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Fungal endophytes in dicotyledonous neotropical trees: patterns of abundance and diversity*

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Fungal endophytes have been found in every plant species examined to date and appear to be important, but largely unquantified, components of fungal biodiversity. Endophytes are especially little known in tropical forest trees, where their abundance and diversity are thought to be greatest. Here, we explore the occurrence of endophytes in a broad diversity of woody, angiospermous taxa in a lowland, moist tropical forest in central Panamá. We use similarity indices to assess host preference and spatial heterogeneity of endophytes associated with two co-occurring, but distantly related, understorey tree species in two sites of that forest, and assess the utility of indices based on frequencies of morphospecies occurrence (Morisita-Horn index) and on presence-absence data (Sørensen's index). We suggest that our understanding of fungal diversity will be enhanced by exploring ecological patterns underlying endophyte occurrence in host species, and discuss methods for assessing the proportion of fungal biodiversity represented by tropical endophytes.

INTRODUCTION

The scale of fungal species diversity is an open debate (Hawksworth 1991, May 1991, Lodge 1997, Fröhlich & Hyde 1999). Although Fungi comprise only *ca* 72,000 described species (Hawksworth *et al*. 1995), estimates of fungal biodiversity range from hundreds of thousands of species (Aptroot 1997) to values as high as 1.5 million (Hawksworth 1991) and greater (e.g. Cannon 1997a). In recent years, emergent data from tropical forests (e.g. Fröhlich & Hyde 1999, Arnold *et al*. 2000) have lent support to these higher estimates of fungal species diversity, suggesting that the number of described species represents but a small fraction of the total number of fungal species thought to exist. Resolution of this debate bears directly upon agriculture, medicine, industry, ecology, and conservation (e.g. Petrini *et al*. 1992, Fox 1993) and depends largely upon further exploration of tropical mycota (Hawksworth 1993).

Tropical fungi are traditionally understudied (Hawksworth 1993, Cannon 1997b), and their taxonomic placement has been confounded, at times, by misidentification according to temperate mycota. Especially difficult to enumerate are cryptic guilds of microfungi, whose presence may go unnoticed and whose ecological roles are little known. Such is the case for fungal endophytes in leaves of tropical trees.

Endophytes are microorganisms that colonise and cause asymptomatic infections in healthy plant tissues (Wilson 1995, Suryanarayanan & Kumaresan 2000). Fungal endophytes are considered at least as ubiquitous as mycorrhizal associations among temperate-zone plants (Carroll 1988), having been found in algae (Hawksworth 1988), mosses (Schulz *et al*. 1993), ferns (Fisher 1996), conifers (e.g. Bernstein & Carroll 1977, Legault, Dessureault & Laflamme 1989), and both monocotyledonous (e.g. Clay 1988, Rodrigues 1994, 1996, Fröhlich, Hyde & Petrini 2000) and dicotyledonous angiosperms (e.g. Petrini, Stone & Carroll 1982, Lodge, Fisher & Sutton 1996, Faeth & Hammon 1997, Rajagopal & Suryanarayanan 2000). Saikkonen *et al*. (1998) reviewed multiple studies showing that individual plants in the temperate zone may harbour dozens of endophyte species, and several recent, quantitative surveys of tropical angiosperms have documented remarkable endophyte richness in individual leaves and trees (e.g. Lodge *et al*. 1996, Arnold *et al*. 2000, Fröhlich *et al*. 2000, Gamboa & Bayman 2001). These surveys suggest that tropical endophytes may contribute substantially to fungal diversity.

In order to place endophyte surveys into a broader context of fungal biodiversity, several parameters of endophyte

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communities must be addressed. We suggest that exploring the occurrence of endophytes in a diversity of tropical forest trees, and assessing endophyte communities for evidence of host preference and spatial variability, will create a much-needed context into which single-species surveys of endophyte diversity may be placed. Here, we discuss the widespread occurrence of fungal endophytes in a diverse assemblage of woody angiosperms in a lowland, moist tropical forest in central Panamá, and describe the frequency with which endophytic fungi were recovered from leaves of three of those tree species. Then, focusing on a large number of endophytic fungi isolated from two of those tree species in a previous survey (Arnold *et al*. 2000), we explore patterns of host preference and spatial heterogeneity using a frequency-based index of similarity (Morisita-Horn index) and a similarity index based on presence-absence data (Sørensen's index). Finally, we discuss several approaches for further exploring tropical endophyte diversity.

