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

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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: Costa Rica, ecology, endophytes, fungi, metabarcoding, phytobiome, phyllosphere, Rubiaceae.

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

59 The mechanisms by which endophytic fungi colonize plant tissues remain a topic of
60 research interest (1–3). Understanding the dynamics that determine their distributions and the
61 environmental constraints shaping their assemblages is crucial. Evidence shows that broad-scale
62 environmental factors such as temperature, elevation, precipitation (4,5), and forest type (6)
63 significantly influence community composition. Additionally, genetic and geographic distance
64 among host trees (7–9), as well as host growth stage and tissue type (10–12), also play crucial
65 roles. Given these factors, endophytes that exhibit recurrence or specificity may play a significant
66 ecological role in forests (13,14). Moreover, under the premise that high diversity maintains the
67 overall integrity of an ecosystem (i.e., biological insurance hypothesis *sensu* Naeem & Li (15))
68 the hidden diversity of endophytes can enhance the fitness of individual trees and, by extension,
69 shape forest composition and demography (16). Efforts to understand endophytic diversity are
70 necessary for predicting their function in association with different tissues, hosts, specific
71 environmental conditions, or entire ecosystems. In this sense, tropical forests offer an ideal setting
72 for studying these patterns and interactions.

73 Rubiaceae is one of the largest and most complex families of angiosperms, comprising over
74 13,000 species that range from weedy herbs to large trees and occupy many habitats and
75 biogeographical regions across all continents (17). However, they are predominantly found in the
76 neotropics (18,19). In Costa Rica, historical data (20), listed 89 genera and 458 species present,
77 but recent updates suggest that this count is underestimated. For example, the genus *Palicourea*
78 alone, previously reported with 44 species, is now known to include 91 (21,22). This increased
79 knowledge highlights the family's extensive diversity. Noteworthy within Rubiaceae are several
80 genera of cultural and economic importance such as *Cinchona*, *Coffea*, *Genipa,* and *Psychotria*.

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

92

93 **METHODS**

94 **Study site**

95 Old-growth forests from two regions in Costa Rica were sampled. Specific sampling locations
96 classified by climatic subregions are shown in Figure 1. Golfito, in the South Pacific, was visited
97 in April 2017. All of the sampling sites in this area are characterized by typical tropical rainforest
98 conditions, with no defined dry season, intense rainfall, and dense evergreen vegetation spanning
99 two to three layers (23). In May 2017, sampling was conducted in Guanacaste, specifically in two
100 distinct regions (North and North Pacific), comprising three subclimates: seasonal dry forest, semi-
101 deciduous forest influenced by monsoons, and the forested slopes of a volcano, influenced by

surrounding mountain systems (23). Climate data, including mean annual temperature, mean annual precipitation and elevation were obtained from the WorldClim database (Table 1) (24).

Sampling

Sampling of leaves and sapwood was conducted following a modified version of the protocol described by Gazis & Chaverri (25). 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.

For 23 of the individuals, which were woody trees with a minimum trunk diameter-at-breast-height of approximately 10 cm, a sterilized knife was used to cut a sliver of outer bark at shoulder height to expose the alburnum and one piece of living sapwood tissue ca. 30 × 5 mm was excised from the exposed area. All collected samples were put in a clean plastic bag and placed on ice for transport to the field workstation for immediate processing. A leaf section of ca. 20 × 4 mm was cut and surface-sterilized using sequential immersions in a 2% sodium hypochlorite solution (1 min), 70% ethanol (30 sec), and sterile distilled water (30 sec). Finally, leaves were dried with autoclaved tissue paper and stored in microtubes containing silica gel before being transferred on

123 ice to the laboratory for DNA extraction (26). Sapwood samples were briefly singed with a flame
124 to eliminate dust and potential surface contaminants introduced during handling, and then placed
125 in microtubes.

126

127 **Sequencing**

128 Samples were transferred to a pre-filled 500-micron Garnet and a 6-mm Zirconium Grinding
129 Satellite bead tube (OPS Diagnostics LLC, New Jersey, USA) and ground using a FastPrep® bead
130 mill (MP Biomedicals, Santa Ana, California, USA). Each tube was treated to a grinding cycle of
131 speed: 6.0 m/s for 45 seconds, or until no visually recognizable fragments remained. Each sample
132 (tissue section) represented a biological replicate, and no technical replicates were performed.
133 Total DNA was extracted using the commercial kit Qiagen DNeasy Plant Mini Kit® according to
134 the manufacturer's instructions (Qiagen, Hilden, Germany). Genomic DNA was sent to Naturalis
135 Biodiversity Centre (NBC, Leiden, The Netherlands) for targeted amplicon metagenomics
136 (metabarcoding) of the ITS2 nrDNA region using Ion PGM Torrent™ technology (Thermo Fisher
137 Scientific, Waltham, MA, USA). The primers used were fITS7 and ITS4, which are fungal specific
138 and with less amplification of plant ITS (27). To ensure accuracy in downstream analyses, a
139 positive control with a non-Costa Rican *Russula galochroa* DNA and a negative control (blank)
140 were included throughout. According to NBC, PCR was performed in a BioRad C1000
141 thermocycler (Hercules, CA, USA), quality of the PCR products was checked with a QIAxcel
142 (Qiagen), PCR products were cleaned with NucleoMag NGS Clean-up and Size Select magnetic
143 beads (Takara Bio Inc., Japan), the pool was run in an Agilent Bioanalyzer (Santa Clara, CA, USA)
144 using a DNA High Sensitivity chip and amplified on the Ion One Touch system (Ion PGM™ Hi-

