Transcriptome and Evolutionary Analysis of *Pseudotrichomonas keilini*, a Free-Living Anaerobic Eukaryote

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Abstract

The early evolution of eukaryotes and their adaptations to low-oxygen environments are fascinating open questions in biology. Genome-scale data from novel eukaryotes, and particularly from free-living lineages, are the key to answering these questions. The Parabasalia are a major group of anaerobic eukaryotes that form the most speciose lineage of Metamonada. The most well-studied are parasitic parabasalids, including *Trichomonas vaginalis* and *Tritrichomonas foetus,* but very little genome-scale data are available for free-living members of the group. Here, we sequenced the transcriptome of *Pseudotrichomonas keilini*, a free-living parabasalian. Comparative genomic analysis indicated that *P. keilini* possesses a metabolism and gene complement that are in many respects similar to its parasitic relative *T. vaginalis* and that in the time since their most recent common ancestor, it is the *T. vaginalis* lineage that has experienced more genomic change, likely due to the transition to a parasitic lifestyle. Features shared between *P. keilini* and *T. vaginalis* include a hydrogenosome (anaerobic mitochondrial homolog) that we predict to function much as in *T. vaginalis* and a complete glycolytic pathway that is likely to represent one of the primary means by which *P. keilini* obtains ATP. Phylogenomic analysis indicates that *P. keilini* branches within a clade of endobiotic parabasalids, consistent with the hypothesis that different parabasalid lineages evolved toward parasitic or free-living lifestyles from an endobiotic, anaerobic, or microaerophilic common ancestor.

Key words: anaerobic eukaryotes, eukaryotic evolution, protist transcriptome, hydrogenosome.

Introduction

Animals, plants, and fungi are well-studied by biologists, and genomes for many lineages are now available. However, most eukaryotic diversity is microbial, and many groups are poorly sampled by genomics and transcriptomics (Sibbald and Archibald 2017). Among unicellular lineages, parasites are best represented by sequencing efforts. These include the parabasalid *Trichomonas vaginalis*, the causative agent of the most common nonviral sexually transmitted disease, trichomoniasis (Donné 1836). *T. vaginalis* infects the genitourinary tracts of 187 million people every year around the world (Menezes et al. 2016) and increases the transmission rate of human immunodeficiency virus (Petrin et al. 1998). There is therefore great interest in the biology of *T. vaginalis* and also in the evolution of parasitism in the whole Parabasalia.

Significance

Eukaryotes are enormously diverse, from animals, plants, and fungi to a range of single-celled forms. But many eukaryotic lineages are poorly sampled by genome-scale sequencing projects, resulting in a paucity of data with which to study eukaryotic evolution. Here, we sequenced and analyzed the transcriptome of *P. keilini,* a free-living, anaerobic eukaryote that is related to the important parasite *Trichomonas vaginalis.* Comparing their gene complements enables us to distinguish features that are common to the broader group—the Parabasalians—from those specific to *Trichomonas* during the evolution of parasitism. The new data also provide a basis for further studies of divergent and fascinating eukaryotic lineages.

There are over 450 described species of parabasalids. The majority are endobiotic, including parasites such as *T. vaginalis* and its relatives that infect other animals (Yamin and Ma 1979; Brugerolle and Lee 2000; Adl et al. 2007; Cepicka et al. 2010). The first free-living parabasalid to be discovered was *P. keilini* (Bishop 1935, 1939). *P. keilini* was originally isolated from pond water in Lincolnshire, UK, and later isolated from mangrove sediments in Japan, a lake in Cyprus (Yubuki et al. 2010); from freshwater sediments in Azerbaijan, sulfurous freshwater spring, and brackish sediments in Greece; and from inland salt marshes in Spain (Céza et al. 2022). As parabasalids are thought to be ancestrally endobiotic (Čepička et al. 2017), several questions naturally arise about a free-living member of the group: Did the free-living lifestyle of *P. keilini* evolve secondarily, or are transitions between free-living and host-associated lifestyles more common than anticipated in parabasalids? Answering these questions fully will require genome-scale data from closely related free-living and host-associated members of the group, alongside further study of their biology. As a step toward providing this knowledge base, we carried out transcriptome sequencing and bioinformatics analyses.