MATERIALS AND METHODS

Study site

Formerly a hilltop within contiguous tropical forest, Barro Colorado Island, Panamá (BCI; ~ 9°9′ N, 79°51′ W) was isolated in 1911 when the Chagres River was dammed for construction of the Panamá Canal (Leigh & Wright 1990). Maintained as a field station by the Smithsonian Tropical Research Institute since 1921, BCI consists of *ca* 1500 ha of moist, semi-deciduous, lowland tropical forest. Roughly two-thirds of the island is covered by mature forest > 400 y old; the remainder of the island consists of late secondary forest approximately 70–100 y old (Croat 1978, Sagers & Coley 1995). In younger forest on BCI, where endophyte surveys were conducted, approximately 110 species of shrubs and trees > 2.5 cm DBH occur per hectare (Lang, Knight & Anderson 1971). The region has a mean annual temperature of 27 °C and receives 2600 mm of precipitation annually, of which 90% falls during the May-December wet season (Leigh, Rand & Windsor 1982).

Endophyte isolations

For surveys of endophyte abundance across species and within leaves (see below), healthy leaves were harvested, washed in running tap water, and processed within four hours of collection. From each washed leaf, we cut 16 adjacent, 1 mm × 2 mm segments of the lamina from midway between the petiole and leaf tip, and between the midvein and margin. Leaf segments were surface-sterilised by sequential washes in 0.525% sodium hypochlorite (2 min) and 70% ethanol (2 min), and then were rinsed with sterile, distilled water and allowed to surface-dry under sterile conditions. This method of surface-sterilisation has been shown to effectively eliminate bacteria, yeasts, fast-growing *Zygomycetes*, and other putatively epiphyllous organisms from endophyte cultures (Arnold *et al*. 2000, see Schulz *et al*. 1993, for a review of surface-sterilisation methods).

We placed leaf segments on Petri dishes containing 2% malt extract agar (MEA), a medium commonly used in endophyte studies (e.g. Carroll, Müller & Sutton 1977, Sherwood-Pike, Stone & Carroll 1985, Schulz *et al*. 1993) and known to yield large numbers of diverse endophytic isolates (Fröhlich *et al*. 2000). We incubated plates at room temperature and with ambient light, assessing each plate for hyphal growth every three days for 21 days.

Proportions of leaves and leaf segments colonised by endophytes

We harvested four healthy, mature leaves (> 6 mo old) from each of three individuals of nine plant species at BCI, choosing host species that represent both a subset of common species at BCI, and a diverse array of host families. In all, representatives of nine plant families were surveyed, including *Anacardium excelsum* (*Anacardiaceae*), *Laetia thamnia* (*Flacourtiaceae*), *Gustavia superba* (*Lecythidaceae*), *Mouriri myrtilloides* (*Melastomataceae*), *Trichilia tuberculata* (*Meliaceae*), *Heisteria concinna* (*Olacaceae*), *Ouratea lucens* (*Ochnaceae*), *Psychotria marginata* (*Rubiaceae*), and *Theobroma cacao* (*Sterculiaceae*). After plating leaf segments as described above, we assessed proportion of leaves colonised by endophytes by counting the number of leaves yielding at least one endophytic fungus.

We further assessed three species in three plant families for proportion of leaf segments colonised by endophytes. For six leaves of each species, we assessed proportion of leaf segments colonised by endophytes for *H. concinna*, *O. lucens*, and *T. cacao* by counting the number of leaf segments per leaf yielding one or more endophytic fungi.

