

The Role of Host Phylogeny Varies in Shaping Microbial Diversity in the Hindguts of Lower Termites

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The hindguts of lower termites and *Cryptocercus* cockroaches are home to a distinct community of archaea, bacteria, and protists (primarily parabasalids and some oxymonads). Within a host species, the composition of these hindgut communities appears relatively stable, but the evolutionary and ecological factors structuring community composition and stability are poorly understood, as are differential impacts of these factors on protists, bacteria, and archaea. We analyzed the microbial composition of parabasalids and bacteria in the hindguts of *Cryptocercus punctulatus* and 23 species spanning 4 families of lower termites by pyrosequencing variable regions of the small-subunit rRNA gene. Especially for the parabasalids, these data revealed undiscovered taxa and provided a phylogenetic basis for a more accurate understanding of diversity, diversification, and community composition. The composition of the parabasalid communities was found to be strongly structured by the phylogeny of their hosts, indicating the importance of historical effects, although exceptions were also identified. Particularly, spirotrichonymphids and trichonymphids likely were transferred between host lineages. In contrast, host phylogeny was not sufficient to explain the majority of bacterial community composition, but the compositions of the *Bacteroidetes*, *Elusimicrobia*, *Tenericutes*, *Spirochaetes*, and *Synergistes* were structured by host phylogeny perhaps due to their symbiotic associations with protists. All together, historical effects probably resulting from vertical inheritance have had a prominent role in structuring the hindgut communities, especially of the parabasalids, but dispersal and environmental acquisition have played a larger role in community composition than previously expected.

Cryptocercus cockroaches and their sister lineage, the lower termites (1), are both dependent on diverse communities of microorganisms in their hindguts to gain nutrition from lignocellulose (reviewed in references 2 and 3). These are fascinating and complex communities dominated by parabasalid (Parabasalia) and oxymonad (Preaxostyla) protists (4) as well as numerous bacterial lineages, including *Actinobacteria*, *Bacteroidetes*, *Elusimicrobia*, *Firmicutes*, *Proteobacteria*, *Spirochaetes*, and *Synergistes* (5–7). Moreover, a large proportion of the bacteria form obligate symbiotic interactions with the protists (see, for example, references 8–14), so the community contains multiple levels of symbiosis. Because of the diversity of symbionts, the hindguts of *Cryptocercus* cockroaches and lower termites can be used as model systems to study the ecology and interactions between protists and bacteria. Here, we investigate the ecological and evolutionary differences between protists and bacteria in structuring community composition. These differences are critical to understanding the diversification and adaptation of microbes, the stability and resilience of community structure, and the maintenance of ecosystem functions, particularly in regard to understanding lignocellulose digestion and the ecology and evolution of the termites.

Protist diversity in the hindguts varies depending on the host species, but related hosts tend to harbor related protists, and the composition of the protist communities for each host species appears to be stable over space and time (4). The symbionts are transmitted among family or colony members through the consumption of proctodeal fluid, which limits the dispersal of symbionts to closely interacting individuals. These observations suggest that the composition of protists in the hindguts is strongly structured by historical (evolutionary) effects due to vertical transmission of the protists and codiversification within their hosts

(15–17). However, this has never been tested using molecular surveys of diversity and phylogenetic approaches. Most of our knowledge on protist diversity in the hindguts is based on morphological descriptions and focused on individual taxa and not the community.

Unlike the protist communities, where over 100 years of morphological examination and decades of molecular characterization provided some expectation of the composition and even conservation between hosts, relatively little is known of the bacteria in lower termites and *Cryptocercus* cockroaches. Recent studies have described the bacterial diversity in the hindguts of *Cryptocercus* cockroaches and a limited representation of lower and higher termites (6, 18–21). Like the protists, the diversity of bacteria is distinct, unique, and consistent compared to other insect gut communities, and historical effects also appear to have played a role in their community structure (18–20). Bacterial diversity, however, has neither been surveyed across the lower termites nor in con-

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junction with the protists to compare the relative influence of historical effects on community structure in these two groups of microorganisms.

To investigate the similarities and differences regulating protist and bacterial community structure, and how each community affects the other at ecological and evolutionary scales, matched descriptions of protist and bacterial diversity are required. We used high-throughput sequencing of a variable region of the small-subunit rRNA gene to obtain comprehensive descriptions of the parabasalid and bacterial diversities in the hindguts of *Cryptocercus* cockroaches and 23 lower termite species. Using these data, we compare the extent by which historical effects have influenced the composition and diversification of parabasalids and bacteria and the role of additional factors in regulating community composition in the hindguts.

MATERIALS AND METHODS

Insect and hindgut collections and DNA extraction. Twenty-three insect species, including representatives from 4 families of lower termites plus *Cryptocercus* cockroaches, were collected (see Table S1 in the supplemental material). The 3 *Cryptocercus* samples were from distinct populations of the *Cryptocercus punctulatus* species complex, many of which have different chromosome numbers (22). The paunch (P3) compartments from hindguts of live insects were dissected, and the contents were collected into Trager's solution U (23) and stored at -20°C . For *Porotermes adamsoni* and *Coptotermes* sp. TN2, the hindgut contents were collected into Trager's solution U and stored in 70% ethanol at -20°C . DNA was extracted from the hindgut contents using the MasterPure Complete DNA and RNA purification kit (Epicentre) by following the manufacturer's protocol. Host DNA was also extracted from either the head or leg of the insect.

Insect barcoding and phylogeny. Host insects were identified based on morphology, and the identity was confirmed by DNA barcoding. For insect samples that were not previously barcoded, an approximately 380-bp fragment of the mitochondrial 16S (large subunit) rRNA gene was PCR amplified and sequenced from head, leg, or hindgut DNA (which contained DNA from both host and symbionts). Template DNA was added to a mixture of 1 \times EconoTaq Plus green (Lucigen) and 5 pmol of the primers LR-J (5'-TTACGCTGTTATCCCTAA-3') and LR-N (5'-CGCCTGTTTATCAAAAACAT-3') for a 25- μl reaction mixture. The following thermal profile was used: 94 $^{\circ}\text{C}$ for 2 min, 35 cycles of 94 $^{\circ}\text{C}$ for 30 s, 50 $^{\circ}\text{C}$ for 1 min, and 72 $^{\circ}\text{C}$ for 1 min, and a final extension at 72 $^{\circ}\text{C}$ for 10 min. The resulting PCR product was cleaned using a silica membrane spin column (Epoch Life Science) with the recommended buffers and Sanger sequenced using BigDye 3.1 (Applied Biosystems). Mitochondrial 16S rRNA sequences from termites previously barcoded were retrieved from GenBank.

The mitochondrial 16S rRNA sequences were aligned using MAFFT L-INS-i (24). The ends of the alignments were trimmed manually. Gblocks was used to remove highly variable and ambiguously aligned sites but allowed smaller final blocks, gap positions, and less strict flanking positions (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) (25). Phylogenetic trees were inferred from maximum likelihood (ML) analysis using RAxML 7.0.4 implementing a general-time-reversible (GTR) model of nucleotide substitution with the Γ (gamma) model of rate heterogeneity (26). Statistical support for the consensus tree was assessed from 1,000 bootstrap replicates.

