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Unusual Mitochondrial Genome Structures throughout the Euglenozoa

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Mitochondrial DNA of Kinetoplastea is composed of different chromosomes, the maxicircle (bearing 'regular' genes) and numerous minicircles (specifying guide RNAs involved in RNA editing). In trypanosomes [Kinetoplastea], DNA circles are compacted into a single dense body, the kinetoplast. This report addresses the question whether multi-chromosome mitochondrial genomes and compacted chromosome organization are restricted to Kinetoplastea or rather occur throughout Euglenozoa, i.e., Kinetoplastea, Euglenida and Diplonemea. To this end, we investigated the diplonemid *Rhynchopus euleeides* and the euglenids *Petalomonas cantuscygni, Peranema trichophorum* and *Entosiphon sulcatum*, using light and electron microscopy and molecular techniques. Our findings together with previously published data show that multi-chromosome mitochondrial genomes prevail across Euglenozoa, while kinetoplast-like mtDNA packaging is confined to trypanosomes.
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Key words: euglenozoan protists; mitochondrial chromosomes; mitochondrial ultrastructure.

Introduction

Mitochondrial genomes are astoundingly diverse across the various eukaryotic lineages. In most animals, fungi and plants, mitochondrial DNAs (mtDNAs) consist of a single chromosome whose physical structure is monomeric circular or multimeric linear (but circular mapping; reviewed in Nosek and Tomaska 2003). Sizes of their mtDNA vary from ~15—20 kbp to several hundred kbp. Gene content, which is essentially independent from genome size, ranges from two dozen in Metazoa to ~50 genes in plants and certain fungi (reviewed in Gray et al. 2004). Studies of protists unveiled the most bacteria-like mitochondrial genome in the flagellate *Reclinomonas americana* (Lang et al. 1997), and an even broader diversity of mitochondrial genome structure than seen in animals, fungi and plants together (Gray et al. 2004). Regarding mtDNA shape, size, conformation and ploidy, anything goes in protist mitochondria (Burger et al. 2003).

The mitochondrial genome of the protist group Kinetoplastea has intrigued the scientific community

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most profoundly. Their mtDNA, referred to as the kinetoplast DNA (kDNA), consists of a maxicircle (present in a dozen copies) and numerous minicircles (present in several thousand copies). The maxicircle generally ranges from 20 to 40 kbp in size depending on the species and encodes typical mitochondrial genes, yet mostly in an encrypted fashion. The numerous types of minicircles, whose sizes vary from 0.5 to 10 kbp across kinetoplastids, encode guide RNAs that serve as templates for decrypting maxicircle genes by a mechanism known as RNA editing (reviewed in e.g., Lukeš et al. 2005).

The physical and spatial structure of kinetoplastid mtDNAs is diverse. First and best described is kDNA of parasitic trypanosomatids including the notorious human pathogens *Trypanosoma* and *Leishmania*. Their mtDNA, designated eu-kDNA, forms a single dense body composed of a compact catenated meshwork of maxi- and minicircles. The free-living *Bodo saltans* possesses pro-kDNA, a single dense body of non-catenated circles. Pan-kDNA, which is found in *Bodo caudatus* and *Cryptobia helicis*, is characterized by loose and large conglomerates of monomeric circles, filling most of the mitochondrial lumen. A fourth type, poly-kDNA, has been reported in *Dimastigella mimosa*, *D. trypaniformis* and *Cruzella marina*. Poly-kDNA consists of monomeric circles packed in multiple bundles that are scattered throughout the mitochondrial lumen. Finally, mega-kDNA of *Trypanoplasma* and *Jarellia* is characterized by tandemly arranged minicircles that are distributed evenly throughout the mitochondrial lumen (Lukeš et al. 2002).

Molecular phylogeny places Kinetoplastida as a sister clade to Diplonemea, with Euglenida branching basally to the two former groups (e.g. Simpson and Roger 2004); all three taxa together form the Euglenozoa. The mitochondrial genomes of both Diplonemea and Euglenida are only poorly characterized, due to several reasons. In the model organism *Euglena gracilis*, mtDNA structure and size remain elusive despite intensive studies (Buetow 1989; Gray et al. 2004; Manning et al. 1971; Nass et al. 1974; Talen et al. 1974; Yasuhira and Simpson 1997); for the other euglenids and for diplonemids, we lack established culture conditions, axenic strains, or specialized organelle isolation protocols. Recent observations suggest that the unusual mitochondrial genome organization seen in kinetoplastids might occur in other Euglenozoa as well. First, a mitochondrial inclusion body reminiscent of a kinetoplast has been discovered in the euglenids *Petalomonas cantuscygni* and *P. mediocanellata* (Leander et al. 2001). Second, we found that mtDNA of the diplonemid *Diplonema papillatum* consists of numerous, circular, 6 and 7 kbp long chromosomes (Marande et al. 2005).

To investigate the architecture and spatial organization of mtDNA across all Euglenozoa, we chose to survey poorly studied euglenids and diplonemids. Within the euglenids, we examined *Petalomonas cantuscygni*, *Peranema trichophorum* and *Entosiphon sulcatum*, heterotrophic members of the allegedly most basal clade, the Heteronematina (Adl et al. 2005). Within diplonemids, we investigated the recently described species *Rhynchopus euleeides* (former *Rhynchopus* sp. 1) (Roy et al. 2007). By employing microscopy and molecular-biology techniques, the presented study sheds light on the elusive mtDNA of these evolutionary and ecologically significant protists.

