Exploring biodiversity dynamics and taxonomy of Rubiaceae fungal endophytes in tropical regions

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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 Rubiaceae. Our hypotheses, centered on the influence of these factors, were tested using targeted amplicon sequencing. Leaves and sapwood samples were collected from old-growth forests in Golfito and Guanacaste, Costa Rica, and fungal diversity was 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 locations, 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 support the highest diversity of terrestrial plants on Earth (1). In these biomes, plant tissues, as a source of labile nutrients and niche space (2), can maintain an extraordinary

48 mosaic of microbial assemblages (3–5), whose distributions influence hosts and drive forest
49 dynamics at diverse levels ranging from mutualism, through commensalism, to pathogenicity
50 (6,7). Plant fungal endophytes that colonize leaves and wood are abundant and diverse (4) but
51 traditionally understudied compared to mycorrhizal and free-living forms. Nevertheless, they play
52 a major role in ecosystems, as endophytes can positively influence plant fitness and health,
53 conferring protection against abiotic stress, herbivores and pathogens (8). Moreover, they might
54 be sources of new bioactive compounds with novel chemical features (9).

55 The mechanisms by which fungi colonize plant tissues remain a topic of research interest
56 (10–12). Endophytes are typically transferred horizontally (13), and therefore, the fungal
57 community composition is affected by the surrounding environmental and geographic factors (14).
58 Understanding the dynamics that determine the distributions and maintenance of species and the
59 underlying environmental constraints that shape the structure and composition of endophytic
60 assemblages is crucial. Endophytes exhibiting recurrence or specificity could play a significant
61 ecological role in forests (15,16). Furthermore, under the premise that high diversity maintains the
62 overall integrity of an ecosystem (i.e., biological insurance hypothesis *sensu* Naeem & Li (1997)),
63 the hidden diversity of endophytes can enhance the fitness of individual trees and, by extension,
64 shape forest composition and demography (9). Efforts to understand endophytic diversity are
65 necessary for predicting their function in association with hosts, specific environmental conditions,
66 or entire ecosystems. In this sense, tropical forests are an ideal setting for studying patterns and
67 interactions of endophytes with host plants.

68 Rubiaceae is one of the largest and most complex families of angiosperms, with over
69 13,000 species. Plants in this group range from weedy herbs to large trees and occupy many
70 habitats and biogeographical regions in all continents (18), but are mainly present in the neotropics

71 (19,20). For instance, according to a 2014 handbook (21), Costa Rica is home to 89 genera and
72 458 species. However, recent enumerations of Central American *Palicourea* spp. (22,23) have
73 identified a higher number of species within this genus — 91 compared to the previously reported
74 44 — suggesting that the total diversity of species may be even greater than previously thought.
75 Notably, Rubiaceae includes several cultural and economically important genera (e.g., *Cinchona*,
76 *Coffea*, *Genipa,* and *Psychotria*).

77 In this study, we investigated endophytic communities at different locations, leaf
78 developmental stages, and between tissue types. We hypothesized that endophytic communities
79 would differ between regions, both in diversity and in composition, as a response to their variable
80 environmental conditions. Secondly, we hypothesized that communities would differ between
81 plant species and that within a tree, endophytes would also differ between young and mature leaves
82 and between leaves and sapwood. In addition to testing these hypotheses, we aimed to understand
83 which fungal groups drove such differences. Characterizing the endophyte communities within
84 Rubiaceae and the processes shaping their assemblages could provide a unique insight into
85 understanding endophyte niche preferences in tropical forests.

86

87 **METHODS**

88 **Study site**

89 Old-growth forests from two locations in Costa Rica were sampled (Figure 1). Golfito, in the South
90 Pacific region, was visited in April 2017. All of the sampling sites in this area are characterized by
91 typical tropical rainforest conditions, with no defined dry season, intense rainfall, and dense
92 evergreen vegetation spanning two to three layers (24). In May 2017, sampling was conducted in
93 Guanacaste, specifically in two distinct regions (North and North Pacific), comprising three

94 subclimates: seasonal dry forest, semi-deciduous forest influenced by monsoons, and the forested
95 slopes of a volcano, influenced by surrounding mountain systems (24). Climate data, including
96 mean annual temperature, mean annual precipitation and elevation were obtained from the
97 WorldClim database (Table 1) (Fick & Hijmans, 2017).
98
99 **Sampling**
100 Young and mature healthy leaves from 50 Rubiaceae plants (distributed in 47 species from 27
101 genera, 10 tribes and 3 subfamilies) were collected (Supplementary Table S1). Plants were
102 identified by a local botanist and vouchers of all individuals were deposited in the University of
103 Costa Rica Herbarium (USJ). Leaves from three different branches per plant were sampled. To
104 differentiate developmental stages, one newly emerged and one mature leaf separated by three
105 nodes were selected.
106 For 23 of the plants, a sterilized knife was used to cut a sliver of outer bark at shoulder
107 height to expose the alburnum and one piece of living sapwood tissue ca. 30 × 5 mm was excised
108 from the exposed area. All collected samples were put in a clean plastic bag and placed on ice for
109 transport to the field workstation for immediate processing. A leaf section of ca. 20 × 4 mm was
110 cut and surface-sterilized using sequential immersions in a 2% sodium hypochlorite solution (1
111 min), 70% ethanol (30 sec), and sterile distilled water (30 sec). Finally, leaves were dried with
112 autoclaved tissue paper, and stored in microtubes containing silica gel before being transferred on
113 ice to the laboratory for DNA extraction. Sapwood samples were only singed and then deposited
114 in the microtubes.

