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Molecular Identification of *Gyrodinium impudicum* and *Gymnodinium sanguineum* by Comparing the Sequences of the Internal Transcribed Spacers 1, 2 and 5.8S Ribosomal DNA

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The sequences coding for the 5.8S rDNA and the internal transcribed spacers (ITS1 and ITS 2) from the isolates of nine isolates of *Gyrodinium impudicum* and two isolates of *Gymnodinium sanguineum* species were amplified, sequenced and compared with the previously known *Alexandrium* species and *Gymnodinium catenatum*. The genetic distance analyses based on the sequence alignment indicated that *Gymnodinium catenatum* and *Gyrodinium impudicum* species were some related, *Alexandrium* species was distant. *G. catenatum* and *G. sanguineum* were quite separate, but these two species belonged to the same genus. *G. impudicum* and *G. catenatum* forming the closet cluster showed some variation in the alignment of ITS regions. The length of ITS1 varied more than that of ITS2 and the length of ITS1 and ITS2 was different for each *G. impudicum, Gymnodinium* and *Alexandrium* species. Also, the length of ITS1 was shorter than that of ITS2. However, on the sequences of *G. sanguineum*, the length of ITS1 was longer about 23 nucleotides than that of ITS2. The phylogenetic analysis and rDNA similarity of *G. impudicum* and *G. catenatum* (59%) is higher than the that of *G. catenatum* and *G. sanguineum* (55%). It was thought that the phylogenetic analysis and the genetic distance revealed that *G. impudicum* and *G. catenatum* were clearly different species and *G. impudicum* may belong to the genus of *Gymnodinium*.

Key words: *Gyrodinium, Gymnodinium*, ITS, rDNA, phylogenetic analysis

Introduction

The marine dinoflagellate genus *Gymnodinium* isolated from Korean coastal waters consists of 5 species (*G. breve, G. catenatum, G. mikimotoi, G. sanguineum* and *G.* A_3). Among them *G. breve* and *G. catenatum* are known to produce toxins. They are regarded as potentially toxic dinoflagellates in Korea (Kim et al., 1997). *Gymnodinium* A_3 was recently isolated from Chungmu and undistinguishable with *Cochlodinium polykrikoides* and *G. catenatum* which have been associated with massive fish kills (Ikeda et al., 1989) under the light microscope because of their morphological similarity (Cho et al., 1998). *Gymnodinium* A_3 was first reported by Iizuka (Hasui et al., 1995), but it was turned out to be *G. impudicum* (Fraga et al., 1995). The identification of *G. impudicum*, toxic *G. catenatum* and harmful *C. polykrikoides* has been done on the basis of ultrastructural morphological features using electron microscopy, but the morphological characteristics were subject to great variation depending on the environmental conditions (Sako et al., 1990; Adachi et al., 1993). Especially, *G. impudicum* has been misidentified in several previous papers as *G. catenatum*. The girdle is a descending left spiral, displaced between 1/3 - 1/4 of the total length of the cell and the sulcus lacks in torsion. These are two reasons why the new species is assigned to *G. impudicum* (Kofoid and Sewzy 1921; Ellegaard et al., 1993).

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Gyrodinium impudicum is generally smaller than *G. catenatum* although the largest *G. impudicum* may overlap the smallest *G. catenatum* in size (Fraga et al., 1995). No toxins were detected in *G. impudicum*. PSP toxins were detected in *G. catenatum* grown in the same culture medium under the same conditions of temperature and light (Mee et al., 1986; Carrada et al., 1991). Therefore, the validity of the taxonomic criteria for identifying species in microalgae is always debatable by algal taxonomists. For example, the genus *Alexandrium* has been split into more than 20 species throughout the world (Adachi et al., 1994), some being associated with PSP. However, it is difficult to differentiate toxic *Alexandrium* species from non-toxic *Alexandrium* species because of their morphological similarity.

In an attempt to resolve the differences between toxic *Alexandrium* and non-toxic *Alexandrium* rapidly, there have been several approaches such as enzyme electrophoresis, toxin profiles, cell surface antigens, surface glycan moieties and restriction fragment length polymorphism (RFLP) analysis (Costas et al., 1995). New approaches have been introduced to eliminate the taxonomic ambiguities and allow the genotypical properties and the nucleic acid sequences (Costas et al. 1995). So far, the sequence analysis has been carried out on two distinct small-subunit (SSU) ribosomal RNA and large-subunit (LSU) for several *Alexandrium* species (Sako et al., 1990, Scholin et al., 1993, Scholin et al., 1994a). Several *Pseudo-nitzschia* species have also been used for SSU rDNA analysis (Miller and Scholin 1996). It has been demonstrated that the sequence analysis of the 'V4' domain of the SSU rDNA region was desirable to determine the phylogenetic placement of the Rapidophyceae because of the variability of this regions on the basis of taxonomic indicator (Tyrrell et al., 1996). The sequence analysis and comparison within species using the targeted SSU rDNA and LSU rDNA is required to attain the unique genotype of the harmful microalgae.

Recently, the internal transcribed spacers (ITS1 and ITS2) containing 5.8S rDNA, has clarified the taxonomic level for many animals, microorganisms, green algae, red algae and diatoms (Adachi et al., 1996). There have been some reports concerning the analysis of *Alexandrium* species using targeted ITS regions (Adachi et al., 1994, 1995, 1996). However, there has been little assessment of the genomic variability at the inter and intra-species levels for the targeted ITS regions. Therefore, we have endeavoured to determine the phylogenetic placement of the genus *Gymnodinum* isolates from Korea as inferred from the ITS regions.

