Université de Montréal

Étude de l'évolution de la structure des génomes mitochondriaux chez les Euglenozoa

par

Joannie Roy

Département de biochimie

Faculté de médecine

Mémoire présenté à la Faculté des études supérieures
en vue de l'obtention du grade de Maître ès Sciences
en Biochimie

Août, 2006

Université de Montréal

Faculté des études supérieures

Ce mémoire intitulé :

Étude de l'évolution de la structure des génomes mitochondriaux chez les Euglenozoa

présenté par :

Joannie Roy

a été évalué par un jury composé des personnes suivantes :

Normand Brisson

président-rapporteur

Gertraud Burger

directeur de recherche

David Morse

membre du jury

RÉSUMÉ

Un des génomes mitochondriaux les plus atypiques connus est celui des Kinetoplastea (Euglenozoa). Leur génome mitochondrial est composé de deux types de molécules : les maxicercles et les minicercles. Chez les trypanosomes (Kinetoplastea), ces molécules sont entrelacées et forment un corps compact, nommée le kinétoplaste. La prévalence de ces caractéristiques chez les autres groupes des Euglenozoa, soit les Diplonemea et les Euglenida, est inconnue. La première partie du travail, établissant les bases préalables à la suivante, porte sur la description de *Rhynchopus euleeides* n. sp. (Diplonemea). La seconde partie concerne la distribution et la structure de l'ADN mitochondrial chez cette espèce ainsi que chez plusieurs autres membres des Euglenida. Les résultats montrent que la présence de chromosomes multiples est une propriété commune aux génomes mitochondriaux des différents membres des Euglenozoa alors que l'organisation spatiale compacte de l'ADN mitochondrial est restreinte au sous-groupe des Kinetoplastea.

Mots clés :
Diplonemea, Euglenida, *Rhynchopus*, *Petalomonas*, *Peranema*, *Entosiphon*, protiste, morphologie, ultrastructure, taxonomie

ABSTRACT

One of the most unusual mitochondrial genomes is that of Kinetoplastea (Euglenozoa), which is composed of two types of DNA molecules: maxicircles and minicircles. In trypanosomes (Kinetoplastea), mitochondrial DNA circles are interlocked into a single compact network, the kinetoplast. Whether these peculiar features prevail throughout Euglenozoa (Kinetoplastea and its neighbour clade: Euglenida and Diplonemea) is unknown. The work first includes a description of *Rhynchopus euleeides* n. sp. (Diplonemea). This provided the basis for an investigation on mitochondrial DNA packaging and structure of this species and several members of Euglenida. Our findings indicate that multiple mitochondrial chromosomes are shared by all Euglenozoa studied, while a compacted spatial organization of mitochondrial DNA is restricted to Kineroplastea.

Key words :
Diplonemea, Euglenida, *Rhynchopus*, *Petalomonas*, *Peranema*, *Entosiphon*, protist, morphology, ultrastructure, taxonomy

TABLE DES MATIÈRES

LISTE DES FIGURES

LISTE DES TABLEAUX

Article 1 :

Article 2 :

LISTE DES SIGLES ET DES ABRÉVIATIONS

~ : environ

° : Degré

µ : micro

A : Adénine

ADN : acide désoxyribonucléique

ADNc : ADN complémentaire

ADNk : kinétoplaste

ADNmt : ADN mitochondrial

ARN : acide ribonucléique

ASCT : acetate :succinate CoA transférase

ASWP : eau de mer artificielle pour protistes (en anglais)

ATCC : American Type Culture Collection (en anglais)

ATP : adénosine triphosphaté

AUF : Agence universitaire de la francophonie

C : celsius

C : cytosine

CCAP : Collection Culture of Algae and Protozoa (en anglais)

cDNA : ADN complémentaire (en anglais)

CIAR : Institut canadien de recherches avancées (en anglais)

CIHR : Instituts de recherche en santé du Canada (en anglais)

DAPI : 4',6'-diamidino-2-phenylindole

EM : microscopie électronique (en anglais)

et al. : locution latine *et alli*. En français : et autres.

FBS : sérum de foetus bovin (en anglais)

Fig. : figure

g : gramme

G : guanine

GA : glutaraldéhyde

gRNA : ARN guide (en anglais)

h : heure

HS : Sérum de cheval (en anglais)

k : kilo

kbp : kilobase (en anglais)

kDNA : kinétoplaste (en anglais)

kpb : kilobase

l : litre

m : milli

M : molaire

min : minute

mtDNA : ADN mitochondrial (en anglais)

n : nano

n. sp. : nouvelle espèce

PB : tampon phosphate (en anglais)

PBS : tampon salin phosphate (en anglais)

PDH : pyruvate deshydrogénase

PFGE : gel d'électrophorèse en champs pulsées (en anglais)

PFO : pyruvate :ferrodoxine oxidoréductase

pH : potentiel hydrogène

PNO : pyruvate :NADP+ oxydoréductase

RNA : acide ribonucléique (en anglais)

rpm : rotation par minute

T : thymine

U : uridine

UGA : Université de l'état de Georgie (en anglais)

V : volt

x g : force gravitationnel

* le genre des noms d'organismes est abrégé à leur première lettre à partir de leur deuxième apparition dans le texte. Par exemple, *Trypanosoma brucei* devient *T. brucei*.

REMERCIEMENTS

Je désire remercier chaleureusement ma directrice de recherche, Gertraud Burger, pour son soutient constant durant ces deux dernières années. Je tiens à la remercier en particulier pour sa rigueur, sa disponibilité, ses encouragements et son encadrement. Elle a grandement participé à la réussite de ce projet.

Ma reconnaissance va également à Franz Lang ainsi qu'aux membres des laboratoires de Gertraud et de Franz pour leur constante disponibilité et pour avoir partagé ces belles années. Plus particulièrement, merci à Shona Teijeiro, William Marande et Lise Forget pour leur participation active dans mes projets.

J'exprime ma gratitude à Julius Lukeš et Drahomíra Faktorová, ainsi qu'aux autres membres de l'équipe de Julius pour leur collaboration étroite et pour m'avoir accueillie en République Tchèque durant l'été 2005. Mon passage là-bas fut très enrichissant tant au niveau professionnel que personnel.

Je remercie spécialement David Morse pour m'avoir parrainée et pour ses encouragements. Merci également au personnel du département de biochimie, du centre Robert-Cedergren et de la maison internationale.

Finalement, la réalisation de ce projet n'aurait pas été possible sans le soutien financier du Ministère de l'éducation pour sa bourse de mobilité, de l'Agence universitaire de la francophonie et des Instituts de recherche en santé du Canada.

1. INTRODUCTION

1.1. La mitochondrie et ses différentes fonctions

La mitochondrie est un organite ubiquitaire chez les cellules eucaryotes aérobies. Engagée dans différentes réactions biochimiques, la mitochondrie est délimitée par une double membrane et possède un bagage génétique indépendant du noyau. Son rôle principal est de supporter le métabolisme énergétique. En effet, le pyruvate, préalablement produit par la glycolyse, y est oxydé grâce à la phosphorylation oxydative et entre ensuite dans le cycle de Krebs. Finalement, les produits de ce cycle permettent la respiration cellulaire, générant en tout jusqu'à 38 moles d'ATP par glucose métabolisé (Saraste 1999).

Chez les eucaryotes anaérobies et aérobies facultatifs, il existe des variantes métaboliques de la mitochondrie classique ainsi que des organites dérivés nommés hydrogénosomes et mitosomes (Andersson et Kurland 1999; Tovar *et al.* 1999). Chez ces espèces, le métabolisme du pyruvate, réalisé soit dans ces organites soit dans le cytoplasme, s'effectue à l'aide de complexes enzymatiques différents dont les produits sont subséquemment convertis grâce à un processus de fermentation (Martin et Müller 1998; Andersson et Kurland 1999; Horner *et al.* 1999; Martin 2005).

Mis à part ce rôle central dans le métabolisme énergétique, la mitochondrie est aussi le lieu de biogenèse des co-facteurs de fer/soufre, importants dans plusieurs réactions enzymatiques majeures et dans le transfert d'électrons (Lill et Kispal 2000). Elle participe aussi à d'autres fonctions cellulaires comme l'homéostasie des ions et l'apoptose (Logan 2006).

1.2. L'origine de la mitochondrie

Malgré cette apparente diversité de fonctions métaboliques, les mitochondries sont issues d'un évènement ancestral commun. Outre la similarité des réactions biochimiques y siégeant et l'histoire évolutive des enzymes impliquées dans le métabolisme énergétique, l'origine ancestrale commune de ces organites est appuyée par certains indices comme la présence d'une double membrane. Cette caractéristique suggère que la mitochondrie provient de l'endocytose d'une bactérie par l'ancêtre de la cellule eucaryote (Figure 1). En fait, c'est en 1890 (Altmann 1890; Williamson 2002) que l'on a pour la première fois émis cette idée. Ce n'est toutefois qu'à partir des années 1960, avec la découverte d'ADN dans les mitochondries (ADNmt) de cellules de mammifères (Nass et Nass 1963; Williamson 2002) que l'hypothèse de l'endosymbiose a été confirmée. Depuis ce temps, l'étude des ADNmt dans les différents règnes du vivant ainsi que de leur contenu en gènes ont permis de reconstruire partiellement l'histoire évolutive de cet organite. Ainsi, la nature des partenaires impliqués et les modalités de cette interaction hôte/symbionte sont de mieux en mieux comprises.

Selon les hypothèses les plus couramment acceptées, c'est la modification de l'environnement ambiant qui aurait poussé les deux partenaires à interagir symbiotiquement. Une de ces hypothèses stipule que l'hôte serait devenu strictement dépendant des déchets métaboliques d'une bactérie productrice d'hydrogène suite à la diminution de ce gaz provenant des sources géologiques (Martin et Muller 1998). Ceci suppose un évènement unique qui aurait eu lieu dans une population il y a environ un milliard d'années. C'est par la suite que les différentes lignées eucaryotes contemporaines auraient divergé pour posséder maintenant soit une mitochondrie, soit des reliques de celle-ci, comme l'hydrogénosome et le mitosome. C'est aussi pendant cette évolution qu'une partie des gènes de l'ancêtre mitochondrial original a migré vers le noyau, d'une manière plus ou moins importante selon l'espèce.

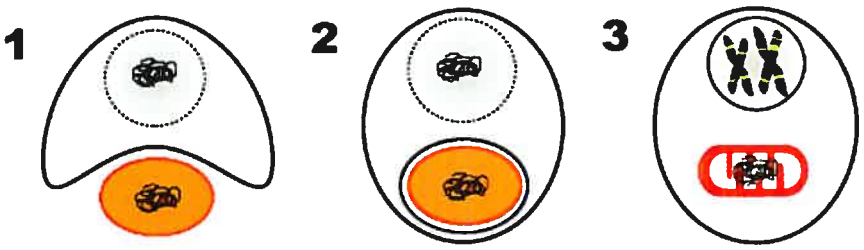


Figure 1. L'endosymbiose de la mitochondrie.

1.3. Les différentes lignées eucaryotes

La classification des eucaryotes et la terminologie employée pour celle-ci ayant changé à plusieurs reprises au cours des années, les taxonomistes ont récemment recommandé et redéfini certains termes, en tenant compte des connaissances actuelles de l'histoire évolutive du vivant (Adl *et al.* 2005; Keeling *et al.* 2005). Les nouveaux schémas reconnaissent cinq à six grands groupes chez les eucaryotes. Chacun de ces groupes est en majorité formé de protistes, desquels ont divergé les lignées d'organismes multicellulaires les plus connues, comme les plantes, les animaux et les champignons. Les protistes, historiquement désignés par les termes protozoaires, protophyta, infusoires, animalcules, protoctista, etc., se définissent communément par exclusion. Selon cette définition, les protistes comprennent les eucaryotes qui ne sont ni des plantes, ni des animaux, ni des champignons.

Selon le schéma (Figure 2) de Keeling et al (2005), qui est communément utilisé, les cinq grands groupes eucaryotes qui prévalent aujourd'hui sont les Plantae, les Chromalveolées, les Unikontes, les Rhizaria et les Excavata. Parmi ceux-ci, certains sous-groupes sont mieux connus. Par exemple, les animaux et les champignons font partie du groupe des Unikontes. Les plantes terrestres et les algues vertes et rouges, quant à elles, appartiennent aux Plantae. On retrouve ensuite les Chromalveolées, dont les espèces les plus connues sont sans doute le

parasite causant la malaria, *Plasmodium* ainsi que les ciliés. Les Rhizaria, pour leur part, comprennent les radiolaires, protistes bien connus des micropaléontologues à cause de leur capacité à se fossiliser. Finalement, il y a les Excavata, dont font partie le parasite causant la maladie du sommeil, *Trypanosoma brucei*, et l'organisme modèle *Euglena gracilis*. C'est aussi dans ce groupe qu'on retrouve les eucaryotes ayant les branches phylogénétiques les plus courtes et les plus basales, tels que les jakobides et les retortamonades.

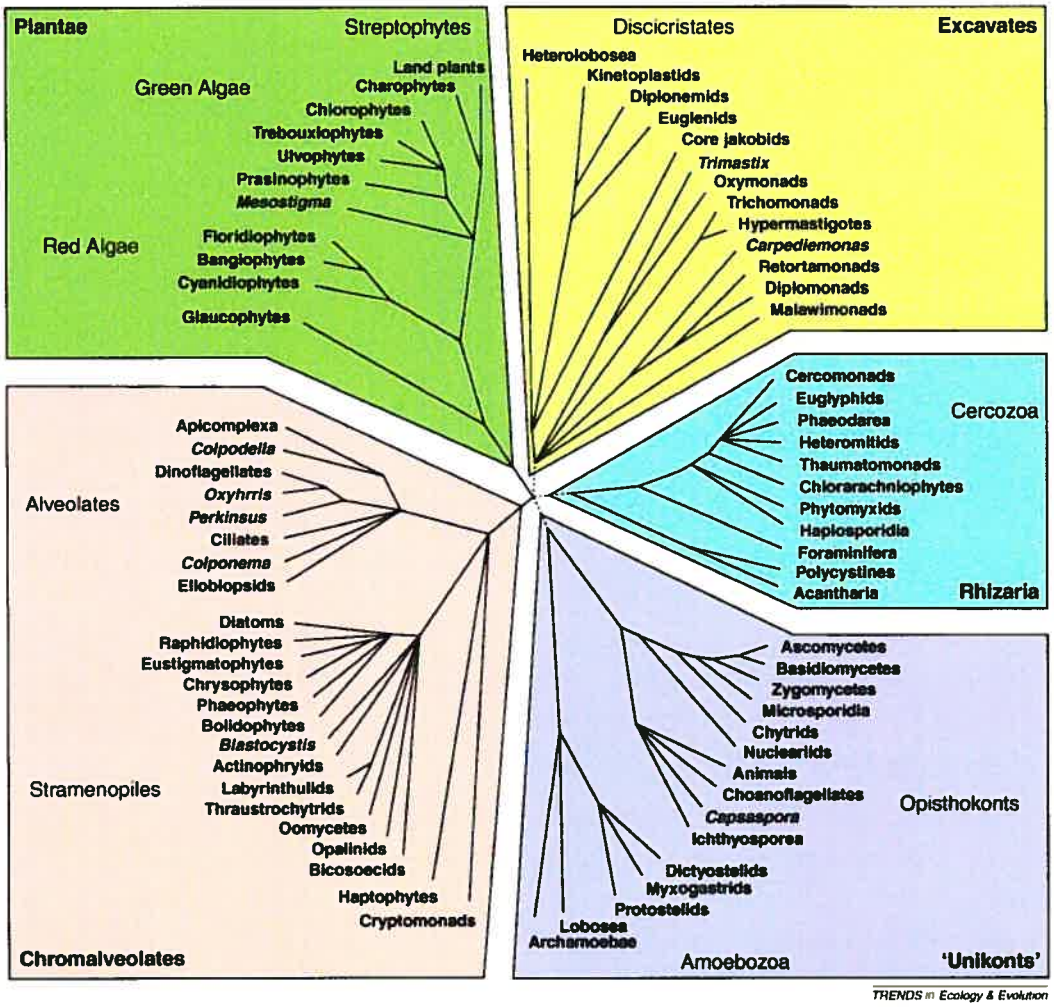


Figure 2. Arbre schématique des grandes lignées eucaryotes (Keeling *et al.* 2005).

1.4. Le génome mitochondrial

Jusqu'à maintenant, c'est chez un protiste membre des Excavata, le jakobide *Reclinomonas americana*, que l'on a retrouvé le génome mitochondrial le plus ressemblant à celui d'une bactérie. Cette découverte a permis de mieux caractériser l'ancêtre commun des mitochondries, puisque le génome de *R. americana* ressemble à celui d'une eubactérie du groupe des alpha-protéobactéries, plus précisément, aux Rickettsiae. Le génome mitochondrial de ce protiste est une molécule circulaire de 69 kpb contenant 97 gènes codant pour des ARN fonctionnels et des protéines (Lang *et al.* 1997).

1.4.1. La structure des génomes mitochondriaux

La similarité du génome mitochondrial à celui d'une eubactérie ne prévaut pas chez tous les organismes. En effet, il existe toute une variété de génomes mitochondriaux. Ceux-ci varient d'une espèce à l'autre selon : la taille du génome, la forme et la conformation, le biais de composition en nucléotides, le nombre de chromosomes, le contenu génique ainsi que le code génétique utilisé dans la traduction.

1.4.1.1.Les animaux, les plantes et les champignons

Suite à la première découverte, chez les mammifères, d'un génome mitochondrial, les chercheurs ont découvert une grande diversité de génomes chez d'autres organismes pluricellulaires, tant chez les animaux que chez les plantes et les champignons. À plusieurs égards, les génomes mitochondriaux des animaux et ceux des plantes terrestres ont évolué dans des directions opposées. D'un côté, chez les animaux, les génomes mitochondriaux se retrouvent sous la forme d'un

monomère circulaire de 14 à 20 kpb qui est principalement composé d'ADN codant. À l'autre extrême, les génomes des plantes, quoique énormes, de 180 à 600 kpb, n'ont qu'une capacité codante de 10-20%. Ils ont une structure complexe, variable selon l'espèce, où les molécules passent parfois d'un état circulaire à linéaire. (Sederoff 1984; Backert *et al.* 1997; Nosek et Tomaska 2003; Lynch *et al.* 2006). Le génome des champignons est quant à lui qualifié de polydispersé, c'est-à-dire composé principalement de molécules linéaires, qui peuvent être arrangées en tandem, de contenu et de complexité variables (Nosek *et al.* 1995; Tomaska *et al.* 2001; Nosek et Tomaska 2003).

