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Heritable Genetic Differences Affect Sunflower Rhizosphere Archaeal, Bacterial, and Fungal Communities Across All Taxonomic Levels

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Abstract

Plant genetics can exert a strong effect on soil rhizosphere microbes. Broad-sense heritability estimates quantify the degree to which plant genotype predicts microbiome composition, enabling characterization of plant genotype effects on rhizosphere microbes. We used 16S rRNA and internal transcribed spacer marker gene sequencing data from rhizosphere soil from 95 field-grown sunflower (*Helianthus annuus*) inbred lines to test the heritability of archaeal, bacterial, and fungal rhizosphere communities, as well as alpha- and beta-diversity metrics and differences in the relative abundance of microbial zero-radius operational taxonomic units (ZOTUs). The majority of taxa passing prevalence and relative abundance cutoffs of 25 and 0.01% were heritable across all taxonomic levels for both prokaryotes (archaea and bacteria) and fungi, with higher heritabilities at finer taxonomic resolution. Metrics of alpha and beta diversity were also heritable, particularly for prokaryotes. Prokaryotic and fungal

communities were both heritable, but there was significantly higher fungal heritability at the ZOTU, genus, and family levels. Prokaryotic richness and fungal richness were only weakly positively correlated, and beta diversity was not correlated, suggesting that sunflower inbred lines affect prokaryotes and fungi in different ways. Our work provides valuable information for crop breeding and provides a general methodological framework for assessing the strength and types of genotype–microbial associations. Because of high heritability for individual microbial taxa, as well as for microbial diversity metrics more broadly, sunflowers are clearly an important species to study the ecology, evolution, and genetics of plant–microbe interactions, and this knowledge can be used to improve sustainability of the crop.

Keywords: assembly, diversity, genotype, heritability, rhizosphere, sunflower

Plant-associated microorganisms play a number of important roles, such as nutrient acquisition, disease resistance, and stress tolerance (Compant et al. 2019; Trivedi et al. 2020). Microbes in

the rhizosphere, the soil directly around and influenced by plant roots, can fix and cycle nitrogen, solubilize phosphorus, affect soil structure, and produce plant-growth-promoting hormones such as indole-3-acetic acid and auxins (Rawat et al. 2021). As such, the soil rhizosphere microbiome, including diverse taxa from all three domains of life, holds great potential for improving agricultural productivity and sustainability in a changing climate. Soil- and plant-associated microbiomes can be manipulated in a number of different ways, such as coating seeds with a microbial slurry prior to planting (O’Callaghan 2016; Rocha et al. 2019), foliar application (Preininger et al. 2018), inoculating soils with key taxa (Kaminsky et al. 2019; O’Callaghan et al. 2022), or breeding plants to select for certain microbial taxa or microbial traits (Clouse and Wagner 2021; Hohmann and Messmer 2017; Nerva et al. 2022). Of these four methods, the latter could potentially be the most effective because it avoids issues with microbial application and establishment, but it has also been the least studied to date. To successfully breed plant cultivars to select for beneficial microbiomes, a more comprehensive and detailed understanding of microbial community inheritance is needed.

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Data availability: ZOTU sequences (16S rRNA gene and ITS) were deposited to NCBI GenBank under BioProject ID PRJNA1200133. Downstream data tables (metadata, ZOTU abundances) and analysis code are publicly available on Zenodo (<https://doi.org/10.5281/zenodo.15412024>).

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The degree to which plants actively control and select their rhizosphere and endosphere microbiomes is still largely unknown, as is the balance of microbial control versus plant control in plant–microbe interactions (Sánchez-Cañizares et al. 2017). Broad differences in rhizosphere microbial communities among plant species and functional groups have been observed (Berg and Smalla 2009; Leff et al. 2018; Matthews et al. 2019), but this can also be attributed to passive effects, as microbes respond to different carbon inputs from litter and exudates. Although it is not surprising that different species of plants would be associated with different microbial communities, it is unclear to what degree different genotypes of the same plant species could select for different rhizosphere microbial communities. Such knowledge is important for agriculture, in which different crop cultivars are commonly used and new cultivars are constantly being developed, tested, and optimized.

When discussing domesticated crop plants, we must first recognize that these plants have been altered significantly over millennia from their wild counterparts and have distinct microbiomes compared with their wild counterparts (Leff et al. 2017; Nerva et al. 2022; Yue et al. 2023). An early finding suggesting the importance of genetics in shaping plant microbiomes was variation in nitrogen fixation by legumes, a key function of microbe–legume interaction in agriculture (Rengel 2002). More recently, next-generation sequencing has built on that work by studying the effects of different crop cultivars on soil rhizosphere communities in maize (Aira et al. 2010; Peiffer et al. 2013), rice (Shenton et al. 2016; Xiong et al. 2021), soybean (Liu et al. 2019; Xu et al. 2009), potato (Brolsma et al. 2017), cotton (Yang et al. 2022), sorghum (Deng et al. 2021), and sunflower (Leff et al. 2017; Pogoda et al. 2024), with most studies demonstrating a strong genotype effect on the rhizosphere. Plant genotype and associated phenotype affect microbial communities, as evidenced by differences across different anatomical areas of the plant, as well as plant development over time (Wagner 2021). Breeding efforts have in turn been directed at manipulating root systems and exudates (Wissuwa et al. 2009). Recent work has sought to identify the specific regions of the plant genome that drive differences in microbial communities. Such studies have been done in the model plant *Arabidopsis thaliana* (Bergelson et al. 2019; Horton et al. 2014) and the crops maize (Meier et al. 2022; Wallace et al. 2018), rice (Roman-Reyna et al. 2019), sorghum (Deng et al. 2021; Sutherland et al. 2022), and sunflower (Pogoda et al. 2024). All of these studies have found associations between certain regions of the plant genome and certain features of the microbial community.

The cultivated sunflower (*Helianthus annuus*) is one of the largest oilseed crops globally (Adeleke and Babalola 2020) and is also valued for human-consumed seeds, birdseed, and microgreens. Sunflowers are amenable to studies on genotype effects due to the wide variety of highly inbred cultivars and high-quality genetic information that exists for many cultivars (Mandel et al. 2014; Pogoda et al. 2021). Additionally, sunflowers are already tolerant of abiotic stresses and may be increasingly cultivated in regions that are affected by drought, salinity, and heat, such as much of the western United States. Lastly, sunflowers offer an interesting study system relative to other crops due to the degree of active and successful introgression of wild-type genes into crop cultivars (Baute et al. 2015; Breton et al. 2010; Zhang et al. 2017).

