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Characteristics of Photosynthetic Pigment Composition of *Gymnodinium mikimotoi* Miyake et Kominami ex Oda

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***Gymnodinium mikimotoi*, a senior synonym of *Gymnodinium nagasakiense* often causes red tides in coastal waters of the western part of Japan. The photosynthetic pigment composition of two strains of *G. mikimotoi* were analyzed by HPLC. They contain chlorophyll c_3 which has not been reported from dinoflagellates. They also contain fucoxanthin, 19′-hexanoyloxyfucoxanthin and 19′-butanoyloxyfucoxanthin as major carotenoides, which are anomalous in dinoflagellates. The pigment composition of *G. mikimotoi* is compared with that of *Gyrodinium aureolum* which occur in European waters and is thought as a conspecific species with *G. mikimotoi* by several authors.**

1. Introduction

Gymnodinium mikimotoi Miyake et Kominami ex Oda is conspecific with *Gymnodinium nagasakiense* Takayama et Adachi and recently concluded as a senior synonym of the latter (Takayama and Matsuoka, 1991). The species often occur in the coastal waters of western part of Japan causing mass mortality of fishes and shellfishes (Takayama and Adachi, 1984; Matsuoka *et al.*, 1989).

Morphological similarity of *G. mikimotoi* with *Gyrodinium aureolum* which occurs in European waters and often associates with fish mortality has been recognized (Tangen and Bjørnland, 1981; Takayama and Adachi, 1984; Taylor, 1985). Tangen and Bjørnland (1981) reported 19′-hexanoyloxyfucoxanthin, which is an anomalous carotenoid in dinoflagellates, as the main carotenoid of *G. aureolum*.

In this paper, we will present the photosynthetic pigment composition of *G. mikimotoi* determined by a reversed-phase high performance liquid chromatograph (HPLC). The pigment composition was compared with that of *G. aureolum*.

2. Materials and Methods

Cultures: Two clonal strains of *G. mikimotoi* were used for this study. Strains 1 and 2 were isolated from Tenma Bay, Wakayama prefecture in 1984 and from Suoh-nada off Fukuoka prefecture in 1985, respectively. The former is the same with *G. nagasakiense* strain Katsuura and the latter is Buzen-'85-2 reported in Partenskey *et al.* (1988). Two centric diatom species of *Skeletonema costatum* and *Chaetoceros affinis* isolated from Tokyo Bay in 1984 were also used for comparative examination. These dinoflagellates and diatoms were grown in the T1 medium (Ogata *et al.*, 1988) and the f/2 medium (Guillard and Ryther, 1962), respectively, at 20 ± 1°C in batch culture. Illumination was provided by cool white fluorescent tubes giving a mean photon flux density of 80 μE m^{-2} s^{-1} with a 12:12 h light dark cycle.

Pigment extraction: Cultured cells were harvested by centrifugation at 4000 g for 10 min and

were extracted the pigments by 100% methanol. Extracted pigments were obtained by filtering through a Columngard-FH filter unit (0.5 μm pore-size, Millipore Corp.) for removing cell debris. Care was taken not to expose the extracted sample to bright light and high temperature above 0°C during the extraction processes.

Pigment analysis: An aliquot of the extract (100 μl) was directly injected into an HPLC (Shimadzu LC-4). Reversed-phase HPLC was performed on a 125 × 4 mm column packed with the octadecyl silica of 5-μm in particle size (Merk Lichrosphere, 100 RP-18e). Pigments were eluted by methanol with water (88:12 v/v) for 9.5 minutes and then by 100% methanol for 30 minutes with the flow rate of 1 ml/min. For the separation of carotenes, methanol with acetone (80:20, v/v) was then applied for 20 minutes.

Detection of pigments: Pigments were detected by a photo-diode array detector (Shimadzu SPD-M1A or SPD-M6A) at 440 nm, and individual pigments were collected from the outlet, manually. After the solvent was evaporated, the absorption spectra of each separated pigment in ethanol, diethyl ether and acetone were examined by using Shimadzu Model MPS 2000 spectrophotometer, and the results were compared with the published ones. Absorption spectra of each pigment of *G. mikimotoi* and diatoms were also compared by using the photo-diode array detector system. Absorbance of each peak was integrated by the system and converted to pigment amount with individual calibration curves made by using following standards and extinction coefficients.

