v3.4.3 "stats" package to investigate the variation in the relative abundance of genera and the soil properties and plant nutrient content across the elevational gradient [45]. Nonmetric multidimensional scaling (NMDS) based on Bray–Curtis dissimilarity was performed using the R v3.4.3 "vegan" package to investigate the differences among groups [46]. Analysis of similarities (ANOSIM) was used to test the statistically significant differences based on OTU richness using the R package "vegan" [46]. Alpha diversity was calculated using the R "vegan" package and was plotted using GraphPad Prism 9 [46]. Additionally, the Bray–Curtis dissimilarities of samples among elevations and at the same elevation were calculated using the R "vegan" package [46]. The Kruskal–Wallis test (for three and more groups) and the Mann–Whitney U test (for two groups) were performed using the R v3.4.3 "stats" package to investigate the significant differences among groups [45]. Functional prediction for fungi and bacteria was achieved using PICRUSt2 [47]. Other diagrams were plotted in GraphPad Prism 9.

3. Results

3.1. Morphological Features

First, the DBH and height of 48 trees at each elevational site were measured. At 2600 m and 2900 m, there were two peaks for DBH and tree height (Figure 1). This revealed that 2600 m and 2900 m are the optimum elevations for the growth of pine trees. The DBH and tree height at 2300 m, 2500 m, 3100 m and 3300 m were significantly lower than those at 2600 m to 2900 m (p = 0.000, Mann–Whitney U test, Figure 1). The greatest DBH and tree height occurred at 2900 m.

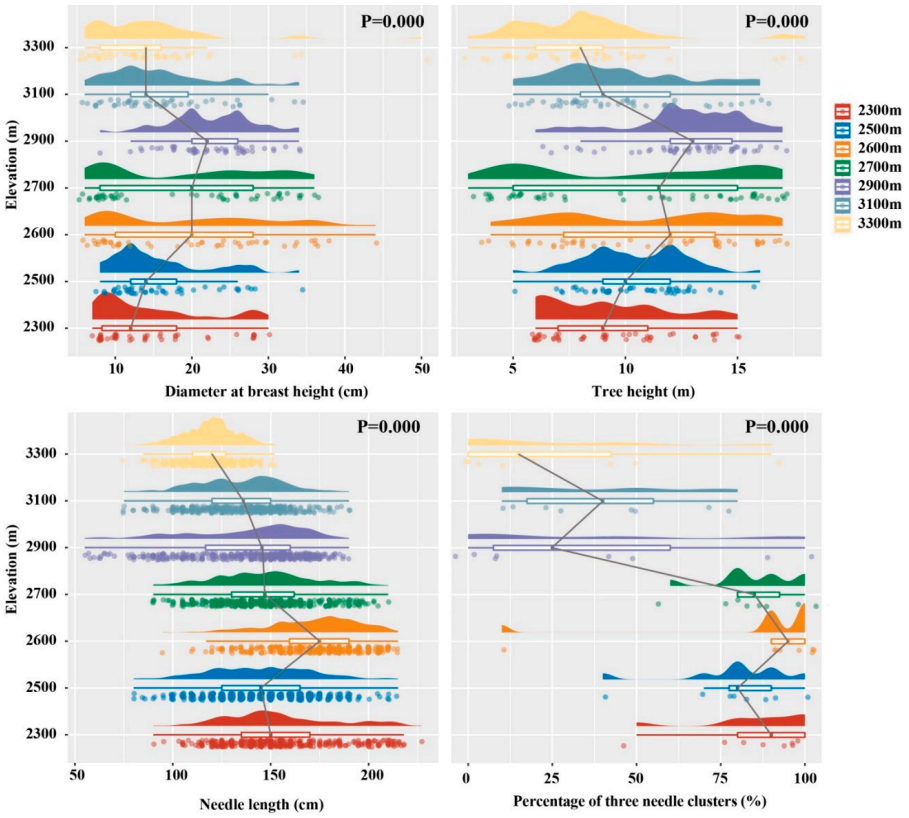

Figure 1. Raincloud plot showing morphological features at different elevations. The half-violin diagram (cloud) shows the kernel density of the data distribution, and the scatter diagram (rain) shows the degree of dispersion. The raincloud plot also includes a box plot (umbrella) and lines (thunder) that link the medians of different groups.

Needle morphological features show relative species stability and can be used to identify coniferous species [48]. The ratios of the three needle clusters from 2300 m to 2700 m were significantly higher than those from 2900 m to 3300 m (p = 0.000, Mann–Whitney U test, Figure 1). The ratios of the three-needle clusters sharply decreased from 2700 m to 2900 m, indicating that the needle morphology switched from *P. yunnanensis*-like to *P. densata*-like (Supplementary Figure S3). Other morphological features were consistent with the switch from the *P. yunnanensis*-like type to the *P. densata*-like type. For example, needle length was longest at 2600 m and then decreased with elevation, and needle diameter and needle cluster diameter increased with elevation (Supplementary Figure S4).

3.2. Soil Chemical Properties and Plant Nutrient Contents

Linear regression was used to investigate the relationship between soil characteristics and the root nutrient contents along the elevational gradient (Figure 2). The organic carbon content of the soil and roots decreased with increasing elevation, and the organic carbon content of the roots had significant negative relationships with elevation ($p < 0.05$). There was no significant difference in the total nitrogen content of roots at different elevations, but there was a significant positive correlation between soil total nitrogen and elevation ($p < 0.05$). The total phosphorus content of the soil increased with increasing elevation, whereas the total phosphorus content of the roots decreased with increasing elevation. The total phosphorus contents of the soil and roots were significantly related to elevation ($p < 0.05$). Finally, the pH value had a significant positive correlation with elevation ($p < 0.05$).

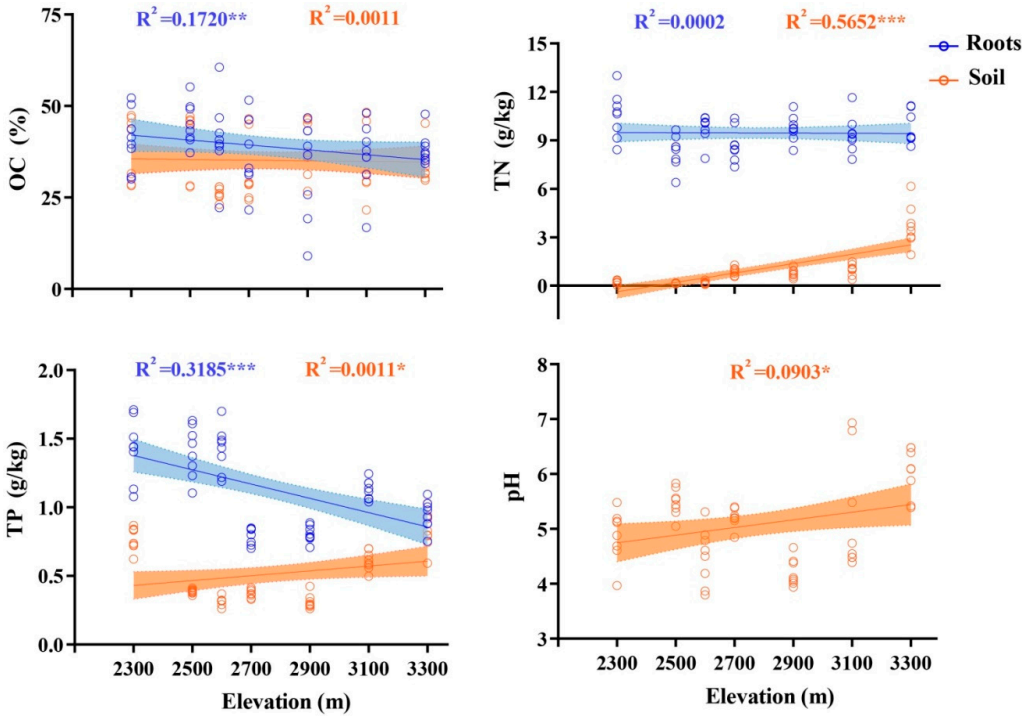

Figure 2. Variation in soil and root chemical properties with elevational gradient. The line indicates the linear regression fit, and the shaded band represents the 95% confidence level. R^2 was employed to determine the models that fit the whole elevational gradient. OC: organic carbon, TN: total nitrogen, TP: total phosphorus. Significant difference: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

