

## DIVERSITY AND HOST RANGE OF FOLIAR FUNGAL ENDOPHYTES: ARE TROPICAL LEAVES BIODIVERSITY HOTSPOTS?

A. ELIZABETH ARNOLD<sup>1</sup> AND F. LUTZONI

*Department of Biology, Duke University, Durham, North Carolina 27708 USA*

**Abstract.** Fungal endophytes are found in asymptomatic photosynthetic tissues of all major lineages of land plants. The ubiquity of these cryptic symbionts is clear, but the scale of their diversity, host range, and geographic distributions are unknown. To explore the putative hyperdiversity of tropical leaf endophytes, we compared endophyte communities along a broad latitudinal gradient from the Canadian arctic to the lowland tropical forest of central Panama. Here, we use molecular sequence data from 1403 endophyte strains to show that endophytes increase in incidence, diversity, and host breadth from arctic to tropical sites. Endophyte communities from higher latitudes are characterized by relatively few species from many different classes of Ascomycota, whereas tropical endophyte assemblages are dominated by a small number of classes with a very large number of endophytic species. The most easily cultivated endophytes from tropical plants have wide host ranges, but communities are dominated by a large number of rare species whose host range is unclear. Even when only the most easily cultured species are considered, leaves of tropical trees represent hotspots of fungal species diversity, containing numerous species not yet recovered from other biomes. The challenge remains to recover and identify those elusive and rarely cultured taxa with narrower host ranges, and to elucidate the ecological roles of these little-known symbionts in tropical forests.

**Key words:** *Ascomycota; Barro Colorado Island; diversity; endophytic fungi; host affinity; ITSrDNA; latitudinal gradient; richness; symbiosis; tropical forests.*

### INTRODUCTION

Comprising interactions that range from mutualism to antagonism, fungal symbioses with plants are key determinants of biomass, nutrient cycling, and ecosystem productivity in terrestrial habitats from the poles to the equator (e.g., Clay and Holah 1999, Hawksworth 2001, Gilbert 2002). Most plant-associated fungi catalogued to date have been recognized because of the fruitbodies they produce in association with their hosts (e.g., plant pathogens, mycorrhizal fungi). Yet plants in all major lineages, including liverworts, mosses, seed-free vascular plants, conifers, and angiosperms, also form cryptic symbioses with fungi that penetrate and persist within healthy aboveground tissues such as leaves. Foliar fungal endophytes (i.e., endophylls or mycophyllas) are a fundamental but frequently overlooked aspect of plant biology: all plant species surveyed thus far harbor one or more endophytic symbionts in their photosynthetic tissues (Stone et al. 2000).

The presence of obligately heterotrophic endophytes within photosynthetic tissues of plants raises the

question of the ecological importance of these cryptic symbionts. Studies of the systemic, maternally inherited endophytes (Clavicipitaceae, Ascomycota) associated with over 300 species of grasses indicate that an array of plant phenotypic traits—including drought tolerance, leaf chemistry, tolerance of heavy metals in soils, and propensity for vegetative reproduction—are directly attributable to the presence of endophytes (Clay and Schardl 2002). However, this model system of endophyte biology, arguably the most familiar to ecologists, represents a special case. The photosynthetic tissues of the vast majority of terrestrial plants are colonized by endophytes that accumulate by contagious spread (i.e., horizontal transmission). These endophytes undergo spatially limited or localized growth within particular tissues, accumulate as tissues age, and comprise a tremendous richness of species spanning at least four phyla of Fungi (see Fröhlich and Hyde 1999, Arnold et al. 2003, 2007; A. E. Arnold, J. Miadlikowska, K. L. Higgins, S. D. Sarvate, P. S. Gugger, A. Way, V. Hofstetter, F. Kauff, and F. Lutzoni, *unpublished manuscript*).

Horizontally transmitted endophytes are frequently thought to have little to no direct effect on the plants they inhabit. However, several recent studies show that plants can respond to endophyte infection in ecologically meaningful ways. Redman et al. (2002) demonstrated that endophytes enhance thermotolerance and salinity tolerance of temperate plants, augmenting their

Manuscript received 20 September 2005; revised 24 April 2006; accepted 2 May 2006. Corresponding Editor: G. S. Gilbert. For reprints of this Special Feature, see footnote 1, p. 539.

<sup>1</sup> Present address: Division of Plant Pathology and Microbiology, Department of Plant Sciences, University of Arizona, Tucson, Arizona 85721 USA.  
E-mail: arnold@ag.arizona.edu

potential to colonize extreme environments (see also Rodriguez et al. 2004). Arnold et al. (2003) showed that infection by a suite of common endophytic fungi increased resistance of a tropical tree, *Theobroma cacao*, to a virulent foliar pathogen (*Phytophthora* sp.). Wagner and Lewis (2000) showed that plants may harbor entomopathogens as endophytes, providing an additional but cryptic defense against insect herbivores. Yet, endophytes also may increase host susceptibility to severe drought (Arnold 2002), and in maize and banana may impair photosynthetic efficiency (Pinto et al. 2001). Together, these effects on host phenotypes raise the intriguing possibility that like lichens, plants represent emergent properties derived from intricate fungal symbioses (see also Atsatt 1988).

At present, it remains difficult to generalize from these studies with regard to the ecological importance of endophytic fungi. Given the remarkable phylogenetic diversity of horizontally transmitted endophytes (Fröhlich and Hyde 1999, Arnold et al. 2007, Higgins et al. 2007; A. E. Arnold, J. Miadlikowska, K. L. Higgins, S. D. Sarvate, P. S. Gugger, A. Way, V. Hofstetter, F. Kauff, and F. Lutzoni, *unpublished manuscript*), it is unlikely that any of these ecological effects is universal among all endophyte–plant associations. To understand the ecology of fungal endophytes requires data regarding fundamental parameters of the endophyte symbiosis. How many species of fungi are capable of forming endophytic associations with a given host? To what degree are these fungi specialized to their hosts? Are endophytic symbionts associated with a given plant lineage consistent over the host's geographic range?

