



# Different assembly mechanisms between prokaryotic and fungal communities in grassland plants and soil

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## Abstract

**Background and Aims** Microorganisms play pivotal roles in plant growth and health. However, it remains unclear whether distinct mechanisms govern the assembly of prokaryotic and fungal communities across plant compartments and soils in grasslands.

**Method** Microbial communities in plant compartments (phyllosphere, leaf endosphere, rhizosphere and root endosphere) and bulk soil of multiple plants from different grasslands were investigated through amplicon sequencing of the 16S rRNA gene and ITS fragment.

**Results** The results showed that the rhizosphere harbored the most diverse fungal communities, while the

alpha diversities of prokaryotic communities did not exhibit a significant difference between the rhizosphere and bulk soil. Moreover, prokaryotic communities across different compartments were subjected to strong selection effects. For instance, there was strong phylogenetic turnover of prokaryotic communities from bulk soil or phyllosphere (two sources of leaf endophyte) to the leaf endosphere. Conversely, fungal communities were affected by strong stochastic effects. Fungi in plant compartments (especially the phyllosphere) were more impacted by geographical characteristics and their interactions with plant genotypes than prokaryotes. Additionally, strong homogeneous selections on the prokaryotes between the phyllosphere and rhizosphere highlighted similar adaptative mechanisms of prokaryotes between the underground and aboveground plant surfaces. The differences between fungi and prokaryotes in plant compartments are closely linked to their functional traits related with fitness for plant niches.

**Conclusion** In summary, distinct mechanisms shape prokaryotic and fungal communities in grassland plant compartments. This research provides significant insights into the community assembly mechanisms of prokaryotes and fungi in grassland plant compartments and ambient soil.

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Chi Liu and Na Li contribute equally to this study.

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## Introduction

Microorganisms can contribute to the growth and health of plants and help plants resist environmental stress (Wang and Song 2022). As an illustration, the symbiotic relationship between plants and arbuscular mycorrhiza entails a mutually beneficial exchange, wherein plants provide the fungi with carbohydrates while the fungi, in turn, enhance the plants' ability to uptake mineral nutrients and resist drought and pathogens (Smith et al. 2010). Plant and microbes form a holobiont to facilitate the survival and growth for both of them (Vandenkoornhuys et al. 2015). Beyond their physiological effects, microbes also exert substantial influence on the plant ecosystem at a large scale. For instance, the diversity of leaf bacteria mediates the relationship between plant diversity and ecosystem function (Laforest-Lapointe et al. 2017). Consequently, investigating the composition and assembly mechanisms of microbial communities in plants has emerged as a prominent topic in ecology. Furthermore, the study of microbial community assembly mechanisms serves as a critical foundation for utilizing synthetic communities in agricultural practices (Ke et al. 2021). For example, organic amendments or inoculation with specific probiotic microbes have been widely employed to improve plant growth and resilience and soil quality in agriculture (Arif et al. 2020).

Plant-microbiome relationships are usually affected by many factors, including plant genotype, plant tissues, and environmental context. It has been shown that prokaryotic communities associated with species of *Agave* are primarily determined by the plant compartment, while the composition of fungal communities is mainly influenced by the biogeography of the host species (Coleman-Derr et al. 2016). Environmental factors can largely affect the plant-microbiome relationship. For example, phyllosphere microbial communities in the subtropical forest exhibit greater abundance and diversity compared to those in tropical and temperate forests (Li et al. 2022). The stability and succession of the rhizosphere microbiota rely on the plant type and soil composition, and microbial composition in bulk soil plays a critical role in the stability of rhizosphere microbiota (Tkacz et al. 2015). Furthermore, community assembly mechanisms for prokaryotes and fungi can shift across different ecosystems (Cao et al. 2022). A plant growth

experiment (Tkacz et al. 2020) indicates that fungal microbiota are primarily influenced by soil, while bacterial microbiota are more profoundly affected by the root fraction compared to soil or plant species. Moreover, spatial factors can also largely impact microbial compositions and structures associated with plants. For example, fungal assembly is more driven by dispersal limitation rather than selection in the rhizosphere soil of *Panax notoginseng* across 26 sites with a median inter-site distance of 208 km (Zhang et al. 2021). In vineyard soils, bacteria are predominantly influenced by deterministic selection, while stochastic processes play a more important role in fungal community assembly (Larsen et al. 2022). In a study exploring the relationships between bacterial and fungal communities in the rhizosphere and root endosphere of *Populus deltoids*, host genotypic distances do not exert a significant effect on microbial communities, whereas soil properties play vital roles in determining microbiome composition (Shakya et al. 2013). Overall, key factors shaping plant-microbiome assembly vary depending on specific systems.

Microbial origins should be considered when evaluating microbial assemblages at the individual plant level. Microbial communities in various plant compartments and bulk soil are not entirely separated. Bacterial communities affiliated with leaves, flowers, and grapes share a higher proportion of taxa with soil rather than with each other, implying that soil can serve as a bacterial reservoir (Zarraonaindia et al. 2015). An extensive taxonomic and functional overlap between the leaf and root microbiota of *Arabidopsis* has been revealed by isolating leaf- and root-derived microbiota that represent the majority of bacterial species (Bai et al. 2015). Furthermore, a common-garden experiment demonstrates that bacteria can reach leaves and flowers of *Arabidopsis thaliana* only from the soil (Massoni et al. 2021). Different plant compartments may have varying selection effects on microbes, which are also regulated by complex environmental and spatial factors. Consequently, sample types, spatial and environmental characteristics must be considered when proposing research hypotheses. For instance, the relationship between the spatial distribution and traits of grass roots and the growth patterns of soil fungi has attracted considerable attention (Sweeney et al. 2020; Hennecke et al. 2023). In addition, plant diversity has stronger linkage with soil fungal diversity than with bacterial diversity across grasslands of northern China (Wang et al. 2022),

meaning that the relationship between soil microbiome and plant depends on the microbial taxonomic group. However, current research on microbial community assembly for different plant compartments often suffers from limited sampling points and organs, thus hindering a comprehensive assessment of assembly characteristics within the plant microbiota across various natural settings. Existing studies have revealed notable discrepancies in community assembly between fungi and bacteria, yet it remains unclear whether these patterns can be applied to multiple compartments of plants.

Present studies generally consider the plant compartment as a factor to decipher its importance in microbial community assembly, along with other factors such as plant species, soil and climatic attributes, and spatial factors (Xiong et al. 2021). The microbiome of plant compartments and the plant itself constitute a self-regulating living system. Controlled experiment and natural investigation experiment are two approaches to study plant-microbiome (Balbin-Suarez et al. 2020; Massoni et al. 2021; Xiong et al. 2021). Natural ungrazed grassland provides a good framework to test some hypotheses on the plant-microbiome relationships. Grasses, especially annual ones, are characterized by their short growth cycles, which render them less susceptible to temporal uncertainties. Natural ungrazed grasslands remain undisturbed by human and livestock interventions, offering the advantage of hosting a diverse array of plant species for convenient sampling. In this study, we studied prokaryotic and fungal communities in bulk soil and plant compartments (phyllosphere, leaf endosphere, rhizosphere, root endosphere) of typical plants growing in the meadow steppe and typical steppe of Inner Mongolia, and the alpine meadow of Qinghai-Tibetan Plateau. We aimed to test two hypotheses: 1) The differences in community assembly between prokaryotes and fungi can be reflected in the selection strength across different plant compartments; 2) Different mechanisms lead to the differences in community assembly patterns between prokaryotes and fungi in grassland plants.

