RESEARCH ARTICLE

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Fungal endophyte communities in the temperate fern *Polystichum munitum* show early colonization and extensive temporal turnover[1]

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PREMISE OF THE STUDY: Fungal endophytes have been shown to colonize all land plants, yet surprisingly little attention has been given to their community composition in ferns. We examined the diversity and temporal turnover of fungal endophytes in foliar tissue of the temperate western sword fern, *Polystichum munitum*, comparing taxa in newly emerged leaflets and in the same fronds after 1 mo of exposure in the field.

METHODS: Utilizing next-generation sequencing, we sampled pinnae from *P. munitum* in spring, 2–3 d after they emerged. We additionally sampled pinnae 30 d later from the same fern blades. From these samples, we sequenced fungal DNA to characterize the initial colonization and temporal turnover of endophytes in the host.

KEY RESULTS: We demonstrate that *P. munitum* is abundantly colonized by endophytes in newly emerged foliar tissue. However, 1 mo later, the community composition undergoes a marked shift: the overall richness of endophytes increases, but the evenness of the community wanes as a single taxon, *Flagellospora fusarioides*, comes to dominate.

CONCLUSIONS: We conclude that *P. munitum* hosts a variety of fungal endophyte taxa, similarly to other land plants. However, the rapid shift of the endophyte community we report is an unprecedented observation. Therefore, we further conclude that repeated sampling should be the standard in endophyte studies, because single sampling events are not sufficient to capture the dynamic nature of these cryptic microfungi.

KEY WORDS culture-independent; Dryopteridaceae; fern microbiota; *Flagellospora fusarioides*; microbial community ecology; microbiome; Nectriaceae; Pacific Northwest; plant–microbe interactions; western sword fern

Fungal endophytes—microfungi that asymptomatically colonize all plants—represent an important component of the plant microbiome (Wilson, 1995; Peršoh, 2015). They are known to produce medically and ecologically relevant compounds (Aly et al., 2010); confer pathogen defense to their host plants in a context-dependent manner (Arnold et al., 2003); and exhibit lability in the symbiotic continuum, often shifting between pathogenic and commensal lifestyles (Delaye et al., 2013; Busby et al., 2015b). However, despite an abundance of information on the small group of clavicipitaceous endophytes that colonize temperate grasses (Clay, 1988; Clay and Schardl, 2002; Gundel et al., 2013), there are very few studies that document the diversity and community structure of nongrass endophytes. Even less is known regarding fungal endophytes that associate with ferns. This is surprising, given that ferns represent the second-largest group of vascular plants (Schuettpelz and Pryer, 2009) and play key ecological functions in many ecosystems (George and Bazzaz, 1999; Coomes et al., 2005). In addition, ferns are seed-free plants with independent, multicellular haploid and diploid life stages, thus creating the potential for distinct microbial colonists that are coupled with unique fern ecology. With this in mind, it is reasonable to assume that ferns may host fungal endosymbionts that are previously undescribed.

Endophyte research in ferns has elucidated novel fungal taxa (Fisher and Punithalingham, 1993) and a significant degree of host specificity (Del Olmo-Ruiz and Arnold, 2014), but it is currently unknown whether newly emerged host tissue is colonized by microfungi—and, if so, what effects these early colonists have on endophyte community composition through time. Additionally, all of the published work on endophyte communities in ferns has relied upon culture-dependent methods (Dreyfuss and Petrini, 1984; Petrini et al., 1992; Fisher and Punithalingham, 1993; Richardson and Currah, 1995; Schmid et al., 1995; Fisher, 1996; Zubek et al., 2010;

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Del Olmo-Ruiz and Arnold, 2014). Because many microfungi cannot be cultivated in vitro, culture-based approaches used to examine endophyte communities likely underestimate the actual diversity of these microbes within plants (Sun and Guo, 2012). Through next-generation sequencing (NGS) technology—which allows for the direct sequencing of environmental samples—we now have the ability to detect unculturable taxa, providing opportunities to document previously unseen diversity (Caporaso et al., 2011). However, limitations do exist in all NGS platforms employed (Lindahl et al., 2013), and sequence databases for fungi are currently biased toward soil-dwelling macrofungi (Kõljalg et al., 2013). Despite current technical shortcomings, researchers have used NGS technology to study microbial diversity across a broad range of habitats (Bálint et al., 2015; Gunawardana et al., 2015; Eusemann et al., 2016). Investigations into the microbiota of understudied plant lineages (e.g., seed-free plants) using NGS technology should also be pursued.