Host preference and spatial heterogeneity

As described by Arnold *et al*. (2000), we marked emerging leaves on nine individuals of *Heisteria concinna* and 10 individuals of *Ouratea lucens* in January-February (early dry season) at two similar, forested sites at BCI. Site I, located near the start of David Fairchild trail, included 10 individuals of *O. lucens* and five of *H. concinna*. Site II, located on Bocanegra trail near marker 30, included four individuals of *H. concinna*, and several (> 4) unmarked individuals of *O. lucens* within 10 m. Sites are separated by *ca* 500 m of intact forest.

From February-July, we harvested 42 leaves of *O. lucens* (all from site I) and 41 leaves of *H. concinna* (17 from site I; 24 from site II). Leaves ranged in age from 0.5 mo to 6 mo. Endophytes were isolated as described in Arnold *et al*. (2000); briefly, leaves were processed as described above, except that from each leaf, we cut 96 leaf segments (1 mm × 2 mm), surface sterilised all, and haphazardly selected 24 segments for plating. This approach increased the probability of encountering many endophyte species per leaf by increasing the area from which leaf segments were drawn.

We subcultured hyphal tips from distinct colonies emerging from leaf segments onto new 2% MEA plates to obtain pure colonies, and stored isolates on MEA slants as vouchers in a living collection. One of us (ZM, with supervision from GSG) conservatively assigned isolates to morphospecies based on ten morphological characters (spore production, spore charac-

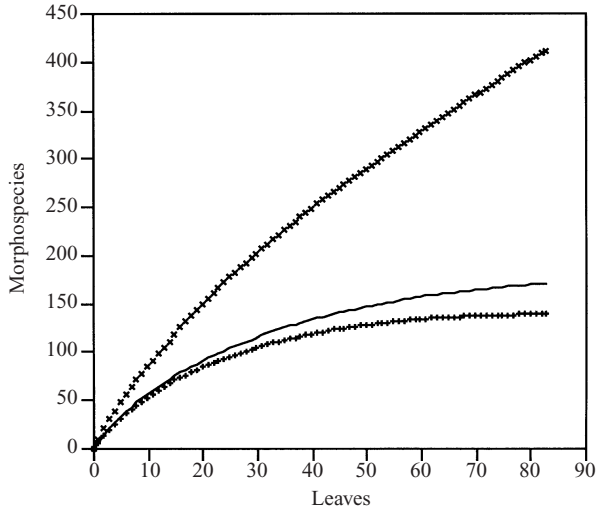

Fig. 1. Species accumulation curves showing the effect of restricting analyses to non-singleton morphospecies: (×) designates the species-accumulation curve for all morphospecies isolated in Arnold *et al.* (2000); (—), those morphospecies occurring in more than one leaf segment; and (+), those morphospecies that occurred in more than one leaf, here defined as non-singletons.

teristics, hyphal height and depth, aerial mycelium form, colony and medium colours, surface texture, margin characters, and growth rates on MEA). We assessed growth characters of isolates that were difficult to assign to morphospecies on a second medium (V8 agar) to assist in morphospecies designations.

For analyses of host preference and spatial heterogeneity in the present paper, we use morphospecies as functional taxonomic units, acknowledging that morphospecies slightly overestimate genetically delimited species (based on preliminary sequence data from ITS1 and ITS2 regions of nuclear rDNA; Arnold *et al.* 2000). Because this overestimate appears to be a function of overassignment of isolates to singleton morphospecies, we restricted analyses to consider only non-singletons, here defined as those morphospecies isolated from 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, and because this approach increased concordance between morphological and molecular taxonomic units (Arnold *et al.* 2000). Effects of restricting analyses to non-singleton morphospecies are shown in species-accumulation curves generated from this dataset (Fig. 1).

Using the freeware program EstimateS (Colwell 1997), we used non-singleton morphospecies to generate species-accumulation curves and similarity indices for endophytes of *O. lucens* and *H. concinna*. We used frequency data (number of isolates of given morphospecies) to calculate Morisita-Horn indices of similarity (MH), and presence-absence data to calculate Sørensen's indices (SI; details regarding calculation of both indices can be found in Magurran 1988 and Colwell 1997). Both indices range in value from 0–1, with 1 indicating entirely coincident samples. These indices were used to assess host preference and spatial heterogeneity by describing similarity of endophyte assemblages within a single host species at two sampling sites, and within a single sampling site for both host species.

