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The deduced primary structure of subunit I from cytochrome *c* oxidase suggests that the genus *Polytomella* shares a common mitochondrial origin with *Chlamydomonas* [1]

Anaid Antaramian [a], Roberto Coria [b], Jorge Ramírez [b], Diego González-Halphen [a,*]

[a] *Departamento de Bioenergética, Instituto de Fisiología Celular, U.N.A.M., Apartado Postal 70-243, Mexico City 045100, Mexico*
[b] *Departamento de Microbiología, Instituto de Fisiología Celular, U.N.A.M., Mexico City, Mexico*

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Abstract

We cloned and sequenced the mitochondrial gene encoding subunit I of cytochrome *c* oxidase (*coxI*) of *Polytomella* spp., a colorless alga related to *Chlamydomonas*. The purpose was to explore whether homology between the two species also exists at the level of a mitochondrial enzyme. The gene is 1512 bp long and contains no introns. The translated protein sequence exhibits 73.8% identity with its *Chlamydomonas reinhardtii* counterpart. The data obtained support the hypothesis that the separation of the colorless alga from the *Chlamydomonas* lineage was a late event in evolution, that occurred after the endosymbiotic process that gave rise to mitochondria.

Keywords: Mitochondrion; Cytochrome *c* oxidase; (*Polytomella*); (*Chlamydomonas*); (Chlorophiceae)

The genus *Polytomella* [1,2] has been classified as a colorless quadriflagellate of the family Chlamydomonadaceae [3,4]. *Polytomella* most likely lost its cell wall and its photosynthetic apparatus [5], therefore it is colorless and naked. Nevertheless, it shares several functional and structural features with the genus *Chlamydomonas* [4,6]. *Polytomella* exhibits large and highly organized mitochondria [7] with a classical electron transport chain [8]. We have chosen this colorless alga to study the mitochondrial respiratory chain components, which have been difficult to characterize in *Chlamydomonas reinhardtii* because of contamination with thylakoid components [9,10]. A highly active bc_1 complex from *Polytomella* spp. has been purified and characterized [11]. The genus *Polytomella* is thought to have evolved from a *Chlamydomonas*-like precursor by losing the cell wall and functional chloroplasts [12]. Molecular evidence supporting the close relationship between *Polytomella agilis* and *C. reinhardtii* has been provided from the nuclear encoded beta-tubulin genes [13], and the 18 S rDNA sequences [14]; moreover, both species exhibit a similar codon usage bias, lacking the use of triplets whose third position is an A [13]. Since the *C. reinhardtii* mitochondrial DNA (mtDNA) has been fully sequenced [15–19] we thought it was of interest to characterize a mitochondrial gene from *Polytomella* spp. We chose the highly-conserved *coxI* gene, which encodes the large subunit of cytochrome *c* oxidase (EC 1.9.3.1) [20] present in all mtDNAs characterized to date. To our knowledge, this is the first report of a mitochondrial gene sequenced from this colorless alga.

Strains and plasmids. *Polytomella* spp. (198.80, E.G. Pringsheim, algae collection from the University of Göttingen, Germany) was stored and maintained under the register number CDBB-951 at the Microbiological Collection of the Department of Biotechnology, CINVESTAV del IPN, Mexico. The alga was grown in the medium described by Wise [3] supplemented with vitamins B_1 (0.06 mg/l) and B_{12} (0.08 mg/l) [21]. *Escherichia coli* DH5α was used to amplify recombinant plasmids. PTZ18R phagemid (Pharmacia) was used for subcloning and sequencing.

MtDNA isolation. Mitochondria from *Polytomella* spp.

Abbreviations: mtDNA, mitochondrial DNA; PCR, polymerase chain reaction.

* Corresponding author. Fax: +52 5 6225611; e-mail: dhalphen@ifcsun1.ifisiol.unam.mx.

[1] The nucleotide sequence data reported in this paper have been submitted to the EMBL/GenBank Data Libraries under the accession number U31972.

