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Lack of evidence for the presence of plastids in the evolutionary history of kinetoplastid protists

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Abstract: The discovery of multiple metabolic enzymes encoded by genes of apparent plant or cyanobacterial origin in trypanosomatids led to the influential hypothesis that the common ancestor of Euglenozoa harboured a plastid that was subsequently lost in kinetoplastids. Here, we critically re-evaluate this hypothesis using an expanded, phylogenetically balanced dataset comprising 299 eukaryotic and 102 bacterial species. We reassess the evolutionary histories of 16 genes encoding proteins previously interpreted as evidence for an ancestral euglenozoan plastid using state-of-the-art maximum-likelihood and Bayesian phylogenetic approaches, supplemented by topology tests. Our analyses reveal that none of the examined genes provides compelling support for a plastid-bearing ancestor of Euglenozoa. Instead, these enzymes display heterogeneous evolutionary origins consistent with multiple independent horizontal gene transfer events between euglenozoans and diverse bacterial (distinct from cyanobacteria) and eukaryotic donors. Only the gene encoding a vacuolar H^+-pyrophosphatase, an electrogenic proton pump, shows limited affinity to chloroplast-bearing lineages, and this signal alone is insufficient to infer plastid ancestry. Taken together, our results strongly suggest a horizontal gene transfer from various non-plastid bearing lineages over the hypothesis of plastid presence in the euglenozoan common ancestor with subsequent loss in kinetoplastids, diplonemids, and non-photosynthetic euglenids.

Keywords: trypanosomatids, horizontal gene transfer, phylogenetics, Euglenozoa

This article contains supporting table and figure (Table S1, Figure S1) online at http://folia.paru.cas.cz/suppl/2026-73-005.pdf

Kinetoplastea is a group of unicellular eukaryotes that, together with euglenids, diplonemids, and symbiontids, comprise the phylum Euglenozoa (Cavalier-Smith 2016). Members of Kinetoplastea include free-living protists, endosymbionts of other unicellular eukaryotes, and parasites of insects, vertebrates, and plants, represented by the family Trypanosomatidae, which includes medically important human pathogens of the genera *Trypanosoma* Gruby, 1843 and *Leishmania* Ross, 1903 (Kostygov et al. 2021). In recent multiprotein-based phylogenetic reconstructions, diplonemids and kinetoplastids form a clade, which is sister to symbiontids; together, these three lineages form a monophyletic group that is sister to euglenids (Lax et al. 2021, Valach et al. 2023).

Within Euglenozoa, euglenophytes (class Euglenophyceae) are the only known photosynthetic lineage, and their plastids are thought to have originated through secondary endosymbiosis involving a pyramimonadalean green alga (Turmel et al. 2009, Jackson et al. 2018). However, phylogenetic analyses have shown that a substantial fraction of plastid-targeted proteins in the model euglenophyte *Euglena gracilis* Klebs, 1883 exhibit phylogenetic affinity to other algal lineages, such as chromophytes, suggesting a more complex evolutionary history (Maruyama et al. 2011, Ponce-Toledo et al. 2018). Consistent with this view, a comprehensive analysis of the plastid proteome of *E. gracilis*, a model organism of considerable biotechnological importance, revealed that approximately 60 plastid proteins, corresponding to about 19% of the inferred algal horizontal gene transfer (HGT) candidates, show phylogenetic affinity to chlorarachniophytes, ochrophytes, and haptophytes (Novák Vanclová et al. 2020).

These genes may have been acquired through multiple, non-mutually exclusive mechanisms. One possibility is repeated gene transfer from ingested prey, consistent with the 'you are what you eat' hypothesis (Doolittle 1998), particularly at the early stages of plastid integration, when ancestral euglenids were likely obligately mixotrophic (Yamaguchi et al. 2012). Alternatively, these genes may have been transferred from a cryptic endosymbiont, via kleptoplasty, or from a transient plastid that preceded the establishment of the extant organelle.

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A recent study of a mixotrophic euglenid *Rapaza viridis* Yamaguchi, Yubuki et Leander 2012, also known as the closest sister taxon to the Euglenophyceae, has revealed the presence of kleptoplasts derived from a specific strain of green alga, *Tetraselmis* sp. (Karnkowska et al. 2023). Nucleus-encoded genes for proteins putatively targeted to the kleptoplast exhibit diverse evolutionary origins, mostly distinct from *Tetraselmis* sp. Importantly, no other lineage within euglenids, or more broadly within Euglenozoa, is currently known to possess plastids.

The identification of numerous metabolic enzymes of putatively plant origin in members of the parasitic flagellates belonging to the genera *Trypanosoma* and *Leishmania* of the family Trypanosomatidae more than two decades ago (Hannaert et al. 2003a) caused a substantial stir in the community (Martin and Borst 2003, Waller et al. 2004). From evolutionary, cell biology and pharmacological perspectives, the prospect of trypanosomatids retaining remnants of a plastid was truly exciting. Moreover, it became instantly reminiscent of an earlier finding of a plastid-derived organelle termed apicoplast in the causative agents of malaria, *Plasmodium* Marchiafava et Celli, 1885, and related apicomplexans (McFadden et al. 1996). The prospects of trypanosomes and leishmanias being potentially sensitive to some herbicides, as is the case of apicomplexan parasites (Dempsey et al. 2013, Corral et al. 2017), were exciting (Hannaert et al. 2003a, Waller et al. 2004).

