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RESEARCH ARTICLE

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Continental sampling reveals core bacterial and environmentally driven fungal leaf endophytes in *Heuchera*

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Abstract

Premise: Endophytic plant-microbe interactions range from mutualistic relationships that confer important ecological and agricultural traits to neutral or quasi-parasitic relationships. In contrast to root-associated endophytes, the role of environmental and host-related factors in the acquisition of leaf endophyte communities at broad spatial and phylogenetic scales remains sparsely studied. We assessed endofoliar diversity to test the hypothesis that membership in these microbial communities is driven primarily by abiotic environment and host phylogeny.

Methods: We used a broad geographic coverage of North America in the genus *Heuchera* L. (Saxifragaceae), representing 32 species and varieties across 161 populations. Bacterial and fungal communities were characterized using 16S and ITS amplicon sequencing, respectively, and standard diversity metrics were calculated. We assembled environmental predictors for microbial diversity at collection sites, including latitude, elevation, temperature, precipitation, and soil parameters.

Results: Assembly patterns differed between bacterial and fungal endophytes. Host phylogeny was significantly associated with bacteria, while geographic distance was the best predictor of fungal community composition. Species richness and phylogenetic diversity were consistent across sites and species, with only fungi showing a response to aridity and precipitation for some metrics. Unlike what has been observed with root-associated microbial communities, in this system microbes show no relationship with pH or other soil factors.

Conclusions: Overall, this work improves our understanding of the large-scale patterns of diversity and community composition in leaf endophytes and highlights the relative significance of environmental and host-related factors in driving different microbial communities within the leaf microbiome.

KEYWORDS

bacteria, community assembly, core microbiome, fungi, Heuchera, leaf endophyte, microbial diversity, plant microbiome, plant-microbe interactions, Saxifragaceae

Plants are hosts to a wide variety of microbial assemblages, including those that spend all or a portion of their lifetime within plant tissues, known as endophytes (Hardoim et al., 2015). Endophytic plant-microbe associations, probably universal across the land plants, confer such positive functional capacities as abiotic stress response, growth promotion, beneficial life history traits, and pathogen or herbivore defense, as well as the potential for negative interactions that can approach pathogenic relationships (Hardoim et al., 2008; Khare et al., 2018; Dini-Andreote, 2020; Trivedi et al., 2020; O'Brien et al., 2021). Endophytic relationships are relatively well characterized in several economically important species such as major pasture grasses (Clay, 1990; Leuchtmann, 1992; Schardl and Tsai, 1992) and crop plants (Fisher and Petrini, 1992; Fisher et al., 1992; Larran et al., 2002; Comby et al., 2016; Correa-Galeote et al., 2018), particularly under regulated experimental conditions. In natural environments, endophyte diversity surveys have been conducted at broad

phylogenetic (Yeoh et al., 2017) and geographic (Yang et al., 2019) scales but have mainly been focused on root-associated microbiomes. These natural surveys show broadly that soil properties are the most important drivers of plant-associated microbiome diversity, much as in free-living soil microbiomes (Thompson et al., 2017; Bahram et al., 2018).

The leaf is a vast ecosystem that supports a diverse range of microbial communities and has been a subject of rapidly rising interest in endophyte research in the past two decades (Harrison and Griffin, 2020). Several studies investigating foliar endophyte communities have documented the roles of host identity, environment, and geographic distance in shaping patterns of leaf endophyte community diversity and assembly (e.g., Zimmerman and Vitousek, 2012; Ding and Melcher, 2016; Huang, 2020; Mina et al., 2020; Luo et al., 2024). However, unlike research on root-associated communities, very few of these studies have investigated these factors within a broad spatial and phylogenetic context, especially in natural systems (Harrison and Griffin, 2020). Hence, our current understanding of the factors that drive patterns of leaf endophyte diversity is primarily based on surveys with a narrow geographic and taxonomic range or a focus on agricultural systems in controlled environments. In addition, most of these studies focus on either fungal or bacterial endophyte communities, limiting our knowledge of the relative influence of host and environmental factors on both microbial communities inside plants.

Host plant phylogeny plays an important and incompletely characterized subsidiary role for both bacterial and fungal communities, a role possibly rooted in shared evolutionary history or conserved plant host traits (Yeoh et al., 2017; Yang et al., 2019). An evolutionary host effect on endophytes may indicate either (1) functional selection of associated microbes by the plant (or vice versa) or (2) shared coevolutionary history between plants and their endophytes. Particularly, a strong case exists for potential host phylogenetic constraints on leaf endophyte communities operating through phylogenetically conserved differences in leaf tissue traits across taxa (Tellez et al., 2022). In addition, there is potential for vertical transmission (particularly well characterized in grasses; Schardl, 2001; Bright and Bulgheresi, 2010) and semi-vertical transmission within hosts through primarily within-population sources of infection (Frank et al., 2017; Kandel et al., 2017).

Abiotic variables have also been linked to differences in patterns of endophyte community diversity and membership, including latitude, elevation, climatic factors, soil properties, and geographic distance. For example, the classic latitudinal diversity gradient pattern has been observed in foliar fungal endophytes, demonstrating increased species richness at lower latitudes (Arnold and Lutzoni, 2007). However, whether bacteria and other microbial communities follow the same pattern is still uncertain, due to the relatively few studies that simultaneously examine both and the differing results among studies. At a local scale, variation in endophyte community structure along elevation has been documented in several host plant species (e.g., Zimmerman and Vitousek, 2012; Cai et al., 2020; Fu et al., 2022) and has been suggested to be connected to differences in vegetation type and, ultimately, climatic factors across the gradient (Huang, 2020). Temperature and precipitation are likewise known to be strongly correlated with endophyte community structure and diversity (Herrera et al., 2011; Giauque and Hawkes, 2013; Glynou et al., 2016), although their influence has been shown to vary between fungal and bacterial communities. For instance, Huang (2020) found mean annual temperature and annual precipitation to be strongly correlated with foliar fungal endophyte community structures in several gymnosperms and *Rhododendron* spp. across forests in Taiwan. By contrast, temperature and precipitation had limited effect on endophytic bacterial communities in examinations of aboveground tissues of several tree species (Firrincieli et al., 2020; Wang et al., 2023).

Soil environment has also been previously suggested as a primary driver of root-associated microbial communities (Yeoh et al., 2017; Barraza et al., 2020; but see Glynou et al., 2016), but the role of edaphic factors for assembling leaf endophyte microbiomes is still sparsely studied. Leaf endophyte communities could be more insulated from the effects of soil physico-chemical properties because of the more controlled environment of internal leaf tissues across varying soil substrates, especially in contrast to rhizosphere communities. The composition of foliar endophyte communities should correspondingly have a stronger response to climatic abiotic factors than to soil substrate properties. Nevertheless, these responses may differ in relation to varying endophyte recruitment processes and contrasting dispersal ecologies of fungal and bacterial endophytes. Indeed, geographic distance has repeatedly been reported as a major determinant of fungal, but not bacterial, endophyte community composition, with dispersal limitation being invoked as a mechanism to explain variation across space (Collado et al., 1999; Langenfeld et al., 2013; Fang et al., 2019).

