Spatio-temporal dynamics of fungal communities and their effects on plants

Kel Cook

Follow this and additional works at: https://digitalrepository.unm.edu/biol_etds

Part of the Biodiversity Commons, Biology Commons, Botany Commons, Bryology Commons, Environmental Microbiology and Microbial Ecology Commons, Molecular Genetics Commons, and the Other Ecology and Evolutionary Biology Commons

Recommended Citation

This Dissertation is brought to you for free and open access by the Electronic Theses and Dissertations at UNM Digital Repository. It has been accepted for inclusion in Biology ETDs by an authorized administrator of UNM Digital Repository. For more information, please contact disc@unm.edu.
Kel Cook  
Candidate  

Biology  
Department  

This dissertation is approved, and it is acceptable in quality and form for publication:  

Approved by the Dissertation Committee:  

Dr. D. Lee Taylor, Chairperson  

Dr. Donald Natvig  

Dr. Scott Collins  

Dr. Jyotsna Sharma
SPATIO-TEMPORAL DYNAMICS OF FUNGAL COMMUNITIES AND THEIR EFFECTS ON PLANTS

by

KEL COOK

Bachelor of Arts in Plant Biology, 2012
Master of Science in Botany, 2015

DISSERTATION

Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy in Biology

The University of New Mexico
Albuquerque, New Mexico

December, 2020
ACKNOWLEDGEMENTS

I must first acknowledge my primary advisor, Dr. Lee Taylor, who has provided support, funding, intellectual guidance, and freedom to pursue new ideas and directions for the past five and a half years. Similarly, I would like to thank my committee members, Dr. Don Natvig, Dr. Scott Collins, and Dr. Jyotsna Sharma, for their valuable critiques and for sharing their scientific expertise.

I owe debt of gratitude to the members of the broader UNM fungal research group, including members of the Taylor, Natvig, and Rudgers labs. Their willingness to listen to various iterations of my research projects and provide feedback has been essential. I must also thank Rae Devan for solving so many lab and analytical problems before I got to them. For computer and statistical advice, I thank Michael Mann and Aaron Robinson. For advice on the greenhouse experiment and piñon pine care, I thank Jessie Marlenee and Annie Montes.

Field work in Costa Rica would not have been possible without extensive support. In particular, I’d like to thank the staff at Universidad de Costa Rica, Jardín Botánico Lankester, and Parque Nacional Tapantí that have assisted us with all of the logistical struggles involved with international field work.

For support at UNM, I must thank CARC for use of their high-performance computers, the staff at the molecular biology facility, including George Rosenberg and Melissa Sanchez, the Dinwiddie lab for allowing us to use their Illumina MiSeq, and greenhouse manager Wes Noe for help with keeping the greenhouse running.

Lastly, I need to thank several people for keeping me sane throughout this endeavor. First, thanks to my family for their continual support and questions along the lines of “So, are you graduating yet?” I have met many wonderful people in my time in Albuquerque, and I must thank Jeremiyah Rodriguez, the musical talent of The Hoodoos and Friends, and the great folks of the UNM graduate student community for support and the good times we’ve had together.
SPATIO-TEMPORAL DYNAMICS OF FUNGAL COMMUNITIES AND THEIR EFFECTS ON PLANTS

by

Kel Cook

Bachelor of Arts in Plant Biology, 2012
Master of Science in Botany, 2015
Doctor of Philosophy in Biology, 2020

ABSTRACT

Fungi perform several critical functions in the environment. Spatiotemporal distributions of fungal communities will mediate when and where these functions happen and how they vary across the landscape. I first explored tropical tree canopy fungal community variation at small spatial scales and documented near total turnover of fungi across sub-meter distances and among adjacent substrates. The second chapter analyzed fungal turnover over the course of three years, where community stability was driven primarily by abundant fungi. In the third chapter, I tested effects of the environment, including host plant and habitat, on canopy fungal communities and found only small effects, indicating high stochasticity. Finally, I used a greenhouse bioassay to demonstrate that microbial spatial variability impacts plant performance. These results show that fungal communities are spatially variable at small scales, this variability is largely stochastic, and fungi are a source of cryptic environmental heterogeneity with impacts on plant community dynamics.
TABLE OF CONTENTS

LIST OF FIGURES ........................................................................................................................................vi
LIST OF TABLES ........................................................................................................................................viii

Chapter 1: Epiphytic fungal communities vary by substrate type and at sub-meter spatial scales ..........1
  Abstract ..................................................................................................................................................1
  Introduction .......................................................................................................................................2
  Materials & Methods .........................................................................................................................5
  Results ...............................................................................................................................................10
  Discussion .........................................................................................................................................18
  Supplemental Figures .........................................................................................................................24
  References .........................................................................................................................................29

Chapter 2: Inter-annual persistence of canopy fungi driven by abundance despite high spatial turnover ....34
  Abstract ...............................................................................................................................................34
  Introduction ......................................................................................................................................35
  Materials & Methods .........................................................................................................................38
  Results ...............................................................................................................................................42
  Discussion .........................................................................................................................................48
  Supplemental Tables and Figures ........................................................................................................53
  References .........................................................................................................................................57

Chapter 3: High diversity but low host specificity of fungi associated with tropical epiphytic cryptogams....61
  Abstract ...............................................................................................................................................61
  Introduction ......................................................................................................................................62
  Materials & Methods .........................................................................................................................64
  Results ...............................................................................................................................................69
  Discussion .........................................................................................................................................76
  Supplemental Tables and Figures ........................................................................................................81
  References .........................................................................................................................................88

Chapter 4: Spatially structured plant-soil feedbacks impact plant growth and mycorrhizal colonization at local scales ....................................................................................................................93
  Abstract ...............................................................................................................................................93
  Introduction .......................................................................................................................................94
  Materials & Methods .........................................................................................................................96
  Results ...............................................................................................................................................102
  Discussion .........................................................................................................................................113
  Supplemental Tables and Figures ........................................................................................................119
  References .........................................................................................................................................126
LIST OF FIGURES

Chapter 1: Epiphytic fungal communities vary by substrate type and at sub-meter spatial scales ........................................1

Figure 1. Alpha diversity by substrate ..........................................................................................................................13
Figure 2. NMDS by substrate .....................................................................................................................................14
Figure 3. First ten cm of distance decay ..................................................................................................................16
Figure 4. Mantel correlograms by substrate ..............................................................................................................17
Figure 5. Venn diagram of variance partitioning analysis ..........................................................................................18
Figure S1. Spatial sampling design ..........................................................................................................................24
Figure S2. Photo of sample core ...............................................................................................................................24
Figure S3. Barplots of fungal taxa by substrate ...........................................................................................................25
Figure S4. Venn diagram of community overlap by substrate ...................................................................................26
Figure S5. Distance decay ...........................................................................................................................................27
Figure S6. Presence-absence distance decay ............................................................................................................28

Chapter 2: Inter-annual persistence of canopy fungi driven by abundance despite high spatial turnover .................34

Figure 1. NMDS by year and substrate .......................................................................................................................43
Figure 2. NMDS ordinations by year ..........................................................................................................................44
Figure 3. Venn diagram of variance partitioning .......................................................................................................45
Figure 4. Boxplot of paired Bray-Curtis dissimilarities ...............................................................................................46
Figure 5. Boxplot of paired partitioned Jaccard dissimilarities ....................................................................................47
Figure 6. OTU reoccurrence rates by initial relative abundance ................................................................................48
Figure S1. Venn diagram of variance partitioning analysis using presence-absence ..............................................53
Figure S2. Boxplots of alpha diversity ......................................................................................................................53
Figure S3. Spatial distance decay by substrate ..........................................................................................................55

Chapter 3: High diversity but low host specificity of fungi associated with tropical epiphytic cryptogams ..........61

Figure 1. Taxonomic barplots by host and treatment .................................................................................................70
Figure 2. NMDS ordination by host type ....................................................................................................................71
Figure 3. NMDS ordinations by host taxon ................................................................................................................73
Figure 4. NMDS ordinations by host and habitat .......................................................................................................74
Figure 5. OTU richness .................................................................................................................................................75
Figure 6. NMDS ordination by sterilization treatment ...............................................................................................75
Figure 7. Boxplot of partitioned beta diversity ..........................................................................................................76
Figure S1. Histogram of occurrence frequency .........................................................................................................81
Figure S2. PA NMDS by host type ............................................................................................................................82
Figure S3. PA NMDS ordination by host taxon ............................................................................................................84
Figure S4. Venn diagrams of community overlap ....................................................................................................85
Figure S5. NMDS ordinations of surface sterilized fungal community ........................................................................86
Figure S6. PA NMDS ordination by host and sterilization treatment ..........................................................................87
Chapter 4: Spatially structured plant-soil feedbacks impact plant growth and mycorrhizal colonization at local scales .......................................................... 93

Figure 1. NMDS ordinations by plot ........................................................................... 104
Figure 2. Barplots of axonomic composition................................................................ 105
Figure 3. Distance decay ............................................................................................ 106
Figure 4. Boxplots of plant biomass and root colonization by plot .............................. 108
Figure 5. Correlograms of blue grama biomass and piñon root colonization ............... 109
Figure 6. Maps of blue grama biomass root colonization ............................................ 110
Figure 7. Boxplot of OTU richness .............................................................................. 111
Figure 8. Effects in pooled versus individual soil samples .......................................... 112
Figure S1. Sampling design for soil cores ................................................................. 119
Figure S2. Boxplots of AM and EM fungi by plot ....................................................... 120
Figure S3. Mantel correlograms for fungi and bacteria .............................................. 121
Figure S4. Maps of piñon pine biomass by core and plot ............................................. 122
Figure S5. Photos of the field sites ............................................................................ 125
LIST OF TABLES
Chapter 1: Epiphytic fungal communities vary by substrate type and at sub-meter spatial scales ..................1
   Table 1. Summary of sequencing data and spatial results .............................................................................12
Chapter 2: Inter-annual persistence of canopy fungi driven by abundance despite high spatial turnover ........34
   Table S1. Comparison of temporal vs spatial community turnover ...........................................................54
   Table S2. Reoccurrence rates by initial relative abundance ......................................................................56
Chapter 3: High diversity but low host specificity of fungi associated with tropical epiphytic cryptogams ...61
   Table 1. Pairwise PERMANOVA results .....................................................................................................72
   Table S1. Sampling design ..........................................................................................................................81
   Table S2. Pairwise PERMANOVA results using PA ..................................................................................83
Chapter 4: Spatially structured plant-soil feedbacks impact plant growth and mycorrhizal colonization at local scales .................................................................................................................................93
   Table S1. Correlations between OTU relative abundance and plant biomass ........................................123
   Table S2. Correlations between OTU relative abundance and pine root colonization ..........................124
Chapter 1: Epiphytic fungal communities vary by substrate type and at sub-meter spatial scales

Abstract

Fungal species have numerous important functions in the environment. Where these functions occur will depend on how fungi are spatially distributed, but spatial structures of fungal communities are largely unknown. This is especially true in hyperdiverse tropical tree canopy systems, which are understudied using high-throughput sequencing technology. Here we explore fungal communities in a Costa Rican tropical rainforest canopy, with a focus on local-scale spatial structure and substrate specificity of fungi. We sampled 135 locations across five tree branches and identified fungi from four substrate types: outer host tree bark, inner bark, dead bryophyte tissue, and living bryophytes. Samples were located between one centimeter and eight meters apart. Fungal community composition and diversity varied among substrate types, even when multiple substrates were in direct contact. Fungi were most diverse in living bryophytes, with 39% of all fungal OTUs found exclusively in this substrate, and the least diverse in inner bark. Fungal communities had significant positive spatial autocorrelation and distance decay of similarity only at distances less than one meter. Similarity among samples declines by half in less than ten centimeters, and even at these short distances, similarities are low with few OTUs shared among samples. These results indicate that community turnover is high and occurs at very small spatial scales, with any two locations sharing very few fungi in common. High heterogeneity of fungal communities in space and among substrates may have important implications for the distributions, population dynamics, and diversity of other tree canopy organisms, including epiphytic plants.
Introduction

Fungi, in their roles as pathogens, saprotrophs, and mycorrhizal mutualists, are important drivers of ecosystem processes, including nutrient cycling (Read & Perez-Moreno, 2003), building soil structure (Rillig & Mummey, 2006), productivity (van der Heijden, Bardgett, & van Straalen, 2008), and structuring plant communities (Klironomos, 2002; van der Heijden et al., 1998), with each fungal taxon impacting these processes differently. If spatial extent of fungal genets tends to be small and community turnover is high, these processes and interactions will also vary at small spatial scales. Thus, variation in fungal community composition at a fine spatial scale may have substantial consequences for larger scale ecological processes, including plant community assembly. Data on fungal community composition and assembly at small scales is very limited.

It is increasingly recognized that microbial communities are heterogeneous at a range of spatial scales. For example, studies of various groups of fungi in terrestrial ecosystems, including arbuscular mycorrhizal fungi (Chaudhary, O’Dell, Rillig, & Johnson, 2014; Mummey & Rillig, 2008; Vannier, Bittebiere, Vandenkoornhuyse, & Mony, 2016), ectomycorrhizal fungi (Genney, Anderson, & Alexander, 2006; Lilleskov, Bruns, Horton, Taylor, & Grogan, 2004; Pickles et al., 2010; Yoshida, Son, Matsushita, Iwamoto, & Hogetsu, 2014), orchid mycorrhizal fungi (Voyron, Ercole, Ghignone, Perotto, & Girlanda, 2017), and foliar endophytes (Higgins, Arnold, Coley, & Kursar, 2014; Koide, Ricks, & Davis, 2017) have demonstrated that these communities are structured spatially and that fungal taxa are patchily distributed. Several studies have reported distance decay of similarity in community composition at scales from tens of centimeters to kilometers (Koide et al., 2017; Lilleskov et al., 2004; Toju, Sato, & Tanabe, 2014; Yoshida et al., 2014). For
example, Mummey & Rillig (2008) found spatial autocorrelation and patchiness in grassland arbuscular mycorrhizal fungi at distances less than 50 centimeters. Spatial patterns are not seen at every scale in every system. For example, Vincent et al. (2016) found no evidence of spatial clustering in rainforest tree leaf endophytes at the scales of tens of meters to hundreds of kilometers. Spatial patterns may be due in part to dispersal limitation (Galante, Horton, & Swaney, 2011; Peay, Garbelotto, & Bruns, 2010). If a study is done at scales larger than the scale at which dispersal probability declines, spatial patterns could be weak or absent. The minimum scales at which significant structure occurs is unknown. Also, prior studies have focused on specific groups of fungi, rather than whole fungal communities, which may have different spatial structures.

Local distributions of fungi can also be affected by substrate specificity. In soils, surficial soil with high organic content and deeper mineral layers contain different arrays of fungi (Rosling et al., 2003; Taylor & Bruns, 1999; Taylor et al., 2014; Tedersoo, Kõljalg, Hallenberg, & Larsson, 2003). Host plant species has been shown to impact community composition of mycorrhizal fungi (Ishida, Nara, & Hogetsu, 2007), endophytes (Hoffman & Arnold, 2008; Thomas, Vandegrift, Roy, Hsieh, & Ju, 2019; Vincent et al., 2016), and bryophilous fungi (Davey, Heimdal, Ohlson, & Kauserud, 2013). Within an individual host plant, fungal community composition, biomass, and species richness can vary between tissue types, such as between photosynthetic and senescent bryophyte tissues (Davey et al., 2013; Davey, Nybakken, Kauserud, & Ohlson, 2009) and the bases and tips of tree leaves (Oono, Rasmussen, & Lefèvre, 2017). These fine-scale differences in fungal distributions among cooccurring substrates have largely been ignored as potential drivers of processes like plant competition and community assembly, nutrient cycling, and disease resistance.
Tropical rainforest trees support a high diversity of epiphytic plants (Benzing, 1990), yet epiphytic fungal communities have been little studied with molecular techniques beyond studies on orchid mycorrhizae (Cevallos, Sánchez-Rodríguez, Decock, Declerck, & Suárez, 2017; Harshani, Senanayake, & Sandamali, 2014; Herrera, Kottke, Molina, Méndez, & Suárez, 2018). Most research on fungal spatial distributions has focused on soils (Bahram, Peay, & Tedersoo, 2015). Compared to soils, tree branches have very different physical and chemical structures. Surfaces inhabitable by fungi are isolated on tree branches in three-dimensional space and surrounded by air, limiting hyphal growth. In comparison, soils are generally continuous, allowing fungi to potentially grow indefinitely (Anderson et al., 2018). Also, soils typically have a surface layer dominated by organic material with a mineral layer below. Epiphytic substrates are almost entirely organic and composed of living organisms, decaying organic material, and host tree bark, while mineral material is minimal to absent. Inorganic nutrients generally have low availability, with occasional pulses of availability related to rainfall and stem-flow (Benzing, 1990). Because of these differences, inferences about patterns of fungal distributions from soil systems have limited applicability to canopy ecosystems. Characterization of fungal distributions and diversity in the epiphytic environment has the potential to provide new insights into the co-existence of diverse plant species in this habitat as well as global patterns of fungal biodiversity.

In this study, we examined the diversity and local scale spatial patterns of fungal communities on homogeneous, neighboring tree branches in a tropical rainforest canopy system. We combine fine scale sampling with high throughput sequencing methods and rigorous spatial statistics to provide compelling evidence of hitherto unappreciated dominance of stochastic dispersal in driving fungal community assembly. Our goals were to
(1) test for differences in fungal community composition and diversity among substrates on tree branches and (2) quantify and characterize fine scale spatial structure of the canopy fungal community.

