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MITOCHONDRIAL DNA IN THE *OOGAMOCHLAMYS* CLADE (CHLOROPHYCEAE): HIGH GC CONTENT AND UNIQUE GENOME ARCHITECTURE FOR GREEN ALGAE[1]

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Most mitochondrial genomes in the green algal phylum Chlorophyta are AT-rich, circular-mapping DNA molecules. However, mitochondrial genomes from the *Reinhardtii* clade of the Chlorophyceae lineage are linear and sometimes fragmented into subgenomic forms. Moreover, *Polytomella capuana*, from the *Reinhardtii* clade, has an elevated GC content (57.2%). In the present study, we examined mitochondrial genome conformation and GC bias in the *Oogamochlamys* clade of the Chlorophyceae, which phylogenetic data suggest is closely related to the *Reinhardtii* clade. Total DNA from selected *Oogamochlamys* taxa, including four *Lobochlamys culleus* (H. Ettl) Pröschold, B. Marin, U. G. Schlöss. et Melkonian strains, *Lobochlamys segnis* (H. Ettl) Pröschold, B. Marin, U. G. Schlöss. et Melkonian, and *Oogamochlamys gigantea* (O. Dill) Pröschold, B. Marin, U. G. Schlöss. et Melkonian, was subjected to Southern blot analyses with *cob* and *cox1* probes, and the results suggest that the mitochondrial genome of these taxa is represented by multiple-sized linear DNA fragments with overlapping homologies. On the basis of these data, we propose that linear mitochondrial DNA with a propensity to become fragmented arose in an ancestor common to the *Reinhardtii* and *Oogamochlamys* clades or even earlier in the evolutionary history of the Chlorophyceae. Analyses of partial *cob* and *cox1* sequences from these *Oogamochlamys* taxa revealed an unusually high GC content (49.9%–65.1%) and provided evidence for the accumulation of *cob* and *cox1* pseudogenes and truncated sequences in the mitochondrial genome of all *L. culleus* strains examined.

***Key index words: cob*; *cox1*; GC content; green algae; *Lobochlamys*; mitochondrial DNA; *Oogamochlamys*; organelle genome architecture; pseudogenes**

***Abbreviations: cob*, gene for apocytochrome b; *cox1*, gene for cytochrome oxidase subunit I; CTAB, cetyltrimethylammonium bromide; CW, clockwise; mtDNA, mitochondrial DNA**

Mitochondrial genomes sampled across eukaryotic lineages are diverse in conformation but rather uniform in nucleotide composition, that is, with few exceptions are AT rich (Gray et al. 2004, Smith and Lee 2008). Circular and linear unit genome-size molecules as well as other complex structures of mitochondrial DNA (mtDNA) have been described (reviewed in Burger et al. 2003b, Bullerwell and Gray 2004, Gray et al. 2004, Bendich 2007); in addition, there are several examples of mitochondrial genomes fragmented into circular or linear subgenomic chromosomes with the most extreme and well-documented examples from the ichthyosporean *Amoebidium parasiticum* (Burger et al. 2003a) and the diplonemid *Diplonema papillatum* (Marande et al. 2005, Marande and Burger 2007) whose mitochondrial fractions contain >100 distinct DNA molecules, which are linear and circular, respectively. The mtDNA of dinoflagellates share with the mtDNAs of *A. parasiticum* and *D. papillatum* the presence of multiple copies of individual genes and gene fragments imbedded within different DNA elements (Norman and Gray 1997, 2001, Chaput et al. 2002, Jackson et al. 2007, Nash et al. 2007, Slamovits et al. 2007); although the structure of the dinoflagellate mtDNAs remains to be determined, it is possible that these elements correspond to different chromosomes, as in *A. parasiticum* and *D. papillatum,* rather than being physically linked on a single chromosome (Jackson et al. 2007, Slamovits et al. 2007, Nash et al. 2008). The AT richness of most mitochondrial genomes is well documented (Bullerwell and Gray 2004, Gray et al. 2004, Smith and Lee 2008), and different models have been put forward to explain this bias. For example, a neutral model proposes that a mutational process favoring AT richness results from a deficiency in DNA repair genes (reviewed by Burger and Lang 2003), while another possibility is that AT richness results from selection for increased efficiency in transcription and DNA replication (Rocha and Danchin 2002, Howe et al. 2003).

The great majority of the completely sequenced mitochondrial genomes from the green algal phylum Chlorophyta, which is composed of the Chlorophyceae, Trebouxiophyceae, Ulvophyceae, and Prasinophyceae, are circular-mapping DNA molecules (Turmel et al. 2007), and at least some of these appear to be unit genome-size circular molecules (Laflamme and Lee 2003). Available

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data, however, suggest that mitochondrial genomes from the *Reinhardtii* clade, in the basal bodies displaced clockwise (CW) group of the Chlorophyceae (Gerloff-Elias et al. 2005), are linear unit genome-sized molecules ranging in size from 16 to 30 kb (Laflamme and Lee 2003, Popescu and Lee 2007, Smith and Lee 2008) with the exception of *Polytomella parva* and *Polytomella* sp. II, which have mitochondrial genomes fragmented into at least two linear pieces (Fan and Lee 2002, Mallet and Lee 2006).

Although most mitochondrial genomes from the Chlorophyta follow the tendency for AT richness, a significant departure can be found in the mitochondrial genome of *Polytomella capuana*, which has an overall base composition of 57.2% GC (Smith and Lee 2008). The GC bias of *P. capuana* mtDNA is the greatest at the most neutrally evolving sites, such as third codon positions (76%), 4-fold degenerate sites (85%), and intergenic regions (68%), and it was suggested that this GC-rich state is the result of biased gene conversion, which has previously been proposed for the origin of GC-rich regions in animal nuclear DNA (Galtier et al. 2001).

A well-supported monophyletic lineage in the CW group of the Chlorophyceae termed the *Oogamochlamys* clade contains two genera, *Oogamochlamys* and *Lobochlamys*, which employ oogamous and isogamous modes of sexual reproduction, respectively (Pröschold et al. 2001). Molecular phylogenetic analyses of chloroplast-encoded LSU rRNA sequence data (Turmel et al. 1993, Buchheim et al. 1996) provide support for this clade being a sister group of the *Reinhardtii* clade, while other phylogenetic analyses (Pröschold et al. 2001, Gerloff-Elias et al. 2005), which use nucleus-encoded SSU rRNA sequence, do not rule out this possibility.

In this study, we chose to survey among selected *Oogamochlamys*-clade taxa *cob* and *cox1* mitochondrial gene sequences and mitochondrial genome structure using agarose gel electrophoresis and Southern blot analysis with *cob* and *cox1* probes. The particular taxa selected include *O. gigantea*, *L. segnis,* and two geographically distinct mating pairs of *L. culleus*. It is hoped that the information gained will clarify whether the mitochondrial genomes of this clade have a simple linear or circular unit genome-sized structure and/or have a GC bias, which may provide insight into the evolution of these mtDNA features in the CW group of the Chlorophyceae.

