Mitochondrial genome conformation and cytochrome respiration-related functions among the CW-group chlorophycean algae

by

Mark Laflamme

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The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "Mitochondrial genome conformation and cytochrome respiration related functions among the CW-group chlorophycean algae" by Mark Laflamme in partial fulfilment for the degree of Doctor of Philosophy.

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To my wife Charline, whose encouragement and absolute support made this dissertation possible.

TABLE OF CONTENTS

Chapter I

Mitochondrial genome conformation among taxa from the CW-group *Volvox*-, *Tetracystis*-, and *Dunaliella*-clades

Chapter II

Mitochondrial DNA of the *Oogamochlamys*-clade taxa *Chlamydomonas segnis* and *Chlamydomonas culleus*: structural features and expression of cytochrome respiration-related functions

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Available data suggest that among green algae (Chlorophyta, *sensu* Sluiman 1985) most taxa have circular-mapping mitochondrial genomes with other taxa having linear genome- or subgenomic-sized mitochondrial DNAs (mtDNA). To date, all examples of the linear mtDNA forms are confined to the *Volvox*-clade (*sensu* Nakayama et al. 1986) of the chlorophycean CW (basal bodies displaced clockwise)-group, although there is no information regarding the conformation of mtDNA from taxa of the *Oogamochlamys*-clade (*sensu* Pröschold et al. 2001), which may be the extant clade most closely related to the *Volvox*-clade. It is not clear, however, if the circular-mapping genomes represent genome-sized circular molecules, if such circular molecules and the linear forms are the predominant *in vivo* mtDNA structures, nor if the linear forms arose only once or multiple times among extant green algal lineages. It was thus of interest to examine the DNA components detected with homologous mtDNA probes following pulsed field gel electrophoresis of total cellular DNA from the chlorophycean CW-group green algal taxa *Chlamydomonas reinhardtii* and *Chlamydomonas moewusii*. The 15.8-kb linear mtDNA of *C. reinhardtii* was the only DNA component detected, and there was no evidence of circular or large linear mtDNA precursors of this 15.8-kb linear form. For *C. moewusii*, which has a circular-mapping 22.9-kb mitochondrial genome, total cellular DNA revealed a mixture of DNA components that hybridized to the mtDNA probe. These components appeared to be circular (relaxed and supercoiled) and genome-sized linear DNA molecules with the latter likely resulting from random double-strand breaks in the circular forms during DNA isolation. In further studies, the mitochondrial genome conformation of additional CW-group taxa was examined using conventional gel electrophoresis and Southern blot analysis with mtDNA probes. The results of these experiments indicate that all taxa from the *Volvox*-clade have genome- or subgenomic-sized linear mtDNAs as their predominant mtDNA form. Interestingly, the mtDNAs of *Chlamydomonas segnis* and *Chlamydomonas culleus*, which affiliate with the *Oogamochlamys*-clade, seem to have an unusual conformation consisting of a heterogeneous collection of molecules with a continuous size distribution spanning the region of the gel corresponding to linear DNAs between 3 and 23 kb. In addition, analysis of PCR generated *cob* and *cox1* sequences suggest these are functional in *C. segnis* but non-functional in *C. culleus*. This result is supported by further experiments that detected *cob* and *cox1* transcripts and a functional cytochrome respiration pathway in *C. segnis* but not *C. culleus*. Finally, in control experiments, *Chlamydomonas moewusii* also scored negative with regard to the detection of *cob* and *cox1* transcripts and cytochrome respiration pathway activity despite the presence of *cob* and *cox1* sequences that look potentially functional.

LIST OF ABBREVIATIONS

°C, degree Celsius

A, adenine

B, = C, G or T

bp, base pair

C, cytosine

cm, centimeter

CW, clockwise

D, = A, G or T

d, day

DNA, deoxyribonucleic acid

DO, directly opposed

EDTA, ethylenediaminetetraacetate

G, guanine

H, = A, C, or T

h, hour

K, keto (large grove) = G or T

kb, kilobase

M, amino (large grove) = A or C

mtDNA, mitochondrial DNA

N, = A, C, G or T

NIES, National Institute for Environmental Studies (Japan)

nt, nucleotide

PAR, photosynthetically active radiation

PCR, polymerase chain reaction

PFGE, pulsed field gel electrophoresis

R, purine = A or G

RNA, ribonucleic acid

rRNA, ribosomal RNA

RT-PCR, reverse transcriptase PCR

S, strong bond = G or C

SAG, Sammlung von Algenkulturen

SDS, sodium dodecyl sulfate

T, thymine

Tris, tris(hydroxymethyl)aminomethane

UTEX, University of Texas Culture Collection

V, = A, C or G

V, volt

W, weak bond = A or T

Y, pyrimidine = C or T

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PREFACE

Some of the data contained in this dissertation has been published previously in the following paper:

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Permission has been obtained to include data presented in the above-mentioned paper as part of this dissertation.

GENERAL INTRODUCTION

I – INTRODUCTORY STATEMENT

The little that is known of mtDNA conformation among taxa from the chlorophycean CW-group of green algae has been inferred mostly from mapping data, and it is currently uncertain whether these genome maps are representative of the major component mtDNAs *in vivo*. The goal of the work described in this dissertation is to obtain physical evidence regarding the diversity of mtDNA conformation among selected taxa from the CW-group. It is hoped the information gained will contribute to our knowledge of this diversity, from a phylogenetic perspective, and ultimately to an understanding of its origin.

II – MITOCHONDRIAL GENOMES AND mtDNA AMONG VARIOUS EUKARYOTIC LINEAGES

Mitochondrial genomes display a remarkable amount of variability in size and structure, both among and within kingdoms of the Eukarya (reviewed in Gray 1999, Gray et al. 1999, Lang et al. 1999). From the 6 kb mitochondrial genome of the malaria parasite, *Plasmodium falciparum*, which encodes only five genes (Feagin 1994), to the 200 – 2400 kb land plant counterparts, which encode 50-70 genes (Ward et al. 1981, Lang et al. 1999), the variability in size and gene content is quite obvious. Since its discovery in

the mid-nineteen-sixties until the mid-nineteen-nineties, the widely held belief was that the mtDNA in nearly all eukaryotes consisted of genome-sized circular molecules. Today it is clear, however, that although genome-sized circular mtDNAs are prevalent in most animal taxa, such molecules are rarely found outside this group (reviewed in Bendich 1993). The mtDNA in most eukaryotic lineages exists as complex assemblages of molecules, and the mechanisms for the replication of these mtDNAs are not obviously linked to the lifestyle of a particular organism. For example, as will be discussed in greater detail below, the distinct mtDNA replication mechanisms employed by land plants and members of the protozoan genus *Plasmodium*, are quite similar. Further, a number of other distinct replication mechanisms are employed among groups of closely related yeast taxa, where even different strains of the same species can differ in this respect.

This section of the general introduction will focus on what is known of the mitochondrial genomes of four lineages within the Eukarya, namely the animals, the fungi, the Apicomplexa and the land plants.

The animals

By comparison to the mitochondrial genomes of other eukaryotic lineages (see below), those of animals show little variation in terms of size, ranging from 10 to 39 kb, and gene content, 36 or 37 genes (Moritz et al.

1987; Pont-Kingdon et al. 2000). In the majority of animal taxa, the mtDNA appears to exist mostly as genome-sized circular molecules (reviewed in Garey and Wolstenholme 1989; Bendich 1993); there are, however, a few exceptions. For example, the mtDNAs from three of the four extant lineages of the phylum Cnidaria exist as genome- or subgenomic-sized linear molecules (Bridge et al. 1992), and the mtDNA of the potato cyst nematode *Globodera pallida* exists as a population of subgenomic-sized circular molecules. It is generally thought that the genome-sized circular forms employ a theta-type [also called expanding-bubble and Cairns-model (Cairns 1963)] replication mechanism (Clayton 1982; Kornbergh, and Baker 1992); there is currently no data regarding the replication of the rare linear mtDNA forms among the animals.

The fungi

The mitochondrial genomes of fungal taxa range in size from about 19- to 190-kb, encode 24 to 43 genes, and the mtDNA conformation is variable among taxa. In rare yeast taxa for example, the mtDNA exists as a linear molecule with terminal repeat sequences, which may or may not have 5' overhangs (Nosek et al. 1995; Nosek et al. 1998). Considerable variation in the number of terminal tandem repeats is responsible for the length heterogeneity observed among this population of mtDNA molecules. It is

thought that maintenance of these "mitochondrial telomeres" is achieved by unequal homologous recombination between the tandem repeats and/or by a telomerase-like activity (Nosek et al. 1995; Nosek et al. 1998) such as described in the ciliate *Tetrahymena* (Morin and Cech 1986; 1988a; b; Burger et al. 2000). A second type of yeast linear mtDNA has been identified; this DNA has terminal inverted repeat sequences with a covalently closed single stranded hairpin (Fukuhara et al. 1983; Dinouël et al. 1993; Nosek et al. 1995), reminiscent of the vaccinia virus telomere (Baroudy and Moss 1982). Although the replication mechanism of this mtDNA form has not been fully elucidated, it almost certainly involves circular intermediates (Dinouël et al. 1993).

In contrast to the rare yeast taxa described above, the mtDNAs of most yeast, as well as taxa from all branches of true fungi and zoosporic moulds, exist as a collection of linear molecules ranging in size from 50 to 150 kb, or two to seven times the mitochondrial genome size. A small fraction of the mtDNA seems to exist as unit genome-sized circles, and this conformer is thought to be the replicative unit that gives rise to the linear DNAs by a rolling-circle (sigma-type) replication mechanism (Maleszka et al.1991; Skelly and Maleszka 1991; Maleszka and Clark-Walker 1992; Maleszka 1993).

The Apicomplexa

The phylum Apicomplexa consists of exclusively parasitic unicellular eukaryotes, and is the group in which the smallest mitochondrial genomes have been identified to date. The first apicomplexan mitochondrial genome described was that of the malaria parasite *Plasmodium yoelii* (Vaidya and Arasu 1987); this genome is 6 kb in size and encodes only five genes (reviewed in Wilson and Williamson 1997). In terms of structure, two distinct types of mtDNAs have been identified in the Apicomplexa. In taxa from the genus *Plasmodium*, the bulk of the mtDNA exists as linear concatemers of the 6-kb sequence. Genome-sized circles, which compose about 1% of the total mtDNA, have also been identified, and these likely give rise to some of the linear concatemers via a rolling-circle replication mechanism (Preiser et al. 1996). In contrast to the situation in yeasts, however, rolling-circles may only account for a small part of mtDNA replication in *Plasmodium*. In addition to the rolling-circles, a phage T4-like recombination dependant replication mechanism is thought to produce to large complex structures, which are then resolved into linear concatenated molecules (Preiser et al. 1996).

Although the gene content of mtDNA from non-malarial taxa from the genus *Theileria* is identical to that of taxa from genus *Plasmodium*, the mtDNA structure is very different and consists of genome-sized linear molecules with terminal inverted repeats (Kairo et al. 1994; reviewed in

Wilson and Williamson 1997). Although nothing is known of the replication of these mtDNAs, the presence of the terminal inverted repeats (telomeres) along with the absence of any linear concatemers or large complex structures indicates a radically different mechanism than described in taxa from the genus *Plasmodium*.

The land plants

The mitochondrial genomes of land plants are the largest such genomes, ranging in size from 200 to 2400 kb (Ward et al. 1981; Lang et al. 1999), and encoding 50 to 70 genes; these are the most structurally complex mtDNAs, and their replication mechanisms are poorly understood.

Mapping data predicts that the mtDNA of most plant taxa is composed of two or more arrangements of a genome-sized circular molecule (the master circle) in addition to subgenomic-sized circular molecules (Palmer and Shields 1984; Klein et al. 1994; reviewed in Albert et al. 1998). The subgenomic-sized molecules are thought to result from intramolecular recombination between direct repeats present in the master circle. The different arrangements of the master circle are thought to result from intermolecular recombination between the subgenomic-sized molecules.

In contrast to the mapping data discussed above, the mtDNAs of land plants exist *in vivo* primarily as two distinct populations, which contain the

same nucleotide sequences, but differ in the size and structural complexity of the DNA molecules. The first population is composed of linear, branched, and rosette-like structures that can be much larger than the genome size. In the liverwort, *Marchantia polymorpha*, the large linear molecules are circularly permuted (Oldenburg and Bendich 2001). This observation, as well as similar observations in the pigweed, *Chenopodium album* (Backert et al. 1996; Backert et al. 1997a; Backert et al. 2000) have led to the proposition that the large mtDNAs structures employ both a recombination-dependent DNA replication mechanism similar to that of phage T4 as well as a rolling-circle replication mechanism (Backert et al. 1997a; Backert et al. 2000; Oldenburg and Bendich 2001). Although little is known of the mechanisms underlying the transmission genetics of the plant mtDNA, it is thought that the large rosette-like structures, which are sometimes observed attached to the mitochondrial membrane, are the heritable genetic units (Backert et al. 1997a; b). The second population is composed mostly of linear DNAs with a continuous size distribution, ranging from about 40- to 200-kb. The origin of this population of mtDNA molecules is poorly understood, though it is thought that these DNAs derive in part from the rolling-circle replication of small circular molecules (Backert et al. 1996; Backert et al. 1997b). In addition, intramolecular recombination between short repeats present in the larger mtDNA forms could account for much of this mtDNA (Small et al.

1989; Backert et al. 1997a); such an hypothesis is supported by mathematical models (Atlan et al. 1993).

III – MITOCHONDRIAL GENOMES AND MTDNA AMONG THE GREEN ALGAE

The mitochondrial genomes among green algal (Chlorophyta, *sensu* Sluiman 1985) taxa are relatively small, ranging from 16 to 75 kb (Table 1), and encoding between 12 and 60 genes. The smallest of these are found in the Chlorophyceae (*sensu* Mattox and Stewart 1984), where two distinct lineages have been identified by 18S rRNA gene sequence data and by the basal body configuration in flagellated cells, which is clockwise in one lineage (CW-group), and directly opposed in the other lineage (DO-group) (Lewis et al. 1992; Friedl 1997).

Mitochondrial gene content and function among CW-group algae

The mitochondrial genomes of three CW-group taxa, namely *Chlamydomonas reinhardtii*, *Chlamydomonas moewusii* and *Chlorococcum capillatum*, have been completely sequenced; these range in size from about 16- to 23-kb and contain only a few genes (c.f. Lang et al. 1999; Nedelcu et al. 2000), including fragmented and scrambled rRNA coding regions, three tRNA

Table 1. Apparent mitochondrial DNA conformation among green algal taxa

Class / group	Strain and source [a]	Size (kb)	Conformation	Reference
Chlorophyceae				
CW-group; *Volvox*-clade	*Chlamydomonas reinhardtii*– UTEX 2244	15.8	linear [b,c]	1,2,3
	Chlamydomonas reinhardtii – UTEX 1062	16.9	linear [b]	4
	Pandorina morum, syngen I – UTEX 854	20 [d]	linear [b]	5
	Polytomella parva – UTEX L193	13.5, 3.5	linear [e]	6
Dunaliella-clade	*Chlorogonium capillatum* – SAG 12-2e	22.7	circular [b]	7
Tetracystis-clade	*Chlamydomonas moewusii* – UTEX 9	22.9	circular [b]	8
	Chlamydomonas moewusii – UTEX 97	22	circular [b]	9
	Chlamydomonas pitschmannii – SAG 1473	16.5	circular [b]	10
DO-group	*Scenedesmus obliquus* – UTEX 78	42.9	circular [b]	11
	Scenedesmus obliquus – KS3/2	42.7	circular [b]	12
Trebouxiophyceae	*Prototheca wickerhamii* – SAG 263-11	55.3	circular [b]	13
	Chlorella N1a	75	circular [b,c]	14
Pedinophyceae	*Pedinomonas minor* – UTEX LB1350	25.1	circular [b]	15
Prasinophyceae	*Platymonas subcordiformis*	43	circular [b]	16
	Nephroselmis olivacea – NIES-484	45.2	circular [b]	15
	Mesostigma viride – NIES-296	42.4	circular [b]	17

CW-group = taxa with basal bodies displaced clockwise; DO-group = taxa with basal bodies directly opposed. The *Volvox*-clade, *Dunaliella*-clade and the *Tetracystis*-clade are based on Nakayama et al. (1996); support for the inclusion of additional taxa in these clades is provided by the results of Buchheim and Chapman (1992), Turmel et al. (1993), Buchheim et al. (1996), and Hepperle et al. (1998). References: 1, Michaelis et al. 1990; 2, Boer and Gray 1991; 3, Vahrenholz et al. 1993; 4, Boynton et al. 1987; 5, Moore and Coleman 1989; 6, Fan and Lee 2002; 7, Kroymann and Zetsche 1998; 8, Denovan-Wright et al. 1998; 9, Denovan-Wright and Lee 1992; 10, Boudreau and Turmel 1995; 11, Nedelcu et al. 2000; 12, Kück et al. 2000; 13, Wolff et al. 1994; 14, Waddle et al. 1990; 15, Turmel et al. 1999; 16, Kessler and Zetsche 1995; 17, Turmel et al. 2002. [a]Strains labeled UTEX derive from the University of Texas Algal Collection; SAG, the Sammlung von Algenkulturen, and NIES, the National Institute for Environmental Forum (Japan). [b]DNA conformation inferred by restriction fragment mapping analysis and/or complete DNA sequence data. [c]DNA conformation observed by electron microscopy. [d]Other syngens were analyzed and have mtDNA sizes ranging from 20 to 38 kb. [e]Two linear pieces of the mitochondrial genome corresponding to 13.5 kb and 3.5 kb have been described.

genes and a conserved set of seven respiratory protein genes. Five of the respiratory protein genes, namely, *nad1*, *nad2*, *nad4*, *nad5*, and *nad6*, encode subunits 1, 2, 4, 5 and 6, respectively, of the rotenone-sensitive NADH : ubiquinone oxidoreductase (complex I), while the remaining two, *cob* and *cox1*, encode apocytochrome b and subunit 1 of cytochrome c oxidase, which are part of complexes III and IV of the cytochrome respiration pathway, respectively (Figure 1). As in higher plants and many fungi, mitochondrial electron transport in *C. reinhardtii* can proceed via two distinct pathways that branch at the level of the ubiquinone pool: the cytochrome pathway and the alternative pathway, the later of which corresponds to the nucleus-encoded alternative oxidase (reviewed in Remacle and Matagne 1998). It has been shown for *C. reinhardtii* that expression of the mitochondrial protein-coding genes is unnecessary for survival under phototrophic growth conditions. For example, mutants of *C. reinhardtii* lacking activity of complex I show a much-reduced heterotrophic growth rate (growth in darkness using acetate as a reduced carbon source) but only a slight reduction in their phototrophic growth rate (Remacle et al. 2001; Cardol et al. 2002). Mutants lacking the activities of complexes III and/or IV of the cytochrome pathway lose their capacity for heterotrophic growth completely, although their phototrophic growth seems barely affected (Matagne et al. 1989; Colin et al. 1995). Perhaps resulting from the lack of any mitochondrial ATP production, mutants lacking the activity of both complexes I and III are viable, although

