

Domestication-driven metaorganism evolution in wheat

Dissertation

zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Christian-Albrechts-Universität zu Kiel

vorgelegt von

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Plön, November, 2019

First referee: Prof. Dr. Eva Holtgrewe Stukenbrock
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Date of oral presentation: 24.01.2020

“Without deviation from the norm progress is not possible.”

Frank Zappa

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Summary of the dissertation:

Plants are not standalone entities but colonized by an immense diversity of eukaryotic and prokaryotic microbes, collectively called the “plant microbiota”. Interest in plant-associated microbiomes has sparked recently, yet our knowledge of microbial variation in plants has remained limited. In my PhD thesis, I studied the impact of domestication on the vertically-transmitted (i.e. seedborne) and environmentally-acquired microbial community assembly in the bread wheat *Triticum aestivum* and its wild progenitors. Furthermore, I disentangled factors shaping seedborne microbial variation in domesticated wheat. Here, I present a novel and comprehensive analysis of the wheat metaorganism based on my analyses of seedborne and environmentally-acquired fungal and bacterial communities of a wide collection of wheat.

Wheat seeds have a low microbial biomass and diversity; therefore they are more prone to technical bias than samples with greater levels of microbial colonization such as leaf and root samples collected from field. In the first chapter, I improved and optimized techniques to increase the accuracy and resolution of amplicon-based sequencing of wheat seeds based on 16S rRNA and ITS marker loci for bacterial and fungal profiling, respectively. As part of this, I increased the efficiency of microbial detection in single seeds by optimizing pre- and post-PCR methods. This allowed me to compare between-sample variation of microbial communities associated with individual seeds and this is further described in chapter 2 and chapter 3.

In the second chapter, I investigated the impact of domestication on the assembly of seedborne and environmentally-acquired microbiota in wheat. Here, I hypothesized that

strong directional selection for few desired traits in the wheat genome and polyploidization events during domestication have led to relaxation in the selection of traits important for plant-microbe interactions. Therefore, I expected to see higher stochasticity in the community composition of seedborne microbes colonizing domesticated wheat compared to the wild wheat (i.e. higher beta diversity in microbial composition among individual domesticated plants). First, I showed that microbial diversity in seeds was low but comparable among different wild and domesticated wheat genotypes collected from Turkey and Germany. Secondly, as a main finding in the study, I demonstrated that despite both wheat species having a comparably low diversity of microbes in seeds, a reduced and more stochastic bacterial community colonizes seedlings of the individual plants of the domesticated wheat *T. aestivum* from Turkey and Germany than the wild wheat *T. dicoccoides*. Finally, I addressed the environmentally-acquired part of the wheat microbiome. I hypothesized that the “ability” of the plant to assemble soil-derived microbial community in seedlings would have been affected by domestication. In brief, independent of the host genotype, different wheat genotypes were able to assemble similarly composed bacterial communities when grown in the same soil. Finally, I also revealed that different eco-evolutionary dynamics govern the assembly of fungal and bacterial communities. Combined, these experiments allowed me to assess the “inherited” versus “acquired” microbial communities and their putative alteration by domestication.

Finally, in the third chapter, I aimed to further dissect the seed microbiota of *T. aestivum*. Considering the role of stochastic events on seedborne microbial assembly, I expected to observe comparable intra-plant (i.e. seeds collected from the same wheat head) and inter-

plant variation (i.e. seeds collected from different wheat heads) in the seedborne microbiota. Furthermore, based on my findings presented in chapter 2, I hypothesized that stochastic processes, for example priority effects, may play a stronger role in seedborne microbial colonization of the domesticated wheat *T. aestivum*, compared to the wild wheat *T. dicoccoides*. Analyzing 16S rRNA and ITS amplicon sequencing data of seedborne microbes colonizing leaves, I showed that variation in seedborne bacterial communities among seeds collected from the same head was not lower than inter-plant variation. Additionally, I found that bacterial diversity decreases over time in the leaves of domesticated wheat – which was not observed in wild wheat. Overall, with this chapter, multilevel comparisons of seedborne microbes in wheat allowed me to propose that intra- and inter-plant variation is comparable in seedborne bacterial communities. However, host type affects the community composition of seedborne microbes colonizing leaves at different plant ages.

Taken together, I showed that microbial diversity and composition in seeds of different wild and domesticated wheat species from Turkey and Germany are not different from each other. Yet, a more diverse bacterial community colonizes the wild wheat compared to the domesticated wheat. Notably, more heterogenous seedborne bacterial communities colonize the domesticated wheat compared to the wild wheat. However, when grown in the same soil type, similar microbial communities colonize the plants independent of the wheat genotype. Moreover, inter- and intra-plant variation is similar to each other in seedborne microbiomes of domesticated wheat. Finally, diversity of seedborne microbial communities in the leaves of domesticated wheat decreases over time whereas it shows

increasing trends in the wild wheat. Overall, the research presented here is a fundamental contribution towards a better understanding of the wheat metaorganism.

Zusammenfassung

Pflanzen sind keine isolierten, sterilen Einheiten, sondern werden von einer immensen Vielfalt an eukaryotischen und prokaryotischen Mikroorganismen besiedelt, die man in ihrer Gesamtheit als „pflanzliche Mikrobiota“ bezeichnet. Obwohl das Interesse an Pflanzen-assoziierten Mikrobiomen stark wächst, wissen wir weiterhin vergleichsweise wenig über die tatsächliche mikrobielle Variation in Pflanzen. Im Rahmen meiner Doktorarbeit habe ich anhand von Weichweizen (*Triticum aestivum*) und wilden Vorgängerarten untersucht, welchen Einfluss Domestikation auf die Zusammensetzung der mikrobiellen Lebensgemeinschaften hat, die vertikal übertragen werden (d.h. samenbürtig sind) und aus der Umwelt aufgenommen werden. Darüber hinaus habe ich Faktoren identifiziert, die einen Einfluss auf die mikrobielle Variation in Samen von Kulturweizen haben. Basierend auf meinen Untersuchungen der pilzlichen und bakteriellen Lebensgemeinschaften, die mit verschiedenen Weizenarten assoziiert leben und samenbürtig sind oder aus der Umwelt stammen, stellt meine Arbeit eine neuartige und umfassende Analyse des Metaorganismus Weizen dar.

Die Biomasse und Vielfalt von Mikroorganismen in den Samen von Weizen sind sehr gering. Ergebnisse für Samenproben sind daher anfälliger für eine Verzerrung durch technische Einflüsse, als Proben von Blatt- oder Wurzelmaterial die allgemein eine stärkere Besiedlung durch Mikroorganismen aufweisen. In Kapitel 1 stelle ich dar, wie ich Methoden für das Profiling von Bakterien- und Pilz-Taxa in Samen von Weizen optimiert habe. Mein Ziel war es dabei, die Genauigkeit und Auflösung der Amplikon-basierten Sequenzierung von 16S rRNA und ITS Marker-Regionen zu erhöhen. Durch Optimierung von Methoden vor und nach der DNA Amplifizierung ist es mir gelungen in einzelnen

Samenkörnern Mikroorganismen mit hoher Effizienz zu detektieren. Dies ermöglichte es mir die mikrobiellen Lebensgemeinschaften in einzelnen Samen miteinander zu vergleichen, was ich in den Kapiteln 2 und 3 beschreibe.

Im zweiten Kapitel untersuche ich den Einfluss von Domestikation auf die Zusammensetzung der samenbürtigen und der aus der Umwelt erworbenen Mikrobiota von Weizen. Ich teste die Hypothese, dass starke gerichtete Selektion für wenige, favorisierte Merkmale und Polyploidisierung während der Domestikation von Weizen dazu geführt haben, dass auf Merkmale die eine Rolle für Interaktionen zwischen Pflanzen und Mikroorganismen spielen, geringerer Selektionsdruck wirkte. Aus diesem Grund erwartete ich, dass die Zusammensetzung der Lebensgemeinschaften von samenbürtige Mikroorganismen die Weichweizen besiedeln stärkere Stochastizität aufweist als bei wilden Weizenarten (d.h. eine höhere Beta-Diversität der mikrobiellen Lebensgemeinschaften bei Individuen der domestizierten Art). Ich zeige, dass die mikrobielle Diversität in den untersuchten Samen insgesamt zwar gering, aber zwischen verschiedenen wilden und domestizieren Weizen-Genotypen aus der Türkei und aus Deutschland vergleichbar ist. Trotz der geringen mikrobiellen Artenvielfalt kann ich weiterhin zeigen, dass *T. aestivum* Keimlinge, die aus Samen aus der Türkei und aus Deutschland angezogen wurden, im Vergleich zu Keimlingen von wildem *T. dicoccoides*, von weniger artenreichen bakteriellen Lebensgemeinschaften besiedelt werden deren Zusammensetzung zufälliger ist. Außerdem setze ich mich mit dem Anteil des Weizen-Mikrobioms auseinander, der aus der Umwelt aufgenommen wird. Ich teste die Hypothese, dass das „Vermögen“ der Pflanze aus dem Boden Mikroorganismen aufzunehmen und daraus eine mikrobielle, Pflanzen-assoziierte Lebensgemeinschaft

zusammensetzen, durch Domestikation beeinflusst wurde. Zusammenfassend habe ich beobachtet, dass sich unabhängig vom Genotyp des Wirts sehr ähnliche bakterielle Lebensgemeinschaften bei verschiedene Weizen Genotypen ausbilden, die in der gleichen Erde kultiviert wurden. Schlussendlich zeige ich, dass unterschiedliche co-evolutionäre Dynamiken auf pilzliche und bakterielle Lebensgemeinschaften einwirken. In der Summe ermöglichen es mir diese Experimente „vererbte“ von „erworbenen“ mikrobiellen Lebensgemeinschaften zu unterscheiden und zu beurteilen, wie sich Domestikation möglicherweise auf sie ausgewirkt hat.

Mein Ziel im Kapitel 3 ist eine tiefere Analyse des Mikrobioms der Samen von *T. aestivum*. Unter Berücksichtigung des Einfluss von Zufallsereignissen auf die Zusammensetzung der samenbürtigen Mikroorganismen erwarte ich, dass die Variation der samenbürtigen Mikrobiota innerhalb einer Pflanze (d.h. aus Samen aus einer Weizenähre) und zwischen verschiedenen Pflanzen (d.h. aus Samen von Ähren verschiedener Pflanzen) vergleichbar ist. Auf Grundlage der Ergebnisse die ich in Kapitel 2 dargestellt habe, stelle ich darüber hinaus die Hypothese auf, dass Zufallsprozesse, wie beispielsweise Prioritätseffekte, bei domestiziertem Weizen *T. aestivum* einen stärkeren Einfluss auf die Besiedlung durch samenbürtige Mikroorganismen haben als bei der nicht-domestizierten Weizenart *T. dicoccoides*. Durch Untersuchung der 16S rRNA- und ITS-Amplikon Sequenzierungsdaten zeige ich, dass die Variation zwischen samenbürtigen bakteriellen Lebensgemeinschaften in Blättern, die aus Samen der gleichen Ähre stammen, nicht geringer ist, als zwischen Lebensgemeinschaften, die aus Samen verschiedener Ähren stammen. Darüber hinaus finde ich, dass sich - anders als bei nicht-domestiziertem Weizen - die bakterielle Diversität in Blättern von Weichweizen

mit der Zeit verringert. Auf Grundlage der Ergebnisse meiner vielschichtigen Untersuchungen die ich in diesem Kapitel präsentiere schlussfolgere ich, dass die Variation von bakteriellen Lebensgemeinschaften, die aus den Samen einer oder verschiedenerer Pflanzen hervorgegangen sind, vergleichbar ist. Dennoch beeinflusst die Wirtspflanze die Zusammensetzung der samenbürtigen Lebensgemeinschaften, die im Verlauf ihrer Entwicklung ihre Blätter besiedeln.

Mit meiner Arbeit zeige ich, dass sich Diversität und Zusammensetzung der Mikrobiota in Samen von domestizierten und wilden Weizenarten aus der Türkei und aus Deutschland nicht unterscheiden. Dennoch, wilde Weizenarten werden von diverseren bakteriellen Lebensgemeinschaften besiedelt als domestizierter Weizen. Samenbürtige bakterielle Gemeinschaften in domestiziertem Weizen sind heterogener als in wilden Arten, allerdings bilden sich ähnliche mikrobielle Lebensgemeinschaften aus, wenn die Pflanzen im gleichen Boden kultiviert werden - unabhängig vom Weizen Genotyp. Darüber hinaus besteht bei domestizierten Weizen kein Unterschied bei der Variation zwischen samenbürtigen Mikrobiomen aus Samen der gleichen Ähre oder von Ähren verschiedener Pfdpflanzen. Die Diversität von samenbürtigen Mikrobiota in den Blättern von *T. aestivum* nimmt mit der Zeit ab, während in nicht-domestiziertem Weizen ein Trend zur Zunahme besteht. Zusammenfassend tragen diese Ergebnisse maßgeblich dazu bei den Metaorganismus Weizen zukünftig besser zu verstehen.

Glossary of Terms

Operational Taxonomic Unit (OTU): OTUs are similar sequence variants of the marker gene sequences clustering together. In this thesis, I used 99% as a threshold to define the cluster identity.

Bacterial feature: “Feature” is the generic term used by the software Qiime2 as an alternative to “OTU”.

Axenic seedlings: In this thesis, I define “axenic seedlings” as the seedlings originating from surface sterilized seeds grown under sterile conditions.

General Introduction

Plants are not standalone organisms but colonized by broad diversity of microorganisms (i.e. *plant microbiota*), collectively with the host called as *plant microbiome* (Schlaeppli and Bulgarelli 2015). From the perspective of the plant, interactions between plants and microbes can fall into a spectrum ranging from pathogenic to symbiotic. Commensal microbes, i.e. microbes that are non-pathogenic to the plant, constitute the majority of the plant microbiota (Vorholt 2012). Plant microbiota inhabiting on (i.e. epiphytes) and inside (i.e. endophytes) of the plant has an impact on the fitness of the plant (Berg and Koskella 2018); (Bulgarelli et al. 2013). For example, plant microbiota fundamentally affect plant function by providing novel nutrients and boosting defense pathways (Friesen et al. 2011). Therefore, microbiota constitute the “functional extension” of the plant (Hassani et al. 2019) and play a major role in buffering of and rapid adaptation to changes in host environment (Haney et al. 2015); (Santos-Medellín et al. 2017). Overlap of selective interests of the plant and microbial partners may confer the coexistence and coevolution of the two entities over long evolutionary timescales (Vandenkoornhuysen et al. 2015). Beneficial interactions such as the ones between *Rhizobium* and legumes and non-leguminous plants (Cao et al. 2017); (Garrido-Oter et al. 2018) or mycorrhizal fungi and different plant species (Parniske 2008); (Choi et al. 2018), where microbes provide nutrients to the plant in exchange for photosynthates, are well-known examples of long term coexistence and coevolution of plant microbiomes (Hassani et al. 2019). On the other hand, vertically-transmitted microbes, for example, seedborne microbes, are especially strong candidates for such long-term associations: they have the potential to shape the coevolution of genetic interactions between plants and microbes because they

are not limited to a single generation but are extended over generations of lifespan of plants (Hassani et al. 2019).

Seedborne microbiota: vertically-transmitting microbes over generations

Plant seeds host diverse microbial communities (Wassermann et al. 2019); (Rybakova et al. 2017); (Barret et al. 2015). Seeds are potential vectors for plant associated microbes allowing them to be transmit from one generation to the next. Thereby the life cycle and biology of microbes and their host are closely associated. Previously, it has been shown that numerous plant pathogens disperse via plant seeds (Schardl et al. 2004). However, theory predicts that at least a fraction of the vertically-transmitting microbes should be in symbiotic relationship with the host suggesting that beneficial interactions can also be maintained over generations (Herre et al. 1999). In support of this scenario, it has been shown that some seedborne microbes produce specific volatiles and antimicrobial products that are important for disease suppression in the plant host (Khalaf and Raizada 2018). For example, the seedborne *Epichloë* is a fungal endophyte that received recognition in sustainable agriculture studies because of its engagement in various beneficial interactions with grasses (Kauppinen et al. 2016). They induce defenses in the plant to prevent pathogen infection by boosting jasmonic-acid pathways (Bastias et al. 2017). Seedborne microbes may thus represent a stronger extent of coadaptation with plant hosts relative to microbes that are taken up from the soil or air.

Knowledge on the seedborne microbes, especially fungal communities of seeds, is relatively limited compared to microbiota of other plant tissues. This limited knowledge

partly stems from technical challenges to handle low microbial biomass and diversity in single seeds. For instance, the model plant species *Arabidopsis thaliana*, producing very small seeds, represents the technical challenge of studying the seedborne microbiota. Studies of seedborne microbial communities have mostly relied on the culture-dependent techniques (i.e. techniques relying on growth of microbes on medium) (Rezki et al. 2018); (Robinson et al. 2016); (Hardoim et al. 2012) or pooling of multiple seeds for culture-independent techniques (Klaedtke et al. 2016); (Robinson et al. 2016); (Adam et al. 2018). These studies identified seedborne microbial compositions and the impact of host genotype and abiotic factors (e.g. terroir) in the microbial community composition of seeds. Furthermore, a few studies provided evidence for the extent of vertical transmission of microbes (Hardoim et al. 2012); (Johnston-Monje and Raizada 2011). Therefore, the available data reveal a shared microbial community in consecutive plant generations. This is notable since it points to a core seedborne microbiota where the plant and its associated microbiota may coevolve over generations and thereby constitute a unit of selection (Vandenkoornhuyse et al. 2015).

Crop domestication as an evolutionary process

Crop domestication is an extensive process involving four main stages (Gaut et al. 2018): i) stewardship and harvesting of wild populations ii) purposeful cultivation of wild populations and selection for desired traits iii) migration of the cultivated forms to novel environments iv) deliberate breeding of the plants to increase the yield of the products. All these stages make crop domestication an ideal model to study evolution because it encompasses a broad spectrum of evolutionary processes (e.g. selection, diversification, speciation, hybridization, introgression) (Meyer and Purugganan 2013). Availability of

the modern and ancestral crop species in nature or in gene banks enables us to reveal major evolutionary changes that occurred in plant genomes. Therefore, for example genes and mutations involved in domestication or gene flow between crops and their wild progenitors can be investigated in detail.

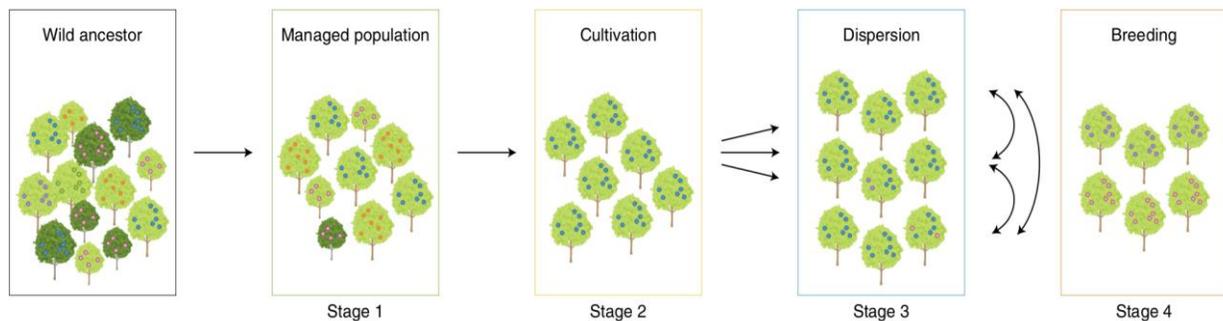


Figure 1: Features of plant diversity during four main stages of plant domestication. Figure adapted from Gaut et al. 2018.

Domesticated plants differ from their wild ancestors in several morphological and physiological traits those of which potentially had been shaped during the domestication process. There is a suite of such traits distinguishing crops and wild progenitors (Doebly et al. 2006). and they are collectively called as *domestication traits*. For example, seed size is a domestication trait that is artificially selected by human cultivation (Purugganan and Fuller 2009). Larger seed size is strongly associated with larger seedlings in several cereals (Pritchard 2000); which potentially confers adaptive advantage to the plant. The loss of shattering in crops is another important trait in plant domestication, since this trait rendered crops dependent on humans for survival and dispersion (Purugganan and Fuller 2009). On the other hand, *diversification traits* are the variation occurred in crop

plants as an outcome of adaptation to distinct local conditions after the completion of the initial domestication event. Shift in the photoperiod in wheat (from winter to spring cultivars) after spreading out from the Fertile Crescent is an example of diversification trait (Doebley et al. 2006).

One important hallmark of domestication in crops is the dramatic reduction of plant genetic diversity. Plant gene pool undergoes through a series of dramatic demographic changes during domestication as a result of two processes: population bottleneck events and strong selection for domestication traits (Doebley et al. 2006). The loss in diversity depends on the initial population size of the wild plant to be domesticated (Eyre-Walker et al. 1998). Moreover, loss of diversity has not been evenly distributed throughout the genome; but accentuated in and around the genomic regions those of which express desired domestication traits (Pont et al. 2019). The decline in host diversity compared to the wild progenitor is estimated to be above 30% on average (Haudry et al. 2007). Selective sweeps are ubiquitous in the genomes of diverse species of crops (Meyer and Purugganan 2013). The impact of the bottlenecks associated with domestication was severe especially in some crops. For example, nucleotide diversity decreased in the bread (*T. aestivum*) and pasta wheat (*T. turgidum*) cultivar by 69% and 84% respectively compared to the wild progenitors (Haudry et al. 2007).

Another important cost of domestication is the accumulation of deleterious mutations in the genome of some crops compared to their wild progenitors (Renaut and Rieseberg 2015); (Wang et al. 2017). For instance, maize harbours more than twice as many fixed deleterious alleles and 10% fewer segregating deleterious alleles than teosinte (Wang et

al. 2017). There are two explanations as to why mutational load accumulates in response to domestication process: i) reduction in population size during the first and second stages of domestication, hence increased efficacy of drift and decreased efficacy of genome-wide purifying selection (Hedrick and Garcia-Dorado 2016) ii) Hitchhiking of deleterious mutations with the selected traits (Kono et al. 2016). These mutations may be preventing crops from achieving fitness (Renaut and Rieseberg 2015).

Impact of domestication on the bread wheat genome

Domestication history of the bread wheat *Triticum aestivum* has been well-documented (Pont et al. 2019); (He et al. 2019); (International Wheat Genome Sequencing Consortium (IWGSC) 2014). The genetic make-up of the modern bread wheat has been shaped by 10,000 years of artificial selection, hybridization, gene flow from the wild progenitors, plant breeding, range expansion, and local adaptation. As a result, today's modern wheat has been selected to thrive in various environments, for example from low to high humidity areas (Pont et al. 2019).

Triticum aestivum was previously proposed to originate from two hybridization events: First, the wild wheat *T. urartu* Tumanian ex Gandilyan (donor of the AA genome) (Feldman and Levy 2012) and an undiscovered species of *Aegilops speltoides* Tausch lineage (donor of the BB genome) hybridized and formed the tetraploid wheat (AABB). Later, a descendant of the resulting tetraploid wheat hybridized with *Aegilops tauschii* Coss (donor of the DD genome). This hybridization formed the allohexaploid (AABBDD)

wheat; which was domesticated to give rise to the modern wheat in the Fertile Crescent region.

Domestication has led to dramatic reduction in wheat population diversity as a result of strong directional selection for the domestication genes and hybridization events (Haudry et al. 2007); (Akhunov et al. 2010); (Pont et al. 2019). Polyploidization resulted in a substantial decrease in diversity in the A and B genomes, but especially in the D genome of bread wheat (Akhunov et al. 2010); possibly because there was gene flow to the A and B genomes from the tetraploid wild emmer while D genome remained isolated after polyploidization (Dvorak et al. 2011). Moreover, following wheat domestication, plant breeding caused a further reduction in genetic diversity of domesticated wheat, once by early breeding improvement (11.7% of reduction) and for a second time following the green revolution (i.e. renovation of agricultural practices in 1950-1960s) (13.3% of reduction) (Pont et al. 2019)). This decrease in diversity as a result of plant breeding has shown to be more severe in the A and B genomes of bread wheat compared to the D genome, suggesting different contribution of these genomes to wheat improvement (Pont et al. 2019).

Gene flow is suggested to be an important mechanism that counteracts the accumulation of deleterious mutations. The A and B genomes of wheat are shown to contain lower frequency of deleterious mutations (compared to synonymous mutations) as a result of gene flow from the tetraploid wild wheat (Dvorak et al. 2011). High load of deleterious mutations in crops is postulated to affect crops performance negatively (Morrell et al. 2011).

Impact of domestication on the plant metaorganism assembly

We do not have a theoretical understanding as to how all those domestication-driven changes in the genomes of plant populations might have affected the evolutionary interactions between plants and their microbial communities. Domestication may have influenced microbial community composition (i.e. diversity and abundances of microbes) and the interactions of plants with their associated microbes (e.g. efficiency of symbiosis, prevalence of pathogenicity). To understand the consequences of plant domestication in microbial assembly is highly important and may enable us to improve crop health. For instance, using such an information, we may rebuild beneficial (and ancestral) interactions between domesticated plants and microbes, and thereby maximize the efficiency of symbiosis among those partners, with an eventual aim of increasing the crop yield. In this context, comparative microbial community analysis of domesticated plants and their wild progenitors are valuable study systems to gain insights into domestication-related effects on plant-associated microbes.

It has been speculated as to how domestication may have changed the assembly of microbial communities; however direct evidence of this speculation is scarce (Pérez-Jaramillo et al. 2018). It is considered that the plant immune system controls the interactions with beneficial microbes as well as the interactions with pathogens (Hacquard et al. 2017). PTI (PAMP-triggered immunity) and ETI (effector-triggered immunity) are two layers of the plant immune system; they are triggered by different microbial gene products yet overlap in physiological responses to a great extent (**Figure 2**) (Ramirez-Prado et al. 2018). It has been shown that the genes that encode proteins related to plant immunity (e.g. PRRs and Nucleotide-Binding Leucine-rich repeat

Receptor (NB-LRR) proteins) coevolve with genes and gene products of the microbes (e.g. (PAMPs/MAMPs) and effectors) to either facilitate or hamper plant-microbe interactions (Hassani et al. 2019). Strong directional selection and polyploidization during domestication may have resulted in a change in the composition and diversity of the immune-system related proteins in plants. This change may have impacted plant-microbe coevolution and interactions, thereby indirectly microbial assembly. Studies investigating the impact of domestication on plant microbiota should thus consider the role of the plant immune system and how this has been altered during domestication.

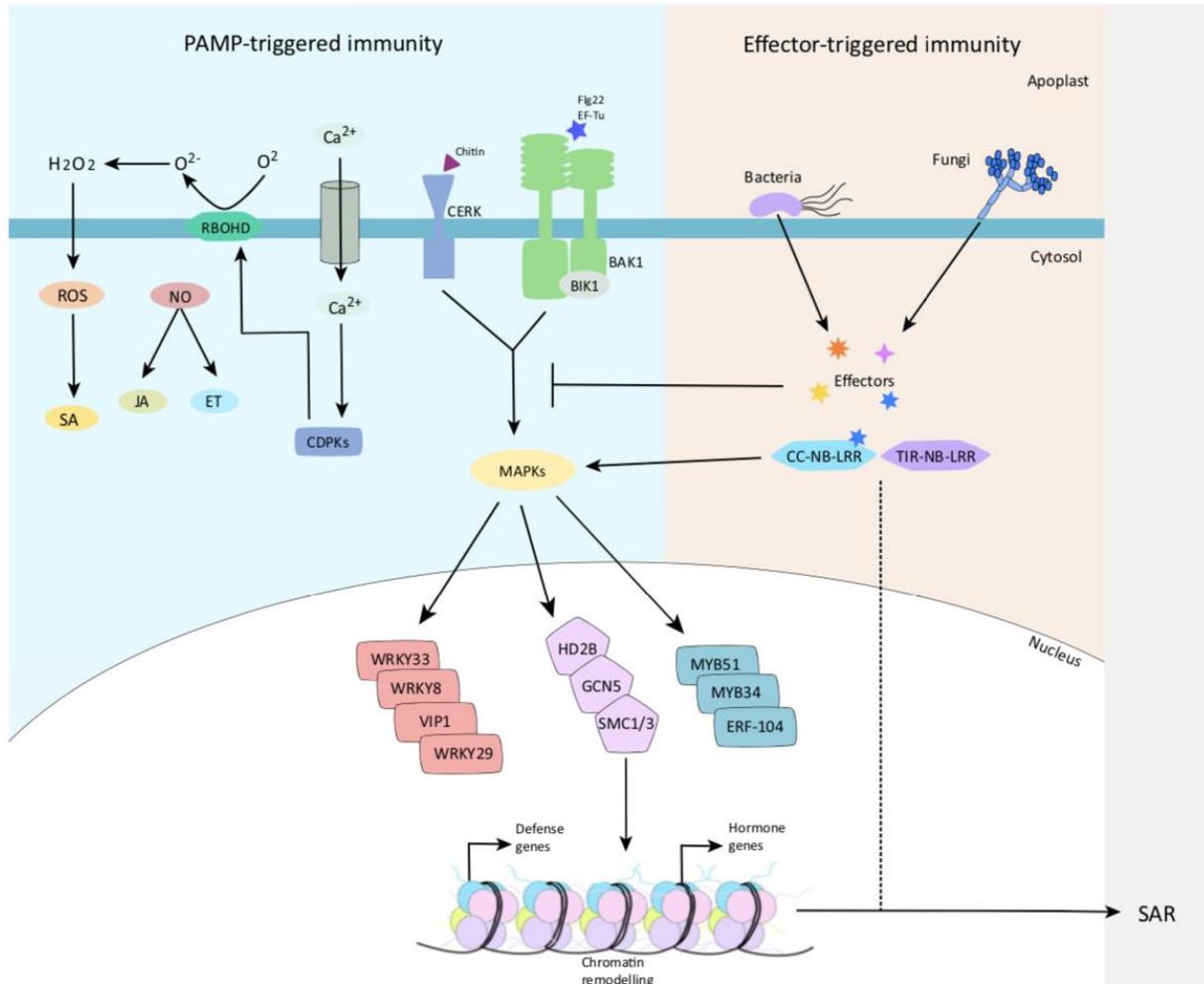


Figure 2: Summary of the innate plant immune system responses. Pathogen-/microbe-associated molecular patterns (PAMPs/MAMPs) detection by Pathogen Recognition Receptors (PRRs) triggers a series of defense responses, called as PAMP-triggered immunity (PTI). Major plant hormones that regulate PTI responses are salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). However, due to the long history of plant-microbe coevolution, microbes usually find ways to suppress PTI. Again by the same reason, plants are able to detect these effectors by R-proteins (CC-NB-LRR and TIR-NB-LRR) and induces the systemic acquired resistance (SAR). This layer of plant immunity is called as effector-triggered immunity (ETI). The activation of common set of genes by PTI and ETI leads to the transcriptional reprogramming. However, usually the defense response triggered by ETI. Figure adapted from Ramirez-Prado, 2018.

Several studies that were carried out in different plant hosts and plant compartments reported contradictory conclusions about the impact of plant domestication on plant-associated microbial communities. Bulgarelli and colleagues observed small but still significant effect of host genotype on the rhizosphere and root-associated microbiota composition when compared between domesticated and wild barley, i.e., *Hordeum vulgare* and *Hordeum spontaneum*, respectively (Bulgarelli et al. 2015). This genotypic effect was apparent not only between domesticated and wild barley, but also among different accessions of the domesticated barley from different domestication levels. However, the observed host genotype effect was only quantitative; in other words, stemming from differences in abundance of microbial taxa in different accessions. Coleman-Derr and colleagues investigated bacterial and fungal communities associated with different plant compartments (soil, rhizosphere, phyllosphere and root and leaf endospheres) in cultivated and native *Agave* species (Coleman-Derr et al. 2016). Investigation of both prokaryotic and eukaryotic community composition allowed the authors to assess the impact of distinct biotic and abiotic factors during plant domestication. Cultivated *Agave* was shown to harbor a lower diversity of bacteria in the rhizosphere and phyllosphere compared to native *Agave* species. However, such decrease in bacterial diversity was not observed in the endosphere. Moreover, fungal communities, that were shown to be determined mostly by biogeography, did not show any pattern in community composition in regard to domestication. In contrast, in recent and ancient wheat ancestors, *Triticum dicoccoides* and *Aegilops sharonensis*, wild grass relatives of wheat were claimed to host more diverse isolated fungal endophytes in seeds and stems which are not detected in the modern wheat, *T. aestivum* (Ofek-Lalzar et al. 2016).

However, because of the technical limitations, there was no further conclusion in this study in regard to the comparison of wild and domesticated wheat microbiota.

In a metaanalysis conducted on the root-associated bacterial community of domesticated and wild plants, it was revealed that domestication has resulted in the same pattern of taxonomic shift in the prokaryotic community composition of roots and rhizosphere in different plant species: Bacteroidetes were consistently enriched in the root and rhizosphere of wild relatives compared to domesticated plants (Pérez-Jaramillo et al. 2018). However, the extent of enrichment was determined by plant taxa. Also, although the phylum Bacteroidetes was commonly enriched in all plants analyzed, different taxa of Bacteroidetes were enriched in the wild relatives of domesticated plants. Bacteroidetes are known for their ability to degrade complex biopolymers (Berlemont and Martiny 2015). Change in the profile of root exudates (e.g. releasing of less complex sugar in cultivated plants), root morphology (e.g. shallower roots in cultivated plants) and environmental factors, and management strategies related to domestication (e.g. usage of pesticides and fertilizers) were proposed to be possible attributes to the enrichment of Bacteroidetes during domestication.

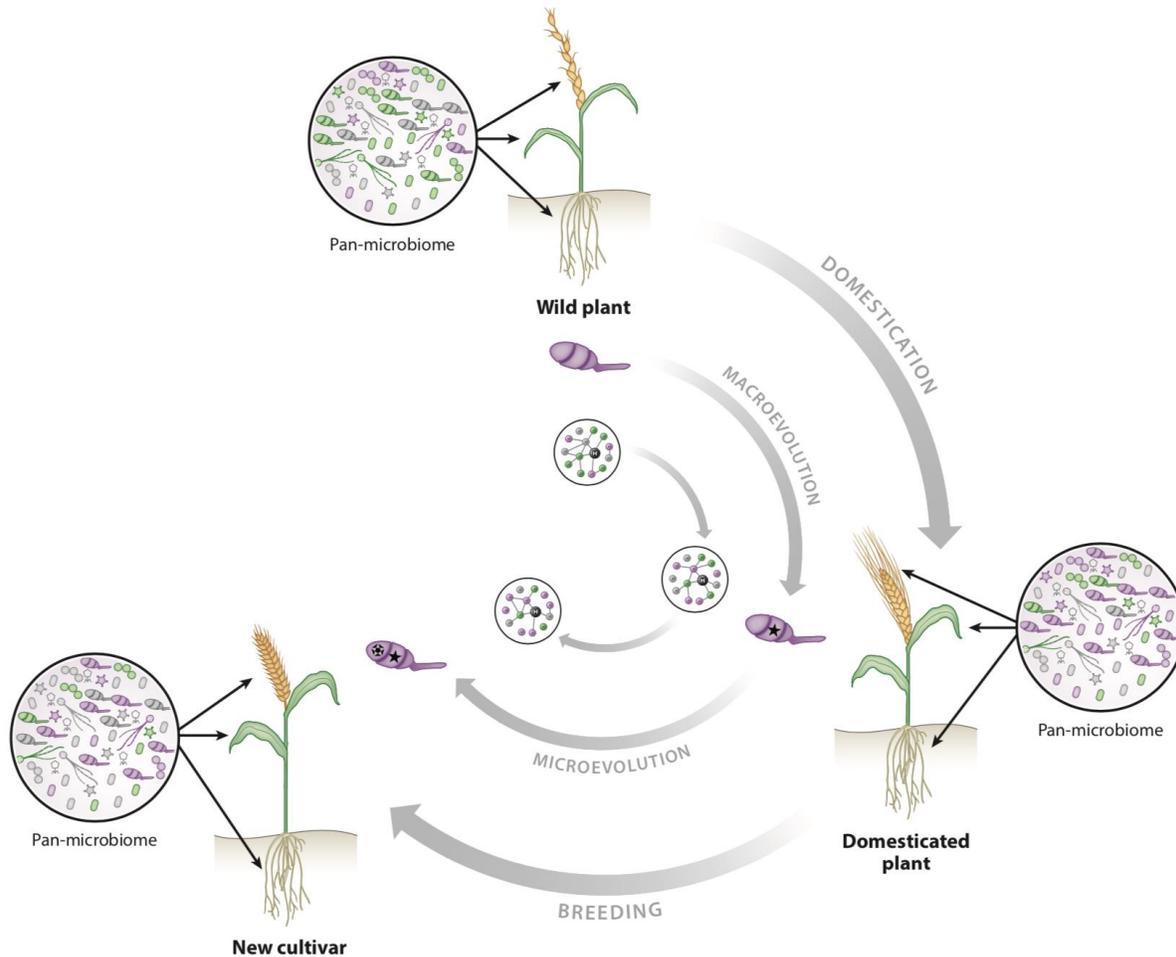


Figure 3: Domestication of plants changed their interactions with microbes

Domestication has impacted microbial diversity and prevalence of microbes. Domestication has also induced new species of microbes (e.g. new pathogens) (macroevolution) and breeding has affected their adaptation at the population level (microevolution). Figure adapted from Hassani, Özkurt et al. 2019.

Domestication may have directly or indirectly affected the prevalence of putative pathogens in agricultural environments **(Figure 3)** (Hassani et al. 2019). In a study where impact of levels of the common sunflower, *Helianthus annuus* domestication in root and rhizosphere-associated microbiota detected, authors claimed shift in composition of fungal but not bacterial communities (Leff et al. 2017). However, neither bacterial and fungal diversity was comparable in different sunflower genotypes from different levels of domestication. Notably, they observed decreased prevalence of putative fungal pathogens in modern sunflower cultivars. In another study conducted on *Beta vulgaris*, wild beet *Beta vulgaris* subsp. *maritima* was observed to harbour more distinct and diverse rhizosphere microbiota compared to modern sugar beet *Beta vulgaris* subsp. *Vulgaris* (Zachow et al. 2014). Furthermore, in comparison to the modern sugar beet, the rhizosphere of wild beet that was colonized with bacteria shows higher performance in coping with abiotic stress and reduced performance in coping with pathogens.

It has been hypothesized earlier that strong selection applied during domestication and recent agricultural activities may have also hindered cooperative interactions of plants with their associated microbes in agricultural systems, this is mainly due to the loss of plant genetic features that function in aiding to engage in interactions with microbes and also due to a decrease in microbial diversity in soil (Pérez-Jaramillo, Mendes, and Raaijmakers 2016). This hypothesis is based on the assumption that plants and their microbiota coevolve by selection favoring microbes that show beneficial effects on the host.

The arbuscular mycorrhizal fungi (AM) and plant interactions are the most prevalent mycorrhizal association in both natural and agricultural environments. Plants devote 4-20% of their photosynthetically fixed carbon to its AM partner (Bago et al. 2000). AM fungi offset this by providing nutrients and water and by protecting plants against pathogens (Sawers et al. 2008). Crops still maintain this ancient symbiosis. However, (Martín-Robles et al. 2018) and colleagues suggested that domestication may have caused a reduction in the efficiency of the symbiosis (Martín-Robles et al. 2018). A comprehensive analysis of mycorrhizal responsiveness across 27 crop species and their wild progenitors was investigated to examine the cooperativeness of AM in different plants (Martín-Robles et al. 2018). Crops responded differently to the AM symbiosis depending on the level of phosphorus (P) in soils on which those plants were grown. Basically, although wild plants benefited highly from AM symbiosis irrespective of P levels, crop plants showed strong engagement with AM symbiosis only in P-limited soils. This result suggests a breakdown in the efficiency of symbiotic relationships between AM and crop plants as a result of domestication. As speculated by Martin-Robles and colleagues, strong directional selection for high yielding crops during domestication might have limited the reallocation of carbohydrates into AM fungi; therefore breaking down the mutualistic interactions with AM fungi (Martín-Robles et al. 2018). Notably, this study also provides evidence that the impact of domestication in plant-microbe interactions may be dependent on the environmental context, critically the impact may be detectable only under certain environmental conditions.

In line with the study of Martin-Robles and colleagues, research on the breadfruit, *Artocarpus* sp., led to a similar conclusion about the impact of domestication on

cooperativeness of the symbionts. It was shown that the response to mycorrhizal fungi declined along a domestication gradient from wild to recently derived breadfruit cultivars (Xing et al. 2012). In general, roots of domesticated breadfruit were shown to harbour fewer but counterintuitively more diverse communities of AM compared to the wild progenitors. The authors proposed that domestication might have unintentionally selected for generalist host traits in breadfruit that are able to engage in interactions with diverse mycorrhiza, since during domestication these plants have been moving between various sites. Therefore, it was reflected as the high diversity of AM fungi in roots of breadfruit along domestication gradient. Concurrent to the study by Martin-Robles and colleagues (Martín-Robles et al. 2018), the authors related this effect to a shift in the reallocation of resources to AM fungi in domesticated plants. Besides supplying nutrients to plants, AM fungi confer protection against pathogens (Maherali and Klironomos 2007). Therefore, reduced reallocation of AM in domesticated plants may confer a higher extent of susceptibility towards pathogens and less resistance to changing environmental conditions.

Interestingly, similar to the trend observed between wild and cultivated plants, the efficiency of symbiosis was reported to decrease in younger cultivars compared to old cultivars of wheat (Hetrick, Wilson, and Cox 1993). Winter wheat cultivars bred before 1950s were reported to show more consistent and strong mycorrhizal dependence compared to wheat cultivars bred later. This finding presumably reflects the impact of recent agricultural activities on the efficiency of mycorrhizal symbiosis. Another case of a recent human interference in plant symbiosis is legume-rhizobium interactions. Roots of legumes (*Acacia* sp.) are colonized by multiple rhizobia strains that confer nitrogen to the

plant in return of carbon from the plant. Different rhizobia strains can significantly differ in the quality of mutualism (defined as the growth performance of any particular rhizobia-host combination) with legumes up to 10-folds induction in the yield (Burdon et al. 1999). Therefore, legumes apply “host sanctions” against less-effective rhizobia in order to engage in a more efficient symbiotic interaction with rhizobia. However, these host sanctions appear to have been relaxed in modern cultivars of legumes compared to old cultivars, presumably as a result of recently applied plant breeding programmes (Kiers et al. 2007). Symbiotic interactions between legumes and rhizobia have been maintained since the domestication of the host, but the efficiency of the symbiotic interaction may have been altered by recent plant breeding programmes.

In sum, we still lack of a theoretical understanding as to how the evolution of the collective (i.e., plant-microbiome) is differentially altered in response to domestication events. Taken together, abovementioned studies show that there is no consistent pattern of effect of plant domestication on host-microbiota assemblies and on cooperativeness of host-microbe interactions. Microbial assembly and host-microbe interactions in crop plants seem to have been driven by a number of factors: 1) anthropogenic interference (i.e. humans, instead of symbionts, providing necessities to plants), 2) domestication syndrome (i.e., inadvertent selection of non-desirable plant traits alongside of beneficial plant traits such as changes in root architecture), 3) environmental factors that have emerged alongside the agricultural activities (e.g. low diversity of soil), and 4) altered symbiotic relationships as observed in the modern cultivars of legumes. However, in other cases, domestication have promoted selection for improved microbiomes, such as enrichment in Bacteroidetes (Pérez-Jaramillo et al. 2018) and beneficial interactions with

microbiomes as observed in the old cultivars of legumes (Kiers et al. 2007). This study aims to improve our understanding on this fundamental question.

Research aims:

As summarized in the general introduction, domestication has impacted microbial community assembly in crop plants. However, so far no consistent pattern of this impact has been revealed. In this thesis, I aimed to focus on the impact of domestication particularly on the seedborne microbial communities of wheat.

