J Mol Evol (1997) 44:341–347

JOURNAL OF MOLECULAR EVOLUTION

Phylogenetic Affinity of Mitochondria of *Euglena gracilis* and Kinetoplastids Using Cytochrome Oxidase I and hsp60

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Received: 5 March 1996 / Accepted: 31 July 1996

Abstract. The mitochondrial DNA-encoded cytochrome oxidase subunit I (COI) gene and the nuclear DNA-encoded hsp60 gene from the euglenoid protozoan *Euglena gracilis* were cloned and sequenced. The COI sequence represents the first example of a mitochondrial genome-encoded gene from this organism. This gene contains seven TGG tryptophan codons and no TGA tryptophan codons, suggesting the use of the universal genetic code. This differs from the situation in the mitochondrion of the related kinetoplastid protozoa, in which TGA codes for tryptophan. In addition, a complete absence of CGN triplets may imply the lack of the corresponding tRNA species. COI cDNAs from *E. gracilis* possess short 5′ and 3′ untranslated transcribed sequences and lack a 3′ poly[A] tail.

The COI gene does not require uridine insertion/deletion RNA editing, as occurs in kinetoplastid mitochondria, to be functional, and no short guide RNA-like molecules could be visualized by labeling total mitochondrial RNA with [α-^{32}P]GTP and guanylyl transferase. In spite of the differences in codon usage and the 3′ end structures of mRNAs, phylogenetic analysis using the COI and hsp60 protein sequences suggests a monophyletic relationship between the mitochondrial genomes of *E. gracilis* and of the kinetoplastids, which is consistent with the phylogenetic relationship of these groups previously obtained using nuclear ribosomal RNA sequences.

Key words: Mitochondrial gene — Codon usage — Mitochondrial phylogeny — *Euglena gracilis* — RNA editing

Introduction

Euglena gracilis is a flagellated protozoan that, according to both morphological (Walne and Kivic 1989; Triemer and Farmer 1991) and molecular (Sogin et al. 1989; Baldauf and Palmer 1993; Nordness et al. 1994; Hashimoto et al. 1995; Henze et al. 1995) data, is monophyletically related to the kinetoplastids. These so-called euglenozoa represent one of the earliest-diverging extant eukaryotic lineages that contain mitochondria (Sogin et al. 1989). *E. gracilis* uniquely contains a chloroplast organelle in addition to a mitochondrion. Although the complete sequence of the chloroplast genome of *E. gracilis* has been determined (Manning et al. 1971; Hallick et al. 1993), little is known about the mitochondrial genome, and what is known appears contradictory on the surface. For example, early electron microscopic evidence and analysis of DNA isolated from mitochondrial fractions both suggested that the mitochondrial DNA is composed of short heterogeneous-sized linear molecules (Nass et al. 1974; Ray and Hanawalt 1965). These results, however, did not agree with the genomic complex-

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GenBank Accession numbers: COI; U49052, Hsp60; U49053

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ity of 60–70 kb derived from Cot analysis and quantitative hybridization experiments (Crouse et al. 1975; Talen et al. 1974). A detailed knowledge of the size, sequence, and genomic organization of the mitochondrial genome of *E. gracilis* is important for understanding the phylogenetic relationships of mitochondria in these lower eukaryotes and the mitochondria of higher eukaryotes such as fungi, animals, and plants. In order to approach these questions we have initiated an investigation of the mitochondrial genome of *E. gracilis.*

In this paper we report the sequences of the mitochondrial-encoded cytochrome oxidase subunit I gene (COI) and the nuclear-encoded hsp60 gene from *E. gracilis*, and we have used these amino acid sequences to investigate the phylogenetic relationship of the *E. gracilis* mitochondrion to other mitochondria.

Materials and Methods

Strain and Medium. Bleached *Euglena gracilis* strain W10BSmL cells were provided by Dr. J.A. Schiff (Brandeis University). The cells were grown at 27°C in Hunter's acidic autotrophic medium (Greenblatt and Schiff 1959).

