Fungal diversity in shade-coffee plantations in Soconusco, Mexico

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Background. As forested natural habitats disappear in the world, traditional, shade-coffee plantations offer an opportunity to conserve biodiversity and ecosystem services. Traditional coffee plantations maintain a diversity of tree species that provide shade for coffee bushes and, at the same time, are important repositories for plants and animals that inhabited the original cloud forest. However, there is still little information about shade-coffee plantation's fungal diversity despite their relevance for ecosystem functioning as decomposers, symbionts and pathogens. Specifically, it is unknown if, and what mycorrhizae-forming fungi can be found on the branches and trunks of coffee bushes and trees, which hold a diversity of epiphytes. Here, we evaluate fungal communities on specific plant microsites on both coffee bushes and shade trees. We investigate the ecological roles played by this diversity, with a special focus on mycorrhizae-forming fungi that may enable the establishment and development of epiphytic plants.

Methods. We collected 48 bark samples from coffee bushes and shade trees (coffee; tree), from four plant microsites (upper and lower trunks, branches and twigs), in two shade-coffee plantations in the Soconusco region in southern Mexico, at different altitudes. We obtained ITS amplicon sequences that served to estimate alpha and beta diversity, to assign taxonomy and to infer the potential ecological role played by the detected taxa.

Results. The bark of shade trees and coffee bushes supported high fungal diversity (3783 amplicon sequence variants). There were no strong associations between community species richness and collection site, plant type or microsite. However, we detected differences in beta diversity between collection sites. All trophic modes defined by FUNGuild database were represented in both plant types. However, when looking into guilds that involve mycorrhizae formation, the CLAM test suggests that coffee bushes are more likely to host taxa that may function as mycorrhizae.

Discussion. We detected high fungal diversity in shade-coffee plantations in Soconusco, Chiapas, possibly remnants of the original cloud forest ecosystem. Several mycorrhiza forming fungi guilds occur on the bark of coffee bushes and shade trees in this agroecosystem, with the potential of supporting epiphyte establishment and development. Thus, traditional coffee cultivation could be part of an integrated strategy for restoration and conservation of epiphytic populations. This is particularly relevant for conservation of threatened species of Orchidaceae that are highly dependent on mycorrhizae formation.

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19 Abstract

20 **Background.** As forested natural habitats disappear in the world, traditional, shade-coffee

21 plantations offer an opportunity to conserve biodiversity and ecosystem services. Traditional

22 coffee plantations maintain a diversity of tree species that provide shade for coffee bushes and, at

23 the same time, are important repositories for plants and animals that inhabited the original cloud

24 forest. However, there is still little information about shade-coffee plantation's fungal diversity

25 despite their relevance for ecosystem functioning as decomposers, symbionts and pathogens.

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27 and trunks of coffee bushes and trees, which hold a diversity of epiphytes. Here, we evaluate

28 fungal communities on specific plant microsites on both coffee bushes and shade trees. We

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30 forming fungi that may enable the establishment and development of epiphytic plants.

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33 plantations in the Soconusco region in southern Mexico, at different altitudes. We obtained ITS

34 amplicon sequences that served to estimate alpha and beta diversity, to assign taxonomy and to

35 infer the potential ecological role played by the detected taxa.

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37 amplicon sequence variants). There were no strong associations between community species

38 richness and collection site, plant type or microsite. However, we detected differences in beta

39 diversity between collection sites. All trophic modes defined by FUNGuild database were

40 represented in both plant types. However, when looking into guilds that involve mycorrhizae

41   formation, the CLAM test suggests that coffee bushes are more likely to host taxa that may

42   function as mycorrhizae.

43   **Discussion.** We detected high fungal diversity in shade-coffee plantations in Soconusco, Chiapas,

44   possibly remnants of the original cloud forest ecosystem. Several mycorrhiza forming fungi

45   guilds occur on the bark of coffee bushes and shade trees in this agroecosystem, with the

46   potential of supporting epiphyte establishment and development. Thus, traditional coffee

47   cultivation could be part of an integrated strategy for restoration and conservation of epiphytic

48   populations. This is particularly relevant for conservation of threatened species of Orchidaceae

49   that are highly dependent on mycorrhizae formation.

50

51   **KEYWORDS:** ITS, mycorrhiza, cloud forest, epiphytes, Chiapas, *Coffea arabica*

52

Introduction

54 Biodiversity is severely threatened by human activities that have transformed natural habitat into

55 agricultural and urban landscapes. Contrary to intensified monocultures, complex

56 agroecosystems, such as shade-coffee plantations, offer a significant opportunity for the

57 conservation of biodiversity (Solis-Montero, Flores-Palacios & Cruz-Angón, 2005; Perfecto et

58 al., 2007) and ecological services like carbon sequestration, soil protection and conservation of

59 native pollinators (Toledo & Moguel, 2012). Additionally, they are low maintenance and low

60 impact agroecosystems, that do not require irreversible changes in their management (Espejo

61 Serna et al., 2005; García-González et al., 2011) and that provide livelihoods for millions of

62 people in more than 50 countries (Wrigley, 1988; Vega, 2008; Amrouk, 2018).

63   Traditional coffee plantations maintain a diversity of native tree species to shade the

64 coffee bushes and are important repositories of the original biodiversity of the cloud forest

65 ecosystem (Moguel & Toledo, 1999). Previous studies have shown that these agroecosystems

66 harbor a high diversity of arthropods (Méndez-Castro & Rao, 2014; Wauters, Fournier &

67 Dekoninck, 2017; Ibarra-Isassi et al., 2021), birds (Mas & Dietsch, 2004; Tejeda-Cruz &

68 Sutherland, 2004; Philpott et al., 2008; MacGregor-Fors et al., 2018; González et al., 2020),

69 mammals (Gallina, Mandujano & Gonzalez-Romero, 1996; Moguel & Toledo, 1999; Caudill,

70 DeClerck & Husband, 2015; Etana et al., 2021) and plants (Cruz-Angón & Greenberg, 2005;

71 González Zamora, Esperón Rodríguez & Barradas, 2016; Alvarez-Alvarez et al., 2021).

72   On the other hand, there is still little information about fungal diversity in traditional

73 coffee plantations despite their relevance for ecosystem functioning as decomposers, symbionts

74 and pathogens (Mueller, Bills & Foster, 2004; Nguyen et al., 2016). Mycorrhiza forming fungi,

75 essential for the establishment and development of some plants, have been detected in soil from

76 shade-coffee plantations (Rao et al., 2020; Jurburg, Shek & McGuire, 2020; Díaz-Ariza, Rivera

77 & Sánchez, 2021). Specifically, it is unknown if, and what mycorrhizae-forming fungi can be

78 found on the branches and trunks of coffee bushes and shade trees, which hold a diversity of

79 epiphytes, some of them endangered, as is the case of some orchids (Moguel & Toledo, 1999;

80 Solis-Montero, Flores-Palacios & Cruz-Angón, 2005; Espejo Serna et al., 2005; Mondragón,

81 Santos-Moreno & Damon, 2009; Damon, 2017).

82   Although we do not understand the mechanisms involved, it is clear that epiphytic

83 orchids have evolved a complex and obligate dependence on certain species of fungi (Selosse et

84 al., 2022). Various authors have demonstrated that the seeds of epiphytic orchids depend upon

85 mycorrhizae forming endophytic fungi to enable the differentiation of the mother cells in the

86 protocorm that develops from the embryo of the seed, thereby completing the process of

87 germination, and that growth ceases and the plant eventually dies in the absence of a mycorrhizal

88 symbiont (Zettler, Delaney & Sunley, 1998; Markovina & McGee, 2000; Pereira et al., 2005).

