Genetic architecture of heritable leaf microbes

Julia A. Boyle[1], Megan. E. Frederickson[1], John R. Stinchcombe[1,2]

1. Department of Ecology and Evolutionary Biology, University of Toronto, 25 Willcocks St., Toronto, ON, Canada, M5S3B2

2. Swedish Collegium for Advanced Study, SE-752 38 Uppsala, Sweden

julia.boyle@mail.utoronto.ca, m.frederickson@utoronto.ca, john.stinchcombe@utoronto.ca

Corresponding author information: Julia Boyle, julia.boyle@mail.utoronto.ca, Department of Ecology and Evolutionary Biology, University of Toronto, 25 Willcocks St., Toronto, ON, Canada, M5S3B2

Abstract

Background

Host-associated microbiomes are shaped by both their environment and host genetics, and often impact host performance. The scale of host genetic variation important to microbes is largely unknown, yet fundamental to the community assembly of host-associated microbiomes, and with implications for the eco-evolutionary dynamics of microbes and hosts. Using *Ipomoea hederacea*, Ivy-leaved morning glory, we generated matrilines differing in quantitative genetic variation and leaf shape, which is controlled by a single Mendelian locus. We then investigated the relative roles of Mendelian and quantitative genetic variation in structuring the leaf microbiome, and how these two sources of genetic variation contributed to microbe heritability.

Results

We found that despite large effects of the environment, both Mendelian and quantitative genetic host variation were important in contributing to microbe heritability, and that the cumulative small effect genomic differences due to matriline explained as much or more microbial variation than a single large effect locus. Furthermore, our results are the first to suggest that leaf shape itself contributes to variation in the abundances of some microbes in the leaf microbiome.

Conclusions

Our results demonstrate the roles of different scales of host genetic variation in the assembly of a natural microbiome. The genetic architecture of plant-associated microbiomes depends on both quantitative genetic variation and Mendelian traits, with similar contributions to microbe heritability. The presence of Mendelian and quantitative genetic variation for host associated microbes suggests that evolution in plant hosts-- at the leaf shape locus, and other quantitative genetic loci-- has the potential to affect microbiome distribution, abundance, and composition.

Keywords: leaf shape, phyllosphere, *Ipomoea hederacea*, microclimate, host age, leaf microbiome, heritability, matriline, Mendelian, quantitative genetics

Background

The community assembly of microbes on a host depends on the environment, the host's traits, and the microbes [1]. Microbes that colonize leaves and other aboveground plant parts, known as the phyllosphere, can disperse to a leaf via air [2], rain [3], or soil [4], after which they experience selection due to conditions on the leaf surface and microbe-microbe interactions [1, 5]. Host genetics play a role in the leaf microbiome; the similarity of leaf microbiomes across species depends on host phylogenetic relatedness [6, 7], and the similarity of microbiomes between individuals of the same species can depend on host intraspecific genetic variation [8–11]. Here we test how a single leaf shape gene affects leaf bacterial communities in *Ipomoea hederacea*, and compare the magnitude of effect from that single locus to that of many small effect loci.

The genetically-based physiological, morphological, or immune traits of hosts can lead to consistent associations between the abundance or community composition of microbes and host genotypes [12–15]. When consistent host-microbe associations are present, the host's microbial phenotype can be considered a heritable host trait, even when the microbes are environmentally-acquired [16–18]. In other words, horizontally-transmitted microbes can be heritable in host populations when host traits have predictable effects on microbial recruitment and alleles for these host traits are transmitted from host parents to host offspring. The heritability of a host's microbial phenotype is important to understanding host evolution and ecology due to microbial effects on host fitness [19–21], and potential reciprocal selection between hosts and microbiomes

[17, 22, 23]. The variation in microbe heritability may have interesting evolutionary repercussions on hosts, given that the phenotypic variance of many host traits can be microbially mediated [17, 18]. Furthermore, if a host's microbial phenotype is due to heritable host genetic variation, then the evolutionary forces affecting those host traits-- selection, drift, mutation, migration, non-random mating-- can in turn affect microbial abundances, distributions, and microbiome composition [24]. Like all heritable traits, host microbial phenotypes can be influenced by few loci of large effect or the cumulative effects of many small effect loci, and understanding microbe heritability necessitates we determine the scale of host variation that matters to microbes.

Mechanisms producing genotype-specific phyllosphere microbiomes are complex and varied. In *Arabidopsis thaliana*, a genome-wide association study of the phyllosphere microbial community found that loci related to defense and plant cell wall integrity affect microbial community variation, while species richness was affected by loci involved with viral reproduction, trichomes, and morphogenesis [9]. Host genotypes may differ in immune genes and disease resistance, which have been linked to differences in switchgrass leaf fungal community structure [25] and inconsistent effects on bacterial and fungal maize phyllospheres [11]. Plant genotypes can also vary in leaf morphology and other leaf attributes; for example, mutations in cuticle formation and ethylene production also affected the microbiome in a synthetic *A. thaliana* phyllosphere [26]. The microclimate of a leaf is determined by many leaf properties including surface temperature, surface area, thickness of the boundary layer of air, chemical composition, trichomes, gas exchange, and nutrients [27]. The boundary layer of air is closest to the leaf surface and has warmer, humid air; non-segmented leaves with larger surface area often have a thicker boundary layer, and thicker boundary layers can impede gas exchange

from the leaves [28]. Compounds and water released by leaves during gas exchange can be an important source of nutrients and resources for phyllosphere microbes [5], and they can impose selection on which microbial species can establish. These findings suggest that leaf morphology, and its effects on microclimates and resources, can potentially affect phyllosphere microbes.

Factors driving phyllosphere microbiome composition may depend strongly on the environment and the interaction between environment and genotype, since the leaf microbiome is primarily environmentally acquired [29]. Environmental factors like weather and seasonality influence which microbes are available to disperse to leaves and with what frequency [29]. Phyllosphere microbes may differ over time due to changing moisture and temperature conditions abiotically filtering for microbes in the air and soil [30], and biotic interactions that emerge at different times, e.g. changing microbe-microbe interactions and new microbes from insects [31] or other plants [32]. Determining the relative roles of environment, phenology, and plant genotype on the microbial community composition and abundance in the phyllosphere is an unresolved empirical challenge.

One unstudied potential source of host genotype influence on the leaf microbiome is leaf shape. Leaf shape is a key morphological difference between plant species [33] and could contribute to interspecific variation in leaf microbiomes. The shape of a leaf determines the temperature and humidity conditions it experiences and even its photosynthetic and gas exchange abilities [34, 35], potentially affecting microbial communities. Comparing the effects of leaf shape on the microbiome between plant species is confounded by other genetic and ecological differences between taxa, and for this reason, it is preferable to use a single species that exhibits intraspecific variation in leaf shape. *Ipomoea hederacea*, Ivy-leaved morning glory, is a flowering annual plant whose leaf shape is determined by a single Mendelian locus [36–38];

homozygous genotypes are either fully lobed or whole, with heterozygotes being partially lobed (Figure 1). In *I. hederacea*, homozygous lobed genotypes have been shown to more protected against extreme temperature changes compared to whole leaves; homozygous lobed leaves remained warmer at night by a mean difference of 0.16-0.22°C, likely due to their altered boundary layer resulting in leaves being more coupled with ambient air temperature [39, 40]. The differences in *Ipomoea hederacea* leaf morphology, and its effects on temperature and boundary layers that affect leaf microclimates, suggest that different leaf shape genotypes could directly determine which colonizing microbes establish on the leaf, indirectly affecting microbe-microbe interactions, and structuring leaf microbial communities as a whole.

