Offprint Botanica Marina Vol. 44, 2001, pp. 57–66

A Comparative Study of the Harmful Dinoflagellates *Cochlodinium polykrikoides* and *Gyrodinium impudicum* Using Transmission Electron Microscopy, Fatty Acid Composition, Carotenoid Content, DNA Quantification and Gene Sequences

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The massive growth of the dinoflagellate alga *Cochlodinium polykrikoides* in Korean coastal waters was first observed in 1982. Thereafter the blooms associated with massive fish kills have been occurring annually, and *C. polykrikoides* is now regarded a harmful red tide organism in Korea. Recently, a new chain-forming *Gyrodinium impudicum*, usually occurring with *Cochlodinium polykrikoides*, was observed. Thus, we need to identify the toxic *C. polykrikoides* from the non-toxic *Gyrodinium impudicum*. Because of a similarity in morphology, it is difficult to distinguish the two species under the light microscope. In this study, comparisons were made using fine-scale features of transverse sections and biochemical analysis including rDNA sequencing targeted ITS regions. The observation of cellular arrangement and structure by transmission electron microscopy showed a similar distribution of the main components in both species, but location of the nucleus and distribution of the chloroplasts were different. Fatty acid composition showed that the long-chain polyunsaturated fatty acid docosahexanoic acid was not present in *Cochlodinium polykrikoides*. These data suggest that fatty acid composition may be a useful biochemical indicator to distinguish the microalgae. The amount of total carotenoids in *Gyrodinium impudicum* was approximately 10 times higher than in *Cochlodinium polykrikoides*. The nuclear DNA content in *Cochlodinium polykrikoides* was twice as high as that in *Gyrodinium impudicum* ($p < 0.06$). Sequence analyses coding for the 5.8S rDNA and ITS regions showed quite different nucleotide alignments with only 55% similarity, the length of the ITS regions also differed. The genetic differences between the two microalgae will allow the development of species specific DNA probes for species differentiation.

Introduction

The dinoflagellate alga *Cochlodinium polykrikoides* Margareff is distributed globally. It is slightly flattened in form and has a characteristic red stigma on the dorsal side of the episome close to the apical groove. The blooms caused by *Cochlodinium polykrikoides* have been associated with massive fish kills and it is regarded as a potentially toxic dinoflagellate in Korea (Kim *et al*. 1997). *Gyrodinium impudicum* Fraga *et* Bravo, recently isolated from Tongyong, Korea, is similar to *Cochlodinium polykrikoides* in morphology and difficult to separate from it under the light microscope. *Gyrodinium impudicum* was first reported as *Gymnodinium* A_3 by Iizuka in Japan (Iizuka 1979). Fraga *et al*. (1995) suggested that the best way to distinguish *Cochlodinium polykrikoides* and *Gyrodinium impudicum* was by the number of cingula on each side of the chain in fixed material. Since the middle 1980s blooms of *Gyrodinium impudicum* in Korean coastal waters have occurred annually in September and October together with *Cochlodinium polykrikoides*.

Some researchers have applied biochemical techniques to harmful algal blooms (HABs), instead of using traditional morphological phenotypes. There are reports on the pigments, fatty acids and sterols of toxic *Gymnodinium catenatum* Graham (Hallegraeff *et al*. 1991), on the biochemical variability of *Prorocentrum lima* (Ehrenberg) Dodge (Morton and Tindall 1995) and the carotenoid composition of *Gyrodinium impudicum* and *Gymnodinium catenatum* (Fraga *et al*. 1995). *Cochlodinium polykrikoides* has had very little biochemical investigation. It was sug-

gested that the use of biochemical characteristics could provide useful information on possible taxonomic links (Partensky *et al*. 1988, Moestrup and Larsen 1990, Hallegraeff *et al*. 1995). In a previous study, we applied DNA fluorochrome 4′-6-diamidino-2-phenolindole (DAPI), which binds specifically to double-stranded DNA, to harmful microalgae in order to compare the DNA feature (Cho *et al*. 1999 b). The amount of light emitted from a particular region of fluorochrome-stained DNA can now be measured either by a microspectrofluorometer or a video interfaced digital image processor (Goff and Coleman 1990). In Korea, Choi *et al*. (1994 b) have applied a video interfaced digital image processor to the quantitative cytology of the life history of *Dasysiphonia chejuensis* Lee *et* West. The nuclear DNA of *Gymnodinium mikimotoi* Miyake *et* Kominami *ex* Oda and *Chattonella* sp. was analysed using the microfluorometer system (Yamaguchi and Imai 1994). However, only a few investigations on the nuclei of *Cochlodinium polykrikoides* and *Gyrodinium impudicum* have been carried out. The recent advances in DNA amplification and sequencing provide a means of determining how closely organisms are related. The new sets of DNA genetic markers that evolve rapidly are now being used, with the expectation that they may provide greater sensitivity for the detection of genetic structure in marine microalgae.

In some cases, the ribosomal DNA sequences were used to clarify the relationship between unique morphotypes and species-level divisions (Scholin *et al*. 1994, Costas *et al*. 1995). Recently, some researchers have used internal transcribed spacer (ITS) regions, which are adjacent to conserved coding regions, to show interspecific and intraspecific ITS sequence variation (Adachi *et al*. 1997).