**Materials & Methods**

*Sample Collection*

This study was conducted in a low montane tropical rainforest in Parque Nacional Tapantí, Cartago Province, Costa Rica along the east bank of the Rio Orosi (9.742°N, 83.784°W, 1300 m elevation) in July of 2015. The riverbank was dominated by *Saurauia montana* (Seem.), which hosts rich epiphyte communities, including bryophytes, mostly consisting of liverworts, lichens, orchids, ferns, and other vascular plants. Samples were collected from 135 points spread across five *S. montana* branches using a 9mm diameter borer. Collection points were situated at a geometric series of increasing distances (Figure S1) producing substantial and relatively even replication of interpoint distances ranging from one centimeter to over eight meters apart. Distances between points on the same branch were measured as linear distance along the branch. Distances between selected points on separate branches were measured with a laser (Leica DISTO D8, Leica Geosystems AG, Switzerland). The remaining distances were extrapolated from known distances assuming linear relationships. While this approach involved some error due to the angling of branches, the error was small (<10 cm in most cases) relative to the distances between points on different branches, which was greater than 2 meters in most cases. Within 24 hours of collection, each sample was dissected into up to four substrate types: inner tree bark, surficial bark, photosynthetic bryophyte tissue, and dead or senescent bryophyte material.
These substrates will henceforth be called inner bark (IB), outer bark (OB), live bryophytes (LB), and dead bryophytes (DB). We removed vascular plant roots and rinsed substrates in sterile water and preserved them in RINAlate (Ambion, ThermoFisher).

**Molecular Methods**

Each sample was rinsed twice with MilliQ water to remove the RNAlater, lyophilized, transferred to a 96-well plate, and ground with two 3.2mm stainless steel beads using a TissueLyser II (Qiagen, Hilden, Germany) at 30hz for 90 sec. Total DNA was extracted from each ground sample with DNeasy 96 Plant kits (Qiagen, Hilden, Germany) using the manufacturer’s protocol beginning at step 7. We amplified the ITS2 region using universal fungal primers 5.8S_Fun (5’ - GTCTGCTGGGCTCGGAGATGTGTTATAAGAGACAGAAAAACTTTYRRCAAYGGA TCWCT 3’) and ITS4_Fun (5’ - TCGTCGGCAGCGTCAGATGTGTTATAAGAGACAGAGCCTCCGCTTATTTGATATG CTTAART 3’), with Nextera adapters added to the core primers (core primers in bold, Taylor et al. 2016). PCR amplification was carried out in 25 μl reactions with 5 μl 5x GoTaq Reaction buffer, 200 μM of each dNTP, 1mM MgCl₂, 0.5 μM of each primer, and 1.25 units of GoTaq polymerase (Promega, Madison, WI, USA). The amplification conditions were an initial denaturation step at 96° for 2 minutes, 27-32 cycles of 94° for 30 seconds, 55° for 40 seconds, and 72° for 2 minutes, and a final 72° elongation step for 10 minutes. PCR products were cleaned using ZR-96 DNA Clean & Concentrator kits (Zymo Research, Irvine, CA, USA). After cleaning, seven cycles of PCR following the above protocol were carried out using oligos at 0.4 μM each to add Illumina adaptor sequences and
sample-specific 6bp barcodes (5’-CAAGCAGAAGACGGCATACGAGAT-NNNNNN-GTCTCGTGGGGCTCGG-3’ and 5’-AATGATACGGCGACCACCGAGATCTACAC-NNNNNN-TCGTCGGCAGCGTC-3’, with Ns representing the variable barcode region). Barcoded samples were pooled at approximately equal concentrations based on gel electrophoresis band brightness and cleaned with Agencourt AmPure XP magnetic beads (Beckman Coulter, Indianapolis, IN, USA). Each library also included a mock community (D. Lee Taylor et al., 2016). Libraries were then sequenced with Illumina MiSeq using the v3 2x300bp chemistry.

**Sequence Processing**

Sequence data were processed primarily with USEARCH v9.2.64 (Edgar, 2013). Paired end reads were first merged using the fastq_mergepairs command. Merged sequences less than 150 bp in length and all unmerged sequences were excluded from further analysis. Remaining primer sequences were removed using cutadapt (Martin, 2011). Reads were then quality filtered to remove all reads with greater than one expected error with the usearch command fastq_filter. Filtered reads were dereplicated with fastx_uniques, and clustered at 97% similarity to form OTUs (operational taxonomic units) using cluster_otus. This step also removed chimeric sequences and OTUs containing only one sequence. All OTUs were then clustered against the UNITE database version 7.1 (Nilsson et al., 2019) at 50% using pick_open_reference_otus.py in QIIME v1.9.1 (Caporaso et al., 2010). OTUs that did not hit a database entry at this level of similarity were treated as likely non-fungal and removed from the dataset. We then assigned the pre-quality filter merged reads to these filtered OTUs with the usearch_global command. Taxonomy was assigned to each OTU with the RDP
Classifier (Wang, Garrity, Tiedje, & Cole, 2007) and the UNITE Fungal ITS training set (version 6.0, Nilsson et al., 2019). Taxonomic assignments with less than 80% confidence were not retained. Abundance and taxonomic data were compiled into an OTU table. All samples with fewer than 1000 reads were removed from the dataset.

**Data Analysis**

**Alpha diversity**

To assess differences in alpha diversity among the four substrates, we calculated OTU richness, the Simpson index, and the Shannon index for each sample. To account for differing sequencing depths among samples, expected richness at 1,000 reads was calculated using the rarefy function in the vegan packages (Oksanen et al., 2019) in R v3.5.1 (R Core Team, 2018). For the Simpson and Shannon indices, we rarefied each substrate sample to 1,000 reads and calculated the indices, repeated this process 1,000 times, and calculated the average indices for each sample. To minimize the impact of potential spatial autocorrelation in alpha diversity, we used a subset of the sampling points, such that all points were at least 45 cm from each other.

Differences in diversity among substrates were analyzed using a Kruskal-Wallis test, and pairwise Wilcoxon rank sum tests were used to test differences between pairs of substrates. Correlations in species richness among substrates within individual sample points were also tested. We used the Bonferroni method to correct p-values for both pairwise Wilcoxon tests and correlations.
Community and spatial analyses

To visualize compositional differences in fungal communities across substrates, we performed two-dimensional non-metric multidimensional scaling (NMDS) using the Bray-Curtis dissimilarity coefficient calculated in the phyloseq (McMurdie & Holmes, 2013) R package with 100 iterations. We visualized the number of OTUs that were shared between substrates based on presence-absence using the VennDiagram R package (Chen, 2018).

We tested for spatial autocorrelation in fungal community composition in each substrate except inner bark, which was omitted from all spatial analysis due to low sample size, using Mantel tests with Pearson correlations with the mantel function in vegan (Oksanen et al., 2019). Statistical significance was determined by permuting the community dissimilarity matrices 999 times. To further understand the scales at which spatial autocorrelation occurs for each substrate, we calculated Mantel correlograms with the mantel.correlog vegan function, again using Person correlations and 999 permutations. Distance classes were determined by program defaults, and p-values were progressively corrected using the Holm method (Holm, 1979).

To examine community turnover and distance decay at the smallest distances, we plotted community similarity using relative abundance data and the Bray-Curtis similarity index against distance between each pair of points for each substrate. We fit lines to the first ten centimeters of data, which were approximately linear, and used these lines to estimate similarity at zero cm and the distance at which similarity declines to half of this initial value. We repeated this process using the Jaccard index in two ways: with complete presence-absence data and with a subset comprising only OTUs making up at least 1% of a sample being counted as present, to minimize the effects of extreme low abundance OTUs.
We used redundancy analysis (RDA) to test effects of space, substrate, and distance along a branch on community composition. To account for differences in sequencing depth between samples, the OTU table was first transformed to relative abundance. We created spatial variables that model the spatial structure of the sampling points using the principal coordinates of neighbor matrices (PCNM) method (Borcard & Legendre, 2002) as implemented in the pcnm function in the vegan package (Oksanen et al., 2019). Spatial variables were derived from the geographic distance matrix, with distances between substrates at the same point set to 0.1 cm. The 46 eigenvectors with positive eigenvalues underwent forward selection with the Blanchet et al. (Blanchet, Legendre, & Borcard, 2008) stopping criterion, retaining 25 variables. To account for effect of position of a point along a branch (near the trunk versus toward the tip), we used distance along a branch, which was scaled from 0, the point closest to the trunk, to 1, the point closest to the tip. We then partitioned variance in community composition explained by the retained spatial variables, substrate type, and distance along branch. Statistical significance of each partition was tested with permutation tests with 999 permutations.

Results

Data Summary

Illumina sequencing of ITS2 amplicons produced 57,748,230 paired-end reads. Of these, 15,866,516 reads passed all filtering steps and belonged to 5,777 non-singleton fungal OTUs at 97% identity (Table 1). Of 533 substrate samples, 153 contained fewer than 1000 passing reads and were excluded. Of these excluded samples, 91 belonged to the inner bark substrate type, likely because fungi occurred in very low abundances in these samples. The
majority of the OTUs (63%) belonged to the Ascomycota, particularly Eurotiomycetes, Leotiomycetes, and Sordariomycetes, while Basidiomycota made up 24.8% of the OTUs (Figure S3). Many OTUs could not be assigned with 80% confidence at any taxonomic level below Fungi: 10.4% were unidentified at the phylum level, and 81.5% could not be assigned to a genus. OTUs that could be identified at ≥80% confidence belonged to a variety of guilds, including wood and litter decay fungi, lichens, orchid mycorrhizal fungi, plant pathogens, and arbuscular mycorrhizal fungi.

Alpha Diversity

There were significant differences among substrates for all three diversity indices (expected richness: $\chi^2 = 45.017, p<0.001$; Shannon: $\chi^2 = 31.86, p<0.001$; Simpson: $\chi^2 = 21.531, p<0.001$). Inner bark had significantly lower Shannon and Simpson indices per sample than the other substrates ($p<0.001$), while there were no significant differences among the others. All substrate pairs had significantly different expected richness, except for outer bark and live bryophytes ($p<0.05$, Figure 1). Live bryophytes had the highest average species richness per sample and the greatest total richness when all samples were combined, while inner bark had the lowest (Table 1). There were no statistically significant ($p<0.05$) correlations in species richness among substrates at the same point.
Table 1. Summary of sequencing data and spatial results, including initial similarity (expected Bray-Curtis similarity at zero cm) and distance at which this initial similarity is halved, by substrate type.

<table>
<thead>
<tr>
<th>Substrate</th>
<th># samples</th>
<th># reads</th>
<th>Mean reads/sample</th>
<th>Mean observed OTUs/sample</th>
<th>Mean inferred OTUs/sample</th>
<th>Initial similarity</th>
<th>Halving distance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Live bryophytes</td>
<td>111</td>
<td>4,164,733</td>
<td>37,520 (± 31,861)</td>
<td>4,429</td>
<td>2.805</td>
<td>0.1093 (p=0.001)</td>
<td>0.279 7 cm</td>
</tr>
<tr>
<td>Dead bryophytes</td>
<td>117</td>
<td>5,114,465</td>
<td>43,713 (± 52,363)</td>
<td>2,055</td>
<td>49.716</td>
<td>0.2174 (p=0.002)</td>
<td>0.513 8 cm</td>
</tr>
<tr>
<td>Outer bark</td>
<td>121</td>
<td>6,015,672</td>
<td>49,716 (± 41,532)</td>
<td>2,830</td>
<td>144.7 (± 62.6)</td>
<td>0.3296 (p=0.001)</td>
<td>0.547 8.3 cm</td>
</tr>
<tr>
<td>Inner bark</td>
<td>31</td>
<td>571,646</td>
<td>18,8440 (± 43,476)</td>
<td>449</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Total</td>
<td>380</td>
<td>5,866,516</td>
<td>41,753 (± 43,597)</td>
<td>5,777</td>
<td>NA</td>
<td>NA</td>
<td>380 31 mm</td>
</tr>
</tbody>
</table>
Figure 1. Alpha diversity by substrate type using expected species richness at 1000 sequences per sample (A) and Simpson (B) and Shannon (C) indices on data rarefied to 1000 sequences. Live bryophytes had the highest per sample expected richness, and inner bark had the lowest diversity according to all three indices.
Community Composition by Substrate

Two-dimensional NMDS ordination provided graphical support for differences in fungal community composition among substrates (Figure 2). Outer bark, dead bryophytes, and live bryophytes separated along the first NMDS axis. Dead bryophytes were clustered between the live bryophytes and outer bark and partially overlapped with them. Inner bark samples did not form a distinct cluster. Notably, the arrangement of the substrates in the ordination mirrors their arrangement in the field, with live bryophytes growing on top of dead bryophytes, which are on the bark surface.

Figure 2. Nonmetric multidimensional scaling (NMDS) ordination plot showing differences in fungal communities among live bryophytes, dead bryophytes, outer bark, and inner bark with 95% confidence ellipses. Stress = 0.2820
Of the 5,777 non-singleton OTUs recovered, 2,468 were found in at least two substrate types (Figure S4), while 319 were present in all four substrates. Live bryophytes had the highest number of unique OTUs, at 2,246, which is 50.7% of all OTUs found in this substrate. Inner bark had only 24 unique OTUs, which made up 5.3% of those found there. The majority (71%) of the OTUs found in inner bark were common to all substrate types.

**Spatial Structure**

Mantel tests showed significant, positive linear correlations between community dissimilarity and distance between points for the three substrates examined (Table 1). Plots of community similarity against geographic distance (Figures 3 and S5) and Mantel correlograms (Figure 4) show dramatic declines in similarity over very short distances. The correlograms show significant positive autocorrelation at only the smallest distance classes. Bray-Curtis similarities tended to be very low, usually less than 0.5, even between adjacent points, and these low initial dissimilarities declined by half within the first ten centimeters (Table 1). Distance decay lines using presence absence data were nearly flat (Figure S6a), but when only high abundance OTUs were considered, they closely resembled the lines using relative abundance (Figure S6b). For all substrates, distance decay leveled off and positive autocorrelation was lost between 30 and 90 centimeters. Distance decay patterns were only apparent within branches; the distances between branches were larger than the distance range over which similarity decays.

Of the three substrates examined, live bryophytes had the lowest similarities at small distances and the lowest initial similarity (Figure 3, Table 1). Outer bark and dead bryophytes had similar distance decay patterns and initial similarities. In the Mantel correlograms, outer bark and dead bryophytes had positive autocorrelation in the first two
distance classes, out to 89 centimeters. Living bryophytes, however, lost positive autocorrelation after the first distance class, at 30 centimeters. All substrates showed negative autocorrelation at some, but not all, larger distance classes.

RDA and variance partitioning revealed that substrate type, position along a branch, and PCNM spatial vectors all have significant relationships with fungal community composition (p<0.001, Figure 5). Of these, the spatial vectors explained the largest portion of the variance, at 7.8%. Position along branch explained the least, and almost half of the variance explained was shared with the spatial vectors. Substrate alone explained 4.3% of the variance. Most of the variance, 87%, was unexplained by any variable, possibly due to the high total number of OTUs and low number shared between most pairs of samples (Figure S6a).

Figure 3. The first ten centimeters of distance decay by substrate type using relative abundance data and Bray-Curtis similarity. Similarity values were low, even at the shortest distances, and decay rapidly over the first ten centimeters. Living bryophytes (LB) had less similarity among samples at short distances than dead bryophytes (DB) or outer bark (OB).
Figure 4. Mantel correlograms demonstrating spatial autocorrelation of fungal community in each substrate type. Filled symbols indicate significant autocorrelation at that distance class. Significant positive autocorrelation is lost after the first or second distance class, which are less than one meter, for all four substrates.
Discussion

Numerous studies have demonstrated patchiness of particular components of the fungal community (e.g. arbuscular mycorrhizal fungi) at a variety of spatial scales from meters to kilometers. Few, however, have combined the power of high-throughput sequencing of total fungal communities with centimeter scale sampling and rigorous geospatial analyses. To our knowledge, this is the first study to apply this suite of methods to tropical canopy branch surfaces, where plants and fungi are hyperdiverse.

Our results reinforce accumulating evidence for spatial structure of fungal communities in soils and other substrates (Bahram et al., 2015; Oono et al., 2017; Thomas et
al., 2019). We document distance decay of community similarity, in which similarity declines rapidly over the first 50 centimeters, a remarkably fine spatial scale (Figures 3 & S5). Even more striking is the limited overlap in species composition among samples, even at small spatial scales. Samples collected 10 centimeters apart or from different substrates from the same location often hosted $\geq 50$ OTUs each but had few to no OTUs in common (Figure S6a). Substrate type and spatial vectors were significant predictors of fungal community composition, but most variation in community composition remained unexplained (Figure 5). Together these results suggest strong dispersal limitation in these communities and an overarching role of stochastic forces in fungal community assembly at spatial scales relevant to plants and other organisms in this system.

Across all sampling points, we found unique community structures across live and dead bryophyte tissues, and surficial and interior host tree bark, despite the facts that these substrates were in physical contact and, in the case of live and dead bryophytes, often intermixed (Figure 2). Similarly, Davey et al. (2012) found fungal community differences between photosynthetic and senescent tissues of terrestrial bryophytes in a boreal forest. This suggests turnover in fungal communities as bryophytes age and die. Substrate, however, explains only a small portion of the variation in the fungal community (Figure 5), likely due to the high spatial turnover that occurs within each substrate type.