Host phylogeny, climate, soil, and geographic distance are all compelling explanatory factors for endophytes, and each has prior evidence in different systems. However, in addition to disagreement among studies (e.g., Langenfeld et al., 2013; Kembel and Mueller, 2014; Glynou et al., 2016; Yeoh et al., 2017; Kivlin et al., 2022; Wang et al., 2023), how associations of plant-associated microbial communities with these factors might change across a wider spatial and phylogenetic scale remains underexplored. For instance, a study system linking population-level and phylogenetic scales (Graham et al., 2018) would provide insight into the phylogenetic level at which host specificity is relevant—are endophyte interactions species-specific, different between major clades, or shared across flowering or even land plants? A multi-scale view would be needed to ask these questions and would also make progress toward linking the incongruent results from phylogenetically broad and single-species surveys performed to date. As advocated by Jung et al. (2021), multi-scale research is also important for generating genotype × environment viewpoints on plant microbiomes and giving researchers additional power to

dissect factors that promote different microbiome assemblages.

Here, we take a novel approach that uses broad geographic coverage of North America within the restricted phylogenetic scope of a recent radiation. Using the host system *Heuchera* L., a cliff-dwelling genus of flowering plants in the family Saxifragaceae with well-characterized phylogenetic relationships and habitat specialization patterns across the genus (Folk et al., 2017, 2018a, 2023), we leverage strong phylogenetic and population sampling to explicitly assess diversity trends at multiple evolutionary levels, from phylogenetic to within-population diversity. We assembled a series of predictors via global environmental layers, including elevation, temperature, precipitation, soil parameters, and latitude. We then used multiple assessments of leaf endophyte diversity to test the hypothesis that these communities, in contrast to root-associated microbiomes, are defined primarily (1) by non-edaphic abiotic environmental variables and (2) by host phylogeny. Finally, we assessed both bacterial and fungal endophyte components to ask whether these communities are shaped (3) by distinct environmental factors.

MATERIALS AND METHODS

Host organism

Heuchera is a genus of ~45 species of flowering plants in Saxifragaceae that is endemic to rock outcrops and montane areas in North America. It occurs from sea level to ~4000 m of elevation across broad temperate environmental gradients including temperate deciduous and evergreen woodland, plains, high alpine scree, and chaparral. Edaphic variation is also high and ranges from strong calciphile taxa (e.g., *H. longiflora*) to some of the most acidic substrates in North America (*H. parviflora* var. *saurensis*), with many narrow endemics particular to specific rock substrates. Hence, this genus forms a robust system for evaluating plant-microbe interactions across strong, continent-level environmental gradients. Aside from small numbers of taxa included in broad surveys (e.g., Jumpponen and Trappe, 1998; Zhang and Yao, 2015), characterizations of arbuscular mycorrhizae (Anneberg and Segraves, 2019), and pharmacognostical evaluations (Wawrosch et al., 2023), endophytic microbial associates are currently unknown for Saxifragaceae.

Sampling

We began with broad species-level sampling across the study group, including 32 of the 64 currently recognized specific and subspecific taxa (50%). Taxa covered are geographically representative of the range of the genus north of Mexico (Figure 1) and include all recognized sections (Folk, 2015). In addition to this broad phylogenetic-aware sampling of the host plant genus, we leveraged population-level sampling from two previous studies on host plant phylogeography in the *Heuchera parviflora* species complex (Folk and Freudenstein, 2015) and the *H. longiflora* complex (Folk et al., 2018b), as well as new sampling performed for this study in the *H. americana* × *H. richardsonii* hybrid zone (see Wells, 1984). The newly sampled taxa were in the *H. americana* group (*H. americana* var. *americana*, *H. americana* var. *hirsuticaulis*, and *H. richardsonii*), the *H. longiflora* group (*H. longiflora* var. *aceroides* and *H. longiflora* var. *longiflora*), and the *H. parviflora* group (*H. missouriensis, H. parviflora* var. *parviflora*, *H. parviflora* var. *saurensis*, and *H. puberula*). Samples were taken from newly expanded leaves collected during peak green season in late spring (May–June). In total, we collected 178 leaf samples, one from each host individual, representing 32 species and varieties of *Heuchera* as well as four closely related outgroup species, with each replicate representing a single leaf. Among the samples, 142 were subjected to bacterial sequencing (a mean of five replicates per host taxon) and 150 to fungal sequencing (a mean of five replicates per host taxon). Sampling is summarized in Figure 1 and Appendices S1 and S2.

DNA extraction

Plant materials were either rapidly frozen at −80°C and subsequently dehydrated or primarily dried in silica-gel prior to extraction. For each DNA extraction, we chose 20–30 mg of tissue from a leaf from a single plant without visible lesions or other obvious disease symptoms. The tissues were incubated for 1 min each in 70% molecular-grade ethanol and 5% bleach to disrupt and eliminate DNA of potential epiphytic microbes, respectively. Tissues were then washed twice in molecular-grade water to remove residual bleach and homogenized with metal beads in a Fisherbrand Bead Mill 24 homogenizer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). We extracted DNA with a standard CTAB protocol (Doyle and Doyle, 1987) with the addition of 90 mg ascorbic acid and 100 mg polyvinylpyrrolidone-40 (PVP-40) per extraction to eliminate plant secondary compounds, per previous optimizations on this plant material (Folk and Freudenstein, 2014). Finally, all extractions were cleaned using a silica column (GeneJET PCR purification kit, Thermo Fisher Scientific) per manufacturer instructions, and extractions were quantified with an Invitrogen Qubit 4 Fluorometer using Qubit Broad Range assay reagents (Thermo Fisher Scientific).

Amplification methods

We used two different amplicon sequencing approaches to characterize both bacterial and fungal communities. Bacterial sequencing was validated in house using primers 515 f and 806r from the Earth Microbiome Project (Thompson et al., 2017) targeting the V4 region of 16S ribosomal DNA and the following thermocycler protocol: initial denaturation at 95°C for