Results

Distribution of mtDNA within the Cell

Rhynchopus cells (Fig. 1, 1A—1C) stained with 4′,6′-diamidino-2-phenylindole (DAPI) reveal abundant mtDNA in a thin (0.25—0.5 μm wide) reticulated pattern. Inspection of different focal planes indicates that the network lines the periphery of the cytoplasm. The peripheral location of mitochondria is confirmed by electron microscopy (see below).

In *Petalomonas* (Fig. 1, 2A—2C), mtDNA is similarly abundant and also distributed in a network pattern. However, in this species, the network branches are thicker (0.5—1.5 μm wide) compared to *Rhynchopus*. Notably, the fluorescence of *Petalomonas* nuclear DNA is weaker than that of mtDNA, regardless of the focal plane. We speculate that a particular chromatin structure or composition renders the nuclear DNA barely accessible to the dye. The area in the cell that conspicuously lacks fluorescence corresponds to the feeding and flagellar apparatuses, in the anterior end of the cell (at the top of Fig. 1, 2B and 2C).

The extra-nuclear DNA of *Entosiphon* (Fig. 1, 3A—3C) appears much less abundant compared to the two above species. Certain areas of the cell harbour small diffusely fluorescent spheres of ~0.2 μm diameter, whereas in other areas, fluorescence is distributed homogenously in a large, cloud-like pattern.

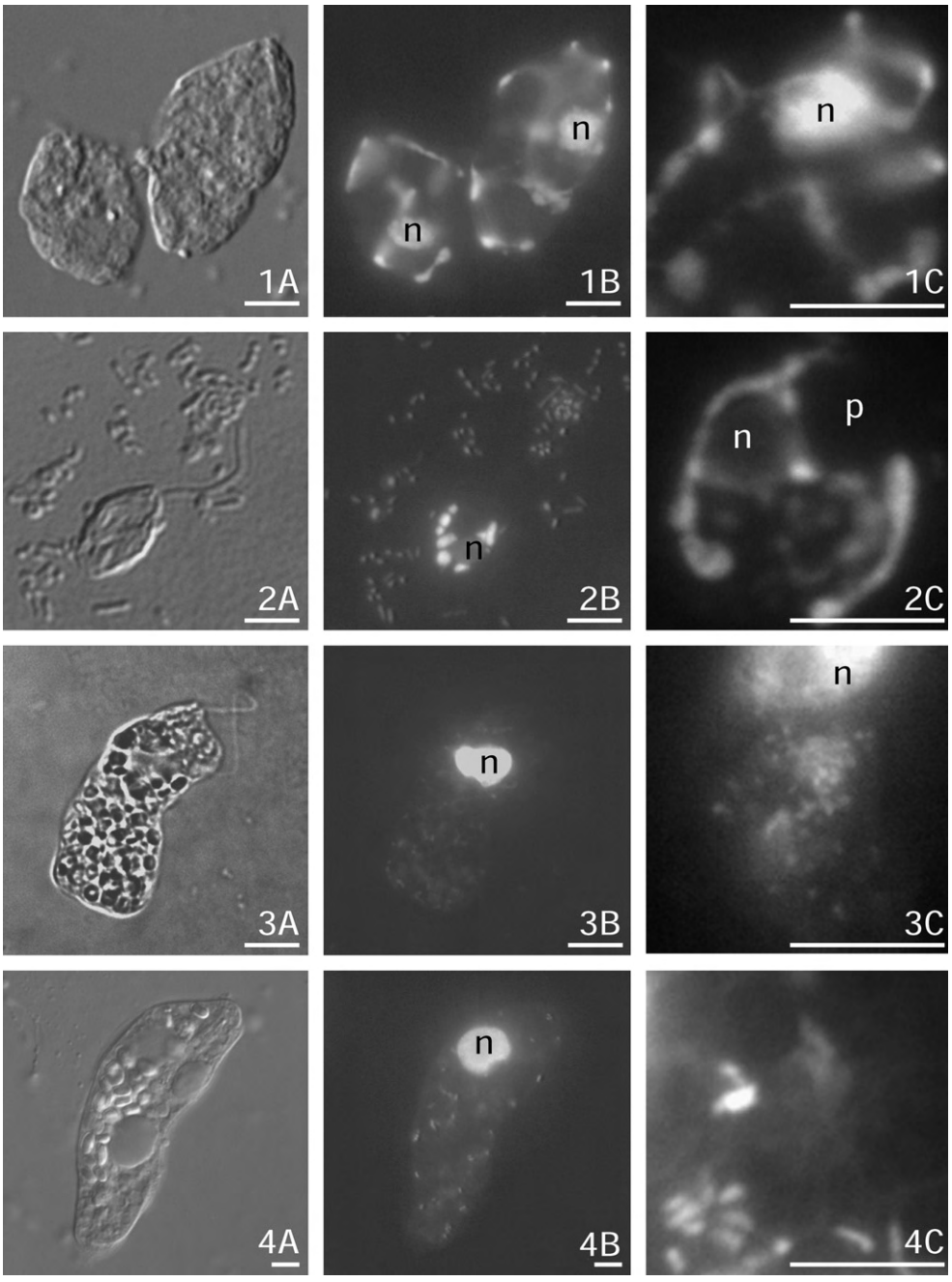

Figure 1. DNA distribution within cells. (1—4, A) Light microscopy of fixed cells stained by DAPI. (1—4, B) Fluorescent microscopy of same cells as in A. (1—4, C) Fluorescent microscopy at higher magnification. (1A—C) *R. euleeides*. (2A—C) *P. cantuscygni*. (3A—3C) *E. sulcatum*. (4A—4C) *P. trichophorum*. (1—2, B+C) DNA is abundant and distributed in a reticulated pattern. (3—4, B+C) DNA is scarce and scattered across the cells. Note stained bacteria in the medium in Fig. 2B. Between 50 and 100 cells of each species were inspected. **n**, nucleus; **p**, sub-apical pocket. Bar = 5 μm.

