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LETTER

Are tropical fungal endophytes hyperdiverse?

A. Elizabeth Arnold,[1] Zuleyka Maynard,[2] Gregory S. Gilbert,[3] Phyllis D. Coley[4] and Thomas A. Kursar[4]

[1]Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ 85721, U.S.A.
E-mail: betsya@u.arizona.edu
[2]Smithsonian Tropical Research Institute, Apartado 2072, Balboa, Republic of Panama.
[3]Department of Environmental Studies, University of California, Santa Cruz, CA 95064, U.S.A.
[4]Department of Biology, University of Utah, Salt Lake City, UT 84112, U.S.A.

Abstract

Fungal endophytes are ubiquitous fungi that inhabit healthy plant tissues without causing disease. Endophytes have been found in every plant species examined to date and may be important, but often overlooked, components of fungal biodiversity. In two sites in a lowland, moist tropical forest of central Panama, we quantified endophyte colonization patterns, richness, host preference, and spatial variation in healthy leaves of two co-occurring, understory tree species [*Heisteria concinna* (Olacaceae) and *Ouratea lucens* (Ochnaceae)]. From 83 leaves, all of which were colonized by endophytes, we isolated 418 endophyte morphospecies (estimated 347 genetically distinct taxa), most of which were represented by only a single isolate (59%). Among morphospecies encountered in more than one leaf (nonsingletons), we found evidence of host preference and spatial heterogeneity using both morphospecies frequencies and presence/absence records. Based on these data, we postulate that tropical endophytes themselves may be hyperdiverse and suggest that extrapolative estimates that exclude them will markedly underestimate fungal species diversity.

Keywords

Barro Colorado Island, fungal endophytes, host preference, Jaccard's index, Ochnaceae, Olacaceae, Panama, tropical forests.

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INTRODUCTION

Fungi comprise only ≈ 72 000 described species, but the true scale of fungal diversity is an open debate (Hawksworth 1991; May 1991; Lodge 1997; Frohlich & Hyde 1999). Its resolution bears important consequences for agriculture, medicine, industry, ecology, and conservation (e.g. Petrini *et al.* 1992; Fox 1993), yet little is known of what may be the greatest pool of fungal diversity: the tropical mycota (Hawksworth 1993), including tropical fungal endophytes.

Endophytes are microorganisms that colonize and cause unapparent, asymptomatic infections in healthy plant tissues (*sensu* Wilson 1995). Endophytic fungi have been found in all plant species examined to date, including algae (Hawksworth 1988), mosses (Schulz *et al.* 1993), ferns (Fisher 1996), and conifers (e.g. Bernstein & Carroll 1977; Legault *et al.* 1989), as well as angiosperms, including grasses (e.g. Clay 1988), palms (Rodrigues 1996; Frohlich & Hyde 1999), and a variety of dicotyledonous shrubs (e.g. Petrini *et al.* 1982) and trees (e.g. Faeth & Hammon 1997). The ubiquity of fungal endophytes, their pharmaceutical potential (Strobel & Long 1998), and evidence that they may act as mutualists with their host plants under certain conditions (e.g. Carroll 1988; Clay 1991) are compelling reasons for their study. Equally intriguing, however, are their implications for estimates of fungal species diversity: multiple studies have documented that individual plants in the temperate zone may harbour dozens of endophyte species (reviewed in Saikkonen *et al.* 1998), suggesting that fungal endophytes may be key components of fungal biodiversity.

Most mycologists agree that fungal diversity likely peaks in tropical forests, where woody angiosperm diversity is also at its highest. Highly diverse Costa Rican (Bills & Polishook 1994) and Puerto Rican leaf-litter fungi (Polishook *et al.* 1996) and decomposers (reviewed in Lodge 1997) corroborate this view. Similarly, 22 endophyte species collected from only three leaves of *Manilkara bidentata* (Sapotaceae) in Puerto Rico (Lodge *et al.* 1996) suggest that tropical endophytes may contribute substantively to fungal diversity. To date, however, large surveys assessing endophyte richness and host preference in tropical dicotyledonous trees, and spatial heterogeneity in a tropical forest, have been lacking, such that the potential contribution of endophytes to fungal biodiversity estimates is little known.