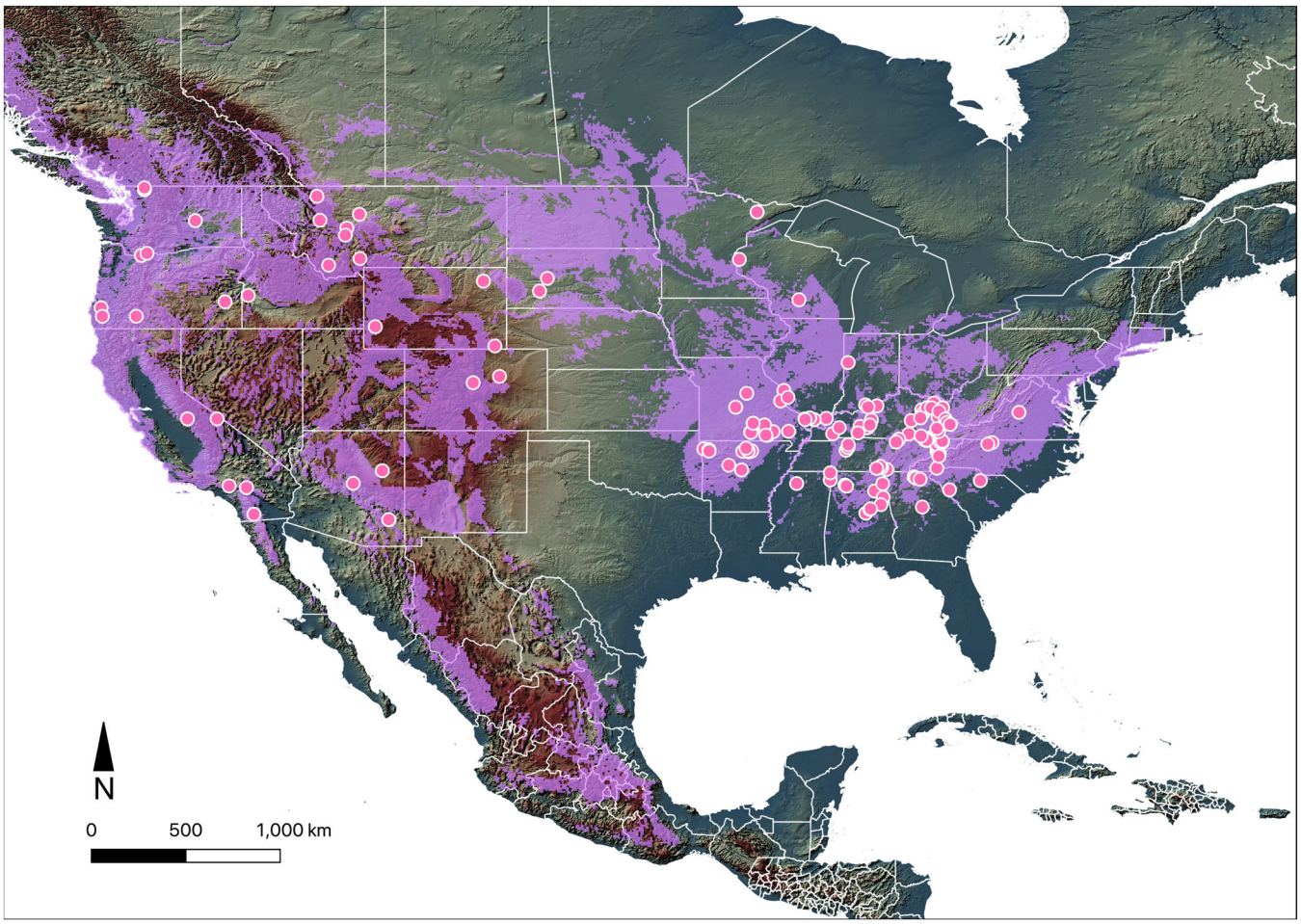

FIGURE 1 Map of *Heuchera* sample locations (pink circles) against genus range (purple shading) based on species distribution modeling data from Folk et al. (2023). Map generated using QGIS version 3.24 (QGIS Development Team, 2021). For sampling data, see Appendices S1 and S2.

3 min, then 35 cycles of 95°C for 45 s, annealing at 52°C for 1 min, and 72°C for 1.5 min, then a final elongation step of 72°C for 10 min. For successful amplicons (see Appendix S1), total DNAs were concentration-normalized and sent to the Michigan State University RTSF Genomics Core (East Lansing, Michigan, USA) for sequencing of 250 bp paired-end reads on a MiSeq System (Illumina, San Diego, California, USA) using a one-step amplification protocol (Kozich et al., 2013). All amplification steps used DreamTAQ Mastermix (Thermo Fisher Scientific), with primer concentrations of 0.5 μM except as noted below, and were performed with filter pipette tips under a dedicated PCR hood that was bleach- and UV-sterilized before each use to minimize contamination.

Fungal characterization used the ITS1 region and the primers ITS1FI2 and ITS2 from Schmidt et al. (2013). To verify the presence of amplifiable DNA, we first validated the presence of the desired product using the primers directly and the following thermocycler protocol: initial denaturation at 95°C for 3 min, then 35 cycles of 95°C for 45 s, annealing at 50°C for 1 min, and 72°C for 1 min, then a final elongation step of 72°C for 10 min. We then reamplified successful samples from total DNA using ITS1FI2 and ITS2 primers that were tagged with 5′ end overhangs specified by the sequencing center using the following thermocycler protocol: initial denaturation at 95°C for 5 min; then 30 cycles of 95°C for 30 s, annealing at 52°C for 30 s, and 72°C for 30 s; then a final elongation step of 72°C for 5 min. Primers for this reaction were at 0.1 μM. Successful amplicons were submitted to the Michigan State University RTSF Genomics Core for a second barcoding amplification and sequencing. Sequencing instrumentation and wet lab precautions followed those for 16S (above).

Sequence processing

We performed sequence analyses in the "QIIME 2" package version 2020.8 (Caporaso et al., 2010; Bolyen et al., 2019). Reads were first denoised via Dada2 (Callahan et al., 2016) in order to error-correct and merge paired-end reads and remove sequence chimeras. As part of this step, primers were trimmed from the 5′ end and, based on Phred quality plots in FastQC (Andrews, 2015), 50 bp were trimmed from the 3′ end of the R2 reads.

For taxonomic classification, we used the Greengenes database (McDonald et al., 2012) for bacterial 16S reads, and the UNITE database (Nilsson et al., 2019) for fungal ITS reads, following recommendations in the QIIME 2 documentation for preparing the taxonomic classifier via a naive Bayesian approach (QIIME 2 module fit-classifier-naive-bayes). We clustered the Greengenes database at 97% and UNITE at 99% identity. We then performed taxonomic classifications of the merged reads against these databases using QIIME 2 module "sklearn" (Pedregosa et al., 2011). For endophyte tissues, 16S and ITS amplicon sequencing approaches were expected to generate host plant DNA sequences due to off-target amplification of organellar 16S rDNA and nuclear ITS, respectively. Based on extensive optimizations, we implemented separate strategies for efficiently removing host DNA from each of these genetic loci. For 16S, we removed host DNA using annotated chloroplast and mitochondrial operational taxonomic unit (OTU) classifications from the Greengenes taxonomy—level 3 (class) and level 5 (family), respectively. For ITS, we customized the UNITE database by adding host plant ITS sequences we have previously generated (Folk and Freudenstein, 2014), and removed host sequences based on level 6 (genus) OTU classifications.

We also performed taxonomic classification for bacterial 16S reads using the updated Greengenes2 database (McDonald et al., 2023) with similar methods to those described above. However, Greengenes2 lacks representation of eukaryotic mitochondrial and chloroplast sequences, which generated very high proportions of unidentified sequences and likely spurious Bacteroidota identifications (see Appendix S3). Both of these most likely reflect incorrect identifications of the organellar genome of the host. Thus, determinations from the original Greengenes database were used for downstream analysis.