The comparison of morphological and biochemical characteristics of Korean isolates of *Cochlodinium polykrikoides* and *Gyrodinium impudicum* and determination of fatty acids and carotenoids were made by use of transmission electron microscopy (TEM). The DNA quantification was carried out in 4′-6-diamidino-2-phenolindole (DAPI), nucleotide sequences (ITS) were obtained. These data will be useful in differentiating *Cochlodinium polykrikoides* from nontoxic *Gyrodinium impudicum* in monitoring programs.

Materials and Methods

Microalgae

Cochlodinium polykrikoides and *Gyrodinium impudicum* for this study were obtained from Kunsan National University, Kunsan, Republic of Korea. The cells were picked out individually under the light microscope with a micropipette or obtained by serial dilution. Clonal cultures were maintained and cultured in f/2-Si medium (Guillard and Ryther 1962) containing an antibiotic mixture (Hasui *et al*. 1995), and grown in a customized culture cabinet (BOD incubator, Japan prod.) at 20 °C under a light intensity of 100 μmol photons m^{-2} s^{-1} from white fluorescent tubes (12:12 h of light:dark cycle). Cultures were maintained in the Harmful Algae Biology Culture Room, National Fisheries Research and Development Institute, Republic of Korea.

Electron microscopy

For the TEM, unpreserved cultures were prepared by filtering through a 0.45 μm cellulose acetate filter, and selected samples were fixed with 2.5 % glutaraldehyde and 1 % osmium tetroxide. The material collected on the filters was subsequently washed with 0.1 M phosphate buffer (pH 7.2). While still on the filter, cells were dehydrated in a stepwise graded ethanol series to 100 % ethanol. Then the filter was placed overnight in two changes of propylene oxide and in a 1:1 mixture of Epon resin and propylene oxide and for 8 h in fresh Epon resin. Thereafter, it was placed in fresh resin again and polymerized for 24 h. Sections were cut with a glass knife approaching the particles on the membrane filter parallel to the membrane surface. Ultra-thin sections (60−90 nm) were cut with a diamond knife, stained with uranyl acetate followed by lead citrate and examined at 80 kV with JEM 1200 EX-II, JEOL (Japan prod.) TEM.

Fatty acid analysis

Phytoplankton samples of *Cochlodinium polykrikoides* and *Gyrodinium impudicum* were grown under standard conditions for one month in 1L plastic culture tanks. The cells were harvested by filtration (Whatman GF/C, 47 mm) and frozen (−20 °C) until analyzed. The modified cold solvent extraction method, described by Folch *et al*. (1957) was used. The filters were suspended in 32 mL of the deionised water for 10 min and the suspension ultrasonicated (400 mL beaker; 5 min: probe sonicator). Chloroform (40 mL) and methanol (20 mL) were added to extract lipid and the filter left to stand overnight. The extract was collected after filtration (Whatman No. 1). The filtrate was evaporated to remove the solvent in a rotary evaporator and the slurry was then dissolved in chloroform and transferred to a separating funnel. The chloroform fraction was cleaned with saturated sodium chloride and distilled water (× 3), then transferred to filter paper containing anhydrate sodium sulfate and evaporated until it became dry. For saponification and methylation, 0.5 M sodium hydroxide (3 mL) was added and refluxed for 5 min, then boron trichloride in methanol was added (3 mL) and boiled for 10 min, and finally n-hexane added and boiled for 1 min. After cooling, saturated sodium chloride (3 mL) was added and mixed by rotation of the flask, then further sodium chloride added. The n-hexane layer was removed, dried with anhydrous sodium sulfate, and resus-

pended and dried under nitrogen, then injected into a Hewlett-Packard 5890A gas chromatograph with flame ionization detection (column: HP-1, 12 m × 0.53 mm; 170 °C initial temperature, 2 °C min^{-1} gradient to 230 °C (5 min); column flow: 3 mL nitrogen min^{-1}; injector 220 °C, detector 230 °C).

Extraction of the carotenoids

The algae were grown in 1L glass containers for 1 month, then harvested by filtration (Whatman GF/C, 47 mm) and frozen (− 20 °C) until required. Carotenoids were extracted by the method of Choi *et al*. (1994 a). Total carotenoid content was determined using the method of MecBeth (1972) and calculated by the following equation:

$$\text{mg/100 g (mg\%)} = \frac{\text{O.D}(\lambda\text{max}) \times \text{vol.} \times 1000}{E^{1\%}_{1\,\text{cm}}(2400) \times \text{weight of sample (g)}}$$

The absorbency detection wavelength was set at 434 nm and recorded on a Shimadzu UV-1200 (Tokyo, Japan).