Peranema cells (Fig. 1, 4A—4C) as well show relatively little DNA staining outside the nucleus. Numerous distinct fluorescent bodies (~1.0 μm length) are scattered all over the cell. These bodies are oval to rod-like in shape and often arranged in rosettes. Electron microscopy confirms that these bodies are indeed mitochondria (see Fig. 2D).

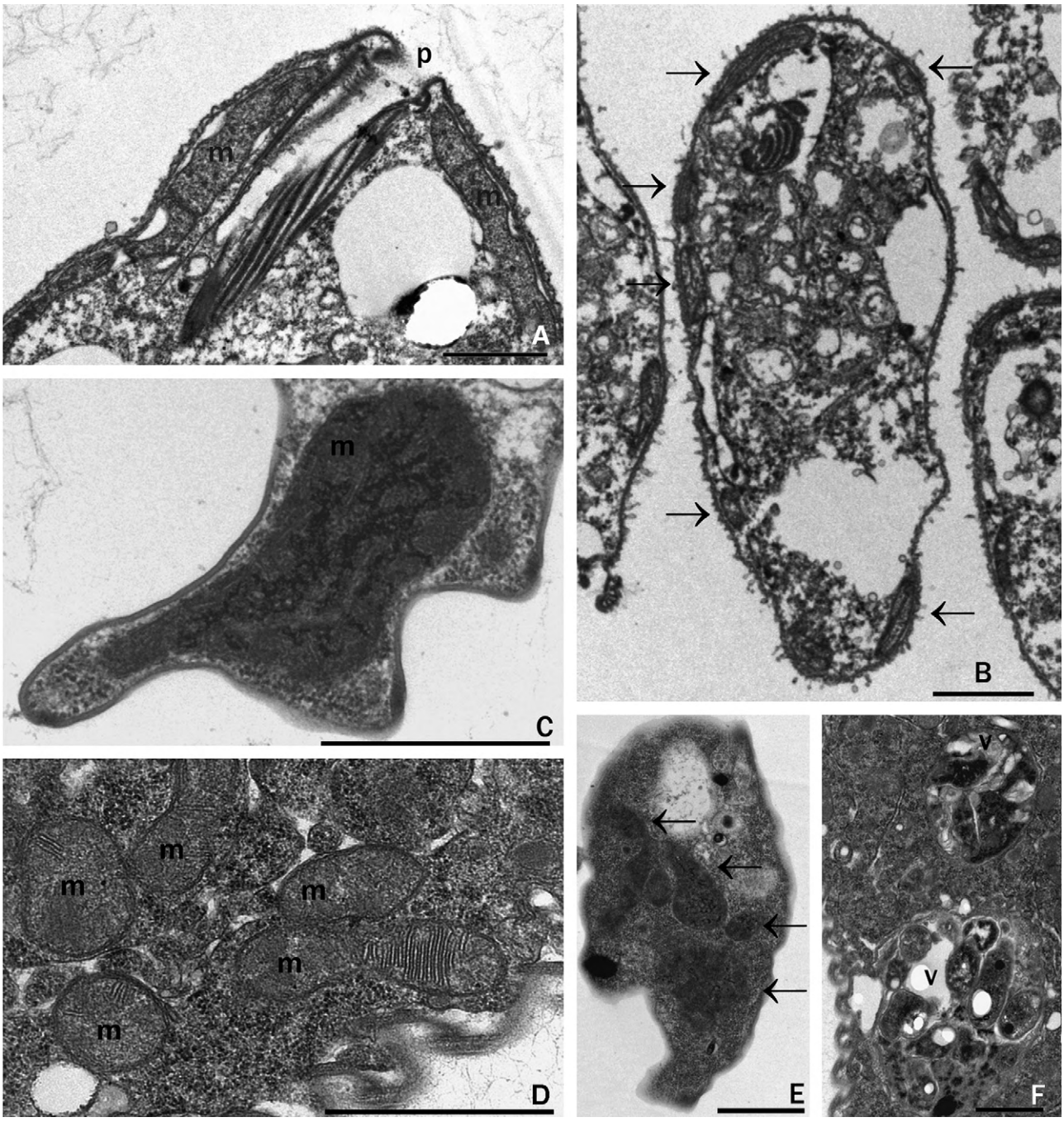

Figure 2. Transmission electron microscopy of cell sections. (A, B) *R. euleeides*. Long and reticulated mitochondrion lining the cell periphery and containing few cristae. (**A**) Apical region. (**B**) Whole cell. Arrows show mitochondrial sections, some with longitudinal cristae. (**C**) *P. cantuscygni*. Transverse section of the cell showing a large and dense mitochondrion with disperse inclusions. (**D**) *P. trichophorum.* Transverse section with numerous sack-shaped mitochondria containing multiple cristae. (**E**) *P. cantuscygni*. Whole cell; arrows show large mitochondrial sections. (**F**) *P. trichophorum*. Transverse section with vesicles, likely food vacuoles, containing dense and rod-shaped bodies, most probably bacteria. About 10—20 sections per species were inspected. **m**, mitochondrion; **p**, sub-apical pocket; **v**, vesicle. Bar $= 1\,\mu m$.