The objectives for this study are : (1) to determine the regions of ribosomal genes which could distinguish between *G. impudicum* and *G. sanguineum* species isolated in Korea, (2) to examine the phylogenetic relationship among the isolated species isolated regarded as potentially toxic dinoflagellates in Korea coast. In this paper, we report the sequence analysis of the 5.8S rDNA, the internal transcribed spacers ITS1 and ITS2 regions of *G. impudicum* and *G. sanguineum*, and the comparision of the phylogenetic tree from *G. impudicum*, *G. catenatum*, *G. sanguineum* and *Alexandrium* species.

Materials and Methods

Sample sources and cultural conditions

The tested microalgae, *G. impudicum* and *G. sanguineum* were the isolates from the coast waters at Chungmu in 1997 and Masan in 1996, respectively (Table 1). The isolation and purification were described as Cho et al. (1998). After a clonal culture was established, cells were maintained and cultured in f/2-Si medium containing an antibiotic mixture (Hasui et al., 1995) at 20℃ under light intensity of 100 μmol m^{-2} s^{-1}. It has been kept in the Harmful Algae Biology Culture Room, National Fisheries Research and Development Institute. The cells of microalgae were harvested from the liquid media by filtering onto Whatman No.1 filter paper and rinsed with distilled deionized water. Before the extraction of DNA, pellets were reserved in the freezer at −20℃.

DNA extraction

Algal DNA was extracted from each sample according to the procedure adapted from the benzyl chloride method (Zhu et al., 1993). Approximately 0.05 g of algal pellets were suspended in 500 $\mu\ell$ of extraction buffer (100 mM Tris-HCl, pH 8.0, 40 mM EDTA), 150 $\mu\ell$ of 10 % (w/v) sodium dodecyl sulphate (SDS) and 300 $\mu\ell$ of benzyl chloride, and incubated at 55℃ for 30 min. Phenol: chloroform: isoamylalchol (25:24:1) was treated twice and RNase (1 mg/mℓ) was added. The algal cells were pelleted and the supernatant mixed with 40 $\mu\ell$ of

Table 1. Isolates of harmful algal species used in this study

Species	Clone	Geographic origin
*G. impudicum**	GI-1	Chungmu, Korea
	GI-2	Chungmu, Korea
	GI-4	Chungmu, Korea
	GI-5	Chungmu, Korea
	GI-6	Chungmu, Korea
	GI-8	Chungmu, Korea
	GI-10	Chungmu, Korea
	GI-cyst	Yousu, Korea
*G. sanguineum**	GS-1	Masan, Korea
	GS-2	Masan, Korea
G. catenatum[a]	PR01	Espinho, Northwest coast, Portugal
	HU02	Huon Estuary, Tasmania, Australia
	HU07	Huon Estuary, Tasmania, Australia
	DE06	Derwent Estuary, Tasmania
A. tamarense[b]	OFX191-A	Ofunato Bay, Japan
	AT4-A	Ofunato Bay, Japan
	304A-B	Mikawa Bay, Japan
	OFX191-B	Ofunato Bay, Japan
	AT4-B	Ofunato Bay, Japan
	304A-B	Mikawa Bay, Japan
A. catenella[b]	TNX22	Tanabe Bay, Japan
	M17	Harima Nada, Japan

*the sequences of *G. impudicum* and *G. sanguineum* have been deposited to the European Molecular Biology Laboratory (EMBL) data library
[a] Data from Adachi et al. (1997).
[b] Data from Adachi et al. (1996).

3 M sodium acetate and incubated on ice for at least 1 h. The DNA was precipitated from the tube at room temperature for 10 min by adding 2.5 volumes of 100% ice-cold ethanol. The pellet was washed with 2 volumes of 70% ethanol and resuspended in distilled water. The DNA was kept at −20℃.

PCR amplification and DNA sequencing

The nuclear rDNA region spanning the ITS1, ITS 2 and 5.8S rRNA gene was amplified by PCR from each strains as described in Table 1. Primers ITS A (5'-CCAAGCTTCTAGATCGTAACAAGGTCCG-TAGGT-3') and ITS B (5'-CCTGCAGTCGACAA-TGCTTAATTCAGCGG-3') were derived from the conserved regions of 18S and 28S rDNA, respectively (Fig. 1). PCR was carried out with Perkin-Elmer model 480 thermocycler using the following program: initial denaturation for 3 min at 95℃, 30 cycles of amplification (denaturation for 30 sec at 95℃, annealing for 30 sec at 50℃, and extention for 1 min at 72℃) and final extention of 5 min at 72℃. The PCR product from the amplification was subjected to preparative electrophoresis in a 1.6% agarose gel in TBE buffer. All PCR products yield only a single visible band. The PCR product was excised from the ethidium bromide stained gel and purified using a QIAGEN gel elution kit (Qiagen, Wartworth CA). Direct sequencing of PCR products was done in an Perkin-Elmer Applied Biosystems ABI 377A sequencer using a PRISM Dye Dideoxy Terminator Cycle Sequencing kit (Perkin Elmer) following the manufacture's protocol. Two primers, ITS A and ITS B, were used for sequencing in both directions. DNA sequences were edited and assembled with the program CLONE MANAGER version 4.0 (Scientific & Educational Software, Stateline, PA).

