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A test of community assembly rules using foliar endophytes from a tropical forest canopy

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11 **Research Article**

12 **A test of community assembly rules using foliar endophytes from a tropical**
13 **forest canopy**

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Accepted Article

56 **Abstract**

- 57 1. Community assembly theory assumes that ecological communities are spatially
58 delimited into patches. Within these patches, coexistence results from environmental
59 filtering, competition, and immigration. Truly delineated communities exist in
60 laboratory studies of microbial cultures in Petri dishes, yet empirical tests conducted
61 in continuous environments often use patches defined by convention as opposed to
62 realised boundaries.
- 63 2. Here we perform a test of ecological community assembly rules using foliar
64 endophyte communities from a tropical rainforest, where leaves are considered as
65 patches for both fungal and bacterial communities. We determined the diversity of
66 fungal and bacterial endophytes using environmental DNA sequencing of 365 top-
67 canopy leaves, collected from 38 host trees belonging to 22 different species across a
68 4-hectare research plot. Three leaves were collected from three or more branches
69 within each tree crown. We tested the effect of host tree species and their level of
70 phylogenetic relatedness on community composition as well as the contribution of
71 geographic distance between leaves to endophyte community diversity.
- 72 3. Endophyte diversity significantly differed across host tree species, as did community
73 composition. Within certain endophytic orders (Xylariales, Rhizobiales) species
74 assemblages significantly differed across host tree species, but this trend was weaker
75 or non-existent in other orders known to contain pathogens and saprotrophs
76 (Polyporales, Solirubrobacterales). Phylogenetically related host tree species displayed
77 more similar endophyte communities than expected by chance, but geographically
78 close trees did not. Consistent with the finding of host-specificity, nearby leaves
79 tended to host more similar communities than distantly positioned ones.
- 80 4. These findings demonstrate that foliar endophytes are structured by dispersal across
81 small spatial scales, but at the scale of the canopy they display patterns of neutral
82 filtering, with only a small part of variation described by host tree differences.
83 Endophyte communities thus act as a model system in evoking the rules predicted by
84 theoretical community ecology.

86 **Keywords**

87 Bacteria; Distribution; French Guiana; Fungi; Metabarcoding; Plant–pathogen interactions;
88 Rainforest;

89 **Introduction**

90 Understanding the mechanisms which allow for the emergence and maintenance of species
91 diversity, and in particular the coexistence of competing species, is a challenge for
92 community ecology (Ricklefs 2004). Theory suggests that local species diversity results from
93 a balance between local constraints, including environmental conditions and competition, and
94 immigration from external sources through dispersal (Vandermeer 1972, Leibold 1995,
95 Hubbell 2001, Condit et al. 2002, Chase et al. 2005). Studies aiming to contrast the relative
96 weight of these mechanisms require spatially discrete communities of identifiable species at a
97 single trophic level, organized in discrete patches, and existing in large enough numbers to
98 allow for replication (Ellwood et al. 2009). Whilst such conditions are achievable for
99 microbiologists conducting laboratory-based experiments on communities grown in Petri
100 dishes (e.g. Maynard et al. 2017), these fail to represent the levels of variability experienced
101 by natural communities (Crowther et al. 2018). Field tests of community assembly rules have
102 often been conducted using trees in tropical rainforests forests, habitats characterized by high
103 levels of species diversity, with large levels of species turnover from one community to the
104 next (Hubbell 2001, Fine et al. 2004). In these studies, communities of trees are defined by
105 convention as opposed to realised boundaries, and as such inferences of structural patterns
106 may be biased, masking the true processes structuring ecological communities.

107 Foliar endophytes inhabit the intercellular layers of leaves, without provoking a
108 symptomatic response from the host plant, although certain species may be latent saprobes or
109 pathogens (de Bary 1886; Carroll 1988; Porrás-Alfaro & Bayman 2011). Within leaves,
110 multiple species coexist in spite of likely competition for the resources available. Because
111 they are constrained to live within leaves, in communities more delineated than other
112 microbial habitats such as soils, they represent a model example of a metacommunity.
113 Colonisation by endophytes is thought to occur soon after the flushing of new leaves, when
114 spores deposit, germinate and subsequently penetrate the leaf cuticle without provoking an
115 antipathogenic response (Arnold and Herre 2003, Griffin & Carson 2015). Initial leaf
116 colonists are likely to maintain their dominance in relation to future colonists, resulting in an
117 effective host defence against pathogens and invertebrate herbivores (Herre et al. 2007; Van
118 Bael et al. 2018), albeit at a cost to the host plant in terms of nitrogen metabolism and
119 photosynthetic rate (Mejia et al. 2014, Christian et al. 2019).

120 Foliar endophytes have been found in all sampled plant hosts (U'Ren et al. 2012), with
121 over 8,000 species of fungi, and 7,000 species of bacteria described as occurring only within
122 leaves according to a recent survey (Griffin & Carson 2018). Tropical regions have shown to
123 support much higher levels of endophyte diversity than temperate regions (Arnold et al. 2000,
124 Arnold & Engelbrecht 2007, Arnold & Lutzoni 2007), although the factors responsible for
125 generating such high levels of diversity remain elusive, as well as the mechanisms that
126 structure communities within leaves. Studies of bacterial endophytes are for the most part
127 limited to their occurrence in agricultural crops or trees in temperate regions (Griffin &
128 Carson 2015, Miyambo et al. 2016, Carrell & Carper 2016). The roles of host tree species
129 (Lodge 1997, Arnold & Herre 2003), dispersal among endophyte communities (Cordier et al.
130 2012, Izuno et al. 2016), and abiotic conditions (Suryanarayanan et al. 2002, 2003,
131 Zimmerman & Vitousek 2012) have all been explored. In these studies, only part of
132 community variation was explained, with the idea of community composition as shaped by
133 neutral processes largely ignored. Indeed, these studies have focused on leaf samples
134 collected from the forest understory, potentially masking the greater levels of wind-driven
135 dispersal in the upper canopy (Gilbert & Reynolds 2005), and restricting the sampling to
136 leaves that contribute little to the overall net primary production of tropical forest ecosystems.
137 Furthermore, the majority of previous studies have relied on techniques which represent
138 endophyte communities based on fungi and bacteria detected following tissue culturing,
139 although it is known that many species do not grow easily on standard culture media (Hyde &
140 Soyong 2008). Environmental DNA (eDNA) sequencing potentially captures a greater
141 breadth of the diversity associated with environmental samples by identifying of observable
142 taxonomic units (OTUs) as proxies for species, and thus is a promising technique for the
143 exploration of endophyte diversity within leaf samples (e.g. Vincent et al. 2016, Del Olmo-
144 Ruiz & Arnold 2017, Haruna et al. 2017).

