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Effects of nutrient supply, herbivory, and host community on fungal endophyte diversity

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Abstract. The microbes contained within free-living organisms can alter host growth, reproduction, and interactions with the environment. In turn, processes occurring at larger scales determine the local biotic and abiotic environment of each host that may affect the diversity and composition of the microbiome community. Here, we examine variation in the diversity and composition of the foliar fungal microbiome in the grass host, *Andropogon gerardii*, across four mesic prairies in the central United States. Composition of fungal endophyte communities differed among sites and among individuals within a site, but was not consistently affected by experimental manipulation of nutrient supply to hosts (*A. gerardii*) or herbivore reduction via fencing. In contrast, mean fungal diversity was similar among sites but was limited by total plant biomass at the plot scale. Our work demonstrates that distributed experiments motivated by ecological theory are a powerful tool to unravel the multiscale processes governing microbial community composition and diversity.

Key words: *biodiversity; community ecology; grasslands; microbiome; Nutrient Network.*

Introduction

Every free-living organism, whether plant or animal, hosts a vast array of microbial symbionts (Qin et al. 2010, Vorholt 2012), and the diversity of these hidden microbiomes can exceed that of free-living organisms (Qin et al. 2010, Hardoim et al. 2015). The composition and diversity of a host's microbiome can alter host physiology, growth, reproduction, and behavior (Rodriguez et al. 2009, Round and Mazmanian 2009, Heijtza et al. 2011, McFall-Ngai et al. 2013, Humphrey et al. 2014, Hardoim et al. 2015). For example, fungal endophytes can provide a wide array of fitness benefits to their plant hosts, including enhanced stress tolerance, resource-use efficiency, and defense against pathogens and herbivores (Rodriguez et al. 2009, Ren et al. 2011, 2014, Busby et al. 2016, Buckley et al. 2019). Despite the importance of the microbiome to each host organism, we lack a predictive understanding of the factors that affect microbiome composition and diversity. Our understanding of the processes governing the assembly of microbiome communities is not limited by data on microbiome community composition, as technological advances have made describing the taxonomic composition of microbiomes relatively straightforward and resulted in a torrent of data and papers (Hug et al. 2016). Nonetheless, most studies to date have been observational and correlational, leaving causal relationships governing microbiome community composition and function difficult to discern within a large suite of covarying factors.

A host's microbiome forms within a complex community and ecosystem context and responds to processes acting at a range of scales from the cellular to the continental (Peay et al. 2010*a*, Borer et al. 2013, 2016). For example, microbial colonization of individual host cells or tissues may preempt or facilitate colonization by subsequent microbial taxa (Joshee et al. 2009, Cordier et al. 2012, Devevey et al. 2015, Fukami 2015). At larger spatial scales, within a local community, the relative abundance of hosts and nonhosts for individual microbial taxa can determine the likelihood of microbial transmission to and colonization of a new host individual (Mitchell et al. 2002, Power and Mitchell 2004, Peay et al. 2010*b*, Belisle et al. 2012, Roche et al. 2012, Borer et al. 2013, Seabloom et al. 2015*a*). At continental scales, the potential pool of microbes available to colonize a host may vary in response to the regional environment (Peay

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et al. 2010*a*, U'Ren et al. 2012, Borer et al. 2013, Barberan et al. 2015) and as a function of continental-scale patterns of air movement (Carotenuto et al. 2017).

In addition to the influences of diverse factors operating at multiple spatial scales, local environmental factors may have direct and indirect effects on microbiome diversity and composition. In particular, nutrient supply to a host can directly alter the composition of microbial symbiont communities by altering host immune responses, host resources available to symbionts, or nutrient-mediated microbial interactions (Smith et al. 2005, Kau et al. 2011, Borer et al. 2013, 2016, Lacroix et al. 2014*b*). In addition to these direct nutrient effects, nutrient supply can alter host community diversity, composition, and productivity, which have been shown to alter the abundance of symbiotic microbes, including pathogens (Mitchell et al. 2002, Johnson et al. 2003, Treseder 2004, Ezenwa et al. 2006, Keesing et al. 2006). For example, concurrent changes in predation and nutrient supply have been observed to induce changes in the microbiome of corals (Shaver et al. 2017), and nutrients and herbivory can induce turnover in the microbial composition within plants (Faeth and Fagan 2002, Pineda et al. 2013). Human activity has increased the input of mineral nutrients into terrestrial ecosystems and dramatically changed the nature of consumer communities globally (Thornton 2010, Foley et al. 2011, Ripple et al. 2015, Steffen et al. 2015), so these two factors are likely to be broadly relevant environmental drivers coupling human activity to changes in the composition and function of microbiome communities within plant hosts.

Here we leverage an existing, regionally replicated experiment to examine the effects of host nutrient supply and host interactions with herbivores on the diversity and composition of the foliar fungal microbiome of *Andropogon gerardii*, a dominant native grass throughout the tallgrass prairie ecosystem. In our analyses, we include data on changes in components of the plant community that we expected to have indirect effects on the diversity and composition of the within-plant microbial community: host abundance (*A. gerardii* cover), plant diversity, total plant biomass, and the proportion of the plant biomass that is composed of live plants and grasses. We combine plant and fungal community data to answer the following three questions.

Q1: What are direct effects of nutrients and consumers on endophyte composition and diversity?

We expect that host nutrient supply (Mitchell et al. 2003, Carvalhais et al. 2013, Seabloom et al. 2013) and herbivory (Borer et al. 2009, David et al. 2016, Saleem et al. 2017) may directly affect within-host microbial communities. For example, the composition of microbial symbiont communities may respond to altered plant tissue chemistry resulting from differences in host nutrient supply (see review in Borer et al. 2016). Herbivores also have the potential to increase colonization by creating wounds or directly serving as vectors (David et al. 2016). Herbivores also may either up- or downregulate plant immune systems that can affect fungal or other endophytes (Traw and Bergelson 2003, Seabloom et al. 2018). The effects of nutrient supply are likely complex because of the potential for interactions between nutrients, herbivores, and their effects on plant endophytes (Bultman et al. 2004, Gruner et al. 2008).

