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RESEARCH ARTICLE

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Complete mitochondrial genomes of the three brown algae (Heterokonta: Phaeophyceae) *Dictyota dichotoma*, *Fucus vesiculosus* and *Desmarestia viridis*

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Abstract We report the complete mitochondrial sequences of three brown algae (*Dictyota dichotoma*, *Fucus vesiculosus* and *Desmarestia viridis*) belonging to three phaeophycean lineages. They have circular mapping organization and contain almost the same set of mitochondrial genes, despite their size differences (31,617, 36,392 and 39,049 bp, respectively). These include the genes for three rRNAs (23S, 16S and 5S), 25–26 tRNAs, 35 known mitochondrial proteins and 3–4 ORFs. This gene set complements two previously studied brown algal mtDNAs, *Pylaiella littoralis* and *Laminaria digitata*. Exceptions to the very similar overall organization include the displacement of *orf*s, tRNA genes and four protein-coding genes found at different locations in the *D. dichotoma* mitochondrial genome. We present a phylogenetic analysis based on ten concatenated genes (7,479 nucleotides) and 29 taxa. Stramenopiles were always monophyletic with heterotrophic species at the base. Results support both multiple primary and multiple secondary acquisitions of plastids.

Keywords Brown algae · Evolution of mitochondria · Stramenopiles · Mitochondrial DNA · Secondary plastids

Abbreviation Mt: Mitochondrial

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Introduction

The stramenopiles (section Heterokonta) encompass both unicellular, e.g., the Bacillariophyceae (diatoms), and multicellular lineages, e.g., the Phaeophyceae (brown algae). They also comprise both heterotrophic species such as Bicosoecida and Oomycetes and autotrophic species including the Bacillariophyceae, Chrysophyceae and Phaeophyceae (Leipe et al. 1994). The plastids of the photosynthetic species arose from a secondary endosymbiosis event involving algae related to extant red algae (Delwiche and Palmer 1997; Medlin et al. 1997). The origin of the host cell that originally acquired the plastid remains unclear. Two main hypotheses have been proposed: first that stramenopiles acquired their photosynthetic lineage late (Saunders et al. 1995; Leipe et al. 1996; Blackwell and Powell 2000); and second that chloroplast acquisition occurred earlier, in a common ancestor of alveolates and stramenopiles, and prior to the stramenopile divergence (Cavalier-Smith 1998; Fast and Keeling 2001; Yoon et al. 2004). This hypothesis implies subsequent multiple losses of chloroplasts within the heterotrophic lineage.

Phylogenetic relationships within the stramenopiles and/or within heterokont algae have been studied using different molecular markers and methods, such as the nuclear small subunit rDNA (e.g., Leipe et al. 1996; Van de Peer et al. 1996; Potter et al.1997; Guillou et al. 1999; Van de Peer et al. 2000), the chloroplast gene *rbc*L

(Daugbjerg and Andersen 1997; Daugbjerg and Guillou 2001), or a combination of both (Sorhannus 2001; Goertzen and Theriot 2003). In these analyses, the nuclear data support the heterokont algae as monophyletic with generally the Bacillariophyceae (including Bolidophyceae) as the most basal group. Exceptionally Oomycetes have been included within the heterokont algae (Van de Peer et al. 2000). Based on further analyses using chloroplast genes, the monophyletic origin of heterokont algal plastids was confirmed with the Bacillariophyceae occupying an intermediate position, associated with the Chrysophyceae.

As the relationship between the photosynthetic and the heterotrophic stramenopiles is still unclear, we decided to reevaluate their phylogeny using a molecular marker common to all of them, e.g., a suite of ten genes from the mitochondrial (not plastidial) genome. Mitochondrial genes provide a valuable alternative to nuclear genes, since the origin of mitochondria is probably concomitant with that of the eukaryotic cell (Martin and Müller 1998; Vellai et al. 1998) and thus are more likely to reflect the evolutionary history of the original host cell. Two recent studies also utilized mitochondrial genes (four genes, 3,708 nucleotides: Sanchez Puerta et al. 2004; five proteins sequences, 1,791 amino acids: Armbrust et al. 2004) and were able to confirm monophyly of the stramenopiles. However, the focus of these studies was the phylogenetic placement of *Emiliania huxleyi* and *Thalassiosira pseudonana*, respectively. Neither of these studies addressed the late–early hypotheses of secondary chloroplast acquisition.

In addition to refining the stability of the broader phylogeny, we were also interested in the evolution of the brown algae and their mitochondrial genomes. The brown algae are characterized by diverse morphologies, ranging from microscopical filaments to huge kelps with complex fronds, tens of meters in length (Patterson 1999). In an earlier study, two brown algal mitochondrial genomes were sequenced, *Pylaiella littoralis* [Ectocarpales] (Oudot-Le Secq et al. 2001), which had been viewed as a representative of the brown algal ancestral condition, and *Laminaria digitata* [Laminariales] (Oudot-Le Secq et al. 2002), which was hypothesized to have evolved more recently. Both were found to be very similar in gene content as well as in gene arrangement, but were quite different with respect to the presence (in *P. littoralis* only) of group II introns and a large insertion of DNA of an unknown origin. In the present study, we sequence and analyze the complete mitochondrial sequences of three additional brown algae *Dictyota dichotoma* [Dictyotales], viewed now as the most ancestral brown algal lineage (Draisma et al. 2001; Rousseau et al. 2001), *Fucus vesiculosus* [Fucales] and *Desmarestia viridis* [Desmarestiales].

We characterize these three new phaeophycean mtDNAs in detail and reassess phylogenetic relationships within the stramenopiles.

Material and methods

Algal sources

Dictyota dichotoma (Hudson) J.V. Lamouroux was cultivated from an isolated individual collected in 1996 at the Pointe de Mousterlin, Brittany, France, by H. Pakker. *Fucus vesiculosus* Linnaeus was collected on 22 October 2001 in front of the Marine Biological Station, in Roscoff, Brittany, France, by M.-P. Oudot-Le Secq. *Desmarestia viridis* (O.F. Müller) J.V. Lamouroux was collected in 1978 in Helgoland by K. Lüning. Frozen tissue from the original algal culture was used.