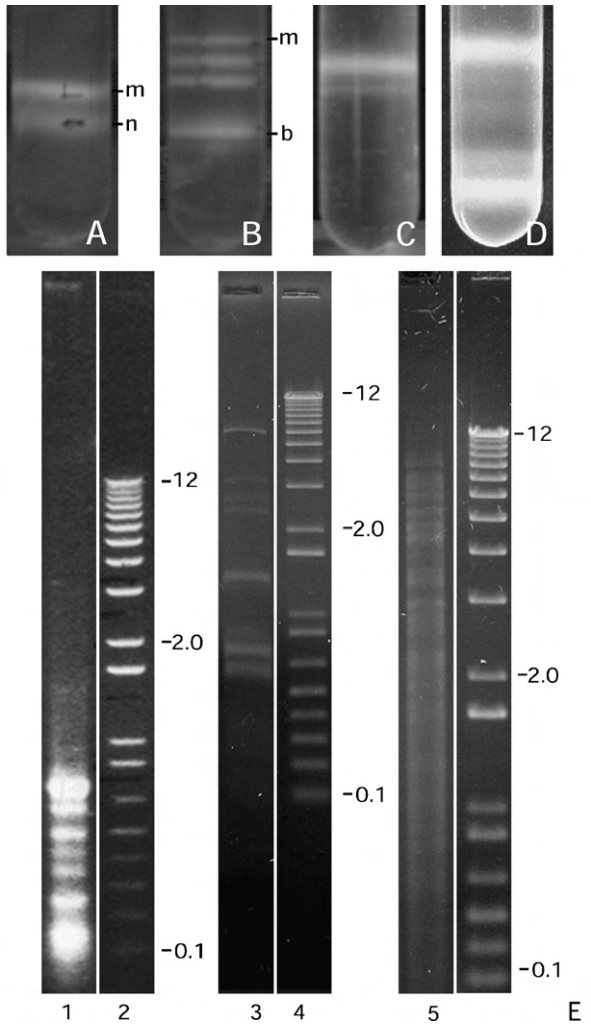

Figure 3. DNA separation and identification. (A—D) CsCl/bisbenzimide gradient. (**A**) *R. euleeides*. Note that mitochondrial DNA is more abundant than nuclear DNA. (**B**) *P. cantuscygni*. The two middle bands were not examined in detail. (**C**) *P. trichophorum*. Mitochondrial DNA corresponds to the second band from top, referred to G+C-rich fraction in the text. A weak band in the middle of the gradient may originate from a bacterial contaminant in the culture. (**D**) *E. sulcatum.* Multiple bands of unknown origin. Note that *E. sulcatum* is fed with bacteria. (**E**) Restriction enzyme digestion patterns. Lanes 2, 4, and 6, molecular weight marker 1 kb+. Lane 1, *R. euleeides* mtDNA (most A+T-rich fraction in A) digested with RsaI. Lane 3, *P. cantuscygni* mtDNA (most A+T-rich fraction in B) digested with RsaI. Lane 5, *P. trichophorum* mtDNA (second band from top in C) digested with SspI. **m**, mitochondrial DNA; **n**, nuclear DNA; **b**, bacterial DNA. Molecular weight in kbp.

Mitochondrial Ultrastructure

Sections of *Rhynchopus* cells inspected by electron microscopy show a reticulated and most likely single mitochondrion. The organelle is positioned at the cell periphery, virtually surrounding the cytoplasm (Fig. 2A, B). The width of mitochondrial sections (0.2—0.6 μm) corresponds to that of the network seen in DAPI staining. The scarce cristae, which can be quite long, are arranged longitudinally, a feature seen in all diplonemids. Earlier more detailed ultrastructural analyses show that mitochondrial cristae in diplonemids are rather lamellar than discoidal, and therefore atypical for Euglenozoa (Kent et al. 1987; Marande et al. 2005; Schnepf 1994; Simpson 1997; Triemer and Farmer 1991; Triemer and Ott 1990; Vickerman 2002). In fact, discoidal cristae are considered a landmark for Euglenozoa and their sister group, the Heterolobosea/Percolozoa (Cavalier-Smith 1993; Nikolaev et al. 2004), and gave rise to the creation of the super group Discicristata (Cavalier-Smith 1998; Patterson 1994).

Petalomonas likely possesses a single mitochondrion as well. The organelle occupies a large portion of the cytoplasm and usually appears in sections as big spheres, often up to 0.8 μm in diameter (Fig. 2C, E). About 50% of the mitochondrial section area consists of irregular, filament-shaped inclusions (0.06—0.14 μm wide). We do not see a ribbon-like pattern in these inclusions as described by others (Leander et al. 2001), probably due to differences in the fixation protocol. According to Leander et al., these inclusion bodies are located exclusively in the anterior end of the cell, close to the flagellar basal body.

Peranema (Fig. 2D) stands out in that it contains numerous, small, distinct mitochondria of oval shape (length 0.2—1.3 μm). Cristae are arranged transversally, occasionally intersecting completely the mitochondrial matrix; in some mitochondria, they are abundant, in others rather scarce. In addition, we observe other spherical compartments (Fig. 2F) of 1.4—3 μm diameter, which include smaller vesicles (0.5—1.3 μm long). These compartments probably represent food vacuoles enclosing bacteria.