## Materials and methods

### Study site descriptions

The samples were collected from three main types of grasslands in China: alpine meadow, meadow

grassland and typical grassland (Table S1). For each sampling site, the geographic information (latitude, longitude and altitude) was recorded with a GPS device. The mean annual temperature (MAT) and mean annual precipitation (MAP) were inferred from the program of DIVA-GIS V7.5 (<http://www.diva-gis.org/>) with 2.5 arc-minutes resolution climate data (WorldClim, <http://www.worldclim.org>) based on the GPS information. Alpine meadow is situated in Naqu, Tibet (92°6'E, 31°16'N), at an elevation of 4499 m. The MAT, MAP and the vegetation coverage are approximately -1.19°C, 397 mm and 97%, respectively. Dominant plant species encompass *Kobresia tibetica*, *Carex thibetica*, *Leymus secalinus*, *Stipa purpurea* etc. Meadow grassland is located in Hulunbeir, Inner Mongolia (120°7'E, 49°21'N), with an elevation of 663 m, an MAT of approximately -1.44°C, a MAP of 383 mm, and a vegetation coverage of 85%. Key plant species include *Leymus chinensis*, *Stipa baicalensis* and *Filifolium sibiricum* (L.) Kitam. Typical grassland is located in Xilinguole, Inner Mongolia (116°38'E, 43°35'N), with an elevation, MAT, MAP, and vegetation coverage of 1244 m, 1.36°C, 334 mm, and 80%, respectively. *Leymus chinensis* and *Stipa grandis* are widely distributed in this area. All sampling locations were selected from conserved, ungrazed areas. This study focused on seven plant species, namely *Stipa purpurea* (SP) in alpine meadow, *Stipa baicalensis* (SB) in meadow grassland, *Stipa grandis* (SG), *Achnatherum pekinense* (AP), *Cleistogenes mucronata* (CM), *Agropyron mongolicum* (AM) and *Sibbaldianthe sericea* (SS) in typical grassland (refer to Table S2). The collected plants encompass species belonging to the same genus, which grow in different grassland types, as well as species from different genera that coexist within the same grassland type (typical steppe). Collecting species with distant phylogenetic relationships within one region can help explore the impact of plant evolutionary distance on communities by controlling for soil and vegetation variability, while collecting species with close phylogenetic relationships across three regions can minimize the influence of plant species differences as much as possible.

### Sample collection and soil properties measurement

The samples were acquired in July, 2015. Initially, an evaluation was conducted to assess the plant diversity

and extent of coverage in each particular site chosen for sampling. The geographical coordinates pertaining to longitude, latitude, and altitude were recorded. Five individual plants were gathered for each species and treated as five replicate samples. For each plant individual, to collect the microbes on leaf surface (phyllosphere, LS), sanitary cotton swabs soaked in sterile saline were utilized for repetitive wiping on both sides of leaves. Subsequently, the entire plant was carefully dug out, and the soil attached to the roots was shaken off. The sterile cotton swabs, also soaked in sterile saline solution, were used to clean the surfaces of diverse roots repeatedly, and collected as the rhizosphere soil (RS). A section of the freshly harvested leaves and roots were cut with disinfected scissors (sterilized with a 75% alcohol solution and subsequently singed) and collected as plant tissue samples. Following this, the bulk soil (S) was obtained and preserved within a 50 mL sterile centrifugal tube. Samples were transported to the laboratory with ice and stored at  $-20^{\circ}\text{C}$  until DNA was extracted. Part of the bulk soil was sieved with a 2 mm mesh and stored at  $4^{\circ}\text{C}$  for analyzing soil properties, including soil pH, electronic conductivity, total organic carbon (TOC), total nitrogen (TN),  $\text{NH}_4^+\text{-N}$ ,  $\text{NO}_3^-\text{-N}$ . All the measurement approaches used have been described in a previously published study (Yao et al. 2014).

#### DNA extraction

The collected cotton swabs were sectioned into centrifugal tubes alongside sterile glass beads. To obtain the root (R) and leaf (L) endophytes, the roots and leaves underwent six cycles of cleaning using PBS solution and were subjected to sterilization. The sterilization process involved washing with sterile Millipore water (30 s), sterilization with 70% (v/v) ethanol (2 min), treatment with sodium hypochlorite solution (2.5% active  $\text{Cl}^-$  with 0.1% Tween 80) (5 min), additional sterilization with 70% (v/v) ethanol (30 s), and concluded with five rinses using sterile Millipore water. Subsequently, the materials were pulverized with liquid nitrogen and deposited into centrifuge tubes along with sterile glass beads. A small quantity of bulk soil was placed into centrifuge tubes with sterile glass beads. All samples were vigorously agitated using a Mini-beadbeater. DNA extraction was performed utilizing the MoBio Powersoil DNA isolation kit (MO BIO Laboratories, Carlsbad, CA, USA)

in accordance with the manufacturer's guidelines, and stored at  $-20^{\circ}\text{C}$  for future use. The genomic DNA of each plant species was extracted using the Rapid Plant Genomic DNA Isolation Kit (Sangon Biotech, Shanghai, China).

#### High-throughput sequencing

The universal primer 515F/909R (Tamaki et al. 2011) and ITS3\_KYO2/ITS4 (Toju et al. 2012) were employed for the amplification of prokaryotic 16S rRNA gene and ITS fragment through polymerase chain reaction (PCR) correspondingly. Primer 515F and ITS4 were linked with 12 unique barcodes at its 5'-end. The 25  $\mu\text{L}$  reaction mixture for amplification comprised 1  $\mu\text{L}$  of each primer (10  $\mu\text{M}$ ), 1  $\mu\text{L}$  template DNA (10 ng/ $\mu\text{L}$ ), 9.5  $\mu\text{L}$  ddH<sub>2</sub>O, and 12.5  $\mu\text{L}$  of MasterMix containing Taq DNA Polymerase, PCR Buffer,  $\text{Mg}^{2+}$ , and dNTPs. Three PCR replications were conducted for each sample. The PCR reaction protocol for the 16S rRNA gene involved an initial step at  $94^{\circ}\text{C}$  for 3 min, followed by 28 cycles of  $94^{\circ}\text{C}$  for 1 min,  $56^{\circ}\text{C}$  for 1 min, and  $72^{\circ}\text{C}$  for 1 min, culminating in a final extension at  $72^{\circ}\text{C}$  for 10 min. In contrast, the PCR reaction protocol for the ITS gene commenced at  $94^{\circ}\text{C}$  for 5 min, followed by 35 cycles of  $94^{\circ}\text{C}$  for 30 s,  $56^{\circ}\text{C}$  for 30 s,  $68^{\circ}\text{C}$  for 45 s, and concluded with a final extension at  $72^{\circ}\text{C}$  for 10 min. Negative controls were implemented to confirm the absence of contamination. Subsequently, PCR products underwent 1% (w/v) agarose gel electrophoresis with  $1.0\times\text{TAE}$  buffer, and the bands were excised and purified using the AxyPrep DNA Gel Extraction Kit (AP-GX-250, Axygen, USA). The PCR product quality was evaluated using a NanoDrop ND-2000 Spectrophotometer (Nano-Drop Technologies Inc., Wilmington, DE). Purified PCR products from all samples were combined in equimolar amounts and subjected to sequencing ( $2\times 300$  bp) using the Illumina Miseq sequencer at the Chengdu Institute of Biology, CAS, China.

#### Sequencing data analysis

Paired-end reads of 16S rDNA and ITS fragments were merged through the utilization of FLASH software (Magoč and Salzberg 2011). Low-quality reads (length  $< 200$  bp, more than two ambiguous bases 'N' and average base quality score  $< 30$ ) were

filtered. Sequences were clustered into operational taxonomic units (OTUs) at a 97% identity threshold using the UPARSE algorithm (Edgar 2013). Potential chimeras in representative sequences were removed by the Uchime algorithm (Edgar et al. 2011). Taxonomic classification was accomplished by utilizing the SILVA (release\_132) database (Quast et al. 2013) for 16S rRNA gene, and the UNITE database (Nilsson et al. 2019) for ITS fragment, with the implementation of the Uclust classifier and default parameters. For 16S rDNA, representative sequences were aligned using PyNAST (Caporaso et al. 2010a), which is embedded in QIIME v1.9.1 (Caporaso et al. 2010b). Phylogenetic tree of prokaryotes was reconstructed based on the maximum likelihood–approximation method in FastTree (Price et al. 2010).

