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Environmental filtering structures fungal endophyte communities in tree bark

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1 **Abstract:** The factors that control the assembly and composition of endophyte communities
2 across plant hosts remains poorly understood. This is especially true for endophyte communities
3 inhabiting inner tree bark, one of the least studied components of the plant microbiome. Here,
4 we test the hypothesis that bark of different tree species acts as an environmental filter
5 structuring endophyte communities, as well as the alternative hypothesis, that bark acts as a
6 passive reservoir that accumulates a diverse assemblage of spores and latent fungal life stages.
7 We develop means of extracting high-quality DNA from surface sterilized tree bark to compile
8 the first culture-independent study of inner bark fungal communities. We sampled a total of 120
9 trees, spanning five dominant overstory species across multiple sites in a mixed temperate
10 hardwood forest. We find that each of the five-tree species harbor unique assemblages of inner
11 bark fungi and that angiosperm and gymnosperm hosts harbor significantly different fungal
12 communities. Chemical components of tree bark (pH, total phenolic content) structure some of
13 the differences detected among fungal communities residing in particular tree species. Inner
14 bark fungal communities were highly diverse (mean of 117-171 OTU per tree) and dominated
15 by a range of Ascomycete fungi living asymptomatically as putative endophytes. Together, our
16 evidence supports the hypothesis that tree bark acts as an environmental filter structuring inner
17 bark fungal communities. The role of these potentially ubiquitous and plant specific fungal
18 communities remains uncertain and merits further study.

19 **Introduction:**

20 Fungal endophytes comprise a critical and ubiquitous component of the plant microbiome,
21 forming cryptic asymptomatic infections in virtually all above and below-ground plant tissues
22 (Rodriguez *et al.,* 2009). Some endophytic fungi may act as mutualists under certain conditions
23 (Christian *et al.,* 2019) by producing a wide range of bioactive compounds, including plant
24 hormones and herbivory deterrents (Strobel & Daisy 2003; Arnold *et al.,* 2003; Porras-Alfaro &
25 Bayman 2011). Paradoxically, other fungi frequently isolated from asymptomatic plant tissue
26 are placed in taxonomic groups typically associated with saprotrophs or plant pathogens, leading
27 to the suggestion that some fungi recovered living as endophytes also occur as a range of other
28 life-styles (Carroll 1988; Lofgren *et al.,* 2018; Selosse *et al.,* 2018).

29 Endophytic fungi dwelling in inner tree bark have been reported for several decades
30 (Webber 1981; Griffith & Boddy 1990); however, despite significant advances in the study of
31 foliar, root, flower and wood endophyte communities, tree bark represents an especially

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32 unexplored component of the plant microbiome (Schulz & Boyle 2005; Rodriguez *et al.,* 2009;
33 Vandenkoornhuyse *et al.,* 2015). Few studies have attempted to characterize the potentially
34 diverse fungal communities that inhabit inner bark (Griffith & Boddy 1990) and the turnover of
35 these communities among plant species and geographic regions is poorly understood (Kowalski
36 & Kehr 1992). Instead, culture-based methods have been used to isolate a subsample of inner
37 bark fungi, primarily focusing on ecologically rare, but medicinally important plant hosts
38 (Griffiith & Boddy 1990; Stierle *et al.,* 1993; Verma *et al.,* 2007; Zhou *et al.,* 2010). The
39 paucity of studies to date may be due to the technical challenge of extracting high-quality DNA
40 from tree bark suitable for PCR amplification (Langrell 2005).

41 Fungal communities in tree bark may hold underappreciated ecological significance, as
42 they inhabit one of the largest plant surfaces by area (Evert 2006) and reside in plant tissue that
43 is frequently the site of devastating insect and associated pathogen attack (*i.e.,* western bark
44 beetle, chestnut blight and Dutch elm disease). For example, some endophytic fungi dwelling in
45 bark can protect trees against Dutch elm disease (Webber 1981), possibly by producing a range
46 of bioactive compounds (Kusari *et al.,* 2012). The structural complexity of tree bark,
47 encompassing the primary phloem, cortex, epidermis and rytiderm (Srivastava 1964), may host
48 relatively diverse communities and potentially unknown fungal taxa (Kowalski & Kehr 1992).
49 A large component of novel diversity within the kingdom Fungi is likely to reside within
50 endophytic communities (Arnold *et al.,* 2000; Rodriguez *et al.,* 2009), however, no study to date
51 has described the diversity or composition of inner bark fungal communities across tree hosts
52 and geographic locales using culture-independent techniques.

53 Host species identity and site characteristics are known to play an important role in the
54 structure of some fungal endophyte communities (Arnold *et al.,* 2000; Hoffman & Arnold 2008;
55 Zimmerman & Vitousek 2012; U'ren *et al.,* 2016; Coleman-Derr *et al.,* 2016; Glynou *et al.,*
56 2018). For example, phylogenetically similar plant species can host more similar communities
57 of foliar endophytes (Arnold *et al.,* 2000; Arnold *et al.,* 2001); nonetheless, the ubiquity of host
58 specificity for endophytic communities colonizing plant tissues other than foliage is very poorly
59 understood (Rodriguez *et al.,* 2009), thereby limiting our understanding of the general processes
60 that structure the assembly of endophytic communities across plant tissues.

61 Large inter and intra-species variation in the chemical and physical attributes of tree bark
62 (Srivastava 1964) suggest that tree species and associated bark chemistry may be a controlling

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63 factor in community membership (Griffith & Boddy 1990: Kowalski & Kehr 1992). Bioactive
64 extracts from tree bark, such as tannins, suberins and alkaloids can vary dramatically across tree
65 species (Srivastava 1964; Verma *et al.,* 2007; Alfredsen *et al.,* 2008). If fungi are metabolically
66 active in inner bark, these compounds could differentially impact community membership,
67 consistent with evidence suggesting environmental filtering (*sensu* Kraft *et al.,* 2015).
68 Alternatively, tree bark could simply physically entrap airborne spores or other latent fungal
69 life-stages (Rodriguez *et al.,* 2009), thereby acting as a passive reservoir for fungi that constitute
70 a range of life-styles (*i.e.,* ectomycorrhizal fungi, saprotrophs, pathogens). This alternative
71 hypothesis is plausible, given that seasonal expansion and contraction of metabolically inactive
72 bark provides an avenue for fungal spores and other latent life stages to become lodged and
73 entombed within the numerous crevices.