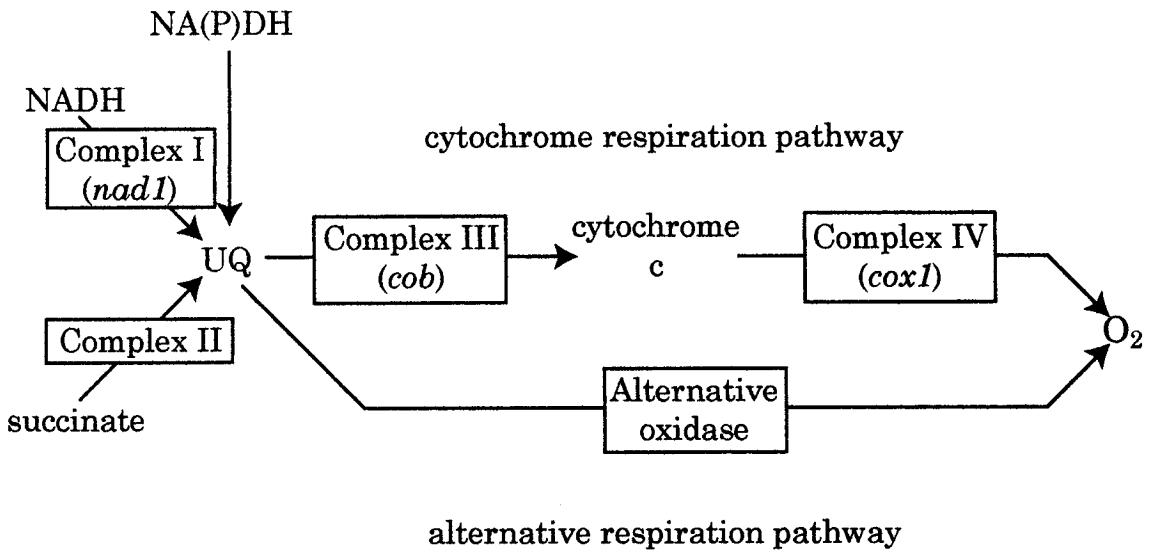


Figure 1. The electron transport chain of *Chlamydomonas reinhardtii*. Complex I: rotenone-sensitive NADH-ubiquinone oxidoreductase; Complex II: succinate-ubiquinone oxidoreductase; Complex III: ubiquinone-cytochrome c oxidoreductase, Complex IV: cytochrome c oxidase; DH: rotenone-insensitive NAD(P)H dehydrogenase. Proton translocation leading to ATP synthesis occurs in complexes I, III and IV. This figure was adapted from Remacle & Matagne (1998).

they lose their capacity for heterotrophic growth and, in addition, show a marked reduction in their phototrophic growth rate (Duby and Matagne 1999).

Structural diversity among green algal mtDNAs

In terms of mtDNA conformation, taxa from most lineages of green algae have circular-mapping mitochondrial genomes, with few examples of taxa having linear mtDNA forms (Table 1). For example, only circular-mapping mitochondrial genomes have been identified in the Trebouxiophyceae (*sensu* Friedl 1995), the Pedinophyceae (*sensu* Moestrup 1991), a class whose phylogenetic position in the green algae is not well resolved, and the Prasinophyceae, a non-monophyletic assemblage of early branching green flagellates (Steinkötter et al.1994). Conversely, evidence for both linear and circular mitochondrial genomes has been obtained for taxa from the Chlorophyceae.

Phylogenetic analysis of 18S rRNA, as well as plastid large subunit rRNA sequences, has revealed at least four distinct clades within the CW-group of the Chlorophyceae, whose phylogenetic relationships are well resolved. These include the *Volvox*-clade, the *Tetracystis*-clade and the *Dunaliella*-clade of Nakayama et al. (1996), and the *Oogamochlamys*-clade of Pröschold et al. (2001) (Figure 2). Nuclear (Buchheim et al.2001), plastid

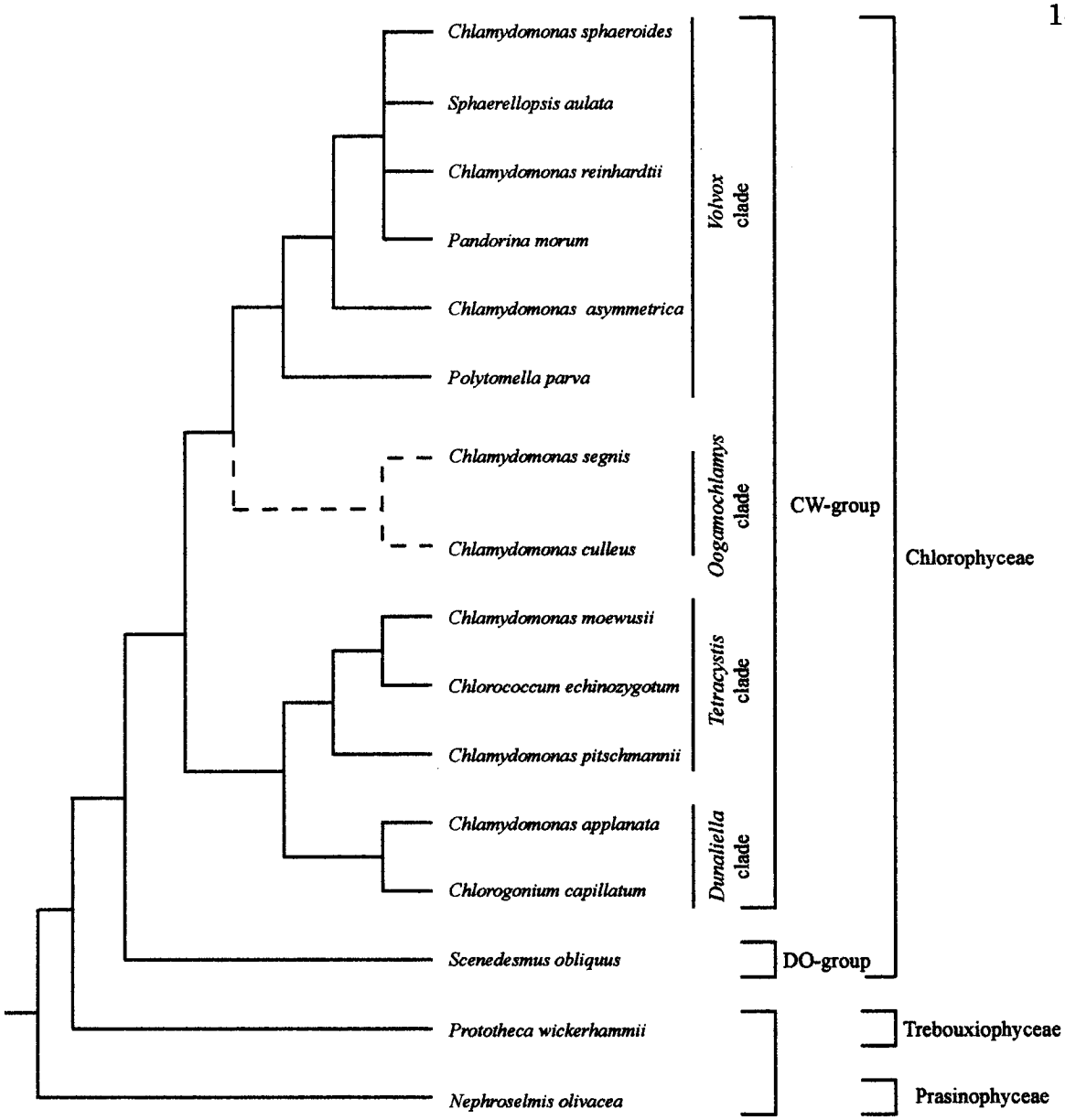


Figure 2. Dendogram depicting four main lineages within the CW-group of green algae. The *Volvox*-clade, *Tetracystis*-clade and *Dunaliella*-clade are as defined by Nakayama et al. (1996), and the *Oogamochlamys*-clade is that of Pröschold et al. (2001). Further phylogenetic inferences are based on the results of Turmel et al. (1993), Buchheim et al. (1996) and Hepperle et al. (1998).

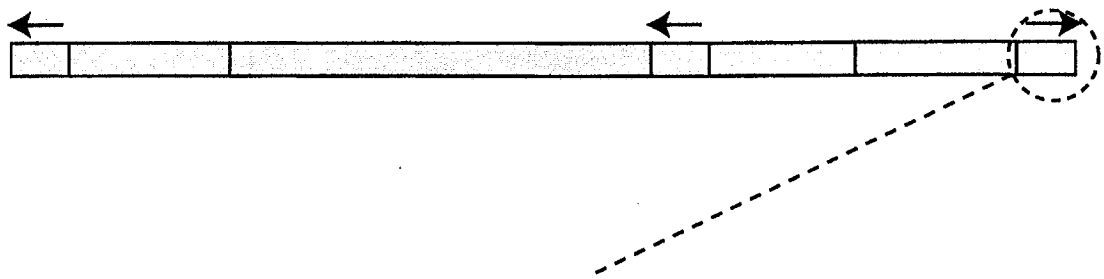
(Buchheim et al.1996), and mitochondrial (Kroymann and Zetsche 1998; Nedelcu et al.2000) DNA sequence data suggest that taxa from the *Tetracystis*-clade and *Dunaliella*-clade shared a common ancestor more recently than did any of these with taxa from the *Volvox*-clade (Figure 2). On the other hand, only the plastid data strongly supports the view that the *Oogamochlamys*-clade is a sister of the *Volvox*-clade, to the exclusion of the *Tetracystis*-clade and the *Dunaliella*-clade. The nuclear and mitochondrial data, however, are not inconsistent with this possibility. The dashed line in Figure 2, connecting the *Oogamochlamys*-clade to the *Volvox*-clade represents this uncertainty.

To date, examples of green algal linear mtDNAs are confined to various strains of *C. reinhardtii* and *Pandorina morum*, as well as the colorless taxon, *Polytomella parva*, all of which affiliate with the *Volvox*-clade of the CW-group (Table 1). In contrast, the mitochondrial genomes of two *C. moewusii* strains and that of *C. capillatum*, which affiliate with the *Tetracystis*-clade and the *Dunaliella*-clade, respectively, are both circular-mapping. Currently, nothing is known of the mtDNA conformation in *Oogamochlamys*-clade taxa.

Features of the CW-group linear mtDNAs

At least three CW-group linear mtDNAs have been identified to date, namely those of *C. reinhardtii* (Ryan et al. 1978; Boer and Gray 1985;

Vahrenholz et al. 1993), *Pandorina morum* (Moore and Coleman 1989), and *Polytomella parva* (Fan and Lee, 2002); complete and nearly complete sequences are available for *C. reinhardtii* and *P. parva*, respectively. The linearity of the *C. reinhardtii* mtDNA has been confirmed both by electron microscopy (Grant and Chang 1978) as well as DNA sequencing (Vahrenholz et al. 1983). This DNA exists in nearly equal amounts of two micro-heterogeneous linear forms (Vahrenholz et al. 1993). The ends of these mtDNAs are composed of identical sequences in inverted orientation, extending over 531 or 532 nucleotides, and have non-complementary 3' tails, which are either 39 or 41 nucleotides long (Figure 3). The outermost 86 nucleotides of the long terminal inverted repeats (LTIRs) are identical to an internal 86 bp sequence, which is located at the 3' end of the L2 rDNA fragment (Vahrenholz et al. 1993). Sequence micro-heterogeneity has also been detected by restriction mapping of the *P. parva* mtDNA (J. Fan, personal communication). Further, restriction mapping as well as partial sequence data have confirmed the presence of LTIRs in *P. morum* (Moore and Coleman 1989) and *P. parva* (Fan and Lee, 2002) respectively, although these sequences share no apparent similarity to that of the *C. reinhardtii* mtDNA. Finally, the mitochondrial genome of *C. reinhardtii* contains a unique open reading frame (ORF), which shows similarity to a reverse transcriptase. This reverse transcriptase-like (RTL) gene is not found in any of the other



5'–TATAGGATCGTGCGAGTCGTAGAAGACGGCTGGTAGGATGCAGATCAACCAAACCGATACTGTTTGCGCACTTAGGCATGCGTAG**T**–3'
3'–ATATCCTAGCACGCTCAGCATCTTCTGCCGACCATCCTACGTCTA–5'

or

5'–TATAGGATCGTGCGAGTCGTAGAAGACGGCTGGTAGGATGCAGATCAACCAAACCGATACTGTTTGCGCACTTAGGCATGCGTAG–3'
3'–ATATCCTAGCACGCTCAGCATCTTCTGCCGACCATCCTACGTCTA**G**–5'

Figure 3. Schematic representation of the *Chlamydomonas reinhardtii* mtDNA (not to scale). Checkered sections represent the Long Terminal Inverted Repeats (LTIRs). Hatched sections represent the outermost 80-nt of the LTIRs, and the internal copy of the 80-nt sequence. Black arrows indicate the direction of the repeat. The magnification shows the sequence of the 80 outermost nucleotides, including the 39- or 41-nt 3' tails. The nucleotides in bold lettering represent the heterogeneity between the mtDNAs.

completely sequenced green algal mitochondrial genomes, or in the nearly complete sequence of the linear *P. parva* mtDNA (Fan and Lee, 2002).Although it has been proposed that RTL plays a role in the replication of the *C. reinhardtii* linear mtDNA (see below), studies have failed to show that the RTL gene product has any reverse transcriptase activity (Faßbender et al. 1994). Moreover, it has been proposed that this unique ORF is no more than the remnant of a degenerate group II intron (Nedelcu and Lee 1998).

Replication of the CW-group mtDNAs

Regions showing similarities to origins of replication in vertebrate mtDNA (Macey 1997) have been found in both *C. moewusii* and *C. capillatum* (Nedelcu and Lee 1998), although there is no experimental evidence to support their use as such *in vivo*. Nonetheless, it seems plausible that if these are indeed circular genome-sized molecules they could replicate using a theta-type replication mechanism (Cairns 1963; Kornbergh and Baker 1993).

On the other hand, the linear mtDNAs of the CW-group algae must have an alternative method of replication. It was first proposed (Grant and Chiang 1980) that the linear mtDNA of *C. reinhardtii* was derived from a circular molecule and that it employed a theta-type replication mechanism. This data is equivocal, however, and there is support for the view that the *C. reinhardtii* mtDNA replicates as a linear molecule (Ma et al. 1992;

Vahrenholz et al. 1993). The 3' overhangs of the *C. reinhardtii* mtDNA are non-complementary and thus re-circularization does not seem plausible, nor does the possibility of a phage T7-like replication mechanism (Watson 1972). The presence of an internal sequence that is identical to the outermost sequence of the LTIRs suggests that some form of hairpin loop may be possible, thus bringing to mind a replication mechanism similar to that of adenoviruses. This seems unlikely, however, given that (i) adenoviruses have proteins covalently attached to the 3' ends of the molecules and (ii) they require a specialized DNA polymerase to initiate replication without conventional RNA primers. In contrast, the ends of the *C. reinhardtii* mtDNA are readily labeled by T4 polynucleotide kinase (Vahrenholz et al. 1993) indicating that there are no attached proteins, and the mitochondrial γ-DNA polymerase (Bolden et al. 1977; Kornbergh and Baker 1992) has been detected in *C. reinhardtii* (Wang 1991), which suggests mtDNA replication uses the standard mitochondrial DNA polymerase. Finally, although the ends of the *C. reinhardtii* mtDNA are inverted repeats, they show no similarity to the telomeric repeats found in other organisms, or to the nuclear telomeres that have been identified in *C. reinhardtii* (Petracek 1990).

