Title: Phylogenetic relationships and spatial distributions of putative fungal pathogens of seedlings across a rainfall gradient in Panama

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Abstract

Despite their importance in structuring plant communities, the identities and spatial distributions of the pathogens impacting wild plant communities are largely unknown. To advance our knowledge of plant-pathogen interactions in tropical forests, I identified likely fungal pathogens from forest sites across a rainfall gradient in Panama and compared the communities of fungi inhabiting a wetter, Atlantic and a drier, Pacific forest (~45 km apart). Seedlings with symptoms of pathogen attack were collected and fungi were isolated from the symptomatic tissue. Based on internal transcribed spacer region sequences, I assigned the fungal isolates to operational taxonomic units (OTUs) and estimated their taxonomic placements. I observed 28 OTUs (defined by 95% sequence similarity); primarily, the genera *Mycoleptodiscus*, *Glomerella*, *Bionectria*, *Diaporthe*, and *Calonectria*. The wetter, Atlantic and drier, Pacific forest sites shared 29% of observed and 56% of non-singleton fungal OTUs, suggesting that, in these forests, the common fungal pathogens of seedlings are relatively widespread, habitat generalists.

Keywords: fungus-plant interactions; pathogen; seedling; tropics; distributions; diversity; tropical forests; trees; Ascomycota

1. Introduction

Fungi are tightly linked to plants as mutualists, pathogens, and decomposers and there is increasing appreciation of the importance of these positive and negative plant-fungal interactions in shaping plant relative abundances (Mangan et al., 2010) and distributions (Nuñez et al., 2009; Defosse et al., 2011; Spear et al., 2015). A plant's ability to colonize new areas may be hindered by the absence of a mutualist (Nuñez et al. 2009), or disease-sensitive plants may be excluded from areas with elevated pathogen pressure (Defosse et al., 2011; Spear et al., 2015). Despite their ubiquity and importance for natural plant communities, basic knowledge about the identities, spatial distributions, and host ranges of phytopathogens in natural systems is limited (but see Davidson, 2000; Augspurger and Wilkinson, 2007; Gilbert and Webb, 2007; Hersh et al., 2012; Schweizer et al., 2013).

To understand how pathogens shape plant communities, it is necessary to identify the pathogens attacking plants and describe their biogeography. The spatial distributions of pathogens influence how disease pressure varies in space and whether or not plants can escape from pathogens. In fact, two widely cited hypotheses have spatially explicit assumptions. Under the Janzen–Connell hypothesis, focused on the maintenance of local plant diversity through distance- and density-dependent pressure from natural enemies, seeds and seedlings perform better when located away from reproductive adults or areas with a high density of conspecifics (Janzen, 1970; Connell, 1971; reviewed in Comita et al., 2014). Under the enemy release hypothesis, successful invasions can be explained by exotic plants escaping their natural enemies (e.g., fungal pathogens), which presumably regulate their populations in their native ranges (Keane and Crawley, 2002; reviewed in Mitchell et al., 2006).

In contrast with the aforementioned hypotheses, which are contingent on a release from pathogens, Spear et al. (2015) provided evidence that, across a rainfall gradient in Panama, seedlings of tree species common in the drier forests are attacked and killed by pathogens in the wetter forests where those dry-forest tree species are normally absent or rare. This suggests that the pathogens attacking seedlings of dry-forest tree species in the wetter forest may be relatively widespread geographically and, by extension, that the tree species cannot spatially escape their pathogens. Given that host-specific pathogens should accumulate in the vicinity of their hosts and that the rainfall gradient is correlated with a distinct turnover of plant species (Pyke et al., 2001), those geographically widespread pathogens must also be capable of damaging multiple host species. An alternative hypothesis for the lack of escape from disease for dry-forest tree species in a forest where they do not naturally occur is that they are being attacked by novel (to them) pathogens that are relatively host-generalized but have spatially restricted ranges. These hypotheses for the lack of escape from disease in geographic space, and their potential discord with commonly invoked hypotheses, further highlight the need for descriptions of the pathogens attacking seedlings and their geographic distributions.

Here, I report the phylogenetic relationships and spatial distributions of fungi likely causing disease in seedlings, to supplement our understanding of plant-pathogen interactions in tropical forests. I used a survey-based approach to: (i) identify fungi likely attacking seedlings in forest sites across a rainfall gradient that spans the Isthmus of Panama; and (ii) compare the communities of putative pathogens in a wetter, Atlantic forest and a drier, Pacific forest. Specifically, fungi were isolated from symptomatic tissue of seedlings and, based on internal transcribed spacer region sequences, I used phylogenetic analyses to infer the taxonomic placement of the isolated fungi. I focused on fungal pathogens that attack seedlings because

mortality during the seedling phase represents a major bottleneck that directly shapes plant communities (Engelbrecht et al., 2007; Comita et al., 2010; Mangan et al., 2010; Baldeck et al., 2014; Green et al., 2014), and because fungi are major agents of seedling mortality in the tropics (Gilbert, 2005).

I surveyed putative fungal pathogens across a rainfall gradient because, for numerous macroorganisms, species distributions are correlated with precipitation gradients. For instance, there is a dramatic turnover in plant species across the same rainfall gradient in Panama (Pyke et al., 2001). Ergo, one might expect fungal pathogens to exhibit turnover across the gradient, with the fungal pathogens in the drier forests differing from those in the wetter forests, in response to changes in the abiotic and/or biotic conditions. However, while the environmental conditions of the wetter, Atlantic and drier, Pacific forests differ, the forests are in relatively close proximity (~45 km apart) and there is evidence that many fungal taxa have large, even cosmopolitan, geographic ranges (Rosendahl et al., 2009; Queloz et al., 2011; Pöhlme et al., 2013; Tedersoo et al., 2014; Davison et al., 2015). Thus, I predicted that the fungal pathogens commonly attacking seedlings are widespread geographically and that the most common fungal pathogens are habitat generalists, inhabiting both the drier and wetter forests.

