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Aerobic mitochondria of parasitic protists: Diverse genomes and complex functions

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ABSTRACT

In this review the main features of the mitochondria of aerobic parasitic protists are discussed. While the best characterized organelles are by far those of kinetoplastid flagellates and *Plasmodium*, we also consider amoebae *Naegleria* and *Acanthamoeba*, a ciliate *Ichthyophthirius* and related lineages. The simplistic view of the mitochondrion as just a power house of the cell has already been abandoned in multicellular organisms and available data indicate that this also does not apply for protists. We discuss in more details the following mitochondrial features: genomes, post-transcriptional processing, translation, biogenesis of iron–sulfur complexes, heme metabolism and the electron transport chain. Substantial differences in all these core mitochondrial features between lineages are compatible with the view that aerobic protists harbor organelles that are more complex and flexible than previously appreciated.

1. Introduction

Aerobic protists represent a diverse array of unicellular organisms, for which the main shared feature is the presence of an aerobic mitochondrion that is, in most cases, capable of oxidative phosphorylation. It also possesses additional essential mitochondrial (mt) pathways, such as fatty acid metabolism, Ca^{2+} homeostasis, biosynthesis of ubiquinone and heme, and the assembly of iron–sulfur clusters. Aerobic unicellular parasites covered by this review fall within three out of five major eukaryotic super-groups, Amoebozoa, Excavata and SAR. In this review, Amoebozoa is represented by the amphizoic genus *Acanthamoeba* causing rare infections in humans. While the majority of amoebozoans is unicellular, this group also includes the well-known model organism *Dictyostelium*, a slime mold capable of producing a macroscopic multicellular stage of aggregated amoeboid cells. Predominantly parasitic Excavata will be extensively discussed, as they are represented by medically relevant *Trypanosoma* and *Leishmania* (Euglenozoa, Kinetoplastea) and *Naegleria* (Heterolobosea). The last, but not least, discussed super-group SAR includes very important human parasites of the genus *Plasmodium* (Alveolata, Apicomplexa) and less known freshwater ectoparasite, *Ichthyophthirius* (Alveolata, Ciliophora), which causes diseases of fish (Fig. 1).[1]

The main goal of this review is to illustrate our current understanding of the structure, organization and replication of mt genome, its gene expression including RNA editing and processing, and finally mt translation. Furthermore, we focus on canonical functions of aerobic mitochondria, such as oxidative phosphorylation, electron transport chain, heme synthesis and iron-sulfur cluster assembly. While by far the most information is available for medically important parasites, we also consider less studied parasites and/or their free-living relatives.

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[1] Throughout the review, we will be using species names only when differences are known within the genera (e.g., *T. brucei* vs *T. cruzi*), otherwise generic names (*Plasmodium*, *Ichthyophthirius*) will be applied.

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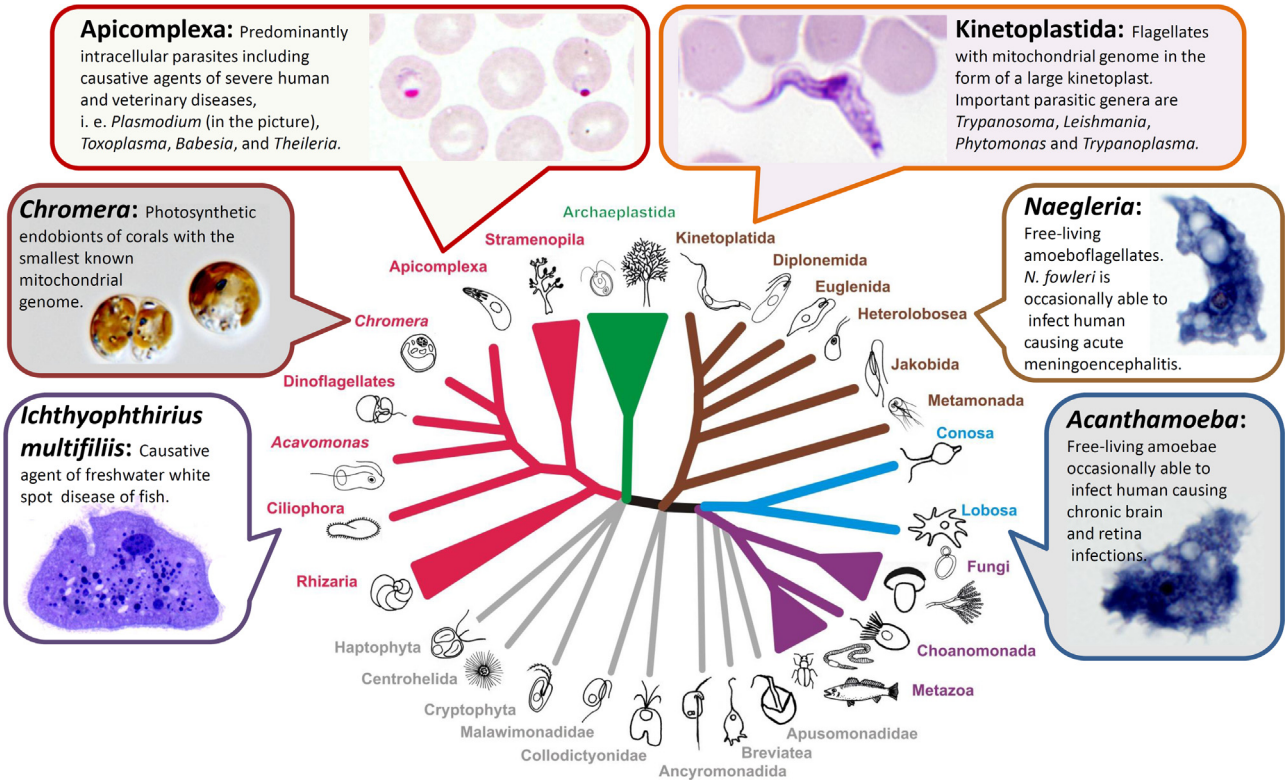

Fig. 1. Schematic tree of eukaryotes. The scheme is based on Adl et al. [14] and contains taxa relevant to the text. Eukaryotic supergroups are color coded—SAR (red), Archaeplastida (green), Excavata (brown), Amoebozoa (blue) and Opisthokonta (violet). Positions of the six main taxa discussed in the review are highlighted by boxes with illustrations. *Chromera* image shows living cocal stages under DIC (courtesy of Miroslav Oborník), *Ichthyophthirius* cell was stained in histological section (courtesy of Martin Kostka), *Trypanosoma* (courtesy of Jan Votýpka) and *Plasmodium* images are from Giemsa stained blood smears, *Acanthamoeba* and *Naegleria* represent hematoxylin stained smears from culture. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2. Mitochondrial genome organization

Mitochondrial genomes are distant derivatives of the genome of an α-proteobacterium that billions of years ago became the pre-mitochondrial endosymbiont in the emerging eukaryotic cell [1]. With the number of genes encoded by mt genomes varying between the minimum of four and the maximum of 106, it always represents only a small fraction of the original endosymbiont gene complement, since the majority of genes has been lost or transferred into the nuclear genome via the process of endosymbiotic gene transfer [2]. However, when the total amount of DNA is considered, some lineages such as the kinetoplastid protists contain up to several megabases (Mbp) of DNA [3], which makes them comparable in size with prokaryotic genomes.