Vahrenholz et al. (1993) have proposed two models to explain mtDNA replication in *C. reinhardtii.* The first model involves the transcription of the internal copy of the repeated sequence, followed by the reverse transcription of this RNA to complete the ends of the molecule (Figure 4). This model

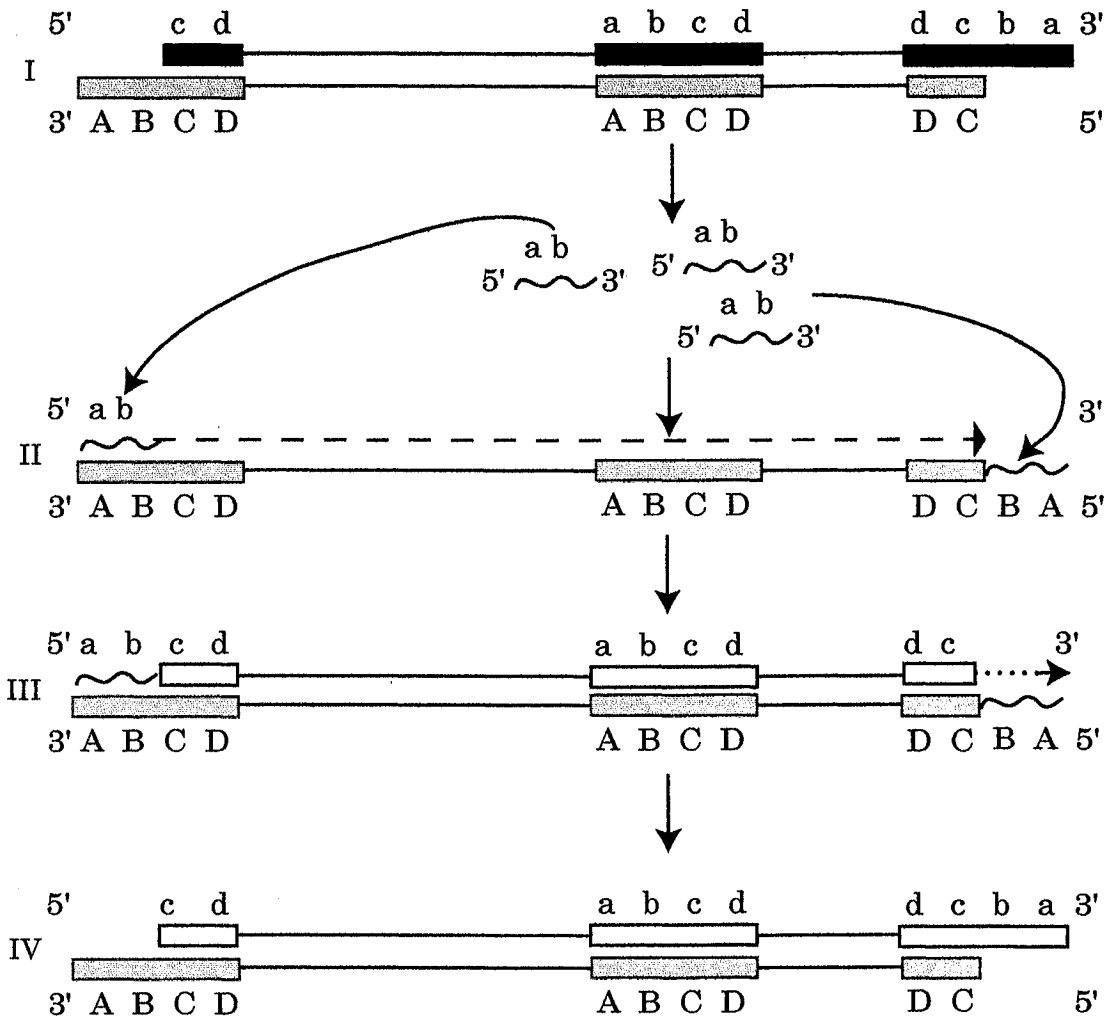


Figure 4. Reverse transcriptase dependant model for the replication of *C. reinhardtii* mtDNA. (I) A transcript [ab] is produced from the internal copy of the repeat. (II) This transcript can serve as an RNA primer, and DNA replication proceeds up to the end of the original DNA strand. Meanwhile a second copy of the ab transcript attaches to the 3' end the original DNA strand. (III) A reverse transcriptase can produce a full-length daughter strand (dotted line). (IV) Removal of the ab RNAs from the RNA-DNA heteroduplex would maintain the 3' overhangs.

seems appealing as it offers a "raison d'être" for the RTL gene, and in addition, it may explain how the 3' overhangs are maintained. Recall, however, that Nedelcu and Lee (1998) have suggested that RTL is the remnant of a group II intron, and probably has no function in the genome, and that Faßbender et al. (1994) have shown that the protein encoded by RTL has no activity in a yeast host, although we cannot exclude the possibility that this protein does function in *Chlamydomonas*. It should be noted, however, that even if RTL does not function in *Chlamydomonas*, the model may still be appropriate, as the mitochondrial DNA polymerase-γ, which likely functions in *C. reinhardtii* (Wang 1991), is known to exhibit reverse transcriptase-like activity (Fridlender 1972). This model may however have another problem, which derives from the micro-heterogeneity discussed earlier. In order to replicate the two differently sized mtDNAs, two different RNAs would have to be transcribed from the same template. The second model proposed for the replication of the *C. reinhardtii* mtDNA involves a site-specific endonuclease and recombination between the repeated sequences (Figure 5). This model is appealing because (i) it is a more general model, which has been suggested in other systems (Heumann, 1976) and (ii) there is no need for a reverse transcriptase. On the other hand, it does not explain the maintenance of the 3' tails, or the micro-heterogeneity among the mtDNAs. Presently, neither model is completely satisfactory, and there are insufficient data to favor one model over the other.

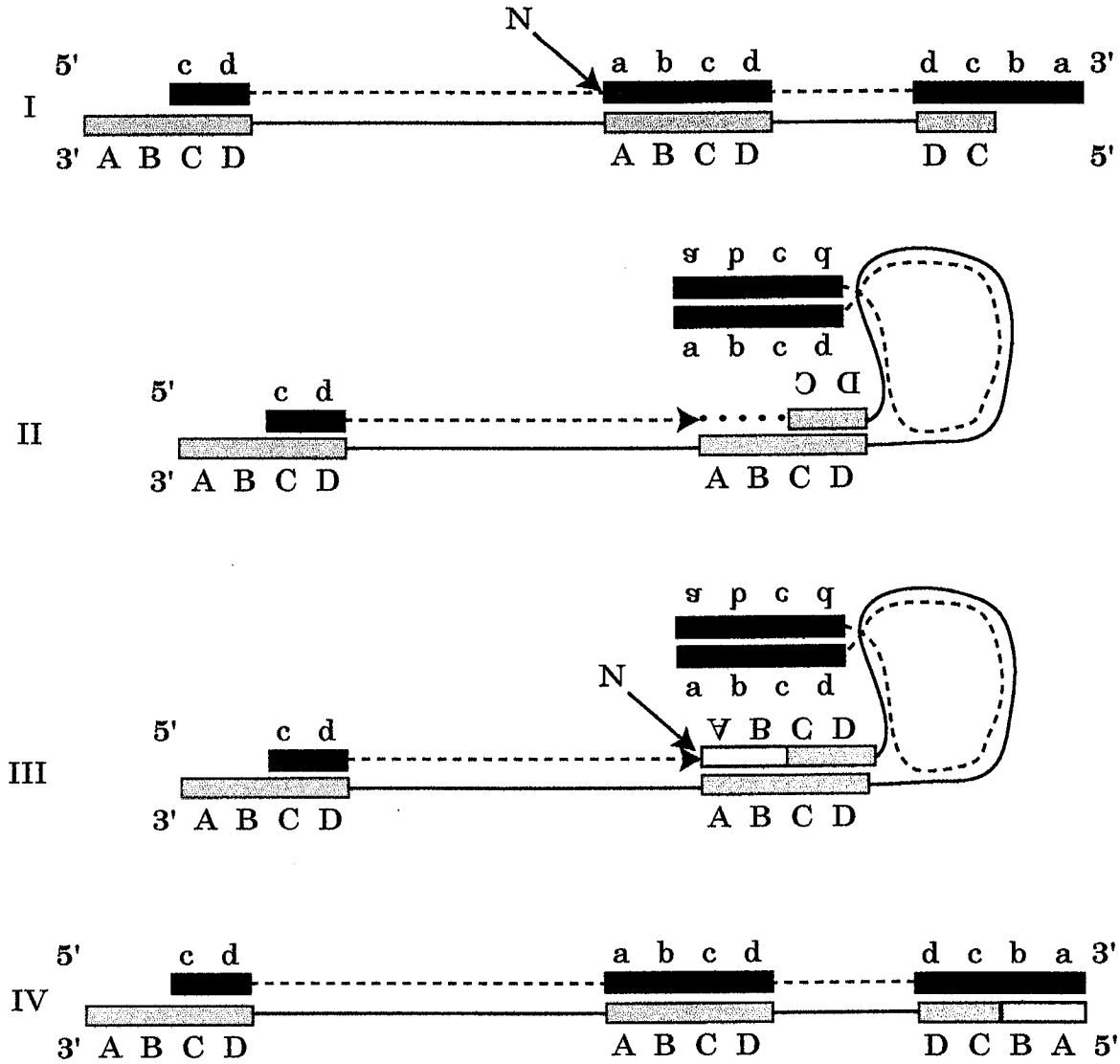


Figure 5. Recombination-dependant model for the replication of *C. reinhardtii* mtDNA. (I) A site-specific endonuclease nicks the double-stranded mtDNA (black arrow). (II) Intra-strand recombination occurs between the internal repeat and one of the external repeats. DNA polymerase can fill in the gap created by the recombination event (dotted line). (III) The endonuclease nicks the DNA again (black arrow). (IV) The resolved product is blunt on one end. The process may be repeated on the other end, producing a blunt-ended molecule.

CHAPTER I

Mitochondrial genome conformation among taxa from the CW-group *Volvox*-, *Tetracystis*- and *Dunaliella*-clades

INTRODUCTION

Information pertinent to mitochondrial genome size and conformation in green algae was discussed in the GENERAL INTRODUCTION and is summarized in Table 1. Recall that all of the linear mitochondrial genomes identified in the Chlorophyta are associated with the chlorophycean "*Volvox*-clade" of Nakayama et al. (1996). The existence of this clade is supported by phylogenetic analyses of nucleus-encoded small and large subunit rRNA (Buchheim et al. 2001) as well as chloroplast LSU rRNA sequence data (Turmel et al. 1993, Buchheim et al. 1996).

Though all green algal mitochondrial genomes characterized outside this clade are circular-mapping, little is known about the *in vivo* structure of these mtDNAs. For example, although restriction fragment analysis reveals a circular-mapping mitochondrial genome for two *Chlamydomonas moewusii* strains (Denovan-Wright and Lee 1992, Lee et al. 1991), Bendich (1993) has commented that these mtDNAs may exist as larger than genome-sized linear molecules. In addition, although the existence of a linear genome-sized mtDNA in *Chlamydomonas reinhardtii* is indisputable (Ryan et al. 1978, Boer et al. 1985, Vahrenholz et al. 1993), the possibility that this mtDNA is produced by the site-specific cleavage of a genome-sized circular (Ryan et al. 1978, Grant and Chang 1980) or concatemeric mtDNA (Bendich 1993) has not been eliminated.

Because of the importance of mitochondrial genome conformation to our understanding of the function and evolution of this genome, the study of mtDNA structure in the CW-group of the Chlorophyceae was undertaken. A variety of gel electrophoretic techniques were employed which provided evidence regarding the *in vivo* conformation of the mtDNA in taxa from this group. The specific questions addressed in this chapter are as follows: (i) Does the mtDNA of *C. reinhardtii* exist in conformations other than the 15.8 kb linear form? (ii) Is the major component mtDNA molecule in *C. moewusii* a circular genome-sized molecule, or does this mtDNA exist mostly as larger than genome-sized linear molecules? (iii) Is linear genome- or subgenomic-sized mtDNA a feature among green algae that is characteristic of and restricted to the *Volvox*-clade of the CW-group?

MATERIALS AND METHODS

Strains, culture conditions, and cell harvesting

The algal strains employed in this study and their sources are listed in Table 2. With the exception of *P. parva*, the only non-photosynthetic taxon employed, all strains were grown under "cool-white" fluorescent bulbs in the minimal medium of Gowans (1960) with twice the concentration of ammonium nitrate and bubbled with 1.5 % CO_2 in air. Unless stated otherwise, these strains were cultured under synchronous growth conditions (alternating 12 h light and 12 h dark periods), and the irradiance level and culture density were adjusted so that cell number increased approximately four-fold during successive cell cycles (Whiteway and Lee 1977). In some instances, identified in the RESULTS section, cells were cultured under exponential growth conditions (continuous light maintained at 250 umol/m^{-2}/s^{-1} PAR). *P. parva* was grown in the medium of Sheeler et al. (1968) and bubbled with air. During the late logarithmic phase of growth in the case of the exponential cultures, and at the onset of the light period in the case of the synchronous cultures, when the cell numbers reached about 4 x 10^6 cells/mL in both instances, cultures were cooled on ice and the cells were harvested by centrifugation (6000 x g) at 4 °C.

Table 2. Taxa used in Chapter I

Taxon	**Source /Comments**
Chlamydomonas sphaeroides – (V)	SAG 25.72, (formerly *C. iyengarii*)
Sphaerellopsis aulata – (V)	SAG 69.72 (formerly *Chlamydomonas gelatinosa*)
Chlamydomonas reinhardtii – (V)	UTEX 2244
Chlamydomonas reinhardtii – (V)	UTEX 2337, = CW15)
Chlamydomonas asymmetrica – (V)	SAG 70.72 (formerly *C. peterfii*)
Polytomella parva – (V)	UTEX L193 (formerly *P. agilis*)
Chlorogonium capillatum – (D)	SAG 12-2e (formerly *C. elongatum*)
Chlamydomonas pitschmannii – (T)	SAG 14.73
Chlamydomonas moewusii – (T)	UTEX 9, (formerly *C. eugametos*)
Chlorococcum echinozygotum – (T)	UTEX 118

V, D, and T identify taxa in the *Volvox*-clade, the *Dunaliella*-clade, and the *Tetracystis*-clade, respectively; see Table 1 for references.

Preparation of total DNA

Total DNA was prepared using a modification of the method of Lemieux et al. (1980). Harvested cells were washed in ice-cold saline-EDTA (150 mM NaCl; 10 mM EDTA, pH 8) and then resuspended in 450 µL of ice cold Buffer A (100 mM Tris-HCl; 150 mM NaCl; 10 mM EDTA, pH 8). Cell lysis was achieved by a 37 °C incubation in 50 µg/mL of proteinase K and 0.5 % SDS for 14-18 h. Ammonium acetate was added to cell lysates at a final concentration of 2.5 M, and these were then extracted twice with one volume of Tris-buffered phenol : chloroform : isoamyl alcohol (24:24:1), followed by ethanol precipitation. The nucleic acid pellet was resuspended in 200 µL of TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA). RNase A was added to a final concentration of 10 µg/mL, and incubated at room temperature for 10 min. Protein material was precipitated with the addition of ammonium acetate to a final concentration of 2.5 M, followed by a 10 min incubation on ice and a one minute centrifugation at 13 000 x g. The DNA was precipitated from the resulting supernatant by the addition of 2.5 volumes of absolute ethanol and a 20 min centrifugation at 13 000 x g. The final DNA pellet was resuspended in 200 µL of TE. To minimize physical damage, all transfers of DNA were done using wide-bored pipette tips, and following precipitation steps, the DNA was allowed to resuspend overnight at room temperature without agitation. DNA was also isolated from strain CW15, a cell-wall deficient

mutant of *C. reinhardtii*, using an in-gel technique (Carle and Olsen 1987). Briefly, cells were embedded in 1 % low melting point agarose (Life Technologies, Rockville, MD), and the agarose plugs were incubated at 37 °C in Buffer A containing 1 mg/mL of proteinase K, then rinsed in 1 % SDS for 14-16 h. A further incubation at 37 °C, in 0.5 % SDS, was performed for 4 h after which the plugs were rinsed briefly in Buffer A and used directly for pulsed field gel electrophoresis (PFGE)

Restriction digests and gel electrophoresis

Unless otherwise indicated, one unit of restriction enzyme per microgram of total DNA was incubated at 37 °C in the buffer recommended by the manufacturer (New England Biolabs, Beverly, MA) for 1 h.

Total DNA (normally 1-3 μg) was fractionated using either PFGE or conventional agarose gel electrophoresis. PFGE was performed using the CHEF-DRII system (Biorad, Hercules, CA) with the following run conditions: 1% PFGE certified agarose (Biorad) in 1X TAE buffer (40 mM Tris-acetate; 1 mM EDTA, pH 8.0), switch times of either 15 or 75 s, and 6 V/cm for 13.5 h. The DNA markers used in these gels were phage λ concatemers (Low Range PFG Marker, New England Biolabs). Conventional gel electrophoresis was performed at room temperature in either 0.6% or 1% agarose NA (Amersham

biosciences, Piscataway, NY) at 0.5 V/cm for 24 h. The DNA markers used in these gels were phage λ DNA digested with *Hin*dIII.

Southern transfer and hybridization

Fractionated DNA was transferred to nylon membranes (Hybond N+, Amersham biosciences) using standard methods (Sambrook et al.1989). The probes used for hybridization analysis were constructed in the following manner: the five largest *Hin*dIII fragments and the 1.2 kb *Pvu*II fragment, which constitute the entire previously cloned mtDNA of *C. moewusii* (= *C. eugametos*, UTEX 9; Denovan-Wright and Lee 1992), were cut out of their vectors and purified by gel electrophoresis, prior to non-radioactive labeling using the AlkPhos Direct Labeling and Detection System (Amersham biosciences). These labeled DNA fragments were mixed in stoichiometric proportions and termed the "*C. moewusii* mtDNA probe." Two DNA fragments (E1 and H1), which constitute approximately 80 % of the previously cloned mitochondrial DNA of *C. reinhardtii* (Boer et al.1985), were labeled, mixed as above, and termed the "*C. reinhardtii* mtDNA probe." Nylon membranes were hybridized with either of these probes at 60 °C for 18 to 24 h, and washed according to the directions of the labeling and detection system. Chemiluminescent detection was then achieved by exposing auto-radiographic film to the membranes.

RESULTS

Search for additional mitochondrial DNA conformations in *C. reinhardtii*

It was of interest to determine if *C. reinhardtii* contains conformations of mtDNA other than the well-characterized 15.8 kb linear form. In these experiments, cells were grown under exponential culture conditions so that all vegetative cell-cycle stages would be represented. Total cellular DNA was fractionated by PFGE at a 15 s switch time after in-liquid cell lysis and DNA purification, in the case of the wild-type strain, and following in-gel lysis, in the case of the cell-wall deficient strain, CW15. Only one hybridizing-component, which migrated as a 16 kb linear molecule, was detected in both samples following Southern blot analysis with the *C. reinhardtii* mtDNA probe, even after overexposure of the films (Figure 6). Similar results were obtained following PFGE at a 75 s switch time, which makes it unlikely that a non-linear mtDNA form was co-migrating by chance with the linear one under both conditions of PFGE.

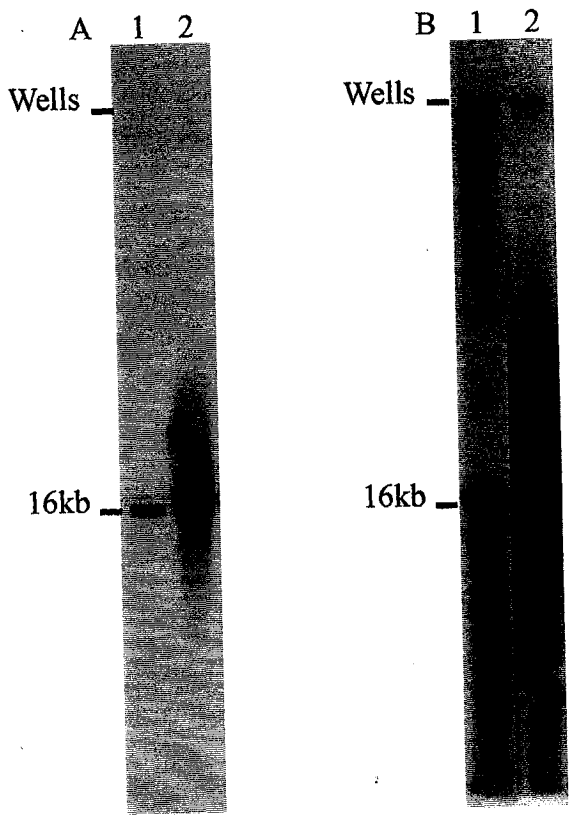


Figure 6. Southern blot analysis of *C. reinhardtii* mtDNA. *C. reinhardtii* wild-type (lane 1) or CW15 (lane 2) DNA was fractionated by PFGE with a 15s switch interval, transferred to a nylon membrane, hybridized with a *C. reinhardtii* probe, and exposed to film for 15 min (A) or 60 min (B).