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prepared as described by Gutiérrez-Cirlos et al. [11], were treated for 15 min with 1 mg/ml DNAse II at room temperature, and centrifuged 15 min at 14 500 × *g*. Mitochondria were incubated for 12 h at 4°C in a buffer containing 10 mM Tris-HCl (pH 8.2), 2% Sarkosil, 10 mM EDTA and 100 mM NaCl [22]. This mixture was centrifuged at 14 500 × *g* and the supernatant was extracted twice with 2 M Tris-HCl (pH 8.0)-saturated phenol, twice with phenol-chloroform and once with chloroform.

Construction of mtDNA libraries. mtDNA from *Polytomella* spp. (10 μg) was digested separately with the restriction enzymes *Eco*RI, *Bam*HI, and *Hin*dIII, and ligated into the pTZ18R vector. Standard recombinant DNA techniques were carried out as described by Sambrook et al. [23]. Transformed *E. coli* DH5α cells were plated on ampicillin-LB, and recombinant plasmids were selected by loss of β-galactosidase activity.

PCR amplification and sequencing of coxI gene. mtDNA (1 μg) from *Polytomella* spp. was used as template for amplification by PCR in the presence of 2 mM $MgCl_2$ and 200 ng of each of the following degenerate deoxyoligonucleotides, synthesized on an Applied Biosystems model 381 A DNA synthesizer. The forward probe 5'-CAC GGT GGA TCC ATG CTA TTG TT(C + T) ATG GTA ATG CC -3' was designed based on the highly conserved sequence HGIIMLLFMVMP, corresponding to residues 60–71 in *C. reinhardtii* cytochrome *c* oxidase subunit I. The backward probe 5'- ATA ATC GGA TCC (A + G)CG (A + G)CG TGG CAT (A + G)CC AAC CAA ACC -3' was designed based on the highly conserved sequence towards the carboxy-terminus region of cytochrome *c* oxidase subunit I (GLAGMPRRMFDY), corresponding to residues 428–439 in *C. reinhardtii*. A *Bam*HI site was included in both deoxyoligonucleotides to facilitate further cloning. For PCR amplification, samples were denatured for 12 min at 94°C, and subjected to 50 cycles of 30 s denaturation at 94°C, 45 s annealing at 55°C and 2 min extension at 72°C. A final extension for 12 min at 72°C

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                                                    (-122) GCACCTTAAATTTTTTACCGAGTTCCTTTACCTGAATTT
AAGCCTTGTTACGACGAACCTGTGTTGGTTGCGGTACAGTTATTTTACAGCAATACCTATGGTCGATATTCTTACACTTTGCA

ATG CGT TGG TTG TAT TCG ACC AAT CAT AAA GAT ATT GGA ATG TTA TAC CTT ATA TTT GCC TTT
Met Arg Trp Leu Tyr Ser Thr Asn His Lys Asp Ile Gly Met Leu Tyr Leu Ile Phe Ala Phe

TTT GGC GGT CTA GTC GGA ACT GGT CTT AGT GTG TTA ATT CGT CTT CAA TTA GCC ACA ACT GGA
Phe Gly Gly Leu Val Gly Thr Gly Leu Ser Val Leu Ile Arg Leu Gln Leu Ala Thr Thr Gly

ACT GGA ATT TTA CAA AAC AAT GGA CAA TTA TTT AAT GTA ATT GTC ACT GGA CAT GGT GTT ATT
Thr Gly Ile Leu Gln Asn Asn Gly Gln Leu Phe Asn Val Ile Val Thr Gly His Gly Val Ile

ATG TTG CTT TTC ATG GTA ATG CCA GCA TTG TTT GGA GGA TTC GGA AAC TAT CTT CTT CCC CTA
Met Leu Leu Phe Met Val Met Pro Ala Leu Phe Gly Gly Phe Gly Asn Tyr Leu Leu Pro Leu

ATG ATA GGT GCT CCT GAT ATG GCT TTT CCT CGT CTA AAC AAT ATT AGC TTT TGG CTC AAC CCA
Met Ile Gly Ala Pro Asp Met Ala Phe Pro Arg Leu Asn Asn Ile Ser Phe Trp Leu Asn Pro

TTT GGA TTC CTC TTG CTT TTG GTT TCT ACT TTG GTA GAG CAG GGG GCT GGA ACT GGT TGG ACT
Phe Gly Phe Leu Leu Leu Leu Val Ser Thr Leu Val Glu Gln Gly Ala Gly Thr Gly Trp Thr