115

116 **Sequencing**

117 Samples were transferred to a pre-filled 500-micron Garnet and a 6-mm Zirconium Grinding
118 Satellite bead tube (OPS Diagnostics LLC, New Jersey, USA) and ground using a FastPrep® bead
119 mill (MP Biomedicals, Santa Ana, California, USA). Each tube was treated to a grinding cycle of
120 speed: 6.0 m/s for 45 seconds, or until no visually recognizable fragments remained. Total DNA
121 was extracted using the QiagenDNeasy Plant Mini Kit® according to the manufacturer's
122 instructions (Qiagen, Hilden, Germany). DNA library preparation, PCR and next generation
123 sequencing of the ITS2 nrDNA region using primers fITS7 and ITS4 (25) were done at Naturalis
124 Biodiversity Centre (Leiden, The Netherlands) using Ion PGM Torrent™ technology (Thermo
125 Fisher Scientific, Waltham, MA, USA). The resulting raw data (fastq files) are deposited in
126 GenBank under the BioProject identifier PRJNA889378.

127

128 **Fungal community analyses**

129 ***ASV classification and taxa assignment.*** Primers and barcodes were trimmed from each read
130 using the command line tool 'cutadapt' (26) and subsequentially processed using R v. 4.0.3 in
131 RStudio v. 2022.12.0+353 (27). Raw reads were subjected to a data curation process using the
132 DADA2 v1.18.0 pipeline (28). Analysis included quality filtering, trimming and chimeras
133 checking. In addition, any spurious contigs, singletons and low abundance reads were also
134 removed using the DADA2 v1.18.0 package (28). The last step of this process assigned DNA
135 reads into Amplicon Sequence Variants (ASV). Taxonomic assignment of ASVs were made by

136 comparing to the UNITE database v8.3 (29) training set from the DECIPHER package, v 2.18.1
137 (30).
138
139 ***Diversity and community structure analyses.*** Statistical analyses and plotting were conducted
140 using Phyloseq v1.38.0 (31), Vegan v2.5.7 (32), Microbiome v1.12.0 (33) and ggplot2 v3.4.1
141 (34) packages in RStudio. Endophyte alpha diversity for each of the datasets was estimated using
142 the Shannon, Simpson, and Chao1 indexes for each variable and transformed to Hill Effective
143 Species Numbers for comparisons by Mann-Whitney U test. Non-metric multidimensional
144 scaling (NMDS) based on Bray-Curtis dissimilarities of ASV abundances was used to visualize
145 community patterns (beta-diversity) using the function 'metaMDS' from Vegan (100 random
146 starts) and statistical differences in endophyte assemblages between different variables (i.e.,
147 location, tissue, and developmental stage) were tested with a PERMANOVA.
148 Relative abundance at family level was calculated and the groups contributing to >1% of
149 the relative abundance averaged from the 6 categories/habitats here evaluated were kept for
150 downstream analyses; and are referred to as the 'top families' onwards. The overall prevalence
151 (i.e., the number of samples in which the taxa appears) of these families was computed. To estimate
152 how strongly they deviate from a random preference among the plant partners available at the
153 study sites, we calculated the d' index of specialization using the bipartite v 2.18 package (35),
154 function 'dfun'. ASVs were statistically classified into habitat generalists or specialists using a
155 multinomial species classification method (CLAMtest) in vegan. Indicator taxa (ASVs with p-
156 values < 0.05) in the different variable categories were identified using the 'multipatt' function in
157 the indicspecies v1.7.12 package (36). Methods regarding Species Accumulation Curves, Mantel