Conformation of *C. moewusii* mitochondrial DNA

It was also of interest to determine the conformation of the mtDNA in *C. moewusii*. As there is no known cell-wall deficient strain of this species, and all attempts to use the in-gel lysis procedure with walled strains failed, the standard in-liquid method of cell lysis and total DNA extraction was employed. Three bands hybridizing with the *C. moewusii* mtDNA probe were visible following fractionation of total *C. moewusii* DNA at the 15 and 75 s pulse times (Figure 7A, lane 1). Based on its mobility relative to linear markers, the fastest migrating component under both gel conditions appeared to be linear 23 kb DNA, the size of the *C. moewusii* mitochondrial genome. The slowest migrating component, designated β, was likely circular DNA since it migrated as would linear DNA of approximately 55 kb at the 15 s pulse-time and as would linear DNA of approximately 135 kb at the 75 s pulse-time. The third DNA component detected by the mtDNA probe, designated α, showed less hybridization than the other two components and migrated as would a linear DNA of about 35 and 40 kb at the 15 and 75 s pulse times, respectively. Following digestion with *Sal*I, a restriction enzyme having a single recognition site in the circular physical map of this taxon's mtDNA (Denovan-Wright et al. 1998), only one hybridizing component was visible in the electrophoretically fractionated total cellular DNA of *C. moewusii*; this DNA component co-migrated with the 23 kb linear marker

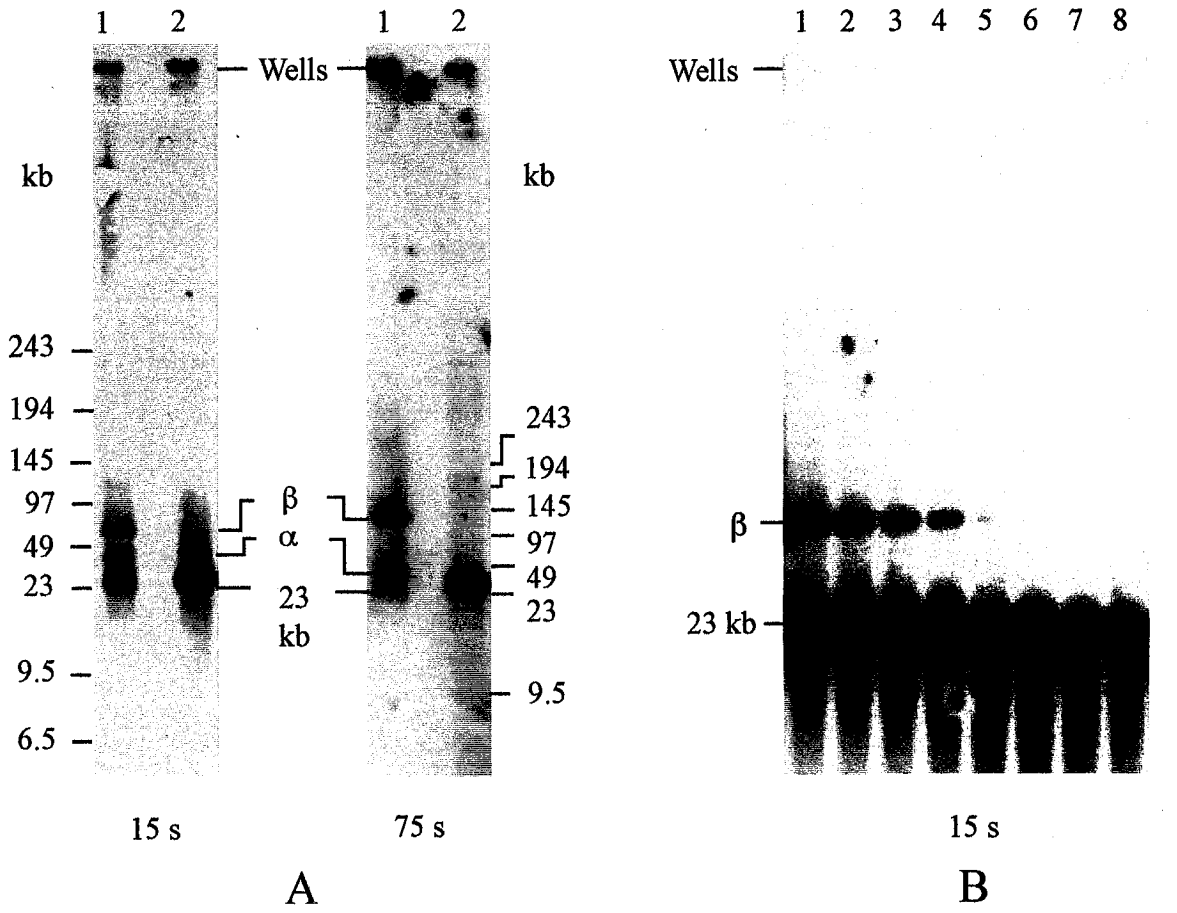


Figure 7. Total DNA from *C. moewusii* fractionated by PFGE. (A) DNA was fractionated at either 15 or 75 s switch intervals as indicated, and hybridized with the *C. moewusii* mtDNA probe: Lanes: 1, undigested DNA; 2, DNA digested with *Sal*I. (B) DNA fractionated by PFGE at a 15 s switch interval and hybridization with the *C. moewusii* mtDNA probe. DNA was digested for 30 min with increasing amounts of *Sal*I. Lanes: 1, DNA undigested; 2 - 8, DNA digested with 0.01, 0.05, 0.1, 0.5, 1 and 2 units of *Sal*I, respectively.

at both pulse times (Figure7A, lane 2) and therefore was likely linear DNA. The disappearance of components α and β, and the increased amount of the 23 kb linear DNA following *Sal*I digestion suggests that these components were circular molecules, likely supercoiled and relaxed circles, respectively, which were converted into the linear 23 kb DNA. Similar results were obtained following digestion with *Xba*I or *Eag*I (data not shown); these restriction enzymes also have only one recognition site in the *C. moewusii* mtDNA physical map, and each cuts at a different region of this map.

To test the hypothesis that the proposed circular forms of *C. moewusii* mtDNA identified in Figure 7A, were genome-sized molecules, total cellular DNA from *C. moewusii* was digested with a range of *Sal*I concentrations such that the lower concentrations produced only partial digestion, and the higher concentrations produced complete digestion. In contrast to the undigested samples shown in Figure 7A, the control DNA sample in Figure 7B had a lower proportion of component β and no detectable component α. This could have resulted from the complete and partial degradation of the α and of the β components, respectively, as a result of the 37 °C incubation in *Sal*I buffer. From the results shown in Figure 7B it is apparent that the gradient of loss of component β, in relation to the increased opportunity for *Sal*I digestion, was not associated with the appearance of any linear DNAs, which had sizes that were multiples of 23 kb, as one might expect from partial digestion of circular multimers. Rather these data suggest that the β–component was

converted directly into genome-sized linear DNA. It is noteworthy that the 23 kb linear DNA in this experiment, in contrast to the sample from Figure 7B, appeared to decrease rather than increase after *Sal*I digestion. This can be explained by the greater proportion, prior to digestion, of linear mtDNA in the sample from Figure 7A compared to the samples from Figure 7B, and the likelihood that the linear mtDNAs in the undigested samples are derived from circular monomers which were broken randomly relative to the single *Sal*I site.

Distribution of CW-taxa with linear mitochondrial genomes

A limited survey of mtDNA conformation was performed, using gel electrophoresis, among selected CW-group taxa with the goal of testing the hypothesis that linear mitochondrial genomes are a feature characteristic of, and restricted to, the *Volvox*-clade of this group. In these experiments, conventional gel electrophoresis was employed using both 0.6% and 1.0% agarose. Total cellular DNA prepared from all taxa examined from the *Volvox*-clade revealed only one band following hybridization with the *C. reinhardtii* mtDNA probe (Figures 8A and C). In each case, these bands co-migrated with the linear size markers, and correspond to sizes of about 14 kb in *P. parva* and *Chlamydomonas asymmetrica* and approximately 25 kb or

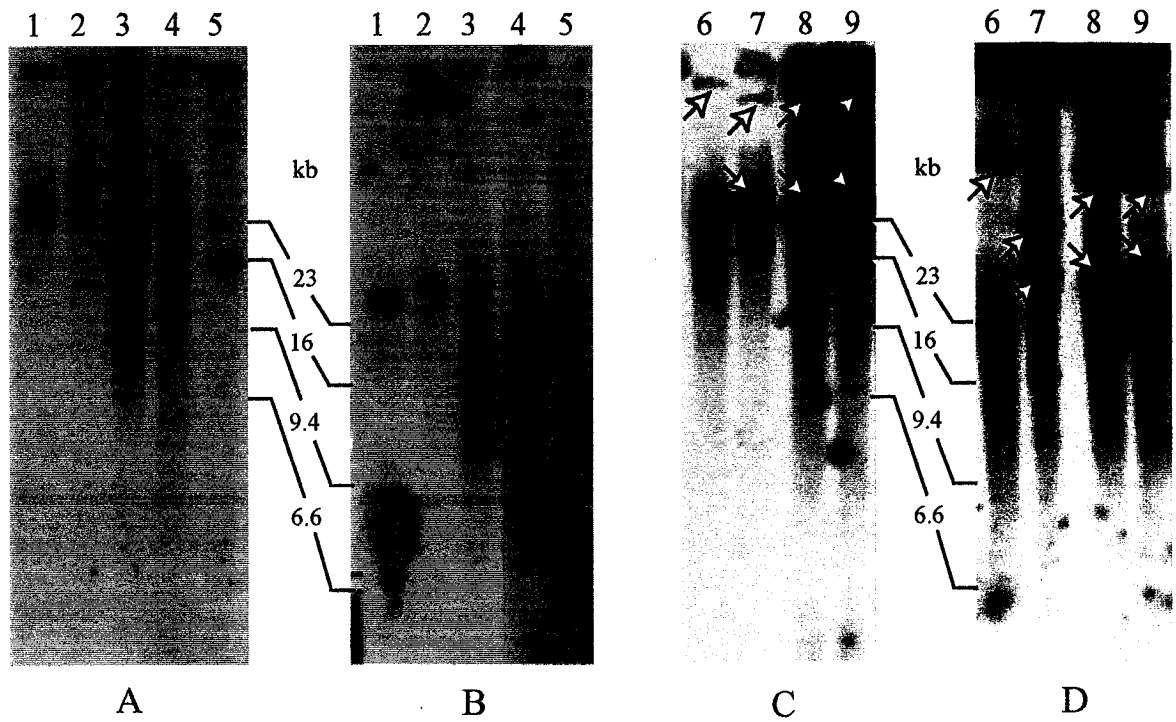


Figure 8. Analysis of mtDNA from various CW-group taxa fractionated by conventional gel electrophoresis. DNA was fractionated through 1.0 % (A and C) or 0.6 % agarose (B and D). Gels (20 cm) were run in 1X TAE at 0.5 V/cm for 24 h; hybridization analysis was done with either the *C. reinhardtii* mtDNA probe (A and B) or the *C. moewusii* mtDNA probe (C and D). The 23-, 9.4- and 6.6-kb pointers correspond to the position of the three largest the 1-*Hin*dIII fragments, whereas the 16-kb pointer corresponds to the position of the 15.8-kb linear DNA of *C. reinhardtii*. Lanes: 1, *C. sphaeroides*; 2, *S. aulata*; 3, *C. reinhardtii*; 4, *C. asymmetrica*; 5, *P. parva*; 6, *C. capillatum*; 7, *C. pitchsmannii*; 8, *C. moewusii*; 9, *C. echinozygotum*. Arrowheads indicate the location of the proposed non-linear mtDNA forms in panels C and D.

more in *Chlamydomonas sphaeroides* and *Sphaerellopsis aulata*: the latter two mtDNAs being at the limit of the linear size range for these gels.

The results of Southern blot analysis of total cellular DNA from taxa outside the *Volvox*-clade, specifically, within the "*Dunaliella*-clade" and the "*Tetracystis*-clade" of Nakayama et al. (1996), following standard gel electrophoresis and using the *C. moewusii* mtDNA probe, are shown in Figures 8B and D. All of these samples, including the one from *C. moewusii*, revealed two or more hybridizing-components. The lower-most band, in each case, co-migrated with linear size markers in both the 0.6% and 1.0% gels; these are assumed to have resulted from randomly broken circles with the smallest one being about 16 kb in *Chlamydomonas pitschmannii* and the largest ones being about 23 kb in *C. moewusii* and *C. capillatum*, the size of the mitochondrial genome maps for each of these taxa (Table 1). DNA from all taxa, with the exception of *C. capillatum*, showed two bands above the compression zone of linear DNA in these gels; these bands likely represent relaxed and supercoiled circular DNA. The *C. capillatum* mtDNA showed only one DNA component above the linear DNA band and this probably represented relaxed circular DNA.

DISCUSSION

Mitochondrial DNA conformation in *C. reinhardtii* and *C. moewusii*

The analyses suggest that the only mtDNA conformation in vegetative cultures of *C. reinhardtii* is its well-characterized 15.8 kb linear DNA molecule. Ryan et al. (1978), using electron microscopy, detected rare (≤1%) circular DNA molecules in mitochondrial-enriched DNA fractions of *C. reinhardtii* similar in density and contour length to that of the linear mtDNA. If such molecules represent circular forms of the *C. reinhardtii* mtDNA, as proposed by Ryan et al. (1978) and Grant and Chiang (1980), and they represent at least 0.1% of the mtDNA, they should have been detected by the Southern blot analyses, especially with the overexposed films. On the other hand, if the fraction of circular mtDNA represents less than 0.1% of the mtDNA and given estimates of mtDNA copy number in *C. reinhardtii* at about 50 molecules per cell (Ryan et al. 1978), such circular molecules would occur, assuming random distribution among cells, in fewer than one of 20 cells. Although Ma et al. (1992) and Duby et al. (2001) obtained a 0.8 kb PCR product which seemingly bridges the ends of the *C. reinhardtii* mtDNA, this product is missing part of the right terminus and may have resulted from a naturally occurring, partly circular mtDNA structure, such as proposed in the

recombination-mediated replication model of Vahrenholz et al, (1993; Figure 5).

The data do not support the possibility that the mtDNA in vegetative cultures of *C. reinhardtii* also exists as large, linear molecules composed of a multimeric array of unit genomes as detected in other systems (Maleszka 1993, Bendich 1996). Such molecules, if present in at least one copy per cell, should have been detected following the Southern blot analyses. The faint hybridization signals, corresponding to the position of the wells in Figure 6, are only visible after overexposure of the film represent much less than one percent of the total hybridization signal.

Restriction fragment mapping (Lee et al. 1991) and DNA sequencing (Denovan-Wright et al. 1998) have shown that two strains of *C. moewusii,* UTEX 97 and UTEX 9, have circular-mapping genomes. Bendich (1993) has pointed out, however, that these data are consistent not only with genome-sized circular mtDNAs, but also with a linear, concatemeric array of unit genomes. The *C. moewusii* DNA samples shown in Figure 7A show hybridization signals in the well regions of the gel corresponding to about 10 to 20 % of the total signal, therefore leaving open the possibility that large multimeric mtDNA molecules are being retained in the wells. It is noted, however, that following *Sal*I digestion, such molecules should have been converted into genome-sized molecules, and no reduction in the amount of well-bound hybridization was detected when such a digestion was performed.

In another, albeit more degraded DNA preparation (Figure 7B, lane 1), no label was detected in the well region and there were no signs of larger than genome-sized degradation products, therefore arguing against the possibility of large multimeric mtDNA molecules in *C. moewusii.*

Rather, the *C. moewusii* mtDNA seems to exist as a mixture of genome-sized circular and linear DNA molecules. Of the two proposed circular conformations detected (α and β), the view that component β is a relaxed circular form and component α is a supercoiled circular form is favored. In support of this possibility, it has been observed that DNA isolated from CsCl gradients, which involves much physical manipulation, or DNA that has been stored for extended periods of time, shows a higher proportion of the linear form and little or no component α (data not shown). In addition, it seems that both forms α and β can be directly converted into linear genome-sized molecules by the action of restriction endonucleases having only one restriction site in the physical map of the *C. moewusii* mtDNA. As for the 23-kb linear mtDNA component observed in the undigested DNA samples, the favored view is that these result from random double-strand breaks in components α and β, which occur during DNA isolation, rather than by the site-specific cleavage of an unknown endonuclease *in vivo.* In the latter case, one would expect that digestion of this linear DNA fraction with restriction enzymes having only one recognition site in the circular mitochondrial genome map would produce two distinct DNA components

with a combined size of 23 kb; this was not observed when the DNA was digested individually with three such restriction enzymes that cut in different regions of the mitochondrial genome map.

Conformation of CW-group mitochondrial DNAs

The hypothesis that genome- or subgenomic-sized linear mtDNAs are characteristic of the *Volvox*-clade and that other CW-group taxa have circular genome-sized mtDNAs was tested. In support of this, DNA from all *Volvox*-clade taxa tested revealed only single hybridizing bands following Southern blot analysis with the mtDNA probe. It is probable that the linear hybridizing-components reflect unit mitochondrial genomes although in the case of *P. parva*, Fan and Lee (2002) have shown that there is at least one additional small, subgenomic mtDNA encoding *nad6*, which is not detected with the *C. reinhardtii* mtDNA probe employed in this study. Although no colonial CW-group algae were included in the present study, it has been demonstrated that the mitochondrial genome from one such taxon, *Pandorina morum*, is linear (Moore and Coleman 1989). In addition, it has been shown that this taxon, along with *Volvox carteri* and a variety of other colonial algae, are closely related to one another and that as a group they form a lineage with *C. reinhardtii* to the exclusion of taxa from the

Dunaliella-clade and the *Tetracystis*-clade (Coleman 1999). Hence, it is likely that all of these taxa have linear mitochondrial genomes.

