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

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MES and DJG designed experiments. MES performed experiments and data analysis. MES and DJG wrote the manuscript.

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.

Introduction

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

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

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

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

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

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

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

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

of transmission.

Materials and methods

Termite collection, maintenance, and identification.

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

Amplification and sequencing of protist and bacterial SSU rRNA genes.

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

washed three times in droplets of TU via micromanipulation, placed in 10μl molecular grade TE Buffer, and then frozen at -20°C.

Frozen protist cells served as templates for PCR reactions in which the 18S rRNA gene of the protist host as well as the V4 hypervariable region of the 16S rRNA gene of bacteria were co-amplified and sequenced. PCR reactions consisted of Phusion® High-fidelity polymerase (1 unit), HF buffer, dNTPs (200 μM), dimethyl sulfoxide (DMSO) (3%), 0.3 μM of each 18S primer (Euk19f, 5'-AYYTGGTTGATYCTGCCA-3' and Euk1772r; 5'-CBGCAGGTTCACCTAC-3') (23), 0.2 μM each of V4 16S primers (515f; 5'-GTGCCAGCMGCCGCGGTAA-3' and 806r; 5'-GGACTACHVGGGTWTCTAAT-3', annealing sequence) (24), and a single protist cell in a final reaction volume of 50 μl. PCR conditions were as follows: Initial denaturation was at 94°C for 3 minutes followed by 35 cycles of 94°C for 45 seconds, 50°C for 60 seconds, 72°C for 2 minutes. Final extension was at 72°C for 10 minutes (25). For *P. vertens* and *D. gracilis* primers 18SFU; 5'-ATGCTTGTCTCAAAGGRYTAAGCCATGC-3' and 18SRU; 5'-CWGGTTCACCWACGGAAACCTTGTTACG-3' were used (26).

PCR products were size-selected by using a 1% agarose gel, purified using the Wizard® SV Gel and PCR Clean-up System (Promega), and quantified using Qubit™ Flourometric quantitation (ThermoFisher Scientific). Barcoded V4 16S rRNA gene amplicons were pooled at 4nM equimolar concentrations and sequenced on an Illumina Miseq (25).

18S rRNA gene amplicons were cloned using the pGEM®-T Easy Vector System (Promega) following the manufacture's protocol, and sequenced by Sanger sequencing. In addition to protist samples, negative controls consisting of TU, TE, and protist-free technical controls were amplified and sequenced. If needed, additional isolated protist cells were used in

18S rRNA gene-only PCR reactions and the amplicons were cloned and sequenced as described above.

V4 16S amplicon filtering, OTU clustering, and oligotyping analysis

V4 16S rRNA gene reads were quality filtered by removing reads that were below an average quality score of Q20 using BBMap (27). Reads were then analyzed using a Quantitative Insights into Microbial Ecology (QIIME™) workflow. Reads were clustered into de novo OTUs at the 95% identity level using the "pick_de_novo_otus.py" script in MACQIIME (version 1.8). OTU percent identity cutoffs were determined by comparing the percent identities of V4 16S SSU rRNA genes of reference *Treponema* spp. and *Endomicrobium* spp. sequences that defined species cutoffs (Supplementary Fig 2). These references were selected as biomarkers for our OTU clustering since *Treponema* and *Endomicrobium* represented the most abundant taxa in our dataset. Sequences were checked for chimeras using the "parallel_indentify_chimeric_seqs.py" with references from the Dictdb (28) and GreenGenes (29) databases.

OTUs were filtered, and contaminates removed, using several criteria. OTUs were deemed contaminates if they were not at least 10 times higher in protists samples compared to negative controls. Second, any OTUs which were not at least (i) ≥0.15% of total read abundance on all individuals of a given protist species or (ii) ≥1.5% of the total read abundance for at least two individuals in a protist species were removed. Sequences which clustered into OTUs that passed this filtering were then further analyzed by oligotyping. Statistical significance in the difference in OTU abundances and heterogeneity across different protist species were tested using the G-Test and PERMANOVA tests respectively.

