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Stable Coexistence or Competitive Exclusion? Fern Endophytes Demonstrate rapid Turnover favoring a dominant fungus

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Title: Stable coexistence or competitive exclusion? Fern endophytes demonstrate rapid turnover favoring a dominant fungus

Running head: Fern endophyte community dynamics

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Abstract

Fungal endophytes are critical members of the plant microbiome, but their community dynamics throughout an entire growing season are underexplored. Additionally, most fungal endophyte research has centered on seed-reproducing hosts, while spore-reproducing plants also host endophytes and may be colonized by unique community members. In order to examine annual fungal endophyte community dynamics in a spore-reproducing host, we explored endophytes in a single population of ferns, *Polystichum munitum*, in the Pacific Northwest. Through metabarcoding, we characterized the community assembly and temporal turnover of foliar endophytes throughout a growing season. From these results, we selected endophytes with outsized representations in sequence data and performed *in vitro* competition assays. Finally, we inoculated sterile fern gametophytes with dominant fungi observed in the field and determined their effects on host performance. Sequencing demonstrated that ferns were colonized by a diverse community of fungal endophytes in newly-emerged tissue, but diversity decreased throughout the season leading to the preponderance of a single fungus in later sampling months. This previously-undescribed endophyte appears to abundantly colonize the host to the detriment of other microfungi. Competition assays on a variety of media types failed to demonstrate that the dominant fungus was competitive against other fungi isolated from the same hosts and inoculation onto sterile fern gametophytes did not alter growth compared to sterile controls suggesting its effects are not antagonistic. The presence of this endophyte in the fern population likely demonstrates a case of repeated colonization driving competitive exclusion of other fungal community members.

Keywords—Catenasporaceae, endophyte competition, fern gametophyte, fungal endophyte, endophyte community, temporal turnover

Introduction

Explorations of the microbial world have revealed that our current understanding of the organism as a biological unit is insufficient, as all macroorganisms are engaged in a plethora of intimate symbioses with microbes (Bordenstein & Theis, 2015; Gilbert, Sapp, & Tauber, 2012). Our newfound awareness of the tremendous bacterial and fungal diversity colonizing multicellular eukaryotes has initiated much excitement regarding their potential functional benefits to hosts

(Busby et al., 2017; Farrar, Bryant, & Cope-Selby, 2014; Gundel, Pérez, Helander, & Saikkonen, 2013). The plant microbiome is no exception, with evidence demonstrating the ability of these microbes to promote plant growth (Khan et al., 2016; Knoth, Kim, Ettl, & Doty, 2014), defend against pathogens and herbivores (Busby et al., 2013; Christian, Herre, Mejia, & Clay, 2017), and provide abiotic stress tolerance (Redman et al., 2011; Rodriguez et al., 2008). One such group of plant-associated microbes—fungal endophytes—are microfungi that colonize intercellular host tissues in an asymptomatic manner (Wilson, 1995) and are broadly classified according to their mode of transmission between hosts and the tissue types in which they reside (Rodriguez, White, Arnold, & Redman, 2009). Current estimates predict the existence of several million undescribed fungal taxa (Blackwell, 2011; Hawksworth & Rossman, 1997)—most of which are plant-associated; therefore, it is clear that our current understanding of fungal endophyte diversity, ecology and natural history is scant at best.

Although an increasing number of studies have demonstrated the existence of mutualistic endophytes under specific conditions (Arnold et al., 2003; Redman et al., 2011; Vidal & Jaber, 2015), there is a recent acknowledgement that most endophytes are commensals that respond to biotic and abiotic conditions in a context-dependent manner (Busby, Ridout, & Newcombe, 2015; May, 2016). Additionally, if we wish to fundamentally understand the community ecology of these diverse plant symbionts, several critical areas must be clarified, including (i) the range of ways they colonize plant tissue and which of these are most common, (ii) what determines their success *in planta*, and (iii) what enables their persistence through time. Our ability to describe terrestrial plant communities in a more comprehensive manner depends upon our understanding of their bacterial and fungal symbionts and how they co-exist on the symbiotic continuum.

Furthermore, since many microbial colonists cannot be cultured *in vitro* (Sun & Guo, 2012), there is high value in conducting metabarcoding analyses of native microbial communities to draw general conclusions about community composition and dynamics (Peay, 2014). Of the metabarcoding studies that have examined endophyte community composition, they most commonly document community composition at a single point in time (Zimmerman and Vitousek 2012, Bálint et al. 2015, but see Kohout et al. 2013, Maciá-Vicente et al. 2020). Although time, labor and financial constraints factor into this reliance on single sampling events, the practice severely limits our understanding of endophyte community dynamics throughout a

host's growing season. Previous metabarcoding work examining temporal turnover has indeed demonstrated a significant difference in community composition over just two sequential sampling months (Younginger & Ballhorn, 2017), but work clearly documenting the annual turnover of plant microbial communities is a large deficiency in the current state of the knowledge in the field.

Despite this deficiency, many useful theoretical frameworks have been developed in the field of community ecology (Leibold et al., 2004; MacArthur & Wilson, 1967; Urban et al., 2008). With recent advancements in sequencing technologies, we now have the opportunity to determine if these same principles of community ecology apply to microorganisms (Borer, Laine, & Seabloom, 2016; Koskella, Hall, & Metcalf, 2017; Mihaljevic, 2012; U'Ren et al., 2019). For example, once microbial colonists arrive to new habitat (i.e. newly emerged tissue)—since most endophytes are horizontally transmitted between hosts (Oono et al., 2014)—it is currently unclear how stochastic or deterministic processes interact to drive realized community composition. These processes could include the timing of arrival of new species (priority effects; Chase, 2003; Fukami, 2015), dispersal limitation (Lee & Hawkes, 2021), residual effects of previous species on their habitat (legacy effects; Cuddington, 2011) or the ability of overlapping generations to recruit offspring in favorable conditions (storage effects; Kennedy, 2010; Warner & Chesson, 1985). Further still, the relative importance of competitive interactions within host tissue after colonization occurs deserves much attention due to its high potential to affect species richness and diversity over time.

Another equally important deficiency in the knowledge of plant microbial communities is the lack of work in a broad range of plant systems. Much of the work on endophytes thus far has understandably focused on economically important host plant systems (e.g. forage grasses, biofuel and food crops; Clay 1988, Redman et al. 2011, Busby et al. 2013, Coleman-Derr et al. 2016), but countless unique and ecologically important microbial interactions remain undiscovered in other understudied plant systems. One such overlooked group, the pteridophytes (ferns and other spore-reproducing vascular plants) are the second-largest group of vascular plants that occupy many important ecological niches and possess independent haploid and diploid life stages (Schuettpelz & Pryer, 2009). The ability of microbial colonists to inhabit the free-living haploid plant could have important implications on this vulnerable life stage of the host. Additionally, whether these haploid colonists are able to transition to the diploid

sporophytic stage remains an outstanding question. Since little work has been conducted on fern-endophyte systems and many outstanding questions remain, we have chosen to utilize a regionally-abundant fern in the Pacific Northwest, western swordfern (*Polystichum munitum*) due to its evergreen growth and tractability as a host plant system in our region.

