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Communities of Endophytic Fungi in Leaves of a Tropical Timber Tree (*Guarea guidonia*: Meliaceae)[1]

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ABSTRACT

Endophytes are microorganisms that live inside plant tissues without causing symptoms of disease. They are a largely unexplored component of biodiversity, especially in the tropics. In this study, leaves from two populations of *Guarea guidonia* trees (Meliaceae) in the Luquillo region, Puerto Rico, were screened for diversity and composition of endophytic fungal communities. A total of 268 leaf fragments from 14 trees were surveyed. Thirty-eight morphospecies of endophytes were found. *Phomopsis, Colletotrichum, Xylaria,* and *Rhizoctonia*-like fungi were the most abundant taxa. Over 95 percent of the leaf pieces had endophytes. Communities had a few abundant species and many species with few individuals. The fungal community from the *Guarea* population in a forest preserve was more diverse than that from a disturbed area. Fungal communities were stratified according to height within a tree, but no differences were found between blade, petiolule, and rachis. The data suggest that the smaller and the more scattered the plant fragments sampled, the higher the probability of approaching real diversity values of endophytic fungal communities.

RESUMEN

Los endófitos son microorganismos que viven dentro de las tejidos vegetales sin manifestarse patológicamente. Ellos constituyen un componente poco explorado de la biodiversidad, especialmente en las zonas tropicales. En este trabajo se estudió la diversidad y composición de las comunidades de hongos endófitos en hojas de dos poblaciones del árbol *Guarea guidonia* (Meliaceae), en la región de Luquillo, Puerto Rico. Un total de 268 fragmentos provenientes de 14 árboles fueron muestreados, encontrándose 38 morfoespecies de hongos endófitos. Los taxones más abundantes fueron *Phomopsis, Colletotrichum, Xylaria,* y *Rhizoctonia.* Más del 95 por ciento de los fragmentos presentó endófitos; las comunidades tenían pocas especies abundantes y muchas especies raras. De las dos poblaciones de *G. guidonia* estudiadas, la situada en una zona más conservada fue más diversa en cuanto a su comunidad de endófitos. Se observó estratificación de acuerdo con la altura del árbol, pero no se observaron diferencias entre la lámina, el peciólulo, y el raquis de las hojas. Los datos sugieren que entre más pequeños y esparcidos sean los fragmentos muestreados, mayor será la probabilidad de aproximarse a los valores reales de diversidad de las comunidades de hongos endófitos.

Key words: endophytic fungi; fungal biodiversity; Guarea; *Puerto Rico.*

De Bary (1866) coined the term endophyte to describe microorganisms that colonize internal plant tissues (cited in Petrini 1991). Carroll (1986) restricted the term endophyte to endosymbionts that do not produce visible disease symptoms on their hosts. Nevertheless, plant–endosymbiont relationships form a complex continuum in which mutualism, saprotrophism, and pathogenicity intergrade (Katz & Lieth 1980, Petrini *et al.* 1982, Clay 1988, Carroll 1991, Fisher & Petrini 1992). Petrini (1991) proposed expanding Carroll's (1986) definition to cover saprotrophs and latent pathogens living within plants while in their asymptomatic state, and this is the definition of endophytes used here.

Most reported endophytes are fungi, but there are endophytic bacteria (Chanway 1996); some protozoans and nematodes probably also live as endophytes. Endophytic fungi have been isolated from all plants that have been examined, and every individual plant is probably host to at least one endophyte (Rodrigues 1996, Strobel 1996). The variety of ecological roles of endophytic fungi, their largely unexplored contribution to biodiversity (Hawksworth 2000), and their potential as a source of bioactive compounds (Petrini *et al.* 1992, Yang *et al.* 1994, Strobel & Long 1998) constitute a strong stimulus to study this group.

