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Phylogenetic analysis of diatom *coxI* genes and implications of a fluctuating GC content on mitochondrial genetic code evolution

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Abstract In order to address the relationships among diatom groups and to investigate possible changes in their mitochondrial (mt) genetic codes, we have analyzed a 1.1-kb region of the cytochrome *c* oxidase subunit I (*coxI*) gene from eight diverse diatom species. A phylogenetic analysis of these *coxI* sequences including representative species of the Phaeophyta, Xanthophyta, Eustigmatophyta and Haptophyta showed that the diatoms (Bacillariophyta) formed a well-supported monophyletic group. Of the eight species investigated, four have been classified together as radial centric diatoms based on morphology. However, in our *coxI* tree, the two radial centrics belonging to the order *Thlassiosirales* (*Skeletonema costatum* and *Thalassiosira nordenskioldii*) were placed as the sister group to the multipolar centric diatoms, while the other two radial centrics (*Melosira ambigua* and *Rhizosolenia setigera*) were in another clade. Also, in two species of the *Tharassiosirales* we found UGA codons that occur at conserved tryptophan (Trp) sites in the *coxI* sequences, strongly indicating that UGA codes for Trp in these diatoms. No evidence of a deviant genetic code was detected in the other analyzed diatom species. There was no apparent relationship between the nucleotide third-position GC content of mtDNA (based on the sequenced *coxI* region) and the presence of a deviant genetic code.

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Introduction

Diatoms (Bacillariophyta) are morphologically classified into three groups: (1) centric diatoms (Coscinodiscophyceae); (2) araphid pennate diatoms (Fragilariophyceae); and (3) raphid pennate diatoms (Bacillariophyceae) (Round and Crawford 1989). This classification, however, is inconsistent with results deduced from recent comparisons of nuclear small subunit ribosomal RNA (SSU rRNA) (Kooistra and Medlin 1996; Medlin et al. 1996). These studies showed that, of these groups, only the raphids were monophyletic, with the centric and araphid diatoms each being paraphyletic lineages.

The codon UGA usually serves as a translational termination signal (stop codon), but in a variety of mitochondrial (mt) genomes it frequently codes for tryptophan (Trp) (Osawa et al. 1992; Inagaki et al. 1998). Inagaki et al. (1998) recently pointed out that re-assignment of the mitochondrial UGA codon for Trp has occurred independently in the lineages of ciliates, kinetoplastids, prymnesiophytes, rhodophytes, and fungi. According to the codon capture theory (Osawa and Jukes 1989), the UGA stop codon must disappear completely from the genome before it can be re-assigned. This disappearance is presumably hastened by directional nucleotide substitution toward AT over GC, but how a specific codon is able to disappear completely from the genome by this mutational pressure alone is not clear. We have shown that the frequency of UGA/Stop codons is directly correlated to the codon third-position GC content in mitochondria, chloroplasts and bacteria (i.e. when the GC content is low, the UGA/Stop codon is more rare than the AT-rich stop codon, UAA) (Inagaki et al. 1998). Therefore, we summarize that the organisms bearing AT-rich genomes could be more prone to evolve alternative genetic codes by UGA

re-assignment. However, it is not always true that organisms bearing UGA/Trp codons have AT-rich genomes. This fact has stimulated discussion on possible alternative mechanisms for such a genetic-code change (Schultz and Yarus 1994, 1996). Here, we show that the mt-genome GC content in diatoms and other algae is not constant; the single identified diatom lineage with a deviant code exhibits moderate, albeit variable, GC content. This apparent flexibility in GC content, and its inferred propensity for change, might explain why the UGA/Trp codon is not limited to AT-rich mt-genomes.

