The complexities of inferring symbiont function: *Paraburkholderia* symbiont dynamics in social amoeba populations and their impacts on the amoeba microbiota

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Abstract

The relationship between the social amoeba *Dictyostelium discoideum* and its endosymbiotic bacteria *Paraburkholderia* provides a model system for studying the development of symbiotic relationships. Laboratory experiments have shown that any of three species of *Paraburkholderia* symbiont allow *D. discoideum* food bacteria to persist through the amoeba lifecycle and survive in amoeba spores, rather than being fully digested. This phenomenon is termed “farming”, as it potentially allows spores dispersed to food poor locations to grow their own. The occurrence and impact of farming in natural populations, however, has been a challenge to measure. Here, we surveyed natural *D. discoideum* populations and found that only one of the three symbiont species, *P. agricolaris,* remained prevalent. We then explored the effect of *Paraburkholderia* on the amoeba microbiota, expecting that by facilitating bacterial food carriage it would diversify the microbiota. Contrary to our expectations, *Paraburkholderia* tended to infectiously dominate the *D. discoideum* microbiota, in some cases decreasing diversity. Similarly, we found little evidence for *Paraburkholderia* facilitating the carriage of particular food bacteria. These findings highlight the complexities of inferring symbiont function in nature and suggest the possibility that *Paraburkholderia* could be playing multiple roles for its host.

Key words: Symbiosis, farming, *Paraburkholderia*, *Dictyostelium discoideum*, amoeba, microbiota

Introduction

Bacterial symbionts have been found to associate with hosts across the tree of life, with often dramatic effects on their ecology and evolution. The roles of bacterial symbionts within their hosts can range from mutualism to parasitism and can vary based on host and environmental contexts, as well as on the length of the association and mode of transmission (R. M. Anderson & May, 1982; Ewald, 1987; Moran, 2006). Amoebae, a polyphyletic group of unicellular eukaryotes, serve as promising model systems for studying symbiotic relationships, particularly the bacterial transition from free-living to intracellular. When amoebae feed on bacteria, a selective pressure is exerted that favors bacteria with mechanisms to resist digestion (Brüssow, 2007; Dunn et al., 2018; Sun et al., 2018). Hence, amoebae serve as intracellular training grounds for bacteria to evolve phagocytosis resisting mechanisms, allowing certain bacteria to occupy amoebae as endosymbionts (Casadevall, 2008; Greub & Raoult, 2004; Molmeret et al., 2005). Bacterial endosymbionts have been commonly identified in *Acanthamoeba* and *Vermamoeba* hosts, including lineages of Chlamydiae, *Proteobacteria*, *Bacteroidetes*, and *Dependentiae* (Delafont et al., 2015; Horn & Wagner, 2004; Schmitz-Esser et al., 2008), although the effects on their hosts are often unclear. For example, while some Chlamydiae symbionts live as energy parasites that are costly to their hosts (Horn, 2008), other Chlamydiae species provide benefits by protecting *Acanthamoeba* from the pathogenic *Legionella* bacteria and enhancing cell motility and growth. (Ishida et al., 2014; König et al., 2019; Maita et al., 2018; Okude et al., 2012). The difficulty of studying the microbial community dynamics in natural environments dampens our understanding of the ecological contexts of amoeba – bacteria interactions, which makes inferring the function of a particular symbiont in its host a challenge.

The social amoeba *Dictyostelium discoideum* has gained traction as a model organism to study host cell – bacteria interactions in laboratory settings. Social amoebae are soil-dwelling protists

that have unique life cycles that involve the transition from a single-cellular state to a multicellular state when their bacterial food source is depleted (Bozzaro, 2019). Tens of thousands of amoeba cells aggregate to ultimately form a fruiting body, which consists of two primary structures: the sorus, a structure used to house spores, and the stalk, which is composed of dead cells that differentiated to support the sorus (Kessin, 2001). It was previously thought that sentinel cells, which perform immune-like functions, cleared all bacteria from the amoeba fruiting body throughout the social cycle (G. Chen et al., 2007), as evidenced by the inability to culture bacteria from many laboratory-isolated *D. discoideum* clones. Recent studies, however, have shown that in nature *D. discoideum* harbors microbiota consisting of both transient and persistent bacterial symbionts (Brock et al., 2018; Sallinger et al., 2021), belonging to the phyla Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes, Chlamydiae, and Acidiobacteria. This recent characterization of the *D. discoideum* microbiota has expanded its potential to serve as a model system for studies pertaining to microbiome formation and maintenance (Farinholt et al., 2019) and some evidence suggests that *D. discoideum* may play an active role in its microbiota formation by secreting lectin proteins that bind bacteria during the social cycle (Dinh et al., 2018).

In natural populations of *D. discoideum*, the most widespread and persistent bacterial symbionts are *Paraburkholderia*, Chlamydiae, and *Amoebophilus* (Haselkorn et al., 2021). Although the functions of these symbionts in natural populations are largely unknown, the *Paraburkholderia* farming symbionts have been the best characterized. The phenomenon of farming was demonstrated primarily in laboratory experiments by comparing the ability of *Paraburkolderia* infected and uninfected *D. discoideum* to grow when a sorus was plated without food bacteria. *Paraburkholderia* are inedible, but they allow bacterial food carriage that is presumed to benefit the amoeba in nature if the spores are dispersed to an area that is scarce in food bacteria, as

the soil could be seeded with the carried bacteria (Brock et al., 2011; DiSalvo et al., 2015; Haselkorn et al., 2019). There is, however, a cost to infection, as *Parburkholderia*-infected *D. discoideum* produce fewer spores in food rich laboratory conditions. While many members of the *Paraburkholderia* and closely related *Burkholderia* genera form symbiotic interactions with a variety of plant, fungi, and insect hosts (Esmaeel et al., 2018; Kaltenpoth & Flórez, 2020; Nazir et al., 2014), only three *Paraburkholderia* species, *P. agricolaris*, *P. bonniea*, and *P. hayleyella* have been found to confer farming and form persistent symbiotic relationships with *D. discoideum* (Haselkorn et al., 2019). The variability in fitness costs and the extent of food carriage that each symbiont permits makes this *Paraburkholderia – D. discoideum* symbiosis context dependent (Khojandi et al., 2019), like many symbiotic relationships (Oliver et al., 2008). Screenings for *Paraburkholderia* in natural *D. discoideum* populations have shown that *P. agricolaris* tends to be the most prevalent, followed by *P. hayleyella* and *P. bonniea* (Haselkorn et al., 2019). Indeed, these differences in symbiotic potential likely translate to different host outcomes, which could be responsible for the differing levels of prevalence between each *Paraburkholderia* species.

Measurements of fitness in laboratory settings have aided our understanding of the consequences of *Paraburkholderia* infection, but its role in natural populations is less clear. To better understand the occurrence and impact of this of this symbiosis, we used PCR based screenings of *D. discoideum* populations to identify current symbiont prevalence, transmission patterns, and genetic diversity. Since symbiont transmission modes play a large role in determining whether a symbiont's evolutionary trajectory will lead to mutualism, benign parasitism, or extreme pathogenicity (R. M. Anderson & May, 1982), we looked for evidence showing whether *P. agricolaris* was environmentally acquired or vertically transmitted between social amoeba hosts. To investigate how *Paraburkholderia* might be conferring bacterial food

121 carriage in a natural setting, we compared the microbiota of infected and uninfected *D.*
122 *discoideum*. We hypothesized that if *Paraburkholderia* was indiscriminately facilitating the
123 carriage of food bacteria, infected amoeba would possess more diverse microbiota.
124 Alternatively, if *Paraburkholderia* was facilitating the carriage of certain food bacteria, perhaps
125 indicative of *D. discoideum* feeding preferences, we hypothesized that *Paraburkholderia*
126 infected amoeba would have microbiota that were similar in composition to each other.
127 Inconsistent with our hypotheses, we found that *Paraburkholderia* tended to dominate the
128 microbiota, often correlating with a decrease in microbiota diversity. Overall, our results provide
129 insight into the natural history of the *Paraburkholderia – D. dicoideum* relationship and further
130 our understanding of the ecological relevance of the farming phenotype.

131

132 **Methods**

133 Soil Collection and Amoeba Culturing

134 We re-sampled natural *D. discoideum* populations in the Virginia Mountain Lake area
135 (Haselkorn et al., 2019, 2021). Soil, sampled from below the leaf litter layer of the forest floor or
136 below rotting logs, was collected from ten different sites at each of four separate locations
137 (Supplemental Table 1) and was plated on hay agar within 96 hours (Supplemental Table 2).
138 After 4-5 days of growth time, Dictyostelid species were morphologically identified via
139 stereomicroscopy (Raper, 2014), and 5-10 sori from each patch were collected with sterile
140 pipette tips and suspended into 250μL of KK2 spore buffer. Every patch of *D. discoideum*,
141 identified by the presence of a basal disc at the base of its stalk, was collected, as well as other
142 social amoeba species, including *D. mucoroides*, *D. giganteum*, *D. purpureum*, *Heterostelium*
143 *pallidum*, and *Polysphondylium violaceum*. We then resuspended fruiting bodies in 100μL of
144 KK2 buffer for “passaging”: replating onto SM/5 agar (Supplemental Table 2) with the addition of

145 200µL of a 1.5 OD_{600nm} suspension of *Klebsiella pneumoniae* food bacteria to increase the
146 quantity of fruiting bodies. Subsets from each amoeba species were sequenced to confirm
147 amoeba identity (See following section).

