Epiphytic fungal communities vary by substrate type and at sub-meter spatial scales

Kel Cook[1], Jyotsna Sharma[2], Andrew Taylor[3], Ian Herriott[4], and D Taylor[1]

[1]University of New Mexico
[2]Texas Tech University
[3]University of Hawai'i at Manoa
[4]University of Alaska Fairbanks

July 23, 2020

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.

Keywords

spatial ecology, bryophytes, epiphytes, distance decay, fungal communities, dispersal limitation

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 *Saurauia*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 involves some error due to the angling of branches, the error is small (<10 cm in most cases) relative to the distances between points on different branches, which is 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 (Figure S2). 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 RNAlater (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 manufacturers protocol beginning at step 7. We amplified the ITS2 region using universal fungal primers 5.8S_Fun (5' -GTCTGCTGGGCTCGGAGATGTGTATAAGAGACAGAAA**AACTTTYRRCAAYGGATCWCT** 3') and ITS4_Fun (5' -TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**AGCCTCCGCTTATTGATATGCTTAART** 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 $MgCl_2$, 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-GTCTCGTGGGCTCGG- 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 removes 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 species richness, the Simpson index, and the Shannon index for each sample. To account for differing sequencing depth between samples, expected richness at 1,000 reads was calculated using the rarefy function in the vegan package (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, possibly 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 could not be identified 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: χ^2= 45.017, p<0.001; Shannon: χ^2= 31.86, p<0.001; Simpson: χ^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.

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.

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 make 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).

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 [?]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 were introduced during sample collection or processing. We found no evidence of pervasive endophytes in the living inner bark of*Saurauia* .

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, be it stochastic dispersal, abiotic environmental factors, and/or unmeasured biotic factors, these drivers do not act on all substrates at the same point together or 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 *Saurauia* . 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-Loucao, 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.

Acknowledgements

This material is based upon work supported by the National Science Foundation under Grant No. DEB-1355155. We would like to thank the UNM Center for Advanced Research Computing, supported in part by the National Science Foundation, for providing the high-performance computing resources used in this work. We thank the staff of Parque Nacional Tapanti and Jardin Botanico Lankester for logistical support in the field.

Literature Cited

Anderson, J. B., Bruhn, J. N., Kasimer, D., Wang, H., Rodrigue, N., & Smith, M. L. (2018). Clonal evolution and genome stability in a 2,500-year-old fungal individual. *BioRxiv* , 377234. doi: 10.1101/377234

Bahram, M., Koljalg, U., Courty, P.-E., Diedhiou, A. G., Kjoller, R., Polme, S., ... Tedersoo, L. (2013). The distance decay of similarity in communities of ectomycorrhizal fungi in different ecosystems and scales. *Journal of Ecology* , *101* (5), 1335–1344. doi: 10.1111/1365-2745.12120

Bahram, M., Peay, K. G., & Tedersoo, L. (2015). Local-scale biogeography and spatiotemporal variability in communities of mycorrhizal fungi. *New Phytologist* , *205* (4), 1454–1463. doi: 10.1111/nph.13206

Barrett, L. G., Kniskern, J. M., Bodenhausen, N., Zhang, W., & Bergelson, J. (2009). Continua of specificity and virulence in plant host–pathogen interactions: causes and consequences. *New Phytologist* , *183* (3), 513–529. doi: 10.1111/j.1469-8137.2009.02927.x

Benzing, D. H. (1990). Vascular epiphytes: general biology and related biota. *Cambridge Tropical Biology Series.*

Blanchet, F. G., Legendre, P., & Borcard, D. (2008). Forward Selection of Explanatory Variables. *Ecology* , *89* (9), 2623–2632. doi: 10.1890/07-0986.1

Borcard, D., & Legendre, P. (2002). All-scale spatial analysis of ecological data by means of principal coordinates of neighbour matrices.*Ecological Modelling* , *153* (1), 51–68. doi: 10.1016/S0304-3800(01)00501-4

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., ... Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods* , *7* (5), 335–336. doi: 10.1038/nmeth.f.303

Carvalho, L. M., Correia, P. M., Ryel, R. J., & Martins-Loucao, M. A. (2003). Spatial variability of arbuscular mycorrhizal fungal spores in two natural plant communities. *Plant and Soil* , *251* (2), 227–236.