Identification and Physical Properties of mtDNA

Buoyant density gradient centrifugation in CsCl plus bisbenzimide allows nucleotide-composition based separation of various genomes present in

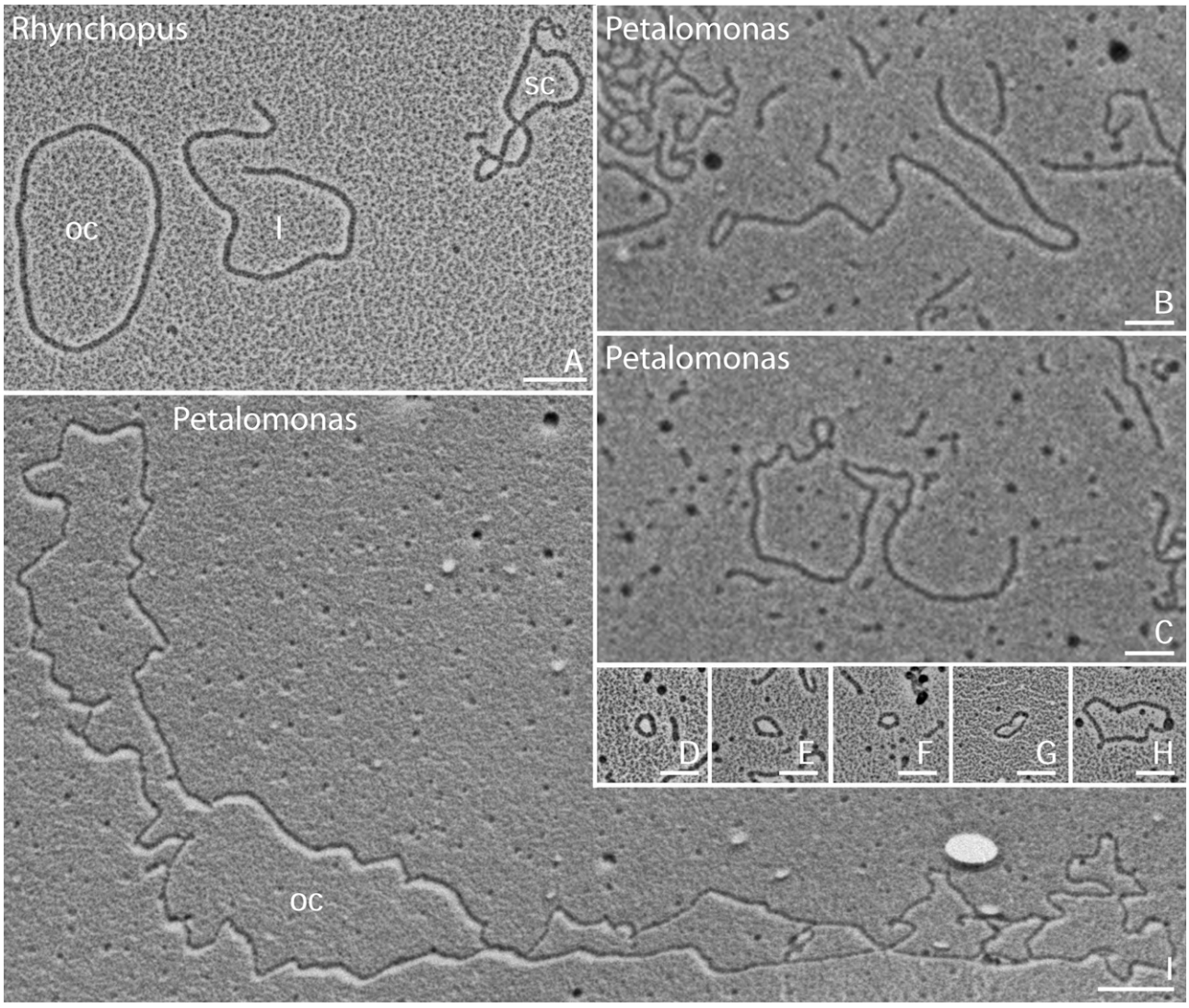

Figure 4. Spread DNA visualized by transmission electron microscopy. (**A**) *R. euleeides*. Mitochondrial chromosomes of 7 kbp in three different conformations. (B—I) *P. cantuscygni*. (**B—C**) Linear molecules with lariat ends. (**D—H**) Small circular molecules of various sizes. (**I**) Large circular molecules. **oc**, open circle; **l**, linear; **sc**, supercoiled conformation. Bar = 200 nm (corresponding to 760 bp using pGEM11 as an internal standard).

the cell. We assessed the sequence complexity of the separated DNA species by restriction enzyme digestion. Putative mtDNA was analyzed further by electron microscopy in order to determine the genome's topology and size.

Total DNA extracted from *Rhynchopus* separates during centrifugation into two similarly broad bands (Fig. 3A). Their subcellular origins were inferred based on the complexity of the restriction enzyme pattern (Fig. 3E, lane 1). The A+T-rich fraction with its simple restriction pattern is considered mtDNA; a similarly low-complexity restriction pattern is seen in *Diplonema* mtDNA and has been confirmed by DNA sequencing. The restriction digest of the *Rhynchopus* G+C-rich fraction displays a 'smear' rather than distinct fragments (data not shown) and is therefore most likely nuclear DNA. Electron microscopy of the mtDNA fraction reveals small circular monomeric molecules (Fig. 4A). Of the >60 individual molecules measured, sizes fall into two distinct classes: One third of $\sim$7 kbp ($\pm$0.1) and two third of 7.7 kbp ($\pm$0.2), with only a small percentage ($\sim$1%) of dimers. At least 20% of the molecules

are supercoiled and ~70% are of relaxed conformation. The residual ~10% are linear molecules that are probably broken secondarily.

