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Communities of fungal endophytes in tropical forest grasses: highly diverse host- and habitat generalists characterized by strong spatial structure

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ABSTRACT

Plant-symbiotic fungi influence the structure and function of all terrestrial ecosystems, but factors shaping their distributions in time and space are rarely well understood. Grasses (Poaceae), which first arose and diversified in tropical forests, harbor diverse but little-studied endophytes in the lowland forests of Panama. We used sequence data for 402 isolates from two sampling years, 11 host species, and 55 microsites at Barro Colorado Island, Panama to investigate the influence of host and habitat (soil type, forest age) in shaping endophyte diversity and composition. In contrast to previous studies, we found no evidence for host- or habitat specificity. Instead, endophytes demonstrated strong spatial structure consistent with dispersal limitation, with community similarity decaying markedly over a scale of hundreds of meters. Spatial structure that is independent of host species and habitat reveals remarkable heterogeneity of endophyte–host associations at small geographic scales and adds an important spatial component to extrapolative estimates of fungal diversity.

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Introduction

A fundamental goal of ecology is to understand the factors that determine the distributions of species. All macroscopic species engage in close biotic interactions with microbes, and the outcomes of such interactions can differ under particular

environmental conditions or as a function of the local pool of interacting species and genotypes (Thompson, 2005; Gallery et al., 2007; Pan et al., 2008; Peay et al., 2010). Therefore, it is important to diagnose not only the geographic distributions of microbial symbionts, but also the underlying abiotic and biotic constraints that shape their ecological associations at

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multiple spatial scales. Plant–microbe symbioses are among the most important biotic forces shaping the structure and function of terrestrial plant communities (e.g., [Read et al., 2004](#)), but in contrast to knowledge regarding the distributions of host plants, relatively little is known regarding the forces that delineate distributions of microbial partners in time and space ([Martiny et al., 2006](#); [Fierer, 2008](#)).

One of earth's most common plant–microbe symbioses is that of endophytic fungi ([Arnold, 2007](#)). Fungal endophytes – defined functionally as fungi that occur within healthy plant tissues without causing overt harm ([Petrini, 1991](#)) – are known from every major lineage of plants, and from all terrestrial biomes. These primarily ascomycetous fungi include one especially species-rich and ubiquitous functional group, the Class 3 endophytes (sensu [Rodriguez et al., 2009](#)). Class 3 endophytes (hereafter, endophytes) typically are horizontally transmitted, form numerous, independent, and highly localized infections in healthy above-ground tissues of plants, and have been recorded from every plant species examined to date ([Rodriguez et al., 2009](#)). In lowland moist forests in the Neotropics, these endophytes are especially diverse: a mature, asymptomatic leaf of a dicotyledonous tree usually hosts more than a dozen cultivable species, with turnover in species composition among leaves, individual plants, and geographically distant sites (e.g., [Lodge et al., 1996](#); [Saikkonen et al., 1998](#); [Arnold et al., 2003](#); [Arnold and Herre, 2003](#); [Arnold and Lutzoni, 2007](#); [Arnold et al., 2009](#)). Their ecological roles have not been studied in most cases, in part because of the complexity of assessing the roles of individual strains in the context of assemblages that can comprise hundreds of endophyte species for a single host plant. However, recent work has begun to reveal important interactions between tropical endophytes and their hosts, including defense against pathogens and herbivores, alteration of photosynthetic efficiency, and changes in water relations (e.g., [Pinto et al., 2000](#); [Arnold et al., 2003](#); [Arnold and Engelbrecht, 2007](#); [Van Bael et al., 2009a, 2009b](#)).

In general, very little is known regarding the factors that shape the distributions of endophytes at local or regional scales. Significant turnover among biomes can be ascribed to differences in plant communities, abiotic factors such as seasonality, and underlying biogeographic history for both plant and fungal partners ([Arnold and Lutzoni, 2007](#); [Arnold et al., 2009](#); [U'Ren et al., 2012](#)). However, relatively little research has been done at a scale appropriate to determine the relative importance of host- and habitat features in shaping local distributions. For tropical endophytes, a growing body of literature indicates that abiotic factors such as relative humidity, exposure to ultraviolet radiation and desiccation, and the density of leaf litter can shape the abundance and composition of inocula at small spatial scales, yielding at least short-term effects on the number of endophyte infections per leaf, their diversity, and their composition ([Rodrigues and Samuels, 1990](#); [Rodrigues, 1994](#); [Lodge et al., 1996](#); [Rodrigues and Dias, 1996](#); [Bayman et al., 1998](#); [Fröhlich and Hyde, 1999](#); [Arnold et al., 2000, 2001](#); [Guo et al., 2001](#); [Kelemu et al., 2001](#); [Gamboa and Bayman, 2001](#); [Cannon and Simmons, 2002](#); [Suryanarayanan et al., 2002](#); [Arnold and Herre, 2003](#); [Arnold and Lutzoni, 2007](#)). However, factors shaping endophyte communities in sites with relatively uniform abiotic and biotic

conditions, such as the understory of intact tropical forests, are not known. Spatial heterogeneity has been detected in several studies within individual forests (e.g., [Arnold et al., 2000](#)), but because such studies typically focus on only a small number of sites, the spatial scale of turnover in endophyte communities within forests has not been determined. Similarly, the interplay of distance and microhabitat conditions such as soil type or land-use history, important in shaping some plant–fungal associations (e.g., [Dumbrell et al., 2010](#)), has not been evaluated. In turn, strict-sense host specificity of tropical endophytes appears to be rare ([Cannon and Simmons, 2002](#); [Suryanarayanan et al., 2002](#); [Pandey et al., 2003](#); [Murali et al., 2007](#); [Arnold and Lutzoni, 2007](#)), although conclusions are somewhat uncertain because signatures of host preference have been reported in some communities (e.g., [Suryanarayanan et al., 2000](#)).