#### Plant marker gene sequencing and phylogenetic analysis

The conservative genes of plants were utilized to identify the taxonomy and phylogenetic relationships among collected plants. The ITS fragment, chloroplast *matK* gene and *rbcL* gene were carefully chosen. The primers were synthesized in accordance with prior researches (Quan and Zhou 2011; Dong et al. 2014). The PCR products were subjected to Sanger sequencing (terminal termination method). Sequences from each gene were aligned employing program MAFFT v7.394 (Kato and Standley 2013) and manually adjusted with the program Bioedit v7.2.5. Subsequently, the aligned sequences from each species were concatenated. The GTR+G+I substitution model was selected according to the jModel-Test 2 software (Darriba et al. 2012). Phylogenetic trees were reconstructed using maximum likelihood approach in the program RAxML v7.2.7 (Stamatakis 2014) with 1000 bootstrap searches. The phylogenetic tree was depicted in Fig. S1.

#### Statistical analysis

All of the statistical analyses were conducted using R (v4.0.0) (R Core Team 2018). The QIIME result files were imported into R using the file2meco package (v0.2.0) (Liu et al. 2022). OTUs that were contaminated or useless were removed based on taxonomic information, including those annotated with mitochondrial or chloroplast information in the 16S

rRNA gene data and those not assigned to the Fungi kingdom in the ITS data. Samples with less than 1000 sequences were removed for both 16S rRNA gene and ITS data. The remaining effective samples were rarefied to 1000 sequences to reduce the sequencing depth effects on the evaluation of community diversity. The correlation network of OTUs was constructed with the `trans_network` class of R `microeco` package (v0.20.0) (Liu et al. 2021). OTUs containing <0.01% of total sequences were removed to increase the robustness. The spearman rho correlation coefficient was calculated, and high and significant coefficients ( $|r_{\text{rho}}| > 0.6$ ,  $P < 0.05$ ) were selected for the network construction. The false discovery rate (FDR) method was adopted for the  $P$  value adjustment. Networks were visualized using the interactive platform Gephi (Bastian et al. 2009). In addition to the network construction for all the data, each correlation network was also constructed for different plant compartments individually with the same threshold as previously described. The edges of networks from different compartments were extracted and merged into one table using the `edge_comp` function of package `meconetcomp` (v0.3.0) (Liu et al. 2023). Then the edge intersections were analyzed and visualized with the `trans_venn` class of `microeco` package. To evaluate the differences of non-random patterns between prokaryotic and fungal communities, we first calculated Bray–Curtis-based Raup–Crick (RC-Bray) metric (Chase et al. 2011) using `cal_rcbray` function (parameters: `runs=1000`, `null.model="independentswap"`) of `trans_nullmodel` class in `microeco` package. The parameter setting means the null model algorithm is “independentswap” (Kembel et al. 2010) with 1000 runs of simulations. The RC-Bray value less than -0.95 or larger than 0.95 represents that the observed community dissimilarity is significantly different from the null expectation (two-tailed test,  $\alpha=0.05$ ) (Chase et al. 2011). Then to further assess the non-random patterns, we calculated the beta net relatedness index (betaNRI) (Fine and Kembel 2011) using the `cal_ses_betampd` function (parameters: `null.model="taxa.labels"`, `runs=1000`, `abundance.weighted=TRUE`) in `trans_nullmodel` class of `microeco` package. Then, the `cal_group_distance` function in `trans_beta` class of `microeco` package was employed to convert the symmetric matrix of RC-Bray and betaNRI to a long-table format with paired distances between any two compartments

of each plant individual. Since betaNRI represents standardized effect values, unlike RC-Bray which carries significance, in order to approximately provide confidence intervals in the betaNRI distributions across different groups, we treated each randomized distribution as a normal distribution, then took a 95% confidence interval, i.e.,  $\pm 1.96$  (Mean  $\pm 1.96$ SD), as the threshold for all simulated distributions (Fine and Kembel 2011). Alpha diversity was estimated with the Shannon–Wiener metric. Bray–Curtis dissimilarity was calculated as taxonomic beta diversity distance matrix. To identify if there were significant differences among different groups on Bray–Curtis dissimilarity matrix, permutational multivariate analysis of variance (PERMANOVA) was performed using adonis function in vegan (Oksanen et al. 2018). The correlations among microbial community dissimilarities with environmental variables were evaluated using the (partial) Mantel test from the mantel function in package vegan. Variance partitioning analysis (VPA) was used to quantify the relative contributions of variables by using the function varpart in package vegan. Climate factors included MAT, MAP and altitude. We did not include spatial distance because the variables derived from principal coordinates of neighbor matrices analysis are strongly correlated with climate factors. Plant factors were represented by the main components (larger than 90% of the total variation) of phylogenetic distance using principal coordinate analysis. Functional prediction was performed using trans\_func class of microeco package. The FAPROTAX database (Louca et al. 2016) was used for the prokaryotic traits prediction, and Tax4Fun method (Ashauer et al. 2015) was adopted for the KEGG pathway prediction of prokaryotic communities. The FungalTraits (Pölme et al. 2020) and FUNGuild (Nguyen et al. 2016) databases were used for the inference of fungal traits. The output of FungalTraits was utilized in the differential abundance test of guilds, because the outcomes of this database exhibit great prediction precision, thereby facilitating the speculation on the mechanisms behind community structure alterations. The output of FUNGuild was employed to investigate the guild constitution of network modules at a superior hierarchical level, owing to the unique advantage of the FUNGuild database in the meta-information. The relative abundance of genera or guilds was fitted using a generalized linear mixed model (GLMM) with the beta distribution

family implemented in the trans\_diff class of microeco package (parameters: method = “glmm”, formula = “Compartment + (1|Plant\_ID)”, family = glmmTMB::beta\_family(link = “logit”).

## Results

### Microbial community compositions

A sum of 1,760,519 and 3,957,224 valid sequences were obtained for the 16S rRNA gene and ITS regions, respectively. After filtering and resampling, a total of 3,137 prokaryotic OTUs (derived from 166 samples) and 2765 fungal OTUs (from 152 samples) were identified. Among the 16S rRNA gene data, 1313 OTUs had detailed classification information at the genus level, with *Haliangium* comprising 40 OTUs. Regarding ITS data, 873 OTUs had genus-level classifications, with *Mortierella* and *Glomus* containing 33 and 27 OTUs, respectively.

Significant variances in microbial community compositions were detected among different plant compartments (Fig. S2). The relative abundances of Proteobacteria in L (32.5%) and R (38.8%) were higher than those in LS (26.3%), RS (22%) and S (19.6%). Conversely, the relative abundances of Acidobacteria in L (5.6%) and R (4.7%) were lower than those in LS (32.1%), RS (20.9%) and S (20.4%). The relative abundances of Bacteroidetes and Firmicutes in L were the highest across all the compartments. Acidobacteria and Verrucomicrobia were the dominant phyla in the LS. The mean relative abundance of Thaumarchaeota in the S (20%) was higher than that in other compartments. With respect to genera, *Bacteroides* was the primary genus in L, while *Bradyrhizobium* and *Burkholderia* were the main genera in LS. *Bradyrhizobium* and *Flavisolibacter* were the dominant genera in R, RS and S. *Lechevalieria* and *Streptomyces* were mainly detected in R, while *Ferruginiter* and *Burkholderia* were mainly found in RS. *Ferruginibacter* and *Candidatus Nitrososphaera* were abundant in S. *Massilia* was primarily found in LS and S of SB (*Stipa baicalensis*) (Fig. S2). Concerning fungi, Ascomycota (60.39%) had the highest relative abundance in all plant compartments. The relative abundances of Basidiomycota in R (37.30%), RS (25.89%) and S (25.27%) were higher than those in L (5.93%) and LS (10.29%). A small

amount of Glomeromycota was found in R, RS and S. *Alternaria* was the dominant genus in L. *Alternaria* and *Portierella* were the dominant genera in the LS, and a small amount of *Archaeorhizomyces* was also found in LS. *Mycena*, *Coniothyrium* and *Agrocybe* was mainly found in R. *Mortierella* was the dominant genus in the RS and S. *Glomus* appeared in the R, RS and S with relative low abundance (Fig. S2). *Hygrocybe* was mainly observed in the R, RS and S of *Stipa*. *Entoloma* was mainly found in the RS and S of *Cleistogenes mucronata*. However, *Clohesyomyces* was mainly detected in the R, RS, and S of *Stipa purpurea*.

