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Single-Cell Transcriptomes and Phylogenomic Analysis of Uncultivated Oxymonads

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ABSTRACT

The oxymonads are anaerobic protist symbionts of animals with spectacular morphological complexity and variability, but because only a handful of species have been cultured, very little data are available for the group. This includes molecular data, with only a couple of species having sequenced genomes or transcriptomes. Oxymonads are divided into five families in a classification that has remained more or less unchanged for 35 years, but the relationships of these families are unknown due to a lack of molecular data from three of them. Here, we used single cell transcriptomics on six species from five genera (*Oxymonas, Streblomastix*, *Pyrsonympha*, *Saccinobaculus*, and *Laeohelix*) to carry out the first phylogenomic analysis of oxymonads that includes all five families. We find a major division between Polymastigidae and Streblomastigidae on one side, and Saccinobaculidae, Oxymonadidae, and Pyrsonymphidae on the other, with Oxymonadidae and Pyrsonymphidae branching as sisters in the latter clade. The phylogenomic tree largely confirms the SSU rRNA tree but provides stronger support at all nodes and suggests a different root. This tree represents a strong starting point to reconstruct the evolution of oxymonads many unusual morphological and molecular characters and to test whether new lineages might represent new families.

1 | Introduction

Oxymonads are a diverse lineage of protists that have evolutionarily divergent characteristics in morphology, genomes, and metabolism. The group remains poorly studied, and relatively few species are available in culture (Karnkowska et al. 2016, Treitli et al. 2018, Novák et al. 2023), all of which belong to one of the five oxymonad families (Polymastigidae). Members of this group are difficult to culture because they are all anaerobes, live in association with animals to some degree, and many have been shown to hold additional layers of symbiotic relationships with specific communities of bacteria and archaea that live inside their cells or on their surface, making their cultivation requirements even more difficult (Hampl 2017). The majority of the considerable morphological diversity of the group is found in one type of symbiotic system, the hindgut of termites and wood-eating cockroaches (Yamin 1979). These hindguts are well-studied models of symbiosis, as the animal host cannot digest the lignocellulose that makes up most of its diet, and depends on complex symbiotic communities of microbes to digest it for them: protists, bacteria, and archaea in the case of the wood-eating cockroaches and all the families of "lower termites", and bacteria, archaea, and sometimes fungi in the case of the "higher" termites, Termitidae (Yamin 1979). Most attention has been focused on the abundant and diverse parabasalian protists in these systems, but *Cryptocercus* and many termites also have oxymonad symbionts. The oxymonads are distinguished by a specialized set of morphological features, and their monophyly was subsequently supported by molecular data; however, like the parabasalians in the same communities,

oxymonads have evolved a large range of sizes and an amazing degree of morphological diversity (Hampl 2017). Known oxymonads range from tiny and relatively simple tetra-flagellates to cells over 100 μm in size, with a wide range of complex structural adaptations, including multiple nuclei, complex symmetry with adherent or free flagella, or highly motile intracellular cytoskeletal structures like the "snake in a bag" axostyle—an intracellular bundle of microtubules that traverse the entire length of the cell (McIntosh et al. 1973)—in *Saccinobaculus* (Cleveland et al. 1934).