Measurement of DNA content using an image analysis system

For staining, tested cells were treated with a microwave for approximately 15 s and added to 1 µg/mL DAPI in Tris buffer 0.5 µg/mL (10 mM Tris, 10 mM EDTA-2Na, 100 mM NaCl, 10 mM 2-mercaptoethylamine hydrochloride, pH = 7.4). After staining, the treated cells were examined under an epifluorescence microscope, Olympus BH2 with a UV filter attachment (excitation, 330−385 nm, emission, 420 nm). To measure the content of DNA, the amount of light emitted from a particular region of fluorochrome-stained DNA was measured by an image analysis system. Some microphotographs stained by DAPI using fluorescence microscope were recorded with a scanner (Sharp, JX-330 p, 24,000 DPI) and directly processed with a personal IBM 586 computer. The DNA content in the nuclei was measured by image analysis software (Optimas 5.1 version for Windows 3.1).

DNA isolation, amplification, sequencing and alignment

Samples were harvested by filtering (Whatman No.1 filter paper) and the filter rinsed with distilled deionized water. Before the extraction of DNA, pellets were frozen (−20 °C) until required. Algal DNA was extracted as described previously (Kim *et al.* 1999). The nuclear rDNA region spanning the ITS-1, ITS-2 and 5.8S rRNA gene was amplified by polymerase chain reaction (PCR) assessment. Primers ITS A(5′-CCAAGCTTCTAGATCGTAACAAGGTCCGTAGGT-3′) and ITS B(5′-CCTGCAGTCGACAATGCTTAATTCAGCGG-3′) were used and derived from the conserved regions of 18S and 28S rDNA, respectively. The fragmentation was carried out with a Perkin-Elmer Model 480 thermocycler using the following program: initial denaturation for 3 min at 95 °C, 30 cycles of amplification (denaturation for 30 s at 95 °C, annealing for 30 s at 50 °C, and extension for 1 min at 72 °C) and final extension of 5 min at 72 °C. The PCR product was stained with ethidium bromide gel and purified by a QIAGEN Gel Elution Kit (Qiagen, Wartworth, CA). Direct sequencing of the PCR products was done in a Perkin-Elmer Applied Biosystems ABI 377A sequencer using a PRISM Dye Dideoxy Terminator Cycle Sequencing Kit (Perkin-Elmer). Sequences were edited and assembled as described previously (Kim *et al*. 1999). The degree of sequence similarity was examined by calculating the nucleotide substitution rates for transitions and transversions using the CLUSTAL W (Thomson *et al*. 1994).

