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Epiphytic fungal communities vary by substrate type and at submetre spatial scales

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Abstract

Fungal species have numerous important environmental functions. Where these functions occur will depend on how fungi are spatially distributed, but the spatial structures of fungal communities are largely unknown, especially in understudied hyperdiverse tropical tree canopy systems. 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. Samples of ~1 cm^3 were collected from 135 points along five adjacent tree branches, with intersample distances from 1 to 800 cm, and dissected into four substrates: outer host tree bark, inner bark, dead bryophytes and living bryophytes. We sequenced the ITS2 region to characterize total fungal communities. 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 operational taxonomic units (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 1 m. Similarity among samples declined by half in less than 10 cm, and even at these short distances, similarities were 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 implications for the distributions, population dynamics and diversity of other tree canopy organisms, including epiphytic plants.

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

1 | INTRODUCTION

Fungi, in their roles as pathogens, saprotrophs and mycorrhizal mutualists, are important drivers of ecosystem processes, including nutrient cycling (Read & Perez-Moreno, 2003), productivity (van der Heijden et al., 2008), building soil structure (Rillig & Mummey, 2006) and structuring plant communities (van der Heijden et al., 1998; Klironomos, 2002), with each fungal taxon impacting these processes differently. If the 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. While data on fungal community composition and assembly at fine scales (centimetres to tens of metres) exist (Bahram et al., 2015; Genney et al., 2006; Oja et al., 2017; Thomas

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et al., 2019), they are limited, with certain habitats, groups of fungi and spatial scales better represented than others.

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 et al., 2014; Mummey & Rillig, 2008; Vannier et al., 2016), ectomycorrhizal fungi (Genney et al., 2006; Lilleskov et al., 2004; Pickles et al., 2010; Yoshida et al., 2014), orchid mycorrhizal fungi (Voyron et al., 2017), foliar endophytes (Higgins et al., 2014; Koide et al., 2017; Thomas et al., 2019) and whole soil fungal communities (Goldmann et al., 2016; Zhang et al., 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 centimetres to kilometres (Koide et al., 2017; Lilleskov et al., 2004; Toju et al., 2014; Yoshida et al., 2014). In a detailed, small-scale study, Mummey and Rillig (2008) found spatial autocorrelation and patchiness in grassland arbuscular mycorrhizal fungi at distances less than 50 cm. However, spatial patterns have not been found 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 metres to hundreds of kilometres. Spatial patterns may be due in part to dispersal limitation (Galante et al., 2011; Peay et al., 2010). If a study is conducted 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 may occur is unknown. Most previous studies have focused on specific groups of fungi rather than whole fungal communities. Fungi have been shown to exhibit competition–colonization tradeoffs (Smith et al., 2018) and have traits that better equip them for different life strategies, much as is seen in plants (Grime, 1977). Groups of fungi with different ecological strategies and traits, including growth morphology and dispersal mechanism, may differ in spatial structure from each other and from the fungal community as a whole. Distance decay has been observed in whole fungal communities, but these studies have tended to focus on larger metre to kilometre spatial scales (Barnes et al., 2016; Goldmann et al., 2016; Thomas et al., 2019; Zhang et al., 2017) or have used older T-RFLP (terminal restriction fragment length polymorphism) methodologies (Sayer et al., 2013).

Local distributions of fungi can also be affected by substrate specificity. In soils, surficial horizons 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 et al., 2003). Fungal communities also differ strongly between plant roots and the surrounding soil (Goldmann et al., 2016). Host plant species has been shown to impact the community composition of mycorrhizal fungi (Ishida et al., 2007), endophytes (Hoffman & Arnold, 2008; Thomas et al., 2019; Vincent et al., 2016), phyllosphere fungi (Kembel & Mueller, 2014) and bryophilous fungi (Davey et al., 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., 2009, 2013) and the bases and tips of tree leaves (Oono et al., 2017). These fine-scale differences in fungal distributions among co-occurring substrates are potential drivers of fungus-mediated processes such as plant competition and community assembly, nutrient cycling, and disease resistance.

Most research on fungal spatial distributions has focused on soils (Bahram et al., 2015), with little work in other systems. Tropical rainforest trees support a high diversity of epiphytic plants (Benzing, 1990), yet fungal communities in this environment have been little studied with molecular techniques beyond studies on orchid mycorrhizae (Cevallos et al., 2017; Harshani et al., 2014; Herrera et al., 2018) and foliar endophytes (Arnold & Lutzoni, 2007; Donald et al., 2020; Vincent et al., 2016; Zimmerman & Vitousek, 2012). 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 grow almost 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). Tree canopies may also be susceptible to different and more frequent disturbances than terrestrial soils. For example, during the 6 years we have worked at our study site, we have witnessed substantial ongoing disturbances in the forms of branch breakage and substrate loss due to wind, rain, floods and epiphyte “landslides.” Disturbance is expected to impact the spatial structure of fungal communities (Craig et al., 2016). 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 coexistence of diverse canopy plant species via creation of cryptic environmental heterogeneity. It may additionally improve our understanding of global patterns of fungal biodiversity.