145 Q™ View OT2 kit), the results were measured on the Qubit (ThermoFisher) for the Ion Sphere™
146 Quality Control kit (ThermoFisher), and finally the sequence run was done on the Ion PGM Torrent
147 using the Ion PGM™ Hi-Q™ View Sequencing kit. The resulting raw data (fastq files) are
148 deposited in GenBank under the BioProject identifier PRJNA889378.

149

150 **Fungal community analyses**

151 ***ASV classification and taxa assignment.*** Primers and barcodes were trimmed from each read
152 using the command line tool 'cutadapt' (28). Subsequent processing was performed in RStudio
153 v. 2022.12.0+353 (29) with R v. 4.0.3, employing the DADA2 v1.18.0 pipeline (30). Raw
154 reads were subjected to data curation aimed at identifying and removing spurious contigs
155 through various filtering processes. For instance, chimeric sequences were removed. Reads with
156 total counts below 0.001% of the total reads and singletons were excluded. Quality filtering
157 included a maximum expected error rate of 2 for both forward and reverse reads, and sequences
158 were trimmed to a minimum length of 100 bp (36). Amplicon Sequence Variants (ASVs) were
159 assigned taxonomically by comparing them to the UNITE database (36) v8.3 (31) training set
160 from the DECIPHER package, v 2.18.1 (32). Only sequences classified as fungi were retained.
161 Additional independent filtering steps included the exclusion of samples with total read counts
162 below 0.001% of the total reads, and taxa with low variance across samples (variance threshold
163 of 1e-5). A prevalence threshold of 1% of total samples was applied, retaining taxa appearing in

164 at least this proportion of samples. The accuracy of the pipeline was validated using the positive
165 controls, and sequences from negative controls were removed to ensure dataset integrity.

166

167 ***Diversity and community structure analyses.*** Statistical analyses and plotting were conducted
168 using the phyloseq v1.38.0 (33), vegan v2.5.7 (34), microbiome v1.12.0 (35) and ggplot2
169 v3.4.1 (36) packages. Endophyte alpha diversity for each dataset was estimated using the
170 Shannon, Simpson, and Chao1 indices for each variable and then transformed to Hill Effective
171 Species Numbers (37,38) for comparisons by Mann-Whitney U test. This transformation
172 integrates species richness and abundance distribution, adjusts sensitivity to rare versus common
173 species, and provides a consistent measure of diversity (43,44). It also allows for more robust
174 comparisons across sites with varying numbers of species and levels of evenness. To visualize
175 community patterns (beta diversity), non-metric multidimensional scaling (NMDS) was
176 performed using the 'metaMDS' function from the vegan package with the 'bray' method and
177 100 random starts. For statistical analyses, data were normalized by total count per sample,
178 converting them into proportions (relative abundances), and Bray-Curtis distances were
179 calculated with the 'vegdist' function. Homogeneity of dispersion was assessed with the
180 'betadisper' function, followed by a permutation test using 'permutest'. Statistical differences in
181 endophyte assemblages across variables (forest regions, tissue types, developmental stages) were
182 evaluated with the 'adonis2' function for PERMANOVA.

183 Relative abundance at the family level was calculated. We retained groups contributing to
184 more than 1% of the average relative abundance across the six categories: forest regions (Golfito
185 and Guanacaste), tissue types (leaf and sapwood) and developmental stages (young and mature).

186 These groups are referred to as the 'top families' in downstream analyses. The overall prevalence
187 (i.e., the number of samples in which the taxa appear) of these families was computed. To estimate
188 how strongly they deviate from a random preference among the plant partners available at the
189 study sites, we calculated the d' index of specialization using the bipartite v 2.18 package (39),
190 function 'dfun'. ASVs were statistically classified as generalists or specialists based on their
191 habitat affinity using a multinomial species classification method (CLAMtest) in vegan. Indicator
192 taxa (ASVs with p-values < 0.05) from the six categories were identified using the 'multipatt'
193 function in the indicspecies v1.7.12 package (40). Methods regarding Species Accumulation
194 Curves, Mantel Correlation Tests, Distance Decay, Canonical Correlation Analysis and the
195 assignment of functional traits are described in Supplementary Methods S2.