Results

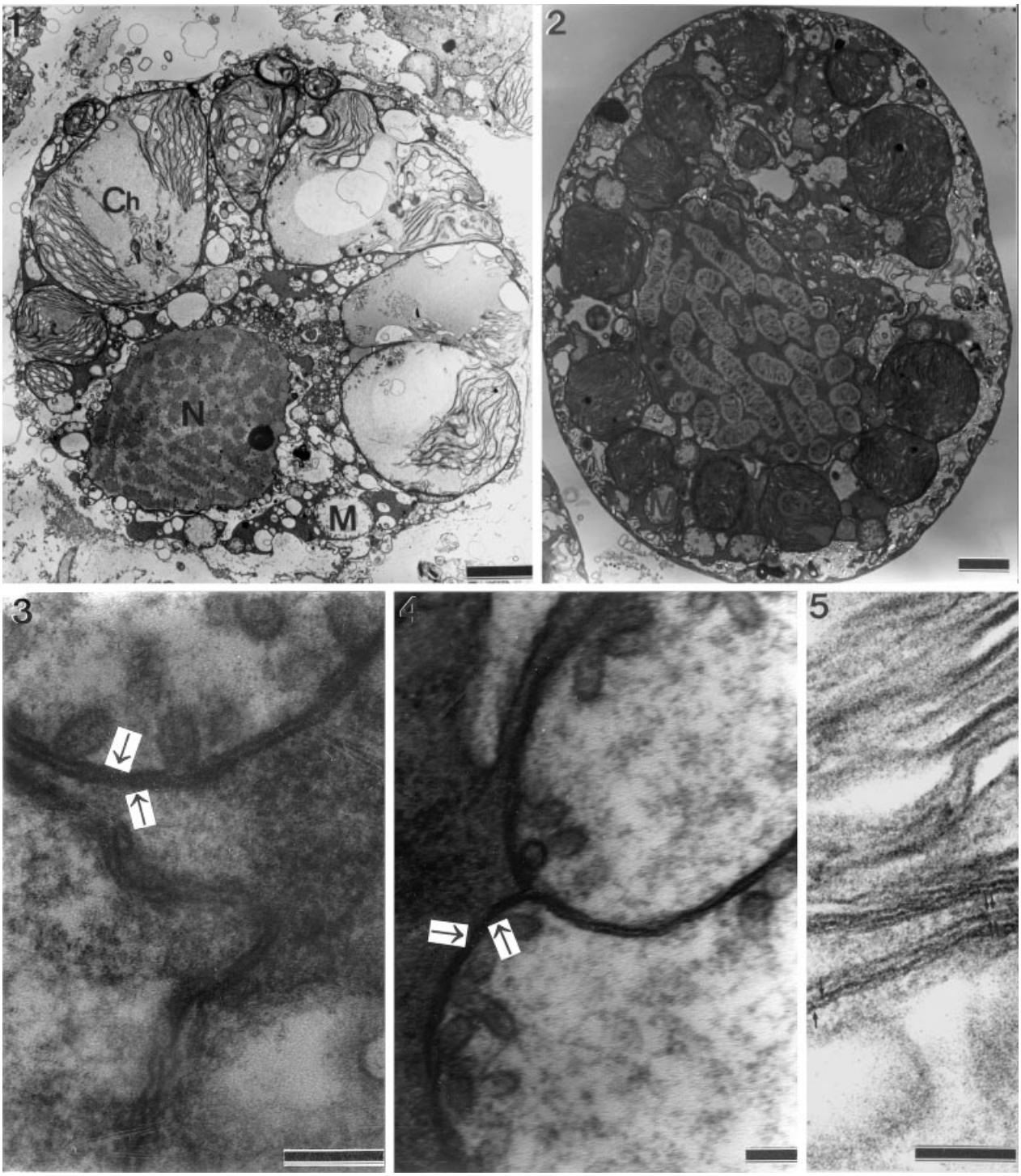
TEM observation

Figure 1 shows a transverse section of *Cochlodinium polykrikoides* and the distribution of the main components of cellular organelles. Cells were enclosed by a single layered plasmalemma and not covered by a cell wall or theca. The endoplasm contained a large nucleus and granular cytoplasm with many mitochondria, whereas the ectoplasm was largely made up of discoid chloroplasts. The nucleus was located in the periphery of the cells and had a rhizostyle connected with the nuclear surface layer. However, in *Gyrodinium impudicum* the nucleus was located almost in the center of the cell (Fig. 2). In Figure 1, each chloroplast is shown to adhere to the cell surface layer, but in *Gyrodinium impudicum* the cell surface layer was separated from the chloroplast (Fig. 2). Mitochondria were surrounded by an electron dense double membrane (arrow) and possessed many tubular cristae in the endoplasm, separated from cytoplasm (Figs. 3, 4). Numerous chloroplasts lie alongside many of the double thylakoid lamellae (single arrow), the lamellae abutting along the entire length of the chloroplast (Fig. 5). Chloroplasts were surrounded by inner and outer membranes (double arrow) and separated from each other (Fig. 5).

Fatty acid composition and carotenoids

The content of saturated and mono-unsaturated fatty acids was similar in both species, but the total content of polyunsaturated fatty acid was 53% in *Cochlodinium polykrikoides* and 48.6% in *Gyrodinium impudicum* (Table I). The algae tested produced a

wide range of unsaturated fatty acids, but did not have any long chain acids (above C_{24}). In addition, both of them produced the essential linolenic acid, as a precursor of eicosapentaenoic acid (about 1%), whereas the amount of linoleic acid, as a precursor of arachidonic acid, was about 7%. They also pro-



Figs 1–5. The dinoflagellates, *Cochlodinium polykrikoides* and *Gyrodinium impudicum* in the transmission electron microscope.
Fig. 1. Transverse section of a whole cell of *Cochlodinium polykrikoides*. Major cellular components as follows: nucleus (N), chloroplast (Ch) and mitochondria (M), (scale bar is 5 μm). Fig. 2. Cross section of whole cell of *Gyrodinium impudicum*. Major cellular components as follows: nucleus (N), chloroplast (Ch) and mitochondria (M), (scale bar is 2 μm). Fig. 3. Mitochondria with double layered membrane (black arrow) and tabular cristae in *Cochlodinium polykrikoides*, (scale bar is 100 nm). Fig. 4. Mitochondria with double layered membrane (black arrow) and tabular cristae in *Gyrodinium impudicum*, (scale bar is 100 nm). Fig. 5. *Gyrodinium impudicum*. Two thylakoid lamellae (single arrow) and chloroplast surrounded by double membrane (double arrow), (scale bar is 100 nm).

Table I. The composition of fatty acids in *Cochlodinium polykrikoides* and *Gyrodinium impudicum*. Fatty acids composition calculatd from the area of the peak (%).

Fatty acid	*Cochlodinium polykrikoides* (%)	*Gyrodinium impudicum* (%)
Saturates		
12:0	3.4	8.8
15:0	0.8	0.5
17:0	0.9	
18:0	6.5	5.1
20:0		0.6
Sum	11.6	15.0
Monounsaturates		
14:1	5.4	8.0
16:1	27.6	28.4
24:1	2.4	
Sum	35.4	36.4
Polyunsaturates		
18:1 (n-6)	21.6	19.3
18:1 (n-3)	8.9	7.0
18:2 (n-6)	7.4	6.7
18:3 (n-3)	1.2	0.8
20:2 (n-6)	6.7	5.0
20:4 (n-3)	1.5	
20:5 (n-3)	3.0	4.1
22:2 (n-6)	2.7	2.4
22:6 (n-3)		3.3
Sum	53.0	48.6
Σ n-3	14.6	15.2
Σ n-6	38.4	33.4
Total	100.0	100.0

The fatty acids are described by *x*:*y* (*n*-*z*), where *x* is the number of carbon atoms, *y* is the number of double bonds, and *z* is the position of the first double bond counting from the methyl end of the molecule.

duced a series of n-3 polyunsaturated fatty acids in similar amounts (about 14%), but the amount of n-6 polyunsaturated fatty acids was different (*Cochlodinium polykrikoides* – 38.4%; *Gyrodinium impudicum* – 33.4%). In particular, the amount of eicosapentaenoic acid was 3% in both species, but the amount of docosahexanoic acid was different, being 3% in *Gyrodinium impudicum* and not found in *Cochlodinium polykrikoides*.