RESULTS

Proportions of leaves and leaf segments colonised by endophytes

We found that all leaves surveyed contained endophytic fungi, regardless of host species or family. On average, we found that 98.7% ± 0.77 (mean ± 1 SE) of leaf segments contained endophytic fungi, and mean values for each species were similar: 97.9% ± 2.08 of leaf segments of *H. concinna* contained endophytes, relative to 99.1% ± 0.69 for *O. lucens* and 99.0% ± 1.04 for *T. cacao*.

Host preference and spatial heterogeneity

We found similar rates of species accumulation in leaves of *Heisteria concinna* and *Ouratea lucens* (Fig. 2). In *H. concinna*, 242 morphospecies were found; in *O. lucens*, 259 morphospecies were found (Arnold *et al.* 2000). Most endophyte morphospecies were rare; of 418 total morphospecies, only 140 morphospecies were found in more than one leaf.

Despite similar patterns of species accumulation, endophytes appear to be segregated according to host species. At site I, where both species were thoroughly sampled, 62% of non-singletons occurred in either *H. concinna* or in *O. lucens*, but not in both host species. With analysis restricted to non-singletons, we found only moderate similarities between host species when using either frequencies (MH = 55.5%) or presence-absence data (SI = 60.7%).

Similarly, in a single host species (*H. concinna*) that was thoroughly sampled at both site I and site II, we found that 48% of non-singletons occurred in only one of two sites, but not in both. Similarity between endophyte assemblages at each site was moderate, whether based on frequencies (MH = 56.4%) or on presence-absence data (SI = 59.8%).

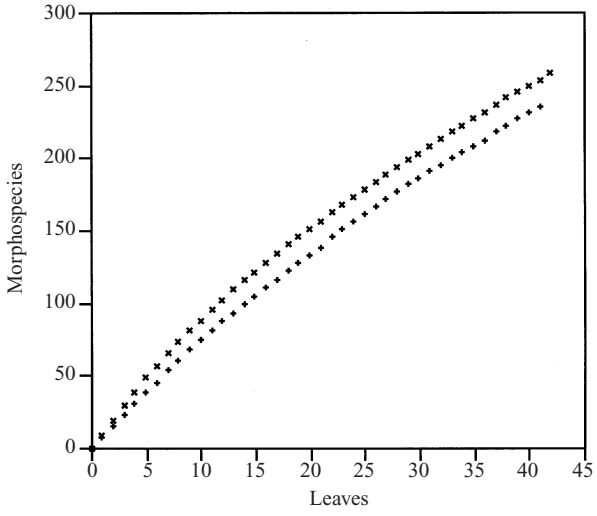

Fig. 2. Species accumulation curves for all endophyte morphospecies isolated from healthy leaves of *Ouratea lucens* (×) and *Heisteria concinna* (+) collected at Barro Colorado Island, Panamá.

DISCUSSION

Proportions of leaves and leaf segments colonised by endophytes

Previous studies have documented endophytic fungi in tropical plants representing the families *Arecaceae* (e.g. Rodrigues & Samuels 1990, Fröhlich *et al*. 2000), *Araceae*, *Bromeliaceae* and *Orchidaceae* (Petrini & Dreyfuss 1981, Richardson & Currah 1995), *Musaceae* (Brown, Hyde & Guest 1998, Photita *et al*. 2001), *Poaceae* (Danielsen & Jensen 1999), *Piperaceae* and *Crassulaceae* (Dreyfuss & Petrini 1984), *Meliaceae* (Rajagopal & Suryanarayanan 2000, Gamboa & Bayman 2001), *Acanthaceae*, *Chenopodiaceae* and *Aizoaceae* (Suryanarayanan & Kumaresan 2000), *Rubiaceae* (Santamaría, Chan & Bayman 2000), *Sapotaceae* (Lodge *et al*. 1996, Bayman *et al*. 1998), *Fabaceae* (Pereira, Azevedo & Petrini 1993), *Araliaceae* (Laessøe & Lodge 1994), *Causarinaceae* (Bayman *et al*. 1998), and *Ochnaceae* and *Olacaceae* (Arnold *et al*. 2000). Here, we document endophytic fungi in tropical representatives of the families *Anacardiaceae*, *Flacourtiaceae*, *Lecythidaceae*, *Melastomataceae*, and *Sterculiaceae*.

