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Use of a Deviant Mitochondrial Genetic Code in Yellow-Green Algae as a Landmark for Segregating Members Within the Phylum

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Abstract. Several algae that were previously classified in the phylum Xanthophyta (yellow-green algae) were assigned in 1971 to a new phylum, Eustigmatophyta. It was anticipated that the number of algae reclassified to Eustigmatophyta would increase. However, due to the fact that the morphological characteristics that segregate eustigmatophytes from other closely related algae can be only obtained through laborious electron microscopic techniques, the number of members in this phylum have increased rather slowly. We attempted, therefore, to segregate two closely related groups of algae, eustigmatophytes and yellow-green algae, on the basis of a molecular phylogenetic tree as a means of providing an alternative method of distinguishing these phyla. We analyzed the mitochondrial cytochrome oxidase subunit I (*COXI*) gene sequences of eight algae classified as xanthophyceans and found that six manifested the expected deviant genetic code where AUA codes for methionine (AUA/Met), but not for isoleucine (AUA/Ile) as in the universal genetic code. The other two, *Monodus* sp. (CCMP 505) and *Ophiocytium majus* (CCAP 855/1), which were presumed to be yellow-green algae, and all the examined eustigmatophytes utilized AUA for Ile. In addition, the phylogenetic tree of *COXI* gene sequences showed that the six yellow-green algae bearing the AUA/Met deviant code composed a tight clade with a bootstrap value of 100%. The phylogenetic tree of the corresponding sequences from *Monodus* sp. and *Ophiocytium majus* and the eustigmatophytes also composed a tight cluster, but with a bootstrap value of 92%. These results strongly suggest that two previously classified members of yellow-green algae belong to the phylum Eustigmatophyta. Therefore, examination of the mitochondrial genetic code in algae appears to be a potentially very useful genetic marker for classifying these organisms, especially when it is considered with the results obtained through a molecular phylogenetic tree.

Key words: Deviant genetic code — Eustigmatophyta — Xanthophyta — Cytochrome oxidase subunit I

Introduction

Eustigmatophytes have been segregated from yellow-green algae (xanthophytes) mainly on the basis of the ultrastructural characteristics of their chloroplasts and flagella (Hibberd and Leedale 1971). These organisms have chlorophyll *a*, but not chlorophyll *b* or *c*, and they do not possess any girdle lamella in their chloroplasts, but bear an eyespot at the anterior end of the motile cell (Hibberd 1989a). Both yellow-green algae and eustigmatophytes are usually rather small and this makes their discrimination difficult using an optical microscope.

The classification of algae on the basis of molecular data is a recent development, and the available sequence data that cover a wide range of algal species are almost solely limited to the nuclear-encoded small subunit of ribosomal RNA (SSU-rRNA) genes (e.g., see Kumar and

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Rzhetsky 1996; van de Peer et al. 1996). However, phylogenetic trees based on DNA or RNA sequences alone may be affected by variations in genomic G + C contents of the analyzed organisms. Considering the above and the expected increment of reliability for a phylogenetic tree when trees based on different functions of genes are inconsistent, we decided to make a phylogenetic tree that covers most of the representative yellow-green algae and some eustigmatophytes based on mitochondrial *COXI* gene sequences.

Superimposition of genetic markers, such as the positions of introns (e.g., Manhart and Palmer 1990) or retroposons (e.g., Takasaki et al. 1994), has been utilized to check the appropriateness of a phylogenetic tree. Among such genetic markers, deviant genetic codes appear to be very reliable because of their stable inheritance. Also, horizontal transfer of a deviant genetic code system appears to be highly unlikely. In terrestrial plant mitochondria, a deviant genetic code has not yet been observed. However, recently, in a marine red-alga (Boyen et al. 1994) and in several colony-forming green-algae (H-Ishimaru et al. 1996), deviant genetic codes have been reported in mitochondria. In this study, we describe another genetic code change in the mitochondria of yellow-green algae (AUA/Met) and demonstrate that it is a potentially useful genetic marker for discrimination between eustigmatophytes and closely related organisms such as yellow-green algae.

This is the first molecular phylogenetic study of eustigmatophytes and xanthophytes through an analysis of mitochondrial DNA sequences and through an examination of the characteristics of their genetic code.