The comparison of the total carotenoids in *Cochlodinium polykrikoides* and *Gyrodinium impudicum* is shown in Figure 6. Although the morphology of these species was similar under the light microscope, carotenoid content was remarkably different. Thus, *Cochlodinium polykrikoides* contained 1.5 mg%, whereas *Gyrodinium impudicum* contained 18.3 mg%.

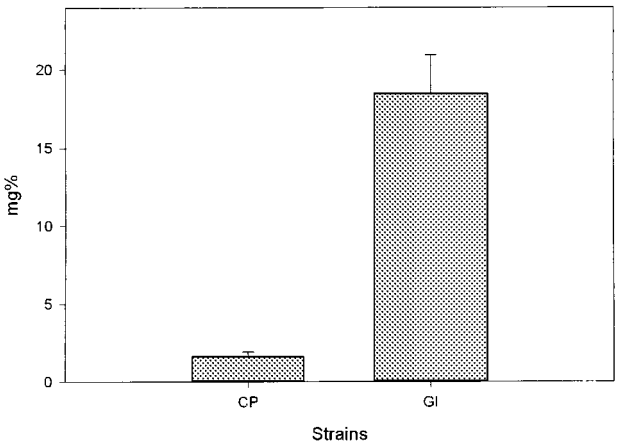


Fig. 6. Carotenoid content of *Cochlodinium polykrikoides* (CP) and *Gyrodinium impudicum* (GI).

Nuclear DNA feature and content

The result of applying the DNA fluorochrome DAPI, specifically binding to double-stranded DNA, to *Cochlodinium polykrikoides* and *Gyrodinium impudicum* is shown in Figure 7. In *Cochlodinium polykrikoides* the DNA-DAPI complexes were concentrated in the periphery of the cell, interconnected by numerous thin filaments of DNA, and emitted fluorescence of a high intensity (Fig. 7 a), but *Gyrodinium impudicum* had a compact appearance in the cell center without any distinct structure inside and was ovoid in appearance (Fig. 7 b). The image analysis system was used to measure the relative nuclear DNA content stained by DAPI. The mean grey level in *Cochlodinium polykrikoides* was twice as large as that in *Gyrodinium impudicum* under the same culture conditions and in the exponential phase ($p < 0.06$ by Student test, Fig. 8).

DNA sequences

Electrophoresis and direct sequencing of each PCR reaction product confirmed that a single product was amplified in each PCR reaction and the size of each product corresponded to the expected rDNA. The alignment of the DNA sequences of the internal transcribed spacers ITS1, ITS2 and 5.8S rDNA are presented in Figure 9. The alignment of *Cochlodinium polykrikoides* and *Gyrodinium impudicum* nucleotide sequences revealed significant differences between the species. There was considerable sequence variation in the ITS1 and ITS2 sequences and little in the regions of the 5.8S rDNA. In particular, the ITS1 was more variable than ITS2 in the two species examined. Table II shows the length of the ITS1, 5.8S and ITS2 regions. The length of the ITS1 region was known to greatly vary in *Cochlodinium polykrikoides* and *Gyrodinium impudicum*, and was 243 bp and 180 bp, respectively. In contrast, the length of the ITS2 region appeared to vary less than ITS1 in both species,

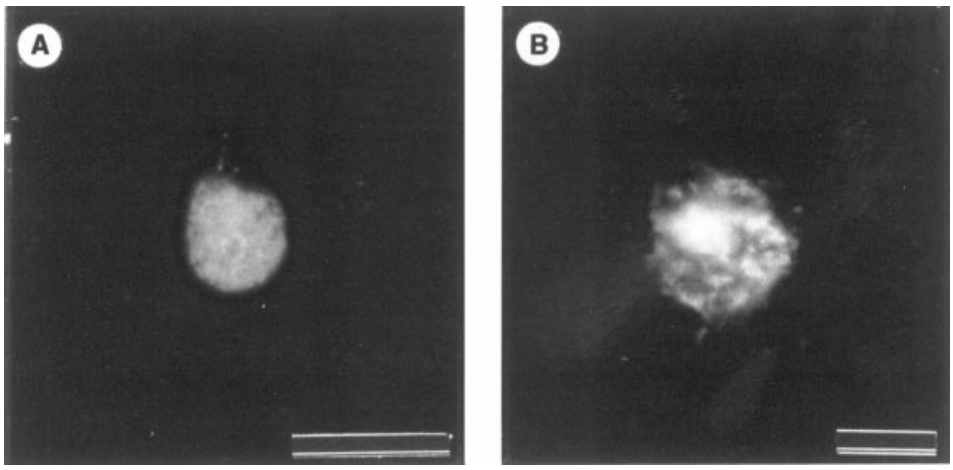


Fig. 7. DAPI stain to show DNA structure. A. *Gyrodinium impudicum* (scale bar is 30 μm), B. *Cochlodinium polykrikoides* (scale bar is 20 μm).