In addition to the paucity of information on fern-associated endophytes, we currently lack knowledge on whether these microfungi are systemically present in newly emerged foliar tissue or if leaves are initially uncolonized and accrue their microbial symbionts through environmental sources. As the leaf lamina unfolds and cellular elongation occurs, new habitat (i.e., leaf tissue) becomes available for microbial colonists. This changing habitat may present a "microsuccessional" space for fungal endosymbionts that deserves attention. If endophytes *are* present in newly emerged foliar tissue, they represent either (1) recent colonists that are superior at dispersal or (2) microbes that existed within the host plant's tissue prior to leaf emergence. It is not yet known whether these early colonists are able to hold their space within leaf tissue through priority effects or instead exist as fugitive species (Hutchinson, 1951) that are outcompeted with the arrival of new and superior competitors.

We endeavored to address the lack of knowledge described above by examining fern-associated endophytes in the temperate host *Polystichum munitum* (Kaulf.) C.Presl (western sword fern) in a culture-independent manner. We chose *P. munitum* as a fern host because of its broad distribution across much of western North America (Soltis and Soltis, 1987) and its evergreen growth in winter months. Rivaled in regional abundance only by *Pteridium aquilinium* (L.) Kuhn (bracken fern), *Polystichum munitum* is a major component of coniferous understories and may serve as an important habitat for endophytic fungi, especially in the winter. We amplified and sequenced fungal DNA directly from fern pinnae that had emerged from the crozier only 2–3 d prior and then repeated the sampling 30 d later on pinnae from the same fern blades. We sought to address whether early stages of fungal symbiosis occur in newly emerged fern croziers and, if so, how these early colonists influence competitive outcomes after 1 mo of environmental exposure. This first look at fern-associated endophytes in an unstudied host plant and understudied plant lineage, in a culture-independent manner, is an important step toward filling a significant gap in the knowledge regarding fungal endophyte community ecology.

MATERIALS AND METHODS

Plant, study site, and sampling—The plant host, *Polystichum munitum* (Dryopteridaceae), is a temperate fern with a distribution ranging from coastal Alaska to southern California and east to Montana (Soltis and Soltis, 1987). It is an evergreen plant that forms dense populations in coniferous understory habitats and is one of the most regionally apparent understory plants in the winter months.

Sampling was conducted at a site in the eastern foothills of the Oregon Coast Range (45.733759 N, 123.18631 W; 310 m above sea level) on 26 April and 26 May, 2014. Weather data covering the range of sampling dates were obtained through the Climate Data Online website (http://ncdc.noaa.gov). In April, when new croziers emerged from the base of the plant, 20 plants (two transects, 10 m apart; all plants ≥2 m apart and not touching) were tagged on four separate blades and an individual pinna was sampled per blade. On the second sampling, another pinna from the same four blades was sampled from each of the same 20 plants in a semirandom pattern (two or three pinnae away from the initial sampling on either side of the blade). Pinnae were obtained by snipping with fine-tipped gardening scissors that were dipped in ethanol and swabbed with a clean Kimwipe (Kimberly Clarke Professional, Roswell, Georgia, USA) between samplings. Pinnae were placed in separate labeled bags and were returned to the laboratory for refrigeration prior to further processing.

Sample processing—All samples were surface sterilized, and total DNA was extracted within 24 h of sampling (U'ren et al., 2014). Individual pinnae were thoroughly rinsed under running tap water for 30 s and then surface sterilized in sequential baths of 95% ethanol, 0.5% sodium hypochlorite, and 70% ethanol (v/v; 10 s, 2 min, 2 min, respectively; Arnold et al., 2003) in a sterile hood. Pinnae were then air dried in the hood on clean Kimwipes before they were placed in sterile 1.7 mL microcentrifuge tubes. Fresh tissue was homogenized in a bead mill (TissueLyser II; Qiagen, Hilden, Germany) immediately preceding DNA extraction with presterilized tungsten carbide beads, according to the manufacturer's instructions. Total DNA was extracted with the Qiagen DNeasy plant mini kit. A negative control was included in each round of DNA extraction and included a presterilized bead. Extracted DNA was quantitated on the Nanodrop spectrophotometer and frozen at −80°C until shipped to the sequencing core. We submitted 20 ng of total DNA per sample, along with a pooled negative control, to Argonne National Laboratory for library preparation and sequencing.

