



A suite of rare microbes interacts with a dominant, heritable, fungal endophyte to influence plant trait expression

Joshua G. Harrison¹  · Lyra P. Beltran² · C. Alex Buerkle¹ · Daniel Cook³ · Dale R. Gardner³ · Thomas L. Parchman² · Simon R. Poulson⁴ · Matthew L. Forister²

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Abstract

Endophytes are microbes that live, for at least a portion of their life history, within plant tissues. Endophyte assemblages are often composed of a few abundant taxa and many infrequently observed, low-biomass taxa that are, in a word, rare. The ways in which most endophytes affect host phenotype are unknown; however, certain dominant endophytes can influence plants in ecologically meaningful ways—including by affecting growth and immune system functioning. In contrast, the effects of rare endophytes on their hosts have been unexplored, including how rare endophytes might interact with abundant endophytes to shape plant phenotype. Here, we manipulate both the suite of rare foliar endophytes (including both fungi and bacteria) and *Alternaria fulva*—a vertically transmitted and usually abundant fungus—within the fabaceous forb *Astragalus lentiginosus*. We report that rare, low-biomass endophytes affected host size and foliar %N, but only when the heritable fungal endophyte (*A. fulva*) was not present. *A. fulva* also reduced plant size and %N, but these deleterious effects on the host could be offset by a negative association we observed between this heritable fungus and a foliar pathogen. These results demonstrate how interactions among endophytic taxa determine the net effects on host plants and suggest that the myriad rare endophytes within plant leaves may be more than a collection of uninfluential, commensal organisms, but instead have meaningful ecological roles.

Introduction

Plants are intimately associated with numerous fungi and bacteria that live within leaves, roots, stems, and other tissues [1, 2]. These microbes, termed endophytes [3] are

ubiquitous and occur in hosts representing all major lineages of plants [4]. Over the last 20 years, it has become clear that dominant endophytic taxa can have dramatic ecological consequences—a finding demonstrated particularly well in studies manipulating the abundance of vertically transmitted fungi occurring within cool-season, perennial grasses [5, 6]. For example, these fungi can influence successional trajectories of vegetation [7, 8], reshape host-associated arthropod assemblages [9], and mediate host reproductive output [10]. In contrast, the ecological roles of rare endophytes—which we define as those taxa that are infrequently encountered and of low biomass—remain largely unexamined, despite that fact that these rare taxa constitute the bulk of biodiversity present within endophyte assemblages. Here, we manipulate both rare and dominant endophytes living within a perennial forb to characterize how these taxa interact and affect host phenotype.

Most endophytes are horizontally transmitted among mature hosts via rainfall, air currents, or arthropods [11, 12] and colonize only a few cubic millimeters of host tissue [13]. Given the low biomass of these rare taxa, it is tempting to downplay their importance. However, examples from

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✉ Joshua G. Harrison
joshua.harrison@uwyo.edu

¹ Department of Botany, University of Wyoming, Laramie, WY, USA

² Ecology, Evolution, and Conservation Biology Program, Biology Department, University of Nevada, Reno, NV, USA

³ Poisonous Plant Research Laboratory, Agricultural Research Service, United States Department of Agriculture, Logan, UT, USA

⁴ Department of Geological Sciences & Engineering, University of Nevada, Reno, NV, USA

macroorganism community ecology demonstrate that certain “keystone” species, despite relatively low abundance, can exert community-wide influence [14]. For instance, beavers are uncommon mammals, yet, by reshaping fluvial geomorphology, they have profound influence on co-occurring aquatic animals, waterfowl, and riparian plants [15]. Similarly, rare endophytes could function as keystone species via several mechanisms, including by influencing the host phenotype, catabolism of low-concentration compounds into products required by other microbial taxa, or synthesis of potent bioactive compounds [16–18].

However, the ecological influence of rare endophytes need not be the purview of just a few species. Instead, minor effects of individual taxa could accrue to the point of assemblage-wide relevance—just as numerous genetic variants, each of minimal influence, commonly underlie phenotypes [19]. For example, an individual endophytic bacterium may trigger a highly localized immune response of negligible importance for the host and co-occurring endophytes. But the combined effects of many bacteria might initiate systemic acquired resistance within plants, with important implications for pathogen resistance and endophyte community assembly [20, 21].

Ascribing ecological influence to endophytic taxa, rare or otherwise, is complicated by a lack of understanding regarding how endophytes mediate plant trait expression [22, 23]. While the effects of certain endophytes on host growth promotion [24] and pathogen resistance [25–27] have attracted attention, few studies have examined endophyte mediation of other traits—including, for example, functional traits such as specific leaf area (e.g. [28]), phenology [29], and foliar elemental concentration [30] (for more, see reviews by [22, 23, 31]). Nevertheless, the handful of studies demonstrating plant trait mediation by endophytes are impressive. For instance, Mejía et al. [32] reported that inoculation of *Theobroma cacao* trees with the widespread, horizontally transmitted, fungal endophyte *Colletotrichum tropicale* affected expression of hundreds of host genes, including upregulation of some involved in the ethylene-driven immune response. These authors also found that inoculation decreased photosynthetic rate, increased leaf cellulose and lignin content, and shifted foliar isotopic ratios of nitrogen (N) and carbon (C). Similarly impressive results were reported by Dupont et al. [33] who found colonization of the grass *Lolium perenne* by the *Epichloë festucae* endophyte affected transcription of one third of host genes (for slightly more tempered, results see [34]). These studies demonstrate the importance of systemic, or otherwise abundant, endophytes on their hosts, but we are unaware of any studies that manipulate the presence of low biomass, non-systemic endophytes to determine the extent to which they have similar effects on host phenotype.

Here, we perturb the microbial consortium within the fabaceous forb *Astragalus lentiginosus* (spotted locoweed) to understand how endophytes belonging to different abundance categories affect plant trait expression. *A. lentiginosus* is a widespread, perennial forb that grows throughout the arid regions of the western United States of America [35]. *A. lentiginosus* exhibits extreme phenotypic variation and has over 40 varietal designations [35], making it the most taxonomically rich plant species in North America [36]. A dominant fungal endophyte present within *A. lentiginosus* is *Alternaria fulva* (Ascomycota: Dothideomycetes: Pleosporaceae: *Alternaria* section *Undifilum* [37–39]). *A. fulva* is a seed-borne endophyte that grows systemically through its host and synthesizes the bioactive alkaloid swainsonine [40]. Consumption of swainsonine-laced tissues by mammalian herbivores can lead to extreme toxicosis and even death [41]. *A. fulva* is prevalent throughout the range of its host, though not all populations of *A. lentiginosus* are colonized by the fungus, and intra-population variation in fungal colonization has also been reported [42].

Alternaria section *Undifilum* fungi have been observed in numerous swainsonine-containing taxa within *Astragalus* and *Oxytropis* that are colloquially called “locoweeds” [39, 43–45]. The nature of the relationship between locoweeds and their seed-borne fungi is somewhat unclear. Swainsonine does not seem to influence certain specialist arthropod herbivores [46, 47], which is suggestive of commensalism [48]. However, recent work supports a more mutualistic relationship between plant and fungus. For instance, Harrison et al. [42] demonstrated, via a DNA sequence-based survey, that swainsonine concentrations and *A. fulva* relative abundance were inversely related to fungal endophyte richness, potentially reducing exposure of hosts to pathogens. In a culture-based survey, Lu et al. [49] reported similar results in two other locoweed species (also see [50] for an analogous phenomenon in a grass-*Neotyphodium* endophyte system). The results from these surveys suggest that vertically transmitted *Alternaria* endophytes can shape fungal endophyte assemblages, though effects on bacterial endophytes are unknown. In addition, Cook et al. [51] demonstrated that *Alternaria* section *Undifilum* endophytes can affect the biomass and protein content of several locoweed taxa, including *A. lentiginosus*. These results suggest *A. fulva* may mediate other host traits as well.