Materials and methods

Diatoms were purchased from the following sources: Center for Culture of Marine Phytoplankton (CCMP, USA), and Culture Collection of Algae and Protozoa (CCAP, UK). The strains used in this study are: *Cylindrotheca closterium* (CCMP 339), *Ditylum brightwellii* (CCAP 1022/2), *Fragilaria striatula* (CCAP 1029/18), *Nitzschia frustulum* (CCMP 558), *Rhizosolenia setigera* (CCMP 1330), *Skeletonema costatum* (CCAP 1077/1B), *Thalassionema nitzschioides* (CCAP 1084/1), and *Thalassiosira nordenskioldii* (CCMP 992). Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Germany) and used as a template for reverse transcriptase. Ready To-Go Beads (Pharmacia Biotech, Sweden) were used for first-strand synthesis with the primer, pB1: 5′-GCWACWACRTARTAWGTRTCRTG-3′ (R = A or G, W = A or T). The first-strand cDNA was converted to double-stranded cDNA by a polymerase chain reaction (PCR) using a second primer, pA1: 5′-TTYTTYGGWCAYCCWGARGTWTA-3′ (Y = T or C). PCR conditions, using a thermal cycler model 9600 (Perkin-Elmer, USA), were: 35 cycles of 30 s at 94 °C, 1 min at 45 °C, and 1 min at 70 °C. Total DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Germany) and employed as a template for PCR with the pC1 and pB1 primer set under the conditions described above. PCR products were cloned into the pT7 Blue T-vector (Novagen, USA). DNA sequencing was carried out by the dye-terminator-cycle sequencing method using a DNA sequencer model 377 (Applied Biosystems, USA). In order to extend 5′-end regions of the genes, PCR was carried out with a highly degenerate AT-rich primer, pC1 (5′-TGGTTNTTYTCNACNAAYCAYAARGAYAT-3′; N = A, C, G or T) with specific primers based on the previously determined DNA sequence. These PCR products were also cloned and sequenced as above. The inferred amino-acid sequences of *coxI* were aligned and phylogenetic analyses were performed using programs in SINCA version 3.0 (Fujitsu System Engineering, Japan); a neighbor-joining (NJ) tree (Saitou and Nei 1987) was inferred using a 2-parameter model (Kimura 1980). Maximum-parsimony analyses were carried out with PAUP version 3.1.1 (Swofford 1991). Unweighted maximum-parsimony trees were obtained using a heuristic search. Bootstrap (BS) re-sampling (500 replicates) was performed to quantify the relative support for branches on the deduced trees (Felsenstein 1985).

Results and discussion

Phylogenetic relationships among diatoms revealed by *coxI* sequences

In Fig. 1 we show a *coxI* phylogenetic tree based on the deduced amino-acid sequence (353 residues) from the diatom genes and the previously published sequences from a diversity of heterokont and haptophyte algae. One of two most-parsimonious (MP) trees is shown; its topology is very similar to the other parsimony tree as well as the neighbor-joining (NJ) distance tree (see below). The phylogeny of *coxI* sequences indicates a robust monophyletic diatom clade (supported by 100% BS), consisting of two basal sub-clades (these are marked 'Clade I' and 'Clade II' in Fig. 1). Clade II is composed of four groups: multipolar centrics, some radial centrics (*Thalassiosirales*), araphid pennates, and raphid pennates. The NJ tree and the two recovered MP trees differ only in the position within Clade II of the araphid pennates, with this group either being connected to the raphid pennate clade (as shown in Fig. 1) or to the other clade (radial/multipolar centrics). This conflict is consistent with the lack of significant bootstrap support for the specific position of araphids. The other basal grouping, Clade I, is solely comprised of two radial centrics: *Melosira ambigua* and *R. setigera* in the Melosirales and Rhizosoleniales, respectively. Notably, the other two radial centrics in our study, *S. costatum* and *T. nordenskioldii* in the order *Thalassiosirales*, were strongly placed in Clade II as the sister group to the multipolar centric diatom, *D. brightwellii* (>81% BS). Thus, our *coxI* tree indicates that the centric diatoms (in general) and the radial centrics (in particular) are paraphyletic groups. Both of these results are consistent with the phylogenetic studies based on small subunit (SSU) rRNA sequences (Kooistra and Medlin 1996; Medlin et al. 1996), and provide independent support for their conclusions.

The UGA codon specifies Trp in the *Thalassiosirales*

We have previously indicated that the mt-genome of one radial centric diatom, *M. ambigua,* utilizes the universal genetic code (Inagaki et al. 1998). Also, no deviant genetic code was detected among six of eight additional diatoms analyzed in this study; these include two species each of raphid pennate diatoms and araphid pennates, a single species representing multipolar centrics, and one (of three studied) radial centrics (*R. setigera* which formed Clade I with *M. ambigua*; Fig. 1). However, in the two radial centric diatoms belonging to Clade II, *S. costatum* and *T. nordenskioldii*, we detected UGA codons in the coding frame of their *coxI* genes. Indeed, these UGA codons appeared specifically at five and three of the eight conserved Trp sites, respectively (Table 1); all of the investigated diatoms had these eight tryptophan sites in their *coxI* sequences. By matching UGA codons present in the cDNA sequences to sequences from PCR fragments (amplified using total DNA as a template), we confirmed these codons were not the result of editing. The codon preference ratio for the UGA/Trp codon in *S. costatum* and *T. nordenskioldii*, is 5/8 (UGA/UGA + UGG) and 3/8, respectively. Thus, it is likely that a UGA/Trp codon would be found in the *coxI* sequences from other diatoms if it was being used: the probability (*P*) that at least