Sequencing—Fungal DNA was amplified with unique sample ID tags, Illumina adaptors, and the primers ITS1F and ITS2—specific for the ITS1 region of rDNA (Smith and Peay, 2014). Following polymerase chain reaction, samples were pooled at a target input of 100 ng sample^{-1}, and 100 μL of the library was cleaned with a column and eluted in 10 μL of PCR water. The cleaned library was diluted to 2 nM for NaOH denaturation and then further diluted to 6.75 pM and loaded onto the Illumina MiSeq 2 × 250 platform with a 10% PhiX control.

Sequence processing and bioinformatics—Sequence data are deposited at NCBI's GenBank (accession PRJNA343984). Raw reads were demultiplexed in QIIME (Caporaso et al., 2011), and reads that did not map to the index barcode file, or those possessing a phred score <20, were discarded (Appendix S1; see Supplemental Data with this article). Any reads containing barcode errors were also discarded. Both forward and paired-end reads were analyzed, but paired-end reads were substantially lower in number following demultiplexing (R1: 7.4M; paired-end: 1.8M) when compared to the forward reads, owing to a decrease in quality toward the end of the reverse read. Analysis with a mock community, prepared in

equimolar concentrations in our laboratory and sequenced on the MiSeq platform, yielded better results in recovering taxa using only the forward reads when compared to paired-end reads (also see Nguyen et al., 2014). Adaptor and primer sites were removed with Cutadapt version 1.10 (Martin, 2011), low-quality base calls were trimmed from the ends of reads with Trimmomatic version 0.36 (Bolger et al., 2014), and reads shorter than 125 bp or possessing homopolymers longer than 9 bp were also discarded from the dataset (Mothur version 1.37.4; Schloss et al., 2009). Chimeric sequences were identified with UCHIME (Edgar et al., 2011) implemented in QIIME using both reference-based and de novo methods, and reads flagged as chimeric from both methods were removed prior to operational taxonomic unit (OTU) clustering. Reads were clustered into OTUs by first removing any that did not match the UNITE fungal database (Kõljalg et al., 2013) at 60% similarity and then were clustered at 97% similarity using reference-based USEARCH clustering (Edgar, 2010). Reads that failed to cluster against the reference-based method were clustered de novo at 97% similarity with UCLUST (Edgar, 2010), and the resultant OTU tables were then merged. Taxonomy was assigned using the BLAST algorithm against the UNITE ITS database. Any taxa that were found in the negative controls from DNA extraction were removed from each sample in the OTU table by the same number of reads (Nguyen et al., 2014).

Statistical analyses—The OTU table (Appendix S2) and associated sample data (Appendices S3 and S4) were analyzed through the R packages phyloseq (McMurdie and Holmes, 2014) and vegan (Oksanen et al., 2011; Appendix S5). Samples, which corresponded to extracted DNA from individual fern pinnae, were pooled by plant within each month (n = 4 plant^{-1}), and these composite samples were used for the remaining analysis. Rarefaction curves were constructed following 999 permutations (Oksanen et al., 2011). Composite samples were then rarefied to 1889 reads—the depth of the sample with the fewest reads—for subsequent analyses. Although recent discussion has questioned the statistical validity of rarefying (McMurdie and Holmes, 2014), work by Weiss et al. (2017) demonstrates that rarefied data clearly reflect underlying biological patterns, while other normalization techniques (e.g., those implemented in DESeq and edgeR) may not be the most appropriate for OTU tables derived from microbial sequencing due to their inherent degree of sparsity (i.e., large number of zeros). Following normalization, alpha diversity metrics were calculated through Hill numbers at the scales of q = 0, 1, 2 (Hill, 1973). These scales represent the exponent of each Hill number, and as the value of q increases, each measure becomes less sensitive to rare taxa. When q = 0, the Hill number refers to OTU richness, q = 1 refers to the exponential of Shannon entropy, and q = 2 refers to the inverse of Simpson's index (Chiu and Chao, 2016).