Our previous work on the sunflower rhizosphere described the genetic underpinnings of heritable bacteria clustered at a 97% genetic similarity threshold (i.e., of the operational taxonomic units, or OTUs, in the rhizosphere) but did not examine fungi or higher taxonomic levels (Pogoda et al. 2024), whereas a broader survey examined genotype-by-environment interactions at the amplicon sequence variant level across the U.S. Great Plains (Bueno de Mesquita et al. 2024). Here, we build on this work by examining

the effects of plant genotype on alpha- and beta-diversity metrics for both prokaryotic (archaea and bacteria) and fungal rhizosphere communities, assessing the broad-sense heritability (the proportion of variance in microbial relative abundances that is due to all genetic effects) of the most prevalent and relatively abundant prokaryotic and fungal taxa at different taxonomic levels, and assessing the degree of deterministic versus stochastic processes in structuring the sunflower rhizosphere microbiome. Determinism in this context means selection driven by plant and environmental factors, whereas stochasticity means that a random subset of microbes from the bulk soil is found in the rhizosphere soil. We hypothesized that (i) microbes at finer-resolution taxonomy (e.g., genus) would be more heritable than at broader-level taxonomy (e.g., phylum), (ii) fungal communities would be more heritable than archaeal and bacterial communities across all taxonomic levels (Leff et al. 2017), and (iii) sunflower rhizosphere microbial communities would be structured primarily by deterministic rather than stochastic processes.

Materials and Methods

Sample collection

Rhizosphere samples were collected from 95 genotypes of sunflower from fields near Carrington, North Dakota, U.S.A., on 10 August 2017 (Table 1). The genotypes are highly inbred, and we consider individuals of the same genotype to be genetically identical. Plots of each genotype consisted of single rows, 6.1 m in length and spaced 0.76 m apart, with a final population of about 20 plants per plot (Pogoda et al. 2024). The sunflowers were all in the same phenophase, just before flowering, at the time of sample collection. Four samples of each genotype (two from each of two randomized, complete block replications) were collected ($n = 380$). Samples were collected by digging up the root ball of an individual plant (approximately $30 \times 30 \times 30$ cm), removing loosely adhering bulk soil, and then vigorously shaking off soil that was directly attached to roots into a 2-mm sieve to remove rocks, roots, and previous crop debris. Sieved soil was then collected in a sterile 50-ml Falcon tube. An aliquot of the rhizosphere soil was stored at -20°C for eventual DNA extraction and sequencing. Chlorophyll content for each sampled plant was measured just prior to rhizosphere sampling with a SPAD meter (Apogee Instruments MC-100, Logan, UT, U.S.A.) and converted to micromoles of chlorophyll per square meter.

DNA extraction, sequencing, and processing

DNA was extracted from 0.250 g of rhizosphere soil with a Qiagen PowerSoil high-throughput 96 kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The V4 region of the 16S rRNA gene was amplified with PCR primers 515F/926R, and the internal transcribed spacer (ITS) region was amplified with PCR primers ITS1f/ITS2, following the Earth Microbiome Project protocols (Thompson et al. 2017). The band size of the amplicons was checked with gel electrophoresis. PCR products were then cleaned and normalized with the SequelPrep normalization kit (Life Technologies Corporation, Carlsbad, CA, U.S.A.). Libraries were then barcoded with 12-base Golay barcodes and pooled for sequencing on an Illumina MiSeq at the BioFrontiers Institute (Boulder, CO, U.S.A.), with 2×250 base pair chemistry.

Raw sequence data were demultiplexed with an in-house Python script (demultiplex_seqs.py, https://github.com/leffj/Jons_bioinfo_code), and adapters were removed with cutadapt (Martin 2011). Reads were then processed with the USEARCH v.10 pipeline to trim and quality-filter reads, merge paired-end sequences, denoise sequences into zero-radius OTUs (ZOTUs) with UNOISE2 (Edgar 2016), and remove chimeras. Taxonomy was assigned

with the ‘assignTaxonomy’ function in the DADA2 R package (Callahan et al. 2016) using the SILVA v.138.1 database for 16S rRNA gene (Quast et al. 2013) and UNITE v.10.0 database for ITS (Abarenkov et al. 2024). Reads assigned to chloroplast, mitochondria, Eukaryota, or unassigned at the domain level were removed from the 16S rRNA gene dataset with the *mctoolsr* R package (Leff 2022). Reads that were not assigned to Fungi at the kingdom level were removed from the ITS dataset. In both datasets, ZOTUs with only one or two sequences across all the samples were removed. The resulting table had a mean of $13,767 \pm 6,730$ standard deviation (SD) reads per sample for 16S rRNA gene and $18,042 \pm 7,084$ SD reads per sample for ITS. ZOTU tables were rarefied (Schloss 2024) to 5,099 reads per sample for 16S rRNA gene (dropping 10 samples) and 4,820 reads per sample for ITS (dropping 1 sample). These numbers were selected to encompass the diversity of the taxa in the samples and not drop too many samples. Following rarefaction, ZOTUs present in only one sample were removed, as, by definition, they would only be present in one genotype. Although some samples were dropped during rarefaction, there were at least three samples for all 95 sunflower genotypes. To standardize comparisons of prokaryotic and fungal communities, only samples present in both datasets after rarefaction were retained ($n = 368$). A Bray–Curtis dissimilarity matrix was calculated on the rarefied 16S rRNA gene and ITS tables and was visualized with principal coordinates analysis.

Statistical analyses

The effects of genotype on calculated alpha-diversity metrics (ZOTU richness and Shannon diversity) and calculated beta-diversity metrics (PCo1 and PCo2, extracted from the principal coordinates analysis), were assessed with a type II analysis of variance (ANOVA) implemented in the *car* R package (Fox and Weisberg 2018), with replicate and genotype as fixed effects. The effects of genotype on taxa from the ZOTU through phyla levels were tested with negative binomial generalized linear models. The broad-sense heritability value for each taxon or diversity metric was the eta-squared effect size of the genotype effect, calculated from the sum of squares from the ANOVA models. More precisely, eta-squared is the sum of squares of the genotype factor divided by the total sum of squares of the model. This corresponds to “broad-sense” heritability rather than narrow-sense heritability, because the plants used were a population of unrelated, highly inbred lines (Wagner 2021). Broad-sense heritability is the proportion of phenotypic variance that is due to all genetic effects (Holland et al. 2002). In other words, this heritability metric represents how much of the variation in each microbial taxon relative abundance is affected by sunflower genetic variation (Morris and Bohannan 2024; van Opstal and Bordenstein 2015; Wagner 2021). Only taxa that were present in >25% of samples and had >0.01% mean relative abundance were tested. This filtering cutoff was performed separately at each taxonomic level. To test for spurious results, null modeling was used to establish expectations based simply on the sample size and number of levels in the grouping factor (i.e., 95) in the absence of biological

effects. Samples were randomly assigned to 95 groups, maintaining the structure of the experimental design (i.e., two samples from each of two randomized, complete block replications). The effect of the grouping variable was tested with type II ANOVA as above (with replicate and “group” as fixed effects), the eta-squared was calculated, and the procedure was repeated 100 times. In other words, in addition to testing the effect of genotype (a factor with 95 levels), we tested the effect of randomized data that is not expected to show significance, as a baseline to determine the effect of random error.

