Oligotyping Bacterial Symbionts of Hindgut Protists Reveals Complex Population Structures and Transmission Trends in the Termite *Reticulitermes flavipes*

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Conflict of interest

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Abstract

The hindgut protists of wood-feeding termites are often colonized by an assemblage of prokaryotic endo- and ectosymbionts. These associations can vary between protist species in a single termite species and between protists in different termite species and are, therefore, hard to characterize. The inability to culture termite associated protists has made it difficult to assess aspects of symbioses such as symbiont-host fidelity, symbiont acquisition, and differences in colonization between individual protists within the same termite host. In this study, we utilize high throughput amplicon sequencing of V4 16S rRNA gene to determine the composition of bacterial symbiont populations associated with single protist cells. Operational Taxonomic Units (OTUs) were generated for protist-associated bacteria at 95% identity level and then further analyzed by oligotyping. The sequence diversity of associated symbionts was then compared within and across different protists species in *Reticulitermes flavipes*. Symbiont populations of Oxymonadida and Parabasilia protist species were significantly different from one another. Importantly, oligotypes corresponding to ectosymbionts were extensively shared between different protist species in many cases. Symbiont signatures such as the expansive host range of these ectosymbionts may be explained by their mode of transmission. Using a fluorescence-based cell assay, we observed the horizontal transmission of ectosymbionts over time. Ectosymbiont transmission was dependent on time, was shown to be an active process, and was non-random with respect to binding locations on some host cells. Although some symbionts were horizontally acquired there are likely to be as yet unidentified mechanisms in place to maintain their specificity to certain protist hosts. Our results suggest that oligotyping of symbionts associated with single protist cells, combined with cell-based transmission assays, can reveal insights into the complexity of their bacterial symbionts' population structures and their host

associations.

Importance

Studying the interactions between the numerous kinds of microbiota within the hindguts of lower termites has been difficult because most members of the community have not yet been cultivated. Specifically, intimacies between hindgut protists and their associated bacteria are thought to be essential for the efficient degradation of lignocellulose, yet these symbioses are poorly understood. In this study, we use culture independent methods to assay the composition of the bacterial symbionts of single, yet-to-be cultivated, protist hosts as well as observe the dynamic nature of their transmission. These methods shed light into the ecology of this multipartite symbiosis by demonstrating with both molecular and cellular data, that some bacterial symbionts are shared across different protist species via horizontal transmission while others maintain fidelity to specific protist hosts.

88

89 **Introduction**

90 The lower termite *R. flavipes* harbors symbionts, from the three domains of life, all of
91 which make significant contributions to the digestion of lignocellulose. These symbionts include
92 uncultivated, hindgut protists of two eukaryotic taxa, Oxymonadida (Class) and Parabasilia
93 (Order) (1, 2). Many of these protists are colonized by both endo- and ectosymbionts from
94 various bacterial taxa (2). These protist-associated bacteria often exhibit complex population
95 structures and occupy different ecological niches on and within their unicellular host.

96 Previous studies have shown that Oxymonadida protists in *Reticulitermes speratus* are
97 co-colonized with *Treponema* ectosymbionts from two distinct phylogenetic clusters (Termite
98 Treponema clusters I and II) (3, 4) as well as a member of the *Bacteroidales* (5). These three
99 ectosymbionts lineages attach by one cell pole to the plasma membrane of their host (6) and
100 exhibit intermixed colonization (5). Other known ectosymbionts include a *Desulfovibrio* species
101 which embeds in the membrane of its Parabasilia host, *Trichonympha* (7). Functional data
102 regarding the nature of these symbioses are limited, but growing. Genome analysis of a
103 *Bacteriodales* ectosymbiont of a *Dinenympha* species found in *R. speratus*, '*Candidatus*
104 Symbiothrix dinenymphae', suggests that it may directly degrade lignocellulose and/or aid its
105 protist host in cellulose degradation (8).

106 The endosymbionts which colonize the cytoplasm of hindgut protists of *Reticulitermes*
107 termites are also composed of several bacterial taxa which vary between different protist and
108 termite species. Some of these include *Endomicrobium* (9), and '*Candidatus* Ancillula' (10). In
109 addition, the nuclei of some hindgut protist species are colonized by *Verrucomicrobia* (11).
110 Genome analysis of some of these endosymbionts suggests that there is convergent evolution for

111 these symbionts to provide support to their unicellular host by synthesizing nutrients absent in
112 the termite's diet (10, 12, 13). Regarding *Endomicrobium*, previous studies have investigated
113 both their population structure and transmission in *Trichonympha* spp. protist hosts. Across
114 various *Trichonympha* spp. these endosymbionts share congruent rRNA gene phylogenies with
115 their host (14) and are composed of a single phylotype (99% sequence identity of their ITS
116 region), which are not shared across different *Trichonympha* spp. (15). These data supported the
117 hypothesis that these endosymbionts are vertically acquired.

118 The associations between termite protists and their symbiotic bacteria are complex and
119 not well understood. For example, in *Reticulitermes* spp. different protist species associate with
120 *Treponema* from the same phylogenetic clusters, but the composition and fidelity of those
121 associations are not resolved beyond those broad phylogenetic groups. Furthermore, assessing
122 the diversity of bacteria which associate with termite protists has been challenging since these
123 protists are not yet cultivated, and are hard to isolate from their termite hosts. Previous studies
124 have overcome these challenges by either using samples which consisted of pooled protist cells
125 (3) or samples in which whole genome amplification (WGA) was performed (15). These
126 methods have yielded novel information, but pooling protist cells limits host and symbiont
127 resolution by depicting the averaged composition of all individuals in a pooled sample and WGA
128 can sometimes bias relative abundance data (16, 17).

129 Here, we used a method in which uncultivated single protist cells, isolated from the
130 hindgut of *R. flavipes*, served as a template for high-throughput amplicon sequencing of the
131 hypervariable V4 region of bacterial symbiont 16S rRNA genes as well as co-amplifying the 18S
132 rRNA gene of the protist host. Using this method, the bacterial symbiont composition of single
133 protist cells can be investigated at high resolution and coverage compared to other methods. In

134 addition, the ability to co-amplify the 18S rRNA gene of individual host cells gives further
135 insight into these complex interactions by determining the relatedness of individual protist cells.
136 We selected six different protist species, four from the genus *Dinenympha* (Oxymonadida),
137 *Pyrsonympha vertens* (Oxymonadida), and *Trichonympha agilis* (Parabasilia). Using these
138 protist species, we tested whether closely related protists, living in the same termite host,
139 associated with similar bacterial symbionts compared to more distantly related protists. We were
140 also able to discern differences in the population structures of the different kinds of bacterial
141 symbionts associated with single protist hosts. Using single isolated protist cells from the hindgut
142 of *R. flavipes* operational taxonomic units (OTUs) were generated at the 95% identity level and
143 the sequences were further characterized by oligotyping. Our results support previous
144 observations in other *Reticulitermes* spp. termites that some hindgut protists share ectosymbiotic
145 *Treponema* but we also extend those observations to another ectosymbiont lineage, '*Ca*.
146 Symbiothrix'. In addition, we show that individuals of a protist species can contain different
147 predominate oligotypes from one another.

148 The observation that different protists species share some ectosymbiont oligotypes
149 suggests that these particular symbionts may be cosmopolitan and horizontally acquired by their
150 protist hosts. To test this hypothesis, we developed an in vitro fluorescence assay which allowed
151 us to detect the horizontal acquisition of ectosymbionts by different protists species. We show
152 that the horizontal transmission of these ectosymbionts required active biological processes and
153 the symbionts exhibited preferential spatial binding to their host cells in some cases. Using this
154 high-resolution molecular approach combined with our transmission assay, we show that almost
155 all symbiont oligotypes are exclusive to either Parabasilia and Oxymonadida protists in *R*.
156 *flavipes* and that their population structures vary with respect to their ecological niche and mode

157 of transmission.

158

159 **Materials and methods**

160

161 **Termite collection, maintenance, and identification.**

162 *R. flavipes* termites were collected using cardboard traps placed under logs, actively colonized by
163 termites, for 2 to 4 weeks in Mansfield Connecticut, USA. Termites were removed from traps
164 and colonies were established in plastic containers containing moistened sterile sand and spruce
165 wood. Species identity of the termites was verified to be *R. flavipes* by solider cast morphology
166 (18), the presence of *Dinenympha gracilis* in the hindguts of worker termites (19, 20), and
167 sequencing of the cytochrome oxidase II gene (Supplementary Fig 1) with primers A-tLEU: 5'-
168 ATGGCAGATTAGTGCAATGG-3' (forward) and B-tLys: 5'-
169 GTTTAAGAGACCAGTACTTG-3' (reverse)(21). For all experiments, only individuals of the
170 worker cast were chosen.