74 This work explores several fundamental questions concerning the community
75 composition and diversity of inner bark fungi spanning a range of common temperate tree
76 species. Foremost, we seek to determine the identity and diversity of fungi inhabiting these
77 tissues. Additionally, we test the hypothesis that tree bark acts as an environmental filter
78 structuring the community membership of inner bark fungi. Finally, we aim to explore whether
79 chemical variation in tree bark can account for differences in fungal community composition.
80 Support for our hypotheses would include evidence demonstrating that fungal communities are
81 more similar among tree species than geographical sites. We also explore an alternative
82 hypothesis that tree bark functions as a passive and random reservoir for dead or inactive fungal
83 spores and hyphae spanning a range of ecological life-styles. Support for this alternative could
84 include site-specific structuring of fungal communities with minimal differences among tree
85 species within a site. Additional support for our alternative hypothesis would include findings
86 that chemical attributes of bark are not meaningfully correlated with fungal community
87 composition, thereby suggesting that inner bark fungal communities are random assemblages of
88 latent fungal life-stages relatively unaffected by bark chemistry. To accomplish our objectives,
89 we develop a novel and high-throughput means of extracting high-quality fungal DNA to
90 compile the first culture-free survey of fungi inhabiting surface sterilized tree bark.

91 ***Methods:***

92 ***Field Collection of Bark Tissue:***

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93 Three sites were identified in Manistee National Forest, Michigan, USA and sampled in May
94 2016. All sites were dominated by plant communities characteristic of mixed temperate
95 hardwood forests (Zak *et al.,* 1988). Two of the sites were geographically proximal (< 2 km),
96 whereas the third site was more than 8 km from the other two sites (Supplementary Figure 1).
97 At each of the three sites, we collected inner bark from the following tree species: black oak
98 (*Quercus velutina)*, white oak (*Quercus alba*), red pine (*Pinus resinosa*), eastern white pine
99 (*Pinus strobus*) and red maple (*Acer rubrum*). Sampled trees were uniformly distributed as
100 mixed natural assemblages and all 40 trees sampled in each site were found within
101 approximately 150m^2. Diamater at breast height (DBH) for oak and pine species ranged from ~
102 0.5 m to 1.8 m. Individuals of red maple were on average smaller and their DBH ranged from
103 0.2 m to 0.6 m. These species span a wide range of phylogenetic breadth and encompass
104 multiple bark morphologies (Plate S1).
105 Within each of the three sites, 8 individual trees from each of the 5 species were sampled
106 (n = 120). Bark samples were taken at DBH, and a ~10 cm^2 sample on the geographic north and
107 south side of each tree was aseptically removed to the depth of the vascular cambium using a
108 sharp, sterilized knife (Plate S1). Samples collected on either side of each tree stem were
109 composited and immediately deposited into a sterile Whirl Pack Bag and placed on ice. Only
110 free-standing trees with healthy foliage and visually asymptomatic bark were sampled. Absolute
111 depth of bark sample varied depending on tree species. Bark was stored at -80°C in the lab, until
112 it was processed for DNA extraction.
113 ***DNA extraction:***
114 Prior to extraction, samples were surface sterilized to remove epiphytic microorganisms using a
115 standard procedure previously employed for tree bark (Fisher &Petrini 1990; Kowalski & Kehr
116 1992; Verma *et al.,* 2007), as well as other plant tissues (Arnold *et al.,* 2007; Zimmerman &
117 Vitousek 2012). No attempt was made to manually remove lichenized fungi, as doing so would
118 damage the sample. The surface sterilizing protocol included submerging the bark sample in
119 95% ethanol for 5 seconds, 0.5% sodium hypochlorite for 2 minutes, 70% ethanol for 2 minutes,
120 rinsing by submerging the sample in sterile deionized water for 2 minutes, and finally an
121 additional minute in fresh sterile deionized water.
122 The following protocol was found to maximize both DNA quality and yield. To
123 mechanically homogenize samples, a 6.35mm flame-sterilized drill bit was used to excavate

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124 surface-sterilized bark samples perpendicular to the naturally exposed surface. Shavings were
125 collected, pooled and then ground in a sterilized mortar for 30 seconds in liquid nitrogen. Pooled
126 bark shavings from each tree were extracted in triplicate; each extraction required 0.1 g of bark
127 shavings weighed into screw top tubes with three 2-mm stainless steel beads. DNA extraction
128 followed a modified version of the MO-BIO DNeasy Plant Mini Kit protocol. Differences from
129 manufacturer protocol include the described upstream mechanical lysis, as well as modifications
130 described below. Chemical lysis of the samples was achieved using the Qiagen Powerlyzer at
131 2,000 rpms for 60 seconds with lysis buffer solution and the RNase A. Instead of an additional
132 500 µl of Buffer AW2, 500 µl of 95% ethanol was added to the spin column and samples were
133 allowed to incubate for five minutes prior to centrifugation for two minutes at 16.1 x 1000 rcf.
134 The flow-through was discarded, and the spin column was returned to the original tube and
135 centrifuged again for one additional minute at 16.1 x 1000 rcf before transferring to a new 1.5
136 ml microcentrifuge tube. After qualitative assessment of DNA using gel electrophoresis, DNA
137 was stored at -20 °C. Assessment of DNA quality was conducted using a Nanodrop
138 Spectrophotometer (Thermo Fisher, USA) and then purified with a MO-BIO PowerClean Kit
139 using the manufacturer's protocol, with minor alterations to prevent ethanol contamination and
140 to concentrate the three extraction replicates. DNA quality was again re-assessed as described
141 above. The Quant-iT PicoGreen dsDNA Assay Kit (LifeTechnologies, CA, USA) and a BioTek
142 SynergyHT Multi-Detection Microplate Reader (BioTek Instruments, VT, USA), were used to
143 quantify DNA concentrations prior to PCR (median = 7.2 ng/µl, SD = 7.34 ng/µl).