196

197 **RESULTS**

198 A total of 6,467,267 reads passed the quality control filters. The mean number of sequences per
199 sample was 58,465 for leaves and 26,989 for sapwood. We identified 3,218 putative taxa (ASVs)
200 belonging to at least 232 different genera (Supplementary Table S3). ASV identification rate was
201 67%, 50% and 33% at the order, family, and genus levels, respectively. The species richness or
202 number of unique ASVs recovered per sample (triplicates, in the case of leaf tissue) ranged from
203 2 to 337, with a mean of 67.

204 All datasets had a high number of single occurrences, and no asymptote was reached in the
205 species accumulation curves (Supplementary Figure S4) indicating that the datasets are
206 incomplete, and the number of samples was not sufficient in capturing the expected endophyte

207 diversity (Hill's q = 0). However, when focusing on dominant species only (Hill's q = 2), all curves,
208 except for sapwood, reached a plateau.

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

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

229 development stages, no effect was detected, there was a high overlap between the fungal
230 communities (Figure 2C), and the PERMANOVA indicated no significant differences (p =
231 0.8122). Fungal endophyte communities did not cluster based on individual plants or at any higher
232 taxonomic level (i.e., genus, tribe, subfamily). The pattern for tribe is shown in Figure 2C, other
233 levels of taxonomy are depicted in Supplementary Figure S5.

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

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

250 that presented very low abundance in Golfito, such as Schizoporaceae and Irpicaceae, were more
251 abundant in Guanacaste and such preference was highlighted by the CLAM test (Figure 3).

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

DISCUSSION

The diversity of fungi found in the leaves and sapwood of tropical plants belonging to the Rubiaceae family did not show discernible patterns of specific host affiliation. Highly abundant 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 (41). 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 (42). 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 (43), and tend to be dominated by few fungal taxa with a broad host range. The latter are predicted to have a widespread availability of

289 suitable partners, displaying loose host affiliation, and occupying geographically and
290 taxonomically disparate plant hosts (44–46), patterns also found in this study (Figure 3).

291 Host jumps or shifts by microbial symbionts usually occur among closely related host taxa
292 and are driven by the exploitation of new adaptive zones (47). Endophytes may display host
293 preferences at the plant family (48) or order (49) level. In our study, we focused exclusively on
294 samples from a single plant family, Rubiaceae, and found that taxonomy did not predict fungal
295 community clustering (Figure 2C, Supplementary Figure S5). However, this does not exclude the
296 possibility of host effects in other contexts, at a finer scale (e.g., within genus) or with more
297 individuals per plant species (50,51).

298

299 **Forest regions shape fungal endophytic communities**

300 The results of our study support the hypothesis that fungal endophytic communities differ between
301 forest regions. Samples from the tropical rainforests of Golfito exhibited higher alpha diversity
302 compared to those from Guanacaste (Table 2), which includes sampling sites classified as tropical
303 dry forest. Notably, these ecosystems experience significant seasonal changes, including extended
304 periods of drought. These shifting conditions lead to the presence of semi-deciduous trees, which
305 alters the availability of host tissues and results in fluctuations in endophyte activity and diversity.
306 For instance, some taxa may become dormant during dry periods, potentially leading to an
307 underestimation of the local pool of endophytes. The temporal distribution of endophytes,
308 documented in both temperate (52) and, more recently, tropical forests (44), supports this
309 phenomenon. Differences between the regions were also evident in community similarity.

310 Moreover, our analysis revealed that endophytic communities in Guanacaste exhibited a more
311 scattered pattern, suggesting higher intra-regional variability among those samples (Figure 2A).
312 This observation aligns with and may be related to the variability in environmental conditions
313 across the region (Table 1; Figure 1).

314 The environment can serve as a filter, regulating which particular taxa can colonize and
315 persist within hosts (42,53). However, in this study, we did not find strong associations between
316 environmental variables (i.e., elevation, temperature, or precipitation) and fungal community
317 composition (Supplementary Figure S7, Supplementary Table S8). This suggests that
318 environmental factors may not be the primary drivers of community variation. Instead, biotic
319 processes such as priority effects may be more predictive of community structure. For instance,
320 horizontally transmitted endophytes must compete with already established horizontally or
321 vertically transmitted microbes, thereby restricting the pool of species able to colonize a given
322 host.