148

149 Symbiont Screening

150 DNA from 15-20 fruiting bodies from the passaged SM/5 plates was extracted using the a
151 Chelex/proteinaseK protocol as in (Haselkorn et al., 2019). PCRs were conducted to screen
152 each sample for *Dictyostelium* DNA by amplifying approximately 500 base pairs of the 18S
153 rRNA gene (Baldauf et al., 2018). To screen for *Paraburkholderia*, we used the
154 *Paraburkholderia* specific primers that amplify a small section of the 16S rRNA gene (Salles et
155 al., 2002). PCR primers and amplification conditions are listed in Supplemental table 3. Each
156 PCR screen included a positive control, two negative PCR controls, and two negative DNA
157 extraction controls to ensure there was no contamination. Positive samples were sent for
158 Sanger sequencing at Eurofins Genomics. Sequences were trimmed using Geneious v8
159 (http://www.geneious.com) (Kearse et al., 2012), and NCBI Blast was used to classify
160 *Paraburkholderia* species. In samples that were identified as either *P. agricolaris*, *P. bonniea*, or
161 *P. hayleyella*, the LepA gene, which allows for better species level identification and haplotype
162 resolution (Haselkorn et al., 2019), was then amplified and sequenced (Spilker et al., 2009).
163 Additionally, we screened for Chlamydiae and *Amoebophilus* using symbiont specific primers for
164 each (Ossewaarde & Meijer, 1999) (Supplemental Table 3).

165

166 *P. agricolaris* phylogeny, Population Genetics, and Co-infection Statistics

167 LepA sequences were used to construct a phylogenetic tree of *P. agricolaris* haplotypes.
168 Overlapping peaks in the DNA chromatogram at a single site were indicative of co-infections

169 with multiple haplotypes and were excluded from analysis because haplotype designations were
170 not able to be resolved. A Maximum Likelihood phylogenetic tree was constructed in MEGA7
171 using the Tamura 3-parameter+G+I model with 1 000 bootstrap replicates (Kumar et al., 2016).
172 Previously identified LepA haplotypes were also placed to determine if these haplotypes had
173 previously been identified (Haselkorn et al., 2019).

174

175 To examine *P. agricolaris* genetic differentiation between *D. discoideum* populations, pairwise
176 F_{ST} values were calculated in DNAsp (v6) (Rozas et al., 2017) using non-coinfected LepA
177 sequences (n=65). Concatenated 16S rRNA and LepA sequences were then used to look for
178 evidence of recombination using the Recombination Detection Program (RDP) (v4) (Martin et
179 al., 2015). Pairwise Fisher exact tests (2x2 contingency tables) between *P. agricolaris*,
180 Chlamydiae, and *Amoebophilus*, were used to investigate ecological co-infection tendencies
181 between symbionts.

182

183 <u>Laboratory inoculations of *D. discoideum* with different *Paraburkholderia* species</u>

184 Separate *P. agricolaris, P. bonniea*, and *P. hayleyella* solutions were made by resuspending
185 stationary phase bacterial colonies from SM/5 plates into KK2 buffer at a concentration of 1.5
186 OD_{600nm} and combined with 1.5 OD_{600nm} *K. pneumoniae* suspensions at a final volume of
187 95%/5% *K.pneumoniae*/*Paraburkholderia.* We then plated 10^5 *D. discoideum* spores (strain
188 QS864) with 200µL of each *Paraburkholderia* solution onto SM/5 plates and incubated at room
189 temperature. Amoebae were collected after one week of growth time, and *Paraburkholderia* was
190 cultured from fruiting bodies to confirm infection. Ten fruiting bodies from each plate were
191 resuspended in KK2 spore buffer and passaged again to confirm persistent *Paraburkholderia*
192 infection. *Paraburkholderia* infected *D. discoideum* lines were then inoculated into 2.5 grams of

193 local soil collected from a natural area maintained by the Department of Biology at the
194 University of Central Arkansas (Conway, AR). Soil contents were subsequently plated as
195 describe above, and *D. discoideum* fruiting bodies were collected directly from these soil pates
196 after one week of growth time.

197

198 DNA Extractions for Microbiota Analyses

199 Approximately 50-100 sori were collected from passaged SM/5 plates (natural infections) or soil
200 plates (lab infections), placed into Hyclone water, and stored at -80°C until DNA was extracted.
201 DNA extractions were performed using the Qiagen DNeasy PowerSoil Pro Kit and the Qiagen
202 DNeasy UltraClean Pro Kit, according to manufacturer's protocols. Samples were then sent for
203 sequencing to Argonne National Laboratories (Lemont, IL, USA). Here, samples were
204 sequenced on the Illumina MiSeq platform using 250bp paired-end cycles. Library preparation
205 and sequencing was performed using the 799F-1115R primer set, which amplifies the V5-V6
206 region of the 16S rRNA gene (Redford et al., 2010), as the more commonly used primers for the
207 V4 region preferentially amplify amoeba mitochondrial DNA (Sallinger et al., 2021). Sequences
208 were returned as FASTQ files with adaptors removed.

209

210 Illumina Sequence Processing

211 Illumina sequence files were demultiplexed and analyzed in QIIME2 (v2020.6). Chimeric
212 sequences were removed, primers were trimmed, and paired-ends were joined using q2-dada2
213 plugin (Callahan et al., 2016; Johnson et al., 2008; McDonald et al., 2012; Sayers et al., 2021).
214 The q2-RESCRIPt plugin was used to curate the SSU SILVA NR99 (version 138) database,
215 and to train a custom V5-V6 amplicon specific classifier (Robeson et al., 2020). The classify-
216 sklearn method was used in the q2-feature-classifier plugin to assign taxonomic names to

217 amplicon sequence variants (ASVs) (Bokulich et al., 2018; Janssen et al., 2018; McKinney,
218 2010). ASVs classified as either mitochondria or chloroplast, as well as ASVs that did not
219 classify to the phylum-level were removed for downstream analysis. ASVs with a percent
220 identity/alignment of less than 0.80/0.80 to the SILVA reference sequences were excluded using
221 the q2-quality-control plugin (Camacho et al., 2009). A phylogenetic tree for downstream
222 analyses was created by inserting ASVs into the Silva 128 reference database using SATé-
223 enabled phylogenetic placement (SEPP), where all fragments were successfully inserted
224 (Janssen et al., 2018). Although the V5-V6 region of 16S rRNA did not provide species level
225 resolution, we identified the genus *Burkholderia-Caballeronia-Paraburkholderia* to represent *P.*
226 *agricolaris* based on Sanger sequences of the more variable LepA gene in our naturally infected
227 study. In laboratory inoculated samples, the genus *Burkholderia-Caballeronia-Paraburkholderia*
228 was identified to represent both *P. agricolaris* and *P. hayleyella* and the genus *Pandorea* was
229 identified to represent *P. bonniea*, based on their high abundance in their respective infection
230 groups.

231

232 Microbiota Diversity Analyses

233 After constructing alpha-rarefaction curves, natural samples were rarefied to a depth of 3 950
234 and laboratory inoculated samples were rarefied to a depth of 4 000 (Weiss et al., 2017). This
235 allowed us to retain 28 *P. agricolaris* infected 16 uninfected natural samples and 19 *P.*
236 *agricolaris*, 9 *P. bonniea*, and 10 *P. hayleyella* artificially infected samples. Alpha-diversity was
237 evaluated by using the q2-diversity plugin to calculate average ASVs, Pielou's evenness, and
238 Faith's phylogenetic diversity (Janssen et al., 2018; Pielou, 1966). Significance of each metric
239 was evaluated using Kruskal-Wallis and pairwise Kruskal-Wallis tests (α=0.05 with FDR q-
240 values) (Kruskal & Wallis, 1952). Beta-diversity was evaluated by using the q2-deicode plugin to
241 calculate the Aitchison distances between non-rarefied sample groups (Martino et al., 2019).

242 Weighted UniFrac and Unweighted UniFrac were also used on rarefied data to evaluate beta-
243 diversity using the q2-diversity plugin (Chang et al., 2011; J. Chen et al., 2012; Faith et al.,
244 1987; C. A. Lozupone et al., 2007; C. Lozupone & Knight, 2005). Ordination plots were
245 generated for each beta-diversity metrics and between group significance was evaluated using
246 PERMANOVA and PERMDISP with 999 permutations and FDR adjusted p-values (M. J.
247 Anderson, 2001). When PERMAONVA and PERMDISP results were both significant, ordination
248 plots were consulted to determine separation between groups. The feature table generated by
249 QIIME2 was uploaded to microbiomeanalyst.ca to perform co-association analyses using the
250 SparCC method.

251

252 **Results**

253 *P. agricolaris* remains prevalent in natural *D. discoideum* populations but does not co-associate
254 with Chlamydiae or *Amoebophilus* endosymbionts

255 To identify the current prevalence of *D. discoideum* endosymbionts, we screened 244 *D.*
256 *discoideum* isolates collected from the Mountain Lake area in 2019 for *Paraburkholderia,*
257 Chlamydiae*,* and *Amoebophilus*. We then compared the prevalence of each endosymbiont in
258 2019 to that of a previous collection in 2000 (Haselkorn et al., 2019, 2021). *P. agricolaris*
259 exhibited a prevalence of 35.1%, up from a 14.9% prevalence in 2000. *P. bonniea* was only
260 found once in our sampling, resulting in a prevalence of 0.79% (down from 2.6% in the 2000
261 collection). *P. hayleyella*, which was previously identified to be 8% prevalent in 2000, was not
262 found in our collection. *P. agricolaris* appeared to be stochastically distributed between each
263 location and collection site, where prevalence varied from less than 15% at location 1 to greater
264 than 60% at location 4 (Figure 1, Supplemental Table 2). Chlamydiae, which was 19% prevalent
265 in 2000, was found to be 58% prevalent in our collection. *Amoebophilus*, 37% prevalent in 2000,

266 was found to be 18% prevalent in our collection. Pairwise Fishers exact tests were used to look
267 for signs of positive or negative co-associations between *P. agricolaris*, Chlamydiae, and
268 *Amoebophilus* at each location. A positive association was observed between *P. agricolaris* and
269 Chlamydiae at location 3 (p=0.0472, 23 expected with 26 observed); however, no other
270 significant associations were found (Supplemental Table 4).