Most knowledge about endophytic fungi comes from temperate regions (Rodrigues 1996, Lodge 1997). Species studied include grasses (Clay 1988), Douglas fir (McCutcheon *et al.* 1993), oak (Fisher

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et al. 1994), chestnut (Bissegger & Sieber 1994), pine (Hata & Futai 1996), and ericaceous plants (Okane *et al.* 1998). Recently, however, attention has been directed to tropical and subtropical plants, including members of the Arecaceae (Rodrigues & Samuels 1990, Rodrigues 1994, Southcott & Johnson 1997), Sapotaceae (Lodge *et al.* 1996, Bayman *et al.* 1998), Orchidaceae and other epiphytes (Richardson & Currah 1995, Bayman *et al.* 1997, Tremblay *et al.* 1998), Fabaceae (Pereira *et al.* 1993), Anacardiaceae (Rodrigues & Samuels 1999), Araliaceae, and Meliaceae (Laessøe & Lodge 1994, Gamboa 1998). These and other studies have established that the composition of the endophytic communities varies among plants and plant organs.

The objective of this study was to compare the structure and composition of the endophytic fungal communities in a tropical tree (*Guarea guidonia* [L]. Sleumer, Meliaceae) from two sites differing in conservation status. Specific questions we asked were: (1) How does sampling strategy affect estimates of endophytic fungal diversity? (2) Is the diversity of fungal endophye communities higher in trees in a protected area than in trees in a disturbed, agricultural setting? (3) Are there differences in diversity and/or taxonomic composition of endophytic fungal communities among levels of the crown within individual trees? (4) Are endophyte communities more similar among different plant species in the same forest than among individuals of the same species from different sites?

MATERIALS AND METHODS

PLANT SPECIES.—*Guarea guidonia* is a tropical timber tree with a range extending from Nicaragua to Argentina, including the Greater Antilles. Its natural habitat is tropical and subtropical wet forest, and although it is not a dominant species, it can reach canopy level and attain up to 30 m in height and 1 m in DBH (diameter at breast height). Seed germination and seedling establishment is higher in shaded, moist places. It is used for furniture, and may have medicinal properties (Liogier 1988, Weaver 1988 and references therein).

STUDY SITE.—Two *G. guidonia* populations were selected from opposite sides of the Luquillo Mountains, Puerto Rico. One population was located at El Verde Biological Station (EV; 18°19′N, 65°49′W; 350 m elev.). El Verde is a former coffee plantation abandoned since the 1920s. Presently, the site is under government protection and native vegetation is abundant. The annual average rainfall is 3450 mm and the average monthly temperature is 21–25°C (Brown *et al.* 1983). This site has been used for studies of endophytes in *Manilkara bidentata* (Lodge *et al.* 1996) and diversity of fungi on leaf litter of *G. guidonia* (Polishook *et al.* 1996). The second population was located at Las Piedras (LP), 20 km southwest of EV (18°13′N, 65°52′W; 130 m elev.). The plant community is highly disturbed, with many *G. guidonia* individuals initially established as living fence posts. In 1997, the only year with available data, the annual rainfall was 1437 mm and the average temperature was 25.7°C.

DESIGN AND SAMPLING.—Following recommendations of Lodge *et al.* (1996), more trees than they used were sampled here. Seven trees spaced at least 20 m apart were chosen from each population of *G. guidonia*. Fully expanded leaflets were sampled between January and October 1997. The sampling unit (SU) was defined as the healthiest, most distal leaflet, its petiolule, and its corresponding rachis segment (Fig. 1). A total of 268 leaf segments were used from each study site.

Two sampling strategies were compared to determine the distribution of endophytes: microdistribution (m) and general (g) distribution. For the microdistribution study, two trees from each study site were selected and one SU from canopy, mid, and basal branches was collected for each tree. General distribution sampling was based on two SUs of basal branches on five different trees from each study site.

