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Characterization of the mitochondrial genome and ribosomal RNAs of the colorless green alga *Polytomella parva*

by

Jinshui Fan

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
2002

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Abstract

The chlorophycean taxa are distinctive from other green algae in that their mitochondrial genome sizes are reduced and their mitochondrial ribosomal RNA genes are fragmented and scrambled. To understand mitochondrial genome evolution in the Chlorophyceae, which needs more information from other taxa, and to develop a system for green algal mitochondrial studies, this work undertook the characterization of the mitochondrial genome and ribosomal RNAs of *Polytomella parva*. This taxon's colorless and cell wall-less features facilitate the isolation of mitochondrial-enriched fractions free of thylakoid membranes. At least two linear mitochondrial DNA (mtDNA) components with sizes of 13.5 and 3.5 kb were detected. Sequences spanning 97 and 86% of these mtDNAs, respectively, revealed that these molecules contain long, at least 1.3 kb, homologous inverted repeat sequences at their termini. The 3.5-kb mtDNA has only one coding region (*nad6*), the functionality of which is supported by both the relative rate at which it has accumulated non-synonymous nucleotide substitutions and its absence from the 13.5-kb mtDNA which encodes nine genes (i.e., large and small subunit rRNA genes, one tRNA gene, and six protein-coding genes). Based on DNA sequence data, it is proposed that a variant start codon, GTG, is utilized by the *P. parva* 13.5-kb mtDNA-encoded gene, *nad5*. Using the relative rate test with *Chlamydomonas moewusii* (= *C. eugametos*) as the outgroup, it is concluded that the non-synonymous nucleotide substitution rate in the mitochondrial protein-coding genes of *P. parva* is on average about 3.3 times that of the *Chlamydomonas reinhardtii* counterparts. RNA from a mitochondrial-enriched preparation was isolated, and the transcripts of the four small subunit (SSU) and eight large subunit (LSU) rRNA-coding regions were characterized by either Northern blot analysis or chemical sequencing. Secondary structure modeling of the SSU and LSU rRNA sequences was performed. The results show that (1) both the mitochondrial SSU and LSU rRNAs of *P. parva* are considerably shorter than their homologs from other green algae, although the main domains typical of conventional rRNAs are conserved, (2) the rRNA fragmentation pattern is different from that of other chlorophycean species but most similar to that of *C. reinhardtii*, among those that have been characterized, (3) three nucleotides are missing from the normally highly conserved GTPase centre of the LSU rRNA, and (4) post-transcriptional modification of the 3'-terminal region of the SSU rRNA is unusual; it has the "eubacterial" 3-methyluridine (corresponding to m^3^U at *Escherichia coli* 16S rRNA position 1498), but lacks the more highly conserved modifications at two adjacent A residues (corresponding to N^6^,N^8^-dimethyladenosine at *E. coli* 16S rRNA positions 1518 and 1519). This research presents the first example of subgenomic mtDNAs of a green alga. The obtained data provide the necessary background for more direct functional studies of the chlorophycean mitochondrial ribosome and rRNAs, using *P. parva* as a model system.
List of Abbreviations

A, adenine
bp, base pair
ATP, adenosine-5'-triphosphate
C, cytosine
°C, degree Celsius
DNA, deoxyribonucleic acid
dNTP, 2'-deoxynucleoside-5'-triphosphate
DTT, dithiothreitol
EDTA, ethylenediaminetetraacetate
g, gram
g, gravity
G, guanine
h, hour
kb, kilobase
L, liter
M, molar
min, minute
ml, milliliter
mM, millimolar
ng, nanogram
nt, nucleotide
OAc, acetate
PCR, polymerase chain reaction
RNA, ribonucleic acid
rpm, revolutions per minute
rRNA, ribosomal RNA
s, second
SDS, sodium dodecyl sulfate
T, thymine
Tris,
tris(hydroxymethyl)aminomethane
U, uracil
µg, microgram
µl, microliter
UV, ultraviolet
V, voltage
vol, volume
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INTRODUCTION
The goal of this dissertation is to characterize the mitochondrial genome and ribosomal RNAs (rRNAs) of *Polytomella parva*, a chlorophycean taxon which lacks a cell wall and a chloroplast. This research subject was chosen with the following considerations. First, the distinctive evolutionary event *Polytomella* experienced, i.e., the secondary loss of its chloroplast, makes it an interesting taxon for study. Second, *Polytomella* provides the advantage of facilitating the isolation of thylakoid membrane-free mitochondrial components and thus is a potential model system for green algal mitochondrial studies. It is hoped that knowledge derived from this research will contribute to this objective. Last, it is also expected that information obtained will eventually contribute to an understanding of the evolutionary mechanisms and pathways of chlorophycean mitochondrial genomes and their rRNA genes, which feature extensive variation and fragmentation, respectively.

A. The genus *Polytomella*

Aragao (1910) first described the unicellular protist he named *Polytomella agilis* and therefore established a new genus. Since the original description of *P. agilis*, a total of seven other *Polytomella* taxa have been described, and these include *Polytomella citri* (Kater, 1925), *Polytomella caeca* typ., *Polytomella caeca* var. minor, *Polytomella papillata* n.sp., *P. parva*, *Polytomella magna*, and *Polytomella capuana* n.sp. (Pringsheim, 1955). These taxa are so morphologically and physiologically similar that some of them could be considered as variants of the same species (Pringsheim, 1955). Later, in fact, *P.*
aigilis, P. citri, and P. parva were identified as the same species (George, 1976). The morphological and physiological similarities among Polytomella taxa support the monophyletic nature of this genus.

Several specific features characteristic of the genus Polytomella have been documented. The vegetative cells are ovoid to spherical in shape, including a hemispherical anterior and a conical posterior part. The cell is surrounded by a delicate membrane without a cell wall. Under the light microscope, a nucleus, storage bodies and two contractile vacuoles are evident. Present at the cell anterior is a papilla, from which four flagella arise with two on each side of the cell. The cells swim fast (Gittleson and Noble, 1973) and exhibit vertical aggregation in the culture (Gittleson and Jahn, 1968). Of the described taxa, only two (P. parva and P. magna n. sp.) have the existence of an eyespot. There is no evidence of chlorophyll in the cell. In the wild, the organisms are usually found flourishing in acidic fresh water teeming with organic substances, which provide the cells with nutrients. Figure 1 presents the generalized depiction of a Polytomella cell.

1. Phylogenetic affiliation of Polytomella

One of the features used for identifying the phylogenetic affiliation of a protist is the nature of storage material in the cell. Doflein (1916) first reported that the storage bodies of P. parva consist of starch, instead of paraglycogen as stated by Aragao (1910). The same conclusion was reached by Kater (1925) with P. citri. In the case of P. caeca, Bourn et al. (1950) even proved that the
storage starch consists of 13-16% amylose and 84-86% amylopectin. Having the starch as the reserve material in *Polystomella* clearly places this taxon as a member of the green algae (Friedl, 1997; Mattox and Stewart, 1984; Melkonian, 1990; Melkonian and Surek, 1995).

The green algae consist of a diversified collection of species, whose phylogenetic relationships continuous to undergo revision. Based on cell ultrastructure, Stewart and Mattox (1975) suggested that the green algae be divided into two main groups: the Charophyceae, with laterally inserted flagella and a multilayered structure microtubular rootlet system, and the rest of the green algae, with the anteriorly inserted flagella and a cruciate "X-2-X-2" microtubular rootlet system. The phylogenetic analysis of nuclear ribosomal small subunit RNA genes (18S rRNA genes) is consistent with this classification scheme (Friedl, 1997). As shown in Figure 2, the Charophyceae *sensu* Stewart and Mattox (1975) and land plants form a lineage, the Streptophyta *sensu* Bremer (1985), and the rest of the green algae form another lineage, the Chlorophyta (*sensu* Sluiman, 1985), which contains four classes: the Prasinophyceae (*sensu* Sluiman, 1985), the Ulvophyceae and the Chlorophyceae (*sensu* Mattox and Stewart, 1984), and the Trebouxiophyceae (*sensu* Friedl, 1995). Within the Chlorophyceae, two evolutionary groups are identified. One of them has the directly opposed (DO), or \(^{6\pi}_{12}\) clock, while the other group has the clockwise (CW), or \(^{\pi}_{7}\) clock, absolute flagellar apparatus orientation, represented by *Scenedesmus obliquus* and *Chlamydomonas*, respectively. It is noticed that the *Chlamydomonas* species spread over the CW-sublineages with
species of other genera, indicating the non monophyletic property of
*Chlamydomonas*, which is also revealed by the morphologic data (Roberts,
1974; Adair et al., 1987) and the sequence analysis of chloroplast ribosomal
large subunit rRNA (Turmel et al., 1993; Buchheim et al., 1996) and 18S rRNA
(Buchheim et al., 1990; Buchheim and Chapman, 1991). Two distinct lineages
within the CW-group are represented by *Chlamydomonas reinhardtii* and
*Chlamydomonas moewusii*, respectively.

a. Ultrastructural, cytological, and biochemical data

In terms of the ultrastructural and cytological data, *Polytomella* is a
member of the Chlorophyta by virtue of the apically inserted flagella and a
cruciate X-2-X-2 type microtubular flagellar root system (Brown et al., 1976a).
Furthermore, although the microtubule organization centers associated with
microtubular flagellar roots (Stearns and Brown, 1981; Stearns and Brown, 1979;
Stearns et al., 1976) suggest a Chlorophyceae affiliation of this genus
(Melkonian, 1990), this class level assignment is weakened by other features of
the flagellar apparatus and mitosis/cytokinesis.

The absolute flagellar apparatus configurations of the green algae seem
very conserved and thus are taken as promising criteria for classification. O'Kelly
and Floyd (1984) proposed that the counterclockwise (CCW), or $11/5$ clock,
flagellar apparatus orientation is primitive and gave rise to the CW one during
the evolution. All the flagellates in the Chlorophyceae have a CW or a DO
orientation, while those in the rest of the Chlorophyta have a CCW orientation
(Mattox and Stewart, 1984; Melkonian, 1990). From time to time, some authors have cited *Polytomella* as a member of the group having the CW flagellar apparatus orientation without proper support. To my knowledge, the flagellar apparatus structure of *Polytomella* has been elucidated only with *P. parva* by Brown et al. (1976a). Four basal bodies are connected by fibers into two offset pairs. In the diagrammatic representation (Figure 10 in the reference), however, both pairs clearly exhibit a CCW, as opposed to a CW orientation. Nakayama et al. (1996a) argued that this is likely a mirror-image reconstruction because the “s” rootlets are drawn on the left side of the basal bodies while they should be located on the right side, and therefore the flagellar apparatus of *P. parva* possibly has a CW absolute orientation. Clearly, caution needs to be observed in order to avoid mirror images (Floyd et al., 1980). The speculations of Nakayama et al., however, seem unjustified based on the following points. First, it was clearly indicated that this figure was drawn in a way so that “it would appear in a view looking down on the anterior part of the cell”, the way the absolute flagellar apparatus configurations are defined (Mattox and Stewart, 1984). Second, to my knowledge, no authors say that the “s” rootlets cannot be located on the left side of the basal bodies, although they were usually drawn on the right side by some authors. Based on the above ultrastructural data, therefore, it is not clear that *Polytomella* has a CW flagellar apparatus.

In the mitosis telophase of the chlorophycean cell, the interzonal spindle collapses and the phycoplast, which consists of microtubules in the cytokinesis plane (Pickett-Heaps and Mcdonald, 1975), develops. The Chlorophyceae share
these features with some species of the Trebouxiophyceae, while the cell in the rest of the green algae has the persistent telophase interzonal spindle and no phycoplast. The only studies on mitosis/cytokinesis of Polytomella were done in *P. parva* by Aragao (1910) and Doflein (1916) and in *P. citri* by Kater (1925). The authors did not specifically mention these features. In the cell division drawings by Aragao, however, a phycoplast-like structure was drawn in the telophase, while those drawings by Doflein and Kater do not depict such a structure. In the drawings of all three authors, especially by Kater, the interzonal spindle was clearly demonstrated in the telophase. In addition, *Polymella* resembles those species having a persistent telophase interzonal spindle in that the two nuclei are distantly separated; in contrast, they are closely located in the cell center in species with collapsing telophase interzonal spindle. Current documentation, therefore, does not support the view that *Polymella* has the mitosis/cytokinesis feature of the Chlorophyceae.

In terms of the biochemical data, the $F_0F_1$-ATPase, which is a mitochondrial membrane-bound complex catalyzing the synthesis of ATP through the protonmotive force generated by electron transfer chain, was isolated and partially characterized from *Polymella* strain, SAG 198.80, hereafter referred to as *Polymella* 198.80. (Atteia et al., 1997). Two of the five subunits of domain $F_1$, in the complex, $\alpha$- and $\beta$-subunits, exhibit features similar to their homologs in *C. reinhardtii* but different from those in other organisms. First, an extension at both the N-terminus of the $\alpha$-subunit and the C-terminus of
the β-subunit was found. Second, the N-terminal regions of both subunits show high sequence similarity in these two taxa: 35 out of 40 and 22 out of 30 amino acid residues are identical in the α- and β-subunit, respectively. Although the authors regarded these data as biochemical evidence for a close relationship between Polytomella 198.80 and C. reinhardtii, it seemed premature to make any conclusion about the specific phylogenetic affiliations of Polytomella without data on these subunits from other chlorophycean taxa.

b. Molecular data

i. Beta tubulin genes

Three beta tubulin genes in P. parva were cloned and sequenced by Conner et al. (1989). These represent the first molecular data obtained for Polytomella. Comparative analyses of these genes with their two counterparts in both C. reinhardtii (Youngblom et al., 1984) and Volvox cateří (Harper and Mages, 1988), a member of the CW-subgroup containing C. reinhardtii (Melkonian and Surek, 1995; Pröschold et al., 2001), reveal some common features among these species. First, all the genes have a bias against the use of codons ending with A. Second, a consistent polyadenylation signal, TGTTAA, is present in these genes. Third, two identical intron positions were found in these genes. Last, the length of protein product predicted for these genes is highly conserved (403 amino acids), and more than 98% of the amino acids are identical. The above evidence was used to argue for a close relationship among
these three species (Conner et al., 1989; Schmitt and Kirk, 1992). Again, as in the case of F,F,-ATPase, this argument is not convincing without the beta tubulin gene data from taxa of other CW-subgroups for comparison.

ii. Mitochondrial rRNA structure

Among the green algal species investigated so far, some variable regions of the mitochondrial ribosomal large and small subunit RNA (LSU and SSU rRNA, respectively) are found to be fragmented in the Chlorophyceae (Boer and Gray, 1988a; Denovan-Wright et al., 1998; Kroymann and Zetsche, 1998; Nedelcu et al., 2000; Nedelcu et al., 1996), but not in taxa from other green algal classes (Wolff et al., 1994) except for Pedinomonas minor (Turmel et al., 1999), whose phylogenetic affiliation is not clear yet. To some extent, therefore, the feature of fragmented mitochondrial rRNA might serve as a marker for the Chlorophyceae. In addition, the corresponding rRNA break regions shared by species could be indicative of phylogenetic relatedness. For instance, the mitochondrial rRNAs are broken in the same corresponding regions in Chorogonium elongatum and C. moewusii (= C. eugametos), but there are different break regions between C. reinhardtii and C. moewusii/C. elongatum. These data are consistent with C. elongatum and C. moewusii forming a clade separated from C. reinhardtii in phylogenetic analysis of concatenated mitochondrial protein sequences (Nedelcu et al., 2000).

In P. parva, one rRNA fragment was detected by Northern blot hybridization
with a probe derived from *C. reinhardtii* mitochondrial rRNA piece L₈ (Nedelcu et al., 1996). Partial mitochondrial DNA sequencing of *Polytomella* 198.80 revealed part of a gene piece that encodes a fragment corresponding to *C. reinhardtii* mitochondrial rRNA piece L₇ (Antaramian et al., 1996), which the authors incorrectly identified as L₄. The 5' break region identified in this gene piece is common to both *Chlamydomonas*/*Chlorogonium* taxa and *Scenedesmus*. These three lines of evidence suggest an affiliation of *Polytomella* with the class Chlorophyceae, but do not provide further phylogenetic resolution within this class.