Q2: What are effects of nutrients and consumers on the local plant community?

Nutrient supply and herbivory also can alter the composition, diversity, and productivity of plant communities (Harpole and Tilman 2007, Harpole et al. 2007, 2016, Borer et al. 2014*b*, Fay et al. 2015), thereby creating the potential for indirect effects on the host microbiome, mediated by changes in the local plant community (Mitchell et al. 2002, Ezenwa et al. 2006, Keesing et al. 2006). In grassland ecosystems, nutrient addition generally reduces plant diversity and increases total plant biomass (Harpole and Tilman 2007, Borer et al. 2014*b*, Fay et al. 2015, Harpole et al. 2016), whereas the effects of herbivore reduction on the productivity, diversity, and composition of plant communities varies widely among sites (Borer et al. 2014*b*, 2017, Seabloom et al. 2015*b*).

Q3: What are indirect effects of local plant community differences on endophyte communities?

Altering the local plant community composition, diversity, and productivity may increase or decrease microbial diversity within a focal host species, depending on the regional diversity of the microbial species pool and the degree to which individual microbial taxa specialize on available plant taxa. Reduced plant diversity may increase the abundance of specialist microbes by increasing relative host or nonhost frequency, as has been shown for plant pathogens (i.e., the dilution effect; Mitchell et al. 2002, Keesing et al. 2006, Scherber et al. 2010, Lacroix et al. 2014*a*). However, reduced plant diversity may lower microbial diversity, if a diverse plant community creates a more diverse pool of potential microbial colonists (i.e., mass effects in metacommunity models; Leibold et al. 2004, Seabloom et al. 2015*a*, Borer et al. 2016). In addition to plant diversity effects, endophytic fungal diversity might be affected by the relative abundance of conspecific plants that contain host-specialist fungi (i.e., percent *A. gerardii* cover) or the abundance of other grasses (i.e., percent grass cover; Keesing et al. 2006). Increased plant biomass may increase microbial diversity simply by providing more available microbial habitat, as predicted by island biogeography or species–area relationships (MacArthur and Wilson 1967, Kuris et al. 1980; but see Kinkel et al. 1987). However, increased plant biomass also may reduce fungal diversity, if the increased biomass increases the dispersal rate of competitively dominant microbes, leading to the loss of competitively inferior species (Nowak and May 1994, Tilman 1994, Tilman et al. 1994, Noble et al. 2011, Seabloom et al. 2015*a*). The effects of plant biomass also may differ, depending on the proportion of live plant biomass hosting biotrophic fungi vs. the dead biomass hosting saprotrophic and necrotrophic fungi.

Materials and Methods

Study system and experimental design

This work was conducted within an existing experiment replicated at four sites in the Great Plains of North America (KY = Kentucky, KS = Kansas, IA = Iowa, MN = Minnesota; Appendix S1: Table S1). These sites were all in tallgrass prairie ecosystems and are a subset of the Nutrient Network (NutNet; nutnet.org) distributed experiment, which was started in 2007 (Borer et al. 2014*a*, 2017). The sites spanned a range of mean annual precipitation (750–1282 mm/yr), mean annual temperature (6.3–13.6°C), and total plant biomass (514–937 g/m^2). Sites also differed in how much of the biomass was composed of grass (20–92%) and live biomass (33–97%). We selected *A. gerardii* as our focal host, because it is widespread across our sites and was the only plant species present in nearly every plot regardless of the treatment. Our focal host species (*A. gerardii*) ranged in cover from 2 to 58%.

The experiment is a full factorial combination of nutrient addition (Control or Fertilized) and vertebrate consumer density (Control or Fenced) for a total of four treatments. The experiment was replicated as a fully randomized block design with three blocks at Kentucky and Kansas, five in Minnesota, and six in Iowa. The nutrient-addition treatment was an addition of 10 g $N \cdot m^{-2} \cdot yr^{-1}$ as time-release urea, 10 g $P \cdot m^{-2} \cdot yr^{-1}$ as triple super phosphate, 10 g $K \cdot m^{-2} \cdot yr^{-1}$ as potassium sulfate, and 100 $g \cdot m^{-2} \cdot yr^{-1}$ of a micronutrient mix (6% Ca, 3% Mg, 12% S, 0.1% B, 1% Cu, 17% Fe, 2.5% Mn, 0.05% Mo, and 1% Zn). N, P, and K were applied annually, and the micronutrient mix was applied once at the start of the study to avoid toxicity of largely immobile micronutrients. Note that ammonium nitrate was used in 2007 instead of urea, but there were no detectable differences between these N sources on plant biomass or diversity (Seabloom et al. 2015*b*). The fences were 2.1 m tall and excluded aboveground, nonclimbing mammalian herbivores. The lower 0.9 m was composed of 1-cm woven wire mesh with a 0.3 m outward-facing flange stapled to the ground to exclude digging animals. The top 1.2 m was composed of spaced rows of woven wire to prohibit large animals from entering the plots. These fences have been shown to alter plant invasions, diversity, and community composition as well total nutrient pools across the global set of NutNet sites (Lind et al. 2013, Borer et al. 2014*b*, Seabloom et al. 2015*b*, Anderson et al. 2018).

A complete pretreatment sampling of the plant and soil community was conducted in 2007 at three of the sites (Kentucky, Kansas, and Minnesota) and in 2008 at the Iowa site. Fencing and nutrients treatment were initiated after the pretreatment sampling, so the sites had either six (Iowa) or seven (Kentucky, Kansas, and Minnesota) years of treatments applied at the time of this study.

Plant sampling

At peak biomass, the cover of all plant species was estimated visually in a 1 × 1 m permanent quadrat located randomly in each plot. Cover estimates included our focal host, *A. gerardii*. At the same time (i.e., at peak biomass), all aboveground plant biomass (live and dead) was collected in two 0.1 × 1 m strips, sorted to separate live (current year's growth) and dead (growth from past years), dried to a constant mass, and weighed to the nearest 0.01 g. Although the plant data are sampled annually, here we use plant cover and biomass data collected in 2014 at the time of fungal endophyte sampling.