2. Methods

2.1. Collection of symptomatic seedlings from forests across a rainfall gradient that spans the Isthmus of Panama

During the rainy seasons of four years (2007, 2010 - 2012), 75 seedlings with observable pathogen damage were collected from seven forest sites across a north to south rainfall gradient that spans the Isthmus of Panama (Fig. S1 and Table 1). The majority (76%) of the seedlings were collected from two forest sites (PNM and SRR), one on the drier, Pacific side of the

Isthmus and one on the wetter, Atlantic side (29 and 28 of the 75 seedlings, respectively; Table 1). Total annual rainfall increases from ≤ 1800 mm of rain year⁻¹ on the drier side to ≥ 3000 mm of rain year⁻¹ on the wetter side (Condit, 1998; Santiago et al., 2004). The rainfall is highly seasonal and total annual rainfall is influenced by the duration of the dry season (ca. 62 d longer on the drier side than the wetter side) and the frequency and duration of dry spells during the wet season (Condit, 1998; Engelbrecht et al., 2006). In addition to differing annual precipitation, the wetter versus drier forests host markedly different plant communities (Pyke et al., 2001), have different soils (Brenes-Arguedas et al., 2008; Condit et al., 2013), and have different understory light levels (Brenes-Arguedas et al., 2011). Also, the best sampled wetter forest site (SRR), located on private property (~ 32 ha), has mixed-age, evergreen forest and is at a higher elevation (200–250 m) than the best sampled drier forest site [Parque Natural Metropolitano (PNM), ~232 ha, elev 50–95 m] that has mixed-age, semi-deciduous forest.

The symptomatic seedlings from which fungi were isolated represent 21 tree species in 11 phylogenetically diverse families (Table S1), including tree species that naturally occur in the wetter, Atlantic and/or the drier, Pacific forests. When possible, (i) seedlings of multiple tree species were collected from the same forest site (1-14 tree species per site, mean = 5), (ii) seedlings of a given tree species were collected from multiple forest sites (1-5 sites per tree species, mean = 1.67), and (iii) multiple seedlings of a given tree species were collected within a single forest site (1-11 seedlings per tree species per site, mean = 2.14). The number of seedlings collected per tree species ranged from one to 18 and not all species were collected from all forest sites (Tables 1 and S1).

Symptomatic seedlings were obtained in two ways: (1) opportunistic collection of naturally occurring, symptomatic seedlings ($N = 10$); and (2) planting seedlings to bait pathogens

from the soil and, in doing so, separate the pathogens from the numerous other organisms in the soil ($N = 65$; Beales, 2012). For the seedling baits, seeds ($N = 43$) or seedlings ($N = 22$) of 19 of the 21 tree species were planted in common gardens in 30 to 40 haphazardly-selected locations per forest site (Tables 1 and S1). In 2007, seeds were germinated in a greenhouse and then the seedlings were transplanted into the forests. In 2010 and 2012, seeds were planted directly in the forests to allow for surface sterilization before planting (as described in Spear et al., 2015). All seedling baits were monitored (weekly or monthly censuses) for symptoms of pathogen attack. The same tree species were planted in the wetter and drier forests to disentangle the effects of host identity and forest site on the fungal pathogens observed and to ensure that the patterns observed are habitat- rather than host-driven.

Live, symptomatic seedlings were collected to maximize the likelihood of isolating the disease-causing fungus or fungi as opposed to saprotrophic fungi which often colonize recently killed plant tissue. Characteristic symptoms of pathogen attack included dark, sunken necrotic lesions on the roots and stem, foliar necrosis, and collapse of the stem at the soil line (i.e., damping off) (Agrios, 2005) and, in some cases, the biotic agent was observed on the seedling (e.g., mycelia).

2.2. Isolation of fungi from symptomatic seedlings

Symptomatic seedlings were collected from the forests and transported to a lab where symptomatic tissue was excised and plated on a selective medium to isolate the disease-causing fungus or fungi. Seedlings were first rinsed under running tap water to remove surface debris. Leaf, stem, and/or root tissue was then excised from the advancing margin of disease, where the causative pathogen is likely to be more abundant or active than secondary, saprotrophic colonizers. Excised tissue pieces were surface sterilized via sequential immersions in 70% EtOH

(2 min), 10% commercial bleach (Clorox, with predilution concentration of 5.25% NaClO; 2 min), and 95% EtOH (30 s) and plated on either MEA (malt extract agar) with 2% chloramphenicol (following Gilbert and Webb, 2007; 59 isolates), water agar (20 isolates), or PARP (pimaricin, ampicillin, rifampicin, and pentachloronitrobenzene) agar (11 isolates). PARP contains antifungals and was originally used with the intention of isolating oomycetes; however, only fungi were cultivated on the PARP plates, suggesting that there was a problem with the antifungals or medium preparation. All five fungal operational taxonomic units (defined by 95% sequence similarity) isolated on PARP were also isolated on MEA and/or water agar. Hyphal growth emerging from the plated plant tissue was subcultured to isolate fungi into pure culture. All plates were maintained in an air-conditioned lab. Living vouchers of these fungal isolates are stored as agar plugs suspended in sterile distilled water with the International Cooperative Biodiversity Group at the Smithsonian Tropical Research Institute, Panama City, Panama.

