Exploring Rubiaceae fungal endophytes across contrasting tropical forests, tree tissues, and developmental stages

Humberto Castillo-González[1, ‡], Jason C. Slot[2], Stephanie Yarwood[3], Priscila Chaverri[4,5]

[1]Department of Plant Science and Landscape Architecture, University of Maryland, College Park, Maryland, 20742, USA.

[2]Department of Plant Pathology, The Ohio State University, Columbus, OH, 43210.

[3]Department of Environmental Science and Technology, University of Maryland, College Park, Maryland, 20742, USA.

[4]Department of Natural Sciences, Bowie State University, Bowie, Maryland, 20715.

[5]Centro de Investigaciones en Productos Naturales (CIPRONA) and Escuela de Biología, Universidad de Costa Rica, 11501-2060, San José, Costa Rica.

Current address: [‡] Chrono-Environnement, Université de Franche-Comté, 25000, Montbéliard, France.

Corresponding author: Priscila Chaverri, phone: +1 (301) 860-3338, email: pchaverri@bowiestate.edu

ORCIDs: H. Castillo-González: https://orcid.org/0000-0003-3460-8404; J. Slot: https://orcid.org/0000-0001-6731-3405; S. Yarwood: https://orcid.org/0000-0003-4593-7908; P. Chaverri: https://orcid.org/0000-0002-8486-6033

Abstract

Fungal endophytes play a pivotal role in tropical forest dynamics, influencing plant fitness through growth stimulation, disease suppression, stress tolerance, and nutrient mobilization. This study investigates the effects of region, leaf developmental stage, and tissue type on endophyte communities in tropical plants. Young and mature leaves were collected from 47 Rubiaceae species, and sapwood from 23 species, in old-growth forests of Golfito and Guanacaste, Costa Rica. Fungal diversity and composition were assessed through metabarcoding of the ITS2 nrDNA region. Most identified ASVs belonged to the phylum Ascomycota. The orders Botryosphaeriales and Glomerellales significantly contributed to endophytic assemblages, without detection of host-specific communities. We observed significant differences in species richness across regions, confirming distinct compositions through beta diversity. No statistically significant variances were found between mature and juvenile leaf tissues. In contrast, leaves exhibited richer and more diverse assemblages than sapwood. As plants experienced diverse environments over time and space, our results may be influenced by changing structural and chemical properties through ontogeny. Given the potential impact of these fungi on agricultural and forest ecosystems, ongoing research is crucial to discern the roles of hosts, endophytes, and other ecological mechanisms in apparent colonization patterns.

Keywords: coffee family, Costa Rica, ecology, metabarcoding, phytobiome, phyllosphere.

INTRODUCTION

Neotropical forests, which support a high diversity of terrestrial plants (1), host an extraordinary mosaic of microbial assemblages within plant tissues, driven by their provision of labile nutrients

48 and niche space (2-5). These microbes play crucial roles in influencing host plants and driving
49 forest dynamics, engaging in interactions that range from mutualism and commensalism to
50 pathogenicity (6,7). Among these microbial groups, endophytes—microorganisms that reside in
51 internal plant tissues without causing disease—are particularly noteworthy. Fungal endophytes,
52 more than their bacterial counterparts, offer numerous benefits to their plant hosts, such as
53 enhanced immunity, improved growth, resistance to abiotic stressors, and the production of
54 bioactive compounds (8,9). In addition, despite the significant diversity of fungal endophytes
55 colonizing leaves and stems (4), they are traditionally understudied compared to root endophytes,
56 mycorrhizae, and free-living forms. Consequently, much remains underexplored regarding their
57 roles in natural ecosystems and their potential applications in improving crop health.

58 The mechanisms by which endophytic fungi colonize plant tissues remain a topic of
59 research interest (10–12). Understanding the dynamics that determine their distributions and the
60 environmental constraints shaping their assemblages is crucial. Evidence shows that broad-scale
61 environmental factors such as temperature, elevation, precipitation (13,14), and forest type (15)
62 significantly influence community composition. Additionally, genetic and geographic distance
63 among host trees (16–18), as well as host growth stage and tissue type (19–21), also play crucial
64 roles. Given these factors, endophytes that exhibit recurrence or specificity may play a significant
65 ecological role in forests (22,23). Moreover, under the premise that high diversity maintains the
66 overall integrity of an ecosystem (i.e., biological insurance hypothesis *sensu* Naeem & Li (1997)),
67 the hidden diversity of endophytes can enhance the fitness of individual trees and, by extension,
68 shape forest composition and demography (8). Efforts to understand endophytic diversity are
69 necessary for predicting their function in association with different tissues, hosts, specific

environmental conditions, or entire ecosystems. In this sense, tropical forests offer an ideal setting for studying these patterns and interactions.

Rubiaceae is one of the largest and most complex families of angiosperms, comprising over 13,000 species that range from weedy herbs to large trees and occupy many habitats and biogeographical regions across all continents (25). However, they are predominantly found in the neotropics (26,27). In Costa Rica, historical data (28), listed 89 genera and 458 species present, but recent updates suggest that this count is underestimated. For example, the genus *Palicourea* alone, previously reported with 44 species, is now known to include 91 (29,30). This increased knowledge highlights the family's extensive diversity. Noteworthy within Rubiaceae are several genera of cultural and economic importance such as *Cinchona*, *Coffea*, *Genipa,* and *Psychotria*.

Characterizing the fungal endophyte communities within Rubiaceae and the factors shaping their assemblages could provide unique insights into understanding their niche preferences in tropical forests. In this study, we investigated diversity patterns across a broad range of plant species, expecting to observe a vast taxonomic and functional diversity (7). We hypothesized that fungal endophytic communities would differ: (i) between forest regions due to varying environmental conditions; (ii) between individuals but showing similarities within closely related plants; and (iii) within a single tree, with differences between young and mature leaves, as well as between leaves and sapwood, exhibiting tissue-specific distributions. To further refine our hypotheses, we aimed to understand which fungal groups drove these differences by investigating whether certain endophytes exhibit cosmopolitan behavior or have significant associations with specific environmental conditions or host characteristics.

METHODS

Study site

Old-growth forests from two regions in Costa Rica were sampled. Specific sampling locations classified by climatic subregions are shown in Figure 1. Golfito, in the South Pacific, was visited in April 2017. All of the sampling sites in this area are characterized by typical tropical rainforest conditions, with no defined dry season, intense rainfall, and dense evergreen vegetation spanning two to three layers (31). In May 2017, sampling was conducted in Guanacaste, specifically in two distinct regions (North and North Pacific), comprising three subclimates: seasonal dry forest, semi-deciduous forest influenced by monsoons, and the forested slopes of a volcano, influenced by surrounding mountain systems (31). Climate data, including mean annual temperature, mean annual precipitation and elevation were obtained from the WorldClim database (Table 1) (Fick & Hijmans, 2017).