Standards: Chlorophyll *a* from *Anacystis nidulans*, chlorophyll *b* from spinach leaves and β-carotene from carot root were obtained from commercially available stocks (Sigma Chemicals). Chlorophyll *c*, fucoxanthin, diadinoxanthin were prepared from cultured diatoms (*Phaeodactylum tricornutum* and *Skeletonema costatum*) in the laboratory. Each pigment was fractionated by the HPLC system mentioned above. The following extinction coefficients (l $g^{-1}cm^{-1}$) were applied for the quantitative calculations: 74.5 at 665 nm in methanol for chlorophyll *a* (MacKinney, 1941); 36.4 at 650 nm in methanol for chlorophyll *b* (MacKinney, 1941); 37.2 at 629.6 nm in acetone for chlorophyll c_2 (Jeffery and Humphrey, 1975); 250 at 450 nm in ethanol for carotenes, diatoxanthin, diadinoxanthin and unknown carotenoids (Goodwin, 1955); 160 at 452 nm in ethanol for fucoxanthin and its derivatives (Jensen, 1966). The value for chlorophyll c_2 was also applied for chlorophyll c_3.

3. Results and Discussion

In HPLC chromatograms of both strains of *G. mikimotoi*, sixteen distinct peaks were observed. A typical chromatogram of strain 1 monitored at a wavelength of 440 nm is shown in Fig. 1. Peak 1 was the absorption of solvent used. Peaks 2 to 14 corresponded to the separated pigments. This chromatogram was compared with that of *S. costatum* of which pigments have already been known (Fig. 1). The relative retention times of peaks of *G. mikimotoi* were compared with those of *S. costatum* and the following correspondence was observed; peak 3 and chlorophyll *c*, 7 and fucoxanthin, 11 and diadinoxanthin, 12 and diatoxanthin, and 14 and chlorophyll *a*.

Light absorption spectra between 400 and 670 nm of the pigments recorded by the diode array detector are shown in Fig. 2. According to the light absorption characteristics of each separated pigments, peaks 2 and 3 were similar to those of chlorophyll *c* and, 4 to 10 were similar to those of fucoxanthin and its derivatives. Peaks 11, 12 and 14 were identical to diadinoxanthin, diatoxanthin and chlorophyll *a*, respectively, but peak 13 was not similar to any known carotenoid.

Light absorption spectra between 400 and 550 nm of the peak 7 and peak 10 pigments of *G.*

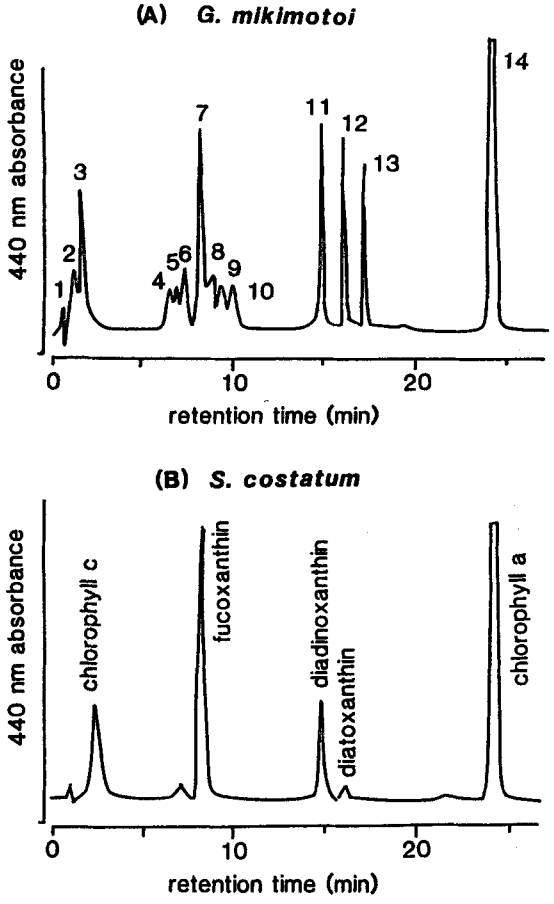

Fig. 1. HPLC absorbance (440) chromatograms of chlorophylls and carotenoids extracted from *Gymnodinium mikimotoi* strain 1 and *Skeletonema costatum*.