145 This study aims to elucidate the factors structuring ecological communities, by
146 characterising the diversity of upper-canopy fungal and bacterial endophytes in a primary
147 rainforest, and shifts in their community composition across phylogenetic and geographical
148 gradients. Because endophytes are restricted to leaf habitats, this system represents an ideal
149 setup to test community ecology theory. Our study makes use of an innovative canopy-access
150 system to sample upper crown leaves from multiple host trees across a fully inventoried 4-ha
151 permanent tree plot. If foliar endophyte communities are structured to a greater extent by their
152 niche, then we would expect to see a strong correlation between the composition of endophyte

153 communities and their host plants. This is to be expected if closely allied host species are
154 more similar with respect to their foliar functional traits than unrelated species (i.e. the traits
155 are phylogenetically conserved as shown by Baraloto et al. (2012) and Courtois et al. (2016)).
156 If dispersal limitation is important in structuring communities, then we would expect to see
157 proximal communities within the canopy following an isolation-by-distance pattern, from
158 leaves sharing the same branch, to those associated with separate tree crowns across the study
159 plot.

160 We hypothesize that at the scale of the 4-ha plot, canopy endophyte assemblages will
161 display patterns of neutral structuration as a result of dispersal limitation. However, when
162 controlling for the scale at which communities assemble, we expect that host tree species will
163 determine endophyte colonization success, and that closely allied host species will match this
164 by having more similar communities than distantly related species. Similarly, we expect that
165 within crowns, proximate leaves will be more similar than distant leaves. In particular, we
166 hypothesize that the response of endophyte occurrence within certain host trees will depend
167 on their level of host specificity, detected when focusing on endophyte community responses
168 within specific fungal and bacterial orders.

169 **Materials and Methods**

170 **Sample Site**

171 Data collection took place at the Nouragues Ecological Research Station in French Guiana,
172 within an area of pristine lowland tropical rainforest protected since 1996. This area is
173 characterised by a high level of floral diversity, with over 1700 recorded angiosperm species
174 (Sabatier, 1990, van der Meer and Bongers, 1996). The reserve experiences mid-day
175 temperatures of 26 °C and rainfall of 2861 mm per year (Réjou-Méchain et al. 2015). It
176 experiences two dry seasons, a longer dry season between September and mid-November, and
177 a shorter dry season in March.

178 Fieldwork described here took place in August 2017. Sampling was conducted in a 4-
179 ha permanent sampling plot adjacent to the Pararé research station (4°02' N, 52°41' W)
180 located on the Arataye tributary of the Approuague River. Access to the canopy was made
181 possible by the 'Canopy Operational Permanent Access System' (COPAS), consisting of a
182 trio of 45 m high pylons set up at the vertices of an equilateral triangle with edges 180 m in

183 length, equipped with a mechanically operated harness which allows for the displacement of
184 an individual anywhere within the upper canopy across the research plot (Fig. 1).

185 Sample design and preparation

186 Leaves were collected from the upper crown of 38 trees, of 22 different species, selected to
187 represent a phylogenetically diverse range of species (Fig. 2 & S.I. 1). A stratified sampling
188 design was conducted for each tree: three leaves were collected from each of a minimum of
189 three branches positioned across the crown. For those trees with a large crown, 4 or 5
190 branches were selected accordingly, so that sampling accurately represented the total spread
191 of the crown. In total this resulted in 365 leaves.

192 Leaves were immediately transported to a field laboratory where they were surface
193 sterilised in order to ensure that sampling consisted of only endophytes. This sterilisation
194 included several steps, first mechanical cleaning and scraping with toothbrushes. Secondly,
195 following from the protocol detailed by Arnold et al. (2000), leaves were soaked in diluted
196 bleach (0,525%) for 2 min before transfer into ethanol (70%) for a further 2 min. This method
197 is the standard in eDNA extraction for endophyte research (e.g. Izuno et al. 2016, Haruna et
198 al. 2018), proving efficient in removing the maximum amount of epiphytic fungi and bacteria
199 from the leaf surface. Finally, leaves were dried with tissue paper, and stored in Ziploc bags
200 containing silica gel before transfer to the lab for DNA extraction.

201 DNA Isolation, PCR amplification and sequencing

202 Two 0.5 cm² pieces of each leaf were placed into a 96 well plate, and a 3mm aluminium bead
203 was added to each well. To facilitate the grinding step, the sealed plates were placed in a -80
204 °C freezer for at least an hour before grinding using a Tissue Lyser (Qiagen, Germany) for 45
205 sec at 30Hz, followed by a centrifugation for 30 seconds at 6000 x g. DNA was extracted
206 from samples using a NucleoSpin Plant II (96) kit (Macherey-Nagel, Düren, Germany)
207 according to the manufacturer protocol modifying only cell lysis time to 1 h and elution
208 volume to 100µL.

