Ecology of microbiomes in natural populations of *Lotus corniculatus*

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Declaration of author contributions

This dissertation "Ecology of microbiomes in natural populations of *Lotus corniculatus*" is my PhD project at University of Tübingen, supervised by Prof. Dr. Eric Kemen and Prof. Dr. Oliver Bossdorf. **Chapters II-IV** of this dissertation include three scientific manuscripts which will be published, each with co-authorships. Following is the declaration of author contributions for each chapter:

Chapter II

Organ-specific microbiomes in natural *Lotus corniculatus* populations: Metacommunity dynamics in the plant endosphere

Katrina Lutap, Juliana Almario, Maryam Mahmoudi, Frank Reis, Oliver Bossdorf, and Eric Kemen

Author Contributions: E.K., O.B., J.A., and **K.L.** conceptualized this work. **K.L.**, F.R., and J.A. collected and processed samples from the field. **K.L.** performed the experiments and data analysis, with M.M. in the machine-learning analysis. **K.L.**, M.M., and E. K. wrote the manuscript, with contributions from all authors. All authors read and approved the final manuscript.

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Chapter III

Organ-specific filtering by abiotic and biotic environmental factors shapes distinct yet overlapping microbial communities across *Lotus corniculatus* roots, shoots, flowers, and seeds

Katrina Lutap, Oliver Bossdorf, and Eric Kemen

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Chapter IV

Plant age and genotype explain variation in the microbiome of natural *Lotus corniculatus* populations

Frank Reis, **Katrina Lutap**, Walter Durka, Jun Hee Jung, Eric Kemen, and Oliver Bossdorf

Author Contributions: O.B., E.K., F.R., and **K.L.** designed the study. F.R. and **K.L.** planned and conducted the field sampling, lab work, and data analysis. W.D. helped with the library preparation and bioinformatics of the ddRAD sequencing. F.R. performed the *Fusarium* experiment and analyzed the data with J.H.J.'s support. F.R., **K.L.**, and O.B. wrote the manuscript with input from all authors. All authors read and approved the final manuscript.

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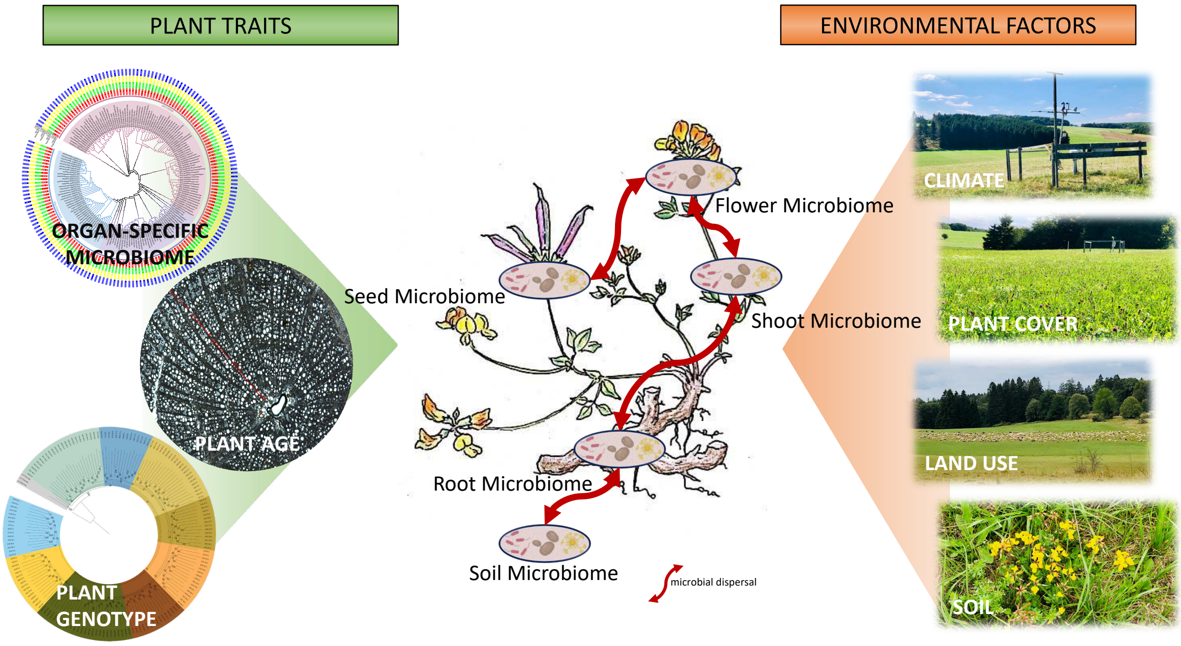
GRAPHICAL ABSTRACT



ABSTRACT

Plants form complex and dynamic interactions with diverse microbial communities of beneficial, neutral, and pathogenic microorganisms spanning multiple phylogenetic kingdoms. The structures of plant-associated microbial communities vary due to a broad range of factors such as plant host, environment, and other assembly processes occurring at multiple tempo-spatial scales. In natural environments plant microbial communities are constantly exposed to such abiotic and biotic perturbations and ecological processes. Thus, to attain a systemic understanding of the ecology of plant microbiomes, it is essential to study microbial communities associated with plants in natural environments and understand the assembly processes that influence patterns of microbial community structures. In this study we examined the ecology of microbiomes in natural populations of *Lotus corniculatus*. Specifically, we investigated the effect of plant organs, plant age and genotype, as well as environmental factors on the diversity and composition of the natural plant microbiome. We collected soil and plant samples for four consecutive years across seven wild populations in Southern Germany and analyzed the bacterial, fungal, and eukaryotic communities associated with soil and *L. corniculatus* roots, shoots, flowers, and seeds.

In this study we established the organ-specificity of plant endophytic communities. Therefore, we used the framework of metacommunity theory of ecology to understand assembly processes that shape community structures by defining microbial communities associated with the roots, shoots, flowers, and seeds as distinct communities linked by dispersal. The plant microbiomes are shaped by selective filtering of the plant organs as demonstrated by the differential abundance and persistence of microbes in roots, shoots, flowers, and seeds. The plant organs can be distinguished with separator microbes that we identified using multi-class support vector machine models. The community structure of the plant microbiomes is further influenced by microbial interactions, as well as by local abiotic and biotic factors. These distinct yet overlapping organ microbial communities are linked and shaped by transmission of microorganisms among the plant compartments and the local environment.

In this study we also observed spatial and temporal variation in *L. corniculatus* microbiomes at multiple scales, therefore we examined how abiotic and biotic factors in the local environment influence the structure of natural microbiomes. We show that plant microbiomes are shaped by a set of environmental factors that are distinct to each plant compartment. Moreover, we found that soil temperature seasonality, soil microbiome composition, air temperature seasonality, plant community richness, and grazing influence the structure and microbial interactions in the plant organs. These environmental factors are different in their association with each compartment's microbiomes, possibly influencing dispersal decisions of microorganisms and consequently contribute in shaping distinct yet overlapping microbiomes across plant organs. We also detected organ-dependent environmental perception in generalist plant microbes as well as in organ biomarker microbes.

Finally, in this study we observed that the natural *L. corniculatus* populations exhibit variations in age and genotype, therefore we investigated the effect of these plant host-related factors on the associated microbial communities. We show that plant age and genotype significantly influence the structure of associated microbiomes. While a large number of taxa are shared among plant genotype and age groups, we identified biomarker microbes that distinguish between host genotypes or age groups through differential abundance analysis. Based on these findings, we isolated *Fusarium* from wild *L. corniculatus* plants and showed that this plant pathogen, which is significantly more abundant in one genotype, elicited stronger response in the offspring of plants of this genotype compared with the offspring of plants of another genotype.

Understanding the ecological processes that shape microbial assembly and community dynamics in natural environments is crucial in leveraging the benefits of plant microbiomes on plant productivity, resilience, and pathogen defense. Knowledge about the organ-specific responses of plant microbiomes to abiotic and biotic perturbations will provide an important framework for targeted control of plant microbiomes in the context of global climate change. Observed patterns on dispersal decisions or habitat choice of microorganisms based on organ-dependent environmental cues and microbial interactions also advance our insight on how beneficial microbes or pathogens survive and persist on specific plant microhabitats or environmental conditions. Finally, knowledge on how plant age and genotype impact associated microbial communities, as well as detection of microbial biomarkers at specific plant age or genotype, provide clues as to how the spread of pathogen can be mitigated in the future or how infection-resistant plants can be developed.

ZUSAMMENFASSUNG

Pflanzen bilden komplexe und dynamische Interaktionen mit vielfältigen mikrobiellen Gemeinschaften aus, die nützliche, neutrale und pathogene Mikroorganismen aus mehreren phylogenetischen Reichen umfassen. Die Struktur pflanzenassoziierter mikrobieller Gemeinschaften variiert aufgrund einer Vielzahl von Faktoren, wie etwa der Wirtspflanze, der Umwelt und anderen Zusammensetzungsprozessen, die auf mehreren zeitlichen und räumlichen Skalen stattfinden. In natürlichen Lebensräumen sind Pflanzenmikrobiome fortlaufend abiotischen und biotischen Einflüssen sowie ökologischen Prozessen ausgesetzt. Um ein systemisches Verständnis der Ökologie von pflanzlichen Mikrobiomen zu erlangen, ist es daher essenziell, mikrobiellen Gemeinschaften in natürlichen Habitaten zu untersuchen und die Prozesse zu verstehen, die die Muster ihrer Zusammensetzung beeinflussen. In dieser Arbeit untersuchen wir die Ökologie von Mikrobiomen in natürlichen Populationen von *Lotus corniculatus*. Wir haben den Einfluss von Pflanzenorganen, Alter und Genotyp der Pflanze sowie von Umweltfaktoren auf die Diversität und Zusammensetzung des natürlichen Pflanzenmikrobioms analysiert. Dafür haben wir in vier aufeinanderfolgenden Jahren Boden- und Pflanzenproben aus sieben Wildpopulationen in Süddeutschland gesammelt und die mikrobiellen Gemeinschaften bestehend aus Bakterien, Pilzen und anderen Eukaryoten im Boden sowie in Wurzeln, Sprossen, Blüten und Samen von *L. corniculatus* untersucht.

Wir konnten in dieser Arbeit nachweisen, dass endophytische mikrobielle Gemeinschaften organspezifisch sind. Angelehnt an die ökologische Theorie der Metagemeinschaft haben wir die Zusammensetzung mikrobieller Gemeinschaften analysiert, indem wir die Gemeinschaften der Wurzeln, Sprossen, Blüten und Samen als voneinander unterscheidbare, aber durch Ausbreitung verbunden betrachtet haben. Das Mikrobiom der Pflanze wird durch eine selektive Filterung der jeweiligen Pflanzenorgane geformt, was sich sowohl in der unterschiedlichen Häufigkeit als auch in der Beständigkeit von Mikroorganismen in den verschiedenen Organen widerspiegelt. Mittels bioinformatischer Analysen (multi-class support vector machine models) ist es uns gelungen Mikroben (Biomarker) zu identifizieren die eine Unterscheidung der Pflanzenorgane ermöglichen. Darüber hinaus wird die Struktur dieser Mikrobiome durch mikrobielle Interaktionen sowie durch lokale abiotische und biotische Faktoren beeinflusst. Deutlich differenzierbare, dennoch teilweise übereinstimmende organspezifische Mikrobiome sind daher über den Austausch von Mikroorganismen zwischen Pflanzenteilen und der Umgebung miteinander verbunden.

Wir haben außerdem räumliche und zeitliche Variationen der Mikrobiome von *L. corniculatus* auf mehreren Ebenen beobachtet und haben daher untersucht, wie biotische und abiotische Faktoren deren Struktur beeinflussen. Unsere Ergebnisse zeigen, dass Pflanzenmikrobiome durch ein spezifisches Set an Umweltfaktoren geprägt werden, das für jedes Pflanzenkompartiment unterschiedlich ist. Insbesondere wirken sich die saisonale Bodentemperatur, die Zusammensetzung des Bodenmikrobioms, die saisonale Lufttemperatur, die Artenvielfalt der Pflanzengemeinschaft sowie Beweidung auf die Struktur und mikrobielle Interaktionen in den Pflanzenorganen aus. Diese Umweltfaktoren beeinflussen die Mikrobiome der einzelnen Kompartimente unterschiedlich, was möglicherweise die Ausbreitung von Mikroorganismen beeinflusst und zur Ausbildung distinkter, jedoch überlappender Mikrobiome beiträgt. Wir konnten zudem einen organspezifischen Einfluss der Umwelt sowohl bei generalistischen Mikroben als auch bei organ-spezifischen Mikroben (Biomarker) feststellen.

Da die natürlichen *L. corniculatus*-Populationen Unterschiede in Alter und Genotyp aufwiesen, haben wir auch den Einfluss dieser wirtsspezifischen Faktoren auf die assoziierten mikrobiellen Gemeinschaften untersucht. Wir konnten zeigen, dass sowohl das Alter als auch der Genotyp der Pflanzen einen signifikanten Einfluss auf die Mikrobiomstruktur haben. Obwohl viele Taxa zwischen Alters- und Genotyp-gruppen geteilt werden, konnten wir durch differentielle Häufigkeitsanalysen Biomarker-Mikroben identifizieren, die sich zwischen verschiedenen Altersgruppen oder Genotypen unterscheiden. Basierend auf diesen Erkenntnissen haben wir *Fusarium* aus wilden *L. corniculatus*-Pflanzen isoliert und konnten zeigen, dass dieses Pflanzenpathogen, das in einem der Genotypen signifikant häufiger vorkam, eine stärkere Abwehrreaktion in den Nachkommen dieses Genotyps auslöst während Nachkommen anderer Genotypen nur schwache Reaktionen gezeigt haben.

Das Verständnis ökologischer Prozesse, die mikrobielle Gemeinschaftsbildung und Dynamik in natürlichen Umgebungen formen, ist entscheidend, um das Potenzial von Pflanzenmikrobiomen bezüglich Produktivität, Resilienz und Krankheitsabwehr von Pflanzen zu nutzen. Erkenntnisse über organspezifische Reaktionen von mikrobiellen Gemeinschaften auf abiotische und biotische Einflüsse liefern ein wichtiges Fundament, um Pflanzenmikrobiome künftig im Kontext des globalen Klimawandels gezielt zu steuern. Beobachtete Muster in der Ausbreitung oder Wahl des Habitats von Mikroorganismen – beeinflusst durch organspezifische Umweltreize und mikrobielle Interaktionen – vertiefen unser Verständnis darüber, wie nützliche Mikroben oder Pathogene auf bestimmten Mikrohabitaten der Pflanze oder unter spezifischen Umweltbedingungen überleben und fortbestehen. Schließlich bietet das Wissen über den Einfluss von Alter und Genotyp der Pflanze auf das Mikrobiom sowie die Identifikation mikrobieller Biomarker Hinweise darauf, wie sich die Ausbreitung von Pathogenen in Zukunft eindämmen lässt oder widerstandsfähige Pflanzen gezielt entwickelt werden können.

CHAPTER I

GENERAL INTRODUCTION

Plants are host to complex and dynamic microbial communities composed of beneficial, neutral, and pathogenic microorganisms that belong to different kingdoms of life such as archaea, bacteria, fungi, protists, and viruses. These plant-microbe interactions are mutually beneficial - microorganisms function in plant growth and development, nutrient acquisition, stress tolerance, and pathogen defense, while the host plants provide habitats and resources for growth and survival of the microorganisms (1). Beyond these associations, plant microbiomes have the potential to be used in agriculture to improve plant health and productivity by manipulating and managing the microbes that live on and inside plants. To leverage such advantages and potentials, an in-depth understanding of the ecological processes that shape plant microbial community structures is important.

Current research on plant microbiomes established that the diverse microbial communities associated with plants are spatially and temporally dynamic at multiple scales. The observed spatial and temporal variations in these plant microbial communities can be attributed to factors such as geographic location, soil properties or land use types, plant genotype, plant compartment, seasonality, and plant age or developmental stage (2-7). Given the significance of plant microbiomes, understanding the factors and mechanisms that drive community assembly and variation is essential. In this work I aim to explore various factors that influence the diversity and structure of plant microbiomes in natural populations. Specifically, I examined how the plant host (*i.e.* plant organs, genotype, and age) and the environment (*i.e.* biotic and abiotic factors) drive community variations in plant microbiomes in natural environments.

Plants harbor distinct organ microbiomes.

One of the main deterministic factors that influence microbial community structure is filtering by the host plant. Plant microbiomes are predominantly shaped by plant compartment, with distinct microbial communities associated with different plant organs. Studies on various plant systems established that organ type is the primary factor that explains variation in microbial community structure, more than other spatio-temporal factors such as sample location, biogeography, climate, host genotype or sex, sampling time, developmental stage or plant age, and disease (8-14). These studies demonstrated that belowground microbial communities significantly differ from those aboveground, and among all plant organs, roots consistently host the most diverse microbial communities.

Plant organ communities are unique in composition and function. Roots are mainly colonized by microbial communities that are distinct from those in the surrounding soils but share a subset of these microbial communities (5, 15-17). Microorganisms that reside in the roots often have beneficial roles in plant growth and development, pathogen protection, stress tolerance, and nutrient acquisition (18). Microbial communities in the plant phyllosphere are generally less diverse compared with root microbiomes, and the associated microorganisms function in stress tolerance, plant growth, biocontrol against pathogens, and in ecosystem functioning such as carbon and nitrogen cycles (19-22). Flower-associated microbial communities are one of the least diverse among plant organ microbiomes (10, 23-25). Flowers harbor a unique community of microorganisms that impact plant-pollinator interactions via multiple ways including influencing floral phenotypes and associating with flower visitors (26-28). Community structure variations in flower-associated microbiomes affect floral display and rewards, nectar composition, pollinator fitness and reproduction, and consequently affect plant-pollinator interactions and dispersal of microbes between plants (26, 29). In various plant species, seeds also harbor one of the least diverse microbial communities (30-32). Seed microbiomes influence seed traits, germination, and growth, and consequently fitness and genetic diversity of plants (33, 34). Seeds play important roles during vertical transmission of microorganisms from one plant generation to the next by being the initial source inoculum of plant microbiomes (32-36).

While many studies described distinct microbial communities in plant organs, there remains a need for a comprehensive understanding of the assembly processes that account for these community patterns. Plant microbial communities are shaped by factors acting at multiple scales across time and space - from populations to individual plants and plant organs (37, 38). Plant microbiomes are also interconnected via transmission of microbes at different levels - from microbial sources outside plant hosts (*i.e.* other plants, animals, and the environment) to transmission within the plant tissues (39, 40). Thus, there needs a framework to simultaneously incorporate the different assembly processes occurring at multiple spatio-temporal scales to establish a more complete understanding of the community dynamics in plant microbiomes. Consequently, it is also essential to study plant microbiomes at different spatio-temporal scales, that is, in the context of all plant organs at multiple sampling sites and years.

Environmental factors impact deterministic and stochastic assembly processes in plant microbiomes.

Plant microbial communities are a collective of complex interactions between plant hosts, microorganisms, and the environment. Along with plant-microbiome interactions, abiotic and biotic factors in the local environment impact the structure of plant microbial communities. Factors such as climate (*i.e.* temperature and precipitation) or land use, as well as potential microbial sources such plant cover and soil microbial communities, shape plant microbial communities (41, 42). While environmental factors influence microbial community patterns at broad geographic scales, these same factors are also perceived differentially by individual plant microhabitats (3, 43, 44). Some of these environmental factors are more relevant to belowground soil or root microbiomes, while others can be more impactful to aboveground plant microbiomes (8, 45-47). To gain a more thorough understanding of the influence of environmental factors on plant microbial communities, it is essential to study microbial community dynamics simultaneously across different plant compartments, exposed to natural environmental conditions at various locations for multiple years. Such information will also expand our knowledge on how environmental factors shape distinct microbial communities associated with different plant organs.

In addition to filtering by plant host and environment, stochastic processes (*e.g.* dispersal) are significant ecological drivers of microbial community assembly. Dispersal patterns of microorganisms across spatial and temporal scales contribute to plant microbial community dynamics, whether via horizontal transmission from the environment or vertical transmission from seeds (33, 48, 49). Fluctuations in habitat conditions (*i.e.* nutrient resource, pH, drought, soil type) can alter likelihood of priority effects and dispersal, as well as vertical transmission of microorganisms (36, 50, 51). Subsequently, survival and establishment of transmitted microorganisms in a plant compartment are then dependent on a combination of assembly factors including plant microhabitat or environmental factors (26, 52). To gain deeper insight on plant microbiome dynamics, understanding the effect of environmental factors on the transmission as well as survival and persistence of microorganisms in the different plant organs is critical. Such knowledge will also broaden our grasp on how microorganisms, especially pathogens, shift from roots to aboveground plant organs or vice versa.

Plant age and genotype influence associated microbiomes.

Plant host filtering drives variation in associated microbial communities when the host's genetic and phenotypic variations impact survival and persistence of microorganisms in the plant. Plant genomic loci associated with plant traits such as cell morphology, defense and immune responses, signaling pathways, and secondary metabolism are known to impact the associated microbial communities (53, 54). While plant microbial communities are known to vary with host genotype, the extent of plant genetic control on microbial communities is crucial information, since microbiome heritability offers opportunities for maximizing beneficial plant-microbe associations through plant breeding and evolution. Furthermore, to acquire better insight into the effect of host genetics on associated microbial communities and to leverage its potential application in the field, it is important to study plant populations with adequate genetic diversity in their natural environments.

Plant age is another source of variation in associated microbial communities. As plants go through developmental stages and ages, changes occur in the plants' physical and chemical defense systems, growth rate, reproduction, physiological processes, stress tolerance, metabolite production, and resource allocation (55-61). These physical and chemical changes consequently influence the plant-associated microbial communities. Only a few studies have explored the impact of plant age on microbial communities. Studies on annual plants showed that tree and bark age influence bacterial and fungal communities (62, 63). Studies on annual plants describe microbial community dynamics through their entire life cycle, while on perennial plants it represents only one growing season and thus there may be inter-growing season variations. Therefore, it is important to study plant age effect in natural environments to take into account natural perturbations and variations between growing seasons. A study on perennial trees aged one to 35 years old showed shifts in microbiome composition during development, but the plants were sampled from identical environments (64). Another study on perennial plants in their native ranges reported variation in bacterial communities in roots and leaves, but the plants are only from two to four years of age (2). Therefore, it is essential to study microbial community patterns in perennial plants with broader age range in natural environments.

***Lotus corniculatus* microbiomes in natural environments.**

Plant microbiomes form complex interactions with plant hosts and their environment; therefore, challenges remain in fully understanding the mechanisms that govern assembly, spatio-temporal variations, and intricate interactions in plant microbial communities, especially in natural conditions. Given the high diversity and complexity of microbial communities in their natural habitats, the ecology of microbiomes *in situ* can only be partially conveyed by *in vitro* studies under laboratory conditions (65). To address these challenges, it is necessary to study plant microbial communities in natural environments, with constant exposure to natural perturbations and interactions, biotic and abiotic elements, and natural microbial sources. Research on plant microbial communities in natural environments is also crucial in harnessing the power of microbiomes as tools for sustainable agriculture via strategies to engineer plants with improved health and productivity.

Lotus corniculatus, a common legume species that is widespread in European grasslands, is an ideal system to study microbial communities in natural environments. *L. corniculatus* is known to host diverse microbes. They form symbiotic relationships with nitrogen-fixing bacteria such as *Rhizobium japonicum*, *Mesorhizobium loti*, and *Rhizobium meliloti*, as well as arbuscular mycorrhiza fungi, which improve plant adaptation in adverse habitats (66-69). They also host non-rhizobial endophytes that promote plant growth (70, 71). *L. corniculatus* in natural populations that are continuously exposed to natural perturbations and microbial sources is also a good plant model system to study the impact of environmental factors on plant microbial communities. These perennial flowering plants can adapt to a broad range of natural environments. They can grow on a wide range of soil conditions, can adapt to different climates, can persist upon grazing and mowing, and are hosts various to insects and bees (66, 67, 72, 73). Furthermore, *L. corniculatus* exhibits a wide genetic diversity - significant genetic variation can be detected in accessions from different populations (74).

Plant host and the environment as drivers of variation in natural microbiomes.

The overarching goal of this work is to study the diversity and structure of microbial communities in natural plant populations. Since the plant microbiome is a collective of intricate interactions between the plant host, associated microorganisms, and the local environment, this work endeavors to study these factors - plant organ, genotype, and age, as well as environmental factors - as drivers of variation in natural microbial communities.

Plants are associated with microbial communities that are distinct in different plant organs. **Chapter II** aims for a deeper insight on the ecological processes that account for such observed organ-specific patterns in natural plant microbiomes. Since the assembly processes that shape plant microbial communities are acting at multiple scales across time and space, a framework is necessary to integrate the different deterministic and stochastic processes that drive plant microbial community dynamics. In this study, the assembly processes that shape microbial communities associated with four plant organs at multiple sites and years are synthesized in the context of metacommunity theory of ecology. Metacommunity theory is the study of distinct communities that are linked by dispersal (75). The assembly processes defined in Vellend's conceptual synthesis of community ecology are integrated into the metacommunity theory - selection, drift, and diversification are shaping each of the distinct communities, which are linked by dispersal (76, 77). This metacommunity concept consolidates the effect of the different ecological processes and their interactions across time and space scales on community structures (78, 79). Studies on plant microbial communities in light of metacommunity ecology are few and recent (80-84). In this study the community dynamics of *L. corniculatus* microbiomes are examined in the framework of metacommunity theory of ecology by defining the microbial communities associated with the roots, shoots, flowers, and seeds as distinct communities linked by transmission of microorganisms from within and outside the plant hosts. The plant organs are unique yet interconnected habitats for microbes, influenced by different assembly processes leading to organ-specific microbial communities (1, 20, 23). By using the metacommunity framework, deterministic and stochastic factors influencing the microbial communities at multiple tempo-spatial scales - from plant populations of multiple years to plant organs - are simultaneously integrated in one ecological framework.

In **Chapter III**, the effect of environmental factors is explored to gain a deeper understanding of the observed spatial and temporal variations in plant microbial communities. Studies have shown that temperature is a dominant driver of diversity patterns in plant microbial communities and suggest that warming causes decrease in microbial biomass (85, 86). It has also been shown that soil heat causes rearrangement patterns in their bacterial communities (87). It has been demonstrated that precipitation induces shifts or increase diversity in their microbial communities (88, 89). Studies on the effect of soil moisture on plant microbial communities are essential in disease control, as it has been found that this soil abiotic factor is important in regulating both root and aboveground diseases (90, 91). The impact of land use intensity on both root and aboveground plant microbial communities has been reported (25, 92). Bacterial, fungal, and oomycete communities in roots and shoots have been shown to respond to plant diversity and composition, and it was observed that fungal pathogen diversity increase with plant species richness (46, 93). Studies established that soil microbes are the main source inoculum for plant microbial communities and variations in the soil microbial communities determine disease outcomes in plants (15, 94). In this study, the diversity and structure of microbial communities associated with the different organs of *L. corniculatus* at multiple sites and years are examined to establish the contribution of abiotic and biotic factors in shaping distinct organ microbiomes. Microbial communities in roots, shoots, flowers, and seeds exposed to natural environmental conditions are simultaneously studied to determine how these abiotic and biotic factors influence stochastic processes such as dispersal of microorganisms, leading to distinct organ microbiomes.

Plant microbial communities vary with host age and genotype. Studies have shown that tree age influence bacterial communities and that age is negatively correlated with diversity (62, 64). It has also been demonstrated that plant genotype shapes rhizosphere and root microbiomes and that in the roots, a significant number of microbes are heritable (54, 95). In previous studies on the effect of plant age and genotype on microbial communities, age is the major factor in shaping the communities, although genetic effect is still significant (63, 95). It was noted that environment effect overcomes plant age in rhizosphere microbial communities (96, 97). Similarly, plant genetic control is weaker compared to location effect on microbial communities (2, 54). **Chapter IV** therefore aims to expand on these observations by examining the extent of heritable variation of plant microbial communities in various populations with broader genetic diversity. In addition, to probe into the effect of host genetics on associated microbial communities in a range of native environments, *L. corniculatus* microbial communities at multiple locations for four years are surveyed. Also in this study, to add more knowledge on how host age shapes plant microbial communities, plants with broader age range are examined by surveying them from various locations and years. To investigate the impact of age on microbial communities associated with perennial plants, which have more than one growing season in the course of their lives, microbial communities in multiple locations and years are analyzed to address variations between growing seasons. For deeper insight, the effect of plant age and genotype on three microbial groups in different plant organs are studied.

Objectives of this thesis

The encompassing goal of this work is to study the diversity and structure of microbial communities associated with the roots, shoots, flowers, and seeds of *L. corniculatus* in natural populations. Specifically, I aimed to investigate the contribution of plant host-related factors (*i.e.* organ, genotype, and age) and environmental factors in shaping the observed spatio-temporal variations in natural microbial communities. In **Chapter II**, I used the framework of metacommunity theory of ecology to understand the assembly processes that account for the community patterns in *L. corniculatus*-associated endophytic communities. In the framework of metacommunity theory, I aimed to establish that plant organs host distinct microbial communities and to identify mechanisms of host selection, microbial interactions, filtering by abiotic and biotic factors, and microbial dispersal processes that consequently lead to assembly of distinct organ microbial communities. In **Chapter III**, I examined the role of various abiotic and biotic factors at multiple sites and years in shaping the distinct yet overlapping microbial communities associated with roots, shoots, flowers, and seeds of *L. corniculatus* in natural populations. I also aimed to dissect how these abiotic and biotic factors influence transmission and establishment of microorganisms between plant organs, resulting in distinct organ microbial communities. In **Chapter IV**, I examined the impact of host age and genotype on the diversity and structure of *L. corniculatus*-associated microbial communities at multiple populations for four years. I also aimed to identify microbial biomarkers that can distinguish between age or genetic groups in plants, as well as to set up proof of concept experiments to test such predictions. To address these objectives, *L. corniculatus* from seven grassland sites of the Biodiversity Exploratories in the Swabian Alps, Germany are collected for four years (98, 99). To survey natural plant microbial communities, I performed amplicon sequencing of microbial 16S rRNA, ITS2, and 18S rRNA genes targeting endophytic communities in the roots, shoots, flowers, and seeds.

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CHAPTER II

ORGAN-SPECIFIC MICROBIOMES IN NATURAL *LOTUS CORNICULATUS* POPULATIONS: METACOMMUNITY DYNAMICS IN THE PLANT ENDOSPHERE

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Organ-specific microbiomes in natural *Lotus corniculatus* populations*:* Metacommunity dynamics in the plant endosphere

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ABSTRACT

The structures of plant microbial communities vary due to a broad range of factors such as host and environmental factors, abiotic and biotic perturbations, and various assembly processes occurring at multiple tempo-spatial scales. In natural environments plant microbial communities are constantly exposed to such perturbations and processes. Thus, to attain a systemic understanding of the ecology of plant microbiomes, it is essential to study assembly processes that influence patterns of microbial community structures in natural environments. In this study we examined bacterial, fungal, and eukaryotic communities in plant organs of *Lotus corniculatus* in natural populations at seven grassland sites for four years. We used the framework of metacommunity theory of ecology to understand assembly processes that shape community structures and variations by defining microbial communities associated with the roots, shoots, flowers, and seeds as distinct communities linked by dispersal. In this study we show the organ-specificity of plant endophytic communities. Our findings suggest that selective filtering by plant organs, microbial interactions, as well as abiotic and biotic factors at tempo-spatial scales result in distinct core microbiomes of plant organs. In addition, transmission of microorganisms from within and outside the plant hosts accounts for the distinct yet overlapping organ microbiomes. We could provide a comprehensive knowledge of the stochastic and deterministic assembly processes that shape plant microbial communities in natural conditions. Understanding these ecological processes is essential for harnessing beneficial effects of plant-associated microbial communities on plant productivity, resilience, and pathogen defense.

Keywords: plant microbiomes, organ-specific microbiomes, metacommunity dynamics, core microbiomes, microbial networks, microbial dispersal, plant-microbe interactions

INTRODUCTION

Plants are associated with diverse microbial communities of beneficial, neutral, and pathogenic microorganisms from across different kingdoms of archaea, bacteria, fungi, protists, and viruses. These plant-microbe interactions can be beneficial for both plant hosts and associated microorganisms - while microorganisms function in plant growth and development, nutrient acquisition, stress tolerance, and pathogen defense, host plants provide habitats and resources for growth and survival of microorganisms (1). These microbes that live within (*i.e.* endophytes) or on the surface (*i.e.* epiphytes) of plants are acquired from seeds via vertical transmission and from biotic and abiotic environments comprising air or wind, rain, soil, insects, and animals via horizontal transmission (2-7). The structures of plant microbial communities vary across plant age and genotypes, geographic locations, soil properties and land use types, developmental stages, and plant compartments (8-12). While many studies on plant microbiomes described these variations in microbial community structures, there remains a need for comprehensive understanding on assembly processes and microbial interactions that account for these community patterns. Current research on plant microbiomes revealed complex associations between plant hosts, microorganisms, and environment, however there are remaining challenges on our understanding of mechanisms that govern community assembly, community structure variations, and intricate interactions in plant microbial communities, especially in natural conditions. Given the high diversity and complexity of microbial communities in their natural habitats, the ecology of microbiomes *in situ* can only be partially conveyed by *in vitro* studies under laboratory conditions (13). To address these challenges, it is crucial to study plant microbiomes in natural environments, with constant exposure to natural perturbations and interactions, biotic and abiotic elements, and natural microbial sources.

Plant microbiomes are shaped by factors acting at multiple scales - from populations to individual plants and plant organs, across time and space (14, 15). Plant microbial communities are also interconnected via transmission of microbes at different levels, from microbial sources outside plant hosts (*i.e* other plants, animals, and environment) to transmission within plant tissues (16, 17). To incorporate simultaneously the community assembly processes occurring at multiple spatial and temporal scales and consequently to attain a more complete understanding of the variations and interactions in plant microbial communities, we examined community dynamics of plant microbiomes in the framework of metacommunity theory of ecology. Metacommunity theory is the study of distinct communities that are linked by dispersal (18). This concept consolidates the effect of different ecological processes and their interactions across space and time scales on community structures (19, 20). The assembly processes defined in Vellend's conceptual synthesis of community ecology are integrated into the metacommunity theory - selection, drift, and diversification are shaping separately each of the distinct communities, which are linked by dispersal (21, 22). Studies on microbial communities in the context of metacommunity ecology are relatively recent. Traditionally, metacommunity theory is used in the study of spatially distinct macrobial communities, such as marine and freshwater habitats, forest communities, moss patches, and insect and aquatic invertebrate metacommunities (23, 24). Application of the metacommunity concept in microbiome research allows for synthesis of both the deterministic and stochastic processes in their roles on shaping microbial communities at multiple temporal and spatial scales. Microbial communities in different ecosystems or sites (*i.e.* soil and freshwater ecosystems), as well as in plant and animal populations, were analyzed as metacommunities (25-29). Research on host-microbiome systems as metacommunities accounts for the interactions of assembly processes and microbial routes within hosts and their outside environments. A few studies on host-microbiomes and their interactions with their environments have been conducted with communities of insects, aquatic and terrestrial animals, and humans (30-36). Studying plant microbiomes as metacommunities takes into account varied plant host characteristics as well as diverse environmental factors and microbial sources influencing at scales ranging from individual plants to populations. Plant-microbiome systems as metacommunities is a fairly new concept (37). Moreover, studies focusing on microbial communities in plant organs in the context of metacommunity ecology are currently few. Investigation of microbial communities associated with roots, flowers, and seeds as discrete communities interacting with their biotic and abiotic environment were recently presented (38-41).

Studying plant organ-associated microbial communities as distinct communities linked with other microbial communities within and outside the plant host holds advantages towards better understanding of complex assembly processes and microbial interactions. Factors influencing the interconnected microbial communities at multiple tempo-spatial scales - from plant populations to individual plants and tissues - are simultaneously integrated in one ecological framework. In the metacommunity context, the plant host encompasses heterogeneous habitats for microbes, in which each of the plant organs are influenced by different assembly processes, biotic interactions, and microbial transmission, shaping distinct plant organ-specific microbial communities (1, 42, 43). Different resources or microhabitat conditions can cause varied growth and survival responses of microorganisms within plant organs, resulting in organ-specific selection for or against a set of microorganisms (14, 17). The abundance-persistence concepts in ecology can be utilized to determine well-adapted microorganisms consistently interacting with host organs across time and space (22, 44-46). Aside from these host-microbe interactions, distinct microbe-microbe interactions can emerge from compartment-specific environments (47, 48). These microbe-microbe interactions can be inferred through correlation networks and consequently microbial hubs that potentially shape microbial community structures can be identified (49-51). Biotic and abiotic factors acting at tempo-spatial scales ranging from plant organs to populations are simultaneously influencing plant microbiomes (14, 16, 17, 52, 53). By inspecting these biotic and abiotic elements at the level of microbial scales, individual plant scales, to population scales (*i.e.* at levels of plant organs, below- and above-ground, and between sites, respectively), we can identify factors that significantly contribute to observed community patterns in plant organ microbiomes. While plant organ microbiomes are distinct, they are interconnected via transmission of microorganisms among plant organs and from outside of plant hosts (1, 14-17, 53). Dispersal events can be inferred by estimating potential microbial sources of plant organ microbiomes (54). The influence of dispersal on microbial communities via priority effects can also be explored by predicting candidate early-arriving microbes which altered community composition (55).

In this study, we investigated the diversity and community composition of microbiomes associated with *Lotus corniculatus* in natural populations. *L. corniculatus*, a common legume species that is widespread in European grasslands, is an ideal system to study assembly processes and variations of microbial communities in natural environments. *L. corniculatus* is a perennial flowering plant that can adapt to a broad range of natural environments - it can grow on different types of soils, is resistant to grazing and mowing, and is host to various insects and bees (56-58). *L. corniculatus* is also known to host diverse microbes, including nitrogen-fixing rhizobia bacteria and arbuscular mycorrhiza fungi which enhance the plant host's ability to adapt in poor habitats, as well as non-rhizobial endophytes that promote plant growth (59-61). In this study, we also examined the assembly processes that account for the community structures and variations in *L. corniculatus*-associated endophytic communities in the framework of metacommunity theory in ecology by defining the microbial communities associated with roots, shoots, flowers, and seeds as distinct communities linked by transmission of microorganisms from within and outside the plant hosts. Specifically, we aimed (i) to establish that plant organs host distinct microbial communities by using diversity measures and predictive models in machine learning (*i.e*. organ-specificity of plant microbial communities); (ii) to identify well-adapted microorganisms selectively filtered by each host plant organ by utilizing abundance-persistence concepts in ecology and machine learning approaches (*i.e.* host selection); (iii) to infer important microbe-microbe interactions in plant organs by constructing correlation networks and predicting hubs in the organ-specific microbial communities (*i.e*. microbial interactions); (iv) to explore at different levels of tempo-spatial scales the factors that contribute to plant organ-specific patterns of microbial communities by using diversity measures across sampling years and sites (*i.e.* biotic and abiotic factors); and finally (v) to predict dispersal events that link distinct plant organ communities by estimating transmission of microorganisms from various microbial sources and predicting microorganisms that potentially have roles in priority effects phenomena (*i.e*. microbial transmission and priority effects). To address these aims, we collected *L. corniculatus* from seven grassland sites in the Swabian Alps, Germany for four years. We performed amplicon sequencing of microbial 16S rRNA, ITS2, and 18S rRNA genes targeting endophytic communities in plant organs. Results are synthesized in the framework of the metacommunity concept to establish a comprehensive understanding of ecological processes that shape plant microbial community dynamics.

METHODS

Collection of *L. corniculatus* samples in Swabian Alps.

To study the ecology of plant microbiomes in natural environments, we collected *Lotus corniculatus* from seven grassland sites in the region of Swabian Alps, Germany for four years (Fig. 4a). These grassland sites of the Biodiversity Exploratories project in southwest Germany have different land use types including unfertilized, mown pastures (AEG3, AEG8, AEG43), fertilized, mown pastures (AEG10, AEG40), and fertilized, mown meadows (AEG17, AEG22) (62-65). We collected plant samples every August-September from year 2018 to 2021 when plants are flowering and producing fruits. We sampled from each site six plants that were randomly distributed throughout the area. We also sampled soil from where plants were uprooted and then pooled together for each site. To characterize endophytic communities in *L. corniculatus* organs, we separated plant samples into roots, shoots, flowers, and seeds and then surface-sterilized them sequentially with sterile water, epiphyte wash (1X TE + 0.1% Triton X-100), 80% ethanol, bleach (2% NaOCl), and finally sterile water. We stored sterilized samples at -20 ^{0}C until processing for DNA extraction.

Amplicon sequencing of *L. corniculatus*-associated bacteria, fungi, and eukaryotes.

We sequenced a total of 700 samples of soil and *L. corniculatus* roots, shoots, flowers, and seeds. We homogenized frozen samples of soil and surface-sterilized roots, shoots, flowers, and seeds in Precellys 24 Tissue Homogenizer (Bertin Technologies) before we extracted DNA using FastDNA^{TM} Spin Kit for Soil (MP Bio) as described in the manufacturer's protocol. Extracted DNA, along with blank samples (*i.e.* water and blank DNA extraction), were used as templates for two-step PCR amplification of bacterial 16S rRNA V5-V7 region, fungal ITS2 region, and eukaryotic 18S rRNA V9 region using primers 799F/1192R, fITS7/ITS4, and F1422/R1797, respectively (Table S1) (51). We designed blocking oligos using R package “AmpStop” to minimize amplification of mitochondrial and chloroplast 16S rRNA, ITS, and 18S rRNA from *L. corniculatus* (Table S1) (66). Amplification products randomized in eight sequencing batches were pooled in equimolar concentrations and purified via magnetic bead clean-up before sequencing on Illumina MiSeq with PhiX control using MiSeq Reagent Kit v3 (600-cycle).

Sequence data processing.

We processed amplicon sequence data of microbial 16S rRNA, ITS2, and 18S rRNA using Mothur as described in Almario *et al*. (Method S1) (52, 67). We taxonomically classified bacterial 16S rRNA, fungal ITS2, and eukaryotic 18S rRNA sequences based on Greengenes database (13_8_99 release), UNITE database (02.02.2019 release), and PR^2 database (version 4.12.0), respectively, with the PhiX genome included in the databases (68-70). For 16S rRNA and 18S rRNA data, Cutadapt was used to remove primer sequences, and for ITS2 data, ITSx was used to remove non-ITS sequences (71, 72).

Diversity and community composition analysis.

For diversity analysis and relative abundance calculations, we used R packages phyloseq, vegan, microbiome, and microeco to analyze OTU tables outputted from Mothur pipeline (73-76). We used Shannon's diversity and Observed species indices to assess alpha-diversity of samples. To check if alpha-diversity measures between samples are significantly different, we tested data whether they are normally distributed via Shapiro-Wilk normality tests and then analyzed using parametric test ANOVA (for normally distributed data) or nonparametric Kruskal-Wallis rank sum test (for non-normal data). We conducted post-hoc analysis using Dunn's test (for Kruskal-Wallis test) or Tukey's HSD (for ANOVA test). For beta-diversity analyses, we used OTU relative abundance tables for Principal Coordinate Analysis (PCoA) ordination of Bray-Curtis dissimilarities between samples. We used PERMANOVA analysis of Bray-Curtis distances to assess significant explanatory variables (*i.e.* plant organ, years of sampling, sampling sites) affecting microbial community structures.

Identification of key organ-specific microbes in *L. corniculatus*.

To determine abundant endophytes in *L. corniculatus* roots, shoots, flowers, and seeds, we identified OTUs with highest relative abundances in each organ (≥1% relative abundance). To determine persistent core microbes in organs, we identified OTUs that occur in at least 90% of the samples across seven sampling sites for four years (≥90% occurrence). To determine hub bacteria, fungi, and eukaryotes in plant organs, we computed correlation networks for each plant organ using SparCC algorithm, as described in Almario *et al*. (Method S2) (52, 77). We used Cytoscape (v.3.9.1) to visualize networks and to calculate network features such as number of nodes and edges, node connectedness (degree), betweenness centrality, and closeness centrality (78). We assigned OTUs that are top 5% in betweenness centrality and closeness centrality scores as hub microbes. We used representative sequences of abundant, core, or hub OTUs for multiple sequence alignment in MUSCLE (Web Form) using default parameters (79). We used resulting alignments in ClustalW format to build neighbor-joining phylogenetic trees in the online tool iTOL (v.6.7.3) (80).

Machine learning classification for diagnosing multi-organ involvement and predicting organ-specific microbes in *L. corniculatus*.

To explore the possibility of a predictive pattern for separation of *L. corniculatus* organs based on microbial composition, we employed a linear classification machine learning model using relative abundance data of bacteria, fungi, and eukaryotes. We utilized one-vs-one approach on the support vector machine with a linear kernel (OneVsOneClassifier(svm.SVC) function in Scikit-learn) to train a binary classification model for separating each organ from others (81). Using Scikit-learn, we randomly selected 67% of samples (n=467) for training and reserved the remaining 33% (n=231) for independent testing and evaluation using the classification_report function, then we visualized confusion matrices using the plot_confusion_matrix function. To identify microbial species that can discriminate each organ from the rest, we employed recursive feature elimination with cross-validation (RFECV function). We calculated the accuracy of the model using K-fold cross-validation (K=10).

Transmission of microbes in *L. corniculatus*.

To determine how *L. corniculatus* organ microbiomes can be linked and influenced by dispersal, we used Sankey diagrams to visualize potential flow of microbes across the soil and plant organs (Method S3). To determine potential origins of organ-associated microbial communities, we used FEAST (Fast Expectation-mAximization microbial Source Tracking) to estimate contribution of potential microbial sources, such as soil, the different plant compartments, or the environment, to each plant organ microbiome (Method S3) (54). To statistically predict potential priority effects phenomena from roots, shoots, flowers, to seeds in *L. corniculatus*, we identified taxa of interest that are potentially involved in such phenomena in plant organs, as described in Debray *et al*. (Method S3) (55).

RESULTS AND DISCUSSION

Endophytic bacterial, fungal, and eukaryotic communities in natural *L. corniculatus* populations are organ-specific.

We surveyed endophytic communities associated with *L. corniculatus* in natural populations by amplicon sequencing of bacteria, fungi, and eukaryotes in 700 samples of soil and plant roots, shoots, flowers, and seeds (Table S2, Table S3). We used blocking oligos which decreased the number of nontarget plant DNA reads (*i.e.* chloroplast, mitochondria, plant ITS2, and plant 18S rRNA) by 90-100% and significantly increased the number of microbial 16S rRNA, ITS2, and 18S rRNA reads (Fig. S1). After processing raw reads and removing unknown and plant sequences, we identified a total of 4,225 16S rRNA, 2,027 ITS2, and 1,773 18S rRNA OTUs clustered based on 97% sequence similarity and were classified into 113 phyla and 1,542 genera (Table S3). Among the plant compartments, roots have the greatest number of OTUs followed by shoots, while there are less number of OTUs detected in flowers and in seeds (Fig. S2a). In all plant compartments, phyla Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes, Acidobacteria, Ascomycota, Basidiomycota, and unclassified Fungi and Eukaryota are the most abundant groups (Fig. 1a). In addition, in roots phyla Chloroflexi and Nematoda are abundant, while in shoots, flowers, and seeds, Arthropoda and unclassified Bacteria dominate. Soil samples have more observed OTUs compared with all plant organs, with groups Actinobacteria, Proteobacteria, Ascomycota, Basidiomycota, and unclassified Fungi as the most abundant phyla (Fig. 1a, Fig. S2a).

Alpha-diversity analysis showed that endophytic communities in roots are the most diverse compared with communities in aboveground plant organs. Shannon diversity index showed that endophytic communities progressively become less diverse from roots to shoots and flowers, and then an increase in bacterial and eukaryotic diversity is observed in seeds

(Fig. 1b, Table S4b). Soil microbial communities are more diverse than microbial communities associated with *L. corniculatus* (Fig. 1b, Table S4b). Beta-diversity analysis revealed that the diverse and stable microbial communities in soil and roots are distinct from the less diverse and more variable communities in shoots, flowers, and seeds. Bray-Curtis dissimilarities-based PCoA showed that soil and root microbial communities are similar in community structures and are distinct from the overlapping endophytic communities in shoots, flowers, and seeds (Fig.1c). The PCoA plots showed a main separation (axis 1) of bacterial communities between above and belowground communities while fungal and eukaryotic communities separate the aboveground organs. Both PCoA and relative abundance profiles of individual samples across sampling sites and years also showed that soil and root microbial communities are more clustered and less dispersed compared with the variable microbial communities in shoots, flowers, and seeds (Fig. S3). PERMANOVA indicated that significant separation of microbial communities was largely explained by plant organ, while sampling years and sites contributed relatively lower to microbial community variations (Fig. S4; Table S4c).

We trained a multi-class support vector machine (SVM) model to determine whether relative abundances of bacterial, fungal, and eukaryotic microbial communities could distinguish between each organ from other organs (all binary possibilities) and soil samples. The performance of the model on the test set showed high accuracy in separating organs and soil samples (accuracy = 77-83%; Table S5). In bacterial and fungal communities, roots and soil samples separated from other groups with higher prediction accuracy compared with aboveground organs, while in eukaryotic communities, seeds are most accurately predicted among all the groups (Fig. 1d). We trained an SVM classifier with recursive feature elimination and cross-validation to identify OTUs that discriminate each organ from the others (without soil samples). Results revealed a subset of 57-166 OTUs that could separate roots, shoots, flowers, and seeds from other organs (Fig. S5).

Based on diversity analyses and predictive models, results show the organ-specificity of endophytic communities in natural *L. corniculatus* populations. Root microbial communities are the most diverse among the plant organ communities. There is significant separation of community structures between aboveground and belowground bacterial communities, while shoot, flower, and seed communities are mainly separated by fungi and eukaryotes. The aboveground endophytic communities are more variable compared with the root communities. Soil microbial communities, which are more diverse than all plant organ communities, have overlapping community composition with the root communities.

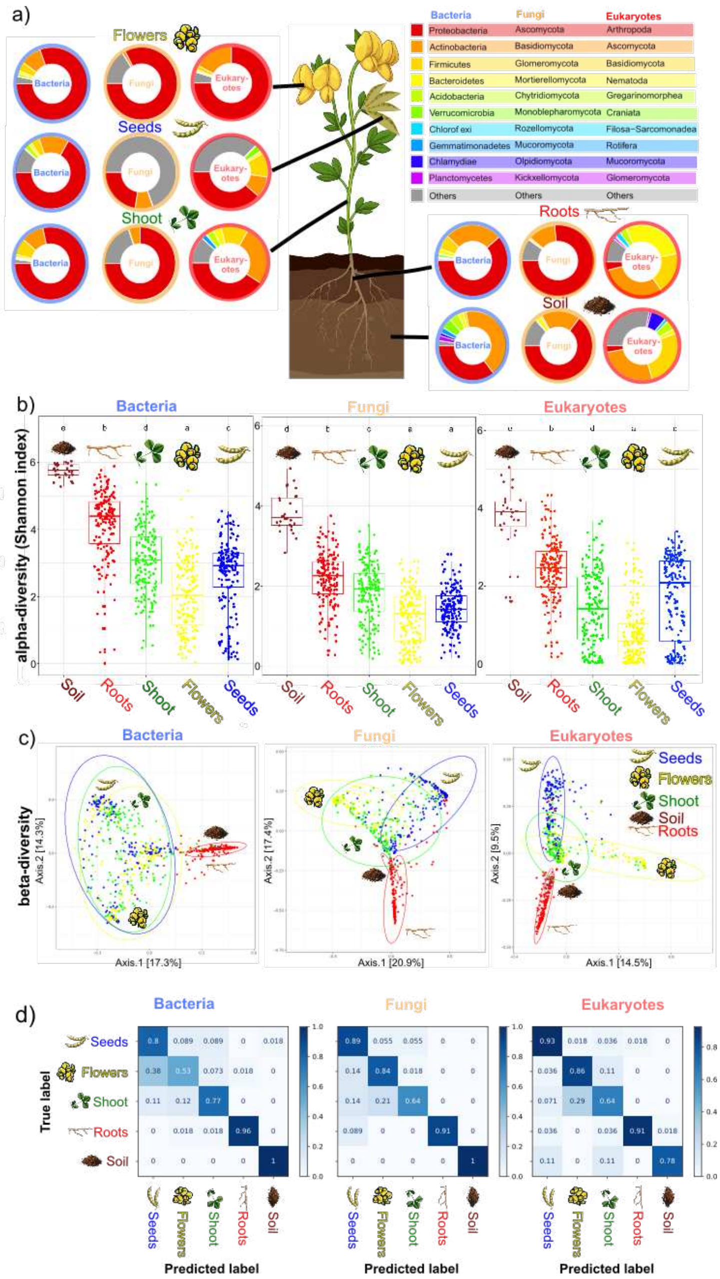

Figure 1. Organ-specificity of plant microbial communities. **(a)** Relative abundance profiles showing the top ten most abundant bacterial, fungal, and eukaryotic phyla in soil and plant organ samples collected from seven grassland sites for four years. The relative abundance of the OTUs for each compartment (*i.e.* soil and plant organs) were aggregated at the phylum level. **(b)** Boxplots of Shannon's α-diversity measurements of bacterial, fungal, and eukaryotic microbial communities associated with soil and plant organs. Shapiro-Wilk normality tests indicated that datasets have non-normal distribution ($p<0.05$) and Kruskal-Wallis rank sum tests were used to test significant differences of α-diversity measurements between soil and plant organ samples. Post-hoc analysis via Dunn's tests indicate that groups are significantly different if letters are not similar (Supplementary Table 4). **(c)** Principal coordinate plots based on Bray-Curtis dissimilarities between bacterial, fungal, and eukaryotic microbial communities associated with all soil and plant organ samples. **(d)** Performance of the multi-class support vector machine (SVM) model on the test set showing high accuracy in separating bacterial, fungal, and eukaryotic communities in organs and soil samples.