We found the greatest species richness, both per sample and in total, in the live bryophyte substrate (Figure 1, Table 1). Greater diversity in live bryophytes may be caused by increased physical and chemical heterogeneity relative to dead bryophytes and tree bark. It may also be related to position of the substrate. When present, live bryophytes were the topmost substrate and could receive more fungal propagules than lower substrates. Live
bryophytes are also the youngest substrate. In a study on pine needle endophytes, Oono et al. (2017) found that the youngest tissue type, needle bases near the tops of trees, had the highest alpha diversity. It is possible that species richness declines with substrate age due to competitive dynamics.

Inner tree bark had the lowest fungal diversity per sample and across the whole substrate (Figure 1, Table 1), and many samples failed to amplify. We infer that fungi had very low abundances in this substrate. Unlike the outer bark, this substrate was living and included sap-filled phloem, which may make it difficult for fungi to survive there. Most of the OTUs found in the inner bark were present in all other substrate types (Figure S4). They may have entered the inner bark from these other substrates or have been introduced during sample collection or processing. We found no evidence of pervasive endophytes in the living inner bark of *S. montana*.

Within individual sampling locations, there was no significant relationship between alpha diversity of fungi in different substrates. In other words, diversity in one substrate could not be used to predict diversity in other substrates at the same point, and there was no tendency to see diversity “hot-spots” or “cold-spots”, where diversity was higher or lower across all substrates. This suggests that whatever drives fungal diversity in this system, be it stochastic dispersal, abiotic environmental factors, and/or unmeasured biotic factors, these drivers do not act on all substrates at the same point in the same way.

Variation in fungal communities in this canopy system occurred at very small spatial scales within each substrate type. In all substrates studied, there was no significant positive autocorrelation observed beyond 90 cm (Figure 4). In live bryophytes, this distance was even smaller; positive autocorrelation was lost, and distance decay began to plateau after 30
cm. One possible explanation for this higher spatial turnover in live bryophytes is the complex three-dimensional structure of bryophyte mats, which could impede hyphal growth and increase surface area relative to the smoother bark of *S. montana*. Live bryophytes, as the topmost substrate layer, may also be more exposed to incoming air- and raindrop-dispersed spores. If airborne propagules are spatially heterogeneous at a small scale, they may drive this pattern. Conversely, homogeneously dispersed propagules would have the opposite effect. Previous research has shown that fungal spores can show spatial structure in the air at the scale of several meters to kilometers (Peay & Bruns, 2014) and in soils from centimeters to meters (Carvalho, Correia, Ryel, & Martins-Loução, 2003; Klironomos, Rillig, & Allen, 1999), but we are unaware of any studies addressing this issue for airborne spores at spatial scales relevant to our study in tropical ecosystems.

It is unclear how much within-substrate spatial patterns are driven by biotic and abiotic environmental heterogeneity and gradients versus dispersal limitation, including dispersal by spores or hyphal growth. Given that we only see spatial structure at the sub-meter scale, similarity between close points may be due to resampling individual genets that have grown vegetatively along the branch. Local spore dispersal may also contribute to spatial patterns, as most spores land near the sporocarp (Galante et al., 2011). The relative contributions of hyphal and spore-based dispersal should be testable by comparing within-branch (with hyphal and spore-based dispersal) and between-branch (with spore dispersal only) spatial patterns. Unfortunately, nearly all between-branch distances in this study were greater than the 90 cm range of spatial autocorrelation, so we could not address this here. Regardless of which dispersal mechanism is more prevalent, genet size in this system is likely small, such that single fungi do not cover entire branches. Further research is also
needed to assess how environmental variables beyond substrate type, such as nutrient concentration, moisture, and host bryophyte species, impact branch-surface fungal communities and the degree to which these drive spatial patterns.

Direct comparisons to other research on spatial patterns of fungi are difficult because measurement of distance decay relationships is sensitive to differences in grain (spatial size of the sampling unit) and extent (scale over which the study takes place) (Nekola & White, 1999), which vary by orders of magnitude among studies. Also, most previous studies focused on specific groups of mycorrhizal fungi, and many used older Sanger sequencing or T-RFLP methods. In contrast, we sampled whole fungal communities with high-throughput sequencing. Despite these limitations, some broad patterns have been found across studies on fungal community spatial structure. Bahram et al. (2013) found that spatial autocorrelation of soil ectomycorrhizal fungi tends to occur at greater distances at low latitudes, often at distances greater than 10 meters. In contrast, we only find autocorrelation at distances less than one meter, though the processes driving spatial structure of soil versus epiphytic fungi likely differ. Some other studies that cover a small spatial extent (centimeters to a few meters) have also found evidence of spatial clustering and autocorrelation of fungi being confined to the sub-meter scale (Mummey & Rillig, 2008; Oja et al., 2017; Tedersoo et al., 2003; Yoshida et al., 2014).

Fungal community variation among substrates and at very small spatial scales may have substantial implications for the epiphytic plant community. Plant propagules or seedlings located less than a meter apart can be exposed to entirely different sets of fungi. These distinct fungal assemblages may have net positive or negative effects on the ability of a seedling to grow and establish. Plant taxa can also differ in their responses to the same
microbes. Orchids, for example, require mycorrhizal fungi to complete their life cycles, but orchid species vary in the fungi they require and in their level of specificity. Even closely related sympatric species can utilize different fungal taxa (Jacquemyn, Brys, Waud, Busschaert, & Lievens, 2015; Shefferson et al., 2007). Pathogenic fungi can also target specific groups of plants, while leaving others unaffected (Barrett, Kniskern, Bodenhausen, Zhang, & Bergelson, 2009). A heterogeneous patchwork of fungi could create isolated “safe sites” for plant establishment, where host specific mutualists are present, and pathogens are absent.

In conclusion, we found extensive turnover of the fungal community at sub-meter spatial scales and among substrates from the same sample point. Small-scale spatial patterns are likely driven by dispersal limitation and other stochastic processes, and they likely have important implications for the plant community. Apparently random variation in plant germination, establishment, and growth rates may have predictable fungal drivers created by the combination of spatial variation and staggering diversity.
Supplemental Figures

Figure S1. Example of spatial sampling design along one branch. Sampling scheme varied slightly among branches due to different branch lengths.

Figure S2. Photo of sample core
Figure S3. Relative abundance of fungal taxa at the phylum (a) and class (b) levels by substrate type and for the whole fungal community.
Figure S4. Venn diagram showing numbers of OTUs shared by each combination of substrates based on presence-absence data. Live bryophytes have the most unique OTUs, while inner bark has the fewest. Most inner bark OTUs are shared with other substrates.
Figure S5. Plots showing distance decay relationships between distance between pairs of samples and fungal community similarity for live bryophytes (a), dead bryophytes (b), and outer bark (c). This uses relative abundance data and Bray-Curtis similarity. Each dot represents a pair of samples, with black dots being pairs on the same branch and colored dots being pairs on different branches. The greatest similarities were found at the smallest distances, generally less than 50 cm.
Figure S6. The first 10 cm of distance decay by substrate using presence-absence data and Jaccard similarity. In (a), all OTUs with at least one read in a sample were counted as present, while in (b), only OTUs making up at least one percent of the reads in a sample were counted as present. Distance decay lines in (a) are nearly flat, likely due to high turnover in very low abundance OTUs. Lines considering only high abundance OTUs in (b) closely resemble the lines created using relative abundance data (Figure 3). Similarities are very low, even at the shortest distances, indicating few shared OTUs.
References


Oono, R., Rasmussen, A., & Lefèvre, E. (2017). Distance decay relationships in foliar fungal endophytes are driven by rare taxa: Distance decay in fungal endophytes. *Environmental Microbiology, 19*(7), 2794–2805. doi: 10.1111/1462-2920.13799


Chapter 2: Inter-annual persistence of canopy fungi driven by abundance despite high spatial turnover

Abstract

While it is now well established that fungal community composition varies spatially at a variety of scales, temporal turnover of fungi is less well understood. While previous work has focused on soil fungi, we studied inter-annual community compositional changes of epiphytic fungi in a rainforest tree canopy. We tracked shifts over the course of three years in three substrate types (live bryophytes, dead bryophytes, and host tree bark) and compared this to intra-annual small-scale spatial turnover. Substrate type had a stronger effect on fungal community than sampling year, with community differentiation by substrate persisting among years. Sampling year had a significant, but very small effect on community composition. Although levels of temporal turnover varied among substrates, the amount of turnover for all substrates was comparable to what is seen at spatial distances between five and nine centimeters for the same substrate. Community stability was largely driven by a few fungi with high relative abundance. Fungi making up at least one percent of a sample were more than twice as likely to persist to subsequent years than making up 0.1% or less. While abundant fungi and their ecological functions are able to persist, most fungi were rare and had low relative abundance, suggesting that most epiphytic fungi are physically small and short lived.
Introduction

Molecular surveys of fungi in the environment have revealed diverse communities with high beta diversity and heterogeneity at a range of spatial scales, from the local to continental (Bahram et al. 2013, 2015, Chaudhary et al. 2014, Oono et al. 2017). While spatial turnover tends to be high, it is largely unknown how stable these communities are over time and how temporal turnover compares to changes with space. Most studies of fungal communities only sample at a single time point and do not capture temporal variation or consider how it may impact the fungal community or its ecological functions. It is also unclear if a sample taken at one time point can be used to predict the composition of fungal communities into the future.

Fungal genets, in the form of continuous vegetative mycelia, have the potential to live for centuries (Anderson et al. 2018), which could promote long term community stability. Turnover of fungal structures like rhizomorphs and hyphae, however, can occur over the span of days to months (Staddon et al. 2003, Treseder et al. 2005, Godbold et al. 2006, Pepe et al. 2018). Because of this, individual fungi might become extirpated from local areas over short time scales. Mycelial death coupled with continuous arrival of new spores or hyphae from the surroundings create the possibility for highly dynamic fungal communities that vary rapidly with little consistency from one year to the next. The level of stability in fungal communities has implications for their ecological functions. High community turnover could result in continuously changing functions that cannot be predicted into the future. While functional redundancy among fungi is possible (Kivlin and Hawkes 2020), some have unique functions. These can include enzymatic activity to degrade and mobilize nutrients from complex organic material (Kyaschenko et al. 2017) or host-specific interactions with host

Longevity and temporal persistence likely vary among fungi. Recent research has found different life history strategies among fungi within the same guild, such as in wood decay fungi (Ovaskainen et al. 2013, Maynard et al. 2019) and mycorrhizal fungi (Lilleskov and Bruns 2003, IJdo et al. 2010, López-García et al. 2014), in which fungi differentially invest in biomass, stress-tolerance, or reproduction. These biological differences could allow some fungal taxa to persist long term, while others appear and disappear rapidly. Environmental conditions also likely play a role, with certain fungi being better suited to different environments or frequent disturbances limiting fungal longevity.

Most studies of fungal temporal stability and turnover have focused on seasonal trends. Fungal community composition (Taylor et al. 2010, Montero Sommerfeld et al. 2013, Bainard et al. 2014), biomass (Voříšková et al. 2014), and enzymatic activity (Voříšková et al. 2014) can all vary intra-annually among seasons. While most seasonal comparisons have focused on temperate or boreal regions with pronounced seasonality, differences in fungal biomass and community composition can also be seen in tropical areas between wetter and drier parts of the year (Kivlin and Hawkes 2016, Reyes et al. 2019). When these studies are conducted over multiple years, they typically find that intra-annual seasonal changes in community composition exceed changes seen among years in the same season (Taylor et al. 2010, Averill et al. 2019).
One way to better understand temporal turnover is to compare it to the turnover in space seen at a single time point. While this is seldom done, it allows insights into how spatial and temporal dynamics separately and together influence fungal communities. When looking at seasonal changes in fungal communities at the continental scale, Averill et al. (2019) found that the difference between seasons at one site could be equivalent to the differences between sites thousands of kilometers apart, suggesting strong seasonal effects on fungi and high community turnover between seasons. At a smaller spatial scale, Izzo et al. (2005) found inter-annual temporal changes in temperate forest ectomycorrhizal fungi were small compared to differences in the community seen across space at the same site. Plots were more similar to each other between years than to nearby plots within the same year. Dominant fungi tended to be more constant among years, suggesting that temporal stability could be driven by only a subset of the fungal community, while other fungi appear and disappear erratically.

In this study we investigated inter-annual turnover of hyperdiverse fungal communities in an epiphytic tree canopy environment. In previous work in this system, we found high spatial turnover at very small, sub-meter distances and among three closely intertwined substrates (Chapter 1). We resampled at the same locations for two additional years, with three years of collections in total, to quantify temporal changes in fungal communities in three different substrates, and we compared this temporal turnover to spatial turnover across small spatial distances within a single year. Additionally, to determine which fungi tend to persist over time, we examined how abundances of individual fungi are related to their persistence to the next year.
Materials & Methods

Sample Collection

We performed this study in Parque Nacional Tapantí in Cartago Province, Costa Rica. The sampling site was located along the east bank of the Rio Orosi (9.742°N, 83.784°W, 1300 m elevation) and consisted of five branches on three Saurauia montana (Actinidaceae) trees. In July 2015 we sampled 135 points along these branches with a 9mm diameter borer for a spatial study (design described in Chapter 1). We returned the subsequent years in July 2016 and 2017 and resampled immediately adjacent to a subset of these points. All resample points were spaced at least 15 cm apart to minimize autocorrelation among points due to spatial proximity. After collection, each core was dissected into three substrate types: live bryophytes (LB), dead bryophytes (DB), and host tree bark (OB). We rinsed substrates in sterile water and removed any vascular plant materials. In 2015 samples were preserved in RNALater (Ambion, ThermoFisher) and later lyophilized upon return to the US, while in 2016 and 2017 they were frozen and lyophilized within a week of collection.

Molecular Biology

We extracted total DNA from each substrate sample using DNeasy 96 Plant kits (Qiagen, Germantown, MD, USA). We used the primers 5.8S_Fun (5’ - AACTTTYRRCAAYGGATCWCT - 3’) and ITS4_Fun (5’ – AGCCTCCGCTTATTGATATGCTTAART - 3’) (Taylor et al. 2016) with attached Nextera adapters (5’ – GTCTGCTGGGCTCGGAGATGTGTATAAGAGACAGAA - 3’ and 5’ - TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG - 3’ respectively). We performed PCR amplification in 25 µl with 5 µl GoTaq reaction buffer, 200 µM of each dNTP, 1 mM
MgCl₂, 0.5 μM of each primer, and 1.25 units of GoTaq polymerase (Promega, Madison, WI, USA). The PCR conditions consisted of an initial denaturation at 96° for two minutes, then 27-32 cycles of denaturation at 94° for 30 seconds, annealing at 55° for 40 seconds, elongation at 72° for two minutes, and a final elongation at 72° for ten minutes. We cleaned 2015 PCR products with ZR-96 DNA Clean & Concentrator kits (Zymo Research, Irvine, CA, USA) and 2016-2017 products with 0.25 μl Exocuclease I (New England Biolabs, Ipswich, MA, USA) per 5 μl of input, incubated at 37° for 15 minutes and then 80° for 15 minutes. We then performed a second PCR to attach unique 6bp barcodes and Illumina adaptors. This PCR was 7 cycles and followed the same protocol as above except the primers (5’ -CAAGCAGAAGACGGCATACGAGAT-NNNNNN-GTCTCGTGGGCTCGG - 3’ and 5’ -AATGATACGGCGACCACCGAGATCTACAC-NNNNNN-TCGTCGGCAGCGTC - 3’, Ns represent variable barcode region) were at 0.4 μM each. We pooled equimolar aliquots of the barcoded samples and purified them with Agencourt AmPure XP beads (Beckman Coulter, Indianapolis, IN, USA). We included a mock community (described in Taylor et al. 2016) in each library and sequenced with an Illumina MiSeq with v3 2x300bp kits.

**Bioinformatics**

We merged paired end reads with USEARCH v9.2.64 (Edgar 2013), excluding merged sequences less than 150 bp and sequences that did not merge. Primer sequences were removed with cutadapt (Martin 2011). We then quality filtered, dereplicated, clustered OTUs at 97% similarity, and removed chimeric OTUs with USEARCH. Only OTUs with at least two sequences were retained. We made taxonomic assignments using the RDP.
Classifier (Wang et al. 2007) and the UNITE database (version 7.2, Nilsson et al. 2019)), keeping only assignments at 80% or above. All OTUs not assigned to the kingdom Fungi were removed from the dataset as likely non-fungal taxa, and samples with fewer than 1000 remaining reads were also excluded.

Statistical Analyses

Effect of year on community

To assess effect of sampling year and substrate type on fungal community we used two-dimensional nonmetric multidimensional scaling (NMDS) with relative abundance data and the Bray-Curtis dissimilarity metric. We also performed NMDS on each substrate separately to examine inter-annual variation within a given substrate type. NMDS was done in the phyloseq package (McMurdie and Holmes 2013) with 100 iterations. All statistical analyses were done in R v3.5.1 (R Core Team 2018).