Several authors have suggested that endophytes are especially diverse in tropical forests (Fröhlich and Hyde 1999, Arnold et al. 2000), and preliminary data gathered by such studies have been used to support arguments for hyperdiversity of Fungi as a whole (Hawksworth 2001). In lowland, moist tropical forests, up to 17 species of endophytes have been recovered from a single leaf, with infection domains typically on the scale of only 2 mm<sup>2</sup> of leaf tissue (Lodge et al. 1996, Gamboa and Bayman 2001). Similar observations have led to the suggestion that there exist more species of endophytes than are currently known in all of Fungi (Dreyfuss and Chapela 1994), and that the majority of the “undiscovered” endophyte diversity occurs in leaves of tropical trees (see Arnold et al. 2000). Yet the endophytes associated with the vast majority of plants in tropical forests have yet to be catalogued, and studies of host specificity and spatial structure—central to extrapolative estimates of diversity—are frequently in conflict. For example, Arnold et al. (2003) found evidence for host specificity of some tropical endophytes, but surveys in French Guyana and India have yielded contradictory results (Cannon and Simmons 2002, Suryanarayanan et al. 2002). As a result, May's (1991) suggestion that tropical fungi are more likely host generalists than are fungi from higher latitudes remains to be evaluated. Similarly, Fröhlich

and Hyde (1999) found that endophyte assemblages in closely related palms differed between New Guinea and Australia, and Arnold et al. (2003) found a strong spatial component underlying the heterogeneity of endophyte communities at five sites across the isthmus of Panama. Yet, Suryanarayanan et al. (2002) found that many endophyte species occurred in multiple forest types in India, suggesting little spatial heterogeneity in endophyte assemblages. The degree to which these different results can be reconciled will only be determined by the application of consistent survey methods and species concepts for studies in multiple sites.

Here, we synthesize the first results of a large-scale survey of endophytic fungi along a latitudinal gradient from the arctic to the tropics, which to date has yielded 8456 endophyte strains from all major lineages of land plants. We use molecular sequence data from 1403 representative strains to examine (1) evidence for a latitudinal gradient of endophyte incidence and diversity; (2) geographic heterogeneity of endophyte assemblages among arctic, boreal, temperate, and tropical sites; and (3) host specificity of endophytes in three forest communities. Our results demonstrate that land plants interact with a tremendous richness of endophytes both within and beyond the tropics. Host specificity is similar in tropical and temperate forests, but increases at higher latitudes. However, even the most frequently recovered tropical endophytes are distinct in terms of their patterns of abundance, diversity, and taxonomic composition relative to other biomes.

## METHODS

### *Study sites, host species, and isolation methods*

The incidence of endophyte infections, defined as the percent of tissue segments containing endophytes, was quantified for 28 host species representing phylogenetically diverse plant taxa that are representative of the aboveground biomass in eight localities (see Appendix A for site descriptions). Study localities included arctic tundra at Iqaluit, Nunavut, Canada (IQN); northern boreal forest at Schefferville, Québec, Canada (SHQ); southern boreal forest at the Mingan Archipelago (MAQ) and Moisie, Québec, Canada (MRQ); temperate semi-deciduous forest at Duke Forest, Durham, North Carolina, USA (DNC); upland Sonoran Desert at Tucson, Arizona, USA (TAZ); southwestern coniferous forest at the Santa Catalina Mountains, near Tucson, Arizona, USA (SCA); and lowland, moist tropical forest at Barro Colorado Island, Panama (BCI). Surveys were conducted at the height of the growing season in the arctic, boreal, and temperate sites, and during the early and late wet season at BCI. A total of 34 host species–site combinations was examined, including liverworts, mosses, seed-free vascular plants, conifers, and angiosperms (Appendix B).

All plant material was sampled for endophytes within 96 h of collection. Healthy leaves or photosynthetic stems with microphylls were washed in running tap

water to remove epiphyllous debris (30 s), patted dry, and cut into small fragments ( $2 \times 1$  mm). Tissue fragments were surface-sterilized using sequential immersion in 95% ethanol (10 s), 10% chlorine bleach (0.525% NaOCl; 2 min), and 70% ethanol (2 min), allowed to surface dry under sterile conditions, and plated on 2% malt extract agar (MEA). Plates were sealed, incubated at room temperature, and scored for fungal growth for up to one year. Emergent hyphae were isolated into pure culture, photographed, and deposited as living vouchers at the Robert L. Gilbertson Mycological Herbarium, University of Arizona (ARIZ).

In each site, we sampled three to nine representative individuals per plant species, and three to nine leaves or stems per individual. In sum, 120–720 2-mm<sup>2</sup> tissue fragments per species per site were examined, corresponding to preliminary data suggesting variable infection frequencies among taxa and sites. In total, 25 340 tissue segments were plated for this study.

#### *Endophyte diversity*

Because most endophytes remained sterile in culture, identification beyond the level of phylum required molecular analysis. Data from the nuclear ribosomal internal transcribed spacer region (ITSrDNA), an approximately 600 base-pair locus frequently used in species-level systematics for fungi, were obtained for 1403 strains of endophytes representing the most common morphotypes in each site (defined by whole-colony morphology, after Arnold et al. [2000]). DNA extraction, PCR, sequencing, and sequence-preparation methods are detailed in Appendix C.

ITSrDNA data cannot be used to infer a phylogenetic species concept for diverse fungal assemblages due to the very rapid rate of evolution of the spacer regions (e.g., Lutzoni et al. 2004). Therefore, we used two recent phylogenetic analyses of endophytic fungi from arctic, boreal, temperate, and tropical sites to infer species boundaries among endophytes (Arnold et al. 2007, Higgins et al. 2007): a 217-taxon tree for Ascomycota + limited Basidiomycota, including 145 temperate endophyte strains; and a 359-taxon tree for Ascomycota containing 118 arctic, boreal, temperate, and tropical endophytes. To conservatively estimate fungal species boundaries, we designated species, genus, or family-level boundaries on each tree on the basis of named (exemplar) taxa only (i.e., without regard to our endophyte strains). We then assessed the position of each endophyte relative to these boundaries, considering endophytes to be distinct from one another if their placement was distal to boundaries delimiting known taxonomic groups based on exemplar taxa alone. We found that 95% ITSrDNA sequence divergence was consistent with endophyte species boundaries inferred using this phylogenetic framework. Therefore, we used 95% sequence similarity for the ITSrDNA region to operationally designate species boundaries. This measure is conservative relative to recently published studies

(e.g., O'Brien et al. 2005) and has the advantage of explicit comparison with phylotypes based on other loci. ITSrDNA genotype groups were delimited using Sequencher 4.2, with the expectation of at least 40% sequence overlap (see Arnold et al. 2007). Hereafter, genotype groups based on 95% ITSrDNA similarity are referred to as species.