Materials and Methods

Organisms

Organisms used in this study were purchased from culture stock centers. Yellow-green algae (Xanthophyta) were *Botrydiopsis alpina* (UTEX 295), *Botrydium granulatum* var. *kolkwitzianum* (CCAP 805/4), *Heterococcus caespitosus* (CCAP 835/2A), *Mischococcus sphaerocephalus* (CCAP 847/1), *Monodus* sp. (CCMP 505), *Ophiocytium majus* (CCAP 855/1), *Tribonema aequale* (CCAP 880/1), and *Vaucheria sessilis* (CCAP 745/1C). Eustigmatophytes were *Eustigmatos magnus* (CCAP 860/2) and *Nannochloropsis oculata* (CCAP 849/1).

These algae were cultured according to the conditions recommended by the respective stock centers that furnished them. About 1 g of cultured cells was harvested and stored at −120°C until used.

Preparation of Total DNA for Polymerase Chain Reaction (PCR)

Frozen cells were pulverized in a Teflon capsule with a tungsten ball by mechanical shaking using a micro-dismembrator (B. Brown, Germany). Total DNA was extracted with phenol after incubation in a SDS/proteinase K solution as described previously (H-Ishimaru et al. 1996). Crude DNA was purified by a resin, GENECLEAN Spin Kit (Bio101, USA), according to the supplier's manual.

Amplification of a Part of COXI Gene by PCR

We designed a set of primers (p1A: 5′ TTYTTYGGNCAYCCNGARGTNTA-3′ and p1C: 5′-TGGTTNTTYTCNACNAAYCAYAARGAYAT-3′; R; A or G, Y; C or T, N; T, C, A, or G) considering the conserved amino acid sites in *coxI* among various organisms.

Amplification was carried out in a 100-μl reaction mixture containing 250 μM of each deoxyribonucleosides, the set of primers described above (1.0 μM each), 2 units of *TaKaRa Ex Taq* DNA polymerase (Takara, Japan) and 0.25 μg of total DNA. A 1.1-kbp DNA fragment was obtained by applying 35 cycles of 1 min at 94°C, 1 min at 50°C, and 2 min at 70°C, using a thermal cycler model 480 (Perkin-Elmer, USA).

Cloning of PCR Products and Sequencing

The expected size of the PCR product was cut out after agarose gel electrophoresis, and cloned into the TA-vector (Invitrogen, USA). Sequence determination was carried out by the dye-terminator cycle sequencing method, using a DNA sequencer model 377A (Perkin-Elmer, USA).

Construction of a COXI *Phylogenetic Tree*

The DNA sequences were analyzed phylogenetically together with the published or registered *COXI* sequences of terrestrial plants, green-algae, and a prymnesiophyte. A phylogenetic tree based on the deduced amino acid sequences was constructed by the neighbor-joining method (Saitou and Nei 1987) utilizing the programs in SINCA (Fujitsu System Engineering, Japan). In this report, AUA was taken as coding for Ile or Met depending on the species (see below). Bootstrap resampling (Felsenstein 1985) (500 times) was carried out to quantify the relative support for branches of the inferred phylogenetic tree.

Results

The phylogenetic tree of the *COXI* gene was constructed using the deduced amino acid sequences generated from the 1059-bp DNA sequenced from the various organisms (Fig. 1). Six out of eight yellow-green algae analyzed in this study made a tight cluster (shown as "Xa," in Fig. 1) with a 100% bootstrap value. These six members had the deviant genetic code, AUA/Met. However, the other two, *Monodus* sp. and *Ophiocytium majus,* which were classified as yellow-green algae, formed a sister group with two typical eustigmatophytes, *Nannochloropsis oculata* and *Eustigmatos magnus,* respectively. The bootstrap values for *Monodus* sp./*N. oculata*-clade and *O. majus*/*E. magnus*-clade were both 100% (Fig. 1). The sister group relationship of these two clades (shown as "Eu" in Fig. 1) was supported by a bootstrap value of 92%.