MATERIALS AND METHODS

Strains and culture conditions. Algal strains used in this study (Table S1 in the supplementary material) were obtained from the Culture Collection of Algae at the University of Texas at Austin (UTEX), the Culture Collection of Algae at the University of Göttingen (SAG), and the Chlamydomonas Center (CC) at Duke University. When subclones of these strains were obtained, they were recovered as individual colonies on agar plates. Unless indicated otherwise, all solid and liquid cultures of *L. culleus*, *L. segnis*, and *O. gigantea* were grown at 22°C on the minimal medium of Gowans (Gowans 1960) modified to contain 0.3 $g \cdot L^{-1}$ NH_4NO_3. Illumination was provided by ''cool-white'' fluorescent bulbs on a 12:12 light:dark (L:D) cycle as described previously (Lemieux et al. 1980). All liquid cultures were supplied with 1% CO_2 in air, and cells were harvested for DNA isolation at the onset of the light period (L-0) once achieving an OD_{750} of 0.25 to 0.35. Cells plated for the scoring of small colonies were grown under continuous illumination (40 μmol photons $\cdot m^{-2} \cdot s^{-1}$ PAR). Acetate-supplemented medium was prepared by adding $CH_3CO_2Na{\cdot}3H_2O$ to a final concentration of 0.2%. Solid media were prepared with 15 $g \cdot L^{-1}$ Difco™ Bacto agar (BD Canada, Mississauga, ON, Canada).

DNA isolation. Cells used for DNA isolation were grown under the above-mentioned conditions and collected by centrifugation (Beckman J2-21 centrifuge; Beckman-Coulter, Mississauga, ON, Canada) at 5,000*g* for 15 min at 4°C. The standard protocol of DNA isolation employed grinding the cell pellets in liquid nitrogen with a mortar and pestle, followed by DNA extraction using the SDS-proteinase K method (Milligan 1992); the resulting samples were resuspended in TE buffer and stored at +4°C or −20°C. In some instances, for comparative purposes, total DNA from *L. segnis* and certain *L. culleus* strains was also prepared by the CTAB (cetyltrimethylammonium bromide) method (Milligan 1992) and with the DNeasy® Plant Mini kit (Qiagen, Mississauga, ON, Canada).

Gel electrophoresis. DNA was fractionated by agarose gel electrophoresis in TAE buffer at room temperature. To determine if different electrophoretic conditions influence the migration of DNA fractions relative to the linear markers, DNA samples were separated in 0.6% agarose at 1.5 $V \cdot cm^{-1}$ for 18 h and at 6 $V \cdot cm^{-1}$ for 3 h. Total DNA, alone and in combination with the lambda/*Hind*III ladder (Fermentas Life Sciences, Burlington, ON, Canada), was fractionated by two-dimensional agarose gel electrophoresis.

Nucleic acid amplification, cloning, and DNA sequencing. Table S2 (in the supplementary material) lists the primer pairs used to amplify *cob* and *cox1* regions. PCR reactions were performed using Platinum® PCR SuperMix High Fidelity kit (Invitrogen, Burlington, ON, Canada), and the amplicons were isolated from gels using the QIAquick gel extraction kit (Qiagen). Directional cloning was accomplished using the TOPO® TA cloning kit (Invitrogen) for amplicons <1 kb; the amplicons >1 kb were cloned using the TOPO® XL PCR cloning kit. Sequencing was carried out by Macrogen (Rockville, MD, USA). The processing of the algal sequences was performed using BioEdit, version 7.0.9 (Hall 1999), MultiAlign (Corpet 1988), and MEGA4 (Tamura et al. 2007). The sequences were deposited in GenBank under the accession numbers AF529310 to AF529316 and FJ393025 to FJ393057.

DNA transfer and Southern blot hybridization. Uncut and in some cases *Hae*II- or *Pvu*II- (Invitrogen) digested DNA was blotted onto Hybond-N+ nylon membranes (GE Healthcare Life Sciences, Piscataway, NJ, USA) using a vacuum blotting system. DNA probes were labeled using the Gene Images AlkPhos Direct Labeling and Detection System (GE Healthcare Bio-Sciences). Hybridization was performed at temperatures ranging from 60°C to 72°C for 12 to 16 h. After hybridization, membranes were washed twice for 10 min in the primary wash solution at the hybridization temperature and then twice in the secondary wash solution for 5 min at room temperature. Probe detection was performed using Super RX medical X-ray film (Fujifilm Medical Systems, Stamford, CT, USA) and an exposure time of 1 to 16 h.

RESULTS

Sequencing of cob *and* cox1 *PCR amplicons generated with degenerate primers.* Using various combinations of degenerate primers (Table S2, Fig. S1 in the supplementary material), partial, functional-like sequences of *cob* were obtained from *L. segnis, O. gigantea,* and all four strains of *L. culleus*; however, sequences with reading-frame-disrupting indels were found in two strains of *L. culleus*, that is, SAG 19.72 and UTEX 1059 (Table S3 in the supplementary material). In the case of *cox1*, the degenerate primers (Table S2, Fig. S1) allowed the amplification of a partial, functional-like sequence from *L. segnis* (GenBank accession no. AF529311) and of an apparently nonfunctional sequence from *L. culleus* SAG 19.72 (AF529313) but failed to generate *cox1* products from the other *L. culleus* strains and from *O. gigantea.* As the use of *cox1* degenerate primers gave inconsistent or spurious results, we decided to employ perfect-match primers in further attempts to obtain amplicons of this gene.

Intergenic PCR. With the goal of obtaining expanded *L. culleus cob* and *cox1* sequences and to get information on the relative orientation of these sequences, we performed PCR experiments with DNA from *L. culleus* SAG 19.72 using various *cob* and *cox1* forward- and reverse-primer combinations (Fig. S1). The only product >1 kb, obtained with the lccoxF1 and lccobR2 primer pair, was gel extracted and cloned. The four clones sequenced (FJ393054–FJ393057) vary in length from 1,860 to 2,054 bp. These *cox1-cob* fragments are flanked by a 3′ half of a *cox1* sequence, extending to the stop codon, and a 5′ truncated *cob* sequence in the same transcriptional orientation. The coding regions of these fragments appear to be part of nonfunctional sequences because of frameshift mutations or truncations. The intergenic region of the different sequences ranges from 512 to 738 bp and is nearly identical except for a ~226 bp indel difference (Fig. S2 in the supplementary material). The *cox1* side of this region contains two highly repeated elements, CCMAGCAGGAG and CATMGTGCA (M = A or C), while the region toward *cob* is represented by long stretches of T and A interspersed with GC repetitive elements.