We found that all leaves of these species contained fungal endophytes. In the literature, quantitative data regarding proportions of leaves colonised by fungal endophytes are rare for tropical woody angiosperms. However, a colonisation rate of 100% exceeds mean values typical of temperate-zone taxa, including evergreen shrubs in western Oregon (78%; Petrini *et al*. 1982), *Pinus banksiana* and *P. resinosa* in Quebec (78%; Legault *et al*. 1989), and *Quercus emoryi* in Arizona (10–100%; Faeth & Hammon 1997). Unfortunately, quantitative surveys of endophyte colonisation patterns may be sensitive to leaf segment size (Carroll 1995), such that variable methodology may prevent precise comparisons across studies; moreover, such comparisons may be confounded by leaf age, canopy cover, sampling season, etc. (Arnold unpubl. data). In general, however, the rarity of 100% leaf colonisation rates among temperate taxa (but see Espinosa-Garcia & Langenheim 1990, and Faeth & Hammon 1997) suggests that proportions of leaves infected in tropical trees generally exceed those of temperate hosts. Moreover, similarity among 10 distantly related hosts at two disparate sites (BCI: nine species discussed here; Puerto Rico: *Manilkara bidentata*, Lodge *et al*. 1996) suggests that colonisation rates among leaves of tropical dicotyledonous trees may be consistently high.

Similarly, few studies have quantified endophyte colonisation patterns in tropical plants. However, among tropical host taxa studied to date, proportions of leaf segments colonised in individual leaves appear to vary widely: Rodrigues (1994) documented that 30% of leaf segments were colonised in fronds of Amazonian palms, whereas Lodge *et al*. (1996) found a greater proportion in *M. bidentata* (90–95%), as did Gamboa & Bayman (2001) for leaves of *Guarea guidonia* (> 95%). Values obtained in the present study (mean = 98.7%) coincide with these higher values, although they exceed the overall mean for *H. concinna* and *O. lucens* (73.9%) reported by Arnold *et al*. (2000). We attribute this disparity to inclusion of very young leaves by Arnold *et al*. (2000); several studies confirm that endophyte infections increase with leaf age (e.g. Bernstein & Carroll 1977, Faeth & Hammon 1997). In general, we expect that mean proportions of leaf segments colonised by endophytes will be greater in tropical rather than temperate plants, but consistent methodology and directly comparable studies are needed to address this expectation.

Host preference and spatial heterogeneity

Among non-singleton morphospecies, we found evidence for both host preference and spatial heterogeneity among endophytes. Support for these patterns was stronger when based on morphospecies frequencies, rather than on presence-absence data, corroborating Lodge's (1997) suggestion that tropical fungi exhibit quantitative preferences, rather than strict specificity *per se*. Arnold *et al*. (2000) reviewed several descriptions of host preference among tropical microfungi, including some tropical *Xylariaceae* (Lodge 1997), ascomycetous leaf-decomposer fungi (Polishook, Bills & Lodge 1996), and leaf-litter fungi (Cornejo, Varela & Wright 1994), suggesting that at least two guilds of microfungi do show evidence of host preference in tropical forests.