Environmental predictor assembly

We used globally interpolated datasets to infer environmental factors at each collection locality. The variables used and their sources were as follows: mean annual temperature (measured in °C) and annual precipitation (in millimeters) (BIOCLIM; Hijmans et al., 2005); aridity (see below); elevation (in meters) (GTOPO30, https://www.usgs.gov/centers/eros/science/usgs-eros-archive-digital-elevation-global-30-arc-second-elevation-gtopo30); and soil pH, sand percent, and carbon content (the last measured in permilles) (SoilGrids; Hengl et al., 2017). An aridity index was calculated as precipitation/potential evapotranspiration (see Middleton et al., 1992) using data from WorldClim2 and Envirem (Fick and Hijmans, 2017; Title and Bemmels, 2018). Note that this aridity index *decreases* with increasing aridity; arid conditions are generally those with index values < 0.5. Environmental values were associated with geolocated sampling localities using scripts published previously (https://github.com/ryanafolk/Saxifragales_spatial_scripts/tree/master/Extract_point_values). Finally, given that varying latitudinal gradients in diversity have been documented for soil (Bahram et al., 2018) and marine microbes (Ibarbalz et al., 2019), we also directly used the latitude of our collecting localities as a predictor.

Community diversity

We used QIIME 2 to generate two primary descriptors of community diversity. First, we characterized measures of overall diversity using Shannon entropy, a diversity measure that includes both taxon presence-absence information and abundance. We then calculated Faith's phylogenetic diversity (PD), which represents the sum of phylogenetic branch lengths connecting a microbial community. We applied these diversity metrics to only the three species groups with strong population sampling to enable comparisons among host taxa with replicate sampling. Given the presence of high levels of host DNA and relatively low endophyte diversity per sample (see Bulgarelli et al., 2013; Kivlin et al., 2022), and despite a high sequencing effort in many samples (Appendix S4), sequence rarefaction was set to 50 for diversity statistics. This number was based on rarefaction analyses conducted at up to 50, 150, 500, and 1000 sequences, respectively, to test the rarefaction that best captures diversity while still including as many samples as possible. For bacterial endophytes, these analyses indicated that 50 non-host sequences (i.e., ~5000 total sequencing reads) sample most of the diversity in the community, as suggested by the rarefaction curves for Shannon diversity reaching a saturation plateau at 50 sequences (see Appendix S5). We also evaluated the impact of sequence rarefaction by repeating downstream analyses with varying sequence cutoffs (no rarefaction and 350 for ITS; 350 and 500 for 16S; see Appendix S6) and found that most results were insensitive to rarefaction. We observed only one difference in terms of significance in fungal Shannon diversity (recovery of a significant effect of latitude at the 350 cutoff), a result that may be due to sampling bias from dropped samples. For fungal endophytes, a cutoff of 50 non-host sequences was relatively substantial and still accounted for variations in sequencing depth between samples (compared to analyses based on non-rarefied data; Appendix S6).

We used both a linear modeling (LM) and a linear mixed-modeling (LMM) framework in R package "lme4" version 1.1.33 (Bates et al., 2015) to understand how these diversity statistics relate separately to environmental drivers and host identity. We constructed models using both the "lm" function in R package "stats" version 4.1.2 (R Core Team, 2021) for LM and the "lmer function" in R package "lme4" version 1.1.33 (Bates et al., 2015) for LMM. For model comparison and selection, we used both manual and automated modeling approaches. For the manual modeling approach, we constructed univariate linear models with each environmental predictor, a null model (in R notation, diversity ~ 1) and a multivariate linear model that included all the predictors. We then performed model selection via

the Akaike information criterion (AIC) using the "aicw" function in the R package "geiger" version 2.0.11 (Pennell et al., 2014) and assessed predictor statistical significance with the "summary.lm" function in R package "stats." For the automated approach, we constructed a linear mixed model with all environmental predictors included as fixed model terms. Host plant species taxonomy was also included as a random term in LMM to separately partition variation attributable to host taxon. We then used the "step" function in R package "lmerTest" version 3.1.3 (Kuznetsova et al., 2017) to perform model selection via AIC and calculate predictor significance using an automated backwards approach. Analyses were performed using R version 4.1.2.

Community composition

In order to characterize differences among microbial communities in terms of taxon composition, we used the UniFrac distance metric, which accounts both for shared taxon presence/absence and for phylogenetic branch length, here including all samples. We used a Mantel testing approach in R package "vegan" version 2.6.4 (Oksanen et al., 2022) to ask whether matrices of UniFrac distance were associated with each of either geographic distance, environment, or host phylogenetic distance. Environment distances were Euclidean distances on the environmental predictors, where two matrices were prepared segregating the environmental predictors into soil (pH, sand percent, and organic carbon content) and non-soil factors (latitude, temperature, precipitation, elevation, and aridity index). Geographic distances were geodesic distances (converted from objects containing longitude and latitude coordinates), calculated using the geodist_vec function in R package "geodist" version 0.0.8 (Padgham, 2021). Since geographic and environmental distances were strongly correlated, we additionally used a partial Mantel approach in R package "vegan" to attempt to control environmental factors for geography. Host phylogenetic distances were patristic distances calculated from the host plant phylogeny of Folk et al. (2017); this was a phylogenetic estimate based on phylogenomic data with complete species-level sampling of the host plants used here. Since that previous phylogeny did not include population-level sampling, population samples were imputed by placing them within the phylogeny based on taxonomic identifications and assuming zero within-taxon branch lengths. Analyses were performed using R version 4.1.2.

RESULTS

Sequencing

For 16S sequencing, we recovered a mean of 236,938 reads per sample across 139 successful samples, with 1737 total bacterial OTUs across all samples and a mean of 97% host DNA prevalence. For ITS sequencing, we recovered a mean of 185,997 reads per sample across 133 successful samples, with a total of 1082 fungal OTUs and a mean of 99% host DNA prevalence; lower fungal diversity compared to bacterial diversity has been documented in leaf endophytes (Bulgarelli et al., 2013).

Taxonomic composition

The five most dominant bacterial phyla by decreasing order of prevalence were Proteobacteria (6–100% per sample), Bacteroidetes (0–83%), Actinobacteria (0–38%), Verrucomicrobia (0–13%), and Cyanobacteria (0–48%) (Figure 2A), as previously reported in other host species (Zarraonaindia et al., 2015; Coleman-Derr et al., 2016; de Souza et al., 2016; Ding and Melcher, 2016; Aydogan et al., 2018; Wemheuer et al., 2019; Mina et al., 2020; Yang et al., 2023). Finer-level classifications of recovered OTUs largely corresponded to typical endophytes documented elsewhere (Hallmann et al., 1997; Rosenblueth and Martínez-Romero, 2006; Miliute et al., 2015; Ding and Melcher, 2016; Afzal et al., 2019; Christian et al., 2021), such as (in decreasing order of overall prevalence for 16S) *Sphingomonas* (which reached highest prevalence at ≤100%), Comamonadaceae, Chitinophagaceae, *Methylobacterium*, *Blastomonas*, *Hymenobacter*, *Pseudomonas*, and Opitutaceae. Similar to other surveys in natural populations (Yeoh et al., 2017), potential diazotrophs (genera *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Frankia*) were observed at low frequencies (≤8% of total 16S reads) in almost all samples (Appendix S7).