CTT TAT CCA CCT TTG AGC GTT CAA GGA AGT GGA AGC AGC ATT GAT CTA GCT ATT TTG AGT TTC
Leu Tyr Pro Pro Leu Ser Val Gln Gly Ser Gly Ser Ser Ile Asp Leu Ala Ile Leu Ser Phe

GAT TTG AAT GGA TTG AGC AGT ATT TTA GGT AGT ATT AAT GTA TTG GTT ACT GCT AAA GGT CTG
Asp Leu Asn Gly Leu Ser Ser Ile Leu Gly Ser Ile Asn Val Leu Val Thr Ala Lys Gly Leu

CGT GCC CCA GGT ATG TCT CTT ATC CAG ATT CCT CTC TTT GTT TAT TCT ATG GTA TTT ACA GCT
Arg Ala Pro Gly Met Ser Leu Ile Gln Ile Pro Leu Phe Val Tyr Ser Met Val Phe Thr Ala

ATT CTT GTA ATT CTC TCT GTA CCA GTT TTG GCA GCT GCT CTA ATT ATG CTT TTG ACA GAT CGT
Ile Leu Val Ile Leu Ser Val Pro Val Leu Ala Ala Ala Leu Ile Met Leu Leu Thr Asp Arg

TCC CTT AAC ACA GCC TAC TTT GTT GAT TCC GGA GAT TTG TTA TTG TAT CAG CAC TTA TTC TGG
Ser Leu Asn Thr Ala Tyr Phe Val Asp Ser Gly Asp Leu Leu Leu Tyr Asn His Leu Phe Trp

TTT TTC GGT CAT CCA GAG GTC TAT ATT TTA ATT CTT CCA GCT TTT GGT GTT ATT AGC AGC ATT
Phe Phe Gly His Pro Glu Val Tyr Ile Leu Ile Leu Pro Ala Phe Gly Leu Ile Ser Ser Ile

ATT AGT TTT TTC AGT AAC AAA CCA GTC TTT GGC GTC ACA GGA ATG ATT TGC GCA ATG GGT GCT
Ile Ser Phe Phe Ser Asn Lys Pro Val Phe Gly Val Thr Gly Met Ile Cys Ala Met Gly Ala

ATC GGT TTG GTC GGT TTC TTA GTG TGG GCT CAC CAT ATG TAC GTT GTC GGT ATG GAC TTG GAC
Ile Gly Leu Val Gly Phe Leu Val Trp Ala His His Met Tyr Val Val Gly Met Asp Leu Asp

ACT GTT GCT TAT TTT ACT AGT GCT TCT ATG ATT ATT GCT ATT CCA ACT GGT ATG AAA GTA TTC
Thr Val Ala Tyr Phe Thr Ser Ala Ser Met Ile Ile Ala Ile Pro Thr Gly Met Lys Val Phe

AGC TGG ATG GCT ACT GCT TAT GCT GGT AAA GTG TAC TTT AGT GTT CCT ATG CTC TAT GCT TTT
Ser Trp Met Ala Thr Ala Tyr Ala Gly Lys Val Tyr Phe Ser Val Pro Met Leu Tyr Ala Phe

GGA TTT TTG GCT TTG TTC ACA ATT GGT GGT GTT ACC GGG GTA GTT TTA GCC AAC GCT GGT GTA
Gly Phe Leu Ala Leu Phe Thr Ile Gly Gly Val Thr Gly Val Val Leu Ala Asn Ala Gly Val

GAT ACC GCT GTT CAT GAC ACT TAT TAC GTT GTC GCT CAC TTT CAT TAC GTG TTA AGC ACT GGT
Asp Thr Ala Val His Asp Thr Tyr Tyr Val Val Ala His Phe His Tyr Val Leu Ser Thr Gly

GCT GTG TTT GCT ATC TTT GCT GGT ATG TAC TTC TAC AGC AAT CTT ATG TTT AAC TTG GGT TAC
Ala Val Phe Ala Ile Phe Ala Gly Met Tyr Phe Tyr Ser Asn Leu Met Phe Asn Leu Gly Tyr