Endophyte diversity was quantified for 21 representative plant taxa in six localities ranging from northern boreal forest to lowland tropical forest (SHQ, MAQ, MRQ, DNC, TAZ, BCI). Hosts that contained endophytes in  $\leq 1\%$  of tissue segments, or for which  $< 15$  isolates have been genotyped, were included in whole-community diversity measures, but not in the diversity assessments for individual host taxa. In sum, diversity was evaluated for endophytes from 23 host species/site combinations, including 1202 genotyped strains from seed-free vascular plants, conifers, and angiosperms (Appendix D). Diversity was measured using Fisher's  $\alpha$ , which is robust for comparisons among samples of different sizes (Leigh 1999). Fisher's  $\alpha$  is defined implicitly by the formula  $S = a \times \ln(1 + n/a)$  where  $S$  is the number of taxa,  $n$  is the number of individuals (defined by numbers of isolates), and  $a$  is Fisher's  $\alpha$  (Leigh 1999).

To assess whole-community richness, we randomly sampled 100 endophyte strains from all genotyped isolates recovered from representative angiosperms in three forests: southern boreal forest at MAQ, temperate forest at DNC, and tropical forest at BCI. Species-accumulation curves were generated for angiosperm-associated endophytes from each forest, and for the entire study (1403 isolates from bryophytes, seed-free vascular plants, conifers, and angiosperms) using EstimateS (*available online*).<sup>2</sup> Total richness for each partition was estimated using the bootstrap estimator, implemented in EstimateS.

Large-scale surveys of microfungi frequently rely on BLAST matches with the NCBI GenBank database for identification. To examine the stability of identifications based on BLAST matches, we compared taxonomic matches at the genus and family levels for the same isolates based on BLAST searches conducted in 2001 and 2005. To confirm taxonomic placement of endophytes, we compared ITSrDNA data against a database of 2058 sequences of endophytic, lichen-associated, and environmental samples (A. E. Arnold, J. Miadlikowska, K. L. Higgins, S. D. Sarvate, P. S. Gugger, A. Way, V. Hofstetter, F. Kauff, and F. Lutzoni, *unpublished manuscript*). Identification at higher taxonomic levels was based on  $\geq 97\%$  ITSrDNA similarity with strains identified through recent phylogenetic analyses (Arnold et al. 2007, Higgins et al. 2007).

Similarity indices based on frequency (Morisita-Horn index) and presence/absence data (Jaccard's index) were

<sup>2</sup> (<http://viceroy.eeb.uconn.edu/EstimateS>)

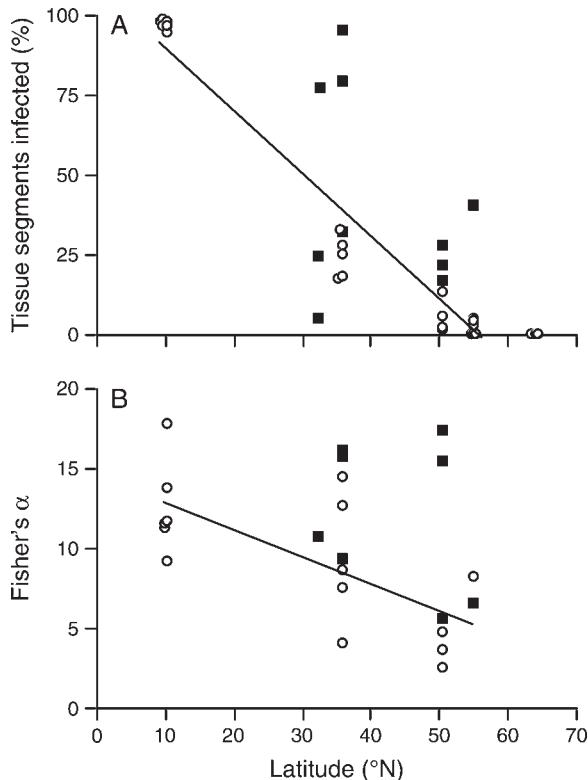


FIG. 1. (A) Latitudinal gradient of endophyte infections. The percentage of tissue fragments (each 2 mm<sup>2</sup>) infected by culturable endophytes for 34 host species/site combinations, representing eight localities ranging from lowland tropical forest (Barro Colorado Island [BCI], Panama) to arctic tundra (near Iqaluit, Nunavut, Canada). Host–site combinations are given in Appendix B. Solid squares indicate conifers; open circles indicate all other hosts. (B) Latitudinal gradient of endophyte diversity. Fisher's  $\alpha$  for 23 host–site combinations, representing 1202 strains of endophytic fungi from six localities ranging from tropical forest at BCI to northern boreal forest (Schefferville, Québec, Canada). Host–site combinations and number of isolates sequenced are given in Appendix D. Solid squares indicate conifers; open circles indicate all other hosts.

used to compare assemblages of endophytes from different sites and hosts (Arnold et al. 2003). Both indices range from 0 (no overlap between assemblages) to 1 (total overlap) and were calculated using EstimateS (see footnote 2) using only those endophyte species that were recovered more than once (nonsingletons).

## RESULTS

### Latitudinal gradient of endophyte infections

Over the entire study, 35.0%  $\pm$  6.5% of tissue segments were infected by culturable endophytes. All plant species contained endophytes within their photosynthetic tissues. Among 28 species of plants in eight localities (34 host species–site combinations), the incidence of endophyte infections decreased linearly from the tropics to the arctic ( $R^2 = 0.74$ ,  $F_{1,32} = 93.30$ ,  $P < 0.0001$ ; Fig. 1A; Appendix B). Whereas endophytes were

recovered from 95.6–99.5% of 2-mm<sup>2</sup> leaf segments examined in lowland, moist tropical forest (BCI), only 1% of leaf segments contained endophytes at the arctic site (IQN). Incidence of endophyte infections in the Sonoran Desert was significantly below expected values given the latitude of the sampling site.