To test the effects of sampling year, spatial location, and substrate type on fungal community composition, we used redundancy analysis (RDA). These analyses were done with both presence-absence and relative abundance transformed fungal data. We included spatial relationships among samples by creating spatial vectors using principal coordinates of neighbor matrices (PCNM) as implemented in the vegan package (Oksanen et al. 2019). Different substrates collected at the same point and time were set at 0.1 cm apart, and samples collected at the same point but in different years were listed as 1 cm apart. The spatial vectors were forward selected with the Blanchet et al. (2008) stopping criterion. We partitioned the variance in fungal community composition among year, substrate, and spatial vectors, and tested significance using permutation tests with 999 permutations.
Alpha diversity

We calculated the expected OTU richness of each sample at 1000 reads of sequencing depth with the rarefy function in vegan (Oksanen et al. 2019). We analyzed log-transformed richness data using a linear mixed-effects model in the lme4 package (Bates et al. 2015). The model included substrate type, sampling year, and their interaction as fixed effects and substrate sample nested withing sampling location as a random effect (this is confusing, need to find a better way to describe the random effect). We tested the significance of each fixed effect with Wald chi-square tests and did pairwise comparisons between years and substrates using the emmeans package (Lenth 2019).

Temporal turnover

We assessed overall interannual change within each sample by calculating the Bray-Curtis and Jaccard dissimilarities between years for each substrate sample. Jaccard dissimilarity was partitioned into nestedness and turnover components according to Baselga (2010) in the betapart package (Baselga et al. 2018). We used a linear mixed-effects model as described above for alpha diversity to test the effects of year, substrate, and their interaction on inter-annual community dissimilarity.

For each year by substrate combination, we calculated the average Bray-Curtis and Jaccard dissimilarity among pairs of samples. We found the spatial distance at which there was a comparable dissimilarity for that substrate as seen in 2015. We used 2015 as our baseline for spatial structure because many more samples were collected over a wider range of distances in 2015 than in subsequent years. Spatial distances and dissimilarities were derived from the linear models fitted to the first ten centimeters of distance decay curved described in Chapter 1.
OTU reoccurrence by relative abundance

To assess the effect of initial OTU relative abundance on likelihood of that OTU being found again in a subsequent year in the same location, we divided relative abundance values into five levels: >0% to 0.01%, >0.01% to 0.1%, >0.1% to 1%, >1% to 10%, and >10%. For each relative abundance category, we calculated the percentage of OTUs that were present the following year in the same sampling location and substrate. This process was repeated for each substrate across all three pairs of years, 2015 to 2016, 2016 to 2017, and 2015 to 2017.

Results

Effect of sampling year

NMDS ordination (Figure 1) showed that the same patterns in fungal community composition initially seen in 2015 persists over two subsequent years. When all years and substrates are plotted together, samples cluster by substrate type rather than by sampling year. When the data are separated by substrate and each substrate is analyzed separately, samples do not cluster by year (Figure 2), suggesting there is no strong effect of sampling year on community composition.

In the RDA and variance partitioning analyses, substrate type, sampling year, and PCNM spatial vectors all had significant effects (p<0.001) on community composition for both presence-absence and relative abundance data. All predictors combined explained 8.2% and 10% of the variation in community composition for presence-absence and relative abundance, respectively. In both cases, year alone explained the least amount of variation, at less than 1% (Figure 3, S1).
Figure 1. NMDS ordination of fungal community composition using relative abundance data by sampling year and substrate. Samples cluster by substrate type but not by year. Stress=0.298
Figure 2. NMDS ordinations of fungal community composition using relative abundance data by sampling year for bark (A, stress=0.306), dead bryophytes (B, stress=0.302), and live bryophytes (C, stress=0.292) with 95% confidence ellipses. None of the three substrates show patterns in fungal community by year.
Figure 3. Venn diagram of variance partitioning analysis using relative abundance data showing effects of substrate type, sampling year, and PCNM spatial vectors on fungal community composition. Residuals=0.918

Alpha diversity

We observed 11,410 OTUs across all substrates and sampling years. Sampling year ($\chi^2=97.0$, $p<0.001$), substrate type ($\chi^2=13.2$, $p=0.001$), and their interaction ($\chi^2=35.0$, $p<0.001$) all had significant effects on OTU richness. Richness increased from 2015 to 2016 ($p<0.001$) but remained steady from 2016 to 2017 (Figure S2). Live bryophytes were more OTU rich than either other substrate in 2015 ($p<0.02$), though their richness was not different from that of dead bryophytes in subsequent years. OTU richness in outer host tree bark changed the least between years. Although overall and per sample OTU richness were high, most OTUs were rare. The majority of OTUs were observed three or fewer times over the course of the study.
Temporal turnover and comparisons with space

Substrate, but not years, had a significant effect on inter-annual Bray Curtis dissimilarity ($\chi^2=31.5, p<0.001$) (Figure 4). For Jaccard dissimilarity, substrate ($\chi^2=48.2, p<0.001$), years ($\chi^2=21.5, p<0.001$), and their interaction ($\chi^2=13.4, p=0.001$) all had significant effects. Live bryophytes had the highest inter-annual Bray Curtis dissimilarity (p=0.001, Figure 4) but was tied with dead bryophytes in Jaccard dissimilarity (Figure 5). Jaccard dissimilarity was composed mostly of OTU turnover, with relatively little nestedness (Figure 5).

![Figure 4](image_url)  
Figure 4. Boxplot of paired Bray-Curtis dissimilarities by substrate and year pair. Each data point is the dissimilarity between samples at the same location in different years. Live bryophyte samples were the most dissimilar over time.

Although substrates varied in amounts of average temporal Bray Curtis dissimilarity, when compared to spatial turnover, these dissimilarities were comparable to similar distances. For example, although live bryophytes had the highest temporal turnover, they
also had the highest levels of spatial turnover. Changes in fungal community composition over the course of one year as quantified by the Bray Curtis index were comparable to changes seen over 5.7 to 8.3 centimeters within a single year (Table S1, Figure S3). Turnover using presence-absence data and the Jaccard index was more variable when compared to space. In one case, with live bryophytes from 2016 to 2017, interannual dissimilarity was lower than the y-intercept value for the spatial distance decay curve. Spatial distances comparable to the temporal dissimilarities varied from -2 to 79 centimeters (Table S1).

![Boxplot of paired partitioned Jaccard dissimilarities by substrate and year pair. Each data point is the dissimilarity between samples at the same location in different years. Nestedness was low, while turnover and total Jaccard dissimilarity were high.](image)

*Relative abundance and persistence*

OTUs at a higher relative abundance persisted to the next year more often than those at lower relative abundances (Figure 6, Table S2). OTUs at most relative abundance levels...
were most likely to persist in dead bryophytes and least likely to persist in live bryophytes. OTU persistence rates were higher for 2015-2016 than in the subsequent pair of years. For most substrate/year pairs, an OTU needed to make up at least one percent of the reads in a sample in order to be present again the next year at least half of the time. OTUs seldom reached this level of relative abundance. Individual OTUs made up one percent or more of a sample only 5.5% of the time and reached ten percent of a sample 0.69% of the time.

![Figure 6](image)

Figure 6. OTU reoccurrence rates at different initial relative abundances by substrate and year pair. OTUs with a higher relative abundance in the first year were more likely to be present in the second year. OTUs in live bryophytes tended to reoccur less of than in other substrates.

**Discussion**

While individual substrate samples varied among years, the effect of sampling year on the overall fungal community was low, smaller than the effects of space or substrate type.
Patterns of fungal specificity among substrates observed in a single year persisted over the duration of the study (Figure 1). Year explained less than one percent of community variation (Figure 3), so while the effect was statistically significant, its biological significance is dubious. This finding suggests that, at a broad, gamma-level scale, the tropical epiphytic fungal community has high stability. The same set of taxa are present from year to year at similar levels of abundance and within the same substrates, though their exact locations within a site may shift. This is also consistent with other findings that inter-annual changes tend to be small compared to intra-annual seasonal ones (Taylor et al. 2010, Averill et al. 2019).

In contrast to community composition, alpha diversity did change among years. There was an increase in per sample OTU richness from 2015 to 2016, but it remained stable from 2016 to 2017 (Figure S2). The reason for this increase is unknown. It could have been related to disturbance caused by our sampling activities the first year or an environmental change. Longer term monitoring is needed to better understand the dynamics and drivers of fungal biodiversity in the epiphytic environment.

If fungal communities varied randomly from year to year, we would expect temporal dissimilarities to match those seen at larger spatial distances, in which there is no spatial autocorrelation and distance decay curves have reached an asymptote (generally >1 meter, Chapter 1). All of the observed distances are well within the range for positive spatial autocorrelation, indicating that communities within individual sampling locations were more similar to each other over time than expected due to chance, and that there is continuity of fungi over the course of multiple years. When Jaccard dissimilarities were partitioned into turnover and nestedness components, turnover predominated. This indicates there was not
just a simple gain or loss of OTUs and fungal diversity with time, but that both were happening simultaneously, resulting in turnover of community members.

Inter-annual turnover within sample locations, as measured by Bray-Curtis and Jaccard dissimilarity varied among substrates (Figure 4, 5). When compared to spatial turnover in the same substrate, however, the spatial distances needed to see the same amount of Bray-Curtis dissimilarity were remarkably similar among substrates, ranging from 5.7 to 8.3 centimeters (Table S1). For example, while live bryophytes had the greatest OTU turnover with time, they also had the highest levels of change across space. Live bryophytes may have the highest inter-annual dissimilarity and OTU turnover because they are actively growing, creating new substrate for fungi to colonize, unlike the underlying dead bryophytes or tree bark, which likely grows slowly. They may also be producing anti-fungal compounds (Banerjee and Sen 1979, Sabovljević et al. 2011) which could limit the growth rate or longevity of fungi. Live bryophytes, when present, are also the top-most substrate layer, leaving them most exposed to incoming fungal spores and disturbance, both of which could increase fungal community turnover. Dead bryophytes and host bark are less exposed and, we expect, more physically and chemically stable, allowing for greater fungal community stability.

Individual fungal OTUs were more likely to persist to the next year if they were at high relative abundance in the first year (Figure 6, Table S2). The least abundant OTUs making up 0.01% or less of a sample persisted to the next year less than 35 percent of the time, depending on the substrate they were present in and the year. In contrast, the most abundant OTUs, making up at least ten percent of a sample, were seen again greater than sixty percent of the time. This suggests that fungi able to establish themselves and
accumulate biomass can maintain their presence for at least a year. Greater persistence in higher abundance fungi may also mean greater stability in ecological functions. Fungi that are more abundant and have greater biomass presumably have more functional significance than low abundance ones represented by only a few reads and that might only be inactive spores. The larger ecological impacts of these more abundant fungi, possibly including decomposition and nutrient cycling, inhibiting plant growth via disease, or promoting plant growth through mycorrhizal symbiosis, can persist and remain stable over multiple years. This temporal stability of high abundance fungi coupled with the high spatial turnover seen in this system (Chapter 1) could create small but persistent patches with greater or lesser suitability for plant germination and growth.

These high relative abundance fungi likely drive the patterns of temporal stability in this system. The spatial distance needed to see the amounts of Jaccard dissimilarity observed among years was much higher than for Bray-Curtis, with one exception with live bryophytes from 2016 to 2017. The Jaccard metric only considers presence-absence and is blind to relative abundance. All fungi, regardless of relative abundance are given the same weight, and the many low abundance, but non-persistent, taxa create high dissimilarity values. With Bray-Curtis, the higher relative abundance taxa, which tend to be more persistent, drive down the dissimilarity values. Without the high abundance OTUs, we would expect much higher dissimilarities and less temporal stability.

The high relative abundance of certain fungi could be due to their early arrival at that location compared to other fungi. Early colonizing microbes, however, do not always persist long term, as has been shown in decaying logs (Chapela and Boddy 1988) and biofilms (Brislawn et al. 2019). The host tree branches used in this study were also several years old,
as we avoided sampling branch tips or twigs, making it unlikely that we were observing early stages of succession. Relative abundances could also be due to environmental factors, with particular fungi being better suited to different environments, as seen with fungi assorting among different substrate types (Figure 1). High and low relative abundances may also be a function of the traits and life histories of different fungi, with some being small, short-lived ruderal species while others are larger with greater longevity. The number of high abundance fungi was low, with the overwhelming majority of OTU observations being less than one percent of a sample. Most fungi were also rare, with the majority of OTUs being observed in three or fewer samples over the course of the study. This, combined with high spatial turnover (Chapter 1), implies that most fungi in the epiphytic environment are physically small and short lived and likely rely on spores for dispersal within and between branches, rather than mycelia.
Supplemental Tables and Figures

Figure S1. Venn diagram of variance partitioning analysis using presence-absence data showing effects of substrate type, sampling year, and PCNM spatial vectors on fungal community composition. Residuals=0.900.

Figure S2. Boxplots of alpha diversity by substrate and year. Alpha diversity was calculated as the expected OTU richness at 1000 reads of sequencing depth. Average diversity increased from 2015 to 2016.
Table S1. Comparison of temporal vs spatial community turnover. Numbers indicate the average temporal dissimilarity by year and substrate type, with numbers in parentheses indicating the spatial distance at which that level of dissimilarity is seen.

<table>
<thead>
<tr>
<th></th>
<th>Live Bryophytes</th>
<th>Dead Bryophytes</th>
<th>Bark</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bray Curtis dissimilarity</strong> – relative abundance</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2015-2016</td>
<td>0.854 (6.7 cm)</td>
<td>0.707 (6.8 cm)</td>
<td>0.663 (6.3 cm)</td>
</tr>
<tr>
<td>2016-2017</td>
<td>0.835 (5.7 cm)</td>
<td>0.744 (8.3 cm)</td>
<td>0.690 (7.1 cm)</td>
</tr>
<tr>
<td>2015-2017</td>
<td>0.902 (9.1 cm)</td>
<td>0.775 (9.5 cm)</td>
<td>0.721 (8.0 cm)</td>
</tr>
<tr>
<td><strong>Jaccard dissimilarity</strong> – presence-absence</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2015-2016</td>
<td>0.984 (56 cm)</td>
<td>0.888 (79 cm)</td>
<td>0.815 (30 cm)</td>
</tr>
<tr>
<td>2016-2017</td>
<td>0.851 (-2 cm)</td>
<td>0.830 (41 cm)</td>
<td>0.817 (31 cm)</td>
</tr>
<tr>
<td>2015-2017</td>
<td>0.909 (77 cm)</td>
<td>0.901 (94 cm)</td>
<td>0.841 (45 cm)</td>
</tr>
</tbody>
</table>
Figure S3. Spatial distance decay by substrate type with Bray-Curtis (A) and Jaccard (B) dissimilarity indices as observed in 2015. Live bryophytes had greater dissimilarities at all distances.
Table S2. Percent of the time an OTU is found the subsequent year based on relative abundance in the initial year.

<table>
<thead>
<tr>
<th></th>
<th>≤ 0.01%</th>
<th>&gt;0.01 – 0.1%</th>
<th>&gt;0.1 – 1%</th>
<th>&gt;1 – 10%</th>
<th>&gt; 10%</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2015 - 2016</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bark</td>
<td>21.79%</td>
<td>32.18%</td>
<td>42.45%</td>
<td>77.30%</td>
<td>90.32%</td>
<td>38.13%</td>
</tr>
<tr>
<td>Dead bryophytes</td>
<td>34.23%</td>
<td>46.02%</td>
<td>58.13%</td>
<td>78.44%</td>
<td>88.89%</td>
<td>49.81%</td>
</tr>
<tr>
<td>Live bryophytes</td>
<td>22.76%</td>
<td>29.90%</td>
<td>54.64%</td>
<td>54.80%</td>
<td>68.09%</td>
<td>31.61%</td>
</tr>
<tr>
<td><strong>2016 - 2017</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bark</td>
<td>12.24%</td>
<td>22.87%</td>
<td>48.12%</td>
<td>73.16%</td>
<td>78.08%</td>
<td>26.33%</td>
</tr>
<tr>
<td>Dead bryophytes</td>
<td>17.20%</td>
<td>31.53%</td>
<td>54.61%</td>
<td>70.05%</td>
<td>80.70%</td>
<td>27.22%</td>
</tr>
<tr>
<td>Live bryophytes</td>
<td>16.10%</td>
<td>27.28%</td>
<td>40.19%</td>
<td>43.26%</td>
<td>63.00%</td>
<td>26.51%</td>
</tr>
<tr>
<td><strong>2015-2017</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bark</td>
<td>16.05%</td>
<td>23.51%</td>
<td>43.79%</td>
<td>67.79%</td>
<td>77.06%</td>
<td>28.73%</td>
</tr>
<tr>
<td>Dead bryophytes</td>
<td>24.42%</td>
<td>33.13%</td>
<td>40.77%</td>
<td>58.88%</td>
<td>77.65%</td>
<td>36.38%</td>
</tr>
<tr>
<td>Live bryophytes</td>
<td>13.92%</td>
<td>16.07%</td>
<td>32.00%</td>
<td>38.92%</td>
<td>55.88%</td>
<td>21.63%</td>
</tr>
</tbody>
</table>
References


Oono, R., A. Rasmussen, and E. Lefèvre. 2017. Distance decay relationships in foliar fungal endophytes are driven by rare taxa: Distance decay in fungal endophytes. Environmental Microbiology 19:2794–2805.