The effect of genotype on community composition (Bray–Curtis dissimilarity) was assessed with permutational multivariate ANOVA implemented with the ‘adonis2’ function in the *vegan* R package (Oksanen et al. 2025; Wagner 2021). Multivariate homogeneity of dispersions was assessed with PERMDISP implemented with the ‘betadisper’ function in *vegan*. Continuous variables (chlorophyll content, ZOTU richness, *Sclerotinia sclerotiorum* pathogen incidence of each genotype in adjacent inoculated plots) were fit to ordinations with the ‘envfit’ function in *vegan*. Note that none of the individuals sampled here was infected with *Sclerotinia*, but we use results from a previously published inoculation experiment at the same site to provide a metric of the variation in resistance to *Sclerotinia* across these 95 genotypes, in which *Sclerotinia* incidence was assessed via a visual disease rating (Pogoda et al. 2024). *Sclerotinia* is a major pathogen of sunflowers and other crops, and *Sclerotinia* resistance is an important plant trait that varies among the 95 sunflower genotypes in this study. Taxa drivers of the ordination were tested with ‘wascores’ in *vegan*.

To assess the relative importance of determinism versus stochasticity in the rhizosphere prokaryotic community, we calculated the nearest taxon index (NTI), based on the phylogenetic distance between each taxon within a sample and its closest relative also found in the sample. This index was then compared with a null expected distribution to determine whether taxa in the dataset were more or less closely related than expected, which would indicate deterministic community assembly (Stegen et al. 2012). First, a phylogenetic tree of the ZOTU sequences was constructed using MUSCLE (Edgar 2004) to align the sequences and then FastTree (Price et al. 2009) to make the tree. Then, NTI was calculated with the *iCAMP* (Ning et al. 2020) R package using the phylogenetic information, as well as the sample by ZOTU relative abundance matrix. ZOTUs that had at least 15 reads across the whole dataset were retained for this analysis ($n = 10,522$). Due to the high degree of variation in the ITS region and difficulties in building accurate phylogenies from ITS sequences, we opted not to perform the NTI analysis on fungal communities. The effect of sunflower genotype on prokaryotic NTI was assessed with ANOVA, and relationships with continuous variables were tested with linear regression. To complement the NTI analysis and further assess assembly mechanisms, we ran Sloan’s neutral model (Sloan et al. 2006) using the ‘ncm’ function in the *pctax* R package (Peng and Jiang 2023). Sloan’s neutral model is a neutral community model that represents a stochastic birth–death immigration process. It is based on the neutral model of Hubbell (2001) but allows for differences in growth rate and accommodates

TABLE 1
Management practices, sample size, climate characteristics (from WorldClim2), and soil characteristics for the Carrington, North Dakota, research site^a

Management		<i>n</i>		Climate				Soil				
Fertilizer	Tillage	16S	ITS	MAT (°C)	Tseas (SD × 100)	MAP (mm)	Pseas (CV)	Clay (%)	OM (%)	pH	NO ₃ [−] (ppm)	P (ppm)
Conventional	Tilled	368	368	4.49	1,277.03	452	64.56	16	3.65	5.7	3.75	16.5

^a MAT, mean annual temperature; Tseas, temperature seasonality; MAP, mean annual precipitation; Pseas, precipitation seasonality; OM, organic matter; P, phosphorus.

large population sizes. The model predicts the relative abundance and frequency of detection based on stochastic processes; taxa that fall above or below the model are suggested to be under positive or negative selection. All figures were made with the *ggplot2* R package (Wickham 2016). All statistical analyses were performed in R version 4.2.3 (R Core Team 2023).

Results

Prokaryotic alpha diversity ranged from 1,676 to 2,463 ZOTUs per sample (mean = 2,100 ± 139 SD), whereas fungal alpha diversity was much lower, ranging from 144 to 284 ZOTUs per sample (mean = 220 ± 21 SD). Prokaryotic ZOTU richness and Shannon diversity were significantly affected by genotype (Fig. 1; Table 2), with mean heritability scores of 0.42 and 0.38, respectively. On the other hand, fungal ZOTU richness was not affected by genotype, and Shannon diversity was only marginally affected by genotype (Fig. 1; Table 2), with mean heritability scores of 0.26 and 0.31, much lower than those for prokaryotes. Prokaryotic and fungal ZOTU richness were significantly but weakly positively associated with each other (linear regression, $R^2 = 0.09$, $P < 0.001$; Supplementary Fig. S1).

Prokaryotic and fungal community composition varied significantly among genotypes (permutational multivariate ANOVA; 16S rRNA gene $F = 52.9$, $R^2 = 0.56$, $P = 0.001$; ITS $F = 69.1$, $R^2 = 0.62$, $P = 0.001$) (Fig. 2). Composition was significantly associated with plant chlorophyll content and ZOTU richness, which were gen-

erally correlated with the second principal coordinate (Fig. 2; envfit $P < 0.05$). Although community dispersion was homogenous in the two randomized blocks (PERMDISP, $P > 0.05$), some genotypes

TABLE 2
Broad-sense heritability (eta-squared for genotype effect) for alpha- and beta-diversity metrics for the 16S rRNA gene and internal transcribed spacer (ITS) datasets^a

Variable	Obs.	Null
16S Richness	0.42***	0.25 ± 0.003
16S Shannon	0.38***	0.24 ± 0.003
16S BC	0.34***	0.26 ± 0.001
16S PCo1	0.53***	0.25 ± 0.003
16S PCo2	0.35***	0.24 ± 0.003
ITS Richness	0.26	0.26 ± 0.003
ITS Shannon	0.31 M	0.25 ± 0.004
ITS BC	0.34***	0.26 ± 0.001
ITS PCo1	0.52***	0.25 ± 0.003
ITS PCo2	0.15*	0.12 ± 0.002

^a *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; M, marginal $P < 0.1$, from analysis of variance or permutational multivariate analysis of variance. Null results are means from 100 bootstraps. BC, Bray–Curtis dissimilarity; Obs., observed eta-squared value.