Chapter 1:

The noise introduced by technical factors during experiments for amplicon sequencing may be more accentuated in low microbial biomass and diversity samples. Another challenge in such samples is the amplification of non-target host DNA, which is decreasing the resolution of microbial sequencing. In the first chapter, I aimed to optimize accurate amplicon-based sequencing analysis of seed and axenic seedling samples that have low microbial density and diversity. For this, I compared and optimized pre- and post-PCR methods. I also aimed to eliminate possible contamination sources and bias during experiments; which may greatly affect the results of the study in low microbial biomass samples. To increase the resolution of microbial profiling, I compared the resolution of two 16S rRNA and two ITS regions. Furthermore, I designed interfering primers that can be used in combination with 16S rRNA global primers to enrich bacterial DNA amplification compared to non-target host DNA amplification.

Chapter 2:

The impact of domestication on the plant microbiota has been poorly understood. In this chapter, I investigated the impact of domestication on fungal and bacterial community assembly in different wild and domesticated wheat genotypes. In this regard, I propagated

wild and domesticated wheat seedlings under sterile conditions and in agricultural and natural soil. Thereby, I studied vertically-transmitted and environmentally-acquired part of the wheat metaorganism. By comparing microbial assembly of domesticated and wild wheat, I aimed to reveal how domestication acts on the seedborne microbes colonizing the wheat and the “ability” of the plant to acquire the microbiota from the environment.

Chapter 3:

In this chapter, I aimed to disentangle different factors shaping seedborne bacterial community assembly in wheat. Here, I examine temporal, spatial and within and between plant variation in seedborne bacterial assembly of wheat. For this, I collected seeds originating from the same and different plants from the same field. I aimed to reveal the stochasticity of the bacterial assembly in seeds by comparing the variation among seedborne bacteria from seeds of the same wheat head and from seeds of different plants. In addition, to reveal the extent of competition among seedborne bacterial communities colonizing the plant, I assessed dynamics of the bacterial assembly in the seedlings of the domesticated wheat during early development of the plant and compared it to the microbial colonization of the wild wheat.

Chapter I- unpublished work

Evaluation and optimization of amplicon-based community profiling methods for low microbial biomass and diversity tissues

Evaluation and optimization of amplicon-based community profiling methods for low microbial biomass and diversity tissues

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Abstract

Amplicon-based sequencing has provided immense knowledge on the microbiomes from various habitats. However, processing samples for the sequencing can introduce contamination and biases that may greatly influence the results. Especially, in low microbial biomass and diversity tissues, the impact of the technical factors may be more accentuated. Therefore, these samples merit special attention to obtain more accurate results. Here, we report our efforts to increase the efficiency in the microbial profiling of wheat seeds and axenic seedlings. First, we compare culture-dependent and independent methods to recover fungal diversity in wheat seedlings. Secondly, we demonstrate the importance of systematic usage of negative controls and randomization of samples in all steps of the experiments. Furthermore, we show that choice of primer region for the sequencing of 16S rRNA and ITS marker loci and usage of primers in combination with interfering primers may significantly enrich the intensity of bacterial band. We also report further methods to optimize the homogenization of the samples, DNA extraction and

pooling of the data. Our findings provide insights into handling seedborne microbial communities; which are low in microbial biomass and diversity compared to the environmental samples. We anticipate our method-based study to be a reference point in the sequencing of 16S rRNA and ITS marker loci in other tissues with low microbial biomass and diversity.

Introduction

Amplicon sequencing is nowadays routinely used for the community analysis of microbiomes (Human Microbiome Project Consortium 2012); (Bahram et al. 2018). Microbial diversity of organisms associated with different hosts was previously studied with PCR based approaches, such as clone library methods (Hayashi, Sakamoto, and Benno 2002); (Hodkinson et al. 2012). However, new sequencing technologies opened the possibility to study associated microbes as communities. Marker loci, like Internal transcribed spacer (ITS) or 16S ribosomal RNA loci (16S rRNA), are the typical targets for amplicon sequencing because they are conserved genomic regions in the fungal and bacterial taxa, respectively, but neutrally evolving; therefore highly variable for taxonomic assignment (Schoch et al. 2012); (Neefs et al. 1993). These markers have revealed an immense microbial diversity in different habitats like the global topsoil, the ocean, plant phyllosphere and the human gut (Bahram et al. 2018); (Sunagawa et al. 2015); (Vorholt 2012); (Human Microbiome Project Consortium 2012).

Despite the immense opportunities that amplicon-based microbial studies provide, the experimental procedures are rife with risks of contamination and bias (Salter et al. 2014); (Sinha et al. 2015); (Pollock et al. 2018). Each methodological step, from sterilizing of the samples to the data analysis, has the potential to introduce biases in the accuracy of analysis of microbial communities. So far, numerous studies have investigated the impact of different factors in microbiota composition and suggested ways to obtain more accurate results (e.g. storage conditions of the samples, sequencing primer design, choice of DNA polymerase) (Lauber et al. 2010); (Fouhy et al. 2016); (Gohl et al. 2016). For example, template DNA concentration used in the PCR libraries is shown to have an effect on

sample profile variability (Kennedy et al. 2014). Amplification of the non-target host DNA, such as 16S rRNA of the plant chloroplast; is one of the major barriers for the spanning of the microbial diversity in samples, especially in plant samples. (M. T. Agler et al. 2016). Batch effects in non-randomized experiments are found to lead erroneous biological conclusions (Leek et al. 2010). The main outcome of these specific methods studies is that we need to consider each step of microbial profiling to standardize the efficiency, repeatability and reliability of results obtained with amplicon-based sequencing technologies.

Plant microbial communities are largely described by the analysis of 16S rRNA and ITS marker loci (e.g. (Durán et al. 2018); (Matthew T. Agler et al. 2016); (Bulgarelli et al. 2012); (Lundberg et al. 2012)). We know that microbial biomass and diversity differ greatly among different plant tissues. For example, up to 10^8 bacterial cells/gram are estimated to be inhabiting on leaf surface (Vorholt 2012). However, in rhizosphere this number is estimated to be 10^{11} per gram (Berendsen et al. 2012). On the other hand, we hypothesize seeds to be low in microbial biomass since they harbor dormant bacterial cells. Seedborne microbes colonizing plant tissues can be studied in a system where plants are grown under sterile conditions, therefore not colonized by environmental microbiota but only the seedborne. We also hypothesize that leaves and roots of these plants (hereafter called as “axenic leaves” and “axenic roots”) should be higher in bacterial biomass compared to seeds since after emergence of the plant, microbes proliferate, therefore increase in amounts. Still, axenic leaves and roots should be comprising low microbial biomass compared to environmental samples since they are only colonized by seedborne microbes.

We need to increase the efficiency of amplicon sequencing protocols to span microbial diversity and composition in tissues with low microbial biomass and diversity. Most of the studies investigating seed-associated microbial communities, processed samples by pooling multiple seeds (e.g. (Klaedtke et al. 2016); (Rezki et al. 2018); (Rybakova et al. 2017)). However, analysis of individual seeds is crucial for the comprehensive and accurate inferences about the microbial community dynamics. This will allow analyses of microbial variation within and between individual plant seeds; hence evaluation of the consistency of variation in each seed.

Another challenge in low microbial biomass tissues is that contamination may constitute a substantial part of the amplified DNA and therefore greatly affect the results of the study. Contamination may originate from various sources: Lab instruments, especially in labs working with different microbial organisms, reagents, human microbiome, cross contamination from other samples and unknown origin. For example, recent studies claimed that human placenta has its own low biomass bacterial community (Zheng et al. 2015) (Aagaard et al. 2014). However, a control study demonstrated that microbial communities of placenta samples and sets of different negative controls were indistinguishable from each other (Lauder et al. 2016). This finding suggested that the described placental microbiome originated from experimental contaminations. Indeed, a recent study found that there is no microbiome in the placenta although placenta may harbour pathogens (Goffau et al. 2019). This example supports that high caution should be taken with low microbial biomass tissues.

Here we aimed to optimize accurate microbiota analysis of plant material that have low microbial density. We developed and optimized a protocol for microbial profiling of seed and axenic seedlings of wheat. First, we compare different growth conditions for culture-dependent isolation of seedborne fungi and confirm that fungal diversity cannot be recovered by culture dependent methods, but rely on culture-independent studies, such as amplicon sequencing. Secondly, we report on methods to detect and eliminate possible contamination sources in low microbial biomass tissues. We provide suggestions for the design of experiments and emphasized the importance of randomization to overcome biases stemming from batch effects, especially in low microbial biomass tissues. Thirdly, we show methods to increase the efficiency of DNA extraction to detect microbial DNA from single seeds and axenic seedlings. We compare the resolution of two 16S rRNA and two ITS regions, and we present interfering primers that can be used in combination with 16S rRNA global primers to overcome host DNA contamination problem. With our approach we can demonstrate an enrichment of the bacterial DNA compared to the host DNA when using interfering primers.

With our pipeline, we were able to obtain higher amplification of the bacterial DNA with the V5-V7 region combined with interfering primers and of the fungal DNA with the ITS1 region. Finally, we suggest to consider length variation in ITS amplicons while pooling the samples at equal concentrations. These results and the optimized pipeline will be valuable for researchers planning future amplicon sequence-based microbial profiling in material with low microbial biomass.

Materials & Methods and Results:

Culture dependent versus independent methods

Microbial diversity in plant microbiomes has been elucidated both by culture-dependent and independent methods (e.g. the 16S and ITS loci). The major limitation of the culture-dependent studies is the part of the microbiome that is not culturable. Nevertheless, we still need microbial colonization assays to expand our knowledge on plant microbiomes from community level analysis to the plant-microbe and microbe-microbe interactions. Therefore, protocols to isolate microbes from different host samples are crucial for experimental studies and hypothesis testing in microbiome research.

Here, we isolated fungi from axenic leaves and roots of the domesticated wheat *Triticum aestivum* and the wild wheat *Triticum dicoccoides* (**Figure 1**). To this end, we harvested axenic seedlings grown for two weeks. We performed the isolation from different media incubated under different conditions: Media used were water, oat, maize, corn meal, rye, %2 malt extract, Mathur's, Czapek-dox, dYT, Sabouraud dextrose, potato dextrose agar (PDA), Reasoner's 2A (R2A) and V8 medium (**Table 1**, see **Supp. Material** for further information). For some of the samples, we crushed the leaves in a Precellys machine (Bertin Instruments, Montigny-le-Bretonneux, France) (6500 rpm, 30 seconds X2 cycles), diluted in 2 mL water and inoculated 100 μ L of the diluted sample to the medium. For others, we cut the samples with a sterilized scissor into 0.5 cm pieces and transferred pieces to different growth media. We incubated the plates under different light conditions (in the dark, 12h light-12h dark cycles in the phytochamber, room light) and temperatures (room temperature and 15 °C).

After observing the growth of fungal colonies, we transferred the colonies from each medium tested into a new medium with antibiotics (Chloramphenicol, 20ng/ μ L).

Fungal colonies were isolated from the roots and incubated in the dark. We managed to isolate fungi only from crushed roots of *T. dicoccoides*. Also, the isolation of fungi from crushed roots was slightly more efficient than from roots cut into pieces (58% of the root samples that we were able to isolate fungi, were crushed samples rather than root pieces). Also, in room temperature, we were able to grow more colonies and faster than 15 °C. However, we were able to grow only one fungal colony from the leaves, which was from *T. aestivum* from the crushed sample grown in the V8 medium in the room conditions.

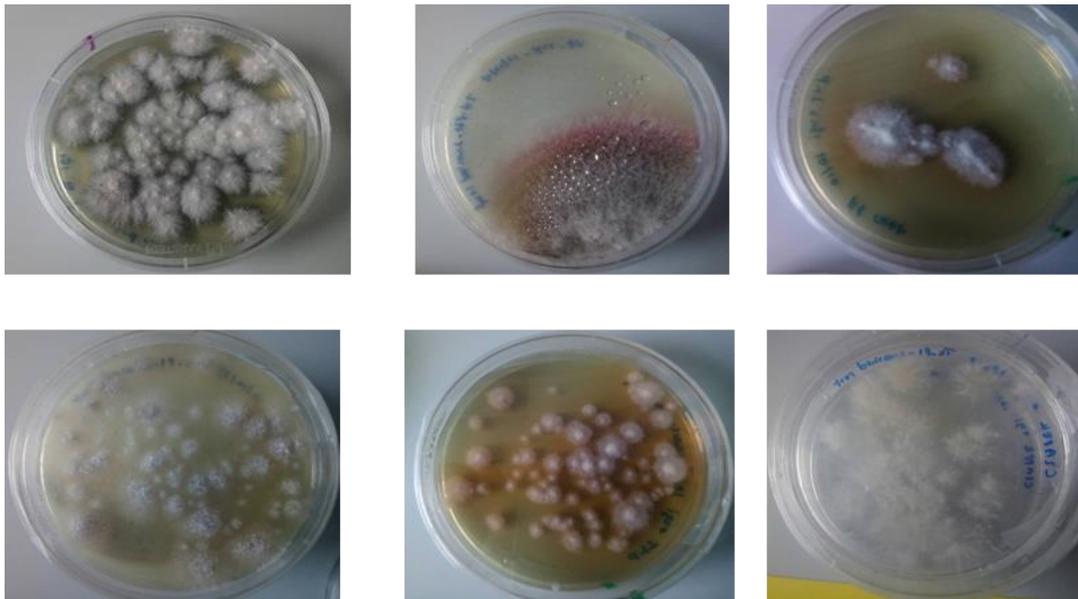


Figure 1: Fungi isolated from axenic seedlings of wheat. We tested different media and conditions to isolate fungi.

We extracted DNA from the fungal isolates by using Qiagen DNA extraction kit (QIAGEN, Hilden, Germany). For this, we first mildly homogenized the fungi in the Precellys (4800 rpm, 15 seconds) and followed the custom protocol of the kit. The isolated fungi were identified by Sanger sequencing. All the DNAs isolated corresponded to the different strains of *Alternaria*; which is the most abundant fungus in different tissues of wheat (Ofek-Lalzar, Gur, and Ben-Moshe 2016).

Eliminating possible bias and contamination sources in low microbial biomass tissues

First step to eliminate contamination is the surface sterilization of the samples. In our studies, to ensure examination of only endophytes or microbes tightly attached to the surface, we mildly washed surface of the wheat seeds. We soaked the seeds shortly in 0.1% Tween20 (Carl Roth GmbH, Karlsruhe, Germany), 80% EtOH (Carl Roth GmbH, Karlsruhe, Germany), 1.2 % NaOCl (Carl Roth GmbH, Karlsruhe, Germany) and washed with water three times. This mild way of sterilization allowed us to keep not only endophytes but also epiphytes that may also have role in the early development of the plant. After the sterilization, we preserved water samples from the last washing step (hereafter called as “wash-off”) and plated them on bacterial and fungal growth medium to examine the efficiency of sterilization (**Figure 2**). Confirming the efficiency of sterilization, we did not detect growth of any colony in the mediums with the wash-offs (**Figure 3**).

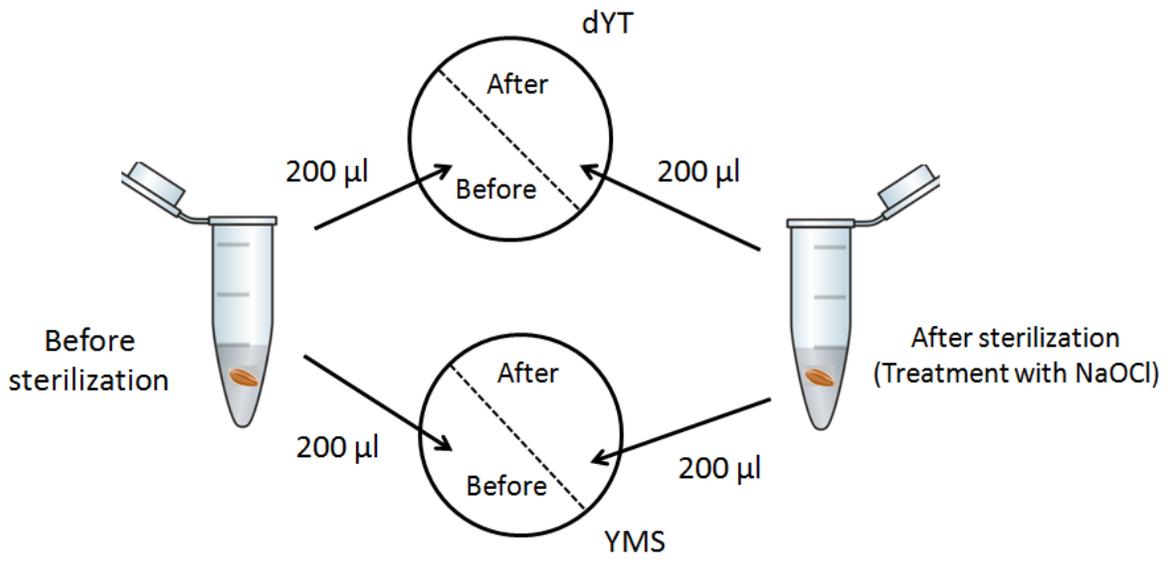


Figure 2: Summary of the surface sterilization step of seeds. Plates contain dYT and YMS growth medium for bacteria and yeast, respectively. "Before" denotes for the plating of the wash-off originating from the first washing of seeds. This is used as a positive control for the sterilization step. "After" denotes for the plating of the wash-off originating from the last washing step, so it is a negative control for the growth of the microbe after the sterilization.

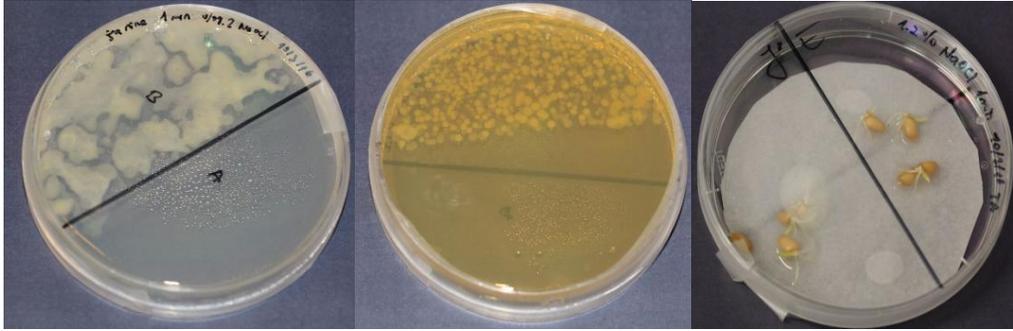


Figure 3: Microbial growth assay for surface sterilization A-B): YMS and dYT plates where growth of bacteria and fungi occurred before sterilization (positive control) while after sterilization there is no growth of any microbe **C)** After sterilization, seeds were able to germinate in a healthy way; which shows sterilization reagents do not leak in the embryo.

Secondly, sequencing of the negative controls from all the steps of the protocols alongside the actual samples is crucial to control for possible contamination from all steps of the experiment; thus for the accurate microbial profiling. For instance, although there was no growth of microbe from the wash-off of sterilization (**Figure 3**), it is possible that there are still uncultured microbes or microbial DNA in the wash-off water. For this reason, we furthermore sequenced the wash-off water alongside samples. In the Chapter 2, I randomly selected three negative controls started from wash-off of the surface sterilization and used as a control until the end of the sequencing. In the Chapter 3, I mixed three negative controls that were randomly selected from three batches of the experiment and sequenced this pooled control sample alongside my samples. I hereby document that, after the removal of the low quality reads and chimeras, no reads remained from the negative controls. This confirmed the absence of any contaminant

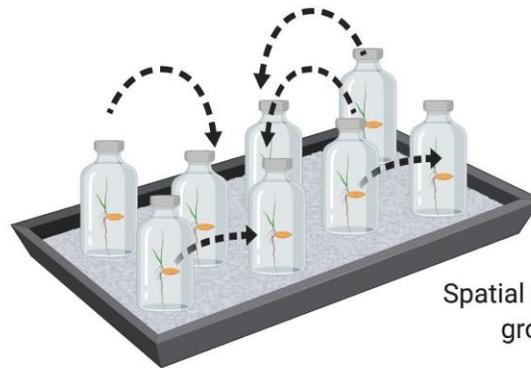
from sterilization, extraction and library preparations that may biased the downstream analysis.

Lastly, batch effects may influence comparisons of microbiome compositions, especially in low microbial biomass tissues. Microbial communities of samples may be more similar to each other compared to the communities of other samples simply because they are processed together (e.g. (Goffau et al. 2019)). To this end, randomization of the samples during all the steps of the microbial profiling can reduce or prevent such batch effects. In our studies, we randomized the location of the growth jars in the growth chamber during the experiments to prevent growth bias stemming from possible variation of the light intensity in the climate chambers (**Figure 4**). Furthermore, we randomized the batch of the samples in DNA extraction. Finally, we randomized the location of the samples in the PCR plates during library preparations. This allowed us to prevent possible batch effects in our final datasets; which was confirmed in downstream data analysis as well. Basically, we did not observe clustering of microbial communities according to DNA extraction, library preparation and sequencing batches.

Surface sterilization

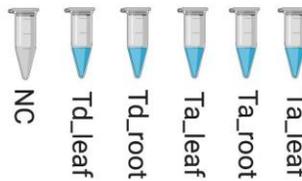
Wash-off water from the sterilization is processed alongside samples as a negative control.

Growth of plants



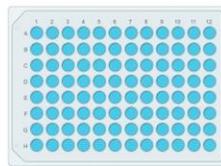
Spatial randomization of growing plants

DNA extraction



Randomization of the plant species and tissues

DNA library preparations



Randomization of the DNA extracts from different batches

Figure 4: Randomization of the samples during microbial profiling. Firstly, seeds were surface sterilized and wash-off water was processed in the next steps alongside plant samples. Sterilized seeds were germinated under sterile conditions in climate chambers. The location of each plant was randomized during growth of the plants to prevent any spatial bias happening in the climate chamber. Harvested plants were processed for DNA extraction. In each batch of the extraction, plant species and tissues used were randomized. Finally, DNA samples coming from different batches were randomized in library preparations.

Sample Homogenization and DNA Extraction

In our study, we show that homogenization of the samples before DNA extraction is an important factor impacting the quality of the amplified microbial DNA in the next step. Effective lysis of the plant tissue and cell wall is required for efficient microbial DNA amplification. On the other hand, vigorous homogenization of the plant sample may also lead to unwanted shearing of DNA (**Figure 5**, first two DNA samples). This effect could potentially decrease the efficiency of the microbial DNA amplification. Therefore, the intensity of the homogenization has to be optimized in a way that prevents shearing host DNA since the sheared DNA can interfere with microbial DNA amplification library preparations, but destroying the tissues.

As suggested by Agler et al. (M. T. Agler et al. 2016), we performed homogenization of the samples in two steps using a Bertin Precellys Instrument (Bertin Instruments, Montigny-le-Bretonneux, France) pre-cooled with liquid nitrogen: First, we disrupted plant material using 4 mm zirconium beads (7500 rpm for 2 x 30 seconds with an in-between pause of 15 seconds). In this step, we simply disrupted the wheat tissue. After treating the samples with Proteinase K and Lysozyme, we next performed a second homogenization where we used 0.1 mm zirconium and 0.5 mm glass beads (6500 rpm for 2 x 30 seconds with a 15 second pause). This step allowed the disruption of the cell wall. We isolated genomic DNA from individual seeds following an extended phenol-chloroform extraction method (see **Supp. Material**) (**Figure 5, 1st gel**). The DNA concentration was varying considerably between samples, however we obtained in general between 9- 54 ng/ μ L per individual seed. After extraction, we cleaned-up the seed DNA using 20% Chelex-100 (Sigma-Aldrich, Taufkirchen, Germany). We show that we can increase the amount of DNA when

we treat the seed DNA with Chelex-100 (**Figure 5, 2nd gel**). We also attempted to isolate DNA from individual seeds by using FastDNA Spin Kit for Soil (Mo Bio Laboratories, Heidelberg, Germany); however with this kit, we failed to isolate any DNA.

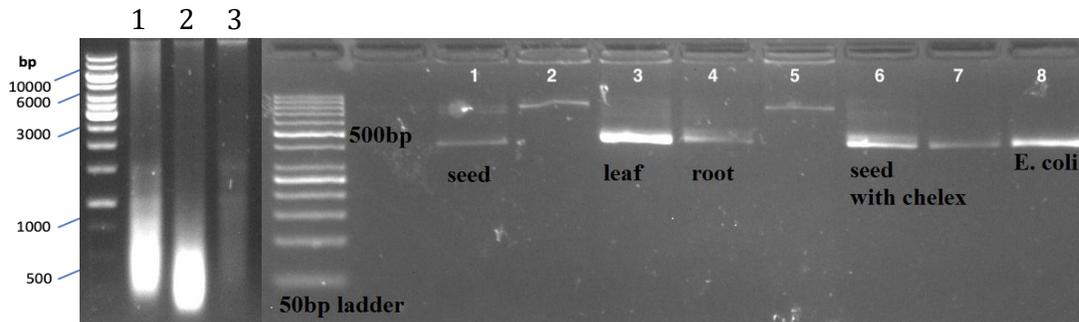


Figure 5: Intensity of homogenization of the plant sample and usage of Chelex-100 are the factors impacting DNA quality. In the first gel, the bands in the lanes 1 and 2 were homogenized with higher intensity program of Precellys compared to the band in the lane 3. As a result, we observe shearing of DNA, seen as bright bands at the 250-750 bp size in the first two samples. In the second gel, we observe that DNA from the same seed sample treated with Chelex-100 (lane 6) results in amplification of the brighter bacterial band compared to non-treated sample band (lane 1). Order of the DNA samples in each lane in the second gel: 1) seed sample non-treated with Chelex-100 amplified with interfering primers 2) leaf sample amplified without interfering primers 3) leaf sample amplified with interfering primers 4) root samples amplified with interfering primers 5) root sample amplified without interfering primers 6) the same seed sample with the lane 1, but treated with Chelex-100 before amplification with interfering primers 7) seed sample amplified with the interfering primers 8) DNA extracted from the *Escherichia coli* isolate used as a positive control.

We also attempted to isolate genomic DNA from axenic leaves and roots with the phenol-chloroform protocol. Although we obtained sufficient concentrations of genomic DNA, we were not able to standardize amplification of microbial DNA; only few samples worked at

the end of the experiment. Remains of the phenol or chloroform after the extraction step may have prevented microbial amplification of the samples. For leaves and roots, we instead use the FastDNA Spin Kit for Soil combined with pre-filled Lysis Matrix E tubes. The concentration of DNA varied between samples ranging between 7- 95 ng/ μ L.

Ultimately, the best methods were the phenol-chloroform method for the extraction of DNA from single seeds and the FastDNA Spin Kit for Soil with Lysis Matrix E for DNA extraction from axenic leaves and roots.

Usage of interfering primers to prevent host contamination

Co-amplification of host DNA is one of the major challenges in plant microbiome studies (M. T. Agler et al. 2016). The V3-V4 and V5-V7 regions of the bacterial 16S rRNA locus are hypervariable regions; therefore often used in plant microbiome studies to assess bacterial diversity (Xu et al. 2018); (Durán et al. 2018) (**Figure 6**). However, chloroplast and mitochondrial DNA can co-amplify with the targeted V3-V4 and V5-V7 region of the bacterial 16S rRNA. In this case, especially if the microbial biomass is low, such as for axenic plant tissues, amplicon sequencing will produce mainly plant sequences. For example, Robertson and colleagues amplified DNA from the wheat seedlings, which resulted in two different amplicon sizes: One plant product at the size of 1,100 bp and one bacterial product at the size of 625 bp (**Figure 7**) (Robinson et al. 2016). Bacterial band was much less abundant than plant band. This made culture-independent sequencing of the seedborne microbiome impossible in this study.

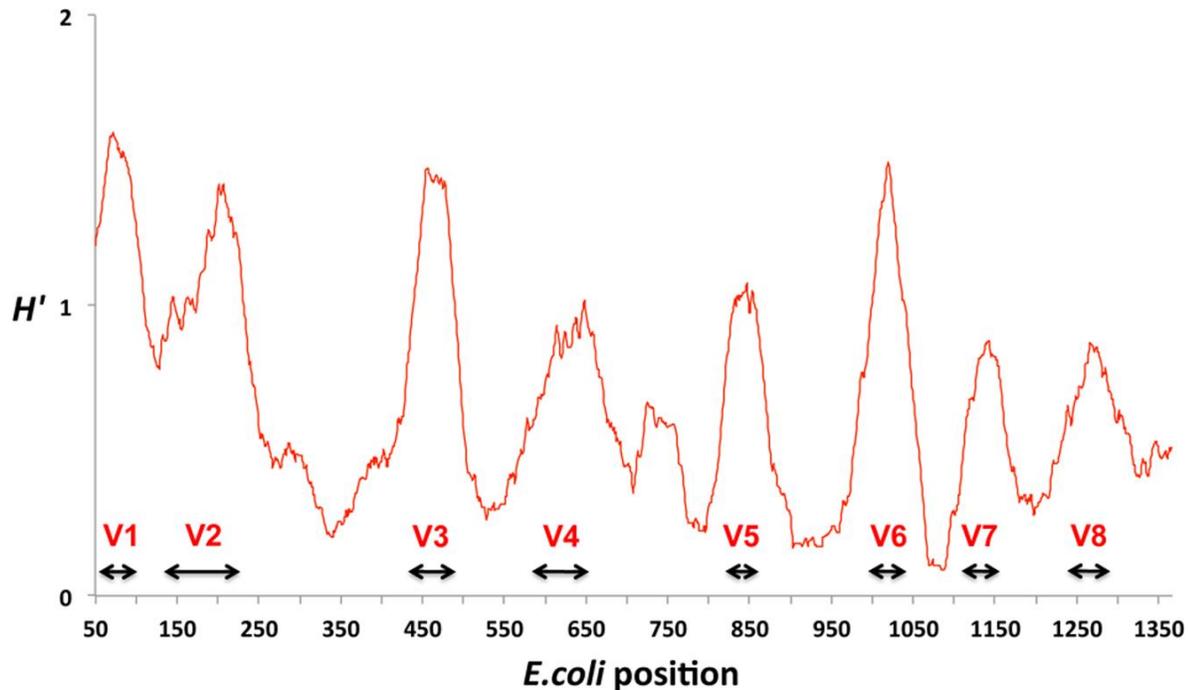


Figure 6: Figure adapted from Vasileiadis et al. 2012 (Vasileiadis et al. 2012). H' denoted Shannon entropy values per nucleic acid base position of the *E.coli* genome.

In this study, we addressed the possible elimination of the co-amplification of plant DNA with interfering primers. Specifically, we used global primers (V3-V4: 341F-806R and V5-V7: 799F-1192R) in combination with interfering primers targeting the V3-V4 and V5-V7 region of the 16S rRNA locus of the host to hinder co-amplification of the wheat chloroplast and mitochondria, respectively (see **Supp. Material**). For this, we followed the interfering primer approach originally developed by Agler and colleagues for *Arabidopsis thaliana* (M. T. Agler et al. 2016). However, we edited the interfering primer sequence based on the corresponding loci.

In a trial sequence, we sequenced both the V3-V4 and V5-V7 region of the 16S rRNA locus amplified from seeds and axenic leaf and axenic root samples. We sequenced 17 samples

in a single-end sequencing MiSeq run for 600 cycles and obtained in total 63866 and 46030 reads respectively for each region. To analyze the percent reduction of co-amplified plant reads when using interfering primers, we calculated the relative abundance of reads assigned to the bacteria kingdom based on the Greengenes database (DeSantis et al. 2006).

Amplification of the V5-V7 region results in plant and bacterial bands of different sizes; therefore making size differentiation of the two product possible on a gel (**Figure 8**). However, the V3-V4 region does not differentiate plant and bacterial bands by size. Based on this, we were able to detect enrichment of the bacterial band only using interfering primers for the V5-V7 region. We excised the enriched bacterial band and sequenced only DNA extracted from this product. Successfully, 97% of the sequenced were bacteria-derived sequence (**Figure 8**). However, we did not succeed with amplification of the V3-V4 region with the interfering primers. Therefore, we used only the V5-V7 region for the further experiments.

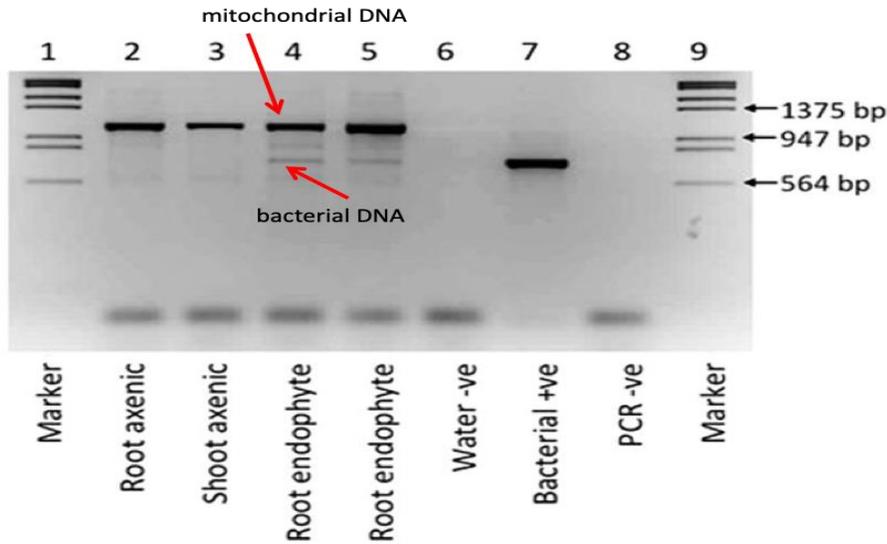


Figure 7: Figure adapted from Robinson et al. 2016. 799f and 1492r primers were used to amplify two products from shoots and roots: a plant mitochondrial product (~1,100 bp) and a bacterial 16S rRNA locus product (~625bp).

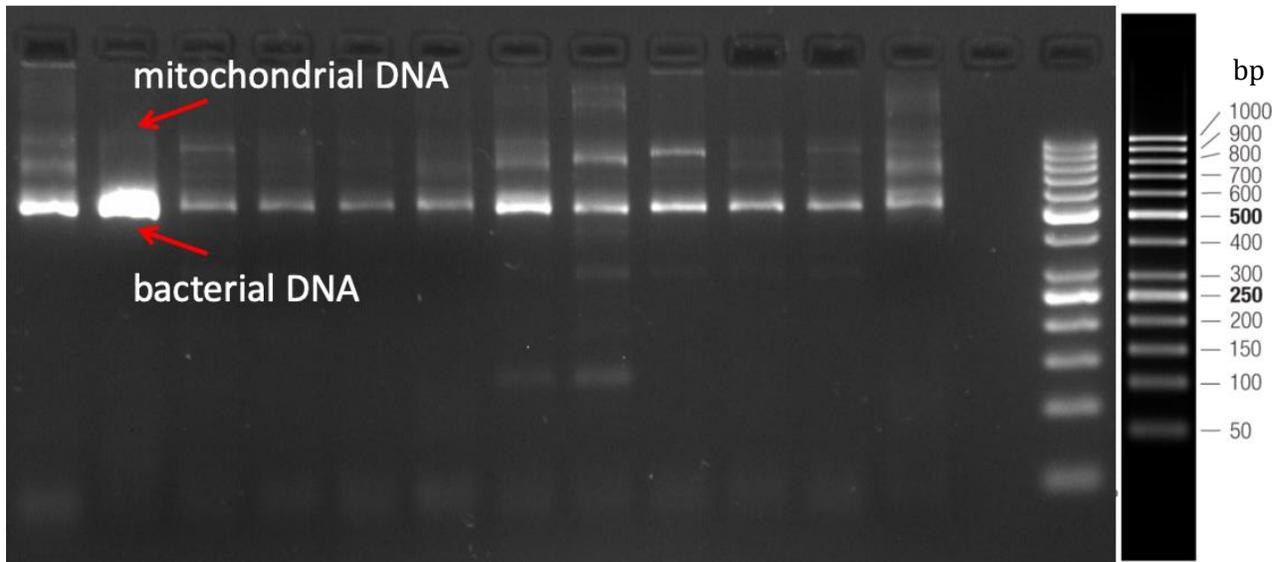


Figure 8: Interfering primers provided the enrichment of bacterial DNA compared to the plant microbial product in wheat seedlings. V5-V7 region of the 16S rRNA locus combined with the designed interfering primers were used to amplify DNA from wheat seedlings.

ITS1 and ITS2 are hypervariable regions of the ITS locus and used in mycobiome studies to reconstruct fungal diversity (Knorr, Jørgensen, and Nicolaisen 2019); (Xu et al. 2018). Co-amplification of the plant DNA can also occur by amplification of the ITS locus; however this phenomenon is minor compared to the 16S rRNA locus based studies (M. T. Agler et al. 2016). To reveal which regions of ITS provided better resolution for our studies, we amplified and sequenced both the ITS1 (ITS1F, ITS2 primers) and ITS2 (ITS7, ITS4 primers) loci with DNA from seeds and axenic leaf and root samples. We sequenced five and nine samples in a single-end sequencing MiSeq run for 600 cycles and obtained in total 148446 and 37316 reads, respectively, for each region. We assigned taxonomic identities based on the UNITE database (Nilsson et al. 2019). Out of 98 taxa, 13 taxa were not assigned to the Fungal kingdom with the ITS reads obtained from our sequencing. However, all the remaining 85 taxa were assigned to the Fungal kingdom. On the other hand, ITS2 reads were assigned to 157 taxa. However, only 95 of these taxa were not assigned to fungi and plant and 34 of them were assigned to the plant reads. Because of the high number of reads assigned to the plant in the ITS2 region, we continued our experiments with the ITS1 region.

Pooling of the DNA library

Pooling of the amplicons at equal concentrations is important for the equal representation of each sample in the sequencing run, in other words for comparable sequencing depth for each sample. Otherwise, differences among samples in diversity might be simply because of the varying number of reads. For example, in the first library we performed, we attempted to sequence samples from tissues with low (i.e. seeds) and high (i.e.

environmental leaves) microbial biomass together; however this complicated the accurate equimolar pooling of the samples. At the end, we obtained low number and quality of reads from the seed samples compared to the leaf samples.

We pooled samples from each PCR plate in equimolar concentrations after quantification of the band intensity on the gel using the software of gel visualizer (BIO RAD, Image Lab™ software 5.2.1). Pools of 16S amplicons were run on the gel and bacterial DNA was cut out of the gel. Therefore, we excised the bacterial band and sequenced only this band. However, the size of the ITS1 locus varies among fungal taxa in contrast to the 16S rRNA locus that is conserved with respect to size among bacteria (e.g. size of the ITS1 region: *Saccharomyces cerevisiae*: 361 bp; *Schizosaccharomyces pombe*: 412 bp) (Korabecna 2007). Therefore, excising gel and sequencing only one band causes deflation of fungal diversity. For this reason, instead of excising the gel, we used the Macherey-Nagel PCR clean-up kit (Macherey-Nagel, Düren, Germany) to eliminate small plant-derived bands and primer dimers in ITS sequencing. The clean-up kit provides a DNA binding buffer that allows to customize the size of the DNAs to be cleaned up. Increasing dilution of the buffer allows to the cleaning of larger DNA fragments. We diluted the buffer to 1:6; whereby we can eliminate non-target DNA but still cover fungal diversity with different sizes of the ITS1 for amplicon sequencing.

Discussion

In this study, we present a pipeline for the accurate analysis of microbial diversity in seeds and axenic seedlings of wheat. First of all, we show that plant microbial community analysis requires culture-independent methods since culture-dependent detection of fungal community gave low number of fungal isolates. Moreover, we show the importance of systematic usage of negative controls and randomization of samples in all steps of the experimental procedure to decrease the contamination and batch effects in the resulting microbial data. Importantly, we propose that best amplification practice should include usage of global primers in combination with interfering primers binding to the homogenous host DNA; thereby enriching the bacterial DNA. We propose that V5-V7 region in combination with interfering primers designed here and the ITS1 region works well to span bacterial and fungal diversity, respectively. We also propose that homogenization of the samples should be optimized with different combinations of bead types and intensity of the beating and be tested for the yield of microbial DNA amplification.

It was recently demonstrated that fungal and bacterial diversity in the seedborne microbiome in wheat is more diverse than suggested by previous studies using both culture-dependent and independent methods (Ofek-Lalzar, Gur, and Ben-Moshe 2016); (Robinson et al. 2016). Ofek-Lalzar and colleagues reported 11 and nine fungal OTUs in multiple seeds of *T. aestivum* and *T. dicoccoides*, using culture-dependent methods; where *Alternaria* spp. was the most prevalent fungal taxa (Ofek-Lalzar, Gur, and Ben-Moshe 2016). Robinson and colleagues isolated only eight genera of bacteria from multiple wheat seeds, dominated by *Erwinia* and *Paenibacillus* (Robinson et al. 2016). In

this study, we were able to culture only several strains of *Alternaria* spp. from multiple wheat seedlings. These are very low numbers compared to the culture-independent analysis of seed microbial communities (Özkurt et al., 2019). Interestingly, as suggested by Robinson and colleagues, antimicrobial puroindolines and high concentrations of phenolic acids existing in the endosphere of wheat seeds may be hindering efficient culturing of microbes associated with seeds (Robinson et al. 2016). Another caveat of culture-dependent methods is that they do not provide information regarding to the abundances of microbes. Thereby analyses of evenness in diversity is impossible. Overall, these findings convincingly show that comprehensive analysis of plant microbial communities require culture-independent methods.

Based on our findings reported here, we also recommend processing negative controls from the first step of the microbial profiling to the last step and sequencing them alongside samples to avoid contaminants and false positive community members. In our datasets, we detected no bacterial or fungal reads amplified from negative controls; showing that seed microbiota did not originate from experimental contaminations. Also, randomization of samples in all steps of the microbial profiling is crucial, especially in low microbial biomass tissues since they are more prone to bias than environmental samples.

Resolution of the amplicon sequencing data is impacted by the region of the marker locus used (Yang, Wang, and Qian 2016). The V3-V4 region of the 16S rRNA locus is widely used in human microbiome studies since it provides high resolution of bacterial taxa (Flemer et al. 2017); (Ross et al. 2018); (Shipitsyna et al. 2013). However, when used in the microbial amplification of plant microbiota, the size of the band that is co-amplified

from the plant chloroplast is indistinguishable from the bacterial band. Therefore, the usage of the V3-V4 primers is impossible for plant samples without interfering primers that prevent amplification of the wheat chloroplast DNA.

There is size differentiation between bacterial and corresponding wheat loci for the primers targeting the V5-V7 region. Here, we aimed to compare the utility of the V3-V4 and V5-V7 regions using them in combination with interfering primers. Unfortunately, we could not make the interfering primers that were designed to target the V3-V4 region of the host work; and all reads were plant sequences rather than bacterial sequence. Therefore, usage of these primers was not an option for our experiments. However, we could successfully amplify and separate the V5-V7 region in combination with interfering primers for the plant samples.

Interference of host DNA also occurs in fungal ITS amplifications; however this interference is less compared to the 16S rRNA amplification. Therefore, we compared the two variable regions ITS1 and ITS2 of the ITS locus without interfering primers. We found that ITS2 reads to a high extent were assigned to plant sequences, and we therefore continued our experiments with the ITS1 region. We also suggest not to excise bands from the gel for the ITS region but instead recover all the fungal bands at different sizes. We propose here that the V5-V7 region in combination with interfering primers and ITS1 region are optimal for microbial profiling of wheat.

To summarize, we reported an optimized and tested pipeline for generation of amplicon data from wheat seeds and axenic seedlings. Based on these methods, we were able to increase the level of microbial resolution to the detection of microbes in individual samples. We anticipate that our pipeline can apply to the microbial profiling of other low microbial diversity and low microbial biomass tissues and opens new perspectives in handling these.

