Novel Chlamydiae and *Amoebophilus* endosymbionts are prevalent in wild isolates of the model social amoeba *Dictyostelium discoideum*

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24 Running Title: Prevalent obligate endosymbionts in a social amoeba

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Summary: Amoebae interact with bacteria in diverse and multifaceted ways. Amoeba predation can serve as a selective pressure for the development of bacterial virulence traits. Bacteria may also adapt to life inside amoebae, resulting in symbiotic relationships (pathogenic or mutualistic). Indeed, particular lineages of obligate bacterial endosymbionts have been found in different amoebae. Here, we screened an extensive collection of *Dictyostelium discoideum* wild isolates for the presence of such bacterial symbionts using PCR primers that identify these endosymbionts. This is the first report of obligate symbionts in this highly-studied amoeba species. They are surprisingly common, identified in 42% of screened isolates (N=730). Members of the Chlamydiae phylum are particularly prevalent, occurring in 27% of the host strains. They are novel and phylogenetically distinct. We also found *Amoebophilus* symbionts in 8% of screened isolates (N=730). Antibiotic-cured amoebae behave similarly to their endosymbiont-infected counterparts, suggesting that endosymbionts do not significantly impact host fitness, at least in the laboratory. We found several natural isolates were co-infected with multiple endosymbionts, with no obvious fitness effects of co-infection under laboratory conditions. The high prevalence and novelty of amoeba endosymbiont clades in the model organism *D. discoideum* opens the door to future research on the significance and mechanisms of amoeba-symbiont interactions.

Introduction:

As voracious microbial predators, free-living amoebae are important shapers of their microbial communities (Clarholm, 1981). This predatory pressure can alter the presence and abundance of specific microbial constituents in the community (Ronn et al., 2002).

50 Amoeba predation is also postulated to play an important role in microbial evolution,
51 particularly in the evolution of bacterial virulence (Matz and Kjelleberg, 2005; Sun et al.,
52 2018). Bacteria that evolve strategies to avoid or survive amoeba predation would be
53 selected for in amoeba rich environments (Erken et al., 2013). Bacteria that are able to
54 enter amoeba cells (via phagocytosis or other entry mechanisms) but avoid subsequent
55 digestion gain access to an attractive intracellular niche. A diverse collection of
56 intracellular bacterial symbionts of amoebae has been found, some of which appear
57 pathogenic, neutral, or beneficial for their amoeba host (DiSalvo et al., 2015; Jeon,
58 1992; König et al., 2019; Maita et al., 2018; Shu et al., 2018; Taylor et al., 2012). Some
59 of these symbionts are important human pathogenic species such as *Mycobacteria,*
60 *Legionella,* and *Chlamydia* (Boamah et al., 2017; Cardenal-Muñoz et al., 2018;
61 Drancourt, 2014; Gomez-Valero and Buchrieser, 2019; Paquet and Charette, 2016;
62 Tosetti et al., 2014). .
63
64 Bacterial endosymbionts of amoebae were first discovered in the diverse, free-living
65 amoeba genus *Acanthamoeba* (Horn and Wagner, 2004). These amoebae are found in
66 soil and freshwater environments and can protect themselves and their bacterial
67 associates by forming cysts under environmentally unfavorable conditions (Rodríguez-
68 Zaragoza, 1994). The presence of such endosymbionts was known long before they
69 could be specifically identified because the inability to isolate obligate associates from
70 their hosts made characterization challenging. Once molecular methods became
71 available, it was clear that these bacteria were from specific lineages in the Chlamydiae,
72 *Proteobacteria*, *Bacteriodetes,* and *Dependentiae* (Delafont et al., 2015; Horn and

Wagner, 2004; König et al., 2019; Maita et al., 2018; Samba-Louaka et al., 2019; Schmitz-Esser et al., 2008). These endosymbionts interact with their amoeba hosts in varied ways. Some inhabit host-derived vacuoles, while others persist in the cytoplasm. A *Neochlamydia* symbiont can protect its host against the detrimental consequences of *Legionella* bacterial infection (Ishida et al., 2014; König et al., 2019; Maita et al., 2018) and other strains have been shown to improve the growth rate and motility of their hosts (Okude et al., 2012). Other known amoeba endosymbionts are from the less-studied genera *Amoebophilus* and *Procabacter*. Genome sequencing of the amoeba symbiont *Candidatus Amoebophilus asiaticus* found no evidence that it could provide novel pathways to supplement host nutrition, but other fitness affects have not been tested (Schmitz-Esser et al., 2010).

Overall, a high proportion of sampled *Acanthamoeba* (25-80%) are infected with endosymbionts and these serve as useful models for understanding how amoebae can serve as training grounds for bacterial intracellular adaptation. The broader distribution of symbiotic bacteria among other amoeba species and their prevalence in natural host populations, however, is unclear (Schmitz-Esser et al., 2008). The potential of amoebae to harbor such bacteria could be of concern to human health. For instance, environmental Chlamydiae have been associated with respiratory diseases and adverse pregnancy outcomes in humans and cattle, although the extent of their importance in causing animal illness is unknown (Borel et al., 2018; Greub, 2009; Taylor-Brown et al., 2015; Taylor-Brown and Polkinghorne, 2017).

Another amoeba that has been used as a model to study host-bacteria interactions is the social amoeba, *Dictyostelium discoideum*. This amoeba, distantly related to *Acanthamoeba*, is terrestrial, living and feeding as single cells in forest soils while the bacterial food supply is plentiful. When food becomes scarce, the amoebae begin a social cycle, which entails thousands of cells aggregating and forming a slug to crawl to the surface of the soil. The cycle culminates in the formation of a fruiting body, made of a ball of spores (sorus) sitting on top of a stalk, which can then be dispersed to more favorable conditions (Kessin, 2001). In many cases, bacteria are cleared from *D. discoideum* cells during the social cycle through innate immune-like mechanisms (Chen et al., 2007). However, vegetative amoebae can be infected with a range of human pathogens such as *Mycobacterium, Bordetella*, and *Legionella* (Bozzaro and Eichinger, 2011; Taylor-Mulneix et al., 2017). As a model system *D. discoideum* has been fruitfully utilized to describe the role of host and pathogen factors in mediating infection mechanisms and virulence (Annesley and Fisher, 2009; Bozzaro et al., 2019; Bozzaro and Eichinger, 2011; Cosson and Soldati, 2008; Steinert, 2011; Steinert and Heuner, 2005).

Recently, a variety of bacterial symbionts have been discovered to colonize *D. discoideum* (Brock et al., 2018; DiSalvo et al., 2015; Haselkorn et al., 2018). The *D. discoideum* microbiome is composed of both persistent and transient bacterial associates (Brock et al., 2018). Three different newly named *Paraburkholderia* species, *P. agricolaris*, *P. hayleyella*, and *P. bonniea* are persistent bacterial symbionts that

119 intracellularly infect vegetative amoebae and spore cells (Brock et al., 2020, 2011;
120 Haselkorn et al., 2018; Khojandi et al., 2019; Shu et al., 2018). While these
121 *Paraburkholderia* themselves do not nourish *D. discoideum*, they confer farming to their
122 hosts by allowing food bacteria to survive in the fruiting body. These food bacteria can
123 then be re-seeded in new environments during spore dispersal, which benefits hosts in
124 food-scarce conditions (Brock et al., 2011; Haselkorn et al., 2018; Khojandi et al., 2019).
125 In natural populations, an average of 25% of *D. discoideum* are infected with
126 *Paraburkholderia* (Haselkorn et al., 2018). Other bacteria, some edible and others not,
127 have the ability to transiently associate with *D. discoideum* even in the absence of
128 *Paraburkholderia* co-infections (Brock et al., 2018). Thus, natural *D. discoideum* hosts a
129 small-scale microbiome and is gaining traction as a simple model system to study the
130 mechanisms of microbiome formation (Dinh et al., 2018; Farinholt et al., 2019).
131 However, all previous sampling of natural *D. discoideum* associates has relied on
132 isolating bacterial colonies from nutrient media plates, which would miss any bacterial
133 species, such as obligate amoeba symbionts, incapable of growing under these
134 conditions.
135
136 Since obligate endosymbionts are common in other free-living amoebae, we
137 endeavored to identify them in *D. discoideum.* Here, we screened a frozen stock
138 collection of 730 natural *D. discoideum* isolates. We first used a 16S rRNA gene
139 amplification strategy with “universal” primers and direct sequencing to detect and
140 identify bacteria within *D. discoideum* spores in a single population. In addition to
141 detecting expected *Paraburkholderia* symbionts, we identified many Chlamydiae and