Conifers frequently contained a higher incidence of cultivable endophytes than expected: values for *Pinus ponderosa* (southwestern coniferous forest at SCA), *Platycladus orientalis* and *Pinus taeda* (temperate forest at DNC), *Pinus banksiana* (southern boreal forest at MRQ), and *Picea mariana* (northern boreal forest at SHQ) lie significantly above the line of fit defined by non-coniferous hosts (Fig. 1A). However, plant lineages were not reliable predictors of the incidence of endophyte infections (comparison among angiosperms, conifers, ferns, and nonvascular plants:  $F_{3,28} = 1.09$ ,  $P = 0.3693$ ).

When endophyte incidence was examined as a function of latitude and annual precipitation, latitude remained significant as a determinant of infection frequency ( $P = 0.0247$ ), whereas annual rainfall was not significant ( $P = 0.0820$ ), and there was no evidence for interaction of these explanatory variables ( $P = 0.3710$ ; for statistical details, see Appendix E). A lack-of-fit test showed that this model was sufficient to explain the observed variation in the incidence of endophyte infections (lack-of-fit  $F_{3,27} = 2.86$ ,  $P = 0.0554$ ).

### Latitudinal gradient of endophyte diversity

Among 21 plant species in six localities (23 host species/site combinations), endophyte diversity decreased linearly from the tropics to northern boreal forest. Diversity of endophytic fungi ranged from Fisher's  $\alpha = 2.6$  for *Empetrum nigrum* (Ericaceae) in southern boreal forest at MAQ to 17.9 for *Trichilia tuberculata* (Meliaceae) in tropical forest at BCI (Appendix D). Coniferous hosts frequently showed higher than expected diversity at higher latitudes (e.g., *Pinus taeda* in temperate forest at DNC, Fisher's  $\alpha = 15.9$ ; *Picea mariana* at MAQ, Fisher's  $\alpha = 17.5$ ). When coniferous plants were excluded from analysis, the remaining diversity values decreased significantly and linearly from the tropics to boreal forests ( $R^2 = 0.47$ ,  $F_{1,13} = 11.3$ ,  $P = 0.0050$ ; Fig. 1B).

Although some coniferous hosts had higher than expected diversity of endophytes (Fig. 1), plant lineage was not a reliable predictor of endophyte diversity (comparisons among angiosperms, conifers, and ferns:  $F_{2,20} = 1.67$ ,  $P = 0.2118$ ). When coniferous hosts with significantly higher-than-expected diversity were excluded and diversity examined as a function of latitude and annual precipitation, latitude remained significant ( $P = 0.0074$ ), whereas annual precipitation was not significant ( $P = 0.1586$ ; for statistical details, see Appendix E). A lack-of-fit test showed that this model was sufficient to explain the observed variation in endophyte diversity

(lack-of-fit  $F_{2,15} = 1.52$ ,  $P = 0.3424$ ). There was no evidence for interaction of these explanatory variables.

#### *Endophytes of angiosperms in three forests*

Species accumulation curves for randomly chosen pools of 100 endophyte strains obtained from angiosperms in three forests increased in richness as a function of decreasing latitude (Fig. 2). At both MAQ (southern boreal forest) and DNC (temperate forest), our sampling of the endophyte community was statistically complete: estimated richness based on bootstrap analyses fell within the 95% confidence interval for observed species richness. At BCI (tropical forest), estimated richness significantly exceeded that recovered by our sampling (Fig. 2), reflecting the high richness of endophytes in this tropical forest site. Diversity of endophytes recovered from angiosperms in these communities increased from southern boreal forest (Fisher's  $\alpha = 9.2$ ) to the temperate zone (Fisher's  $\alpha = 25.7$ ) to the tropics (Fisher's  $\alpha = 30.9$ ).

#### *Taxonomic composition of endophytic fungi*

Representative endophytes from all forests were dominated by Ascomycota, with the Dothideomycetes especially prevalent in boreal forest, and the Sordariomycetes predominating at DNC and BCI (Fig. 2). Whereas species richness and diversity increased markedly from boreal forest to the tropics, the number of fungal classes represented by endophytes decreased from boreal forest to BCI (Fig. 2). Representative endophytes inhabiting angiosperms at MAQ included six classes, including several early-diverging lineages of Ascomycota (Saccharomycotina and Pezizomycetes). Five classes were recovered at DNC, with the percent representation of Sordariomycetes and Dothideomycetes intermediate between the boreal and tropical sites. At BCI, three classes were represented by endophytes (Fig. 2). Tropical endophyte assemblages were especially dominated by species within the Phyllachorales, Xylariales, Diaporthales, and Hypocreales (Sordariomycetes), and by Dothideomycetes affiliated with *Botryosphaeria* (Appendix F).

Comparison of BLAST searches of the NCBI GenBank database performed for the same sequences in 2001 and 2005 demonstrated instability in genus- and family-level matches (Appendix G). Family-level identification, based on highest BLAST affinity in 2001, changed for five of 14 isolates (35.7%) when compared against GenBank in 2005. Genus-level identification changed for 11 of 14 isolates (78.6%). All matches in 2005 were confirmed by recent phylogenetic analyses (Arnold et al. 2007, Higgins et al. 2007).