Sampling units were surface-sterilized by immersion in tap water (30 sec), 0.05 percent Tween-20 (30 sec with agitation), 75 percent ethanol (1 min), 2.6 percent sodium hypoclorite (50% Clorox®, 3 min), and 75 percent ethanol (30 sec) (Lodge *et al.* 1996). For the microdistribution study, one 10 × 6 mm segment from the leaflet blade was cut and further subdivided into 15 2 × 2 mm segments (Fig. 1), which were individually placed in petri plates containing half-strength potato dextrose agar (PDA) with 50 ppm penicillin, streptomycin, and tetracycline added after autoclaving. The petiolule and one rachis piece from each SU were halved longitudinally, cut into four segments 2 mm long, and plated on PDA (Fig. 1). For general distribution, five 2 × 10 mm segments were cut parallel to the midrib (Fig. 1) and plated on PDA. Petiolule and rachis pieces were treated as in microdistribution sampling (Fig. 1). Petri plates were incubated in a culture chamber with a cycle of 12 hours light/dark at 20 to 22°C. Isolated fungi were subcultured and identified to the genus level. Fungi that could not be identified on PDA

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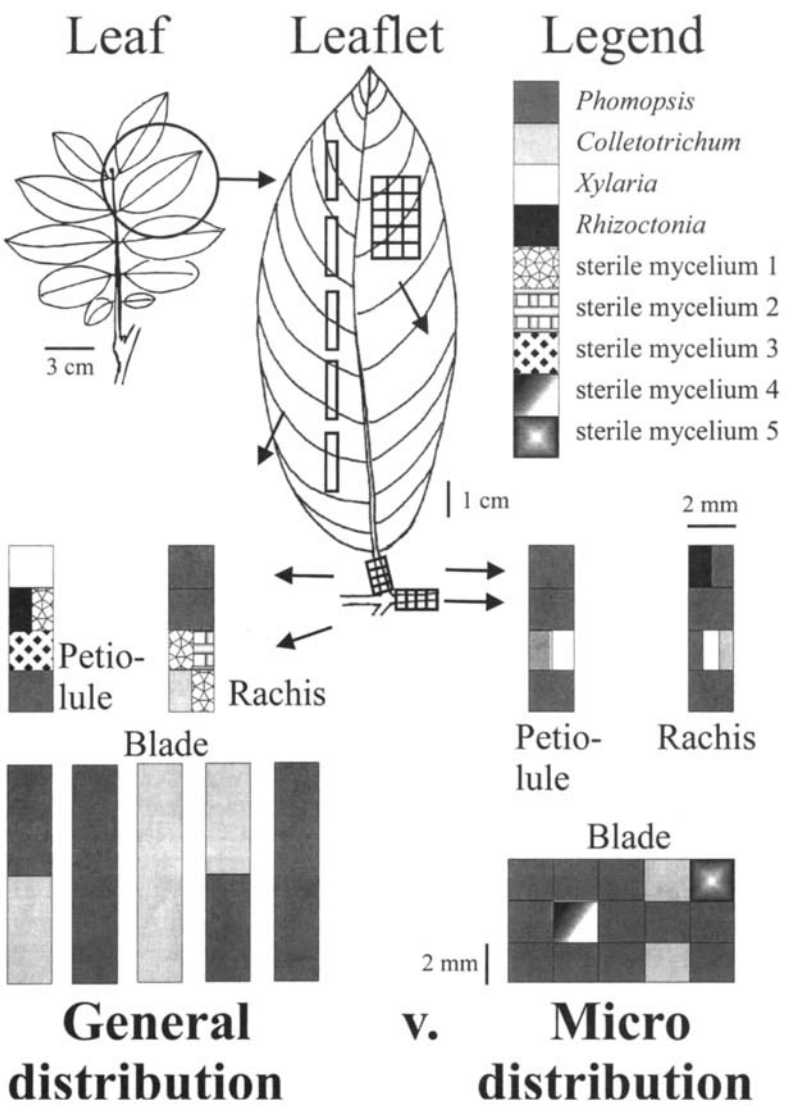

FIGURE 1. Schematic representation of sampling method and distribution maps. Top left, general aspect of *Guarea guidonia* leaf; top center, leaflet showing areas sampled; bottom left, general distribution map from a typical leaf; bottom right, microdistribution map from a typical leaf.

were transferred to malt extract agar (Difco), 5 percent V8 vegetable juice agar (Cotty 1989), and 2 percent water agar to promote sporulation.