In the present work, we examined the temporal turnover of endophyte communities over an entire growing season and further examined microbe-microbe competitive interactions and plant-microbe functional interactions through three main components: a metabarcoding observational study, a culture-based competition assay and a culture-based fern gametophyte bioassay. We sought to clarify whether endophyte communities demonstrate significant temporal turnover throughout the growing season. Additionally, we explored whether any notable fungal endophytes colonize fern hosts at the specific study site, and if so, how these taxa interact in the presence of competition to affect the host plant in axenic inoculations. The present work is a comprehensive examination of fern microbial communities growing in a coniferous understory in the Coast Range of Western Oregon, USA. We hope the results presented herein will encourage other researchers to determine whether similar phenomena are observable in other endophyte-host plant systems, regions and biomes.

Materials and Methods

Study site location and establishment—

The field survey was conducted in the Oregon Coast Range at 45.73376 N, 123.18631 W from April 26th, 2014 to January 26, 2015. In the spring, 4 fronds were tagged on 20 plants of *Polystichum munitum* (Kaulf.) C.Presl (western swordfern) along two transects, each 100 m long and separated by 50 m. No two sample plants were touching and were at least 3 m apart. Sampling was conducted approximately every 30 days for 10 months total.

Sampling protocol, surface sterilization, and culturing—

At the time of establishment of the study site, one leaflet (i.e. pinna; the unit of sampling) per frond (N = 80 per month) was isolated with shears that were sanitized between each sampling. Leaflets were placed in a separate, clean envelope and transported to the laboratory within 2 hours for refrigeration. Within 24 hours of sampling, leaflets were surface sterilized in a sterile hood and allowed to dry on a fresh Kimwipe (see Arnold et al. 2003 for sterilization methods). One hundred mg of each leaflet was utilized for DNA extraction and remaining tissue was plated

onto malt extract agar (MEA) medium (2% w/v) for culture-based analysis. After a period of 7 days, any fungi emerging from the surface sterilized leaflets were re-isolated onto fresh MEA plates, creating axenic cultures. Axenic cultures were maintained in ambient conditions until the conclusion of the 10-month sampling period, after which they were utilized for *in vitro* competition assays (below).

Metabarcoding DNA extraction and library preparation—

Following each monthly sampling event, fresh tissue was homogenized in a bead mill and total DNA was extracted with the Qiagen DNeasy plant mini kit (Qiagen, Hilden, Germany). A negative control was included in each round of DNA extraction and included a pre-sterilized bead. Extracted DNA was stored at -80° C until library preparation. Fungal DNA was amplified utilizing the reaction and cycling parameters as specified (Appendix S1). Reactions were verified with electrophoresis. Fungal DNA from each sample was amplified in duplicate, pooled and cleaned with the Agencourt AMPure XP kit (Beckman Coulter, CA, USA). Final concentrations of cleaned PCR products were verified with the Qubit Fluorometer (Life Technologies, CA, USA) according to the manufacturer's instructions. Ten ng of each cleaned sample was added to the final library, diluted to 10 nM and sequenced on the Illumina MiSeq platform at 2 x 250 bp with a 30% PhiX spike-in added to the sequencing run. Each sequencing run (5 total) comprised two months of samples from the same 20 plants (n = 160), extraction controls from each month (n = 2), pooled PCR negative controls (n = 1), and a positive control (i.e. mock community) comprised 30 different fungal taxa from the phyla Basidiomycota and Ascomycota obtained from our region and pooled in equimolar amounts.

Within-plant endophyte colonization—

We additionally sampled host rhizome, rachis and leaflet tissue on four different individuals (N = 12) 100 m away from the same population at the conclusion of the monthly survey in order to determine if the same taxa found in the metabarcoding study were also present in other plant compartments. Sampling was conducted on 10/14/2015 and samples were surface sterilized and sequenced in an identical manner to the metabarcoding study above.

Sequence processing and bioinformatics—

Raw sequence data is deposited at NCBI's Sequence Read Archive (SRA) under the following accession: PRJNA343984. The first two months of sequence data that documented early endophyte colonization patterns were previously published, but were included in the analysis

here to clarify community dynamics over an entire growing season (Younginger & Ballhorn, 2017). Forward and reverse reads from each sequencing run were merged with USEARCH v10.0.240 (Edgar & Flyvbjerg, 2015) and overlapping regions that did not match on the forward and reverse reads by at least 90% were discarded. PhiX sequences and any reads with an expected error threshold greater than 1.0 were removed (4.4% of total reads) with USEARCH and re-oriented on the forward strand with the UNITE database (v8.2; Nilsson et al. 2018). Following these sequence filtering steps, 14.8 M sequences remained for the analysis. Sequences were denoised into zero-radius operational taxonomic units (zOTUs; also known as exact or amplicon sequence variants (ESVs or ASVs, respectively)) using the UNOISE3 algorithm implemented in USEARCH (Edgar, 2016). For the sake of simplicity, we will hereafter refer to ASVs as OTUs in this study owing to the utility and widespread use of the latter abbreviation and the fact that ASVs refer to a more specific instance of an OTU. Taxonomic assignments were made through the SINTAX algorithm (Edgar, 2016) using the RDP ITS database (v2 2016; Cole et al. 2014) and added to the OTU matrix. The RDP ITS database was utilized for taxonomic assignments in lieu of a more recent version of the UNITE database since assignments were made with the former at a greater proportion. Any reads from negative controls that mapped to taxa were summed (across all negative controls) and this number of reads was removed from actual samples in the final OTU matrix by subtraction (Nguyen, Smith, Peay, & Kennedy, 2014; Younginger & Ballhorn, 2017). The metabarcoding component of this study revealed a dominant endophyte that rapidly colonized all host plants at the study site (OTU1) and an isolate of this taxon was further examined through *in vitro* competition and gametophyte assays (below).