Here, we describe colonization patterns, richness, host preference and spatial variability of fungal endophytes in healthy leaves of two sympatric tree species in two sites in

a lowland tropical forest. We explore the consistency of these data with comparable studies of microfungi at different tropical sites. We then assess the implications of endophyte richness for fungal biodiversity estimates.

MATERIALS AND METHODS

Endophyte sampling

At Barro Colorado Island, Panama [BCI; ~9–9′N, 79–51′W; see Leigh *et al.* (1995) for a thorough site description], we examined endophyte communities associated with leaves of *Heisteria concinna* Standl. (Olacaceae) and *Ouratea lucens* (H.B.K.) Engler (Ochnaceae), two distantly related, but co-occurring, species of understory trees typical of lowland, semideciduous Panamanian forest (Croat 1978). In January–February (early dry season) we marked emerging leaves on nine individuals of *H. concinna* and 10 individuals of *O. lucens*. Marked individuals of both species occurred together in two apparently similar, forested sites separated by $\approx$ 500 m of intact forest: site I (David Fairchild trail near 0 m: 10 individuals of *O. lucens*; five of *H. concinna*) and site II (Bocanegra trail near 30 m: four individuals of *H. concinna*, with several [> 4] unmarked individuals of *O. lucens* within 10 m).

From February to July we harvested healthy, 0.5–6-month-old leaves of *H. concinna* ($n = 41$ leaves: 17 from site I; 24 from site II) and *O. lucens* ($n = 42$ leaves, all from site I). Within 4 h of harvesting, each leaf was washed in running tap water and processed: from midway between the petiole and leaf tip, and between the midvein and the margin of each leaf, we cut 96 adjacent, 1 mm × 2 mm segments from the lamina and surface-sterilized them in 0.525% sodium hypochlorite (2 min) and 70% ethanol (2 min). (The absence of strictly epiphytic bacteria, yeasts, and fast-growing Zygomycetes from our plates suggested that this method of surface-sterilization was effective.) We then arbitrarily selected 24 segments and placed them on Petri dishes containing 2% malt extract agar (MEA), a general medium commonly used in endophyte studies (e.g. Carroll *et al.* 1977; Sherwood-Pike *et al.* 1985; Schulz *et al.* 1993) and known to yield large numbers of endophytic isolates and species relative to other media (Frohlich & Hyde 1999). Plates were incubated on laboratory benches at room temperature with ambient light. Every 3 days for 21 days, we assessed each leaf segment for fungal growth. Hyphal tips from distinct colonies emerging from leaf segments were subcultured on new 2% MEA plates to obtain pure colonies.

Once in pure culture, isolates were vouchered on MEA slants in a living collection. We then conservatively assigned all isolates to morphospecies based on 10 morphological characters, including spore production, spore characteristics, hyphal height and depth, aerial mycelium form, colony and medium colours, surface texture, margin characters, and growth rates on MEA. In order to eliminate effects imposed by different researchers, one of us (ZM) was responsible for all morphospecies designations (with supervision by GG). Discrete colour matches were based on paint samples, and growth characters of isolates that were difficult to assign to morphospecies were assessed on a second culture medium (V8 agar) to assist qualitatively in morphospecies designations.

Those seven characters with a finite number of character states (spore production, hyphal height and depth, aerial mycelium form, surface texture, margin characters, growth rates) had 7.8 ± 2.8 (mean $\pm$ 1 SE) possible states. A power analysis of our morphospecies criteria suggests that, based on these characters alone (i.e. excluding characters based on colour and spore morphology), a minimum of 78 125 unique trait combinations were possible.