142 *Amoebophilus* sequences, and a few *Procabacter* sequences in screened samples. To
143 determine the extent of Chlamydiae and *Amoebophilus* symbionts in our collection we
144 then used symbiont specific PCR screening. From each of these prevalent bacterial
145 lineages, we found genetically distinct novel species and were able to visualize
146 representative endosymbiont cells within spores via electron microscopy. We also used
147 antibiotics to cure natural hosts of Chlamydiae and *Amoebophilus* and compared host
148 fitness to uncured counterparts. Finally, we looked at the fitness effects of co-infections
149 since co-infections occur in nature.
150

151 **Results:**

152 *Obligate endosymbionts are common in wild-collected clones of* Dictyostelium
153 discoideum

154 To investigate the presence and prevalence of obligate bacterial symbionts in *D.*
155 *discoideum*, we screened a frozen collection of 730 natural *D. discoideum* isolates for
156 bacterial DNA. The vast majority of our screened isolates were collected from the
157 Eastern half of the United States (Fig. 1a) and had previously been screened for
158 *Paraburkholderia* (Haselkorn, *et al.* 2018, Table S1). This included 16 populations with
159 seven or more individuals collected. Our largest collection from Virginia consists of
160 about 200 individuals collected in 2000 and in 2014. Across all locations we found over
161 41% of samples harbored at least one obligate endosymbiont. Specifically, 117 (~16%)
162 were infected with *Amoebophilus* and 198 (~27%) with Chlamydiae. Sixteen (2.1%)
163 were co-infected with both *Amoebophilus* and Chlamydiae (Fig. 1b). Two isolates (both

164 from Virginia) were infected with an obligate bacterium identified as belonging to the
165 genus *Procabacter*.
166
167 *The* Procabacter, Amoebophilus*, and Chlamydiae endosymbionts of* D. discoideum *are*
168 *novel and phylogenetically distinct from known symbionts.*
169 For *Procabacter*, *Amoebophilus*, and the three most prevalent Chamydiae strains (those
170 occurring in more than one individual) we sequenced the full length 16S rRNA for our *D.*
171 *discoideum* endosymbionts to better determine their evolutionary relationships. In all
172 cases, the 16S rRNA haplotypes were novel and highly diverged, ranging from 6-12%
173 sequence difference from other sequences currently in GenBank or SILVA (Table S2).
174 For the *Procabacter* and *Amoebophilus* endosymbionts, only a single haplotype was
175 found for each. The *Procabacter* endosymbiont of *D. discoideum* is sister to all of the
176 *Procabacter* endosymbionts of *Acanthamoeba* (Fig. 2a). While it is most closely related
177 to this genus, forming a clade with 100% bootstrap support, the 16S rRNA sequence is
178 only a 93% match. A similar pattern is seen for the *Amoebophilus* symbiont of *D.*
179 *discoideum* (Fig. 2b.). This symbiont groups most closely with the putative
180 *Amoebophilus* symbiont from genome assembly of the recently sequenced
181 *Dictyostelium polycephalum.* This Dictyostelid symbiont clade is sister to the
182 *Amoebophilus asiaticus* endosymbionts of *Acanthamoeba*, and is 6% diverged at the
183 16S rRNA gene (Table S2).
184
185 There are 8 distinct and novel haplotypes of *D. discoideum* endosymbionts in the
186 Chlamydiae. For the three most prevalent haplotypes (1, 2, and 3), we sequenced the

187 full 16S rRNA gene to reconstruct a more resolved phylogeny which included their
188 nearest neighbors from the SILVA database and representatives from many
189 Chlamydiae families (Fig. 2c). There are currently 6 formally named and 8 proposed
190 Chlamydiae families (Pillonel et al., 2018; Taylor-Brown et al., 2015), and several other
191 recently discovered novel clades from marine sediments (Dharamshi et al., 2020). The
192 *D. discoideum* Chlamydiae endosymbionts do not appear to be closely related (<97%
193 sequence match) to any of these groups. Haplotypes 1 and 2 and 4 form their own well-
194 supported clade, grouping with the *Rhabdochlamydiaceae* and *Ca.*
195 Arenachlamydiaceae, sister to the *Simkaniaceae*. Many of the internal nodes on this
196 phylogeny have low bootstrap support, making more detailed phylogenetic inferences
197 impossible. This *D. discoideum* endosymbiont clade, however, does have only a 91%
198 identity to its closest phylogenetic neighbor and may represent a new genus or family of
199 Chamydiae. Greater than 10% sequence divergence at the full length 16S rRNA gene
200 has been proposed as family level difference (Everett et al., 1999), although full genome
201 sequences may be necessary to make this assertion. Detailed searches for this most
202 common Chlamydiae haplotype 1 in the IMNGS database returned 13 matches (>99%
203 sequence identity), mostly as “uncultured bacteria” from soil metagenomic studies
204 (Table S3). One such study was in the Northeast United States (Massachusetts), in a
205 region close to where we sampled *D. discoideum*, and it is likely that amoeba
206 endosymbionts are being sequenced as part of these metagenomes.
207
208 Our other Chlamydiae haplotypes are scattered throughout the phylogeny. Haplotypes 3
209 and 5 form a well-supported clade and may be sister to *Rhabdochlamydiaceae*/ *Ca.*

210 Arenachlamydiaceae/ *Simkaniaceae* families. These haplotypes have a greater than
211 11% nucleotide difference from its nearest neighbors on the phylogeny (and in the
212 SILVA and NCBI databases, Table S2) and may represent a novel family as well.
213 Haplotypes 6, 7 and 8 fall in different places in the phylogeny and do not group closely
214 with any other taxa, although these haplotypes are based on only 220bp of sequence
215 information so their taxonomic identity is less clear. Finally, while the newly discovered
216 marine Chlamydiae lineages (Dharamshi et al., 2020) were not included in this
217 phylogeny, when we aligned our Chlamydiae haplotypes with these sequences, the
218 closest match had only an 89% sequence identity.
219
220 *Distribution of endosymbionts across populations*
221 While the average overall prevalence of infection of Chlamydiae endosymbionts was
222 27%, it varied among populations, ranging from 0% to 75% (Table S1). The *D.*
223 *discoideum* Chlamydiae haplotypes 1, 2 and 3 were the most prevalent in natural
224 populations. Haplotype 1 represented 79.8% of all *D. discoideum* Chlamydiae
225 infections, while haplotype 2 was 13.6% and haplotype 3 was 3.5%. Haplotypes 4-8
226 were represented only once. Haplotype 1 was found in 12 populations, haplotype 2 was
227 found in 8 different populations, and haplotype 3 was found in three populations. The
228 diversity of Chlamydiae strains was the highest in the Virginia Mountain Lake collection
229 from 2000, which had six of the eight haplotypes (1-6), and the second highest diversity
230 of Chlamydiae haplotypes was found in the Texas Houston Arboretum collection (1, 2, 3
231 and 8). Interestingly, haplotype 1 was the only haplotype circulating in the most recent
232 2014 Virginia Mountain Lake collection. While haplotype diversity was lower in this

233 collection, Chlamydiae prevalence (for all haplotypes) in the region increased from 20%
234 in 2000 to 38% in 2014.
235
236 The one strain of *Amoebophilus* found to infect *D. discoideum* was distributed across 9
237 populations, although at low prevalence, often with just a single individual infected in
238 many locations. This endosymbiont was generally less prevalent than Chlamydiae, with
239 the exception of the 2000 Virginia Mountain Lake population, where it infected 37% of
240 the population. *Amoebophilus* prevalence in this region, however, decreased to 10% in
241 2014. *Amoebophilus* was also relatively prevalent in the geographically-distant Texas
242 Houston Arboretum population, infecting 23% of the population.
243
244 *Obligate endosymbionts can be visualized within reproductive host spore cells*
245 Transmission electron microscopy (TEM) revealed bacterial cells inside developed
246 spores from representative *Amoebophilus,* Chlamydiae*,* and *Procabacter* host isolates
247 (Fig. 3). Symbiont cells were detectable in the majority of symbiont host spores
248 visualized by TEM. Each symbiont appeared to have gram-negative type cell walls but
249 displayed unique morphologies within spore cells. *Amoebophilus* endosymbionts were
250 rod-like, measured 0.3-0.5μm in width and 0.5-1.3μm in length, and appeared to be
251 distributed throughout the cytoplasm within a host membrane. *Procabacter*
252 endosymbionts were rod shaped, measured 0.25-0.5μm in width and 0.75-1.5μm in
253 length, and in many cases were surrounded by a host membrane. Chlamydiae
254 endosymbionts appeared as dense wrinkled spheres measuring 0.3-0.6μm that were
255 distributed throughout the cytoplasm.