Sequencing of cox1 *from the four strains of* L. culleus. Based on the additional sequence at the 3′ end of *cox1* obtained from the *L. culleus* SAG 19.72 *cox1-cob* clones described above, a new perfect-match primer lccoxR2 was designed and used in conjunction with lccoxF2 to amplify *cox1* sequences from all four *L. culleus* strains employed in this study (Table S4 in the supplementary material). Functional-like *cox1* sequences were identified only in *L. culleus* strains SAG 18.72 and UTEX 1060, while *cox1* sequences with numerous indels and substitutions were found in all four strains of *L. culleus.*

Analysis of cob *sequence heterogeneity in* L. culleus *strains.* Early experiments indicated that *cob* sequence heterogeneity of *L. culleus* SAG 19.72 can be revealed by sequencing or by running the PCR products on high-percentage agarose gels. Using the exact-match *cob* primers lccobF1/lccobR1 (Table S2, Fig. S1), three distinct-sized PCR products of ~520, 460, and 410 bp (Fig. S3 in the supplementary material) were identified for SAG 19.72 following thermal gradient PCR. This PCR product-size heterogeneity observed within the parental stock could have been the result of heterogeneity among cells that arose since the origin of this strain, over 60 years ago, likely from a single vegetative cell, as is the common practice (Harris 2009, pp. 261–2). We therefore repeated this experiment with several subclones of this stock; however, no reduction in PCR product-size heterogeneity was observed among the subclones. Moreover, the distinct gel fractionation pattern of PCR products is identical to that of UTEX 1058 (data not shown), reported to be the same strain as SAG 19.72. Several cloned products that correspond in size to each distinct-sized PCR product were sequenced from SAG 19.72 and one of its subclones. The alignment of these sequences, which correspond to nucleotides 315 to 729 in *Chlamydomonas reinhardtii cob*, allowed the identification of four sequence types; three (FJ393033, FJ393034, and FJ393035) exhibit numerous indels and in some cases nucleotide substitutions, and one (FJ393035) encodes a potentially functional amino-acid sequence based on alignment with the *C. reinhardtii* counterpart. Complex and unique *cob* PCR-amplicon patterns (data not shown) were also revealed with *L. culleus* strain SAG 18.72 (reported as crossable with SAG 19.72) and strains SAG 1059 and 1060 (reported as crossable with each other but not with either SAG 18.72 or 19.72), but these PCR products were not analyzed by sequencing.

Analysis of cox1 *sequence heterogeneity in* L. culleus *SAG 19.72.* A similar PCR approach was used to assess *cox1* sequence heterogeneity in *L. culleus* SAG 19.72 using the exact-match *cox1*, primers lccoxF/lccoxR (Table S2, Fig. S1). In this case, only one amplicon was evident following electrophoresis (data not shown). This band was gel extracted, and several clones were obtained and sequenced. Alignment of these sequences, which correspond to nucleotides 667 to 1,101 in *C. reinhardtii cox1*, revealed three sequence types; one (FJ393051) encodes a potentially functional amino-acid sequence based on alignment with the *C. reinhardtii* counterpart, while the other two (FJ393050, FJ393052) exhibit indels and/or nucleotide substitutions.

GC content of cob *and* cox1 *fragments in* L. culleus, L. segnis, *and* O. gigantea *and of the intergenic regions in* L. culleus. The GC content of functional- and nonfunctional-like *cob* and *cox1* sequences is between

61.0% and 65.1% in the four *L. culleus* strains, which is higher than the value observed in the *cob* and *cox1* gene fragments of *L. segnis* and the *cob* gene fragment of *O. gigantea*, which are between 49.9% and 54.8% (Table S5 in the supplementary material). The only noncoding DNA sequences available are the four *cox1-cob* intergenic sequences from *L. culleus* (SAG 19.72), which, at 58.4%–59.6% GC, are slightly lower in GC content than the *cob* and *cox1* gene-fragment coding regions from the four *L. culleus* strains (Table S5). Table 1 shows that the inflated GC content in the concatenated functional-like *cob* and *cox1* fragments from the four *L. culleus* strains is almost exclusively the result of high GC content (88.8%–93.2%) at the third codon positions. This value compares to 75.2, 70.1, and 50.0% GC at the third-codon position in the corresponding coding regions of *cox1* and *cob* from *P. capuana*, *L. segnis,* and *C. reinhardtii*, respectively, and 60.5% GC from the comparable *cob* sequence of *O. gigantea*.

Cob- *and* cox1*-hybridizing DNA in* L. culleus, L. segnis, *and* O. gigantea. Southern blot analysis of total DNA from the original stocks of *L. culleus* (SAG 19.72 and 18.72, and UTEX 10.59 and 10.60) as well as from subclones of these stocks was performed using functional-like *cob* and *cox1* probes obtained from *L. culleus* SAG 19.72. These experiments indicate that (1) the *cob* probe produces different hybridization patterns than the *cox1* probe; (2) the hybridizing pattern of a subclone may or may not resemble that of the parental strain; (3) all strains and subclones show multiple bands after hybridization with *cob* and *cox1* probes except for the UTEX 1060 strain and its subclone, which display only one *cob*-hybridizing band; and (4) DNA from the UTEX 1059 and 1060 strains and their subclones show stronger hybridization signals than does the DNA from SAG 19.72 and 18.72 and their derivatives, especially SAG 18.72.1, despite loading roughly similar amounts of total DNA from the former strains compared to the latter ones as seen by the ethidium-bromide staining (Fig. 1). The samples with low levels of gel-bound hybridization do not seem to result from a preferential retention of mtDNA in the wells because they do not appear to show a consistent excess of well-bound hybridization. Finally, all *cob*- and *cox1*-hybridizing fragments appear to have comigrated with the linear DNA markers following electrophoresis at 1.5 $V \cdot cm^{-1}$ for 18 h (Fig. 1) and 6 $V \cdot cm^{-1}$ for 3 h (data not shown), therefore supporting their conformation as linear molecules (Johnson and Grossman 1977).

Southern blot analysis of total DNA from the *L. segnis* parental stock with the *L. segnis cob* and *cox1* probes revealed a smear ranging from ~3 to 20 kb (Fig. 2). Similar results were obtained when the total DNA was obtained from subclones of this stock (data not shown). Southern blot analysis of *Hae*II- or *Pvu*II-digested *L. segnis* DNA from the parental stock with the *cob* and *cox1* probes was performed to determine if the DNA regions hybridizing to these probes occurred in various genomic contexts. The multiple fragments detected with these probes following digestion of the target DNA with either enzyme support this possibility (Fig. 2). Although the *cob* and *cox1* probes, for the most part, appeared to identify the same set of *Hae*II or *Pvu*II fragments, there were some probe-specific fragment differences for each digest. Two-dimensional agarose gel electrophoresis was used to gain information about the structural features of the *L. segnis cob*- and *cox1*-hybridizing DNA. This technique employs different electrophoresis conditions in the two dimensions so that nonlinear DNA molecules should not comigrate with linear reference markers in both dimensions (Trigueros et al. 2001, Gunnarsson et al. 2004). The arc formed by the population of *cob*-hybridizing *L. segnis* DNA fragments superimposes with the arc formed by the linear reference markers, therefore supporting the hypothesis that the *cob*-hybridizing *L. segnis* DNA is a population of linear molecules (Fig. 3).