Although no plastid remnants have been detected in trypanosomatids, it has been proposed that their common ancestor with euglenids harboured a plastid that was subsequently lost in the trypanosomatid lineage, potentially explaining the presence of genes whose closest homologues occur in plant and algal chloroplasts or cytosol, as well as in cyanobacteria (Hannaert et al. 2003a). The key support for the trypanosomatid parasites harbouring a photosynthetic plastid at some stage of their evolutionary history came from phylogenetic analyses performed using gene sequences, at that time available only from *Trypanosoma brucei* Plimmer et Bradford, 1899 and, to lesser extent, from *Leishmania major* Yakimoff et Schokhor, 1914 (Hannaert et al. 2003a).

Here, we critically re-evaluate this conclusion using advanced methodology on a substantially expanded dataset that includes free-living relatives of trypanosomatids, namely euglenids, diplonemids, and kinetoplastids (Kostygov et al. 2021). As detailed below, we conclude that the available evidence no longer supports the intriguing possibility of a remnant plastid and argue that it shall not be considered plausible, as is still frequently the case (Colasante et al. 2018, Novák Vanclová et al. 2020, Olson et al. 2020, Dhumal et al. 2022).

MATERIALS AND METHODS

Protein identification

The genomes and predicted proteins for 102 bacterial and 299 eukaryotic species (including 121 euglenozoans) were downloaded from the sources listed in Table S1. Among euglenozoan genome- and transcriptome-derived proteomes, only 91 with ≤ 70% missing universal eukaryotic single-copy orthologs (BUSCOs) were selected for the reference dataset (Table S1). The completeness of the proteomes was assessed using BUSCO v.5.4.3 and the 'euglenozoa_odb10' reference database (Manni et al. 2021).

Proteins of *Trypanosoma brucei* encoded by genes of putative plant origin from Hannaert et al. (2003a) were downloaded from TriTrypDB release 63: 6-phosphogluconate dehydrogenase (6PGDH; Tb927.9.12110), alternative oxidase (AOX; Tb927.10.7090), adenylate kinase (ADK; Tb927.10.830), fructose-1,6-bisphosphate aldolase (ALD; Tb927.10.5620), glucose-6-phosphate dehydrogenase (G6PDH; Tb927.10.2490), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Tb927.6.4280), glycerol-3-phosphate dehydrogenase (G3PDH; Tb927.8.3530), phosphoglycerate kinase (PGK; Tb927.1.700), phosphoglycerate mutase (iPGM; Tb927.10.7930), sedoheptulose-1,7-bisphosphatase (SBPase; Tb927.2.5800), superoxide dismutase (SOD; Tb927.11.15020), vacuolar H^+-pyrophosphatase ($V\text{-}H^+$-PPase; Tb927.8.7980), α-hydroxyacid dehydrogenase (AHADH; Tb927.11.11250), and ω-6-fatty acid desaturase (Tb927.2.3080). The sequences for two proteins, acyl carrier protein and 6-phosphogluconolactonase, were omitted from the analyses as trimmed alignment was too short and could not be used for obtaining well-resolved phylogenetic trees.

For the identification of homologues of these genes in the dataset, *T. brucei* proteins were used as queries for BLASTp and tBLASTn searches (Camacho et al. 2009) with the *e*-value thresholds specified for each protein in Fig. S1, and other parameters at their default values.

Phylogenetic inference

Putative homologues were aligned with MAFFT v.7.490 (Katoh and Standley 2013) using L-INS-I algorithm and other settings left as default. Alignments were trimmed with trimAl v.1.4rev15 (Capella-Gutiérrez et al. 2009) with the '-gt 0.8' option. The sequences with ≥ 10% gaps in trimmed alignment were removed from further analyses. The remaining sequences were realigned, and the alignment was trimmed, using the software and parameters mentioned above. Maximum likelihood (ML) phylogenetic trees were inferred in IQ-TREE v.2.1.0 with automatic selection of the amino acid substitution model by the built-in ModelFinder and branch support estimated by ultrafast bootstrap method with 1,000 replicates. The following additional models were included with 'madd' option: C10-60, LG4M, LG4X, LG+C20+F+G, LG+C40+F+G, and LG+C60+F+G. Bayesian trees were inferred using MrBayes v.3.2.7a under mixed model prior and rate variation across sites according to a gamma distribution.

The analysis was run using four independent chains for a minimum of 1,000,000 generations (or longer if standard deviation of split frequencies was higher than 0.01) with sampling every 100th of them, and discarding 25% samples as burn-in. For the initial PGK dataset the standard deviation of split frequencies did not fall below 0.05 and the results of Bayesian reconstruction were not considered. For AOX, PGK, and $V\text{-}H^+$-PPase, an additional phylogenetic tree was constructed using a subset of the original sequences that formed a well-supported clade in the initial analysis, with the aim of improving resolution of the branching order between euglenozoan and non-euglenozoan sequences.