Morphospecies concept

Use of morphospecies to designate functional taxonomic units is common in field surveys for small, cryptic, or highly diverse taxa, especially in tropical sites (e.g. Finlay *et al.* 1996; Oliver & Beattie 1996; Longino & Colwell 1997; Bruhl *et al.* 1998; Finlay 1998; Lawton *et al.* 1998; McWilliam & Death 1998; Zanuncio *et al.* 1998). Similarly, many endophyte studies, especially in tropical sites, include a relatively large proportion of taxa designated only as morphospecies (e.g. Lodge *et al.* 1996; Frohlich & Hyde 1999). Although morphospecies generally are used to group fungal isolates that do not sporulate in culture (mycelia sterilia), we chose a morphospecies approach for cataloguing all fungal endophytes obtained in the present study due to the large number of isolates and a paucity of taxonomic resources for identifying Neotropical microfungi (Polishook *et al.* 1996). Despite frequent use of morphospecies in endophyte surveys, however, statements describing the accuracy with which fungal morphospecies approximate true species are absent from the literature. Moreover, given recent advances in fungal taxonomy, even that subset of tropical endophytes that might be assigned to known species on the basis of spore morphology might not represent stable taxa once subjected to molecular analysis (e.g. O'Donnell 1992; Aptroot 1997; Jacobs & Rehner 1998). For these reasons, we deemed necessary an approach combining both molecular and morphological characters. We then assessed concordance between our morphospecies and genetic species in two ways.

First, the relationship between our morphospecies and genetically delineated species was examined in a separate study, in which fungal isolates from four tropical and two temperate-zone leaf-litter samples were characterized by the morphospecies criteria used in the present study, and by sequence data from the internal transcribed spacer regions 1 and 2 (nuclear rDNA, ITS-1 and ITS-2). On average, three-fourths of morphological types represented precise or conservative descriptions of ITS-based taxa (defined by a phylogenetic species concept, *sensu* Vilgalys 1991; Schardl & Leuchtmann 1999; Taylor *et al.* 1999). Overall, morphospecies of leaf-litter fungi overestimated richness based on ITS-based taxa by 17.1% $\pm$ 10.3%, with slightly better concordance among four tropical samples alone (Gilbert *et al.* unpublished results).

We are uncertain whether patterns of morphological and molecular correspondence among leaf-litter fungi are similar for fungal endophytes (see Discussion). Therefore, in order to more precisely assess the utility of our morphospecies concept for tropical endophytes, we are examining morphological and molecular concordance among endophytes obtained in the present study. Following Raeder & Broda (1985), we have extracted total cellular DNA from lyophilized mycelia and have amplified total genomic DNA using primers ITS-4 and ITS-5 (White *et al.* 1990) and PCR protocols described in Rehner & Uecker (1994). To date we have sequenced ITS-1 and ITS-2 for 43 endophyte isolates representing 27 morphospecies. We submitted ITS sequences for all 27 morphospecies to Genbank BLAST searches and recorded the most probable taxonomic match for each sequence; in cases in which multiple matches were equally probable, we recorded the lowest taxonomic level shared by those disparate matches. Because Genbank lacks sequence data for most fungi at the species level, we could not estimate the number of species in our sample based on species-specific matches. Instead, we assessed the number of genera, families, orders, and classes matched by our isolates.

Among 27 morphospecies, we found high-probability matches with members of nine genera representing eight families (Lasiosphaeriaceae, Botryosphaeriaceae, Xylariaceae, Trichocomaceae, Hypocreaceae, Amphisphaeriaceae, Herpotrichiellaceae, Sclerotiniaceae), seven orders (Sordariales, Pleosporales, Xylariales, Hypocreales, Eurotiales, Chaetothyriales, Leotiales), and four traditional classes of Ascomycotina (Discomycetes, Pyrenomycetes, Loculoascomycetes, Plectomycetes). Because our molecular data are preliminary, use of a phylogenetic species concept is not yet feasible for designating species numbers. However, the broad representation of ascomycetous taxa among only 27 morphospecies suggests high upper-level diversity among isolates obtained in this study.