256

257 Amoebophilus *and Chlamydiae can be eliminated from host isolates without significantly*

258 *impacting host fitness*

259 We did not observe any obvious defects in the growth and development of Chlamydiae

260 and *Amoebophilus* infected hosts. To better assess the impact of these endosymbionts

261 on their hosts, we measured the reproductive fitness of representative host isolates

262 before and after endosymbiont curing (Fig. 4). We eliminated Chlamydiae and

263 *Amoebophilus* from four of their natural hosts through culturing for two social cycles on

264 antibiotic saturated plates. After antibiotic exposure and resumption of development on

265 normal media, we confirmed stable loss of endosymbionts via endosymbiont-specific

266 PCR screening (Fig. 4a). We next cultured $1x10^5$ spores from antibiotic-treated and

267 untreated *D. discoideum* counterparts on nutrient media with *Klebsiella pneumoniae*

268 food bacteria. Following culmination of fruiting body development after a week of

269 incubation, we harvested and measured total spore contents (Fig. 4b). We found no

270 significant differences in spore productivity according to endosymbiont status (one-way

271 analysis of variance, ($F(5,42)=1.106$, $p=0.372$). Thus, symbiont infections in these

272 natural host isolates have no significant impact on host spore productivity under these

273 conditions. We could not assess the fitness impact of Chlamydiae or *Amoebophilus* in

274 new host amoebae as our attempts to establish infections in new hosts have been thus

275 far unsuccessful.

276

277 Amoebophilus *and Chlamydiae infections do not alter the fitness of hosts during*

278 *exposure to* Paraburkholderia

279 Since co-infections with unculturable symbionts and *Paraburkholderia* occur in the wild,
280 we wanted to determine whether these co-infections would result in a different fitness
281 outcome than *Paraburkholderia* infections alone. We tested the fitness impact of
282 Chlamydiae and *Amoebophilus* during *Paraburkholderia* exposure using the same
283 strategy as above, but with the addition of 5% by volume of the indicated
284 *Paraburkholderia* strain to food bacteria prior to plating (Fig. 5). These *Paraburkholderia*
285 symbionts range from highly detrimental (*P. hayleyella* strain 11), to intermediately
286 detrimental (*P. agricolaris* strains 159 and 70), to neutral (*B. bonniea* strain 859) under
287 the culturing conditions used for this assay (Haselkorn et al., 2018; Khojandi et al.,
288 2019; Shu et al., 2018). We again observed that exposure to distinct *Paraburkholderia*
289 strains significantly influenced spore productivity for both *Amoebophilus* ($F(3,60)=36.87$,
290 $p=<0.001$) and Chlamydiae ($F(3,60)=28.52$, $p=<0.001$) originating host lines (Fig. 6).
291 However, as with single unculturable symbiont infections, we found that presence or
292 absence of *Amoebophilus* or Chlamydiae had no significant impact on spore
293 productivity during exposure to *P. hayleyella*-11 ($F(5,42)=1.171$, $p=0.339$), *B.*
294 *agricolaris*-159 ($F(5,42)=0.985$, $p=0.438$), *P. agricolaris*-70 ($F(5,42)=1.095$, $p=0.377$), or
295 *P. bonniea-859* ($F(5,42)=0.661$, $p=0.655$) (Fig. 5). Thus, under these conditions the
296 fitness impact of *Paraburkholderia* symbiosis on host spore productivity does not appear
297 to be altered (for better or worse) by co-infections with obligate endosymbionts.
298
299 *Detection of positive associations between Chlamydiae and* Paraburkholderia *and*
300 Amoebophilus *and* Paraburkholderia *and a negative association between* Amoebophilus
301 *and Chlamydiae*

302 Our earlier culture-based screening of this *D. discoideum* natural collection found that
303 25% of isolates were infected with *Paraburkholderia* symbionts (Brock et al., 2020;
304 Haselkorn et al., 2018). For this new round of sequence-based screening, we identified
305 multiple instances of *Paraburkholderia-Amoebophilus* and *Paraburkholderia-*
306 Chlamydiae co-infections in addition to the *Amoebophilus*-Chlamydiae co-infections
307 from above. Specifically, 42 isolates (6%) were co-infected with *Paraburkholderia* and
308 *Amoebophilus*, 25 (3.5%) with *Paraburkholderia* and Chlamydiae, and 9 (1%) were co-
309 infected with all three symbiont types (Fig. 1b). Co-infection prevalence of the different
310 endosymbionts were tested in our three highest sampled populations: Texas-Houston
311 Arboretum, Virginia Mountain Lake 2000, and Virginia Mountain Lake 2014. Pairwise
312 Fisher Exact tests showed a small positive association between Chlamydiae and
313 *Paraburkholderia* in the Texas population (p=0.0132, 15 observed with 10 expected) but
314 not the Virginia populations. There was a positive association between
315 *Paraburkholderia* and *Amoebophilus* in the Virginia 2000 collection (p=0.000277, 43
316 observed with 30 expected) and a negative association between Chlamydiae and
317 *Amoebophilus* in the Virginia 2014 collection (p=0.000024, none observed with 10
318 expected).
319
320 **Discussion:**
321 Many microorganisms, particularly those that are obligately host-associated, are often
322 overlooked because they are unculturable in standard laboratory conditions. The
323 presence of obligate bacterial symbionts in free-living amoebae has been known for
324 some time, particularly for *Acanthamoeba* (Fritsche et al., 1993; Hall and Voelz, 1985;

325 Jeon and Jeon, 1976; Proca-Ciobanu et al., 1975). The list of amoebal endosymbionts
326 and their host species has since expanded but obligate endosymbionts have not been
327 previously described in the social amoeba *D. discoideum* (Corsaro et al., 2010; Delafont
328 et al., 2015; Horn and Wagner, 2004; Samba-Louaka et al., 2019; Schmitz-Esser et al.,
329 2008; Schulz et al., 2014). Here, we screened a collection of 730 *D. discoideum* isolates
330 using a PCR detection strategy in order to identify bacterial associates that could be
331 missed using conventional culturing techniques. Furthermore, to our knowledge, this is
332 the largest collection of amoebae from natural populations to be screened, facilitated by
333 the relative ease in which social amoeba fruiting bodies can be sampled. Here, we find
334 that obligate symbionts are quite common among *D. discoideum* collected from the wild,
335 with bacterial sequences of unculturable bacteria present in 301 isolates (41.2%). All
336 but two of these unculturable bacterial sequences aligned most closely to *Amoebophilus*
337 or *Chlamydiae* species. The identification of these bacteria in *D. discoideum* reveals
338 that this valuable eukaryotic model organism is a natural vector of environmental
339 bacteria. This opens a new role for *D. discoideum* as a research tool for the
340 investigation of how bacterial endosymbionts evolve to live inside hosts, how they affect
341 the fitness of their amoeba host, and how they interact with other bacteria in the
342 amoeba microbiome.
343
344 The intracellular morphology of endosymbionts in *D. discoideum* spores were in some
345 cases similar to previous observations of related endosymbionts in other amoebae.
346 *Amoebophilus* cells in *D. discoideum* spores appeared similar to previous observations
347 of *Amoebophilus* symbionts in *Acanthamoeba sp*. (Horn et al., 2001; Schmitz-Esser et

348 al., 2010). The localization of *Procabacter* endosymbionts within a membrane bound
349 vacuole has also been observed for Candidatus *Procabacter* sp. OEW1 in
350 *Acanthamoeba* spp. host cells (Heinz et al., 2007). However, this differs from a previous
351 report on *Procabacter* symbionts in *Acanthamoeba* trophozoites and cysts wherein
352 bacterial cells were not found to be enclosed in vacuoles (Fritsche et al., 2002). The
353 wrinkled morphology of Chlamydiae cells in *D. discoideum* spores is somewhat
354 reminiscent of the spiny structure of *Chlamydia*-like symbionts in *Acanthamoeba,*
355 *Naegleria,* and *Hartmannella* host species (Casson et al., 2008; Corsaro and Greub,
356 2006; Horn et al., 2000). However, these morphological observations may be artifacts of
357 (or exaggerated by) sample preparation prior to TEM and may be better resolved using
358 cryo-electron tomography (Huang et al., 2010; Santarella-Mellwig et al., 2013).
359
360 *Chlamydiae*-like organisms typically have two or more morphotypes that correspond to
361 distinct developmental stages. Elementary bodies are the infectious form that can be
362 transmitted extracellularly from cell to cell. Once internalized, elementary bodies
363 differentiate into reticulate bodies that typically replicate within inclusion vesicles,
364 although some environmental Chlamydiae can be found directly in the cytosol
365 (Bayramova et al., 2018; Benamar et al., 2017; Bou Khalil et al., 2017; Collingro et al.,
366 2020; Horn, 2008; Nylund et al., 2018). However, in *D. discoideum* host spores, we only
367 visualize one morphology that does not appear to be in an inclusion but is rather
368 distributed throughout the cytosol. It is unclear what morphotype this represents and
369 whether other morphotypes exist in *D. discoideum* prior to spore formation. It is possible