144 **Polymerase Chain Reaction:**

145 The ITS2 region was amplified using Illumina dual-indexed primers 5.8S Fun and ITS4 Fun
146 (Taylor *et al.,* 2016). The forward and reverse primer each contained the appropriate Illumina
147 Nextera adaptor, linker sequence and error correcting Golay barcode for use with the Illumina
148 MiSeq platform. All PCR reactions were performed in triplicate following Taylor *et al.* (2016),
149 using Phusion High Fidelity DNA Polymerase and master mix (New England BioLabs, MA,
150 USA). Samples with high concentrations of DNA were diluted for a target template
151 concentration of 0.78 – 16.5 ng/µl (mean = 8.8 ng/µl). Each PCR reaction contained 6 µL High
152 Fidelity Phusion 5X buffer, 0.75 µL each primer (10 µM initial concentration), 0.42 µL dNTPs
153 (20 mM initial concentration of each dNTP), 1.5 µL of template DNA and 0.23 µL of Taq (2U/
154 µL) brought to a final volume of 20 µL with molecular-grade water. PCR conditions consisted

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155 of an initial denaturation step at 94°C for 3 minutes, followed by 27 cycles of the following: 30s
156 at 94°C, 45s at 57°C, and 90s at 72°C followed by a final extension step of 72 °C for 10
157 minutes. PCR amplification was successful for 118/120 of all tree samples. Replicate PCR
158 products were pooled and equimolar concentrations of DNA were sequenced at The University
159 of Michigan, Microbial Systems Molecular Biology Laboratory using a full run of Illumina
160 MiSeq (2 x 250bp); PhiX oligonucleotides were spiked for base diversity.

161 **Bioinformatic Analyses:**

162 All sequence processing was performed using QIIME 1.9.1. A total of 18,051,236 raw reads
163 were demultiplexed and assigned to unique samples. Reads were then joined using the
164 *multiple_join_paired_ends.py* script using a conservative 100 bp requirement of sequence
165 overlap (settings –j 100, -p 25). Reads that were unable to be joined were discarded, while
166 joined reads were then subjected to strict quality filtering (-q 19 -p 0.75 -r 3; *sensu* Taylor *et al.,*
167 2016). Sequences that were not assigned to Fungi or samples with less than 1000 sequences
168 were removed, resulting in one red pine sample being discarded from our analyses. Average
169 joined read length was 321 bp, with a total of 4,442,861 sequences. Reads were clustered to
170 operational taxonomic units (OTUs), using the reference based USEARCH (v6; Edgar *et al.,*
171 2013) algorithm with 97% sequence similarity; chimera detection was also performed
172 simultaneously with reference-based detection. Taxonomic identity was assigned using the top
173 BLAST match with the *assign_taxonomy.py* function in QIIME, using the UNITE fungal
174 reference set (Kõljalg *et al.* 2013). After processing, there was a median of 3.617×10^5
175 sequences per sample (SD $=1.476 \times 10^4$). Rarefying was conducted using the
176 *single_rarefaction.py* command, with 3900 sequences (Figure S2-3). OTUs that appeared less
177 than twice across all samples were removed, and the resulting BIOM file was used for all
178 subsequent statistical analyses (McDonald *et al.,* 2012). 25 OTUs comprised 61% of unknown
179 sequences and these OTU were manually assigned using BLAST; manually assigned OTU were
180 not amended to the dataset as identified taxa (Supplementary Table 4).

181 **Chemical Characteristics of Bark:**

182 Chemical characteristics were measured on bark samples that were not subjected to surface
183 sterilization. Samples were ground and homogenized using a grinder (Krups, USA). Carbon and
184 N concentrations were determined on a subset of ground samples using a LECO TrueMac CN-

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185 analyzer. Bark pH was measured by mixing 1g of ground material with 100 mL of deionized
186 water; after 1 hr, samples were filtered, and pH was measured with a glass electrode. Finally,
187 total phenolic content (TPC) of the bark was determined using a procedure described by
188 Ainsworth & Gillespie (2007). The assay employs the Folin-Ciocalteu reagent, which
189 transforms in color upon accepting electrons from phenolic moieties, making it a general
190 measure of TPC and other oxidizing substrates. Briefly, 22.5 mg ± 2 mg of ground bark sample
191 was extracted in 80% v/v methanol by vortexing at maximum power for 20 minutes. Samples
192 were then combined with Folin-Ciocalteu reagent and alkalized with 10% filtered sodium
193 carbonate solution. Samples were analyzed in technical triplicates using a microplate reader at
194 765 nm (Bio-Tek, USA) with tannic acid as a standard (Ainsworth & Gillespie, 2007).
195 **Statistical Analysis:**
196 Diversity measures (Chao1, inverse simpson) were calculated and compared using two-way
197 ANOVA, with site (n = 3) and tree species (n = 5) as fixed factors. Bray-Curtis dissimilarity
198 matrices were generated and principle coordinate analysis (PCoA) plots were used to visualize
199 differences in community composition. To exclusively study fungi inhabiting inner bark,
200 lichenized genera were removed by culling OTU that matched to a database of lichenized fungal
201 genera (Lücking *et al.,* 2016). We then tested for differences in fungal community composition
202 between samples using site and tree species as factors using permutational analysis of variance
203 (PERMANOVA) with Bray-Curtis distance matrices; this analysis calculates a test statistic by
204 comparing dissimilarities between interclass and intraclass objects (Paliy & Shankar 2016),
205 implemented using *vegan v. 2.5-4* (Oskanen *et al.,* 2007). To test if centroid location drove
206 significant differences among sites and species, a multivariate test of homogeneity of group
207 differences was conducted using the BETADISPER command in vegan, followed with Tukey's
208 HSD test of pairwise differences between group means. These analyses were repeated after
209 collapsing OTUs into fungal orders and when OTU that could not be assigned at the level of
210 fungal order were removed from the dataset. Differences between fungal communities
211 inhabiting angiosperm and gymnosperms hosts were also compared using PERMANOVA and
212 BETADISPER tests as above. Average linkage hierarchical clustering, using unweighted
213 arithmetic average clustering (UPGMA) was conducted using the *hclust* command after
214 computing Bray-Curtis matrices to further visualize differences (Legendre & Legendre 2012).