For fungi, by far the most dominant phylum was Ascomycota (only missing in a single sample; otherwise, 5–100%), with Basidiomycota (0–44%, absent in a slight majority of samples), Olpidiomycota (0–77%, absent in most samples), and Mucoromycota (<1%) as minor community members (Figure 2B), similar to close relative *Saxifraga* (Zhang and Yao, 2015) and other plants (Zimmerman and Vitousek, 2012; Jin et al., 2013; Fan et al., 2020; Pang et al., 2022). As with bacteria, fungal fine-level OTU designations generally contain previously documented endophytes (Fisher et al., 1992; Araújo et al., 2001; Gamboa and Bayman, 2001; Romero et al., 2001; Zimmerman and Vitousek, 2012; Douanla-Meli et al., 2013; Jin et al., 2013; Matsumura and Fukuda, 2013; Zhang and Yao, 2015; Fang et al., 2019; Fan et al., 2020; Pang et al., 2022); in order of decreasing abundance the most prevalent were *Penicillium*, Pleosporaceae, *Septoria*, and *Alternaria* (all four ≤100% abundance), *Mycosphaerella*, *Tetracladium*, *Ramularia*, and *Colletotrichum* (Appendix S8).

Drivers of leaf endophyte diversity

Using both linear-model and mixed-model frameworks, we tested for a role of climate, soil environment, latitude, elevation, and species identity on leaf endophyte diversity as measured by Shannon entropy and Faith's PD. For bacteria, we found the null model was favored for both diversity

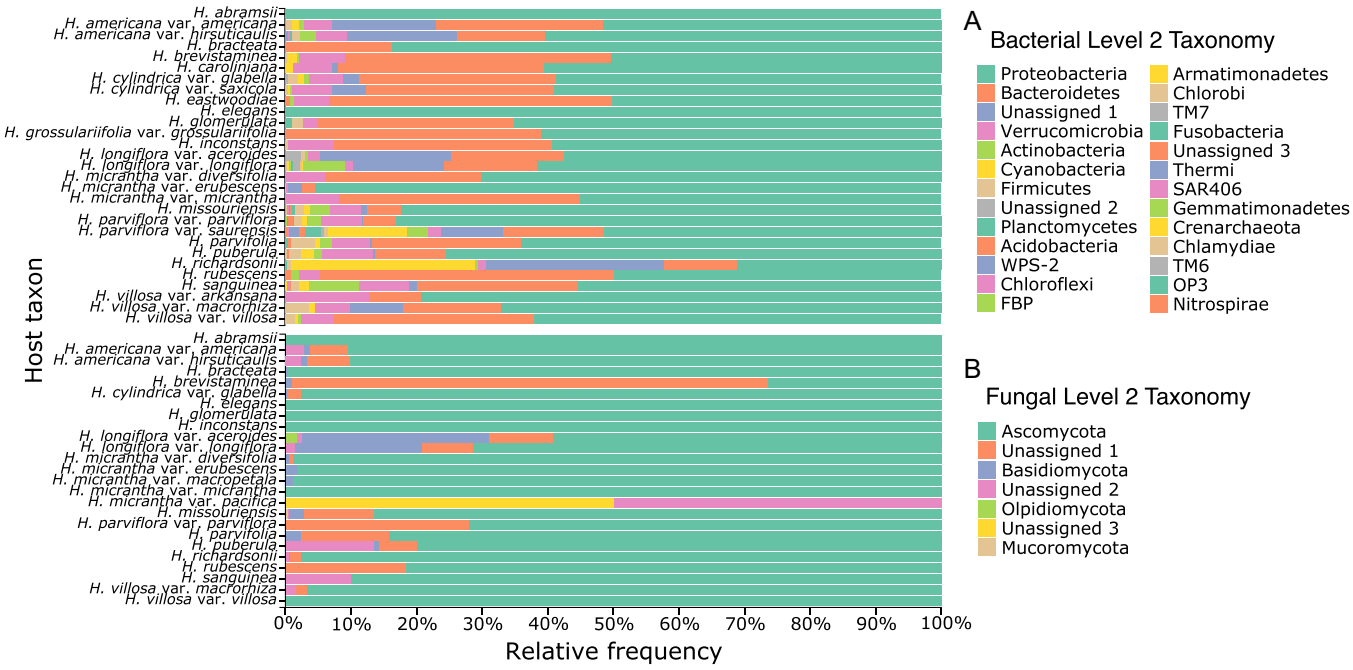

FIGURE 2 Foliar (A) bacterial and (B) fungal endophyte phylum-level diversity and relative frequency (%) across host (*Heuchera*) plant taxa.

metrics in either modeling framework, meaning that bacterial leaf endophyte diversity metrics were insensitive to the predictors we measured (Appendix S6). However, for fungi, manual modeling indicated that precipitation and aridity were significant predictors of Shannon diversity for fungal endophytes ($P = 0.0106$ and 0.0108, respectively; Table 1 and Figure 3), while aridity index was marginally significant for Faith's PD ($P = 0.05$; Table 1). Automated model selection likewise favored a model with only precipitation as the predictor (Shannon diversity ~ precipitation; $P = 0.0106$); aridity was not identified in the best model, perhaps due to collinearity with precipitation or due to the random host term (Appendix S6). The null model was favored for Faith's PD (Faith's PD ~ 1; Appendix S6). Considering potential host effects, based on examination of endophyte diversity vs. host identity boxplots (Figure 4), the only species group that showed distinctness in Shannon diversity or Faith's PD was the *H. parviflora* group, although this difference was not significant (16S: ANOVA, $F_{3,26} = 1.60$ and 1.80, $P = 0.212$ and 0.173, respectively; ITS: $F_{2,14} = 1.53$ and 0.74, $P = 0.25$ and 0.50, respectively; Figure 4); taxa in the other two species groups had near-identical means.

Drivers of leaf endophyte community composition

Using microbial UniFrac distances as a characterization of leaf endophyte community composition, we asked whether communities were associated with any of three potential drivers: geography (that is, isolation-by-distance), soil (pH, sand percent, and organic carbon content) or non-soil environment (latitude, temperature, precipitation, elevation, and aridity index), and host phylogeny. For bacteria, we found that only host phylogeny was significant (Mantel test, $P = 0.002$; Table 2 and Figure 5A). For fungi, we found that geography (Mantel test, $P < 0.001$; Table 2 and Figure 5B) and both soil and non-soil environment (Mantel test, $P = 0.03$ and 0.01, respectively; Appendix S6) were significantly associated with UniFrac distance. Given that we found spatial autocorrelation among both sets of environmental predictors (Mantel test, both $P < 0.05$), we controlled for geography using a partial Mantel approach. We found after this correction that both soil and non-soil environment were no longer significant ($P = 0.36$ and 0.43, respectively; Table 2) for fungi, indicating that geography was the best predictor of fungal community composition and the effect of environment independent of geography was negligible.

DISCUSSION

Here, to address a need for broad-scale spatial and phylogenetic studies investigating patterns of leaf endophyte diversity, we assessed the role of environmental and host-related factors in acquiring foliar endophyte microbiomes across a widely sampled geographic distribution and host phylogeny in the flowering plant genus *Heuchera* across North America. We found that fungal endophyte diversity was significantly greater in wetter sites, but bacterial endophyte diversity was consistent across environmental gradients and host species. Fungal and bacterial community structures were therefore independently predicted by distinct factors. Dissimilarity between fungal communities

TABLE 1 Results from manual modeling approach with Akaike information criterion (AIC) weights, ΔAIC, R^2, and *P*-values of linear models describing relationships between predictors and foliar fungal endophyte diversity metrics. Significance codes: *** 0, ** 0.001, * 0.01, † 0.05, (none) 0.1.