209 PCRs were performed using two primers; ITS1 nuclear rDNA primers to target fungi
210 (Fwd: ITS5 GGAAGTAAAAGTCGTAACAAGG (Epp et al. 2012) and a modified version
211 of Rev: 5.8S_Fungi CAAGAGATCCGTTGTTGAAAGTK, Taberlet et al. 2018), and 16S
212 rDNA (V5-V6) primers to target bacteria (Bact01 primers - Fwd:

213 GGATTAGATACCCTGGTAGT and Rev: CACGACACGAGCTGACG (Fliegerova et al.
214 2014)). To discriminate samples after sequencing, forward and reverse primers were
215 synthesized with a combination of two-different 8-nucleotide tags per sample, following a
216 double-indexing strategy (Binladen et al. 2007). As such, each PCR was amplified with a
217 unique combination of tagged primers. Each PCR reaction was performed in a total volume of
218 20 μ l and comprised 10 μ l of AmpliTaq GoldMaster Mix (Life Technologies, Carlsbad, CA,
219 USA), 5.84 μ L of nuclease-free water AmbionTM (Invitrogen, Waltham, Massachusetts,
220 USA), 0.25 μ M of each primer, 3.2 μ g of BSA (Roche Diagnostic, Basel, Switzerland), and 2
221 μ l of DNA template. Two PCRs per sample were performed under the following conditions:
222 Polymerase reactivation 10 min at 95°C, followed by 35 (fungi) or 30 (bacteria) cycles of 30
223 sec at 95°C, 30 sec at 55°C (fungi) or 57°C (bacteria) and 1 min at 72°C; followed by a final
224 step of 7 min at 72°C. Two wells in each plate were filled with water, to act as PCR negative
225 controls. Eight wells positioned randomly across each plate were left empty (with no PCR
226 products) to act as sequencing controls (non-used tag combinations).

227 Amplicons were pooled and libraries were prepared with the TruSeqNano PCR free
228 Illumina kit and were sequenced on Paired-end (2X250 bp) in the Illumina Miseq platform
229 (Illumina, San Diego, CA, USA) at the INRA Genotoul-GetPlaGe core facility (Toulouse,
230 France) using the Paired-end MiSeq Reagent Kit V3 (Illumina, San Diego, CA, USA),
231 following the manufacturer's instructions.

232 Sequence data curation

233 The generated reads were subject to a data-curation pipeline using scripts in R (R
234 Development Core Team, 2013), and the OBITools package (Boyer et al. 2016). Firstly,
235 'Illuminapairedend' was used for paired-end read assembly where an exact alignment
236 algorithm assigns a quality score for each nucleotide position, generating a score for each read
237 based on the number of mismatches, followed by 'ngsfilter' which removes primer and tag
238 sequences and assigns reads to samples. Here, reads with any primers with more than two
239 mismatches, and any tags with 1 or more mismatches are removed. 'obiuniq' then
240 dereplicates only those sequences which are strictly identical. 'obigreb' removes sequences of
241 low quality (containing Ns or with a pair alignment score below 50). Finally sequences with
242 only one read across the global dataset (singletons) were removed, since these represent
243 potential sequencing artefacts. 'sumacust' was then used to generate Operational Taxonomic
244 Units (OTUs), where pairwise sequence dissimilarity using the raw number of mismatches

245 was calculated, and where those sequences $\geq 97\%$ or more were assigned to the same OTU, a
246 threshold commonly applied to delimitate proxies for during molecular analysis of fungi in
247 particular (Nilsson et al., 2008; Coissac 2012). For each OTU, the sequence with the highest
248 number of reads in each cluster was assigned as the seed sequence, used for subsequent
249 taxonomic assignment.

250 Taxonomic assignment of OTUs was conducted using reference databases with the
251 'ecotag' function, which uses global alignment of sequences against full-length references.
252 For fungi the reference database was obtained by running an in-silico PCR with the ecoPCR
253 program (Ficetola et al., 2010) on Genbank (release 197; <ftp://ftp.ncbi.nlm.nih.gov/genbank>)
254 using the primer pairs employed here. Taxonomic assignment yielding the highest similarity
255 score was kept, with similarity scores for fungi ranging from 0.5 to 1 with sequences in ncbi.
256 For Bacteria, taxonomic assignment was conducted using the SILVA database (Quast et al.
257 2012), with similarity parameters kept at 0.97, higher than fungi since bacterial sequence
258 databases are much more complete than for fungi (Truong et al., 2017). This approach
259 allowed for the elimination of a certain number of chimeras which are likely to have formed
260 during PCR. In particular, taxonomic identification facilitated later analysis, by distinguishing
261 between orders known to contain OTUs corresponding to endophytic fungi (Xylariales,
262 Capnodiales, Chaetothyriales, Phyllachorales, Diaporthales, Sordariales, Pleosporales,
263 Hypocreales) against orders which are known to contain generalist saprotrophs (Polyporales,
264 Eurotiales, Corticiales). Similarly, bacterial endophyte orders which have strict relationships
265 with plants (e.g. Rhizobiales, Acetobacterales, Sphingomonadales) could be compared with
266 orders known to contain more generalist bacteria (e.g. Solirubrobacteriales,
267 Pseudonocardiales, Acidobacteriales).

268 Although initial processing of sequences using OBITools removes a large number of
269 erroneous sequences introduced to the dataset during sequencing, further processing based on
270 methodological controls can remove additional erroneous sequences and contaminants. OTUs
271 which could represent contaminants arising during extraction and during PCR (present within
272 the PCR negative controls) were removed from the dataset by comparing occurrence in
273 controls with that in samples. Potential contaminants were detected by calculating the mean
274 sum of reads for each OTU in PCR negative controls, and sequencing controls (blanks)
275 divided by the mean sum of reads across both these controls and samples. For each OTU, the
276 acceptable level of OTU abundance present in PCR negative controls was set to be 5% or less

277 than the total number of reads. Above this threshold, an OTU was more likely to be a
278 contaminant, and as such it was excluded. OTUs which only occur in negative controls were
279 also removed. Finally, all sequence data associated with blank controls was removed from the
280 datasets.