***L. corniculatus* core microbiomes are shaped by host selection.**

To identify key microbes which can explain the identified organ-specific patterns in community structures of *L. corniculatus* microbiomes, we examined species abundance, an ecological pattern that is observed in macrobial and microbial communities (Fig. 2, Table S6). Except for *Pantoea* and *Pseudomonas*, which are abundant in both above- and below-ground plant organs, the most abundant OTUs (relative abundance (RA) > 1%) in root microbial communities are distinct from the set of abundant OTUs in aboveground microbial communities. Similarly in microbial communities of shoots, flowers, and seeds, the most abundant OTUs (RA > 1% in at least one plant organ) are mostly the same and enriched specifically in the aboveground microbial communities. The observed similarity in the sets of abundant taxa accounted for the observed overlapping community structures of the aboveground plant organ communities despite high variability throughout sampling years and sites (Fig.1c, Fig. S3). Thus, the overlapping community structure in shoots, flowers, and seeds, as well as the distinction between aboveground and belowground plant organ community structure, can be largely explained by the most abundant species.

Persistence of species over time and space is also an ecological pattern that is important in structuring macrobial and microbial communities. We identified persistent organ-specific ‘core’ microbes that can be detected in 90% of *L. corniculatus* organ samples collected across all seven sites and four years (Fig. 2, Table S7). In roots we found 56 bacterial, seven fungal, and seven eukaryotic core OTUs, which consists 1% of all 7,504 root OTUs. Many of the core species identified in roots are also abundant species in roots. In shoots there are seven bacterial, four fungal, and four eukaryotic core OTUs (0.3% of 5,223 total shoot OTUs), while in flowers and seeds there are seven (0.2% of 3,706 total flower OTUs) and eight (0.3% of 3,706 total seed OTUs) core OTUs, respectively. Likewise, most of the core species in aboveground communities are also abundant in their respective plant organ microbial communities. The smaller core communities in shoots, flowers, and seeds suggest fluctuating aboveground microbial communities, in contrast with the more stable root communities with a relatively larger core community. Meanwhile, *Pseudomonas* and *Cladosporium* are persistent over time and space in all plant organs, suggesting stable associations and high adaptation to the plant endophytic environment.

To determine microbes that distinguish between *L. corniculatus* organs, we used multi-class support vector machine (SVM) model that predicts OTUs that are highly associated with each organ (Fig. S5). In root microbial communities, 84 separator OTUs were identified with the root compartment. Among these OTUs are abundant species in roots and are persistent throughout years and sites of collection (Fig. 2, Table S7). *Pseudomonas, Phyllobacterium, Frankia, Mesorhizobium, Cryptosporangium, Steroidobacter, Rhizobium,* and *Bosea* are abundant and core bacteria that showed high association with roots. Abundant and core fungi such as *Exophiala, Cadophora,* and *Dactylonectria* are also distinctive of root microbial communities. In shoot microbial communities there are 102 separator OTUs identified, among these OTUs are core and abundant species *Bacillus*, *Agrobacterium* and *Alternaria* (Fig. 2, Table S7). There are 57 OTUs that are highly associated with the flower compartment including abundant and core fungi *Cladosporium* and insects *Pezothrips* (Fig. 2, Table S7). There are 166 OTUs that are distinctive of seed microbial communities (Fig. 2, Table S7). *Pseudomonas, Ralstonia,* and *Cladosporium* are abundant and persistent species that are highly associated with seeds. Different resources or habitat conditions in plant organs could result in the filtering of different groups of microorganisms adapted to organ-specific features, consequently resulting in organ-specific distinctive microbial communities (82-95). These microorganisms that are selectively filtered by each of the plant organs are likely specialized microbes that are able to thrive in the unique physical microstructures of the organs while improving plant growth, nutrient uptake, and resistance to stress and diseases They could supply nutrients while in turn benefitting from resources in particular organs. Alternatively, they could be pathogens that hijack the host's genetic resources.

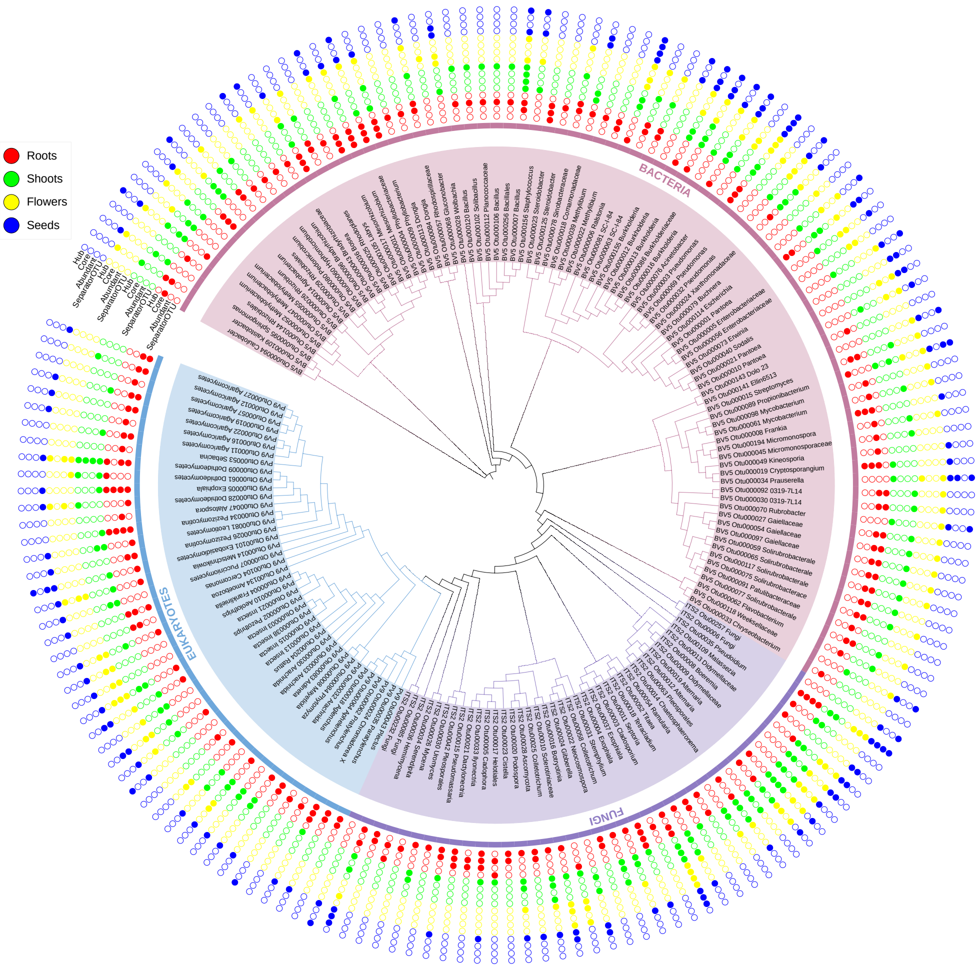

Figure 2. Organ-specific microbes identified via abundance, persistence, and predictive machine learning models. Neighbor-joining phylogenetic tree of top abundant OTUs (relative abundance in the plant organ microbial communities > 1%) and core OTUs (detected in 90% of the organ samples). Abundant and core OTUs that are either separator OTUs (all identified separator OTUs using multi-class support vector machine (SVM) model are detailed in Supplementary Fig. 5) or hubs (hubs identified using microbial networks are detailed in Fig. 3 and Supplementary Fig. 6) are also indicated.

Community structures of *L. corniculatus* microbiomes are influenced by microbial interactions.

Species interactions are also important in maintaining structures of macrobial and microbial communities. Microbial species uniquely adapted to a particular organ environment that can significantly affect microbial interactions consequently can also affect organ-specific microbial community structuring. To infer potential microbe-microbe interactions and to identify hubs in the plant organ communities, we built correlation networks based on species abundances (Fig. 3). In general, root microbial community networks are more complex than networks of aboveground organs. In addition, bacterial networks are more complex compared with fungal and eukaryotic networks in all plant organs. The number of nodes are consistently highest in roots in all bacterial, fungal, and eukaryotic networks, followed by microbial community networks of shoots, flowers, and seeds. (Fig. 3b). Root networks also have the highest number of edges, which correspond to significant correlations between microbes, compared with aboveground organ networks (Fig. 3c). Based on ANOVA and Tukey's HSD, the nodes of root microbiome networks have the highest number of interactions among all the organ microbiome networks (Fig. 3d). Aboveground, shoot microbiome networks have higher node connectedness compared with flower and seed networks, which have node connectedness that are not significantly different. There are more nodes that are shared between shoots and flowers in bacterial and fungal networks compared with other organ-to-organ networks, while in eukaryotic networks the root-to-shoot networks have the highest number of shared nodes (Fig. 3e).

Hub microbes, which are highly connected with other microbes in the network, potentially have essential roles in plant-microbe and microbe-microbe interactions and thus shape the community structure (51). We identified hub microbes which can significantly influence microbial community structures in each plant organ based on betweenness centrality and closeness centrality scores (Fig. 2, Fig. S6, Table S7). Many among the hub microbes are abundant and persistent across seven sampling sites throughout four years. In root microbial communities there are 101 bacterial, 39 fungal, and 32 eukaryotic hubs, which comprise 2% of total root OTUs. Many of the inferred hubs are also machine learning-predicted separator microbes in roots, including the hub fungi *Exophiala* that are both abundant and persistent species in roots. Shoot microbial communities comprise 97 bacterial, 33 fungal, and 13 eukaryotic hubs (3% of all shoot OTUs). Separator microbes for the shoot compartment *Bacillus*, *Alternaria*, and Dothideomycetes are hub microbes that are both persistent and abundant in shoots. Flower-associated microbial communities have 90 bacterial, 23 fungal, and 8 eukaryotic hubs, which consists 3% of total flower OTUs. Expectedly, most of the hubs are separator microbes, including *Cladosporium,* species that are also abundant and persistent in flower compartments. In seed microbial communities, there are 61 bacterial, 14 fungal, and 4 eukaryotic hubs, which is 2% of total seed OTUs. *Pseudomonas* and *Ralstonia* are hub microbes that are both core, abundant, and seed separator microbes. Among the predicted hubs in the plant organs are microbes that are abundant, or persistent across sampling years and sites, and highly associated with the respective organ compartments and are thus important in diversity, stability, and organ-specificity of the microbial communities. These hub microbes either as pathogens or beneficial endophytes play important roles in microbial interactions and can be important in shaping microbial communities of each plant organ (84, 88-95).

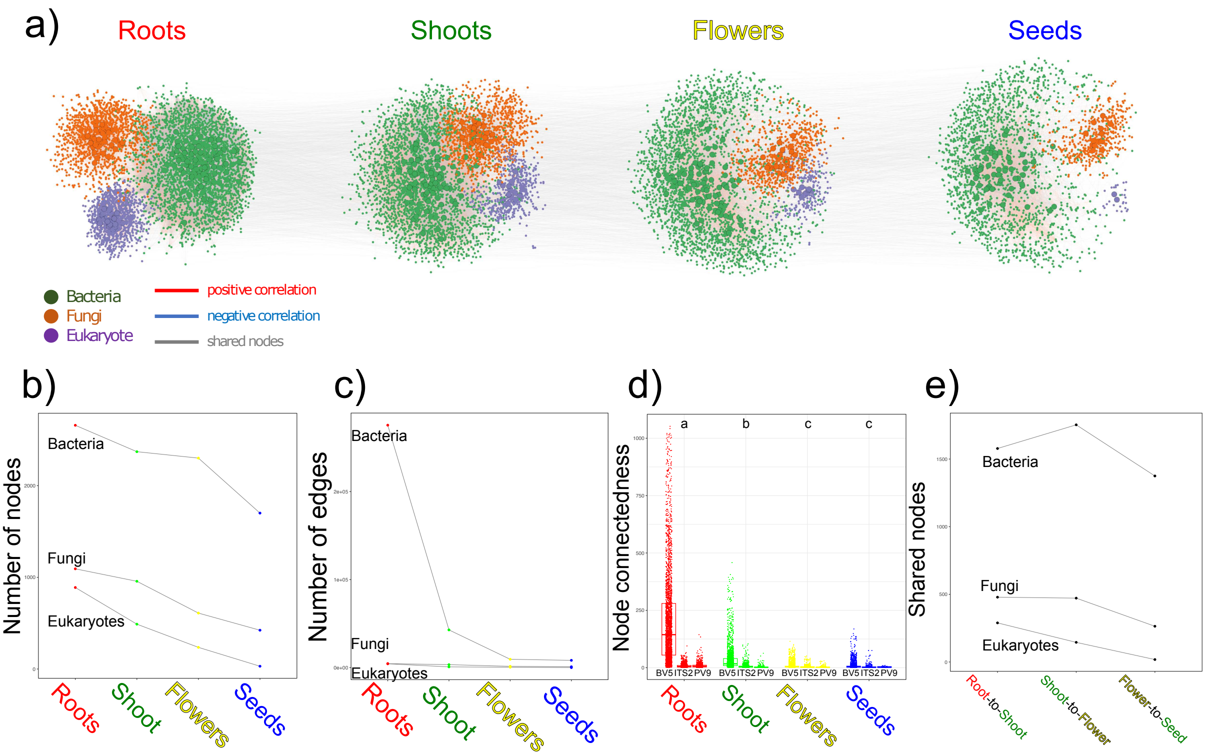

Figure 3. Microbial interactions in organ-specific plant microbiomes. (a) Correlation networks based on species abundance of bacterial (green), fungal (orange), and eukaryotic (purple) microbes of each plant organ. Nodes are OTUs and edges represent potential microbe-microbe interactions. Significant positive interactions are colored red, while negative interactions are blue ($P<0.001$). Hub microbes of each organ microbial communities are represented by bigger and bordered nodes. Grey lines that connect each organ microbiome network show connections of nodes/OTUs that are shared with the next organ. Network characteristics such as number of **(b)** nodes and **(c)** edges, **(d)** node connectedness (degree), and **(e)** nodes shared between organs are used to describe the networks. Overall **(d)** node connectedness (node degree of all bacterial (BV5), fungal (ITS2), and eukaryotic (PV9) networks) were compared between organ microbiome networks via ANOVA and Tukey's HSD.

Variation in *L. corniculatus* microbiomes are driven by biotic and abiotic factors across time and space.

Beta-diversity analysis of microbiomes in *L. corniculatus* natural populations revealed that plant organs largely contributed to microbial community variation, while years of collection and sampling sites showed smaller effects (Fig.1c, Fig. S4, Table S4c). To further dissect other factors that can influence community structure in *L. corniculatus* microbiomes, we analysed diversity and community composition on each plant organ across sampling years and sites. Throughout sampling sites and years, *L. corniculatus* plants as a whole maintained significantly different levels of microbiome alpha-diversity (except the fungal community), and at least one of the bacterial, fungal, or eukaryotic communities of the plant organs exhibited significantly different diversities (Fig. S7, Fig. 4b-g). Relative abundance profiles of the most abundant bacterial, fungal, and eukaryotic groups in the plants also vary across all sampling sites and years (Fig. S8). Specifically, in roots the diversity of microbial communities is similar across all sampling plots, and while the diversity of fungal and eukaryotic communities are also similar across all sampling years, root bacterial communities exhibited different alpha-diversities throughout the years (Fig. 4b-g). In contrast, microbial communities in aboveground plant organs have significantly different alpha-diversities throughout the years, except in fungal communities of all aboveground organs and in shoot eukaryotic communities (Fig. 4e-g). At least one of the bacterial or fungal communities associated with shoots, flowers, and seeds showed significantly different alpha-diversities across different sampling sites, while eukaryotic communities are similar (Fig. 4b-d).

Beta-diversity analysis of *L. corniculatus* microbial communities indicated that years of collection accounted more on the observed variation in bacterial communities compared to sampling sites (Fig. S4, Table S4c). Specifically, while in roots both variables have almost equal effects on variation, the influence of years of collection is higher in the aboveground microbial communities (Fig. 4h, Fig. S9a). These observations show that bacterial communities in roots are relatively more stable across all years and sites, while aboveground communities varied through years of collection, consistent with the relative abundance profiles of bacterial communities in plant organs throughout all years and sites (Fig. S3a-b). *L. corniculatus* are perennial plants, hence the roots could have maintained stable bacterial communities over the years, while in aboveground plant organs the communities are more variable due to consistent perturbations in the sampling sites such as mowing or animal grazing. The recurrent emergence of shoots, flowers, and seeds amidst such perturbations potentially contributed to the variation in bacterial communities in these organs (96, 97). On the other hand, fungal and eukaryotic community structures in all plant organs except seeds are influenced to a larger extent by sampling sites (Fig. 4i-j, Fig. S9b-c). The larger effect of sampling sites suggests that local environmental conditions and soil microbial communities of the different grassland sites have larger influence on the variation of fungal and eukaryotic communities associated with *L. corniculatus* roots, shoots, and flowers. Bacterial, fungal, and eukaryotic community variations in the soil are also influenced largely by sampling sites (Fig. 4h-j, Fig. S9), hence soil microbiomes could have contributed to the beta-diversity patterns observed in the plant organs. Disturbances in the local environments like temperature fluctuations, wind, UV levels, and precipitation, and consequently plant adaptations to these changes, can also potentially augment to the variations of fungal and eukaryotic communities in roots, shoots, and flowers. Insect visitors present in the sampling sites, especially pollinators, can also affect microbial community composition in plants (98). Thus, observed patterns of diversity and composition could be attributed to abiotic factors and biotic interactions acting at different spatial and temporal scales, that is, at the level between roots and aboveground plant organs to the level of sampling locations or years.

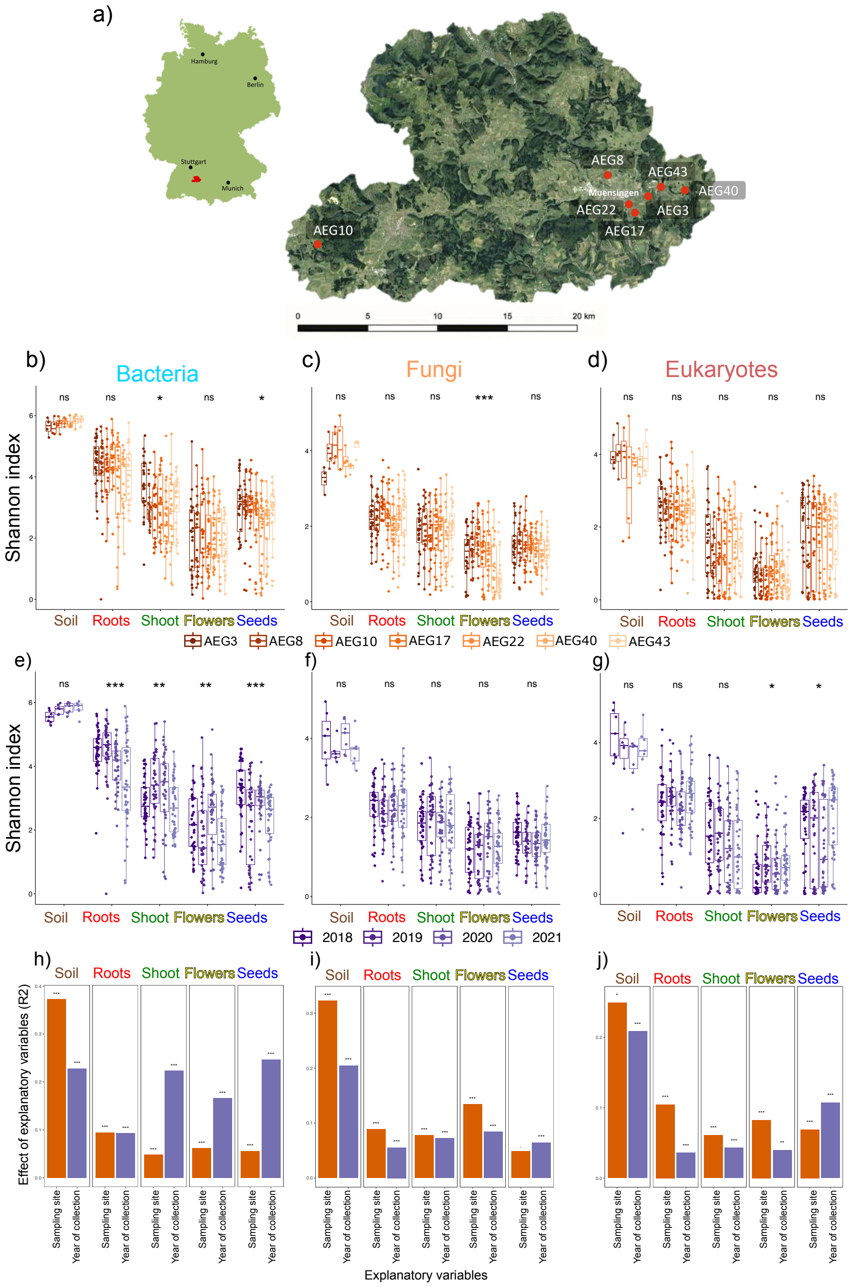

Figure 4. Diversity and community composition of plant organ-associated microbiomes in *Lotus corniculatus* populations in seven grassland sites for four years. (a) Map of the seven grassland plots in the Swabian Alps, Germany where samples were collected from years 2018-2021. α-diversity (Shannon index) of the bacterial, fungal, and eukaryotic microbial communities associated with soil and plant organs were compared between **(b-d)** sampling sites or **(e-g)** collection years using Kruskal-Wallis significance test. The effect of explanatory variables (*i.e.* sampling sites, year of collection) on the β-diversity of soil- and plant organ-associated microbial communities were also assessed using **(h-j)** PERMANOVA.

L. corniculatus microbiomes are distinct communities linked and influenced by dispersal.

To investigate how *L. corniculatus* recruits microbes to assemble into organ-specific communities, we compared OTUs detected across soil and plant organs (Fig. 5a). The distinct but overlapping plant organ microbiomes share a subset of their communities. Most OTUs that are present in plant organs are also detected in soil samples. With increasing distance from the soil, soil-detected OTUs in the plant organs decrease (Fig. S10). Only a small proportion of the core communities in roots are transmitted to upper plant organs. Core microbes *Pseudomonas* and *Cladosporium* are potentially good disperser microbes that are consistently transmitted and maintained throughout the interconnected plant organs (Fig. 2). Mostly, the OTUs in plant organs are transmitted from neighboring compartments and a lesser proportion are from other sources, presumably from the local environment. These observations suggest that there are similarities between plant organ microbial communities due to initial colonizer microbes from soil, while variations in community composition are accumulated through various microbial sources from the environment as well as from other plant compartments. Thus, plants establish their organ-specific microbiomes by recruitment and selecting microbes from soil and their environment as well as by transmission of microbes from other plant compartments.

To further verify these observations, we used FEAST to determine potential origins of the organ-specific microbiomes (Fig. 5b). FEAST was used to estimate the contribution of potential microbial sources, such as soil, the different plant compartments, or the environment, to each plant organ microbiome. Since in FEAST the source and sink communities are preassigned and consequently the direction of transmission is not determined, we tracked the transmission at multiple directions (Fig. S11). We observed that there is potential dispersal of microbes from various microbial sources and multiple directions. A large proportion of plant microbes are dispersed between plant compartments (13%-92% of microbes in sink organs are from other plant compartments). Soil microbes are transmitted to all plant organs, and aboveground plant organs tend to have lesser soil microbes compared with roots. Certain fractions of plant organ microbiomes were assigned by FEAST to “unknown sources”, which can be other potential microbial sources from the environment such as insects, pollinators, animals, rain, wind, or soil splashes. These calculations showed that dispersal occurs between plant organ microbiomes as well from soil and environment and thus influences community structures of these organ-specific microbiomes.

Dispersal can also affect microbial community diversity through arrival history. A set of early-arriving species can impact assembly of communities by changing resources or environmental conditions, in a historically-contingent community assembly called priority effects. Debray *et al.* presented an approach to statistically predict from a natural microbiome dataset which taxa are potentially involved in influencing succession during community assembly (55). Based on such approach, we identified candidate early-arriving OTUs that may have inhibited or facilitated establishment of other OTUs by examining if their abundance in plant organs correlated with altered community composition. Changes in relative abundance of some of the key OTUs in plant organs consequently showed changes in community structure (Fig. 5c-d, Fig. S12). For instance, relative abundances of *Phyllobacterium* and *Mesorhizobium* contributed to the variation of microbial communities in roots, while relative abundances of *Pantoea*, *Ralstonia*, and *Burkholderia* accounted for the microbial community variations in aboveground organs. *Phyllobacterium* and *Mesorhizobium* are abundant, persistent, and highly associated microbes in roots (Fig. 2). *Pantoea* are abundant throughout the whole plant and are core microorganisms in shoots, while *Ralstonia* and *Burkholderia* are key microbes (*i.e.* either abundant, core, hub, or separator microbes) in most of aboveground organs (Fig. 2). The prediction of these taxa and their potential roles during priority effects phenomenon enables future experimental manipulation of arrival history in complex natural microbial communities.

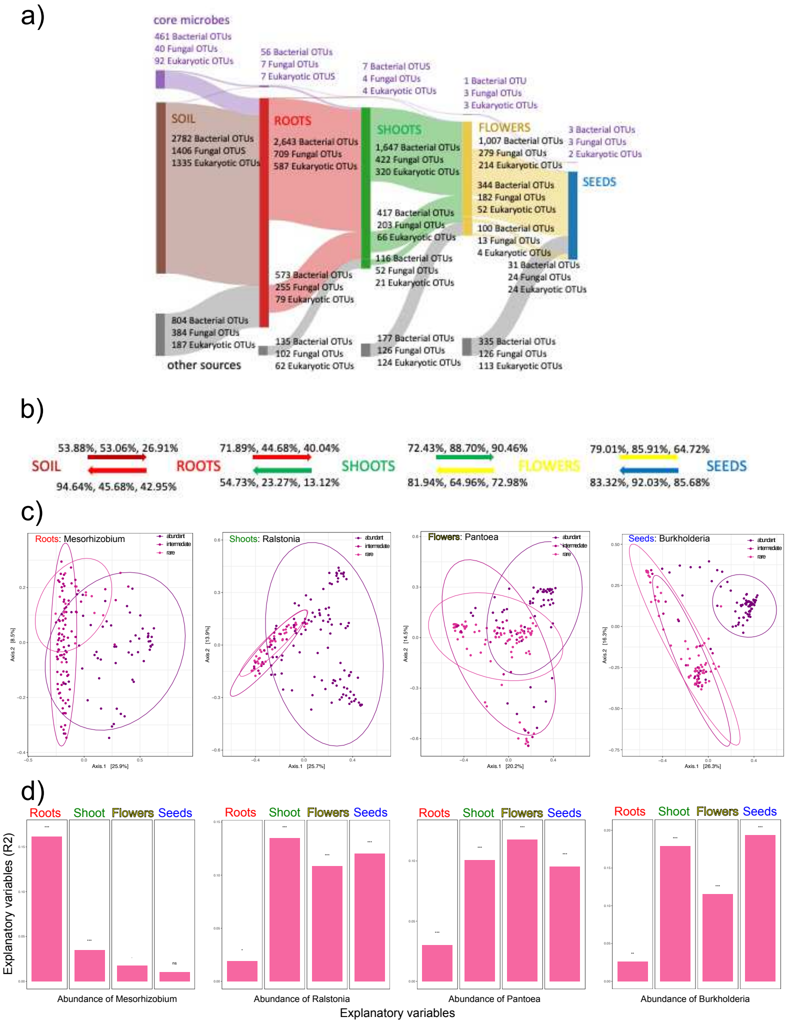

Figure 5. Recruitment of microbes from soil and environment to establish organ-specific microbiomes in *Lotus corniculatus*. (a) Sankey diagram of shared microbes between neighboring plant compartments as well as from other sources (*i.e.* from local environment). Nodes of the diagram represent different potential microbial sources (*i.e.* soil, plant organs, and environment/others) and arcs correspond to the number of bacterial, fungal, and eukaryotic OTUs shared between nodes. The number of core OTUs in each plant organ are

displayed in purple nodes and arcs in the diagram. **(b)** FEAST was also used to estimate the contribution (% bacterial, fungal, and eukaryotic contribution, respectively) of potential microbial sources to each plant organ microbiome. In FEAST the direction of microbial transmission is preassigned, thus calculation of microbial contribution was tracked at both directions. For more details of transmission at multiple directions, see Supplementary Fig. 11. **(c)** PCoA of Bray-Curtis dissimilarities of plant organ microbial communities and **(d)** PERMANOVA using abundance of candidate early-arriving OTU as explanatory variable of community composition variation. (c) and (d) show that candidate early-arriving OTUs (BV5_OTU11_*Mesorhizobium*, BV5_OTU6_*Ralstonia*, BV5_OTU1_*Pantoea*, BV5_OTU16_*Burkholderia*) potentially altered the microbial community composition of roots, shoots, flowers, and seeds, respectively, based on their abundance in the plant organ microbial communities. (Explanatory variables: abundant OTU: Relative abundance in each plant organ microbial community (RA) >= 0.01; intermediate OTU: RA >= 0.001 and < 0.01; rare OTU: RA < 0.001).

CONCLUSIONS

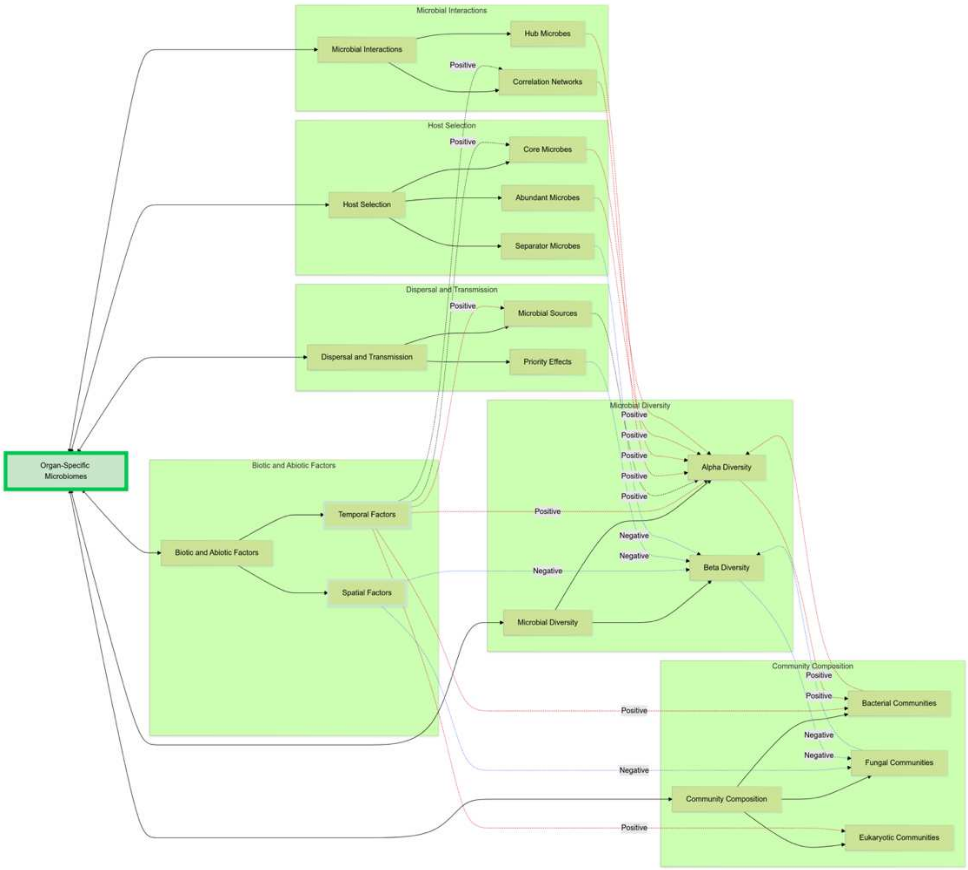

Figure 6. Ecological processes at different tempo-spatial scales that shape metacommunity dynamics in organ-specific *Lotus corniculatus* microbial communities. This figure integrates results from multiyear, multisite sequencing of bacterial, fungal, and eukaryotic communities in *L. corniculatus*, summarizing how spatial, temporal, and ecological processes shape organ-specific microbiomes within a metacommunity framework. Arrows indicate effects supported by results in the manuscript. Solid lines represent hierarchical relationships in the framework (*e.g.*, microbial diversity as a component of organ-specificity), while dotted lines indicate

functional effects extracted from observed data: red for positive influences and blue for negative influences. For instance, temporal factors positively influenced alpha diversity in aboveground plant parts, whereas spatial factors had a stronger effect on the beta diversity of fungal and eukaryotic communities. Transmission processes such as microbial sourcing and priority effects further shaped organ-specific microbiomes by introducing stochastic variation and assembly history-dependent patterns.

We examined the diversity and community composition of *Lotus corniculatus* microbiomes in natural populations in the framework of metacommunity theory to gain broader insight on the ecology of plant microbiomes *in situ*. In this study we showed the organ-specificity of endophytic communities of *L. corniculatus* and established an overview of the assembly processes at tempo-spatial scales that account for the community patterns observed in plant microbiomes in natural populations (Fig. 6). Analysis of community composition and diversity of *L. corniculatus* microbiomes revealed that plant organs are the main source of variation in microbial community structure, while sampling years and sites contributed less. It has been shown that plant compartments contribute more in shaping microbial community composition than geographical locations or sampling times (96, 99, 100). Other studies found that plant compartments account more for associated bacterial community composition, while geographical locations of host plants rather determine fungal community composition (9, 101). Plant organs select for a group of microorganisms that developed adaptive traits to successfully inhabit their unique microenvironments, and in *L. corniculatus* organs abundant and persistent microorganisms are beneficial microbes that enhance growth and fitness, or pathogens that utilize host's genetic and physiological resources. In the same way, hub microbes in *L. corniculatus* organs are abundant and persistent microbes that are either known plant pathogens or beneficial to plant hosts.

L. corniculatus organs host distinct but overlapping microbial communities linked via transmission of microorganisms within the plant host and the outside environment, which signifies that the organs are discrete ecological niches that are interconnected with each other and with the environment. *L. corniculatus* organ-associated microorganisms can also potentially influence community composition during dispersal via priority effects. Consistent with previous observations, the root microbiomes are the most diverse communities among the plant organ communities and have distinct but overlapping community composition with soil microbiomes (42, 100-102). The overlapping and less diverse aboveground microbiomes that are distinct from root and soil microbiomes demonstrated the compositionally-nested characteristic of microbial communities observed in several plant species, where the aboveground communities are subsets of the more diverse belowground communities (9, 100, 101). While the main source of microbiome variation is plant compartment, biotic and abiotic factors from the environment also contribute to patterns of community structures in *L. corniculatus* microbiomes. Plant microbial community structure is also shaped by environmental gradients such as local site conditions, land use, and soil properties, as well as biotic elements like pollinators, insects, and local fauna (10, 98, 103-105). Abiotic and biotic elements in the environment acting at multiple temporal and spatial scales affect *L.*

corniculatus microbiomes - while different sampling times and locations affected plant microbial community composition, environmental factors acting at different plant compartments also attributed to the more diverse and stable root microbial communities that are distinct from the less diverse and more variable but overlapping endophytic communities in shoots, flowers, and seeds.

In this study we present bases for future experimentation to explore mechanisms on how key members of *L. corniculatus* organ microbiomes influence community dynamics and species interactions. Variations in community diversity and composition observed at tempo-spatial scales (*i.e.* from differences between root and aboveground plant microbiomes to between year/site variations) provided basis for more in-depth investigation of the crucial roles of abiotic environmental factors, such as soil properties like land use history and soil chemistry, climate and temperature differences in soil and aboveground, or elevation gradients, in shaping these community patterns. This study also provided a requisite basis to test predictions on important agents of horizontal transmission in the environment such as pollinators or on seed microbiomes as initial colonizers during vertical transmission. While in this study we focused on selective filtering by plant organs, microbial interactions, and environmental factors, as well as on stochastic transmission of microorganisms from various sources, other factors such as plant genotype diversity, wider range of geographic locations, or other ecological processes such as genetic drift and diversification of microbiome members additionally cause variations in community dynamics of plant-associated microbiomes. Given the functions of plant-associated microbial communities in plant growth, stress tolerance, and protection, reconstruction of plant microbiomes offers prospects to maximize their beneficial effects for plant productivity, resilience, and pathogen defense. To successfully control plant microbiomes in the field, there remains a need for a comprehensive knowledge of the ecological processes and microbial interactions that shape microbial community dynamics and assembly in natural environments.

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AUTHOR CONTRIBUTIONS: E.K., O.B., J.A., and K.L. conceptualized this work. K.L., F.R., and J.A. collected and processed samples from the field. K.L. performed the experiments and data analysis, with M.M. in the machine-learning analysis. K.L., M.M., and E. K. wrote the manuscript, with contributions from all authors. All authors read and approved the final manuscript.

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DATA AVAILABILITY: Primers and blocking oligos used in this study are listed on Table S1. Sequencing data, metadata, OTU tables, and scripts are available on https://gitlab.nfdi4plants.de/kalutap/ecology-of-the-core-microbiome-in-natural-lotus-corniculatus-populations and on Biodiversity Exploratories Information System (BExIS) (https://www.bexis.uni-jena.de/) under Dataset ID 31836.

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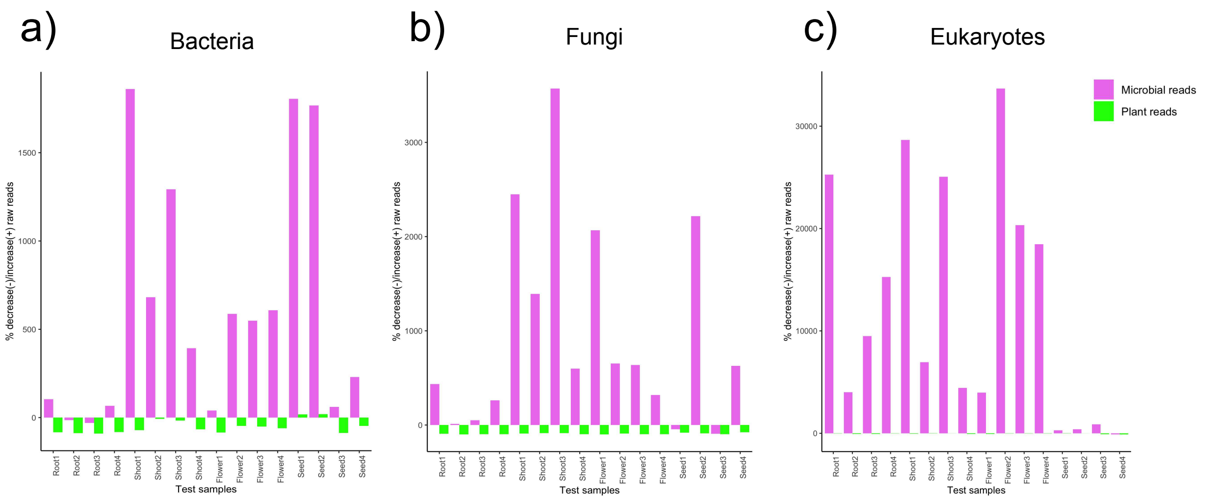
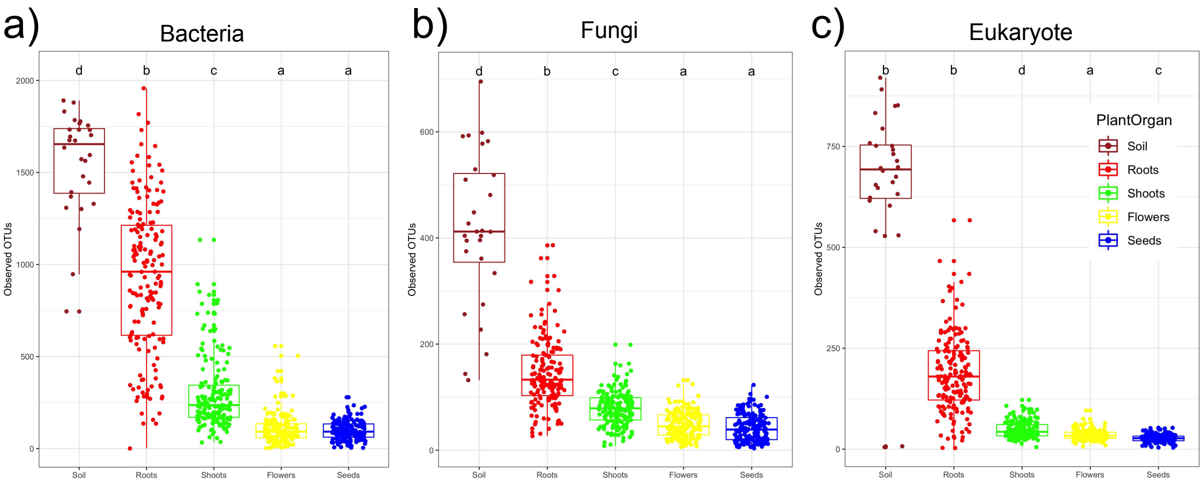
SUPPLEMENTARY MATERIALS

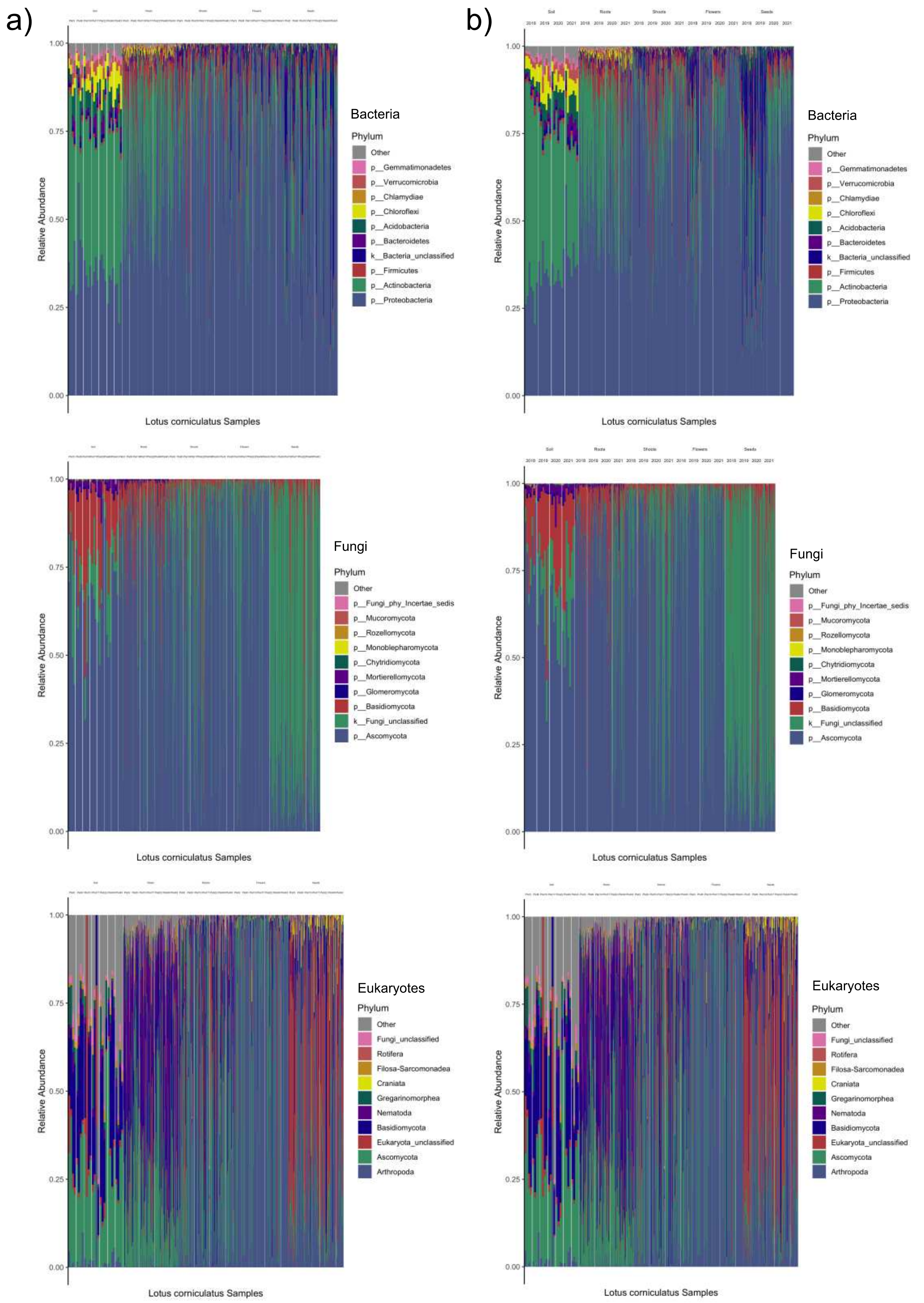
SUPPLEMENTARY FIGURES



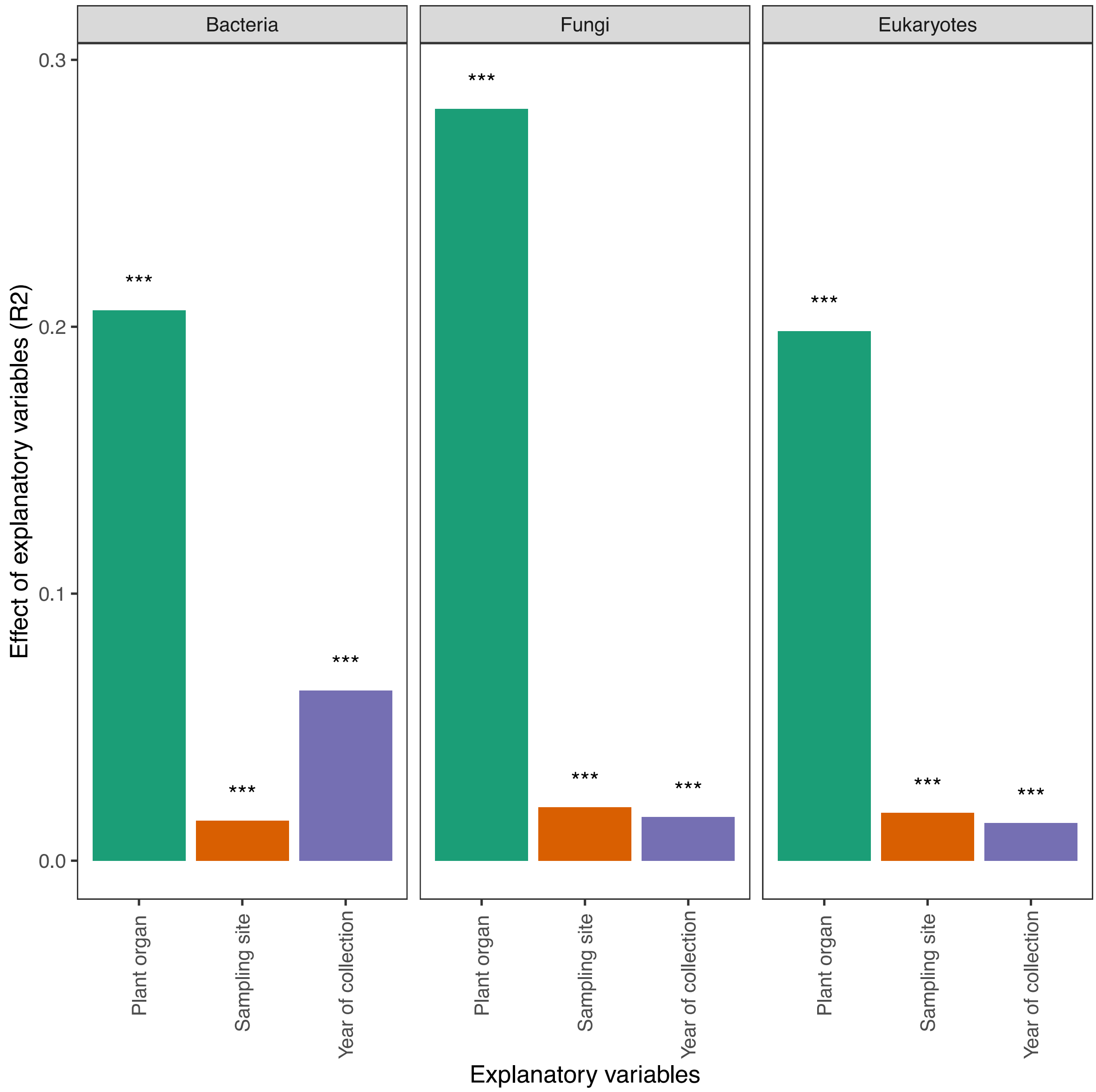
Supplementary Figure 1. Increase of target **(a)** bacterial 16S rRNA, **(b)** fungal ITS2, and **(c)** eukaryotic 18S rRNA reads and decrease of corresponding reads of nontarget host *Lotus corniculatus* chloroplast, mitochondria, plant ITS2, and plant 18S rRNA when blocking oligos are used during library preparation of test sequencing samples.



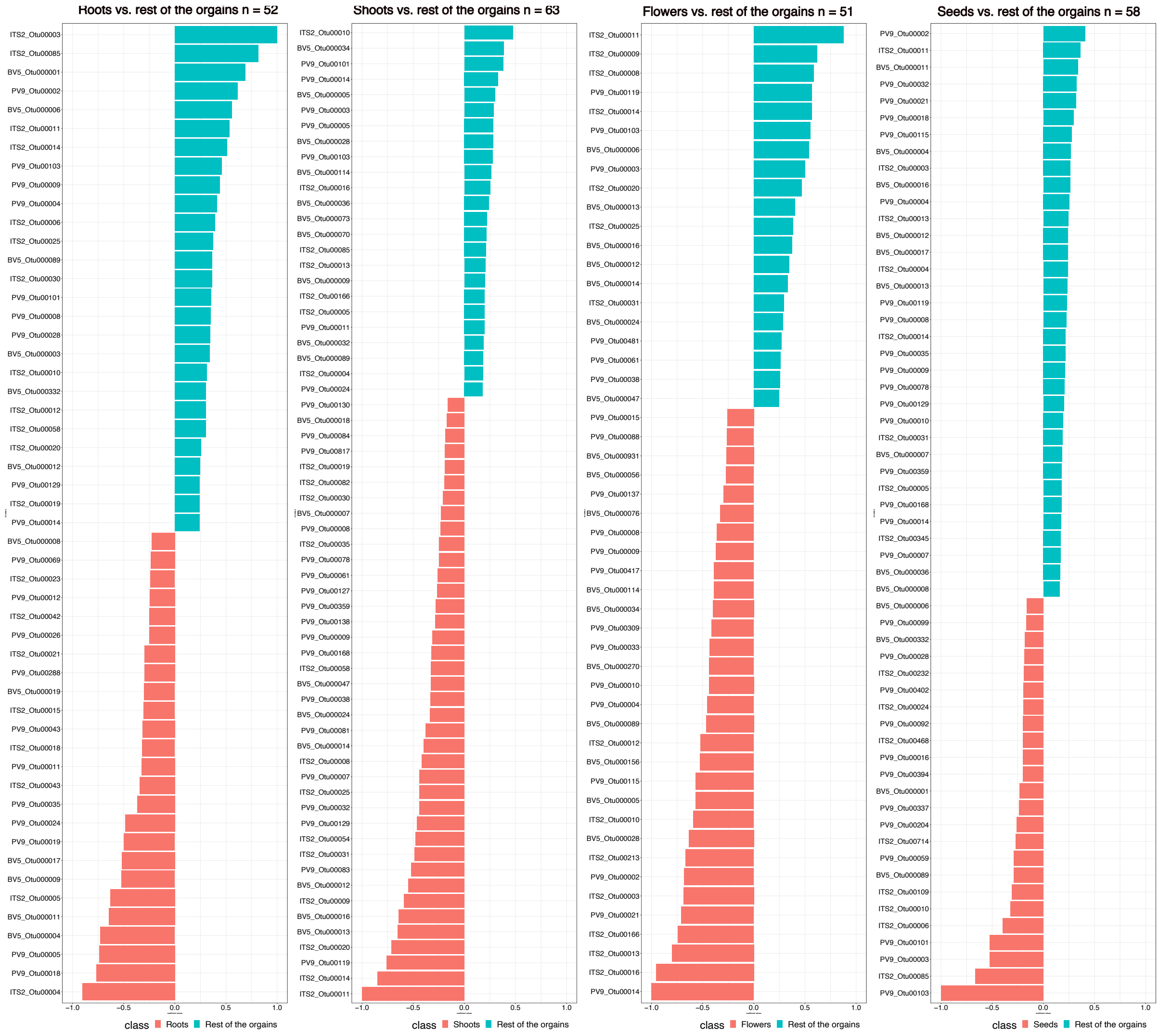
Supplementary Figure 2. Boxplots of Observed OTUs of **(a)** bacterial, **(b)** fungal, and **(c)** eukaryotic microbial communities associated with soil and *Lotus corniculatus* plant organs. Datasets have non-normal distribution based on Shapiro-Wilk normality tests (*P*<0.05) thus Kruskal-Wallis rank sum tests were used to test significant differences of α-diversity measurements between soil and plant organ samples. Compartments are significantly different if letters are not similar based on post-hoc analysis via Dunn's (Supplementary Table 4).