DNA preparation and PCR procedures

Algal tissue was ground in liquid nitrogen and total DNA extracted in a buffer containing 100 mM Tris–HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 0.1% (w/v) PVPP, 0.2% (v/v) β-mercaptoethanol and 2% (w/v) CTAB. After two chloroform-isoamyl alcohol (24:1, v/v) extractions, the aqueous DNA solution was purified with the Sephaglas BandPrep Kit (Pharmacia), following the manufacturer's instructions. These total DNAs were used as a template to amplify the mitochondrial DNAs. Primer design was based initially on *Pylaiella littoralis* and *Laminaria digitata* mitochondrial sequences and on multiple alignments of mitochondrial and bacterial sequences of homologous genes; and secondarily on the specific algal sequence under study. PCR experiments were performed either in a Cetus DNA thermo cycler (Perkin Elmer) or in a Mastercycler gradient cycler (Eppendorf) with Ready-to-Go beads (Taq, Amersham Pharmacia Biotech), 2 mM of each primer and 0.5–2 μL of total DNA (1–20 ng). The reaction profile was as follows: initial denaturation at 95°C for 1–3 min; ten cycles of denaturation at 95°C for 30 s, annealing at 46–62°C (depending on primer sequences) for 1 min, and elongation step at 72°C for 20 s–3 min (depending on the expected size of the amplified fragment); followed by 20–30 cycles of denaturation at 95°C for 30 s, annealing step at 46–62°C for 30 s, and elongation at 72°C for 20 sec–3 min; and a final elongation at 72°C for 10 min. When the expected size of the amplified fragment was above 4 kb the Expand Long Template PCR System (Roche) was used, according to the manufacturer protocols. PCR products were loaded on 0.8–1.5% agarose gels; fragments were cut from the gel. The DNA was extracted from gel slices and was purified using the Wizard® PCR Preps DNA Purification System (Promega) following the manufacturer's directions.

DNA sequencing

Direct sequencing of PCR products was performed using the PCR primers and additional internal primers

with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems). Sequencing reactions were run on an ABI 377 automated sequencer (PE Applied Biosystems). Sequences, read on both strands, were assembled using the BioEdit Sequence Alignment Editor version 5.0.9 (Hall 1999).

Sequences and alignments

The complete sequences of the three mitochondrial genomes are available from GenBank under the following accession number: *F. vesiculosus*, AY494079; *D. dichotoma*, AY500368; and *D. viridis* AY500367. The three new mitochondrial sequences were manually aligned with those of *P. littoralis* and *L. digitata* using BioEdit Sequence Alignment Editor versions 5.0.9 and 6.0.7 (Hall 1999). The nucleotide sequences of the ten mitochondrial genes (*atp6*, *atp9*, *cob*, *cox1–3*, *nad1*, *nad3–4* and *nad4L*) from 22 selected organisms and the bacterial counterparts of these genes from two α-proteobacteria (Table 1) were aligned with those of the five brown algal mitochondrial sequences. Regions too ambiguous to align were excluded. The dataset is 7,479 nucleotide positions long with *atp6* (543), *atp9* (213), *cob* (1,083), *cox1* (1,437), *cox2* (726), *cox3* (780), *nad1* (906), *nad3* (339), *nad4* (1,200) and *nad4L* (252).

Phylogenetic analysis

Bayesian maximum likelihood analysis was performed using MrBayes version 3 (Ronquist and Huelsenbeck 2003). Models of DNA evolution were determined with Modeltest 3.06 (Posada and Crandall 1998) and PAUP (Swofford 2003), based on Hierarchical Likelihood Ratio Tests, run on each partition (e.g., individual gene alignment). Table 2 summarizes the models chosen. The following settings were used: nst (number of substitution types) = 6 (models TVM and GTR), with gamma-distributed rates across sites and proportion of invariable sites when required. We allowed the set of parameters to be different for each partition. Introduced gaps were treated as missing data in subsequent analyses. The different parameters from the phylogenetic models were estimated during the phylogenetic analysis. Four chains were run; trees were sampled every 100 of the 2,000,000 generations and the 1,000 first trees were discarded as burn-in.

Table 1 Organisms included in the phylogenetic study

Species[a]	Classification	Accession no.[b]
Desmarestia viridis	Stramenopiles, Phaeophyceae	**AY500367**
Dictyota dichotoma	Stramenopiles, Phaeophyceae	**AY500368**
Fucus vesiculosus	Stramenopiles, Phaeophyceae	**AY494079**
Laminaria digitata	Stramenopiles, Phaeophyceae	AJ344328
Pylaiella littoralis	Stramenopiles, Phaeophyceae	AJ277126
Thalassiosira pseudonana	Stramenopiles, Bacillariophyceae	DQ186202
Chrysodidymus synuroideus	Stramenopiles, Chrysophyceae	AF222718
Ochromonas danica	Stramenopiles, Chrysophyceae	AF287134
Cafeteria roenbergensis	Stramenopiles, Bicosoecida	AF193903
Thraustochytrium aureum	Stramenopiles, Labyrinthulida	AF288091
Phytophthora infestans	Stramenopiles, Oomycetes	U17009
Saprolegnia ferax	Stramenopiles, Oomycetes	AY534144
Emiliania huxleyi	Haptophyceae, Isochrysidales	AY342361
Rhodomonas salina	Cryptophyta, Cryptomonadaceae	AF288090
Cyanidioschyzon merolae	Rhodophyta, Bangiophyceae	D89861
Porphyra purpurea	Rhodophyta, Bangiophyceae	AF114794
Chondrus crispus	Rhodophyta, Florideophyceae	Z47547
Nephroselmis olivacea	Viridiplantae, Chlorophyta, Prasinophyceae	AF110138
Prototheca wickerhamii	Viridiplantae, Chlorophyta, Trebouxiophyceae	PWU02970
Chara vulgaris	Viridiplantae, Streptophyta, Characeae	AY267353
Marchantia polymorpha	Viridiplantae, Streptophyta, Marchantiaceae	M68929
Malawimonas jakobiformis	Malawimonadidae	AF295546
Reclinomonas americana	Jakobidae	AF007261
Monosiga brevicollis	Choanoflagellida, Codonosigidae	AF538053
Schizophyllum commune	Fungi, Basidiomycota	AF402141
Allomyces macrogynus	Fungi, Chytridiomycota	U41288
Acanthamoeba castellanii	Acanthamoebidae	U12386
Mesorhizobium loti MAFF303099	α-proteobacteria, Rhizobiales	BA000012
Rickettsia prowazekii str. Madrid E	α-proteobacteria, Rickettsiales	AJ235269

[a]Underlined species are photosynthetic with chloroplasts derived from primary endosymbiotic events; double underlined species have chloroplasts from secondary origin

[b]Numbers in bold are those of the sequences obtained in this study

Table 2 Evolutionary models chosen by Modeltest

Gene	Model
atp6	GTR + I + G
atp9	GTR + I + G
cob	TVM + I + G
cox1	GTR + I + G
cox2	TVM + G
cox3	TVM + I + G
nad1	GTR + I + G
nad3	TVM + I + G
nad4	GTR + I + G
nad4L	GTR + G

GTR General time-reversible model, *I* Proportion of invariable sites, *G* shape parameter of the gamma distribution, *TVM* Trans-Versional model.

Results and discussion

Characterization of mt genomes in *Dictyota dichotoma*, *Fucus vesiculosus* and *Desmarestia viridis*, with references to those of *Pylaiella littoralis* and *Laminaria digitata*

The *D. dichotoma*, *F. vesiculosus* and *D. viridis* mtDNAs have a circular mapping organization. The three mtDNA maps are depicted in Fig. 1. Their sizes are: 31,617 bp (*D. dichotoma*), 36,392 bp (*F. vesiculosus*) and 39,049 bp (*D. viridis*). Different characteristics of the genomes, such as their A + T content for different categories of sequences and figures about spacer sequences and overlaps are summarized in Table 3. All these values are in the same range as those of *P. littoralis* and *L. digitata* (Oudot-Le Secq et al. 2001, 2002).