158 Correlation Tests, Distance Decay, Canonical Correlation Analysis and the assignment of
159 functional traits are described in Supplementary Methods S2.
160
161 **RESULTS**
162 A total of 6,467,267 reads passed the quality control filters. The mean number of sequences per
163 sample was 58465 for leaves and 26989 for sapwood. We identified 3,218 putative taxa (ASVs)
164 belonging to at least 232 different genera (Supplementary Table S3). ASV identification rate was
165 67%, 50% and 33% at the order, family, and genus levels, respectively. The species richness or
166 number of unique ASVs recovered per sample (triplicates, in the case of leaf tissue) ranged from
167 2 to 337, with a mean of 67.
168 All datasets had a high number of single occurrences, and no asymptote was reached in the
169 species accumulation curves (Supplementary Figure S4) indicating that the datasets are
170 incomplete, and the number of samples was not sufficient in capturing the expected endophyte
171 diversity (Hill's $q = 0$). However, when focusing on dominant species only (Hill's $q = 2$), all curves,
172 except for sapwood, reached a plateau.
173 Fungal endophyte assemblages differed between locations and tissue types (Table 2).
174 Samples originating from Golfito exhibited consistently higher alpha diversity indices compared
175 to those from Guanacaste, as supported by a Hill numbers comparison ($q=0,1,2$) that demonstrated
176 statistical differences for $q=0$ ($p \leq 0.05$) and $q=1$ ($p \leq 0.01$). Notably, leaf and sapwood tissues
177 were found to harbor distinct fungal communities, with leaves exhibiting significantly richer
178 assemblages ($p \leq 0.001$ in all instances). Turning to the developmental stage, the taxa richness
179 index ($q=0$) was notably higher in mature leaves. However, the other two diversity indices ($q=1$,

180 q=2) showed slightly greater diversity in new leaves, though these differences were not statistically
181 significant.

182 Using non-metric multidimensional scaling (NMDS) we observed differences between
183 location and dispersion of points, with Guanacaste samples appearing more scattered (Figure 2A);
184 three outliers were removed for visualization purposes. Community dissimilarities between these
185 variables were corroborated via PERMANOVA, which indicated significant separation in fungal
186 assemblages when comparing between the different locations ($p < 0.001$). In the case of tissue
187 type, points from leaf and bark communities were dispersed differently in the NMDS analysis
188 (Figure 2B) and presented significant heteroscedasticity ($p < 0.01$), which has been shown to affect
189 PERMANOVA testing ($p < 0.001$). No clear location effect was observed in the NMDS plot;
190 however, the dispersion of the points varied: leaf samples converged on the interior of the cluster
191 while sapwood samples, being more variable, were found mostly around it; two samples were
192 removed for better visualization (figure 2B). Considering just the leaves at the two different
193 development stages, no effect was detected, there was a high overlap between the fungal
194 communities (figure 2C), and the PERMANOVA indicated no significant differences ($p = 0.8122$).
195 Fungal endophyte communities did not cluster based on individual plants or at any higher
196 taxonomic level (i.e., genera, tribe, subfamily). The pattern for tribe is shown in Figure 2C, other
197 levels of taxonomy are depicted in Supplementary Figure S5.

198 The phylum Ascomycota was the most abundant, representing 83% of all fungal ASVs.
199 Within this group, the most abundant and prevalent classes were Sordariomycetes followed by
200 Dothideomycetes. Tremellomycetes and Agaricomycetes were the most abundant and prevalent
201 Basidiomycota, making up 8.2% of the ASVs. We characterized endophytic assemblages
202 dominated by a few abundant dominant taxa and many rare taxonomic groups. For example, we

203 identified 156 different families (Supplementary Table S6) from which Glomerellaceae,
204 Phyllostictaceae and Mycosphaerellaceae composed the bulk of biodiversity (45.14%) present
205 within the endophytic assemblages (Figure 3).

206 The most abundant fungal families were not the most prevalent in all cases (e.g.,
207 Xylariaceae), but they tended to correspond (Figure 3). The top 12 families comprised 59% of the
208 total relative abundance. With the d' index, we determined that the most prevalent and abundant
209 families (i.e., Glomerellaceae and Phyllostictaceae) had values closer to 0 (no specialization). This
210 indicates that members of these fungal families interact with a broad host range. In contrast,
211 Irpicaceae, had a low prevalence and presented a value closer to 1 (perfect specialist). Interesting
212 is the case of Apiosporaceae, that despite a low prevalence, showed little specialization. The major
213 fungal families were similar between Golfito and Guanacaste (Figure 3), but some of the groups
214 that presented very low abundance in Golfito, such as Schizoporaceae and Irpicaceae, were more
215 abundant in Guanacaste and such preference was highlighted by the CLAM test (Figure 3).

216 We did not find indicator species among the top 12 families in the sapwood communities.
217 In contrast, some indicator species were identified for leaves. For example, Phyllostictaceae,
218 despite being the second most abundant and prevalent family overall, was not found in sapwood.
219 Sporocadaceae and Nectriaceae, both of which had very low abundance in the foliar tissues,
220 comprised around 10% of the relative abundance in sapwood samples. As a matter of fact, most
221 families with less than 5% relative abundance in leaves clearly presented higher values in sapwood,
222 except for Diaporthaceae and Xylariaceae. The CLAM test classified most families as specialists
223 in one or the other habitat, except for Cladosporiaceae, which happened to be the only group that
224 did not show habitat preference in any of the evaluated instances (Figure 3). Interestingly, some
225 of the families preferred sapwood over leaves but displayed a preference for a developmental stage