Sampling

Young and mature healthy leaves from 50 Rubiaceae individuals (distributed in 47 species from 27 genera, 10 tribes and 3 subfamilies) were collected (Supplementary Table S1). The taxonomy was assessed by a local botanist, and vouchers of all individuals were deposited in the University of Costa Rica Herbarium (USJ). We sampled plants as we encountered them, aiming to include as many different species as possible. For each individual sampled, fully expanded leaves were collected from three randomly selected branches, chosen from various parts of the crown. To differentiate developmental stages, we selected one newly emerged and one mature leaf per branch, totaling six leaves per plant.

114 For 23 of the individuals, which were woody trees with a minimum trunk diameter-at-
115 breast-height of approximately 10 cm, a sterilized knife was used to cut a sliver of outer bark at
116 shoulder height to expose the alburnum and one piece of living sapwood tissue ca. 30 × 5 mm was
117 excised from the exposed area. All collected samples were put in a clean plastic bag and placed on
118 ice for transport to the field workstation for immediate processing. A leaf section of ca. 20 × 4 mm
119 was cut and surface-sterilized using sequential immersions in a 2% sodium hypochlorite solution
120 (1 min), 70% ethanol (30 sec), and sterile distilled water (30 sec). Finally, leaves were dried with
121 autoclaved tissue paper, and stored in microtubes containing silica gel before being transferred on
122 ice to the laboratory for DNA extraction (32). Sapwood samples were briefly singed with a flame
123 to eliminate dust and potential surface contaminants introduced during handling, and then placed
124 in microtubes.
125
126 **Sequencing**
127 Samples were transferred to a pre-filled 500-micron Garnet and a 6-mm Zirconium Grinding
128 Satellite bead tube (OPS Diagnostics LLC, New Jersey, USA) and ground using a FastPrep® bead
129 mill (MP Biomedicals, Santa Ana, California, USA). Each tube was treated to a grinding cycle of
130 speed: 6.0 m/s for 45 seconds, or until no visually recognizable fragments remained. Each sample
131 (tissue section) represented a biological replicate, and no technical replicates were performed.
132 Total DNA was extracted using the commercial kit Qiagen DNeasy Plant Mini Kit® according to
133 the manufacturer's instructions (Qiagen, Hilden, Germany). Genomic DNA was sent to Naturalis
134 Biodiversity Centre (NBC, Leiden, The Netherlands) for targeted amplicon metagenomics
135 (metabarcoding) of the ITS2 nrDNA region using Ion PGM Torrent™ technology (Thermo Fisher
136 Scientific, Waltham, MA, USA). The primers used were fITS7 and ITS4, which are fungal specific

137 and with less amplification of plant ITS (33). To ensure accuracy in downstream analyses, a
138 positive control with a non-Costa Rican *Russula galochroa* DNA and a negative control (blank)
139 were included throughout. According to NBC, PCR was performed in a BioRad C1000
140 thermocycler (Hercules, CA, USA), checked the quality of the PCR products with a QIAxcel
141 (Qiagen), cleaned PCR products with NucleoMag NGS Clean-up and Size Select magnetic beads
142 (Takara Bio Inc., Japan), ran the pool in an Agilent Bioanalyzer (Santa Clara, CA, USA) using a
143 DNA High Sensitivity chip and amplified on the Ion One Touch system (Ion PGM™ Hi-Q™ View
144 OT2 kit), results measured on the Qubit (ThermoFisher) for the Ion Sphere™ Quality Control kit
145 (ThermoFisher), and finally the sequence run was done on the Ion PGM Torrent using the Ion
146 PGM™ Hi-Q™ View Sequencing kit. The resulting raw data (fastq files) are deposited in
147 GenBank under the BioProject identifier PRJNA889378.
148
149 **Fungal community analyses**
150 ***ASV classification and taxa assignment.*** Primers and barcodes were trimmed from each read
151 using the command line tool 'cutadapt' (34). Subsequent processing was performed in RStudio
152 v. 2022.12.0+353 (35) with R v. 4.0.3, employing the DADA2 v1.18.0 pipeline (36). Raw
153 reads were subjected to data curation aimed at identifying and removing spurious contigs
154 through various filtering processes. For instance, chimeric sequences were removed. Reads with
155 total counts below 0.001% of the total reads and singletons were excluded. Quality filtering
156 included a maximum expected error rate of 2 for both forward and reverse reads, and sequences
157 were trimmed to a minimum length of 100 bp. (36)Amplicon Sequence Variants (ASVs) were
158 assigned taxonomically by comparing them to the UNITE database (36) v8.3 (37) training set
159 from the DECIPHER package, v 2.18.1 (38). Only sequences classified as fungi were retained.

160 Additional independent filtering steps included the exclusion of samples with total read counts
161 below 0.001% of the total reads, and taxa with low variance across samples (variance threshold
162 of 1e-5). A prevalence threshold of 1% of total samples was applied, retaining taxa appearing in
163 at least this proportion of samples. The accuracy of the pipeline was validated using the positive
164 controls, and sequences from negative controls were removed to ensure dataset integrity.
165
166 ***Diversity and community structure analyses.*** Statistical analyses and plotting were conducted
167 using the phyloseq v1.38.0 (39), vegan v2.5.7 (40), microbiome v1.12.0 (41) and ggplot2
168 v3.4.1 (42) packages. Endophyte alpha diversity for each dataset was estimated using the
169 Shannon, Simpson, and Chao1 indices for each variable and then transformed to Hill Effective
170 Species Numbers (43,44) for comparisons by Mann-Whitney U test. This transformation
171 integrates species richness and abundance distribution, adjusts sensitivity to rare versus common
172 species, and provides a consistent measure of diversity (43,44). It also allows for more robust
173 comparisons across sites with varying numbers of species and levels of evenness. To visualize
174 community patterns (beta diversity), non-metric multidimensional scaling (NMDS) was
175 performed using the 'metaMDS' function from the vegan package with the 'bray' method and
176 100 random starts. For statistical analyses, data were normalized by total count per sample,
177 converting them into proportions (relative abundances), and Bray-Curtis distances were
178 calculated with the 'vegdist' function. Homogeneity of dispersion was assessed with the
179 'betadisper' function, followed by a permutation test using 'permutest'. Statistical differences in

180 endophyte assemblages across variables (forest regions, tissue types, developmental stages)
181 were evaluated with the 'adonis2' function for PERMANOVA.