Two of the overlapping regions are exactly conserved among the three mtDNAs described here, as well as in *P. littoralis* and *L. digitata*; these are found between the genes encoding the ribosomal proteins *rps8*, *rpl6* and *rps2*. Both involve the start codon ATG and a stop codon (TGA or TAA); the *rps8–rpl6* overlap is 4 bp long, ATGA, and the *rpl6–rps2* overlap is only 1 bp long, A (in the context: taAtg).

More than 60 putative coding regions were identified in each of the three mtDNAs (Table 4), basically the same as in *P. littoralis* and *L. digitata* mtDNAs. These are the genes for three rRNAs (23S, 16S and 5S), for 25 tRNAs in the case of *D. dichotoma* or 26 for *F. vesiculosus* and *D. viridis*, for 35 proteins identified by sequence homology, as well as for one common ORF (also encoded in *P. littoralis* and *L. digitata* mtDNAs). *Fucus vesiculosus* and *D. viridis* share another related ORF, despite its size difference (379 and 622 amino acids, respectively). Finally, *D. dichotoma* and *D. viridis* each encode one more unique unknown *orf*, *orf54* and *orf211*, respectively. None of the three new mtDNAs contain introns, nor any insertion with a phage-like RNA polymerase gene as has been described in the *P. littoralis* genome (Costa et al. 1997; Rousvoal et al. 1998; Oudot-Le Secq et al. 2001). At present *P. littoralis* is the only known brown alga that shows these unusual features in its mitochondrial genome.

The gene order is very well-conserved between the three new brown algae mtDNAs and those of *P. littoralis* and *L. digitata*, although exceptions exist (Fig. 2). These are mainly due to *orf*s not present in all the mtDNAs (see gray triangles in Fig. 2), to tRNA genes (see dashed lines) and to a few genes coding for proteins (*atp8*, *atp9*, *rpl31* and *rps10*) in the *D. dichotoma* mtDNA compared to the other mtDNAs (see black lines).

In order to calculate pairwise identity scores, a nucleotide alignment of the five complete mitochondrial genomes was restricted to the genes, conserved *orf*s and corresponding spacers, found at the same location in all the brown algal mtDNAs (i.e., those that are found at the same horizontal level in the five maps, Fig. 2). The resulting alignment contains 34,501 positions. Four out of the five mtDNAs share from 69 to 75% identity (Table 5), whereas the *D. dichotoma* sequence is only 57–58% identical to the others. When the *cox2* insertion region, absent in *D. dichotoma* (see below), is removed from the alignment it reaches 64% identity with the others (not shown).

Genetic code

The universal genetic code seems to be used in all of these mtDNAs, based on protein sequence alignments. RNA editing does not appear to be necessary.

A gene, *rps14* in *D. dichotoma* and a putative *orf*, *orf379* in *F. vesiculosus*, use GTG as initiation codon, and two other *orf*s, *orf211* in *D. viridis* and *orf37* in *D. dichotoma* use TTG. GTG and TTG are commonly used as alternative start codons in the mitochondrial genetic code of different animal groups (e.g., invertebrates, molds, protozoans and coelenterates), in the plant plastidial code and in the bacterial code (Elzanowski and Ostell 2000). All the codons are used to encode proteins (Supplementary material). Those ending in A or T outnumbered the synonymous codons ending in G or C, as expected for A + T rich genomes. All of the three stop codons are used (see Table 5), with a marked preference for TAA of 64% (*D. dichotoma*) and 83% (*F. vesiculosus* and *D. viridis*).

rRNA genes

The 5S rRNA gene is encoded in the three new mtDNAs. These three genes share with the *L. digitata* 5S gene, a putative insertion between stems V and I that may form a hairpin that is absent in *P. littoralis* (a representation of the potential folding of the five *rrn5* is provided as Supplementary Material). Stems I are shorter and weaker than usual, as was the case for the *P. littoralis* and *L. digitata* genes. The *rns* genes are well conserved and their potential folding is in general