89 After the initial germination and development stages, the association appears to become

90 facultative and highly variable across species, seasons and distributions on a local, but not on a

91 geographical scale (McCormick, Whigham & Canchani-Viruet, 2018) and further research is

92 required to establish patterns and tendencies.

93   These fungi can be observed as hyphal coils, or pelotons, within cells of the roots and

94 are therefore termed endophytes, but furthermore they form an intimate mycorrhizal association

95 with the orchid plant. We use the term mycorrhizae forming endophytic fungi, instead of the

96 more usual term Orchid Mycorrhizal Fungi (OMF), first, to distinguish them from the networks

97 of ectomycorrhizal fungal that associate with the external surfaces of plant roots, especially trees,

98 and second, to avoid the idea that the fungi that associate with Orchidaceae form a small, well-

99 defined group of fungal species.

100  Epiphytes germinate and complete their development on the bark of the branches,

101 trunks and even the twigs of trees, bushes and lianas in forest ecosystems and we can assume that

102 the necessary fungi are present on the bark surfaces and that the spatial distribution and niche

103 preferences of these potentially mycorrhizal fungi will then influence the distribution of

104 epiphytic orchids on those surfaces. Complex, variable and incompletely defined spatial

105 distributions have been observed for endophytic mycorrhizal fungi associated with terrestrial

106 orchids in soils and epiphytic orchids on bark surfaces. The OMF (equivalent to our mycorrhizae

107 forming epiphytic fungi) evaluated by (Petrolli et al., 2021) were spatially related to orchid roots,

108 however, to the contrary, Kartzinel et al. (2013) found that sowing the seeds of a rare epiphytic

109 orchid close to or far from mature conspecific plants did not affect germination success. It should

110 be noted that Petrolli et al. (2021) focused on young trees, and only two individuals, and as

111 commented by Kartzinel (2013), we know that the microbial community and environmental

112 conditions found on older trees tend to be more favorable for the establishment of epiphytic

113 orchids.

114  Here, we evaluate fungal communities on specific plant microsites on both coffee

115 bushes and shade trees. We investigate the ecological roles played by this diversity, with a

116 special focus on mycorrhizae-forming fungi that may enable the establishment and development

117 of epiphytic plants.

118

Materials & Methods

Study area

The study area is in the southeast of Mexico, within a biodiversity hotspot (Mittermeier et al., 2011; CONABIO, 2012) including the Tacaná-Boquerón Biological Corridor, considered to be the second most important region for orchid species richness in Mexico (Arriaga et al., 2000; Solano-Gómez et al., 2016). Samples were collected from two localities, with mountainous, sloping terrain, separated by more than 50km, in the Soconusco region in the state of Chiapas and chosen because of their differing characteristics (e.g. altitude), in April 2017. "Los Hermanitos" (hereinafter referred to as H) is situated at 15°06'02.8" N and 92°19'14.9" W, at 537 m.a.s.l., towards the lowest altitude tolerated by *Coffea arabica*, whereas "Benito Juárez El Plan" (hereinafter referred to as B) is situated at 15°05'0.517" N and 92°08'14.806" W, and at a much higher elevation, 1500 m.a.s.l. In each locality, four coffee bushes and two shade trees (hereinafter coffee, tree, respectively) were selected, with four microsites each: twig, branch, upper trunk (near the origin of the first branch), and lower, or base of the trunk. In both sites, the overstory, including the trees sampled, were mature *Inga micheliana* (Fabaceae) and the original forest trees would have first been thinned out about 80 years ago and then replaced by *I. micheliana* within the last 20 or 30 years, although in most coffee plantations in the region some original forest tree species are retained. A 6 x 7 cm bark sample, 2-5 mm thick, was taken from each microsite (n= 48), using a previously disinfected scalpel. Each bark sample was individually placed in a labeled plastic bag and transported to the laboratory in an icebox with ice. We obtained verbal permission to collect samples from the land owners: Mr. Marco Polo Zamora Martínez, Predio Las Bugambilias, Finca Los Hermanitos, Tapachula, Chiapas; Mr. Bonifasio Morales Ortíz, field in Ejido Benito Juárez El Plan, Cacahoatán, Chiapas.

142

143   **Laboratory procedures**

144   To conserve the microorganisms present on the bark surface, the samples were not surface

145   sterilized. Genomic DNA was extracted from the surface of the 48 bark samples following a

146   CTAB (Hexadecyltrimethylammonium bromide) based protocol (Díaz-Cárdenas et al., 2008).

147   DNA quality was checked on a 1% agarose gel, stained with SYBR® Green and visualized

148   under UV light. Using the ITS3F (GCATCGATGAAGAACGCAGC) and 4R

149   (TCCTCCGCTTATTGATATG) primer pair (White et al., 1990), an ITS2 amplicon library was

150   built and run on an Illumina MiSeq instrument at Macrogen Inc. in Seoul, South Korea to

151   produce 300 bp paired-end sequences.

152

153   **Bioinformatics and statistical analyses**

154   Sequence quality was assessed with FastQC v0.11.8 (Andrews, 2018) and summarized with

155   MultiQC (Ewels et al., 2016). Adapters and primer fragments were removed with CUTADAPT

156   version 1.18 (Martin, 2011). The resulting trimmed sequences were imported into and analyzed

157   with the package QIIME2 version q2cli 2019.1.0 (Bolyen et al., 2019). The ITSxpress plugin

158   was used to remove the conserved regions flanking ITS2 to improve accuracy in taxonomic

159   assignment (Rivers et al., 2018) and considering models for all taxa. Although the amplicon

160   library was designed to amplify fungal markers, this 'all taxa' approach was designed to give a

161   glimpse of the eukaryotic diversity associated with the selected microsites. The DADA2

162   (Callahan et al., 2016) plugin was used to de-noise sequences, correct errors in marginal

163   sequences, join paired-end reads and remove chimeric and singleton sequences. We used default

164   values for parameters, except for removing reads with more than six errors (max-ee =6). The

165 DADA2 plugin produced a feature table and a file with representative sequences (i.e. amplicon

166 sequence variants -ASVs). Results and statistics were visually assessed with QIIME2-view

167 (https://view.qiime2.org/). All ASVs were classified using the feature-classifier plugin and using

168 the UNITE 8.0 database (Nilsson et al., 2019) for QIIME version 18.11.2018 with trimmed

169 reference sequences for all eukaryotes (https://dx.doi.org/10.15156/BIO/786335). The QIIME

170 classifier was trained with this database and the 'fit-classifier-naive-bayes' method.

171 Representative sequences were then classified using the 'sklearn' method. Heatmaps were

172 generated with R (R Core Team, 2017) to better visualize diversity at class level.

173   Alpha (Shannon Index) and beta diversity (Bray-Curtis distance) metrics were

174 calculated with the q2-diversity plugin, which rarefies according to a user specified depth,

175 aiming to choose a value that is as high as possible while excluding as few samples as possible.

176 Additionally, tests for associations between alpha diversity and collection site, plant type,

177 microsite type (Group 1 – G1 from now onwards), and plant type-microsite combinations (Group

178 2 – G2 from now onwards) were carried out. Similar associations were investigated but using

179 beta diversity parameters.

180   Modifications to this pipeline were implemented to analyze diversity patterns in Fungi

181 only. To that end, ITSxpress was run again specifying Fungi as the focus taxa. Taxonomic

182 classification was carried out with the previously trained classifier. The resulting table was used

183 to filter the feature table and include only sequences assigned to the Fungi kingdom. Alpha

184 (Shannon Index) and beta diversity (Bray-Curtis distance) metrics were calculated using this

185 filtered table as input. For these calculations we used the parameter 'sampling-depth'

186 implemented in QIIME2 to randomly subsample the counts in each sample, without replacement,

187 so that each sample in the resulting table has even sampling. Following QIIME2 guidelines and

188   after checking the filtered feature table, we chose a sampling depth value that was as high as

189   possible while aiming to lose as few samples as possible.