3.3. Soil Chemical Properties and Plant Nutrient Contents

In total, 119 samples from seven elevations were sequenced and all samples were first subsampled to the minimized read sample, i.e., 96,322 reads for fungi and 45,860 reads for bacteria. The rarefaction curve reached saturation at the minimized number of reads (Supplementary Figure S5).

For fungi, there were two dominant phyla, i.e., Ascomycota (48.87%) and Basidiomycota (47.02%). Dominant genera included *Geminibasidium* (9.41%), *Sebacina* (8.60%) and *Russula* (8.50%) (Supplementary Figure S6). For bacteria, three known phyla accounted for more than 8%, i.e., Proteobacteria (41.58%), Actinobacteria (36.57%) and Firmicutes (8.15%). Thirteen genera accounted for more than 1% and the top five known genera were *Mycobacterium* (10.41%), *Bradyrhizobium* (8.97%), *Candidatus Phytoplasma* (4.67%), *Rhodoplanes* (1.99%) and *Acidibacter* (1.80%) (Supplementary Figure S6).

3.4. Differentiation of the Pine Root Microbiome at Different Elevations

According to the sequencing results, Ascomycota and Basidiomycota were the predominant phyla in roots, accounting for 58.95% and 39.08%, respectively (Supplementary Figure S6). There were five genera accounting for more than 5%, i.e., unclassified genera from Helotiales (15.60%), *Russula* (8.46%), *Sebacina* (8.44%) and unclassified genera from Hysteriales (7.72%). Additionally, Ascomycota and Basidiomycota were the predominant phyla in rhizosphere soil, accounting for 39.70% and 54.32%, respectively (Supplementary Figure S6). *Geminibasidium* (17.51%), *Sebacina* (8.77%), *Russula* (8.66%), *Penicillium* (6.53%), *Oidiodendron* (5.82%) and *Pseudeurotium* (5.36%) were the predominant genera.

For bacteria, Proteobacteria (40.19%), Actinobacteriota (37.53%) and Firmicutes (14.16%) were the predominant phyla in roots. *Mycobacterium* (11.33%), *Candidatus Phytoplasma* (9.92%) and *Bradyrhizobium* (9.92%) were the predominant genera. Proteobacteria and Actinobacteriota were the predominant phyla in roots, accounting for 42.74% and 35.68%, respectively (Supplementary Figure S6). There were three genera accounting for more than 5%, i.e., *Bradyrhizobium* (9.91%), *Mycobacterium* (9.58%) and an unclassified genus from Xanthobacteraceae (8.53%). In general, the variations in microbes in roots and rhizosphere soil were distinct at different elevations.

PCoA was used to evaluate the microbial communities recruited across the elevational gradient. Both rhizosphere soil and root microbial communities were significantly different along the elevational gradient (Figure 3, $p < 0.05$). The distribution of bulk soil groups on PC1 was wide for both fungi and bacteria (Figure 3, Supplementary Figure S7). The degree of overlap of bacterial microbial communities was higher than that of the fungal microbial community in both rhizosphere soil and root endophytes (Figure 3). The elevational changes in microbial communities had similar characteristics; that is, the microbial groups at a certain elevation overlapped more with those at a similar elevation. However, there were a few exceptions; for example, the root bacterial community at 2300 m overlapped more with that at high elevations (3100 m and 3300 m).

Furthermore, we determined the linear regression between elevation and the dominant genera (>1.0%) in roots and soil. There were 15 bacterial genera that had significant variation among the elevational sites. *Mycobacterium* and two other genera in roots, *Burkholderia-Caballeronia-Paraburkholderia* and six other genera in rhizosphere soil decreased with elevation, whereas unclassified Xanthobacteraceae, *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* in roots, *Mycobacterium* and two other genera in rhizosphere soil increased with elevation (Figure 4). Similarly, 11 fungal genera had significant variation among the elevational sites (Figure 4). There was partial consistency between OTUs and genera. For example, bacterial OTU1587 (*Mycobacterium*) and OTU9758 (*Rhodoplanes*) in roots decreased with elevation, whereas OTU13052 (*Mycobacterium*) in rhizosphere soil increased with elevation (Supplementary Figure S8).

3.5. Strategies for the Differential Recruitment of Root Endophytes by Related Pines

According to morphological features, the pines were divided into two types: the *P. yunnanensis*-like type (2300–2700 m) and the *P. densata*-like type (2900–3300 m). The endophytic fungal and bacterial communities of these two types were significantly different (Figure 5a,b, $p = 0.001$), indicating different recruited microbiome strategies between the two types.