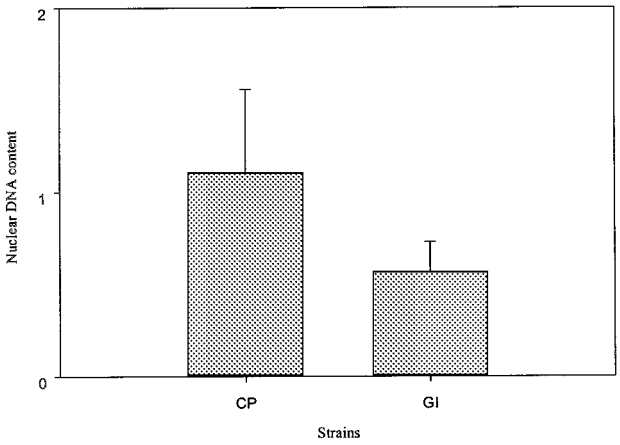


Fig. 8. The relative DNA contents stained by DAPI using an image analysis system. CP: *Cochlodinium polykrikoides*, GI: *Gyrodinium impudicum*

and *Gyrodinium impudicum* contained more longer sequences in the ITS2 region than *Cochlodinium polykrikoides*. *Cochlodinium polykrikoides* and *Gyrodinium impudicum* had different nucleotide lengths, 585 bp and 560 bp, respectively. Due to variability in the ITS regions, the conserved 5.8S was very small and had a very high homology compared with the ITS DNA homology. The aligned ITS1,2 and 5.8S regions between *Cochlodinium polykrikoides* and *Gyrodinium impudicum* had a 55% sequence similarity.

Discussion

Morphological description

The taxonomy of the unarmoured dinoflagellate species is based mainly on the general morphology of the cell. The position of the cingulum and the proportion of displacement of cingulum against the total cell length are important characteristics in distinguishing the two genera. However, it is very difficult to differentiate between *Cochlodinium polykrikoides* and *Gyrodinium impudicum* under the optical microscope, as each strain had almost identical ovoid to spherical cells. In an attempt to resolve the differentiation between them, we have tested the application of FITC-conjugated lectin to *Cochlodinium polykrikoides* and *Gyrodinium impudicum*, and found that coral tree agglutinin (ECA), snail agglutinin (HPA) and wheat agglutinin (WGA) were able to discriminate between the two species (Cho *et al.* 1998). Different lectin binding profiles indicated that cellular glycan moieties differed at the cell surface. Both strains were fundamentally the same in their internal structure, except for the position of the nucleus and the chloroplast attachment to cell surface. There are no pyrenoid or mucocysts present in either *Cochlodinium polykrikoides* or *Gyrodinium impudicum*. These are present in the raphidoflagellates for example: *Chattonella* spp., and *Fibrocapsa japonica* Toriumi *et* Takano (Cho *et al.* 1999 a). Hara and Chihara (1982) observed that mitochondria showed different shapes depending on cored cristae, but both microalgae examined had the same tubular cristae in the endoplasm.

Biochemical and genetic comparison

Both *Gyrodinium* cf. *aureolum* Huburt, *sensu* Braarud and Heimdal (Chang 1996) and *Gymnodinium mikimotoi*, which are morphologically similar, were characterized by the presence of 19′-hexanoyloxyfucoxanthin as a major carotenoid. In addition, Moestrup and Larsen (1990) suggested that peridinin played an

Table II. Length of the 5.8S rDNA and ITS regions of *Cochlodinium polykrikoides* and *Gyrodinium impudicum*.

Microalgae	ITS1 (bp)	5.8S (bp)	ITS2 (bp)	Total (bp)
Cochlodinium polykrikoides	243	160	182	585
Gyrodinium impudicum	180	162	218	560