In vitro competition assays—

DNA from the aforementioned axenic cultures of fungi, generated from the same host plants, was extracted with the REDExtract-N-Amp kit (Sigma-Aldrich, MO, USA) following the manufacturer's instructions and amplified utilizing the reaction and cycling parameters as specified (Appendix S1). Extracted DNA was submitted for Sanger sequencing (Functional Biosciences, WI, USA). Raw sequences were removed of forward and reverse primers, concatenated through Geneious v10.0.5 (Kearse et al., 2012), and exported to a single fasta file. Representative OTU sequences from the high-throughput sequencing (HTS) dataset were then aligned to culture sequences through the BLASTn algorithm v2.2.28 (Camacho et al., 2009). The

output was sorted by percent sequence similarity and then by the length of the match. Any sequences that did not possess 100% sequence similarity over at least 150 bp of the HTS read were discarded. Any cultures that possessed a 100% sequence match to a given OTU were then grouped together and compared by morphology and sequence similarity. We then performed an additional alignment in Geneious with the MAFFT v1.3.7 algorithm on the HTS and culture-based sequences to confirm that the HTS OTUs only mapped to a single cultured species unambiguously, across the 150 bp read. A total of 11 of these isolates were then transferred to fresh MEA medium to generate starting material for *in vitro* competition assays two months after completion of field sampling.

After one additional month of growth, cultures were competed against the dominant OTU from the metabarcoding study by placing a 6 mm punch into fresh plates with lignocellulose agar (2% w/v, adjusted to pH 5.5; Sharma and Pandey 2010) at an equal distance from the edge of the plate. This medium was chosen for the assays to mimic colonization of a new host and due to the most favorable growth of the dominant OTU1 in preliminary trials. We tested the growth of OTU1 in 5 additional types of media optimized for slow-growing fungi (Ferrari et al. 2011), including one medium made with extracts from lyophilized *P. munitum* tissue and agar (Sarhan et al., 2016), but none of these media yielded more rapid growth than lignocellulose agar. Plates were sealed and placed in a cardboard box for one month. After this incubation period, plates were removed and photographed on a table with 3 cm gridlines for analysis with a macro lens on a digital single-lens reflex camera (Canon, Tokyo, Japan). Plates were then scored by calculating the difference in growth of the competitor versus the growth of OTU1 in the direction towards each other (Schwelm et al., 2009; Wardle & Parkinson, 1992). All measurements were conducted with the FIJI version of ImageJ software v2.0.0 (Rueden et al., 2017; Schindelin et al., 2012), results were analyzed in R and figures were generated with ggplot2.

Gametophyte assays—

Spore-bearing leaves of *P. munitum* were collected from the same plants after the HTS observational study was complete on 9/18/17. Leaves were dried in a closed container with silica gel. After two weeks of drying, leaves were scraped of sori and spores were collected in a sterile falcon tube. Ten mg aliquots of these spores were then placed in sterile 2 mL Eppendorf tubes and soaked overnight in autoclaved DDH_2O on a shaker rotating at 40 rpm. Spores were spun at 2000 rpm for 3 minutes and the supernatant was decanted. Spores were sterilized by immersion

in a solution of 5.5% NaOCl and 0.1% Tween 20 (v/v) and vortexed for 10 minutes (Fernandez, Bertrand, & Sanchez-Tames, 1993). Samples were rinsed three times with PCR water with a 2000 rpm centrifuge step (3 min) between each rinse. Spores were then washed with 70% EtOH (v/v) for 30 sec and rinsed with PCR water and centrifuged twice more. Spores were re-suspended in PCR water and diluted to 3500 spores mL^{-1}. Fifty mL of the spore suspension were plated onto Modified Parker/Thompson's Basal Nutrient Medium (Klekowski, 1969), stored at 21° C with indirect sunlight for a period of two months and were visually inspected with a stereo microscope (Leica, IL, USA) for visible contamination prior to fungal treatments.

Suspensions of fungal conidia were created from cultures of the dominant endophyte (OTU1). A culture of *Plectania milleri* served as an additional source of fungal inoculum. We chose this isolate due to its robust growth and frequency of isolation from the same host plants. Suspensions were generated by flooding culture dishes with 10 mL of 0.05% Tween 80 and hyphae were scraped with autoclaved microscope slides twice. This material was poured into a 15 mL sterile falcon tube packed with autoclaved cotton balls, creating a filter for fungal fragments. Cotton was removed from the filtered suspension and falcon tubes were spun in a centrifuge at 2000 rpm for five minutes. The supernatant was discarded and suspensions were rinsed with sterile DDH_2O and centrifuged twice more. Conidia were resuspended in 10 mL of sterile DDH_2O and diluted to 1×10^5 cells mL^{-1}. One hundred µL of the respective suspensions (OTU1 and *Plectania milleri*) were pipetted onto treatment groups (ca. 1×10^4 conidia) and 100 µL of sterile DDH_2O was pipetted onto controls (N = 30). Changes in surface area were recorded after a period of 0, 4 and 8 weeks by generating images with a digital camera as described above (*in vitro* competition assays). Surface area (cm^2) was analyzed in ImageJ with a custom macro which converted images to RGB, and utilizing the blue image only, thresholding to highlight only gametophytic tissue. Thresholding was manually adjusted to ensure consistent capture of tissue if necessary. At the conclusion of the experiment, total DNA was extracted from each gametophyte and submitted for Sanger sequencing. Resultant sequencing reads were aligned in Geneious and compared to sequences generated from source cultures. All treatments were confirmed to be axenic with the appropriate fungal strain and control gametophytes yielded no fungal sequences.

Statistics—

For the metabarcoding temporal study, the OTU matrix generated from sequencing, and associated metadata were imported into the R v4.0.0 (R core team 2016) packages phyloseq v1.32.0 (McMurdie & Holmes, 2014) and vegan v2.5.6 (Oksanen et al., 2011) for statistical analyses and figure generation with ggplot2 v3.3.0 (Wickham, 2009). Samples were grouped by plant in R (i.e., 4 samples per plant within each month were summed) prior to any statistical analyses as pooling samples computationally (versus physically) has been shown to result in greater richness recovered from sampling efforts (Song et al., 2015). Twenty plants (the unit of replication after pooling) were included in each month. Following pooling, samples were rarefied by randomly sampling without replacement to 15,000 sequences per sample. This resulted in the loss of 11 samples (10 from April, 1 from June) and 45 OTUs, which was an acceptable compromise for more robust conclusions of community dynamics. Due to the large loss of samples from April, which was likely the result of low fungal template DNA in newly-emerged tissues, the sequencing month of April was removed from the final analysis, but analyses with the month of April are still included in supplemental materials (Appendices S2, S3 & S4). Alpha diversity metrics were calculated with Hill numbers at the scales of q = 0, 1, and 2 (Hill, 1973, Chiu & Chao, 2016). Differences in each alpha diversity measure between sampling months were examined with repeated-measures ANOVA by specifying sampling month as the grouping variable, each diversity measurement as the dependent variable and the plant individuals as an error stratum, thereby removing the variance attributed to plant individuals in the final model (Chambers, Freeny, & Heiberger, 2017). An ordination plot of community composition, grouped by plants within each sampling month, was generated through non-metric multidimensional scaling (NMDS) with a square root transformation and Wisconsin double standardization following the generation of a Bray-Curtis dissimilarity matrix. Significant differences between sampling months were examined with a repeated-measures permutational multivariate analysis of variance (PERMANOVA; Anderson 2001, Anderson and Walsh 2017) following 999 permutations with Bray-Curtis dissimilarity while again treating plant individuals as an error stratum. Additionally, an examination of significant differences between the dispersion of group centroids (corresponding to sampling month), visualized in ordination, was determined through a multivariate version of Levene's test for variance homogeneity, implemented in the betadisper function in the Vegan R Package (Anderson, 2006, Oksanen et al., 2011). Following ordination, sequencing read numbers were converted to relative abundance and low abundance taxa (less

