A Study of the Cockroach Gut Flagellates in Genus *Lophomonas* with Single Cell
Approaches

by

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ABSTRACT

Lophomonas is a genus of flagellated parabasalids that exist as commensal symbionts in the hindguts of a variety of pest cockroaches. The genus contains two species: *Lophomonas blattarum* and *Lophomonas striata*. The two species differ by way of bacterial ectosymbionts that attach to the outside of *L. striata*, giving rise to a striated and spindle-shaped appearance. As the attachment of bacterial symbionts prohibits *L. striata* from taking up large food particles in the same manner as *L. blattarum,* it is likely the two species differ in which metabolic genes they possess. Here, a comparison of transcriptomes between the two *Lophomonas* species show slight differences between the species. Metagenomic analysis of *L. striata* also presents the possibility of *L. striata* ectosymbionts as belonging to the genus *Parabacteroides*.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Introduction

The metamonads are a group of flagellated, anaerobic protists, all of which

lack classical mitochondria; instead, greatly reducing their mitochondria to

hydrogenosomes or becoming amitochondriate altogether (Cavalier-Smith 2003). The

phyla Metamonada consists of three major phyla: Preaxostyla (which includes the order

Oxymonadida), Fornicata and Parabasalia (Adl et al. 2019). Metamonads can be free-

living, but the majority live in other organisms, including human parasites like *Giardia*.

The parabasalids, a major lineage of metamonads, constitute a clade of anaerobic

flagellated protists characterized by cellular features such as the presence of parabasal

bodies, which are dense Golgi complexes associated with flagella (Čepička et al. 2016).

Most parabasalids exist as symbionts of a wide variety of animal hosts, typically residing

in their digestive tracts. Some of the most notable parabasalids are pathogens of humans

and livestock, including the sexually transmitted human parasite *Trichomonas vaginalis*

and the bird parasite *Histomonas meleagridis*. Most parabasalids inhabit the hindguts of

termites, where they aid their hosts in lignocellulose digestion of the wood of which the

termites are so fond (Brune 2014). Related to the termite-specific protists is the genus

Lophomonas, found in the hindguts of cockroaches.

Lophomonas blattarum Stein was first discovered in the hindgut of the Oriental

cockroach *Blatta orientalis* as a commensal flagellate (Stein 1860). Morphologically, *L.

blattarum* cells are typically round or egg-like in shape, 20-60 µm in length and possess a

bountiful tuft of anterior flagella, numbering about 50 flagella per cell attached to an

1

intracellular calyx (Kudo 1926b). *Lophomonas striata* Bütschli was discovered about 18 years after *L. blattarum* was first described and was also found residing in the hindgut of *B. orientalis* (Bütschli 1878). Cells of *L. striata* are about 30-40 µm long and, like *L. blattarum*, possess an anterior tuft of flagella; however, *L. striata* are more carrot-shaped and striated due to the fusiform bacteria that attach to the outer membrane of the cell (Kudo 1926a).

Both species of *Lophomonas* can be found as commensal flagellates in a variety of different pest species of cockroach, though their presence is not always observed, suggesting the relationship between protist and cockroach is non-obligate, at least from the perspective of the host (Martínez-Girón et al. 2017). *L. blattarum* is known to ingest both starch and yeast cells (Kudo 1926b). This seems to be another major difference between the *Lophomonas* species, as *L. striata* has never been observed phagocytosing material, possibly due to the size and shape constraint placed on the cell by its ectosymbionts (Kudo 1926a). This suggests major metabolic differences between *L. blattarum* and *L. striata*, especially in tandem with the bacterial symbioses present with the latter.

Notably, *L. blattarum* has also been implicated in bronchopulmonary infection in immunocompromised humans, with cases of "lophomoniasis" reported since the 1990s (Xue et al. 2014). Diagnosis of lophomoniasis is usually done morphologically, which occasionally leads to misdiagnosis when respiratory ciliated cells are misidentified as *L. blattarum* (Meng et al. 2019). Lophomoniasis, then, is controversial, and some question whether *L. blattarum* is actually found in human lungs (Li & Gao 2016). Molecular

identification of *L. blattarum* in humans has previously been described (Fakhar et al.

2019), but molecular phylogenetic analysis reveals that those human-derived

Lophomonas sequences branch with Trichomonadida, evolutionarily distant from the

Trichonymphida that cockroach-derived *Lophomonas* branches with (Nguyen et al. 2023,

Appendix A). Since the order Lophomonadida branches sister to Trichonymphida, it is

possible that Lophomonadida represents a more basal order of Parabasalia alongside

Trichonymphida (Figure 1).
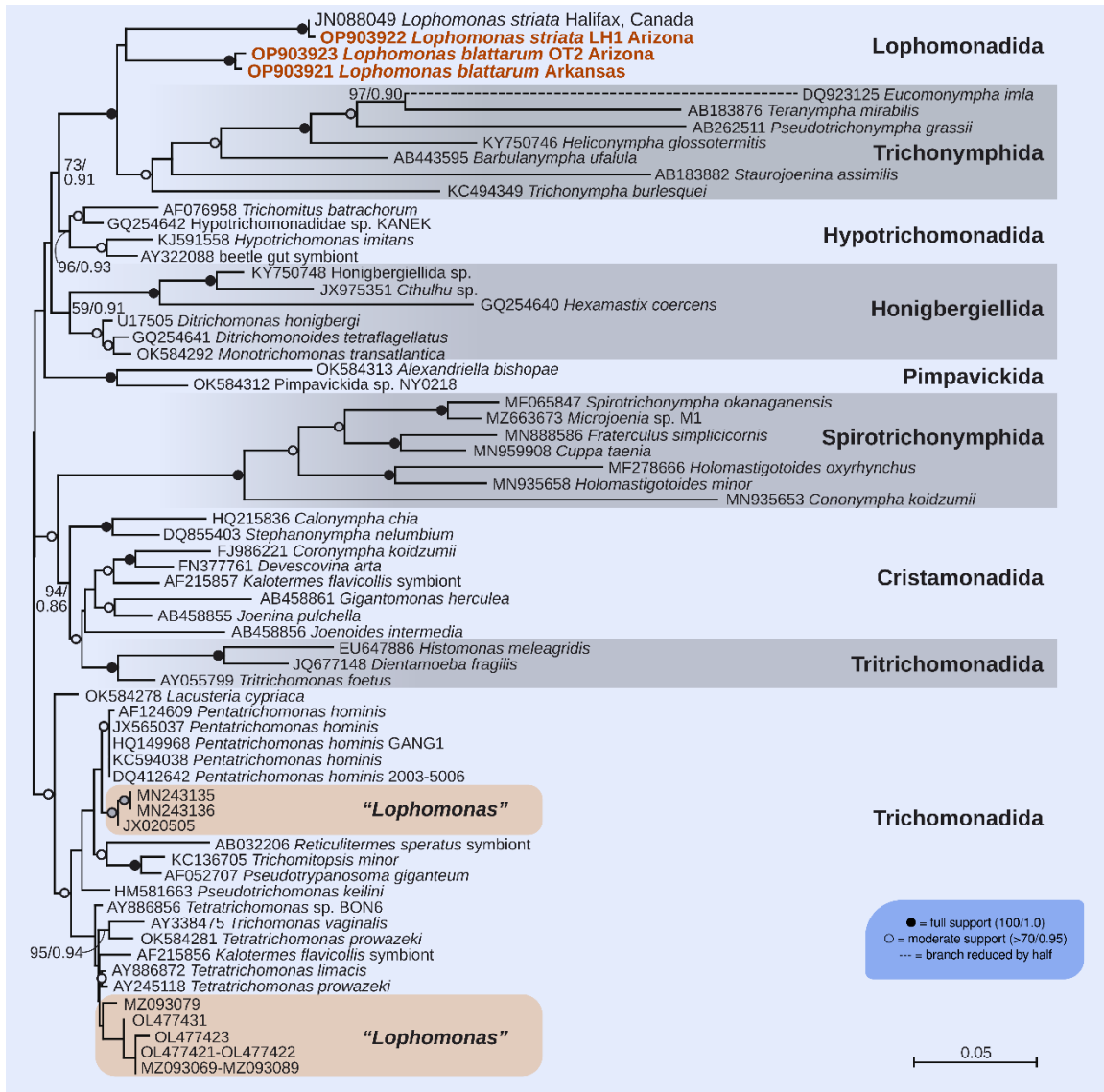
Fig. 1. Phylogenetic position of *Lophomonas* in Parabasalia. Maximum-likelihood 18S rRNA tree of *Lophomonas* within Parabasalia, alongside sequences from supposedly human-derived "*Lophomonas*" cells. Bolded branches indicated sequences from cockroach-derived *Lophomonas* cells; sequences with an orange background indicate sequences derived from human samples. Filled circles indicate full support, empty circles indicate moderate support and dashed branches indicate that the length has been reduced in half. Figure modified from Nguyen et al. 2023.