323 Additionally, neutral processes, such as dispersal limitation and ecological drift, also play
324 a significant role in the distribution pattern of endophytes (54–56). Successful host colonization
325 and taxa distribution depend on factors such as dispersal opportunities and movement restrictions
326 across different environments (57). Consequently, the similarity in species composition between
327 ecological communities typically decreases with increasing distance, a phenomenon known as
328 distance decay (58). Although dispersal limitation is not considered strong for endophytes,
329 distance decay has been reported with varying magnitudes (59–61). Our study found a marginally
330 significant interaction between geographic location and community structuring (Supplementary
331 Table S8). Moreover, the endophytic communities were more strongly correlated with the

332 geographic distance than environmental parameters (Supplementary Table S8), despite no clear
333 evidence of distance decay (Supplementary Figure S9).

334 Both Golfito and Guanacaste shared the same dominant generalist taxa (Figure 3),
335 characterized by high prevalence and abundance across a variety of plant hosts. In contrast, some
336 fungi exhibited specialist behavior, such as Irpicaceae in Guanacaste samples, and Apiosporaceae
337 in Golfito. This suggests that while generalists are broadly distributed, specialized interactions are
338 more localized, likely due to habitat- and niche- specific factors. For instance, the availability of
339 suitable plant hosts in a given ecosystem may limit the geographical distribution these specialized
340 taxa (57,62). This differentiation underscores the role of niche processes in shaping fungal
341 communities, leading to variations in both taxonomic composition and functional traits. At this
342 broad spatial scale, environmental heterogeneity between regions may influence trait values more
343 than to taxonomy, producing community variations that are not solely explained by taxonomic
344 classifications (63,64).

345

346 **Tissue type predicts endophyte community variations**

347 The phyllosphere is a dynamic and heterogeneous environment, whose surfaces are often
348 considered relatively oligotrophic (65). The ability to colonize plant tissues is largely conditioned
349 by complex physical-chemical interactions with their hosts. For example, fungi must pass through
350 several lines of defense (66) and resist the host production of antimicrobial compounds (54,67).
351 Results from the present study support our hypothesis, and previous studies, that endophyte
352 communities are structured by plant tissue (43,68,69). We observed large differences in

353 endophytes between leaves and sapwood. Leaves harbored more robust and rich assemblages than
354 sapwood, although the latter is considered rich in organic nutrients and is a target of many different
355 organisms (70). We observed little overlap between foliar and sapwood endophytes, suggesting
356 spatial heterogeneity across plant compartments (71–73).

357 While most of the top families of fungi were present in both tissues, they differed in relative
358 abundance and were not dominated by the same taxa. The most abundant family in sapwood was
359 Sporocadaceae, and not surprisingly, both families belonging to the Basidiomycota in the top 12
360 families preferred sapwood according to the CLAM test (Figure 3). In the present study,
361 Phyllostictaceae, the second most abundant family on the dataset was not detected in sapwood,
362 indicating an affinity of this taxon towards foliar tissues. Meanwhile, other families (i.e.,
363 Nectriaceae, Apiosporaceae and Irpicaceae) almost imperceptible in leaves, presented higher
364 abundance in sapwood, suggesting that tissue type correlates with selective enrichments of specific
365 endophytes (74,75), and some fungi may not be able to persist or disperse between different tissues.

366 Specificity of fungal endophytes in bark has been previously reported (76). These fungi
367 hold great but underappreciated ecological significance, for they inhabit large surfaces often
368 targeted by insects and pathogens (77,78) and represent a passive reservoir of spores and latent life
369 that will eventually play an important role in wood decay dynamics (79,80). Several studies on
370 endophytic community profiles suggest broad correlations between diversity and select defense
371 mechanisms in bark (81), e.g., reaction zones, the vascular cambium, and the transpiration stream
372 in the sapwood (82). Furthermore, the presence of certain fluids, such as sap, can also act as a
373 barrier depending on their levels of specific compounds (83,84). The sapwood of trees consists of
374 dead xylem cells, and the presence of air-filled vessels and pits can create physical barriers that

375 limit endophyte movement within the tissue (85,86). Similarly, the negative pressure within the
376 xylem can hinder endophytes from colonizing this tissue (87), as they may not be able to penetrate
377 the vessel walls or survive under conditions of low oxygen and nutrient availability and high-water
378 stress (88,89). Therefore, bark acts as an environmental filter structuring inner bark fungal
379 communities (79) which might explain the differences in community richness, diversity and
380 composition observed in this study.