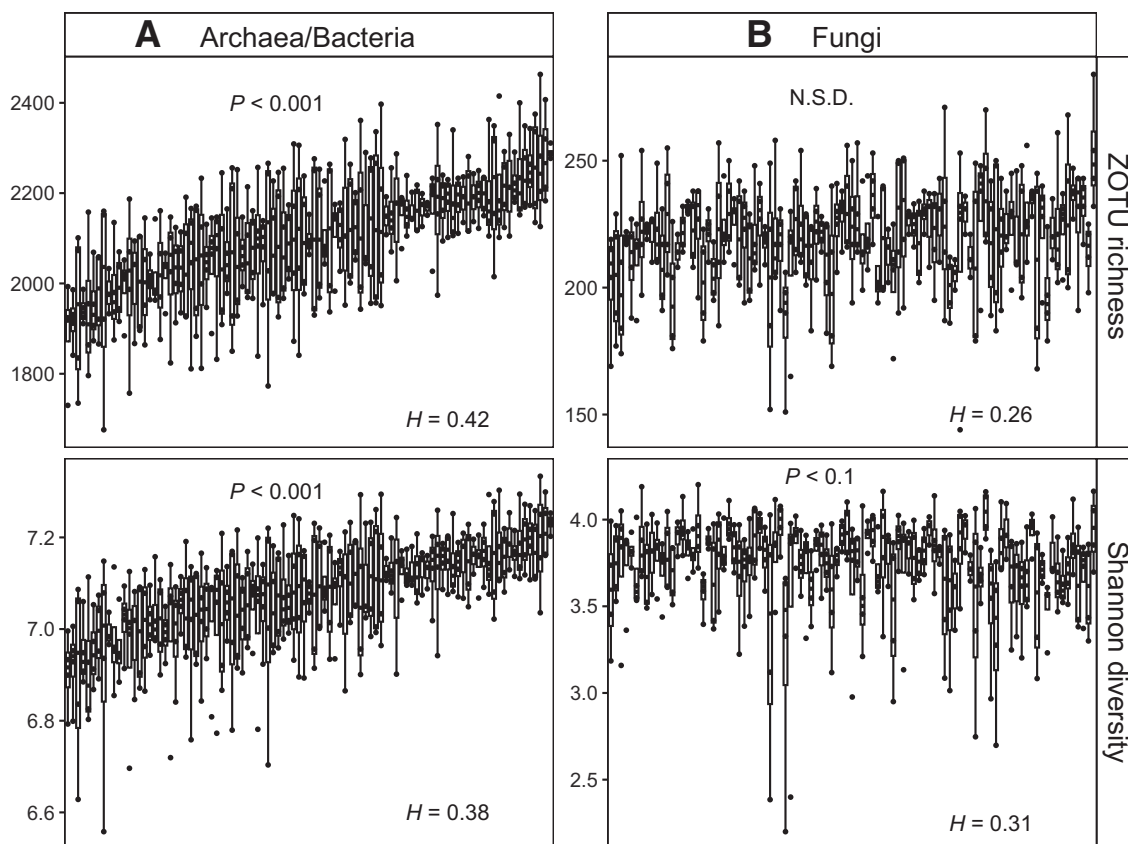


Fig. 1. Alpha diversity (zero-radius operational taxonomic unit [ZOTU] richness and Shannon diversity) of **A**, archaeal and bacterial (16S rRNA gene) and **B**, fungal (internal transcribed spacer [ITS]) communities in rhizosphere soils across 95 sunflower genotypes in Carrington, North Dakota. The broad-sense heritability score H and analysis of variance P values are shown for each panel. The x axis is sorted by overall ZOTU richness (archaea and bacteria plus fungi) and is the same in both panel columns. Note that the y axis scale varies among panels. N.S.D., not significantly different.

had more variable prokaryotic and fungal communities than others (PERMDISP, $P < 0.05$). Prokaryotic and fungal community dissimilarity were not significantly correlated with each other (Mantel test, $r = -0.03$, $P = 0.95$; Supplementary Fig. S2); pairwise genotype comparisons with the most dissimilar prokaryotic communities did not necessarily have the most dissimilar fungal communities, and vice versa. The first two principal coordinates of prokaryotic and fungal community composition were both significantly heritable, especially PCo1, with heritabilities of 0.53 and 0.52, respectively (Table 2). The top prokaryotic phyla across the whole dataset were Proteobacteria (mean relative abundance 24%), Acidobacteria (17%), Actinobacteriota (16%), Verrucomicrobiota (10%), and Bacteroidota (6%) (Supplementary Fig. S3). The top fungal classes were Sordariomycetes (35%), Dothideomycetes (10%), Mortierellomycetes (9%), Agaricomycetes (6%), and Tremellomycetes (4%) (Supplementary Fig. S4).

The heritability of prevalent and relatively abundant taxa from ZOTU to the phylum level ranged from 0.18 to 0.66, with significant effects of genotype on the majority of taxa tested and with means >0.3 in all cases (Fig. 3; Table 3). Although prokaryotic alpha diversity was more heritable than fungal alpha diversity, the heritability of the relative abundances of individual fungal ZOTUs, genera, and families was significantly greater than that of prokaryotic ZOTUs, genera, and families (t test, $P = 0.004$, 0.01, and 0.003, respectively). At the order, class, and phylum levels, there were no significant differences between fungal and prokaryotic heritability (Fig. 3; Table 3). The heritability of relative abundances of taxa that passed the prevalence and relative abundance cutoff for both prokaryotes and fungi, at all taxonomic levels, was significantly

greater than predicted by null models, except for fungal classes and fungal phyla. The difference between observed heritability and null expectations was greater for prokaryotes than fungi, but the percentage of taxa that had significant heritability out of those that were tested was greater for fungi than for prokaryotes. Mean heritability scores were highest at the ZOTU level and declined with decreasing taxonomic resolution (Table 3).

Most ($>50\%$) of the significantly heritable prokaryotic ZOTUs were bacteria from the Proteobacteria, Acidobacteriota, and Actinobacteriota phyla, and the proportion of significantly heritable ZOTUs from these phyla was higher than the proportion of ZOTUs from those phyla in the whole dataset (Fig. 4). Most ($>75\%$) of the significantly heritable fungal ZOTUs were from the Ascomycota, Basidiomycota, and Mortierellomycota phyla, which were also the three phyla with the most ZOTUs in the whole dataset. However, there were relatively more Ascomycota and Mortierellomycota ZOTUs and relatively fewer Basidiomycota ZOTUs that both passed the prevalence and relative abundance cutoff to be tested for heritability and that were significantly heritable (Fig. 4).

The mean NTI value across the whole dataset was 1.42 ± 1.39 SD (Fig. 5). As this value was significantly greater than zero (t test, $P < 0.05$, 95% confidence interval 1.28 to 1.56), the prokaryotic rhizosphere communities were on average phylogenetically clustered (Stegen et al. 2012). NTI values varied significantly by sunflower genotype (ANOVA, $P < 0.05$) but were not significantly related to *Sclerotinia* resistance, chlorophyll content, ZOTU richness, or Shannon diversity (linear regression, $P > 0.05$). Individual communities ranged from highly phylogenetically overdispersed (minimum NTI = -4.19) to highly phylogenetically clustered