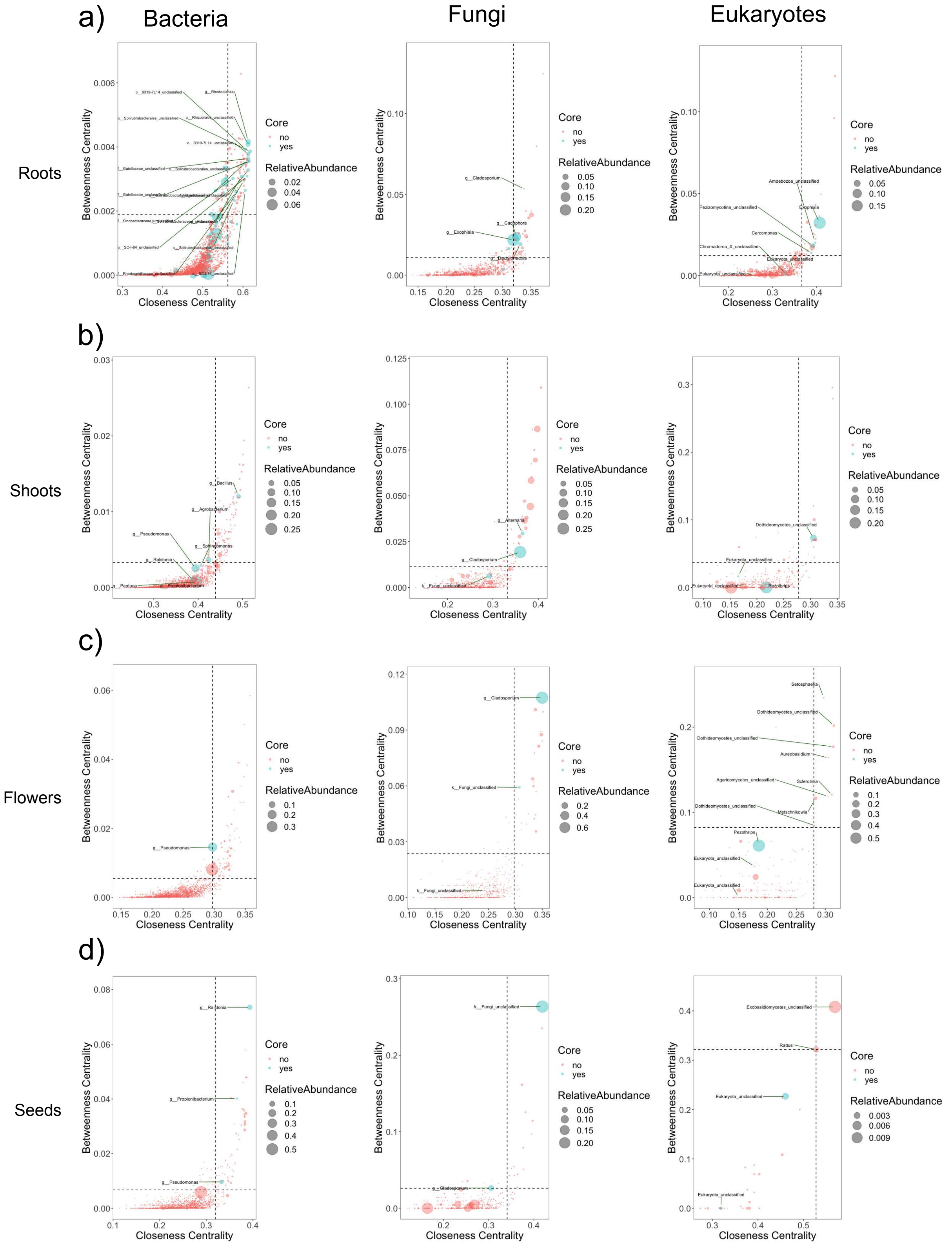
Supplementary Figure 3. Relative abundance of the top ten most abundant bacterial, fungal, and eukaryotic phyla detected in all soil and plant samples collected from **(a)** seven grassland sites for **(b)** four years. The relative abundance of the OTUs for each sample were aggregated at the phylum level.



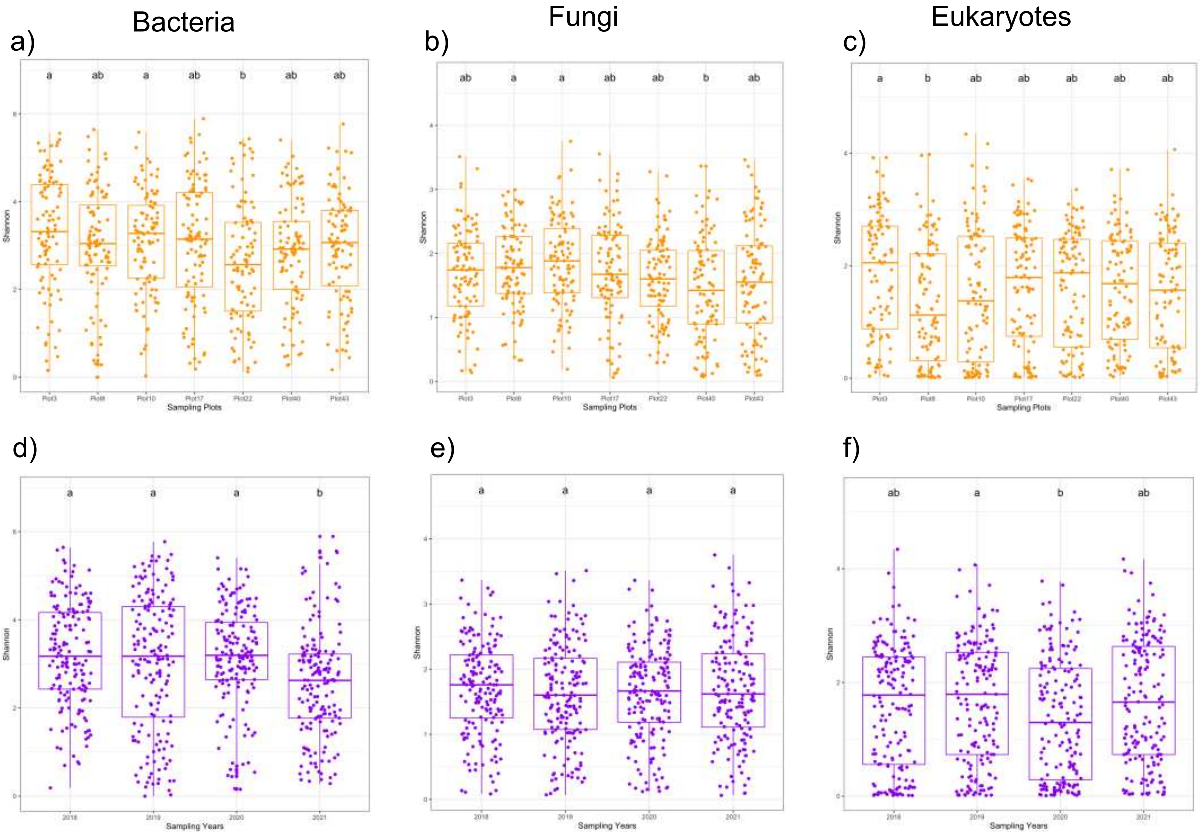
Supplementary Figure 4. Barplots of R^2 statistic from PERMANOVA showing the percentage of variance that can be explained by factors such as plant organ, year of collection, and sampling sites (Supplementary Table 4).



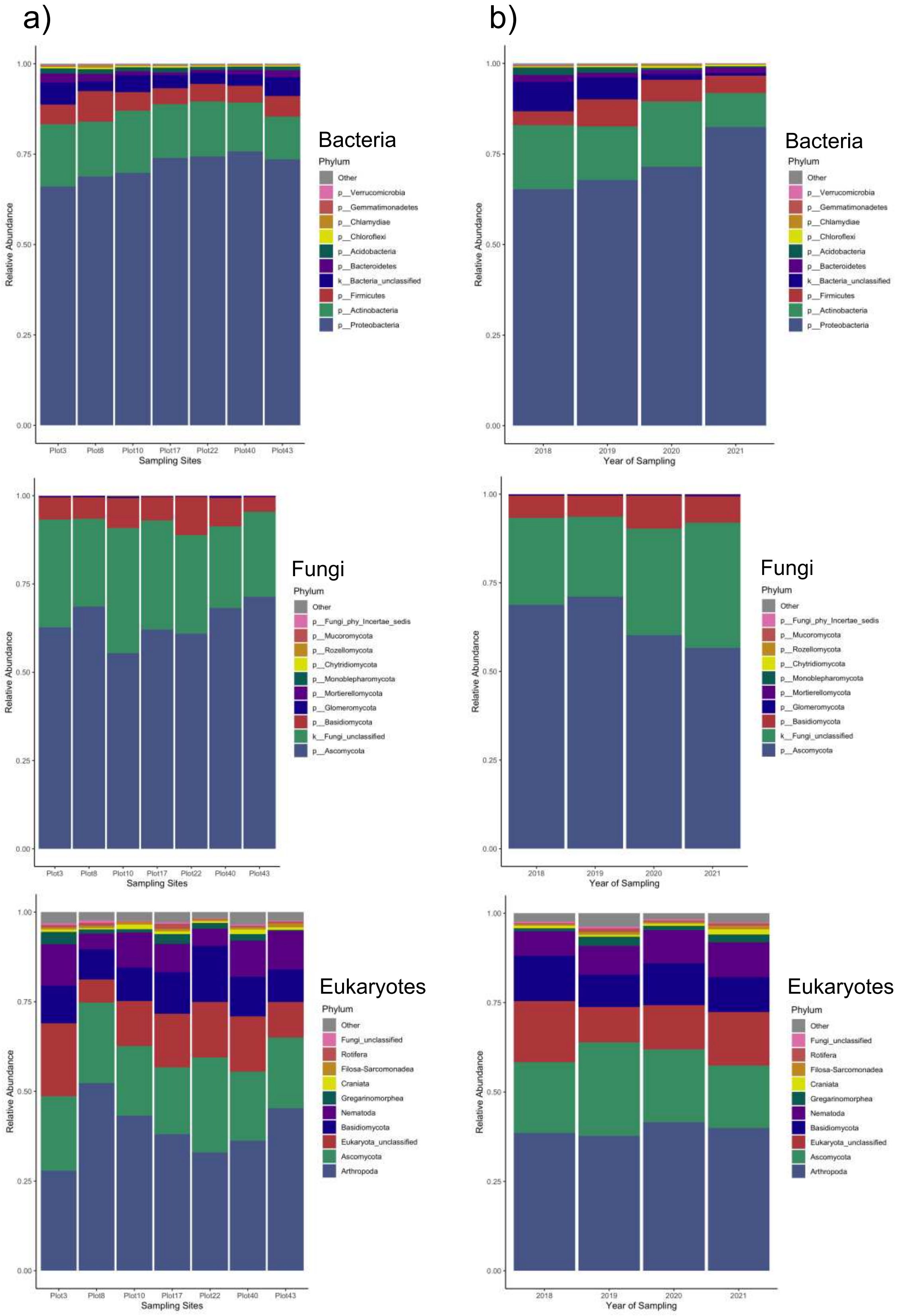
Supplementary Figure 5. Discriminatory OTUs that distinguish each organ from others. The OTUs were identified using an SVM classifier with recursive feature elimination and cross-validation. A total of 84, 102, 57, and 166 OTUs for roots, shoots, flowers, and seeds, respectively, were identified. The x-axis values represent the coefficient value of each OTU in separating a specific organ (values < 0) from the rest (values > 0). Only OTUs with absolute coefficient values greater than 0.5 are included.



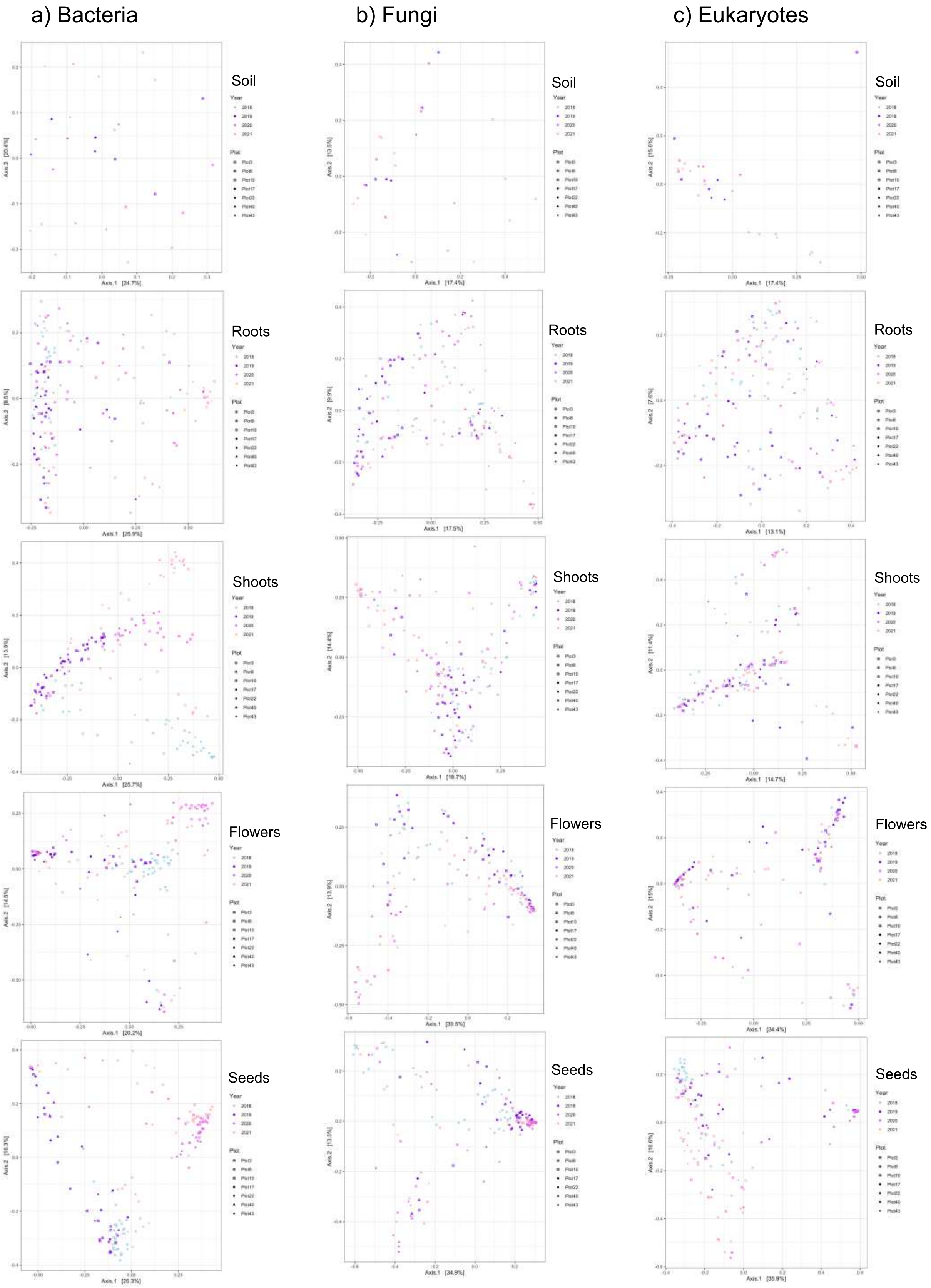
Supplementary Figure 6. Microbial hubs of **(a)** root, **(b)** shoot, **(c)** flower, and **(d)** seed microbiomes. Bacterial, fungal, and eukaryotic hubs were identified based on their high betweenness centrality and closeness centrality in correlation networks calculated with FastSpar (Fig. 3). Dotted lines mark the OTUs that are top 5% in betweenness centrality and closeness centrality scores and thus assigned as hub microbes. Size of the circles represent the OTU abundance and green circles are the core microbes.



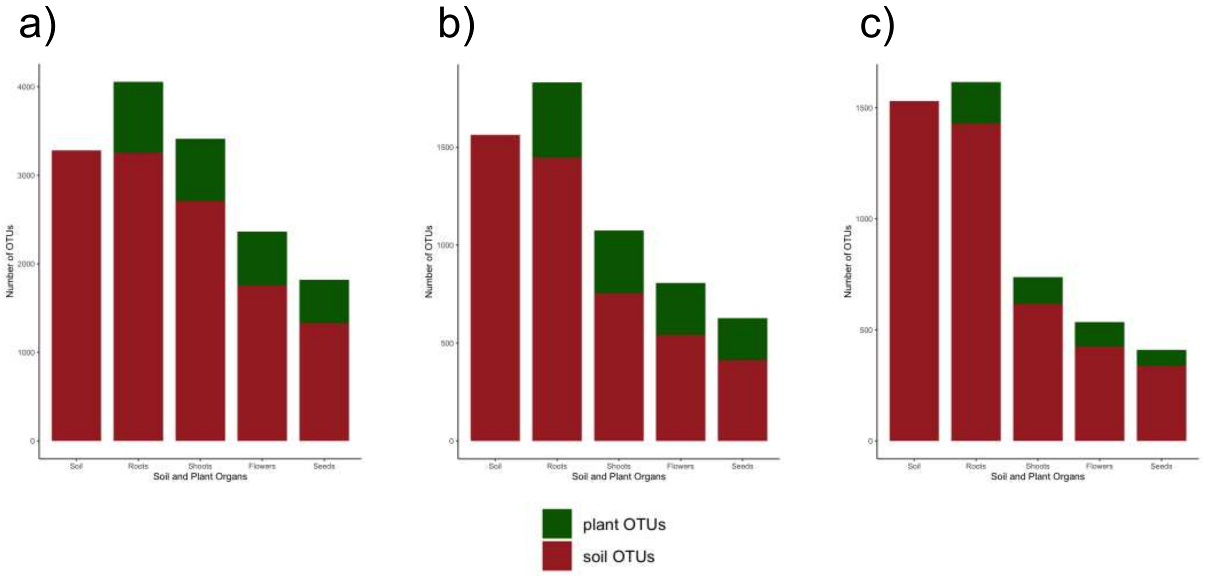
Supplementary Figure 7. Boxplots of Shannon's α-diversity measurements of bacterial, fungal, and eukaryotic microbial communities associated with whole plants. Shapiro-Wilk normality and Kruskal-Wallis rank sum tests were used to test significant differences of Shannon measurements between **(a-c)** sampling sites and (**d-f)** sampling years. Post-hoc analysis via Dunn's tests indicate that groups are significantly different if letters are not similar.



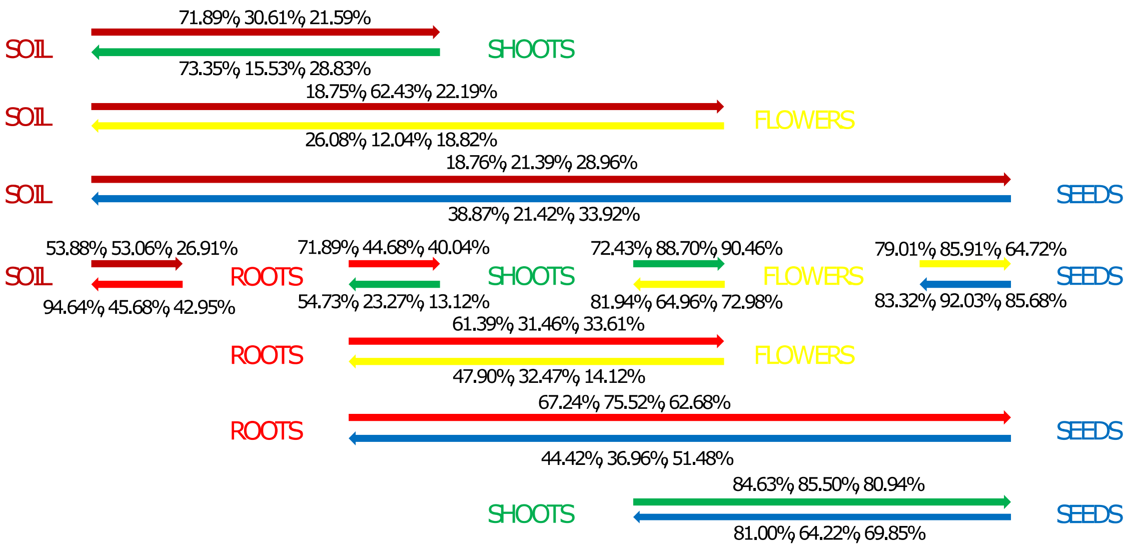
Supplementary Figure 8. Relative abundance profiles showing the top most abundant bacterial, fungal, and eukaryotic phyla in plant samples collected from **(a)** seven grassland sites for **(b)** four years. The relative abundance of the OTUs for each site or year were aggregated at the phylum level.



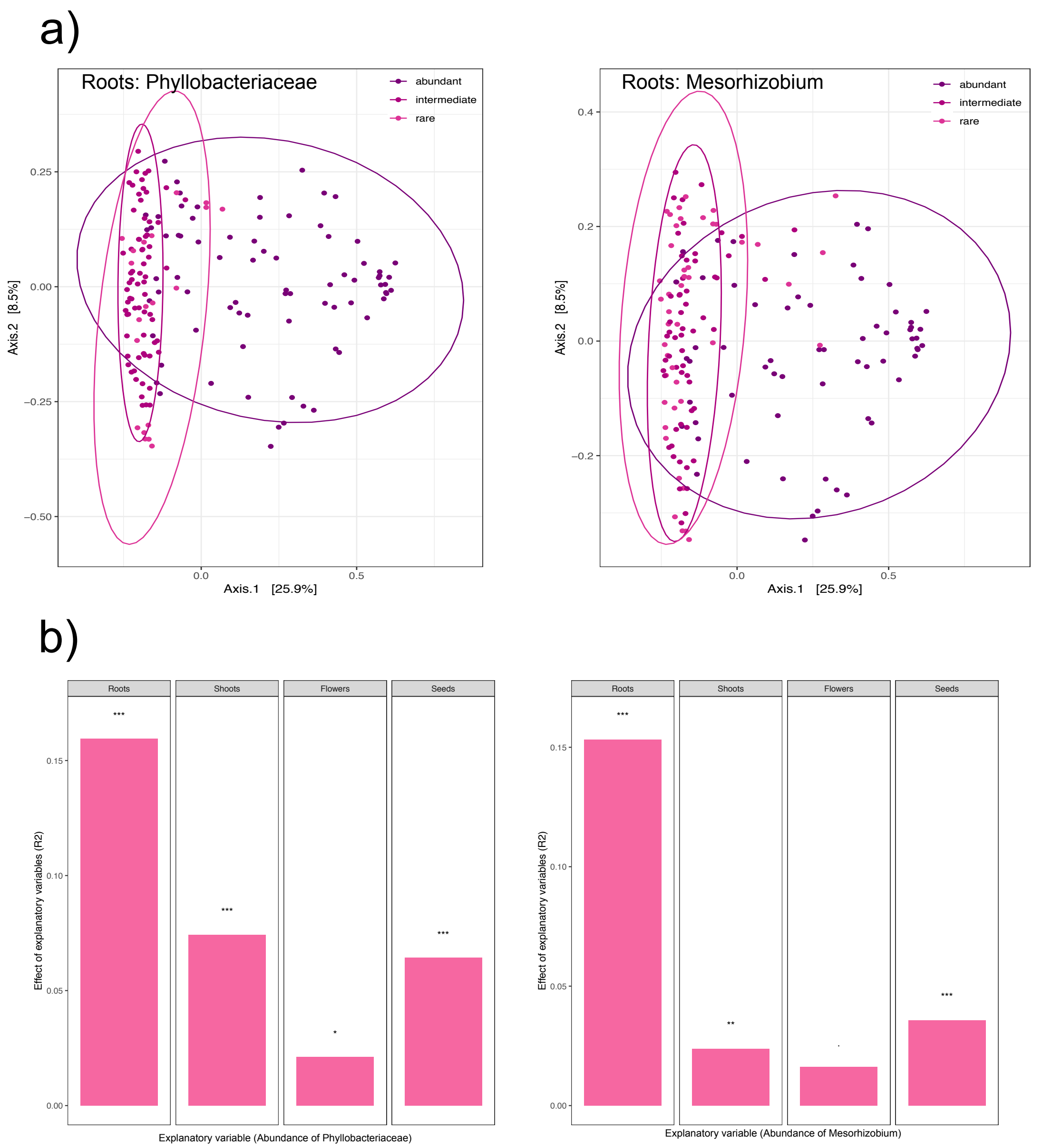
Supplementary Figure 9. Principal coordinate plots based on Bray-Curtis dissimilarities between **(a)** bacterial, **(b)** fungal, and **(c)** eukaryotic microbial communities associated with soil and plant organ samples.



Supplementary Figure 10. Distribution of soil and plant **(a)** bacterial, **(b)** fungal, and **(c)** eukaryotic OTUs in soil and plant organs. OTUs are designated as soil OTUs if they are found in soil samples, and the rest of non-soil OTUs are designated as plant OTUs.



Supplementary Figure 11. FEAST calculations of source contribution to the sink microbiomes. Microbial transmission was tracked at multiple directions of sources and sinks. The contribution of the source to the sink microbiomes are in percentages of the total bacterial, fungal, and eukaryotic sink microbiomes, respectively.



Supplementary Figure 12. **(a)** PCoA of Bray-Curtis dissimilarities of root microbial communities and **(b)** PERMANOVA using abundance of candidate early-arriving OTU as explanatory variable of community composition variation. **(a)** and **(b)** show that candidate early-arriving OTUs (BV5_OTU4_*Phyllobacteriaceae,* BV5_OTU17_*Mesorhizobium*) potentially altered the microbial community composition in the roots based on their abundance. (Explanatory variables: abundant OTU: Relative abundance in the root microbial community (RA) >= 0.01; intermediate OTU: RA >= 0.001 and < 0.01; rare OTU: RA < 0.001).

SUPPLEMENTARY TABLES

Supplementary Table 1. Primers and blocking oligos used in this study.

Primer name	Primer sequence (5’-to-3’ orientation)
799F	AACMGGATTAGATACCCKG
1192R	ACGTCATCCCCACCTTCC
fITS7	GTGARTCATCGAATCTTTG
ITS4	TCCTCCGCTTATTGATATGC
F1422	ATAACAGGTCTGTGATGCCC
R1797	TGATCCTTCTGCAGGTTCACCTAC
clamp1_BV5_mitoF	GATGAGTGTTCGCCCTTGGTCTACGTGGAT
clamp1_BV5_mitoR	CTGCTCAGGGTTCCAAACTCAACGTTGGCA
clamp1_ITS2_F	AACCATTAGGTCGAGGGCACGTCTGCCTGG
clamp1_ITS2_R	TGAGMGYGGTTACACCACGCATGCGGGTCT
clamp9_PV9_F	GATGTATTCAACGAGTCTATAGCCTTGGCC
Clamp15_PV9_R	TCTCACAACGTCGCAGGCAGCGAACCGCCC

Supplementary Table 2. Basic statistics of the Illumina sequencing data. Amplicon sequencing of the microbial 16S rRNA, ITS2, and 18S rRNA genes in a total of 700 samples of soil and *Lotus corniculatus* roots, shoots, flowers, and seeds, along with blank samples collected from seven grassland sites in the Swabian Alps, Germany for four years.

Sequencing runs	Sequence reads	Sequence length	%GC
RunLotus1	15,367,467	300	52
RunLotus2	19,049,132	300	52
RunLotus3	19,931,059	300	52.5
RunLotus4	18,326,536	300	52.5
RunLotus6	21,112,668	301	53
RunLotus7	20,429,103	301	52.5
RunLotus8	20,404,891	301	53
RunLotus9	21,088,582	301	52.5
	155,709,438 (total)		

Supplementary Table 3. Summary of sequence data after processing by mothur and phyloseq.

Locus	Domain	Subdomain	Kingdom	Phylum	Subphylum	Class	Order	Family	Genus	Species	OTUs
16S rRNA	-	-	Bacteria	25	-	95	182	335	586	671	4,225
ITS2	-	-	Fungi	15	-	51	129	280	531	788	2,027
18S rRNA	Eukaryota	10	26	73	122	197	-	-	425	486	1,773

Supplementary Table 4. Statistical analysis of alpha- and beta-diversity measurements

(a) Shapiro-Wilk normality tests

α-diversity index	Statistic (W)	p-value	normality
16S rRNA_Shannon	0.98747	1.091e-05	non-normal distribution
16S rRNA_Observed	0.75878	< 2.2e-16	non-normal distribution
ITS2_Shannon	0.98424	7.343e-07	non-normal distribution
ITS2_Observed	0.70022	< 2.2e-16	non-normal distribution
18S rRNA_Shannon	0.95252	3.048e-14	non-normal distribution
18S rRNA_Observed	0.56119	< 2.2e-16	non-normal distribution

(b) Kruskal-Wallis rank sum tests by soil/plant organs and post-hoc analysis via Dunn's test

α-diversity index	Statistic (K-W chi-squared)	p-value	significance
16S rRNA_Shannon	295.25	< 2.2e-16	significant differences
16S rRNA_Observed	498.33	< 2.2e-16	significant differences
ITS2_Shannon	237.31	< 2.2e-16	significant differences
ITS2_Observed	397.04	< 2.2e-16	significant differences
18S rRNA_Shannon	258.82	< 2.2e-16	significant differences
18S rRNA_Observed	423.6	< 2.2e-16	significant differences

(c) Permutational multivariate analysis of variance (PERMANOVA) of variables plant organ, collection year, and sampling sites.

Permutation test for adonis under reduced model
Terms added sequentially (first to last)
Permutation: free
Number of permutations: 999

16S rRNA					
	Df	SumOfSqs	R2	F	Pr(>F)
PlantOrgan	4	55.372	0.20622	49.3158	0.001 ***
Year	3	17.103	0.06370	20.3100	0.001 ***
Plot	6	4.029	0.01501	2.3923	0.001 ***
Residual	684	192.001	0.71507		
Total	697	268.506	1.00000		
ITS2					
	Df	SumOfSqs	R2	F	Pr(>F)
PlantOrgan	4	76.128	0.28167	70.9434	0.001 ***
Year	3	4.441	0.01643	5.5178	0.001 ***
Plot	6	5.408	0.02001	3.3598	0.001 ***
Residual	687	184.303	0.68190		
Total	700	270.280	1.00000		
18S rRNA					
	Df	SumOfSqs	R2	F	Pr(>F)
PlantOrgan	4	60.755	0.19845	44.2882	0.001 ***
Year	3	4.310	0.01408	4.1894	0.001 ***
Plot	6	5.475	0.01788	2.6606	0.001 ***
Residual	687	235.610	0.76959		

Total	700	306.151	1.00000		

Significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Abbreviations: Df ‘degrees of freedom’; SumsOfSqs ‘Sums of Squares’; F ‘F statistic’; R2 ‘R2 statistic’; Pr(>F) ‘p-values’.

Supplementary Table 5. Performance tests of the SVM models.

Taxa	Organ		precision	recall	f1-score	support
Bacteria	Seeds	0	0.625	0.80357143	0.703125	56
Bacteria	Flowers	1	0.69047619	0.52727273	0.59793814	55
Bacteria	Shoots	2	0.81132075	0.76785714	0.78899083	56
Bacteria	Roots	3	0.98148148	0.96363636	0.97247706	55
Bacteria	Soil	4	0.9	1	0.94736842	9
Bacteria		accuracy	0.77489177	0.77489177	0.77489177	0.77489177
Bacteria		macro avg	0.80165569	0.81246753	0.80197989	231
Bacteria		weighted avg	0.78134907	0.77489177	0.77254389	231
Taxa			precision	recall	f1-score	support
Fungi	Seeds	0	0.7	0.89090909	0.784	55
Fungi	Flowers	1	0.75806452	0.83928571	0.79661017	56
Fungi	Shoots	2	0.9	0.64285714	0.75	56
Fungi	Roots	3	1	0.91071429	0.95327103	56
Fungi	Soil	4	1	1	1	9
Fungi		accuracy	0.82758621	0.82758621	0.82758621	0.82758621
Fungi		macro avg	0.8716129	0.85675325	0.85677624	232
Fungi		weighted avg	0.84634316	0.82758621	0.82807477	232
Taxa	Organ		precision	recall	f1-score	support
Eukaryote	Seeds	0	0.85	0.92727273	0.88695652	55
Eukaryote	Flowers	1	0.73846154	0.85714286	0.79338843	56
Eukaryote	Shoots	2	0.76595745	0.64285714	0.69902913	56
Eukaryote	Roots	3	0.98076923	0.91071429	0.94444444	56
Eukaryote	Soil	4	0.875	0.77777778	0.82352941	9
Eukaryote		accuracy	0.83189655	0.83189655	0.83189655	0.83189655
Eukaryote		macro avg	0.84203764	0.82315296	0.82946959	232
Eukaryote		weighted avg	0.8353256	0.83189655	0.8304252	232

Supplementary Table 6. Abundant OTUs (**relative abundance > 1%**) in *Lotus corniculatus* organs.

		Relative abundance			
OTU	Genus	Roots	Shoots	Flowers	Seeds
Bacteria					
BV5_Otu000001	*Pantoea*	**0.01498769**	**0.25475602**	**0.39135219**	**0.53456217**
BV5_Otu000003	*Pseudomonas*	**0.01419121**	**0.10772585**	**0.20539426**	**0.0531343**

BV5_Otu000004	*Phyllobacteriaceae*	**0.07248534**	0.00054067	0.00086442	0.00054539
BV5_Otu000005	*Enterobacteriaceae*	2.45E-05	0.00237893	**0.12226135**	**0.01422907**
BV5_Otu000006	*Ralstonia*	0.0005517	**0.06358809**	**0.01562879**	**0.07815603**
BV5_Otu000007	*Bacillus*	**0.03395148**	**0.02838181**	0.00241461	0.00069461
BV5_Otu000008	*Frankia*	**0.04906623**	0.00023149	3.93E-05	1.57E-05
BV5_Otu000009	*Phyllobacterium*	**0.03919923**	0.00068449	0.00030655	7.77E-05
BV5_Otu000010	*Pantoea*	0.00091647	**0.0124075**	**0.01680126**	**0.02267523**
BV5_Otu000011	*Mesorhizobium*	**0.02558929**	0.00063515	0.00072956	7.94E-05
BV5_Otu000012	*Burkholderia*	0.00164728	**0.03042322**	0.00972065	**0.01644205**
BV5_Otu000013	*Burkholderia*	0.00083817	**0.03141899**	0.00876367	**0.01736878**
BV5_Otu000014	*Agrobacterium*	0.00501434	**0.03865919**	0.00136009	0.00281248
BV5_Otu000015	*Streptomyces*	**0.02373183**	0.00045353	9.95E-05	0.00011431
BV5_Otu000016	*Burkholderia*	0.00074167	**0.0303996**	0.00767331	**0.01618725**
BV5_Otu000017	*Mesorhizobium*	**0.02286487**	0.00031075	0.00065989	0.00025132
BV5_Otu000018	*Comamonadaceae*	0.0062342	**0.01769185**	0.00033791	**0.02457407**
BV5_Otu000019	*Cryptosporangium*	**0.02155104**	0.00015905	0.00010301	2.01E-05
BV5_Otu000021	*Pantoea*	0.00052872	0.00695622	**0.01308926**	**0.01592197**
BV5_Otu000022	*Methylibium*	**0.019637**	0.00022047	7.02E-05	1.83E-05
BV5_Otu000023	*Steroidobacter*	**0.01899183**	0.00025301	5.92E-05	4.80E-05
BV5_Otu000024	*Xanthomonadaceae*	0.00477739	**0.02151746**	0.00025778	0.00059513
BV5_Otu000028	*Wolbachia*	1.01E-06	0.0014283	**0.01519235**	**0.01760352**
BV5_Otu000032	*Pseudomonas*	0.00045571	0.00352115	**0.01337044**	0.00700109
BV5_Otu000040	*Sodalis*	0.00184406	1.26E-05	5.47E-06	**0.02810647**
BV5_Otu000056	*Enterobacteriaceae*	0.00108648	0.00033332	**0.01385366**	3.49E-05
BV5_Otu000068	*Gluconobacter*	2.27E-06	5.25E-06	**0.0128056**	1.75E-06
Fungi					
ITS2_Otu00003	*Cladosporium*	0.00108977	**0.2671789**	**0.76240753**	**0.04540433**
ITS2_Otu00004	*Exophiala*	**0.23861407**	0.00042552	0.00037056	0.00037517
ITS2_Otu00005	*Cadophora*	**0.12629519**	0.00027117	0.0001305	0.00026054
ITS2_Otu00006	*Fungi*	**0.01084811**	**0.04378309**	**0.01783285**	**0.24165332**
ITS2_Otu00008	*Boeremia*	0.00022675	**0.06855963**	0.00358989	**0.15808927**
ITS2_Otu00009	*Didymellaceae*	0.00791914	**0.0633385**	**0.01207996**	0.00837013
ITS2_Otu00010	*Sclerotiniaceae*	1.36E-05	2.38E-05	**0.03687987**	**0.20319659**
ITS2_Otu00011	*Septoria*	4.2685E-05	**0.09075922**	5.94E-05	0.0002119
ITS2_Otu00012	*Alternaria*	4.83E-05	**0.02015062**	**0.03302806**	**0.0907913**
ITS2_Otu00013	*Didymellaceae*	0.00023233	**0.02319715**	**0.03025786**	**0.03560474**
ITS2_Otu00014	*Chaetosphaeronema*	0.00059344	**0.07000393**	4.40E-05	0.00016153
ITS2_Otu00015	*Pseudomassaria*	**0.02789404**	4.14E-05	3.9402E-05	2.95E-05
ITS2_Otu00016	*Botryotinia*	2.97E-05	0.00139567	**0.04266852**	0.00042554
ITS2_Otu00017	*Helotiales*	**0.02200804**	0.00236582	1.64E-05	9.90E-05
ITS2_Otu00018	*Tetracladium*	**0.02145356**	1.11E-05	1.59E-05	1.74E-05
ITS2_Otu00019	*Alternaria*	4.93E-05	**0.01773128**	**0.01641507**	**0.03986536**
ITS2_Otu00020	*Podospora*	1.12E-05	**0.04191523**	0.00152255	3.47E-05
ITS2_Otu00021	*Dactylonectria*	**0.01881724**	1.4032E-05	1.59E-05	4.86E-05
ITS2_Otu00022	*Neocosmospora*	**0.0187555**	2.68E-05	4.10E-06	1.39E-05
ITS2_Otu00023	*Cistella*	**0.01668841**	8.16E-06	5.79E-05	3.65E-05

ITS2_Otu00024	*Gibberella*	0.00182659	**0.01768527**	0.00076435	**0.07204492**
ITS2_Otu00025	*Colletotrichum*	0.00125693	**0.03415043**	0.00052657	0.00015979
ITS2_Otu00026	*Mycena*	**0.0148273**	6.79E-05	1.25E-05	1.74E-05
ITS2_Otu00028	*Ascomycota*	**0.01283799**	0.00064285	0.00170248	5.21E-05
ITS2_Otu00030	*Uromyces*	1.29E-06	**0.03014028**	0.00010317	2.78E-05
ITS2_Otu00031	*Stemphylium*	0.00164826	**0.02476839**	4.30E-05	0.00101609
ITS2_Otu00085	*Fungi*	0.00087376	0.00188744	0.00146493	**0.01086953**
ITS2_Otu00109	*Malassezia*	8.0501E-05	0.0006931	0.0005539	**0.02153931**
Eukaryotes					
PV9_Otu00002	*Pezothrips*	0.00127649	**0.22407626**	**0.5935281**	0.00544734
PV9_Otu00003	*Insecta*	0.0001378	0.00840221	0.00466225	**0.9106856**
PV9_Otu00004	*Frankliniella*	0.00015456	**0.09482595**	**0.12677873**	0.00044784
PV9_Otu00005	*Exophiala*	**0.18068931**	0.00018327	6.80E-05	9.03E-05
PV9_Otu00007	*Pucciniomycetes*	0.00063188	**0.23242558**	0.0020743	0.00016626
PV9_Otu00008	*Mayetiola*	0.00012911	**0.07492251**	**0.0488326**	0.00012514
PV9_Otu00009	*Dothideomycetes*	**0.02789218**	**0.06098462**	**0.01528368**	0.00410025
PV9_Otu00010	*Aeolothrips*	2.76E-05	0.00940713	**0.05010144**	3.66E-05
PV9_Otu00011	*Agaricomycetes*	**0.05681411**	6.27E-05	2.94E-05	3.22E-05
PV9_Otu00012	*Agaricomycetes*	**0.04652027**	0.00029151	1.51E-05	5.99E-05
PV9_Otu00013	*Insecta*	1.18E-05	**0.03836716**	**0.02424527**	0.00065611
PV9_Otu00014	*Metschnikowia*	1.24E-05	0.00027306	**0.0343287**	5.10E-05
PV9_Otu00015	*Insecta*	1.64E-05	**0.01191882**	**0.02390365**	**0.02006059**
PV9_Otu00016	*Agaricomycetes*	**0.03859289**	0.0001064	5.70E-06	0.00031286
PV9_Otu00018	*Aglenchus*	**0.03959254**	3.69E-05	1.12E-05	0.00012067
PV9_Otu00019	*Agaricomycetes*	**0.02896228**	0.00206027	1.56E-05	1.07E-05
PV9_Otu00021	*Insecta*	1.64E-05	0.00815929	**0.01215686**	1.79E-05
PV9_Otu00022	*Agaricomycetes*	**0.03146715**	9.84E-06	4.61E-06	4.92E-05
PV9_Otu00024	*Chromadorea*	**0.02882231**	5.66E-05	1.51E-05	2.41E-05
PV9_Otu00026	*Pezizomycotina*	**0.02743875**	1.23E-05	8.78E-06	2.32E-05
PV9_Otu00032	*Arachnida*	4.03E-06	**0.04404551**	0.00080172	1.34E-05
PV9_Otu00033	*Arachnida*	3.26E-05	7.38E-06	**0.01642066**	1.79E-05
PV9_Otu00038	*Insecta*	6.52E-06	**0.0440246**	1.54E-06	2.86E-05
PV9_Otu00081	*Leotiomycetes*	3.57E-05	**0.01225707**	0.00010707	9.83E-06
PV9_Otu00101	*Exobasidiomycetes*	0.00018094	0.00033456	0.00011497	**0.01129961**

Supplementary Table 7. Key microbes (abundant OTUs: relative abundance > 1%; core OTUs: persistent in 90% of samples; hub OTUs; separator OTUs: predicted by SVM model) in *Lotus corniculatus* organs.

(a) Roots

	Separator	Abundant	Core	Hub
BV5_Otu000001_Pantoea	yes	yes	no	no
BV5_Otu000003_Pseudomonas	yes	yes	yes	no
BV5_Otu000004_Phyllobacteriaceae	yes	yes	yes	no
BV5_Otu000005_Enterobacteriaceae	no	no	no	no
BV5_Otu000006_Ralstonia	yes	no	no	no

BV5_Otu000007_Bacillus	no	yes	yes	no
BV5_Otu000008_Frankia	yes	yes	yes	no
BV5_Otu000009_Phyllobacterium	yes	yes	yes	no
BV5_Otu000010_Pantoea	no	no	no	no
BV5_Otu000011_Mesorhizobium	yes	yes	yes	no
BV5_Otu000012_Burkholderia	yes	no	no	no
BV5_Otu000013_Burkholderia	yes	no	no	no
BV5_Otu000014_Agrobacterium	no	no	no	no
BV5_Otu000015_Streptomyces	no	yes	yes	no
BV5_Otu000016_Burkholderia	yes	no	no	no
BV5_Otu000017_Mesorhizobium	yes	yes	yes	no
BV5_Otu000018_Comamonadaceae	no	no	yes	no
BV5_Otu000019_Cryptosporangium	yes	yes	yes	no
BV5_Otu000021_Pantoea	no	no	no	no
BV5_Otu000022_Methylibium	no	yes	yes	no
BV5_Otu000023_Steroidobacter	yes	yes	yes	no
BV5_Otu000024_Xanthomonadaceae	no	no	yes	no
BV5_Otu000025_Rhodoplanes	no	no	yes	yes
BV5_Otu000026_Rhizobium	yes	yes	yes	no
BV5_Otu000027_Gaiellaceae	no	no	yes	yes
BV5_Otu000028_Wolbachia	no	no	no	no
BV5_Otu000029_Rhizobiales	no	no	yes	yes
BV5_Otu000030_0319-7L14	no	no	yes	yes
BV5_Otu000032_Pseudomonas	no	no	no	no
BV5_Otu000033_Chryseobacterium	no	yes	no	no
BV5_Otu000034_Prauserella	no	no	no	no
BV5_Otu000036_Bosea	yes	yes	yes	no
BV5_Otu000039_Methylibium	no	no	yes	no
BV5_Otu000040_Sodalis	no	no	no	no
BV5_Otu000043_Bradyrhizobiaceae	no	no	yes	no
BV5_Otu000045_Micromonosporaceae	no	no	yes	no
BV5_Otu000047_Methylobacterium	no	no	no	no
BV5_Otu000049_Kineosporia	no	no	yes	no
BV5_Otu000052_Methylobacterium	yes	no	no	no
BV5_Otu000054_Gaiellaceae	no	no	yes	yes
BV5_Otu000055_Methylobacterium	no	no	no	no
BV5_Otu000056_Enterobacteriaceae	no	no	no	no
BV5_Otu000057_Rhodospirillaceae	no	no	yes	yes
BV5_Otu000059_Solirubrobacterales	no	no	yes	yes
BV5_Otu000060_Hyphomicrobium	no	no	yes	no
BV5_Otu000061_Mycobacterium	no	no	yes	no
BV5_Otu000062_Flavobacterium	no	no	yes	no
BV5_Otu000063_SC-I-84	no	no	yes	yes
BV5_Otu000065_Solirubrobacterales	no	no	yes	yes
BV5_Otu000068_Gluconobacter	no	no	no	no
BV5_Otu000069_Pseudomonas	no	no	no	no

BV5_Otu000070_Rubrobacter	no	no	no	no
BV5_Otu000073_Erwinia	no	no	no	no
BV5_Otu000075_Solirubrobacteraceae	no	no	yes	yes
BV5_Otu000076_Acinetobacter	no	no	no	no
BV5_Otu000077_Solirubrobacterales	no	no	yes	yes
BV5_Otu000078_Sinobacteraceae	no	no	yes	yes
BV5_Otu000079_Buchnera	no	no	no	no
BV5_Otu000080_Pedomicrobium	no	no	yes	no
BV5_Otu000081_SC-I-84	no	no	yes	yes
BV5_Otu000084_Dongia	no	no	yes	no
BV5_Otu000086_Burkholderiaceae	no	no	no	no
BV5_Otu000089_Propionibacterium	yes	no	no	no
BV5_Otu000091_Patulibacteraceae	no	no	yes	no
BV5_Otu000092_0319-7L14	no	no	yes	yes
BV5_Otu000094_Caulobacter	no	no	yes	no
BV5_Otu000095_Sphingomonas	no	no	no	no
BV5_Otu000096_Bradyrhizobium	no	no	yes	no
BV5_Otu000097_Gaiellaceae	no	no	yes	yes
BV5_Otu000098_Mycobacterium	no	no	yes	no
BV5_Otu000102_Solibacillus	no	no	yes	no
BV5_Otu000105_Labrys	no	no	yes	no
BV5_Otu000106_Bacillus	no	no	yes	no
BV5_Otu000109_Kaistobacter	no	no	yes	yes
BV5_Otu000112_Planococcaceae	no	no	yes	no
BV5_Otu000113_Dongia	no	no	yes	no
BV5_Otu000114_Escherichia	no	no	no	no
BV5_Otu000117_Solirubrobacterales	no	no	yes	yes
BV5_Otu000118_[Weeksellaceae]	no	no	no	no
BV5_Otu000120_Bacillus	no	no	yes	no
BV5_Otu000125_Steroidobacter	no	no	yes	no
BV5_Otu000141_Ellin6513	no	no	no	no
BV5_Otu000143_Dolo	no	no	yes	no
BV5_Otu000144_Rhizobiales	no	no	yes	no
BV5_Otu000155_Burkholderia	no	no	no	no
BV5_Otu000156_Staphylococcus	no	no	no	no
BV5_Otu000194_Micromonospora	no	no	yes	no
BV5_Otu000256_Bacillales	no	no	yes	no
BV5_Otu000713_Bacteria	no	no	no	no
ITS2_Otu00003_Cladosporium	yes	no	yes	yes
ITS2_Otu00004_Exophiala	yes	yes	yes	yes
ITS2_Otu00005_Cadophora	yes	yes	yes	no
ITS2_Otu00006_Fungi	yes	yes	yes	yes
ITS2_Otu00008_Boeremia	no	no	no	no
ITS2_Otu00009_Didymellaceae	no	no	no	no
ITS2_Otu00010_Sclerotiniaceae	yes	no	no	no
ITS2_Otu00011_Septoria	yes	no	no	no

ITS2_Otu00012_Alternaria	yes	no	no	yes
ITS2_Otu00013_Didymellaceae	no	no	no	yes
ITS2_Otu00014_Chaetosphaeronema	yes	no	no	yes
ITS2_Otu00015_Pseudomassaria	yes	yes	no	no
ITS2_Otu00016_Botryotinia	no	no	no	yes
ITS2_Otu00017_Helotiales	no	yes	no	yes
ITS2_Otu00018_Tetracladium	yes	yes	no	no
ITS2_Otu00019_Alternaria	yes	no	no	no
ITS2_Otu00020_Podospora	yes	no	no	no
ITS2_Otu00021_Dactylonectria	yes	yes	yes	no
ITS2_Otu00022_Neocosmospora	yes	yes	no	no
ITS2_Otu00023_Cistella	yes	yes	no	no
ITS2_Otu00024_Gibberella	no	no	no	no
ITS2_Otu00025_Colletotrichum	yes	no	no	no
ITS2_Otu00026_Mycena	no	yes	no	no
ITS2_Otu00028_Ascomycota	yes	yes	no	no
ITS2_Otu00030_Uromyces	yes	no	no	no
ITS2_Otu00031_Stemphylium	yes	no	no	no
ITS2_Otu00033_Ilyonectria	no	yes	yes	no
ITS2_Otu00034_Serendipita	no	yes	no	yes
ITS2_Otu00035_Pseudoidium	yes	no	no	no
ITS2_Otu00036_Hemimycena	yes	yes	no	no
ITS2_Otu00037_Exophiala	no	yes	no	no
ITS2_Otu00042_Pleosporales	yes	no	no	no
ITS2_Otu00052_Titaea	yes	no	no	yes
ITS2_Otu00054_Ramularia	no	no	no	no
ITS2_Otu00058_Colletotrichum	yes	no	no	no
ITS2_Otu00063_Pleosporales	no	no	no	no
ITS2_Otu00085_Fungi	yes	no	yes	no
ITS2_Otu00109_Malassezia	yes	no	no	no
ITS2_Otu00232_Fungi	no	no	no	no
ITS2_Otu00257_Fungi	no	no	no	no
PV9_Otu00002_Pezothrips	yes	no	no	no
PV9_Otu00003_Insecta	yes	no	no	no
PV9_Otu00004_Frankliniella	yes	no	no	no
PV9_Otu00005_Exophiala	yes	yes	yes	yes
PV9_Otu00007_Pucciniomycetes	no	no	no	no
PV9_Otu00008_Mayetiola	yes	no	no	no
PV9_Otu00009_Dothideomycetes	yes	yes	no	yes
PV9_Otu00010_Aeolothrips	no	no	no	no
PV9_Otu00011_Agaricomycetes	yes	yes	no	no
PV9_Otu00012_Agaricomycetes	yes	yes	no	no
PV9_Otu00013_Insecta	yes	no	no	no
PV9_Otu00014_Metschnikowia	yes	no	no	no
PV9_Otu00015_Insecta	no	no	no	no
PV9_Otu00016_Agaricomycetes	no	yes	no	no

PV9_Otu00018_Aglenchus	yes	yes	no	no
PV9_Otu00019_Agaricomycetes	yes	yes	no	no
PV9_Otu00021_Insecta	no	no	no	no
PV9_Otu00022_Agaricomycetes	yes	yes	no	no
PV9_Otu00024_Chromadorea_X	yes	yes	yes	no
PV9_Otu00026_Pezizomycotina	yes	yes	no	no
PV9_Otu00027_Agaricomycetes	yes	yes	no	no
PV9_Otu00028_Dothideomycetes	yes	no	no	no
PV9_Otu00030_Arachnida	no	yes	no	no
PV9_Otu00032_Arachnida	yes	no	no	no
PV9_Otu00033_Arachnida	yes	no	no	no
PV9_Otu00034_Pezizomycotina	yes	yes	yes	yes
PV9_Otu00035_Paratylenchus	yes	yes	no	no
PV9_Otu00038_Insecta	no	no	no	no
PV9_Otu00043_Plectus	yes	yes	no	no
PV9_Otu00047_Alatospora	no	yes	no	no
PV9_Otu00053_Sebacina	no	yes	no	no
PV9_Otu00057_Agaricomycetes	no	yes	no	no
PV9_Otu00061_Dothideomycetes	no	no	no	yes
PV9_Otu00062_Pratylenchus	no	yes	no	no
PV9_Otu00064_Aphelenchus	no	yes	no	no
PV9_Otu00081_Leotiomycetes	yes	no	no	no
PV9_Otu00083_Adineta	no	no	no	no
PV9_Otu00084_Phytomyza	no	no	no	no
PV9_Otu00101_Exobasidiomycetes	yes	no	no	no
PV9_Otu00103_Eukaryota	yes	no	yes	no
PV9_Otu00104_Cercomonas	yes	no	yes	yes
PV9_Otu00134_Amoebozoa	no	no	yes	yes
PV9_Otu00204_Rattus	no	no	no	no
PV9_Otu00337_Eukaryota	no	no	yes	no

(b) Shoots

	Separator	Abundant	Core	Hub
BV5_Otu000001_Pantoea	no	yes	yes	no
BV5_Otu000003_Pseudomonas	no	yes	yes	no
BV5_Otu000004_Phyllobacteriaceae	yes	no	no	no
BV5_Otu000005_Enterobacteriaceae	yes	no	no	no
BV5_Otu000006_Ralstonia	no	yes	yes	no
BV5_Otu000007_Bacillus	yes	yes	yes	yes
BV5_Otu000008_Frankia	no	no	no	no
BV5_Otu000009_Phyllobacterium	yes	no	no	yes
BV5_Otu000010_Pantoea	no	yes	no	no
BV5_Otu000011_Mesorhizobium	no	no	no	yes
BV5_Otu000012_Burkholderia	yes	yes	no	no
BV5_Otu000013_Burkholderia	yes	yes	no	yes
BV5_Otu000014_Agrobacterium	yes	yes	yes	no