381 The canopy cover of trees creates dynamic microclimates that affect endophyte distribution
382 and growth by modifying temperature, humidity, and wind conditions (90–92). Fungi release
383 spores from within and upon canopies, that are then dispersed onto vegetation and exposed to
384 various elements (93). During precipitation, for example, natural events such as throughfall and
385 stemflow transport bioaerosols and spores through different habitats within a plant microbiome,
386 including bark and leaves (94). The accumulation of endophyte propagules is then influenced by
387 surface area, as plant structures of various sizes interact differently with rainwater and wind
388 (95,96). Woody stems can be challenging to saturate with water due to their fiber content,
389 cylindrical shape and thickness (97,98). Additionally, the large surface area on certain tree species,
390 often covered in small grooves or ridges, increases the number of accumulation areas, such as
391 fissures, cracks, tree hollows, and dendrotelmata (99,100). These structures enable the storage of
392 a vast number of spores and serve as reservoirs for fungi, offering wide niches for their survival
393 (101). In fact, a recent study found that large water-insoluble particles (e.g., conidia, spores) tend
394 to accumulate more on bole surfaces than on leaves or branches (102). Although these spaces and
395 other structures, such as lenticels, may act as entry points for fungal endophytes, complex polymers
396 in this tissue, including lignin and suberin, pose significant barriers to endophyte colonization
397 (103–105).

398 In foliar tissue, water droplets containing spores can adhere to trichomes, epidermal cell
399 grooves, and the curvature of leaves (94), and use stomata, trichomes, and other structures to enter
400 the tissue (106–108). However, the hydrophobic nature of the cuticle causes aerial depositions to
401 be washed off during rainfalls, leading to self-cleaning surfaces (94). Despite leaves being rich in
402 bioactive compounds with antifungal properties such as phenolics, alkaloids and terpenes
403 (109,110), endophytes thrive in these environments due to high moisture content, and nutrient
404 availability.

405

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

407 Generally, newly emerged leaves in woody plants start endophyte-free but rapidly become
408 colonized by fungal spores (111). In this study, differences in foliar endophyte richness due to leaf
409 developmental stage (young vs. mature) were not consistent across diversity indices (Table 2). We
410 predicted that new leaves would present lower endophyte abundance due to hypothetical higher
411 concentration of chemical defenses (112–114). Ontogeny-related shifts in endophyte diversity
412 could also be anticipated due to various changes in tissue characteristics, including genetic traits,
413 chemistry and topology (65), plant hormonal or physiological properties, and physical
414 characteristics, such as tissue structure and geometry (115). However, it is important to note that
415 these factors were not directly measured.

416 Our beta diversity analyses (Figures 2B, 2C) show that leaf developmental stage was not a
417 strong predictor of the observed community compositions. Defense syndromes in plants are
418 usually associated to strategies of resource conservation vs resource acquisition (e.g., young vs

419 mature leaves) (116). In fact, some studies have found large qualitative and quantitative differences
420 in metabolomic composition between expanding and mature leaves (117) (i.e., Optimal Defense
421 Theory); however, this is not universally observed (118). Research in tropical trees has found low
422 variation in defense traits and weak shifts in secondary chemistry during leaf maturation (110).
423 This aligns with the "grow fast, die fast" hypothesis, which suggests that plants in these biomes
424 adjust carbon allocation to balance early growth against defenses due to shorter leaf lifespans
425 (119,120). Similarly, the resource availability hypothesis suggests that plants in resource-limited
426 environments (e.g., boreal regions) invest more in constitutive defenses than those in resource-rich
427 habitats (e.g., tropical regions) (121). This implies that plants in the tropics may not necessarily
428 experience strong selective pressure to evolve higher levels of chemical defense against biotic
429 threats (122).

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

440 Endophytes interact with both the internal environment of the host plant and the broader
441 external abiotic conditions, that influence their distribution and functions in complex ways (123).
442 We observed intriguing patterns in the composition and diversity of foliar endophytes in tropical
443 Rubiaceae. However, important caveats must be considered when interpreting these findings.
444 Although our analysis accounted for certain environmental parameters, we did not measure other
445 significant factors such as shade and light availability (124,125), humidity (126,127), wind (128),
446 ground topography (129), and soil characteristics (e.g., carbon content, texture, and
447 physicochemical properties) (130,131). For instance, stable and functional soils, like those of
448 tropical forests, are likely to host robust microbial reservoirs (132), facilitating the horizontal
449 transfer of endophytes to the phyllosphere, adding another layer of complexity to plant-endophyte
450 interactions.

451 Moreover, when accounting for geographic factors and habitat effects, it is crucial to
452 acknowledge the potential influence of other unmeasured variables, including forest structure and
453 composition, plant density (111), tree diversity, climatic gradients (123,133), and landscape-scale
454 elements (134). Temporal factors, such as forest succession and seasonal variations also contribute
455 (135–137). Moreover, the host's age (138), genetics, architecture and spatial structure (111,139)
456 introduce additional complexity that we did not capture. Nonetheless, the diverse nature of the
457 observed endophytes implies that no single ecological effect is universal. While the determined
458 patterns may be constrained by the specific environmental and climatic factors herein considered,
459 endophyte-plant interactions represent a novel dimension of niche differentiation for coexisting
460 tree species (132), still highly unexplored and nuanced by the intricate and context-dependent
461 trade-offs shaping these communities.