Preparation of Mitochondrial DNA and RNA. Mitochondria were isolated by the method of Chaudhary and Merret (1984). Late-log or stationary-phase cells were treated with trypsin to create spheroplasts, which were disrupted by hypotonic treatment. After differential centrifugation, the crude mitochondrial fraction was further purified by Percoll gradient isopycnic centrifugation. Isolated mitochondria were resuspended in 2 ml of 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM Na_2EDTA, and lysed by the addition of 200 ml of 10% SDS. The lysate was extracted twice with phenol-chloroform (1:1, vol:vol) and precipitated with ethanol. QUIAGEN columns were used to remove coprecipitated polysaccharide, and the extract was digested with RNase A or DNase I, to obtain, respectively, DNA or RNA. In some experiments, Hoechst-CsCl ultracentrifugation was also used for purification of mitochondrial DNA from total cellular DNA (Simpson 1979).

Estimation of Genomic Complexity by Quantitative Hybridization. The same amount of purified total mitochondrial DNA and a randomly cloned fragment were serially diluted and spotted on Hybond N+ membrane (Amersham). The membrane was probed with the same cloned fragment. The genomic complexity was calculated from the relative intensity of the signal based on the assumption that the cloned fragment is unique in the mitochondrial genome.

In vitro Capping of RNAs. Purified mitochondrial RNA from *E. gracilis* (4 μg), from *Leishmania tarentolae* (2.5 μg), or the mixture of them was incubated at 65°C for 10 min and chilled on ice. The incubation mixture containing 20 μCi of [α−^{32}P]GTP and 4 U of guanylyl transferase (Gibco/BRL) in 60 mM Tris-HCl (pH8.0), 6 mM $MgCl_2$, and 10 mM dithiothreitol was incubated at 37°C for 60 min. The products were analyzed on an 10% polyacrylamide 8 M urea gel.

Heterologous Southern Hybridization With L. tarentolae *Gene Probes.* About 5 μg of purified mitochondrial DNA was digested with an appropriate restriction enzyme. Gel electrophoresis and blotting were performed using standard procedures (Sambrook et al. 1989). The blots were hybridized at 60 C with a ^{32}P-labeled PCR-amplified *L. tarentolae* COI gene fragment (nt 11,677–12,836 of GenBank entry LEIKPMAX), a PCR-amplified CYb gene fragment (nt 5430–6476), or a PCR-amplified 12S rRNA gene fragment (nt 438–1600) and washed twice with 2 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate) 0.1% SDS at 55°C for 15 min each.

Cloning of COI-encoding genomic fragment. Mitochondrial DNA was digested with *Sau*3AI or *Taq* I and cloned into pBluescript KS− (Stratagene). Several hundred clones were screened with the same PCR-amplified *L. tarentolae* COI gene fragment as used in the Southern hybridization, and positive clones were selected and sequenced.

Cloning of hsp60 cDNA. The internal region of the hsp60 gene was amplified by RT-PCR with S2110 (5′-CCGGATCCGTNAARGC-NCCNGGNTTYGGNGA-3′) and S2112 (5′-CCGAATTCATNCC-NCCCATNCCNCCCAT-3′), cloned, and sequenced. The 5′ and 3′ ends of the mRNA were cloned using the RACE procedure (see below).

5′ and 3′ RACE. 5′ RACE was performed as previously described (Edwards et al. 1991). Briefly, cDNA was synthesized with S1216 (5′-CTATACCAACAGGCATAATA-3′) for COI mRNA from mitochondrial RNA or S2121 (5′-TCCGCCAGTGAAGATAGCAA-3′) for hsp60 mRNA from cytoplasmic poly[A] RNA, and ligated to an ''anchor'' oligonucleotide S1859 (5′-p-CACGAATTCACTATCGATTC-TGGAACCTTCAGAGG-NH_2-3′). The anchor-ligated cDNA was PCR amplified with a nested mRNA specific oligonucleotide S1714 (5′-CGCGGATCCATGTATAGTAATCCAA-3′) for COI mRNA or S2120 (5′-CCCGGATCCTGCAGCATGTTGGTCTTGTT-3′) for hsp60 mRNA and an antisense-anchor oligonucleotide, S1408 (5′-GTTCCAGAATCGATAGTGAATTCGT-3′). For 3′ RACE of COI, a poly[C] tail was added to the 3′ end of total mitochondrial RNA with poly[A] polymerase (Gibco/BRL), and cDNA synthesis was primed with S1680 (5′-AAGGATCCGGGGGGGGGGGGGG-3′) by using SuperScript II reverse transcriptase (Gibco/BRL). The 3′ end of the COI cDNA was PCR amplified with S1680 and an internal-specific primer S1217 (5′-AGTTCCTGAATATGCTGATA-3′). 3′ RACE for hsp60 was performed with S1569 (5′-CCGGATCCTTTTTTTTTTT-TTTTTTTTT-3′) and two nested internal primers, S2134 (5′-CAGCAGGGCAAATATGTCAA-3′) and S2119 (5′-CCCGAATTC-GGATCATCGACCCTGCCAAA-3′).