Phylogenetic analysis

The analyzed nuclear rDNA sequences were submitted to the European Molecular Biology Laboratory (EMBL) data library. They were aligned with the sequences of the related strains such as *G. catenatum* PR01, HU02, HU07, DE06 (Adachi et al., 1997), *A. catenella* M17, TNX22 and *A. tamarense* OFX191-A, AT4-A, 304A-A, OFX191-B, AT4-B, 302 A-B (Adachi et al., 1996).

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). The determined sequences were as follow: *G. impudicum*, AF 131074 : *G. sanginum*, AF 131075. The species having same sequences was registrated as only one species. Phylogenetic analyses were inferred by neighbor-joining method (Miller and Scholin 1996). The strength of the internal branches from the resulting trees were statistically tested by bootstrap analysis from 1,000 bootstrap replications (Felsenstein 1985).

Results

Sequence alignments

Electrophoresis and direct sequencing of each PCR reaction product confirmed that a single

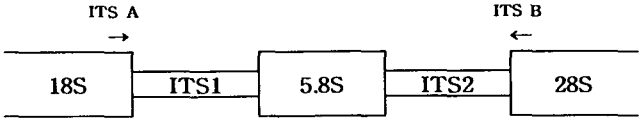


Fig. 1. A map of the ribosomal DNA region containing ITS1, ITS2 and the 5.8S rDNA gene (referenced from Adachi et al., 1994). Arrows indicate the positions of the primers used for PCR and sequence analysis.