190   The feature table containing only Fungi sequences was imported into R to perform

191   further diversity analyses and create visualizations. The library ampvis2 v2.7.27(Andersen et al.,

192   2018) and amplicon v1.14.2 (Liu et al., 2021) were used to filter samples with a minimum of

193   10,000 reads and rarefy at two different depths (20,000 and 12,408 reads), calculate alpha

194   diversity and create box plots. To test if there were significant differences between alpha

195   diversity among collection sites, plant type, microsite type (Group 1), and plant type-microsite

196   combinations (Group 2) we performed a Tukey range test. Additionally, to visualize differences

197   in beta diversity among groups (same as above), we performed a non-metric multidimensional

198   scaling (NMDS) analysis based on the Bray-Curtis distance and tested for differences in fungal

199   composition among groups with the PERMANOVA method, applying the adonis function

200   implemented in vegan (Oksanen et al., 2018) and included in the package amplicon.

201   To obtain a list of species that are associated with particular microsites (or combinations

202   of those), we performed an indicator species analysis using the R package indicspecies v1.7.12

203   (De Caceres & Legendre, 2009) with the function multipatt executing 999 permutations.

204   Sequences classified with QIIME2 were submitted to the FUNGuild v1.0 database

205   (Nguyen et al., 2016) to assign functional annotation. We created boxplots with R (R Core Team,

206   2017) to visualize the trophic mode distribution on the different microsites. To investigate a

207   possible association between fungal taxa with known mycorrhizal potential and coffee bushes or

208   shade trees, we applied the CLAM test (Chazdon et al., 2011) as implemented in the R v.3.4.1

209   program vegan v2.5-3 (Oksanen et al., 2018), to perform a classification of habitat

210   specialists/generalists, and those too rare to classify, in two distinct habitat types (i.e. coffee and

211   trees), minimizing bias due to differences in sampling intensities. We classified all ASVs

212   identified as belonging to the Fungi kingdom, Fungi genera and mycorrhizal guilds. We used a

213   coverage limit of 10, a 'supermajority' (2/3) specialization threshold, and a significance alpha

214   value of 0.0025 to correct for multiple comparisons.

215

216   ## Results

217   DNA was successfully purified from all 48 samples. The number of sequences obtained, number

218   of ASVs, microsite type and plant type are shown in Supplementary File 1. Between 142,544 –

219   256,570 sequences were obtained for each sample. After ITSxpress trimming for all taxa,

220   filtering, and DADA2 denoising, between 9,357 – 140,012 sequences were retained. When

221   selecting only Fungi with ITSxpress, followed by filtering and DADA2 denoising, between

222   2,884 – 128,158 sequences were retained.

223   Diversity metrics obtained with QIIME2 for the 'All taxa' dataset were calculated at a

224   sampling depth of 77,000 reads. At this depth, the alpha-rarefaction curves plateaued, indicating

225   that sampling was representative for all plant-microsite treatments, except in 'Tree Twigs'

226   (Supplementary File 1, Figure S1). Group significance tests suggested that there was no strong

227   association between community richness (i.e. Shannon Index) and collection site (B and H),

228   plant type (coffee; tree), G1 (microsite) and G2 (plant type-microsite) combinations. To detect if

229   samples within a group were more similar to each other than to samples from other groups,

230   PERMANOVA pairwise tests were carried out. To determine which specific pairs differed from

231   one another we used the Bray-Curtis distance obtained with QIIME2. Significant differences

232   were obtained when comparing collection site (B vs H, p=0.004) and plant type (coffee; tree,

233   p=0.001). No significant differences were obtained within G1 nor within G2. Visualizations for

234   alpha and beta diversity associations are presented in Supplementary File 1, Figure S3.

235   Some of the sequences that passed the filter for only Fungi sequences applied by

236   ITSxpress (i.e. 4414 ASVs), were not actually classified as belonging to the Fungi kingdom,

237   according to the QIIME2 classifier (Supplementary Files 2 and 3). They included Alveolata (53),

238   Chromista (14), Protista (254), Rhizaria (5), Viridiplantae (5), and unassigned sequences (300).

239   Thus, another filter was applied in QIIME2 to consider only ASVs tagged as Fungi resulting in

240   3783 ASVs. Only 75% of these Fungi ASVs were assigned to phylum, 29% to genus, and 16%

241   to species level. The most widespread fungi, classified with the BLAST tool implemented in

242   QIIME2-view, (i.e. detected in 25% - 45% of samples) were *Cladosporium tenuissimum*,

243   *Lasiodiplodia*, *Fusarium* and *Pestalotiopsis*, which are considered as common pathogens. On the

244   other hand, the species indicator analysis detected 11 ASVs associated with only five microsites,

245   or their combinations on the same plant type. All except one (i.e. *Bulleribasidium*), belonged to

246   Ascomycota, and included several pathogens, endophytic and lichen forming taxa (Table 1).

247   Three occurred in coffee bushes and the rest in shade trees. A further 29 ASVs were associated

248   with 11 microsites or their combinations, however they could not be taxonomically assignment

249   beyond family level (Supplementary File S2)

250   Regarding this fungal diversity, alpha-rarefaction curves plateaued when considering a

251   sequencing depth of 20,000, indicating that sampling was representative for all plant-microsite

252   combinations (Figure S2). Comparisons performed with the Tukey test did not detect any

253   significant difference in alpha diversity (Shannon and Simpson indices), among microsites when

254   rarefying at 12,408 (Figure S3) and 20,000 (Figure 1). Similarly, there were no strong

255   associations between community species richness, and any of: collection site, plant type, G1 or

256   G2, as calculated with QIIME2 with the Shannon index and the Kruskal-Wallis test.

257   On the other hand, significant differences in species composition were obtained when

258   comparing collection sites (B vs H, p=0.001) with the Bray-Curtis distance and the

259   PERMANOVA method implemented in QIIME2. No significant differences were obtained

260   between plant types, within G1, or within G2 (Supplementary File 1, Figure S4, S5 and S6).

261   These results were confirmed by the NMDS and adonis tests implemented in R (Figure 2). There

262   was a clear separation between the samples collected in the two localities (adonis R=0.036, p =

263   0.001). Significant results were also obtained when comparing groups defined by plant type

264   (coffee; trees, adonis R=0.026, p =0.002), however there was an overlap between these groups

265   when observing the NMDS graph (Figure 2b). When comparing branch, twig, trunk high and

266   trunk low, there were only significant differences when 'twig' was part of the comparison (twig

267   vs trunk low, adonis R=0.055, p=0.001; twig vs trunk high, adonis R = 0.052, p = 0.005; twig vs

268   branch adonis R = 0.048, p 0.08). When comparing microsites, we detected significant

269   differences in beta diversity when comparing the twigs and trunks of coffee bushes (coffee twig

270   vs coffee trunk low, adonis R= 0.08, p= 0.001; coffee twig vs coffee trunk high, adonis R= 0.08,

271   p=0.012), and when comparing tree twig vs tree trunk low (adonis R= 0.16, p= 0.027). Similarly,

272   beta diversity between tree twig and other sites on coffee bushes was significantly different (tree

273   twig vs coffee trunk high, adonis R= 0.105, p= 0.044; tree twig vs coffee branch, adonis R=

274   0.105, p= 0.003; coffee trunk low vs tree twig, adonis R= 0.106, p= 0.005). We also detected

275   differences in beta diversity between branches of both plant types (tree branch vs coffee branch,

276   adonis R= 0.101, p=0.019). Beta diversity of samples collected from coffee trunk low and high

277   was significantly different from beta diversity of tree branches (adonis R= 0.101, p= 0.013;

278   adonis R= 0.103, p= 0.05). Beta diversity was also significantly different between coffee trunk

279   high and tree trunk low (adonis R= 0.1, p= 0.035).