226 when found in foliar tissues: Schizoporaceae, Irpicaceae, and Apiosporaceae. Within
227 developmental stage, less than half of the families showed habitat preference or specialization and
228 only two were recognized as indicators for mature leaves and none for new. Some of the families
229 exhibited habitat preference in all the evaluated instances, for example, Mycosphaerellaceae and
230 Xylariaceae preferred Golfito, leaf tissue, and mature stages; while Schizoporaceae and Irpicaceae
231 preferred Guanacaste, sapwood, and new leaves (Figure 3).
232
233 **DISCUSSION**
234 A fungus' niche preference is largely determined by the interaction between genotype and
235 environment (37). Therefore, it can vary among forests that differ in plant community composition
236 and biogeographic and environmental conditions. Communities of endophytes with a broad host
237 range may be completely shaped by the environment, while for those with host restrictions the
238 plant might exert a stronger influence (38). Results from this study support previous findings that
239 in high-diversity natural systems, such as tropical forests, the endophyte communities are typically
240 composed of numerous taxa of low relative abundances (39), and tend to be dominated by few
241 fungal taxa with a broad host range. The latter are predicted to have a widespread availability of
242 suitable partners, displaying loose host affiliation, and occupying geographically and
243 taxonomically disparate plant hosts (4,5,40), patterns seen also in our current study (Figure 3).
244 Host jumps or shifts by microbial symbionts usually occur among closely related host taxa
245 and are driven by the exploitation of new adaptive zones (41). Endophytes may display host
246 preferences at the plant family (42) or order (43) level. In our study, since all the samples belonged
247 to Rubiaceae, factors other than plant taxonomy shaped the community. However, deeper sampling

248 might reveal an effect of host on a finer scale (e.g., within genus) as it has been previously reported
249 (44).

250 The results of our study support the hypothesis that the composition of endophyte
251 communities is influenced by geographic location. Samples from the tropical rainforests of Golfito
252 displayed higher alpha diversity compared to those from Guanacaste, which includes regions
253 classified as tropical dry forest. These variations were evident not only in terms of richness (see
254 Table 2) but also in community similarity. It is noteworthy that the temporal distribution of
255 endophytes has been observed in both temperate (45) and, more recently, tropical forests (4). This
256 observation is relevant to our study because in Guanacaste, the presence of semi-deciduous trees
257 and the occurrence of extended periods of drought due to seasonality may lead to some taxa
258 remaining dormant during specific periods, potentially resulting in an underestimation of the local
259 pool of endophyte species in Guanacaste.

260 However, our findings align with the distribution patterns observed in higher organisms,
261 indicating that environmental factors play a crucial role in shaping biodiversity. Guanacaste
262 exhibited a more scattered pattern, suggesting higher variability in the communities in those
263 samples (refer to Figure 2A). This observation could be attributed to the differences and variability
264 in environmental conditions (see Table 1) across the region (refer to Figure 1).

265 The environment can serve as a filter and regulate which particular taxa are capable of
266 colonizing and persisting within the hosts (38,46). In general, we did not find strong associations
267 between environmental variables (i.e., elevation, temperature, or precipitation) and the variation
268 in fungal communities (Supplementary Figure S7, Supplementary Table S8), suggesting that
269 neutral processes (e.g., dispersal limitation) are also important in the distribution pattern of
270 endophytes (47–49). Since endophyte transmission occurs mostly horizontally, host colonization

271 and taxa distribution depend on dispersal and successful establishment in the plant (50). This
272 process restricts the pool of species available to colonize a given host, hence, similarity of species
273 composition between ecological communities typically decreases as distance increases, a
274 phenomenon known as distance decay (51).

275 Dispersal limitation is not considered strong for endophytes, but distance decay has been
276 previously reported both absent (52) and documented at different magnitudes (53,54). Our study
277 suggests that there was a marginally significant interaction between geographic location and
278 community structuring (Supplementary Table S8). Moreover, the endophytic communities were
279 more strongly correlated with the geographic distance than with the environmental parameters
280 (Supplementary Table S8), however, no evidence of distance decay was observed (Supplementary
281 Figure S9).

282 Both Golfito and Guanacaste were dominated by host generalists, characterized by high
283 prevalence and abundance, contrasting what appears to be local restricted specialists, such as
284 Irpicaceae in Guanacaste, or Apiosporaceae in Golfito. The availability of suitable hosts might be
285 the limiting factor in the geographical distribution of the examined taxa (50,55). However, niche
286 processes filter communities producing variations in taxonomic groups and functional traits, and
287 it would not be unlikely for environmental heterogeneity to be more closely related to trait values
288 than to taxonomy, at this broad spatial scale (56,57).