370 that additional Chlamydiae developmental stages occur in vegetative amoebae and that
371 only one form is favored within metabolically inert host spores.
372
373 Out of the 251 amoeba isolates from the 2014 Virginia Mountain Lake collection, we
374 identified only two infected with a bacterium with sequence homology to *Procabacter*,
375 suggesting that *D. discoideum* is not a preferred natural host for this endosymbiont. Yet,
376 this *D. discoideum Procabacter* lineage possibly represents an important link for
377 understanding the evolution and adaptation of *Procabacter* to amoeba hosts, as the
378 phylogenetic placement of the novel *D. discoideum Procabacter* lineage is sister to the
379 rest of the *Procabacter* species infecting *Acanthamoeba.* Comparative genome
380 sequencing of this clade, as well as future research into the impact of these infections
381 on their hosts and more extensive wild isolate screenings will better inform our
382 understanding of the significance of these infections in *D. discoideum.*
383
384 We identified multiple *D. discoideum* infected with an *Amoebophilus* species that most
385 closely matched Ca. *Amoebophilus asiaticus*, an endosymbiont originally discovered in
386 *Acanthamoeba* (Horn et al., 2001). Similar to the *D. discoideum Procabacter* symbionts,
387 the *D. discoideum Amoebophilus* symbiont lineage is sister to the rest of the
388 *Amoebophilus* clade. The fact that it groups most closely with a putative *Amoebophilus*
389 symbiont discovered in the genome assembly of *Coremiostelium polycephalum,*
390 formerly called *Dictyostelium polycephalum* (Sheikh et al., 2018) suggests that there
391 may be some host specificity of *Amoebophilus* among social amoebae, although
392 sampling of additional amoeba species would be necessary to elucidate this pattern.

393 The next most closely related bacteria to the *Amoebophilus* genus are the insect
394 facultative endosymbionts of the *Cardinium* genus (Santos-Garcia et al., 2014). These
395 bacteria infect up to 7% of all insect species and have diverse effects on their hosts
396 ranging from reproductive parasitism to nutritional supplementation and defense against
397 parasites. Comparative genome sequencing has uncovered potential routes of
398 adaptation from free-living marine bacteria to either an insect or amoeba host (Santos-
399 Garcia et al., 2014). Including this additional *Amoebophilus* species in comparative
400 genome analysis could further elucidate this process and highlight potential amoeba
401 species-specific adaptations. *Acanthamoeba* and *Dictyostelium* are evolutionarily
402 distant hosts with different lifestyles, thus specific bacterial adaptations may have
403 evolved.
404
405 The global significance of *Amoebophilus* infections is under active investigation.
406 *Amoebophilus* have been associated with coral microbiomes and bovine digital
407 dermatitis lesions, which may simply be an indicator that their host amoebae are
408 present in these samples (Apprill et al., 2016; Choi et al., 2009; Horn et al., 2001;
409 Huggett and Apprill, 2019; Zinicola et al., 2015). In amoebae, the full extent of this
410 bacterium's affect is unknown. The moderately reduced genome of Ca. *Amoebophilus*
411 *asiaticus* lacks important metabolic pathways but contains a high number of proteins
412 with eukaryotic domains that are thought to be essential for host cell interactions
413 (Schmitz-Esser et al., 2010). Genes associated with host cell exploitation, such as
414 toxins, antimicrobial factors, and a unique secretion system, suggest that Ca.
415 *Amoebophilus asiaticus* is more parasitic than mutualistic (Böck et al., 2017; Penz et al.,

416 2010; Schmitz-Esser et al., 2010). We did not observe an obvious fitness cost of hosting
417 *Amoebophilus* via our spore productivity assays. However, this measures just one
418 aspect of host fitness in defined laboratory conditions and it remains possible that these
419 symbionts may impact host fitness in important ways at different life stages or under
420 different environmental conditions.
421
422 Chlamydiae endosymbionts were the most prevalent in the screened collection, with
423 approximately 27% of *D. discoideum* isolates infected. Environmental Chlamydiae
424 appear to be diverse and ubiquitous and many Chlamydiae endosymbionts associate
425 with a wide range of protozoa and other organisms (Corsaro et al., 2013; Corsaro and
426 Venditti, 2006; Coulon et al., 2012). The incredible genetic diversity within the phylum
427 Chlamydiae suggests ancient interactions with eukaryotes, with estimates that the
428 association is 700 million years old (Greub and Raoult, 2003). The diversity of
429 Chlamydiae, as estimated from various 16S rRNA genome databases and sequence
430 read archives, ranges anywhere from 1157-1483 different families (Collingro et al.,
431 2020). This type of screening, however, does not identify the hosts, so insight into
432 adaptation to particular hosts is limited. In this study we have identified eight novel
433 lineages, potentially representing two novel families, both with closely related
434 haplotypes suggesting ongoing evolution within the *D. discoideum* host. The separation
435 of these novel *D. discoideum* Chlamydiae lineages from the other amoeba lineages
436 suggests a long-standing association and possible co-adaptation. As our attempts to
437 transfer *Amoebophilus* or Chlamydiae endosymbionts to new *D. discoideum* hosts were
438 unsuccessful, we could not probe the potential role of host-symbiont adaptation in

439 fitness outcomes. This strengthens the speculation that these symbionts have been
440 associated with their specific host lines for a long period of time but also raises the
441 question of how they were originally acquired. Other lab experiments have shown that
442 some of these Chlamydiae strains are easily transferred across different host genera
443 while others cannot be used to successfully re-infect even their original host amoebae
444 (Corsaro et al., 2013; Coulon et al., 2012; Okude et al., 2012). A previous transfer of a
445 *Protochlamydia* strain to new hosts resulted in a decreased growth rate, suggesting
446 mutual co-evolution between the endosymbiont and its original host (Okude et al.,
447 2012).
448
449 Similar to our results for *Amoebophilus*-infected hosts, we did not find any obvious
450 fitness costs from Chlamydiae infection of *D. discoideum* under standard lab conditions
451 for a subset of *D. discoideum* isolates. This does not, however, rule out the possibility
452 that these symbionts may impart significant impacts on their hosts which may be life-
453 stage, environmental, or genotypically context-dependent (Taylor-Brown et al., 2015).
454 Studies of the impact of *Chlamydiae* endosymbionts on their amoeba hosts suggest that
455 the range of outcomes spans the parasite to mutualist continuum (Hayashi et al., 2012).
456 The high prevalence and widespread distribution of particular haplotypes, like haplotype
457 1, may suggest ecological relevance. It is unclear whether the abundance of particular
458 endosymbiont haplotypes represents an epidemic of a new genotype or selective
459 sweeps due to beneficial effects on their hosts under particular environmental
460 conditions, as seen in insect symbionts (Cockburn et al., 2013; Himler et al., 2011).
461 Depending on the temperature, one *Parachlamydia* endosymbiont of *Acanthamoeba*

462 can either be lytic or endosymbiotic (Greub et al., 2003). The Chlamydiae endosymbiont
463 S13 does not significantly harm its *Acanthamoeba* host and instead has been shown to
464 protect it from *Legionella pneumophila* infection (Ishida et al., 2014; König et al., 2019;
465 Maita et al., 2018; Matsuo et al., 2009; Okude et al., 2012). A *Protochlamydia*
466 endosymbiont has been shown to increase the migration rate, actin density, and growth
467 rate of its native host but had an adverse effect when transferred to non-native hosts
468 (Okude et al., 2012). Overall, future investigations into the impact of these symbionts on
469 hosts using diverse fitness assays and a range of culture conditions will help to
470 elucidate their significance in infected host amoebae.
471
472 At least 11.4% (83 out of 730) of these *D. discoideum* are co-infected with some
473 combination of *Chlamydiae*, *Amoebophilus,* or *Paraburkholderia*. The lack of detectable
474 fitness costs from co-infection with unculturable endosymbionts and *Paraburkholderia* in
475 the laboratory is consistent with random associations found in several populations. In
476 different environments, however, other microbe-microbe interactions may have an effect
477 that favors certain combinations of symbionts, as is seen in aphids (Rock et al., 2018).
478 Chlamydiae occur in co-infections less often than would be expected with
479 *Amoebophilus* in one *D. discoideum* population, consistent with the observation that
480 Chlamydiae interact negatively with the *Legionella* pathogen in *Acanthamoeba*. On the
481 other hand, *Paraburkholderia* and *Amoebophilus* are positively associated in one
482 population. *Paraburkholderia* symbionts of *D. discoideum* have been shown to allow
483 other food bacteria to enter into the amoeba spores (Brock et al 2011, DiSalvo et al
484 2015), and perhaps a similar positive interaction occurs in this case. This effect may