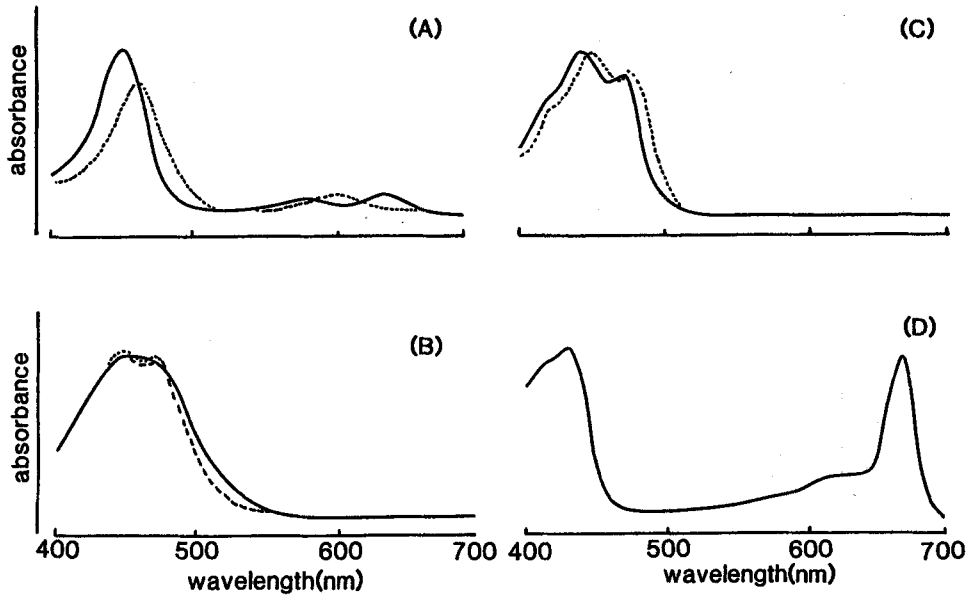

Fig. 2. Visible absorption spectra of HPLC separated pigments from *Gymnodinium mikimotoi* strain 1 obtained by diode-array detector. (A) peak 2 (···), 3 (—). (B) peak 6 (- - -), 7 (—), 10 (···). (C) peak 11 (—), 12 (···). (D) peak 14 (—).

mikimotoi strain 1 were determined in acetone, ethanol and diethyl ether, and results are shown in Fig. 3 in comparison with the absorption spectra of fucoxanthin isolated from *Pavlova lutheri* (Wright and Jeffery, 1987). The presence of absorption maximum at 446 nm and an inflection at 470 nm was further evidences that the peak 7 pigment of *G. mikimotoi* was identified as fucoxanthin.

Light absorption spectra of the peak 10 in the three solvents are shown in Fig. 4 in comparison with the absorption spectra of 19′-hexanoyloxyfucoxanthin extracted from *Emiliania huxleyi* (Jeffery and Wright, 1987). The absorption spectra of peak 10 pigment were similar to those of 19′-hexanoyloxyfucoxanthin in respect to the primary maximum at 444 nm and an obvious secondary maximum at 470 nm.

According to the light absorbance characteristics and relative retention times compared with published information (Wright and Jeffery, 1987; Bidigare, 1989), the pigments of peaks 4 and 5 were thought as unknown fucoxanthin derivatives, and the peak 6 was identified as 19′-butanoyloxyfucoxanthin, the peak 8 was cis-19′-butanoyloxyfucoxanthin, and peak 9 was cis-fucoxanthin.

Two strains of *G. mikimotoi*, strains 1 and 2 were investigated fucoxanthin derivatives at different growth phase, logarithmic and stationary (Table 1). Both strains had the same number of fucoxanthin derivative components. There were some differences in the contents of each fucoxanthin derivative between strains 1 and 2 such as large contents of fucoxanthin as much as 40–50% in the strain 1 compared to those of 10–19% in the strain 2. The contents of fucoxanthin derivatives did not show remarkable differences in these two growth phases.

Several species of *Gymnodinium* and *Gyrodinium aureolum* are known as anomalously pigmented dinoflagellates containing fucoxanthin derivatives (see Kite and Dodge, 1988) instead of peridinin, the major carotenoid in most photosynthetic dinoflagellates (Johansen *et al.*, 1974). *G. mikimotoi* is shown to belong to the member of anomalousy pigmented dinoflagellates.