Oligotyping analysis was performed for bacterial taxa of interest using the pipeline and

software developed by Eren and coworkers (30). Reads were extracted for bacterial taxa of interest using the "q2oligo.py" script and then reads were padded with gap characters to ensure equal length using the "o-pad-with-gaps" command. The reads were then assessed for Shannon entropy using the "entropy-analysis" command to identify informative base positions based on their sequence variation. Oligotyping was then performed using the "oligotype" command with the minimum substantive abundance parameter (-M) set at 20, and the minimum abundance for at least one sample parameter (-a) set a 1.0. The oligotyping analysis was iteratively performed including high entropy (>0.2) base positions until the oligotypes for each taxon had converged, meaning that further analysis would not generate additional oligotypes. Oligotypes were then manually filtered by excluding those from samples which were comprised of a single read and/or were below 0.1% in their relative abundance.

Oligotyping was also performed on V4 16S rRNA gene reads from samples consisting of the free-living bacterial fractions (fractions enriched in bacterial cells not attached to protists) from three individual termite hindguts. Hindguts were dissected and ruptured as described above. The bacterial fraction of their hindgut contents was prepared by centrifugation. Total hindgut contents were centrifuged at 3,000 rpms for 90 seconds to separate large cells (protist cells) from smaller bacterial cells. This free-living bacterial fraction (supernatant) was then centrifuged at 13,000 rpms for 90 seconds followed by three washes in TU and resuspended in molecular grade TE buffer. Bacterial V4 16S rRNA genes were amplified and sequenced as described above. All reads from these samples were processed using the same pipeline as the reads from single protist cells.

Phylogenetic analysis of SSU rRNA genes

For bacterial OTUs, representative sequences (centroid sequences) of *Treponema*, *Bacteriodales* and '*Ca*. Endomicrobium' were aligned to full length 16S rRNA gene reference sequences of each taxa using MUSCLE (31). Appropriate evolutionary models were determined for each alignment using jModelTest (32, 33) and phylogenetic trees were generated using RAxML (34) using the full alignment length. This phylogenetic analysis of the centroid sequences from OTUs revealed that the QIIME workflow mistakenly designated a certain *Bacteriodales* OTU as '*Candidatus* Azobacteroides', which should have been designated as '*Ca*. Symbiothrix'. The 18S rRNA genes obtained by this study were also aligned to reference sequences using MUSCLE and the full alignment was used to make a phylogenetic tree with RAxML.

Scanning electron microscopy

Scanning electron microscopy (SEM) was used to investigate the morphology of hindgut protists and their ectosymbionts. Protist cells were collected by low spin centrifugation as described above and fixed in 2% glutaraldehyde in TU (pH 7) for 1 hour at RT in an anaerobic chamber. The samples were deposited onto poly-L-lysine coated silicon wafer chips (Prod No. 16008, Ted Pella Inc.), washed with 80 mM Na cacodylate buffer (pH 7), and post-fixed in 2% osmium tetroxide at RT for 1 hour. The cells were rinsed twice for 5 minutes in distilled water then dehydrated in serial concentrations of ethanol (30%, 50%, 70%, 95%, 100%, 5 min each), and critical point dried (931GL, Tousimis). Samples were then mounted on SEM stubs using silver paint, sputter coated with Palladium (E5100, Polaron), and examined using a scanning electron microscope (Nova NanoSEM 450, FEI).