TABLE 1. Base composition in the *Oogamochlamys*-clade taxa *Lobochlamys culleus* (*Lc*), *Lobochlamys segnis* (*Ls*), and *Oogamochlamys gigantea* (*Og*). The coding-region base composition was determined for concatenated *cox1* and *cob* fragments except for *O. gigantea*, where only a *cob* fragment was obtained. For *Polytomella capuana* (*Pc*) and *Chlamydomonas reinhardtii* (*Cr*), base composition was calculated using the sequences corresponding to those employed for the *L. culleus* strains and *L. segnis*.

	Coding regions					Codon site position														
						Site 1					Site 2					Site 3				
	%A	%T	%C	%G	%GC	%A	%T	%C	%G	%GC	%A	%T	%C	%G	%GC	%A	%T	%C	%G	%GC
Lc SAG 19.72	14.8	22.8	25.6	36.8	62.4	26.1	20.4	22.5	30.9	53.4	17.1	42.3	19.8	20.7	40.5	1.2	5.7	34.3	58.7	93.0
Lc SAG 18.72	14.4	22.6	24.8	38.1	62.9	24.6	21.7	22.3	31.3	53.6	16.7	41.1	19.8	22.3	42.1	1.9	5.0	32.4	60.8	93.2
Lc UTEX 1059	15.5	22.6	25.1	36.7	61.8	25.1	20.3	24.6	29.9	54.5	18.7	39.0	20.3	21.9	42.2	2.7	8.6	30.5	58.3	88.8
Lc UTEX 1060	14.6	24.1	24.4	36.9	61.3	24.2	23.2	21.7	30.9	52.6	20.3	41.1	20.3	21.9	41.9	2.9	7.9	31.3	57.8	89.2
Ls	16.5	29.3	27.8	26.4	54.2	25.4	21.8	22.0	30.8	52.8	17.6	42.5	21.0	18.8	39.8	6.4	23.5	40.4	29.7	70.1
Og	11.1	39.0	17.3	32.6	49.9	16.6	35.3	18.2	29.9	48.1	15.5	43.3	23.5	17.6	41.1	1.1	38.5	10.2	50.3	60.5
Pc (1560 nt)	17.0	28.1	28.7	26.3	55.0	25.6	24.2	18.3	31.9	50.2	19.4	41.7	22.5	16.3	38.8	6.0	18.3	45.2	30.6	75.2
Cr (1566 nt)	19.3	34.1	23.8	22.8	46.6	25.5	25.7	16.9	32	48.9	17.2	41.8	21.3	19.7	41.0	15.1	34.9	33.3	16.7	50.0

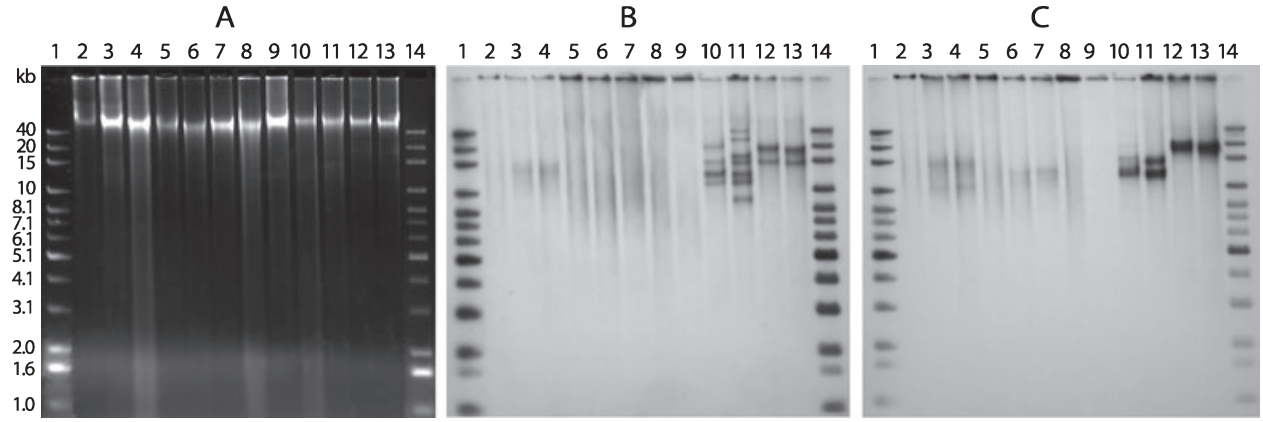

FIG. 1. Southern blot analysis of total DNA from *Lobochlamys culleus* strains fractionated by 0.6% agarose gel electrophoresis at 1.5 V · cm^{-1} for 18 h. (A) Ethidium-bromide-staining patterns. (B) Hybridization results with the *L. culleus* SAG 19.72 *cob* probe. (C) Hybridization results with the *L. culleus* SAG 19.72 *cox1* probe. Lanes 1 and 14, 1 kb DNA extension ladder (Invitrogen); lanes 2 and 3, strain SAG 19.72 with intentionally different amounts of DNA loaded per lane; lanes 4–7, subclones 19.72.10, 19.72.1, 19.72.2, 19.72.3; lanes 8 and 9, strain SAG 18.72 and subclone 18.72.1; lanes 10 and 11, strain UTEX 1059 and subclone 1059.1; lanes 12 and 13, strain UTEX 1060 and subclone 1060.1.

Southern blot analysis of total DNA from *O. gigantea* with either the *L. culleus* SAG 19.72 *cox1* (Fig. 4) or *cob* (data not shown) probes revealed three bands. None of these bands appears to correspond to simple linear molecules. When electrophoresis was performed at low voltage (1.5 V · cm^{-1} for 18 h), these bands migrated as linear molecules of ~7, 5, and 3.5 kb, respectively; the 7 kb band giving the faintest signal, and the 3.5 kb band the strongest. However, following digestion with *Pvu*II, the *cox1* (Fig. 4) and *cob* (data not shown) probes each identified three bands with relative stoichiometries paralleling the undigested bands and corresponding to molecules of ~5.5, 4, and 2.3 kb relative to the linear markers. When electrophoresis of undigested and digested *O. gigantea* DNA samples was performed at high voltage (6 V · cm^{-1} for 3 h), the presumed corresponding bands identified by order and stoichiometry in both the digested and undigested samples appeared to migrate more slowly relative to the linear markers, therefore suggesting structures other than simple linear molecules (data not shown).