	Faith's PD					Shannon diversity			
Model	**AIC**	**ΔAIC**	**R^2**	***P***	**Model**	**AIC**	**ΔAIC**	**R^2**	***P***
Aridity index	190.30	0.00	0.06	0.05†	Precipitation	184.58	0.00	0.10	0.01*
Precipitation	190.74	0.44	0.05	0.07	Aridity index	184.63	0.05	0.10	0.01*
Null	192.14	1.84	0.00	0.36	Soil pH	188.63	4.04	0.04	0.10
Latitude	192.94	2.64	0.02	0.28	Latitude	188.69	4.11	0.04	0.11
Elevation	193.17	2.86	0.02	0.33	Soil sand %	188.91	4.32	0.03	0.12
Soil sand %	193.26	2.95	0.01	0.36	Null	189.39	4.80	0.00	0.35
Soil pH	193.50	3.20	0.01	0.43	Elevation	189.65	5.07	0.03	0.20
Soil carbon	193.74	3.44	0.01	0.54	Temperature	190.96	6.38	0.007	0.52
Temperature	193.89	3.58	0.004	0.62	Soil carbon	191.13	6.55	0.004	0.62
Host species	202.44	12.14	0.06	0.85	Host species	192.69	8.11	0.15	0.20
All predictors	207.41	17.11	−0.04	0.64	All predictors	196.01	11.43	0.09	0.18

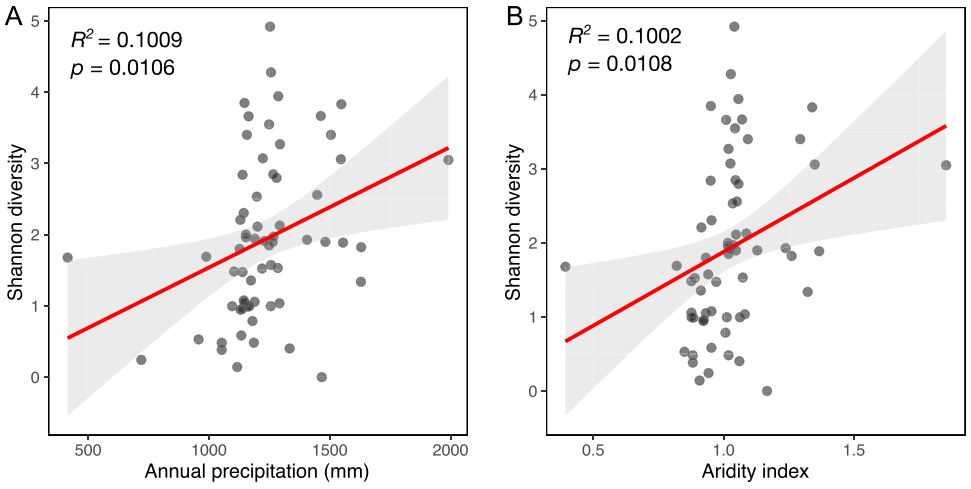

FIGURE 3 Foliar fungal endophyte Shannon diversity with respect to (A) annual precipitation (mm) and (B) aridity index. Gray shading around the regression line indicates 95% confidence interval.

increased as geographic distance between hosts increased, whereas bacterial communities were not related to distance or environment but were more similar in more closely related host taxa. Neither fungal nor bacterial endophyte communities exhibited a significant relationship with soil pH or other soil environmental factors.

Our finding that host phylogeny significantly influences bacterial (but not fungal) community structure suggests a level of host control over bacterial community colonization of internal leaf tissues that is not evident in fungal communities. Similar patterns have been reported in smaller-scale studies, demonstrating that leaf endophytic bacterial communities are chiefly controlled by host identity (Ding et al., 2013; Mina et al., 2020), as well as showing that host biogeography and other abiotic factors play a minor role in bacterial community assembly (Coleman-Derr et al., 2016). Bacterial diversity, on the other hand, was remarkably consistent across host species and all environmental variables measured. This pattern is consistent with previous work across host plants in which abiotic factors have little or no influence on leaf bacterial richness and abundance. For example, several studies have shown that precipitation, temperature, and elevation generally does not exert a significant effect on bacterial diversity (Hirano et al., 1996; Copeland et al., 2015; Wemheuer et al., 2020; Stone and Jackson, 2019, 2021). Additionally, there is very limited knowledge about bacterial endophyte diversity patterns along latitudinal gradients. While a latitudinal diversity gradient pattern has been observed previously in leaf fungal endophytes (see Arnold and Lutzoni, 2007), this was not a significant predictor