than a 3 x 10^{-5} proportion of the total dataset) were removed for easier visualization in the taxonomy plot. For the gametophyte assay, differences in gametophyte surface area (from gametophyte assays) were determined between weeks 0 and 4 and weeks 0 and 8 for each treatment and tested with one-way ANOVA following an examination of data distributions to fulfill parametric assumptions.

Results

Rarefaction curves and alpha diversity estimates—

For the metabarcoding study, we observed a total richness of 861 OTUs across all sampling months. Rarefaction curves reveal a sharp decrease in richness from May (when foliar tissue is first fully emerged) to June (Fig. 1). This richness dramatically falls in July and remains below 150 OTUs for the remainder of the sampling months. For the remaining sampling months (August through January), the richness at the study site declined to 50 OTUs in August and increased to 141 OTUs by the end of the sampling period. This decline in total richness was accompanied by a concomitant increase in the number of reads from the dominant taxon, OTU1. A further examination of alpha diversity through Hill numbers (Bálint et al., 2015; Chiu & Chao, 2016; Younginger & Ballhorn, 2017) demonstrated the greatest median richness in May ($q = 0$; $F_{8,144} = 103.5$, $p < 0.001$; Fig. 2). When $q \geq 1$ (exponential of Shannon entropy and the inverse of the Simpson index, respectively), the greatest diversity observed across sampling months occurred in May; beyond this sampling month, diversity remained close to one (June through January; Appendix S5) due to the presence of OTU1 which becomes dominant in the plant population beyond May.

Beta diversity and ordination—

Overall, the ordination plot shows a high degree of beta diversity in the first two months of the growing season, with very little beta diversity between the remaining months. A significant difference in community composition was observed in fern endophyte communities across the entire growing season (PERMANOVA: Pseudo−$F_{8,170} = 9.61$ $p < 0.001$; Fig. 3). Pairwise comparisons of sampling months with PERMANOVA demonstrated that all months were significantly different from one another with the exception of August versus September and December versus October, November and January (Appendix S6 & S7). Additionally, significant differences in dispersion (i.e. community heterogeneity) were observed between sampling

months (PERMDISP2; Pseudo−$F_{8,170} = 33.33$ $p < 0.001$) with May and June being the most dispersed. Pairwise comparisons in community dispersion revealed no difference from the months of September onward with the exception of September versus October (Appendix S8 & S9). In June, less dissimilarity is observed between host plants as endophyte communities become more homogenous across the study site. In June and May, the endophyte communities appear distinct from other sampling months. For the remaining sampling months (July – January), there is much overlap of community composition between plants *within* a given sampling month and nearly complete overlap *between* sampling months. This result is driven by the presence of OTU1 and the exclusion of other taxa.

An examination of the taxonomy of the endophyte community shows identical trends in the unfiltered and filtered datasets (i.e. any OTU > 3 x 10^{-5} of the total dataset; Appendix S10). In newly emerged fern leaves examined in the metabarcoding study, the endophyte community is colonized most abundantly by taxa from three fungal classes: the Agaricomycetes, Leotiomycetes and Sordariomycetes (Fig. 4). After one month of exposure in the field, the foliar tissue of the host is abundantly colonized by OTU1 (assigned to *Collophora* Damm and Crous within the Leotiomycetes). All other taxa decline in relative abundance through time, yet OTU1 comprises nearly 100% of sequencing reads from July – January.

Within-plant endophyte colonization—

The dominant OTU1 was also found in the pinnae, rachises and rhizomes of the host plant, though in lower relative abundance in all three plant compartments than seen in the full metabarcoding study (assigned to the *Collophora* genus of Leotiomycetes; Appendix S11). OTU1 ranked seventh in relative abundance in aboveground host tissue, which differs from the results found in the metabarcoding temporal study. Based on these differences, we cannot rule out that the sharp turnover of OTU1 in the temporal study may have been caused by the repeated tissue sampling that created a disturbance which OTU1 was able to exploit in contrast to the previously undisturbed plants described here. The belowground rhizome tissue had a greater representation of OTUs from Glomeromycota including the *Glomus* and *Rhizophagus* genera while the aboveground tissue had a greater representation of OTUs from the Agaricomycetes and Leotiomycetes as seen previously. Finally, ordination plots demonstrated the existence of distinct and homogeneous endophyte communities in host rhizome tissue with a high degree of endophyte overlap in aboveground tissues (rachises and leaflets) but more heterogeneity overall

(Appendix S12). While a lower relative abundance of OTU1 was found in foliar tissue from this group of plants, it still comprised the seventh-highest read count of all taxa found in these plants, 100 m from the temporal study site. Due to the low sample sizes of this component of the work, statistical conclusions were not drawn, but figures are included for a summary of intraplant endophyte variation in this host population.

In vitro competition assays—

All of the 11 taxa grown in culture with OTU1 demonstrated greater growth rates over one month (Fig. 5). In fact, 5 of the 11 taxa tested exhibited a two-fold greater growth rate than OTU1 on LCA medium. These results were not restricted to LCA, as preliminary trials were conducted between OTU1 and several taxa on MEA, potato dextrose agar (PDA), Reasoner's 2A agar (R2A), Rose Bengal agar and a medium made from 4 g of lyophilized and ground *P. munitum* tissue with 20 g of agar L^{-1} (2% w/v; fern medium), all confirming that OTU1 performs poorly in competition assays owing to slow growth rates *in vitro*. In fact, even over an entire year of competitive conditions, OTU1 remains covered by the hyphae of every competitor that it was tested against.

Gametophyte assays—

At four weeks, neither OTU1- or *P. milleri*-inoculated gametophytes of *P. munitum* show a significant difference in surface area (cm^2) when compared to sterile water controls, though there is a slight increase in surface area in OTU-inoculated plants (one-way ANOVA: $F_{2,27} = 2.19$, $p = 0.131$; Fig. 6). At eight weeks, there is a negligible increase in surface area observed in OTU1-inoculated gametophytes relative to controls with more variability seen in both groups of plants overall, but this result is not significant. In contrast, gametophytes inoculated with *P. milleri* show a significant reduction in surface area over 8 weeks to below baseline values ($F_{2,27} = 18.87$, $p < 0.001$).