The structural organization of mt genomes is highly variable, aptly summed up as "anything goes" [4]. It includes classical circular or circular mapping DNA molecules, as well as linear ones of various sizes, sometimes flanked by terminal repeats or telomeres, and populations of DNA circles catenated into a network. Fig. 2 summarizes the coding content of mitochondria from eukaryotic groups that are the focus of this review. Most protists, including their parasitic representatives, exceed animal mt genomes in both the gene number and size [5]. Typical animal mt genomes encode only 13 protein-coding genes, which are located on a ~16 kb-long circle. For instance, the mt genomes of free-living flagellates from the groups Jakobida (Excavata) exhibit the largest known coding capacity, with *Andalucia godoyi* being the current record holder encoding 66 protein-coding genes, 6 open reading frames (ORFs) of unknown function, 29 genes for tRNAs, 3 rRNAs and 2 RNA molecules important for translation and RNA processing [6]. Jakobid mitochondria bear some primitive features reminiscent of bacterial genomes, such as genes organized in bacteria-like operones and eubacterial type RNA polymerase [6]. On the other hand, some protist groups, such as the alveolates (dinoflagellates, apicomplexans), reached the other extreme, with their mt genomes reduced to only three subunits of respiratory chain complexes and two fragmented rRNAs [7].

It is apparent that the reduction of the mt genome size and its coding capacity are not correlated with the parasitic way of life—in terms of gene content, the smallest genomes are actually found also in the free-living protists (e.g., dinoflagellates), while the largest mt genomes in terms of the DNA content are present in parasitic kinetoplastids [3,8–10]. Should there be any trait that correlates with the gene content of mt genomes, then it is the presence and composition of the electron transport chain (ETC). Only those derivatives of mitochondria that do not contain any membrane-bound component of the ETC, namely the hydrogenosomes, mitosomes and various intermediates thereof, do not contain mt genomes. Notably, when the ETC complexes I, III and IV are present, at least one subunit of each complex is encoded in the mt genome. Several arguments have been put forward to explain this fact. Some assume that structural features of these proteins make their transport from the cytosol difficult and so transfer of their genes into the nucleus has never been successful [11]. Others postulate that their transport across the mt membranes is feasible, but that the N-terminal hydrophobic part of some ETC subunits (e.g., cox 1, cyB, atp6) causes mis-targeting to endoplasmic reticulum [12]. Finally, a widely debated hypothesis assumes that the expression of individual mt-encoded subunits enables fine-tuned regulation of the ETC, and consequently the whole organelle [13].

The structure and composition of different mt genomes were best studied in medically relevant parasitic protists and their close

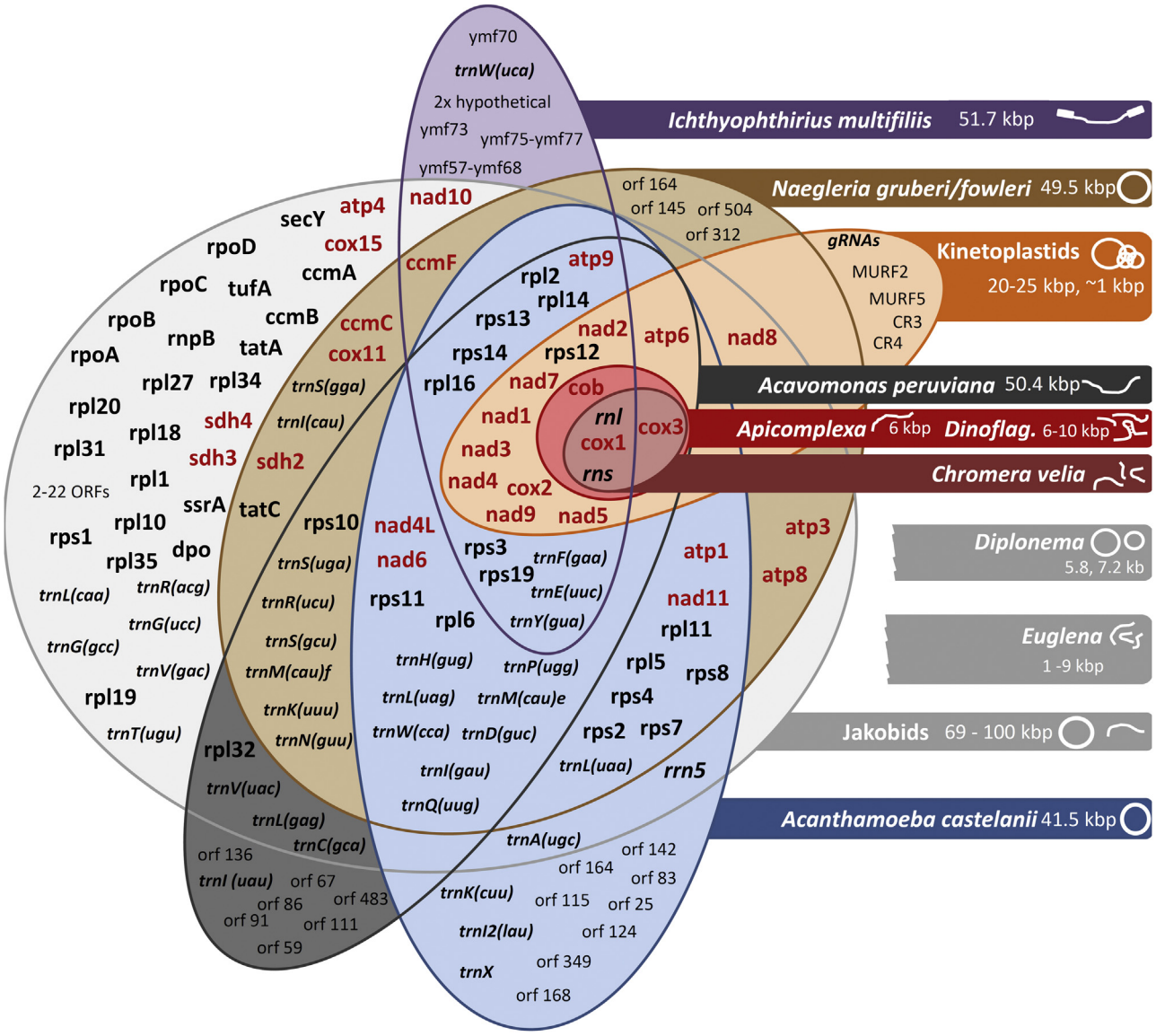

Fig. 2. Schematic representation of coding capacities, structures and sizes of mitochondrial genomes. Genomes of taxa covered by this review are shown in colors, their relatives are shown in shades of grey. Protein-coding genes with known function are given in bold, genes for proteins involved in the electron transport chain are given in red, rRNA and tRNA genes are given in italics. Labels indicate the structures and sizes of DNA molecules constituting the genomes. The genome coding capacity of *Diplonema papilatum* and *Euglena gracilis* is not known. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

relatives. The amoeboflagellate *Naegleria*, a member of Heterolobosea, within the supergroup Excavata [14], is a distant relative of the above-mentioned jakobids [6]. The 49 kb-long circular genome of the human pathogen *Naegleria fowleri* is almost identical to the non-pathogenic *Naegleria gruberi*, both being relatively gene-rich, containing 46 protein-coding genes, 20 tRNAs and 3 rRNAs [15,16]. The only other available heterolobosean mt genome is that of free-living *Acrasis kona*. Despite its large size, it codes for less genes, and at least 11 genes, which were retained in the mt genome of *Naegleria*, have been transferred into the *A. kona* nucleus [17].