1.4.1.2.Les protistes

C'est chez les génomes mitochondriaux des protistes que les plus grandes variations de structure ont eu lieu, à l'image de la diversité biologique qu'ils représentent eux-mêmes aujourd'hui. Par exemple, le génome de l'apicomplexe *P. falciparum*, l'agent causant le paludisme, est le plus petit génome connu présentement, composé d'une molécule linéaire de 6 kpb (Feagin *et al.* 1991; Feagin 1992; 2000) et codant pour trois protéines et deux ARN ribosomaux. À l'opposé, le génome de *Amoebidium parasiticum* est d'environ 200 kpb et est composé de quelques centaines de chromosomes linéaires (Lang *et al.* 2002; Burger *et al.* 2003).

1.4.1.2.1. Les Euglenozoa

Parmi toute cette variété observée chez les protistes, c'est celle des trypanosomes qui a intrigué le plus profondément les scientifiques. Les trypanosomes font partie des Kinetoplastea, eux-mêmes sous-ensemble des Euglenozoa qui eux, appartiennent aux Excavata. On retrouve trois sous-groupes chez les Euglenozoa : les Kinetoplastea, les Diplonema et les Euglenida (Figure 3). Les espèces les plus

étudiées sont, comme ci-haut mentionné, le parasite causant la maladie du sommeil en Afrique, *T. brucei*, celui causant la maladie de Chagas en Amérique du Sud, *T. cruzi*, et ceux responsables de la leishmaniose, *Leishmania sp.* De plus, parmi les quelques autres membres des Euglenozoa connus, notons le flagellé photosynthétique *E. gracilis*, membre du sous-groupe des Euglenida, qui sert communément de modèle en biologie.

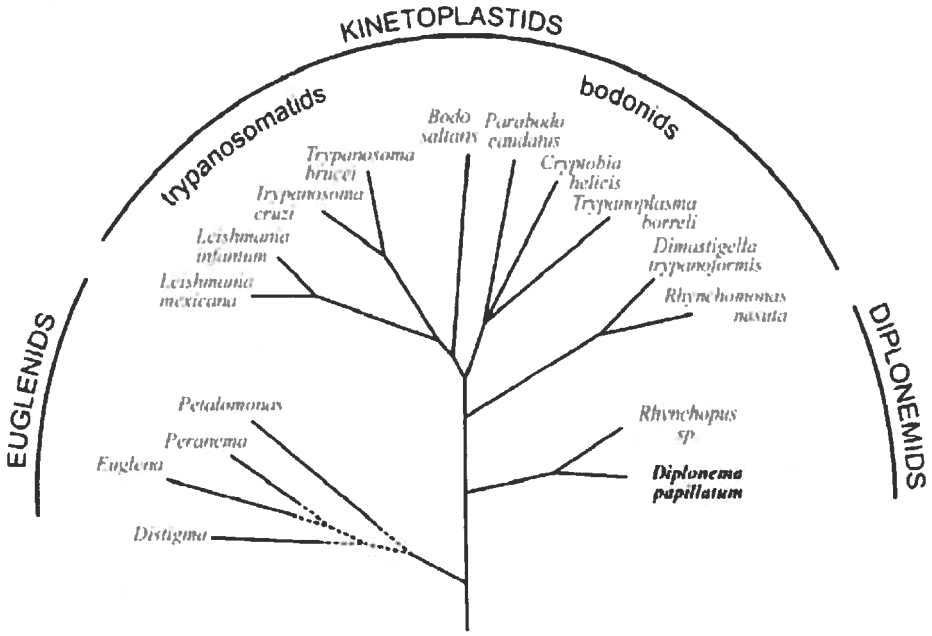


Figure 3. Arbre schématique des Euglenozoa (modifié d'après Marande et al. 2005)

1.4.1.2.1.1.Les Kinetoplastea

Chez les trypanosomes, le génome mitochondrial est composé d'un grand chromosome circulaire (appelé maxicercle) et de milliers de différents petits chromosomes circulaires (appelés minicercles). Le maxicercle est d'une taille de 20 à 40 kpb alors que la multitude de minicercles mesurent de 0,5 à 10 kpb. Ces molécules sont concaténées entre elles pour former un réseau dense qui s'appelle

le kinétoplaste (ADNk). Par ailleurs, les gènes sont encodés sur les maxicercles et leurs ARN messagers respectifs sont massivement édités (RNA editing) afin de rendre la traduction possible. Cette édition, effectuée par l'éditosome, consiste en une insertion et/ou une délétion d'uracile. La matrice d'ARN du complexe de l'éditosome est nommée l'ARN guide et est encodée sur les minicercles (Lukes *et al.* 2002; Simpson *et al.* 2002).

La composition et la structure de l'ADNk varient peu au sein des différentes espèces de trypanosomes. Toutefois, des variantes sont retrouvées chez les membres du taxon voisin, les bodonides, qui forment avec les trypanosomes le groupe des Kinetoplastea. Malgré ces quelques exceptions chez les Kinetoplastea, peu d'autres membres des Euglenozoa sont connus du point de vue de leur génome mitochondrial.

1.4.1.2.1.2.Les Diplonemea

Récemment, un génome mitochondrial unique a été mis en évidence chez *Diplonema papillatum*, du groupe des Diplonemea (Euglenozoa), des flagellés phagotrophes et osmotrophes. En effet, chez *D. papillatum*, le génome est composé de plusieurs molécules circulaires de 6 et 7 kpb (Marande *et al.* 2005). De plus, les gènes encodés sur ces chromosomes sont fragmentés (un fragment par gène) et un mécanisme d'épissage en *trans* des ARN messagers est nécessaire à leur maturation complète. Toutefois, contrairement au kinétoplaste, ces molécules ne forment pas de corps dense visible au microscope. L'autre genre appartenant aux Diplonemea est *Rhynchopus*. Il s'agit d'un genre peu étudié dont quelques espèces seulement ont été décrites ultrastructuralement.

1.4.1.2.1.3.Les Euglenida

Chez les Euglenida, seulement le génome mitochondrial de *E. gracilis* a été étudié. Malgré de nombreuses études (Manning *et al.* 1971; Nass *et al.* 1974; Talen *et al.* 1974; Buetow 1989; Hayashi et Ueda 1989; Hayashi-Isimaru *et al.* 1993; Tessier *et al.* 1997; Yasuhira et Simpson 1997; Gray *et al.* 2004), la structure de ce génome est peu comprise. En effet, selon le groupe de recherche, la taille des molécules observées varie de 1 à 70 kpb. Par contre, toutes les études décrivent les molécules comme étant majoritairement linéaires et complexes. Mis à part cette description partielle du génome de *E. gracilis*, la seule autre information disponible dans la littérature à propos du génome mitochondrial des Euglenida est l'observation par microscopie électronique, chez deux espèces de *Petalomonas*, d'une structure dense semblable à celle des kinétoplastes (Leander *et al.* 2001).

1.5. Hypothèse de travail

La quantité d'information disponible relative à la mitochondrie et à la diversité de son génome est biaisée d'un groupe phylogénique à l'autre. Ce problème s'observe tant, à grande échelle, entre des grands groupes taxonomiques, qu'à plus petite échelle, à travers certains sous-groupes. Par exemple, les plantes, les animaux et les champignons sont beaucoup plus étudiés que les protistes. Le même problème s'applique également aux trois sous-groupes des Euglenozoa, où les génomes mitochondriaux des Kinetoplastea sont beaucoup mieux caractérisés que ceux des Diplonemea et des Euglenida. L'objectif de ce mémoire est donc d'établir l'étendue de la diversité des génomes mitochondriaux retrouvées à travers les Euglenozoa. Plus précisément, les questions abordées dans ce travail sont les suivantes : i- Le type de chromosomes circulaires multiples retrouvés chez *D. papillatum* est-il unique à cette espèce ou est-il répandu à travers les genres

composant les Diplonemea? ii- L'existence d'un génome mitochondrial sous forme d'un kinétoplaste est-elle restreinte qu'aux Kinetoplastea?

La systématique des Diplonemea est controversée et se fonde principalement sur quelques membres du genre *Diplonema*. Ainsi, avant de qualifier la structure du génome mitochondrial retrouvé chez les Diplonemea, il est nécessaire de clarifier les relations regroupant les deux genres qui le composent, soit *Diplonema* et *Rhynchopus*. À ce sujet, certains auteurs ont déjà soulevé la nécessité de recueillir plus d'informations morphologiques et moléculaires sur le genre *Rhynchopus*. Simpson (1997) et Von der Heyden (2004) s'entendent sur la difficulté actuelle d'établir la distinction entre les deux genres. Afin d'éclaircir cette problématique, le premier article présenté dans la section suivante met à jour les connaissances disponibles sur les Diplonemea, plus particulièrement sur la distinction entre les deux genres, en décrivant la nouvelle espèce *Rhynchopus euleeides* n. sp. Cet article permet ainsi d'établir les bases sur lesquels s'appuie l'analyse comparative subséquente des génomes mitochondriaux.

Le deuxième article présente et compare des données sur la structure physique du génome mitochondrial, tant chez *R. euleeides* que chez certaines espèces du groupe des Euglenida (*P. cantuscygni*, *P. trichophorum* et *E. sulcatum*). Les génomes mitochondriaux de *R. euleeides* et de *D. papillatum* sont premièrement comparés. Ensuite, l'étude des génomes des Euglenida permet de mettre en perspective ce groupe par rapport aux deux autres taxons voisins, les Diplonemea et les Kinetoplastea.

2. RÉSULTATS

ACCORD DES COAUTEURS

1. Identification de l'étudiante et du programme

Joannie Roy, M. SC. Biochimie

2. Description de l'Article

Joannie Roy, Drahomíra Faktorová, Oldřich Benada, Julius Lukeš et Gertraud Burger. 'Rhynchopus euleeides n. sp. (Diplonemea), a Free-living, Marine Euglenozoan', soumis à la revue 'Journal of Eukaryotic Microbiology' le 7 avril 2006.

3. Déclaration de tous les coauteurs autres que l'étudiant

À titre de coauteur de l'article identifié ci-dessus, je suis d'accord pour que **Joannie Roy** inclue cet article dans **son mémoire de maîtrise** qui a pour titre **Étude de l'évolution de la structure des génomes mitochondriaux chez les Euglenozoa.**

Coauteur	Signature	Date
Drahomíra Faktorová	[redacted]	30/05/2006
Gertraud Burger	[redacted]	31 May 06
Julius Lukeš	[redacted]	7/6/06
Oldřich Benada	[redacted]	14/7/06

Description of *Rhynchopus euleeides* n. sp. (Diplonemea), a Free-living Marine Euglenozoan

JOANNIE ROY[a.1], DRAHOMÍRA FAKTOROVÁ[b.1.2], OLDŘICH BENADA[c], JULIUS LUKEŠ[b] and GERTRAUD BURGER[a.d]

[a]*Centre Robert Cedergren, Bioinformatics & Genomics, Département de biochimie, Université de Montréal, Montréal, QC, H3T 1J4, Canada*

[b]*Biology Centre, Institute of Parasitology, Czech Academy of Science, and Faculty of Biology, University of South Bohemia, 37005 České Budějovice (Budweis), Czech Republic*

[c]*Institute of Microbiology, Czech Academy of Sciences, 142 20 Prague 4, Czech Republic*

[d]*Canadian Institute for Advanced Research, Program in Evolutionary Biology*

Corresponding author: G. Burger, Université de Montréal, 2900 Boul. Édouard-Montpetit, Montréal QC, H3T 1J4 Canada - Telephone: (514) 343-7936; FAX: (514) 343-2210; e-mail: [redacted]

[1] These authors contributed equally to this work.

[2] Previous name Drahomíra Novotná.

ABSTRACT. We describe *Rhynchopus euleeides* n. sp., using light and electron microscopy. The free-living flagellate, which has been isolated earlier from a marine habitat, can be grown axenically in a rich medium. In the trophic stage, cells display radical changes in form (metaboly); at rest they are lemon-shaped. Gliding is the predominant manner of locomotion. The two flagella are short stubs of unequal length, and typically concealed in the pocket. Swarmer cells, which form only occasionally, are smaller, disk-shaped, with two flagella of more than two-times the body length. Flagella have conventional axonemes, but appear to lack a paraxonemal rod. Cells have a single sub-apical opening that is decorated with a prominent apical papillum. Both the flagellar pocket and the adjacent feeding apparatus merge together into this opening. The most likely single peripherally located mitochondrion is reticulated and contains only a few lamellar cristae. Mitochondrial DNA is abundant and evenly distributed throughout the organelle. Morphological synapomorphies confirm the affiliation of the species with the genus *Rhynchopus* (Diplonemea, Euglenozoa). We discuss the characters which distinguish *Rhynchopus* from *Diplonema*, and the validity of the two genera.

Key Words: Diplonemea, Euglenozoa, flagellates, morphology, ultrastructure, taxonomy.

The genus *Rhynchopus* was first described in 1948, based on the observation of a single species, *Rhynchopus amitus* (Skuja 1948; see also Al-Qassab et al. 2002). This free-living flagellate was isolated from pelagic water in the Erken Lake of the east coast of Sweden. Up to now, the only member of this genus that has been characterised ultrastructurally is the parasitic *Rhynchopus coscinodiscivorus*, which feeds on the cytoplasm of planktonic diatoms (Schnepf 1994). Otherwise, only sketchy descriptions are available for a few additional isolates, some reported as parasites of clams and lobster larvae, and others free-living (Bodammer and Sawyer 1981; Kent et al. 1987; Simpson 1997; Vickerman 2000). In fact, given the scarce details of these descriptions, it is difficult to verify whether these specimens are *Rhynchopus* spp. or rather *Diplonema* spp.

Initially, *Rhynchopus* was considered an unusual euglenid and placed in the Euglenida (Skuja 1948). Since then, classification of this group has undergone numerous modifications (in the following, we will use the taxonomical scheme of Adl et al. (2005)). Most importantly, the genus *Rhynchopus* has been removed from the order Euglenida and placed, together with the genus *Diplonema*, - which now also comprises species previously named *Isonema* (Schuster et al. 1968; Patterson and Brugerolle 1988; Triemer and Ott 1990), into the Diplonemea sensu Cavalier-Smith, 1993 (Simpson 1997). Now, Diplonemea and Euglenida, together with Kinetoplastea (commonly named diplonemids, euglenids and kinetoplastids), constitute the phylum Euglenozoa. A higher-level assemblage unites Euglenozoa and Heterolobosea into Discicristata (Patterson 1994; Cavalier-Smith 1998). Furthermore, Euglenozoa, Heterolobosea and jakobids have been merged into Excavata (Simpson and Patterson 2001; Adl et al. 2005). However, solid phylogenetic evidence for the monophyly of these higher order groupings remains to be demonstrated.

Here, we describe in detail the morphology of *Rhynchopus euleeides* n. sp., determined by light and electron microscopy. Based on several features, which set this organism apart from the other members of the genus, we propose the creation of a new species. With this report, we aim to fill in a critical lack of morphological information about *Rhynchopus* and diplonemids in general, a lack already

underlined by others a decade ago (Schnepf 1994; Simpson 1997). A detailed morphological description of this group is most timely. Due to the recent discovery of a unique mitochondrial gene and genome structure in *Diplonema papillatum* (Marande et al. 2005), diplonemids are now attracting attention beyond the protist community.

MATERIALS AND METHODS

Isolation and cultivation. *Rhynchopus euleeides* n. sp. (previously named '*Rhynchopus* sp.1' - ATCC 50226) was obtained from the American Type Culture Collection, where it was deposited by T. Nerad in 1986. Cells were 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/liter thiamine-HCl), and trace metal elements (4.36 µg/liter Na_2EDTA, 3.15 µg/liter $FeCl_3 \cdot 6\ H_2O$, 9.8 µg/liter $CuSO_4 \cdot 5H_2O$, 22 µg/liter $ZnS0_4 \cdot 7H_2O$, 10 µg/liter $CoCl_2 \cdot 6H_2O$, 18 µg/liter $MnCl_2 \cdot 4H_2O$, 6.3 µg/liter $NaMoO_4 \cdot 2H_2O$), supplemented with 10% horse (HS) or foetal bovine (FBS) serum. Cultures were grown without shaking at room temperature in adhesion-treated flasks (Corning). A cell scraper (Sarstedt) was used for harvesting the cells from the flask bottom. A mix of antibiotics (160 µg/ml streptomycin, 160 µg/ml kanamycin, 280 µg/ml penicillin-G (Wisent)) was added for long-term maintenance of the strain, but not for cultures used in experiments. Stocks were conserved in liquid nitrogen in modified artificial sea water containing 5% dimethyl sulfoxide. For the test of phagotrophy, the serum was replaced by either 0.1-1% of crystallized egg yolk or ~10^5/ml bacteria (a mix of *Enterobacter aerogenes*, *Silicibacter* sp., *Bacillus* sp. and *Pseudomonas* sp.). The titre was determined by counting the cells in a hemacytomer.

Light and fluorescent microscopy. Live cells were observed with an inverted Nikon Eclipse TE2000-U microscope and pictures were taken with MetaMorph 6.3r3 (Molecular Devices). For DAPI (4',6'-diamidino-2-phenylindole)-staining, cells were fixed for 10 min at room temperature in 4% paraformaldehyde diluted

in 3.3 % artificial sea water. Fixation was stopped by spinning the cells down and resuspending them in 3.3% artificial sea water. 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 phosphate-buffered saline (PBS) pH 7.2 for 5 min. The stained cells 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, cells were washed twice in artificial sea water, centrifuged at low-speed (~1200 x g), and fixed following two different protocols: (i) 2% glutaraldehyde (GA) in 0.25 M phosphate buffer (PB) pH 7.2 overnight at 4 °C; (ii) same as (i) but the concentration of PB was 0.1 M. After fixation, cells were washed in the respective buffer supplemented with 4% glucose, pelleted, and embedded in 2% agarose. Post-fixation was done with 2% OsO_4 in PB for 2 h at room temperature, followed by a washing step with PB. After dehydration in graded series of ethanol, the cells were embedded in Epon-Araldite. Ultrathin sections were stained with lead citrate and uranyl acetate and examined under a JEOL JEM 1010 microscope.

For scanning electron microscopy observations, cells were grown on coverslips for a few hours, fixed in 5% glutaraldehyde in 0.25M PB pH 7.2 for 5 min at 37 °C in a PELCO 3440 Max laboratory Microwave Oven (Ted Pella; Reddin, CA), washed in the same buffer, followed by a post-fixation step with 1% osmium tetroxide in 0.1M PBS, pH 7.2 for 5 min and dehydrated in graded series of ethanol. Coverslips were air-dried, mounted on aluminum stubs with conductive carbon paint (SPI supplies) and observed without any further treatment with a JEOL JSM-7400F high resolution Field Emission Scanning Electron Microscope in the secondary mode at 1.5 kV.