Two Ile codons, AUU and AUC, appeared at conserved Ile sites in all the analyzed species. However, the

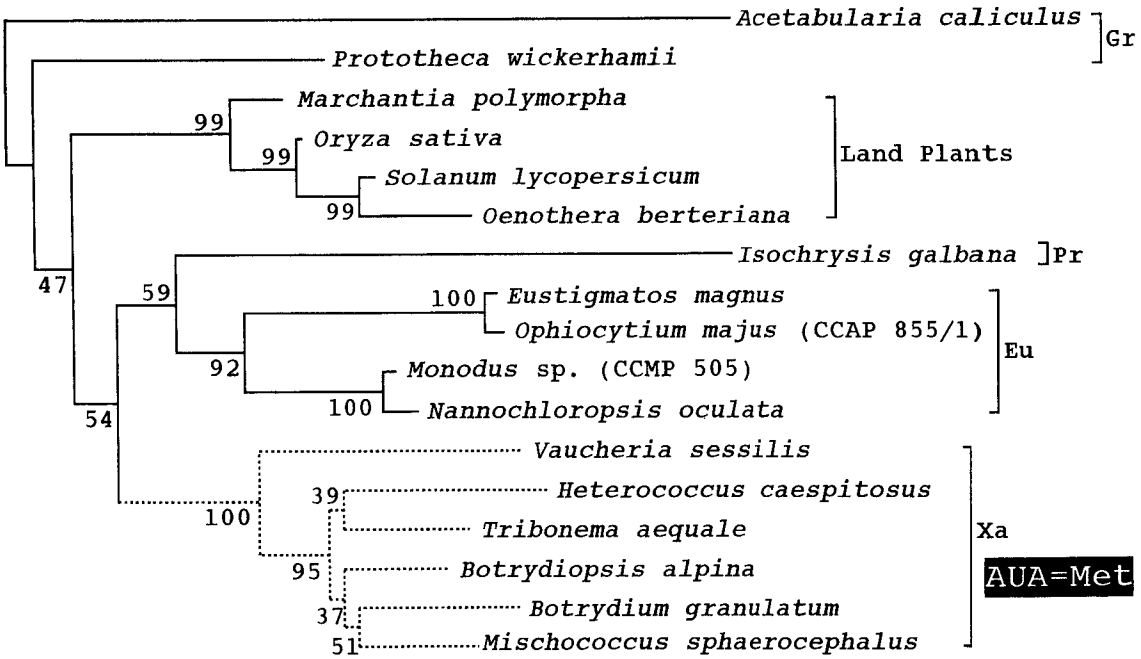

Fig. 1. Phylogenetic tree based on the deduced amino acid sequences from 1059 bp of DNA sequenced from the *COXI* gene of various algae. The lineage expected to bear a deviant genetic code AUA for methionine is shown by the dotted line. Evolutionary distance was calculated by Kimura's two parameters method (Kimura 1980), and the tree was constructed by the neighbor joining method (Saitou and Nei 1987). *Abbreviations:* Gr, green algae; Pr, prymnesiophyte; Eu, eustigmatophytes; and Xa, xanthophytes. Accession numbers for data base are as follows: *A. caliculus* (*AB003088*), *P. wickerhamii* (*U02970*), *M. polymorpha* (*M68929*), *O. sativa* (*M57903*), *S. lycopersicum* (*X54738*), *O. berteriana* (*X05465*), *I. galbana* (*AB000119*), *E. magnus* (*AB000205*), *O. majus* (*AB000210*), *Monodus* sp. (*AB000207*), *N. oculata* (*AB000209*), *V. sessilis* (*AB000212*), *H. caespitosus* (*AB000206*), *T. aequale* (*AB000211*), *B. alpina* (*AB000203*), *B. granulatum* (*AB000204*), *M. sphaerocephalus* (*AB000208*).

other Ile codon, AUA specifically appeared at conserved Met sites (Table 1) in six of eight yellow-green algae that formed the Xa-clade in the *COXI* tree. On the other hand, in the other two yellow-green algae (*Monodus* sp. and *Ophiocytium majus*) and in two eustigmatophytes (*Eustigmatos magnus* and *Nannochloropsis oculata*) that made up the Eu-clade (Fig. 1), the AUA codon appeared at conserved Ile sites (Table 2). In the sequenced *COXI* region, there are 20 conserved ATA sites for yellow-green algae that make up the Xa-clade (Table 1). Among these 20 sites, 13 clearly corresponded to AUG/Met sites in the algae that were used for the comparison (shown in boldface in Table 1). On the other hand, only two sites corresponded to Ile (shown underscored in Table 1). At other sites, the corresponding amino acid is not well conserved, and thus no valuable information could be obtained.