Community composition between sampling months was compared through significance testing, ordination, and visualizations of taxonomic assignments to each OTU. Permutation-based multivariate analysis of variance (PERMANOVA) using Bray-Curtis dissimilarity was applied to examine whether the endophyte community composition between newly emerged pinnae and those 1 mo older was significantly different following 999 permutations. To visualize patterns of dissimilarity in endophyte community composition between sampling months in ordination space, we applied nonmetric multidimensional scaling (NMDS) utilizing Bray-Curtis dissimilarity with 100 random starts. Taxonomic assignments were plotted through phyloseq using families as the grouping variable and genera to fill the bars. Additionally, taxonomy was visualized with individual plants as the grouping variable, within each sampling month, with genera filling the bars. All read numbers were converted to relative abundance prior to plotting taxonomy, and any taxa that were <0.03% of the total dataset were excluded in taxonomy plots.

RESULTS

Alpha diversity/OTU richness—Rarefaction curves on the non-normalized data demonstrate that greater richness was found in leaflets that were 1 mo old than in those that were newly emerged (Fig. 1). Asymptotes were not reached in rarefaction curves, indicating that our sampling effort did not capture the full richness present in our study site (common in microbial NGS datasets). Newly emerged leaflets contained a total of 153 OTUs from 20 plants. One month later, a total of 264 OTUs were obtained from pinnae on the same fern blades on the same 20 plants. Hill numbers, which were calculated from the rarefied data, found greater richness (q = 0) in pinnae that were 1 mo old than in newly emerged pinnae (median = 35 vs. 13.5 OTUs plant^{-1}, respectively; Fig. 2). However, at the scales of q = 1 (exponential of Shannon's entropy) and q = 2 (inverse of Simpson's index), which place increasing weight on common taxa, there was little difference in diversity between the two sampling months.

β diversity/dissimilarity—A significant difference was found in community composition between sampling months (PERMANOVA: $F_{1,38}$ = 22.825, P < 0.001, R^2 = 0.375). Ordination of community dissimilarity through NMDS showed no overlap of the 95% confidence interval ellipses, with markedly greater dispersion in samples obtained from newly emerged leaflets than in leaflets that were 1 mo old (Fig. 3).

Taxonomy—A comparison of taxonomic assignments given to OTUs across the two sampling months documented a large proportion of reads comprising three fungal families: Nectriaceae, Sebacinaceae,

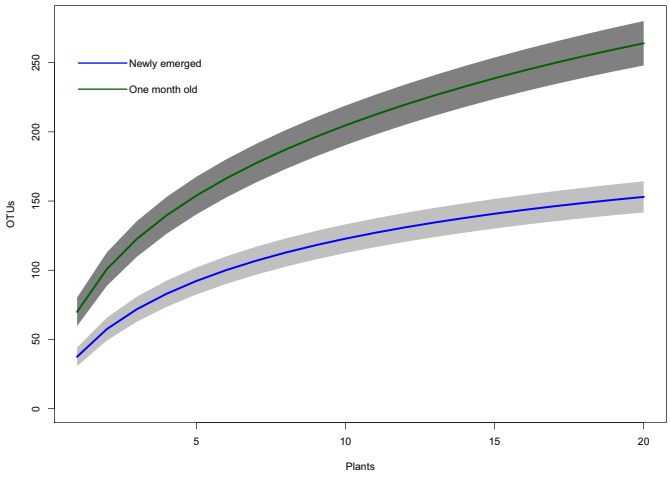

FIGURE 1 Rarefaction curves of observed operational taxonomic units (OTUs) in *Polystichum munitum*. Lines represent the median number of OTUs after 999 permutations plotted against the number of plants within each sampling month. Shaded areas represent the 95% confidence interval about the median.