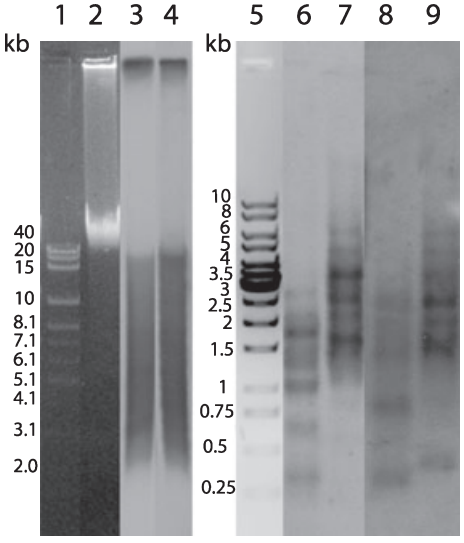

FIG. 2. Southern blot analysis of total DNA from *Lobochlamys segnis*. Lanes 1 and 2, DNA samples fractionated by 0.6% agarose gel electrophoresis at 6 V · cm^{-1} for 2 h and stained with ethidium bromide. Lane 1, 1 kb DNA extension ladder (Invitrogen); lane 2, undigested *L. segnis* DNA; lanes 3 and 4, the same as lane 2, following hybridization with the *L. segnis cob* and *cox1* probes, respectively; lanes 5–9, DNA samples fractionated by 0.8% agarose gel electrophoresis at 10 V · cm^{-1} for 1 h. Lane 5, 1 kb Gene Ruler ladder (Fermentas Life Sciences); lanes 6–9, *L. segnis* DNA digested with *Hae*III (lanes 6 and 8) or *Pvu*II (lanes 7 and 9) following hybridization with the *L. segnis cob* (lanes 6 and 7) and *cox1* probes (lanes 8 and 9).

Origin of cob *and* cox1 *sequence heterogeneity in* L. culleus *SAG 19.72.* Southern blot analysis with the *cob* and *cox1* probes indicates that the bulk of the hybridizing fraction from SAG 19.72 and its clonal derivatives is composed of DNA molecules <20 kb in length (presumably, mtDNA). However, the possibility remains that some of the *cob* and *cox1* sequences that exhibit numerous indels are part of pseudogenes present at low copy number in the high-molecular-weight fraction, containing mostly nuclear DNA, rather than in the lower-molecular-weight DNA, which should be enriched for mtDNA. To test this idea, gel-purified high- and low-molecular-weight fractions were analyzed by PCR and Southern blot analysis. PCR with the lccobF/lccobR primers was performed at 20, 25, and 30 cycles using a fixed concentration of three different DNA samples; these included gel-extracted DNA from the high- (>40 kb) and the low- (<10 to 18 kb) molecular-weight fractions and total DNA. After 25 cycles, bands were detected from the low-molecular-weight and total DNA fractions, but no bands were

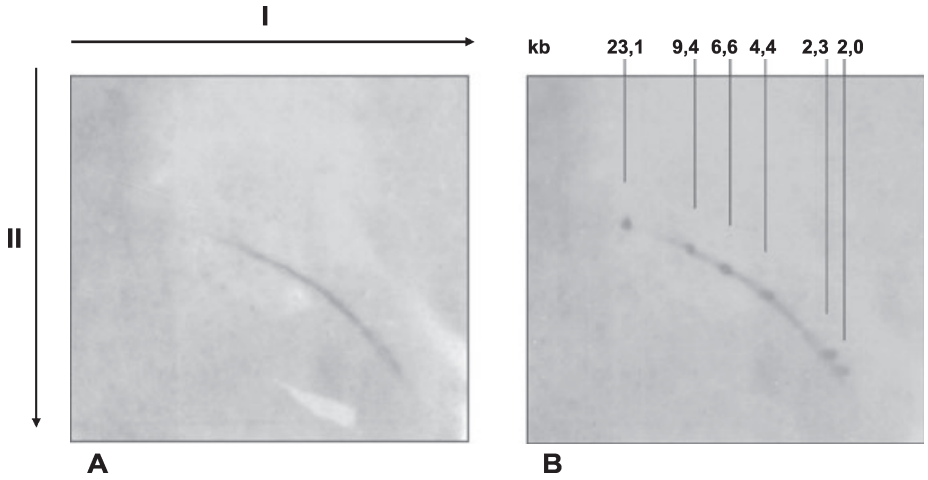

FIG. 3. Southern blot analysis of total DNA from *Lobochlamys segnis* separated by two-dimensional agarose gel electrophoresis. I: direction of migration in the first dimension (0.4% agarose at 1 V · cm^{-1} for 18 h); II: direction of migration in the second dimension (1% agarose at 4 V · cm^{-1} for 4 h). (A) Hybridization using the *L. segnis cob* probe; (B) hybridization using the *L. segnis cob* probe and labeled markers (Lambda DNA/*Hind*III marker, Fermentas Life Sciences).

observed from the high-molecular-weight fraction. After 30 cycles, bands were detected from each of the DNA fractions; however, the quantity of the bands originating from the high-molecular-weight, gel-extracted DNA was noticeably lower. Southern blot analysis using *cob* and *cox1* probes and the three aforementioned DNA samples, undigested and cleaved with the restriction enzyme *Pvu*II, gave results similar to those obtained by PCR—that is, strong signals from the gel-purified, low-molecular-weight fraction and total DNA and faint or no signals from the gel-purified, high-molecular-weight fraction (Fig. 5).

Growth characteristics of the L. culleus *and* L. segnis *strains.* In contrast to the *L. segnis* stock, none of the *L. culleus* stocks employed was capable of continuous growth in the dark or elevated growth in the light when acetate was added to the medium (data not shown). In addition, a striking feature of all the *L. culleus* stocks, not evident for *L. segnis*, was the tendency to segregate mutants that formed small colonies (Fig. S4 in the supplementary material) on agar plates similar in appearance to the intercalating-agent-induced minute colonies described in *C. reinhardtii* (Alexander et al. 1974, Remacle and Matagne 1998). The minute-like colonies in *L. culleus,* which formed on both minimal and acetate-supplemented media, were noticeably bleached and dead after about a month of culture, and their proportions among the parental stocks and their subclones varied from ~1% to approximately half of the colony-forming units plated. We have no information on *O. gigantea* with respect to these features. When growing cells for DNA extraction, it was noticed that at least some of the *L. culleus* parental stocks or subclones, particularly those that segregated the highest frequency of the lethal small-colony mutants, exhibited noticeably slower growth rates than *L. segnis* and *C. reinhardtii* under the same photoautotrophic culture conditions.

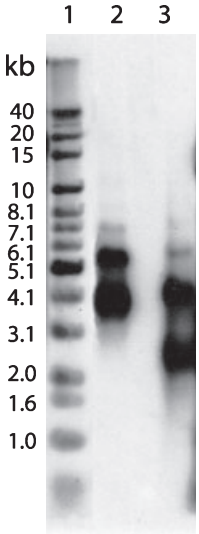

FIG. 4. Southern blot analysis of total DNA from *Oogamochlamys gigantea.* Lanes 1–3, DNA samples run on a 0.6% agarose gel at 1.5 V · cm^{-1} for 18 h and hybridized with the *Lobochlamys culleus* SAG 19.72 *cox1* probe. Lane 1, 1 kb DNA extension ladder (Invitrogen); lane 2, undigested DNA from *Oogamochlamys gigantea*; lane 3, DNA from *O. gigantea* digested with the restriction enzyme *Pvu*II.