In contrast, all taxa examined from the *Dunaliella*-clade and the *Tetracystis*-clade, with the exception of *C. capillatum*, had three DNA components detected by the *C. moewusii* mtDNA probe. These bands likely correspond to open- and closed-circular mtDNA molecules, along with linear genome-sized mtDNA molecules that were derived from the circular forms during DNA isolation, as suggested for *C. moewusii*. It is expected that under the electrophoretic conditions used here, the faster migrating (lower) non-linear DNA is a supercoiled circular DNA, and the slower migrating (upper) non-linear DNA is a relaxed circular DNA. Consistent with this possibility, it was noted that the faster migrating non-linear form of the 16 kb mtDNA of *C. pitchsmannii* migrated to approximately the same position as the faster migrating non-linear form of the 23 kb mtDNA of *C. moewusii*, an observation that could be explained by the unpredictable migration of supercoiled molecules through agarose gels, which is dependant not only on the size of the molecules but also on their superhelical density (Johnson and Grossman 1977). The single non-linear mtDNA form of *C. capillatum* is probably the open circular form with the closed circular form being absent as a result of DNA degradation. It is also noted that although the physical map of the *C. capillatum* mtDNA is smaller than that of *C. moewusii* (Table 1), the *C. capillatum* linear mtDNA migrated slower than the linear mtDNA of *C.*

moewusii. This could be explained by a migration retardation of the *C. capillatum* DNA due to the intentional overloading of this sample in order to obtain an appropriate hybridization signal.

Origin of linear mtDNAs among the CW-group algae

Available data are consistent with the hypothesis that the linear, genome- or sub-genomic-sized mtDNA conformation in green algae is limited to the *Volvox*-clade of the chlorophycean CW-group and that this linear DNA was derived from an ancestral circular form. This circular form appears to have been maintained in all other CW-group taxa examined and, based on mapping data, possibly all other green algae. In an attempt to determine if the origin of the linear mtDNA form occurred at the base of the *Volvox*-clade or earlier in the evolution of the CW-group, it will be necessary to examine mtDNA conformation in other extant clades, which are closely related to the *Volvox*-clade, such as the "*Oogamochlamys*-clade" of Pröschold et al. (2001).

It has been suggested (Nedelcu and Lee 1998b) that linearization of mtDNA within the chlorophycean class may have been the result of incidental recombination between a circular genome-sized mtDNA and a linear episome with terminal inverted repeat sequences, such as described in some strains of maize (Schardl et al. 1984). The apparently rare occurrence of extant clades with linear mitochondrial genomes in green algae is in contrast

to the situation in yeast where taxa with linear- or circular-mapping mtDNAs are interspersed on phylogenetic trees (Fukuhara et al. 1993, Nosek et al. 1998), therefore suggesting a different mechanism or mechanisms for the origin of the linear mtDNA forms in yeast.

Aside from the need for a special mechanism to replicate the 5' ends of any linear DNA, the long-term biological significance of having a linear mitochondrial genome in the green algae or any other group of organisms is not clear. Based on available data, there seems to have been no effect of mtDNA linearization in the CW-group on the size or gene content of this DNA (Nedelcu and Lee 1998a, Fan and Lee 2002). As more linear and circular mitochondrial genomes from this group are sequenced, it will be of interest to determine if there is any correlation between mtDNA conformation and the rate and pattern of its evolution. For example, it has been suggested that in yeast, gene rearrangements occur much less frequently among mitochondrial genomes with a particular type of linear structure compared to those that are circular (Drissi et al. 1994, Nosek et al. 1998). Current information from CW-group taxa, however, reveals coding regions in the mtDNA to be highly rearranged both among and between taxa with linear or circular mitochondrial genomes (Nedelcu and Lee 1998, Fan and Lee 2002).

Chapter II

Mitochondrial DNA of the *Oogamochlamys*-clade taxa *Chlamydomonas segnis* and *Chlamydomonas culleus*: structural features and expression of cytochrome respiration-related functions

INTRODUCTION

The results presented in Chapter I are consistent with the mtDNA being predominantly distinct genome- or subgenomic-sized linear molecules in all *Volvox*-clade taxa examined, and genome-sized circular molecules in all taxa examined outside this clade. The linear mtDNAs identified are thought to be linear genome-sized molecules, although in the case of *P. parva*, which branches at the base of this *Volvox*-clade (compare Nakayama et al. 1996 and Pröschold et al. 2001) the mitochondrial genome is in at least two linear pieces (Fan and Lee, 2002). This apparently unique or rare origin of linear mtDNAs among extant green algal lineages led to the proposition that linearization of the ancestral, circular mitochondrial genome occurred at or near the base of the *Volvox*-clade in the evolution of the CW-group. This is in contrast to the situation in yeast, where taxa with linear or circular genome-sized DNAs are dispersed on phylogenetic trees (Fukuhara et al. 1993; Nosek et al. 1998), suggesting the shift from circular to linear mtDNA may have occurred multiple times in these taxa.

In order to investigate further the timing of mtDNA linearization during the evolution of the CW-group, the electrophoretic behavior of mtDNA-hybridizing components from the CW-group *Oogamochlamys*-clade (Pröschold et al. 2001) taxa, *C. segnis* and *C. culleus*, were examined. Molecular phylogenetic analysis of chloroplast-encoded large subunit rRNA

sequence data (Turmel et al. 1993; Buchheim et al. 1996) provides strong statistical support for this group being a sister clade of the *Volvox*-clade.

This chapter is concerned with the following two questions: (i) does the mtDNA of the *Oogamochlamys*-clade taxa exist mostly as genome- or subgenomic-sized linear molecules, as in the *Volvox*-clade taxa, or as genome-sized circular molecules as observed in all other CW-group taxa examined? (ii) are the *Oogamochlamys*-clade taxa mitochondrial genomes functional in terms of cytochrome respiration and related functions? The latter question spawned from the unusual mtDNA structure that seems to exist in these *Oogamochlamys*-clade taxa.

MATERIALS AND METHODS

Strains, culture conditions, and preparation of total nucleic acids

The algal strains employed in this study and their sources are listed in Table 3. The procedures employed in culturing and harvesting cells as well as in the isolation and purification of total DNA were described in Chapter I. Isolation of total RNA was achieved by grinding approximately 200 mg of cells in liquid nitrogen and using the resulting powder with the RNeasy plant RNA isolation system (Qiagen, Valencia, CA) following the recommendations of the manufacturer.

Gel electrophoresis and hybridization analysis

Total DNA (normally 1-3 μg) was fractionated by agarose electrophoresis and transferred to nylon membranes as described in Chapter I. PCR amplicons of the *cob* genes from *C. reinhardtii* or *C. culleus* were labeled non-radioactively using the AlkPhos Direct Labeling and Detection System (Amersham Biosciences) and employed as probes for hybridization analysis. These probes will hereafter be termed the "*C. reinhardtii-cob*" and the "*C. culleus-cob*" probes, respectively. Nylon membranes were hybridized in the buffer supplied for 18 to 24 h and washed according to the directions of the labeling and detection system, prior to exposure of the film.

Table 3. Taxa used in Chapter II.

Strains	Source[a] /Comments
Chlamydomonas culleus[a]	UTEX 1057 (formerly *C. frankii*)
Chlamydomonas moewusii	UTEX 9 (formerly *C. eugametos*)
Chlamydomonas pitschmannii	SAG 14.73
Chlamydomonas reinhardtii	UTEX 2244
Chlamydomonas segnis[a]	UTEX 1905 (formerly *C. pallidostigmatica*)

[a]The names *Lobochlamys culleus* and *Lobochlamys segnis* have recently been proposed for *C. culleus* and *C. segnis* respectively (Pröschold et al. 2001).

Nucleic acid amplification, cloning and DNA sequencing

The following primer pairs were designed for amplification of *cob*, *cox1* and *nad1* sequences (Table 4): the *cob*-78 / *cob*-280 pair, amplifies the DNA corresponding to the region between codons 78 and 280 of the *C. reinhardtii cob* gene product, the *cox1*-75 / *cox1*-448 pair amplifies the region between codons 75 and 448 of the *C. reinhardtii cox1* gene product, and the *nad1*-25 / *nad1*-263 amplifies the region between codons 25 and 263 of the *C. reinhardtii nad1* gene product. The design of these primers was based on multiple sequence alignments for these genes from a range of green plants. PCR amplifications of total cellular DNA were carried out in a Geneamp 2400 thermal cycler (Perkin-Elmer / Applied Biosystems, Foster City, CA) using 25 µL reaction mixtures and reagents from Amersham Biosciences. One microgram of total cellular DNA template was initially denatured at 94 °C for 3 min, then amplified by 35 cycles of 30 s denaturation at 94 °C, 30 s annealing at 50 - 65 °C, and extension at 72 °C for 1 min; a final extension period of 5 min at 72 °C followed the cycling. Reverse transcriptase-PCR (RT-PCR) reactions were carried out using Ready-to-go RT-PCR beads (Amersham Biosciences) and the conditions specified by the manufacturer.

Table 4. Primers employed for the amplification *cob*, *cox1* and *nad1* sequences

Primer name	Primer sequence
cob–78	5'–GGTTGGWTAYTKCGTTATDYNCAYGCDAAY–3'
cob–280	5'–ATAGCATATACCCRWARRARRTACCAYTC–3'
cox1–75	5'–ATGCCTGCATTAATNGGWGGHTTYGGKAAY–3'
cox1–448	5'–TCWGGATAATCRRGMATWCKNCKWGGCAT–3'
nad1–25	5'–MMMRTWATGGCWWSTATGCAACG–3'
nad1–263	5'–AATTGRTCRTAWCGATAACGTGG–3'

Bases, A = Adenine; C = Cytosine; G = Guanine; T = Thymine. Degenerate bases, B = C, G or T; D = A, G or T; H = A, C or T; K = G or T; M = A or C; N = A, C, G, or T; R = A or G; S = G or C; V = A, C or G; W = A or T; Y = C or T.

Cloning of amplicons was achieved by blunt end ligations into *smaI* digested pUC18, and transformation of *E. coli* DH5α using standard procedures (Sambrook et al. 1989).

DNA sequencing of both strands (except where indicated otherwise), was performed at the Centre for Applied Genomics, Hospital for Sick Children, in Toronto, Canada. Sequence data were compiled and edited using DNA Strider version 1.1 (Marck 1988). Sequence similarity searches were performed using the BLAST network services (Altschul et al. 1990), provided by the National Center for Biotechnology Information. Alignments of nucleotide and deduced amino acid sequences were done with Clustal W version 1.74, (Thompson et al. 1994). Some sequence alignments were examined and optimized manually using SeqVu 1.0.1 (J. Gardner, Garvan Institute of Medical Research, Sydney, Australia).

Capacity for heterotrophic growth and detection of cytochrome respiration

Cells actively growing in liquid Tris-Phosphate (TP) medium (Harris 1985) under standard phototrophic conditions (Chapter I) were transferred to agar plates made with either TP or TRIS-acetate phosphate (TAP) medium (Harris 1985). TP plates were incubated under 100 $\mu mol/m^{-2}/s^{-1}$ of continuous light, while TAP plates were incubated in the dark. Capacity for

heterotrophic growth was determined qualitatively by comparing cell growth under both conditions after about 7 d.

Detection of respiration via the cytochrome pathway was achieved using a tetrazolium overlay technique originally developed in yeast by Ogur et al. (1957) and adapted for *Chlamydomonas* by Dorthu et al. (1992). Briefly, cells are grown for 2 to 3 d in continuous light (100 $\mu mol/m^{-2}/s^{-1}$) on TAP plates. The test medium is composed of 1.5 % agar in 0.07 M phosphate buffer, pH 7.0, and supplemented with 0.1% 2,3,5-triphenyltetrazolium chloride (TTC). The assay was performed by pouring 15 mL of test medium at 50 °C over the 2- to 3-day-old plates. These plates were then incubated in the dark at 27 °C for 5 to 8 h, after which respiratory-competent cells became purple whereas respiratory-deficient cells remained green.

RESULTS

Hybridization analysis

Following fractionation by agarose gel electrophoresis, total cellular DNA from *C. culleus* and *C. segnis*, as well as *C. reinhardtii* included as a control, was subjected to Southern blot analysis using the *C. reinhardtii-cob* and *C. culleus-cob* probes. Under relaxed hybridization conditions, using the *C. reinhardtii-cob* probe, only a single-hybridizing component corresponding to a linear DNA of 16 kb was detected in the *C. reinhardtii* preparation (Figure 9A, lane 1), while the DNAs prepared from *C. culleus* and *C. segnis* revealed a diffuse signal spanning most of the region between the 3- and 23-kb linear size markers (Figure 9A, lanes 2 and 3); no well-bound hybridization signals were observed. Under the same hybridization conditions, but using the *C. culleus-cob* probe, a similar diffuse signal was obtained with DNA samples recovered from *C. culleus* and *C. segnis* (Figure 9B, lanes 1 and 2) as well as with DNA extracted from equal mixtures of *C. culleus* and *C. reinhardtii* cells, or of *C. segnis* and *C. reinhardtii* cells (Figure 9B, lanes 3 and 4). Re-probing of this blot with the *C. reinhardtii-cob* probe under more stringent hybridization conditions revealed no signs of degradation of the *C. reinhardtii* 16-kb mtDNA (Figure 9C, lanes 3 and 4).

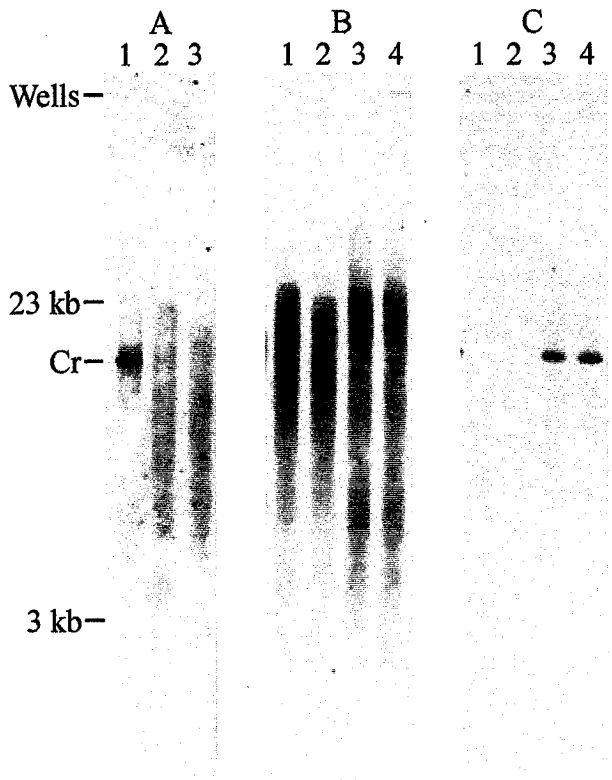


Figure 9. Southern blot analysis of total DNA from *C. reinhardtii*, *C. culleus* and *C. segnis* with mtDNA probes. DNA was fractionated through 20 cm, 0.8 % agarose gels at 0.5 V/cm for 24 h. Autoluminograms were obtained by hybridization at (A) 55 °C with the *C. reinhardtii-cob* probe; lanes: 1, *C. reinhardtii*; 2, *C. culleus*; 3, *C. segnis*. (B) 55 °C with the *C. culleus-cob* probe; lanes: 1, *C. culleus*; 2, *C. segnis*; 3, mixture of *C. reinhardtii* and *C. culleus*; 4, mixture of *C. reinhardtii* and *C. segnis*. (C) The membrane from B was stripped and re-probed at 65 °C with the *C. reinhardtii-cob* probe.

Fractionation of *C. culleus* and *C. segnis* DNAs by PFGE (15 s switch interval, 6 V/cm, 13.5 h), which could resolve linear DNA markers as large as 243 kb, confirmed that the largest mtDNA-hybridizing components co-migrated with linear DNAs of about 23 kb (Figure 10). Finally, no distinct bands were observed following digestion of the *C. segnis* and *C. culleus* DNAs with *Acc*I, *Eco*RI, *Eco*RV and *Hin*dIII individually; rather, these revealed a diffuse hybridization signal with a higher proportion of the smaller sized molecules (Figure 11).

Sequence analysis of *cox1* and *cob* PCR products

PCR products were generated from *C. segnis* whole cell DNA with the *cob* and *cox1* degenerate primers, and these were sequenced directly. As shown in Table 5, the partial *C. segnis cob* and *cox1* nucleotide sequences are GC-rich relative to other CW-group algae, however, they share high identity, 66 and 73 % respectively, with the aligned regions of their *C. reinhardtii* counterparts; the deduced amino acid sequences of these same regions are 78 and 82 % identical to their *C. reinhardtii* counterparts.

A PCR product generated from *C. culleus* whole cell DNA with the *cob* degenerate primers was sequenced directly. Alignment of this sequence with its *C. reinhardtii* counterpart shows 68 % identity at the nucleotide level

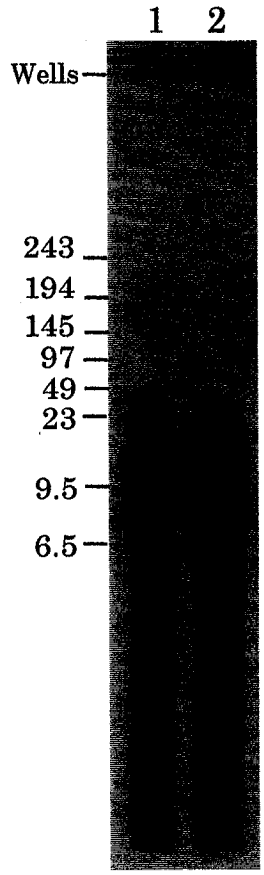


Figure 10. Southern blot analysis of total DNA from *C. segnis* and *C. culleus*. Total unrestricted DNA from *C. segnis* (lane 1) and *C. culleus* (lane 2) was fractionated by PFGE, transferred to a nylon membrane and hybridized to the *C. culleus-cob* probe.