BV5_Otu000015_Streptomyces	no	no	no	yes
BV5_Otu000016_Burkholderia	yes	yes	no	no
BV5_Otu000017_Mesorhizobium	no	no	no	no
BV5_Otu000018_Comamonadaceae	yes	yes	no	no
BV5_Otu000019_Cryptosporangium	yes	no	no	no
BV5_Otu000021_Pantoea	no	no	no	no
BV5_Otu000022_Methylibium	no	no	no	no
BV5_Otu000023_Steroidobacter	no	no	no	no
BV5_Otu000024_Xanthomonadaceae	yes	yes	no	yes
BV5_Otu000025_Rhodoplanes	no	no	no	yes
BV5_Otu000026_Rhizobium	no	no	no	no
BV5_Otu000027_Gaiellaceae	no	no	no	yes
BV5_Otu000028_Wolbachia	yes	no	no	no
BV5_Otu000029_Rhizobiales	no	no	no	yes
BV5_Otu000030_0319-7L14	no	no	no	yes
BV5_Otu000032_Pseudomonas	yes	no	no	no
BV5_Otu000033_Chryseobacterium	no	no	no	no
BV5_Otu000034_Prauserella	yes	no	no	no
BV5_Otu000036_Bosea	yes	no	no	yes
BV5_Otu000039_Methylibium	no	no	no	no
BV5_Otu000040_Sodalis	no	no	no	no
BV5_Otu000043_Bradyrhizobiaceae	no	no	no	no
BV5_Otu000045_Micromonosporaceae	no	no	no	yes
BV5_Otu000047_Methylobacterium	yes	yes	no	no
BV5_Otu000049_Kineosporia	no	no	no	no
BV5_Otu000052_Methylobacterium	yes	yes	no	yes
BV5_Otu000054_Gaiellaceae	no	no	no	yes
BV5_Otu000055_Methylobacterium	yes	no	no	yes
BV5_Otu000056_Enterobacteriaceae	no	no	no	no
BV5_Otu000057_Rhodospirillaceae	no	no	no	yes
BV5_Otu000059_Solirubrobacterales	no	no	no	yes
BV5_Otu000060_Hyphomicrobium	no	no	no	no
BV5_Otu000061_Mycobacterium	no	no	no	yes
BV5_Otu000062_Flavobacterium	no	no	no	yes
BV5_Otu000063_SC-I-84	no	no	no	yes
BV5_Otu000065_Solirubrobacterales	no	no	no	yes
BV5_Otu000068_Gluconobacter	no	no	no	no
BV5_Otu000069_Pseudomonas	no	no	no	no
BV5_Otu000070_Rubrobacter	yes	no	no	no
BV5_Otu000073_Erwinia	yes	no	no	no
BV5_Otu000075_Solirubrobacteraceae	no	no	no	yes
BV5_Otu000076_Acinetobacter	yes	no	no	no
BV5_Otu000077_Solirubrobacterales	no	no	no	yes
BV5_Otu000078_Sinobacteraceae	no	no	no	yes
BV5_Otu000079_Buchnera	no	no	no	no
BV5_Otu000080_Pedomicrobium	no	no	no	yes

BV5_Otu000081_SC-I-84	no	no	no	yes
BV5_Otu000084_Dongia	no	no	no	no
BV5_Otu000086_Burkholderiaceae	yes	no	no	no
BV5_Otu000089_Propionibacterium	yes	no	yes	no
BV5_Otu000091_Patulibacteraceae	no	no	no	no
BV5_Otu000092_0319-7L14	no	no	no	yes
BV5_Otu000094_Caulobacter	no	no	no	no
BV5_Otu000095_Sphingomonas	yes	no	yes	no
BV5_Otu000096_Bradyrhizobium	no	no	no	no
BV5_Otu000097_Gaiellaceae	no	no	no	yes
BV5_Otu000098_Mycobacterium	no	no	no	no
BV5_Otu000102_Solibacillus	no	no	no	yes
BV5_Otu000105_Labrys	no	no	no	no
BV5_Otu000106_Bacillus	no	no	no	yes
BV5_Otu000109_Kaistobacter	no	no	no	yes
BV5_Otu000112_Planococcaceae	no	no	no	yes
BV5_Otu000113_Dongia	no	no	no	no
BV5_Otu000114_Escherichia	yes	no	no	no
BV5_Otu000117_Solirubrobacterales	no	no	no	yes
BV5_Otu000118_[Weeksellaceae]	yes	no	no	no
BV5_Otu000120_Bacillus	no	no	no	yes
BV5_Otu000125_Steroidobacter	no	no	no	no
BV5_Otu000141_Ellin6513	no	no	no	no
BV5_Otu000143_Dolo	no	no	no	no
BV5_Otu000144_Rhizobiales	no	no	no	no
BV5_Otu000155_Burkholderia	no	no	no	no
BV5_Otu000156_Staphylococcus	yes	no	no	no
BV5_Otu000194_Micromonospora	no	no	no	yes
BV5_Otu000256_Bacillales	no	no	no	yes
BV5_Otu000713_Bacteria	no	no	no	no
ITS2_Otu00003_Cladosporium	no	yes	yes	yes
ITS2_Otu00004_Exophiala	yes	no	no	no
ITS2_Otu00005_Cadophora	yes	no	no	no
ITS2_Otu00006_Fungi	no	yes	yes	no
ITS2_Otu00008_Boeremia	yes	yes	no	yes
ITS2_Otu00009_Didymellaceae	yes	yes	no	yes
ITS2_Otu00010_Sclerotiniaceae	yes	no	no	no
ITS2_Otu00011_Septoria	yes	yes	no	yes
ITS2_Otu00012_Alternaria	no	yes	no	yes
ITS2_Otu00013_Didymellaceae	yes	yes	no	no
ITS2_Otu00014_Chaetosphaeronema	yes	yes	no	yes
ITS2_Otu00015_Pseudomassaria	yes	no	no	no
ITS2_Otu00016_Botryotinia	yes	no	no	no
ITS2_Otu00017_Helotiales	yes	no	no	no
ITS2_Otu00018_Tetracladium	yes	no	no	no
ITS2_Otu00019_Alternaria	yes	yes	yes	yes

ITS2_Otu00020_Podospora	yes	yes	no	no
ITS2_Otu00021_Dactylonectria	no	no	no	no
ITS2_Otu00022_Neocosmospora	no	no	no	no
ITS2_Otu00023_Cistella	no	no	no	no
ITS2_Otu00024_Gibberella	no	yes	no	yes
ITS2_Otu00025_Colletotrichum	yes	yes	no	yes
ITS2_Otu00026_Mycena	no	no	no	no
ITS2_Otu00028_Ascomycota	no	no	no	no
ITS2_Otu00030_Uromyces	yes	yes	no	no
ITS2_Otu00031_Stemphylium	yes	yes	no	no
ITS2_Otu00033_Ilyonectria	no	no	no	no
ITS2_Otu00034_Serendipita	no	no	no	no
ITS2_Otu00035_Pseudoidium	yes	yes	no	no
ITS2_Otu00036_Hemimycena	no	no	no	no
ITS2_Otu00037_Exophiala	no	no	no	no
ITS2_Otu00042_Pleosporales	no	no	no	no
ITS2_Otu00052_Titaea	no	no	no	no
ITS2_Otu00054_Ramularia	yes	yes	no	no
ITS2_Otu00058_Colletotrichum	yes	yes	no	yes
ITS2_Otu00063_Pleosporales	yes	yes	no	yes
ITS2_Otu00085_Fungi	yes	no	yes	no
ITS2_Otu00109_Malassezia	no	no	no	no
ITS2_Otu00232_Fungi	no	no	no	no
ITS2_Otu00257_Fungi	no	no	no	no
PV9_Otu00002_Pezothrips	no	yes	yes	no
PV9_Otu00003_Insecta	yes	no	no	no
PV9_Otu00004_Frankliniella	no	yes	no	no
PV9_Otu00005_Exophiala	yes	no	no	no
PV9_Otu00007_Pucciniomycetes	yes	yes	no	no
PV9_Otu00008_Mayetiola	yes	yes	no	no
PV9_Otu00009_Dothideomycetes	yes	yes	yes	yes
PV9_Otu00010_Aeolothrips	no	no	no	no
PV9_Otu00011_Agaricomycetes	yes	no	no	no
PV9_Otu00012_Agaricomycetes	no	no	no	yes
PV9_Otu00013_Insecta	no	yes	no	no
PV9_Otu00014_Metschnikowia	yes	no	no	no
PV9_Otu00015_Insecta	no	yes	no	no
PV9_Otu00016_Agaricomycetes	no	no	no	no
PV9_Otu00018_Aglenchus	no	no	no	no
PV9_Otu00019_Agaricomycetes	yes	no	no	no
PV9_Otu00021_Insecta	no	no	no	no
PV9_Otu00022_Agaricomycetes	no	no	no	no
PV9_Otu00024_Chromadorea_X	yes	no	no	no
PV9_Otu00026_Pezizomycotina	no	no	no	no
PV9_Otu00027_Agaricomycetes	no	no	no	no
PV9_Otu00028_Dothideomycetes	no	no	no	yes

PV9_Otu00030_Arachnida	no	no	no	no
PV9_Otu00032_Arachnida	yes	yes	no	no
PV9_Otu00033_Arachnida	no	no	no	no
PV9_Otu00034_Pezizomycotina	yes	no	no	no
PV9_Otu00035_Paratylenchus	no	no	no	no
PV9_Otu00038_Insecta	yes	yes	no	no
PV9_Otu00043_Plectus	no	no	no	no
PV9_Otu00047_Alatospora	no	no	no	no
PV9_Otu00053_Sebacina	no	no	no	no
PV9_Otu00057_Agaricomycetes	no	no	no	no
PV9_Otu00061_Dothideomycetes	yes	no	no	yes
PV9_Otu00062_Pratylenchus	no	no	no	no
PV9_Otu00064_Aphelenchus	no	no	no	no
PV9_Otu00081_Leotiomycetes	yes	yes	no	no
PV9_Otu00083_Adineta	yes	no	no	yes
PV9_Otu00084_Phytomyza	yes	yes	no	no
PV9_Otu00101_Exobasidiomycetes	yes	no	no	no
PV9_Otu00103_Eukaryota	yes	no	yes	no
PV9_Otu00104_Cercomonas	no	no	no	no
PV9_Otu00134_Amoebozoa	no	no	no	no
PV9_Otu00204_Rattus	no	no	no	no
PV9_Otu00337_Eukaryota	no	no	yes	no

(c) Flowers

	Separator	Abundant	Core	Hub
BV5_Otu000001_Pantoea	no	yes	no	no
BV5_Otu000003_Pseudomonas	no	yes	yes	yes
BV5_Otu000004_Phyllobacteriaceae	no	no	no	yes
BV5_Otu000005_Enterobacteriaceae	yes	yes	no	no
BV5_Otu000006_Ralstonia	yes	yes	no	yes
BV5_Otu000007_Bacillus	yes	no	no	yes
BV5_Otu000008_Frankia	no	no	no	no
BV5_Otu000009_Phyllobacterium	no	no	no	yes
BV5_Otu000010_Pantoea	no	yes	no	yes
BV5_Otu000011_Mesorhizobium	no	no	no	yes
BV5_Otu000012_Burkholderia	yes	no	no	yes
BV5_Otu000013_Burkholderia	yes	no	no	yes
BV5_Otu000014_Agrobacterium	yes	no	no	no
BV5_Otu000015_Streptomyces	no	no	no	yes
BV5_Otu000016_Burkholderia	yes	no	no	yes
BV5_Otu000017_Mesorhizobium	no	no	no	yes
BV5_Otu000018_Comamonadaceae	no	no	no	no
BV5_Otu000019_Cryptosporangium	no	no	no	no
BV5_Otu000021_Pantoea	no	yes	no	yes
BV5_Otu000022_Methylibium	no	no	no	no
BV5_Otu000023_Steroidobacter	no	no	no	no

BV5_Otu000024_Xanthomonadaceae	yes	no	no	no
BV5_Otu000025_Rhodoplanes	no	no	no	yes
BV5_Otu000026_Rhizobium	no	no	no	yes
BV5_Otu000027_Gaiellaceae	no	no	no	yes
BV5_Otu000028_Wolbachia	yes	yes	no	no
BV5_Otu000029_Rhizobiales	no	no	no	yes
BV5_Otu000030_0319-7L14	no	no	no	yes
BV5_Otu000032_Pseudomonas	yes	yes	no	yes
BV5_Otu000033_Chryseobacterium	no	no	no	no
BV5_Otu000034_Prauserella	yes	no	no	yes
BV5_Otu000036_Bosea	yes	no	no	no
BV5_Otu000039_Methylibium	no	no	no	no
BV5_Otu000040_Sodalis	no	no	no	no
BV5_Otu000043_Bradyrhizobiaceae	no	no	no	yes
BV5_Otu000045_Micromonosporaceae	no	no	no	no
BV5_Otu000047_Methylobacterium	yes	no	no	no
BV5_Otu000049_Kineosporia	no	no	no	no
BV5_Otu000052_Methylobacterium	no	no	no	yes
BV5_Otu000054_Gaiellaceae	no	no	no	yes
BV5_Otu000055_Methylobacterium	no	no	no	yes
BV5_Otu000056_Enterobacteriaceae	yes	yes	no	no
BV5_Otu000057_Rhodospirillaceae	no	no	no	yes
BV5_Otu000059_Solirubrobacterales	no	no	no	yes
BV5_Otu000060_Hyphomicrobium	no	no	no	no
BV5_Otu000061_Mycobacterium	no	no	no	yes
BV5_Otu000062_Flavobacterium	no	no	no	no
BV5_Otu000063_SC-I-84	no	no	no	yes
BV5_Otu000065_Solirubrobacterales	no	no	no	yes
BV5_Otu000068_Gluconobacter	no	yes	no	no
BV5_Otu000069_Pseudomonas	no	yes	no	no
BV5_Otu000070_Rubrobacter	no	no	no	yes
BV5_Otu000073_Erwinia	no	no	no	yes
BV5_Otu000075_Solirubrobacteraceae	no	no	no	yes
BV5_Otu000076_Acinetobacter	yes	yes	no	no
BV5_Otu000077_Solirubrobacterales	no	no	no	yes
BV5_Otu000078_Sinobacteraceae	no	no	no	yes
BV5_Otu000079_Buchnera	no	yes	no	no
BV5_Otu000080_Pedomicrobium	no	no	no	no
BV5_Otu000081_SC-I-84	no	no	no	yes
BV5_Otu000084_Dongia	no	no	no	no
BV5_Otu000086_Burkholderiaceae	no	no	no	yes
BV5_Otu000089_Propionibacterium	yes	no	no	yes
BV5_Otu000091_Patulibacteraceae	no	no	no	no
BV5_Otu000092_0319-7L14	no	no	no	yes
BV5_Otu000094_Caulobacter	no	no	no	no
BV5_Otu000095_Sphingomonas	no	no	no	yes

BV5_Otu000096_Bradyrhizobium	no	no	no	yes
BV5_Otu000097_Gaiellaceae	no	no	no	yes
BV5_Otu000098_Mycobacterium	no	no	no	yes
BV5_Otu000102_Solibacillus	no	no	no	no
BV5_Otu000105_Labrys	no	no	no	no
BV5_Otu000106_Bacillus	no	no	no	no
BV5_Otu000109_Kaistobacter	no	no	no	yes
BV5_Otu000112_Planococcaceae	no	no	no	no
BV5_Otu000113_Dongia	no	no	no	no
BV5_Otu000114_Escherichia	yes	no	no	yes
BV5_Otu000117_Solirubrobacterales	no	no	no	no
BV5_Otu000118_[Weeksellaceae]	no	no	no	yes
BV5_Otu000120_Bacillus	no	no	no	no
BV5_Otu000125_Steroidobacter	no	no	no	no
BV5_Otu000141_Ellin6513	no	no	no	yes
BV5_Otu000143_Dolo	no	no	no	no
BV5_Otu000144_Rhizobiales	no	no	no	no
BV5_Otu000155_Burkholderia	no	no	no	no
BV5_Otu000156_Staphylococcus	yes	no	no	no
BV5_Otu000194_Micromonospora	no	no	no	yes
BV5_Otu000256_Bacillales	no	no	no	no
BV5_Otu000713_Bacteria	no	no	no	no
ITS2_Otu00003_Cladosporium	yes	yes	yes	yes
ITS2_Otu00004_Exophiala	no	no	no	no
ITS2_Otu00005_Cadophora	no	no	no	no
ITS2_Otu00006_Fungi	no	yes	yes	yes
ITS2_Otu00008_Boeremia	yes	no	no	yes
ITS2_Otu00009_Didymellaceae	yes	yes	no	yes
ITS2_Otu00010_Sclerotiniaceae	yes	yes	no	yes
ITS2_Otu00011_Septoria	yes	no	no	no
ITS2_Otu00012_Alternaria	yes	yes	no	yes
ITS2_Otu00013_Didymellaceae	yes	yes	no	yes
ITS2_Otu00014_Chaetosphaeronema	yes	no	no	no
ITS2_Otu00015_Pseudomassaria	no	no	no	no
ITS2_Otu00016_Botryotinia	yes	yes	no	yes
ITS2_Otu00017_Helotiales	no	no	no	no
ITS2_Otu00018_Tetracladium	no	no	no	no
ITS2_Otu00019_Alternaria	no	yes	no	yes
ITS2_Otu00020_Podospora	yes	no	no	no
ITS2_Otu00021_Dactylonectria	no	no	no	no
ITS2_Otu00022_Neocosmospora	no	no	no	no
ITS2_Otu00023_Cistella	no	no	no	no
ITS2_Otu00024_Gibberella	no	no	no	no
ITS2_Otu00025_Colletotrichum	yes	no	no	no
ITS2_Otu00026_Mycena	no	no	no	no
ITS2_Otu00028_Ascomycota	no	no	no	no

ITS2_Otu00030_Uromyces	no	no	no	no
ITS2_Otu00031_Stemphylium	yes	no	no	no
ITS2_Otu00033_Ilyonectria	no	no	no	no
ITS2_Otu00034_Serendipita	no	no	no	no
ITS2_Otu00035_Pseudoidium	no	no	no	no
ITS2_Otu00036_Hemimycena	no	no	no	no
ITS2_Otu00037_Exophiala	no	no	no	no
ITS2_Otu00042_Pleosporales	no	no	no	no
ITS2_Otu00052_Titaea	no	no	no	no
ITS2_Otu00054_Ramularia	yes	no	no	no
ITS2_Otu00058_Colletotrichum	no	no	no	no
ITS2_Otu00063_Pleosporales	no	no	no	no
ITS2_Otu00085_Fungi	no	no	yes	no
ITS2_Otu00109_Malassezia	no	no	no	no
ITS2_Otu00232_Fungi	no	no	no	no
ITS2_Otu00257_Fungi	no	no	no	no
PV9_Otu00002_Pezothrips	yes	yes	yes	no
PV9_Otu00003_Insecta	yes	no	no	no
PV9_Otu00004_Frankliniella	yes	yes	no	no
PV9_Otu00005_Exophiala	no	no	no	no
PV9_Otu00007_Pucciniomycetes	no	no	no	no
PV9_Otu00008_Mayetiola	yes	yes	no	no
PV9_Otu00009_Dothideomycetes	yes	yes	no	yes
PV9_Otu00010_Aeolothrips	yes	yes	no	no
PV9_Otu00011_Agaricomycetes	no	no	no	no
PV9_Otu00012_Agaricomycetes	no	no	no	no
PV9_Otu00013_Insecta	no	yes	no	no
PV9_Otu00014_Metschnikowia	yes	yes	no	yes
PV9_Otu00015_Insecta	yes	yes	no	no
PV9_Otu00016_Agaricomycetes	no	no	no	no
PV9_Otu00018_Aglenchus	no	no	no	no
PV9_Otu00019_Agaricomycetes	no	no	no	no
PV9_Otu00021_Insecta	yes	yes	no	no
PV9_Otu00022_Agaricomycetes	no	no	no	no
PV9_Otu00024_Chromadorea_X	no	no	no	no
PV9_Otu00026_Pezizomycotina	no	no	no	no
PV9_Otu00027_Agaricomycetes	no	no	no	no
PV9_Otu00028_Dothideomycetes	no	yes	no	yes
PV9_Otu00030_Arachnida	no	no	no	no
PV9_Otu00032_Arachnida	no	no	no	no
PV9_Otu00033_Arachnida	yes	yes	no	no
PV9_Otu00034_Pezizomycotina	no	no	no	no
PV9_Otu00035_Paratylenchus	no	no	no	no
PV9_Otu00038_Insecta	yes	no	no	no
PV9_Otu00043_Plectus	no	no	no	no
PV9_Otu00047_Alatospora	no	no	no	no

PV9_Otu00053_Sebacina	no	no	no	no
PV9_Otu00057_Agaricomycetes	no	no	no	no
PV9_Otu00061_Dothideomycetes	yes	no	no	no
PV9_Otu00062_Pratylenchus	no	no	no	no
PV9_Otu00064_Aphelenchus	no	no	no	no
PV9_Otu00081_Leotiomycetes	no	no	no	no
PV9_Otu00083_Adineta	no	no	no	no
PV9_Otu00084_Phytomyza	no	no	no	no
PV9_Otu00101_Exobasidiomycetes	no	no	no	no
PV9_Otu00103_Eukaryota	yes	no	yes	no
PV9_Otu00104_Cercomonas	no	no	no	no
PV9_Otu00134_Amoebozoa	no	no	no	no
PV9_Otu00204_Rattus	no	no	no	no
PV9_Otu00337_Eukaryota	no	no	yes	no

(d) Seeds

	Separator	Abundant	Core	Hub
BV5_Otu000001_Pantoea	yes	yes	no	no
BV5_Otu000003_Pseudomonas	yes	yes	yes	yes
BV5_Otu000004_Phyllobacteriaceae	yes	no	no	yes
BV5_Otu000005_Enterobacteriaceae	no	yes	no	no
BV5_Otu000006_Ralstonia	yes	yes	yes	yes
BV5_Otu000007_Bacillus	yes	no	no	yes
BV5_Otu000008_Frankia	yes	no	no	no
BV5_Otu000009_Phyllobacterium	yes	no	no	no
BV5_Otu000010_Pantoea	no	yes	no	no
BV5_Otu000011_Mesorhizobium	yes	no	no	no
BV5_Otu000012_Burkholderia	yes	yes	no	yes
BV5_Otu000013_Burkholderia	yes	yes	no	yes
BV5_Otu000014_Agrobacterium	no	no	no	yes
BV5_Otu000015_Streptomyces	no	no	no	no
BV5_Otu000016_Burkholderia	yes	yes	no	yes
BV5_Otu000017_Mesorhizobium	yes	no	no	yes
BV5_Otu000018_Comamonadaceae	yes	yes	no	no
BV5_Otu000019_Cryptosporangium	yes	no	no	no
BV5_Otu000021_Pantoea	yes	yes	no	no
BV5_Otu000022_Methylibium	no	no	no	no
BV5_Otu000023_Steroidobacter	no	no	no	no
BV5_Otu000024_Xanthomonadaceae	yes	no	no	no
BV5_Otu000025_Rhodoplanes	no	no	no	no
BV5_Otu000026_Rhizobium	yes	no	no	no
BV5_Otu000027_Gaiellaceae	no	no	no	no
BV5_Otu000028_Wolbachia	yes	yes	no	no
BV5_Otu000029_Rhizobiales	no	no	no	no
BV5_Otu000030_0319-7L14	no	no	no	no
BV5_Otu000032_Pseudomonas	yes	no	no	yes

BV5_Otu000033_Chryseobacterium	no	no	no	no
BV5_Otu000034_Prauserella	yes	yes	no	yes
BV5_Otu000036_Bosea	yes	no	no	no
BV5_Otu000039_Methylibium	no	no	no	no
BV5_Otu000040_Sodalis	yes	yes	no	no
BV5_Otu000043_Bradyrhizobiaceae	no	no	no	yes
BV5_Otu000045_Micromonosporaceae	no	no	no	no
BV5_Otu000047_Methylobacterium	no	no	no	no
BV5_Otu000049_Kineosporia	yes	no	no	no
BV5_Otu000052_Methylobacterium	yes	no	no	yes
BV5_Otu000054_Gaiellaceae	no	no	no	no
BV5_Otu000055_Methylobacterium	no	no	no	no
BV5_Otu000056_Enterobacteriaceae	no	no	no	no
BV5_Otu000057_Rhodospirillaceae	no	no	no	yes
BV5_Otu000059_Solirubrobacterales	no	no	no	no
BV5_Otu000060_Hyphomicrobium	no	no	no	no
BV5_Otu000061_Mycobacterium	no	no	no	no
BV5_Otu000062_Flavobacterium	no	no	no	no
BV5_Otu000063_SC-I-84	no	no	no	no
BV5_Otu000065_Solirubrobacterales	no	no	no	no
BV5_Otu000068_Gluconobacter	no	no	no	no
BV5_Otu000069_Pseudomonas	no	no	no	no
BV5_Otu000070_Rubrobacter	yes	no	no	yes
BV5_Otu000073_Erwinia	yes	no	no	yes
BV5_Otu000075_Solirubrobacteraceae	no	no	no	no
BV5_Otu000076_Acinetobacter	no	no	no	no
BV5_Otu000077_Solirubrobacterales	no	no	no	no
BV5_Otu000078_Sinobacteraceae	no	no	no	no
BV5_Otu000079_Buchnera	no	no	no	no
BV5_Otu000080_Pedomicrobium	no	no	no	no
BV5_Otu000081_SC-I-84	no	no	no	no
BV5_Otu000084_Dongia	no	no	no	no
BV5_Otu000086_Burkholderiaceae	yes	no	no	yes
BV5_Otu000089_Propionibacterium	yes	no	yes	yes
BV5_Otu000091_Patulibacteraceae	no	no	no	no
BV5_Otu000092_0319-7L14	no	no	no	no
BV5_Otu000094_Caulobacter	no	no	no	no
BV5_Otu000095_Sphingomonas	yes	no	no	no
BV5_Otu000096_Bradyrhizobium	no	no	no	yes
BV5_Otu000097_Gaiellaceae	no	no	no	no
BV5_Otu000098_Mycobacterium	no	no	no	no
BV5_Otu000102_Solibacillus	no	no	no	no
BV5_Otu000105_Labrys	no	no	no	no
BV5_Otu000106_Bacillus	no	no	no	no
BV5_Otu000109_Kaistobacter	no	no	no	no
BV5_Otu000112_Planococcaceae	no	no	no	no

BV5_Otu000113_Dongia	no	no	no	no
BV5_Otu000114_Escherichia	no	no	no	yes
BV5_Otu000117_Solirubrobacterales	no	no	no	no
BV5_Otu000118_[Weeksellaceae]	yes	no	no	yes
BV5_Otu000120_Bacillus	no	no	no	no
BV5_Otu000125_Steroidobacter	no	no	no	no
BV5_Otu000141_Ellin6513	yes	no	no	yes
BV5_Otu000143_Dolo	no	no	no	no
BV5_Otu000144_Rhizobiales	no	no	no	no
BV5_Otu000155_Burkholderia	yes	no	no	yes
BV5_Otu000156_Staphylococcus	yes	no	no	yes
BV5_Otu000194_Micromonospora	no	no	no	no
BV5_Otu000256_Bacillales	no	no	no	no
BV5_Otu000713_Bacteria	yes	no	no	yes
ITS2_Otu00003_Cladosporium	yes	yes	yes	no
ITS2_Otu00004_Exophiala	yes	no	no	no
ITS2_Otu00005_Cadophora	yes	no	no	no
ITS2_Otu00006_Fungi	yes	yes	yes	yes
ITS2_Otu00008_Boeremia	yes	yes	no	no
ITS2_Otu00009_Didymellaceae	yes	no	no	no
ITS2_Otu00010_Sclerotiniaceae	yes	yes	no	no
ITS2_Otu00011_Septoria	yes	no	no	no
ITS2_Otu00012_Alternaria	no	yes	no	no
ITS2_Otu00013_Didymellaceae	yes	yes	no	no
ITS2_Otu00014_Chaetosphaeronema	yes	no	no	no
ITS2_Otu00015_Pseudomassaria	yes	no	no	no
ITS2_Otu00016_Botryotinia	no	no	no	no
ITS2_Otu00017_Helotiales	no	no	no	no
ITS2_Otu00018_Tetracladium	no	no	no	no
ITS2_Otu00019_Alternaria	no	yes	no	no
ITS2_Otu00020_Podospora	yes	no	no	no
ITS2_Otu00021_Dactylonectria	no	no	no	no
ITS2_Otu00022_Neocosmospora	no	no	no	no
ITS2_Otu00023_Cistella	no	no	no	no
ITS2_Otu00024_Gibberella	yes	yes	no	no
ITS2_Otu00025_Colletotrichum	yes	no	no	no
ITS2_Otu00026_Mycena	no	no	no	no
ITS2_Otu00028_Ascomycota	yes	no	no	no
ITS2_Otu00030_Uromyces	no	no	no	no
ITS2_Otu00031_Stemphylium	yes	no	no	no
ITS2_Otu00033_Ilyonectria	no	no	no	no
ITS2_Otu00034_Serendipita	no	no	no	no
ITS2_Otu00035_Pseudoidium	no	no	no	no
ITS2_Otu00036_Hemimycena	no	no	no	no
ITS2_Otu00037_Exophiala	no	no	no	no
ITS2_Otu00042_Pleosporales	no	no	no	no

ITS2_Otu00052_Titaea	no	no	no	no
ITS2_Otu00054_Ramularia	no	no	no	no
ITS2_Otu00058_Colletotrichum	no	no	no	no
ITS2_Otu00063_Pleosporales	no	no	no	no
ITS2_Otu00085_Fungi	yes	yes	yes	no
ITS2_Otu00109_Malassezia	yes	yes	no	no
ITS2_Otu00232_Fungi	yes	no	no	yes
ITS2_Otu00257_Fungi	yes	no	no	yes
PV9_Otu00002_Pezothrips	yes	no	no	no
PV9_Otu00003_Insecta	yes	yes	no	no
PV9_Otu00004_Frankliniella	yes	no	no	no
PV9_Otu00005_Exophiala	yes	no	no	no
PV9_Otu00007_Pucciniomycetes	yes	no	no	no
PV9_Otu00008_Mayetiola	yes	no	no	no
PV9_Otu00009_Dothideomycetes	yes	no	no	no
PV9_Otu00010_Aeolothrips	yes	no	no	no
PV9_Otu00011_Agaricomycetes	no	no	no	no
PV9_Otu00012_Agaricomycetes	no	no	no	no
PV9_Otu00013_Insecta	yes	no	no	no
PV9_Otu00014_Metschnikowia	yes	no	no	no
PV9_Otu00015_Insecta	yes	yes	no	no
PV9_Otu00016_Agaricomycetes	yes	no	no	no
PV9_Otu00018_Aglenchus	yes	no	no	no
PV9_Otu00019_Agaricomycetes	yes	no	no	no
PV9_Otu00021_Insecta	yes	no	no	no
PV9_Otu00022_Agaricomycetes	yes	no	no	no
PV9_Otu00024_Chromadorea_X	yes	no	no	no
PV9_Otu00026_Pezizomycotina	yes	no	no	no
PV9_Otu00027_Agaricomycetes	yes	no	no	no
PV9_Otu00028_Dothideomycetes	yes	no	no	no
PV9_Otu00030_Arachnida	no	no	no	no
PV9_Otu00032_Arachnida	yes	no	no	no
PV9_Otu00033_Arachnida	no	no	no	no
PV9_Otu00034_Pezizomycotina	no	no	no	no
PV9_Otu00035_Paratylenchus	yes	no	no	no
PV9_Otu00038_Insecta	no	no	no	no
PV9_Otu00043_Plectus	no	no	no	no
PV9_Otu00047_Alatospora	no	no	no	no
PV9_Otu00053_Sebacina	no	no	no	no
PV9_Otu00057_Agaricomycetes	no	no	no	no
PV9_Otu00061_Dothideomycetes	no	no	no	no
PV9_Otu00062_Pratylenchus	no	no	no	no
PV9_Otu00064_Aphelenchus	no	no	no	yes
PV9_Otu00081_Leotiomycetes	yes	no	no	no
PV9_Otu00083_Adineta	yes	no	no	no
PV9_Otu00084_Phytomyza	yes	no	no	no

PV9_Otu00101_Exobasidiomycetes	yes	yes	no	yes
PV9_Otu00103_Eukaryota	yes	no	yes	no
PV9_Otu00104_Cercomonas	yes	no	no	no
PV9_Otu00134_Amoebozoa	no	no	no	no
PV9_Otu00204_Rattus	yes	no	no	yes
PV9_Otu00337_Eukaryota	yes	no	yes	no

SUPPLEMENTARY METHODS

Supplementary Method 1. Sequence data processing using Mothur.

We processed amplicon sequence data of microbial 16S rRNA, ITS2, and 18S rRNA using Mothur as described in Almario *et al*. (1, 2). In brief, processing of amplicon reads include forming contigs by pairing single-end reads (*make.contigs*), quality filtering for paired reads that are 100-600 bases long with at least 5 bases overlap (*screen.seqs*), demultiplexing (*rename.seqs*), dereplication (*unique.seqs, count.seqs*), detecting and removing chimera using VSEARCH (*chimera.vsearch, remove.seqs*), classifying sequences *(classify.seqs*), OTU clustering at 97% sequence similarity threshold using dgc method (*cluster*), abundance filtering for OTUs with more than 50 reads (*split.abund*), classifying OTUs (*classify.otu*), creating OTU tables (*make.shared*), removing OTUs classified as chloroplast, mitochondria, *Arabidopsis*, Embryophyceae, unknown, and PhiX sequences (*remove.lineage*), and identifying OTU representative sequences based on abundance (*get.oturep*).

Supplementary Method 2. Identification of hub microbes in *L. corniculatus*.

To determine hub bacteria, fungi, and eukaryotes in plant organs, correlation networks for each plant organ were computed using SparCC algorithm, as described in Almario *et al*. (1, 3). In brief, OTU absolute count tables with low abundance OTUs removed were used as input for FastSpar, a parallelizable implementation of SparCC algorithm, to calculate correlations (4). P-values were calculated from 1,000 bootstraps and resulting correlations with $P<0.001$ were used for constructing networks.

Supplementary Method 3. Transmission of microbes in *L. corniculatus*.

To determine how *L. corniculatus* organ microbiomes can be linked and influenced by dispersal, we used Sankey diagrams to visualize potential flow of microbes across the soil and plant organs. Nodes of the diagrams represent different potential microbial sources (*i.e.* soil, plant organs, and environment/others) and arcs correspond to the number of OTUs shared between nodes.

To determine potential origins of organ-associated microbial communities, we used FEAST (Fast Expectation-mAximization microbial Source Tracking) to estimate contribution of potential microbial sources, such as soil, the different plant compartments, or the environment, to each plant organ microbiome (5). In the FEAST analysis we used multidirectional approach where microbiomes from plant organs or soil samples can be both sources or sinks. We implemented FEAST in R using default parameters for 1,000 iterations and we tested sinks with the same group of sources (*i.e.* all source microbiomes throughout seven sites for four years). In FEAST a proportion of the sink microbiomes is also potentially transmitted from "unknown sources", which can be other microbial sources that are not the assigned source in the analysis.

To statistically predict potential priority effects phenomena from roots, shoots, flowers, to seeds in *L. corniculatus*, we identified taxa of interest that are potentially involved in such phenomena in plant organs, as described in Debray *et al*. (6). We examined abundant, core, hub, or machine learning-predicted OTUs if changes in their relative abundance in plant organs correlate with changes in microbial community structure. We used PCoA of Bray-Curtis dissimilarities of plant organ microbial communities and PERMANOVA to assess the influence of these OTUs on community structure when they are abundant or rare (*i.e.* abundant OTUs: Relative abundance in each plant organ (RA) >= 0.01; intermediate OTUs: RA >= 0.001 and < 0.01; rare OTUs: RA < 0.001).

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CHAPTER III

ORGAN-SPECIFIC FILTERING BY ABIOTIC AND BIOTIC ENVIRONMENTAL FACTORS SHAPES DISTINCT YET OVERLAPPING MICROBIAL COMMUNITIES ACROSS *LOTUS CORNICULATUS* ROOTS, SHOOTS, FLOWERS, AND SEEDS

Katrina Lutap, Oliver Bossdorf, and Eric Kemen

Organ-specific filtering by abiotic and biotic environmental factors shapes distinct yet overlapping microbial communities across *Lotus corniculatus* roots, shoots, flowers, and seeds

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ABSTRACT

Plant microbiome assembly is modulated via filtering by the host plant and local environment as well as stochastic processes like microbial dispersal. *Lotus corniculatus* in natural populations that are continuously exposed to natural perturbations and microbial sources is an ideal plant model system to study the ecological processes that structure the distinct yet overlapping microbial communities in plant organs. We observed spatial and temporal variation in microbiomes associated with *L. corniculatus* roots, shoots, flowers, and seeds across seven grassland sites for four years. In this study we examined how abiotic and biotic factors in the local environment throughout multiple years contribute to the structure of microbiomes associated with *L. corniculatus* populations. We show that plant microbiomes are shaped by a set of environmental factors that are distinct to each plant compartment. These environmental factors either directly influence the plant microbiomes or by indirectly affecting them via other biotic factors. The environmental factors soil temperature seasonality, soil microbiome composition, air temperature seasonality, plant community richness, and grazing are found to influence the structure and microbial interactions in the plant organs, and are different in relationships with microbiomes with each compartment, possibly influencing dispersal decisions of microorganisms and consequently contribute in shaping distinct yet overlapping microbiomes across plant organs. *Burkholderia* and *Sulfuritalea*, plant-associated microbes that are highly correlated with environmental variables across all plant organs, respond to environmental variables differently depending on their organ microhabitat. This organ-dependent environmental perception is also observed in biomarker microbes in roots, shoots, and flowers, such as the rhizobial symbiont

Mesorhizobium, leaf pathogen *Setosphaeria*, and necrotroph *Botryotinia*, respectively. Our knowledge about the organ-specific response of plant microbiomes to abiotic and biotic perturbations will equip us with a framework to understand and engineer plant microbiomes in the context of global climate change. The observed patterns on dispersal decisions or habitat choice based on organ-dependent environmental cues and microbial interactions in plant microbiomes also advance our insights on how beneficial microbes or pathogens survive and persist on specific plant microhabitat and environmental conditions.

Keywords: plant microbiomes, organ-specific microbiomes, environmental factors, microbial networks, structural equation modeling, plant-microbe interactions

INTRODUCTION

Plants are host to diverse and dynamic microbial communities and oftentimes these associations provide reciprocal benefits. Plants equip microorganisms with habitat and resources for survival and growth, while the associated microorganisms are crucial in plant growth and nutrition, plant health and defense, or stress tolerance (1). Beyond these relationships, plant microbiomes have the potential to be used as tools for manipulation and management of plants with improved health and productivity. To leverage such potential, an in depth understanding of the assembly processes that shape plant community structure and interactions is important. Current research has established a spatially and temporally dynamic microbial communities associated with plants. The observed spatial and temporal variations in these plant microbial communities can be attributed to factors such as geographic location, soil properties, plant genotype, plant compartment, seasonality, and plant age or developmental stages (2-7). Given their significant roles, understanding the mechanisms that drive community assembly and variation in plant microbiomes is essential.

One of the main deterministic factors that influence microbial community assembly is filtering by the host plant and local environment. Plant host filtering drives variation in diversity and composition of associated microbial communities when genetic and phenotypic variations impact survival and persistence of microorganisms in the host plant. Plant genomic loci associated with plant traits such as cell morphology, defense and immune responses, signaling pathways, and secondary metabolism affect variation of the associated microbial communities (8, 9). Morphological and chemical differences between plant organs also result in distinct microbial communities due to selective pressure by the unique microhabitat conditions (10-13). In addition to plant organ and genotype, filtering by abiotic and biotic factors in the local environment impacts the structure of plant microbial communities. Factors such as climate (*i.e.* temperature, precipitation) or land use, as well as potential microbial sources such plant cover and soil microbial composition, influence plant microbiomes (14, 15). As environmental factors influence microbial community patterns at broad geographic scales, these same factors are also perceived differentially by individual plant microhabitats (16-18). Some of these environmental factors are more relevant to belowground soil or root microbiomes, while others can be more impactful to aboveground plant microbiomes (3, 19-21). One key challenge therefore is to study microbial community dynamics in the different plant compartments simultaneously on whole plants exposed to natural environmental conditions at various sites for multiple years.

Along with environmental filtering, stochastic processes such as dispersal are also significant ecological drivers of microbial community assembly. Dispersal patterns of microorganisms across spatial and temporal scales contribute to plant microbiome dynamics, whether via horizontal transmission from the environment or vertical transmission from seeds (13, 22, 23). Changes in environmental conditions (*i.e.* nutrient resource, pH, drought, soil type) can alter likelihood of priority effects and dispersal, as well as vertical transmission of microorganisms (24-26). Survival and establishment of transmitted microorganisms in a plant compartment are then dependent on a combination of assembly factors including plant microhabitat or environmental factors (12, 27). Knowledge on the effect of environmental factors on the transmission as well as survival and persistence of microorganisms in the plant organs is critical in understanding plant microbiome dynamics. Such information will also improve our insight on how microorganisms, especially pathogens, shift from roots to aboveground plant organs or vice versa.

Thus, it is important to investigate how environmental factors shape distinct microbial communities associated with different plant organs and how these abiotic and biotic factors influence transmission and establishment of microorganisms from the roots to aboveground plant compartments. *L. corniculatus* in natural populations that are continuously exposed to natural perturbations and microbial sources is a good plant model system to study the impact of environmental factors on the structure of plant microbial communities. *L. corniculatus,* a legume species that is ubiquitous in European grasslands, can withstand a broad range of natural environments. *L. corniculatus* is a perennial flowering plant that can grow on a wide range of soil conditions, can adapt to different climates, can persist upon grazing and mowing, and hosts various insects and bees (28-31). *L. corniculatus* forms symbiotic relationships with nitrogen-fixing bacteria such as *Rhizobium japonicum*, *Mesorhizobium loti*, and *Rhizobium meliloti*, as well as arbuscular mycorrhiza fungi, which improve plant adaptation in adverse habitats (28, 29, 32, 33). *L. corniculatus* also hosts non-rhizobial endophytes that promote plant growth (34, 35). In our previous study on *L. corniculatus*, we showed the spatial and temporal variation of plant-associated microbial communities at multiple scales - the diversity and community composition vary across sampling sites and years, as well as in different plant compartments (36). We demonstrated the transmission of microorganisms between the plant organs and from other outside microbial sources which contributed to distinct yet overlapping microbial communities in the roots, shoots, flowers, and seeds (36). Although it was established that the plant organs are the primary driver of microbial community structure, it is not clear how abiotic and biotic factors as well as microbial sources from the local environment can also influence diversity and composition of the plant-associated microbial communities. Therefore, *L. corniculatus* in natural environments is an ideal system to study the roles of abiotic and biotic factors on the ecological processes that structure and maintain distinct microbial communities in different plant compartments.

In this study, we investigated the effect of environmental factors on plant-associated microbial communities. We examined the role of various abiotic and biotic factors in shaping the distinct yet overlapping microbial communities associated with roots, shoots, flowers, and seeds of *L. corniculatus* in natural populations. Specifically, we aimed (i) to establish that plant microbial community variation occur at multiple spatial and temporal scales - from sampling site and year to the level of plant organs; (ii) to show that plant microbial communities are shaped by a subset of environmental factors that are distinct with each plant organ; (iii) to identify plant organ microbe biomarkers and determine if these microorganisms differently respond to environmental factors at different plant compartments; (iv) to demonstrate that environmental factors influence transmission and establishment of microorganisms between plant organs; and finally (v) to utilize structural equation modeling to synthesize differential effects of environmental factors that contribute to structuring distinct microbial communities in plant organs. To address these objectives, we performed amplicon sequencing of microbial 16S rRNA, ITS2, and 18S rRNA genes targeting endophytic communities in roots, shoots, flowers, and seeds of *L. corniculatus* collected from seven grassland sites in the Swabian Alps, Germany for four years. Results aimed to establish an overarching understanding on how differential environmental filtering shapes the microbial communities associated with the different plant compartments.

METHODS

Collection and processing of *L. corniculatus*

To study the effect of local environmental factors on plant microbiomes in natural environment, we collected a total of 168 *L. corniculatus* plants and 28 soil samples from seven wild populations in the region of Swabian Alps, Germany for four years (Fig. 1a). Every year in the summer (August/September 2018-2021), we randomly sampled six plants that are flowering and fruit-producing from each of all seven sites. We took soil samples from the spot where we sampled the plants, and then pooled together for each site. To study the endophytic communities associated with the roots, shoots, flowers, and seeds, we surface-sterilized the plant organs sequentially with sterile water, epiphyte wash (1X TE + 0.1% Triton X-100), 80% ethanol, bleach (2% NaOCl), and sterile water. We stored the sterilized samples at -20 ^{0}C until we process them for DNA extraction.

DNA extraction, library preparation, and amplicon sequencing

We first homogenized frozen roots, shoots, flowers, seeds, and soil samples in Precellys 24 Tissue Homogenizer (Bertin Technologies) and then we extracted DNA using FastDNA^{TM} Spin Kit for Soil (MP Bio) following the manufacturer's protocol. We performed two-step PCR amplification of the bacterial 16S rRNA V5-V7 region, fungal ITS2 region, and eukaryotic 18S rRNA V9 region using primers 799F/1192R, fITS7/ITS4, and F1422/R1797, respectively, on extracted DNA samples (Table S1) (37). We included blank samples (*i.e.* water and blank DNA extraction) as controls during library preparation. We also used blocking oligos, designed using R package "AmpStop", to minimize amplification of mitochondrial and chloroplast 16S rRNA, ITS, and 18S rRNA from *L. corniculatus* (Table S1) (38). We pooled the amplification products in equimolar concentrations, purified by magnetic bead clean-up, and randomized in eight sequencing batches. We sequenced them on Illumina MiSeq with PhiX control using MiSeq Reagent Kit v3 (600-cycle).

Processing of sequence data

We processed microbial 16S rRNA, ITS2, and 18S rRNA amplicon sequences using Mothur as described in Almario *et al.* (5, 39). In brief, we processed amplicon reads by using the following commands: *make.contigs* (contigs formation by pairing single-end reads), *screen.seqs* (quality filtering for 100-600 bases long paired reads with at least 5 bases overlap), *rename.seqs* (demultiplexing), *unique.seqs* and *count.seqs* (dereplication), *chimera.vsearch* and *remove.seqs* (detection and removal of chimera via VSEARCH), *classify.seqs* (classification of sequences), *cluster* (clustering of OTU at 97% sequence similarity threshold via the dgc method), *split.abund* (abundance filtering for OTUs with more than 50 reads), *classify.otu* (OTU classification), *make.shared* (creation of OTU tables), *remove.lineage* (removal of OTUs classified as chloroplast, mitochondria, *Arabidopsis*, *Embryophyceae*, unknown, and PhiX sequences), and *get.oturep* (identification OTU representative sequences based on abundance). We taxonomically classified bacterial 16S rRNA, fungal ITS2, and eukaryotic 18S rRNA sequences based on Greengenes database (13_8_99 release), UNITE database (02.02.2019 release), and PR2 database (version 4.12.0), respectively, which included the PhiX genome (40-42). We used Cutadapt to remove primer sequences in 16S rRNA and 18S rRNA data, while we used ITSx to remove non-ITS sequences in ITS2 data (43, 44).

Environmental variables

This study is part of Biodiversity Exploratories (www.biodiversity-exploratories.de), an open research platform for biodiversity and ecosystem research in Germany (45). We collected samples for four years in the Swabian Alps from seven grassland sites, which have different land use types including unfertilized, mown pastures (AEG3, AEG8, AEG43), fertilized, mown pastures (AEG10, AEG40), and fertilized, mown meadows (AEG17, AEG22) (46-48). We obtained data on climatic variables (*i.e.* air temperature, precipitation, soil temperature, soil moisture) for the period of September 2017 to August 2021 from the Biodiversity Exploratories database (Biodiversity Exploratories Instrumentation Project (BExIS dataset ID 19007)). In this study we used climate values that include (i) annual mean temperature (average monthly air temperature, at 10 cm and 200 cm aboveground); (ii) temperature seasonality (amount of air temperature variation over a year, at 10 cm and 200 cm aboveground); (iii) annual precipitation (sum of all total monthly precipitation, in mm); (iv) precipitation seasonality (measure of the variation in monthly total precipitation over the course of the year, in mm); (v) soil annual mean temperature (average monthly soil temperature, at 5 cm and 10 cm below surface); (vi) soil temperature seasonality (amount of soil temperature variation over a year, at 5 cm and 10 cm below surface); (vii) annual soil moisture (sum of all total monthly soil moisture, at 10 cm below surface); and (viii) soil moisture seasonality (measure of the variation in monthly total soil moisture over the course of the year, at 10 cm below surface). For air temperature and soil temperature, we calculated the climate values from September of previous year to August of the year of sampling. For precipitation and soil moisture, we calculated climate values over the period of Water Year (*i.e.* October of previous year to September of the year of sampling). We also obtained vegetation records of the grassland sites from 2018 to 2021 from BExIS (BExIS dataset ID 31389, 20766) (49, 50). We used Shannon's diversity and Richness indices to measure alpha-diversity and species richness of plant communities, respectively. To assess plant community composition, we performed principal component analysis (PCA) and then used PCA axis 1 and 2 scores as proxy for plant community composition. For soil microbial communities, we used Shannon's diversity and Chao1 indices to measure the alpha-diversity and species richness, respectively. To assess soil microbial community composition, we performed principal coordinate analysis (PCoA) ordination of Bray-Curtis dissimilarities between soil samples and used PCoA axis 1 and 2 scores as proxy for soil microbial community composition. For diversity and community composition analysis of both soil microbial and plant communities, we used vegan and phyloseq R packages (51-53).

Diversity and composition of *L. corniculatus* microbiomes

To calculate diversity, composition, and relative abundance profiles of microbial communities, we used R packages phyloseq, vegan, microbiome, and microeco to analyze OTU tables generated from Mothur sequence processing (51-55). For alpha-diversity analyses, we used Shannon's diversity and Chao1 indices. To assess if alpha-diversity measures are significantly different between samples, we checked for normality of the data using Shapiro-Wilk normality tests, and then analyzed using the parametric test ANOVA (for normally distributed data) or the nonparametric Kruskal-Wallis rank sum test (for non-normal data). We conducted post-hoc analysis using Dunn's test (for Kruskal-Wallis test) or Tukey's HSD (for ANOVA test). For beta-diversity analyses, we performed Principal Coordinate Analysis (PCoA) ordination of Bray-Curtis dissimilarities between samples, then used PERMANOVA analysis of the Bray-Curtis distances to assess significant effect of explanatory variables (*i.e.* plant organ, year of collection, sampling site) on microbial community structures. We used merged data from bacterial 16S rRNA, fungal ITS2, and eukaryotic 18S rRNA OTU tables in all analyses.

Environmental factors as explanatory variables of microbiome composition

To identify the main environmental factors that shape microbial community structure in *L. corniculatus* plant organs, we used the trans_env class of the microeco R package to analyze the merged bacterial 16S rRNA, fungal ITS2, and eukaryotic 18S rRNA datasets (55).

We inspected the significant differences of the environmental variables across sampling sites or years using ANOVA. We used Bray-Curtis distance-based RDA analysis (dbRDA) with feature selection (*i.e.* forward selection method) for explanatory environmental variables to each plant organ microbial community and calculated the adjusted R-squared of the RDA model that explains variation of community composition. We also calculated the significance of the RDA models that include the identified subset of environmental variables which best explain microbial community composition, and the contribution of such environmental variables to the model via ANOVA and vegan:envfit, respectively.

Association of organ biomarker microbes with environmental variables

To identify bacteria, fungi, and eukaryotes that distinguish between plant organs, we used the trans_diff class of the microeco R package (55). We used linear discriminant analysis effect size (LEfSe) ($P < 0.001$, LDA score ≥ 4) to identify biomarker genera for *L. corniculatus* roots, shoots, flowers, and seeds (56). Then we used the trans_env class of the microeco R package to test for correlations between environmental variables and relative abundances of the identified biomarker microbes (55). We used heat map to visualize the Pearson correlation coefficients.

Microbial community networks with environmental factors

To examine interactions between plant organ microbiomes and environmental factors, we performed network analysis based on correlations of OTU abundances and environmental variables. Using the trans_network class of the microeco R package, we calculated Pearson correlation coefficients of environmental variables and OTUs filtered with abundance threshold (relative abundance ≥ 0.0001) (55). We used the computed correlation coefficients ($P < 0.0001$) to build and analyse microbial community networks in Cytoscape_v3.10.1 (57). We also used the trans_env class of microeco R package to calculate correlations and to build heatmaps of bacteria, fungal, and eukaryotic genera that are highly correlated with environmental variables (55).

Multigroup structural equation modelling

We used multigroup structural equation modelling (SEM) to understand the direct and indirect relationships of environmental factors with *L. corniculatus* microbiomes. We used a generalized linear model with gaussian distribution to investigate abiotic and biotic environmental variables as predictors of the response variable, plant microbiome composition. We grouped environmental variables into categories, including precipitation, air temperature, land use intensity, soil moisture, soil temperature, plant cover, and soil microbial diversity, and then we employed model selection to identify representative variables for multigroup SEM. For model selection, we first identified multicolinearity between variables by calculating Spearman correlation coefficients and variance inflation factors (VIF), then we excluded variables flagged as highly correlated (absolute correlation > 0.8) and with high VIF values (VIF > 5). The best-fitting model with all the representative variables was identified using stepwise selection in both forward and backward directions based on Akaike Information Criterion (AIC). To calculate standardized path coefficients, we fitted the selected GLM model in the R package piecewiseSEM with plant organs as a grouping variable and scale as type of variable standardization (58). We did all analyses in R version 4.2.1 (51).

RESULTS

Spatial and temporal variation in microbial communities associated with *L. corniculatus* roots, shoots, flowers, and seeds.

Plant-associated microbial communities are continuously exposed to dispersal processes and filtering by local environmental conditions and host plant. These combinations of stochastic and deterministic processes occur at varying temporal and spatial scales - acting at the levels of plant compartments, above and belowground, or geographical distances, as well as at the levels of diurnal cycles to seasons. In *L. corniculatus*, plant organs are the main source of variation in the associated microbial communities ($R^2 = 0.20800$, $P < 0.001$; Fig. 1bc, Fig. S1a). In addition to plant organs, sampling sites and year of collection also contributed to composition variation in bacterial, fungal, and eukaryotic communities associated with *L. corniculatus* (Fig. 1bc, Fig. S1a). The effect of year of sample collection in explaining microbial community composition variation ($R^2 = 0.02647$, $P < 0.001$) is higher than sampling site ($R^2 = 0.02022$, $P < 0.001$).

Significant differences in microbial community diversity are attributed to year of sample collection but not sampling site. The Shannon diversity ($P < 0.05$) of microbial communities associated with the aboveground flower and seed microbial communities vary between collection years but not in the roots and shoots (Fig. 1e). On the other hand, the diversity of the microbial communities are stable across the different sites (Fig. S1c). Meanwhile, year of sample collection contributed to significant differences in microbial community richness in all organs, and sampling site only contributed to significant differences in richness in shoot microbial communities. Chao1 richness index ($P < 0.05$) of microbial communities associated with both above- and below-ground plant organs significantly differ between collection years, and between sampling sites for shoot microbial communities (Fig. 1f, Fig. S1d).