There were eight conserved ATA sites in the same sequenced region of the four algae (Table 2) that make up the Eu-clade. Six out of eight sites clearly correspond to Ile, and the other two sites also corresponded in most cases to Ile. Therefore, for the six yellow-green algae described above, the AUA codon is most probably used for Met, and not for Ile, as found in the universal genetic code. On the other hand, this codon is used for Ile in the Eu-clade that is composed of the two eustigmatophytes (*Eustigmatos magnus* and *Nannochloropsis oculata*) and the two yellow-green algae (*Monodus* sp. and *Ophiocytium majus*). Therefore, *Monodus* sp. (CCMP 505) and *Ophiocytium majus* (CCAP 855/1) are most certainly members of the phylum Eustigmatophyta, in contradiction to their traditional classification as members of the phylum Xanthophyta. This is the first report of a deviant genetic codon, AUA, for Met in mitochondria from photosynthetic protists.

Discussion

Eustigmatophyta is a phylum that was derived in 1971 (Hibberd and Leedale 1971) to accommodate members of the unicellular coccoid xanthophytes that manifested some unique, fine structures in their chloroplasts and flagella, and possessed characteristic composition in their photosynthetic pigments. Only six species were originally included in the group, and the number has been slowly increasing (Lee and Bold 1973; Antia et al. 1975; Hibberd 1981). However, it is expected that some other members of Xanthophyta might still require reclassification. Considering the recent rapid development of PCR techniques, the construction of a molecular phylogenetic tree would provide an alternative means of classifying certain algae other than the laborious observation of a series of sections by electron microscopy.

Both *Mischococcus* and *Ophiocytium* are genera belonging to the same order, Michococcales, in the phylum

Table 1. ATA sites in species comprising the Xa-clade

Site no.[a]		14	18	**52**	56	**58**	73	**79**	**84**	**158**	163
Land Plant	*Marchantia polymorpha*	M	F	**M**	M	**M**	L	**M**	**L**	**M**	L
Green Alga	*Prototheca wickerhamii*	L	F	**M**	M	**M**	L	**M**	**L**	**M**	M
Eustigmatophytes and Xanthophytes											
Eu[b]	*Eustigmatos magnus*	A	L	**M**	F	**M**	M	**M**	**M**	**M**	I
	Nannochloropsis oculata	A	L	**M**	F	**M**	M	**M**	**M**	**M**	M
	Monodus sp.	A	L	**M**	F	**M**	M	**M**	**M**	**M**	M
	Ophiocytium majus	A	L	**M**	F	**M**	M	**M**	**M**	**M**	I
Xa[c]	*Botrydiopsis alpina*	ATA	M	**ATA**	M	**M**	ATA	**ATA**	**L**	**ATA**	ATA
	Botrydium granulatum	ATA	ATA	**M**	ATA	**ATA**	ATA	**ATA**	**ATA**	**ATA**	ATA
	Heterococcus caespitosus	ATA	L	**ATA**	ATA	**ATA**	ATA	**ATA**	**ATA**	**ATA**	ATA
	Mischococcus sphaerocephalus	L	ATA	**ATA**	ATA	**ATA**	ATA	**ATA**	**ATA**	**ATA**	ATA
	Tribonema aequale	ATA	ATA	**ATA**	ATA	**ATA**	M	**ATA**	**ATA**	**ATA**	ATA
	Vaucheria sessilis	ATA	L	**ATA**	ATA	**ATA**	ATA	**ATA**	**L**	**ATA**	L

Table 1. Continued

Site no.[a]		**165**	**195**	**259**	**263**	264	**278**	**296**	306	**324**	343
Land Plant	*Marchantia polymorpha*	**M**	**M**	**M**	**M**	I	**M**	**M**	I	**M**	V
Green Alga	*Prototheca wickerhamii*	**M**	**M**	**M**	**M**	C	**M**	**M**	I	**M**	V
Eustigmatophytes and Xanthophytes											
Eu[b]	*Eustigmatos magnus*	**M**	**M**	**M**	**M**	L	**M**	**M**	I	**M**	V
	Nannochloropsis oculata	**M**	**M**	**M**	**M**	L	**M**	**M**	I	**M**	V
	Monodus sp.	**M**	**M**	**M**	**M**	L	**M**	**M**	I	**M**	V
	Ophiocytium majus	**M**	**M**	**M**	**M**	L	**M**	**M**	I	**M**	V
Xa[c]	*Botrydiopsis alpina*	**ATA**	**ATA**	**ATA**	**ATA**	I	**M**	**ATA**	I	**ATA**	ATA
	Botrydium granulatum	**ATA**	**ATA**	**ATA**	**ATA**	I	**ATA**	**ATA**	ATA	**M**	ATA
	Heterococcus caespitosus	**ATA**	**ATA**	**ATA**	**ATA**	ATA	**M**	**ATA**	I	**M**	M
	Mischococcus sphaerocephalus	**ATA**	**ATA**	**ATA**	**ATA**	I	**ATA**	**ATA**	I	**M**	ATA
	Tribonema aequale	**L**	**M**	**ATA**	**M**	I	**ATA**	**ATA**	I	**ATA**	ATA
	Vaucheria sessilis	**ATA**	**ATA**	**ATA**	**ATA**	I	**M**	**ATA**	I	**ATA**	ATA