Due to difficulties in culturing oxymonads, there are genomic and/or transcriptomic data from only two oxymonad families: *Monocercomonas* and *Blattamonas* (Polymastigidae) and *Streblomastix* Streblomastigidae (Karnkowska et al. 2016; Treitli et al. 2018, 2019; Novák et al. 2023). These data were key to revealing their unique status as eukaryotes that have completely lost mitochondria, and their biochemical relationships with surface symbionts, respectively. But with genomic data from only two lineages, we cannot reconstruct the evolution of the many other interesting characters in the group, since we do not have a robust phylogenetic framework for comparative analyses. A few studies have attempted to infer relationships through single gene trees (Moriya et al. 2003; Heiss and Keeling 2006; Treitli et al. 2018; Radek et al. 2019), but these studies are based on few genes and, while topologies are relatively consistent, support remains low. Addressing the relationships between these five oxymonad families will require a well-supported phylogenomic tree with representatives of most of the diversity. Indeed, the lack of progress with oxymonads is reflected in the relative stasis of their taxonomy. Where other protist groups have seen major revisions with the application of molecular and especially genomic data to phylogenies, oxymonad taxonomy has gone virtually unchanged since the classical treatment by Brugerolle in 1990 (Brugerolle and Lee 2000) and an updated treatment by Hampl (Hampl 2017). In both schemes, oxymonad diversity is represented by five families: Polymastigidae, Saccinobaculidae, Pyrsonymphidae, Streblomastigidae, and Oxymonadidae, with the major difference in 35 years being two genera, *Opisthomitus* and *Oxynympha*, that are not clearly associated with any known family (Hollande and Carruette-Valentin 1970; Radek et al. 2014, 2019). With genomic data from only two of the five families, it is impossible to be certain of how well the current classification really represents the diversity: known species may not fall into the groups that they have been proposed to belong to, and yet-to-be-described species might fall outside these groups. Nevertheless, this five-family scheme is currently the best starting point we have to explore the diversity of oxymonads; therefore, we have sequenced single cell transcriptomes from representatives of type genera from all of the missing families, and carried out phylogenomic analyses to determine their branching order. We combined microscopic observations with single-cell transcriptomics from manually isolated cells representing six species from five genera, and four of the families. Together with existing genomic data, the addition of these new transcriptomes works to 'fill in the blanks' for all five currently recognized families. The resulting tree shows well-supported relationships between several subgroups, providing the first phylogenomic framework for oxymonad diversity.

2 | Materials and Methods

2.1 | Host Collection and Symbiont Identification

Neotermes costaseca was collected in Chacra y Mar, Perú in June 2014, and *Oxymonas* was collected from this specimen. *Reticulitermes hesperus* was collected on Galiano Island, Canada in July 2014, and *Pyrsonympha* and *Laeohelix* were collected from this specimen. *Zootermopsis angusticollis* was collected in Vancouver, Canada in August 2014, and *Streblomastix* was collected from this specimen. The wood-eating cockroach, *Cryptocercus punctulatus*, was collected in North Carolina, USA in June 2015, and *Saccinobaculus* was collected from this specimen. Symbiotic oxymonads were identified by microscopy as fitting the overall description of genera already known to be in that host species, or in the case of *N. costaseca* to be in closely related species that had been investigated previously, as this is a relatively new species whose symbionts have not been formally described (Scheffrahn 2018).

2.2 | Single-Cell Transcriptomics

Hindgut contents were observed on a Leica DMIL microscope, and cells of interest were isolated with a microcapillary pipet and washed with Trager's Medium U a minimum of three times before being isolated in individual tubes with lysis buffer (Picelli et al. 2014). For each sample, cDNA was extracted according to the Smart-seq2 protocol (Kolisko et al. 2014; Picelli et al. 2014). Illumina Nextera Flex or XT library preparations were performed by the Sequencing and Bioinformatics Consortium, University of British Columbia, and sequenced on Illumina NextSeq or MiSeq platforms. Resulting forward and reverse raw reads were trimmed in Cutadapt (Martin 2011) before assembly with rnaSPAdes v3.15.1 (Bankevich et al. 2012). Nucleotide assemblies were converted to amino acid sequences using TransDecoder v5.5.0 for open reading frame identification and annotation (Haas et al. 2013) and BLASTP (Poux et al. 2017) was used to search against the UniProt database with an *e*-value threshold of ≤1 e10-5. Even though all cDNA was amplified independently on different dates, the possibility of cross-contamination between individual datasets still remained. In order to reduce this, WinstonCleaner (https://github.com/kolecko007/WinstonCleaner) was used to identify and remove potential cross-contaminating sequences, using the software's default parameters. Both original full assemblies, cleaned assemblies and files containing specific threshold settings for each dataset pair are deposited in Figshare (DOI: 10.6084/m9.figshare.31148839). Transcriptomes are available in GenBank under accession PRJNA1433164.

2.3 | Inference of Genetic Code

To determine genetic codes, the transcripts were searched against the BLASTN database to identify highly expressed genes with broad coverage (alpha- and beta-tubulin), and aligned using the -linsi option of mafft v.7.520 (Katoh and Standley 2013) with published sequences with known genetic codes. Alignments were then opened and manually inspected using Geneious' nucleotide translation function to determine

which code would result in the appropriate protein translation, which was tested via BLASTP (Poux et al. 2017). Phylogenetic analysis (Figure S2) was done to confirm the origin of the genes and revealed contamination from an unidentified lineage that used a noncanonical genetic code where TAR encodes glutamine in the *Saccinobaculus* libraries, while sequences attributed to *Saccinobaculus* itself used the universal code (Figure S2). The Proteome prediction for the multigene datasets used TransDecoder v5.5.0.; in the case of *Streblomastix* the -G Hexamita option was used.