#### *Host affinity and geographic structure of endophyte communities*

Overall, 277 species were recovered from 1403 sequenced strains representing endophytes of common plants in arctic, boreal, temperate, and tropical localities

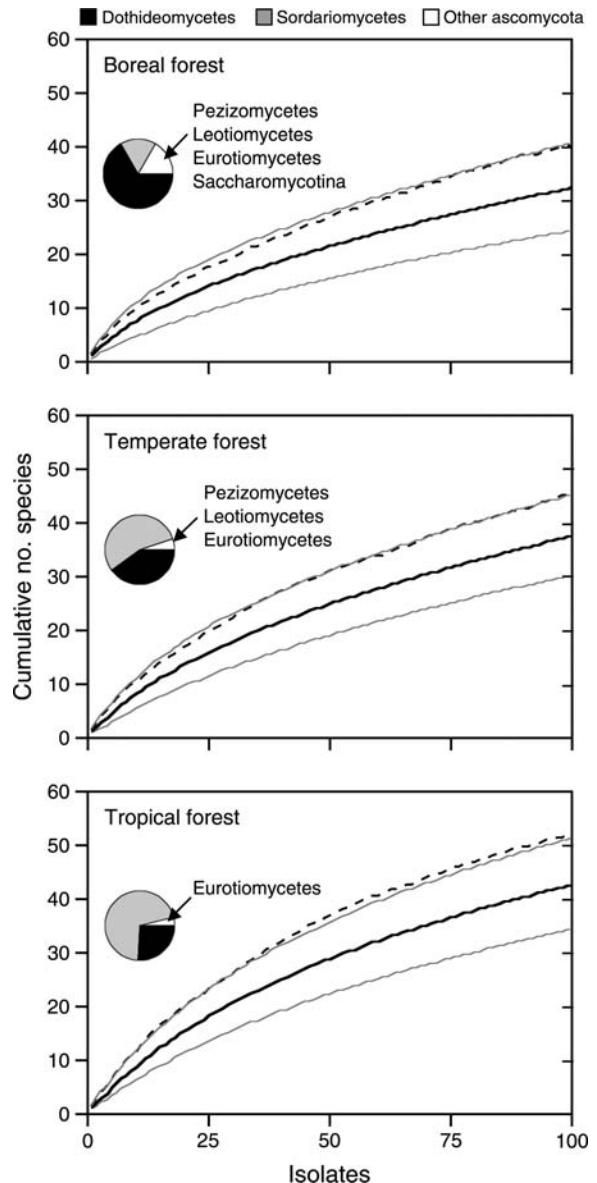


FIG. 2. Species accumulation curves and bootstrap estimates of total richness for random samples of 100 endophyte strains isolated from 3–6 angiosperm species in each of three sites: southern boreal forest (Mingan Archipelago, MAQ), temperate forest (Duke Forest, DNC), and lowland, moist tropical forest (BCI). Inset pie charts indicate the taxonomic distribution of endophytes among classes of Ascomycota in each site, determined by comparison of ITSrDNA sequence data with endophytes identified using phylogenetic analyses. Solid black lines indicate observed richness; solid gray lines indicate 95% CI around the observed richness; and black dashed lines indicate bootstrap estimates of total species richness inferred using EstimateS (see footnote 2).

(Fisher's  $\alpha = 103.1$ ). The species accumulation curve remains non-asymptotic, and estimated richness significantly exceeds the richness captured by our sampling to date (Fig. 3).

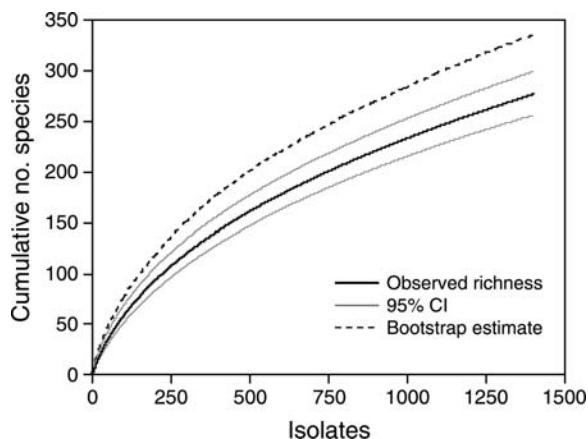


FIG. 3. Species accumulation curve and estimated richness for 277 putative species recovered from 1403 strains of endophytic fungi isolated from healthy, aboveground foliage of representative arctic, boreal, temperate, and tropical plants. The solid black line indicates observed richness; solid gray lines indicate 95% CI around the observed richness; and the black dashed line indicates bootstrap estimates of total species richness inferred using EstimateS (see footnote 2).

Only three endophyte species were found at a frequency exceeding 5% of isolates. All were collected only in boreal and temperate sites and were members of a single class (Leotiomycetes). The most common species (Rhytismataceae, *Lophodermium* sp. 1) accounted for 102 isolates (7.3%) and was found in conifers and angiosperms in temperate (DNC) and boreal (MAQ, SHQ) sites. *Lophodermium* sp. 2 accounted for 93 isolates (6.6%) and was recovered from *Pinus taeda* at DNC and *P. banksiana* in southern boreal forest at MRQ. Leotiaceae sp. 1 (Leotiales) accounted for 85 isolates (6.0%) and was found in *P. taeda* at DNC, *P. banksiana* at MRQ, and *Picea mariana* at SHQ. Overall, 138 species (49.8%) were found only once (singletons). Among representative endophytes of angiosperms, 59.0%, 37.5%, and 51.6% of species were singletons in boreal, temperate, and tropical sites, respectively.

Among species recovered more than once (nonsingletons;  $N = 139$  species, represented by 1265 strains), 71.2% were recovered from only one broadly defined biogeographic region (tropical forest; sites in the temperate zone; or arctic/boreal sites). The frequency with which species were recovered from only one site was significantly greater than expected given a random distribution among regions (64.8%, estimated from subsamples from 1000 pseudoreplicates using species recovered at least three times, corresponding to three regions;  $T = -4.73$ ,  $P < 0.0001$ ). Twenty-four species occurred in both arctic/boreal and temperate localities (Jaccard's index = 0.203; Morisita-Horn index = 0.169), and 14 occurred in both temperate and tropical sites (Jaccard's index = 0.133; Morisita-Horn index = 0.220). Only two genotypes occurred in both arctic/boreal and tropical sites (Jaccard's index = 0.021; Morisita-Horn

index = 0.003). Over the entire data set (including singletons), 76.2% of endophyte species found at BCI were recovered only from that site. Similarly, 75% percent of species from the temperate zone were found only in temperate hosts, and 77.9% of endophyte species recovered in high latitude sites were found only in boreal and arctic plants.