When the structure of mt genomes is concerned, perhaps the most complex one evolved in another lineage of excavate protists, the medically highly relevant kinetoplastid flagellates. Their mt DNA, termed kinetoplast (k) DNA, is invariably composed of thousands of circular molecules, which are, depending on the species, either relaxed or supercoiled, and free or catenated into a compact network [3,10]. In some kinetoplastids, the kDNA is extremely large and may represent the biggest organellar genomes known so far [18]. In the best studied kinetoplastid, *Trypanosoma brucei*, the kDNA is composed of maxi- and minicircles interlocked into probably two independent, yet mutually catenated networks [8]. Thousands of minicircles (~1 kb in size) representing the majority of kDNA, are variable in sequence and encode guide (g) RNAs necessary for mRNA maturation (see below) [19]. Few dozen copies of maxicircles (~25 kb in size) represent equivalents of a typical mt genome and are believed to form a homogeneous population. In both strands of their coding region, maxicircles carry 20 genes, whose order is highly conserved among trypanosomatids of the genera *Trypanosoma*, *Leishmania*, and *Leptomonas*. They comprise the small (SSU) and large subunit (LSU) rRNA genes, a gene encoding ribosomal protein, 13 genes coding for ETC subunits, and 4 ORFs of unknown function [20–22]. Interestingly, no tRNA gene was identified in the kDNA and thus likely all tRNA molecules must be imported from the cytosol (see also below) [23]. Much less is known about the kDNA of the free-living and parasitic bodonids, other than it is mostly non-catenated, highly repetitive and freely distributed throughout the mt lumen [3].

Mitochondrial genomes of diplonemids and euglenids, sister lineages of kinetoplastids, are also very unusual and consist of populations of circular and linear molecules, respectively [24,25]. In the case of *Diplonema papilatum*, the circular molecules encode pieces of genes and the mature mRNA has to be formed by massive *trans*-splicing [26,27]. Somewhat unexpectedly, the mt genome of *Euglena gracilis* contains just 5 protein-coding genes, the transcripts of which do undergo neither RNA editing, nor splicing [28].

The mt genome of *Acanthamoeba castellanii* is a 41 kb-long circular molecule carrying 43 protein-coding genes, 16 tRNAs and LSU, SSU and 5S subunit of rRNA, with all but two genes encoded on one DNA strand [29,30]. Furthermore, its mt genome uses modified genetic code, in which the canonical stop codon UGA encodes tryptophan. A curious case of partial endosymbiotic gene transfer of a cox1 gene has been noted, in which the C-terminal part has been transferred to the nuclear genome, while the N-terminal piece remained in the mt genome, where it is fused with the neighboring cox2 [31]. The *A. castellanii* mt genome is relatively similar to the genomes of other amoebozoans, such as the slime mold *Dictyostelium*, as they share the same genome structure, the cox1–cox2 fusion, the group I introns of the same sequence and position, and similar gene composition, although the gene order is very different [32]. This comparison supports the opinion that there is no correlation between the genome size and structure and the way of life of the protists.

Some protist lineages possess linear mt genomes. For instance, the few sequenced mt genomes of ciliates are all composed of relatively large 40–70 kb linear molecules capped by telomeric repeats. In the region before telomeres are located inverted repeats containing protein-coding and/or RNA-coding genes [33]. The mt genome of parasitic *Ichthyophthirius multifiliis* is in all respects typical for a ciliate, and very similar to its free-living relative *Tetrahymena pyriformis* [34,35]. It contains 41 protein-coding genes, 5 tRNA genes, and genes for SSU and LSU rRNA subunit, which are, same as in *Tetrahymena*, split into two pieces.

Apicomplexans, dinoflagellates and related lineages contain the smallest mt genomes known. This drastic reduction apparently took place in their common ancestor after the split from the lineage of free-living colponemid flagellate *Acavomonas*, which harbors a gene-rich mt genome [36]. Mitochondrial genomes of dinoflagellates and apicomplexans invariably encode only cox1, cox3, cyB and fragmented rRNA genes, while the mt genome organization and structure vary. For instance, the mitochondria of the coccidian *Eimeria* and the haemosporidians *Plasmodium* and *Leucocytozoon* harbor a circular and/or tandemly repeated linear elements ~6 kb in size, while in the related piroplasmids, *Babesia* and *Theileria*, the ~6 kb-long linear element is present only in a single copy [37]. Furthermore, the mt genomes of dinoflagellates consist of heterogeneous population of linear DNA molecules with relatively large non-coding regions containing loop-forming repeats and coding parts that contain complete, truncated and in the case of cox3 fragmented genes, which require *trans*-splicing. Interestingly, all transcripts undergo extensive editing of numerous types and use non-canonical start and stop codons [38,39]. The most reduced mt genome is harbored by the photosynthetic alveolate *Chromera velia*, which consist of short linear molecules that encode cox1, a highly divergent cox3 and fragmented LSU and SSU rRNA genes [40] (Fig. 2).

3. Replication of mitochondrial genomes

Despite huge variability in the mt DNA structure, the replication machinery remains quite similar in most mitochondria [41,42]. From studies in yeasts and plants, recombination driven replication emerged as the predominant mode of replication of linear chromosomes [41,43,44] and closely resembles that of T4 bacteriophage [45]. Only a relatively narrow set of proteins was suggested to function in the mt DNA replication. In metazoans this set is composed of DNA polymerase γ, twinkle DNA helicase, mt RNA polymerase, single-stranded DNA binding protein, RNase H1, DNA ligase III and topoisomerases [46]. Up to date, no primase was found in metazoans, and it is widely accepted that its role is fulfilled by mt RNA polymerase.

For most aerobic parasitic protists the composition of their mt replication machinery is unknown. The one exception is *Acanthamoeba*, since the following mt DNA replication proteins were identified in its mt proteome: DNA polymerase A, twinkle DNA helicase, RecQ family ATP-dependent DNA helicase, Pif1 DNA helicase, DNA ligase, topoisomerases II and III and few others [47]. Hence, *Acanthamoeba* seems to possess more or less standard mt replication machinery. In *Plasmodium* and other apicomplexans, which carry mt genomes in the form of linear or circularly permutated chromosomes [48,49], a recombination driven mode of replication has also been described [50]. Ciliates, which are related to apicomplexans and represented here by parasitic *Ichthyophthirius*, are likely to share this type of replication. Moreover, their linear chromosomes contain telomere-like structures similar to those described in the yeast *Candida parapsilosis*, which are important for the recombination driven replication [35,43,51].