We examined the relative importance of hosts and habitat characteristics in structuring endophyte communities in a lowland tropical forest. We focused on grasses (Poaceae), which first arose and diversified in the shaded margins of tropical forests ([Kellogg, 2001](#)). In a companion paper ([Higgins et al., 2011](#)), we showed that grasses in the forest understory harbor highly diverse Class 3 endophytes, rather than the well-studied clavicipitaceous endophytes that characterize many pasture, woodland, and domesticated forage grasses (i.e., Class 1 endophytes, sensu [Rodriguez et al., 2009](#)). Our finding of host generalism among fungal communities as a whole, and in more detailed analyses of two common genera, *Colletotrichum* and *Anthostomella* ([Higgins et al., 2011](#)), prompted us to explore other ecological factors that might influence endophyte assemblages. Here, we use sequence data from cultivable fungi obtained from two sampling years and 55 geographically proximate sites to investigate soil type and forest age as factors that may structure endophyte communities.

Materials and methods

This study was conducted at Barro Colorado Island, Panama (BCI; ~9°9' N, 79°51'W), a former hilltop isolated from mainland forests by the creation of Gatun Lake in 1914. The island is composed of mature forest (>400 yr old) and secondary forest in areas cleared approximately 100 yr ago. It has been protected as a research reserve since 1923 and maintained by the Smithsonian Institution since 1946. For a full site description see [Leigh et al. \(1996\)](#).

As detailed in [Higgins et al. \(2011\)](#), we focused on 11 locally common species representing six subfamilies of Poaceae (sensu [Barker et al., 2001](#)) ([Table 1](#)). All are perennial and occur frequently in the understory of primary and secondary forest at BCI ([Croat, 1978](#)). In addition to representing a phylogenetically diverse array of species, focal hosts represented subfamilies that arose and persisted in forest environments (Anomochlooideae, Pharoideae, Bambusoideae, Ehrhartoideae), and subfamilies that ancestrally transitioned to open environments (Centothecoideae, Panicoideae) ([Kellogg, 2001](#)).

Fifty-five study sites were selected to maximize coverage of the 1400 ha island ([Supplementary Appendix 1, Fig 1](#)). For each site we collected information on soil type ([Fig 1](#)) and forest age

Table 1 – Results of a culture-based survey of endophytic fungi associated with healthy foliage of eleven species of grasses in the forest understory at Barro Colorado Island, Panama (see Higgins et al., 2011, where these values were first presented; these are included here to provide context for analyses of spatial, interannual, edaphic, and forest-type effects). Columns indicate currently recognized subfamilies (Barker et al., 2001), plant species, the number of leaves sampled, isolation frequency (mean \pm SE, calculated from the proportion of leaf segments per individual from which a fungus was isolated in culture), the number of OTU (based on 99% nrITS-partial LSU sequence identity), and diversity (Fisher's alpha) based on OTU.

Subfamily	Species	Leaves sampled	Isolation frequency	Fisher's alpha
Anomochlooideae	<i>Streptochaeta spicata</i>	16	0.51 \pm 0.27	22.2
Pharoidaeae	<i>Pharus latifolius</i>	18	0.58 \pm 0.38	17.5
Bambusoideae	<i>Chusquea simpliciflora</i>	18	0.80 \pm 0.16	34.7
	<i>Lithachne pauciflora</i>	18	0.44 \pm 0.31	43.1
	<i>Olyra latifolia</i>	18	0.66 \pm 0.26	16.6
	<i>Rhipidoeladum racemiflorum</i>	15	0.91 \pm 0.10	28.4
	<i>Streptogyna americana</i>	18	0.73 \pm 0.15	26.2
Ehrhartoideae	<i>Orthoclada laxa</i>	17	0.53 \pm 0.19	14.6
Panicoideae	<i>Ichnanthus pallens</i>	18	0.67 \pm 0.29	15.1
	<i>Oplismenus hirtellus</i>	18	0.90 \pm 0.16	47.5
	<i>Panicum pilosum</i>	18	0.61 \pm 0.29	18.2
	Total	192	–	60.1
	Mean \pm SD	–	0.67 \pm 0.16	25.9 \pm 11.5

Isolation frequency and diversity did not differ significantly among subfamilies (comparisons based only on Bambusoideae and Panicoideae, from which multiple species were sampled; $F_{1,5} = 0.0294$, $P = 0.8705$, $F_{1,5} = 0.1197$, $P = 0.7434$, respectively).

(mature or secondary) from the literature (Leigh et al., 1996; Baillie et al., 2007). Common grasses at BCI are restricted to areas with light to medium canopy cover and relatively open areas of the understory (Croat, 1978), such that all sites were similar with regard to these microhabitat features.