Fluorescent symbiont transmission assays

For all tranmission assays, experiments were carried out in an anaerobic chamber with gas composition as described above. Hindguts were dissected from termites, ruptured with sterile forceps, and their contents were collected in anaerobic buffer containing anaerobic water with resazurin (1μg/ml), sodium thioglycolate (0.5g/L), and sodium bicarbonate (0.1M) pH 8.0 (Pedro et al., 2004). Samples were then fractionated by low spin centrifugation (3,000 rpm for 90 seconds) to separate protists and from bacteria which were unattached to protists. Each fraction was then washed three times in buffer by centrifugation at either 3,000 rpm (for protist fraction) or 13,000 rpm (for bacterial fraction) for 90 seconds. The washed fractions were then split into two equal volume groups and stained with either Texas Red®-X succinimidyl ester (TRSE, Molecular Probes™) or AlexaFlour 488 succinimidyl ester (SE488, Molecular Probes™) at 10μg/ml for 1 hour at room temperature (RT) in the dark in the anaerobic chamber. Dye conjugation was done per manufacturer's instructions. Stained cells were then washed 3 times in TU with reduced glutathione serving as a stop reagent for the amine reactive dyes. Protist and bacterial fractions were combined to produced two samples (Red-TRSE-stained and Green-SE-488 stained).

To assay for symbiont acquisition by protists, the two samples (Red-TRSE-stained and Green-SE-488 stained) were combined and monitored for the horizontal acquisition of new bacteria which was evident by heterogeneity in fluorescent signals of bacteria on individual protists. Samples were taken at various time points (0, 3, 15, and 20 hours), fixed with 2% formaldehyde, and viewed using a Nikon TE300 Eclipse microscope. Alternatively, fixed samples were mounted in ProLong™ Diamond Antifade Mountant (ThermoFisher) and imaged using a Nikon A1R Spectral Confocal microscope. To test if symbiont acquisition required biologically active processes, this assay was repeated with the addition of either tetracycline

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

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

Sequence submission

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

Results

Morphological and phylogenetic diversity of hindgut protists

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

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

OTU composition of the bacterial symbionts of individual hindgut protists

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

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

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

and *D*. species I & II. Interestingly, *D*. *gracilis* and *D*. *fimbriata* lacked these endosymbiotic bacteria. *Verrucomicrobia* symbionts, known to colonize the nuclei of some protists, were found associated with two of the protists species we sampled (*D*. *fimbriata* & *D*. species I).

Desulfovibrio symbionts were composed of a single OTU and only found associated with *D*. species II. Symbionts of this bacterial taxon were previously found embedded in the plasma membrane of *T*. *agilis* in a different termite, *R*. *speratus* (7). Surprisingly the samples of *T*. *agilis* cells that we examined from *R*. *flavipes* did not contain any reads from *Desulfovibrio* bacteria.

Other bacterial taxa included *Bacteriodales*, which were clustered into two OTUs (*Bacteriodales* OTU1 and OTU2). Two of three individual *P*. *vertens* cells (cells D and F) contained *Bacteriodales* OTU1 while the second OTU was found associated with all three single cells of the *D*. species II cells used in this study. The remaining OTUs corresponded to *Sediminibacterium* which was found across all the *Dinenympha* spp. and *Ruminococcaceae* which was found associated with *D*. species I.

Distribution of symbiont oligotypes across individual protist hosts

The diversity and distribution of certain bacterial taxa were further investigated using an oligotyping analysis which provides a higher-resolution survey of the diversity within bacterial OTUs (30). For example, protist-associated *Treponema* symbionts were initially clustered into 8 OTUs, at the 95% identity level, were further clustered into 42 distinct oligotypes (Fig 3A). Most *Treponema* oligotypes (34 out of 42) were extensively shared across different protist species with only 8 oligotypes being exclusive to a single protist species. One *Treponema* oligotype in particular (TO1) was found on a single *T*. *agilis* cell (cell B) but also on every *Dinenympha* spp. 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,

4).