[a] The deduced amino acid sequence was numbered, assigning the first codon of the sequenced *COXI* region for the site no. 1.
[b] Eu-clade consisting algae (see text, and Fig. 1).
[c] Xa-clade consisting algae (see text, and Fig. 1).

Table 2. ATA sites in species comprising the Eu-clade

Site No.		62	**134**	**149**	**152**	**240**	**241**	**266**	285
Land Plant	*Marchantia polymorpha*	I	**I**	**I**	**I**	**I**	**I**	**I**	V
Green Alga	*Prototheca wickerhamii*	M	**I**	**I**	**I**	**I**	**I**	**I**	I
Eustigmatophytes and Xanthophytes									
Eu	*Eustigmatos magnus*	ATA	**I**	**ATA**	**ATA**	**I**	**ATA**	**I**	V
	Nannochloropsis oculata	I	**ATA**	**I**	**I**	**I**	**I**	**I**	ATA
	Monodus sp.	I	**ATA**	**I**	**I**	**I**	**I**	**I**	I
	Ophiocytium majus	ATA	**I**	**ATA**	**ATA**	**ATA**	**I**	**ATA**	V
Xa	*Botrydiopsis alpina*	I	**I**	**I**	**I**	**I**	**I**	**I**	I
	Botrydium granulatum	I	**I**	**I**	**I**	**I**	**I**	**I**	I
	Heterococcus caespitosus	I	**I**	**I**	**I**	**I**	**I**	**I**	I
	Mischococcus sphaerocephalus	I	**I**	**I**	**I**	**I**	**I**	**I**	I
	Tribonema aequale	I	**I**	**I**	**I**	**I**	**I**	**I**	V
	Vaucheria sessilis	I	**I**	**I**	**I**	**I**	**I**	**I**	I

Xanthophyta (yellow-green algae) (Hibberd 1989b). However, in our *COXI* tree *Ophiocytium majus* showed a distant relationship with *Mischococcus sphaerocephalus,* and a close relationship with *Eustigmatos magnus,* which is a representative of the eustigmatophytes (Fig. 1). The stable pairing of *Eustigmatos* (eustigmatophyte) and *Ophiocytium* (yellow-green alga) (bootstrap value 100%), suggests that *Ophiocytium majus* (CCAP 855/1) is a member of eustigmatophytes. Another instance of a stable pair consisted of a yellow-green alga and an eustigmatophyte was shown in Fig. 1. *Nannochloropsis oculata* (eustigmatophyte) and *Monodus* sp. (yellow-

green alga) possessed a bootstrap value of 100%. The suggested very close relationship of *Monodus* sp. to an eustigmatophyte appeared reasonable, since some species that have been reclassified as members of the eustigmatophytes once belonged to the xanthophycean genus, *Monodus* (Hibberd 1981). The reclassification of *Ophiocytium majus* and *Monodus* sp. as eustigmatophytes is not only supported by the *COXI* tree but also by the amino acid assignment of the AUA codon to either Ile or Met. (Tables 1, 2). Further study of their cell structures and pigment composition now appears essential to confirm the results obtained in this study, especially for *Ophiocytium majus,* because it has been considered until now to be a typical yellow-green alga. The relationships between clades, Xa, Eu, Pr (prymnesiophyte), terrestrial plants and Gr (green-algae) are not obvious because the bootstrap value for each node is not significantly high (Fig. 1).