Each host species was collected from six sites with the exception of *Rhipidoeladum racemiflorum*, which was collected from five (Supplementary Appendix 1). Each collection site for a given species was as distant as possible from other collection sites for conspecifics (average distance = 2.3 km). When available, multiple species were collected from the same site.

Tissue collection and processing

Mature, healthy leaves were collected as described in Higgins et al. (2011). Briefly, in the early rainy season (May–Jul.) of 2006 and 2007 one healthy, asymptomatic leaf from each of three individuals per species per site (with a few exceptions, Supplementary Appendix 1) was collected in a clean plastic bag, transported to the lab, and processed within 6 hr. Each leaf was rinsed with running tap water, cut into 2 mm² pieces, and surface sterilized using sequential washes of 70% EtOH (2 min), 10% commercial bleach (Clorox, with pre-dilution concentration of 5.25% NaClO₂; 2 min) and 95% EtOH (30 s) (see Arnold and Herre, 2003; Arnold and Lutzoni, 2007). Fifteen segments were chosen haphazardly from each leaf and plated onto 2% malt extract agar (MEA), which supports growth by diverse endophytes (Fröhlich and Hyde, 1999; Arnold et al., 2000). Imprints of treated leaf pieces onto 2% MEA yielded no fungal growth following incubation for 14 d, confirming that surface-sterilization was effective (Arnold et al., 2009). Plates were incubated at room temperature under natural light and dark cycles, and emergent growth isolated into pure culture on 2% MEA. Living vouchers were deposited with the International Cooperative Biodiversity Group at the Smithsonian Tropical Research Institute, Panama City, Panama.

Molecular analyses

Four hundred and two representative isolates were selected on the basis of culture morphology for molecular analysis. Isolates selected for sequencing represented all host species and sites, as well as both study years (Supplementary Appendix 1). Representatives of all morphotypes were sequenced, and morphotypes were sequenced in proportion to their abundance (Arnold, 2002).

DNA was extracted from fresh mycelium following Arnold and Lutzoni (2007). The nuclear ribosomal internal transcribed spacers and 5.8s gene (nrITS) and the first 600 bp of the large ribosomal subunit (partial LSU) were amplified by PCR as a single fragment (nrITS-partial LSU) following Higgins et al. (2011). SYBR Green staining of gel-electrophoresed products showed a single band for each. Amplicons were cleaned, normalized, and sequenced in both directions on an AB 3730XL (Applied Biosystems, Foster City, CA) using PCR primers (5 μ M) at the University of Arizona Genomics and Technology Core. Reads were assembled and bases called automatically using *phred* and *phrap* (Ewing et al., 1998) with automation by Mesquite (Maddison and Maddison, 2007). Base calls and sequence trimming were verified by manual inspection in Sequencher v4.5 (Gene Codes Corporation, Ann Arbor, MI). Consensus sequences were submitted to BLAST searches for preliminary identification at higher taxonomic levels (Supplementary Appendices 2 and 3) and archived at GenBank under accessions EU686744–EU687191.

Sequences were grouped into operational taxonomic units (OTU) based on percent sequence identity (Arnold et al., 2007; Arnold and Lutzoni, 2007; Hoffman and Arnold, 2008; U'Ren et al., 2009, 2010; Higgins et al., 2011). Because the precise amount of sequence identity within vs. between biological species of tropical endophytes is not known, and at best provides only a proxy for delineating species or other taxonomic units, analyses were conducted using OTU defined by 95%