4

Since *Lophomonas* is a parabasalid, the genus is closely related to many termite-associated protists, most of which are obligate symbionts. One termite protist, the oxymonad *Streblomastix strix,* is known to harbor many bacterial ectosymbionts that may aid in lignocellulose digestion and nitrogen fixation, bolstering the metabolic capacities of both the protist and termite hosts (Treitli et al. 2019). This is not uncommon among termite protists, and similar metabolic relationships have also been studied in the *Reticulitermes* oxymonad symbiont *Dinenympha* and its bacterial symbionts, in which the bacterial ectosymbionts are suspected to aid in lignocellulose digestion (Yuki et al. 2015). Likewise, it would not be unexpected to find synonymous interactions between *L. striata* and its ectosymbionts. Interestingly, termite protists with bacterial ectosymbionts share similar cell structure to *L. striata; Hoplonympha, L. striata* and even the more distantly related *S. strix,* for instance, appear star-shaped in cross-sections, with bacteria that adhere to the folds of the cell membrane (Beams et al. 1960; Noda et al. 2006).

The aim of this study was to develop a pipeline for processing, assembling and annotating *Lophomonas* transcriptomes, and compare the transcriptomes of both *L. blattarum* and *L. striata* to determine any major differences between them, especially in regard to metabolic genes, like those encoding carbohydrate enzymes. Additionally, the ectosymbionts of *L. striata* were of particular interest, and metagenomic analysis of a single-cell whole genome amplification was carried out in an attempt to characterize the bacteria present on the cell. The *L. striata* transcriptome assembly had an abundance of bacterial transcripts present, more so than that of *L. blattarum*, some of which may have come from *Parabacteroides* genomes present in the metagenomic assembly.

Methods

Sample collection and *Lophomonas* isolation

   Blatta orientalis were captured in Tempe, Arizona in October 2018 and

Shelfordella lateralis were captured in Gilbert, Arizona in July 2022. All cockroaches

had their hindguts removed and suspended in Ringer's solution (8.5 g NaCl, 0.20 g KCl,

0.20 g CaCl2, 0.10 g NaHCO3 per liter, HiMedia Laboratories). Both *L. blattarum* and *L.

striata* were seen in *Blatta orientalis*; only *L. striata* was observed in *Shelfordella

lateralis*. Individual *Lophomonas* cells were identified by morphology (Figure 1) and

isolated by drawn-glass pipette. Cells were isolated on an AxioVert inverted microscope

and photographed using an Axiocam 105 color camera (Zeiss). After isolation, each cell

was washed twice with fresh Ringer's buffer solution and placed into 0.2 mL tubes with

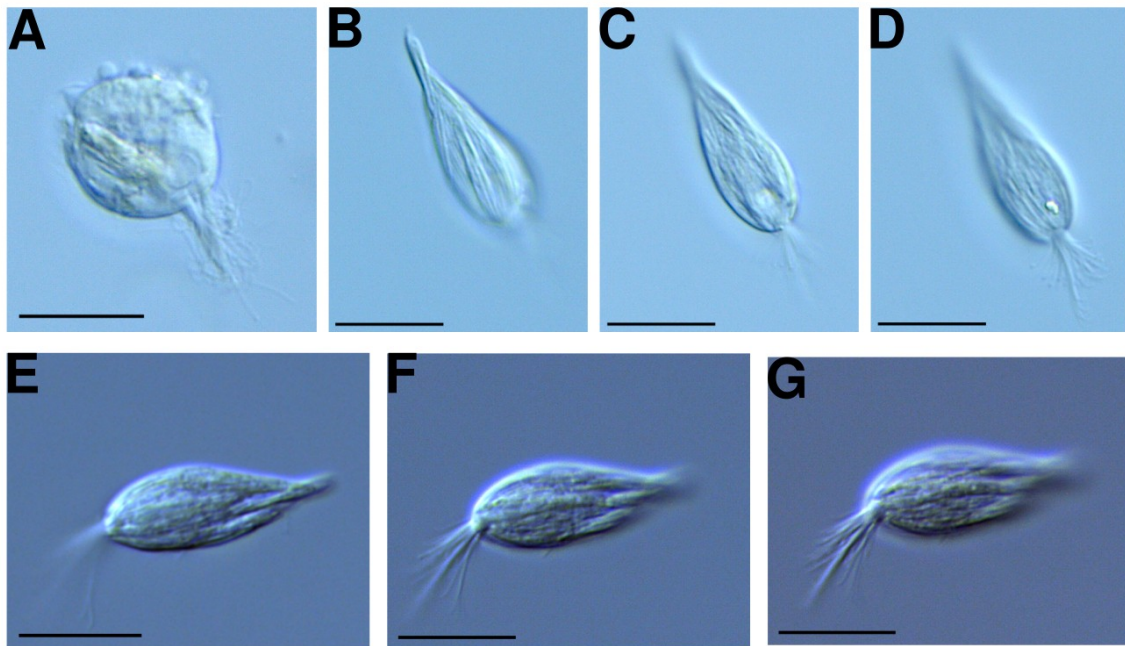50 μL acetone. All samples were stored at -20°C until processing.