Phylogenetic Analysis. Amino acid sequences of COI or the bacterial homologues CoxA and CtaD from *Drosophila melanogaster* (GenBank accession number U37541), *Homo sapiens* (V00662), *Caenorhabditis elegans* (X54252, S93745), *Schizosaccharomyces pombe* (X54421, X00886, X02819, X15738), *Saccharomyces cerevisiae* (M97514), *Trypanosoma brucei* (M14820, M15560), *E. gracilis* (this work; U49052), *Zea mays* (X02660), *Chlamydomonas reinhardtii* (X54860, K02967, M22649, M25124–M25130, X04381, X13456, X14606), *Paracoccus denitrificans* (X05829 for CtaDI, Y07533 for CtaDII), *Rhodobacter sphaeroides* (X62645), *Rhizobium leguminosarum* (X74341), and *Bradyrhizobium japonicum* (X54800) were aligned by the CLUSTALW program with manual refinement, and 490 internal sites with an unambiguous alignment were used for the analysis. For the organisms in which COI mRNA is subjected to RNA editing, the amino acid sequences inferred from the fully edited mRNA sequences were used. Amino acid sequences of hsp60 or the bacterial homologue groEL from *Caenorhabditis elegans* (L36035), *Homo sapiens* (M34664), *Saccharomyces cerevisiae* (M33301), *Schizosaccharomyces pombe* (D50609), *Zea mays* (Z12114), *Trypanosoma brucei* (L43797), *E. gracilis* (this work; U49053), *Rickettsia tsutsugamushi* (M31887), *Rhizobium meliloti* (M94192), *Rhodobacter sphaeroides* (U37639), *Escherichia coli* (X07850), *Pseudomonas aeruginosa* (S77424), *Helicobacter pylori* (X73840), *Bacilus subtilis* (M81132), and *Chlamydia trachomatis* (M31739) were also aligned, and 500 internal sites were used for the analysis. Maximum-likelihood analysis was performed by using the PROTML program (ver. 2.2) contained in the MOLYPHY-

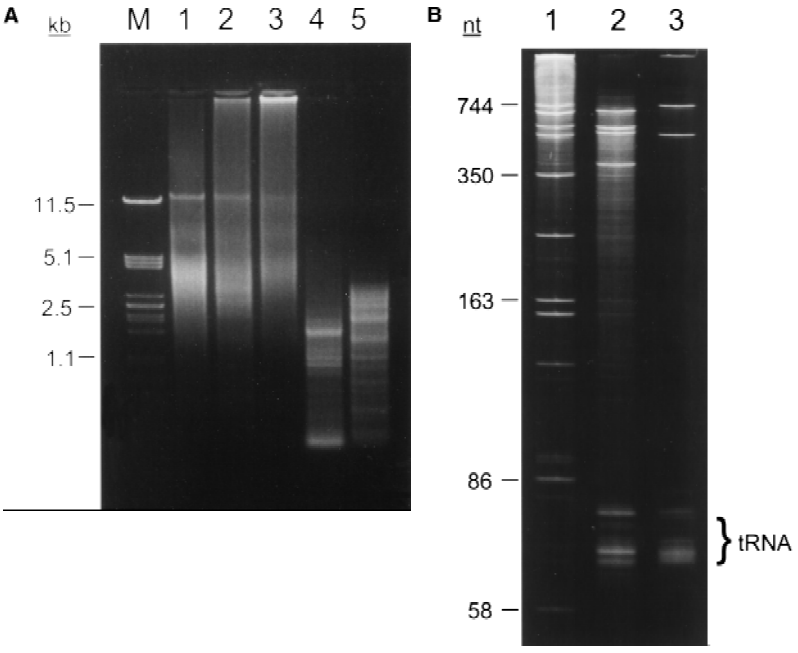

Fig. 1. **A** Ethidium-bromide-stained agarose gel of mitochondrial DNA from *E. gracilis* W10BSmL strain digested with several restriction enzymes. *M,* lambda DNA digested with *Pst* I. Lane *1,* undigested *E. gracilis* mitochondrial DNA; lane *2, Eco* RI digestion; lane *3, Bam* HI digestion; lane *4, Sau*3A I digestion; lane *5, Taq* I digestion. **B** 6% polyacrylamide 8 M urea gel electrophoresis of mitochondrial RNA. Lane *1, E. gracilis* total cell RNA; lane *2, E. gracilis* mitochondrial RNA; lane *3, L. tarentolae* mitochondrial RNA.