DATA ANALYSIS.—Diversity (*i.e.*, heterogeneity) was measured for each community by the Shannon-Wiener function (H′; Krebs 1989). Individual components of diversity (richness and evenness) were estimated by jackknife and evenness indices (Ŝ and J′, respectively). Similarity between and within endophytic communities was measured with the Morisita index (Krebs 1989). To estimate the extent to which fungal diversity was revealed by the sampling, both cumulative species and relative abundance species curves were constructed. Additionally, the relative abundance curves were compared to a theoretical model of species abundance, lognormal distribution, using the Kolmogorov-Smirnov test for goodness of fit.

RESULTS

OVERALL DIVERSITY OF ENDOPHYTES.—Leaves of *G. guidonia* were rich in diversity of endophytic fungi.

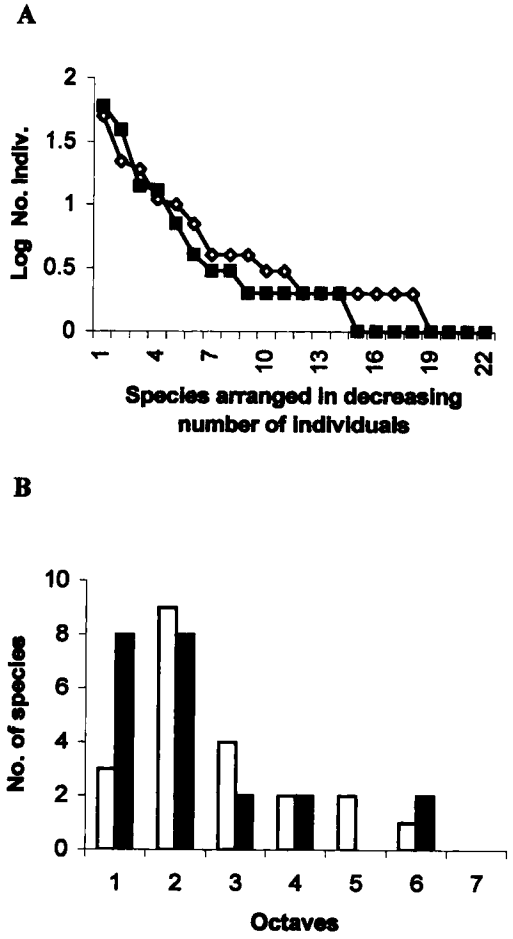

FIGURE 2. Endophytic fungi in *Guarea guidonia* leaves from EV. (A) Log transformed abundance species curve: squares correspond to EVg and diamonds to EVm. (B) Preston's octave scale: EVg (black) and EVm (white).

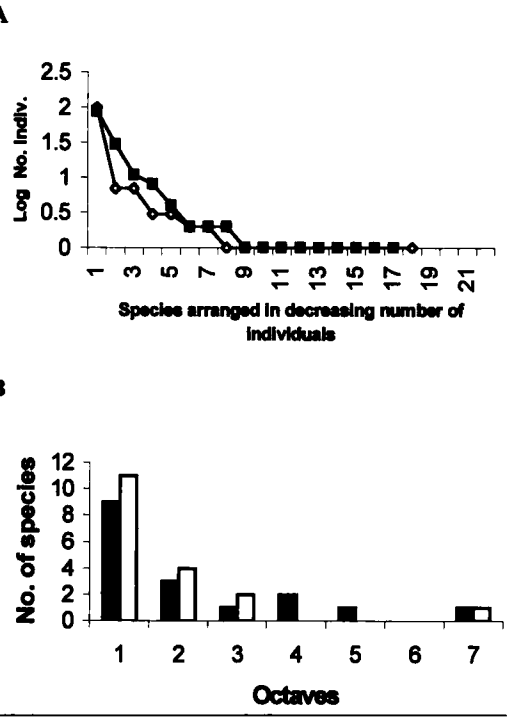

FIGURE 3. Endophytic fungi in *Guarea guidonia* leaves from LP. (A) Log transformed abundance species curve: squares correspond to LPg and diamonds to LPm. (B) Preston's octave scale: LPg (black) and LPm (white).