In contrast to other aerobic protists, the unique kDNA replication machinery of kinetoplastid parasites is understood to a considerable detail. Unlike the mt DNA in most other eukaryotes, the kDNA network replicates only once per cell cycle and prior to nuclear replication [8,10]. The kDNA replication is tightly coordinated with that of the flagellar basal body, with which the kinetoplast is physically connected via a protein assembly called the tripartite attachment complex [52,53]. Both kDNA maxi- and minicircles are replicated uni-directionally via theta structures, yet only the former DNA species are replicated within the network [54], whereas minicircles are released from the kDNA network into the kinetoflagellar zone prior to their replication [54,55]. Subsequently, the replicated minicircles migrate into two antipodal sites, where they are reattached into the growing network by topoisomerase II action. Due to the unusual complexity of replication, maintenance and regulation of the kDNA network, the number of proteins involved in these processes is estimated to be over 150, which is several times more than estimated for other eukaryotes [8]. Consequently, many replication proteins are kinetoplastid-specific, and/or frequently diversified into several paralogs from a single copy present in most eukaryotes [56,57]. For instance, at least two ligases, two primases and seven DNA helicases are required for the kDNA replication in *T. brucei* [58–61]. Strikingly, there are at least six mt DNA polymerases, from which one is related to polymerase κ and two to polymerase β that are typically involved in nuclear DNA damage repair. The remaining polymerases are derived from the bacterial polymerase I [62,63].

We may speculate that the diverse origin of proteins involved in mt genome replication could have specified the replication process in certain lineages. Accordingly, the mt genome organization simply reflects the capability of the replication machinery to multiply such a structure. For example, the acquirement of mt proteins originally involved in DNA repair or in the replication of bacteriophage DNA could have led to recombinant driven replication followed by an establishment of linear chromosomes, a feature typical for apicomplexans and ciliates.

4. Mitochondrial RNA metabolism

As described above, during eukaryotic evolution a massive reduction of the mt genome content occurred probably in a step-

by-step manner, with a great many of the α-proteobacterial genes being lost or transferred to the nucleus. Together with some remarkably complex and often evolutionary unrelated RNA processing mechanisms, mt protein and RNA import machineries have been acquired in order to maintain gene expression within the organelle. Prominent among those processes, which probably emerged due to the relaxed constraints on the mt gene expression, are various forms of RNA editing, defined as programmed alterations of the nucleotide sequence of an RNA species relative to the sequence of the encoding DNA [64]. RNA editing became widespread among aerobic protists probably thanks to mechanisms postulated in the model of constructive neutral evolution [65].

In kinetoplastids, RNA editing involves massive insertion and/or deletions of uridine residues into mRNA precursors. By correcting numerous frameshifts, introducing start and stop codons and often adding substantial fraction of the coding sequence, RNA editing restores the ORFs of most mt-encoded mRNAs [19]. Hundreds of small antisense gRNAs, encoded by the kDNA minicircles [66], specify the sites of editing via complementary base pairing. Numerous multiprotein and extremely dynamic (sub)-complexes provide an array of enzymatic activities needed to achieve this type of RNA editing [9,19].

Recently, a combination of in silico and proteomic approaches revealed numerous ribonucleases and helicases in the mitochondrion of *Acanthamoeba*, suggesting the presence of a complex RNA metabolism. Moreover, an unusually high number of mitochondrion-targeted pentatricopeptide repeat (PPR) proteins suggests possible occurrence of mRNA editing in its organelle [47]. The PPR proteins, considered to be uniquely adapted to recognizing sites for RNA editing in mitochondria and plastids of plants, were recently discovered also in *N. gruberi*. Indeed, the transcriptomic analysis revealed two sites of C–U editing in its cox1 and cox3 transcripts, which restore highly evolutionary conserved amino acid identities [67]. While in the mitochondrion of apicomplexans, mRNA editing is lacking, it is very abundant and extremely complex in their sister group of dinoflagellates [68].

In addition to mRNA editing, also some mt tRNAs undergo various mechanistically and evolutionary unrelated editing events, which expand decoding capacity of the mt genome, as well as the possibility to correct non-functional gene sequences on the post-transcriptional level [69,70]. A single mt tRNAs modification, likely linked to extensive tRNA editing, was described in kinetoplastids. In its anti-codon, the imported tryptophanyl-tRNA ($tRNA^{Trp}$) is subject to specific C–U editing, which allows decoding the frequent mt tryptophan codons [23]. Furthermore, the imported $tRNA^{Trp}$ undergoes an unusual thiolation at position 33, which negatively regulates the level of edited tRNA, providing an optimal ratio of edited vs unedited tRNAs [71]. Recently, wyosine/wybutosine modification of $tRNA^{Phe}$, which prevents frameshifting during decoding of UUU and UUC codons in the cytosol of archaeans and eukaryotes, was identified in the *T. brucei* mitochondrion. The unique localization of wyosine-$tRNA^{Phe}$ supposedly helps with decoding of the slippery U-rich mt transcripts generated by the extensive kinetoplastid-specific type of U-insertion/deletion RNA editing [72].

In most organisms, tRNA genes have been partially or entirely transferred from the mitochondrion to the nucleus. In order to maintain functional mt translation, distinct mechanisms of tRNA import have evolved, likely independently [70]. A number of studies have investigated factors and/or mechanisms required for the import of tRNAs, but so far, the only common denominator of various tRNA import mechanisms is the need for ATP as energy source [73].

Two groups of parasitic protists, namely kinetoplastids and apicomplexans, represent extreme cases of complete loss of tRNA genes from their mt genomes. As a consequence, they must import all tRNAs from the cytosol [73–75]. Moreover, in order to charge free tRNAs with amino acids within the organelle, the mitochondrion of *T. brucei* also imports a complete set of amino acyl tRNA synthetases [76,77]. In contrast, in *Plasmodium* and *Toxoplasma* aminoacyl tRNA synthethases have been localized only to the cytosol and the relict plastid (apicoplast), but not to the mitochondrion [78,79]. Consequently, it has been hypothesized that tRNAs are imported from the cytosol into the mitochondrion in their aminoacylated state, which can be subsequently used only once in the organellar translation elongation [80]. Indeed, one of four phenylalanyl-tRNA synthetases (PheRS), which are encoded by the *Plasmodium* genome, is actually targeted to its mitochondrion. Interestingly, this mt PheRS is exclusively present in *Plasmodium*, as it was not found in other apicomplexans. The recombinant mt PheRS of *Plasmodium* was shown to aminoacetylate tRNA in vitro, however, its role in vivo is more likely connected to phenylalanine level regulation, since the key enzyme of this pathway (phenylalanine hydroxylase) is missing from its genome [81].

In contrast to *Plasmodium* that lacks mt-encoded tRNAs, the mt genome of *Acanthamoeba* encodes 16 tRNA genes, of which 13 undergo 5′ editing within the acceptor stem [29,82]. This includes a removal of 5′ mismatches by unknown enzymes and repair of truncated tRNA by a 3′–5′ nucleotidyl transferase, so far characterized in *Saccharomyces* and *Dictyostelium*, a relative of *Acanthamoeba* [69]. Interestingly, the mt genome of *N. fowleri* contains a set of 23 tRNA genes, a full set for functional organellar translation [16]. This is not a common feature for heterolobosean protists though, as the closely related *Acrasis* holds an incomplete repertoire of only 9 mt-encoded tRNAs, with the remaining tRNAs being likely imported into the organelle [17]. A common feature of these two parasites is the mismatches in the 1st thru 3rd positions of the amino acid acceptor stem of several tRNAs. In order to obtain mature and functional tRNAs, this non-Watson–Crick base-pairing is perhaps corrected by mt 5′ tRNA editing [69,83].