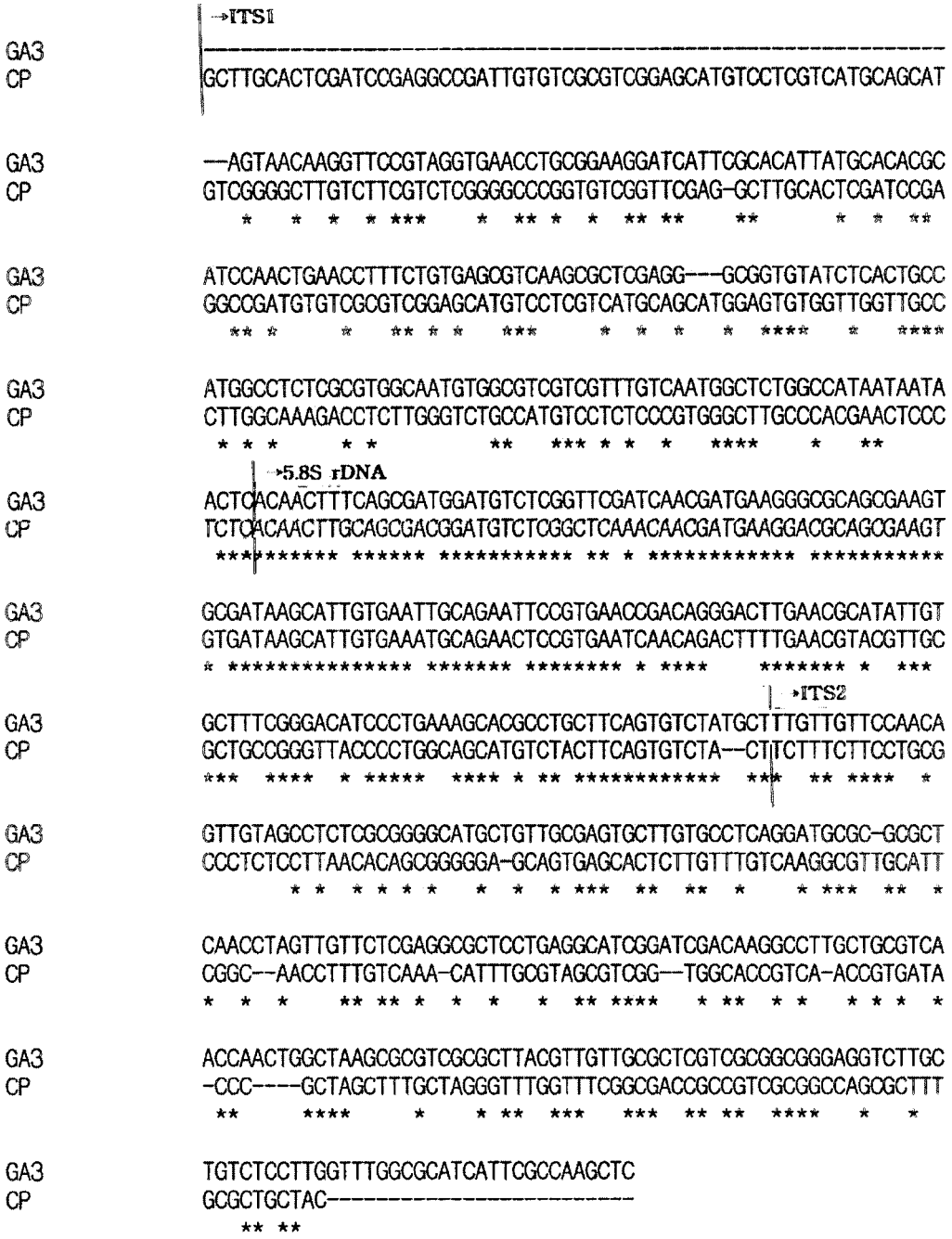


Fig. 9. Sequence alignment of the ITS1,2 and 5.8S rDNA regions. The alignment was generated by the multiple alignment program CLUSTAL W using a gap weight of 3.0 and a gap length weight of 0.1 ITS1 spans from 1 to 240 bp; the 5.8S coding region was from 261 to 421 bp; and ITS2 was from 421 to 700 bp. A hyphen represents a gap and a period represents a base identical to that of the top sequence. An asterisk represents an identical sequence on vertical lines. The sources of the sequences were as follows: GA3: *Gyrodinium impudicum*, CP: *Cochlodinium polykrikoides*.

important role as a tool for differentiation between the closely related species, *Gymnodinium breve* Davis and *Gymnodinium mikimotoi*. Fatty acid and sterol data can also provide useful chemotaxonomic information on microalgal relationships (Hallegraeff *et al*. 1991, Suzuki *et al*. 1997). Cho *et al*. (1999 a) suggested that fatty acids might be useful as a comparative chemotaxonomic tool for the raphidophyte group. Budge *et al*. (1997) suggested that the fatty acid composition of toxic *Pseudo-nitzschia* and non-toxic *Pseudo-nitzschia pungens* (Grunow *ex* Cleve) Halse differed considerable and provided a useful means of differentiating between them. Fatty acids also play a role as biomarkers for the toxic *Alexandrium fundyense* Balech and non-toxic *Scrippsiella trochoidea* (Stein) Loeblich. Therefore chemotaxonomic markers can and also play a key role in differentiating between morphologically indistinguish-

able species. From our data on fatty acid composition (Table I), both *Cochlodinium polykrikoides* and *Gyrodinium impudicum* had a high abundance of palmitic acid and a series of n-3 and n-6 polyunsaturated fatty acids, which gave a similar fatty acid profile. However, the abundance of the long-chain polyunsaturated fatty acid docosahexanoic acid differed between the strains, and might be used to differentiate between these organisms. Cho *et al*. (1999 a) reported the different abundance of eicosapentaenoic acid for two strains of *Fibrocapsa japonica* that might be useful as comparative chemotaxomic tools. However, in *Cochlodinium polykrikoides* and *Gyrodinium impudicum* there was a similar abundance of eicosahexanoic acid, but 22:6 (n-3) was found to differ. *Gymnodinium catenatum*, which is morphologically similar to *Cochlodinium polykrikoides* and *Gyrodinium impudicum*, contained a larger amount of 20:5 (n-3) and 22:6 (n-3) (approximately 10 times greater than that of *Cochlodinium polykrikoides* and *Gyrodinium impudicum*, Hallegraeff *et al*. 1991, Table I). Therefore, it is possible to differentiate toxic *Gymnodinium catenatum* from non-toxic *Gyrodinium impudicum* by the amount of long-chain fatty acids.