To assess the relative contributions of quantitative and Mendelian genetic variation we first examined if a single large effect Mendelian locus underlying leaf shape generates leaf microbiome differences within a host species. Then, we estimated the heritability of microbes due to many small effect loci that differ between plant lines– in other words, the amount of microbiome variation attributable to quantitative genetic causes. Finally, we compared the relative importance of host genotype to other factors such as germination time, and therefore host age, to assess the role of the environment in structuring leaf microbiomes.

Methods

Study system and crossing design

Ipomoea hederacea, Ivy-leaved morning glory, is a flowering annual plant, commonly found in eastern North America in roadside ditches and agricultural fields. It is predominantly selfing [41], and dies with the first hard frost in autumn. We used seeds derived from a cross by Campitelli and Stinchcombe [39], where individuals from the two alternate homozygous leaf

shape phenotypes (i.e., fully lobed or whole) were collected from North Carolina, USA, selfed for seven generations to generate homozygous parents (P1), and then crossed with each other (Figure 1). A single F1 was allowed to self-fertilize, producing F2 progeny. We scored F2 plants for leaf shape, and allowed them to self-fertilize; we refer to all the selfed progeny of an individual F2 plant as a "matriline." We used F3 seeds, set by F2 plants we had identified as heterozygous for leaf shape, as our experimental seeds. In total, we used seeds from 82 matrilines. As expected, genotype frequencies did not significantly differ from a 1:2:1 ratio for leaf shape ($\square^2_{(2, 218)} = 3$, $p = 0.22$), with 55 plants with whole leaves, 112 plants with partially lobed leaves, and 51 plants with fully lobed leaves.

Our breeding design has three consequences for leaf microbiomes, which we return to in the Discussion. First, variation between matrilines is due to the combined effects of loci that differed between the original parents of the cross and the effects of recombination. Second, because of the effects of recombination, significant differences between leaf shape genotypes are due to either the effects of the leaf shape locus itself or linked loci not broken up by recombination. Third, within a matriline, we expect 25% of the loci that differed between the parents to still be heterozygous, which is considerably more within-matriline genetic variation than would be found in inbred lines or recombinant inbred lines (RILs), potentially reducing our power and making our estimates of microbe heritability more conservative.

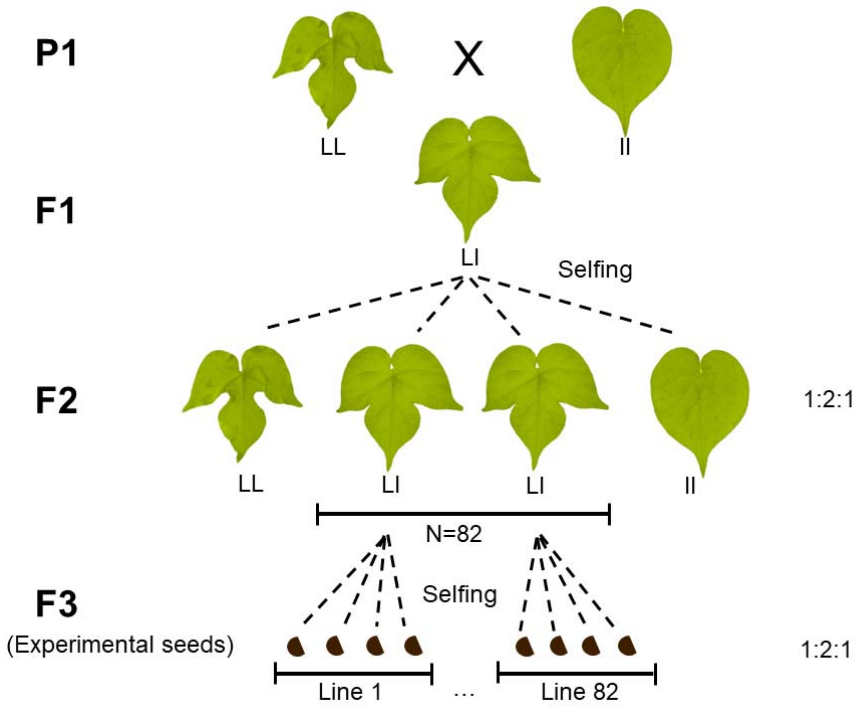

Figure 1. *Ipomoea hederacea* leaf polymorphism and crossing design. Leaf shape is determined by a single Mendelian locus. First, both homozygous genotypes were collected from North Carolina, USA and selfed for seven generations to create the parents (P1). To generate the plants used in the field experiment, the two homozygous P1 individuals were crossed, then heterozygote offspring were selfed for two more generations. Our experiment used F3 plants from 82 matrilines. Photos taken by Julia Boyle, with brightness and colors edited for style and clarity.

Field site and experimental design

In 2021, we planted a total of 240 *I. hederacea* seeds from 82 matrilines (2-3 seeds/matriline) in a common garden at Koffler Scientific Reserve (www.ksr.utoronto.ca) in Ontario, Canada. We plowed and disked an old field for the common garden site, and we assigned morning glories in 3 blocks of 80 plants each, planted 1 m apart with a 6 ft tall bamboo

pole to climb. We scarified seeds then planted them in a greenhouse in peat pots containing soil from the field. We planted the first cohort on June 4th and a second cohort on July 2nd, because of poor germination in the first cohort. After the majority of plants in a given cohort germinated (approximately one week), we transplanted the pots into the field. The 218 surviving plants consisted of 83 individuals from the first cohort, and 135 individuals from the second cohort. There were no significant differences in the frequencies of leaf shape genotypes between the two cohorts ($\square^2_{(2, 218)} = 0.31$, $p = 0.86$; Table S1). The common garden was moderately weeded until the *I. hederacea* plants had established; neighboring plants in the common garden were predominantly *Cirsium arvense*, Canada thistle. On September 1st, we collected similarly-sized leaves within one foot from the ground by cutting them with sterilized scissors into a sterile plastic bag, after which they were stored in a -80°C freezer until DNA extraction. We avoided leaves that looked very young or very old, as well as leaves with herbivory damage. At the time of leaf collection, plants in the first germination cohort were 89 days old with 96% of plants flowering, and plants in the second germination cohort were 61 days old with 49% of plants flowering. We photographed collected leaves against a $1cm^2$ grid background, and measured leaf surface area and shape using ImageJ [42]. From these photographs, we calculated leaf circularity as $4\pi(\text{Area}/\text{Perimeter}^2)$, which has a value between 0 and 1, with 1 indicating a perfect circle.

Sequencing and QIIME2 analysis

We performed extractions using the whole leaves with QIAGEN DNeasy® PowerSoil® Pro Kits; both epiphytic and endophytic microbes were extracted. We sent samples to Génome Québec (Montréal, Canada) for Illumina MiSeq PE 250bp 16S rRNA gene amplicon sequencing on the conserved hypervariable V4 region (primer pair 515F-806R). We used Quantitative

Insights Into Microbial Ecology 2 (QIIME2) v.2022.2 [43] to trim the sequences for quality and we denoised the sequences with DADA2 [44]. Samples had a median read frequency of 35,436 reads. Using QIIME2, we removed amplicon sequence variants (ASVs) that had fewer than 10 reads across all samples, and assigned taxonomy using the 'sklearn' feature classifier and Greengenes 16S rRNA gene V4 region reference [45], then filtered out reads assigned as cyanobacteria and mitochondria to remove plant DNA. After these steps, we had 4,934 ASVs across the 218 leaf samples, with samples having a median read frequency of 4,523 reads. After visualizing a rarefaction curve, we created a rarefied dataset with 4,695 ASVs total across 183 samples, with 1809 reads/sample; the first and second cohorts retained 77 and 106 samples respectively. Finally, we constructed a phylogeny using QIIME2's MAFFT [46] and FastTree 2 [47] to obtain a rooted tree.