Based on both presence/absence and frequency data, similarity among endophyte communities associated with angiosperm hosts within DNC (temperate forest) and BCI (tropical forest) were low, ranging from 0.065 to 0.120 (Jaccard's index) and 0.039 to 0.220 (Morisita-Horn index). However, assemblages of endophytes in common angiosperms were significantly more similar among hosts within DNC and BCI than among hosts in southern boreal forest at MAQ (Wilcoxon signed-rank test,  $\alpha = 0.05$ ; Fig. 4). At lower latitudes, the most commonly recovered endophytes had the broadest host ranges: host range (proportion of surveyed hosts in which a species was present) was positively associated with the incidence of particular species at BCI ( $R^2 = 0.81$ ;  $F_{1,52} = 221.75$ ,  $P < 0.0001$ ) and DNC ( $R^2 = 0.18$ ;  $F_{1,54} = 11.64$ ,  $P = 0.0011$ ), but not at MAQ ( $R^2 = 0.06$ ;  $F_{1,30} = 2.10$ ,  $P = 0.1569$ ).

#### DISCUSSION

Terrestrial plants engage in symbioses with a tremendously diverse array of endophytic fungal species. Given the geographic range encompassed by this study, sampling ~1400 isolates was insufficient to adequately capture the richness of cultivable endophytes (observed richness, 277 species; estimated richness, 335 species; Fig. 3). In agreement with previous studies, endophyte

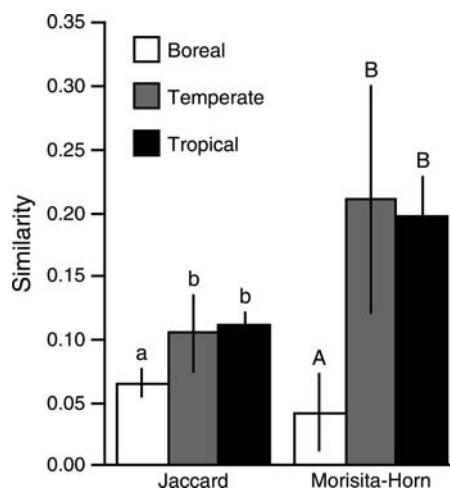


FIG. 4. Similarity (mean  $\pm$  SE) for all pairwise comparisons of nonsingleton endophytes isolated from representative angiosperms in southern boreal forest (Mingan Archipelago, MAQ), temperate forest (Duke Forest, DNC), and tropical forest (BCI), using presence/absence (Jaccard's index) and frequency data (Morisita-Horn index). Different letters indicate significantly different means within each index.

communities at small and large spatial scales were characterized by a large number of singletons (species that occurred only once; see Arnold et al. 2000), including 138 of the 277 species recovered. The host- and geographic specificity of these rare species remains unknown. More generally, the ecological roles of the vast majority of endophytes recovered in this study have yet to be explored.

Our surveys show that plants experience fundamentally different symbiotic environments according to their biogeographic locality. The incidence of endophyte infections increased from the arctic to the tropics, with <1% to >99% of tissue segments harboring endophytes (Fig. 1A). Like many organisms, endofoliar symbionts also increase in diversity with decreasing latitude, both in terms of assemblages associated with individual hosts (Fig. 1B) and at the community level (Fig. 2). The majority of species found in broadly defined geographic regions (arctic/boreal, temperate, and tropical sites) were unique to those areas, and even when singletons were excluded from analysis, 71.2% of species were unique to only a single biogeographic zone. Representative angiosperms at a high-latitude site harbor communities of endophytes that include at least six classes of Ascomycota, but are characterized by lower diversity at the species level than are lower-latitude sites. In contrast, endophytes of representative host plants in lowland Panama are very species-rich, but represent only three fungal classes. Intermediate levels of class- and species-diversity were observed in a mesic temperate forest (DNC). To our knowledge, few studies have documented an inverse relationship between phylogenetic diversity (here defined as class-level diversity) and species diversity in fungal communities.

We used similarity in endophyte assemblages among angiosperms in each site, and the proportion of sampled hosts in which an endophyte species was recovered, as indices of host specificity. Levels of host specificity were similar in a temperate forest (DNC) and at BCI, but these forests were characterized by lower host specificity than southern boreal forest (MAQ) (Fig. 4). In particular, the prevalence of closely related symbionts among distantly related tree species in Panama contrasts with the large number of distantly related symbionts inhabiting closely related species in boreal forest (e.g., multiple species of Ericaceae). However, the steep species accumulation curves observed in this study (Figs. 2, 3) are indicative of statistically incomplete sampling, suggesting that conclusions regarding host specificity and spatial structure remain tentative at best. Excluding singletons permits more adequate examination of the sampled communities: species accumulation curves for nonsingletons are typically asymptotic (Arnold, unpublished data). Due to the high frequency of rare species, however, excluding singletons often results in analyses that are based on less than half of the observed species in a given host or site. Thus, a major challenge remains: to characterize the host affinity and

spatial structure of rare and/or singleton species. While clearly important for understanding the ecology of tropical endophyte communities, this is also important at higher-latitude sites, which contain large numbers of singleton species as well.

May (1991) suggested that Hawksworth's (1991) estimate of 1.5 million species of fungi on a global scale overestimates fungal species richness, in part because the postulated ratio of vascular plant species to fungal species (1:6, based on data from the temperate zone) may overestimate host specificity of fungi in tropical forests. We found that the most frequently isolated and readily identifiable endophytes in tropical plants are also those with broadest host ranges. Largely generalist species of *Xylaria*, *Colletotrichum*, *Phomopsis*, *Fusarium*, and *Botryosphaeria* typically grow rapidly and competitively on the nonselective, plant-based media frequently used in survey work (e.g., malt extract agar, potato dextrose agar, cornmeal agar [e.g., Lodge et al. 1996, Fröhlich and Hyde 1999, Arnold et al. 2000]). Are these truly the most common endophytes, or simply those that grow most rapidly in culture? The greatest diversity of tropical endophytes—and the greatest host specificity—likely lie in the endophytes that do not grow rapidly or sporulate on nonselective media: these may be more specialized symbionts with narrower host ranges, reduced competitive ability when growing on a substrate other than the host, and more distinctive life histories. Recent work in the temperate zone has shown that culture-free methods such as direct (environmental) PCR of foliage will recover an endophyte community complementary to that recovered via culturing alone, with the common genera listed above never recovered using the direct PCR approach (Arnold et al. 2007). These methods need to be applied to tropical forest plants to fully elucidate the composition, diversity, and host range of the foliar endophyte community.