5. Mitochondrial ribosomes

Due to the presence of protein-coding genes in the mt DNA, a typical eukaryotic cell must retain a complete mt translation machinery comprised of mt ribosomes, tRNAs and additional proteins. While mt rRNAs are invariably encoded by mt DNA, genes for mt ribosomal proteins were mostly transferred into the nucleus with the corresponding proteins being consequently imported into the organelle. Not surprisingly, mt ribosomes of aerobic protists studied so far share more features with their bacterial counterparts than with the cytosolic ribosomes [84]. Nevertheless, the composition of the mt ribosomes in different phylogenetic lineages is quite diverse [85], as exemplified by variously reduced or fragmented rRNAs prone to domain loss and by acquisition of novel proteins [86]. An increase in the protein/RNA ratio is responsible for a low sedimentation values (S) of mt ribosomes. Still, despite the extensive variation in rRNA length and protein composition, the overall structure and appearance of the mt ribosomes remains quite conserved [87].

Kinetoplastids are the only aerobic protists with experimentally established composition of the mt ribosomes [88–90]. In other protists, the composition of mt ribosomes has not been addressed directly yet and can only be estimated from a comparative analysis of their mt proteomes and genomes with well-known mt ribosomes [85,86]. *Trypanosoma* and *Leishmania* contain the smallest mt ribosomes known to date, sedimenting at 50S, and composed of 30S SSU and 40S LSU [91]. Furthermore, their mt rRNAs are extremely short sedimenting at only 9S and 12S, respectively [92] (Fig. 3). Although the rRNAs fold into a characteristic secondary structure,

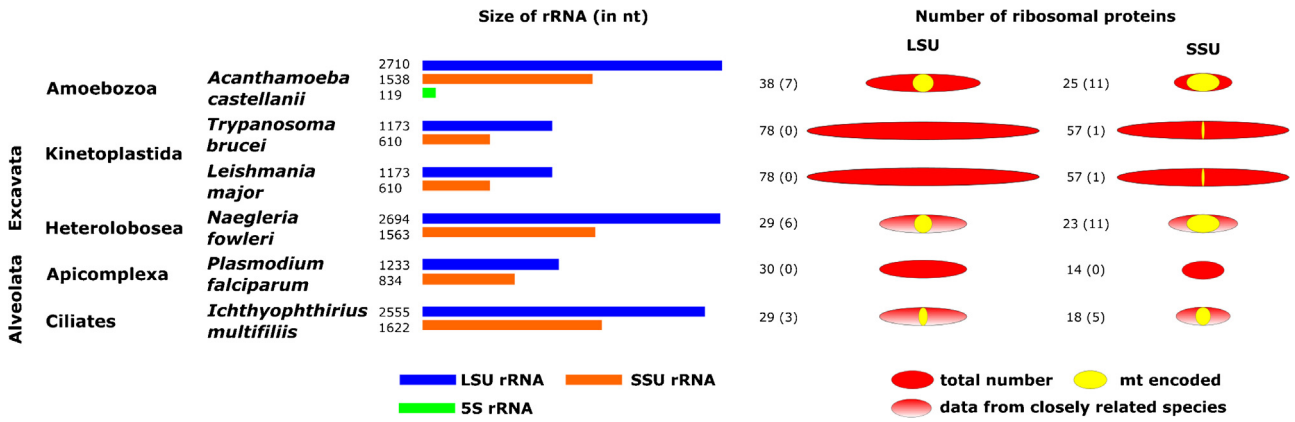

Fig. 3. Schematic representation of mature rRNA lengths and ribosomal proteins numbers in respective species. The sizes of rRNA species are shown in nucleotides (nt). The numbers of known ribosomal proteins are indicated on the left. The numbers in brackets represent ribosomal proteins encoded by the mt genome. Due to the lack of information about the nuclear-encoded mt ribosomal proteins in *N. fowleri* and *I. multifiliis*, data are shown for their closest relatives *N. gruberi* and *T. thermophyla*, respectively.

some domains underwent drastic reduction or were completely lost. For example, one such deletion is behind the resistance to chloramphenicol, a typical inhibitor of mt translation [93]. The observed shrinkage of rRNAs is thought to be at least partially compensated by the recruitment of additional proteins, which is exemplified by these extremely protein-rich mt ribosomes [89]. On the other hand, acquisition of novel proteins followed by rRNA shrinkage can also be explained by constructive neutral evolution, a theory that offers a reasonable explanation for other complex processes like RNA editing [94].

Furthermore, the mt ribosomes of kinetoplastids exhibit another unique feature as their 9S rRNA, a subset of ribosomal proteins and several PPR proteins assemble into a ribonucleoprotein complex named 45S SSU* [87,90]. This particle was shown to be essential for mt proteosynthesis, albeit its exact role is still not completely understood [95].

In contrast to kinetoplastids, another members of the Excavata, both *Naegleria* species, bear non-reduced mt rRNAs and its mt ribosomes contain at least 11 LSU and 6 SSU subunits encoded by the mt genome (Figs. 2 and 3) [16,86]. Interestingly, a homologue of only 1 out of 95 kinetoplastid specific mt ribosomal protein was identified in the *N. gruberi* nuclear genome, suggesting very distinct composition of this protist's mt ribosome. Nevertheless, the presence of an increased number of PPR domain-containing proteins indicates some similarities with the kinetoplastid translation machinery [15]. Increased number of mt PPR proteins, documented in kinetoplastids and *N. gruberi*, is also shared with the amoebozoan *Acanthamoeba* [47]. Furthermore, its mt translation machinery is characterized by additional features, such as the unique presence of mt-encoded 5S rRNAs and introns in genes encoding mt rRNAs [96,97] (Fig. 3). These introns are similar to those from yeast and likewise probably capable of self-splicing [98].

Curiously, although the apicomplexans are medically extremely important, experimental data regarding their mt ribosomes is lacking. On the other hand, their short and highly fragmented mt rRNAs have been well characterized. They are split into fragments as short as 23 nt, which are scattered all over the mt genome, making them the most fragmented rRNAs known [7,49]. Since these fragments are post-transcriptionally processed only by oligoadenylation, they are most likely not spliced together to form a mature full-length rRNA molecule. In the case of *Plasmodium*, 15 LSU and 12 SSU rRNA fragments were identified totaling 1.2 kb and 0.8 kb, respectively, with all the domains directly involved in protein synthesis being retained [7] (Fig. 3). In addition, the reduced apicomplexan mt genome lacks any ribosomal proteins, which thus have to be imported from the cytosol [86,99]. Less extensive fragmentation was identified in the ciliate *Ichthyophthirius*, the mt SSU and LSU rRNAs of which are split into two fragments, with additional duplication of the LSU rRNA [35]. The coding capacity of ciliates is greater than that of apicomplexans, with 8 mt ribosomal proteins being retained in their mt genome [35,86].