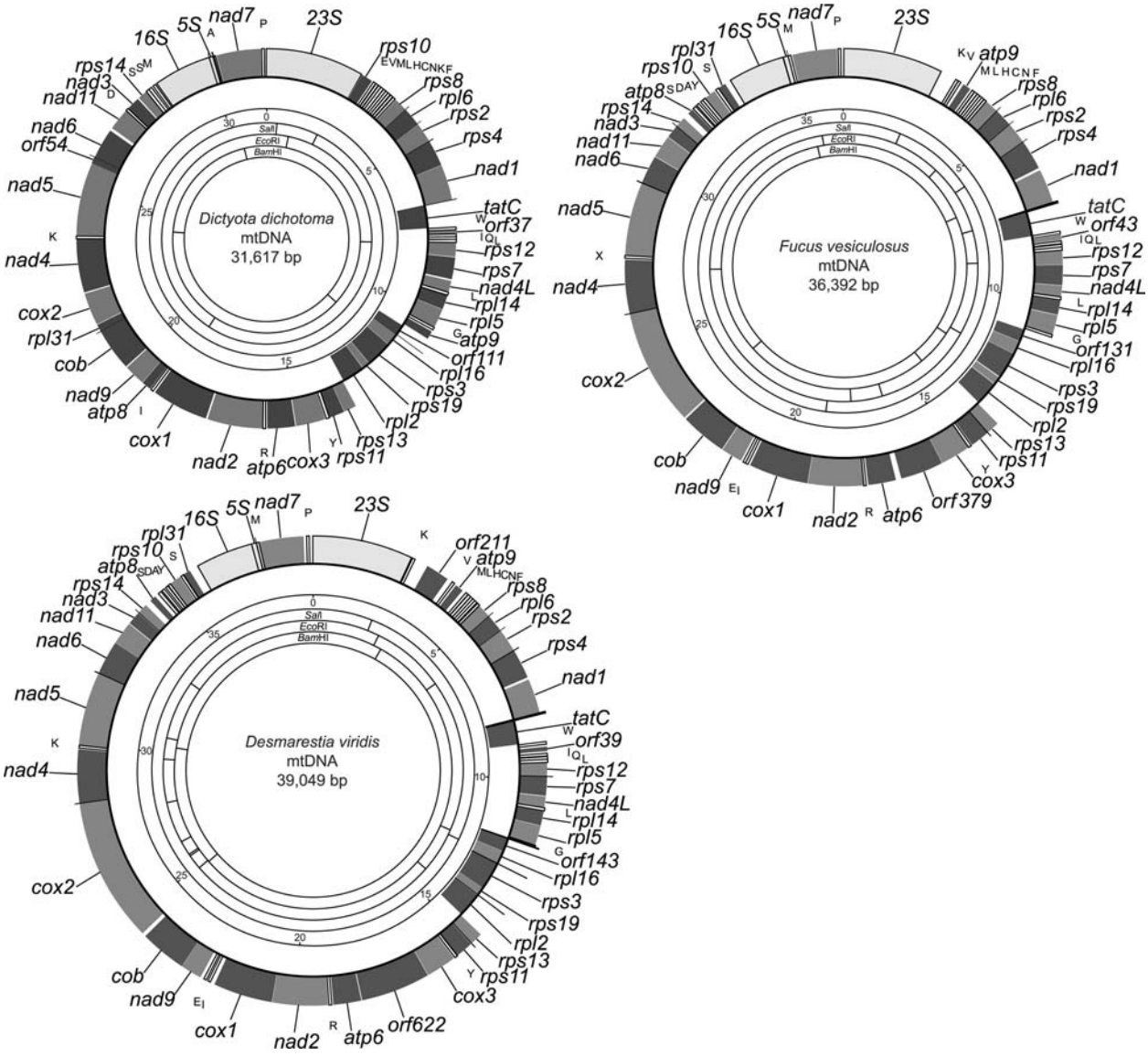


Fig. 1 Physical map and gene organization of the *Dictyota dichotoma*, *Fucus vesiculosus* and *Desmarestia viridis* mtDNAs (a color version is provided as supplementary data). Genes and *orfs* are depicted as blocks, with gene abbreviations listed in Table 4. The gene blocks shown outside and inside are transcribed clockwise and counter-clockwise, respectively. Transfer RNA genes (*trn*) are indicated as *white boxes* surrounded by *black lines*, their names are indicated by the amino acid (one-letter code) they specify (see Fig. 3). *Thin black bars* between gene blocks indicate gene overlaps. The *three inner rings* show the restriction fragments generated by *Bam*H1, *Eco*R1 and *Sal*1, respectively. On the *black circle*, between genes and restriction maps, the scale size is shown (in kilobases)

Table 3 Characteristics of *Dictyota dichotoma*, *Fucus vesiculosus* and *Desmarestia viridis* mtDNAs

Characteristic	*D. dichotoma*	*F. vesiculosus*	*D. viridis*
Size (bp)	31,617	36,392	39,049
Overall A + T content (%)	63.5	65.6	63.4
Protein A + T content (%)	65.4	66.7	64.0
Spacer A + T content (%)	76.5	77.3	74.1
rDNA A + T content (%)	58.9	57.8	55.7
Spacer content (%)	3.2	5.6	6.1
Spacer size (bp)	1–74	1–422	1–385
Avg. spacer size (bp)	18.8	35	43.8
Overlapping genes (bp)	12	9	13
Overlap size (bp)	1–30	1–66	1–60
Average overlap size (bp)	8.3	13.4	15.2

Table 4 Coding regions identified in *Dictyota dichotoma*, *Fucus vesiculosus* and *Desmarestia viridis* mtDNAs

Genes and *orf*s	*D. dichotoma*	*F. vesiculosus*	*D. viridis*
Ribosomal RNA genes: 3			
rnl (23S rRNA)	+	+	+
rns (16S rRNA)	+	+	+
rrn5 (5S rRNA)	+	+	+
Transfer RNA genes (See Figs. 3, 4)	25	26	26
Ribosomal protein genes			
Small subunit (*rps*): 11 (*rps2–4, rps7, rps8, rps10–14, rps19*)	+	+	+
Large subunit (*rpl*): 6 (*rpl2, rpl5, rpl6, rpl14, rpl16, rpl31*)	+	+	+
Complex I (NADH dehydrogenase genes): 10 (*nad1–7*, *nad9*, *nad11*)	+	+	+
Complex III (apocytochrome b): 1 (*cob*)	+	+	+
Complex IV (cytochrome oxidase genes): 3 (*cox1*, *cox2*, *cox3*)	+	+	+
Complex V (F_0-ATPase genes): 3 (*atp6*, *atp8*, *atp9*)	+	+	+
Sec-independent protein translocation pathway gene: 1(*tatC*) [a]	+	+	+
Unidentified conserved *orf*s	*orf*37	*orf*43	*orf*39
	*orf*111	*orf*131	*orf*143
		*orf*379	*orf*622
Unidentified unique *orf*s	*orf*54		*orf*211
Total	66	67	68

[a]Corresponds to gene RP782 of unknown product of *Rickettsia prowazekii*; mitochondrial *orfs* homologous to the *tatC* gene, called also *ymf16*: ORF262 *Chondrus crispus*, ORFx angiosperm, ORF244 *Marchantia polymorpha*, ORF234.1 *Prototheca wickerhamii*, ORF260 *Reclinomonas americana*. Plastidial *orfs* homologous to the *tatC* gene: *ycf43*, ORF263 *Odontella sinensis*, ORF254 *Porphyra purpurea*

accordance with the eubacterial model (Wuyts et al. 2002). The same can be said about the *rnl* genes although some of their variable areas differ from one species to the other.