At BCI, the most common endophytes are found in host plants with very different phylogenetic positions, leaf longevity, leaf expansion rates, chemical defenses, and structural components (see Arnold 2002). This argues for relative homogeneity in the distribution of common endophytes across the forest, with occurrence of given species effectively random with regard to available hosts. However, the dominant endophytes typically differ among co-occurring plant species (Arnold et al. 2000, 2003). This non-random distribution argues for selectivity in the establishment of endophyte symbioses. Roles of leaf chemistry have been investigated previously and may be key to shaping endophyte communities in given hosts (Arnold and Herre 2003). Other factors, such as competitive interactions among endophytes, remain to be evaluated.

Importantly, the occurrence of given fungi in multiple hosts does not imply equality in terms of interactions with those hosts. Previously documented growth responses to leaf chemistry, and the correlation of these results with endophyte isolation frequencies from

different hosts (Arnold et al. 2003), suggest that interactions between given plant and fungal taxa are likely unique. Whether the outcomes of such interactions are stable in time and space, and/or are influenced by other endophytes that may co-occur with focal species in individual leaves, remain to be evaluated. Recent analyses have shown that endophytism is an evolutionarily labile state, characterized by frequent transitions to and from pathogenicity over the history of the Ascomycota (A. E. Arnold, J. Miadlikowska, K. L. Higgins, S. D. Sarvate, P. S. Gugger, A. Way, V. Hofstetter, F. Kauff, and F. Lutzoni, *unpublished manuscript*). Pathogens with complex life histories frequently manifest different ecological states in different hosts (Agrios 1997), but the potential for particular endophytes to play different ecological roles in different hosts remains an exciting question. In particular, the ability of endophytes to act as avirulent and perhaps mutualistic symbionts of one host, but as virulent pathogens of another species or genotype, is especially intriguing.

A critical next step for assessing these and other topics of interest to ecologists lies in developing methods to adequately designate biologically meaningful taxonomic units for these frequently sterile microfungi. One frequently used method for estimating species boundaries among endophytes is to rely on BLAST searches of the NCBI GenBank database to identify isolates on the basis of molecular sequence data (e.g., Arnold et al. 2000, Guo et al. 2000). However, BLAST matches are based on non-evolutionary matching criteria, are subject to error due to mis-identified sequences, can be difficult to interpret when all top matches are unidentified isolates or environmental samples, and are limited to those fungi present in GenBank (21 075 ITSrDNA sequences in early 2004 [Lutzoni et al. 2004]). We found that BLAST analyses performed at different times can differ markedly in terms of inferred genus- and family-level identifications for the same sequences. Although phylogenetic species concepts are likely sensitive to a similar issue (reflecting the increasing availability of multigene datasets for many species over time), phylogeny-based taxonomy provides the tool needed to positively identify the closest relatives of endophytes.

When species boundaries are conservatively estimated using the methods outlined here, it becomes clear that the tropical moist forest at BCI harbors a remarkably high diversity of endophytic fungi. Endophytes associated with only a few leaves of a single tropical host species (Fisher's  $\alpha = 9.3\text{--}17.9$ ; Appendix D) approximate the species diversity represented by all plant stems  $>10$  cm diameter at breast height (dbh) on a representative hectare of high-elevation neotropical forest (e.g., Volcan Barva, Costa Rica, at 2000 m; cited in Leigh [1999]). By sampling 100 representative endophyte strains from tropical foliage (corresponding to 100 leaf fragments, each measuring  $2\text{ mm}^2$ ) we recovered diversity consistent with that of all trees  $\geq 20$  cm dbh on the 50-ha Forest

Dynamics Plot at BCI (Leigh 1999). The majority of endophyte species recovered at BCI were found only once, and were unique relative to samples in larger geographic regions in both the temperate zone and boreal and arctic sites. All biomes studied here have unique and diverse endophyte communities, providing support for arguments of fungal hyperdiversity raised by Hawksworth and others (see Hawksworth 2001). However, tropical trees, and the leaves that they bear, appear to be special hotspots of fungal species diversity.

#### ACKNOWLEDGMENTS

This research was supported by an NSF Postdoctoral Fellowship in Microbial Biology to A. E. Arnold (DEB-0200413), NSF DEB-0228668 (Assembling the Tree of Life grant) to F. Lutzoni, NSF CAREER grant DEB-0133891 to F. Lutzoni, NSF DEB-0343953 to A. E. Arnold and J. Dalling, NSF DEB-9902346 to L. A. McDade and A. E. Arnold, and the College of Agriculture and Life Sciences at the University of Arizona. We thank S. D. Sarvate, K. L. Higgins, M. Hoffman, and F. Kauff for technical assistance, the Arnold and Lutzoni labs for helpful discussion, L. A. McDade for support and guidance during the development of this work (A. E. Arnold), and N. Dénomée, O. Choulik, and the Smithsonian Tropical Research Institute for logistical support. We are especially grateful to G. Gilbert for helpful comments on the manuscript and for the invitation to contribute this paper.