289 The phyllosphere is a dynamic and heterogeneous environment, whose surfaces are often
290 considered relatively oligotrophic (58). The ability to colonize plant tissues is largely conditioned
291 by complex physical-chemical interactions with their hosts. For example, fungi must pass through
292 several lines of defense (59) and resist the host production of antimicrobial compounds (47,60).
293 Results from the present study support our hypothesis, and previous studies, that endophyte

294 communities are structured by plant tissue (39,61,62). We observed large differences in
295 endophytes between tissue types (leaves vs. sapwood). Leaves harbored more robust and rich
296 assemblages than sapwood, although the latter is considered rich in organic nutrients and is a target
297 of many different organisms (63). We observed little overlap between foliar and sapwood
298 endophytes, suggesting spatial heterogeneity across plant compartments (64–66).

299 While most of the top families of fungi were present in both tissues, they differed in relative
300 abundance and were not dominated by the same taxa. The most abundant family in sapwood was
301 Sporocadaceae, and not surprisingly, both families belonging to the Basidiomycota in the top 12
302 families preferred sapwood according to the CLAM test (Figure 3). In the present study,
303 Phyllostictaceae, the second most abundant family on the dataset was not detected in sapwood,
304 indicating an affinity of this taxon towards foliar tissues. Meanwhile, other families (i.e.,
305 Nectriaceae, Apiosporaceae and Irpicaceae) almost imperceptible in leaves, presented higher
306 abundance in sapwood, suggesting that tissue type correlates with selective enrichments of specific
307 endophytes (67,68).

308 Specificity of fungal endophytes in bark has been previously reported (69). These fungi
309 hold great but underappreciated ecological significance, for they inhabit large surfaces often
310 targeted by insects and pathogens (70,71) and represent a passive reservoir of spores and latent life
311 that will eventually play an important role in wood decay dynamics (72,73). Several studies on
312 endophytic community profiles suggest broad correlations between diversity and select defense
313 mechanisms in bark (74), e.g., reaction zones, the vascular cambium, and the transpiration stream
314 in the sapwood (75). Furthermore, the presence of certain fluids, such as sap, can also act as a
315 barrier depending on their levels of specific compounds (76,77). The sapwood of trees consists of
316 dead xylem cells, and the presence of air-filled vessels and pits can create physical barriers that

317 limit endophyte movement within the tissue (78,79). Similarly, the negative pressure within the
318 xylem can hinder endophytes from colonizing this tissue (80), as they may not be able to penetrate
319 the vessel walls or survive under conditions of low oxygen and nutrient availability and high-water
320 stress (81,82). Therefore, bark acts as an environmental filter structuring inner bark fungal
321 communities (72) which might explain the differences in community richness, diversity and
322 composition observed in this study.

323 The canopy cover of trees creates dynamic microclimates that affect endophyte distribution
324 and growth by modifying temperature, humidity, and wind conditions (83–85). Fungi release
325 spores from within and upon canopies, that are then dispersed onto vegetation and exposed to
326 various elements (86). During precipitation, for example, natural events such as throughfall and
327 stemflow transport bioaerosols and spores through different habitats within a plant microbiome,
328 including bark and leaves (87). The accumulation of endophyte propagules is then influenced by
329 surface area, as plant structures of various sizes interact differently with rainwater and wind (6,88).
330 Woody stems can be challenging to saturate with water due to their fiber content, cylindrical shape
331 and thickness (89,90). Additionally, the large surface area on certain tree species, often covered in
332 small grooves or ridges, increases the number of accumulation areas, such as fissures, cracks, tree
333 hollows, and dendrotelmata (91,92). These structures enable the storage of a vast number of spores
334 and serve as reservoirs for fungi, offering wide niches for their survival (93). In fact, a recent study
335 found that large water-insoluble particles (e.g., conidia, spores) tend to accumulate more on bole
336 surfaces than on leaves or branches (94). Although these spaces and other structures, such as
337 lenticels, may act as entry points for fungal endophytes, complex polymers in this tissue, including
338 lignin and suberin, pose significant barriers to endophyte colonization (95–97).