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215 Canonical correspondence analysis (CCA) was used to visualize the variation (inertia)
216 explained by the chemical attributes of the bark substrate (*e.g.,* pH, TPC, N), using Hellinger
217 transformed species abundance matrices at both the OTU and order level (Legendre &
218 Gallagher 2001). To further test how the chemical characteristics of bark affected the
219 composition of the fungal community, we fitted generalized linear models (GLMs) with the
220 'manyglm' function in MVABUND v. 4.0.1 (Wang *et al.,* 2017) and performed multivariate
221 analyses of deviance with Hellinger transformed OTU abundances (i.e. ANOVA for models
222 with non-normal error distributions; Warton *et al.,* 2015). GLM's explicitly model the mean-
223 variance relationship of ecological counts, assuming a negative binomial distribution (Warton *et*
224 *al.,* 2015), and can be employed to reveal the strength and significance of the relationship
225 between a predictor and the response variable (Paliy & Shankar 2016). This analysis was carried
226 out after removing lichenized OTU and we also performed this test on all fungal orders
227 comprising more than 1% of all sequences. All chemical parameters (pH, N, TPC) were
228 included in the model without interaction components (Cuellar-Gempeler & Leibold 2019). The
229 effect of predictor variables was quantified using likelihood-ratio tests (ANOVA, pit trap
230 resampling, 999 bootstraps) and Bonferroni correction. We emphasize that the CCA analysis
231 serves as a visualization of the statistically corroborated GLM results (Bálint *et al.,* 2015).
232 Differences in the chemical attributes of trees were compared using two-way ANOVA, with site
233 and species as main effects. Finally, observed fungal diversity was regressed against the
234 chemical attributes of the bark (N, TPC, and pH) and fit with linear or polynomial models. All
235 analyses were conducted in R v. 3.5.3 (R Core Team, 2019).

236 **Results**

237 **Sequencing Yield:**

238 Sequence-based rarefaction curves were nearly asymptotic for most individual samples (Figure
239 S2) and tree species (Figure S3), implying that sequencing depth was largely adequate to
240 capture the diversity of fungi encountered in our samples. In total, 1945 fungal OTUs were
241 identified in the rarefied dataset (clustered at 97% similarity).

242 **Variation among tree species in inner bark fungal communities:**

243 Tree species was a highly significant factor structuring the composition of inner bark fungal
244 communities (lichenized fungi removed; PERMANOVA: Tree species: $F_{4,116} =$ 17.40, *P* =
245 0.0001, $R^2 = 0.37$; Figure 1). Multivariate homogeneity of group dispersion of tree species

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246 effects was not significant (ANOVA: $F_{4,116} = 0.94$, $P = 0.4$), indicating that differences in fungal
247 community dispersion among groups (tree species) was not driving the observed effect of tree
248 species on fungal community composition. Site was also a significant predictor of fungal
249 community composition ($F_{2,116} = 1.64$, $P = 0.018$, $R^2 = 0.032$), and there was a significant site
250 by species interaction on fungal community composition ($F_{8,116} = 1.38$, $P = 0.01$). Interestingly,
251 sites that were geographically closest to one another did not generally have more similar fungal
252 communities (Figure S4). Finally, in accordance with the clustering analysis, there were
253 significant differences between groups, when trees were grouped as angiosperms or
254 gymnosperms and lichenized OTU were removed ($F_{1,116} = 3.23$, $P = 0.0003$).
255 Tree species remained a highly significant factor when fungi were grouped at the level
256 of order, and orders dominated by lichenized fungi were removed (PERMANOVA: $F_{4,116} =$
257 24.68, $P = 0.0001$, $R^2 = 0.47$). We could not, however, confirm that this was not driven by
258 greater within-group (tree species) dispersion of fungal community composition (ANOVA:
259 $F_{4,116} = 7.93$, $P = 0.00001$). Moreover, for fungal orders, no significant differences in fungal
260 community similarity among sites was detected ($P = 0.074$). Finally, when OTU that could not
261 be identified at the level of order were removed, we found qualitatively similar results as for the
262 full dataset (lichenized fungi removed): Tree species: $F_{4,116} = 16.13$, $P = 0.0001$, Site: $F_{2,116}$
263 =1.48, $P = 0.045$; Site x Species: $F_{8,116} = 1.32$, $P = 0.0169$.
264 The chemical characteristics of tree bark varied significantly among tree species
265 (ANOVA: all $P < 0.001$); neither site nor a site by species interaction occurred in our analysis,
266 indicating that bark chemical characteristics were species specific (Figure: S7-S9). Finally,
267 Generalized Linear Models revealed a significant effect of both pH and Total Phenolic Content
268 on fungal community composition (pH: Deviance = 388.9, $P = 0.001$); (TPC: Deviance = 331.8;
269 $P = 0.001$), but not N content ($P > 0.5$). The relative significance of these predictor variables
270 also varied among abundant fungal orders (Table S5).

271 **Fungal Diversity:**

272 White pine hosted the most diverse assemblages of inner bark fungi (mean = 171 OTUs, SE =
273 6.4), whereas red pine (mean = 117 OTUs, SE = 12.4) was the least diverse (Table 1). Two-way
274 ANOVA revealed highly significant differences among plant species in fungal α-diversity for
275 both inverse Simpson ($P = 0.0001$), and Chao1 measures ($P = 0.0001$) (Table 1). These results
276 were robust across sampling locations, as no significant differences in tree level α-diversity was

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277 observed among sites ($P > 0.5$).

278 Fungal communities were heavily dominated by the Ascomycota, which comprised 75%
279 of all OTUs. At the Class level, Dothideomycetes and Eurotiomycetes were present across all
280 tree species composing between 10 to 27% of all OTU. A total of 97 fungal orders were
281 detected across all tree species, the distribution of these orders varied among tree species
282 (Figure 2, Table S1). More than 41% of all fungal OTU detected in red pine belonged to the
283 order Rhytismatales, however this order was nearly absent in all other tree species (Figure 2).
284 Other fungal orders such as the Helotiales, were present across all tree species (Table S1). The
285 25 most abundant unidentified OTU comprised 61% of all unidentified sequences (Table S4).
286 Although, no attempt was made to mechanically remove lichenized tissue prior to DNA
287 extraction, lichenized OTUs made up a relatively small proportion of our dataset, composing
288 18% of all genera observed and only 7% of all rarefied sequences.