171

172 **Amplification and sequencing of protist and bacterial SSU rRNA genes.**

173 Samples consisting of single protist cells were prepared from termites in an anaerobic chamber
174 with atmospheric content of CO_2 5.5%, H_2 5.5%, and N_2 89%. Hindguts were dissected and then
175 ruptured in Trager's solution U (TU) (22). Hindgut contents were then washed by centrifugation
176 at 3,000 rpm for 90 seconds, and then resuspended in TU for a total of three washes. Samples
177 were then diluted and spotted on glass slides treated with RNase AWAY® Reagent (Life
178 Technologies) and UV light. Individual protist cells were isolated using a micromanipulator
179 (Eppendorf CellTram® Vario) equipped with a hand-drawn glass capillary. Protists cells were

180 washed three times in droplets of TU via micromanipulation, placed in 10μl molecular grade TE
181 Buffer, and then frozen at -20°C.
182 Frozen protist cells served as templates for PCR reactions in which the 18S rRNA gene
183 of the protist host as well as the V4 hypervariable region of the 16S rRNA gene of bacteria were
184 co-amplified and sequenced. PCR reactions consisted of Phusion® High-fidelity polymerase (1
185 unit), HF buffer, dNTPs (200 μM), dimethyl sulfoxide (DMSO) (3%), 0.3 μM of each 18S
186 primer (Euk19f, 5'-AYYTGGTTGATYCTGCCA-3' and Euk1772r; 5'-
187 CBGCAGGTTCACCTAC-3') (23), 0.2 μM each of V4 16S primers (515f; 5'-
188 GTGCCAGCMGCCGCGGTAA-3' and 806r; 5'-GGACTACHVGGGTWTCTAAT-3',
189 annealing sequence) (24), and a single protist cell in a final reaction volume of 50 μl. PCR
190 conditions were as follows: Initial denaturation was at 94°C for 3 minutes followed by 35 cycles
191 of 94°C for 45 seconds, 50°C for 60 seconds, 72°C for 2 minutes. Final extension was at 72°C for
192 10 minutes (25). For *P. vertens* and *D. gracilis* primers 18SFU; 5'-
193 ATGCTTGTCTCAAAGGRYTAAGCCATGC-3' and 18SRU; 5'-
194 CWGGTTCACCWACGGAAACCTTGTTACG-3' were used (26).
195 PCR products were size-selected by using a 1% agarose gel, purified using the Wizard®
196 SV Gel and PCR Clean-up System (Promega), and quantified using Qubit™ Flourometric
197 quantitation (ThermoFisher Scientific). Barcoded V4 16S rRNA gene amplicons were pooled at
198 4nM equimolar concentrations and sequenced on an Illumina Miseq (25).
199 18S rRNA gene amplicons were cloned using the pGEM®-T Easy Vector System
200 (Promega) following the manufacture's protocol, and sequenced by Sanger sequencing. In
201 addition to protist samples, negative controls consisting of TU, TE, and protist-free technical
202 controls were amplified and sequenced. If needed, additional isolated protist cells were used in

203 18S rRNA gene-only PCR reactions and the amplicons were cloned and sequenced as described
204 above.
205
206 **V4 16S amplicon filtering, OTU clustering, and oligotyping analysis**
207 V4 16S rRNA gene reads were quality filtered by removing reads that were below an average
208 quality score of Q20 using BBMap (27). Reads were then analyzed using a Quantitative Insights
209 into Microbial Ecology (QIIME™) workflow. Reads were clustered into de novo OTUs at the
210 95% identity level using the "pick_de_novo_otus.py" script in MACQIIME (version 1.8). OTU
211 percent identity cutoffs were determined by comparing the percent identities of V4 16S SSU
212 rRNA genes of reference *Treponema* spp. and *Endomicrobium* spp. sequences that defined
213 species cutoffs (Supplementary Fig 2). These references were selected as biomarkers for our
214 OTU clustering since *Treponema* and *Endomicrobium* represented the most abundant taxa in our
215 dataset. Sequences were checked for chimeras using the "parallel_indentify_chimeric_seqs.py"
216 with references from the Dictdb (28) and GreenGenes (29) databases.
217 OTUs were filtered, and contaminates removed, using several criteria. OTUs were deemed
218 contaminates if they were not at least 10 times higher in protists samples compared to negative
219 controls. Second, any OTUs which were not at least (i) ≥0.15% of total read abundance on all
220 individuals of a given protist species or (ii) ≥1.5% of the total read abundance for at least two
221 individuals in a protist species were removed. Sequences which clustered into OTUs that passed
222 this filtering were then further analyzed by oligotyping. Statistical significance in the difference
223 in OTU abundances and heterogeneity across different protist species were tested using the G-
224 Test and PERMANOVA tests respectively.
225 Oligotyping analysis was performed for bacterial taxa of interest using the pipeline and

226 software developed by Eren and coworkers (30). Reads were extracted for bacterial taxa of
227 interest using the "q2oligo.py" script and then reads were padded with gap characters to ensure
228 equal length using the "o-pad-with-gaps" command. The reads were then assessed for Shannon
229 entropy using the "entropy-analysis" command to identify informative base positions based on
230 their sequence variation. Oligotyping was then performed using the "oligotype" command with
231 the minimum substantive abundance parameter (-M) set at 20, and the minimum abundance for
232 at least one sample parameter (-a) set a 1.0. The oligotyping analysis was iteratively performed
233 including high entropy (>0.2) base positions until the oligotypes for each taxon had converged,
234 meaning that further analysis would not generate additional oligotypes. Oligotypes were then
235 manually filtered by excluding those from samples which were comprised of a single read and/or
236 were below 0.1% in their relative abundance.
237 Oligotyping was also performed on V4 16S rRNA gene reads from samples consisting of
238 the free-living bacterial fractions (fractions enriched in bacterial cells not attached to protists)
239 from three individual termite hindguts. Hindguts were dissected and ruptured as described above.
240 The bacterial fraction of their hindgut contents was prepared by centrifugation. Total hindgut
241 contents were centrifuged at 3,000 rpms for 90 seconds to separate large cells (protist cells) from
242 smaller bacterial cells. This free-living bacterial fraction (supernatant) was then centrifuged at
243 13,000 rpms for 90 seconds followed by three washes in TU and resuspended in molecular grade
244 TE buffer. Bacterial V4 16S rRNA genes were amplified and sequenced as described above. All
245 reads from these samples were processed using the same pipeline as the reads from single protist
246 cells.
247
248 **Phylogenetic analysis of SSU rRNA genes**

249 For bacterial OTUs, representative sequences (centroid sequences) of *Treponema*, *Bacteriodales*
250 and '*Ca*. Endomicrobium' were aligned to full length 16S rRNA gene reference sequences of
251 each taxa using MUSCLE (31). Appropriate evolutionary models were determined for each
252 alignment using jModelTest (32, 33) and phylogenetic trees were generated using RAxML (34)
253 using the full alignment length. This phylogenetic analysis of the centroid sequences from OTUs
254 revealed that the QIIME workflow mistakenly designated a certain *Bacteriodales* OTU as
255 '*Candidatus* Azobacteroides', which should have been designated as '*Ca*. Symbiothrix'. The 18S
256 rRNA genes obtained by this study were also aligned to reference sequences using MUSCLE and
257 the full alignment was used to make a phylogenetic tree with RAxML.
258
259 **Scanning electron microscopy**
260 Scanning electron microscopy (SEM) was used to investigate the morphology of hindgut protists
261 and their ectosymbionts. Protist cells were collected by low spin centrifugation as described
262 above and fixed in 2% glutaraldehyde in TU (pH 7) for 1 hour at RT in an anaerobic chamber.
263 The samples were deposited onto poly-L-lysine coated silicon wafer chips (Prod No. 16008, Ted
264 Pella Inc.), washed with 80 mM Na cacodylate buffer (pH 7), and post-fixed in 2% osmium
265 tetroxide at RT for 1 hour. The cells were rinsed twice for 5 minutes in distilled water then
266 dehydrated in serial concentrations of ethanol (30%, 50%, 70%, 95%, 100%, 5 min each), and
267 critical point dried (931GL, Tousimis). Samples were then mounted on SEM stubs using silver
268 paint, sputter coated with Palladium (E5100, Polaron), and examined using a scanning electron
269 microscope (Nova NanoSEM 450, FEI).
270
271 **Fluorescent symbiont transmission assays**