In differentiating morphospecies, we cultivated all isolates under conditions as uniform as could be achieved in the laboratory, grouped problematic isolates with growth characters from two distinct media, delineated morphospecies on the basis of morphological characters traditionally used in fungal taxonomy, and were conservative in our morphospecies designations. Our preliminary sequence data suggest a high degree of upper-level taxonomic diversity among a small subset of isolates, and support from Gilbert *et al.* (unpublished data) suggests that our morphospecies concept, although slightly overestimating genetic species, is generally effective. For these reasons, we use morphospecies as functional taxonomic units in this paper. Moreover, because singletons (see below) are more prone to erroneous segregation as unique forms than are nonsingletons, we expect nonsingleton morphospecies to represent relatively stable taxa. We therefore use only nonsingletons in the analyses that follow, and expect our results to be robust to changes in absolute species numbers.

Analyses

We assessed all nonsingleton morphospecies for evidence of host preference and spatial variability by randomizing morphospecies frequencies and comparing observed frequencies with which morphospecies were restricted to only one host species or site against frequencies derived from 1000 randomizations. For all analyses, singletons included those morphospecies isolated from only one leaf segment, as well as those represented by multiple isolates from only a single leaf; nonsingletons were defined as those morphospecies occurring in more than one leaf. We chose this conservative approach because independence among multiple isolates of a given morphospecies within individual leaves could not be determined with certainty.

We then used presence/absence data for nonsingletons to calculate Jaccard's index (JI), a measure of similarity between pairs of samples (*sensu* Polishook *et al.* 1996).

$$JI = a/(a + b + c) \quad (1)$$

where a represents the number of species occurring in both samples, b represents the number of species restricted to sample 1, and c represents the number of species restricted to sample 2. JI ranges from 0 (no taxa shared) to 1 (all taxa shared).

RESULTS

From 83 leaves (1992 leaf segments), we obtained 1472 isolates representing 418 fungal morphospecies. Cultural characteristics suggest that these endophytes include broad diversity of Ascomycotina (including traditional

Deuteromycotina). All leaves were colonized by endophytic fungi (100%), as were 73.9% of leaf segments. Overall, leaves contained 10.5 ± 0.48 (mean $\pm$ 1 SE) morphospecies per 24 segments, and new morphospecies accumulated rapidly with each additional leaf sampled (Fig. 1). A total of 242 morphospecies occurred in *H. concinna* and 259 occurred in *O. lucens*, but most endophyte morphospecies were rare: 246 (59%) appeared in only one 2 mm^2 leaf segment.

At site I, where both host species were thoroughly sampled ($n = 17$ leaves of *H. concinna*; $n = 42$ leaves of *O. lucens*), 47 of 76 nonsingleton morphospecies (62%) occurred in either *H. concinna* or in *O. lucens*, but not in both ($P = 0.004$), and similarity across host species was intermediate (JI = 0.59). Similarly, in a single host species (*H. concinna*) that was thoroughly sampled at both site I ($n = 17$ leaves) and site II ($n = 24$ leaves), 29 of 61 nonsingletons (48%) occurred in only one of two sites, but not in both ($P = 0.048$). We found that similarity between endophyte assemblages at each site was moderately low (JI = 0.48).

DISCUSSION

Estimated number of genetically distinct taxa

In this survey, we isolated 418 unique morphological types of endophytic fungi. However, a strictly morphological approach to delineating taxa of tropical microfungi is limited by the prevalence of undescribed species, subtle differences among taxa with relatively few phenotypic characters, delayed or absent sporulation in culture, and convergence by disparate taxa into similar morphological forms. Preliminary sequence data suggest that at least nine genera representing eight families and seven orders are represented among the first 27 morphospecies sequenced; still, the number of true fungal species remains to be addressed.