289 Second-order polynomial fit revealed a significant correlation between TPC and fungal
290 richness ($P = 0.001$, adjusted $R^2 = 0.17$) revealing highest fungal richness at intermediate TPC
291 values (Figure S6). Although significant, pH accounted for a small proportion of variance in
292 fungal richness, with diversity peaking in the range of pH 4-5 ($P = 0.053$, $R^2 = 0.051$; Figure
293 S6).

294 **Discussion**:

295 Several lines of evidence support the hypothesis that tree species acts as a strong
296 environmental filter structuring inner bark fungal communities and that these diverse
297 communities are dominated by metabolically active endophytic fungi in asymptomatic surface
298 sterilized plant tissue (Hardoim *et al.*, 2015). Tree species accounted for 37-47% of total
299 variation among fungal communities, depending upon the taxonomic level of fungal
300 communities examined, whereas sampling site location explained only 1.8% of total variation in
301 community composition. These results suggest that inner bark communities are not random
302 assemblages of fungi subject to site level differences in spore dispersal. Instead, heterogeneity in
303 the bark of different tree species acts as an environmental filter structuring distinct endophytic
304 communities.

305 Our results are consistent with previous culture based studies that compared the identify
306 and frequency of inner bark fungi isolated from different tree species (Griffith & Boddy 1990;
307 Kowalski & Kehr 1992). Griffith and Boddy found that very high incidence of endophytic

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308 infection (1990), and Kowalski and Kehr (1992), found that many fungal taxa could only be
309 isolated from specific tree species. These authors suggested that some endophytic fungi may
310 have strong host specificity, particularly between angiosperm and gymnosperm hosts (Kowalski
311 & Kehr 1992). We extend these results by sampling a much larger proportion of the total fungal
312 community encountered within inner bark across a range of common tree species, showing that
313 angiosperm and gymnosperms trees possess significantly different fungal communities.
314 Moreover, we also demonstrate that differences in fungal community structure across tree hosts
315 are maintained at deeper phylogenetic levels (i.e. when fungal communities are grouped at the
316 level of order). Together, our results are broadly congruent with evidence documenting strong
317 turnover of foliar and root endophytic fungal communities across plant hosts (Arnold *et al.,*
318 2000; Higgins *et al.,* 2006; Hoffman & Arnold 2008). As the first such study of its kind,
319 additional sampling across a broader environmental gradient and geographic extent, may also
320 reveal sampling sites (and climatic differences) as relatively strong drivers of inner bark fungal
321 community composition (Webber 1981; Hoffman & Arnold 2008; Zimmerman & Vitousek
322 2012).

323 Both pH and TPC were significant predictors of fungal community composition, and
324 were also significant predictors for many of the abundant fungal orders detected here.
325 Accordingly, both pH and TPC represent potential axes of variation that may structure some
326 proportion of the differences detected in fungal community composition. We note that our assay
327 of TPC represents a general and non-specific measure of phenolic and antioxidant compounds
328 present in inner bark, preventing us from determining the specific compound classes that may
329 impact fungal community composition (Ainsworth & Gillespie 2007). It is critical to note that
330 bark pH, TPC and N appear to be strongly confounded with tree species identity (Figure S7-S9).
331 As a result, we cannot definitively conclude that our measures of bark chemical characteristics
332 drive observed variation among fungal communities without explicit experimental conformation
333 that disentangles these parameters from other host specific effects. Mechanistic determination of
334 the role of TPC and pH in environmental filtering *sensu stricto* (Kraft *et al.,* 2015), would
335 delineate whether fungi colonizing inner bark differentially tolerate the apparently distinct
336 abiotic conditions found across different plant species. These tests, however, are beyond the
337 scope of the current study.

338 Many of the dominant fungal lineages we detected asymptomatically in inner bark have

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339 previously been reported to include a range of endophytic, pathogenic and saprobic lifestyles.
340 For example, the Helotiales and Caetothyriales were both abundant fungal orders recovered
341 from inner tree bark; these taxonomic groups are known to harbor a diverse range of endophytes
342 inhabiting roots and leaves, as well as a range of plant pathogens and saprotrophs (Tedersoo *et*
343 *al.,* 2009; Texieira *et al.,* 2014) For example, the Herpotrichiellaceae (Caetothyriales) and the
344 Dermateaceae (Helotiales) were detected across all hosts in our study; these enigmatic fungi are
345 sometimes detected as root endophytes in a range of environments and plant hosts (Allen *et al.,*
346 2003; Obase & Matsuda 2014; Chen *et al.,* 2015; Jumponnen *et al.,* 2017). Similarly, the
347 Tricholomataceae, a fungal family enriched in white oak bark, is known to include a range of
348 foliar endophytes as well as potent plant pathogens (Lana *et al.,* 2011). The genus *Mycena* was
349 also found to be dominant in oak bark; *Mycena* are well known as a saprotrophic white rot
350 fungi, but can also be detected as an endophyte in tree roots (Kernaghan & Patriquin 2011).
351 Intriguingly, *Mycena* has previously been collected on the bark of live tropical trees (Desjardin
352 *et al.,* 2007), but to the best of our knowledge, this is the first known occurrence of this genus
353 inhabiting surface sterilized inner bark. Additionally, *Therrya* (class Leotiomycetes, order
354 Rhytismatales) was found in very high abundance in Red Pine bark (~ 41% of all OTU); this
355 genus has previously been reported as associated with the bark and stems of pine and other
356 coniferous trees where it occurs as a putative endophyte, as a virulent pathogen (Funk 1980) or
357 as a saprotroph on standing dead branches (Minter 1996; Solheim *et al.,* 2012). Together these
358 observations suggest that *Therrya* may persist as a wider range of lifestyles than previously
359 known. Similarly, *Lepteutypa* was highly abundant but almost exclusively found in white oak
360 bark; members of this genus are known to form cankers in tree bark, however no discernable
361 cankers were present in any of our sampled bark tissue (Swart 1973). This may be the first
362 report of this genus growing asymptomatically in inner bark. Finally, white oak bark was
363 enriched in fungi belonging to the Xylariales, a result consistent with previous reports of
364 endophytic *Xylaria* in oak bark tissue (Griffith & Boddy 1990; Collado *et al.,* 2001; Davis *et al.,*
365 2003). *Xylaria* is also frequently reported as a saprotroph and has been postulated to 'wait' for
366 plant senescence to opportunistically decay plant tissue, rarely or never conferring a mutualistic
367 benefit to the plant host (Davis *et al.,* 2003).