272 For all tranmission assays, experiments were carried out in an anaerobic chamber with gas
273 composition as described above. Hindguts were dissected from termites, ruptured with sterile
274 forceps, and their contents were collected in anaerobic buffer containing anaerobic water with
275 resazurin (1μg/ml), sodium thioglycolate (0.5g/L), and sodium bicarbonate (0.1M) pH 8.0 (Pedro
276 et al., 2004). Samples were then fractionated by low spin centrifugation (3,000 rpm for 90
277 seconds) to separate protists and from bacteria which were unattached to protists. Each fraction
278 was then washed three times in buffer by centrifugation at either 3,000 rpm (for protist fraction)
279 or 13,000 rpm (for bacterial fraction) for 90 seconds. The washed fractions were then split into
280 two equal volume groups and stained with either Texas Red®-X succinimidyl ester (TRSE,
281 Molecular Probes™) or AlexaFlour 488 succinimidyl ester (SE488, Molecular Probes™) at
282 10μg/ml for 1 hour at room temperature (RT) in the dark in the anaerobic chamber. Dye
283 conjugation was done per manufacturer's instructions. Stained cells were then washed 3 times in
284 TU with reduced glutathione serving as a stop reagent for the amine reactive dyes. Protist and
285 bacterial fractions were combined to produced two samples (Red-TRSE-stained and Green-SE-
286 488 stained).
287 To assay for symbiont acquisition by protists, the two samples (Red-TRSE-stained and
288 Green-SE-488 stained) were combined and monitored for the horizontal acquisition of new
289 bacteria which was evident by heterogeneity in fluorescent signals of bacteria on individual
290 protists. Samples were taken at various time points (0, 3, 15, and 20 hours), fixed with 2%
291 formaldehyde, and viewed using a Nikon TE300 Eclipse microscope. Alternatively, fixed
292 samples were mounted in ProLong™ Diamond Antifade Mountant (ThermoFisher) and imaged
293 using a Nikon A1R Spectral Confocal microscope. To test if symbiont acquisition required
294 biologically active processes, this assay was repeated with the addition of either tetracycline

295 10μg/ml or cycloheximide 10μg/ml to each sample 1 hour prior to the start of the assay and
296 compared to a no treatment control. In addition, anaerobic symbionts were killed by exposure to
297 atmospheric oxygen, labeled with propidium iodide (PI), and mixed with live cells to assay for
298 the binding of dead bacteria to live protist hosts.

299 The fluorescent assay was then used to investigate whether or ectosymbionts could come
300 from the free-living (unattached) pool of bacteria. Hindgut contents were fractionated into
301 bacterial or protist fractions and stained with TRSE as described above. These TRSE labeled
302 fractions where then added to a SE-488 stained protist cell fractions and incubated in an
303 anaerobic chamber as described above. Samples were taken at 15 hours post the start of the
304 assay, fixed, and viewed as described above.

305

306 **Sequence submission**

307 In progress. All 18S rRNA gene sequences derived from protists have been submitted to the
308 National Center for Biotechnology Information (NCBI) Genbank under accession numbers
309 MH174292 – MH174303 as well as the termite mitochondrial cytochrome oxidase II gene
310 (accession number MH171305). Sequences of the bacterial V4 16S gene amplicons are being
311 submitted to the NCBI Sequence Read Archive (SRA).

312

313 **Results**

314 **Morphological and phylogenetic diversity of hindgut protists**

315 The morphology of protists used in this study was investigated using both light microscopy and
316 SEM. These data along with their 18S rRNA gene phylogeny indicated that these protists
317 consisted of five different species: *T. agilis*, *P. vertens*, *D. gracilis*, *D. fimbriata*, and two

318 uncharacterized *Dinenympha* species (I & II). We obtained near-full length or partial (>1 kb) 18S
319 rRNA genes sequences from individual protist cells, aligned them to references sequences, and
320 reconstructed their phylogeny using RAxML. Undescribed species such as *Dinenympha* species I
321 & II clustered within other *Dinenympha* sequences supporting that they are indeed members of
322 that genus (Fig 1). Differential interference contrast (DIC) micrographs of representative
323 morphotypes of each protist species used in this study are provided as Supplementary Fig 4.
324

325 **OTU composition of the bacterial symbionts of individual hindgut protists**

326 Despite residing in the same termite host, the community of bacterial symbionts found on
327 *Trichonympha*, *Pyrsonympha*, and *Dinenympha* species were significantly different from one
328 another (PERMANOVA f=2.21 p=0.001) (Supplementary Fig 2). Even when an OTU was
329 present on different protist species, the relative abundance of that OTU was significantly
330 different across those protists (G-test, Bonferroni $p \leq 0.05$). Based on these observations we
331 concluded that associations between these protists and their bacterial symbionts are not random
332 in *R. flavipes*.

333 With the exception of one OTU (*Treponema* OTU4), all other *Treponema* OTUs were
334 exclusive to either Oxymonadida or Parabasilia hosts (Fig 2). Interestingly, these OTUs
335 correspond to ectosymbiotic *Treponema* which are known to colonize *T. agilis* and various
336 *Dinenympha* species in *Reticulitermes* termites. The other known ectosymbiotic lineage '*Ca.*
337 Symbiothrix', was composed of a single OTU and was found at various relative abundancies
338 across each *Dinenympha* individual and to the exclusion of other protists species.

339 The *Endomicrobium* symbionts clustered into two OTUs (*Endomicrobium* OTU1 &
340 OTU2). The first OTU was found only in *T. agilis* cells while the second was found in *P. vertens*

341 and *D*. species I & II. Interestingly, *D. gracilis* and *D. fimbriata* lacked these endosymbiotic
342 bacteria. *Verrucomicrobia* symbionts, known to colonize the nuclei of some protists, were found
343 associated with two of the protists species we sampled (*D. fimbriata* & *D*. species I).
344 *Desulfovibrio* symbionts were composed of a single OTU and only found associated with
345 *D*. species II. Symbionts of this bacterial taxon were previously found embedded in the plasma
346 membrane of *T. agilis* in a different termite, *R. speratus* (7). Surprisingly the samples of *T. agilis*
347 cells that we examined from *R. flavipes* did not contain any reads from *Desulfovibrio* bacteria.
348 Other bacterial taxa included *Bacteriodales*, which were clustered into two OTUs
349 (*Bacteriodales* OTU1 and OTU2). Two of three individual *P. vertens* cells (cells D and F)
350 contained *Bacteriodales* OTU1 while the second OTU was found associated with all three single
351 cells of the *D*. species II cells used in this study. The remaining OTUs corresponded to
352 *Sediminibacterium* which was found across all the *Dinenympha* spp. and *Ruminococcaceae*
353 which was found associated with *D*. species I.
354
355 **Distribution of symbiont oligotypes across individual protist hosts**
356 The diversity and distribution of certain bacterial taxa were further investigated using an
357 oligotyping analysis which provides a higher-resolution survey of the diversity within bacterial
358 OTUs (30). For example, protist-associated *Treponema* symbionts were initially clustered into 8
359 OTUs, at the 95% identity level, were further clustered into 42 distinct oligotypes (Fig 3A). Most
360 *Treponema* oligotypes (34 out of 42) were extensively shared across different protist species
361 with only 8 oligotypes being exclusive to a single protist species. One *Treponema* oligotype in
362 particular (TO1) was found on a single *T. agilis* cell (cell B) but also on every *Dinenympha* spp.
363 cell used in this study. Another oligotype (TO15) was shared among all *T. agilis* cells but only