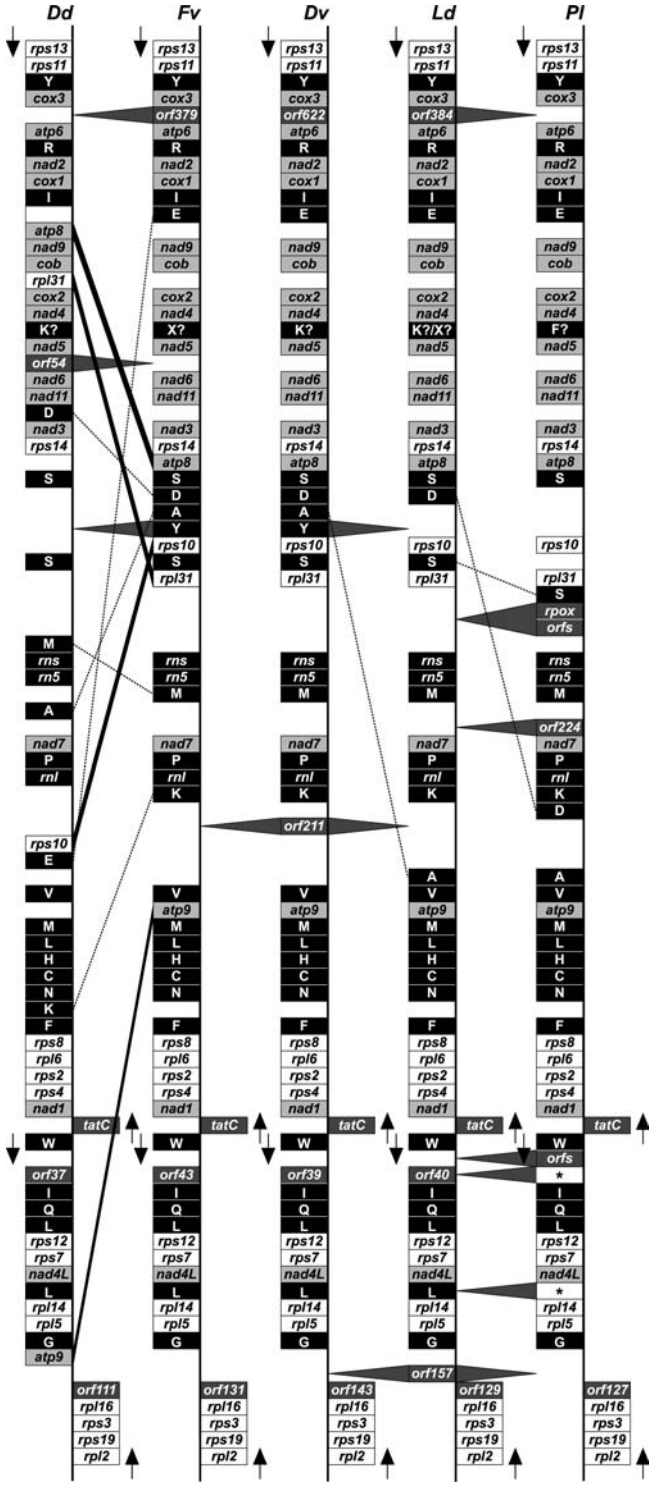
trn genes

The *D. dichotoma*, *F. vesiculosus* and *D. viridis* mtDNAs share 24 potential *trn* genes with *L. digitata* mtDNA (Fig. 3) that can fold, following the standard cloverleaf secondary structure (Sprinzl et al. 1998) and fit the L-shape, except in the $tRNA^{Arg}$ [ucu] from *D. dichotoma*, where the position 48 is not a pyrimidine but an adenine (Zagryadskaya, Kotlova and Steinberg 2004). The $tRNA^{Leu}$ [caa] gene cannot be depicted in the *P. littoralis* mtDNA (see the star in Fig. 2), though the region still shares high sequence homology with the *trn* sequence, it lacks the main conserved features (Oudot-Le Secq et al. 2002). Another region, common to the five mtDNAs, but labeled differentially in the different algal mtDNAs (Figs. 2 and 4a), "K?" in *D. dichotoma*, *D. viridis* and *L. digitata*, "X?" in *F. vesiculosus* and *L. digitata*, or "F?" in *P. littoralis*, can fold according to the cloverleaf model. As shown in Fig. 4a, this region has a high similarity level with the *trn* Glu sequence, but some of the so-called invariant positions do not follow the consensus (gray shading in Fig. 4a). In addition, the anticodon positions are not the same in all the five mtDNAs. They are TTT for a *trn* Lys in *D. dichotoma* and *D. viridis*. The anticodon in *F. vesiculosus* mtDNA is TTA, which should "decode" the stop codon TAA (the most frequently used stop codon in the genome). It is even more complex in the *P. littoralis* and *L. digitata* cases; in the first, the anticodon loop is one nucleotide longer than usual, leading to "AAAA", the *trn* Phe (F), while one nucleotide is missing in the second, leading to two possibilities for the anticodon position: TTT (*trn* Lys) as in *D. dichotoma* and *D. viridis* or TTA as in *F. vesiculosus*. The *P. littoralis* and *L. digitata* sequences also have an insertion of 16 and 15 nucleotides, respectively, in the D loop (see Fig. 4). Finally, since the five mtDNAs encode well-conserved, canonical genes for both tRNA Lys and Phe, this noncanonical gene should

Table 5 Pairwise identities of mtDNA sequences

	P. littoralis	*L. digitata*	*D. viridis*	*F. vesiculosus*	*D. dichotoma*
P. littoralis	–				
L. digitata	73 % [a]	–			
D. viridis	71 %	75 %	–		
F. vesiculosus	69 %	70 %	72 %	–	
D. dichotoma	57 %	57 %	57 %	58 %	-

Pairwise identity between sequences were calculated in BioEdit v6.0.7 (Hall 1999)
The alignment of the five mtDNAs was restricted to genes and spacers found at the same location, e.g., all the genes, *orfs* and corresponding spacers that are highlighted in Fig.2 by any kind of line were excluded. This alignment totals 34,501 positions (32,599 nucleotides for *P. littoralis*, 33,355 for *L. digitata*, 33,407 for *D. viridis*, 32,353 for *F. vesiculosus* and 29,684 for *D. dichotoma*)
[a]In Oudot-Le Secq et al. (2002), the alignment was less reduced, leading to 71% identity



◀

Fig. 2 Gene order comparison between the mtDNAs of *Dictyota dichotoma* (*Dd*), *Fucus vesiculosus* (*Fv*), *Desmarestia viridis* (*Dv*), *Laminaria digitata* (*Ld*) and *Pylaiella littoralis* (*Pl*) mtDNAs (colored version in supplementary material). On these linear representations of mtDNA gene contents, only the gene order is taken into account; spaces between two genes are there only to allow horizontal alignment and to help visual comparison. Genes on a same horizontal line are homologous. *White blocks* depict ribosomal protein encoding genes; *light gray blocks* (*black writing*) depict genes coding for proteins involved in respiratory chains; *dark gray blocks* (*white writing*) depict other protein encoding genes and *orfs*; *black blocks* depict RNA genes (tRNA and rRNA genes). *Arrows* indicate transcription direction. *Gray triangles* highlight presence/absence of a gene between two mtDNAs. *Thick black lines* highlight the different locations of protein encoding genes between *D. dichotoma* and other mtDNAs. *Dashed black lines* highlight *trn* different locations. The two asterisks in *P. littoralis* mtDNA indicate that although tRNA Leu and *orf40* are not coded anymore, the corresponding regions still share high sequence homology with the *L. digitata* sequence

not be necessary to encode a tRNA. On the other hand, the fact that the cloverleaf folding is conserved, when the other tRNA's mandatory positions are not, may suggest that this pseudogene has now gained a new function. It could serve as punctuation marks, for the process of a large mRNA encompassing the surrounding genes. It is known that *trns* play such a role in fungi mt genome (Burger et al. 1985) and animal mt genomes (Clayton 1984). The case of a pseudogene that could have gained such a function has been described recently in the mt genome of a fish (Mabuchi et al. 2004). Furthermore, a long mRNA encompassing at least *cox2*, *nad4* and *nad5* genes has been evidenced by a northern experiment (M.-P. Oudot-Le Secq and Loiseaux-de Goër, unpublished data) in *P. littoralis*.

Fucus vesiculosus and *D. viridis* mtDNA share one additional putative tRNA gene (Fig. 4b), that of $tRNA^{Tyr}$ [aua]. Both sequences display deviations from the consensus (gray shading). Another $tRNA^{Tyr}$ gene, but with [gua] as anticodon, is shared by the five mtDNAs. We do not know if these noncanonical *trns* are transcribed and used or if they are remains or intermediates of the evolutionary process. In the first case, the sequences seem to be related to those of *trn* Glu and could have gained a new function as a processing signal, but in the latter (*trn* Tyr), there is no such obvious origin (not shown). Since all the codons are necessary to code the protein genes, the *trn* set encoded in the brown algal mtDNAs is not sufficient to decode them all; the lacking tRNAs have to be imported from the cytosol to mitochondria, as it is the case in many organisms (Schneider and Maréchal-Drouard 2000).