Fig. 2. Lophomonas cell morphologies. (A-G) Differential interference contrast light microscopy of the *Lophomonas* cells isolated and used in this study. (A) *L. blattarum* cell OT2 isolated from *Blatta orientalis*. (B-D) *L. striata* cell LH1 isolated from *Blatta orientalis* in three optical sections. (E-G) *L. striata* cell isolated from *Shelfordella lateralis* in three optical sections. All scale bars 20 are μm.

Lophomonas cDNA synthesis and whole genome amplification

L. blattarum* and *L. striata* cells isolated from *Blatta orientalis* underwent cDNA synthesis and amplification. Tubes containing cells in acetone were held at 65°C until dry, at which point whole transcriptome amplification was performed using the SmartSeq2 protocol as previously described (Picelli et al. 2014). Libraries for Illumina sequencing were prepared at the Arizona State University Genomics Core Facility using the LTP kit (Kapa Biosystems). DNA was sheared to approximately 600bp fragments using Covaris M220 ultrasonicator, then end repaired and A-tailed as described in the Kapa protocol. Illumina-compatible adapters with unique indexes (IDT #00989130v2) were ligated on each sample separately. Adapter-ligated molecules were purified using Kapa Pure Beads and amplified with Kapa HIFI enzyme (Kapa Biosystems). Each library was then analyzed for fragment size on a TapeStation (Agilent) and quantified by qPCR using the Kapa Library Quantification Kit on a Quantstudio 5 (ThermoFisher Scientific). Single cell libraries were then pooled at equimolar concentrations and sequenced on the NovaSeq platform (Illumina) using 2x150 (SP300) chemistry at the Translational Genomics Research Institute (TGen, Phoenix, AZ). Species identification was confirmed as described in Appendix A.

The *L. striata* cell isolated from *Shelfordella lateralis* was subjected to whole genome amplification. Multiple displacement amplification (MDA) was performed using the REPLI-g Advanced DNA Single Cell Kit (Qiagen) according to manufacturer

7

instructions. Amplified products were purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) as per manufacturer instructions. Purified MDA products were sent to Psomagen for whole genome sequencing on the NovaSeq platform (Illumina) using 2x150 (SP300) chemistry.
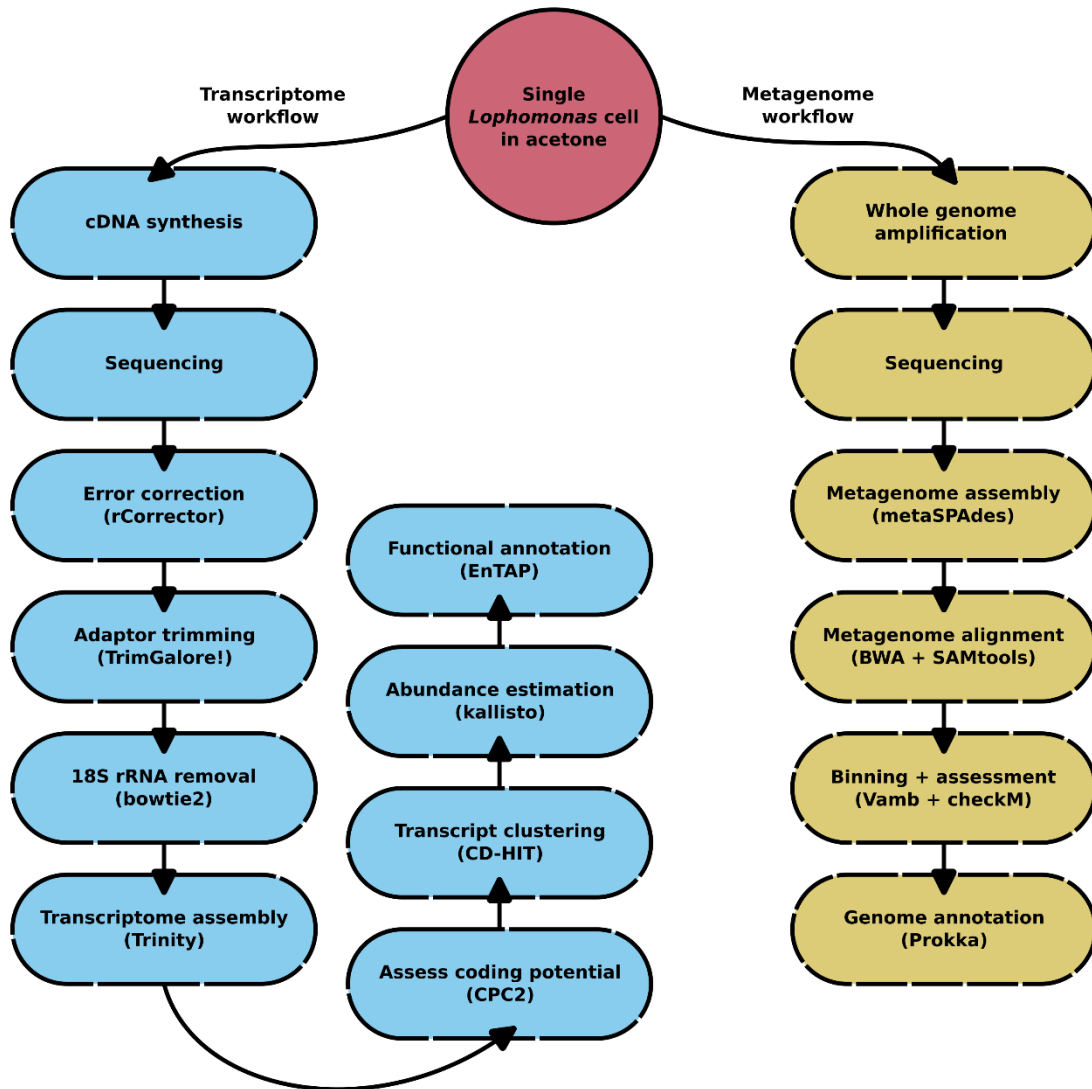

Fig. 3. Summary of workflows following single cell isolation. Left describes the transcriptome process from cDNA synthesis, leading up to functional annotation and

8

right describes the metagenome process from whole genome amplification to genome annotation.