Statistical analysis

For statistical analysis, we used R v4.2.0 [48], with the tidyverse [49], lmerTest [50], phyloseq [51], vegan [52], and microbiome [53] packages. The general model structure we used included leaf shape genotype and germination cohort as fixed effects, and random effects of block and matriline, unless otherwise specified. All linear models were adjusted with type III ANOVAs, calculated in the car package [54].

First, we compared leaf shape surface area and circularity between genotypes using linear mixed effect models. Next, we identified the core microbiota by filtering the rarefied dataset to genera found in at least 80% of samples. To assess the effect of leaf shape and germination cohort on microbial composition, we calculated a weighted UniFrac distance matrix using rarefied data, then we used an adonis2 PERMANOVA (999 permutations) followed by a

permutational test of dispersion on significant groups using betadisper. The vegan package does not allow for random effects, so we did not include block and matriline. To confirm that germination cohort sample sizes did not affect our results, we also downsampled to equal cohort sizes 100 times, and used the same permutation tests for composition and dispersion (Figure S3). To visualize the community, we used a weighted UniFrac Principal Coordinate Analysis (PCoA) using rarefied relative abundance data.

We estimated broad-sense heritability (H^2) of microbial community phenotypes and taxonomic groups using linear mixed effects models, taking a similar approach as Wagner et al. [10] and Grieneisen et al. [14]. Here, the random effects of block and matriline explain variance in the microbe abundance phenotype after accounting for the mean effects of the germination cohort and leaf shape, meaning our estimates of H^2 are conditioned on the fixed effects of the germination cohort and leaf shape (as is commonly the case [55]). To estimate H^2 from the random effects, we calculated the ratio of matriline genetic variance (V_G) to the sum of matriline, spatial block, and residual variance ($V_P = V_G + V_{Block} + V_E$). We generated the community phenotypes using rarefied data and included observed species richness (base 10 log-transformed), evenness, Shannon diversity, and the first three axes of the weighted UniFrac PCoA as the response variables in individual linear mixed effects models. To estimate the heritability of taxonomic groups in a more compositionally aware way [56], we aggregated the non-rarefied dataset to genera that were in at least 30% of samples, added 1 to each abundance, and center log ratio transformed the abundance matrix. Next, we fit a linear mixed effects model to the abundance of each genus that was in at least 30% of samples. To assess the statistical significance of the matriline effect, we compared the log-likelihood of our models with and without line as a random effect; the difference in log-likelihoods is X^2 distributed, with one

degree of freedom [57]. Finally, solely for the purposes of a qualitative comparison of the magnitude of variance explained by the leaf shape locus, matriline effects, spatial blocks, and residual variation, we used the same mixed effects model except with leaf shape as a random effect.

Results

Genotype morphology

Leaf shape genotypes of *I. hederacea* differed quantitatively. Leaf shape genotypes differed significantly in measures of circularity (*Wald* $X^2_{(2,218)}$=1520, p<0.001; Table S2), with the whole genotype having the highest circularity followed by heterozygotes (Figure S1). While there was a trend for leaf surface area to be the largest in whole leaves and lowest in the lobed leaves, the difference was not significantly predicted by leaf shape genotype (*Wald* $X^2_{(2,218)}$=4.15, p>0.05; Table S2) or germination cohort (*Wald* $X^2_{(1,218)}$=2.22, p>0.05; Table S2) (Figure S1). As we specifically chose to sample similarly sized leaves, with the intent of minimizing differences in total leaf area, these results suggest our sampling strategy worked and that we can attribute differences in microbes due to leaf shape as not simply being the result of differences in leaf area.

Community composition and diversity

The core *I. hederacea* leaf microbes were the genera *Methylobacterium*, *Sphingomonas*, *Deinococcus*, *Pseudomonas*, *Arthrobacter*, and *Hymenobacter*. Communities were structured by the high relative abundances of *Methylobacterium* and *Sphingomonas* genera (Figure 3 and 4), and their relative abundances were negatively correlated (Figure S2). The first PCoA axis was

strongly positively correlated with *Methylobacterium* relative abundance while the second axis was strongly negatively correlated with *Sphingomonas* relative abundance (Figure S2).

Germination cohort was the main driver of overall microbial community composition. While microbiomes from both cohorts overlapped in composition, they were significantly different from each other ($F_{(1,182)}$=10.4, p=0.001; Table S3); older plants from the first cohort had more *Methylobacterium*, and younger plants from the second cohort had more *Sphingomonas* (Figure 4ACD). The younger cohort's microbiomes were more distinct from one another, creating significantly higher dispersion ($F_{(1,182)}$=10.4, p=0.001; Table S3). Germination cohorts always remained significantly different in composition and dispersion when cohort sample sizes were equalized (Figure S3), suggesting this is a true biological phenomenon. Germination cohort significantly predicted observed richness (*Wald* $X^2_{(1,182)}$=10.1, p=0.001; Table S4), with older plants having higher ASV richness than younger plants, but it did not predict Shannon diversity (*Wald* $X^2_{(1,182)}$=3.33, p>0.05; Table S4) or evenness (*Wald* $X^2_{(1,182)}$=2.96, p>0.05; Table S4).

Despite differences in morphology and microclimate that we *a priori* predicted to affect the leaf microbiome, we did not detect strong leaf shape effects on community composition as a whole. Leaf shape did not significantly structure composition ($F_{(2,182)}$=1.83, p>0.05; Table S3), or affect Shannon diversity, evenness, or observed richness (*Wald* $X^2_{(2,182)}$<3.97, p>0.05 for all; Table S4). While whole community composition was not affected, we still identified microbial genera significantly affected by leaf shape, as discussed below.

Heritable microbes and community phenotypes

Microbial community phenotypes did not have a strong genetic basis, yet we still discovered significant heritable variation in some microbial traits. Community phenotypes had

low broad-sense heritability ($0 \leq H^2 \leq \sim 0.05$) with the third axis of the PCoA having the highest H^2, followed by Shannon diversity (Figure 2A). All community phenotypes with non-zero H^2 were significantly heritable, as the models' log-likelihoods were improved by including matriline; community phenotypes with no H^2 (evenness and PCoA axis 2) showed no difference in fit when matriline was included (Figure S4). The main effect of the germination cohort significantly affected observed richness, as previously described, as well as the first two axes of the PCoA (*Wald* $X^2_{(1,182)}>6.71$, $p<0.01$ for both axes; Table S4), whereas leaf shape did not significantly affect community phenotypes (*Wald* $X^2_{(2,182)}<4.85$, $p>0.05$ for all; Table S4). In our qualitative comparison of the magnitudes of variances explained, plant matriline had a larger effect on community phenotypes' H^2 than leaf shape, with the exception of observed richness (Figure 2C, Table S5).