While isolating fungi directly from the advancing margin of symptomatic tissue should increase the likelihood of isolating the disease-causing fungus or fungi, the fungi isolated are not necessarily the causative pathogens. Saprotrophic fungi often colonize recently killed plant tissue and may outgrow the disease-causing pathogen(s) in culture. Traditionally, phytopathologists establish causation by inoculating healthy plants with the putative pathogen, in order to generate the symptoms originally observed, and by re-isolating the putative pathogen (fulfilling Koch's postulates; Agrios 2005). I did not conduct proof of pathogenicity in this study. Furthermore, while I did estimate the taxonomic placements of the fungal isolates (methods described in subsequent Methods sections), fungi cannot be reliably classified as pathogens based on their taxonomic affiliations because members of a given genus often represent a range of lifestyles, including pathogens, mutualistic endophytes, and saprotrophs (Delaye et al. 2013). As such, I

cautiously refer to all fungi isolated in this study as “likely” or “putative” pathogens and all interpretations and discussions of the fungal communities should be treated with similar caution.

2.3. Molecular analyses

DNA from 90 isolates was used for molecular identification based on the nuclear ribosomal internal transcribed spacer (ITS) region (ca. 600 base pairs), which is the accepted fungal DNA barcode (Schoch et al., 2012). Fragments of fungal mycelia were collected from each isolate and preserved in sodium dodecyl sulfate (SDS) extraction buffer for up to 1 y. As symptomatic seedlings were collected over multiple years, the generation of DNA sequence data was completed over multiple years and by multiple labs, including the Arnold Lab at the University of Arizona, the International Cooperative Biodiversity Group at the Smithsonian Tropical Research Institute (STRI), and the molecular research lab at STRI’s Naos Marine Laboratories (see Fig. S2 for details about the primers used). The extraction of DNA from mycelia, PCR amplification, and bidirectional sequencing methods followed each lab’s specific protocols (Higginbotham et al., 2014; Sandberg et al., 2014).

Forward and reverse sequence reads were assembled using Sequencher 5.2 (Gene Codes, Ann Arbor, MI, USA). For seven isolates, either the forward and reverse reads failed to form a consensus region of DNA or only the forward read successfully amplified, resulting in unidirectional reads. For those seven isolates, I used the forward read to estimate taxonomic placement and assign membership in operational taxonomic units. All ITS sequences were manually trimmed and edited in Sequencher 5.2. Sequence data will be made publicly available in the National Center for Biotechnology Information (NCBI) GenBank database (accessions XXXX-XXXX).

2.4. Phylogenetic analyses

To estimate the taxonomic placements of the fungal isolates, I referenced the 84 edited consensus sequences and six unidirectional reads against the fungal ITS DNA sequences in the GenBank database via the NCBI's nucleotide Basic Local Alignment Search Tool (BLASTn) algorithm (accessed September 2014; Altschul et al., 1990) and then conducted maximum likelihood and Bayesian phylogenetic analyses (following Higginbotham et al., 2014). First, I identified groups of apparently closely related isolates by aligning the sequences for all 90 isolates in the web-based Multiple Sequence Comparison by Log- Expectation (MUSCLE) tool (Edgar, 2004). I visually inspected that alignment to identify clusters of sequences that aligned well to one another. A cluster of sequences that aligned easily was treated as a group of closely related fungal isolates; hereafter referred to as “dataset”. There were 31 datasets in total and each dataset contained from one to 13 of the 90 fungal isolates (Table S2). The ITS sequences for all isolates in a given dataset were referenced against sequences in GenBank and the top 50 BLASTn matches for each were downloaded. The top 50 matches for all isolates in a given dataset were compiled and then redundant sequences and sequences from potentially misidentified strains and/or unvouchered specimens were removed. Whenever possible, I included at least one sequence from a reliable culture collection (e.g., ATCC - American Type Culture Collection, Manassas, Virginia) in the compilation of sequences for a dataset. Based on the named BLASTn matches for a dataset, I selected outgroups by consulting the 10th edition of the Dictionary of Fungi (Kirk et al., 2008) and published phylogenetic studies. Sequence data for the outgroups were acquired from GenBank. Each dataset was then individually aligned in MUSCLE and the alignments were trimmed to relatively consistent starting and ending points in Mesquite 2.75 (Maddison and Maddison, 2011).

To develop phylogenetic hypotheses using maximum likelihood (ML) and Bayesian methods, it is necessary to specify a model of nucleotide substitution. For each dataset, different models of substitution were compared in R ver. 3.3.0 (R Core Team, 2016) using the ‘modelTest’ function in the package ‘phangorn’ (Schliep, 2011). For 28 of the 31 datasets, the general time reversible (GTR) model with gamma distributed rate variation among sites (G) and a proportion of invariable sites (I) had the most support. For trees AB, AD, and H (see Table S2 and the Appendix in the Supplementary Materials), the best model of substitution was GTR+I.

Phylogenetic trees were inferred by: (i) maximum likelihood (support determined by 100 bootstrap replicates; starting tree generated by a fast ML stepwise-addition algorithm) using the Genetic Algorithm for Rapid Likelihood Inference web service (GARLI 2.1) hosted at molecularevolution.org (Zwickl, 2006; Bazinet et al., 2014); and by (ii) Bayesian methods (5 million generations, four chains, two runs, random starting trees, sampling every 1,000th tree, and the first 25 % of samples from the cold chain discarded as burn-in) using MrBayes ver. 3.2.2 (Ronquist and Huelsenbeck, 2003) accessed via the CIPRES Science Gateway web portal (Miller et al., 2010). The trees were visualized in FigTree ver. 1.4.2 (Rambaut, 2007). The topologies reflect the majority rule consensus trees based on maximum likelihood analyses and support for each clade is presented as ML bootstrap values ($\geq 50\%$) and Bayesian posterior probabilities ($\geq 50\%$). The resulting trees are included in the Supplementary Appendix.