Discussion

This study clearly demonstrates the temporally-dynamic nature of endophyte communities in a fern host system. Further, the metabarcoding study coupled with culture-based competition and gametophyte assays has revealed a notable fungal taxon that plays an outsized role in the host endophyte community and does not exert deleterious effects on the host at its gametophytic stage. While we cannot explicitly provide a mechanistic explanation for this result, future work

in this study system should experimentally test whether priority effects, dispersal limitation, ecological heterogeneity or other factors are predominant drivers of the observed colonization patterns of OTU1 in this system. Still, previous work has documented that OTU1 quickly spreads throughout the host population and is either able to outcompete neighboring taxa through repeated colonization and competitive exclusion—which was not supported by the *in vitro* competition results—or its spread is somehow facilitated by the host plant (Younginger & Ballhorn, 2017).

Results from the metabarcoding study contrast sharply with results from other plant systems (Jumpponen & Jones, 2010; Peñuelas, Rico, Ogaya, Jump, & Terradas, 2012; Peršoh, 2013; Davey, Heegaard, Halvorsen, Ohlson, & Kauserud, 2012) as the richness of fungal OTUs initially increases within the fern host early in the growing season, then after two months, richness rapidly declines until there is effectively one dominant OTU found at the study site. It is clear that endophytes that colonize this temperate fern population display different community dynamics than would be expected through time (Peršoh, 2015). Specifically, as newly-emerged foliar tissue is exposed to fungal inoculum, richness should increase over time until an equilibrium is reached and competitive coexistence prevails (Arnold & Herre, 2003; Jumpponen & Jones, 2010; Levins & Culver, 1971). While early colonization by fungal taxa was previously observed in newly-emerged fern pinnae, just 2 – 3 days old (April; Younginger & Ballhorn, 2017), there is far more heterogeneity between plants which is likely the result of patchy colonization. An increase in richness is observed one month after foliar tissue has emerged in May, as presumed, but the colonization of OTU1 is associated with decreasing richness at the study site in the remaining sampling months. By July, OTU1 comprises most of the sequencing reads and this trend continues throughout the remainder of the growing season. Although sequencing read numbers may be a poor estimate of actual abundance due to PCR and sequencing biases (Amend, Seifert, & Bruns, 2010; Nguyen et al., 2014), the incidence of OTU1 across all plants and sampling months after April likely reflects the sharp temporal turnover and exclusion occurring at the study site.

Metabarcoding temporal turnover—

A closer examination of the temporal turnover of endophyte community composition documents several intriguing trends. First, a significant difference is found in community composition through time. This basic result is unsurprising, yet a deeper look at what is driving this

significant difference reveals a distinctive pattern that can be partitioned into two components: changes in community composition between months and between plants. When comparing months in ordination space, the total community at the site in May is dissimilar from all other months throughout the season and grows progressively more similar between months as a result of the increase in OTU1 and a concomitant decrease in all other taxa. Between plants, early colonizing taxa are highly patchy in newly-emerged tissue resulting in heterogeneous communities on a small scale but become less heterogeneous in July and extremely homogenous throughout the remainder of the growing season in a pattern indicative of microsuccession. Therefore, we can summarily state that the early fern endophyte community at this study site is rich and uneven, and this rapidly changes to a highly homogenous community between plants and through time. From a statistical perspective, a difference in group dispersions in ordination plays a role in the significant difference in community composition through time. Additionally, the fact that endophyte communities are distinct—in ordination space—in the months of May and June compared to all other sampling months, and May and June are also fairly distinct from one another, we conclude that the endophyte community in this plant population changes through time and becomes more similar between hosts. While the tissue sampling regime may have played a role in the homogenization of the endophyte community through time, we have recovered OTU1 in both the same, and different, host plants from the study site in subsequent years, and have also found this OTU in host plants across the Pacific Northwest, U.S.A (unpublished). We conclude that this OTU is ecologically relevant and not merely an opportunistic artifact of our sampling effort. It is currently unclear if the sharp reduction in richness is found in other populations of *P. munitum* or in other fern species, as no other culture independent studies have examined fern endophyte communities. Furthermore, there is no other metabarcoding work, to the best of our knowledge, examining the temporal turnover of fungal endophytes monthly, over an entire growing season in any plant host species making generalizations impossible at this stage.

Metabarcoding taxonomy—

Taxonomic assignments of HTS sequencing reads show a higher representation of fungi from Basidiomycota in May than expected, as it is currently presumed that most symbiotrophic endophytes belong to the phylum Ascomycota (Arnold et al., 2009; Higgins, Arnold, Miadlikowska, Sarvate, & Lutzoni, 2007). Early in the growing season, we observe a greater

representation of fungal endophytes from the Agaricomycetes and Microbotryomycetes of the phylum Basidiomycota which includes mushroom-forming saprotrophs and ectomycorrhizal fungi (Hibbett et al. 2007) – our dataset includes endophytes from the Sebacinaceae and Geastrales family and order, respectively, that are predominantly saprotrophs (Wells and Oberwinkler 1982, Hosaka et al. 2006), and the Leotiomycetes of phylum Ascomycota; the most diverse phylum commonly known as sac fungi – our dataset includes *Collophora* of Tympanidaceae, a genus commonly associated with pathogens of woody plants and the best match to OTU1 (Lorch et al. 2011) and *Articulospora*, an aquatic hyphomycete (Baschien et al. 2013). We additionally recovered sequencing reads early in the growing season from the Sordariomycetes of phylum Ascomycota; including *Fusicolla aquaeductuum* of Nectriaceae; a plant pathogen (Gräfenhan et al. 2011). Although it may be initially surprising to find taxonomic assignments from such a diverse range of fungi with trophic modes dissimilar to endophytism, the short sequencing reads of HTS technology are known to be better suited for community-based inferences than taxonomic assignments (Peay, 2014) and these assignments may be precise only to higher taxonomic ranks—e.g., to family or genus rather than species. We are confident that our surface-sterilization procedure removes spores and other non-endophytic fungal material that may have been deposited on leaf surfaces by nearby fungal reproductive structures (U'Ren et al., 2019), meaning that the results observed early in the growing season are indicative of ephemeral taxa exploiting an uncolonized habitat (i.e. newly emerged leaf tissue). The sharp temporal turnover that happens in just 30 days (from May to June), results in a reduction of endophytes from the Agaricomycetes and Dothideomycetes, with an increase in representation from the Leotiomycetes. These taxonomic assignments more closely align with current observations of most symbiotrophic endophytes belonging to Ascomycota (R J Rodriguez et al., 2009). Although OTU1 was assigned to the genus *Collophora* using the most recently annotated database from RDP (June 2016; Cole et al. 2014), the highest similarity of the representative sequence for OTU1 to the database sequence was only 86.2% at 91.2% query coverage. This representative sequence is, however, identical to the ITS1 region of an isolate obtained from the same plants in culture-based work. This isolate is part of a currently undescribed family of fungi within the Helotiales (Leotiomycetes) which will be named Catenasporaceae in a forthcoming paper by the authors (Younginger et al. *under revision*). Additionally, OTU41 is also identical to the ITS1 region of another isolate obtained from the study plants which will be described as

another species within Catenasporaceae. Both of these taxa were isolated multiple times from the study plants during the course of the culture-based work.