A single mature leaf from three or four individual *A. gerardii* plants was collected in 2–4 blocks at each site in August of 2014, for a total of 170 samples (KY = 44, KS = 45, IA = 32, MN = 49) with 8–15 plants sampled per treatment at each site. The collected leaf was the second leaf above the uppermost senescent leaf. Leaves were stored in a cooler (about 4°C) until they were surface-sterilized in the lab by immersing them for 1 min each in water, 75% ethanol, 0.4125% sodium hypochlorite (bleach solution), 75% ethanol, and sterile distilled water. Following surface sterilization, samples were stored at −80°C. We tested this sterilization protocol prior to sample collection by placing sterilized and unsterilized grass leaves onto sterile culture plates for a few minutes, removing the leaves, and monitoring the plates for fungal growth. We did not observe any fungal growth in plates which had been in contact with sterile leaves.

Fungal DNA extraction and sequencing

Leaves were ground in liquid nitrogen with a mortar and pestle, total genomic DNA was extracted using the Qiagen Plant Mini Extraction Kit (Qiagen N.V., Venlo, the Netherlands), and standardized to 20 ng/μL. Fungal genomic libraries were made by amplifying the ITS region as in Nguyen et al. (2015). Each sample was barcoded with unique 7 base-pair (bp) sequences and the ITS1 region was amplified with the standard primers ITS1f (5′- AATGATACGGCACCACCGAGATCTACAC-GG-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2 (5′-CAAGCAGAAGACGGCATACGAGAT-barcode-CG-GCTGCGTTCTTCATCGATGC-3′). The ITS1 primer includes an Illumina Nextera adaptor, linker sequence, and a bar code. Polymerase chain reaction (PCR) was done in triplicate using Roche FastStart High Fidelity Taq (Roche, Indianapolis, Indiana, USA) with annealing temperatures at 51°, 53° and 55°C. PCRs with no visible bands were repeated once. Two negative controls (distilled water instead of DNA) were included in every PCR set. Amplicons from the triplicate PCR reactions were pooled for each sample, purified using the QIAQuick Purification Kit (Qiagen N.V., Venlo, the Netherlands), and quantified using the Quant-iT® dsDNA HS Assay kit in a Qubit Fluorometer (Thermo Fisher, Waltham, Massachusetts, USA). Equal amounts of these purified libraries (25 ng) were pooled and sequenced in Illumina MiSeq at the University of Minnesota Genomics Center (UMGC).

Sequence data from the MiSeq runs were combined and analyzed using an analytical pipeline adapted from Nguyen et al. (2015). Adapter and distal priming sites were removed using cutadapt v1.7.1 (Martin 2011) and Trimmomatic v 0.32 (Bolger et al. 2014). Short sequences, homopolymers, and sequences containing ambiguous bases were removed using Mothur v.1.34.4 (Schloss et al. 2009). The clean sequences were then dereplicated and clustered into operational taxonomic units (OTUs) at a 97% cutoff, with chimera sequences removed using USEARCH (Edgar 2010) followed by additional re-clustering using UCLUST (Edgar 2010) implemented in Qiime (Caporaso et al. 2010). Nguyen et al. (2015) found that combining clustering algorithms (i.e., chain-picking) was more stringent and more accurately recovered the fungal taxa in a constructed mock community than using a single clustering method. After removing singleton OTUs, a representative sequence for each OTU was selected and assigned a taxonomy using BLAST (Basic Local Alignment Search Tool) against the UNITE fungal database v 7.0 with BLAST v 2.2.28+ (Camacho et al. 2009). Only annotations >80% identity and >80% alignment length were retained. The pooled OTU counts from the distilled water negative controls were subtracted from each sample. The 748 reads from the distilled water controls represented a fraction of the total number of reads (<0.0001) and were clustered into 27 OTUs with a median abundance of 4.5 reads per sample. Seventy-two percent of the negative control reads were classified as a single OTU matching *Phoma calidophila* (Ascomycota, Dothideomycetes, Pleosporales). Four samples with less than 1,000 reads were removed from the analyses. OTUs that could not be assigned matches against the UNITE database were excluded from the taxonomy report, but included in statistical analyses.

Statistical analysis

In assessing plant and fungal diversity, we use the effective number of species based on the probability of interspecific encounter ($\mathrm{ENS_{PIE}}$). $\mathrm{ENS_{PIE}}$ provides a more scale-independent measure of potential biodiversity effects than richness and is much more robust to the effect of rare species than species richness (Chase and Knight 2013). These properties are particularly important for amplicon sequencing data, in which a large number of unique sequences are observed at low frequencies and the number of reads obtained per sample may vary greatly. $\mathrm{ENS_{PIE}}$ estimates the number of equally abundant species and is equivalent to the inverse Simpson's index of diversity. We calculated $\mathrm{ENS_{PIE}}$ as $1/\sum_{i=1}^{S} p_i^2$, where S is the total number of species and p_i is the proportion of the community represented by species i (Chase and Knight 2013). In our fungal data, $\mathrm{ENS_{PIE}}$ is positively correlated with rarefied richness ($r = 0.64$), evenness ($r = 0.79$), and Shannon's diversity ($r = 0.78$) across all samples in our data, and results are qualitatively similar when using different metrics. Note that our diversity metrics are calculated using OTUs as opposed to named species.

In our analyses of the direct effects of the experimental nutrient and fencing treatments on the endophyte diversity (Q1) and the local plant community (Q2), we used linear regression with site included as a fixed effect (Appendix S1: Table S3). When we incorporated the indirect effects of the plant community into our models (Q3), we used a multimodel inference and model averaging (Grueber et al. 2011) as there could be a number of similarly informative models due to the correlation among the plant community responses (e.g., plant diversity and productivity). To do this, we first fit a mixed-effects model using the lmer function in the lme4 package as the base model for the model averaging. In this model, Site, Block, and Plot were treated as nested random effects. The fixed effects in the model were Site, the experimental treatments (Nutrient Addition and Fencing), attributes of the plant community (log plant Biomass, proportion live biomass, proportion grass biomass, log plant diversity, and log *A. gerardii* cover), and all first-order interactions among the fixed effects. We then used the dredge function in the MuMIn library to fit all possible models and the model.avg function to estimate parameter values, errors, and Akaike information criterion ($\mathrm{AIC_c}$) –weighted importance for all models within four $\mathrm{AIC_c}$ units of the top model (Grueber et al. 2011). The experimental treatments were included in all 8,740 models assessed by the dredge function.