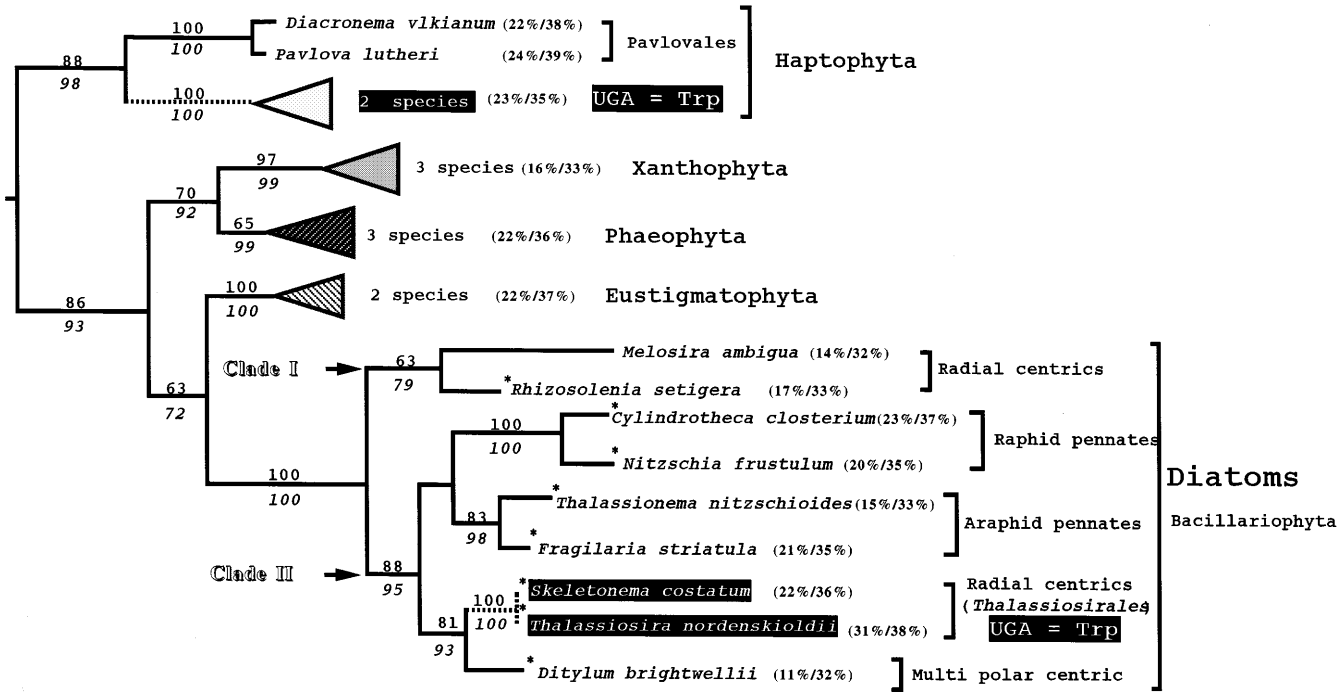

Fig. 1 Phylogenetic tree of *coxI* amino-acid sequences. The tree shown was constructed by maximum parsimony. The *coxI* sequence from the protist, *Acanthamoeba castellanii* (Burger et al. 1995, GenBank accession number, U12386) was used as the outgroup. Bootstrap values above 50% are indicated. Values indicated in *Arabic characters* (and above the branches) are based on parsimony and those indicated in *italics* (below the branches) were based on distance (NJ). Clades indicated by *dotted lines* use the UGA codon for Trp. Species names for which a *coxI* sequence was determined in this study are noted with asterisks (*). The GC content (percentage of GC at codon third positions) for each species or the average GC content for the phylum is given *in parentheses* before the shill, while the overall GC content of the *coxI* gene is after the shill. The species and GenBank accession numbers for the *coxI* sequences used in this study are: (Haptophyta) *Isochrysis galbana* (AB000119); *Phaeocystis pouchetii* (AB000120); *Diacronema vlkianum* (AB009420), *Pavlova lutheri* (AF045691) (Hayashi-Ishimaru et al. 1997 and unpublished results); (Xanthophyta) *Botrydium granulatum* (AB000204); *Mischococcus sphaerocephalus* (AB0002208); *Vaucheria sessilis* (AB000212); (Eustigmatophyta) *Nannochloropsis oculata* (AB000209); *Nannochloropsis* sp. (AB000207) (Ehara et al. 1997); (Phaeophyta) *Chorda filum* (AFO37991); *Ectocarpus* sp. (AF037994); *Undaria pinnatifida* (AF037993) (Ehara et al. 1998); (Bacillariophyta) *Melosira ambigua* (AB009418; Inagaki et al. 1998), *Cylindrotheca closterium* (AB020222), *Ditylum brightwellii* (AB020223), *Fragilaria striatula* (AB020224), *Nitzschia frustulum* (AB020225), *Rhizosolenia setigera* (AB020226), *Skeletonema costatum* (AB020227), *Thalassionema nitzschioides* (AB020228) and *Thalassiosira nordenskioldii* (AB020229) (this study)

one UGA codon appears in the eight Trp sites is high [i.e. 1–(ratio of UGG/Trp codon)^8, e.g. P is 0.9996 when the UGA:UGG (Q) = 5:3; $P = 0.996$/Q = 1:1; $P = 0.90$/Q = 1:3). Therefore, most probably those diatoms whose *coxI* sequences did not contain UGA/Trp codons use the universal genetic code.