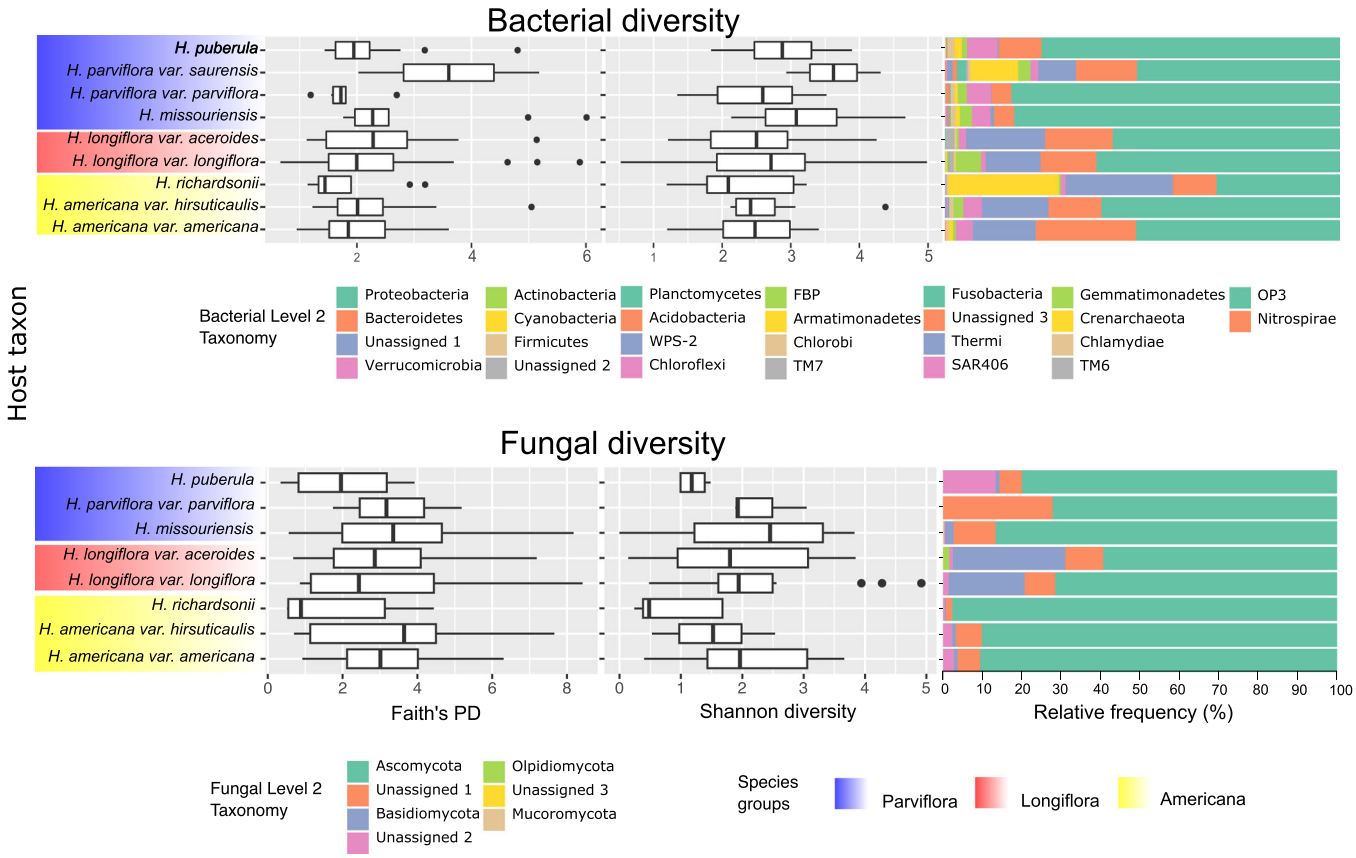

FIGURE 4 Boxplots of foliar microbial endophyte Faith's phylogenetic diversity and Shannon diversity with relative frequency (%) across strongly sampled host (*Heuchera*) taxa.

TABLE 2 Results of Mantel correlation tests between microbial endophyte community UniFrac distances and predictor distances (values for soil and non-soil environment for fungal endophytes are based on partial Mantel test results). Soil environment = soil pH, sand percent, and organic carbon content. Non-soil environment = latitude, temperature, precipitation, elevation, and aridity index. Mantel statistic *r*-values are based on Pearson's product-moment correlation. Significance codes: *** 0, ** 0.001, * 0.01, † 0.05, (none) 0.1.

	Fungal endophytes		Bacterial endophytes	
	r	*P*	*r*	*P*
Geographic distance	0.150	0.0003***	−0.037	0.715
Host phylogeny	0.024	0.221	0.141	0.002**
Soil environment	0.014	0.359	−0.096	0.934
Non-soil environment	0.007	0.425	−0.028	0.651

of endofoliar fungal diversity in this study. Thus, our study, while limited to North America, serves as initial evidence that leaf bacterial endophyte species and phylogenetic diversity do not follow a latitudinal diversity gradient.

The observed relationship of host phylogeny with bacteria may be indicative of a deliberate choice of beneficial microbes by the host plant (or vice versa) or a mutual evolutionary relationship between host plants and their bacterial endophytes. Processes such as vertical transmission of bacterial endophytes from parent to progeny via seed (Bergna et al., 2018; Zhang et al., 2022) demonstrate an intimate association between endophytes and host plant species. In particularly intimate endophyte relationships, the external environment may be less important than host-specific recruitment and maintenance of bacterial communities in shaping patterns of community composition and diversity across host plant taxa. Our results reveal a microbial community for the host genus *Heuchera* consisting of a set of bacterial endophytes that form a stable and close relationship with the host and are less affected by spatial processes and external environmental factors. These results are therefore consistent with the model of a core bacterial microbiome (i.e., a set of bacterial taxa that may be exclusively and consistently recruited to confer positive host functional traits, as well as ecological and evolutionary advantages; Risely, 2020). Identifying which underlying recruitment processes explain the relationship of bacterial endophytes to the host should be subject to further investigation.

Our observation that isolation-by-distance was significant for fungi and not bacteria is a remarkable parallel to recent global-scale work on soil microbiomes (Bahram et al., 2018), where both environmental parameters and geographic distance significantly determined fungal diversity. This contrasting pattern has also been revealed previously by small-scale

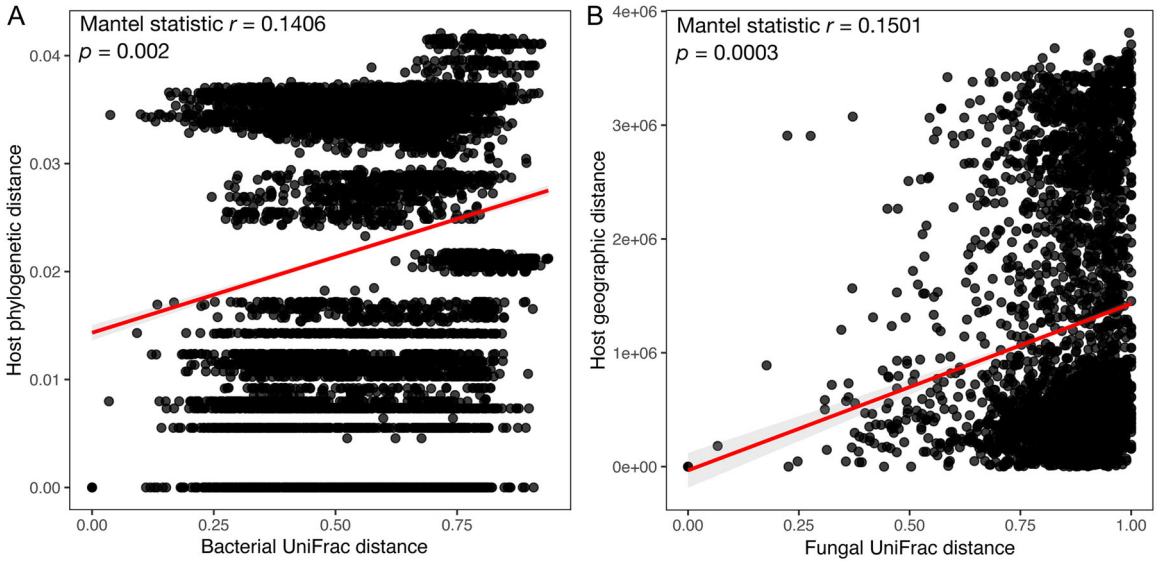

FIGURE 5 Correlation of host (A) phylogenetic distance with bacterial community UniFrac distance and (B) geographic distance with fungal community UniFrac distance based on Mantel test statistics. Linear relationship is represented by the red trend line; gray shading around the regression line indicates 95% confidence interval.

comparative investigations that reported distinct drivers of microbial community composition between bacteria and fungi, the latter influenced by geographic distance more than bacteria (Shakya et al., 2013; Coleman-Derr et al., 2016; Wei et al., 2022). For example, foliar fungal endophyte community structure was found to be strongly correlated with geographic distance in several oak species (and other plant hosts; see Langenfeld et al., 2013; Coleman-Derr et al., 2016); the similarity of spatially adjacent sites was independent of host habitat and phylogeny, as well as changes in climatic and environmental conditions (Collado et al., 1999; Lau et al., 2013; Koide et al., 2017). This repeatedly observed pattern may reflect distinct dispersal ecologies of fungal and bacterial endophytes in *Heuchera*. Foliar fungal endophytes are usually horizontally transmitted as spores or small pieces of hyphae via air (Rodriguez et al., 2009), which suggests that geographic location plays a significant role in endophytic community recruitment. Dispersal limitation, in particular, may be one of the possible explanations for spatial effects in fungi; Zhang and colleagues (2021) found strong evidence supporting the "size-dispersal" hypothesis, which states that larger fungi are more dispersal constrained than smaller bacterial microorganisms. This can lead to greater geographic heterogeneity of fungal endophyte communities and, as a result, community similarity declines with growing geographic distance. As previously noted, we used a partial Mantel approach to attempt to control environmental factors for geography given the spatial autocorrelation we observed. Collinearity of environmental and geographic predictors is challenging to tease apart regardless of the modeling framework, but the tests provided valuable insights into the roles of these predictors in community recruitment.