In vitro competition assays—

One potential explanation for the remarkable increase in abundance of OTU1 relative to other endophytes in the metabarcoding study is that it is highly competitive once colonizing the host; however, based on the results of the *in vitro* competition assays, we can only conclude that OTU1 is not highly competitive in culture. We performed the competition assays on lignocellulose agar (LCA) medium since OTU1 was observed to grow most rapidly, but we also examined the growth of OTU1 in isolation and with competitors in 5 other types of media, some of which are optimal for slow-growing fungi (Ferrari et al. 2011) and one generated from lyophilized *P. munitum* tissue in an effort to mimic conditions within host tissue (Sarhan et al., 2016). OTU1 was still rapidly outcompeted by other isolates obtained from the same study site (data not shown). We acknowledge that *in vitro* work does not truly mimic conditions *in planta* and OTU1 may behave in a more competitive manner in living tissue. We predict that the results of the metabarcoding study are most-likely explained by the following hypotheses which are not mutually exclusive: (i) the abundance of senescent host tissue surrounding the plants serves as a reservoir of inoculum that allows for repeated colonization events, (ii) other vectors including rain and canopy throughfall allow for colonization (very little herbivory was observed throughout the study making this type of vector unlikely), (iii) the host plant provides a poor habitat for endophytes—as ferns are high in antimicrobial tannins (Mehltreter, Walker, & Sharpe, 2010)—and OTU1 is able to exploit this sub-optimal host to its advantage, though we did not examine changes in host foliar chemistry and OTU1 grew slowly on the fern medium compared to other endophytes, and finally, (iv) the host or other endophytes facilitate the colonization and/or spread of OTU1 through some other unknown mechanism. Although all of the above are possible, a particularly intriguing explanation involves the senescent material serving as an inoculum source (hypothesis (i); storage effects; Kennedy 2010). The senescent fern material is the product of growth in previous years (2 – 5 years old), may play a role in suppressing understory plant competitors and could be a way for OTU1 to re-colonize living tissue as an endophyte. Once the host plant tissue senesces, OTU1 (which is already present as an endophyte) could switch to a saprotrophic lifestyle where it undergoes sexual reproduction, fruiting, and re-colonization of the living host (Song, Kennedy, Liew, & Schilling, 2017).

Isolations of fruiting bodies on senescent material obtained from the exact same plants in the metabarcoding study has confirmed that these endophytes are indeed utilizing the leaf litter for sexual reproduction (Younginger et al. *under revision*). If this were the case, it would conform to the Foraging Ascomycete hypothesis, which posits that many fungi utilize the endophytic lifestyle as a method to tolerate unfavorable seasons or disperse to distant sites while carrying out a saprobic lifestyle to complete sexual reproduction and re-colonize hosts as an endophyte (Thomas, Vandegrift, Ludden, Carroll, & Roy, 2016). It is also currently acknowledged that there is remarkable lifestyle lability observed in fungal endophyte lineages, with some taxa existing as latent pathogens (Carroll, 1988), aquatic hyphomycetes (Chauvet, Cornut, Sridhar, Selosse, & Bärlocher, 2016; Sokolski, Piché, Chauvet, & Bérubé, 2006), or endolichenic fungi (Arnold et al., 2009).

Gametophyte assays—

Since a marked increase of a single taxon at the study site—comprised of a healthy and dense population of *P. munitum*—may indicate a mutualistic interaction occurring between the symbiont and host, we sought to elucidate whether OTU1 acts antagonistically or beneficially towards its host in sterile conditions. We chose to examine these potential outcomes in the haploid, gametophytic stage of *P. munitum* since generating an abundance of sterile plants is tractable with relatively straightforward spore sterilization, and also because the recruitment of juvenile ferns in nature requires the gametophytic stage, making the assay ecologically relevant. We hypothesized that if OTU1 is present at the study site in the abundance indicated in both culture-dependent and –independent methods, it is also likely to colonize the gametophytic stage. Based on the results of the experiment, we conclude that OTU1 is, at minimum, a commensal, especially when compared to the effects of *P. milleri*—a saprotroph initially discovered in leaf litter in Idaho (Paden & Tylutki, 1969)—which was also isolated from the same fern population. We observed greater variability in OTU1-inoculated plants after 8 weeks, but also observed a greater increase in surface area relative to controls, though this result was not statistically significant. It is possible that if the experiment were run for an additional four weeks, we would have found a significant difference in the change in surface area relative to controls, though *P. munitum* gametophytes are likely transitioning to a sporophytic stage in nature beyond this time point (Migliaro & Gabriel Y Galán, 2012). Additionally, plants treated with *P. milleri* were greatly antagonized and we risked losing an opportunity to confirm our treatment success via

sequencing. It should be noted that *P. milleri* has only previously been described as a saprotroph, thus we are unable to clarify whether its antagonistic effects are found in other host species when living endophytically (Paden & Tylutki, 1969). However, we chose to also utilize *P. milleri* conidia in our fungal suspensions due to its frequent isolation from the same host plants at the Coast Range study site.

We extracted total DNA from the gametophytes after 8 weeks to confirm that inoculated plants were axenic and control plants were sterile. Sequencing confirmed that our protocol was robust and all plants contained their respective endophyte and no other fungi, while control plants remained free of fungi. It is possible that the presence of ungerminated conidia from the fungal suspensions led to the conclusion that inoculated plants were colonized; however, we took particular care to gently pluck gametophytes from the culture dish without also removing medium. Furthermore, due to the fact that both sources of inocula were initially isolated as endophytes from the same sporophytic hosts, we suspect that both were capable of colonizing gametophytes, leading to the observed results. The fact that we did not observe a significant difference in the change in surface area of OTU1-inoculated gametophytes, relative to controls, does not mean that we can confidently state that OTU1 is a commensal, as the absence of proof is not the same as proof of the absence (Zapalski, 2011). It is important to consider that if OTU1 is able to colonize the gametophytic stage of *P. munitum* in nature, it may enable its host to evade pathogenic fungi, thereby altering the evolutionary trajectory of both partners in a highly context-dependent manner.