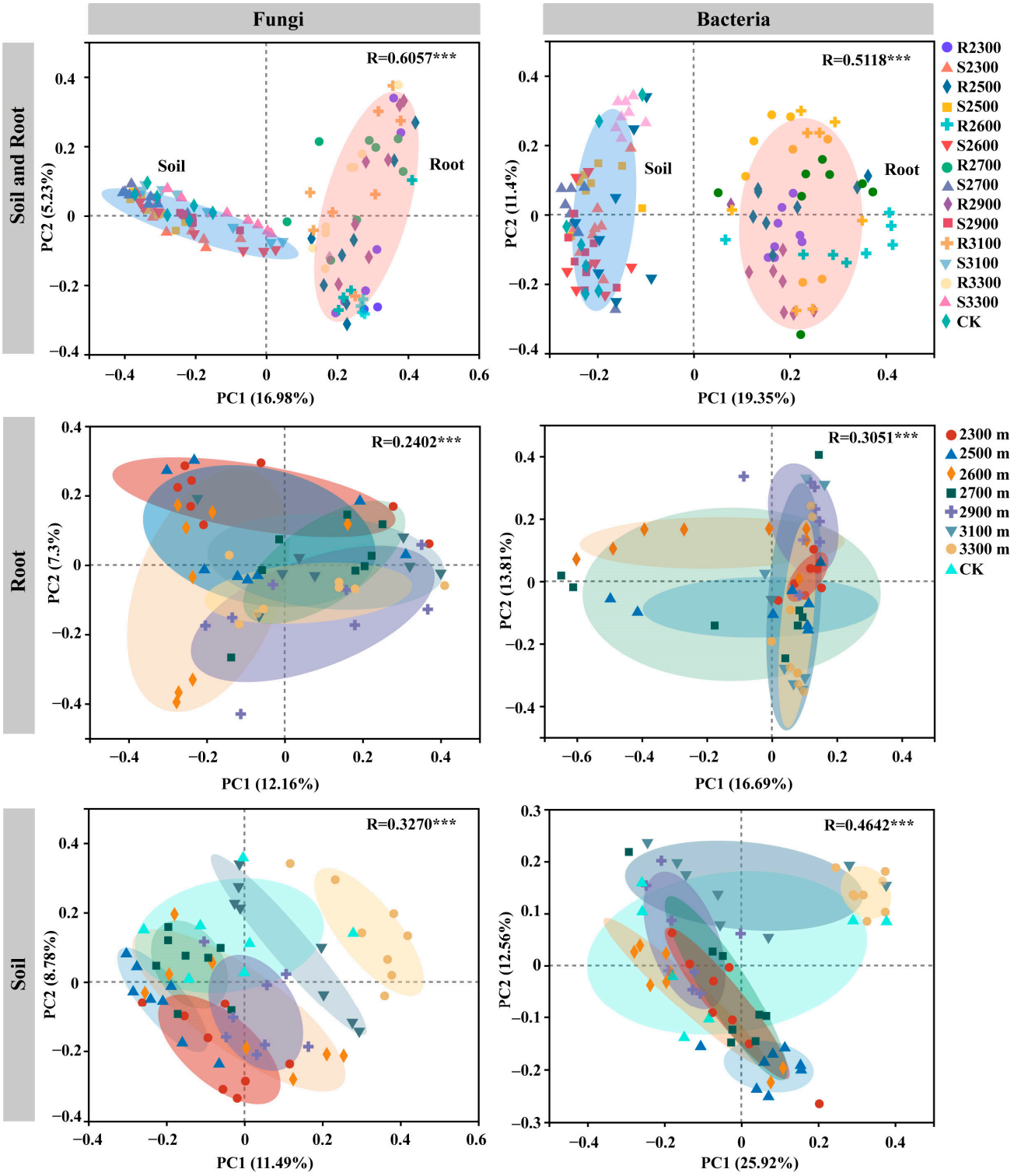

Figure 3. PCoA of the microbial communities of pine roots at different elevations. Significant difference: *** $p \leq 0.001$.

Then, we further analyzed the different microbes recruited by the two types. The *P. yunnanensis*-like type recruited more Mucoromycota and Ascomycota: Hysteriales, *Niesslia*, Herpotrichiellaceae, *Archaeorhizomyces*; Basidiomycota: *Helvellosebacina*, etc. Meanwhile, the *P. yunnanensis*-like type recruited more Actinobacteriota: *Actinophytocola*, *Pseudonocardia*, *Solirubrobacter*, etc. (Figure 5c–f). However, the *P. densata*-like type recruited more Ascomycota: Dothideomycetes, *Ilyonectria*; Basidiomycota: *Hydropus*, *Mycenella*, as well as

Proteobacteria: *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* clade, *Hyphomicrobium*, *Rhodomicrobium*, *Rhizobacter*, *Steroidobacter*; Firmicutes: *Paenibacillus*, etc. In general, different pine trees had different recruitment strategies regarding the microbial communities, and the species composition and quantity of microbes varied greatly (Figure 5c–f).

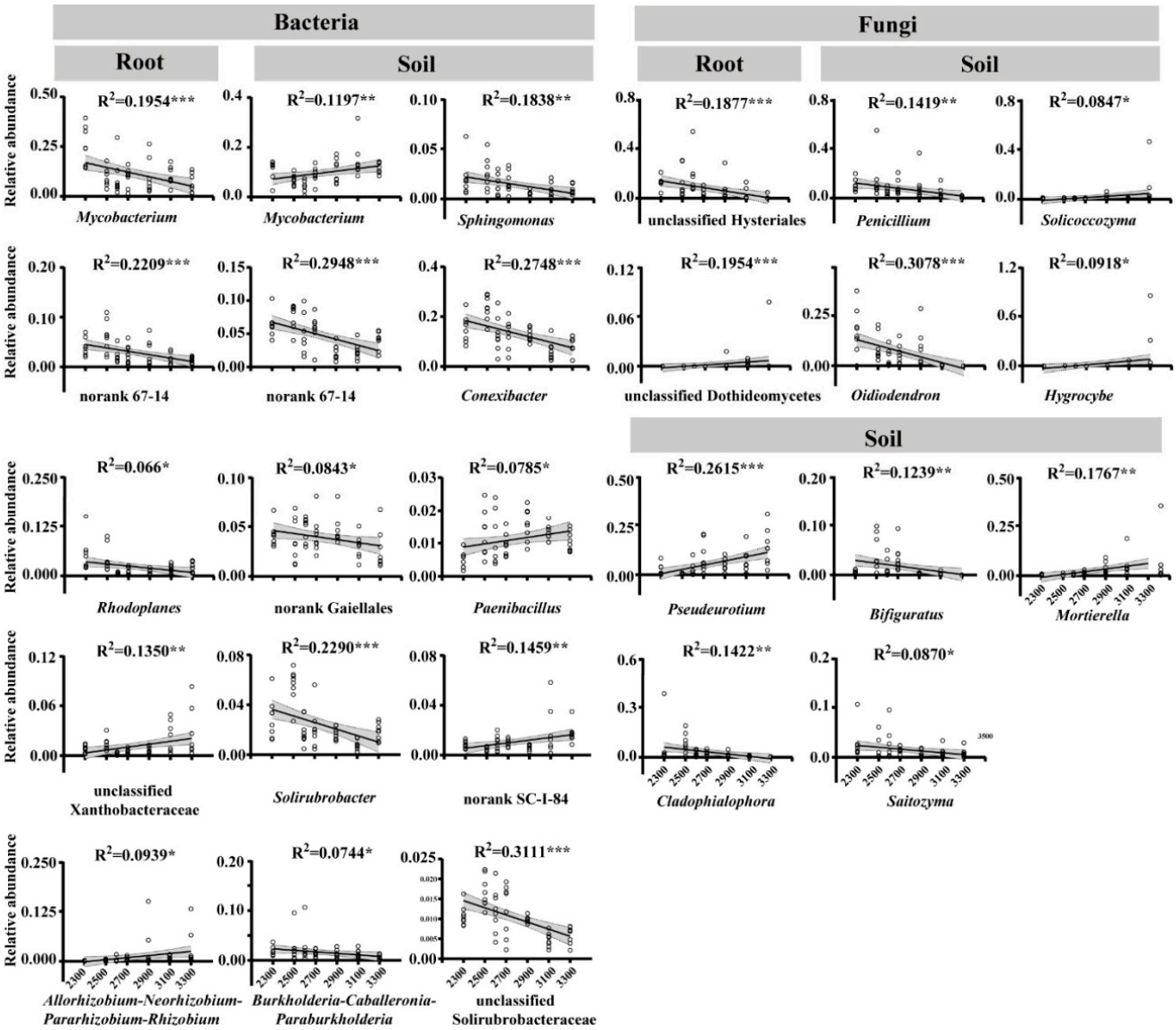

Figure 4. Variation in the relative abundance of genera with elevational gradient. The line indicates the linear regression fit, and the shaded band represents the 95% confidence level. R^2 was employed to determine the models that fit the whole elevational gradient. Significant difference: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Comparative analysis of bacterial KEGG pathways from samples of two types and carbohydrate metabolism (e.g., propanoate metabolism, glycolysis/gluconeogenesis, and starch and sucrose metabolism) and lipid metabolism were enriched in the *P. yunnanensis*-like type, and environmental adaptation, cellular community prokaryotes (e.g., quorum sensing), vitamins and signal transduction (e.g., two-component system) and metabolism of cofactors and vitamins (e.g., porphyrin and chlorophyll metabolism, pantothenate and CoA biosynthesis, folate biosynthesis) were enriched in the *P. densata*-like type (Figure 6a, Supplementary Figure S9). For fungal MetaCyc pathways, the ubiquinol biosynthesis and phosphatidylglycerol biosynthesis were enriched in *P. yunnanensis*-like type, and octane oxidation was enriched in the *P. densata*-like type (Figure 6b).