Here, we present a largely complete transcriptome dataset of *P. keilini*, a free-living anaerobic parabasalid isolated from salt marsh sediment in Spain. We perform phylogenetic analyses to place *Pseudotrichomonas* in the eukaryotic tree and use comparative genomics to trace gene content and hydrogenosome evolution within Parabasalia. We envisage that the *P. keilini* transcriptome will be of use in further analyses of early eukaryote genomic and metabolic evolution.

Transcriptome of *P. keilini*

We isolated and cultured *P. keilini* (supplementary fig. S1), then extracted RNA and sequenced it on the Illumina MiSeq and NovaSeq6000 platforms (see supplementary Methods, Supplementary Material online); reads were deposited at the NCBI Short Read Archive (PRJNA884676). Transcriptome reads were assembled into 137,389 transcripts using Trinity RNA-Seq (Haas et al. 2013), and predicted proteins were obtained using TransDecoder. We performed basic local alignment search tool-based filtering to remove contaminants from the assembly (see supplementary Methods, Supplementary Material online, supplementary table S1, Supplementary Material online), and clustered identical overlapping proteins to obtain 18,851 nonredundant predicted proteins, of which functions could be predicted for 12,811.

To evaluate the completeness of the *P. keilini* transcriptome, we conducted a benchmarking universal single copy orthologs (BUSCO) analysis (Simão et al. 2015) of *P. keilini* using a set of conserved eukaryotic genes and compared it to BUSCO values for other published Metamonada (supplementary table S2, Supplementary Material online). These datasets (a mixture of transcriptomes and genomes) had a median completeness of 47% of BUSCOs, with a 95% confidence interval ranging from 35.29 to 46.71. Our *P. keilini* transcriptome includes complete protein-coding genes for 47% of BUSCOs, the median value, and the same as some other free-living metamonads such as *Anaeramoeba flamelloides*. By comparison, the completely sequenced *T. vaginalis* genome encodes 53% of BUSCOs. Overall, we concluded that we had produced a near-complete transcriptome that is likely to be informative about the evolution and metabolism of *P. keilini.*

P. keilini branches within a clade of parasitic parabasalids

To investigate the relationship of *P. keilini* to other parabasalids and metamonads, we used SpeciesRax (Morel et al. 2022) to infer a species tree including *P. keilini*, its closest parabasalid relatives, and a representative sample of other eukaryotic lineages, using 13,346 gene families clustered from 43 genomes (Fig. 1) using Broccoli (Derelle et al. 2020; supplementary Methods, Supplementary Material online). Interestingly, the phylogeny indicates that *P. keilini* branches within a clade of parasitic parabasalids with very good support, as the sister lineage to a clade comprising *T. vaginalis*, *Trichomonas gallinae*, and *Trichomonas tenax* (extended quadripartition internode certainty [EQPIC] 0.53, reflecting good agreement between this species tree node and the underlying quartets of the input gene family trees). The cattle