By removing embryos from the seed coat, the abundance of *A. fulva* in plant tissues can be greatly reduced or eliminated [52]. We used this approach to manipulate the abundance of *A. fulva* in *A. lentiginosus* plants to experimentally test the aforementioned antagonistic relationship between *A. fulva* and co-occurring endophytes and explore how *A. fulva* affects various host traits, including size, leaflet area, specific leaf area, foliar C and N, phenology,

and nitrogen fixation in the rhizosphere. For a subset of focal plants, we applied an inoculum slurry to leaf surfaces to boost exposure to rare, horizontally transmitted endophytes. We applied these manipulations in a full factorial design to compare how endophytes of differing abundance categories shape host plant phenotype and explore how a dominant endophyte might influence co-occurring fungi and bacteria.

Methods

Field experiment

During the early spring of 2017, seeds of *A. lentiginosus* var. *wahweapensis* from the Henry Mts. in Utah, USA (collected in 2005 from a population known to possess *A. fulva*) were lightly scarified, left to imbibe deionized water overnight and then germinated indoors in a mix of humus, compost, and topsoil sourced from the Reno, NV region. To reduce the relative abundance of the vertically transmitted fungal endophyte, *A. fulva* embryos were excised from a subset of seeds prior to planting, as per [52]. Notably, this treatment can eliminate the presence of *A. fulva* from plants altogether, but occasionally only reduces *A. fulva* abundance. Seedlings were grown at ambient temperature under a 16:8 (light:dark) daily lighting regime and watered with dechlorinated tap water. Individuals from different treatments were interspersed haphazardly and not allowed to touch one another. Seedlings were periodically reorganized to avoid any influence of subtly differing conditions across the growth area. To speed growth, Miracle-Gro (Scotts Miracle-Gro Company, Marysville, OH) was applied several times to all replicates during the first month of growth. To control for possible confounding effects of seed coat removal, embryos were excised from a subset of seeds and planted along with potato dextrose agar (PDA) that was sterile, or that was colonized by *A. fulva*, which had been cultured from intact seeds. These control seedlings were planted several weeks later than other seedlings, due to slow growth of *A. fulva* cultures.

In early June, seedlings were installed in five gallon pots filled with equal parts locally sourced humus and topsoil and placed in an abandoned, largely denuded field near the University of Nevada, Reno. No *Astragalus* taxa were observed growing within this field and the plants that were present in the field were mostly non-native forbs. *A. lentiginosus* grows in a wide variety of settings, including roadsides and disturbed areas not unlike our site. A total of 300 plants were installed (between 54 and 68 per treatment group, see Table 1). Pots were organized randomly with respect to treatment and were placed one meter apart so plants never touched one another. Dechlorinated water was

Table 1 Number of *Astragalus lentiginosus* plants installed in field experiment for each treatment group.

<i>A. fulva</i> reduced	Inoculum applied	Sample size
Experimental plants		
Yes	Yes	62
Yes	No	68
No	Yes	54
No	No	54
Control plants installed with agar		
Yes	Yes	13
Yes	No	13
No	Yes	18
No	No	18

Treatments were applied to plants in a full factorial design and included reduction of the relative abundance of the vertically transmitted fungal endophyte, *Alternaria fulva* (through embryo excision) and foliar application of regionally sourced, endophyte inocula. To control for effects of seed coat removal, embryos were excised from seed coats and planted alongside agar that was either sterile or contained *A. fulva*.

applied as needed to all plants at the same time (typically every other day, except during the heat of summer when watering was conducted daily). Every 2 weeks a slurry of microbial inocula (described below) was sprayed on leaves of half of the plants. A solution with identical surfactant, but no microbial inoculum, was applied to untreated plants. Plants were left in the field from early June through mid-September, at which point leaves were removed for sequencing and culturing.

Inoculum preparation

Twenty morphologically unique, reproductive fungal isolates were obtained from the following woody shrubs growing near Reno, NV: *Artemisia tridentata*, *Ericameria nauseosa*, *Prunus andersonii*, and *Tetradymia canescens*. These shrubs are abundant throughout the Great Basin Desert and, consequently, we reasoned they contained horizontally transmitted, foliar microbes likely to be regularly encountered by *A. lentiginosus*. Indeed, sequencing revealed that most of the fungal taxa within the inoculum were observed in the wild-collected *A. lentiginosus* individuals examined in [42] (for details see the Supplemental Material). Individual shrubs to be sampled were selected haphazardly. We did not use leaves from *Astragalus* species to avoid inoculating plants with *A. fulva* and thus obviating our treatment to reduce this fungus. Leaves were cut into sections (of several mm²) and placed on PDA and the resulting microbial growth isolated and subcultured over 2 weeks. Spores from isolates were removed and suspended in deionized water and 0.0001% TWEEN 20 (Sigma-Aldrich), a detergent that functioned as a surfactant. A

haemocytometer was used to dilute the suspension to $\sim 100,000$ spores ml^{-1} . This concentration was chosen because it produced no obvious negative symptoms in *A. lentiginosus* seedlings during preliminary experiments. Aseptic technique was used throughout culturing and inoculum preparation. Two aliquots of inoculum were sequenced to identify the constituent microbial taxa. We specifically targeted fungi for culturing, but sequencing revealed that bacteria were also present within the inoculum.

Plant trait measurement

All plant traits were measured concomitant with sample collection for foliar microbiome characterization. Plant size was measured as the minimum size box that would enclose the plant. This was calculated as the product of the width of the plant at its widest point, the width of the plant perpendicular to that point, and plant height. Phenological state and number of leaves were characterized for each plant. Area and specific leaf area (SLA; leaflet area divided by mass of leaflet) were measured for three dried leaflets per plant and averaged. Two healthy leaflets were removed from 8–12 leaves per plant, rinsed with tap water, dried in a laminar flow hood (<12 h total) and frozen until further processing. These leaflets were then parsed for microbiome characterization, swainsonine quantification, and carbon (C) and nitrogen (N) analysis. Swainsonine concentration in ~ 50 mg of dried, ground foliar tissue was measured using an LC–MS/MS approach described in [53]. Briefly, an 18 h extraction in 2% acetic acid with agitation was followed by centrifugation. Supernatant was added to 20 mM ammonium acetate and subjected to LC–MS/MS analysis. Percent C and N and ^{14}N : ^{15}N isotopic ratios in 3–4 mg dried foliar tissue, were measured by the Nevada Stable Isotope Laboratory using a Micromass Isoprime stable isotope ratio mass spectrometer (Elementar, Stockport, UK) and a Eurovector elemental analyzer (Eurovector, Pavia, Italy). The percentage of nitrogen in tissues due to fixation alone (Ndfa) was calculated as per [54] through comparison with samples from co-occurring *Chenopodium album*, which is not known to harbor nitrogen fixing rhizosphere bacteria.

Sequence and culture-based characterization of the foliar microbiome

We characterized endophytic assemblages through both culturing and DNA sequencing, thus affording us insight into the effects of treatment on microbial assemblages via two complimentary measurement techniques. For our culture-based assay, we choose three leaflets per plant. Leaflets were surface sterilized, cut into 3–4 pieces, and

plated onto PDA using aseptic technique. Surface sterilization involved rinsing in 95% ethanol for 30 s, followed by 2 min in 10% sodium hypochlorite solution, 2 min in 70% in ethanol, and a final rinse with deionized water. Preliminary experiments confirmed the success of this surface sterilization technique. Cultures were grown in the dark at ambient temperatures for 1.5 months. Microbial growth (either fungal or bacterial) was isolated, subcultured, and the number of morphologically unique cultures and the percentage of leaf pieces colonized recorded. Cultures corresponding to *A. fulva* were identified visually through comparison to *A. fulva* cultures grown from seeds used for this experiment and through sequencing (we did not sequence the other cultures for logistical reasons).