364 found on one *D*. species II cell (cell O). These data suggest that almost all *Treponema* are
365 exclusive to either Parabasilia or Oxymonadida hosts with most being shared across the different
366 *Dinenympha* spp. but only a few shared between Oxymonadida protists and *T. agilis*.
367 The sequence variation between these *Treponema* oligotypes varied from as a few as one
368 base pair (bp) difference to as many as 55 bp differences over their V4 16S rRNA gene sequence
369 (Fig 3A). At the sequence level, the three oligotypes which were shared between *T. agilis* and the
370 *Dinenympha* spp. (TO1, TO15, & TO16) were more similar to one another than they were to
371 other oligotypes which are not shared between those protist hosts. For example, TO1 was 5 bp
372 different from TO16 but was 55 bp different from TO10 which is an oligotype only found on *T.*
373 *agilis* cells. This indicates that sequence similarities among different *Treponema* oligotypes does
374 not directly correspond to their associations with a particular protist species.
375 The '*Ca*. Symbiothrix' oligotypes shared a similar population structure to that of the
376 *Treponema* symbionts. Although the sequences corresponding to these ectosymbionts clustered
377 into a single OTU, they were further classified into eight oligotypes (Fig 3B). These oligotypes
378 were between 1 and 5 bp different from one another across their V4 16S rRNA gene sequence
379 and were shared across different *Dinenympha* spp. hosts. Most *Dinenympha* spp. cells were co-
380 colonized by multiple '*Ca*. Symbiothrix' oligotypes but some individuals (cells G, H, J, and T)
381 were colonized by predominately one '*Ca*. Symbiothrix' oligotype.
382 The *Endomicrobium* sequences, which initially clustered into two OTUs, where further
383 classified into 8 oligotypes (Fig 3C). *T. agilis* cells were colonized by two predominate
384 *Endomicrobium* oligotypes (EO1 and EO2). Interestingly although all of the *T. agilis* cells
385 contained these two oligotypes, within each host cell only one oligotype was predominate (≥99%
386 in relative abundance). Two of the three *T. agilis* cells (cells A and B) were both predominately

387 colonized by EO1 while the third cell (cell C) was colonized by EO2. These two oligotypes
388 differed by 1 bp from one another but were between 11 to 15 bp different from the
389 *Endomicrobium* oligotypes found in *Dinenympha* spp. and *P. vertens* samples.
390 The *Endomicrobium* that were associated with the different Oxymonadida protists were
391 classified into six different oligotypes (Fig 3C). In *P. vertens* cells, there was one predominate
392 oligotype (EO3) which was exclusive to that protist species and was at least 99% in their relative
393 abundance. One *P. vertens* cell (cell F), had an additional *Endomicrobium* oligotype (EO4)
394 which found at low abundance (0.9%) and not in the other *P. vertens* cells (cells D and E)
395 however, it was found in one *Dinenympha* species I cell (cell R).
396 With the exception of one cell (cell Q), all *Dinenympha* spp. cells which had
397 *Endomicrobium* symbionts were also colonized by a predominate oligotype. Interestingly one
398 *Endomicrobium* oligotype (EO8) was present at various abundancies across multiple
399 *Dinenympha* spp. cells (cells O, P, Q, R, S). EO8 was the only *Endomicrobium* oligotype found
400 associated with cell S but it was also found associated with other *Dinenympha* species such as
401 cell Q, where it was at almost equal abundances with another oligotype (EO7). Thus, all
402 *Endomicrobium* oligotypes except EO4 and EO8, were restricted to a single host species.
403
404 **Phylogenetic diversity of protist-associated *Treponema, Bacteriodales*, and *Endomicrobium***
405 The *Treponema* represent the most diverse bacterial taxa associated with the protists investigated
406 in this study. Collectively they comprised 8 OTUs and 42 oligotypes. Using SEM, multiple
407 morphologically distinct *Treponema*, which were indentified by the presence of endoflagella,
408 were observed to be attached to single protist cells (Fig 4B & 4C), supporting that these
409 ectosymbiont populations are heterogeneous in their composition, as has been seen by others (3,

410 4).

411 The diversity of these *Treponema* was further investigated by aligning representative
412 sequences from each OTU to full length 16S rRNA gene reference sequences and generating a
413 16S rRNA gene phylogeny. As seen in *R. speratus*, hindgut protists are co-colonized with
414 members from both termite *Treponema* clusters I & II (3) (Fig 4A). Although these two
415 *Treponema* clusters were present at equal relative abundances on each protist species, their
416 overall abundance differed across protist hosts (2way ANOVA p=0.009) (Fig 4D). For example,
417 cluster II *Treponema* were significantly higher in their relative abundance on *D. fimbriata*
418 compared to *T. agilis*, *D. gracilis*, and *D.* species I.

419 The 16S rRNA gene phylogeny of the two *Endomicrobium* OTUs obtained from this
420 study was also investigated. One OTU (*Endomicrobium* OTU1) was exclusive to *T. agilis*, while
421 the second (*Endomicrobium* OTU2) was found in both *P. vertens* and two species of
422 *Dinenympha* (Fig 1A). Each of these OTUs clustered with reference sequences derived from
423 hindgut protist samples from other termite species. *Endomicrobium* OTU1 clustered with
424 '*Candidatus* Endomicrobium trichonymphae' (9, 13) references while the second OTU clustered
425 with '*Candidatus* Endomicrobium pyrsonymphae' (9) reference sequences (Supplementary Fig
426 3). These data support that the *Endomicrobium* are vertically acquired by their host since they
427 clustered in accordance to their host protist species which supports previous studies (14, 15).

428

429 **Horizontal transmission of ectosymbionts**

430 The OTU clustering and oligotyping analysis of bacterial symbionts of single protist cells
431 suggested that some ectosymbiont types (*Treponema* and '*Ca*. Symbiothrix) associated with
432 multiple protist species. We hypothesized that these molecular data indicated symbiont sharing

433 and may come about through the horizontal transmission of ectosymbionts. An in vitro
434 fluorescence-based assay was developed to test this hypothesis. Protists and bacterial from the
435 hindgut of *R. flavipes* were stained with either TRSE (red fluorescence) or SE488 (green
436 fluorescence), mixed together, and the transfer or acquisition of new ectosymbionts was assayed
437 over time. Since protists began the experiment with ectosymbiont populations that were
438 homogeneous in their fluorescent label, newly acquired ectosymbionts were evident based on
439 fluorescent heterogeneity of ectosymbiotic bacteria. Transmission observed in this assay should
440 represent only half of the total transfer events since we could not distinguish newly acquired
441 ectosymbionts which were the same color as the majority of the cells on the host. Over time
442 many species of protist hosts including *T. agilis* and several species of *Dinenympha* acquired an
443 increasing number of horizontally transferred ectosymbionts (Fig 5), which were visibly attached
444 to host's plasma membrane and not entangled in flagella or other bacterial cells (Fig 5). These
445 data support the hypothesis that ectosymbionts of hindgut protist can be horizontally acquired.
446 In well characterized symbioses in which symbionts are horizontally transmitted, several
447 active biological processes are involved. These include changes in the gene expression of the
448 symbiont so that it can properly recognize and occupy its niche on or in its host (36, 37). To
449 determine if ectosymbiont acquisition by hindgut protists requires active processes, we tested
450 whether inhibiting protein synthesis would affect ectosymbiont transmission. The assay was
451 repeated with the addition of either tetracycline or cycloheximide and compared to a no-
452 treatment control. Tetracycline was chosen as the bacteriostatic agent due to previous reports that
453 termite-associated *Spirochaetes* and *Bacteriodetes* decreased in their relative abundance after
454 tetracycline treatment, suggesting that they were sensitive to that antibiotic (38). Cycloheximide
455 has been used to target protein synthesis across different protist taxa (39, 40) and was used in this

456 study in an attempt to interfere with translation in the hindgut protist of *R. flavipes*. Over time,
457 samples which were treated with tetracycline had significantly fewer protists that acquired new
458 ectosymbionts compared to the control (15 hours p=0.06, 20 hours p=0.02) (Fig 6A). These data
459 indicate that inhibiting protein synthesis in the ectosymbionts affected their ability to be
460 horizontally acquired by their protist hosts. Samples which were treated with cycloheximide
461 were not significantly different from the control.

462 In addition to inhibiting protein synthesis, we exposed samples consisting of both protists
463 and bacteria to atmospheric oxygen for several hours, which killed strictly anaerobic organisms.
464 We confirmed that oxygen killed both ectosymbiont and free-living bacteria by labeling with
465 propidium iodide (PI) which labels cells which have died (41) (Fig 6B and 6C). These PI-labeled
466 cells were then added to live samples to assay for the binding of dead ectosymbionts to protist
467 hosts. In these experiments, we did not observe the binding of dead ectosymbionts to live protist
468 cells (n= 4 independent experiments) (Fig 6D). We concluded from these experiments that the
469 horizontal transmission of ectosymbionts requires live ectosymbionts and active translation.
470 These data also support that the horizontal transmission observed in our assays is not due to non-
471 specific binding.

472 We noticed that during these experiments, most newly acquired ectosymbionts appeared
473 to bind to the anterior end of *Dinenympha* species II. To determine if this was true, or if binding
474 was random, newly attached ectosymbionts were counted along the length of this protist species.
475 The resulting data supports that newly acquired ectosymbionts bound more frequently towards
476 the anterior cell pole of *D*. species II (Pearson's R p=0.0005) (Fig 6E-6I) than the posterior cell
477 pole. This increase in frequency at one cell pole compared to the other was not observed in other
478 *Dinenympha* species (Fig 6E). Since this cell pole is lacking flagella from the host cell, is it

479 unlikely that this increased binding is due to entanglement. These data support that the binding of
480 ectosymbionts to protist hosts is not a random event, and that in *Dinenympha* species II there is a
481 preferred region for the acquisition of new, horizontally acquired ectosymbionts.