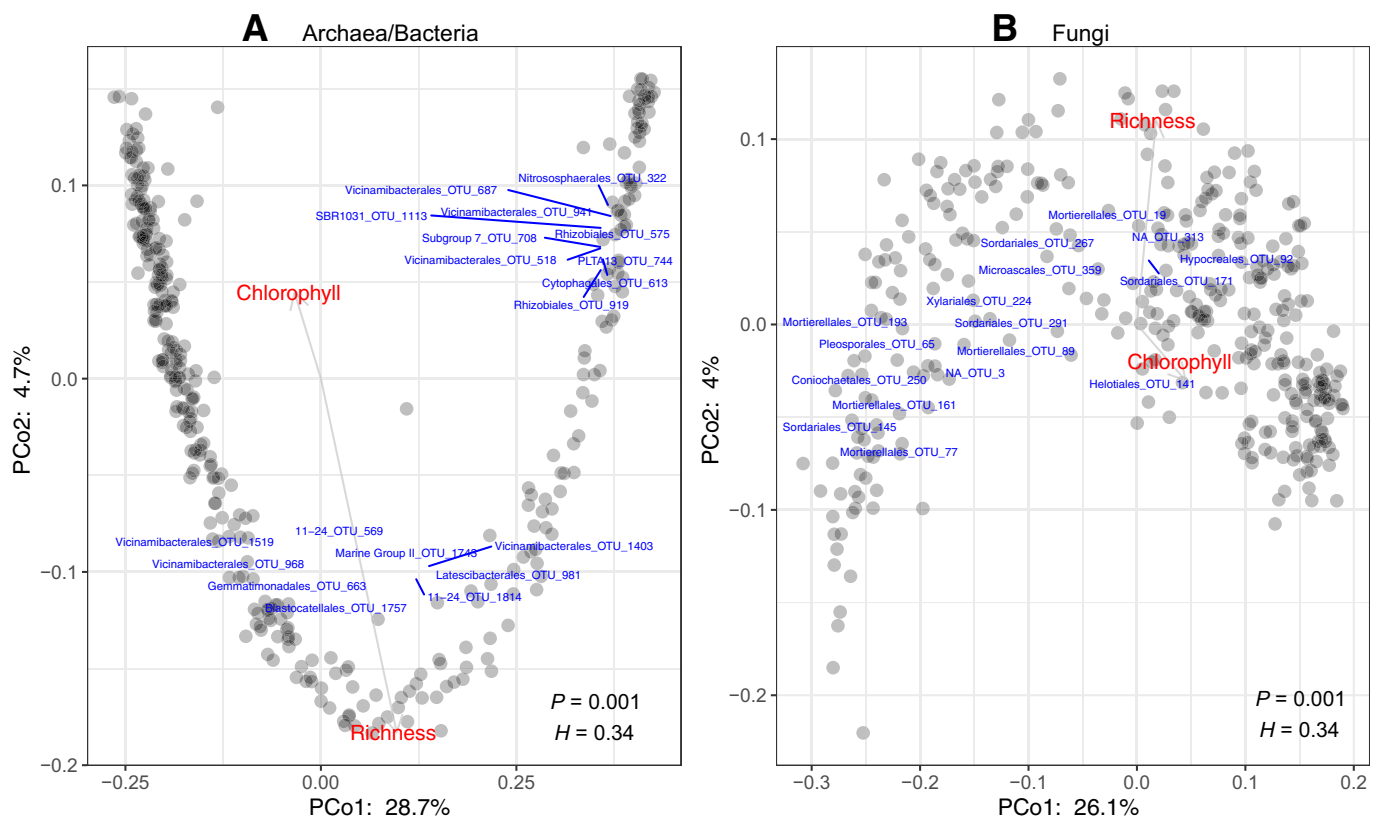


Fig. 2. Principal coordinates analysis (PCoA) of Bray–Curtis dissimilarities for **A**, archaea and bacteria and **B**, fungi. Vectors and red text represent loadings of the sample-level variables chlorophyll content and zero-radius operational taxonomic unit (ZOTU) richness. Blue text shows the loadings of the 10 taxa with the highest PCo1 loading and 10 taxa with the highest PCo2 loadings for both prokaryotes ($n = 19$ taxa shown, 1 taxon was in the top 10 of both axes) and fungi ($n = 16$ taxa shown, 4 taxa were in the top 10 of both axes). Note that the axes scales differ between panels.

(maximum NTI = 5.07) to stochastically assembled with no phylogenetic clustering (NTI approximately 0).

The majority of prokaryotic and fungal ZOTUs (94 and 57%, respectively) were under positive selection relative to Sloan's neutral model (Fig. 6). There were smaller proportions of taxa that fit the neutral model (3 and 26%, respectively) or were under the neutral model, suggesting negative selection (3 and 17%, respectively). The proportion of neutral ZOTUs was greater than the proportion of negatively selected ZOTUs for fungi, whereas it was about the same for prokaryotes (Fig. 6).

Discussion

Here, we used microbial marker gene sequencing from diverse sunflower genotypes to assess the broad-sense heritability of sunflower soil rhizosphere prokaryotic and fungal communities. Alpha and beta diversity varied significantly across the 95 sunflower genotypes, and most of the prevalent and relatively abundant taxa were significantly heritable, especially for fungi. Heritability was greatest at the finest taxonomic level (ZOTU); decreases in heritability at higher taxonomic resolutions could be due to higher taxonomic levels containing multiple distinct functional groups and diverse

ZOTUs with different relative abundance patterns. Communities were on average phylogenetically clustered, but there was substantial variation in NTI values, including communities with no phylogenetic clustering and a few communities that were phylogenetically overdispersed.

At a broader level, plant functional group and species have been shown to have a significant influence on soil microbiomes (Berg and Smalla 2009; Dassen et al. 2017; Garbeva et al. 2008). Finer-level effects of different plant cultivars are less well studied but could be important for agriculture, in which targeted breeding leads to the development of different cultivars of the same crop that have certain benefits. At one site with 95 genotypes and thus a high degree of genetic diversity, it was not surprising to detect the genotype effects observed in our study, even though all samples were from the same plant species. Nonetheless, it was surprising to see just how much variation could be explained by sunflower genotype. This could be partially due to the local site conditions and genetic-by-environment interactions that result in genotype being a relatively more important driver of microbial community composition at some sites compared with others. To put this result in context, this study site (Carrington, ND, U.S.A.) had among the greatest variation in microbial rhizosphere community composition out of 15 sites across the U.S. Great