182 Relative abundance at the family level was calculated. We retained groups contributing to
183 more than 1% of the average relative abundance across the six categories: forest regions (Golfito
184 and Guanacaste), tissue types (leaf and sapwood) and developmental stages (young and mature).
185 These groups are referred to as the 'top families' in downstream analyses. The overall prevalence
186 (i.e., the number of samples in which the taxa appear) of these families was computed. To estimate
187 how strongly they deviate from a random preference among the plant partners available at the
188 study sites, we calculated the d' index of specialization using the bipartite v 2.18 package (45),
189 function 'dfun'. ASVs were statistically classified as generalists or specialists based on their
190 habitat affinity using a multinomial species classification method (CLAMtest) in vegan. Indicator
191 taxa (ASVs with p-values < 0.05) from the six categories were identified using the 'multipatt'
192 function in the indicspecies v1.7.12 package (46). Methods regarding Species Accumulation
193 Curves, Mantel Correlation Tests, Distance Decay, Canonical Correlation Analysis and the
194 assignment of functional traits are described in Supplementary Methods S2.

195

196 **RESULTS**

197 A total of 6,467,267 reads passed the quality control filters. The mean number of sequences per
198 sample was 58,465 for leaves and 26,989 for sapwood. We identified 3,218 putative taxa (ASVs)
199 belonging to at least 232 different genera (Supplementary Table S3). ASV identification rate was
200 67%, 50% and 33% at the order, family, and genus levels, respectively. The species richness or
201 number of unique ASVs recovered per sample (triplicates, in the case of leaf tissue) ranged from
202 2 to 337, with a mean of 67.

203 All datasets had a high number of single occurrences, and no asymptote was reached in the
204 species accumulation curves (Supplementary Figure S4) indicating that the datasets are
205 incomplete, and the number of samples was not sufficient in capturing the expected endophyte
206 diversity (Hill's $q = 0$). However, when focusing on dominant species only (Hill's $q = 2$), all curves,
207 except for sapwood, reached a plateau.

208 Fungal endophyte assemblages differed between forest regions and tissue types (Table 2).
209 Samples originating from Golfito exhibited consistently higher alpha diversity indices compared
210 to those from Guanacaste, as supported by a Hill numbers comparison ($q=0,1,2$) that demonstrated
211 statistical differences for $q=0$ ($p \leq 0.05$) and $q=1$ ($p \leq 0.01$). Notably, leaf and sapwood tissues
212 were found to harbor distinct fungal communities, with leaves exhibiting significantly richer
213 assemblages ($p \leq 0.001$ in all instances). Regarding the developmental stage, the taxa richness
214 index ($q=0$) was significantly higher in mature leaves compared to new leaves. In contrast, the
215 other two diversity indices ($q=1$, $q=2$) showed slightly greater diversity in new leaves, though these
216 differences were not statistically significant.

217 Using non-metric multidimensional scaling (NMDS) we observed differences between
218 location and dispersion of points, with Guanacaste samples appearing more scattered (Figure 2A);
219 three outliers were removed for visualization purposes. Community dissimilarities between these
220 variables were corroborated via PERMANOVA, which indicated significant separation in fungal
221 assemblages when comparing between the different forest regions ($p < 0.001$). In the case of tissue
222 type, points from leaf and bark communities were dispersed differently in the NMDS analysis
223 (Figure 2B) and presented significant heteroscedasticity ($p < 0.01$), which has been shown to affect
224 PERMANOVA testing ($p < 0.001$). No clear location effect was observed in the NMDS plot;
225 however, the dispersion of the points varied: leaf samples converged on the interior of the cluster

226 while sapwood samples, being more variable, were found mostly around it; two samples were
227 removed for better visualization (figure 2B). Considering just the leaves at the two different
228 development stages, no effect was detected, there was a high overlap between the fungal
229 communities (figure 2C), and the PERMANOVA indicated no significant differences ($p = 0.8122$).
230 Fungal endophyte communities did not cluster based on individual plants or at any higher
231 taxonomic level (i.e., genus, tribe, subfamily). The pattern for tribe is shown in Figure 2C, other
232 levels of taxonomy are depicted in Supplementary Figure S5.

233 The phylum Ascomycota was the most abundant, representing 83% of all fungal ASVs.
234 Within this group, the most abundant and prevalent classes were Sordariomycetes followed by
235 Dothideomycetes. Tremellomycetes and Agaricomycetes were the most abundant and prevalent
236 Basidiomycota, making up 8.2% of the ASVs. We characterized endophytic assemblages
237 dominated by a few abundant dominant taxa and many rare taxonomic groups. For example, we
238 identified 156 different families (Supplementary Table S6) from which Glomerellaceae,
239 Phyllostictaceae and Mycosphaerellaceae composed the bulk of biodiversity (45.14%) present
240 within the endophytic assemblages (Figure 3).

241 The most abundant fungal families were not the most prevalent in all cases (e.g.,
242 Xylariaceae), but they tended to correspond (Figure 3). The top 12 families comprised 59% of the
243 total relative abundance. With the d' index, we determined that the most prevalent and abundant
244 families (i.e., Glomerellaceae and Phyllostictaceae) had values closer to 0 (no specialization). This
245 indicates that members of these fungal families interact with a broad host range. In contrast,
246 Irpicaceae, had a low prevalence and presented a value closer to 1 (perfect specialist). Interesting
247 is the case of Apiosporaceae, that despite a low prevalence, showed little specialization. The major
248 fungal families were similar between Golfito and Guanacaste (Figure 3), but some of the groups