Total DNA extracted from *Petalomonas* forms four bands in the CsCl gradient (Fig. 3B). The restriction digest pattern of the most A+T-rich fraction is of low complexity, indicating that this band consists of mtDNA (Fig. 3E, lane 3). Electron microscopy of spread A+T-rich DNA reveals mostly linear molecules of up to 40 kbp in size, some of which possessing lariat ends (Fig. 4B—C). A few molecules form circles of 1—2.5 kbp (Fig. 4D—H). Among more than hundred molecules screened, we found two instances of a circular 40-kbp molecule (Fig. 4I). The two middle bands of the CsCl gradient show 'smeary' restriction patterns (data not shown) and have not been characterized further. Finally, the lowest band must originate from the food bacterium, because it has the same buoyant density as *Pseudomonas fluorescens*, on which *Petalomonas* was grown.

In *Peranema*, two distinct bands occur in the CsCl gradient (Fig. 3C). In this organism, mtDNA corresponds most probably to the second band from top, the more G+C-rich fraction, because the complexity of its restriction pattern is lower than that of the A+T-rich fraction (Fig. 3E, lane 5). Electron microscopy of the G+C-rich fraction shows exclusively linear DNA molecules, with sizes of 1—75 kbp in approximately equimolar distribution (~100 molecules were measured; data not shown). The relatively high background in the restriction pattern suggests that the putative mtDNA fraction is contaminated by nuclear DNA. It is unclear whether the broad range of molecule sizes seen in electron microscopy is the authentic form of mtDNA or mainly broken nuclear DNA.

Total DNA obtained from *Entosiphon* separates into approximately six bands by CsCl gradient centrifugation (Fig. 3D). All fractions produce a homogeneously smeary restriction digest pattern, regardless of the enzyme used (data not shown). At present it is unknown which of the isolated fractions contains mtDNA.

Discussion

Euglenozoa, United by their Dissimilar Mitochondria?

We report here a survey of mitochondria and their genomes from poorly studied Euglenozoa: the diplonemid *Rhynchopus euleeides* and the euglenids *Entosiphon sulcatum*, *Peranema trichophorum* and *Petalomonas cantuscygni*. Several experimental approaches were employed in this study. Transmission electron microscopy of cell sections shows the localization of mitochondria within the cell and the shape of their cristae. Fluorescence microscopy of cells stained with DAPI reveals mtDNA and its distribution within the organelle. CsCl density centrifugation separates cellular DNA species based on their A+T-content. Restriction digestion indicates the sequence complexity of DNA species and therefore allows the distinction between nuclear (highly complex) and mitochondrial (less complex) origin. Finally, electron microscopy of spread mtDNA determines the shape and length of the molecules.

Localization of Mitochondria within the Cell: Mitochondria of *Peranema*, *Petalomonas* and *Rhynchopus* are confined to the cell periphery, sometimes tightly lining the inner side of the plasma membrane. The same has been reported earlier for mitochondria of *Entosiphon* (Triemer and Farmer 1991) and of other euglenid and diplonemid species (Belhadri and Brugerolle 1992; Cann and Pennick 1986; Hayashi and Ueda 1989; Hilenski and Walne 1985a, 1985b; Leander et al. 2001; Leedale 1967; Leedale and Buetow 1970; Marande et al. 2005; Mignot 1966; Pellegrini 1980a, 1980b; Roy et al. 2007). Yet, not all Euglenozoa share this feature. In kinetoplastids, the size, ultrastructure and intracellular location of the mitochondrion often varies during the life cycle, and the organelle appears physically connected with the basal body (Liu et al. 2005; Shapiro and Englund 1995). Variable morphology of mitochondrial cristae in Euglenozoa questions the utility of this character for high-level taxonomic classification.

Number of Mitochondria per Cell: Kinetoplastids are not the only euglenozoans with a single mitochondrion per cell; the same applies to diplonemids (this report and Marande et al. 2005) and most euglenids. For example, cells of *Petalomonas* and *Entosiphon* apparently contain a single organelle. In addition, earlier studies describe a single ('giant') mitochondrion in *E. gracilis* (Pellegrini 1980b). However, *Peranema* is an exception. As we demonstrate here, this organism possesses multiple, small, clearly distinct organelles (see also Hall 2005).

DNA Organization and Distribution within Mitochondria: Mitochondrial DNA is distributed homogenously throughout the mitochondrial lumen in the diplonemids *Diplonema* and *Rhynchopus* (Marande et al. 2005) as well as in the

euglenid *Peranema*. In *Petalomonas*, the situation is more complicated. Mitochondrial DNA fluorescence is conspicuously absent in the anterior (flagellated) end of *Petalomonas* cells (Fig. 1, 2C), which coincides with the region confining mitochondrial inclusion bodies (Leander et al. 2001). We conclude that the electron-density of these inclusions is not due to compacted mtDNA. Therefore, a structural homology of inclusion bodies and kinetoplasts as proposed by Leander and co-workers is most unlikely. *Entosiphon* mtDNA has a much different appearance, occurring in numerous small agglomerates, termed nucleoids, reminiscent of what has been reported in *E. gracilis* (Hayashi and Ueda 1989; Hayashi-Isimaru et al. 1993). While DAPI-stained *Euglena* nucleoids are 0.7—1.3 μm in diameter and occur in small thread-like clusters, those of *Entosiphon* are less than half that size. Taken together, mtDNA of diplonemids and euglenids is generally dispersed and sometimes organized in tiny bodies, whereas kinetoplast-like mtDNA packaging remains an exclusive character of trypanosomes.