GAT GAA AAC AAA GGT ACT GTT CAA TTC CTG TTG TTC TTC TTA GGT GTT AAC TTG ACT TTC TTC
Asp Glu Asn Lys Gly Thr Val Gln Phe Leu Leu Phe Phe Leu Gly Val Asn Leu Thr Phe Phe

CCT CAG CAT TTC TTG GGT TTG GCT GGT ATG CCT CGT CGT ATG TTC GAT TAC CGT GAC GCC TTC
Pro Gln His Phe Leu Gly Leu Ala Gly Met Pro Arg Arg Met Phe Asp Tyr Arg Asp Ala Phe

ACT GGT TTA AAT TTA CTG TCT TCT TAT GGT GCT TTG GTT AGT TTT ACT AGT CTT TTG TAT GCA
Thr Gly Leu Asn Leu Leu Ser Ser Tyr Gly Ala Leu Val Ser Phe Thr Ser Leu Leu Tyr Ala

GGA ACA GTC TTT ACC CCT GCT CCA GCA CTT TAC CAG AAC CGC ACC AGT ACT AGT CTT GAG TGG
Gly Thr Val Phe Thr Pro Ala Pro Ala Leu Tyr Gln Asn Arg Thr Ser Thr Ser Leu Glu Trp

TTG TTG CCA GCT ACT CCA GCC TTC CAT ACT TTC TCT GAA GTT CCA GTA CTT CGC GTA GCT TAA
Leu Leu Pro Ala Thr Pro Ala Phe His Thr Phe Ser Glu Val Pro Val Leu Arg Val Ala  *

TCATGTCGCTTTATCTTTTTATGTTATTTTTGCAGCCTCTTTCTTTATCTTTTGTTTCACGCTCTTTTTTCTAAGATTTCCTCT
CTTATGATGTCTTTTTGCTACTCTTTGGTCGG (1626)
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Fig. 1. Nucleotide sequence of cytochrome *c* oxidase subunit I from *Polytomella* spp. Start and stop codons of the *coxI* gene are underlined.

was carried out. The same conditions of PCR were used to amplify the 1.1 kb product, using as template the 14 different plasmids from the mtDNA-*Hin*dIII library. Clone H1 from the *Hin*dIII library was completely sequenced (3.1kb), using the kit Seq Ver 2 from **USB**, based on the method of Sanger et al. [24].

Southern analysis. The 1.1 kb PCR product was transferred to a nylon membrane and hybridized with the biotinylated probe of *coxI* gene from *C. reinhardtii* (a fragment from stock P-85 from *C. reinhardtii* CC-125) kindly provided by Dr. Elizabeth H. Harris (*Chlamydomonas* Genetics Center, Department of Botany, Duke University). The hybridization was carried out at 55°C, and the nylon membrane was washed twice with 5 × SSC containing 0.5% SDS, and once with 1 × SSC containing 1% SDS at the same temperature, and exposed overnight with a XR-OMAT-AR Kodak film.

Sequence analysis. Sequences were analyzed using the GCG Sequence Analysis Software Package (Genetics Computer Group, Madison, WI). Alignments were carried out with the Pileup program [25] using sequences in the Swissprot data bank. Cladograms were constructed with the program Evolutionary Analysis (GCG package), and distances were corrected according to Kimura [26]. The terms similarity and homology are used as suggested by Reeck et al. [27].

Characterization of the coxI gene from Polytomella spp. Two degenerate deoxyoligonucleotides were designed based on highly conserved regions of the *coxI* gene. With these probes, a PCR-amplification product of 1.1 kb was obtained using mtDNA from *Polytomella* spp. as a template. Three mtDNA libraries were constructed and screened by PCR-mediated amplification, and a clone (1H) that gave rise to a product of 1.1 kb was identified in the *Hin*dIII library. The two 1.1 kb products gave positive hybridization with the *cox*I gene biotinylated probe from *C. reinhardtii* (data supplied for review but not shown). Clone 1H from *Polytomella* spp. was completely sequenced; it contained three ORFs. The middle ORF was identified as the full length *coxI* gene. The partial sequences of the genes flanking the *coxI* gene allowed the preliminary identification of a gene homologous to L4 (fragment 4 of the large subunit of rRNA), and in the same orientation than the *coxI* gene, a fragment homologous to *nad4* (a gene encoding for subunit 4 of NADH-ubiquinone reductase) of the linear mtDNA from *C. reinhardtii*. The presence of a gene homologous to L4 suggests that the ribosomal RNA genes may also be fragmented and scrambled in the mtDNA of *Polytomella* spp., sharing this peculiar pattern with other Chlamydomonadales [28].