In assessing the composition of the fungal community (Q1 and Q3), we used permutational multivariate analysis of variance (PERMANOVA) using the adonis function in the vegan library. PERMANOVA compares distance matrices and uses permutation tests to determine significance and is analogous to redundancy analysis and multivariate analysis of variance (MANOVA) (Legendre and Anderson 1999, Anderson 2001, McArdle and Anderson 2001). In our analyses, we first rarefied the community matrix to 1,000 reads using the rarefy function in the vegan library prior to calculating the Bray-Curtis and Jaccard distance matrices that were the input to the PERMANOVA. PERMANOVA statistics are based on 999 permutations of the distance matrices. The PERMANOVA model included the nested effects of Site, Block within Site, Plot within Block, and Plant within Plot; the experimental treatments (Nutrient

Addition and Fencing); and attributes of the plant community (log plant Biomass, proportion live biomass, proportion grass biomass, log plant diversity, and log *A. gerardii* cover; Appendix S1: Table S1).

We compared individual OTU responses by extracting the linear coefficients (slopes) from the PERMANOVA for each OTU. These coefficients are equivalent to the regression coefficients in a univariate linear regression and summarize the change in abundance of each OTU in response to the independent variables in the model (e.g., nutrient addition, fencing, plant diversity, and plant biomass). We assembled these coefficients into a data matrix in which each row contained the regression coefficients for a single OTU. We used these data as an input to a principal components analysis (PCA) based on a correlation matrix. This analysis quantifies correlations among the effects of the experimental treatment and plant community variables across the entire fungal community. In this PERMANOVA, we treated site as a strata as opposed to a fixed effect, which constrained permutations to samples within a site, thereby allowing us to estimate OTU responses averaged across all sites.

All statistical analyses were conducted in R (R Core Team, 2017).

RESULTS

We obtained 13,833,199 DNA sequences from the 170 *A. gerardii* leaf samples (3,416–209,325 reads per sample; mean = 81,372) that could be identified as fungi, and detected 2,769 unique fungal OTUs. Of these fungal taxa, 97% had matches at the Phylum level, 88% at the Class level, 83% at the Order level, 71% at the Family level, and 61% at the Genus level. Note that all OTUs identified as Fungi (matched and unmatched to a lower taxonomic level) were used in subsequent analyses. These OTUs were from 6 phyla, 22 classes, and 64 orders (Appendix S1: Fig. S2). OTU frequencies by phylum were as follows: Ascomycota (70%), Basidiomycota (24%), Chytridiomycota (11%), Zygomycota (1.1%), Glomeromycota (1%), and Rozellomycota (0.4%).

Q1: What are direct effects of nutrients and consumers on endophyte composition and diversity?

Mean fungal endophyte diversity did not differ among sites ($F = 1.932$; $P = 0.146$), and there were no consistent, direct effects of the nutrient or fencing treatments on fungal diversity ($P > 0.700$; Appendix S1: Table S3; Fig. 1). However, nutrient addition had strong effects on fungal diversity at some sites ($F = 4.345$; $P = 0.012$ for Site:Nutrient interaction), strongly reducing fungal diversity at the Minnesota site, but increasing fungal diversity at the Kentucky site (Appendix S1: Fig. S1). In contrast to fungal diversity, there were significant effects of nutrient addition and herbivore fencing on fungal community composition ($P < 0.05$; Appendix S1: Table S4; Fig. 3). However, these effects varied between sites (Fig. 3), probably due to the large differences in fungal community composition at the different sites or to differential effects across the sites. The largest source of variation in the fungal community was spatial variation (i.e., differences among sites, block, plots, and plants within plot; Fig. 3). Notably, differences among plants within individual plots accounted for more variation than experimental treatments or the local plant community composition (Fig. 3; Appendix S1: Table S4; Fig. S2). Although spatial variation in fungal species composition among the communities was substantial, species of the classes Dothidiomycete and Sordariomycete were most common at all four sites. In addition, the Tremellomycetes, not often reported in foliar fungal communities, were common (Appendix S1: Fig. S2).

Q2: What are effects of nutrients and consumers on the local plant community?

Sites differed in all measures of the focal plant community structure (Appendix S1: Tables S1 and S2). Generally, nutrient addition decreased plant diversity ($F = 13.304$; $P = 0.001$), increased plant biomass ($F = 6.142$; $P = 0.019$), and reduced the proportion of grasses ($F = 8.053$, $P = 0.008$) across all sites (Appendix S1: Table S3; Fig. 1). There was a strong interaction between nutrient addition and fencing on total plant biomass ($F = 8.053$; $P = 0.013$), such that nutrient addition induced a greater increase in total (live + dead) plant biomass outside of fences (Appendix S1: Table S3; Fig. 1).

Q3: What are indirect effects of local plant community change on endophyte communities?

Although the effects of nutrient and fencing treatments on fungal endophyte diversity within our focal host (*A. gerardii*) varied widely among sites, the plant community had consistent and significant effects on endophyte diversity. The strongest effect was the decline in fungal diversity associated with increasing total plant mass ($z = 2.46$; $P = 0.014$; Appendix S1: Table S2; Fig. 2). Furthermore, fertilization effects were strongly dependent on whether the plant community was dominated by live plants or dead biomass ($z = 2.95$; $P = 0.003$; Appendix S1: Table S2; Fig. 2). Specifically, fertilization decreased fungal diversity in plant communities with a low proportion of live biomass relative to litter, but increased fungal diversity in plots that had a high proportion of live biomass. However, live biomass primarily differed among sites (Appendix S1: Table S1), so this response may reflect other among-site drivers of microbial diversity.