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Supplementary material

Preparation of the growth media for the isolation of fungi (for 1L of medium):

R2A medium: 0.500 gram of casein acid hydolysate, 0.500 gram of yeast extract, 0.500 gram of protease peptone, 0.500 gram of dextrose, 0.500 gram of starch, 0.300 gram of K_2PO_4 , 0.024 gram of $MgSO_4$, 0.300 gram of sodium pyruvate and 15.000 gram of agar

dYT medium: 8.0 gram of tryptone, 5.0 gram of yeast extract and 2.5 gram of NaCl

V8 medium: 150 mL of V8 juice, 3 gram of $CaCO_3$ and 15 gram of agar

Corn meal medium: 2 gram of corn meal fusion and 15 gram of agar (Sigma-Aldrich, Taufkirchen, Germany)

%2 malt extract medium: 20 gram of malt extract and 15 gram of agar

Mathur's medium: 2.00 gram of peptone, 1.22 gram of $MgSO_4$, 2.72 gram of K_2HPO_4 , 2.80 gram of sucrose and 20 gram of agar

Water medium: 15 gram of agar

Czapek-dox medium: 50 gram of Czapek-dox agar mix (Sigma-Aldrich, Taufkirchen, Germany)

PDA medium: 39 gram of PDA mix (Sigma-Aldrich, Taufkirchen, Germany)

Sabouraud dextrose medium: 30 gram of Sabouraud dextrose agar mix (Sigma-Aldrich, Taufkirchen, Germany)

Oat medium: 60 gram of oat fusion and 15 gram of agar

Rye medium: 60 gram of rye fusion and 15 gram of agar

Supp Table 1: Overview of the growth media that we were able isolate fungi

	Root pieces	Crushed Root	Leaf pieces	Crushed Leaf
Water	X	X		
R2A	✓	X		
V8	✓	✓		✓
Mathur's	✓	✓		
Oat	✓	✓		
Rye	X	X		
Corn meal	✓	✓		
PDA	✓	✓		
Czapek-dox	✓	✓		
Sabouraud dextrose	✓	✓		
dYT	✓	✓		
%2 malt	✓	✓		

Homogenization and DNA Extraction from single seeds:

We transferred seed samples into 7mL tubes containing four of 2.5 mm sterilized zirconium beads. We cooled a Bertin Precellys Instrument (Bertin Instruments, Montigny-le-Bretonneux, France) with a liquid nitrogen poured inside the Cryolys unit (Bertin Instruments, Montigny-le-Bretonneux, France). We crushed samples at 7500 rpm

for 2 x 30 seconds with an in-between pause of 15 seconds. We added 0.6 mL of DNA extraction buffer (0.5% SDS, 50 mM TRIS buffer at pH 8, 200 mM NaCl, 2mM EDTA) and 50 µg/mL of Lysozyme and 10 µg/mL of Proteinase K to the crushed samples. Next, we incubated the samples for 45 min at 37°C. After incubation, samples were transferred into new tubes containing 200 mg of 0.1 mm zirconium and 200 mg of 0.5 mm glass beads and homogenized the samples again with a Bertin Precellys instrument (Bertin Instruments, Montigny-le-Bretonneux, France) (6500 rpm for 2 x 30 seconds with a 15 second pause). After the second homogenization, we added 10 µg/mL RNase to each sample and incubated at 37°C for 45 minutes. After the incubation, we centrifuged the samples at 13000 rpm for 2 minutes to remove beads and non-homogenized tissue. We cleaned up the nucleic acids with phenol/chloroform/isoamyl alcohol (25:24:1) for three times, and chloroform/isoamyl alcohol (24:1) one time, then precipitated by adding 1/10th of the samples volume of 3 M sodium acetate and 2.5x volume 100% ethanol. We then centrifuged at 4°C at 13000 rpm for 30 minutes. Afterwards, we washed the pellet with 70% ethanol. Finally, we washed the pellets in 100 µL of sterile water and stored at -20 °C until further usage. As a final step, we treated the extracted DNA extracted with 1:1 with 20% Chelex-100 (Sigma-Aldrich, Taufkirchen, Germany) for 30 minutes to remove potential PCR inhibitors.

16S rRNA and ITS universal and interfering primers:

Bacteria - 16S V5-V7	Primer ID	Primer sequence
Forward	799F	AACMGGATTAGATACCCCKG
Reverse	1192R	ACGTCATCCCCACCTTCC
Bacteria - 16S V3-V4	Primer ID	Primer sequence
Forward	B341F	CCTACGGGAGGCAGCAG
Reverse	B806R	GGACTACHVGGGTWTCTAAT
Fungi - ITS1	Primer ID	Primer sequence
Forward	ITS1F	CTTGGTCATTTAGAGGAAGTAA
Reverse	ITS2	GCTGCGTTCTTCATCGATGC
Fungi - ITS 2	Primer ID	Primer sequence
Forward	fITS7	GTGARTCATCGAATCTTTG
Reverse	ITS4	TCCTCCGCTTATTGATATGC
Chloroplast - 16S V3-V4		Primer Sequence
Forward	V3CBF	GAGGTGGAAGGCCTACGGGTCGTCAA CTTC
Reverse	V3CBR	TGTCAGTGTCGGCCCAGCAGAGTGCT TTCG
Mitochondria - 16S V5-V7		Primer Sequence
Forward	V5MBF	GGATCAGGGGCCAGCTAACGCGTGA AACA
Reverse	V5MBR	CGGAGCGGGCGCGTACTATTACCAC TACG

Conditions for the amplification of marker genes:

ITS sequences			V5/7 Nested PCR		
Temp	Time	Cycle	Temp	Time	Cycle
98°C	30 sec		98°C	30 sec	
98°C	10 sec	<u>1st PCR:</u> X 20 <u>2nd PCR:</u> X15	98°C	10 sec	<u>1st PCR:</u> X 15 <u>2nd PCR:</u> X20
55 °C	30 sec		55°C	45 sec	
72 °C	30 sec		72 °C	30 sec	
72 °C	5 min		72 °C	5 min	
4 °C	∞		4 °C	∞	

Chapter II- preprint

Higher stochasticity of microbiota composition in seedlings of domesticated wheat compared to wild wheat

1 **Higher stochasticity of microbiota composition in seedlings of domesticated wheat**
2 **compared to wild wheat**

3

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23 **Keywords**

24 Microbial profiling, seedborne plant microbiota, endophytes, *Triticum aestivum*,
25 domestication, co-adaptation

26 **Abstract**

27 Plants constitute an ecological niche for microbial communities that colonize different
28 plant tissues and explore the plant habitat for reproduction and dispersal. The
29 association of microbiota and plant may be altered by ecological and evolutionary
30 changes in the host population. Seedborne microbiota, expected to be largely vertically-
31 transferred, have the potential to co-adapt with their host over generations. Reduced
32 host diversity because of strong directional selection and polyploidization during plant
33 domestication and cultivation may have impacted the assembly and transmission of
34 seed-associated microbiota. Nonetheless, the effect of plant domestication on the
35 diversity of their associated microbes is poorly understood. Here we show that microbial
36 communities in domesticated wheat, *Triticum aestivum*, are less diverse but more
37 inconsistent among individual plants compared to the wild wheat species, *T.*
38 *dicoccoides*. We found that diversity of microbes in seeds overall is low, but comparable
39 in different wheat species, independent of their genetic and geographic origin.
40 However, the diversity of seedborne microbiota that colonize the roots and leaves of
41 the young seedling is significantly reduced in domesticated wheat genotypes. Moreover,
42 we observe a higher variability between replicates of *T. aestivum* suggesting a stronger
43 effect of chance events in microbial colonization and assembly. We also propagated wild
44 and domesticated wheat in two different soils and found that different factors govern
45 the assembly of soil-derived and seedborne microbial communities. Overall, our results
46 demonstrate that the role of stochastic processes in seedborne microbial community
47 assembly is larger in domesticated wheat compared to the wild wheat. We suggest that
48 the directional selection on the plant host and polyploidization events during
49 domestication may have decreased the degree of wheat-microbiota interactions and
50 consequently led to a decreased stable core microbiota.

51

52 **Introduction**

53 Plants coexist with a large diversity of microorganisms. Most of the plant-associated
54 microbiota is acquired from the environment, while a smaller component is vertically
55 inherited e.g. via the seed (1), (2). Plant-microbe interactions range from parasitic or
56 neutral to beneficial whereby the microbiota can contribute to increased nutrient
57 uptake, stress tolerance and disease resistance (3), (4), (5). There is a growing attention
58 on plant microbiota and its role in the future improvement of agricultural plant
59 production. However, the underlying plant traits that govern plant microbial assembly
60 and maintenance are still poorly understood.

61 Interactions and co-evolution of plants with their associated pathogens and mutualists
62 have been intensively studied (6), (7), (8). It is well-known that the plant immune system
63 plays a fundamental role in the interaction with both pathogens and mutualists (9), (10).
64 Consequently, genes encoding immune related proteins co-evolve with microbial-
65 produced proteins to either abort or facilitate interactions (11). To which extent plants
66 co-evolve with their associated microbiota has so far little been addressed. Plant-
67 microbe co-evolution may be pronounced for seed-associated microbes that co-exist
68 with their host over multiple generations. Seeds constitute a microbial niche for
69 dispersion and transmission over multiple host generations. Also, seedborne microbes
70 may harbour competitive advantages compared to the environmentally introduced
71 microbes because they are already established in the plant niche during early
72 colonization of the plant. Knowledge on seed-associated microbes, notably seedborne
73 fungi, is relatively limited compared to microbiota associated with other plant tissues
74 such as leaves and roots (12), (13). This is partly due to technical challenges of handling
75 single seeds and the extraction of sufficient amounts of microbial DNA. For example, the
76 model species *Arabidopsis thaliana* produces very small seeds that has limited detailed
77 studies of the seedborne microbiota in this species (14). Thus, most studies of
78 seedborne microbial communities have used culture dependent techniques or pooled
79 multiple seeds for culture independent methods (2), (13), (15), (16), (17). While these
80 studies have provided insight into overall diversity of seedborne microbiota, they have

81 not allowed high-resolution analyses of microbial diversity within individual seeds, nor
82 to which extent these microbial taxa co-evolve with the plant host.

83 In this study, we have assessed microbial communities of individual seeds across several
84 wild and domesticated wheat species. Specifically, we have asked to which extent the
85 seedborne microbial communities of closely related plant species differ and investigated
86 differences that may reflect divergent co-adaptation of the microbiota. We focused our
87 study on wheat, which represents an ideal model system to study the impact of recent
88 artificial plant selection associated with domestication and genetic plant divergence on
89 plant associated microbiota. Bread wheat, *Triticum aestivum*, was domesticated in the
90 Fertile Crescent 10-12.000 years ago and the domestication history has been well
91 characterized (18), (19), (20). Moreover, the underlying genetics of wheat domestication
92 has been described in details, including bottlenecks in the wheat diversity following
93 strong directional selection and polyploidization (21), (20). More recently, comparative
94 genome analyses have allowed identification of domestication signatures along the *T.*
95 *aestivum* genome (22). *T. aestivum* has been dispersed worldwide with wheat
96 cultivation and constitute a major crop on all continents (23). Wild relatives of the
97 hexaploid wheat *T. aestivum* originate in the Near East and can be found in natural
98 grassland vegetation, including tetraploid emmer wheat, *Triticum dicoccoides*, and
99 diploid einkorn, *Triticum boeoticum* and red wild einkorn *Triticum urartu* (24), (25). The
100 well-documented domestication history and close relatedness of wild and domesticated
101 wheat provide an optimal framework for comparative analyses of plant associated
102 microbial communities. Moreover, it allows us to address the consequences of plant
103 domestication on seed-associated microbial communities.

104 We hypothesized that strong directional selection during wheat domestication has
105 impacted genetic factors involved in microbial assembly, for example immune related
106 genes. If the plant genotype exerts a considerable impact on the plant associated
107 microbiota, we would then expect to observe differences in microbial diversity and
108 community composition. To address this hypothesis, we focused our study on both
109 bacterial and fungal endophytes of wheat seedlings. We show that domestication did

110 not entail a loss of microbial diversity in seeds, but rather in the diversity of early
111 colonizers of the domesticated plant. We also addressed the alteration of microbiota
112 assembly when wheat seeds were propagated in soil and we demonstrate that soil is a
113 main determinant of microbial diversity. Moreover, in leaves of wild wheat seedlings
114 show less variation between replicates when grown in natural soil, consistent with the
115 observation from axenic wheat seedlings. On the other hand, the soil type is a main
116 determinant in microbial community composition. Finally, we also suggest that the
117 biodiversity of wheat-associated fungal community is governed by different selection
118 regimes in comparison to the bacterial community.

119

120 **Material and Methods**

121 **Seed Collections:**

122 Our study built on a unique collection of wheat material including three wild species *T.*
123 *dicoccoides* (2n=28), *T. boeoticum* (2n=14), *T. urartu* (2n=14) collected in the Near East
124 and domesticated bread wheat *T. aestivum* (2n=42) collected in the Near East and North
125 Germany (Suppl Fig. 1 and Suppl Table 1). We here refer collectively to these five wheat
126 species and cultivars as wheat “genotypes”. More precisely, wild wheat accessions were
127 sampled in a South-East region of Turkey, a region located in the Fertile Crescent and
128 known to be the natural environment of these three wild wheat progenitors. Moreover,
129 the region is considered to be a site of early domestication and cultivation of bread
130 wheat *T. aestivum* (25). Our seed collections of the wild wheat represent two
131 geographical populations of the wheat in central-eastern Turkish-Iraqi race (24). Seeds
132 of the wild wheat were collected from one of the centers of massive stands in Karacadağ
133 (provinces of Şanlıurfa and Diyarbakır) and Kartal-Karadağ (province of Gaziantep) in the
134 South-East region of Turkey at different nearby fields in three years; 2004, 2005, and
135 2006 (Suppl Table1- Suppl Fig. 1) (24).

136 Seeds of domesticated wheat *T. aestivum* were obtained from a local farm in the same
137 region where the wild wheat was collected. The *T. aestivum* genotype from Turkey is a

138 winter wheat and local landrace of Kışlak, province of Hatay, and it has not been treated
139 with chemicals by the farmer and only with a minimum amount of fertilizer (personal
140 comm. by Nufel Gündüz, 2017). Also, we collected seeds from a modern winter wheat
141 cultivar, Benchmark (IG Pflanzenzucht, Ismaning, Germany) originating from an
142 experimental farm in Schleswig-Holstein, Germany. In contrast to the Turkish *T.*
143 *aestivum*, this inbred cultivar has been treated with chemicals during seed-production.
144 Seeds of both *T. aestivum* genotypes were collected in 2017.

145

146 **Processing of the seed, leaves and roots:**

147 To ensure that we only isolate microbial DNA from the interior of seeds and tightly
148 attached to the surface, we mildly surface semi-sterilized the seeds before DNA
149 extraction. Seeds were surface-sterilized by shortly soaking them in TritonX 0.1%, 80%
150 EtOH and 1.2% bleach followed by three washes with nuclease-free water. Three
151 randomly selected samples from the wash-off water were also processed for sequencing
152 as sterilization controls alongside the sterilized seeds.

153 Sterilized seeds were frozen by utilizing the Cryolys cooling unit and homogenized with a
154 Precellys Evolution Tissue Homogenizer (Bertin Instruments, Montigny-le-Bretonneux,
155 France). DNA was extracted from single seeds following a phenol-chloroform extraction
156 protocol (see Suppl. Text). This method was developed from a previously established
157 protocol for *Arabidopsis thaliana* (26), and here optimized to increase the efficiency of
158 extraction of bacterial and fungal DNA from single seeds. Three randomly selected
159 negative controls (i.e. blanks) of DNA extraction were also sequenced alongside the seed
160 samples. Processing of DNA extracts and sequencing is described below.

161 We further addressed the colonization dynamics of the seedborne microorganisms in an
162 *in-vitro* experiment where we germinated seeds under sterilized conditions in closed
163 sterile jars to assess microbial diversity in leaves and roots. In brief, seeds from three
164 wheat genotypes (*T. aestivum* from Turkey and Germany and *T. dicoccoides* from
165 Turkey) (same seed populations used to characterize seedborne microbial diversity)

166 were surface sterilized and germinated under sterile conditions with 16h light/8h dark
167 cycles at 15°C (n=4-8 per population) in a climate chamber (Percival plant growth
168 chambers, CLF PlantClimatics GmbH, Wertingen, Germany). In the sterile jars, plants
169 were grown in a nutrient-rich PNM medium (see Suppl. Text). We let seedlings develop
170 for two weeks, which corresponds to the emergence of the second leaf. About six
171 centimeter of two leaves and multiple leaves and roots of two weeks old seedlings were
172 harvested with sterile forceps and processed for DNA extraction. DNA extraction was
173 performed using the PowerPlant Pro DNA Isolation Kit (Mo Bio Laboratories, Heidelberg,
174 Germany) according to the manufacturer's instructions.

175

176 **Transplant Soil Experiments:**

177 To address if domestication has entailed a change in the ability of plants to associate
178 with microbial communities, we reciprocally transplanted domesticated and wild wheat
179 (*T. aestivum* from Germany and Turkey and *T. dicoccoides* from Turkey) in a German
180 agricultural soil and a natural soil from a region of the Fertile Crescent in Turkey. Both
181 soil types were mixed with 5% peat and sifted with a sieve. We propagated seedlings
182 from surface sterilized seeds in the two soil types in the climate chamber. We harvested
183 leaves and roots as described above for the axenically propagated seedlings (n=6-8 per
184 wheat accession- soil type combination). Additionally, three pots per soil type were
185 filled with soil without plants and used as controls that were processed alongside
186 samples. The position of each pot was being changed during the experiment to
187 randomize any spatial bias. After two weeks, leaves and roots were harvested with
188 sterile forceps and scissors and mildly washed with water, 1% PBS and 1% PBS + 0.02%
189 Tween20 to remove loosely attached microbes and soil particles from the roots. Finally,
190 samples were processed for DNA extraction. DNA extraction was performed using the
191 PowerPlant Pro DNA Isolation Kit (MoBio Laboratories, Heidelberg, Germany) according
192 to the manufacturer's instructions.

193

194 Sequencing of amplicons

195 The V5-V7 sequence of the bacterial 16S ribosomal RNA (16S rRNA gene) and a
196 sequence of the fungal ribosomal internal transcribed spacer (ITS1) region were
197 amplified using the primer combinations 799F-1192R and ITS1F-ITS2 to assess bacterial
198 and fungal diversity, respectively (27), (28). Bacterial and fungal sequences were
199 amplified with a two-step PCR protocol. In the first PCR step, interfering primers were
200 utilized to enrich amplification of the 16S rRNA and preventing unintended co-
201 amplification of the DNA. These interfering primers were originally developed by Agler
202 and co-workers for microbial community analyses of *A. thaliana* (26). Here, we modified
203 the interfering primers to target the corresponding wheat loci and changed the PCR
204 protocol to optimize primer interference (Suppl. Text). In the second step of PCR,
205 reverse primers barcoded with 12 base pair indexes and unique to each sample were
206 used as barcodes to multiplex different samples in one sequencing run (Metabion
207 International AG, Planegg, Germany). The primer setup used here was taken from Agler
208 et al. 2016 (26). Three PCR replicates for each sample were used as technical replicates
209 in each step and subsequently merged at the end of each PCR.

210 Finally, amplicon libraries were quantified fluorescently with the Invitrogen Qubit 3.0
211 Fluorometer (Thermo Fisher Scientific, Darmstadt, Germany). 16S and ITS amplicons
212 were combined in equimolar concentrations in combined libraries. During DNA
213 extraction as well as during library preparation, samples were randomized to prevent
214 any possible batch effect. The combined libraries were paired-end sequenced for 600
215 cycles on an Illumina MiSeq machine at the sequencing facility of Max Planck Institute
216 for Evolutionary Biology, Plön, Germany.

217

218 Data Analysis

219 Raw reads were demultiplexed and converted into fastq files for downstream analysis
220 using the bcl2fastq Conversion Software of Illumina (Illumina, bcl2fastq Conversion
221 Software v2.20.0.422). We followed the QIIME2 version 2019.1 pipeline to preprocess

222 and filter the fastq files (29). In brief, the conserved flanking regions of the ITS reads
223 were trimmed with the q2-itsxpress plugin integrated into QIIME2 (30). Afterwards, the
224 DADA2 software package also integrated into QIIME2 was used to correct and truncate
225 sequences and filter chimeric reads for 16S reads (31). However, ITS reads were not
226 truncated but only corrected and filtered as recommended by the q2-itsxpress plugin
227 tutorial (<https://forum.qiime2.org/t/q2-itsxpress-a-tutorial-on-a-qiime-2-plugin-to-trim-its-sequences/5780>). After filtering and denoising, no fungal or bacterial features
228 remained in the negative controls of DNA extraction and sterilization. Alpha diversity
229 rarefaction plots for each sample confirm that a sufficient depth of coverage of the 16S
230 and ITS datasets were achieved for both seeds and seedlings (Suppl Fig. 2). Further
231 details regarding the amplification and sequencing is included in the supplementary
232 materials and methods (see the Suppl Text).
233

234 For the taxonomic classification of 16S and ITS datasets, we used the Greengenes13.8
235 and UNITE 7.2 databases, respectively (32), (33). We utilized the q2-feature-classifier
236 plugin of QIIME2 to extract the reference sequences from the databases and train the
237 Naïve Bayes classifier (34). We extracted the target sequence of the 799F-1192R primer
238 pairs from the Greengenes13.8 database. However, we did not extract the target
239 sequences of ITS primers but used the full reference sequences as suggested by the q2-
240 feature-classifier tutorial (<https://docs.qiime2.org/2018.6/tutorials/feature-classifier/>).
241 Next, we trained the Naïve Bayes classifier based on the reference sequences and
242 taxonomy. Finally, the resulting feature table was used to determine taxonomic relative
243 abundances and for the subsequent statistical analyses of beta-diversity.

244 Downstream analyses were conducted with the “phyloseq”, “vegan”, “ampvis2” and
245 “ggplot2” R packages or custom R scripts (35), ((36)), ((37)), ((38)), ((39)). Samples with
246 fewer than 1000 reads for 16S and 200 reads for ITS were excluded from the resulting
247 table. Moreover, taxonomically unassigned reads at the kingdom level and reads
248 assigned to mitochondrial or other plant sequences were excluded for further analyses.
249 Further information about the summary of the data before and after filtering is available
250 in the supplementary material (Suppl Table 2). Before estimating alpha diversity indices,

251 the samples were rarefied to even depth. Alpha diversity indices of the samples were
252 estimated with Shannon diversity metrics and using observed number of features (i.e.
253 richness) as a metrics. The significance of differences in diversity among wheat groups
254 and pairwise multiple comparisons between wheat species were tested using a Kruskal-
255 Wallis test (`krus.test` in R) and Conover's test in the "PMCMR" package where we
256 corrected the p-values with the "holm" correction method (40).

257 To compare the composition of communities and abundances of microbial taxa among
258 different populations of wheat hosts, the counts from the feature tables were
259 normalized by the `cumNorm` function in the "metagenomeSeq" package (41). We
260 computed the Jaccard, Bray-Curtis and unweighted UniFrac distances to compare the
261 structure of bacterial communities among/between samples. First metrics account for
262 the absence/presence, second for both absence/presence and abundances whereas
263 UniFrac metrics incorporates phylogenetic relatedness of bacterial communities into the
264 calculation of distances. For fungal communities, we only used Bray-Curtis and Jaccard
265 metrics. We did not report phylogeny-based metrics for the fungal data because
266 sequence length variation in ITS may lead to erroneously inference of phylogeny. Beta
267 diversity distance matrices were used for principal coordinate analysis (PCoA).
268 Permutational multivariate analysis of variance analysis (PERMANOVA) was performed
269 to test the significance of the effect of soil type and host type and their interactions in
270 the microbial community composition ("`adonis`" function in the "vegan" package in R).

271

272 **Results**

273 **Domestication of wheat has negligible effect on seedborne microbial diversity**

274 To compare the diversity of seedborne microbiota between domesticated and wild
275 wheat we firstly used the three wild wheat species *T. dicoccoides*, *T. urartu* and *T.*
276 *boeoticum* and two genotypes of domesticated wheat *T. aestivum*, one landrace from
277 Turkey and an inbred cultivar from Germany (Suppl Table 1). For *T. dicoccoides* we used
278 genotypes from four different populations in South-East Turkey (Suppl Table 1). Our

279 method of seed surface sterilization allowed us to assess microbial diversity exclusively
280 of the seedborne microbiota of individual seeds. We extracted DNA from individual
281 seeds and amplified microbial DNA using both bacterial and fungal specific primers (16S
282 rRNA gene, V5-7 regions and ITS, ITS1 region, respectively) (Suppl Table 3 and Suppl
283 Text).

284 Measures of alpha diversity (within sample diversity) show an overall low diversity of
285 microbial taxa in the wheat seeds and notably a low diversity of seedborne fungal taxa
286 compared to other plant tissues like leaves and roots. On average, in 58 samples, we
287 found 68.7 bacterial features and 5.3 fungal features (corresponding to a Shannon Index
288 of 2.6 and 0.8 for bacteria and fungi, respectively) (Fig. 1A and 1B). Notably, pairwise
289 comparisons of alpha diversity among the different wheat genotypes showed no
290 difference. Specifically, we did not observe any difference in microbial diversity
291 associated with seeds of wild and domesticated wheat (Kruskal-Wallis test, Richness: $p=$
292 0.8029 and 0.1924; Shannon diversity: $p=$ 0.6728 and 0.2530 for bacterial and fungal
293 communities, respectively) or among the different wheat genotypes (Kruskal-Wallis test,
294 Richness: $p=$ 0.2421 and 0.4481; Shannon diversity: $p=$ 0.6826 and 0.7191 for bacterial
295 and fungal communities, respectively).

296 Taken together, our estimates of alpha diversity in different wheat accessions and
297 species suggest that domestication has not entailed a loss of diversity in the seedborne
298 microbiota.

299

300 **Different composition of microbial communities associated with different wheat** 301 **species**

302 We next investigated the composition of microbial communities associated with the
303 wheat seeds. Comparisons of beta-diversity (between sample variation), showed that
304 the seedborne bacterial and fungal communities cluster independently of the wheat
305 species (Suppl Fig. 3). Notably, the seedborne microbial communities of *T. aestivum*
306 accessions from Germany and Turkey cluster together although the first represents a

307 highly inbred modern cultivar and the second a local Turkish landrace.

308 We further characterized and compared the identity and abundances of microbial taxa.
309 To this end, we aggregated the assigned taxonomy of each microbial feature to the
310 family level. First, we assessed the distribution of major bacterial groups associated with
311 seeds of the different wheat genotypes. We found a considerable variability among
312 replicates of the same wheat genotype (on average 12.1% of bacterial features at the
313 family level exist in all replicates of the same wheat genotype). Hereby, the wheat seed
314 microbiota was mostly dominated by Proteobacteria and to lesser extent by Firmicutes,
315 Actinobacteria and Bacteroidetes (Fig. 2A). These results are in accordance with
316 previous studies of seed-associated bacteria of crop (e.g. maize, barley, rice) (42)(16),
317 (42), (43)(16) and non crop plants (e.g. radish) (2), (44). However, at lower taxonomic
318 levels, we observed differences in abundances of several microbes among the different
319 wheat genotypes (Fig. 2A). For example, the Halomonadaceae family, including bacteria
320 known to promote plant salt tolerance and growth (45), represent a substantial
321 proportion of the bacterial community in the seeds of wild wheat (17.6-22.9%) but a
322 small proportion of the domesticated wheat seed microbiome (5.2-7%).

323 Among the fungal taxa, we also found a considerable variability among replicates of the
324 same wheat genotype (on average 17.4% of fungal features at the family level exist in all
325 replicates of the same wheat genotype). Fungal communities were dominated by
326 Ascomycetes (Fig. 2B). Notably fungi in the order Pleosporales are abundant in the
327 wheat seeds, including species of *Alternaria* that are highly abundant in seeds of *T.*
328 *aestivum* and previously also shown to dominate wheat endophyte communities (46).
329 Interestingly, Trichosphaeriaceae and Chaetomiceae were detected to be the most
330 prevalent two fungal families in *T. boeoticum* (33.5% & 29.9%) and *T. urartu* (53.5% &
331 24.3%), were not detected in other wheat species.

332 In summary, while microbial alpha diversity is comparable among domesticated and
333 wild wheat species, we report differences in the taxonomic composition of bacterial and
334 fungal seedborne communities. These findings indicate that although domesticaion has

335 a minor effect on the overall microbial community richness, it may have impacted the
336 structure of these communities.

337

338 **Axenic seedlings of wild wheat are colonized by more diverse bacterial communities**
339 **than domesticated wheat**

340 We hypothesized that a proportion of the seedborne microbiota colonizes the plant
341 seedling after seed germination. In order to compare microbial diversity and community
342 composition of these early colonizers in domesticated and wild wheat, we set up an
343 experiment using seeds of the German *T. aestivum* cultivar, Turkish *T. aestivum* landrace
344 and the Turkish *T. dicoccoides* genotypes. We germinated surface-sterilized seeds and
345 propagated these under sterile conditions. We harvested leaves and roots of the
346 seedlings two weeks after seed germination, including a total of 32 plant samples
347 consisting of roots and leaves (4-8 replicates per wheat and per tissue) and used these
348 samples to profile bacterial and fungal communities.

349 Analyses of the bacterial microbiota revealed a total of 589 and 632 different bacterial
350 features in leaves and roots (after filtering and rarefaction) (Suppl Table2). The analysis
351 revealed that bacterial communities associated to the roots of *T. dicoccoides* are
352 significantly more diverse compared to the communities associated with the Turkish and
353 German *T. aestivum* genotypes (pairwise alpha diversity comparisons, Kruskal Wallis,
354 Richness: $p= 0.0023$ and $p= 0.0023$; Shannon index: $p= 0.0021$; $p= 0.0027$, respectively).
355 Additionally, the leaves of *T. dicoccoides* hosted more diverse bacterial communities
356 compared to domesticated wheat from Turkey (Kruskal Wallis, Richness: $p= 0.0280$;
357 Shannon index: $p= 0.0066$) (Fig. 3A). Taken together, significantly more diverse
358 seedborne bacterial community was transmitting into leaves and roots in wild wheat
359 compared to the domesticated wheat.

360 Roots and leaves of the wheat seedlings were colonized by few fungal taxa, and we
361 obtained in total only 98 and 74 unique fungal features in leaves and roots (after
362 filtering and rarefaction). In contrast to the striking difference we observed for bacterial

363 communities in wild and domesticated wheat, we observed no significant difference in
364 the diversity of fungal colonizers suggesting that different processes determine the
365 colonization of bacterial and fungal endophytes (Fig. 3B).

366 We next compared the identity and abundances of the microbial communities of seeds
367 and seedlings. Overall, the same bacterial and fungal phyla were dominant in seeds as
368 well as in leaves and roots, however we observed some significant shifts in microbial
369 abundance (Fig. 4A). For example, Comamonadaceae, Halomonadaceae, Vibronaceae
370 and several other bacterial families enriched in seeds of both wild and domesticated
371 wheat, did not colonize roots of the German *T. aestivum* accession. Furthermore,
372 Paenibacillaceae was only present at very low abundance (0-0.1%) in seeds but a
373 dominant colonizer of roots of *T. aestivum* from Germany (26.3%).

374 Fungal ascomycete taxa were the most abundant in the wheat seedlings (Fig 4B).
375 Notably, Pleosporales were abundant colonizers of seedlings of both wild and
376 domesticated wheat from Turkey and Germany. Aureobasidiaceae were abundant only
377 in the seedlings of *T. aestivum* from Turkey (25.4% in leaves and 17.2% in roots), but not
378 in other wheat. Other abundant seedborne fungi were not detected in the leaves and
379 roots of the wheat seedlings. For example, Mycosphaerellaceae (40% in *T. aestivum* from
380 Germany), Saccharomycetaceae (37.5% in *T. dicoccoides*); Aspergillaceae (17.3% in *T.*
381 *dicoccoides*) found to be abundant in the seedborne communities were either absent or
382 only present at low relative abundance in the seedlings. Together, these results
383 demonstrate a difference in the assembly of seedborne bacterial, but not fungal
384 communities in wild and domesticated wheat seedlings. Moreover, our results indicate
385 that more diverse microbial communities are sustained in root and leaves of wild wheat
386 seedlings compared to domesticated wheat.

387

388 **Axenically grown domesticated wheat seedlings assemble less homogeneous**
389 **microbial communities**

390 Our analyses of bacterial diversity in seeds and seedlings of *T. aestivum* and *T.*

391 *dicoccoides* revealed a considerably fewer diversity in the replicates of *T. aestivum*
392 seedlings (Fig. 3A). We further examined between-sample variation by computing Bray-
393 Curtis and Jaccard and Unweighted UniFrac distance metrics (Fig. 5, and Suppl Fig. 3).
394 Our results show that replicates of seedborne bacterial communities of the wild wheat
395 and domesticated wheat from Turkey and Germany cluster together in the PCoAs (Fig.
396 5A and Suppl Fig. 3D-E). However, the bacterial colonizers of *T. dicoccoides* are distinct
397 from the seedborne community and there is less variation among replicates of root and
398 leaf communities. In contrast, in the domesticated *T. aestivum* wheat from Germany
399 and Turkey, we observed more heterogeneous microbial communities associated with
400 the leaves and roots (Fig. 5A). Pairwise comparisons of Bray-Curtis distances confirmed
401 the distinct community composition of the wild and domesticated wheat: replicates of
402 *T. dicoccoides* seedlings were colonized by notably more similar bacterial communities
403 in comparison to replicates of *T. aestivum* seedlings (Fig. 5B).

404 Additionally, we compared variability in fungal communities among seed and seedling
405 replicates. In contrast to the seedborne bacterial communities of wheat, we found
406 considerably more difference among replicates of seedborne fungi as well as colonizers
407 of roots and leaves (Fig. 5C-D and Suppl Fig. 3D). Overall, these findings also support
408 that different processes govern the assembly of bacterial and fungal communities in
409 seeds and seedlings of wheat. We moreover conclude that wheat domestication and
410 plant polyploidization did not entail a modification of the seedborne microbial diversity,
411 but rather affected the diversity and composition of leaf and root colonizers.

412

413 **Domestication has not changed the assembly of soil-derived root microbiota**

414 In their natural environment, plant seedlings are also colonized by microorganisms from
415 the soil (47). To investigate if seedlings of wild and domesticated wheat assemble
416 different microbial communities from soil, we set up an experiment with the same
417 wheat genotypes used above. We germinated seeds and propagated seedlings of *T.*
418 *dicoccoides* and *T. aestivum* in two different soils: an agricultural soil from Germany and

419 a natural soil obtained from a location in the South-East region of Turkey close to the
420 sampling site where the wild wheat accessions were obtained. We propagated the three
421 wheat genotypes (*T. dicoccoides* from Turkey and *T. aestivum* from Turkey and from
422 Germany) independently in the agricultural and the natural soil (6-8 replicate plants per
423 wheat-soil combination). The seeds used here were surface sterilized as in the
424 experiments described above.

425 Our results reveal that soil type rather than plant genotype is a main determinant of
426 bacterial community structure in roots of the wheat seedlings (using a PERMANOVA
427 test, explained by 61.38% of the between-sample variation; $p= 0.001$) (Fig. 6A-B).
428 However, for the leaf-associated microbial communities, the wheat genotypes explain a
429 significant proportion of the bacterial diversity (for soil type 13.54%; $p= 0.001$, for wheat
430 accession 6.09%; $p= 0.020$ and for the interaction of soil and wheat accession 5.31%; $p=$
431 0.066) (Fig. 6 and Suppl Fig. 12).

432 In general, plants grown in the agricultural soil were colonized by more diverse bacterial
433 communities in leaves and roots (Fig. 6C and 6D). In contrast to the axenic experiment,
434 we did not detect a difference in alpha diversity between leaves of wild and
435 domesticated wheat when seedlings were propagated in the soil. However, in
436 accordance with our observations from the axenic experiment, we observe less variation
437 between replicates of *T. dicoccoides* compared to domesticated *T. aestivum* when the
438 seedlings were propagated in the natural soil. On the other hand, we observe a
439 significant difference in alpha diversity between microbial communities of *T. dicoccoides*
440 and the German accession of *T. aestivum* propagated in the agricultural soil. Hereby, the
441 bacterial diversity is higher in roots of the wild wheat when compared to the
442 domesticated wheat. We speculate that the higher diversity of the bacterial
443 communities associated with *T. dicoccoides* in the foreign soil originates from novel
444 plant-microbe interactions.

445 The composition of fungal communities was assessed only in roots of the three wheat
446 genotypes (Suppl Fig. 10 and Suppl Fig. 11). Our results show that soil type but not

447 wheat genotype is also the main determinant of fungal community structure associated
448 with the wheat roots (PERMANOVA test, %8.52 of the between-sample variation; $p=$
449 0.001). Notably, when the seedlings were propagated in the natural soil, they were
450 colonized by significantly more diverse fungal communities ($p= 3.71 \times 10^{-6}$ and $p= 8.90 \times$
451 10^{-4} for richness and Shannon Index, respectively) (Suppl Fig. 11A-B). Moreover, based
452 on analyses of pairwise Bray Curtis distances we found that fungal communities are
453 more similar among replicates when seedlings were propagated in the natural soil
454 compared to the agricultural soil (Suppl Fig. 11C). Also, the wild wheat were colonized
455 by less homogenous fungal communities compared to the domesticated wheat in both
456 soil types. Taken together, the differences in diversity and community composition of
457 fungal wheat colonizers had little effect of the wheat genotype, but a significant effect
458 of the soil type.

459 We next compared the identity and abundances of microbial taxa in the seedlings of the
460 wheat seedlings propagated in soil. Clearly, roots and leaves exhibited distinct bacterial
461 composition when compared to the bacterial communities of the bulk soil implying
462 specificity related to plant colonization (Fig. 6A and Suppl Fig. 13A). However, in general
463 microbial composition was similar among the three different wheat genotypes when
464 grown in the same soil type (Suppl Fig. 13A). Basically, roots were dominated by two
465 bacterial families; Oxalobacteraceae (18.6-26.5%), Streptomyetaceae (27-32.6%) (Suppl
466 Fig. 13A) where Streptomyetaceae were the dominant colonizer of roots when
467 seedlings were growing in the agricultural soil (40.2- 49.7%) (Suppl Fig. 13C). On the
468 other hand, leaves were colonized by other bacterial families: Oxalobacteraceae (12.2-
469 22.2%), Comamonadaceae (7.4-19.9%), Rhizobiaceae (12.1-21.3%), Halomonadaceae
470 (13.6-15.9%), Vibronaceae (8.4-14.7%). Notably, the two bacterial families
471 Halomonadaceae and Vibronaceae were highly abundant phyllosphere colonizers in
472 leaves propagated in both soil types (Suppl Fig. 13B), although they were not detected
473 in the bulk soil or roots (0-0.1%). On the other hand, these bacteria were also prevalent
474 members of the seedborne leaf community suggesting that they may have originated
475 from the seeds (Fig. 4A).

476 Also the fungal communities were similar among the different wheat genotypes when
477 grown in the same soil type (Suppl Fig. 14). The most prevalent member of seedborne
478 fungal communities, Pleosporaceae, were still detectable in the roots of plants
479 propagated in the natural soil (10.8- 16.6%). However, plants grown in the agricultural
480 soil were mostly colonized by the fungal taxa in the family Pseudeurotiaceae (28.4-
481 33.9%).

482

483 **Discussion**

484 Plant domestication has entailed a significant loss of genetic diversity, as well as
485 physiological and anatomical changes in the selected species (e.g. 18). In this study we
486 have addressed to which extent domestication has changed the seedborne microbiota
487 composition and the potential of wheat to assemble environmental (i.e. soil) microbial
488 communities. We combined experimental assays with microbial profiling of a unique
489 collection of wheat genotypes from a region in the Fertile Crescent, the center of origin
490 of wheat. We specifically optimized our protocols of DNA extraction and microbial
491 amplification to characterize the microbial community of individual seeds.

492 Comparing microbial communities associated with seeds, we showed significant
493 differences in the composition of the bacterial communities in wild and domesticated
494 wheat. This finding is consistent with previous studies, which have also revealed some
495 differences in microbial community composition of domesticated plants and their wild
496 relatives (48), (47), (49). Leff and co-workers investigated the impact of domestication
497 on seedborne microbial communities of different sunflower accessions. Consistent with
498 our results, they did not detect differences in fungal diversity between modern and wild
499 sunflower and they demonstrated a minimal vertical transmission of fungal endophytes
500 from seeds to roots of the developing seedling (30).

501 A growing body of evidence suggests that different layers of the plant immune system
502 play significant roles in shaping the plant microbiota (50). Plants receptors that are
503 involved in microbial recognition and “management” involve Pathogen Recognition
504 Receptors (PRRs) and Nucleotide-Binding Leucine-rich repeat Receptor (NB-LRR)

505 proteins (51). Wheat domestication and polyploidization may have conferred a change
506 in the composition and diversity of these immune-related proteins and thereby
507 indirectly impacted the assembly of the wheat microbiome. Comparative genome
508 studies of domesticated and wild wheat have identified signatures of domestication in
509 the *T. aestivum* genome, which may correlate with microbial community assembly,
510 including changes in the repertoire of genes involved in signaling, hormone production
511 and metal accumulation (52). We speculate that different microbial community
512 composition in German and Turkish *T. aestivum*, and the wild relative *T. dicoccoides*
513 reflect genetic differences among these wheat genotypes. We note that such changes
514 notably have impacted the composition of bacterial communities and to a much lesser
515 extent fungal communities. Interestingly, we observe little difference among seed and
516 soil-derived fungal communities of the wild and domesticated wheat species. This
517 suggests that different traits and mechanisms are responsible for the assembly of
518 bacterial and fungal plant-associated communities.

519 The seedborne microbiome of wheat has previously been investigated primarily with
520 culture-dependent methods (15), (46). Robinson and colleagues characterized
521 seedborne microbial community in roots and shoots of the axenically grown seedlings
522 by isolation and cultivation of microbiota (12). This study allowed them to characterize
523 only eight bacterial taxa that could be defined at the genus level. Olfek-Lalzar also
524 studied fungal endophytes in seeds of wild and domesticated wheat also using a culture-
525 dependent method. They were able to isolate and identify 31 OTUs from 100 seeds; of
526 these fungi more than half occurred only one time. In our study based on culture-
527 independent methods and optimized molecular protocols, we were able to identify
528 significantly higher diversity of bacteria and fungi than previously reported in wheat
529 seeds (Suppl Table 2). Our data thereby provides an extended resource for further
530 research of the wheat “core” microbiota, as well as species-specific microbial partners
531 of modern and wild wheat.

532 An underlying assumption in our research of the seedborne microbiota is that a
533 significant proportion of these microorganisms later represent endophytes in the wheat

534 plant. To qualitatively and quantitatively characterize the early seedling colonizers we
535 propagated seedlings under sterile conditions and assessed microbial diversity in leaves
536 and roots of the young seedlings. A striking finding from this experiment was 1) a higher
537 diversity of bacterial colonizers in the wild wheat *T. dicoccoides* compared to *T.*
538 *aestivum*, and 2) an inconsistent community composition among replicates in *T.*
539 *aestivum* seedlings. We speculate that stochastic processes (e.g. priority effects) play a
540 stronger role in *T. aestivum* and result in the dominance of random bacterial taxa in
541 different replicates. We see this effect both in the Turkish landrace of *T. aestivum* as
542 well as in the inbred German wheat cultivar Benchmark, suggesting that the effect is
543 consistent among different *T. aestivum* genotypes. We speculate that domestication
544 may have entailed less selective constraints on plant traits that contribute to microbial
545 assembly. Consequently, non-deterministic events could play a larger role in the
546 assembly of microbial communities associated with domesticated wheat. Nonetheless,
547 we note that the 16S and ITS data only allow a low-resolution analysis of microbial
548 diversity and a more comprehensive investigation of plant colonization will require
549 strain specific markers or specifically labeled strains.