For iPGM and ω-6-fatty acid desaturase, where kinetoplastid and diplonemids sequences unexpectedly did not form a monophyletic group, we tested alternative hypotheses where the re-

spective sequences were constrained to form a clade using IQ-TREE v.2.1.0 (-g option). For each constrained topology, an ML tree was inferred under the same substitution model used for the unconstrained analysis. Site-wise log-likelihoods were calculated for the unconstrained and each constrained topology, and statistical support for alternative topologies was assessed with the approximately unbiased (AU) test implemented in IQ-TREE. These tests were performed based on RELL resampling with 10,000 replicates. Significance was evaluated at $\alpha = 0.05$, and constrained topologies with *p*-values below this threshold were considered significantly worse than the best-fitting (unconstrained) topology.

RESULTS AND DISCUSSION

Hannaert et al. (2003a) use the phylogenetic positions of 16 *Trypanosoma brucei* genes involved in glycolysis, the hexose-monophosphate pathway, and other processes, which show a close relationship to cyanobacteria and eukaryotic lineages with primary plastids, as evidence for the presence of a plastid in the common ancestor of kinetoplastids and euglenids. In this study, we use a phylogenetically balanced dataset and modern phylogenetic inference methods to demonstrate that there is currently no evidence to support the claim that all euglenozoans, including kinetoplastids, shared a plastid at any point in their evolutionary history. Instead, the data are more consistent with the model, where organisms acquire genes through HGT from various sources. In the case of photosynthetic euglenids, this might have facilitated the acquisition of secondary plastids, while in kinetoplastids and diplonemids, the corresponding enzymes may have been repurposed for different functions, such as within the glycosome. Below, we will dissect the phylogeny of the genes in question, subdivided based on their appurtenance to metabolic pathways.

Glycolytic enzymes

Several genes encoding glycolytic enzymes demonstrated phylogenetic affiliation to cyanobacterial and plant sequences (Hannaert et al. 2003a). Fructose-1,6-bisphosphate aldolase (ALD) converts fructose-1,6-bisphosphate into glyceraldehyde-3-phosphate and dihydroxyacetone phosphate (Pirovich et al. 2021). While euglenophytes possess both the cytosolic and plastid-targeted class I enzymes, the homologue in trypanosomatids and diplonemids has a recognisable peroxisomal targeting signal (PTS2) at its N-terminus (Chudzik et al. 2000) and appears to be localised in the glycosome. Unlike Hannaert et al. (2003a), but similar to Rogers and Keeling (2004), we did not find any phylogenetic relationship between kinetoplastid and diplonemid ALD and their homologues from plants. Moreover, they do not share a common origin with the sequences found in euglenids (Fig. S1A).

Phosphoglycerate kinase (PGK) is an enzyme that catalyses the reversible transfer of a phosphate group from 1,3-bisphosphoglycerate to ADP, resulting in the production of 3-phosphoglycerate and ATP (Rojas-Pirela et al. 2020). Our analysis points to bacterial (distinct from cyanobacteria) origin for both the glycosomal and cytosolic PGKs in diplonemids and kinetoplastids, as well as for the cytosolic PGK in euglenids (Figs. 1 and 2A; Fig. S1B). The chloroplastic PGK in euglenids clusters with the cytosolic PGKs from other eukaryotes and is likely encoded by an ancestral eukaryotic gene retargeted to the plastid (Fig. S1B). Sequences from cyanobacteria, chloroplastids (= Viridiplantae, or 'green' lineage, including chlorophytes, streptophytes, and prasinodermophytes), stramenopiles, glaucophytes, rhodophytes, cryptophytes, some alveolates, and haptophytes form a separate clade (Fig. S1B). Two additional diplonemid PGKs (from *Diplonema japonicum* Kito, 1976 and *Artemidia motanka* Prokopchuk, Tashyreva et Lukeš, 2019) clustering with the bacterial sequences originate from transcripts that do not contain a spliced leader sequence and thus likely represent contaminants (100% identity at the protein level to the sequences from Bacilli).

The enzyme 2,3-bisphosphoglycerate-independent phosphoglycerate mutase (iPGM) was identified in all analysed Euglenozoa except for euglenids, where only putative contaminant sequences were found in very few species (Fig. 1). It appears that the respective gene is present in some bacteria (distinct from cyanobacteria), which likely served as a source from which it was acquired by eukaryotes. A sister relationship between diplonemid and chloroplastid sequences is indicative of the HGT between these two lineages. Alternatively, they may have acquired the iPGM gene from the same or closely related donors. Topology tests using constrained trees did not reject alternative hypotheses in which diplonemid and kinetoplastid iPGMs form a clade sister to a monophyletic group comprising the CRuMs member *Rigifila ramosa* Yabuki, Ishida et Cavalier-Smith, 2013 and chloroplastids (AU *p*-value = 0.232), or a clade sister to chloroplastids (AU *p*-value = 0.290). Thus, a close phylogenetic affinity among diplonemid, kinetoplastid, and chloroplastid iPGMs remains plausible, although less supported than the unconstrained topology in which diplonemids and kinetoplastids do not cluster together (Fig. S1C).