In this study, we examined the diversity and local-scale spatial patterns of fungal communities on homogeneous, neighbouring tree branches in a tropical rainforest canopy system. We combine centimetre-scale sampling with deep metabarcode sequencing (>50 million sequences generated) and spatial statistics, including principal coordinates of neighbour matrices, to provide evidence of hitherto unappreciated dominance of stochastic dispersal in driving fungal community assembly. Our goals were to (i) test for differences in diversity and fungal community and functional group composition among substrates on tree branches, and (ii) quantify and characterize fine scale spatial structure of the canopy fungal community. We predict that fungal diversity, community composition and guild composition will differ among canopy substrates. More specifically, we hypothesize that the highest diversity will occur in living bryophytes because this substrate contains multiple

metabolically active bryophyte species, which may contain more fungal niches than other substrates; we expect the lowest diversity in host tree bark, which was sampled from a single species. Saprotrophic fungi are expected to be most diverse in dead bryophytes. We also hypothesize that the overall fungal community turnover will peak at small spatial scales of centimetres to metres. Bryophytes at this site are diverse, heterogeneous, and grow and turn over quickly. We therefore hypothesize that fungal turnover will occur at a smaller spatial scale in living bryophytes than in the more stable host tree bark. Further, we predict that spatial structure will differ among fungal lineages. We hypothesize that members of the Ascomycota, many of which reproduce asexually with conidia, will have greater turnover and smaller patch size than members of the Basidiomycota, which often spread via mycelial growth. Testing these hypotheses will provide insights into whether fungal taxonomy or the above-mentioned traits are predictive of spatial structure.

2 | MATERIALS AND METHODS

2.1 | Sample collection

This study was conducted in a low montane rainforest in Parque Nacional Tapantí, Cartago Province, Costa Rica, along the east bank of the Rio Orosi (9.742°N, 83.784°W, 1,300 m elevation) in July 2015. The riverbank was dominated by *Saurauia montana* (Seem.), which hosts rich epiphyte communities. Epiphytes included mosses and liverworts, lichens, assorted ferns, orchids, bromeliads, Melastomataceae, Araceae and other vascular plants (Ingram et al., 1996). Samples were collected from 135 points spread across five branches on three neighbouring *Saurauia* trees using a 9-mm-diametre borer. Collection points were situated at a geometric series of increasing distances (Figure 1) producing relatively even replication of interpoint distances ranging from 1 cm to over 8 m apart. Distances between points on the same branch were measured as the linear distance along the branch. Distances between selected points on separate branches were measured with a laser (Leica DISTO D8, Leica Geosystems AG). The remaining distances were extrapolated from known distances assuming linear relationships. While this approach involves some error due to the angling of branches, we think the error is small relative to the distances between points on different branches, which is greater than 2 m in most cases. Within 24 hr of collection, each sample was dissected into up to four substrate types: inner host tree bark (inner bark, IB), surficial host tree bark (outer bark, OB), photosynthetic bryophyte tissue (live bryophytes, LB), and dead or senescent bryophyte material (dead bryophytes, DB; Figure 1). Live bryophytes were distinguished from dead based on green colour and intactness of tissues. Vascular plant roots were not included in any of the substrates, and any roots found in the sample cores were removed to prevent sequencing root-associated fungi. Substrates were rinsed in sterile water and preserved in RNAlater (Ambion, ThermoFisher).

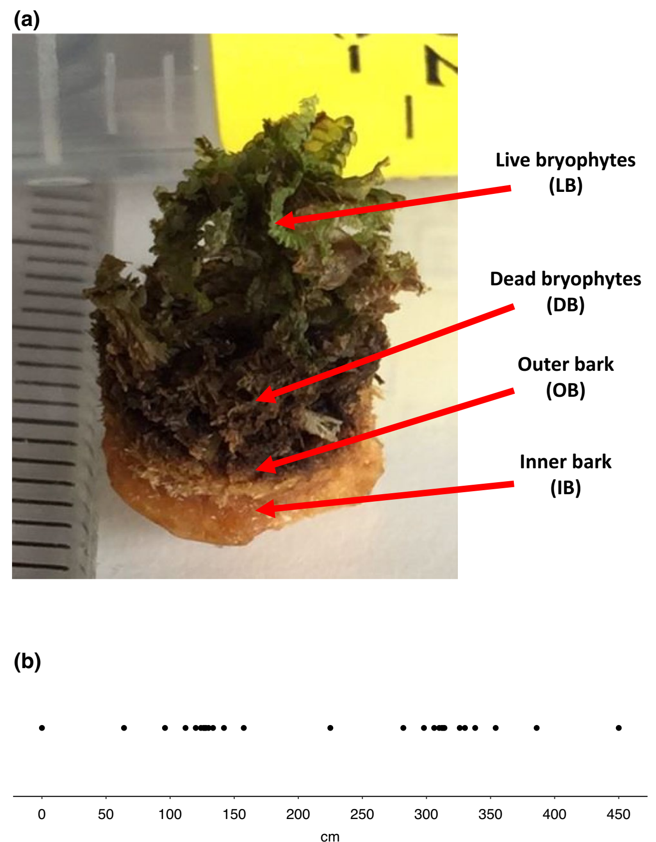

FIGURE 1 Sampling design, including photo of a sample core (a) and an example of spatial sampling design along one branch (b). Sampling scheme varied slightly among branches due to different branch lengths