DISCUSSION

Fragmented mitochondrial genome structure in the Lobochlamys. The results presented here suggest that the *Oogamochlamys* clade of the CW group of the Chlorophyceae contains the most unusual and diverse mitochondrial genome structures so far described for any green algal group. It seems that all of the taxa analyzed have a multipartite mitochondrial genome composed mostly of linear subgenomic fragments with overlapping homologies. For *L. segnis*, the level of fragmentation seems to be the most extreme of those examined; the *cob* and *cox1* probes formed a smear following Southern blot analysis, much like the mitochondrial gene-specific hybridization signals observed in similar experiments with dinoflagellate DNA (e.g., Jackson et al.

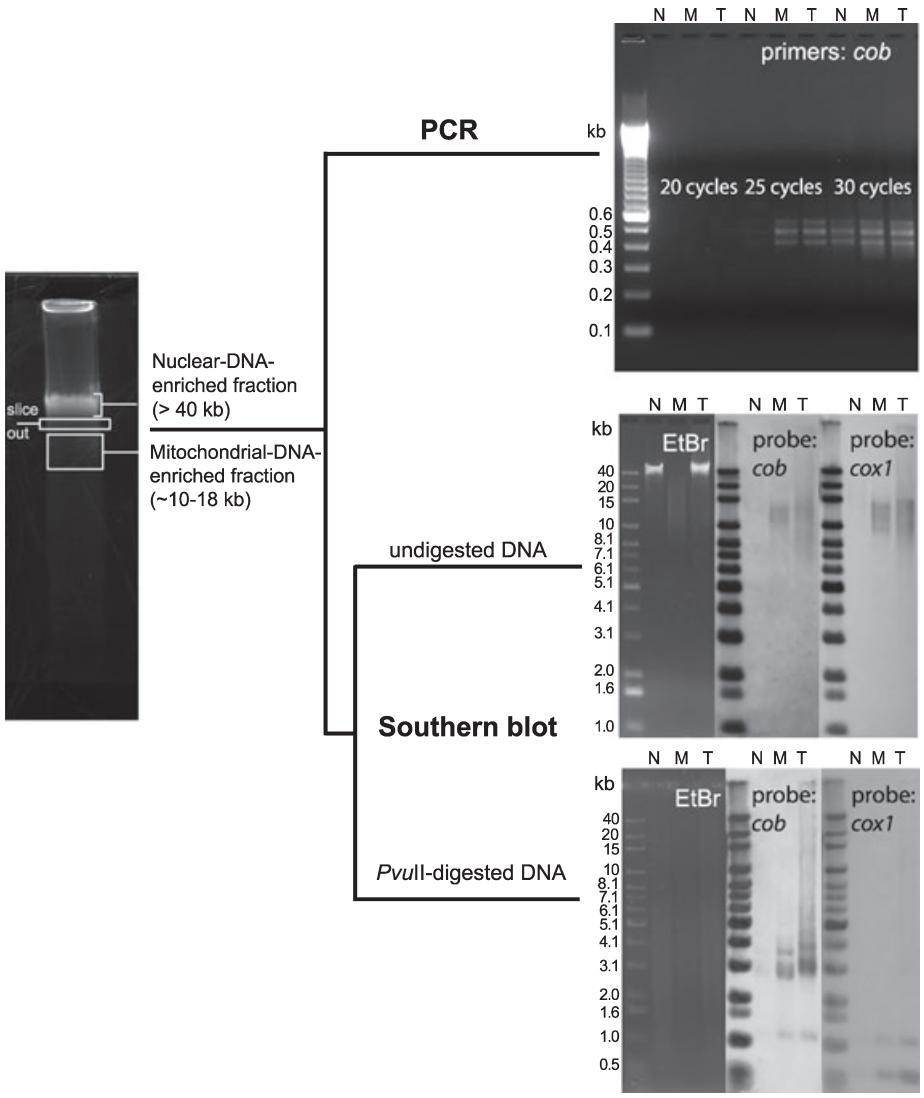

FIG. 5. Evidence that *cob* and *cox1* sequences are derived from the mtDNA of *Lobochlamys culleus* SAG 19.72. Experiments were performed with nuclear-DNA-enriched (N) and mitochondrial-DNA-enriched (M) fractions and with total DNA (T). PCR for 20, 25, and 30 cycles using *cob* primers was performed with the three DNA samples, and the products were subjected to agarose gel electrophoresis. The same three DNA samples, both undigested and *Pvu*II digested, were subjected to Southern blot analysis with *cob* and *cox1* probes. Size markers include the Invitrogen 100 bp DNA ladder (upper right panel) and 1 kb DNA extension ladder (middle and lower right panels). EtBr, ethidium bromide.

2007) with hardly any individual mtDNA bands being visible. The high end of this smear corresponded to molecules of ~20 kb, an expected size of an intact CW-group mitochondrial genome (Laflamme and Lee 2003). In the case of *L. culleus* SAG 19.72 and 18.72, the *cob* and *cox1* probes yielded similar results but sometimes identified individual bands, typically corresponding to ~15 kb or less, and these varied in size depending on the probe and the particular isolate. It seems unlikely that the polydispersed *cob*- and *cox1*-hybridizing patterns observed for these taxa were the result of enzymatic degradation of the mtDNA, because the nonhybridizing DNA (predominately nuclear DNA) visualized by ethidium-bromide staining showed no sign of degradation, and total DNA from *L. segnis* and *L. culleus* SAG 19.72 and 18.72 prepared by two additional procedures (see Materials and Methods) gave the same hybridization results. Moreover, in similar hybridization experiments with mtDNA probes, the intact 15.8 kb mtDNA of *C. reinhardtii* was identified in preparations of total DNA copurified from mixtures of *C. reinhardtii* and *L. segnis* or *L. culleus* SAG 19.72 cells (our unpublished data). We are less confident, however, in excluding the possibility that the wide size range of mtDNA recovered from these algae results from a particular vulnerability of their mitochondrial genomes to breakage during isolation; this could result, for example, from an excess of single-stranded regions associated with high recombinational activity or a particular mode of replication (Backert et al. 1997). Interestingly, the electrophoretic patterns of *cob*- and *cox1*-hybridizing DNA in *L. culleus* UTEX 1059 and 1060 are much less complex and more discrete than those from the other *L. culleus* pair but nevertheless indicate multiple discrete linear DNA forms with some overlapping homologies. At least one of the bands in UTEX 1059 has an apparent size of ~40 kb or approximately double the size of a typical

intact CW-group mitochondrial genome. Finally, the situation for the *cob*- and *cox1*-hybridizing bands in *O. gigantea* is less clear. All three of these bands hybridized to both probes and appeared to have apparent sizes relative to linear markers that were slightly larger in the high-voltage gels than in the low-voltage gels, and the same was true of the three faster-migrating bands visible following *Pvu*II digestion. Our hypothesis is that the undigested DNA molecules in this taxon correspond to linear chromosomes with some secondary structure at the telomeres, such as proposed for the mtDNA of *P. capuana* (Smith and Lee 2008). Such structures could affect their migration relative to linear DNA markers under the two gel conditions, and this could also apply to the identified *Pvu*II digestion products, which could each contain one telomere. Our data seem inconsistent with a circular conformation of the *O. gigantea* mtDNA. We conclude, therefore, that much of the mtDNA in the *Oogamochlamys* clade is linear and that this trait may have been present in the common ancestor of this clade and the *Reinhardtii* clade or even earlier.