DNA was extracted from three surface-sterilized, dried, and ground leaflets per plant using DNeasy plant mini kits (Qiagen, Hilden, Germany). Extraction blanks for each kit were used as negative controls. Dual-indexed libraries were made at the University of Wyoming and were sequenced on the Illumina NovaSeq platform (paired-end 2×250 ; San Diego, CA, USA) by Psomagen, Inc. (Rockville, MD, USA). To characterize bacterial assemblages, the 16S (V4) locus was amplified using the 515–806 primer pair [55], while for fungal assemblages the ITS1 locus was amplified using the ITS1f-ITS2 primer pair [56]. A synthetic DNA internal standard (ISD) was added to template DNA prior to library creation [57]. In addition, unique synthetic DNAs of our own design were added to each sample to allow cross-contamination to be detected (*sensu* [58]; we refer to these oligos as “coligos,” which is short for cross-contamination checking oligos). A mock community consisting of eight bacteria and two fungi was also sequenced (Zymo Research, Irvine, CA, USA) as a positive control. PCR was performed in duplicate and unique index sequences were ligated onto each PCR replicate, thus allowing us to determine technical variation due to PCR. For full library preparation details see the Supplemental Material.

Sequence data were demultiplexed using a custom perl script that used Levenshtein distances to correct errors in index sequences and assign reads to samples. Primers and Illumina adapters were removed using cutadapt v1.13-py27 (Martin 2011) and poly-G tails removed using fastp v0.21.0 (poly-G tails occurred due to a lack of signal for very short-template molecules, such as our coligos [see above]);. Paired-end reads were merged using vsearch v2.9.0 [59, 60] with staggering allowed, a minimum of ten overlapping bases, and a maximum of 12 mismatches in the overlapping region. The probability of base-calling errors within merged reads was estimated and those reads expected to include more than one error were discarded.

Using vsearch, unique reads were identified and clustered into OTUs (operational taxonomic units) using the ‘cluster unoise’ algorithm [61] with a minimum of

12 sequences required, each with a minimum length of 56 nucleotides. OTUs clustered via this algorithm may differ by as little as a single nucleotide and are sometimes referred to as exact sequence variants, or ESVs [62]. There is an ongoing dialog regarding the use of ESVs for fungi; for a justification of our approach see the Supplemental Material.

Chimeras were detected using the ‘uchime3 denovo’ algorithm and removed. For ITS sequences, those reads that did not merge were concatenated and processed separately, but identically, as those that did merge. OTUs made from shorter, merged reads were aligned to those made using the longer, concatenated reads and any short OTUs that aligned were not considered. We chose this approach for ITS reads because in preliminary work we discovered that, for some taxa, the ITS1 region was too lengthy to allow paired-end sequences to merge. After removing chimeras, OTUs, both long and short, were combined and an OTU table was made via aligning both merged and concatenated, unmerged reads to OTUs using the ‘usearch global’ algorithm.

Taxonomic hypotheses for OTUs were generated using the SINTAX algorithm [63] and the UNITE (v7.2; [64]) and Ribosomal Database Project database (RDP; v16; [65]) for fungi and bacteria, respectively. Read counts for OTUs corresponding to the ISD were summed for each replicate to allow normalization by the ISD. For 16S data, host plastid DNA was identified through comparison to all fabaceous chloroplasts available from the NCBI nucleotide database (accessed Feb 9, 2016; [42]). For ITS data, host DNA was identified through matching known *A. lentiginosus* and *Oxytropis* sp. (sister taxon to *Astragalus*) ITS sequences to OTUs. For both 16S and ITS datasets, reads for all plant OTUs were summed. In addition, for the 16S data, OTUs for mitochondrial DNA were identified using the MIDORI database [66] and removed from the OTU table. For both 16S and ITS data, those few OTUs that were not classified to any taxon were removed from the data. If >5% of the total reads for an OTU were in negative controls, then these OTUs were deemed possible contaminants and discarded (30 fungal OTUs and 2 bacterial OTUs were discarded). OTUs corresponding to *A. fulva* were identified through comparison to GenBank accession JX827264.1 and those corresponding to *Leveillula taurica* with accession MT472005.1.

Statistical analysis

We analyzed sequence count data via a hierarchical Bayesian modeling (HBM) framework that provides estimates of proportional relative abundance for each microbial taxon [67, 68]. The model estimates parameters of replicate-specific, multinomial distributions that describe taxon proportions (p parameters) and Dirichlet parameters that describe proportion estimates for the entire sampling group. This

method shares information among replicates for more accurate parameter estimation and allows propagation of uncertainty in parameter estimates to downstream analyses (for a full description, see [68]). Rarefaction is not needed when using this modeling approach, because proportion estimates are used for downstream analyses and because estimates for each replicate are informed by data from all other replicates due to the hierarchical nature of the model. Modeling was conducted in the R computing environment (R Core Team [69]) using the CNVRG v0.2R package [70]. CNVRG is a user-friendly wrapper for implementing Dirichlet-multinomial modeling with rStan [71], which itself is an interface to the Stan model specification software [72]. We used the Hamiltonian Monte Carlo sampling algorithm to characterize posterior probability distributions (PPDs). We took 1000 samples from posteriors, with a thinning rate of two, after a burn-in of 500 samples. Model convergence was confirmed via the Gelman-Rubin statistic (all parameters had a statistic very near one [73]). To account for compositionality, we divided the proportion estimate for each taxon in a replicate by the proportion of reads assigned to the ISD for that replicate. By placing the relative abundances of all taxa on the scale of the ISD, we were able to better compare taxon abundances among treatment groups (for more details of the problem of compositionality and how an ISD can help see [57, 74]).

To measure the extent to which OTUs differed in relative abundance among treatment groups, PPDs for Dirichlet parameters for each OTU and treatment group were subtracted. This generated a PPD of the difference in that parameter between any two treatment groups. If 5% or less of the density of that PPD was on either side of zero, then we deemed a treatment-associated shift in microbial relative abundance was credible. Means of PPDs for parameters of interest were used as point estimates for those parameters.

Species equivalents of Shannon’s entropy and Simpson’s diversity [75] were calculated using CNVRG for the treatment group as a whole and for each replicate. To estimate diversity equivalencies for a treatment group, the equivalency was calculated for *each* sample of the Dirichlet distribution characterizing microbial relative abundances within that treatment group. This generated a PPD of diversity, thus propagating uncertainty in relative abundance estimates into estimates of treatment group diversity (for a similar approach see [76]). To determine how diversity equivalents differed between treatment groups, the overlap of PPDs for each group was examined (as per above). Diversity equivalents were also estimated for each replicate so that these estimates could function as the response in a linear model testing for associations between plant trait variation and shifts in microbial diversity (see below). To estimate diversity for each replicate, the means of PPDs of multinomial parameters for that replicate were

calculated (recall that these parameters estimated proportional microbial relative abundance) and diversity equivalencies were calculated for the resulting vector.

HBM was also used to estimate differences among treatment groups in the mean values of plant traits, sequence-based estimates of microbial diversity, and culture richness. Each response variable was modeled as a draw from a normal distribution characteristic of the sampling group as per [77]. The mean (μ) and variance (σ^2) of this distribution was estimated through sharing of information among replicates. The prior distribution for μ was a normal distribution centered at zero with a precision of 0.0001 (variance = 10,000). The prior distribution for σ^2 was a uniform distribution from 0 to 100 (for full model specification see provided R code). MCMC sampling and tests for credible effects of treatment via PPD overlap were conducted as described above. For these analyses, we used the JAGS model specification language [78] as implemented via rjags v4-6 [79].