485 outweigh some of the negative interactions of Chlamydiae with other bacteria, as in one
486 population there was positive association with Chlamydiae and *Paraburkholderia*.
487 *Paraburkholderia* symbionts are easily transferred to new hosts via co-culturing and can
488 be found both intracellularly and extracellularly in amoeba cells and structures (DiSalvo
489 et al., 2015; Haselkorn et al., 2018; Shu et al., 2018). *Paraburkholderia* infections in new
490 hosts may be detrimental or beneficial depending on symbiont genotype and
491 environmental context (Haselkorn et al., 2018; Khojandi et al., 2019). Endosymbiont co-
492 infection is not unique to *D. discoideum,* as previous and alternative isolation methods
493 are allowing for identification of co-infections in *Acanthamoeba* (Heinz et al., 2007;
494 Lagkouvardos et al., 2014).
495
496 The growing list of bacterial symbionts of free-living amoebae attests to their attractiveness
497 as an intracellular niche for bacterial symbionts. The similarities between amoebae and
498 animal macrophages, as well as the shared intracellular entrance and survival
499 mechanisms employed by endosymbionts and pathogens, further highlight the potential
500 medical and research relevancy of amoebae-symbiosis systems. The discovery of a high
501 prevalence of infection and novel lineages of common amoebae symbiont clades in the
502 model organism *D. discoideum* opens the door to future research on the ecological
503 significance and mechanisms of amoeba-symbiont interactions.
504
505 **Methods:**
506 <u>Bacterial Strains and Cultural Conditions:</u>

507 We used *Klebsiella pneumoniae* throughout as our standard *D. discoideum* food supply.
508 For *Paraburkholderia*-unculturable co-infection experiments, we used the
509 *Paraburkholderia* symbionts *P. hayleyella*-11, *P. agricolaris*-70, *P. agricolaris*-159, and
510 *B. bonniea*-859. We grew bacteria on SM/5 agar plates [2 g glucose, 2 g BactoPeptone,
511 2 g yeast extract, 0.2 g $MgCl_2$, 1.9 g $KHPO_4$, 1 g $K2HPO_5$, and 15 g agar per liter,
512 premixed from Formedium] at room temperature before resuspending them in KK2
513 buffer [2.25 g $KH2PO_4$ (Sigma-Aldrich) and 0.67 g $K2HPO_4$ (Fisher Scientific) per liter].
514 We then adjusted bacterial suspension to a final OD_{600nm} of 1.5. We plated amoebae
515 spores with 200µl of bacterial suspensions for all culturing conditions.
516
517 Symbiont Screening
518 Total DNA from *D. discoideum* was harvested by suspending 15-25 sori in a 5% chelex
519 solution with 10µg proteinase K, incubating samples for 240 minutes at 56°C, 30
520 minutes at 98°C, and removing DNA supernatant from chelex beads. PCR with
521 eukaryotic specific primers was run as a DNA extraction control, and symbiont specific
522 primers were used to detect symbionts in each sample (Table S2) (Corsaro et al., 2002;
523 Ossewaarde and Meijer, 1999). For all primer sets we used a touchdown PCR protocol
524 consisting of 94°C for 30 s, 68°C-55°C for 45 x (dropping 1 degree per cycle) for 14
525 cycles, and 72°C for 1 min, followed by 94°C, 55°C, 72°C for 20 cycles. To confirm
526 infection we ran PCR samples on a one percent agarose gel with TAE buffer and
527 imaged on a transilluminator. PCR products were sent for sequencing (Genewiz), and
528 the resulting Sanger sequences were cleaned using the program Geneious v8
529 (http://www.geneious.com (Kearse et al., 2012)). We use NCBI Blast to initially identify

530 the bacterial species/strain based on highest sequence similarity. Sequences have
531 been deposited to GenBank under accession numbers (MT596490-MT596496).
532
533 Amoebae Clone Types and Culture Conditions:
534 Amoebae were cultured from freezer stocks by resuspending frozen spores onto SM/5
535 agar plates with 200µl of food *K. pneumoniae* suspensions. Plates were incubated at
536 room temperature under low lights and ~60% humidity for approximately 1 week (or until
537 fruiting body development).
538
539 For antibiotic curing and spore quantification, we used four uninfected, four
540 *Amoebophilus* infected, and four Chlamydiae infected host lines. Uninfected
541 representatives were QS864, QS970, QS1003 and QS1069 (highlighted in yellow in
542 Table S1). *Amoebophilus* infected representatives included QS851, QS1002, QS1011,
543 and QS1017 (highlighted in green in Table S1). Chlamydiae infected representatives
544 included QS866, QS991, QS1023, and QS1089 (highlighted in blue in Table S1). For all
545 experiments, we initiated experimental conditions from pre-developed fruiting bodies by
546 suspending ~8-15 sorus contents in KK2. We then plated 10^5 spores onto an SM/5 plate
547 with 200µl of bacterial suspensions (*K. pneumoniae* only or with 95% *K. pneumoniae/*
548 5% *Paraburkholderia* mixtures by volume for co-infection experiments). Plates were
549 then incubated as described above for the time indicated for each experimental
550 condition.
551
552 Curing of Symbiont Infections:

553 We treated uninfected, Chlamydiae-infected, and *Amoebophilus* infected representative
554 isolates with rifampacin by plating ~10^2 spores with 400µl *K. pneumoniae* on SM/5
555 medium containing 30µg/ml rifampacin. Plates were incubated until fruiting body
556 formation. Sori from grown fruiting bodies were then harvested and replated for a
557 second round of antibiotic exposure (using the same strategy as described above).
558 Following fruiting body formation, spores were harvested and transferred to SM/5 with
559 *K. pneumoniae* and incubated. Following fruiting body formation on non-antibiotic
560 plates, we extracted total sorus content DNA and checked for symbiont infection using
561 the Chlamydiae-specific and *Amoebophilus*-specific PCR protocol described above.
562
563 Total Spore Productivity Assay:
564 To determine total spore productivity of antibiotic treated and untreated *D. discoideum*
565 isolates we plated 10^5 spores on SM/5 agar with 200µl of *K. pneumoniae* and incubated
566 at room temperature. Five days after plating, total plate contents were collected into
567 ~10ml KK_2 + NP-40 alternative detergent solution. Solutions were then diluted
568 appropriately (~20x) and spores quantified via hemocytometer counts. We measured
569 and averaged over at least three replicates. To determine spore productivity after
570 exposure to *Paraburkholderia*, we conducted the assay as described but plated spores
571 with 200µl of a 95/5% by volume mixture of *K. pneumoniae/Paraburkholderia* cell
572 suspensions.
573
574 Statistics

575 Significant positive or negative associations between co-infecting endosymbionts were
576 determined using a Fisher Exact test (2x2 contingency table) on pairwise comparisons
577 in our three most highly sampled populations. Statistical analyses for spore productivity
578 experiments were performed in R 3.6.0. Comparisons were done using one-way
579 ANOVA's with antibiotic treatment as the independent variable.
580
581 Transmission Electron Microscopy
582 Complete processing and imaging of spores by transmission electron microscopy was
583 performed by Wandy Beatty at the Washington University Molecular Microbiology
584 Imaging Facility. *D. discoideum* isolates imaged included QS864, QS851, QS886,
585 QS1040. Briefly, spores were harvested in KK2 buffer and fixed with 2%
586 paraformaldehyde with 2.5% glutaraldehyde (Polysciences Inc., Warrington, PA) in 100
587 mM cacodylate buffer (pH 7.2) for 1-3 hrs. at room temperature. We washed samples
588 with cacodylate buffer and postfixed in 1% osmium tetroxide (Polysciences Inc.) for 1 hr
589 before rinsing in dH_2O prior to *en bloc* staining with 1% aqueous uranyl acetate (Ted
590 Pella Inc., Redding, CA) for 1 hr. We dehydrated samples in increasing concentrations
591 of ethanol and embedded them in Eponate 12 resin (Ted Pella Inc.). Sections of 95 nm
592 were cut with a Leica Ultracut UCT ultramicrotome (Leica Microsystems Inc.,
593 Bannockburn, IL), stained with uranyl acetate and lead citrate, and viewed on a JEOL
594 1200 EX transmission electron microscope (JEOL USA Inc., Peabody, MA) equipped
595 with an AMT 8 megapixel digital camera (Advanced Microscopy Techniques, Woburn,
596 MA).