281 All OTUs which were identified as non-fungal following taxonomic assignment were
282 removed from the fungal database (S.I. 2). Bacterial sequences which were identified as
283 originating from ribosomes or chloroplasts were removed from the bacterial dataset, since
284 these likely represented plant host genetic material rather than that of bacterial endophytes
285 (S.I. 3). It is noted that future studies of bacterial endophytes should use primers more adapted
286 to reduce this effect (e.g. Beckers et al. 2016). PCR replicates were then pooled. We excluded
287 fungal samples with less than 100 reads from subsequent analysis. These low-quality samples
288 likely result from PCR amplification failure. This filter was chosen as a result of the majority
289 of samples averaging a much higher read count. This resulted in the removal of 14 samples,
290 with subsequent analysis for the fungal communities performed on a dataset of 351 leaves. In
291 comparison, bacterial read counts per sample were dominated by ribosome and chloroplast
292 sequences. Once these were removed, all samples containing a minimum of 1 read were kept
293 in the dataset, resulting in the removal of 8 leaves, with subsequent analysis for the bacterial
294 communities performed on a dataset of 357 leaves.

295 Following clustering of sequence reads with a 97% similarity cut-off, 11,026 OTUs
296 (67,338,264 reads) were obtained for fungi, and 2,859 OTUs for bacteria (12,746,769 reads).
297 Removing OTUs occurring disproportionately in the negative controls and blank PCR wells
298 brought the number of OTUs down to 10,303 for fungi, and to 2,438 for bacteria. Removal of
299 non-fungal OTUs brought the total number of OTUs down to 10,160 (S.I. 2), and 1,953 OTUs
300 for bacteria (S.I. 3). Subsequent analysis was performed on these reduced datasets.

301 Statistical analysis

302 In order to establish the relative effects of host tree niche creation and dispersal limitation,
303 leaf sample endophyte diversity was classified and community composition at multiple spatial
304 and phylogenetic scales was determined. All statistical analysis was performed using Rstudio
305 version 1.1.442 (R Development Core Team, 2013).

306 To quantify endophyte diversity, rarefaction curves for each PCR based on the
307 number of reads were compiled using the ‘rarefy’ function in the vegan package (Oksanen et
308 al. 2013). These curves demonstrated whether sequencing depth was sufficient in representing
309 the diversity of selected host tree endophyte diversity, defined by whether curves reached
310 asymptote. Species accumulation curves were also prepared using the ‘specaccum’ function,
311 which determine whether the number of leaves sampled for each species was sufficient in
312 capturing endophyte diversity. Subsequent analysis was conducted on a standardised dataset,
313 whereby the observed dataset was resampled so as to have the same relative abundance of
314 OTUs, but for read count per sample to be equal, using the first quantile of the dataset as the
315 new total read count per sample. Endophyte alpha diversity for each of the datasets (351
316 leaves for fungi, 357 for bacteria) was characterized by calculating a Shannon Index for each
317 sample, selected to neither over nor under represent rare OTUs. This was converted to
318 effective number of species, to reflect the number of equally-common species required to give
319 a particular value (Jost 2006).

320 The effect of host tree species in influencing endophyte alpha diversity was
321 determined using ANOVA, with Tukey Post Hoc tests revealing which species-by-species
322 comparisons were significant. To further understand within communities which OTU groups
323 differed significantly from one host tree species to the next, data were converted to presence
324 absence, and aggregated at the order level, chosen since taxonomic assignment for fungi in
325 particular was best at this level, and to allow for enough replication to statistically test for
326 patterns of variation, using ANOVA.

327 To determine host species effects on community composition and beta diversity,
328 variability in endophyte community composition was examined across all 351 leaf samples.
329 Bray-Curtis similarity measures were used to construct a community distance matrix based on
330 the observed endophyte communities within each sample. The variance in distance between
331 groups of interest was compared with a nonparametric null distribution based on 999
332 permutations of the observed data. The effect of host tree species on endophyte community
333 composition was tested on the complete dataset ($n = 351$ for fungi, 357 for bacteria). The
334 effect of individual host tree, and branch within each tree on endophyte community
335 composition was tested on a reduced similarity matrix composed of samples collected from
336 only 3 branches per tree ($n = 327$ for fungi, 331 for bacteria). These were evaluated with
337 permutational multivariate analysis of variance (PERMANOVA; Anderson 2013), using the
338 ‘adonis’ function in the vegan package (Oksanen et al. 2013). To visualise the variation

339 explained by host tree individual, host tree species, and branch identity, partitioning of
340 variance was conducted using the 'varpart' function in vegan.

341 To explore whether observed variance was common across all fungal endophytes, or
342 whether it differed across OTUs within different orders PERMANOVA were performed on
343 two subsets of the data; one containing only OTUs identified as belonging to the order
344 Xylariales (well documented endophytes, see Rodriguez et al., 2009), the other containing
345 OTUs identified to order Polyporales (order known to contain pathogens). Similarly, for
346 bacteria, analysis was rerun on a subset of the data, comprising those OTUs identified to the
347 order Rhizobiales (an order known to contain plant associated bacteria) and another data set
348 comprised of OTUs identified to the order Solirubrobacterales (an order known to contain
349 generalist bacteria).

350 To determine the influence of phylogenetic and geographic distance of the host tree
351 species on the endophytic communities, OTU reads for each sample from the same tree were
352 summed, reducing the full datasets for both fungi and bacteria down to 38 samples. DNA read
353 counts were normalised across all trees so as to have a balanced dataset. Bray-Curtis
354 similarity of each tree endophyte community was determined and compiled in a community
355 similarity matrix. A phylogenetic tree representing all of the species sampled here was
356 prepared using the ape package in R (Fig. 3; Paradis et al. 2004), by pruning a species-level
357 phylogenetic hypothesis published elsewhere (Baraloto et al. 2012). Terminal nodes
358 representing members of the same species, i.e. where more than one tree was sampled, were
359 included as polytomies with an intraspecific edge distance of 0.5 between all, so as to be very
360 low in comparison to the interspecific edge distances elsewhere in the phylogeny. The
361 resulting edge distances were then used to prepare a phylogenetic distance matrix.

362 A second distance matrix was prepared comparing the geographic distance between
363 each of the 38 trees across the 4-ha sampling plot in metres. Both matrices were directly
364 compared with the endophyte community similarity matrices by performing partial Mantel
365 tests based on Pearson's product-moment correlation, using the function mantel.partial in the
366 ecodist package (Goslee & Urban 2007). In each case, the effect of one distance metric was
367 controlled for when determining the effect of the other.