2.4 | Phylogenetic Analysis

One SSU rRNA sequence was extracted from each of the six new transcriptomes and aligned with 150 orthologues from other oxymonads available from GenBank as well as sequences from *Trimastix* and *Paratrimastix*, nonoxymonad metamonads, as outgroups. The unaligned and untrimmed dataset has been deposited to Figshare repository (DOI: 10.6084/m9.figshare.31148839). The alignment was performed using the accurate -linsi option of mafft v.7.520 (Katoh and Standley 2013), and the character matrix was only trimmed at the ends, removing columns with 50% or more missing data; the final analyses used 158 sequences and 5332 sites. The maximum likelihood inference was performed on IQ-TREE v. 1.6.12 (Nguyen et al. 2015) using the TIM3 + F + I + G4 model, selected as the best fitting according to the BIC parameter. 1000 standard nonparametric bootstrap pseudoreplices were run for statistical support. For the multigene dataset, the six new proteomes predicted from the single-cell transcriptomes were input into PhyloFisher v1.2.15 [55] as a custom dataset. Each of the 240 single-gene trees generated was manually checked as part of the PhyloFisher pipeline to identify contaminants, paralogous, or otherwise aberrant sequences, which were omitted from the final dataset. The final dataset consisted of 194 genes (51,145 amino acid sites) from 18 metamonad taxa (including nine nonoxymonad metamonads acting as an outgroup). Taxa and genes were chosen to maximize coverage for the oxymonads, while retaining breadth across the tree. A maximum-likelihood phylogeny of the final dataset was generated using IQ-TREE2 v. 2.2.0 under the LG + C60 + G model with 1000 ultrafast bootstraps. Bayesian analysis on the same dataset used PhyloBayes-MPI v 4.1c and the CAT-GTR model. Four chains were run in parallel, with a sampling frequency every five trees until each chain surpassed 8000 iterations. While all four trees resulted in identical topology, only two of the four chains converged with a max difference under 0.03 (maxdiff = 0.006). Clade frequency over 50% from the four parallel runs was used to generate a posterior consensus. All corresponding orthologs and paralogs used in Phylofisher analysis are deposited in the Figshare repository (DOI: 10.6084/m9.figshare.31148839).

3 | Results and Discussion

3.1 | Single-Cell Transcriptomes From Oxymonads

Oxymonads were isolated from wood-eating insect hosts in which they were previously described to be part of the symbiotic community (Yamin 1979), and specifically from three different "lower" termites, *Neotermes costaseca*, *Reticulitermes hesperus*, and *Zootermopsis angusticollis*, as well as the wood-eating cockroach, *Cryptocercus punctulatus*. Oxymonad symbionts were identified based on their morphological similarity to described species known from the same hosts, and 4–10 cells were isolated from each of five genera: *Oxymonas*, *Streblomastix*, *Pyrsonympha*, *Laeohelix*, and *Saccinobaculus*, which represent a wide range of oxymonad morphologies (Figure 1). Several of the single-cell transcriptomes were consistently found to have a higher-than-normal level of contamination compared with other single-cell transcriptomes (e.g., see Mathur et al. 2021; Cooney et al. 2023). For this reason, in instances where we had multiple transcriptomes from the same species, we kept only the representative with the lowest amount of contamination. Ultimately, we focused specifically on orthology assignments for genes in the phylogenomic dataset, where such assignments can be made with a higher degree of confidence. We did not attempt to address specific functional questions with genes that have been sampled less deeply or that do not have well-studied phylogenies, as these would require substantial contamination detection that would be difficult with the available data. Future studies on specific pathways that use these data will likely require corroboration from several oxymonads.