597 Phylogenetic Tree Construction

598 The *Procabacter* full length 16S rRNA phylogeny was created using the top ten NCBI
599 Blast results for the *Procabacter* endosymbiont of D. *discoideum* sequence and
600 representative species from the phylum *Betaproteobacteria*. The species were chosen
601 using the NCBI taxonomy database and 16S rRNA sequences were obtained from the
602 NCBI nucleotide database as well as the three top hits to the SILVA database. A
603 sequence from *Rickettsia rickettsii* was also obtained from the NCBI nucleotide
604 database to serve as an outgroup. Sequences were imported into Mega7 (Tamura et
605 al, 2013) and aligned with MUSCLE. All sequences were then trimmed to a uniform
606 1396 bp. The Kimura 2-parameter model with a discrete Gamma distribution was
607 selected using the "Find Best DNA/Protein Models" command in Mega7. A Maximum
608 Likelihood tree was then constructed with 1000 bootstrap replicates.
609
610 The *Amoebophilus* full length 16S rRNA phylogeny was created using the top five NCBI
611 Blast results for the *Amoebophilus* endosymbiont of *D. discoideum* sequence, a
612 sequence from the recently sequenced *Dictyostelium polycephalum* genome assembly,
613 a sequence from the related endosymbiont *Candidatus Cardinium hertigii*, and
614 representative species from the order *Cytophagales*. The species were chosen using
615 the phylogeny in Hahnke et al., (2016) as a guide, and full length 16S rRNA sequences
616 were obtained from the NCBI nucleotide database (Hahnke et al., 2016) as well as the
617 three top hits from the SILVA database. A sequence from *Bacteroides fragilis* was also
618 obtained from the NCBI nucleotide database to serve as an outgroup. Sequences were
619 imported into Mega7, aligned with MUSCLE, and trimmed to a uniform 1364 bp. The
620 Kimura 2-parameter model with a discrete Gamma distribution was selected using the

621 “Find Best DNA/Protein Models” command in Mega7. A Maximum Likelihood tree was
622 then constructed with 1000 bootstrap replicates.
623
624 The Chlamydiae full length 16S rRNA phylogeny was created using 1-2 representative
625 species from the nine families in the phylum Chlamydiae from Taylor-Brown et al.,
626 (2015), 1 from *Ca.* Arenachlamydiaceae (Pillonel et al. 2018), and the three top hits for
627 each haplotype from the SILVA database. Full length 16S rRNA sequences of chosen
628 species were obtained from both the NCBI nucleotide and SILVA databases.
629 Sequences were imported into Mega7 and aligned with MUSCLE, and then trimmed to
630 1354 bp. The Tamura 3-parameter model with a discrete Gamma distribution was
631 selected for the short tree, and the General Time Reversal model with a discrete
632 Gamma distribution and invariant sites was selected for the full-length tree using the
633 “Find Best DNA/Protein Models” command in Mega7. A Maximum Likelihood tree was
634 then constructed for each with 1000 bootstrap replicates.
635
636 <u>Verification of symbiont strain novelty</u>
637 In order to verify that, to the best of our knowledge, these *D. discoideum* Chlamydiae
638 lineages are novel, we searched the SILVA database (in addition to the NCBI Blast
639 database) for nearest neighbors. A table of the sequence identities to the SILVA
640 database for all of our full length 16S rRNA endosymbiont haplotypes is included as
641 Supplemental Table 2. Two recent papers (Pillonel et al., 2018 and Dharamshi et al.,
642 2020) have newly discovered Chlamydiae lineages so we compared our 16S rRNA
643 sequences to theirs where possible. The phylogenetic analyses from the Pillonel et al.

644 paper were done from metagenomic sequences, which often lack identified 16S rRNA
645 genes, and thus it was not always possible to compare directly. We did use BLAST to
646 query our sequences against these metagenomes in NCBI and in no case did we find a
647 match with >85% sequence identity, if a match was found at all. For the Dharamshi et
648 al. paper we separately aligned our sequences (full length Chlamydiae haplotypes 1-3)
649 with their publicly available 16S rRNA sequences. We ran a Neighbor-joining
650 phylogenetic analysis in MEGA 7 and computed a pairwise distance matrix. Finally, we
651 searched the IMNGS database (Lagkouvardos et al. 2016), which is designed to query
652 sequence read archives from NCBI for 16S rRNA sequences, for matches to our most
653 common *D. discoideum* Chlamydiae haplotype.
654

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662

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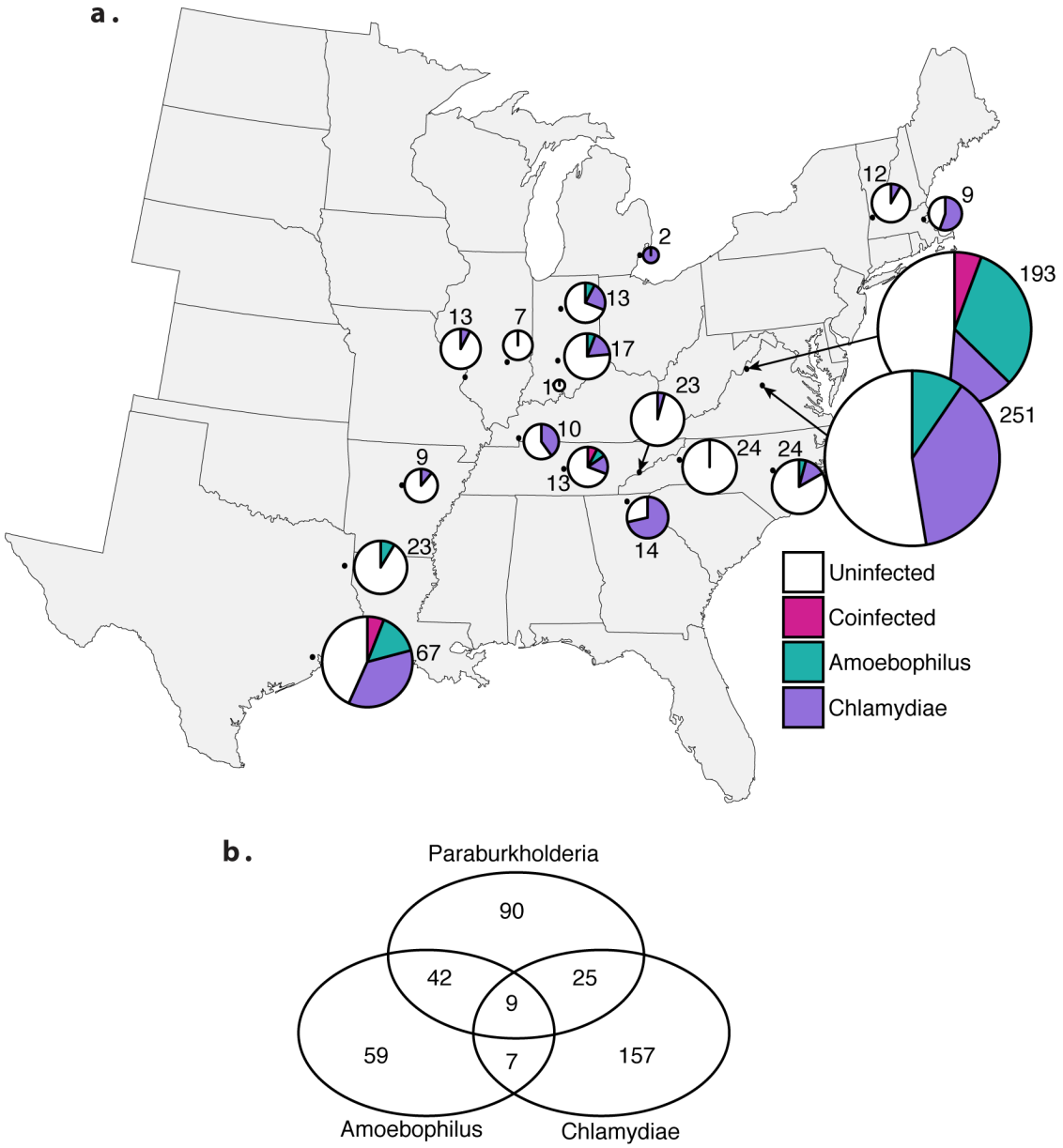
965 **Table and Figure Legends:**



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967 **Figure 1. Unculturable symbiont prevalence in natural *D. discoideum* populations.**

968 (a) Pie charts indicate Chlamydiae, *Amoebophilus*, and Chlamydiae-*Amoebophilus* co-