280   A heatmap at class level was created to evaluate possible trends in taxon frequency,

281   according to sample, plant type or microsite (Figure 3). Agaricomycetes (Basidiomycota) was

282   the most relatively abundant class (52.8% of total reads), particularly in a cluster containing

283   mostly coffee bush samples (lower cluster in dendrogram). These fungi comprise a wide

284   spectrum of ecological functions. The second most abundant (19.47%) were Dothideomycetes

285   (Ascomycota), mostly endophytic or saprobes, with higher abundance in the upper cluster that

286   includes both coffee bush and shade tree bark samples. Other Ascomycota classes are relatively

287   abundant in samples in the upper cluster and have several ecological functions were

288   Sordariomycetes (8.25%), Eurotiomycetes (5.11%), Lecanoromycetes (4.99 %), Leotiomycetes

289   (3.38%), Orbiliomycetes (3.33 %), together with Tremellomycetes (1.48%, Basidiomycota).

290   ASVs considered as Fungi, were assigned to nine trophic modes using FUNGuild. All

291   trophic modes were sampled in both plant types (Figure 4), except pathogen-saprotroph-

292   symbiont (only on coffee twigs and trunk) and saprotroph-pathotroph-symbiotroph (on trunk

293   lower area). However, these two annotations might be redundant in the FUNGuild database and

294   actually belong to the pathotroph-saprotroph-symbiont classification. Symbiotrophs show higher

295   relative abundance in coffee bushes than in shade trees –except for tree twigs. When looking into

296   guilds that involve mycorrhizae formation, some occur only on coffee bushes or have a higher

297   frequency than in samples from shade trees (Figure 5). This may suggest that, although both

298   types of plants harbor similar diversity, coffee bushes are more likely to host taxa that may

299   function as mycorrhizae. All orchid mycorrhiza sequences were detected on 'coffee trunk low',

300   with a relative sequence abundance of 0.0036. Table 2 shows the total occurrence of sequences

301   classified as mycorrhizal fungi, stating the lowest common taxonomy assigned and ecological

302   description.

303   We applied the CLAM test to only include ASVs tagged as belonging to the Fungi

304 Kingdom (3,783) resulting in 1,648 ASVs classified as coffee bush specialists and 1,099 as

305 shade tree specialists, representing 43.6% and 29.1%, respectively. Only 3.8% ASVs were

306 classified as generalists and 23.6% as too rare to be classified. On the other hand, 111 of the 302

307 identified genera were classified as coffee bush specialists and 85 as shade tree specialists, with

308 58 being generalist and 49 too rare to be classified. We were also interested in investigating if

309 any of the mycorrhizal guilds could be classified as coffee bush or shade tree specialists. The

310 CLAM test suggests that 50% (6 guilds) of the mycorrhizal fungi can be classified as coffee bush

311 specialists and 25% (3 guilds) as shade tree specialists, whereas two guilds are generalists and

312 one is too rare to be classified. Table 3 shows the mycorrhizal guilds and their classification.

313   The data that support the findings of this study are openly available in GenBank at

314 https://www.ncbi.nlm.nih.gov/genbank/, under the Bioproject PRJNA610266. Commands and

315 scripts used to run bioinformatics analyses are available from

316 https://github.com/zarzamora23/Fungi-ITS-analyses.git

317

Discussion

319 An understanding of the microbiome of agroecosystems is essential to guide management

320 strategies and ensure sustainability (Toju et al., 2018). Some epiphytic plants (e.g. orchids) are

321 primarily dependent upon mycorrhiza-forming endophytic fungi and later dependent upon other

322 organisms, such as specialist pollinators, to establish stable, persistent populations. Traditional

323 coffee plantations, which host a variety of native species of trees, offer a unique opportunity for

324 the conservation of epiphytic plants and other elements of the original native flora and fauna.

325 However, recently, in the region of Soconusco, management practices designed to increase

326   production (e.g., monoculture shade, heavy pruning or elimination of shade trees, use of new,

327   dwarf coffee varieties, removal of moss and epiphytes, and the use of agrochemicals) have been

328   shown to endanger the local flora and fauna that had adapted to the original traditional, shaded

329   coffee agroecosystem (Perfecto et al., 1996; Moguel & Toledo, 1999) during the one and a half

330   centuries since the introduction of coffee into the Soconusco region. Here, we investigated

331   fungal diversity on the coffee bushes themselves and on the trees providing shade in two

332   traditional coffee plantations at different altitudes, to detect taxa with the potential to form

333   mycorrhizal interactions with epiphytic plants.

334   Fungal diversity in general was high in both coffee bushes and shade trees in shade-

335   coffee plantations in the Soconusco region. We identified 3,783 ASVs classified as Fungi,

336   representing more OTUs than those detected in other bark studies carried out in temperate forests

337   (e.g. 2,207 by Hagge et al., 2019; 1,945 by Pellitier et al., 2019). However, the data are not

338   entirely comparable as, unlike our study, they were interested in fungal colonization within the

339   bark, and the external surface was sterilized prior to sampling. On the other hand, another study

340   focusing on orchid mycorrhizal fungi in orchid roots and non-sterilized bark samples (trunk, fork

341   and branch) of tropical, urban trees (Izuddin et al., 2019) detected 26 orchid mycorrhizal fungi,

342   belonging to Ceratobasidiaceae, Serendipitaceae and Tulasnellaceae. The fork microsite had the

343   highest abundance, whereas the branches had the highest diversity and the trunks the lowest.

344   Ceratobasidiaceae were associated with the roots of three orchid species, Serendipitaceae with

345   six and Tulasnellaceae with two orchid species. They also found non-*Rhizoctonia* fungi shared

346   by bark and orchid roots and these could also prove to have mycorrhizal function (Ascomycetes

347   – *Fusarium, Lachnum, Curvularia*; Basidiomycetes – *Mycena, Marasmius*).

348   Agaricomycetes and Dothideomycetes were the most relatively abundant classes on the

349   collected bark samples. The latter was also highly abundant in a study analyzing the soil fungal

350   community in coffee plantations and a nearby forest in El Salvador (Rao et al., 2020). Classes

351   Sordariomyctes, Agaricomycetes, Lecanomyctes, Euromycetes were also highly abundant,

352   coinciding with those detected in high abundance in our study (Rao et al., 2020). This reflects

353   some similarities between fungal communities in coffee plantations in southern Mexico and

354   coffee plantations and forests in El Salvador.

355   We detected several taxa forming endo, ecto and arbuscular mycorrhizal fungi

356   belonging to 13 families (Table 2), suggesting that shade tree and coffee bush bark in the shade-

357   coffee plantations could serve as reservoir of fungi needed for the establishment and

358   development of epiphytic plants. We did not detect significant differences in alpha diversity

359   among sites, plant type or microsite. However, there are significant differences in beta diversity

360   among sites, plant type and some microsites. Differences in beta diversity, and thus in

361   community composition, between sites could be associated with differences in altitude (Gómez-

362   Hernández et al., 2012; Ogwu et al., 2019; Arias Mota & Heredia Abarca, 2020). It is possible

363   that some species have more affinity for higher altitudes (537 vs 1500 m. a. s. l.), but sampling at

364   intermediate sites would help to understand the effect of altitude and other environmental

365   variables on fungal diversity and community composition. Distance from conserved or perturbed

366   areas might be an important factor influencing community composition as this may affect the re-

367   colonization potential of the sites (López-Quintero et al., 2012; Hazard et al., 2013). Benito

368   Juárez el Plan is situated 200 m away from areas still covered with original vegetation, whereas

369   Los Hermanitos is several kilometers away. On the other hand, it is unlikely that time since land

370   use changed has influenced beta diversity as both sites were transformed into shade-coffee

371   plantations around the same time, more than 50 years ago.