2.2 package (Adachi and Hasegawa 1992) and a semiconstrained tree with the JTT amino acid substitution matrix (Jones et al., 1992). Bootstrap values were estimated by the RELL method, which is implemented in PROTML (Kishino et al. 1990).

Results

Mitochondrial DNA from E. gracilis *Has a Genomic Complexity of Approximately 60 kb but Is Isolated as Short Linear Fragments*

DNA isolated from purified mitochondrial fractions of *E. gracilis* by several methods proved to consist mainly of short heterogeneous-sized linear fragments migrating at approximately 5 kb in agarose (Fig. 1A).

However, quantitative hybridization with a mitochondrial DNA-specific probe confirmed the previously reported (Talen et al. 1974) complexity of the mitochondrial genome of approximately 60–70 kb (data not shown).

CHEF gel analysis of total cell DNA obtained from cells embedded in agarose blocks was also performed to determine the in situ size and conformation of the mitochondrial DNA (data not shown). The gel was blotted and hybridized with a cloned COI gene fragment (see below). The mitochondrial DNA remained at the origin even after the introduction of strand breakage by γ-ray irradiation of the agarose blocks prior to electrophoresis. As a control for the extent of strand breakage by the γ-irradiation, the circular chloroplast DNA was found to migrate through the CHEF gel as 140-kb linear fragments (data not shown). This may be related to the observed fragmentation of isolated mitochondrial DNA, but this was not investigated further. Apparently undegraded mitochondrial RNA with a slight contamination of cytoplasmic RNA could be recovered from the purified mitochondrial fraction (Fig. 1B). Four distinct high-molecular-weight putative rRNA bands and several bands in the tRNA region were observed.

Apparent Absence of Cappable RNA Species in Mitochondria

In mitochondria of kinetoplastids short uridylylated RNA molecules called guide RNAs mediate the uridine insertion/deletion type of RNA editing (Simpson et al. 1993). It is known that these guide RNA molecules can be capped in vitro with guanylyl transferase and GTP (Blum and Simpson 1990). To search for similar RNA species in *E. gracilis* mitochondria, we performed in vitro capping with purified mitochondrial RNA from *E. gracilis.* We were not able to detect any capping of RNAs (data not shown). When the purified mitochondrial RNA preparation was added to a capping reaction of the purified RNA from the kinetoplastid *L. tarentolae,* it did not show any inhibitory effect, suggesting an absence of contaminants in the RNA preparation from *E. gracilis* that might affect the reaction (data not shown).

Codon Usage of COI Gene

Cytochrome oxidase I (COI), cytochrome *b* (Cyb), and 12S ribosomal RNA gene probes prepared from *L. tarentolae* mitochondrial maxicircle DNA were used in Southern hybridizations to search for homologous genes in *Sau*3A I–digested mitochondrial DNA from *E. gracilis.* The only positive was a hybridization of the *L. tarentolae*

Table 1. Codon Usage of *Euglena gracilis* COI

UUU	Phe	34	UCU	Ser	28	UAU	Tyr	27	UGU	Cys	3
UUC	Phe	8	UCC	Ser	0	UAC	Tyr	0	UGC	Cys	0
UUA	Leu	55	UCA	Ser	3	UAA	Stop	0	UGA	(Stop)	0
UUG	Leu	0	UCG	Ser	0	UAG	Stop	0	UGG	Trp	7
CUU	Leu	2	CCU	Pro	10	CAU	His	19	CGU	Arg	0
CUC	Leu	0	CCC	Pro	0	CAC	His	0	CGC	Arg	0
CUA	Leu	0	CCA	Pro	5	CAA	Gln	3	CGA	Arg	0
CUG	Leu	0	CCG	Pro	0	CAG	Gln	0	CGG	Arg	0
AUU	Ile	29	ACU	Thr	18	AAU	Asn	24	AGU	Ser	15
AUC	Ile	0	ACC	Thr	1	AAC	Asn	0	AGC	Ser	0
AUA	Ile	38	ACA	Thr	12	AAA	Lys	7	AGA	Arg	13
AUG	Met	22	ACG	Thr	0	AAG	Lys	1	AGG	Arg	0
GUU	Val	20	GCU	Ala	14	GAU	Asp	11	GGU	Gly	31
GUC	Val	0	GCC	Ala	0	GAC	Asp	1	GGC	Gly	0
GUA	Val	15	GCA	Ala	0	GAA	Glu	7	GGA	Gly	12
GUG	Val	0	GCG	Ala	0	GAG	Glu	0	GGG	Gly	0