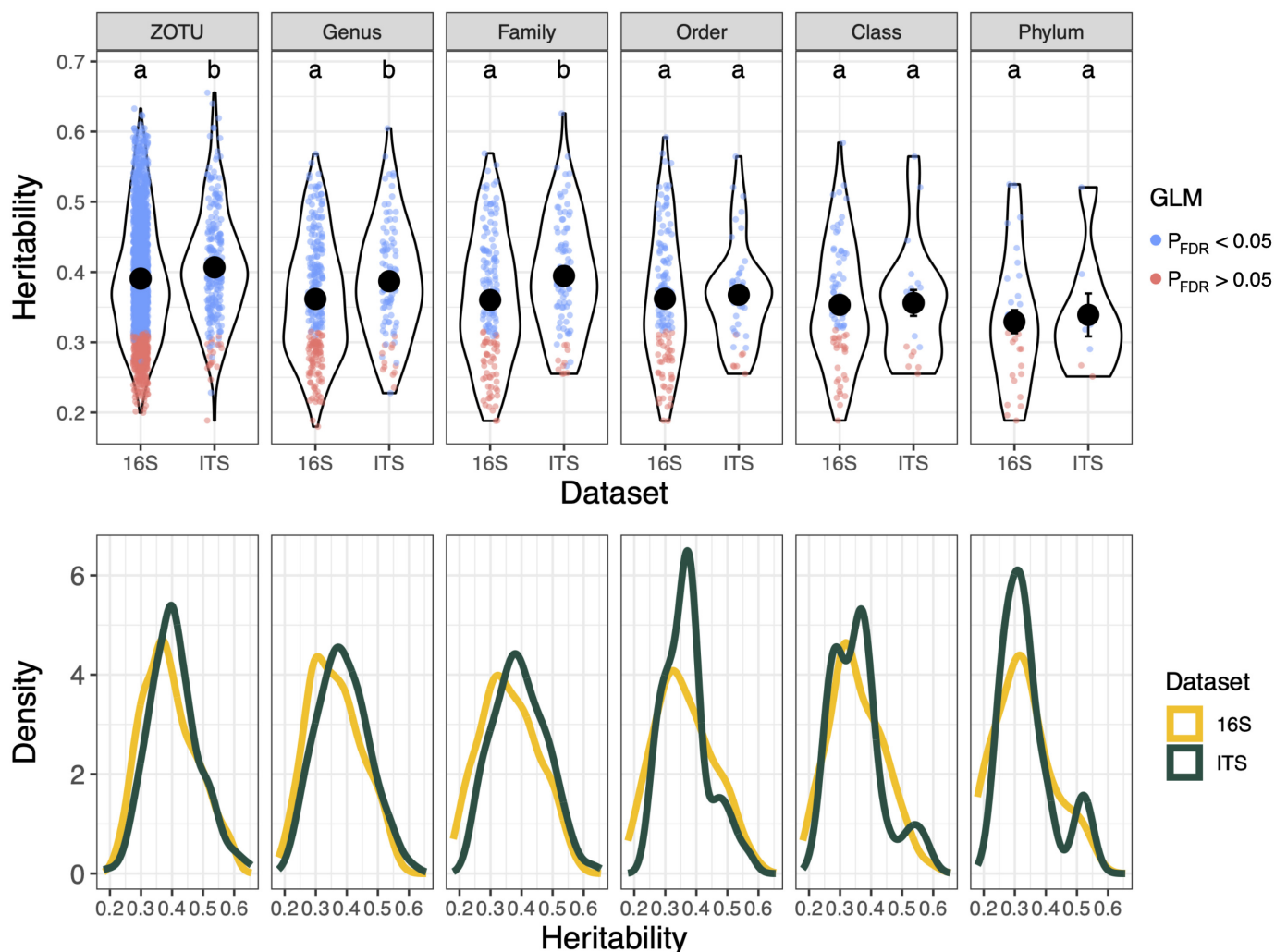


Fig. 3. Broad-sense heritability of archaeal and bacterial taxa (16S rRNA gene) and fungal taxa (internal transcribed spacer [ITS]) at varying taxonomic levels (top row). In all cases, the taxon prevalence and abundance cutoff to be tested was 25 and 0.01%, respectively. Different letters represent significant differences in heritability between 16S rRNA gene and ITS results within each panel. Also shown (bottom row) are the kernel densities for the heritability results shown in the top row, where peaks correspond to the most frequent values in the data. GLM, generalized linear model.

Plains (Bueno de Mesquita et al. 2024). Although the 95 genotypes in our study are all domesticated and not recent crop-wild hybrids, they still include substantial variation in phenotypic traits such as height, seed size, oil content, and branching pattern. In other crops,

some studies have detected significant effects with only two or three genotypes (Aira et al. 2010; Xu et al. 2009). The effect of genotype can depend on the environment (Bueno de Mesquita et al. 2024), the crop species (Matthews et al. 2019), the degree of domestication

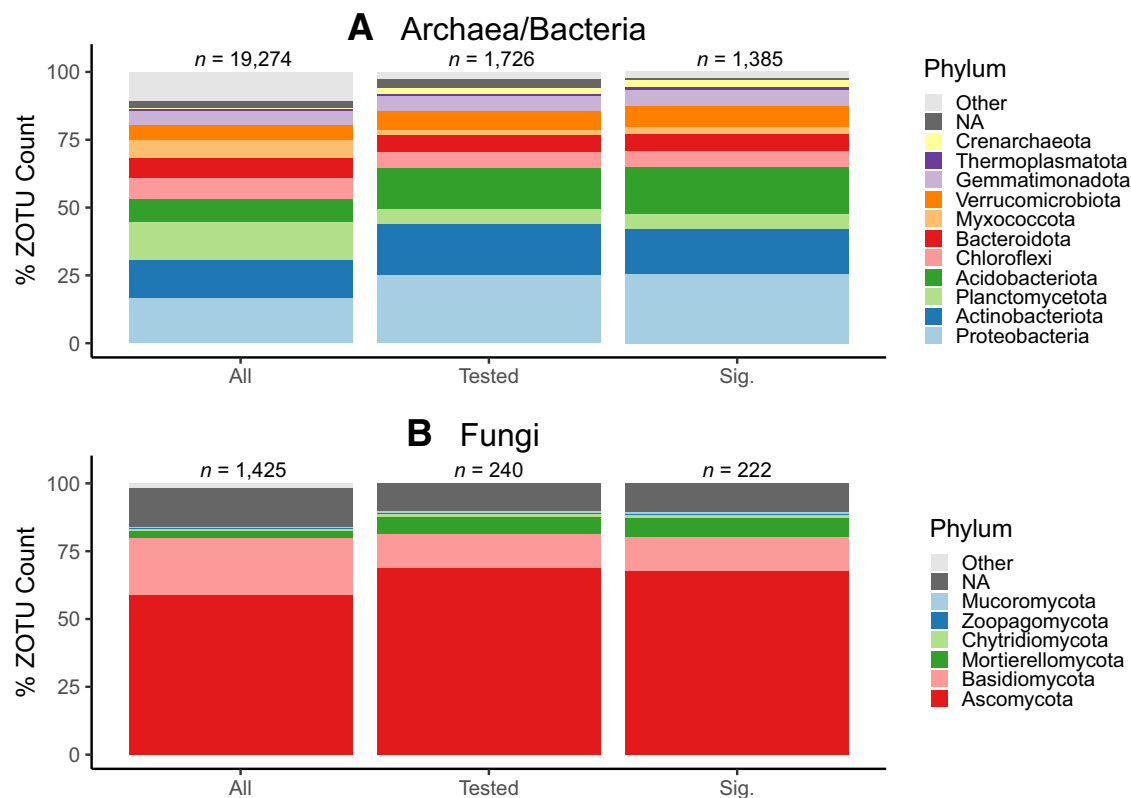


Fig. 4. Percent counts of zero-radius operational taxonomic units (ZOTUs) by phylum for the whole dataset (“All”), the subset of ZOTUs that passed a 25% prevalence and 0.01% relative abundance cutoff (“Tested”), and the subset of those that were significantly affected by plant genotype (“Sig.”). For archaea/bacteria, the top 11 phyla in the “Sig.” group are shown, whereas others are grouped into “Other.” For fungi, all 6 phyla present in the “Sig.” group are shown, whereas others are grouped into “Other” for the “All” group, which contained some other phyla. In both cases, stacked bars and legends are ordered by relative abundance in the “All” group, from bottom to top. The “n” refers to the number of ZOTUs, not the sample size, which in all cases was 368.