550 Domestication and plant breeding have involved strong artificial selection of desired
551 crop traits. For several domesticated species it is demonstrated that a negative
552 consequence of domestication is a severe loss of genetic variation and an accumulation
553 of deleterious mutations (53), (54), (21). These “domestication costs” may have reduced
554 local adaptation of crop plants to their environment, including the local environmental
555 microbiota. We addressed signatures of plant adaptation to the soil microbiota in the
556 Turkish *T. dicoccoides* using local soil from Turkey. We compared plant microbial
557 diversity of this local combination to a foreign combination of *T. dicoccoides* in a
558 German soil. Moreover, we also assessed microbial diversity of two *T. aestivum* wheat in
559 two soils. Although the two soils comprise different geochemical properties, they were
560 comparable in their overall microbial diversity. Notably, we find that soil rather than
561 plant genotype determines the composition of root associated bacterial communities
562 suggesting that the “plant-selected” proportion of the soil-derived microbiota overall is

563 small. Interestingly, we observe a stronger effect of the plant genotype on the bacterial
564 phyllosphere community than on the root-associated bacterial communities. We
565 speculate that the phyllosphere imply a strong selection on the associated microbiota.
566 More detailed analyses of microbial diversity e.g. based on metagenome sequencing or
567 microbial population genomic data is needed to study plant-microbe co-adaptation.
568 In conclusion, the present study provides new insights into the microbial community
569 composition and colonization of domesticated and wild wheat. Our findings indicate an
570 increased role of chance events and priority effects on seedling colonization. We
571 moreover speculate that the difference between wild and domesticated wheat reflect
572 changes in the plant immune system conferred by artificial selection and
573 polyploidization during domestication and crop improvement. Future crop breeding
574 strategies should account for microbial diversity and the ability of crops to assemble and
575 maintain beneficial microbial communities. Such efforts will rely on research of plant-
576 microbial co-adaptation and the underlying mechanisms that determine microbial plant
577 colonization.

578

579 **Acknowledgements:**

580 The authors would like to thank Eric Kemen for helpful discussions, employees of the
581 experimental farm Hohenschulen of Kiel University and Doreen Landermann for
582 technical assistance. The authors also thank the organizers of the EMBO workshop
583 „Plant Microbiota 2017“ for their input. This work was funded by the DFG Collaborative
584 Research Centre (CRC) 1182 “Origin and Function of Metaorganisms” and the
585 International Max Planck Research School for Evolutionary Biology. The authors declare
586 no competing interests.

587

588 **Author Contributions:**

589 The study was conceived and designed by EÖ, EHS and TD. Seed collections and soil
590 from Turkey were provided by HÖ and US. The experiments were performed by EÖ and

591 MAH. Amplicon sequencing was performed by SK. Data analysis was conducted by EÖ
592 and TD. The manuscript was written by EÖ and EHS. All authors read the manuscript.

593

594 **Conflict of Interest:**

595 The authors declare no conflict of interest.

596

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- 763
- 764

765 **Figure legends:**

766 **Figure 1: Alpha diversity in the seedborne microbiota of different wheat genotypes:**
767 **Seeds of different wild and domesticated wheat harbour comparable microbial**
768 **diversity**

769 Alpha diversity of **A)** bacterial and **B)** fungal features in the seeds of different wheat. *T.*
770 *boeoticum* (Tb), *T. urartu* (Tu), *T. dicoccoides* (Td), *T. aestivum* from Turkey (Ta_Tr) and
771 *T. aestivum* from Germany (Ta_De). Each dot in the boxplots show the microbial
772 diversity of a single seed. Pairwise comparisons of alpha diversity showed no significant
773 difference between wheat genotypes (Conover's Test: Richness p-values: 0.41-1.00 and
774 1.00, Shannon diversity p-values: 1.00 for bacteria and fungi, respectively).

775

776 **Figure 2: Composition of the seedborne microbiota across different wheat genotypes**

777 Mean relative abundances of the most abundant **A)** twenty bacterial features, and **B)**
778 fifteen most abundant fungal features at the family level in seeds of different wheat
779 species. Color for each feature ranges from blue (minimum 0) to red with higher relative
780 abundance values. (Abbreviations on figure, L: landrace, C: cultivar, IS: Incertae sedis
781 taxa).

782

783 **Figure 3: Significantly more diverse bacterial but not fungal communities are**
784 **colonizing the wild wheat**

785 Microbial diversity in different tissues of axenically-grown wheat: Alpha diversity of **A)**
786 bacterial and **B)** fungal taxa in seeds, leaves and roots of the wild wheat *T. dicoccoides*,
787 *T. aestivum* from Turkey and *T. aestivum* from Germany respectively. Global p-values for
788 each tissue are shown in green and p-values of pairwise comparisons are in black color.
789 (* < 0.05; ** < 0.005; ns= non-significant)

790

791 **Figure 4: Composition of the microbiota in the axenic seedlings across different wheat**
792 **genotypes**

793 Mean relative abundances of the most abundant twenty **A)** bacterial and **B)** fungal
794 features at the family level in seeds, leaves and roots of the German *T. aestivum*
795 genotype Ta_De, the Turkish *T. aestivum* genotype Ta_Tr and the wild wheat *T.*
796 *dicoccoides* genotype Td, respectively. Colors for each taxon illustrate relative
797 abundance and ranges from blue (minimum 0) to red with higher relative abundance
798 values. (Abbreviation, IS: Incertae sedis taxa).

799

800 **Figure 5: Wild wheat is colonized by more homogenous bacterial communities among**
801 **different individual plants compared to domesticated wheat in roots**

802 **A-B)** Bray-Curtis distance metrics based PCoAs of **A)** bacterial **C)** fungal communities of
803 each seed, leaf and root sample from the axenic experiment. **B and D)** Estimated
804 pairwise Bray-Curtis distances of **B)** bacterial and **D)** fungal communities in the
805 replicates of each wheat genotype for each tissue. Global p-values for each tissue are
806 shown in green and p-values of pairwise comparisons are in black color. (** <0.005; ***
807 < 0.0005; ns= non-significant)

808

809 **Figure 6: Soil type in roots, both soil type and wheat genotype in leaves are the**
810 **determinants of the bacterial community**

811 **A)** Bray-Curtis distances based PCoA of bacterial communities of plants grown in soil **B)**
812 Summary statistics for the beta-diversity and significant alpha diversity comparisons of
813 the bacterial communities **C-D)** Interaction plots showing alpha and beta diversity
814 comparisons of bacterial communities in **C)** leaves and **D)** roots of different wheat
815 grown in different soil types.

816

817 **List of Supplementary Material**

818 **Supplementary Text**

819

820 **Suppl Table 1: Detailed information about the wheat collections**

821 **Suppl Table 2: Summary of the sampling and sequencing data**

822 "b.r." denotes number of microbial features before filtering and rarefying while "a.r."
823 denotes number of features after rarefaction.

824 **Suppl Table 3: Sequence of the V5-7 and ITS1 primers used for amplification and**
825 **sequencing**

826

827 **Suppl Figure 1: Location of the seed collections**

828 **A)** Samples were collected from Turkey and Germany **B)** Location of fields from South-
829 East region of Turkey and North Germany where seeds were collected. Zoomed version
830 of the locations was depicted to make visualization easier.

831 **Supp Figure 2: Alpha rarefaction curves for bacterial and fungal communities of each**
832 **sample collection of each experiment A)** 16S seed samples **B)** ITS seed samples **C)** 16S
833 axenic leaves and roots **D)** ITS axenic leaves and roots **E)** 16S leaf samples grown in soil
834 and **F)** ITS root samples grown in soil **G)** ITS roots grown in the soil

835 **Suppl Figure 3: UniFrac and Jaccard distances based PCoAs of the microbial community**
836 **in seeds and axenic seedlings A)** Bray-Curtis PCoA- seed-associated bacteria and fungi
837 **B)** Jaccard PCoA- seed-associated bacteria and fungi **C)** Unweighted UniFrac PCoA- seed-
838 associated fungi **D)** Jaccard PCoA- axenic seedling-associated bacteria and fungi **E)**
839 Unweighted UniFrac PCoA- axenic seedling-associated bacteria

840 **Suppl Figure 4: Pairwise Bray-Curtis distances of the bacterial communities in the**
841 **replicates of seeds**

842 Each dot shows pairwise distance between two replicates. Red boxplots show distances
843 of the microbial communities between samples of the same wheat genotype. Black
844 boxplots show distances of the bacterial communities between samples of two
845 corresponding wheat genotypes indicated.

846 **Suppl Figure 5: Pairwise Unweighted UniFrac distances of the bacterial communities**
847 **oft he replicates of seeds**

848 Each dot shows pairwise distance between two replicates. Red boxplots show distances
849 of the microbial communities between samples of the same wheat genotype. Black
850 boxplots show distances of the bacterial communities between samples of two
851 corresponding wheat genotypes indicated.

852 **Suppl Figure 6: Pairwise Bray-Curtis distances of the bacterial communities in the**
853 **replicates of seeds and axenic seedlings**

854 Each dot shows pairwise distance between two replicates. Red boxplots show distances
855 of the microbial communities between samples of the same wheat genotype. Black
856 boxplots show distances of the bacterial communities between samples of two
857 corresponding wheat genotypes indicated.

858 **Suppl Figure 7: Pairwise Unweighted UniFrac distances of the bacterial communities in**
859 **the replicates of seeds and axenic seedlings**

860 Each dot shows pairwise distance between two replicates. Red boxplots show distances
861 of the microbial communities between samples of the same wheat genotype. Black
862 boxplots show distances of the bacterial communities between samples of two
863 corresponding wheat genotypes indicated.

864 **Suppl Figure 8: Pairwise Bray-Curtis distances of the fungal communities in the**
865 **replicates of seeds**

866 Each dot shows pairwise distance between two replicates. Red boxplots show distances
867 of the microbial communities between samples of the same wheat genotype. Black

868 boxplots show distances of the bacterial communities between samples of two
869 corresponding wheat genotypes indicated.

870 **Suppl Figure 9: Pairwise Bray-Curtis distances of the fungal communities in the**
871 **replicates of seeds and axenic seedlings**

872 Each dot shows pairwise distance between two replicates. Red boxplots show distances
873 of the microbial communities between samples of the same wheat genotype. Black
874 boxplots show distances of the bacterial communities between samples of two
875 corresponding wheat genotypes indicated.

876 **Suppl Figure 10: Bray-Curtis distances based PCoA of the fungal communities of roots**
877 **grown in the agricultural and natural soil**

878 Data based on ITS amplicon data from *Triticum aestivum* (Ta_De and Ta_Tr) and
879 *Triticum dicoccoides* (Td).

880 **Suppl Figure 11: Interaction plots of fungal communities of root samples grown in the**
881 **soil from agriculture and wild**

882 Data based on ITS amplicon data from *Triticum aestivum* (Ta_De and Ta_Tr) and
883 *Triticum dicoccoides* (Td).

884 **Suppl Figure 12: Interaction plots showing Shannon Index estimates of bacterial**
885 **communities in seedlings grown in soil**

886 Data based on 16S amplicon data from *Triticum aestivum* (Ta_De and Ta_Tr) and
887 *Triticum dicoccoides* (Td).

888 **Suppl Figure 13: Composition of the bacterial community in seedlings colonized by the**
889 **soil-derived microbiota A) Bacterial community in different tissues B) Leaf-associated**
890 **bacterial community in plants grown in agricultural and natural soil C) Root-associated**
891 **bacterial community in plants grown in agricultural and natural soil**

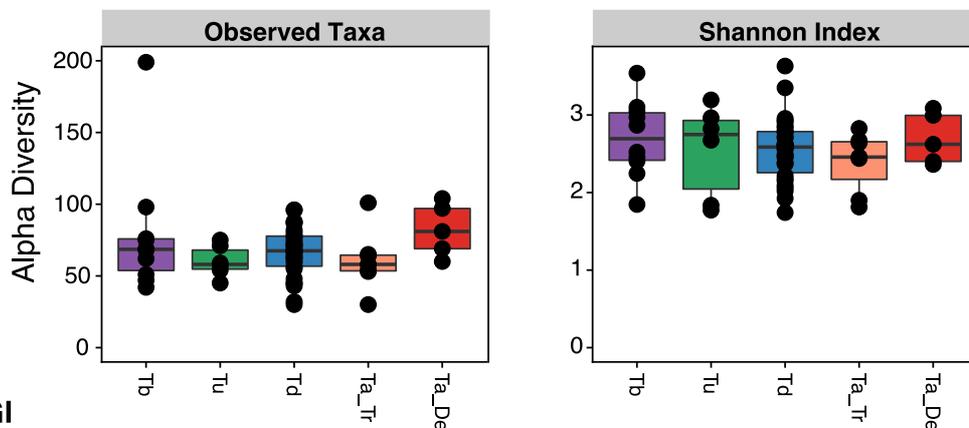
892 **Suppl Figure 14: Composition of the fungal community in roots colonized by the soil-**
893 **derived microbiota**

894 Data based on mean read abundances of fungal features. Analyses based on ITS
895 amplicon data from *Triticum aestivum* (Ta_De and Ta_Tr) and *Triticum dicoccoides* (Td).
896 (IS) stands for the “Incertae sedis” taxa.

897

BACTERIA

A



FUNGI

B

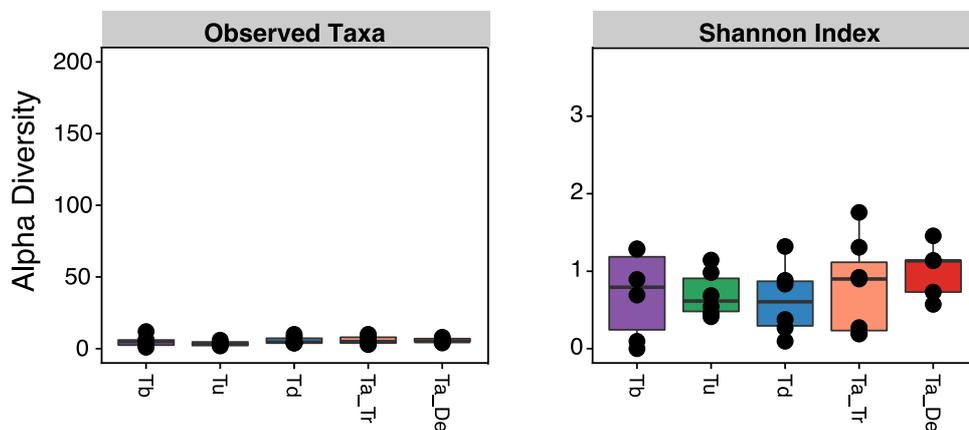


Figure 1

A

	Tb	Tu	Td	Ta_Tr	Ta_De
Proteobacteria: Pseudomonadaceae -	14.6	24.4	23.5	32	16.9
Proteobacteria: Halomonadaceae -	22.6	17.6	22.9	7	5.2
Proteobacteria: Vibrionaceae -	19.6	15.8	18.5	4.5	3.4
Proteobacteria: Comamonadaceae -	12.9	9.3	13.5	17	32.7
Proteobacteria: Oxalobacteraceae -	4	2.8	5.9	7	15.2
Proteobacteria: Enterobacteriaceae -	1	1.6	1.8	27.4	9.2
Firmicutes: Bacillaceae -	11	13.1	0	0	0
Proteobacteria: Neisseriaceae -	2.6	2.8	1.8	0.3	0.3
Proteobacteria: Xanthomonadaceae -	2.3	3.7	1.6	0.3	0.6
Proteobacteria: Shewanellaceae -	0.9	1.1	1.6	1.4	1.1
Proteobacteria: [Chromatiaceae] -	0.9	1	1.4	0.3	0.4
Proteobacteria: Caulobacteraceae -	1.3	0.3	1.3	0.7	0
Tenericutes: Achleplasmataceae -	0	0	1.9	0	0
Firmicutes: Streptococcaceae -	1.2	1.3	1	0.2	0.1
Actinobacteria: Microbacteriaceae -	0.3	0.1	0.1	0.5	8
Firmicutes: Enterococcaceae -	1.3	1.2	0.6	0	0
Proteobacteria: Sphingomonadaceae -	0.6	0.2	0.6	0.3	0.4
Bacteroidetes: [Weeksellaceae] -	0.6	0.3	0.2	0.1	3.2
Proteobacteria: Methylobacteriaceae -	0.2	0.5	0.4	0.3	0
Firmicutes: Aerococcaceae -	0.2	0.4	0.3	0.1	0.1

Wild wheat

(L)

(C)

B

	Tb	Tu	Td	Ta_Tr	Ta_De
Ascomycota: Pleosporales_(S) -	1.4	2.3	11.3	57.8	39.3
Ascomycota: Trichosphaeriaceae -	33.5	53.5	0	0	0
Ascomycota: Pleosporaceae -	19.6	11.2	16.2	18.5	0.1
Ascomycota: Chaetomiaceae -	29.9	24.3	0	0	0
Ascomycota: Mycosphaerellaceae -	0	0	16	2.9	40
Ascomycota: Saccharomycesaceae -	0	2.1	37.5	1.5	0
Ascomycota: Cladosporiaceae -	12.7	0.9	0.1	1.3	14.7
Ascomycota: Aspergillaceae -	0.5	5.7	17.3	0	0
Ascomycota: Aureobasidiaceae -	0	0	0	10.3	0.1
Ascomycota: Phaeosphaeriaceae -	0	0	0	5	0.1
Ascomycota: Nectriaceae -	0	0	0	0	5.5
Mucoromycota: Rhizopodaceae -	2.1	0	0.9	0	0
Ascomycota: Dothioraceae -	0	0	0	2	0
Ascomycota: Botryosphaeriaceae -	0	0	0.6	0	0
Ascomycota: Didymellaceae -	0	0	0	0.3	0.1

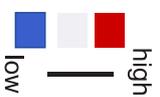


Figure 2

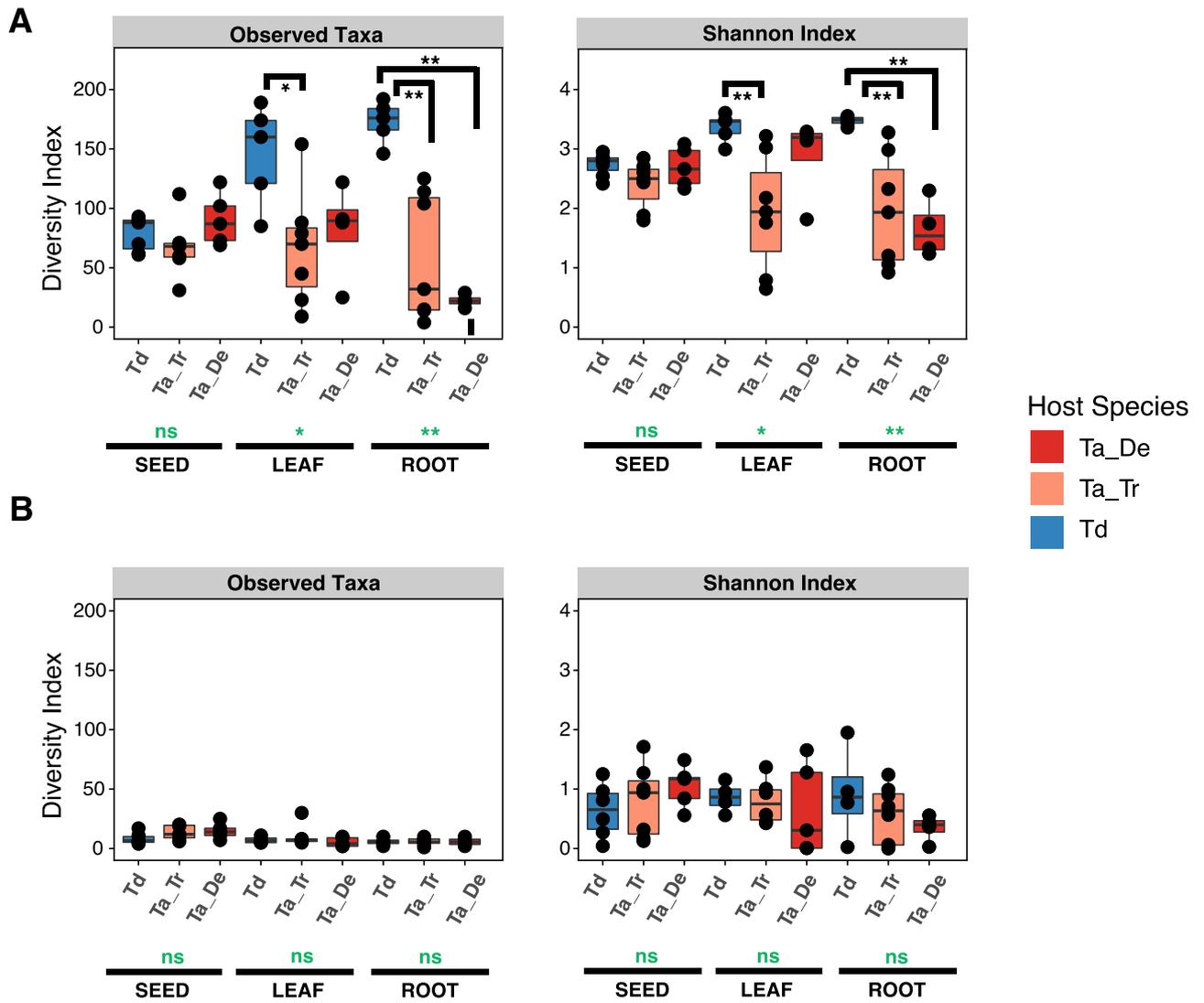


Figure 3

A

	SEED			LEAF			ROOT		
	Td	Ta_Tr	Ta_De	Td	Ta_Tr	Ta_De	Td	Ta_Tr	Ta_De
Proteobacteria: Enterobacteriaceae -	2.4	27.4	9.2	4.1	29.7	24.9	2.2	44.4	24.6
Proteobacteria: Pseudomonadaceae -	23.6	31.9	16.9	7.6	29.4	2.9	6.1	22.6	15.9
Proteobacteria: Comamonadaceae -	17.7	17	32.7	15.2	5.3	14.9	20.4	3.5	0
Proteobacteria: Oxalobacteraceae -	9.2	7	15.2	21.3	6.8	18.2	18.9	3.8	28.6
Proteobacteria: Halomonadaceae -	20.7	7	5.2	18.7	6	8.6	20	8.3	0
Proteobacteria: Vibrionaceae -	14.6	4.5	3.4	15.4	6	9.4	17.7	9.7	0
Firmicutes: Paenibacillaceae -	0.1	0	0.1	1.4	10.7	2.6	1.1	0.1	26.3
Proteobacteria: Burkholderiaceae -	0	0	0.8	6.5	1.8	4.1	5.4	1.6	0
Actinobacteria: Microbacteriaceae -	0.1	0.5	8	0.6	0.4	1.4	0.2	2.9	1
Proteobacteria: [Chromatiaceae]	1.1	0.3	0.4	1.7	0.5	0.7	1.4	1	0
Proteobacteria: Xanthomonadaceae -	1.5	0.3	0.6	1	0.3	0.8	1.3	0.6	0
[Thermi]: Deinococcaceae -	0	0	0.1	0	0	7.4	0	0	0.6
Proteobacteria: Sphingomonadaceae -	1.1	0.3	0.4	1.5	0.2	1.2	0.3	0	1.1
Proteobacteria: Stenaneliaceae -	1.6	1.4	1.1	0.2	0	0	0.2	0	0
Proteobacteria: Neisseriaceae -	1.2	0.3	0.3	0.9	0.3	0.6	1	0.4	0
Firmicutes: Streptococcaceae -	0.9	0.2	0.1	0.7	0.2	0.2	0.8	0.3	0
Bacteroidetes: [Weeksellaceae]	0.3	0.1	3.2	0.1	0	0	0.1	0	0
Proteobacteria: Caulobacteraceae -	1.6	0.7	0	0.2	0	0.1	0.2	0	0
Proteobacteria: Methylobacteriaceae -	0.5	0.3	0	0.1	0.5	0.3	0.1	0	0.5
Proteobacteria: Methylocystaceae -	0	0	0	0.1	0.8	0.2	0.1	0	0.7

B

	SEED			LEAF			ROOT		
	Td	Ta_Tr	Ta_De	Td	Ta_Tr	Ta_De	Td	Ta_Tr	Ta_De
Ascomycota: Pleosporales f.(IS) -	11.3	57.8	39.3	43.9	27.3	49.7	46.7	36.2	78.4
Ascomycota: Pleosporaceae -	16.2	18.5	0.1	37.4	13.7	40.1	31.2	29.4	25.2
Ascomycota: Aureobasidiaceae -	0	10.3	0.1	0	25.4	0	0	17.2	0.7
Ascomycota: Mycosphaerellaceae -	16	2.9	40	0	0	1.3	0	0	0
Ascomycota: Saccharomycetaceae -	37.5	1.5	0	0	0	0	0	0	0
Basidiomycota: Sportidiobolaceae -	0	0.2	0	0	13.8	6.1	1.7	10	0.1
Ascomycota: Cladosporiaceae -	0.1	1.3	14.7	2.3	5.7	0.7	1.7	0.1	0.4
Ascomycota: Aspergillaceae -	17.3	0	0	1.1	0.2	0	1.6	0	0
Ascomycota: Saccharomycetales f.(IS) -	0	0	0	6.5	3.1	0	5	0	0
Basidiomycota: Bulleribasidiaceae -	0	0	0	0	0	0	0	7.1	0
Ascomycota: Nectriaceae -	0	0	5.5	0.4	3.4	0	0	0	0
Basidiomycota: Phaffomycetaceae -	0	0	0	0	0.9	0	0	0	0
Ascomycota: Phaeosphaeriaceae -	0	5	0.1	0	0	0	8.9	0	0
Ascomycota: Chaetomiaceae -	0	0	0	6.5	0	0	0	0	0
Ascomycota: Didymellaceae -	0	0.3	0.1	0	1.4	0	3	0	0
Ascomycota: Trichosphaeriaceae -	0	0	0	0	3.4	0	0	0	0
Ascomycota: Dothioraceae -	0	2	0	0	0	0	0	0	0
Basidiomycota: Meruliaceae -	0	0	0	0	1.7	0	0	0	0
Ascomycota: Ophiocordycipitaceae -	0	0	0	0	0	1.2	0	0	0
Mucromycota: Rhizopodaceae -	0.9	0	0	0	0	0	0	0	0

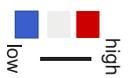


Figure 4

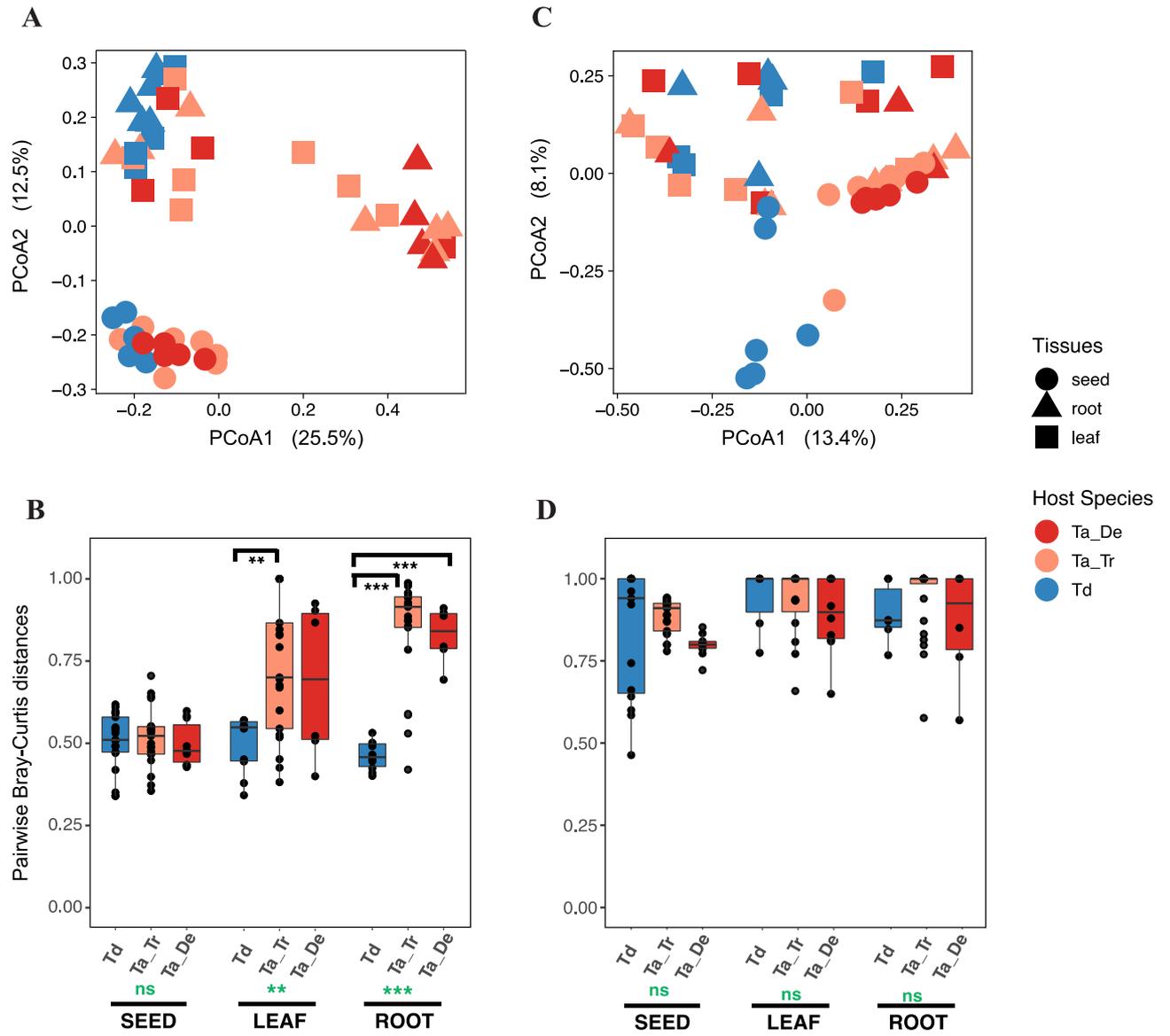


Figure 5

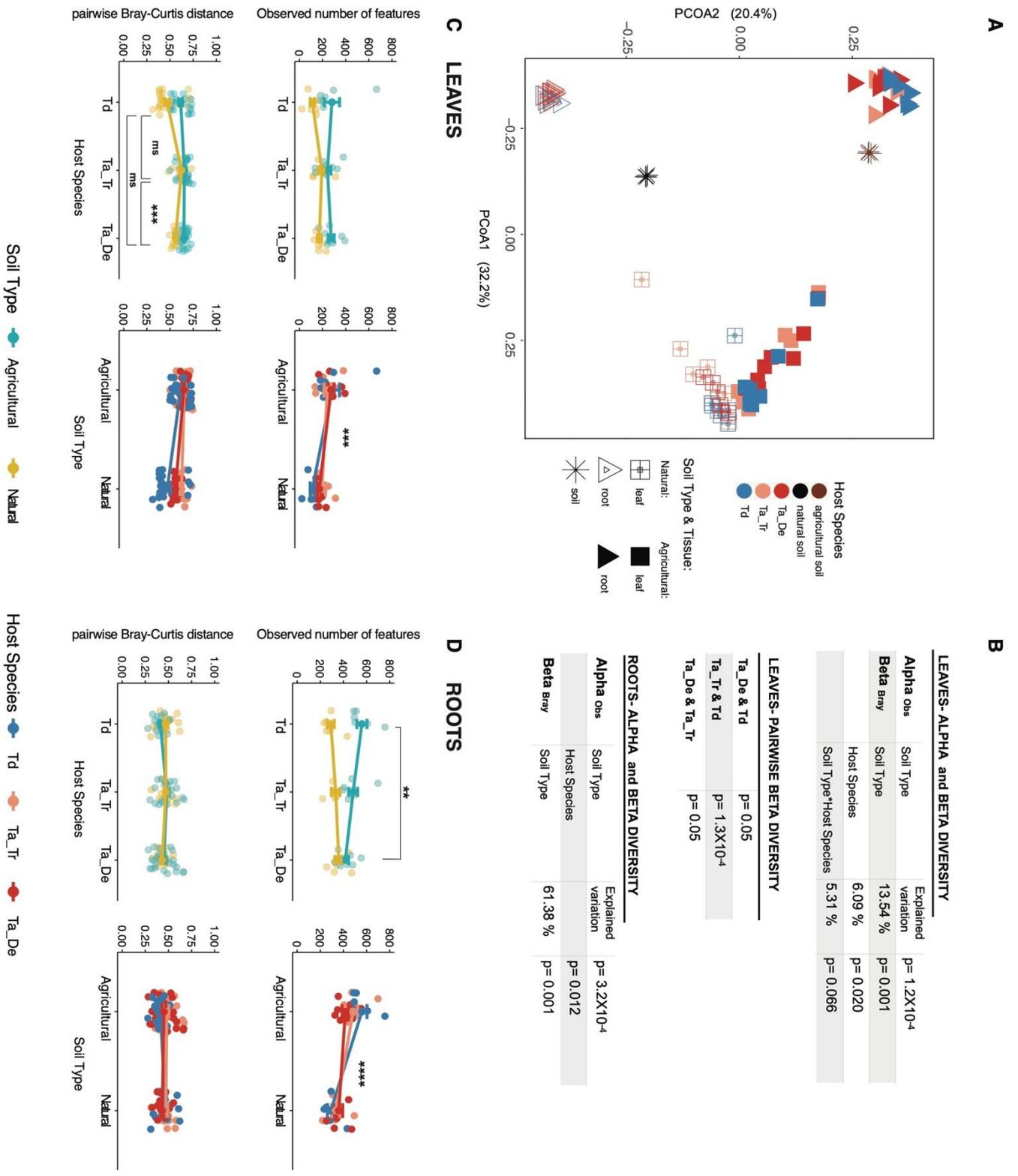
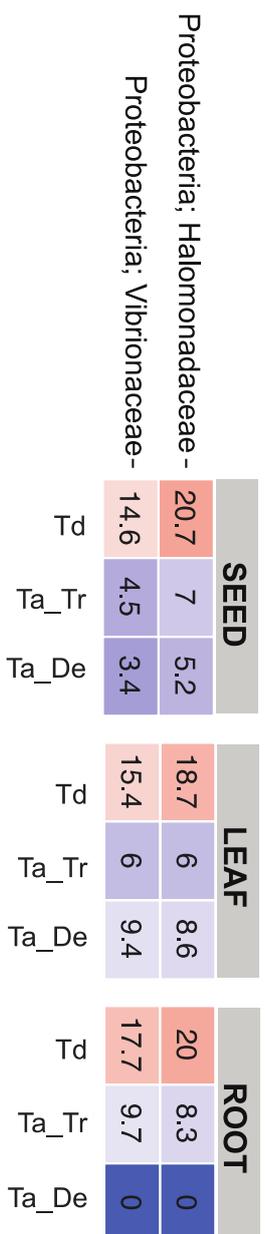


Figure 6

A Seedborne bacteria



B Soil-derived bacteria

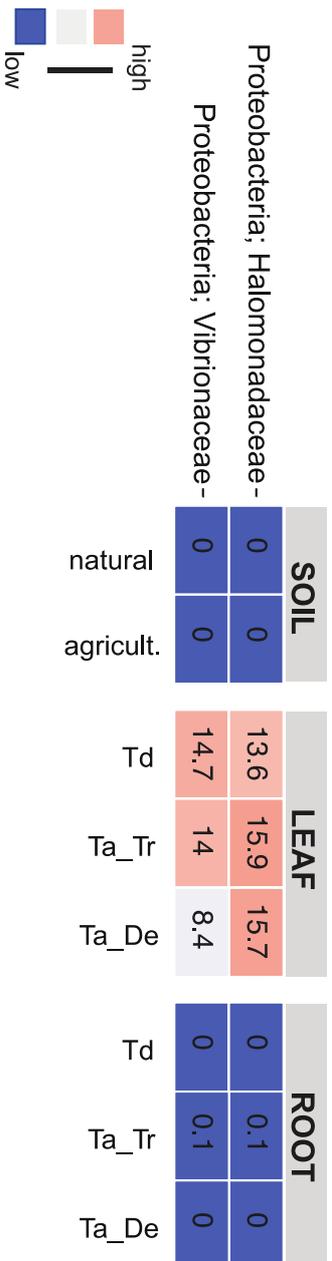


Figure 7

1 **Higher stochasticity of microbiota composition in seedlings of domesticated wheat**
2 **compared to wild wheat**

3

4 Ezgi Özkurt, M. Amine Hassani, Uğur Sesiz, Sven Künzel, Tal Dagan, Hakan Özkan, Eva H.
5 Stukenbrock

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7

8 **Supplementary methods**

9

10 **Origin of the wheat material**

11

12 Seed collections of *Triticum dicoccoides*, *Triticum boeoticum* and *Triticum urartu* were
13 collected from different locations in South- East Turkey in previous years (2004, 2005, 2006)
14 (Supp. Table 1). Seeds of *T. aestivum* from Turkey was collected by the farmer from a field in
15 Kışlak, Antakya in 2017 (Supp. Table 1) and stored in a dry and cold room. We obtained these
16 seeds in the same year from the farmer and we stored them at 4 °C until further usage.
17 Seeds of *T. aestivum* from Germany (Benchmark cv.) were collected from the experimental
18 farm of the Kiel University in Hohenschulen, Schleswig-Holstein in 2017. All the seeds in the
19 collections were stored at 4 °C until further usage and processed all the seeds in the
20 collections in 2018.

21

22

23 **Surface sterilization of seeds**

24 Seeds of *Triticum aestivum*, *Triticum dicoccoides*, *Triticum boeoticum* and *Triticum urartu*
25 were washed with sterile miliQ water. Later, each seed was treated shortly with 0.1%
26 TritonX and washed with water, then 80% EtOH and washed with water and lastly 1.2 %
27 Sodium Hypochlorite (NaClO) and finally washed with water three times. Wash-off water
28 from some seeds were kept and sequenced. Some seeds were immediately processed for
29 DNA extraction while others were grown in sterile jars.

30

31

32

33 **Axenic propagation of leaves and roots**

34 After sterilization, seeds were directly transferred into the plant minimal medium (PNM). 1L
35 of PNM medium is composed 1 mL of of 500 mM Potassium Nitrate, 1mL of KH₂PO₄ stock
36 solution (5g/ 100mL), 1 mL of K₂HPO₄ stock solution (2.5 g/100mL), 1mL of 2M Magnesium
37 Sulfate (MgSO₄ X 7 H₂O), 1 mL of 200 mM Calcium Nitrate, 2.5 mL of Fe-EDTA and 1mL of
38 Sodium Chloride stock solution (2.5 g/100 mL). The pH of the solution was adjusted to 6.2
39 and the medium was solidified by adding Gelrite. Finally, the medium was autoclaved at 121
40 °C for 15 minutes. After autoclaving, 10 mL 1M filtered MES was added to the medium. The
41 medium was transferred into sterile jars and after it was solidified, sterilized seeds were
42 transferred into the medium. Seeds were germinated in sterile jars in a climate chamber
43 (Percival plant growth chambers, CLF PlantClimatics GmbH, Wertingen, Germany). Light
44 period of 16/8 hours, temperature 15 °C) for two weeks corresponding to the second leaf
45 growth stage.

46

47

48 **DNA Extraction from seeds and seedling material**

49

50 We followed a similar method to the Phenol-Chloroform extraction method customized by
51 Agler et al, 2016 (1). For DNA extractions, seed, leaf and root materials was processed as
52 follows: Single seeds were transferred into 7mL tubes containing sterilized zirconium beads.
53 Approximately six cm of leaves and six cm of multiple roots were used for DNA extraction.
54 We crushed samples with Bertin Precellys Instrument (Bertin Instruments, Montigny-le-
55 Bretonneux, France) pre-cooled with liquid nitrogen at 7500 rpm for 2 x 30 seconds with an
56 in-between pause of 15 seconds. To the crushed samples, we added 0.6 mL of DNA
57 extraction buffer (0.5% SDS, 50 mM TRIS buffer at pH 8, 200 mM NaCl, 2mM EDTA). Next, we
58 added 50 µg/mL of Lysozyme and 10 µg/mL of Proteinase K and incubated the samples for
59 45 min at 37°C. After incubation, samples were transferred into new tubes containing 0.1
60 mm zirconium and 0.5mm glass beads and beat-treated using Bertin Precellys (Bertin
61 Instruments, Montigny-le-Bretonneux, France) at 6500 rpm for 2 x 30 seconds with a 15

62 second pause. 10 µg/mL RNase was added to each sample before incubation at 37°C for 45
63 minutes. We next centrifuged the samples at 13000 rpm for 2 minutes to remove beads and
64 non-homogenized tissue. The nucleic acids were cleaned up with
65 phenol/chloroform/isoamyl alcohol (25:24:1) for three times, and chloroform/isoamyl
66 alcohol (24:1) one time, then precipitated by adding 1/10th of the sample volume of 3 M
67 sodium acetate and 2.5x volume 100% ethanol. Samples were then centrifuged at 4°C at
68 13000 rpm for 30 minutes. Afterwards, the pellet was washed with 70% ethanol. Finally,
69 pellets were washed in 100 µL of sterile water and stored at -20 °C until further usage. DNA
70 extracted from seeds was treated with 1:1 with 20% Chelex-100 for 30 minutes to remove
71 potential PCR inhibitors. Harvested leaves and roots were processed with the PowerSoil DNA
72 kit (Mo Bio Laboratories, Heidelberg, Germany) according to the manufacturer's instructions
73 (See Material and Methods).

74

75

76 **DNA library preparation**

77

78 We prepared libraries for replicates of individual seed as well as leaf and root samples,
79 respectively. In addition, we included three negative controls of DNA extraction and three
80 wash-off controls from the surface sterilization to the libraries.

81 PCR amplification was performed in two steps (Suppl. Table 1 and 2). For 16S, we used the
82 interfering primer approach developed by Agler and co-workers for *Arabidopsis thaliana*
83 (Suppl. Table 3). However, we modified the primers according to the wheat genome (2). In
84 the first step, universal primers primers Bacteria V5/V6/V7: B799F/ B1192R and for fungi
85 ITS1: ITS1F/ITS2 were used to amplify the targeted regions (Suppl Table 3). Interfering
86 primers for the 16S region were added in this step. Triplicates of each reaction were
87 performed for both first and second steps. First PCR was run for 15 and 20 cycles for 16S and
88 ITS, respectively. After the first reactions, three replicates were combined. 10 µL of each PCR
89 product was cleaned with 0.5 µL Antarctic phosphatase and 0.5 µL Exonuclease I in 1.22 µL
90 Antarctic phosphatase buffer (New England Biolabs GMBH, Frankfurt, Germany) at 37 °C for
91 30 minutes followed by 80 °C for 15 min. The product of the cleaned samples was used as a
92 template for the second PCR in which unique barcodes for sequencing of 12bp length were

93 added as well. Second PCRs were run for 20 and 15 cycles for 16S and ITS, respectively
 94 including in total 35 cycles for both 16S and ITS reactions.