Host genetics had a stronger influence on the abundance of microbial genera than community phenotypes, with both quantitative genetic and Mendelian traits affecting microbe abundance. Considering the genera in at least 30% of samples, we identified eight genera with non-zero and significant broad-sense heritability. The most heritable genera ($0.05 \leq H^2 \leq 0.07$) were *Nocardioides*, *Kineococcus*, *Pseudomonas*, and *Agrobacterium* (Figure 2B). Our likelihood ratio test results showed that including plant matriline improved model fit for 10 out of 15 genera (which included all genera with non-zero H^2), there was no difference in fit for 3 genera (*Deinococcus*, *Hymenobacter*, *Sphingomonas*), and slightly reduced model fit for 2 genera (*Actinotelluria*, *Rubellimicrobium*) (Figure S4). The germination cohort significantly affected the abundance of more than half of the most common genera (*Wald* $X^2_{(1,218)}>3.83$, $p<0.05$ for all; Table S6, Figure 2B). Four genera, *Sphingomonas*, *Nocardioides*, *Methylobacterium* and

Agrobacterium, had significant main effects of leaf shape on their phenotype (*Wald* $X^2_{(2,218)}>7.03$, $p<0.05$ for all; Table S6, Figure 2B). When we examined whether the magnitude of plant matriline variance was qualitatively comparable to leaf shape locus, we found that these four genera had equal to larger magnitudes of variance attributable to leaf shape than plant matriline (Figure 2D, Table S7). In heritable genera with a non-significant main effect of leaf shape, plant matriline explained more variance than leaf shape (Figure 2D, Table S7), as expected.

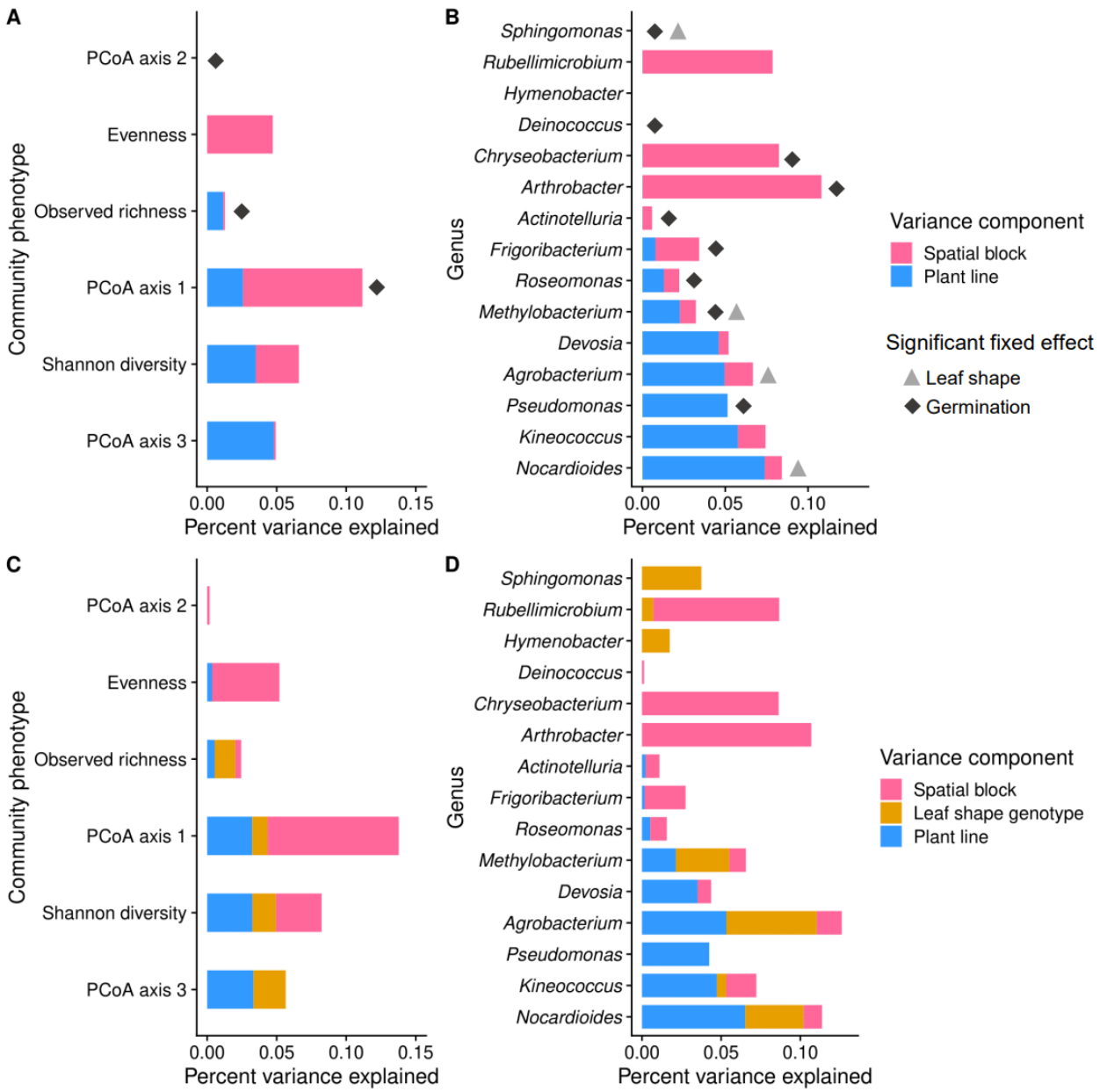

Figure 2. Broad-sense heritability (H^2) of community phenotypes and common microbial genera. Genera are ordered by percent variance explained by plant matriline in the H^2 model. A and B: H^2 of community phenotypes and genera in at least 30% of samples. H^2 was calculated as the percent variation explained by genetic line compared to the total of matriline, spatial block, and residual variances. Significant fixed effects of germination cohort and leaf shape are indicated by diamonds and triangles respectively. C and D: H^2 of community phenotypes and

genera in at least 30% of samples, but with leaf shape as a random effect to directly compare the magnitude of variance explained to that of matriline.

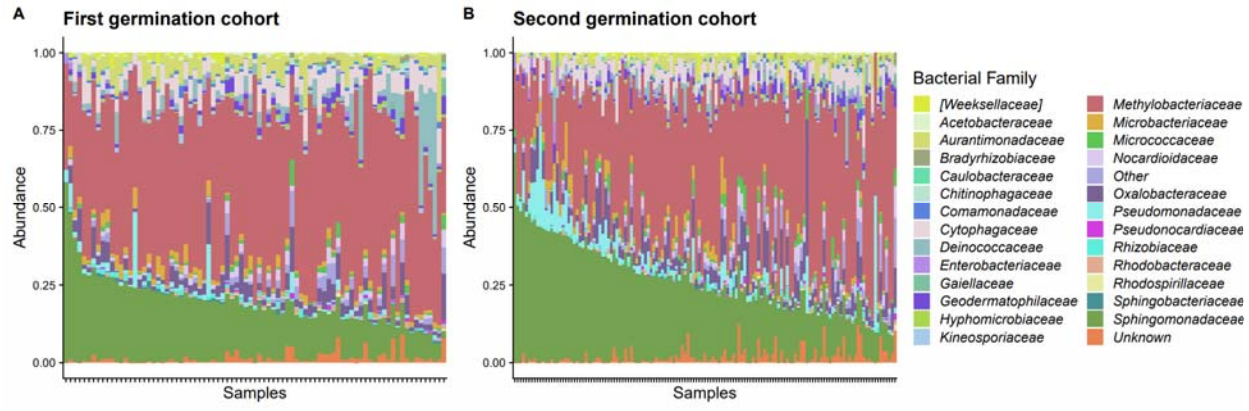

Figure 3. Relative abundance of bacterial families in the *Ipomoea hederacea* leaf microbiome, indicated by color. Samples are ordered by *Sphingomonadaceae* abundance and grouped by germination cohort. The first germination cohort (A) was 89 days old (n=83) and the second germination cohort (B) was 61 days old (n=135) at the time of sampling. For this visualization we included only families in at least 30% of samples and at least 1% relative abundance.