Decontamination and filtering for phylogenomics yielded six transcriptomes from 10 single cells (in the case of *Saccinobaculus doroaxostylus* three transcriptomes were merged and in the case of *Streblomastix strix* two were) with coverage between 51% and 94.3% of a 194-gene dataset ultimately used to infer the trees. From each transcriptome, we also specifically identified the SSU rRNA sequence and used these to construct a phylogeny (Figure S1) to confirm that the dominant SSU rRNA corresponded to the expected species based on the morphology of the collected cell or to determine the genus if species-level identification was not possible. All isolated cells were found to fall into the expected position in the tree (Figure S1). In the case of pyrsonymphids, a species-level identification was not possible with the microscope used for single-cell isolation since the distinguishing characters are not visible at that resolution: the host was proposed to harbor at least three different species, and diagnostic molecular markers are not available to distinguish them (Dacks et al. 2001; Coots et al. 2025). The two isolated cells were found to fall into distinct positions within the Pyrsonymphidae in the SSU rRNA tree, one belonging to the genus *Pyrsonympha* and the other to the genus *Laeohelix*. Neither exactly match any described species from this host (Figure S1), so we refer to them as *Pyrsonympha* sp. and *Laeohelix* sp. For *Saccinobaculus*, we sampled multiple cells of two morphologically distinguishable types, some corresponding to the *Saccinobaculus ambloaxostylus* morphology and some corresponding to the *Saccinobaculus doroaxostylus* morphology (Cleveland et al. 1939). Although these are distinguishable from one another, it is not clear that each morphology corresponds to a single species (de Koning et al. 2008). Transcriptome data from all three cells matching the *S. doroaxostylus* morphology proved to be highly similar and related to *S. doroaxostylus* in SSU rRNA trees (Figure S1). Other genes from these cells also shared a high degree of identity, and thus these transcriptomes were merged for phylogenomic analyses. Transcriptomes from cells matching the description of *S. ambloaxostylus* proved more