95

96 **Table 1: PCR conditions for the V5-7 amplification**

97

V5-V7				
<u>1st PCR:</u>		<u>1st PCR</u>		
		<u>conditions:</u>		
10 µL	Phusion Master Mix	98°C	30s	
1 µL	Forward Primer	98°C	10s	
1 µL	Reverse Primer	55°C	45s	X15 cycles
2 µL	Interfering Forward Primer	72°C	30s	
2 µL	Interfering Reverse Primer	72°C	5m	
0.5 µL	DMSO			
3 µL	DNA			
0.5 µL	H2O			
Clean-up with ExoI and Antarctic Phosphatase	10 µL of the 1st PCR product is cleaned-up	37°C 80°C	30m 15m	
<u>2nd PCR:</u>		<u>2nd PCR</u>		
		<u>conditions:</u>		
10 µL	Phusion Master	98°C	30s	

	Mix			
1 µL	Forward Primer	98°C	10s	
1 µL	Barcoded Reverse Primer	55°C	45s	X20 cycles
0.5 µL	DMSO	72°C	30s	
3 µL	Clean-up product	72°C	5m	
4.5 µL	H2O			

98

99

100

101 **Table2: PCR conditions for the ITS1-ITS2 amplification**

102

ITS1F-ITS2				
<u>1st PCR:</u>		<u>1st PCR conditions:</u>		
10 µL	Phusion Master Mix	98°C	30s	
1 µL	Forward Primer	98°C	10s	
1 µL	Reverse Primer	55°C	30s	X20 cycles
0.5 µL	DMSO	72°C	30s	
1 µL	DNA	72°C	5m	
6.5 µL	H2O			
Clean-up with ExoI and Antartic	10 µL of the 1st PCR product is	37°C 80°C	30m 15m	

Phosphatase	cleaned-up			
2nd PCR:		2nd PCR conditions:		
10 µL	Phusion Master Mix	98°C	30s	
1 µL	Forward Primer	98°C	10s	
1 µL	Barcoded Reverse Primer	55°C	30s	X15 cycles
0.5 µL	DMSO	72°C	30s	
1 µL	Clean-up product	72°C	5m	
6.5 µL	H2O			

103

104 Finally, PCR products of ITS and 16S amplifications were sub-pooled in equal concentrations
105 where the concentration of each sample was estimated using the software of gel visualizer
106 (BIO RAD, Image Lab™ software 5.2.1). Subpools of 16S amplicons were run on the gel and
107 bacterial DNA was cut out of the gel. DNA concentration of the subpools were quantified
108 with Qubit. Then, we combined subpools in equal concentrations into final pools. Finally, we
109 quantified the final concentration of the final pool with Qubit 3.0 Fluorometer (Thermo
110 Fisher Scientific, Darmstadt, Germany).

111

112

113 **Amplicon Sequencing**

114 The final DNA pool spiked with 10% PhiX genomic DNA (Illumina, San Diego, CA)
115 was used for MiSeq sequencing (Illumina, San Diego, CA) using the MiSeq Reagent Kit v3
116 (Illumina, San Diego, CA). 0.5 µM of forward, reverse and index sequencing primers
117 complementary to the linker/primer region of the concatenated primers were added

118 together (sequence of primers are available in Suppl Table 3). Paired-end sequencing was
119 performed for 600 cycles for 301 bp amplicon size according to the Illumina instructions
120 (<http://os.bioprotocol.org/attached/file/20171217/miseq%20denature%20dilute%20libraries%20guide%2015039740%2003.pdf>).
121

122

123

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131

Table S1: Summary information about the wheat seed collections

Year	Location	Species	Latitude	Longitude
2017	Turkey,Kışlak	<i>Triticum aestivum</i> (non-treated)	35° 58' 21.8"	36° 07' 41.8"
2017	Germany	<i>T. aestivum</i> cultivar Benchmark, treated)	54°18'50.9"	9°59'42.2"
2005	West population	<i>Triticum boeoticum</i>	37° 19' 31"	37° 09' 28"
2006	West population	<i>T. boeoticum</i>	36°54'29"	37°11'49"
2006	West population	<i>Triticum dicoccoides</i>	37°20'19"	37°16'50"
2006	West population	<i>T. dicoccoides</i>	37°19'50"	37°18'51"
2004	East population	<i>T. dicoccoides</i>	37°50'40"	39°47'58"
2004	East population	<i>Triticum uratu</i>	37°50'40"	39°47'58"
2004	East population	<i>T. dicoccoides</i>	37°47'31"	39°57'18"

Table S2: Summary amplicon sequencing data

	No of samples	Total no of reads	Average no of reads	No of OTUs before filtering	No of OTUs after rarefaction
Seeds- bacteria	58	2356761	40633,81	1441	1157
Seeds-fungi	30	2621026	87367,53	272	124
Axenic leaves- bacteria	16	633975	84526	760	589
Axenic leaves- fungi	15	719618	47974,53	119	98
Axenic roots- bacteria	16	663813	41488,31	828	632
Axenic roots- fungi	16	2309064	144316,5	113	74
Leaves- bacteria	42	2460327	58579,21	3864	3676
Roots- bacteria	39	1690934	43357,28	3184	3100
Roots-fungi	42	1545621	36800,5	3757	3737
Natural soil- bacteria	3	100190	33396.67	1258	1248
Agricultural soil- bacteria	3	82333	27444,33	1270	1266

Table S3: Sequence of the V5-7 and ITS1 primers used for amplification and sequencing

16S, ITS universal and interfering primers

Universal Primers- PCR1

Bacteria - V5/V6/V7	Primer ID	Primer sequence
Forward	799F	AACMGGATTAGATACCKG
Reverse	1192R	ACGTCATCCCCACCTTCC

Fungi ITS 1- PCR1

Fungi - ITS1	Primer ID	Primer sequence
Forward	ITS1F	CTTGGTCATTTAGAGGAAGTAA
Reverse	ITS2	GCTGCGTTCTTCATCGATGC

Interfering Primers: Mitochondria - V5/V6/V7

Forward	V5MBF	GGATCAGGGGCCAGCTAACGCGTGAAACA
Reverse	V5MBR	CGGAGCGGGGCGCGTACTATTACCACTACG

16S, ITS PCR2 primers

Bacteria	Primer ID	Primer sequence
Forward	B5-F	AATGATACGGCGACCACCGAGATCTACACTGCGACTGGCGAACMGGATTAGATACCKG
Reverse	B5-R	CAAGCAGAAGACGGCATACGAGATXXXXXXXXXXXXCAGCCATTTAGTGTCACGTCATCCCCACCTTCC
Fungi	Primer ID	Primer sequence
Forward	FITS1-F	AATGATACGGCGACCACCGAGATCTACACTCACGCGCAGGCTTGGTCATTTAGAGGAAGTAA
Reverse	FITS1-R	CAAGCAGAAGACGGCATACGAGATXXXXXXXXXXXXCGTACTGTGGAGAGCTGCGTTCTTCATCGATGC

XXXXXXXXXXXX: 12bp barcode sequence

<u>16S, ITS sequencing primers</u>	-	-
Bacteria - V5/V6/V7	Primer ID	Primer sequence
Forward	B5-R1	ACGACTGCGACTGGCGAACMGGATTAGATACCC
Reverse	B5-R2	CAGCCATTTAGTGTCACGTCATCCCCACCTTCC
Index	B5-R3	GGAAGGTGGGGATGACGTGACACTAAATGGCTG

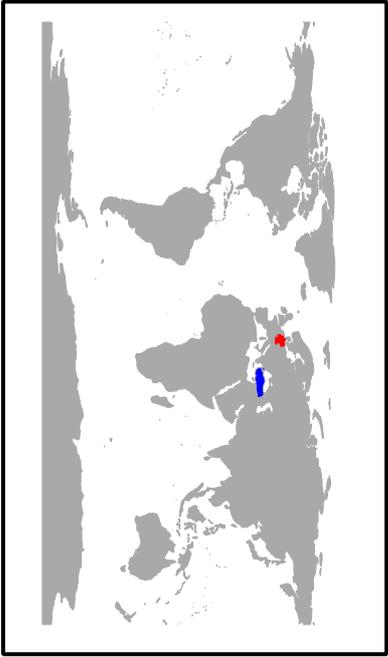
Fungi ITS 1- PCR1

Fungi - ITS1	Primer ID	Primer sequence
Forward	ITS-R1	TCACGCGCAGGCTTGGTCATTTAGAGGAAGTAA
Reverse	ITS-R2	CGTACTGTGGAGAGCTGCGTTCTTCATCGATGC
Index	ITS-R3	GCATCGATGAAGAACGCAGCTCTCCACAGTACG

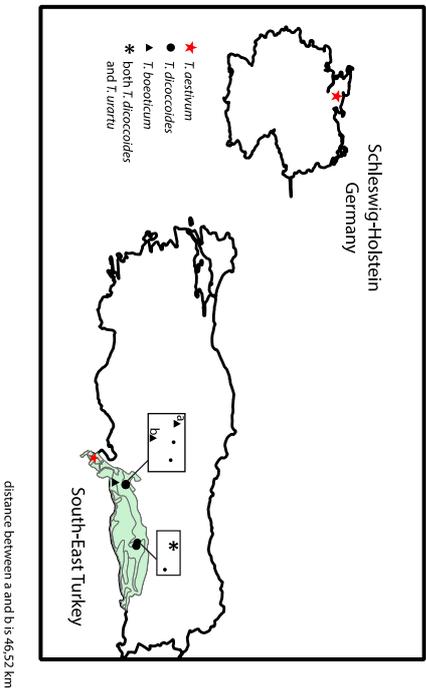
Table S4- S7 is available here: <https://www.biorxiv.org/content/10.1101/685164v1.supplementary-material>

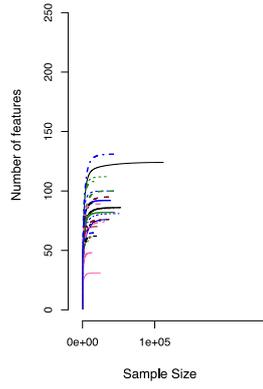
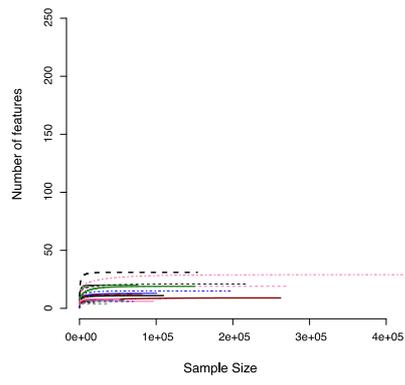
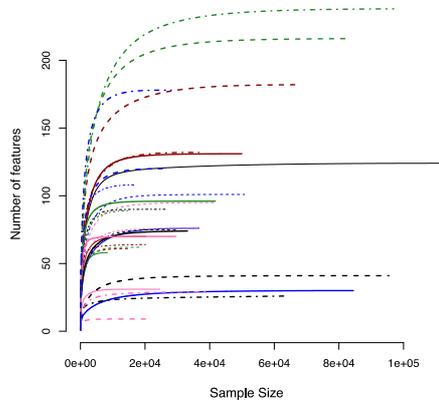
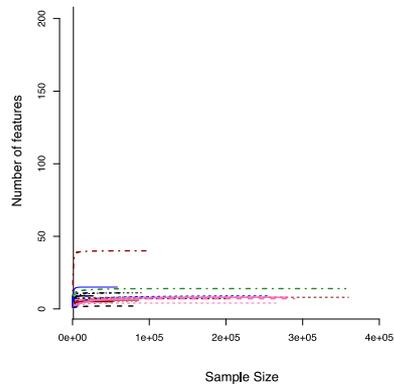
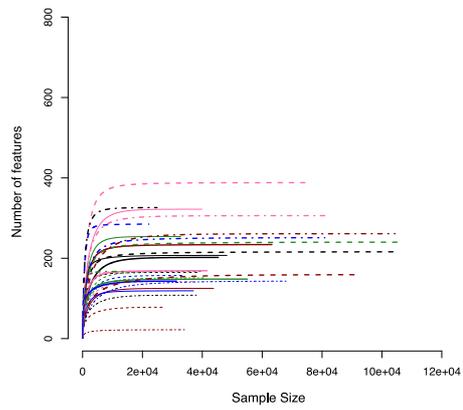
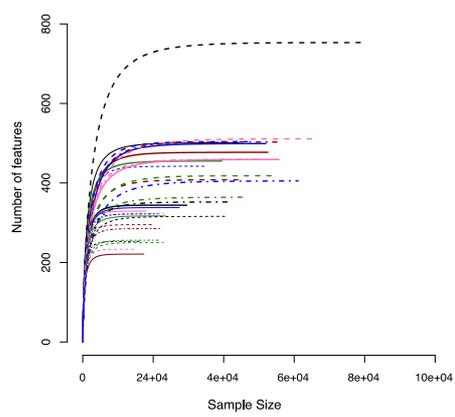
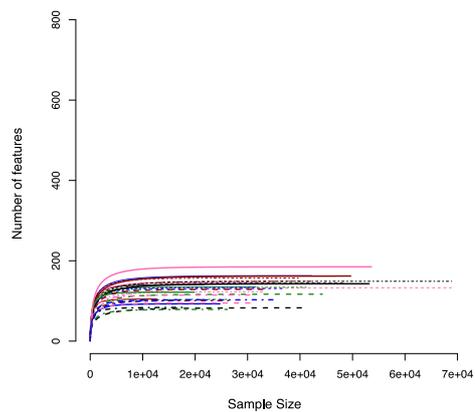
Supp. Fig1