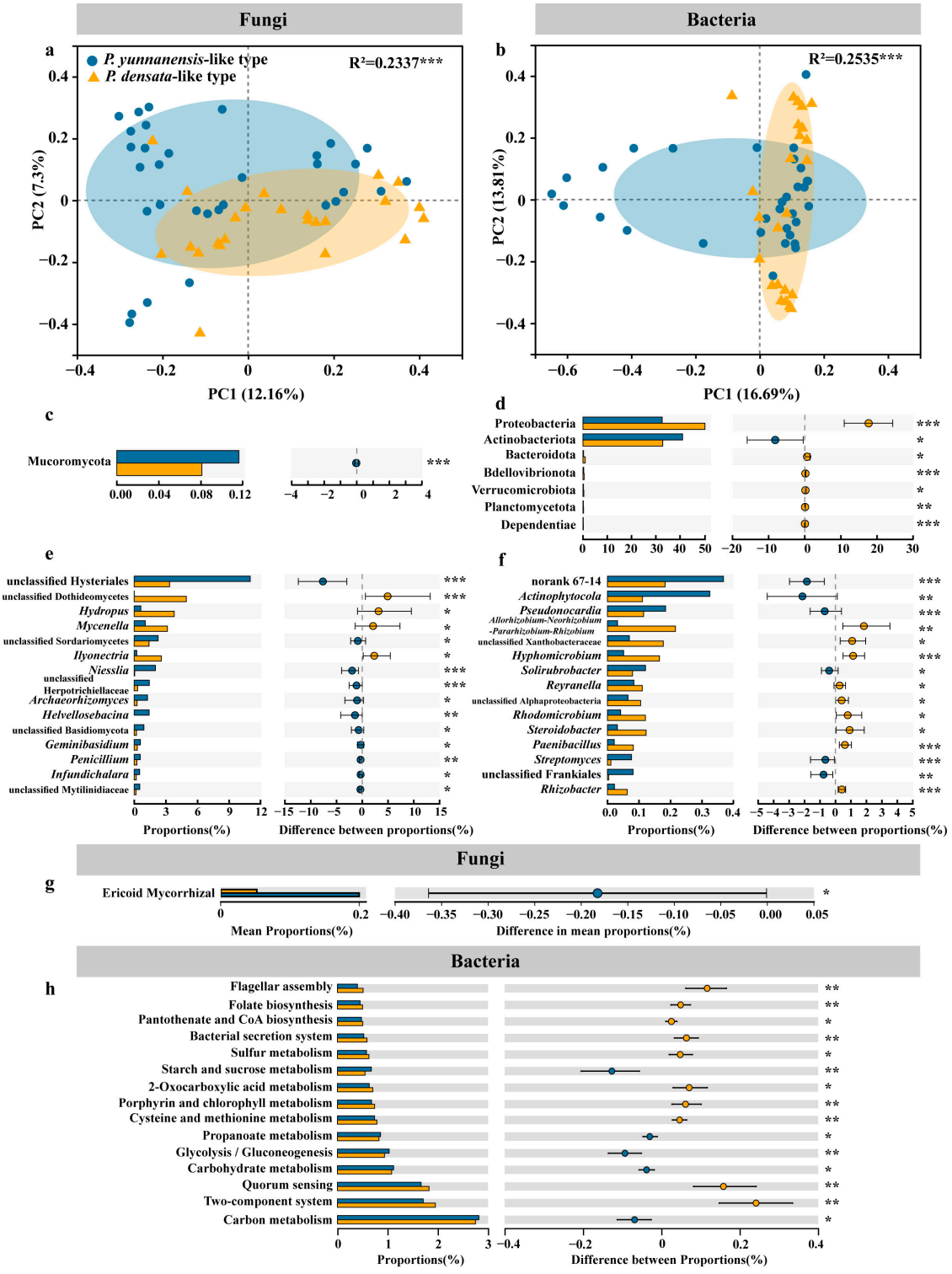

Figure 5. Differentiation of endophytic microbial communities and predicted KEGG or MetaCyc functions predicted by PICRUSt2 between the *Pinus yunnanensis*-like type and *Pinus densata*-like type. The PCoA of the root endophytic fungal (**a**) and bacterial (**b**) communities of different pine types. The different groups between the *P. yunnanensis*-like type and *P. densata*-like type at the phylum (**c**,**d**) and genus (**e**,**f**) levels. The different functions between rhizosphere soil and roots predicted by FUNGuild (**g**) and PICRUSt2 (**h**). Mann–Whitney U tests were used to evaluate differences in the abundance of pine root endophytes. Significant difference: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. "U-" represents "unclassified-".