The evolutionary relationships between the insects were also visualized by generating a pairwise distance matrix of the percent dissimilarity between the mitochondrial 16S rRNA sequences and conducting a principal coordinate analysis (PCoA). This PCoA plot was compared to the PCoA plots generated from the microbial communities (see below).

18S and 16S rRNA pyrotag sequencing, error correction, and operational taxonomic unit (OTU) clustering and curation. The diversities of protist and bacterial hindgut microbes in each termite sample were

determined separately by tag-encoded Roche 454 FLX+ titanium sequencing. For protists, the V4-to-V5 region of the 18S (small subunit) rRNA gene was amplified using the primers Euk560F (5'-CCAGCASCY GCGGTAATWCC-3') and Euk1055R (5'-CGGCCATGCACCACC-3'). For bacteria, primers 28F (5'-GAGTTTGTATCNTGGCTCAG-3') and 519R (5'-GTNTTACNGCGGCKGCTG-3') were used to amplify the V1-to-V2 region of the 16S (small subunit) rRNA gene. PCR amplification and sequencing were performed by the Research and Testing Laboratory (Lubbock, TX, USA) as described previously (27, 28).

The 16S rRNA and 18S rRNA sequences were analyzed separately, but by using identical methods, except where indicated below. As implemented in QIIME (29), AmpliconNoise and Perseus were used to remove sequencing errors, PCR errors, and reads that were chimeric (30). The denoised sequences were clustered using UClust (31) with a 97% similarity threshold to designate OTUs. OTUs represented by at least 5 sequence reads were retained for further analysis. The longest sequence of the cluster was chosen as the representative OTU. The OTUs were assigned a taxonomic designation using RDP Classifier (32). Reference taxonomies were obtained from the GreenGenes (v. 4_feb_2011) and Silva (v. r104) databases for 16S and 18S analyses, respectively.

From the 236 OTUs identified from the 18S rRNA sequence data, OTUs classified as *Metazoa* (17 OTUs, all of which corresponded to insect host sequences), *Fungi* (2 OTUs), *Bacteria* (8 OTUs), and *Archaea* (2 OTUs) were removed, as well as two potentially chimeric OTUs, identified based on divergent blastn hits along the length of the sequence to the nonredundant (nr) database in GenBank. To filter out cross-contamination between samples, OTUs were considered only if they were represented by at least 3 sequences in a sample.

Sequences from parabasalid protists dominated the 18S rRNA data set (97.9% of the sequences). Oxymonads are also known to be present in several hosts examined, but the 18S rRNA genes of oxymonads are unusually long and often refractory to molecular analysis (33–35), so we expected the oxymonads to be underrepresented. Accordingly, we removed oxymonad OTUs from the analyses (23 *Saccinobaculus* OTUs from *Cryptocercus* cockroaches and one *Dinenympha* OTU and one *Pyronympha* OTU from the hindgut of *Reticulitermes okanaganensis*) and also 4 potential diplomonad OTUs (found in *Cryptocercus* hindguts), to focus exclusively on the parabasalids which in any case dominate these communities.

For the OTUs identified from the 16S rRNA sequence data, a blastn search (E value cutoff = $1\text{e}-50$, percent identity cutoff = 95%) to GenBank's nr database was used to identify potential contaminants in the data. This strict search revealed 7 OTUs that hit bacteria found on human or mouse skin as well as a single OTU corresponding to *Blattabacterium*, an endosymbiont found in *Cryptocercus* fat bodies, and 1 OTU matching a bacterium found in human blood. These OTUs were removed from further analysis. An additional chimeric OTU was also removed.

Phylogenetic and community analyses. The parabasalid 18S rRNA OTU sequences were aligned using MAFFT L-INS-i. Gblocks was used to remove ambiguously aligned positions, and a phylogenetic tree was calculated using RAxML and a GTR-GAMMA nucleotide substitution model.

The bacterial 16S rRNA OTU sequences were aligned using MAFFT L-INS-i. Alignment positions that were gaps in greater than 80% of the sequences and the top 10% of positions with the most entropy (uncertainty) were removed using QIIME. RAxML with a GTR-GAMMA model was used to build a phylogenetic tree. Additional alignments and phylogenetic trees were made with sequences from OTUs belonging to specific bacterial phyla or classes.

Although the statistical support was not robust, 18S rRNA and 16S rRNA phylogenetic trees always recovered clusters containing OTUs with the same classification (as assigned by the RDP Classifier). Unclassified OTUs found within these clusters were manually assigned the same class-level taxonomy.

The OTU read abundances for each sample were subsampled (rarefied) to normalize the total abundance across all samples. Rarefaction curves were calculated using the Chao-1 estimator as implemented in

QIIME. Using total or normalized OTU abundances, unweighted UniFrac distances (36) between communities were calculated using QIIME, and beta diversity was examined using PCoA from the vegan package (<http://CRAN.R-project.org/package=vegan>) (37), as implemented in R v. 1.16 (www.r-project.org). PCoA plots were compared to each other using Procrustes analyses. PROTEST, a permutation test, was used to test the significance of the Procrustes analyses. Shannon-Wiener diversity index and Pielou's evenness index were also calculated using vegan. Topiary Explorer (38) was used to obtain subtrees and color branches from phylogenetic trees. OTU networks were visualized using Cytoscape v.2.8.2 (39).

Nucleotide sequence accession numbers. Host mitochondrial 16S rRNA gene sequences were deposited in the NCBI nucleotide database (GenBank) under accession numbers KJ438360 to KJ438378. 454 sequence data have been deposited in the NCBI sequence read archive (SRA) under the BioProject accession number PRJNA238270 and study accession number SRP038994.

RESULTS

Insect phylogeny. Host identifications were confirmed by DNA barcoding the mitochondrial 16S rRNA gene, and the barcode phylogeny was compared with other cockroach/termite phylogenies. Morphology and barcode data allowed most termites to be identified to the species level. For all termites except *Neotermes jouteli*, the phylogeny from the barcode data matched expectations predicated on morphology-based identifications (Fig. 1A). Phylogenetic analysis confirmed that each host insect fell into one of 6 distinct groups that were consistent with established phylogeny (40), essentially corresponding with their taxonomic family: Cryptocercidae, Kalotermitidae, Stolotermitidae, Archotermopsidae, Rhinotermitidae (excluding *Reticulitermes*), and *Reticulitermes* (Fig. 1A). The genus *Reticulitermes* branched as a sister to other members of its family, Rhinotermitidae, and, as other studies have shown, *Heterotermes* clustered with *Coptotermes* (40–42). The *Reticulitermes* and the rhinotermitids formed two distinct, strongly supported clusters, so we treated them as separate groups for analyzing the symbiont communities. *Porotermes* and *Zootermopsis* are members of different families but are considered close relatives and were once classified together within the same family, Termopsidae, albeit in different subfamilies. The kalotermitid termites grouped together, but with low support.