249 that presented very low abundance in Golfito, such as Schizoporaceae and Irpicaceae, were more
250 abundant in Guanacaste and such preference was highlighted by the CLAM test (Figure 3).
251 We did not find indicator species among the top 12 families in the sapwood communities.
252 In contrast, some indicator species were identified for leaves. For example, Phyllostictaceae,
253 despite being the second most abundant and prevalent family overall, was not found in sapwood.
254 Sporocadaceae and Nectriaceae, both of which had very low abundance in the foliar tissues,
255 comprised around 10% of the relative abundance in sapwood samples. As a matter of fact, most
256 families with less than 5% relative abundance in leaves clearly presented higher values in sapwood,
257 except for Diaporthaceae and Xylariaceae. The CLAM test classified most families as specialists
258 in one or the other habitat, except for Cladosporiaceae, which happened to be the only group that
259 did not show habitat preference in any of the evaluated instances (Figure 3). Interestingly, some
260 of the families preferred sapwood over leaves but displayed a preference for a developmental stage
261 when found in foliar tissues: Schizoporaceae, Irpicaceae, and Apiosporaceae. Within
262 developmental stage, less than half of the families showed habitat preference or specialization and
263 only two were recognized as indicators for mature leaves and none for new. Some of the families
264 exhibited habitat preference in all the evaluated instances, for example, Mycosphaerellaceae and
265 Xylariaceae preferred Golfito, leaf tissue, and mature stages; while Schizoporaceae and Irpicaceae
266 preferred Guanacaste, sapwood, and new leaves (Figure 3).
267
268 **DISCUSSION**
269 The diversity of fungi found in the leaves and sapwood of tropical plants belonging to the
270 Rubiaceae family did not show discernible patterns of specific host affiliation. Highly abundant
271 community members seemed to be consistently present within the microbiome across different

habitats and diverse host species. Fungal communities in leaves appear to be stable in early tissue development and persist in mature stages. As expected, tissues contained distinct habitats for symbionts. The mechanisms of community assemblage at regional scales are not clear. We found weak evidence for dispersal limitation and environmental partitioning; therefore, the observed patterns may be a result of unknown environmental variables that are correlated with the different geographic locations.

Cosmopolitan fungi dominate hyperdiverse endophyte communities

A fungus' niche preference is largely determined by the interaction between genotype and environment (47). Therefore, it can vary among forests that differ in plant community composition and biogeographic and environmental conditions. Communities of endophytes with a broad host range may be completely shaped by the environment, while for those with host restrictions the plant might exert a stronger influence (48). Results from this study support previous findings that in high-diversity natural systems, such as tropical forests, the endophyte communities are typically composed of numerous taxa of low relative abundances (49), and tend to be dominated by few fungal taxa with a broad host range. The latter are predicted to have a widespread availability of suitable partners, displaying loose host affiliation, and occupying geographically and taxonomically disparate plant hosts (4,5,50), patterns also found in this study (Figure 3).

Host jumps or shifts by microbial symbionts usually occur among closely related host taxa and are driven by the exploitation of new adaptive zones (51). Endophytes may display host preferences at the plant family (52) or order (53) level. In our study, we focused exclusively on samples from a single plant family, Rubiaceae, and found that taxonomy did not predict fungal

294 community clustering (Figure 2C, Supplementary Figure S5). However, this does not exclude the
295 possibility of host effects in other contexts, at a finer scale (e.g., within genus) or with more
296 individuals per plant species (54,55).

297

298 **Forest regions shape fungal endophytic communities**

299 The results of our study support the hypothesis that fungal endophytic communities differ between
300 forest regions. Samples from the tropical rainforests of Golfito exhibited higher alpha diversity
301 compared to those from Guanacaste (Table 2), which includes sampling sites classified as tropical
302 dry forest. Notably, these ecosystems experience significant seasonal changes, including extended
303 periods of drought. These shifting conditions lead to the presence of semi-deciduous trees, which
304 alters the availability of host tissues and results in fluctuations in endophyte activity and diversity.
305 For instance, some taxa may become dormant during dry periods, potentially leading to an
306 underestimation of the local pool of endophytes. The temporal distribution of endophytes,
307 documented in both temperate (56) and, more recently, tropical forests (4), supports this
308 phenomenon. Differences between the regions were also evident in community similarity.
309 Moreover, our analysis revealed that endophytic communities in Guanacaste exhibited a more
310 scattered pattern, suggesting higher intra-regional variability among those samples (Figure 2A).
311 This observation aligns with and may be related to the variability in environmental conditions
312 across the region (Table 1; Figure 1).

313 The environment can serve as a filter, regulating which particular taxa can colonize and
314 persist within hosts (48,57). However, in this study, we did not find strong associations between
315 environmental variables (i.e., elevation, temperature, or precipitation) and fungal community

316 composition (Supplementary Figure S7, Supplementary Table S8). This suggests that
317 environmental factors may not be the primary drivers of community variation. Instead, biotic
318 processes such as priority effects may be more predictive of community structure. For instance,
319 horizontally transmitted endophytes must compete with already established horizontally or
320 vertically transmitted microbes, thereby restricting the pool of species able to colonize a given
321 host.

322 Additionally, neutral processes, such as dispersal limitation and ecological drift, also play
323 a significant role in the distribution pattern of endophytes (58–60). Successful host colonization
324 and taxa distribution depend on factors such as dispersal opportunities and movement restrictions
325 across different environments (61).(61) Consequently, the similarity in species composition
326 between ecological communities typically decreases with increasing distance, a phenomenon
327 known as distance decay (62). Although dispersal limitation is not considered strong for
328 endophytes, distance decay has been reported with varying magnitudes (63) (64,65). Our study
329 found a marginally significant interaction between geographic location and community structuring
330 (Supplementary Table S8). Moreover, the endophytic communities were more strongly correlated
331 with the geographic distance than environmental parameters (Supplementary Table S8), despite
332 no clear evidence of distance decay (Supplementary Figure S9).

333 Both Golfito and Guanacaste shared the same dominant generalist taxa (Figure 3),
334 characterized by high prevalence and abundance across a variety of plant hosts. In contrast, some
335 fungi exhibited specialist behavior, such as Irpicaceae in Guanacaste samples, and Apiosporaceae
336 in Golfito. This suggests that while generalists are broadly distributed, specialized interactions are
337 more localized, likely due to habitat- and niche- specific factors. For instance, the availability of
338 suitable plant hosts in a given ecosystem may limit the geographical distribution these specialized

339 taxa (61,66). This differentiation underscores the role of niche processes in shaping fungal
340 communities, leading to variations in both taxonomic composition and functional traits. At this
341 broad spatial scale, environmental heterogeneity between regions may influence trait values more
342 than to taxonomy, producing community variations that are not solely explained by taxonomic
343 classifications (67,68).

344

345 **Tissue type predicts endophyte community variations**

346 The phyllosphere is a dynamic and heterogeneous environment, whose surfaces are often
347 considered relatively oligotrophic (69). The ability to colonize plant tissues is largely conditioned
348 by complex physical-chemical interactions with their hosts. For example, fungi must pass through
349 several lines of defense (70) and resist the host production of antimicrobial compounds (58,71).
350 Results from the present study support our hypothesis, and previous studies, that endophyte
351 communities are structured by plant tissue (49,72,73). We observed large differences in
352 endophytes between leaves and sapwood. Leaves harbored more robust and rich assemblages than
353 sapwood, although the latter is considered rich in organic nutrients and is a target of many different
354 organisms (74). We observed little overlap between foliar and sapwood endophytes, suggesting
355 spatial heterogeneity across plant compartments (75–77).