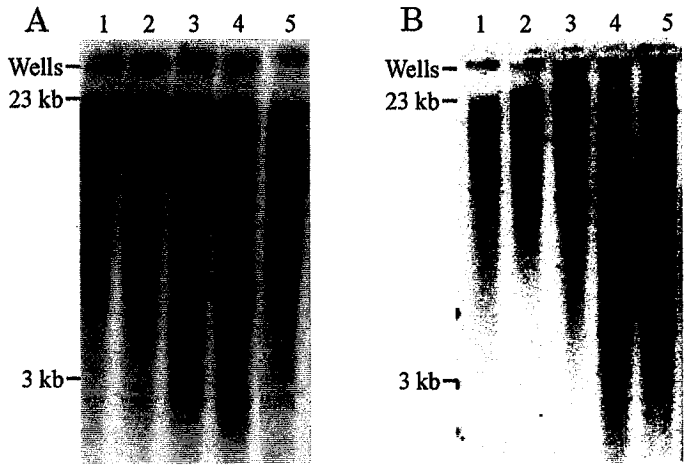


Figure 11. Southern blot analysis of total DNA from *C. segnis* and *C. culleus*, following restriction digestion. *C. culleus* (A) and *C. segnis* (B) DNA was fractionated in 1.0% agarose gels in 1X TAE at 3V/cm for 4 h, transferred to nylon membranes and hybridized with the *C. culleus-cob* probe. Lanes: 1, undigested; 2, *Eco*RV; 3, *Hin*dIII, 4, *Eco*RI, 5, *Acc*I

Table 5. GC content of partial *cob* and *cox1* sequences and percent identity of nucleotide and deduced amino acid sequences of selected CW-group taxa relative to the *C. reinhardtii* counterparts.

	Cr		Cs		Cc		Cm		Cap	
	cob	*cox1*	*cob*	*cox1*	*cob*	*cox1*	*cob*	*cox1*	*cob*	*cox1*
(%) identity (nucleotide)	—	—	66	73	68	77[a]	67	70	74	74
(%) identity (amino acid)	—	—	78	82	63[b]	60[c]	67	80	74	79
GC content (%)	47	47	54	55	61	61	32	36	36	41

The nucleotides compared correspond to positions 234 to 840 of the *C. reinhardtii cob* gene and position 225 to 1344 of the *C. reinhardtii cox1* gene. This represents approximately 55% and 75% of the *C. reinhardtii cob* and *cox1* genes, respectively. Percent identities were calculated by pairwise comparison to the *C. reinhardtii* sequences, while excluding all gapped positions. [a]Based on the sequence of the PCR clone. [b]The percent identity rises to 88% if only the inframe portion (positions 16 to 150) of the *C. culleus* sequence are compared. [c]Amino acid sequence was deduced from the most plausible reading frame of the PCR clone sequence. Abbreviations: Cr, *C. reinhardtii*; Cs, *C. segnis*; Cc, *C. culleus*; Cm, *C. moewusii*; Cap, *Chlorogonium capillatum*.

(Figure 12), but only 63 % identity at the deduced amino acid level (Figure 13). The lower level of amino acid identity, compared to the *C. segnis* – *C. reinhardtii* comparison, results from reading frame shifts associated with seven 1-nt deletions in the *C. culleus* sequence relative to the *C. reinhardtii* counterpart. In addition, two potential in-frame stop codons (TGA) were identified in the *C. culleus* sequence at positions corresponding to methionine residues in the *cob* genes of other CW-group algae.

PCR of whole cell *C. culleus* DNA using *cox1* degenerate primers produced a single component of about 1.1 kb, as observed on 1 % agarose gels; attempts to sequence this product were unsuccessful because of sequence heterogeneity. A clone prepared from this PCR-generated DNA yielded a 1063-nt sequence, and alignment of this sequence with the corresponding region of the *C. reinhardtii cox1* sequence revealed homology between the two sequences, i.e., about 77 % identity of the aligned regions. There were, however, 17 insertion/deletion (indel) differences between the two sequences; 14 of these, with sizes ranging from 1- to 18-nt, had the shorter form in *C. culleus*, whereas three, with sizes from 5- to 19-nt, had the longer form in *C. culleus* (Figure 14).

With the goal of exploring the potential heterogeneity of *cox1* sequences in *C. culleus*, the PCR was repeated using exact-match primers

Figure 12. Multiple sequence alignment of the partial *cob* nucleotide sequences from *C. segnis* (Cseg) and *C. culleus* (Ccul) with the corresponding sequences of *C. reinhardtii* (Crei), *C. moewusii* (Cmoe) and *C. capillatum* (Ccap).

Note the *C. culleus* reading frame is first disrupted at nucleotide position 6 of the sequence, and is reestablished by the fifth, one nucleotide deletion at nucleotide position 83. The seventh, one nucleotide deletion disrupts the frame once again at nucleotide position 457; there is presently insufficient sequence data to determine whether the reading frame is reestablished further downstream.

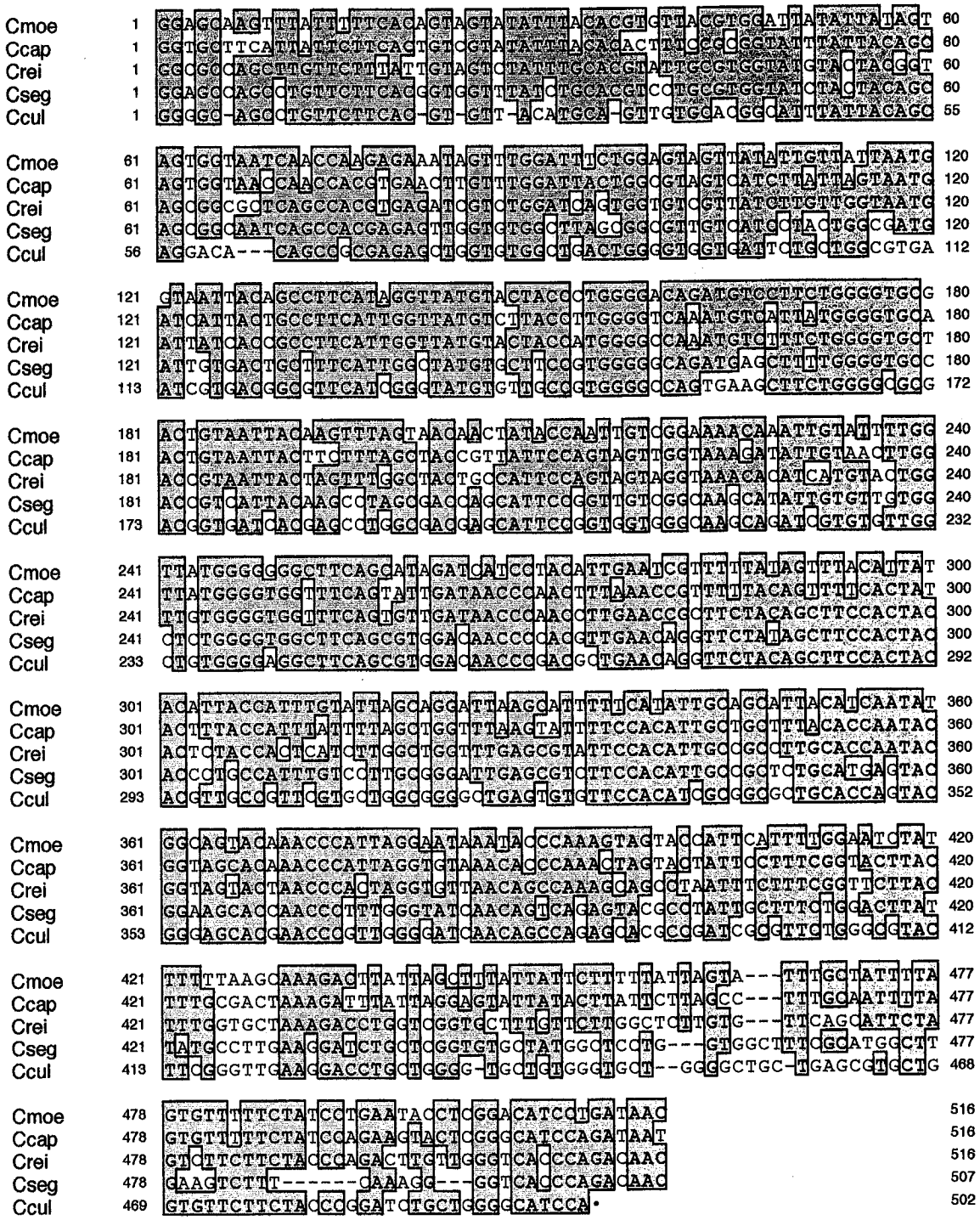


Figure 12

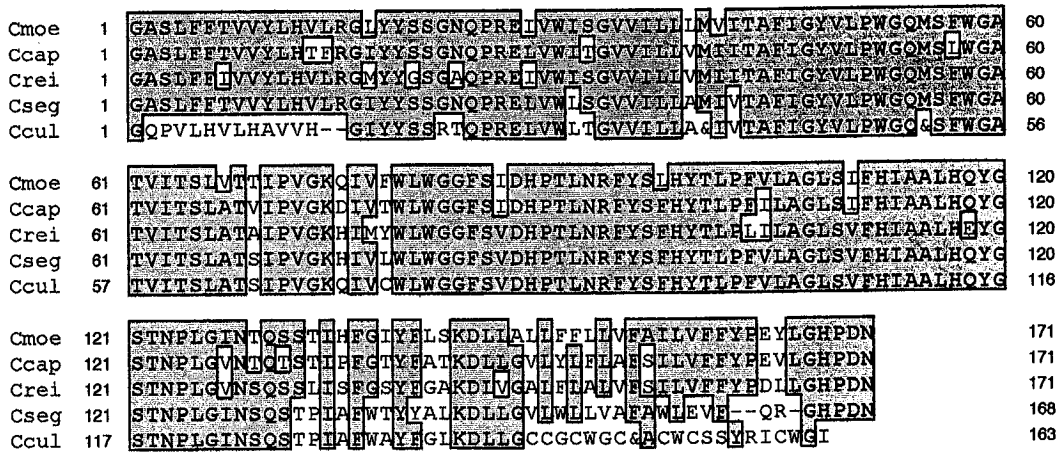


Figure 13. Multiple sequence alignments of the partial *cob* deduced amino acid sequences from *C. segnis* and *C. culleus* with the corresponding sequences of *C. reinhardtii*, *C. moewusii* and *C. capillatum*. Abbreviations are as in Figure 12. Dashes represent gaped regions and ampersands (&) correspond to "TGA" stop codons present in the *C. culleus* sequence.

Figure 14. Multiple sequence alignment of three partial *cox1* sequences from *C. culleus*, with the corresponding *C. segnis* and *C. reinhardtii* sequences. Ccul-1 corresponds to the cloned 1.1-kb *cox1* amplicon obtained by PCR of total *C. culleus* DNA using the *cox1* 75 and *cox1* 448 primers. Ccul-2 and Ccul-3 correspond to the 1.0- and 0.9-kb products, respectively, that were obtained by use of exact match primers based on the sequence on Ccul-1; these amplicons were sequenced directly. Solid dots represent regions where no sequence data has been obtained whereas dashes represent gaps due to indels.

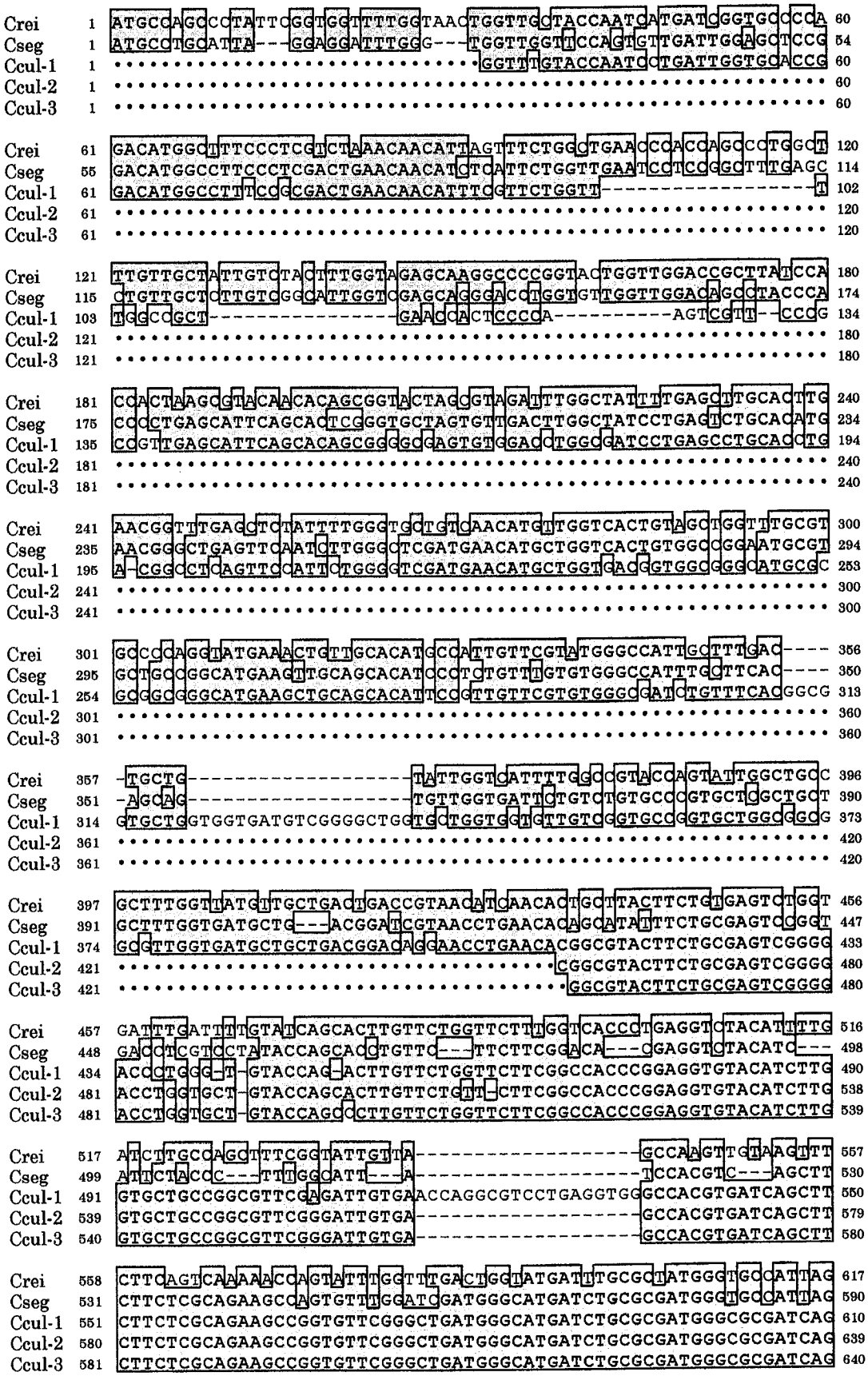


Figure 14

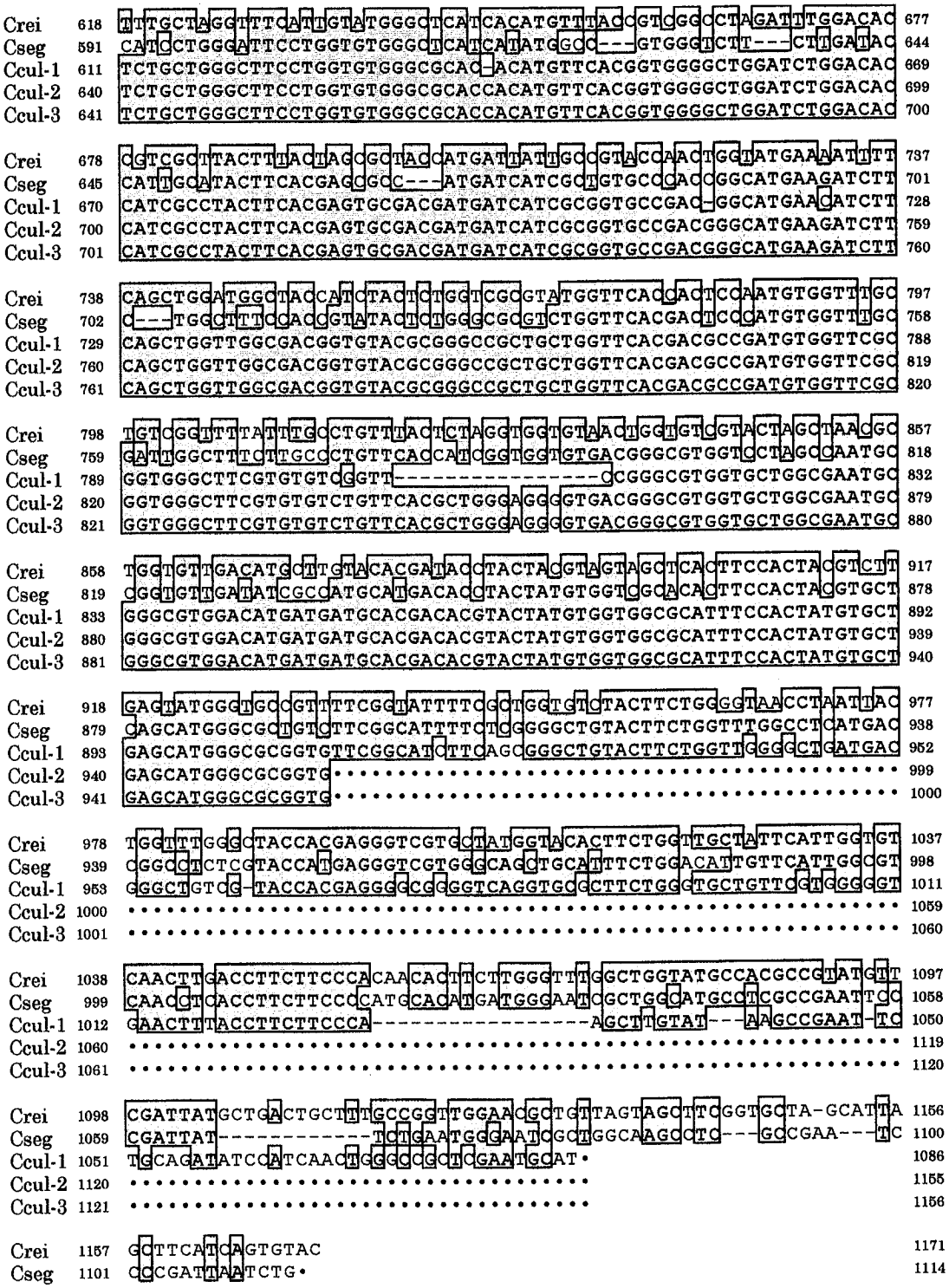


Figure 14 (continued)

based on the sequence data obtained from the *C. culleus cox1* PCR clone. The DNA recovered was characterized on high-resolution (4 %) agarose gels, and two size-classes of products, namely, 0.9 and 1.0 kb, were resolved. Although several hundred nucleotides could be determined for each product by direct sequencing using the reverse primer, only 33-nt were obtained using the forward primer with the 0.9-kb product and no sequence was obtained using the forward primer of the 1.0-kb product. The DNA sequences determined using the reverse primer for the 0.9- and 1.0-kb PCR products were identical to each other except for a single 1-nt indel. Interestingly, multiple sequence alignments of the 0.9- and the 1.0-kb *cox1* PCR products and the corresponding region in *C. reinhardtii*, reveal only one 1-nt deletion in the *C. culleus* sequences relative to nucleotide position 468 of the *C. reinhardtii* sequence; this deletion is also present in the *C. culleus cox1* PCR clone (Figure 14).