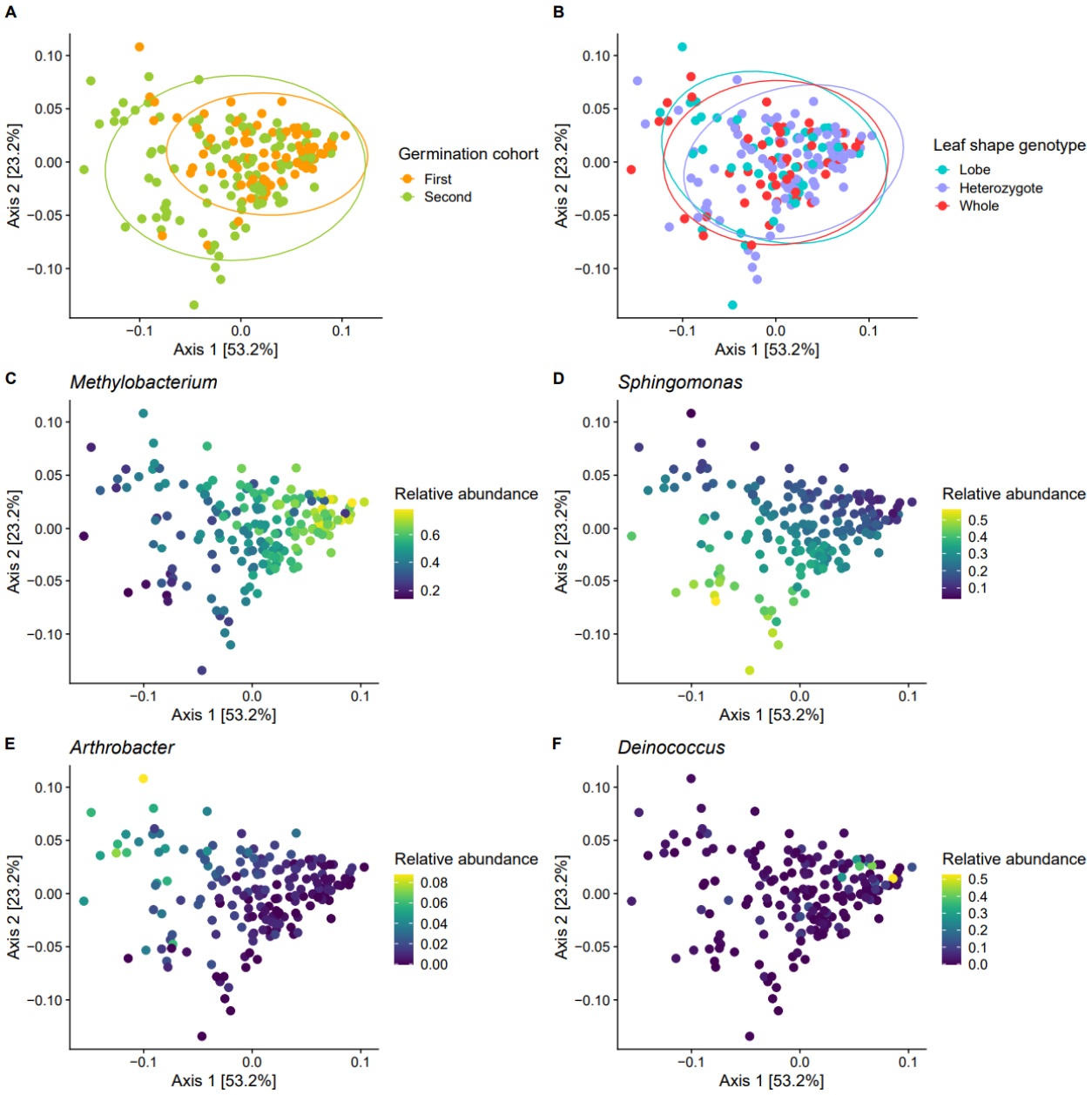

Figure 4. Weighted UniFrac PCoA of *I. hederacea* leaf microbiomes. Germination cohort significantly predicted community composition (A), while leaf shape was non-significant (B). Core microbiota structured communities (C-F). For C-F, note that the relative abundance legend scales differ.

Discussion

Microbes can be considered an extended host phenotype with potentially adaptive functions for the host [58], and heritable microbes are more likely to consistently affect host phenotype and fitness through time. Here we examined the relative contributions of Mendelian and quantitative genetic variation to the heritability of host-associated microbes to determine what types of host genetic variation mattered to microbial ecology. We found that both Mendelian and quantitative genetic host variation contribute to microbe heritability, and that the cumulative small effect genomic differences due to matriline explained as much or more variation than a single large effect locus. We were able to identify heritable microbial variation despite a large effect of germination timing, which incorporated host age and initial microbial environment, and which was a major factor structuring community composition. We discuss the implications of our results below.

Genetic influence on the microbiome

There was genetic variation at several scales mediating how well microbes can establish and perform in the phyllosphere. It is important to note that, as always, our estimates of heritability are specific to the population [59] and conditioned on fixed effects in the model [55], in our case, the germination cohort and leaf shape. A single leaf shape gene significantly explained the abundance of several common microbes, suggesting the genetic differences in leaf microclimate (or other, unknown features of the leaf shape genotypes) impact microbial establishment and persistence, and can contribute to intraspecific differences in the phyllosphere. When comparing the magnitude of effect, both leaf shape and matriline were important factors influencing microbe abundance, although plant matriline affected more of the microbes tested. Community phenotypes like species diversity and weighted UniFrac distances showed lower

heritability than individual genera, but since the majority of microbes are not likely to be heritable in an environmentally acquired microbiome, very low heritability at a community scale was perhaps unsurprising. Our estimates of heritability are low to commensurate compared to other heritable microbiome studies (summarized in Table S8; [10, 12–14, 16, 60–64]; while the range of heritabilities for plant-associated microbes across other studies was as broad as H^2=0-1, the average significant heritability of microbes and community phenotypes was usually low ($H^2 \leq 0.10$) (Table S8). Our results therefore follow the trend of low heritability for environmentally acquired microbes, with a strong influence of the environment.

In our experiment, each plant line represented a different mosaic of the original parental genotypes, similar in concept to recombinant inbred lines (RILs). However, because the seeds we used in our experiment were F3 individuals, approximately 25% of the loci initially heterozygous in the F1 (i.e., those that differed between the parents) remain heterozygous within matrilines. One consequence of this is that there is genetic variation between matrilines due to the effects of recombination, and genetic variation within matrilines (due to heterozygosity and segregation within a matriline). These effects make it more difficult to detect quantitative genetic variation in microbial phenotypes, especially compared to RIL populations that have been made homozygous within lines. Our results may thus underestimate the prevalence and magnitude of H^2 due to reduced power. Furthermore, our crossing design included only two parental genotypes, potentially limiting the amount of genetic variation segregating in the cross. For instance, had we sampled more genotypes to create MAGIC or Nested Association Mapping lines [65, 66], more genetic variation would be captured in our population. It is difficult to determine, *a priori*, whether this would lead to increased or decreased estimates of genetic variation. On the one hand, two parental genotypes is a limited sample of the genetic variation found in many

populations. Although putatively neutral genetic variation in *I. hederacea* populations is often quite low [67], there is frequently quantitative genetic variation [68, 69]. On the other hand, the selfing rate in *I. hederacea* is quite high (~93%) [67, 70]. Rare outcrossing events, followed by selfing, may produce recombinant populations not that dissimilar from our experimental population. Similar arguments have been made for *A. thaliana* [71, 72], which is also highly selfing. As a qualitative investigation of the influence of including limited genetic variation in our crossing design, we gathered estimates of H^2 for life history and quantitative traits in *Arabidopsis thaliana*, specifically comparing studies that used either multiple accessions or RILs. These data (Table S9), while an imperfect comparison, suggest that if anything, our estimates of heritability could be under-estimated from using two parental lines in the initial cross.