To identify the core microbiome of plant species, we extracted 16S rRNA and ITS datasets for each plant species and removed samples associated with bulk soil (S). We defined the core OTU for each plant as those with an occurrence frequency greater than 50% in the samples and an average abundance greater than 0.1%. Subsequently, we analyzed the unique and common core OTUs among the plants. Among the total core OTUs (224, derived from 7 plant species) of prokaryotes, the shared core OTUs (217) accounted for 96.9% (Fig. S3). These OTUs were mainly derived from genera *Arthrobacter*, *Terrimonas*, *Acidibacter*, *Sphingomonas*, and *Flavisolibacter*. Regarding fungi, the shared core OTUs (45) accounted for 53.6% of the total core OTUs (84) (Fig. S4). These OTUs mainly originated from genera *Phaeococcomyces*, *Mortierella*, and *Umbelopsis*. *Stipa purpurea* possessed a unique core OTU that originated from the Capnodiales order. When only considering three *Stipa* species from different regions, the results are also similar (Fig. S3 and S4). These findings indicate that fungi have fewer core OTUs across different plant species than prokaryotes.

#### Alpha diversity of prokaryotes and fungi

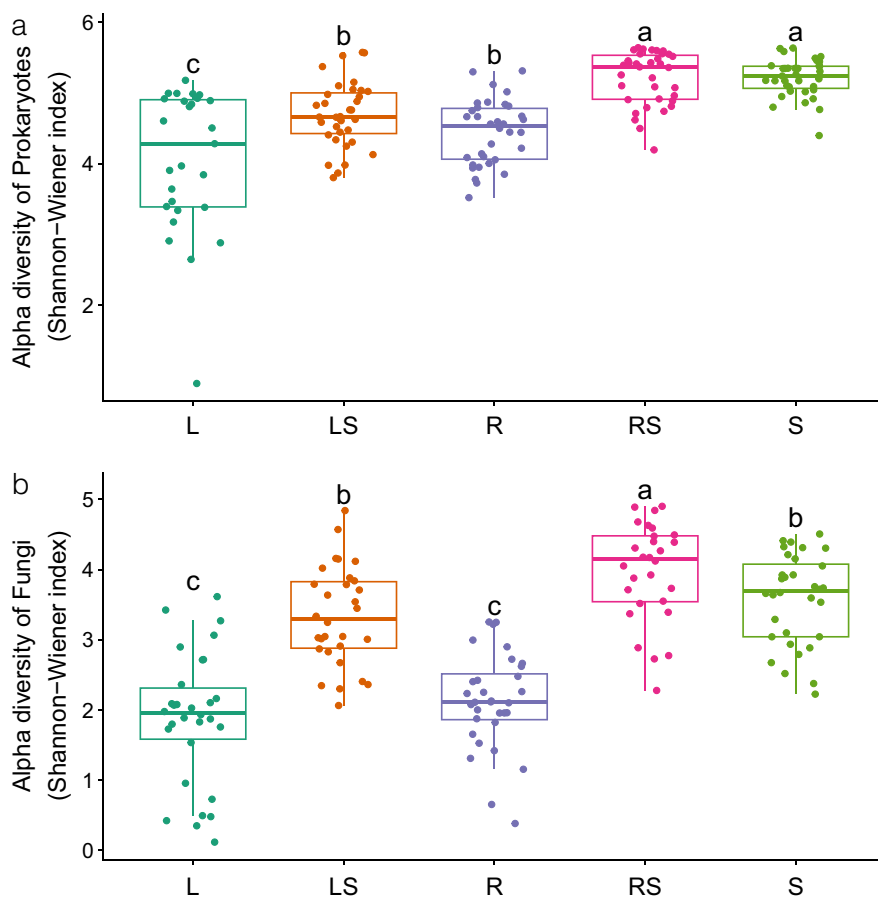
The alpha diversity patterns differed notably between prokaryotes and fungi (Fig. 1). For fungal communities, RS exhibited the highest Shannon–Wiener index value (Mean: 3.97), which was significantly greater ( $P < 0.05$ , ANOVA with Duncan’s new multiple range test) than S (Mean: 3.58). However, no significant difference was observed between RS and S in prokaryotic communities ( $P > 0.05$ ). When considering the endophytes, the alpha diversity of prokaryotes in R (Mean: 4.44) was significantly larger ( $P < 0.05$ ) than

that in L (Mean: 4.04). However, such a discrepancy was not observed within the fungal communities.

#### Beta diversity of prokaryotes and fungi among plant compartments and soil

To investigate the non-random patterns of prokaryotic and fungal communities across various plant compartments, we first computed RC-Bray metric. Then, the matrix was converted to distances between samples from any two compartments in each plant individual. We summarized the percentages of significant values ( $< -0.95$  or  $> 0.95$ ) against all the values related to each compartment. For example, the “L” in the results (Fig. 2a) denotes the percentage of significant values in “L vs LS”, “L vs RS”, “L vs R” and “L vs S” divided by the total values in these four groups. The results showed that RS in prokaryotes has the highest value (12.2%) compared to other compartments (Fig. 2a). It is also higher than the value of RS in fungi (3.8%). For L and LS, the value was also higher in prokaryotes than in fungi (Fig. 2a), meaning that prokaryotic communities in L, LS and RS have stronger non-random patterns than fungal communities. To further reveal which type of selection effect dominated the non-random patterns in prokaryotes, we calculated the phylogenetic beta diversity for prokaryotes along with null model, i.e. betaNRI. Notably, prokaryotes displayed strong homogeneous selection effects from RS to LS and from S to RS (Fig. 2b). The communities from S to RS, LS or L all underwent strong variable selections. Furthermore, clustering analysis demonstrated distinct community similarities among plant compartments for prokaryotes and fungi (Fig. S5). In summary, prokaryotes and fungi exhibited diverse community assembly patterns across different compartments and bulk soil. Moving forward, we conducted PerMANOVA analysis based on the Bray–Curtis metric to examine whether plant species and compartments significantly influenced microbial community structures. All plant species, compartments, and their interactions exhibited significant effects on the structure of prokaryotic communities (Plant species:  $R^2=0.06$ ,  $F=2.75$ ,  $P=0.001$ ; Compartment:  $R^2=0.33$ ,  $F=23.45$ ,  $P=0.001$ ). Similar patterns were also detected for fungal community structure (Plant species:  $R^2=0.15$ ,  $F=6.96$ ,  $P=0.001$ ; Compartment:  $R^2=0.19$ ,  $F=13.38$ ,  $P=0.001$ ).