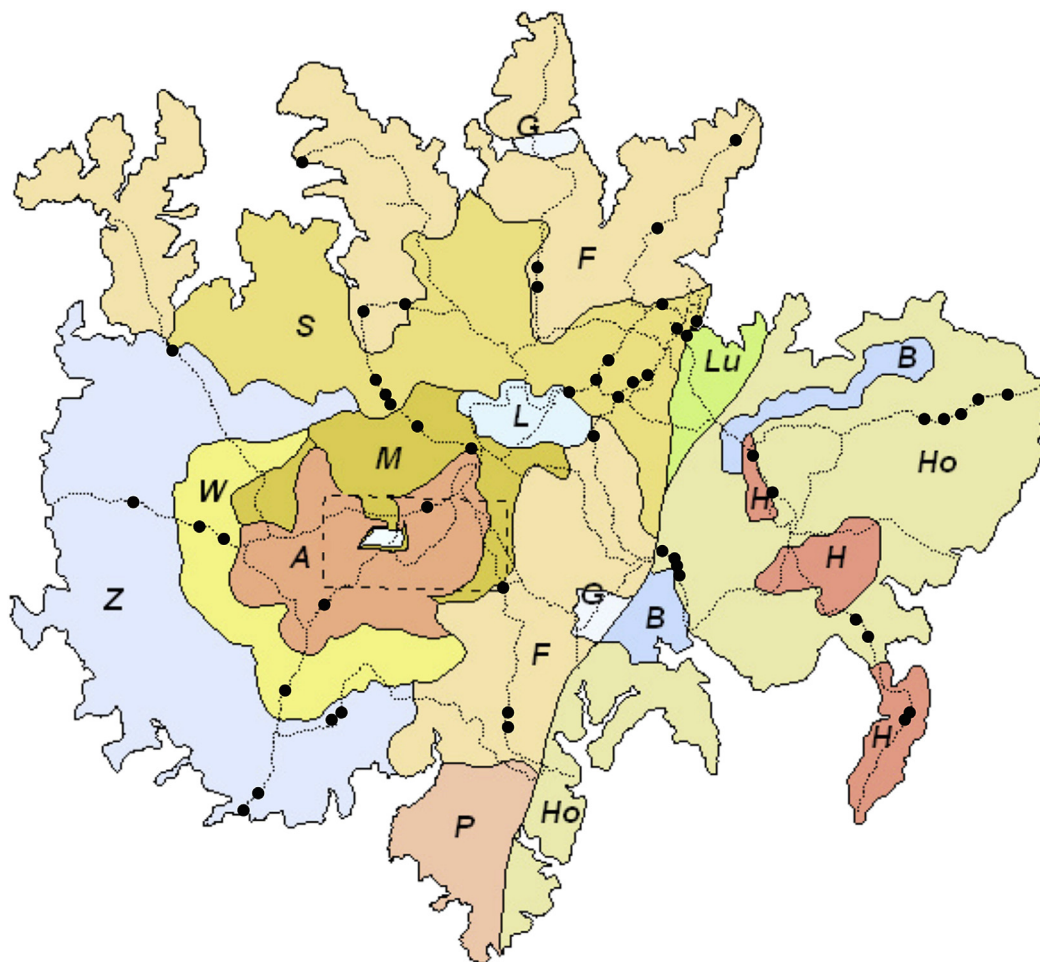


Fig 1 – Map of soil types of Barro Colorado Island, with 55 unique collection sites for this study marked with dots. Soil type abbreviations are based on nearby trail names (see Baillie et al., 2007, which contains complete description of characteristics of each site). Soil types are abbreviated as follows: A – AVA, B – Barbour, F – Fairchild, G – Gross, H – Harvard, Ho – Hood, L – Lake, Lu – Lutz, M – Marron, P – Poacher, S – Standley, W – Wetmore, and Z – Zetek.

sequence similarity, previously validated as a proxy for species in four common genera of tropical endophytes (U'Ren et al., 2009), 97% similarity (O'Brien et al., 2005), and 99% similarity, which provides genotype-level resolution while still allowing for minor sequencing error (Gallery et al., 2007). Because we examined host and site preferences at a relatively fine scale, we report analyses based on 99% sequence similarity. Our conclusions did not change when 95% and 97% sequence similarity were used (data not shown). Genotype groups were assembled in Sequencher v4.5, following Arnold et al. (2007, 2009), Higgins et al. (2007, 2011) and U'Ren et al. (2009, 2010, 2012).

Statistical analyses

Statistical analyses were conducted in R v2.9.0 (R Development Core Team, 2008) using the *ape*, *cluster*, *vegan*, *MCMCpack*, *qvalue*, and *LabDSV* packages (<http://www.cran.r-project.org>). The endophyte community of each host/site combination was defined as all sequenced isolates recovered from that host species in that site. Diversity was measured as Fisher's alpha, following Higgins et al. (2011) and Arnold and Lutzoni (2007). Because sampling intensity for sequencing

differed slightly among hosts and sites, matrices of genotype distributions among communities were transformed to presence/absence data and included only those genotypes occurring in at least two communities (McCune and Grace, 2002). A similarity value was calculated for every pair of communities using the one-complement of the Bray/Curtis (Sørensen) dissimilarity index (Legendre and Legendre, 1998; McCune and Grace, 2002). Pairwise physical distance was estimated using a scaled map and ranged from <3 m (two hosts in the same location) to 5.4 km (opposite sides of the island).

We used three methods to test the predictions that endophyte assemblages from the same host species, soil type, or forest type would be more similar to each other than those from different host species, soil-, or forest types. First, host preference was examined using a cluster analysis based on the Bray/Curtis dissimilarity index for each pair of communities. Clusters were determined using hierarchical clustering with Lance–Williams flexible beta linkage, where $\beta = -0.25$ (Kaufman and Rousseeuw, 1990; McCune and Grace, 2002).

Second, we evaluated the effects of host species, soil type, and forest type using multiresponse permutation procedures (MRPP) and Analysis of Similarity (ANOSIM). MRPP tests the

similarity (or homogeneity) of group members against the mean of all groups, and determines if groups are more or less similar than expected by chance. Positive values of the chance-corrected within-group agreement statistic (A) indicate more similarity within groups than would be expected by chance (with $A = 1$ as the highest possible value). ANOSIM is similar in execution but uses rank similarity scores instead of raw scores, with the ANOSIM R value interpreted similarly to the MRPP A statistic (McCune and Grace, 2002). The year in which the cultures were obtained (2006 or 2007) also was tested as a predictor of high community similarity. Indicator Species Analysis (ISA) was used to evaluate the respective contributions of individual genotypes to the patterns observed by ANOSIM and MRPP. A genotype's indicator value was calculated as the product of its relative abundance in one group (compared to all groups) and its relative frequency in all communities in the group. Significance was determined by comparing the observed indicator value to those obtained from randomizing species among groups 1 000 times (McCune and Grace, 2002).