339 In foliar tissue, water droplets containing spores can adhere to trichomes, epidermal cell
340 grooves, and the curvature of leaves (87), and use stomata, trichomes, and other structures to enter
341 the tissue (98–100). However, the hydrophobic nature of the cuticle causes aerial depositions to
342 be washed off during rainfalls, leading to self-cleaning surfaces (87). Despite leaves being rich in
343 bioactive compounds with antifungal properties such as phenolics, alkaloids and terpenes
344 (101,102), endophytes thrive in these environments due to high moisture content and nutrient
345 availability. In this study, differences in foliar endophyte richness due to leaf developmental stage
346 (young vs. mature) was not consistent across various diversity indices (Table 2). Although new
347 leaves were predicted to present lower abundance of endophytes in response to a speculative higher
348 concentration of chemical defenses (103,104), our findings suggest that ontogeny-related shifts in
349 endophyte diversity resulting from changes in genetic traits, tissue chemistry and topology (58),
350 plant hormonal and physiological properties, and/or physical characteristics, such as tissue
351 structure and geometry (105), were not strong enough to significantly impact the overall
352 community composition. Finding also supported by our beta diversity analyses (Figures 2B, 2C).
353 Some studies have found large qualitative and quantitative differences in metabolomic
354 composition between expanding and mature leaves (106) (i.e., Optimal Defense Theory), but this
355 is not always the case (107). Research in tropical trees has found low variation in defense traits
356 and weak shifts in secondary chemistry during leaf maturation (102). It has been documented that
357 the turnover times of leaf pools across these biomes exhibit an inverse relationship to growth
358 ("grow fast, die fast" hypothesis) (108). Some theories suggest that this trade-off happens in
359 agreement with the plant investment strategies, adjusting carbon allocation to balance early growth
360 against defenses (108,109). Plants in the tropics do not necessarily experience strong selective
361 pressure to evolve higher levels of chemical defense against biotic threats (110). We assessed taxa

362 relative abundance and noticed no significantly higher rates of foliar endophytic fungi increasing
363 with developmental stage. Both habitats were dominated by the same families, which can be
364 considered prominent components of foliar endophyte communities in Rubiaceae tropical plants
365 and are likely to play a significant role in ecosystem functioning. The CLAM test identified
366 Xylariaceae and Mycosphaerellaceae as the two families that displayed a preference for the mature
367 leaves' habitat. These families are commonly known as foliar endophytes, but they also include
368 pathogens, litter decomposers, and wood saprotrophs (Supplementary Figure S10).

369 With the imminent threat of climate change and the constant challenges in conservation
370 and agriculture, endophytes have the potential to transform the way we think about and manage
371 plant stress resistance (111–113). Understanding their distributions and functioning in association
372 with different host plants and ecosystems, and their response to environmental disturbances, is key
373 to predicting the potential impact of many of these fungi in agricultural ecosystems and climate
374 change science. How to translate this acquired knowledge for forest conservation and agricultural
375 management remains a challenge. There are still cryptic and undiscovered taxa, whose presence
376 has gone unnoticed and whose ecological roles are unknown. It is imperative to continue the efforts
377 to understand the degree to which apparent patterns of host colonization and selection for diverse
378 endophytes are dictated by the host, the endophyte or other ecological mechanisms.

379

380 **CONCLUSIONS**

381 The diversity of fungi found in the phyllosphere (leaves and sapwood) of tropical plants belonging
382 to the Rubiaceae family did not show discernible patterns of specific host affiliation. High
383 abundance community members seemed to be consistently present within the microbiome across
384 different habitats and diverse host species. Fungal communities in leaves appear to be stable in

early tissue development and persist on mature stages. As expected, tissues contained distinct habitats for symbionts. The mechanisms of community assemblage at regional scales are not clear. Since we found weak evidence for dispersal limitation and environmental partitioning, the observed patterns may be a result of unknown environmental variables that are correlated with the different geographic locations.

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. Dr. Maile Neel for her feedback, 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.

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DATA ACCESSIBILITY

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

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.

FIGURES AND TABLES

Figure 1. Map of Costa Rica showing climatic subregions and sample locations. Made per request by the National Meteorological Institute in Costa Rica. (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).