The relative abundance profiles of the top ten bacterial, fungal, and eukaryotic phyla in the plant organs also vary between year of collection and sampling sites (Fig. 1d, Fig. S1b, Fig. S2). Therefore, the composition, diversity, and richness of microbial communities associated with *L. corniculatus* roots, shoots, flowers, and seeds are significantly affected by either spatial and temporal factors. While microbial community heterogeneity is observed between plant compartments, and especially more discernible between above- versus below-ground plant organs, observed differences in plant microbial community structures between sampling sites or years suggest that abiotic and biotic factors are acting on microbial community assembly processes at different levels of spatial and temporal scales.

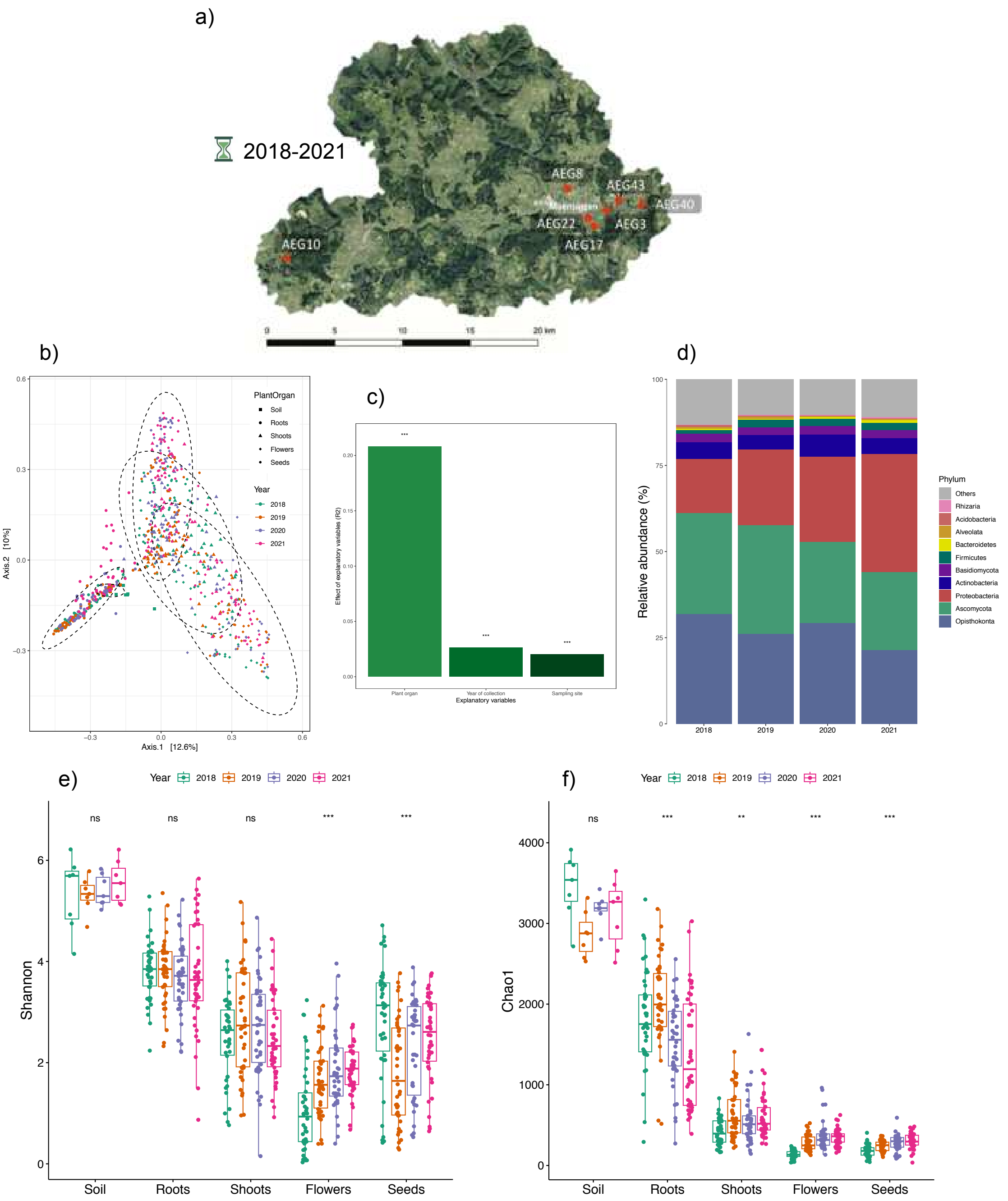

Figure 1. Spatial and temporal variation in *Lotus corniculatus* microbiomes. (a) Map of seven grassland sites in the Swabian Alps where plant and soil samples were collected from 2018-2021. For diversity and composition analysis, we used merged data from bacterial 16S rRNA, fungal ITS2, and eukaryotic 18S rRNA OTU tables: (b) Principal coordinate analysis based on Bray-Curtis dissimilarities between soil and plant organ microbiomes. (c) R2 statistics from PERMANOVA show the percentage of variance that can be explained by factors such as plant organ, year of collection, and sampling sites. (d) Relative abundance of ten most abundant phyla per year of collection in plants collected from seven grassland sites for four years. (e) Shannon and (f) Chao1 diversity of soil and plant organ microbiomes were compared between sampling years using Kruskal-Wallis significance test.

Microbial community composition in *L. corniculatus* are shaped by a subset of environmental factors that are distinct with each plant organ.

To delve deeper into the processes that contribute to the observed spatial and temporal variation in *L. corniculatus*-associated microbial communities, we examined a set of environmental variables documented from seven sites (AEG3, AEG8, AEG10, AEG17, AEG22, AEG40, AEG43) of the Biodiversity Exploratories in the Swabian Alps during four years of sampling (Summers of 2018-2021) (Fig. 1a, Fig. S3-4). First, biotic and abiotic factors in the soil such as soil moisture and temperature, as well as soil microbial community composition, diversity, and richness were both taken into account (Fig. S3a-c, Fig. S4a-c). For aboveground biotic and abiotic factors, air temperature and precipitation, as well as plant community composition, diversity, and richness were analyzed (Fig. S3d-f, Fig. S4d-f). Finally, the land use intensity (LUI) in the sampling sites, including the LUI index components grazing, mowing, and fertilization, were examined (Fig. S3g, Fig. S4g). These environmental variables significantly differ between either sampling sites or years. Soil climate variables such as annual moisture, moisture seasonality, annual mean temperature, and temperature seasonality, significantly differ both among sampling sites and years. Likewise, the soil microbial community composition, diversity, and richness are also significantly different between sampling sites and years. Aboveground climate factors such as annual precipitation and annual mean temperature also vary between sampling sites and years, and climate indices such as precipitation and temperature seasonality that do not vary among sampling sites are significantly different throughout sampling years. Similarly, the composition, richness, and diversity of vegetation cover in the grassland sites vary among the seven plots, but only the plant community composition and richness significantly differ among the sampling years. The LUI of the sampling sites ranges from unfertilized and fertilized mown pastures to fertilized mown meadows. LUI and index components (*i.e.* fertilization, mowing, grazing), are significantly different between sampling sites, but not between years (except mowing).

Since each plant organ may perceive a set of environmental stimuli different from other compartments, the environmental factors that are most important in shaping the plant-associated microbial communities are most likely different across roots, shoots, flowers, and seeds. To determine the main environmental factors that are important in shaping microbial community composition in each plant organ, we applied Bray-Curtis distance-based RDA analysis (dbRDA) for explanatory environmental variables separately for each organ microbial communities. Feature selection based on forward selection method was used to identify a subset of environmental variables included in the RDA model that best explains the microbial community composition. The significance of the model and the contribution of the environmental variables to the model were also calculated (*i.e.* via ANOVA and vegan:envfit, respectively).

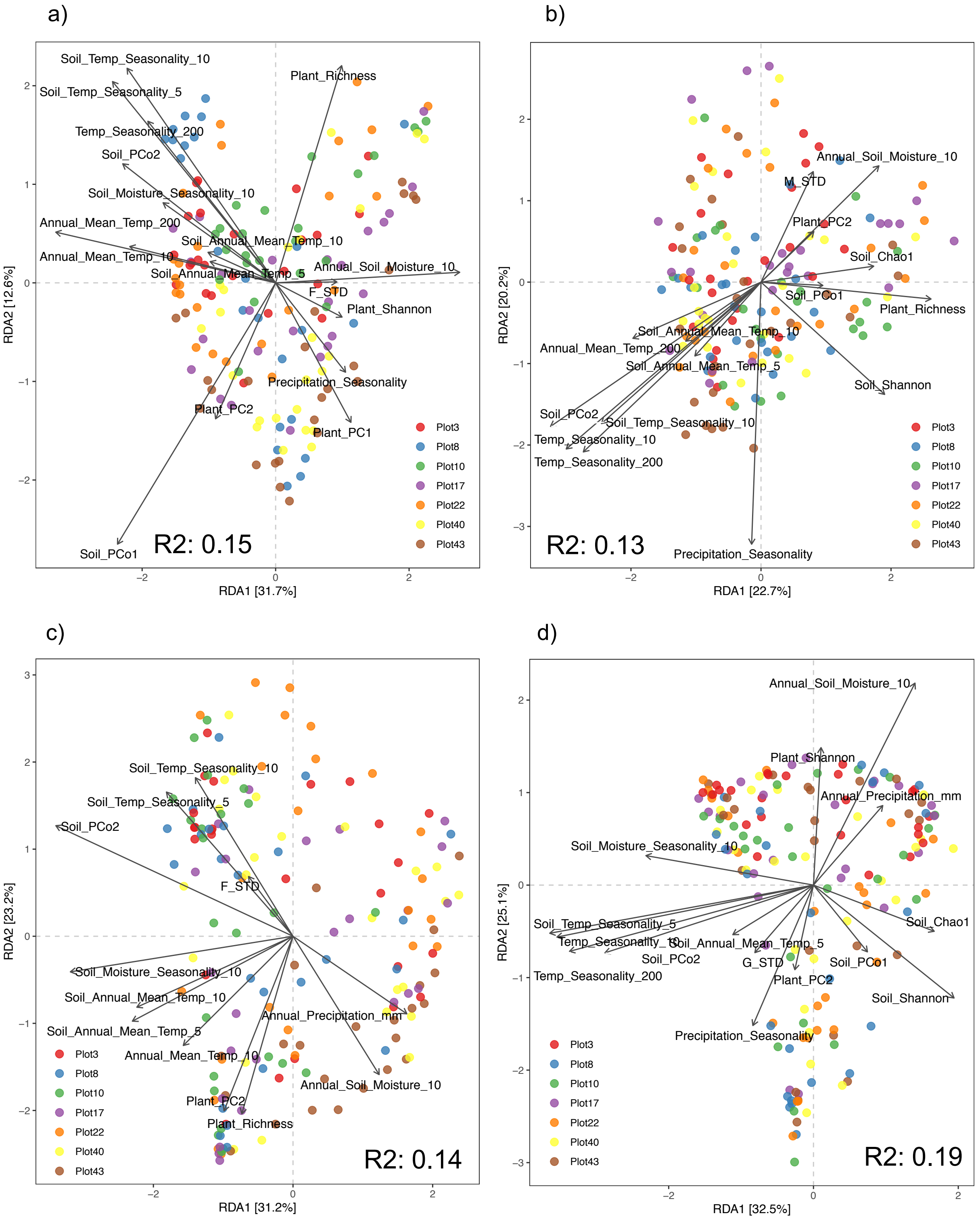

Figure 2. *Lotus corniculatus* microbiomes are shaped by a subset of environmental variables that are different with each plant organ. Bray-Curtis distance-based RDA analysis (dbRDA) of (a) root, (b) shoot, (c) flower, and (d) seed microbiomes, with vectors representing environmental variables which were feature-selected based on forward selection method. Variance (adjusted R^2) of microbial communities explained by the feature-selected environmental variables included in the significant RDA models ($P < 0.05$) are indicated.

In general, microbial communities associated with *L. corniculatus* are shaped by all environmental factors with varying importance for each plant organ. For all plant organs, significant models (*P* < 0.05) include a subset of environmental factors that explain up to 19% variation in microbial community composition. A combination of biotic and abiotic factors explains 15% of variation in root microbial community composition (Fig. 2a). The composition of bacterial, fungal, and eukaryotic communities are mainly shaped by microbiome composition and temperature seasonality in the soil as well as richness of vegetation cover (Table S2). Meanwhile the microbial community composition in shoots are primarily driven by abiotic factors both in soil and aboveground, such as temperature and precipitation seasonality (Fig. 2b, Table S2). The set of environmental factors included in the significant model accounts for 13% of the variation in shoot microbial communities. From the model comprising a combination of factors that explains 14% of microbial community composition variation, the most important environmental drivers in shaping microbial community composition in flowers are biotic and abiotic components in the soil - microbial community composition, moisture, and temperature (Fig. 2c, Table S2). Among the set of environmental variables that account for 19% of variation, seed-associated microbial community composition are best explained by abiotic factors, such as temperature seasonality in the soil and aboveground (Fig. 2d, Table S2). Only the aboveground microbial communities are significantly affected by soil microbiome richness and diversity, as well as annual precipitation and mean temperature (Fig. 2). Plant organs are shaped by different land use indicators - root- and flower-associated microbial communities are significantly affected by fertilization, while shoot and flower microbiomes are significantly shaped by mowing and grazing, respectively (Fig. 2). Therefore, variation in microbial communities associated with different plant organs are shaped by a set of environmental perturbations that are distinct to each plant compartment.

Organ biomarker microbes selectively respond to environmental factors in different *L. corniculatus* organs.

Plant-associated microbial communities below- and aboveground are exposed to similar local environmental conditions but are perceived at different scales, and these perturbations, in addition to other plant host selection and stochastic processes, consequently lead to the distinct but overlapping community structures of microbial communities associated with the roots, shoots, flowers, and seeds (Fig. 1). To probe if environmental factors are perceived by microorganisms differently in different plant compartments, we assessed if the abundance of organ biomarker microbes are correlated with environmental factors. Biomarker microbes, which are differentially abundant microbes that account for the differences between plant organ microbial communities, were identified via LEfSe analysis (Fig. 3ace). The relationship between the abundance in each plant organ of these biomarker microbes and environmental variables were calculated using Pearson correlation.

In the roots, the abundance of root biomarker bacteria *Mesorhizobium* and *Cryptosporangium* significantly correlated with environmental variables, but showed no significant correlation in other plant organs (Fig. 3b, Fig. S6). Most of the environmental variables important in shaping root microbial community composition, such as temperature seasonality in the soil and aboveground air, soil moisture seasonality, and soil microbial composition, are negatively correlated with the abundance of *Mesorhizobium* in the roots (Fig. 2a). *Cryptosporangium* abundance are significantly correlated with soil moisture and plant diversity, which are variables that also significantly shape root microbial communities (Fig. 2a). In the shoots, shoot biomarker fungi *Setosphaeria* abundance is positively correlated with soil microbial community composition, while in the roots their abundance is negatively correlated with soil microbial community richness and diversity (Fig. 3d, Fig. S7). These biotic factors (*i.e.* soil microbes) are relevant factors in shaping shoot microbial community composition (Fig. 2b). Abundance of flower biomarker microbes *Wolbachia* and *Botryotinia* are significantly correlated with environmental variables in the flowers but not in other plant organs (Fig. 3ac, Fig. S6-7). *Wolbachia* abundance is positively correlated with microbial community composition and moisture seasonality in the soil, temperature seasonality both in the soil and aboveground, and precipitation seasonality - all important variables influencing microbial communities of all plant organs (Fig. 2). *Botryotinia* abundance also significantly correlated with microbial community composition and temperature seasonality in soil as well as temperature and precipitation aboveground.

The abundance of aboveground biomarker microbes for the shoot (*i.e. Prauserella, Pantoea, Agrobacterium, Septoria, Chaetosphaeronema*), flower (*i.e Pseudomonas, Cladosporium, Alternaria*), and seed (*i.e. Propionibacterium, Malassezia*) microbial communities are significantly correlated with environmental factors in the aboveground plant organs but not in the roots (Fig. 3, Fig. S6-8). Meanwhile there are also biomarker microbes, such as the shoot biomarker *Burkholderia* and seed biomarker *Ralstonia,* in which their abundance in all plant compartments are significantly associated with environmental factors (Fig. 3b, Fig. S6). Some microorganisms that are important members of the plant organ microbial communities respond to environmental perturbations only in the corresponding plant organ but not in other compartments, therefore highlighting the role of a set of environmental variables that are important in shaping plant organ microbial communities but otherwise have weak effect in other plant compartments. Many aboveground biomarker microorganisms are also receptive to environmental factors only in aboveground compartments and not in the roots. On the other hand, some biomarker microorganisms perceive and are affected by such environmental variables anywhere in the plants.

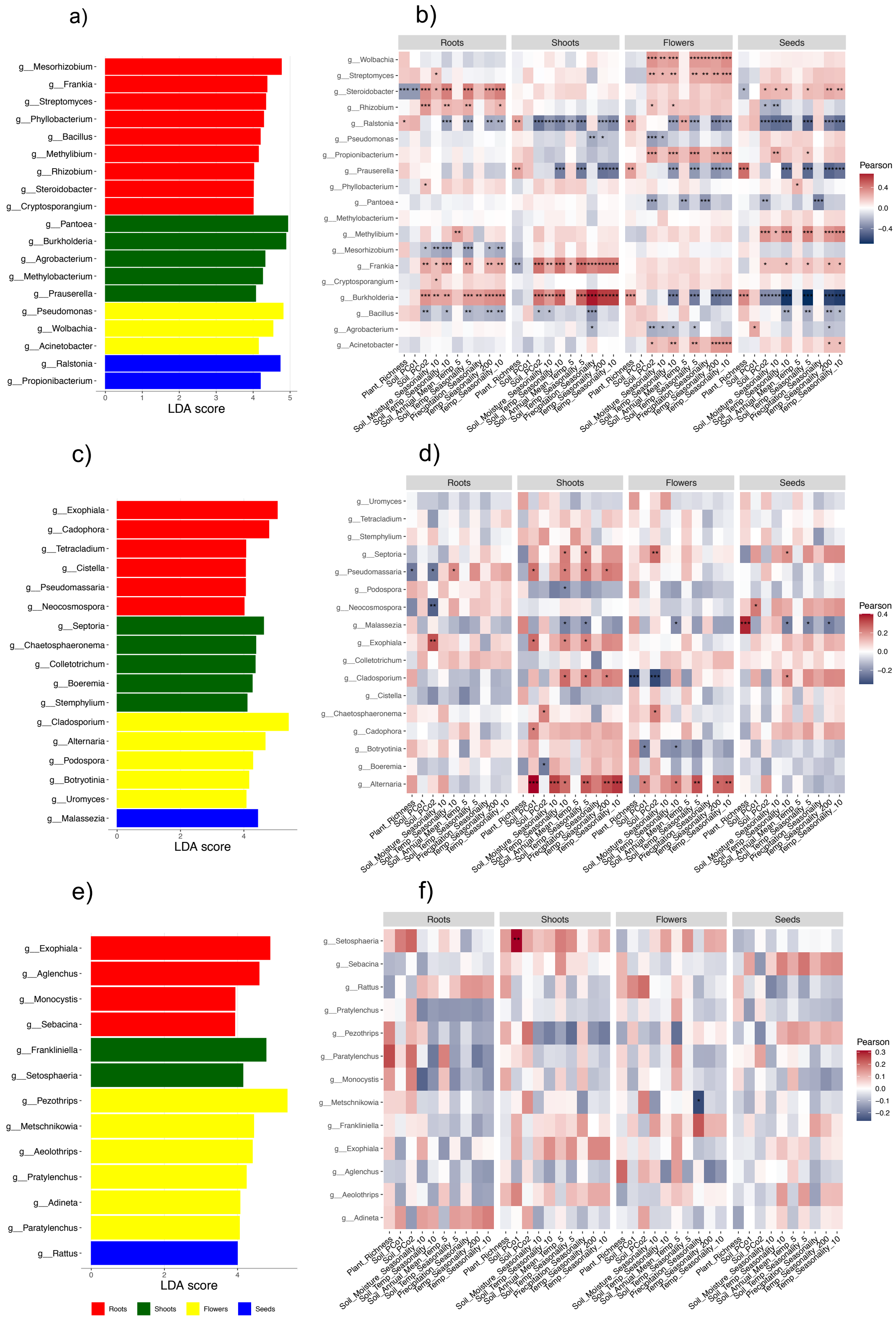

Figure 3. ***Lotus corniculatus* organ biomarker microbes respond differently to environmental variables in different plant organs.** LEfSe analysis to identify differentially abundant (a) bacterial, (c) fungal, and (e) eukaryotic biomarkers ($P < 0.001$, LDA score ≥ 4) of the plant organ microbiomes. Heatmap of Pearson correlations between selected environmental variables and relative abundances of the identified (b) bacterial, (d) fungal, and (f) eukaryotic organ biomarker microbes in roots, shoots, flowers, and seeds.

Organ-specific filtering by environmental factors influence transmission and establishment of microorganisms in different *L. corniculatus* organs.

Distinct *L. corniculatus* organ microbiomes are overlapping and interconnected via transmission of microorganisms between plant compartments and from outside environment (36). The microbial communities in roots, shoots, flowers, and seeds are shaped by a set of environmental factors that are distinct to each plant compartment. To examine how environmental factors can influence transmission of microorganisms within plants and how these factors contribute to assembly of these microbial communities to become organ-specific, we analyzed how local biotic and abiotic factors shape community structure of plant organs, specifically looking into how these factors impact microbial interactions in the different plant organs. To analyze interactions between environmental factors and plant organ microbiomes, we performed network analysis based on Pearson correlation coefficients of OTU abundances and environmental variables and analyzed co-occurrence patterns between microbial taxa and abiotic and biotic factors (Fig. 4). After applying abundance threshold (relative abundance ≥ 0.0001) and criteria for correlation ($P < 0.0001$), the complexity of root microbial network is highest with 708 nodes and 14,977 edges, followed by shoot microbial network with 338 nodes and 4,014 edges, seed microbial network with 159 nodes and 1,358 edges, and finally flower microbial network with the least number of nodes (119) and edges (429) (Table S3). The root microbial network has more positive correlations (73% of total significant correlations) than negative correlations (27%) with environmental variables, while the shoot microbial network is evenly positively and negatively correlated (51% and 49%, respectively), and both the flower and seed microbial networks have less positive correlations (20% and 26%, respectively) than negative correlations (80% and 74%, respectively) with environmental variables.

The environmental factors that impact the structure of microbial community networks in the plants differentially affect the plant compartments (Fig. 4). Thirty percent of the OTUs in the plant microbiome network are significantly correlated with environmental variables (*i.e*. specifically 29%, 29%, 31%, and 35% of the OTUs in the root, shoot, flower, and seed microbiome networks, respectively). Among the soil climate variables, soil temperature seasonality has the highest number of significant correlations. Most of the correlations between soil temperature seasonality and microbes in the roots are positive, while in the aboveground plant parts the correlations are negative. Soil microbiome composition also showed high number of correlations and is mostly positively correlated with root microbes and negatively correlated with microbes in aboveground organs. Among the climate variables aboveground, temperature seasonality has the most number of significant correlations. In the roots and shoots, temperature seasonality is primarily positively correlated with the microbes, in contrast it is negatively correlated with microbes in the flowers and seeds. Plant community richness is also highly correlated with the microbes in the plant, and while it is mostly negatively correlated with the root microbes, in the aboveground plant parts it is primarily positively correlated. The relationship between LUI and microbe abundances differs across different plant organs - it is negatively correlated in the roots, positively correlated in the shoots, and no correlation in flowers and seeds. Among the LUI components, grazing has the highest number of correlations with the plant microbes, and is positively correlated with the microbes in all plant organs. Overall, soil temperature seasonality, soil microbiome composition, temperature seasonality, plant community richness, and grazing are the most relevant environmental variables in the microbial networks, and such environmental variables often differ in relationships with microbes in the roots compared with aboveground microbes in shoots, flowers, and seeds.

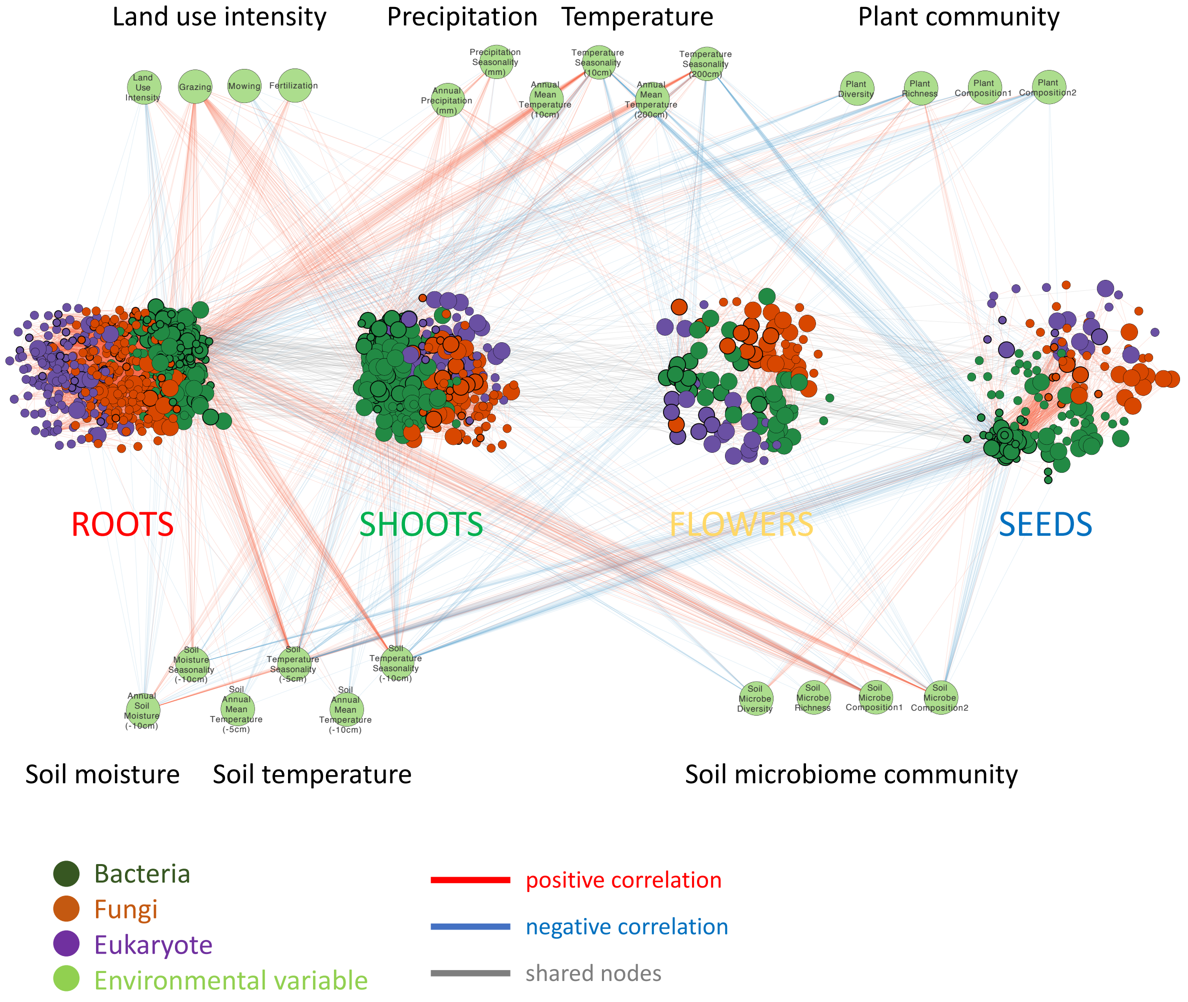

Figure 4. Environmental factors influence microbial interactions in *Lotus corniculatus* organ microbiomes. Root, shoot, flower, and seed networks based on Pearson correlations ($P < 0.0001$) of OTU abundances and environmental variables. Bigger-sized nodes are shared between adjacent plant compartments and nodes with wider border are significantly correlated with environmental variables.

Throughout continual transmission of microorganisms across plant compartments, the microorganisms that eventually survive and persist in each organ depend on a combination of host and environment conditions, including abiotic and biotic factors. The contrast in relationship between environmental factors and microbe abundances in a particular organ compared with other plant organs possibly played essential role in shaping the distinct plant organ communities. In the root network, 19% of the OTUs are potentially transmitted to the shoot network, while 26% of the shoot network OTUs are transmitted to the flower network, and 51% of the flower network OTUs goes to the seed network (Fig. 4). Among the transmitted microbes to the shoot network, 34% are significantly correlated with environmental variables, while 19% and 36% of the microbes that goes to the flower and seed networks, respectively, have significant interactions with environmental variables. For instance, *Burkholderia* and *Sulfuritalea*, which are microorganisms that are present in all organs and thus are potentially transmitted across organs, are highly correlated with environmental variables (Fig. S9). *Burkholderia* and *Sulfuritalea* abundances are positively correlated with soil temperature seasonality, soil microbiome composition, and temperature seasonality in the roots and shoots, and shifted to negative relationship in flowers and seeds. Meanwhile their abundances are positively correlated with plant community richness in the flowers and seeds. This is consistent with the observed patterns that the association between microbial abundances and environmental factors shifts when microorganisms are transmitted from roots to aboveground plant compartments.

Structural equation modeling infers direct and indirect environmental effects that shape microbial community composition in *L. corniculatus* organs.

The multigroup SEM, with plant organ as grouping variable, aims to estimate direct and indirect effects of environmental factors on microbial community composition in roots, shoots, flowers, and seeds (Fig. 5). Through SEM we assessed the direct influence of abiotic (precipitation, air and soil temperature, soil moisture, and LUI) and biotic (community composition of plant cover and soil microbiome) environmental factors on plant microbial community composition, as well as indirect influence of the abiotic factors via their effect on such biotic factors (Fig. 5a). We built a SEM model (Fisher's C = 2.032, P = 0.362, Fig. 5b) that estimated the direct effect of the abiotic variables - annual precipitation, temperature seasonality at 200 cm aboveground, LUI, annual soil moisture at 10 cm below surface, and soil annual mean temperature at 10 cm below surface - on plant microbial community composition (PCoA axis 2 scores). The indirect effect of these abiotic variables through their influence on the biotic variables - plant cover composition (PCA axis 2 scores) and soil microbiome community composition (PCoA axis 2 scores) - was also evaluated.

We found that precipitation and plant community composition directly increase community composition of plant microbiomes, while temperature (air and soil) and soil microbiome community composition negatively affect plant microbiome community composition. Soil moisture positively affects microbiome community composition in roots, shoots, and seeds, but negatively influences flower microbiomes. LUI has no direct effect on plant microbiomes, but it can indirectly influence them via its negative and positive relationship with plant cover and soil microbiomes, respectively. Thus, through multigroup SEM we establish that environmental factors differentially affect the different plant organs and possibly dispersal decisions of microorganisms. The calculated path coefficients, although significant, are low and may explain a small proportion of variation in plant microbiome community composition.

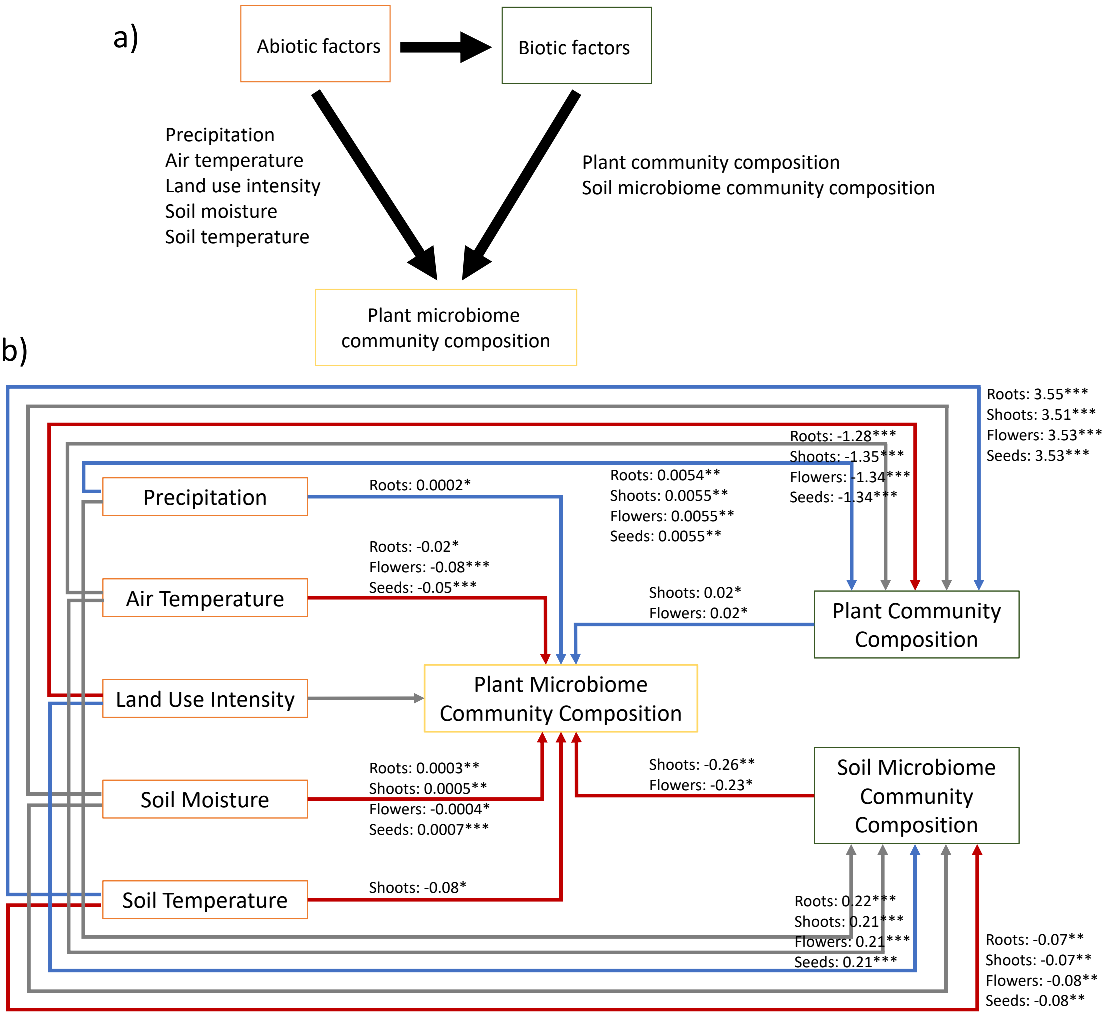

Figure 5. Multigroup SEM shows direct and indirect effects of environmental factors on *L. corniculatus* organs. (a) Causal hypothesis for the multigroup SEM which proposes the direct and indirect (via biotic factors) effects of abiotic environmental factors on plant microbiome community composition. b) Multigroup SEM, with plant organs as grouping variable, show significant path coefficients to estimate the effects of precipitation (annual precipitation), air temperature (temperature seasonality at 200 cm aboveground), land use intensity (LUI), soil moisture (annual soil moisture at 10 cm below surface), soil temperature (soil annual mean temperature at 10 cm below surface), plant cover composition (PCA axis 2 scores), and soil microbiome community composition (PCoA axis 2 scores) on plant microbiome community composition (PCoA axis 2 scores). Blue arrows indicate positive and red arrows indicate negative path coefficients. Gray arrows indicate nonsignificant path coefficients but are included in the significant model. Only significant path coefficients are shown: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

DISCUSSION

L. corniculatus microbial communities are distinct yet overlapping across roots, shoots, flowers, and seeds. In addition to organ-specificity of the microbiomes, we observed spatial and temporal variations across multiple sites and years. While plant organs are the primary source of microbiome variation, this study aims to further explore other factors that account for the observed spatial and temporal variations. Specifically, we examined how the abiotic and biotic factors in the local environment throughout multiple years contribute to the structure of microbial communities associated with *L. corniculatus* populations. We observed that plant organ microbial communities are shaped by a set of environmental factors that are distinct to each plant compartment. These environmental factors influence plant microbiomes, either by directly affecting them or indirectly through their influence on other biotic factors. Dispersal of microorganisms within plant compartments and from surrounding environment, and afterwards organ-specific filtering by biotic and abiotic factors, contribute in shaping distinct and overlapping microbial communities across plant organs. Subsequent establishment and persistence of microbial communities after dispersal are dependent on their differential perception of environmental conditions in each plant organ.

In general, *L. corniculatus* microbial communities are shaped by every local abiotic and biotic factor with varying importance for each plant organ. In the roots, both abiotic and biotic factors in the soil, such as microbiome composition and temperature, primarily explain variation in microbial communities. It is established that soil microbiomes are the primary reservoir of microorganisms that are recruited towards and enter the roots (10, 19, 59). Temperature changes in the soil directly influence root microbial community composition by regulating which microbial taxa survive and dominate in the community and indirectly by shifting the community structure of the soil microbial sources and by impacting recruitment of microbes due to changes in root exudation patterns (60-62). Vegetation cover is also found to influence soil and root microbial communities mainly by affecting soil properties (63, 64). In the shoots, abiotic factors temperature and precipitation largely explain microbial community structure. Temperature, both in soil and aboveground, significantly impacts leaf microbiomes by shifting increase or decrease of beneficial and pathogenic microbial taxa (65-68). Similarly, precipitation also impacts abundance of plant pathogens, and can serve as microbial reservoir of phyllosphere microbiota (69, 70). Microbial communities in the flowers are primarily explained by both abiotic and biotic components of the soil. Soil microbes have been found to reach and colonize flower tissues (19, 71). Conditions in the soil like temperature shape soil microbiomes as well as impact recruitment and selection of microbes by the roots and thus indirectly filter microbes that eventually reach the flowers (72, 73). Generally, changes in abiotic conditions like temperature and moisture induce changes in vegetative and reproductive plant tissues and their associated microbial communities (74). Seed microbial communities are mainly affected by temperature, both in soil and aboveground. The environment including abiotic components like temperature affects the structure of seed-associated microbial communities by significant enrichment of microbial taxa leading to changes in co-occurrence patterns (75, 76). Furthermore, LUI components fertilization, mowing, or grazing differently affect *L. corniculatus* organs. Microbial communities in roots are impacted by fertilization, while shoot microbial communities are affected by mowing. Nitrogen fertilization regulates soil microbe abundance, root exudation, and microbe recruitment resulting in enrichment of microbial groups and genes involved in nitrogen cycle in the roots (77-79). Long-term mowing impacts leaf microbial community structure with variation in leaf functional traits and enrichment of microbial taxa like Actinobacteria (80). Flower microbial communities are influenced by fertilization and grazing. Fertilization can modify the chemical properties such as N and C availability in the phyllosphere, and is found to alter microbial community diversity in flowers associated with enriched microbial groups (81). Disturbances like grazing also alter flower microbial communities by creating stress and physical changes in the flower microhabitats or by introduction of microbes by grazing animals, resulting in shift in abundances of some microbial genera (81).

In the *L. corniculatus* endophytic metacommunities, transmission of microorganisms between plant organs is determined by environmental factors along with the differing organ microhabitats and microbial interactions, resulting in distinct yet overlapping microbial communities of the roots, shoots, flowers, and seeds that are linked by dispersal. Organisms evaluate their dispersal decisions based on conditions suitable for their survival and reproduction, considering ecological cues such as abiotic conditions and species interactions (82). In *L. corniculatus*, the environmental factors soil temperature seasonality, soil microbiome composition, air temperature seasonality, plant community richness, and grazing, which are found to influence the structure and microbial interactions in the organs and are different in association per organ microbial communities, possibly influence dispersal decisions of microorganisms whether to settle or leave the plant compartments. Indeed, soil microbial communities, temperature in soil and aboveground, plant communities, and grazing are important in structuring plant-associated microbial communities (19, 59, 63-68, 81, 83, 84). *Burkholderia*, a genus that includes plant-associated microbes that can either be pathogenic, neutral, or beneficial to their hosts, are microbes in *L. corniculatus* that are highly correlated with environmental variables across all plant organs and respond to such environmental variables differently depending on their organ microhabitat (85). *Burkholderia* abundance fluctuate in response to environmental factors depending on the habitat, for instance, their abundance are positively associated with temperature in soil and aboveground as well as soil microbiome composition in the roots and shoots while negatively correlated in the flowers and seeds. This organ-dependent environmental perception is also observed in the rhizobial symbiont *Mesorhizobium*, leaf pathogen *Setosphaeria*, and necrotroph *Botryotinia*, which are biomarker microbes in roots, shoots, and flowers, respectively (86-88). Such biomarker microbes are abundant and persistent in their corresponding organ microhabitats and their habitat choice is influenced by their differential response to environmental factors in different plant compartments. It is established that abiotic and biotic perturbations can impact chemical and morphological properties in plant compartments, thus can explain the differential response of microbes to environmental factors in different plant organs (89, 90). Therefore, dispersal decisions or habitat choice, and consequently structure of microbial communities, depend on the interactions of microhabitats, key microbes, and environmental factors. This also highlights the role of a set of environmental variables that are important in shaping microbial communities in a plant organ that is different compared with other plant compartments.

It has been previously shown that LUI impacts bacterial diversity in roots, leaves, and flowers, however in our study the effect of the individual LUI component grazing on *L. corniculatus* microbiome structure is more pronounced, probably due to low variation in LUI among the seven sampling sites (81, 91). Through SEM approach we detect the indirect influence of LUI on plant microbiomes via their effects on plant cover and soil microbiomes. While we examined soil and aboveground abiotic (*i.e.* temperature and moisture/precipitation) and biotic factors (*i.e.* soil microbiomes, vegetation cover), as well as LUI (*i.e.* fertilization, grazing, mowing), other environmental variables like humidity, solar radiation, or edaphic factors such as soil pH and soil C:N also influence plant microbiomes (19, 92, 93). The observed patterns on dispersal decisions or habitat choice based on organ-dependent environmental cues and microbial interactions in *L. corniculatus* microbiomes advance our insights on how beneficial microbes or pathogens survive and reproduce on specific plant microhabitat and environmental conditions, providing basis for further investigations to test how to mitigate pathogen spread or to engineer perturbation-resistant microbiomes. Our knowledge on the organ-specific response of plant microbiomes to abiotic and biotic factors will equip us with a framework to understand and manipulate plant microbiomes experiencing climate change.

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AUTHOR CONTRIBUTIONS: All authors conceptualized this work. K.L. collected and processed samples from the field, and performed the experiments and data analysis. K.L. wrote the manuscript with contributions from all authors. All authors read and approved the final manuscript.

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DATA AVAILABILITY: Primers and blocking oligos used in this study are listed on Table S1. Sequencing data, metadata, OTU tables, and scripts are available on https://gitlab.nfdi4plants.de/kalutap/ecology-of-the-core-microbiome-in-natural-lotus-corniculatus-populations and on Biodiversity Exploratories Information System (BExIS) (https://www.bexis.uni-jena.de/) under Dataset ID 31836. Datasets of environmental variables used but not generated in this study are also available on BExIS with their dataset IDs stated in Methods.

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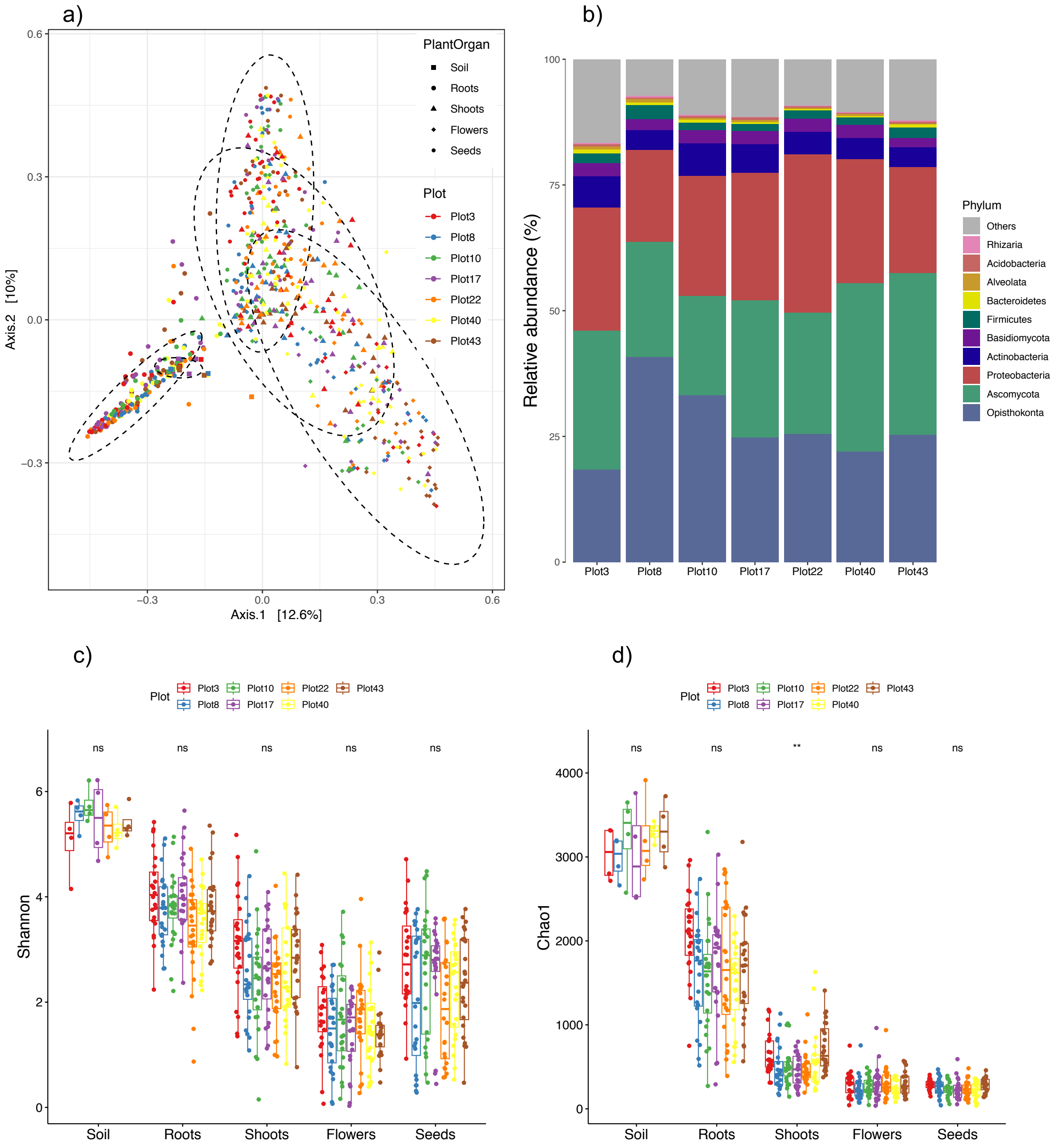
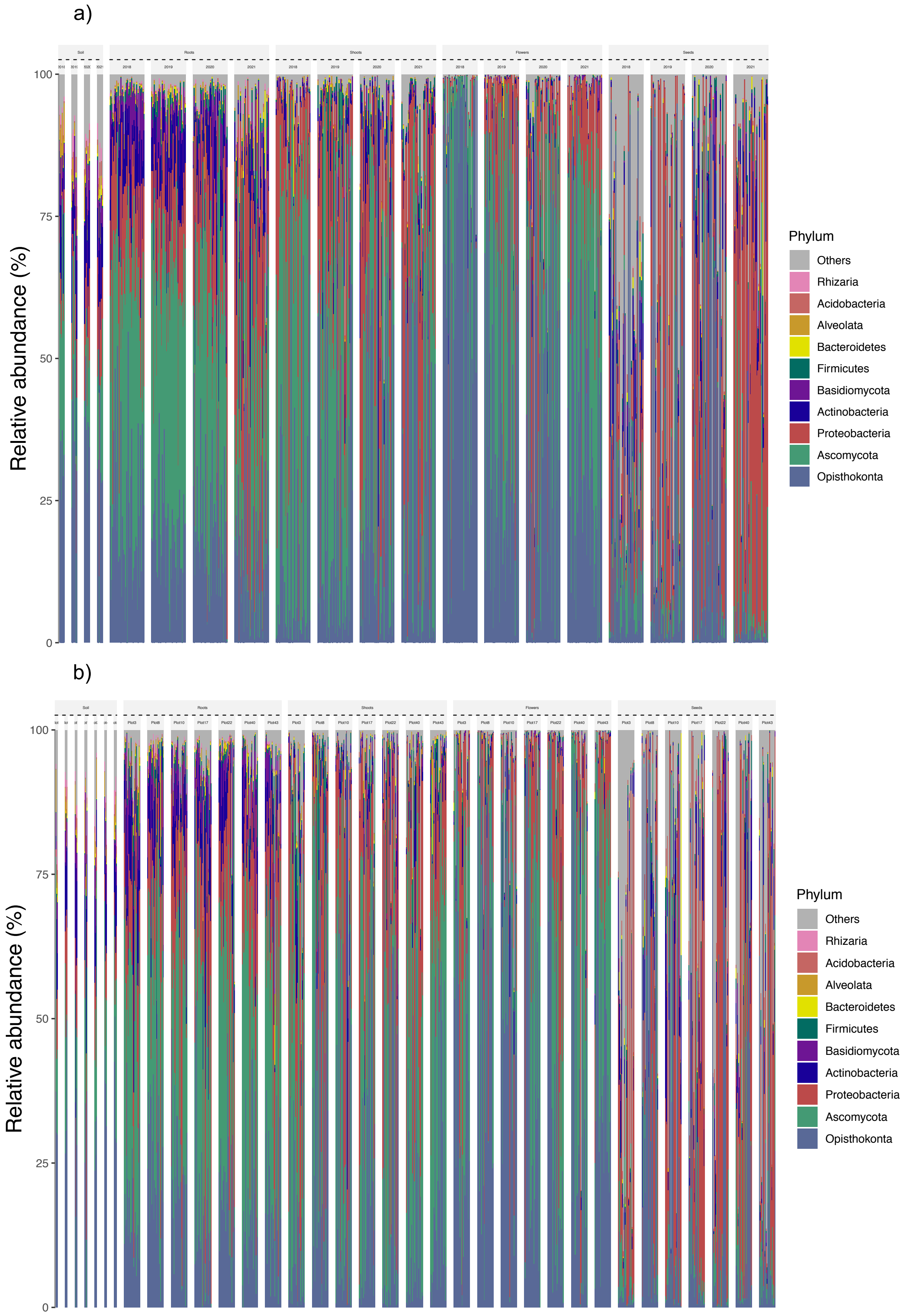
SUPPLEMENTARY MATERIALS

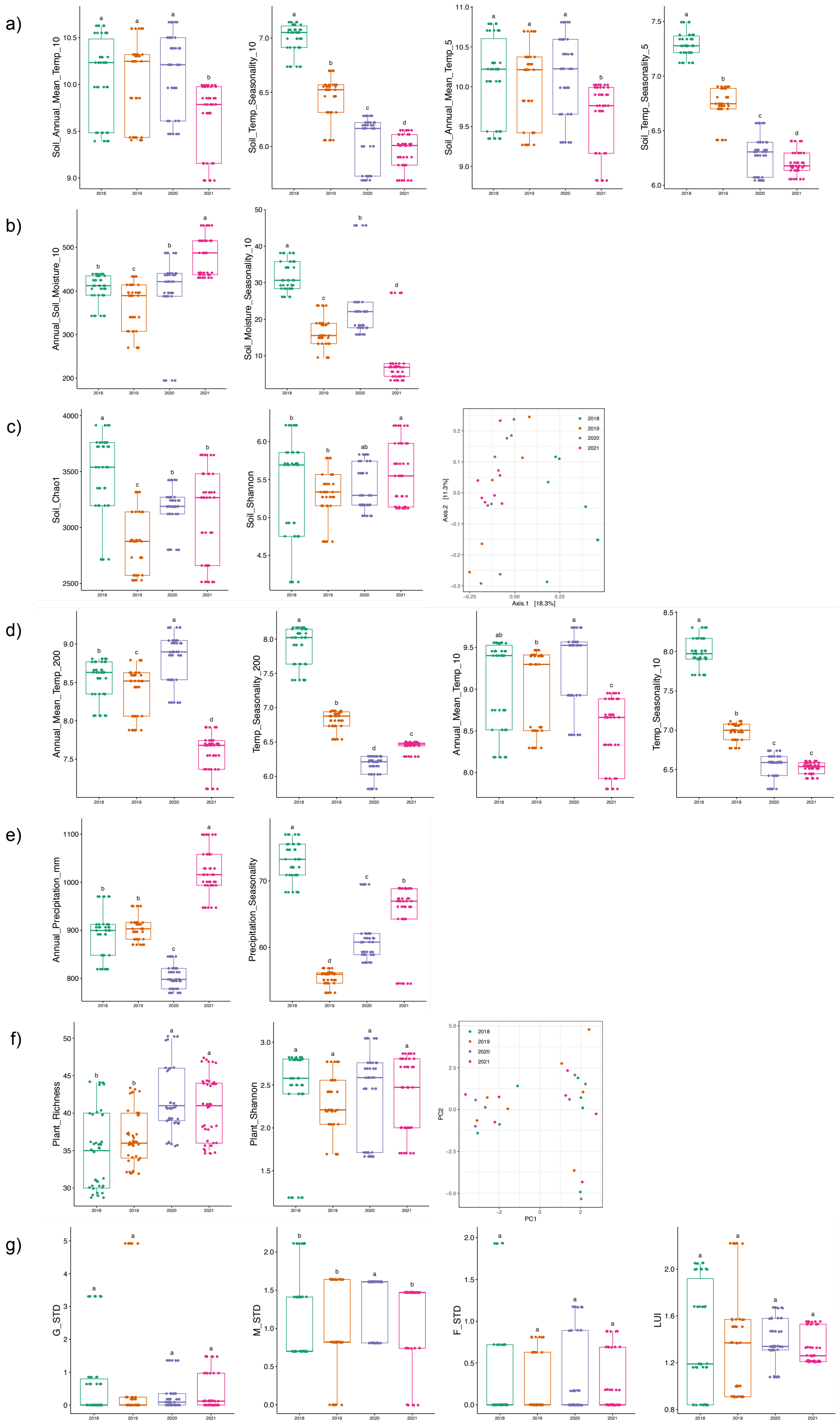
SUPPLEMENTARY FIGURES



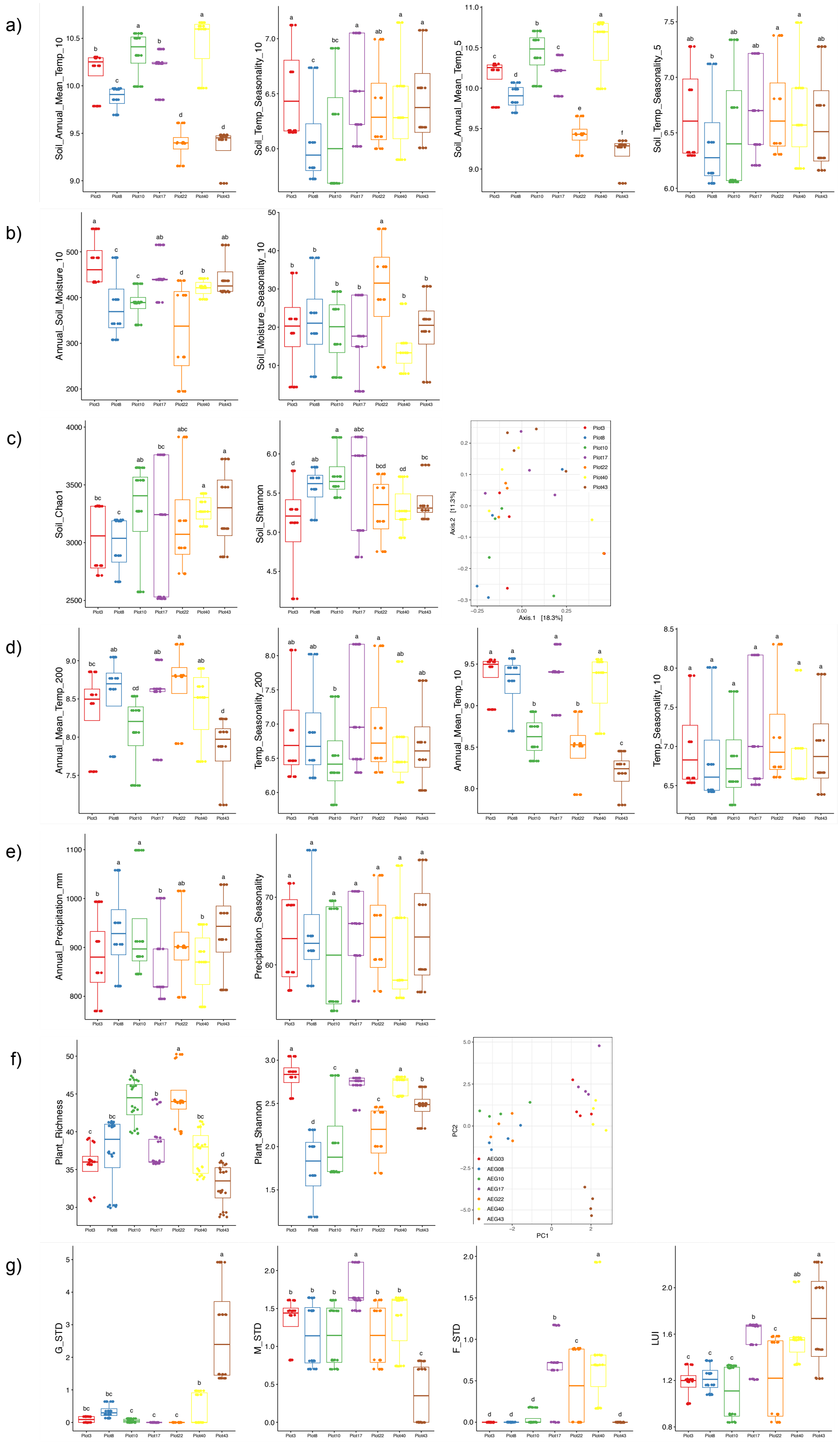
Supplementary Figure 1. Diversity and composition analysis based on merged data from bacterial 16S rRNA, fungal ITS2, and eukaryotic 18S rRNA OTU tables. (a) Principal coordinate analysis based on Bray-Curtis dissimilarities between soil and plant organ microbiomes. (b) Relative abundance of ten most abundant phyla per sampling site in plants collected from seven grassland sites for four years. (c) Shannon and (d) Chao1 diversity of soil and plant organ microbiomes were compared between sampling sites using Kruskal-Wallis significance test.