Endophyte community composition also was affected by attributes of the local plant community (Appendix S1: Table S4; Fig. 3), and there were consistent responses among fungal taxa to the treatments and plant community, with most (86%) of the variation in effects of

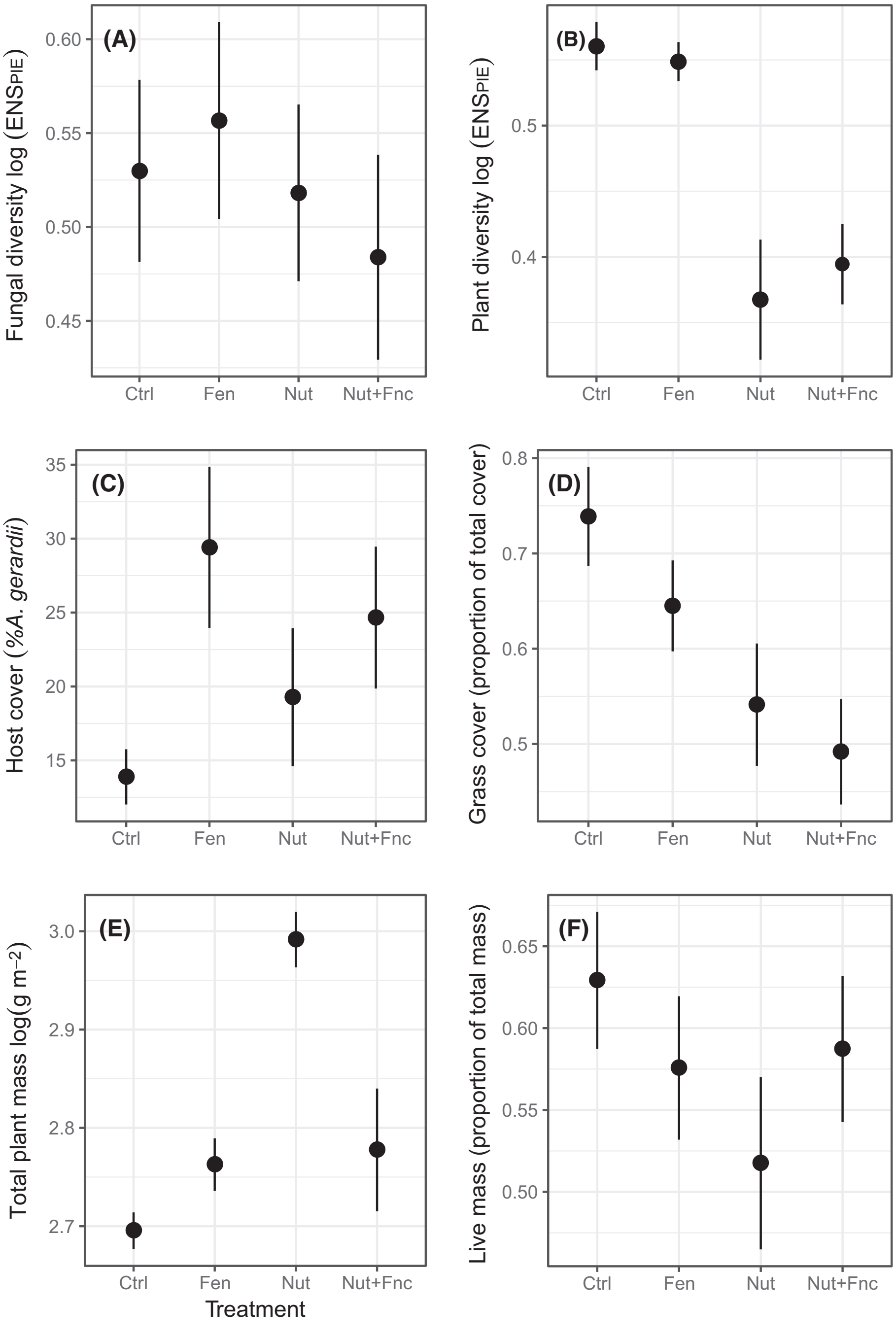

FIG. 1. Effects of herbivore fencing and nutrient addition on leaf fungal endophyte diversity and plant communities at four mesic prairie sites. Treatment abbreviations are as follows: Ctrl = control, Fen = fencing treatment, Nut = nutrient treatment, Nut+Fen = nutrient and fencing treatment. (A) Fungal diversity is measured in the leaves of the grass, *Andropogon gerardii*. Plant community responses are (B) vascular plant diversity, (C) percent cover of the focal host plant (*A. gerardii*), (D) proportion cover of all grasses, total; (E) total aboveground biomass (live + dead), and (F) proportion live plant mass (live mass divided by total mass). Error bars represent 1 ± SEM.

nutrients, fencing, and plant community on individual OTUs accounted for by two principal components (PC1 and PC2; Fig. 4). Most (67%) of the variation among the OTUs was accounted for by PC1, which differentiated among fungal OTUs based on a contrast between the positive effects of total plant biomass, the combined nutrient and fencing treatment, and plant diversity vs. the negative effects of host (*A. gerardii*) cover, live biomass, nutrient addition alone, and fencing alone. An additional 19% of the variation among OTU responses was accounted for by PC2, a contrast between positive effects of plant diversity, the negative effects of grass cover, and the combined nutrient and fencing treatment.