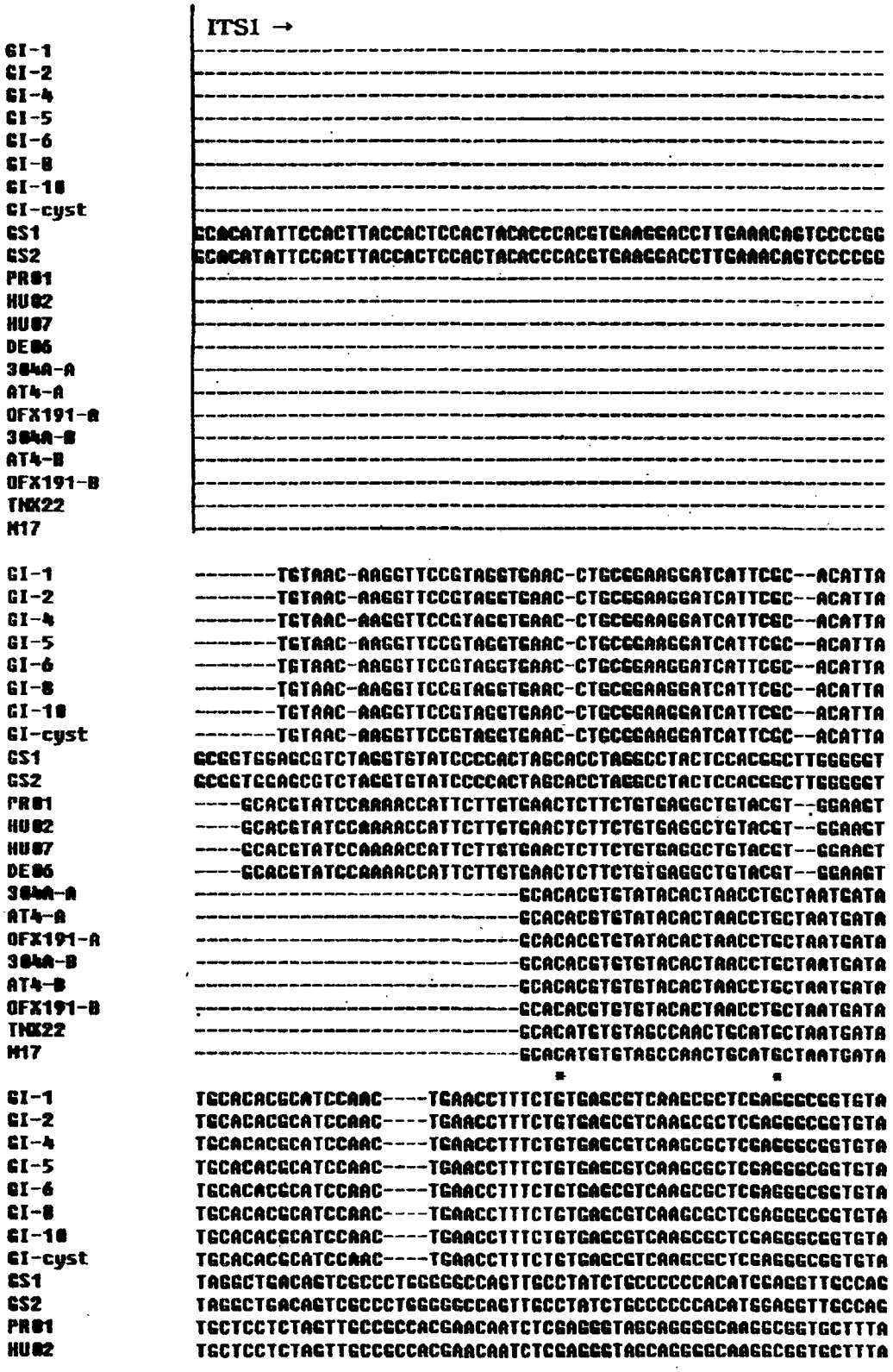
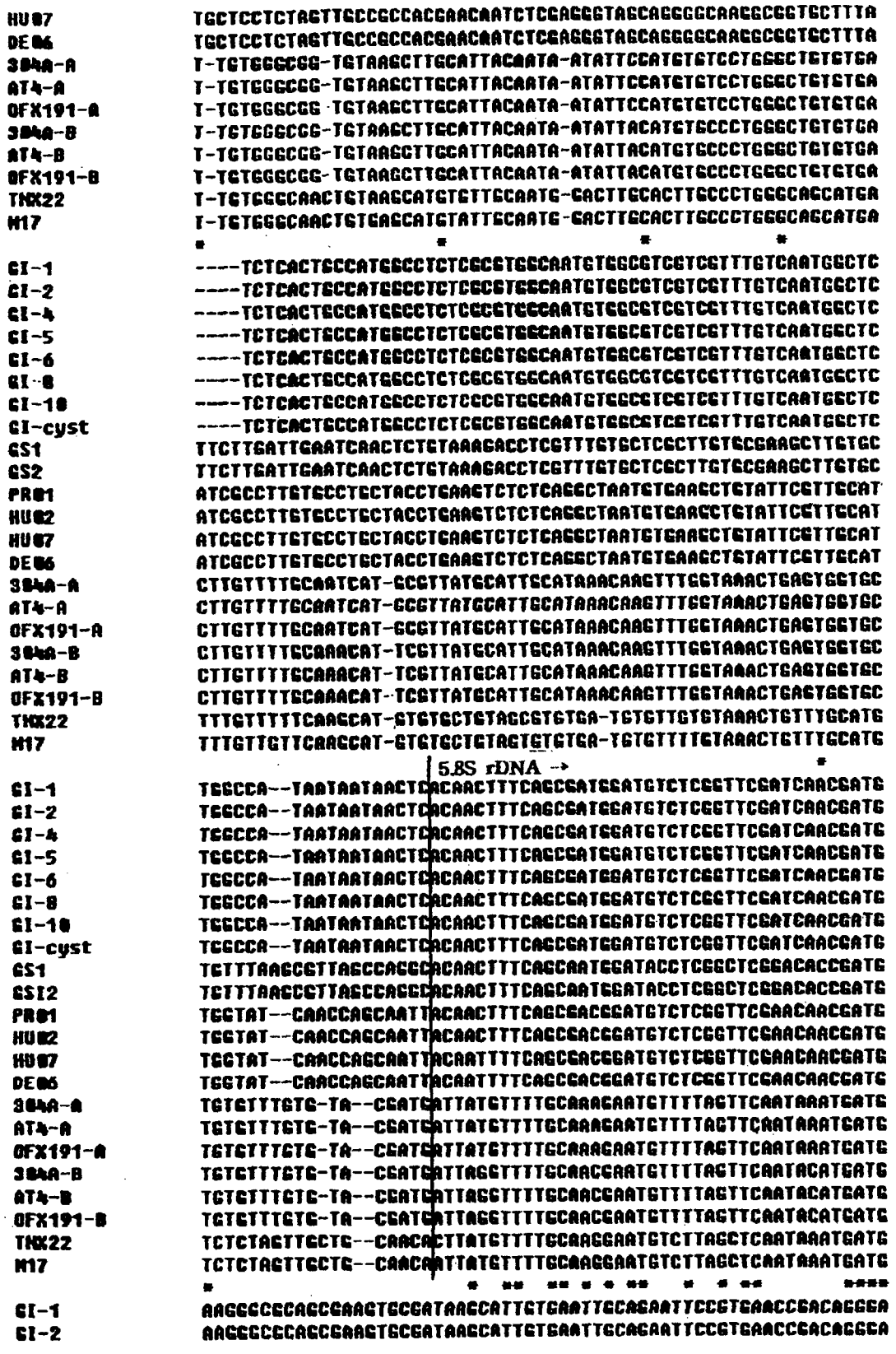