RESULTS

Light microscopical observations. The feeding behaviour of *Rhynchopus euleeides* n. sp. depends on available nutrients. In a serum-based medium, cells feed osmotrophically; at high concentrations of serum (~10%), the culture turns blackish due to an unidentified compound secreted by the cells once they reach the stationary phase (~ 10^7 cells/ml). Alternatively, in a medium containing crystallized egg yolk or bacteria, the flagellates will feed by phagotrophy. Generation time of axenic cultures in modified artificial sea water supplemented with 10% serum is approximately 8 hours. Division occurs longitudinally, from the anterior to the posterior end (Fig. 1).

Trophic cells at rest are typically 10 -- 25 μm long and 4 -- 8 μm wide in the center, of almost symmetrical elliptical shape, yet flattened dorso-ventrally (Fig. 7--9). Cells glide on the surface, but with increasing cell density, a small percentage of them will float and cluster together. Locomotion of the trophic stage is accompanied by peristaltic-like contractions, expansions and contortions of the body, termed 'metaboly'. The anterior portion of the cell appears to be responsible for motion and steering (Fig. 2--6). Cysts, which form readily in aging cultures, are rounder and smaller than trophic cells. Free-swimming ('swarmer') cells are rare, and have been observed only at two occasions both in starved cultures. Swarmer cells are ~5 μm long and rather disk-shaped. While the flagella are invisible in trophic and cyst stage, they can be clearly distinguished in the swarmers stage, where they are about 2.5 times longer than the body and used for fast propulsion (Fig. 32).

Fluorescent microscopy of DAPI-stained cells reveals the nucleus, enclosing a single, less fluorescent nucleolus. A large amount of mitochondrial DNA (mtDNA) is seen peripherally, apparently distributed uniformly throughout the reticulated organelle (Fig. 7, 8). Clearly, a kinetoplast-like structure is absent.

Electron microscopical observations. Scanning microscopy shows that the cell surface is smooth, although in some young cells, the posterior end displays a

spiral-like pattern (data not shown). A single opening is located sub-apically, with its rim folded into a lip and protruding at the dorsal side to form an apical papillum (Fig. 9, 10). In all >100 cells inspected, only a single opening has been observed. Flagella remain concealed in glider cells from young and axenic cultures, but a structure resembling a single, short, barely emerging flagellum has sometimes been observed in an older, non-axenic culture (data not shown).

Transmission microscopy proved to be difficult, since the cells' ultrastructure collapsed easily during fixation, probably due to their poor resistance to osmotic change. Among the different protocols tested, the fixation with GA diluted in 0.25M PB yielded the best results. The obtained images show that cells possess a sub-apical cavity that encloses the feeding apparatus and the flagellar apparatus. Longitudinal sections of the whole cell reveal that both apparatuses occupy up to half of the total body length (Fig. 11, 12, 21, see scale bar), and are generally oriented parallel to one another (Fig. 14), but depending on the cell shape, they may appear as well in perpendicular orientation (Fig. 15). The two flagella, which are inserted in their pocket in parallel orientation (Fig. 12, 17, 18), consist of conventional 9 (2) + 2 axonemes and lack a paraxonemal rod (Fig. 13--17). Transversal sections often show only one flagellum within the pocket, indicating that the two flagella are of unequal length (Fig. 14, 16). The flagellar root system features a transitional zone bounded by two plates (Fig. 12, 13, 15, 17, 18). The two basal bodies are supported by surrounding microtubules (Fig. 12, 18).

The feeding apparatus, which is positioned adjacent to the flagellar pocket, displays a cytopharyngeal component that is coated on the interior with vanes (ribs) and supporting rods on one side, and with a row of multidirectional microtubules on the opposite side (Fig. 14, 15, 19--22). In a longitudinal view, the cytopharynx appears as a horn-like structure, through which nutrients enter the cell (Fig. 21).

Beneath the cell membrane lies a single layer of tightly packed microtubules oriented longitudinally. This component of the cytoskeleton is thought to be a major player in metaboly and locomotion (Fig. 23, 24). The (most likely single) mitochondrion that coates the inner side of the cell membrane has a reticulated

shape and encloses sparse and mostly longitudinally oriented lamellar cristae (Fig. 25, 26). A fibrillar array of mtDNA was not detected, corroborating the absence of a kinetoplast-like structure. The Golgi apparatus generally assumes a linear form, but occurs also in a circularized form (Fig. 27, 28). The round nucleus contains a single centrally located electron-dense nucleolus and chromosomes that seem to be permanently condensed (Fig. 29). No structures resembling trichocysts or extrusomes have been observed.

DISCUSSION

Rhynchopus sp. 1 (ATCC 50226) is a species widely used in molecular phylogenies that aim at the elucidation of the evolutionary history of Euglenozoa (Moreira et al. 2001; Busse and Preisfeld 2002; Simpson et al. 2002; Busse and Preisfeld 2003; Simpson et al. 2004; von der Heyden et al. 2004). In this report, we fill a gap in the knowledge of this key flagellate by providing a detailed morphological description, summarized in Fig. 30--33. The new name we propose, *Rhynchopus euleeides* n. sp., means 'maggot-like' and is inspired by the cell shape, movement and inclination to congregate.

Members of the genus *Rhynchopus* bear in their trophic stage two short flagella that are barely emerging from, or completely buried in, the flagellar pocket, while in the swarmer stage, flagella are long and fully motile (Vickerman 2000; von der Heyden et al. 2004). The features that distinguish individual species within the genus are manifold (see Table 1). First, the habitat varies. Many members of the genus, including *R. euleeides* n. sp., live in marine environments, whereas the type species *R. amitus* occurs in fresh water. We propose that *R. amitus* derives from a marine taxon, since the Erken Lake, from which it has been isolated, was originally part of the Baltic sea and turned into an enclosed basin only relatively recently (Weyhenmeyer 1998). Second, the lifestyle of *Rhynchopus* species is quite diverse. Some members are free-living (*Rhynchopus* sp. 2 (ATCC50230), *R. amitus, R. euleeides* n. sp.), *R. coscinodiscivorus* is an intracellular parasite of

diatoms, and several poorly described species were reported to be ectoparasites or ectocommensals (Bodammer and Sawyer 1981; Kent et al. 1987; Simpson 1997; Vickerman 2000). Yet, it is possible that these latter taxa are only opportunistic parasites rather than obligate ones. Third, the flagella are quite diverse across the genus. In the trophic stage, they are of about equal length in *R. coscinodiscivorus*, while in *R. euleeides* n. sp., one flagellum appears to be shorter than the other. Notably, the axoneme is arranged irregularly in *R. coscinodiscivorus* (Schnepf 1994), whereas in *R. euleeides* n. sp., it displays a canonical organization. Finally, what sets *R. euleeides* n. sp. conspicuously apart from the well-described *R. amitus* and *R. coscinodiscivorus* is a regular lemon-like shape of its resting cells, contrasting with the pear-shaped cells of the latter two species. Taken together, the particular combination of ecological, morphological and ultrastructural characteristics observed in *R. euleeides* n. sp. qualifies this taxon clearly as a new species.

Diplonemea, comprising by the genera *Rhynchopus* and *Diplonema*, share several features. Both display pronounced metaboly, feature an oval to sack-like cell shape at rest, possess two short flagella, and contain an exceptionally large amount of mt DNA ((Maslov et al. 1999; Marande et al. 2005); this work). In addition, the single reticulated mitochondrion of diplonemids encloses only a few lamellar, parallel-arranged cristae (with the exception of *Diplonema ambulator*, which was reported to have numerous cristae (Triemer and Ott 1990)). Moreover, the flagellar and feeding apparatuses are very similar in *Rhynchopus* and *Diplonema* (Montegut-Felkner and Triemer 1994; Schnepf 1994; Montegut-Felkner and Triemer 1996). Members of both genera have their feeding apparatus longitudinally arranged and connected with the flagellar apparatus (Triemer and Ott 1990). This complex structure has been first classified as a type II feeding apparatus known from euglenids (Triemer and Farmer 1991a; Linton and Triemer 2001), but a detailed three-dimensional ultrastructural analysis did not support this notion (Montegut-Felkner and Triemer 1996).

Given the synapomorphies of *Rhynchopus* and *Diplonema* described above, it is difficult to identify features that unambiguously distinguish the two genera.

Morphological distinctions are usually based on the relative length of their two flagella, which are unequal in length in most *Rhynchopus* species, but equal in all *Diplonema* species described so far. Some authors consider the occurrence of a swarmer stage with fully motile flagella as an additionnal distinctive trait of the genus *Rhynchopus* (von der Heyden et al. 2004), but this character is of limited practical use for taxonomic identification, as swarmers form rarely, at least in *R. euleeides* n. sp. As summarized in Table 1, the available morphological information on *Diplonema* and *Rhynchopus* species is too limited to rigorously distinguish the two genera. However, our results indicate that the presence of a single sub-apical opening could be a feature characteristic for *Rhynchopus*, since all *Diplonema* species examined by scanning electron microscopy were reported to have two separate openings (Porter 1973; Triemer and Farmer 1991b). To confirm this hypothesis, a comprehensive scanning electron microscopy study of *Rhynchopus* and *Diplonema* species will be necessary.

Rhynchopus and *Diplonema* were initially placed in the Euglenida (Griessmann 1914; Skuja 1948). Indeed, there are several features common to euglenids and diplonemids, yet, most are also shared with kinetoplastids. For example, the feeding and flagellar apparatuses are organized in a similar manner, as are the flagella. Moreover, all three groups possess a microtubule-reinforced feeding apparatus, a flagellar root system consisting of two basal bodies and a microtubular root system (Simpson 1997). Similarly, the giant reticulated mitochondrion including long and parallel lamellar cristae appears to be wide-spread in Euglenozoa (Simpson 1997; Simpson et al. 2002), although earlier reports describe mitochondria of kinetoplastids and euglenids as being of moderate size and containing densely packed and rather short discoidal cristae (Brugerolle et al. 1979; Pellegrini 1980; Farmer and Triemer 1994).

The phylogenetic relationships within the Euglenozoa remain controversial (Moreira et al. 2001; Busse and Preisfeld 2002; Simpson et al. 2002; Busse and Preisfeld 2003; Moreira et al. 2004; Simpson and Roger 2004; von der Heyden et al. 2004; Marande et al. 2005). Recent single-gene phylogenies provide reasonable support for the sister relationship of Diplonemea and Kinetoplastea, with

Euglenida branching prior to the divergence of the two former clades (Simpson and Roger 2004). Yet, *Rhynchopus* and *Diplonema* are not always recovered as sister groups, with *Rhynchopus* species sometimes emerging from within the genus *Diplonema* (von der Heyden et al. 2004). This means that at present, neither morphological nor molecular phylogeny data support a clear-cut distinction between these genera, raising the question whether *Diplonema* and *Rhynchopus* should be merged. Resolving this issue will require comparative ultrastructural studies of previously characterized taxa, as suggested above, as well as phylogenetic analyses based on multiple protein-coding genes.

The relationships within Diplonemea might also be revealed by comparative mitochondrial genomics. As we have shown recently, the mitochondrial genome of *D. papillatum* has a unique multi-partite structure with gene fragments encoded on distinct circular chromosomes (Marande et al. 2005). Interestingly, a similar genome structure is found in *R. euleeides* n. sp. (W. Marande, J. Roy and G. Burger, unpubl. results). Work is in progress to determine how broadly this unusual kind of genome and gene structure is distributed across Diplonemea and related flagellates.

Taxonomic summary

Phylum Euglenozoa Cavalier-Smith, 1981

Class Diplonemea Cavalier-Smith, 1993

Genus *Rhynchopus* Skuja, 1948

Rhynchopus euleeides, n. sp. Roy, Faktorová, Lukeš et Burger 2006

Diagnosis. We describe '*Rhynchopus* sp.1' (ATCC 50226) and rename it *Rhynchopus euleeides* n. sp. The description is based on morphological and physiological data.

Description. In the trophic stage, cells are slightly elongated and 10 -- 25 μm in length by 4 -- 8 μm in width. In resting cells, the anterior and posterior ends are symmetrical. Cells display metaboly and move by gliding. Flagella are concealed. When starved, the swarmer stage appears with two fully motile flagella used for

swimming. Flagellar and feeding apparatuses are arranged parallel to the longitudinal axis of the cell and merge together into a single sub-apical opening. The mitochondrion, lining the cell periphery, has few and long longitudinally oriented cristae of lamellar shape. In serum-supplemented medium, a black pigment is secreted as the culture ages.

***In vitro* cultivation.** Axenic cultures can be obtained in modified artificial sea water medium, supplemented with mineral trace elements, vitamins and serum. Cells should be grown in adhesion-treated culture flask in horizontal position, with passage to new medium twice a week. Alternatively, serum can be replaced with bacteria or crystallised egg yolk, resulting in slower grow rate.

Type locality. Isolated from sea water, New Bedford, Maine, USA.

Etymology. *Rhynchopus euleeides* n. sp. has been named for its similarity to maggots in shape, movement and gregarious behaviour.

Type material. The type culture has been deposited by T.A. Nerad in 1986 at the American Type Culture Collection, under the accession number ATCC 50226. The hapantotype has been deposited at the International Protozoan Type Slide Collection under the accession number USNM 1091283.

ACKNOWLEDGMENTS

This research was supported by a grant (MOP-79309) from the Canadian Institutes of Health Research (CIHR) Genetics Program to G.B., by fellowships of the Agence universitaire de la francophonie (AUF), the Ministry of Education of Québec and CIHR to J.R., by grants 5022302 and Z60220518 from the Grant Agency of the Czech Academy of Sciences and grant 6007665801 from the Ministry of Education of the Czech Republic to J.L., and by Institutional Research Concept Z50200510 to O.B. We gratefully acknowledge the Canadian Institute for Advanced Research (CIAR) Program for Evolutionary Biology, for salary and interaction support to G.B. We also thank Shona Teijeiro (Université de Montréal) for the art work, and the electronic microscopy unit of Antonio Nanci (Médecine

dentaire, Université de Montréal) for usage of their facility. J.R. thanks Hugo S. Doyer (Université de Montréal) and Jean-Michel Lavoie (Université Laval) for etymological advice.

LITERATURE CITED

Adl, S. M., Simpson, A. G., Farmer, M. A., Andersen, R. A., Anderson, O. R., Barta, J. R., Bowser, S. S., Brugerolle, G., Fensome, R. A., Fredericq, S., James, T. Y., Karpov, S., Kugrens, P., Krug, J., Lane, C. E., Lewis, L. A., Lodge, J., Lynn, D. H., Mann, D. G., McCourt, R. M., Mendoza, L., Moestrup, O., Mozley-Standridge, S. E., Nerad, T. A., Shearer, C. A., Smirnov, A. V., Spiegel, F. W. & Taylor, M. F. 2005. The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. *J. Eukaryot. Microbiol.*, **52**:399-451.

Al-Qassab, S., Lee , W. J., Murray, S., Simpson, A. G. B. & Patterson, D. J. 2002. Flagellates from Stromatolites and Surrounding Sediments in Shark Bay, Western Australia. *Acta Protozool.*, **41**:91-144.

Bodammer, J. E. & Sawyer, T. K. 1981. Aufwuchs protozoa and bacteria on the gills of the rock crab, *Cancer irroratus* Say: a survey by light and electron microscopy. *J. Protozool.*, **28**:35-46.

Brugerolle, G., Lom, J., Nohýnková, E. & Joyon, L. 1979. Comparaison et évolution des structures cellulaires chez plusieurs espèces de bodonidés et cryptobiidés appartenant aux genres *Bodo*, *Cryptobia* et *Trypanoplasma* (Kinetoplastida, Mastigophora). *Protistologica*, **15**:197-221.

Busse, I. & Preisfeld, A. 2002. Phylogenetic position of *Rhynchopus* sp. and *Diplonema ambulator* as indicated by analyses of eulenozoan small subunit ribosomal DNA. *Gene*, **284**:83-91.

Busse, I. & Preisfeld, A. 2003. Systematics of primary osmotrophic euglenids: a molecular approach to the phylogeny of *Distigma* and *Astasia* (Euglenozoa). *Int. J. Syst. Evol. Microbiol.*, **53**:617-24.

Cavalier-Smith, T. 1998. A revised six-kingdom system of life. *Biol. Rev. Camb. Philos. Soc.*, **73**:203-66.

Farmer, M. A. & Triemer, R. E. 1994. An ultrastructural study of *Lentomonas applanatum* (Preisig) n.g. (Euglenida). *J. Eukaryot. Microbiol.*, **41**:112-119.

Griessmann, K. 1914. Ueber marine Flagellaten. *Arch. Protistenkd.*, **32**:1-78.

Kent, M. L., Elston, R. A., Nerad, T. A. & Sawer, T. K. 1987. An Isonema-like flagellate (Protozoa: Mastigophora) infection in larval Geoduck clams, *Panope abrupta*. *J. Invertebr. Pathol.*, **50**:221-229.

Linton, E. W. & Triemer, R. E. 2001. Reconstruction of the flagellar apparatus in *Ploeotia costata* (Euglenozoa) and its relationship to other euglenoid flagellar apparatuses. *J. Eukaryot. Microbiol.*, **48**:88-94.

Marande, W., Lukeš, J. & Burger, G. 2005. Unique mitochondrial genome structure in diplonemids, the sister group of kinetoplastids. *Eukaryot. Cell*, **4**:1137-46.

Maslov, D. A., Yasuhira, S. & Simpson, L. 1999. Phylogenetic affinities of *Diplonema* within the Euglenozoa as inferred from the SSU rRNA gene and partial COI protein sequences. *Protist*, **150**:33-42.

Montegut-Felkner, A. & Triemer, R. 1994. Phylogeny of *Diplonema ambulator* (Larsen and Patterson) 1. Homologies of the flagellar apparatus. *Europ. J. Protistol.*, **30**:227-237.

Montegut-Felkner, A. & Triemer, R. 1996. Phylogeny of *Diplonema ambulator* (Larsen and Patterson) 2. Homologies of the feeding apparatus. *Europ. J. Protistol.*, **32**:64-76.

Moreira, D., Lopez-Garcia, P. & Rodriguez-Valera, F. 2001. New insights into the phylogenetic position of diplonemids: G+C content bias, differences of evolutionary rate and a new environmental sequence. *Int. J. Syst. Evol. Microbiol.*, **51**:2211-9.

Moreira, D., Lopez-Garcia, P. & Vickerman, K. 2004. An updated view of kinetoplastid phylogeny using environmental sequences and a closer outgroup: proposal for a new classification of the class Kinetoplastea. *Int. J. Syst. Evol. Microbiol.*, **54**:1861-75.

Patterson, D. J. 1994. Protozoa: Evolution and Systematics. *In:* Hausmann, K. & Hülsmann, N. (eds.), Progress in Protozoology. Gustav Fischer Verlag, Stuttgart - Jena - New York. pp. 1-14.