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

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

Horizontal transmission of ectosymbionts

The OTU clustering and oligotyping analysis of bacterial symbionts of single protist cells suggested that some ectosymbiont types (*Treponema* and '*Ca*. Symbiothrix) associated with 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

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

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

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

Discussion

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

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

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

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

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

The observation that two *Endomicrobium* oligotypes (EO4 and EO8) were found on different protist species was surprising. Since previous reports documented the strict vertical transmission of these endosymbionts across *Trichonympha* spp. hosts, one would expect that the *Endomicrobium* oligotypes to be specific to a single protist host species. However, our oligotyping data suggests that at least two these oligotypes were found on different protist 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

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

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

The observations that we could (i) detect protist-associated *Treponema* oligotypes in samples enriched in free-living bacteria and (ii) detect horizontal transmission of new ectosymbiont from the free-living bacterial cell fractions, supports that these ectosymbionts may live both in the free-living state and as protist-bound ectosymbionts. Since not all the protist-associated ectosymbiont oligotypes were detected in the free-living bacterial fractions we cannot 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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Figure legends

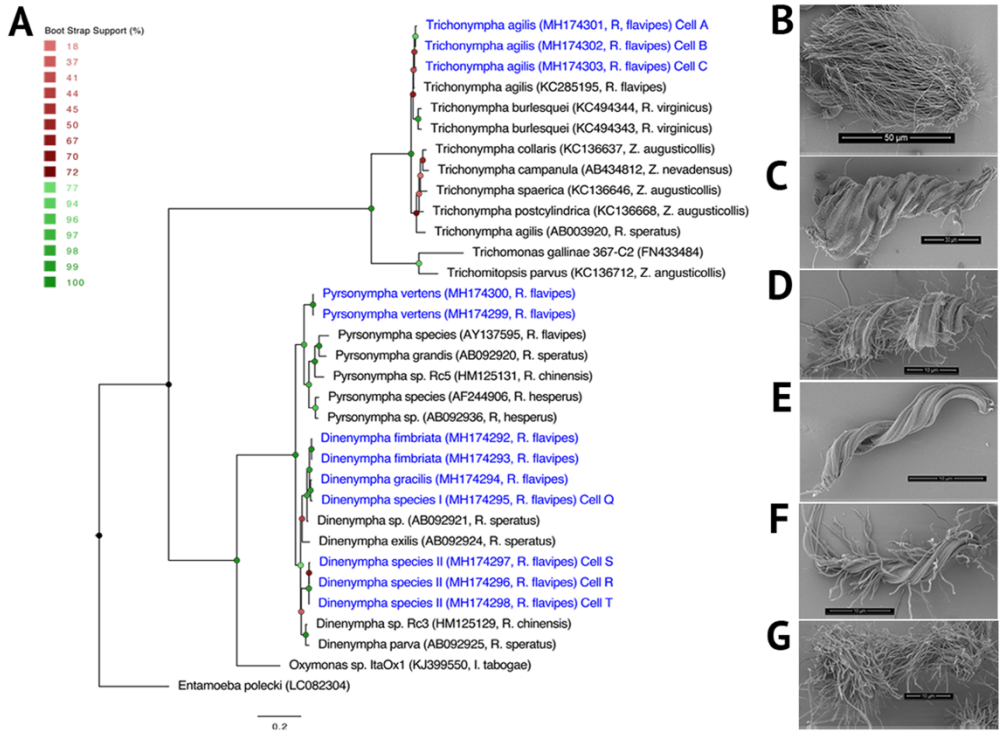
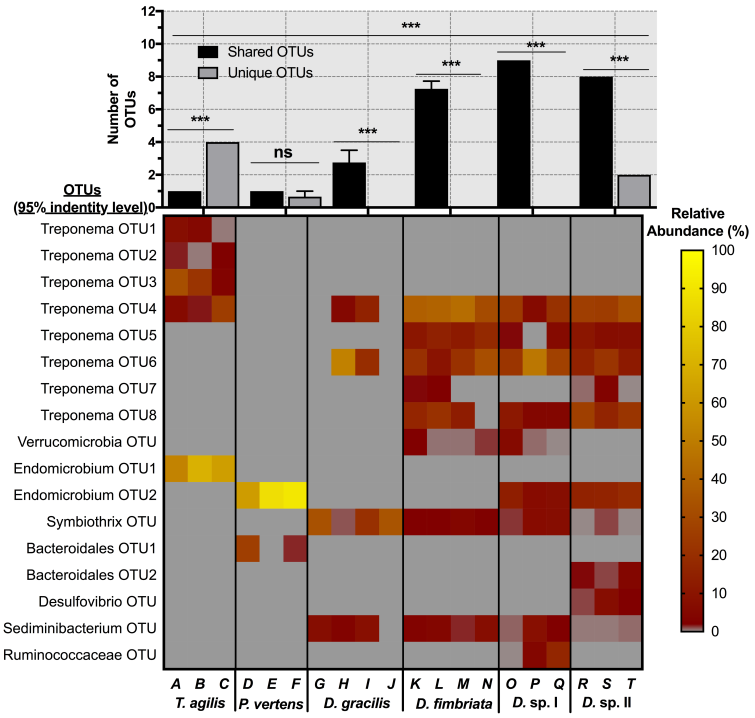