TABLE 3						
Broad-sense heritability of relative abundances of 16S rRNA gene (archaeal and bacterial) and internal transcribed spacer (ITS; fungal) taxa at different taxonomic levels, for those passing a 25% prevalence and 0.01% relative abundance cutoff ^a						
Variable	# Tested	# Sig.	% Sig.	Obs.	Null	NT
16S ZOTUs	1,726	1,385	80.2	0.39 ± 0.002	0.30 ± 0.0009	**
ITS ZOTUs	240	222	92.5	0.41 ± 0.005	0.34 ± 0.0005	**
16S Genus	256	169	66.0	0.36 ± 0.005	0.29 ± 0.0007	**
ITS Genus	96	84	87.5	0.39 ± 0.008	0.34 ± 0.0004	*
16S Family	155	104	67.1	0.36 ± 0.007	0.28 ± 0.0009	**
ITS Family	87	77	88.5	0.39 ± 0.009	0.35 ± 0.0005	*
16S Order	127	85	66.9	0.36 ± 0.008	0.28 ± 0.0010	**
ITS Order	43	36	83.7	0.37 ± 0.011	0.32 ± 0.0006	*
16S Class	72	45	62.5	0.35 ± 0.010	0.27 ± 0.0010	**
ITS Class	20	14	70.0	0.36 ± 0.018	0.32 ± 0.0008	N.S.
16S Phylum	31	16	51.6	0.33 ± 0.016	0.27 ± 0.0010	*
ITS Phylum	8	6	75.0	0.34 ± 0.031	0.32 ± 0.0010	N.S.

^a NT, null test with 100 bootstraps. **, all 100; *, 95 to 99/100 significantly different from observed (Obs.); N.S., Obs. not significantly different from null. # Significant (Sig.) is based on negative binomial generalized linear model with $P_{FDR} < 0.05$.

(Leff et al. 2017), and how genetically or phenotypically diverse they are, particularly for traits important for soil microbes, such as tissue C:N or exudate composition and production. We have not quantified the diversity or quantity of root exudates on these genotypes or isolated their effect on microbial communities, which is an avenue for future research. Exudates and other root traits have been shown to exert a major influence on the rhizosphere microbiome, including richness and composition (Zhalnina et al. 2018). For example, plants with a greater diversity of exudates and a greater diversity of root architecture can harbor more diverse microbiomes. Although all of the samples were taken from one site and therefore were exposed to the same climate and soil type, we cannot rule out that microbial relative abundances were also modulated by plot-to-plot microenvironment differences, microbe–microbe interactions, and random variation, not related to plant genotypes. We considered these effects by conducting null model tests of heritability, which confirmed that significant effects of genotype were greater than expected by chance.

Here, we examined broad-sense heritability and found that fungal taxa were generally more heritable than bacterial and archaeal taxa. Previous work found greater narrow-sense heritability in 16S rRNA gene compared with ITS communities, but not broad-sense heritability (Park et al. 2023). However, host effects were stronger on fungi than bacteria in *Arabidopsis thaliana* rhizospheres (Bergelson et al. 2019), similar to results in sunflowers (Leff et al. 2017). Furthermore, our findings that fungal and prokaryotic richness were only weakly correlated and beta diversity was not significantly correlated suggest that sunflower genotypes may affect prokaryotes and fungi differently, as has been previously suggested (Leff et al. 2017).

If microbial recruitment into the rhizosphere is at least partially controlled by the plant, it follows that rhizosphere microbial communities would be expected to be phylogenetically clustered rather than overdispersed or randomly assembled. Our data partially sup-

port this hypothesis, as communities on average were phylogenetically clustered according to the nearest taxon index, and a majority of ZOTUs were under positive selection relative to Sloan's neutral model. However, there were only 127 of 368 communities that had NTI values >2, which is typically used as a cutoff to determine significant phylogenetic clustering (Stegen et al. 2012). There are several reasons the NTI values were not higher than they were. First, selective pressure from the plant is only one of several factors contributing to rhizosphere community assembly, with environmental factors, dispersal, and stochasticity also playing roles (Li et al. 2024; Nuccio et al. 2016; Scott et al. 1995). Second, the rhizosphere is the most diverse host-associated habitat on Earth, and soils and sediments are among the most diverse free-living habitats (Thompson et al. 2017). The bulk soil contains thousands of microbial taxa; some taxa could randomly disperse from the bulk soil to the rhizosphere soil, and a subset of those could be able to persist there regardless of plant traits. Other rhizosphere studies that calculated NTI have found variable results, ranging from phylogenetic clustering (Vieira et al. 2020; Zhang et al. 2020) to phylogenetic overdispersion (Fan et al. 2017).

Because our study was performed on a domesticated crop species, it is crucial to consider the role of domestication and agricultural conditions in the observed results. Domestication has had myriad effects on plant–microbe interactions, and it is likely that many beneficial plant–microbe interactions were lost during domestication (Nerva et al. 2022), whereas interactions that influence important phenotypes in domesticated agricultural settings would be more likely to be kept. This is a particularly interesting aspect of this group of sunflowers, as some genotypes contain more recent introductions of wild genes and traits from the wild. Previous genomic work on sunflowers showed that all 80 examined cultivar genomes contained one or more introgressions from wild populations, with every chromosome having evidence of introgression in at least one modern