Light absorption spectra of the pigment peaks 2 and 3 were shown in Fig. 5. Absorption spectrum of the fast eluting polar fraction (peak 2) in acetone appeared to be identical with that of chlorophyll c_3 isolated from *E. huxleyi* (Jeffery and Wright, 1987) with three distinctive peaks at 451.7 nm, 584.3 nm and 626.7 nm. The slow eluting fraction of peak 3 had an identical spectrum to that of chlorophyll c_2 with three peaks at 449.1 nm, 580.7 nm and 629.3 nm. Chlorophyll c_3 has been found in eight species of bacillariophytes out of 51 observed, prymnesiophytes such as *Pavlova* sp., *Pavlova salina*, *E. huxleyi*, *Phaeocystis pouchetii* (see

Table 1. Comparisons of fucoxanthin derivatives of the two strains of *Gymnodinium mikimotoi* strain 1 and 2. All the values are represented as percentages against the total fucoxanthin derivatives.

Fucoxanthin derivatives	Strain 1		Strain 2	
	logarithmic	stationary	logarithmic	stationary
unknown 1	5.3	7.4	17.0	13.1
unknown 2	4.7	8.1	18.1	13.1
19′-butanoyloxyfucoxanthin	11.1	12.6	26.6	21.8
fucoxanthin	51.6	42.2	10.6	19.4
cis-19′-butanoyloxyfucoxanthin	8.4	10.4	8.5	8.7
cis-fucoxanthin	8.4	9.6	8.5	8.3
19′-hexanoyloxyfucoxanthin	10.5	9.6	10.6	15.5

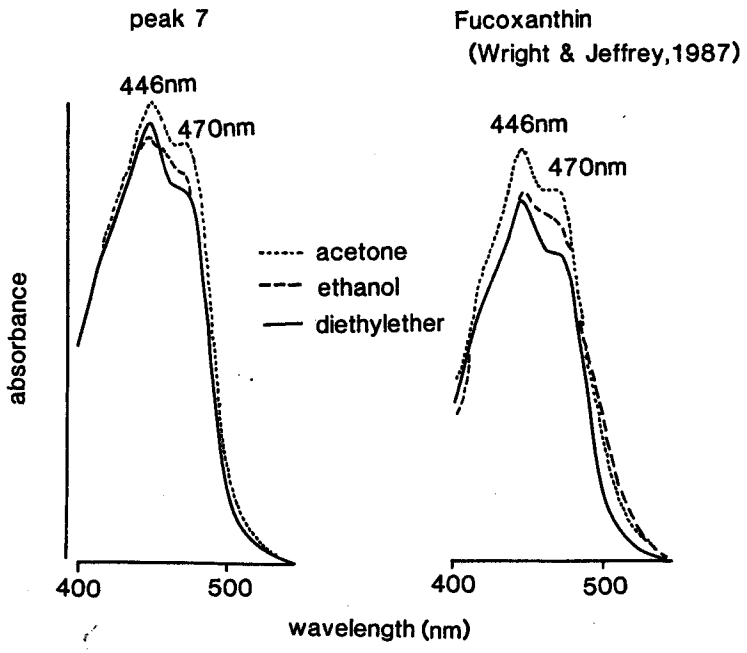

Fig. 3. Visible absorption spectra of HPLC separated pigment (peak 7) from *Gymnodinium mikimotoi* in acetone, ethanol and diethyl ether, and comparison with fucoxanthin from *Pavlova lutheri* (Wright and Jeffrey, 1987).