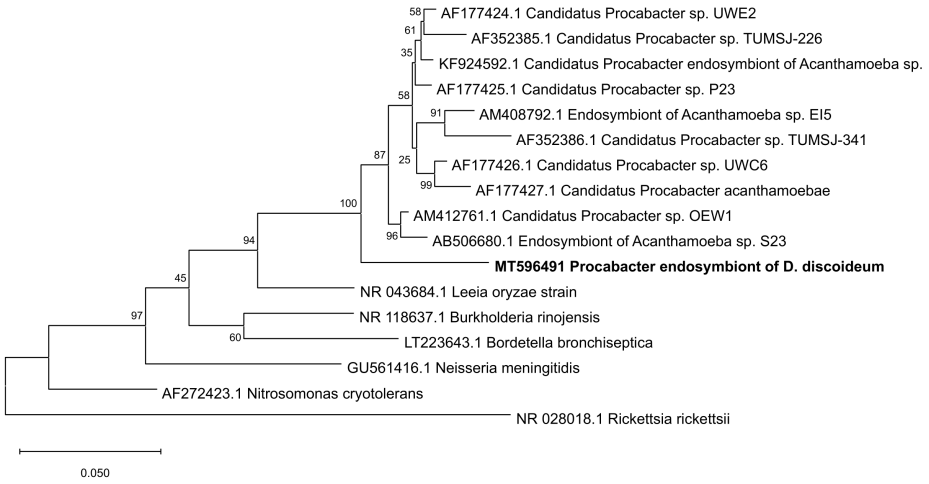
969 infection patterns in respective populations of *D. discoideum* isolates from across the

970 Eastern United States. *Procabacter*, due to its low prevalence, is not included. Numbers

971 next to pie charts indicate the number of isolates for each population. (b) Of all symbiont

positive amoebae isolates, several are co-infected with more than one symbiont species, represented by the Venn diagram indicating the number of isolates infected with *Paraburkholderia, Amoebophilus,* and Chlamydiae.

a.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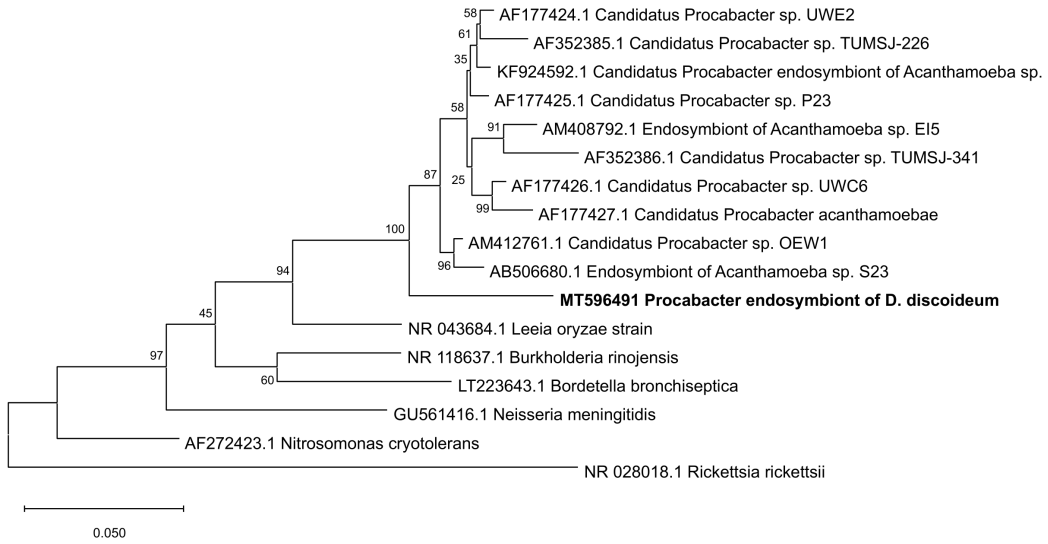
AF177424.1 Candidatus Procabacter sp. UWE2
AF352385.1 Candidatus Procabacter sp. TUMSJ-226
KF924592.1 Candidatus Procabacter endosymbiont of Acanthamoeba sp.
AF177425.1 Candidatus Procabacter sp. P23
AM408792.1 Endosymbiont of Acanthamoeba sp. EI5
AF352386.1 Candidatus Procabacter sp. TUMSJ-341
AF177426.1 Candidatus Procabacter sp. UWC6
AF177427.1 Candidatus Procabacter acanthamoebae
AM412761.1 Candidatus Procabacter sp. OEW1
AB506680.1 Endosymbiont of Acanthamoeba sp. S23
MT596491 Procabacter endosymbiont of D. discoideum
NR 043684.1 Leeia oryzae strain
NR 118637.1 Burkholderia rinojensis
LT223643.1 Bordetella bronchiseptica
GU561416.1 Neisseria meningitidis
AF272423.1 Nitrosomonas cryotolerans
NR 028018.1 Rickettsia rickettsii
0.050

1011 b.



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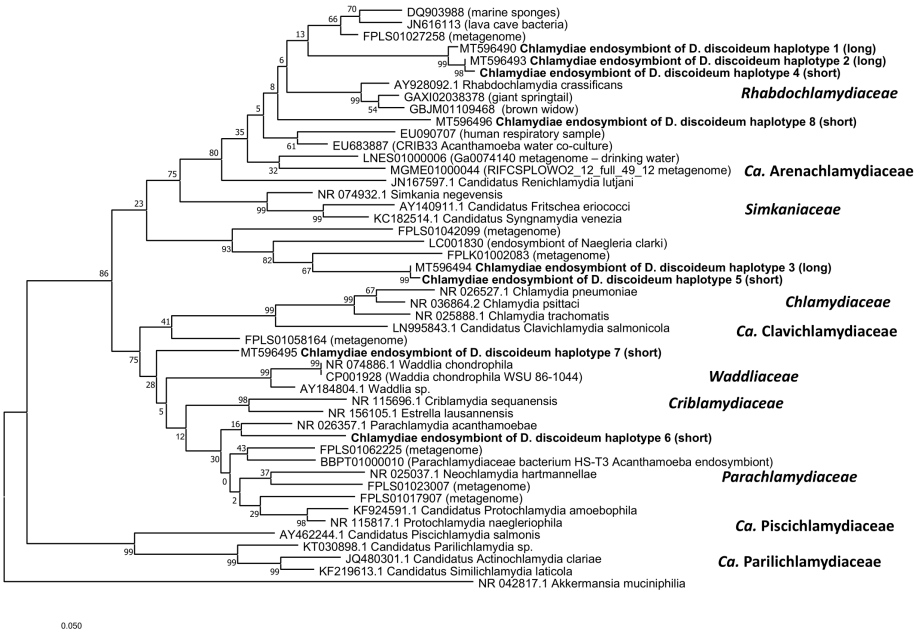
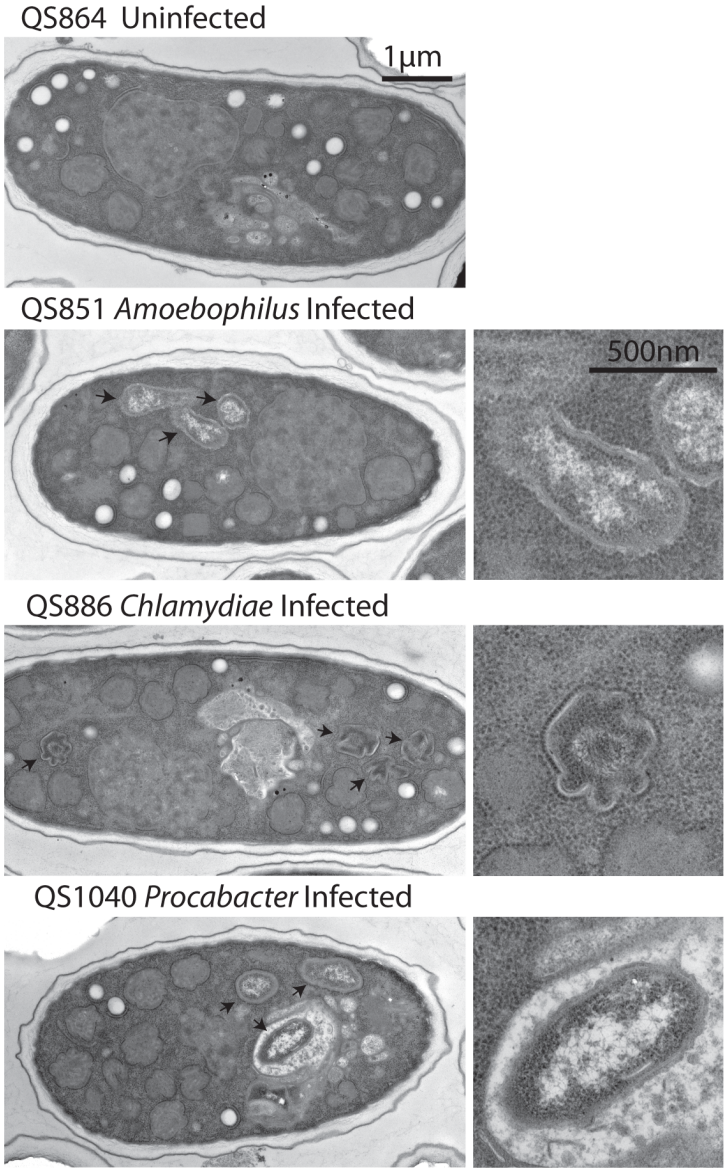


Figure 2. Unculturable endosymbionts of *Dictyostelium* are related to unculturable endosymbionts of other amoebae. 16S rRNA phylogenies of *Procobacter* (a) *Amoebophilus* (b) and Chlamydiae (c & d) endosymbionts constructed using the Maximum Likelihood method in Mega7, with 1000 bootstrap replicates. Family names, where applicable are delineated in bold italics.