The DNA sequence of the *coxI* gene from *Polytomella* spp. is illustrated in Fig. 1, it shows a continuous open reading frame (ORF) of 1512 base pairs in its full length. As in *C. reinhardtii*, the gene is not interrupted by intervening sequences, in contrast to homologous genes from *Podospora anserina* [29] and fungi [30–32]. Nucleotide

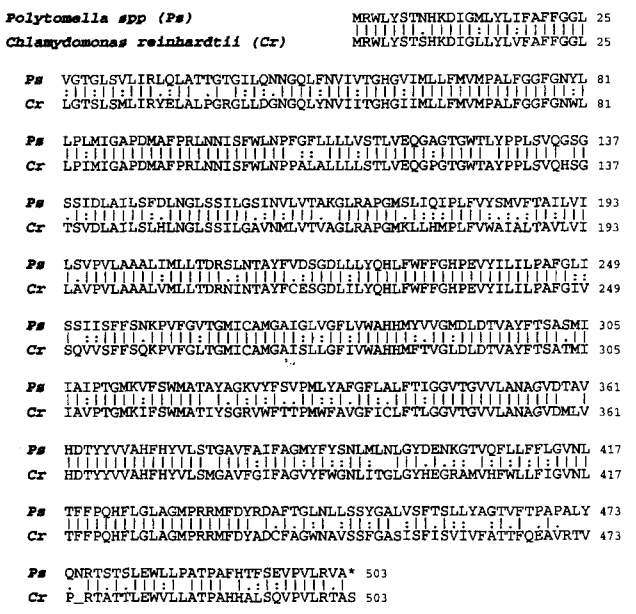

Fig. 2. Sequence alignment of the predicted cytochrome *c* oxidase subunit I gene from *Polytomella* spp. (Ps) with that from *C. reinhardtii* (Cr). Numbering refers to the *Polytomella* spp. sequence. Amino acid identities are indicated by (|); conserved amino acids by colons (:) and semi-conserved residues by periods (.).

sequence comparison of *coxI* genes from *Polytomella* spp. and *C. reinhardtii* revealed 68.7% identity within the open reading frame.

Translation of the DNA sequence predicts a protein of 503 residues with a molecular mass of 54 781 Da. The alignment of the amino acid sequences of cytochrome *c* oxidase subunit I from *Polytomella* and *C. reinhardtii* [33] (Fig. 2) revealed an identity of 73.8% and a similarity of 87.9%. The similarity between the two subunits I of the cytochrome *c* oxidase is extremely high, and extends over the complete sequence. The predicted apoprotein of subunit I of cytochrome *c* oxidase from *Polytomella* spp. contains the 6 invariant histidine residues (H60, H235, H284, H285, H370, H372) that are known to be the ligands for heme *a*, and the Cu_B-heme a_3 binuclear center [34]. Its hydropathy profile is similar to those of cytochrome *c* oxidase subunit I from other organisms, and fits the current model of 12 transmembrane helices [35,36]. Fig. 3 shows a cladogram generated from cytochrome oxidase subunit I sequences. The result obtained showed that *Polytomella* spp. *coxI* sequence clearly affiliates with its *C. reinhardtii* homolog.