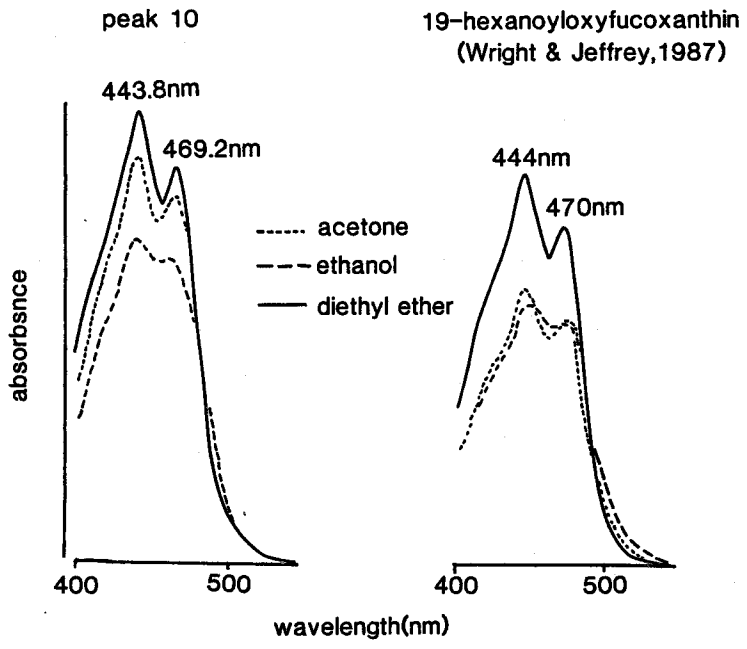

Fig. 4. Visible absorption spectra of HPLC separated pigment (peak 10) from *Gymnodinium mikimotoi* in acetone, ethanol and diethyl ether and comparison with 19′-hexanoyloxyfucoxanthin from *Emiliania huxleyi* (Wright and Jeffrey, 1987).

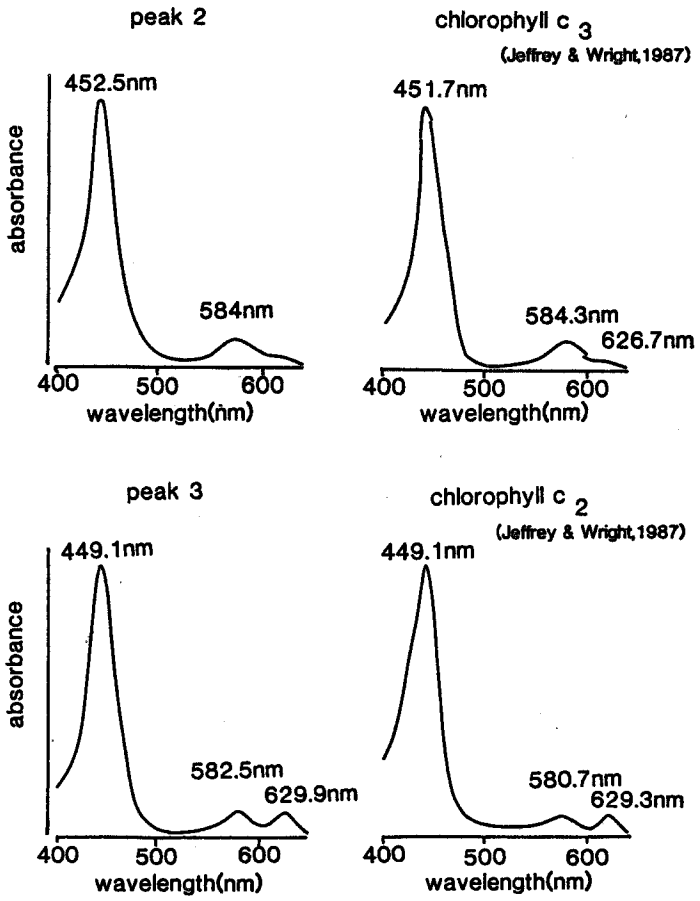

Fig. 5. Visible absorption spectra of HPLC separated pigment of peak 2 (fast eluting fraction) and peak 3 (slow eluting fraction) in acetone, and comparison with chlorophyll c_3 from *Emiliania huxleyi* (Wright and Jeffrey, 1987), and chlorophyll c_2 from *E. huxleyi* (Wright and Jeffrey, 1987).

Stauber and Jeffery, 1988) and a chrysophyte, *Pelagococcus subviridis* (Vesk and Jeffery, 1987). This report first provides the evidence for a dinoflagellate containing chlorophyll c_3.

The pigment compositions of *G. mikimotoi* and *Gyrodinium aureolum* which was investigated by Tangen and Bjørnland (1981) are compared (Table 2). The major carotenoid of *G. aureolum* is 19′-hexanoyloxyfucoxanthin and its content was 45.8% of chlorophyll *a* and about 70% of total carotenoids (Tangen and Bjørnland, 1981). Although the thin-layer chromatographic plate they used did not have an enough performance to separate fucoxanthin and 19′-hexanoyloxyfucoxanthin, they concluded the major carotenoid as 19′-hexanoyloxyfucoxanthin from following bases: (1) the absorption spectrum had a pronounced minimum between two peaks which was absent in fucoxanthin (see Figs. 3 and 4); (2) mass spectral peaks were in satisfactory accordance with the spectrum of 19′-hexanoyloxyfucoxanthin from *Coccolithus* (*Emiliania*) *huxleyi* reported by Arpin *et al.* (1976). However, from the result of mass spectrum analysis, Tangen and Bjørnland (1981) suggested the contamination of fucoxanthin in the 19′-hexanoyloxyfucoxanthin fraction of *G. aureolum*. Mass spectral peaks were at m/e 772 (mo-