2.2 | 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 3.2-mm stainless steel beads using a TissueLyser II (Qiagen) at 30 Hz for 90 s. Total DNA was extracted from each ground sample with DNeasy 96 Plant kits (Qiagen) using the manufacturer's 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**-3′), with Nextera adapters added to the core primers (core primers in bold, Taylor et al., 2016). Polymerase chain reaction (PCR) amplification was carried out in 25-μl reactions with 5 μl 5× GoTaq Reaction buffer, 200 μM of each dNTP, 1 mM $MgCl_2$, 0.5 μM of each primer and 1.25 units of GoTaq polymerase (Promega). The amplification conditions were an initial denaturation step at 96°C for 2 min, 27–32 cycles of 94°C for 30 s, 55°C for 40 s and 72°C for 2 min, and a final 72°C elongation step for 10 min. PCR products were cleaned using ZR-96 DNA Clean & Concentrator kits (Zymo Research). 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 6-bp indexes (5′-C

AAGCAGAAGACGGCATACGAGAT-NNNNNN-GTCTCGTGGGCTCGG-3′ and 5′-AATGATACGGCGACCACCGAGATCTACAC-NNNNNN-TCGTCGGCAGCGTC-3′, with Ns representing the variable index region). Indexed samples were pooled at approximately equal concentrations based on gel electrophoresis band brightness into three libraries and cleaned with Agencourt AmPure XP magnetic beads (Beckman Coulter). Each library included a mock community (Taylor et al., 2016). Libraries were then sequenced with Illumina MiSeq using the v3 2 × 300-bp chemistry.

2.3 | Sequence processing

Sequence data were processed primarily with USEARCH version 9.2.64 (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 7.0 (QIIME release, sh_refs_qiime_ver7_97_s_31.01.2016.fasta, Rolf Henrik Nilsson et al., 2019) at 50% using pick_open_reference_otus.py in QIIME version 1.9.1 (Caporaso et al., 2010) with USEARCH version 6.1 (Edgar, 2010). OTUs that did not hit a database entry at this level of similarity were treated as probably nonfungal and removed from the data set. We then assigned the prequality filter merged reads to these filtered OTUs with the usearch_global command.

Taxonomy was assigned to each OTU with SINTAX (Edgar, 2016) using the UNITE database (version 8.2 USEARCH release, Nilsson et al., 2019) and with PROTAX (Abarenkov et al., 2018). OTUs with taxonomic assignments with less than 50% bootstrap confidence as determined by SINTAX were not retained. To improve the taxonomic placement of dominant, poorly identified OTUs, we selected the 100 most abundant OTUs that were unplaced at the order or finer levels. We performed BLASTN searches of GenBank for these OTUs and downloaded related sequences, prioritizing accessions that were "fully identified" *sensu* Nilsson et al. (2006). OTU sequences and selected BLAST matches were then aligned in ALIVIEW (Larsson, 2014) using MUSCLE (Edgar, 2004), long ends missing in most sequences were removed, and maximum-likelihood trees were estimated with GARLI 1.9 (Zwickl, 2006) using default settings. We then visually evaluated trees to identify clades that encompassed our OTUs. Taxonomic placements were based on the consensus identity of the ancestral node in these encompassing clades. This algorithm is similar to the least common ancestor method employed by MEGAN (Huson et al., 2007) except that occasional outlier sequences were ignored. Tree-based taxonomic refinements were combined with all other placements obtained from PROTAX (Abarenkov et al., 2018) and SINTAX (Edgar, 2016). We used the FungalTraits database (Põlme et al., 2020) to assign primary lifestyles and growth forms to OTUs with taxonomic assignments to the genus level with the exception of Glomeromycota, which we listed as arbuscular mycorrhizal and filamentous mycelium even when sub-Phylum taxonomy was unknown. Abundance and taxonomic data were compiled into an OTU table.

2.4 | Data analysis

2.4.1 | 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, we used the rarefy function in the VEGAN package (Oksanen et al., 2019) to calculate expected OTU richness if samples were rarefied to 1,000 reads. R version 3.5.1 (R Core Team, 2018) was used for this and all other statistical analyses. For the Simpson and Shannon indices, we resampled each substrate sample to 1,000 reads using the rrarefy VEGAN function 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 analysed using a Kruskal–Wallis test, and pairwise Wilcoxon rank sum tests were used to test differences between pairs of substrates. This was repeated separately to test for differences in diversity among selected primary lifestyles (animal parasites, litter saprotrophs, soil saprotrophs, wood saprotrophs, plant pathogens and lichenized fungi) and growth morphologies (filamentous mycelium, dimorphic yeasts and yeasts) as assigned by FungalTraits and for Ascomycota and Basidiomycota OTUs. Analyses on these subsets of OTUs were conducted using raw observed OTU richness for LB, DB and OB from the complete data set, rather than the reduced set described above. To assess if individual sampling locations tend to have higher or lower diversity across all substrates present at that point, correlations in OTU richness among substrates within individual sample points were also tested.