Evolutionary implications

We found similar magnitudes of variance in microbial phenotypes attributable to plant line and leaf shape genotype for several common bacterial genera, which is important because there is the potential for different evolutionary forces to act on quantitative genetic loci versus Mendelian loci. For example, Mendelian traits like leaf shape have high potential to show sampling effects due to drift, especially in small populations [73]. In contrast, drift is much less likely to produce a change in quantitative traits which are influenced by many genes [73]. Thus, drift can potentially lead to bigger effects on leaf shape-associated microbes. Furthermore, high selfing in *I. hederacea* will reduce the frequency of heterozygous leaf shape genotypes by as much as 50% per generation. In this way, the mating system may have outsized effects on microbes linked to Mendelian traits. If natural selection acts on host traits, as it appears to with *I.*

hederacea's leaf shape [38], there may be indirect effects of selection on the microbial phenotype in the host population.

The microbes we identified as having heritable variation in abundance are also linked to host performance and fitness in other systems. These findings from other systems suggest intriguing hypotheses for further study. For example, one of the most heritable genera that was also significantly affected by leaf shape, *Agrobacterium*, has pathogens linked to causing tumor-like galls in plants [74], suggesting a potential link of leaf shape to disease risk. *Methylobacterium* spp. are linked to improving plant growth [75], and increased growth rate improves fitness in *I. hederacea* [39]. *Pseudomonas* spp. have a wide range of positive or negative effects on plant performance [76]. The most heritable genus *Nocardioides* is commonly endophytic on plant leaves and stems [77, 78] and has species capable of fixing nitrogen [79] and reducing nitrate [80]. Evaluating whether any of these microbial genera which have effects on host performance in other systems do so in *I. hederacea* will require further empirical work. If heritable microbes affect plant fitness, then there is higher potential for reciprocal selection between the microbes and plant hosts.

Environmental and phenological effects on the leaf microbiome

Community-wide differences between germination cohorts could be due to seasonal differences in what microbes were present in the plant's early life and the amount of time selection had to act on the microbial communities. Input of new microbes to the phyllosphere can be low relative to environments like soil rhizospheres, and these sources may change across the growing season [29]. The cumulative input of microbes may matter as well, since we found that the older plant phyllospheres had a higher species richness than younger plants; this is possibly

because the leaves themselves were older and had more time to collect rare microbes dispersing from the environment. Additionally, as plants age and change phenologically, this may change the leaf environment for microbes [81–83]. Whilst in a time of growth and development, young plants have weaker immune responses [84], meaning they may impose weaker selection on their phyllospheres. Thus, the disparate microbiomes on young plants could be due to weaker selection from the environment and host over a shorter time. In contrast, older plants showed more similar and clustered microbiomes suggesting that over time there is selection on microbes by the *I. hederacea* leaf microclimate, and the microbial community converges. The merging community compositions between young and old plants could suggest microbial succession occurred in the phyllosphere [85, 86].

The large effect of germination cohort is not unexpected, given that most studies on microbiome heritability find a very large effect of environment and host age on the microbiome [10, 12, 16, 87]. For example, Walters et al. [16] found that plant age was the largest driver of the maize rhizosphere, but in one year they nonetheless found 143 heritable root microbes with H^2=0.15-0.25. While our results suggest genera like *Methylobacterium* and *Sphingomonas* were significantly affected by host age and initial microbial environment, host genotype still significantly mediated these environmental and phenological changes in microbe abundance. Our results add to recent evidence that the assembly process of host-associated microbiomes is governed by both stochastic forces and host-based selective forces [87, 88].

Conclusions

Our results show that while *I. hederacea* leaf microbe composition differences were primarily shaped by host age and the environment, there exists a heritable subset of core

microbes in the *I. hederacea* microbiome. A Mendelian trait and quantitative genetic variation across matrilines explained similar amounts of variation in microbial abundance, with implications for plant-microbe eco-evolutionary dynamics across time. Furthermore, we show for the first time that leaf shape itself may contribute to differences in phyllosphere microbial abundances within a species.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Funding

Funding sources include NSERC graduate funding to JAB and NSERC discovery grants to MEF and JRS.

Authors' contributions

JAB, MEF, and JRS collaborated on the ideas; JAB, MEF, and JRS designed the methodology, JAB performed the experiments, and JAB, MEF, and JRS wrote and edited the manuscript. JAB, MEF, and JRS analyzed the data. All authors contributed to this manuscript and approved its publication.

Acknowledgements

We thank Brandon Campitelli for generating the morning glory lines and the staff of Koffler Scientific Reserve in 2021 for helping to maintain the field site. We acknowledge Génome

Québec (Montréal, Canada) for sequencing samples. Finally, we gratefully acknowledge the Swedish Collegium for Advanced Study, and Jennifer Mack, Natuschka Lee, Iva Lučić, and Arthur Asseraf, for support.

References

1. Leveau JH. A brief from the leaf: latest research to inform our understanding of the phyllosphere microbiome. Curr Opin Microbiol. 2019;49:41–9.

2. Vokou D, Vareli K, Zarali E, Karamanoli K, Constantinidou HIA, Monokrousos N, et al. Exploring Biodiversity in the Bacterial Community of the Mediterranean Phyllosphere and its Relationship with Airborne Bacteria. Microb Ecol. 2012;64:714–24.

3. Lindow SE. Role of Immigration and Other Processes in Determining Epiphytic Bacterial Populations. In: Morris CE, Nicot PC, Nguyen-The C, editors. Aerial Plant Surface Microbiology. Boston, MA: Springer, Boston, MA; 1996. p. 155–68.

4. Massoni J, Bortfeld-Miller M, Widmer A, Vorholt JA. Capacity of soil bacteria to reach the phyllosphere and convergence of floral communities despite soil microbiota variation. Proc Natl Acad Sci U S A. 2021;118:e2100150118.

5. Dastogeer KMG, Tumpa FH, Sultana A, Akter MA, Chakraborty A. Plant microbiome–an account of the factors that shape community composition and diversity. Curr Plant Biol. 2020;23:100161.

6. Redford AJ, Bowers RM, Knight R, Linhart Y, Fierer N. The ecology of the phyllosphere: geographic and phylogenetic variability in the distribution of bacteria on tree leaves. Environ

Microbiol. 2010;12:2885–93.

7. Kembel SW, O'Connor TK, Arnold HK, Hubbell SP, Wright SJ, Green JL. Relationships between phyllosphere bacterial communities and plant functional traits in a neotropical forest. Proc Natl Acad Sci. 2014;111:13715–20.

8. Bodenhausen N, Horton MW, Bergelson J. Bacterial Communities Associated with the Leaves and the Roots of Arabidopsis thaliana. PLoS One. 2013;8:e56329.

9. Horton MW, Bodenhausen N, Beilsmith K, Meng D, Muegge BD, Subramanian S, et al. Genome-wide association study of Arabidopsis thaliana leaf microbial community. Nat Commun. 2014;5:5320.

10. Wagner MR, Lundberg DS, Del Rio TG, Tringe SG, Dangl JL, Mitchell-Olds T. Host genotype and age shape the leaf and root microbiomes of a wild perennial plant. Nat Commun. 2016;7:12151.