Fig. 2 Continued



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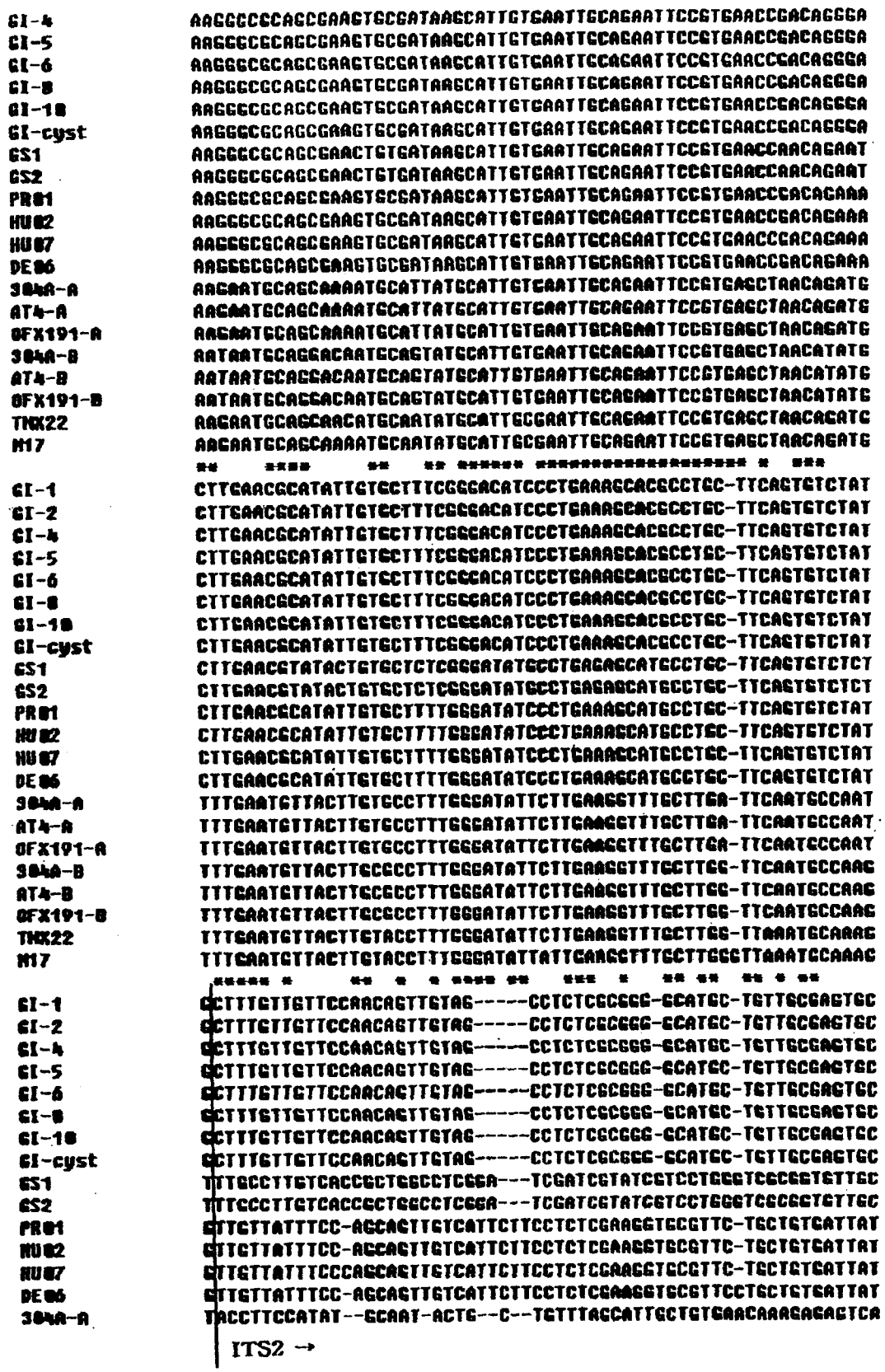

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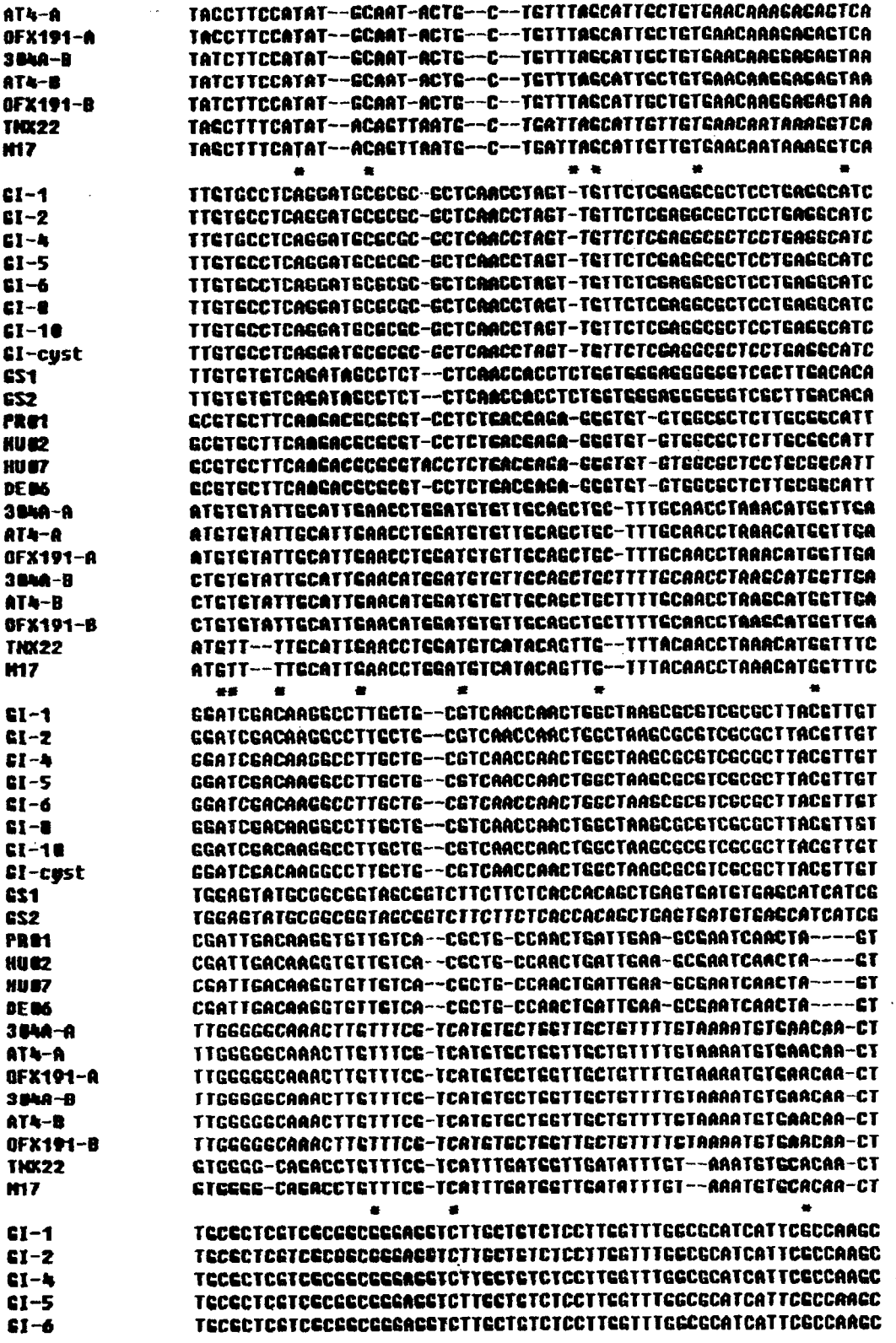