Patterson, D. J. & Brugerolle, G. 1988. The ultrastructural identity of *Stephanopogon apogon* and the relatedness of this genus to the other kinds of protists. *Europ. J. Protistol.*, **23**:279-290.

Pellegrini, M. 1980. Three-dimensional reconstruction of organelles in *Euglena gracilis* Z. II. Qualitative and quantitative changes of chloroplasts and mitochondrial reticulum in synchronous cultures during bleaching. *J. Cell. Sci.*, **46**:313-40.

Porter, D. 1973. *Isonema papillatum* sp. n., a new colorless marine flagellate: A light- and electron microscopic study. *J. Protozool.*, **20**:351-6.

Schnepf, E. 1994. Light and electron microscopical observations in *Rhynchopus coscinodiscivorus* spec. nov., a colorless, phagotrophic euglenozoon with concealed flagella. *Arch. Protistenkd.*, **144**:63-74.

Schuster, F., Goldstein, S. & Hershenov, B. 1968. Ultrastructure of a flagellate, *Isonema nigricans* nov. gen. nov. sp., from a polluted marine habitat. *Protistologica*, **4**:141-154.

Simpson, A. G. B. 1997. The identity and composition of the Euglenozoa. *Arch. Protistenkd.*, **148**:318-28.

Simpson, A. G. B., Gill, E. E., Callahan, H. A., Litaker, R. W. & Roger, A. J. 2004. Early evolution within kinetoplastids (Euglenozoa), and the late emergence of trypanosomatids. *Protist*, **155**:407-22.

Simpson, A. G. B., Lukeš, J. & Roger, A. J. 2002. The evolutionary history of kinetoplastids and their kinetoplasts. *Mol. Biol. Evol.*, **19**:2071-83.

Simpson, A. G. B. & Patterson, D. J. 2001. On core jakobids and excavate taxa: the ultrastructure of *Jakoba incarcerata*. *J. Eukaryot. Microbiol.*, **48**:480-92.

Simpson, A. G. B. & Roger, A. J. 2004. Protein phylogenies robustly resolve the deep-level relationships within Euglenozoa. *Mol. Phylogenet. Evol.*, **30**:201-12.

Skuja, H. 1948. Taxonomie des Phytoplanktons einiger Seen in Uppland, Schweden. *Symb. Bot. Upsal.*, **9**:5-399.

Triemer, R. & Farmer, M. A. 1991a. The ultrastructural organization of the heterotrophic euglenids and its evolutionary implications. *In:* Patterson, D. & Larsen, J. (eds.), The biology of free-living heterotrophic flagellates. Clarendon Press, Oxford, U.K. pp. 185-205.

Triemer, R. E. & Farmer, M. A. 1991b. An ultrastructural comparison of the mitotic apparatus, feeding apparatus, flagellar apparatus and cytoskeleton in euglenoids and kinetoplastids. *Protoplasma*, **164**:91-104.

Triemer, R. E. & Ott, D. W. 1990. Ultrastructure of *Diplonema ambulator* Larsen & Patterson (Euglenozoa) and its relationship to *Isonema*. *Europ. J. Protistol.*, **25**:316-320.

Vickerman, K. 2000. Diplonemids. *In:* Lee, J. J., Leedale, G. F. & Bradbury, P. (eds.), An illustrated guide to the protozoa. Allen Press, Lawrence, Kansas. pp. 1157-1159.

von der Heyden, S., Chao, E. E., Vickerman, K. & Cavalier-Smith, T. 2004. Ribosomal RNA phylogeny of bodonid and diplonemid flagellates and the evolution of Euglenozoa. *J. Eukaryot. Microbiol.*, **51**:402-16.

Weyhenmeyer, G. A. 1998. Resuspension in lakes and its ecological impact - a review. *Arch. Hydrobiol.*, **51**:185-200.

FIGURE LEGENDS

Fig. **1--10.** Light and scanning electron microscopy of *Rhynchopus euleeides*, n. sp. Cell morphology and DNA distribution. **1.** Final stage of cell division. Anterior end is indicated by an asterisk. **2--6.** Movement of a single cell (direction of the movement is shown by arrows), time interval 7 s. Note large refractile vacuoles. **7.** DAPI-stained cell revealing nuclear (N) and mitochondrial (arrowheads) DNA. **8.** Same cell as in Fig. 7 in Nomarski interference contrast. **9.** Whole cell displaying a smooth surface. **10.** Enlarged anterior part of the cell showing the sub-apical pocket (O) and the apical papillum (P). Bar = 10 μm (Fig. 1-8) and 2 μm (Fig 9-10).

Fig. **11--18.** Transmission electron microscopy of *Rhynchopus euleeides*, n. sp. Architecture of the flagellar and feeding apparatuses. **11.** Longitudinal section through the anterior part of the cell showing the feeding apparatus (FA) that is supported by multiple fibres (arrows) and the apical papillum (P) surrounding the sub-apical opening (O). **12.** Longitudinal section through the anterior part of the cell showing the flagellar pocket (Fl) re-inforced apically by fibers (arrows), the apical papillum (P), the sub-apical pocket (O), the transitional plate (arrowheads), the basal body (B) and the flagellar root (R). **13.** Detail of Fig. 12 showing the transitional zone of the flagellum (arrowheads). **14.** Transverse section of the flagellar pocket (Fl) and the feeding apparatus (FA), both structures being supported by fibres (arrows). **15.** Section through the flagellar and feeding apparatuses. Transitional plates (arrowheads) and supporting fibres (arrows) are visible. **16.** Transverse section of a 9 (2) + 2 ordered axoneme. Only a single flagellum was seen in this section. **17.** Transverse section of the flagellar pocket containing two flagella. The left flagellum shows the transitional zone, characterized by a 9 (2) + 0 tubular arrangement. **18.** Section through the flagellar pocket, basal bodies (B) and roots (R) of both flagella. Note the transitional plates

(arrowheads). Bar = 500 nm; the fixation protocol (i) was used (see Materials and Methods).

Fig. **19--29**. Transmission electron microscopy of *Rhynchopus euleeides*, n. sp. Architecture of feeding apparatus, organelles, and cytoskeleton. **19.** The feeding apparatus (FA) at the very apical end of the cell. Note the prominent fibres surrounding the cytostome (arrow) and the structured apical papillum (P). **20.** Oblique view of the feeding apparatus (FA) showing its horn shape. Note the apical papillum (P) **21.** Longitudinal view of the apical papillum (P) merged with the feeding apparatus that forms a J-shaped tube lined by two-directional fibres (arrows). **22.** Transverse section of the feeding apparatus (FA) containing a number of microtubules and plicate vanes (arrows) surrounding the central cytostome. **23.** Tranverse section of the cytoskeletal corset (C) of the cell. **24.** Longitudinal section of the cytoskeletal corset (C). **25.** Longitudinally sectioned peripherally-located mitochondrion (M) with prominent cristae (arrow). **26.** Cross-sectioned peripherally-located mitochondrion (M) with scarse cristae (arrows). **27.** Linear form of the Golgi apparatus (G). **28.** Multimembraneous vesicles, interpreted as circularized Golgi apparatus (G). **29.** Transverse section of the cell showing a large nucleus (N) with condensed chromosomes and a prominent nucleolus (nu). Bar = 500 nm; the fixation protocols (i) (Fig. 19--24, 27-29) and (ii) (Fig. 25, 26) were used (see Materials and Methods).

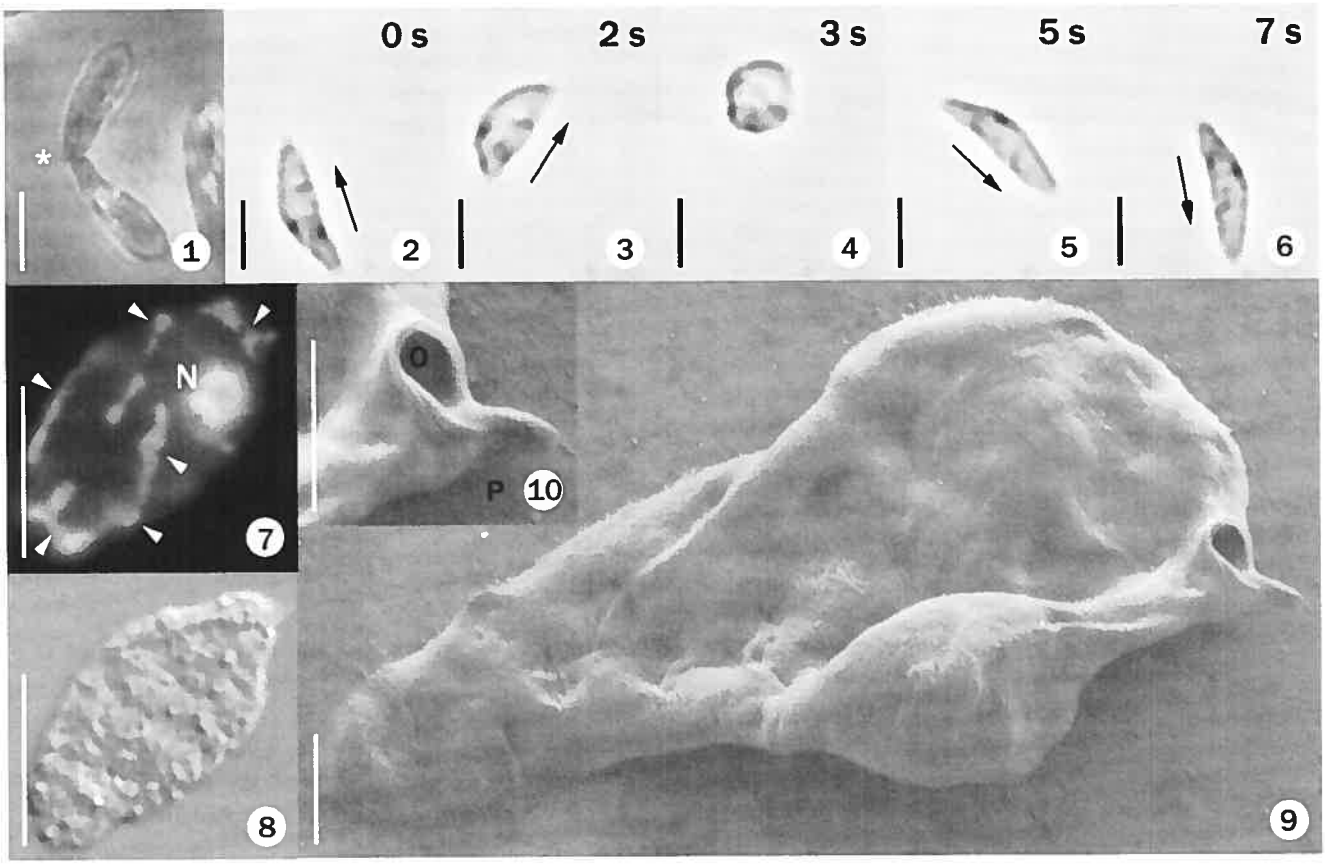
Fig. **30--33**. Schematic three-dimensional representation of *Rhynchopus euleeides* n. sp. **30**. Outer view of a cell in the trophic stage. Cell shape suggests snapshot in movement. Note the single sub-apical opening. **31**. Top view of the anterior end of the cell represented in Fig. 30 illustrating its dorso-ventral flatness. **32**. Swarmer stage. Note its small size and long flagella. **33**. Inner view of the cell represented in Fig. 30 and 31. Note that the flagellar and feeding apparatuses merge together into a single microtubule-reinforced area. Bar = 2 μm.

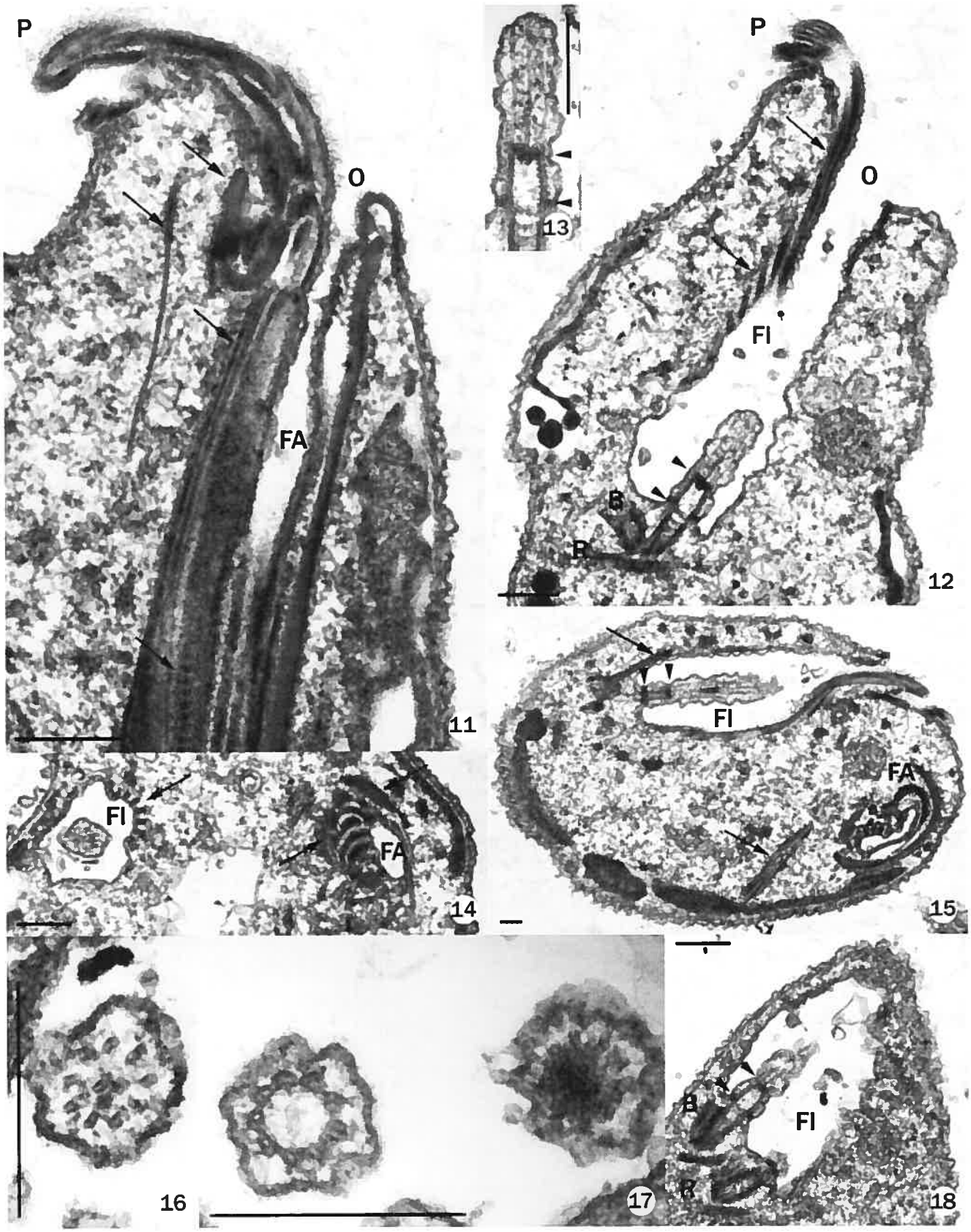
Table 1. Comparative features of the described Diplonemea species.

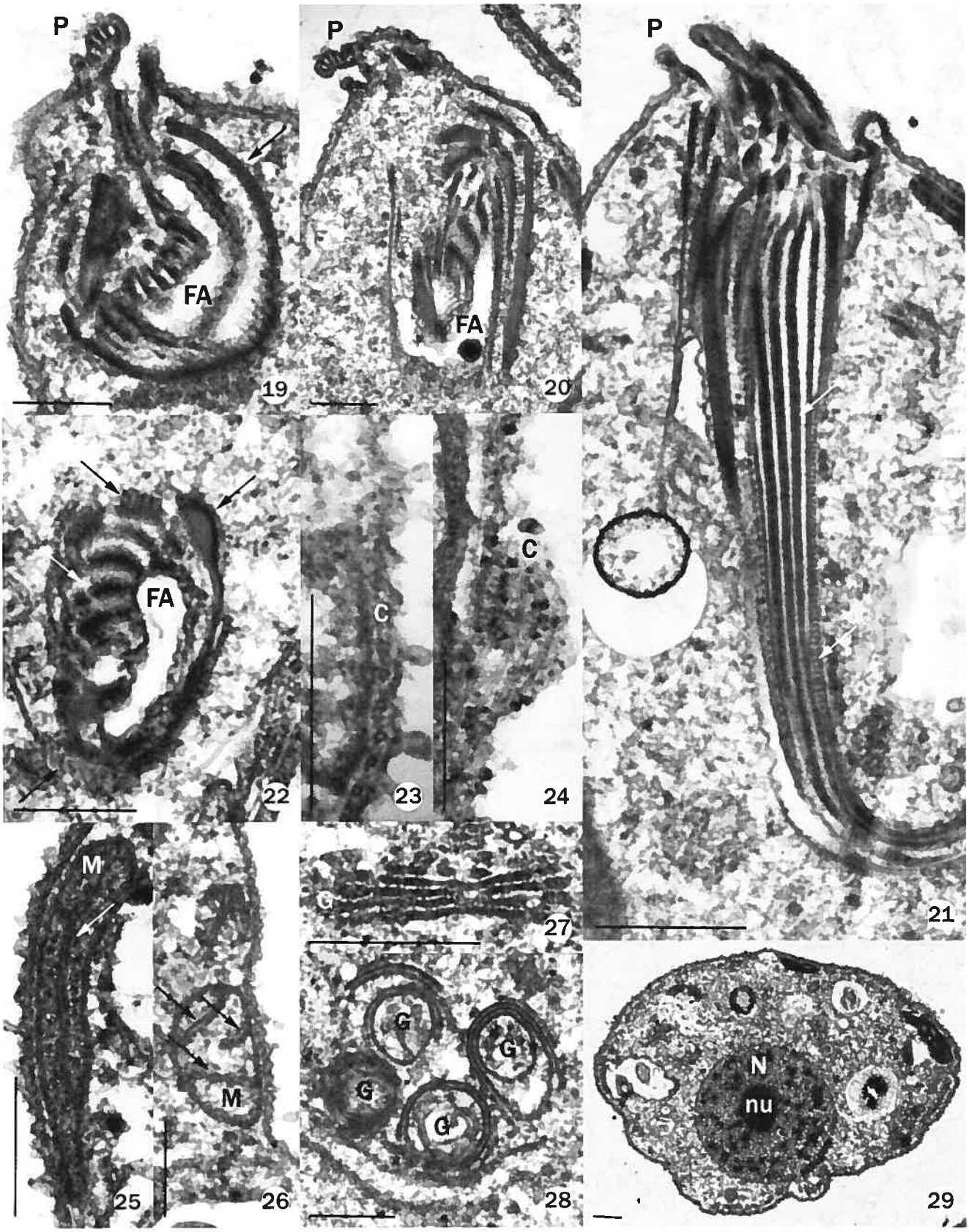
Species	Description	Habitat	Parasi-tism[a]	Pigment produc-tion	Swarmer stage	Cell dimensions (µm)	Apical papillum	Open-ing	Equal length of flagella	Emerging length of flagella (µm)	Ordered axoneme	Paraxo-nemal rod	Tricho-cyst
R.. euleeides	this report	marine	no	yes	yes	10-25 x 4-8	yes	1	no (T)	0 (T); ~12 (S)	yes (T)	no (T)	no
R. amitus	Skuja 1948[b]	fresh water, marine	no[b]	?	yes	20-25[b] x 7-9	yes	?	?	0 (T); ~5-11 (S)	?	?	?
R. coscino-discovorus	Schnepf 1994[b]	marine	yes	?	?	20-25 x 10-12	yes	?	yes (T)	?	no (T)	no (T)	no
R. sp.2 ATCC 50230	see Simpson 1997	marine	?	?	yes	?	?	?	?	?	yes (S)	yes (S)	?
D. breviciliata	Griessmann 1913	marine	no	?	no	28-35 x 8-10	yes	?	yes	~8-10	?	?	?
D. nigricans	Schuster et al. 1968	marine	no	yes	no	40-50 x 3-7	no	?	yes	3	yes	no	yes
D. papillatum	Porter 1973	marine	no	no	no	10-24 x 4-8	yes	2	yes	5.5-7	yes	no	no
D. ambulator	Larsen & Patterson 1990[c]	fresh water, marine	yes	yes	no	17-24 x 6-10	yes	2	yes	2-3	yes	no	no
D. metabolicum	Larsen & Patterson 1990	marine	?	?	no	30-48 x ?	no	?	?	~10	?	?	?