Last, we constructed design matrices of each of the categorical factors (soil type, forest type, year) to use Mantel tests, which examine correlation between symmetric distance or similarity matrices. The values in design matrices indicate whether the comparison is between groups (0) or within a group (1). Because soil type and forest age are spatially auto-correlated (Leigh et al., 1996; Baillie et al., 2007), partial Mantel tests were used to examine effects of total environmental similarity (a design matrix combining the soil and forest type information for each site) on endophyte community similarity while controlling for distances between sites (McCune and Grace, 2002). A partial Mantel test also was used to determine the effect of year while accounting for distance between sites, as many sites were spatially aggregated within a sampling period. Significance of p -values was corrected for multiple comparisons as appropriate: only p -values with the positive false discovery rate q -value < 0.05 were considered significant (Benjamini and Hochberg, 1995; Storey, 2002, 2003).

Results

As reported in our previous study (Higgins et al., 2011), culturable endophytes were recovered from every plant examined and from 2 264 of 2 925 tissue segments overall (77.4% of segments; mean, $67.4 \pm 16\%$ per species; Table 1). Isolation frequency did not differ significantly among hosts (Table 1). A representative sample of 402 sequenced isolates, including isolates from both sampling years and all morphotypes, host species, and sites, comprised 94 putative species (Fisher's alpha = 38.3, based on 95% sequence similarity groups) and 124 genotypes (Fisher's alpha = 60.1, based on 99% sequence similarity groups; Table 1, Supplementary Appendix 2). Bootstrap analyses indicated an estimated richness of 155 genotypes, of which 80% were recovered. Seventy-six genotypes were recovered only once (61.3%). Genotypic diversity per host species ranged from Fisher's alpha = 14.6–47.5 (mean \pm SD = 25.9 ± 11.5) and did not differ significantly among host lineages that were sampled sufficiently for comparison (Table 1). BLAST comparisons revealed higher-level taxonomy

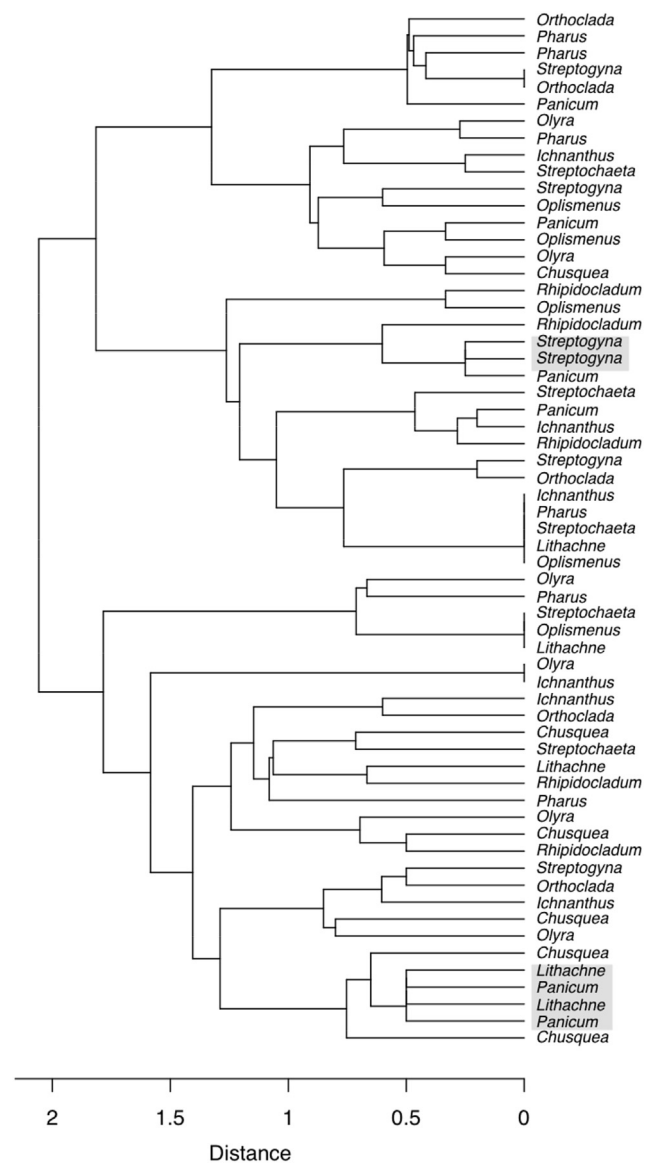


Fig 2 – Results of cluster analysis based on dissimilarity values between pairs of endophyte communities. Each community represents an independent collection of an individual of a given host species within a site, and is labeled with the genus of the plant host. The x-axis represents the distance between clusters as calculated by the Lance–Williams flexible equation with $\beta = -0.25$. Gray shading indicates communities that display closest affinity to assemblages isolated from the same host plant.

for 377 isolates (Supplementary Appendices 2 and 3), with the remainder having ambiguous or unknown placement. Members of at least three classes and 20 families of Ascomycota were recovered (Supplementary Appendix 3).