Although mitochondrial genomes fragmented into linear and circular pieces have been described outside the green algae (reviewed by Gray et al. 2004 and, for more recent examples, see Marande et al. 2005, Gibson et al. 2007), within the green algae such descriptions are so far restricted to the bipartite linear mitochondrial genome of *P. parva* and some other *Polytomella* taxa (Fan and Lee 2002, Mallet and Lee 2006), which are members of the *Reinhardtii* clade. However, in the case of *Polytomella* mtDNA, all known examples of mitochondrial genome fragmentation seem to involve small subgenomic elements containing the *nad6* coding region.

Recombinationally active mtDNA in L. culleus. Another interesting attribute of mtDNA of *L. culleus* strains, and maybe of other taxa in the *Oogamochlamys* clade, is their apparent structural variability, as revealed by differences in size and number of fragments recorded among the different strains and between subclones and the original stock of the same strain. This characteristic is especially obvious among *L. culleus* SAG 19.72 and UTEX 1059 and their subclones; for example, in strain UTEX 1059, the *cob* probe revealed four mtDNA fragments in the parental stock DNA, while in the subclone DNA, seven fragments were obvious (Fig. 1). The structural complexities and inter- and intrastrain variations in *L. culleus* mtDNA may be the result of recombinational processes between repeat sequences, as suggested for the mtDNA of land plants (Backert et al. 1997, Kanazawa et al. 1998) and dinoflagellates (Norman and Gray 1997, 2001, Jackson et al. 2007, Nash et al. 2008).

Evidence of GC-rich mtDNA in Oogamochlamys*-clade taxa.* Base-composition analyses of the concatenated *cox1*- and *cob*-sequence data obtained in this study (only *cob* in the case *O. gigantea*) suggest that the mtDNA of *Oogamochlamys*-clade taxa is GC biased relative to that of other green algal species. Among the *Oogamochlamys*-clade taxa examined, the *cob*- and *cox1*-coding regions have the highest GC content in the four strains of *L. culleus* with values of at least 61%. This compares to 55% GC in the comparable regions of *P. capuana* mtDNA, which has the highest GC content of all the complete mitochondrial genome sequences deposited in GenBank, and 46.6% GC in the corresponding regions of *C. reinhardtii* mtDNA, known to have the second-most GC-rich complete mitochondrial genome sequence among green algae outside the *Oogamochlamys* clade (D. R. Smith and R. W. Lee, unpublished data). As in *P. capuana*, the elevated %GC in the *L. culleus* and other *Oogamochlamys* mtDNA sequences is most prominent at third-codon positions in protein-coding genes, potentially among the most neutrally evolving positions in these mtDNAs. As proposed earlier for *P. capuana* mtDNA (Smith and Lee 2008), we suggest that the high GC content of the *Oogamochlamys* mtDNA in general and the *L. culleus* mtDNA in particular is the result of a biased gene conversion mechanism whereby mismatches in heteroduplexed recombination intermediates are repaired preferentially to GC (Galtier and Duret 2007). An abundance of mtDNA recombination and a high incidence of cells heteroplasmic for single nucleotide polymorphisms could contribute to an elevated level of GC-biased gene conversion in these algae, especially in *L. culleus*. A high GC content of mitochondrial genomes in the *Oogamochlamys*-clade taxa needs to be confirmed by sequencing larger sections of these genomes.

Evidence for pseudogenes in the mtDNA of L. culleus. Sequence analysis of *cob* and *cox1* amplicons produced with different primer combinations revealed multiple *cob* and *cox1* sequence types from *L. culleus* strains, but only one type of each for *L. segnis* and one type of *cob* for *O. gigantea*. While in *L. segnis* and *O. gigantea* all these partial sequences seem to correspond to a functional-like type, in the case of *L. culleus* strains, several *cob* and *cox1* partial sequences appear to be nonfunctional because of numerous frame-shifting indels. The results of reverse transcription-PCR (RT–PCR) experiments with *cob* primers support the hypothesis that these putative functional and nonfunctional sequences are transcribed (our unpublished data). *Cob* and *cox1* are genes encoded in the mitochondrial genome of all green algae and land plants characterized to date, and we have no reason to suspect that *L. culleus, L. segnis,* and *O. gigantea* are different in this regard. However, the cellular location of the apparently nonfunctional *cob* and *cox1* sequences of *L. culleus* strains is less clear. Although the maintenance of mitochondrial pseudogenes or gene fragments in the mitochondrial genomes of diverse lineages is now well documented (Bensasson et al. 2001, Bur-

ger et al. 2003a, Richly and Leister 2004, Gibson et al. 2007, Jackson et al. 2007, Waters et al. 2008), there are also numerous reports of nucleus-encoded mitochondrial pseudogenes (numts) in many taxa (Bensasson et al. 2001, Pereira and Baker 2004, Richly and Leister 2004) including *C. reinhardtii* (the *C. reinhardtii* database at http://genome.jgi-psf.org/Chlre4/Chlre4.home.html). Our Southern blot hybridization experiments with *L. culleus* SAG 19.72 total DNA and gel-enriched, high- and low-molecular-weight fractions (Fig. 5) indicate that the *cob*- and *cox1*-hybridizing DNA is restricted to the low-molecular-weight fraction in the gel and is therefore likely mtDNA. Such experiments, however, do not eliminate the possibility that one or more of the apparently nonfunctional *cob* and *cox1* sequence variants are derived from numts present at much lower copy number than mitochondrial-encoded homologues. If true, some of the district-size *cob* amplicons detected by gel electrophoresis should have appeared after a lower number of PCR cycles when employing a nuclear-DNA-enriched fraction compared to an mtDNA-enriched fraction or total DNA; on the contrary, the nuclear-DNA-enriched fraction required at least five more cycles before any *cob* amplicons were visible. These hybridization and PCR experiments favor the hypothesis that most, if not all, *L. culleus cob* pseudogenes are mitochondrial encoded; however, this conclusion needs to be substantiated by more extensive sequence analysis.