Similar groupings were recovered using a PCoA of genetic distances (see Fig. S1 in the supplemental material). As other studies have shown, the phylogeny of the termites is not fully resolved, such as the placement of the Kalotermitidae relative to other families and the paraphyly of the Rhinotermitidae (40, 43, 44), so correlation of the PCoAs was used to assess the relationship between insect hosts and their hindgut communities instead of cladogenesis (see below).

Alpha diversity of parabasalids and bacteria. A total of 116,617 18S rRNA and 124,142 16S rRNA sequences were obtained, resulting in 236 18S rRNA OTUs and 1,902 16S rRNA OTUs after removing erroneous sequences, chimeras, and rare OTUs with less than 5 sequences. From these, manually curated data sets were established by removing potentially cross-contaminating OTUs or, from the 18S rRNA OTUs, nonparabasalid symbionts, resulting in an exclusively parabasalid data set of 172 OTUs (86,721 sequences) and a bacterial data set of 1,889 OTUs (122,516 sequences) (see Materials and Methods for details). From these final data sets, rarefaction curves were asymptotic, indicating that the parabasalid and bacterial communities were sufficiently sampled (see Fig. S2 in the supplemental material).

The rhinotermitids hosted hindgut communities with significantly lower bacterial diversity than the other host groups (one-way analysis of variance, both richness and evenness, $P = 4.9 \times 10^{-7}$; pairwise t tests, P values of < 0.0005) (see Table S1 in the supplemental material). Along with *Porotermes adamsoni* (Stolotermitidae), these hindgut communities were dominated by singular OTUs that were likely from symbiotic bacteria of their hindgut protists, as discussed below. Within the parabasalids, however, diversity indices did not correlate with insect host phylogenetic groups, tested by a one-way analysis of variance (richness, $P = 0.142$; evenness, $P = 0.742$).

Compared to each other, parabasalid and bacterial diversity indices also were not correlated, either positively or negatively, when tested by linear regression analysis (richness, $r^2 = 0.07$, $P = 0.19$; evenness, $r^2 = 0.09$, $P = 0.13$). *Cryptocercus* and termopsid species contained the highest diversity of parabasalid taxa, whereas bacterial diversity was highest in the kalotermitids and *Reticulitermes* species (see Table S1 in the supplemental material). *Incisitermes banksi* and *Incisitermes schwarzi* hosted the lowest diversity of parabasalids, harboring only 2 OTUs each.

Parabasalid endemism and diversity. The parabasalid taxa found in the hindguts mostly fell within one of five classes: Cristamonadea, Spirotrichonympha, Trichomonadea, Trichonympha, and Tritrichomonadea (Fig. 1B and 2A and B). Seventeen remaining OTUs were questionably assigned (marked with a question mark) or could not be assigned to an existing order. These OTUs likely represent lineages that lack molecular data, and their phylogenetic relationships are poorly understood. The vast majority of parabasalid OTUs were either unique to a host species (75% of OTUs) or restricted to their host group (Fig. 3A), suggesting that host species harbor highly endemic symbiont communities. Only a single OTU, belonging to the Trichomonadea, was shared in hindguts across host groups, between *Porotermes adamsoni* from the Stolotermitidae and *Coptotermes testaceus* from the Rhinotermitidae, but was the least abundant member in either community (0.03% and 3% of the sequence reads, respectively). In addition, only 45% of the parabasalid OTU sequences were at least 95% similar to known 18S rRNA sequences in GenBank, and the number of OTUs generally exceeded the number of known described species in a host (see Table S1 in the supplemental material).

Host phylogeny structures parabasalid community composition. The similarities of the parabasalid communities were compared by multivariate analyses using a phylogenetic metric (UniFrac). The communities clustered based on the phylogenetic groupings of their hosts (Fig. 3B) and not by their geographic location (see Fig. S3 in the supplemental material). This indicates that historical effects, primarily vertical transmission and the diversification of parabasalids within host lineages, and not dispersal or environmental acquisition, were the likely explanations for this pattern.

Distinct clusters were evident for the parabasalid communities from the hindguts of *Cryptocercus* spp., *Reticulitermes* spp., *Zootermopsis* populations, and the rhinotermitids (Fig. 3B). The parabasalid communities from kalotermitid termites also clustered together, but not as tightly. This loose clustering was also observed for the genetic distances of the kalotermitids (see Fig. S1 in the supplemental material), which may reflect their being a more diverse group or that they were much better sampled.