### iii. *cox2* and *cox3*

The gene content of the mitochondrial genome is rather different among the green algae (Gray, 1999; Nedelcu et al., 2000). This is likely as a result of differential mitochondrial gene transfer to the nuclear genetic compartment (Gray, 1995). In all the three CW-group species whose mitochondrial genomes have been completely sequenced, *cox2* and *cox3* are two of those mitochondrially originating genes missing from their mitochondrial DNA (Michaelis et al., 1990; Denovan-Wright et al., 1998; Kroymann and Zetsche, 1998). These two genes were recently identified in both *C. reinhardtii* and *Polytomella* 198.80 (Pérez-Martinez et al., 2000, 2001). Sequence analysis of these genes reveals some features characteristic of nuclear encoded mitochondrial genes (Brennicke et al., 1993; Claros et al., 1995). Namely, they
have a mitochondrion-targeting sequence-encoding region, the codon usage is different from that of mitochondrially located genes, a polyadenylation signal is present, and the encoded proteins have lower hydrophobicity than the mitochondrially encoded counterparts in other organisms. These features, plus the evidence from Southern blot hybridization, make it believable that cox2 and cox3 are located in nuclear DNA in both C. reinhardtii and Polytomella 198.80. Because S. obliquus still has these two genes in its mitochondrial genome (Nedelcu et al., 2000), it is reasonable to suggest that cox2 and cox3 were transferred from the mitochondrion to the nucleus in the CW-group ancestor after its separation from the ancestor of the DO-group. This character, therefore, could be used to support Polytomella as a member of the CW-group. This conclusion also draws support from the cox2 structure. While cox2 is a single continuous gene in S. obliquus and other non-chlorophycean green algal taxa so far characterized, it is split into two pieces in both Polytomella 198.80 and C. reinhardtii.

iv. cox1 and cob

The two sequenced mitochondrially located genes, cox1 and cob, from Polytomella 198.80 (Antaramian et al., 1998; Antaramian et al., 1996) encode amino acids that are 73.8% and 56% identical, respectively, to those encoded by their counterparts in C. reinhardtii. The two phylogenetic trees reconstructed by the authors, however, could not provide much information on the phylogeny of Polytomella because, other than C. reinhardtii, the appropriate species were not
included. The species closest to the CW-group that are included are *Marchantia polymorpha* (a liverwort) in the *cob* tree and *Prototheca wickerhamii* (Trebouxiophyceae) in the *cox1* tree. Although *Polytomella* was grouped with *C. reinhardtii* in both trees, the only conclusions that can be drawn are that *Polytomella* is a member of the green algae from the *cob* tree, or of the Chlorophyceae from the *cox1* tree. Figure 3 and Figure 4 show the alignments of the sequences of amino acids encoded by *cox1* and *cob*, respectively, among chlorophycean species. There are five and two significant blocks (shaded) in Figure 3 and Figure 4, respectively, where *Polytomella* 198.80 shares the deletions with all three CW-group species relative to the DO-group species *S. obliquus*. Thus, these alignments suggest that *Polytomella* be a member of the CW-group.

**v. 18S rRNA gene**

In the 18S rRNA gene trees constructed with the methods of neighbor joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) (Melkonian and Surek, 1995; Nakayama et al., 1996a), *Polytomella* affiliates with the CW-sublineage containing *C. reinhardtii*. In the two 18S rRNA gene trees constructed with the methods of NJ and MP, *Polytomella* is also a member of the *C. reinhardtii* clade and is separated from other clades including the *C. moewusii* clade (Nakayama et al., 1996b). The close relationship between *Polytomella* and *C. reinhardtii* is supported by the high bootstrap values (89% to 100%) uniting this clade following phylogenetic analysis by all three methods.
In summary, the current non molecular data provide little information on the phylogenetic affiliation of *Polytomella*, although it was treated as a member of the Chlorophyceae (Melkonian, 1990). The molecular data now, however, strongly suggest that *Polytomella* is a member of the Chlorophyceae and in the *C. reinhardtii* sublineage of the CW-group. Nevertheless, compared with other CW-group species, *Polytomella* has some distinct characteristics, which imply some evolutionary trends.

2. Evolution of *Polytomella*

a. Colorlessness and heterotrophy

Several lines of evidence support the view that colorlessness in *Polytomella* is a derived trait resulting from the degeneration of the chloroplast. First, the electronic microscopy of *Polytomella* cell shows a plastid-like structure (Brown et al., 1976a, b, c; Webster, et al., 1968). In the electron microscopic (EM) photograph by Moore et al. (1970) this structure was demonstrated to be bound by double membranes, a character of green algal chloroplasts. Inside the structure, no intact thylakoids exist. The plastid-like structure forms a layer beneath a mitochondrial layer except in the anterior part of the cell. This structure is reminiscent of the situation in *C. reinhardtii* where the cup-shaped chloroplast is also surrounded by a layer of mitochondria. The very thin feature of the plastid-like structure argues for the degeneration of the chloroplast over time. Second, like green plants, *Polytomella* has starch as a reserve polysaccharide. These facts imply a plant-like history of *Polytomella*. Last,
degeneration of the chloroplast into a nonphotosynthetic plastid has happened independently on many occasions in other algae (Round, 1980) and flowering plants (Wolfe et al., 1992a).

The degeneration of the chloroplast in *Polytomella* could be hypothesized as going through the following steps. The last green ancestor of *Polytomella* should have maintained heterotrophic ability, which could be justified by the fact that *C. reinhardtii* and many other chlorophycean taxa have the ability to grow in the dark (Harris, 1989). Photosynthetic capacity, therefore, was likely dispensable during the evolution of the *Polytomella* lineage, if it lived in an environment rich in organic substances. Thus, mutations in the genes involved in the structure or function of the chloroplast gradually may have accumulated in the population. Some environmental factors like limited illumination may have enhanced this process. At some transitional stages, the cell was probably relying primarily on a heterotrophic lifestyle, with some support from a low level of photosynthesis in the chloroplast. Eventually, the chloroplast could have lost its photosynthetic capacity as a result of multiple mutations, thereby forcing the organism to rely solely on heterotrophy.

b. Eyespot

Most species of the CW-group have an eyespot in the anterior lobe of the cell (Harris, 1989; Mattox and Stewart, 1984; Melkonian, 1990). Of the examined *Polytomella* strains, however, only two have an eyespot.

The eyespot is believed to be responsible for two types of phototactic
behaviour in flagellated cells: positive and negative taxis (reviewed by Melkonian, 1984). This is supported by the fact that an eyespot containing strain, P. magna, shows these behaviors, while the eyespot-lacking Polytomella strains do not (Melkonian, 1984). It is proposed that the eyespot apparatus in green algae was acquired shortly after the ancestral green flagellate evolved from a zooflagellate (Melkonian, 1982), thus enabling the cell to be more efficient in photosynthesis (Boscov and Feinleib, 1979; Foster and Smyth, 1980; Melkonian and Robenek, 1979). For instance, the eyespot can lead the cell to a location where suitable light for photosynthesis is available.

The evolutionary trend of the eyespot in Polytomella could be summarized as follows. First, as mentioned before, the genus Polytomella is most likely monophyletic. The ancestor of the Polytomella lineage likely had an eyespot inherited from its green ancestor, because an eyespot still exists in some Polytomella species. Second, the ensuing eyespot loss in some Polytomella species is probably related to their heterotrophic character. This could also draw support from the fact that members of the Polytoma genus, another heterotrophic group of chlorophycean green algae also lack an eyespot. It could be imagined that, because photosynthesis is not necessary for these organisms to survive, mutations in eyespot-related genes would have accumulated without selection pressure. Third, eyespot loss is an on-going process, and all the Polytomella species will eventually be without the eyespot apparatus.
c. Cell covering and cytoskeleton, mitochondrion, and mitosis/cytokinesis

The *Polytomella* cell is covered by just one plasma membrane. According to Mattox and Stewart (1977), a cell wall was present in the ancestor of *Chlamydomonas*-like algae. In this sense, *Polytomella* experienced a secondary loss of its cell wall. This phenomenon is also exemplified by many wall-deficient and wall-less *C. reinhardtii* mutants and is not uncommon among naturally occurring chlorophycean taxa (Harris, 1989). The cell wall in green algae, living in freshwater environments, provides resistance to osmotic stress under conditions of low osmolarity. As *Polytomella* inhabits aquatic environments of high osmolarity, a result of an abundance of organic molecules and other solutes, there probably was no selective pressure to maintain a cell wall in vegetative cells. In fact, losing the cell wall could have provided the *Polytomella* ancestor with a selective advantage as a result of increased permeability to dissolved organic food sources and a greater energetic efficiency as a result of not having to generate an unneeded structure, factors which would have allowed a faster growth rate than observed in walled strains.

It seems, however, that *Polytomella* has developed some structural features to compensate for the fragility associated with the lack of a cell wall. For example, all the other green algae investigated have no more than four flagellar rootlets. *Polytomella*, however, has eight, from which cytoplasmic microtubules originate (Brown, 1968; Brown et al., 1976a; Stern and Brown, 1979, 1981). These massive cytoplasmic microtubules form a layered structure just beneath the plasma membrane. In addition, one giant basket-like mitochondrion in
Polytomella might provide additional structural support. This mitochondrion lies close to the plasma membrane and occupies 19% of the cell volume in Polytomella (Moore and Burton, 1974), compared to a mitochondrial volume of only 1-3% in C. reinhardtii (Boynton et al., 1972; Harris, 1989).

In primitively wall-less green algal species, like those in the Ulvophyceae, the cell and spindle elongate during mitosis so that the chromosomes can be pulled towards opposite ends of the cell. In walled species, however, the elongation is restricted, and phycoplast develops during telophase to separate the chromosomes. Thus, the collapsing interzonal spindle and the phycoplast are proposed to have developed coordinately with a cell wall over evolutionary time (Mattox and Stewart, 1977). As mentioned before, current data do not support the occurrence of collapsing spindle in Polytomella: there is a chance that a reverse process occurred in Polytomella after the loss of its cell wall.

d. Life cycle

The life cycle of Polytomella reportedly includes three stages: asexual reproduction by binary cellular fission, sexual reproduction, and encystment/excystment. The latter two prove rather interesting when compared to the life cycle of other CW-group species.

i. Sexual reproduction

In the first literature on Polytomella, Aragao (1910) reported the existence of sexual reproduction in P. parva. Latter, Doflein (1916) and Kater (1925)
challenged this conclusion by arguing that the observed so-called cell
conjugation was likely in fact cell division. Decades later, Lewis et al. (1974)
reported the discovery of sexual reproduction in P. caeca with the method of
microculture, which can trace individual cells under the microscope. According to
their description, the sexual life history of Polytomella differs from that of both C.
reinhardtii and C. moewusii in two respects. First, Polytomella is homothallic,
while C. reinhardtii and C. moewusii are heterothallic, however other taxa related
the later two taxa appear to be homothallic (Harris, 1989). Second, the zygote in
C. reinhardtii and C. moewusii usually encysts into a dormant zygospore, which
protects it from adverse environmental conditions (Mattox and Stewart, 1984). In
contrast, the zygote stage of Polytomella is rather transient: the formation of a
zygospore is not observed. The zygote remains quadriflagellated and divides into
four daughter cells 3-3.5 h later after its formation. It remains unknown why
Polytomella evolved these two different features however speculation is possible.
For example, homothalism could increase the chance of finding a mate under
conditions of low population density although this might decrease the chance for
recombination with unrelated individuals. The lack of a zygospore wall on
Polytomella zygotes could indicate that this taxon does not couple the initiation of
sex with conditions of environmental stress so that the presence of such a thick-
walled zygote is unnecessary especially if there is another mechanism of
producing resting spores as discussed below.
ii. Encystment/excystment

In contrast to *C. reinhardtii* and *C. moewusii*, the vegetative cells of *Polytomella* experience encystment. This phenomenon has been extensively documented in terms of cell physiology and ultrastructure (Gittleson et al., 1969; Lewis et al., 1974; Sheeler et al., 1970). The cyst has four layers of walls (Brown et al., 1976b) and can survive for a long period of time without division (Reed et al., 1976). It can develop back into vegetative cells through the process of excystment when placed in fresh medium (Kahn and Moore, 1971). The encystment/excystment process provides taxa with opportunities to survive harsh environmental conditions such as desiccation, starvation, etc. This is especially important in the case of *Polytomella* because the naked vegetative cells are fragile, and their cell growth is, by virtue of their heterotrophic existence, dependent on a rich source of organic molecules.

It is very interesting that encystment takes place shortly after culture inoculation and throughout the stationary phase, independent of the physiological state of cells and the availability of nutrients in the medium (Sheeler et al., 1970). On the other hand, excystment does not take place in the medium, in which cysts are formed even though nutrients are still available in this medium, unless cysts are transferred to fresh medium. Selection of these features appears to represent the best solution for the preservation of this taxon.

e. Nuclear DNA composition

Studies on DNA density profiles in cesium chloride gradients showed that
the density of the main DNA band, which is assumed to be nuclear DNA, is similar in *C. reinhardtii* and *C. moewusii*, but significantly higher than the counterparts in *Polytomella* species (Table 1). Calculations based on the equation of Schildkraut et al. (1962) indicate that the expected mean percentage of guanine and cytosine, i.e., GC content, of *C. reinhardtii* and *C. moewusii* nuclear DNAs is 64% and 59%, respectively, values which are considerably higher than the corresponding values of *P. caeca* and *P. parva*, which are 37% and 42%, respectively. These observations suggest that nuclear DNA in *Polytomella* may have evolved towards a lower GC content. Beta tubulin gene introns of *P. parva* and *C. reinhardtii* are ideal candidates for further confirmation of this assumption using comparative analyses. First, introns are supposed to have lower functional constraints than exons and may therefore more readily reveal mutational biases resulting in base compositional change. Second, as mentioned before, the genes of each species have two corresponding introns that were most likely inherited from a common ancestors and are thus comparable. It is noticed in Table 2 that the GC content of the homologous introns at position 132 is much lower in *P. parva* (from 18.4% to 25.9%) than in *C. reinhardtii* (61.9% and 62.5%). The same trend also exists in the homologous introns at position 57 (from 28.1% to 34.3% in *P. parva*, but 56.2% and 58.2% in *C. reinhardtii*), as well as in the other apparently non homologous introns.

The GC content variation among genomes has been explained in three ways. (1) High temperature and ultraviolet (UV) radiation could select genomes with high GC content because GC base pairs are more thermostable than AT
base pairs in DNA (Argos et al., 1979; Kagawa et al., 1984; Kushiro et al., 1987), and UV can create T-T dimers (Singer and Ames, 1970). (2) The biased tRNA abundance may influence the GC content by determining codon usage pattern in protein genes (Ikemura, 1981, 1982). (3) The difference between mutation rate of A or T to G or C and of G or C to A or T has been proposed as a factor governing the genome base composition (Muto and Osawa, 1987; Sueoka, 1962, 1988). The first two ways do not seem to account for the case of Polytomella vs. C. reinhardtii convincingly since there are no such apparent environmental condition differences between the two species, and the GC content of beta tubulin gene introns, which is not supposed to be affected by tRNA abundance, shows the same trend as that of the genomes. Rather, the low GC content of Polytomella nuclear genome could be explained by the mutation bias to A and T.

The causes of biased A•T/G•C mutation pressure are still to be revealed. Bird (1980) reported that methylation of cytosine residues to form 5-methylcytosine can promote GC to At transition and thus abundant methylase activity is a potential cause of high AT content in DNA. According to Dujon (1981), uracil is derived frequently from the deamination of cytosine in DNA and is excised by a repair mechanism. If, however, the relevant repair mechanism is deficient or the deamination enzymes are abundant, uracil is expected to accumulate in DNA, and thus there exist a potential of GC to AT transition. There is no direct evidence regarding either of the two aspects in Polytomella.
B. Mitochondrial genomes

It is now commonly accepted that mitochondrion emerged by a process called endosymbiosis during which a cell host (a nucleus-containing eukaryote or an archaebacterium) engulfed a bacterial symbiont, giving rise to mitochondria. This theory has gained strong support from contemporary genomics data, which point to an α-proteobacterial ancestry of mitochondria and suggest that they arose from a single endosymbiosis event (Gray et al., 1999; Gray et al., 1998; Lang et al., 1998). To date, the most bacteria-like mitochondrial genome has been found in the protozoon *Reclinomonas americana* (Lang et al., 1997), while the eubacterial genome most resembling mitochondrial genomes is found in *Rickettsia prowazekii* (Andersson et al., 1998).