368 In order to determine whether the presence of OTUs within the leaves of a host tree
369 represented true host specificity, and to avoid issues associated with low sample size (e.g.

370 Cordier et al. 2012, Novotny et al. 2002), a metric was calculated to determine the probability
371 that OTU presence within a single host species occurred by chance. Using the bipartite
372 package (Dormann et al. 2008), the degree of each OTU (i.e. the number of leaf samples
373 within which they occur) was calculated. Host tree name was replaced by a numerical value
374 (1-22 for each sampled host), before a random sample of host tree for each of these degrees
375 was determined. The standard deviation of the randomly generated host tree numbers was
376 then calculated. Each time this equalled zero (i.e. the degrees were sampled as the same
377 species), a count of 1 was assigned. This process was reiterated 1000 times, with the count
378 tally of iterations divided by 1000. The calculated metric thus ranged from 0-1, with 0
379 representing no chance that an OTU occurred in sampled host by chance, and 1 representing
380 certainty that OTUs occurred in sampled host by chance. For OTUs with a degree of 1, such
381 a calculation was not possible. To determine the importance of rare OTUs in generating
382 patterns detected during analysis, the percentage of all OTUs only occurring in each host tree
383 was calculated.

384 **Results**

385 Endophyte α -diversity

386 Average OTU richness per leaf was calculated to be 168 for fungi, ranging from 15 to 1018,
387 and 46.3 for bacteria, ranging from 1 to 504. Fungal OTU identification rate was 45%, 36%,
388 and 19% at the order, family, and genus level, respectively. Bacterial OTU identification was
389 98%, 88%, and 59% at the order, family, and genus level, respectively. OTU accumulation
390 curves indicated that sampling was representative of the total endophyte community for fungi,
391 but to a lesser extent for bacteria (S.I.4). Sequencing depth varied across samples (see S.I. 5),
392 justifying standardisation approaches when calculating alpha diversity metrics.

393 Per-leaf endophyte diversity was dependent on the host tree with above average
394 numbers of OTUs only occurring in specific host trees (Fig. 2). Significant differences in host
395 tree Shannon diversity indices were detected for both fungi (ANOVA $F_{21,335} = 4.42$, $p =$
396 <0.001), and to a lesser extent for bacteria (ANOVA $F_{21,336} = 1.82$ $p = 0.016$). Tukey HSD
397 revealed that leaves collected from *Carapa surinamensis*, *Dussia discolor*, *Inga huberi* and
398 *Virola kwatae* had significantly higher diversity indices than those collected from other trees
399 for fungi, whilst for bacteria, only *Virola kwatae* was found to be significantly higher in

400 diversity than *Eperua rubiginosa*, with all other host species not differing significantly in their
401 bacterial diversity (Fig. 2).

402 The relative occurrence of certain fungal orders (using OTU occurrence aggregated
403 for each order) differed significantly from one host tree to the next (Fig. 3; S.I. 6).

404 Host-tree effects on β -diversity

405 When examining the effects of host tree identity on leaf endophyte community composition,
406 PERMANOVA revealed the significant contribution of both species (Fungi: $F = 2.65$, $p =$
407 0.001 ; Bacteria: $F = 1.97$, $p = 0.001$) and nested individual trees (Fungi: $F = 1.58$, $p = 0.001$;
408 Bacteria: $F = 1.3$, $p = 0.001$) in contributing to variation (Table 1).

409 In addition to the importance of individual host tree (Fungi: $F = 2.25$, $p = 0.001$;
410 Bacteria: $F = 1.70$, $p = 0.001$), PERMANOVA also demonstrated a significant effect of
411 nested branch (Fungi: $F = 1.08$, $p = 0.001$; Bacteria: $F = 1.12$, $p = 0.001$) in determining
412 endophyte community composition (Table 2). Although host tree is a significant contributor,
413 it only explains a part of the variation amongst communities, as revealed by the associated R^2
414 values (Table 1). Partitioning of variance demonstrated the cumulative effects of species, host
415 individual and branch only accounted for 12% of variation for fungi, and 10% of variation for
416 bacteria (Fig. 4)

417 Endophyte order-specific effects on β -diversity

418 When determining the effects of host tree species on specific orders of fungi, PERMANOVA
419 demonstrated a significant effect for Xylariales ($F = 1.78$, $p = 0.001$; Table 1) but not for
420 Polyporales ($F = 1.01$, $p = 0.434$; Table 1). When determining the effects of host tree species
421 on specific orders of bacteria, PERMANOVA demonstrated a significant effect for both
422 Rhizobiales ($F = 1.92$, $p = 0.001$; Table 1) and Solirubrobacterales ($F = 1.5$, $p = 0.007$; Table
423 1).

424 Phylogenetic and geographic effects on β -diversity

425 Mantel tests revealed a significant correlation between endophyte community composition
426 and phylogenetic distance of the host tree species for both fungi ($r = 0.1251$, $p = 0.044$), and
427 bacteria ($r = 0.227$, $p = 0.009$). Both groups displayed no significant correlation between tree

428 endophyte community composition and geographic distance (Fungi: $r = 0.091$, $p = 0.064$;
429 Bacteria: $r = -0.062$, $p = 0.783$).

430 OTU host specificity

431 Out of the 10158 detected fungal OTUs, 5542 occurred in only one host tree. Of these, 4982
432 only occurred in one leaf and as such host specificity could not be determined. For the
433 remaining 560 OTUs, when accounting for the probability that their occurrence within a host
434 was determined by chance with a cut off of 0.05, only 81 OTUs demonstrated true host
435 specificity. This usually corresponded to OTUs occurring in 3 or more leaves of host trees of
436 the same species, encompassing a broad taxonomy of fungal species (S. I. 7).