To evaluate associations between plant traits and microbial diversity, linear models were created in a HBM framework. Beta coefficients for plant traits were estimated for each treatment group, with a prior sampled from a normal distribution centered at the estimated across-treatment effect of each trait and a precision estimated across all treatments. Hyperpriors for beta coefficients were normal distributions centered at zero with a precision of 0.0001 (for full model specification see R code provided; also see [42]). Means of PPDs for each beta coefficient were used as point estimates of the effect of that covariate. The proportion of the PPD for each beta coefficient that did not overlap zero was used to determine certainty of a non-zero effect. Prior to modeling, missing values in covariates were imputed using the random-forest algorithm [80] as implemented by the randomForest R package [81]. When models were run without imputing data, results were similar to those reported here. To determine effects of treatment on microbial assemblages, as a whole, principal coordinates analysis (PCoA) and PERMANOVA were conducted on Bray–Curtis transformed tables of point estimates of proportions (derived as described above) for microbial taxa.

We chose not to report effects of treatment on endophyte richness using sequence data. To explain, when a dominant taxon is present (such as *A. fulva* or *L. taurica*) within a replicate it captures much of the sequencer's bandwidth for that replicate. Therefore, that replicate would have fewer reads available to allocate to the other taxa present, which would result in spuriously lower richness. Consequently, to assay effects of treatment on richness, we relied on culturing data.

We omitted from all analyses ten plants for which seed coat removal did not reduce *A. fulva* as ascertained via swainsonine concentration (this compound is not known to

be produced by the host plant), culturing, or molecular data. We also omitted plants with no evidence of *A. fulva* occurrence from the *A. fulva* positive treatment group, because *A. fulva* is known to be incompletely transmitted between generations [82]. Analyses were repeated while retaining all of these plants in their original treatment groups and the results obtained were qualitatively similar to those presented here.

Results

Sequencing summary and microbial diversity description

After removing host, ISD, coligo (oligos for accounting of cross-contamination), and contaminant reads and applying our stringent quality control approach, we retained for analysis 7,417,832 reads from 2292 fungal OTUs and 76,900 reads from 642 bacterial OTUs (from over 20 million 16S reads, most of which were from host organelles; for full details see the Supplemental Material).

The majority of fungal OTUs (which were specified at single nucleotide variation resolution) belonged to the Ascomycota (86%). Many OTUs were assigned to *L. taurica* (991 OTUs) and *A. fulva* (101 OTUs). A variety of bacterial taxa were observed, including many members of Acidobacteria, Actinobacteria, Proteobacteria, Firmicutes, and Chloroflexi. The most abundant bacterial OTUs were assigned to the Lactobacillaceae, Bacillaceae, Listeriaceae, and Staphylococcaceae.

Effects of the vertically transmitted fungus on the host and co-occurring microbes

Treatment to reduce the relative abundance of the dominant, heritable fungus *A. fulva* from *A. lentiginosus* plants was successful as evidenced by read counts (Table S1), swainsonine concentrations (Fig. 1), and culturing (Fig. 2). *A. fulva* presence influenced plant phenotype—colonized plants were much smaller and had fewer leaves than uncolonized plants (Fig. 1, S1). The negative effect of *A. fulva* on plant size was observed in the second year of monitoring as well (Fig. S2). Foliar N was affected by both *A. fulva* and rare microbes—plants without *A. fulva* and that were untreated with inoculum had elevated %N in their leaves. Moreover, *A. fulva* generally increased the δ^{15} (ratio of N isotopes) and reduced NDFA, a proxy for rhizosphere nitrogen fixation activity, though these effects were less pronounced than some other effects on host phenotype (Table 2). The effects of *A. fulva* colonization were generally similar for plants grown from embryos planted alongside *A. fulva* infected agar (see Methods, for details of this control treatment), thus

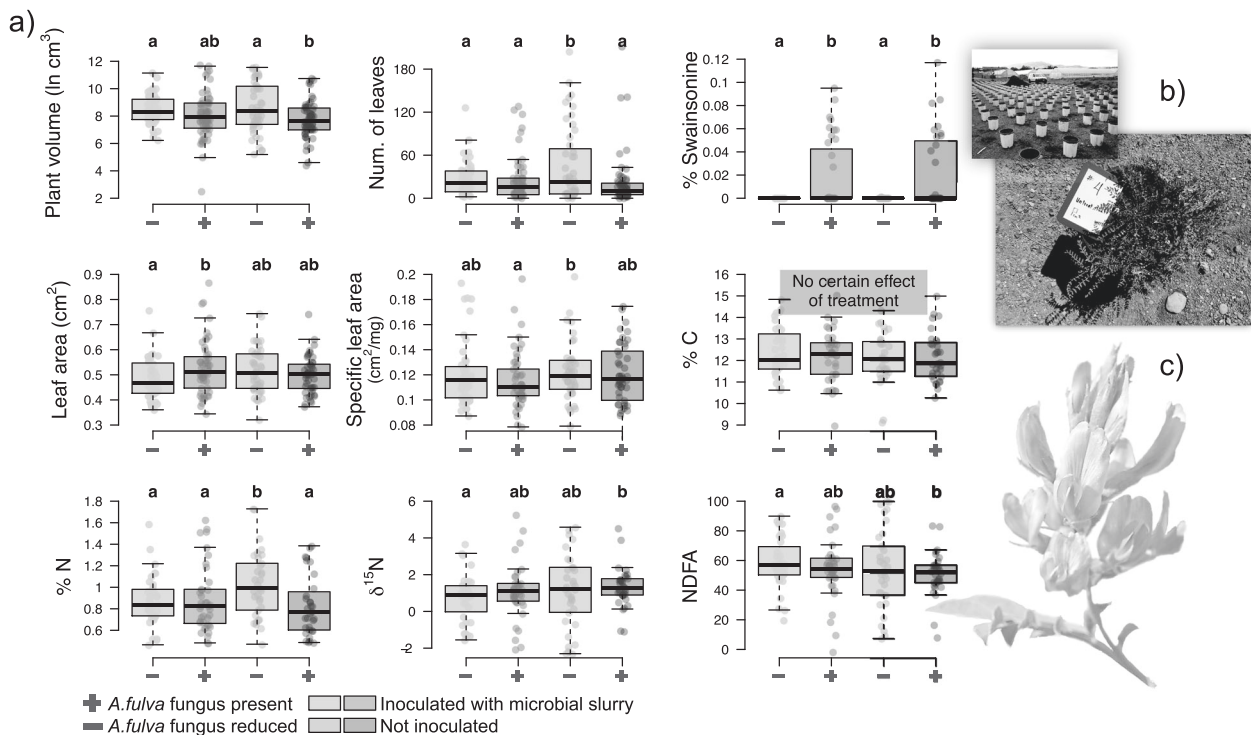


Fig. 1 Variation in *Astragalus lentiginosus* traits among treatment groups. a + and – symbols on the x axis represent treatment to reduce the relative abundance of the vertically transmitted fungus, *Alternaria fulva*. Boxes shaded blue denote treatment with endophyte inoculum slurry and boxes shaded yellow denote plants that did not receive the slurry. Percentage of N, C, and swainsonine refer to foliar dry mass composition. Differences in mean trait values among treatment groups were determined through a hierarchical Bayesian analysis. Credible

differences ($\geq 95\%$ difference in posterior probability distributions of parameter estimates) among treatment groups are designated through the letters above each boxplot. For estimates of mean trait values for each treatment group see Table 2. Boxplots summarize the data and describe interquartile range with a horizontal line denoting the median. Whiskers extend to the 10th and 90th percentiles. b shows an overview of the experimental installation and c depicts the inflorescence of *A. lentiginosus*. All photos by J. Harrison (color figure online).

the observed results are not due to the confounding influence of seed coat removal (Fig. S1). We did not observe an effect of *A. fulva* colonization on %C, or phenology (using Fisher's exact test to examine flowering status at time of harvest).