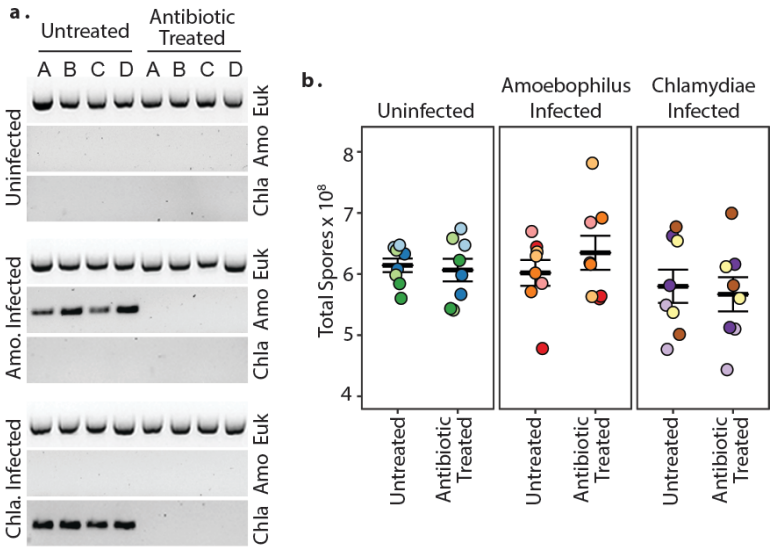
1033

1034 **Figure 3. Unculturable symbionts have distinct morphologies in infected spores.**

1035 Transmission electron micrographs of spores from the indicated representative

1036 uninfected and infected *D. discoideum* isolates. Images on the right show close-ups of

1037 intracellular bacteria.



1038

1039 **Figure 4. Elimination of *Amoebophilus* and Chlamydiae from natural hosts with**

1040 **antibiotics does not impact host reproductive fitness.** Four uninfected,

1041 *Amoebophilus*-infected, and Chlamydiae-infected representative host isolates were

1042 treated with rifampicin to eliminate symbionts (a) and reproductive fitness (b) was

1043 assessed. Symbiont presence is indicated by amplification with *Amoebophilus*-specific

1044 or Chlamydiae-specific PCR and DNA gel electrophoresis (with Eukaryote specific gene

1045 amplification serving as an internal control) (a). Reproductive fitness was assessed by

1046 quantifying total spore productivity of *D. discoideum* cultures after completion of one

1047 social cycle (b). Individual *D. discoideum* isolates are represented by point color.

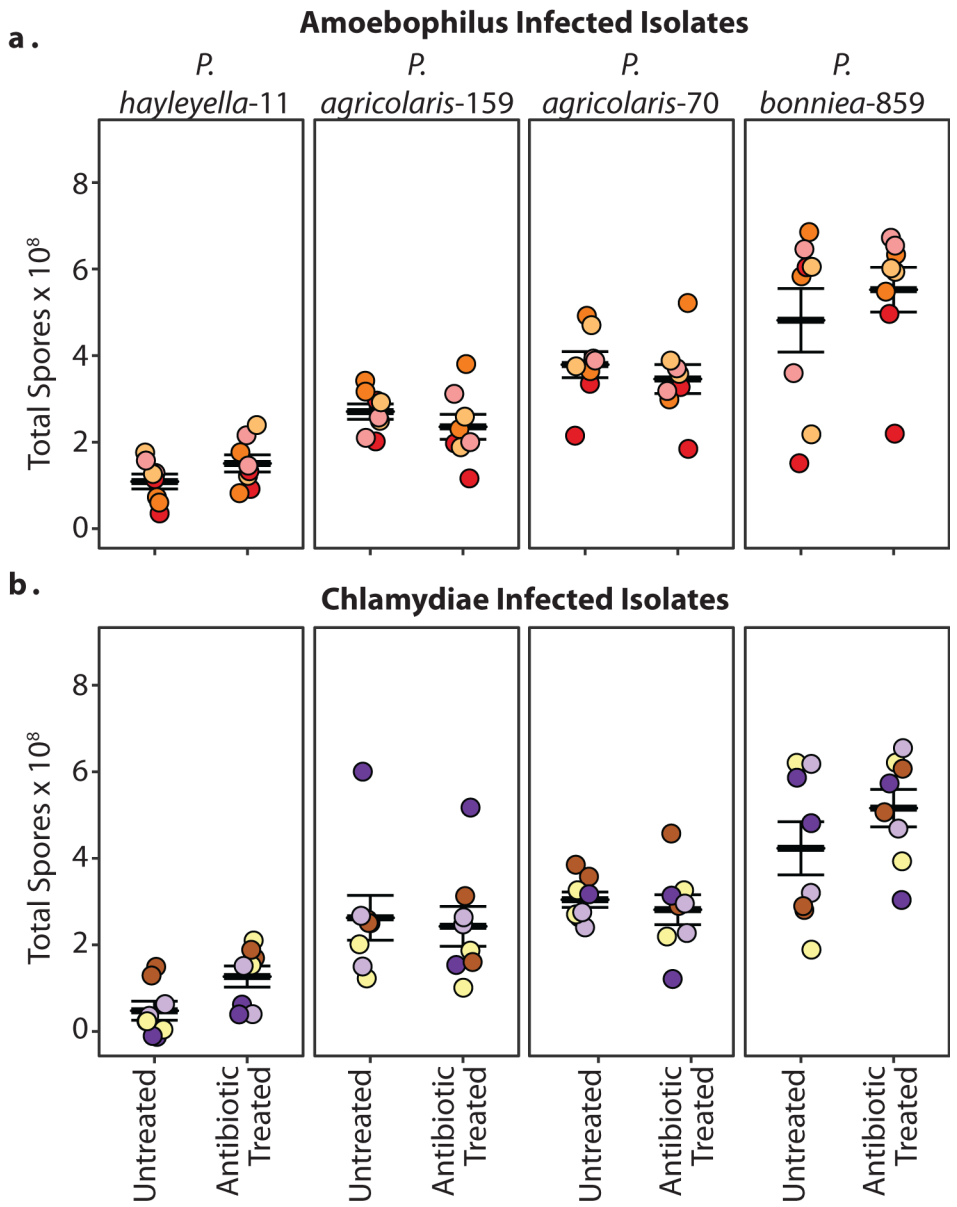


Figure 5. *Amoebophilus* and Chlamydiae infections do not impact the reproductive fitness of hosts co-infected with *Paraburkholderia* symbionts. Reproductive fitness was quantified for four untreated and antibiotic-cured *Amoebophilus*-infected (a) and Chlamydiae-infected isolates (b) following social cycle completion after exposure to the indicated *Paraburkholderia* symbiont strains. Individual *D. discoideum* isolates are represented by point color.

1055 **Table S1. Table of Screened *D. discoideum* Natural Isolates.** Table lists each *D.*
1056 *discoideum* isolate by identification number and location of collection. PCR amplification
1057 of *Dictyostelium*, *Paraburkholderia*, *Amoebophilus*, or Chlamydiae specific DNA is
1058 indicated with a 1 (for positive amplification) or 0 (for negative amplification). Samples
1059 used for fitness assays are highlighted in yellow.
1060
1061 **Table S2. SILVA Database Classification of Symbiont Haplotypes.** Percent
1062 sequence identity to closest hit in the database for the five full length 16S rRNA
1063 sequence haplotypes and their least common ancestor taxonomic classification.
1064
1065 **Table S3. IMNGS database >99% matches for Chlamydiae Endosymbiont of *D.***
1066 ***discoideum* Haplotype 1.** Includes metagenome sample type and collection location.
1067
1068 **Table S4. PCR Primers Used for this Study.** PCR primer names, sequences, and
1069 associated references.
1070