2.4.2 | Community and spatial analyses

To visualize compositional differences in fungal communities across substrates, we performed two-dimensional nonmetric multidimensional scaling (NMDS) using the Bray–Curtis dissimilarity coefficient calculated in the phyloseq (McMurdie & Holmes, 2013) R package with 100 iterations. Data were first transformed to relative abundance to account for differences in sequencing depth among samples. This also gives less weight to OTUs with very low abundance and any possible trace contaminants. We visualized the number of

TABLE 1 Summary of sequencing data and spatial results by substrate and across all substrates. This includes number of samples and sequence reads that pass all filtering steps, the mean number of reads per sample, number of OTUs found in a substrate, mean numbers of OTUs per sample, Mantel test results, initial similarity (expected Bray–Curtis similarity at 0 cm) and distance at which this initial similarity is halved

Substrate	No. of samples	No. of reads	Mean reads per sample	Observed OTUs	Mean OTUs per sample	Mantel's *r*	Initial similarity	Halving distance
Live bryophytes	111	4,164,733	37,520 (± 31,861)	4429	186.0 (± 155.8)	0.1093 ($p = .001$)	0.279	7 cm
Dead bryophytes	117	5,114,465	43,713 (± 52,363)	2055	108.1 (± 46.6)	0.2174 ($p = .002$)	0.513	8 cm
Outer bark	121	6,015,672	49,716 (± 41,532)	2830	144.7 (± 62.6)	0.3296 ($p = .001$)	0.547	8.3 cm
Inner bark	31	571,646	18,8440 (± 43,476)	449	27.9 (± 29.0)	NA	NA	NA
Total	380	5,866,516	41,753 (± 43,597)	5777	135.9 (± 104.8)	NA	NA	NA

OTUs that were shared between substrates based on presence–absence using the VennDiagram R package (Chen, 2018). We also constructed barplots comparing phylum-level taxonomy and trophic mode of OTUs across substrates. We tested differences in ratio between Ascomycota and Basidiomycota relative abundance among substrates using a Kruskal–Wallis test and pairwise Wilcoxon rank sum tests.

We tested for spatial autocorrelation in overall fungal community composition in each substrate except IB, 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). These tested for correlations between matrices of sample dissimilarity (calculated using relative abundance OTU data and the Bray–Curtis dissimilarity metric) and spatial distances between sampling points. 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 Pearson correlations and 999 permutations. Distance classes were determined by program defaults, and *p*-values were progressively corrected using the Holm method (Holm, 1979). We repeated this with the individual primary lifestyles, growth morphologies and phyla mentioned above. As the relative abundance of each of these subgroups varied among samples, sometimes dramatically, we used presence–absence data and the Jaccard dissimilarity metric when analysing these data subsets. Samples that completely lacked OTUs from a particular subgroup were excluded from those analyses.

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 fitted and compared linear regression lines to the first 10 cm of data, which were approximately linear, and used these lines to estimate similarity at 0 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 also constructed and compared distance decay curves out to 100 cm separately for Ascomycota and Basidiomycota OTUs.

We used redundancy analysis (RDA) to test effects of spatial structure, branch, substrate and distance along a branch on whole 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 neighbour 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 geographical 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. (2008) stopping criterion, retaining 25 variables. To account for effect of position of a point along a branch (near the trunk, where points tend to be more sheltered and shaded, vs. toward the tip, where samples are more exposed), 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, branch, substrate type and distance along branch. The statistical significance of each partition was tested with permutation tests with 999 permutations.

3 | RESULTS

3.1 | 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 nonsingleton fungal OTUs at 97% identity (Table 1). Of 533 substrate samples, 153 contained fewer than 1,000 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 (62.8%) belonged to the Ascomycota, particularly Eurotiomycetes, Leotiomycetes and Sordariomycetes, while Basidiomycota made up 23.0% of the OTUs (Figure S1). Many could

not be identified with 50% confidence at any taxonomic level below Fungi: 13.1% were unidentified at the phylum level, and 70.9% could not be assigned to a genus. Each substrate contained an assortment of fungi with different lifestyles (Figure S2), including animal parasites (157 OTUs), plant pathogens (272 OTUs), soil saprotrophs (155 OTUs), litter saprotrophs (272 OTUs), wood saprotrophs (455 OTUs), root endophytes (16 OTUs) and lichenized fungi (73 OTUs). The majority of OTUs and reads could not be assigned to primary lifestyles despite efforts to improve taxonomic assignments using phylogenetic methods.

3.2 | Alpha diversity

There were significant differences among substrates for all three diversity indices (expected OTU richness: $\chi^2 = 45.017$, $p < .001$; Shannon: $\chi^2 = 31.86$, $p < .001$; Simpson: $\chi^2 = 21.531$, $p < .001$). Inner bark had significantly lower Shannon and Simpson indices per sample than the other substrates ($p < .001$), while there were no significant differences among the others. All substrate pairs had significantly different expected richness, except for outer bark and live bryophytes ($p < .05$, Figure 2). In agreement with our hypothesis, 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 ($p < .05$) correlations in species richness among substrates at the same point. In other words, the OTU richness found in a single live bryophyte sample was not predictive of the richness in the underlying dead bryophyte material or bark surface at the same point.

As we predicted, there were significant differences in OTU richness among substrates for all primary lifestyles, growth forms and phyla ($p < .01$). For most groups, DB had lower richness than LB or OB, which were not significantly different. Exceptions were lichens, for which LB had the greatest per-sample OTU richness and OB had the lowest, and for filamentous mycelium and Basidiomycota, which were most diverse in OB. Yeasts were significantly more diverse in LB than DB, but no other differences were significant.