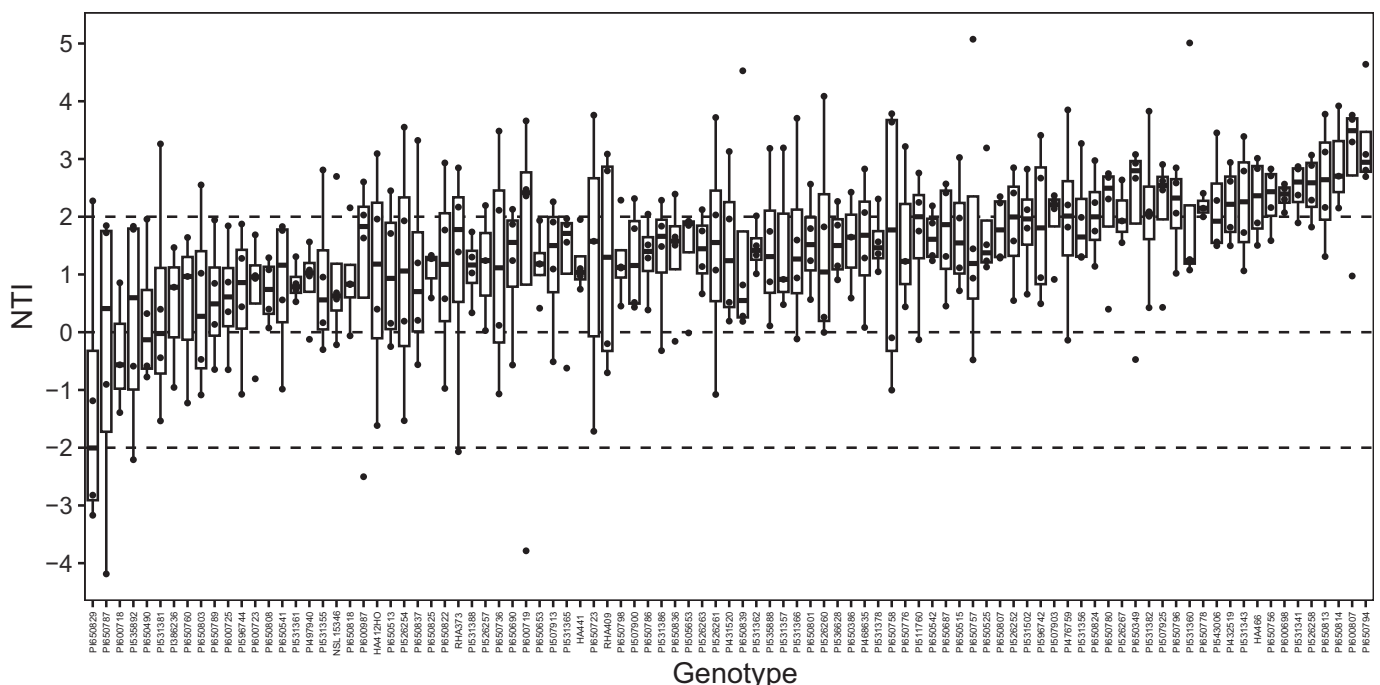


Fig. 5. Nearest taxon index (NTI) values for each sample across the 95 sunflower genotypes. Dashed lines are shown at 0, 2, and –2 for reference. Values above 2 are considered significantly phylogenetically clustered, whereas values below –2 are considered phylogenetically overdispersed. Values close to zero represent stochastic community composition.

line, and that cumulatively, unique introgressions were found on approximately 10% of the cultivated sunflower genome (Baute et al. 2015). Similarly, agricultural conditions (irrigation, fertilization) can also have a major impact on plant–microbe interactions. For example, plant–mycorrhizae interactions have been shown to exist on a continuum from mutualism in low phosphorus conditions to parasitism in high phosphorus conditions (Johnson et al. 1997). Plant genetic associations with microbes were shown to differ in high N versus low N environments in maize, with greater genotype effects in the low N environment (Meier et al. 2022). This makes sense because plant effects such as root exudates could be overwhelmed by an already nutrient-rich environment. Exudation itself is also modulated by nitrogen starvation (Novak et al. 2024) and phosphate availability (Brisson et al. 2022). These results are relevant to our field site, which has active conventional agricultural management, including fertilization and use of herbicides and pesticides.

Here, we show that at one location, 95 different sunflower genotypes were associated with considerable variation in ZOTU richness, Shannon diversity, and overall community composition in both prokaryotes and fungi. There were many taxa across all taxonomic levels that were heritable, with fungi showing higher broad-sense heritability than prokaryotes. Prokaryotic rhizosphere communities were on average phylogenetically clustered, which is in line with the idea that the plant plays a deterministic role in shaping the microbial community, to an extent. Whether the heritable variation observed here is mappable to distinct genomic regions in sunflower should be the focus of future work using genome-wide association studies. Disease resistance is a major goal of crop breeding, with two broad options available: we can breed for disease resistance with minimal effects on the microbiome (Wagner et al. 2020), or we can try to breed to select for beneficial microbes that antagonize pathogens (Bergelson et al. 2021). Whatever strategy is employed should take a holistic view of microbial functions

beyond disease resistance, as well as of other plant phenotypic traits besides disease resistance. Our work contributes to foundational understanding that will underpin future efforts in breeding for beneficial plant–microbe interactions.

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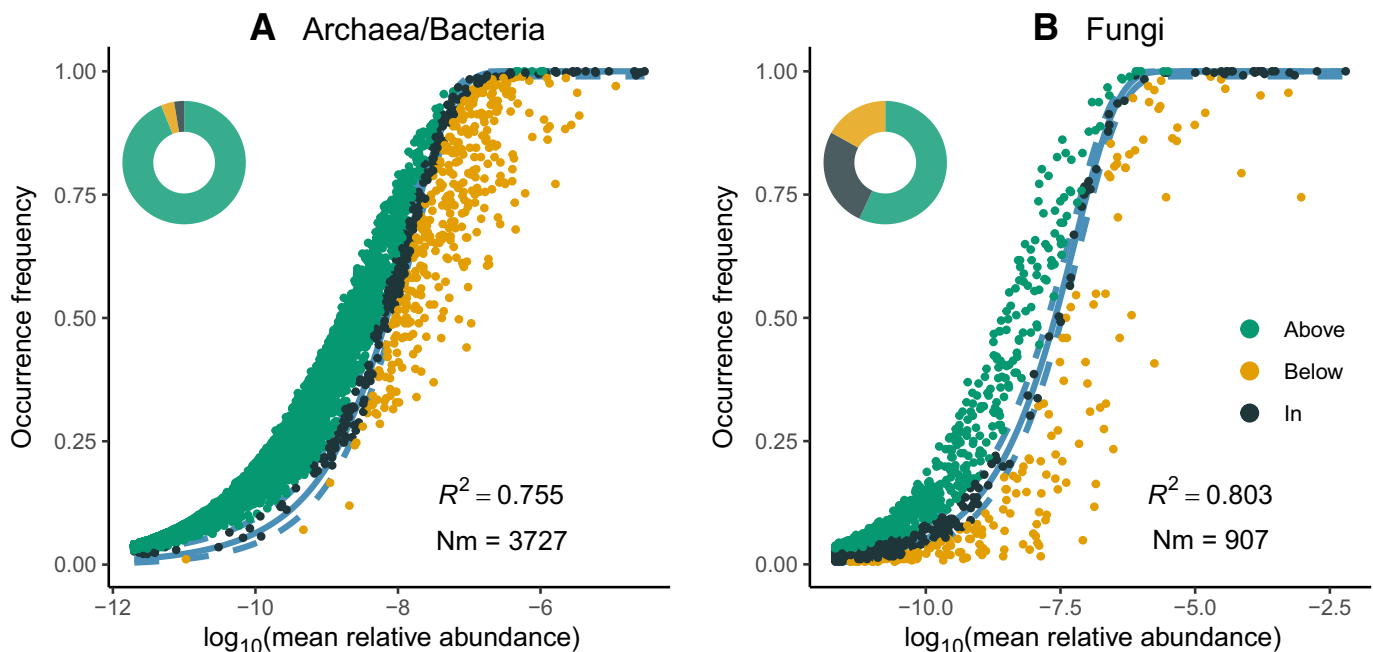


Fig. 6. Sloan's neutral model for **A**, archaea/bacteria and **B**, fungi. In both datasets, only zero-radius operational taxonomic units (ZOTUs) with at least 15 reads across the whole dataset were included ($n = 10,522$ and $1,425$, respectively). Inset pies show the proportion of ZOTUs that were above (positive selection), below (negative selection), or in (no selection) the neutral model. The solid blue line represents the best fit to the neutral model, and the dashed blue lines are the 95% confidence intervals. The R^2 is the fit to the neutral model, and the Nm is the metacommunity size times immigration.

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