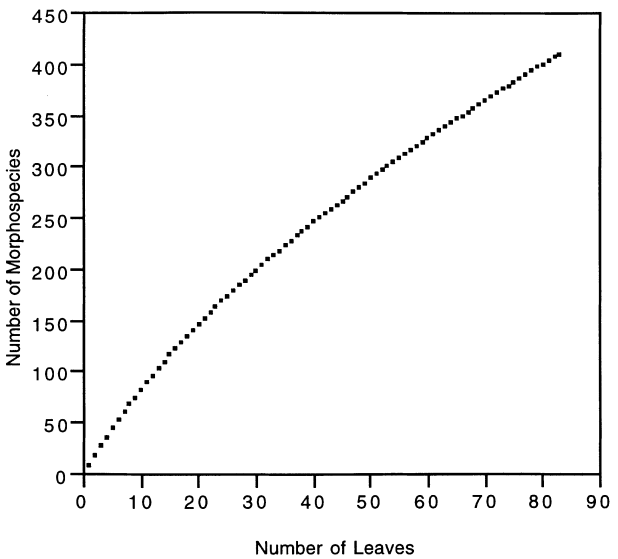

Figure 1 Species accumulation curve for endophyte morphospecies isolated from healthy leaves of *Heisteria concinna* ($n = 41$) and *Ouratea lucens* ($n = 42$) in two forested sites at Barro Colorado Island, Panama. Leaf order was randomized 100 times.

To the best of our knowledge, no estimate relating endophyte morphospecies to genetic species has been presented in the literature, despite frequent use of morphospecies in endophyte studies (e.g. Gaylord *et al.* 1996; Polishook *et al.* 1996; Schulthess & Faeth 1998; Frohlich & Hyde 1999). We therefore apply the relationship given by Gilbert *et al.* (unpublished data) for leaf-litter fungi, and estimate that 418 endophyte morphospecies correspond to $\approx$ 347 genetically distinct taxa (range 305–389).

We are uncertain whether correspondence between morphological and molecular taxa among leaf-litter fungi applies equally to fungal endophytes: although these guilds are comprised largely of Ascomycotina and often have fungal families and genera in common, they appear to share few fungal species in tropical forests (Lodge 1997; Frohlich & Hyde 1999). However, extensive surveys suggest significant overlap between temperate-zone endophytes and leaf-litter fungi (G. Carroll, personal communication). Whether nonoverlap between tropical leaf-litter and endophyte species is a true pattern, or is instead an artifact of highly diverse assemblages that have not been thoroughly sampled, is unclear. However, based on general ecological and higher-level taxonomic similarities between leaf-litter and endophytic fungi, we regard 347 genetic species as a reasonable working estimate of endophyte richness at the genetic level.

Colonization patterns

Few studies have quantified endophyte colonization patterns in tropical plants. However, among tropical host taxa studied to date, proportions of leaf segments colonized in individual leaves vary widely: whereas Rodrigues (1994) found few leaf segments of Amazonian palms colonized by endophytes (30%), Lodge *et al.* (1996) found a greater proportion in *M. bidentata* (90%–95%) than were found in the present study (73.9%). We attribute the latter disparity to the presence of very young leaves in our sample: Lodge *et al.* (1996) sampled only fully expanded leaves, and several studies confirm that endophyte infections increase with leaf age (e.g. Bernstein & Carroll 1977).

In contrast, among tropical host taxa surveyed to date, proportions of leaves colonized by endophytes appear relatively constant: we found an equivalent proportion of