Transcriptome assembly and annotation

De novo assembly was carried out as described by the Harvard FAS Informatics group (https://informatics.fas.harvard.edu/best-practices-for-de-novo-transcriptome-assembly-with-trinity.html). rCorrector v1.0.5 was used to correct erroneous k-mers in the raw reads (Song & Florea, 2015). Any reads deemed uncorrectable by rCorrector were removed with TranscriptomeAssemblyTools (https://github.com/harvardinformatics/TranscriptomeAssemblyTools). After k-mer correction, adaptors and low-quality bases were trimmed by TrimGalore! v0.6.0 with cutadapt v4.3, filtering out reads with phred scores less than 5 or lengths less than 36 base pairs (Martin, 2011). Using bowtie2 v2.5.1, *Lophomonas* 18S sequences downloaded from SILVA were mapped onto the trimmed to remove unwanted rRNA reads (Langmead & Salzberg, 2012). The processed reads were then assembled using Trinity v2.15.1 (Grabherr et al., 2011). Assembly quality was assessed using bowtie2 to map reads back onto the Trinity assembly. BUSCO v5.4.7 was used to estimate transcriptome completeness with the eukaryota odb10 database (Manni et al., 2021).

Transcript coding potential was calculated using the CPC2 web server; any transcripts deemed "non-coding" were tossed to further remove any non-mRNA reads, including rRNA and tRNA (Kang et al., 2017; Kong et al., 2007). CD-HIT v4.8.1 was used to cluster transcripts to reduce contig redundancy (Fu et al., 2012; W. Li & Godzik, 2006). Kallisto v0.46.2 was used to estimate transcript abundance; representative

9

sequences were sorted by descending transcripts per million (TPM) and the top 200 sequences were extracted from that list (Bray et al., 2016). Annotation of those 200 sequences was done through EnTAP v0.10.8 in blastp mode against the National Center for Biotechnology Information non-redundant database (NCBI-nr) and the UniProtKB Swiss-Prot database with manual curation (Hart et al., 2020). After manual decontamination, DIAMOND v2.1.8 was used to align translated transcripts against the eukaryotic orthologous group (KOG) database (Buchfink et al., 2015). A summary of this process can be seen in Figure 2.

Metagenome assembly, binning and annotation

Assembly of the metagenome was carried out using metaSPAdes v3.15.3 (Prjibelski et al., 2020). Post-assembly quality assessment was done through QUAST v5.2.0 (Mikheenko et al., 2018). BWA v0.7.17 and SAMtools v1.9 were used for metagenomic alignment (Danecek et al., 2021; H. Li, 2013). Alignment files generated from BWA and SAMtools were used by Vamb v4.1.3 for binning (Nissen et al., 2021). CheckM v1.2.2 was used to assess bin quality in *lineage_wf* mode (Parks et al., 2015). Bins of interest were annotated using Prokka v1.14.6 (Seemann, 2014). Annotated 50S ribosomal protein genes were subject to blastx searches against the NCBI-nr database in an attempt to elucidate species identity.

Results

10

Transcriptome quality assessment and functional annotation

Transcriptome assemblies were generated for both *L. blattarum* and *L. striata*, though *L. striata* was more deeply sequenced, resulting in a higher number of transcripts (over three-fold) and higher total length (more than double). Estimated transcriptome completeness was very low (Table 1), as both assemblies were estimated to be less than 10% complete. GC contents of both assemblies were low, though the *L. striata* assembly had a noticeably higher GC content.

Table 1. Summary of *Lophomonas* transcriptome assembly features. LH1 corresponds to *Lophomonas striata* and OT2 corresponds to *Lophomonas blattarum*. Number of transcripts, total length and GC content taken from Trinity. Completeness taken from BUSCO and number of clusters taken from CD-HIT.

Sample	No. of transcripts	Total length, bp	GC content, %	Completeness, %	No. of clusters
LH1	23,290	13,905,808	45.14	7.40%	16,063
OT2	7,380	5,432,563	38.77	4.30%	5,075

After functional annotation of the top 200 most highly expressed transcripts of both assemblies, all transcripts with fungal, plant or animal origins were removed, as were any transcripts that could not be functionally annotated. This reduced the number of *L. striata* (LH1) transcripts to 156, and the number of *L. blattarum* (OT2) transcripts to 170. However, after the removal of transcripts with bacterial taxonomic assignment, the number of LH1 transcripts dropped to 84—less than half of the original list of 200 transcripts. Bacteria seemed to make up less of the OT2 assembly, as 123 protistan transcripts remained after discarding transcripts with bacterial assignment. Percentage-wise, following removal of fungal, plant and animal transcripts, 46.5% of the remaining LH1 transcripts were bacterial, compared to 27.5% for the OT2 assembly.

11

Each of the remaining transcripts were of protistan origin, with metamonads as the top blastp hit for every transcript. Interestingly, all of the top hits for the *L. blattarum* assembly were parabasalids, including *Trichonympha agilis*, *Trichomonas vaginalis*, and *Histomonas meleagridis*. Though the majority of the *L. striata* transcripts had similar results, some of the transcripts in the assembly had oxymonads as their top hits, accounting for approximately 17% of the remaining transcripts. The oxymonads included *Streblomastix strix* and *Monocercomonoides exilis*.

Some of the remaining annotated transcripts were hypothetical proteins with no known function and could not be used in functional analysis. After annotation with KOG categories and discardment of transcripts without clear functions, only 64 of the original 200 LH1 transcripts remained. Likewise, 90 of the original 200 OT2 transcripts remained. Only the top 64 most highly expressed of the 90 OT2 transcripts were used for comparison between the two assemblies.

Since very few transcripts were used in functional analysis, the most highly expressed transcripts between the two *Lophomonas* assemblies were generally similar (Figure 3). Though the *L. striata* assembly had significantly more annotated ribosomal protein transcripts, this is likely due to the very low completeness of both transcriptome assemblies. The number of annotated transcripts related to metabolism were low in both assemblies, with 8 LH1 and 14 OT2 transcripts falling under KOG metabolic categories.