Cevallos, S., Sanchez-Rodriguez, A., Decock, C., Declerck, S., & Suarez, J. P. (2017). Are there keystone mycorrhizal fungi associated to tropical epiphytic orchids? *Mycorrhiza* , *27* (3), 225–232. doi: 10.1007/s00572-016-0746-8

Chaudhary, V. B., O'Dell, T. E., Rillig, M. C., & Johnson, N. C. (2014). Multiscale patterns of arbuscular mycorrhizal fungal abundance and diversity in semiarid shrublands. *Fungal Ecology* , *12* , 32–43. doi: 10.1016/j.funeco.2014.06.003

Chen, H. (2018). *VennDiagram: Generate High-Resolution Venn and Euler Plots* . Retrieved from https://CRAN.R-project.org/package=VennDiagram

Davey, Marie L., Heegaard, E., Halvorsen, R., Ohlson, M., & Kauserud, H. (2012). Seasonal trends in the biomass and structure of bryophyte-associated fungal communities explored by 454 pyrosequencing.*New Phytologist* , *195* (4), 844–856. doi: 10.1111/j.1469-8137.2012.04215.x

Davey, Marie L., Heimdal, R., Ohlson, M., & Kauserud, H. (2013). Host- and tissue-specificity of moss-associated Galerina and Mycena determined from amplicon pyrosequencing data. *Fungal Ecology* , *6* (3), 179–186. doi: 10.1016/j.funeco.2013.02.003

Davey, M.L., Nybakken, L., Kauserud, H., & Ohlson, M. (2009). Fungal biomass associated with the phyllosphere of bryophytes and vascular plants. *Mycological Research* , *113* (11), 1254–1260. doi: 10.1016/j.mycres.2009.08.001

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nature Methods* , *10* (10), 996–998. doi: 10.1038/nmeth.2604

Galante, T. E., Horton, T. R., & Swaney, D. P. (2011). 95% of basidiospores fall within 1 m of the cap: a field-and modeling-based study. *Mycologia* , *103* (6), 1175–1183. doi: 10.3852/10-388

Genney, D. R., Anderson, I. C., & Alexander, I. J. (2006). Fine-scale distribution of pine ectomycorrhizas and their extramatrical mycelium.*New Phytologist* , *170* (2), 381–390. doi: 10.1111/j.1469-8137.2006.01669.x

Harshani, H. C., Senanayake, S., & Sandamali, H. (2014). Host tree specificity and seed germination of Dendrobium aphyllum (Roxb.) C.E.C. Fisch. in Sri Lanka. *Journal of the National Science Foundation of Sri Lanka* , *42* (1), 71. doi: 10.4038/jnsfsr.v42i1.6682

Herrera, P., Kottke, I., Molina, M. C., Mendez, M., & Suarez, J. P. (2018). Generalism in the interaction of Tulasnellaceae mycobionts with orchids characterizes a biodiversity hotspot in the tropical Andes of Southern Ecuador. *Mycoscience* , *59* (1), 38–48. doi: 10.1016/j.myc.2017.08.003

Higgins, K. L., Arnold, A. E., Coley, P. D., & Kursar, T. A. (2014). Communities of fungal endophytes in tropical forest grasses: highly diverse host- and habitat generalists characterized by strong spatial structure. *Fungal Ecology* , *8* , 1–11. doi: 10.1016/j.funeco.2013.12.005

Hoffman, M. T., & Arnold, A. E. (2008). Geographic locality and host identity shape fungal endophyte communities in cupressaceous trees.*Mycological Research* , *112* (3), 331–344. doi: 10.1016/j.mycres.2007.10.014

Holm, S. (1979). A Simple Sequentially Rejective Multiple Test Procedure. *Scandinavian Journal of Statistics* , *6* (2), 65–70. Retrieved from JSTOR.

Ishida, T. A., Nara, K., & Hogetsu, T. (2007). Host effects on ectomycorrhizal fungal communities: insight from eight host species in mixed conifer?broadleaf forests. *New Phytologist* , *174* (2), 430–440. doi: 10.1111/j.1469-8137.2007.02016.x

Jacquemyn, H., Brys, R., Waud, M., Busschaert, P., & Lievens, B. (2015). Mycorrhizal networks and coexistence in species-rich orchid communities. *New Phytologist* , *206* (3), 1127–1134. doi: 10.1111/nph.13281

Klironomos, J. N. (2002). Feedback with soil biota contributes to plant rarity and invasiveness in communities. *Nature* , *417* (6884), 67–70. doi: 10.1038/417067a

Klironomos, J. N., Rillig, M. C., & Allen, M. F. (1999). Designing belowground field experiments with the help of semi-variance and power analyses. *Applied Soil Ecology* , *12* (3), 227–238. doi: 10.1016/S0929-1393(99)00014-1

Koide, R. T., Ricks, K. D., & Davis, E. R. (2017). Climate and dispersal influence the structure of leaf fungal endophyte communities of Quercus gambelii in the eastern Great Basin, USA. *Fungal Ecology* , *30* , 19–28. doi: 10.1016/j.funeco.2017.08.002

Lilleskov, E. A., Bruns, T. D., Horton, T. R., Taylor, D. L., & Grogan, P. (2004). Detection of forest stand-level spatial structure in ectomycorrhizal fungal communities. *FEMS Microbiology Ecology* ,*49* (2), 319–332. doi: 10.1016/j.femsec.2004.04.004

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.Journal* , *17* (1), 10–12. doi: 10.14806/ej.17.1.200

McMurdie, P. J., & Holmes, S. (2013). phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE* , *8* (4), e61217.