The evolution of D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is highly complex, involving multiple gene losses and gains, including acquisitions by the HGT between prokaryotes and eukaryotes (Martin and Cerff 2017). GAPDH sequences comprise a large and diverse family of multifunctional proteins that participate in glycolysis and Calvin cycle (Henry et al. 2015). To obtain a well-resolved phylogenetic tree, we focused on the GAPDH homologues closely related to the glycosomal GAPDH (gGAPDH) of kinetoplastids (referred to as GAPC in the literature), which catalyses the conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate (Petersen et al. 2003, Hamilton et al. 2004).

Our analysis suggests that diplonemids and some euglenids (Fig. S1D) have retained an ancestral eukaryotic GAPDH, while trypanosomatids and all analysed euglenids possess a GAPDH gene acquired horizontally from bacteria (the closest branches include but are not limited to cyanobacteria). In trypanosomatids, the corresponding proteins are localised to the glycosome, while in euglenids, they are found in the cytosol (Michels et al. 1991, Henze et al. 1995). Some trypanosomatids (e.g., *Leishmania*, *Porcisia*, and *Trypanosoma* spp.) possess an additional cytosolic GAPDH, with the respective gene closely related to the bacterial

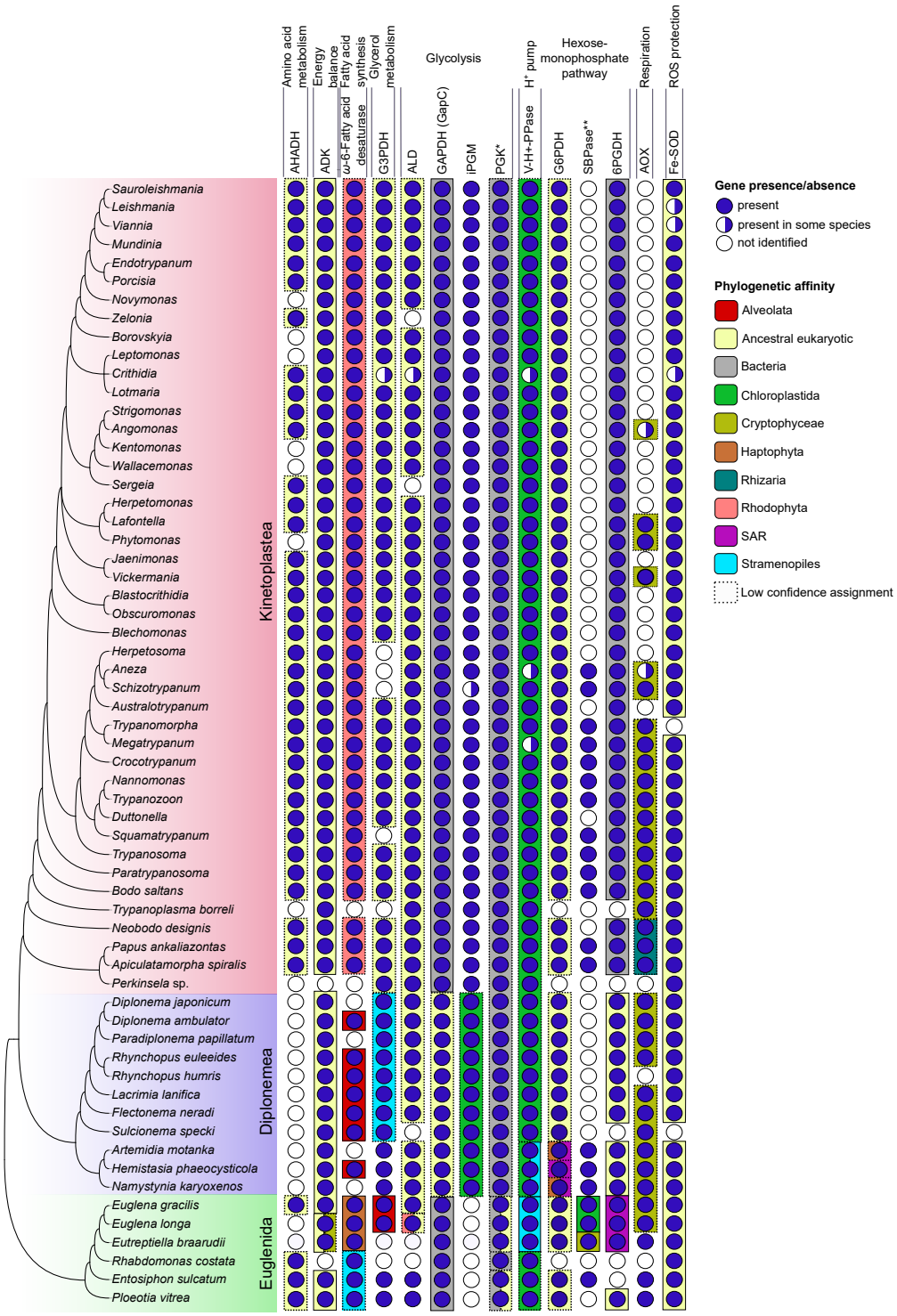

Fig. 1. Phylogenetic affinity and distribution of 'plant-like' euglenozoan genes. Gene presence/absence is indicated with coloured circles: blue indicates gene presence, white – absence, white-blue – presence in at least one species in the group. Colour-filled boxes indicate genes' phylogenetic affinity (in case of several homologues with different phylogenetic affinity, several background colours are used): red – Alveolata, yellow – ancestral eukaryotic gene, grey – bacteria, green – Chloroplastida, olive green – Cryptophyceae, brown – Haptophyta, teal – Rhizaria, pink – Rhodophyta, violet – SAR (stramenopiles, alveolates, and rhizarians), light blue – stramenopiles. In case of low confidence assignment (low branch support values or support by only one method of phylogenetic inference) the box borders are shown with dashed lines. Proteins are grouped by their function (indicated at the top). *Abbreviations*: 6PGDH – 6-phosphogluconate dehydrogenase; ADK – adenylate kinase; AHADH – α-hydroxyacid dehydrogenase; ALD – fructose-1,6-bisphosphate aldolase; AOX – alternative oxidase; $V\text{-}H^+$-PPase – vacuolar H+-pyrophosphatase; G3PDH – glycerol-3-phosphate dehydrogenase; G6PDH – glucose-6-phosphate dehydrogenase; GAPDH – glyceraldehyde 3-phosphate dehydrogenase; iPGM – cofactor-independent phosphoglycerate mutase; PGK – phosphoglycerate kinase; SBPase – sedoheptulose-1,7-bisphosphatase; SOD – superoxide dismutase. Cladogram of Euglenozoa is based on Kostygov et al. (2021, 2024). * – only a maximum likelihood tree is available; ** – assignment to hexose-monophosphate pathway is putative. This figure includes data only for the selected reference species with high quality genomes/transcriptomes marked with an asterisk in Table S1.

homologues (Fig. S1D). The analysis by Qian and Keeling (2001) suggests the presence of a diplonemid GAPA/B gene, which was acquired from a bacterium and does not cluster with the chloroplast GAPDH of *Euglena gracilis*, supporting the independent origin of the respective genes.

Hexose-monophosphate pathway enzymes