271

272 <u>*P. agricolaris* is genetically diverse and shows evidence of recombination and environmental</u>
273 <u>acquisition</u>

274 To gain insight into the natural and co-evolutionary history of *P. agricolaris* and *D. discoideum,*
275 we investigated *P. agricolaris* haplotype diversity, genetic recombination tendencies, and
276 transmission patterns. Phylogenetic analyses of LepA sequences (n=65) revealed ten distinct
277 haplotypes, and placement of previous *P. agricolaris* sequences showed that 7 of these
278 haplotypes have not been previously identified (Figure 2a) (Haselkorn et al., 2019). Haplotype 7
279 was the only haplotype found in amoebae from multiple locations and pairwise F_{ST} comparisons
280 showed that the locations harboring haplotype 7 were the most similar in terms of *P. agricolaris*
281 genetic composition (Supplemental table 4). Furthermore, up to 5 different haplotypes were
282 identified in amoebae from a single soil sample (Supplemental Table 5). Using concatenated
283 16S rRNA and LepA sequences we identified two putative inter-locus recombination events,
284 both between haplotypes 2 and 9. We used the presence of a *P. agricolaris* haplotype infecting
285 multiple amoebae species as evidence of environmental acquisition. In addition to infecting *D.*
286 *discoideum,* haplotype 3 was identified in *D. purpureum,* haplotype 4 was identified in *D.*
287 *mucoroides*, haplotype 8 was identified in *H. pallidum*, and haplotype 7 was identified in *D.*
288 *giganteum, H. pallidum, P. violaceum*, and *D. mucoroides* (Figure 2).

289

290 *Paraburkholderia* symbionts dominate the *D. discoideum* microbiota and infection correlates
291 with a general decrease in microbiota diversity

292 Our investigation of the impacts of *Paraburkholderia* on the natural *D. discoideum* microbiota
293 was limited to *P. agricolaris*, as *P. bonniea* and *P. hayleyella* were not found. Overall, denoising
294 of 16S rRNA sequences resulted in 130 amplicon sequence variants (ASVs) with a total
295 frequency of 1 573 424 reads. These were represented by 6 phyla, 9 classes, 33 orders, 49
296 families, and 57 genera. The majority of sequences belonged to the genus *Burkholderia-*
297 *Caballeronia-Paraburkholderia* (57.16%), which was identified to represent *P. agricolaris* based
298 on Sanger sequences of the more variable LepA gene. Although we did not find *P. hayleyella* or
299 *P. bonniea* infected *D. discoideum* in nature, we were able to gain insights on their impacts by
300 culturing artificially infected *D. discoideum* on local soil. This yielded 168 ASVs with a total
301 frequency of 2 135 808 reads. These were represented by 10 phyla, 15 classes, 49 orders, 77
302 families, and 140 genera.

303

304 If *Paraburkholderia* infection was somehow facilitating the carriage of random environmental
305 bacteria, we posited that *Paraburkholderia*-infected *D. discoideum* would have a more diverse
306 microbiota than uninfected ones. However, we found that *P. agricolaris* tended to dominate the
307 *D. discoideum* microbiota, accounting for an average of 71.35% of bacterial community
308 abundance, for greater than 90% of reads in the majority (16/28) of infected samples, and for
309 76.86% of all reads (Figure 3a). The *D. discoideum* microbiota evenness and richness was not
310 significantly altered by *P. agricolaris* infection (Pielou's Evenness, p=0.213399; observed ASVs,
311 p=0.376987). However, *D. discoideum* infected with *P. agricolaris* did possess less
312 phylogenetically diverse microbiota than uninfected *D. discoideum* (Faith's Phylogenetic
313 Diversity, p=0.048117) (Figure 4a). Laboratory inoculation of each *Paraburkholderia* species
314 into *D. discoideum* yielded similar results. *P. agricolaris*, *P. bonniea*, and *P. hayleyella* each had

315 abundances of greater than 90% in 13/19, 8/9, and 10/10 infected *D. discoideum* isolates,
316 respectively (Figure 3b). Consistent with the trend in *Paraburkholderia* abundance, laboratory
317 inoculation with each of the *Paraburkholderia* species resulted in significant reductions in *D.*
318 *discoideum* microbiota evenness, richness, and phylogenetic diversity (Pielou's Evenness, $p \leq$
319 0.001515; observed features (ASVs), $p \leq 0.041061$; Faith's Phylogenetic Diversity, $p \leq$
320 0.041759) (Figure 4b). *P. hayleyella* infection significantly reduced microbiota evenness when
321 compared to *P. agricolaris* ($p = 0.041457$), but no other pairwise differences were observed
322 between each *Paraburkholderia* species (Figure 4b).

323

324 Overabundance of *Paraburkholderia* alters the composition and structure of the *D. discoideum*
325 microbiota

326 While *Paraburkholderia* infection did not diversify the *D. discoideum* microbiota, we thought it
327 possible that *Paraburkholderia* might facilitate the carriage of specific food bacteria, altering the
328 microbiota structure and/or composition. ß-diversity comparisons using weighted UniFrac, which
329 measures microbial community structure by considering the phylogenetic relatedness and
330 relative abundances of each ASV, showed that the microbiota of infected *D. discoideum* were
331 similar to each other, and dissimilar to the microbiota of uninfected *D. discoideum*
332 (PERMANOVA, pseudo-F=10.5405, p=0.001; PERMDISP, F=8.97892, p=0.01; where
333 significant PERMDISP results were negated by the clear grouping of *P. agricolaris* infected
334 microbiota in PCoA space) (Figure 5a). This is not surprising given the high relative abundance
335 of *Parburkholderia*. ß-diversity comparisons using unweighted UniFrac, which measures
336 microbial community composition by only considering phylogenetic relatedness and presence or
337 absence of ASVs showed the effect of *Paraburkholderia* without directly considering its relative
338 abundance. Here we also saw significant differences between *P. agricolaris* and infected and
339 uninfected amoebae (PERMANOVA, pseudo-F=3.3698, p=0.002; PERMDISP, F=14.0089,

340 P=0.003 where significant PERMDISP results were negated by a clear distinction between
341 infected and uninfected samples upon PCoA visualization). (Figure 5b). The impact, however, is
342 unlikely due to carriage of specific food bacteria, but rather that the high relative abundance of
343 *P. agricolaris* corresponds with a reduction in microbiota phylogenetic diversity (Figure 4a).

344

345 The contribution of *P. agricolaris* to the similarity between infected *D. discoideum* samples is
346 more quantitatively highlighted via Robust Aitchison analysis, which is a novel beta-diversity
347 metric that is more adept at handling the sparsity of microbial community data. Here, Aitchison
348 analysis uses centered log ratio transformations as opposed to relative abundances (which can
349 often yield spurious results). Pairing log ratio transformation with the compositional Aitchison
350 distances allows for better interpretations of microbiome composition and also yields insight into
351 salient members of microbial communities that are responsible for sample clustering [60]. Along
352 with showing significant differences between *P. agricolaris* infected and uninfected *D.*
353 *discoideum* microbiota (PERMANOVA, pseudo-F=9.28284, p=0.001; PERMDISP, F=0.655692,
354 p=0.392) Robust Aitcheson PCA highlights the influence of *P. agricolaris* (indicated by labeled
355 vector) on the grouping of infected *D. discoideum* microbiota (Figure 5c).

356

357 *D. discoideum* inoculated with *Paraburkholderia* in the laboratory showed similar, yet more
358 pronounced, differences when compared to uninfected *D. discoideum*. *D. discoideum* microbiota
359 structure, determined using weighted UniFrac, was significantly altered by infection with *P.*
360 *agricolaris*, *P. hayleyella*, or *P. bonniea* when compared to uninfected *D. discoideum*
361 (PERMANOVA, pseudo-F ≥ 7.384689, p ≤ 0.007, PERMDISP, F=26.8176, p=0.001 where
362 significant PERMDISP results were negated by clear PCoA grouping) (Figure 6a). Likewise, *D.*
363 *discoideum* microbiota composition, evaluated using unweighted UniFrac, was altered by

364 infection with either *P. agricolaris*, *P. hayleyella*, or *P. bonniea* (PERMANOVA, pseudo-F ≥
365 4.200470, p ≤ 0.002, PERMDISP, F=2.30245, p=0.11) (Figure 6b). Microbiota compositional
366 comparisons using Aitchison distances showed a clear distinction between the microbiota of
367 *Paraburkholderia* infected amoebae and uninfected amoebae (PERMANOVA, pseudo-
368 F=22.282, p=0.001, PERMDISP, F=8.9081, p=0.001). Furthermore, Aitchison PCA highlights
369 the contribution of each *Paraburkholderia* species to microbiota composition (Figure 6c). These
370 results are consistent with the reduction in evenness, richness, and phylogenetic diversity
371 observed in α-diversity comparisons.

372

373 <u>*P. agricolaris* positively associates with *Rhizobiales* in the natural *D. discoideum* microbiota</u>

374 Although we did not find indication of an enhancement in bacterial food carriage by
375 *Paraburkholderia* via compositional comparisons, we reasoned that food carriage could be
376 indicated by specific co-associations with *P. agricolaris.* Therefore, we performed co-association
377 analyses using the SparCC method, which accounts for bacterial presence and abundance, and
378 is adept at handling the sparsity of microbial community data. We identified only one positive
379 association between *P. agricolaris,* represented by the order Burkholderiales, and the order
380 Rhizobiales (p=0.0297), and no significant negative association. Overall, significant associations
381 between other members of the amoeba microbiota were scarce (Figure 7).