Mummey, D. L., & Rillig, M. C. (2008). Spatial characterization of arbuscular mycorrhizal fungal molecular diversity at the submetre scale in a temperate grassland: Arbuscular mycorrhizal fungal molecular diversity. *FEMS Microbiology Ecology* , *64* (2), 260–270. doi: 10.1111/j.1574-6941.2008.00475.x

Nekola, J., & White, P. (1999). Nekola JC, White PS.. The distance decay of similarity in biogeography and ecology. J Biogeogr 26: 867-878.*Journal of Biogeography* , *26* , 867–878. doi: 10.1046/j.1365-2699.1999.00305.x

Nilsson, R. H., Larsson, K.-H., Taylor, A. F. S., Bengtsson-Palme, J., Jeppesen, T. S., Schigel, D., ... Abarenkov, K. (2019). The UNITE database for molecular identification of fungi: handling dark taxa and parallel taxonomic classifications. *Nucleic Acids Research* ,*47* (D1), D259–D264. doi: 10.1093/nar/gky1022

Oja, J., Vahtra, J., Bahram, M., Kohout, P., Kull, T., Rannap, R., ... Tedersoo, L. (2017). Local-scale spatial structure and community composition of orchid mycorrhizal fungi in semi-natural grasslands. *Mycorrhiza* , *27* (4), 355–367. doi: 10.1007/s00572-016-0755-7

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., ... Wagner, H. (2019). *vegan: Community Ecology Package* . Retrieved from https://CRAN.R-project.org/package=vegan

Oono, R., Rasmussen, A., & Lefevre, 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

Peay, K. G., & Bruns, T. D. (2014). Spore dispersal of basidiomycete fungi at the landscape scale is driven by stochastic and deterministic processes and generates variability in plant-fungal interactions.*New Phytologist* , *204* (1), 180–191. doi: 10.1111/nph.12906

Peay, K. G., Garbelotto, M., & Bruns, T. D. (2010). Evidence of dispersal limitation in soil microorganisms: Isolation reduces species richness on mycorrhizal tree islands. *Ecology* , *91* (12), 3631–3640. Retrieved from JSTOR.

Pickles, B. J., Genney, D. R., Potts, J. M., Lennon, J. J., Anderson, I. C., & Alexander, I. J. (2010). Spatial and temporal ecology of Scots pine ectomycorrhizas. *New Phytologist* , *186* (3), 755–768. doi: 10.1111/j.1469-8137.2010.03204.x

R Core Team. (2018). *R: A Language and Environment for Statistical Computing* . Retrieved from https://www.R-project.org/

Read, D., & Perez-Moreno, J. (2003). Mycorrhizas and nutrient cycling in ecosystems–a journey towards relevance? *New Phytologist* ,*157* (3), 475–492.

Rillig, M. C., & Mummey, D. L. (2006). Mycorrhizas and soil structure.*New Phytologist* , *171* (1), 41–53. doi: 10.1111/j.1469-8137.2006.01750.x

Rosling, A., Landeweert, R., Lindahl, B. D., Larsson, K.-H., Kuyper, T. W., Taylor, A. F. S., & Finlay, R. D. (2003). Vertical distribution of ectomycorrhizal fungal taxa in a podzol soil profile. *New Phytologist* , *159* (3), 775–783. doi: 10.1046/j.1469-8137.2003.00829.x

Shefferson, R. P., Taylor, D. L., Weiss, M., Garnica, S., McCormick, M. K., Adams, S., ... Lee, Y.-I. (2007). THE EVOLUTIONARY HISTORY OF MYCORRHIZAL SPECIFICITY AMONG LADY'S SLIPPER ORCHIDS. *Evolution* ,*61* (6), 1380–1390. doi: 10.1111/j.1558-5646.2007.00112.x

Taylor, D. L., & Bruns, T. D. (1999). Community structure of ectomycorrhizal fungi in a Pinus muricata forest: minimal overlap between the mature forest and resistant propagule communities.*Molecular Ecology* , *8* (11), 1837–1850. doi: 10.1046/j.1365-294x.1999.00773.x

Taylor, D. Lee, Hollingsworth, T. N., McFarland, J. W., Lennon, N. J., Nusbaum, C., & Ruess, R. W. (2014). A first comprehensive census of fungi in soil reveals both hyperdiversity and fine-scale niche partitioning. *Ecological Monographs* , *84* (1), 3–20. doi: 10.1890/12-1693.1