A Procrustes analysis of the PCoA plots of the parabasalid com-

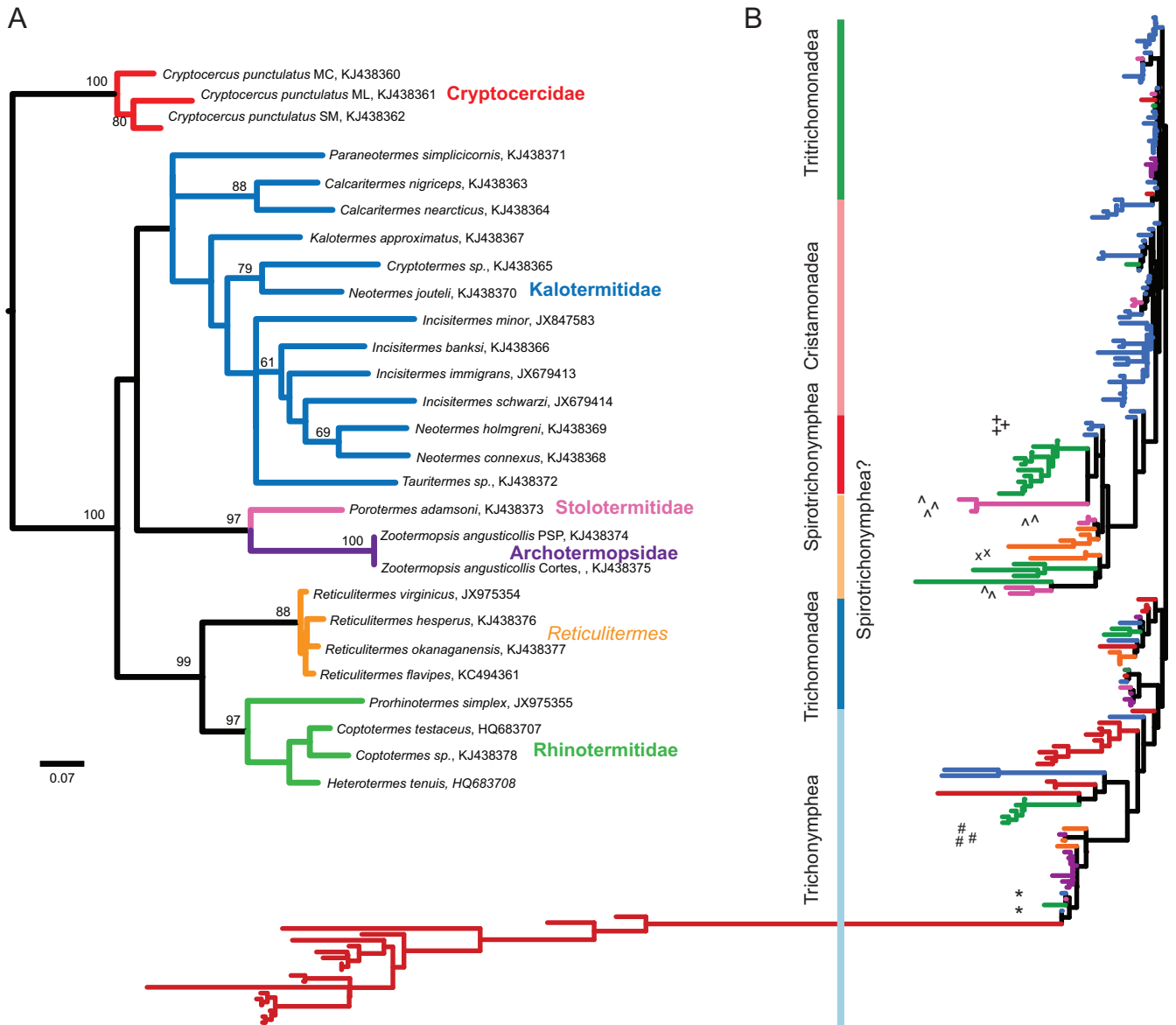


FIG 1 (A) Phylogenetic tree of the insects sampled for this study based on an alignment of mitochondrial 16S rRNA sequences and calculated using maximum likelihood. Bootstrap values at nodes with greater than 60% support are shown. The color of the branches indicates the grouping of the insects into 6 lineages, which roughly correspond to their taxonomic families. GenBank accession numbers follow the species names. (B) Maximum likelihood tree from an alignment (255 positions) of parasitoid OTU sequences. The branches are colored corresponding to panel A, indicating the insect host lineage for the majority of sequences represented by an OTU. *, *Trichonympha* OTUs from *Incisitermes* species; #, *Trichonympha* OTUs from *Reticulitermes* species; ^, Spirotrichonympha-like OTUs from *Porotermes adamsoni* that likely originated from horizontal transfer; +, Spirotrichonympha OTUs from *Paraneotermes simplicicornis* that are possibly basal or horizontally transferred; x, divergent OTUs classified as “Trichomonadea?” in *Reticulitermes*.

munities with the mitochondrial 16S genetic distances of their hosts further demonstrates the congruence between parasitoid community structure and host evolution and the dominance of historical effects in structuring the community composition (Fig. 4A).

A phylogenetic tree of the OTU sequences reveals the overall pattern of diversification and radiation of the parasitoid symbionts within host lineages (Fig. 1B). Of the major parasitoid taxa, the Trichonympha appear to have radiated early, in the ancestor of *Cryptocercus* cockroaches and the termites, and also later diversified within specific termite lineages, such as *Trichonympha* within the archotermopsids and *Pseudotriconympha* within the

rhinotermitids. The Cristamonadea have almost exclusively diversified within the kalotermitids, and the Spirotrichonympha likely originated and diversified alongside the rhinotermitids.

Supporting this pattern of diversification, Trichonympha diversity was highest in *Cryptocercus*, while kalotermitids harbored the greatest diversity of Cristamonadea but very few Trichonympha (Fig. 2A). Outside of the kalotermitids, Cristamonadea OTUs were very rare, being found in the stolotermitid *Porotermes adamsoni* and the rhinotermitid *Coptotermes* sp. Termites from the Rhinotermitidae hosted most of the diversity of Spirotrichonympha, but the hindguts of *Paraneotermes simplicicornis* and