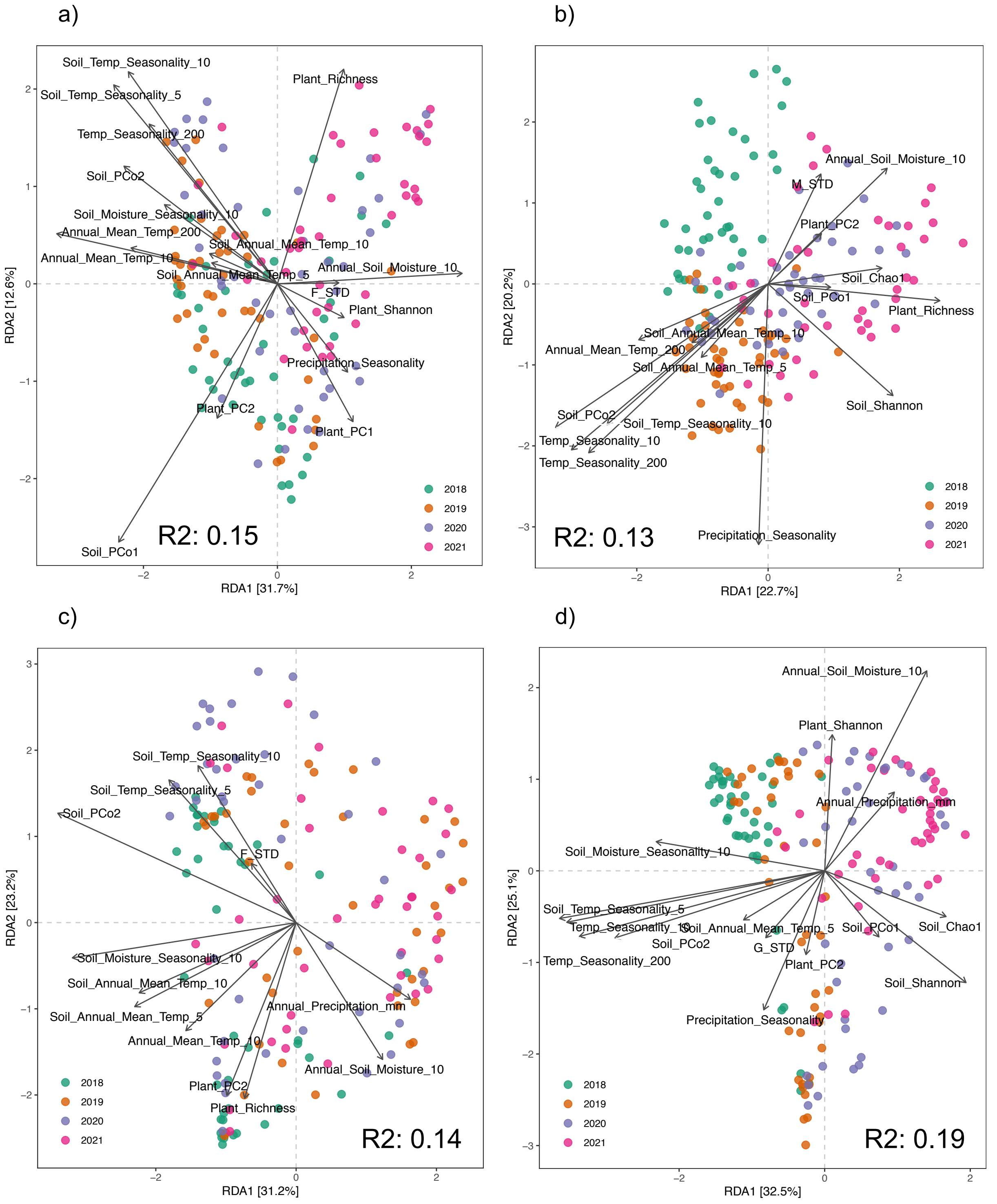
Supplementary Figure 2. Relative abundance of ten most abundant phyla in soil, root, shoot, flower, and seed samples collected for (a) four years from (b) seven grassland sites.



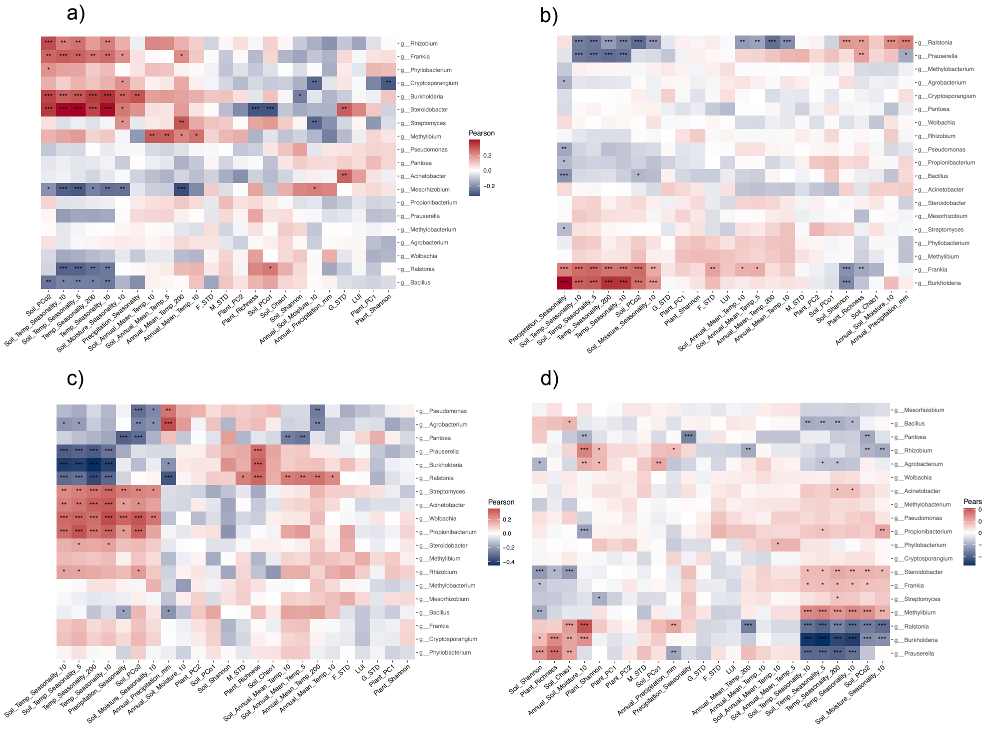
Supplementary Figure 3. Local environmental conditions during four years of sampling. (a) Soil temperature (soil annual mean temperature, soil temperature seasonality, at 5 cm and 10 cm below surface); (b) soil moisture (annual soil moisture, soil moisture seasonality, at 10 cm below surface); (c) soil microbiome composition (Shannon's diversity, Chao1 indices, PCoA); (d) air temperature (annual mean temperature, temperature seasonality, at 10 cm and 200 cm aboveground); (e) precipitation (annual precipitation, precipitation seasonality); (f) plant community composition (Shannon's diversity, Richness, PCA); and (g) land use intensity (fertilization, grazing, mowing) were compared between years of collection using ANOVA.



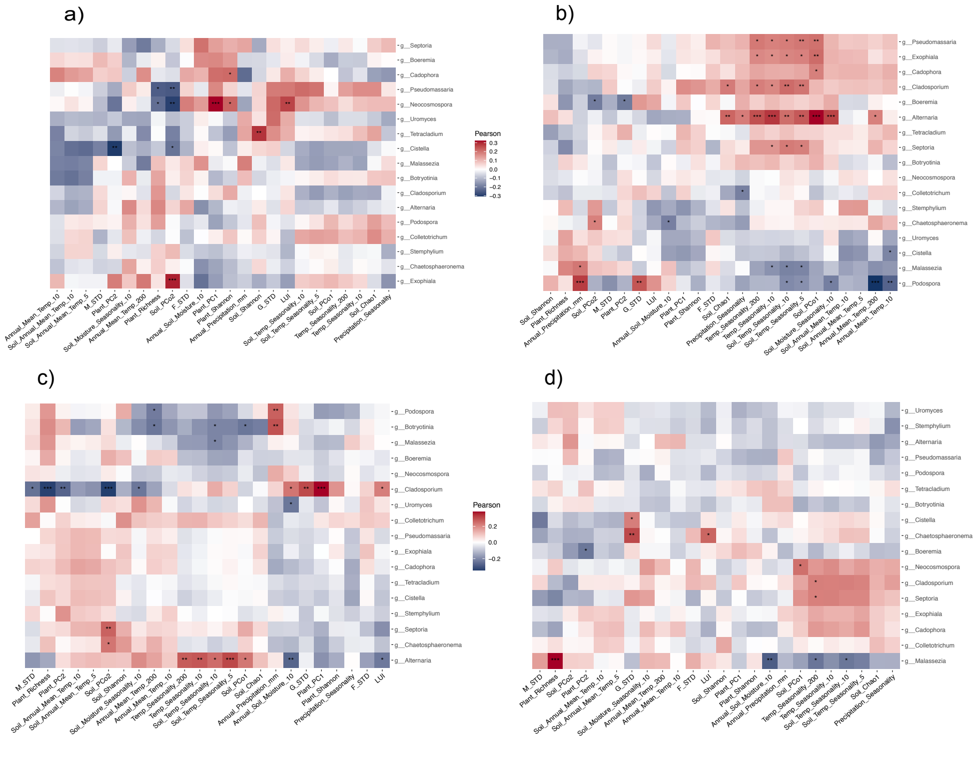
Supplementary Figure 4. Local environmental conditions across seven grassland sites. (a) Soil temperature (soil annual mean temperature, soil temperature seasonality, at 5 cm and 10 cm below surface); (b) soil moisture (annual soil moisture, soil moisture seasonality, at 10 cm below surface); (c) soil microbiome composition (Shannon's diversity, Chao1 indices, PCoA); (d) air temperature (annual mean temperature, temperature seasonality, at 10 cm and 200 cm aboveground); (e) precipitation (annual precipitation, precipitation seasonality); (f) plant community composition (Shannon's diversity, Richness, PCA); and (g) land use intensity (fertilization, grazing, mowing) were compared between sampling sites using ANOVA.



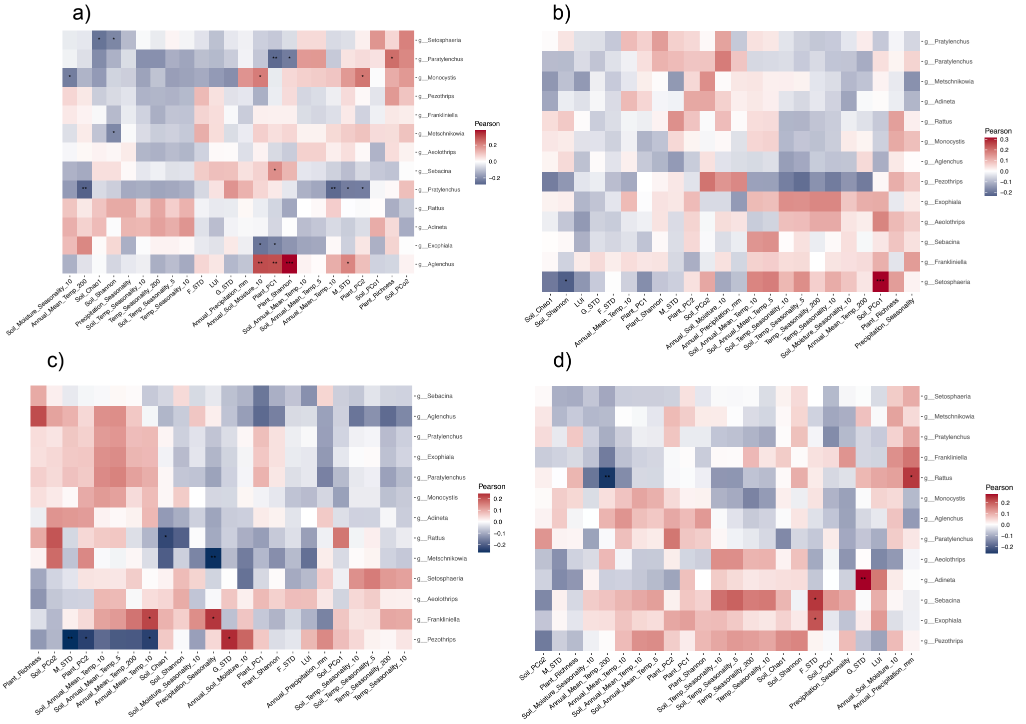
Supplementary Figure 5. Bray-Curtis distance-based RDA analysis (dbRDA) of (a) root, (b) shoot, (c) flower, and (d) seed microbiomes, with vectors representing environmental variables which were feature-selected based on forward selection method. Variance (adjusted R^2) of microbial communities explained by the feature-selected environmental variables included in the significant RDA models ($P < 0.05$) are indicated.



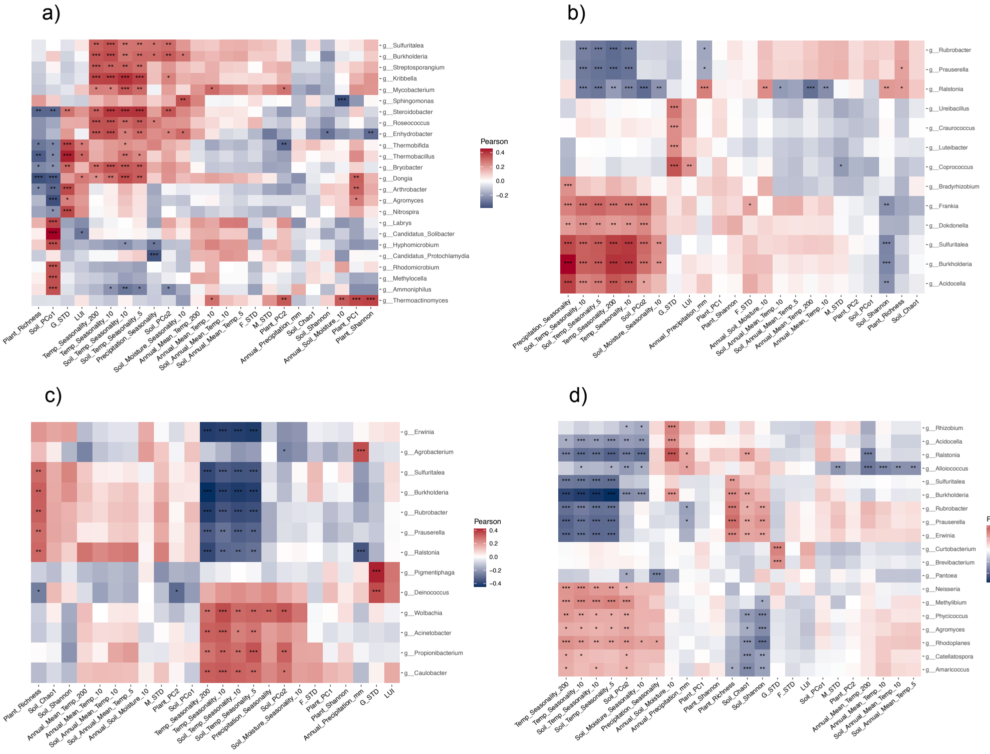
Supplementary Figure 6. Heatmap of Pearson correlations between all environmental variables and relative abundances of the identified bacterial organ biomarker microbes (in Fig. 3a) in (a) roots, (b) shoots, (c) flowers, and (d) seeds.



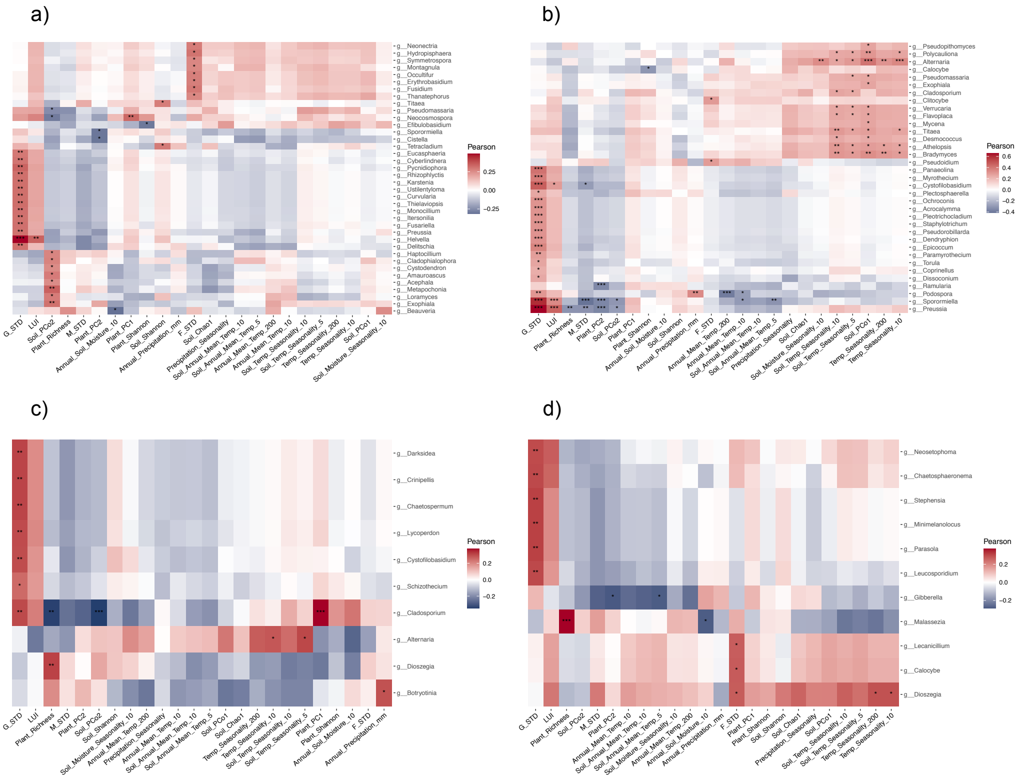
Supplementary Figure 7. Heatmap of Pearson correlations between all environmental variables and relative abundances of the identified fungal organ biomarker microbes (in Fig. 3c) in (a) roots, (b) shoots, (c) flowers, and (d) seeds.



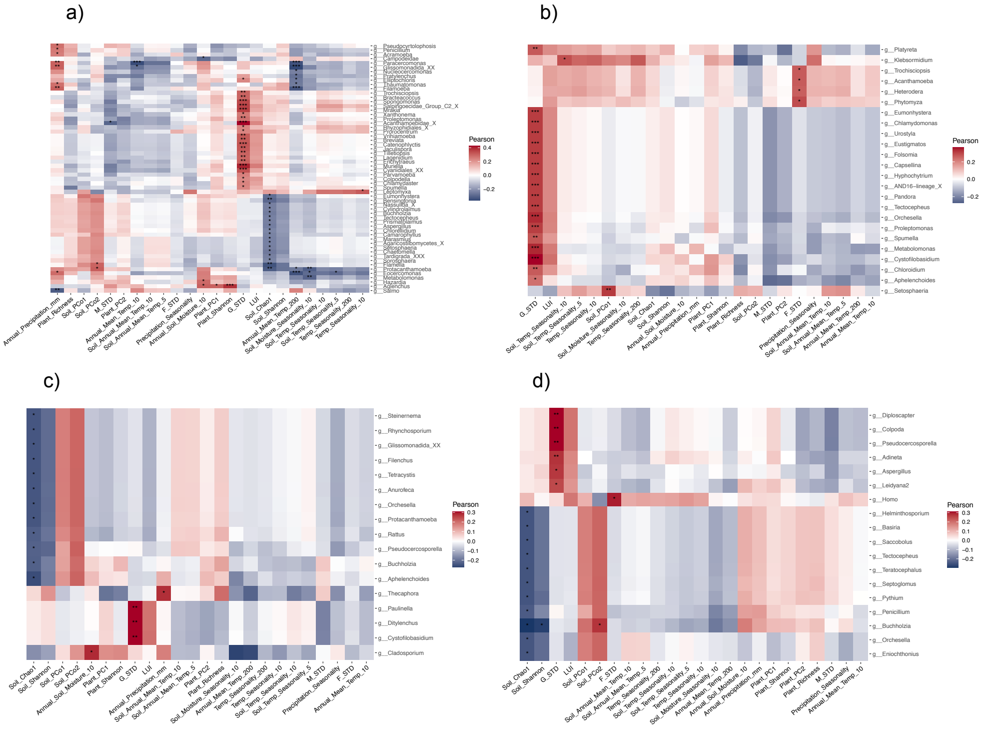
Supplementary Figure 8. Heatmap of Pearson correlations between all environmental variables and relative abundances of the identified eukaryotic organ biomarker microbes (in Fig. 3e) in (a) roots, (b) shoots, (c) flowers, and (d) seeds.



Supplementary Figure 9. Heatmap of Pearson correlations between all environmental variables and relative abundances of highly correlated bacterial genera in (a) roots, (b) shoots, (c) flowers, and (d) seeds.



Supplementary Figure 10. Heatmap of Pearson correlations between all environmental variables and relative abundances of highly correlated fungal genera in (a) roots, (b) shoots, (c) flowers, and (d) seeds.



Supplementary Figure 11. Heatmap of Pearson correlations between all environmental variables and relative abundances of highly correlated eukaryotic genera in (a) roots, (b) shoots, (c) flowers, and (d) seeds.

SUPPLEMENTARY TABLES

Supplementary Table 1. Primers and blocking oligos used in this study.

Primer name	Primer sequence (5'-to-3' orientation)
799F	AACMGGATTAGATACCCKG
1192R	ACGTCATCCCCACCTTCC
fITS7	GTGARTCATCGAATCTTTG
ITS4	TCCTCCGCTTATTGATATGC
F1422	ATAACAGGTCTGTGATGCCC
R1797	TGATCCTTCTGCAGGTTCACCTAC
clamp1_BV5_mitoF	GATGAGTGTTCGCCCTTGGTCTACGTGGAT
clamp1_BV5_mitoR	CTGCTCAGGGTTCCAAACTCAACGTTGGCA
clamp1_ITS2_F	AACCATTAGGTCGAGGGCACGTCTGCCTGG
clamp1_ITS2_R	TGAGMGYGGTTACACCACGCATGCGGGTCT
clamp9_PV9_F	GATGTATTCAACGAGTCTATAGCCTTGGCC
clamp15_PV9_R	TCTCACAACGTCGCAGGCAGCGAACCGCCC

Supplementary Table 2. Contribution of environmental variables to the dbRDA model (vegan:envfit).

a) ROOTS

	dbRDA1	dbRDA2	r2	Pr(>r)	
G_STD	0.21124	-0.97743	0.1169	0.001	***
M_STD	0.09815	0.99517	0.0174	0.243	
F_STD	0.99293	-0.11867	0.0094	0.451	
LUI	0.51492	-0.85724	0.1028	0.001	***
Plant_PC1	0.47555	-0.87969	0.1156	0.001	***
Plant_PC2	-0.42413	0.9056	0.1	0.001	***
Plant_Richness	0.1337	0.99102	0.2162	0.001	***
Plant_Shannon	0.81892	-0.5739	0.0185	0.206	
Soil_PCo1	-0.50667	0.86214	0.4562	0.001	***
Soil_PCo2	-0.74359	-0.66863	0.1295	0.001	***
Soil_Chao1	0.78725	0.61664	0.0282	0.111	
Soil_Shannon	0.87638	0.48162	0.0445	0.03	*
Annual_Soil_Moisture_10	0.9976	-0.06925	0.1202	0.001	***
Soil_Moisture_Seasonality_10	-0.78114	-0.62436	0.0627	0.009	**
Soil_Annual_Mean_Temp_10	-0.91791	-0.39678	0.0146	0.279	
Soil_Temp_Seasonality_10	-0.44233	-0.89685	0.2605	0.001	***
Soil_Annual_Mean_Temp_5	-0.96703	-0.25468	0.0121	0.348	
Soil_Temp_Seasonality_5	-0.52027	-0.854	0.2504	0.001	***
Annual_Precipitation_mm	0.86112	0.5084	0.0677	0.002	**
Precipitation_Seasonality	0.5805	-0.81426	0.0544	0.008	**
Annual_Mean_Temp_200	-0.98985	-0.14212	0.1757	0.001	***
Temp_Seasonality_200	-0.50787	-0.86143	0.1563	0.001	***
Annual_Mean_Temp_10	-0.98637	-0.16453	0.0753	0.002	**
Temp_Seasonality_10	-0.45047	-0.89279	0.2141	0.001	***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Permutation: free
Number of permutations: 999

b) SHOOTS

	dbRDA1	dbRDA2	r2	Pr(>r)	
G_STD	-0.58521	-0.81089	0.0641	0.007	**
M_STD	0.36054	0.93274	0.0681	0.006	**
F_STD	-0.09965	0.99502	0.0383	0.038	*
LUI	-0.9992	-0.03996	0.0041	0.747	
Plant_PC1	-0.715	0.69913	0.0335	0.055	.
Plant_PC2	0.76024	0.64964	0.0132	0.329	
Plant_Richness	0.96952	-0.24502	0.2294	0.001	***
Plant_Shannon	-0.347	0.93786	0.0724	0.002	**
Soil_PCo1	0.97843	-0.20659	0.0093	0.472	
Soil_PCo2	-0.7466	0.66527	0.3741	0.001	***
Soil_Chao1	0.99957	-0.02929	0.0827	0.001	***
Soil_Shannon	0.74073	-0.6718	0.2178	0.001	***
Annual_Soil_Moisture_10	0.74701	0.66482	0.1556	0.001	***
Soil_Moisture_Seasonality_10	-0.74603	0.66591	0.2245	0.001	***
Soil_Annual_Mean_Temp_10	-0.77495	0.63202	0.0534	0.008	**
Soil_Temp_Seasonality_10	-0.80379	0.59492	0.5583	0.001	***
Soil_Annual_Mean_Temp_5	-0.68778	0.72592	0.0553	0.009	**
Soil_Temp_Seasonality_5	-0.81162	0.58418	0.5534	0.001	***
Annual_Precipitation_mm	0.93389	-0.35755	0.094	0.001	***
Precipitation_Seasonality	-0.17916	0.98382	0.4413	0.001	***
Annual_Mean_Temp_200	-0.87845	0.47784	0.1507	0.001	***
Temp_Seasonality_200	-0.72809	0.68548	0.503	0.001	***
Annual_Mean_Temp_10	-0.75412	0.65674	0.0945	0.001	***
Temp_Seasonality_10	-0.75467	0.65611	0.556	0.001	***

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
Permutation: free
Number of permutations: 999

c) FLOWERS

	dbRDA1	dbRDA2	r2	Pr(>r)	
G_STD	0.69153	-0.72234	0.0869	0.001	***
M_STD	-0.61855	0.78575	0.1046	0.001	***
F_STD	-0.75279	-0.65826	0.001	0.94	
LUI	0.83544	-0.54959	0.017	0.25	
Plant_PC1	0.9274	-0.37406	0.0654	0.006	**
Plant_PC2	-0.50014	0.86594	0.0772	0.001	***
Plant_Richness	-0.37901	0.92539	0.0717	0.003	**
Plant_Shannon	0.99952	-0.03111	0.0116	0.38	
Soil_PCo1	-0.79307	0.60912	0.1019	0.001	***
Soil_PCo2	-0.95056	-0.31053	0.2485	0.001	***
Soil_Chao1	0.66223	0.7493	0.0046	0.692	

Soil_Shannon	0.90306	0.42951	0.0904	0.001	***
Annual_Soil_Moisture_10	0.68503	-0.72852	0.0599	0.009	**
Soil_Moisture_Seasonality_10	-0.99694	0.07823	0.1877	0.001	***
Soil_Annual_Mean_Temp_10	-0.96463	0.26362	0.0939	0.001	***
Soil_Temp_Seasonality_10	-0.6913	-0.72257	0.0879	0.002	**
Soil_Annual_Mean_Temp_5	-0.95162	0.30727	0.1046	0.001	***
Soil_Temp_Seasonality_5	-0.80047	-0.59937	0.104	0.001	***
Annual_Precipitation_mm	0.92017	-0.39153	0.0503	0.013	*
Precipitation_Seasonality	-0.85354	-0.52102	0.0853	0.001	***
Annual_Mean_Temp_200	-0.92559	0.37854	0.096	0.002	**
Temp_Seasonality_200	-0.79935	-0.60087	0.1167	0.001	***
Annual_Mean_Temp_10	-0.8427	0.53838	0.0607	0.004	**
Temp_Seasonality_10	-0.82283	-0.56829	0.1463	0.001	***

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
Permutation: free
Number of permutations: 999

d) SEEDS

	dbRDA1	dbRDA2	r2	Pr(>r)	
G_STD	-0.84721	0.53125	0.0255	0.133	
M_STD	0.9546	-0.29788	0.0143	0.32	
F_STD	-0.86137	0.50797	0.0064	0.589	
LUI	-0.45251	0.89176	0.0098	0.458	
Plant_PC1	-0.19806	0.98019	0.0603	0.005	**
Plant_PC2	-0.36462	0.93116	0.0117	0.391	
Plant_Richness	0.97919	-0.20294	0.1711	0.001	***
Plant_Shannon	0.11269	0.99363	0.0624	0.004	**
Soil_PCo1	0.82915	-0.55903	0.0204	0.166	
Soil_PCo2	-0.98586	0.16756	0.3926	0.001	***
Soil_Chao1	0.98026	-0.19773	0.1152	0.001	***
Soil_Shannon	0.91754	-0.39765	0.2109	0.001	***
Annual_Soil_Moisture_10	0.67946	0.73372	0.2564	0.001	***
Soil_Moisture_Seasonality_10	-0.99465	-0.10327	0.2353	0.001	***
Soil_Annual_Mean_Temp_10	-0.94176	0.33628	0.0486	0.017	*
Soil_Temp_Seasonality_10	-0.98997	0.14126	0.5906	0.001	***
Soil_Annual_Mean_Temp_5	-0.94977	0.31293	0.0428	0.028	*
Soil_Temp_Seasonality_5	-0.99579	0.09162	0.6236	0.001	***
Annual_Precipitation_mm	0.8461	0.53303	0.0468	0.021	*
Precipitation_Seasonality	-0.61599	0.78775	0.0972	0.001	***
Annual_Mean_Temp_200	-0.92024	-0.39136	0.1414	0.001	***
Temp_Seasonality_200	-0.99023	0.13943	0.5399	0.001	***
Annual_Mean_Temp_10	-0.98051	0.19647	0.0375	0.041	*
Temp_Seasonality_10	-0.99474	0.10244	0.5907	0.001	***

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
Permutation: free
Number of permutations: 999

Supplementary Table 3. Summary statistics of plant organ microbiome networks generated by network analyzer in Cytoscape 3.10.1.

Summary Statistics	Roots	Shoots	Flowers	Seeds
Number of nodes	708	338	119	159
Number of edges	14977	4014	429	1358
Average number of neighbors	42.308	23.751	8.286	17.082
Network diameter	6	7	9	7
Network radius	4	4	5	4
Characteristic path length	2.522	2.794	3.718	2.924
Clustering coefficient	0.487	0.561	0.563	0.643
Network density	0.06	0.07	0.085	0.108
Network heterogeneity	0.869	0.72	0.575	0.953
Network centralization	0.191	0.162	0.102	0.23

CHAPTER IV

PLANT AGE AND GENOTYPE EXPLAIN VARIATION IN THE MICROBIOME OF NATURAL *LOTUS CORNICULATUS* POPULATIONS

Frank Reis, Katrina Lutap, Walter Durka, Jun Hee Jung, Eric Kemen, and Oliver Bossdorf

Plant age and genotype explain variation in the microbiome of natural *Lotus corniculatus* populations

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ABSTRACT

In natural populations, plants are associated with a huge diversity of bacteria, fungi and other microbes. There is usually substantial microbiome variation between different plant individuals and populations, and the drivers of this variation are still poorly understood, particularly in wild plants. Here, we were interested in the potential for plant genotype and plant age to explain intraspecific variation in plant microbiome. In seven natural populations, we genotyped a total of 168 plants over four years, determined their ages through herb chronology, and then sequenced their root, shoot, flower and seed microbiomes. We found that plant genotypes differed both in the diversity and composition of microbes, and that some microbial taxa were associated with particular plant genotypes - a result that we experimentally confirmed for one of the observed genotype-microbe associations. The genotype effects tended to be strongest and most consistent for plant-associated bacteria, with the largest plant genotype differences in the microbiome diversity of flowers and seeds. In contrast to the widespread plant genotype effects, we found less evidence for an effect of plant age on microbiome diversity: the age of plants explained variation in fungi diversity, and it was associated with the abundance of several microbial taxa. Our study indicates that the genotype of a plant and - to a lesser degree its age - influence the diversity and composition of plant-associated microbiota, even in complex natural environments.

Keywords: bacteria, fungi, herb chronology, metagenomics, microbial ecology, plant-microbe interactions

INTRODUCTION

Plants are colonized by a wide range of bacterial, fungal and eukaryotic microorganisms collectively called the plant microbiome. The diversity and composition of this microbiome is strongly shaped by the plant species (1,2) and the surrounding microbial community, especially the soil microbiome (3,4), but there are many other biotic and abiotic factors that are known to influence plant microbiome variation among and within species, including climate (5,6), soil characteristics (7), land use (8,9), herbivory (10,11), or plant disease (12–16). Besides this plethora of external factors, the plant microbiome can also be influenced by the plant itself. Variations in gene expression and plant traits can result in changing physical and chemical properties of the plants, and thus habitat conditions for the microbes, which in turn can alter microbiome diversity and composition. Previous studies have shown that the plant microbiome indeed varies across different plant tissue types such as roots, shoots, or flowers (17–19) and also between the inside (endophytic) and outside (epiphytic) microbiome (19,20). Two other potentially important factors creating microbiome variation among plants of the same species are their genetic differences, and their differences in age or developmental stage.

It is well established that the genetic variation in plants can change pathogen resistance (21–23), and there is also good evidence that it can influence the overall composition of the plant microbiome, *e.g*. in soybean (24), potato (10), black cottonwood (25). While the studies mentioned above were all under controlled lab conditions, there is also evidence from more natural common garden experiments, *e.g*. for plant genotype effects on the microbiome of *Medicago truncatula* (26) and *Boechera stricta* (27). So far the research on these questions has been largely restricted to crops and model species, and to usually short-term experiments, whereas studies on wild plants and natural populations remain rare. Still, extrapolating lab results about plant-microbe interactions to natural environments is often challenging (28) because in natural populations plant microbiomes are influenced by a much larger complexity of biotic and abiotic factors. A true understanding of plant genotype effects, and their relative importance, therefore requires to also study plant genotype-microbiome relationships in natural populations.

Another potentially important but so far very little studied intrinsic factor creating intraspecific variation in plant microbiomes is the age or developmental stage of a plant. As a plant grows and develops, it undergoes physiological and biochemical changes that influence important functional traits, such as defense against herbivory (29,30) or immune responses to pathogens (31). These changes across different life stages will inevitably also influence plant-microbiome interactions (32). For example, the root-associated microbiome of rice varies between the vegetative phase and later life stages (33), and very similar results were found for soybean (34) and sugarcane (35). For annual plants like rice or soybean, such a comparison between vegetative and reproductive phase covers their entire life cycle. However, many plants, in particular wild ones, are perennial, which creates additional possibilities for age-related microbiome variation *e.g.* through slower successional or competitive replacement processes. So far, there are hardly any studies of perennial plants examining relationships between plant age and plant microbiome. An exception is the study by Wagner *et al.* (27) who found substantial changes in root (and to a lesser extent leaf) bacterial communities when comparing two- and four-year-old *Boechera stricta* plants in the common garden, suggesting that significant microbiome changes can also occur at later life stages of perennials. Considering that most plants are perennial, we clearly need more studies relating plant age to plant microbiome in longer-lived plants.

Understanding the influences of plant age and genotype on microbiome composition may be important not only from a fundamental scientific perspective but also for agriculture and plant conservation, for instance if it helps to better manage beneficial crop microbiomes that increase yield production, stress tolerance, or pathogen resistance. The last point is particularly important given that pathogens are a huge threat to global food security (36). More generally, understanding the drivers and consequences of plant microbiome diversity may support the management of plant populations in rapidly changing environments, and the effects of plant age and genotype on plant microbiomes - particularly under realistic conditions - are important elements of this.

Here, we combined amplicon-based metagenomics with herb chronology and plant genotyping to study relationships between plant age and genotype, and the diversity and composition of plant microbiomes within and among natural plant populations. Our study organism was *Lotus corniculatus*, a widely distributed perennial legume that grows naturally in a broad range of environments in temperate Eurasia and is an important food source for many pollinators, such as bumblebees, honeybees, and many wild bees as well as butterflies, flies, and beetles (37–39). As a nitrogen fixer it is part of a complex plant-microbe symbiosis influencing the nutrient dynamics of grassland ecosystems (40), and it therefore plays an important role in agriculture. As *L. corniculatus* is known to have a broad life span range at least up to 15 years (41); (Anna Roeder & Christiane Roscher, unpublished data), and it is known to harbour significant intraspecific genetic diversity (42,43), the species is a suitable system for asking questions about effects of plant age and genotype. Specifically, we wanted to answer the following questions:

1. Do plant age and genotype affect the diversity of the plant microbiome, and if yes how do these effects vary between different plant tissues and microbial groups?

2. Are plant age and genotype related to the composition (beta diversity) of the plant microbiome, and how are these relationships influenced by the plant tissue and microbial groups?

3. Which specific microbial taxa are most differentiated between plants of different ages and genotypes, and can we confirm some of these findings experimentally?

To answer these questions, we collected *L. corniculatus* plants from seven semi-natural grasslands in the Swabian Jura region in Germany over four consecutive years, and we then determined the ages and genotype of all plants, and analysed their bacterial, fungal, and eukaryotic microbes separately for roots, shoots, flowers, and seeds.

METHODS

Plant material

We collected *L. corniculatus* plants from seven extensively managed meadows in the Swabian Alb region of southern Germany (Fig. 1a, Tab. S1) for four consecutive years from 2018 to 2021. All collections were done in the late summer (August/September), when the plants were still flowering but already carried some ripe fruits, so that both flowers and seeds could be sampled. In each of the seven populations, we carefully excavated six entire plants, resulting in a total of 4 years x 7 populations x 6 plants = 168 plants. In the lab, we divided the plants into roots, shoots, flowers and seeds, and surface sterilized all plant parts. Therefore, we washed them by shaking in Falcon tubes (50 ml for roots and shoots; 25 ml for flowers) and 1.5 ml Eppendorf tubes for seeds for 30 s with autoclaved ultrapure water (UPW), for 60 s with epiphyte wash solution (0.1 % Triton X-100 in 1x TE buffer), for 30 s with 80% EtOH and for 30 s with 2 % NaOCl. After that, we rinsed the samples three times (seeds: five times) for 10 s with UPW, and froze them.

Determination of plant ages

To determine the ages of the sampled plants, we cut 3 cm of the root crown just below the hypocotyl (Fig. 1b), the oldest part of the plant root, from all plants and preserved them in 70% ethanol. In the lab, we placed these root samples into a microtome (Reichert, Vienna, Austria) and sliced them into transverse sections. Very small roots with a diameter smaller than 3 mm were clamped using a piece of wood. To harden the specimens and prevent them from drying out and cracking, all specimens and the blade were kept moist with 99% ethanol during the cutting process. The root crown slices were then fixed on a microscope slide in a 1:1 mixture of water and glycerol and covered with a cover glass. We used a microscope to count the number of annual rings, with ring boundaries defined by marginal parenchyma, semi-ring porosity, or both (44) (Fig. 1b). Where necessary we used polarized light to improve the visibility of the annual rings. Since the annual rings were not equally distinct in all specimens, we classified the distinctness of the rings into 1 = very good, 2 = intermediate, 3 = poor and 4 = no annual rings visible (Tab. S2) and used only classes 1 to 3 for our analyses. For the statistical analysis we decided to group the plants into 5 age groups since the low number of replicates for the very young (2–3 years) and very old plants (6–13 years). Therefore we grouped the plants into age categories: younger than 4, 4, 5, 6, and older than 6 years (Fig. 1d).

Plant genotyping

To genotype the sampled plants, and assess their relatedness, we employed ddRAD (45), a reduced-representation sequencing approach. We extracted plant DNA from all shoot samples using the QIAGEN DNeasy Plant Mini Kit (QIAGEN, 2019). We then double-digested the DNA and followed the library preparation protocol of Peterson *et al.* (45) with small modifications as given in Durka *et al.* (43). Multiplexed samples were sequenced paired-end

(PE 150) on Illumina Novaseq 6000 platform. After sequencing, we demultiplexed the reads using process_radtags from the Stacks 2.0 pipeline (46) resulting in an average of 6.31 million reads retained per sample. We assembled reads, including three outgroup samples of *L. tenuis* and *L. pedunculatus* from Durka *et al.* (43) and called SNPs using dDocent 2.7.8 (47,48) with default parameters, except of using 0.90 as ClusteringSimilarity, filtered according to O'Leary *et al.* (47) introducing a mean depth threshold of 5, and a minor allele frequency threshold of 0.05 and keeping only 1 SNP per contig. The resulting VCF file was converted into a fasta file (containing only the SNPs for each sample) with PGDSpider version 2.1.1.5 (49). To construct a Maximum Likelihood tree we used the program IQ-TREE release 2.0.3 (50). We used ModelFinder to select the best-fitting substitution model based on the Bayesian Information Criterion (BIC) (51) which identified the General Time Reversible (GTR) model with empirical base frequencies (F), a proportion of invariable sites (I), and gamma-distributed rate heterogeneity with four categories (G4) as the best-fit model (GTR+F+I+G4). To assess branch support, we also performed ultrafast bootstrap approximation (UFBoot) with 1,000 replicates (52). Finally, we used TreeCluster version 1.0.4 (53) to classify the SNP-based genotypes into a more restricted number of genetic groups (later used in our analyses), based on the total branch length of all leaves in the cluster (Sum Branch) with a threshold of 3.1. We visualized the phylogenetic tree using Interactive Tree of Life (iTOL) (54) and coloured the samples by their genetic group assignments. From here on we will refer to these nine genetic groups as genotypes.

Microbiome sequencing

To prepare the plant samples for microbiome sequencing, we first homogenized all frozen samples of soil, roots, shoots, flowers, and seeds with a Precellys 24 Tissue Homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France), and then extracted the DNA using the FastDNA Spin Kit for Soil (MP Biomedicals, Irvine, CA, USA). We then performed a two-step PCR targeting the bacterial 16S rRNA V5-V7 region, the fungal ITS2 region, and the eukaryotic 18S rRNA V9 region, using the primers 799F/1192R, fITS7/ITS4, and F1422/R1797, respectively (Tab. S3) (20). As controls we used blank samples (UPW and blank DNA extraction). To reduce the amplification of mitochondrial and chloroplast rRNA sequences from *L. corniculatus*, we incorporated blocking oligos designed with the R package *AmpStop* (Tab. S3) (55,56). We pooled the amplified products in equal concentrations, purified them with magnetic bead clean-up, and randomly assigned each to one of eight sequencing batches. We sequenced all pools on Illumina MiSeq platform with PhiX control, using the MiSeq Reagent Kit v3 (600-cycle). We then processed all microbial 16S rRNA, ITS2, and 18S rRNA amplicon sequences using Mothur (55,57,58). To remove the primer sequences from 16S rRNA and 18S rRNA data we used Cutadapt, and to remove non-ITS sequences from ITS2 data we used ITSx (59,60). For the taxonomic classification of the bacterial 16S rRNA reads, we used the Greengenes database (13_8_99 release), for fungal ITS2 reads the UNITE database (02.02.2019 release), and for eukaryotic 18S rRNA the PR2 database (version 4.12.0), all of which included the PhiX genome (61–63).

Data analysis

We carried out all data analyses in R Studio 2024.12.1 (64), and we used *dplyr* for structuring and manipulating data frames (65) and *ggplot2* package (66) for creating figures. We used the *vegan* package (67) for calculating the alpha diversity (Shannon index) of bacterial, fungal, or eukaryotic microbiomes of each tissue in each plant, and then linear models to test for the effects of plant age or genotype. To account for variation in other factors, all models included the plant organ, and its respective interaction with plant genotype or plant age as well as sample location and year.

In addition to variation in microbial alpha diversity, we also analysed the compositional turnover (beta diversity) between different samples, using the R packages *vegan*, *phyloseq*, and *microbiome* (67–69). We first used the OTU relative abundances for a Principal Coordinate Analysis (PCoA) of the Bray-Curtis dissimilarities between samples, and then a PERMANOVA to test how much of the variation in microbial community composition could be explained by plant age, plant genotype, or their interaction. We also visualised the relative abundance profiles of the ten most abundant classes of bacteria, fungi or eukaryotes for the different *L. corniculatus* genotypes or age groups, using the *microeco* package (68). To further understand the abundance patterns of the three different main groups of microbes, we also created graphs of the frequencies of different levels of commonness of OTUs, occurring in one to nine genotypes, or in one to five age groups.

To identify specific bacterial, fungal, or eukaryotic taxa that were associated with particular plant genotypes or plant age groups, we used the R packages *microbiomeMarker* and *phyloseq* (69,70). We used linear discriminant analysis effect size (LEfSe) (p-value < 0.05, LDA score ≥ 2) (71) to identify microbial genera that were significantly associated with particular *L. corniculatus* genotypes or age groups, either separately for each plant organ, or across organs.

Genotype-specificity experiment

While LEfSe analyses can suggest a role of host genotype for specific microbial taxa, formal proof of genotype-specificity is only possible with infection experiments. Although a comprehensive test of our LEfSe results was beyond our means, we exemplarily tested one LEfSe result for the fungal pathogen *Gibberella*/*Fusarium* that showed a strong genotype association. We isolated *Fusarium* strains from *L. corniculatus* shoots collected around Tübingen using standard surface sterilization and plating on *Fusarium*-selective medium (72). The detailed protocol can be found in the Supplementary Methods (Method S1). We used the isolated *Fusarium* strain to experimentally inoculate daughter plants of our genotype 1 - associated with high *Fusarium* abundances in LEfSe - versus plants of the (presumably) less susceptible genotype 8. To break seed dormancy, we submerged the seeds of the two genotypes in liquid nitrogen for five minutes and then germinated them on wet tissue paper at 21°C/18°C with a 16 h/8 h light/dark cycle in a growth chamber. After seven days we transplanted the germinated seeds into individual pots filled with standard potting soil. For each genotype, we used six plants for the infection treatment and six plants as controls. For spore production, we transferred five 1 cm² pieces of Potato Dextrose Agar media with the isolated *Fusarium* in 250 ml of liquid mung bean media (73) and incubated them for four days in the dark at 28°C at 150 RPM. For the plant infection, we filtered the suspension through autoclaved cheesecloth, counted the spores using a counting chamber and standardized the suspension to 10^6 spores/ml. To infect the plants, we dipped the entire shoots of the infection treatment plants in the spore suspension, whereas the shoots of the control plants were dipped in UPW. Two weeks after the infection we harvested all plants and quantified infection intensities through image analysis. For each plant, we disassembled all shoots and leaves and scanned them with a photo scanner (Epson Perfection V600) at high resolution (~ 35 MP). We used ImageJ (74) to mask the plant stems and restrict analyses to the leaf area, and used the OpenCV2 package (75) in Python v3.17 (76) to filter all green (HSV thresholds of 5,70,70 and 75,250,215) and yellow pixels (HSV thresholds of 0,70,71 and 30,234,234) in these images. We used the number of yellow and green pixels as a proxy for the plant health, since leaf wilt and chlorosis are the most common symptoms of a *Fusarium* infection (77). We tested the genotype-specificity of the *Fusarium* infection with a linear model that used the number of yellow pixels as a response variable and included infection treatment, plant genotype, and their interaction as categorical explanatory variables, and the number of green pixels as a covariate. To improve the model fit, we log-transformed the yellow to green pixel ratio prior to the analysis.

RESULTS

The ddRAD sequencing identified a total of 5739 SNPs across all plant individuals. The Maximum Likelihood tree with best score (LL score = -733814, not shown) and consensus tree (LL score = -733810; Fig. S1) agree in all major branches. Both trees are robust, with most bootstrap values above 50%. Based on the branch lengths of the ML tree, the TreeCluster algorithm grouped the plants into nine genotype groups, each represented by 14 to 26 samples (Fig. 1b, Fig. S1). While six of these groups were significantly overrepresented in some sites (Fig. 1c, $\chi2$ test, $P < 0.001$), all groups occurred across multiple sites (3-6 sites, average 4.4 sites), usually with multiple replicates per site, and the use of the genotype groups therefore allowed us to test for their microbiome associations at least partly independently from site variation. The estimated ages of the same plant individuals ranged from two to 13 years, with most plants four to six years old (Fig. 1d). We found no relationship between plant age and sampling year ($\chi2$ test, $P = 0.076$), but there was a statistical association between plant age and sampling site ($\chi2$ test, $P < 0.001$), with an overrepresentation of the younger than four-year-old plants in one of the sites. Thus, plant age was only to a small extent confounded with site, allowing us to test for its (largely) independent association with microbiome.