368 Observations that identical fungal isolates can be both hypervirulent pathogens or
369 persist as asymptomatic endophytes (Lana *et al.,* 2011; Sakalidis *et al.,* 2011; Jumponnen *et al.,*

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370 2017) depending upon plant host conditions or coevolutionary history (Lofgren *et al.,* 2018),
371 support the proposition that fungi isolated living endophytically in plant tissue can undergo
372 lifestyle switching (Caroll 1988; Selosse *et al.,* 2018). Our work significantly expands current
373 understanding of the identity and ecology of fungi inhabiting inner bark and contributes to the
374 evolving interpretation of fungal niches, showing that potentially saprobic or pathogenic fungi
375 may persist asymptomatically in inner bark tissue.

376 Although our study did not attempt to isolate fungal mycelium, we postulate that a
377 significant proportion of the fungal taxa studied here may be actively growing as hyphae. While
378 it is possible for the physical attributes of different tree species' bark to differentially entrap
379 fungal spores or other dormant fungal life stages, thereby driving the strong differences in
380 fungal communities detected among tree species, there are multiple reasons to suggest that this
381 is unlikely and that bark does not solely act as a passive sieve for fungi. Spores are an especially
382 durable and resistant fungal life history stage (Bruns *et al.,* 2009), making it unlikely that
383 communities of spores or other latent life stages would persist differentially across a range of
384 bark pH and bark phenolic contents. Reports of actively growing endophytic mycelium, isolated
385 from surface sterilized elm (Webber 1981), pacific yew (Stierle *et al.,*1993), beech, oak (Griffith
386 & Boddy 1990), pine and larch bark (Kowalski & Kehr 1992), indirectly support these claims.
387 Understanding the potential seasonal turnover of these communities will strengthen our
388 understanding of the natural history of these organisms and their role in inner tree bark
389 (Younginger & Ballhorn 2017).

390 We found a mean range of 22-247 fungal OTUs per tree. These results are remarkably
391 consistent with other well replicated, next-generation sequencing studies of fungal endophyte
392 communities. For example, Zimmerman and Vitousek (2012) studied foliar endophytes in a
393 single tree species across a substantial environmental gradient in Hawaii and found a range of
394 40-257 OTUs per tree. Our methodology likely detected many fungal taxa that are typically
395 isolated with very low frequency when culturing techniques are employed (Kowalski & Kehr
396 1992; Hoffman *et al.,* 2006). Given that diversity estimates generated from molecular sequence
397 data are notoriously coupled with specific sequence processing choices (Dickie 2010; Nguyen *et*
398 *al.,* 2015; Taylor *et al.,* 2016), we caution against over interpretation of the absolute magnitude
399 of OTU diversity detected here, while maintaining that inner bark fungal communities are
400 reasonably diverse.

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401 Diverse communities of inner bark fungi appear to be ubiquitous across a range of plant
402 taxa and their presence may have ecological implications for our understanding of plant defense
403 and decay dynamics. The potential role of a subset of these fungi in plant defense, as has been
404 previously shown for some inner bark fungal endophytes (Webber & Hedger 1984; Verma *et*
405 *al.,* 2007; Alfredsen *et al.,* 2008), remains an intriguing area of research. Future studies that
406 explore the distribution and function of these fungi across plant hosts and geographic locales
407 will assist in our understanding of the dynamics of the growing number of plant diseases that
408 penetrate through tree bark to reach the vascular tissue (Webber 1981; Bentz *et al.,* 2010; Herms
409 & McCullough 2014). Additionally, the unique inner bark fungal assemblages found in each
410 tree species may impact subsequent decay dynamics. The role of inner bark fungi as immediate
411 colonizers of dead plant tissue is plausible (Selosse *et al.,* 2018), especially given the potentially
412 saprotrophic capacity of some of the dominant fungi found here. Distinct fungal assemblages
413 across tree species, may drive divergent decay trajectories if they impact the establishment and
414 activity of subsequently arriving wood decay fungi by exerting priority effects (Fukami 2015).
415 Consistent with this reasoning, wood endophyte communities have been shown to impact the
416 community assembly and function of subsequently arriving saprotrophic fungi by producing a
417 range of bioactive exudates (Heilmann-Clausen & Boddy 2005; Cline *et al.,* 2018).

418 **Conclusion**:

419 This work highlights that inner tree bark harbors non-random fungal assemblages and that these
420 communities are an underappreciated and potentially ubiquitous component of the plant
421 microbiome (Vandenkoornhuyse *et al.,* 2015). By studying the inner bark of different tree
422 species across multiple study sites we provide strong evidence that tree bark of different species
423 and evolutionary histories can serve as an environmental filter structuring fungal community
424 membership. The range of dominant fungal lineages found here include potential plant
425 pathogens and saprotrophs detected asymptomatically as endophytic fungi in inner tree bark;
426 these results support the ‘dual-niche’ flexibility of many endophytic fungi (Selosse *et al.,* 2018).
427 Finally, our study suggests that inner bark fungal communities are comparatively as diverse as
428 some foliar endophyte communities, and the large proportion of unidentified fungal taxa
429 detected in our study may reflect the understudied nature of this plant tissue.
430

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440 wrote the manuscript and analyzed the data. PTP and SOS developed the DNA extraction
441 methodology. All authors contributed to the preparation of the final version of this paper.
442