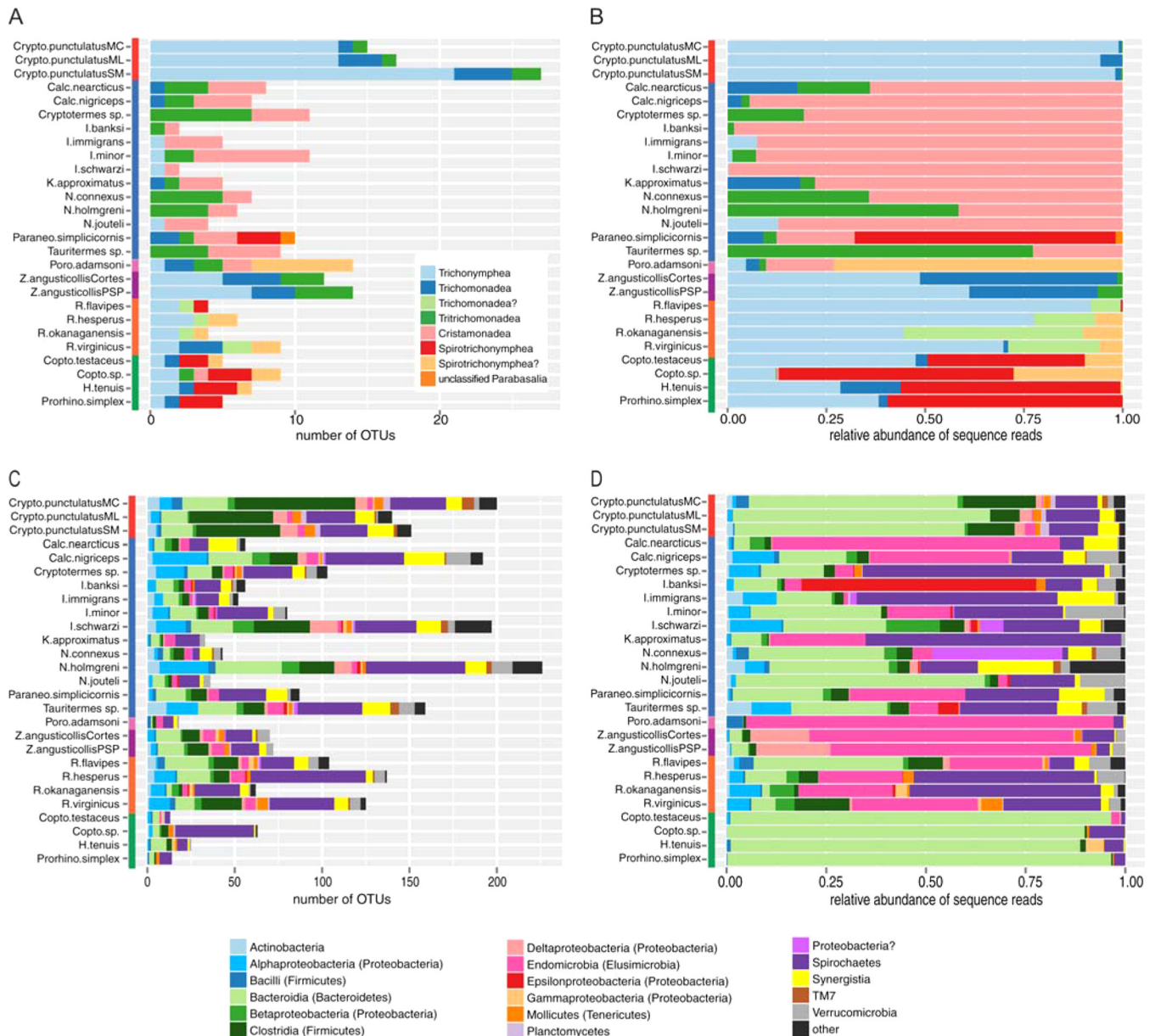


FIG 2 (A) Number of OTUs for a taxonomic class of parasabalids in the hindguts of *Cryptocercus* cockroaches and lower termites; (B) relative abundance of parasabalid sequence reads, classified by taxonomic class, with figure legend as shown in panel A; (C) number of OTUs for a taxonomic phylum or class (phylum) of bacteria in the hindguts of *Cryptocercus* cockroaches and lower termites; (D) relative abundance of bacterial sequence reads classified by taxonomic phylum or class (phylum). Figure legends for panels C and D are below the figures. For all figures, OTUs with questionable classifications are labeled with a question mark. The color of the bars next to the host names indicate the phylogenetic group of the insect corresponding to Fig. 1A: red, Cryptocercidae; blue, Kalotermitidae; pink, Stolotermitidae; purple, Archotermopsidae; orange, *Reticulitermes*; and green, Rhinotermitidae.

Porotermes adamsoni also harbored spirotrichonymphid OTUs despite the fact that these were not found in the other kalotermitid and termopsid termites.

Exceptions to host phylogeny structuring parasabalid communities. Not all of the parasabalid communities clustered alongside communities from related hosts, and examining symbiont phylogeny provided some potential explanations for these exceptions. For example, the communities from two kalotermitid species, *Incisitermes immigrans* and *I. schwarzi*, clustered closer to the communities from *Zootermopsis* than to other kalotermitids (Fig. 3B). From the phylogeny, these hosts can be seen to harbor *Tricho-*

nympha taxa related to those found in *Porotermes* and *Zootermopsis* (Fig. 1B). The removal of these *Trichonympha* OTUs from the *I. immigrans* and *I. schwarzi* data resulted in these communities clustering with the rest of the kalotermitids (see Fig. S4A in the supplemental material). Likewise, the parasabalid community of *Porotermes adamsoni* clustered away from its relative *Zootermopsis*, which was due to a number of Spirotrichonympha-like taxa in *P. adamsoni* (see Fig. S4B).

Other exceptions were noted that did not affect the clustering of the communities as a whole. The *Paraneotermes simplicicornis* community clustered with the rest of the kalotermitids as ex-

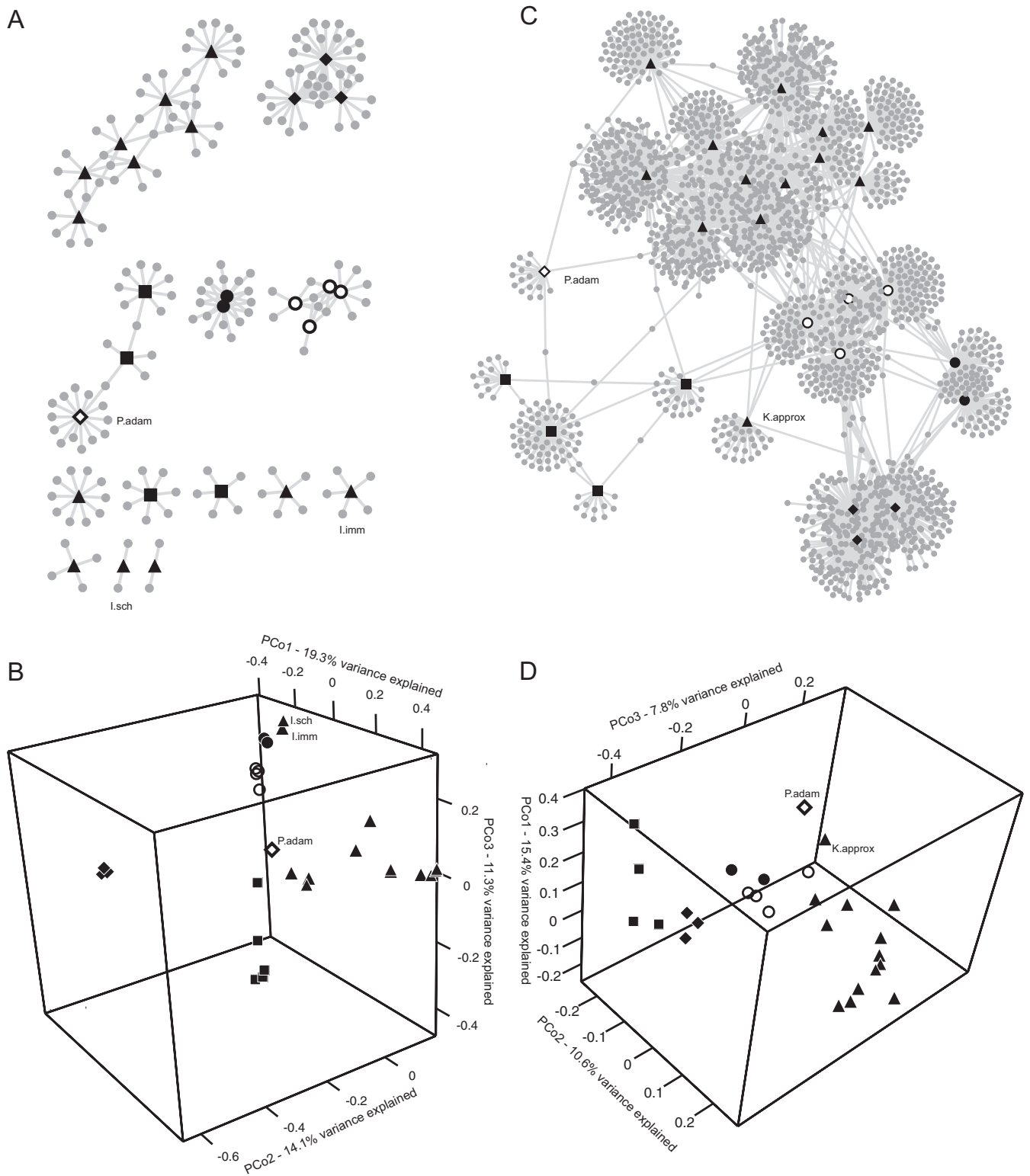


FIG 3 (A) Occurrence network of parasitoid OTUs in the hindguts of *Cryptocercus* cockroaches and lower termites; (B) PCoA using unweighted UniFrac distances between parasitoid communities from the hindguts of *Cryptocercus* cockroaches and lower termites; (C and D) the bacterial communities as described for panels A and B. For panels B and D, similar results were obtained from subsampled, normalized OTU abundances. The communities are represented by symbols based on the phylogenetic grouping of the host insect: solid diamonds, Cryptoceridae; solid triangles, Kalotermitidae; open diamond, Stolotermitidae; solid circles, Archotermopsidae; open circles, *Reticulitermes*; and solid squares, Rhinotermitidae. Communities that did not cluster with communities from related hosts are labeled. I.imm, *Incisitermes immigrans*; I.sch, *Incisitermes schwarzi*; K.approx, *Kaloterms approximatus*; P.adam, *Porotermes adamsoni*.