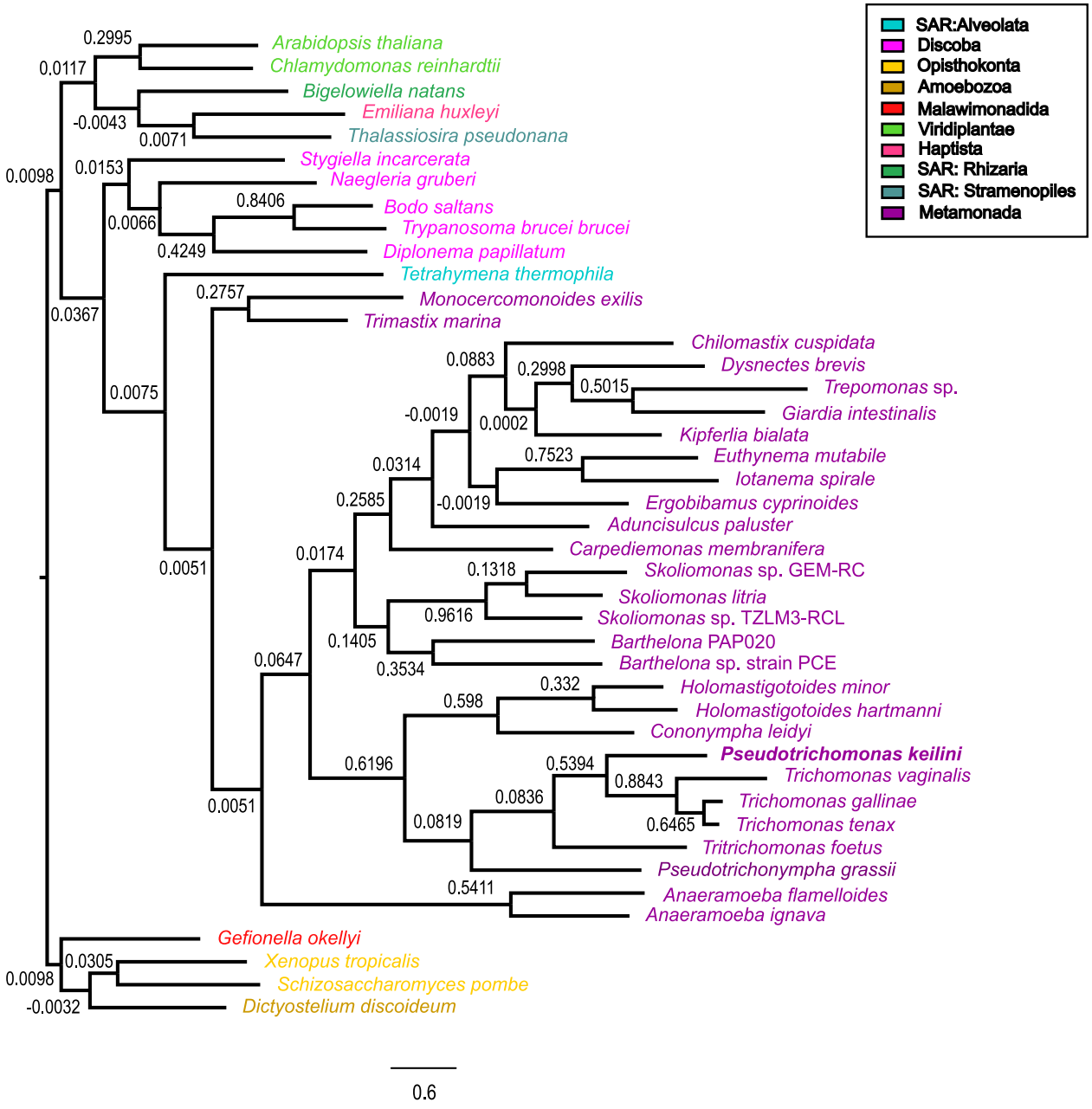

Fig. 1. Species tree of *P. keilini* among parabasalids and metamonads. SpeciesRax estimated species tree from 13,346 gene family trees, clustered from 43 genomes. Branch supports are EQPIC scores (Morel et al. 2022), reflecting the degree of agreement between quartets in the input gene trees and the inferred maximum likelihood species tree; >0.5 denotes high support). *P. keilini* groups robustly with the parasitic parabasalids *T. vaginalis, T. gallinae*, and *T. tenax*. Branch lengths in units of mean expected substitutions per site. Species are colored based on their taxonomic groups as indicated in the color code box on the right.

and feline parasite *T. foetus* branches sister to this *P. keilini-T. vaginalis* clade (Fig. 1). The phylogeny suggests either that *P. keilini* evolved a free-living lifestyle from a parasitic ancestor, or alternatively that there have been at least two transitions to parasitism within this clade: once in the ancestor of *T. foetus* and once in the ancestor of the *Trichomonas* clade. However, our phylogeny lacks many free-living and commensal endobiotic Parabasalia due to the lack of molecular data, and definitively testing between these hypotheses will require denser genomic sampling of related lineages. In 18S rRNA phylogenies, *Pseudotrichomonas* and *Lacusteria* are the two deepest branches within the order Trichomonadida. The position of Trichomonadida within Parabasalia is not resolved, but it might be the sister clade of Honigbergiellida, which contains

several free-living species. The endobiotic *Trichomitus batrachorum* branches distantly and belongs to yet another order, the Hypotrichomonadida (Céza et al. 2022). Parasitic and free-living species seem to have arisen multiple times each within the predominantly commensal endobiotic Parabasalia.