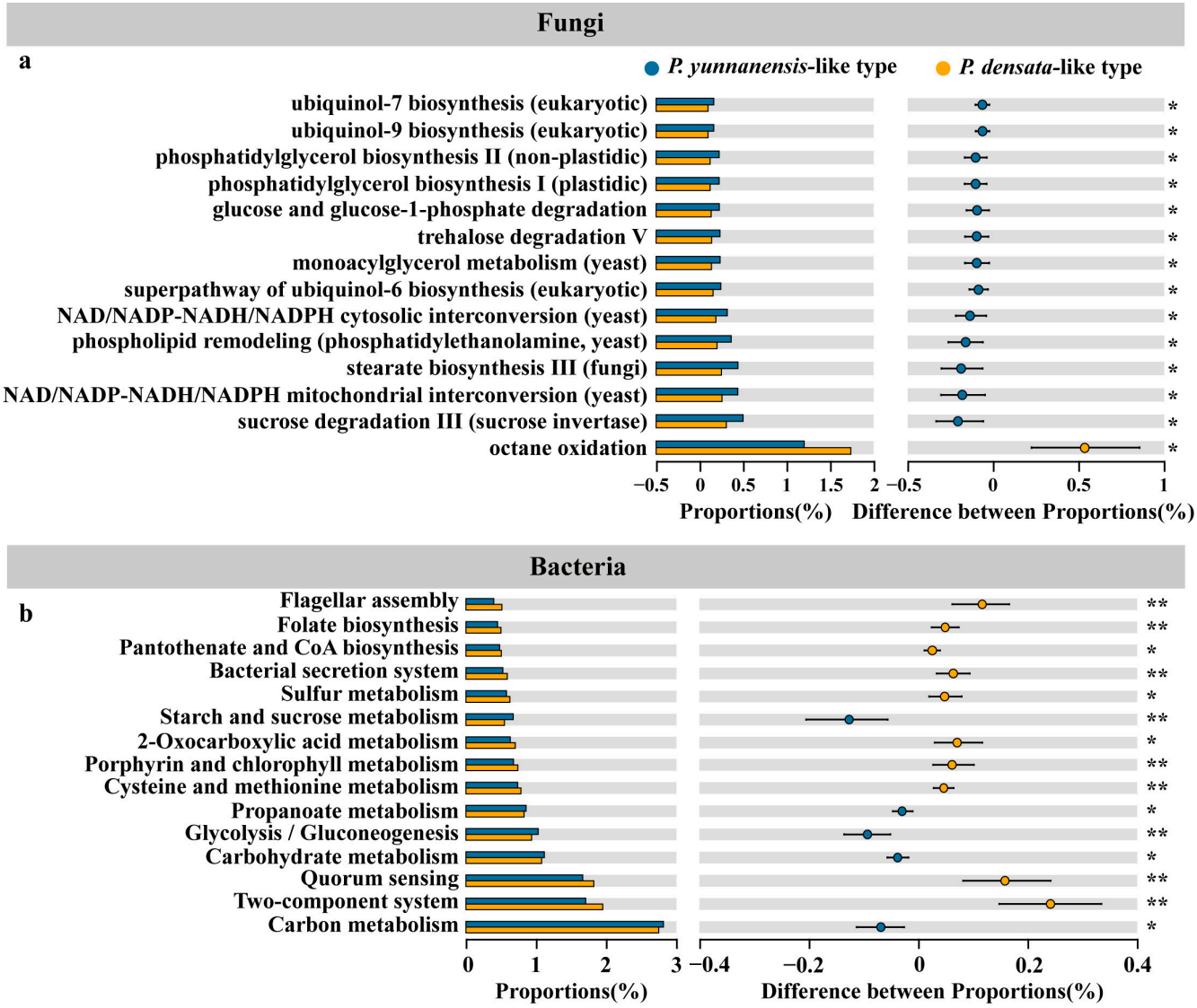

Figure 6. Differentiation of predicted KEGG or MetaCyc functions between the *P. yunnanensis*-like type and *P. densata*-like type. The different fungal (**a**) and bacterial (**b**) functions between the *P. yunnanensis*-like type and *P. densata*-like type predicted by PICRUSt2. Mann–Whitney U tests were used to evaluate differences in the abundance in the abundance of pine root endophytes. Significant difference: * $p \leq 0.05$, ** $p \leq 0.01$.

3.6. Pines Recruited More Diverse Microbial Communities from Similar Soil Microbial Communities

To explore the origin of root endophytes, the alpha and beta diversities of the root and rhizosphere soil microbial communities were measured. First, the rarefaction curve showed that the species number in rhizosphere soils was higher than that in roots (Supplementary Figure S5). Similarly, the alpha diversity of rhizosphere soils was significantly higher than that of roots ($p < 0.001$, Mann–Whitney U test). Moreover, we verified that the microbial communities were completely divided into root and rhizosphere soil groups in both fungi and bacteria (Figure 7, $p = 0.001$ for Bray–Curtis dissimilarity), explained primarily by isolation sources (roots vs. rhizosphere soils) rather than by elevation sites, although there were differences in soil characteristics and climate among the elevation sampling sites.

We inferred that these pines might enrich specific microbial communities from rhizosphere soils and cause a significant transformation of the endosphere microbial community. Therefore, the proportion of root endophytes enriched in rhizosphere soil was measured. The results showed that 70% of the fungi in the roots accounted for only 9.66% of the

corresponding OTUs in the rhizosphere soil. Similarly, 70% of the bacteria in the roots accounted for 38.90% of the corresponding OTUs in the rhizosphere soil (Figure 8a,b). Additionally, the different microbes at the phylum and genus levels were determined between the root and soil groups. The fungi switched from Basidiomycota predominant in the rhizosphere to Ascomycota predominant in the root endosphere, caused by Helotiales, Hysteriales, *Venturia*, *Phialocephala* and Sordariomycetes, belonging to Ascomycota, which were enriched in roots from the soil microbiome pool (Figure 8c–f). The bacteria were switched from Acidobacteriota, Myxococcota and Chloroflexi predominant in the rhizosphere to Firmicutes predominant in the root endosphere, caused by Firmicutes: *Candidatus Phytoplasma* enriched in roots from the soil microbiome pool. Additionally, the genera Proteobacteria: *Acidibacter*, Actinobacteriota: *Acidothermus*, Actinobacteriota: *Actinophytocola* and Firmicutes: *Streptococcus* were enriched in roots (Figure 8c–f).

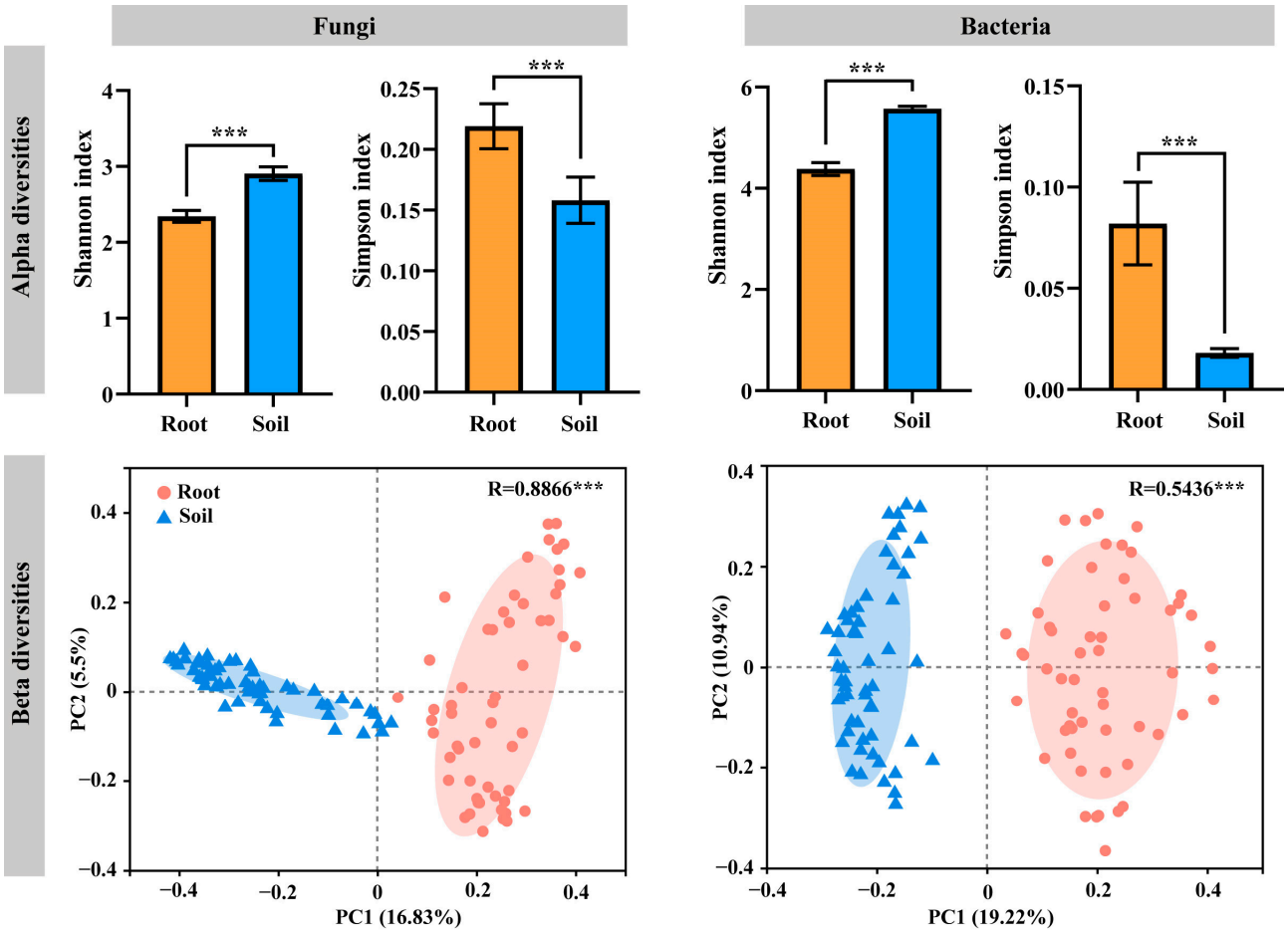

Figure 7. Alpha and beta diversities of microbial communities in rhizosphere soil and roots. Mann–Whitney U tests were used to evaluate differences between rhizosphere soil and roots. The error bar represents one standard error. Significant difference: *** $p \leq 0.001$.