Fig. 2 Continued

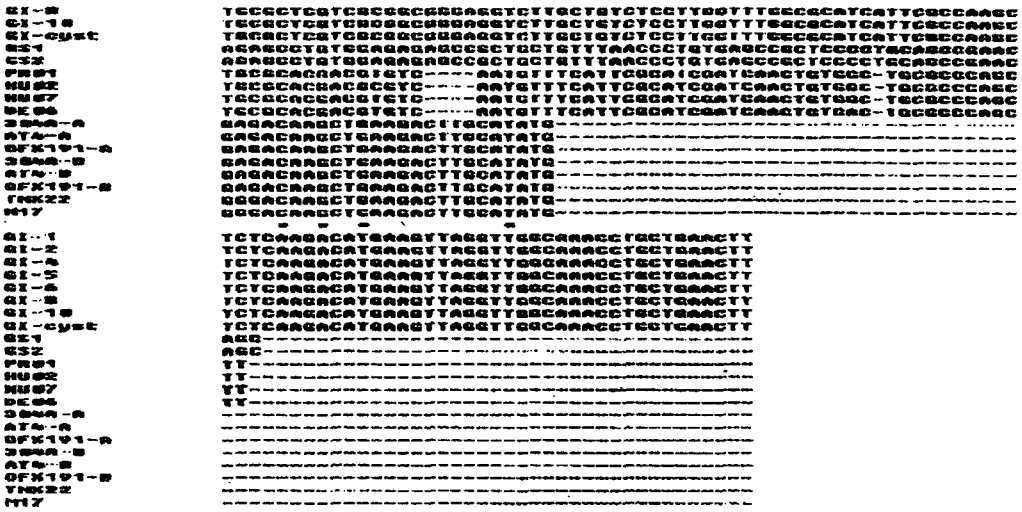

Fig. 2. The alignment of the sequences of the 5.8S rDNA with the flanking internal transcribed spacers ITS1 and ITS2. The alignment was generated by the multiple alignment program CLUSTAL W using a gap weight of 3.0 and a gap length weight of 0.1. ITS1 spans from 1 to 240 bp; the 5.8S coding region is from 261 to 421 bp; and ITS2 is 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 source of each sequence are as follows: GI for G, impudicum isolates GI-1, 2, 4, 5, 6, 8, 10, cyst in this study (AF131074); GS for *G. sanguineum* isolate GS-1, 2 in this study (AF131075); PR01, HU02, HU07, DE06 for *G. catenatum* isolate (Adahi et al., 1997); 304A-A, AT4-A, OFX191-A, 304A-B, AT4-A, OFX191-B for *A. tamarense* (Adachi et al., 1994); TNX22, M17 for *A. catenella*.

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 is shown in Fig. 2. There is considerable sequence variation in the ITS sequences and little in the regions of the 5.8S rDNA. The internal transcribed spacers contain most of the sequence variation. Especially, the ITS1 is more variable than ITS2 compared with all species used in this study. The length of ITS1 varied more than that of ITS2 and each *G. impudicum, G. sanguineum, Gymnodinium* and *Alexandrium* species had different size in total length (Table 2). The size of ITS1 ranged from 165 to 260 bp and the nucleotide sequences were aligned in 261 positions, of which 255 bases (98%) were variable and 7 (2.0 %) were conserved regions. *Gymnodinium* and *Gyrodinium* species have longer sequences in total length than *Alexandrium* species. The total length of the PCR product of *Alexandrium* is 70 to 150 bp shorter than the *Gymodinium* species. The ITS1 of *G. sanguineum* appeared to have about 60 bp insertion compared with the other *Gymnodinium, Gyrodinium* and *Alexandrium* species. The ITS2 ranged from 180 to 268 bp and the sequences were aligned in 279 positions, of which 261 bases (93.5 %) variable and 18 (6.5%) were phylogenetically conserved regions. The conserved regions were very

Table 2. Nucleotide length of the internal transcribed spacers1, 2 and 5.8S rDNA

		Length (bp)			
		ITS1	5.8S	ITS2	Total
G. impudicum[a)]		179	160	268	607
G. sanguineum[b)]		260	160	237	657
G. catenatum[c)]	PR01	192	160	225	577
	HU02	192	160	225	577
	HU07	192	160	227	579
	DE06	192	160	226	578
A. tamarense[d)]	304A-A	165	160	184	509
	AT4-A	165	160	184	509
	OFX191-A	165	160	184	509
	304A-B	165	160	184	509
	AT4-B	165	160	185	510
	OFX191-B	165	160	185	510
A. catenella[d)]	TNX22	166	160	180	506
	M17	166	161	180	507