382

383 <u>Chlamydiae is abundant in the natural *D. discoideum* microbiota but does not affect its diversity</u>

384 Given the high prevalence of Chlamydiae in *D. discoideum* populations, we investigated how it
385 might impact the natural *D. discoideum* microbiota. We found that the phylum Chlamydiae
386 accounted for 21.57% of all sequence reads, making it the second most abundant group. The
387 average abundance of Chlamydiae across infected samples was 43.34%, where Chlamydiae

388 accounted for 36.86% of all reads in infected samples and for greater than 90% of reads in 8/25
389 infected samples (Figure 3a). Chlamydiae infection was not correlated with microbiota
390 evenness, richness, or phylogenetic diversity (Pielou's Evenness, p=0.731175; observed
391 features (ASVs), p=0.257608; Faith's Phylogenetic Diversity, p=0.213492). However,
392 Chlamydiae infected *D. discoideum* did have distinct microbiota compositions and structures,
393 measured by Weighted UniFrac (PERMANOVA, pseudo-F=5.38621, p=0.004, PERMDISP,
394 F=0.164404, p=0.660) and unweighted UniFrac (PERMANOVA, pseudo-F=4.31874, p=0.003,
395 PERMDISP, F=8.00349, p=0.011) respectively.

396

397 **Discussion**

398 While previous studies of the *Paraburkholderia – D. discoideum* symbiosis have worked with
399 samples isolated from nature, exploration of the farming phenomenon has been primarily
400 laboratory based. Therefore, we aimed to investigate this interaction as it might occur in a more
401 natural context. It has been previously established that *Paraburkholderia* is prevalent in *D.*
402 *discoideum* populations (Haselkorn et al., 2019). Here, we expand upon these findings by
403 quantifying *Paraburkholderia* prevalence nearly twenty years after an initial sampling. Our
404 recent survey of natural *D. discoideum* populations showed little to no incidences of *P. bonniea*
405 and *P. hayleyella* infection, yet *P. agricolaris* remained prevalent. Furthermore, we found
406 evidence that *P. agricolaris* is environmentally acquired in nature, which is supported by its high
407 degree of genetic diversity. While the presumed benefit of farming could drive this symbionts'
408 persistence, overall, we did not find support for our hypothesis that the *Paraburkholderia*
409 farming symbiont would increase the diversity of *D. discoideum* microbiota. Rather, *P.*
410 *agricolaris* tended to infectiously dominate the *D. discoideum* microbiota in nature. Artificial
411 inoculation of each farming symbiont recapitulated this pattern, where *P. bonniea* and *P.*

412 *hayleyella* also infectiously dominated the *D. discoideum* microbiota, making it unclear if food
413 carriage is ecologically relevant.

414

415 It is a challenge to quantify the farming phenotype in nature, as measurement of the phenotype
416 has primarily been done in the lab. Laboratory tests of farming are generally in a controlled
417 environment, starting with *D. discoideum* clones being passaged and cleared of natural
418 transient bacteria and then provided with an ample supply of specific types of food bacteria
419 (Brock et al., 2011). *Paraburkholderia* confers secondary food carriage under these conditions,
420 increasing the microbial diversity. This food carriage is mostly extracellular and varies by
421 *Paraburkholderia* genotype and food bacterial species (Khojandi et al., 2019; Miller et al., 2020).
422 Recent work has shown that *Paraburkholderia* symbionts inhibit phagosome acidification (Tian
423 et al., 2022), suggesting that food carriage could be mediated by the creation of a more
424 hospitable environment for other bacteria that get ingested . In the soil environment, however,
425 many fruiting bodies contain microbiota, both with and without *Paraburkholderia* (Brock et al.,
426 2018; Sallinger et al., 2021), and in our study, *Paraburkholderia* did not increase the diversity of
427 this microbiota. In fact, in our lab infection assay, where fruiting bodies were collected directly
428 from the microbially diverse soil environment, *Paraburkholderia* significantly decreased overall
429 diversity.

430

431 Other effects that *Paraburkholderia* have on the microbiota are less clear. Although
432 *Paraburkholderia* does not increase the diversity of the microbiome, we cannot rule out the
433 possibility that it causes an increase in overall microbial load, which could allow an increase in
434 other edible bacteria. We did observe that *Paraburkholderia* infected amoebae had
435 compositionally similar microbiota, which could be consistent with farming if *Paraburkholderia*

436 favored the carriage of particular bacteria more likely to be food. These compositional changes,
437 however, were most likely due to a decrease in phylogenetic diversity correlated with
438 *Paraburkholderia* infection. Given the abundant natural microbiota of *D. discoideum* and other
439 social amoeba species in the absence of *Paraburkholderia* infection, the potential benefits of
440 food carriage are likely not specific to *Paraburkholderia* infected amoebas. The only other
441 significant effect of *Paraburkholderia* on the microbiota was its positive co-association with
442 Rhizobiales. While the implications of this association are unclear, it merits further investigation,
443 given observations of similar patterns in experimental studies (Khojandi et al., 2019) and the
444 importance of Rhizobiales in the soil environment.

445

446

447 *Paraburkholderia* may have alternative benefits which increase host fitness. A previous study
448 has demonstrated that *D. discoideum* spore production is significantly reduced when exposed to
449 the toxin ethidium bromide but is not impacted under the same conditions when harboring
450 *Paraburkholderia* (Brock et al., 2016). While this study suggested that farming associated
451 bacteria over-compensated for fewer sentinel cells produced by *Paraburkholderia* infected
452 hosts, the dominance of *Paraburkholderia* in the amoeba microbiota could suggest
453 *Paraburkholderia* itself plays a role in detoxification. Such roles are often assumed by
454 *Burkholderia* symbionts of both insects and fungi. For example, the bean bug *Riptortus*
455 *pedestris* harbors *Burkholderia* symbionts that protect their host from the harmful effects of the
456 insecticide fenitrothion, and other *Burkholderia* symbionts protect their fungal hosts from a
457 variety of anti-fungal agents (Kikuchi et al., 2012; Nazir et al., 2014). Alternatively, it is possible
458 that *P. agricolaris* competitively excludes other potentially detrimental bacteria from infecting the
459 amoeba fruiting body, just as the certain *Burkholderia* symbionts out compete non-native
460 symbionts in the crypt of the bean bug *Riptortus pedestris* (Itoh et al., 2019).

There is conflicting evidence as to whether these *Paraburkholderia-D. discoideum* symbioses are co-evolved mutualisms. The prevalence of *P. agricolaris* in nature is consistent with beneficial effects that outweighs any cost of infection, as would be seen in a mutualism. However, this high degree of prevalence could also be attributed to a more parasitic relationship. Likewise, finding almost no incidence of *P. bonniea* or *P. hayleyella* infection could indicate their detriment or lack of relevance to host fitness. More time points would be needed to establish this trend ecologically. Indeed, some *P. hayleyella* have a demonstrably large fitness cost in laboratory assays, though *P. bonniea* has not been reported to significantly impact *D. discoideum* spore production (Haselkorn et al., 2019; Khojandi et al., 2019). Many long term co-evolved mutualisms lack horizontal transmission, and the symbiont genomes degrade as they adapt to living solely inside their host and vertical transmission (Moran, 1996). However, *P. agricolaris* possesses a larger genome than both *P. bonniea* and *P. hayleyella* (Brock et al., 2020). Due to this larger genome, *P. agricolaris* can utilize a wider diversity of carbon sources than its counterparts, which makes it more similar to other free-living *Paraburkholderia* and *Burkholderia* relatives. It also infects a fewer proportion of spores than the other *Paraburkholderia* symbiont species (Khojandi et al., 2019; Miller et al., 2020), suggesting that it is not strongly dependent on vertically transmission. *P. bonniea* and *P. hayleyella*, which infect a higher proportion of spores in laboratory experiments, may be more reliant on vertical transmission in nature as their smaller genomes suggest they may not be as capable of living freely in the soil.

It is also possible that *P. agricolaris* is simply prevalent in *D. discoideum* populations because it is prevalent in, and subsequently acquired from, the soil environment. This is supported by the findings of Sallinger *et al.* (Sallinger et al., 2021), where *P. agricolaris* was detectable in

486 relatively high frequency in bulk soil sample from which amoebae were cultured. Environmental
487 acquisition could explain the high genetic diversity of *P. agricolaris* in natural populations, which
488 differs from the pattern of a single beneficial haplotype sweeping through a population after
489 initial infection, as often occurs in insect symbionts (Cockburn et al., 2013; Himler et al., 2011).
490 Here, *P. agricolaris* would not be subjected to the population bottleneck and subsequent loss of
491 genetic diversity elicited by vertical transmission. Additionally, a wider diversity of *P. agricolaris*
492 haplotypes could be acquired from the soil upon initial infection. The intracellular and
493 extracellular occupation of *P. agricolaris* makes it both horizontally and vertically transmissible
494 (Khojandi et al., 2019), a pattern that has been observed in insects, where the oriental chinch
495 bug obtains its *Burkholderia* symbiont from both parent and environment (Itoh et al., 2014). It is
496 also consistent with the observation of these same *Paraburkholderia* haplotypes infecting other,
497 distantly related co-occurring species of social amoebae. This acquisition could be driven by
498 either the host (Rashidi & Ostrowski, 2019) or bacteria (Shu et al., 2018). The ease of
499 environmental acquisition of *P. agricolaris* may ultimately lessen the pressure to develop and
500 maintain vertical transmission and limit co-evolution. Such patterns have also been observed in
501 symbioses between *Burkholderia* and broad-headed insects, where acquisition of *Burkholderia*
502 from the environment dampens co-evolutionary dynamics (Garcia et al., 2014; Kikuchi et al.,
503 2007).

504

505 Recent evidence has also suggested that environmental factors could impact *Paraburkholderia*
506 prevalence, where *P. hayleyella* infection provided additional detriment to *D. discoideum* under
507 artificial warming conditions (Shu et al., 2020). Therefore, our findings regarding symbiont
508 prevalence may not be generalizable to other *D. discoideum* populations occurring in different
509 soil environments. However, the environmental parameters that impact this symbiosis merit
510 further investigation. Even within our sub-sampling the symbiont prevalence was highly variable,

511 suggesting that differences in microhabitat may have large effects. Additional sampling of
512 different geographic locations at different times of year may help to elucidate these patterns.