Taylor, D. Lee, Walters, W. A., Lennon, N. J., Bochicchio, J., Krohn, A., Caporaso, J. G., & Pennanen, T. (2016). Accurate Estimation of Fungal Diversity and Abundance through Improved Lineage-Specific Primers Optimized for Illumina Amplicon Sequencing. *Applied and Environmental Microbiology* , *82* (24), 7217–7226. doi: 10.1128/AEM.02576-16

Tedersoo, L., Koljalg, U., Hallenberg, N., & Larsson, K.-H. (2003). Fine scale distribution of ectomycorrhizal fungi and roots across substrate layers including coarse woody debris in a mixed forest.*New Phytologist* , *159* (1), 153–165. doi: 10.1046/j.1469-8137.2003.00792.x

Thomas, D., Vandegrift, R., Roy, B., Hsieh, H.-M., & Ju, Y.-M. (2019). Spatial patterns of fungal endophytes in a subtropical montane rainforest of northern Taiwan. *Fungal Ecology* , *39* , 316–327. doi: 10.1016/j.funeco.2018.12.012

Toju, H., Sato, H., & Tanabe, A. S. (2014). Diversity and Spatial Structure of Belowground Plant–Fungal Symbiosis in a Mixed Subtropical Forest of Ectomycorrhizal and Arbuscular Mycorrhizal Plants. *PLoS ONE* , *9* (1), e86566. doi: 10.1371/journal.pone.0086566

van der Heijden, M. G.A, Klironomos, J. N., Ursic, M., Moutoglis, P., Streitwolf-Engel, R., Boller, T., ... Sanders, I. R. (1998). Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. *Nature* , *396* (6706), 69–72.

van der Heijden, Marcel G. A., Bardgett, R. D., & van Straalen, N. M. (2008). The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecology Letters* ,*11* (3), 296–310. doi: 10.1111/j.1461-0248.2007.01139.x

Vannier, N., Bittebiere, A.-K., Vandenkoornhuyse, P., & Mony, C. (2016). AM fungi patchiness and the clonal growth of Glechoma hederacea in heterogeneous environments. *Scientific Reports* , *6* (1). doi: 10.1038/srep37852

Vincent, J. B., Weiblen, G. D., & May, G. (2016). Host associations and beta diversity of fungal endophyte communities in New Guinea rainforest trees. *Molecular Ecology* , *25* (3), 825–841. doi: 10.1111/mec.13510

Voyron, S., Ercole, E., Ghignone, S., Perotto, S., & Girlanda, M. (2017). Fine-scale spatial distribution of orchid mycorrhizal fungi in the soil of host-rich grasslands. *New Phytologist* , *213* (3), 1428–1439. doi: 10.1111/nph.14286

Wang, Q., Garrity, G. M., Tiedje, J. M., & Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology* ,*73* (16), 5261–5267. doi: 10.1128/AEM.00062-07

Yoshida, N., Son, J. A., Matsushita, N., Iwamoto, K., & Hogetsu, T. (2014). Fine-scale distribution of ectomycorrhizal fungi colonizing Tsuga diversifolia seedlings growing on rocks in a subalpine Abies veitchii forest. *Mycorrhiza* , *24* (4), 247–257. doi: 10.1007/s00572-013-0535-6

Data accessibility:

We plan to submit DNA sequences to Genbank SRA

Author contributions

Kel Cook: performed research, analyzed data, wrote the paper

Jyotsna Sharma: designed research, performed research

Andrew Taylor: designed research, performed research, analyzed data, edited the paper

Ian Herriott: performed research

Lee Taylor: designed research, performed research, edited the paper

Tables

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.

Substrate	Number of samples	Number of reads	Mean reads/sample	Observed OTUs	Mean OTUs/sample
Live bryophytes	111	4,164,733	37520 (± 31,861)	4,429	186.0 (± 155.8)
Dead bryophytes	117	5,114,465	43,713 (± 52,363)	2,055	108.1 (± 46.6)
Outer bark	121	6,015,672	49,716 (± 41,532)	2,830	144.7 (± 62.6)
Inner bark	31	571,646	18,8440 (± 43,476)	449	27.9 (± 29.0)
Total	380	5,866,516	41,753 (± 43,597)	5,777	135.9 (± 104.8)

Figure Legends

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 have the highest per sample expected richness, and inner bark has the lowest diversity according to all three indices.

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

Figure 3. The first ten centimeters of distance decay by substrate type using relative abundance data and Bray-Curtis similarity. Similarity values are low, even at the shortest distances, and decay rapidly over the first ten centimeters. Living bryophytes have less similarity among samples at short distances than the other substrates.

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.

Figure 5. Venn diagram of variance partitioning analysis showing the effects of substrate type, proximal to distal position along a branch, and the PCNM vectors on fungal community composition.