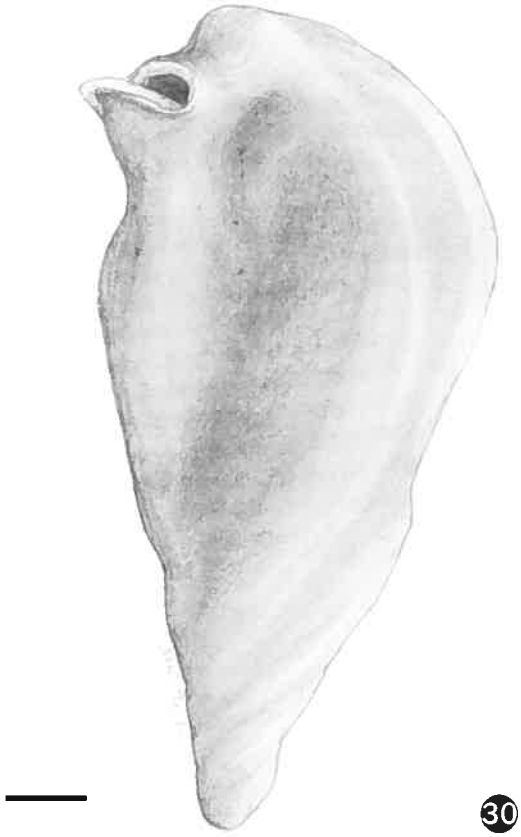
? , no information available; T, trophic stage; S, swarmer stage.

[a], data are lacking to distinguish between opportunistic and obligate parasitism; [b], see also Al-Qassab et al. 2002; [c], see also Triemer & Ott, 1990

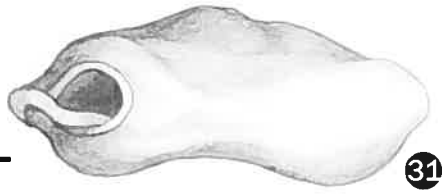

0 s
2 s
3 s
5 s
7 s
*
1
2
3
4
5
6
N
7
O
P
10
8
9


P
O
FA
11
13
P
O
Fl
B
P
12
Fl
FA
15
Fl
FA
14
16
17
B
Fl
18

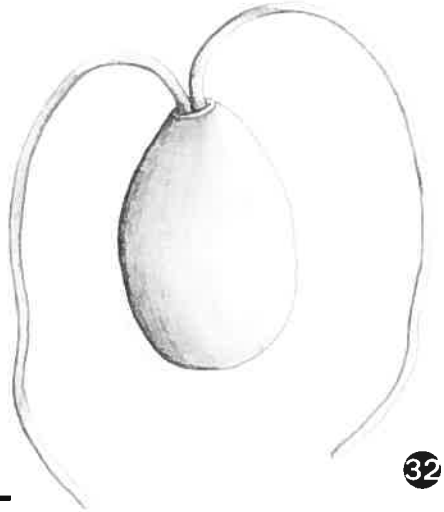

P
FA
19
P
FA
20
P
21
FA
22
C
C
23
C
24
M
M
25
26
27
G
G
G
G
28
N
nu
29



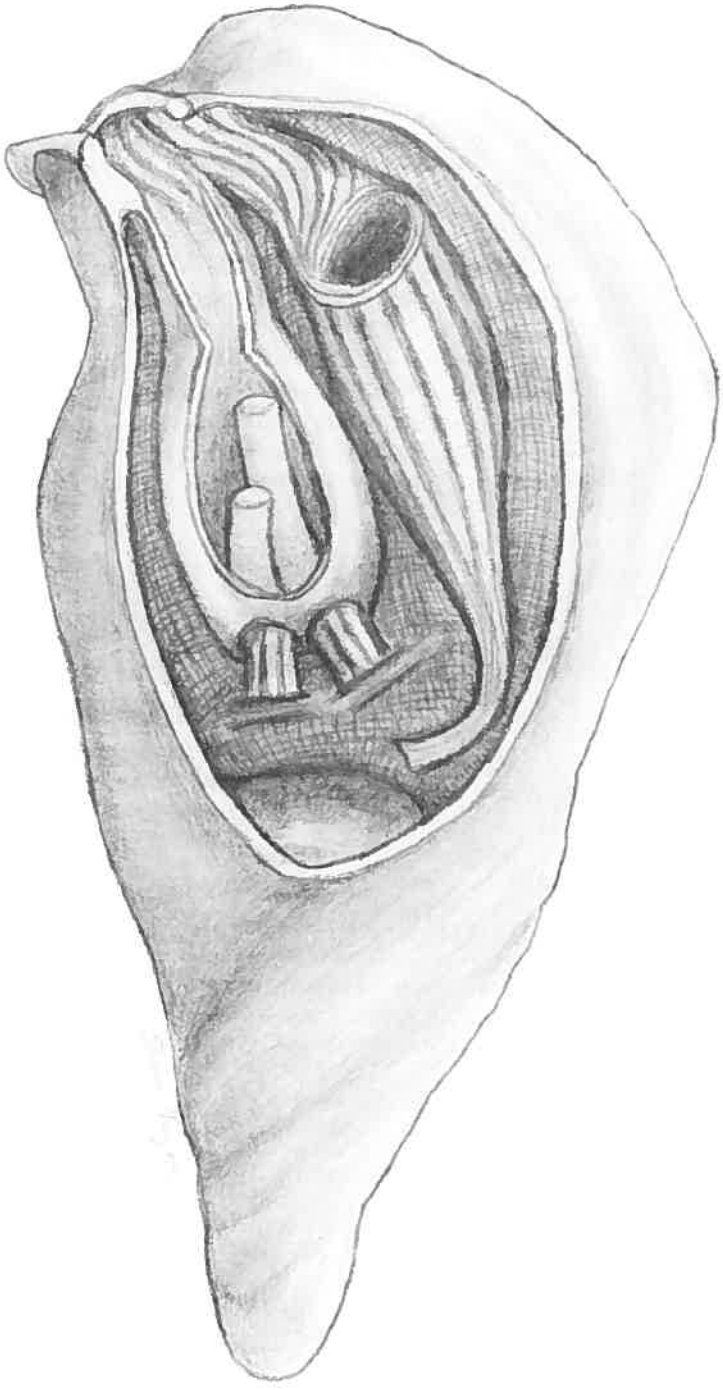
30



31



32



33

ACCORD DES COAUTEURS

1. Identification de l'étudiante et du programme

Joannie Roy, M. SC. Biochimie

2. Description de l'Article

Joannie Roy, Drahomíra Faktorová, Julius Lukeš et Gertraud Burger. 'Unusual Mitochondria Genome Structure throughout Euglenozoa', en préparation pour la soumission à la revue 'Protist'.

3. Déclaration de tous les coauteurs autres que l'étudiant

À titre de coauteur de l'article identifié ci-dessus, je suis d'accord pour que **Joannie Roy** inclue cet article dans **son mémoire de maîtrise** qui a pour titre **Étude de l'évolution de la structure des génomes mitochondriaux chez les Euglenozoa.**

DRAHOMÍRA FAKTOROVÁ [redacted] August 11, 2006

Coauteur Signature Date

Julius Lukeš [redacted] August 11, 2006

Coauteur Date

[redacted]

Gertraud Burger [redacted] 23 Aug 2006

Coauteur Signature Date

Unusual Mitochondrial Genome Structure throughout Euglenozoa

Running title: Euglenozoan Mitochondrial Genomes

Joannie Roy[a], Drahomíra Faktorová[b], Julius Lukeš[b], and Gertraud Burger[a,1]

[a]Centre Robert Cedergren, Bioinformatics & Genomics, Département de biochimie, Université de Montréal, Montréal, QC, H3T 1J4, Canada

[b]Biology Centre, Institute of Parasitology, Czech Academy of Sciences and Faculty of Biology, University of South Bohemia, 370 05 České Budějovice (Budweis), Czech Republic

[1] Corresponding author; fax 1 (514) 343-2210 e-mail [redacted]

One of the most unusual mitochondrial genomes has been found in Kinetoplastea. While typical mitochondrial DNA is made up of a single chromosome, that of Kinetoplastea is composed of multiple chromosomes: The maxicircle that bears 'regular' genes, and numerous minicircles that specify guide RNAs involved in RNA editing. In trypanosomatids (Kinetoplastea), DNA circles are compacted into a single dense body, the kinetoplast. In this report, we address the question whether mitochondrial genomes with multiple chromosomes and aggregated spatial organization are restricted to Kinetoplastea or prevail throughout the whole Euglenozoa - Kinetoplastea, Euglenida and Diplonemea. Until now, we have examined the Diplonemea - *Rhynchopus euleeides* and the Euglenida - *Petalomonas cantuscygni, Peranema trichophorum* and *Entosiphon sulcatum*, using light and electron microscopy and molecular techniques. Our findings indicate that compact mtDNA packaging is confined to trypanosomatids, while multi-chromosome mitochondrial DNA exists in all euglenozoan species studied so far.

Key words: Diplonemea, Euglenida, Kinetoplastea, *Rhynchopus*, *Petalomonas*, *Peranema*

Introduction

Given a common alpha-proteobacterial ancestor of all contemporary mitochondria, a wide variety of evolutionary pressures must have triggered the tremendous diversity observed across eukaryotic lineages. In the most extensively studied taxa, genome structure and gene content vary from monomeric circular molecules of 14-20 kbp coding for two dozen of genes in Metazoa (Wolstenholme 1992), to multimeric linear, circular-mapping molecules of enormous size (Nosek and Tomaska 2003), but containing only up to twice as many genes in plants (Backert et al. 1997). More recent extension of these studies to protists not only provided striking support for the endosymbiont theory of the origin of mitochondria by the discovery of the most bacteria-like mitochondrial genome in *Reclinomonas americana*, but also revealed an astounding diversity of mitochondrial genome structures (Lang et al. 1999; Burger et al. 2003; Gray et al. 2004). Regarding shape, size, conformation or ploidy of protist mitochondrial genome, anything goes.

Mitochondrial genomes of the protist group Kinetoplastea have intrigued the scientific community most profoundly. Their mitochondrial DNA (mtDNA) referred to as the kinetoplast DNA (kDNA) consists of a maxicircle, which generally ranges from 20 to 40 kbp in size depending on the species, and encodes typical mitochondrial genes that are encrypted. Kinetoplasts also contain numerous minicircles of a size varying, across kinetoplastids, between 0.5 to 10 kbp and encode guide RNAs, the decryption's template used in RNA editing (Lukes et al. 2002).

The physical architecture of kDNA (the kinetoplast), observable by transmission electron microscopy, is manifold across kinetoplastids (Table 1). The first and best described structure is the eu-kDNA of *Trypanosoma* and *Crithidia*, a single dense body consisting of a highly organized catenated network of maxi and minicircles. In *Bodo saltans*, a pro-kDNA has been described, whose single dense body is composed of tightly packed, but un-catenated circles. Moreover, in *Bodo*

caudatus and *Cryptobia helicis*, a pan-kDNA is present, characterized by loose and large conglomerates of monomeric circles, filling most of the mitochondrial lumen. A fourth structure, poly-kDNA, which consists of monomeric circles packed in multiple bundles that are scattered throughout the mitochondrial lumen, is found in *Dimastigella mimosa*, *D. trypaniformis* and *Cruzella marina*. Finally, an unusual form of kDNA is the mega-kDNA, present in *Trypanoplasma* and *Jarellia,* where the DNA is distributed evenly in the lumen but where the minicircles are tandemly link together. Apart from their structure, an interesting characteristic of the kinetoplast is its physical connection to the basal bodies of the flagella and its segregation during cell growth (Ogbadoyi et al. 2003).

Recently, two important findings brought to light the possibility that the muti-chromosome organization observed in kinetoplastids might be prevalent in all three euglenozoan clade, i.e., also in diplonemids and euglenids. Indeed, Leander et al, (2001) observed by electron microscopy an inclusion body in mitochondria of *Petalomonas cantuscygni* and *P. mediocanellata*, reminiscent of a kinetoplast. In addition, we recently described mitochondrial chromosomes of 6 and 7 kbp in *Diplonema papillatum* (Marande et al. 2005). In this case, no kinetoplast-like structure was observed but instead, we found that the mitochondrial chromosomes are monomeric circles evenly distributed throughout the mitochondrial lumen.

To investigate whether multi-particle mitochondrial chromosomes are indeed prevalent across all Euglenozoa, we chose to survey the poorly studied euglenids and diplonemids. Within the euglenids, we examined: *Petalomonas cantuscygni*, *Peranema trichophorum* and *Entosiphon sulcatum*, three heterothrophic members of the most basal clade, the Heteronematina (Adl et al. 2005). Within diplonemids, we examined *Rhynchopus euleeides* which was recently described using light, transmission and scanning electron microscopy (Roy et al. submitted). It was formally named *Rhynchopus* sp 1 ATTC 50226 and will be referred in this article to *Rhynchopus euleeides*. Together with the previously characterized *D. papillatum*, both diplonemid genera will then be surveyed.

Here we report the structure of mitochondria and the physical and molecular organization of mtDNA in these Euglenozoa species using fluorescent microscopy, electron microscopy and molecular biology techniques.

Results

mtDNA Distribution within the Cells

For detection and quantification of mtDNA of Euglenozoa, we used DAPI which binds to A+T-rich DNA preferentially by minor-groove interaction (Manzini et al. 1983; Trotta et al. 2003).

Rhynchopus cells stained with DAPI (Fig. 1, 1A-C) show abundant mtDNA in a thin (250-500 nm wide) reticulated pattern. Inspection of different focal planes indicates that the network lines the periphery of the cytoplasm, which is confirmed by electron microscopy (see below).

In *Petalomonas* (Fig. 1, 2A-C), we observed a distribution of DNA similar to *Rhynchopus.* Again, large amounts of mtDNA form a dense, but much thicker (0.5-1.5 μm wide) network meandering through the cell. Surprisingly, the fluorescence of mtDNA is much stronger than that of the nucleus, regardless of the focal plane. The area in the cell that systematically lacks fluorescence corresponds to the feeding and flagellar apparatuses.

Compared to the two above species, the extra-nuclear DNA of *Entosiphon* (Fig. 1, 3A-C) and *Peranema* (Fig. 1, 4A-C) appears much less abundant. *Entosiphon* displays small diffusely fluorescent spheres of ~ 0.2 μm diameter in certain areas of the cell, whereas in others, the fluorescence pattern is voluminous and cloudy. *Peranema* cells reveal fluorescent bodies of ~1.0 μm length, with distinct oval to rod-like shapes often arranged in rosettes. Electron microscopy confirms that these bodies are indeed mitochondria (see Fig 2D).

Mitochondrial Ultrastructure in Diplonemids and Euglenids: multiple monomeric circles

Cell sections of *Rhynchopus* clearly show that the reticulated and most likely single mitochondrion lies at the periphery of the cell, surrounding almost totally the cytoplasm (Fig. 2A, 2B). The width of sectioned mitochondria (0.2-0.6 μm) corresponds to that of the network seen in DAPI staining. The scarce cristae are sometimes very long and arranged longitudinally (see also Roy et al. submitted).

The *Petalomonas* mitochondrion occupies a large proportion of the cytoplasm and usually appears in the sections as big spheres of diameters varying from 0.2 to 0.8 μm (Fig 2C, 2E). Mitochondria enclose ribbon-shaped inclusions (0.6 to 1.4 μm wide) occupying about 50% of the section.

Peranema (Fig 2D) contains numerous mitochondria of oval shape, with a length varying from 0.2 to 1.3 μm. The cristae are often numerous and intersect completely the mitochondrial matrix. In addition to the mitochondria, we observed other spherical compartments (Fig. 2F) of 1.4 to 3 μm diameter, which contain vesicles of 0.5 to 1.3 μm length and probably represent food vacuoles enclosing bacteria.

In none of the examined species did we detect a dense body resembling a kinetoplast, nor was observed a connection between the base of the flagellar apparatus and the mitochondrion.

Spatial Properties and Architecture of mtDNA

To separate DNA according to nucleotide composition, we used buoyant density gradient centrifugation with cesium chloride and bisbenzimide. This latter compound intercalates specifically in A+T-rich regions, thus decreasing the density of DNA. This allows separation of various genomes present in the same cell (or culture), provided that the nucleotide composition is homogenous within a genome and different between them. After separation, the DNA size and topology

has been assessed by restriction enzyme digestion patterns and by electron microscopy of spread DNA.

Rhynchopus DNA (Fig. 3A) separates into two bands, whose genomic origins were inferred based on the complexity of the restriction enzyme pattern (Fig 3E, line 1) and later by DNA sequencing. The A+T-rich DNA corresponds to mtDNA, forming a band nearly as broad in the gradient as the more G+C-rich nuclear DNA. Electron microscopy of the mtDNA fraction shows small circular monomeric molecules. The molecules sizes fall into two distinct classes; one around 7 kbp (± 0.1) and the second one, on average, 7.7 kbp (± 0.2); a very small percentage (<1%) is dimeric. The conformation of at least 20% of the molecules is supercoiled, 70% is relaxed, and the rest is linear, probably broken during manipulations.