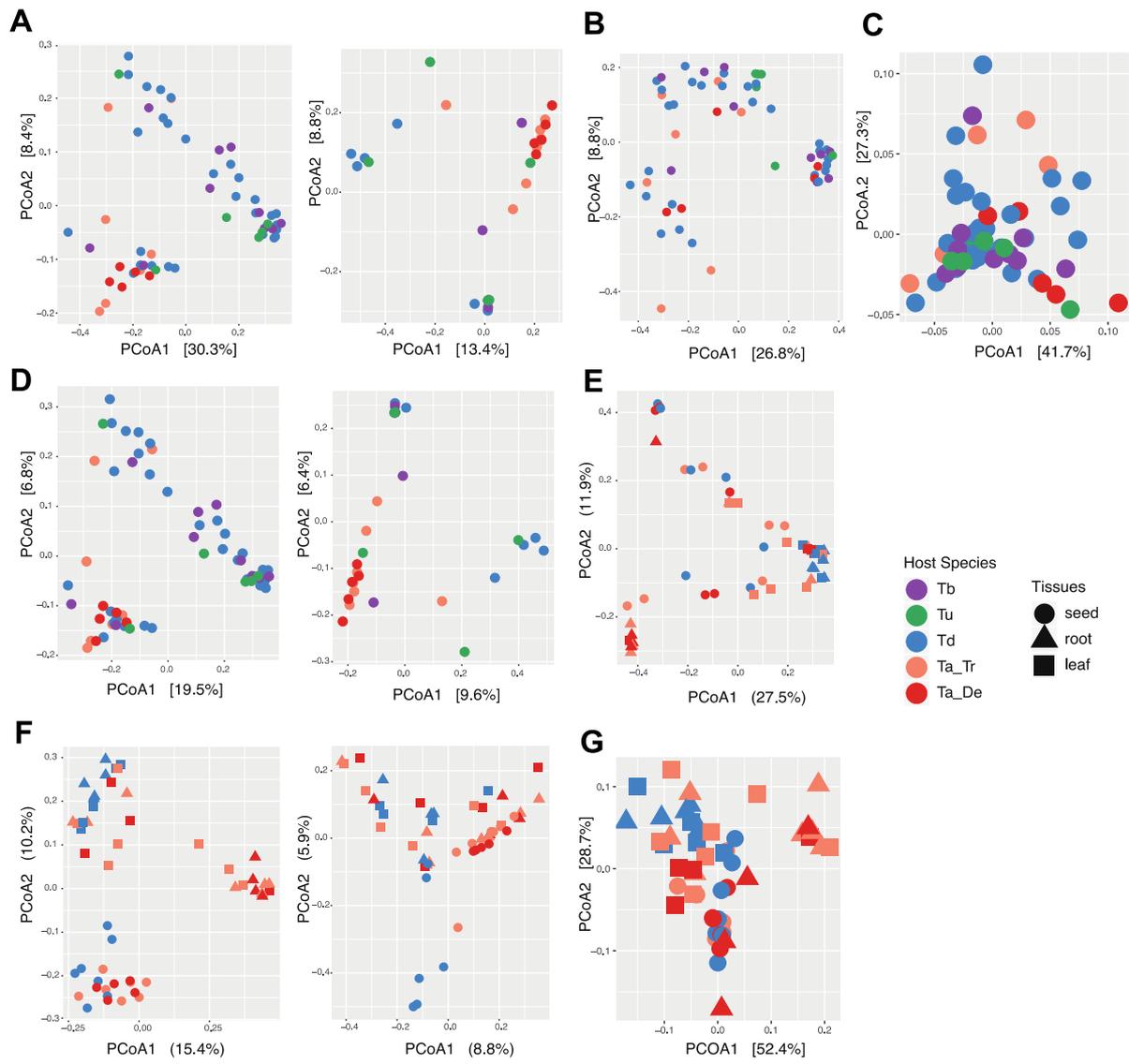
A



B

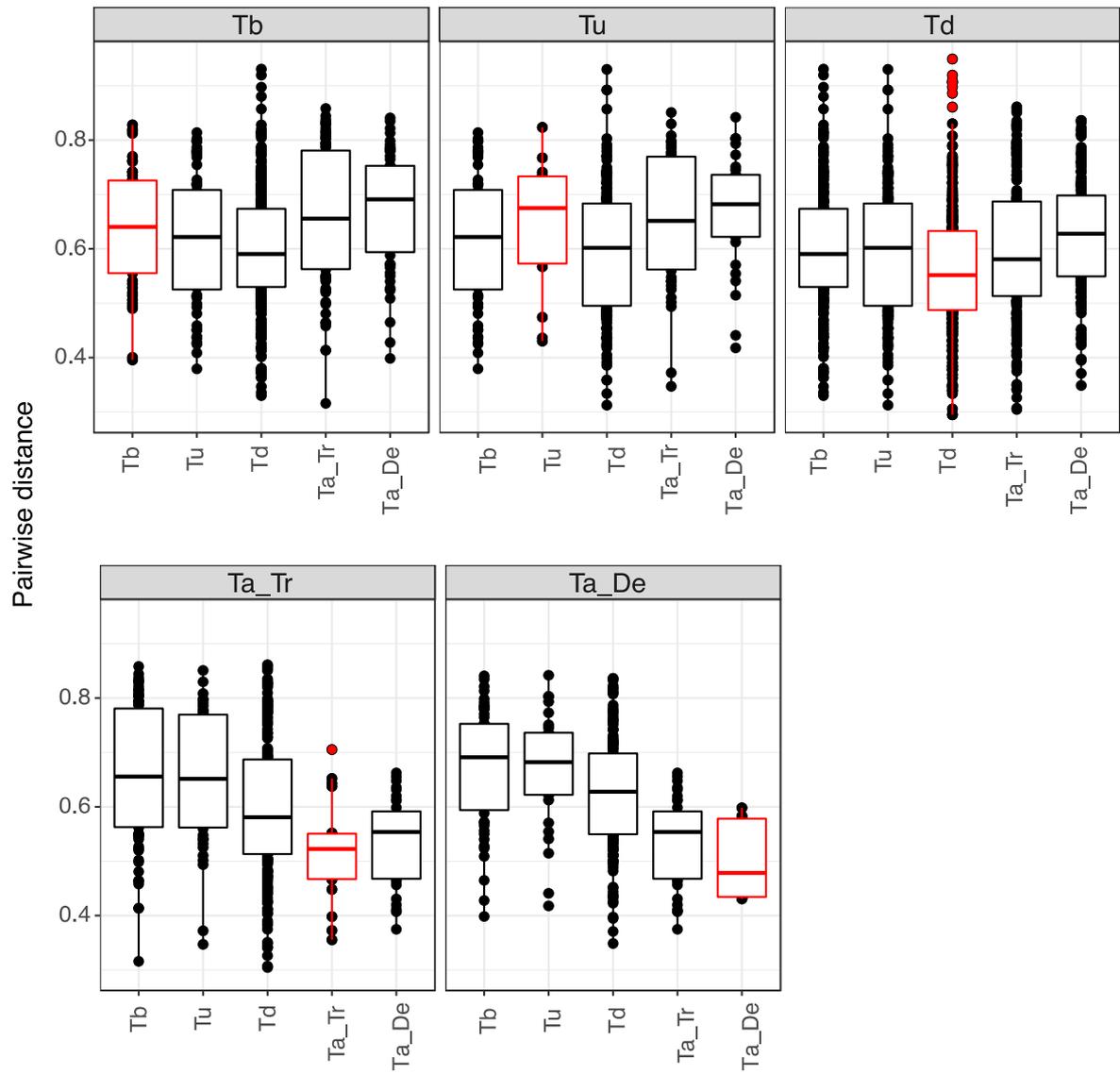


A**B****C****D****E****F****G**



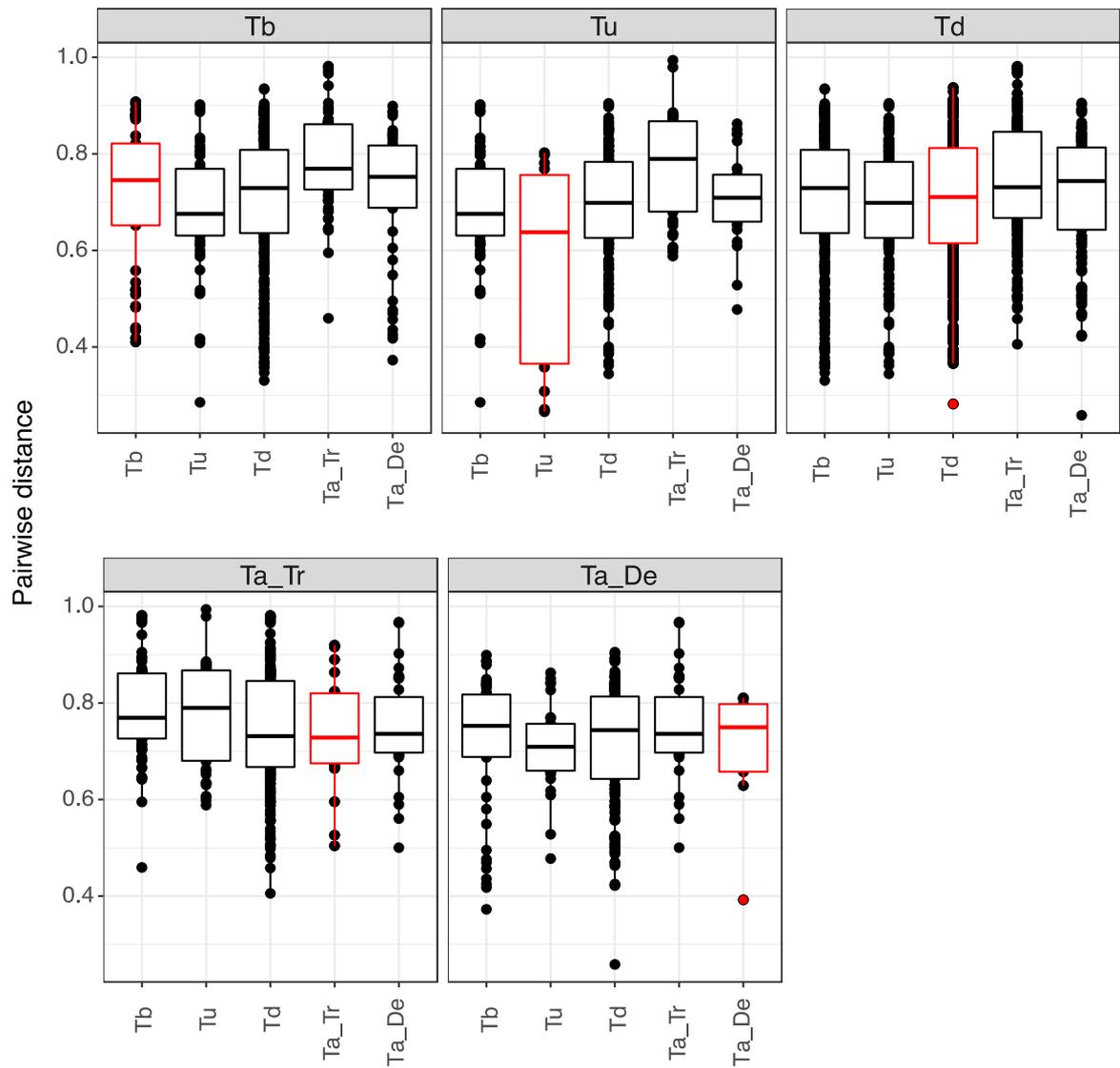
Supp. Fig3

Distance Metric = Bray-Curtis

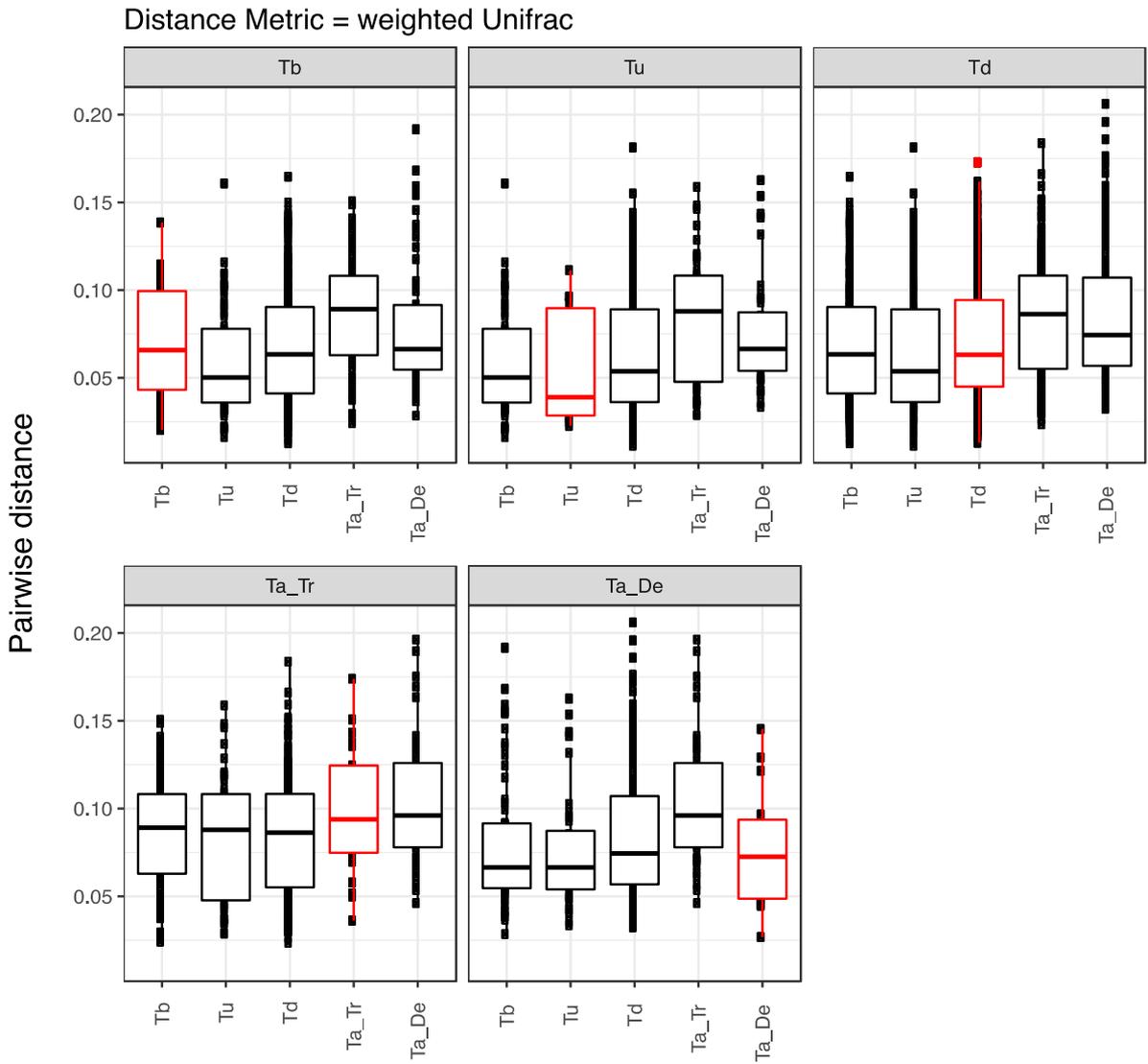


Supp. Fig4

Distance Metric = unweighted UniFrac

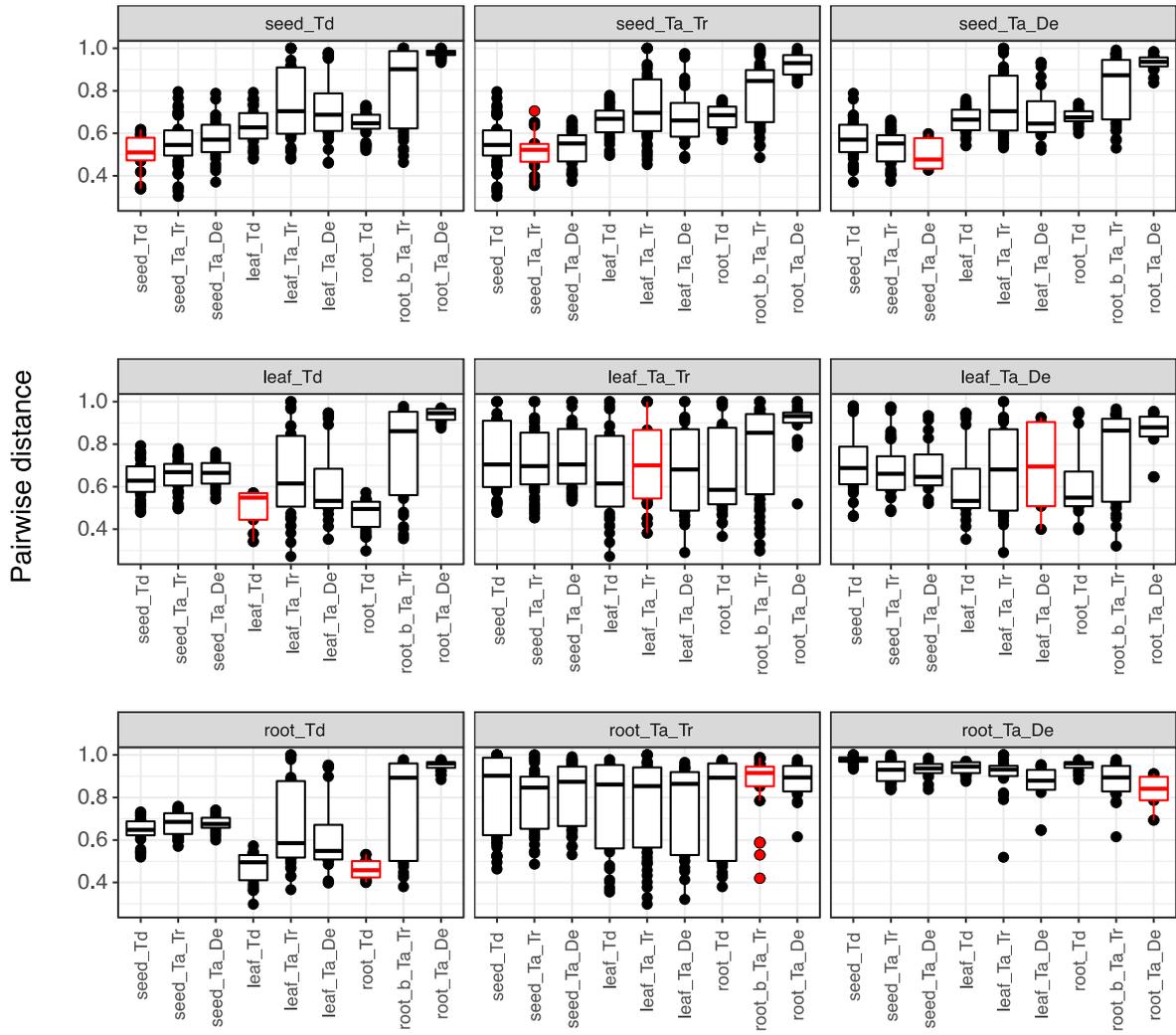


Supp. Fig5



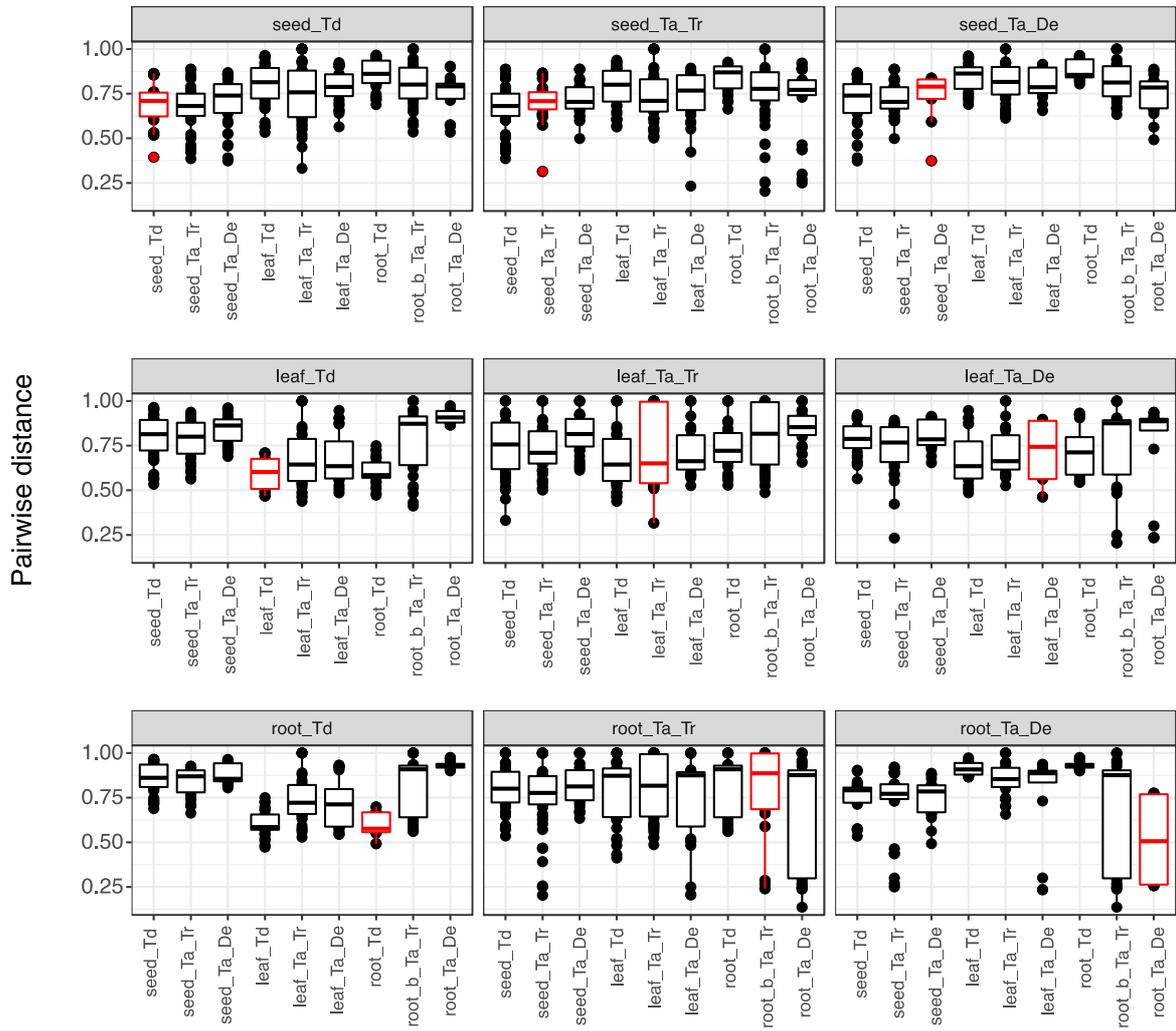
Supp. Fig6

Distance Metric = Bray-Curtis



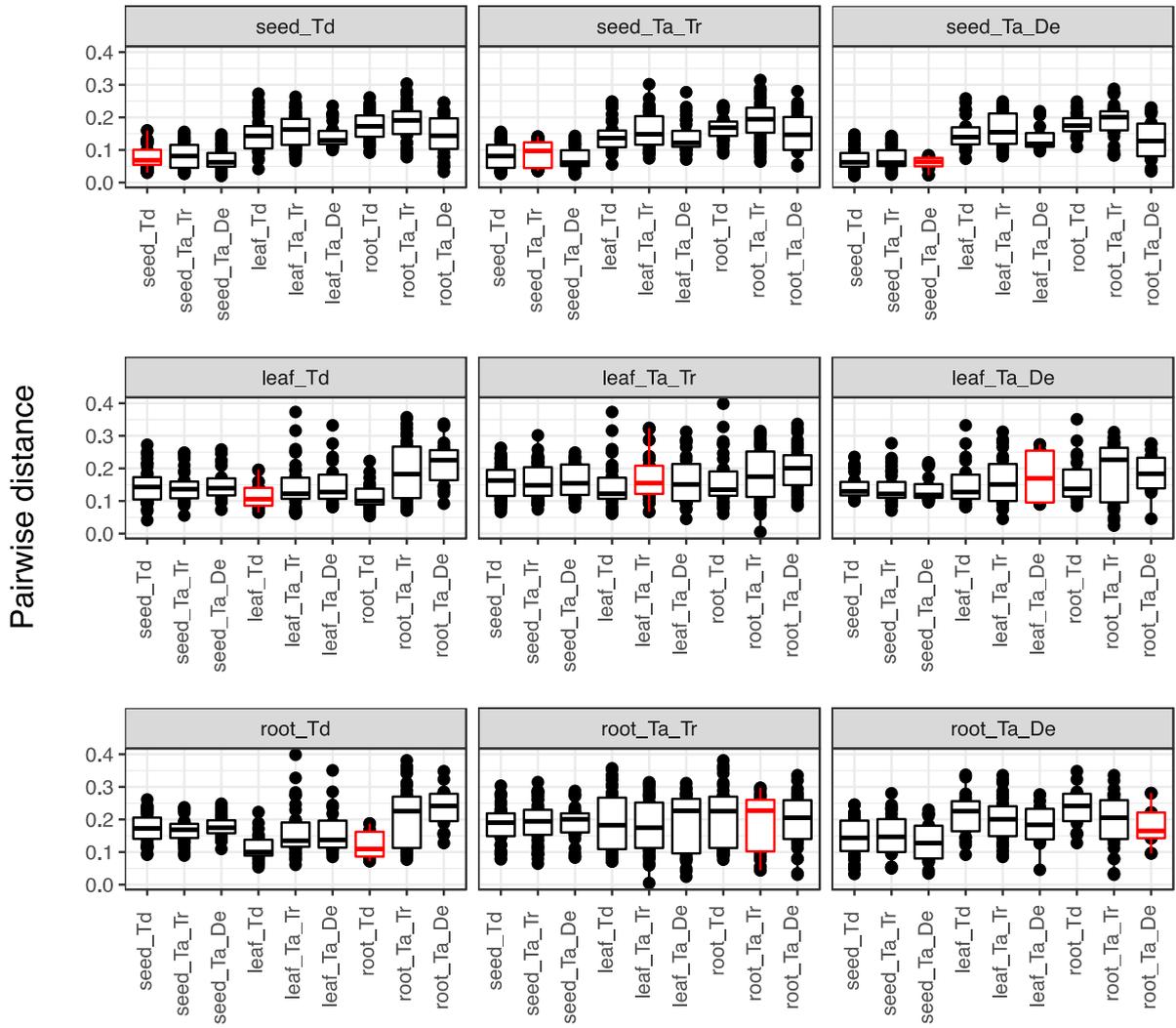
Supp. Fig7

Distance Metric = unweighted Unifrac



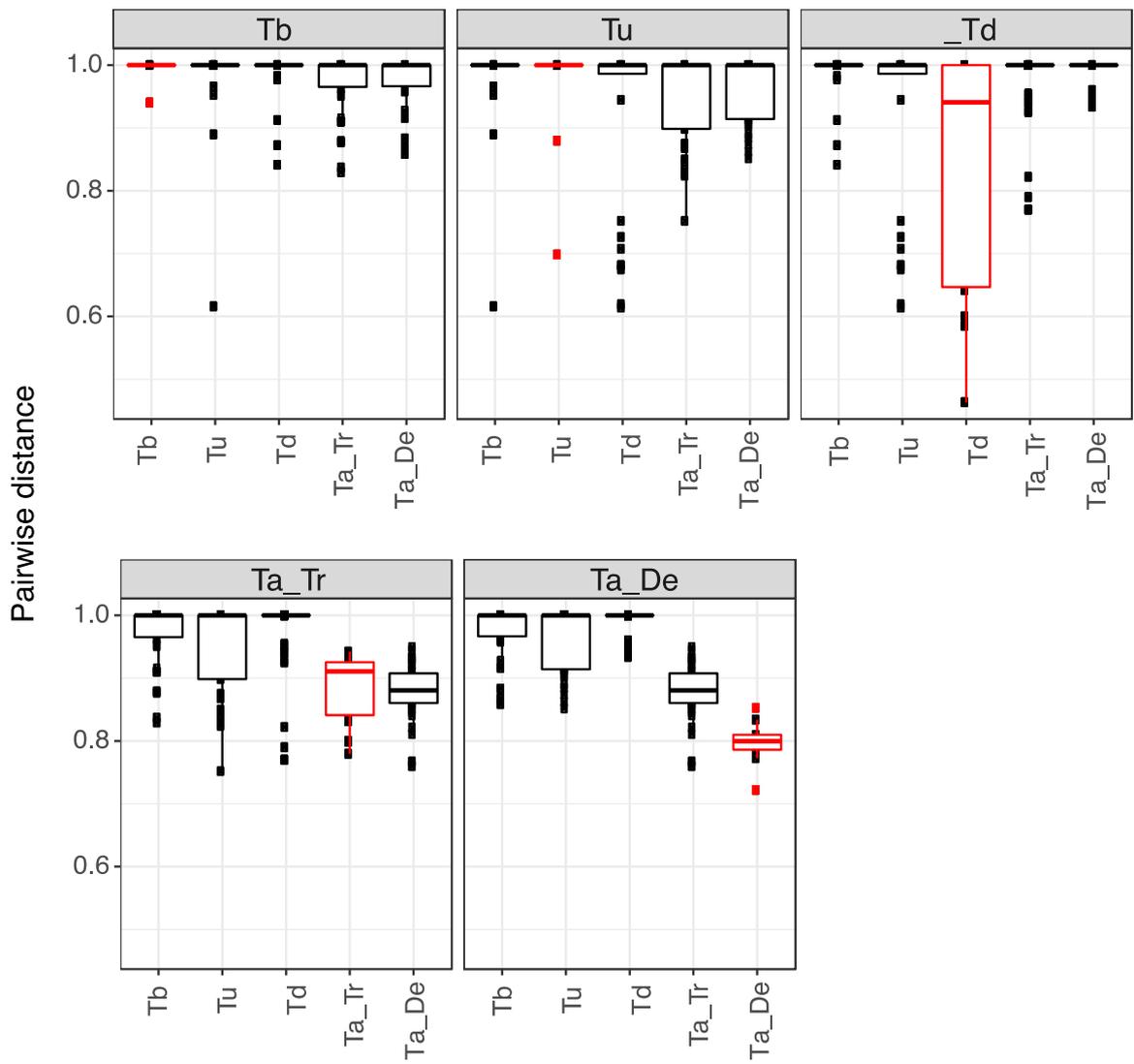
Supp. Fig8

Distance Metric = weighted Unifrac



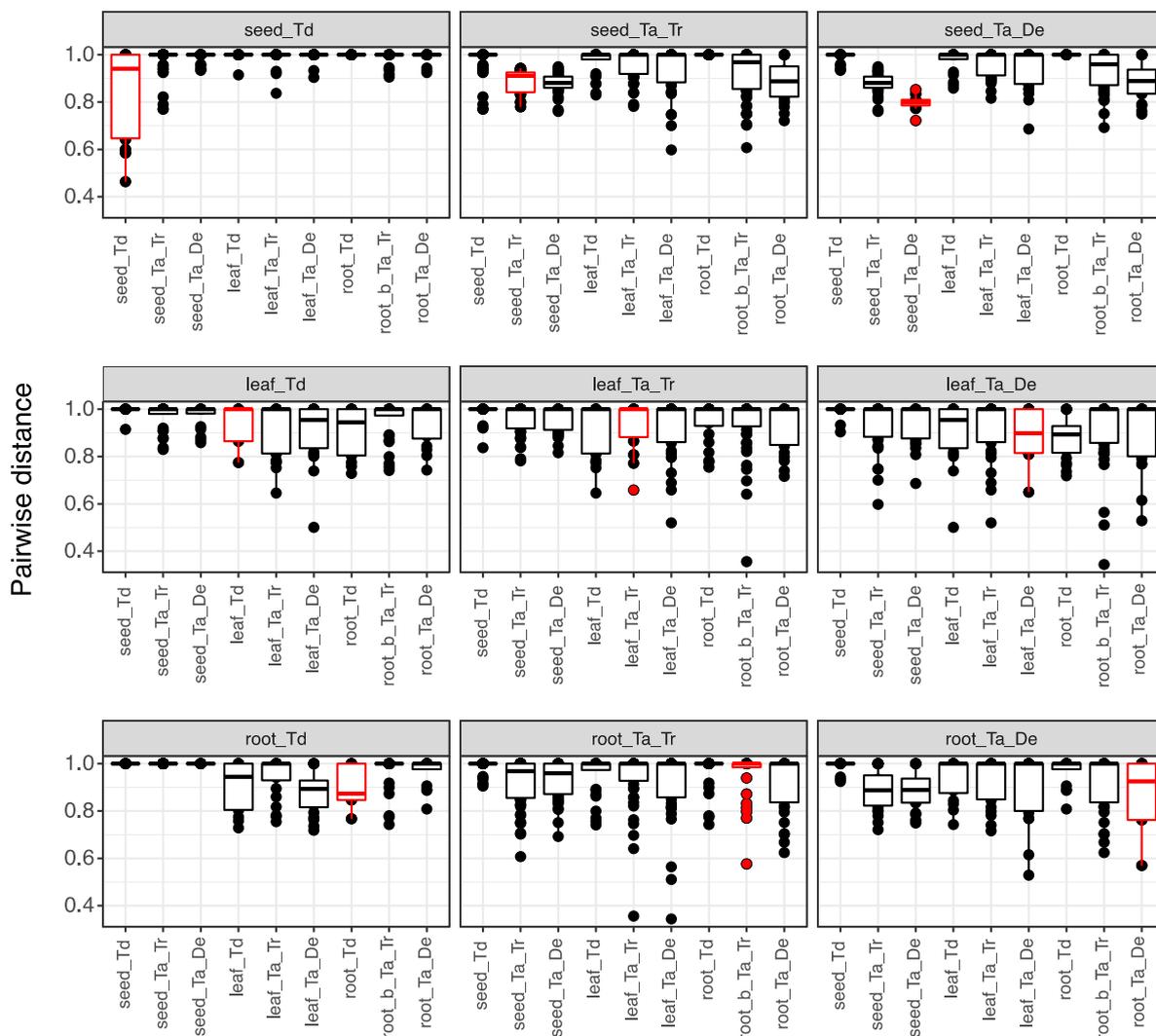
Supp. Fig9

Distance Metric = Bray-Curtis

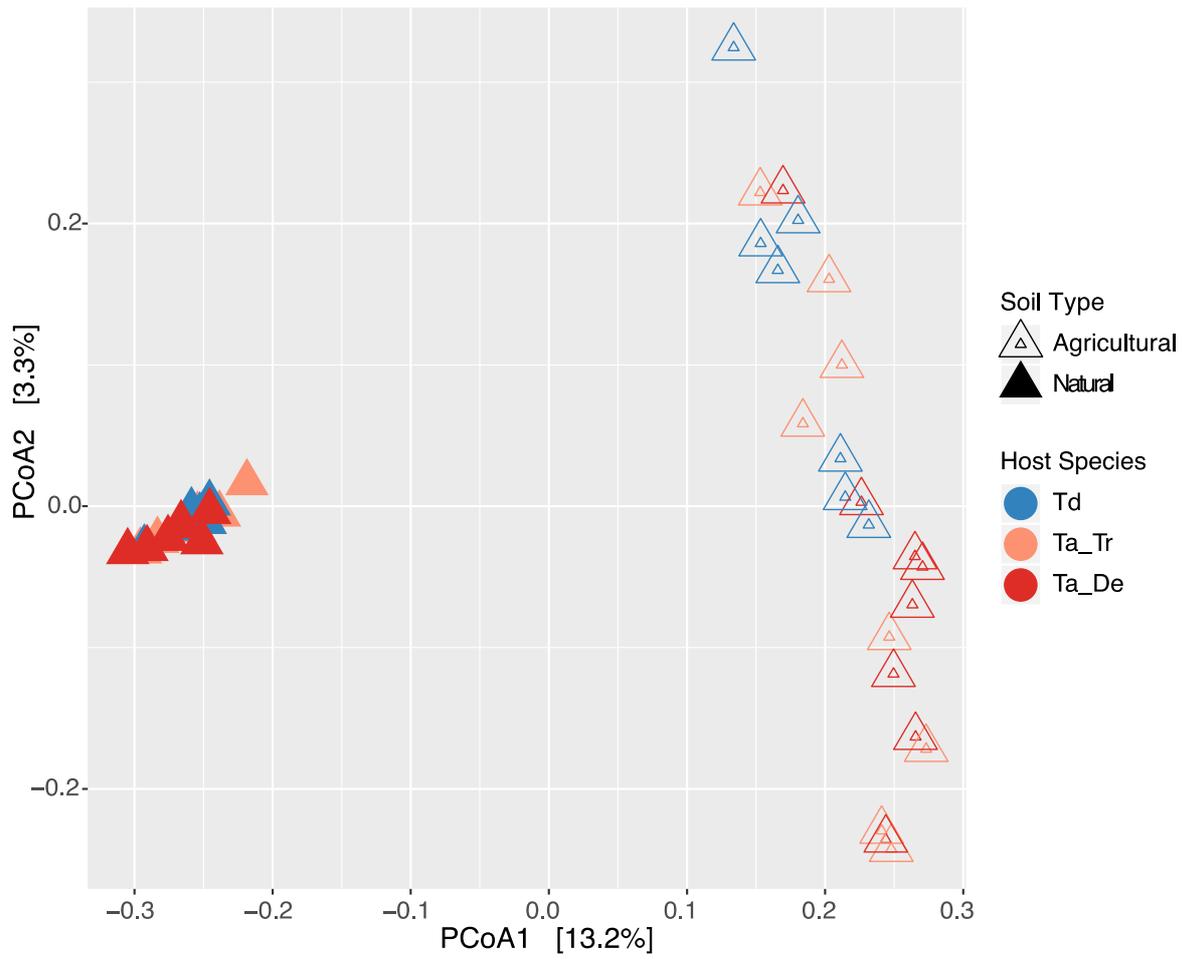


Supp. Fig10

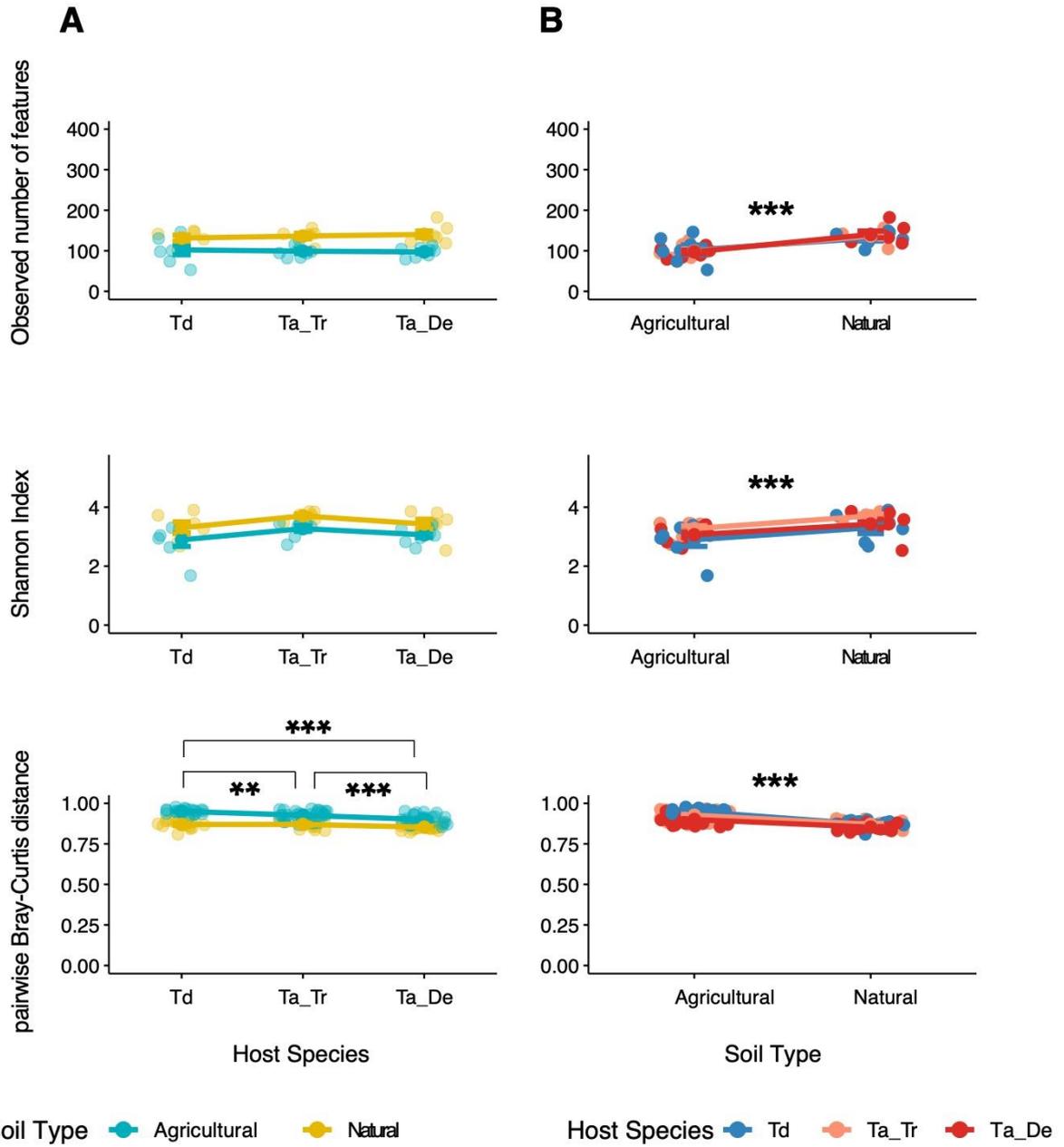
Distance Metric = Bray-Curtis



Supp. Fig11

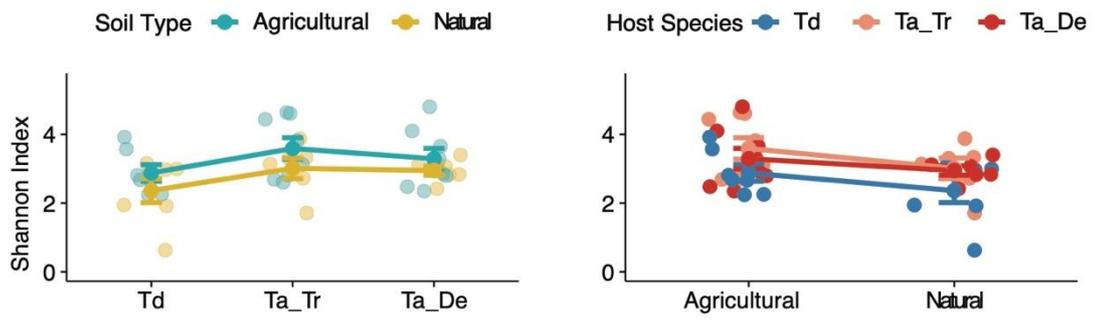


Supp. Fig12

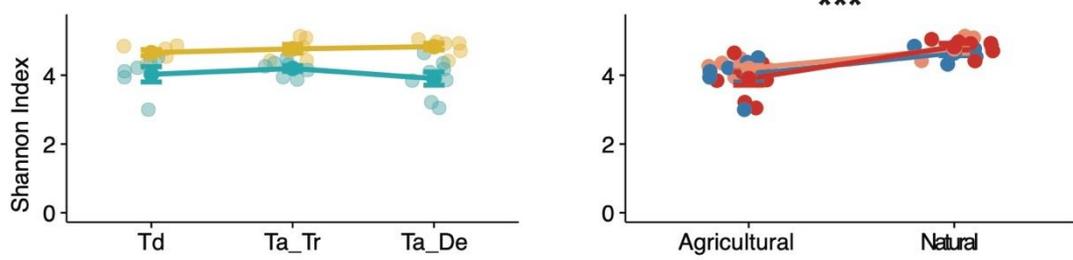


Supp. Fig13

A) LEAVES



B) ROOTS

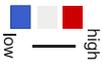


Supp. Fig14

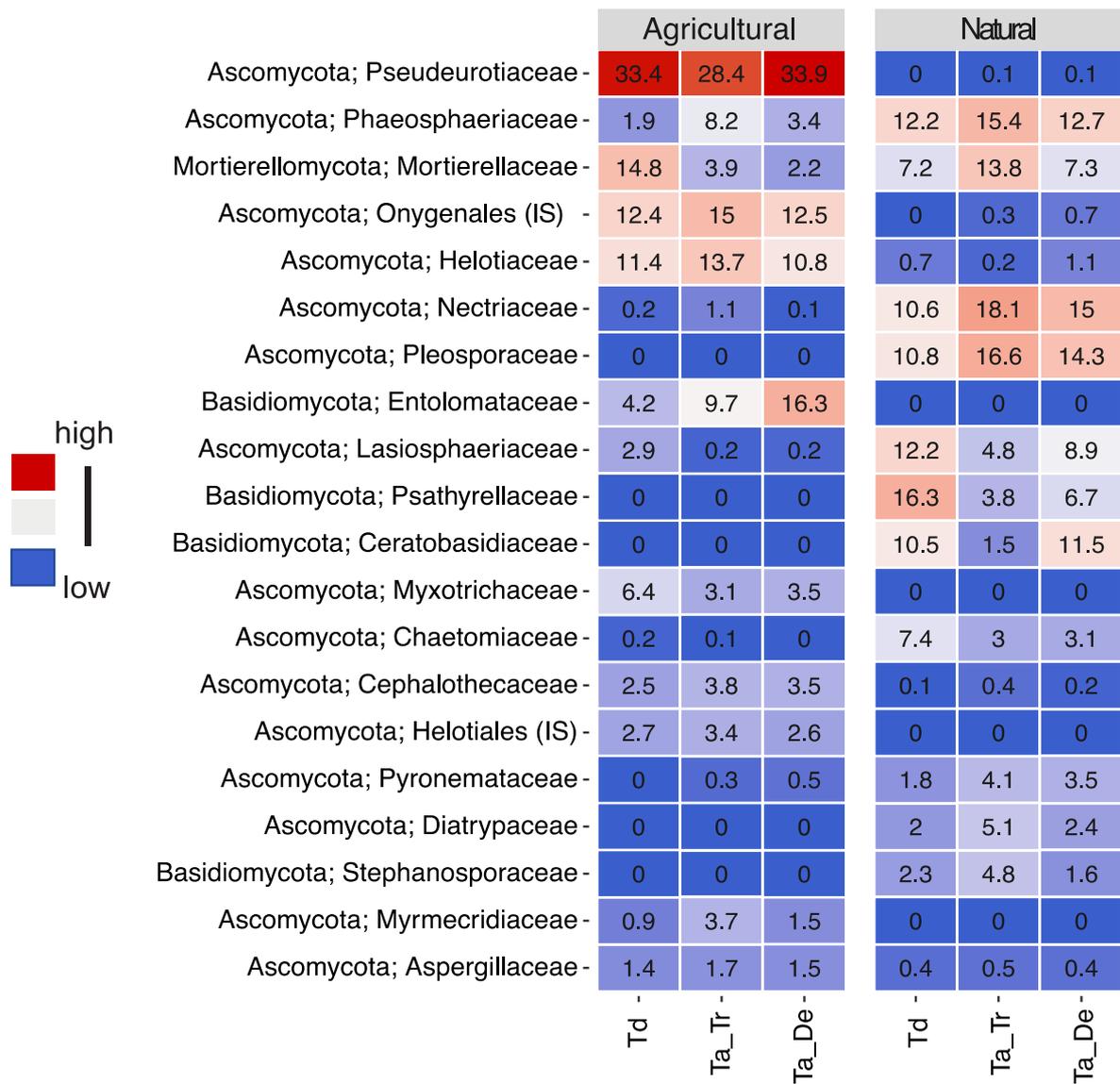
	Leaf			Root			Soil	
	Td	Ta_Tr	Ta_De	Td	Ta_Tr	Ta_De	natural soil	agr. soil
Proteobacteria: Oxalobacteraceae	222	12.2	21.1	18.6	26.5	24.4	1	0.9
Actinobacteria: Streptomycetaceae	0.3	2	3	39.3	30	29.4	0.7	10.3
Proteobacteria: Comamonadaceae	7.4	19.9	16.1	8.3	9.8	8.8	2	3.5
Proteobacteria: Rhizobiaceae	213.3	12.9	12.1	1	1.3	1.1	0.2	0
Proteobacteria: Halomonadaceae	13.6	15.9	15.7	0	0.1	0	0	0
Proteobacteria: Vibrionaceae	14.7	14	8.4	0	0.1	0	0	0
Actinobacteria: Micromonosporaceae	0.1	0.5	0.6	5.6	5.9	7.7	3.4	1.9
Actinobacteria: Nocardioidaceae	0.2	0.9	1.5	5.1	4.2	4	4	5.4
Bacteroidetes: Flavobacteriaceae	0.2	0.4	0.2	5.9	4.8	4.7	0.3	2.1
Proteobacteria: Xanthomonadaceae	1.3	1.6	1.3	3	2.7	2.7	1.5	5.9
Proteobacteria: Burkholderiaceae	3.5	2	3.2	0.4	0.5	0.3	0.1	2.1
Proteobacteria: Pseudomonadaceae	3.5	1.6	3.1	0.3	0.3	0.4	0.2	0.5
Proteobacteria: Neisseriaceae	2.7	1.7	1.6	0.6	0.6	0.5	0.2	0.9
Proteobacteria: Pseudonocardiaceae	0	0.2	0.2	0.5	0.5	0.4	0.2	0.2
Actinobacteria: Geodermatophilaceae	0.2	0.4	0.3	1.4	1.4	1.4	0.7	6.9
Proteobacteria: Hyphomicrobiaceae	0	0.1	0.1	2.5	1.8	1.9	1	0.4
Actinobacteria: Actinosynnemataceae	0	0.1	0.1	1.7	1.6	1.4	1	1.6
Proteobacteria: Caulobacteraceae	0.1	0.2	0.2	0.3	0.2	0.2	12.9	0.1
Actinobacteria: Rubrobacteraceae	0	0.2	0.1	1	0.8	0.9	1.4	1.5
Actinobacteria: Microbacteriaceae	0.1	0.2	0.3	0	0.1	0	0	0
Proteobacteria: Chromatiales	1.4	1.5	0.6	0	0.1	0	0	0

	Agricultural			Natural		
	Td	Ta_Tr	Ta_De	Td	Ta_Tr	Ta_De
Proteobacteria: Oxalobacteraceae	4.1	20.3	36.2	0.3	1.3	1
Proteobacteria: Rhizobiaceae	0.1	0.1	0	42.5	29.6	28.2
Proteobacteria: Halomonadaceae	11.2	10.5	8.1	16.1	23.2	25.8
Proteobacteria: Comamonadaceae	10.4	21.3	17.1	4.4	18.1	14.8
Proteobacteria: Vibrionaceae	8.1	16.3	4.5	21.2	11	13.6
Proteobacteria: Burkholderiaceae	7	2.9	5.6	0	0.7	0.1
Proteobacteria: Phyllobacteriaceae	0.1	0.1	0.2	7	3.7	6.9
Proteobacteria: Pseudomonadaceae	4.4	2.6	2.4	1.1	0.4	0.4
Actinobacteria: Streptomycetaceae	0.5	3.1	5.1	0	0.6	0.1
Proteobacteria: Xanthomonadaceae	1.5	2.2	1.9	1	0.8	0.6
Proteobacteria: (Chromatiales)	0.6	2.1	0.2	2.9	0.8	1.3
Proteobacteria: Neisseriaceae	1.5	1.7	1.8	0.7	0.5	0.7
Actinobacteria: Nocardioidaceae	0.5	1.2	2.4	0	0.6	0.3
Actinobacteria: Propionibacteriaceae	0.8	2.1	0.8	0.1	0.3	0.3
Firmicutes: Streptococcaceae	0.8	1.1	0.7	0.8	0.3	0.9
Proteobacteria: Ethnobaracteriaceae	0.5	1.1	0.5	0.7	0.4	0.5
Proteobacteria: Methylobacteriaceae	0.5	0.9	0.5	0.3	0.7	0.5
Actinobacteria: Micromonosporaceae	0.1	0.5	1	0.1	0.5	0.1
Firmicutes: Staphylococcaceae	0.3	0.7	0.7	0	0.2	0.2
Proteobacteria: Methylophilaceae	0.2	0.5	0.6	0.1	0.4	0.2

	Agricultural			Natural		
	Td	Ta_Tr	Ta_De	Td	Ta_Tr	Ta_De
Actinobacteria: Streptomycetaceae	4.7	40.2	40.2	15.5	11.5	8.5
Actinobacteria: Oxalobacteraceae	11.6	22	23.3	25.7	31.8	25.9
Proteobacteria: Comamonadaceae	2.7	5.2	3.3	14	15	16.1
Actinobacteria: Micromonosporaceae	3.6	3.5	3.3	7.6	8.8	13.6
Bacteroidetes: Flavobacteriaceae	0.5	0.5	0.3	11.2	9.4	10.5
Actinobacteria: Nocardioidaceae	8	6.2	5.4	2.2	1.8	2.1
Proteobacteria: Xanthomonadaceae	4.1	3.6	3.1	2	1.6	2.2
Actinobacteria: Actinosynnemataceae	1.4	0.9	0.5	3.7	2.9	3.9
Proteobacteria: Caulobacteraceae	1.7	1.5	1	1.7	1.7	1.8
Proteobacteria: Hyphomicrobiaceae	2	2	1.7	0.7	0.7	0.9
Proteobacteria: Rhizobiaceae	0.6	1.2	0.8	1.5	1.4	1.5
Actinobacteria: Thermomonosporaceae	1	1.2	1.1	1.1	1.3	0.8
Actinobacteria: Microbacteriaceae	1	0.8	0.7	0.9	0.8	1.2
Actinobacteria: Pemiconosporaceae	0.4	0.5	0.3	1.7	0.9	1.4
Chloroflexi: (Korlethrixaceae)	0.4	0.4	0.4	0.7	1.4	1.4
Actinobacteria: Pseudonocardiaceae	0.3	0.4	0.1	1.1	1.1	0.9
Proteobacteria: Pseudomonadaceae	0.5	0.4	0.2	0.6	0.6	0.8
Firmicutes: Paenibacillaceae	0.8	1.3	0.8	0	0	0
Proteobacteria: Bradyrhizobiaceae	0.8	0.7	0.5	0.3	0.4	0.4
Actinobacteria: Micrococcaceae	0.5	0.2	0.5	1	0.5	0.4



Supp. Fig15



Supp. Fig16

Chapter III- unpublished work

Diassembling the seed microbiota

Disassembling the seed microbiota

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Keywords

Microbial profiling, plant microbiomes, seed microbiomes, vertical transmission, *Triticum aestivum*

Abstract

Plant seed microbiome composition may be shaped by multiple factors but the relative contribution of host type and different environmental factors remains elusive. Here we examined seedborne bacterial communities of wheat samples collected from the same plants, different plants and different locations of the same field in Germany. In addition, we compared these to the bacterial communities of wild wheat seeds from the Fertile Crescent region over time. For this, we germinated seeds under axenic conditions and detected bacterial communities colonizing leaves over different ages of the leaf. We showed that host type and developmental time are the main driver of the bacterial community composition in the seedborne bacterial communities of wheat leaves. We further revealed that microbial diversity is decreasing over time in the domesticated wheat but not in the wild wheat. Also, microbial composition was stable over time in the domesticated wheat, but not in the wild wheat. We suggest that this decrease in diversity may be because of higher competition among seedborne microbes of domesticated wheat compared to the wild wheat. Overall, multilevel comparisons of seedborne bacterial communities allowed us to disentangle factors shaping the microbial variation.

Introduction

Plants live together with a large diversity of microorganisms and engage in various interactions with them. Although some of these interactions are pathogenic (Mansfield et al. 2012) or neutral to the plant (Vorholt 2012), others are beneficial (Vorholt 2012) and increase the ability of the plant to cope with biotic and abiotic stress, promote growth and protect against pathogens. The ability to associate with beneficial microorganisms may thereby be an adaptive trait to the plant. For example, *Colletotrichum tofieldiae*, a fungal root endophyte of *Arabidopsis thaliana*, cooperates with the plant in low phosphorous conditions by supplying the roots with phosphorous (Hiruma et al. 2016). In this way, the fungi boosts the growth of the *A. thaliana* under stress conditions. There is plethora of such examples (e.g. (Ritpitakphong et al. 2016); (Vurukonda et al. 2016); (Berg and Koskella 2018)). These beneficial plant-microbe associations may be exploited in an agricultural and commercial context to increase the resilience of crop plants allowing them to adapt into new environments and to prevent yield losses caused by pathogen infection or abiotic stress (Busby et al. 2017). However, efforts to explore the beneficial effects of plant-microbe interactions must acknowledge the complexity of plant microbiome assembly and rely on a detailed understanding of environmental and genetic factors shaping microbial communities and plant-microbe and microbe-microbe interactions.

Plant seeds are not standalone entities, but harbour a diversity of endophytes (Özkurt et al., 2019). Since seedborne microbes are the first colonizers of the plant seedling, occupying plant tissues before other microbes are taken up from the environment, they may have fundamental roles in the early development of the plant. Indeed, seedborne

endophytes aid in rock weathering and plant growth in cacti, thereby support establishment of the early plant in barren deserts (Puente et al. 2009). Controlling and manipulating seedborne microbiome could allow to exploit the beneficial functions of these microbiota. For this, first we need to understand the determinants of seedborne microbial assembly as well as the long-term and short-term dynamics in microbial community of seeds.

Seed microbial assembly may be altered by environmental and genetic differences in the host population. Research so far show that plant genotype, environment and stochastic effects might have a role in recruitment of microbes in seeds (Klaedtke et al. 2016); (Rybakova et al. 2017); (Rezki et al. 2018). Previously, the environmental factor “terroir” was proposed to be a key driver of the assembly in seed-associated microbes of common bean (Klaedtke et al. 2016). Here, terroir was defined as the combination of human practices and environmental conditions. However, different bean cultivars did not explain variation in seed-associated communities. On the other hand, seedborne bacterial communities of different barley (Yang et al. 2017) and oilseed rape cultivars (Rybakova et al. 2017) showed strong host-genotype dependency. However, this distinction among cultivars may be explained by the different soil types where cultivars had grown. In another study, microbial communities of radish was profiled across three successive plant generations (Rezki et al. 2018). High variability in seed community composition was detected from plants having the same genotype and coming from the same field. This suggest that neutral processes such as ecological drift or dispersion rather than selection may be an important factor in explaining seed-associated microbiota. Although we have now some knowledge on the determinants of seedborne microbial communities, we still

do not have an overview of the temporal, spatial and individual variation and impact of agricultural treatments on the seedborne microbial communities of plants.

In this study, we addressed the impact of the host and environment on the seedborne microbiome in wheat. Notably we focused on variability among individual seeds of the same plant and of different plants growing in the same environment. To this end, we studied microbes inside seeds as well as seedborne bacterial communities colonizing leaves. Firstly, to investigate stochasticity of the seedborne microbiome in a field, we detected individual variation among seedborne microbes originating from the same plant and different plants of the domesticated wheat *Triticum aestivum*. Finally, we detected spatial variation in seed bacterial community by comparing bacterial assembly in seeds collected from different locations within a field. Secondly, we examined temporal variation in the seedborne microbial colonization among different developmental stages of the leaves in domesticated wheat seedlings. In addition, we compared microbial diversity in the domesticated wheat seedlings to microbial diversity in seedlings of the wild wheat relative *T. dicoccoides*. Here, based on previous observations (Özkurt et al., 2019), we hypothesized that competitive microbial dynamics govern the domesticated wheat but not the wild wheat relative. In this case, we expected to observe decreasing microbial diversity in the domesticated wheat cultivar, but not in the wild wheat species. Multilevel comparisons of seedborne bacterial communities permitted us to disassemble microbial variation at different scales.

Material and Methods

Seed Collections

Our study built on a collection of the *T. aestivum* Quintus spring cultivar seeds (W.von Borries Eckendorf, Leopoldshöhe, Germany) collected from an experimental farm of the Kiel University in Kiel, North Germany (N 54 18 36, E 9 58 48) in August 2018. More precisely, we sampled plant seeds from three different plots from the same and different plants (**Figure 1**). Therefore, in our collections we included 1) seeds originating from the same plant, 2) seeds originating from different plants but the same plot and finally 3) seeds from different plants collected at different plots (**Figure 1**). Plants growing in the field were treated with different fertilizer and pesticides (e.g. Magnesium oxide, N-Gabe, Agent, Biscaya etc.). Seeds collected from plot1, are located near the outside border of the field (**Figure 1A**), which is nearby a sugar beet field. These two fields were separated by a dust road. Therefore, seeds collected from plot1 may have been more impacted by outside interactions than seeds collected from plot2 and plot3.

In addition to domesticated wheat, we included the wild wheat *T. dicoccoides* in our collections as an outgroup having a different genotype and originating from a different environment than the domesticated wheat. Here, we refer to *T. aestivum* and *T. dicoccoides* as being a different “host type”. The seed material for these samples were collected in 2006 from South East Turkey (N37 15 33, E37 29 03), which is in the Fertile Crescent region, where is proposed to be the origin of domesticated bread wheat (Salamini et al. 2002)

Experimental Design

In this study, we aimed to gain insight into the ecological processes that shape the microbiome in a field site. For this, we investigated the relative influences of different factors in community assembly processes. We basically investigated the environmental, host and age component of the variation in the seedborne bacterial assembly of the wheat. By growing seeds under axenic conditions at different growth times, we examined the variation in seedborne leaf-microbial assembly at different ages of the wheat seedlings. Our aim was to address potential competition among microbes by describing changes in microbiota development.

For the spatial component, we compared seedborne microbial community in different plants of domesticated wheat. More precisely, we compared seedborne microbial assembly originating from the seeds of the same plant and among plants from the same plot and among plants from different plots. For the host component, we compared microbial communities between seeds of the wild and domesticated wheat.

To characterize the composition and dynamics of seedborne microbial communities, we first surface-sterilized seeds and propagated them under axenic conditions in sterile jars (**Figure 1B**). To ensure only elimination of the contaminants but keeping microbes from the interior of seeds and tightly attached to the surface, seeds were mildly surface-sterilized. Some of the individual seeds of domesticated wheat were processed for DNA extraction and 16S rRNA sequencing whereas others were propagated in closed and sterile jars. Finally, we merged the sequencing data of the wild seeds from a previous study to our dataset and analyzed it along our samples (**Table 1**) (Özkurt et al., 2019). We propagated some of the surface-sterilized seeds of domesticated and wild wheat in sterile

PNM medium with 16h light/8h dark cycles at 15°C in a climate chamber (Percival plant growth chambers, CLF PlantClimatics GmbH, Wertingen, Germany). These axenically-grown seedlings were harvested at different ages (T1: three days (Domesticated: n=18 from three different plants; Wild: n=5), T2: 14 days (Domesticated: n=11 from two different plants), T3: 28 days (Domesticated: n=15 from three different plants; Wild: n=6)). It must be noted that we have not included the time point T2 of the wild wheat in this work because of limited number of seed material we obtained from the Fertile Crescent region. Also, seed collections of the wild wheat are originating from the same field, but we do not have the track of the plant origin (i.e. whether seeds are originating from the same plant or different plants).

Three days of growth corresponds to 3-4 cm growth of leaf, two weeks corresponds to the emergence of the 2nd leaf and four weeks to the emergence of four leaves (**Figure 1B**). Abnormally grown plants were excluded from the rest of the experiment. We further extracted DNA from harvested leaves. Three randomly selected negative controls of sterilization and DNA extraction were combined and sequenced alongside samples as a control. Genomic DNA isolated from the seed and leaf samples and the negative control were used to amplify the V5-V7 variable region of the 16S rRNA gene with PCR primers targeting the 799F/1192R region (Özkurt et al. 2019). We followed the QIIME2 version 2019.1 pipeline to preprocess and filter the amplicon data (Bolyen et al. 2019). In brief, libraries were paired-end sequenced for 2x300 cycles on an Illumina MiSeq machine at the sequencing facility of Max Planck Institute for Evolutionary Biology, Plön, Germany (Illumina, San Diego, CA).

All the samples were randomized during growth of the plants, DNA extraction and library

preparations. Further information about growth medium, DNA extraction and sequencing was done as in Özkurt et al. (Özkurt et al., 2019).

Data Analysis

DADA2 package integrated into the QIIME2 was used to correct and filter chimeric reads (Callahan et al. 2016) (Bolyen et al. 2019). The filtered reads were processed for the taxonomic classification based on the 16S sequence database Greengenes13.8 (DeSantis et al. 2006). Downstream analyses were conducted with the R environment (version 3.5.2) (R Core Team 2018) with these packages and custom scripts: “phyloseq”, “vegan”, “ampvis2” and “ggplot2” (McMurdie and Holmes 2013); (Oksanen et al., 2019); (Andersen et al., 2018); (Wilkinson 2011). Further information on the amplicon data analysis can be found in (Özkurt et al., 2019).

We rarefied the OTU table to its even depth whereby all the samples had the same library size (“rarefy_even_depth” function in the “phyloseq” package in R) (McMurdie and Holmes 2013). Based on the rarefied table, we computed the observed number of features, Shannon index and inverse Simpson index to estimate within sample diversity. The significance of differences in diversity among samples were tested using a Kruskal-Wallis test (krus.test in R). We normalized the OTU counts with the cumulative sum scaling normalization method (cumNorm function in the “metagenomeSeq” package in R) (Paulson et al. 2013). We measured Bray-Curtis and weighted/unweighted UniFrac distances based on the normalized counts to estimate between-sample variation among microbial communities. The estimated values were used for principal component analysis (PCoA). The determinants of the between-sample variation were estimated using

“Permutational multivariate analysis of variance analysis” (PERMANOVA) (“adonis” function in the “vegan” package in R) (Oksanen et al., 2019).

Results

Sequencing Summary

To describe the diversity of bacteria associated with seeds of wild and cultivated wheat, we sequenced 16S rRNA region of the domesticated wheat seeds and axenic leaves of domesticated and wild wheat from different ages. In total, our analyses of the seedborne microbiota included 82 samples, 28 seed samples and 54 leaf samples (*T. aestivum*: T1: 18, T2: 11, T3: 15 replicates; *T. dicoccoides*: T1: 5, T3: 5 replicates) From these samples, we obtained 15,479,202 reads in total. After removing low quality and chimeric reads, as well as non-bacterial sequences, 4,016,538 and 4,479,110 reads remained from the seed and leaf sequencing, respectively (**Table 1**). This corresponded to a total of 6649 OTUs in 82 samples. After rarefaction of the OTU table to its even depth, 4964 OTUs remained. No reads remained in the negative controls after the quality control and filtering and where thus excluded from the further analyses. Alpha diversity rarefaction plots for each sample confirmed that a sufficient depth of sequencing was achieved for all the samples (**Suppl Fig. 1**).

Seedborne microbial community gradually decrease in diversity over time in the domesticated wheat but not in the wild wheat

To temporarily monitor microbial diversity in seedborne bacterial community after plant colonization, we compared alpha diversity of seedborne microbiota in seeds and leaves of wild and domesticated wheat based on the filtered and rarefied OTU table. In the case of niche overlap among microbes (i.e. overlap in resource use), thus high competition, we expected a decrease in microbial community diversity over time. Leaves of both wheat species were categorized according to the time of sampling and developmental stage that we defined as T1 (3-4 days), T2 (14 days) and T3 (28 days) for the domesticated, T1 (3-4 days) and T3 (28 days) for the wild wheat. We used “Observed OTUs” (number of unique OTUs), the Shannon index (considers both diversity and evenness) and the inverse Simpson index (considering more weight to more abundant species) to estimate microbial diversity in each sample.

Notably, we observed a significant gradual decrease in bacterial diversity over time in the leaves of domesticated but not in wild wheat (Kruskal Wallis Test: Observed $p=0.0007008$; inverse Simpson $p=0.000007617$) (**Figure 2**). In fact, we observed a trend of increasing microbial diversity by time in the wild wheat. This may reflect higher competition among microbes in the domesticated wheat compared to the wild wheat; therefore leading to a decreased diversity by time.

In brief, bacterial diversity was gradually decreasing over time in the leaves of the cultivar whereas it was showing increasing trends in the wild wheat.

Wild wheat but not domesticated wheat harbors temporally dynamic microbial composition

To further define the bacterial community associated with wheat, we investigated the taxonomic composition of samples in detail. First, we assessed major bacterial groups and their prevalence associated with the seeds and leaves of domesticated and wild wheat. To this end, we aggregated the assigned taxonomy of each bacterial OTU to the family level. In accordance with the previous studies on seedborne microbiota of different plants (Yang et al. 2017); (Adam et al. 2018); (Barret et al. 2015); (Özkurt et al., 2019), Proteobacteria was the major phylum in the seeds and leaves of wheat. At the finer taxonomic level, the microbial community was primarily dominated by the bacterial families Pseudomonadaceae (33.1%) and Comamonadaceae (29.7%) in the wild seeds (i.e. T₀). However, at the first time point after the leaves emerged (i.e. T₁); the community composition shifted and was dominated by Oxalobacteraceae (28.6%) (**Figure 3A**). In contrast to the wild wheat, community composition did not change significantly in domesticated wheat from T₀ to T₁; where Halomonadaceae (29.6% & 22.9, respectively) and Vibrionaceae (38% & 17.4%, respectively) were the most prevalent bacterial families. While both bacterial families remained as predominant members throughout the wheat development in domesticated wheat; predominant taxa changed over developmental times in wild wheat. Notably, at four weeks (i.e. T₃), bacterial composition of wheat leaves converged to highly similar compositions in wild and domesticated host types. Halomonadaceae (30.8% & 30.9%), Vibrionaceae (23.1% & 24.4%) and Enterobacteriaceae (13.3% & 12.5%) were the most dominant bacterial families at this time point in both hosts.

Taken together, during seedling emergence, bacterial communities were dominated by different bacterial families in wild and domesticated wheat. However, while bacterial composition was relatively stable over time in seedlings of the domesticated wheat, seedlings of wild wheat were colonized by more dynamic microbiota, comprising different families at different time points. Interestingly, at the last time point, T3, community composition however converged more similar communities in the two host types.

Core bacterial OTUs are rare even among samples of the same plant

Our dataset of seedborne bacteria in wheat seeds and leaves allowed us to identify the occurrence of eventual core microbial species. To this end, we determined the sets of OTUs that are common or distinct among different sample categories: i) between wild and domesticated wheat over time (i.e. variation over time among host types) (**Figure 3B**) ii) over time during three time points in the leaves of domesticated wheat (i.e. temporal variation) (**Figure 3C**) iii) among seeds collected at different locations of the field (i.e. spatial variation) (**Figure 3D**) iv) leaf samples originating from the same or different plants (i.e. variation within and among heads of individual plants) (**Figure 3E**). We created Venn diagrams comparing each category, where we denote shared and exclusive bacterial taxa (**Figure 3B-D**). In brief, bacterial OTUs shared among the groups of each category are denoted as “core” (e.g. **Figure 3C**, bacteria that shared in T1, T2, T3); whereas OTUs specific to some categories as “exclusive” (e.g. **Figure 3C**, OTUs appeared only in T1 or bacteria shared between T1 and T2 but not T3). Otherwise, OTUs detected only in some individuals of the categories were denoted as a part of the “non-core” OTUs (i.e. basically all OTUs that are not part of “core” and “exclusive” bacteria).

Overall, substantially small part of the bacterial OTUs could be categorized as core microbiota members, while the majority of the OTUs appeared in only some individuals, i.e. as non-core OTUs (**Figure 3B-D**). Still, a small number of shared bacteria between wild and domesticated was colonizing leaves consistently at different developmental time (T1: 8 OTUs; T3: 14 OTUs) (**Figure 3B**). Together, these OTUs comprised 4.43% and 7.84% of all the reads in T1 and T3, respectively. Six of the OTUs were consistently detected in all developmental times in both wheat. These OTUs were classified to the Neisseriaceae, Pseudomonadaceae families and *Stenotrophomonas*, *Vibrio*, and *Halomonas* (2 OTUs) genus.

Leaves of the domesticated wheat collected at different time points had only few but its exclusive bacterial taxa (**Figure 3C**). For example, in T1 some OTUs with unknown bacterial taxa were comprised only at that time point while disappeared in later growth stages. Also, T2 was exclusively including OTUs from different bacterial taxa (e.g. *Ralstonia*, *Propionibacterium* and unknown taxa) that did not appear at T1 or T3. Finally, at T3, two OTUs belonging to the Vibrionaceae family appeared in T3, although they were not detected in earlier stages. In total 24 OTUs formed the core OTUs in the leaves of *T. aestivum*. Although these OTUs constitute only 0.05% of all OTUs in the leaves of *T. aestivum*, they correspond to 12.32% of all reads in the domesticated leaves.

Overall, leaves of the wild and domesticated wheat shared a small (4.43% of the community) core bacterial community during seedling development. Also, seeds of *T. aestivum* shared small number of distinct OTUs (24 OTUs out of 4648 OTUs) during seedling development, but they constituted an important amount of the bacterial reads (12.32%).

Seed-associated bacterial communities vary with the location of the plant in a field

We compared bacterial communities of seeds of the *T. aestivum* cultivar Quintus collected from different areas of the field (i.e. plot1, plot2, plot3) (**Figure 1A**). Seeds of the plant collected from plot1 was depleted of several bacterial taxa found with plants of the two other plots (i.e. exclusive OTUs) (**Figure 3D**). Some of the depleted OTUs included the bacterial families (e.g. Enterobacteriaceae, Pseudomonadaceae, Neisseriaceae) appeared as part of core microbial community between wild and domesticated wheat and among developmental times. Thus, we concluded that location of the plant in the field might impact diversity of bacterial communities in seeds, leading to the depletion of non-core bacterial taxa.

Samples of the same plants are not more similar to each other in bacterial community composition compared to the samples of different plants

To reveal whether samples originating from the same host plant share more similarity in microbial composition in comparison to samples collected from different plants, we analyzed microbial data of the axenic leaves of the same developmental time from the same and different plants. Only a few OTUs were shared among samples originating from the same plant host; thus constituting the core microbiome of the individual plant (**Supp. Figure 2**). On the other hand, among leaves originating from two different plants of the same plot, we found a similar proportion of shared OTUs. When the same comparison was done among three different plants of the same plot, no OTUs fulfilled the criteria of being present in all samples at relative abundances higher than 0.1%. In conclusion,

generally, the variation within the same plant was not less than among different plants of *T. aestivum* (**Supp. Figure 2**).

Taking together our results show that wild wheat but not domesticated wheat harbours temporally dynamic bacterial communities. To summarize: i) Wild and domesticated wheat were colonized by a small shared bacterial community. In general, we observed that small number of core OTUs shared among different comparisons. Instead, the majority of the taxa were observed only in some individuals. ii) Albeit being small, a core community of bacteria comprising %12.32 of all reads were persistent over three developmental times in the domesticated wheat (temporal variation). iii) We observed depletion of exclusive bacterial taxa in the seeds collected from plot1 compared to the seeds collected from other locations in the field (spatial variation). iv) Finally, variation in bacterial community among seeds collected from the same head was not lower than inter-plant variation (individual variation).