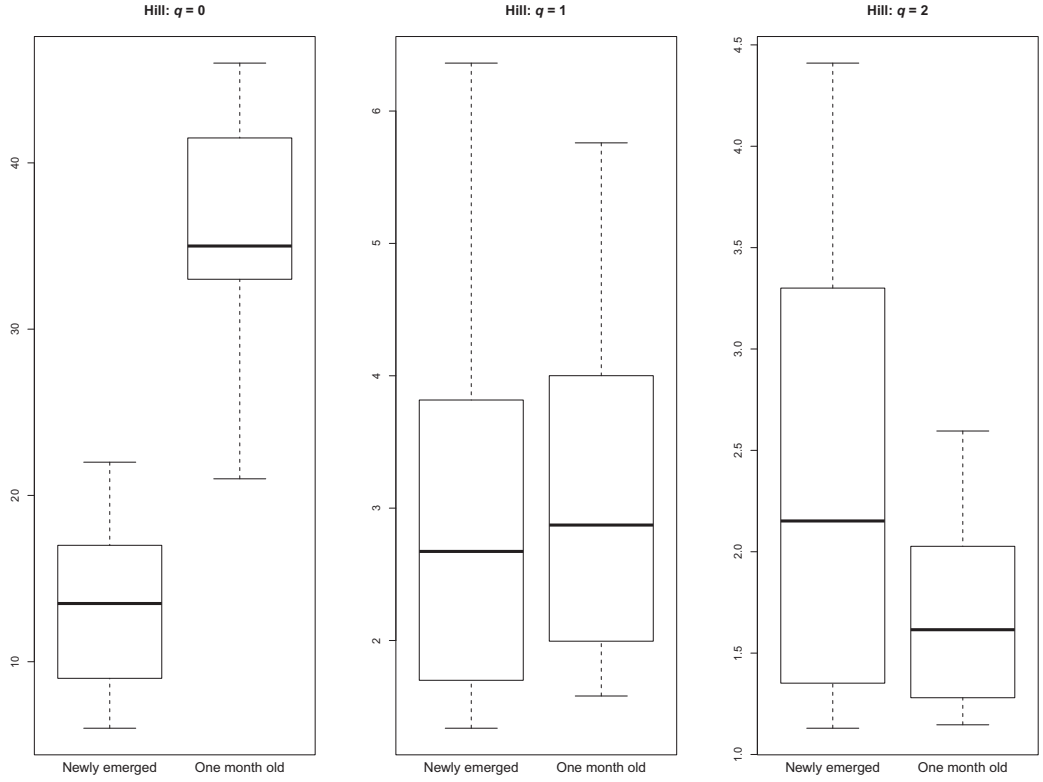

FIGURE 2 Hill numbers of diversity for endophyte communities in *Polystichum munitum*. Each plot represents increasing scales of the exponent *q*, which places more emphasis on common taxa. A scale of $q = 0$ is equivalent to OTU richness, while scales of $q = 1$ and 2 are equivalent to the exponential of Shannon's entropy and the inverse of Simpson's index, respectively. Boxes represent the upper and lower quartiles, thick lines represent the median, and whiskers represent the upper and lower deciles for each diversity estimate within each plant and sampling month. Outliers were observed for $q = 0$ (33 for plant 11.4), $q = 1$ (22 for plant 12.5), and $q = 2$ (3 and 13 for plants 10.5 and 12.5, respectively) but were not included for figure clarity.

and Helotiaceae (Fig. 4A, B). In newly emerged pinnae, most of the reads contained genera in the three aforementioned families, but following 1 mo of environmental exposure, a reduction in the relative abundance of OTUs from Sebacinaceae and Helotiaceae occurred with a substantial increase in the relative abundance of a single taxon in Nectriaceae. This OTU, which aligned as *Flagellospora fusarioides* following BLAST taxonomic assignment, was found in all samples in both sampling months but comprised only 1.56% of reads in newly emerged tissue and 69.10% of reads 1 mo later.

DISCUSSION

Our culture-independent analysis of the endophyte community in the temperate fern host *P. munitum* revealed that this plant system is abundantly colonized by endophytic fungi, to a similar extent as other land plants studied thus far (Jumpponen and Jones, 2010; Zimmerman and Vitousek, 2012; Busby et al., 2015a). Fungal sequences were obtained from most of the samples of newly emerged pinnae, providing evidence of early colonization of fungi into new vegetative tissue. A surprising result, and one yet to be observed in other culture-independent studies, is the rapid shift in community composition over only 30 d, in which a single OTU, which is likely related to *F. fusarioides*, comprises the greatest relative abundance when compared to other taxa. The temporal turnover of endophytes in this fern host is punctuated and results in a sharp decline in the evenness of fungi after 1 mo of environmental exposure. This observation may or may not be unique to fern host systems, and future work will need to address this phenomenon directly across plant taxa.