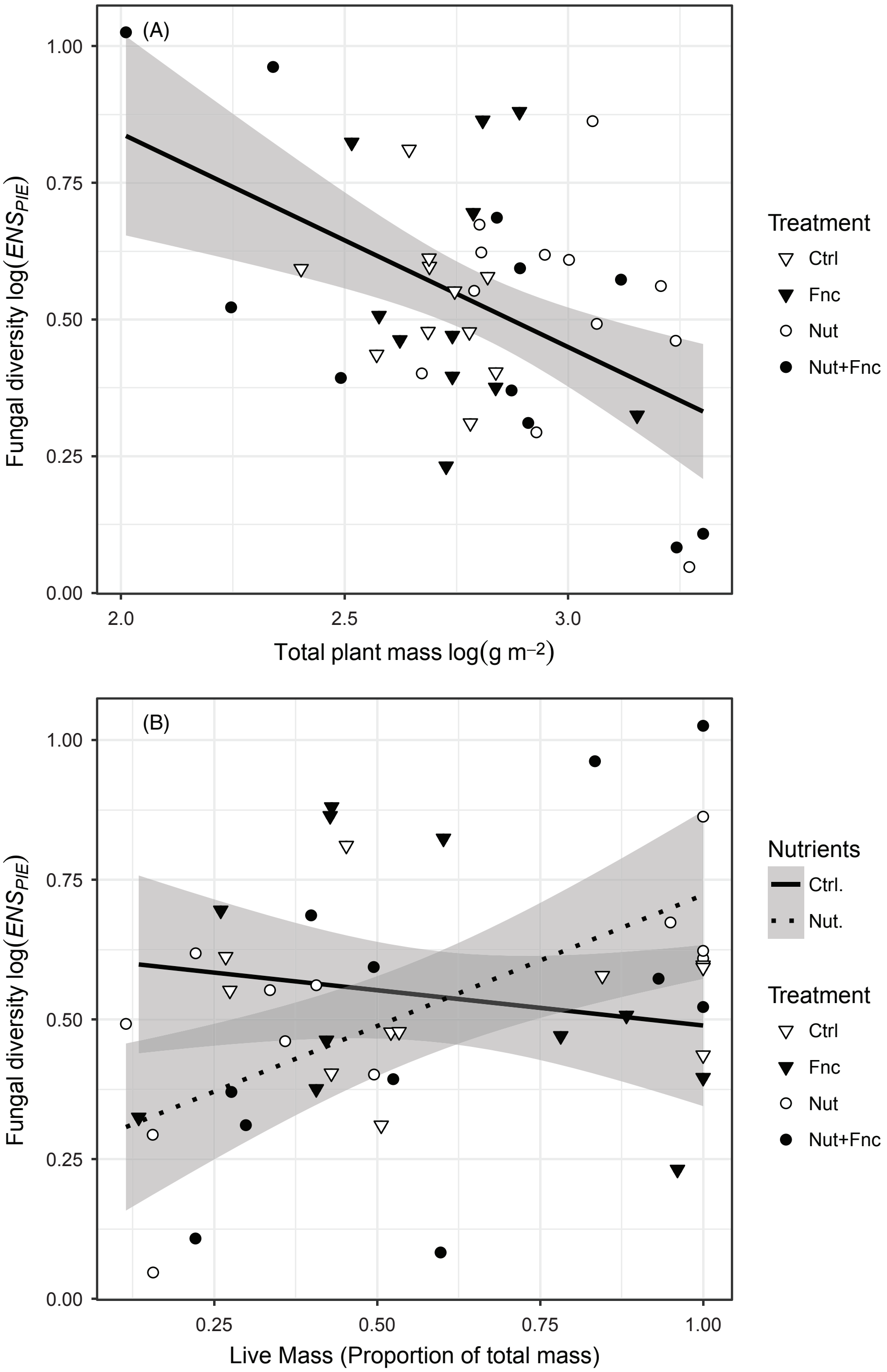

Fig. 2. Effects of (A) total plant biomass and (B) proportion live plant biomass on fungal endophyte diversity in the grass *Andropogon gerardii.* Treatment abbreviations are as follows: Ctrl = control, Fen = fencing treatment, nut = nutrient treatment, Nut+Fen = nutrient and fencing treatment. Shaded areas represent 95% confidence limits.

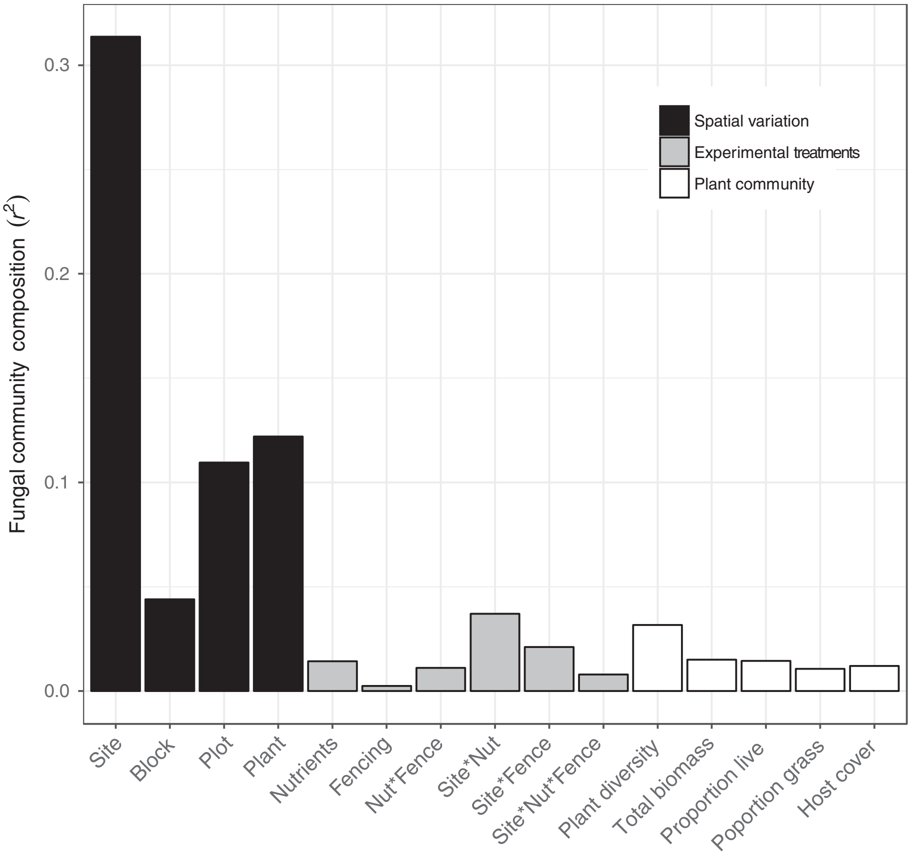

FIG. 3. Amount of the variation (r^2) in fungal endophyte communities in leaves of the grass *Andropogon gerardii* accounted for by spatial variation, nutrient addition (Nut) and herbivore fencing (Fence) treatments, and the plant community. Results are from a PERMANOVA using Bray-Curtis distance and 999 permutations of the data. Spatial variation represents differences among sites (Site), blocks within a site (Block), plots within a block (Plot), and plants within a plot (Plant). Plant community measures are vascular plant diversity, total aboveground biomass (live + dead), proportion live plant mass (live mass divided by total mass), proportion biomass of grasses (grass mass divided by total mass), and percent cover of the focal host plant (*A. gerardii*).