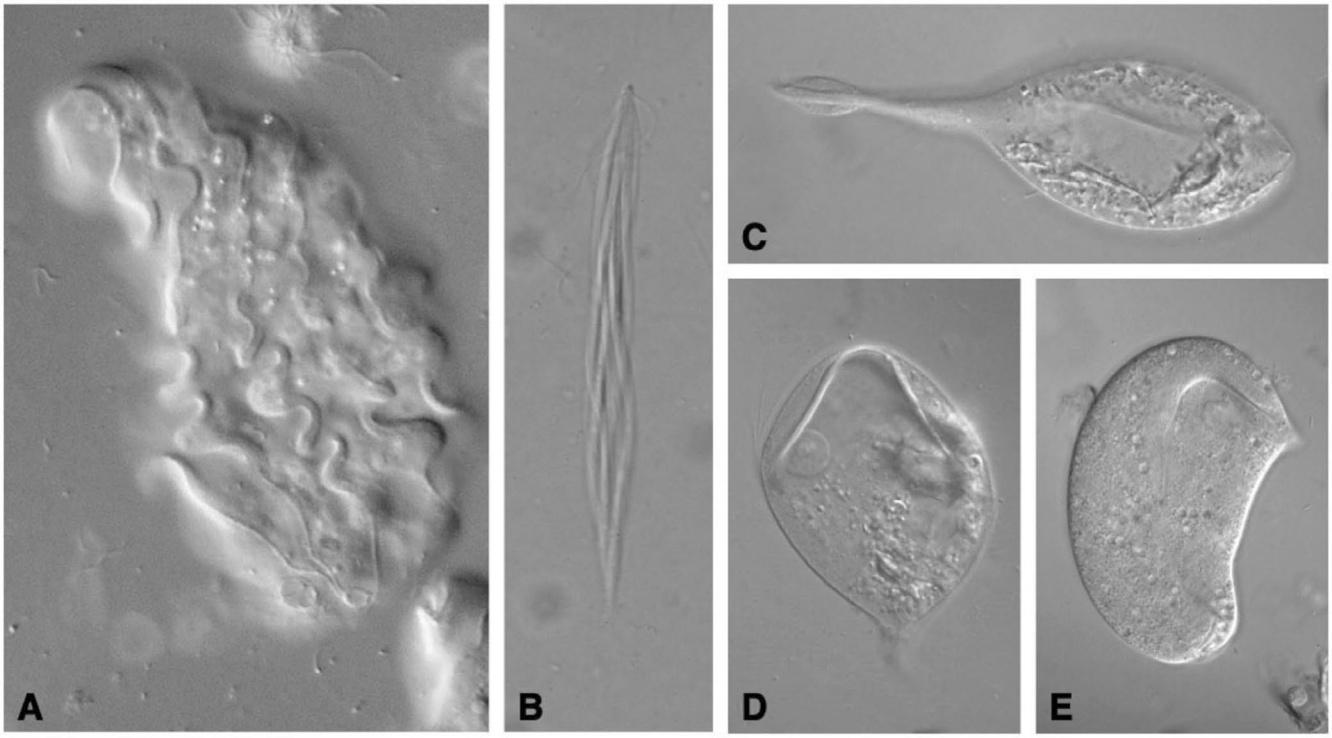

FIGURE 1 | Representative organisms from oxymonad genera, showing their high degree of morphological diversity. (A) *Pyrsonympha*, a large (100–150 μm) member of the Pyrsonymphidae with a characteristically twisted cell body and long flagella wrapped around the surface along distinctive helix-shaped ridges; (B) *Streblomastix*, a member of the Streblomastigidae, needle-shaped (100–300 μm long) and with prominent, regularly spaced ridges that provide a larger surface for the ectosymbiotic bacteria that cover its body; (C). *Oxymonas*, a member of the Oxymonadidae with an extended anterior holdfast used to attach to the host's gut epithelium (90–130 μm long excluding the retractable holdfast); (D) *Saccinobaculus ambloaxostylus*, a member of the Saccinobaculidae; and (E) *Saccinobaculus doroaxostylus*, a member of the Saccinobaculidae; both *Saccinobaculus* species (100–200 μm long) display a prominent, thick, but extremely flexible and mobile microtubular structure, the axostyle, whose movement gave the genus its name (the "snake-in-the-bag").

difficult, as they were highly contaminated and most data sets were discarded. Even the dataset with the highest coverage for the intended organism was still found to have multiple SSU rRNAs, but one phylotype had much higher coverage and was closely related to existing *S. ambloaxostylus* sequences, while the coverage of the other phylotype was lower by at least a factor of five (most by a factor of > 100), and all fell into a paraphyletic cluster of sequences also related to *S. ambloaxostylus*. It is impossible to discern from this if there were multiple related species of *Saccinobaculus* in these libraries or if the genomes of *Saccinobaculus* cells are especially complex in gene families. For protein-coding genes, we typically observed only a single sequence, so one dataset was retained to represent *S. ambloaxostylus*. For *Oxymonas* sp. and *Streblomastix strix*, a single phylotype was found that fell in the expected position in the tree: related to another *Oxymonas* from another *Neotermes* host and to *S. strix* from *Z. angusticollis*, respectively.

3.2 | Phylogenomic Relationships Between Five Families of Oxymonads

The phylogenomic tree (Figure 2) unsurprisingly recovers the monophyly of oxymonads with full support. More interestingly, however, it shows highly supported relationships between all five formally recognized subgroups (Brugerolle and Lee 2000; Hampl 2017). The root of the tree divides oxymonads into two major subdivisions, separated with strong bootstrap support (100% and 99%). One is composed of the Polymastigidae and Streblomastigidae, whereas the other is composed of the Saccinobaculidae, Pyrsonymphidae, and Oxymonadidae, the relative relationships of which are also fully supported, with the latter two families being sister lineages.

The topology of the phylogenomic tree is very similar to the SSU rRNA topology, with two significant differences. First, the root of the oxymonads in the SSU rRNA trees typically places the Streblomastigidae as the sister of all other oxymonads (albeit with low statistical support). In the phylogenomic tree, the root falls instead between the Streblomastigidae + Polymastigidae and the other groups, a result strongly supported by bootstrap values. The other major difference is the high levels of support for the overall branching order of the groups. Even lineages recovered in both analyses (e.g., the large group uniting Saccinobaculidae, Oxymonadidae, and Pyrsonymphidae) are unsupported in SSU rRNA analyses but well supported in phylogenomic trees, providing an important confirmation for the overall structure of the tree. Notably, both trees leave the monophyly of Polymastigidae as dubious, especially where it concerns the position of *Blattamonas*. In short, the topology of SSU and phylogenomic tree are highly similar, except for the root, but every node uniting two or