Table 2. Comparisons of chlorophylls and carotenoids of *Gymnodinium mikimotoi* strain 1 and *Gyrodinium aureolum* (Tangen and Bjørnland, 1981). All the values are represented as weight percentages against chlorophyll *a*.

pigments	*G. mikimotoi*	*G. auleorum*
chlorophylls		
chlorophyll *a*	100.0	100.0
chlorophyll c_3	1.1	
chlorophyll c_2	7.3	10.6*
carotenoids		
fucoxanthin derivatives		
unknown 1	2.1	
unknown 2	2.0	
19′-butanoyloxyfucoxanthin	4.4	
fucoxanthin	27.0	
cis-19′-butanoyloxyfucoxanthin	3.6	
cis-fucoxanthin	3.9	
19′-hexanoyloxyfucoxanthin	6.7	45.8
diadinoxanthin	13.2	2.1
unknown carotenoid 1		3.5
unknown carotenoid 2		1.6
diatoxanthin	8.8	7.1
unknown carotenoid 3	9.0	2.8
β, β-carotene	2.4	1.0
β, ε-carotene	0.6*	0.3

*not investigated in detail.

lecular ion of 19′-hexanoyloxyfucoxanthin), 756, 754 (M-H_2O), 738, 736, 726, 658, 656, 640, 624, 622, 580, 562, 544 and 536. They suggested that the following peaks originated from fucoxanthin; m/e 658 (molecular ion), 640 (M-H_2O) and 622 (M-$2H_2O$). The peak at m/e 726 corresponds to M-H_2O peak of 19′-butanoyloxyfucoxantin. Because M-H_2O peak is much stronger than molecular ion peak in the case of 19′-hexanoyloxyfucoxanthin (Arpin *et al.*, 1976), 19′-butanoyloxyfucoxanthin might also be contaminated in the 19′-hexanoyloxyfucoxanthin fraction of *G. aureolum*. We assume that 19′-hexanoyloxyfucoxanthin is a major component but fucoxanthin and also 19′-butanoyloxyfucoxanthin are contained as minor components of carotenoids in *G. aureolum*. On the contrary, 19′-hexanoyloxyfucoxanthin is one of many fucoxanthin derivatives in *G. mikimotoi* comprising only 10 to 16% of the total amount (Table 1).

Loeblich (1984) suggested the plastids of dinoflagellates that possess a major pigment other than peridinin (e.g. fucoxanthin or 19′-hexanoyloxyfucoxanthin) are derived from a past symbiosis with an eukaryotic alga. Since *Emiliania huxleyi* contains 19′-hexanoyloxyfucoxanthin as a main carotenoid and fucoxanthin and 19′-butanoyloxyfucoxanthin in a trace amount (Wright and Jeferry, 1987), we may assume an alga with *Emiliania*-like pigment composition as an origin of plastid of *G. aureolum* as Tangen and Bjørnland (1981) did. The dominance of 19′-butanoyloxyfucoxanthin in carotenoids is known from crysophytes, *Pelagococcus subviridis* and *Aureococcus anophagefferens* and the prymnesiophyte, *Phaeocystis pouchetii* (Wright and

Jeferry, 1987). Especially the pigment system of *G. mikimotoi* resembles to one strain of *P. subviridis* which contains chlorophyll c_3, fucoxanthin and its two 19′-derivatives (strain CS-99 in Wright and Jeferry, 1987). We may also assume an alga with *Pelagococcus*-like pigment composition as an origin of chloroplast of *G. mikimotoi*.

Partensky *et al.* (1988) reported a significant discrepancy in DNA contents of *Gymnodinium nagasakiense* (*mikimotoi*) and *Gymnodinium* cf. *nagasakiense* (= *Gyrodinium* cf. *aureolum*). Reanalysis of the pigment composition of *G. aureolum* by HPLC is necessary for further discussion on the relationship between these two species.

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