In the case of potato mitochondria (Weber et al. 1990), it has been shown that one of the two isoleucine tRNAs have a modified base 2-lysylcytidine (2-lysyl C) in the first position of the anticodon (tRNA(Ile/LAU)), which results in AUA being translated as Ile. This modified base is identical with that found in the isoleucine tRNA of *Escherichia coli* (Muramatsu et al. 1988a) that also translates AUA as Ile. In addition, it has been shown that when 2-lysyl C is replaced with unmodified C, the identity of this Ile-tRNA changes from Ile to Met, and the tRNA translates only the AUG codon as Met (Muramatsu et al. 1988b). In mitochondria of most animals, in which both AUA and AUG are used for Met, it is believed to be highly likely that only one *Met-tRNA* gene with the anticodon CAU exists, and that the first anticodon base (C) of the tRNA is not modified to 2-lysyl C. This type of mitochondrial *Met-tRNA* has an extra unpaired nucleotide within the base-paired T-stem, and it was suggested this nucleotide allows pairing of the anticodon CAU with the codon AUA (Sibler et al. 1985). In the mitochondria of the blue mussel, *Mytilus edulis* (Hoffmann et al. 1992), and in the mitochondria of the nematode *Ascaris suum,* a Met-tRNA with a modified anticodon *CAU (*C = 5-formylcytidine) is probably responsible for the formation of a wobble pair with AUA (Moriya et al. 1994). Therefore, sequencing of the mitochondrial Met-tRNA of yellow-green algae, including any modifications, is essential in understanding what occurred in the ancestral change of AUA from Ile to Met. The amino-acid assignment of AUA seems to change rather easily in animals, because it has been reported that assignment of this codon underwent atavism, that is, AUA reverted from Met to Ile in the mitochondria of planaria (Bessho et al. 1991) and echinoderms (Himeno et al. 1987; Jacobs et al. 1988; Cantatore 1989) independently. This is in contrast to the plant kingdom where the assignment of this codon seems rather stable—only one assignment change seems to have occurred in an ancestor of the yellow-green algae. Indeed, AUA is used for Ile throughout land plants (for review, Osawa et al. 1992), green-algae (H-Ishimaru et al. 1996), prymnesiophytes [Hayashi-Ishimaru et al. unpublished; the COXI sequences for five prymnesiophytes have been submitted under DDBJ accession numbers AB000117-000120, AB000213], a red-alga (Boyen et al. 1994) and eustigmatophytes (this study). Therefore, AUA/Met appears to be a very useful landmark for the segregation of yellow-green algae from other groups of algae and, in particular, from the closely related eustigmatophytes.

Algae, including eustigmatophytes and yellow-green algae, but excluding red- and green-algae, usually possess chloroplasts that are bounded by three or four membranes. This peculiar number of outer membranes has promoted speculation that such chloroplasts might be descended from endosymbiotic algae (e.g., Gibbs 1981, 1993). There exists not only circumstantial evidence but also convincing data that this is the case for *Euglena gracilis* (Morden et al. 1992), cryptomonads (yellow-algae) (Douglas et al. 1991, 1992; Eschbach et al. 1991), and chlorarachniophytes (amoeboid algae) (McFadden et al. 1994; van de Peer et al. 1996). In *Euglena gracilis,* the capture of chloroplasts from a green-algae was suggested by comparison of a tree based on SSU-rRNA and trees based on chloroplastidal genes (Morden et al. 1992). In the former tree, *E. gracilis* was positioned as a protozoa which is close to kinetoplastid protozoa (Kumar and Rzhetsky 1996), while in the latter tree it was assigned as an organism that is very close to green-algae (Morden et al. 1992; van de Peer et al. 1996). This discordance on the phylogenetic position of *E. gracilis* was explained by lateral transfer of chloroplasts from a green-alga through endosymbiosis (Morden et al. 1992; Palmer and Delwiche 1996). The possibility of mitochondrial replacement between a host cell and a symbiont was not examined, because a mitochondrial phylogenetic tree that encompasses a wide range of algae was not available. However, now it is possible to compare a nuclear-encoded SSU-rRNA tree (van de Peer et al. 1996) and a mitochondrially encoded *COXI* gene tree, even though only a limited number of algae are available. Such a comparison between our *COXI* tree and a nuclear-encoded SSU-rRNA tree did not show any obvious differences, which is statistically significant, between the two trees. Therefore, considering that the acquisition of chloroplasts from endosymbiotic algae did occur, replacement of the host mitochondria with the endosymbiotic ones seems highly unlikely in the eustigmatophytes and yellow-green algae examined in this study.

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