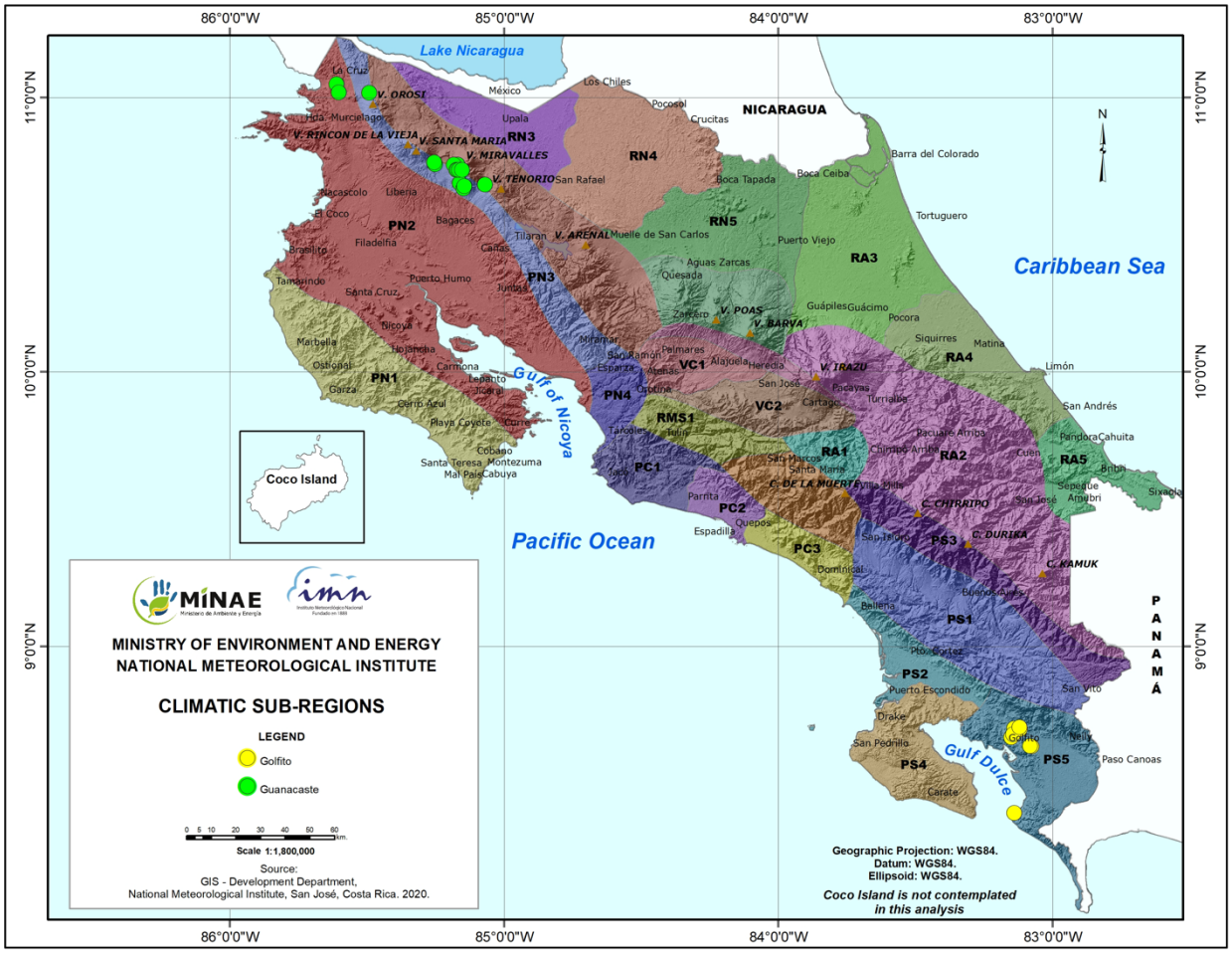


Figure 2. NMDS of Bray Curtis distances showing dissimilarities between the structure of endophyte community assemblages from (A) different locations, (B) different tissues and (C) different plant tribes, developmental stage represented here by shape.