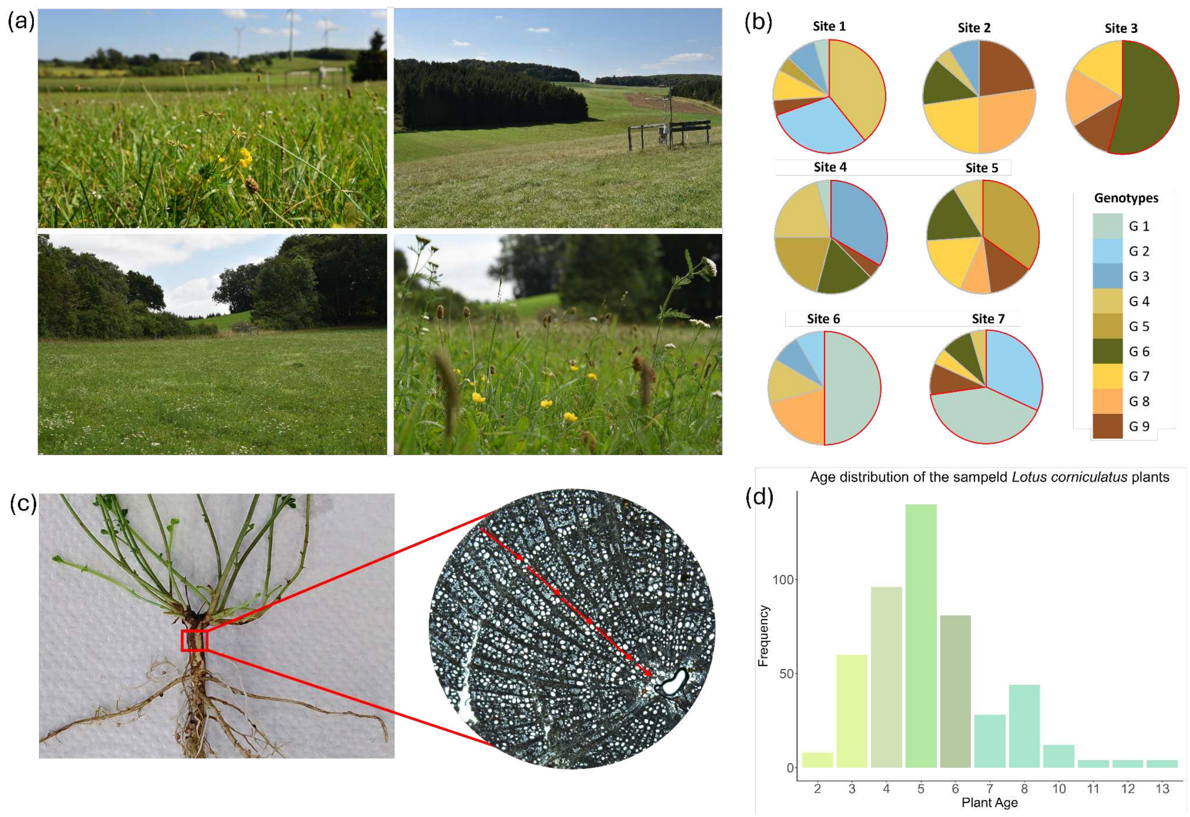

Figure 1. Sampling region, genotypic diversity, and determination of plant age**. a)** Photos of four of the sampling sites in southwest Germany. **b)** Frequencies of *L. corniculatus* genotypes in the seven studied populations. The red borders indicate cases where genotypes are significantly overrepresented in specific populations. **c)** The root crown of a *L. corniculatus* plant, and its cross-section, with the growth rings used to assess plant age. **d)** Age distribution of all sampled plants based on herb chronology. The colours indicate the five age groups we created for the statistical analysis.

Microbiome diversity differs between plant genotypes and ages

Across all sampling sites, years, plant individuals, and plant organs (672 samples), the metagenome sequencing identified a total of 4225 bacterial (16S rRNA), 2027 fungal (ITS2 rRNA), and 1773 eukaryotic (18S rRNA) OTUs, clustered at 97% sequence similarity, and classified into 113 phyla and 1542 genera. The most abundant bacterial classes we detected in the *L. corniculatus* plants were Gammaproteobacteria, Alphaproteobacteria, Betaproteobacteria, Actinobacteria, and Bacilli (Fig. 3a). The most abundant fungal classes are Dothideomycetes, Leotiomycetes, Eurotiomycetes, Sordariomycetes, and the most abundant eukaryotic classes are Insecta, Dothideomycetes, Chromadorea_X, and Agaricomycetes (Fig. 3b,c).

Part of the observed microbial diversity differed systematically between plant organs, genotypes, and age groups. Specifically, the variation in Shannon diversity (= within-sample) was highly significant between the different plant organs of all genotypes and age groups. In all cases, root diversity was substantially higher than that of other organs, followed by shoot and flower diversity. Notably, seed diversity was higher than that of flowers (Fig. 2a-f, Fig. S2).

Both bacterial and fungal communities differed significantly among plant genotypes, with some genotypes harboring consistently higher bacterial or fungal diversity than others, across plant organs (Fig. 2a,b). We also found a significant interaction between genotype and plant organ for bacterial diversity: genotype differences were much more pronounced in *L. corniculstus* flowers and seeds than in shoots and roots (Fig. 2a). A similar, albeit not statistically significant, pattern was visible for fungal diversity, where also the genotype differences were greatest in flowers (Fig. 2b). In contrast to the flower and seed microbiome, the microbial diversity of roots and shoots was much less variable between plant genotypes, and there were also no genotype differences in the diversity of eukaryotes (Fig. 2c). When comparing the microbiome diversity of different plant age groups, we found a significant age effect for fungal diversity, with younger plants harbouring a higher diversity of fungal microbes than older ones (Fig. 2e). However, there were no plant age effects on bacterial or eukaryote diversity (Fig. 2d,f).

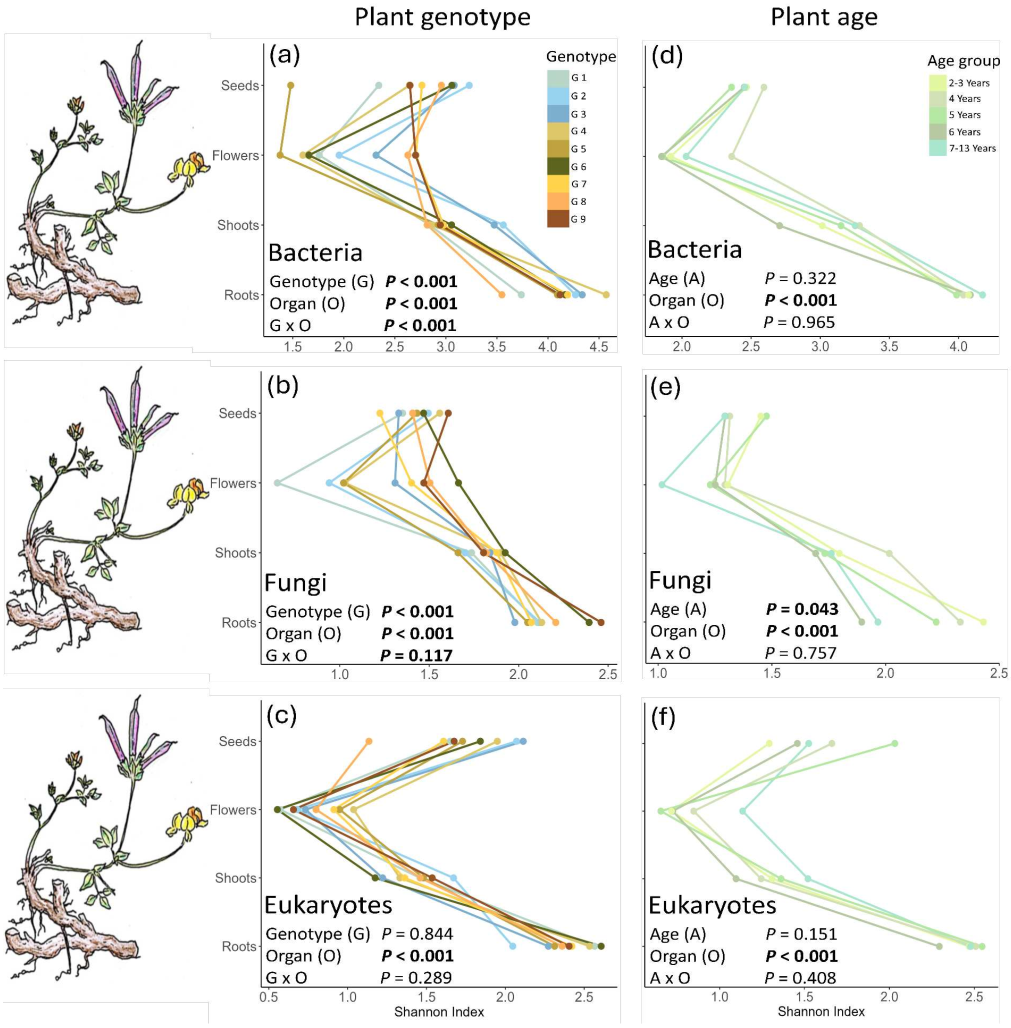

Figure 2: The average alpha diversity of bacterial, fungal and eukaryotic microbial communities associated with different genotypes (panels a - c) or different age groups (panels d - f) of *L. corniculatus* across seven semi-natural grasslands. Illustrations by S. Nicolai Rühl.

Plant genotype and age explain variation in microbiome composition

The age and genotypes of plants were not only associated with the diversity but also the composition of their microbiome. A large number of taxa associated with *L. corniculatus* were shared among all genotypes and age groups, but we also found taxa that were less widely distributed, or even occurred in only one genotype or age group. Interestingly, the patterns of microbial commonness and rarity strongly differed between bacteria on the one side, and fungi and eukaryotes on the other side. In bacteria the majority of OTUs (2572 out of 4225) occurred in all nine genotypes, while OTUs that occurred in only one or few genotypes were rare. In fungi and eukaryotes, such less widely distributed taxa were much more frequent (Fig. 3g). The results were similar when we examined OTU distribution across age groups: almost 80% of all bacterial OTUs occurred in all age groups, and those that occurred in only one or few age groups were rare, whereas the patterns were much more even in fungi and eukaryotes (Fig. 3h).

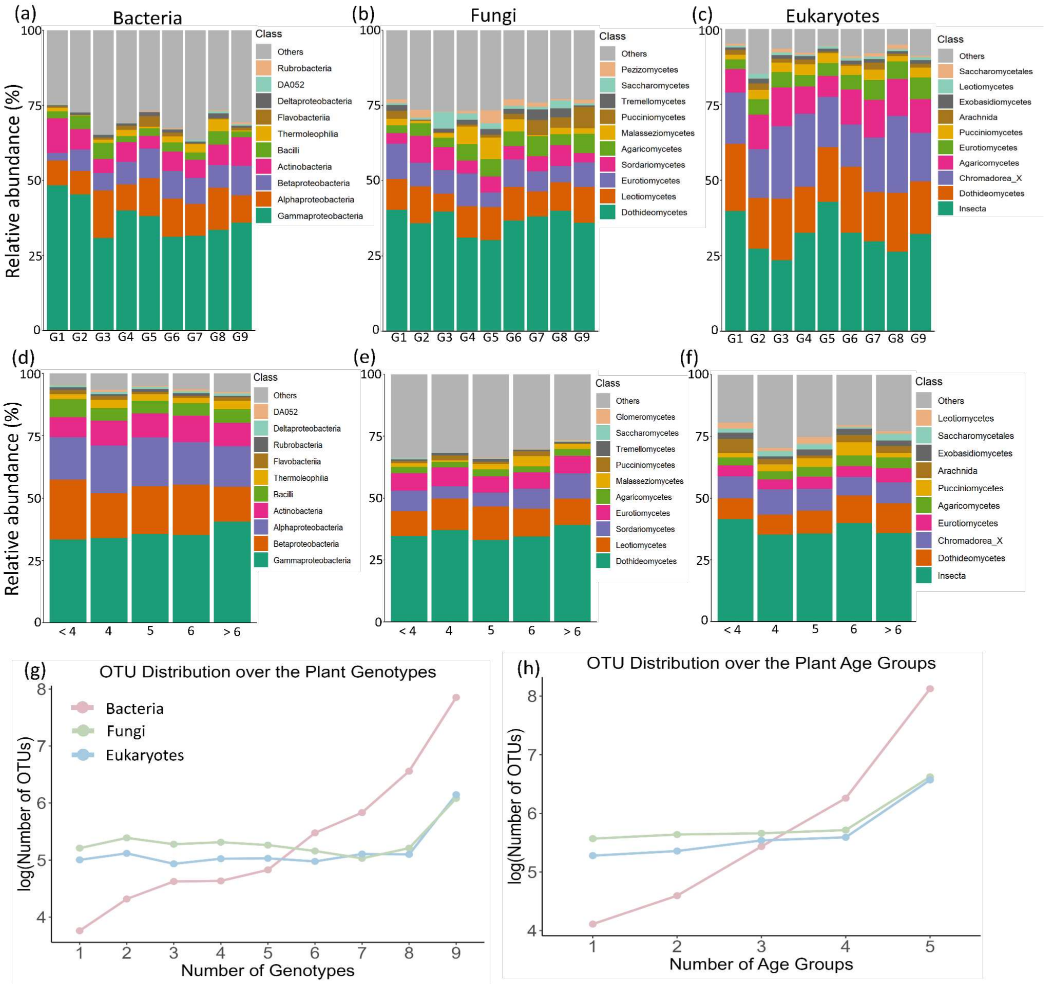

Figure 3: Relative abundance profiles of microbial communities associated with different genotypes (G1 - G9; panels a-c) or ages (panels e-g) of *L. corniculatus*, separately for bacteria, fungi and eukaryotes. We restricted the plots to the ten most abundant classes in each microbial group. Panels (d) and (h) summarize the frequencies of different levels of commonness across genotypes or ages in the three microbial groups.

The most consistent genotype effects were in roots and shoots, where the PERMANOVA identified significant plant genotype effects in the composition of all three microbial groups, with around 10% of the variation in community composition explained by plant genotype. In addition, there were significant genotype effects for bacterial communities in flowers and seeds, and for fungal communities in flowers (Tab. 1, Fig. S3). Interestingly, these genotype effects in flowers and seeds were even stronger, with over 15% variance explained.

We found no significant main effects of plant age on the composition of bacteria, fungal, and eukaryotic communities in any of the four plant organs (Tab. 1). However, there were significant plant age by genotype interactions for fungal communities in roots and shoots, and eukaryotic communities in roots, indicating a complex interplay between plant genotype and age in explaining microbial community composition in these cases.

Table 1: Results of PERMANOVA analyses testing the effects of plant genotype, plant age, or their interaction, on the Bray-Curtis dissimilarities of microbial communities associated with the roots, shoots, flowers, and seeds of *L. corniculatus* across seven semi-natural grasslands in SW Germany.

	Bacteria											
	Roots			Shoots			Flowers			Seeds		
	R^2	*F*	*Pr(>F)*	R^2	*F*	*Pr(>F)*	R^2	*F*	*Pr(>F)*	R^2	*F*	*Pr(>F)*
Plant genotype	0.102	1.53	**0.009**	0.103	1.57	**0.004**	0.174	2.67	**0.001**	0.160	2.59	**0.001**
Plant age	0.033	0.99	0.455	0.043	1.3	0.107	0.026	0.81	0.732	0.049	1.55	0.064
Genotpye x Age	0.234	1.04	0.309	0.231	1.04	0.277	0.180	0.82	0.956	0.204	0.98	0.549
Residual	0.632			0.623			0.620			0.588		
	Fungi											
	Roots			Shoots			Flowers			Seeds		
	R^2	*F*	*Pr(>F)*	R^2	*F*	*Pr(>F)*	R^2	*F*	*Pr(>F)*	R^2	*F*	*Pr(>F)*
Plant genotype	0.092	1.41	**0.005**	0.108	1.61	**0.001**	0.173	3.02	**0.001**	0.070	1.04	0.385
Plant age	0.029	0.90	0.71	0.034	1.06	0.351	0.042	1.48	0.103	0.025	0.74	0.844
Genotpye x Age	0.264	1.21	**0.012**	0.248	1.15	**0.036**	0.239	1.23	0.079	0.258	1.12	0.385
Residual	0.615			0.615			0.545			0.646		
	Eukaryotes											
	Roots			Shoots			Flowers			Seeds		
	R^2	*F*	*Pr(>F)*	R^2	*F*	*Pr(>F)*	R^2	*F*	*Pr(>F)*	R^2	*F*	*Pr(>F)*
Plant genotype	0.107	1.66	**0.001**	0.090	1.38	**0.008**	0.082	1.21	0.139	0.073	1.13	0.235
Plant age	0.035	1.07	0.264	0.037	1.13	0.257	0.029	0.85	0.641	0.046	1.41	0.076
Genotpye x Age	0.246	1.13	**0.021**	0.245	1.11	0.067	0.244	1.07	0.308	0.264	1.20	**0.043**
Residual	0.612			0.628			0.645			0.617		

Specific microbial taxa associated with plant genotypes and age groups

While a large number of taxa are shared among the plant genotypes and age groups, the LEfSe analyses revealed that some microbe taxa were significantly associated with particular individual plant genotypes or age groups (Fig. 4). Interestingly, these patterns of differential abundance were generally stronger in bacteria and fungi than in eukaryotic microbes, and they also tended to be more frequent with regard to plant genotypes (Fig. 4a) than for plant age groups (Fig. 4b), corroborating the PERMANOVA results with more frequent plant genotype than plant age effects (Tab. 1). Moreover, the LEfSe analyses also found that some plant genotypes and age groups showed much larger numbers of specific microbial taxa associations (e.g. genotypes 2, 3, 7 and 8, and age groups 2/3 and 5) than others. When analysed separately for each plant organ, the LEfSE results differed significantly between organs, both for plant genotypes and plant age groups (Tab. S4), indicating that the observed microbial taxa associations are to some degree organ-specific.

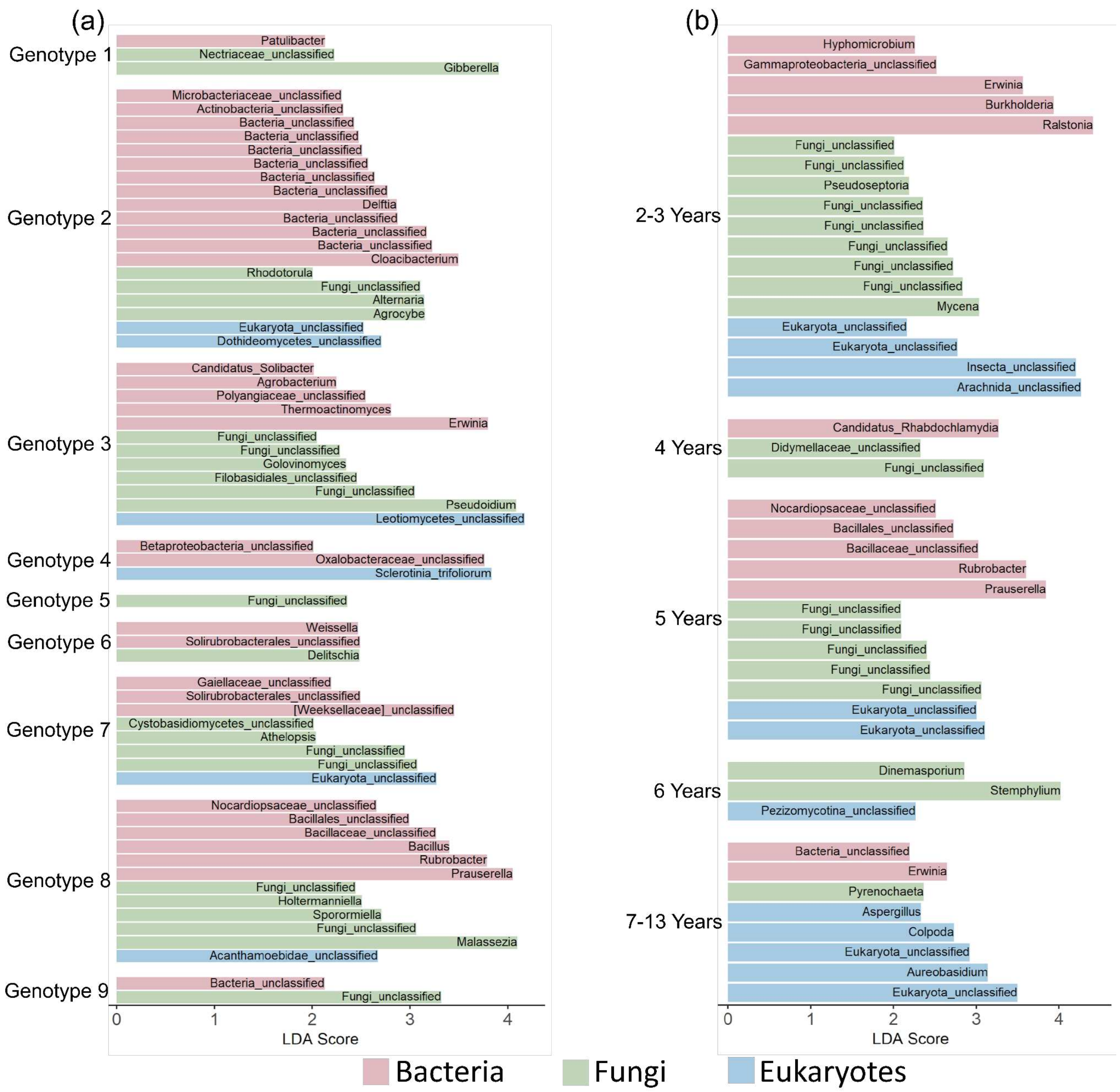

Figure 4: Microbial taxa significantly associated with specific genotypes (a) or age groups (b) of *L. corniculatus*, as identified through LefSe analyses. The three colours represent separate LefSe analyses conducted for bacteria, fungi, and eukaryotes, which were analysed independently. These groups are presented together to provide a clearer overview of microbial variation across genotypes and age groups; however, the LDA score of taxa from different groups cannot be directly compared with each other. The raw results can be found in the appendix (Fig. S4).

Our experimental test of one of the observed LEfSe results, the overabundance of *Gibberella/Fusarium* associated with *L. corniculatus* genotype 1, confirmed the results from the field. In both tested genotypes, the chlorotic area (Fig. 5b) increased after infection with

Fusarium (Fig. 5a) compared to the control treatment. However, the strength of this effect strongly varied between the genotypes. In the more susceptible genotype 1 the chlorotic area increased on average over 10-fold, whereas in genotype 8 the average chlorosis only doubled in infected plants compared to control plants (Fig. 5c). These differences in effect sizes were also confirmed by a significant genotype by treatment effect in the linear model (see Fig. 5 for details).

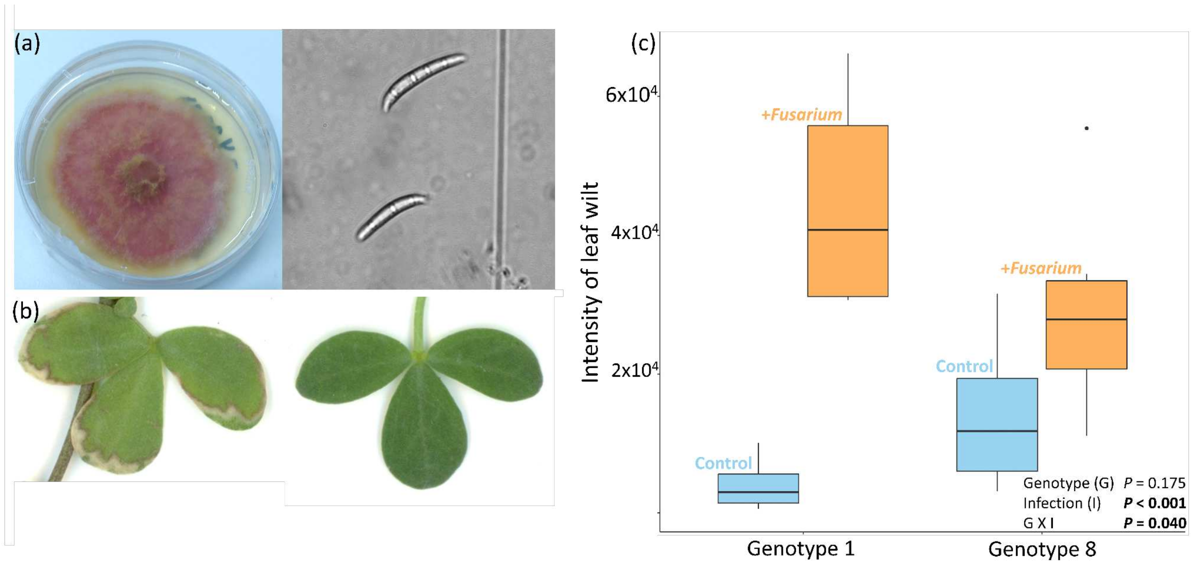

Figure 5: Experimental test of genotype-specificity of *Fusarium* infection of *L. corniculatus*. **a)** Isolated *Fusarium* in plate cultivation and microscopic image of *Fusarium* spores. **b)** Typical diseased and healthy leaves of *L. corniculatus*. **c)** Average intensities of leaf wilt of two *L. corniculatus* genotypes, with or without experimental *Fusarium* infection.

DISCUSSION

Plants harbour an astonishing diversity of microbes, but the factors shaping these complex communities throughout a plant's life—particularly in natural populations—remain poorly understood. Here, we show that in natural *L. corniculatus* populations both the age and genotype of plants is associated with changes in the diversity and composition of their associated microbiome. This is the case for the entire microbiome but also for individual microbial taxa that are significantly associated with specific genotypes or age groups.

Effect of plant age and genotype on microbiome diversity

We generally found large differences in the average microbiome diversity of different plant organs, a result that has already been described in detail elsewhere (55), and that likely reflects the different properties of plant organs as habitats for microbes, and their different modes of connectedness with the environment (78). Because of this large variation, we included the plant organs as factors in our analysis, or ran analyses separately for different organs.

The alpha diversity of bacterial and fungal microorganisms differed significantly among the studied *L. corniculatus* genotypes. Interestingly, this effect was particularly strong in the flowers where we observed the strongest among-genotype variation in bacteria and fungi, whereas the microbiome diversity of roots and shoots was much more stable across genotypes. A possible explanation for this could be that flower traits are generally thought to be under stronger genetic control than vegetative plant traits (79), and that these traits can influence the microbiome, particularly if they affect the abundance and diversity of flower visitors (80), which are known to influence the flower microbiome (81,82). For example, nectar secretion can vary among *L. corniculatus* genotypes (83), potentially influencing the rate of flower visitation. In the study area, the flower visitors of *L. corniculatus* include various bee species—bumblebees, honeybees, and wild bees—but also butterflies (primarily Lycaenidae) and sometimes flies and beetles (39). This broad range of pollinators may play a role in the observed flower microbiome variation between different plant genotypes. Moreover, the flower microbiome is closely linked to the seed microbiome, particularly for bacteria (84–87), which may explain why we also found considerably among-genotype variation for bacteria in seeds.

Among-genotype variation in microbiome was generally lower in roots and shoots than in flowers and seeds. It is well known that the root microbiome is strongly influenced, in part through affecting root traits, by different environmental factors such as soil type, structure, and pH, and by the surrounding soil microbiome (1,3,4,88,89). Together, this may ‘override’ the influence of host genetics and reduce its relative impact on root microbiomes and, as a consequence, also the neighboring shoot microbiomes.

Compared to the variation among genotypes, there was less variation in microbiome diversity between plants of different ages. Only for the Shannon diversity of fungi there were significant differences between age groups. This is interesting, as many fungi play important roles as plant symbionts or pathogens (90–92), and their interactions with plants are strongly influenced by the plant immune system. The immune response of plants changes throughout their development (31,93), and this could explain plant age-related variation in fungal microbiome diversity. Our result show that in principle plant age can also influence plant microbiome diversity, although to a lesser degree than plant genotype, and our evidence is limited to fungal microorganisms.

We should point out that comparisons of plant ages and genotypes in natural populations are not without challenges. In a field study, variation in plant age and genotype is inevitably confounded with variation in environment. We tried to account for this by aggregating both genotypes and ages into broader groups, which allowed ‘replication’ across populations. However, there was still statistical overrepresentation of some genotype and age groups in some populations. Thus, in spite of our efforts both factors have not been fully independent of the sampling population, and we therefore cannot rule out that some of the observed genotype or age effects are overestimates.

Effect of plant genotype and age on microbiome composition

We found that different *L. corniculatus* genotypes were also associated with variation in the composition of the microbiome, *i.e.* genetic variation in the host not only affected the alpha diversity but also the beta diversity of the microbes. Again, the extent of these effects strongly depended on the plant organ: in roots and shoots the composition of all three major microbial groups (bacteria, fungi, and eukaryotes) was affected by plant genotype, whereas in flowers only the composition of bacteria and fungi, and in the seeds only that of bacteria, showed significant plant genotype associations. A plethora of genetically variable plant traits are known to affect the plant microbiome and could thus be underlying these observed composition changes. For example, root exudates are an important determinant of the rhizosphere microbiome (94,95), and can vary across plant genotypes (96,97). Intraspecific variation in leaf morphology and leaf chemistry has been shown to influence microbial communities in the phyllosphere (98,99), and variation in plant defense genes is strongly affecting plant colonisation by specific pathogens (100,101).

In contrast to plant genotype, we found little evidence for effects of plant age on the composition of microbial communities. Only in fungi and eukaryotes, and mainly in roots and shoots, we found a significant plant age by genotype interaction, *i.e.* age effects were inconsistent and differed among genotypes, or were restricted to particular genotypes. This was counter to our expectation of age-related changes in species composition because of gene expression and functional trait changes during plant development (93). One possible reason for our findings could be that most species composition changes happen at early plant ages, and that the main factors shaping the microbiome such as root exudates (102) stabilize after this initial phase, so that the microbial communities reach an equilibrium fairly early. Unfortunately our youngest sampled plants were two years old, so we could not compare with one-year-old plants, to understand the early-stage dynamics better. Clearly, a better understanding of these questions, and of the observed plant age by genotype interactions, requires controlled experiments with longer-term observation of the colonisation dynamics of different plant genotypes under standardized environmental conditions.

When looking at the overall frequency patterns of microbes, we found that although many taxa were shared across all plant genotypes and ages, there were still significant numbers of taxa that occurred only in a subset of plant genotypes or age groups, and there was an intriguing difference between bacteria on the one hand, and fungi and eukaryotes on the other hand. While in bacteria the level of generalism was particularly high, *i.e.* a large fraction of taxa occurred everywhere, frequency distributions of taxa were much more even in fungi and eukaryotes, showing a greater dispersal rates of bacteria than of non-bacteria microbes. Bacteria generally possess dispersal mechanisms unavailable to other microorganisms, allowing them to spread more efficiently. For instance, they can rapidly disperse through fungal hyphae in soil (103) and, in general, exhibit much higher dispersal rates than fungal microbes (104). In contrast, fungal dispersal is more strongly constrained by potential dispersal limitation (105), which may further contribute to the broader distribution of bacterial taxa.

Associations of microbial taxa with specific plant genotypes and age groups

The differential abundance analysis identified a number of significant associations of microbial taxa with specific plant genotypes. Interestingly, these associations were not randomly distributed, but some plant genotypes harboured many more specific microbial taxa than others. Plant genotypes can differ in their production of secondary metabolites, which influence the recruitment or inhibition of specific microbes (106). Additionally, modifications in root morphology can limit microbial attachment and interaction, reducing the presence of specialized microbial associations in the root (107). Changes in these chemical and morphological traits can require greater levels of specialization of associated microbes.

If we look at the genotype associations in more detail, we find that several mutualistic bacteria, such as *Bacillus* (108–110), primarily soil-associated bacteria like *Cloacibacterium*, *Prauserella,* and *Rubrobacter*, and some growth-promoting fungi like *Agrocybe* (111,112) were overrepresented in some plant genotypes. Similar host genotype effects on *Bacillus* have been observed in other studies, *e.g.* in maize cultivars resistant to corn stalk rot versus non-resistant cultivars (113), or in sweet pepper cultivars (109). While plant interactions with the soil-associated bacteria are not yet fully understood, previous studies have shown that the plant genotype can influence the abundance of specific soil microbes in the rhizosphere (114), and that plants actively recruit genotype-specific beneficial soil microbes (115). We also found several pathogens with differential abundance among plant genotypes, *e.g. Erwinia* bacteria responsible for soft rot (116) and the fungal pathogens *Sclerotinia*, *Alternaria*, *Pseudoidium*, *Golovinomyces,* and *Gibberella*. Our results corroborate previous studies with different plant species that also found genetic variation in plant resistance to these pathogens (117–120). In general, plant genetic effects on pathogen resistance are well-known phenomena, and a key topic in agriculture and crop management (121). They also play an important role in natural populations, and are cornerstones of the geographic mosaic of coevolution observed in many wild species (122–124).

We also found that some microbial taxa were significantly overrepresented in particular plant age groups, *e.g.* bacteria from the genera *Burkholderia, Erwinia,* and *Ralstonia,* and fungi from the genus *Mycena* in the youngest plant group. *Burkholderia* bacteria are known for their growth-promoting effects (115) and have been shown to benefit maize plants during germination and seedling development (125). Although the studied *L. corniculatus* plants were already beyond these initial stages of development, the observed higher abundances of *Burkholderia* in young plants suggest that these bacteria also play a role in early *L. corniculatus* growth and development. *Mycena* is a saprotrophic fungus opportunistically invading plant roots (126), and *Erwinia* and *Ralstonia* are bacterial pathogens causing wilt in various plant species. The increased abundances of these taxa in young *L. corniculatus* plants suggests these may have less-developed immune system (127,128) or other differences that make them more susceptible to invasion, *e.g.* root physiology (129). There were also microbial taxa that were particularly abundant in older plants, *e.g.* the pathogenic fungus *Pyrenochaeta* which is known to cause root lesions (130), and which we found overrepresented in plants older than six years. Our results confirm a previous study with oilseed rape that also observed a higher abundance of *Pyrenochaeta* in later plant developmental stages (131). In general, our data show that while plant age does not have strong effects on the overall composition of microbes, there are some significant influences at the level of individual microbial taxa, even in a long-lived plant such as *L. corniculatus*.

It is important to keep in mind that all of the results discussed above are statistical associations between microbial taxa and plant genotypes/age groups in the field, but no formal proof of causal relationships. The latter require manipulative experiments, and to obtain such formal proof, at least for one example of an observed significant genotype-microbe association, we conducted a small lab experiment testing the susceptibility of two of the sampled *L. corniculatus* genotypes to the fungal pathogen *Fusarium* (teleomorph *Gibberella*) under controlled conditions. As expected, the genotype with overabundance of *Fusarium* in the LEfSe analysis developed significantly greater leaf wilt when infected with *Fusarium* in the lab than another plant genotype that had not shown the same abundance of *Fusarium* in the field. This strongly indicates that the observed plant genotype association of *Fusarium* is indeed true and reflects genetically-based plant variation.

CONCLUSION

Our findings show that host genotype is an important driver of natural microbiome diversity of *L. corniculatus*. This influence is evident not only in overall microbial diversity and composition but also in the abundance of specific microbial genera across different genotypes. We also found evidence of plant age influencing the fungal diversity. Additionally, the abundance of specific microbial genera varies between the different host age groups. With varied genetics or age, the physiology and requirements of the plants change, and due to the close interaction between the microbiome and the host, this can result in an altered optimal microbiome composition. Therefore, it is valuable to combine insights from controlled laboratory experiments with data from natural environments to gain a more comprehensive understanding of the impact of genotype and age on the microbiome. Overall, our results show that genotype, and to a lesser extent age, play important roles in shaping the microbiome diversity and composition of natural *L. corniculatus* populations. This knowledge can also help optimize plant health, improve crop yields, and enhance ecosystem resilience which is crucial for effective ecosystem management and plant breeding practices.

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AUTHOR CONTRIBUTIONS: O.B., E.K., F.R., and K.L. designed the study; F.R. and K.L. planned and conducted the field sampling, lab work and data analyses; W.D. helped with the library preparation and bioinformatics of the ddRAD sequencing; F.R. performed the *Fusarium* experiment and analyzed the data with the support of J.H.J.; F.R., K.L., and O.B. wrote the manuscript with input from all authors. All authors read and approved the final manuscript.

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DATA AVAILABILITY: Primers and blocking oligos used in this study are listed on Table S3. Sequencing data, metadata, OTU tables, scripts, and vcf files are available on https://gitlab.nfdi4plants.de/kalutap/ecology-of-the-core-microbiome-in-natural-lotus-corniculatus-populations and on Biodiversity Exploratories Information System (BExIS) (https://www.bexis.uni-jena.de/) under Dataset ID 31836.

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APPENDIX

SUPPLEMENTARY METHODS

Supplementary Methods (Method S1): *Fusarium* isolation protocol

We surface-sterilized shoot tissue from plants in different health conditions by washing it for 30 seconds with 2% sodium hypochlorite and rinsing it three times with UPW. The washed shoots were then homogenized in UPW and plated on *Fusarium*-selective medium (Jung *et al.*, 2013) slightly modified to 20 g/L dextrose, 15 g/L agar, 4 g/L potato extract, 50 mg/L kanamycin, 20 mg/L toxoflavin per 10 µl of the solution. After incubation at 30°C for 18h, we transplanted the hyphal tips of all growing fungi to fresh plates, extracted the DNA of these pure cultivations using the FastDNA^{TM} Spin Kit for Soil (MP Biomedicals), and Sanger sequenced it at the ITS2 marker for identification.

SUPPLEMENTARY FIGURES

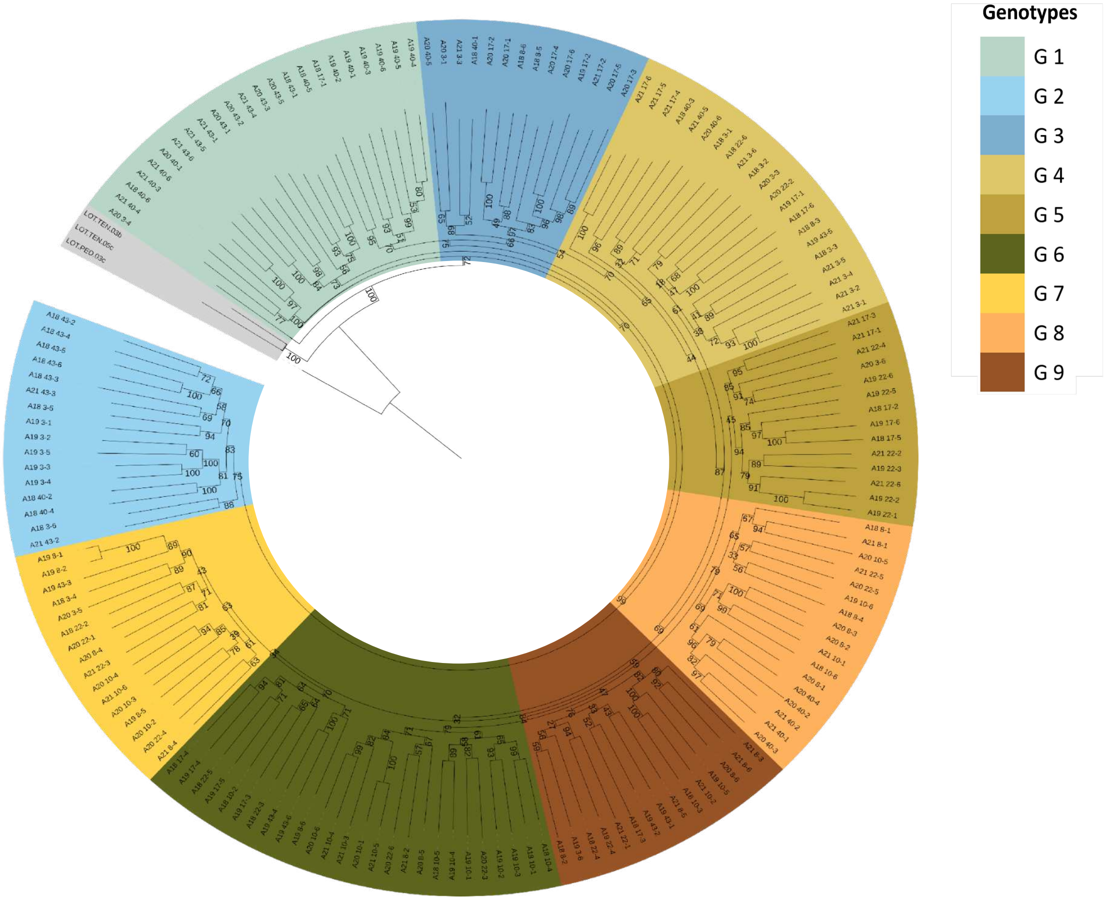

Figure S1: Bootstrap consensus tree derived from 1,000 replicates using IQ-TREE. Branches are supported if present in ≥50% of bootstrap replicates. Coloured by the different genotypes identified using IQ-tree.

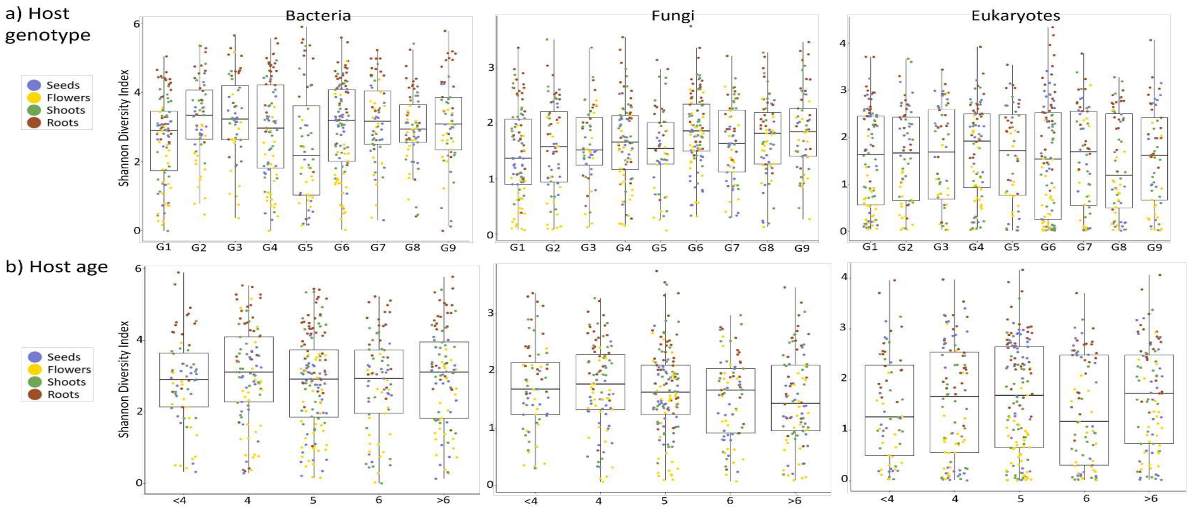

Figure S2: a) Box plots of the alpha diversity of bacterial, fungal and eukaryotic microbial communities associated the nine host genotypes coloured by plant organs. **b)** Box plots of the alpha diversity of bacterial, fungal and eukaryotic microbial communities associated the five host age groups coloured by plant organs.

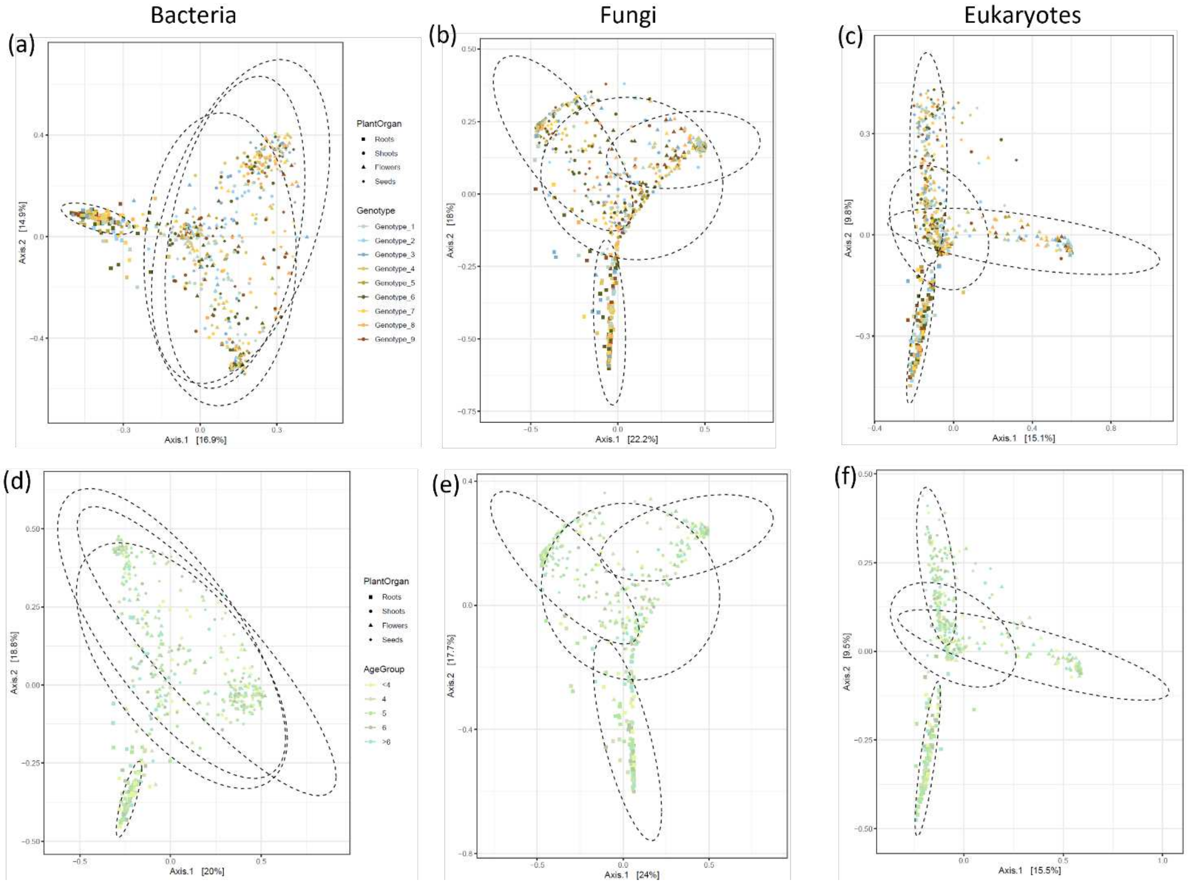

Figure S3: PCoA based on Bray-Curtis dissimilarities of the bacterial **(a,d),** fungal **(b,e)** and eukaryotic **(e,f)** communities associated the different plant genotypes and age groups.

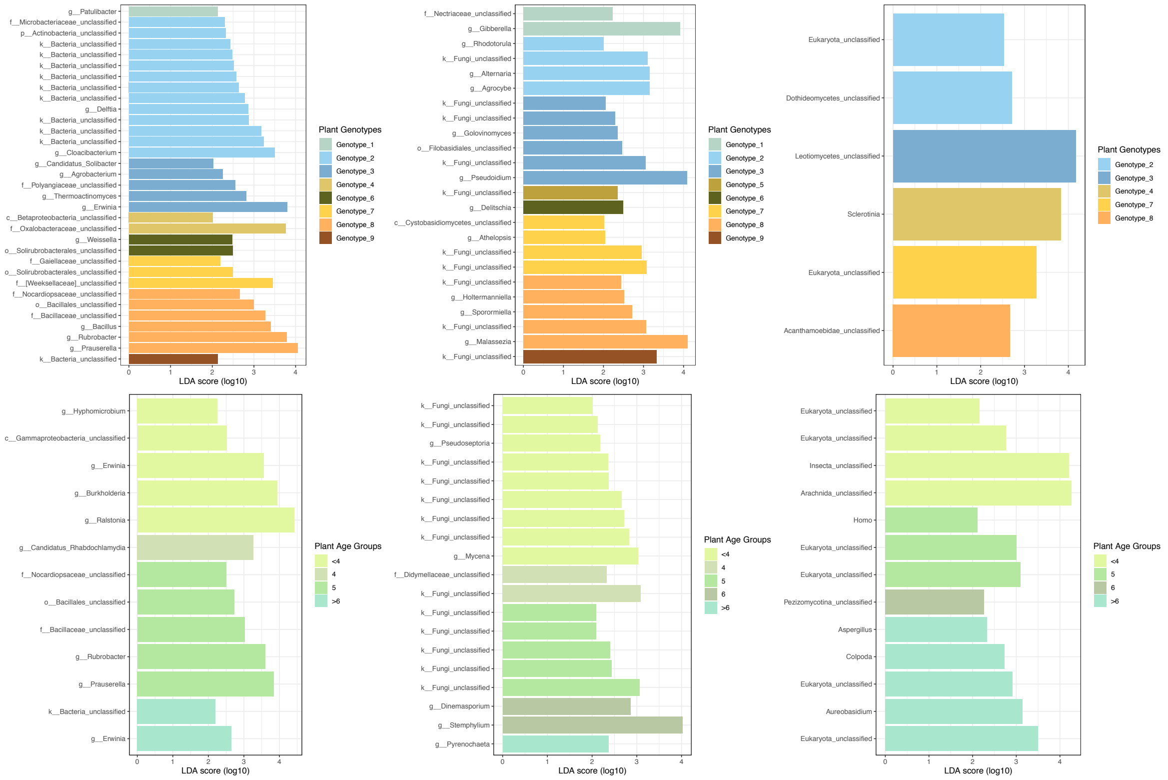

Figure S4: LefSe to identify differentially abundant OTUs in different genotypes and age groups of *L. corniculatus* plants.

SUPPLEMENTARY TABLES

Table S1: Coordinates of the seven sampling locations.

Site	Latitude	Longitude
1	48.41	9.53
2	48.42	9.49
3	48.38	9.21
4	48.40	9.52
5	48.40	9.51
6	48.41	9.57
7	48.41	9.54

Table S2: Age determination results including quality scores.

Sampled 2019			Sampled 2020			Sampled 2021		
Plant-ID	Age	Quality score	Plant-ID	Age	Quality score	Plant-ID	Age	Quality score
Lot19_3-1	4	2	Lot20_3-1	4	1	Lot21_3-1	5	2
Lot19_3-2	5	3	Lot20_3-2	5	2	Lot21_3-2	3	2
Lot19_3-3	5	2	Lot20_3-3	5	2	Lot21_3-3	4	1
Lot19_3-4	7	3	Lot20_3-4	4	3	Lot21_3-4	3	1
Lot19_3-5	11	2	Lot20_3-5	4	1	Lot21_3-5	4	1
Lot19_3-6	4	2	Lot20_3-6	5	2	Lot21_3-6	8	2
Lot19_8-1	4	2	Lot20_8-1	3	3	Lot21_8-1	4	3
Lot19_8-2	8	1	Lot20_8-2	2	2	Lot21_8-2	3	3
Lot19_8-3	N/A	4	Lot20_8-3	2	2	Lot21_8-3	4	3
Lot19_8-4	4	1	Lot20_8-4	4	3	Lot21_8-4	3	3
Lot19_8-5	N/A	4	Lot20_8-5	6	2	Lot21_8-5	3	2
Lot19_8-6	6	2	Lot20_8-6	3	1	Lot21_8-6	4	3
Lot19_10-1	5	1	Lot20_10-1	5	3	Lot21_10-1	8	2
Lot19_10-2	4	1	Lot20_10-2	5	2	Lot21_10-2	3	2
Lot19_10-3	N/A	4	Lot20_10-3	6	3	Lot21_10-3	5	1
Lot19_10-4	6	3	Lot20_10-4	7	2	Lot21_10-4	5	3
Lot19_10-5	8	2	Lot20_10-5	5	3	Lot21_10-5	5	1
Lot19_10-6	6	2	Lot20_10-6	4	2	Lot21_10-6	7	2
Lot19_17-1	3	1	Lot20_17-1	5	1	Lot21_17-1	3	3
Lot19_17-2	4	3	Lot20_17-2	4	2	Lot21_17-2	5	2
Lot19_17-3	3	1	Lot20_17-3	4	3	Lot21_17-3	4	1
Lot19_17-4	N/A	4	Lot20_17-4	5	3	Lot21_17-4	6	1
Lot19_17-5	8	3	Lot20_17-5	5	2	Lot21_17-5	6	1
Lot19_17-6	6	2	Lot20_17-6	5	1	Lot21_17-6	5	2
Lot19_22-1	5	1	Lot20_22-1	5	1	Lot21_22-1	5	2
Lot19_22-2	5	2	Lot20_22-2	5	2	Lot21_22-2	5	2
Lot19_22-3	5	3	Lot20_22-3	6	2	Lot21_22-3	6	3
Lot19_22-4	8	3	Lot20_22-4	3	2	Lot21_22-4	4	2
Lot19_22-5	5	2	Lot20_22-5	6	1	Lot21_22-5	4	1
Lot19_22-6	6	2	Lot20_22-6	5	2	Lot21_22-6	4	3
Lot19_40-1	6	3	Lot20_40-1	3	1	Lot21_40-1	6	1
Lot19_40-2	10	3	Lot20_40-2	7	3	Lot21_40-2	8	2
Lot19_40-3	12	3	Lot20_40-3	5	1	Lot21_40-3	N/A	4
Lot19_40-4	10	3	Lot20_40-4	6	3	Lot21_40-4	7	2
Lot19_40-5	8	2	Lot20_40-5	7	2	Lot21_40-5	N/A	4
Lot19_40-6	6	2	Lot20_40-6	6	2	Lot21_40-6	3	2
Lot19_43-1	8	3	Lot20_43-1	6	3	Lot21_43-1	8	1
Lot19_43-2	8	2	Lot20_43-2	6	1	Lot21_43-2	3	1
Lot19_43-3	6	2	Lot20_43-3	5	3	Lot21_43-3	5	1
Lot19_43-4	4	2	Lot20_43-4	5	3	Lot21_43-4	10	2

Lot19_43-5	13	2	Lot20_43-5	7	2	Lot21_43-5	5	2
Lot19_43-6	5	2	Lot20_43-6	4	2	Lot21_43-6	5	2

Table S3: Primers and blocking oligos used for microbiome sequencing.

Primer name	Primer sequence (5’-to-3’ orientation)
799F	AACMGGATTAGATACCCKG
1192R	ACGTCATCCCCACCTTCC
fITS7	GTGARTCATCGAATCTTTG
ITS4	TCCTCCGCTTATTGATATGC
F1422	ATAACAGGTCTGTGATGCCC
R1797	TGATCCTTCTGCAGGTTCACCTAC
clamp1_BV5_mitoF	GATGAGTGTTCGCCCTTGGTCTACGTGGAT
clamp1_BV5_mitoR	CTGCTCAGGGTTCCAAACTCAACGTTGGCA
clamp1_ITS2_F	AACCATTAGGTCGAGGGCACGTCTGCCTGG
clamp1_ITS2_R	TGAGMGYGGTTACACCACGCATGCGGGTCT
clamp9_PV9_F	GATGTATTCAACGAGTCTATAGCCTTGGCC
clamp15_PV9_R	TCTCACAACGTCGCAGGCAGCGAACCGCCC

Table S4: Lefse to identify differentially abundant OTUs in different genotypes of *L. corniculatus* plants by plant organs.