Protein-encoding genes and *orfs*

The identified mitochondrial protein-encoding gene set is the same in the five mtDNAs studied so far (Table 4). These are: genes for the three first subunits of the cytochrome oxidase (*cox1–3*); *cob*, which encodes apocytochrome B; ten genes encoding ten subunits of the NADH dehydrogenase *nad1–7, 4L, 9* and a short *nad11* gene; three genes encoding three subunits of the ATPase, *atp6, 8* and *9*; and seventeen ribosomal protein-coding genes. The *cox2* gene contains a large in-frame insertion of 2,994 bp in *D. viridis*, comparable in size and related to those found in the *cox2* genes of *P. littoralis* (2,973 bp) and *L. digitata* (2,979 nucleotides). The insert also exists in the *F. vesiculosus cox2* gene, with a slightly

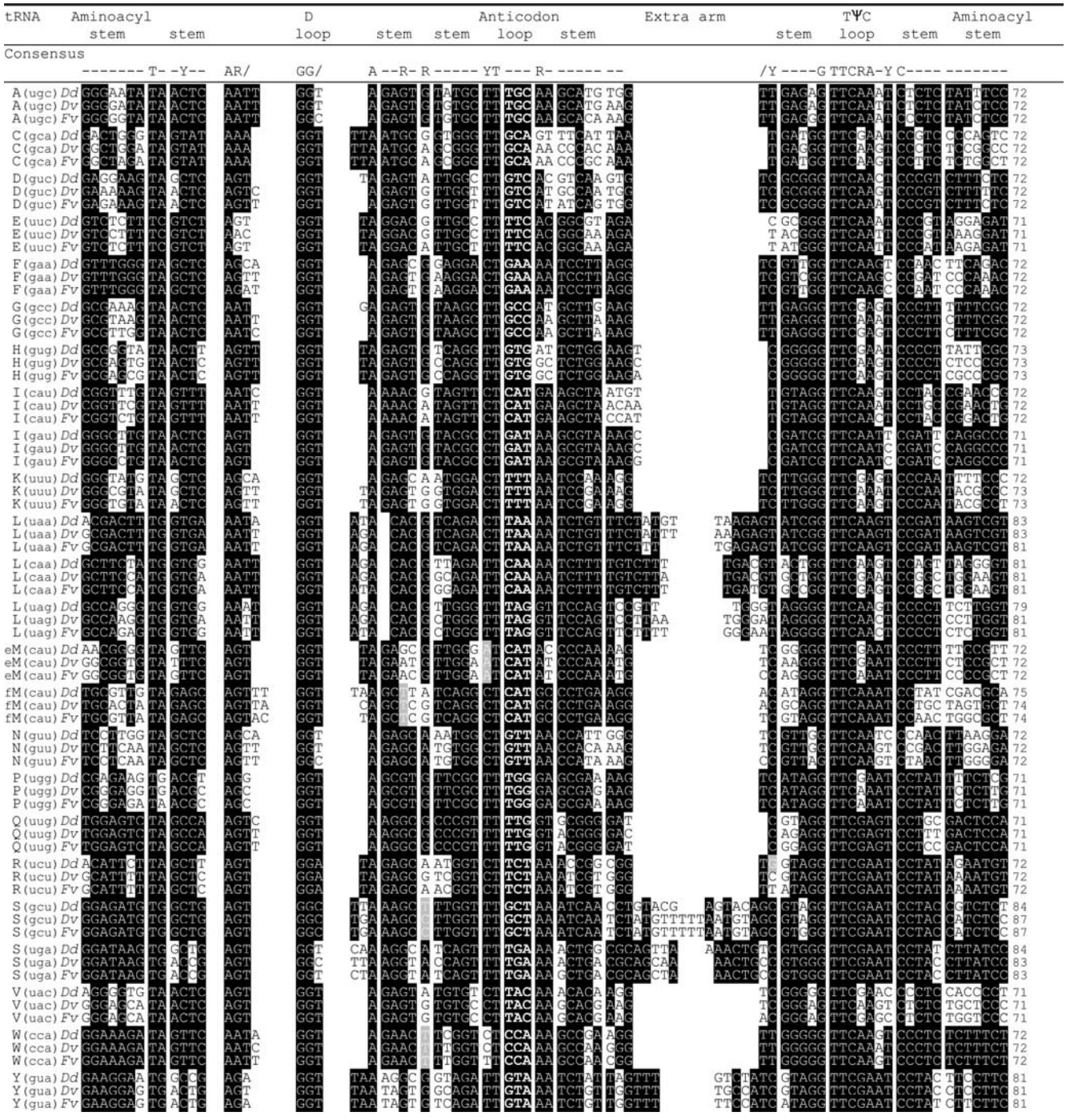


Fig. 3 Nucleotide sequences of the 24 common tRNA genes found in the mitochondrial genomes of *D. dictyota* (*Dd*), *D. viridis* (*Dv*) and *F. vesiculosus* (*Fv*). Anticodons are shown in *bold*. *Black shading* highlights identities between the three sequences for each *trn*. The *upper line* (Consensus) shows the invariant and semi-invariant nucleotides found in tRNA genes, *gray shading* indicates positions that do not fit the consensus

smaller size (2,283 bp) but is absent in the *D. dichotoma* gene. The insert is also absent in *Sphacellaria* (unpublished data). These insertions show a conserved leucine-zipper-like region and may prove useful as phylogenetic markers within brown algae. Pearson et al. (2001) sequenced a 648 bp long fragment identical to part of the *F. vesiculosus cox2*-insertion, starting 61 bp after the beginning of the insertion (only the first 3 bp are not the same) and finishing just upstream from the leucine-zipper-like region. This fragment was found among the clones obtained by the screening of a cDNA library generated by suppression subtractive hybridization for *F. vesiculosus* undergoing mild desiccation stress. Northern analysis did not show a clear over-expression after desiccation (Pearson et al. 2001). This suggests a possible excision of the insertion in the *cox2* mRNA, but