Evidence for a functional cytochrome pathway. Mutations that disrupt the activity of *cob* and *cox1* genes in *C. reinhardtii* lack a functional cyanide-sensitive cytochrome pathway and lose the ability to grow in the dark on acetate-supplemented medium, but their growth under photoautotrophic conditions is hardly affected (Remacle and Matagne 1998, Duby and Matagne 1999, Remacle et al. 2001). Functional-like partial *cob* and *cox1* sequences were recovered from almost all *L. culleus* strains studied; however, we did not obtain complete sequences of these genes and, therefore, cannot be confident based on this information alone that these strains contain functional copies of *cob* and *cox1*. Moreover, unlike *L. segnis*, none of the *L. culleus* strains could grow in the dark on acetate medium, so they could potentially lack functional *cob* and *cox1*. Nonetheless, we found clear support for a cyanide-sensitive cytochrome pathway and biochemical evidence for similar levels of cytochrome c oxidase activity (our unpublished data) in both *L. segnis* and *L. culleus* SAG 19.72. These findings support the presence of functional *cob* and *cox1* genes in these taxa and undermine the possibility that *cob* and *cox1* pseudogenes in *L. culleus* are directly linked to the inability of these strains to grow in the dark on acetate medium. This inability to grow heterotrophically on acetate medium, also reported for several species of *Chlamydomonas* (Harris 2009, pp. 176–8), could result from causes other than deficiencies in cyanide-sensitive respiration including, for example, defects in enzymes involved in acetate metabolism (Wiseman et al. 1977).

Maintenance of pseudogenes. One may ask why *cob* and *cox1* pseudogenes in *L. culleus* strains are maintained along with functional forms of these genes. As mentioned above, mutations in *C. reinhardtii* rendering the cytochrome pathway nonfunctional have little effect on growth under photoautotrophic conditions. If the same applies to *L. culleus*, one might expect natural selection to be ineffective in removing these apparently nonfunctional *cox1* and *cob* forms from *L. culleus* populations, especially if population sizes are small, which reduces the power of natural selection, and if these pseudogenes are associated with truncated DNA elements having a replicative advantage (Taylor et al. 2002). Moreover, even in mammals (Schon et al. 2000) and the nematode *Caenorhabditis elegans* (Tsang and Lemire 2002, Liau et al. 2007), for example, where mitochondrial-encoded cytochrome-pathway genes are essential for cellular function, cells can operate with a mixture of nonfunctional and functional gene copies in the mitochondria until some critical threshold proportion of the mutant form is reached.

Mitochondrial dysfunction in L. culleus*?* When streaked on agar plates, all four strains of *L. culleus* used in this study segregated lethal small-colony mutants. Similar small-colony mutants termed ''minutes'' have been recovered from wildtype *C. reinhardtii* following treatment with the intercalating agents acroflavin or ethidium bromide. These mutants have altered mitochondrial structure and function, are inherited in a non-Mendelian fashion, and are associated with the specific loss of mtDNA (Alexander et al. 1974, Gillham et al. 1987). The *C. reinhardtii* minute mutations share similarity with intercalating-agent-induced and spontaneously occurring vegetative petite mutations, described many years earlier in the budding yeast *Saccharomyces cerevisiae*, where mtDNA sustains major alterations or is lost entirely (Gillham 1978, pp. 171–203). One obvious explanation for the occurrence of *L. culleus* small-colony mutants is that they result from a critical accumulation of defects in mtDNA. However, the accumulation of *cox1* and *cob* pseudogenes in *L. culleus* cannot by itself explain the occurrence of the lethal small-colony mutants, because loss of cyanide-sensitive respiration alone should have little effect on growth under phototrophic conditions as discussed earlier. Rather, we hypothesize that the lethal small colonies result from a more general array of alterations in mtDNA, and their critical accumulation leads to a termination of essential mitochondrial functions. These mutations could include, for example, alterations of mtDNA telomeres, RNA processing sites, and coding regions for structural RNAs and certain respiratory proteins. With a better knowledge of the *L. culleus* mtDNA sequence, it might be possible to assay the level of

these proposed defective alleles in pools of *L. culleus* small-colony mutants and wildtype-looking colonies, as has been done with minute- and normal-colony segregants of *C. reinhardtii* heteroplasmic for mtDNA deficiencies (Randolph-Anderson et al. 1993), and therefore test for a connection between the accumulation of defective mtDNA and the lethal small-colony phenotype in *L. culleus*. Finally, this work raises the question, what is or are the factors contributing to the accumulation of indels in the mtDNA of *L. culleus*? Does a high GC content and/or an abundance of direct repeats in the mtDNA of *L. culleus* make this genome more prone to the formation of indels? Does *L. culleus* contain deficiencies in nucleus-encoded mtDNA repair and damage tolerance pathways, which may be responsible for an elevated incidence of these mutations? In mice, for example, short direct repeats in mtDNA, especially if rich in G and C, are thought to promote the occurrence of deletions in this DNA through replication jumping (Chung et al. 1996). The existence of nuclear genes affecting repair and damage tolerance in mtDNA have been described in different systems including *S. cerevisiae*, where mutations affecting these functions have been implicated in the occurrence of vegetative petite colonies (Chen and Clark-Walker 2000, O'Rourke et al. 2005, Cheng et al. 2007).

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Supplementary Material

The following supplementary material is available for this article:

Figure S1. The position of the primers used in the amplification of *cox1* and *cob* sequences from *Lobochlamys culleus* SAG 19.72 and *Lobochlamys segnis* UTEX 1905 relative to full-length *cox1* and *cob* sequences from *Chlamydomonas reinhardtii*.

Figure S2. Schematic drawing using GATA (Nix and Eisen 2005) of the sequence of two fragments (clone 1, 1,860 bp; clone 2, 2,054 bp) containing a *Lobochlamys culleus* SAG 19.72 *cox1-cob* region. In black are the regions conserved, and in white the regions that show the least sequence conservation. The lines between clones indicate the position of divergent sequences.

Figure S3. Profile of *cob* amplicons from *Lobochlamys culleus* following PCR amplification with a gradient of annealing temperatures from 65°C (lane 1) to 55°C (lane 8) and using the perfect match primers lccobF1/lccobR1 (Table S2, Fig. S1). Lane M, 100 bp DNA size markers (Invitrogen).

Figure S4. Small colony mutants of a subclone of *Lobochlamys culleus* SAG 18.72. A cell suspension was streaked on minimal agar medium and maintained for 10 d at room temperature under continuous ''cool white'' fluorescent lighting at 40 μmol photons · m^{-2} · s^{-1} PAR.

Table S1. Algal strains and sources.

Table S2. Primers used for the amplification of *cob* and *cox1* sequences from *Lobochlamys culleus, Lobochlamys segnis*, and *Oogamochlamys gigantea*.

Table S3. Cloned *cob* sequences from various *Oogamochlamys*-clade taxa obtained by amplification with *cob* universal primers.

Table S4. Cloned *cox1* sequences from various *Lobochlamys culleus* strains obtained by amplification with lccoxF2 and lccoxR2 primers.

Table S5. GC content and the size of various sequences obtained from *Lobochlamys culleus, Lobochlamys segnis*, and *Oogamochlamys gigantea*.

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