Conclusions—

This study highlights a striking case of a plant-associated microbial community that runs counter to our current understanding of the temporal turnover of endophytes in nature (Peñuelas et al., 2012). Although it is presumed that endophyte communities should show increased richness and diversity through time, eventually reaching a stable equilibrium of coexistence (Arnold & Herre, 2003; Jumpponen & Jones, 2010), this appears to be the exact opposite in the host population and future work should address whether this is the case in other fern systems. Although we did not specifically examine the role of abundant fruiting bodies from OTU1 observed at the study site, storage effects from fungal inoculum in senescent host tissue may also be driving the observed dominance of OTU1 (Chesson, 2000; Kennedy, 2010). Additionally, although we demonstrate that OTU1 is an inferior competitor in culture conditions, we predict that it relies

upon extensive colonization events from senescent material early in the season and is somehow able to better exploit the habitat created by the host to drive out neighboring endophyte taxa. Finally, we clearly show that OTU1 is at least a commensal on the gametophytic stage of its host, and when considered in the context of abundant fungal pathogens that may antagonize plants in nature, could enable its host to evade pathogens at this vulnerable stage of its life cycle. Taken together, these results clearly show a previously undescribed phenomenon occurring in fern endophyte communities. It is vitally important that we encourage research foci on endophyte communities to move beyond traditional plant systems of economic importance, since exciting and ecologically-relevant endophyte interactions are occurring in other hosts, waiting to be discovered.

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Data Accessibility:

- DNA sequences: NCBI SRA: PRJNA343984
- All informatics processing steps are included in a Jupyter notebook file available on GitHub (github.com/byounginger/publication_code) and Dryad (https://doi.org/10.5061/dryad.05qfttf1c).
- All R code and input files are available on GitHub (github.com/byounginger/publication_code) and Dryad (https://doi.org/10.5061/dryad.05qfttf1c).

Author Contributions

The study design was conceived by B.S. Younginger and D.J. Ballhorn. Study site establishment, sample collection and molecular biology were conducted by B.S. Younginger, N.U. Stewart and M.A. Balkan. All authors contributed to the manuscript.

Figures with captions

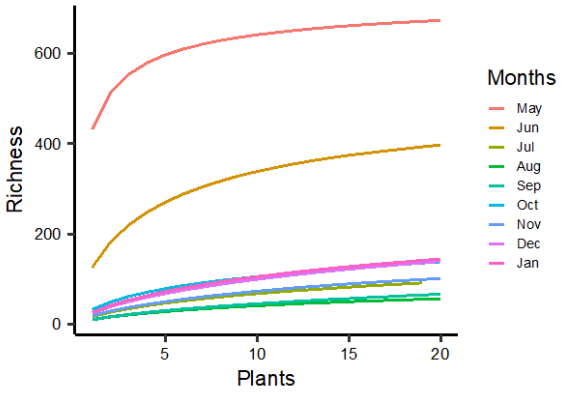

Figure 1. Rarefaction curves of operational taxonomic unit (OTU) richness of each sampling month in the metabarcoding study. Curves represent mean OTU richness following 999 permutations (n = 20 plants per month).

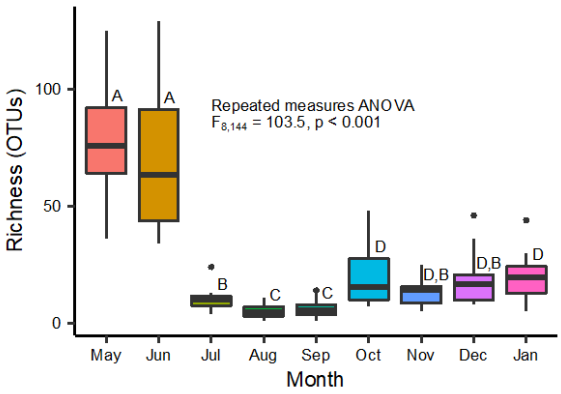

Figure 2. Hill measures of alpha diversity at the scale of q = 0 (richness) for each sampling month in the metabarcoding study. Differences in Hill measures were examined with repeated-measures ANOVA and Tukey's HSD post-hoc analysis. Boxplots represent the median value (middle line) for each plant within each month, boxes represent upper and lower quartiles and lines represent upper and lower deciles (n = 20 plants per month).

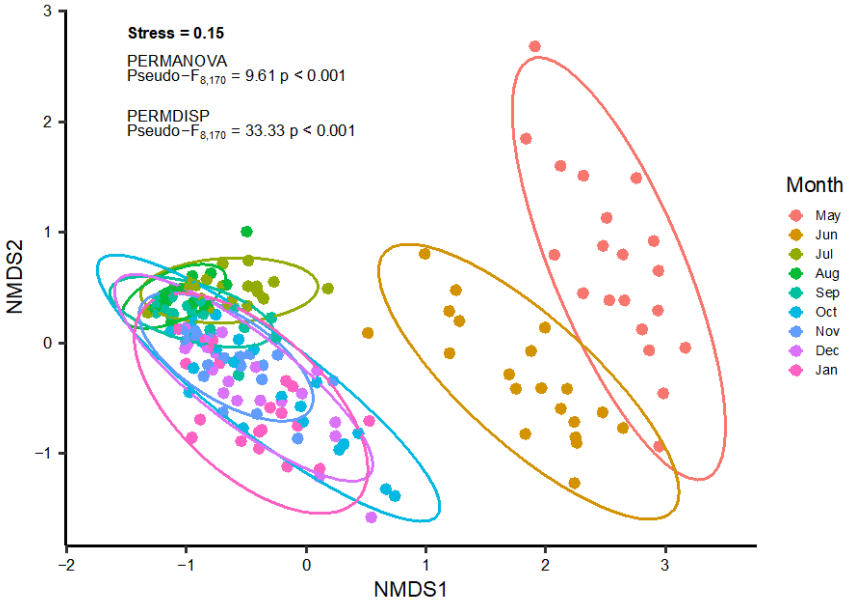

Figure 3. Non-metric multidimensional scaling (NMDS) of endophyte community composition within each sampling month in the metabarcoding study. Values were square root transformed and Wisconsin double standardized following the generation of a Bray-Curtis dissimilarity matrix from the OTU table. Points represent each plant at the study site, colored by the sampling month and ellipses represent 95% confidence intervals. Stress is equal to 0.15. Differences in community composition throughout the sampling period were examined with a repeated-measures permutational multivariate analysis of variance (PERMANOVA) following 999 permutations of Bray-Curtis dissimilarity while holding plant individuals as an error stratum. Significant differences in group dispersion about each centroid were tested with a multivariate version of Levene's test for variance homogeneity (PERMDISP2; n = 20 plants per month).