356 While most of the top families of fungi were present in both tissues, they differed in relative
357 abundance and were not dominated by the same taxa. The most abundant family in sapwood was
358 Sporocadaceae, and not surprisingly, both families belonging to the Basidiomycota in the top 12
359 families preferred sapwood according to the CLAM test (Figure 3). In the present study,
360 Phyllostictaceae, the second most abundant family on the dataset was not detected in sapwood,

361 indicating an affinity of this taxon towards foliar tissues. Meanwhile, other families (i.e.,
362 Nectriaceae, Apiosporaceae and Irpicaceae) almost imperceptible in leaves, presented higher
363 abundance in sapwood, suggesting that tissue type correlates with selective enrichments of specific
364 endophytes (78,79), and some fungi may not be able to persist or disperse between different tissues.

365 Specificity of fungal endophytes in bark has been previously reported (80). These fungi
366 hold great but underappreciated ecological significance, for they inhabit large surfaces often
367 targeted by insects and pathogens (81,82) and represent a passive reservoir of spores and latent life
368 that will eventually play an important role in wood decay dynamics (83,84). Several studies on
369 endophytic community profiles suggest broad correlations between diversity and select defense
370 mechanisms in bark (85), e.g., reaction zones, the vascular cambium, and the transpiration stream
371 in the sapwood (86). Furthermore, the presence of certain fluids, such as sap, can also act as a
372 barrier depending on their levels of specific compounds (87,88). The sapwood of trees consists of
373 dead xylem cells, and the presence of air-filled vessels and pits can create physical barriers that
374 limit endophyte movement within the tissue (89,90). Similarly, the negative pressure within the
375 xylem can hinder endophytes from colonizing this tissue (91), as they may not be able to penetrate
376 the vessel walls or survive under conditions of low oxygen and nutrient availability and high-water
377 stress (92,93). Therefore, bark acts as an environmental filter structuring inner bark fungal
378 communities (83) which might explain the differences in community richness, diversity and
379 composition observed in this study.

380 The canopy cover of trees creates dynamic microclimates that affect endophyte distribution
381 and growth by modifying temperature, humidity, and wind conditions (94–96). Fungi release
382 spores from within and upon canopies, that are then dispersed onto vegetation and exposed to
383 various elements (97). During precipitation, for example, natural events such as throughfall and

384 stemflow transport bioaerosols and spores through different habitats within a plant microbiome,
385 including bark and leaves (98). The accumulation of endophyte propagules is then influenced by
386 surface area, as plant structures of various sizes interact differently with rainwater and wind (6,99).
387 Woody stems can be challenging to saturate with water due to their fiber content, cylindrical shape
388 and thickness (100,101). Additionally, the large surface area on certain tree species, often covered
389 in small grooves or ridges, increases the number of accumulation areas, such as fissures, cracks,
390 tree hollows, and dendrotelmata (102,103). These structures enable the storage of a vast number
391 of spores and serve as reservoirs for fungi, offering wide niches for their survival (104). In fact, a
392 recent study found that large water-insoluble particles (e.g., conidia, spores) tend to accumulate
393 more on bole surfaces than on leaves or branches (105). Although these spaces and other structures,
394 such as lenticels, may act as entry points for fungal endophytes, complex polymers in this tissue,
395 including lignin and suberin, pose significant barriers to endophyte colonization (106–108).
396 In foliar tissue, water droplets containing spores can adhere to trichomes, epidermal cell
397 grooves, and the curvature of leaves (98), and use stomata, trichomes, and other structures to enter
398 the tissue (109–111). However, the hydrophobic nature of the cuticle causes aerial depositions to
399 be washed off during rainfalls, leading to self-cleaning surfaces (98). Despite leaves being rich in
400 bioactive compounds with antifungal properties such as phenolics, alkaloids and terpenes
401 (112,113), endophytes thrive in these environments due to high moisture content, and nutrient
402 availability.

403

404 **Leaf developmental stage does not drive endophyte community differences**

405 Generally, newly emerged leaves in woody plants start endophyte-free but rapidly become
406 colonized by fungal spores (114). In this study, differences in foliar endophyte richness due to leaf
407 developmental stage (young vs. mature) were not consistent across diversity indices (Table 2). We
408 predicted that new leaves would present lower endophyte abundance due to hypothetical higher
409 concentration of chemical defenses (115–117). Ontogeny-related shifts in endophyte diversity
410 could also be anticipated due to various changes in tissue characteristics, including genetic traits,
411 chemistry and topology (69), plant hormonal or physiological properties, and physical
412 characteristics, such as tissue structure and geometry (118). However, it is important to note that
413 these factors were not directly measured.

414 Our beta diversity analyses (Figures 2B, 2C) show that leaf developmental stage was not a
415 strong predictor of the observed community compositions. Defense syndromes in plants are
416 usually associated to strategies of resource conservation vs resource acquisition (e.g., young vs
417 mature leaves) (119). In fact, some studies have found large qualitative and quantitative differences
418 in metabolomic composition between expanding and mature leaves (120) (i.e., Optimal Defense
419 Theory); however, this is not universally observed (121). Research in tropical trees has found low
420 variation in defense traits and weak shifts in secondary chemistry during leaf maturation (113).
421 This aligns with the "grow fast, die fast" hypothesis, which suggests that plants in these biomes
422 adjust carbon allocation to balance early growth against defenses due to shorter leaf lifespans
423 (122,123). Similarly, the resource availability hypothesis suggests that plants in resource-limited
424 environments (e.g., boreal regions) invest more in constitutive defenses than those in resource-rich
425 habitats (e.g., tropical regions) (124). This implies that plants in the tropics may not necessarily
426 experience strong selective pressure to evolve higher levels of chemical defense against biotic
427 threats (125).

428 Consequently, tropical plants may not exhibit significant variations in the phytochemical
429 profiles of young versus mature leaves. This could account for the absence of significant
430 differences in endophyte communities across developmental stages, as we observed no increase in
431 endophyte relative abundance with tissue maturity. Additionally, both habitats were dominated by
432 the same families, which can be considered prominent components of foliar endophyte
433 communities in Rubiaceae tropical plants and are likely to play a significant role in ecosystem
434 functioning. The CLAM test identified Xylariaceae and Mycosphaerellaceae as the two families
435 that displayed a preference for the mature leaves' habitat. These families are commonly known as
436 foliar endophytes, but they also include pathogens, litter decomposers, and wood saprotrophs
437 (Supplementary Figure S10).