In a previous report we applied the fluorochrome DAPI to some microalgae in order to compare DAN features and have concluded that DAPI might be a possible tool to identify morphologically similar marine phytoplankton (Cho *et al*. 1999 b). Figure 7 shows that the location of nucleids stained by DAPI in *Cochlodinium polykrioides* and *Gyrodinium impudicum* was quite different, as well as the position of the nucleus, observed in transverse sections (Figs 1−5). Choi *et al*. (1994 b) suggested that a video interfaced digital image processor was able to measure the DNA content stained by DAPI and provide quantitative cytology for the life history of a marine red alga. *Gyrodinium impudicum* showed significantly larger amounts of total carotenoids than did *Cochlodinium polykrikoides* (Fig. 6), and the DNA content was different ($p < 0.06$) from *Cochlodinium polykrikoides* which contained approximately twice as much DNA as *Gyrodinium impudicum* (Fig. 8). It appears that DNA-DAPI complex morphology may be able to differentiate between target microalgae, and the nuclear DNA content stained by DAPI using an image analysis system could play an important role as a chemotaxonomic tool in Korean microalgae monitoring events in the future.

The reason why we have selected the alignment of nucleotide ITS regions is that ITS is located between 18S rDNA and 28S rDNA, which include conserved 5.8S rDNA and contain both considerable sequence variation and phylogenetically conserved regions. Due to these points of ITS variation and high conservation, some researchers have used them as a taxonomic tool at intra-species levels (Steane *et al*. 1991, Coleman *et al*. 1994), as well as inter-species levels in various algae (Goff *et al*. 1994). According to Adachi *et al*. (1997), the nuclear ITS spacer regions of *Gymnodinium catenatum* provide phylogenetically informative and *G. catenatum*-specific genetic markers. The genus *Alexandrium* consists of several toxic and non-toxic species, but it is very difficult to discriminate between them under the light microscope. Recently, several investigators have analyzed the ITS sequence including conserved 5.8S rDNA regions and have generated alignment of ITS1, 2, and the 5.8S rDNA fragment (Adachi *et al*. 1996). Adachi *et al*. (1996) suggested that the ITS region from *Alexandrium* was a useful genetic marker at the species and population level, and in particular ITS was thought to be a promising source for nuclear molecular markers and to prepare DNA probes specific to *Alexandrium*. *Gymnodinium catenatum* which causes paralytic shellfish poisoning (PSP) like *Alexandrium* is a common dinoflagellate in Korea, but it is difficult to differentiate between *Gymnodinium catenatum* and *Gyrodinium impudicum* without observation of the fine-scale morphology. However, Costas *et al*. (1995) reported that *Gymnodinium catenatum* and *Gyrodinium impudicum* were divergent on the basis of sequence analyses of the large subunit RNA gene fragment. Likewise, we reported previously that *Gymnodinium catenatum* and *Gyrodinium impudicum* targeted ITS regions were clearly an aid for identification based on the phylogenetic analysis and the genetic distance (Kim *et al*. 1999). In this study, we compared the sequence analyses of the 5.8S rDNA and ITS regions between *Cochlodinium polykrikoides* and *Gyrodinium impudicum* and found quite different sequence alignments and lengths of the ITS regions (Fig. 9). Similarity was only 55%, which is a great distance genetically, despite the morphological similarity. Therefore, genetically *Cochlodinium polykrikoides* is very different from *Gyrodinium impudicum* and we are able to propose promising species specific DNA probes designed from these regions for the harmful *Cochlodinium polykrikoides* and non-toxic *Gyrodinium impudicum*. Furthermore, a specific oligonucleotide DNA probe for *Cochlodinium polykrikoides* should allow detection of the resting cyst in Korean coastal waters.

Our conclusion is that although *Cochlodinium polykrikoides* and *Gyrodinium impudicum* are morphologically similar, biochemical data such as fatty acids, total carotenoids and nuclear DNA quantification indicate a different biochemical composition and content, and chemotaxonomic information discussed in this study may play an important role in their discrimination. The rDNA with ITS region sequence analysis confirmed a long genetic distance between the species, and we will establish a species specific DNA probe targeted to *Cochlodinium polykrikoides* in order to distinguish harmful *Cochlodinium polykrikoides* and non-toxic *Gyrodinium impudicum* to complement monitoring and prediction systems in Korean coastal waters.

Acknowledgements

The authors thank Dr H. J. Jeong, Kunsan National University for providing the isolate of *Cochlodinium polykrikoides* samples used in this study. We also appreciate Dr C. K. Lee, Harmful Algal Research Division, National Fisheries Research and Development Institute, and Dr J. K. Park, Inje University for their many contributions to the culturing efforts. We are especially grateful to Keug Rae Jo and Yung Sook Kim, Inje University for skillful assistance of electron microscopy and to M. G. Ha, Korea Basic Science Institute for assistance with computer analyses and useful discussions. We are grateful to two anonymous reviewers for suggestions and comments that improved the manuscript.

Accepted 19 September 2000.

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