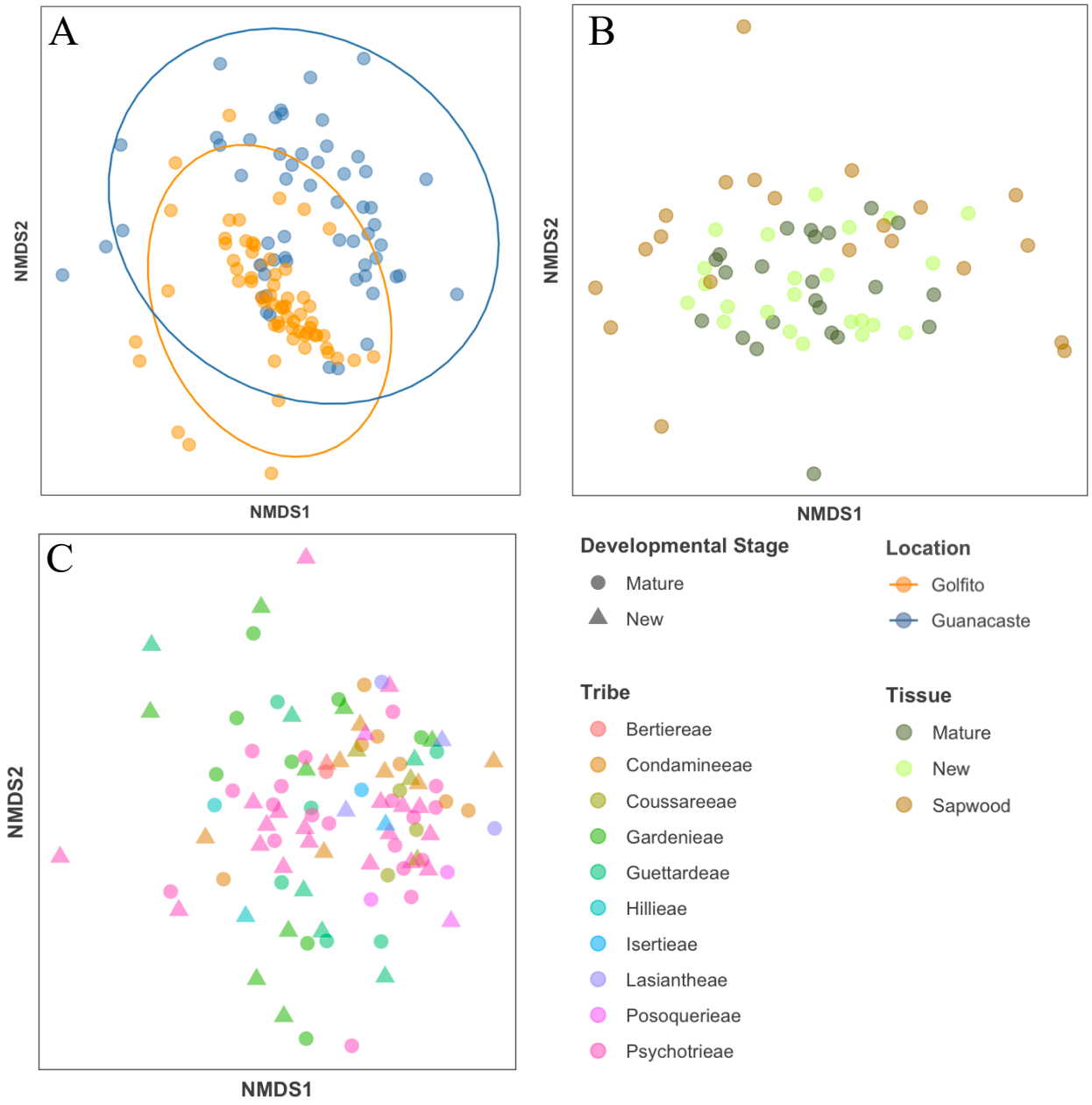
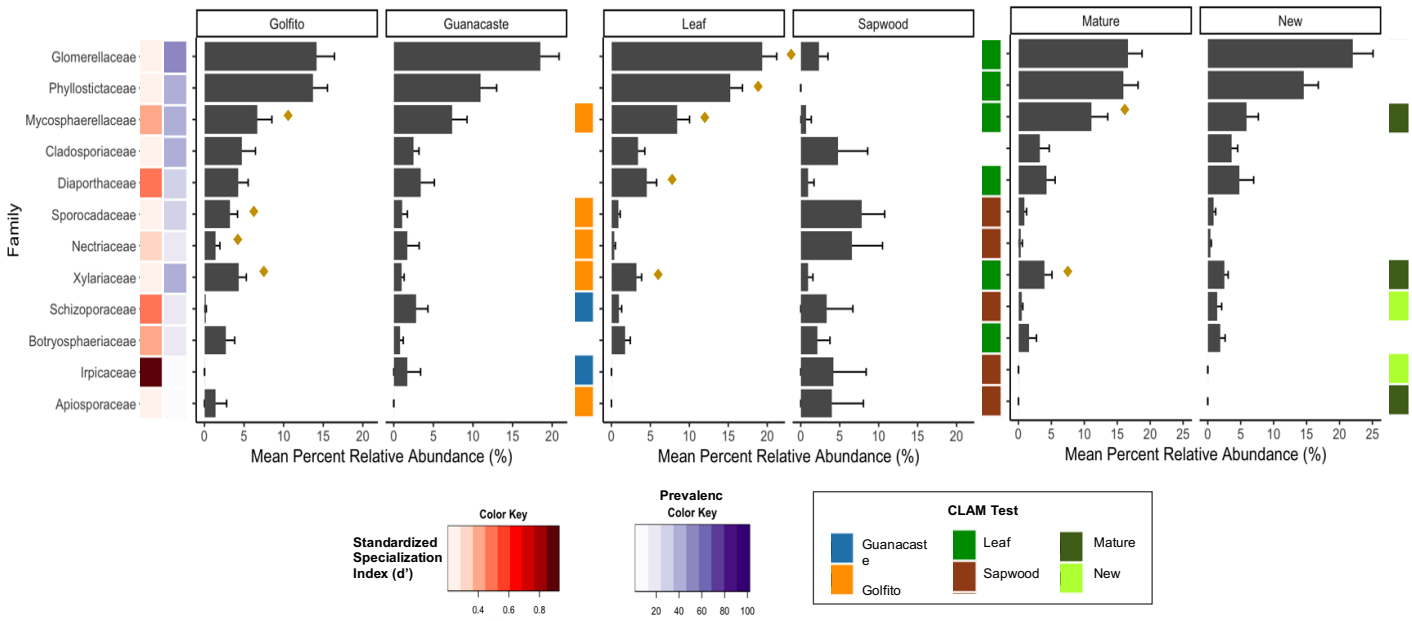


Figure 3. Prevalence and mean percent relative abundance of the 12 most abundant families weighting all habitats within location, tissue type and foliar developmental stage. Families are organized based on abundance, high to low, top to bottom. Mean abundance is plotted with error bars representing the 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 or generalization is indicated by the CLAM test.



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

811 (± SE).

Location	Sites	Plants (n)	Annual Mean Temperature (ºC)	Annual Precipitation (mm)	Elevation (m)
Guanacaste	15	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

812

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