513

514 Like *Paraburkholderia*, we found that Chlamydiae is highly prevalent in natural populations,
515 though the reasons for this are less clear. Laboratory studies of Chlamydiae in *D. discoideum*
516 found no fitness cost of infection (Haselkorn et al., 2021), but potential benefits have yet to be
517 explored. Like *Paraburkholderia*, Chlamydiae was overabundant in the microbiota that it
518 occupied, resulting in compositional uniformity. Co-association analyses within the microbiota
519 showed positive associations between the order Chlamydiales and the orders Flavobacteriales
520 and Peptostreptococcal, suggesting that this bacterium could be altering the social amoeba
521 microbiota in meaningful ways. The high prevalence of Chlamydiae in natural populations
522 suggests an ecological relevance for this symbiont that is worth further studying.

523

524 Overall, the variability regarding *P. agricolaris* localization, prevalence, transmission patterns,
525 genetic diversity, and costs of infection might indicate movement along the parasitism –
526 mutualism spectrum. Such dynamics can be difficult to study in more complex systems. Thus,
527 the *D. discoideum* – *Paraburkholderia* symbiosis, as well as the *D. discoideum* microbiota as a
528 whole, offers a useful model for understanding symbiotic development. Future studies that aim
529 to identify specific environmental parameters and soil conditions that either facilitate or hinder
530 this association will be useful in providing further ecological contextualization. Furthermore,
531 future studies that aim to investigate additional functions of *Paraburkholderia* will further our
532 understanding of the context dependent nature of symbiotic interactions in general.

533

534

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Data Accessibility

All sequences used to confirm *Paraburkholderia* infection and identity in natural *D. discoideum* populations have been uploaded to GenBank under accession numbers MZ395652 - MZ395810. Sequences used for *P. agricolaris* haplotype analyses have been uploaded under accession numbers MZ399634 - MZ399696. Next-generation 16S rRNA sequences obtained for microbiota comparisons have been uploaded as BioProject PRJNA737516. Sequences used to confirm amoeba species identity were uploaded to GenBank under accession numbers MZ396209-MZ396243.

Author Contributions

TSH and JGD designed research. TSH, MH, HO, and JGD performed research. JGD and MSR analyzed data. TSH and JGD wrote the paper with edits from MH and MSR.

801 **Figure Legends:**

802 **Figure 1. *Paraburkholderia* and Chlamydiae are prevalent in natural *D. discoideum***
803 **populations**. Pie charts indicate single and co-infection prevalence rates for each site sampled
804 in the Mountain Lake, Virginia area.

805 **Figure. 2. Phylogenetic evidence for environmental acquisition of *P. agricolaris.***
806 Maximum likelihood phylogenetic tree of *P. agricolaris* haplotypes constructed from the LepA
807 housekeeping gene. Colored boxes represent every amoeba species found to contain the
808 corresponding haplotype. Multiple amoebae species harboring the same haplotype was used as
809 indication of environmental acquisition of *P. agricolaris.*

810 **Figure. 3. *Paraburkholderia* infectiously dominates the *D. discoideum* microbiota**. Bar
811 plots show the members of the most abundant taxa within the microbiota of A) Naturally infected
812 *D. discoideum* isolates and B) artificially infected *D. discoideum* isolates.

813 **Figure. 4. *Paraburkholderia* does not diversify the *D. dicoideum* microbiota.** Alpha-
814 diversity box plots showing the Pielou's evenness, observed ASVs, and Faith's phylogenetic
815 diversity for A) isolates naturally infected with *Paraburkholderia* and B) isolates artificially
816 infected with each *Paraburkholderia* symbiont. Boxes span the first and third quartiles and
817 whiskers represent ± 1.5 IRQ. Letters shared in common denote no significant difference
818 (Kruskal-Wallis α=0.05 after FDR correction.

819 **Figure 5. High degrees of *P. agricolaris* abundance results in similar microbiota**
820 **compositions among infected amoebae.** Beta-diversity ordination plots comparing the
821 microbiota of *P. agricolaris* infected and uninfected *D. discoideum*, where each point represents
822 an individual isolates microbiota and the closer points are to each other, the more similar their
823 microbial communities. Kernel density estimate plots along the x and y axes indicate the
824 distribution of points along the respective axes and highlight each groups concentration in
825 ordination space. A) Weighted UniFrac PCoA plot showing the difference between infected and
826 uninfected microbiota structures. B) Unweighted UniFrac PCoA showing the difference in
827 microbiota compositions. C) Aitchison PCA plot showing the contribution of key bacterial taxa to
828 sample clustering.

829 **Figure 6. Over-abundance of each *Paraburkholderia* species in the microbiota results in**
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839 **Figure 7. *P. agricolaris* positively associates with members of the order Rhizobiales.**
840 Heatmap showing significant co-associations between bacterial orders within the natural *D.*
841 *discoideum* microbiota. *P. agricolaris* is represented by the order Burkholderiales.

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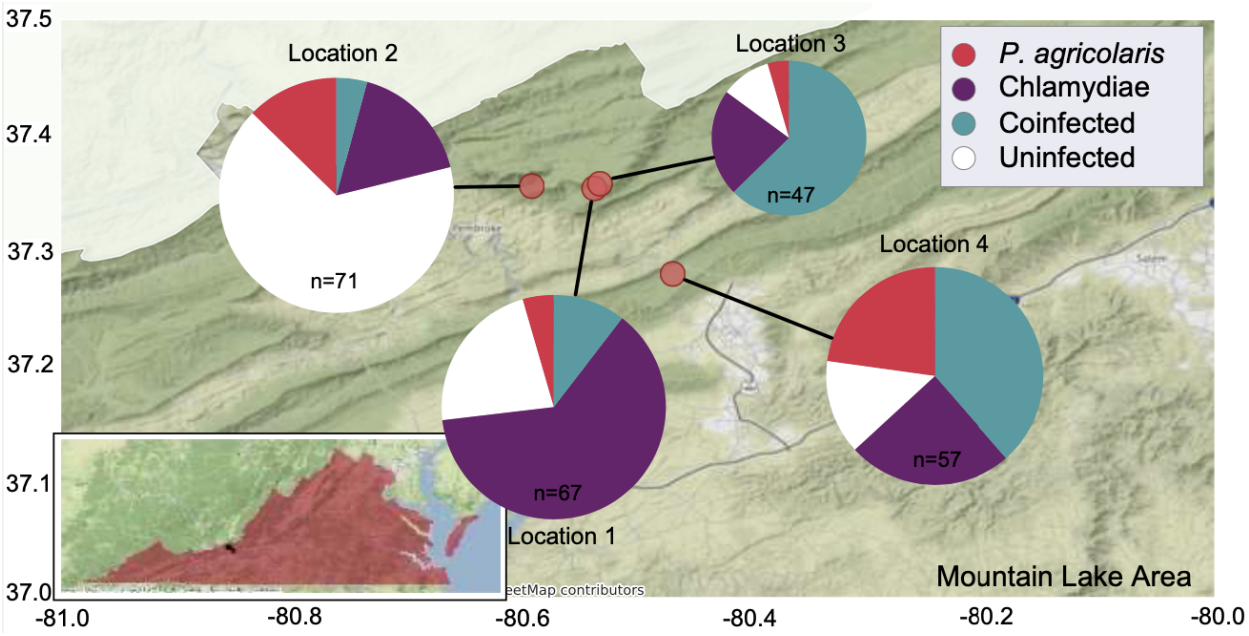
Figures



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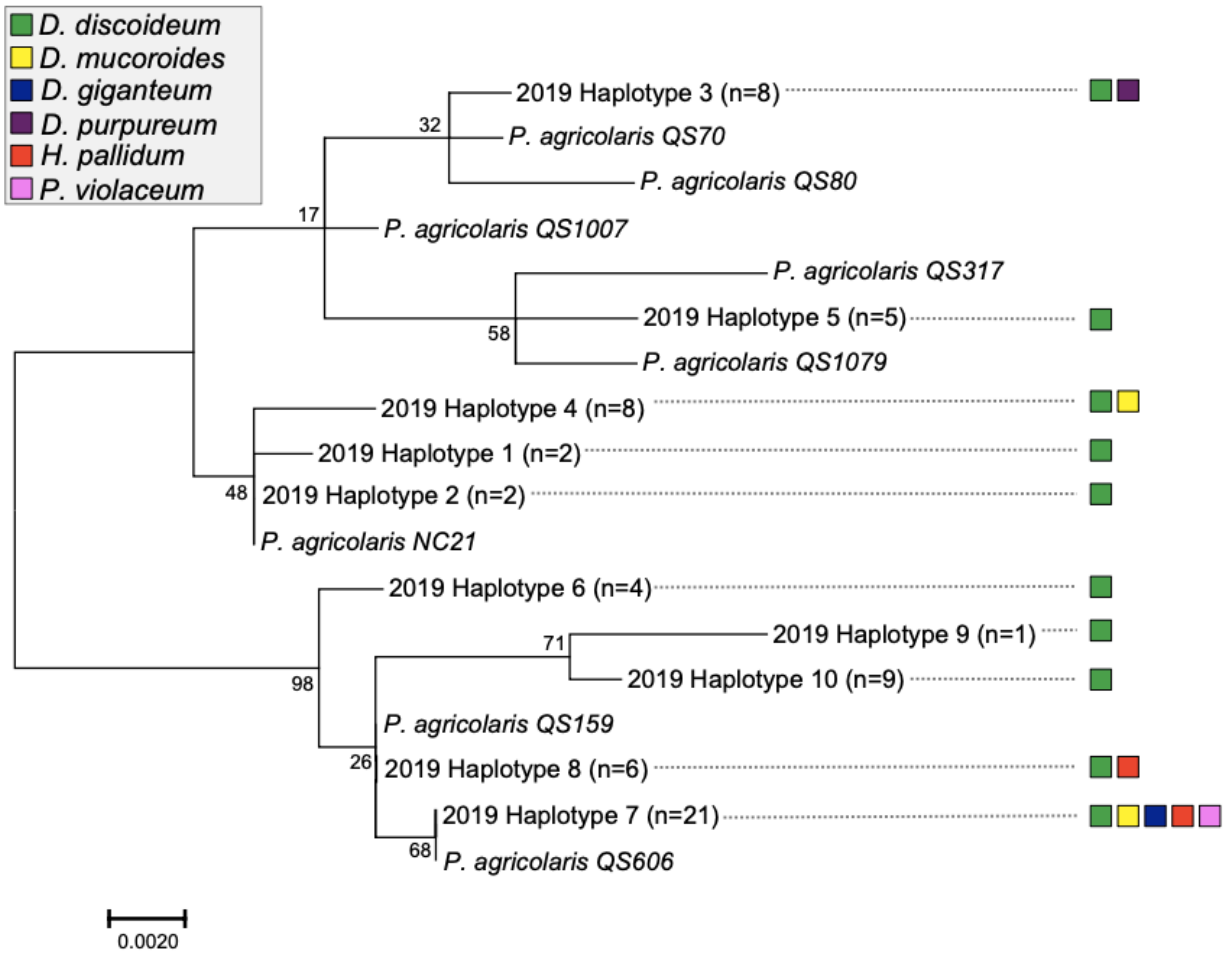