Comparative analysis of bacterial KEGG pathways from samples of root and rhizosphere soil showed that carbohydrate metabolism (e.g., pyruvate metabolism, glycolysis/gluconeogenesis and propanoate metabolism), energy metabolism (e.g., methane metabolism) and lipid metabolism were enriched in roots, while the metabolism of cofactors and vitamins, signal transduction (e.g., two-component system) and cellular community prokaryotes were enriched in rhizosphere soil (Figure 9a, Supplementary Figure S10). For fungal MetaCyc pathways, the pyrimidine deoxyribonucleotides, superpathway of adenosine nucleotides and palmitate biosynthesis were enriched in rhizosphere soil and aerobic respiration, TCA cycle II et al. were enriched in roots (Figure 9b).

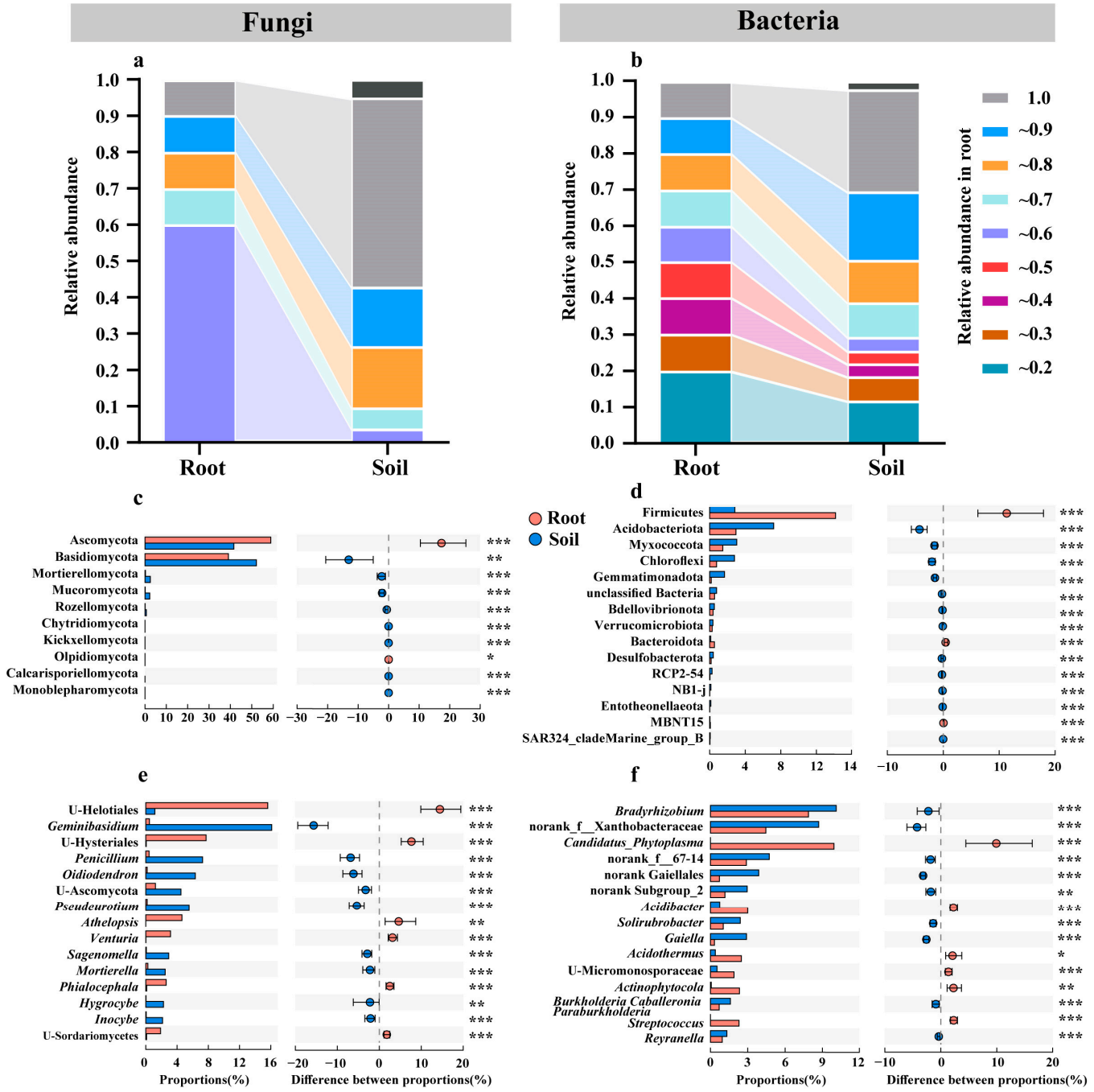

Figure 8. Differentiation of microbial communities in rhizosphere soil and roots. The proportion of root endophytic fungi (**a**) and bacteria (**b**) enriched in rhizosphere soil. The different groups between rhizosphere soil and roots at the phylum (**c**,**d**) and genus (**e**,**f**) levels. Mann–Whitney U tests were used to evaluate differences in the abundance of rhizosphere soil and roots. Significant difference: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. "U-" represents "unclassified-".

Based on the PCoA results, we found that the microbial communities in the rhizosphere soil were more similar than those in the roots (Figure 7). To quantify this, the distance based on Bray–Curtis dissimilarity among elevations and at specific elevations was measured. The results showed that the dissimilarities of endophytes were significantly higher than those of rhizosphere soil in both fungal and bacterial communities based on all elevational samples (Figure 10a,c, $p < 0.001$). Additionally, the dissimilarities in the microbial community among elevations were higher than those at a particular elevation,

indicating possible differences in microbial communities among elevations and species (Figure 10b,d, $p < 0.001$). Compared with fungal communities, bacterial communities were more similar in both roots (bacteria:fungi$_{dissimilarity}$ = 0.734:0.883) and rhizosphere soil (bacteria:fungi$_{dissimilarity}$ = 0.539:0.807) (Figure 8).