A. fulva presence modestly affected diversity of co-occurring bacterial and fungal endophytes (Fig. 3 and Table S2). Species equivalents of Shannon's entropy for both bacteria and fungi increased in plants colonized by *A. fulva* (Fig. 3), but the opposite was true for equivalents of Simpson's diversity. Simpson's diversity index places more weight on abundant taxa than does the Shannon index [83].

We also observed a negative association between *A. fulva* and *L. taurica*. *L. taurica* is a powdery mildew (Erysiphaceae) known to colonize numerous plant species, including *A. lentiginosus* [42]. *L. taurica* was the most abundant fungus sequenced and dropped in relative abundance when *A. fulva* was present (Fig. 2 and Table S1). This negative association was also observed visually, as we noted a powdery mildew infection on the leaves of a subset of the plants used for this experiment, and infections were less severe in plants colonized by *A. fulva* (Fig. S3).

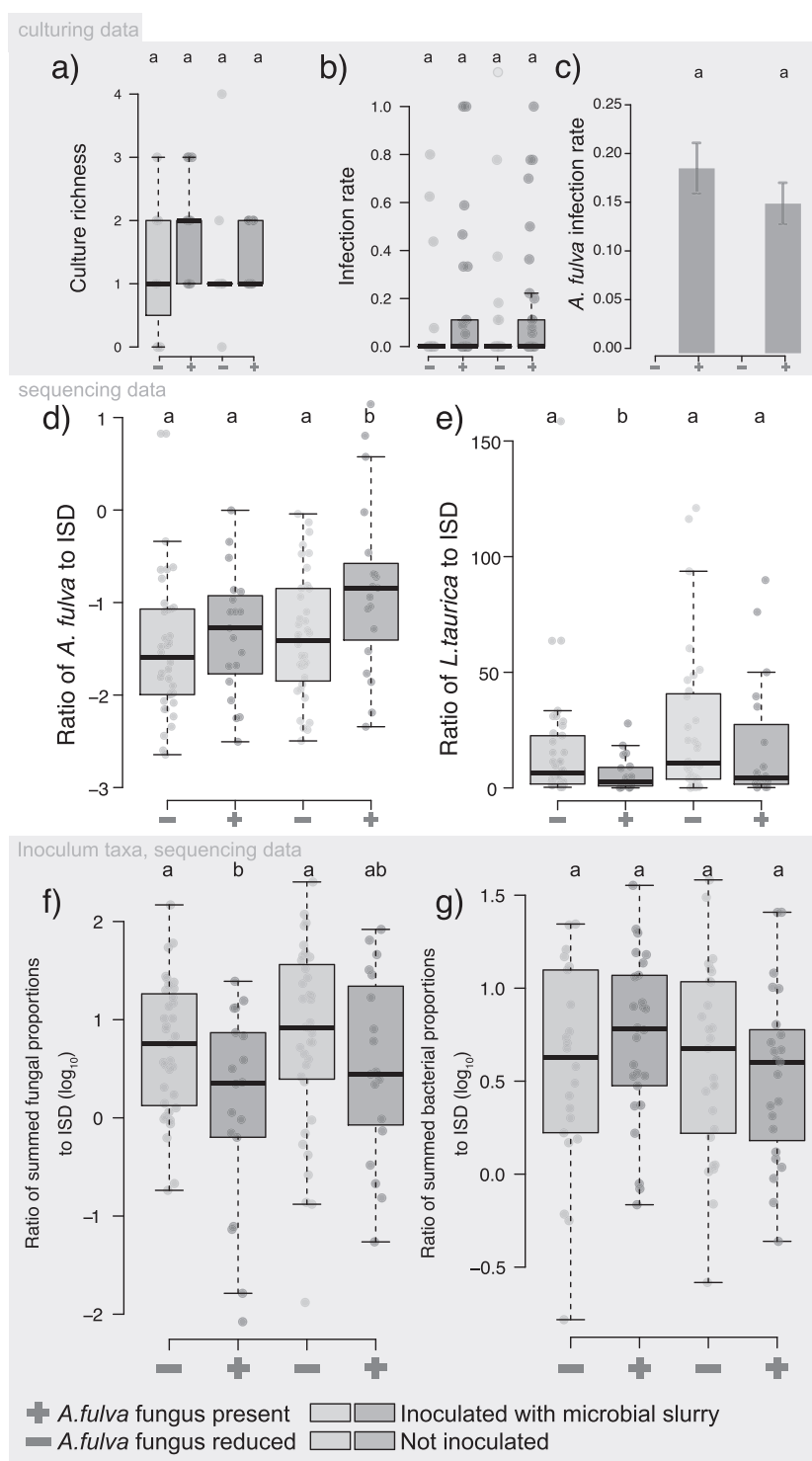
For less abundant fungi (those less than 0.04% of total reads), which accounted for all fungal OTUs aside from *A. fulva* and *L. taurica*, we observed a modest increase in absolute abundance when *A. fulva* was present (Fig. 4), which was likely responsible for the increase in Shannon's diversity with *A. fulva* infection.

The influence of horizontally transmitted endophytes on hosts

The inoculum slurry applied to plants was created from 20 morphologically distinct cultures derived from shrubs common in the Great Basin Desert. Sequencing revealed the slurry was composed of 20 fungal and 260 bacterial OTUs, including many taxa that have been observed in wild-collected plants (for a full description, see the Supplemental Material). Inoculation was successful, as shown by the effects of treatment discussed elsewhere. Additional evidence for inoculation success was that 55 of the bacterial OTUs and 3 of the fungal OTUs were sequenced from plants used in this experiment and in almost all cases, inoculum application led to an increase in the read counts

Fig. 2 Influence of treatment on microbial assemblages within *Astragalus lentiginosus*.

Boxes shaded blue denote treatment with endophyte inoculum slurry; boxes shaded yellow denote plants that did not receive the slurry. The top row contains data from culturing microbes from leaves of host plants. Data shown include culture richness (a), the proportion of leaves infected with microbes other than *A. fulva* (b), and the rate of *A. fulva* infection (c). Cultured taxa were not identified to species, but were nearly all fungi. The middle row depicts the effect of treatment on *Alternaria fulva* (d) and *Leveillula taurica* (e) abundance (estimated as the ratio of the proportion of reads for either taxon to the internal standard [ISD]); these were the two most abundant fungal taxa present. The bottom row shows abundances of fungal (f) and bacterial (g) taxa that were in the inoculum mixture used to treat plants. Credible differences among treatment groups are denoted through the letters above each boxplot. Boxplots summarize the data and describe interquartile range with a horizontal line denoting the median. Whiskers extend to the 10th and 90th percentiles. Treatment to reduce *Alternaria fulva* is shown as *A. fulva* −, with a + denoting lack of treatment to reduce this taxon (color figure online).



obtained for those taxa (in 87% of bacterial taxa and two out of the three fungal taxa). Fungi present in inoculum that were also observed in treated plants included *Preussia* sp. (the most abundant fungus in the inoculum), *L. taurica*, and *Penicillium* sp., while successful bacterial colonizers were predominantly members of Proteobacteria, Actinobacteria, and Firmicutes. Inoculum application had modest effects on

overall Shannon's and Simpson's diversity (Fig. 3). Inoculum application did not lead to significantly different centroids in PCoA ordinations (Figs. S4 and S5).

Inoculum application had no visibly pathogenic effects on plants—they appeared healthy and leaves had no evidence of necrosis. However, inoculum application did influence plant phenotype, but only when the dominant

Table 2 Trait values for *Astragalus lentiginosus* individuals in each treatment group.