11. Wagner MR, Busby PE, Balint□Kurti P. Analysis of leaf microbiome composition of near□isogenic maize lines differing in broad□spectrum disease resistance. New Phytol. 2019;225:2152–65.

12. Peiffer JA, Spor A, Koren O, Jin Z, Tringe SG, Dangl JL, et al. Diversity and heritability of the maize rhizosphere microbiome under field conditions. Proc Natl Acad Sci U S A. 2013;110:6548–53.

13. Deng S, Caddell DF, Xu G, Dahlen L, Washington L, Yang J, et al. Genome wide association study reveals plant loci controlling heritability of the rhizosphere microbiome. ISME

J 2021 1511. 2021;15:3181–94.

14. Grieneisen L, Dasari M, Gould TJ, Björk JR, Grenier JC, Yotova V, et al. Gut microbiome heritability is nearly universal but environmentally contingent. Science (80-). 2021;373:181–6.

15. Wagner MR, Tang C, Salvato F, Clouse KM, Bartlett A, Vintila S, et al. Microbe-dependent heterosis in maize. Proc Natl Acad Sci. 2021;118:e2021965118.

16. Walters WA, Jin Z, Youngblut N, Wallace JG, Sutter J, Zhang W, et al. Large-scale replicated field study of maize rhizosphere identifies heritable microbes. Proc Natl Acad Sci U S A. 2018;115:7368–73.

17. Henry LP, Bruijning M, Forsberg SKG, Ayroles JF. The microbiome extends host evolutionary potential. Nat Commun. 2021;12:5141.

18. Mueller UG, Linksvayer TA. Microbiome breeding: conceptual and practical issues. Trends Microbiol. 2022;30:997–1011.

19. Wagner MR, Lundberg DS, Coleman-Derr D, Tringe SG, Dangl JL, Mitchell-Olds T. Natural soil microbes alter flowering phenology and the intensity of selection on flowering time in a wild Arabidopsis relative. Ecol Lett. 2014;17:717–26.

20. Petersen C, Hamerich IK, Adair KL, Griem-Krey H, Torres Oliva M, Hoeppner MP, et al. Host and microbiome jointly contribute to environmental adaptation. ISME J. 2023;17:1953–65.

21. Pogoda CS, Keepers KG, Reinert S, Talukder ZI, Smart BC, Attia Z, et al. Heritable differences in abundance of bacterial rhizosphere taxa are correlated with fungal necrotrophic pathogen resistance. Mol Ecol. 2023;00:1–14.

22. Baltrus DA. Adaptation, specialization, and coevolution within phytobiomes. Curr Opin Plant Biol. 2017;38:109–16.

23. Koskella B, Bergelson J. The study of host–microbiome (co)evolution across levels of selection. Philos Trans R Soc B. 2020;375:20190604.

24. Johnson MTJ, Stinchcombe JR. An emerging synthesis between community ecology and evolutionary biology. Trends Ecol Evol. 2007;22:250–7.

25. VanWallendael A, Benucci GMN, da Costa PB, Fraser L, Sreedasyam A, Fritschi F, et al. Host genotype controls ecological change in the leaf fungal microbiome. PLOS Biol. 2022;20:e3001681.

26. Bodenhausen N, Bortfeld-Miller M, Ackermann M, Vorholt JA. A Synthetic Community Approach Reveals Plant Genotypes Affecting the Phyllosphere Microbiota. PLOS Genet. 2014;10:e1004283.

27. Pincebourde S, Woods HA. Climate uncertainty on leaf surfaces: the biophysics of leaf microclimates and their consequences for leaf-dwelling organisms. Funct Ecol. 2012;26:844–53.

28. Runkle E. The Boundary Layer and Its Importance. Greenhouse Product News. 2016;:34.

29. Vacher C, Hampe A, Porté AJ, Sauer U, Compant S, Morris CE. The Phyllosphere: Microbial Jungle at the Plant–Climate Interface. Annu Rev Ecol Evol Syst. 2016;47:1–24.

30. Núñez A, García AM, Moreno DA, Guantes R. Seasonal changes dominate long-term variability of the urban air microbiome across space and time. Environ Int. 2021;150:106423.

31. Humphrey PT, Nguyen TT, Villalobos MM, Whiteman NK. Diversity and abundance of

phyllosphere bacteria are linked to insect herbivory. Mol Ecol. 2014;23:1497–515.

32. Meyer KM, Porch R, Muscettola IE, Luisa A, Vasconcelos S, Sherman JK, et al. Plant neighborhood shapes diversity and reduces interspecific variation of the phyllosphere microbiome. ISME J 2022. 2022;:1–12.

33. Nicotra AB, Leigh A, Boyce CK, Jones CS, Niklas KJ, Royer DL, et al. The evolution and functional significance of leaf shape in the angiosperms. Functional Plant Biology. 2011;38:535–52.

34. Givnish TJ. Comparative Studies Of Leaf Form: Assessing The Relative Roles Of Selective Pressures And Phylogenetic Constraints. New Phytol. 1987;106:131–60.

35. Yates MJ, Verboom GA, Rebelo AG, Cramer MD. Ecophysiological significance of leaf size variation in Proteaceae from the Cape Floristic Region. Funct Ecol. 2010;24:485–92.

36. Elmore CD. Mode of Reproduction and Inheritance of Leaf Shape in Ipomoea hederacea . Weed Sci. 1986;34:391–5.

37. Bright KL, Rausher MD. Natural selection on a leaf-shape polymorphism in the ivyleaf morning glory (Ipomoea hederacea). Evolution (N Y). 2008;62:1978–90.

38. Campitelli BE, Stinchcombe JR. Natural selection maintains a single-locus leaf shape cline in Ivyleaf morning glory, Ipomoea hederacea. Mol Ecol. 2013;22:552–64.

39. Campitelli BE, Stinchcombe JR. Testing potential selective agents acting on leaf shape in Ipomoea hederacea: Predictions based on an adaptive leaf shape cline. Ecol Evol. 2013;3:2409–23.

40. Campitelli BE, Gorton AJ, Ostevik KL, Stinchcombe JR. The effect of leaf shape on the thermoregulation and frost tolerance of an annual vine, Ipomoea hederacea (Convolvulaceae). Am J Bot. 2013;100:2175–82.

41. Hull-Sanders HM, Eubanks MD, Carr DE. Inbreeding depression and selfing rate of Ipomoea hederacea var. integriuscula (Convolvulaceae). Am J Bot. 2005;92:1871–7.

42. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods. 2012;9:671–5.

43. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat Biotechnol 2019 378. 2019;37:852–7.

44. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-resolution sample inference from Illumina amplicon data. Nat Methods. 2016;13:581–3.

45. McDonald D, Price MN, Goodrich J, Nawrocki EP, Desantis TZ, Probst A, et al. An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. ISME J . 2011;6:610–8.

46. Katoh K, Misawa K, Kuma KI, Miyata T. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res. 2002;30:3059–66.

47. Price MN, Dehal PS, Arkin AP. FastTree 2 – Approximately Maximum-Likelihood Trees for Large Alignments. PLoS One. 2010;5:e9490.

48. R Core Team. R: A language and environment for statistical computing. 2022.

49. Wickham H, Averick M, Bryan J, Chang W, McGowan L, François R, et al. Welcome to the Tidyverse. J Open Source Softw. 2019;4:1686.

50. Kuznetsova A, Brockhoff PB, Christensen RHB. lmerTest Package: Tests in Linear Mixed Effects Models . J Stat Softw. 2017;82:1–26.