2.5. Assigning operational taxonomic units

The 90 ITS sequences were grouped into operational taxonomic units (OTUs) based on different thresholds of sequence similarity (90, 95, 97, and 99%) and at least 40% sequence overlap using the program Sequencher 5.2. Only those sequences ≥ 350 bp were included and six of the 90 sequences were unidirectional reads. U’Ren et al. (2009) found 95% sequence

similarity to be appropriate for delimiting species boundaries for some of the taxa recovered in this study. Thus, the summary statistics and ecological analyses reported herein are based on OTUs defined by 95% sequence similarity. Results based on 90, 97, and 99% sequence similarity are reported in the Supplementary Materials (Tables S3 and S4; Figs. S3 and S4).

2.6. Ecological analyses

I extrapolated OTU accumulation curves using the “specaccum” function (Oksanen et al., 2013). The curves incorporate fungi from all 75 seedlings collected from the forest sites and represent the mean accumulation of OTUs for 100 randomizations of seedling order derived from the observed richness. I plotted the OTU abundance distributions based on Fisher's log-series using the “fisherfit” function (Oksanen et al., 2013).

Here, I report observed richness, the number of singletons and their contribution to the observed richness, abundance-based estimates of extrapolated richness [Chao1 (classic formula) and Abundance Coverage-based Estimator (ACE)], and the diversity index Fisher's alpha for: (i) fungi isolated from symptomatic seedlings collected from all seven forest sites spanning the precipitation gradient and the isthmus; (ii) fungi isolated from symptomatic seedlings collected from the best sampled forest site on the drier, Pacific side of the isthmus (PNM; Table 1); and (iii) fungi isolated from symptomatic seedlings collected from the best sampled forest site on the wetter, Atlantic side of the isthmus (SRR; Table 1). Diversity was measured by Fisher's alpha (α) because it is reasonably robust for comparing unequal sample sizes and because it is less sensitive to small sample sizes than other diversity indices (e.g., Simpson's index; Condit et al., 1996; Leigh, 1999; Magurran, 2004).

To explore a potential relationship between annual precipitation and the community composition and spatial distributions of putative fungal pathogens, I compared the fungi isolated

from diseased seedlings collected from the best sampled drier forest site to the fungi isolated from diseased seedlings collected from the best sampled wetter forest site (PNM and SRR, respectively; Table 1). A variety of seedling collection and fungal isolation methods were used to generate the 90 fungal isolates in this study and seedlings of all tree species were not harvested from both the drier and wetter forests (Tables 1 and S1). To avoid potential confounding effects from methodological differences, I used a subset of the 90 isolates to compare the wetter and drier forests. The subset ($N = 44$) includes only fungal isolates: (i) cultivated on MEA; (ii) from seedlings that originated from surface-sterilized seeds planted to bait pathogens; and (iii) from the six tree species for which at least one seedling of each species was collected in both the wetter and drier forest sites (SRR and PNM, respectively; see Table S1).

I used three methods to assess community similarity for the putative pathogens in the best sampled drier forest site (PNM) and the best sampled wetter forest site (SRR; Table 1). I first used the classic Jaccard index of similarity, which is based on incidence data (presence/absence, ignoring relative abundance), to facilitate comparison with other studies. The Jaccard index of similarity ranges from 0 to 1, with 0 indicating no overlap in the OTUs present and 1 indicating complete overlap. Second, I used Chao's abundance-based Jaccard similarity index, which was developed to reduce the bias (generally, an underestimation of similarity) associated with small sample sizes, unequal sampling, and diverse assemblages with a large proportion of rare taxa by accounting for unseen taxa (Chao et al., 2005). Third, I used a nonparametric analysis of variance using distance matrices (adonis) with 999 permutations and Chao as the method to calculate pairwise distances (Chao takes into account unseen taxa) to test for differences in the two groups' centroids. Because 'adonis' is sensitive to dispersion or spread effects (Anderson, 2001), I tested whether dispersion differs between wetter and drier forest sites (homogeneity of

variance) using the ‘betadisper’ function. The significance of the fitted model was analyzed with a standard parametric ANOVA. The putative fungal pathogen communities in the drier and wetter forest sites have homogenous dispersions (average distance to median: drier forest = 0.53, wetter forest = 0.61; $F_{1,10} = 1.03$, $P = 0.335$). For the adonis and betadisper analyses, the frequencies of fungal OTUs were summed for each host species by site combination [e.g., *Anacardium excelsum* seedlings collected from the drier forest site (PNM) would be one experimental unit].

Ecological analyses were performed in R ver. 3.3.0 (R Core Team, 2016) using the packages ‘vegan’ (Oksanen et al., 2013) and ‘SpadeR’ (Chao et al., 2016).

3. Results and discussion

3. 1. Identities of the putative fungal pathogens

Culturable fungi were isolated from 75 seedlings with observable symptoms of pathogen attack, yielding 90 isolates (Table S2). Initial investigations of taxonomic placement were made by referencing the sequence data against those sequences deposited in GenBank via BLASTn. Fifty (56%) of the 90 isolates had top matches to named strains (Table S2). The top matches to named strains tentatively suggested placement in the Sordariomycetes (Diaporthales, Hypocreales, and Xylariales), Dothideomycetes (Botryosphaeriales and Capnodiales), and Eurotiomycetes (Eurotiales).