437

438 Out of the 1954 detected bacterial OTUs, 901 occurred in only one host tree. Of these, 737
439 only occurred in one leaf and as such host specificity could not be determined. For the
440 remaining 164 OTUs, when accounting for the probability that their occurrence within a host
441 was determined by chance with a cut off of 0.05, only 14 OTUs demonstrated true host
442 specificity. This usually corresponded to OTUs occurring in 3 or more leaves of host trees of
443 the same species, encompassing a broad taxonomy of bacterial species (S. I. 7).

444

445 Conversely, no single OTU occurred across all sampled leaves, and only 31 fungal OTUs and
446 3 bacterial OTUs occurred in more than half of the total leaf number.

447

448 **Discussion**

449 Tropical foliar endophytes represent an ideal model system to explore the factors shaping
450 ecological communities. The high levels of both α - and β -diversity found in this study reveal
451 the relative importance of host tree contributions to endophyte niche and dispersal limitation
452 across spatial scales. In spite of acknowledged biases introduced by PCR methods and
453 clustering thresholds (Dickie 2010, Lindner & Banik 2011), and a potentially low level of
454 bacteria detection as a result of a primer which also amplified chloroplast and mitochondrial
455 sequences, eDNA methods used here reveal levels of endophyte diversity an order of
456 magnitude higher than culture-based methods, with OTU taxonomic identification and
457 relative abundance consistent with previous studies of tropical canopy microbial communities
458 (e.g. Izuno et al. 2016, Haruna et al. 2018).

459 Differences in endophyte diversity and community composition were detected at the
460 host tree level for fungi. Given this trend was consistent with tree phylogenetic relatedness, it
461 is likely that endophyte colonization success is dependent on shifts in leaf traits across host
462 tree species, such as cuticle thickness or tannin levels (Arnold and Herre 2003). Carapa
463 surinamensis, Dussia discolor, Inga huberi and Virola kwatae trees displayed higher levels of
464 fungal endophyte diversity than other species present under similar conditions, and given the
465 known role of certain tropical endophytes as symbionts (e.g. Arnold et al. 2003; Bae et al.
466 2009; Van Bael et al. 2009; Christian et al. 2019), such variation in the endophytes present
467 could have functional consequences on host plant health for canopy leaves.

468 When observing the diversity of endophytes associated with host tree species at the
469 order level, clear differences in the relative abundance of certain groups are apparent. Certain
470 orders of endophytes differed significantly across different host species (e.g. Xylariales, for
471 fungi; Rhizobiales for bacteria), whilst orders characterized by OTUs known to occur across a
472 wide range of habitat types (e.g. Polyporales for fungi; Solirubrobacterales for bacteria) did
473 not differ significantly in their occurrence among host tree species. Such a finding mirrors
474 that of Peay et al. (2013), which showed that soil fungal communities with lineages
475 containing pathogens and mycorrhizal fungi correlated with tree species richness of tropical
476 forest plots, whilst saprotrophs did not. Nevertheless, at the 4-ha plot scale, although
477 significant, host tree only explained a small proportion of the variation in endophyte
478 community composition, suggesting the contribution of other factors.

479 Limitations in dispersal ability are known to have consequences in structuring spore
480 dependent ecological communities (Peay et al. 2012). Here, distance between leaves was
481 found to result in significant differences in endophyte community assembly, although this was
482 scale dependent. When aggregating samples at the host tree individual level, no significant
483 correlation was found between tree endophyte community composition and their proximity to
484 one another within the 4-ha plot. At the scale of a single tree crown however, leaves
485 positioned on the same branch contained endophyte communities more similar to one another
486 than those found on an adjacent branch. This result could reflect differences in the age of
487 leaves, given that leaves occurring on the same branch will be more similar in age, with
488 consequences for leaf chemistry and community establishment. The result could also suggest
489 an effect of dispersal limitation in determining endophyte community composition within a
490 tropical forest upper canopy, albeit only detectable at scales of less than 10 m. Beyond this, in
491 absence of the physical limitations to dispersal such as in the understory (e.g. Arnold et al.

492 2003, Izuno et al. 2016), of a strong environmental gradients (e.g. Suryanarayanan et al. 2002,
493 Zimmerman & Vitousek 2012), and because of high rates of spore dispersal (e.g. Peay et al.
494 2012) tropical forest canopies are likely to display communities characterised by high turn-
495 over. This contrasts with the findings of low species turnover at both local (10-100m) and
496 regional scales (100km) in a tropical forest understory for fungal endophytes (Vincent et al.
497 2016). Here, authors used culturing to detect fungal OTUs and detected high numbers of
498 generalist OTUs across their sampled host trees and sites. In comparison, our use of
499 metabarcoding fails to detect high numbers of generalists, and instead reveals the importance
500 of large numbers of rare OTUs.

501 Partitioning of the variance in endophyte community composition revealed that at the
502 canopy scale, only a small part of total variation in endophyte assemblage could be explained
503 by the summed effects of branch, host tree, and host species (12% for fungi, 10% for
504 bacteria). Thus, although significant, each of these factors are limited in explaining the high
505 levels of variation in the system. Instead, leaf endophyte assemblages are largely
506 characterized by rare OTUs which occur sporadically within samples, the sum of which
507 represents a large part of the total diversity within the canopy. Indeed, when accounting for
508 the probability of chance in determining the presence of an OTU within a host tree, for both
509 fungi and bacteria only 0.7% of detected OTUs could truly be considered host specific.
510 Conversely, no OTUs were so generalist that they occurred across all samples, and only a
511 small proportion occurred in more than half of all sampled leaves. In spite of the relatively
512 small sample size here, this finding counters the idea of a 'core microbiome' for endophytes
513 (e.g. Kembel et al. 2014), at least in the context of this study. Thus, the detected variation
514 across host species is likely to be driven by the differences in the relative abundance of a
515 small number of generalist OTUs and the numerous rare OTUs.