1. Mitochondrial genome diversity

Despite their common origin, mitochondrial genomes show extensive variability in conformation, size, and gene content (excluding unique and intron-encoded open reading frames) between and within diverse lineages. Animal mitochondrial genomes are relatively conserved. They are usually a single circular DNA molecule of around 16 kb in length, which encodes 37 genes (Boore, 1999). These genomes are compactly organized, having little or no intergenic sequences. Mitochondrial genomes in land plants range in size from about 180 to 2400 kb, encode about 50 to 70 genes (Kubo et al., 2000; Lang et al., 1999), and have a very complex and not well understood in vivo structural organization (Backert et al., 1997; Bendich, 1993; Oldenburg and Bendich,
2001) despite the presence of physical maps, which suggest that master circular forms can give rise to subgenomic circular forms by intramolecular recombination (Fauron et al., 1995; Palmer and Shields, 1984; Unseld et al., 1997). In addition, identified functional genes only account for a small portion of the plant mitochondrial genome. Fungal mitochondrial genomes are mostly circular with sizes ranging from about 28 to 100 kb and encode 24 to 43 genes (Lang et al., 1999). In the lineage of protists, the mitochondrial genomes in *Plasmodium*, the causative agent of malaria, encode only five genes in a 6-kb element, which is repeated in variably-sized tandem arrays (Feagin, 1994; Wilson and Williamson, 1997); in comparison, the circular mitochondrial genomes of *R. americana* and *Jakoba libera* encode 94 genes and is 100 kb in length, respectively (Lang et al., 1999).

Moreover, examples of subgenomic, presumably autonomously replicating, circular or linear mitochondrial DNA (mtDNA) forms that encode standard mitochondrial genes have been identified in some mesozoan (Watanabe et al., 1999) and metazoan (Armstrong et al., 2000; Bridge et al., 1992; Pont-Kingdon et al., 2000) animals. These mtDNAs differ from the special classes of small circular mtDNA molecules found in the single mitochondrion (kinetoplast) of trypanosomal protozoa, that encode only guide RNAs used to edit transcripts produced by the main mtDNA (Shapiro and Englund, 1995) and the senDNA or other subgenomic circular mtDNA forms associated with senescent or particular mutant strains of filamentous fungi, respectively (Griffiths, 1992).
2. Mitochondrial genomes in green algae

To date, mtDNAs from eight taxa representing diverse lineages of the green algae have been sequenced to completion (Table 3). Although the green algae comprise a distinct evolutionary lineage (Freidl, 1997), most of these eight genomes fall into one of two extremes with respect to genome size, gene content, gene structure and rate of overall sequence divergence. One type described as "reduced-derived" (Lang et al., 1999) has been identified in three Chlamydomonas-like taxa from the lineage of the CW-group, i.e., C. reinhardtii (Boer and Gray, 1991; Michaelis et al., 1990), C. moewusii (Denovan-Wright et al., 1998) and Chlorogonium elongatum (= C. capillatum) (Kroymann and Zetsche, 1998), and P. minor (Turmel et al., 1999), a unicellular green alga of uncertain phylogenetic placement in the Chlorophyta. These 16 to 25-kb genomes feature a considerable loss of protein-coding and tRNA genes relative to animal, fungal, and plant counterparts, the lack of a 5S rRNA gene and ribosomal protein-coding genes, discontinuous LSU and SSU rRNA-coding regions, and a high rate of overall sequence divergence. In contrast, mitochondrial genomes described as "ancestral" (Lang et al., 1999) have been identified in P. wickerhamii (Wolff et al., 1994), Nephroselmis olivacea (Turmel et al., 1999), and Mesostigma viride (Turmel et al., 2002), members of non-chlorophycean green algal lineages, with sizes ranging from of ca. 42 to 55 kb, respectively. These mitochondrial genomes feature a greatly expanded repertoire of protein-coding and tRNA genes, conventional, continuous LSU and SSU rRNA-coding regions, the existence of a 5S rRNA gene and ribosomal
protein-coding genes, and a lower rate of overall sequence evolution compared to the reduced-derived type. The ca. 43-kb mitochondrial genome of S. obliquus, a taxon associated with the chlorophycean group of green algae but from a distinct lineage compared to the Chlamydomonas-like algae, has characteristics of both aforementioned green algal mitochondrial genome types, such as the lack of a 5S rRNA gene and ribosomal protein-coding genes, discontinuous LSU and SSU rRNA-coding regions, and overall rates of sequence evolution similar to the reduced-derived form but a more complex assemblage of protein-coding and tRNA genes like the ancestral form (Kück et al., 2000; Nedelcu et al., 2000). Finally, the genes are extensively rearranged among the above investigated green algal mitochondrial genomes, although some conservation was observed in cases of C. moewusii and Chlorogonium (Kroymann and Zetsche, 1998).

With regard to conformation, seven of the eight completely sequenced green algal mtDNAs are circular-mapping, which presumably reflects an in vivo circular conformation of the mtDNA (Laflamme and Lee, 2002). However, the mtDNA of C. reinhardtii, a member of the Volvox clade (sensu Nakayama et al., 1996b) within the CW-group of the chlorophycean lineage, is a linear 15.8-kb molecule with a 580- or 581-bp sequence at one terminus that is repeated in an inverted orientation at the other terminus (Vahrenholz et al., 1993); mitochondrial genomes from other members of the Volvox clade that have been characterized by gel electrophoresis are also linear mtDNAs (Moore and Coleman, 1989; Laflamme and Lee, 2002).

Despite all the diversity in mitochondrial genomes so far described for
green algae, there is no evidence to date of any taxon from this group with standard mitochondrial genes associated with separate subgenomic mtDNAs as found in some other lineages.

C. Discontinuous mitochondrial ribosomal RNA genes

Ribosomal RNA, together with ribosomal protein, constitutes the ribosome in which protein synthesis is carried out. Since the discovery of RNA catalytic activity (Cech et al., 1981) and the revealing of potential 16S rRNA secondary structure (Noller and Woese, 1981), the traditional view that rRNA is mainly a structural component of ribosome has ever been challenged. Instead, it is now understood that rRNA plays active roles in protein synthesis, including mRNA selection, tRNA binding, proofreading, peptidyl transferase function, etc. (Gray and Schnare, 1996). It has been suggested that the first ribosome was essentially RNA.

Despite its increasingly convincing functional roles, broad diversity has been observed in rRNA. For example, although LSU and SSU rRNAs are usually continuous polyanucleotides, discontinuous ones have been reported in prokaryotes and eukaryotic nucleocyttoplasm, chloroplast, and mitochondria. These rRNA transcript pieces are the results of disrupted LSU and SSU rRNA genes (Gray and Schnare, 1996). This phenomenon is particularly prominent in mitochondria.
1. Distribution of discontinuous mitochondrial rRNA genes

In mitochondria, discontinuous rRNA genes were observed in various protist lineages. The mitochondrial SSU rRNA gene of *Tetrahymena falciparum* (Schnare et al., 1986) and LSU rRNA gene of *Paramaecium aurelia* (Seilhamer et al., 1984) are each disrupted into two modules. Interestingly, the two LSU rDNA modules in *T. falciparum* are scrambled relative to the order in which their transcript counterparts of *E. coli* are arranged (Heinonen et al., 1987). Discontinuous and scrambled mitochondrial rRNA genes were also found in *Plasmodium sp.* (Feagin et al., 1992; Vaidya et al., 1989) and *Theileria parva* (Kairo et al., 1994), which are encoded on both DNA strands.

Current studies revealed a gradual evolutionary trend of green algal mitochondrial rRNA gene discontinuity. The taxa, which occupy the phylogenetically basal positions and contain the ancestral type of mitochondrial genome, such as *P. wickerhamii* of the Tribuxiophyceae (Wolff et al., 1994) and *N. olivacea* (Turmel et al., 1999) and *M. viride* (Turmel et al., 2002) of the Prisinoophyceae, have conventional continuous mitochondrial LSU and SSU rRNA genes. However, both mitochondrial LSU and SSU rRNA genes are fragmented in the advanced Chlorophyceae class. Within it, the taxa with reduced-derived type of mitochondrial genomes demonstrate a higher degree of mitochondrial rRNA genes fragmentation. For example, the mitochondrial LSU rRNA gene is fragmented into eight and six pieces in *C. reinhardti* (Boer and Gray, 1988a) and *C. moewusii/Chlorogonium* (Denovan-Wright and Lee, 1994; Kroymann and Zetsche, 1998), respectively, and the mitochondrial SSU rRNA
gene is fragmented into four and three pieces in *C. reinhardtii* and *C. moewusii/Chlorogonium*, respectively. In comparison, *Scenedesmus*, a taxon with a mitochondrial genome of an intermediate evolutionary stage, has mitochondrial LSU and SSU rRNA genes, which are disrupted into two and four pieces (Nedelcu et al., 2000). In addition, the mitochondrial LSU rRNA gene of the phylogenetically uncertain taxon *Pedinomonas* consists of two fragments. In most of the above cases, these rDNA modules are scrambled and interspersed with each other and/or with other coding regions. Fragmented mitochondrial genes are also predicted in other chlorophyceans based on Northern blot analysis (Denovan-Wright et al., 1996; Nedelcu et al., 1996).

2. Functional implications of discontinuous and scrambled mitochondrial rRNA gene transcripts in green algae

Several lines of evidence support the functionality of fragmented mitochondrial LSU and SSU rRNA gene transcripts in green algae, although this has not yet been directly demonstrated. First, in *C. reinhardtii*, transcripts of these genes are in significantly higher abundance compared to mitochondrially encoded protein-coding genes in the same organism (Boer and Gray, 1988a). Second, rDNA sequences predict secondary structures of LSU and SSU rRNAs formed by intra- and intermolecular base pairing of rRNA fragments. In these structures the universally conserved domains of continuous counterparts are contained, and all the break points were found to reside in the regions variable among sources in terms of sequence and secondary structure (Boer and Gray,
1988a; Denovan-Wright and Lee, 1994; Nedelcu et al., 2000). Third, by means of Northern blot analysis, ribosomal association of these rRNA pieces was demonstrated in *C. moewusii* (Denovan-Wright and Lee, 1995). Last, the observations that the well-studied eukaryotic cytoplasmic ribosomes have a 5.8S rRNA and that chloroplast ribosomes of higher plants have a 4.5S rRNA (Gray and Schnare, 1990; and references therein), which correspond to the 5′- and 3′-end of *E. coli* LSU rRNA, respectively, indicate that the function of rRNA is not constrained by its continuity in at least the variable regions.

Co-transcription (Boer and Gray, 1988a) and scrambling of the rRNA genes will not allow the coupling of transcription, post-transcriptional processing, and ribosomal assembly as in both prokaryotes and eukaryotes (Nomura et al., 1984). This implies that the formation of noncovalent LSU and SSU rRNA network may occur after the maturation of rRNA fragments; ribosomal proteins might play a role in the process rRNA of assembly (Boer and Gray, 1988a).

3. Potential factors and mechanisms involved in the fragmentation and scrambling of mitochondrial rRNA genes in green algae

Gray and Schnare (1996) suggested that discontinuous rRNA genes were derived from conventional continuous sequences, which in turn originated from discontinuous ancestor sequences as a result of the loss of processing internal transcribed spacers. The sporadic appearance of discontinuous rRNA genes among different lineages indicates their independent occurrences. Yet, the factors and mechanisms responsible for the fragmentation and scrambling still
remain largely unclear despite a few proposals put forward for green algal mitochondria.

In the *C. reinhardtii* mitochondrial genome, a reverse transcriptase-like coding region (*rfl*) was found. This prompted Boer and Gray (1988b) to hypothesize that the rRNA genes were originally discontinuous, but the rDNA modules were in the conventional order, and that they became scrambled following the copying of the rRNA pieces by reverse transcriptase activity, insertion of cDNA into the mitochondrial genome, and recombination between duplicated copies of rDNA modules. However, no similar *rfl* sequence has been found in any other mitochondrial genomes that contain scrambled rRNA genes. Moreover, the product of *rfl* in *C. reinhardtii* seems not to have reverse transcriptase activity (Faßbender et al., 1994). Denovan-Wright and Lee (1994) proposed that scrambling of rRNA genes has resulted from recombination between nonhomologous regions such as the dispersed short repeated elements found in the mtDNAs of *C. reinhardtii* (Boer and Gray, 1991) and *C. moewusii* (Denovan-Wright and Lee, 1994). According to the above authors, rRNA gene fragmentation occurred prior to scrambling. Nedelcu (1998) proposed a process that would have resulted in the simultaneous fragmentation and scrambling of rRNA genes, i.e., recombination between inverted repeats located in the variable regions of rRNA gene and its flanking regions.
MATERIALS AND METHODS
A. Strain and cell culture conditions

*P. parva* (UTEX L 193) was obtained from the University of Texas at Austin Culture Collection. The medium for the growth of the strain contained 0.1% tryptone, 0.2% yeast extract, and 0.2% sodium acetate (Sheeler et al., 1968). The strain stock was maintained at room temperature by transferring cysts into tubes containing 10 ml of fresh medium once every two to three weeks. Cultures were routinely plated on LB-agar medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1.5% agar) (Shambrock et al., 1989) to ensure the absence of microbial contaminants. Cells were cultured at 25 °C with shaking for small cultures (100-250 ml) or mild aeration for larger cultures (5-15 L). They were harvested by centrifugation (2000 × g) at 4 °C for 15 min, in the late logarithmic phase of growth (OD$_{750}$ = 0.45) for DNA isolation or in the logarithmic phase (OD$_{750}$ = 0.31) for RNA isolation.

B. Preparation of mitochondrial-enriched fractions

Mitochondrial-enriched fractions to be used for DNA isolation were prepared by procedure B of Ryan et al. (1978). Cells from 16 L of culture were washed twice in buffer A (0.3 M mannitol, 3 mM EDTA, 0.1% bovine serum albumin (BSA), 1 mM 2-mercaptoethanol, 50 mM Tris-HCl, pH 8.0). After being resuspended in 123 ml of buffer A, cells were disintegrated by passing through a chilled French pressure cell at 500 psi. The resulting suspension was diluted with 123 ml of buffer A, followed by centrifugation at 1000 × g for 15 min. The pellet was resuspended in 61 ml of buffer A, followed by centrifugation under the same
conditions. The combined supernatants were centrifuged at 10,000 × g for 20 min. The resulting pellet was resuspended in 30 ml of buffer A and treated with 50 ug/ml DNAse I (code DPRF, Worthington) on ice for 90 min. After being diluted with 124 ml of Buffer B (0.15 M NaCl, 0.1 M EDTA, 10 mM Tris-HCl, pH 8.0), the mixture was centrifuged at 10,000 × g for 20 min to collect mitochondrial-enriched fraction. Unless indicated otherwise, all steps were performed at 4°C.

For RNA isolation, the protocol of mitochondrial preparation described by Spencer et al. (1992) was followed with modifications. A cell pellet from 6 L of culture was washed once in buffer C (50 mM Tris-HCl, pH 8.0, 300 mM mannitol, 0.1% BSA, 1 mM β-mercaptoethanol, 20 mM EDTA). After resuspension in 30 ml of buffer C, the cells were transferred to a 40 ml glass homogenizer (Kontes Glass Co.) and disturbed manually with a pestle until 90% of them were broken. Following centrifugation (1,000 × g) for 10 min, the supernatant was saved and subjected to another centrifugation (12,000 × g) for 20 min to collect crude mitochondria. The resulting pellet was resuspended in 2 ml of buffer C and loaded onto a gradient consisting of equal volume of 1.15 and 1.55 M sucrose (in buffer C). The gradient was centrifuged (25,000 rpm, Backman SW 41 Ti rotor) for 1 h. The band at the interface of 1.15 and 1.55 M sucrose layers was removed with a 18 gauge needle with a 90° bended. The fraction obtained was slowly diluted with buffer D (50 mM Tris-HCl, pH 8.0, 20 mM EDTA), followed by centrifugation (12,000 × g) for 15 min. All steps were carried out at 4°C.
C. DNA isolation

The DNA isolation method of Ryan et al. (1978) was adapted. Whole cell and mitochondrial-enriched pellets were lysed in buffer B containing 2% sarkosyl, 1% SDS, and 0.5 mg/ml proteinase K (Boehringer Mannheim) at 50°C for 1 h. The lysate was extracted once with equal volume of phenol (equilibrated to pH ≥ 8.0 with Tris buffer) and twice with equal volume of chloroform/isoamyl alcohol (24/1, vol/vol). The final aqueous phase was precipitated in two volumes of absolute ethanol at -20°C for 2 h or longer. The precipitate was collected by centrifugation (12,000 × g) at 4°C for 20 min. After being air-dried, the pellet was dissolved in TES buffer (1 mM EDTA, 0.15 M NaCl, 10 mM Tris-HCl, pH 8.0), followed by digestion at 37°C for 1 h with RNase A (25 ng/ml) (Amersham Pharmacia Biotech), which was pre-treated in boiling water for 15 min. Non-DNA materials were removed by centrifugation (12,000 × g) for 1 min after precipitation at room temperature in the presence of 2.5 M NH₄OAc. DNA was then precipitated twice with ethanol, air-dried, and redissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). There was no further DNA purification step employing preparative CsCl gradient centrifugation as described by Ryan et al. (1978).