Chapter 3: High diversity but low host specificity of fungi associated with tropical epiphytic cryptogams

Abstract

Epiphytic plants, which grow on other plants for support, make up a large portion of Earth’s plant diversity. Like other plants, their surfaces and interiors are colonized by diverse assemblages of fungi, which can benefit their hosts by increasing tolerance for abiotic stressors and resistance to disease. Fungal communities associated with epiphytic plants and the processes that structure these communities remain understudied. To address this, we characterized the fungi associated with seven epiphytic cryptogamic plant taxa in a Costa Rican rainforest and examined the effects of host identity and microhabitat on external and endophytic fungal communities. We found low host specificity for both external and endophytic fungi and weak differentiation between epiphytic and neighboring epilithic plant hosts. High turnover in fungi within and between hosts and habitats suggests that epiphytic plant-associated fungal communities are highly diverse and are structured by stochastic processes.
**Introduction**

Epiphytic plants make up about 10% of the land plant flora (Madison 1977) and are especially diverse and abundant in tropical forests (Benzing 1990, Nieder et al. 2001). Common epiphytes include liverworts, mosses, ferns, bromeliads, and orchids. Epiphytes face additional challenges compared to their counterparts that grow on the ground, including lack of access to water and nutrients stored in the soil, and they are highly vulnerable to climate change and land use change (Benzing 1998, Nadkarni and Solano 2002, Zotz and Bader 2009). Interactions with symbiotic fungi likely help ameliorate these stresses. Despite the prevalence and diversity of epiphytic plants, the fungi associated with them and the factors influencing community structure of these fungi are largely unknown.

Plants host a broad range of fungi within their tissues and on their surfaces, and these fungi have a diverse array of ecological functions. The most widely known known are mycorrhizal fungi, which form mutualisms with plant roots and exchange nutrients scavenged from the substrate for photosynthate from the plant. Other fungi are plant pathogens that cause disease in the host plant. Some endophytic fungi live entirely inside the host plant without causing disease symptoms. Their function is often mysterious, but some endophytes have been shown to increase host plant tolerance to abiotic stress (Waller et al. 2005, Wu et al. 2006) and defense against herbivores (Clay et al. 1985, Bamisile et al. 2018) and pathogenic microbes (Arnold et al. 2003, Liu et al. 2007, Wiewióra et al. 2015). Fungi associated with plants, be they mutualistic, pathogenic, or commensal, have been observed in all major lineages of land plants, including the seed plants, ferns, lycopods, and bryophytes. Surveys of fungi in epiphytes have largely been focused on mycorrhizal fungi in vascular

Fungi associated with plants can vary in several ways, including among host plant species. Host specificity is seen among fungi associated with terrestrial boreal bryophytes (Kauserud et al. 2008), tree bark endophytes (Pellitier et al. 2019), and among fungi associated with roots (Shefferson et al. 2007, Toju et al. 2013) and leaves (Gange et al. 2007, Moricca et al. 2012, Karimi et al. 2012, Kembel and Mueller 2014) of vascular plants. Within individual hosts, fungi vary among tissues, such as between roots and shoots of vascular plants (Wearn et al. 2012) and between living and dead or senescent bryophyte tissue (Davey et al. 2013b, Chapter 1). Fungal variation by host can be due to differences in chemical composition among host tissues (Pellitier et al. 2019). Fungi can also differ within a single host species depending on the habitat of the host plant. For example, plant-associated fungal communities can vary between rural and urban areas (Matsumura and Fukuda 2013), along elevation gradients (Davey et al. 2013a), between forests of different ages (Davey et al. 2014), and along continental-scale geographic gradients (Nelson and Shaw 2019). These geographical differences can be driven by dispersal limitation (Higgins et al. 2014) or variation in environmental conditions, like rainfall and temperature (Zimmerman and Vitousek 2012). It remains to be seen if fungi associated with epiphytic plants follow the same complex distribution patterns as those observed in ground-dwelling plants.

The epiphytic plant community may host a large, unexplored diversity of fungi. Previous work using culturing (Arnold et al. 2000, Arnold and Lutzoni 2007) and sequence based approaches (Donald et al. 2020) have found high diversity of Ascomycota in tropical tree leaf endophytes. Fungal taxa were frequently rare, with the majority being found in only
one or a few leaf samples. Research on fungi in epiphytic substrates has documented high fungal species richness and spatial turnover over short, sub-meter distances (Chapter 1), which might be explained in part by high host specificity and community variation among epiphytic plants.

In this study, we sequenced fungi associated with seven co-occurring epiphytic plants, including five bryophytes and two groups of ferns, to test whether fungal communities differ among host plants. To determine if differences in fungi were greater for endophytes than for the entire fungal community and if endophytes had greater host specificity, we took subsamples of three bryophyte taxa and surface sterilized them. This allowed us to distinguish endophytic fungi from the external or coincidental fungi found in unsterilized plants. For two of the bryophytes, we collected the same taxa on both branches and nearby rocks to assess the effect of microhabitat on fungal community composition. We expected fungal communities to segregate by host plant, with particularly strong differences between fern and bryophyte hosts, and for these differences to be strongest when considering only endophytes as opposed to epiphytic, coincidental and endophytic fungi. We predicted that endophytic fungi would be a nested subset of those found of the corresponding unsterilized plants. We also expected to find large differences between plants living in the epiphytic environment versus those living on rocks.

**Materials & Methods**

*Sample Collection and Processing*

This study took place in Parque Nacional Tapantí in Cartago Province, Costa Rica along a 500 m transect on the east bank of Rio Orosi (9.742°N, 83.784°W, 1300 m
elevation), in a low montane rainforest. In July 2016 and July 2018, we collected small epiphytic plants, including liverworts (*Trichocolea* and *Plagiochila*), mosses (*Prionodon, Thuidium*, and *Orthostichopsis*), and ferns (*Elaphoglossum peltatum* and *Hymenophyllaceae*), from the branches of *Saurauia montana* trees. *Elaphoglossum* samples were separated into leaf and root/rhizome subsamples. In January 2019 we collected eight additional samples of *Thuidium* and *Plagiochila*, and four *Trichocolea*. Four each of the *Thuidium* and *Plagiochila* were epiliths collected from nearby rocks. Each of the 2019 samples, both epiphytic and epilithic, were split into two subsamples. One subsample was rinsed thoroughly in distilled water. This treatment was aimed to remove most incidental spores and hyphae but retain a significant portion of taxa growing on the plant tissues. The other half of these samples were surface sterilized by immersion for 30 seconds in 96% ethanol, 90 seconds in 10% bleach, 30 seconds in 70% ethanol, and three rinses in deionized water. The goal of this treatment was to remove the bulk of incidental and external fungi, leaving primarily true endophytes. Essentially the same procedure has been used for isolating endophytic fungi in culture (Arnold et al. 2000). After collection and processing, samples were either stored in RNALater (Ambion, ThermoFisher) or lyophilized. For a summary of sampling design and sample sizes, see Table S1.

We extracted total DNA from each sample using DNeasy 96 Plant kits (Qiagen, Germantown, MD, USA). We identified host plants and confirmed our morphotypes by sequencing the *rbcL* region. We used the primers *rbcL-Z1* (5’-ATGTCACCACAAACAGARACTAAAGC-3’, modified from Kress and Erickson 2007) and *a_r* (5’-CTTCTGCTACAAAAATAAGAATCGATCTC-3’, Kress and Erickson 2007). We performed PCR amplifications in 25 μl reactions with 5 μl GoTaq reaction buffer, 0.2 μM of
each primer, 200 μM of each dNTP, 1 mM MgCl₂, and 1.25 units of GoTaq polymerase (Promega, Madison, WI, USA) with the following thermocycler conditions: initial denaturation at 96° for two minutes, thirty five cycles of 94° for thirty seconds, 50° for 45 seconds, and 72° for 90 seconds, followed by a final elongation at 72° for eight minutes. We sequenced rbcL using rbcL-Z1 as the sequencing primer.

To sequence the fungal ITS2 region in each plant sample, we used the primers 5.8S_Fun (5’ - AACTTTYRRAAYGGATCWCT - 3’) and ITS4_Fun (5’ – AGCCTCCGGCTTTATGATATGCTTAART - 3’) (Taylor et al. 2016) with attached adapters (5’ – GTCTGCTGGGCTCGGAGATGTGTATAAGAGACAGAAA - 3’ and 5’ - TCGTCCGGCAGCGTGTATAAGAGACAG - 3’ respectively). We performed PCR as described above for rbcL, with the following differences: 0.5 μM of each primer was used, the PCR annealing step was at 55° for forty seconds, the final elongation step lasted ten minutes, and only thirty cycles. We cleaned the PCR products using 0.25 μl Exonuclease I (New England Biolabs, Ipswich, MA, USA) per 5μl of sample incubated at 37° for 15 minutes and 80° for 15 minutes. We then added sample specific 6bp barcodes and Illumina adaptors with a second, seven-cycle PCR reaction following the same procedure as above, with the exception of the primers (5’ - CAAGCAGAAGACGGCATACGAGAT-NNNNNN-GTCTCGTGGGCTCGG - 3’ and 5’ - AATGATACGGGCAGCACCACCA-NNNNNN-TCGTCCGGCAGCGT - 3’, Ns represent variable barcode region, at 0.4 μM each). A mock community (described in Taylor et al. 2016) was added to each run to validate parameter choices in later bioinformatic steps. The barcoded samples and mock communities were quantified and pooled in equal amounts then purified with Agencourt
AmPure XP beads (Beckman Coulter, Indianapolis, IN, USA) and sequenced on the Illumina MiSeq platform using the 2x300bp kit.

**Bioinformatics**

ITS2 data were processed in USEARCH v9.2.64 (Edgar 2013). We merged paired-end reads and excluded sequences that did not successfully merge or had a merged length of less than 150 bp. We removed primer sequences with cutadapt (Martin 2011) and then quality filtered, dereplicated, and clustered reads at 97% similarity into OTUs (operational taxonomic units). The clustering step also removed chimeric OTUs. We excluded any OTUs represented by only a single sequence. Taxonomic assignments were made using SINTAX (Edgar 2016) with the UNITE v.8.2 (Abarenkov et al. 2020) database. Assignments with less than 80% confidence were dropped. We removed all OTUs assigned to a kingdom other than Fungi and OTUs classified as *Malassezia*, a common contaminant, and we dropped all samples with fewer than 1000 remaining sequences. *Elaphoglossum* leaf and root/rhizome subsamples were determined not to differ by organ (PERMANOVA F=0.135, p=0.135) and were combined into single samples. *rbcL* sequences were quality trimmed using CodonCode Aligner (CodonCode Corp., Dedham, MA, USA) and identified by searching for similar sequences in GenBank (Benson et al. 2017) using the BLAST algorithm (Altschul et al. 1990).

**Statistical analysis**

All analyses were performed in R v3.5.1 (R Core Team 2018) in the vegan (Oksanen et al. 2019) and phyloseq (McMurdie and Holmes 2013) packages. To account for
differences in sequencing depth among samples, OTU counts were transformed in two ways: relative abundance (RA) and presence-absence (PA). Analyses were done for both data transformations.

We visualized differences in fungal community composition among host plants, between surface sterilized and un-sterilized plants, and between habitats using nonmetric multidimensional scaling (NMDS) ordinations. We tested for differences among groups using permutational multivariate analysis of variance (PERMANOVA) as implemented in the adonis2 function (McArdle and Anderson 2001) in the vegan package. To test for differences between pairs of host plants, we used pairwise PERMANOVAs. During pairwise tests, we adjusted p-values to account for multiple testing using the Holm method (Holm 1979).

We visualized the overlap in fungal communities among unsterilized, epiphytic host taxa using Venn diagrams generated with the VennDiagram R package (Chen 2018). We tested if the number of OTUs unique to single host plant taxa (host specific OTUs) and number of OTUs shared across all hosts (generalist OTUs) were significantly different from what would be expected by chance with a permutation test. In this test, we took each sample and randomly reassigned it to a new host taxon, keeping the numbers of each host taxon constant. We then calculated the number OTUs that were specific to one of these randomized hosts and the number of those present in all the hosts. This process was repeated 1000 times, and then the actual observed values were compared to the generated distributions to assess statistical significance. This was done for all seven host taxa and for just the bryophytes.
We tested the effects of surface sterilization on OTU diversity using a paired t-test. We calculated alpha diversity in each sample by estimating the number of OTUs present at a sequencing depth of 1000 reads using the rarefy function in the vegan package. We tested for a difference in sample dispersion (i.e. the degree of dissimilarity among communities in a set of samples) between surface sterilized and unsterilized samples using the PERMDISP2 procedure, as implemented in the betadisper function in vegan. We partitioned beta diversity, in the form of the Jaccard metric, between paired surface sterilized and unsterilized samples into nestedness and turnover components using betapart (Baselga et al. 2018).

Results
Epiphytic fungal communities were highly diverse, with 11,860 OTUs found across all samples (n=71). Most of these OTUs were rare and found in only one or two samples (Figure S1). The majority or plurality of fungal OTUs in every unsterilized host plant taxon belonged to the Ascomycota (Figure 1). The second most abundant group was the Basidiomycota. In surface sterilized samples, the percentage of Ascomycota was even higher, making up over two thirds of all reads (Figure 1). Other phyla each made up less than one percent of all OTUs. 4827 OTUs could not be assigned to a phylum at 80% confidence.
Fungal community composition varied significantly by host plant (RA: $F=1.823$, $p=0.001$, $R^2=0.159$, PA: $F=1.404$, $p=0.001$, $R^2=0.127$) when only epiphytic, unsterilized samples were considered. Community composition also differed by whether the host plant was a fern or bryophyte (RA: $F=2.635$, $p=0.001$, $R^2=0.04$, PA: $F=1.908$, $p=0.002$, $R^2=0.0294$, Figure 2, S2). When testing differences between pairs of host plant taxa, all differences were significant when using RA data, with the exception of Orthostichopsis-Prionodon (Table 1). In contrast, only four of 21 pairs were significantly different when using PA data (Table S2). Ordinations suggested limited differences in fungal communities among host taxa, with high overlap between clusters (Figure 3, S3).
Each host taxon contained some unique OTUs (Figures S4). 902 OTUs were shared across all hosts, and 1154 were shared among all bryophyte hosts. Neither of these numbers are significantly different from what would be expected by chance (p=0.384 and p=0.25, respectively). The number of OTUs unique to a single host taxon was not significantly different from chance when using either the entire epiphytic, unsterilized data set or just the bryophytes.

Figure 2. NMDS ordination comparing unsterilized RA fungal community composition of bryophytes and ferns with 95% confidence ellipses. The host plant groups form two overlapping clusters. Stress=0.272
Table 1. Pairwise PERMANOVA results by host plant using RA data. Bolded values indicate statistically significant results.

<table>
<thead>
<tr>
<th>Plant Family</th>
<th>Hymenophyllaceae</th>
<th>Elaphoglossum</th>
<th>Thuidium</th>
<th>Plagiochila</th>
<th>Trichocolea</th>
<th>Orthostichopsis</th>
<th>Prionodon</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>1.74</td>
<td>1.87</td>
<td>2.35</td>
<td>2.53</td>
<td>2.58</td>
<td>2.16</td>
<td>2.06</td>
</tr>
<tr>
<td>p</td>
<td>0.02</td>
<td>0.02</td>
<td>0.021</td>
<td>0.021</td>
<td>0.021</td>
<td>0.028</td>
<td>0.027</td>
</tr>
<tr>
<td>R²</td>
<td>0.088</td>
<td>0.135</td>
<td>0.117</td>
<td>0.117</td>
<td>0.109</td>
<td>0.177</td>
<td>0.158</td>
</tr>
</tbody>
</table>

Note: RA = Randomized ANOVA
Figure 3. NMDS ordinations of unsterilized RA fungal community composition by bryophyte (A, stress=0.266) and fern (B, stress=0.157) host with 95% confidence ellipses. Bryophytes do not show strong clustering by host, while ferns form overlapping clusters.

Fungal communities differed significantly between epiphytic and epilithic environments for unsterilized Plagiochila and Thuidium (RA: F=1.69, p=0.003, R²=0.051, PA: F=1.65, p=0.004, R²=0.051). When analyzing RA, the effect of host (F=2.445, p=0.001, R²=0.073) was stronger than the effect of habitat, and there was no significant interaction
with host identity (Figure 4). Effects of habitat were not significant for surface sterilized samples (Figure S5).

As expected, OTU richness was significantly lower in surface sterilized subsamples than in their unsterilized counterparts ($t=26.915$, df=18, $p<0.001$, Figure 5). There was significantly more dispersion in unsterilized samples using both RA ($F=15.928$, $p<0.001$) and PA ($F=73.072$, $p<0.001$) data, and this can also be seen in the ordinations of these samples (Figure 6, S6). Fungal communities differed significantly by host type in surface sterilized samples (RA: $F=1.30$, $p=0.002$, $R^2=0.140$, PA: $F=1.39$, $p=0.001$, $R^2=0.148$). Jaccard dissimilarity between paired sterile and unsterile samples was high, averaging 0.935, meaning there were few shared OTUs between sample pairs. When Jaccard dissimilarities were partitioned into turnover and nestedness components, turnover predominated (Figure 7).

![Figure 4. NMDS ordinations of unsterilized RA fungal community composition by host and habitat. Samples cluster by host more strongly than by habitat. Stress=0.2044](image-url)
Figure 5. OTU richness, calculated as the expected number of OTUs at a sequencing depth of 1000 reads, in surface-sterilized and unsterilized bryophyte samples. Sterilized samples had greatly reduced diversity compared to their unsterilized counterparts.