51. McMurdie PJ, Holmes S. phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLoS One. 2013;8:e61217.

52. Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, Mcglinn D, et al. Package "vegan" Version 2.5-7. 2020.

53. Lahti L, Shetty S. microbiome R package. 2019. https://bioconductor.org/packages/release/bioc/html/microbiome.html. Accessed 3 Apr 2023.

54. Fox J, Weisberg Sanford. An R Companion to Applied Regression. 3rd edition. Sage Publications; 2019.

55. Wilson AJ. Why h2 does not always equal Va / Vp□? J Evol Biol. 2008;21:647–50.

56. Bruijning M, Ayroles JF, Henry LP, Koskella B, Meyer KM, Metcalf CJE. Relative abundance data can misrepresent heritability of the microbiome. Microbiome. 2023;11:222.

57. Buse A. The Likelihood Ratio, Wald, and Lagrange Multiplier Tests: An Expository Note. Am Stat. 1982;36:153–7.

58. Friesen ML, Porter SS, Stark SC, Von Wettberg EJ, Sachs JL, Martinez-Romero E. Microbially Mediated Plant Functional Traits. Annu Rev Ecol Evol Syst. 2011;42:23–46.

59. Falconer D, Mackay T. Introduction to Quantitative Genetics. 4th edition. England: Prentice Hall, Harlow; 1996.

60. Schweitzer JA, Bailey JK, Fischer DG, LeRoy CJ, Lonsdorf E V., Whitham TG, et al. Plant–Soil–Microorganism Interactions: Heritable Relationship Between Plant Genotype And Associated Soil Microorganisms. Ecology. 2008;89:773–81.

61. Goodrich JK, Waters JL, Poole AC, Sutter JL, Koren O, Blekhman R, et al. Human Genetics Shape the Gut Microbiome. Cell. 2014;159:789–99.

62. Wallace JG, Kremling KA, Kovar LL, Buckler ES. Quantitative genetics of the maize leaf microbiome. Phytobiomes J. 2018;2:208–24.

63. John Wallace R, Sasson G, Garnsworthy PC, Tapio I, Gregson E, Bani P, et al. A heritable subset of the core rumen microbiome dictates dairy cow productivity and emissions. Sci Adv. 2019;5:8391–4.

64. Edwards JA, Saran UB, Bonnette J, MacQueen A, Yin J, Nguyen T uyen, et al. Genetic determinants of switchgrass-root-associated microbiota in field sites spanning its natural range. Curr Biol. 2023;33:1926-1938.e6.

65. Cavanagh C, Morell M, Mackay I, Powell W. From mutations to MAGIC: resources for gene discovery, validation and delivery in crop plants. Curr Opin Plant Biol. 2008;11:215–21.

66. Yu J, Holland JB, McMullen MD, Buckler ES. Genetic Design and Statistical Power of Nested Association Mapping in Maize. Genetics. 2008;178:539–51.

67. Campitelli BE, Stinchcombe JR. Population dynamics and evolutionary history of the weedy

vine Ipomoea hederacea in North America. G3 Genes, Genomes, Genet. 2014;4:1407–16.

68. Stock AJ, Campitelli BE, Stinchcombe JR. Quantitative genetic variance and multivariate clines in the Ivyleaf morning glory, Ipomoea hederacea. Philos Trans R Soc B Biol Sci. 2014;369:20130259.

69. Henry GA, Stinchcombe JR. G-matrix stability in clinally diverging populations of an annual weed. Evolution (N Y). 2023;77:49–62.

70. Ennos RA. Quantitative studies of the mating system in two sympatric species of Ipomoea (Convolvulaceae). Genetica. 1981;57:93–8.

71. Weinig C, Dorn LA, Kane NC, German ZM, Halldorsdottir SS, Ungerer MC, et al. Heterogeneous Selection at Specific Loci in Natural Environments in *Arabidopsis thaliana*. Genetics. 2003;165:321–9.

72. Weinig C, Stinchcombe JR, Schmitt J. Evolutionary Genetics Of Resistance And Tolerance To Natural Herbivory In Arabidopsis thaliana. Evolution (N Y). 2003;57:1270–80.

73. Vasemägi A. The Adaptive Hypothesis of Clinal Variation Revisited: Single-Locus Clines as a Result of Spatially Restricted Gene Flow. Genetics. 2006;173:2411–4.

74. Lippincott JA, Lippincott BB. Tumour-initiating Ability and Nutrition in the Genus Agrobacterium. J Gen Microbiol. 1969;59:57–75.

75. Lee HS, Madhaiyan M, Kim CW, Choi SJ, Chung KY, Sa TM. Physiological enhancement of early growth of rice seedlings (Oryza sativa L.) by production of phytohormone of N2-fixing methylotrophic isolates. Biol Fertil Soils. 2006;42:402–8.

76. Wang NR, Wiesmann CL, Melnyk RA, Hossain SS, Chi M-H, Martens K, et al. Commensal Pseudomonas fluorescens Strains Protect Arabidopsis from Closely Related Pseudomonas Pathogens in a Colonization-Dependent Manner. MBio. 2022;13:e02892-21.

77. Kämpfer P, Glaeser SP, McInroy JA, Busse H-J. Nocardioides zeicaulis sp. nov., an endophyte actinobacterium of maize. Int J Syst Evol Microbiol. 2016;66:1869–74.

78. Wang P, Kong X, Chen H, Xiao Y, Liu H, Li X, et al. Exploration of Intrinsic Microbial Community Modulators in the Rice Endosphere Indicates a Key Role of Distinct Bacterial Taxa Across Different Cultivars. Front Microbiol. 2021;12:629852.

79. Nafis A, Raklami A, Bechtaoui N, El Khalloufi F, El Alaoui A, Glick BR, et al. Actinobacteria from Extreme Niches in Morocco and Their Plant Growth-Promoting Potentials. Diversity. 2019;11:139.

80. Dastager SG, Lee J-C, Pandey A, Kim C-J. Nocardioides mesophilus sp. nov., isolated from soil. Int J Syst Evol Microbiol. 2010;60:2288–92.

81. Escudero A, Mediavilla S. Decline in photosynthetic nitrogen use efficiency with leaf age and nitrogen resorption as determinants of leaf life span. J Ecol. 2003;91:880–9.

82. England JR, Attiwill PM. Changes in leaf morphology and anatomy with tree age and height in the broadleaved evergreen species, Eucalyptus regnans F. Muell. Trees. 2006;20:79.

83. Pantin F, Simonneau T, Muller B. Coming of leaf age: control of growth by hydraulics and metabolics during leaf ontogeny. New Phytol. 2012;196:349–66.

84. van Butselaar T, Van den Ackerveken G. Salicylic Acid Steers the Growth–Immunity

Tradeoff. Trends Plant Sci. 2020;25:566–76.

85. Redford AJ, Fierer N. Bacterial succession on the leaf surface: a novel system for studying successional dynamics. Microb Ecol. 2009;58:189–98.

86. Copeland JK, Yuan L, Layeghifard M, Wang PW, Guttman DS. Seasonal Community Succession of the Phyllosphere Microbiome. Mol Plant-Microbe Interact. 2015;28:274–85.

87. Dove NC, Veach AM, Muchero W, Wahl T, Stegen JC, Schadt CW, et al. Assembly of the Populus Microbiome Is Temporally Dynamic and Determined by Selective and Stochastic Factors . mSphere. 2021;6.

88. Xu CCY, Lemoine J, Albert A, Whirter É Mac, Barrett RDH. Community assembly of the human piercing microbiome. Proc R Soc B Biol Sci. 2023;290.