[a,b)] It appeared only one strain because their sequences were same.
[c)] Data from Adachi et al. (1997).
[d)] Data from Adachi et al. (1996).

small, because the ITS regions were very variable in intra-species. Therefore, many variable regions were on the ITS regions, because the sequences of *G. impudicum*, the genus *Gymnodinium* and the genus *Alexandrium* were compared one another. On the other hand, 5.8S rDNA had generally constant size in the 160 bp exception of *A. catenella* M17. *A. catenella* M17 had one insertion on 5.8S rDNA. The DNA homology appeared very low on the ITS1 and ITS2, while the DNA homology was very high on 5.8S rDNA. *G. impudicum* and *G. catenatum* showed 59% homology, which was higher than that of *G. catenatum* and *G. sanguineum* (55%). All the sequence from vegetative cell and cyst are idential in all the isolates of *G. impudicum*.

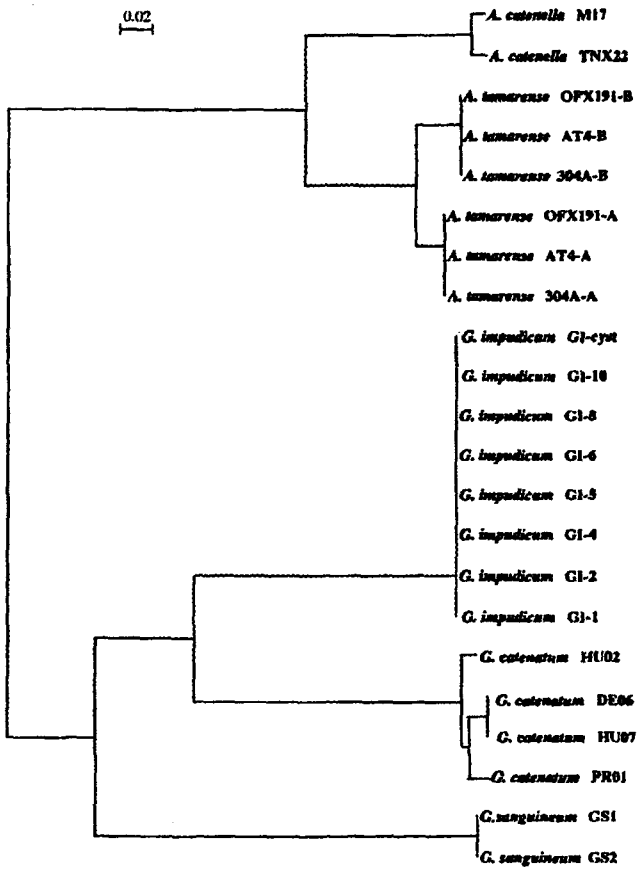

Fig. 3. Phylogenetic tree of *G. impudicum*, *Gymnodinium* species and *Alexandrium* species inferred from the nucleotide sequences of the internal transcribed spacers ITS1, ITS2 and 5.8S rDNA. This tree were obtained by the neighbor-joining methods and the alignment of the sequences showed in Fig. 2.

Phylogenetic analyses of the ITS1, 5.8S rDNA and ITS2 aligned sequences

One phylogenetic tree requiring 658 changes was found in the original data set composed of the 5.8S rDNA and both internal transcribed spacer sequences (Fig. 3). The census tree generated by neighbor-joining methods of 1,000 bootstrap replications of this data set had an identical topology to that of the most phylogenetic tree and indicated that the majority of the branches were favoured at high level of confidence. All of the *G. impudicum* and *Gymnodinium* species formed a cluster with a low ratio (about 60%). Especially, *G. impudicum*, formed a near cluster with *G. catenatum*, known as the genus *Gymnodinium*. In other words, phylogenetic relationship of *G. impudicum* and *G. catenatum* was closer than the same genus of *G. sanguineum*. *Alexandrium* species formed another cluster which located in much distance. The position of *G. impudicum* was confirmed as being the basal to the *Gymnodinium* cluster. *G. impudicum* occurred outside of *Gymnodinium* in the 1,000 trees used to generate the bootstrap consensus tree. *G. impudicum* is more closely related to *G. catenatum* than to *G. sanguineum*. The genus *Alexandrium* is completely a different cluster, compared with *G. impudicum* and *Gymnodinium* species.

Genetic distances

A matrix of Kimura genetic distances (Kimura 1980) between pairs of samples calculated from the entire 658 bp of the 5.8S rDNA, ITS1 and ITS2 sequences were given in Table 3. The Jin & Nei model (1990), which uses the gamma distribution to estimate the change in the rate of substitution at different sites, gave very similar genetic distances and these values were also very similar to those obtained from the Kimura model (1980). The genetic distances between *G. impudicumis* and *G. catenatum* species were quite closer than those of *G. catenatum* and *G. sanguineum*. Also, the genetic distances between *Alexandrium* and *Gymnodinium* species is very distant.