Cytochrome respiration-related functions

Activity of the cytochrome respiration pathway, capacity for heterotrophic growth, and the presence of *cob* and *cox1* transcripts (Figure 15) were scored in *C. segnis* and *C. culleus* as well as in additional selected CW-group taxa, and these results are summarized in Table 6. *C. segnis* scored positive for all of these features, as did the wild-type strains of *C.*

reinhardtii and *C. pitchsmannii*. Conversely, *C. culleus* scored negative for all these features, and surprisingly, so did *C. moewusii*. Products of the expected size were obtained by PCR amplification of total DNA from *C. culleus* and *C. moewusii* (data not shown), indicating that the degenerate primers used should have been able to amplify the *cob* and *cox1* transcripts if these were present in the RNA preparations.

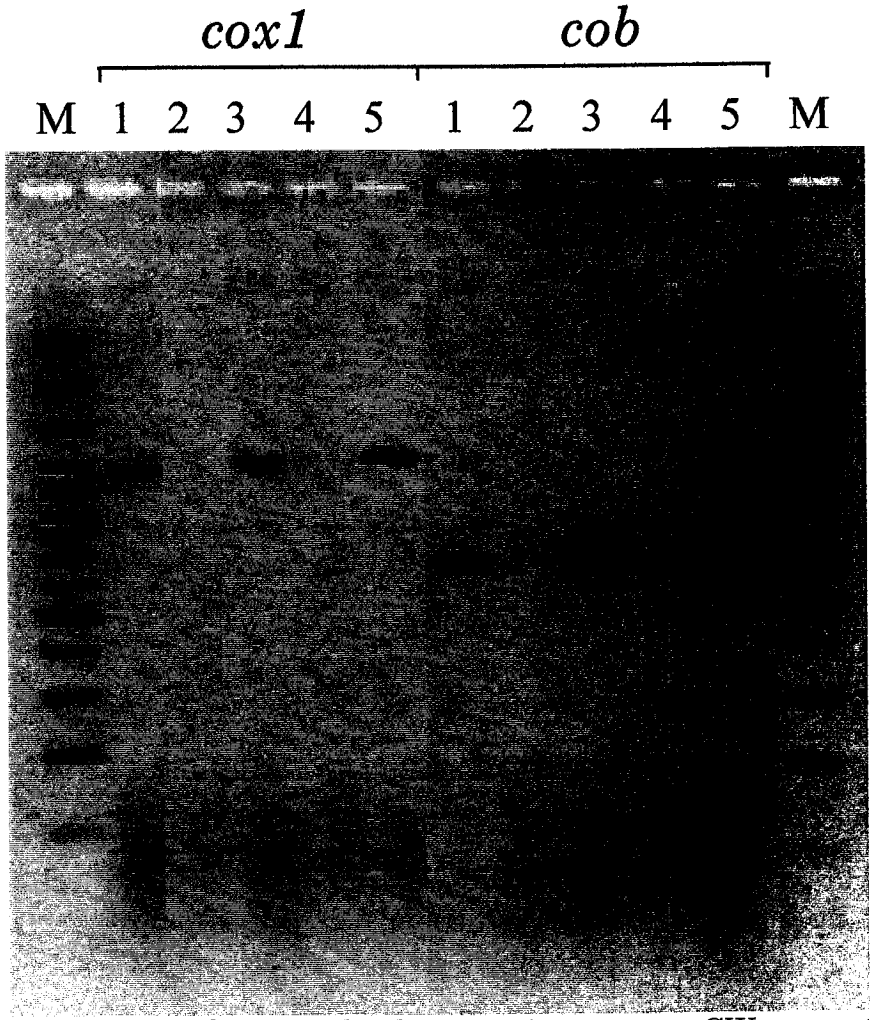


Figure15. Detection of *cox1* and *cob* transcripts among CW-group taxa by RT-PCR. RT-PCRs were performed using total cellular RNA from various CW-group taxa using the *cox1* and *cob* primer sets as described in the Materials and Methods. Following the RT-PCR, 5μL of each sample was fractionated in a 2% agarose and the gel was visualized by UV fluorescence after ethidium bromide staining. The image contrast was inverted for printing purposes. Lanes: M, DNA size marker; 1, *C. reinhardtii*; 2, *C. moewusii*; 3, *C. pitschmannii*; 4, *C. culleus*, 5, *C. segnis*.

Table 6. Detection of *cob* and *cox1* transcripts, capacity for heterotrophic growth, and cytochrome respiration in various CW-group taxa.

	cob		*cox1*		Heterotrophic	Cytochrome
Taxon	PCR	RT-PCR	PCR	RT-PCR	Growth	Respiration
Cs	+	+	+	+	+	+
Cc	+	—	+	—	—	—
Cr	+	+	+	+	+	+
Cp	+	+	+	+	+	+
Cm	+	—	+	—	—	—

The columns PCR and RT-PCR indicate whether or not *cob* and *cox1* primer-specific amplicons were obtained from whole cell DNA or RNA, respectively. Heterotrophic growth indicates a taxon's capacity to grow on medium containing acetate, in the absence of light, and cytochrome respiration indicates whether or not the cytochrome respiration pathway is active, as assayed by the reduction of TTC to red formazan. Abbreviations: Cs, *C. segnis*; Cc, *C. culleus*; Cr, *C. reinhardtii*; Cp, *C. pitschmannii*; Cm, *C. moewusii*.

DISCUSSION

Mitochondrial DNA conformation in *C. segnis* and *C. culleus*

Southern blot analyses of total DNA from *C. segnis* and *C. culleus* using mtDNA probes gave results in accordance with the view that these DNAs consist of a heterogeneous collection of linear or small circular DNAs, or a mixture of the two, with a continuous size distribution spanning the region between the 3- and 23-kb linear size markers. The presence of larger circular DNAs (≥10 kb) seems unlikely as these would have migrated in the gels employed to a position above the compression zone for linear DNA, and hybridizing DNA was not observed in this region of the gels. If the DNA in the upper region of the *C. segnis* and *C. culleus* hybridization signal is linear DNA, such molecules are within the mitochondrial genome size range expected for CW-group taxa (Chapter I); hybridization signals lower in the smear could correspond to subgenomic-sized forms. It seems that degradation by endogenous nucleases cannot explain the hybridization results obtained with the *C. segnis* and *C. culleus* DNAs, as DNA isolated from mixtures of *C. segnis* or *C. culleus* cells with *C. reinhardtii* cells revealed the intact *C. reinhardtii* 16-kb mtDNA following hybridization analysis.

The diffuse hybridization signals observed for *C. segnis* and *C. culleus* are reminiscent of the signals obtained in Southern blot hybridization

experiments with mtDNA probes using the total cellular DNA from other biological systems, including fungi, apicomplexa, land plants, and the dinoflagellate *Crypthecodinium cohnii*; the smeared signal observed in these systems has been proposed to result from the rolling-circle and/or T4-like recombination-dependant replication mechanisms thought to be employed by their mtDNAs (Bendich 1993; Wilson and Williamson 1997; Backert et al. 2000; Norman and Gray 2001). At the present time we know little about the structure or origin of the heterogeneously-sized population of *C. segnis* and *C. culleus* mtDNAs. It appears, however, that their origin cannot be explained simply by the employment of a rolling-circle mechanism of mtDNA replication, as this type of replication commonly produces a smeared signal corresponding to linear concatemers much larger than the genome size, and restriction digests of these concatenated DNAs usually produces the restriction pattern expected for the unit genome. Rather, available information regarding the mtDNAs of *C. segnis* and *C. culleus* including the apparent size range of the heterogeneous mtDNA elements and the presence of multiple versions of the *cox1* elements suggests some similarities with the mtDNA of *Crypthecodinium cohnii* (Norman and Gray 1997; Norman and Gray, 2001). For *Crypthecodinium cohnii* these features are thought to result from a recombination-dependent replication mechanism and an abundance of short repeat sequences, the latter of which has also been documented for some CW-group mtDNAs (Boer and Gray 1991; Nedelcu and Lee 1998).

Recombination-dependent replication mechanisms in most systems, however, produce complex DNA structures which are much larger than the genome size, and no candidate structures have been observed for *C. segnis*, *C. culleus* or *Crypthecodinium cohnii*. On the other hand, it is possible that such structures are resolved and/or degraded very quickly in these taxa, so that their steady state level is undetectable by the hybridization assays employed. Clearly, further characterization of the *C. segnis*, *C. culleus* and *Crypthecodinium cohnii* mtDNAs are needed before the extent of the similarities and differences in the origin of these structures can be assessed.

Available evidence led to the suggestion that linear mtDNAs may have arisen only once among extant lineages of the chlorophycean CW-group and possibly the green algae, and that this event occurred at the base of the *Volvox*-clade or earlier in the evolution of the CW-group (Chapter I). Southern blot analysis with mtDNA probes using DNA from the *Oogamochlamys*-clade taxa *C. segnis* and *C. culleus* was thus performed, with the expectation that these would yield hybridization patterns consistent with either genome- or sub-genomic-sized linear mtDNAs forms characteristic of *Volvox*-clade taxa or genome-sized circular forms of non-*Volvox*-clade taxa, as observed previously. Rather a third mtDNA conformation was uncovered, which is unlike any observed in CW-group taxa. Still, it is interesting that abundant genome-sized circular mtDNA forms are a feature of all CW-group taxa examined except those associated with the *Volvox*-clade and the

Oogamochlamys-clade. Given the suggested close relationship between these clades (Turmel et al. 1993; Buchheim et al. 1996), I favor the hypothesis that the mtDNAs of *C. segnis* and *C. culleus* are a heterogeneous population of linear molecules and the loss of genome-sized circular mtDNA occurred in a common ancestor of these clades. Future work should focus on testing the possibility that the mtDNA is indeed linear in the *Oogamochlamys*-clade taxa. Further it will be important to examine the mtDNAs of taxa from other closely related clades such as the "*Chloromonas*-clade" and the "*Radiosa*-clade" of Pröschold et al.(2001), which may have specific evolutionary connections with the *Oogamochlamys*-clade and the *Volvox*-clade.

Cob and *cox1* sequences of *C. culleus* and *C. segnis*

Analysis of the *cob* and *cox1* sequences of *C. culleus* suggests that these are pseudogenes and that the cytochrome respiration pathway is not functional in this taxon. Though these sequences show homology to the functional genes in *C. reinhardtii*, they contain multiple frameshift-causing indels. In addition, if the *cob* sequence were transcribed, it would contain at least two in-frame stop codons. With regards to *cox1*, multiple sequences have been amplified, yet none of these seem capable of encoding a functional protein. Note also, that no *cob* or *cox1* transcripts were detected using RT-PCR, a further indication that these are pseudogenes.

In contrast to what is observed with *C. culleus*, analysis of the *C. segnis cob* and *cox1* sequences reveals no frameshifts, and transcripts from both genes were detected by RT-PCR, therefore supporting the functionality of these genes. High identity of both the nucleotide and deduced amino acid sequences relative to their *C. reinhardtii* counterparts is evident, although the *C. segnis* sequences show a higher codon bias towards codons that end in G or C, which is consistent with their higher GC content.

Cytochrome respiration-related functions among the CW-group algae

The presence of putative *cob* and *cox1* pseudogenes in *C. culleus* can lead one to question whether this taxon has a functional cytochrome respiration pathway. As *C. reinhardtii* mutants deficient in cytochrome respiration lose their capacity for heterotrophic growth, this ability as well as the ability to reduce TTC, which is diagnostic of cytochrome respiration (Dorthu et al. 1992), was tested in *C. culleus* and *C. segnis*. Although *C. segnis* scored positive in both assays, *C. culleus*, in each case, scored negative. Moreover RT-PCR detected *cob* and *cox1* transcripts from *C. segnis* but not from *C. culleus*. As both *C. segnis* and *C. culleus* appear to have similar mtDNA structures, and only *C. culleus* seems to be deficient in cytochrome respiration-related functions, one could conclude that this loss of function is not associated with the unusual mtDNA structure in this taxon.

Surprisingly, *C. moewusii* showed no signs of cytochrome respiration activity and no detectable *cob* or *cox1* transcripts despite the fact these sequences revealed no features characteristic of pseudogenes (Denovan-Wright et al. 1998). *Nad1* transcripts from *C. moewusii*, however, were amplified by RT-PCR; the presence of these transcripts indicates that complex I activity and mitochondrial ATP production are possible in this taxon (Duby et al. 1999; Cardol et al.2002). Although *C. culleus nad1* transcripts were not detected by RT-PCR, this result is uninformative as the same primers used in this reaction failed to amplify a *nad1* PCR product from *C. culleus*.

It has been shown for *C. reinhardtii* that cytochrome respiration is only necessary under heterotrophic growth conditions (reviewed in Remacle and Matagne 1998). Thus, it may not be unexpected to find naturally occurring taxa that are deficient for cytochrome respiration, particularly if these existed in light-rich environments that were low in dissolved organic matter. It would be interesting to determine if loss of cytochrome pathway functions and the lack of *cob* and *cox1* gene expression is a common feature among taxa that cannot grow heterotrophically, and if such phenotypes are selectively neutral or perhaps even advantageous under some environmental conditions, as suggested by Wetherell (1958).

GENERAL DISCUSSION

In terms of the size and reduced coding capacity of the CW-group mitochondrial genomes (Nedelcu et al. 2000), one might say that these are more "animal-like" than "plant-like". As animals are the only group in which most taxa have been shown to contain abundant genome-sized mtDNA molecules, it was initially tempting to say that the CW-group mtDNAs resembled most closely those of animals in terms of their simple structure. The mtDNA structure in *C. segnis* and *C. culleus*, however, clearly does not support this view. Rather, it seems that the structure of the mtDNA in *C. segnis* and *C. culleus* adds to the view that the mtDNA in most eukaryotes is composed of a heterogeneous collection of molecules. Assuming the alpha-proteobacterial origin of mitochondria (Margulis 1970; Gray and Doolittle 1982), and assuming a circular DNA in this bacterium, it is a difficult task to explain from an evolutionary perspective that only few lineages among the Eukarya have genome-sized circular mtDNA as their predominant mtDNA form.

Though green algal phylogenies are continually being deciphered, the phylogenetic framework for this group is rather solid, and obvious trends can be identified in the course of green algal mtDNA evolution. For example, there is an obvious reduction in mitochondrial genome size and coding capacity in taxa from the Chlorophyceae relative to those of the Trebouxiophyceae and the Prasinophyceae, which are a heterogeneous collection of early branching taxa. Within the Chlorophyceae, there is a

further reduction observed in taxa from the CW-group relative to the one taxon examined from the DO-group (Nedelcu et al. 2000; Kück et al. 2000). Another interesting observation is the progression of mitochondrial rDNA fragmentation; the mitochondrial rDNA genes are continuous in the Prasinophyceae and Trebouxiophyceae, fragmented into a few pieces in the DO-group of the Chlorophyceae, and highly fragmented in the CW-group of the Chlorophyceae (Nedelcu 1997). Finally, the present study suggests that one may eventually be able to define a pathway for the evolution of mtDNA structure in this group. The working hypothesis could be that the ancestral mtDNA type is a genome-sized circular molecule, and that this mtDNA type was maintained throughout green algal evolution up to a point, near the origins of the *Volvox*-clade and the *Oogamochlamys*-clade.

It would be of interest to determine the structure of the heterogeneous population of mtDNAs observed in *C. segnis* and *C. culleus*. It seems unlikely that further characterization could be accomplished by electron microscopic techniques, as it has proven very difficult to obtain pure mtDNA from walled strains of CW-group taxa, although one might be able to isolate cell-wall deficient mutants of these taxa. Conversely, two-dimensional gel electrophoresis combined with Southern blot analysis has proven useful for the separation and characterization of various mtDNA conformations in land plant (Oldenburg and Bendich, 2001) and apicomplexan taxa (Preiser et al. 1996). A more complete view of the mtDNA structure in *C. segnis* and *C.*

culleus might shed some light on the mechanism of replication employed by these DNAs, their origin during cell growth and the evolutionary origin of their unusual features.

Finally, it seems ironic that one of the more intriguing results of this dissertation is not related to mtDNA structure, but to mitochondrial genome function. The absence of cytochrome respiration and the lack of expression of *cob* and *cox1* genes in vegetative cultures of *C. moewusii* was a surprising result, as the sequence of these genes does not suggest that they have been under relaxed evolutionary constraints. It thus seems likely that either (i) the *cob* and *cox1* sequences became inactive (pseudogenes) very recently in the evolutionary origin of *C. moewusii*, so that no substitutions have yet occurred, or (ii) these genes are indeed functional and are expressed either under growth conditions not employed here or during non-vegetative life-cycle stages, such as zygote maturation. In either case, these results merit further study. It may turn out that the shift from active to inactive cytochrome respiration pathways can occur via relatively simple mechanisms in the green algae.

APPENDIX

Introduction

Two distinct studies are included in this appendix. The first describes the fractionation of total cellular DNA from *C. culleus* by Cesium chloride gradient centrifugation, and the second is a phylogenetic analysis of CW-group taxa based on partial mitochondrial *cob* sequences.

Materials and Methods

Cesium chloride gradients

A 5-L culture of *C. culleus* was grown under synchronous growth conditions and harvested by centrifugation as described in Chapter I. Total DNA was extracted as described in Chapter I, adjusting for volume, and the final DNA pellet was resuspended in 5 mL of TE. This was added to 35.1 g of centrifugation grade CsCl (Gibco Life Sciences), and the final volume was adjusted to 40 mL with TE. This mixture was supplemented with 0.05 $mg \cdot mL^{-1}$ (final) of bisbenzimide, and mixed on a rotary shaker for 15 min prior to centrifugation. Forty milliliter quick-seal centrifugation tubes were spun in a vTi50 vertical rotor (Beckman) at 40 000 rpm for 24 h, and the resulting gradients were examined with UV illumination. The observed bands were extracted from the tube through an 18-gauge needle, and the

recovered DNA was subjected to multiple rounds of ethanol precipitation to remove contaminating CsCl, prior to storage in TE buffer.