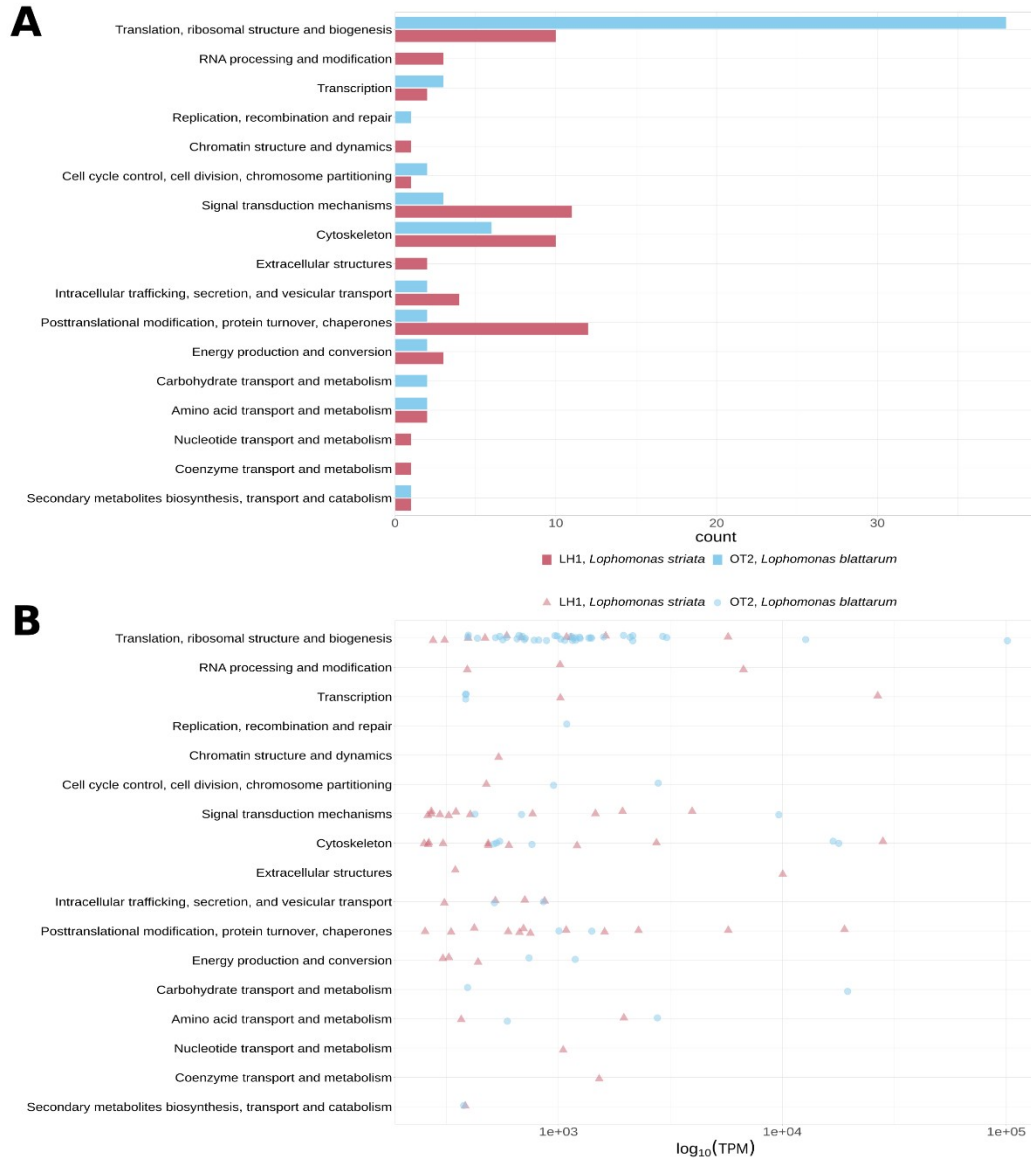*Fig. 4.* Comparison of KOG annotations between *Lophomonas* transcriptomes. (A) Counts of each functional category in the top 64 annotated transcripts of both species. (B) Expression values in transcripts per million (TPM) for each functional category for the top 64 annotation transcripts of both species. Pink bars and triangles represent LH1, *L. striata*; blue bars and circles represent OT2, *L. blattarum.*

13

Metagenome quality assessment and genome annotation

After metagenome assembly through SPAdes, assembly metrics were generated

with QUAST, reporting 47,701 scaffolds over 500 bp long and a total length of

89,916,942 bp. The GC content was somewhat low, at 39.05%. Binning revealed four

bins with over 90% estimated completeness and relatively low contamination; three bins

had less than 10% estimated contamination (Table 2).

Table 2. Summary of *L. striata* metagenome bins. Completeness and contamination
calculated from CheckM. Contigs, total bases and number of CDS taken from Prokka.
List of possible organisms taken from top blastx hits from annotated 50S ribosomal
protein encoding genes.

Bin ID	Completeness, %	Contamination, %	No. of contigs	Total bases	No. of CDS	Possible organism
bin3	100	11.91	236	4,576,387	3,779	*Parabacteroides*
bin1	99.57	0	51	4,883,495	4,635	Hyphomicrobiales
bin5	96.63	5.62	117	1,743,020	1,607	*Endomicrobium*
bin2	94.05	1.26	17	617,181	580	*Blattabacterium*

Genome annotation through Prokka revealed two bins with over 100 contigs each,

both of which contained annotated 16S and 23S ribosomal RNA genes. The other two

bins of interest had less than 100 contings each, and neither of them had annotated small

subunit (SSU) ribosomal RNA genes, despite the relatively high estimated completeness

of both bins, though one bin had an annotated 5S ribosomal RNA gene. Due to the lack

of SSUs in half of the bins of interest, an attempt at species identification was done by

querying translated 50S ribosomal protein encoding genes against the NCBI-nr database.

The bin with the highest estimated completeness (bin3) had matches with

Parabacteroides species, all of which had over 90% amino acid sequence similarity.

Other potential bacteria present in the metagenome include *Endomicrobium* sp. and

Blattabacterium cuenoti, as there were annotated ribosomal protein genes from bins that matched these organisms with over 90% sequence identity. One bin of interest (bin1) had an estimated completeness of nearly 100% and 0% estimated contamination, but a variety of different organisms appeared in translated nucleotide searches, including *Paramesorhizobium* and *Brucella* species, with sequence identities greater than 90% for all of them.

Discussion

Though the transcriptome assemblies generated from the *Lophomonas* cells in this study were of very low estimated completeness, it has been reported that BUSCO can underestimate completeness of metamonad genomes due to the high divergence of BUSCO homologs in metamonads (Salas-Leiva et al., 2021). Even considering this, however, transcriptome completeness of under 10% is devastatingly low, and more *Lophomonas* samples should be sequenced in hopes of assembling more complete transcriptomes. Of the assemblies, many of the sequences could not be functionally annotated, even in the top 200 most highly expressed transcripts, though this may not be unsurprising, as an estimated 40-60% of predicted microbial genes have no known function (Vanni et al., 2022). Initially, only the top 200 most highly expressed transcripts were selected for functional annotation for ease of comparison, but species contamination and sequences without functional annotation reduced this amount tremendously and led

to an inadequate dataset. Instead, functional annotation should be done on complete transcriptomes to better understand the two species.