DNA extracted from *Petalomonas* forms four bands in CsCl (Fig. 3B). The restriction enzyme pattern of the most A+T-rich band is of low complexity, indicating that it is mtDNA (Fig 3E, line 3). The lowest band has the same density as *Pseudomonas fluorescens*, on which *Petalomonas* was fed. DNA spreading of the A+T-rich band revealed mostly linear mtDNA molecules (Fig. 4B-C), - some with lariat ends, up to 50 kbp in size, but also some circles of 1-2.5 kbp (Fig. 4D-E). Among the few hundreds molecules screened, we observed two instances of a circular 40-kbp molecule (Fig. 4I). The two middle bands of the CsCl gradient have not been characterized yet.

In *Peranema*, because of the low-complexity restriction pattern (Fig 3E, line 5), the G+C-rich band is most likely mtDNA (Fig. 3C). In electron microscopy, it consists of linear molecules of random size (1-75 kbp, data not shown). No assessment of *Entosiphon* DNA was possible, because all bands separated by CsCl gradient (Fig 3D), after digesion, appeared as a smear, regardless of the restriction enzymes used (data not shown).

Discussion

Euglenozoa, United by their Mitochondria

Euglenozoa mitochondria are highly unusual in many regards. Most members of the three groups, Kinetoplastea, Diplonemea and Euglenida, possess a single mitochondrion (Table 1). Moreover, the mitochondrion of most euglenids and diplonemids is reticulated, lining the inner surface of the cell (Mignot 1966; Leedale 1967; Leedale and Buetow 1970; Pellegrini 1980b; a; Hilenski and Walne 1985a; b; Cann and Pennick 1986; Hayashi and Ueda 1989; Belhadri and Brugerolle 1992; Leander et al. 2001; Marande et al. 2005)(Roy et al, submitted). Notable exceptions are *Peranema trichophorum* and *Entosiphon sulcatum,* which possess multiple mitochondria. In all Euglenozoa studied so far, mtDNA is composed of multiple chromosomes. In most cases, the DNA of mitochondria accounts for high proportion of the fluorescence compared to the nucleus. Organization of mtDNA in dense bodies seems to be confined to Trypanosoma.

Not only the morphology, but also the metabolism of euglenozoan mitochondria is unusual. For example, Euglenozoa do not feature the classical aerobic metabolism where pyruvate is converted by pyruvate dehydrogenase (PDH) via acetyl-CoA to CO_2 through the Krebs cycle (Ginger 2005). Trypanosomes, although obligate aerobic, are characterized, in culture, by a dysfunctional Krebs cycle and the presence of the acetate:succinate CoA transferase (ASCT), a pathway typical for anaerobic mitochondria and hydrogenosomes. The energy metabolism in *E. gracilis* mitochondria is a similar hybrid of aerobic and anaerobic pathways. Here, pyruvate is processed via the constitutive pyruvate:NADP+ oxidoreductase (PNO), a fusion enzyme similar to the pyruvate:ferrodoxin oxidoreductases (PFO), otherwise found in anaerobic mitochondria. This particularity is not restricted to *E. gracilis* since we found a PNO homologue also in a *P. trichophorum* cDNA library (Genbank number

XXXX). To our knowledge, energy metabolism in diplonemids has not been examined yet.

Diplonemids Circular and Monomeric Mitochondrial Genome

The diplonemids, mostly free-living fresh or salt water flagellates, have been first described in the early 19th century. However, the inclusion of *Rhynchopus* in diplonemids, as well as their affiliation to Euglenozoa, is much more recent. One of the characteristics of *Rhynchopus* and *Diplonema* is the distinct shape of mitochondrial cristae which are conspicuously scarce and arranged longitudinally (Kent et al. 1987; Triemer and Ott 1990; Triemer and Farmer 1991; Schnepf 1994; Simpson 1997; Vickerman 2000; Marande et al. 2005). In both genera, mitochondrial chromosomes are small, circular, and monomeric (present results and Marande et al. 2005). We have shown that mitochondrial genes of *D. papillatum* display a unique structure with each chromosome carrying a single gene fragment and requiring trans-splicing to produce mRNAs. Ongoing research in our laboratory indicates that genome architecture and gene structure are shared by *R. euleeides* and other *Diplonema* species (Marande, unpublished). The main difference is that in *D. papillatum*, mitochondrial chromosomes are smaller than those of *R. euleeides* - 5.9 and 7.2 kbp, versus 7.0 and 7.7 kbp, and that supercoils make up only a minor fraction (Marande et al. 2005). Another interesting difference between the two examined species is the relative base composition of their genomes. In *D. papillatum*, the nuclear genome is more A+T rich than the mitochondrial one, while *R. euleeides* shows the opposite.

Enigmatic mitochondrial genome structure in euglenids

The distribution and the structure of the mtDNA of the euglenid model species *E. gracilis* is still unclear. In *E. gracilis*, the mtDNA forms small thread-shaped clusters, termed nucleoids, which, in immunoelectron microscopy, are surrounded

by a region denser than the mitochondrial matrix. When observed by DAPI, the nucleoids are spherical or ovoid in shape and 0.70-1.30 μm in diameter (Hayashi and Ueda 1989; Hayashi-Isimaru et al. 1993). In this regard, the DNA distribution observed in *Peranema trichophorum* and *E. sulcatum* is similar to that in *E. gracilis*. In *Petalomonas cantuscygni*, mtDNA is of much different appearance, apparently filling the whole mitochondrion. In contrast, *Petalomonas* mitochondria contain an inclusion body that is reminiscent of a kinetoplast (Leander et al. 2001), although our data show that in this euglenid, mtDNA is distributed throughout the reticulated mitochondrion, rather than in clusters. The electron-dense region within this mitochondrion appears to contain mtDNA, but according to the fluorescence pattern observed using DAPI, it is not likely that DNA accounts for this high electron-density, as is the case in trypanosomes.

Many studies have been published on the mitochondrial chromosomes of *E. gracilis*. Observations include a collection of diverse small linear molecules and some rare circular molecules of uncertain origins (Manning et al. 1971; Nass et al. 1974; Talen et al. 1974; Buetow 1989; Yasuhira and Simpson 1997; Gray et al. 2004). To date, there is no clear consensus on the length and the complexity of this genome. The length range varies from less than 1 kbp to up to 60-70 kbp. However, all these independent studies agree on the difficulty to study *E. gracilis* mtDNA. Similarly, the genome of *Petalomonas cantuscygni* is though to be highly complex. It most likely consists of a large circular chromosome plus a few smaller ones, as seen by EM. The *in vivo* conformation of the *P. cantuscygni* mitochondrial genome might be composed of polydisperse, circularly permuted, linear molecules, as in plants and fungi. This would explain the scarceness of large circles and the presence of lariat-ends. The presence of small circles could then be explained by the resolution of the lariat species, which might represent replication intermediaries.

Provided that the assignment of mtDNA of *Peranema trichophorum* is correct, its mtDNA is large and of high complexity comparable to that described for *E. gracilis* (Buetow 1989; Gray et al. 2004). Pulsed-field gel electrophoresis

(PFGE) experiments could possibly confirm (or refute) the presence of different size populations of molecules in this euglenid.

Despite numerous studies, only a few genes have been identify so far in the mitochondrial genome of *E. gracilis*. Split LSU, SSU (Gray et al. 2004) and some tRNAs (Buetow 1989) have been reported, while only the sequence of *cox1* (Tessier et al. 1997; Yasuhira and Simpson 1997) is available. Moreover, the DNA is A+T rich with patches of G+C rich regions (see latter citations). In *Petalomonas cantuscygni*, sequencing data from a library constructed with the most A+T-rich DNA are consistent with a ~40 kbp circular molecule with additional smaller ones (Roy et al., unpublished). However, despite a total of ~50 kbp and >25 kbp contiguous sequences, only a partial gene fragment (*atp6*) could be annotated with confidence. This sheds light on the complexity of expected genome structure. Finally, in the case of *Peranema trichophorum*, the construction of a mtDNA library, using the G+C-rich band isolated from CsCl/bisbenzimide gradient, failed. This library contains unrecognizable sequences and also some related to the Wolbachieae or Rickettsieae group and other intracellular or free-living alpha-proteobacteria. Whether these bacteria are endosymbionts, ectosymbionts, or a contaminant of the culture is unknown at the current time.

Concluding remarks

In diplonemids and euglenids, our findings show that abundant, circular multi-chromosome mtDNA is much more widespread across Euglenozoa than was thought before. What remains unclear is the time of apperance of these commun features in the course of evolution and whether they are linked to one another.

Trying to answer questions about genome structure, coding content, expression and function of these mitochondria is not a trivial task, but once the phylogenic relationships within the three groups are resolved with confidence, things will be facilitated. Phylogenetic knowledge will also help to obtain clues about the origin of RNA editing that prevails in kinetoplastids. Having that in mind, we have started to sequence cDNAs from *Diplonema*, *Rhynchopus*,

Peranema and *Petalomonas* in order to unveil their mitochondrial gene structure and content. Due to their apparent complexity, ingenious approaches will be needed in order to solve the puzzle of euglenozoan mitochondrial genomes.

Methods

Culture: *Rhynchopus euleeides* n. sp. (previously named '*Rhynchopus* sp.1' - ATCC 50226) was obtained from the American Type Culture Collection. Cells were 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/liter thiamine-HCl), and trace metal elements (4.36 µg/liter Na_2EDTA, 3.15 µg/liter $FeCl_3 \cdot 6\ H_2O$, 9.8 µg/liter $CuSO_4 \cdot 5H_2O$, 22 µg/liter $ZnS0_4 \cdot 7H_2O$, 10 µg/liter $CoCl_2 \cdot 6H_2O$, 18 µg/liter $MnCl_2 \cdot 4H_2O$, 6.3 µg/liter $NaMoO_4 \cdot 2H_2O$), supplemented with 10% horse (HS) or fetal bovine (FBS) serum. *Peranema trichophorum* – CCAP 1260/1B was obtained from Collection Culture of Algae and Protozoa. They were grown axenically in KNOP medium as described in Saito et al. (2003) but using 0.01-0.1% crystallized egg yolk as nutrient source. *Petalomonas cantuscygni* - CCAP 1259/1 was obtained from Collection Culture of Algae and Protozoa. It was cultivated in ASWP medium consisting of 3.3% sea salts, 5% soil extract, 5 mg/litre tricine and minerals (5.63 mg/liter $NaNO_3$, 0.23 mg/liter Na_2HPO_4, 0.19 mg/liter K_2HPO_4) and supplemented with 10^4/ml of *Pseudomonas fluorescens*. *Entosiphon sulcatum* (kindly provided by Mark Farmer (UGA)) was grown in KNOP medium supplemented with Timothy hay infusion and *Silicibacter sp.* A barley seed was added to keep bacteria growing.

DAPI (4',6'-diamidino-2-phenylindole)-staining: Cells were fixed for 10 min at room temperature in 4% paraformaldehyde diluted in either artificial sea water or PBS. Fixation was stopped by spinning the cells down and resuspending them in respective 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 phosphate-buffered saline (PBS) for 5 min. The stained cells 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 the cell, cultures were washed twice in artificial sea water or PBS, centrifuged at low-speed (~1200 x g) and fixed in 2% glutaraldehyde (GA) 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. They were post-fixed with 2% OsO4 in PB for 2 h at room temperature and then washed in 0.25 M PB. After dehydration in graded series of ethanol, the cells were embedded in Epon-Araldite. Ultrathin sections were stained with lead citrate and uranyl acetate and examined under a JEOL JEM 1010 microscope.

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

DNA isolation: 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). DNA was extracted by 1mg/ml pronase E (Sigma) and 3% N-laurosyl-sarcosine. DNA was purified by series of phenol-chloroform extraction and ethanol precipitation. DNA was resuspended in TE. 10 ug/ml bisbenzimide (Hoechst 33258) and 1,1g/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 needle 18G1. Dilution and high speed

centrifugation were used to remove the salt. Isopropyl alcohol precipitation was used to remove bisbenzimide. Assessment of the mitochondrial band was confirmed by restriction enzyme pattern (RsaI for *P. cantuscygni* and *R. euleeides*, SspI for *P. trichophorum*).

Acknowledgments

This research was supported by a grant MOP-79309, from the Canadian Institutes of Health Research (CIHR), Genetics Program, to G.B., fellowships of the Agence univeristaire de la francophonie (AUF), the Ministry of Education of Québec and CIHR to J.R., by grants 5022302 and Z60220518 from the Grant Agency of the Czech Academy of Sciences and grant 6007665801 from the Ministry of Education of the Czech Republic, to J.L. We gratefully acknowledge the Canadian Institute for Advanced Research (CIAR), Program for Evolutionary Biology, for salary and interaction support to G.B. and to D.F. We gratefully thank O. Benada (Institute of Microbiology, Prague, Czech Republic) for assistance in electron microscopy of mtDNA and William Marande for sharing unpublished data.

References

Adl, S. M., Simpson, A. G., Farmer, M. A., Andersen, R. A., Anderson, O. R., Barta, J. R., Bowser, S. S., Brugerolle, G., Fensome, R. A., Fredericq, S., James, T. Y., Karpov, S., Kugrens, P., Krug, J., Lane, C. E., Lewis, L. A., Lodge, J., Lynn, D. H., Mann, D. G., McCourt, R. M., Mendoza, L., Moestrup, O., Mozley-Standridge, S. E., Nerad, T. A., Shearer, C. A., Smirnov, A. V., Spiegel, F. W. & Taylor, M. F. 2005. The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. *J. Eukaryot. Microbiol.*, **52**:399-451.

Backert, S., Nielsen, B. L. & Borner, T. 1997. The mystery of the rings: structure and replication of mitochondrial genomes from higher plants. *Trends Plant Sci*, **2**:477-483.

Belhadri, A. & Brugerolle, G. 1992. Morphogenesis of the Feeding Apparatus of *Entosiphon sulcatum* - an Immunofluorescence and Ultrastructural-Study. *Protoplasma*, **168**:125-135.

Blom, D., de Haan, A., van den Berg, M., Sloof, P., Jirku, M., Lukes, J. & Benne, R. 1998. RNA editing in the free-living bodonid *Bodo saltans*. *Nucleic Acids Res*, **26**:1205-13.

Borst, P., Fase-Fowler, F., Weijers, P. J., Barry, J. D., Tetley, L. & Vickerman, K. 1985. Kinetoplast DNA from *Trypanosoma vivax* and *T. congolense*. *Mol Biochem Parasitol*, **15**:129-42.

Borst, P., Hoeijmakers, J. H. J. & Hajduk, S. L. 1981. Structure, Function and Evolution of Kinetoplast DNA. *Parasitology*, **82**:81-&.

Buetow, D. E. 1989. The mitochondrion. *In* Inc, A. P. (ed.), The biology of *Euglena*, San Diego. p. 247-314.

Burger, G., Gray, M. W. & Lang, B. F. 2003. Mitochondrial genomes - anything goes. *Trends Genet, in press*.

Cann, J. P. & Pennick, N. C. 1986. Observations on *Petalomonas cantuscygni* n. sp., a New Halo-Tolerant Strain. *Archiv Fur Protistenkunde*, **132**:63-71.

Cheng, D. & Simpson, L. 1978. Isolation and characterization of kinetoplast DNA and RNA of Phytomonas davidi. *Plasmid*, **1**:297-315.

Coggins, L. W. 1987. Preparation of nucleic acids for electron microscopy. *In* Sommerville, J. & Scheer, U. (ed.), EM in molecular biology; a practical approach. Oxford press, Washington. p. 1-28.

Fergusson, L. & Davis, R. 1978. Quantitative electron microscopy of nucleic acids. *In* Koehler, J. K. (ed.), Advanced Techniques in Biological Electron Microscopy II. Springer Verlag, Berlin. p. 123-171.

Ginger, M. L. 2005. Trypanosomatid biology and euglenozoan evolution: new insights and shifting paradigms revealed through genome sequencing. *Protist*, **156**:377-92.

Gray, M. W., Lang, B. F. & Burger, G. 2004. Mitochondria of protists. *Annu Rev Genet*, **38**:477-524.

Hajduk, S. L., Siqueira, A. M. & Vickerman, K. 1986. Kinetoplast DNA of Bodo caudatus: a noncatenated structure. *Mol Cell Biol*, **6**:4372-8.

Hayashi, Y. & Ueda, K. 1989. The shape of mitochondria and the number of mitochondrial nucleoids during the cell cycle of *Euglena gracilis*. *J Cell Sci*, **93**:565-570.

Hayashi-Isimaru, Y., Ueda, K. & Nonaka, M. 1993. Detection of DNA in the nucleoids of chloroplasts and mitochondria in *Euglena gracilis* by immunoelectron microscopy. *J Cell Sci*, **105**:1159-1164.

Hilenski, L. L. & Walne, P. L. 1985a. Ultrastructure of the Flagella of the Colorless Phagotroph *Peranema trichophorum* (Euglenophyceae) .1. Flagellar Mastigonemes. *Journal of Phycology*, **21**:114-125.

Hilenski, L. L. & Walne, P. L. 1985b. Ultrastructure of the Flagella of the Colorless Phagotroph *Peranema trichophorum* (Euglenophyceae) .2. Flagellar Roots. *Journal of Phycology*, **21**:125-134.

Kent, M. L., Elston, R. A., Nerad, T. A. & Sawer, T. K. 1987. An *Isonema*-like flagellate (Protozoa: Mastigophora) infection in larval Geoduck clams, *Panope abrupta*. *J. Invertebr. Pathol.*, **50**:221-229.

Lang, B. F., Gray, M. W. & Burger, G. 1999. Mitochondrial genome evolution and the origin of eukaryotes. *Annu Rev Genet*, **33**:351-97.

Leander, B. S., Triemer, R. E. & Farmer, M. A. 2001. Character evolution in heterotrophic euglenids. *European Journal of Protistology*, **37**:337-356.

Leedale, G. F. 1967. Euglenida-euglenophyta. *Annu Rev Microbiol*, **21**:31-48.

Leedale, G. F. & Buetow, D. E. 1970. Observations on the mitochondrial reticulum in living *Euglena gracilis*. *Cytobiologie*, **1**:195-202.

Lukes, J., Arts, G. J., van den Burg, J., de Haan, A., Opperdoes, F., Sloof, P. & Benne, R. 1994. Novel pattern of editing regions in mitochondrial transcripts of the cryptobiid Trypanoplasma borreli. *EMBO J*, **13**:5086-98.

Lukes, J., Guilbride, D. L., Votypka, J., Zikova, A., Benne, R. & Englund, P. T. 2002. Kinetoplast DNA network: evolution of an improbable structure. *Eukaryot Cell*, **1**:495-502.