D. RNA isolation

All the chemicals used were RNase-free grade. To minimize potential RNase contamination, water was treated with 0.1% diethyl pyrocarbonate (DEPC) (Sambrook et al., 1989); glassware was baked at 180°C overnight;
plasticware was either baked at 120°C overnight if it could sustain the
temperature or soaked in 3% hydrogen peroxide for 30 min, followed by rinsing
with DEPC-treated water. All steps were carried out at 4°C.

For the isolation of total cellular RNA, cells were washed once in buffer E
(50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 50 mM EDTA) and lysed in three
volumes of buffer E containing 2% sarkosyl. The lysate was extracted twice with
an equal volume of water-saturated phenol and twice with an equal volume of
chloroform/isoamyl alcohol (24/1, vol/vol). After the addition of a 0.1 volume of 3
M NaOAc (pH 5.2), the aqueous phase was precipitated with 2.5 volumes of
absolute ethanol at -20°C overnight. RNA was collected by centrifugation
(12,000 × g) for 20 min.

For the isolation of mitochondrial-enriched RNA, a mitochondrial-enriched
pellet prepared from 6 L of culture was resuspended in 9 ml of buffer F (10 mM
Tris-HCl, pH 8.5, 50 mM KCl, 10 mM MgCl₂) and lysed by the addition to the
suspension of 1 ml of 20% Triton X-100 (in buffer F). After centrifugation
(10,000 × g) for 10 min, the supernatant was saved, and SDS was added to a
final concentration of 2%. Subsequent RNA extraction from the lysate followed
the steps described above.

E. Electrophoretic analysis of DNA

DNA samples in five volumes were mixed with one volume of a 6X stock
of loading buffer (100 mM EDTA, pH 8.0, 15% Ficoll, 0.1% xylene cyanol, 0.1%
bromophenol blue) and loaded in agarose gels made with TAE buffer (40 mM
Tris-Acetate, pH 8.0, 1 mM EDTA). Electrophoresis was performed in TAE buffer. Fractionated DNA was stained with ethidium bromide (0.5 ug/ml) before being visualized with a UV light.

F. Electrophoretic analysis of RNA

RNA samples in two volumes were mixed with one volume of a 3X stock of loading buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 7 M urea, 0.05% bromophenol blue, 0.05% xylene cyanol) and heated at 90°C for 3 min, followed by cooling on ice for 5 min. The denatured RNA was loaded in 8% polyacrylamide (19 acrylamide:1 N, N'-methylenebisacrylamide) denaturing slab gels (20 cm x 20 cm x 0.15 cm), which contained 7 M urea and were made in 0.5 × TBE buffer (45 mM Tris-Borate, 1 mM EDTA) (Sambrook et al., 1989). Electrophoresis was carried out at 350 V for 4 h. RNA was visualized with a UV light after being stained with ethidium bromide (0.5 ug/ml).

G. Polymerase chain reaction (PCR) and reverse transcriptase-PCR (RT-PCR)

PCR experiments were performed in a thermal cycler (Geneamp PCR System 2400, Perkin-Elmer) using total cellular DNA as the template and reagents from MBI Fermentas. Each reaction (50 µl) contained 1 ug total cellular DNA, 2.5 mM MgCl₂, 0.2 mM dNTPs, 2 units of Taq DNA polymerase and 1 mM primers. DNA was initially denatured at 94°C for 3 min and amplified by 40
cycles, each involving denaturation at 94°C for 45 s, annealing at 50°C for 30 s and extension at 72°C for 2 min. There was a final extension period at 72°C for 7 min. According to the manufacturer's instructions, RT-PCR was performed using "RT-PCR Beads" (Amersham Pharmacia Biotech) which contains dNTPs, reverse transcriptase, and stabilizers.

H. Recovery of DNA from agarose gels and PCR reactions

DNA isolated from a mitochondrial-enriched preparation was fractionated by agarose (1%) gel electrophoresis. The 13.5- and 3.5-kb bands, presumed to be mtDNAs, were cut from the gels. The DNA was recovered using the GFX PCR DNA and Gel Band Purification kit (Amersham Pharmacia Biotech) following the manufacture's instruction. This kit uses a chaotropic agent that denatures proteins, dissolves agarose, and promotes the binding of double-stranded DNA (100 bp to 48 kb in length) to a glass fiber matrix. After proteins and salt contaminants were washed away, the DNA was eluted in a low ionic strength buffer. The same kit was used to purify PCR products.

I. DNA cloning

1. Preparation of competent cells

Chemically competent cells were prepared aseptically according to Sambrook et al. (1989) with some modifications. A freezer stock of E. coli strain XL1-Blue MCF²(Stratagene) was streaked onto an LB plate and incubated at 37°C overnight. A single colony from the plate was inoculated in 10 ml of LB
medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) (Shambrook et al., 1989) at 37°C overnight with shaking at 250 rpm. 0.5 ml of the culture was transferred to 50 ml of LB medium and grown at 37°C with shaking at 250 rpm until the OD$_{600}$ reached 0.4 (~2.5 h). The culture was chilled on ice for 10 min and then centrifuged (3,000 × g) at 4°C for 15 min to collect cells. The cell pellet was resuspended in 20 ml of chilled 0.1 M CaCl$_2$ and kept on ice for 20 min. After being collected by centrifugation (3,000 × g) at 4°C for 15 min, the cells were resuspended in 2 ml of chilled 0.1 M CaCl$_2$ and then distributed into 60 µl aliquots in microcentrifuge tubes. The aliquots of competent cells were kept on ice and were usable for up to 2 days.

2. Ligation of DNA into a vector

a. Ligation of genomic DNA

The recovered 13.5- and 3.5-kb DNAs were digested with HindIII and EcoRI (Amersham Pharmacia Biotech), respectively, following the instructions provided by the manufacturer.

The vectors were prepared as follows. Plasmid DNA pBluescript II SK$^+$ (Stratagene) was digested with HindIII and EcoRI separately. 4 µg of restricted plasmid DNA was dephosphorylated with 0.1 unit of alkaline phosphatase (Amersham Pharmacia Biotech) at 37°C for 30 min and then treated at 85°C for 15 min to inactivate the enzyme. After the mixture was extracted with phenol once, the DNA was precipitated with ethanol in the presence of 0.2 M NaCl and dissolved in TE buffer.
Ligation reactions were carried out in 10 μl of reaction mixtures consisting of 1X OPA+ buffer (10 mM Tris-acetate, pH 7.5, 10 mM Mg(OAc)$_2$. 50 mM KOAc), 1.3 mM ATP, 0.2 unit of T$_4$ ligase (Amersham Pharmacia Biotech), 25 ng genomic DNA and 12 ng vector digested with the same restriction enzymes. Reaction mixtures were incubated at 10°C for 3 h and then treated at 65°C for 10 min.

b. Ligation of PCR products

Purified PCR products were treated to blunt the ends as follows. 1 μg DNA was mixed with 10 μl of 10X polishing buffer (0.5 M Tris-HCl, pH 7.5, 10 mM MgCl$_2$, 10 mM DTT, 0.5 mg/ml BSA, 0.2 mM dNTP, 10 mM ATP), 5 units of Klenow fragment of E. coli DNA polymerase I (Amersham Pharmacia Biotech) and 6 units of T$_4$ polynucleotide kinase (Amersham Pharmacia Biotech). Water was added to reach a final reaction volume of 100 μl. The reaction was carried out at 37°C overnight. DNA was purified by phenol extraction and recovered by ethanol precipitation as stated above.

Linearized (with Smal) and dephosphorelated plasmid pUC18 (pUC18 Smal I/BAP, Amersham Pharmacia Biotech) was used as the vector. 15 μl of ligation mixture (100 ng blunt-ended PCR products, 50 ng vector, 1.3 mM ATP, 0.4 unit of T$_4$ ligase, 1X OPA+) was incubated at 10°C for 8 h and then treated at 65°C for 10 min.

3. Transformation
2 μl of the ligation mixture was added to 60 μl of competent cells. After 20 min on ice, the ligation/competent cell mixture was incubated at 42°C for 90 s, followed by chilling on ice for 3 min. Subsequently, 540 μl of SOC medium (2% tryptone, 0.5% yeast extract, 0.05% NaCl, 2.5 mM KCl, 20 mM glucose) was added to the transformed cell mixture, which was then incubated at 37°C for 1 h. Up to 300 ml of the cell mixture was spread onto individual LB plates containing ampicillin, 5-bromo-4-chloro-3-indoly-β-D-galactoside (X-gal) and isopropylthio-β-D-galactoside (IPTG) (Sambrook et al., 1989). The plates were incubated at 37°C overnight.

4. Screening

Transformed cells were selected for ampicillin resistance conferred by the plasmid, and the cells harbouring the recombinant plasmids were further selected for their inability to conduct alpha complementation (Sambrook et al., 1989). The recombinant plasmids were isolated by the minipreparation method (see below), and those containing inserts of the 13.5- and 3.5-kb mtDNAs were identified by Southern blot hybridization analysis (see below) using these DNAs as probes.

5. Alkaline minipreparation of plasmids

Recombinant plasmids were extracted from the host cells by the alkaline lysis preparation method (adapted from Sambrook et al., 1989). Single colonies were inoculated in 15 ml-tubes containing 3 ml of LB medium with 100 μg/ml
ampicillin and grown at 37°C overnight with shaking. The cells were collected in microcentrifuge tubes, frozen at -20°C for 2 h, and then resuspended in 100 µl of buffer, which consisted of 25 µl of 1 M Tris-HCl, pH 8.3, 20 µl of 0.5 M EDTA, 45 µl of 20% glucose and 10 µl of 10 mg/ml lysozyme. After incubation on ice for 5 min, the cells were lysed at room temperature for 5 min by adding 200 µl of a solution containing 0.2 M NaOH and 1% SDS. Subsequently, 150 µl of 3 M KOAc (pH 5.0) was added into the cell lysate. The suspension was incubated on ice for 5 min, followed by centrifugation (12,000 × g) for 8 min. 1 µl of RNase A (1 mg/ml, Amersham Pharmacia Biotech) was added to the supernatant, and the digestion was carried out at 37°C for 1 h. Following that, 12.5 µl of 10 M NH₄OAc was added to precipitate non-DNA materials (15 min on ice followed by centrifugation at 10,000 × g for 30s). Plasmids were recovered by ethanol precipitation after phenol extraction. Plasmids prepared in this way were used for purposes including DNA sequencing.

J. DNA transfer and Southern blot hybridization

After fractionation by agarose gel electrophoresis, DNA was transferred to a nylon membrane (Hybond N+, Amersham Pharmacia Biotech) using the capillary transfer method (Sambrook et al., 1989) with 0.4 M NaOH as the transfer solution. The membrane was then baked at 80°C for 2 h. The Alkphos Direct Labelling and Detection System kit (Amersham Pharmacia Biotech) was employed for prob labeling and hybridization. DNA probes were labelled at 37°C for 30 min. Hybridization was carried out in plastic bags overnight in 60°C water
bath with shaking. The membranes were washed twice in primary wash buffer (2 M urea, 0.1% SDS, 50 mM NaH$_2$PO$_4$, pH 7.0, 150 mM NaCl, 1 mM MgCl$_2$) at 60°C for 10 min and twice in secondary wash buffer (1 M Tris, 2 M NaCl) at room temperature for 5 min. Chemiluminescent detection was achieved by exposing auto-radiographic film to the membrane. For re-probing, the membrane was stripped in 0.5% SDS at 70°C for about 4 h and checked for completeness of signal removal.

**K. RNA transfer and Northern blot hybridization**

RNA was loaded and fractionated in several lanes of a 8% polyacrylamide gel and was then blotted onto a nylon membrane (Hybond-N+, Amersham Pharmacia Biotech) by the method of capillary transfer (Sambrook et al., 1989), using 0.5 mM NaOH as the transfer solution. The membrane was baked at 80°C for 2 h and then cut into strips, each of which corresponds to a lane of the gel. The Alkphos Direct Labeling and Detection System kit (Amersham Pharmacia Biotech) was employed for probe labeling and hybridization. Oligodeoxynucleotide probes (listed in Table 4) were labeled at 37°C for 2.5 h or longer. Hybridization was carried out in a hybridization oven at 38°C overnight. The membrane strips were washed twice in the primary wash buffer at 38°C for 10 min and twice in the secondary wash buffer at room temperature for 5 min, followed by signal detection as stated above.

**L. DNA sequencing**
Cloned and in some cases PCR amplified mtDNA segments were sequenced commercially (Dalhousie University-NRC Institute for Marine Biosciences Joint Laboratory, Halifax, or Center for Applied Genomics, Hospital for Sick Children, Toronto) on both strands using LICOR 4200 (LICOR; dye primers), or ABI 373 or 377 (PE-Applied Biosystems; dye terminators) automated DNA sequencers. In the case of PCR products, sequences were obtained either directly from the product or from two independent clones. The method of primer walk was employed to achieve the full sequence of DNA wherever necessary.

M. RNA labelling and sequencing

The experiments were performed by M.N. Schnare. RNA isolated from the mitochondrial-enriched fraction and semi-purified mitochondrial ribosomes (Denovan-Wright and Lee, 1995) of P. parva was 3'-end-labeled with $[^{32}P]pCp$ using RNA ligase (Peattie, 1979). The end-labeled mitochondrial rRNAs were fractionated in 6% polyacrylamide gels (33 cm × 40 cm × 0.05 cm) at 1,700 V and eluted according to Schnare et al (1983). The partial chemical degradation method (Peattie, 1979) was used to determine the 3'-terminal sequence of mitochondrial rRNAs.

N. Data analysis

The BLAST network services (Altschul et al., 1990) provided at the National Center for Biotechnology Information (NCBI) were used for sequence
similarity searches. The program Gene Runner (Hastings Software) was used for sequence editing and compiling. Multiple DNA and protein sequence alignments were performed using the program CLUSTAL W, version 1.7 (Thompson et al., 1994). rRNA sequences were aligned manually based on proposed secondary structure, and the identity between sequences were calculated using CLUSTAL W, version 1.7 (Thompson et al., 1994). Codon usage was analysed using the program Codons 1.4 (Lloyd and Sharp, 1992).

Relative rate tests were performed according to Graur and Li (2000) as shown in Figure 4. The program RRTree, version 1.1.10 (Robison-Rechavi and Huchon, 2000) was employed for calculation of nucleotide substitution between sequences.
RESULTS
A. Mitochondrial genome of *P. parva*

1. Identification, size, and conformation of *P. parva* mtDNAs

Following fractionation by agarose gel electrophoresis, DNA from the mitochondrial-enriched fraction of *P. parva* revealed two prominent components that were barely visible or not visible, respectively, in the total cellular DNA preparation (Fig. 6). The two components consistently corresponded to sizes of 13.5 and 3.5 kb relative to linear DNA size markers when the concentration of agarose was either 1% or 0.6% (Fig. 7). These results support the linear conformation of the two DNA species (Johnson and Grossman 1977). By means of genomic DNA cloning and in some cases PCR amplification, DNA segments collectively spanning 97 and 86% of the 13.5- and 3.5-kb DNAs, respectively, were recovered and sequenced, thereby yielding two partial physical and gene maps (Fig. 8). The inverted repeat structure of the termini of the two partial maps together with my inability to recover either clones or PCR products bridging the ends of the 13.5- or 3.5-kb molecules further argue that these DNAs are linear molecules with unique ends. Considering the source of the two DNAs and the coding regions they contain, it is concluded that the 13.5- and 3.5-kb DNAs are components of the *P. parva* mitochondrial genome. Sequences of the 13.5- and 3.5-kb mtDNAs are deposited in GenBank under the accession numbers AY062933 and AY062934, respectively.