More broadly, the maximum likelihood SpeciesRax topology recovered the monophyly of Metamonada (including Parabasalia, Fornicata, Preaxostyla, *Barthelona*, *Skoliomonas*, and Anaeramoebae), although the deep relationships between these lineages were poorly resolved, with EQPIC scores close to 0 for the deepest splits within Metamonada, representing conflicting support from gene family trees. Alternative approaches such as concatenation (Stairs et al. 2021; Williamson et al. 2024) or reconciliation methods that model gene tree uncertainty (Cerón-Romero et al. 2022; Morel et al. 2024) may be required to resolve these deep branches of the eukaryotic tree.

Gene content evolution in *P. keilini* and its relatives

To investigate the evolutionary origins of the *P. keilini* genome, we mapped gene family evolution on the inferred eukaryotic species tree using a phylogenetic birth–death model implemented in Count (Csűös 2010), summarized in Fig. 2 (see also supplementary Methods, Supplementary Material online). The analysis suggested that the common ancestor of Parabasalia and Fornicata had a relatively small genome (2,975 gene families), with extensive gene gain in the parabasalid lineage after its divergence from the common ancestor with Fornicata. This is consistent with previous reports of gene family expansions in Parabasalia (Oyhenart and Breccia 2014; Handrich et al. 2019; Maciejowski et al. 2023). The common ancestor of *P. keilini* and *T. vaginalis* was inferred to have a gene repertoire comparable in size to that of *P. keilini* (5,280 gene families), with additional gene gains in the *T. vaginalis* lineage after its divergence from *T. gallinae* and *T. tenax*. Functional annotation of genes gained in the *T. vaginalis* lineage highlighted a role for binding to cellular components of the urogenital tract, a crucial step for extracellular parasite survival (Pereira-Neves and Benchimol 2007) (supplementary table S5, Supplementary Material online). Another key parasitic function can be seen through the presence of genes binding to spectrin, a protein found on the host cell surface that acts as the main gateway for target cell degradation (Fiori et al. 1997). These results are consistent with the view that the *T. vaginalis* lineage adapted to a parasitic lifestyle after divergence from its common ancestor with *P. keilini*, in part through the gene gains mapped here.

The maximum likelihood species tree contained some heterodox features that were poorly supported by EQPIC scores, including the branching of the ciliate *Tetrahymena thermophila* with metamonads rather than stramenopiles, alveolates and rhizarians (EQPIC 0.0075), and a deep-branching position of Anaeramoebae within metamonads (0.0051) rather than as sister to Parabasalia (Stairs et al. 2021). We therefore performed a sensitivity analysis in which we fit the phylogenetic birth–death model to a species tree edited to more closely reflect these current views of eukaryotic relationships (reviewed in Burki et al. 2020); see supplementary fig. S2, Supplementary Material online for the alternative tree. The results were closely similar.

The hydrogenosome of *P. keilini*

Metamonads are ancestrally anaerobic, and characterized metamonads have a range of reduced mitochondrial homologs or mitochondria-related organelles (MROs; Stairs et al. 2015). One of the best-characterized MROs is the hydrogenosome of *T. vaginalis*, and so we sought to investigate the MRO of *P. keilini* and to compare it with that of its close anaerobic, parasitic relative. To do so, we searched the *P. keilini* protein set for proteins previously implicated in MRO function and metabolism (Stairs et al. 2015), proteins that have been localized to the hydrogenosome in *T. vaginalis* (569 proteins, Schneider et al. 2011) and other hallmark mitochondrial proteins, including the mitochondrial carrier family of transporters.