DISCUSSION

We found a highly diverse fungal endophyte community in the host species, *A. gerardii*. Average levels of diversity within *A. gerardii* did not differ among the sites we sampled across the eastern Great Plains Region, despite large differences in abiotic and biotic conditions among these sites. Nutrient addition had strong but highly site-specific effects on fungal diversity, increasing fungal diversity at our northernmost site in Minnesota but decreasing fungal diversity at our southernmost site in Kentucky. Nutrient addition and herbivore exclusion also had strong effects on aspects of the local plant community that we expected to affect fungal endophyte diversity within our focal host species, including total plant biomass and proportion of the plant biomass composed of live plants. In contrast to fungal diversity, fungal community composition within individual leaves was highly variable at all spatial scales ranging from among sites to among plants within a plot. Although this high spatial variation dominated the composition of endophytic fungal microbiomes, there were detectable effects of the fertilization and fencing treatments and the plant community on fungal community composition, though these effects varied among sites.

Although endophyte community composition was highly variable at all spatial scales, we found that fungal diversity within a single *A. gerardii* individual was surprisingly consistent across the sites in our region, suggesting general constraints on the total number of unique taxa that can occur in a host individual at one time. This similarity is particularly striking given the high degree of compositional turnover among sites, plots, and even plants within a single plot in our study. Broadly speaking, ecological processes that govern diversity within a host individual likely fall into one of few broad categories (Vellend 2010, Noble et al. 2011, Seabloom et al. 2015*a*, Borer et al. 2016): colonization and extinction dynamics (Kuris et al. 1980, Kinkel et al. 1987, Belisle et al. 2012), local selection processes acting on fitness differences or frequency dependence within a host (Adler et al. 2007), and drift (Adler et al. 2007).

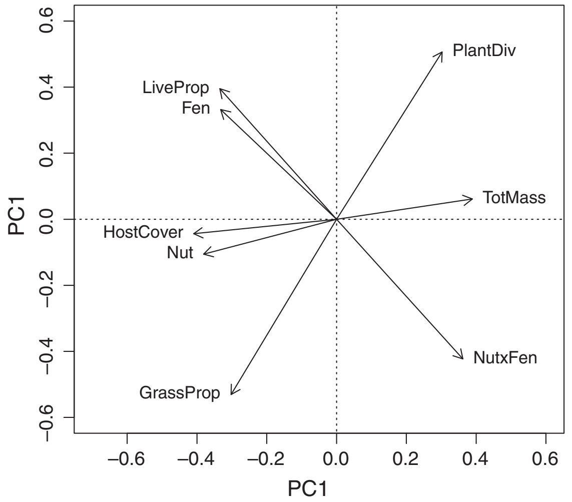

FIG. 4. Principal components analysis (PCA) showing the correlation among the effects of nutrient addition, herbivore fencing, and plant community on the change in abundance of 2,769 individual fungal endophyte taxa from the leaves of the grass, *Andropogon gerardii*. The PCA is based on eight variables that summarize the effects of each parameter in a linear regression on the abundance of individual fungal OTUs. Parameter abbreviations are as follows: Fen = fencing treatment, Nut = nutrient treatment, Nut×Fen = nutrient and fencing treatment interaction, TotMass = total plant biomass, LiveProp = proportion live biomass, GrassProp = proportion grass biomass. HostCover = percent host (*A. gerardii*) cover, PlantDiv = plant diversity. The first two principal components, shown here, account for 81% of the variation in regression parameters among the fungal taxa.

Other studies find a combination of these processes acting at different scales. For example, a study of the crayfish microbiome demonstrated that the bacteria on the carapace reflect the microbial community in the environment, whereas bacteria on gills are far more consistent, less diverse, and associated with host function (Skelton et al. 2017). The data in our study represent a single snapshot in time; repeated sampling would provide greater insight into temporal turnover in these endophyte communities.

Although the diversity of the microbiome may be directly affected by environmental factors such as nutrient supply and resulting nutrient content of a host, or herbivory and predation pressure (Smith and Holt 1996, Smith et al. 2005, Cumming and Guegan 2006, Borer et al. 2013, David et al. 2016), these factors also may affect the diversity, composition, and productivity of the local community and have indirect effects on microbial communities within individual hosts (Mitchell et al. 2002, Ezenwa et al. 2006, Keesing et al. 2006). In our study, we found that direction and strength of the effects of nutrient supply on fungal endophyte diversity within individual hosts differed strongly among sites. This site dependence may reflect the importance of environmental context in governing host–endophyte interactions, as has been demonstrated in other perennial grass hosts (Ren et al. 2011, 2014, Busby et al. 2016, Whitaker et al. 2018, Buckley et al. 2019). In contrast to the direct effects of host nutrient content on endophyte diversity, we found that effects apparently mediated by changes in the local plant community were much more consistent. Notably, fungal diversity declined with total plant biomass across all sites and treatments. The strong coupling between the microbial and plant communities has been observed in other experimental and observational studies (Kowalchuk et al. 2002, Peay et al. 2013, Leff et al. 2015, Prober et al. 2015), and highlights the need for stronger linkages between microbial, community, and ecosystem ecology.