leaves colonized by endophytes in *H. concinna* and *O. lucens* (100%) as did Lodge *et al.* (1996) in leaves of *M. bidentata*. These data appear to exceed typical mean values for temperate-zone surveys (e.g. ≈ 78% for evergreen shrubs in western Oregon, Petrini *et al.* 1982; ≈ 78% for *Pinus banksiana* and *P. resinosa* in Quebec, Legault *et al.* 1989); however, quantitative surveys of endophyte colonization patterns may be sensitive to leaf segment size (Carroll 1995), such that variable methodology among temperate-zone studies prevents precise comparisons with the present study. Such comparisons may be confounded further by unexplored effects of leaf age, phylogenetic affinity among hosts, microclimate, or sampling season. However, the rarity of 100% leaf colonization rates among a wide range of temperate taxa (but see Espinosa-Garcia & Langenheim 1990) suggests that proportions of leaves infected in tropical trees likely exceed those of temperate hosts. Moreover, similarity among three distantly related hosts (*H. concinna*, *O. lucens*, and *M. bidentata*) in two disparate sites (Puerto Rico and Panama) suggests consistently high colonization rates among and within leaves of tropical dicotyledonous trees.

Endophyte richness

Although we isolated a large number of endophyte morphospecies in the present survey, the steep slope and lack of an asymptote in the morphospecies-accumulation curve (Fig. 1) suggests that sampling of the endophyte community in these hosts, and in this tropical forest, is far from complete. These data suggest remarkable diversity among endophytes of two host trees in a lowland Panamanian forest.

In order to assess the consistency of endophyte diversity across tropical sites and species, we compared our data with those of Frohlich & Hyde (1999), who quantified endophyte infections in *Licuala* spp. (Arecaceae) in Australia and Brunei Darussalam (Borneo). Based on endophytes isolated during three sampling periods, and on estimates of unsampled taxa derived using Preston's octave-scale method, the authors estimate that 140 species of endophytic fungi were associated with three individuals of *Licuala* sp. in Brunei Darussalam. For comparison, we adjusted the number of endophytic morphospecies isolated from leaves of *H. concinna* (242 morphospecies) and *O. lucens* (259 morphospecies) by applying the ratio of genetic species:morphospecies derived from Gilbert *et al.* (unpublished results). We estimate that in our sample, ≈ 200 endophyte species (range: 176–225 species) were associated with nine individuals of *H. concinna*, and ≈ 215 endophyte species (range: 188–240 species) were associated with 10 individuals of *O. lucens*. These values are conservative, representing only that subset of leaves that was collected and sampled for endophytes. Relative to Frohlich & Hyde (1999), however, we sampled from a larger number of individuals and collected endophytes during 6 months of continuous sampling. For these reasons, we consider the data presented here to be comparable with the results of Frohlich & Hyde (1999).

We also compared our data with the results of Lodge *et al.* (1996), whose work represents one of the only published surveys of endophyte diversity in a tropical dicotyledonous tree. Using one leaf per each of three trees, and sampling from 50 leaf segments per leaf, Lodge *et al.* (1996) isolated 22 endophyte species from three leaves of *M. bidentata* in the Luquillo Mountains of Puerto Rico. Using Preston's method, Lodge *et al.* (1996) estimated that as many as 25–28 species of endophytes were actually present in their sampled leaves. For comparison, we grouped morphospecies records for sets of three mature leaves of *H. concinna* that were each harvested within 10 days of one another approximately 6 months after budbreak ($n = 5$ sets). On average, we found 29.8 ± 2.01 (mean $\pm$ 1 SE) endophyte morphospecies per three leaves (estimated 25 genetically distinct species). Moreover, we found that individual leaves contained 8–19 morphospecies; these values are consistent with Lodge *et al.*'s (1996) findings from *M. bidentata* (12, 12, and 17 species/leaf). Thus, on the basis of small samples of leaves, our findings of endophyte richness are consistent with those of Lodge *et al.* (1996). Such similarity among distantly related tree species at two tropical sites suggests a consistent pattern of high richness for tropical endophytes.

Host preference and spatial variation

Although we found evidence for host preference and spatial heterogeneity among endophytes, support for those patterns was much stronger when based on morphospecies frequencies than on presence/absence data. These results corroborate Lodge's (1997) suggestion that in contrast to traditional concepts of host- and site specificity, host-preference and spatial patterns among tropical fungi are described more accurately by differences in relative abundance than by presence/absence alone. Because published data comparing tropical endophyte abundances in naturally sympatric, dicotyledonous hosts and sites are unavailable, we are uncertain whether most endophytes show similar patterns of host preference and spatial variability in tropical forests. However, inferences can be drawn from other guilds of tropical microfungi.