482 After observing the horizontal transmission of ectosymbionts we decided to sequence the
483 bacterial V4 16S rRNA gene from the free-living bacterial fraction of three hindgut samples to
484 see if we could detect protist-associated oligotypes in those fractions. In each of the three free-
485 living bacterial fractions we detected protist-associated *Treponema* oligotypes which collectively
486 accounted for 82.8% – 92% of the total *Treponema* reads generated from the free-living bacterial
487 fractions (Supplemental Fig 5A). The majority (74% - 82.8% in relative abundance) of these
488 reads had the same V4 oligotypes as protist-associated *Treponema* found associated with
489 *Dinenympha* spp. hosts. The remaining oligotypes were those shared between *T. agilis* and the
490 *Dinenympha* species. We did not observe any reads corresponding to the *Treponema* oligotypes
491 which are exclusively associated to *T. agilis* in these bacterial fractions. Reads corresponding to
492 '*Ca*. Symbiothrix' were also absent in these bacterial fractions.

493 After detecting ectosymbiont oligotypes in the free-living bacterial fractions of hindguts,
494 we used our fluorescence assay to determine if newly attached ectosymbionts could also transfer
495 to protists from the pool of free-living bacteria. In these assays, horizontal transmission was seen
496 from ectosymbionts from both the free-living bacterial fraction containing unattached bacterial
497 cells, as well as from the protist cell fractions, where bacteria were mainly attached to protist
498 (Supplementary Fig 5B). There was no significant difference between the percentage of protist
499 cells that acquired new ectosymbionts from these two cell fractions.

500

501

502 **Discussion**

503

504 In this study, we show that in the termite *R. flavipes*, the associations between hindgut protists
505 and their symbiotic bacteria exhibit specificity in different aspects of their interactions including
506 (i) host range, (ii) transmission, and (iii) population structures. Most bacterial OTUs and
507 oligotypes were exclusive to either Parabasilia or Oxymonadida protists. The sharing of several
508 *Treponema* and '*Ca*. Symbiothrix' oligotypes across all *Dinenympha* protists led to the
509 hypothesis that these ectosymbionts can be horizontally acquired. This was experimentally
510 supported using a fluorescence-based assay, which allowed us to visualize the acquisition of new
511 ectosymbionts by protist cells over time, and test whether acquisition required biologically active
512 processes.

513 Using single protist cells as templets for high throughput amplicon sequencing allowed us
514 to detect how symbiont populations varied between individual host cells. Despite occurring in
515 the same hindgut, associations between protist cells and their symbionts exhibited varying levels
516 of fidelity. For example, even though many ectosymbiont oligotypes were shared across closely
517 related *Dinenympha* species, only a few were shared with *Trichonympha* or *Pyrsonympha*. This
518 suggests that there must be mechanisms that result in, or ensure, specificity between these
519 bacterial symbionts and their protist hosts.

520 The oligotyping analysis provided a high-resolution characterization of the population
521 structures and transmission trends of the bacterial symbionts of hindgut protists. For example,
522 individual *T. agilis* cells differed in which *Endomicrobium* oligotype was predominate. Since the
523 two *T. agilis* cells (cells A and B) which share the same predominate *Endomicrobium* oligotype
524 (EO1) were more similar to one another in their 18S rRNA gene sequence than they were to the

525 third cell (cell C), these differences in their symbiont populations may reflect host divergence.
526 Previous studies have already demonstrated the possibility that what was thought to be a single
527 species of *T. agilis* in *R. flavipes* is likely more than one species (42).

528 Overall, the population structures of these protist-associated bacteria differed from one
529 another with respect to the ecological niche that they occupied on or within their protist hosts.
530 For example, ectosymbiotic bacteria such as the *Treponema* and '*Ca*. Symbiothrix' presented
531 more sequence diversity compared to the intracellular *Endomicrobium* or *Verrucomicrobia*
532 symbionts. This may be due to different ecological factors encountered across the intracellular or
533 extracellular niches. For example, the extracellular environment is likely to be dynamic
534 compared to the cytoplasm of a protist host. Ectosymbionts may experience differences in
535 available nutrients and be subject to greater competition compared to endosymbionts. Supporting
536 this, the ectosymbiont '*Ca*. Symbiothrix dinenymphae', does not show evidence of genome
537 reduction and encodes many genes evolved in polysaccharide degradation, and the uptake of
538 various sugar monomers (8) suggesting that these ectosymbionts may need the genomic and
539 metabolic flexibility to utilize different carbon sources when available. Such selective pressures
540 may be responsible to the increased diversity seen in protist-associated ectosymbionts
541 populations compared to the populations of endosymbiotic bacteria.

542 The observation that two *Endomicrobium* oligotypes (EO4 and EO8) were found on
543 different protist species was surprising. Since previous reports documented the strict vertical
544 transmission of these endosymbionts across *Trichonympha* spp. hosts, one would expect that the
545 *Endomicrobium* oligotypes to be specific to a single protist host species. However, our
546 oligotyping data suggests that at least two these oligotypes were found on different protist
547 species. Since only one of the three *P. vertens* samples contained EO4, it is likely that this

548 oligotype represents contamination. One possible source of contamination could be that these
549 *Endomicrobium* cells were taken up during feeding (either directly, or indirectly along with
550 wood particles). The contamination could have also come from technical reasons, such as the
551 carry-over of bacterial cells or DNA during micromanipulation.

552 In lower termites, protist-associated *Treponema* are members of both termite *Treponema*
553 cluster I or II. The divergence of these *Treponema* clusters is not due to the phylogeny of their
554 termite, or protist, hosts as both clusters contain *Treponema* found associated with various
555 termites and protists species. Members of both clusters co-colonize individual protist hosts in *R.*
556 *flavipes* as they do in *R. speratus* and other termites (3, 4). In this work, we found that organisms
557 from the two *Treponema* clusters were present on individual protists at roughly equal portions,
558 however their relative abundance was significantly different across protist species (Fig 3C).

559 The associations of *Treponema* from these two clusters with protists may give an insight
560 into how the physiologies of organisms in the clusters might differ. It has been hypothesized that
561 symbiosis between hindgut protists and their ectosymbiotic *Treponema* involves syntrophic
562 exchange of reduced fermentation end products. This hypothesis stems from the observation that
563 cultivated strains of *Treponema primitia* belonging to cluster I have been shown to consume CO_2
564 and H_2 in reductive acetogenesis, as well as fix nitrogen (3, 43–45). If protists-associated
565 *Treponema*, from cluster I are also acetogens, then they could provide their host with a necessary
566 H_2 and CO_2 sink.

567 Less is known of the cluster II *Treponema* since there are currently no cultured members.
568 However, it is thought that their metabolism may be different from those in cluster I. These
569 *Treponema* may aid in the hydrolysis of cellulose or other plant-derived polysaccharides since
570 some of their close relatives carry out similar functions in other environments (4). This

571 difference in metabolism between the two *Treponema* clusters may provide the selective pressure
572 needed to maintain both at equal proportions on individual protist cells.

573 The ectosymbiont populations of hindgut protists in *R. flavipes* we studied were dynamic
574 as demonstrated by the fact that protists acquired new ectosymbionts over time. This horizontal
575 transmission of ectosymbionts required active bacterial processes because it was lowered in the
576 presence of tetracycline. However, it could not be determined if inhibition of bacterial translation
577 by tetracycline was directly involved in inhibiting the transmission. For example, tetracycline
578 may have inhibited translation of proteins involved in host binding, or the effect could be due to
579 a decrease in proteins involved in motility, other cellular processes, or an increased in bacterial
580 mortality. To further test that host-binding is an active process and not passive, oxygen-killed
581 hindgut bacteria were stained with PI and added to live samples. In these experiments, dead
582 bacteria were never observed to be attached to live protist hosts.

583 That ectosymbionts bound preferentially to the anterior cell pole of *D*. species II
584 suggested that there was spatial specificity to the process. This spatial specificity was not
585 observed on other *Dinenympha* host cells. The cause of this specificity is not known, but may be
586 the result of new cell membrane, or binding structures for ectosymbionts, being added to the host
587 at the anterior pole. Since these protist cells are morphologically polarized, it could also be that
588 there are some protist functions or signals that are specific to that cell pole that allowed
589 ectosymbionts to bind more readily than the other cell pole.