Glucose-6-phosphate dehydrogenase (G6PDH), the first enzyme of the hexose-monophosphate pathway (HMP), present in trypanosomatids, euglenids, and the family Diplonemidae does not show affiliation to plant sequences and instead appears to represent an ancestral eukaryotic gene (Fig. 1; Fig. S1E). Hemistasiidae spp. have two homologues of this gene, which form two distinct well-supported monophyletic groups with the sequences of SAR (Stramenopila, Alveolata, and Rhizaria) and haptophytes.

Sedoheptulose-1,7-bisphosphatase (SBPase) is an enzyme involved in the Calvin-Benson cycle within the chloroplasts of photosynthetic eukaryotes, where it catalyses the hydrolysis of sedoheptulose-1,7-bisphosphate into sedoheptulose-7-phosphate (Gutle et al. 2016). Surprisingly, its homologue has been identified in a range of non-photosynthetic eukaryotes, including trypanosomatids, ascomycetes, and alveolates (Teich et al. 2007). Yet experimental data addressing the role of this protein in these heterotrophs is still lacking.

It has been hypothesised that this glycosomal enzyme participates in an alternative pentose phosphate pathway variant in trypanosomes (Hannaert et al. 2003b). However, while SBPase retains its canonical enzymatic activity, this hypothetical pathway is unlikely to be functional in the bloodstream stage of *T. brucei* (Kovářová et al. 2024). In Kinetoplastea, SBPase is present in the genus *Trypanosoma*, the early-branching trypanosomatid *Paratrypanosoma confusum* Votýpka et Lukeš, 2013, the eubodonid *Bodo saltans* Ehrenberg, 1832, and the free-living Prokinetoplastina (Fig. 1).

All these kinetoplastid SBPases form a well-supported clade branching off the phylogenetic tree near the alveolate homologues with moderate support (Fig. S1F). Euglenophyte SBPases appear to be of two different origins, with the sequences from *Euglena* spp. and *Eutreptiella* spp. nested within Chloroplastidae and Cryptophyceae, respectively (Fig. S1F). In diplonemids, SBPase distribution is restricted to the family Hemistasiidae, with the respective sequences clustering separately from other euglenozoan homologues.

We identified 6-phosphogluconate dehydrogenase (6PGDH), an enzyme that converts 6-phosphogluconate to ribulose 5-phosphate (Jakkula et al. 2021), in nearly all analysed euglenozoan genomes (Fig. 1). In *T. brucei*, the respective protein predominantly localises to the cytosol (Hanau et al. 2013, Billington et al. 2023). Hannaert et al. (2003a) suggested that the respective gene is of cyanobacterial origin, while another study (Andersson and Roger 2002) and our analyses indicate its affinity to eubacteria distinct from cyanobacteria. Maruyama et al. (2008) found that the sequence from *Paradiplonema papillatum* (Tashyreva, Simpson, Horák et Lukeš, 2022) belongs to the clade of ancestral eukaryotic sequences likely originating from eubacteria. In contrast, the euglenid homologues were probably acquired via HGT from stramenopiles and, along with their donor group, belong to the 'cyanobacterial 6PGDH' clade (Fig. S1G).

Our analysis, which includes additional diplonemid and euglenid sequences, supports this observation and demonstrates that several heterotrophic euglenids *Ploeotia vitrea* (Dujardin, 1841), *Petalomonas cantuscygni* Cann et Pennick, 1986, and *Notosolenus urceolatus* Larsen et Patterson, 1990 lack 6PGDH of stramenopile origin and instead possess homologues affiliated with the diplonemid sequences in the ancestral eukaryotic clade. This finding further highlights the convoluted history of this gene in Euglenozoa and suggests that there is no single origin of 6PGDH in kinetoplastids, diplonemids, and euglenids.