I used phylogenetic analyses to assign taxonomic placements with greater confidence. Identifications based on the top BLAST hits agreed with the phylogenetically-informed estimations for 39 of the 90 isolates (Table S2). Of the 39 congruencies, the phylogenetic analyses provided greater taxonomic resolution than the top BLAST match in eight cases (Table S2). For six of the fungal isolates, the top BLAST match appears to be misidentified at the

genus-level based on the phylogenetic analyses (Table S2). For seven of the isolates, their top BLAST hits were identified to genus and, based on the phylogenetic analyses, I could only confidently make family-level assignments (Table S2). Finally, of the 40 top hits that had no taxonomic information, I was able to assign 33 to genus, one to family, and six to class, as a result of the phylogenetic analyses (Table S2).

Based on the phylogenetic estimations, all 90 fungal isolates belonged to the largest phylum of Fungi, Ascomycota, and its largest subphylum, Pezizomycotina (Kirk et al., 2008). Ascomycota make up the majority of described fungi and are ecologically diverse, including saprotrophs involved in decomposition and nutrient cycling, mutualistic endophytes, and pathogens of plants (Kirk et al., 2008). The majority of the fungal isolates were Sordariomycetes (92%) and the rest were Dothideomycetes (4%) and Eurotiomycetes (3%). Thirteen of the 83 fungal isolates classified as Sordariomycetes were estimated to be members of the genus *Mycoleptodiscus* (Magnaporthaceae), which has uncertain placement within the Sordariomycetes (Kirk et al., 2008). Two of the isolates classified as Sordariomycetes were not closely related to any reliable, named sequences in GenBank (see tree G in the Supplementary Appendix). Isolates TBA262 (532 bp) and TBA050 (527 bp) had 94 and 95% sequence similarity, respectively, to their closest, cultured matches in GenBank, identified as leaf litter ascomycetes (Table S2). Isolates TBA262 and TBA050 may represent unknown taxa or described taxa that have not yet been sequenced. Further analyses are required to clarify their taxonomic affiliations.

The 90 fungal isolates represented nine orders and 12 families (Table 2). Sixty-seven of the 90 isolates (74%) could confidently be assigned to 16 genera (Tables 2 and S2). The Hypocreales was the most commonly observed order (41% of the isolates), followed by the Magnaporthales (14% of isolates) (Table 2). The five genera most frequently isolated from

diseased seedlings, *Mycoleptodiscus*, *Glomerella*, *Bionectria*, *Diaporthe*, and *Calonectria* (Table 2), include species known to be pathogens of plants and overlap with fungal pathogens of seedlings in a temperate forest (specifically, *Gomerella*, *Bionectria*, and *Diaporthe*; Hersh et al., 2012).

3.2. Richness and diversity of the putative fungal pathogens

The 90 fungal isolates, from 75 seedlings, represented 28 operational taxonomic units (OTUs), defined by 95% sequence similarity. The abundance distribution exhibited a hollow-curve shape, a common pattern for a wide variety of ecological communities (Magurran, 2004). Most of the fungal OTUs were rare (20 of the 28 OTUs were isolated less than five times) and relatively few were abundant, with the two most frequently isolated OTUs comprising 28% of the isolates (Fig. 1A). Diversity, as measured by Fisher's α , was 13.93 (95% confidence interval = 8.40, 22.49). The accumulation curve for this study was non-asymptotic (Fig. 1B), 19 (68%) of the 28 OTUs detected were singletons or doubletons (Fig. 1A), and the abundance-based extrapolated richness values exceeded observed richness (Table 3). Non-asymptotic curves are characteristic of taxa rich systems, such as fungal endophytes in tropical forests (Higgins et al., 2011), and are indicative of incomplete sampling. Greater sampling is necessary to yield realistic richness estimates. As such, any conclusions about richness, diversity, community similarity, and distributions should be treated as tentative.

3.3. Richness and diversity of the putative fungal pathogens in a drier, Pacific forest vs. a wetter, Atlantic forest

Considering a subset of isolates ($N = 44$) collected with standardized methods (only fungi isolated on MEA, from seedlings that originated from planted, surface-sterilized seeds, and from the six tree species for which at least one seedling of each species was collected in both forests),

12 fungal OTUs were collected from the best sampled drier forest site on the Pacific side of the isthmus (PNM; $N = 29$ isolates) and 10 OTUs were collected from the best sampled wetter forest site on the Atlantic side of the isthmus (SRR; $N = 15$ isolates). Based on OTUs defined by 95% sequence similarity and accounting for the unequal sample sizes and rare and unseen OTUs, there was tentative support for a richer and more diverse community of putative fungal pathogens in the wetter forest than the drier forest [Chao 1 estimated richness: 21.4 (95% confidence interval = 12, 73.8) vs. 18 (12.9, 50.8), respectively; Fisher's α : 13.1 (4.4, 44) vs. 7.7 (3.3, 16.9); Tables 3 and S3). Richness correlates positively with precipitation for all soil fungi across the same rainfall gradient in Panama (McGuire et al., 2012), for fungal endophyte communities across a rainfall gradient in Hawaii (Zimmerman and Vitousek, 2012), and for soil fungi at the global scale (Tedersoo et al., 2014). However, the diversity and estimated richness values for the wetter versus drier forests should be interpreted cautiously given the small sample sizes and overlapping confidence intervals. Furthermore, greater estimated richness in the wetter vs. drier forest was not a consistent pattern across the different sequence similarity thresholds (Table S3). Additional sampling is needed to draw firm conclusions about the richness and diversity of fungal pathogens in the wetter versus drier forests of Panama.