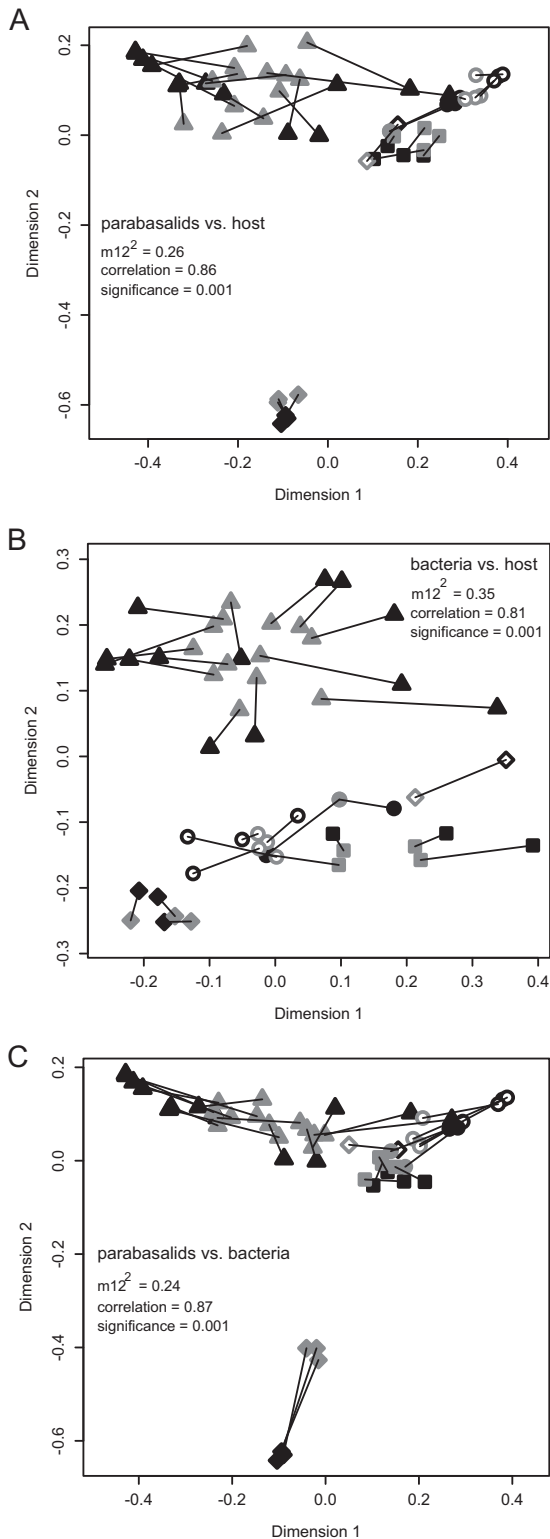


FIG 4 (A) Procrustes analysis comparing the PCoA plots of the parabasalid communities (black symbols) and the genetic distances of their hosts' mitochondrial 16S rRNA sequences (gray symbols); (B) Procrustes analysis comparing the bacterial communities (black symbols) and their hosts (gray symbols); (C) Procrustes analysis comparing the parabasalid (black symbols) and bacterial (gray symbols) communities. Symbols as described for Fig. 3.

pected, but unlike the other kalotermitids, *P. simplicicornis* harbored Spirotrichonymphea taxa. These OTUs were on short branches basal to the rest of the Spirotrichonymphea (Fig. 1B).

Reticulitermes species also harbored parabasalids that were phylogenetically distinct from those in their sister lineage, the rhinotermitids (Fig. 1B). In particular, *Reticulitermes* species harbored *Trichonympha* symbionts that were closely related to those found in the Archotermopsidae, whereas the other rhinotermitids all harbored *Pseudotriconympha* symbionts (except for a *Trichonympha* OTU that was found in *Coptotermes* sp.). *Reticulitermes* species also did not harbor Spirotrichonymphea taxa that were characteristic in the rhinotermitids, but both host lineages harbored Spirotrichonymphea-like OTUs. These OTUs, however, were highly divergent (2 were classified as "Trichomonadea?"), and the observed phylogenetic relationships may be due to long-branch attraction.

Bacterial diversity. Similar to recent investigations of lower termites (6, 18–21), the bacterial communities were dominated by a combination of *Bacteroidetes* (34.7% of the sequences and 15.5% of the OTUs), *Endomicrobia* (*Elusimicrobia*) (14.7 and 4.0%), and *Spirochaetes* (18.4 and 28.4%) (Fig. 2C and D). For the *Bacteroidetes* and *Endomicrobia* in particular, a very high proportion of these sequences belonged to relatively few OTUs. In contrast, most of the diversity in the hindguts is represented by OTUs of low sequence abundance, including taxa from the *Actinobacteria*, *Firmicutes* (*Clostridia* and *Bacilli*), *Planctomycetes*, *Proteobacteria*, *Synergistes*, *Tenericutes* (*Mollicutes*), TM7, and *Verrucomicrobia* (Fig. 2C and D). A small proportion of the sequences (0.5%), representing 19.6% of the OTUs, were sufficiently divergent from known sequences that they could not be assigned to a phylum.

Bacterial community not as strongly influenced by historical effects. Compared to the parabasalids, similar proportions of bacterial OTUs were unique to a host species (78%) or were shared among unrelated hosts (2%) (Fig. 3C). In contrast to the parabasalids, however, the taxonomic diversity of hindgut bacteria was not obviously consistent with host phylogeny. The rhinotermitids tended to lack *Elusimicrobia* and *Synergistes*, but this was not absolute (Fig. 2C). The other two dominant lineages, *Spirochaetes* and *Bacteroidetes*, were found in every hindgut sampled. Even upon examining the phylogenetic trees of individual bacterial phyla (which did not necessitate accurate classification), no termite lineages could be singled out as hosting a particular lineage of bacteria.

Despite the lack of obvious bacterial lineages occurring specifically within a host lineage, the bacterial communities did cluster based on the phylogeny of their hosts (Fig. 3D and 4B), indicating that historical effects had some influence on community composition. When the bacterial and parabasalid PCoA plots were compared with one another, the clustering of the bacterial communities also corresponded with that of the parabasalids (Fig. 4C).