FIG 1 Phylogenetic and morphological diversity of hindgut protist species from *R. flavipes*. (**A**) Phylogenetic tree of 18S rRNA genes from single protists cells. Four 18S rRNA genes from single protist clustered to known references sequences (*D. fimbriata*, *D. gracilis*, *P. vertens*, and

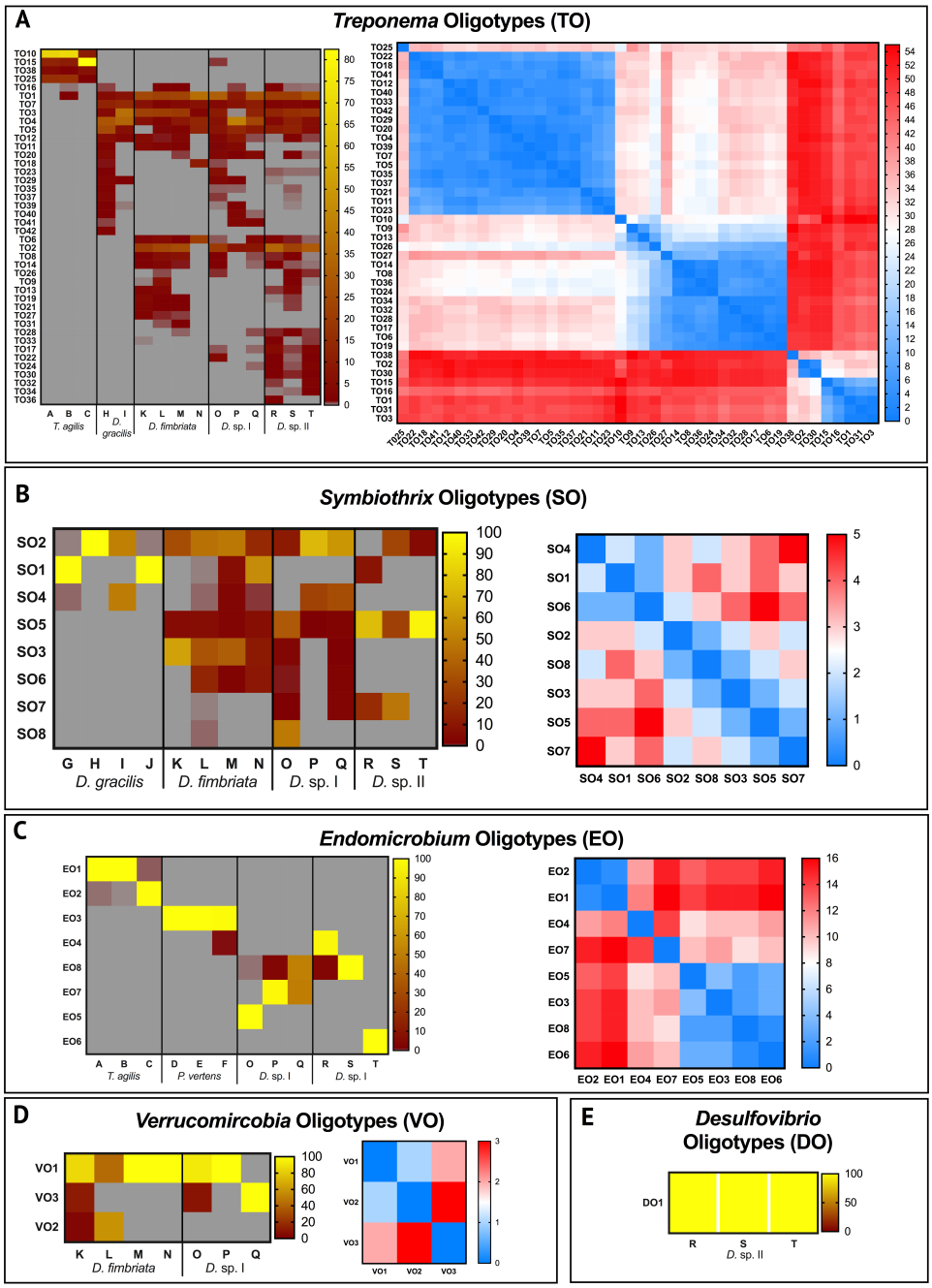
792 *T. agilis*). Other previously undescribed protists, *D.* species I and *D.* species II, clustered within
793 the genus *Dinenympha*. Taxa in blue represents sequences obtained by this study. Sequences
794 from cells which are designated by a letter represent individuals in which the 18s rRNA gene
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