2. General features of *P. parva* mitochondrial genome

**Base composition** The available DNA sequences of the 13.5- and 3.5-
kb mtDNAs of *P. parva* indicate an overall A + T content of 59.5 and 57.3%, respectively. Compared to other CW-group chlorophycean taxa, these values are well below the A + T content of the entire mtDNA of *C. moewusii* (65.4%) and intermediate between that of the entire mtDNAs of *C. reinhardtii* (54.8%) and *Chlorogonium* (62.2%). The A + T content difference between coding and non-coding regions is insignificant in the 13.5-kb mtDNA (60.1 and 57%, respectively), but more prominent in the 3.5-kb mtDNA (65.3 and 55.9%, respectively).

**Gene content** Coding regions identified in the two *P. parva* mtDNAs include seven respiratory chain protein-coding genes, one tRNA gene, and LSU and SSU rRNA genes (Table 5). Neither a 5S rRNA nor ribosomal protein-coding genes were found. The only tRNA gene currently identified encodes tRNA$^{\text{met}}$. Predicted secondary structure of this tRNA$^{\text{met}}$ (Fig. 9) shows a base-pair between nucleotides 1 and 72, thus suggesting that it is an elongator rather than an initiator (Newton et al. 1999).

**Gene structure** All the genes in the two mtDNAs of *P. parva* are intron-free. The protein-coding genes encode amino acid sequences that are more similar in length to their homologs in *C. reinhardtii* than in *C. moewusii*. The LSU and SSU rRNA genes, which are located in the 13.5-kb mtDNA, are discontinuous and scrambled as the homologs in all other well characterized chlorophycean green algal mtDNAs (Nedelcu et al., 2000 and references therein). At least eight (*ml_a through _h*) and four (*ms_a through _d*) LSU and SSU rRNA-coding modules, respectively, have been identified (Table 5; Fig. 8).
They are scattered over a 8-kb stretch of DNA and interspersed with each other and/or with other coding sequences. Interestingly, \textit{ms\_a} and \textit{ml\_g} are located transcriptionally opposite to the rest of the LSU and SSU rDNA modules and thus show a feature not previously reported in the mitochondrial DNA of green algae, however, rRNA coding regions in the apicomplexans \textit{Plasmodium} and \textit{Theileria} are distributed on both DNA strands (Feagin 1994).

\textbf{Genome structure} All of the coding regions identified are in the 13.5-kb mtDNA except for \textit{nad6}, which is in the 3.5-kb mtDNA. As in the mtDNA of \textit{C. reinhardtii} (reviewed by Michaelis et al., 1990), the coding regions in the \textit{P. parva} 13.5-kb mtDNA are compactly organized with minimal intergenic spacers. In case of \textit{P. parva} 13.5-kb mtDNA, most of the intergenic spacers range from 0 to 38 bp in length except for one of 69 bp. Unlike other green algal species, no apparent repeats are detected in the intergenic spacers of \textit{P. parva} 13.5-kb mtDNA. Excluding the terminal sequences, the coding regions account for 97.4% of the sequence, in comparison to 90.4% in \textit{C. reinhardtii}. The genes are arranged into two unequally-sized clusters, one of which is in the opposite transcriptional orientation from the other.

3. Genetic code and codon usage

Although most of the protein-coding genes in the two identified mtDNAs of \textit{P. parva} appear to have a standard ATG start codon, it is inferred from the DNA sequence data that the mitochondria of this taxon utilize an unusual start codon for \textit{nad5}. Two initiation codons have been proposed for \textit{C. reinhardtii nad5}, with
the one of Boer and Gray (1986) being 63 nucleotides downstream of the one proposed by Vahrenholz et al. (1985). Multiple alignment of the predicted amino acid sequences of *P. parva* and *C. reinhardtii* nad5 (Fig. 10) is consistent only with the downstream start codon position of this gene in *C. reinhardtii*. However, the *P. parva* gene revealed a GTG rather than an ATG codon at the corresponding position; this corresponds to the 15th nucleotide following the termination of the *ms-c* module, as determined by RNA sequencing (see below).

Table 6 shows the codon usage in the seven mitochondrial protein-coding genes of *P. parva*. According to the standard genetic code, all the sense codons are used except for three of the six coding for arginine, i.e., CGG, AGA and AGG. Of the three termination codons, only TAA is utilized. Among the four-codon families, codons ending with T or A greatly outnumbered those ending with G or C.

4. Flanking sequences of the 13.5- and 3.5-kb mtDNAs

The alignments of the available terminal sequences derived from the 13.5- and 3.5-kb mtDNAs reveal an homologous inverted repeat sequence of almost 1.3 kb in the two DNAs (Fig. 11). The left and right repeat sequences in the 13.5-kb mtDNA and the right repeat sequence in the 3.5-kb mtDNA start immediately downstream of *cob, nad1*, and *nad6*, respectively. The left repeat of the 3.5-kb mtDNA starts 43 bp upstream of *nad6*, and a stem-loop structure can be modeled from this 43-bp sequence (Fig. 12). The four copies of the repeat sequence show only occasional differences in sequence, and a 43-bp sequence
present in the right repeat of the 13.5-kb mtDNA is missing from the other three copies of the repeat. It is noteworthy that single copies of the 7-bp sequence TGCGCAC are located at one end of and immediately following this extra 43-bp sequence therefore suggesting its loss from the other three terminal repeat regions by unequal crossing over, intrastrand deletion, or slipped-strand mispairing (Graur and Li, 2000). Interestingly, the inverted repeat sequence in both the 13.5- and 3.5-kb mtDNA molecules contain two copies of a 42-bp direct sub-repeat, which are separated from each other by a 197-bp sequence. No open reading frame having a potential coding capacity of more than 70 amino acids was detected in the sequenced part of the inverted repeat regions. Following BLAST searches no sequence in any of these regions was found to be significantly similar to any sequence in GenBank at the level of either protein or DNA.

5. Southern blot hybridization analysis of *P. parva* mtDNAs

Southern blot hybridization experiments of *P. parva* total cellular and mitochondrial-enriched DNA preparations with *P. parva* mtDNA probes confirm the homology between the termini of the 13.5- and 3.5-kb mtDNA maps as well as the absence of *nad6* from the 13.5-kb mtDNA. The clone containing 13.5-kb mtDNA fragment H54, which contains *cob*, *ms_a*, and part of *ml_g*, detected the 13.5 but not the 3.5-kb mtDNA. The clone containing 3.5-kb mtDNA fragment E12 (Fig. 8), which contains part of the two inverted repeat regions and *nad6*, detected both the 13.5- and the 3.5-kb mtDNA components. Finally, the PCR
product, derived from nad6, detected the 3.5- but not the 13.5-kb mtDNA (Fig. 13). The last two probes gave additional discrete signals in the mitochondrial-enriched DNA preparation (and in more exposed blots of total cellular DNA) at positions corresponding to linear DNA molecules of about 2.1 and 1.8 kb. These results, which have been observed consistently with independent DNA samples, imply the existence of additional small mtDNA molecules, which harbor nad6 sequence.

6. Comparisons of cob and cox1 between P. parva and Polytomella 198.80

Sequences of cob and cox1 have been reported for Polytomella 198.80 (Antaramian et al., 1996, 1998), and these reveal few differences with the counterparts in P. parva. The length of cob and cox1 are 1034 bp and 1512 bp, respectively, in the two taxa, and between the cob and cox1 homologs only 2 and 5 nucleotide substitution differences have been detected, respectively. In addition, no differences were noted between the two taxa for the 122 nucleotides upstream of cox1, which includes part of the rRNA fragment L-7-coding region (not L-4 as suggested by Antaramian et al., 1996) and 114 nucleotides downstream of cox1 into the 5' end of nad4. Considering the high rate of evolution of Polytomella mtDNA (see below), it is likely that P. parva and Polytomella 198.80 are conspecific.

7. Evolutionary rate analysis of protein-coding genes

Nucleotide substitution levels for seven protein-coding genes encoded in
mtDNA were estimated for all pair wise comparisons between homologs of *P. parva*, *C. reinhardtii*, and *C. moewusii*. Levels of synonymous substitution were saturated between all homologous gene sequences and therefore could not be calculated. Differences in the number of non-synonymous substitutions were estimated (Table 7), and these were used to calculate the rate of non-synonymous substitution between homologous mitochondrial genes in the *P. parva* lineage relative to the *C. reinhardtii* lineage using *C. moewusii* as the outgroup (Nakayama et al., 1996b; Buchheim et al., 1996; Pröschold et al., 2001). The value of $K_{13}$ (number of non-synonymous substitutions between *P. parva* and *C. moewusii*)-$K_{23}$ (number of non-synonymous substitutions between *C. reinhardtii* and *C. moewusii*), for each of the mitochondrial genes compared, is consistently positive and more than five times the standard error, indicating that the non-synonymous substitution rate difference between the *P. parva* and *C. reinhardtii* lineages for these genes is highly significant. The non-synonymous substitution rate of the protein-coding genes in the *P. parva* lineage averages about 3.3 times that of the homologs in the *C. reinhardtii* lineage, with the lowest value being 2.5 times for *cob* and the highest value being 4.4 times for *nad1*. Interestingly, *nad6*, the only gene identified in the 3.5-kb mtDNA, has a non-synonymous substitution rate ratio of 2.8 between the two lineages, which is not remarkable compared to the other mitochondrial genes characterized.
B. Mitochondrial ribosomal RNAs of *P. parva*

1. Identification of mitochondrial rRNA fragments

Total cellular and mitochondrial-enriched RNA of *P. parva* were subjected to polyacrylamide gel electrophoresis (Fig. 14). The total cellular RNA sample revealed prominent components typical of the nucleocytoplasm, including 25/28S, 18S, 5.8S, and 5S rRNAs, as well as tRNAs. In addition, the RNA from the mitochondrial-enriched fraction contained, several potential mitochondrial rRNA species not visible in the total cellular RNA preparation that had sizes significantly smaller than the 25/28S and 18S rRNAs.

Either 3′-terminal RNA sequencing (Fig. 15) or Northern blot analysis (Fig. 16) was employed in an attempt to establish the correspondence between each of the *P. parva* mitochondrial rRNA-coding modules identified previously by DNA sequencing (Fig. 8 and Table 8) and the putative mitochondrial rRNA components observed in Figure 14. 3′-terminal chemical sequencing data was obtained for five RNA species, thereby establishing that each of these corresponds to a transcript of one of the rRNA-coding modules (Fig. 15 and Table 8). Northern blot analysis with individual probes derived from the remaining seven rRNA-coding modules each identified a single transcript (Fig. 16). Five of these transcripts corresponded in size to an RNA component detected in the mitochondrial-enriched fraction of Figure 14. The other two transcripts, L2 and L4, were not detected by ethidium bromide staining; the former fragment did not stain well because of its small size, and the latter fragment comigrated with the tRNAs. In addition, it is noted that the rRNA pairs S4/L8 and
$S_2/L_3$ could not be well separated in standard 8% polyacrylamide gels due to their similar sizes within the pairs (Fig. 14), but could be differentiated using 6% polyacrylamide sequencing gels (data not shown). Due to their similar sizes, rRNA fragments $L_1$, $L_6$, and $S_1$ also co-migrated (Fig. 14 and Fig. 16).

Estimates of the length of the 12 $P$. parva mitochondrial rRNA species and the corresponding $E$. coli coordinates are summarized in Table 8.

Approximate locations of the 5'- and 3'-ends of these mitochondrial rRNAs were estimated on the basis of their sizes as determined by gel electrophoresis, secondary structure modeling and the proximity of neighboring coding regions as determined by DNA sequencing. The 3'-ends of rRNA species $L_3$, $L_5$, $L_7$, $S_3$, and $S_4$ were precisely located by direct chemical sequencing. The derived size of each rRNA fragment (Table 8) is consistent with the size of the corresponding rDNA module identified previously by DNA sequencing, therefore arguing against the presence of additional points of discontinuity within any of the coding modules. The size of $S_2$ was estimated at 179 nucleotides by secondary structure comparison and cannot be much larger when the positions of flanking genes are considered; it therefore seems likely that its electrophoretic mobility is artifactually slow since it migrates near the 200 nucleotides marker in polyacrylamide gels (Fig. 14 and Fig. 16).

2. Chemical cleavages of rRNA fragment $S_4$

Sequencing of the 3'-terminus of the $P$. parva mitochondrial rRNA fragment $S_4$, which corresponds to the 3'-end region of SSU rRNA from other
sources, revealed two sites where chemical cleavage was unusual compared to other sites in this rRNA (Fig. 15). The residue located 45 nucleotides from the 3'-end, where the DNA sequence predicts a U, exhibited an extraordinarily strong C-specific chemical cleavage. This enhanced cleavage is diagnostic for 3-methyluridine (m^3U) in the RNA as was observed, for example, at the homologous position in SSU rRNA from E. coli, wheat mitochondria, and Euglena gracilis chloroplasts (Schnare and Gray 1982; Schnare et al. 1992). In addition, the residue located 27 nucleotides from the 3'-terminus, where the DNA sequence predicts a G, was resistant to G-specific chemical cleavage. This lack of cleavage, however, may not indicate post-transcriptional modification since there are examples where some unmodified G residues give blanks in the G lane of chemical sequencing gels (Peattie 1979; M.N.Schnare, unpublished data).

Many SSU rRNAs have two adjacent N^6,N^6-dimethyladenosine residues (corresponding to E. coli 16S rRNA positions 1518 and 1519) that are resistant to A-specific chemical cleavage (Schnare and Gray, 1982). I observed normal chemical cleavage at the homologous sites in P. parva SSU rRNA (24 and 25 nucleotides from the 3'-terminus of S_2 rRNA, Fig. 15), indicating that these two residues are not modified to generate the highly conserved dimethyladenosines.

3. Potential structures of P. parva mitochondrial SSU and LSU rRNAs

Figure 17 shows the proposed mitochondrial SSU and LSU rRNA secondary structures of P. parva based on the sequences of the rDNA modules and modeled after the proposed secondary structures of their respective E. coli
homologs (Cannone et al., 2002). The mitochondrial SSU and LSU rRNA fragments of *P. parva* have combined lengths of 979 and 1,556 nucleotides, respectively. In these structures, theoretically, the four SSU and eight LSU rRNA pieces could be brought together through intermolecular base-pairing to form structures containing three and six domains in the SSU and LSU rRNA, respectively, as in other fragmented chlorophycean counterparts (Boer and Gray, 1988a; Denovan-Wright and Lee, 1994).

The *P. parva* mitochondrial rRNAs appear structurally conventional throughout most of the evolutionarily conserved structural and functional cores (see Cannone et al. (2002) for structure conservation diagrams) but deletions, relative to the corresponding regions of *C. reinhardtii* (Boer and Gray, 1988a) and *C. moewusii* (Denovan-Wright and Lee, 1994) mitochondrial and *E. coli* (Cannone et al., 2002) rRNA, occur in three regions not reported previously to be variable. First, three nucleotides are missing from the GTPase center predicted for *P. parva* mitochondrial LSU rRNA; this center is otherwise rather conserved relative to the counterparts from *E. coli* as well as *C. reinhardtii* and *C. moewusii* mitochondria. Second, the 431-436 region of SSU rRNA lacks seven nucleotides. Third, two sequences in the stem that results from the interaction of LSU rRNA fragments $L_6$ and $L_7$ are eliminated. Although the DNA used for sequencing the region encoding the GTPase center was obtained by PCR (Fig. 8), the possibility of amplification errors was minimized by a parallel PCR reaction with *C. reinhardtii* DNA, which produced a product having the same sequence found for cloned DNA that had not been PCR amplified (Boer and
Gray, 1988a). In addition, a cDNA of the P. parva mitochondrial GTPase center was obtained by RT-PCR, and its sequence exactly matches the DNA prepared by PCR, thus supporting the absence of the three nucleotides in the GTPase center of P. parva LSU rRNA. All DNA sequences were determined for both strands.