Forest age and soil type

A simple Mantel test revealed a significant correlation between the design matrix of overall environmental similarity

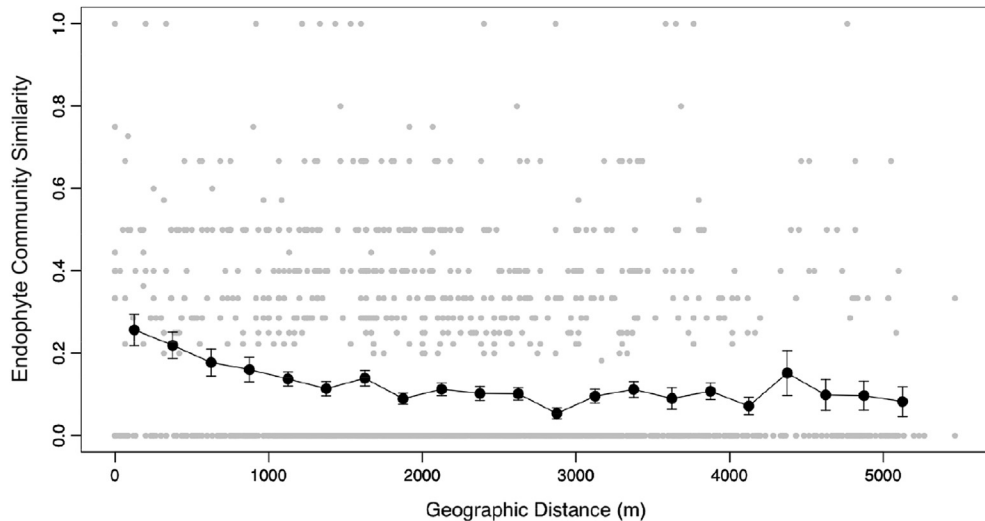


Fig 3 – Similarity of endophyte communities (based on presence/absence data for nonsingleton OTU) between pairs of host–site combinations vs. the physical distance between sites (gray circles). Physical distance was segmented into classes corresponding to consecutive 250 m intervals (0–249 m, 250–499 m, etc.), and the mean similarity of each class is shown with a filled black circle. Means are connected with lines to emphasize the decline in similarity as distance between sites increases ($p < 0.01$, Mantel test). Bars around circles represent standard errors.

(soil- and forest type) and the similarity matrix of fungal assemblage composition (Mantel's $r = 0.07$, $p < 0.05$). By itself, forest type had a small but significant effect on the similarity of endophyte assemblages (MRPP $A = 0.02$, $p < 0.005$; ANOSIM $R = 0.09$, $p < 0.01$). Within-soil-type similarity was significantly lower than between-soil-type similarity (MRPP $A = 0.03$, $p < 0.05$), but this result was not supported by ANOSIM. However, forest types and soil types were partially auto-correlated in a spatial fashion (data not shown). When the design matrix of environmental similarity was examined with a partial Mantel test to take intersite distances into account, the relationship between community similarity and environmental similarity became nonsignificant (partial Mantel's $r = 0.05$, $p = 0.90$). Likewise, partial Mantel tests on separate design matrices of forest- and soil-type similarity revealed nonsignificant effects in each case (forest: $r = 0.04$, $p = 0.83$; soil: $r = 0.04$, $p = 0.88$).

Host affinity

Cluster analysis revealed no structuring of endophyte communities by their hosts. In general, assemblages did not cluster according to host taxonomy (Fig 2, Table 1), with communities from the same host species grouping together only three times. In each case they were members of unresolved clusters containing assemblages from grasses in different species and subfamilies (Fig 2). ANOSIM and MRPP confirmed that community similarity did not differ significantly within vs. among host species (ANOSIM, $p = 0.74$; MRPP, $p = 0.80$). Indicator Species Analysis confirmed that no fungal genotype was a significant indicator of any host plant species (data not shown).

Spatial structure

Distance between sites significantly influenced the similarity of endophyte assemblages. Communities in greater proximity to each other were more similar than those that were more distant (Fig 3; Mantel's $r = -0.20$, $p = 0.001$), with marked decay in similarity over relatively small distances (hundreds of meters; see Fig 3). A partial Mantel test that took environmental similarity (soil and forest type) into account still revealed a significant effect of distance between sites (partial Mantel's $r = -0.19$, $p < 0.001$). When the same procedure was used to account for the effects of year, the distance effect remained significant (partial Mantel's $r = -0.20$, $p < 0.001$).

Interannual variation

Communities sampled during the same year were significantly more similar to each other than expected by chance (MRPP $A = 0.05$, $p < 0.001$; ANOSIM $R = 0.14$, $p < 0.01$). A simple Mantel test confirmed a significant relationship between community similarity and year (Mantel's $r = 0.13$, $p < 0.01$). This result persisted even after distance between collection sites was taken into account (reflecting clustered sampling during each year; partial Mantel's $r = 0.12$, $p < 0.01$).

Substantial differences in community structure were observed between years. Among 45 genotypes that occurred in at least two sites, four appeared only in 2006, and 19 were recovered only in 2007 (Supplementary Appendix 2). Although the most common genotype (genotype 5, Xylariales) was dominant in both years, the next five most abundant genotypes were each found in only one sampling year. Only five of 124 genotypes were significant indicators of year, including two for 2006 (genotypes 3 and 30, with indicator values of 0.41

and 0.23, respectively) and three for 2007 (genotypes 17, 4, and 18, with indicator values of 0.49, 0.26, and 0.19) (for estimated taxonomic placement, see [Supplementary Appendix 2](#)). The remaining year-specific genotypes were not considered significant indicators because they were recovered too rarely during a given year.