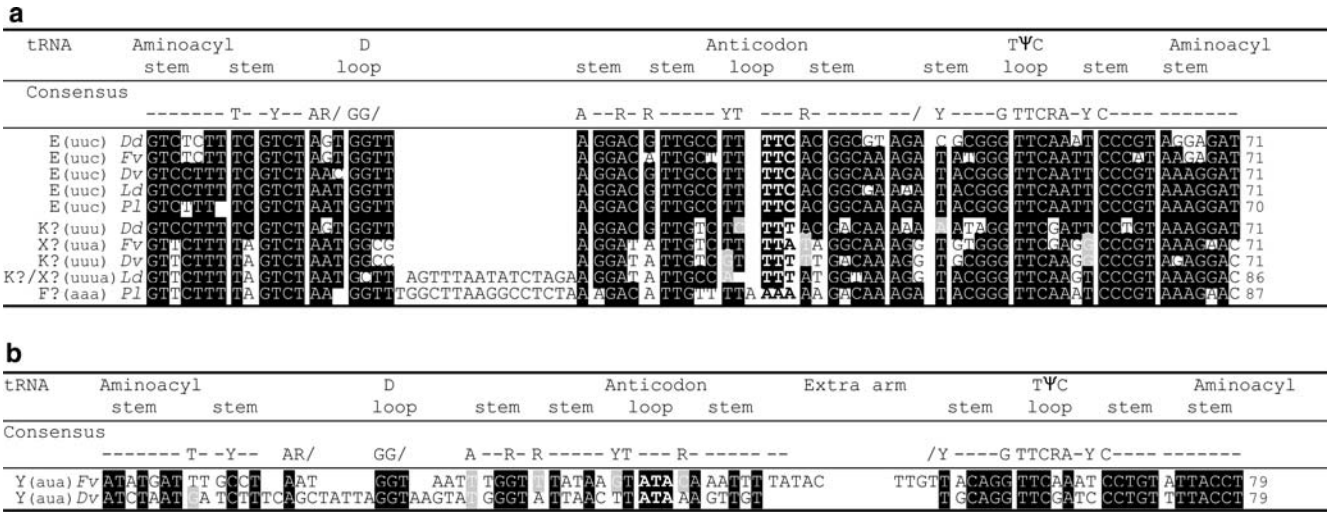


Fig. 4 Nucleotide sequences of two putative tRNA genes. **a** Comparison between the *trn* Glu (E) sequences of the five brown algal mtDNAs with a putative tRNA gene, found within the five genomes at a same location. **b** Sequence of an additional *trn* Tyr (Y) found in *D. viridis* and *F. vesiculosus* mtDNAs

would be in contradiction with the results obtained previously (Oudot-Le Secq et al. 2001). An AT-rich sequence downstream of the cloned fragment on the *F. vesiculosus* genome, i.e., AAAAAAAATTAGAAAAA could explain the presence of this clone after selection of full-length poly-A^+ mRNAs.

The gene encoding a protein transporter component of the *secY*-independent pathway, *tatC* (previously called *ymf16*), is present, always in the reverse transcriptional orientation, in the five brown algal mtDNAs. These genomes all encode one related *orf*, *orf111* (*D. dichotoma*), *orf131* (*F. vesiculosus*), *orf143* (*D. viridis*), *orf129* (*L. digitata*) and *orf127* (*P. littoralis*), which seems specific to brown algal mtDNAs. No similarity with other *orfs* was found in other algae or other organisms that have been found up till now. Between the *trn* Trp and *trn* Ile, another small *orf* is present in four out of the five genomes. These *orf43* (*F. vesiculosus*), *orf39* (*D. viridis*) and *orf40* (*L. digitata*), even if small, are very well conserved, while *orf37* (*D. dichotoma*) is different. No similarity with other *orfs* has been found by Blast. In *P. littoralis* three longer *orfs* precede a region that could be a pseudogene of this ORF. *F. vesiculosus* and *D. viridis* share another common *orf* with *L. digitata*, despite size differences (379, 622 and 384 amino acids encoded, respectively). Finally, *D. dichotoma* and *D. viridis* each encode one more unique unknown *orf*, *orf54* and *orf211*, respectively.

Repeats

Despite the great similarity between the brown algal mtDNAs, they do not share any common inverted repeats. The longest repeats found in each mtDNA are mutually different, both in size and location (not shown). Two of these harbor a different stem–loop of 23 nucleotides, one between *trn* Trp and *trn* Ile (in *orf37*) in *D. dichotoma*, with a eight nucleotide long loop, and the other between the *trn* Glu and *nad9* genes in the case of *D. viridis*, with a six nucleotide long loop. At the same location, *L. digitata* also has an imperfect inverted repeat (one bulge) of 15 nucleotides. But the largest inverted repeat is found at the end of the *D. dichotoma rps7* gene. This gene, which is about 100 nucleotide longer that its counterparts, harbors a 30 nucleotide long stem-loop, covering the end of the gene in such way that the stop codon is located in the middle of the second repeat.

Phylogenetic relationships of stramenopiles based on mt genes

Phylogenetic reconstructions were carried out using a concatenation of ten mitochondrial protein-encoding genes (*atp6*, *atp9*, *cob*, *cox1–3*, *nad1*, *nad3–4* and *nad4L*) from a set of 29 species (Table 1). The 12 stramenopiles comprise: four heterotrophs (one Bicosoecida, one Labyrinthulida and two Oomycetes), two Chrysophyceae, the Bacillariophyceae *Thalassiosira pseudonana* (Armbrust et al. 2004) and the five Phaeophyceae, *D. dichotoma*, *F. vesiculosus*, *D. viridis*, *L. digitata* and *P. littoralis*. These represent all of the complete stramenopile mtDNAs currently available. Other representatives from eukaryotic lineages included: the Haptophyceae *Emiliania huxleyi* (Sanchez Puerta et al. 2004), the Cryptophyceae *Rhodomonas salina*, three Rhodophyceae, two Chlorophyta, two Streptophyta, one Jakobidae, one Malawimonanididae, the Choanoflagellida *Monosiga brevicollis* (Burger et al. 2003), two fungi and the amoeboid protozoon *Acanthamoeba castellanii* (Burger et al. 1995); finally, two α-proteobacteria were used as outgroup.