438 Endophytes interact with both the internal environment of the host plant and the broader
439 external abiotic conditions, that influence their distribution and functions in complex ways (126).
440 We observed intriguing patterns in the composition and diversity of foliar endophytes in tropical
441 Rubiaceae. However, important caveats must be considered when interpreting these findings.
442 Although our analysis accounted for certain environmental parameters, we did not measure other
443 significant factors such as shade and light availability (127,128), humidity (129,130), wind (131),
444 ground topography (132), and soil characteristics (e.g., carbon content, texture, and
445 physicochemical properties) (133,134). For instance, stable and functional soils, like those of
446 tropical forests, are likely to host robust microbial reservoirs (135), facilitating the horizontal
447 transfer of endophytes to the phyllosphere, adding another layer of complexity to plant-endophyte
448 interactions.

449 Moreover, when accounting for geographic factors and habitat effects, it is crucial to
450 acknowledge the potential influence of other unmeasured variables, including forest structure and

451 composition, plant density (114), tree diversity, climatic gradients (126,136), and landscape-scale
452 elements (137). Temporal factors, such as forest succession and seasonal variations also contribute
453 (138–140). Moreover, the host's age (141), genetics, architecture and spatial structure (114,142)
454 introduce additional complexity that we did not capture. Nonetheless, the diverse nature of the
455 observed endophytes implies that no single ecological effect is universal. While the determined
456 patterns may be constrained by the specific environmental and climatic factors herein considered,
457 endophyte-plant interactions represent a novel dimension of niche differentiation for coexisting
458 tree species (135), still highly unexplored and nuanced by the intricate and context-dependent
459 trade-offs shaping these communities.

460 With the imminent threat of climate change and the constant challenges in ecosystem
461 management and agriculture, endophytes have the potential to transform the way we think about
462 and manage plant stress resistance (143–145). Understanding their distributions and functioning
463 in association with different host plants and ecosystems, and their response to environmental
464 disturbances, is key to predicting the potential impact of many of these fungi in managed and
465 natural ecosystems. How to translate this acquired knowledge into practical applications for forest
466 conservation and agricultural practices remains a challenge. Many cryptic and undiscovered taxa,
467 whose ecological roles are still unknown, await exploration. In the future, functional group and
468 single trait approaches could offer valuable insights into how ecosystem and plant traits influence
469 endophytic fungi, and how these microorganisms, in turn, affect host and ecosystem functions. It
470 is imperative to continue the efforts to understand the degree to which apparent patterns of host
471 colonization and selection for diverse endophytes are dictated by the host, the endophyte or other
472 ecological mechanisms.
473

ACKNOWLEDGEMENTS

We thank botanist Pedro Juarez-Retana for the plant identification, Efraín Escudero-Leyva for his help in sample collection, and Wilber Alvarado for assistance in the field. Dr. Gloriana Chaverri and Dr. Óscar Quirós from Fundación ProSur, for the access to sites in Golfito and lab space during fieldtrips. Samples were collected under the Institutional Biodiversity Commission (University of Costa Rica) resolutions VI07723-2018 and VI5152-2016, and Costa Rica's National System of Conservation Areas (SINAC), permit Nº ACG-PI-017-2017. Thanks to Dr. Maile Neel for her feedback; and Dr. David Zelený and Joseph Murphy for help with bioinformatics.

FUNDING

This study was supported by the U.S. National Science Foundation grant DEB-1638976 to P. Chaverri.

SUPPLEMENTARY INFORMATION

Supplementary information is publicly available in Zenodo https://doi.org/10.5281/zenodo.13892068

Supplementary Table S1. List of putative plant species collected based on morphology. In bold are the species collected twice.

Supplementary Methods S2.

Supplementary Table S3. Taxonomic assignment (7 ranks) of all ASVs identified in the dataset, plus their functional traits, can be found publicly available on the Github repository https://github.com/humb15/RuFEnTroFo.

Supplementary Figure S4. Species accumulation and diversity curves for endophytes sampled

497 from (A) young leaves (YL), mature leaves (ML) and sapwood (Sa) tissues, and; (B) Golfito and
498 Guanacaste forests. Metrics include richness (q = 0), Shannon (q = 1) and Simpson (q = 2).
499 **Supplementary Figure S5**. NMDS of Bray Curtis distances showing the distribution of
500 endophytic community assemblages according to (A) genus within tribes and (B) tribes within
501 subfamilies.
502 **Supplementary Table S6**. List of identified fungal families among endophyte communities.
503 **Supplementary Figure S7**. CCA analysis for correlation between Amplicon Single Variants
504 distribution and environmental variables: Elevation, Annual Mean Temperature and Annual
505 Precipitation). Red crosses represent ASVs and black circles represent samples. p-values:
506 Elevation 0.001, AMT 0.073, AP 0.119; CCA1 0.007, CCA2 0.143.
507 **Supplementary Table S8**. Mantel correlation values for community dissimilarity and geographic
508 and environmental factors. Based on Spearman, p-value reported.
509 **Supplementary Figure S9**. Distance decay of taxonomic similarity along spatial distance, every
510 point represents pairwise similarities between samples. Panels A: combined; B: Golfito; C:
511 Guanacaste.
512 **Supplementary Figure S10**. Main lifestyles (primary and secondary) of the genera identified
513 among the top 12 families.
514
515 **DATA AVAILABILITY**
516 The resulting raw data (fastq files) and supporting metadata are deposited and publicly available
517 in GenBank under the BioProject identifier PRJNA889378.
518
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913
914 **AUTHOR CONTRIBUTIONS**

Conceptualization: P.C., H.C., J.S.; Formal analysis: H.C.; Funding acquisition: P.C., J.S.; Investigation: H.C.; Methodology: H.C., P.C., S.Y.; Project administration: P.C., S.Y.; Resources: P.C.; Supervision: P.C., S.Y.; Validation: P.C., S.Y., J.S., H.C.; Visualization: H.C., S.Y; Writing: H.C., J.S., S.Y., P.C.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

FIGURES AND TABLES

Figure 1. Map of Costa Rica showing climatic subregions and sample locations. Legend: RN1 – Eastern slopes of the Guanacaste and Tilarán mountain range (very humid subtropical forest), PN2 – North Pacific Central subregion (tropical dry forest), PN3 – Slopes and foothills of the Guanacaste and Tilarán mountain range subregion (monsoon-influenced rainforest), PS5 – Pacific slopes of the Talamanca mountain range (low montane rainforest). GPS locations for each site: **Guanacaste:** 10.668483 N, -85.147333 W; 10.677167 N, -85.143889 W; 10.681444 N, -85.068889 W; 10.686972 N, -85.161389 W; 10.735542 N, -85.151997 W; 10.735675 N, -85.163583 W; 10.735928 N, -85.168861 W; 10.736069 N, -85.173694 W; 10.754444 N, -85.169244 W; 10.754583 N, -85.182722 W; 10.755889 N, -85.251833 W; 10.762333 N, -85.254639 W; 11.01725 N, -85.490778 W; 11.018633 N, -85.601944 W; **Golfito**: 8.392333 N, -83.13863 W; 8.635881 N, -83.075394 W; 8.6367222 N, -83.0815556 W; 8.668781 N, -83.149222 W; 8.679278 N, -83.143139 W; 8.698944 N, -83.118911 W; 8.701139 N, -83.138722 W; 8.706139 N, -83.119631 W. Figure created by the National Meteorological Institute of Costa Rica at our request based on Solano & Villalobos (2001).