Lukes, J., Jirku, M., Avliyakulov, N. & Benada, O. 1998. Pankinetoplast DNA structure in a primitive bodonid flagellate, *Cryptobia helicis*. *Embo J*, **17**:838-46.

Manning, J. E., Wolstenholme, D. R., Ryan, R. S., Hunter, J. A. & Richards, O. C. 1971. Circular chloroplast DNA from *Euglena gracilis*. *Proc Natl Acad Sci U S A*, **68**:1169-73.

Manzini, G., Barcellona, M. L., Avitabile, M. & Quadrifoglio, F. 1983. Interaction of diamidino-2-phenylindole (DAPI) with natural and synthetic nucleic acids. *Nucleic Acids Res*, **11**:8861-76.

Marande, W., Lukeš, J. & Burger, G. 2005. Unique mitochondrial genome structure in diplonemids, the sister group of kinetoplastids. *Eukaryot. Cell*, **4**:1137-46.

Maslov, D. A., Hollar, L., Haghighat, P. & Nawathean, P. 1998. Demonstration of mRNA editing and localization of guide RNA genes in kinetoplast-mitochondria of the plant trypanosomatid *Phytomonas serpens*. *Mol Biochem Parasitol*, **93**:225-36.

Maslov, D. A. & Simpson, L. 1994. RNA editing and mitochondrial genomic organization in the cryptobiid kinetoplastid protozoan *Trypanoplasma borreli*. *Mol Cell Biol*, **14**:8174-82.

Mignot, J. 1966. Structure et ultrastructure de quelques euglenomonadines. *Protistologica*, **2**:51-117.

Nass, M. M., Schori, L., Ben-Shaul, Y. & Edelman, M. 1974. Size and configuration of mitochondrial DNA in *Euglena gracilis*. *Biochim Biophys Acta*, **374**:283-91.

Nosek, J. & Tomaska, L. 2003. Mitochondrial genome diversity: evolution of the molecular architecture and replication strategy. *Curr Genet*, **44**:73-84.

Ogbadoyi, E. O., Robinson, D. R. & Gull, K. 2003. A high-order trans-membrane structural linkage is responsible for mitochondrial genome positioning and segregation by flagellar basal bodies in trypanosomes. *Mol Biol Cell*, **14**:1769-79.

Pellegrini, M. 1980a. Three-dimensional reconstruction of organelles in *Euglena gracilis* Z. I. Qualitative and quantitative changes of chloroplasts and

mitochondrial reticulum in synchronous photoautotrophic culture. *J Cell Sci*, **43**:137-66.

Pellegrini, M. 1980b. Three-dimensional reconstruction of organelles in *Euglena gracilis* Z. II. Qualitative and quantitative changes of chloroplasts and mitochondrial reticulum in synchronous cultures during bleaching. *J Cell Sci*, **46**:313-40.

Riou, G. F. & Saucier, J. M. 1979. Characterization of the molecular components in kinetoplast-mitochondrial DNA of *Trypanosoma equiperdum*. Comparative study of the dyskinetoplastic and wild strains. *J Cell Biol*, **82**:248-63.

Saito, A., Suetomo, Y., Arikawa, M., Omura, G., Khan, S. M., Kakuta, S., Suzaki, E., Kataoka, K. & Suzaki, T. 2003. Gliding movement in *Peranema trichophorum* is powered by flagellar surface motility. *Cell Motil Cytoskeleton*, **55**:244-53.

Schnepf, E. 1994. Light and electron microscopical observations in *Rhynchopus coscinodiscivorus* spec. nov., a colorless, phagotrophic euglenozoon with concealed flagella. *Arch. Protistenkd.*, **144**:63-74.

Simpson, A. G. B. 1997. The identity and composition of the Euglenozoa. *Arch. Protistenkd.*, **148**:318-28.

Stolba, P., Jirku, M. & Lukes, J. 2001. Polykinetoplast DNA structure in *Dimastigella trypaniformis* and *Dimastigella mimosa* (Kinetoplastida). *Mol Biochem Parasitol*, **113**:323-6.

Sturm, N. R. & Simpson, L. 1990. Partially edited mRNAs for cytochrome b and subunit III of cytochrome oxidase from *Leishmania tarentolae* mitochondria: RNA editing intermediates. *Cell*, **61**:871-8.

Talen, J. L., Sanders, J. P. & Flavell, R. A. 1974. Genetic complexity of mitochondrial DNA from *Euglena gracilis*. *Biochim Biophys Acta*, **374**:129-35.

Tessier, L. H., van der Speck, H., Gualberto, J. M. & Grienenberger, J. M. 1997. The *cox1* gene from *Euglena gracilis*: a protist mitochondrial gene without introns and genetic code modifications. *Curr Genet*, **31**:208-13.

Triemer, R. & Farmer, M. A. 1991. The ultrastructural organization of the heterotrophic euglenids and its evolutionary implications. *In* Patterson, D. &

Larsen, J. (ed.), The biology of free-living heterotrophic flagellates. Clarendon Press, Oxford, U.K. p. 185-205.

Triemer, R. E. & Ott, D. W. 1990. Ultrastructure of *Diplonema ambulator* Larsen & Patterson (Euglenozoa) and its relationship to *Isonema*. *Europ. J. Protistol.*, **25**:316-320.

Trotta, E., Del Grosso, N., Erba, M., Melino, S., Cicero, D. & Paci, M. 2003. Interaction of DAPI with individual strands of trinucleotide repeats. Effects of replication in vitro of the AAT x ATT triplet. *Eur J Biochem*, **270**:4755-61.

Vickerman, K. 2000. Diplonemids. *In* Lee, J. J., Leedale, G. F. & Bradbury, P. (ed.), An illustrated guide to the protozoa. Allen Press, Lawrence, Kansas. p. 1157-1159.

Wolstenholme, D. R. 1992. Animal mitochondrial DNA: structure and evolution. *Int Rev Cytol*, **141**:173-216.

Yasuhira, S. & Simpson, L. 1995. Minicircle-encoded guide RNAs from *Crithidia fasciculata*. *Rna*, **1**:634-43.

Yasuhira, S. & Simpson, L. 1997. Phylogenetic affinity of mitochondria of *Euglena gracilis* and kinetoplastids using cytochrome oxidase I and hsp60. *J Mol Evol*, **44**:341-7.

Yurchenko, V., Hobza, R., Benada, O. & Lukes, J. 1999. *Trypanosoma avium*: large minicircles in the kinetoplast DNA. *Exp Parasitol*, **92**:215-8.

Zikova, A., Vancova, M., Jirku, M. & Lukes, J. 2003. *Cruzella marina* (Bodonina, Kinetoplastida): non-catenated structure of poly-kinetoplast DNA. *Exp Parasitol*, **104**:159-61.

Figure Legends

Figure 1. DNA distribution within cells. (1-4, A) Light microscopy of stained cells. (1-4, B) Same cells as in A, observed by fluorescent microscopy. (1-4, C) Fluorescent microscopy at higher magnification. (1A-C) *R. euleeides*. (2A-C) *P. cantuscygni*. (3A-C) *E. sulcatum*. (4A-C) *P. trichophorum*. (1A-2C) DNA is abundant and distributed in a reticulated pattern. (3A-4C) DNA is scarce and scattered across the cells. Note stained surrounding bacteria in 2B. Fixed cells were stained by DAPI. n, nucleus; p, sub-apical pocket. Bar = 5 μm.

Figure 2. Transmission electron microscopy of mitochondria and other vesicles. (A-B) *R. euleeides*. Long and reticulated mitochondrion lining the cell periphery and containing very few cristae. (A) Apical region. (B) Whole cell. Arrows show mitochondrion 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, probably bacteria. m, mitochondrion; p, sub-apical pocket; v, vesicle. Bar =1 μm.

Figure 3. DNA separation and identification. (A-D) CsCl/bisbenzimide gradient. (A) *R. euleeides*. Note that mitochondrial DNA is more abundant than nuclear (B) *P. cantuscygni*. The two middle bands were not successfully examined. (C) *P. trichophorum*. Here, mitochondrial DNA is in the G+C rich band. (D) *E. sulcatum*. Three bands of yet unknown subcellular nature. Note that *E. sulcatum* is bacteria fed. (E) Restriction enzyme digestion patterns. Lanes 2, 4, and 6, 1 kb+ molecular weight marker. Lane 1, *R. euleeides* mtDNA digested with RsaI. Lane 3, *P. cantuscygni* mtDNA digested with RsaI. Lane 5, *P. trichophorum* mtDNA

digested with SspI. m, mitochondrial DNA; n, nuclear DNA; b, bacterial DNA. Molecular weight in kbp.

Figure 4. DNA spreading observed by electron microscopy. (A) *R. euleeides*. mtDNA molecules 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).

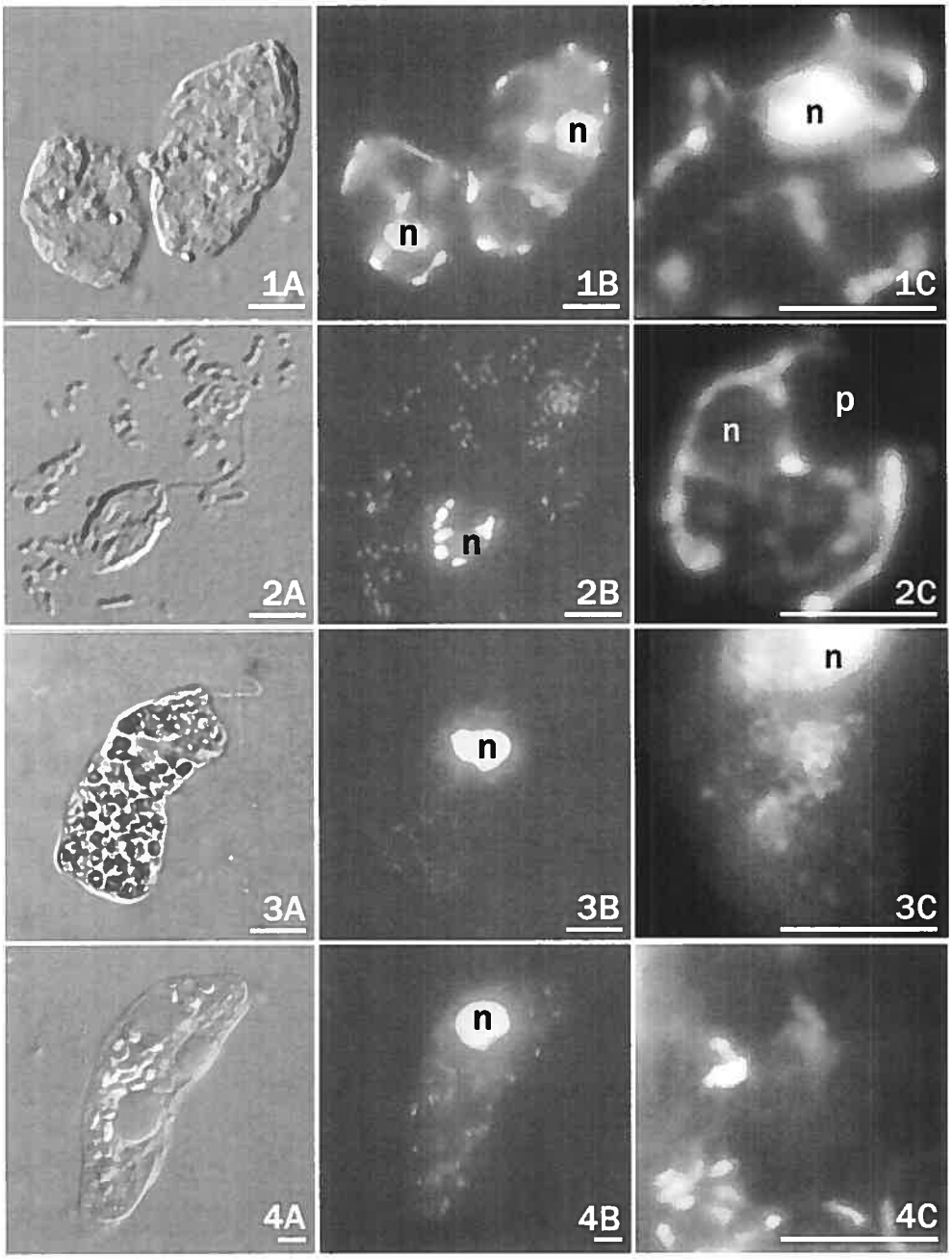
digested with SspI. m, mitochondrial DNA; n, nuclear DNA; b, bacterial DNA. Molecular weight in kbp.

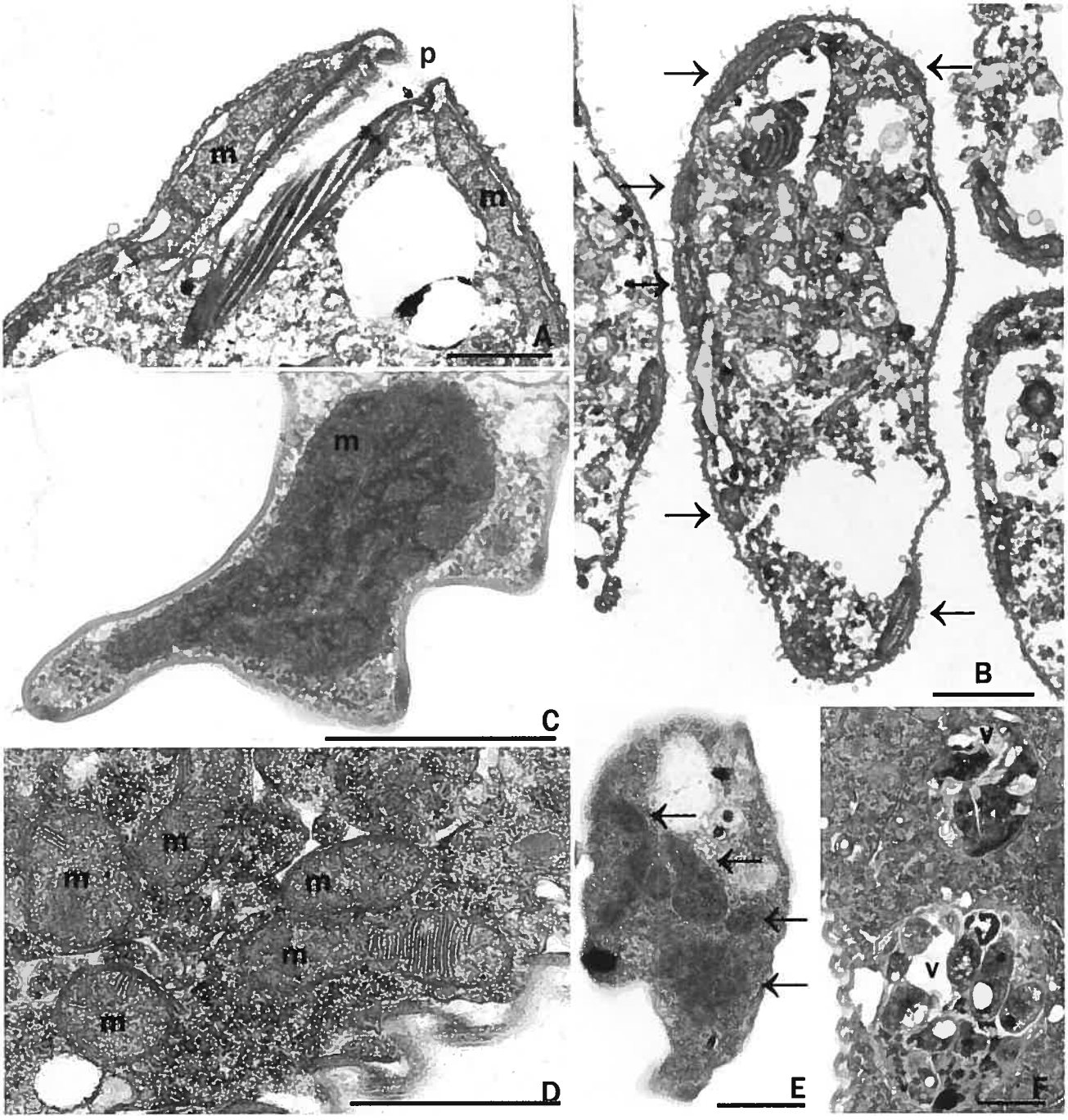
Figure 4. DNA spreading observed by election microscopy. (A) *R. euleeides*. mtDNA molecules of 7 kbp in three different conformations (B-I) *P. cantuscygni*. (B-C) Linear molecules with lariat ends. (D-H) Small circular molecules of various sizes. (I) Large circular molecules. oc, open circle; l, linear;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

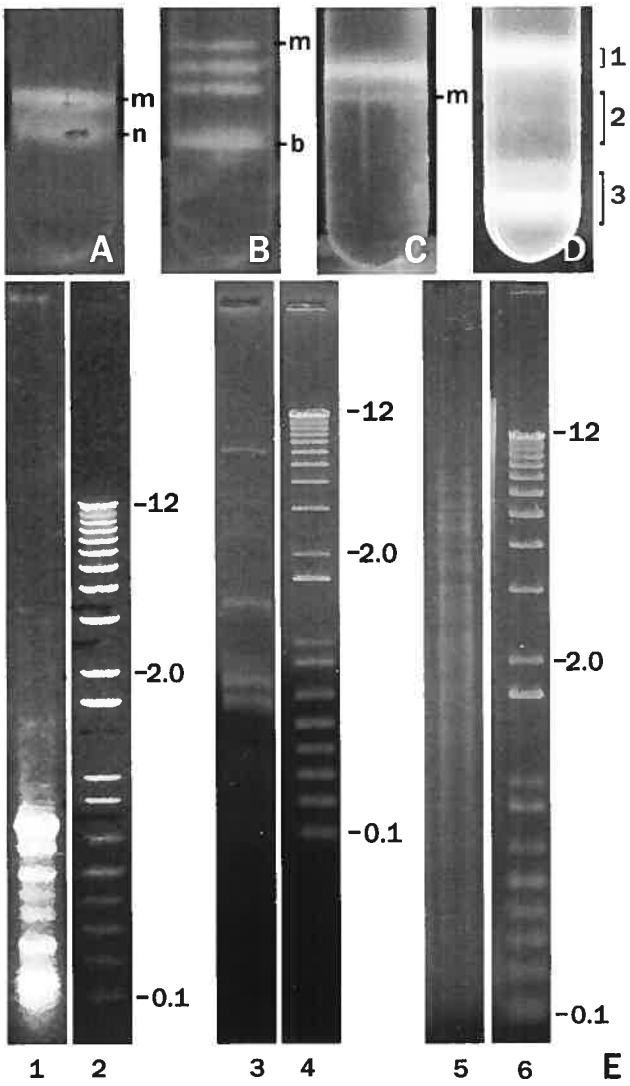
Table 1. Comparaison of mtDNA within Euglenozoa