Host type and seedling age are the main determinants of the seedborne bacterial community composition in leaves

Our analyses of alpha-diversity revealed a clear difference between wild and domesticated wheat in diversity of seedborne microbial communities of leaves from different time points. We further examined between-sample variation in composition of microbial communities. To do this, we calculated three measures of beta diversity: Bray-Curtis (a measure that considers presence/absence of the taxa and relative abundances), weighted UniFrac distances (a measure that considers relative abundances and phylogenetic

relatedness) and unweighted UniFrac distances (a measure that considers presence/absence of the taxa and phylogenetic relatedness) (**Figure 4**).

Ordination analysis based on distance matrices revealed distinct clustering of wild and domesticated seedborne microbial communities. Host type (13.6%) was the main driver of the variation in community composition in leaves (PERMANOVA: $p= 0.001$). Here, host type refers to the two wheat species, one being domesticated the other wild. Host type was followed by seedling age (9.06%) as the second determinant of the variation in the leaves of the domesticated wheat (PERMANOVA: $p= 0.046$). Finally, the interaction of the host type and seedling age explained %3.59 of the variation (PERMANOVA: $p= 0.020$).

As for the leaves, we find that for seeds host type (29.11%) is the main driver of the community composition (PERMANOVA: $p= 0.001$). In addition, spatial factors (i.e. the plot in the field where the plants were collected) of the seeds (15.59%) was marginally explaining the variation (PERMANOVA: $p= 0.078$). Notably, for both leaves and seeds the plant origin (i.e. being originated from the same plant or different plants) did not explain the variation in beta diversity. In other words, for *T. aestivum* variation among samples of the same plants and of different plants were not differing from each other.

Taken together, host type and developmental time were the determinants of the variation in bacterial community composition in the leaves. In the seeds, both host type and spatial origin of the plant were explaining the variation. However, plant origin of the seeds and leaves did not explain between-sample variation.

Discussion

In this study, we experimentally monitored temporal changes in the composition and diversity of the seedborne microbiome in seedlings of domesticated and wild wheat, starting from dormant microbial communities associated with seeds to the 28 days old leaf. Hereby, we created a temporal map of microbial composition and diversity during early seedling development of domesticated and wild wheat. To our knowledge, this is the first study comparing seedborne microbial communities over time and among seeds originating from the head of the same plant and from different plants.

Temporal dynamics of seedborne bacterial communities has been investigated previously by Liu and colleagues in maize grains (Liu et al. 2013). However, the study addressed bacteria associated with different developmental stages of grains (i.e. milky and dough stages), but not the microbes colonizing the plant as here. Also, the study used clonal libraries technique. In our study we addressed the seedborne microbes colonizing the plant over time in wheat, using amplicon-based sequencing.

One of the most important findings in this study was that diversity of the microbial community decreases by time in *T. aestivum* but not in *T. dicoccoides* (**Figure 2**). In contrast, microbial diversity slightly increased over time in *T. dicoccoides*. We speculate that competition (e.g. the impact of numerical priority effects) might be higher in the seedborne microbes of *T. aestivum* compared to *T. dicoccoides*; thereby conferring decreased diversity over time. The observed pattern in *T. dicoccoides* may suggest the occurrence of less antagonistic effects among the colonizing microorganisms. We speculate that this pattern may reflect more cooperative seedborne microbial interactions in *T. dicoccoides* compared to *T. aestivum*.

Moreover, we observed shifts in the community composition in the wild wheat at T1; which represents the earliest time point in our dataset after the emergence of the first leaf. Basically, bacterial communities were initially dominated by Pseudomonadaceae and Comamonadaceae inside seeds of *T. dicoccoides*. However, during ageing of the seedling there was a change in microbial abundance, first an increased abundance of Oxalobacteraceae and finally of Halomonadaceae and Vibrionaceae at the later stages. However, microbial composition of domesticated wheat, being dominated by the same bacterial families, was stable during seedling development. This suggests that microbial communities in the wild wheat species may be undergoing dynamic changes of the vertically acquired microbiota, which do not occur in the domesticated wheat species.

Microbes being part of core microbiota imply that they are well-adapted to the host; therefore they potentially have major roles in plant health. Seeds collected from one location of the field, plot1, was lacking some OTUs compared to the seeds originating from other locations in the field, which are plot2 and plot3 (**Figure 1A, Supp. Figure 2**). These OTUs were not part of the core OTUs, but rather exclusive OTUs which are shared between other plots or exclusive to the other plots. This area in the field is next to a sugar beet field and tractor way spreading pesticides and fungicides. Here, we speculate that the area may have been exposed to other treatments, including different pesticides and fungicides that have indirectly impacted the microbial communities of wheat. Previously, Perazzolli and colleagues suggested that fungicide treatment may affect richness and diversity of bacterial communities in grapevine phyllosphere (Perazzolli et al. 2014). Moreover, some of the depleted OTUs belonging to the bacterial families (e.g. Enterobacteriaceae, Pseudomonadaceae, Neisseriaceae) appeared as part of core

microbial community in the leaves of wild and domesticated wheat in different developmental times. The detection of bacterial groups in this study cannot be functionally informative, however, the consistent presence of certain groups over the developmental times in the leaf samples may indicate that they are an important part of the wheat development.

Lastly, our analysis of between-sample variation reveals that host type and developmental time are the main determinants of seedborne bacterial composition in leaves. We also showed that the location of the plant in a field is a factor explaining variation in bacterial community inside seeds. On the other hand, seedborne microbial communities in leaves originating from the same or different plants grouped together. Overall, these results suggest that seedborne bacterial communities associated with plants in an agricultural field are heterogenous; however homogenous in nearby plants. However, host type, defined as different genotypes and different field sites affect community composition. Also, bacterial community at different time points during early seedling development shift by time, especially in the wild wheat species.

We conclude that temporal dynamics of microbial community is different in domesticated wheat compared to the wild wheat. The differences noted in the seedborne microbiota of wild and domesticated wheat could potentially influence the plant in different ways: For example, they could impact the efficiency of vertical transmission or cooperation of vertically-transmitting microbes with the host plants; which may ultimately impact the fitness of the host. Acknowledgement of the community dynamics (e.g. extent of competition among microbial members) could provide opportunities to modulate the seed microbiota with precision and thereby improve the agricultural practices. Further

research will be required to experimentally validate the difference in the extent of competition among microbes between wild and domesticated wheat.

Acknowledgements:

The authors would like to thank Amine Hassani for helpful discussions, Doreen Landermann for helping the collection the Quintus seeds, employees of the experimental farm Hohenschulen of Kiel University. This work was funded by the DFG Collaborative Research Centre (CRC) 1182 “Origin and Function of Metaorganisms” and the International Max Planck Research School for Evolutionary Biology. The authors declare no competing interests.

Author Contributions:

The study was conceived and designed by EÖ and EHS. Domesticated seeds were collected by EÖ. Wild seed collections from Turkey were collected and provided by HÖ. The experiments were performed by EÖ and ZM. Data analysis was conducted by EÖ. The manuscript was written by EÖ and EHS. All authors read and confirmed the manuscript.

Conflict of Interest:

The authors declare no conflict of interest.

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Figure legends:

Figure 1: **A)** Sampling design: Seeds of the Quintus cv. were collected from three different plots. Each plot is in the area of 1m². In addition to the Quintus cv., we studied seeds and axenic leaves of the wild wheat collected from the Fertile Crescent region **B)** Experimental design of the study: Seeds collected from the same and different plants (1), surface-sterilized (2) and were processed for DNA extraction (3) and 16S sequencing (4). Some of these seeds (5) were germinated under sterile conditions over three time points (6), harvested (7) and processed for DNA extraction (8) and 16S sequencing (9).

Figure 2: Alpha-diversity estimates of the seeds and leaves from different plant ages in domesticated and wild wheat: Microbial diversity is decreasing over time in the domesticated wheat (Observed: $p < 0.005$; Shannon: p is not significant; inverse Simpson: $p < 0.00005$).

Bacterial diversity was estimated in each sample by observed number of OTUs, Shannon index and inverse Simpson estimate metrics. Each dot in the plots represents bacterial diversity in each individual sample.

Figure 3: Shared or distinct bacterial composition compared among different sample categories. In the Venn diagrams, OTUs were denoted at the genus level if the annotation is available. If not, they were annotated at the family level. **A)** Composition of the bacterial communities across developmental times in the domesticated and wild wheat. Bacterial OTUs were aggregated at the family level. Mean relative abundances of the most abundant ten bacterial families were depicted. Color for each feature ranges from blue (minimum 0) to red with higher relative abundance values. **B)** Core bacterial taxa shared between domesticated and wild wheat at each developmental time. **C)** Bacterial OTUs shared or

distinct among developmental stages of the domesticated wheat leaves. **D)** OTUs shared or distinct in the seeds of the plants collected from different plots in the same field.

Figure 4: Host type and developmental time are the determinants of the seedborne microbial communities in leaves

A) Bray-Curtis **B)** Weighted **C)** Unweighted UniFrac distances based PCoA of bacterial communities of seeds and axenic leaves **D)** Summary statistics for the PERMANOVA results of the Bray-Curtis comparisons of bacterial communities.

Table legends:

Table 1: Summary of the sequencing

Supplementary figures and tables:

Supp. Figure 1: Alpha-rarefaction curves of each sample. Each sample reaches saturation in sequencing depth.

Supp. Figure 2: Number of OTUs: Shared in the axenic wheat leaves whose seeds are originating from the same plant; shared in the axenic wheat leaves originating from two; and three different plants collected from the same plot.

A

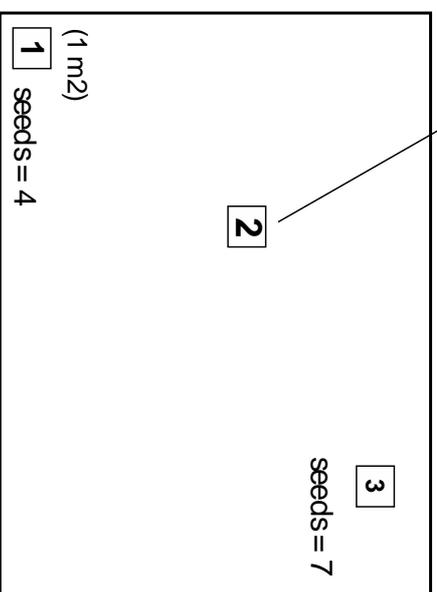
Wild wheat: 6 seeds, 10 leaves

Quintus cultivar:

Plant1: 6 seeds, 21 leaves (7 T1 + 8 T2+ 6 T3)

Plant2: 6 seeds, 14 leaves (5 T1+ 6 T2)

Plant3: 5 seeds, 14 leaves (5 T1+ 5 T2+ 4 T3)



B

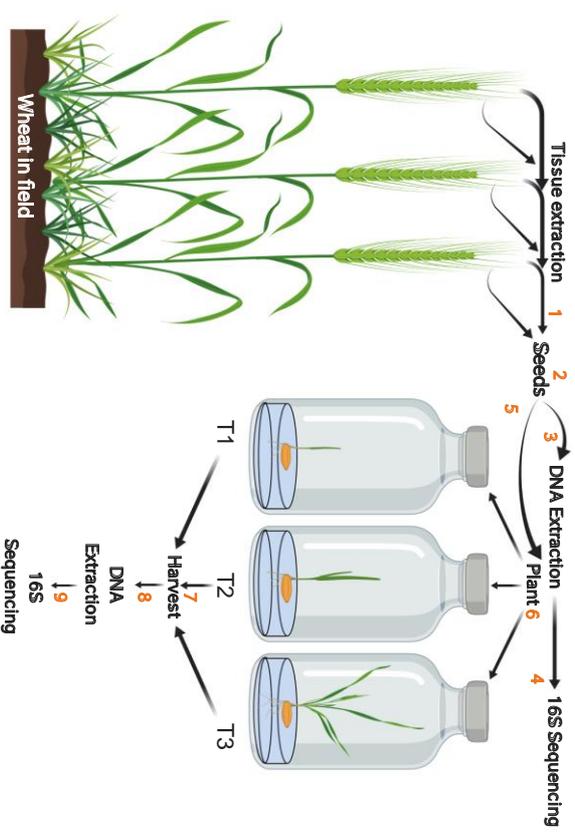
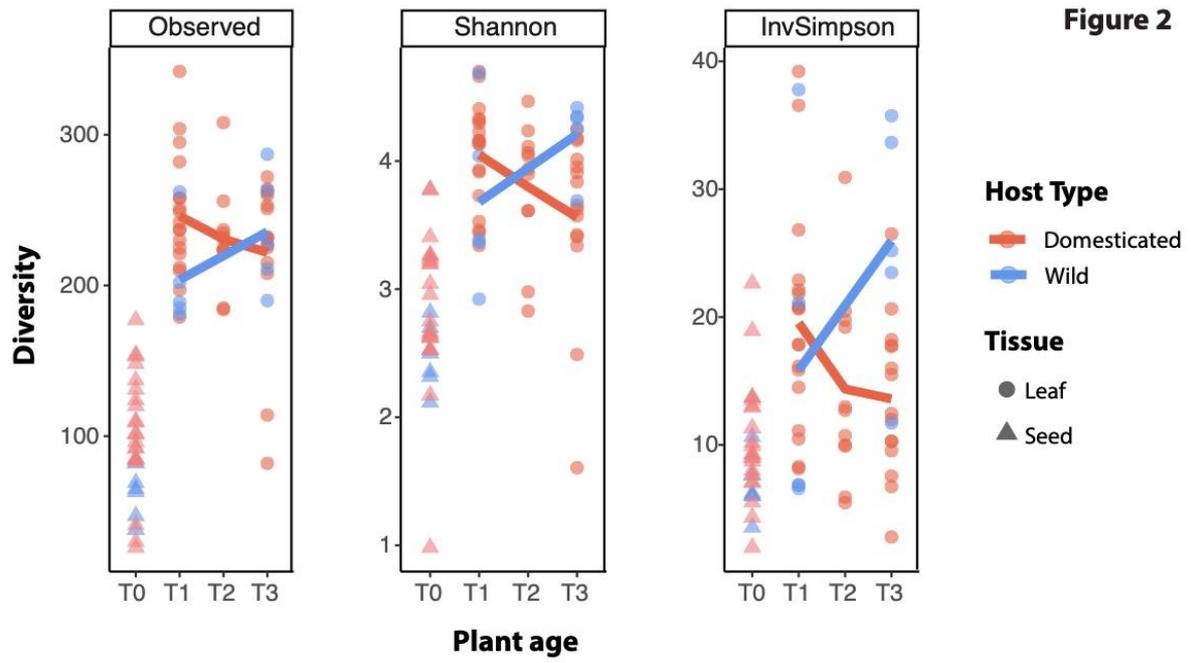
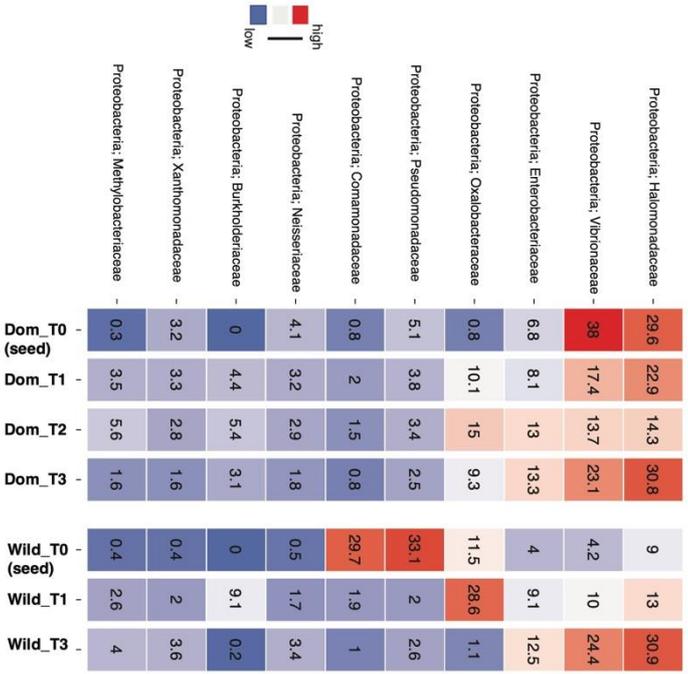


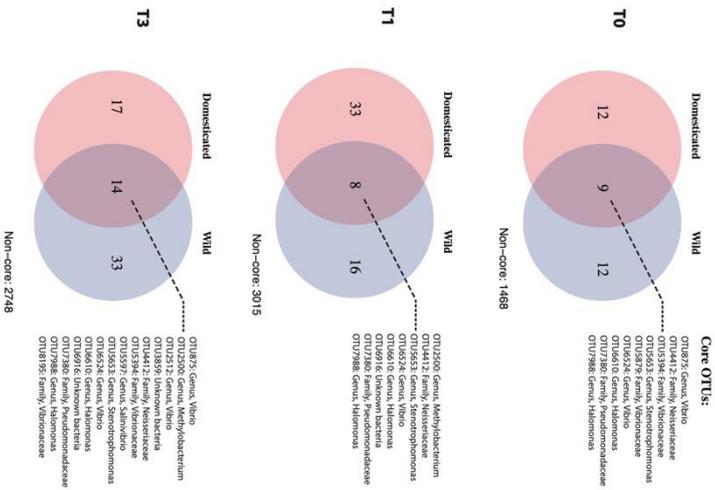
Figure 1



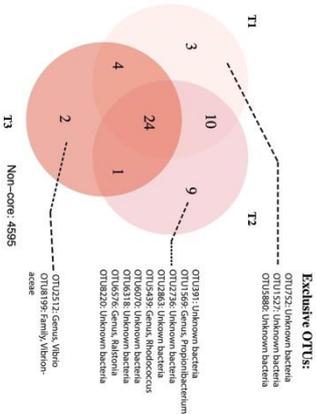
A



B



C



D

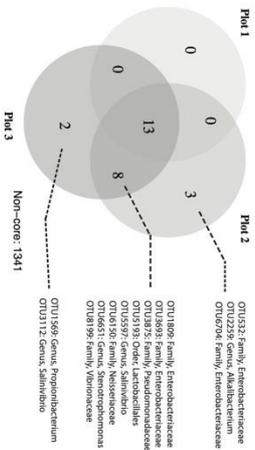
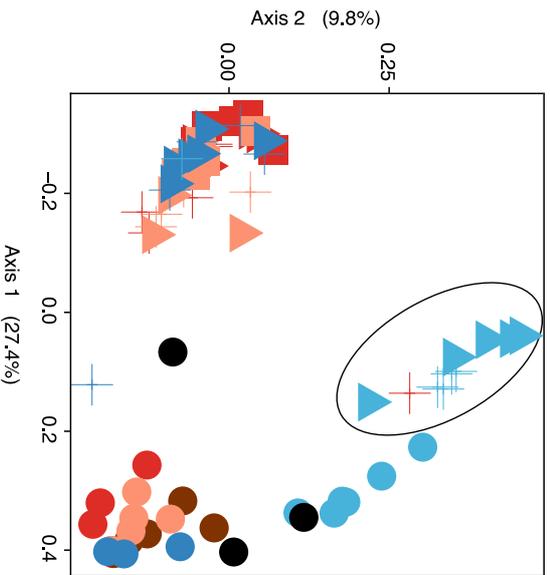
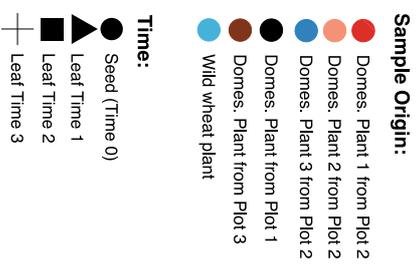
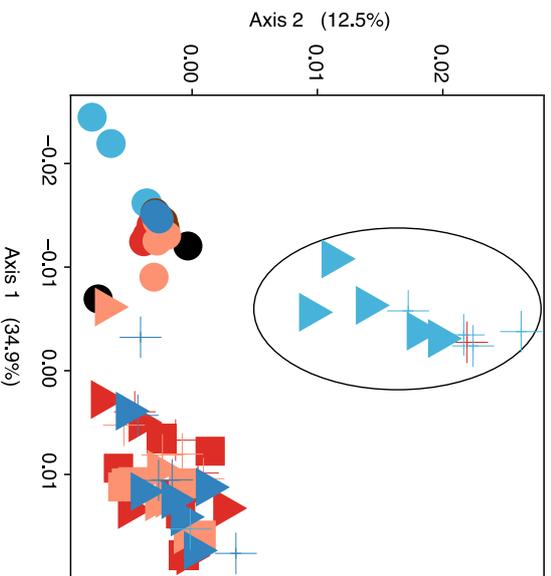


Figure 3

Bray-Curtis



Weighted UniFrac



Unweighted UniFrac

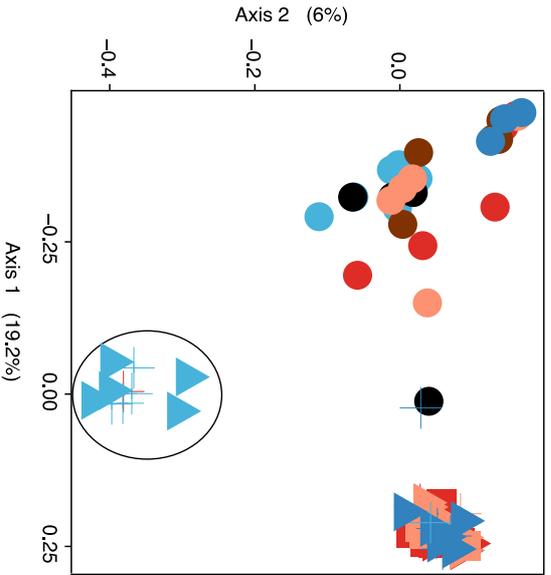


Figure 4

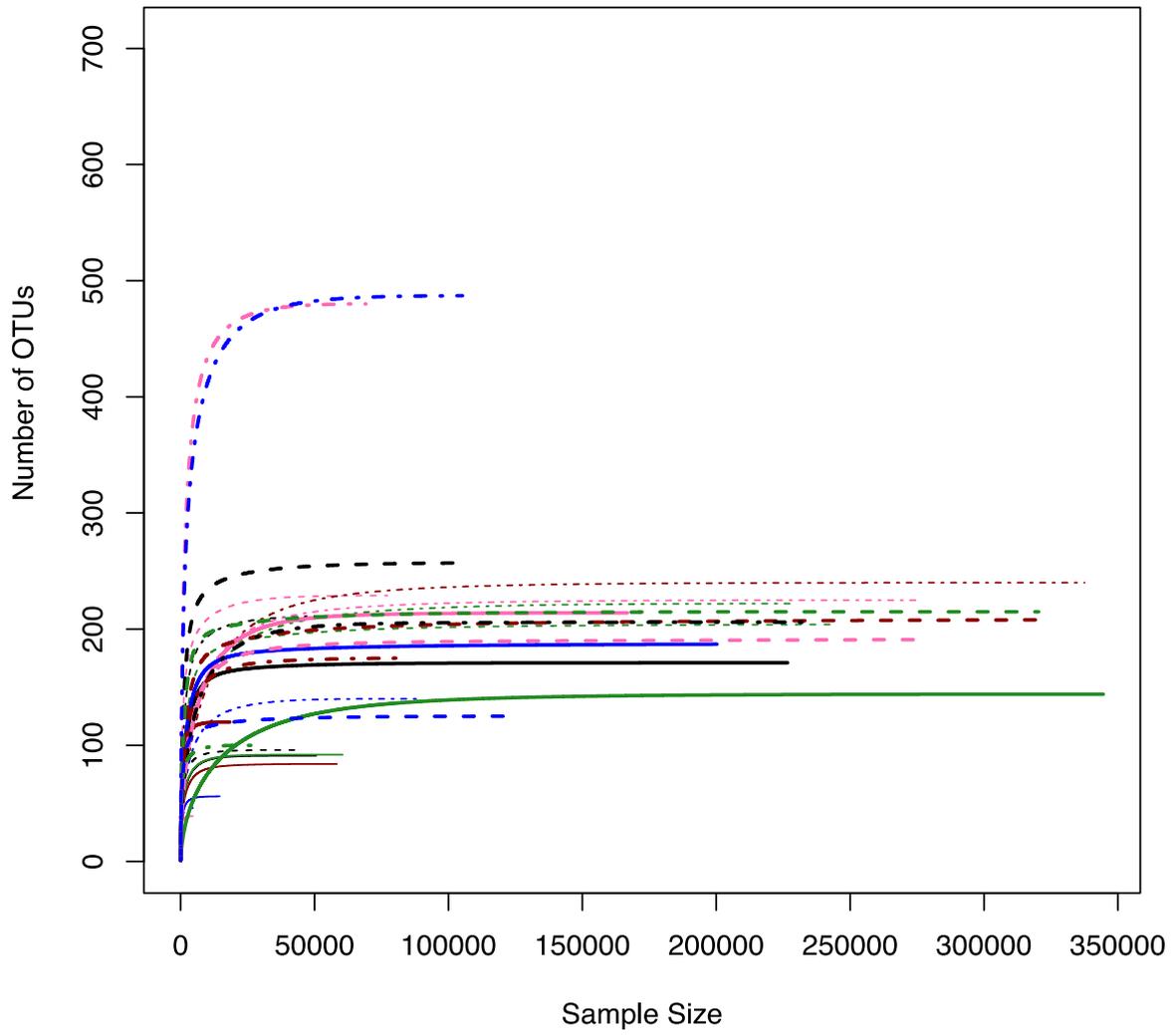
LEAVES-BRAY-CURTIS DIVERSITY

Factors	Explained variation	Significance
Host Type	13.16 %	p = 0.001
Plant Origin	4.45 %	p = 0.426
Developmental Time	9.06 %	p = 0.046
Host Type * Dev. Time	3.59 %	p = 0.020

SEEDS-BRAY-CURTIS DIVERSITY

Factors	Explained variation	Significance
Host Type	29.11 %	p = 0.001
Plant Origin	6.37 %	p = 0.751
Plot	15.59 %	p = 0.078

Supp. Figure 1



Supp. Figure 2

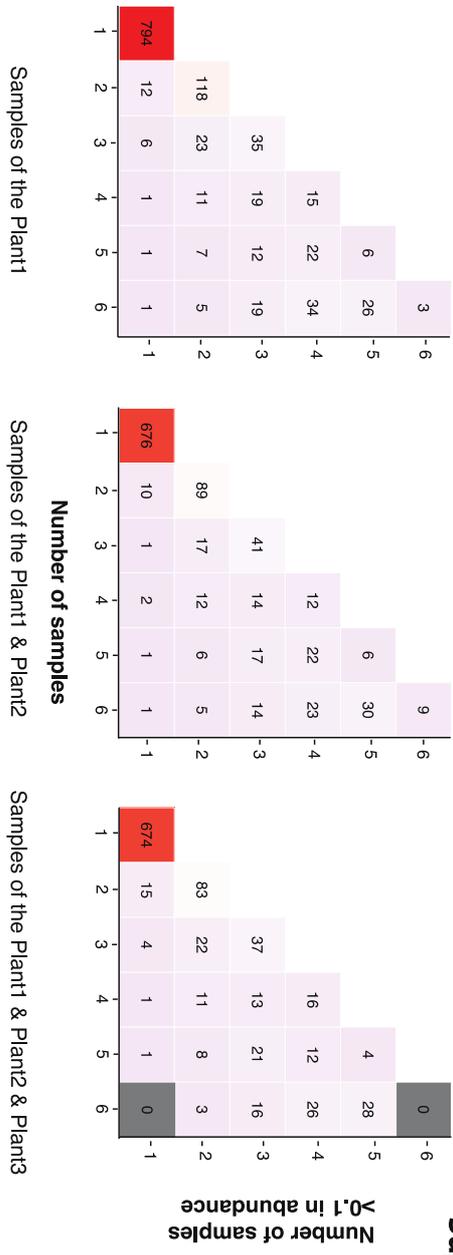


Table 1: Summary amplicon sequencing data

	No of samples	Total no of reads	Average no of reads	Minimum number of reads	Total number of features
Seeds- domesticated	22	3785755	172079.77	2608	1367
Seeds- wild	6	230783	38463.83	4517	239
Leaves- domesticated T1	18	1505267	83625.94	28262	2469
Leaves- domesticated T2	11	900893	81899.36	25938	1522
Leaves- domesticated T3	15	1451800	96786.67	32240	2269
Leaves-wild T1	5	318692	63738.4	56727	848
Leaves-wild T3	5	302458	60491.6	20732	1060

Annex: published work

Interactions and coadaptation in metaorganisms

Interactions and Coadaptation in Plant Metaorganisms

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Annu. Rev. Phytopathol. 2019. 57:483–503

First published as a Review in Advance on
July 26, 2019

The *Annual Review of Phytopathology* is online at
phyto.annualreviews.org

<https://doi.org/10.1146/annurev-phyto-082718-100008>

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Keywords

plant microbiota, microbial host specialization, seedborne microbes, plant domestication, community analyses

Abstract

Plants associate with a wide diversity of microorganisms. Some microorganisms engage in intimate associations with the plant host, collectively forming a metaorganism. Such close coexistence with plants requires specific adaptations that allow microorganisms to overcome plant defenses and inhabit plant tissues during growth and reproduction. New data suggest that the plant immune system has a broader role beyond pathogen recognition and also plays an important role in the community assembly of the associated microorganism. We propose that core microorganisms undergo coadaptation with their plant host, notably in response to the plant immune system allowing them to persist and propagate in their host. Microorganisms, which are vertically transmitted from generation to generation via plant seeds, putatively compose highly adapted species and may have plant-beneficial functions. The extent to which plant domestication has impacted the underlying genetics of plant–microbe associations remains poorly understood. We propose that the ability of domesticated plants to select and maintain advantageous microbial partners may have been affected. In this review, we discuss factors that impact plant metaorganism assembly and function. We underline the importance of microbe–microbe interactions in plant tissues, as they are still poorly studied but may have a great impact on plant health.

INTRODUCTION

A broad diversity of microorganisms, including viruses, bacteria, fungi, and oomycetes, colonize plant tissues (19). Some microbial species propagate on plant surfaces, whereas others engage in more intimate associations with the plant host to colonize the inter- or intracellular space of plant tissues (**Figure 1**). Some of these microorganisms are pathogens that cause disease, whereas others are mutualistic symbionts that promote plant growth via the exchange of nutrients. In between is a range of interaction types, including a community of commensal microorganisms with no visible effects on plant fitness and some microorganisms with minor beneficial or pathogenic effects. These microorganisms have specialized to overcome plant immune responses, otherwise induced by invading microorganisms, to explore plant tissues for their own growth and reproduction (52).

A growing field of research focuses on the diversity and function of plant-associated microbial communities, and many studies have provided evidence for the beneficial role of microbial associations beyond the relevance of individual symbionts. The plant microbiota may constitute an important component in rapid adaptation of plants to changes in their environment such as fluctuations in nutrient and water availability as well as biotic and abiotic stresses. Consequently, the evolution of certain traits of the plant may be tightly linked with the diversity and evolution of the associated microbiota. In some animal systems, the high extent of mutual coadaptation and

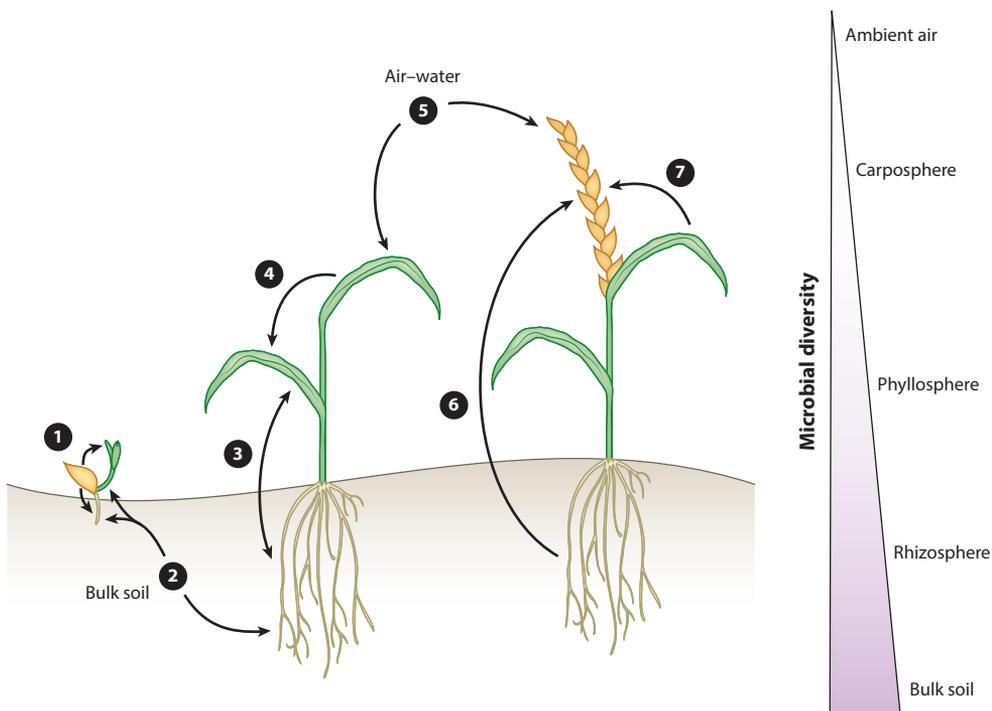


Figure 1

Plants acquire their microbiota from different inoculum sources. **1** Seeds harbor microorganisms that can colonize the nascent plant root and leaf tissues. **2** Most of the root microbiota and, to a lesser extent, the leaf microbiota are recruited from the bulk soil. **3** It is proposed that certain microbes can translocate from their respective tissue as well as from neighboring plants. **4** Microbes in the phyllosphere can be transferred between leaves of the same plant or to leaves of neighboring plants. **5** Airborne microbes and precipitation are recognized as sources of inoculum for leaves and seeds. The seed microbiota mainly resemble the **6** leaf and **7** root microbiota.

interdependency of a eukaryotic host and its microbiota has provided the basis for the metaorganism concept (16, 129). Importantly, the term metaorganism encompasses the host and its associated microbes as a single evolutionary unit in which the macro- and microorganisms form a network of symbiotic interactions that affect their evolutionary trajectories (48, 116). This concept is debated, as the presence of host-associated microbiota alone does not provide sufficient evidence for the evolution of beneficial symbiosis (82). However, a fraction of the plant microbiota may have coexisted and coevolved over long evolutionary times and thereby constitutes a component of the plant metaorganism. Such long-term associations may, for example, include microbial members, such as seedborne microbial species that are vertically transmitted across plant generations.

In this review, we focus on plant metaorganisms and microbial adaptations to the plant-associated lifestyle. These may notably involve adaptations to the plant immune system to avoid or tolerate induced immune responses. In particular, we address the occurrence of vertically transmitted microbial communities, including species that have adapted and become specialized to their plant host habitat. Such core microbial species may constitute organisms that shape the evolution of plant pathogens and mutualists of the same species. We propose that further research into microbial interactions and coadaptation is essential to understanding the role of plant microbiota in disease resistance and their potential role in plant protection.

THE PLANT MICROBIOTA: A FUNCTIONAL EXTENSION OF HOST TRAITS

The metaorganism concept implies the existence of synergistic interdependence of the multicellular host with its associated bacteria, archaea, fungi, and numerous other microbial and eukaryotic species. Indeed, mutualistic symbiotic associations are widely documented in the plant kingdom, and multiple studies have provided insight into the underlying mechanisms of growth promotion and increased disease resistance conferred by microbiota members. These “functional extensions” of the plant metaorganism are mediated by a reservoir of microbial genes, some of which represent specific adaptations to coexistence with plants (42, 83, 90). Microbiota-mediated plant traits are of paramount importance to plants, as has been illustrated notably in many studies of the symbiosis between *Rhizobium* and legumes (reviewed in 21, 89, 128) and between mycorrhizal fungi and diverse plant species (reviewed in 24, 87). These prominent symbiosis examples have demonstrated the outcome of prolonged coexistence and coevolution whereby microbes provide nutrients to their host in exchange for photosynthates.

Recent functional studies of less prominent microbiota members show a diversity of other microbial taxa with similar significant influence on plant health. Two independent studies showed that the nonmycorrhizal plant *Arabidopsis thaliana* can receive phosphorus (P) from the ascomycete fungus *Colletotrichum tofieldiae* (54) and from the basidiomycete fungus *Serendipita indica* (7). Almario and colleagues studied the root mycobiota of a wild relative of *A. thaliana*, *Arabis alpina*, that is capable of thriving in alpine P-limited soils (5). The authors demonstrated that the roots of *A. alpina* harbor a distinct mycobiota and described 15 fungal taxa as members of the core microbiota. Among these fungal species, *A. alpina* associates with a beneficial ascomycete fungus that belongs to the Helotiales. This particular association was shown to increase the tolerance of the host plant to the P-limited soils (5). It is plausible that adaptation of *A. alpina* to its nutrient-limited habitat has involved positive selection of traits that promote the association with specific microbial partners to facilitate P-uptake.

Similarly, nonlegume plants have evolved symbiotic interactions with other diazotrophic bacteria than *Rhizobium* to fix atmospheric nitrogen (reviewed in 100). For instance, the association between the nonlegume plant *Parasponia* spp. and the actinomycete bacterium *Frankia* spp. (3), the

association between the cyanobacterium *Nostoc* spp. and several plant species belonging to Gunneraceae or Cycadaceae (1), and the endosymbiotic associations of the Proteobacteria *Azospirillum* spp., *Azoarcus* spp., and *Herbaspirillum* with the roots of diverse plant species, including cereals (32, 95).

A recent study conducted by Tarquino and colleagues further corroborated these data (111). The authors investigated the role of leaf-associated microbiota of the marine seagrass *Posidonia sinuosa* in facilitating nitrogen (N) uptake from a pool of dissolved organic nitrogen (111). Through amino acid mineralization, the phyllospheric microbes provided the essential element N to *P. sinuosa*, thereby promoting overall plant productivity by an increased nutrient uptake. The plant microbiota may alternatively promote plant growth by induction of master transcriptional regulators that activate phosphate stress responses and repress the immune system (22). Together, these examples point to new groups of plant-associated microbial taxa, which have adapted to plant symbiosis. It will be interesting in the future to assess the evolution of these symbioses and the underlying molecular basis of plant–microbe interactions.

In plant metaorganisms, microbial functions define interactions with not only the host but also the other associated microbial partners. Recent research of plant–microbe interactions aimed to extend the complexity of the studied systems by gradually increasing microbial diversity using synthetic communities that highly resemble the host microbiota. Synergistic interaction among community members may be influencing growth-promoting effects, as recently demonstrated by Garrido-Oter and colleagues (45). These authors addressed root-growth promotion mediated by Rhizobiales, which are bacterial members of the core root microbiota of a wide range of plant species (105, 126). The study revealed that a large set of Rhizobiales strains promote root growth of the non-nodulating plant species *A. thaliana*. The observed phenotype was independent of whether or not the particular tested strain encoded the *nod* and *nif* genes producing enzymes involved in the fixation of atmospheric nitrogen. Further analyses revealed that one specific strain could tune down the immune system. Remarkably, not all Rhizobiales strains were able to modulate the plant immune system but they could act in a synergistic manner when used in a tripartite gnotobiotic or sterilized plant system. The study constitutes an example of how microbiota members can collectively modulate plant growth in natural settings, and it suggests that prolonged coexistence and synergetic interactions may be important drivers of microbial interactions and coevolution. Although this study remains reductionist compared to natural environments, it demonstrates the relevance of synthetic microbial communities to study synergistic interactions and plant metaorganism function (120).

In a recent study, Durán and colleagues followed the same experimental logic by increasing complexity of the microbial inoculum added to a gnotobiotic system, including oomycete and fungal species derived from the roots of *A. thaliana* (38). The authors used a synthetic community experiment (65) to assess the role of each group (i.e., bacterial, fungal, and oomycete) of the root microbiota in altering the host fitness. Although all microbial taxa were root derived, the authors found that a microbial root community consisting of only fungi and oomycete partners significantly compromised the growth of *A. thaliana* (38). By restoring the bacterial fraction, the authors could rescue the plant and further demonstrated that plant protection is common among the bacterial members of the root microbiota (38). Taken together, these recent studies provide insights into the function and microbial synergies of plant metaorganisms, and they underline the importance of the microbiota and microbial interactions in plant fitness. It is worth highlighting that although plants have coadapted with diverse microbial communities, proper homeostasis of the microbiota is critical for the host fitness. A perturbed microbiota may result in pathobiota that negatively impact plant fitness rather than promoting it (8). It is still poorly understood how microbiota homeostasis is achieved and how it is maintained during plant development and

environmental changes, but it is likely that the plant immune system plays a central role in defining the composition of the plant-associated microbiota.

Although most research of plant–microbe interactions has focused on the binary relationship of one microorganism and its plant host, much less is known about the interaction of plant pathogens or mutualists with other plant-associated microorganisms. There is, however, an increasing appreciation that plant pathogen resistance is greatly impacted by the composition and collective functions of the microbial community (16). This implies that an important but little explored component of plant–pathogen interactions also involves direct and indirect interactions among microbial partners and between these and the plant host.

Microbial community studies based on the amplification and sequencing of specific DNA markers such as the bacterial 16S ribosomal RNA sequence and the fungal internal transcribed spacer (ITS) region have provided insight into the taxonomic diversity of microorganisms for a wide variety of plant species. Furthermore, microbial community analyses have allowed predictions of positive and negative associations among microbial species and the identification of hub species that particularly shape community structure (2).

Microbial interactions have also been experimentally investigated. Gnotobiotic plant systems with synthetic community experiments have shed light on functional properties of individual community members as well as functional properties of collaborative associations of the microbiota. Most of this research has so far been conducted with *A. thaliana* as a model plant and has demonstrated the functional relevance of keystone bacterial species with a major impact on community compositions and/or functions and microbial synergistic interactions in these communities (e.g., 13, 38, 67, 73).

Plant pathogens and mutualists coexist, interact, and compete with these microbial communities from the point of epiphytic growth and infection to the endophytic colonization. An important topic still to be addressed is how these microbial interactions impact disease development, immune signaling, and microbial community composition. We still lack knowledge about the extent of coevolution of plant-associated microorganisms and, notably, whether and how invading pathogens or mutualists coevolve with plant-associated microbial communities. These are important questions that can be addressed in future studies by integrating knowledge from the field of molecular phytopathology with comparative and functional plant microbiota studies.

THE ROLE OF MOLECULAR PLANT–MICROBE INTERACTIONS IN METAORGANISM ASSEMBLY

The extent to which plants control the assembly of microbial communities to select microbial partners, which together constitute the most beneficial community, is still an open question. From studies of plant–pathogen and plant–mutualist interactions, we have gained detailed insight into the underlying basis of molecular interactions of plants and pathogens. Different components of the plant immune system define the ability of pathogens and mutualist partners like mycorrhizal fungi and nitrogen-fixing bacteria to colonize infected tissues. Interestingly, the establishment of mutualistic interactions with, e.g., *Rhizobium* or mycorrhizal fungi is, in large part, also defined by the same components of the plant immune system that are involved in plant–pathogen interactions, suggesting a more general role of the immune system in managing microbial associations. The plant immune system is described in several excellent reviews (e.g., 30, 58, 130), and we here summarize only key aspects and point to important perspectives in terms of the plant immune system and microbiota assembly.

Plants have developed cell surface immune receptors [pattern recognition receptors (PRRs)] for detection of microbial-derived molecules, which are produced by a broad spectrum of microbes

(131). Recognition of these pathogen-/microbe-associated molecular patterns (PAMPs/MAMPs) activates PRRs and initiates the first layer of induced plant defenses (after the physical barriers of plant surfaces). PAMP-triggered immunity (PTI) is sufficient to restrict the growth of most infecting microbes and is likely to also have a large impact on the assembly of the microbiota of the plant (49). Some microbes have evolved so-called effector proteins, which allow the microbial species to overcome plant defense responses, often by interfering with PTI (71). The composition of effectors is typically highly strain-specific and based on specific protein-protein interactions. Therefore, effector proteins and molecules also act as determinants of the host range of the effector-producing microbe (35). Plants produce intracellular receptors, so-called resistance (R) proteins, which most often comprise leucine-rich repeats (LRRs), a central nucleotide-binding site (NBS), and a variable amino-terminal domain. These R proteins can define resistance toward microbes that have evaded PTI by detection of effector proteins or effector-modified plant proteins. Activation of intracellular R proteins results in the induction of a second layer of defense, effector-triggered immunity (ETI) (74).