Up to nine fungal taxa were isolated from a 60 mm^2 leaf section subdivided into 15 2 × 2 mm fragments ($\bar{x}$ = 5.5). At least one fungus was isolated from 98.5 and 95.5 percent of leaf fragments from EV and LP, respectively. Additionally, 19.4 and 14.1 percent of leaf fragments from EV and LP yielded more than one fungus. Species were densely aggregated at a very fine scale (Fig. 1). All leaf parts examined (blade, petiolule, and rachis) had a diverse endophyte flora. No taxon was found exclusively in any one of the three leaf parts, except for species found only once, in which case no correlation could be postulated.

Thirty-eight morphospecies were found, including four distinct taxa of *Colletotrichum,* three of *Phomopsis,* two of *Xylaria,* two of *Rhizoctonia,* and one each of *Trichoderma, Curvularia,* and *Pestalotia.* Twenty-four morphospecies could not be identified due to lack of spore production or distinctive structures, but these only constituted 22 percent of the isolates found.

Endophytic communities in *G. guidonia* fit a lognormal distribution model, with a few species abundant and most species relatively rare (Figs. 2 and 3; Table 1; Krebs 1989). The lognormal model for estimating richness (number of species) did not differ from the observed data (Kolmogorov-Smirnov: $P > 0.05$; Krebs 1989). Species richness was also estimated with the jackknife index, which is based on the number of unique species (*i.e.,* species found in only one SU; Krebs 1989, Lodge *et al.* 1996). Except in one case, the jackknife index gave a higher number of species than the lognormal distribution (Table 2). The cumulative species abundance curves did not reach saturation, as indicated by the absence of an asymptote (Fig. 4).

SCALES OF SAMPLING: MICRO VERSUS GENERAL.—In general, more species were isolated from a 10 × 2 mm leaf piece with microdistribution sampling

TABLE 1. *Species and number of isolates of endophytic fungi within leaves of* Guarea guidonia *per strata and sampling site. EV = El Verde; LP = Las Piedras.*

Species	Microdistribution sampling						General sampling	
	Low branch EV	Mid branch EV	Canopy branch EV	Low branch LP	Mid branch LP	Canopy branch LP	EV	LP
Colletotrichum sp. 1	1	7	2	1	2		2	11
Colletotrichum sp. 2	2	2	3				4	
Colletotrichum sp. 3			2	1		1		
Colletotrichum sp. 4	10	5	4	6	1		14	30
Phomopsis sp. 1	25	16	9	29	40	29	60	88
Phomopsis sp. 2		4		1				
Phomopsis sp. 3		2					2	
Xylaria sp.						1	13	1
Xylaria arbuscula							1	
Rhizoctonia sp. 1					1		1	1
Rhizoctonia sp. 2	2	2	18	4	1	2	3	4
Pestalotia sp.							1	1
Trichoderma sp.	1	2					1	1
Curvularia sp.								1
Unknown sterile mycelia	12	10	14	3	6	4	61	18
Total no. of spp.	10	12	13	9	9	8	22	17
Total no. of isolates	53	50	52	45	51	37	163	156

than in an equal area with general sampling. In the microdistribution study, each 10 × 2 mm piece was cut into five 4 mm^2 pieces, giving an edge/area ratio almost two times higher than in the general distribution study. Microdistribution gave higher estimates of diversity (H') in EV than did general distribution sampling (Table 2); however, in LP, the two sampling strategies gave very similar results.