Phylogenetic analysis

The taxa included in this part of the study are listed in Table 8. PCR amplifications and sequencing of the amplicons was achieved using the *cob*-78 / *cob*-280 primer pair and the conditions described in Chapter 2. As the *cob* sequence of *C. culleus* appears to shift in and out of frame (Figure 13), only the in-frame portion of this sequence was employed for this phylogenetic reconstruction. This data set is thus composed of 127 amino acid residues, which correspond to positions 108 to 235 of the *C. reinhardtii cob* sequence. The neighbor joining and parsimony trees were generated using the "Neighbor" and "Prot-pars" modules of the Phylip package 3.5c (Felsenstein 1993), respectively, and are based on distance matrices calculated by the "protdist" module of Phylip. Bootstrap resampling analyses (1000 data sets) were performed using the seqboot module of Phylip to assess branch support in these trees. The maximum likelihood tree was produced using tree-puzzle (Schmidt et al. 2002).

Table 7. Taxa employed in appendix

Strain / Source	**Source of sequence data**
Chlamydomonas reinhardtii – UTEX 2244	GB # NC001638
Chlamydomonas incerta – SAG 7.73	P. Jarman, unpublished
Chlamydomonas globosa – CC 3349	This study
Chlamydomonas culleus – UTEX 1057	This study
Chlamydomonas segnis – UTEX 1905	This study
Chlamydomonas moewusii – UTEX 9	GB # NC001872
Chlamydomonas pitchsmannii – SAG 14.73	This study
Chlamydomonas assymetrica – SAG 70.72	This study
Chlamydomonas smithii – UTEX 1062	GB # X55305
Chlamydomonas debaryana – UTEX 569	P. Jarman, unpublished
Chlamydomonas zebra – SAG 10.63	P. Jarman, unpublished
Chlorogonium capillatum – UTEX 118	GB # Y07814
Nephroselmis olivacea – NIES-296	GB # AF110138

Results and Discussion

Cesium chloride gradients

Following centrifugation, two high intensity bands, referred to as "upper" and "lower" based on their position in the gradient, were visible under UV illumination; one low intensity band, referred to as "middle", was also observed. The DNA from each of these three regions of the gradient was purified, and an aliquot of each was used in Southern blot analysis with the C. *culleus-cob* probe (see Chapter II). Intriguingly, the DNA isolated from each fraction of the gradient showed diffuse signals of approximately equal intensity (Figure 16, lanes 1) and amplicons of the *cob* sequence were obtained from each fraction by PCR (Figure 16, lanes 3), suggesting that the mtDNA is present over a broad region of the gradient. This is in contrast to the situation in *C. reinhardtii*, where CsCl fractionation of total DNA also produces three visible bands although in this case the mtDNA is found only in the lower portion of the top band (Ryan et al. 1973; Whiteway and Lee 1977). *Eco*RI digestion of the DNA from each fraction revealed no distinct bands (Figure 16, lanes 2), as discussed in Chapter II.

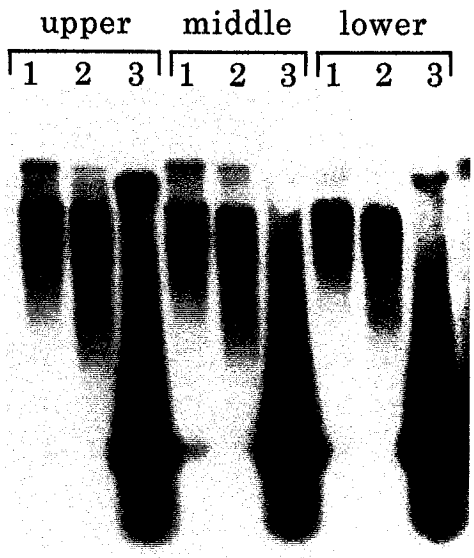


Figure 16. Southern hybridization of CsCl fractionated DNA from *C. culleus*. The probed employed was the *C. culleus-cob* probe described in Chapter II. Lanes: 1, undigested DNA; 2, DNA digested with *Eco*RI; 3, PCR amplicon of the *cob* sequence, obtained from each CsCl fraction. Upper, middle and lower refer to the positions of the bands in the CsCl gradient.

The broad distribution of *C. culleus* mtDNA in CsCl gradients, as described here, is similar to that observed for the mtDNA of the dinoflagellate *Crypthecodinium cohnii* (Norman and Gray 1997) and the apicomplexan *P. falciparum* (Preiser et al. 1997). As discussed in earlier sections of this dissertation, the mtDNAs from all these taxa reveal diffuse electrophoretic migration patterns which may result from size heterogeneity associated with recombination-dependant replication mechanisms. In the case of *P. falciparum* evidence has been obtained that suggests the broad distribution of mtDNA in the CsCl gradients results in part from the presence of single stranded DNAs that are attached to duplexes (Preiser et al. 1997); such DNA forms further support the proposed recombination-dependant replication mechanism (Preiser et al. 1997; GENERAL INTRODUCTION). The broad distribution of *Crypthecodinium cohnii* and *C. culleus* mtDNA in CsCl gradients may indicate similar replication mechanisms in these taxa.

Phylogenetic analysis of CW-group mitochondrial *cob* sequence.

Cob sequences were obtained from taxa representing the four CW-group lineages that were studied in Chapters I and II, and phylogenetic analysis of these sequences was done using neighbor joining, parsimony and maximum likelihood methods. The prasinophyte *Nephroselmis olivacea* was

employed as the out-group. Multiple alignments of *cob* sequences were produced and a section totaling 127 amino acids was employed for the analysis. Although these preliminary results are based on a small data set, the neighbor joining, parsimony and maximum likelihood trees (Figures 17, 18, and 19, respectively) show the same topologies, and in each case, all taxa affiliate to the lineages predicted by the nuclear and/or plasmid data (see Figure 2 and references therein). The parsimony and maximum likelihood trees provide reasonable statistical support for the view that the *Volvox*-clade and the *Oogamochlamys*-clade are sisters to the exclusion of the *Tetracystis*-clade and the *Dunaliella*-clade; the neighbor-joining tree is also in agreement with this view, though the result is less statistically robust. Thus, based on this small data set, the mitochondrial *cob* data are in agreement with the nuclear and plastid data.

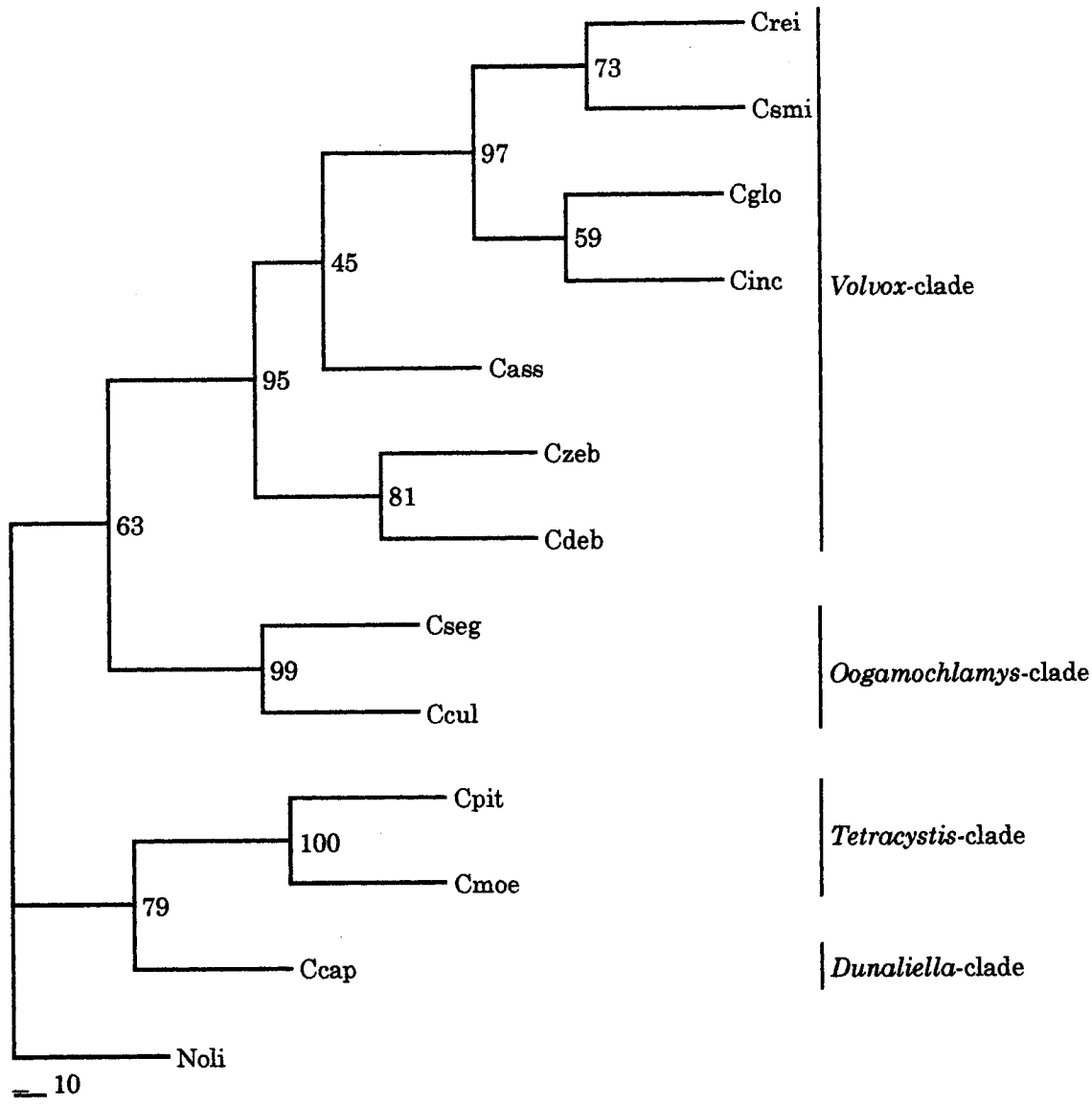


Figure 17. Phylogenetic analysis of *cob* sequences by neighbor joining. Abbreviations: Csmi, *C. smithii*; Crei, *C. reinhardtii*; Cinc, *C. incerta*; Cglo, *C. globosa*; Cass, *C. assymetrica*; Cdeb, *C. debaryana*; Czeb, *C. zebra*; Ccul; *C. culleus*; Cseg, *C. segnis*; Cmoe, *C. moewusii*; Cpit; *C. pitchsmannii*; Ccap, *C. capillatum*; Noli, *N. olivacea*.

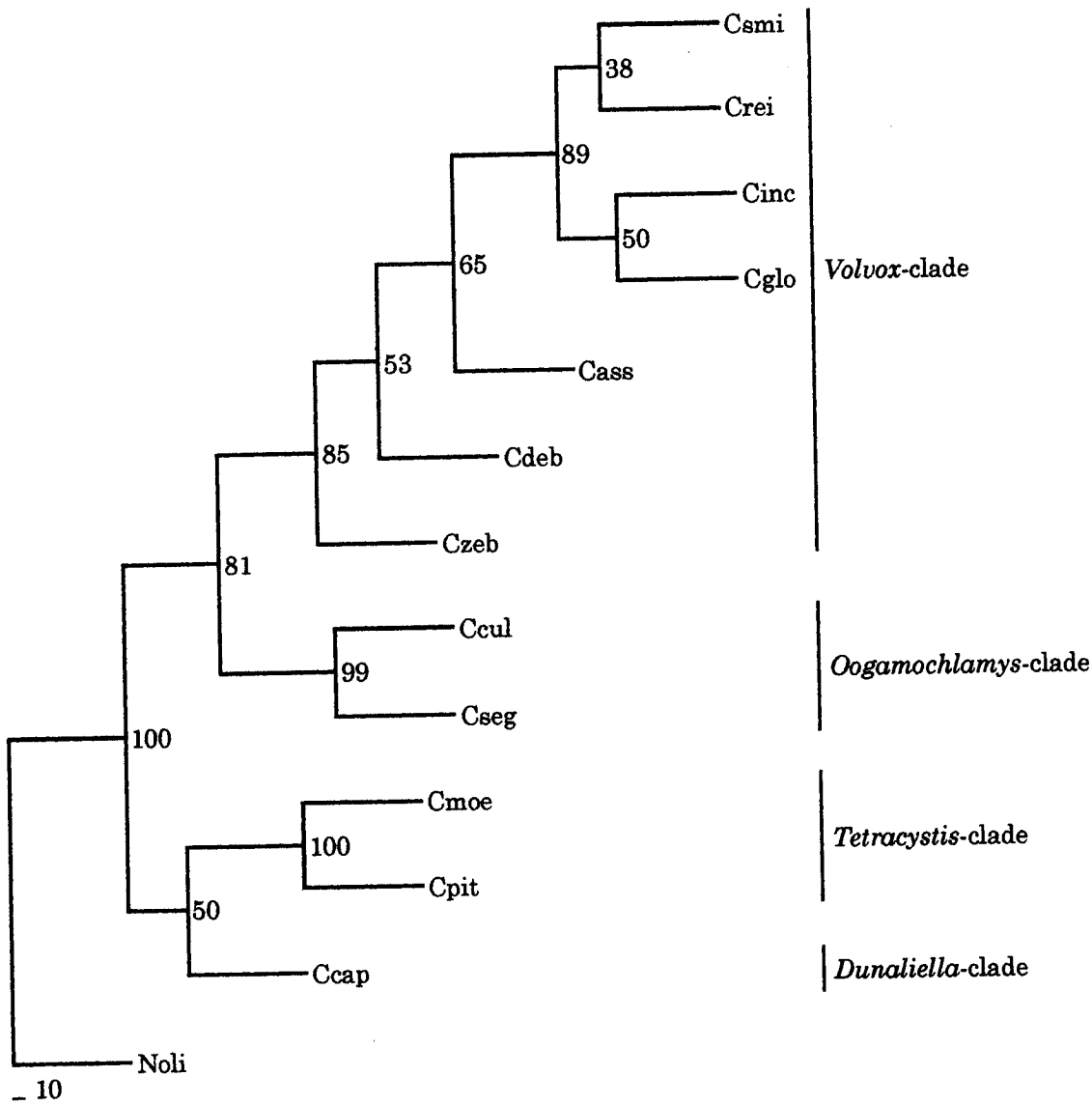


Figure 18. Phylogenetic analysis of *cob* sequences by parsimony. Abbreviations are as in Figure 17.

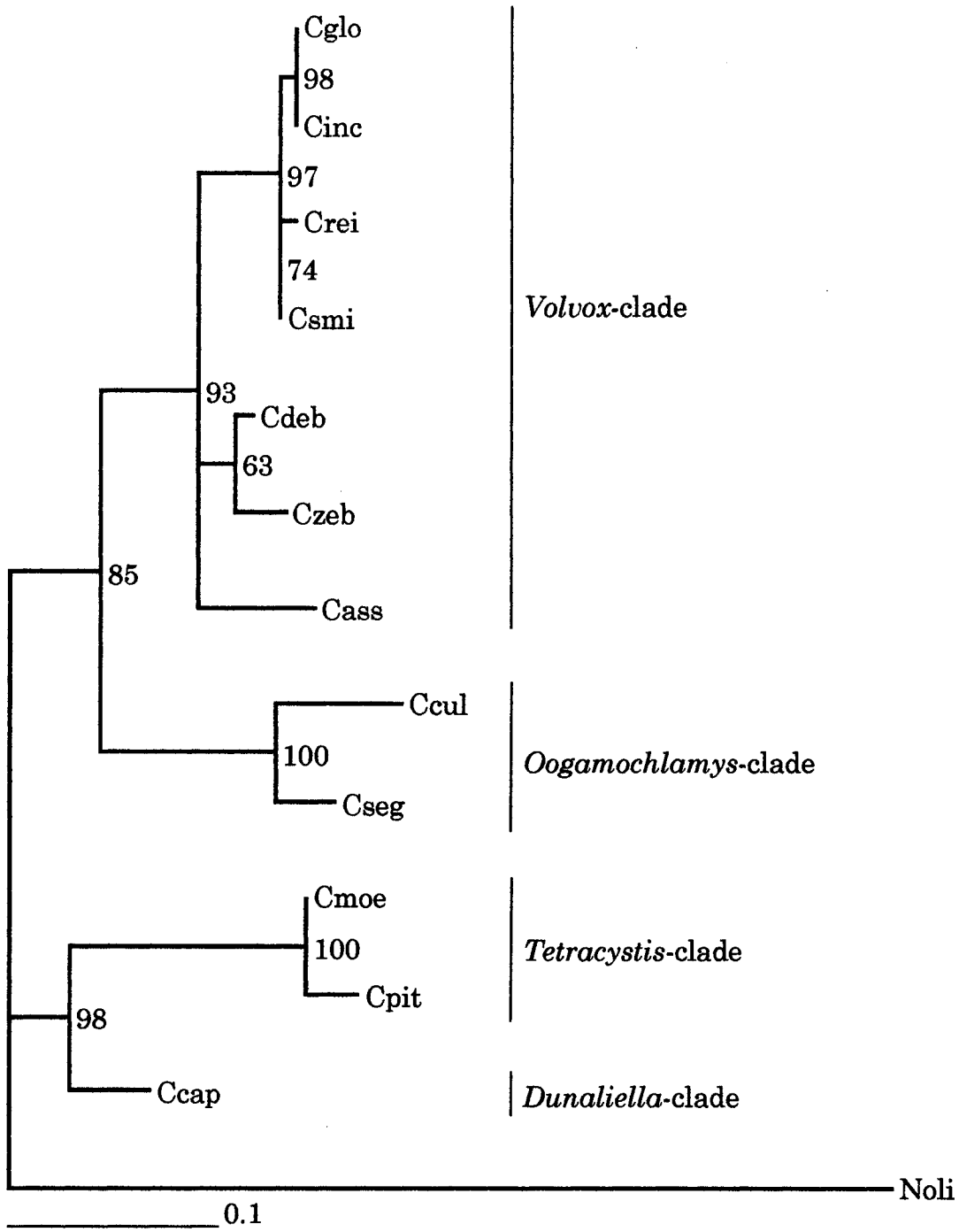


Figure 19. Phylogenetic analysis of cob sequences by maximum likelihood. Abbreviations are as in Figure 17.

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