The clade of *S. costatum* + *T. nordenskioldii* (UGA/Trp) was well-resolved (>81% BS) as a sister group to the multipolar centric diatom, *D. brightwellii*, a species which appears to use the universal code. This is the third example of the re-assignment of the UGA codon within a traditionally described phylum. In the Haptophyta, three of four orders bear a deviant genetic code (UGA/Trp) (Hayashi-Ishimaru et al. 1997), while two species belonging to the order *Pavlovales* utilize the universal code (Fig. 1; see also Inagaki et al. 1998). Also, in the Rhodophyta, *Chondrus crispus* bears UGA/Trp (Boyen et al. 1994), while *Cyanidium caldarium* uses the universal genetic code (Viehmann et al. 1996).

Directional nucleotide substitution and genetic-code evolution in mitochondria

It has been suggested that directional nucleotide substitution toward AT over GC is essential for genetic-code changes (discussed in Inagaki et al. 1998). We have shown that the frequency of the UGA stop codons in bacteria and chloroplasts is clearly correlated with the codon third-position GC content (Inagaki et al. 1998). Therefore, we investigated whether there is any correlation between the codon third-position GC content and the presence of the deviant code in diatoms and other yellow algae. The calculated codon third-position GC content (herein, simply 'GC') and the overall GC content of the *coxI* sequences are both indicated in Fig. 1 (shown in parentheses; codon third-position/overall GC content). Among the diatoms, there is no apparent tendency for the species bearing UGA/Trp to be low GC (in fact the *Thalassiosirales* are two of the three highest for GC). This lack of correlation is also apparent for both the haptophytes and rhodophytes which use UGA/Trp [e.g. *C. crispus* (UGA/Trp) is 17% GC, while *C. caldarium* (UGA/Stop) is 16% GC)]. Further, the diversity of GC indicated in Fig. 1 suggests that the GC

Table 1 Presence of UGA codons at conserved tryptophan sites in diatom COXI genes

Species		Site no.[a]							
		68	90	113	173	223	274	309	314
Bacillariophyta									
Centric diatoms	*Ditylum brightwellii*	W[b]	W	W	W	W	W	W	W
	Melosira ambigua	W	W	W	W	W	W	W	W
	Rhizosolenia setigera	W	W	W	W	W	W	W	W
Tharassiosirales	*Skeletonema costatum*	UGA	W	UGA	W	W	UGA	UGA	UGA
	Thalassiosira nordenskioldii	UGA	UGA	W	W	W	UGA	W	W
Raphid diatoms	*Nitzschia frustulum*	W	W	W	W	W	W	W	W
	Cylindrotheca closterium	W	W	W	W	W	W	W	W
Araphid diatoms	*Fragilaria striatula*	W	W	W	W	W	W	W	W
	Thalassionema nitzschioides	W	W	W	W	W	W	W	W