**Fig. 1** Microbial alpha diversity. Different lowercase letters denote significant differences among different groups tested by ANOVA analysis with Duncan's new multiple range test using duncan.test function of agricolae package ( $P < 0.05$ ). L: leaf endophyte; LS: phyllosphere; R: root endophyte; RS: rhizosphere; S: bulk soil

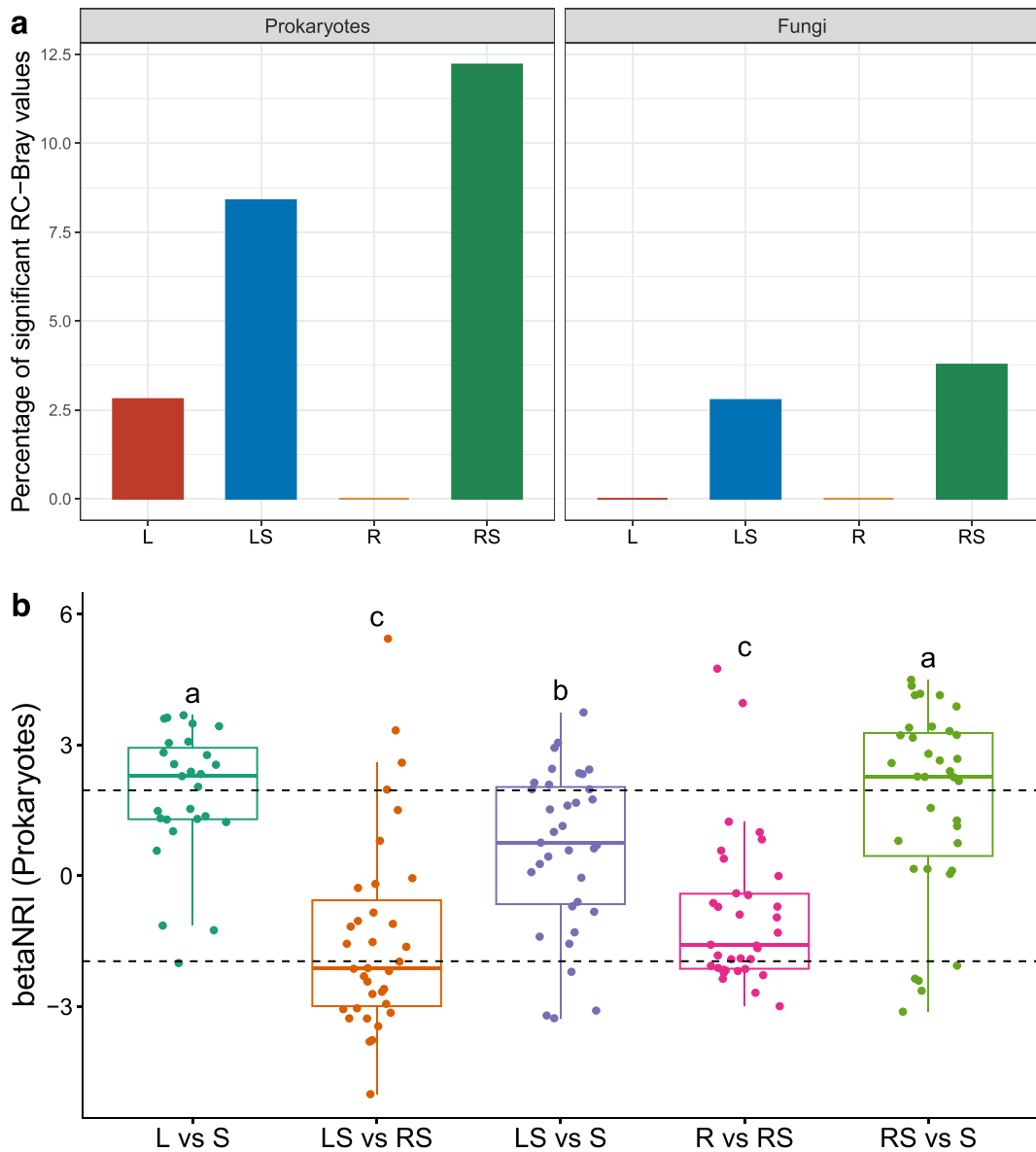


### Microbial co-occurrence patterns across plant compartments and soil

Species co-occurrence pattern is an important community characteristic that can connect the non-random patterns of communities to species distributions and traits. The network analyses were conducted for prokaryotes and fungi to reveal the species co-occurrence relationships. Under the same network construction conditions, the prokaryotic network (Fig. 3a) showed higher density (0.086) and average degree (23.2) than the fungal network (density: 0.016; average degree: 6.2; Fig. 3d). The co-occurrence patterns of prokaryotic OTUs in compartment L were particular, with high abundances (Fig. 3b) of OTUs in module 2 and their characteristic traits (Fig. 3c), which can be attributed to the unique leaf habitat provided for prokaryotes. Notably, a substantial proportion of OTUs in module 2 within compartment L possessed the characteristic traits of anaerobic

chemoheterotrophy and fermentation (Fig. 3c), while module 1 was dominated by taxa exhibiting aerobic chemoheterotrophy with high abundance in compartments LS and RS. For fungi, within the three modules containing the highest number of OTUs, module 3 exhibited a greater abundance of taxa in compartment L compared to the other modules (Fig. 3e), alongside relatively higher ratios of potential plant pathogens (Fig. 3f). Individual networks were constructed for each compartment, allowing for a comparative analysis of the impact of plant niches on co-occurrence relationships. Noteworthy observations include the presence of numerous unique edges in RS-L, RS-LS, and R-L within prokaryotic networks (Fig. 3g). As for fungi, the intersection groups RS-LS, RS-S, and LS-L displayed the largest number of unique edges (Fig. 3h). These differences further underscore the fact that the selection effects of niches on fungal co-occurrence relationships are more intricately associated with spatial distances between plant compartments, while



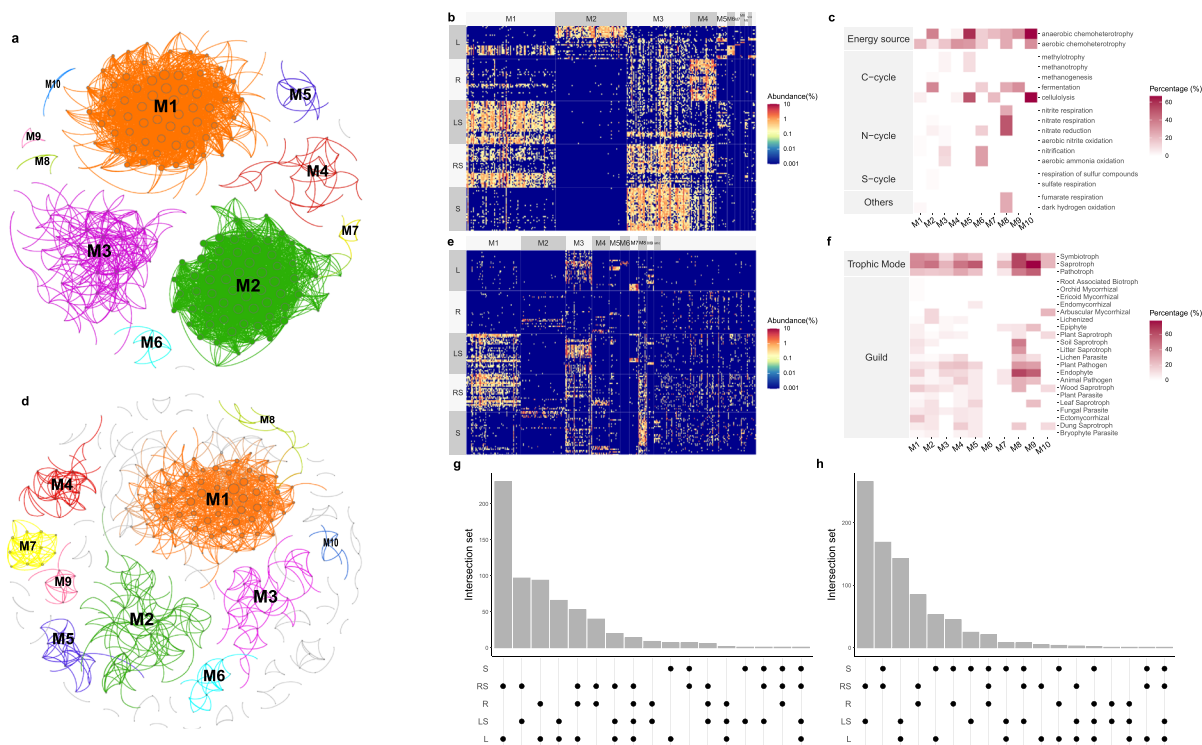


**Fig. 2** Non-random patterns of communities across plant compartments and soil. **a**: RC-Bray matrix was converted to distances between samples from any two compartments in each plant individual. The percentage denote significant values ( $<-0.95$  or  $>0.95$ ) against all the values related with each compartment. For example, the value of L represents the percentage of significant values in “L vs LS”, “L vs RS”, “L vs R” and “L vs S” divided by the total values in these four groups. **b**: Each point represents the value of the indicator between two niches (as shown on the horizontal axis) derived from an indi-