3.4. Similarity of the putative fungal pathogen communities in a drier, Pacific forest vs. a wetter, Atlantic forest

The communities of putative fungal pathogens in the wetter and drier forest sites were remarkably similar. Considering the subset of isolates collected with standardized methods from the two best sampled forest sites (PNM and SRR; $N = 44$), the wetter and drier forest sites shared five (29%) of the 17 observed OTUs (Fig. 2A; classic Jaccard similarity coefficient = 0.29; Table S4) and five (56%) of the 9 observed, non-singleton OTUs (Fig. 2B). Taking into consideration

rare and unseen OTUs, the similarity of the two communities was even greater (Chao-Jaccard abundance-based similarity index = 0.63, standard error = 0.16; Table S4). An overlap in putative fungal pathogen OTUs between the wetter and drier forest sites was still observed when considering a single host species, *Dalbergia retusa*. Five of the 10 fungal OTUs isolated from *D. retusa* seedlings were unique to the drier forest (PNM), three were unique to the wetter forest (SRR), and two (20%) were collected from both the drier and wetter forests [$N = 20$ isolates from 18 seedlings; 12 isolates were from 11 seedlings collected in the drier forest site (PNM) and 8 isolates were from 7 seedlings collected in the wetter forest site (SRR)]. Finally, based on an analysis of variance using a matrix of pairwise distances, the two communities of putative pathogens did not significantly differ in their community structure (adonis: $F_{1,10} = 1.19$, $P = 0.293$, $R^2 = 0.11$).

For those fungal isolates for which genus-level taxonomic assignments could be confidently made, five of the 15 genera were shared between the best sampled wetter and drier forests (SRR and PNM, respectively; Tables 1 and S2). *Mycoleptodiscus* and *Bionectria* were among the most commonly isolated genera in both the wetter and drier forests. Four fungal genera were only observed in the wetter forest (*Mycosphaerella*, *Talaromyces*, *Hypocrea*, and *Calonectria*) and two fungal genera were only observed in the drier forest (*Emericella* and *Pestalotiopsis*).

3.5. The most common putative fungal pathogens of seedlings in these tropical forests of Panama are habitat and host generalists.

Consistent with the observed fungal community similarity between the best sampled wetter and drier forest sites (SRR and PNM, respectively), I found evidence for broad geographic distributions for the fungal OTUs. The most commonly observed fungal OTU, *Mycoleptodiscus*

sp., was isolated from seedlings collected from four forest sites, spanning the rainfall gradient and isthmus (see OTU 2 in Table S2). These results are surprising for a number of reasons. First, the likelihood of observing the same OTU in multiple forests seems low given the estimated richness of fungi in tropical forests and the limited sampling of this study. Second, the environmental conditions of the wetter and drier forests differ in a variety of ways. In addition to differing in annual rainfall, the wetter and drier forests differ in their understory light levels, deciduousness, soils, plant community composition, and elevation (Pyke et al. 2001; Santiago et al. 2004; Engelbrecht et al., 2006; Brenes-Arguedas et al., 2008; Brenes-Arguedas et al., 2011; Condit et al., 2013). Third, the overlap between the putative fungal pathogen communities in the wetter and drier forests (29% of observed OTUs shared) was considerably greater than that of the tree communities in the wetter and drier forests (forest inventory plots 50 km apart share ca. 1-15% of their tree species; Condit et al., 2002). Yet, the community similarity and unexpectedly broad spatial distributions observed in this study have also been observed for root-associated fungi in North America (Queloz et al., 2011).

The relatively broad geographic distributions of the fungal OTUs suggest that at least some of the putative fungal pathogens produce propagules conducive to long-distance dispersal. Although fungal propagules are dispersed passively and most travel very short distances from their source, the likelihood that at least one propagule will travel a long distance and establish a new population is high given the number of propagules produced (Martiny et al., 2006). Furthermore, long-distance dispersal is possible via strong winds, moving water bodies, dust storms, co-migration with host plants, animals (especially birds), and human activities (Ristaino and Gumpertz, 2000; Brown and Hovmøller, 2002; Davidson et al., 2005; Hyder et al., 2009; Rosendahl et al., 2009; Kennedy et al., 2011; Pölme et al., 2013; Egan and Klironomos, 2014;

Peterson et al. 2014; Davison et al. 2015). Conversely, the relatively wide spatial distributions of the fungal pathogens may not be due to long-distance dispersal but rather to numerous short-distance dispersals over a long period of time, eventually leading to relatively wide ranges.

Given that there is very little overlap in the tree species present in the wetter, Atlantic and the drier, Pacific forests (Pyke et al., 2001; Condit et al., 2002), the observed overlap between the putative pathogen communities inhabiting a wetter forest (SRR) and a drier forest (PNM) suggests that the fungi are capable of infecting more than one tree species (i.e., the geographic distributions of the fungi are not limited by the availability of a single host). Concordantly, the most commonly observed OTU, *Mycoleptodiscus* sp. (OTU 2), was isolated from seedlings of seven tree species in six families (Table S2). Similarly, OTU 8, a *Bionectria*, was isolated from seedlings of five tree species in five families (Table S2). In fact, 15 (54%) of the 28 observed fungal OTUs and 15 (83%) of the 18 non-singleton OTUs were isolated from two or more of the 11 tree families represented by the collected seedlings. These results suggest low specificity at the family level; lower than that observed for fungal pathogens of leaves in the same region of Panama (only 29.9% of cross-inoculations between heterofamilial tree species resulted in disease; Gilbert and Webb, 2007). Low specificity was observed for isolates from both the best sampled drier and the best sampled wetter forest sites. Considering all isolates from the best sampled drier forest (PNM; $N = 35$), six of the 15 observed fungal OTUs were isolated from more than one tree species; one of the six multi-host OTUs (OTU 4, *Sordariomycetes* sp.) was isolated from five tree species (Table S2). Likewise, considering all isolates from the best sampled wetter forest (SRR; $N = 35$), seven of the 20 observed fungal OTUs were isolated from more than one tree species, with up to four host species for a given OTU (e.g., OTU 2, *Mycoleptodiscus* sp.; Table S2). Host generalism is further supported by some of the putative