COI probe to a 300-bp *Sau*3AI fragment at a low stringency. This fragment was cloned and sequenced.

The sequence contained an open reading frame, the predicted amino acid sequence of which showed a similarity with the COI genes of other organisms. A 2.8-kb *Taq* I fragment which hybridized to the cloned COI fragment was subsequently obtained. This fragment contained a single open reading frame of 1,485 bp, which corresponded to the entire COI gene.

The frequency of each triplet codon in this gene is shown in Table 1. The overall codon usage shows a bias for AU at the third codon position, reflecting the 76% AT content of the gene. For the arginine and leucine codons, in which the first position is degenerate, A (arginine) or U (leucine) is preferred to C at the first position. In the case of the arginine codons, only AGA triplets are used, and no CGN triplet occurs within the COI reading frame. Since there is only a single species of tRNA that reads the CGN codon in the mitochondria of other organisms (Heckman et al. 1980), the lack of the CGN triplet in the COI gene might imply the absence of a corresponding tRNA in the mitochondria of *E. gracilis.*

A remarkable feature of the codon usage of the COI gene in *E. gracilis* is that all seven deduced tryptophan residues are specified by TGG codons rather than TGA, which is frequently used to specify tryptophan in the mitochondrial genomes of animals, fungi, or protists, including the related kinetoplastid protozoa (Maslov and Simpson 1994; de la Cruz et al. 1984). Although we have only analyzed a single mitochondrial gene, it is likely that *E. gracilis* mitochondria use the universal codon for tryptophan.

We were not able to find any other open reading frames in the 2.8-kb *Taq* I fragment other than COI gene. Although it does not exclude the possibility of the existence of heavily edited genes in those regions, it is more likely that genes are loosely packed in their mitochondrial genome, consistent with the relatively large genome size.

The COI mRNA Lacks a 3′ Poly[A] Tail and Has Short 5′ and 3′ Untranslated Sequences

The 5′ and 3′ ends of the COI mRNA transcripts were cloned by the RACE procedure and sequenced. The most frequent 5′ end (six out of eight clones) was a uridine nucleotide located nine nucleotides upstream from the first encoded methionine (Fig. 2A).

The initial attempts to amplify the 3′ end of the COI mRNA by oligo[dT]-primed cDNA synthesis were not successful. This was attributed to an absence of a poly[A] tail at the 3′ end of the mRNA, which was also suggested by an inability to purify poly[A] RNA from total *E. gracilis* mitochondrial RNA by standard procedures. To investigate this possibility, a poly[C] tail was added to the 3′ end of mitochondrial RNAs with poly[A] polymerase, and RT-PCR was performed, using oligo[dG] as a 3′ primer and an internal sequence as a 5′ primer. All seven clones analyzed contained 28 nucleotide genomically encoded 3′ untranslated sequences (3′ UTR) and identical 3′ ends (Fig. 2B). These sequences also confirmed the absence of a poly[A] tail between 3′ UTR and the added poly[C] tail.

Mitochondrial Phylogeny Inferred From Mitochondrial DNA-Derived COI and Nuclear DNA-Derived hsp60 Sequences

Hsp60 is a nuclear genome-encoded mitochondrial protein which has been successfully used to investigate mitochondrial phylogeny in other organisms (Clark et al. 1995; Viale and Arakaki 1994). A full-length cDNA of the hsp60 gene from *E. gracilis* was obtained by PCR using degenerate oligonucleotides. The 5′ and 3′ ends of the hsp60 mRNA were obtained by 5′ and 3′ RACE procedures. The predicted 569-amino-acid sequence showed a high similarity with known hsp60 and groEL sequences (data not shown).