	Inoc. treated, <i>A. fulva</i> –	Inoc. treated, <i>A. fulva</i> +	No inoculum, <i>A. fulva</i> –	No inoculum, <i>A. fulva</i> +
Size (cm ³)	9456 (9430,9485)	12417 (12393,12443)	20932 (20904,20,962)	6502 (6478,6528)
Leaves	26.84 (20.17,33.32)	24.78 (16.74,32.54)	45.21 (30.29,60.24)	21.86 (11.55,31.43)
Leaflet area (cm ²)	0.49 (0.46,0.51)	0.53 (0.5,0.56)	0.52 (0.49,0.55)	0.5 (0.48,0.52)
SLA (cm ² mg ⁻¹)	0.12 (0.11,0.13)	0.11 (0.11,0.12)	0.12 (0.11,0.13)	0.12 (0.11,0.13)
δ N15	0.78 (0.33,1.18)	1.09 (0.59,1.59)	1.1 (0.42,1.79)	1.32 (0.89,1.74)
%swainsonine	0 (0,0)	0.02 (0.01,0.03)	0 (0,0)	0.02 (0.01,0.03)
%N	0.87 (0.8,0.96)	0.89 (0.79,1)	1.03 (0.92,1.13)	0.83 (0.72,0.93)
%C	12.4 (12.01,12.78)	12.23 (11.82,12.65)	12.12 (11.71,12.53)	12.15 (11.74,12.55)
NDFA	58.56 (52.6,64.45)	54.24 (47.4,60.68)	54 (44.7,63.79)	51.24 (45.79,56.7)

Values shown are means of posterior probability distributions of the mean for each trait with 95% credible intervals in parentheses. A plus after *Alternaria fulva* means we did not attempt to reduce *A. fulva* abundance through embryo excision. Minus symbols mean *A. fulva* was reduced.

fungus *A. fulva* was not present. For instance, inoculum application reduced leaf count (by approximately 50%) and foliar %N (Table 2), but this was only apparent for plants without *A. fulva* (Fig. 1). Inoculum treatment had minor, idiosyncratic effects on trait variation, sometimes reducing and other times increasing variation (Table S3). For %N and plant size, the directionality of the effect of inoculum on trait variation depended on *A. fulva* treatment, with inoculation increasing trait variation when *A. fulva* was reduced, but decreasing it otherwise. In general, associations between the diversity of horizontally transmitted endophytes, either bacterial or fungal, with plant trait variation were weak and often limited to a specific treatment group (Tables S4 and S5).

Discussion

Foliar endophyte assemblages are typically composed of a few dominant taxa and numerous taxa of low relative abundances that occupy a small proportion of their host's tissues (we refer to these as rare taxa [13, 84]). Ecological relevance is often considered the domain of abundant microbial taxa, because of their greater biomass and prevalence. However, our results suggest that characterizing the overall effects of endophyte assemblages may require study of rare taxa. Indeed, we report that a suite of rare endophytes affected host size and foliar N content, among other traits. We also observed that the influence of these taxa was attenuated by the presence of a dominant fungal endophyte, which itself mediated host plant phenotype.

It is important to note that, regardless of treatment, host plants appeared healthy to the eye—their tissues were green, and, in many cases, they fruited successfully during the first and second years of growth. Thus, both *A. fulva* and co-occurring microbes meet the criterion of living asymptotically within plant tissues necessary for

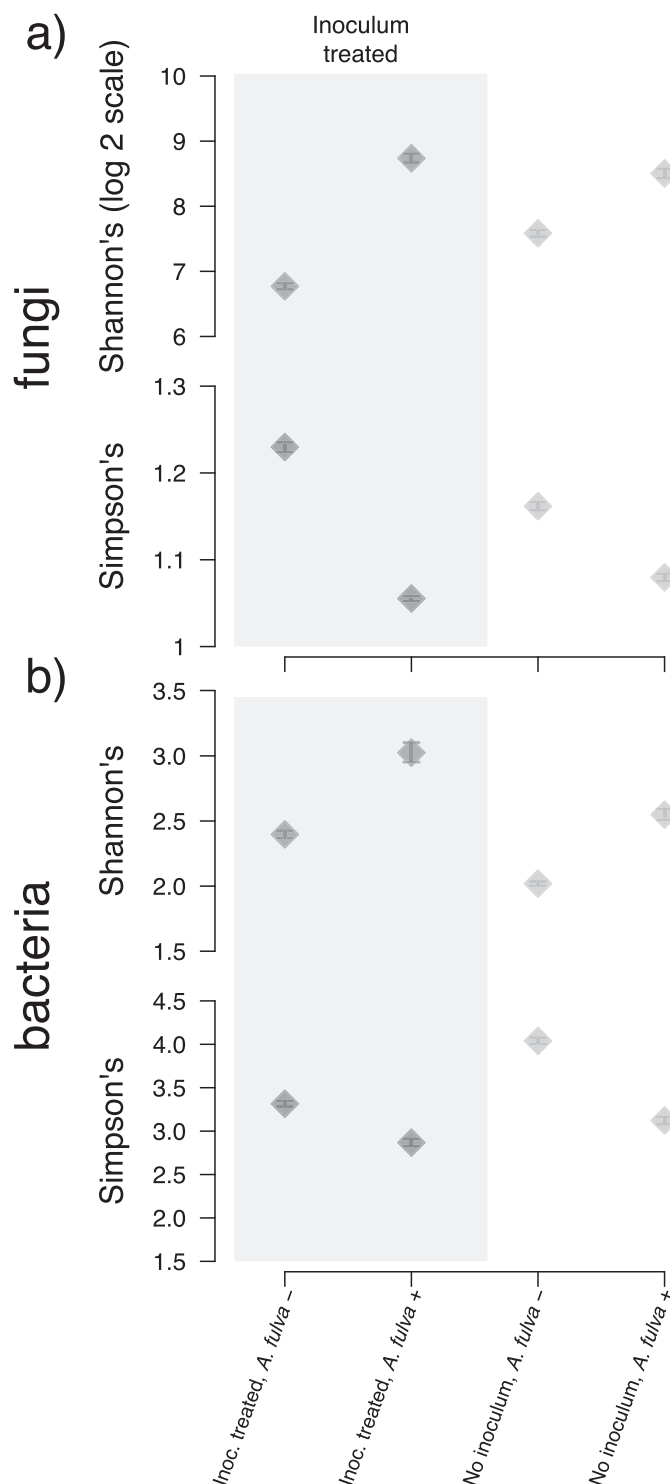
designation as endophytic taxa rather than obligate pathogens [3].

Aside from *A. fulva* and *L. taurica*, all other microbial taxa observed, be they fungal or bacterial, were rare, as shown through read counts (Fig. 4) and culture-determined infection rate (Fig. 2b). Microbial taxa within the inoculum mixture were of similarly very low abundance in plants (Fig. 2). Indeed, the abundance of all these taxa combined was generally about that of the ISD, which was spiked into samples with the minuscule concentration of 0.03 pg/μL. Thus, it was surprising that we saw fairly dramatic effects of treatment with the inoculum.

We were not able to attribute the effects of the inoculum to specific taxa, but this was by design. We were curious what the overall effects of a complex endophyte assemblage might be for plants grown in nature—thus, we were attempting to provide a different perspective than that offered by gnotobiotic studies in laboratory settings. Many of the taxa in our inoculum mixture have been observed in wild plants, with low relative abundances [42], including possible pathogens such as *Preussia*. Thus, it seems very plausible that the effects of the inoculum mixture that we observed could occur in wild populations of *A. lentiginosus*.