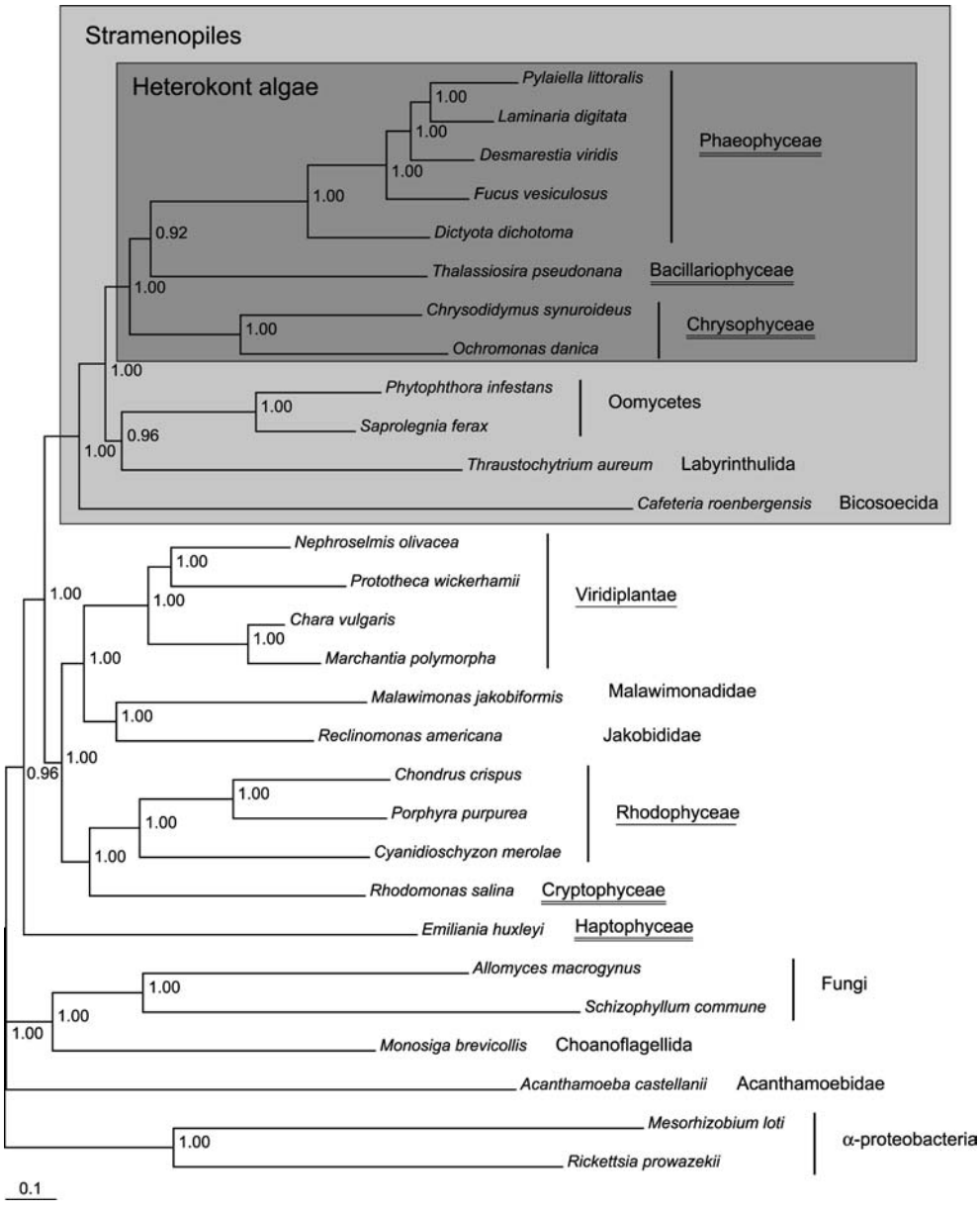


Fig. 5 Phylogenetic tree constructed from mtDNA nucleotide sequences of ten genes (*atp6*, *atp9*, *cob*, *cox1-3*, *nad1*, *nad3-4*, *nad4L*, 7,479 positions) from 29 species, using Bayesian phylogenetic inference. Posterior probabilities are indicated at the nodes. The dataset was partitioned into genes, and each partition analyzed with the appropriate DNA evolutionary model, as determined by Modeltest (Table 2). The *light gray area* highlights the stramenopiles and the *dark gray area* the heterokont algae. *Single* and *double underlinings* highlight organisms with primary and secondary plastids, respectively

The overall topology of the tree (Fig. 5) is very well supported by posterior probabilities of 1.00 for a majority of nodes. The stramenopiles form a monophyletic group in which the photosynthetic species cluster together. The Chrysophyceae emerge first, followed by *T. pseudonana* and the Phaeophyceae (posterior probability = 0.92). The relationships between phaeophycean species are in accordance with previous studies based on nuclear rDNA data (Rousseau and de Reviers 1999; Rousseau et al. 2001) and on nuclear and plastidial combined data (Draisma et al. 2001).

Within the heterotrophic stramenopile species, *Cafeteria roenbergensis* (Bicosoecida) is the most basal followed by *Thraustochytrium aureum* (Labyrinthulida) and Oomycetes (posterior probability = 0.96) as a sister group of the algal species.

A complex sister lineage to that of the heterokonts comprises the ''green'' lineage (Viridiplantae) plus *R. americana* and *M. jakobiformis* on one hand, and the red algae plus the cryptophyte *Rhodomonas salina* on the other. The haptophyte *Emiliania huxleyi* diverges before these, with a posterior probability of 0.96, separated from the well-conserved fungi-choanoflagellate clade and *A. castellanii* (Acanthamoebidae). In this analysis, the association of *R. americana* and *M. jakobiformis* with the green lineage separated from the parallel association between the cryptophyte and the red lineage is an argument in favor of two primary endosymbiotic events at the origin of primary chloroplasts, since Viridiplantae and Rhodophyceae do not form a monophyletic group. The presence of this clade (Viridiplantae, *R. Americana–M. jakobiformis*, Rhodophyceae, Cryptophyceae) be-

tween stramenopiles and haptophytes does not support the single acquisition of secondary plastids by a common ancestor of these lineages. The monophyly of the heterokont algae also suggests a secondary endosymbiotic event, which would have occurred in a heterotrophic stramenopile.

Until additional mitochondrial genomic data become available for more stramenopiles—such as representatives from the labyrinthulid/thraustochytrid group, the opalinids or the free living *Developayella elegans*, which are supposed to be close to the Oomycetes (Massana et al. 2002), for the heterotrophic heterokonts, and the Synurophyceae, Eustigmatophyceae, Xanthophyceae, Raphidophyceae and Pelagophyceae, for the algae—this conclusion remains provisional.

Conclusion

Although brown algae display great morphological and physiological differences (e.g., diversity of life cycles), the brown algal mitochondrial genomes studied so far are similar and alignable. The main common features of *P. littoralis* and *L. digitata* mtDNAs are shared by the other brown algal mtDNAs. These are the presence of rare mitochondrial encoded genes, such as *rrn5*, the presence of many ribosomal protein subunit encoded genes, and the unusually short *nad11* gene, where only the first third, corresponding to the well-known Fe–S binding domain of the protein is encoded. A large in-frame insertion is found in the *cox2* gene, although of variable size, in all but the *D. dichotoma* mtDNA. It will be interesting to investigate more species and determine if this insertion was acquired after the *D. dichotoma* divergence or earlier and lost secondarily.

Phylogenetic relationships based on a set of ten mitochondrial genes support previous analyses based on single and double-gene studies (Rousseau and de Reviers 1999; Draisma et al. 2001) that utilized nuclear and chloroplast loci. This result is also corroborated by Fig. 2, showing the rearrangements necessary to switch from one genome organization to the others and indicating that gene order may be a phylogenetically informative character (Boore and Brown 1998; Rokas and Holland 2000; Sankoff et al. 2000). Indeed the phylogenetic order of modifications implied by Fig. 2 is the same as that given by the trees, i.e., *D. dichotoma*, *F. vesiculosus*, *D. viridis* and *L. digitata*–*P. littoralis*.

As genome sequencing becomes faster and cheaper, the switch to longer alignments as well as the utilization of gene order and indels should provide greater resolution in deeper branches. Finally, the finding that phaeophycean mitogenomes are remarkably similar provides encouragement for the development of useful genetic markers for species and population-level studies.

Electronic supplementary material

An electronic appendix to this article provides color versions of Figs. 1 and 2, codon usage of mitochondrial encoded protein genes and the potential folding of 5S rRNAs, on the Current Genetics' web site.

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