This analysis confirmed that *P. keilini* possesses a hydrogenosome that, in many respects, is similar to that of *T. vaginalis*: both organisms encode the same subset of 12 hallmark proteins drawn from a larger set found in a wide range of MROs across the eukaryotic tree of life (Stairs et al. 2015), including the key enzymes needed to reduce protons to molecular hydrogen via the oxidative decarboxylation of pyruvate (pyruvate-ferredoxin oxidoreductase, [FeFe]-hydrogenase, and the associated maturases) (supplementary fig. S4, Supplementary Material online, supplementary tables S2 to S5, Supplementary Material online). Overall, our analyses suggest that *P. keilini* has all the enzymes needed to carry out glycolysis, with the resulting pyruvate imported into the hydrogenosome for further catabolism (Fig. 3).

The transition from mitochondrion to hydrogenosome was previously inferred to have occurred in the common ancestor of all metamonads (Leger et al. 2017). This is consistent with our findings, in that phylogenetic analysis of the key hydrogenosomal enzymes recovered a monophyletic clade of parabasalids in each of the trees (supplementary figs. S3 to S14, Supplementary Material online). In total, the *P. keilini* transcriptome encodes orthologues of 487/569 *T. vaginalis* proteins localized to the hydrogenosome by proteomics (Schneider et al. 2011). Both organisms also encode the same complement of six paralogous mitochondrial carrier family proteins, suggesting that the requirements for transport into and out of the MRO are similar in each case (see supplementary