The decline in fungal diversity with increasing total plant biomass provides insight into some of the processes governing endophyte diversity in these grasslands. Island biogeography theory has been applied to microbial, pathogen, and parasite systems, and would predict that the increase in available biomass, i.e., microbial habitat, should lead to an increase in diversity (Kuris et al. 1980, Kinkel 1997). Island biogeography theory assumes that all species (i.e., microbes) have identical fitness and that richness is driven strictly by the balance of colonization and extinction of identical species (MacArthur and Wilson 1967). In contrast, models that incorporate fitness differences or strong competitive hierarchies predict that increased dispersal can lead to a loss of diversity because of exclusion of competitively inferior species (Nowak and May 1994, Tilman 1994, Noble et al. 2011, Seabloom et al. 2015*a*). It may be in our system that the increased plant biomass led to more rapid spread of competitively dominant fungi, leading to a decline in fungal diversity within a host individual. Note that richness, diversity, and evenness are all highly correlated in our data. Alternately, the higher biomass may have changed the microenvironment (e.g., increased humidity) in the plots in ways that favored a smaller subset of fungal taxa. It is also possible that the plant hosts outgrow fungal colonists in highly productive communities. More detailed studies would be needed to resolve the importance of these potential mechanisms.

We did not find evidence for direct effects of host (*A. gerardii*) abundance or plant diversity on endophyte diversity, although such host–endophyte diversity correlations have been found in other studies of foliar fungal endophytes. For example, fungal endophyte incidence and diversity tracks plant diversity across latitudinal clines in tree foliage (Arnold and Lutzoni 2007). Similarly, coffee planted in diverse plantations had greater foliar fungal diversity than leaves from plants in lower-diversity agroecosystems (Saucedo-Garcia et al. 2014). The association between diversity of plants and diversity of endophytes could arise through a variety of pathways, including host specialization (Joshee et al. 2009), strong environmental filtering (Skelton et al. 2017), or dispersal limitation (Oono et al. 2017). Where endophytes are host generalists or not dispersal limited, this diversity association may break down (Vincent et al. 2016).

We note that our study quantified diversity as the probability of interspecific encounter (Chase and Knight 2013), which is less sensitive to extremely rare species, whereas many of the studies cited here are based on richness or rarefied richness, which do not explicitly account for abundance distributions in the community. Although our results were similar if we used endophyte diversity or rarefied richness, we suggest that the abundance-weighted metric provides two advantages for multiscale ecological studies, though documenting total endophyte diversity is also critical. First, abundance-weighted metrics provide a more consistent metric of diversity among communities that differ in sampling intensity (i.e., number of reads; Chase and Knight 2013). They also provide a strong link between diversity, species interaction intensity, and community function (Chase and Knight 2013).

In our system, although we have evidence for dispersal limitation, either the range of diversity is not wide enough to detect a coupling between plant and microbial diversity or host generalists dominate the microbial communities in our focal grassland species. In our experiment, we did not directly manipulate local plant diversity; existing plant diversity experiments would provide strong tests of the interactions between local plant community diversity and endophyte diversity within individual hosts. In addition, controlled laboratory experiments using endophyte cultures would likely provide more mechanistic insight into the microbe–microbe and plant–microbe interactions that ultimately govern endophyte community diversity.

Recent technological advances have opened a new window into microbial communities, and perhaps the most striking result arising from these new data is the high levels of diversity and variability among communities. Consistent with previous work (Arnold and Lutzoni 2007, U'Ren et al. 2012, Zimmerman and Vitousek 2012, Vincent et al. 2016, Whitaker et al. 2018), we found highly diverse communities of fungal endophytes. Although the variation in these communities was dominated by turnover at large spatial scales (i.e., among sites), almost a quarter of the community variability occurred among plants within a single 5 × 5 m plot. The enormous variation in foliar fungal endophytes within and among sites is similar to other studies (Zimmerman and Vitousek 2012, Higgins et al. 2014, Hardoim et al. 2015, Whitaker et al. 2018), suggesting that hyperdiversity at multiple scales is an emerging paradigm rather than an exception. These results highlight the need for empirical and theoretical approaches that incorporate the full range in scale from the individual to the biogeographic, if we are to understand the processes that govern the formation and maintenance of microbial communities (Peay et al. 2010*a*, Borer et al. 2013, 2016).

A mechanistic understanding of the causal factors affecting microbiome diversity and composition remains out of grasp, despite a wealth of data. We suggest that this understanding is limited by the lack of tightly coupled experimentation and theory that originally moved community ecology from an observational to a predictive science (Simberloff and Wilson 1969, Tilman 1977, Costello et al. 2012). Although observational studies are an important starting point to understanding the drivers of microbiome diversity and composition, experimentation is critical to determining underlying drivers, and predicting the effects of environmental change. For example, environmental nutrient supply can alter microbial symbiont communities through a variety of mechanisms, including changes in host immune response, microbial fitness, or among-microbe interactions (Smith et al. 2005, Kau et al. 2011, Borer et al. 2013, 2016, Lacroix et al. 2014*b*). However, large-scale environmental gradients in nutrients can be correlated with many other factors (e.g., climate, local plant diversity, and host species abundance) that also can change microbial communities (Mittelbach et al. 2001, Seabloom et al. 2010, Lacroix et al. 2014*a*). For this reason, distributed experiments that are replicated at biogeographic scales can be powerful tools for studying the underlying drivers of microbial communities generally (Leff et al. 2015, Prober et al. 2015) and have great potential to deepen our knowledge of processes determining the composition of microbiomes. Furthermore, existing community ecology theory, and metacommunity theory in particular, may provide the needed conceptual framework through which we can understand the general processes that govern the assembly of microbiome communities (Costello et al. 2012, Seabloom et al. 2015*a*, Borer et al. 2016).

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at http://onlinelibrary.wiley.com/doi/10.1002/ecy.2758/suppinfo

DATA AVAILABILITY

Data are available from the Environmental Data Initiative: https://doi.org/10.6073/pasta/a99564dd7604f33df9936ae21b8e08e0