vidual plant. The horizontal dotted line represents the approximate 95% confidence intervals around the null expectation ( $\text{Mean} \pm 1.96\text{SD}$ ). Different lowercase letters denote significant differences among different groups tested by ANOVA analysis with Duncan’s new multiple range test using `duncan.test` function of `agricolae` package ( $P < 0.05$ ). betaNRI: beta net relatedness index; RC-Bray: Bray–Curtis-based Raup–Crick index; L: leaf endophyte; LS: phyllosphere; R: root endophyte; RS: rhizosphere; S: bulk soil

the coexistence relationship of prokaryotes exhibits stronger site specificity. In summary, the coexistence

patterns across plant compartments exhibited significant variations between prokaryotes and fungi.



**Fig. 3** Network analysis of prokaryotic and fungal communities. **a**: Network visualization of all the prokaryotic communities using Gephi software. **b**: Prokaryotic abundance in different modules. **c**: Prokaryotic OTU percentages with different functions (predicted with the FAPROTAX database) in modules. **d**: Network visualization of all the fungal communities using Gephi software. **e**: Fungal abundance in different modules. **f**: Fungal OTU percentages with different functions

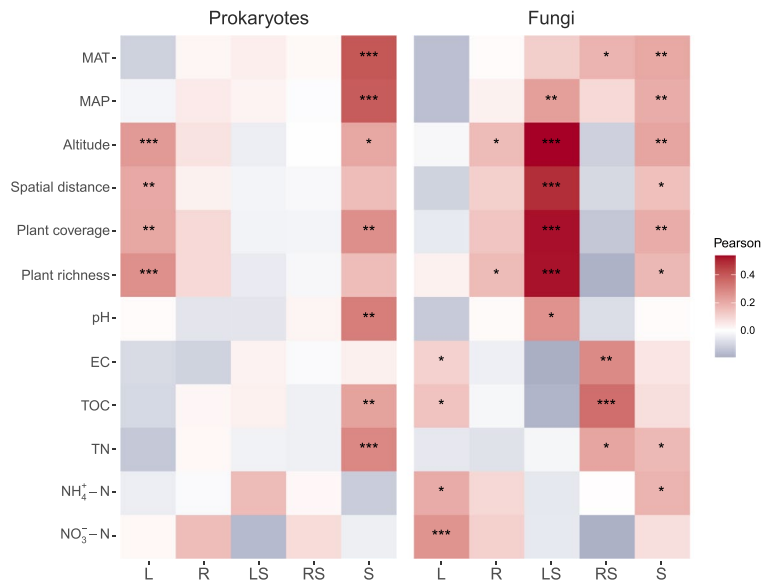
(predicted with the FUNGuild database) in modules. **g**: The edge intersections of five networks, constructed for prokaryotic communities of five compartments, respectively. **h**: The edge intersections of five networks, constructed for fungal communities of five compartments, respectively. L: leaf endophyte; LS: phyllosphere; R: root endophyte; RS: rhizosphere; S: bulk soil

The factors shaping prokaryotic and fungal communities in different compartments

To explore the effects of environmental factors on microbial communities in different plant compartments, Mantel test and partial Mantel test were used (Fig. 4, Figs. S7 and S8). The results demonstrated that fungal communities in LS were significantly correlated with plant richness ( $R=0.52$ ,  $P=0.001$ , partial Mantel test with Pearson correlation), plant coverage ( $R=0.53$ ,  $P=0.001$ ) and spatial distance ( $R=0.48$ ,  $P=0.001$ ) (Fig. 4b and Fig. S7). In prokaryotes, these environmental factors mainly affected the communities of L (Fig. 4a), while only a limited number of factors were significantly correlated with prokaryotic communities of R, LS, and RS. When considering three species (collected from

three regions) of *Stipa* genera, the partial Mantel test still exhibited a similar trend (Fig. S8). Subsequently, VPA was performed to quantify the relative contributions of different factors to the changes in microbial communities of different plant compartments (Fig. 5). The result showed that climate factors alone contributed the most to the variations in fungal communities of LS (5.7%) across all plant compartments. Plant (phylogenetic distance) alone accounted for a higher degree of variation in fungal communities of L (24.8%) compared to other factors and compartments. Notably, the interactions of plant and climate factors explained over 10% of the variations in communities within S (prokaryotes 15.6%, fungi 14.5%), L (fungi 18.7%), and LS (fungi 22.3%). In summary, it is apparent that fungal communities are more

**Fig. 4** Partial Mantel test between Bray–Curtis distance matrix and environmental factors. Environmental factor data were converted into Euclidean distances for partial Mantel test analysis. L: leaf endophyte; LS: phyllosphere; R: root endophyte; RS: rhizosphere; S: bulk soil. \*:  $P < 0.05$ , \*\*:  $P < 0.01$ ; \*\*\*:  $P < 0.001$ . MAT: mean annual temperature; MAP: mean annual precipitation; EC: electrical conductivity; TOC: total organic carbon; TN: total nitrogen



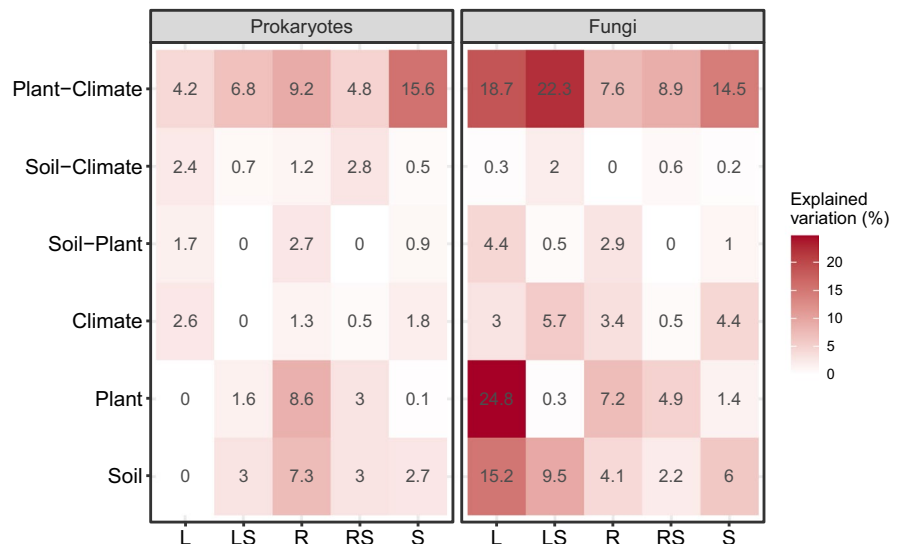
profoundly influenced by plant and climate factors compared to prokaryotic communities.