Other enzymes

The enzyme α-hydroxyacid dehydrogenase (AHADH), a member of the broader malate dehydrogenase family, is involved in aromatic amino acid metabolism and showed phylogenetic affinity to malate dehydrogenases from the plant cytosol (Hannaert et al. 2003a). We identified the respective homologue in most kinetoplastids and euglenids (Fig. 1), and it likely has a cytosolic localisation in *T. brucei*, although some signal was detected in the nucleoplasm and flagellar cytoplasm (Billington et al. 2023). While the homologue is absent from diplonemids (Fig. 1), the euglenid and trypanosomatid sequences form a well-supported clade that does not show close affinity to sequences from Chloroplastida (Fig. S1H).

Trypanosoma brucei adenylate kinase (ADK), reported by Hannaert et al. (2003a) to be closely related to the homologue from maize chloroplasts, was identified in all analysed Euglenozoa, except the highly reduced *Perkinsela* sp., where it is likely genuinely lacking, while its absence in *Rhabdomonas costata* (Pringsheim, 1942) is possibly caused by missing data (Fig. 1). According to TrypTag, the *T. brucei* protein localises into the paraflagellar rod (Billington et al. 2023). In our analysis, the ADK sequences form a clade that does not show strong affinity to any chloroplast-bearing lineages (Fig. S1I). It is worth noting that some euglenids, such as *Euglena longa* (Marin et Melkonian 2003) and *Eutreptiella braarudii* Throndsen, 1969, possess an additional ADK homologue, which forms a sister group to the sequences of Cryptophyceae (Fig. S1I), albeit with rather low support and only by the maximum-likelihood method (bootstrap value 73%).

An enzyme of glycerol metabolism, glycerol-3-phosphate dehydrogenase (G3PDH), in Euglenozoa appears to be of common eukaryotic origin, showing no close affiliation to the bacterial sequences contrary to what was reported previously (Akinyi et al. 2008). Diplonemid and euglenid sequences fall within the same eukaryotic clade, but they form a monophyletic group neither with each other nor with kinetoplastids, but constitute a sister groups to stramenopiles and alveolates, respectively (Fig. S1J).

Alternative oxidase (AOX) is a cytochrome-independent terminal oxidase, which is an integral component of the respiratory chain of some bacteria and a range of eukaryotes

(Pennisi et al. 2016). Hannaert et al. (2003a) reported that the *T. brucei* AOX clusters with its plant homologues, yet our analysis and that of Pennisi et al. (2016) do not support this relationship (Fig. S1K). The remaining AOX sequences form a well-supported clade with homologues from stramenopiles, rhizarians, and cryptophytes (Fig. S1K).

As judged by the presence of AOX in multiple distantly related eukaryotic lineages, it appears that AOX was already present in the Last Eukaryotic Common Ancestor (LECA). Since AOX is present in nearly all euglenids and diplonemids, as well as in kinetoplastids outside the family Trypanosomatidae, it is plausible that the common ancestor of Euglenozoa also possessed it. This gene is present in most members of the genus *Trypanosoma*, whereas its distribution is patchy in other trypanosomatids, with only *P. confusum*, *Angomonas deanei* Teixeira et Camargo, 2011 and members of the genera *Lafontella* Kostygov et Yurchenko, 2015, *Phytomonas* Donovan, 1909, and *Vickermania* Kostygov et Yurchenko, 2020 retaining it.

Phylogenetic analysis of omega-6-fatty acid desaturases, which are involved in the biosynthesis of polyunsaturated fatty acids, has suggested that the trypanosomatid homologues are closely related to their plant counterparts (Hannaert et al. 2003a), while another study proposed that they are more related to fungi than plants (Alloatti and Uttaro 2011). Our analysis shows that the kinetoplastid omega-6-fatty acid desaturases form a well-supported clade with fungi and rhodophytes, but prominently not with diplonemids (Fig. S1L). According to the topology test with the constraint imposing monophyly on diplonemids and kinetoplastids, such topology can be rejected (AU p-value < 0.05), suggesting that the kinetoplastid and diplonemid sequences indeed have distinct origins. Moreover, the euglenid sequences fall into three distinct sub-clades only very distantly related to any other euglenozoans, with two of them forming strongly supported groups with haptophytes, and the third one having no obvious close relatives.

We were unable to resolve the phylogenetic position of iron-containing superoxide dismutase (Fe-SOD) with confidence, with the euglenozoan clade branching as a sister to alveolates (Fig. S1M). Additionally, diplonemids belonging to the family Hemistasiidae exhibit a divergent Fe-SOD that does not cluster with any other euglenozoan sequences.

Among all analysed genes, only the one encoding vacuolar H+-pyrophosphatase ($V\text{-}H^+$-PPase) shows clear affinity to the chloroplastid sequences, albeit this relationship was inferred only through maximum-likelihood analysis (Fig. 2B; Fig. S1N). While this gene was for a long time associated only with plants and phototrophic bacteria, it was subsequently identified in several non-photosynthetic protists (Drozdowicz and Rea 2001, Perez-Castineira et al. 2002), including trypanosomatids, where $V\text{-}H^+$-PPase functions as a proton pump in acidocalcisomes (Lemercier et al. 2002).