Comparisons involving 775 and 1174 nucleotides of mitochondrial SSU and LSU rDNA, respectively, that could be aligned among P. parva, C. reinhardtii, and C. moewusii, revealed a nucleotide identity of 61% and 69%, respectively, between P. parva and C. reinhardtii, and an identity of 58% and 68%, respectively, between P. parva and C. moewusii. These results underscore the considerable evolutionary divergence, at the primary sequence level, between the P. parva mitochondrial rDNA sequences and those of C. reinhardtii and C. moewusii.
DISCUSSION
A. Genome structure and organization

Two linear mtDNA components from *P. parva* with sizes of 13.5 and 3.5 kb were identified and partially characterized. This represents the first description of subgenomic mtDNAs from a green alga. The standard, apparently required gene, *nad6*, is the only gene identified in the 3.5-kb mtDNA; the absence of *nad6* from the 13.5-kb mtDNA argues for the functionality of this DNA. Moreover, for *nad6*, the ratio of the number of non-synonymous substitutions in the *P. parva* lineage to that of the *C. reinhardtii* lineage is within the range of the other mtDNA-encoded genes, thus supporting the view that *nad6* is under normal evolutionary constraints and therefore functional. A potential stem-loop structure in the region immediately upstream of *nad6* might play some role in the initiation of *nad6* transcription, as similar potential structures have been identified upstream of genes in the minicircular mtDNAs of the mesozoan animal *Dicyema misakienne* (Watanabe et al., 1999). Interestingly, no similar potential structure was identified in the 13.5-kb mtDNA molecule of *P. parva*. However, two potential promoter sequences for bidirectional transcription initiation were identified between *mlg* and *cox1* in *P. parva* 13.5-kb mtDNA. One, 5'-ATATTCTTA-3', is located nine nucleotides upstream of *cox1*, and the other, 5'-GTATTGCTG-3', is located five nucleotides upstream of *mlg*. These sequences show similarity to the consensus promoter sequence in the mtDNA of fungi (Tracy and Stern, 1995) as well as the potential promoters identified upstream of *cox1* and *nad5* (Duby et al., 2001) in the region of bidirectional transcription initiation proposed for the *C. reinhardtii* mtDNA (Gray and Boer, 1988).

Examples of subgenomic mtDNAs, most of which are circular-mapping,
have been described in other eukaryotic lineages, with varying degrees of completeness. *P. parva*, however, seems to offer the only clear example of a mitochondrial genome containing subgenomic linear DNA molecules that harbor standard mitochondrial coding regions and that share homologous inverted repeat ends. Certain hydrozoan taxa have been shown to contain two ca. 8-kb linear mtDNA molecules in contrast to the single 14- to 17-kb linear mtDNA found in most hydrozoans (Warrior and Gall, 1985; Bridge et al., 1992); nevertheless, except for a 3.2-kb sequence at an end of one of the two linear mtDNAs from *Hydra attenuata* (Pont-Kingdon et al., 2000), these genomes are not well characterized.

Based on the available evidence, it can not be decided conclusively at the present time whether or not the 13.5- and 3.5-kb mtDNAs of *P. parva* replicate autonomously. Southern blot hybridization experiments (Fig. 13) using probes specific to the 13.5- or 3.5-kb mtDNAs both revealed signals in the well regions of the gel almost equivalent in intensity to those of the migrating 13.5- and 3.5-kb components. These signals could have resulted from (1) 13.5- and 3.5-kb linear molecules that were trapped in the well regions possibly by nuclear DNA and/or impurities or (2) one or more larger replicative forms of mtDNA from which the 13.5- and 3.5-kb sequences are normally excised. The former possibility is favoured because attempts to obtain PCR products connecting the 13.5- and 3.5-kb mtDNAs or bridging the ends of each of these DNA components were unsuccessful.

If the 13.5- and 3.5-kb linear DNAs are not derived from some larger
replicative form(s) and replicate autonomously as linear molecules, they would require a mechanism to replicate their 5'-ends like any other linear DNA capable of replication. MtDNA telomeres from a variety of organisms have evolved a diversity of mechanisms aimed at solving this problem as revealed by their distinct structures (reviewed by Nosek et al., 1998). The available information does not enable one to propose a specific telomeric mechanism that might be employed by the mtDNAs of *P. parva*, and it is unclear as to the possible role in this potential process, if any, that could be played by the direct sub-repeat sequences common to the four copies of the inverted repeat sequence. In the absence of sequence at the very termini of the 13.5- and 3.5-kb mtDNAs, one can not rule out the possibility that these direct sub-repeat sequences share sequence identity with the outermost termini of the *P. parva* mtDNAs and have a role in telomere maintenance, as proposed for the internal 86-bp repeat of the outermost inverted repeat sequence in *C. reinhardtii* mtDNA (Vahrenholz et al., 1993; Duby et al., 2001).

**B. Gene content, structure, and order**

The gene content and discontinuous structure of the LSU and SSU rRNA-coding regions in the *P. parva* mitochondrial genome are typical of the "reduced-derived" type of mtDNA identified in *C. reinhardtii* (Boer and Gray, 1988a; Michaelis et al., 1990), *Chlorogonium capillatum* (= *C. elongatum* SAG 12-2e) (Kroymann and Zetsche, 1998), and *C. moewusii* (UTEX 9) (Denovan-Wright et al., 1998), except for two tRNA genes, *tmW(cca)* and *tmQ(uug)*, not identified in
the *P. parva* mtDNA; a reverse transcriptase-like coding region (*rtl*) (Boer and Gray, 1988b), possibly a degenerate group II intron (Nedelcu and Lee, 1998) so far identified only in the mtDNA of *C. reinhardtii*, has also not yet been detected in the *P. parva* mtDNA. The missing tRNA coding regions could not be identified in the sequenced portion of the two *P. parva* mtDNAs using the program tRNAscan SE 1.21 (Lowe and Eddy, 1997), and there appears to be no remaining space outside of the inverted repeat sequence regions of the 13.5- and 3.5-kb mtDNAs that could accommodate the expected ca. 75-bp coding regions. Moreover, it appears that the short DNA segments currently unsequenced at each end of the two identified *P. parva* mtDNAs are also part of the terminal inverted repeats and have no coding function. Although transfer of *tmW*(caa) and *tmQ*(uug) to the nucleus in *P. parva* is possible, an alternative explanation is that they are encoded in one or two additional as yet unidentified mtDNA(s).

Genes in the 13.5-kb mtDNA of *P. parva* are entirely rearranged relative to other completely sequenced mtDNAs of chlorophycean taxa, i.e., *C. reinhardtii*, *C. moewusii*, *Chlorogonium*, and *Scenedesmus*, except the cluster of *nad4* and *nad2*, which is conserved in *C. moewusii* mtDNA. In contrast, mtDNAs of *C. moewusii* and *Chlorogonium* share three gene clusters (Kroymann and Zetsche, 1998; Denovan-Wright et al., 1998).

### C. Genetic code and codon usage

Based on the DNA sequence, a non-standard start codon, GTG, is
predicted in *nad5* of *P. parva* 13.5-kb mtDNA. Evidence has been reported that mitochondria in several lineages use non-standard initiation codons, and in many cases this includes GTG, as, for example, in the protist *Tetrahymena pyriformis* (Edqvist et al., 2000). Although there is no previous report for the use of unusual start codons in green algal mitochondria, other non-standard codons appear to be used: the standard termination codons TGA and TAG are used as a sense codon specifying tryptophan and leucine in *Pedinomonas* (Turmel et al., 1999) and some chlorophyceans (Hayashi-Ishimaru et al., 1996), respectively. In *Scenedesmus*, the conventional termination codon TAG and serine codon TCA specify leucine and translational termination signal, respectively (Kück et al., 2000; Nedelcu et al., 2000). The possibility that G to A editing could modify the GTG codon to ATG in the *nad5* transcript of *P. parva* has not formally been eliminated, however, this seems unlikely at the present time because RNA editing has not yet been reported in green algal mitochondria and G to A editing is rare, having only recently been detected in HIV-1 viral transcripts (Bourara et al., 2000).

The codon usage pattern in the same set of seven mitochondrial standard protein-coding genes shows both similarity and difference between *P. parva*, *C. reinhardtii*, and *C. moewusii*. Like homologs of *C. reinhardtii* and *C. moewusii* (Denovan-Wright et al., 1998), *P. parva* mitochondrial sequences preferentially use synonymous codons ending with A or T in four-codon families, thus in agreement with the high A + T content of these chlorophycean mtDNAs. However, in comparison with thirteen and two unused codons in the sequences
of *C. reinhardtii* and *C. moewusii*, respectively, five codons are absent in the *P. parva* homologs; among them, TGA is used by both *Chlamydomonas* species. The two unused codons in *P. parva* mitochondrial protein-coding genes, i.e., the arginine codon AGA and the termination codon TGA, have an A in the third position. This observation seems inconsistent with the high A + T content of *P. parva* mtDNA. Rather, the absence of AGA and TGA could be explained by the lack or low abundance of corresponding tRNA and specific binding release factor, respectively.

**D. Elevated evolutionary rate of *P. parva* mitochondrial protein-coding genes**

On average, the non-synonymous substitution rate in mitochondrial protein-coding genes is about 3.3 times greater in the *P. parva* lineage compared to the *C. reinhardtii* one. *P. parva* mitochondrial protein-coding genes, therefore, in terms of nucleotide substitutions that cause amino acid change, seem to be evolving conspicuously faster than those in *C. reinhardtii*. Interestingly, the same trend is observed in phylogenetic trees based on 18S rDNA sequences (Nakayama et al., 1996a, b; Pröschold et al., 2001), therefore suggesting that the higher evolutionary rate is characteristic of the *P. parva* lineage rather than a particular genetic compartment of the lineage. Such a lineage effect could be explained by (1) a greater number of mutations, potentially because of a greater number of generations, or (2) a higher probability of mutation fixation, possibly because of a smaller population size (Pringsheim, 1955), or both, relative to the
C. reinhardtii lineage. It is noteworthy that an accelerated rate of evolution in rRNA genes residing in the nuclear, mitochondrial, and plastid compartments has also been observed in some non-photosynthetic holoparasitic plants (Wolfe et al., 1992a; Duff and Nickrent, 1997 and references therein).

E. General structure of P. parva mitochondrial rRNAs

One of the most distinguishing features of the fragmented rRNAs of P. parva is their combined SSU rRNA and LSU rRNA lengths, which are considerably shorter than the fragmented and continuous rRNA counterparts so far characterized from other green algae (Table 9). The most parsimonious explanation of these data is that P. parva mitochondrial rRNA-coding regions evolved from longer versions of these genes similar to those described from other green algae. Although this proposed loss of sequence occurred in both the conserved and variable regions of the P. parva mitochondrial rDNA, most of this reduction, using the mitochondrial rDNA sequences of C. reinhardtii and C. moewusii as references, occurred in the variable regions. This is illustrated by the observations that even though the variable regions account for a minority of the green algal mitochondrial SSU and LSU rDNA lengths, they account for 68% and 73% of the overall length reduction of the P. parva SSU and LSU rRNAs, respectively, relative to the C. reinhardtii counterparts, and 62% and 56% for the SSU and LSU rRNAs, respectively, relative to the C. moewusii counterparts. In addition, the mitochondrial rRNA of P. parva does not have the large insertions found in the mitochondrial SSU and LSU rRNAs of C. reinhardtii (Boer and Gray,
1988a) and the SSU rRNA of C. moewusii (Denovan-Wright and Lee, 1994), which are located in regions not previously identified as variable.

The observed small size of the P. parva mitochondrial rRNA-coding regions seems not to be a result of random events but rather a trend for smaller SSU and LSU rRNA-coding regions in the lineage leading to P. parva. Evidence for such a trend is supported by length reductions, some of which are drastic, in most SSU and LSU rRNA variable regions relative to the C. reinhardtii and/or C. moewusii counterparts as shown in Figure 17. Moreover, additional deletions occur in P. parva mitochondrial rRNA in three conserved regions (Fig. 17), as indicated earlier in the RESULTS and discussed below.

The driving force behind the loss of sequence from the P. parva mitochondrial rRNA genes compared to other green algae is not clear, but it could be the result of a higher rate of deletion mutations in P. parva mtDNA possibly through slip strand mispairing (Graur and Li, 2000) and/or a higher probability that such mutations are fixed in the P. parva population possibly in response to enhanced selective pressures for a smaller mitochondrial rDNA coding regions in P. parva. It seems unlikely, however, that P. parva's obligate heterotrophic form of nutrition is directly related to these processes as the other obligate heterotrophic species listed in Table 9, namely Prototheca wickerhamii, has the second largest mitochondrial rRNA-coding regions among this group. It is noteworthy, however, that P. parva can multiply with a doubling time of less than 5 h (Sheeler et al., 1970), which is faster than the upper limit of growth reported for both C. reinhardtii and C. moewusii (Harris, 1989) and likely most or
all of the other taxa listed in Table 9, therefore suggesting a possible connection between growth rate and reduced mitochondrial rRNA gene size.

Finally, no RNAs corresponding in sequence to the two abundant RNA species, L2c and L3a, from *C. reinhardtii* mitochondria, which were thought initially to be components of this alga's mitochondrial LSU rRNA were identified (Boer and Gray, 1988a). Homologous mitochondrial RNAs have also not been identified in *C. moewusi*i (Denovan-Wright and Lee, 1994), so it now appears unlikely that these are components of *C. reinhardtii* mitochondrial ribosomes.

**F. GTPase center of *P. parva* mitochondrial rRNA**

The GTPase center, which corresponds to positions 1051-1108 of *E. coli* LSU rRNA and has well defined function of ribosomal protein and elongation factor binding (Thompson, 1996), is very highly conserved in terms of its 58 nucleotides length and proposed secondary structure. However, due to the reduced length of the *P. parva* GTPase center (55 nucleotides), two base pairs are not possible, corresponding to pair 1058:1080 and pair 1082:1086 in the secondary structure model for the *E. coli* GTPase center (Fig. 18). Comparative (Ryan and Draper, 1991), thermodynamic (Laing and Draper, 1994), and crystal structure (Conn et al., 1999) studies emphasize the fundamental importance of the 1082:1086 base pair. It is noted, however, that disruption of this base pair also occurs in the mitochondrial rRNA of trypanosomatid protozoa and metazoan animals (Cannone et al., 2002), all of which have highly reduced LSU rRNA sequence lengths.
In addition, the C1090 of the *P. parva* GTPase center is also noteworthy. The crystal structure of the eubacterial GTPase center shows a base triple A1089-A1090-U1101 in which A1089 N3 is hydrogen-bonded to A1090 N6 in a so called "A-A sidestep" (Conn et al., 1999). In the mitochondria of both *C. reinhardtii* (Boer and Gray, 1988a) and *C. moewusii* (Denovan-Wright and Lee, 1994), this base triple is conserved. The importance of A1090 in *C. reinhardtii* is further suggested by the observation that a nucleotide change at this site may contribute to the suppression of a single nucleotide frameshift mutation in the mitochondrial gene *cox1* of *C. reinhardtii* possibly because of the absence of the "A-A side step" (Matagne and Baurain, 2001). Because position 1090 is a C while position 1101 is still a U in the mitochondrial LSU rRNA of *P. parva*, such a base triple and "A-A sidestep" can not be expected.

G. Unusual deletions in the other two conserved regions of SSU and LSU rRNAs

Length reduction in SSU rRNA fragment *S*₅ near coordinates 431-436, which correspond to the *E. coli* SSU rRNA positions of 722-733, altered the potential secondary structure in this region. Evidence has been shown in *E. coli* by different approaches that binding of ribosomal proteins occurs within or in the proximity of this region (see Zimmermann, 1996 for a review). When 16S rRNA carrying C726G mutation was expressed *in vivo*, dramatic consequences developed, i.e., induction of heat shock proteins, production of novel peptides, and changes in levels of proteins (Prescott and Dahlberg, 1990). It was
determined that this mutation affects the binding affinity and translation termination (Prescott and Göringer, 1991).

Two sequences in the LSU region of interaction between L₆ and L₇, which correspond to positions 1299-1302 and 1626-1641 of E.coli LSU rRNA, are deleted. It has been shown that parts of the missing sequences are in a region involved in ribosomal protein binding (Raué et al., 1990).