When comparing the community composition between months, we conclude that there is substantial temporal turnover of endophytes in the *P. munitum* fern host at this Oregon Coast Range study site. Samples from newly emerged pinnae often contained lower read numbers (which is likely the result of low fungal template concentrations in the samples) and, after normalization, fewer observed OTUs overall when compared to pinnae 1 mo later. This may be the product of either less time for environmental sources of fungi to colonize the fern tissue or less time for fungi existing within the fern rachises a priori to colonize the emerging pinnae. Although we did not specifically examine whether seasonal changes in fungal inoculum or host-tissue maturation was responsible for the observed difference in community composition, future investigations could clarify the origin of these taxa and the similarity to taxa found in mature leaflets at the same point in time. Arnold and Herre (2003) observed a similar trend whereby leaf age affected the abundance of fungal taxa recovered in a culture-based analysis of *Theobroma cacao* endophytes. Increased canopy cover also resulted in a greater percentage of leaf segments colonized by fungi in that study. All the host plants examined in the present study grew underneath a dense canopy of *Pseudotsuga menziesii* (Douglas fir), and—although we did not examine differences in endophyte relative abundance in habitats without a canopy—the marked increase in fungal OTUs after 1 mo may have been the result of sporulation events from overhead sources. Additionally, rain did fall at this study site between the sampling dates (7.8 cm over 30 d), providing an additional vector for fungal propagules (Suryanarayanan et al., 2002), but no insect or mammalian herbivory was observed on any of the plants between sampling dates. Given that a similar trend of increased colonization over time was found in work on tropical fern endophytes (Arnold and Herre, 2003), this suggests a general applicability of the findings reported in the present study, conducted in a temperate fern host.

The *F. fusarioides* taxon that quickly overtook the endophyte community in our study was present in the newly emerged pinnae of all the plants analyzed, but in low relative abundance when compared to other OTUs. Following 1 mo of environmental exposure, this fungus comprised most of the sequencing reads. We