Figure 6. NMDS ordination of RA fungal community composition by host and sterilization treatment and 95% confidence ellipses base of sterilization treatment. Samples form distinct clusters based on sterilization. Stress=0.195
Figure 7. Boxplot of partitioned beta diversity among paired unsterilized-surface sterilized bryophyte samples. Overall Jaccard dissimilarities between sample pairs were high. Turnover tended to be higher than the nestedness component, indicating lack of shared OTUs.

Discussion

Fungal OTU richness was very high in epiphytic plant hosts, with 11,860 found across all sampled plants. While metabarcoding studies are notorious for oversplitting microbial taxa, we argue that our results are likely to be reasonably accurate due to our use of a mock community and careful parameter tuning in USEARCH. Diversity was also high within individual plant samples, with un-sterilized samples typically containing upward of 200 OTUs (ALPHA BOXPLOT), though most of these OTUs were rare (Figure S1). Ascomycota OTUs made up the majority of reads in most samples and made up an even higher proportion of the fungal community in surface sterilized samples. The dominance of Ascomycota is consistent with past studies on endophytes (Arnold 2007, Rodriguez et al. 2009, Del Olmo-Ruiz and Arnold 2017) and leaf associated fungi (Jumpponen and Jones 2009, Davey et al. 2012, Kembel and Mueller 2014). Glomeromycota were present in some samples, though typically at levels below one percent RA. They have previously been
observed in epiphytic plants (Janos 1993, Rabatin et al. 1993) and may be forming arbuscular mycorrhizal associations with the ferns in this study, but cytological studies would be required to confirm this. About forty percent of OTUs could not be identified at the phylum level, suggesting that fungal diversity in this system is poorly catalogued.

While fungal community composition did vary significantly among host plant taxa, differences were small, with high overlap among plants (Figure 3). Most pairwise comparisons between hosts using PA data were not significant, indicating a lack of differences in OTUs present among hosts. While each host did have some unique fungal OTUs (Figure S4), the number of these unique fungi is no greater than expected by chance given the rarity of most OTUs. Differences when using RA data, on the other hand, were more often significant, though explanatory power of host was still small. This is especially true among bryophytes, where $R^2$ values were 0.09 or less. Greater differences in RA versus PA suggests that hosts share many fungal taxa, but these fungi differ in abundance based on host. This might reflect a fairly uniform influx of propagules, in which more common taxa are found in several hosts, but differences in biomass, and hence RA, of actively growing OTUs are related to host-fungus interactions. Differences in fungal communities were larger between ferns and bryophytes, possibly due to differences in biochemistry between these two distantly related groups of plants.

Other researchers have documented high host specificity of endophytic or leaf-associated fungi; for example, Kembel and Mueller (2014) were able to explain over half of the variation in tropical tree leaf fungal community with host plant taxonomy. High host specificity, however, is not universal. Bayman et al. (1997), for example, found higher variation in fungal endophytes within single plants than across host orchid species.
Similarly, Del Olmo-Ruiz and Arnold (2017) and Donald et al. (2020) found that tropical plants differed in endophytic fungal communities, but host specificity was low. It is possible that many fungi in this environment are generalists without strong host preferences. This pattern has been found with culturable angiosperm leaf endophytes, where host breadth was lower in the tropics than in temperate or boreal regions (Arnold and Lutzoni 2007). This may be due to the high plant diversity found in the tropics, in which density of any particular host plant tends to be low, making it difficult for specialized fungi to find their preferred hosts. This may be especially applicable in epiphytes because they are spatially separated on different tree branches. The complex three-dimensional architecture of the epiphytic habitat likely limits hyphal spread among host plants. Specificity can also be low in polar regions, however, as has been seen in root endophytes (Botnen et al. 2014). Another possible explanation for low specificity is lack of differences in the traits of the host plants, particularly the bryophytes. More research on bryophyte biochemistry would be needed to tie differences in fungi with host chemical traits.

Contrary to our expectations, differences in fungal communities between epiphytic and epilithic bryophytes were small (Figure 4). These communities did not differ when only considering endophytic fungi in the surface sterilized samples. Research on variation in fungal associations between microhabitats at the same site in the tropics are very limited, so we can make few comparisons. Del Olmo-Ruiz and Arnold (2014) examined differences in fungal endophytes in various fern species and did not find a difference between epiphytic and terrestrial ferns, though this study involved different host species in each habitat. Climate, including temperature and precipitation, can have impacts on endophyte community composition (Zimmerman and Vitousek 2012, Giauque and Hawkes 2016, Barge et al. 2019),
though the epiphytes and epiliths in our study were located at the same site and exposed to the same climate. Plants in both habitats were likely exposed to the same species pool of airborne fungal propagules, and abiotic conditions were similar enough between the two habitats to permit some of the same plants to grow in both. Abiotic differences may have been too small for environmental filtering to have a substantial effect on fungal communities.

Fungal diversity was much lower in surface sterilized plants than was observed in unsterilized plants, as expected (Figure 5). Endophytic fungal communities in surface sterilized samples were more different from one another than were un-sterilized ones (Figure 6). This is contrary to our prediction that endophytes would be more consistent both within and between hosts due to increased specialization that is presumably needed to function as an endophyte. On the other hand, this finding is consistent with the idea that the unsterilized community more closely resembles a homogeneous airborne propagule community than does the endophyte community. The surface sterilized plants also did not contain nested subsets of the fungi recovered from their un-sterilized counterparts, but had additional fungi not found in the other (Figure 7). This may be due to the high, microscale spatial turnover in fungal communities and intra-host variation (Chapters 1 and 2). Our sampling method was destructive, and the exact same plant material cannot be used for both sterilized and un-sterilized sampling. Thus, if endophytes in our system have high turnover within individual plants or between nearby plants (see e.g. Bayman et al. 1997, Oono et al. 2017), this could help explain the limited nestedness and high turnover between our sterile-unsterile sample pairs. It might also be that fungi are much more abundant on plant surfaces, swamping out the signal from endophytes. Also, because bryophytes lack a cuticle, we may have inadvertently killed a portion of the endophytes with our sterilization procedure.
Overall, our data suggests low specificity in cryptogamic plant-fungal interactions in a tropical epiphyte system. Host specificity was low in our studied bryophyte and fern taxa, even when only endophytes were considered. Abiotic environment, in the form of an epiphytic versus epilithic habitat, also had little effect on the fungal community. The community was highly diverse, with high turnover between samples that could not be explained by host or environment. In a previous study of this system, fungi detected in living bryophytes were found to have high spatial turnover at very small spatial scales of less than one meter (Chapter 1). The earlier spatial study together with the present study suggest that tropical epiphytic fungal communities are hyper-diverse with extremely stochastic community assembly and a lack of strong biotic or abiotic drivers at the local scale.
Supplemental Tables and Figures

Table S1. Sampling design and sample sizes by year, host, and habitat. Numbers in parentheses indicate number of surface sterilized subsamples.

<table>
<thead>
<tr>
<th>Host plant</th>
<th>Number of samples by year and habitat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hymenophyllaceae</td>
<td>5</td>
</tr>
<tr>
<td>Elaphoglossum</td>
<td>4</td>
</tr>
<tr>
<td>Thuidium</td>
<td>6</td>
</tr>
<tr>
<td>Plagiochila</td>
<td>6</td>
</tr>
<tr>
<td>Trichocolea</td>
<td>5</td>
</tr>
<tr>
<td>Orthostichopsis</td>
<td>4</td>
</tr>
<tr>
<td>Prionodon</td>
<td>5</td>
</tr>
</tbody>
</table>

Figure S1. Histogram of occurrence frequency for fungal OTUs. Data from surface sterilized subsamples was omitted. The majority of OTUs were found in only one or two samples.
Figure S2. NMDS ordination comparing unsterilized PA fungal community composition of bryophytes and ferns with 95% confidence ellipses. The ferns form a cluster nested within the bryophytes. Stress=0.172
Table S2. **Pairwise PERMANOVA results by host plant using PA data.** Bolded boxes indicate statistically significant results.

<table>
<thead>
<tr>
<th>Host Plant</th>
<th>F = 0.83, P = 0.753</th>
<th>F = 1.13, P = 0.721</th>
<th>F = 1.17, P = 0.721</th>
<th>F = 1.19, P = 0.721</th>
<th>F = 1.01, P = 0.721</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hymenophyllaceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elaphoglossum</td>
<td>F = 0.721, P = 0.410</td>
<td>F = 1.32, P = 0.396</td>
<td>F = 1.32, P = 0.396</td>
<td>F = 1.32, P = 0.396</td>
<td></td>
</tr>
<tr>
<td>Thuidium</td>
<td>F = 0.112, P = 0.736</td>
<td>F = 1.32, P = 0.169</td>
<td>F = 1.32, P = 0.169</td>
<td>F = 1.32, P = 0.169</td>
<td></td>
</tr>
<tr>
<td>Plagiochila</td>
<td>F = 0.866, P = 0.080</td>
<td>F = 1.32, P = 0.105</td>
<td>F = 1.32, P = 0.105</td>
<td>F = 1.32, P = 0.105</td>
<td></td>
</tr>
<tr>
<td>Trichocolea</td>
<td>F = 0.64, P = 0.169</td>
<td>F = 1.49, P = 0.015</td>
<td>F = 1.49, P = 0.015</td>
<td>F = 1.49, P = 0.015</td>
<td></td>
</tr>
<tr>
<td>Prionodon</td>
<td>F = 0.83, P = 0.753</td>
<td>F = 1.13, P = 0.721</td>
<td>F = 1.13, P = 0.721</td>
<td>F = 1.13, P = 0.721</td>
<td>F = 1.13, P = 0.721</td>
</tr>
</tbody>
</table>

**Orthostichopsis**
Figure S3. NMDS ordinations of unsterilized PA fungal community composition by bryophyte (A, stress=0.162) and fern (B, stress=0.152) host with 95% confidence ellipses. Bryophytes do not show strong clustering by host, and *Elaphoglossum* are nested within Hymenophyllaceae.
Figure S4. Venn diagrams showing overlap in OTU composition by plant host for ferns (A) and bryophytes (B). Numbers in the circles indicate numbers of OTUs shared by different sets of host taxa. Abundance of OTUs is not considered, only presence.
Figure S5. NMDS ordinations of surface sterilized fungal community composition by host and habitat for RA (A, stress=0.188) and PA (B, stress=0.209) data. Neither ordination shows strong clustering patterns.
Figure S6. NMDS ordination of PA fungal community composition by host and sterilization treatment with 95% confidence ellipses by sterilization treatment. Samples form distinct clusters based on sterilization. Stress=0.121
References


Chen, H. 2018. VennDiagram: Generate High-Resolution Venn and Euler Plots.


Oono, R., A. Rasmussen, and E. Lefèvre. 2017. Distance decay relationships in foliar fungal endophytes are driven by rare taxa: Distance decay in fungal endophytes. Environmental Microbiology 19:2794–2805.


Chapter 4: Spatially structured plant-soil feedbacks impact plant growth and mycorrhizal colonization at local scales

Abstract

Plant-soil feedbacks are widely regarded as important drivers of plant diversity and coexistence. The soil microbial communities that interact with plants are frequently spatially heterogeneous at small scales in natural environments. Impacts of microbial spatial structure on plant-soil feedbacks and plant growth are potentially dramatic but remain to be demonstrated. Here we carried out a greenhouse experiment using field-collected soils to connect naturally occurring microbial heterogeneity to variation in plant growth responses. We found that biomass in a grass species and ectomycorrhizal root colonization in a tree responded to differences in soil microbial communities and that these responses varied at the same spatial scales as the microbial communities. Although microbial effects on biomass tended to be negative and root colonization low, some soils had positive biomass and root colonization effects, and these effects were spatially structured. Microbial spatial heterogeneity may thus create heterogeneous plant-soil feedback effects and play a role in maintaining plant coexistence.
Introduction

The plant-soil feedback (PSF) concept has revolutionized plant ecology by acknowledging and quantifying feedbacks between plants and soil microbes that shape plant population dynamics, community composition, and productivity (Klironomos 2002, Reynolds et al. 2003, Kulmatiski et al. 2008, Mangan et al. 2010, van der Putten et al. 2013, 2016). PSF occurs when plants alter the properties of the soil, typically by affecting the microbial community, which in turn impacts the future performance of the same or other plant species in the same soil. These feedbacks can be quantified using experiments and the mathematical framework introduced by Bever et al. (1997). Theoretical and experimental work on PSF has shown that it plays a key role in maintaining coexistence among plant species, which is an ongoing problem in theoretical ecology (Silvertown 2004, Bever et al. 2010, Crawford et al. 2019). Coexistence can be promoted by negative PSF, in which plants grown in soil conditioned by conspecifics do poorly compared to those grown in soils conditioned by competitors (Bonanomi et al. 2005, Chung and Rudgers 2016, Eppinga et al. 2018, Mack et al. 2019), leading to negative frequency dependence mediated by the belowground community, typically due to the activities of specialized pathogens (Packer and Clay 2000, Petermann et al. 2008, Bever et al. 2015).

PSF models and experiments usually assume that soil communities are homogeneous and well-mixed (Bever 2003), but empirical data have shown this is not the case. Prokaryotes and fungi in soil can have non-uniform spatial distributions at nearly every scale that has been examined, from centimeters to thousands of kilometers (Nunan et al. 2002, 2003, Wallenius et al. 2011, Bahram et al. 2013, 2015, Averill et al. 2019). Plant roots are of limited size and will only come in contact with microbes in a constrained area, meaning
individuals may be exposed to very different groups of soil microbes, even within the same site. To account for variability in soils, researchers will sometimes mix soils from several sites, plots, or conditioning plants to get an “average” soil for a site or treatment. This practice has been subject to debate (Reinhart and Rinella 2016, Gundale et al. 2017, Rinella and Reinhart 2017, 2019, Teste et al. 2019), and these homogenized microbial communities may not be representative of those that plants will encounter in the field.

Consequences of small-scale spatial microbial heterogeneity for plant growth in natural settings are completely unknown but are potentially of great importance. The limited available research on the effects of spatial heterogeneity on PSF have focused on simulation studies (Bever et al. 1997, Mack and Bever 2014, Abbott et al. 2015) or mesocosm studies in which non-uniform microbial distributions were artificially created (Brandt et al. 2013, Burns and Brandt 2014, Wubs and Bezemer 2016, 2018, Burns et al. 2017). PSF effects in nature might not be constant, but instead vary within a range of values in a spatially structured way. Localized patches with higher abundances of specialized pathogens could reduce plant fitness and increase mortality (Packer and Clay 2000), while nearby patches may have taxon-specific mutualists which increase recruitment and growth of some plants, but not others (Abbott et al. 2015), encouraging plant coexistence via environmental heterogeneity. Uniting the PSF framework with the large body of empirical support for microbial spatial structure may provide a major step toward understanding plant population dynamics and coexistence.

Here, we perform a greenhouse experiment using field-collected soils to test the hypotheses that spatial structure in soil microbial communities may strongly impact the effects of soils on plant growth and that the use of homogenized “average” microbial communities may be misleading with respect to the dynamics of natural systems. We report
strong evidence for spatial structure in PSF for two coexisting keystone arid land plants that host distinct mycorrhizal communities. We also show that these microbe-mediated spatial patterns correspond to the spatial structure of microbial communities in these same soils.

**Materials & Methods**

*Study system and soil collection*

In October 2019 we collected soils from a private ranch in northeastern New Mexico (35.47°N, 100.62°W, 1950m elevation). The ranch contains a mosaic of grassland (GL) and piñon-juniper woodland. GL was dominated by blue grama (*Bouteloua gracilis*), a perennial grass that forms arbuscular mycorrhizal associations, intermixed with other grasses, forbs, and cacti. PJ was dominated by piñon pine (*Pinus edulis*), an ectomycorrhizal tree, and one-seed juniper (*Juniperus monosperma*), an arbuscular mycorrhizal tree, with an understory containing sparse grasses (including blue grama) and forbs, cacti, and oaks (*Quercus*). One fifty centimeter by four meter plot was established in each of these two habitats. Thirty-two sampling locations were set up in each plot in a spatially structured design (Figure S1). All sampling locations in both plots were 15 cm or less away from a blue grama plant, and PJ samples were all between one and three meters from an adult piñon. Soil cores of 5 cm diameter and 25 cm depth, a scale we consider appropriate for the size and rooting area of blue grama and piñon pine seedlings, were collected at each sampling point. Each soil core was homogenized and sieved though a 2 mm mesh to remove rocks and large root fragments. Three soil pools were constructed by pooling equal volumes of soils from all 32 GL soils, all 32 PJ soils, and all 74 soils from both plots. Half of each of the pools was removed and sterilized to serve as negative controls.
Greenhouse experiment

Plants were grown in 164 ml conainers (Stuewe & Sons, Tangent, Oregon, USA) with the bottoms blocked with a sterile cotton ball. The growing medium included 100 ml of sterilized sand (sterilized though two four-hour autoclave treatments) covered by a layer of 15 ml of the individual soil sample, pool, or sterile control, which was then covered by a thin layer of sterilized sand to prevent cross contamination between pots. The amount of live soil was low compared to sand to minimize the effects of any differences between soils in structure or chemistry on the plants, as has been done in other studies (Brinkman et al. 2010, Hendriks et al. 2013, Teste et al. 2017, Gundale et al. 2019). Additionally, we fertilized all plants with an AeroGarden liquid plant food solution (AeroGarden, Boulder, CO, USA). Two milliliters of the fertilizer (4-3-6) were mixed with one gallon of water (final concentration: 53 ppm nitrogen, 40 ppm P₂O₅, 79ppm K₂O, 13 ppm calcium, 6.6 ppm magnesium), and each plant received five milliliters of this solution every other week. Plants were watered daily.