The mitochondrial DNA-encoded COI sequence and

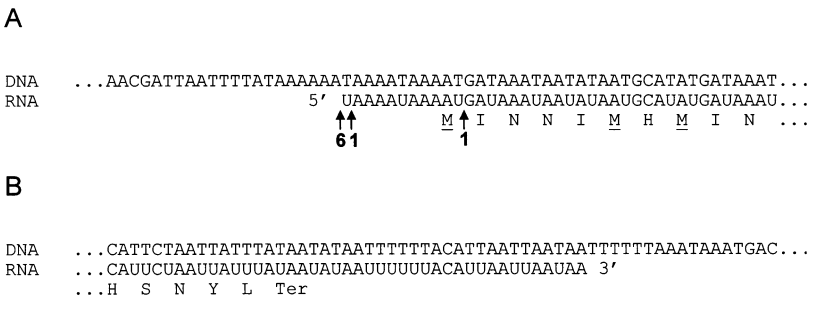

Fig. 2. **A** 5′ end of COI mRNA (RNA). The genomic sequence (DNA) and the deduced amino acid sequence are also shown. Methionines which are potential translation initiation codons are *underlined. Arrows and numbers* indicate the positions of the 5′ end and the number of RACE clones. **B** 3′ end of COI mRNA (RNA). The genomic sequence is also shown (DNA). All clones analyzed had the same 3′ end (see text).

the nuclear DNA-encoded hsp60 sequence were used to investigate the phylogenetic relationships of the euglenoid mitochondrion. As shown in Fig. 3, both the COI and hsp60 data support the affinity of *E. gracilis* mitochondria to kinetoplastid *Trypanosoma brucei* with a high bootstrap value, in agreement with the nuclear rRNA phylogeny (Sogin et al. 1989). These results provide evidence for monophyly of mitochondria from euglenoids and kinetoplastids.

Discussion

We report here the cloning and sequencing of the COI gene from *E. gracilis,* which represents the first sequence information from the mitochondrial genome of this organism. Interestingly, the COI gene possesses features that are distinct from mitochondrial genes of kinetoplastids, including an exclusive use of the TGG triplet to encode tryptophan residues and the lack of a poly[A] tail at the 3′ end of the mRNA. The universal code is used in mitochondria of several organisms, including all land plants investigated, *Dictyostelium discoideum* (Cole and Williams 1994; Angata et al. 1995), *Schizosaccharomyces pombe,* and *Physarum polycephalum* (Gott et al. 1993). It is known that mitochondrial mRNAs of plants also lack a poly[A] tail (Gray 1992), but the occurrence of this feature has not been extensively investigated in other organisms. These data are possibly suggestive of a close evolutionary relationship between euglenoid mitochondria and mitochondria of one of these organisms.

Although cytoplasmic rRNA phylogeny suggests a close relationship of euglenoid protozoa and kinetoplastids (Sogin et al. 1989), there is good evidence that the chloroplast of euglenoids was obtained in evolution by a secondary symbiosis of eukaryotic alga (Morden et al. 1992; Martin et al. 1992; Gibbs 1978). This raises the possibility that the mitochondrial genome of the euglenoids or some portion thereof was obtained by lateral transfer from the eukaryotic endosymbiont. However, our phylogenetic analysis with the mitochondrial-encoded COI and the nuclear-encoded hsp60 sequences clearly shows an affinity of mitochondria from euglenoids with those from kinetoplastids, which is consistent with phylogenetic reconstructions based on morphological and rRNA data (Sogin et al. 1989; Walne and Kivic 1989; Triemer and Farmer 1991).

These results suggest that the mitochondrial genome coexisted with the nuclear genome since the establishment of primary symbiosis in the euglenoids. Therefore, those mitochondrial gene characters shared by euglenoids and other organisms are either due to independent origins or represent primitive mitochondrial characters that remained unchanged in several lineages. Further investigation will be needed to clarify these possibilities.

The failure to isolate high-molecular-weight mitochondrial DNA from purified mitochondrial fractions from *E. gracilis* could be due to nuclease activity that survives guanidinium thiocyanate inactivation or to an in vivo fragmentation. Manning et al. (1971) observed linear molecules varying between 1 and 19 mm from a mitochondrial fraction of *E. gracilis,* the larger of which would be in the size range expected from the complexity studies. The lack of migration of the *E. gracilis* mitochondrial DNA in CHEF gels even after the introduction of strand breakage by γ-irradiation suggests either an unusual in situ conformation of the mitochondrial DNA or a tight interaction of the DNA with proteins which is not easily disrupted by incubation of the agarose-embedded cells in an SDS-proteinase solution. The in vivo conformation of the mitochondrial DNA of *E. gracilis* remains to be elucidated.