With the imminent threat of climate change and the constant challenges in ecosystem management and agriculture, endophytes have the potential to transform the way we think about and manage plant stress resistance (140–142). Understanding their distributions and functioning in association with different host plants and ecosystems, and their response to environmental disturbances, is key to predicting the potential impact of many of these fungi in managed and natural ecosystems. How to translate this acquired knowledge into practical applications for forest conservation and agricultural practices remains a challenge. Many cryptic and undiscovered taxa, whose ecological roles are still unknown, await exploration. In the future, functional group and single trait approaches could offer valuable insights into how ecosystem and plant traits influence endophytic fungi, and how these microorganisms, in turn, affect host and ecosystem functions. It is imperative to continue the efforts to understand the degree to which apparent patterns of host colonization and selection for diverse endophytes are dictated by the host, the endophyte or other ecological mechanisms.

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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 from (A) young leaves (YL), mature leaves (ML) and sapwood (Sa) tissues, and; (B) Golfito and

500 Guanacaste forests. Metrics include richness (q = 0), Shannon (q = 1) and Simpson (q = 2).

501 **Supplementary Figure S5**. NMDS of Bray Curtis distances showing the distribution of

502 endophytic community assemblages according to (A) genus within tribes and (B) tribes within

503 subfamilies.

504 **Supplementary Table S6**. List of identified fungal families among endophyte communities.

505 **Supplementary Figure S7**. CCA analysis for correlation between Amplicon Single Variants

506 distribution and environmental variables: Elevation, Annual Mean Temperature and Annual

507 Precipitation). Red crosses represent ASVs and black circles represent samples. p-values:

508 Elevation 0.001, AMT 0.073, AP 0.119; CCA1 0.007, CCA2 0.143.

509 **Supplementary Table S8**. Mantel correlation values for community dissimilarity and geographic

510 and environmental factors. Based on Spearman, p-value reported.

511 **Supplementary Figure S9**. Distance decay of taxonomic similarity along spatial distance, every

512 point represents pairwise similarities between samples. Panels A: combined; B: Golfito; C:

513 Guanacaste.

514 **Supplementary Figure S10**. Main lifestyles (primary and secondary) of the genera identified

515 among the top 12 families.

516

517 **DATA AVAILABILITY**

518 The resulting raw data (fastq files) and supporting metadata are deposited and publicly available
519 in GenBank under the BioProject identifier PRJNA889378.

520

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901 **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).