In addition to spatial and host-related factors, previous studies have documented the significant role that environmental factors play in driving patterns of microbial diversity (Zimmerman and Vitousek, 2012; Giauque and Hawkes, 2016). Leaf endophytes, by contrast, may be relatively buffered from the external environment by host protection and internal physiology (Wang et al., 2023), but foliar endophyte diversity data at broader spatial and phylogenetic scales are needed to further test this hypothesis. The results of our continental sampling across the host plant genus *Heuchera* show that precipitation factors were the most important environmental predictors of leaf fungal endophyte diversity (see also Zimmerman and Vitousek, 2012; Penner and Sapir, 2021), whereas bacterial communities were unrelated to the environmental factors tested here (see also Hirano et al., 1996; Copeland et al., 2015; Stone and Jackson, 2019, 2021; Wemheuer et al., 2020). This suggests an important role of climate, particularly water availability, in shaping broad patterns of fungal diversity within leaf tissues. Water availability may impact fungal species richness and abundance in terms of influencing organismal metabolism, growth and survival, and resource availability, as reported in several investigations (Hirano and Upper, 2000; Williams and Rice, 2007; Manzoni et al., 2012). In addition, for fungal endophytes that are primarily horizontally transmitted through spores from the environment (Rodriguez et al., 2009), precipitation may also be directly involved in the dispersal and successful recruitment of these endophytes. Rainfall may release and disperse tiny fungal spores from the environment and surrounding plants toward other plants, particularly on leaf tissues with small wounds that facilitate easier entry. Increased moisture in the air also causes stomata to open, providing access points for dispersed fungal spores to infiltrate the leaf internal tissues. Furthermore, small droplets of water on leaf surfaces may be a prerequisite for some fungal spores to initiate germination and subsequent infection of leaf tissues (Bald, 1952).

We also demonstrate here that the *Heuchera* leaf endophyte microbiome shows no relationship with the soil environment, a contrast to what has been observed in rhizosphere and root endophyte communities (Fierer and Jackson, 2006; Baker et al., 2009; Afzal et al., 2011; Bokati et al., 2016). Van Bael et al. (2017) similarly suggest that soil environment gradients do not significantly influence foliar endophyte diversity and community assembly. This may be due to host buffering against edaphic conditions in the more insulated internal leaf environments that microbial communities inhabit. Indeed, in a recent work by Zhou et al. (2023b), soil salinity was found to determine endophytic bacterial communities in roots but not in leaves, where host leaf metabolism is thought to have more control over community assembly.

Our work also derives substantially from silica-dried collections, an approach used previously to characterize legume nodule microbiomes (Johnson, 2019). That we recovered, as major community components, numerous bacterial and fungal genera previously known to be typical plant endophytes indicates that useful insights can be derived from diverse preservation strategies, although we did not compare tissue preservation effects in our analyses. Easy-to-use preservation approaches are especially suitable for widely spread and inaccessible field sites for broad geographic surveys. Herbarium materials prepared under less controlled conditions than those used here have been the subject of several studies. Daru et al. (2018) and Bieker et al. (2020) were able to obtain useful endophyte microbiome data from herbarium specimens, although with higher quantities of exogenous DNA due to inconsistent mounting and storage procedures. However, materials from herbaria may prove useful in future studies to track how endophytic communities might change through time. In addition to herbaria, large, preserved tissue resources exist in several museums and other institutions as well as individual labs that would, together with a similar approach to ecological predictor assembly via georeferences, enable broad-scale surveys of endophyte diversity potentially beyond the scale of purpose-collected microbial materials. In terms of challenges, our results were consistent with earlier research reporting remarkably low endophyte abundance in leaf samples, as well as reduced diversity compared to root tissue (Kivlin et al., 2022; Zhou et al., 2023a). Endophyte abundance in leaves may just be inherently too low to prevent nonspecific amplification even when specific primers are used, because the host overwhelms the PCR reaction. These findings point to the possibility that leaves naturally have far fewer microbial endophytes, in terms of both quantity and diversity, than other tissues of the plant body.

CONCLUSIONS

The roles of host, climate, and soil factors in driving the assembly of leaf endophyte communities have been relatively less studied at broad scales, yet a broader scope is needed to resolve disagreement among studies performed at narrower scales. Overall, this work improves our understanding of the large-scale patterns of diversity and community composition in leaf endophytes using an evolutionary framework, and highlights differences in how environmental and host-related factors shape bacterial and fungal communities within the leaf microbiome. Our results for bacteria suggest a core microbiome primarily shaped by host phylogeny, whereas for fungi our data highlighted geographic distance and precipitation factors as the best predictors of community composition. Host-mediated selection of core microbiomes and dispersal limitation may respectively underlie these differences, although further study is needed to identify specific mechanisms. The present study also introduces silica-dried collection as an effective and efficient preservation approach for broad-scale leaf microbiome studies. Our findings highlight the value of in-depth clade-based microbiome research and the intricacy of microbiome assembly within certain plant organs.

AUTHOR CONTRIBUTIONS

R.A.F. conceived this study (with the assistance of D.J.P.). N.J.E.-W. and R.A.F. conducted the fieldwork. R.C., S.S.-D., and K.B. performed the wet lab work (in consultation with C.M.S. and H.R.J.). D.J.P. and R.A.F. analyzed the data and wrote the manuscript. All authors contributed to all drafts and gave final approval for publication.

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DATA AVAILABILITY STATEMENT

All sequence data and raw reads are deposited in the Sequencing Read Archive, under BioProject PRJNA1123281. Scripts used for implementing QIIME 2, diversity, and statistical analyses are available on GitHub (https://github.com/dexcemp/heuchera_microbiome).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1. Sampling location and host taxonomy.

Appendix S2. (A) Number of samples per host taxon and per sequencing type investigated in this study; (B) sampling summary of host taxonomy and populations with mean number of replicates per host taxon and population.

Appendix S3. Bacterial phylum-level diversity and relative frequency across samples using the updated Greengenes2 database.

Appendix S4. (A) Fungal and (B) bacterial sequence frequency per sample.

Appendix S5. Shannon diversity alpha rarefaction curves for (A) bacterial 16S and (B) fungal ITS sequences.

Appendix S6. Results of downstream diversity analysis, including no rarefaction and varying sequence cutoffs.

Appendix S7. Bacterial genus-level diversity and relative frequency across samples.

Appendix S8. Fungal genus-level diversity and relative frequency across samples.

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