Once again, the *Porotermes adamsoni* bacterial community stood out because it did not cluster with that of *Zootermopsis* as expected based on host phylogeny (Fig. 3D). Instead, its community clustered closer to that of *Kalotermes approximatus*, which also did not cluster as expected with the other kalotermitid termites. Although different sets of shared or unique taxa were tested, the diversity of the bacteria in the *P. adamsoni* and *K. approximatus* communities was sufficiently distinct that we could not identify specific taxa or OTUs that were causing these communities to

TABLE 1 Procrustes analysis of hindgut bacterial diversity compared to the host's mitochondrial 16S rRNA sequence^a

Bacterial class/phylum	m12 ²	Correlation	Significance
<i>Actinobacteria</i>	0.452	0.741	0.007
<i>Alphaproteobacteria</i> (Proteobacteria)	0.489	0.715	0.033
<i>Bacilli</i> (Firmicutes)	0.495	0.711	0.015
<i>Bacteroidetes</i>	0.369	0.795	0.001
<i>Betaproteobacteria</i> (Proteobacteria)	0.447	0.744	0.001
<i>Clostridia</i> (Firmicutes)	0.424	0.759	0.002
<i>Deltaproteobacteria</i> (Proteobacteria)	0.400	0.775	0.006
<i>Endomicrobia</i> (Elusimicrobia)	0.320	0.824	0.001
<i>Mollicutes</i> (Tenericutes)	0.276	0.851	0.001
<i>Spirochaetes</i>	0.321	0.824	0.001
<i>Synergistes</i>	0.393	0.779	0.001
TM7	0.516	0.695	0.118
<i>Verrucomicrobia</i>	0.467	0.730	0.008

^a Bacterial OTUs were split into their respective class (or phylum) before principal coordinate analyses were conducted. Only bacterial classes that had at least one sample with at least 5 OTUs were analyzed. Bolded are the bacterial classes that were the most significant in the Procrustes analysis (m12² values of <0.400, correlation values of >0.775, and significance values of ≤0.001).

cluster away from their relatives or driving them closer to nonrelatives.

The bacterial communities were split into separate data sets based on the assigned phylum or class of the OTUs to examine whether the clustering was driven by specific bacterial lineages. *Endomicrobia*, *Mollicutes*, and *Spirochaetes* communities had the strongest correspondence with host phylogeny, while *Alphaproteobacteria*, *Bacilli*, and TM7 communities had the least significant associations (Table 1).

Bacterial symbionts of protists. Using a blastn search of GenBank's nonredundant nucleotide database, we identified OTUs closely related to those of confirmed protist symbionts and found that bacterial symbionts of protists comprised a major fraction of many of the hindgut communities. For the rhinotermitids, each hindgut was completely dominated by a single but different *Bacteroidales* (*Bacteroidetes*) OTU (94.4% of the 16S rRNA sequences in *Coptotermes testaceus*, 89.4% in *Coptotermes* sp., 81.7% in *Heterotermes tenuis*, and 95.6% in *Prorhinotermes simplex*). Three out of these 4 OTUs shared at least 95% similarity with endosymbionts of the protist *Pseudotriconympha* known from termites of the same host genera (45, 46). Likewise, 8 out of 10 *Endomicrobia* OTUs in the hindguts of *Zootermopsis angusticollis* (comprising 83.0% of the 16S rRNA sequences) were at least 95% similar to endosymbionts of the parabasalid *Trichonympha* in the related termites *Hodotermopsis sjoestedti* and *Zootermopsis nevadensis* (47, 48). The *Porotermes adamsoni* hindgut was also dominated by *Endomicrobia* (4 OTUs representing 91.8% of the 16S rRNA sequences), and a single OTU related to *Bacteroidetes* ectosymbionts of *Barbulanympha* represented 51.3% of the 16S rRNA sequences from the hindguts of *Cryptocercus* cockroaches.

In total, 3.6% of the OTUs (25.3% of the sequences) shared at least 95% similarity with known bacterial symbionts of hindgut protists represented in GenBank. Many more OTUs were also likely symbionts of protists but have not been previously studied or were divergent from those in GenBank.

DISCUSSION

Weighing historical effects with horizontal transfer and ecological and stochastic effects on community composition. The pro-

tist symbionts in the hindguts of *Cryptocercus* cockroaches and lower termites were long suspected to have codiversified with their hosts (15, 17, 49). The common ancestor of *Cryptocercus* cockroaches and termites is thought to have evolved a dependence on a specialized consortium of gut microbes that allowed it to gain nutrition from the consumption of wood (16, 50). The transmission of this gut consortium was ensured via coprophagy and by the development of proctodeal trophallaxis, which is the direct transfer of hindgut fluid from the rectum of a donor to the mouth of the recipient and dependent on some degree of social behavior in the host. Over evolutionary time, this mode of symbiont transmission further confined the dispersal of hindgut microbes to closely interacting individuals, such as within a family or colony, resulting in the diversification of the symbionts within their hosts from the ancestral gut consortia.

Using deep pyrotag sequencing to describe the parabasalid and bacterial diversity in the hindguts of *Cryptocercus punctulatus* and 23 species representing the major lineages of lower termites, we have provided molecular evidence that host phylogeny structures microbial community composition. The data suggest that host-symbiont codiversification not only took place but was the prevailing mode of evolution between the origin of termites and the diversification of the parabasalids. However, the composition of symbionts was not entirely due to these historical effects. Exceptions were observed, particularly for the Spirotrichonympha, the Trichonympha, and the rare bacteria. Instead of strict vertical transmission, these symbionts were likely transferred between distantly related hosts. Ecological and stochastic effects, such as selection for the most beneficial symbionts given the diet of their host or incomplete transmission of the symbiont community, may also partly explain the composition of communities that deviate from their expected distribution based on host phylogeny.

The occurrence of *Spirotrichonympha*-like protists in the hindguts of *Porotermes adamsoni* and *Paraneotermes simplicicornis* was not consistent with diversification within their host lineage but possibly due to transfer from rhinotermitids. Alternatively, *P. simplicicornis* may be harboring slowly evolving basal spirotrichonymphids that were not retained in other kalotermitids. Spirotrichonympha have also been documented in termites from the Hodotermitidae (4), which are close relatives of *Porotermes adamsoni*. Interestingly, *P. simplicicornis* shares nesting and feeding behaviors that are similar to those of rhinotermitids and hodotermitids. Unlike other kalotermitids which feed and nest in drywood, *P. simplicicornis* is subterranean and forages on dampwood at or near its nest (intermediate nester). Many rhinotermitids are also subterranean intermediate nesters that forage on dampwood, while other rhinotermitids and the hodotermitids are subterranean separate-piece nesters foraging for food that is distinct from the nesting substrate and includes wood, grass, or detritus (51, 52). The presence of Spirotrichonympha in these hindguts might possibly play a role in these ecological similarities, or the similar habitats may have facilitated the movement of symbionts.