Comparing the two assemblies, it is of note that the GC content of the *L. striata* assembly is significantly higher than that of the *L. blattarum* assembly. Low GC contents are expected of parabasalids, with *Trichomonas vaginalis* reporting a genomic GC content of 33% (Carlton et al., 2007). This number is similar to the 38% GC content of the *L. blattarum* assembly, but is much lower than the 45% GC content of the *L. striata* assembly. The higher GC content may be due to the presence of bacterial ectosymbionts associated with *L. striata*. The presence of oxymonad transcripts in the *L. striata* assembly is intriguing, as *Streblomastix strix*, like *L. striata*, is known to host bacterial ectosymbionts, though the two species are distantly related (Treitli et al., 2019). The functional annotations of these sequences ranged from ribosomal proteins and histone H4 proteins to adenosine deaminases. Other oxymonad sequences were annotated as transferases and transporter proteins, including oligosaccharyltransferases, oxysterol-binding proteins and ABC transporters. It is possible that the transcripts annotated as oxymonad in origin are in fact bacterial and from the ectosymbionts of the protists; alternatively, it could be a case of convergent evolution, as both species possess bacterial ectosymbionts. Of course, the oxymonad transcripts could easily be contamination from other organisms in the cockroach hindgut, as *Monocercomonoides* has not been reported to have ectosymbionts, but was nonetheless included in taxonomic matches in the LH1 assembly.

16

Both assemblies had annotations in the KOG categories of amino acid transport and metabolism; energy production and conversion; nucleotide transport and metabolism; and secondary metabolites biosynthesis, transport and catabolism. The *L. blattarum* assembly also had transcripts associated with carbohydrate transport and metabolism, as well as lipid transport and metabolism. Annotated metabolic transcripts in common with both assemblies include hydrogenosomal NADH dehydrogenase, multidrug ABC transporters and amino acid transporters. Additional transcript annotations for *L. striata* include ones for ornithine carbamoyltransferase, S-adenosylmethionine synthetase, adenosine deaminase, succinate-CoA synthetase and guanylate cyclase. Metabolic transcript annotations specific in the *L. blattarum* assembly include ones for glycosyl hydrolase, family 19; prosaposin; alcohol dehydrogenase; choline-like transporter; hydroxymethylglutanyl-CoA synthetase; and ornithine decarboxylase. Unfortunately, any significant differences truly present between the two species cannot be ascertained due to the incomplete transcriptomes and rather noisy data.

Of the bacterial metagenomic bins, the presence of *Parabacteroides*, especially in the most complete bin, is expected, as it is commonly found in the hindguts of cockroaches, inhabiting a similar role to *Bacteroides* bacteria, as both contain genes involved in carbohydrate metabolism, including glycosyl hydrolases (Dukes et al., 2023). Bacteroidales species are also common as protist ectosymbionts, as ectosymbionts of fellow parabasalids *Devescovina* and *Hoplonympha* are also bacteria in the order Bacteroidales (Noda et al., 2006). The presence of *Blattabacterium* is also unsurprising,

17

as it is a known obligate endosymbiont of nearly all cockroach species, though usually found inhabiting fat cells of fat bodies, not the hindgut (GIER, 1936).

The presence of *Endomicrobium* in the metagenomic bins is exciting, as it has previously been described as an endosymbiont of related protists, including *Trichonympha agilis* (Stingl et al., 2005). However, there have also been free-living species of *Endomicrobium* described in *Reticulitermes* termites (Zheng et al., 2016). It is unclear whether the *Endomicrobium* found in the metagenome is present as an endosymbiont of *L. striata* or as a free-living bacterium within the cockroach gut. The Hyphomicrobiales bacteria may exist in the cockroach as diazotrophs, as many bacteria in this order are able to fix nitrogen; notabtly, bacteria of this order have yet to be observed in termite hindguts.

Though a better understanding of *Lophomonas* and its bacterial neighbors in the cockroach hindgut has been gained through the transcriptome and metagenome assemblies produced here, much work still needs to be done in assembling higher quality transcriptomes. Metagenomic pathway analysis of the bacterial metagenomes would also be promising, especially after aligning the *L. striata* transcriptome against that of the *Parabacteroides* genome to determine if any transcripts in the *L. striata* assembly can be attributed to what are likely its bacterial ectosymbionts

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APPENDIX A

THE PHYLOGENETIC POSITION OF *LOPHOMONAS*

True molecular phylogenetic position of the cockroach gut commensal *Lophomonas blattarum* (Lophomonadida, Parabasalia)

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ABSTRACT
Lophomonas blattarum is a facultative commensal gut dweller of common pest cockroaches. Its cells are roughly spherical in shape with an apical tuft of ~50 flagella. Controversially, it has been implicated in human respiratory infections based on light microscopic observations of similarly shaped cells in sputum or bronchoalveolar lavage fluid. Here, we have sequenced the 18S rRNA gene of *L. blattarum* and its sole congener, *Lophomonas striata,* isolated from cockroaches. Both species branch in a fully supported clade with Trichonymphida, consistent with a previous study of *L. striata,* but not consistent with sequences from human samples attributed to *L. blattarum*.

Keywords

dyspnea; empyema; flagellate; hypermastigote; lophomoniasis; pleural trichomonosis; symbiosis; *Tetratrichomonas*, trichomonad

INTRODUCTION

Lophomonas blattarum Stein is a flagellate that lives in the guts of common pest cockroaches, specifically *Blatta orientalis* (its type host), *Periplaneta americana*, and *Blattella germanica* (Stein 1860; Semans 1943). Its local prevalence among host individuals varies from less than 10% to nearly 50%, indicating that it is not an obligate symbiont for the host (Kudo 1926a; Tsai and Cahill 1970). It is also not likely to be a parasite, because it is regularly found in seemingly healthy hosts (Martínez-Girón et al. 2017), though no studies have yet tested its health or fitness effects directly. *Lophomonas blattarum* feeds by phagocytosing yeast cells, starch grains, and microsporidian spores, but typically not bacteria (Kudo 1926a). Its abundance within the gut is influenced by the diet of its host: on a high-protein diet, *L. blattarum* will die after a few weeks, but on a high-carbohydrate diet, its numbers increase (Armer 1944). It has a very low tolerance for oxygen, even relative to the ciliates and amoebae that coinhabit the anoxic to microoxic habitats of the cockroach gut (Cleveland 1925; Hoyte 1961).

  Lophomonas blattarum cells are spherical to pyriform in shape, measure 15-30 µm in length, and bear a characteristic tuft of ~50 flagella (Fig. 1, A, B) (Kudo 1926a). These flagella are inserted along a horseshoe shape on the cell apex that is subtended by a microtubular cup-like structure (calyx or chalice) that holds the nucleus at the anterior end of the cell. At the base of the calyx, the microtubules come together to form an axial rod, the axostyle, that extends the length of the cell, sometimes forming a small bump or point on the otherwise rounded posterior pole of the cell (Kudo 1926a). One other species in this genus is known: *Lophomonas striata* Bütschli (Bütschli 1878). It shares the calyx, axostyle, and apical tuft of flagella that characterize *L. blattarum*, but its cells are longer, 30-60 µm in length, and spindle-shaped, due to their covering of ectosymbiotic bacteria (Fig. 1, C-E) (Kudo 1926b; Beams et al. 1960). This covering prevents *L. striata* from ingesting large particles, though it may ingest and digest its ectosymbionts, as has been shown for the similarly bacteria-coated termite symbionts *Hoplonympha* and *Streblomastix* (Beams et al. 1960; Leander and Keeling 2004; Noda et al. 2006).