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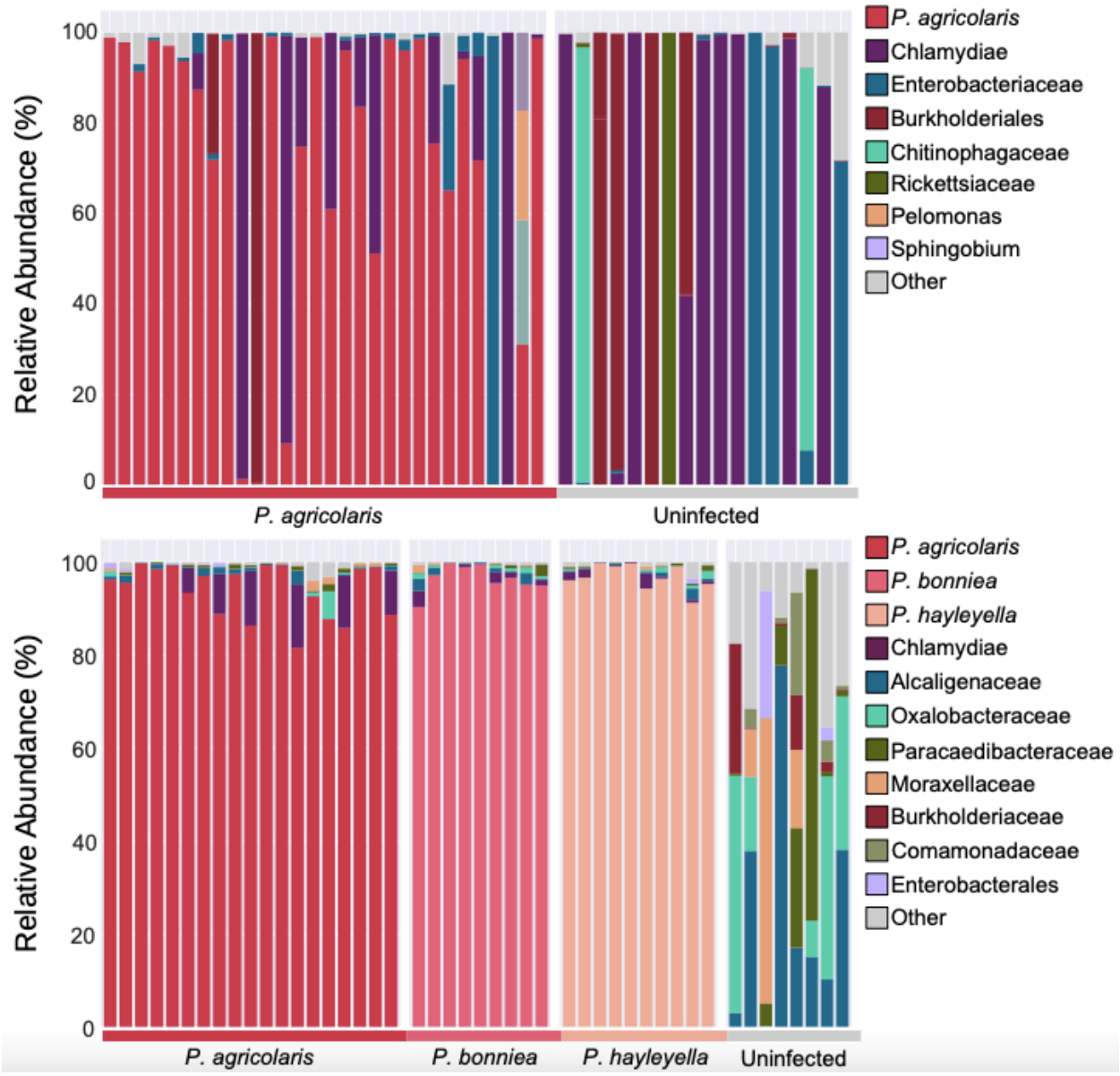

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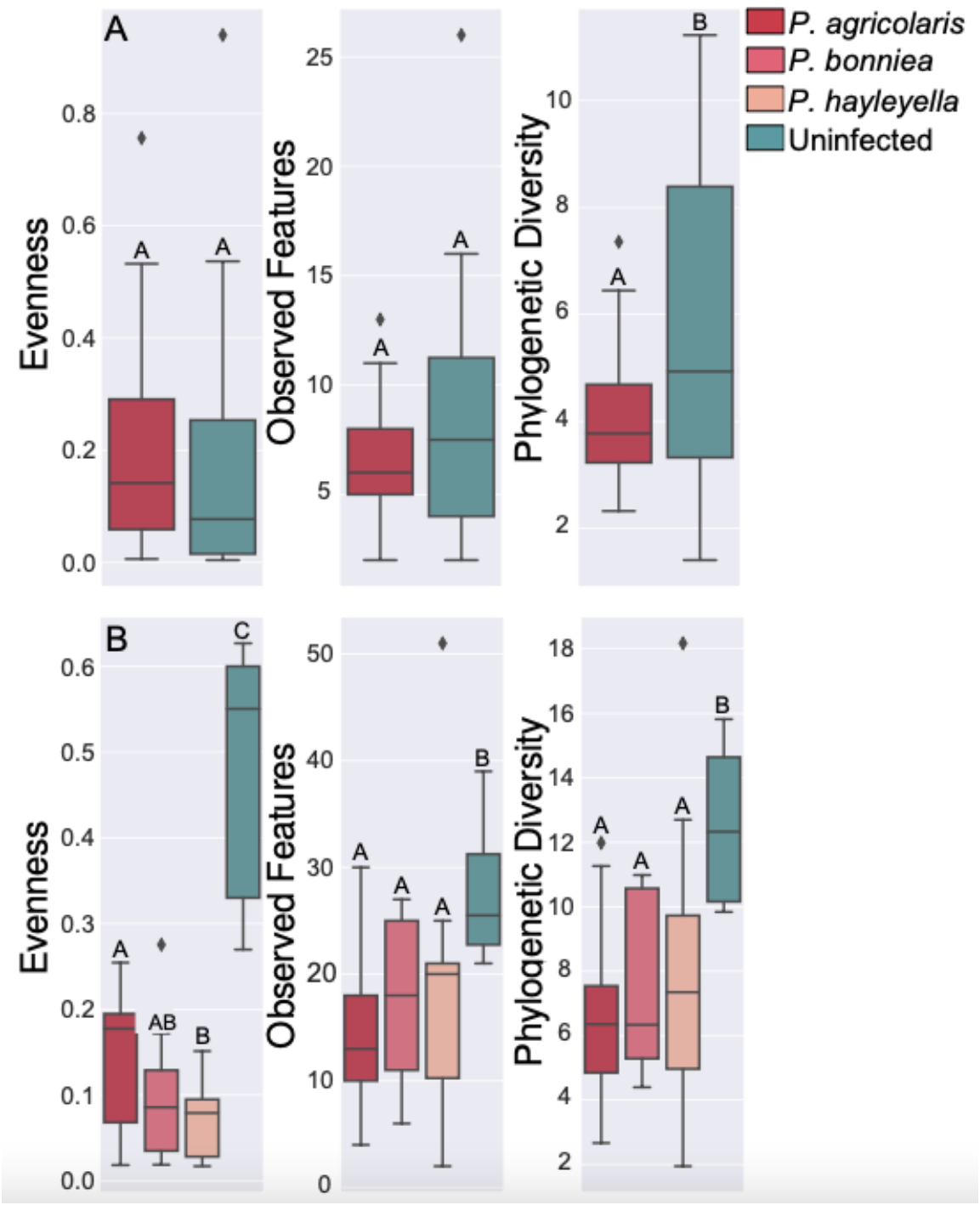