372   The NMDS plot does not show a clear distinction between samples collected on coffee

373   bushes and shade trees, but species composition was significantly differently according to the

374   PERMANOVA and adonis tests. Fungi community composition in the microsite 'twig' showed

375   significant differences from other microsites more often than other microsites, holding the

376   highest number of exclusive taxa as demonstrated with the indicator species test. A possible

377   explanation for differences in beta diversity in microsites and presence of indicator species might

378   be a result of differences in bark texture of the coffee bushes and shade trees, as well as

379   differences in water retention, pH or chemical composition that can be more favorable for some

380   species of fungi than others (Pecoraro et al., 2021).

381   Interestingly, some of the 'indicator species' are considered as pathogens; this kind of

382   function receives particular attention due to its economic importance and might be the reason

383   why they could be identified. However, there were other 26 ASVs classified as indicator species

384   in our analysis that could not be taxonomically assigned (Supplementary File 2). In addition to

385   the ecological roles that the different fungi species are playing in the coffee plantations, it is

386   worth mentioning that some of them – or their relatives – have biotechnological potential (e.g.

387   *Tolypocladium*; Borel, Kis & Beveridge, 1995) which adds another ecosystem service provided

388   by shade-coffee plantations in Soconusco, and other regions of the world, in areas previously

389   covered by cloud forest. A biogeographic study of fungi in the Neotropical Cloud Forest

390   (NTCF), resulted in a species list and distribution records comprising 2962 species (Del Olmo-

391   Ruiz et al., 2017). We detected 40 of those species in the shade-coffee plantations in Soconusco,

392   which are probably a remnant of the diversity held by the original cloud forest. However, this is

393   likely an underestimation as most of the ASVs in our study could not be assigned to species

394   level. On the other hand, we detected 19 ASVs classified as Glomeromycota that comprise

395   arbuscular mycorrhizae. Interestingly, Del Olmo Ruíz et al. (2017), defined an area of

396   endemicity of cloud forest in the Chiapas Highlands based on the distribution of six species of

397   Glomeromycota. The authors hypothesize that these arbuscular mycorrhizae fungi and their

398   association with epiphytic bromeliads are relevant for community assembly in the cloud forest.

399   Del Olmo Ruíz et al. (2017) found that Ascomycota and Basidiomycota were the most abundant

400   phyla in the cloud forest in the Mesoamerica region. These Fungi phyla were the most relatively

401   abundant phyla in our study and in the Fungi community described by Rao et al. (2020) in soil

402   samples from a shade-coffee plantation in El Salvador.

403   All trophic modes considered in the FUNGuild database are present in the collection

404   sites and on microsites. As we did not sterilize the bark samples, the fungal community reflects

405   the available organisms in the collection site and surrounding areas (Ovaskainen et al., 2020).

406   Symbiotrophs have higher abundances on coffee bushes microsites than on shade trees. The

407   presence of fungi with this trophic mode on bark is essential for the establishment of epiphytic

408   plants (McCormick, Whigham & Canchani-Viruet, 2018). Although, establishment also depends

409   on the entire fungal community, for example competitors, other symbionts enabling plant

410   growth, and the availability of nutrients produced by other fungi, etc. (Pecoraro et al., 2018).

411   Thus, shade-coffee plantations still hold some of the fungal diversity of the cloud forest,

412   allowing the occurrence of important ecological processes such as mycorrhizal formation and

413   epiphyte development. The type and number of mycorrhizal fungi detected depends greatly on

414   taxonomic assignment, and this is limited by the available databases. In turn, this affects guild

415   assignment and classification as specialists or generalists. Thus, it is likely that the diversity of

416   mycorrhiza and other fungi we detected is an underestimation, which should serve to encourage

417   further study of the ecology, taxonomy and molecular identification of these taxa.

418

Conclusions

420 We detected high fungal diversity in shade-coffee plantations in Soconusco, Chiapas, possibly

421 including remnants of the original cloud forest ecosystem. Several mycorrhiza forming fungi

422 guilds occur on the bark of coffee bushes and shade trees in this agroecosystem, with the

423 potential of supporting epiphyte establishment and development. We are working towards

424 developing an integrated strategy for a return to traditional coffee cultivation (Perfecto &

425 Armbrecht, 2002; Harvey et al., 2008), in protected areas in the region of Soconusco, with

426 particular emphasis on the restoration and conservation of epiphytic plants (e.g. orchids) within

427 this agroecosystem (Hietz, 2005; Toledo & Moguel, 2012, p.; Toledo-Aceves et al., 2013). It is

428 paramount to restore microorganisms that facilitate and promote ecosystem health in general and

429 the various stages of plant development.

430

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Tables

719 **Table 1.** Fungal taxa associated with bark microsites in coffee bushes and shade trees according

720 to an indicator species analysis. Only taxa that were classified to genus and species are shown.

721 **Table 2.** Total occurrence of sequences classified as mycorrhizal fungi by FUNGuild in bark

722 samples from four microsites on coffee bushes and shade trees in two shade-coffee plantations in

723 Soconusco, Mexico.

724 **Table 3.** CLAM classification of mycorrhiza forming fungi guilds as specialist, generalist or

725 rare, detected in bark from coffee bushes and shade trees in two shade-coffee plantations in

726 Soconusco, Mexico.

727

Figure Legends

729 **Figure 1.** Boxplots showing Shannon Index for Fungi alpha diversity detected on bark samples,

730 according to collection site, plant type, sampled plant part and microsite in two shade-coffee

731 plantations. B: Benito Juárez El Plan, H: Hermanitos; CTw: coffee twig; CTh: coffee trunk high;

732 CTl: coffee trunk low; CBr: coffee branch; TTw: tree twig; TTh: tree trunk high; TTl: tree trunk

733 low; TBr: tree branch

734 **Figure 2.** NMDS plots showing groupings of bark samples according to a) collection site; b)

735 plant type; c) sampled plant part; and d) microsite. Notice the clear separation associated with the

736 collection site. B: Benito Juárez El Plan; H: Hermanitos

737 **Figure 3.** Heatmap showing the frequency of sequences assigned to Fungi Classes in samples

738 taken from coffee bushes and shade trees in two shade-coffee plantations in Soconusco, Chiapas,

739 Mexico.

740   **Figure 4.** Boxplots showing trophic mode frequency per microsite according to FUNGuild

741   analysis. Bark samples were taken from coffee bushes and shade trees in two shade-coffee

742   plantations in Soconusco, Mexico. Relative read abundance given in log scale.

743   **Figure 5.** Mycorrhizal guild frequency per plant type, detected in bark samples obtained from

744   four microsites on coffee bushes and shade trees in two shade-coffee plantations in Soconusco,

745   Chiapas, Mexico.

746

Fungal taxa associated with bark microsites in coffee bushes and shade trees according to an indicator species analysis. Only taxa that were classified to genus and species are shown.

A: Ascomycota; B: Basidiomycota; CTw: coffe twig; CTh: coffee trunk high; CTl: coffee trunk low; CBr: coffee branch; TTw: tree twig; TBr: tree branch

1 **Table 1.** Fungal taxa associated with bark microsites in coffee bushes and shade trees according to an indicator species analysis.
2 Only taxa that were classified to genus and species are shown.