The COI gene of *E. gracilis* contained a continuous open reading frame and did not require any RNA editing of the uridine insertion/deletion type which occurs in the kinetoplast-mitochondrion of kinetoplastid protozoa to create translatable open reading frames. In addition, no evidence for the presence of guide RNA-like molecules, which contain the edited sequence information in kinetoplastid mitochondria, was obtained by 5′ end-capping of mitochondrial RNA in vitro with [α-^{32}P]GTP and guanylyl transferase. The COI gene is unedited in all analyzed trypanosomatids but is extensively edited in the related cryptobiid kinetoplastid protozoan, *Trypanoplasma borreli* (Maslov et al. 1994). This evidence suggests that the uridine insertion/deletion type of RNA editing may not occur in the mitochondria of *E. gracilis,* but this must remain an open question pending analysis of additional mitochondrial genes.

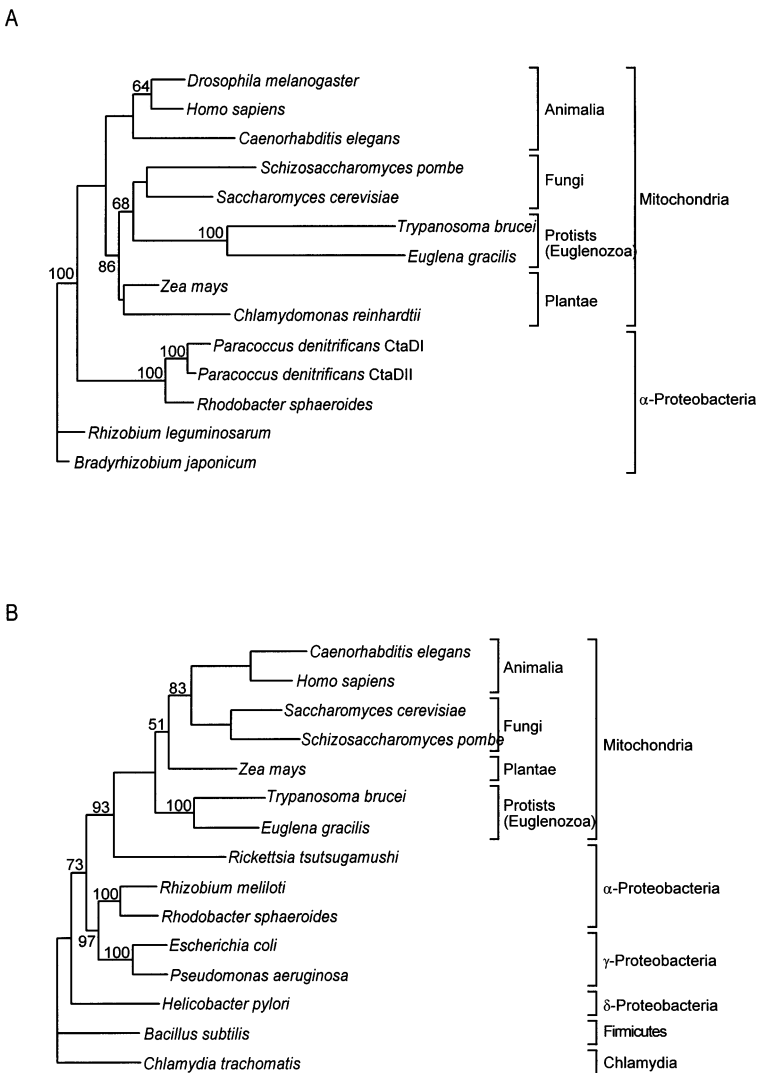

Fig. 3. Phylogenetic reconstructions of two mitochondrial genes from several eukaryotic lineages determined by the maximum likelihood method. **A** COI tree. **B** Hsp60 tree. Estimated bootstrap parameters for each node are indicated. The nodes without bootstrap values were assumed in reconstruction. The ln likelihood values are −8,474.31 and −10,095.96 for **A** and **B,** respectively.

Acknowledgments. We thank J. A. Schiff for providing the *E. gracilis* strains and M. W. Gray for information on universal codon usage in other mitochondrial systems. We also thank all the members of the Simpson Lab for advice and discussion.

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