It seems unlikely that unintended consequences of treatment induced the results we observed since we applied a mock treatment to account for the effects of leaf wetting and surfactant application and we controlled for the effect of embryo excision from seed coats. However, we considered two caveats to our results. First, it is possible that our inoculum mixture was biased toward endophytes that grow rapidly in culture. If that was the case, then perhaps an inoculum mixture containing slower-growing taxa, which plausibly could include more biotrophic taxa, would have different effects on the host than those we observed. Second, our plants were reared with regular watering. This was by necessity because the plants were grown in pots as opposed to planted directly in the soil. Pots provided many

Fig. 3 Microbial diversity was influenced by treatment. Effect of treatment on fungal (a) and bacterial diversity (b) as estimated from sequencing data. Treatments are noted on the x axis. Points denote the means of posterior probability distributions (PPD) of diversity entropy equivalencies. 95% high density intervals for these PPDs were very narrow but are shown superimposed on each estimate. Points shaded blue denote treatment groups receiving the endophyte inoculum slurry; points shaded yellow denote groups that did not receive the slurry. Treatment to reduce *Alternaria fulva* is shown as *A. fulva* –, with a + denoting lack of treatment to reduce this taxon (color figure online).

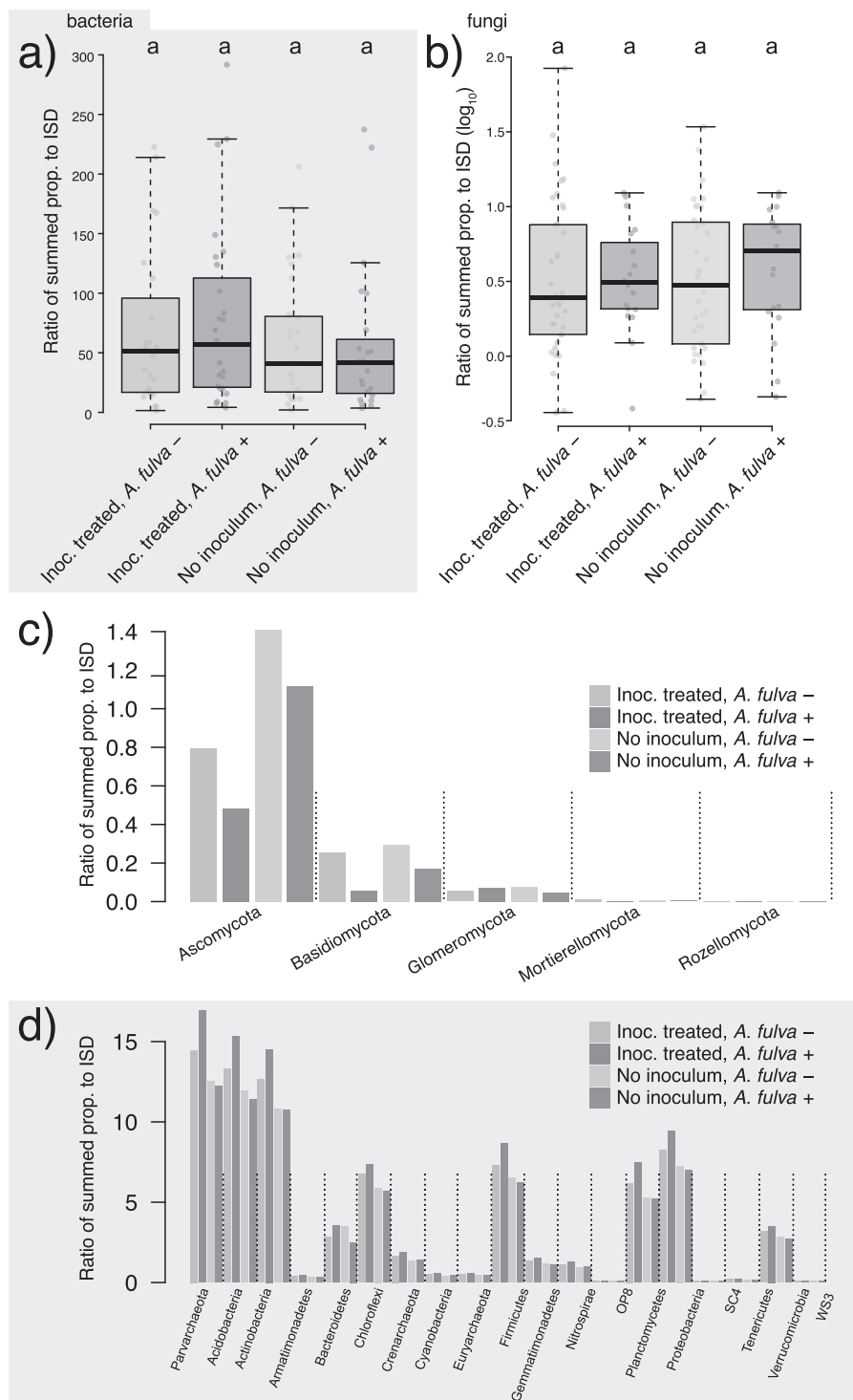


logistical advantages, but their use led to a heating of the soil and a rapid draining of water that would have killed many plants, if they did not receive water that was supplemental to precipitation. It is unclear what effects watering might have had on our results, though we note that we were careful to avoid wetting leaves during watering as leaf wetness can influence microbial colonization.

Ecological roles of rare endophytes

The effects of rare endophytes that we observed likely have implications for host fitness. Certainly the approximately 50% reduction in plant size and leaf number that we report could lead to reduced seed output (Table 2) and it seems likely that the effects on foliar N and leaf morphology we

Fig. 4 Most of the microbial biodiversity present in this experiment was low abundance. The top row shows the ratio of bacterial (a) and fungal (b) proportions to the internal standard (ISD; not including *A. fulva* and *L. taurica*, which were more abundant), with proportions for all taxa summed by treatment group. The proportion of reads for all bacterial taxa was less than 3% for each treatment group (the other reads were from host organelle DNA). As for fungi, the most abundant taxon, besides *A. fulva* and *L. taurica*, was represented by less than 5% of the fungal reads in any given treatment group and, when averaged across groups, the relative abundance of this taxon was less than a percent. Most fungal taxa in most treatment groups composed less than 0.02% of reads. The average abundances of fungal phyla by treatment group are shown on the middle row (c) and abundances of bacterial phyla are shown on the bottom row (d). Blue shading denotes treatment groups receiving the endophyte inoculum slurry, whereas groups that did not receive the slurry are shaded yellow. Boxplots summarize the data and describe interquartile range with a horizontal line denoting the median. Whiskers extend to the 10th and 90th percentiles. Treatment to reduce *Alternaria fulva* is shown as *A. fulva* –, with a + denoting lack of treatment to reduce this taxon (color figure online).



observed could also influence host fitness. Beyond the direct influence of trait variation to plant persistence and reproductive output, the marked shifts in phenotype that we observed could have indirect effects on host-associated organisms, such as arthropods, which also affect host fitness. For instance, size and foliar %N are often strong predictors of variation in insect assemblages and herbivory

across plant species [85, 86], thus shifts in these traits induced by low-biomass microbial taxa could have cascading effects on arthropod communities.

We considered two possibilities for the distribution of ecological influence among rare endophytic taxa. Specifically, influence could be limited to several keystone taxa or could be cumulative, such that a quorum must be reached

before the combined effect of rare endophytes induces a response by the host. We were unable to satisfactorily resolve these two non-mutually exclusive hypotheses. However, the quorum hypothesis might lead to a negative association between microbial diversity and plant size, because higher microbial diversity would occur when taxa were more evenly distributed, each with a role to play. We did not unequivocally observe such a negative association (Tables S4 and S5). Indeed, we found that most rare endophytes occurred infrequently in samples, suggesting that if a quorum was present and responsible for the shifts in host phenotype observed, then that quorum must be easily met and be composed of very little total biomass that does not manifest in notable shifts in diversity.