Discussion

Sequence analyses of the 5.8S rDNA and ITS regions of 8 isolates of *G. impudicum* from Chungmu and 2 isolates of *G. sanguineum* from

Table 3. Genetic distances between pairs of algal species calculated by the Kimura two-parameter model from the combined sequence data for 5.8S rDNA, ITS1 and ITS2

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1														
2	0.404													
3	0.295	0.424												
4	0.293	0.423	0.002											
5	0.294	0.425	0.003	0.005										
6	0.297	0.429	0.003	0.005	0.003									
7	0.477	0.535	0.506	0.506	0.508	0.505								
8	0.477	0.535	0.506	0.506	0.508	0.505	0.000							
9	0.477	0.535	0.506	0.506	0.508	0.505	0.000	0.000						
10	0.493	0.551	0.516	0.516	0.520	0.517	0.042	0.042	0.042					
11	0.493	0.551	0.516	0.516	0.520	0.517	0.042	0.042	0.042	0.000				
12	0.493	0.551	0.516	0.516	0.520	0.517	0.042	0.042	0.042	0.000	0.000			
13	0.486	0.558	0.515	0.515	0.517	0.514	0.191	0.191	0.191	0.211	0.211	0.211		
14	0.486	0.554	0.517	0.517	0.517	0.516	0.188	0.188	0.188	0.207	0.207	0.207	0.016	

(658 bp)

1, *G. impudicum*; 2, *G. sanguineum*; 3, *G. catenatum* PR01; 4. *G. catenatum* HU02; 5, *G. catenatum* HU07; 6, *G. catenatum* DE06; 7, *A. tamarense* 304A-A; 8, *A. tamarense* AT4-A; 9, *A. tamarense* OFX191-A; 10, *A. tamarense* 304A-B; 11, *A. tamarense* AT4-B; 12, *A. tamarense* OFX191-B; 13, *A. catenella* TNX22; 14, *A. catenella* M17

Masan in Korea clarified that the length and sequence of these isolates within the species was very constant. The length variations of the ITS regions have been found 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). The length of ITS2 in *G. impudicum* is longer than that of ITS1 by 89 bp (Table 2). However, the length of ITS1 was longer in *G. sanguineum* by 23 bp than that of ITS2. Especially, *G. sanguineum* in the length of ITS1 has longer by 95 bp than *A. tamarense* which had the shortest nucleotide length. *G. impudicum* reached 268 nucleotide in the ITS2. While all strains used in this study reached 160 nuceotides in 5.8S rDNA except of *A. catenella* M17 which had 161 bp. Namely, the length of ITS regions plays important role in algal taxonomy.

Also, taxonomists consider currently *G. impudicum* and *G. catenatum* to be in separate *Gymnodiniaceae* family, the genus *Gyrodinium* and the *Gymnodinium*, respectly, with main differential criterion being in the size and the position of girdle. The girdle in the *Gymnodinium* species is located in the equatorial region of the cell, which is little and less than one fifth of the cell length, or no displacement. Species with a girdle displacement of one fifth of the cell length or larger are referred to *Gyrodinium* (Kofoid and Sewzy 1921). Also, no PSP was found in *G. impudicum* which has caused blooms in several areas but no associated harmful effects have been reported, while *G. catenatum* is a toxin chain-forming dinoflagellate that causes shellfish poisoning at its type locality (Fraga et al., 1995). However, generic assignment is often arbitrary, as several species of *Gymnodinium* have a girdle displacement of about one fifth of the length. But, the phylogenetic trees based on rDNA sequences support clearly the molecular differences and the taxonomic view in these two species (Fig. 3). All of the Gymnodiniaceae family examined, *G. impudicum, G. catenatum, G. sanguineum*, form the poly-phyletic group that is further subdivided in three, and the genus *Alexandrium* form a different cluster completely. Surprisingly, *Gyrodinium impudicum* isolated from Chungmu is more closely related to the *G. catenatum* species than *G. sanguineum* (Fig. 3.). *Gyrodinium impudicum* and *G. catenatum*, which have little morphological differences, form a near cluster of closely related organisms with small genetic distance from *G. sanguineum* (Table 4), although the genus is different each other. The nucleotide sequences of the 5.8S rDNA were highly conserved among the inter-species in this study, but those of the two ITS regions were variable. In general, the coding regions of the rDNA, such as the 5.8S DNA, are highly conserved and suited for phylogenetic studies

among distantly related taxa. On the other hand, the non-coding regions, such as the ITS regions or the intergenic region (IGR) are more variable and suited for phylogenetic studies among closely related taxa.

The present results show that this concept also applies to the algae. The three genera sequences extracted from the paper of Adachi et al. (1994, 1996) revealed a distantly related tree to one another on the basis of the morphology. *Gyrodinium impucium* and *G. catenatum* are different in molecular phylogeny, although these are similar in morphology. The nucleotide identities of ITS regions was relatively low among the three genera, and the sequences were ambiguously aligned. When they are compared, the identities are low, because the species have much differences in morphology and molecular phylogeny. On the other hand, the alignment of the 5.8S rDNA showed that only 44 of 161 site were variable including one gap. This result suggests that the 5.8S region is suitable for phylogenetic studies of distantly related algae.

In conclusion, we have distinguished between *G. impudicum* and *G. catenatum* by amplifying rDNA with sequence analysis. It is important that *G. impudicum* belonged to the other genus, is closely related to *G. catenatum*. In another words, these two species may belong to the same genus. Further, improvement may be required to evaluate more other conserved regions to confirm their relationship based on genetic similarity.

Acknowledgments

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