H. Mitochondrial rRNA break points

All the interrupted points, three and seven in P. parva mitochondrial SSU and LSU rRNAs, respectively, are located in regions previously identified as variable in sequence and secondary structure in other rRNAs (Gray and Schnare, 1996). Figure 19 shows the comparison of mitochondrial SSU and LSU rRNA discontinuity patterns among the Chlamydomonas-like chlorophycean algae. The number and position of broken variable regions in LSU rRNA is identical between P. parva and C. reinhardtii homologs (Boer and Gray, 1988a), while these two organelles share two out of three break points in their SSU rRNAs. The other break point in P. parva SSU rRNA (separating S₁ and S₂ in the structure models) is shared with C. moewusii/Chlorogonium (Denovan-Wright and Lee, 1994; Kroymann and Zetsche, 1998). Overall the fragmentation patterns of P. parva mitochondrial rRNAs are more similar to those of C. reinhardtii than to their counterparts of C. moewusii. The one common broken variable region between P. parva and C. moewusii/Chlorogonium is likely due to convergent evolution and may indicate a particular susceptibility of this variable
region to becoming discontinuous.

I. Post-transcriptional modification of *P. parva* mitochondrial SSU rRNA 3'-terminus

Information about the post-transcriptional modification pattern of rRNA is limited because of technical challenges involved in identifying and localizing modifications in large RNAs (Maden et al., 1995). However, direct chemical sequencing of 3'-end-labeled SSU rRNA has proven useful for evaluating the presence or absence of the "eubacteria specific" 3-methyluridine (corresponding to m^3^U at *E. coli* 16S rRNA position 1498) and the two adjacent N^6^,N^6^-dimethyladenosine residues (corresponding to *E. coli* 16S rRNA positions 1518 and 1519) that are found in both eubacterial and eukaryotic cytoplasmic SSU rRNA (Schnare and Gray, 1982; Schnare et al., 1992). The m^3^U and the two m^6^A modifications are present in wheat (Schnare and Gray, 1982) and *Acanthamoeba castellanii* (Lonergan and Gray, 1994) mitochondrial rRNAs, two systems in which the rRNA sequences/structures have retained striking similarity to their eubacterial homologs (Spencer et al., 1984; Lonergan and Gray, 1994). The more divergent mitochondrial rRNAs from *Tetrahymena pyriformis* (Schnare et al., 1986) and fungi (Klootwijk et al., 1975; Lambowitz and Luck, 1976) are missing all three of these modifications, while the short SSU rRNAs from animal mitochondria have the two m^6^A modifications but do not have the m^3^U (Baer and Dubin, 1980, 1981; Dubin and HsuChen, 1983). The data presented here for *P. parva* mitochondria provide the first example of any SSU rRNA that has the
"eubacterial" m³U without also having at least one of the adjacent m⁶₂A modifications (note that *E. gracilis* chloroplast 16S rRNA has the m³U but has only one of the m⁶₂A residues (Schnare et al., 1992)).

The biological function of modified nucleosides is far from being appreciated in rRNA. As site-specific modifications require an expensive investment in terms of genetic information, energy, and material resources, that the modified nucleosides play important roles in rRNA function is predictable from the evolutionary perspective. This was testified in some mutants. In *Saccharomyces cerevisiae* a mutation reducing transcription activity of a gene, whose product is involved in the methylation of a nucleoside of mitochondrial peptidyltranferase center, reduced the formation of functional LSU (Sirum-Connolly and Mason, 1993). In *E. coli*, a loss of modification in 16S rRNA conferred antibiotic resistance (Helser et al., 1971). In vitro experiments carried out with *E. coli* 16S rRNA showed that absence of methylation at two adjacent adenosines, dimethylation of which are highly conserved as discussed above, reduced ribosomal activity (Cunningham et al., 1990). It is suggested that the role of the two modified A residues is possibly to "fine-tune" the efficiency of translational initiation and fidelity, as well as subunit association (Van Knippenberg, 1985).

**J. Phylogenetic implications**

The mitochondrial genome of *P. parva*, in comparison to the three completely sequenced CW-group counterparts, is most like the one from *C.
reinhardtii. First, the linear structure of the *P. parva* 13.5- and 3.5-kb mtDNAs resembles that of the unit genome-sized counterpart in *C. reinhardtii*, although the inverted repeat sequence in the *P. parva* mtDNAs (at least ca. 1300 bp) is much longer than the inverted repeat counterpart in *C. reinhardtii* (531 or 532 bp). Second, the positions of break points in the SSU and LSU rRNA-coding regions of *P. parva* 13.5-kb mtDNA are clearly more similar to those of homologs in the *C. reinhardtii* than *C. moewusii* or *Chlorogonium* mtDNA. Third, no introns were identified in any of the coding regions of *P. parva* mtDNAs, and in this respect this mitochondrial genome resembles those of *C. reinhardtii* strains UTEX 2337 and 1062, which have no or only one intron (Colleaux et al., 1990), respectively, and contrasts with the more “intron-rich” mitochondrial genomes of *C. moewusii* (Denovan-Wright et al., 1998) and *Chlorogonium* (Kroymann and Zetsche, 1998). In addition, although gene order in the 13.5-kb mtDNA of *P. parva*, which contains all but one of the genes so far identified in its mitochondrial genome, seems highly rearranged relative to the linear mtDNA of *C. reinhardtii*, as well as the circular-mapping mtDNAs of *C. moewusii* and *Chlorogonium*, it is noted that the terminal genes on the *C. reinhardtii* mtDNA map, *cob* and *nad1*, are also located at similar positions in the *P. parva* 13.5-kb mtDNA. Finally, genes are encoded in only one mtDNA strand in *C. moewusii* and *Chlorogonium*, however in the mtDNA of *C. reinhardtii* and the 13.5-kb mtDNA of *P. parva*, two opposite transcriptional orientations are observed, and interestingly, *cox1* is the first gene in the right transcriptional orientation of both taxa. Overall, these data are consistent with phylogenetic analysis based on 18S
rDNA sequence data, which place *P. parva* in the *Volvox* clade of CW-group taxa, which includes *C. reinhardtii* but excludes *C. moewusii* and *Chlorogonium* (Buchheim et al., 1996; Nakayama et al., 1996b; Pröschold et al., 2001).

**K. Conclusions and future directions**

The subgenomic feature of the *P. parva* mitochondrial genome, which has not been found in other green algal species, indicates that this genome followed a distinct evolutionary pathway and adds to the previously revealed extensive diversity of green algal mitochondrial genomes. In addition to the fragmentation of mitochondrial rRNAs revealed in other green algal taxa, this study uncovered other interesting features of the *P. parva* mitochondrial rRNAs, i.e., significantly smaller sizes relative to their homologs in other green algal taxa, deletions in regions that are conserved and demonstrated to be functionally important in other systems, such as the GTPase center, and the lack of two highly conserved post-transcriptional modification sites of SSU rRNA. The data obtained provides background information for the study of green algal mitochondria using *P. parva* as a model system, including the direct study of fragmented mitochondrial rRNA function.

Some interesting topics regarding the mitochondrial genome of *P. parva* warrant further study. First, Southern blot analysis revealed the presence of additional small mtDNAs. It would be of interest to look into the potential coding capacity of these DNA molecules, which might carry sequences such as the two tRNA genes that exist in all of the eight completely sequenced mitochondrial
genomes of green algae, but have not been found in the *P. parva* mtDNA. Using sucrose gradient fractionation, these were successfully isolated by this investigator; the same method could be used in further studies. Second, although current results favour the view that the subgenomic mtDNAs replicate autonomously, further experimentation is needed to confirm the absence of larger mtDNA replicative precursors. Last, it is important to obtain the terminal sequences of these linear mtDNAs as this information might suggest the mechanism of replication of these termini.

With respect to the *P. parva* mitochondrial ribosomes and rRNAs, two aspects of research deserve to be considered. First, isolation of mitochondrial ribosomes for functional studies could be achieved by fractionating ribosomes from a mitochondrial-enriched pellet (see Appendix). Second, more extensive sequencing of the mitochondrial SSU and LSU rRNAs could reveal additional information regarding post-transcriptional modification patterns. Data on rRNA post-transcriptional modification are very limited, especially in mitochondria. Among the limiting factors are the large size of rRNAs, which hampers the acquisition of their sequences by direct RNA sequencing, as well the difficulty of isolating relatively pure mitochondrial rRNAs, in the case of green plants. The mitochondrial SSU and LSU rRNAs of *P. parva* are certainly good candidates for such research because they consist of small pieces which, as demonstrated in this study, can be isolated as distinct species.
APPENDIX
Fractionation and RNA analysis of *P. parva* ribosomes

Material and methods

1. Strain and cell culture conditions

   The strain and cell culture conditions are described in the MATERIALS AND METHODS. Cells were harvested in the logarithmic phase (OD_{750} = 0.31) by centrifugation (2000 × g) at 4°C for 15 min.

2. Fractionation of and RNA extraction from ribosomes

   All steps were carried out at 4°C. Cells were washed once in TD buffer (133 mM NaCl, 5 mM KCl, 0.7 mM Na_{2}HPO_{4}, 25 mM Tris-HCl, pH 7.5). After being suspended in Buffer G (25 mM KCl, 25 mM Mg_{2}Cl, 25 mM Tris-HCl, pH 7.5), the cells were disintegrated by manual homogenisation in the presence of 20% Triton X-100. Centrifugation (12,000 × g) was carried out for 10 min, and the resulting supernatant containing total ribosomes was saved for subsequent ribosomal fractionation.

   The sucrose gradient was prepared by sequentially loading 5 ml of 39, 34, 29, 24, 19, 14, and 9% sucrose (in buffer G) in a polyallomer centrifuge tube (25 × 89 mm, Beckman). The tube was then set for 10 to 24 h.

   Total ribosomes (60 A_{260} units in 1.2 ml) were loaded on the top of the sucrose gradient and centrifuged at 23,500 rpm in a Beckman SW28 rotor for 14 h. Fractionation of the gradient was performed using a syringe pump to upwardly displace the gradient with 60% sucrose as the chasing solution. Fractions of 0.4 ml were collected and analysed spectrophotometrically at 260 nm. RNA
Results and discussion

Total ribosomes of *P. parva* were subjected to centrifugation in a sucrose density-gradient containing 25 mM Mg\(^{2+}\). After fractionation of this gradient, two distinct, a major and a minor, UV absorption peaks were observed (Fig. 20). RNA extracted from the fractions of the two peaks exhibited different electrophoretic patterns in a polyacrylamide gel (Fig. 21). The fractions of the major peak show RNA bands typical of the nucleocytoplasm, supporting the nucleocytoplasmic origin of ribosomes in this peak. RNA extracted from the fractions of the minor peak contains mainly RNA species with a size corresponding to nucleocytoplasmic SSU rRNA. In addition, some small RNA species that were not observed in RNA prepared from the major peak appeared, but in significantly lower proportion. These small RNA species have sizes corresponding to mitochondrial rRNA fragments L\(_3\), L\(_7\), L\(_8\), S\(_3\), and S\(_4\) (Fig. 14).

These preliminary results indicate that the minor absorption peak contains mitochondrial ribosomes that co-sediment with nucleocytoplasmic ribosomal small subunit fraction, thus implying that they have similar S values. Because the S value of nucleocytoplasmic ribosomes is relatively constant among different lineages (Curgy, 1985) and the cytoplasmic ribosomal small subunit of *C. reinhardtii*, a close relative of *Polytomella*, is 37-40S (Harris 1989), this value might represent an approximate S value for the *P. parva* mitochondrial ribosomes. This estimate, however, needs to be confirmed as it is considerably
smaller than the S value of mitochondrial ribosomes in other species (Curgy, 1985), including *C. moewosii*, whose mitochondrial ribosomes have been reported to be 60-66S (Denovan-Wright and Lee, 1995).
### Table 1. Density and GC content of the bain DNA band in *C. reinhardtii*, *C. moewusii*, *P. caeca*, and *P. parva*

<table>
<thead>
<tr>
<th>Taxa</th>
<th>Density (g/cm³)(^a)</th>
<th>G + C (%)(^b)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. reinhardtii</em></td>
<td>1.723</td>
<td>64</td>
<td>Lemieux et al. 1980; Robreau and LeGal 1975; Kieras and Chiang 1971</td>
</tr>
<tr>
<td><em>C. moewusii</em></td>
<td>1.718</td>
<td>59</td>
<td>Lemiex et al. 1980</td>
</tr>
<tr>
<td><em>P. caeca</em></td>
<td>1.696</td>
<td>37</td>
<td>Kieras and Chiang 1971</td>
</tr>
<tr>
<td><em>P. parva</em></td>
<td>1.701</td>
<td>42</td>
<td>Kieras and Chiang 1971</td>
</tr>
</tbody>
</table>

\(^a\) The DNA density is referred to the references.

\(^b\) The GC content is calculated according to the equation (Schildkraut et al. 1962): \((\rho - 1.660 \text{ g/cm}^3)/0.098\), where \(\rho\) is the density of DNA.
### Table 2. Intron GC content (%) of beta tubulin genes

<table>
<thead>
<tr>
<th>Intron Location</th>
<th>( P.\ parva )</th>
<th>( C.\ reinhardtii )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \beta_1 )</td>
<td>( \beta_2 )</td>
</tr>
<tr>
<td>6/7 (^b)</td>
<td>35.5</td>
<td>35.5</td>
</tr>
<tr>
<td>8/9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15/16</td>
<td>44.9</td>
<td>32.3</td>
</tr>
<tr>
<td>57 (^c)</td>
<td>33.8</td>
<td>28.1</td>
</tr>
<tr>
<td>132</td>
<td>18.4</td>
<td>25.9</td>
</tr>
</tbody>
</table>

\(^a\) There are three and two beta tubulin genes reported in \( P.\ parva \) and \( C.\ reinhardtii \), respectively. The exons of all these genes are well conserved in length (403 codons). Intron locations are indicated by the positions of exon codons where they reside. Only two introns appear at homologous sites in the two taxa.

\(^b\) \( i/j \) = between codon \( i \) and \( j \).

\(^c\) \( k \) = within codon \( k \).
<table>
<thead>
<tr>
<th>Taxa</th>
<th>Class</th>
<th>Accession Number</th>
<th>Map</th>
<th>Genome Size (kb)</th>
<th>A + T (%)</th>
<th>Total Genes</th>
<th>Respiratory protein genes</th>
<th>Ribosomal protein genes</th>
<th>rRNA genes</th>
<th>tRNA genes</th>
<th>Free ORFs</th>
<th>Intron ORFs</th>
<th>Group I Introns</th>
<th>Group II Introns</th>
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<tr>
<td>C. reinhardtii</td>
<td>Chlorophyceae</td>
<td>U03843</td>
<td>Linear</td>
<td>15.758</td>
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<td>C. moewusii</td>
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<td>C. elongatium</td>
<td>Chlorophyceae</td>
<td>Y07814 Y13643 Y13644</td>
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<td>0</td>
<td>0</td>
<td>1</td>
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<td>5' TTATCTCATAGTGAAAGCTAGGGGCAAGAC 3'</td>
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<tr>
<td>$S_2$</td>
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</tr>
<tr>
<td>$L_1$</td>
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<tr>
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<td></td>
</tr>
<tr>
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<td>$L_8$</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Table 5. Coding regions\(^a\) identified in the mitochondrial genome of *P. parva***

A. Ribosomal RNA genes\(^b\) (2)
   - Small subunit rRNA in pieces *ms\_a, ms\_b, ms\_c, ms\_d*
   - Large subunit rRNA in pieces *ml\_a, ml\_b, ml\_c, ml\_d, ml\_e, ml\_f, ml\_g, ml\_h*

B. Transfer RNA genes (1)
   - *trnM(caa)*

C. Respiratory chain genes (7)
   - NADH dehydrogenase (*nad1, nad2, nad4, nad5, nad6*)
   - Apocytochrome b (*cob*)
   - Cytochrome oxidase (*cox1*)

---

\(^a\) All the coding regions are located in the 13.5 kb mtDNA, except for *nad6*, which is encoded in the 3.5 kb mtDNA.