3.3 | Community composition by substrate

Two-dimensional NMDS ordination provided graphical support for our expectation that fungal community composition differs among substrates and that fungal taxa display some substrate specificity or preference in the canopy habitat (Figure 3). 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.

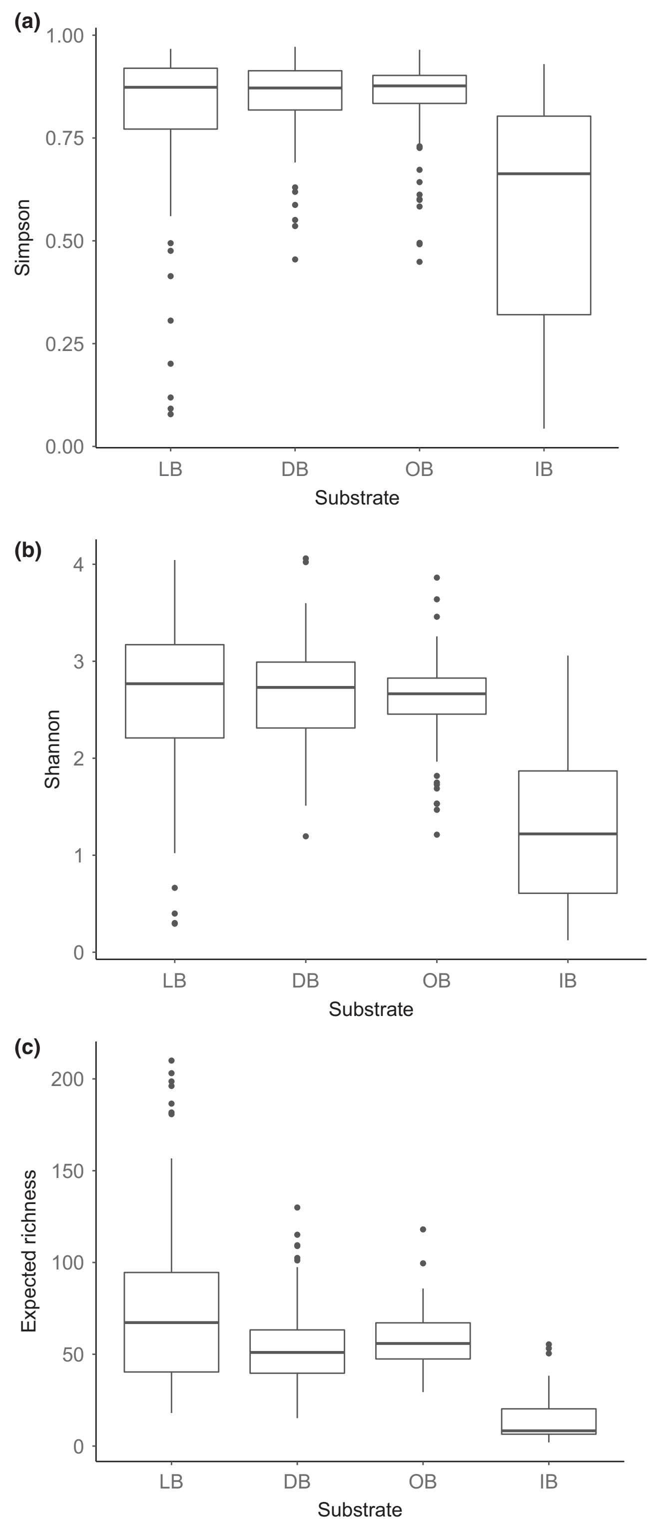

FIGURE 2 Alpha diversity by substrate type (live bryophytes = LB, dead bryophytes = DB, outer bark = OB, inner bark = IB) using expected species richness at 1,000 sequences per sample (a) and Simpson (b) and Shannon (c) indices on data rarefied to 1000 sequences. LB have the highest per-sample expected richness, and IB has the lowest diversity according to all three indices

Of the 5777 nonsingleton OTUs recovered, 2,468 were found in at least two substrate types (Figure S3), 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.

The substrates differed in their ratios of Ascomycota to Basidiomycota reads ($p < .001$, Figure S4), with LB having a higher ratio than OB or DB, which did not differ. Most fungal reads could not be assigned to a primary lifestyle with FungalTraits, limiting our ability to infer differences among substrates, though lichenized fungi appear to be more abundant in LB and litter saprotrophs more abundant in DB (Figure S2).