Micro- and general distribution studies revealed different species composition for the same location. In EV, there were five species found with microdistribution that were not isolated with general distribution, and another five species that were exclusive to the general distribution study. For LP, the numbers of species unique to each sampling method were eight and eight, respectively. At both sites, a *Colletotrichum* and a *Phomopsis* were found only in micro-sampling, and a *Pestalotia* was found only in general sampling. Thus, either sampling strategy alone would have missed some of the fungi that were found.

COMPARISON BETWEEN SITES.—More species were found in EV than in LP (Table 2). According to values for species richness, heterogeneity, and evenness, EV had a more diverse community of endophytes than LP (Table 2). The Morisita index was used to estimate similarity between sites and between micro- and general distribution (Table 3). Communities from different sites differed more than communities obtained with different sam-

TABLE 2. *Measures of diversity for endophytic foliar communities from two populations of* Guarea guidonia. *Two sampling methodologies (micro and general) were employed. EV = El Verde; LP = Las Piedras.*

	EV micro-sampling	EV general sampling	LP micro-sampling	LP general sampling
Observed species	21	22	18	17
Lognormal estimate	23	36	21	21
Jackknife estimate (Š)	28	33	28	26
Diversity (H')	3.37	3.18	1.78	2.21
Evenness (J')	0.76	0.71	0.42	0.54

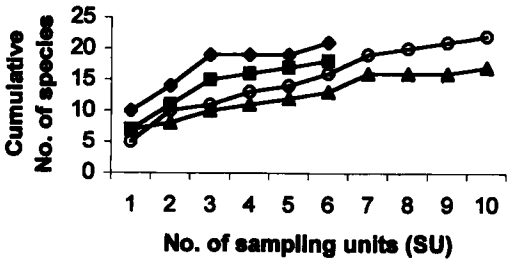

FIGURE 4. Cumulative species curve for endophytic communities in *Guarea guidonia* leaves. Diamonds correspond to EVm, circles to EVg, squares to LPm, and triangles to LPg.

TABLE 3. *Morisita similarity index among endophytic fungal communities in* Guarea guidonia. *EV = El Verde; LP = Las Piedras.*

	EV micro	EV general	LP micro	LP general
EV micro	—	0.87	0.73	
EV gen.		—		0.85
LP micro			—	0.95
LP gen.				—

pling strategies. Microdistribution revealed more differences between sites than did general distribution.

VERTICAL DISTRIBUTION.—Microdistribution samples were taken at three levels in each site: basal, mid, and canopy. Two SUs were taken at each level and heterogeneity (*i.e.*, diversity = H′), richness, evenness, and similarity were estimated (Table 4). Diversity was higher for EV than LP at each tree level. Diversity and number of species increased with tree height in EV, but tended to decrease with tree height in LP (Table 4). According to the jackknife estimate of richness, the LP community was more under-sampled than EV. Evenness in EV was almost twice that of LP for middle and canopy levels, and canopy level in LP was the poorest community (Table 4). Whereas Morisita similarity between lower versus canopy communities in EV was relatively low (0.54), the similarity between lower versus canopy for LP was high (0.98; Table 5). Overall similarity was higher among communities at canopy levels in LP than for communities from EV (Table 5).

COMPARISON BETWEEN PLANT SPECIES.—At the community level, fungi found in *G. guidonia* in EV were distinct from those found in *M. bidentata* at the same site, but highly similar to those found in *Guarea* from LP. Endophytic communities in *G. guidonia* were dominated by *Phomopsis* and *Colletotrichum* in EV as well as in LP, while the three most common fungi in *M. bidentata* were species of *Xylaria* (Lodge *et al.* 1996, Bayman *et al.* 1998). *Xylaria* species comprised more than half the isolates from *Manilkara* but only 2.7 percent of those from *Guarea*. Similarly, *Fusarium* was common in *Manilkara* but was not isolated from *Guarea*. In contrast, *Phomopsis* was the most common genus in *Guarea* (51% of isolates) but was only isolated six times from *Manilkara* (Table 1; Lodge *et al.* 1996).