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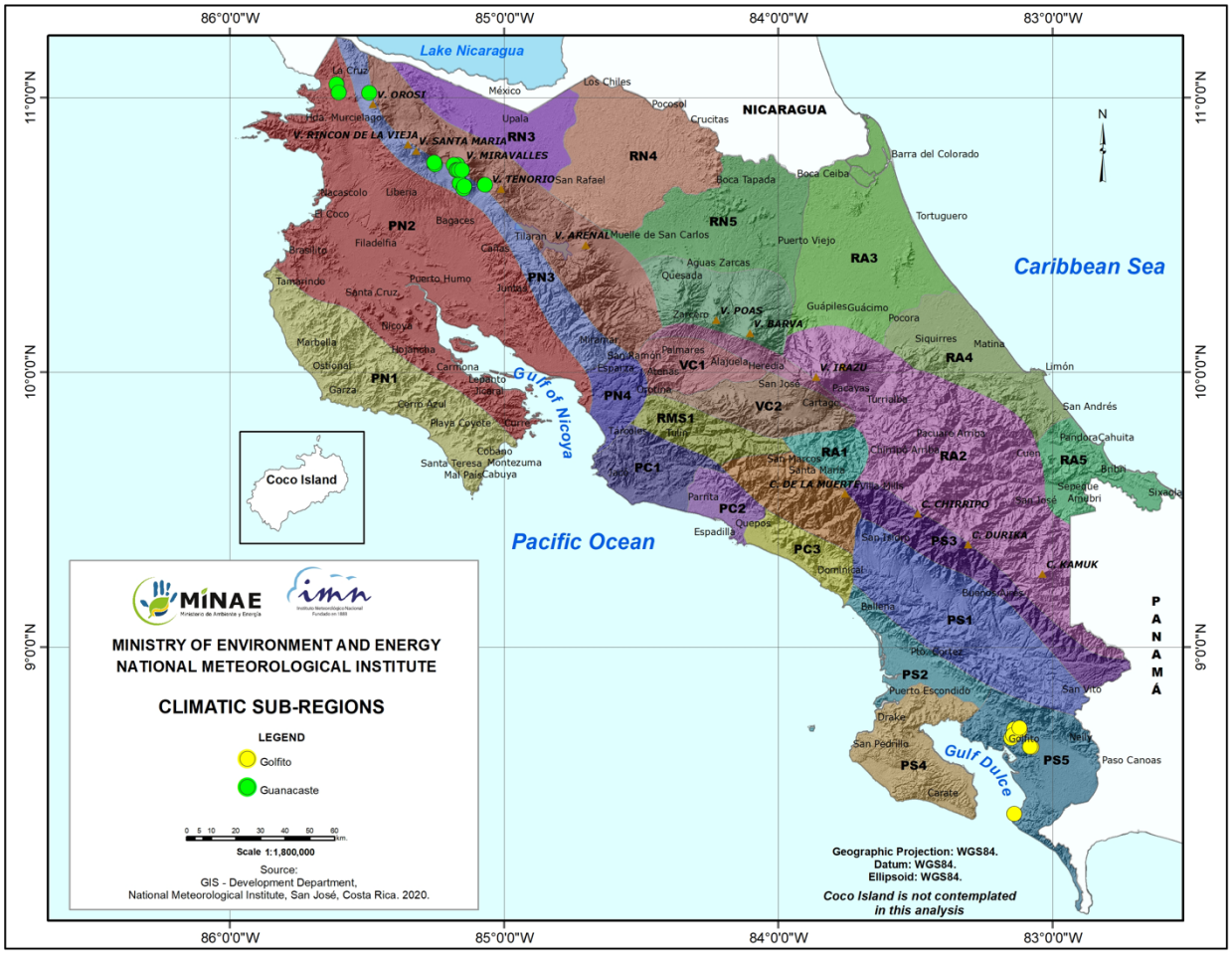
86°0'0"W
85°0'0"W
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Lake Nicaragua
NICARAGUA
Caribbean Sea
Pacific Ocean
Gulf of Nicoya
Gulf Dulce
PANAMA
Coco Island
V. OROSI
V. RINCON DE LA VIEJA
V. SANTA MARIA
V. MIRAVALLES
V. TENORIO
V. ARENAL
V. POAS
V. BARVA
V. IRAZU
C. DE LA MUERTE
C. CHIRRIPO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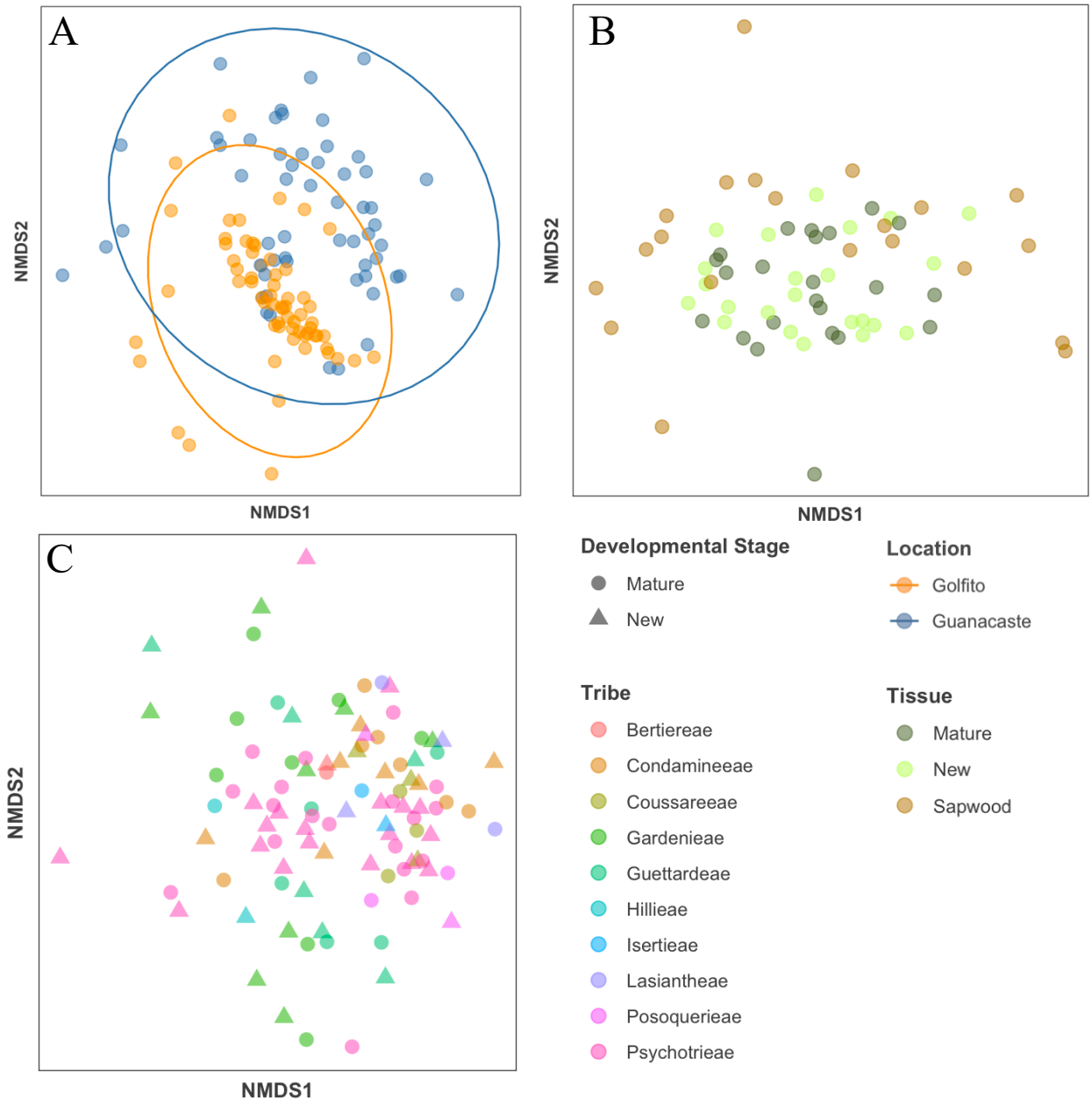
MINAE
Ministerio de Ambiente y Energía
imn
Instituto Meteorológico Nacional
Fundado en 1888
MINISTRY OF ENVIRONMENT AND ENERGY
NATIONAL METEOROLOGICAL INSTITUTE
CLIMATIC SUB-REGIONS
LEGEND
Golfito
Guanacaste
0 5 10 20 30 40 50 60 km.
Scale 1:1,800,000
Source:
GIS - Development Department,
National Meteorological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