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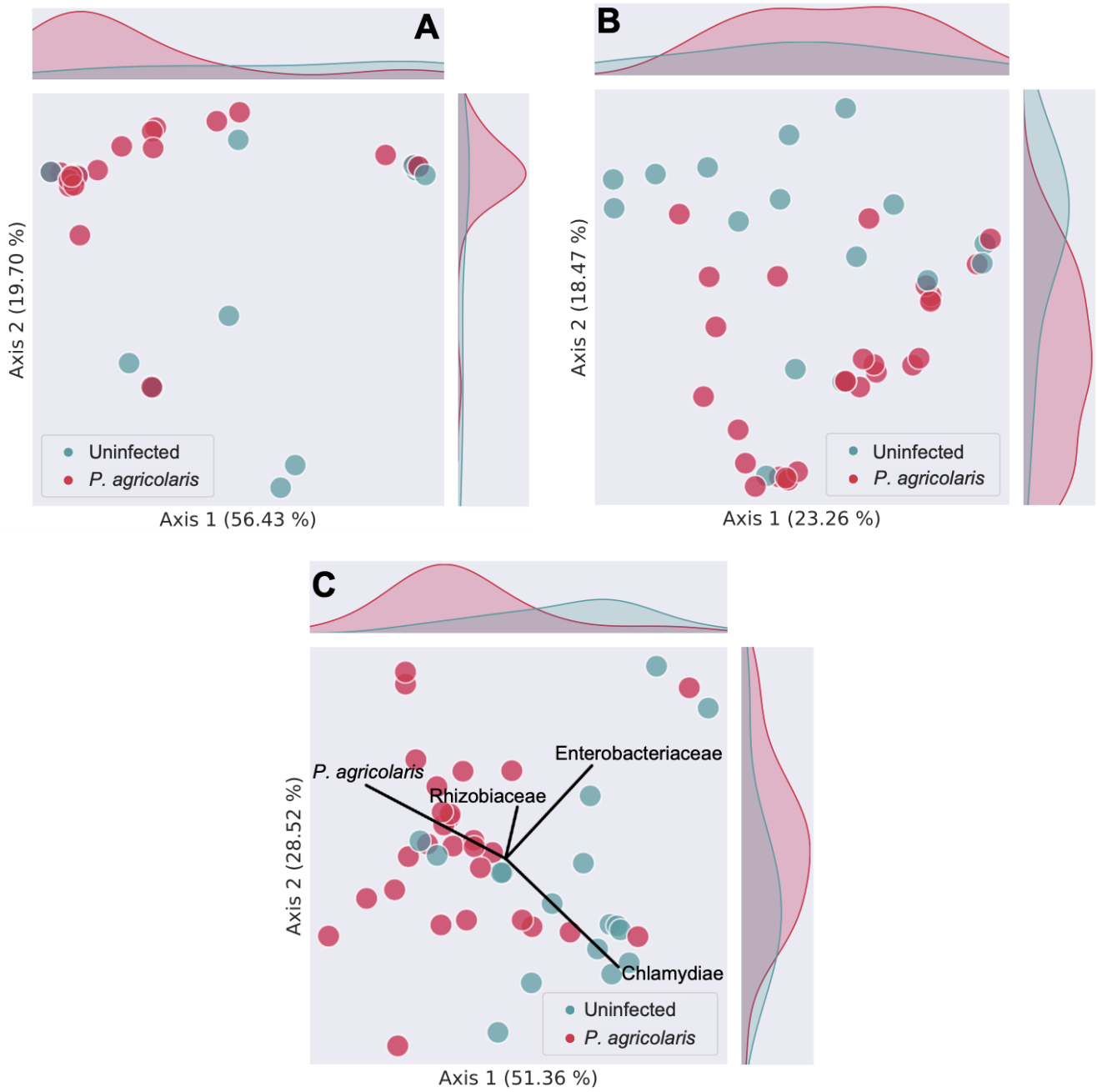

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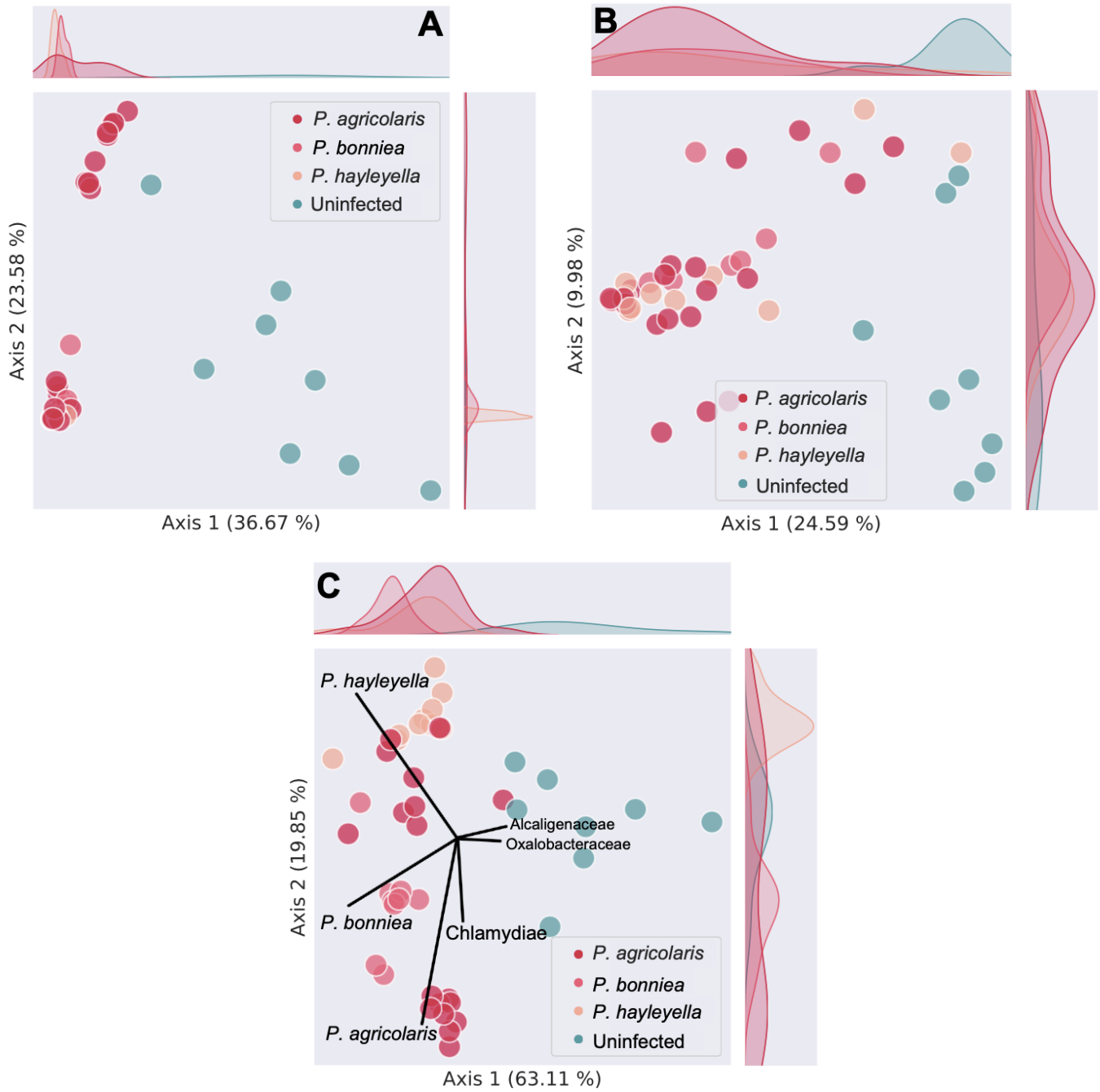

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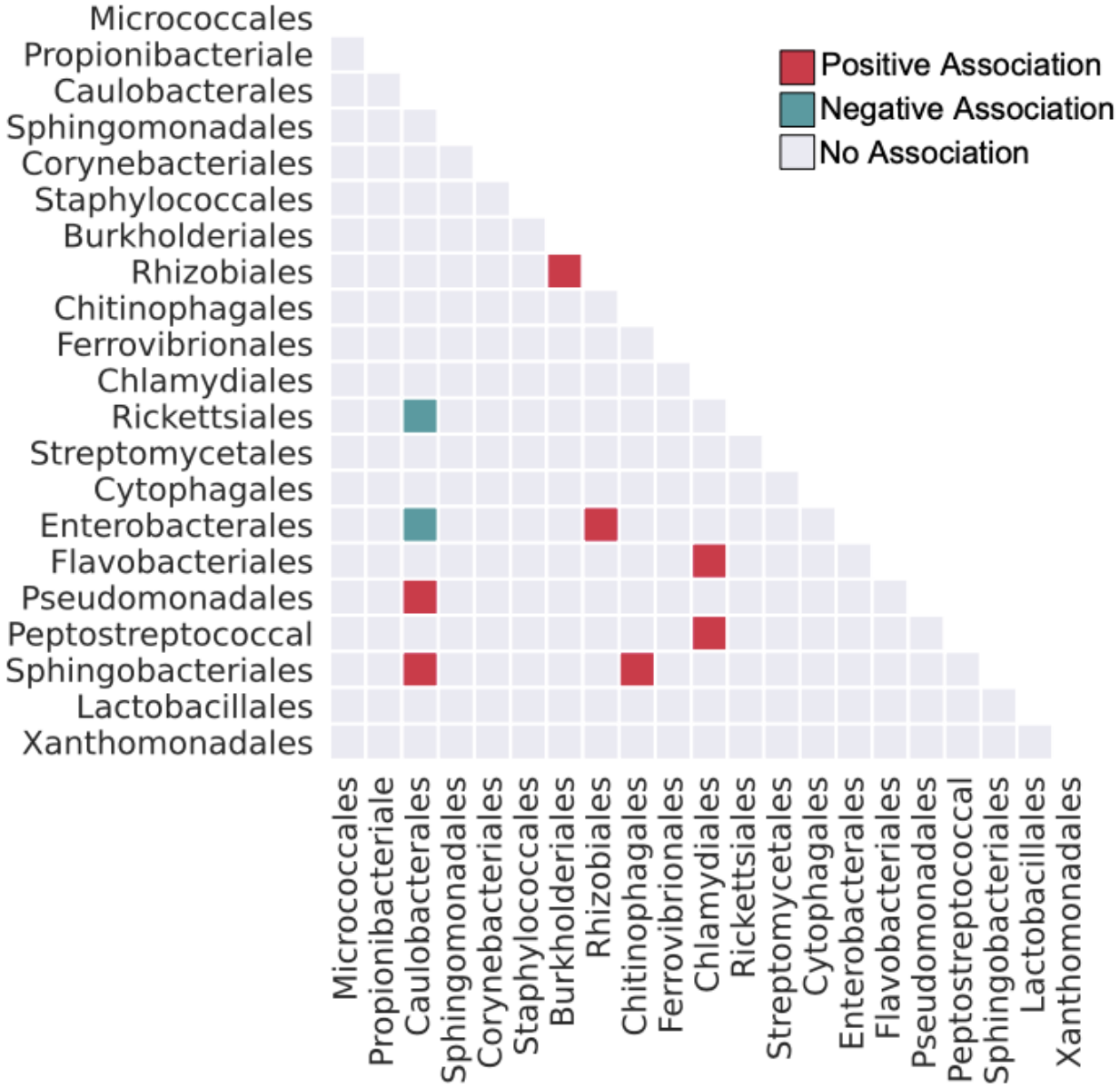