#### LITERATURE CITED

- Agrios, G. N. 1997. Plant pathology. Academic Press, San Diego, California, USA.
- Arnold, A. E. 2002. Tropical endophytes: diversity and ecology. Dissertation, University of Arizona, Tucson, Arizona, USA.
- Arnold, A. E., D. A. Henk, R. L. Eells, F. Lutzoni, and R. Vilgalys. 2007. Diversity of foliar fungal endophytes in loblolly pine inferred by culturing and environmental PCR. *Mycologia*, *in press*.
- Arnold, A. E., and E. A. Herre. 2003. Canopy cover and leaf age affect colonization by tropical fungal endophytes: ecological pattern and process in *Theobroma cacao* (Malvaceae). *Mycologia* 95:388–389.
- Arnold, A. E., Z. Maynard, G. S. Gilbert, P. D. Coley, and T. A. Kursar. 2000. Are tropical fungal endophytes hyperdiverse? *Ecology Letters* 3:267–274.
- Arnold, A. E., L. C. Mejía, D. Killo, E. I. Rojas, Z. Maynard, N. Robbins, and E. A. Herre. 2003. Fungal endophytes limit pathogen damage in a tropical tree. *Proceedings of the National Academy of Sciences (USA)* 100:15649–15654.
- Atsatt, P. N. 1988. Are vascular plants inside-out lichens? *Ecology* 69:17–23.
- Cannon, P. F., and C. M. Simmons. 2002. Diversity and host preference of leaf endophytic fungi in the Iwokrama Forest Reserve, Guyana. *Mycologia* 94:210–220.
- Clay, K., and J. Holah. 1999. Fungal endophyte symbiosis and plant diversity in successional fields. *Science* 285:1742–1744.
- Clay, K., and C. Schardl. 2002. Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. *American Naturalist* 160:S99–127.
- Dreyfuss, M. M., and I. H. Chapela. 1994. Potential of fungi in the discovery of novel, low-molecular weight pharmaceuticals. Pages 44–80 *in* V. P. Gullo, editor. *The discovery of natural products with therapeutic potential*. Butterworth-Heinemann, London, UK.
- Fröhlich, J., and K. D. Hyde. 1999. Biodiversity of palm fungi in the tropics: are global fungal diversity estimates realistic? *Biodiversity and Conservation* 8:977–1004.
- Gamboa, M. A., and P. Bayman. 2001. Communities of endophytic fungi in leaves of a tropical timber tree (*Guarea guidonia*: Meliaceae). *Biotropica* 33:352–360.

- Gilbert, G. S. 2002. Evolutionary ecology of plant diseases in natural ecosystems. *Annual Review of Phytopathology* 40: 13–43.
- Guo, L. D., K. D. Hyde, and E. C. Y. Liew. 2000. Identification of endophytic fungi from *Livistona chinensis* based on morphology and rDNA sequences. *New Phytologist* 147: 617–630.
- Hawksworth, D. L. 1991. The fungal dimension of biodiversity: magnitude, significance, and conservation. *Mycological Research* 95:641–655.
- Hawksworth, D. L. 2001. The magnitude of fungal diversity: the 1.5 million species estimate revisited. *Mycological Research* 105:1422–1432.
- Higgins, K. L., A. E. Arnold, J. Miadlikowska, S. D. Sarvate, and F. Lutzoni. 2007. Phylogenetic relationships, host affinity, and geographic structure of boreal and arctic endophytes from three major plant lineages. *Molecular Phylogenetics and Evolution*, *in press*.
- Leigh, E. G., Jr. 1999. *Tropical forest ecology*. Oxford University Press, Oxford, UK.
- Lodge, D. J., P. J. Fisher, and B. C. Sutton. 1996. Endophytic fungi of *Manilkara bidentata* leaves in Puerto Rico. *Mycologia* 88:733–738.
- Lutzoni, F., et al. 2004. Assembling the fungal tree of life: progress, classification, and evolution of subcellular traits. *American Journal of Botany* 91:1446–1480.
- May, R. M. 1991. A fondness for fungi. *Nature* 352:475–476.
- O'Brien, H. E., J. L. Parrent, J. A. Jackson, J.-M. Moncalvo, and R. Vilgalys. 2005. Fungal community analysis by large-scale sequencing of environmental samples. *Applied and Environmental Microbiology* 71:5544–5550.
- Pinto, L. S. R. C., J. L. Azevedo, J. O. Pereira, M. L. C. Vieira, and C. A. Labate. 2001. Symptomless infection of banana and maize by endophytic fungi impairs photosynthetic efficiency. *New Phytologist* 147:609–615.
- Redman, R. S., K. B. Sheehan, R. G. Stout, R. J. Rodriguez, and J. M. Henson. 2002. Thermotolerance conferred to plant host and fungal endophyte during mutualistic symbiosis. *Science* 298:1581.
- Rodriguez, R. J., R. S. Redman, and J. M. Henson. 2004. The role of fungal symbioses in the adaptation of plants to high stress environments. *Mitigation and Adaptation Strategies for Global Change* 9:261–272.
- Stone, J. K., C. W. Bacon, and J. F. White, Jr. 2000. An overview of endophytic microbes: endophytism defined. Pages 3–29 *in* C. W. Bacon and J. F. White, editors. *Microbial endophytes*. Marcel Dekker, New York, New York, USA.
- Suryanarayanan, T. S., T. S. Murali, and G. Venkatesan. 2002. Occurrence and distribution of fungal endophytes in tropical forests across a rainfall gradient. *Canadian Journal of Botany* 80:818–826.
- Wagner, B. L., and L. C. Lewis. 2000. Colonization of corn, *Zea mays*, by the entomopathogenic fungus *Beauveria bassiana*. *Applied and Environmental Microbiology* 66: 3468–3473.

#### APPENDIX A

Site descriptions for eight arctic, boreal, temperate, and tropical localities surveyed for endophytic fungi (*Ecological Archives* E088-032-A1).

#### APPENDIX B

Study sites, hosts, and incidence of endophyte infection among 2-mm<sup>2</sup> segments of photosynthetic tissues for 34 host species–site combinations (*Ecological Archives* E088-032-A2).

#### APPENDIX C

Methods for DNA extraction, PCR, sequencing, and sequence assembly (*Ecological Archives* E088-032-A3).

#### APPENDIX D

Sites, hosts, sampling intensity, species richness, and diversity (Fisher's alpha) for 1202 strains of foliar endophytes from 21 host species in tropical, temperate, and boreal localities (*Ecological Archives* E088-032-A4).

#### APPENDIX E

Statistical tables for regression analyses regarding effects of latitude and annual rainfall on endophyte incidence and diversity (*Ecological Archives* E088-032-A5).

#### APPENDIX F

Taxonomic distribution of orders of Ascomycota among representative strains of endophytic fungi recovered from woody angiosperms in lowland, moist, tropical forest at Barro Colorado Island, Panama (BCI) (*Ecological Archives* E088-032-A6).

#### APPENDIX G

Instability in BLAST-based identifications at the genus and family levels for foliar endophytes from two focal tropical hosts (*Heisteria concinna*, *Ouratea lucens*) (*Ecological Archives* E088-032-A7).