  Because of its many flagella, *Lophomonas* was included in the traditional order Hypermastigida (Grassi and Foà 1911). It is the only hypermastigote to inhabit a host other than termites or their sister lineage, the wood-feeding cockroach *Cryptocercus* (Brugerolle and Lee 2000). The Hypermastigida is now known to be polyphyletic, and its former members are distributed across three orders: Trichonymphida, Spirotrichonymphida, and Cristamonadida (Čepička et al. 2010, 2017). *Lophomonas* was initially included in the Cristamonadida because its apical tuft of flagella makes it superficially similar to the joeniids, which are included in Cristamonadida based on similarities in basal body and microtubule arrangement between *Joenina* and *Devescovina* (Brugerolle and Patterson 2001). However, molecular analyses later placed *L. striata* sister to Trichonymphida, indicating that apical flagellar tufts arose independently in *Lophomonas* and Cristamonadida (Gile and Slamovits 2012). Today *Lophomonas* is the only genus in its order Lophomonadida (Cavalier-Smith 2013; Čepička et al. 2017).

Lophomonas blattarum has been implicated in human bronchopulmonary infection, with the first case reported in China in 1992 (Guozhong 2008). Since then, many more cases have been reported, at first mostly in China, but more recently from Mexico, Venezuela, Peru, Spain, Turkey, and Iran (Martínez-Girón and Doganci 2010; Zerpa et al. 2010; Kilimcioglu et al. 2014; Fakhar et al. 2019; Elena Villagrán-Herrera et al. 2020). Diagnosis of infection is typically made by observing cells with an apical tuft of flagella in samples of sputum or bronchoalveolar lavage fluid (Li and Gao 2016). However, ciliated epithelial cells, and fragments of them called ciliocytophthoria, look superficially like *L. blattarum* under the light microscope (Gelardi and Ciprandi 2019), leading some authors to question the identification of *Lophomonas* in medical case studies (Martínez-Girón and van Woerden 2014; Li and Gao 2016). Recent studies claim to have confirmed the diagnosis of *L. blattarum* infection by molecular methods (Fakhar et al. 2019, 2021; Mokhtarian et al. 2022), but the short fragments of the 18S rRNA gene produced in these studies are highly similar to the Trichomonadida *Pentatrichomonas* or *Tetratrichomonas,* which are evolutionarily distant from Lophomonadida (Čepička et al. 2017).

In this study we identified true *L. blattarum* in its type host and obtained 18S rRNA gene sequences. *L. blattarum* branches sister to *L. striata,* as expected from their morphology and ultrastructure, but without statistical support. Our results indicate that previously deposited *'L. blattarum'* sequences in GenBank are misidentified.

METHODS
Lophomonas isolation and culture
Blatta orientalis (Oriental cockroach) were captured in Tempe, Arizona (33.4208, -111.9328) in October, 2018, and Fayetteville, Arkansas (36.06518, -94.165275) in June, 2012.

Cockroaches collected in Arizona were dissected and their hindgut contents suspended in Ringer's solution (8.5 g NaCl, 0.20 g KCl, 0.20 g $CaCl_2$, 0.10 g $NaHCO_3$ per liter, HiMedia Laboratories). Individual *L. blattarum* and *L. striata* cells were identified by their clearly distinct morphologies and isolated by drawn-glass micropipette on an AxioVert inverted microscope and photographed with an Axiocam 105 color camera (Zeiss). Occasionally both species were observed in the same host individual. Each single cell was washed twice in fresh Ringer's buffer and ejected into a 0.2 ml tube containing 50 µl acetone and stored at -20 ºC until processing.

Blatta orientalis collected in Arkansas were dissected and macerated in sterile insect buffered saline (9.0 g NaCl, 0.2 g KCl, 0.27 g $CaCl_2$, 4.0 g glucose per liter, buffered to pH 7.2 with $NaHCO_3$), then inoculated into a variety of biphasic media. *Lophomonas blattarum* grew best in 15 ml polypropylene conical tubes containing a 3 ml solid slant of inspissated horse serum (85 °C for 1.5-2 h) with a 3 ml liquid overlay of ATCC 802 medium, plus 200 µl of heat-inactivated donor horse serum and 2 drops of Fleishman's yeast (50 mg/ml in dH_2O stock solution). Cultures were maintained at room temperature in sealed tubes and passed into fresh medium every 4-7 days. Cysts formed in cultures older than 5 days. To separate *L. blattarum* from the abundant yeast in the medium, a pool of ~20 *L. blattarum* cells was manually isolated by drawn-glass micropipette using a Zeiss Axiovert 200 M inverted microscope. Cells were washed in

medium U (Trager 1934) and ejected into a 1.5 ml tube for immediate DNA extraction using the MasterPure DNA Purification Kit (Epicentre, Madison, Wisconsin, USA).

Lophomonas cDNA synthesis and amplification

Whole transcriptome amplification was carried out using the SmartSeq2 protocol using poly-dT primers for first strand synthesis and a template-switching oligo to enable sequencing of the 5' end of each transcript (Picelli et al. 2014). Each 0.2 ml tube holding a single cell was incubated at 65 ºC until the acetone evaporated (~5 min) and the SmartSeq2 protocol was carried out directly on dry cells.

The 18S rRNA gene was amplified from the DNA and the two cDNA templates using Econotaq Plus Green PCR Master Mix (Lucigen) and the universal eukaryotic primers PF1 5'- TGCGCTACCTGGTTGATCCTGCC-3' and FAD4 5'-TGATCCTTCTGCAGGTTCACCTAC-3', as described previously (Gile and Slamovits 2012). PCR products were visually inspected on an agarose gel, and bands of the expected size, 1500 bp, were purified using the NucleoSpin Gel and PCR Clean-Up kit (Macherey-Nagel) and cloned using the TOPO® TA Cloning® Kit with One Shot® TOP10 chemically competent *E. coli* (ThermoFisher Scientific). Multiple clones from each PCR reaction were sequenced completely on both strands and found to be nearly identical in sequence (99%, 2-15 nucleotides different in each pairwise comparison). The nucleotide differences among clones are genuine as no ambiguities were seen within each individual bi-directionally sequenced clone. Intragenomic 18S sequence variability is expected in Parabasalia (Taerum et al. 2018; Noda et al. 2023). A single, representative sequence from each template was included in phylogenetic analyses and submitted to NCBI GenBank under accessions OP903921 (*L. blattarum* from Arkansas), OP903922 (*L. striata* cell LH1 from Arizona), and OP903923 (*L. blattarum* cell OT2 from Arizona).