Although Lodge (1997) discussed host generalism among tropical leaf-decaying Agaricales (Basidiomycotina) and ascomycetous wood-decay fungi, she noted that some tropical Xylariaceae (Ascomycotina) are restricted to

single genera or families of host plants in Puerto Rico. Similarly, in a quantitative study, Polishook *et al.* (1996) found evidence for host preference among highly diverse, ascomycetous leaf-decomposer fungi in a Puerto Rican forest: among isolates from two host species in two sites, 58% of nonsingletons were restricted to one species, and similarity between host species was low (JI = 0.26, 0.32). In comparison, endophytes in the present survey are comparable in their degree of host preference (62%), but appear more similar across hosts (JI = 0.59). We attribute the disparity in JI to differing definitions of nonsingletons: Polishook *et al.* (1996) may have underestimated similarity by including among nonsingletons those morphospecies represented by multiple isolates from only a single leaf. In contrast, our more conservative approach increases JI and would overestimate similarity if, in a given leaf, some proportion of multiple isolates of a given morphospecies represent independent infections initiated by more than one fungal propagule. In general, however, our findings of host preference are comparable with those of Polishook *et al.* (1996) and corroborate Cornejo *et al.*'s (1994) survey of Panamanian leaf-litter fungi.

Spatial variability has not been thoroughly explored for tropical microfungi and may be difficult to discern in studies in which stratum, substrate, or host preferences confound spatial patterns. However, concentrating on two co-occurring host species, Polishook *et al.* (1996) found evidence for spatial heterogeneity among leaf-litter fungi, citing low similarity across sites separated by 200 m (JI = 0.34, 0.38). Our results are consistent, suggesting moderately low similarity among endophytes from a single host species across sites separated by 500 m (conservative JI = 0.48). As Lodge (1997) suggests, relative abundances of morphospecies may further elucidate patterns of specificity; together, however, these studies suggest that host preference and spatial variability may be prevalent among at least two highly diverse guilds of tropical microfungi.

Implications for fungal biodiversity estimates

Hawksworth (1991) postulated the existence of 1.5×10^6 fungal species, an estimate based on the observation that roughly six fungal species associate with each plant species in the British flora. Skeptics, citing low rates of new species descriptions from little-known tropical mycofloras, argued that this value overestimates fungal diversity (May 1991). Frohlich & Hyde (1999), however, have suggested that 33 fungal species associate with individual palm species in Borneo and Australia, while other authors have postulated that there may be at least one million species of endophytic fungi alone (Dreyfuss & Chapela 1994). Our survey excluded an array of fungal symbionts (strictly epiphyllous, actively pathogenic, saprophytic, and mycorrhizal fungi), sampled from only a single stratum in a single forest, and may have overlooked unculturable or medium-specific endophytes; still, we encountered 418 morphospecies (estimated 347 genetic species) from two host species in two sites. We found evidence for host preference and spatial variability among those morphospecies and suggest that patterns of richness and preference may be consistent across host species and tropical sites.

Based on these observations, we suggest that tropical endophytes comprise an important and quantifiable component of fungal biodiversity. The high richness we found leads us to postulate that tropical endophytes themselves may be hyperdiverse, but further sampling within and among host species and sites is required to assess the true number of endophytic species. Although further research is needed to determine the appropriate order of magnitude, we expect that continuing exploration of fungal species diversity in tropical forests, and consideration of little-explored guilds such as fungal endophytes, will demonstrate that 1.5×10^6 species markedly underestimates fungal biodiversity.

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BIOSKETCH

A. Elizabeth Arnold is a tropical ecologist focusing on plant–fungus interactions. Her interests include the diversity and ecological roles of endophytes in tropical trees, fungal biodiversity, and community ecology.

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