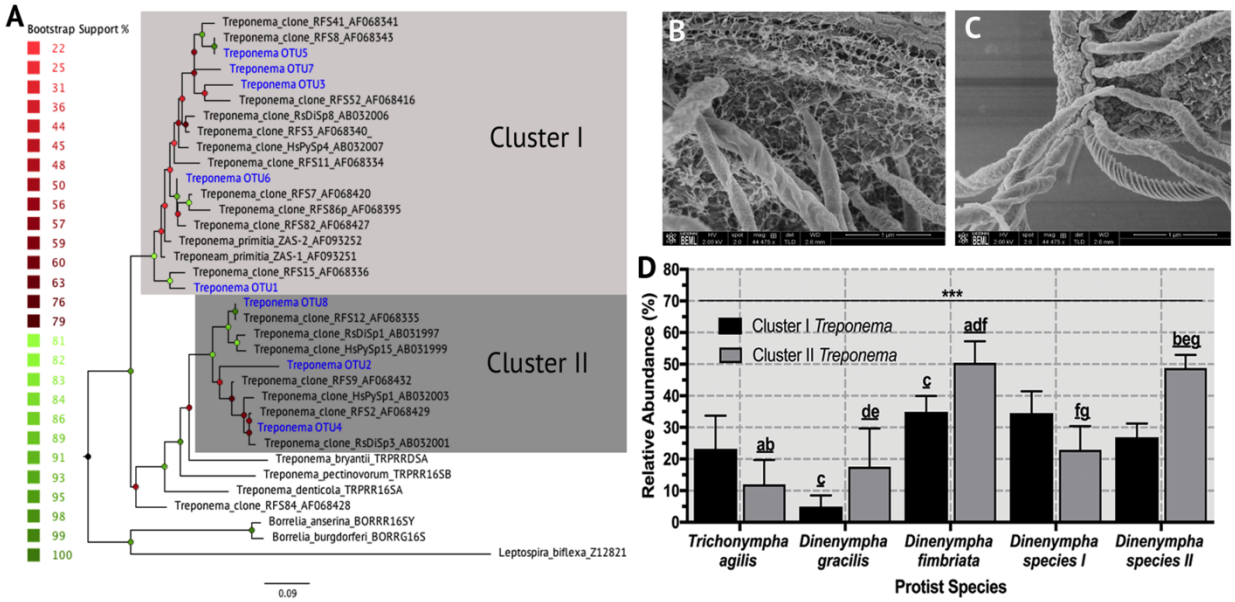
protist species. Statistical significance across protist species determined by 2-way ANOVA and within a given protist species using T-test (*** p=0.001, **p=0.02, * p≤0.05). Heat map represents the relative abundance of each OTU in a single protist sample. The relative abundance of each OTU was significantly different between *Trichonympha*, *Dinenympha*, and *Pyrsonympha* hosts (G-test, Bonferroni p≤0.05).