516 Due to their presence in well-defined sampling units, foliar endophytes represent an
517 ideal example of community assembly within which to test assembly rules for betadiversity in
518 tropical habitats. Communities associated with leaves collected in the upper canopy are
519 known to be distinct from those found at lower levels (Izuno et al. 2016), but the findings
520 presented here demonstrate the scale dependant factors contributing to their composition.
521 When carefully controlling for community scale, and sampling intensity, community
522 assembly becomes clearly non-neutral in leaf-associated fungi and bacteria, but not in
523 opportunistic species which may tend to be rare and found across a range of samples
524 (Magurran & Henderson 2003). Given the emerging knowledge surrounding their role as

525 symbionts, the community structure of endophytes within the canopy layer is likely to play an
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539 preparation; JD, MR and JC performed data analysis.

540 **Data Archive** – Data available from the Dryad Digital Repository:

541 <https://doi.org/10.5061/dryad.XXXXXXX>

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Table 1: PERMANOVA analysis to determine the effect of host tree species and nested host tree individual in determining fungal and bacterial endophyte community composition. Analysis was performed on community dissimilarity matrixes constructed from six separate datasets; one containing all fungal OTUs detected in the analysis of 351 leaves; one containing only OTUs identified as Xylariales; one containing only OTUs identified as Polyporales; one containing all bacterial OTUs detected in the analysis of 358 leaves; one containing only OTUs identified as Rhizobiales and one containing only OTUs identified as Solirubrobacterales.

| | Fungi | 351 leaf data | | | | Xylariales data | | | | Polyporales data | | | | Bacteria | 358 leaf data | | | | Rhizobiales data | | | | Solirubrobacterales | | | |
|-----------|-------|---------------|------|------|-------|-----------------|------|------|-------|------------------|------|------|-------|----------|---------------|------|------|-------|------------------|------|------|-------|---------------------|------|------|-------|
| | | df | F | R2 | p | df | F | R2 | p | df | F | R2 | p | | df | F | R2 | p | df | F | R2 | p | df | F | R2 | p |
| Sp | | 21 | 2.65 | 0.14 | 0.001 | 21 | 1.78 | 0.11 | 0.001 | 21 | 1.01 | 0.12 | 0.434 | | 21 | 1.97 | 0.11 | 0.001 | 21 | 1.92 | 0.18 | 0.001 | 15 | 1.5 | 0.35 | 0.007 |
| Sp: Tree | | 16 | 1.58 | 0.06 | 0.001 | 16 | 1.32 | 0.06 | 0.001 | 16 | 0.91 | 0.09 | 0.855 | | 16 | 1.3 | 0.05 | 0.001 | 15 | 1.28 | 0.09 | 0.001 | 9 | 1.09 | 0.15 | 0.336 |
| Residuals | | 313 | | 0.79 | | 287 | | 0.83 | | 134 | | 0.79 | | | 320 | | 0.83 | | 166 | | 0.73 | | 31 | | 0.5 | |
| Total | | 350 | | 1 | | 324 | | 1 | | 171 | | 1 | | | 357 | | 1 | | 202 | | 1 | | 55 | | 1 | |

Table 2: PERMANOVA analysis to determine the effect of host tree individual and nested branch in determining endophyte community composition. Analysis was performed on community dissimilarity matrixes constructed from a dataset comprised of 327 leaves for fungi, 331 for bacteria.

| | Fungi | | | | Bacteria | | | |
|--------------|-------|------|------|------|----------|------|------|------|
| | df | F | R2 | p | df | F | R2 | p |
| Tree | 37 | 2.25 | 0.22 | .001 | 37 | 1.70 | 0.17 | .001 |
| Tree: Branch | 75 | 1.08 | 0.21 | .001 | 75 | 1.12 | 0.23 | .001 |
| Residuals | 214 | | 0.57 | | 218 | | 0.60 | |
| Total | 326 | | 1 | | 330 | | 1 | |

Figure 1: The Canopy Operational Permanent Access (COPAS) system, allowing access for unrestricted access to the upper canopy. This was used to sample leaves from which endophyte communities were extracted.



Figure 2: The effective Shannon diversity index of each sampled tree species, based on the of OTUs within each leaf sample. The fungal dataset is based on 351 leaves and the bacterial dataset is based on 357 leaves. Trees are arranged by phylogenetic relatedness.