Discussion

We examined factors shaping endophyte communities in eleven species of tropical forest grasses at Barro Colorado Island, Panama, including representatives of six ecologically and phylogenetically diverse subfamilies of Poaceae. We found no systematic differences in isolation frequency or diversity of endophytes among hosts, nor evidence for host specificity at the level of grass species or subfamily. No significant effects of forest age or soil type on endophyte communities were observed once intersite distance was taken into account. Instead, our analyses demonstrate strong spatial structure in endophyte assemblages, with marked decay of similarity between sites with increasing distance ([Fig 3](#)). Appreciable disparities were observed among sites separated by hundreds of meters, with localities ca. 1 km from one another as different as those ca. 5 km apart. Previous studies have shown that similarity of endophyte communities decreases between sites over larger geographic distances (e.g., [Arnold et al., 2003](#); [Arnold and Lutzoni, 2007](#); [Hoffman and Arnold, 2008](#); [U'Ren et al., 2012](#)), but our study is unique in having a spatially explicit sampling design and an appropriate geographic scale to reveal spatial structure consistent with dispersal limitation in a single study area, and the first to clearly differentiate such effects from habitat- or host-driven patterns.

The global diversity of fungi has been discussed actively for the past 20 yr, with special attention to extrapolative estimates of species richness based on host- and geographic specificity (e.g., [Hawksworth, 1991, 2001](#)). Host generalism and a lack of detectable habitat selectivity due to edaphic or forest characteristics appear to suggest that high estimates of fungal richness be treated with caution. However, although non-specific with regard to hosts, forest age, and soil conditions, the rich community of endophytes observed in these tropical grasses suggests that even host- and habitat generalists can display local spatial structure that will contribute substantially to biodiversity at regional and larger scales.

Habitat- and host generalism

Because soil type and land-use history influence plant community structure in tropical forests (e.g., [Croat, 1978](#)), we anticipated that endophyte communities would reflect underlying differences in soil composition and forest age (see also [Gamboa and Bayman, 2001](#); [Arnold et al., 2003](#)). However, once effects of soil- and forest type were corrected for spatial autocorrelation, neither significantly influenced endophyte community structure. Due to the limited range of conditions in which tropical understory grasses can be found at BCI ([Croat, 1978](#)), we were unable to compare microhabitats that differed in light or leaf litter density, which can influence inoculum density, infection frequency, and endophyte

diversity (e.g., [Arnold and Herre, 2003](#); [Herre et al., 2007](#)). At present, our data reveal the relative insensitivity of these horizontally transmitted, highly diverse endophytes to major edaphic characteristics and forest age, and contrast with belowground symbionts such as arbuscular mycorrhizal fungi ([Dumbrell et al., 2010](#)) and broader communities of soil microbes ([Fierer and Jackson, 2006](#)).

Although host preference may be observed among some endophytes of distantly related tropical trees that differ markedly in leaf-defense syndromes (e.g., [Arnold and Herre, 2003](#)), mounting evidence suggests that tropical endophytes rarely demonstrate strict-sense host specificity (e.g., [Cannon and Simmons, 2002](#); [Murali et al., 2007](#); see also inoculation experiments presented in [Arnold et al., 2003](#); [Mejía et al., 2008](#)). Such host generalism is consistent with [May's \(1991\)](#) suggestion that strong host affinity will be rare in communities containing a high diversity of potential host plants, and [Arnold and Lutzoni's \(2007\)](#) observation of lower host affinity among endophytes of angiosperms in tropical vs. boreal sites. Our data provide additional evidence of host generalism of tropical endophytes at the levels of host species and subfamily. Recent analyses further suggest that many of the genotypes recovered here also are found in leaves of sympatric dicotyledonous trees, and that unculturable endophytes of these grasses also are host-generalists ([Higgins et al., 2011](#)).

Because many assemblages of plant-associated fungi are structured by habitat- and host traits ([Suryanarayanan et al., 2000](#); [Arnold et al., 2003](#); [Pan et al., 2008](#); [Saunders and Kohn, 2009](#); [U'Ren et al., 2010](#); see also [Dumbrell et al., 2010](#)), we evaluated our conclusions in light of several challenges to assessing ecological associations of endophytes. Among the most significant is the need to sample with statistical sufficiency to support analyses of host- or habitat preference ([Arnold, 2007](#)). Our recovery of ca. 80% of expected genotypic diversity, both overall and from each host species (see [Higgins et al., 2011](#)); the consistent dominance of a single genotype among hosts (genotype 5, [Supplementary Appendix 2](#)); and the absence of even a nonsignificant trend for higher similarity of communities within vs. among habitats or hosts support our conclusions of generalism.