Comparison of phylogenetically closely related free-living and parasitic lineages within alveolates and excavates reveals that short mt rRNAs are confined to parasitic kinetoplastids and apicomplexans. The trend toward extensive reduction of mt rRNAs, accompanied by the elimination of ribosomal protein from the mt genome in highly specialized parasitic lineages of aerobic protists is summarized in Fig. 3.

6. Electron transport chain

Extensive studies of mitochondria from several uni- and multicellular model organisms broke the long-standing monolithic view of the organelle as being just a power house of the cell. Although the generation of ATP is therefore not anymore perceived as its sole role, it is functionally linked to mt ETC. The multi-subunit protein complexes of ETC couple the transfer of electrons to their final acceptor oxygen with the proton translocation across the inner mt membrane [100]. The generated electrochemical gradient (Δym) is absolutely crucial for mt protein import and ion homeostasis but it can also be used for ATP synthesis by F_oF_1 ATP synthase.

The activity, role and purpose of the mitochondrion in aerobic protists are especially intriguing, since the physiological state of the organelle appears to be directed by the environment the cell occupies. Moreover, the majority of protists including parasites develop in a great variety of different environmental niches, where they are confronted by dramatic changes in their nutrition supply to which they have to promptly adapt [101]. To act well under these challenges, aerobic parasitic protists often possess well equipped mitochondrion that enables them to quickly and efficiently remodel various mt activities during their life cycle. As the functional plasticity of the mitochondrion is a key metabolic adaption, to support their complex life style most parasitic protists evolved a complex organelle, often with branched ETC and additional enzymes.

In most eukaryotes, the ETC is composed of four multi-subunit membrane-bound complexes; complex I (NADH:ubiquinone oxidoreductase), complex II (succinate:ubiquinone dehydrogenase), complex III (ubiquinol:cytochrome *c* reductase) and complex IV (cytochrome *c* oxidase), with two electron carriers (ubiquinone and cytochrome *c*) functioning between them. In addition to these conventional complexes, aerobic protists including para-

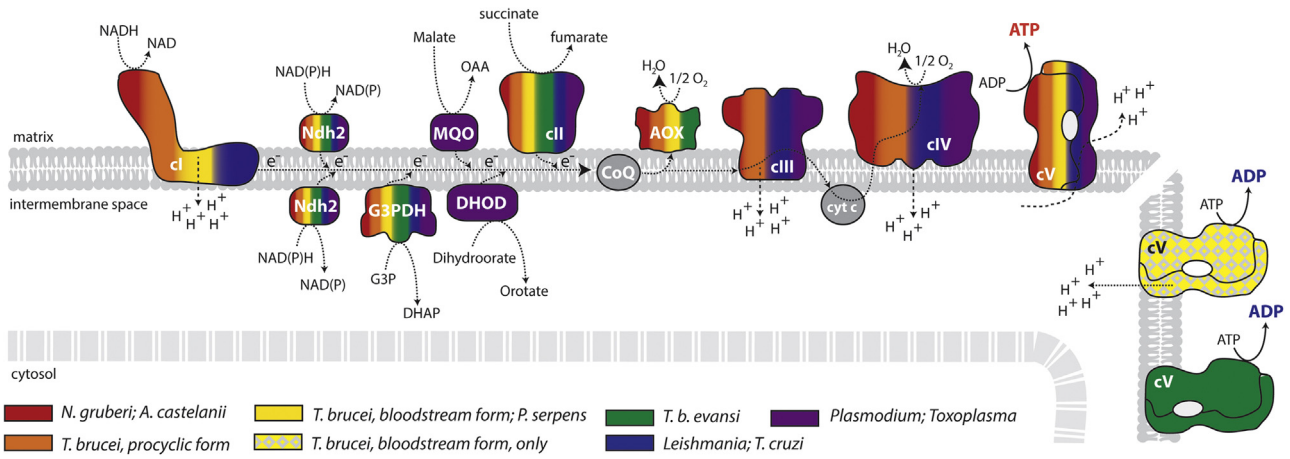

Fig. 4. Mitochondrial respiratory chain of selected aerobic parasitic protists. The presence of certain ETC complexes is illustrated by a colored strip representing following species: *Naegleria*, *Acanthamoeba* (both red), *T. brucei* insect stage (orange), *T. brucei* bloodstream stage, *Phytomonas* (both yellow), *T. b. evansi* (green), *Leishmania* and *T. cruzi* (both blue), and *Plasmodium* and *Toxoplasma* (both purple). The electrons enter the ETC via complex I (cI), an alternative dehydrogenase (Ndh2), glycerol-3-phosphate dehydrogenase (G3PDH), complex II (cII), malate:quinone oxidoreductase (MQO) and/or dihydroorotate dehydrogenase (DHOD), from which they are further transferred to coenzyme Q (CoQ) to form ubiquinol. Then the electrons can be passed directly to alternative oxidase (AOX) or complex III (cIII). Cytochrome *c* (cyt *c*) is the next electron acceptor, which donates electrons to complex IV (cIV). Complexes I, III and IV couple the transfer of electrons with proton translocation across the inner mt membrane to generate an electrochemical gradient. The energy of this gradient is then harnessed by F_oF_1 ATP synthase to generate ATP. In *T. b. evansi* (green) and *T. brucei* bloodstream form (yellow with grey diamonds) this complex works in reverse in order to maintain the $\Delta\psi$m in the absence of complexes III and IV, as illustrated on the right side of the bent membrane. OAA—oxaloacetate. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sites discussed here developed more complex ETCs. The additional enzymes contribute to electron entry, such as glycerol-3-phosphate dehydrogenase (G3PDH), type-II alternative NAD(P)H dehydrogenase (Ndh2), dihydroorotate dehydrogenase (DHOD) and malate:quinone oxidoreductase (MQO), and also to electron exit as some parasites encode in their genomes an alternative oxidase (AOX). The canonical ETC is present in the free-living ciliate *Tetrahymena* [102], and in *Acanthamoeba* and *N. gruberi* although these two protists also possess Ndh2, G3PDH and AOX. Moreover, these protists have the capacity to generate energy using typical hydrogenosomal pathways, even thought the importance and exact regulation of these enzymatic reactions is still incompletely understood [30,47,103]. Noteworthy modifications in the composition and activity of ETC are also observed in digenetic parasites such as *Trypanosoma*, *Leishmania* and *Plasmodium* (Fig. 4).

Complex I is the largest ETC enzyme and catalyzes transfer of electrons from reduced molecules of NADH to ubiquinone with the concomitant translocation of protons across the mt inner membrane [104]. Interestingly, this complex seems to retain only limited activity in kinetoplastids [105], and is under culture conditions dispensable for both life stages of *T. brucei* [106,107], *Trypanosoma cruzi* [108], and the insect stage of *Leishmania* [109,110]. However, *Leishmania* amastigotes are in vitro sensitive to the complex I inhibitor, rotenone [111], suggesting more significant role of this complex in their adaption to the mammalian host. Complex I was also shown to be active in *Phytomonas* [112,113], a plant parasite lacking complexes III and IV due to specific deletion in its kDNA maxicircle [114]. Since its $\Delta\psi$m is sensitive to rotenone, it is evident that in *Phytomonas* complex I is the only respiratory complex able to maintain the $\Delta\psi$m [115].