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Supplemental Tables

Supplemental Table 1: Soil Sample Metadata

Soil Sample	Location Name	GPS Coordinates	Description	Soil Temperature °C
Vial Sample 1	Blueberry Ridge Trail	N 37.357 W 80.359	Under log w/ foliage	16.5
Vial Sample 2	Indian Trail	N 37.357 W 80.534	Under foliage	14.6
Vial Sample 3	Indian Trail	N 37.358 W 80.533	Under and within rotting log	15
Vial Sample 4	Indian Trail	N 37.358 W 80.533	Under and within rotting log	14.5
Vial Sample 5	Indian Trail	N 37.357 W 80.534	Inside bowl of rotting stump	17
Vial Sample 6	Bald Knob Trail	N 37.354 W 80.537	Under log	14.5
Vial Sample 7	Bald Knob Trail	N 37.354 W 80.538	Inside of log	16
Bag Sample 1	Bald Knob Trail	N 37.353 W 80.537	Under log	17
Bag Sample 2	Bald Knob Trail	N 37.353 W 80.538	Under ground foliage	15.1
Bag Sample 3	Rocky Sink Road	N 37. 339 W 80.505	Under ground foliage	17
Bag Sample 4	Ricky Sink Road	N 37.340 W 80. 506	Under ground foliage	20
Bag Sample 5	Cougar Lane	N 37.356 W 80.464	Under ground foliage	17.2
Vial Sample 8	Cougar Lane	N 37.356 W 80.464	Under ground foliage	17.2
Vial Sample 9	Clover Hollow Road	N 37.315 W 80.497	Inside of rotting stump	N/A
Bag Sample 6	Cascade Falls	N 37.353 W 80.598	Under log	16.2
Bag Sample 7	Cascade Falls	N 37.354 W 80.598	Under ground foliage near log	17
Vial Sample 10	Cascade Falls	N 37.355 W 80.595	Under log, no foliage	18
Vial Sample 11	Cascade Falls	N 37.355 W 80.595	In log, no foliage	19.4
Vial Sample 12	Cascade Falls	N 37.356 W 80.594	Under log and foliage, near water	18.5
Vial Sample 13	Cascade Falls	N 37.356 W 80.592	Within divot in living tree	18.5
Bag Sample 8	Cascade Falls	N 37.356 W 80.592	Under ground foliage near log	16.1
Vial Sample 14	Cascade Falls	N 37.358 W 80.589	Under log and foliage	14.5
Vial Sample 15	Cascade Falls	N 37.359 W 80.588	Under log and foliage	14.5
Bag Sample 9	Cascade Falls	N 37.359 W 80.588	Under log and foliage	14.5
Vial Sample 16	Cascade Falls	N 37.361 W 80.586	Beside rotting stump	17.5
Bag Sample 10	Cascade Falls	N 37.361 W 80.586	Next to rotting log, under foliage, ontop of rocks	16.6
Vial Sample 17	Cascade Falls	N 37.361 W 80.586	Under rotting log and foliage	19
Bag Sample 11	Pandapas	N 37.281 W 80.470	Under foliage with pine mixed in	17.8
Vial Sample 18	Pandapas	N 37.281 W 80.470	Under foliage with pine mixed in	17.8
Vial Sample 19	Pandapas	N 37.281 W 80.470	Under rotting tree near water	20.3
Vial Sample 20	Pandapas	N 37.280 W 80.470	Under foliage, many roots present	16.5
Vial Sample 21	Pandapas	N 37.280 W 80.467	Between multiple decomposing logs, under foliage	18.5
Bag Sample 12	Pandapas	N 37.282 W 80.465	Between multiple decomposing logs, under foliage	18
Vial Sample 22	Pandapas	N 37.280 W 80.467	Between multiple decomposing logs, under foliage	18.5
Bag Sample 13	Pandapas	N 37.280 W 80.472	Beside rotting log, under foliage, many roots present	16.5
Vial Sample 23	Pandapas	N 37.280 W 80.472	Inside of rotting stump	17.5

956 **Supplemental Table 2:** Microbiological Media Preparation and KK2 extraction buffer

Hay agar ingredients (per liter)	Hay agar preparation protocol
15 g hay	1. Boil 15 g of hay in 1.5 L of DI H2O
1.5 L deionized H2O	2. Filter out hay from solution
1.5 g KH2PO4	3. Add each ingredient to hay infused solution
0.62 g Na2HPO4	4. Autoclave hay infused solution
15 g agar	5. Pipette 30 mL of solution into 100 mm petri dish

SM/5 agar ingredients (per liter)	SM/5 agar preparation protocol
2 g glucose	1. Mix each ingredient into 1 L of deionized water
2 g BactoPeptone	2. Autoclave solution
2 g yeast extract	3. Pipette 30 mL of solution into 100 mm petri dish
0.2 g MgCl2	
1.9 g KHPO4	
1 g KH2PO4	
15 g agar	

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KK2 Spore Buffer ingredient (per liter)
2.25 g KH2PO4
0.67 g K2HPO4
1.0 L deionized water

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968 **Supplemental Table 3:** Primer Sets

Primer	Sequence	Reference
D307F	5'-GTTTGGCCTACCATGGTTGTAA-3'	Baldauf *et. al.,* 2018
D862R	5'-GGGSGTTCATATTGGGGCG-3'	Baldauf *et. al.,* 2018
Burk3F	5'-CTGCGAAAGCCGGAT-3'	Salles *et. al.,* 2002
Burk3R	5'-TGCCATACTCTAGCYYGC-3'	Salles *et. al.,* 2002
LepAF	5'-CTSATCATCGAYTCSTGGTTCG-3'	Spilker *et. al.,* 2009
LepAR	5'-CGRTATTCCTTGAACTCGTARTCC-3'	Spilker *et. al.,* 2009
CHL16SFOR	5'-CGTGGATGAGGCATGCAAGTCGA-3'	Ossewaarde and Meijer, 1999
CHL16SREV	5'-CAATCTCTCAATCCGCCTAGACGTCTTA	Ossewaarde and Meijer, 1999
AmoF	5'-AAAACCCTTGCGAGGGTTCTTGAAG-3'	Haselkorn *et. al.,* 2020
AmoR	5'-GCCTTCGCTGTTGGTATTCCTTATGG-3'	Haselkorn *et. al.,* 2020
799F	5'-AACMGGATTAGATACCCKG-3'	Chelius & Triplett, 2001
1115R	5'-AGGGTTGCGCTCGTTG-3'	Reysenbach & Pace, 1995
Protocol:		

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970 **Supplemental Table 4:** Prevalence, co-associations, and genetic community comparisons of
971 symbiont screenings

Location	Location name	*P. agricolaris* prevalence (%)	Chlamydiae prevalence (%)	Amoebophilus prevalence (%)
1	Bald Knob Trail	14.93	74.63	31.34
2	Cascade Falls	16.9	21.13	14.08
3	Indian Trail	59.57	82.98	14.89
4	Pandapas	61.4	63.16	10.53
5	Total	35.12	57.85	18.18

Bald Knob Trail

Fisher Exact Test for Coassociation	Observed Coinfections	Expected Coinfections	Significance (p value)
P. agricolaris - Chlamydiae	7	7.313	1
P. agricolaris - Amoebophilus	5	3.134	0.2659
Chlamydiae - Amoebophilus	16	15.36	0.7745

Cascade Falls

Fisher Exact Test for Coassociation	Observed Coinfections	Expected Coinfections	Significance (p value)
P. agricolaris - Chlamydiae	3	2.54	0.708
P. agricolaris - Amoebophilus	2	1.35	0.6152
Chlamydiae - Amoebophilus	3	2.11	0.4309

Indian Trail

Fisher Exact Test for Coassociation	Observed Coinfections	Expected Coinfections	Significance (p value)
P. agricolaris - Chlamydiae	26	23.23	0.0472
P. agricolaris - Amoebophilus	5	4.17	0.6845
Chlamydiae - Amoebophilus	6	5.809	1

Pandapas

Fisher Exact Test for Coassociation	Observed Coinfections	Expected Coinfections	Significance (p value)
P. agricolaris - Chlamydiae	22	22.11	1
P. agricolaris - Amoebophilus	5	3.684	0.3889
Chlamydiae - Amoebophilus	5	3.789	0.3974

***P. agricolaris* populations Fst values**	Column1	Column2
Population 1	**Population 2**	**Fst**
Bald Knob Trail	Cascade Falls	0.68667
Bald Knob Trail	Indian Trail	0.88679
Bald Knob Trail	Pandapas	0.87908
Cascade Falls	Indian Trail	0.13982
Cascade Falls	Pandapas	0.6532
Indian Trail	Pandapas	0.77522

972 **Supplemental Table 5:** Soil samples and corresponding *P. agricolaris* haplotypes

Location	Soil Sample Code	*P. agricolaris* haplotypes present
Bald Knob Trail	1B	10
Cascade Falls	2B1	5,6,7
Indian Trail	3A1	5,6,7
Indian Trail	3A2	7
Indian Trail	3B	7,8
Pandapas	4A1	1,2,3,4,5
Pandapas	4A2	1,3,4
Pandapas	4A3	4

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