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



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

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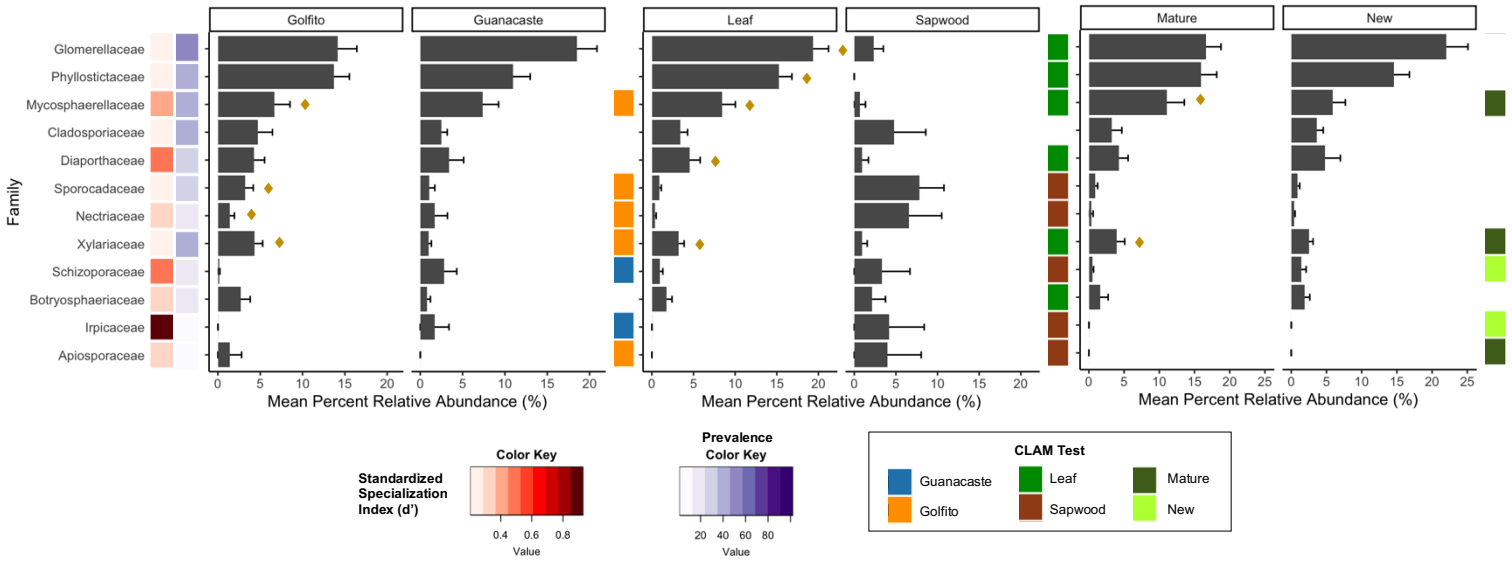

937 **Table 1.** Characterization of collecting sites, including mean environmental variables in locations

938 (± 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

939

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