3.4 | Spatial structure

Mantel tests showed significant, positive linear correlations between overall fungal community dissimilarity and distance

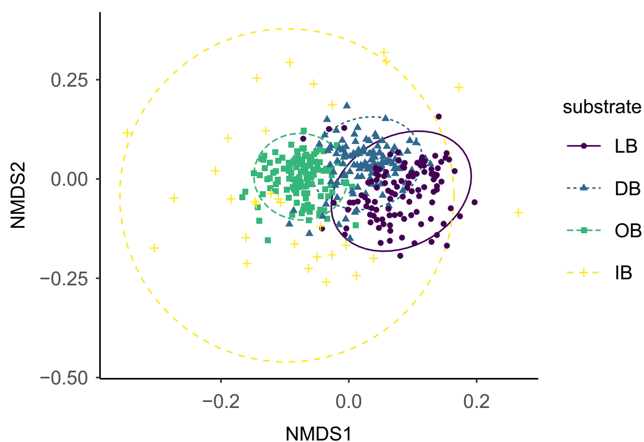

FIGURE 3 Nonmetric multidimensional scaling (NMDS) ordination plot showing differences in fungal communities among LB, DB, OB and IB with 95% confidence ellipses. Stress = 0.2820

between points for the three substrates examined (Table 1). As we predicted, plots of community similarity against geographical distance (Figure 4; Figure S5) and Mantel correlograms (Figure 5) show dramatic declines in similarity over very short distances. The correlograms show significant positive autocorrelation, with community composition being more similar among samples than expected by chance, only at the smallest distance classes, up to 89 cm depending on the substrate type. Bray–Curtis similarities tended to be very low even between adjacent points. The initial similarities (modelled Bray–Curtis similarity at a distance of 0 cm) for LB, DB and OB were 0.279, 0.513 and 0.547, respectively. These low initial similarities declined by half within the first 10 cm (Table 1). Distance decay lines using presence–absence data had slopes near zero (DB: −0.0036, LB: −0.0018, OB: −0.0076; Figure S6a), but when only OTUs representing at least 1% of a sample were considered, they closely resembled the greater slopes seen when using relative abundance (presence–absence DB: −0.0159, LB: −0.0125, OB: −0.0262; relative abundance DB: −0.0253, LB: −0.0198, OB: −0.0333; Figure S6b). For all substrates, distance decay levelled off and positive autocorrelation was lost between 30 and 90 cm. Distance decay patterns were only apparent within branches; nearly all the distances between branches were larger than the distance range over which similarity decays.

In agreement with our hypothesis, LB had the lowest similarities at small distances and the lowest initial similarity (Figure 4, Table 1). OB and DB had similar distance decay patterns and initial similarities. In the Mantel correlograms, OB and DB had positive autocorrelation in the first two distance classes, out to 89 cm. LB, however, lost positive autocorrelation after the first distance class, at 30 cm. All substrates showed negative autocorrelation at some, but not all, larger distance classes.

Spatial patterns of individual groups of fungi, as assigned to primary lifestyles and growth forms using FungalTraits, frequently differed from the general trends seen in the entire fungal community (Figures S7–S15). For example, LB had significant positive autocorrelation out to the third distance class of 147 cm for soil saprotrophs (Figure S11) while the other substrates only had positive

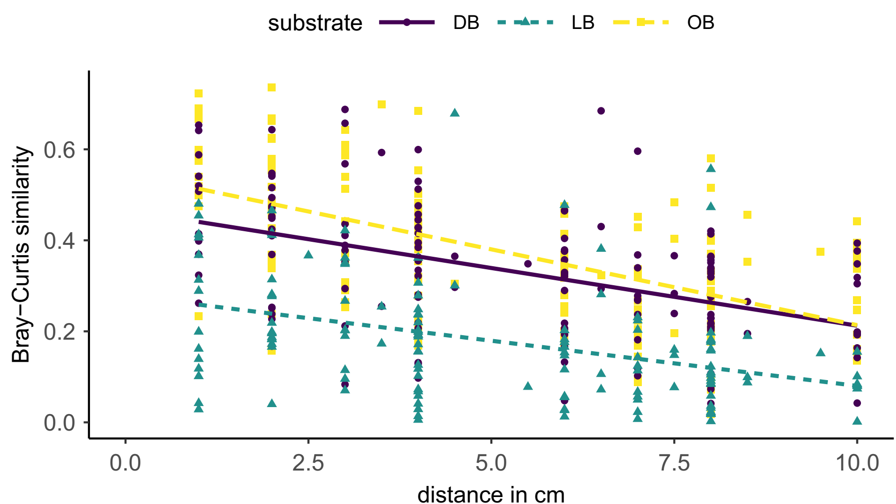

FIGURE 4 The first 10 cm of distance decay by substrate type using relative abundance data and Bray–Curtis similarity. Similarity values are low, even at the shortest distances, and decay rapidly over the first 10 cm. LB have less similarity among samples at short distances than the other substrates