CTw	A: Glomerellales, *Colletotrichum gigasporum*	Genus globally distributed, on various plants as epiphyte, saprobe, endophyte and pathogen. It has been reported on coffee (Douanla-Meli & Unger, 2017).
	B: Tremellales, *Bulleribasidium*	11 species, some parasitize other fungi (e.g. *Cladosporium sp.*) (Sampaio et al., 2002).
CBr, CTh, CTl	A: Hypocreales, *Tolypocladium*	Parasites of truffle-like fungi, soil saprotrophs, plant endophytes, pathogens of insects, nematodes, rotifers (Borel et al., 1976; Yu et al., 2021).
TTw	A: Chaetothyriales, *Strelitziana africana*	Isolated from leaves of Strelitzia in South Africa (Arzanlou & Crous, 2006).
	A: Capnodiales, *Cladosporium*	Plant pathogens, fungi parasites, soil inhabitants. Spores are wind dispersed and abundant (Parbery, 1969).
	A: Ostropales, *Absconditella rubra*	Genus of lichenized fungi (Czarnota, 2016).
	A: Capnodiales, *Pseudocercospora norchiensis*	Leaf pathogens on many economically and ecologically important plant species, including *Eucalyptus* and neotropical species (Pérez et al., 2013).
	A: Pleosporales, *Periconia*	Plant and occasional human pathogens, saprobes. Genus reported in cloud forest of Veracruz, Mexico (Arias Mota & Heredia Abarca, 2020).
TBr	A: Xylariales, *Pestalotiopsis*	Plant pathogens; they have been reported on coffee plants (Song et al., 2013).

	A: Xylariales, *Biscogniauxia*	Some species are tree pathogens (Nugent et al., 2005).
TBr, TTw	A: Xylariales, *Pseudopestalotiopsis simitheae*	Isolated from *Pandanus*; pathogenes, considered a threat to tropical hosts; reported in a wide range of hosts across the world (Nozawa et al., 2017; Gualberto et al., 2021).

3 A: Ascomycota; B: Basidiomycota; CTw: coffe twig; CTh: coffee trunk high; CTl: coffee trunk low; CBr: coffee branch; TTw: tree
4 twig; TBr: tree branch

Total occurrence of sequences classified as mycorrhizal fungi by FUNGuild in bark samples from four microsites on coffee bushes and shade trees in two shade-coffee plantations in Soconusco, Mexico.

CTw: coffe twig; CTh: coffee trunk high; CTl: coffee trunk low; CBr: coffee branch; TTw: tree twig; TTh: tree trunk high; TTl: tree trunk low; TBr: tree branch

1   **Table 2.** Total occurrence of sequences classified as mycorrhizal fungi by FUNGuild in bark samples from four microsites on coffee
2   bushes and shade trees in two shade-coffee plantations in Soconusco, Mexico.

Sequences	Guild	Lowest Taxonomy	Microsite
8,555	Ectomycorrhizal-Fungal Parasite-Plant Pathogen-Wood Saprotroph	Helotiaceae	CTw,CTl,CTh,CBr, TTl
6,667	Endomycorrhizal-Plant Pathogen-Undefined Saprotroph	Ceratobasidiaceae	CTw,CTl,CTh,CBr, TTl,TTh,TBr
4,287	Ectomycorrhizal-Fungal Parasite	*Delicatula integrella*	CTl,TTl
1,649	Arbuscular Mycorrhizal	Diversisporales (*Acaulospora lacunosa*), Glomerales (*Rhizophagus*), Archaeosporales, Gigasporales	CTl,CTh,TTl,TTh
637	Orchid Mycorrhizal	*Serendipita*	CTl
429	Ectomycorrhizal-Fungal Parasite-Soil Saprotroph-Undefined Saprotroph	*Entoloma*	CTw,CTl,CTh,TTh ,TBr
120	Bryophyte Parasite-Ectomycorrhizal-Ericoid Mycorrhizal-Undefined Saprotroph	*Rhizoscyphus monotropae*	CTl
117	Bryophyte Parasite-Lichen Parasite-Ectomycorrhizal-Ericoid Mycorrhizal-Undefined Saprotroph	*Pezizella*	CTl,CTh,CBr
106	Ectomycorrhizal-Wood Saprotroph	*Tropicoporus linteus*	CTl,CTh
97	Orchid Mycorrhizal-Plant Pathogen-Wood Saprotroph	*Rhizoctonia fusispora*	TTl
93	Ectomycorrhizal	Sebacinales, *Endogone*	CTl, CTh
10	Bryophyte Parasite-Ectomycorrhizal-Ericoid Mycorrhizal-Undefined Saprotroph-Wood Saprotroph	*Pezoloma ericae*	CTh

3   CTw: coffe twig; CTh: coffee trunk high; CTl: coffee trunk low; CBr: coffee branch; TTw: tree twig; TTh: tree trunk high; TTl: tree
4   trunk low; TBr: tree branch

CLAM classification of mycorrhiza forming fungi guilds as specialist, generalist or rare, detected in bark from coffee bushes and shade trees in two shade-coffee plantations in Soconusco, Mexico.

1 **Table 3.** CLAM classification of mycorrhiza forming fungi guilds as specialist, generalist or
2 rare, detected in bark from coffee bushes and shade trees in two shade-coffee plantations in
3 Soconusco, Mexico.

Guild type	Coffee bushes (total sequences)	Shade trees (total sequences)	Classes
Arbuscular mycorrhizal	900	749	Specialist: tree
Ectomycorrhizal	93	0	Specialist: coffee
Endomycorrhizal, plant pathogen, undefined saprotroph	2,418	4,249	Specialist: tree
Ectomycorrhizal, fungal parasite, soil saprotroph, undefined saprotroph	300	129	Generalist
Ectomycorrhizal, fungal parasite, plant pathogen, wood saprotroph	8,530	25	Specialist: coffee
Ectomycorrhizal, wood saprotroph	106	0	Specialist: coffee
Ectomycorrhizal, fungal parasite	3,671	616	Generalist
Orchid mycorrhizal, plant pathogen, wood, saprotroph	0	97	Specialist: tree
Orchid mycorrhizal	637	0	Specialist: coffee
Bryophyte parasite, ectomycorrhizal, ericoid, mycorrhizal, undefined saprotroph	120	0	Specialist: coffee
Bryophyte parasite, ectomycorrhizal, ericoid, mycorrhizal, undefined saprotroph, wood saprotroph	10	0	Too rare
Bryophyte parasite, lichen parasite, ectomycorrhizal, ericoid mycorrhizal, undefined saprotroph	117	0	Specialist: coffee

Figure 1

Boxplots showing Shannon Index for Fungi alpha diversity detected on bark samples, according to collection site, plant type, sampled plant part and microsite in two shade-coffee plantations