fungus pathogens isolated from tree seedlings having high sequence similarity with endophytic fungi isolated from Neotropical grasses (e.g., isolate ERS058; see tree E in Appendix; Table S2; Higgins et al., 2011).

While host-specialized pathogens receive considerable attention for their hypothesized role in the maintenance of diversity in tropical forests, the results of this study support the hypothesis that host-generalized fungal pathogens are common in tropical forests. Given the relative rarity of tree species in diverse tropical forests and the passive dispersal of fungal pathogens, selection should favor fungal pathogens capable of attacking multiple hosts (May 1991). Likewise, there is experimental evidence that fungal pathogens of plants in tropical forests are able to attack multiple host species (Gilbert and Webb, 2007; Schweizer et al., 2013; Spear, *in prep*).

3.6. Conclusions and future directions

I observed support for my predictions that fungal pathogens attacking seedlings are relatively widespread geographically and that there is overlap between the fungal pathogen communities inhabiting the wetter, Atlantic and drier, Pacific forests. There is also evidence that the putative fungal pathogens are multi-host and capable of attacking seedlings in a variety of plant families, supporting wide host ranges. However, experimental assessments of host specificity are needed. Together, these results suggest that relatively widespread, generalist fungal pathogens are attacking seedlings in the tropical forests of Panama.

Despite their postulated importance in regulating the local diversity, spatial turnover, and regional diversity of tropical plant communities (Gilbert 2005; Mangan et al., 2010; Bagchi et al., 2014; Spear et al., 2015), fungal pathogen communities in natural systems remain poorly understood. This study of the phylogenetic relationships, geographic distributions, and

community composition of putative fungal pathogens in forests spanning a rainfall gradient is an important contribution to our understanding of plant-pathogen interactions in tropical forests. Fungal pathogens with broad geographic distributions and wide host ranges may mean that plant hosts cannot escape disease in geographic space, which has important implications for the distance-dependent assumptions of the Janzen-Connell hypothesis and the enemy release hypothesis (Janzen, 1970; Connell, 1971; Keane and Crawley, 2002). Future work, including evaluating the richness and diversity of fungal pathogens in the wetter versus drier forests of Panama, should involve more intensive sampling in this system. Culture-based isolation and culture-independent, direct amplification of fungal DNA from plant tissue (*sensu* Arnold et al., 2007; Higgins et al., 2011) should be used in combination for a comprehensive description of the fungi attacking seedlings.

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Fig. 1. (A) Relative abundance distribution, based on Fisher's log-series, and (B) accumulation of fungal OTUs, defined by 95% sequence similarity, from seedlings with pathogen-caused damage collected from tropical forests in Panama. The solid, black curve in the relative abundance plot (A) represents the expected number of OTUs based on the observed abundance (bars) ($N = 90$ isolates). Most of the fungal OTUs were rare and relatively few were abundant. The non-asymptotic accumulation curve (B) represents the mean accumulation of OTUs for 100 randomizations of seedling order derived from the observed richness ($N = 75$ seedlings) and is indicative of high diversity and incomplete sampling.

Fig. 2. Community similarity (overlap) for fungal OTUs isolated from seedlings with pathogen-caused damage in a drier forest (PNM, $N = 29$ isolates) versus a wetter forest (SRR, $N = 15$ isolates). Considering all 17 fungal OTUs observed in the drier and wetter forests (A), five were unique to the wetter forest (white), seven were unique to the drier forest (dark grey), and five were observed in both the drier and wetter forests (light grey intersection) (classic Jaccard similarity coefficient = 0.29; Chao-Jaccard abundance-based similarity index = 0.63). Only considering the nine non-singleton fungal OTUs observed in the drier and wetter forests (B), one was unique to the wetter forest (white), three were unique to the drier forest (dark grey), and five were observed in both the drier and wetter forests (light grey intersection).

Table 1. Seedlings with pathogen-caused damage were collected from tropical forests that span a rainfall gradient and the Isthmus of Panama (see Fig. S1). The columns include the seven forest sites, each site's approximate location and annual precipitation, the number of seedlings and host species collected, the method(s) used to obtain symptomatic seedlings, which included opportunistic collection of naturally occurring seedlings and baiting pathogens from the soil by planting seedlings or surface-sterilized seeds, the number of fungal isolates cultured from the seedlings, the number of fungal OTUs (defined by 95% sequence similarity) observed, and the number of singletons and their contribution to the observed number of OTUs.