In contrast to the highly diverse microbial-produced effector molecules, MAMPs are considered to be highly conserved. Microbial molecules, i.e., MAMPs, that can trigger PTI responses include bacterial flagellin, elongation factor Tu (EF-Tu), lipopolysaccharides (LPSs), peptidoglycans, and fungal chitin (14). MAMP recognition is an evolutionarily ancient trait of land plants, as orthologs of the MAMP flg22 (a 22-amino acid epitope of flagellin) receptor FLS2 exhibit a high degree of conservation among genomes of phylogenetically distant plants (14). Interestingly, some plants, including a subset of Solanaceae plants and rice, have other receptors for flagellin detection, i.e., they act in an FLS2-independent manner (25, 53, 60). Likewise, multiple immunogenic epitopes of flagellin and other MAMPs have been identified among diverse plant-associated microbial species, pointing to convergent evolution of MAMP recognition in plants (and eukaryotes in general; see 85). However, the recognition of other MAMPs is limited to certain plant species reflected by the restricted distribution of several PRRs among land plants. For example, the receptors for EF-Tu and LPS (the PRR proteins EFR and LORE, respectively) are restricted to Brassicaceae plants (66, 91).

We still lack a detailed understanding of the regulation and role of PTI in microbiota assembly. Notably, the large amounts of microbes that are associated with plants raise the question of whether the plant immune system is constantly triggered by microorganisms growing on plant surfaces. Abundance or microbial load seems to matter, as demonstrated by the observation that complete lack of PTI responses in *A. thaliana* leads to uncontrolled bacterial growth in leaves (123). Because PRRs have a certain affinity toward their ligand (the MAMP), plants may regulate the strength of the defense responses based on the detected amount of the respective MAMP (4, 40). In addition, PRR complexes often share coreceptors, and different MAMPs lead to the activation of the same downstream signaling cascades (37, 131). Therefore, a higher diversity of MAMPs does not necessarily lead to a stronger response.

Notably, although in theory every cell is able to detect and respond to microbial infection, tissues with a high risk for microbial entrance, like plant roots and shoots, show higher expression levels of FLS2 and most likely other PRRs (9). These tissues might react with a stronger, more cost-intensive defense response due to their higher numbers of receptors without impacting the entire plant. After activation, PRRs like FLS2 and EFR are internalized quickly and directed to degradation (10, 80). This mechanism might prevent an overreaction in response to MAMP recognition. PRR degradation is, however, a transient process, and plants do not stay insensitive to the respective MAMP. In conclusion, it is assumed that PTI is triggered more often than ETI, which is induced only by invading pathogens. The latter usually results in a stronger defense reaction called the hypersensitive response (HR), which is associated with cell death. Not having HR

triggered by default during PTI could be a safety mechanism of the plant to prevent the drastic consequences of continuous HR.

Microbial adaptation to the coexistence with plants may entail strong selection pressure on genes involved in the production of MAMPs to avoid PTI responses. There is growing evidence that weakly immunogenic alleles of MAMPs, including flagellin, have evolved in some plant-associated microbes by the accumulation of genetic variation in MAMP epitopes of less structural or functional importance (25). For example, flagellin of the plant pathogen *Agrobacterium tumefaciens* is not immunogenic and enables plant infection without recognition by the plant immune system (40). The same is true for the flagellin sequence of the nitrogen-fixing *Rhizobium meliloti*, which is exceptionally divergent from the conserved flg22 and does not trigger PTI, whereas this is not the case for flagellin derived from *Escherichia coli* (40).

This observed variation at the level of MAMP production and MAMP recognition suggests that the community structure of the plant-associated microbiota may be determined to a large extent by the presence/absence of detectable MAMP epitopes. A current working hypothesis further states that epitopes that can be recognized by *A. thaliana* are rare among microbiota members, whereas epitopes that cannot be detected by this plant are more frequently present (49). Therefore, the composition of PRRs of a given plant determines the association of microbial partners.

As mentioned above, the interaction of plants with mutualistic symbionts is based on the same immune-related pathways as the interaction of plants with pathogens. Mutualistic symbionts also produce effector proteins that interfere with ETI; however, at the level of PTI, there already appears to be recognition of the beneficial microbial partner. This recognition is, for some associations, based on symbiosis molecules that are highly conserved across kingdoms. Bacterial cell walls mainly contain peptidoglycan polymers, whereas fungi are recognized by plants via their main cell wall component, chitin. Interestingly, both cell wall polymers contain *N*-acetylglucosamine (GlcNAc) units and trigger PTI. In contrast, a common feature between plant-beneficial arbuscular mycorrhizal (AM) fungi and rhizobia are their signals for symbiosis, which are also based on GlcNAc but have lipid modifications (131). These lipochitoooligosaccharides (LCOs) can differ in length, lipid type, and additional modifications, presenting the possibility for plant host specificity (46). The combination of these symbiosis signals and effector proteins promotes colonization of host plants (29, 63), whereas activation of plant immune responses due to effector recognition restricts the establishment of symbiotic interactions with the plant. It is plausible that the overall balance between these two sets of signals and microbial niche compatibility determines whether or not a particular microorganism can become established in the plant. The downregulation of plant defenses during symbiosis with rhizobia again emphasizes the important role of the immune system during plant colonization (47) and hints at a role for plant defense responses (or the absence of these) during the establishment of endophytic associations and the plant microbiome in general (67). The plant immune system can therefore also be seen as a plant tool to control the accommodation of beneficial microbes and for monitoring of microbial load (49).

Finally, an important factor that should not be ignored when considering bacterial load and its consequences for the plant immune system is the detection and interference with quorum sensing (QS) by the plant. QS in gram-negative bacteria—plant pathogens as well as commensals—is dependent on secreted *N*-acyl homoserine lactones (AHLs). AHLs of root-associated microbes can alter immune responses in a tissue-specific manner and are able to induce systemic resistance responses in many plant species (79, 104, 108). Whether an AHL has a beneficial or a negative effect on the plant is dependent on the type of the AHL, its concentration, and the time of exposure. In addition, plants can also secrete QS-mimicking compounds (44, 79). Plant-derived AHL mimics have the potential to interfere with QS in the associated bacteria, giving the plant the ability to control bacterial gene expression and, consequently, the performance of the plant-associated

microbes. The production and secretion of plant-derived AHL mimics may be regulated by the presence of other bacteria (44), suggesting an important role for the microbial community structure in appropriate plant responses. This also suggests that coevolution of microbes and the host immune system is needed for a successful plant–microbe association and the establishment of the plant metaorganism.

VERTICAL INHERITANCE OF MICROBIAL PARTNERS: THE SEEDBORNE MICROBIOTA OF PLANTS

Plants acquire the vast majority of their microbiota from the environment, notably the soil. However, a smaller fraction is acquired vertically from the mother plant, for example via seeds. We here consider that vertically transmitted microbial communities are core members of the plant metaorganism and that prolonged symbiotic associations, one plant generation after the other, can shape plant–microbe coevolution of interacting genes. Microorganisms that are taken up from the environment may be adapted to plant colonization but may show a lesser extent of coevolution compared to vertically transmitted species. Seeds can harbor diverse microbial communities, as shown via studies of diverse plant species. These seedborne microorganisms may play a fundamental role in the early development of the plant (98, 114). For the microorganism, the seed provides an efficient means of dispersal and transmission from one plant generation to the next. It has long been known that many plant pathogens are dispersed via crop seeds, e.g., the ascomycete *Aspergillus flavus* and the basidiomycete *Rhizoctonia solani*. However, commensal and beneficial microorganisms have also been detected in plant seeds, including species of fungi such as beneficial species of *Alternaria* and *Epichloë* and bacteria such as *Paenibacillus* and *Curtobacterium* (51, 103, 114). During plant development, seedborne and vertically transmitted microorganisms may have a competitive advantage in comparison to microorganisms that are taken up from the environment, as they are already established in the seedling as it develops (97, 98, 109). However, so far only a few studies have addressed the persistence of seedborne microorganisms during plant development, including mainly studies of the fungal grass endophyte *Epichloë* (103).

It is plausible that plants have evolved mechanisms to influence the communities of seedborne microorganisms to propagate beneficial associations. Indeed, some seedborne endophytes have been shown to express functional traits related to disease suppression, e.g., the production of specific volatiles, extracellular ribonucleases, and antimicrobial compounds (62). These plant-associated traits may reflect stronger coadaptation with the plant host of vertically transmitted microbial partners compared to microorganisms that are taken up from the environment.

Studies of seed-associated microbes are relatively limited compared with other plant-associated microorganisms. This limited knowledge stems, in part, from the technical challenges of experimentally handling the seeds of many plant species, including seeds of the model species *A. thaliana*, which produces very small seeds. Research on seedborne microbial communities has been based notably on studies of crop species such as rice, maize, and wheat (e.g., 50, 57, 98). These studies have addressed community structures and the impact of plant genotypes and abiotic factors (e.g., soil type) in shaping microbial diversity in seeds. Furthermore, a few studies have addressed the extent of vertical transmission across generations (50, 57). Vertical inheritance of a core microbial community across generations has, for example, been suggested for species of maize for which some microbial partners have persisted during domestication and in managed environments (57). In another study, almost half of the endophyte operational taxonomic units (OTUs) detected in seeds of rice were also found in a following generation of seeds (50), indicating a vertically transmitted core microbial community. Yet a limitation of these studies is the taxonomic resolution

of microbial partners. The studies were all based on amplicon sequencing techniques using the 16S ribosomal gene, which does not allow a fine-scale resolution of microbial species diversity. In fact, solid evidence for vertical transmission would require identification of the same strain, e.g., a fluorescent labeled endophyte expressing green fluorescent protein, which can be detected and isolated in subsequent plant generations (114). Although more experimental studies are needed to study mechanisms of vertical transmission of plant-associated microorganisms, the available data demonstrating the same OTUs in consecutive seed generations are remarkable, as they point to core members of the plant metaorganism where the plant and its associated microbes putatively evolve in parallel and constitute a unit of selection (116).

Lastly, seeds are not the only route for vertical transmission of endophytes. In an elegant study by Vannier and coworkers, the asexual plant *Glechoma hederacea* was used to study the extent of vertical transmission of endophytes from mother to daughter plants via internodes (118). The existence of similar phylum richness of mother and daughter plants supported the hypothesis that internodes could provide a means for vertical transmission of endophytes. In the experiment, mother plants were induced to develop new ramets in separate pots and the microbial composition of daughter plants was investigated. A diversity of fungi and bacteria was vertically transmitted to the new generation of plants. However, an intriguing finding from the experiment was that only a specific subset of the original pool of endophytes was transmitted to the daughter plants of *G. hederacea*, suggesting a filtering mechanism that acts on the microbiota during transmission.

Whether these communities comprise microorganisms that have a functional relevance or mainly comprise species that explore the plant seed as a means of dispersal remains elusive. We still need more comprehensive studies describing the composition and stability of seed-associated communities, the various factors shaping them, and their colonization routes. More importantly, after having a solid knowledge about community composition, we need to define the functionality of the individual community members in the later stages of plant development by experimental studies and analyses of whole genomes. Controlling and manipulating the relationship between plants and their seed-associated microbiota could permit the exploitation of the beneficial functions of these microbiota in agriculture. However, to do so, we need a more detailed understanding of the seed-associated microbial community composition and the factors shaping microbial maintenance of plant generations as well as the long-term and short-term community dynamics and coadaptation.

THE ENVIRONMENTAL CONTRIBUTION TO PLANT MICROBIOTA ASSEMBLY

One of the most important sources of microbial partners is the substrate in which the plant grows, most often the soil (17, 41, 73, 121) (**Figure 1**). Soil not only serves as a supportive growth matrix but also constitutes the main seeding source of a plant's associated microbes, some of which extend the plant functional repertoire. It is therefore not surprising that soil physical and chemical characteristics are the main driving forces that shape rhizospheric and root-endospheric microbial communities (11, 18, 73, 107). Regarding the prokaryotic fraction of the plant microbiota, it has been shown that plant compartments (68, 83, 122), host genotype (13, 92, 105), immune system (67), and developmental stages (23, 55), as well as season (27), shape the structure of the associated microbial communities. Interestingly, biogeography has a more prominent role in shaping communities of plant-associated fungi compared with communities of plant-associated bacteria, which may highlight a stronger constraint on dispersal for soilborne fungi than that for bacteria (15, 26, 110). In contrast to belowground plant tissue, aboveground plant tissue is subject to various microbial sources such as air, soil, and precipitation (83). Although it is acknowledged that

plants nest diverse microbes, plant-microbial diversity is limited compared to the bulk soil. The microbiota surveys of several non-crop plants like *A. thaliana* and its relatives (6, 34, 105) and crop plants like maize (88), barley (17), rice (39), and citrus (125) have demonstrated that the “plant filter” favors Proteobacteria and Actinobacteria and, to a lesser extent, Bacteroidetes and Firmicutes (6, 125). Although less attention has been paid to the plant mycobiota, a few studies have employed culture-independent methods to study plant-associated fungi. Similar to bacteria, only a few lineages at the phylum level of Ascomycota and Basidiomycota dominate the fungal plant microbiota, including sister species of known plant-pathogenic fungi (**Table 1**) (5, 77, 112, 115, 122).

THE IMPACT OF DOMESTICATION AND AGRICULTURE ON THE PLANT METAORGANISM

Domestication of crops has entailed a strong directional selection of a few desired plant traits and has caused dramatic losses in plant genetic diversity (33). The extent to which domestication has affected plant-microbe interactions is less well understood. However, given the protective function of some microbial partners, a better understanding of the diversity and function of microbial species associated with crop plants may provide an important aspect of future crop-protection strategies. Comparative studies of microbial communities associated with crop species and their wild progenitors can provide valuable insights into domestication-related effects on plant-associated microbes.

There is ample evidence that plant genotype is one factor that determines microbial community composition (**Figure 2**). Domestication has led to the evolution of new species and these domesticated species have been further optimized by plant breeding (**Figure 2**). Studies of plant pathogens have demonstrated that both macro- and microevolutionary processes have been affected by domestication and agriculture. Crop domestication has given rise to new pathogen species (macroevolution), and agricultural systems greatly impact rates of adaptation at the population level (microevolution) (81). The extent to which human-mediated selection for desired plant traits has impacted the interaction with microorganisms is less clear but is a fundamental aspect of improving crops of the future.

Studies of genome evolution in domesticated and wild species have demonstrated a cost of domestication whereby domesticated species have accumulated more deleterious and nonadvantageous mutations relative to their wild relatives (72, 93). Furthermore, plant breeding prevents local adaptation of crops with their environment, including the local microbiota. It is possible that these factors, the evolutionary cost of domestication, and the absence of local adaptation generate plant genotypes that are less fit in terms of selective assembly of beneficial plant-microbe communities. Comparative community studies may reflect such effects; however, a deeper knowledge of the underlying genetic traits responsible for microbial community assembly and adaptation is needed to fully understand the impact of domestication on plant traits involved in these processes.

Bulgarelli and coworkers studied the microbial diversity associated with roots of domesticated and wild accessions of barley, *Hordeum vulgare* and *Hordeum spontaneum*, respectively (17). The study identified a small but significant effect of plant genotype on bacterial communities of the root and rhizosphere. However, this genotype effect was apparent between not only wild and domesticated accessions but also different domesticated accessions. In a study of microbial diversity in wild and domesticated wheat species, Ofek-Lalzar and coworkers characterized the diversity of fungal endophytes by sequencing of the ITS locus from isolated fungal strains (86). They demonstrated considerably higher diversity of fungal OTUs in wild wheat species compared to domesticated wheat, suggesting that domestication has impacted plant-fungus interactions. Similarly, in a study based on 16S sequencing of bacterial OTUs, Zachow and coworkers demonstrated a higher

Table 1 Plant-associated fungal endophytes

Host species	Host common name	Host family	Host type	Host clade	Annual or perennial	Tissue type	Taxonomic level of fungi	Most common fungi	References
<i>Triticum aestivum</i>	Bread wheat	Poaceae	Domesticated crop	Monocots	Annual	Leaf	Genus	<i>Zyzocephala</i> , <i>Dioszegia</i> , <i>Cladosporium</i> , <i>Cryptococcus</i>	59
<i>T. aestivum</i>	Bread wheat	Poaceae	Domesticated crop	Monocots	Annual	Leaf	Genus	<i>Zyzocephala</i> , <i>Dioszegia</i> , <i>Cryptococcus</i> , <i>Sporobolomyces</i> , <i>Blumeria</i>	101
<i>Hordeum vulgare</i>	Barley	Poaceae	Domesticated crop	Monocots	Annual	Leaf	Genus	<i>Sporobolomyces</i> , <i>Ascochyta</i>	102
<i>Avena sativa</i>	Oat	Poaceae	Domesticated crop	Monocots	Annual	Leaf	Genus	<i>Sporobolomyces</i> , <i>Cladosporium</i> , <i>Cryptococcus</i>	102
<i>Secale cereale</i>	Rye	Poaceae	Domesticated crop	Monocots	Annual	Leaf	Genus	<i>Sporobolomyces</i> , <i>Cladosporium</i> , <i>Cryptococcus</i>	102
<i>Triticum</i> × Secale	Triticale	Poaceae	Domesticated crop	Monocots	Annual	Leaf	Genus	<i>Sporobolomyces</i> , <i>Cladosporium</i> , <i>Cryptococcus</i>	102
<i>Picea abies</i>	Norway spruce tree	Pinaceae	Tree	Gymnosperms	Perennial	Root	Genus	<i>Phialocephala</i>	64
<i>Acer pseudo-platanus</i>	Sycamore maple	Sapindaceae	Tree	Eudicots	Perennial	Leaf	Order	<i>Venturiales</i> , <i>Erysiphales</i> , <i>Capnoidiales</i>	106
<i>Fraxinus excelsior</i>	European ash	Oleaceae	Tree	Eudicots	Perennial	Leaf	Order	<i>Venturiales</i> , <i>Erysiphales</i> , <i>Pleosporales</i>	106
<i>Fraxinus ornus</i>	Flowering ash	Oleaceae	Tree	Eudicots	Perennial	Leaf	Order	<i>Venturiales</i> , <i>Erysiphales</i>	106
<i>Olea europaea</i>	Olive	Oleaceae	Ornamental plant	Eudicots	Perennial	Leaf	Genus	<i>Cladosporium</i>	75
<i>Draecena draco</i>	Canary Islands dragon tree	Asparagaceae	Ornamental plant	Monocots	Perennial	Leaf	Genus	<i>Cladosporium</i>	75

(Continued)

Table 1 (Continued)

Host species	Host common name	Host family	Host type	Host clade	Annual or perennial	Tissue type	Taxonomic level of fungi	Most common fungi	References
<i>Chlorophytum comosum</i>	Spider plant/airplane plant	Asparagaceae	Ornamental plant	Monocots	Perennial	Leaf	Genus	<i>Cladosporium</i>	75
<i>Aloe arborescens</i>	Krantz aloe	Asphodelaceae	Ornamental plant	Monocots	Perennial	Leaf	Genus	<i>Cryptococcus</i>	75
<i>Musa acuminata</i>	Blood bananas	Musaceae	Ornamental plant	Monocots	Perennial	Leaf	Genus	<i>Wallenia</i>	75
<i>Cynomorium songaricum</i>	Suo yang	Cynomoriaceae	Wild parasitic plant	Eudicots	Perennial	Root	Genus	<i>Gibberella</i>	28
<i>Nitroaria tangutorum</i>	NA	Nitriariaceae	Wild shrub	Eudicots	Perennial	Root	Genus	<i>Fusarium</i>	28
<i>Arabidopsis thaliana</i>	<i>Arabidopsis</i>	Brassicaceae	Wild cress	Eudicots	Annual	Root	Class	<i>Dothidomycetes</i> , <i>Leotiomycetes</i> , <i>Sordariomycetes</i> , <i>Agaricomycetes</i>	38
<i>Arabis alpina</i>	Alpine rockcress	Brassicaceae	Wild cress	Eudicots	Perennial	Root	Order	<i>Helotiales</i>	5
<i>Helianthus annuus</i>	Sunflower	Asteraceae	Domesticated and wild strains	Eudicots	Annual	Root	Order	<i>Olpidiales</i> , <i>Hypocreales</i>	69
<i>H. annuus</i>	Sunflower	Asteraceae	Domesticated and wild strains	Eudicots	Annual	Seed	Order	<i>Pleosporales</i>	69
<i>Raphanus sativus</i>	Radish	Brassicaceae	Domesticated crop	Eudicots	Annual	Seed	Genus	<i>Alternaria</i> , <i>Cladosporium</i>	94

Abbreviations: NA, not available.

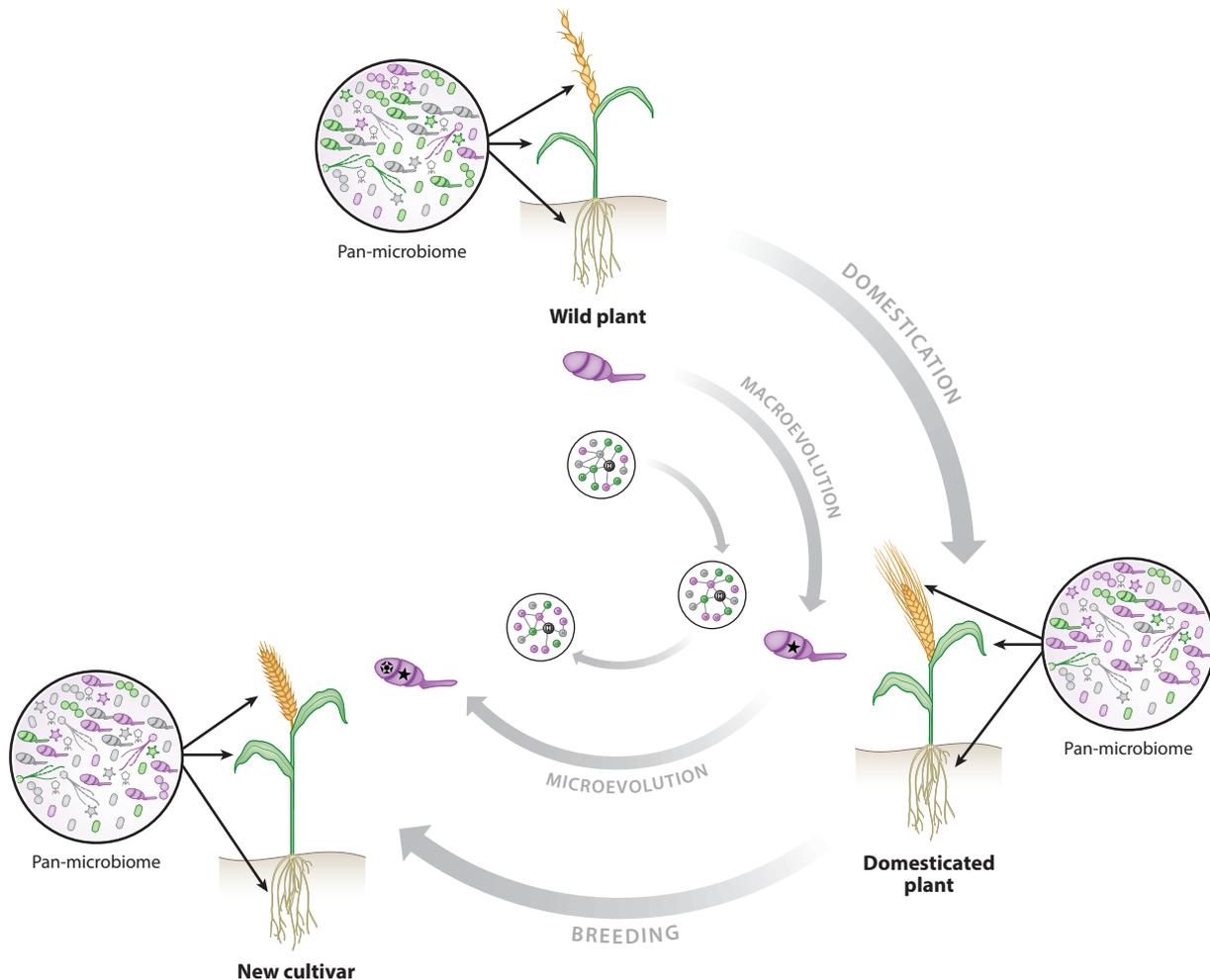


Figure 2

Plants are colonized by diverse microbial communities in their below- and aboveground tissues. The union between these microbial communities constitutes the pan-microbiome. Domestication of crop plants has altered their morphology and physiology and also the structure and function of their associated microbiota. Domestication and breeding have led to increased crop yield and the use of pathogen-resistant cultivars. These processes are concomitant with pathogen macro- and microevolution, leading to more virulent strains and evolution of their effector gene repertoires. We propose that these alterations of the plant host and its associated microbiota also alter plant-microbe and microbe-microbe interactions.

diversity of microbial OTUs in wild beet, *Beta vulgaris* subsp. *maritima*, compared to domesticated sugar beet, *Beta vulgaris* subsp. *vulgaris* (127). It is, however, still unclear why microbial diversity of individual plants is reduced in these domesticated species.

Functional studies of beneficial microbial symbionts of domesticated and wild plants have primarily focused on AM fungi and nitrogen-fixing bacteria in the genus *Rhizobium*. In an extensive experimental setup, a comprehensive analysis of mycorrhizal responsiveness across 27 crop species and their wild progenitors was conducted (78). Crops responded differently to AM symbiosis in P-rich and P-poor soils: Wild plants irrespective of P level benefited highly from AM symbiosis, whereas crop plants showed strong engagement with AM symbiosis in P-limited soils only.

Likewise, domestication of breadfruit was shown to have impacted mycorrhizal communities (124). Domesticated breadfruit forms mycorrhizal associations with more distinct species of AM fungi; however, the overall colonization of AM fungi is considerably lower compared to colonization of wild breadfruit roots. This more generalist symbiosis trait may reflect the movement of breadfruit across different soil types and climate conditions during domestication, selecting for the ability to form mycorrhizal associations with more diverse fungal communities. Together, these findings indicate a lower efficiency of symbiotic relationships of crop plants compared to their wild relatives. It is possible that selection for high-yielding crops has limited the reallocation of carbohydrates to AM fungi, thereby reducing the extent of symbiosis (78).

The selection of beneficial nitrogen-fixing *Rhizobium* bacteria was furthermore shown to be more relaxed in domesticated leguminosa species compared to wild species. Consequently, domesticated species are more prone to form symbiosis with less effective rhizobia strains. But domestication may, in other species, have selected for more efficient symbiotic interactions (84). Inoculated with the same *Rhizobium*, cultivated soybeans surpassed wild soybeans in the nodule symbiotic performance parameters, including total tissue weight, total nodule number, total nitrogen, and total ureides accumulation. Higher nitrogen-fixation capacity is an important trait to promote the size of plants and their seeds, and domestication-mediated selection may indirectly have selected for improved symbiosis capacity in soybean.

To understand the impact of plant domestication on microbial assembly and function, we need clear definitions of plant metaorganisms, including core microbial members and keystone species. The examples summarized above suggest that there is no consistent pattern of domestication effects on microbial community assemblies of plants. Microbial communities of crop plants may to a large extent be defined by anthropogenic interference (i.e., humans instead of symbionts providing necessities to the plant), domestication syndromes (i.e., inadvertent selection of nondesirable plant traits alongside beneficial plant traits such as changes in root architecture), or environmental factors that emerged alongside agricultural activities (i.e., reduced diversity of soil biotic and abiotic variables). However, in some species, domestication may also have implied selection for improved microbial community assembly or symbiotic interactions, as observed in soybean.

THEORETICAL MODELS ON THE ASSEMBLY OF THE PLANT MICROBIOTA

Microbial communities are assembled *de novo* in each plant and change in composition according to plant age and interactions of the plant with its environment. Much of the research on plant-microbial communities has aimed to identify genetic or environmental factors that determine microbial community compositions. As outlined above, the assembly of microbial communities is affected by multiple factors (2, 117); however, there is also a component of stochasticity in the microbial community assembly that is less well understood.

Two categories of processes in microbial community assembly can be considered: (*a*) selective (deterministic) processes, in which different microorganisms occur in distinct environments because of differences in their relative ecological fitnesses, and (*b*) neutral processes, in which the occurrence of different microorganisms in a distinct environment is independent of their fitness (99). Neutral and deterministic processes have been extensively addressed in community studies of macroorganisms, e.g., plants and corals (36, 96). Only recently, with the development of sequencing-based techniques for microbial community profiling, have neutral processes also been considered in microbiome studies, e.g., in the human-associated microbiome (56, 119).

In a seminal plant experimental study, Maignien and coworkers observed predictable dynamics of taxa presence/absence in microbial communities of replicates of the *A. thaliana* phyllosphere

(76). During early developmental stages, microbial communities reflected airborne communities, whereas at later stages all phyllosphere replicates converged to similar communities over time. The reproducible and persistent microbial composition suggested strong selection imposed on the phyllosphere communities. Nevertheless, the abundance of microbes showed a high variation correlated with spatial variation in the experimental setup, suggesting that, in addition to selection, neutral processes, in particular involving microbial dispersal, played a role in the *A. thaliana* microbial communities.

Besides stochastic patterns of microbial dispersal, assembly history, i.e., the temporal sequence of community assembly, also plays a role in community assembly (31, 43, 61). The effects caused by the assembly history of a community are also referred to as priority effects, whereby early microbial colonizers might affect the establishment and growth of late colonizers. The identity of the early colonizers might be stochastic or dependent on specific functional traits; however, their presence may define the ability of other microbes to establish in the environment. In this case, even a small change in the order of arrival of species could cause highly idiosyncratic community composition under the same environmental conditions. The importance of priority effects in microbial communities has been demonstrated notably for wood-inhabiting fungi for which variation in community composition was defined by the order of species joining the community (31). Priority effects have also been demonstrated experimentally in the sticky monkey flower *Mimulus aurantiacus*, in which the final microbiota community composition in floral nectar was defined by the order in which microbial species were introduced in synthetic community experiments (113).

Finally, the competitive lottery model has been proposed to explain the unexpected lack of similarity in community composition, e.g., in bacterial communities associated with the marine macroalgae *Ulva australis* (20). The competitive lottery model argues that habitats are colonized by guilds of functionally equivalent species but randomly by the taxa that happen to arrive first. In this respect, the model considers the redundancy of microbial functions and embraces both neutral and deterministic processes. Here, we underline the need for considering neutral processes in microbial community studies and in the interpretation of community variation. Neutral processes merit attention not only because they are an unexplained part of the variation but also because they might be exploited to be ecologically informative. Accounting for neutral processes will help scientists to distinguish randomly occurring and consistently present microorganisms and may support the definition of core microbiota and metaorganism members.

CONCLUSIONS

Research on plant–microbe interactions and coadaptation has primarily focused on pathogenic microorganisms and mutualistic symbionts such as AM fungi and *Rhizobium* bacteria. These studies document the pivotal role of the plant immune system in both the defeat of pathogens and the establishment of beneficial symbiosis. New research has, however, extended this view of the plant immune system and suggests a broader role of plant immune receptors and signaling components in the overall assembly of microbial communities (49). Given the large diversity of plant-associated microorganisms, this implies a complex network of immune signaling to simultaneously promote and suppress, respectively, beneficial and harmful organisms. More molecular and experimental research is required to understand the complex interaction of diverse microbial communities with the plant immune system.

Evolution of the plant immune system is shaped by interactions with microorganisms. Among the most rapidly evolving plant genes are genes encoding nucleotide binding–leucine-rich-repeat (NB-LRR) proteins (R proteins) that diversify in response to the diversification of plant-pathogen effectors (12). Likewise, genes encoding effector proteins are among the most rapidly evolving

pathogenicity genes. These genes are often encoded in specific genome compartments enriched with transposable elements allowing for their rapid diversification (81). A survey of several hundred bacterial genomes indicated that the plant immune system also shapes genome evolution of commensal microorganisms (70). This genome-based study identified a large number of genes with domains predicted to interact with plant receptors. The extent to which these interactions also drive bacterial diversification and coevolution is, however, unclear. Integrating population genetics approaches and population genomics data will in the future allow us to better assess microbial adaptation in plant metaorganisms.

Domestication, plant breeding, and agricultural practices have likely impacted the evolutionary dynamics of plant–microbe interactions. In modern agriculture, local adaptation of plants to co-existing microbial communities is hindered by intensive breeding. It is furthermore possible that plant breeding has influenced the ability of plants to host the most beneficial microbial communities. Increasing our understanding of microbial coevolution and plant metaorganism function is crucial for the development of new crop-protection strategies based on beneficial microbial interactions.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

Research on plant microbiota in the groups of E.H.S. and T.D. is supported by the DFG Collaborative Research Centre “Function and Origin of Metaorganisms” (SFB1182).

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Errata

An online log of corrections to *Annual Review of Phytopathology* articles may be found at <http://www.annualreviews.org/errata/phyto>

Concluding remarks and perspectives:

This study is, to the best of my knowledge, the most comprehensive study analyzing wheat microbial communities spanning a wide collection of genotypes in details. In this thesis, I used wild and domesticated *Triticum* species as models to study vertical-transmitting (seedborne) and environmentally-acquired (soil-derived) microbiota of plants. I analyzed bacterial and fungal communities associated with seeds and seedlings of different wheat species. Also, I studied soil-derived microbial assembly of wild and domesticated wheat seedlings grown in agricultural and natural soil. Plant domestication, breeding and agricultural activities may have altered plant-microbe interactions. Comparative studies of crop and wild progenitor microbiomes are crucial to gain insights into the domestication-related changes in crop microbiomes and to harness microbiomes for sustainability in agriculture.

In chapter 1, I solved the problem of amplifying microbial DNA from individual seed and axenic seedling samples; which are low in microbial diversity and biomass. The protocols I optimized here allowed me to improve the resolution and accuracy of microbial diversity analyses. Usage of the V5-V7 region of the 16S rRNA locus in combination with interfering primers designed in this study prevented host DNA amplification and enriched bacterial amplification. As a result, the quality of the amplicon dataset significantly increased. Without using interfering primers, all sequencing reads ended up being host-derived. In contrast, when using this primer combination in my libraries, 98.6% of the reads were classified as bacteria in the dataset in chapter 2. Furthermore, I confirmed that there was no microbial contamination in DNA isolates and PCR products by systematically using negative controls in each step, and sequencing the negative controls alongside samples. I

also optimized homogenization and DNA extraction from seeds and seedlings so that working with individual plant samples was possible. As a result of all these optimizations, I was able to analyze the stochasticity of microbial compositions among replicates of individual plants in chapter 2 and to analyze and compare variation in microbial communities in seeds originating from the same or different plants in chapter 3.

My findings in chapter 2 provide novel insights into the impact of domestication on plant-microbe interactions. First, domestication may have conferred a strong role of stochastic processes in the assembly of vertical transmitting microbes. Second, domestication has not affected the ability of the plant to assemble soil-derived microbial diversity in leaves and roots. Third, bacterial diversity and consistency was different, but fungal community composition was comparable, between wild and domesticated wheat. This suggests that different eco-evolutionary dynamics govern fungal and bacterial communities. However, there is limited literature studying the possible persistence of the seedborne microbes in the plant leaves and roots after colonization by the environmentally-acquired microbiota. Finally, I show that the two bacterial families Halomonadaceae and Vibrionaceae very likely originated from seeds and persisted in leaves after host colonization of the soil-derived microbes. However, tracking microbes over generations at the strain level will provide further knowledge about the persistence of seedborne microbes over generations. Overall, these four points of my study provided fundamental contribution towards better understanding of wheat microbiota and impact of wheat domestication on microbial assemblies.

An intriguing question is how domestication led to an increased effect of stochasticity in microbiota assembly. Based on the aforementioned findings, I speculate that alterations in the plant immune system during domestication, including strong selection and polyploidization events, may have conferred changes in the way plants associate with coexisting microbiota. Alterations in the genetic repertoire of immunity may have relaxed selection on the genes regulating plant-microbe interactions in crops, leading to more stochastic and low-diversity bacterial communities.

In chapter 3, I aimed to reveal factors that explain a higher microbial variation in domesticated wheat, as explored in chapter 2. For this, I disassembled the seed microbiome based on temporal factors (i.e. plant age), spatial factors (i.e. location in the field), and plant origin (i.e. originating from the same or different plants). The novel finding of this study is that intra- and inter-plant variation was comparable in seedborne microbial communities. This comparison has never been done before and was possible thanks to the techniques that I developed in chapters 1 and 2.

In chapter 3, I compared seedborne microbial diversity and composition between wild and domesticated wheat species over time. In the domesticated wheat *T. aestivum*, seedborne bacterial diversity gradually decreased over time in the leaves. In contrast, seedborne bacterial diversity increased in the wild species *T. dicoccoides*. Furthermore, microbial composition of the domesticated wheat *T. aestivum* was stable during seedling development. In other words, diversity was dominated by the same bacterial family over time. However, communities of the wild wheat *T. dicoccoides* changed during development.

Which traits make vertically-transmitting microbes different than other microbes and what is their functional relevance to plants? For future research, I would study the population genetics of the vertically-transmitted microbes. I would examine functions of positively-selected genes in the vertically-transmitted microbes and compare them to homologous genes among environmentally-acquired microbes. This would help me to discover traits that are essential for vertical transmission in microbes and to predict the functional relevance of these microbial traits. Furthermore, composing synthetic microbial communities and performing recolonization experiments would further validate the functional relevance of these microbes. Transplanting seedborne microbes between wild wheat and wheat cultivars and measuring the impact of the microbes to the plant fitness in different plant backgrounds may allow to optimize seed microbial communities for the optimized plant health.

Author contributions

1st Chapter:

This chapter was conceived and designed by Ezgi Özkurt (EÖ) and Eva H. Stukenbrock (EHS). The optimizations were performed by EÖ. Data analysis was conducted by EÖ. The manuscript was written by EÖ and EHS.

2nd Chapter:

This chapter will be submitted to the mBio journal and available as a preprint in BioRxiv (**doi:** <https://doi.org/10.1101/685164>). The study was conceived and designed by EÖ, EHS and Tal Dagan (TD). Seed collections and soil from Turkey were provided by Hakan Özkan (HÖ) and Uğur Sesiz. The experiments were performed by EÖ and Mohamed Amine Hassani. Amplicon sequencing was performed by Sven Künzel. Data analysis was conducted by EÖ and TD. The manuscript was written by EÖ and EHS.

3rd Chapter:

This chapter will be submitted to the Environmental Microbiology. The study was conceived and designed by EÖ and EHS. Domesticated seeds were collected by EÖ. Wild seed collections from Turkey were collected and provided by HÖ. The experiments were performed by EÖ and Zaywa Mariush. Data analysis was conducted by EÖ. The manuscript was written by EÖ and EHS.

I also contributed to a review paper as a second author. I wrote three sections and compiled a table and edited the manuscript:

Interactions and Coadaptation in Plant Metaorganisms

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Annual Review of Phytopathology 2019 57:1, 483-503

Acknowledgements:

I am very grateful to my supervisor Eva Stukenbrock for giving me the opportunity to work with her. I am thankful to her for giving me the flexibility, support and guidance during my PhD. I learnt a lot during my scientific voyage with her. Thank you for being resistant to me who is a fierce debater of ideas.

I thank my TAC committee members Tal Dagan and Julien Dutheil, the joint microbiota meeting people, Ryszard Soluch, Nils Huelter and Amine Hassani for fruitful discussions helped me to develop ideas.

I am grateful to all current and past members of the Environmental Genomics group for sharing their great knowledge on all aspects of fungi with me. Special thanks to Janine Haueisen for translating the summary into German.

I should also acknowledge Ozan, Primrose, Malavi and Gillian for the proofreading of this thesis, wiping up Turkish language inspirations in my texts.

I should particularly acknowledge Gündüz and Kök families, Jet Hacer and Hakan Özkan for their immense effort to provide me wheat seeds from Turkey.

I thank to all friends who made my life in Plön much better and memorable. Ana Isabel, the words are not enough but just a summary to describe you. Thank you for helping me to leave drama out of life by being my friend. Do not forget, you are a hope for the kindness and goodness in society. Fede, I am writing these words from your house in your pijamas. You are not only an amazing scientist but also an equally good friend. I am sure your existence will warm my heart even from Paris to Norwich. Your obstinate

friend is always here to laugh and cry together. Karen, my first Bolivian friend, thank you being such a nice person. We talked for hours on various subjects and I enjoyed in each bit of these conversations. I also owe some laughters to Ana Teles and Malavi. Ana, your energy has no end, enough for everybody. Malavi, I should admit that some of your jokes are funny. So many things to say, but in summary: Love you bacı! And I will always miss you so much.. See you next year in the EEBST. Zahra, I want to acknowledge you both for you being a human being with values and for cooking amazing helwa for me. Gillian, thanks for being Gillian! Maintain yourself, you are cool in a quirky sort of way. Tania and Jennifer, thanks a lot for the occasional Friday coffee sessions. And Tania you are a premium cook and person. (Ultra)Wiolat, I should really appreciate your immense effort to finish the rice pudding. Vandana, for being cheerful and positive all the times. Gönensin Ozan, thanks a lot for sharing your friendship and furniture with me.

I want to thank to all people of my office for adopting me to the bird environment. I do not forget for my second officemates, Ana Teles and Fede for hosting me very kindly during the writing of this thesis. The Cat Empire group (Filipa, Karen, Tania, Ana Teles, Carolina, Maria), these were memorable times in Bremen, many thanks!

My friends were still with me with their all support when I a moving out Plön: Artemis, Dimitris, Karen, Tania and Maria, thank you so much for your immense help. How I would be able to move without you?

Başak, Ezgi and Gizem, my all the time supportive friends. We shared relatively short but lasting times in my first year in Plön. Başak, so many things to tell. In brief, thanks a lot for entertaining me by telling stories to relax me and cooking amazing food for

me. To Jatin, we shared many things together in the first days of dark and cold Plön, thanks cherry. I also want to acknowledge the Turkish community here: Onur, Gökçe and Çağdaş, thank you for making me feel at home. Dimitris you also made me feel at home somehow. Onur Erk, thanks for the postcards in various languages.

How can I forget about my previous labmates "Somel Birlik" and my scientific father Mehmet. Thanks for making me feel that you are always here with me. Thanks to Dilek Koptekin for listening me and my jokes. To Ezgi Altınışık for being an amazing namesake, friend, colleague and comrade.

To Cansu and Vedat, although being far from each other, your existence warms my heart in any part of the world. Cheers for the oldie goldie crazy days!

I thank my family (my parents Melek and Hüseyin, my siblings Tolga and Zeynep and my littles Derin and Deniz). Mum, thanks for listening about my thesis with great interest and supporting me all the time and more importantly thanks for being my mum. Canım annem!

I specially thank you dear Emrah. I have so many things to tell you but I have nowhere to begin. Thank you for being my love, my close friend, my family, my company and everything. Thank you for waiting me for so long... Canımın yongası!

Lastly, I would like to acknowledge SciHub for sharing scientific knowledge with all the world.

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Declaration

Hereby I declare that:

i. apart from my supervisor's guidance, the content and design of this dissertation is the product of my own work. The coauthors' contributions to specific chapters are listed in the Author Contributions section of this thesis;

ii. this thesis has not already been submitted either partially or wholly as part of a doctoral degree to another examination body;

iii. the preparation of the thesis has been subjected to the Rules of Good Scientific Practice of the German Research Foundation;

iv. I have not had any academic degree withdrawn.

Plön, November 27, 2019

Ezgi Özkurt