811

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

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

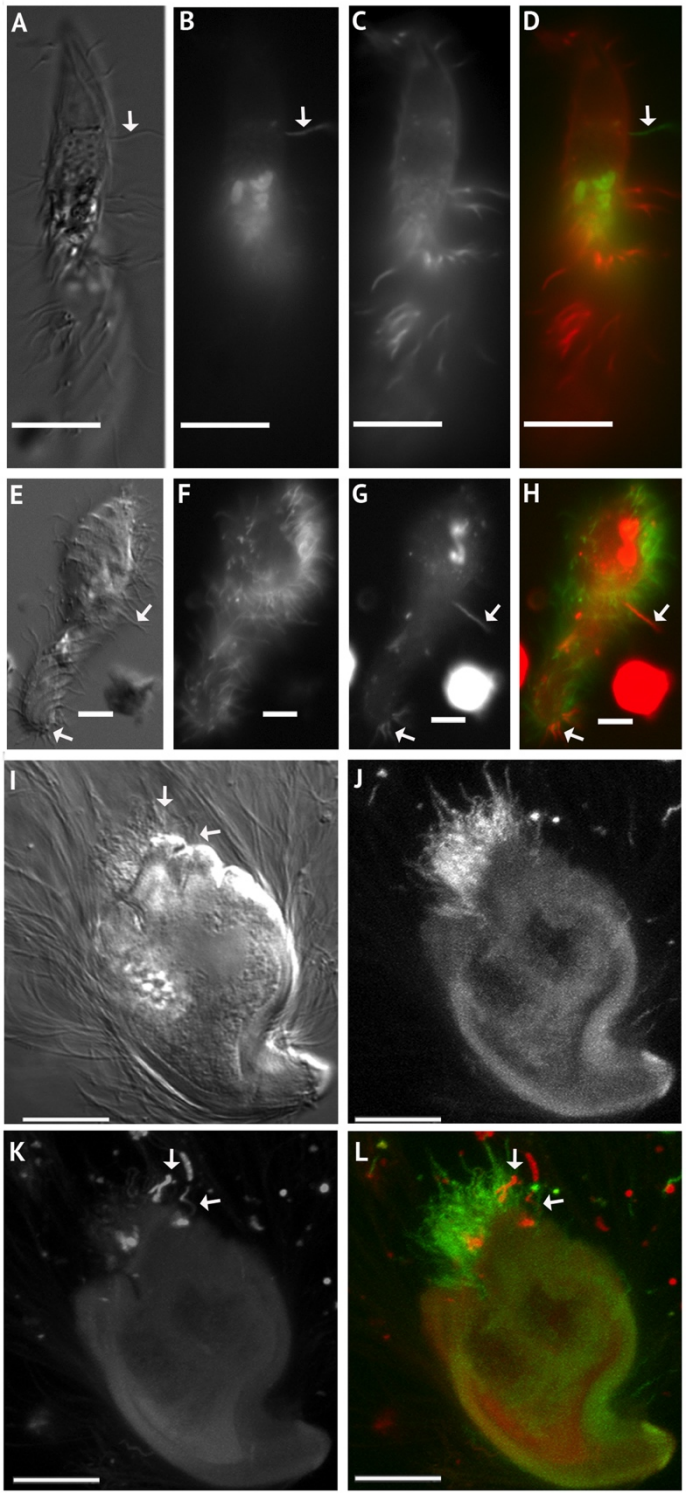


820

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

829 in blue represent sequences obtained by this study, for example any bars labeled with “a” are

830 significantly different from each other.