Forest	Approx. location	Annual precip. (mm)	No. of seedlings (& hosts) collected	Collection method (no. of seedlings)	No. of fungal isolates	No. of fungal OTUs	No. of singletons (% of OTUs)
1. Parque Natural Metropolitano (PNM) ^{b,d}	8°59'36.62"N, 79°32'36.17"W	1,800 ^c	29 (7)	opportunistic (4), seed planted (25)	35	15	6 (40%)
2. Gunn Hill in Ciudad de Saber (formerly Fort Clayton; FC) ^a	9°0'50"N, 79°35'W	2,010 ^f	7 (6)	seedling planted (7)	8	5	2 (40%)
3. Sendero del Charco (SC) ^c	9°4'52.93"N, 79°39'57.92"W	N/A	2 (1)	opportunistic (2)	2	2	2 (100%)
4. Sendero Camino de Cruces (CC) ^c	9°6'6.44"N, 79°36'56.45"W	N/A	1 (1)	opportunistic (1)	1	1	1 (100%)
5. Barro Colorado Island, Barro Colorado Nature Monument (BCI) ^c	9° 9'21.90"N, 79°51'5.80"W	2,600 ^g	3 (3)	opportunistic (3)	3	3	3 (100%)
6. Buena Vista Peninsula, Barro Colorado Nature Monument (BV) ^a	9°10'46.20"N, 79°49'54.88"W	2,600 ^g	5 (3)	seedling planted (5)	6	4	2 (50%)
7. Santa Rita Ridge (SRR) ^{a,b,d}	9°20'03.71" N, 79°46'39.96"W	3,000 ^c	28 (14)	seedling planted (10), seed planted (18)	35	20	12 (60%)

Year(s) in which the forest was a study site: ^a2007, ^b2010, ^c2011, ^d2012.

References for precipitation values: ^cSantiago et al. (2004), ^fBrenes-Arguedas et al. (2013), ^gWindsor (1990).

N/A = not available.

Table 2. Taxonomic affiliations of the fungi isolated from seedlings with pathogen-caused damage. All of the isolates ($N = 90$) belonged to the phylum Ascomycota and the subphylum Pezizomycotina. While the fungi were isolated from symptomatic tissue and most were likely to be phytopathogens, those in the class Eurotiomycetes (listed in grey) were unlikely to be pathogenic to plants. For each taxonomic group, the number of isolates observed is listed in parentheses. In some cases, the teleomorphic name for a genus is followed by the anamorphic name, separated by a backslash.

Class	Order	Family	Genus
Sordariomycetes (83) ^a	Chaetosphaeriales (1)	Chaetosphaeriaceae (1)	unknown (1)
	Diaporthales (6)	Diaporthaceae (6)	<i>Diaporthe/Phomopsis</i> (6)
	Glomerellales (8)	Glomerellaceae (8)	<i>Glomerella/</i>
			<i>Colletotrichum</i> (8)
	Hypocreales (37)	Bionectriaceae (7)	<i>Bionectria/</i>
			<i>Clonostachys</i> (7)
		Hypocreaceae (2)	<i>Hypocrea/Trichoderma</i> (2)
		Nectriaceae (28)	<i>Calonectria/</i>
			<i>Cylindrocladium</i> (5)
			<i>Gibberella/Fusarium</i> (2)
			<i>Glionectria/</i>
			<i>Gliocladiopsis</i> (4)
			<i>Leuconectria/</i>
			<i>Gliocephalotrichum</i> (1)
			<i>Nectria/Fusarium</i> (3)
			<i>Nectricladiella/</i>
			<i>Cylindrocladiella</i> (4)
			<i>Neonectria/</i>
			<i>Cylindrocarpon</i> (4)
			unknown (5)
	Magnaporthales (13)	Magnaporthaceae (13)	<i>Mycoleptodiscus</i> (13)
	Xylariales (8) ^b	Amphisphaeriaceae (4)	<i>Pestalotiopsis</i> (4)
		Xylariaceae (2)	unknown (2)
		unknown (2)	unknown (2)
	unknown (10)	unknown (10)	unknown (10)
Dothideomycetes (4)	Botryosphaeriales (2)	Botryosphaeriaceae (2)	unknown (2)
	Capnodiales (2)	Mycosphaerellaceae (2)	<i>Mycosphaerella</i> (1)
Eurotiomycetes (3)	Eurotiales (3)	Trichocomaceae (3)	unknown (1)
			<i>Emericella/Aspergillus</i> (1)
			<i>Talaromyces/</i>
			<i>Penicillium</i> (2)

^a *incertae sedis* for 15 of the 83 isolates.

^b *incertae sedis* for 2 of the 8 isolates.

Table 3. Richness and diversity of fungal operational taxonomic units (OTUs), defined by 95% sequence similarity, observed for isolates from all forest sites and for a subset of isolates from one drier and one wetter forest site (PNM and SRR, respectively; Table 1). Columns include the number of seedlings collected, the number of fungal isolates from those seedlings, observed richness of fungal OTUs, the number of singletons and their contribution to the observed richness, the abundance-based Chao1 and Abundance-based Coverage Estimator (ACE) estimates of the extrapolated richness (95% confidence interval), and the Fisher's α diversity index (95% confidence interval).

	No. of seedlings collected	Fungal isolates	OTUs obs.	No. of singletons (% of OTUs)	Chao1 (CI)	ACE (CI)	Fisher's α (CI)
All forests	75 (21 tree species, 11 families)	90	28	10 (36%)	33.5 (29.3, 50.8)	37.3 (30.9, 57.9)	13.9 (8.4, 22.5)
Drier forest (PNM)	24 (6 tree species, 5 families)	29	12	5 (42%)	18 (12.9, 50.8)	15.8 (12.7, 31.6)	7.7 (3.3, 16.9)
Wetter forest (SRR)	13 (6 tree species, 5 families)	15	10	7 (70%)	21.4 (12, 73.8)	24.4 (13, 78.4)	13.1 (4.4, 44)