Due to expected differences in leaf chemistry, we were surprised to see similar patterns of species accumulation for endophytes in leaves of *Ouratea lucens* and *Heisteria concinna*: in tropical forests, leaf expansion rates (i.e. rate of growth from budbreak to maturity) are generally associated with differences in leaf chemistry, such that leaves that expand quickly tend to contain lower levels of terpenoids, phenols, and tannins than do leaves that expand slowly (Becker 1981, Coley & Aide 1991, Coley & Barone 1996). At BCI, *O. lucens* produces leaves that expand rapidly, reaching full size in only 8 d (Aide 1993). In contrast, *H. concinna* produces leaves that expand slowly, reaching full size in 35 d (Coley, pers. comm.). Several studies have shown that tropical species with fast-expanding leaves have higher mean rates of damage by herbivores than do species with slowly expanding leaves (e.g. Aide & Lodoño 1989, Coley 1983, Coley & Kursar 1996), and extracts from fast-expanding leaves tend to support more fungal growth in lab assays than do extracts from species with slowly expanding leaves (Coley & Barone 1996). For this reason, we expected that leaves of a fast-expanding species such as *O. lucens* might harbour more endophytes than would leaves of *H. concinna*. However, rates of species accumulation for these two species are similar. We expect that factors such as leaf chemistry instead might underlie patterns of host preference.

Unfortunately, spatial variability has not been thoroughly explored for tropical microfungi and may be difficult to discern should stratum, substrate, or host preference confound spatial patterns. In the present study, we excluded the confounding variable of host preference by assessing similarity of endophytes associated with a single host species in two sampling sites. We found strong evidence of spatial heterogeneity among endophytes of *H. concinna* despite apparent similarity of the two sites. Such spatial segregation may be due, in part, to limited dispersal capabilities. Nonrandom distributions of tropical microfungi have been noted by Cornejo *et al*. (1994) and Polishook *et al*. (1996) and may be prevalent among endophytic fungi in tropical forests.

Because extrapolative estimates of biodiversity such as

those used by Hawksworth (1991) are based in part on estimates of host specificity, further exploration of tropical endophyte communities is warranted to determine proportions of endophytes truly specialised to given hosts. We suggest that in estimates of species diversity, spatial heterogeneity also should be considered, as nonrandom distributions of fungi may confound the accuracy of such estimates.

Behaviour of similarity indices

Both Morisita-Horn and Sørensen's indices suggest that endophytes are nonrandomly distributed with respect to host and site in the present study. Considering only non-singleton morphospecies likely inflates similarities by excluding rare and unique species; this effect should be especially pronounced for Sørensen's index, in which similarity is based on presence-absence data alone. We found that for non-singletons, both the Morisita-Horn index (55.5%) and Sørensen's index (60.7%) suggested moderate similarities among endophyte assemblages in two host species at a single site. We compared these values with similarity indices calculated for all morphospecies (including singletons), and found that similarity was much lower when calculated with Sørensen's index (23.5%) than with the Morisita-Horn index, which was largely unchanged (58.1%). Sørensen's index appears much more sensitive to the inclusion of rare species, and although it circumvents difficulties in determining what constitutes an endophytic individual, it may be difficult to interpret in communities in which singletons are prevalent.

CONCLUSIONS

Fungal endophytes in tropical trees represent an important and quantifiable component of fungal biodiversity. Highly abundant in a broad assemblage of host species, fungal endophytes appear to be highly diverse in a wide array of tropical angiosperms (e.g. Lodge *et al*. 1996, Arnold *et al*. 2000, Fröhlich *et al*. 2000, Gamboa & Bayman 2001). Moreover, recent studies based in Australia (Fröhlich & Hyde 2000), India (Rajagopal & Suryanarayanan 2000), China (Brown *et al*. 1998), Borneo (Fröhlich *et al*. 2000), Brazil (Rodrigues 1994), Puerto Rico (Bayman *et al*. 1998), Costa Rica (Danielsen & Jensen 1999), and Panamá (Arnold *et al*. 2000) suggest that endophyte research has become a global enterprise. However, disparities in methodology, as well as phylogenetic differences among host taxa, obscure comparisons across studies such that large-scale patterns of tropical fungal diversity are difficult to discern. We suggest that research based on consistent methods, and drawing first from pantropical species, would assist in determining the degree of gamma diversity present among endophytes; at the same time, multi-species studies assessing local host preference and spatial heterogeneity would elucidate ecological patterns underlying traditional surveys of endophytes associated with single host species. Although much further research is needed to determine the true number of fungal species, we conclude that tropical endophytes are an important component of diversity estimates and contribute substantially to fungal biodiversity.

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