938

86°0'0"W
85°0'0"W
84°0'0"W
83°0'0"W
11°0'0"N
10°0'0"N
9°0'0"N
Lake Nicaragua
NICARAGUA
Caribbean Sea
Pacific Ocean
Gulf of Nicoya
Gulf Dulce
PANAMA
Coco Island
V. OROSI
V. RINCON DE LA VIEJA
V. SANTA MARIA
V. MIRAVALLES
V. TENORIO
V. ARENAL
V. POAS
V. BARVA
V. IRAZU
C. DE LA MUERTE
C. CHIRRIPO
C. DURIKA
C. KAMUK
RN3
RN4
RN5
PN1
PN2
PN3
PN4
VC1
VC2
RMS1
RA1
RA2
RA3
RA4
RA5
PC1
PC2
PC3
PS1
PS2
PS3
PS4
PS5
La Cruz
México
Los Chiles
Pocosol
Crucitas
Upala
Hda. Murciélago
Liberia
San Rafael
Boca Tapada
Boca Ceiba
Barra del Colorado
Tortuguero
Puerto Viejo
Muelle de San Carlos
Aguas Zarcas
Quesada
Guápiles
Guácimo
Pocora
Siquirres
Matina
Limón
San Andrés
Pandora
Cahuita
Bribri
Sixaola
Sepecue
Amubri
San José
Cuen
Bacuare Arriba
Chirripo Arriba
Turrialba
Cartago
Pocosay
San Ramón
Palmares
Heredia
Alajuela
Esparza
Atenas
Orotina
Tarcoles
Jacó
Parrita
Quepos
Espadilla
Dominical
Bellena
Buenos Aires
Pto. Cortez
Puerto Escondido
San Vito
Drake
San Pedrillo
Golfito
Neily
Paso Canoas
Carate
Bagaces
Filadelfia
Cañas
Tilarán
Brasilito
Tamarindo
Puerto Humo
Santa Cruz
Juntas
Bíjagua
Hojancha
Nicoya
Marbella
Ostional
Garza
Carmona
Lepanto
Jicaral
Corro Azul
Playa Coyote
Cóbano
Santa Teresa
Mal País
Montezuma
Cabuya
Cacascolo
El Coco
Miramar
Coordinates: 86°0'0"W, 85°0'0"W, 84°0'0"W, 83°0'0"W; 11°0'0"N, 10°0'0"N, 9°0'0"N
MINAE
Ministerio de Ambiente y Energía
imn
Instituto Meteorológico Nacional
Fundado en 1888
MINISTRY OF ENVIRONMENT AND ENERGY
NATIONAL METEOROLOGICAL INSTITUTE
CLIMATIC SUB-REGIONS
LEGEND
Golfito
Guanacaste
0 5 10 20 30 40 50 60 km.
Scale 1:1,800,000
Source:
GIS - Development Department,
National Meteorological Institute, San José, Costa Rica. 2020.
Geographic Projection: WGS84.
Datum: WGS84.
Ellipsoid: WGS84.
Coco Island is not contemplated
in this analysis

939 **Figure 2.** NMDS of Bray Curtis distances showing dissimilarities between the structure of
940 endophyte community assemblages from (A) different locations, (B) different tissues and (C)
941 different plant tribes, developmental stage represented here by shape.