\(^b\) The rDNA modules are named in the 5' to 3' order in which their transcript counterparts appear in conventional LSU and SSU rRNAs.
Table 6. Codon usage of seven protein-coding genes in the *P. parva* mitochondrial genome

<table>
<thead>
<tr>
<th>Codon</th>
<th>AA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No&lt;sup&gt;b&lt;/sup&gt;</th>
<th>%&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Codon</th>
<th>AA</th>
<th>No</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Ala</td>
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<td>7</td>
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<tr>
<td>GCC</td>
<td>(&quot;)</td>
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<td>16</td>
<td>CTA</td>
<td>(&quot;)</td>
<td>60</td>
<td>14</td>
</tr>
<tr>
<td>GCA</td>
<td>(&quot;)</td>
<td>46</td>
<td>21</td>
<td>CTG</td>
<td>(&quot;)</td>
<td>29</td>
<td>7</td>
</tr>
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<td>6</td>
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<td>Arg</td>
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<td>31</td>
<td>AAG</td>
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<td>10</td>
<td>16</td>
</tr>
<tr>
<td>CGC</td>
<td>(&quot;)</td>
<td>13</td>
<td>25</td>
<td>ATG(GTG)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Met</td>
<td>76 (1)</td>
<td>99(1)</td>
</tr>
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<td>44</td>
<td>TTT</td>
<td>Phe</td>
<td>140</td>
<td>65</td>
</tr>
<tr>
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<td>0</td>
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<td>0</td>
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<td>0</td>
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<td>AAT</td>
<td>Asn</td>
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<td>57</td>
<td>CCA</td>
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<td>47</td>
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<tr>
<td>AAC</td>
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<td>43</td>
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<td>46</td>
<td>TCC</td>
<td>(&quot;)</td>
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<td>7</td>
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<td>Thr</td>
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<td>13</td>
<td>28</td>
<td>ACC</td>
<td>(&quot;)</td>
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<tr>
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</tr>
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<td>GCC</td>
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<td>ACG</td>
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<td>55</td>
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<td>CAC</td>
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<td>19</td>
<td>44</td>
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<td>38</td>
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<td>13</td>
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<td>(&quot;)</td>
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<td>33</td>
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<td>21</td>
<td>TGA</td>
<td>(&quot;)</td>
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<td>0</td>
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</tbody>
</table>

<sup>a</sup> AA, amino acid.

<sup>b</sup> No, total number of codon used in the seven sequences.

<sup>c</sup> %, percentage in homologous codons.

<sup>d</sup> Codons not used are indicated in bold.

<sup>e</sup> GTG is used as an initiation codon in *nad5*. 
Table 7. Differences in the number of nonsynonymous substitutions per 100 nonsynonymous sites and the relative rates of nonsynonymous substitutions in mitochondrial genes between *P. parva* (species 1) and *C. reinhardii* (species 2) with *C. moewusii* (species 3) as a reference.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Nucleotides Compared</th>
<th>$K_{12}$</th>
<th>$K_{13}$</th>
<th>$K_{23}$</th>
<th>$K_{13}-K_{23}$±SE</th>
<th>Rate Ratio</th>
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<td>1110</td>
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<td>37.0</td>
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<td>16.6±2.7</td>
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<td>26.1</td>
<td>16.4</td>
<td>9.7±1.7</td>
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<td>26.0±3.3</td>
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<td>88.0</td>
<td>39.4</td>
<td>48.5±7.0</td>
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<td>67.7</td>
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<td>36.3±4.1</td>
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<td>62.7</td>
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<td>29.5±3.5</td>
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<td>432</td>
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<td>62.7</td>
<td>31.8</td>
<td>30.9±6.8</td>
<td>2.8</td>
</tr>
</tbody>
</table>

$K_{ij}$ = number of substitutions per 100 non-synonymous sites between species i and j.

SE = standard error.

The ratio of the rate in the *P. parva* lineage to the rate in the *C. reinhardii* lineage (Graur and Li, 2000).
Table 8. *P. parva* mitochondrial rRNA genes and their transcripts: estimated length, approximate coordinates of corresponding regions in *E. coli* homologs and identifying methods

<table>
<thead>
<tr>
<th>rRNA Genes</th>
<th>rRNAs</th>
<th>Length (Nucleotides)</th>
<th>Homologous Regions in <em>E. coli</em></th>
<th>Identifying Method <em>a</em></th>
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<td>SSU</td>
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*N* = Northern blot hybridization with oligonucleotide probes derived from corresponding gene modules; *S* = 3'-terminal sequencing.


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<td>2,085 a</td>
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*Length of LSU rRNA does not include L_{26} and L_{39} (Boer and Gray, 1988).*
Figure 1. Generalized depiction of a *Polytomella* cell. Modified from Pringsheim (1955).
Figure 2. Schematic presentation of green plant phylogeny based on 18S rDNA sequences. Two main lineages, the Streptophyta and the Chlorophyta, are revealed. The Streptophyta consists of the Charophyceae and land plants, and the Chlorophyta consists of the Prasinophyceae, the Ulvophyceae, the Trebouxiophyceae and the Chlorophyceae. The Chlorophyceae is further divided into two groups: the DO-group with the directly opposed flagellar apparatus orientation and the CW-group with the clockwise flagellar apparatus orientation, represented by *Scenedesmus* and *Chlamydomonas*, respectively. The *Chlamydomonas* species spread over the CW-subgroups, reflecting the non monophyletic property of this genus. *C. reinhardtii* and *C. moewusii* represent two of the CW-groups. Dashed lines denote other CW-subgroups.
Figure 3. Multiple alignment of amino acid sequences encoded by cry1 in different green algal species. The alignment was carried out using CLUSTAL W (1.7) (Thompson et al., 1994). Asterisks indicate positions in which an identical amino acid appears in all the five sequences. cel, Chlorogonium elongatum; ce, Chlamydomonas moewusii; cr, Chlamydomonas reinhardtii; ps, Polytomella 198.80; so, Scenedesmus obliquus. The shaded regions imply insertion/deletion difference between S. obliquus and other species.
Figure 4. Multiple alignment of amino acid sequences encoded by cob in different green algal species. Other explanations are the same as in Figure 3.
Figure 5. Demonstration of relative rate tests (adopted from Graur and Li (2000)).

Assuming that species 3 is an outgroup to species 1 and 2, and 0 is the common ancestor of species 1 and 2, the number of substitutions (K) between species can be written as:

\[ K_{13} = K_{01} + K_{03} \]
\[ K_{23} = K_{02} + K_{03} \]
\[ K_{12} = K_{01} + K_{02} \]

Therefore, the number of substitutions in lineage 1 and 2 can be deduced from the above three equations as:

\[ K_{01} = (K_{13} + K_{12} - K_{23}) / 2 \]
\[ K_{02} = (K_{12} + K_{23} - K_{13}) / 2 \]
Figure 6. Agarose (1%) gel electrophoresis of *P. parva* DNA isolated from total cellular (T) and mitochondrial-enriched (M) fractions. The DNA sizes indicated are based on lambda DNA *Hind*III and *Bst*EII fragments (MBI Fermentas).
Figure 7. Southern blot analysis of *P. parva* mtDNAs. Total cellular DNA of *P. parva* was fractionated in 1.0% and 0.6% gels and blotted (A and B, respectively). Probes used consist of clones H54 and E12 (Fig. 8). M-linear DNA markers.

T-Total cellular DNA of *P. parva*. Homology between the vector sequence and the lambda (M1) and plasmid (M2) DNA-derived markers (MBI Fermentas) accounts for the hybridization signals associated with these markers.
Figure 8. Partial physical and gene maps of *P. parva* 13.5- and 3.5-kb mtDNAs based on sequences obtained from 13,135 bp of the 13.5-kb mtDNA and 3,018 bp of the 3.5-kb mtDNA. Most of these sequences were obtained from cloned *HindIII* or *EcoRI* restriction fragments of the two mtDNAs, indicated above and below the respective maps. The specific linkage of H54, H11, H50 and H24 in the 13.5-kb mtDNA are based on gene continuity between the fragments. The sequences of the two internal uncloned regions of the 13.5-kb mtDNA were obtained from PCR products that were produced with primers designed from the flanking cloned fragments. The sequences flanking the left terminus of the 13.5-kb mtDNA and the two termini of the 3.5-kb mtDNA were obtained from PCR products produced with primer pairs including in each case an outside primer designed from the 3’ region of fragment H51 as shown in Figure 11 and an inner sequence of the closest cloned region. For gene abbreviations see Table 5. Half arrows indicate directions of gene transcription. Thick solid arrows near the ends of the maps denote terminal inverted repeats; the two flags within these regions represent two direct sub-repeats. Shading depicts the homologous feature of the repeat sequences between the two mtDNAs. Dashed arrows at the very ends represent the predicted and unsequenced termini of the 13.5- and 3.5-kb mtDNAs. Restriction sites shown are: A, *Aval*; B, *BglII*; E, *EcoRI*; H, *HindIII*; S, *SalI*. 
Figure 9. Predicted secondary structure of tRNA\textsuperscript{met} encoded in \textit{P. parva}

mitochondrial genome. Nucleotides are numbered according to Sprinzl et al. (1996).
Figure 10. A, Multiple alignment of N-terminal part of NAD5 between *P. parva* and *C. reinhardtii*. Asterisks indicate positions in which an identical amino acid appears in the two sequences. B, Sequence of *P. parva* nad5 5' part and its upstream region, and corresponding coding capacity. & and # represent stop codons. The arrow indicates the 3'-end of SSU rRNA fragment S3, which was defined by direct RNA sequencing.
Figure 11. Multiple alignment of the available flanking sequences in the 13.5- and 3.5-kb mtDNAs of *P. parva*. Sequences begin immediately (a) downstream of *cob* in the 13.5-kb mtDNA, (b) downstream of *nad1* in the 13.5-kb mtDNA, (c) upstream of *nad6* in the 3.5-kb mtDNA, and (d) downstream of *nad6* in the 3.5-kb mtDNA. Sequence a and the 3'-part of sequences c and d were obtained by PCR as described in Figure 8; the 3' primer sequence employed was complementary to the nucleotides of sequence b surrounded by a rectangle. A 43-bp sequence immediately upstream of *nad6*, that is missing from the other three repeat regions, can be modeled into a stem-loop structure (Fig.12). A 44-bp sequence is present in the repeat region downstream of *nad1*, that is missing from the other three repeat regions; single copies of a 7-bp sequence located at one end of and immediately following the other end of this extra 44-bp sequence are underlined. Shaded areas indicate two direct repeats within each copy of the inverted repeat sequence. Asterisks indicate positions in which an identical base appears in all the four sequences.
Figure 11
Figure 12. A potential stem-loop structure prior to the *nad6* sequence located in *P. parva* 3.5 kb mtDNA. IR, inverted repeat.
**Figure 13.** Southern blot hybridization analysis of the *P. parva* 13.5- and 3.5-kb mtDNAs. A, ethidium bromide staining pattern and B, C, and D, Southern blot analysis with clone H54, clone E12, and a PCR product of *nad6*, respectively. DNA markers derived from plasmid digests (MBI Fermentas) (lane 1), total cellular DNA (lane 2), and DNA extracted from a mitochondrial-enriched pellet (lane 3) were fractionated by agarose (1%) gel electrophoresis. Homology between the vector sequence of clones H54 and E12 and the plasmid-derived DNA markers accounts for the hybridization signals associated with these markers in B and C.
**Figure 14.** Electrophoretic profile of *P. parva* total (T) and mitochondrial -enriched (M) RNA in an 8% polyacrylamide gel. Positions of nucleocytoplasmic rRNAs including 25/28S, 18S, 5.8S, 5S rRNA and tRNAs, are indicated. Positions of size markers (Sigma) are also shown. Arrows point to the positions of the five mitochondrial rRNA species whose 3'-termini were sequenced (Fig. 15). Lines with a dot at one end indicate the positions of Northern blot hybridization signals obtained with oligonucleotide probes derived from the other seven mitochondrial rDNA modules (Fig. 16).
Figure 15. Autoradiograms of 20% sequencing gels showing the resolution of limited chemical digests of five 3’-end-labeled *P. parva* mitochondrial rRNA species. These RNAs were identified as transcripts of rDNA modules *ml_c*, *ml_e*, *ml_g*, *ms_c*, and *ms_d*, and are designated L₃, L₅, L₇, S₃, and S₄, respectively. In the gel for S₄, the arrows indicate the positions of reduced G-lane and enhanced C-lane cleavage; this sequence is numbered from the 3’-terminus.
Figure 15
Figure 16. Northern blot hybridization analysis to identify *P. parva* mitochondrial rRNA species. RNA species detected with oligonucleotide probes derived from rDNA modules *ml_a*, *ml_b*, *ml_d*, *ml_f*, *ml_h*, *ms_a*, and *ms_b* are designated L1, L2, L4, L6, L8, S1, and S2, respectively. Locations of size markers (Sigma) are indicated.
Figure 17. Potential secondary structures of *P. parva* mitochondrial rRNAs. (A) SSU rRNA, (B) 5' half and (C) 3' half of the LSU rRNA. The structures are constituted by four and eight RNA species in the case of SSU and LSU rRNAs, respectively, whose 5'- and 3'-termini are indicated. Roman numerals denote the three and six domains of SSU and LSU rRNA, respectively. Thin lines define regions variable among rRNAs of *P. parva*, *C. reinhardtii*, and *C. moewusii* mitochondria and *E. coli*; number of nucleotides in these regions is indicated for *P. parva* (Pp), *C. reinhardtii* (Cr), and *C. moewusii* (Cm). The GTPase center of the LSU rRNA is enclosed by thick lines. Small squares, each of which represents a nucleotide, describe structures that are conserved in *C. reinhardtii* and *C. moewusii* mitochondria and in *E. coli*, but altered or absent in the mitochondria of *P. parva*; the square in brackets indicates an extra nucleotide in *E. coli*. 
Figure 18. Comparison of potential GTPase center structure in the LSU rRNA of A, *E. coli* and B, *P. parva* mitochondria. The *E. coli* sequence is numbered according to its position in the full length LSU rRNA. The filled circles represent three nucleotides missing from the corresponding positions of the *P. parva* mitochondrial GTPase center.
**Figure 19.** Comparison of mitochondrial rRNA fragmentation patterns among *Chlamydomonas*-like algae. The rRNAs are drawn to the scale of the *E. coli* homologs. The arrow and arrowheads indicate the break points unique to *P. parva/C. moewusii/Chlorogonium* and *P. parva/C. reinhardtii*, respectively.
Figure 20. Sucrose gradient fractionation profile of *P. parva* ribosomes. Each dot represents a fraction (0.4 ml). The fractions are numbered in ascending order from the top of the gradient. Two distinct absorption peaks are indicated by letters A and B.
Figure 21. Polyacrylamide gel (8%) electrophoretic patterns of RNA isolated from *P. parva* ribosomes. Lanes 1-3, serial amounts of RNA isolated from peak A; lane 4, RNA isolated from peak B (Figure 20). Positions of nucleocytoplasmic (25/28S, 18S, 5.8S, and 5S) and mitochondrial (L3, L7, L8, S3, and S4) rRNAs are indicated.


Boer, P. H. and Gray, M. W. (1988b) Genes encoding a subunit of respiratory NADH dehydrogenase (ND1) and a reverse transcriptase-like protein (RTL) are linked to ribosomal RNA gene pieces in *Chlamydomonas reinhardtii* mitochondrial DNA. *EMBO J.* **7**, 3501-3508.


Gray, M. W., Lang, B. F., Cedergren, R., Golding, G. B., Lemieux, C., Sankoff,
D., Turmel, M., Brossard, N., Delage, E., Littlejohn, t. G., Plante, l., Rioux,
gene content in protist mitochondrial DNAs. *Nucleic Acids Res.* **26**, 865-
878.

Zimmermann, R. A. and Dahlberg, A. E. (eds.), *Ribosomal RNA: Structure,
Evolution, processing, and Function in Protein Biosynthesis*. CRC Press,
Boca Raton, FL., pp. 49-69.

rRNA. In Hill, W., Dahlberg, A., Garrett, R. A., Moore, P. B., Schlessinger,
Evolution*. American Society for Microbiology, Washington, D.C., pp. 589-
597.


Hayashi-Ishimaru, Y., Ohama, T., Kawatsu, Y., Nakamura, K. and Osawa, S.

Rearranged coding segments, separated by a transfer RNA gene, specify
the two parts of a discontinuous large subunit ribosomal RNA in

Helser, T. L., Davies, J. E. and Dahlberg, J. E. (1971) Chang in methylation of
16S ribosomal RNA associated with mutation to kasugamycin resistance

Ikemura, T. (1982) Correlation between the abundance of yeast transfer RNAs
and the occurrence of the respective codons in protein genes. Differences in synonymous codon choice patterns of yeast and Escherichia coli with reference to the abundance of isoaccepting transfer RNAs. J. Mol. Biol. 15, 573-579.