TABLE 5. *Morisita index of similarity among endophytic fungal communities in* Guarea guidonia. *EV = El Verde; LP = Las Piedras.*

	Low branch EV	Mid branch EV	Canopy branch EV	Low branch LP	Mid branch LP	Canopy branch LP
LBEV	—	0.89	0.54	0.97		
MBEV		—	0.61		0.7	
CBEV			—			0.41
LBLP				—	0.98	0.98
MBLP					—	1.01
CBLP						—

DISCUSSION

DIVERSITY AND FREQUENCY OF ENDOPHYTES.—Based on available data, a high rate of infection is typical of tropical trees (Rodrigues 1994, Lodge *et al.* 1996, Southcott & Johnson 1997, Bayman *et al.* 1998). The percentage of infected plant fragments reported here for *G. guidonia* (>95%) is similar to data from *M. bidentata* trees in the same stand as our EV population (Lodge *et al.* 1996), and is higher than infection percentage reported for tropical non-arboreal species (Southcott & Johnson 1997) or temperate trees (Carroll & Carroll 1978, Wilson & Carroll 1994).

Xylaria species were the dominant endophytes in *M. bidentata* (Lodge *et al.* 1996, Bayman *et al.*

TABLE 4. *Measures of diversity for communities of endophytic fungi in* Guarea guidonia *from different tree levels. EV = El Verde; LP = Las Piedras.*

	Low branch EV	Mid branch EV	Canopy branch EV	Low branch LP	Mid branch LP	Canopy branch LP
Diversity (H′)	2.4	3.09	3.08	1.83	1.39	1.34
Evenness (J′)	0.72	0.86	0.83	0.58	0.42	0.45
Jackknife estimate (Š)	11	13	16	11	13	10
Observed species	10	12	13	9	10	8

1998). Nevertheless, *Xylaria* was not the dominant taxon in *G. guidonia*. *Xylaria meliacearum* and *X. guareae,* two species described as decomposers of *G. guidonia* by Laessøe & Lodge (1994), were not found in our study.

Species abundance in fungal assemblages generally follows a lognormal pattern (Lussenhop 1981 cited in Dix & Webster 1995). Relative species abundance for *G. guidonia* (Figs. 2 and 3) fit a lognormal model of distribution. The community, however, was under-sampled, as shown by Preston's scales for each community (Figs. 2 and 3) and the cumulative species curve (Fig. 4). The modal octave was included within the transformed sample only for the EVg community (Fig. 2B); for other samples, there is no certainty that the leftmost octave is the modal one. Nevertheless, the large tail to the right indicates that these communities are characterized by many rare species.

It is clear that the number of SUs used was insufficient to saturate the species-effort curve (Fig. 4), but it appears that adding one or two more SUs would provide an adequate sample. The jackknife estimate gave higher values than the lognormal model (Table 2). From that value, it appears that 66–75 percent of the total species present in EV were found and 64–66 percent in LP were detected. Of course, this only includes species that grow in culture media under the conditions used.

Scales of sampling: micro versus general.—The distribution maps (Fig. 1) demonstrate that leaf endophytes in *G. guidonia* infect petiolule, rachis, and blade, and have a fine-scale pattern of species distribution. This fine-scale distribution of endophytes has already been shown to exist in tropical and temperate plants (Carroll 1995, Lodge *et al.* 1996), and clearly indicates that sampling strategy, especially size of plant fragments, is very important for estimating diversity of endophytic fungal communities. From plant fragments as small as 2 × 2 mm, we isolated up to three morphospecies (Fig. 1). Increasing the total edge of the fragment may have allowed a larger number of species to grow out of the leaf, with less interference from other endophytes.