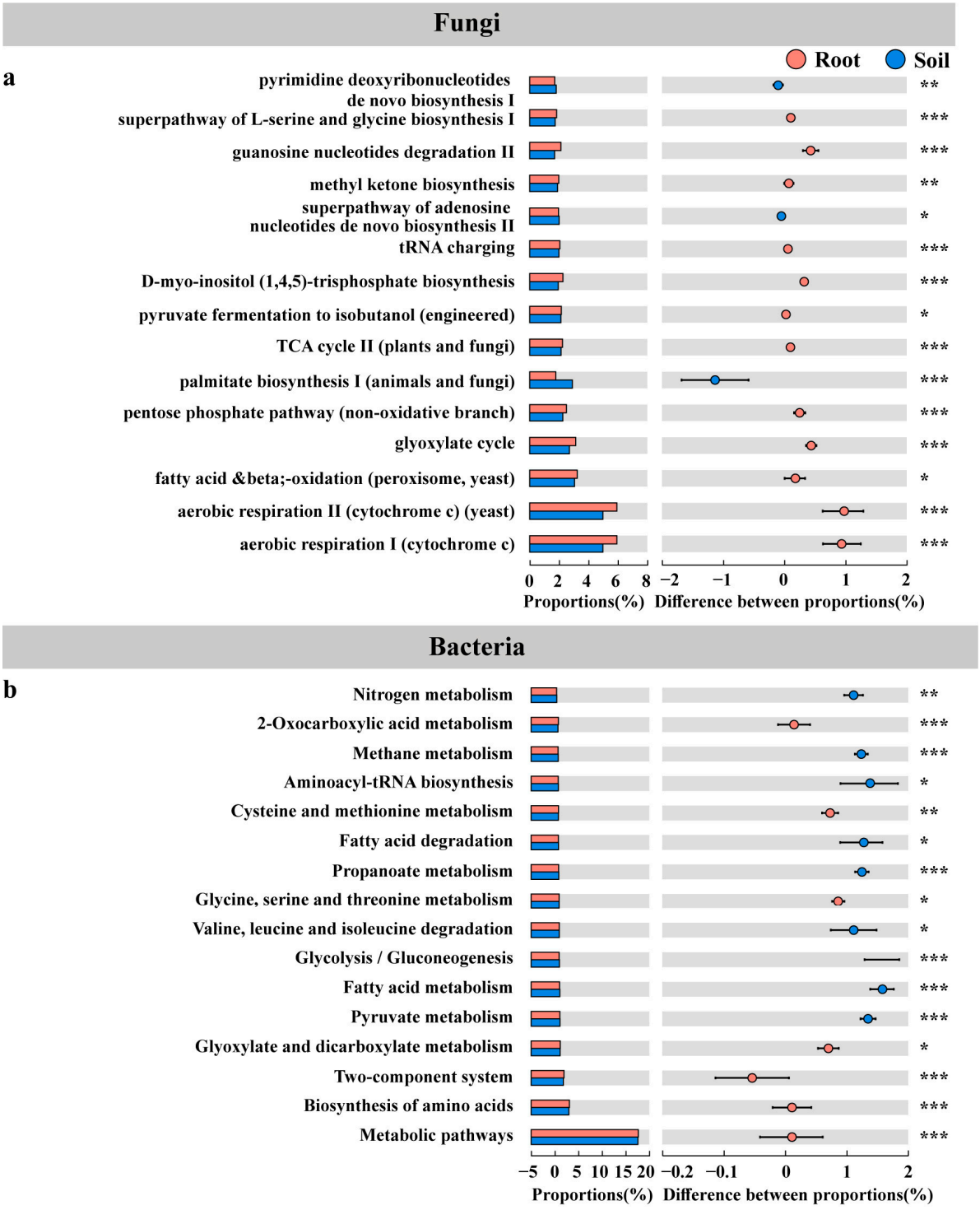

Figure 9. Differentiation of predicted KEGG or MetaCyc functions predicted by PICRUSt2 in rhizosphere soil and roots. The different fungal (**a**) and bacterial (**b**) functions between rhizosphere soil and roots predicted by PICRUSt2. Mann–Whitney U tests were used to evaluate differences in the abundance of rhizosphere soil and roots. Significant difference: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

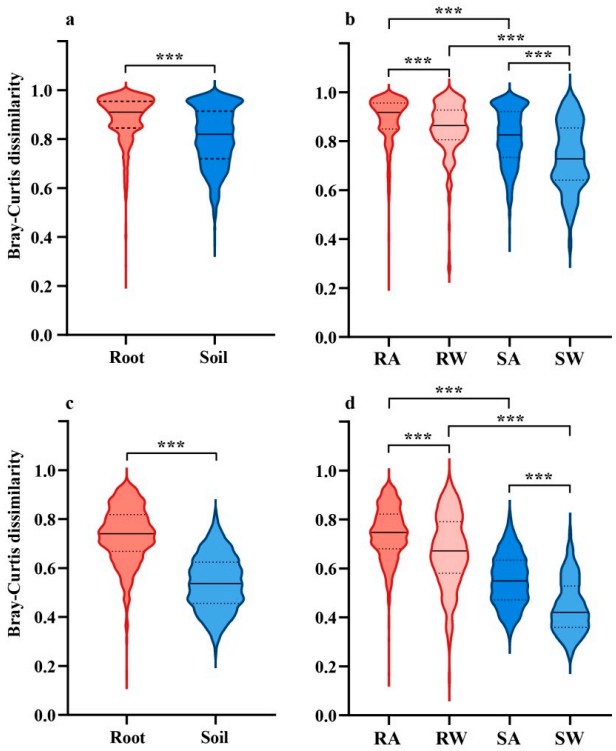

Figure 10. Dissimilarities of microbial communities in rhizosphere soil and roots based on Bray–Curtis dissimilarity. The fungal community (**a**) and bacterial community (**c**) dissimilarities between roots and soil in all samples. The fungal community (**b**) and bacterial community (**d**) dissimilarities separately calculated among elevations and at a particular elevation. RA: Root communities among elevations, RW: Root communities at a particular elevation, SA: Soil communities among elevations, SW: Soil communities at a particular elevation. Mann–Whitney U tests were used to evaluate differences in the abundance of rhizosphere soil and roots. Significant difference: *** $p \leq 0.001$.

4. Discussion

Pinus densata is considered a homoploid hybrid speciation (HHS) type species that originated from the putative parent species *P. tabuliformis* and *P. yunnanensis* [14]. The intermediate morphology of pine hybrids is contributed by both parental species, and hybrid derivatives are more similar to the parent with higher genetic contribution, resulting in cryptic introgression [49]. Xing et al., (2014) reported that in mixed zones, needle morphological features show relative species stability and can be used to identify coniferous species, and the ratios of three-needle clusters were 46.67% and 26.09% in *P. yunnanensis* and *P. densata*, respectively [48]. In this study, we also found significant morphological changes, especially a change from three-needle clusters to two-needle clusters (Figure 1). The ratio of the three-needle clusters changed from 83.75% (2300–2700 m) to 34.58% (2900–3300 m) on average, showing the change from the *P. yunnanensis*-like type to the *P. densata*-like type. Additionally, the decrease in needle length and increase in needle diameter from 2300 m to 3300 m indicated the more efficient water use of *P. densata*-like types. This drought tolerance trait would likely promote its ecological separation from its parental species and benefit the high-elevation adaptability of two-needle pines [50].

Interestingly, we found that the transition of three-needle pines to two-needle pines occurred very quickly, from 2700 m to 2900 m, with the ratio of three-needle clusters decreasing from 85.0% to 37.5%, whereas we previously thought the conversion might be gradual and slow. In other words, a pine tree quickly completes the transition from three needles to two needles, thus efficiently completing its adaptation to the environment. In addition, pine trees at 2600 m to 2900 m had greater DBHs and tree heights, which also shows the effectiveness of this rapid transition. The sampling sites in a few studies also showed a rapid switch between *P. yunnanensis* and *P. densata* in the heart of the Hengduan

Mountains [23,24]. In particular, pines at 2900 m had the greatest tree height and DBH, revealing that two-needle pines were better adapted to this mixed zone.