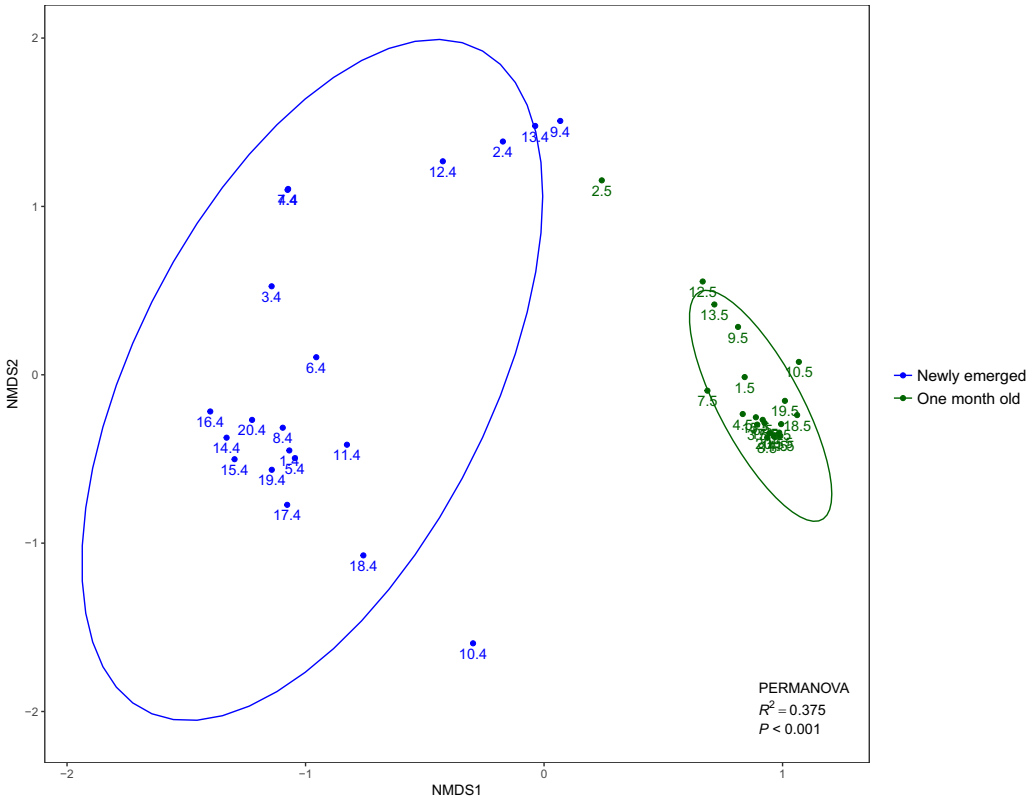

FIGURE 3 Nonmetric multidimensional scaling (NMDS) of fungal endophyte operational taxonomic units (OTUs) in *Polystichum munitum*. The Bray-Curtis dissimilarity metric, following 999 permutations, was used to determine the degree of dissimilarity in multivariate data between samples and plotted in two dimensions. Blue points represent OTUs from newly emerged fern pinnae, and green points are OTUs from pinnae 1 mo later. Each point is from pooled sequences of 4 samples plant^{-1}. Ellipses represent 95% confidence interval about the centroid of each grouping variable (sampling month). Stress is equal to 0.16.

acknowledge that sequencing reads do not reflect true abundance in NGS data sets, due to PCR biases during library preparation and additional bias during sequencing (Amend et al., 2010; Nguyen et al., 2014). Despite these potential biases, it is reasonable to assume that the trend of increasing relative abundance of the *F. fusarioides* OTU, observed across all samples and plants, reflects the underlying biology at the study site.

Although more fungal taxa were found overall in pinnae exposed for 1 mo, the observed shift in community composition favoring *F. fusarioides* was likely the result of (1) superior competition on the part of this taxon in comparison to other endophytes, (2) repeated colonization events from some source of spores, or (3) an unknown mechanism by the fern host favoring this fungus. Whether *F. fusarioides* provides some adaptive advantage to its host remains to be elucidated in future functional studies. We have recovered the taxon *F. fusarioides* on four occasions from the same plants and study site, and forthcoming in vitro competition assays will determine whether this endophyte performs similarly on artificial media containing plant extracts from *Polystichum munitum* and also from other plant species that co-occur at the study site (Mejía et al., 2008; Sarhan et al., 2016).

Although BLAST results from the UNITE and GenBank databases match the highly abundant OTU with *F. fusarioides* (Nectriaceae), this result should be treated with caution. The authority documents this fungus as an aquatic hyphomycete of decaying alder leaves (Ingold, 1942). Although individuals of *Alnus rubra* grow within 50 m of the study site, all samples were obtained from healthy, aboveground, surface-sterilized *P. munitum* tissue. We are currently working toward a more accurate identification of this OTU isolate through the assembly of longer contigs of the entire ITS region and through the morphological identification of conidiophores. It should be noted that plant-associated fungi are known to demonstrate switching from saprobic to endophytic lifestyles (Delaye et al., 2013), so this OTU may be either a closely related taxon to *F. fusarioides* or a novel observation of an endophytic lifestyle of *F. fusarioides* in a temperate fern host. Additionally, although we are calling the fungal taxa observed from our sequencing efforts "fungal endophytes," the potential exists for some of these taxa to be epiphytic fungi (i.e., living both endophytically and epiphytically on the same leaflet) or recalitrant fungal propagules that were not removed from our surface-sterilization steps (Busby et al., 2015b). Taxonomic observations show that some of our OTUs are closely related to the lichenic genera *Acarospora* and *Ochrolechia*. In the future, sequencing controls that are generated from the surface-sterilizing solutions would address whether the OTUs observed are indeed phyllospheric fungi.

This first culture-independent study of endophytic fungi in ferns demonstrates a high degree of colonization in newly emerged foliar tissue in spring. The substantial temporal turnover of endophytes following 1 mo of environmental exposure is unlike any observed in the literature, to the best of our knowledge. In addition, such a marked shift in community composition that favors a single fungal taxon at the expense of others is an unexpected result that deserves more attention in other ferns. We conclude that repeated sampling should be the norm in future endophyte studies, because conclusions drawn from single sampling events may not accurately capture the dynamic nature of communities of microfungi residing cryptically within host plants.