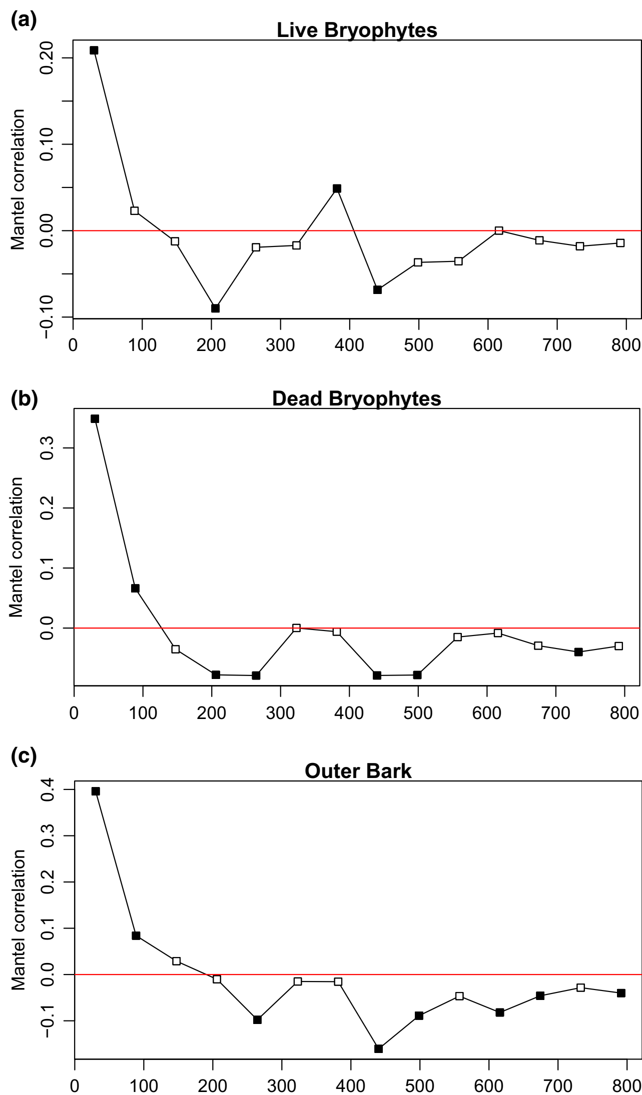

FIGURE 5 Mantel correlograms demonstrating spatial autocorrelation of the fungal community in LB (a), DB (b) and OB (c). Filled symbols indicate significant autocorrelation at that distance class. Significant positive autocorrelation is lost after the first or second distance class, which are less than 1 m, for all three substrates

autocorrelation in the first class of 30 cm. Filamentous mycelia followed similar patterns as the whole fungal community except in DB, for which they only had positive autocorrelation in the first distance class and not the second (Figure S13). Yeasts did not have significant autocorrelation in LB at all, while they did have significant autocorrelation in the first distance class for OB and DB (Figure S15). Inability to assign most fungal reads to a primary lifestyle or growth form limits our ability to fully understand the spatial structures of these groups, as many OTUs were excluded from these analyses. Further, some primary lifestyles and growth forms included few OTUs and samples relative to the whole data set. This may further impact spatial analyses.

Ascomycota followed the same general spatial trend in all three substrates, with positive autocorrelation out to the first two distance classes (Figure S16). Basidiomycota spatial structure differed among substrates with positive autocorrelation only at the first distance class in LB, to the second in OB and to the third in DB (Figure S17). In OB and LB, Ascomycota communities had greater average similarity among samples than Basidiomycota at all distances and both phyla had similar decay in similarity over 100 cm (Figure S18). In DB, however, distance decay lines for these two phyla had markedly differing slopes, with Basidiomycota having a steeper slope. At smaller distances Basidiomycota communities had greater intersample similarity than Ascomycota, but this trend was reversed at larger distances (Figure S18). Overall, these results did not support our hypothesis that Ascomycota would display spatial structure at a finer scale than Basidiomycota.

RDA and variance partitioning revealed that substrate type, branch and PCNM spatial vectors have significant relationships with total fungal community composition ($p < .001$, Figure 6). Position along branch did not have a significant relationship independent of the other partitions ($p > .05$). Of these, the spatial vectors explained the largest portion of the variance, at 7.8%. Position along branch explained the least, and most of the variance explained was shared with branch and the spatial vectors. Most of the variation explained by branch was also shared with the spatial vectors. Substrate alone explained 4.3% of the variance. Most of the variance, 85.9%, was unexplained by any variable.

4 | 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 metres to kilometres and among hosts or substrates (Bahram et al., 2015; Davey et al., 2013; Koide et al., 2017; Mummey & Rillig, 2008). Few, however, have combined the power of high-throughput sequencing of total fungal communities with centimetre-scale sampling and geospatial analyses. To our knowledge, this is the first study to apply this suite of methods to tropical canopy branch surfaces.

Our study revealed 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 1). In agreement with our results, 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 6), probably due to the high spatial turnover that occurs within each substrate type.

In accordance with our hypothesis, we found the greatest species richness, both per sample and in total, in the live bryophyte substrate (Figure 2, Table 1). Greater diversity in LB might be related to the complex three-dimensional structure of living bryophytes (Rice et al., 2014), heterogeneous tissues that can host specialized fungal pathogens and

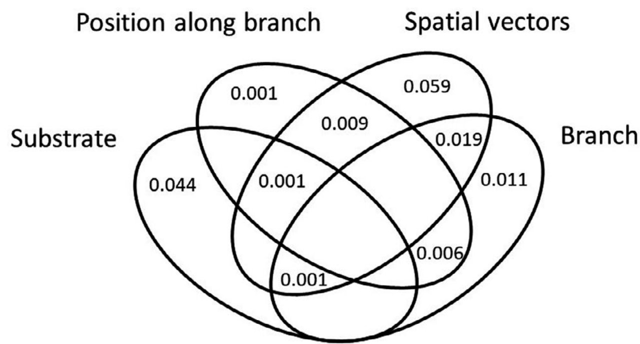

FIGURE 6 Venn diagram of variance partitioning analysis showing the effects of substrate type, branch, proximity to distal position along a branch, and the PCNM vectors on fungal community composition. Partitions without numbers explain <0.1% of the variation

parasites (Boehm & McLaughlin, 1988; Davey & Currah, 2006), and active antifungal defences (Andersson et al., 2005; Mekuria et al., 2005). It may also be related to position of the substrate. When present, LB were the topmost substrate and could receive more fungal propagules than lower substrates. LB are also the youngest substrate, as underlying DB and tree bark must necessarily be older. In a study of 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. In other systems, priority effects have been shown to influence fungal community dynamics (Kennedy & Bruns, 2005; Leopold et al., 2017). An important area for future work is to measure the degree to which fungal species arrival is stochastic or deterministic and the degree to which competition or other forces shape subsequent community composition.