A comparison of the pattern of codon utilization for cytochrome *c* oxidase subunit I of *Polytomella* spp. with that of *C. reinhardtii*, is shown in Fig. 4. As in *Chlamydomonas*, a significant bias is found in each codon family; nevertheless 7 codons that are not used in *C. reinhardtii*, are used in *Polytomella* spp. *coxI* gene: UUA (L), CUC (L), GUG (V), ACA (T), GCA (A), AAU (N) and GGA (G). As in *C. reinhardtii*, among the 'absent' codons is

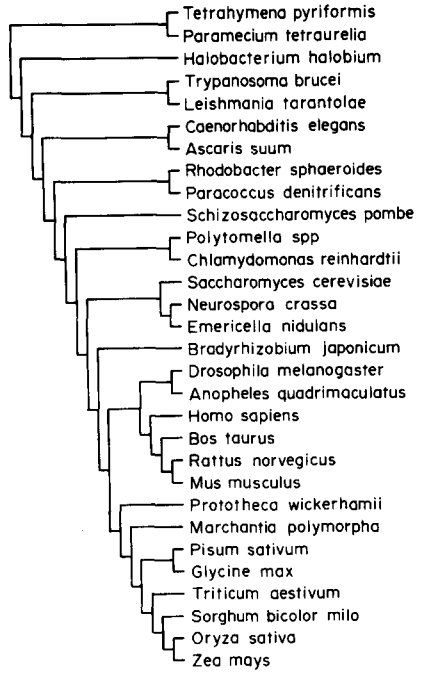

Fig. 3. Phylogenetic analysis of cytochrome *c* oxidase subunit I sequence. To construct the cladogram, the amino acid sequences of cytochrome *c* oxidase subunit I were compared among different organisms (SWISSPROT data bank), including the one of *Polytomella* spp. obtained in this study.

CGG, which in plant mitochondria codes for W rather than R [37,38]. Interestingly, in the 15.8 kb mtDNA of *C. reinhardtii* only 3 tRNA genes (for W, Q and M) have been detected; therefore, at least 17 to 20 tRNAs have to be imported from the cytoplasm to support intra-mitochondrial protein synthesis [19]. The presence of a more balanced mitochondrial codon usage in *Polytomella* spp., in contrast with the extreme codon bias of *C. reinhardtii*, leads us to hypothesize that a more larger genome, encoding more than three tRNA genes, will be found in *Polytomella* spp.

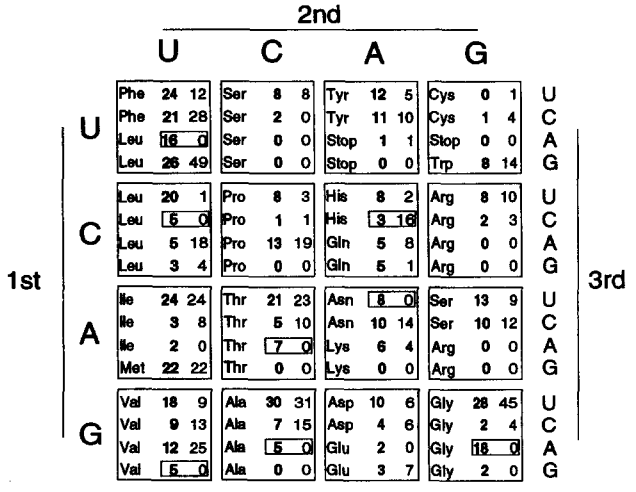

Fig. 4. Codon usage of the mtDNA sequences of *coxI* from *Polytomella* spp. (this study) and from *C. reinhardtii* [33]. Boxes indicate conspicuous differences in the codon usage.

This work describes the cloning and sequencing of a new gene of the *coxI* family, and to our knowledge, the first mitochondrial gene sequenced from the colorless alga *Polytomella* spp. The data obtained supports the hypothesis of Round [12], which states that the separation of the colorless alga such as *Polytomella* from the *Chlamydomonas* lineage was a late event in evolution, that took place long after the endosymbiotic process that gave rise to mitochondria. The high similarity of the mitochondrial *coxI* sequences, the clustering of *Polytomella* and *Chlamydomonas* in the constructed phylogenetic tree, the presence of a similar mitochondrial codon usage, and the suggested presence of fragmented and scrambled ribosomal RNA genes in *Polytomella*, strongly support the idea of a common mitochondrial ancestor for both species. We conclude that the genus *Polytomella* is a unique model to use in the study of the mitochondrial respiratory complexes in the Chlamydomonadales, and to understand the process and evolutionary significance of secondary loss of organelles among protists.

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