B: Benito Juárez El Plan, H: Hermanitos; CTw: coffee twig; CTh: coffee trunk high; CTl: coffee trunk low; CBr: coffee branch; TTw: tree twig; TTh: tree trunk high; TTl: tree trunk low; TBr: tree branch
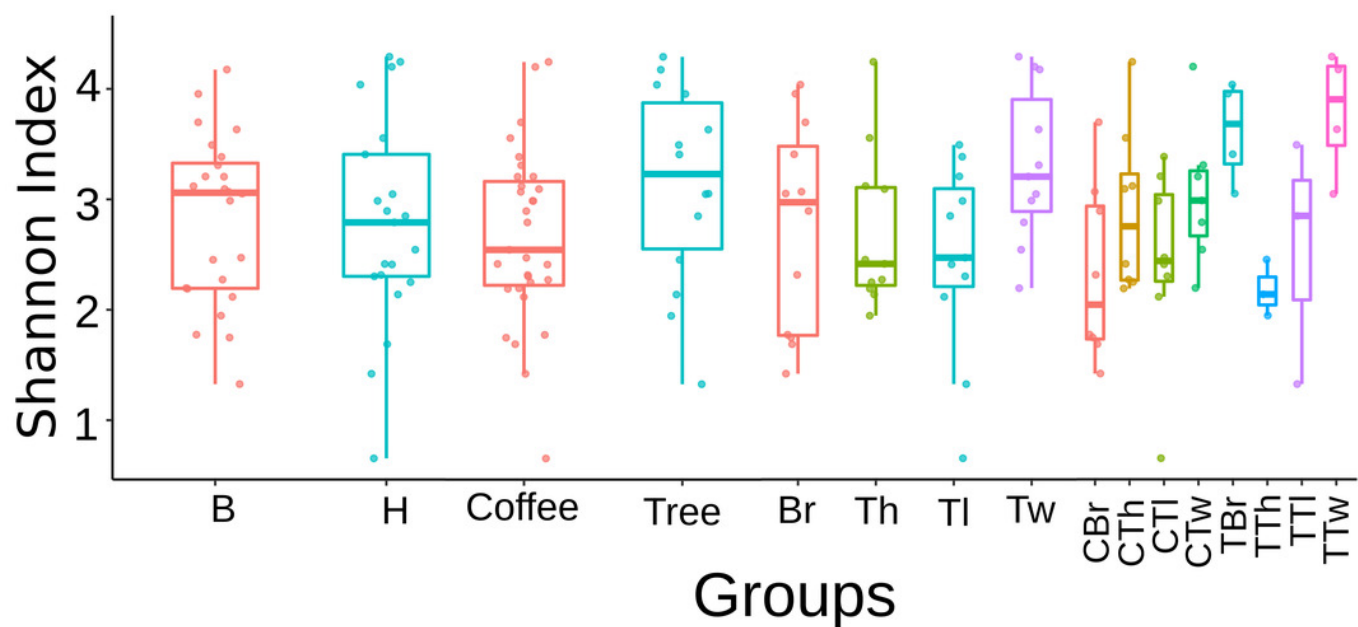
Figure 2

NMDS plots showing groupings of bark samples according to a) collection site; b) plant type; c) sampled plant part; and d) microsite. Notice the clear separation associated with the collection site.

B: Benito Juárez El Plan; H: Hermanitos
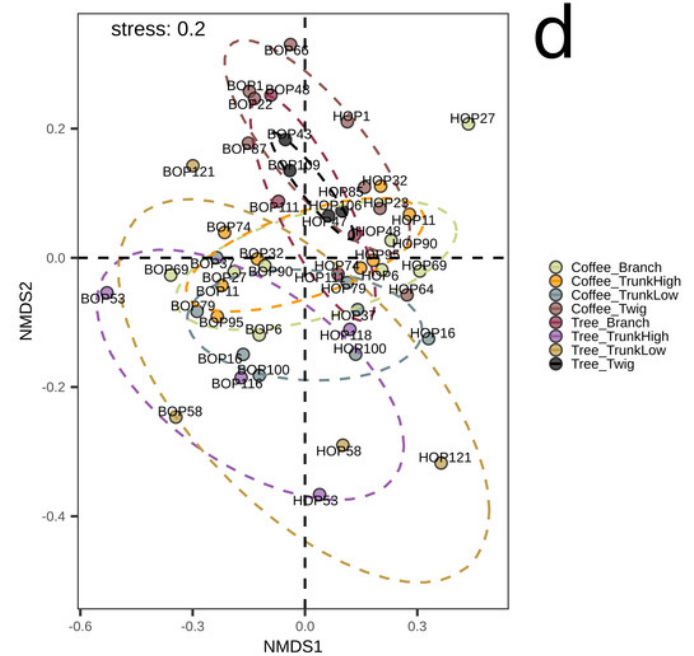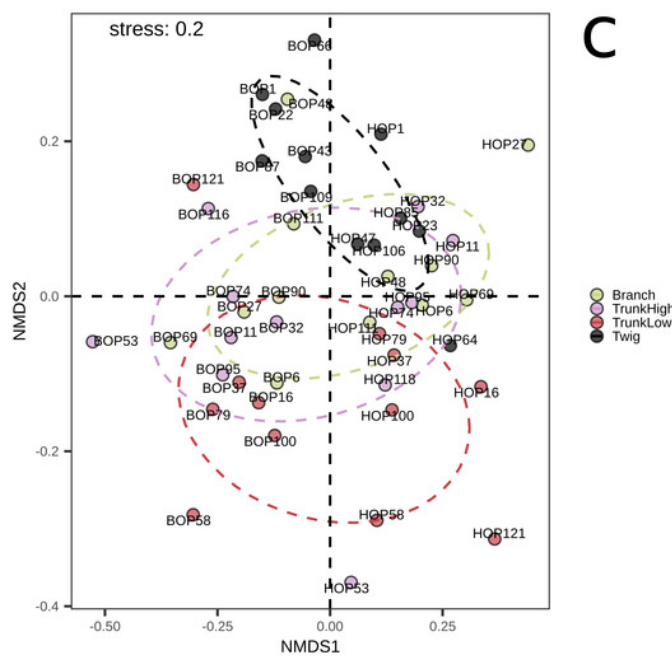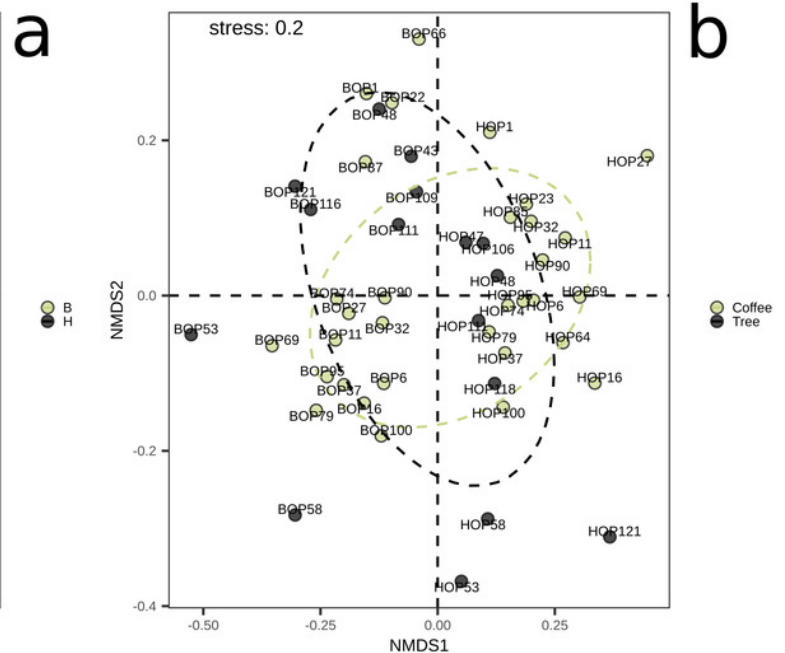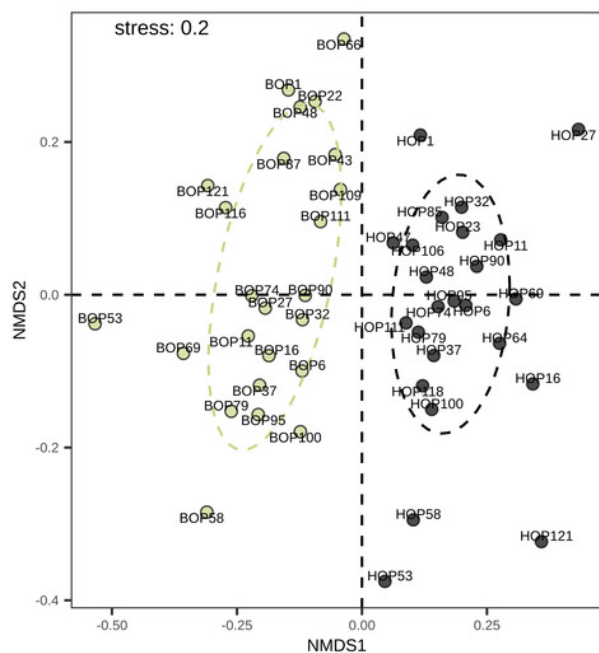
Figure 3