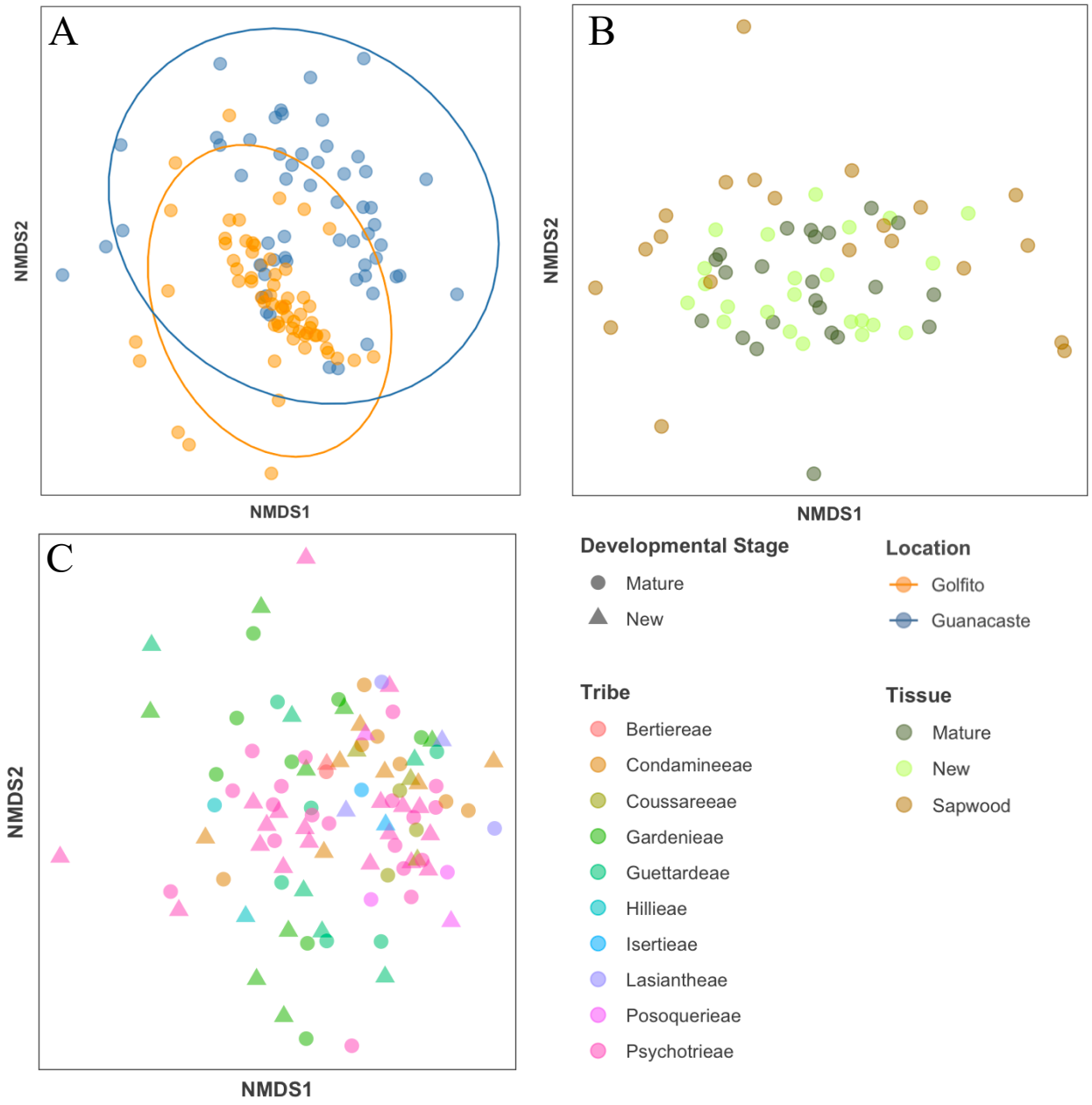
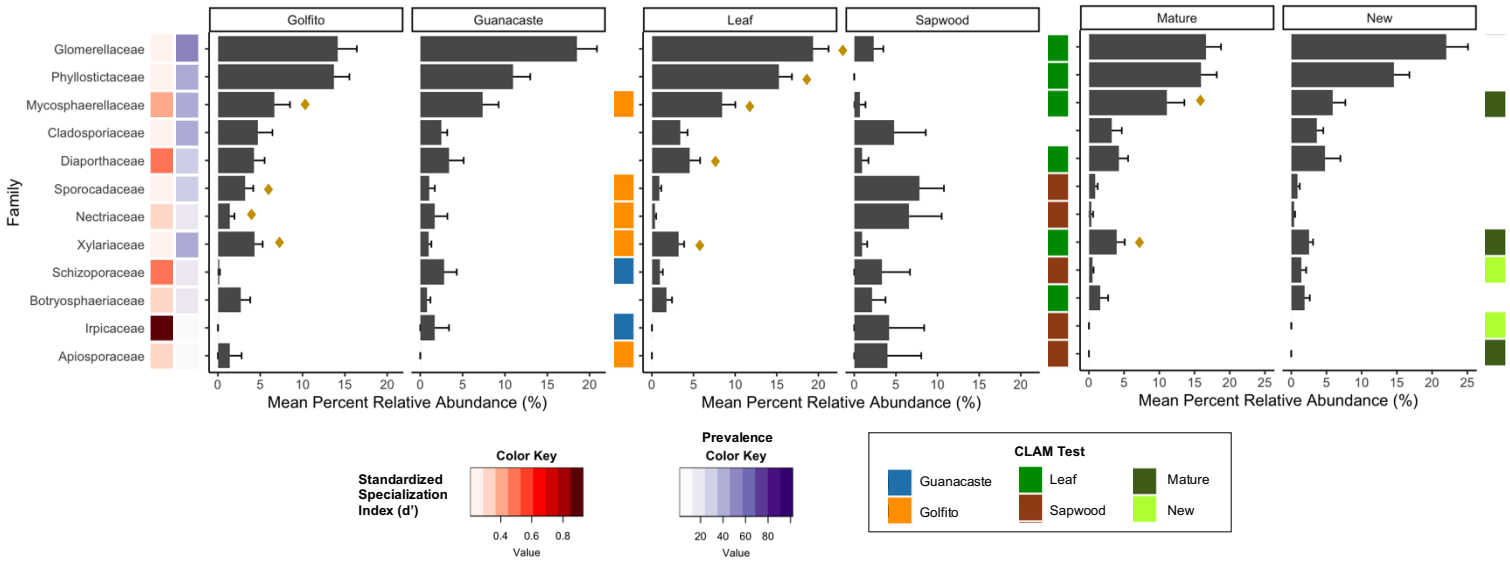


Figure 3. Prevalence and mean percent relative abundance of the 12 most abundant families weighting all habitats within location, tissue type and foliar developmental stage. Families are organized based on abundance, high to low, top to bottom. Mean abundance is plotted with error bars representing the standard error. d' index calculates how specialized the identified taxa are in relation to the 47 plant putative species. Denoted with a diamond symbol (♦) are the families with indicator species within the different habitats; and habitat specialization is indicated by the CLAM test.



950 **Table 1.** Characterization of collecting sites, including mean environmental variables in locations

951 (± SE).

Location	Sites	Plants (n)	Annual Mean Temperature (ºC)	Annual Precipitation (mm)	Elevation (m)
Guanacaste	14	23	22.94 ± 0.12	2718.32 ± 45.03	749.56 ± 41.72
Golfito	8	27	24.65 ± 0.19	3522.09 ± 21.17	124.18 ± 12.23

952

953 **Table 2.** Alpha diversity represented by Hill Effective Species Numbers (± SE) for the evaluated
954 variables. Hill numbers represent taxa richness (Chao1), Shannon diversity (the exponential of
955 Shannon entropy), and Simpson diversity (the inverse of Simpson index). Mann-Whitney U test
956 p-values reported.

Variable	**Category**	**Sample size**	**Obs. Species richness**	**Shared number of ASVs**	**Taxa richness (q = 0)**	**Shannon diversity (q =1)**	**Simpson diversity (q = 2)**
Location	Golfito	66	2146	315	79.52 ± 7.91	25.41 ± 2.26	13.75 ± 1.08
	Guanacaste	57	1387		51.81 ± 6.69	20.06 ± 2.38	11.48 ± 1.21
	p-value				**0.00655**	**0.04821**	0.07629
Tissue type	Leaf	46	1514	96	61.89 ± 7.03	21.22 ± 2.61	11.84 ± 1.38
	Sapwood	23	240		11.83 ± 1.64	1.57 ± 0.17	0.66 ± 0.05
	p-value				**4.99E-06**	**8.22E-06**	**2.41E-04**
Development stage	Mature	50	2253	912	92.40 ± 8.56	22.80 ± 2.51	14.31 ± 1.22
	New	50	1756		66.18 ± 7.87	25.62 ± 2.63	14.56 ± 1.31
	p-value				**0.0067**	0.4628	1.0000

957