Seeds were acquired from Plants of the Southwest (Santa Fe, NM, USA). Blue grama seeds were treated to kill endophytes by suspending them in water and incubating them at 55°C for 15 minutes. They were then surface sterilized by soaking them in 70% ethanol for two minutes, 2% hypochlorite bleach for two minutes, and then rinsing in sterile water four times. Piñon seeds were surface sterilized by soaking in a 1% hypochlorite bleach solution for 90 seconds followed by three rinses in sterile water. Blue grama seeds were sown directly into prepared containers with six seeds per pot, and then thinned to one per pot after germination. Piñon seeds were first germinated in trays of sterilized vermiculite and then
transferred into containers. For each individual soil, pool, and negative control, six replicate pots for each species were used.

After 15 weeks for blue grama and seven months for piñon, we harvested the plants, dried them, and measured dry biomass. For piñon, we also recorded percent colonization of ectomycorrhizal fungi. This was measured by recording the number of root tips colonized out of 50 per seedling, or less if there were fewer root tips. Plants that failed to germinate or died over the course of the experiment were excluded.

*Molecular biology and bioinformatics*

A subset of each individual soil sample and each pool was set aside for sequencing. These subsamples were lyophilized, and we extracted DNA using DNEasy PowerSoil kits (Qiagen, Germantown, MD, USA). For each sample we amplified and sequenced both fungi and bacteria. For fungi, we used the primers ITS4_Fun (5’ – AGCCTCCGCTTATTGATATGCTTAART - 3’) and 5.8S_Fun (5’ - AACTTTYRRAAYGGATCWCT - 3’) to amplify the ITS2 region (Taylor et al. 2016), while for bacteria we amplified the 16S V4 region with 515F (Parada) (5’ – GTGYCAGCMGCCGCGGTAA -3’) (Parada et al. 2016) and 806R (Apprill) (5’ – GGACTACNVGGGTWTCTAAT -3’) (Apprill et al. 2015). We added 5’ adapter regions to both the forward and reverse primers in each set (forward: 5’ - TCGTCGGCAGCGTCAGATGTAGTATAAGAGACAG - 3’ and reverse: 5’ – GTCTGCTGGCTCAGAGATGTAGTATAAGAGACAGAAA - 3’). We performed PCR in 25 μl reactions containing 5 μl GoTaq reaction buffer, 200 μM of each dNTP, 1 mM MgCl₂, 0.5 μM of each primer, and 1.25 units of GoTaq polymerase (Promega, Madison, WI, USA).
PCR conditions for fungi consisted of an initial denaturation at 96° for two minutes, then 28 cycles of denaturation at 94° for 30 seconds, annealing at 55° for 40 seconds, elongation at 72° for two minutes, and a final elongation at 72° for ten minutes. For bacteria it involved a three-minute initial denaturation at 96°, 28 cycles of 94° for 45 seconds, 50° for one minute, 72° for 90 seconds, and a final 72° for ten minutes. We removed residual primers from the PCR products using 0.25 μl of Exonuclease I (New England Biolabs, Ipswich, MA, USA) per 5 μl of sample incubated at 37° for 15 minutes and then 80° for another 15 minutes. After cleaning, we performed a second PCR to attach Illumina adapters and 6 bp sample-specific barcodes. This PCR followed the same protocol as the fungal PCR listed above except it was seven cycles and the primers (5’ - AATGATACGGGCGACCACCGAGATCTACAC-NNNNNN-TCGTCGCGACGCAGTC - 3’ and 5’ -CAAGCAGAAGACGGCATACGAGAT-NNNNNN-GTCTCGTGTTGGCTCGG - 3’, Ns represent variable barcode region) were at 0.4 μM each. We pooled the barcoded samples in equal quantities, purified them with Agencourt AmPure XP beads (Beckman Coulter, Indianapolis, IN, USA), and sequenced them with Illumina MiSeq using the 2x300 bp kit.

ITS2 and 16S sequence data were processed separately in USEARCH v9.2.64 (Edgar 2013). We merged paired end reads, dropping sequences that had a merged length of less than 150 bp or did not successfully merge. We removed primer regions with cutadapt (Martin 2011), quality filtered sequences with greater than one expected error, dereplicated, and clustered the remaining reads into OTUs (operational taxonomic units). This clustering step also removed chimeric OTUs and excluded any singleton OTUs. We then constructed OTU tables by clustering the primer-trimmed sequences against the OTUs at 97% identity. We assigned taxonomy to the OTUs using SINTAX (Edgar 2016) and the UNITE v8.2
database (Abarenkov et al. 2020) for fungi and the RDP training set v16 (Cole et al. 2014) for bacteria. Only taxonomic assignments with at least 80% confidence were retained. We dropped all OTUs assigned to Viridiplantae. For the fungal OTUs, we assigned them to guilds using FUNGuild (Nguyen et al. 2016).

**Statistical analysis**

We performed all analyses in R v3.5.1 (R Core Team 2018) with the vegan (Oksanen et al. 2019), phyloseq (McMurdie and Holmes 2013), and ape (Paradis and Schliep 2018) packages. To account for differences in sequencing depth among samples, all OTU counts were converted to relative abundance. We visualized differences in fungal and bacterial communities between GL and PJ using non-metric multidimensional ordination (NMDS). We tested for community differences using permutational multivariate analysis of variance (PERMANOVA) with the adonis2 function (McArdle and Anderson 2001) in vegan. We tested differences in per-sample relative abundance in ectomycorrhizal and arbuscular mycorrhizal fungi between plots using Wilcoxon tests.

We tested for spatial structure in microbial communities using Mantel tests by examining correlations between spatial distance matrices and community dissimilarity matrices. Community dissimilarities were calculated using the Bray-Curtis metric. We examined spatial autocorrelation at different spatial distances using Mantel correlograms, constructed separately for each plot. We visualized distance decay for fungi and bacteria for each habitat by plotting spatial distances between points against community similarity. We fitted linear regressions to each curve and calculated the slope and intercept for each.
To test the impact of microbial inoculum on plant biomass and pine root colonization, we used a permutation test. We randomly reassigned each plant, excluding those grown in pooled soils or sterile controls, to a soil core, calculated the mean mass for each of these randomized cores, calculated the standard deviation of these means, and repeated this process 1000 times. The actual standard deviation of the means was compared to the distribution of the randomized ones to calculate statistical significance. All subsequent analyses using plant biomass or root colonization used the mean value for a soil core rather than individual plant measurements unless otherwise noted.

We calculated feedback on plant biomass using the ratio between biomass in home soils (GL for grass and PJ for pine) versus the mean value in the away soil (PJ for grass and GL for pine; [home-mean away]/mean away). We used t-tests to test if these values were significantly different from zero. This process was repeated using the mean in sterilized soils in place of away soils to assess positive or negative effects of microbial inoculum on plant growth. To test for spatial autocorrelation in plant response, we calculated Moran’s I (Moran 1950) for biomasses and root colonization in each plot using, using spatial locations of the soil cores to calculate distances. We plotted correlograms showing correlation at different spatial distances using the ncf package (Bjornstad 2018). We also tested the correlation between individual OTUs and plant biomass and root colonization by calculating Spearman correlation coefficients between OTU relative abundance and biomass/colonization for each soil core. P-values were adjusted for multiple testing using the Holm method (Holm 1979). For root colonization, only data from the PJ plot was used due to very low rates of ectomycorrhizal colonization of pine in GL.
We compared the microbial alpha diversity between pooled soil samples and individual soil samples using Wilcoxon tests. To account for differences in sequencing depth among samples, we estimated OTU richness at 1000 reads using the rarefy function in vegan (Oksanen et al. 2019). To test whether different conclusions about plant-soil feedback and effects of inoculum on biomass and colonization were reached when using pooled soils instead of individual soil samples, we repeated the analyses of plant-soil feedback above using pooled soil data. We compared these results to those from individual soils. We also tested differences in biomass and root colonization between pooled and individual soil samples with Wilcoxon tests.

Results

Microbial communities

High-throughput sequencing revealed 2599 fungal OTUs and 15,117 bacterial OTUs. These communities differed significantly between GL and PJ (fungi: F=18.387, p=0.001, R²=0.232, bacteria: F=29.048, p=0.001, R²=0.319). In the ordinations (Figure 1), the two habitats formed distinct clusters, further supporting differences between them in microbial composition. The two plots also differed in taxonomic composition. For fungi, GL was dominated by Ascomycota, which made up about 70% of all reads. PJ had a much greater proportion of Basidiomycota (Figure 2a). Both arbuscular and ecto-mycorrhizal fungi were present in both plots. Relative abundances of arbuscular mycorrhizal fungi were consistently low, making up less than one percent of the fungal reads, though higher in GL (W=889, p<0.001; Figure S2a). Ectomycorrhizal fungi were much more abundant in PJ, frequently making up ten or more percent of a sample, while they usually made up less than one percent
Abundant ectomycorrhizal genera included *Amanita, Geopora, Tricholoma, Hygrophorus, Tomentella, Russula,* and *Inocybe.* For bacteria, GL contained more Actinobacteria, while PJ had a greater proportion of Proteobacteria (Figure 2b). About 25% of fungal reads and 15% of bacterial could not be assigned at the phylum level.

Mantel tests revealed significant correlation between spatial distances and community dissimilarities for both fungi (GL: $r=0.31$, $p=0.001$, PJ: $r=0.54$, $p=0.001$) and bacteria (GL: $r=0.39$, $p=0.001$, PJ: $r=0.57$, $p=0.001$) in both plots. In GL, fungi showed significant positive spatial autocorrelation only at the smallest distances of less than 30 cm but had positive autocorrelation out to 108 cm in PJ (Figure S3a, b). Bacteria had significant positive autocorrelation in the first two distance classes, up to 69 cm, in GL, and up to 108 cm in PJ (Figure S3c, d).

Bacteria had a significantly higher intercept in distance decay than fungi in both habitats ($P<0.001$) and a greater community similarity at all spatial distances observed (Figure 3), indicating that bacterial communities were more similar to each other within a plot than fungal communities were. Bacteria did not differ in intercept or slope between the habitats ($p>0.05$), while fungi did, with a higher intercept and greater slope in PJ than in GL ($p<0.001$).
Figure 1. NMDS ordination of fungal (a, stress=0.129) and bacterial (b, stress=0.098) OTUs by plot with 95% confidence ellipses. There was strong differentiation between grassland and woodland communities for both groups of microbes.
Figure 2. Taxonomic composition of fungal (a) and bacterial (b) reads by plot. Within a plot, all soil cores were weighed equally. Basidiomycota and Proteobacteria made up a greater portion of reads in PJ than in GL, while GL had more Ascomycota and Actinobacteria.
Figure 3. Distance decay in community similarity by taxonomic group and plot. Each point represents a comparison between a pair of samples. Similarity in community composition between samples declined with spatial distance between them. Fungal communities were less similar at all distances than the bacterial ones were.

**Plant biomass and root colonization**

Response of blue grama biomass to microbial inoculum did not differ significantly between plots (t=1.50, p=0.14). It did, however, vary more among individual soil cores than expected by chance (p<0.001), indicating that soil core had an effect on plant growth. Soils from both plots had average negative effects on blue grama biomass relative to sterile controls on average (GL: t=-2.62, p=0.013, PJ: t=-4.65, p<0.001, Figure 4a), though this varied by core, with some having greater biomass than the sterilized soils. Piñon pine had significant negative PSF (t=-2.223, p=0.0337), with trees having slightly lower biomass when grown in their home PJ soils compared to sterilized soils (t=-2.642, p=0.0128), while pines grown with GL inoculum were not different from the sterile controls (t=-0.507, p=0.616; Figure 2b). Piñon root colonization differed between plots, with much higher colonization in PJ (Figure 4c). Only two out of 169 GL pines had ectomycorrhizal root tips,
compared to 56 pines in PJ soils. No colonization was observed in the sterile soils. Pine root colonization (p<0.001), but not biomass (p=0.554), varied more than expected by chance. Biomass for blue grama had positive spatial autocorrelation in both plots (GL: Moran’s I=0.361, p<0.001, PJ: Moran’s I=0.491, p<0.001). This autocorrelation was only significant at small distances of less than one meter (Figure 5a, b, Figure 6a). Pine biomass did not show spatial autocorrelation at any of the distances sampled in this study (GL: Moran’s I=-0.0323, p=0.84, PJ: Moran’s I=-0.0625, p=0.6, Figure S4), but root colonization did demonstrate spatial autocorrelation in the PJ plot (Moran’s I= 0.2868, p<0.001) at distances of up to one meter (Figure 5c). Colonization tended to be higher on the western side of the plot (Figure 6b).

We found 22 OTUs that were significantly correlated with blue grama biomass. Two of these were positive, while 20 were negative (Table S1). Only one OTU was correlated with pine biomass, a species of Ascomycota that could not be identified to a lower level. Several fungal and bacterial OTUs were correlated with ectomycorrhizal root colonization, including a known ectomycorrhizal fungus, Geopora sp. In contrast to biomass, most of the correlations with root colonization were positive (Table S2).
Figure 4. Boxplots of plant biomass (a and b) and root colonization (c) by plot. Each data point is the average of all plants grown in soil from a single core. Microbial inoculum had a negative effect on blue grama growth relative to the sterile control, while there was a slight negative effect for piñon pine in PJ. Root colonization was much higher in PJ, with only two plants having colonization in GL.
Figure 5. Correlograms showing spatial autocorrelation using Moran’s I of blue grama biomass by plot (a and b) and piñon pine root colonization in PJ (c) at different distance classes (in cm). Solid points indicate significant positive (>0) or negative (<0) autocorrelation. Significant positive autocorrelation can be seen at small distances, generally less than one meter. Pine biomass did not have autocorrelation in either plot (data not shown).
Figure 6. Maps of blue grama biomass (a) and percent root colonization (b) by core and plot. Values are the means obtained from plants grown in each soil core, with shade and size of the dots indicating the magnitude of response. Biomass is presented relative to sterile controls, (actual mass – sterile mass)/sterile mass. Spatial structure is apparent for blue grama biomass in both plots and for pine root colonization in PJ, but not GL, where colonization was almost absent. Locations on the map indicate the collection location for the soils; plants were grown in the greenhouse. The bottom of each plot faces north, and plot dimensions are in centimeters. Plots are not shown to scale.
Effect of soil pooling

The pooled soils had greater OTU richness than the individual soil samples for both fungi (W=178, p=0.011) and bacteria (W=169, p=0.028, Figure 7). Pooled soils had a strong negative effect on blue grama biomass, and these effects were more negative than the average for individual soils (W=1043.5, p=0.029, Figure 8a). These negative effects, however, were not different between GL and PJ (t=1.98, p=0.14), as was seen with individual soils. Pine biomass did not differ between pooled and individual soils (W=2128.5, p=0.295; Figure 8b). No difference in blue grama biomass was seen between plots when using pooled samples (t=-0.914, p=0.397), as was seen with individual soils. While the mean root colonization was greater in pooled than individual soil samples (Figure 8c), this difference was only marginally significant (W=549.5, p=0.0788), possibly due to low sample size for the pooled samples (n=6).

Figure 7. Boxplot of bacterial and fungal OTU richness (calculated as the number of OTUs at 1000 reads) in individual and pooled soil samples. Bacteria were more OTU rich than fungi, and pooled soil samples contained more OTUs on average than the individual soils did.
Figure 8. Comparison of microbial inoculum effects in pooled versus individual soil samples on blue grama biomass (a), piñon pine biomass (b), and pine root colonization (c). Data points represent individual plants, not averages. Pooled soils had a more negative effect on blue grama growth than the individual soils, while pools had increased root colonization in pines.
Discussion

Spatial heterogeneity of soil microbes has long been theorized to play an important role in plant coexistence and PSF (Bever et al. 1997), but this has not been shown in natural environments. Our study demonstrated empirically that spatial structure of naturally occurring soil microbial communities can impact plant performance. Blue grama biomass and piñon root colonization varied in non-random, spatially structured ways at the same spatial scales at which soil bacterial and fungal communities varied in composition. Although effects of microbial inoculum on blue grama tended to be negative (Figure 4a), a subset of the locations in each plot had greater growth than the sterile controls (Figure 6a). Similarly, pine root colonization was uneven across the PJ plot (Figure 6b), with some locations having high colonization while others had little to none. Pines are considered obligately ectomycorrhizal, and they perform poorly and fail to establish when compatible fungi are absent (Wright 1957, Trappe 1977, Smith and Read 2010). Thus, pine seedlings that germinate and begin to grow in areas with high root colonization rates may have an advantage relative to pines without early access to mycorrhizae (Blaudez et al. 2000, Tibbett and Sanders 2002, Kipfer et al. 2012, Guerrero-Galán et al. 2019). This heterogeneous patchwork of soil microbes and plant responses to those microbes renders some locations more amenable to blue grama or piñon pine establishment and growth, while adjacent locations are less hospitable, creating “safe sites” that could favor these plants over their competitors. Spots with strong negative microbial impacts, however, may render the resident more vulnerable to mortality and make the location susceptible to invasion by other taxa. Thus, microbial heterogeneity can create spatial variation in environmental suitability and
localized source-sink metapopulation dynamics, which can foster plant coexistence and biodiversity (Chesson 2000, Amarasekare and Nisbet 2001, Amarasekare 2003).