Euglenozoan Mitochondrial Genomes: from Unconventional to Intractable

In *Rhynchopus*, we discovered an unusual mitochondrial genome architecture, consisting of circular 7.0 and 7.7 kbp chromosomes. As published earlier, *D. papillatum* mtDNA has a similar genome structure, but the circles are somewhat smaller (Marande et al. 2005). In addition, our preliminary results show that *Rhynchopus* displays a likewise unconventional fragmentation of mitochondrial genes as *D. papillatum*, with single exons encoded on each chromosome. A radical difference between *R. euleeides* and *D. papillatum* is the nucleotide composition of their mtDNAs. *Rhynchopus* mtDNA has a typical A+T-content (60%), while that of *D. papillatum* is unusually rich in G+C (46.5% A+T), — even more G+C-rich than its nuclear genome.

The mitochondrial genome of *Petalomonas* appears conventional at first sight. In electron microscopy, we observe predominantly linear but also a small number of circular ~40-kbp molecules, plus a few small circles and lariats. In vivo, *Petalomonas* mtDNA consists probably of circularly permutated, linear chromosomes (which would occur circular mapping in restriction analysis and DNA sequencing). The various forms observed could be generated by rolling circle (sigma) replication as described for plants and fungi (Backert et al. 1997; Maleszka et al. 1991). Preliminary sequencing of a library constructed with the bona fide mtDNA is consistent with a ~40 kbp circular-mapping chromosome (Roy et al., unpublished). However, despite ~25 kbp of contiguous sequence, only part of a single gene (*atp6*, encoding a subunit of ATP synthase) could be annotated with confidence. If *atp6* is present in *Petalomonas* mtDNA, then this genome should also contain the highly conserved genes *cob*, *cox1, cox2 and cox3* (specifying apocytochrome b and three subunits of cytochrome c oxidase; see GOBASE at http://gobase.bcm.umontreal.ca/ 'Gene distribution' page (O'Brien et al. 2006). Therefore, we suspect massive gene encryption and RNA editing, exceeding in extent what is seen in trypanosomatid mitochondria.

The presumptive mtDNA of *Peranema* appears larger in restriction analysis than that of the other taxa included in our study. Length measurement by electron microscopy shows a wide range of molecule sizes. One explanation therefore is that mtDNA is contaminated with high-complexity DNA of nuclear origin. DNA sequencing of this material yielded mostly sequences without recognizable coding content, which may indicate either nuclear DNA or, alternatively, massively encrypted mtDNA. The few significant hits found match genes from Wolbachieae, Rickettsieae, and other intracellular and free-living alpha-proteobacteria. Whether these latter sequences originate from endo/ectosymbionts of *Peranema* or from an undetected commensal in the culture needs to be determined. In brief, the size and topology of the *Peranema* mitochondrial genome remains obscure.

DNA extracted from *Entosiphon* cultures separates into six bands in CsCl gradient centrifugation. Several of these fractions may be of bacterial origin, because this euglenid is fed on *Silicibacter* and moreover, the culture is not monoxenic. Yet, none of the various DNA fractions yields distinct restriction fragments. This was unexpected. Even if mtDNA would be contaminated with high-complexity DNA, restriction digests should still reveal a low-complexity pattern overlaying a 'smeary' background. Since all DNA fractions look similarly high-complex in restriction analysis, *Entosiphon* mtDNA remains unidentified.

Despite numerous and long-lasting studies, data on *Euglena* mtDNA are confusing. Reports describe collections of diverse linear and sometimes circular molecules, ranging in length from less than 1 kbp to up to 70 kbp, and some authors evoke experimental artefacts accounting for the

various genome structures observed (Buetow 1989; Gray et al. 2004; Manning et al. 1971; Nass et al. 1974; Talen et al. 1974; Yasuhira and Simpson 1997). The only mitochondrial genes identified so far in *E. gracilis* encode fragmented LSU and SSU rRNAs (Gray et al. 2004; see also Buetow 1989) and subunit 1 of cytochrome oxidase (Tessier et al. 1997; Yasuhira and Simpson 1997). In summary, the architecture of *Euglena* mtDNA remains elusive. Clues may come ultimately from comparative studies of less derived euglenids.

Taxonomic Coverage of Euglenozoa

Mitochondrial genomes of Kinetoplastida and particularly the pathogenic trypanosomes, have been well surveyed as outlined in the Introduction (Lukeš et al. 2005; Stuart et al. 2005). For Diplonemea, mtDNA information is now available for both genera *Diplonema* and *Rhynchopus*, the latter data reported here. Within Euglenida, we add data on *Petalomonas*, which is a member of Sphenomonadales. Notably, this species is the most basal euglenid in numerous molecular phylogenies (although with moderate statistical support; e.g., Moreira et al. 2001; von der Heyden et al. 2004). Moreover, we provide information on *Peranema* and *Entosiphon*, which belong to the Heteronematales. Together, Heteronematales and Sphenomonadales form the Heteronematina, which is one of the three large euglenid groups. *Euglena*, with mtDNA data published by others, belongs to Euglenea, the second, large group. Only for the third large euglenid group Aphagea, with its genera *Distigma* and *Rhabdomonas,* are mtDNA data still lacking. By filling in critical information on diplonemids and euglenids, the presented study has added significant taxonomic breadth to the coverage of mtDNA diversity in Euglenozoa.

Concluding Remarks

We show here that mitochondrial multi-chromosome genomes, and probably gene encryption and RNA editing as well, are much more widespread in Euglenozoa than has been previously thought. This report also illustrates the challenges of unravelling genome structure and coding content in Euglenozoa mitochondria. In order to solve the remaining puzzles, experimental approaches must be most resourceful in order to match the exuberant inventiveness of mitochondrial evolution.