[a] Numbering of sites is based on the 353 amino-acid sequence (1059 bp) of *Ditylum brightwellii*
[b] "W" is coded by UGG

content in mt-genomes can change readily. In support of this, the difference in GC content between the two diatoms using UGA/Trp (*S. costatum* and *T. nordenskioldii*) is striking – 22% vs 31% – yet they have no inferred amino-acid differences in *coxI*. Moreover, their sister group, represented by *D. brightwellii* (using UGA/Stop), is only 11% GC. Therefore, one possible scenario is that the mtDNAs now having UGA/Trp codons were previously AT-rich (perhaps as low as 10–15%), a bias which promoted the complete disappearance of the UGA/Trp codon from the genome. Consistent with this, UGA is used as a stop codon either rarely or not at all in many AT-rich mt-genomes (Nakamura et al. 1997), while an UAA stop codon is utilized much more frequently (Inagaki et al. 1998). The occurrence of stop codons in mt-genomes is quite limited [the largest number, so far known, is 67 in *Recliromonas americana* (Lang et al. 1997)] compared to that of most sense codons. Therefore a specific stop codon could easily disappear from the whole genome through its replacement with synonymous codons. The expected ratio of UGA stop codons to all stop codons (UGA/UGA + UAG + UAA) experiences a precipitous decline with a lowered GC content: the ratio of the UGA stop codon is 0.2 when the GC content is 30% and drops to 0.03 when the GC content is 10% (Inagaki et al. 1998). For example, the colorless green alga, *Prototheca wickerhamii*, is 12% GC (based on 36 genes), yet no UGA codon appears in its complete mt-genome. Additionally, only one UGA stop codon is present in the entire mt-genome of the protozoan, *R. americana* (10% GC, 67 genes) (Lang et al. 1997), and only two UGA stop codons are used in *Dictyostelium discoideum* (14% GC, 36 genes) (Nakamura et al. 1997). Interestingly, once the UGA codon is captured as a Trp codon, it appears to rarely revert, perhaps due to its preferred use over the alternative UGG/Trp codon; in nearly all mt-genomes using both UGG and UGA Trp codons, their codon ratio (UGA/UGG) is approximately 5/1, regardless of GC content (Inagaki et al. 1998). However, recently it has become clear that there are some exceptional cases. In the mt-genomes of the octocoral *Sarcophyton glaucum* (Beaton et al. 1998), the hexocoral *Metridium senile* (Beagley et al. 1998), the amoeboid protozoon *Acanthamoeba castellanii* (Burger et al. 1995), the nematode *Ascaris suum* (Okimoto et al. 1990) and the fluke *Fasciola hepatica* (Garey et al. 1989), a UGG/Trp codon is used much more frequently than the cognate UGA/Trp. Such exceptional codon selection against the use of UGA/Trp might be due to: (1) the $tRNA^{Trp}$ de-coding UGG codons much more efficiently than the cognate UGA codons, or (2) there being a prominent bias toward G predominating over A in the sense strand, with the translational efficiency for UGG/Trp and UGA/Trp codons being almost the same. Preferential use of UGG/Trp in the amoeboid protozoon *A. castellanii* (Burger et al. 1995) might be due to the $tRNA^{Trp}$ bearing the anticodon 5′-CCA-3′, which should only be able to pair with a UGG codon. It is assumed that a mis-match predicted in the anticodon stem might allow de-coding of both cognate codons even with the anticodon 5′-CCA-3′ (Burger et al. 1995). In the case of the nematode *A. suum* and the fluke *F. hepatica*, the bias of G over A in the sense strand (Garey et al. 1989; Okimoto et al. 1992) is the most probable reason for the infrequent occurrence of UGA/Trp compared to UGG/Trp. The reason for the unusual selection against the use of UGA/Trp, notwithstanding the A + T biases in the sense strand, in *S. glaucum* (Beaton et al. 1988; Pont-Kingdon et al. 1998) and *M. senile* (Pont-Kingdon et al. 1994, 1998) remains unclear. Studies of the relative translational efficiency of the Trp synonymous codons (UGG and UGA), and assessment of the recognition ability of the peptide chain release factor for the canonical stop codons (UAA and UAG) and also UGA/Trp will be essential, considering the theory of codon reassignment through ambiguous translation (Schultz and Yarus 1994, 1996).

The evidence presented here from diatom *coxI* sequences continues to support the hypothesis that UGA codons have separately evolved to code for Trp in various mitochondrial lineages. Further, the disappearance of UGA stop codons was most likely promoted by the generally low, but fluctuating, GC content in mt-genomes.

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