Our analysis identified its homologues in nearly all Euglenozoa (Fig. 1), even in the extremely reduced *Perkinsela* sp. (Tanifuji et al. 2017), yet the previously reported sister relationship with the alveolate sequences (Hannaert et al. 2003a) was not confirmed (Fig. 2B; Fig. S1N). Euglenozoan $V\text{-}H^+$-PPases form two distinct clades on the phylogenetic tree: one encompassing all three main lineages (mainly photosynthetic representatives among euglenids) and clustering sister to Chloroplastida, while the second clade includes Hemistasiidae and heterotrophic euglenids, and its branching shows a sister relationship to stramenopiles (Fig. S1N). The sister relationship of Hemistasiidae and photosynthetic euglenids with stramenopiles indicates the possibility of HGT involving these two lineages.

Taken together, our phylogenetic trees based on a taxonomically balanced dataset and inferred using a set of currently available phylogenetic methods do not support the hypothesis that the euglenozoan common ancestor possessed a primary plastid. Instead, genes previously reported to show close affinity to the cyanobacterial or plant homologues display no close relationship to these lineages and exhibit heterogeneous evolutionary origins, including that by putative HGT events involving euglenozoans, stramenopiles, alveolates, and bacteria distinct from cyanobacteria.

Based on an analysis of a phylogenetically restricted dataset, as well as the phylogenetic methods available at that time, Hannaert et al. (2003a) concluded that the euglenozoan ancestor possessed a plastid. Using a much broader and phylogenetically balanced dataset, as well as cutting edge bioinformatic methods, our analysis does not support this conclusion. Combined, phylogenetic data presented here suggest that the genes on which the hypothesis of early plastid acquisition in the euglenozoan evolution was based originate from numerous sources, showing a complex and often different pattern in euglenids, kinetoplastids, and diplonemids.

For example, euglenozoan PGK and trypanosomatid 6PGDH sequences affiliate with bacteria distinct from cyanobacteria. Similarly, GAPDH displays multiple independent acquisitions by euglenozoans, with trypanosomatids and euglenids harbouring bacterial-derived homologues that differ in subcellular localisation between these two lineages. Among genes analysed herein, only those encoding $V\text{-}H^+$-PPase, an electrogenic proton pump well-known for its diverse functions in plants (Segami et al. 2018), shows some affinity to the chloroplastid sequences, although with a limited support only by the Bayesian method and with undefined direction of the putative HGT. Collectively, our data indicate that the set of "plant-like" genes of trypanosomatids reported by Hannaert et al. (2003a), is, in fact, a mosaic of acquisitions from different sources distinct from primary plastid-bearing lineages and cyanobacteria. Although a systematic, genome-wide survey of genes putatively acquired through horizontal gene transfer in Euglenozoa is still lacking, existing studies largely focused on the HGT events in individual euglenozoan species (Ienne et al. 2012, Valach et al. 2023) did not identify any evidence for an excessive flow of genes from the plastid-bearing lineages.

Let us combine the rather robust phylogenetic data with morphological evidence, which became available since the Hannaert et al. (2003a) study, that further does not speak in favour of kinetoplastid flagellates ever possessing a plastid. Most proteins with the plant-like phylogenetic signal were localised in the glycosome (Gualdrón-López et al. 2012), with this organellar localisation confirmed recently (Bill-