The NADH oxidation activity of complex I might be bypassed by the acquisition of Ndh2. Alternative dehydrogenases are single polypeptide enzymes that catalyze the transfer of electrons from NAD(P)H to ubiquinone without proton translocation [116]. These enzymes are able to oxidize NAD(P)H produced either in the cytosol or mt matrix, depending on the orientation of the NADH binding site, which may differ among species of the same group [117,118]. Apicomplexan parasites completely lack complex I activity and their genomes did not reveal any evidence for complex I presence [119]. *Plasmodium* spp. possess a single Ndh2 facing the cytosol [120], while *Toxoplasma* expresses two Ndh2 isoforms, both facing the mt matrix [121]. Due to the essentiality of Ndh2 for apicomplexans [122] and owing to its absence in mammalian cells, Ndh2 is emerging as an attractive antimalarial drug target [123,124]. In its mt inner membrane, *T. brucei* also possesses Ndh2, which is essential for the insect stage [125]. Since this enzyme seems to face the intermembrane space, it is possibly involved in reoxidation of the cytosolic NADH, and thus the assumption that *T. brucei* Ndh2 functionally replaced the activity of complex I has yet to be experimentally verified [125].

Complex II, another major electron entry to the ETC, is present in most aerobic protists, functionally connecting ETC either with the canonical tricarboxylic (TCA) cycle, as in *Plasmodium*, *Toxoplasma* and other apicomplexans [126], or with an incomplete version of TCA in the insect stage of *T. brucei* [127,128]. A prominent feature of the branched ETC of all the discussed parasites is the implication of additional mt dehydrogenases that represent additional electron donors. One such omnipresent dehydrogenase is mt G3PDH, an enzyme facing the intermembrane space, which transfers electrons from reduced compounds in the cytosol [129,130]. Importantly, in the bloodstream stage of *T. brucei* it has an irreplaceable role in glycerol-3-phosphate: dihydroxyacetone phosphate (G3P:DHAP) shuttle that controls NAD+/NADH ratio in the glycosome, a specialized organelle that compartmentalizes first seven glycolytic reactions [131]. The G3P:DHAP shuttle transfers electrons from glycerol-3-phosphate to ubiquinone, resulting in the production of ubiquinol, which is then oxidized by AOX. This pathway represents the simplest ETC and is typical only for the bloodstream stage of *T. brucei* [132,133].

Apicomplexans possess two other dehydrogenases: DHOD, which is an essential enzyme involved in pyrimidine synthesis, and the membrane-bound MQO responsible for transferring electrons from the TCA cycle intermediates to the ETC [130]. Two studies of in vitro cultured erythrocytic stages supported a conclusion that the only essential function of the *Plasmodium* ETC is to regenerate ubiquinone, as this molecule is required by DHOD [134,135]. These results are, however, confronted by in vivo-obtained data, which identified Ndh2 as a potential drug target [117,136]. Nevertheless, the importance of the ETC in *Plasmodium* is exemplified

by the potent anti-malarial action of atovaquone and antimycin A, both inhibitors of complex III [137].

Respiratory complexes III and IV are two final enzymes, with the former one catalyzing the reduction of cytochrome *c*, which is then oxidized by complex IV and the electrons are being passed to molecular oxygen, producing water. Interestingly, these two heme-containing complexes are absent from the mitochondrion of *Phytomonas* and the bloodstream stage of *T. brucei* [133]. Complex III, but not complex IV is uniquely absent in the phototropic relative of apicomplexan parasites *Chromera* and thus its branched ETC is divided into two independently operating pathways [40,49].

Not only the entry of electrons is branched in a number of aerobic protists, but also their exit can be directed to two different oxidases. In addition to classical complex IV, the plant-like cyanide-insensitive alternative oxidase (AOX) is present in *T. brucei*, *N. gruberi*, *Acanthamoeba* and *Chromera* [40,103,138,139]. Similarly to Ndh2, AOX fulfills its function without translocating protons across the mt inner membrane. For the bloodstream stage of *T. brucei*, AOX is the only terminal oxidase and thus represents a promising target for chemotherapeutic development [140]. Insect stage of *T. brucei*, as well as *Acanthamoeba* and *N. gruberi* contain both terminal oxidases, allowing them to redirect electrons between proton-pumping and radical oxygen species (ROS)-producing complexes III and IV and the single-subunit AOX.

All the discussed parasites encode in their nuclear and mt genomes genes that allow them to produce, at least in theory, ATP via oxidative phosphorylation (OXPHOS). While the need to produce ATP this way is dictated by the availability of different nutrients in their environment, parasitic protists retain an enormous metabolic flexibility to quickly adapt to changes in their energy suppliers. For example, OXPHOS becomes attenuated by high concentration of glucose, as was proposed for *Leishmania* promastigotes, *T. cruzi* trypomastigotes [141,142] and erythrocytic stage of *Plasmodium* [130,143]. It is tempting to speculate that such an attenuation of OXPHOS leads to decreased ROS production and thus to reduced oxidative stress.

When amino acids and fatty acids become the metabolites taken up predominantly, OXPHOS becomes essential for maintaining the cellular ATP levels as exemplified by the *Toxoplasma* tachyzoites [144], the insect stages of *T. brucei* and *Plasmodium* [145,146], and the *T. cruzi* and *Leishmania* amastigotes [133,147,148]. Interestingly, contrary to the transcriptomic and metabolomic studies, recent work using in vitro axenically grown *Leishmania* amastigotes implicated a dispensable role of OXPHOS for this mammalian-dwelling stage [149]. However, since these parasites were grown in media containing a high concentration of glucose, the observations arising from this study may just reflect the versatility and flexibility of the energy metabolism, as shown previously for the insect stage of *T. brucei* [150]. A remarkable fine tuning of OXPHOS was observed in *T. cruzi* trypomastigotes, in which the increased activity of complexes II and III along with the simultaneous reduction of complex IV activity caused an electron bottleneck that resulted in an electron leak and thus ROS formation. This oxidative pre-conditioning may help to improve protection of the amastigotes against the host immune system [141].

An extreme case of adaption is represented by the bloodstream stage of *T. brucei*, which depends solely on ATP produced by glycolysis and lacks the OXPHOS pathway including proton pumping complexes III and IV. Interestingly, these cells maintain the essential $\Delta\psi m$ by the hydrolytic activity of F_oF_1 ATPase with the concomitant proton translocation across the mt inner membrane [151,152]. Moreover, the naturally emerging "petite" mutant of *T. brucei* [153], *Trypanosoma b. evansi*, which partially or completely lost kDNA, generates the $\Delta\psi m$ electrogenically using ATP hydrolysis coupled to the ATP^{4-}/ADP^{3-} exchange by an ATP/ADP carrier [152,154].