Enriched genera and guilds in plant compartments compared to bulk soil

In order to further explore the selective effects of different plant compartments on the soil species pool, a GLMM based on the beta distribution family was utilized to model the relative abundance (0–1) of various genera or guilds (Fig. 6). Concerning the 16S rRNA data (Fig. 6a), the genera *Xiphinematobacter*

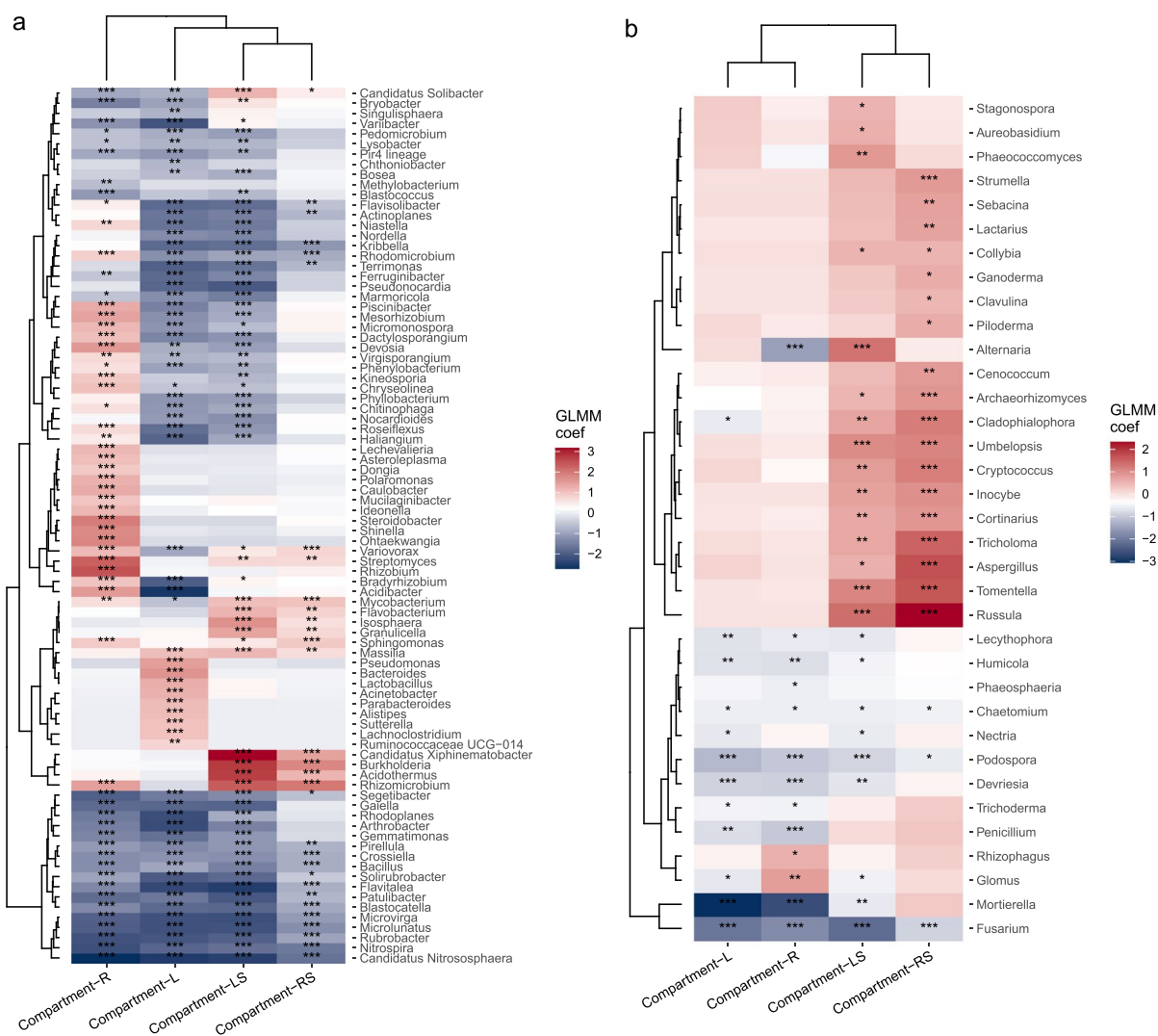
(LS:  $\text{coef} = 3.18$ ,  $P = 3.33\text{e-}40$ ), *Bacteroides* (L:  $\text{coef} = 1.63$ ,  $P = 9.60\text{e-}10$ ), *Rhizomicrobium* (RS:  $\text{coef} = 1.99$ ,  $P = 6.19\text{e-}18$ ), and *Rhizobium* (R:  $\text{coef} = 2.47$ ,  $P = 4.06\text{e-}26$ ) exhibited the highest relative abundances in different plant compartments compared to S. Notably, compartment R displayed a distinct overall trend for prokaryotes compared to the other groups according to the clustering analyses of model coefficients (Fig. 6a), while in the fungal results, R demonstrated a more similar trend to L (Fig. 6b). Regarding fungi data, LS and RS exhibited a greater number of significantly upregulated

**Fig. 5** Variation partitioning analysis for different plant compartments. L: leaf endophyte; LS: phyllosphere; R: root endophyte; RS: rhizosphere; S: bulk soil



genera compared to L and R, such as *Russula* (LS: coef=1.37,  $P=2.19\text{e-}06$ ; RS: coef=2.34,  $P=1.65\text{e-}14$ ). The selective effects exerted by different plant compartments on microorganisms may be closely related to their functional traits. Subsequently, the GLMM was further employed to analyze functional guilds based on functional predictions (Fig. S9). We found that the changes of guilds from soil to different plant compartments were consistent with the results of genera. These results reflected the selective effects

specific to certain plant compartments. For instance, L favored a larger proportion of guilds related to anaerobic chemoheterotrophy (coef=2.09,  $P=1.33\text{e-}12$ ) and fermentation (coef=1.94,  $P=4.84\text{e-}14$ ) among prokaryotes. R favored a greater presence of guilds associated with cellulolysis (coef=1.29,  $P=1.74\text{e-}10$ ) and nitrogen fixation (coef=0.99,  $P=2.81\text{e-}12$ ) among prokaryotes. In terms of fungal guilds, LS and RS showed the highest increase in the animal-associated guild (LS: coef=2.15,



**Fig. 6** Differential abundance test of genera across plant compartments and bulk soil. **a**: The effects of plant compartments on the genera of prokaryotes relative to bulk soil revealed by the generalized linear mixed model (GLMM). The family function is the beta distribution function, and the link function is

the 'logit' function. The reference of all the compartments is bulk soil (S). **b**: The effects of plant compartments on the genera of fungi revealed by the GLMM. L: leaf endophyte; LS: phyllosphere; R: root endophyte; RS: rhizosphere. \*:  $P < 0.05$ , \*\*:  $P < 0.01$ , \*\*\*:  $P < 0.001$

$P=1.46e-16$ ) and Ectomycorrhiza\_exploration\_type-contact guild (RS: coef=2.36,  $P=1.74e-15$ ).

## Discussion

Different community assembly patterns and mechanisms between prokaryotes and fungi in plant compartments

Many investigations have elucidated the strong selections of plant compartments on microbial communities. However, the distinctions in the assembly of prokaryotic and fungal communities across various plant compartments at a large spatial scale remain elusive. Within the realm of natural grassland plants, our study revealed divergent patterns in the alpha diversity (Fig. 1), beta diversity (Fig. 2, Figs. S5, and S6), and species co-occurrences (Fig. 3) between prokaryotes and fungi. Notably, prokaryotes exhibited a higher prevalence of shared core OTUs across plant species compared to fungi (Fig. S3 and S4). Furthermore, the geographical factors and soil physical–chemical properties contributing to this divergence differed (Fig. 4 and Fig. 5). Significantly, our research addressed a critical gap in understanding the community assembly mechanisms for prokaryotes and fungi by investigating the interconnections among plant compartments (Figs. 2, and 3, and Fig. S6) within natural grassland plants. Many studies have demonstrated that the factors shaping prokaryotic and fungal communities differ (Coleman-Derr et al. 2016; Tkacz et al. 2020; Li et al. 2022). However, disparate methodologies and materials have precluded the establishment of consistent conclusions. Our findings indicated that prokaryotic community assembly patterns varied from one compartment to another (Fig. 2), closely intertwined with abiotic factors and prokaryotic functions and traits specific to different plant compartments. Notably, our results verified the first hypothesis that the differences of community assembly between prokaryotes and fungi could be reflected in the selection strength across different plant compartments. Nevertheless, the current dataset does not permit a comprehensive dissection of the relative contributions of abiotic factors influencing species movements. An example of controlled studies illuminating the selective mechanisms across plant organs is the profound impact of root exudates

on microbial selection, resulting in substantial shifts in the microbial community from S to RS (Cheps-ergon and Moleleki 2023). In addition, it has been observed that flavones in root exudates can enrich rhizosphere Oxalobacteraceae, thereby enhancing maize performance under nitrogen deprivation (Yu et al. 2021). We contend that the findings of this study warrant further controlled experiments with ingenious design to elucidate the dynamics of species movement among different plant compartments, including transitions from S to RS, from RS to R, and from S to LS.