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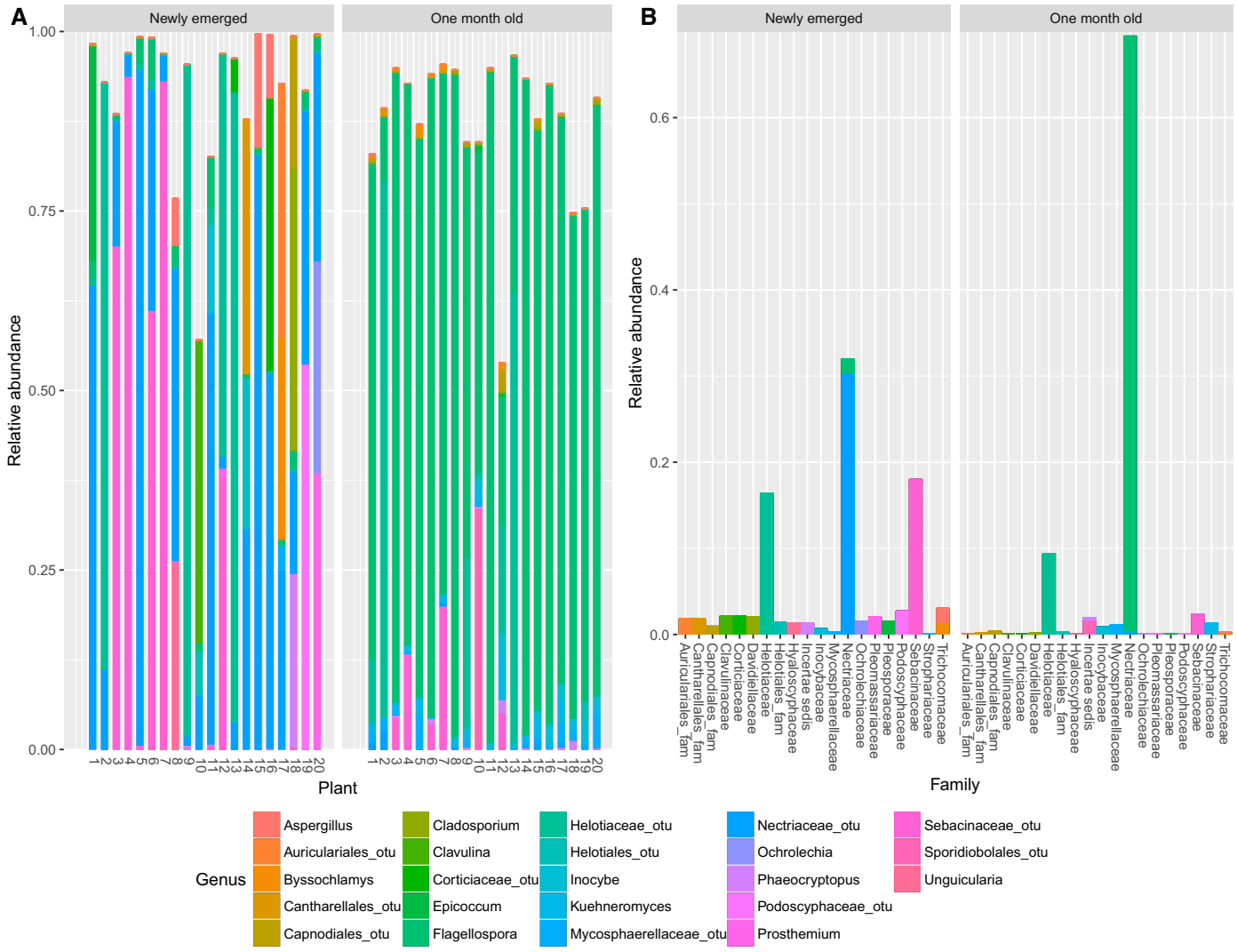

FIGURE 4 Taxonomy and relative abundance of fungal endophytes in *P. munitum* over two sampling months. Height of the bars represents relative abundance of operational taxonomic units within each sampling month, separated by (A) plant or (B) fungal family on the *x*-axis and filled by fungal genera. Any taxa that represented <0.03% of overall relative abundance were omitted from the plots.

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