Inner tree bark had the lowest fungal diversity per sample and across the whole substrate (Figure 2, Table 1), and many samples failed to amplify or were dominated by plant sequences. We infer that fungi had very low abundances in this substrate. Unlike OB, 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 IB were present in all other substrate types (Figure S3). They may have entered the inner bark from these other substrates or were introduced as contaminants during sample collection or processing. We found no evidence of pervasive endophytes in the living inner bark of *Saurauia*.

As we predicted, different fungal primary lifestyles, growth forms and phyla were not distributed evenly among substrates. Lichen richness was highest in LB, consistent with that substrate being the topmost layer where lichens would have access to sunlight. Wood saprotrophs and Basidiomycota were more diverse in OB, as would be expected. Contrary to our hypothesis, saprotrophic groups were not most diverse in DB, though these results may reflect the large number of OTUs that could not be assigned to a primary lifestyle and were excluded from analysis.

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 the forces that drive fungal diversity, be they stochastic dispersal, abiotic environmental factors and/or unmeasured biotic factors, do not act on all substrates at the same point in the same way.

We hypothesized that community spatial structure and distance decay would be observed at distances of centimetres to metres. We document distance decay of community similarity at even smaller spatial scales than expected, with similarity declining rapidly over the first 50 cm (Figure 4; Figure S5). In all substrates studied, there was no significant positive autocorrelation observed beyond 90 cm (Figure 5) for the whole fungal community. In LB, 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 metres to kilometres (Peay & Bruns, 2014) and in soils from centimetres to metres (Carvalho et al., 2003; Klironomos et al., 1999), but we are unaware of any studies addressing this issue for airborne spores at spatial scales relevant to our study in tropical ecosystems.

Even more striking than the small scale of spatial structures is the limited overlap in species composition among samples. Samples collected 10 cm apart often hosted ≥50 OTUs each but had few to no OTUs in common (Figure S6). This was especially pronounced in LB, which had the lowest intersample similarity at all distances. The environmental variable we used to explain intrasubstrate community patterns, distance along the branch, which can act as a proxy for other variables such as light exposure and moisture, was a poor predictor of community composition (Figure 6). This suggests that stochastic processes such as dispersal limitation play a strong role in structuring communities. Given that we only see spatial structure at the submetre 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, most 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 probably small, such that single fungal individuals 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.

In addition to differences among substrates, spatial structure varied among fungal taxonomic and functional groups. Detectable spatial structure was lacking in some groups, such as yeasts in live bryophytes (Figure S15). Contrary to our hypothesis, Ascomycota did not consistently display spatial structure at smaller scales than Basidiomycota, and Ascomycota communities tended to be more similar to each other at any given distance than Basidiomycota communities (Figure S18). While Ascomycota spatial trends were similar across LB, DB and OB, Basidiomycota varied between these three substrates. This indicates spatial structure may be driven, in part, by biological and ecological differences among groups of fungi. While these probably include body size and dispersal mechanism, they may also include ecological interactions such as competition and mutualism and the spatial arrangement of different resources in the environment.

While our results reinforce accumulating evidence for spatial structure and distance decay of similarity of fungal communities in soils and other substrates (Bahram et al., 2015; Oono et al., 2017; Thomas et al., 2019), direct comparisons with other research on spatial patterns of fungi are difficult. Measurements of distance–decay relationships are 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 m. In contrast, we only find autocorrelation at distances less than 1 m, though the processes driving spatial structure of soil vs. epiphytic fungi probably differ. Some other studies that cover a small spatial extent (centimetres to a few metres) have also found evidence of spatial clustering and autocorrelation of fungi being confined to the submetre scale (Mummey & Rillig, 2008; Oja et al., 2017; Tedersoo et al., 2003; Yoshida et al., 2014) or within-branch scale (Donald et al., 2020).

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 1 m 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 et al., 2015; Shefferson et al., 2007). Pathogenic fungi can also target specific groups of plants, while leaving others unaffected (Barrett et al., 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 submetre spatial scales and among substrates from the same sample point. Small-scale spatial patterns are probably driven by dispersal limitation and other stochastic processes, and they probably 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.

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AUTHOR CONTRIBUTIONS

K.C.: performed research, analysed data, wrote the paper. J.S.: designed research, performed research. A.T.: designed research, performed research, analysed data, edited the paper. I.H.: performed research. L.T.: designed research, performed research, edited the paper.

BENEFITS GENERATED

Benefits from this research accrue from the sharing of our molecular data on a public database as described above.

DATA AVAILABILITY STATEMENT

Raw sequences generated by Illumina MiSeq and OTU representative sequences are available on GenBank through BioProject PRJNA762332.

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