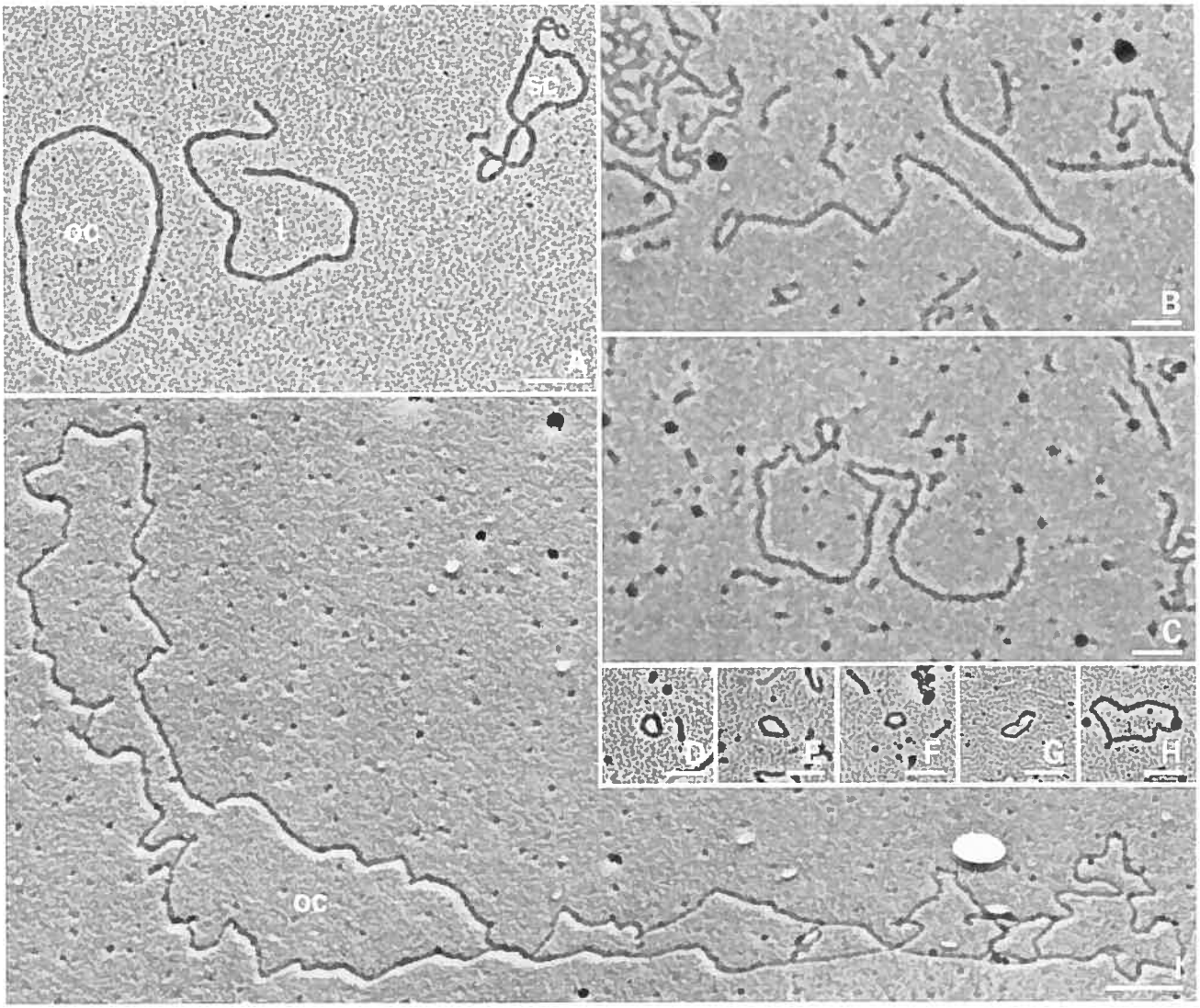
Taxon	Mitochondrial shape	mtDNA distribution	Chromosome topology	Chromosome number	Size (kbp)	Reference
Kinetoplastea - trypanosomatids						
Trypanosoma brucei	Elongated	Eu-kDNA	Minicircle	Multitude	1.0	1
			Maxicircle	Single	21-27	
T. vivax	Elongated	Eu-kDNA	Minicircle	Multitude	0.5	2
			Maxicircle	Single	23	
T. avium	Elongated	Eu-kDNA	Minicircle	Multitude	10	3
			Maxicircle	Single	ND	
T. cruzi	Elongated	Eu-kDNA	Minicircle	Multitude	1.4	4
			Maxicircle	Single	40	
T. mega	Elongated	Eu-kDNA	Minicircle	Multitude	2.3	4
			Maxicircle	Single	24.5	
T. equiperdum	Elongated	Eu-kDNA	Minicircle	Multitude	0.97	5
			Maxicircle	Single	24	
Leishmania tarentolae	Elongated	Eu-kDNA	Minicircle	Multitude	0.9	6
			Maxicircle	Single	21	
Crithidia fasciculata	ND	Eu-kDNA	Minicircle	Multitude	2.5	1
			Maxicircle	Single	38	
C. luciliae	ND	ND	Minicircle	Multitude	2.5	4
			Maxicircle	Single	35	
Phytomonas serpens	ND	Eu-kDNA	Minicircle	Multitude	1.5	7
			Maxicircle	Single	27-36	
P. davidi	ND	ND	Minicircle	Multitude	1.1	8
			Maxicircle	Single	36.5	
Herpetomonas muscarum	ND	ND	Minicircle	Multitude	1.1	4
			Maxicircle	Single	32	
H. ingenoplastis	ND	ND	Minicircle	Single	17-23	4
			Maxicircle	Single	36	
Kinetoplastea - bodonids						
Bodo saltans	Elongated	Pro-kDNA	Minicircle	Multitude	1.4	9
			Maxicircle	Single	70	
Cryptobia helicis	Elongated	Pan-kDNA	Minicircle	Multitude	4.2	10
			Maxicircle	Single	43	
Dimastigella trypaniformis	ND	Poly-kDNA	Minicircle	Multitude	1.45	11
			Maxicircle	Single	ND	
Trypanoplasma borreli	ND	Mega-kDNA	Minicircle	Multitude	200	12-13
			Maxicircle	Single	80	
P. caudatus	ND	Pan-kDNA?	Minicircle	Multitude	10-12	14
			Maxicircle	Single	19	
Cruzella marina	ND	Poly-kDNA	Minicircle	Multitude	2.0	15
			Maxicircle	Single	ND	
Diplonemea						
D. papillatum	Thick reticulated peripheral	Disperse	A-class	Multitude	6	16
			B-class	Multitude	7	
R. euleeides	Thin reticulated peripheral	Disperse	1^{e} class	Multitude	7	this study
			2^{e} class	Multitude	7.7	
Euglenida						
E. gracilis	Reticulated	Nucleoids	Linear	Multitude	1-70	17
P. cantuscygni	Thick reticulated	Disperse	Small circle	Multitude	1.0-1.5	this study
			Big circle	ND	~40	
			Linear	Multitude	1-50	
P. trichophorum	Numerous spheres	Nucleoids	Linear	ND	2-75	this study
E. sulcatum	Numerous spheres	Nucleoids	ND	ND	ND	this study

1, Yasuhira and Simpson 1995; 2, Borst et al. 1985; 3, Yurchenko et al. 1999; 4, Borst et al. 1981; 5, Riou and Saucier 1979; 6, Sturm and Simpson 1990; 7, Maslov et al. 1998; 8, Cheng and Simpson 1978; 9, Blom et al. 1998; 10, Lukes et al. 1998; 11, Stolba et al. 2001; 12, Lukes et al. 1994; 13, Maslov et Simpson 1994; 14, Hajduk et al. 1986; 15, Zikova et al. 2003; 16, Marande et al. 2005.


1A
n
1B
n
1C
2A
n
2B
n
p
2C
3A
n
3B
n
3C
4A
n
4B
4C


p
m
m
A
m
C
B
m
m
m
m
m
D
E
v
v
F


m
n
A
m
b
B
m
C
1
2
3
D
12
2.0
0.1
1
2
3
4
5
6
E


OC
L
SC
A
B
C
D
E
F
G
H
OC
I

3. CONCLUSION

La pertinence de la description d'une nouvelle espèce du genre *Rhynchopus* a été mise en évidence dans le premier article présenté. En effet, les connaissances disponibles dans la littérature portant sur les membres de ce groupe, de même que sur ceux des Diplonemea en général, étaient dispersées et peu actuelles, tant au niveau de l'organisation structurale que des caractéristiques génétiques de ce groupe. La description de *R. euleeides* a permis de consolider l'idée que les deux genres des Diplonemea, *Rhynchopus* et *Diplonema*, sont très similaires à plusieurs égards. Ainsi, la morphologie générale, la locomotion par 'métabolie' ainsi que l'ultrastructure des appareils flagellaires et digestifs sont très semblables d'une espèce à l'autre. Toutefois, c'est l'ouverture sub-apicale unique et la présence d'une phase de dispersion qui distingue le genre *Rhynchopus*.

Le second article a permis d'établir et de caractériser la diversité de structures de génomes mitochondriaux retrouvés dans le groupe des Euglenozoa. Cet article a mis l'emphase sur la nécessité de récolter des données sur les génomes mitochondriaux d'espèces représentatives des trois groupes des Euglenozoa. De la distribution de l'ADN mitochondrial et de la nature structurale du génome, se dégagent certaines tendances reliant les membres du groupe. Premièrement, la topologie circulaire des chromosomes est une caractéristique commune au moins des Kinetoplastea et des Diplonemea. De plus, malgré le fait que chez la majorité des Euglenozoa l'ADN mitochondrial soit abondant et composé de multiples chromosomes, la présence d'un kinétoplaste est restreinte aux Kinetoplastea.

Suite aux travaux présentés dans ces deux articles, plusieurs perspectives pourront être explorées. Premièrement, l'analyse et la comparaison des génomes mitochondriaux des différentes espèces de Diplonemea permettra de déterminer si toutes les espèces nécessitent l'épisssage en *trans* des gènes fragmentés tel que

retrouvé chez *D. papillatum*, et si oui, de comprendre les mécanismes qui le régissent. Deuxièmement, le séquençage de banques d'ADNc et d'ADN mitochondrial de toutes les espèces étudiées ici fournira des données phylogénétiques qui pourront s'intégrer aux connaissances sur la structure des génomes mitochondriaux. Ceci permettra alors une meilleure compréhension du parcours évolutif qui a modelé la structure et les mécanismes d'expression sophistiqués des génomes mitochondriaux des Euglenozoa.

SOURCES DOCUMENTAIRES

Adl, S. M., Simpson, A. G., Farmer, M. A., Andersen, R. A., Anderson, O. R., Barta, J. R., Bowser, S. S., Brugerolle, G., Fensome, R. A., Fredericq, S., James, T. Y., Karpov, S., Kugrens, P., Krug, J., Lane, C. E., Lewis, L. A., Lodge, J., Lynn, D. H., Mann, D. G., McCourt, R. M., Mendoza, L., Moestrup, O., Mozley-Standridge, S. E., Nerad, T. A., Shearer, C. A., Smirnov, A. V., Spiegel, F. W. & Taylor, M. F. 2005. The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. *J. Eukaryot. Microbiol.*, **52**:399-451.

Altmann, R. 1890. Die Elementarorganismen und ihe Beziehungen zu den Zellen. *Dans* Comp., V. editor, Leipzig. p.

Andersson, S. G. & Kurland, C. G. 1999. Origins of mitochondria and hydrogenosomes. *Curr Opin Microbiol*, **2**:535-41.

Backert, S., Nielsen, B. L. & Borner, T. 1997. The mystery of the rings: structure and replication of mitochondrial genomes from higher plants. *Trends Plant Sci*, **2**:477-483.

Buetow, D. E. 1989. The mitochondrion. *Dans* Inc, A. P. (ed.), The biology of *Euglena*, San Diego. p. 247-314.

Burger, G., Forget, L., Zhu, Y., Gray, M. W. & Lang, B. F. 2003. Unique mitochondrial genome architecture in unicellular relatives of animals. *Proc Natl Acad Sci U S A*, **100**:892-7.

Feagin, J. E. 1992. The 6-kb element of *Plasmodium falciparum* encodes mitochondrial cytochrome genes. *Mol Biochem Parasitol*, **52**:145-8.

Feagin, J. E. 2000. Mitochondrial genome diversity in parasites. *Int J Parasitol*, **30**:371-90.

Feagin, J. E., Gardner, M. J., Williamson, D. H. & Wilson, R. J. 1991. The putative mitochondrial genome of *Plasmodium falciparum*. *J Protozool*, **38**:243-5.

Gray, M. W., Lang, B. F. & Burger, G. 2004. Mitochondria of protists. *Annu Rev Genet*, **38**:477-524.

Hayashi, Y. & Ueda, K. 1989. The shape of mitochondria and the number of mitochondrial nucleoids during the cell cycle of *Euglena gracilis*. *J Cell Sci*, **93**:565-570.

Hayashi-Isimaru, Y., Ueda, K. & Nonaka, M. 1993. Detection of DNA in the nucleoids of chloroplasts and mitochondria in *Euglena gracilis* by immunoelectron microscopy. *J Cell Sci*, **105**:1159-1164.

Horner, D. S., Hirt, R. P. & Embley, T. M. 1999. A single eubacterial origin of eukaryotic pyruvate: ferredoxin oxidoreductase genes: implications for the evolution of anaerobic eukaryotes. *Mol Biol Evol*, **16**:1280-91.

Keeling, P. J., Burger, G., Durnford, D. G., Lang, B. F., Lee, R. W., Pearlman, R. E., Roger, A. J. & Gray, M. W. 2005. The tree of eukaryotes. *Trends in Ecology & Evolution*, **20**:670-676.

Lang, B. F., Burger, G., O'Kelly, C. J., Cedergren, R., Golding, G. B., Lemieux, C., Sankoff, D., Turmel, M. & Gray, M. W. 1997. An ancestral mitochondrial DNA resembling a eubacterial genome in miniature. *Nature*, **387**:493-7.

Lang, B. F., O'Kelly, C., Nerad, T., Gray, M. W. & Burger, G. 2002. The closest unicellular relatives of animals. *Curr Biol*, **12**:1773-8.

Leander, B. S., Triemer, R. E. & Farmer, M. A. 2001. Character evolution in heterotrophic euglenids. *European Journal of Protistology*, **37**:337-356.

Lill, R. & Kispal, G. 2000. Maturation of cellular Fe-S proteins: an essential function of mitochondria. *Trends Biochem Sci*, **25**:352-6.

Logan, D. C. 2006. The mitochondrial compartment. *J Exp Bot*, **57**:1225-43.

Lukes, J., Guilbride, D. L., Votypka, J., Zikova, A., Benne, R. & Englund, P. T. 2002. Kinetoplast DNA network: evolution of an improbable structure. *Eukaryot Cell*, **1**:495-502.

Lynch, M., Koskella, B. & Schaack, S. 2006. Mutation pressure and the evolution of organelle genomic architecture. *Science*, **311**:1727-30.

Manning, J. E., Wolstenholme, D. R., Ryan, R. S., Hunter, J. A. & Richards, O. C. 1971. Circular chloroplast DNA from *Euglena gracilis*. *Proc Natl Acad Sci U S A*, **68**:1169-73.

Marande, W., Lukeš, J. & Burger, G. 2005. Unique mitochondrial genome structure in diplonemids, the sister group of kinetoplastids. *Eukaryot. Cell*, **4**:1137-46.

Martin, W. 2005. The missing link between hydrogenosomes and mitochondria. *Trends Microbiol*, **13**:457-9.

Martin, W. & Muller, M. 1998. The hydrogen hypothesis for the first eukaryote. *Nature*, **392**:37-41.

Martin, W. & Müller, M. 1998. The hydrogen hypothesis for the first eukaryote. *Nature*, **392**:37-41.

Nass, M. M. & Nass, S. 1963. Intramitochondrial Fibers with DNA Characteristics. I. Fixation and Electron Staining Reactions. *J Cell Biol*, **19**:593-611.

Nass, M. M., Schori, L., Ben-Shaul, Y. & Edelman, M. 1974. Size and configuration of mitochondrial DNA in *Euglena gracilis*. *Biochim Biophys Acta*, **374**:283-91.

Nosek, J., Dinouel, N., Kovac, L. & Fukuhara, H. 1995. Linear mitochondrial DNAs from yeasts: telomeres with large tandem repetitions. *Mol Gen Genet*, **247**:61-72.

Nosek, J. & Tomaska, L. 2003. Mitochondrial genome diversity: evolution of the molecular architecture and replication strategy. *Curr Genet*, **44**:73-84.

Saraste, M. 1999. Oxidative phosphorylation at the fin de siecle. *Science*, **283**:1488-93.

Sederoff, R. R. 1984. Structural variation in mitochondrial DNA. *Adv Genet*, **22**:1-108.

Simpson, A. G. B. 1997. The identity and composition of the Euglenozoa. *Arch. Protistenkd.*, **148**:318-28.

Simpson, A. G. B., Lukeš, J. & Roger, A. J. 2002. The evolutionary history of kinetoplastids and their kinetoplasts. *Mol. Biol. Evol.*, **19**:2071-83.

Talen, J. L., Sanders, J. P. & Flavell, R. A. 1974. Genetic complexity of mitochondrial DNA from *Euglena gracilis*. *Biochim Biophys Acta*, **374**:129-35.

Tessier, L. H., van der Speck, H., Gualberto, J. M. & Grienenberger, J. M. 1997. The *cox1* gene from *Euglena gracilis*: a protist mitochondrial gene without introns and genetic code modifications. *Curr Genet*, **31**:208-13.

Tomaska, L., Makhov, A. M., Nosek, J., Kucejova, B. & Griffith, J. D. 2001. Electron microscopic analysis supports a dual role for the mitochondrial telomere-binding protein of *Candida parapsilosis*. *J Mol Biol*, **305**:61-9.

Tovar, J., Fischer, A. & Clark, C. G. 1999. The mitosome, a novel organelle related to mitochondria in the amitochondrial parasite *Entamoeba histolytica*. *Mol Microbiol*, **32**:1013-21.

von der Heyden, S., Chao, E. E., Vickerman, K. & Cavalier-Smith, T. 2004. Ribosomal RNA phylogeny of bodonid and diplonemid flagellates and the evolution of Euglenozoa. *J. Eukaryot. Microbiol.*, **51**:402-16.

Williamson, D. 2002. Timeline - The curious history of yeast mitochondrial DNA. *Nature Reviews Genetics*, **3**:475-481.

Yasuhira, S. & Simpson, L. 1997. Phylogenetic affinity of mitochondria of *Euglena gracilis* and kinetoplastids using cytochrome oxidase I and hsp60. *J Mol Evol*, **44**:341-7.