443 **Data Accessibility Statement:**
444 Raw sequences and associated metadata have been deposited in SRA. Bioproject:
445 PRJNA546283, samples: SRR9205176 - SRR9205. All other metadata is accessible at
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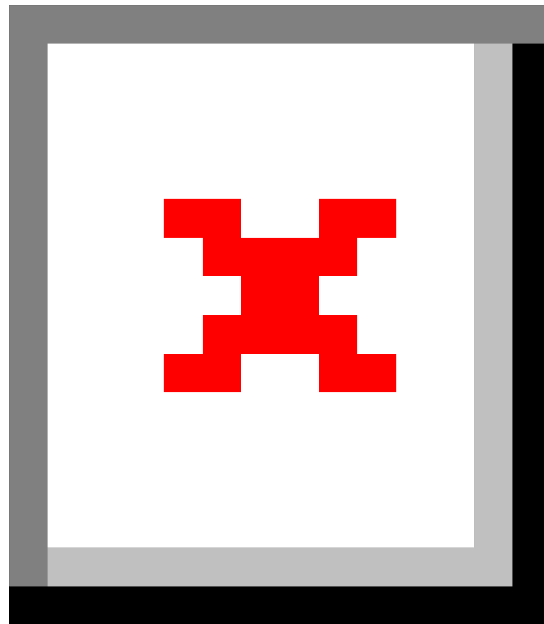

Figure 1. Principle Coordinates Analysis (PCoA) constructed using Bray-Curtis distance matrices. Fungal OTU exclude lichenized fungi. Individual points represent inner bark fungal communities from individual trees (colors) and sites (shapes). Plotted tree species labels represent centroid locations. Differences among tree species explains approximately 20 times more variation in fungal community composition than does site. BO = Black Oak, WO = White Oak, RM = Red Maple, WP = White Pine, RP = Red Pine.

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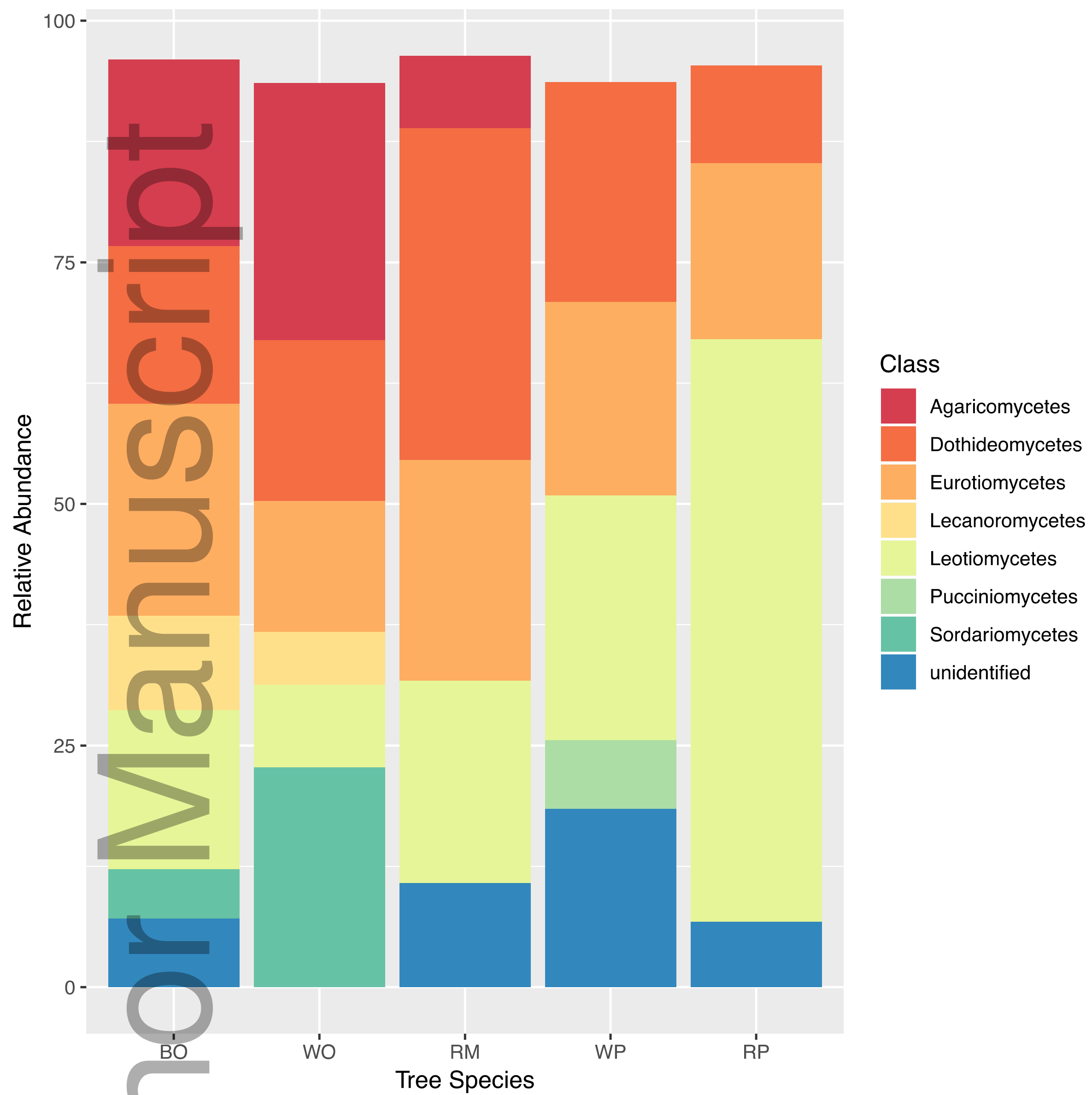