Phylogenetic Analysis

New clone sequences were trimmed of vectors and assembled using Geneious R9 (Kearse et al. 2012). New and previously published sequences were aligned using MAFFT v. 7.205 (Katoh and Standley 2013) with iterative refinement using the L-INS-I option. Ambiguously aligned sites were removed using TrimAl v1.2 under the "automated 1" option (Capella-Gutiérrez et al. 2009). The final alignment had 75 sequences and 1133 sites.

Maximum likelihood (ML) and Bayesian phylogenetic analyses were performed using IQ-TREE v. 1.6.12 (Nguyen et al. 2015; Trifinopoulos et al. 2016) and MrBayes v. 3.2.6 (Ronquist et al. 2012) respectively. ML analyses used the GTR+F+R5 model as specified by ModelFinder implemented in IQ-TREE (Kalyaanamoorthy et al. 2017). Support for nodes was assessed from 1,000 ultrafast bootstrap replicates (Hoang et al. 2018). Bayesian analyses were carried out under the GTR model with four evolutionary rate categories approximated by a gamma distribution. Two independent chains, sampled once every 100 generations, were run until they converged (the average standard deviation of partition frequency values between the chains dropped below 0.01). Convergence was reached after 420,000 generations. The first 25% of trees were then discarded as burn-in and majority rule consensus trees were computed from the remaining 6302 trees from both runs.

RESULTS & DISCUSSION

We obtained 3 new *Lophomonas* 18S rRNA gene sequences: one *L. blattarum* 18S from a culture established in Arkansas (OP903921), one *L. blattarum* 18S from a single cell isolated in Arizona (OP903923), and one *L. striata* 18S, also from a single cell isolated in Arizona (OP903922) (Fig. 1, A-E). The two new *L. blattarum* sequences were highly similar to one another (99% identical, with 17 nucleotides different) despite coming from cockroaches in Arizona and Arkansas, roughly 1,880 km apart. Likewise, the new 18S sequence from *L. striata* was 98.5% identical (22 nucleotides different) to the previously published *L. striata* sequence obtained from cockroaches in Halifax, Canada, more than 5,000 km distant and from different host species: *Periplaneta americana* in Halifax, and *Blatta orientalis* in Arizona. This confirms morphological reports that *L. striata* inhabits multiple host species (Stein 1860; Semans 1943).

In our phylogenetic analyses (Fig. 1, F) all new sequences branched in a robustly supported clade with Trichonymphida, consistent with the previous analysis of *L. striata* (Gile and Slamovits 2012). The expected sister relationship between *L. blattarum* and *L. striata* was recovered in both ML and Bayesian analyses, but with very low statistical support (75% ultrafast bootstrap, 0.72 posterior probability). Their shared branch is very short, in contrast to their long individual branches, suggesting a relatively ancient divergence.

Several sequences attributed to *L. blattarum*, allegedly originating from humans, have been deposited in NCBI GenBank in the past decade. These 18S sequences do not originate from either *L. blattarum* or *L. striata* (Fig 1F), and barring the unlikely polyphyly of the genus *Lophomonas*, they likely come from other, unrelated parabasalids. JX020505 was deposited in 2012 under the title "First report of bronchopulmonary infection caused by *Lophomonas blattarum* in Thailand". No article is linked to the submission, so it is unclear what type of sample was analyzed or what methods were used. The sequence is 99% identical to previously published *Pentatrichomonas hominis* sequences. The other 17 sequences (MN243135-36, MZ093069-79, OL477421-23, and OL477431) were obtained by PCR from human bronchoalveolar lavage fluid or nasal secretions using primers designed to match the sequence from Thailand and *L. striata* (Fakhar et al. 2019, 2021; Mokhtarian et al. 2022). In our phylogenetic analyses, all of these sequences robustly branch within Trichomonadida (Fig. 1, F), either sister to *P. hominis* or with *Tetratrichomonas*.

Tetratrichomonas are known inhabitants of human lungs (Mantini et al. 2009; Lin et al. 2019). Several strains isolated from humans have been characterized using both morphological and molecular methods (Kutisova et al. 2005). One phylotype, so far detected only from humans, was named *Tetratrichomonas empyemagena* (Lopez-Escamilla et al. 2013). Much of the diversity of *Tetratrichomonas* is not captured in our 18S rRNA gene phylogeny because many strains have been characterized by sequencing the ITS region only, including *T. empyemagena* (Lopez-Escamilla et al. 2013). *Pentatrichomonas hominis* is normally found in human intestines, not lungs, but it has been detected in lung samples in two case reports (Leterrier et al. 2012; Yao and Ketzis 2018; Duboucher 2021).

In conclusion, our 18S data indicate that *L. blattarum* is closely related to *L. striata,* as expected, though their sisterhood is not strongly supported with this single-gene analysis. Nevertheless, a fully supported node links both *Lophomonas* species with Trichonymphida. These data further indicate that no true *L. blattarum* sequences have yet been published from human lung samples. It remains to be seen whether this is due to a failure to amplify *Lophomonas* sequences with primers designed from a *P. hominis*-like sequence or simply because no *Lophomonas* was present in the samples.

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FIGURE LEGEND

Figure 1. *Lophomonas blattarum* morphology and phylogenetic position. **A-E.** Differential interference contrast light micrographs of *Lophomonas* cells isolated in this study. **A.** *L. blattarum* cell from culture. Ingested yeast cells fill the cytoplasm (y). **B.** *L. blattarum* cell OT2, isolated from *Blatta orientalis* in Arizona. The nucleus (n) in its calyx is visible within the cell directly beneath the flagellar bundle (fb). **C-E**. *L. striata* cell LH1 in three optical sections collectively showing the posterior cell pole, fusiform ectosymbiotic bacteria, and apical nucleus (n) and flagellar bundle (fb). All scale bars 20 μm. **F.** Maximum likelihood unrooted phylogeny of 18S rRNA gene sequences from all 9 orders of Parabasalia. New sequences obtained in this study are indicated by bold text. Statistical support at nodes is shown where >50% for ultrafast bootstrap replicates and >0.8 for posterior probability. Full statistical support is indicated by filled circles; moderate to strong statistical support for nodes (at least 70% BS and at least 0.95 PP) is indicated by open circles. The dashed line indicates that the branch for *Eucomonympha imla* has been reduced by half. Grey boxes with rounded corners at bottom indicate sequences derived from human samples that were deposited in GenBank under the organism name '*Lophomonas*'.

APPENDIX B

PERMISSION TO PREVIOUSLY PUBLISHED ARTICLE

All co-authors of the article as it appears in Appendix A have granted permission for the use of the paper in this thesis.