It is now widely accepted that the establishment of plant-associated microbial communities is not random but is controlled by specific assembly rules [51]. Exogenous driving factors, such as soil characteristics, soil quality [52,53], seasons [54,55] and climate, are characterized by changing the microbial pool to change the microbial composition of plant roots. Endogenous drivers, including the plant host type [39,56], plant parts [57,58], plant development stage [59] and plant immunity [60], are characterized by the process of host-microbe selection in a specific soil microbial pool. While the soil microbial pool is the result of exogenous factors, the bacterial and fungal communities of plant roots (both rhizosphere and endophytic microbes) are subsets of the microbial community in the soil [58,61]. Unlike a study at a larger geographic scale [62], in this study, we limited the geographic variation of the microbial pool by focusing our study on a single mountain. Thus, the differences in root endophytic microbes should be mainly due to the microbe recruitment strategies of different pines and their adaptation to the microenvironment, rather than due to the differences in the soil microbial pool in the geographical environment. For the selection of sample plots, Mao and Wang established the distribution model of *P. densata* and its parents by combining extensive field investigations and geographic information system-based analyses, and the results show that environmental adaptation and geographic isolation can maintain and strengthen the differentiation of species. At the same time, it is pointed out that the large environmental heterogeneity also leads to the difficulty of quantitative assessment on the geographical scale [23]. In this study, it was easy to find a large area of pure *P. densata* forest in the sampling area, but it was difficult to find a mountain ecosystem with the characteristics of a mixed zone and a 1000 m elevation difference. Therefore, this paper only discusses one mountain ecosystem, and the results will have some reference value.

Although there are many bacterial groups in nature, the major bacterial groups and their abundance distribution are similar in bulk soil and rhizosphere soil at the phylum level, i.e., Proteobacteria, Actinobacteria and Acidobacteria. The root endophytic bacteria were mainly composed of Proteobacteria and Actinobacteria. However, the abundance of Firmicutes in roots was approximately five times that in rhizosphere soil, which indicated that there were more Firmicutes in roots as endophytic bacteria, similar to the findings reported by Trivedi et al., (2020) [63]. Compared with bacteria, other root endophytic fungi also play important roles and have the ecological function of helping plants absorb phosphorus [64–66]. The fungi that colonize plant roots are mainly Ascomycota and Basidiomycota [63]. Functionally, endophytic bacteria are already in the host plant, mainly performing metabolism and immune-related functions [67], while rhizosphere soil bacteria act as connectors between the rhizosphere and the external environment. The functions performed are mainly related to external communication and the exchange of substances (molecular communication in the rhizosphere). Both rhizosphere and root endophytic fungi were related to energy metabolism and environmental adaptation.

Similar to most studies, the microbial community structure in the rhizosphere and roots of plants showed distinct differences at different elevations (Figure 3), suggesting that elevation is an important driver of microbial community structure [2,29,68]. Some beneficial microorganisms, such as the drought-tolerant indicator bacterial group *Rhodoplanes* [69], *Bradyrhizobium* and others, decreased with elevation, suggesting a gradual deterioration of environmental conditions. Similarly, the fungus *Oidiodendron*, as a group of ericoid mycorrhizal fungi that can increase plant yield and drought resistance, decreased with elevation [70]. Overall, the effects of the environment and host plants on microorganisms exhibit complex variations.

In this study, we also measured the soil chemical content and nutrient elements in roots and found that root organic carbon, total phosphorus, soil total nitrogen, total phosphorus and pH showed a strong correlation with the elevational gradient (Supplementary Figure S11). The positive correlation between the soil nitrogen content and elevation was consistent with that reported in a previous study and may be related to land cover transformation [71].

Then, we further studied the effects of soil properties and plant nutrient contents on the microbial community. For the driving force of the soil microbial community structure, soil organic carbon, total nitrogen, total phosphorus and pH had significant effects, among which nitrogen and pH were the most important factors affecting the change in soil microbial communities with elevation (Supplementary Figure S12) [5–7,72]. For the distribution of the rhizosphere soil and root endophytic microbiome at elevation, the organic carbon, total nitrogen and total phosphorus contents of soil and roots were the main driving factors, which indicated that elevation affected both biotic and abiotic factors [2,3], eventually affecting the recruitment of microbes to pine trees (Supplementary Figure S11).

We divided all samples into root samples and soil samples for analysis. Additionally, we divided the root endophytes into *P. yunnanensis*-like types and *P. densata*-like types according to their morphological features, and then the characteristics of the microbial community and recruitment strategy were analyzed. Interestingly, although the microbial alpha diversity of soil was significantly higher than that of root endophytes, the differentiation of root endophyte communities in different samples was significantly greater than that of the soil microbiome, revealing that different pine trees recruited different microbiomes from similar microbiome pools in rhizosphere soil to adapt quickly to the environment (Figures 6–10). Similarly, a previous study also showed that the endophytic microbial community of roots exhibited strong correspondence with the host plant phylogeny, but this was not the case in the rhizosphere [73]. This also indicates that the root system selected a different microbial community from the rhizosphere soil microbes to adapt to the environment. The result of this recruitment was that 70% of the fungi in the roots accounted for only 9.66% of the corresponding OTUs in the rhizosphere soil. Similarly, 70% of the bacteria in the roots accounted for 38.90% of the corresponding OTUs in the rhizosphere soil. These results suggested that the recruitment of the root endophytes is not random and that the "host might have selected" more different microbes even more than the elevational difference [33]. The *P. yunnanensis*-like type recruited more Mucoromycota and Actinobacteriota. Meanwhile, the *P. densata*-like type recruited more Proteobacteria. For the function of bacteria, the root endophytic bacteria enriched in the *P. densata*-like type have similar functions to the rhizosphere soil microorganisms mentioned above, mainly by environmental adaptation, quorum sensing and the metabolism of auxiliary factors and vitamins. It is possible that the metabolism related to the environmental adaptation function is more active. The endophytes in the roots of the *P. yunnanensis*-like type were mainly involved in carbohydrate and lipid metabolism. In terms of fungal function, root endophytic fungi of *P. yunnanensis*-like type were related to energy metabolism and fungi of *P. densata*-like type were related to utilization of n-alkanes. Overall, the construction of the pine root microbiome is a complex coevolution and interaction between the host and microbes [74].

5. Conclusions

To the best of our knowledge, this is one of the few studies that covered the elevational changes in microbial community recruitment strategies across homoploid hybrid species [14]. Although it is largely unknown how plant evolution has shaped root microbial communities and, in turn, how these microbes affect plant ecology, such as the ability to mitigate biotic and abiotic stressors [73], this study provides insights into environmental adaptive strategies during species evolution. In brief, this paper studied the climatic adaptation strategies of the hybrid species in the mixed zone of *P. yunnanensis* and *P. densata* in terms of morphology and chemical and microbial recruitment. The results showed that the hybrid species rapidly changed the morphology of the needles and recruited different microbiomes from similar soil microbial pools in a small elevation range to adapt to biotic and abiotic influences driven by elevation. This study provides new insights and perspectives for understanding the mechanism of homoploid hybridization and the adaptation mechanism of species in coniferous forests on mountain plateaus.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f14040685/s1, Figure S1: workflow of sample collection; Figure S2: sampling sites; Figure S3: morphological features of pine needles at different elevations; Figure S4: raincloud plot showing needle morphological features at different elevations; Figure S5: rarefaction curves for different groups of samples; Figure S6: composition and relative abundance of microbial communities at the phylum and genus levels; Figure S7: the distribution of samples at different elevations on the PC1 axis analysed by PCoA; Figure S8: variation in OTU abundance with altitudinal gradient; Figure S9: the different groups between the two types of pines at the OTU level and functions of bacteria at the KEGG 2 level; Figure S10: the different groups between rhizosphere soil and roots at the OTU level and functions of bacteria at the KEGG 2 level; Figure S11: RDA/CCA of the microbial communities of different pine types; Figure S12: RDA/CCA of the microbial communities of rhizosphere soil and roots; Table S1: sampling site information; Table S2: Plant information for sampling sites at different elevations.

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