Genotype							
OTU	**enrich_genotype**	**organ**	**ef_lda**	**pvalue**	**padj**	**Locus**	**Genus**
Otu000030	Genotype_4	roots	3.589	<0.001	<0.001	16S	o__0319-7L14_unclassified
Otu000074	Genotype_8	roots	3.739	0.003	0.003	16S	f__Sinobacteraceae_unclassified
Otu000075	Genotype_3	roots	3.346	<0.001	<0.001	16S	f__Solirubrobacteraceae_unclassified
Otu000098	Genotype_3	roots	3.184	<0.001	<0.001	16S	g__Mycobacterium
Otu000103	Genotype_4	roots	3.052	0.004	0.004	16S	g__Kribbella
Otu000107	Genotype_1	roots	3.638	0.037	0.037	16S	f__Sphingomonadaceae_unclassified
Otu000110	Genotype_7	roots	3.423	0.000	0.000	16S	o__Solirubrobacterales_unclassified
Otu000129	Genotype_4	roots	2.890	<0.001	<0.001	16S	f__Gaiellaceae_unclassified
Otu000133	Genotype_1	roots	3.399	0.001	0.001	16S	g__Agrobacterium
Otu000162	Genotype_5	roots	2.747	0.001	0.001	16S	f__[Entotheonellaceae]_unclassified
Otu000167	Genotype_6	roots	2.895	0.016	0.016	16S	o__Solirubrobacterales_unclassified
Otu000193	Genotype_5	roots	2.737	0.000	0.000	16S	f__EB1017_unclassified
Otu000215	Genotype_5	roots	2.596	0.002	0.002	16S	f__Gaiellaceae_unclassified
Otu000244	Genotype_5	roots	2.659	<0.001	<0.001	16S	g__Amaricoccus
Otu000254	Genotype_2	roots	2.981	<0.001	<0.001	16S	c_Gammaproteobacteria_unclassified
Otu000260	Genotype_4	roots	2.683	<0.001	<0.001	16S	o__Solirubrobacterales_unclassified
Otu000263	Genotype_4	roots	2.507	<0.001	<0.001	16S	f__C111_unclassified
Otu000273	Genotype_5	roots	2.203	0.016	0.016	16S	c__PAUC37f_unclassified
Otu000282	Genotype_6	roots	2.485	0.001	0.001	16S	f__Micromonosporaceae_unclassified
Otu000283	Genotype_2	roots	2.648	0.008	0.008	16S	f__Gaiellaceae_unclassified
Otu000289	Genotype_1	roots	2.539	0.001	0.001	16S	g__Marmoricola
Otu000338	Genotype_9	roots	2.966	0.006	0.006	16S	f__Haliangiaceae_unclassified
Otu000351	Genotype_4	roots	2.310	<0.001	<0.001	16S	o__Acidimicrobiales_unclassified

Otu000364	Genotype_3	roots	2.408	<0.001	<0.001	16S	f__AKIW874_unclassified
Otu000368	Genotype_4	roots	2.385	<0.001	<0.001	16S	o__Acidimicrobiales_unclassified
Otu000370	Genotype_3	roots	2.479	0.001	0.001	16S	c__S085_unclassified
Otu000373	Genotype_2	roots	2.852	<0.001	<0.001	16S	p__Actinobacteria_unclassified
Otu000386	Genotype_5	roots	2.358	<0.001	<0.001	16S	f__EB1017_unclassified
Otu000423	Genotype_5	roots	2.425	<0.001	<0.001	16S	g__Balneimonas
Otu000425	Genotype_3	roots	2.514	0.000	0.000	16S	g__Thermoactinomyces
Otu000436	Genotype_3	roots	2.303	0.002	0.002	16S	g__Coprococcus
Otu000467	Genotype_3	roots	2.656	0.002	0.002	16S	f__Comamonadaceae_unclassified
Otu000474	Genotype_9	roots	3.100	<0.001	<0.001	16S	g__Sphingomonas
Otu000516	Genotype_4	roots	2.096	<0.001	<0.001	16S	g__Candidatus_Solibacter
Otu000536	Genotype_8	roots	2.365	0.035	0.035	16S	f__Dolo_23_unclassified
Otu000545	Genotype_8	roots	2.650	<0.001	<0.001	16S	g__Actinoallomurus
Otu000567	Genotype_2	roots	2.038	<0.001	<0.001	16S	f__EB1017_unclassified
Otu000573	Genotype_5	roots	2.085	<0.001	<0.001	16S	o__Acidimicrobiales_unclassified
Otu000592	Genotype_9	roots	2.088	0.016	0.016	16S	f__Conexibacteraceae_unclassified
Otu000644	Genotype_5	roots	2.362	<0.001	<0.001	16S	f__Cystobacterineae_unclassified
Otu000656	Genotype_5	roots	2.133	<0.001	<0.001	16S	o__Micrococcales_unclassified
Otu000669	Genotype_8	roots	2.470	0.006	0.006	16S	f__Caulobacteraceae_unclassified
Otu000690	Genotype_7	roots	2.575	0.002	0.002	16S	c_Gammaproteobacteria_unclassified
Otu000734	Genotype_5	roots	2.029	0.014	0.014	16S	o__NB1-j_unclassified
Otu000764	Genotype_2	roots	2.011	0.004	0.004	16S	f__Haliangiaceae_unclassified
Otu000812	Genotype_2	roots	2.423	<0.001	<0.001	16S	f__Polyangiaceae_unclassified
Otu000974	Genotype_2	roots	2.077	0.026	0.026	16S	f__Beijerinckiaceae_unclassified
Otu001053	Genotype_8	roots	2.559	0.002	0.002	16S	c__SJA-4_unclassified
Otu001075	Genotype_2	roots	2.479	<0.001	<0.001	16S	g__Rahnella
Otu001147	Genotype_3	roots	2.025	<0.001	<0.001	16S	o__Bacillales_unclassified
Otu001210	Genotype_5	roots	2.091	0.024	0.024	16S	g__Candidatus_Protochlamydia
Otu00004	Genotype_5	roots	5.014	0.027	0.027	ITS2	g__Exophiala
Otu00035	Genotype_7	roots	2.227	0.007	0.007	ITS2	g__Pseudoidium
Otu00038	Genotype_1	roots	4.110	0.017	0.017	ITS2	f__Bionectriaceae_unclassified
Otu00068	Genotype_2	roots	3.920	<0.001	<0.001	ITS2	g__Agrocybe
Otu00139	Genotype_3	roots	3.419	0.007	0.007	ITS2	g__Leohumicola
Otu00300	Genotype_1	roots	3.000	0.013	0.013	ITS2	g__Delitschia
Otu00774	Genotype_1	roots	2.588	<0.001	<0.001	ITS2	g__Dendryphion
Otu01037	Genotype_5	roots	2.037	0.002	0.002	ITS2	g__Gremmenia
Otu01062	Genotype_9	roots	2.006	0.002	0.002	ITS2	g__Microdochium
Otu00023	Genotype_6	roots	2.829	0.006	0.006	18S	Penicillium
Otu00024	Genotype_1	roots	4.438	0.004	0.004	18S	Chromadorea_X_unclassified
Otu00129	Genotype_4	roots	2.948	0.004	0.004	18S	Gregarinidae_unclassified
Otu00154	Genotype_3	roots	2.636	0.012	0.012	18S	Chytridiomycotina_unclassified
Otu00196	Genotype_2	roots	3.218	<0.001	<0.001	18S	Dothideomycetes_unclassified
Otu00208	Genotype_8	roots	3.401	0.022	0.022	18S	Acanthamoebidae_unclassified
Otu00306	Genotype_6	roots	2.922	0.002	0.002	18S	Assulina
Otu00314	Genotype_2	roots	2.355	0.015	0.015	18S	Trichosporon
Otu00505	Genotype_6	roots	2.313	0.037	0.037	18S	Ramicandelaber

Otu00510	Genotype_9	roots	2.552	0.005	0.005	18S	Eukaryota_unclassified
Otu00770	Genotype_3	roots	3.275	0.020	0.020	18S	Eukaryota_unclassified
Otu000009	Genotype_3	shoots	2.987	0.010	0.010	16S	g__Phyllobacterium
Otu000017	Genotype_2	shoots	2.617	<0.001	<0.001	16S	g__Mesorhizobium
Otu000034	Genotype_3	shoots	4.025	0.001	0.001	16S	g__Prauserella
Otu000062	Genotype_2	shoots	3.000	0.030	0.030	16S	g__Flavobacterium
Otu000070	Genotype_3	shoots	3.739	0.002	0.002	16S	g__Rubrobacter
Otu000073	Genotype_3	shoots	3.857	<0.001	<0.001	16S	g__Erwinia
Otu000118	Genotype_8	shoots	3.148	0.011	0.011	16S	f__[Weeksellaceae]_unclassified
Otu000151	Genotype_3	shoots	3.351	0.001	0.001	16S	g__Bacillus
Otu000466	Genotype_2	shoots	2.792	0.002	0.002	16S	g__Massilia
Otu000938	Genotype_5	shoots	2.025	0.001	0.001	16S	f__Sporichthyaceae_unclassified
Otu001023	Genotype_2	shoots	2.012	0.013	0.013	16S	f__Xanthomonadaceae_unclassified
Otu001029	Genotype_1	shoots	2.250	0.000	0.000	16S	g__Microbacterium
Otu001174	Genotype_9	shoots	2.080	0.012	0.012	16S	f__Planococcaceae_unclassified
Otu001270	Genotype_1	shoots	2.387	<0.001	<0.001	16S	f__Nocardiaceae_unclassified
Otu001782	Genotype_2	shoots	2.658	0.000	0.000	16S	g__Buchnera
Otu001914	Genotype_2	shoots	2.352	<0.001	<0.001	16S	f__Oxalobacteraceae_unclassified
Otu002076	Genotype_2	shoots	2.022	<0.001	<0.001	16S	f__Actinosynnemataceae_unclassified
Otu003094	Genotype_6	shoots	2.137	<0.001	<0.001	16S	g__Pseudomonas
Otu00004	Genotype_2	shoots	3.176	0.003	0.003	ITS2	g__Exophiala
Otu00026	Genotype_3	shoots	2.411	<0.001	<0.001	ITS2	g__Mycena
Otu00103	Genotype_2	shoots	2.376	0.000	0.000	ITS2	g__Athelopsis
Otu00122	Genotype_2	shoots	3.164	<0.001	<0.001	ITS2	g__Plectosphaerella
Otu00312	Genotype_7	shoots	3.324	0.006	0.006	ITS2	g__Dissoconium
Otu00381	Genotype_9	shoots	2.886	<0.001	<0.001	ITS2	g__Cystofilobasidium
Otu00386	Genotype_2	shoots	2.021	0.005	0.005	ITS2	f__Lentitheciaceae_unclassified
Otu00562	Genotype_1	shoots	2.729	<0.001	<0.001	ITS2	o__Xylariales_unclassified
Otu01017	Genotype_2	shoots	2.427	0.001	0.001	ITS2	g__Desmococcus
Otu01075	Genotype_7	shoots	2.665	0.003	0.003	ITS2	g__Golovinomyces
Otu01359	Genotype_5	shoots	2.390	0.000	0.000	ITS2	o__Pleosporales_unclassified
Otu01433	Genotype_2	shoots	2.005	0.002	0.002	ITS2	f__Stachybotryaceae_unclassified
Otu01689	Genotype_2	shoots	2.096	0.000	0.000	ITS2	g__unclassified_Verrucariaceae
Otu01708	Genotype_5	shoots	2.227	0.000	0.000	ITS2	g__Puccinia
Otu01710	Genotype_2	shoots	2.235	0.000	0.000	ITS2	o__Hypocreales_unclassified
Otu01762	Genotype_3	shoots	2.072	<0.001	<0.001	ITS2	g__Thanatephorus
Otu02224	Genotype_2	shoots	2.039	<0.001	<0.001	ITS2	g__Hypoxylon
Otu00043	Genotype_2	shoots	3.314	0.032	0.032	18S	Plectus
Otu00308	Genotype_9	shoots	3.054	<0.001	<0.001	18S	Microdochium
Otu02170	Genotype_2	shoots	2.556	0.000	0.000	18S	Klebsormidium
Otu000016	Genotype_8	flowers	4.265	<0.001	<0.001	16S	g__Burkholderia
Otu000024	Genotype_3	flowers	2.793	0.043	0.043	16S	f__Xanthomonadaceae_unclassified
Otu000025	Genotype_1	flowers	3.196	0.035	0.035	16S	g__Rhodoplanes
Otu000034	Genotype_8	flowers	4.140	<0.001	<0.001	16S	g__Prauserella
Otu000070	Genotype_8	flowers	3.843	<0.001	<0.001	16S	g__Rubrobacter
Otu000073	Genotype_8	flowers	3.893	<0.001	<0.001	16S	g__Erwinia

Otu000082	Genotype_8	flowers	3.730	<0.001	<0.001	16S	p__Proteobacteria_unclassified
Otu000086	Genotype_8	flowers	3.599	<0.001	<0.001	16S	f__Burkholderiaceae_unclassified
Otu000096	Genotype_7	flowers	2.767	0.009	0.009	16S	g__Bradyrhizobium
Otu000115	Genotype_2	flowers	3.033	0.029	0.029	16S	g__Massilia
Otu000151	Genotype_8	flowers	3.536	<0.001	<0.001	16S	g__Bacillus
Otu000291	Genotype_8	flowers	2.941	0.004	0.004	16S	g__Methylotenera
Otu000404	Genotype_6	flowers	2.339	0.003	0.003	16S	f__Sinobacteraceae_unclassified
Otu001113	Genotype_2	flowers	2.511	0.001	0.001	16S	c_Gammaproteobacteria_unclassified
Otu001383	Genotype_7	flowers	2.113	<0.001	<0.001	16S	o__MIZ46_unclassified
Otu004213	Genotype_2	flowers	2.629	<0.001	<0.001	16S	g__Deinococcus
Otu00273	Genotype_2	flowers	3.742	<0.001	<0.001	ITS2	g__Alternaria
Otu00543	Genotype_8	flowers	3.115	<0.001	<0.001	ITS2	k__Fungi_unclassified
Otu01410	Genotype_7	flowers	2.273	<0.001	<0.001	ITS2	k__Fungi_unclassified
Otu02368	Genotype_2	flowers	2.398	<0.001	<0.001	ITS2	g__Rhodotorula
Otu00099	Genotype_8	flowers	4.061	0.011	0.011	18S	Sclerotinia
Otu000126	Genotype_4	seeds	2.395	0.024	0.024	16S	g__Rhizobium
Otu000141	Genotype_2	seeds	4.070	<0.001	<0.001	16S	o__Ellin6513_unclassified
Otu000332	Genotype_2	seeds	4.604	0.011	0.011	16S	k__Bacteria_unclassified
Otu000493	Genotype_5	seeds	2.306	<0.001	<0.001	16S	g__Pantoea
Otu000630	Genotype_5	seeds	2.115	0.003	0.003	16S	g__Pantoea
Otu000713	Genotype_6	seeds	3.654	<0.001	<0.001	16S	k__Bacteria_unclassified
Otu001330	Genotype_2	seeds	3.344	<0.001	<0.001	16S	k__Bacteria_unclassified
Otu001840	Genotype_2	seeds	3.150	0.001	0.001	16S	k__Bacteria_unclassified
Otu002150	Genotype_2	seeds	3.025	0.001	0.001	16S	k__Bacteria_unclassified
Otu00321	Genotype_8	seeds	4.259	0.024	0.024	ITS2	g__Malassezia
Otu00410	Genotype_2	seeds	3.392	0.005	0.005	ITS2	k__Fungi_unclassified
Otu00472	Genotype_2	seeds	3.583	<0.001	<0.001	ITS2	k__Fungi_unclassified
Otu00603	Genotype_2	seeds	3.529	0.009	0.009	ITS2	k__Fungi_unclassified
Otu00990	Genotype_3	seeds	3.127	0.005	0.005	ITS2	k__Fungi_unclassified

Age

OTU	**enrich_age**	**organ**	**ef_lda**	**pvalue**	**padj**	**Locus**	**Genus**
Otu000093	>6	roots	3.680	0.002	0.002	16S	f__Xanthobacteraceae_unclassified
Otu000295	<4	roots	2.716	0.015	0.015	16S	g__Hyphomicrobium
Otu000396	<4	roots	3.184	0.020	0.020	16S	c__Gammaproteobacteria_unclassified
Otu000421	>6	roots	2.634	0.003	0.003	16S	g__Steroidobacter
Otu000575	>6	roots	2.282	0.029	0.029	16S	f__C111_unclassified
Otu000889	<4	roots	2.184	0.013	0.013	16S	g__Bacillus
Otu001115	<4	roots	2.086	0.046	0.046	16S	o__B07_WMSP1_unclassified
Otu001130	>6	roots	2.007	0.036	0.036	16S	o__Phycisphaerales_unclassified
Otu001174	<4	roots	2.045	0.048	0.048	16S	f__Planococcaceae_unclassified
Otu001384	6	roots	2.061	0.047	0.047	16S	f__Flavobacteriaceae_unclassified
Otu001569	<4	roots	2.164	0.043	0.043	16S	g__Bdellovibrio
Otu001627	>6	roots	2.164	<0.001	<0.001	16S	g__Criblamydia
Otu00152	<4	roots	3.717	0.008	0.008	ITS2	g__Mycena
Otu00246	4	roots	2.817	0.032	0.032	ITS2	p__Ascomycota_unclassified
Otu00255	>6	roots	2.964	0.006	0.006	ITS2	g__Pyrenochaeta

Otu00816	>6	roots	2.285	0.002	0.002	ITS3	g__Rhizophagus
Otu00072	6	roots	4.353	0.018	0.018	18S	Helicotylenchus
Otu00223	4	roots	3.070	0.044	0.044	18S	Pezizomycotina_unclassified
Otu00413	>6	roots	2.628	0.004	0.004	18S	Pezizomycotina_unclassified
Otu00469	5	roots	2.097	0.030	0.030	18S	Syncephalis
Otu00577	<4	roots	2.292	0.037	0.037	18S	Chromadorea_X_unclassified
Otu01077	<4	roots	2.005	<0.001	<0.001	18S	Paulinella
Otu000033	>6	shoots	3.370	0.045	0.045	16S	g__Chryseobacterium
Otu000043	4	shoots	2.542	0.001	0.001	16S	f__Bradyrhizobiaceae_unclassified
Otu000070	4	shoots	3.672	0.007	0.007	16S	g__Rubrobacter
Otu000186	>6	shoots	2.484	0.034	0.034	16S	c__S085_unclassified
Otu000191	>6	shoots	2.835	0.046	0.046	16S	f__Gaiellaceae_unclassified
Otu000370	>6	shoots	2.106	0.020	0.020	16S	c__S085_unclassified
Otu000511	>6	shoots	2.798	0.013	0.013	16S	g__Sphingomonas
Otu000560	>6	shoots	2.203	0.030	0.030	16S	g__Nocardioides
Otu000568	4	shoots	2.360	0.028	0.028	16S	g__Streptomyces
Otu000977	>6	shoots	2.130	0.013	0.013	16S	f__Gaiellaceae_unclassified
Otu001155	<4	shoots	2.117	0.040	0.040	16S	g__Paenibacillus
Otu001242	>6	shoots	2.368	0.003	0.003	16S	g__Tissierella_Soehngenia
Otu001934	>6	shoots	2.766	0.015	0.015	16S	g__Actinoplanes
Otu00007	6	shoots	2.734	0.017	0.017	ITS2	g__Acremonium
Otu00008	>6	shoots	4.517	0.009	0.009	ITS2	g__Boeremia
Otu00090	6	shoots	3.435	0.025	0.025	ITS2	g__Nectriella
Otu00559	4	shoots	2.710	0.018	0.018	ITS3	f__Didymellaceae_unclassified
Otu01220	<4	shoots	2.739	0.002	0.002	ITS3	g__Pseudoseptoria
Otu00294	>6	shoots	3.251	0.008	0.008	18S	Panagrolaimus
Otu01388	>6	shoots	2.312	0.002	0.002	18S	Panagrolaimus
Otu000112	>6	flowers	3.019	<0.001	<0.001	16S	f__Planococcaceae_unclassified
Otu000140	4	flowers	2.855	0.002	0.002	16S	g__Frigoribacterium
Otu000276	4	flowers	2.684	0.010	0.010	16S	g__Nocardioides
Otu001033	4	flowers	2.284	0.001	0.001	16S	g__Chryseobacterium
Otu001273	4	flowers	2.237	0.032	0.032	16S	k__Bacteria_unclassified
Otu002511	6	flowers	2.041	<0.001	<0.001	16S	k__Bacteria_unclassified
Otu00007	>6	flowers	2.733	0.006	0.006	ITS2	g__Acremonium
Otu00488	6	flowers	2.924	0.032	0.032	ITS2	k__Fungi_unclassified
Otu00610	5	flowers	3.457	0.039	0.039	ITS2	g__Cenococcum
Otu00558	>6	flowers	3.549	0.011	0.011	18S	Eukaryota_unclassified
Otu00770	4	flowers	2.837	0.049	0.049	18S	Eukaryota_unclassified
Otu000074	4	seeds	2.651	0.024	0.024	16S	f__Sinobacteraceae_unclassified
Otu000122	4	seeds	2.185	0.015	0.015	16S	f__Gaiellaceae_unclassified
Otu000275	>6	seeds	3.189	0.022	0.022	16S	f__Burkholderiaceae_unclassified
Otu000289	<4	seeds	2.131	0.018	0.018	16S	g__Marmoricola
Otu000819	<4	seeds	2.032	<0.001	<0.001	16S	o__Bacillales_unclassified
Otu001353	6	seeds	2.621	0.020	0.020	16S	g__Cupriavidus
Otu001638	>6	seeds	3.030	0.036	0.036	16S	k__Bacteria_unclassified
Otu002021	>6	seeds	2.835	0.003	0.003	16S	k__Bacteria_unclassified

Otu002029	<4	seeds	2.063	0.037	0.037	16S	g__Mesorhizobium
Otu00058	5	seeds	2.276	0.010	0.010	ITS2	g__Colletotrichum
Otu01315	<4	seeds	2.558	0.036	0.036	ITS2	k__Fungi_unclassified
Otu01406	>6	seeds	2.772	0.008	0.008	ITS2	k__Fungi_unclassified
Otu01771	<4	seeds	2.377	0.002	0.002	ITS3	k__Fungi_unclassified

CHAPTER V

SYNTHESIS

In this work I examined the diversity and structure of microbial communities associated with *Lotus corniculatus* in natural populations in order to gain a broader understanding of the ecology of plant microbiomes *in situ.* Bacterial, fungal, and eukaryotic communities in plant organs of *L. corniculatus* from seven grassland sites were surveyed for four years. As with most plant species, *L. corniculatus* root microbial communities are the most diverse among the plant organ communities, and have distinct but overlapping community composition with soil microbial communities (1-4). The less diverse aboveground *L. corniculatus* microbial communities are distinct from root and soil communities, and as is also observed in several plant species, have compositionally-nested characteristic where the aboveground communities are subsets of the more diverse belowground communities (1, 4, 5). *L. corniculatus* shoots, flowers, and seeds harbor microbial communities that are overlapping and more variable than root microbial communities. Plant organ is the main source of structure variation in *L. corniculatus* microbial communities, while year of collection and sampling site contributed less. Meanwhile, the effect of sampling location and year on microbial community variation is stronger than the effect of plant age and genotype. Previous studies have established that plant compartments contribute more in shaping microbial community composition than geographical locations or times of sampling (4, 6, 7). Studies also found that plant age and genetic control are weaker compared with the contribution of location to microbial community variation (8, 9). In this work, I studied the complex interactions between the plant host, its associated microorganisms, and the local environment. Specifically, this work aimed to study factors such as plant organ, genotype, and age, as well as environmental factors as drivers of variation in microbial communities in natural environments.

In **Chapter II**, the organ-specificity of plant endophytic communities was demonstrated. The framework of metacommunity theory of ecology was utilized to synthesize assembly processes that shape plant microbial community structures by assigning root, shoot, flower, and seed microbial communities as distinct communities linked by microbial dispersal. Findings suggest that selective filtering by plant organs, microbial interactions, as well as abiotic and biotic factors at tempo-spatial scales result in distinct core microbial communities of plant organs. Transmission of microorganisms from within and outside the plant hosts also account for the distinct yet overlapping organ microbial communities. In **Chapter III**, we investigated how abiotic and biotic factors in several local environments throughout multiple years contribute to the structure of plant microbial communities, based on the premise of the observed spatial and temporal variations in *L. corniculatus* microbial communities in **Chapter II**. We found that plant microbial communities are shaped by a set of environmental factors that are distinct to each plant organ. Environmental factors that influence the structure and microbial interactions in the plant organs potentially influence dispersal decisions of microorganisms resulting in distinct yet overlapping microbial communities of plant organs, as also observed in **Chapter II**. Organ-dependent environmental perception was also detected in plant-associated microbes, observed both in organ biomarker microbes and in generalist microbes that are highly associated with environmental variables. In **Chapter IV**, we observed variations in age and genotype in *L. corniculatus* populations. Contingent on this observation, we examined how host age and genotype influence plant microbial communities. We found that both age and genotype contribute to variations in diversity and composition of plant microbial communities. While a large number of taxa are shared among plant ages and genotypes, differentially abundant microbes that can be used to distinguish between host age groups or genotypes were identified.

Metacommunity dynamics of plant endophytic communities

L. corniculatus roots, shoots, flowers, and seeds host distinct but overlapping microbial communities which signifies that the organs are discrete ecological niches that are interconnected with each other and with their environment. In **Chapter II**, the assembly processes at tempo-spatial scales that influence patterns in plant microbial communities were consolidated in the framework of metacommunity theory in ecology wherein microbial communities in the roots, shoots, flowers, and seeds were defined as distinct communities linked by transmission of microorganisms within the plant host and outside environment. Results suggest that metacommunity dynamics of plant endophytic communities are governed by selective filtering of plant organs, microbial interactions, abiotic and biotic factors at tempo-spatial scales, and microbial dispersal.

Core microbiomes are shaped by selective filtering of organs.

Roots are mainly colonised by microbial communities that, while distinct, share a subset of microbial communities with surrounding soils (3, 10-12). In *L. corniculatus* roots, endophytic communities are mostly distinct from soil microbial communities, even with apparent overlap in community composition. Such overlap in community composition can be observed when majority of root endophytes are subsets of the surrounding soil microbial communities that were filtered by the root microenvironment. It was previously determined that root-specific endophytic communities are assembled via a two-step selection process wherein the root exudates prompt enrichment of soil microbes in the rhizosphere through a root-microbial interaction called rhizodeposition and subsequently the root compartments select for groups of species that thrive and persist in the root microenvironment (13). The mechanisms on how root microenvironments filter for symbionts and commensals and against pathogens are not completely understood, however it has been shown that host immune system and competition or cooperation among the microorganisms are the main selective forces that determine the assembly of root microbial communities (3, 14, 15). Microorganisms that reside in the roots often have beneficial roles in plant growth and development, pathogen protection, stress tolerance, and nutrient acquisition (13). Important microbes detected in *L. corniculatus* roots mostly originate from soil or water, and they potentially provide nutrients or bioactive compounds that enhance growth and fitness of host plants, while these microbes successfully inhabit the roots by utilizing substrates or forming physical microstructures in the root environments. Other key microbes in *L. corniculatus* roots are either pathogenic, parasitic, or root feeders.

Microorganisms that are associated with the plant phyllosphere function in stress tolerance, plant growth, biocontrol against pathogens, and in ecosystem functioning such as carbon and nitrogen cycles (16). In addition to being beneficial, microorganisms inhabiting the stems and leaves should have the ability to adapt to the shoot microenvironments. Microorganisms that are abundant and persist in the leaves and stems are regulated by the plant host‘s physiological and physico-chemical features - the shoots' physiological properties such as nutrient sources and defense signals can determine the group of microorganisms that can colonise and thrive, while the physical characteristics of shoots such as leaf structure and surface waxiness can help establish interactions between colonising microorganisms and the host plant (17, 18). Abundant and persistent microorganisms in *L. corniculatus* shoots are either beneficial microbes that increase plant fitness or pathogens that can utilize the plant host's genetic and physiological resources.

Microbes that colonize flowers should have the ability to adapt to their harsh microenvironments and should be able to metabolize nutrients that are uniquely found in these microenvironments (19, 20). The chemistry in flower microenvironments, with the abundance of volatile chemicals, secondary metabolites, antimicrobials, and immune response molecules, determine which microorganisms can survive (19, 21-24). The distinctive morphological and chemical traits of flowers also regulate flower visitors and consequently the microbes that are transmitted to the flowers (25, 26). The unique community of microorganisms in flowers impact plant-pollinator interactions via multiple ways including influencing floral phenotypes and associating with flower visitors (21, 27, 28). Thus community structure variations in flower-associated microbial communities affect floral display and rewards, nectar composition, pollinator fitness and reproduction, and consequently affect plant-pollinator interactions and dispersal of microbes between plants (21, 29). In *L. corniculatus* flowers, key microorganisms are specialists that abundantly grow in the flower environments by utilizing floral resources such as nectar while they also produce compounds that impact floral scent and phenotype crucial for attracting floral visitors. Flowers are also one of main points of contact with insects and animals, and thus insects like thrips and endosymbionts transmitted by these insects, as well as bacteria transmitted from humans or animals via interkingdom inter-kingdom horizontal transmission are commonly detected in *L. corniculatus* flowers.

Dispersal of microorganisms from flowers to seeds are influenced by both the flower and seed phenotypes - presence of pollen, stigma microtopography, and seed microstructures determine which microbes are effectively transmitted into the seeds (30). In addition to physical filtering by flower and seed microstructures, the seed microenvironment then applies selective pressures to retain groups of seed-specific microbes. Seed-specific microbial communities adapt to osmotic pressure and water stress, physical and chemical defenses, or available nutrients in the seed microenvironments (30-32). Key microorganisms found in *L. corniculatus* seeds are either known plant pathogens or beneficial microbes that increase plant host fitness.

Microbiome structures are influenced by microbial interactions.

Hub microbes which are important in microbial interactions and consequently in structuring organ-specific microbial communities in *L. corniculatus* are either pathogens or beneficial endophytes. In roots, hubs *Rhodoplanes*, *Exophiala*, and *Cadophora*, which originate from the rhizosphere soil, benefit host plants, while *Dactylonectria* are known root pathogens (33-36). *Rhodoplanes* are phototrophic bacteria that are able to fix nitrogen, while *Exophiala* and *Cadophora* enhance host plant fitness (33-35).

Microbial interactions influence patterns of community structure in stems and leaves (37). To access resources and survive in fluctuating aboveground plant parts, microorganisms need to either compete or cooperate with other community members (18). Hub microbes with high level of interactions with other community members have been shown to impact structure of microbial communities in leaves. *Albugo*, an oomycete pathogen in *Arabidopsis thaliana*, is a hub microbe that can change leaf microbiome structures and inoculation on leaves can lead to decreased microbiome diversity (38). In *L. corniculatus* shoots, *Bacillus*, *Cladosporium*, and *Alternaria* are hub microbes that are abundant and persistent across sampling sites and years. *Bacillus*, which benefit host plants in various ways such as producing bioactive compounds or controlling quorum sensing, and thus are important in resistance and protection against pathogens, are also hub microbes in *A. thaliana* leaf microbial communities (39, 40). On the other hand, *Cladosporium* and *Alternaria* are known plant pathogens but there are also nonpathogenic species that are abundant and beneficial in healthy plants (41-44).

Because of competition for restrictive resources and nutrients in flowers, microbial communities usually have low species richness with a small number of abundant species (19, 23). *L. corniculatus* flowers are colonized by low diversity microbial communities, with hub microbes that are known flower specialists, beneficial microbes, or plant pathogens. *Metschnikowia* and *Pseudomonas* are flower specialists that affect flower phenotypes via production of organic compounds (21, 45). *Ralstonia* and *Botryotinia* are known plant pathogens, while *Cladosporium*, *Alternaria*, *Burkholderia*, and *Pseudomonas* can be both pathogens or beneficial microbes in various flowers (21, 41-44, 46-50).

Microbial interactions such as competition for space and resources are one of the main drivers of selection in seeds (51, 52). *Ralstonia,* which are hub microbes in both flowers and seeds in *L. corniculatus*, are known plant pathogens (46). Other seed hub microbes are

Pseudomonas and *Burkholderia* which are either pathogenic or beneficial to plants, and *Prauserella* which are endophytic actinobacteria that were previously isolated from roots and stems (48, 53-56). *Dioszegia,* which are one of the discriminative yeasts in *L. corniculatus* seeds, have also been shown to have important roles in microbial interactions in *Arabidopsis* leaf microbial communities (38).

Microbiome variations are driven by abiotic and biotic factors at tempo-spatial scales.

Root microbial communities are more diverse and stable than aboveground microbial communities. Nevertheless, they can still be susceptible to biotic and abiotic perturbations belowground such as fluctuations in soil properties, nutrient cycling, or soil-borne pathogens which specifically impact microbial community structures in the roots (57-59). The less diverse and more variable aboveground plant microbial communities are also susceptible to fluctuating environmental factors such as continuous changes in temperature, water, nutrients, or UV levels, which consequently filter for microorganisms that can adapt to such challenging environments (60-63). Consistent day-night fluctuations of aboveground abiotic conditions can elicit assembly of variable microbiomes in the phyllosphere (18, 64, 65). Plant microbial community structure are also shaped by environmental gradients such as local site conditions or land use and soil properties, as well as biotic elements like pollinators, insects, and local fauna (21, 66-69). Numerous perturbations from humans or animals such as mowing or grazing could also affect aboveground plant microbial communities as a result of the unstable habitats and constant reemergence of microbial communities (6, 70). Shoot microenvironments therefore selects for a group of microorganisms that have developed adaptive traits to tolerate and protect from various abiotic stresses (17, 71). Similarly, stresses from the environment such as UV exposure, temperature fluctuations, pollutants, presence of pathogens, or animal disturbances also filter for microorganisms that can colonize flowers (20, 21, 72, 73). Comparable abiotic elements such as precipitation, pollutants, or water and nutrient availability that impacts the aboveground plant parts also influence community structures of seed microbial communities (74-76).

In *L. corniculatus*, abundant microorganisms for each plant organ are mostly similar aboveground, but are distinct from the abundant microorganisms in the roots. The aboveground plant organs were exposed to the same external environment allowing similar abundant microorganisms to survive and thrive in the shoots, flowers, and seeds, resulting in microbial communities that are overlapping in community composition. In addition, microbial community composition in the shoots, flowers, and seeds are more variable and have relatively smaller core communities as opposed to the stable microbial communities in roots. These patterns of microbial community structures can also be attributed to the fluctuating abiotic factors specifically impacting the aboveground plant parts. In general, abiotic and biotic elements in the environment acting at multiple temporal and spatial scales shape *L. corniculatus* microbial communities - from different sampling time and locations to different plant compartments.

Organ microbiomes are distinct communities linked by dispersal.

Stochastic transmission processes impact microbial community patterns in *L. corniculatus* organs. In addition to soil microorganisms, the primary reservoir of root microbial communities, microorganisms from other plant organs are transmitted to *L. corniculatus* roots. Stochastic processes such as priority effects phenomenon potentially contribute to the community composition in *L. corniculatus* root microbial communities, as have previously observed in other plant species (15, 77). Key *L. corniculatus* root microbes *Phyllobacterium* and *Mesorhizobium*, which are nitrogen-fixing bacteria generally found in root nodules, potentially influence community composition via priority effects.

Dispersal of microorganisms from seeds via vertical transmission or from the environment such as soil, wind, rain, animals, or insects via horizontal transmission contribute to shoot microbial community composition (18, 78-83). *L. corniculatus* organs have overlapping microbial community composition due to constant dispersal of microorganisms from various sources such as soil, abiotic elements, or other plant tissues, and subsequently the aboveground microbial communities become distinct from root microbial communities due to filtering by the combination of shoot microenvironment, microbial interactions, and aboveground environmental factors. Abundant and core microorganisms in *L. corniculatus* shoots like the pathogens *Pantoea*, *Ralstonia*, and *Burkholderia* potentially influence community composition via priority effects. Priority effects has also been shown in *A. thaliana* phyllosphere wherein the growth of the members of the microbial communities are dependent on the arrival order of some keystone microbes (84).

Flowers are usually transient and repeatedly re-emerge, hence continual stochastic transmission of microorganisms from various sources throughout developmental stages can result to variability of associated microbial communities (73, 85). Dispersal of microorganisms from various microbial sources such as soil, wind, water, or animal visitors contribute to the community composition of the microbial communities of aboveground plant parts of *L. corniculatus* including flowers, and are likely contributing to their overlap in community compositions. In addition, the flowers themselves via pollinators are main agents of dispersal of microorganisms between plants (86-88). These various dispersal processes can also affect flower microbial community structures via historically contingent assembly, as seen in flowers of some plant species where the microenvironment chemistry and resources were modified by microorganisms and thus late-arriving microorganisms are inhibited (89-91). In *L. corniculatus*, the abundant and hub microbe *Ralstonia* affects microbial community structure in flowers potentially via priority effects.

Mechanisms of microbial dispersal within plants and from the environment into the seeds have been described - seeds can acquire microorganisms from other plant tissues via vascular and nonvascular tissues, from flowers via the stigma, or microorganisms can also be dispersed into the seeds from the outside environment (92, 93). *Malassezia* and *Propionibacterium* are distinctive species in *L. corniculatus* seeds that have been most likely transmitted from humans, and the highly associated *Sodalis* are insect symbionts that are possibly transmitted to the seeds by insects in contact with the flowers or seeds (94-96). In *L. corniculatus*, seed microbial communities collected from different sites for several years are variable and have small core community. Such variable microbial communities in the seeds constitute microorganisms that are dispersed from potential sources such as other plant tissues, soil, and microbial sources from the environment. The arrival order of key microbes *Pantoea*, *Ralstonia*, or *Burkholderia* pathogens potentially contribute to the variable microbial community structure. Seeds also play important roles during vertical transmission of microorganisms from one plant generation to the next by being the initial source inocula of plant microbial communities (78, 79, 92, 97, 98). Key microorganisms in *L. corniculatus* seeds like *Pantoea* and *Gibberella* have been previously shown to be vertically transmitted to next generation seedlings (98, 99).

Organ-specific filtering by abiotic and biotic environmental factors in plant endophytic communities

In **Chapter II** it was shown that while plant organs are the primary source of microbial community variation, spatial and temporal variations across multiple sites and years were also observed. **Chapter III** therefore aimed to further explore other factors that account for the observed spatial and temporal variations. Abiotic (*i.e.,* precipitation, air and soil temperature, soil moisture, and land use intensity and its components fertilization, grazing, and mowing) and biotic (*i.e.,* plant communities and soil microbial communities) environmental factors across multiple sites and years - and how they shape microbial community structures in *L. corniculatus* populations - were examined. We found that plant organ microbial communities are shaped by a set of environmental factors that are distinct to each plant compartment. Also, in **Chapter II** it was demonstrated that there is transmission of microorganisms between plant organs and from other microbial sources in the environment. Therefore, in addition to selective filtering by the plant organ, it is found in **Chapter III** that organ-dependent environmental factor perception influence dispersal decisions or habitat choice of microorganisms, leading to distinct yet overlapping microbial communities in the roots, shoots, flowers, and seeds. Differential perception of environmental conditions in each plant organ is also observed both in organ biomarker microbes and in environmental variable-associated generalist microbes.

Plant microbiomes are shaped by a set of environmental factors that are distinct with each organ.

Variations in *L. corniculatus* root microbial communities are primarily explained by both abiotic and biotic factors in the soil, such as microbiome composition and temperature. Soil microbial communities are the primary reservoirs of microorganisms that are recruited towards the roots (12, 13, 68). Temperature changes in the soil directly influence root microbial community composition by regulating which microbial taxa survive and dominate in the roots and indirectly by shifting the community structure of the soil microbial sources as well as by impacting recruitment of microbes due to changes in root exudation patterns (100-102). In addition, vegetation cover also influences *L. corniculatus* root microbial communities, just like in other plant species by affecting soil properties and consequently soil microbial sources (103, 104).

Aboveground *L. corniculatus* microbial communities are mainly affected by microbial sources and abiotic factors both in soil and aboveground. In shoots, temperature and precipitation largely explain microbial community structure. Temperature, both in soil and aboveground, shifts increase or decrease of beneficial and pathogenic microbial taxa in leaves (105-108). Precipitation also impacts abundance of plant pathogens, and can serve as microbial reservoir of phyllosphere microbial communities (109, 110). Just like in roots, *L. corniculatus* flower microbial communities are primarily shaped by both abiotic and biotic components of the soil. Soil microbes are transmitted to and eventually colonize flower tissues (68, 111). Thus, conditions in the soil like temperature indirectly affect flower microbial communities by impacting soil microbial community composition and by affecting recruitment and selection of microbes by the roots (112, 113). Changes in temperature and moisture also induce changes in vegetative and reproductive plant tissues and their associated microbial communities (27). In *L. corniculatus* seeds, microbial communities are mainly affected by temperature, both in soil and aboveground. Abiotic components in the local environment including temperature generally affects the structure of seed-associated microbial communities by significant enrichment of microbial taxa leading to changes in co-occurrence patterns (114, 115).

Organ-specific filtering by environmental factors influence transmission and establishment of microorganisms in plant microbiomes.

Distinct organ microbial communities in *L. corniculatus* are interconnected via transmission of microorganisms between plant compartments and from microbial sources in the local environment. Organisms evaluate their dispersal decisions and habitat choices based on conditions suitable for their survival and reproduction, considering ecological cues such as abiotic conditions and species interactions (116). Soil and air temperature, soil microbiome composition, plant community richness, and grazing, influence the structure and microbial interactions in *L. corniculatus* organs. These environmental factors differ in association with each organ microbial community, possibly influencing dispersal decisions of microorganisms whether to settle or leave the plant compartments. It has indeed been found that soil microbial communities, temperature in soil and aboveground, plant communities, and grazing are important in structuring plant-associated microbial communities (12, 68, 70, 103-108, 117, 118). Therefore, in plant endophytic metacommunities, the combination of selective filtering by plant organs, organ-dependent environmental factor perception, and organ-specific microbial interactions govern dispersal decisions and habitat choices of microorganisms, resulting in distinct yet overlapping microbial communities in roots, shoots, flowers, and seeds. These insights are crucial in understanding how microorganisms, especially pathogens, disperse from roots to aboveground plant organs and vice versa.

Plant microbes selectively respond to environmental factors dependent of organ habitat.

L. corniculatus-associated microbes that are highly correlated with environmental variables across all plant organs, such as *Burkholderia* and *Sulfuritalea*, respond differently to environmental variables depending on their organ habitat. *Burkholderia* and *Sulfuritalea* abundances fluctuate in response to environmental factors depending on the habitat, for instance, their abundance in the roots and shoots are positively associated with soil and aboveground temperature as well as soil microbiome composition, while in flowers and seeds their abundance are negatively correlated with these same environmental variables. *Burkholderia* are either plant pathogens or beneficial endophytes that promote growth with capabilities for nitrogen fixation and nodulation, quorum sensing, or degradation of aromatic compounds (48, 119).

Organ-dependent environmental perception is also observed in root, shoot, and flower biomarker microbes, such as the rhizobial symbiont *Mesorhizobium*, leaf pathogen *Setosphaeria*, and necrotroph *Botryotinia*, respectively (120-122). These biomarker microbes are abundant and persistent in their corresponding organ habitats, and their habitat choices are possibly influenced by their differential response to environmental factors in different plant compartments. Abiotic and biotic perturbations can impact chemical and morphological properties in plant compartments, and thus can explain the differential response of microbes to environmental factors in different plant organs (123, 124). Therefore, dispersal decisions or habitat choices, and consequently structure of microbial communities, depend on the interaction of microhabitat, key microbes, and environmental factors. Adding to this complex synergy is the set of key environmental variables that differ in their contribution to microbial community variation with each plant compartment.

Plant age and genotype and their influence on endophytic communities

In **Chapter II** and **III**, the diversity and structure of microbial communities in natural populations of *L. corniculatus* were described, and **Chapter IV** details that the plant populations exhibit variation in age and genotype. While sampling time is independent of plant age, sampling site has small effect. Similarly, sampling site has small effect on the distribution of plant genotypes, however, while most genetic groups are overrepresented in some sampling populations, they are widely distributed across populations. In these populations, plant age and genotype are independent. Previous studies on natural *L. corniculatus* populations have shown genetic diversity among and within populations, and that age structure ranges from seedlings to 14 years (125-127). Contingent on these observations, the effect of plant host factors age and genotype on the associated microbial communities was studied.

In **Chapter II** and **III**, it was detailed how plant organs and environmental factors contribute to spatial and temporal variation in plant microbial communities, therefore in **Chapter IV** the influence of plant age and genotype on microbial communities was investigated. Plant age and genotype significantly influence the diversity and structure of the associated microbial communities, albeit less than the contribution of plant organ, sampling site, or sampling year. A large number of taxa are shared among plant ages and genotypes; however, differentially abundant microbial taxa can distinguish between host age groups or genotypes. *Fusarium*, a plant pathogen that was isolated from wild *L. corniculatus*, is a differentially abundant biomarker of one genotype and elicited stronger response to infection in the offspring plants of this particular genotype compared to another genotype.

Plant age shapes composition of associated microbiomes.

Studies have shown that plant age influences bacterial and fungal communities, and in trees found to be negatively correlated with bacterial diversity (9, 128-130). In *L. corniculatus,* the age of plants contributes to the variation in composition of associated microbial communities, but not to their richness and diversity. By two years of age, the plants already harbor microbial communities that are stable in diversity and richness, while community composition of the aboveground bacterial communities shifts at different ages. In **Chapter II** it was previously shown that perennial *L. corniculatus* plants maintain stable bacterial communities in the roots over the years, while in aboveground plant organs, bacterial communities are variable potentially due to perturbations in the local environment, including mowing or animal grazing that prompt constant shoot regeneration. In rice, it was observed that the root bacterial and archaeal communities shift in composition during the early phase and then stabilize for the rest of the life cycle (131). It also has been noted that environmental factors can overcome the effect of age in shaping plant microbial communities (8, 132).

The larger proportion of microbial taxa in the communities are shared between plant age groups, however there is still noticeable variation in composition between the age groups. Both in whole plants or organs, there are shifts in abundances of the most abundant bacterial, fungal, and eukaryotic classes among plants of different age groups. At the OTU level, some low abundance and low occurrence taxa that are exclusively detected in each age group are potentially plant pathogens, including *Drechslera*, *Sarocladium*, *Gliophorus*, *Mycena*, and *Eremothecium*. Differentially abundant microbes can be also be used as biomarkers to distinguish between plant age groups, both at the level of whole plant or organs. Plants aged less than four years harbor differentially abundant *Ralstonia*, *Burkholderia*, and *Erwinia*, while five-year-old plants are enriched with *Rubrobacter* and *Prauserella*, and those that are aged more than six years are abundant with *Aureobasidium*. Some fungal pathogens are also enriched at a particular lifetime of the plants - plants aged less than four years are enriched with *Mycena* and *Pseudoseptoria*, while six-year-old plants harbor abundant *Stemphylium*. It was similarly demonstrated in trees and other perennial plants that increased abundance of microbial groups corresponded with tree age and secondary metabolite synthesis (129, 130). It was also observed that pathogen frequency is associated with plant age in flowering plant populations (133). It was also noted that fungal disease incidence in plants also corresponded with population age (134).

Plant genotype shapes diversity and composition of associated microbiomes.

It has been established that plant genotype influences plant microbial communities and a significant number of plant microbes are heritable (135-138). In *L. corniculatus*, the genotype of plants influences the diversity, richness, and composition of associated microbial communities. The effect of host genotype on diversity is stronger in bacterial and fungal communities compared with eukaryotic communities. In the same way, plant genotype has a weak effect on the composition of eukaryotic communities in flowers and seeds. A study previously showed that the host genotype's effect on microbial community variation is mostly limited to roots and leaves, and no effect on seeds (139). The weak effect of plant genotype on seed microbial communities may be masked by environmental factors (74). Research on plant genotype and the eukaryotic fraction outside of the fungal groups is rare, but a study on the impact of host genotype on rhizosphere eukaryotic communities observed that the host genotype's influence is stronger in fungal communities compared with other eukaryotic groups (140). In *L. corniculatus*, host genotype influence on microbial community diversity, richness, and composition varies with different plant organs. These observations mirror what are demonstrated in **Chapter II** and **Chapter III** where organ filtering and organ-dependent perception of environmental factors were noted. In other grasses and trees, the impact of host genotype on microbial communities are stronger in one plant compartment than the others (139, 141).

Similar with plant age, there is a large proportion of microbial taxa in the communities that are shared among *L. corniculatus* genotypes. Nonetheless the abundance of the most abundant bacterial, fungal, and eukaryotic groups varies among the plant genotypes, both in organs or in plants entirely. Some microbial taxa that are unique to genotype groups are present in low occurrence and abundance, and are potential plant pathogens (*e.g., Volutella, Paraphoma, Clavaria, Gliophorus, Leptosphaeria, Marasmius, Drechslera, Mycena,* and *Eremothecium*). Both at the level of whole plant or organs, some microbes are significantly more abundant in one genotype compared to other genotypes and can thus be utilized as biomarkers that can differentiate between the genotypes. These genotype biomarkers include *Cloacibacterium, Agrocybe, Erwinia, Sclerotinia, Prauserella, Rubrobacter*, and *Bacillus*. Some plant genotypes have genotype biomarkers that are potentially plant pathogens such as *Gibberella, Alternaria, Pseudoidium, Golovinomyces,* and *Malassezia*. Expectedly in other trees, host genotype affects the abundance of certain fungal and bacterial groups, such as plant pathogens or microbes with potential biocontrol and plant growth promotion properties (130, 141). In a proof-of-concept experiment, the pathogen *Fusarium* (teleomorph *Gibberella*), a *L. corniculatus* genotype biomarker, elicited greater infection susceptibility in offspring of a genotype associated with differentially abundant *Fusarium* compared with offspring of another genotype. Genotype-based resistance and susceptibility to *Fusarium* has been detected in rice and corn, leading to identification of genes and pathways associated with pathogen response (142, 143).

Plant age and genotype interact with plant organ, sampling location, and sampling time to shape associated microbiomes.

Plant age and genotype, which are examined in **Chapter IV**, interact with other factors plant organ, sampling location, and sampling time, which are studied more in detail in **Chapters II** and **III**, to shape microbial communities in natural plant populations. For instance, the composition of fungal communities in roots and shoots of *L. corniculatus* are impacted by genotype-by-age interactions. Moreover, the effect of host age and genotype on microbial community composition varies by plant organ. Finally, plant age and genotype differently influence the structure of associated microbial communities in different sampling sites and years. In addition, the impacts of sampling location and time on plant age and genotype effect on microbial communities vary by plant organ.

In previous studies, plant age is the major factor in shaping the associated microbial communities, although genetic effect is still significant (130, 136). This is in contrast to the observations on *L. corniculatus* populations, where host genotype contributes more to microbial community variation than plant age. It was shown in many studies that environment effect is stronger than the effect of plant age and genotype on plant microbial communities (8, 9, 74, 132, 135). However, one study observed that plant genetics contribute more to microbial community variance than ecological factors (137). In terms of temporal factors, it was found that the effect of host genotype on root microbial communities are highly contingent on time of sampling (144). Similarly, the impact of plant genotype on microbial communities are largely conditional with plant compartment (139, 141, 144). These findings demonstrate the complex synergy of plant traits and the local environment in the structuring of plant microbial communities.

Conclusions

In this work, I consolidated the complex interactions between the plant host, its associated microorganisms, and the local environment. This work is a study of factors plant organ, genotype, and age, as well as environmental factors, as drivers of variation in microbial communities in natural environments. **Chapter II** demonstrated the organ-specificity of plant endophytic communities and established an overview of the assembly processes at spatial and temporal scales that account for the community patterns observed in plant microbial communities in natural environments. The framework of metacommunity theory consolidated deterministic and stochastic assembly processes, including selective filtering by plant organs, microbial interactions, abiotic and biotic environmental factors, and microbial dispersal, that shaped distinct yet overlapping plant organ microbial communities. In addition to plant organs (**Chapter II**), which are the primary sources of variation, environmental factors (**Chapter III**) at multiple sites over the years contributed to the observed spatial and temporal variations at multiple scales in plant microbial communities. Organ-dependent perception of abiotic and biotic factors in the local environment also contributes to habitat filtering and dispersal decisions of microorganisms, leading to organ-specificity patterns of plant microbial communities. **Chapter IV** established that plant age and genotype contribute to the variations in associated microbial communities, albeit less than the contribution of plant organ, sampling site, or sampling year. The larger proportion of microbial communities are shared among plant ages and genotypes; however, differentially abundant biomarker microbes can be used to distinguish between host age groups or genotypes. All these described processes in **Chapters II** to **IV** - on how various host plant and environmental factors shape patterns in microbial communities - are observed in key organ microbes including known plant symbionts and pathogens.

This work presents a foundation for more in-depth studies that will test the patterns and predictions established from studying microbial communities in natural populations. *In situ* data that took into account natural perturbations combined with controlled laboratory experiments would provide a more overarching understanding of the mechanisms that shape plant microbial communities. While we included in this work various factors, observations can be refined by studying plant populations in a wider range of geographic locations with broader land use intensity, elevation gradients, or plant genetic diversity. Other environmental variables known to influence microbial communities such as soil chemistry and edaphic factors, solar radiation, or humidity would expand the perspective on the impact of the local environment on plant microbial communities. Examining other ecological processes that also contribute to microbial community variation, such as genetic drift or diversification of microbial community members, would also provide further insight.

Given the important roles of microbial communities in plant growth, stress tolerance, and protection, their reconstruction offers prospects to maximize their beneficial effects. Understanding the ecological processes that shape microbial community dynamics and assembly in natural environments is essential for harnessing their beneficial effects for plant productivity, resilience, and pathogen defense. Knowledge about the organ-specific response of plant microbial communities to abiotic and biotic perturbations will equip us with a framework to understand and engineer plant microbial communities in the context of global climate change. Observed patterns on dispersal decisions or habitat choice of microorganisms based on organ-dependent environmental cues and microbial interactions can also advance our insights on how beneficial microbes or pathogens survive and persist on specific plant microhabitats and environmental conditions. Insights on how plant age/genotype impact microbial communities and on plant age/genotype microbial biomarkers provide clues as to how pathogen spread can be mitigated in the future or how to develop infection-resistant plants. The collective knowledge gained from this work expands the foundation for sustainable agriculture management and practices that incorporate improved crop yields, perturbation-resilient plants, and optimized plant health.

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