Heatmap showing the frequency of sequences assigned to Fungi Classes in samples taken from coffee bushes and shade trees in two shade-coffee plantations in Soconusco, Chiapas, Mexico.
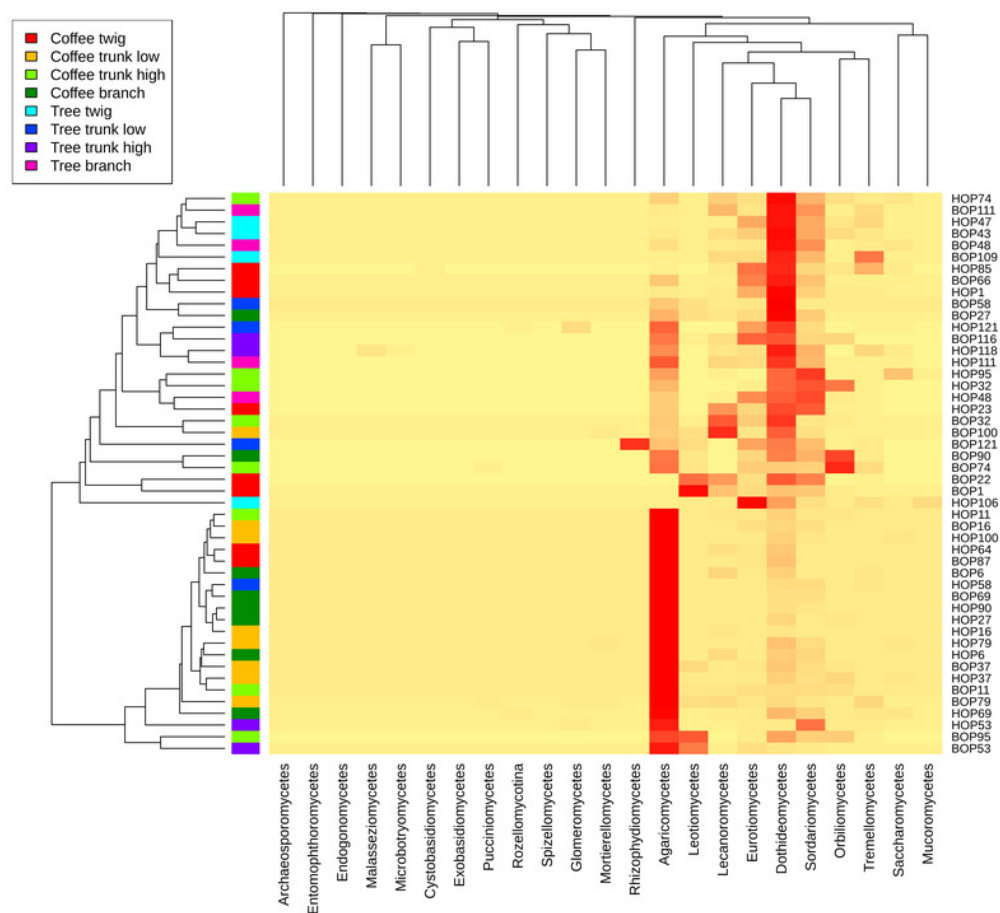
Figure 4

Boxplots showing trophic mode frequency per microsite according to FUNGuild analysis. Bark samples were taken from coffee bushes and shade trees in two shade-coffee plantations in Soconusco, Mexico.

Relative read abundance given in log scale.
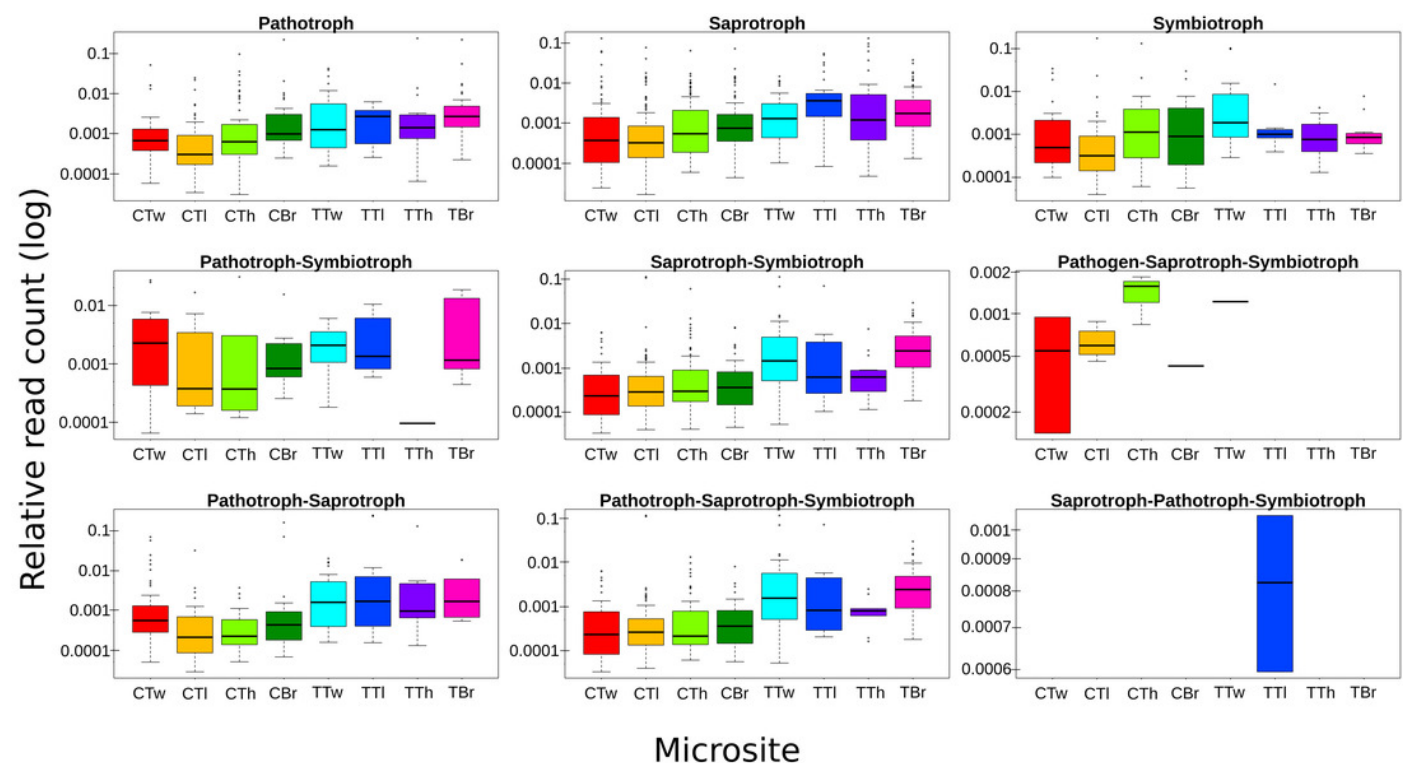
Figure 5

Mycorrhizal guild frequency per plant type, detected in bark samples obtained from four microsites on coffee bushes and shade trees in two shade-coffee plantations in Soconusco, Chiapas, Mexico.