Geographical factors, soil physicochemical properties and plant genotypes together shape plant-microbiome patterns

Local dispersal plays a pivotal role in the assembly of the phyllosphere microbiome, and demographic factors such as the identity of nearby neighbors and biomass or age are significant determinants of phyllosphere microbiome diversity (Meyer et al. 2022). This may partly account for the strong correlations between plant biomass, coverage, and the fungal phyllosphere microbiome (Fig. 4). It is speculated that the dispersal from nearby neighbor plants significantly influences the assembly of the fungal community. However, these findings were insufficient in explaining why the prokaryotic communities in LS and RS exhibited homogeneous selection effects. The source of phyllosphere microorganisms is diverse, including soil, air, neighboring plants, herbivores, and insects (Xu et al. 2022). Nonetheless, the source of prokaryotic communities in RS is generally considered to be the soil (Trivedi et al. 2020). Thus, it is inferred that the homogeneous selection effects on prokaryotic communities between LS and RS are likely determined primarily by the plant secretion metabolites, as the plant exudates, particularly secondary metabolites, exert substantial selective effects on prokaryotes (Pang et al. 2021). Although we cannot definitively determine which sources primarily shape the prokaryotes and fungi, according to our results, it is hypothesized that fungi are more influenced by surrounding soil properties than prokaryotes, as fungal communities are subject to more stochastic effects. Previous studies have demonstrated that plant genotypes impose strong selection on microbiomes (Morella et al. 2020). Our findings also verified that plant

genotypes had a greater influence on fungi compared to prokaryotes (Fig. 5). In addition to the plant compartments considered in this study, the heterogeneity (differences in habitats) of aboveground plant organs is generally underestimated (Junker and Keller 2015). Consequently, future investigations should focus on the heterogeneity of the plant itself. Our findings were consistent with previous finding that stochastic processes predominantly govern the assembly of soil fungal communities in grasslands in northwestern China (Guo et al. 2022). Furthermore, our research supplements existing studies on grasslands by suggesting that the relationships between fungi and compartments are likely to be less deterministic relative to prokaryotic communities within different grass compartments. Additionally, the fluctuations in grassland vegetation biomass due to climate and soil conditions can indirectly influence microbial communities by affecting the grass root physiology and morphology. For example, the spatial and morphological traits of grass roots can greatly impact the composition of the rhizosphere fungal community (Sweeney et al. 2020; Hennecke et al. 2023). It is noteworthy that the design of this study is unbalanced across the three sampling areas. We carefully addressed the potential impact of this imbalance on the interpretation of the results related with soil, space and climate factors through analysis using multiple methods (Figs. 4 and 6, Fig. S3, S4 and S8). However, we cannot guarantee that environmental factors in different regions will have similar effects on multiple plant species other than those under the *Stipa* genus sampled in three regions of this study. Therefore, future related studies may need to increase the number of sampled plant species to enhance confidence. In summary, geographical factors, soil physicochemical properties, and plant genotypes are critical determinants influencing the assembly of prokaryotes and fungi in plant compartments, and this supported our second hypothesis.

Functional traits of prokaryotes and fungi partly determine the assembly patterns across plant compartments

Functional analysis highlighted the linkage between prokaryotic assembly in plant compartments and their functional traits (Figs. 3c, and 6c, and Fig. S10). The phyllosphere microbiome has been recognized

to possess great importance in regard to plant traits (Kembel et al. 2014; Zhu et al. 2022). It is more conserved than previously acknowledged and predominated by generalist bacteria that have adapted to environmental heterogeneity through evolutionarily conserved traits (Massoni et al. 2020). Schäfer et al. investigated the interactions among the phyllosphere microbiota, established genotype–phenotype relationships, and revealed the significance of microbial interactions and functions in the phyllosphere (Schäfer et al. 2022). Functional differentiation is the primary reason for the heterogeneous selection between L and LS for prokaryotes (Fig. 2c). We found that only the prokaryotes in L exhibit strong selection effects due to the special niche within the leaf (Fig. 3). The strong phylogenetic turnover of prokaryotic communities between S and RS, as shown by betaNRI (Fig. 2a), suggested that microbes from S to RS may be attributed to the conserved adaptive mechanisms and functional traits related to the rhizosphere, such as high ABC transporters in RS relative to S (Fig. S10). Functional redundancy is crucial for maintaining ecosystem functions. Prokaryotes generally exhibit extreme complexity in terms of the redundancy for each function, such as the ability to utilize substrates and produce hormones promoting plant growth (Trivedi et al. 2020). It is important for future studies to investigate how species replacement occurs within a plant compartment related to functional redundancy.

Compared to prokaryotes, we know little about the metabolic traits of fungi. According to our results, LS and RS had more OTUs belonging to ectomycorrhizal and endophyte in M1 of the network than in M2 (Fig. 3) and higher abundances of those guilds than other compartments (Fig. 6), implying that fungal traits are also crucial to community assembly. However, it is still unclear to what extent fungal functional redundancy can affect their assembly patterns in plant compartments. In addition to the factors considered in this study, other factors, including plant growth stages, can exert strong selection effects on the microbial community. It is unknown whether the findings of this study can be extrapolated to other ecosystem studies. Therefore, this topic deserves further study to disentangle the effects of plant growth dynamics and microbial dispersal on plant-microbiome interactions. Additionally, more plant samples from broader species, genera, or orders may be required to

decipher prokaryotic and fungal community assembly processes and key driving factors (Toju et al. 2019). Therefore, functional and phenotypic characteristics constitute the biological basis for the differential distribution of prokaryotes and fungi in plant compartments.

## Conclusion

This study revealed significant distinctions in the community assembly patterns and potential mechanisms between prokaryotes and fungi in multiple compartments of grassland plants. Prokaryotes and fungi showed large differences in the core OTUs distribution, alpha diversity, beta diversity and species co-occurrences relationships. Compared to other compartments, phyllosphere fungi were more strongly influenced by geographical and vegetation factors. In contrast, leaf endophytes in prokaryotic communities were more influenced by various environmental factors. Phyllosphere and rhizosphere prokaryotic communities were subjected to strong homogeneous selection. These differences between fungi and prokaryotes are closely linked to their functional traits and biological characteristics. Generally, the assembly of fungal communities in different plant compartments is primarily influenced by local environmental factors such as soil and vegetation, while prokaryotic communities in different plant parts is more closely related to the plants themselves. Our sampling was conducted in protected, non-grazed grasslands. It is valuable to investigate whether plant–microbe associations under different grazing conditions yield consistent conclusions with the present study. Furthermore, our findings provide guiding insights for practical applications. For instance, when applying bacterial and fungal agents, their adaptability and specificity in different plant compartments should be considered.

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**Authors' contribution** The initial idea was conceived by Chi Liu, Xiangzhen Li and Minjie Yao. The experiments were conducted by Chi Liu, Na Li and Minjie Yao. The data analysis was performed by Chi Liu, Na Li and Min Xiao. The original

manuscript was written by Chi Liu and Minjie Yao, and revised by all the authors.

**Data availability** The original sequences are available in National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the BioProject accession number PRJNA782821.

## Declarations

**Conflict of interests** The authors declare that they have no known conflicting interests that could affect the work reported herein.

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