Alternatively, infrequently observed, keystone taxa could have caused the treatment effects we report, as these taxa, by definition, exert greater influence than would be predicted from their low biomass. For instance, a localized infection by a keystone taxon could have effects that spread throughout the host (e.g., through hormone stimulation), yet that taxon would not be present in the majority of leaves sequenced from that host. This concept suggests limitations of the common practice of *in silico* identification of keystone taxa as those taxa that are prevalent among samples, such that their removal from co-occurrence networks causes a shift in network topology [87–89]. We reiterate the non-exclusivity of the keystone and quorum hypotheses and suggest disentangling the two represents a profitable line of inquiry for future work.

Effect of *Alternaria fulva*, the heritable fungus

In addition to the influence of rare endophytes, we observed that the dominant, vertically transmitted fungal endophyte *A. fulva* also reduced host size and foliar %N (Fig. 1; consistent with Cook et al. [51]). Inhibition of host growth by *A. fulva* is perplexing, because the fungus is vertically transmitted in seeds and, therefore, its fitness is tied to that of its host. Larger *A. lentiginosus* plants generally produce more seeds (J. Harrison, personal observation) and, thus, selection should operate against mechanisms by which *A. fulva* reduces host growth. On the other hand, *A. fulva* grows very slowly in culture [90, 91] and fast-growing plants could possibly outpace hyphal growth. If the fungus cannot grow fast enough to reach seeds before they mature, then its direct fitness is zero. Consequently, constraining host growth may improve fungal fitness, because it would allow time for hyphae to reach reproductive structures. This hypothesis awaits further testing.

Another intriguing possibility is that a fungal-induced reduction in plant size could actually improve plant longevity in extreme conditions and thereby lead to a positive, time-averaged, effect on fungal fitness. For several native

plants in the Great Basin, small stature paradoxically facilitates the ability to withstand drought and competition from invasive annual grasses [92, 93]. Thus, it is possible that plants colonized by *Alternaria* endophytes could better survive the harsh desert climate, providing both the plant and the fungus more opportunity to reproduce. Interestingly, previous work has shown that *Alternaria* endophytes do not reduce plant size in locoweeds that are drought-stressed [94] and that swainsonine concentration can increase during drought stress. Thus, perhaps the negative affect of *A. fulva* on host size we observed here, in a well-watered, controlled setting, would not play out in drought-stressed wild populations.

The potential fitness costs imposed by *A. fulva* on its host may be ameliorated by the negative association we observed between *A. fulva* and the most abundant pathogen present, *L. taurica* (Fig. 2). These results support the hypothesis posed by Lu et al. [49] that the *Alternaria* spp. occurring within *Astragalus* and *Oxytropis* act as mutualists to their hosts by restricting pathogen exposure. *A. lentiginosus* is a plant of frequently disturbed, climatically variable, arid landscapes and it is likely that pathogen pressure in such locales is particularly damaging, because the lack of resources could impede recovery from tissue loss. The same rationale has inspired the growth-rate hypothesis in the literature characterizing interactions between plants and insect herbivores [95]. This hypothesis predicts plants growing in resource poor conditions will recuperate from herbivory slowly, and thus benefit from investment in phytochemical defenses that would otherwise be too costly. Similarly, tolls imposed by *A. fulva* on *A. lentiginosus* may be acceptable to the host given the harshness of the arid American West.

Our results compliment recent research presented in Christian et al. [30] showing that endophytes, and interactions between endophytes and pathogens, can alter N distribution and uptake in plants. The study did not demonstrate endophyte-induced shifts in %N content at the whole plant level (such as those we observed here), but it did show that endophytes influenced N uptake in plants and affected N distribution among leaves (also see [32]). We also observed credible, treatment-induced shifts in $\delta^{15}\text{N}$ and nitrogen fixation in the rhizosphere (NDFA) (Fig. 1 and Table 2), but we also found that rare endophytes reduced total foliar %N when *A. fulva* was not present to attenuate their effects. When taken together with previous work [30, 32], our results suggest that the effects of horizontally transmitted endophytes on foliar N depend on host taxon and individual, abiotic context (e.g. N availability), and interactions with other microbiota, and, when these factors align, the effect of endophytes on N allocation within hosts can be noteworthy. Notably, we saw some subtle trends that bear further examination; specifically, reducing *A. fulva* colonization was associated with higher NDFA. Thus, the

work we present here, when coupled with the results in Christian et al. [30], suggest that foliar endophytes may affect N fixation, which typically happens below ground. This suggests that interactions among microbes in the phyllosphere can influence what happens in the rhizosphere.

Other considerations: a milieu of interactions and experimental design

Our study demonstrates the ecological consequences of interactions among microbes [18], as evidenced by a negligible effect of inoculum application for plants colonized by *A. fulva* and the negative association between *A. fulva* and *L. taurica*. We suggest that these results are not likely due to direct competition for resources between *A. fulva* and other microbes—the disparity in leaf size and microbe size is too great and there seems to be enough healthy leaf tissue for all parties (based on the lack of visual infection symptoms in our plants). Even for *A. fulva*, which grows systemically through its host, physical encounters with co-occurring microbes are probably rare—with the possible exception of encounters with *L. taurica*. We note that it is plausible that swainsonine could inhibit growth of competing microbes, as the molecule inhibits the action of alpha-mannosidase [40], which is used by fungi to process oligosaccharides [96]. However, it is unknown if swainsonine is exuded into plant tissues or is instead retained within fungal cells, if the latter, then it is not clear how the compound would affect co-occurring microbes. We suggest that indirect mediation of microbe–microbe interactions by the host is more likely. Indeed, gene expression studies in several perennial grasses [33, 34, 97] and in *Theobroma cacao* [32] have demonstrated an upregulation in the host immune response after colonization by endophytes (see [98]). To speculate, it is possible that *A. fulva* similarly primes the host immune response, which could negatively affect co-occurring microbes.

To account for any adverse effects of seed coat removal, we planted control seeds alongside sterile agar or agar inoculated with *A. fulva*. This technique was successful as shown by culturing results (Fig. 2), swainsonine concentrations (Fig. S1), and sequencing output (Fig. 2). The results we observed from control plants were very similar to those from treated plants, except for foliar C and N concentrations, which were more variable among controls. Most manipulative studies of vertically transmitted fungal endophytes attempt to kill fungi within seeds through either heat treatment (e.g. [99]), long-term storage (e.g. [7]), or fungicide application (e.g. [100]). While studies manipulating endophytes via these techniques have been of critical importance, it is possible that these treatments could have undesirable consequences that are hard to control for and that could obscure effects of endophyte reduction.

Consequently, we suggest others consider the approach we used here when seeds from endophyte-free plants are not available.

Conclusion

Our results suggest that rare, low-biomass endophytic taxa can have marked influence on their hosts and that these effects may be mediated by co-occurring dominant microbial taxa. It remains to be seen how often rare endophytic taxa affect host phenotypes in other systems. However, given that every study of endophytic biodiversity with which we are familiar shows a steep rank-abundance curve, with many taxa of low relative abundances, it seems plausible that the cumulative role of rare microbes across hosts could be substantial.

We also hope that a more careful consideration of different taxa (common and rare) within endophyte assemblages will illuminate parallels with diversity–ecosystem function studies of macroscopic organisms where community-wide effects of rare taxa have been demonstrated [101]. As biodiversity declines, such connections across scales of observation could provide impetus for conserving rare taxa, large and small, as important contributors to ecosystem processes.

Data availability

All scripts, plant trait data, and processed sequence data are available at: <https://github.com/JHarrisonEcoEvo/RareMicrobes>. Raw data are hosted by the University of Wyoming. MiSeq data used during preliminary work for this experiment can be found at: <https://doi.org/10.15786/r9xy-6x03> while NovaSeq data analyzed herein can be found at: <https://hdl.handle.net/20.500.11919/7166>.

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Author contributions JGH, LPB, and MLF conducted the field experiment. LPB and JGH performed culturing. JGH executed analyses. Analytical chemistry was conducted by DC and DRG. All authors contributed to experimental design and manuscript preparation.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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