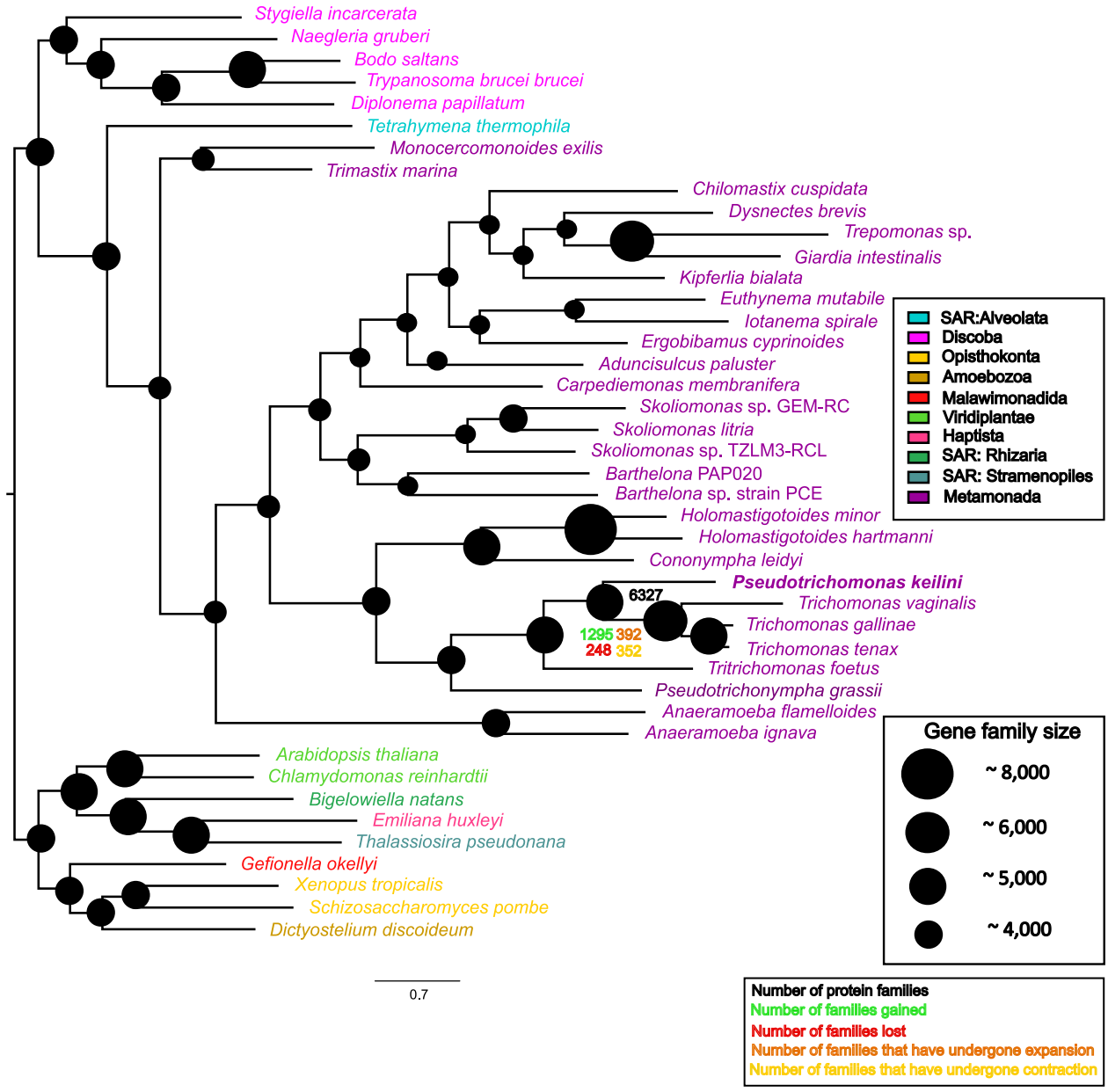

Fig. 2. Gene family evolution in parabasalids and metamonads. We used a phylogenetic birth–death model implemented in Count (Csűös 2010) to map gene family evolution onto the inferred species tree. Numbers and the diameter of circles indicate gene family repertoire size at ancestral nodes, while family gains, losses, expansions, and contractions are plotted for the *T. vaginalis, T. gallinae*, and *T. tenax* lineages after their divergence from *P. keilini*. The analysis was also performed on a species tree manually edited to reflect the consensus view of deep eukaryotic relationships (supplementary fig. S2, Supplementary Material online), with closely similar results for gene content evolution within metamonads.

Supplementary Material for more discussion on hydrogenosomal import in *P. keilini*).

The biosynthesis of iron–sulfur (Fe–S) clusters is perhaps the most widely (although not universally) conserved function of mitochondria and MROs (Stairs et al. 2015). The *P. keilini* hydrogenosome carries out Fe–S cluster biosynthesis using the ISC pathway, as in *T. vaginalis.* We detected orthologues of all the ISC enzymes present in *T. vaginalis* and *Naegleria gruberi* in the *P. keilini* transcriptome. Interestingly, *P. keilini* shares an iron–sulfur flavoprotein of bacterial origin used in the detoxification of reactive oxygen species with *T. vaginalis* and one other eukaryotic anaerobe, *Entamoeba histolytica* (Peña-Diaz and Lukeš 2018).

In sum, we sequenced the transcriptome of a free-living anaerobic parabasalid, *P. keilini*. Although free-living, the gene content, metabolism, and hydrogenosome of *P. keilini*