Reticulitermes species harbor protist communities that are phylogenetically distinct from their rhinotermitid relatives, including *Trichonympha* and divergent Spirotrichonympha-like taxa but not typical Spirotrichonympha. Furthermore, *Reticulitermes* species but not the other rhinotermitids are known to harbor oxymonad protists (53). The distinctness of the parabasalid community in the hindguts of *Reticulitermes* species, in partic-

ular the occurrence of *Trichonympha* closely related to those in Archotermopsidae, suggests that symbiont transfer has had a significant role in structuring these communities (49).

It is also likely that *Trichonympha* symbionts were transferred from stolotermitids to *Incisitermes*. However, because the evolutionary relationship of the Kalotermitidae relative to the termopsids and rhinotermitids is not resolved and the monophyly of the Rhinotermitidae is in dispute (40, 44), it is also possible that these *Trichonympha* symbionts have been retained in these host species from the ancestral consortium due to an ecological advantage or stochasticity but have gone extinct in their relatives.

The influence of symbiotic associations with protists, environmental acquisition, or dispersal on bacterial community composition. Compared to the protists, the influence of historical effects on bacterial community composition was not as clearly evident. Host phylogeny corresponded with the diversity of the dominant bacterial phyla, the *Bacteroidetes*, *Endomicrobia*, and *Spirochaetes*, but, except for the *Mollicutes* and *Synergistes*, not with the more rare lineages. Most of the bacterial OTUs in the hindguts were unique to a host species, which suggests that these symbionts have become specialized to the *Cryptocercus*/termite hindgut environment and are unlikely to have been acquired from the external environment. However, without knowing the dispersal, evolutionary, and extinction rates of bacteria, the unique occurrence of a symbiont is not evidence of codiversification or host specificity. Differences in the bacterial communities from lower and higher termites are more likely driven by dietary changes and the environmental acquisition of symbionts (18, 19).

Bacteroidetes, *Endomicrobia*, *Spirochaetes*, and *Synergistes*, the bacterial lineages that showed the strongest correlation with host phylogeny, can be free-living in the hindguts, but many are protist symbionts (see, for example, references 9, 10, 13, 14, and 54–56). Bacteria from the *Actinobacteria* and *Deltaproteobacteria* are also known to have symbiotic associations with protists (48, 57). In previous studies (12, 45), and as we have shown here, greater than 80% of the bacteria can be symbionts of hindgut protists. The bacterial symbionts likely support the metabolic activities of their protist hosts by providing nitrogenous compounds through amino acid synthesis or nitrogen fixation or as a sink for hydrogen produced during lignocellulose fermentation (46, 58, 59). They have also been shown to assist in motility (14, 60).

It seems likely that the importance of historical effects on bacterial composition is driven by, or at least bolstered by, symbiotic associations between bacteria and protists, where the protists have codiversified with their insect host. If these associations in the hindguts were established early or prior to the evolution of *Cryptocercus* cockroaches and lower termites, three levels of coevolution and codiversification have occurred, between the insect, protist, and bacterium. Codiversification has been shown between species of rhinotermitid termites, one of their hindgut protists, *Pseudotriconympha*, and their *Bacteroidales* endosymbionts (61). Codiversification has also been demonstrated between multiple species of the parabasalid *Trichonympha* and their *Elusimicrobia* endosymbionts (47) and between devescovinids and their *Bacteroidales* ectosymbionts (62). However, the three-tiered codiversification structure is broken if ancestral bacteria are replaced by a different symbiont or the protist-bacterium symbioses are more recently established (10, 63).

Host phylogeny does not seem to structure the less abundant bacteria, even those with relatively high diversity, such as the *Fir-*

micutes. Rare taxa can represent a major proportion of the diversity in the hindguts but may be part of a transient community of bacteria whose presence in the hindgut fluctuates (18). Indeed, it is not clear whether the composition of the bacterial community is stable and consistent in most or all individual insects in a colony, as is typical for the protists in termite hindguts. For the rare taxa, dispersal and environmental acquisition are probably more significant in structuring the communities. These bacteria may have more generalized or nonessential functions and can be absent or more easily replaced in the ecosystem by other bacteria (20). These bacteria may also be more resilient to survival outside the hindgut and more easily transferred among hosts.

Diversity and endemism. Parabasalid diversity in the hindguts of lower termites and *Cryptocercus* cockroaches is often incompletely described and largely based on morphology. The 18S rRNA pyrotag sequencing provided comprehensive and unbiased descriptions of parabasalid diversity in the hindguts and revealed many undiscovered taxa. The majority of the 18S rRNA OTUs were divergent (<95% similar) from known parabasalid sequences in molecular databases. In addition, the number of parabasalid OTUs often exceeded the number of known described species in the hindguts of lower termites and *Cryptocercus* cockroaches (see Table S1 in the supplemental material) or the taxonomic composition at the species or genus level differed. But in some rare cases, even if the overall number of OTUs observed exceeded the number of known species for a host, there was also evidence of overclassification within particular subgroups (where the number of OTUs was smaller than the number of described species). Nevertheless, for each host, we have likely uncovered novel and/or cryptic species or obtained molecular data from species known only through morphology. More importantly, these molecular surveys provide a phylogenetic basis for a more accurate understanding of parabasalid diversity, diversification, and community composition.

Although the hindguts and relevant environments have not been sampled exhaustively, it is likely that many of the hindgut microbes are endemic to a host species. Most of the symbiont OTUs were unique to a host species or shared only among closely related hosts (and the cutoff we applied might not capture sister species-level divergence). Especially for the parabasalids, the endemism is due to diversification within specific host families. For example, most of the existing diversity of Cristamonadea, Spirotrichonympha, and Trichonympha results from radiation within the hindguts of specific host lineages, that is, the kalotermitids, rhinotermitids, and *Cryptocercus*, respectively. This diversification can result in several closely related species existing in a single host species (64).

The ecological niches of most species of symbionts have yet to be identified, so the factors driving this diversification are not known. Microenvironments, such as oxygen or pH gradients, within the different compartments of the hindgut or food particle size may have a role (65, 66). Moreover, the microbial community itself can create these microenvironments (67). Biochemical coordination of the symbiont community with their host to extract nutrition also likely influences symbiont diversification (68, 69). The biology of the symbionts must also complement the biology and behavior of the host. For example, symbionts of *Cryptocercus punctulatus* can encyst and are retained through molts, whereas in termites, symbionts die during molts and must be reacquired from nestmates (70). The gut structure, nesting style, and foraging

behaviors of the termites may also impact the diversification of the symbiotic community (71). Given that speciation has occurred within the environment of a host hindgut, these hindgut communities provide an interesting system to examine the mechanisms of sympatric speciation.

Conclusions. The protist communities in the hindguts of *Cryptocercus* cockroaches and the lower termites are heavily structured by host phylogeny due to historical effects, but only a portion of the bacterial communities shared this characteristic. Correlation of the bacteria with host phylogeny may have occurred indirectly through their associations with protists, and direct interactions with their hosts were not a significant force. Even in these clear cases of vertical transmission, where historical effects were expected to dominate microbial community structure, a significant proportion of the bacterial community remained strongly influenced by dispersal and stochasticity compared to the protists.

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