Wide ranges of plant responses were all found within the very small spatial extent of our plots, each of which was only 2 m² in area. Recent mesocosm studies using heterogeneous soils suggest that the spatial scale at which soils vary can impact their effects on the plant community (Wubs and Bezemer 2016, 2018). If soils vary on very small scales, plants can grow to quickly exceed the scale of microbial variation, spreading their roots into new microbial communities and encountering new pathogens or mutualists. Thus, the spatial scales of microbial community variation, plant response variation, and the root architecture and growth rates of different plant species all interact to determine effects of soil heterogeneity on plant communities. Temporal turnover of microbes also needs to be considered. Microbial communities change with time (Kivlin et al. 2018, Chapter 2), and rapid introductions and extirpations of microbes could overwhelm spatial effects. Slower changes could still create “moving targets”, in which favorable sites shift in space over time. The plants themselves impact temporal changes in microbes, for example by accumulating pathogens in surrounding soil (Mangla and Callaway 2008) or selectively cultivating mutualists (Simms et al. 2006, Kiers et al. 2011).

Pooled soil samples were not representative of individual soils in either microbial community composition or plant response. Pooled soils had greater bacterial and fungal diversity than the individual soils (Figure 7), which is unsurprising considering the pools combined the heterogeneous soils from throughout the plots. All microbes, including those that are limited to one or a few soil cores, should be present in the pools, even if at low abundances. Our inability to detect all taxa from across the plots in the pools is likely due to
limited volumes of soil used in DNA extractions as well as limited sequencing depths. Effects of the pools on blue grama growth were more negative than the average for the individual soils (Figure 8a), suggesting more frequent antagonistic interactions in the pools. This might have occurred because patchily distributed pathogens end up in the pooled samples. This can be seen as equivalent to the ‘sampling effect’ highlighted by Wardle and others in their critiques of many studies of the relationship between biodiversity and ecosystem function (Huston 1997, Tilman et al. 1997, Wardle 1999). Piñon pine had greater root colonization in pools than individual soils, in which some cores had little to no colonization (Figure 8c). These results are further evidence that combined soils are not “average” soils and should not be used as such, as has been pointed out by others (Reinhart and Rinella 2016, Rinella and Reinhart 2017, 2019, Burns et al. 2017, Peacher and Meiners 2020). Sampling of field soils for PSF experiments should be carried out with consideration of microbial spatial heterogeneity. Homogenization of soils might, however, be useful in some cases, such as restoration efforts for ectomycorrhizal plants like piñon, by more evenly distributing beneficial mutualists.

Microbial inoculum had little effect on piñon pine biomass (Figure 4b, Figure S4). We attribute this to the ontogeny of piñon, which is slow-growing with relatively large seeds. Early growth was likely dependent on nutrient stores from the seeds, with lesser contributions from photosynthesis and soil nutrients. This is opposed to blue grama, which has much smaller seeds and is fast growing, with two of the plants in our greenhouse experiment flowering over the course of only 15 weeks. Piñon biomass in PJ was slightly lower than in GL (Figure 4b), possibly due to the plants investing carbon to form associations with ectomycorrhizal fungi. Piñon, and other K-selected plants, likely needs to be grown for
longer periods to fully assess the effects of microbial inoculation. Benefits of ectomycorrhizas, for example, may only become apparent later in the life of the plant or when exposed to abiotic stress, which was minimized in the greenhouse. In a similar bioassay with piñon pine, seedlings grown in live soil for 6 months also had lower biomass than those grown in sterile soils (Montes and Taylor, manuscript in prep). However, in another study in which we grew piñon for 1.5 years, seedlings in live soils with ectomycorrhizal colonization achieved twice the biomass of seedlings in sterile control soil (Olivas and Taylor, unpublished data). Ectomycorrhizal fungi and root colonization were almost entirely absent in the GL plot (Figure S2b, Figure 6b), which would likely reduce the survival of piñon in these areas and limit invasion of pines into regions without live plants hosting compatible fungi (Gehring and Whitham 1994). This has major implications for the resilience of semi-arid forests, which have experienced massive die-offs in recent decades worldwide that have been related to ongoing climate change (Breshears et al. 2005, Allen 2007, Allen et al. 2010).

Although both bacterial and fungal communities differed starkly between the plots (Figure 1), we found little evidence for feedback between blue grama and piñon pine. Blue grama performed equally in both plots. This may be because blue grama was present in both plots, though at much lower density in PJ, allowing specialist pathogens to occur in both plots. Similarly, pine biomass did not differ strongly between plots, though root colonization did, as noted above. This difference in biomass between plots may be due to the different mycorrhizal types of these two plants. Feedback between plants of differing mycorrhizal guilds tends to be neutral to positive, rather than negative (Crawford et al. 2019). PSF by itself may not play a decisive role in maintaining coexistence between these two plant
species, though the lack of ectomycorrhizal fungi in GL could inhibit pine invasion into grasslands.

Both bacterial and fungal communities exhibited significant spatial structure and distance decay in our plots (Figure 3, S3). The plots were not monocultures and did not have perfect plant homogeneity (Figure S5), as such conditions are rare in natural settings, so microbial spatial turnover was likely due to a combination of environmental heterogeneity, including differences in soil conditioning by plants, and stochastic processes like dispersal. Bacterial communities had greater overall and per sample OTU richness than fungal communities (Figure 7), but they had lower beta diversity. When examining distance decay, bacteria had greater community similarities than fungi at all distances observed (Figure 3). Similar patterns have been seen in other systems and are often attributed to differences in size and dispersal ability between the two groups (Kaspari et al. 2010, Prévost-Bouré et al. 2014, Vacher et al. 2016). Slopes of distance decay did not differ substantially among microbe groups or plots, except for fungi in PJ, which had a steeper slope and higher intercept.

By using several individual soils and sequencing the microbes in them, we have begun to crack open the “black box” of effects of specific microbes on plants (Tiedje et al. 1999, Horton and Bruns 2001, van der Putten et al. 2016). Microbial communities are not explicitly examined in most PSF studies, though there are some exceptions (Rigg et al. 2016, Semchenko et al. 2018). Several OTUs were positively and negatively correlated with blue grama biomass and pine root colonization (Table S1, S2). Effects of microbial inoculation on blue grama tended to be negative (Figure 4a), as is often seen in grasslands (Kulmatiski et al. 2008) and has been shown for this species in particular (Chung and Rudgers 2016), and
correlations with individual microbes were also mostly negative. While many microbes were poorly identified, some included known plant pathogens, such as an Ophiostomataceae OTU. An arbuscular mycorrhizal Glomeraceae OTU had a strong negative correlation as well, suggesting this mycorrhizal symbiosis was functioning as a parasitism rather than mutualism (Johnson et al. 1997), at least under the conditions of our greenhouse experiment. Several taxa were positively correlated with pine root ectomycorrhizal colonization, including Geopora, a known ectomycorrhizal fungus. Bacteria positively correlated with root colonization could have associated with the ectomycorrhizal fungi themselves or with the tree roots in the soil that hosts them and/or be mycorrhizal helper bacteria, which have found in previous studies (Garbaye and Bowen 1987).

In conclusion, we show that naturally occurring heterogeneity of soil microbial communities can have profound consequences for plant growth and establishment, and this may have important consequences for plant community dynamics and coexistence. Further research is needed to determine the effects of heterogeneity on community level processes and how it interacts with environmental variables known to impact PSF, such as nutrient availability (Bennett and Klironomos 2019, in ’t Zandt et al. 2019), climatic conditions (Classen et al. 2015, Ren et al. 2015), and stress (Beals et al. 2020), which were not varied in our greenhouse experiment but are likely important in the field. Measurement and inclusion of microbial composition and its spatial structure is likely to improve the predictive capacity of PSF theory and our ability to explain plant coexistence in a changing world.
Supplemental Tables and Figures

Figure S1. Sampling design for soil cores in each plot. Diagram is not to scale. The bottom of the plot faces north.
Figure S2. Boxplots of relative abundance of arbuscular mycorrhizal fungi (a) and ectomycorrhizal fungi (b) by plot. Assignments to mycorrhizal type were made using FUNguild. Each data point is an individual soil core. Arbuscular mycorrhizal fungi had low per-sample abundances and were more abundant in GL. Ectomycorrhizal fungi had low abundances in GL but were much more abundant in PJ.
Figure S3. Mantel correlograms for fungi (a and b) and bacteria (c and d) in each plot by distance class (in cm). Filled points indicate significant autocorrelation. Both groups significant positive spatial autocorrelation at smaller distances, generally under one meter. Range of spatial autocorrelation was larger in PJ than GL for both taxa.
Figure S4. Maps of piñon pine biomass by core and plot. Values are the means of each core, with shade and size of the dots indicating the magnitude of response. Biomass is presented relative to sterile controls, (actual mass – sterile mass)/sterile mass. No spatial structure is seen in pine biomass in either plot. Locations on the map indicate the collection location for the soils; plants were grown in the greenhouse. The bottom of each plot faces north, and plot dimensions are in centimeters. Plots are not shown to scale.
Table S1. Spearman correlations between OTU relative abundance and plant biomass for blue grama and piñon pine.

<table>
<thead>
<tr>
<th>OTU</th>
<th>Taxonomy</th>
<th>Spearman’s Rho</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blue grama</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OTU34</td>
<td>Ascomycota</td>
<td>+0.548</td>
</tr>
<tr>
<td>OTU379</td>
<td>Glomeraceae</td>
<td>-0.518</td>
</tr>
<tr>
<td>OTU734</td>
<td>Ophiostomataceae</td>
<td>-0.453</td>
</tr>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OTU7928</td>
<td>Bacteria</td>
<td>+0.542</td>
</tr>
<tr>
<td>OTU1789</td>
<td>Bacteria</td>
<td>-0.614</td>
</tr>
<tr>
<td>OTU8787</td>
<td>Acidimicrobiales</td>
<td>-0.577</td>
</tr>
<tr>
<td>OTU497</td>
<td>Bacteria</td>
<td>-0.524</td>
</tr>
<tr>
<td>OTU3416</td>
<td>Rhodospirillaceae</td>
<td>-0.519</td>
</tr>
<tr>
<td>OTU12322</td>
<td>Acidobacteria_Gp16</td>
<td>-0.512</td>
</tr>
<tr>
<td>OTU4691</td>
<td>Actinobacteria</td>
<td>-0.508</td>
</tr>
<tr>
<td>OTU2320</td>
<td>Gaiella</td>
<td>-0.503</td>
</tr>
<tr>
<td>OTU2032</td>
<td>Bacteria</td>
<td>-0.503</td>
</tr>
<tr>
<td>OTU6705</td>
<td>Acidobacteria_Gp16</td>
<td>-0.502</td>
</tr>
<tr>
<td>OTU6483</td>
<td>Alphaproteobacteria</td>
<td>-0.501</td>
</tr>
<tr>
<td>OTU1501</td>
<td>Rhodospirillaceae</td>
<td>-0.494</td>
</tr>
<tr>
<td>OTU2050</td>
<td>Gammaproteobacteria</td>
<td>-0.490</td>
</tr>
<tr>
<td>OTU2939</td>
<td>Actinobacteria</td>
<td>-0.490</td>
</tr>
<tr>
<td>OTU5140</td>
<td>Actinobacteria</td>
<td>-0.489</td>
</tr>
<tr>
<td>OTU1373</td>
<td>Bacteria</td>
<td>-0.488</td>
</tr>
<tr>
<td>OTU273</td>
<td>Acidobacteria_Gp10</td>
<td>-0.485</td>
</tr>
<tr>
<td>OTU2555</td>
<td>Rhodospirillales</td>
<td>-0.484</td>
</tr>
<tr>
<td><strong>Piñon pine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fungal OTU2344</td>
<td>Ascomycota</td>
<td>+0.425</td>
</tr>
</tbody>
</table>
Table S2. Spearman correlations between OTU relative abundance and pine root colonization in PJ.

<table>
<thead>
<tr>
<th>OTU</th>
<th>Taxonomy</th>
<th>Spearman’s Rho</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fungi</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OTU331</td>
<td>Unidentified</td>
<td>+0.717</td>
</tr>
<tr>
<td>OTU1748</td>
<td>Chaetothyriales</td>
<td>+0.685</td>
</tr>
<tr>
<td>OTU1208</td>
<td>Unidentified</td>
<td>+0.682</td>
</tr>
<tr>
<td>OTU28</td>
<td><em>Geopora</em></td>
<td>+0.660</td>
</tr>
<tr>
<td>OTU1346</td>
<td><em>Ascomycota</em></td>
<td>+0.649</td>
</tr>
<tr>
<td>OTU355</td>
<td><em>Glomeraceae</em></td>
<td>+0.647</td>
</tr>
<tr>
<td>OTU655</td>
<td>Unidentified</td>
<td>+0.646</td>
</tr>
<tr>
<td>OTU53</td>
<td>Pleosporales</td>
<td>+0.642</td>
</tr>
<tr>
<td>OTU173</td>
<td>Pleosporales</td>
<td>+0.639</td>
</tr>
<tr>
<td>OTU667</td>
<td>Unidentified</td>
<td>+0.632</td>
</tr>
<tr>
<td>OTU182</td>
<td>Helotiales</td>
<td>+0.632</td>
</tr>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OTU2479</td>
<td>Micromonosporaceae</td>
<td>+0.770</td>
</tr>
<tr>
<td>OTU1467</td>
<td>Proteobacteria</td>
<td>+0.770</td>
</tr>
<tr>
<td>OTU2687</td>
<td>Bacteria</td>
<td>+0.747</td>
</tr>
<tr>
<td>OTU1733</td>
<td>Bacteria</td>
<td>+0.733</td>
</tr>
<tr>
<td>OTU610</td>
<td>Acidobacteria_Gp6</td>
<td>+0.730</td>
</tr>
<tr>
<td>OTU1414</td>
<td><em>Armatimonas</em></td>
<td>+0.723</td>
</tr>
<tr>
<td>OTU2512</td>
<td><em>Actinoplanes</em></td>
<td>+0.721</td>
</tr>
<tr>
<td>OTU2512</td>
<td>Alphaproteobacteria</td>
<td>+0.719</td>
</tr>
<tr>
<td>OTU184</td>
<td>Acetobacteraceae</td>
<td>+0.713</td>
</tr>
<tr>
<td>OTU177</td>
<td>Bacteria</td>
<td>+0.707</td>
</tr>
<tr>
<td>OTU6976</td>
<td>Acidobacteria_Gp3</td>
<td>+0.707</td>
</tr>
<tr>
<td>OTU246</td>
<td>Alphaproteobacteria</td>
<td>+0.705</td>
</tr>
<tr>
<td>OTU2384</td>
<td>Micromonosporaceae</td>
<td>+0.705</td>
</tr>
<tr>
<td>OTU229</td>
<td>Acetobacteraceae</td>
<td>+0.704</td>
</tr>
<tr>
<td>OTU316</td>
<td><em>Skermanella</em></td>
<td>+0.700</td>
</tr>
<tr>
<td>OTU1709</td>
<td>Bacteroidetes</td>
<td>-0.695</td>
</tr>
<tr>
<td>OTU5843</td>
<td>Micromonosporaceae</td>
<td>+0.694</td>
</tr>
<tr>
<td>OTU944</td>
<td>Bacteria</td>
<td>+0.693</td>
</tr>
<tr>
<td>OTU759</td>
<td>Acidobacteria_Gp10</td>
<td>+0.691</td>
</tr>
<tr>
<td>OTU621</td>
<td>Sphingomonadaceae</td>
<td>+0.691</td>
</tr>
<tr>
<td>OTU6478</td>
<td><em>Rubrobacter</em></td>
<td>+0.690</td>
</tr>
<tr>
<td>OTU14677</td>
<td>Rhizobiales</td>
<td>+0.690</td>
</tr>
<tr>
<td>OTU552</td>
<td>Actinobacteria</td>
<td>+0.688</td>
</tr>
<tr>
<td>OTU1781</td>
<td>Bacteria</td>
<td>+0.686</td>
</tr>
<tr>
<td>OTU14932</td>
<td>Actinobacteria</td>
<td>+0.686</td>
</tr>
<tr>
<td>OTU892</td>
<td>Alphaproteobacteria</td>
<td>+0.685</td>
</tr>
<tr>
<td>OTU6936</td>
<td><em>Pseudonocardia</em></td>
<td>+0.684</td>
</tr>
<tr>
<td>OTU2320</td>
<td><em>Gaiella</em></td>
<td>+0.683</td>
</tr>
<tr>
<td>OTU446</td>
<td>Bacteria</td>
<td>+0.680</td>
</tr>
<tr>
<td>OTU1471</td>
<td><em>Streptomyces</em></td>
<td>-0.709</td>
</tr>
<tr>
<td>OTU7395</td>
<td>Bacteria</td>
<td>-0.676</td>
</tr>
</tbody>
</table>
Figure S5. Photos of the field sites, including the GL site, complete with flags marking soil core sites (a), and a nearby piñon-juniper woodland site (b). The pictured PJ site is not the exact location used in this study but is in the same vicinity and has a similar appearance.
References


Bjornstad, O. N. 2018. ncf: Spatial Covariance Functions.