831

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

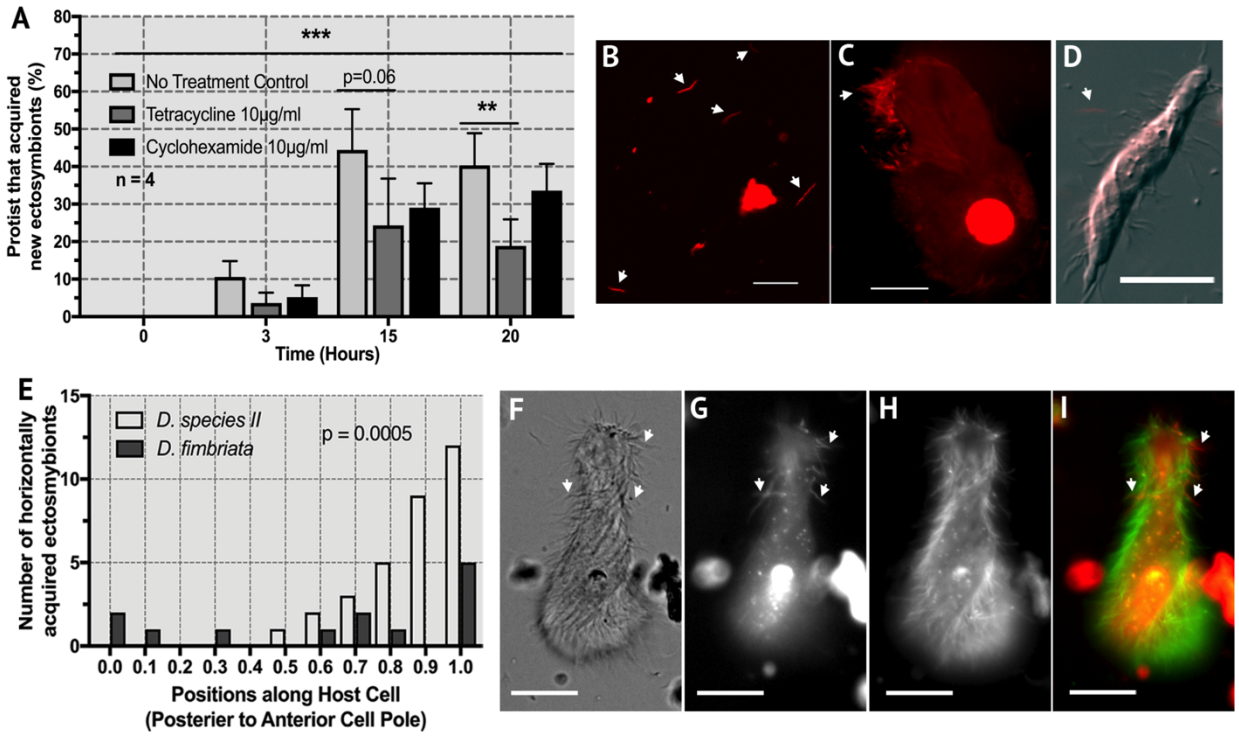


FIG 6 Horizontal transmission of ectosymbionts involves active processes and is non-random. (A) Transmission is dependent on time as the percentage of protists which acquired new ectosymbionts significantly increased over time (2way ANOVA, ***p=0.001). (**A**) The addition of tetracycline significantly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

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