A second challenge lies in the high richness of endophyte communities: many species or OTU often are recovered only once (e.g., [Arnold et al., 2000](#); [Davis and Shaw, 2008](#); [Gazis and Chaverri, 2010](#)). Analyses of host- or habitat preference necessarily exclude these singletons, such that our conclusions are based on fewer than half of the OTU recovered here – and thus reflect only the distributions of the most common genotypes. Culture-free methods such as direct-PCR and cloning have been proposed to overcome this problem, but genotypes recovered rarely in culture are not necessarily found more frequently when these methods are used in tandem ([Arnold et al., 2007](#); [Higgins et al., 2011](#)). The difficulty of assigning ecological affinities to rare taxa in endophyte surveys may be resolved through next-generation sequencing approaches, at least to a point (see [Jumpponen and Jones, 2009](#)). In the meantime, phylogenetic studies suggest that regardless of the method of observation, rarely recovered endophytes often are closely related to common genotypes or phylotypes (frequently conspecific or congeneric; if not, then representing the same families and orders; see [Arnold et al., 2007](#); [Higgins](#)

et al., 2011, but see Gazis et al., 2012). To our knowledge, rare and common species thus appear to share relatively recent evolutionary histories rather than representing markedly different lineages in the fungal tree of life, and may have similar patterns of host- and habitat generalism.

A third challenge lies in evaluating occurrence vs. incidence – that is, whether community similarity should be evaluated based on presence/absence data (occurrence), or on the abundance of fungi in each sample (incidence; see Arnold et al., 2000, 2001; U'Ren et al., 2010). Lodge (1997), among others, suggested that tropical fungal communities more clearly exhibit host affinity when examined in terms of relative abundance rather than presence/absence alone (see also Arnold and Herre, 2003). We used conservative presence/absence measures because our estimates of sequence abundance were not strictly representative of total culture abundance (i.e., a slightly larger proportion of isolates was sequenced for some morphotypes relative to others). However, when analyses were performed using isolation frequencies, we found no evidence for host structuring in these communities (data not shown). These results were corroborated in our evaluation of two common genera (*Colletotrichum* and *Anthostomella*) in previous work (Higgins et al., 2011).

Finally, investigating ecological associations requires data that provide the proper level of resolution to distinguish biologically meaningful taxonomic units. The nrITS-partial LSU region used here evolves at different rates among fungi, and although useful in distinguishing species and intraspecific strains in many taxa, it can be uninformative at these levels in many cases (e.g., Rojas et al., 2010). Therefore, we may have overlooked some fine-scale host- or habitat affinity by artificially “lumping” distinct organisms into a single OTU. In such a case we would expect the effects of distance to be obscured as well, yet distance-based effects were strong (Fig 3). In turn, phylogenetic analyses of two of the most common genera obtained in this survey reveal that although an individual OTU can contain several well-supported clades, these clades do not show any notable host- or habitat affinity (Higgins et al., 2011). Future work using microsatellite markers or other fine-scale tools may detect population-level associations that are ecologically meaningful (e.g., Oono et al., 2011) but at present our dataset does not reveal such patterns.

Taken together our results suggest that endophytes associated with grasses in this tropical forest are diverse host-generalists that are not strongly sensitive to forest age or edaphic characteristics, and are capable of infecting not only a wide array of phylogenetically diverse grasses (this study) but leaves of dicotyledonous trees in the same forest as well (Higgins et al., 2011).

Strong spatial structure

When effects of forest age and soil type were taken into account, our data revealed strong spatial heterogeneity in endophyte communities. Regardless of host or habitat traits, similarity of communities decayed significantly over the scale of only a few hundred meters. Although similarity of endophyte communities was typically low even within the same site (mean similarity < 0.30, Fig 3), similarity declined linearly to <0.10 at a distance of ca. 1 km between sampling sites.

Arnold et al. (2003) reported a striking decline in similarity of endophyte assemblages at larger spatial scales (up to 350 km), but did not sample densely enough within localities to reveal such fine-scale decay. That study reported higher similarity values overall because their analyses relied on isolation frequency data, used morphotypes rather than genotypes as OTU, and excluded zero values, which were considered here.

Our observation of marked decay of similarity over relatively small distances is consistent with dispersal limitation, long considered important in shaping the distributions of many macroscopic organisms but rarely invoked as a force that defines ranges of microbial species (see discussion in deWit and Bouvier, 2006; Finlay, 2002; Whittaker et al., 2003; Peay et al., 2010). Recent developments in molecular ecology and systematics have fostered a new perspective regarding distributions of superficially similar prokaryotes and microbial eukaryotes at multiple scales (e.g., Telford et al., 2006), and have begun to indicate that small-scale spatial heterogeneity is an important component of microbial community ecology. Our data support re-evaluation of the potentially major importance of dispersal limitation in contributing to local, regional, and large-scale diversity of microbial species, including endophytic fungi.

Interannual variation

We observed striking differences in endophyte assemblages in the 2 yr of our study. At present we lack sufficient information to determine whether these differences are part of a dynamic cycle of population change driven by environmental factors around a relatively stable underlying distribution, or if endophyte distributions are undergoing random “ecological drift” over time. Studies capturing a greater number of endophytes with replicated sampling structure over multi-year periods are needed but have not yet been conducted in any biome. We anticipate that such studies will reveal, as in the case of macrofungi (e.g., Straatsma et al., 2001; Mueller et al., 2004), far greater richness than is observed in any short-term study, and will provide new perspectives on the distribution – and remarkable scale – of fungal biodiversity.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.funeco.2013.12.005>.

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