590 Protists from *R. flavipes*, and other lower termites cannot yet be cultured and this results
591 in some limitations in the ectosymbiont acquisition assays. After 20 hours, most protist cells
592 have died and lysed during the in-vitro experiments. This limited the time over which the assay
593 could be conducted. Because of this, we could not determine if ectosymbionts could also be

594 vertically transmitted during protist cell division. For vertical transmission, ectosymbionts would
595 have to remain attached to a dividing host cell such that the daughter cells directly inherit the
596 ectosymbionts of the parent cell. We have not yet witnessed actively dividing hindgut protists
597 but there is no evidence to suggest that they would have to shed their ectosymbionts prior to, or
598 during, cell division. We also could not discern if the acquisition of ectosymbionts required
599 active protein synthesis by the protist hosts. We found that cycloheximide did not significantly
600 affect ectosymbiont acquisition. However, it may still be the case that protein synthesis by the
601 protist host is required for acquisition. For example, it could be that the rate of protein turnover
602 in these protists is slow and that 20 hours was not enough time to detect an effect. It is also
603 possible that protists are not sensitive enough to cycloheximide for it to completely inhibit
604 protein synthesis.

605 The fact that at least some of these ectosymbionts can be horizontally transmitted raised
606 the question as to whether these bacteria were obligately associated with their protist host. In
607 order to be horizontally transmitted the bacterium must be free-living (unattached) at some point.
608 Since *Treponema* compose the majority of bacteria in the hindgut of *R. flavipes* (25, 46) and are
609 frequently observed unattached in the luminal contents, specific facultative associations between
610 protists and free-living *Treponema* which can become ectosymbionts could not be ruled out.

611 The observations that we could (i) detect protist-associated *Treponema* oligotypes in
612 samples enriched in free-living bacteria and (ii) detect horizontal transmission of new
613 ectosymbiont from the free-living bacterial cell fractions, supports that these ectosymbionts may
614 live both in the free-living state and as protist-bound ectosymbionts. Since not all the protist-
615 associated ectosymbiont oligotypes were detected in the free-living bacterial fractions we cannot
616 rule out the possibility that some ectosymbionts are obligate symbionts and perhaps, vertically

617 acquired. We could not detect any reads belonging to '*Ca*. Symbiothrix' in the free-living
618 bacterial fractions of individual termites, thus they may represent ectosymbionts which are
619 obligate or vertically acquired. Using fluorescent in situ hybridization (FISH) with oligotype
620 specific probes may help to resolve which symbionts are horizontal transmitted. However,
621 because as many as 25 ectosymbiont oligotypes associated with a single protist cell (as is the
622 case with *D*. species II), designing and testing that many FISH probes would be impractical.
623 The assay to detect horizontal transmission of ectosymbionts was useful for revealing
624 new information about the interactions between prokaryotes and protists in the termite hindgut
625 community. The possibility of horizontal transmission of ectosymbionts between protists or
626 between the pool of free-living bacteria and protists may explain how these ectosymbiont
627 populations maintain their heterogeneous colonization of protists. Also, it may provide
628 information as to how some ectosymbiont types associate with high specificity; these may not
629 participate in horizontal transfer and may, instead, be acquired vertically.
630 Of course, specificity can occur even if bacteria can be horizontally acquired.
631 Specificity during transfer must result from mechanisms that encourage the binding of some
632 symbionts and/or discourage the binding of others, perhaps through specific attachment factors
633 made the host, the symbiont or by both.
634
635 **Conflict of interest**
636 The authors declare no conflict of interest.
637
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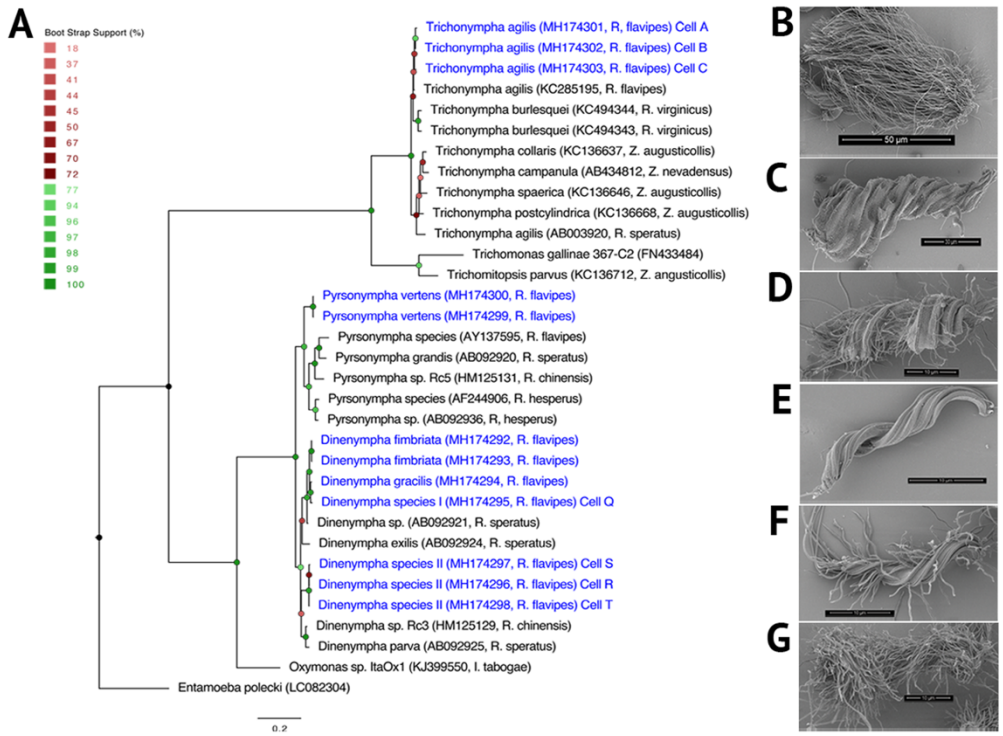
786

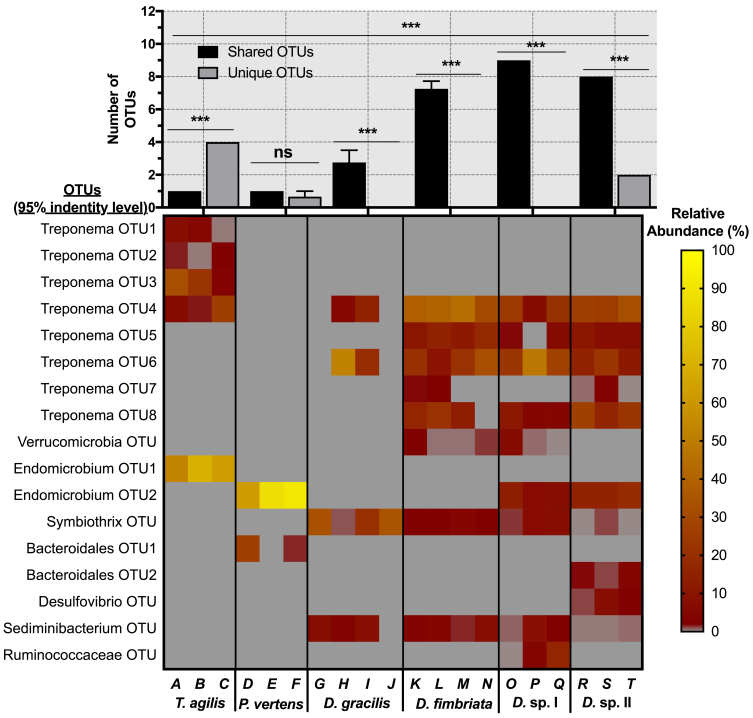
787 **Figure legends**



788

789 **FIG 1** Phylogenetic and morphological diversity of hindgut protist species from *R. flavipes*. (**A**)
790 Phylogenetic tree of 18S rRNA genes from single protists cells. Four 18S rRNA genes from
791 single protist clustered to known references sequences (*D. fimbriata*, *D. gracilis*, *P. vertens*, and

792 *T. agilis*). Other previously undescribed protists, *D.* species I and *D.* species II, clustered within
793 the genus *Dinenympha*. Taxa in blue represents sequences obtained by this study. Sequences
794 from cells which are designated by a letter represent individuals in which the 18s rRNA gene
795 was co-amplified with the bacterial V4 16S rRNA gene. Tip labels include the protist name,
796 accession number, and host termite species. Scanning electron micrographs of representative
797 individuals of each protist species ((B) *T. agilis*, (C) *P. vertens*, (D) *D.* species I, (E) *D. gracilis*,
798 (F) *D. fimbriata*, and (G) *D.* species II). Scale bars represent 50μm (C), 30μm (D), and 10μm (E
799 – G).