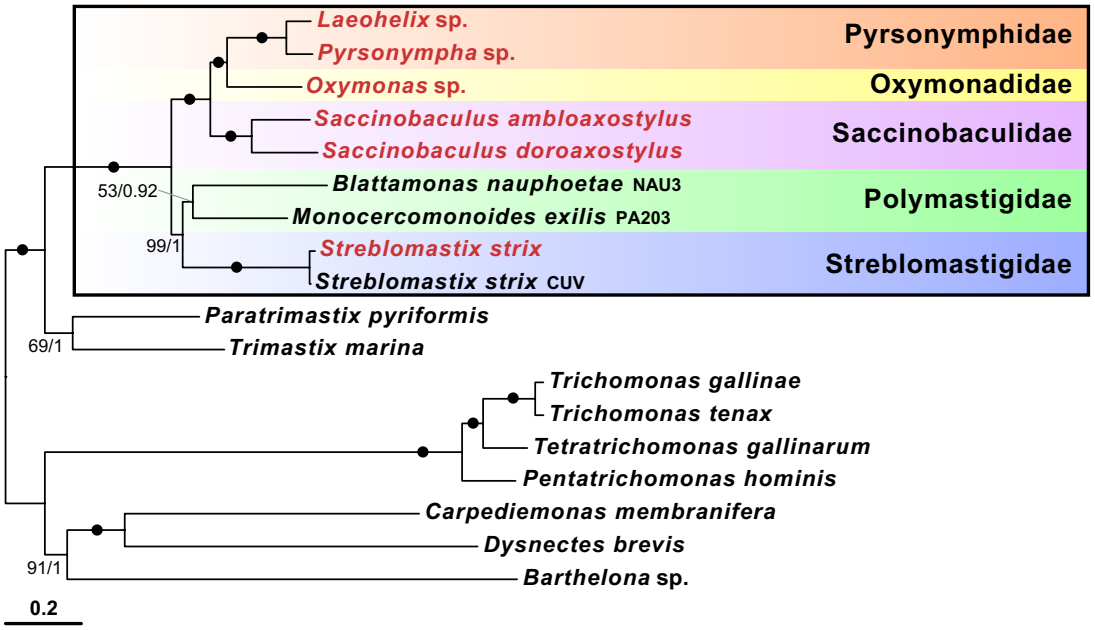

FIGURE 2 | Maximum Likelihood phylogenomic tree of oxymonads based on 194 genes and 51,145 amino acid positions. Oxymonads are boxed at the top, and outgroups consist of closely related metamonads. The five known families of oxymonads are labeled and colored. Numbers at nodes indicate maximum-likelihood nonparametric bootstrap support and Bayesian posterior probabilities.

more families is unsupported in the SSU tree and strongly supported in the phylogenomic tree.

Based on the SSU rRNA tree, two genera that remain unclassified can be predicted to fall in specific positions in the phylogenomic tree when data will become available: *Opisthomitus* is strongly supported as the sister to Pyrsonymphidae, as noted previously (Radek et al. 2014), and *Oxynympha* is closely related to a clade of unidentified environmental sequences simply referred to as "Oxymonadida sp."; together, they appear to be the sister group of the Saccinobaculidae (Radek et al. 2019). It is also noteworthy that *Trimastix* and *Paratrimastix* form a monophyletic group sister to oxymonads in our analyses, which conflicts with previously published topologies of the group (Zhang et al. 2015; Leger et al. 2017; Wiśniewska et al. 2024). However, this part of the tree is not well supported in any analysis, so which possibility is more likely is currently unclear.