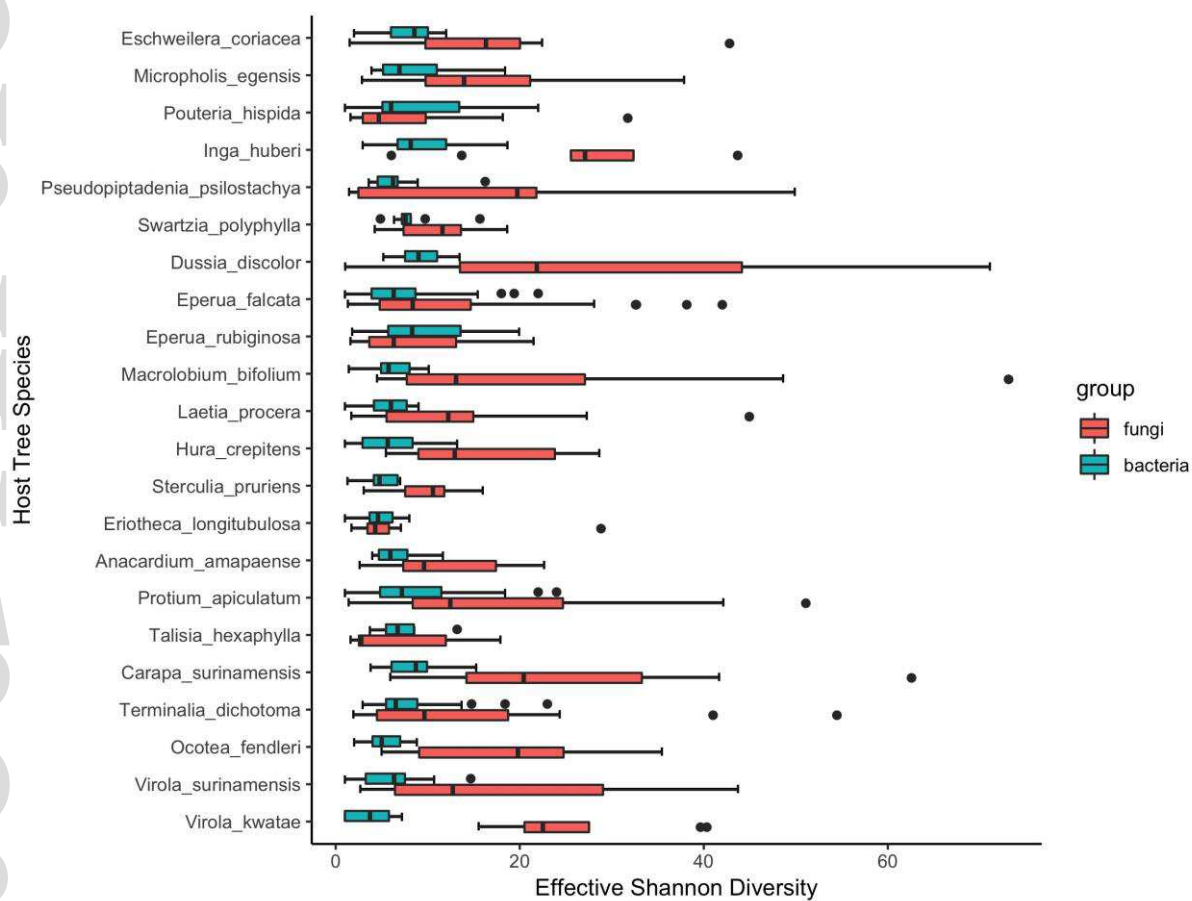


Figure 3: The relative occurrence of identified fungal OTUs in host trees when classed by order for a) fungi and b) bacteria. Non-identified OTUs are not included here, but represent on average 50% of all detected fungal OTUs for each sample. Host tree species are arranged by order of phylogenetic relatedness, modified from Baraloto et al. (2012). Orders are arranged in order to represent their decreasing relative occurrence from left to right, indicated by a shift from red to yellow.

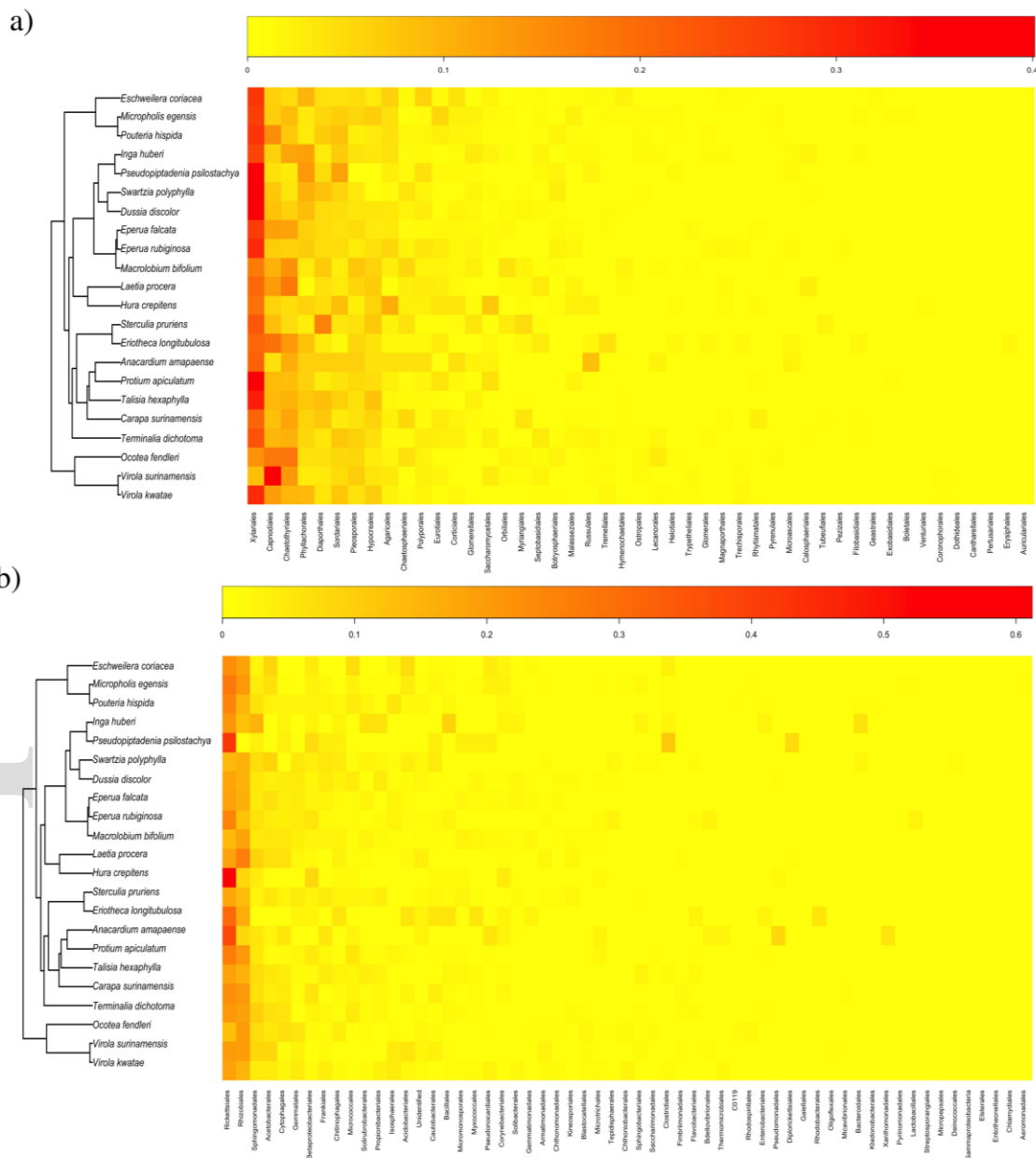


Figure 4: Partitioning of variance of endophyte community composition of leaves explained by host individual, host species and host branch for a) fungi and b) bacteria. Residuals indicate the amount of total variation unexplained by these factors.