800

801 **FIG 2** Diversity and distribution of protist-associated bacterial OTUs. Average number of
802 unique and shared OTUs across different protist species and the relative abundance of each
803 OTU. OTUs represent sequence clustering at the 95% identity level. Bar charts represent the
804 average number of unique or shared OTUs across the different protist species with error bars
805 representing the standard error of the mean. For example, *T. agilis* shares one OTU with other

806 protist species. Statistical significance across protist species determined by 2-way ANOVA and
807 within a given protist species using T-test (*** $p=0.001$, **$p=0.02$, * $p\leq0.05$). Heat map
808 represents the relative abundance of each OTU in a single protist sample. The relative abundance
809 of each OTU was significantly different between *Trichonympha*, *Dinenympha*, and *Pyrsonympha*
810 hosts (G-test, Bonferroni $p\leq0.05$).

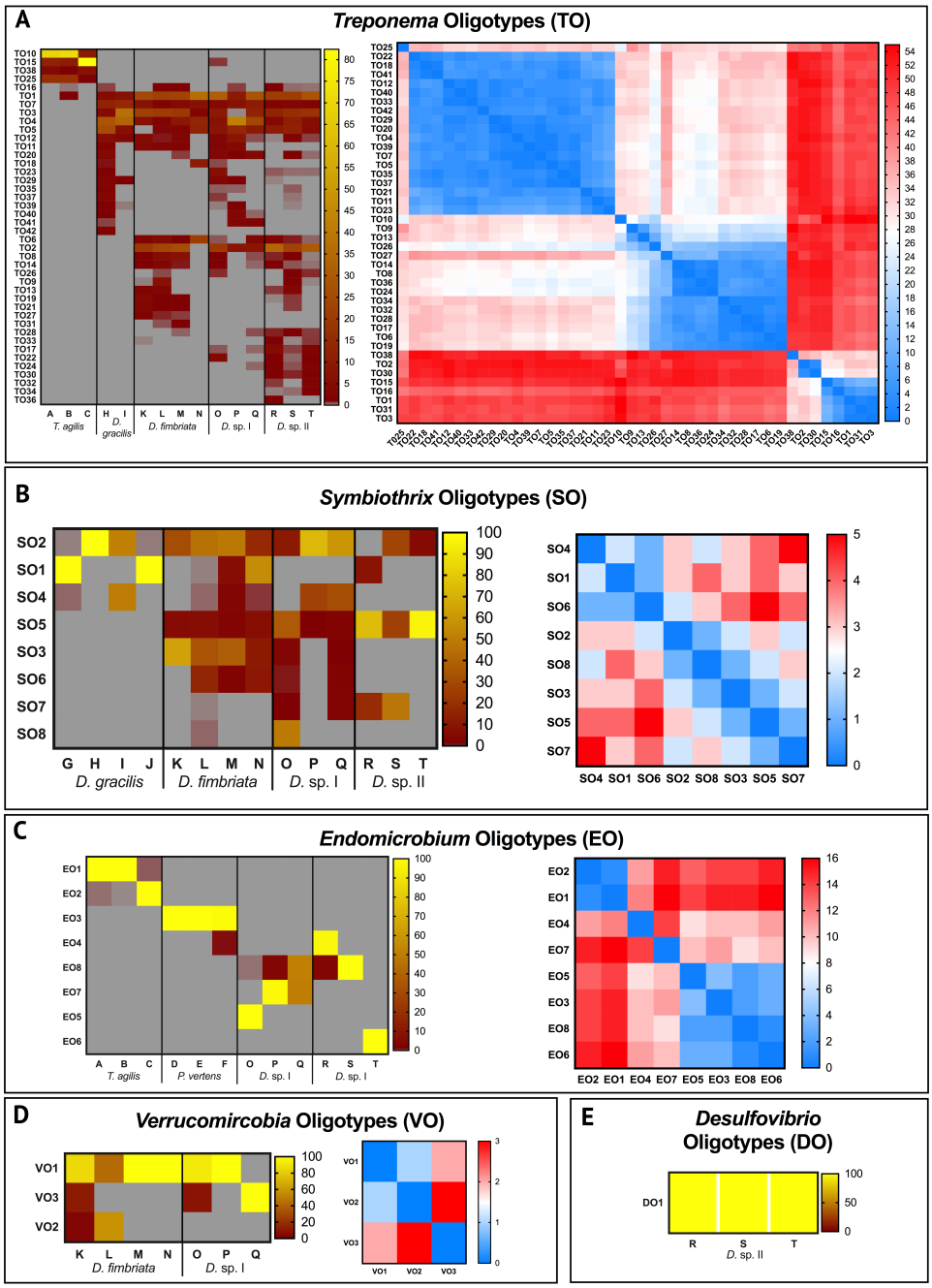


FIG 3 Oligotypes of bacterial symbionts of hindgut protists. Heat maps (Red and Yellow) represent the relative abundance of each oligotype as a percent of the total reads for that bacterial taxon for each protist cell. Heat maps (Red and Blue) depict the number of base pair differences between each oligotype per bacterial taxon. Oligotypes in which the pairwise number of

differences are represented as blue cells are more similar to one another while red cells depict more dissimilar pairwise comparisons. (A) *Treponema*, (B) '*Ca*. Symbiothrix', (C) *Endomicrobium*, (D) *Verrucomicrobia*, and (E) *Desulfovibrio* oligotypes across individual protist cell samples.