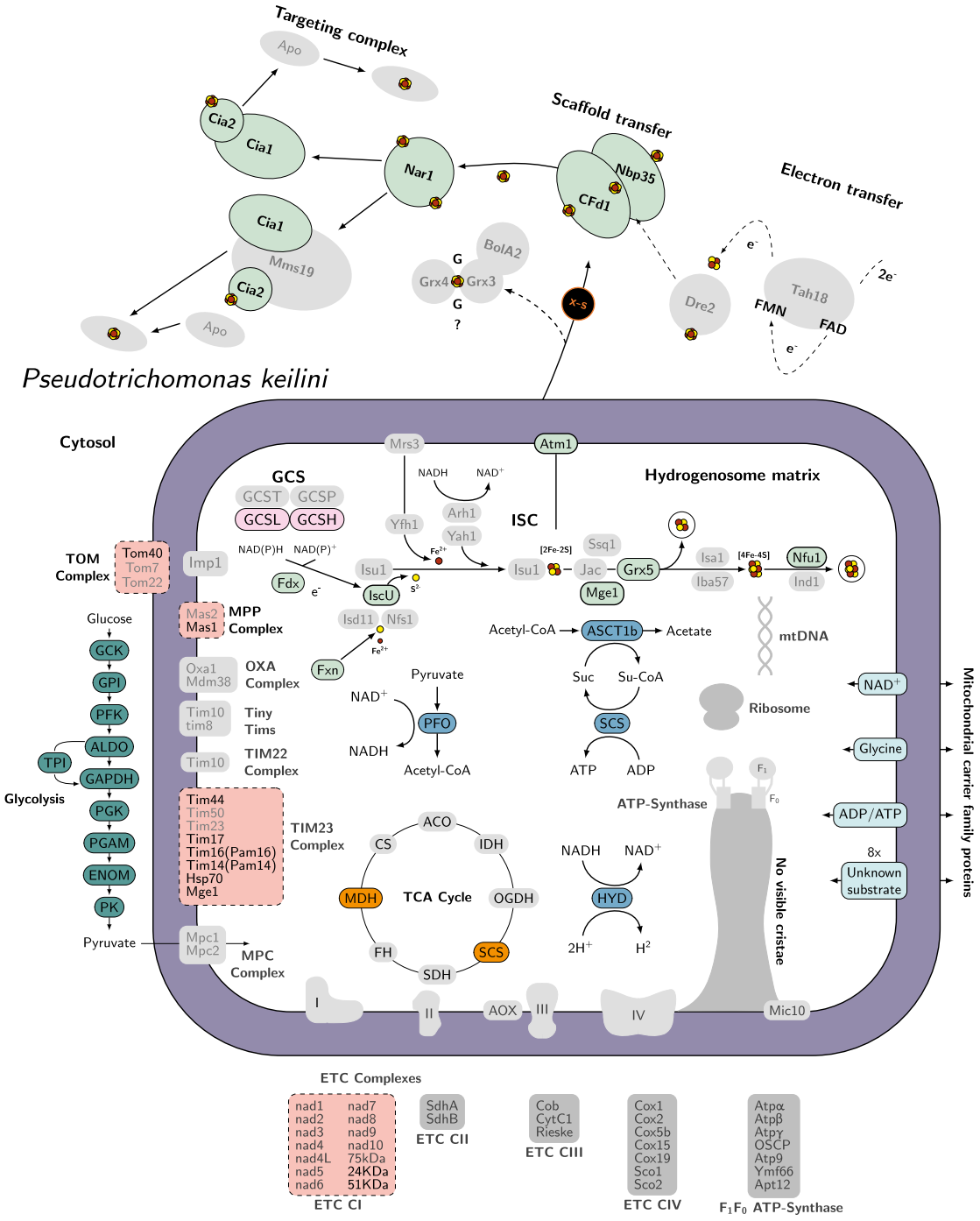

Fig. 3. Predicted metabolic pathways in the *P. keilini* hydrogenosome. Key enzymatic reactions are depicted, including glycolysis, the main pathway used to produce pyruvate, which is then oxidized by the tricarboxylic acid (TCA) cycle in the hydrogenosome. The translocases of the outer and inner mitochondrial membranes (TOM and TIM, respectively) and the electron transport chain (ETC) subunits that were detected in complex I are also indicated; note that the subunits identified (NuoE and NuoF) are common in anaerobes, and are involved in other processes beyond oxidative phosphorylation (Hrdy et al. 2004; Stairs et al. 2021). Boxes with solid outlines represent complexes for which all subunits were identified, dashed outlines represent complexes with some subunits identified, and boxes in gray with no outline indicate complexes with no subunits identified. Figure design is adapted from Peña-Diaz and Lukeš (2018) and Lewis et al. (2019).

are in many ways similar to its parasitic relative, *T. vaginalis*. The phylogenetic position of *P. keilini* in the species tree inferred from a large sample of protein-coding genes is consistent with the numerous transitions between free-living and host-associated lifestyles that have been suggested by 18S rRNA gene trees. Based on the analysis of BUSCO gene content, the transcriptome is likely to be largely complete and will represent a useful resource for future comparative analyses of parabasalids and eukaryotic evolution more broadly.

Supplementary Material

Supplementary material is available at *Genome Biology and Evolution* online.

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Data Availability

Read data has been deposited in the NCBI SRA (accession number PRJNA884676). The transcriptome assembly, gene models, and annotations are available in a FigShare repository: https://doi.org/10.6084/m9.figshare.26528119.v1.

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