Nevertheless, small SUs units are not the complete solution to sampling problems. Some fungi found repeatedly in the general distribution study were never found when smaller leaf pieces were used, and vice versa. There have been no prior reports of two sampling scales used simultaneously for studying endophytic communities. Changes in size of SUs are presented in only two studies, and although an increase in percentage of infection was observed when SU size was larger, the number of recorded species was less (Carroll 1995, Lodge *et al.* 1996).

The microdistribution study gave a higher estimate of diversity (H′) in EV, while the general distribution study showed higher diversity in LP (Table 2). These results can be explained as follows: in EV, there were more species and higher evenness than in LP, and a more complex mosaic of distribution of species and individuals is therefore expected. On the other hand, in LP, there were fewer species and individuals than in EV, which may have decreased aggregation and competition among fungi. It is not clear whether contiguous fragments containing the same fungus represent different individuals or multiple sampling of the same colony, or whether different fungi in the same fragment are in physical contact or are spatially separated. These questions may have important implications for interactions among endophytes and for production of fungal secondary metabolites (Bayman *et al.* 1998).

Comparison between sites.—The dominant endophytes were the same in both sites, and differences in diversity were due to the presence of rare species. El Verde is a much more protected and diverse plant community than LP, and heterogeneity and evenness were higher for EV than for LP (Table 2). This is not surprising. Rodrigues (1994) reported that surrounding vegetation is an important source of inoculum for endophytes, which may explain differences between EV and LP; however, annual rainfall differed between EV and LP, and effects of rainfall on endophytic community composition are not known. Since only two sites were compared, it is not clear if differences were attributable to conservation status, climate, or other factors.

The Morisita index showed that similarity within communities is higher than between communities, as is evident when comparing EVg versus EVm and LPg versus LPm (Table 5). It appears that the endophytic fungal communities in *G. guidonia* are relatively constant, independent of habitat. The Morisita index is appropiate for estimating similarity between endophytic fungal communities because it takes account of the relative abundance of species and not just presence/absence. This is useful for comparing communities with many rare species.

Vertical distribution.—Studies of endophyte distribution within tree crowns are rare, and conflicting results have been found. In Douglas fir, no cor-

relation was found between the degree of infection and the height of the crown, whereas in Balsam fir, a negative correlation was found between height and density of infections (Bernstein & Carroll 1977, Johnson & Whitney 1989). In *G. guidonia,* diversity (H′) increased from the lower canopy to middle canopy in EV, but not in LP (Table 4). Each strata in EV was more diverse than its counterpart in LP, with the canopy in LP being the poorest community. Since there was no forest surrounding the LP canopy, high radiation on leaves at this level may have induced water stress and contributed to lower endophyte loads.

Implications for biodiversity.—This study has direct implications for the study of endophytic fungi and their contributions to biodiversity. First, small fragments may yield more endophyte taxa than large fragments, at least in diverse communities. Second, using leaf fragments of different sizes (and varying isolation procedures in other ways) may favor different groups of fungi and thus increase diversity. Third, a cumulative species curve should be constructed to determine when enough SUs have been included. Fourth, sampling protocols should include several canopy levels, since leaves from the canopy may have a different endophyte composition than leaves from basal branches. Fifth, there may be a correlation between plant diversity in an area and endophyte diversity, which merits further study.

Identification of endophytes to species level is critical for studies of host specificity and biodiversity; however, many of these fungi do not sporulate in culture, and are thus very difficult to identify. Many belong to form genera such as *Phomopsis* for which taxonomy is so confused that specific epithets are arbitrary. DNA sequence analysis of such fungi may provide means to classify them and to compare results between labs in different areas; our lab and several others are doing this. Given the richness of endophytic fungal communities in *G. guidonia* and other tropical plants, and differences in endophyte communities between trees growing in the same site (*e.g., G. guidonia* and *M. bidentata* in EV), it is possible that endophyte biodiversity is much greater than currently believed (Hawksworth 2000).

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