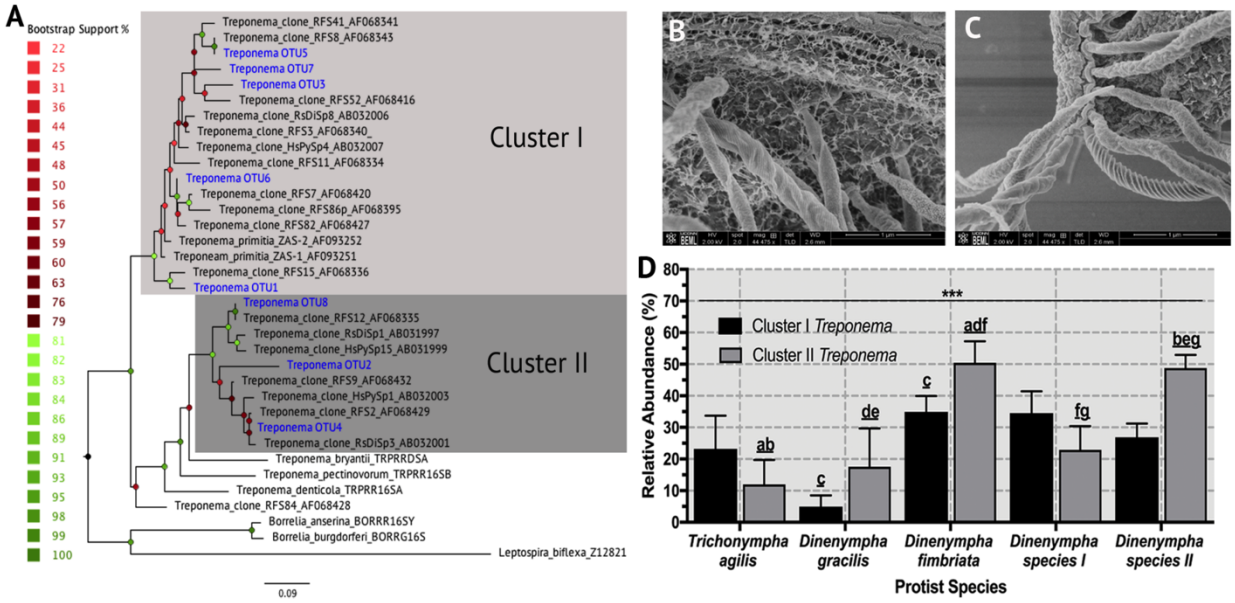
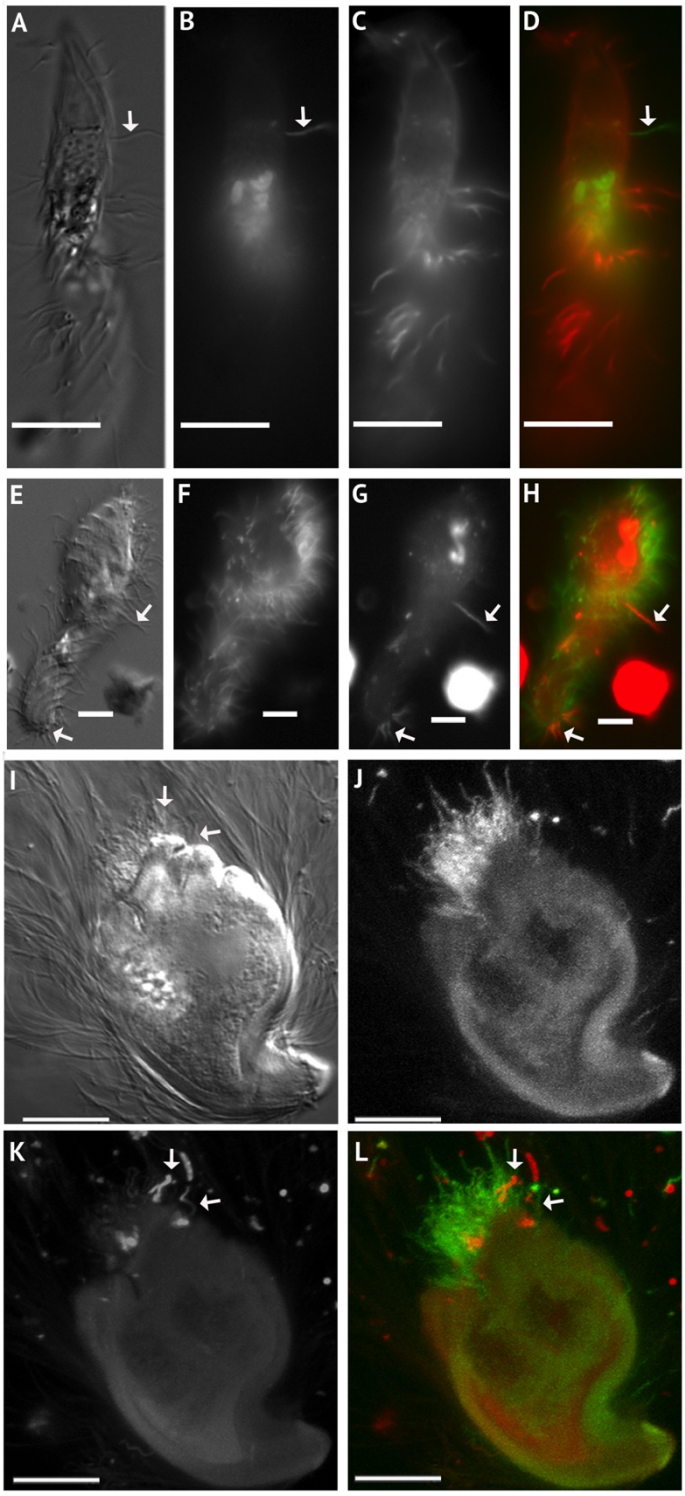
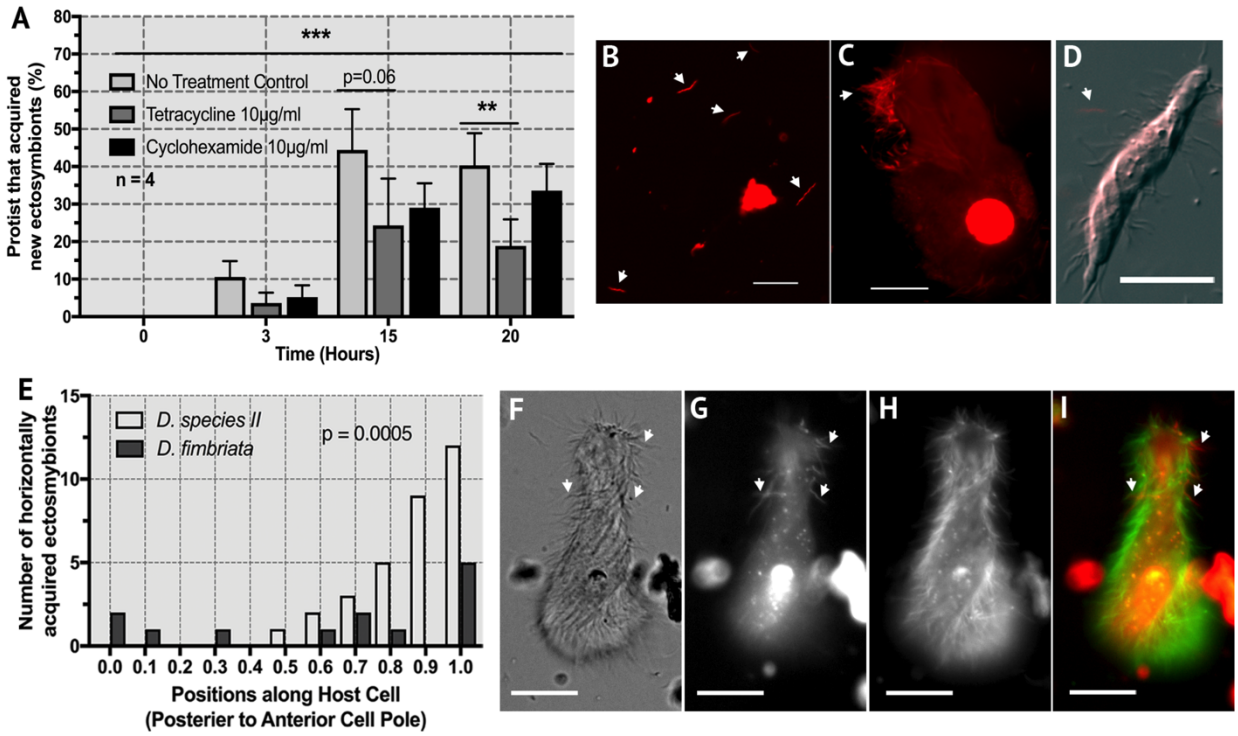


FIG 4. Phylogenetic and morphological diversity of protist-associated *Treponema*. (A) Maximum likelihood tree showing that representative sequences from each *Treponema* OTU clustered within either Termite *Treponema* cluster I or cluster II. (B and C) The morphological diversity of *Treponema* on single protist hosts can be seen using SEM, supporting their heterogeneous population structure (*D*. species I shown). (D) The relative abundance of the different *Treponema* clusters was not different from one another on a single host species however, there was significant difference across different protist species (2way ANOVA, *** p=0.001). Letters represent significant differences between protist species (T-test, $p<0.05$). Taxa

in blue represent sequences obtained by this study, for example any bars labeled with “a” are significantly different from each other.



832 **FIG 5** Horizontal transmission of ectosymbionts across different protist species. DIC and
833 fluorescence micrographs of hindgut protist and their ectosymbionts stained with either TRSE
834 (shown red) or SE-488 (shown green) at Time=12 hours of fluorescent assay. Overtime several
835 different protist species including (A-D) *D. fimbriata*, (E - H) *D.* species II, and (I – L) *T. agilis*
836 acquired new ectosymbionts. Micrographs are arranged from left to right as DIC, SE488, TRSE,
837 and merged (SE488 and TRSE) for each protist. Fluorescence micrographs J – L are maximum
838 intensity Z-projections. Arrows point to horizontally acquired ectosymbionts and scale bars
839 represent 10μm.



840

841 **FIG 6** Horizontal transmission of ectosymbionts involves active processes and is non-random.
842 (A) Transmission is dependent on time as the percentage of protists which acquired new
843 ectosymbionts significantly increased over time (2way ANOVA, ***p=0.001). (**A**) The addition
844 of tetracycline significantly lowered the percentage of protists at acquired new ectosymbionts (T-

845 test, p=0.06 at Time=15 hours, and **p=0.02 at T=20 hours) while the addition of cycloheximide
846 had no significant effect. (B - D) Micrographs of PI stained cells. Exposing hindgut contents to
847 O_2 killed hindgut bacteria (B and C) which did not bind to live protist cells (D) (arrows point to
848 O_2 killed bacteria). (E) Significantly more ectosymbionts (Pearson's R, p=0.0005) bound towards
849 the anterior cell pole compared to the posterior cell pole on *D*. species II however, this binding
850 characteristic was not seen in other *Dinenympha* species. (F - I) Fluorescence and DIC
851 micrograph of *D*. species II stained with amine reactive dyes (G TRSE, H SE488), showing
852 increased binding of new ectosymbionts (arrows) toward the anterior cell pole. Scale bars
853 represent 10μm.