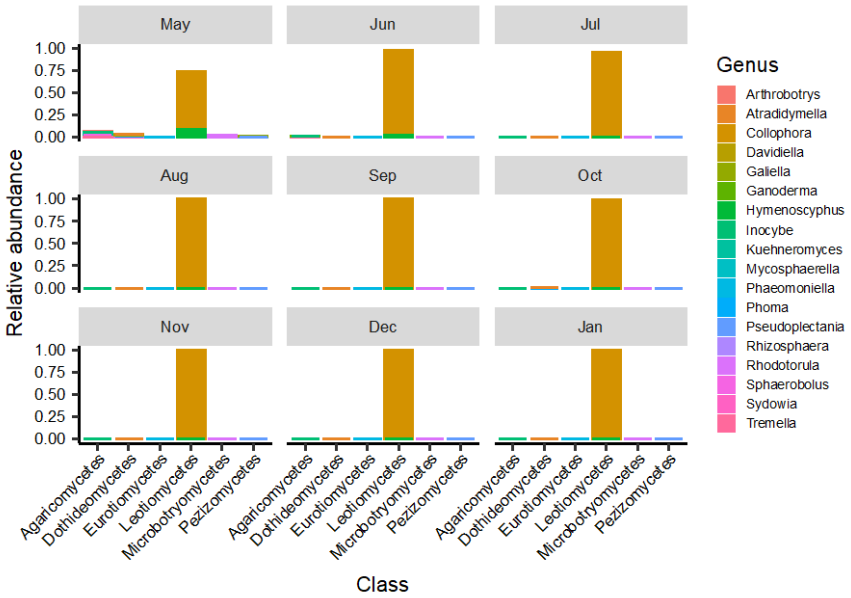

Figure 4. Taxonomy of endophyte community composition within each sampling month of the metabarcoding study. Colored bars represent the relative abundance of genera found within each fungal class (x-axis). Any taxa that represent less than 3 x 10^{-5} of the total dataset were removed for easier visualization (n = 20 plants per month).

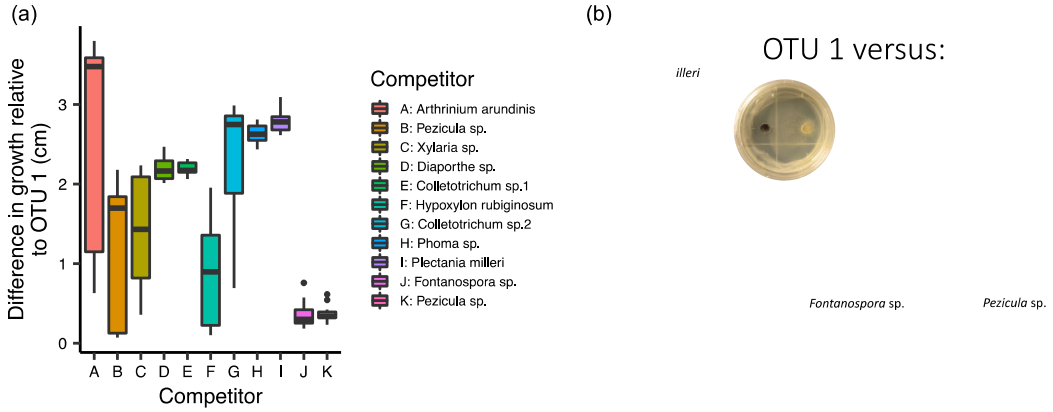

Figure 5. Results of *in vitro* competition assays of OTU1 versus 11 other strains recovered from the same plants examined in the metabarcoding study. (a) Boxes represent the difference in growth of each competitor relative to the growth of OTU1 in the direction towards one another in the culture dish. Boxplots represent the median value (middle line) for each pairwise comparison, boxes represent upper and lower quartiles and lines represent upper and lower deciles (n = 12 replicates per competitor; 132 plates total). (b) Representative assay plates of each competitor (right) growing in the presence of OTU1 (left) on lignocellulose agar (LCA).

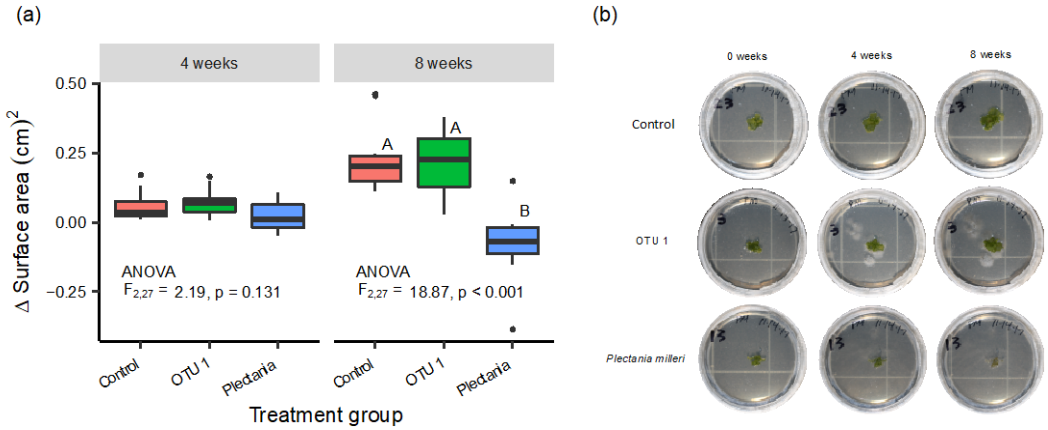

Figure 6. The change in surface area (cm^2) of *P. munitum* gametophytes treated with conidia suspensions generated from fungal cultures initially isolated from the host plants examined in the metabarcoding study (OTU1 and *Plectania milleri*) or with sterile DDH_2O (control). Spores from the fern host were harvested, surface sterilized and plated on Modified Parker/Thompson's Basal Nutrient Medium and allow to grow for two months in indirect sunlight prior to treatments. Images were taken at 0, 4 and 8 weeks after inoculation and surface area was analyzed with ImageJ through a custom macro. Differences in surface area were determined by subtracting the

surface area at week 0 from week 4 and also at week 8. Differences were examined with one-way ANOVA at 4 and 8 weeks and further analyzed with Tukey's post-hoc HSD. (a) Boxplots represent the median change in surface area (middle line) for treatment groups at 4 and 8 weeks, boxes represent upper and lower quartiles and lines represent upper and lower deciles (N = 30). (b) Representative images of gametophyte assays. Columns correspond to weeks post-inoculation and rows represent treatment groups.