Methods

Culture: *Rhynchopus euleeides* n. sp. (ATCC 50226, previously named '*Rhynchopus* sp.1') was purchased from the American Type Culture Collection. The organism was cultivated axenically in modified artificial sea water consisting of 3.3% sea salts (Instant Ocean), vitamins (0.5 μg/ml biotin, 0.5 μg/ml B12, 100 μg/l thiamine-HCl), and trace metal elements (4.36 μg/l Na_2EDTA, 3.15 μg/l $FeCl_3$ $6H_2O$, 9.8 μg/l $CuSO_4$ $5H_2O$, 22 μg/l $ZnSO_4$ $7H_2O$, 10 μg/l $CoCl_2$ $6H_2O$, 18 μg/l $MnCl_2$ $4H_2O$, 6.3 μg/l $NaMoO_4$ $2H_2O$), supplemented with 10% horse or fetal bovine serum. *Peranema trichophorum* (CCAP 1260/1B) was purchased from the Culture Collection of Algae and Protozoa (Oban, Scotland). The organism was grown axenically in KNOP medium as described by Saito et al. (2003), but supplemented by 0.01—0.1% crystallized egg yolk as a nutrient source (Saranak and Foster 2005). *Petalomonas cantuscygni* (CCAP 1259/1) was also purchased from the Culture Collection of Algae and Protozoa. This euglenid was cultivated in ASWP medium consisting of 3.3% sea salts, 5% soil extract, 5 mg/l tricine and minerals (5.63 mg/l $NaNO_3$, 0.23 mg/l Na_2HPO_4, 0.19 mg/l K_2HPO_4) and supplemented with 10^4/ml of the feeder bacterium *Pseudomonas fluorescens. Entosiphon sulcatum* (kindly provided by Mark Farmer (University of Georgia, Athens)) was cultured in KNOP medium supplemented with Timothy hay infusion (hay purchased from the pet shop R.C. Hagen Inc., Montreal) and *Silicibacter sp.* A barley seed was added to sustain bacterial growth.

DAPI (4′,6′-diamidino-2-phenylindole)-staining: For detection and quantification of euglenozoan mtDNA, we used DAPI which binds preferentially to A+T-rich DNA by minor-groove interaction (Manzini et al. 1983; Trotta et al. 2003). Cells were fixed for 10 min at room temperature in 4% paraformaldehyde diluted in either artificial sea water or phosphate-buffered saline (PBS). Fixation was stopped by spinning the cells down and resuspending them subsequently in appropriate buffers. After the cells were allowed to adhere onto poly-L-lysine-coated slides for 2 h in a humidity chamber, the slides were stained with 1 μg/ml DAPI in PBS for 5 min. The stained slides were washed, mounted with the antifade reagent (0.233 g 1,4-diazabicyclo-(2,2,2)octane; 1 ml 0.2 M Tris—HCl, pH 8.0; 9 ml glycerol) and examined with a Zeiss Axioplan 100 microscope.

Electron microscopy: For transmission electron microscopy of cells, cultures were washed twice in artificial seawater or PBS, centrifuged at

low-speed (~1200 × *g*) and fixed in 2% glutaraldehyde in 0.25 M phosphate buffer (PB) overnight at 4 °C. After fixation, cells were washed in buffer supplemented with 4% glucose, pelleted and embedded in 2% agarose. Cells were post-fixed with 2% OsO_4 in PB for 2 h at room temperature and then washed in 0.25 M PB. After dehydration in graded series of ethanol, cells were embedded in PolyBed (Polysciences). Ultra thin sections were stained with lead citrate and uranyl acetate and examined under a JEOL JEM 1010 microscope.

For transmission electron microscopy of DNA, serial dilutions of DNA (0.1, 0.01, 0.001 mg/ml) were precipitated by cytochrome *c* (0.1 mg/ml) in 0.5 M ammonium acetate solution. DNA was transferred on clean 200-mesh grids coated with 3.5% collodium using a hypophase bath of 0.25 M ammonium acetate. Grids were shadowed by Pt80:Pd20 and carbon coated. Observations were conducted using a Philips CM electron microscope. The cytochrome-*c* method is described in detail elsewhere (Coggins 1987; Fergusson and Davis 1978). The precise magnification of molecules was determined by replica granting (Balzers). The contour length of molecules was measured using a curvimeter. Plasmid pGEM11 served as a size standard for the conversion from μm to kbp. A mean error was calculated for size distribution of *Rhynchopus* chromosomes.

DNA extraction: Confluent axenic or monoxenic cells were collected with a cell scraper, pelleted at low speed and washed in NET-50 buffer (100 mM NaCl, 50 mM EDTA, 1 mM Tris, pH 8.0). DNA was extracted by 1 mg/ml pronase E (Sigma) and 3% N-laurosyl-sarcosine. DNA was purified by series of phenol-chloroform extraction and ethanol precipitation.

CsCl/bisbenzimide centrifugation: Buoyant density gradient centrifugation in CsCl plus bisbenzimide (Hoechst dye 33258) separates DNAs based on nucleotide composition. Bisbenzimide intercalates specifically in A+T-rich regions of DNA, thus decreasing its density. DNA was resuspended in TE and 10 μg/ml bisbenzimide and 1.1 g/ml of CsCl was carefully added. Samples were centrifuged at 45,000 rpm in a StepSaver65V13 rotor for 48 hours at 21 °C. Bands were collected with a syringe needle of size 18G1. Salts were removed by dilution and high-speed centrifugation. Isopropyl alcohol precipitation removed bisbenzimide. DNA was digested by restriction enzymes *Rsa*I (*P. cantuscygni* and *R. euleeides*) and *Ssp*I (*P. trichophorum*).

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