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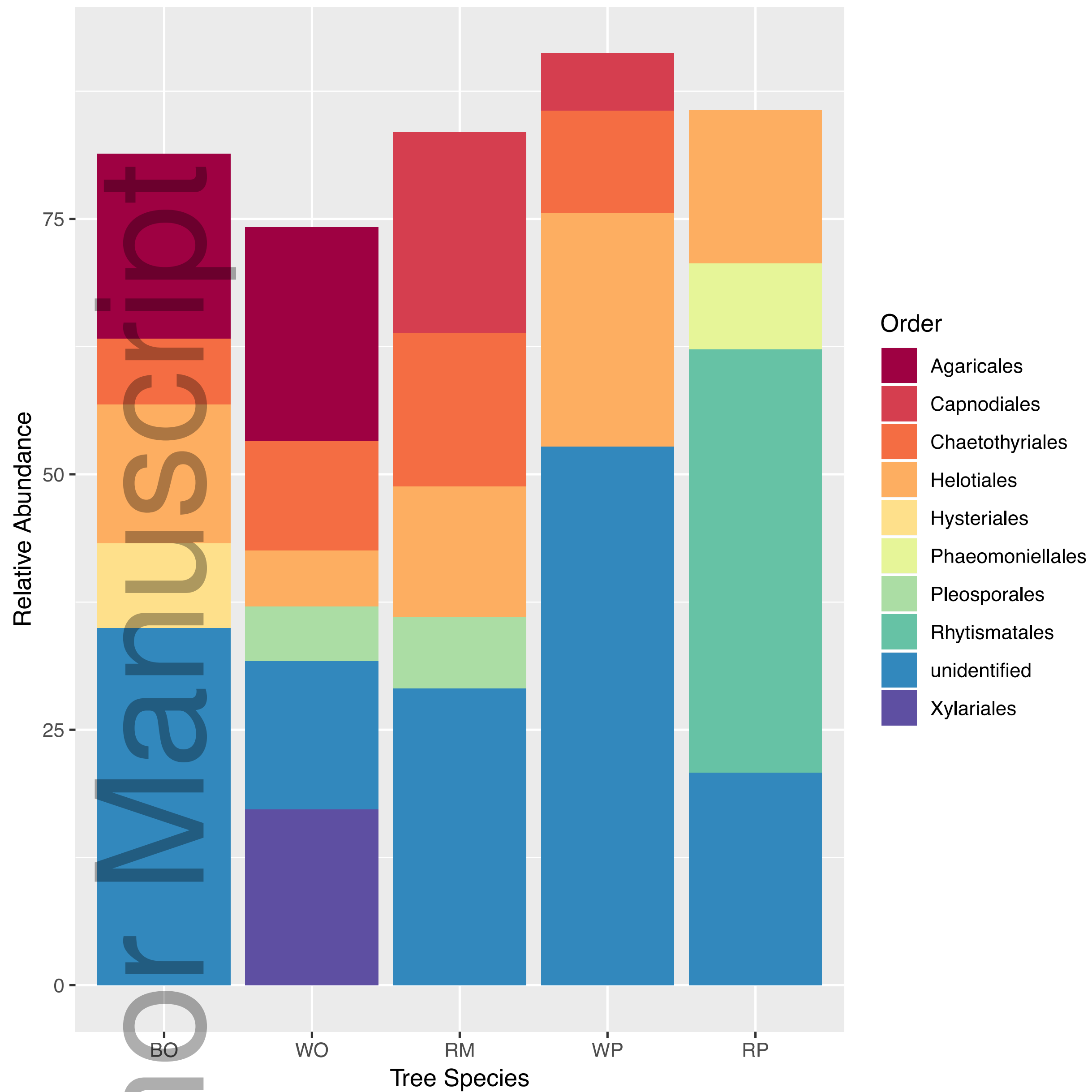

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Figure 2. Taxonomic composition of fungal a. classes and b. orders. Data presented exclude lichenized fungi. Relative abundance is calculated as the average proportion of DNA sequences found within each tree species assigned to each order, comprising more than 5 % of all sequences.

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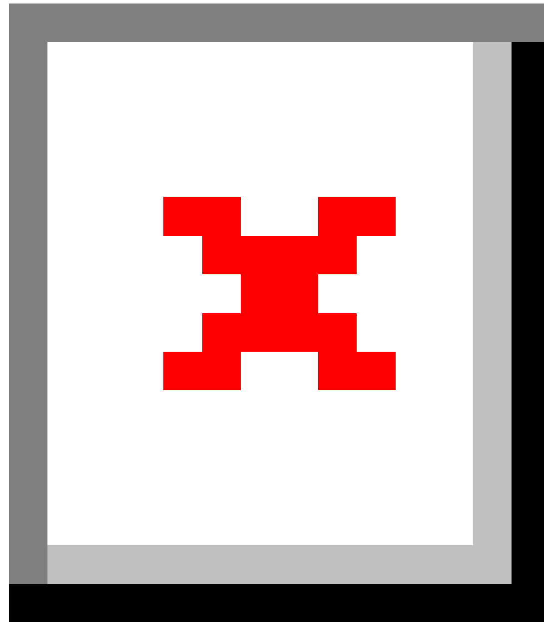

Figure 3. Canonical correspondence analysis (CCA) depicting differences among inner bark fungal communities across five tree species. Points represent fungal communities sampled from individual trees, with Hellinger transformed OTU counts. Ordination is constrained by chemical parameters (pH, N content and Total Phenolic Content (TPC)) which together explain 11.8% of overall variance (inertia). Plotted fungal order names are scaled centroid coordinates for weighted distances comprising more than 1% of all OTU. Axis percentages depict constrained variation. The proximity between plant species and fungal orders labels can be understood as the

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probability of occurrence in the bark of a given tree species. BO = Black Oak, WO = White Oak, RM = Red Maple, WP = White Pine, RP = Red Pine.

Table 1. Inner bark fungal diversity measures for the studied tree species with lichenized fungi removed. Superscripts denote significant differences at P <0.05

Tree Species	Observed OTU	SE	Chao1	Inverse Simpson
White Oak	122.27^{b}	8.97	178.19^{b}	$11.80^{a,b}$
Black Oak	169.42^{b}	7.13	239.26^{a}	$16.16^{c,e}$
Red Maple	146.00^{a}	5.72	221.03^{a}	9.91^{b}
Red Pine	113.70^{b}	12.39	182.93^{b}	4.35^{d}
White Pine	171.13^{a}	6.37	261.82^{a}	$15.10^{a,e}$

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Tree Species	Observed OTU	SE	Chao1	Inverse Simpson
White Oak	122.27[b]	8.97	178.19[b]	11.80[a,b]
Black Oak	169.42[b]	7.13	239.26[a]	16.16[c,e]
Red Maple	146.00[a]	5.72	221.03[a]	9.91[b]
Red Pine	113.70[b]	12.39	182.93[b]	4.35[d]
White Pine	171.13[a]	6.37	261.82[a]	15.10[a,e]

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