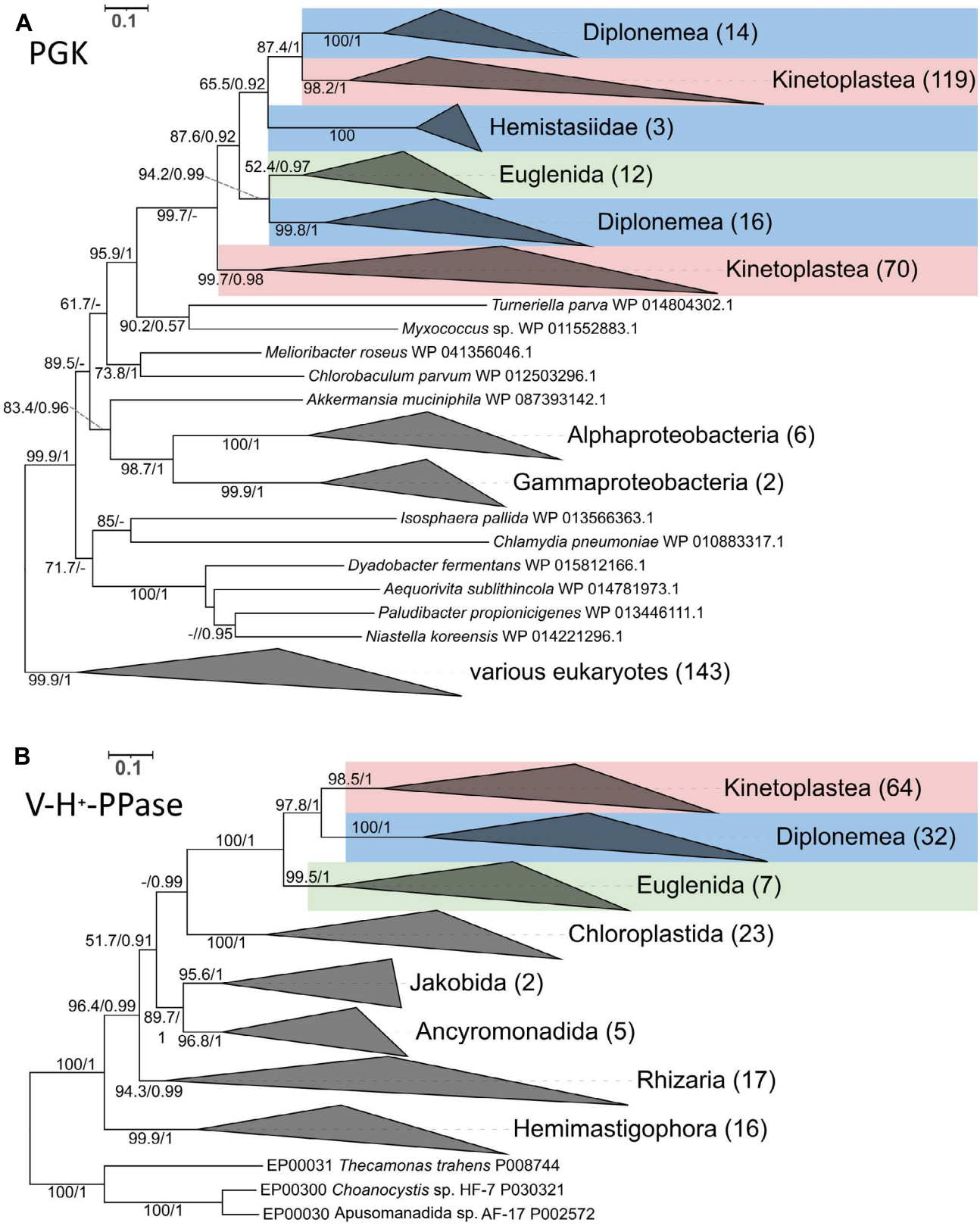

Fig. 2. Maximum-likelihood phylogenetic trees for phosphoglycerate kinase (PGK) (panel A) and vacuolar H+-pyrophosphatase (V-H+-PPase) (B). The phylogenetic tree for PGK is consistent with the horizontal transfer from bacteria (distinct form cyanobacteria) to the euglenozoan common ancestor. V-H+-PPase demonstrates phylogenetic affinity to the homologues from chloroplastids (with support only from the Bayesian inference), although the direction of potential horizontal gene transfer cannot be established. Numbers at the branches: ultrafast bootstrap support (%) / posterior probability. The amino acid substitution model was LG+I+G4 for both proteins. Only bootstrap support values ≥ 50% and posterior probabilities ≥ 0.5 are shown. The scale bar represents 0.1 substitutions per site. Diplonemid, kinetoplastid, and euglenid sequences are shown on blue, pink, and green background, respectively.

ington et al. 2023). Furthermore, the prospect of a plastid reduced to a tiny inconspicuous vesicle that guided intracellular localisation in *Trypanosoma brucei* of YCF45, an unknown function protein typically associated with the chloroplast (Hallick and Bairoch 1994), remained unfulfilled since, depending on the used start codon, the protein localised to the mitochondrion (Týč et al. 2010). Moreover, despite the expectations, no more genes with a strong plastid-like phylogenetic signal surfaced from the now comprehensive and well-annotated genomes of *T. brucei* and numerous other trypanosomatids and bodonids. It was also argued that the acquisition of a chloroplast has a (lasting) impact on the structure of the accepting cell. The profound morphological differences between the plastid-bearing euglenids and parasitic trypanosomatids were used as a supporting argument against the plastid-early hypothesis, i.e., the plastid-bearing common ancestor of euglenids and kinetoplastids (Leander 2004). The vast phylogenetic data currently available strongly point to the acquisition of a secondary green plastid by endosymbiosis only following the divergence of the euglenid lineage from the other euglenozoans, namely diplonemids and kinetoplastids (Karnkowska et al. 2023).

Finally, the kinetoplastid parasites are not the only group where prospects of a relict plastid that was lost in extant species came to naught. Indeed, oomycetes and goniomonads have also been predicted to carry at some point a plastid but a careful phylogenetic and other analyses did not support this claim (Wang et al. 2017, Cenci et al. 2018). Furthermore, even in cases with strong evidence for a lost organelle, such as the mitochondrial loss in an oxymonad (Karnkowska et al. 2016) and the plastid loss in a dinoflagellate (Gornik et al. 2015), there are unexpectedly limited genomic remnants left in the nuclei of these protists secondarily devoid of an organelle (Gawryluk et al. 2019, Novák et al. 2023). It has been recently argued that this should not be 'unexpected' after all, since selection does not seem to favour the retention of these genes (Keeling 2024). We conclude that trypanosomatids, for good reasons considered as exemplary non-conventional unicells (Lukeš et al. 2023), for once behave as standard eukaryotes that do not hide a reduced plastid or convincing signs of its presence at any point in the evolutionary history.

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