3.3 | A Cryptic Oxymonad and the Distribution of a Noncanonical Genetic Code

One interesting characteristic of oxymonads is their genetic code. *Streblomastix* was previously shown to have evolved a noncanonical genetic code where TAR encodes glutamine (Keeling and Leander 2003). Other families use or are presumed to use the universal code, but in an early analysis of the symbiotic community of the cockroach *Cryptocercus*, gene sequences were assigned to an unidentified oxymonad that was not obviously related to *Streblomastix*, but nevertheless used the same noncanonical code (de Koning et al. 2008). We detected that same lineage here as well: in *Saccinobaculus* transcriptomes we identified low abundance variants that appear to be a contamination derived from an oxymonad with TAR codons at glutamine positions (see Materials and Methods). To confirm this, we analyzed particularly highly expressed genes with relatively well-supported phylogenies, specifically alpha- and beta-tubulin. Both tubulin phylogenies showed that homologues with the noncanonical codons branch in a position distinct from "true" *Saccinobaculus* homologs and are instead closely related to environmental clones from *Cryptocercus* that also have the same code (Figure S2). The most plausible source of these genes is one of the three understudied oxymonad genera purported to live in *Cryptocercus* with no molecular data definitively linked to them: *Notila*, *Paranotila*, or the small flagellate described by Cleveland as *Monocercomonoides globus* (Cleveland et al. 1934). If this is *M. globus*, the genus designation is most likely incorrect since they share the same genetic code as *S. strix* and are probably more closely related to Streblomastigidae than to Polymastigidae. The homologues with noncanonical codons are not clearly monophyletic in the tubulin trees (Figure S2), but the trees are not well supported and are unrooted: we predict they will prove to be monophyletic when fully characterized.

3.4 | Concluding Remarks

The data described here allow for the reconstruction of a well-resolved phylogenomic tree representing all the currently known families of Oxymonada, which is a nice step forward for such a poorly studied group. However, we still have much to learn not only about the biology of these organisms and their diversity beyond the gross morphology of the cells, but also their phylogenetic diversity and the detailed structure of the tree. A better picture of some aspects of their functional diversity could be gleaned from genomic data, for example, an examination of metabolism diversity, and tests of whether the absence of mitochondria applies to the whole group (we saw no evidence for mitochondrion-targeted proteins, but considering the high level of contamination, we cannot draw definitive conclusions).

Advancing our understanding of the oxymonad phylogeny will also require genomic data from a greater diversity of oxymonads for two other reasons. First, the known diversity of the five families is quite different: Streblomastigidae, for instance, is a small group with only one described species, while Polymastigidae includes six known genera (Hampl 2017; Treitli et al. 2018), and two Pyrsonymphidae genera have recently been split into several (Coots et al. 2025)—keeping in mind that the diversity of all five families is certainly underrepresented by such numbers. To test the monophyly of the families, it would be important to sample more genera within each. Second, it is equally or more important to sample other genera that might fall outside these groups and represent new families. *Oxynympha* and *Opisthomitus* (Hollande and Carruette-Valentin 1970; Radek et al. 2014, 2019) are already known possibilities: to test whether they fall into the predicted positions in the phylogenomic tree or if they affect the placement of the root, they need to be reisolated and added to a phylogenomic analysis. The cryptic data from *Cryptocercus* also suggest an interesting avenue to study: their genetic code and position in the alpha-tubulin trees suggests more than one poorly studied taxon whose position in the tree would affect how we might interpret the evolution of the code in the lineage, a good example of how a well-sampled and well-supported tree can help interpret character evolution.

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Data Availability Statement

The data that support the findings of this study are openly available in GenBank at https://www.ncbi.nlm.nih.gov/genbank/.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** Maximum likelihood phylogenetic tree of oxymonads based on publicly available small subunit ribosomal RNA (SSU rRNA) genes and transcripts. Genera are indicated to the right, and families are indicated by colored boxes. SSU rRNA from single-cell transcriptomes are shown in red with red arrows. Numbers at nodes indicate standard nonparametric bootstrap support (values below 70% are not shown). The scale bar represents 0.1 substitutions. **Figure S2:** Unrooted maximum-likelihood phylogeny of oxymonad alpha-tubulin (top) and beta-tubulin (bottom) comparing genes from novel transcriptomes with orthologs from publicly available data. Numbers at nodes indicate ultrafast bootstrap support (values below 70% not shown). Tubulins from transcriptomes generated in this study are in bold, and genes from *Saccinobaculus* libraries are in color, blue for true *Saccinobaculus* genes, and red for genes from the contaminating oxymonad. The genetic code for each gene is shown to the right, where TAR = stop is the universal code and TAR = Q is the noncanonical genetic code. Asterisks denote published taxa in which stop codons were not included, and as such are inferred from the absence of TAR at glutamine positions. The trees were not rooted at the position suggested by the phylogenomic analysis because these trees include taxa not represented in the phylogenomic tree that fall close to this root, leaving several possible roots all consistent with the phylogenomic tree.