Whether fully developed or in a reduced state, the mitochondria are key contributors to the vitality and flexibility of aerobic parasitic protists. Inhibition of any of these essential pathways, especially those that significantly diverged or are even absent from the mitochondria of their mammalian hosts, is consequently in the spotlight for anti-parasitic drug development.

7. Iron–sulfur cluster assembly

Regardless of how far the reduction of the aerobic mitochondrion progressed in various (mostly anaerobic) protist lineages, one metabolic pathway is invariably retained—the iron–sulfur (Fe–S) cluster assembly. Fe–S clusters belong to the most ancestral cofactors and are likely present in every extant cell. Intriguingly, despite their generally relatively simple chemical structure, their biosynthesis is highly complex and each cell has to synthesize all clusters incorporated into its proteins [155]. A typical eukaryotic complement of proteins requiring Fe–S clusters for their activity exceeds 100 proteins, many of which are involved in essential cellular processes, such as DNA replication and maintenance, translation, respiration etc. [156–158].

Although some recent studies indicate that the extra-mitochondrial Fe–S proteins could be matured without the involvement of the mitochondrion [159,160], the organelle still remains central for their overall biosynthesis, which is initiated by the release of sulfur from a cysteine by cysteine desulfurase. This is followed by the engagement of frataxin as a sulfur-transfer-enhancer and possible iron-acquisitor [161,162]. The Fe–S clusters are synthesized on a conserved scaffold protein with dedicated participation of the ETC [163]. All mt components of the canonical eukaryotic pathway are present in *T. brucei* and other kinetoplastids [164], proving that its complexity did not substantially increase with multicellularity. One interesting exception is the capacity of the conserved intermembrane space protein Erv1 to export precursors for cytosolic Fe–S assembly in the absence of Mia40, its functional partner in multicellular organisms as well as yeast [165]. However, the key component of the Fe–S cluster export machinery, Atm, fulfills its expected function in *T. brucei* [166], where the clusters are incorporated into the extra-mitochondrial proteins via a conserved cytosolic Fe–S protein assembly (CIA) pathway [167].

Due to its active respiratory chain rich in the Fe–S cluster-containing proteins, the insect stage of *T. brucei* requires substantially more clusters in its mitochondrion than the bloodstream stage, for the organelle of which no Fe–S cluster-dependent enzymatic activity is known [168]. Hence, while in the insect stage only a fraction of mitochondrion-generated sulfur precursors is exported to the cytosol, it appears that in the bloodstream stage most of them leave the organelle [9].

Much less is known about the Fe–S cluster assembly of the mitochondrion of other aerobic protists including the notorious *Plasmodium*, which requires the ETC for the regeneration of ubiquinone [134] and thus synthesizes required clusters within the mitochondrion. Moreover, it retains components of both the mt export and CIA machineries, and also synthesizes clusters via a sulfur mobilization pathway in its non-photosynthetic plastid, the apicoplast [169].

8. Heme metabolism

Heme is a ubiquitous prosthetic group required by all extant cells, although at least one exception is known [170]. It is either synthesized by a tetrapyrrole biosynthesis pathway or, much less frequently, scavenged from the environment [171]. Heme is usually part of proteins participating in ETC and redox reactions. It is mostly associated with an aerobic lifestyle, as anaerobes pos-

sess only very few hemoproteins [172]. Several parasitic aerobes have lost the heme synthesis pathway, although since the loss predated the switch to the parasitic life style, it was probably not triggered by the easy access to heme from the host. As heme auxotrophs, *Trypanosoma* species rely on obtaining this cofactor from their hosts, whereas *Leishmania* species regained the last three steps of heme synthesis by horizontal gene transfer from a γ-proteobacterium, which are localized in its mitochondrion [173]. At the same time, *Leishmania* imports heme from the host via leishmania heme response 1 protein [174], while the bloodstream stage of *T. brucei* was shown to acquire heme via the haptoglobin–hemoglobin receptor [175]. How is heme imported into the mitochondrion of *T. brucei*, where most of it is required as a cofactor of cytochromes, remains unknown, although Mdl is becoming a promising candidate of the organellar import [166].

The situation is quite different in another prominent aerobic protist, *Plasmodium*. Although this blood-dwelling parasite has an unlimited access to host heme, *Plasmodium* and related apicomplexans retained an active biosynthesis pathway [176]. The erythrocytic stages import heme but a vast majority of it is sequestered as crystals of insoluble hemozoin in the food vacuole [177], probably due to the fact that the parasite lost the capacity to detoxify it [178]. Most cellular heme is likely needed in the mt respiratory chain as in other non-photosynthetic organisms. Yet on the contrary to kinetoplastids, the cofactor is also synthesized by a heme biosynthesis pathway that winds thru the mitochondrion, the cytosol and the apicoplast [173,179]. While it was assumed that all mitochondrion-localized heme is a product of this pathway, latest results indicate that in the intraerythrocytic part of the *Plasmodium* life cycle, at least a fraction of the mt heme is host-derived, scavenged via an unknown mechanism from the food vacuole [180]. Remarkably, the *Plasmodium* heme synthesis pathway was recently shown to be essential in the mosquito stages but not in the mammalian stages [181].

The photosynthetic alveolate *Chromera* evolved a unique way of heme synthesis. Although being a full phototroph, it synthesizes heme via the originally heterotrophic C4 pathway, similar to the non-photosynthetic apicomplexans [49,182]. Based on analyses of their genomes, the heme synthesis pathway seems to be absent in *N. gruberi* [15], whereas it has been retained by *Acanthamoeba* [183]. Whatever the mechanism via which aerobic parasitic protists obtain heme, they have to balance between its essentiality for key mt pathway and its overall toxic properties.

9. Conclusions

Protists are the most diverse group of extant eukaryotes and their mitochondria reflect that. Indeed, these organelles widely differ in aerobic protistan parasites, and seem to invariably contain only three processes. The first one is the assembly of Fe–S clusters performed by a highly conserved pathway. The second process is the transport of electrons from reductive equivalents generated in the mitochondrial matrix or cytosol to the terminal acceptor—oxygen. The flow of electrons via ETC of various design is mostly accompanied by the generation of ATP. In some cases, however, when ATP is not produced, ETC serves other purposes, such as balancing the NAD^+/NADH ratio in the glycosome or reducing intermediates in pyrimidine synthesis. It is very likely that it is the ETC that constraints all these mitochondria to retain their genomes and gene expression systems, which is their third common feature. Interestingly, the genetic element of the organelle became a particularly inventive evolutionary playground, with some protists (e.g., *Naegleria*) remaining conservative, as they encode all standard components of gene expression on a circular mapping chromosome, while others minimized the coding capacity, import almost all components from the cytosol and structurally modified their mitochondrial genomes. Particularly striking examples of departures from the standard arrangement are represented by ribosomes assembled from fragmented rRNAs or baroque RNA editing of a handful of mt transcripts that depends on dozens of dedicated proteins imported from the cytosol, as well as arguably the most complex genomic structure.

Research on mitochondria of parasitic protists uncovered that rather than a general trend for simplification, these organelles exhibit diverse outputs of evolutionary experiments, frequently fine-tuned to special life strategies of their bearers. Studies of protist mitochondria not only broaden our view of how flexible they can be, but also disclose potential drug targets that may help to control these dangerous parasites.

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