Korean Journal of Life Science
Vol. 12. No. 6. 821~834, 2002

Phylogenetic position of five Korean strains of *Alexandrium tamarense* (Dinophyceae), based on internal transcribed spacers ITS1 and ITS2 including nuclear-encoded 5.8S rRNA gene sequences

Eun Seob Cho*[1], Sam Geun Lee and Iksoo Kim[2]

[1]*Harmful Algal Research Division, National Fisheries Research and Development Institute, Busan 619-900, Korea*
[2]*Department of Sericulture and Entomology, National Institute of Agricultural Science and Technology, RDA, Suwon 441-100, Korea*

Abstract

In order to measure the inter- and intraspecific genetic divergences within the genus *Alexandrium*, the variations within the internal transcribed spacer (ITS1 and ITS2) regions and 5.8S ribosomal RNA gene of eight *Alexandrium* species were examined for 33 strains from diverse geographical locations by direct sequencing. Five isolates of *A. tamarense* (AT-2, AT-6, AT-10, AT-A and AT-B) from Jinhae Bay, Korea were found to be completely identical to a Japanese strain OFX151-A. The length of the amplified ITS1-5.8S-ITS2 region varied from 481 nucleotides (in *A. margalefi*) to 528 nucleotides (in *A. affine* CU1-1). ITS1 and ITS2 nucleotide lengths were negatively correlated, whereas a positive correlation was found between their G+C content. The degree of sequence divergence ranged from 0.3% (1 bp) to a maximum of 53% (305 bp). Pairwise sequence comparisons revealed a small degree of divergence between *A. tamarense* and *A. fundyense* isolates (1.2 - 2.3% = 6-12 bp), but a high degree of divergence between *A. tamarense* and *A. catenella* (19.8% = 102 bp), and between *A. catenella* and *A. fundyense* (19.7%). Although most nodes were weakly supported by bootstrap values, some types tend to form independent molecular groups. *A. catenella* isolates also formed an independent molecular sub-group, with relatively strong bootstrap values (94% or 85% and 79% or 98%, respectively in PAUP and NJ trees). Interestingly, *A. cohorticula* and *A. frateculus* always clustered within the same sub-group, this result being supported by strong bootstrap values. Our results indicate that the ITS regions provide useful informations on hierarchical population genetic structure and a high phylogenetic resolution in intraspecific and interspecific *Alexandrium* population.

Key words – *Alexandrium tamarense*, ITS, molecular analysis, phylogeny, PSP, taxonomy

Introduction

The genus *Alexandrium* is distributed widely in cold temperate waters and is consisted of as many as 30 species to date. This genus is linked to paralytic shellfish poisoning (PSP) events throughout the world [18,30]. In Korea, *A. tamarense* also produces toxins responsible for PSP in coastal waters. PSP contaminated mussels and oysters has a serious impact on shellfish farmers and the trade of marine products [10]. As *Alexandrium* species are primarily identified on the basis of fine-scale morphological features [15], their taxonomy still presents considerable difficulties owing to their high variability and polymorphism at different sampling sites, and even in cultures [9,25,26]. Moreover, criteria used to classify these

*To whom all correspondence should be addressed
Tel : 34-91-394-3769, Fax : 34-91-394-3769
E-mail : eseob@vet.ucm.es

species differ by taxonomists[7,15,31,34]. Currently molecular methods have been extensively employed in phylogenetic and taxonomic studies of the genus *Alexandrium*, using a variety of molecular techniques based on genomic variation, such as restriction fragment length polymorphism (RFLP) analysis 1 and DNA sequence analysis of various ribosomal regions [7,28,29].

Ribosomal RNA genes are tandemly repeated multigene familes containing both genic and nongenic, or spacer regions. Each repeat unit contains a copy of the 18S, 5.8S, and 28S rDNA and an intergenic spacer (IGS). The 5.8S rDNA gene is typically flanked by a bipartite internal transcribed spacers (ITS), the ITS1 and ITS2, which separates the 5.8S rDNA from the 18S and 28S genes, respectively. The nuclear rRNA genes, and the more rapidly evolving internal spacer regions, are attractive candidates for genetic markers at higher and lower taxonomic levels[19]. The ITS regions are generally considered to be under low functional constraint, and are treated as typical non-functional spacer sequences[16]. Adachi *et al.* suggested that the ITS regions from *Alexandrium* were contributed to provide reliable information concerning intra- and inter-specific variation and phylogeny[1-3]. We previously have attempted to determine the extent of genetic variations among various harmful dinoflagellates of the Korean coastal waters using molecular phylogenetic analysis of the ITS regions [11-13, 22]. We also showed the important role played by the ITS region sequences as a taxonomic tool for determining the phylogenetic relationship between morphologically similar *Gyrodinium impudicum* and *Gymnodinium catenatum*[22].

A recent study showed that the toxin content of five strains of *A. tamarense* showed a significant difference among five strains isolated from the same seawater sample[10]. Unfortunately, DNA sequence data are not available for any isolate until now. The purpose of this study is to assess the phylogenetic relationships among different isolates of the genus *Alexandrium*, with a special focus on the generic concept through the comparison of various geographic samples of *A. tamarense* obtained from Korea, Japan and United States. We also attempted to assess the usefulness of the ITS regions as a genetic marker for *Alexandrium* species by sequencing five isolates of *A. tamarense* collected from Korea and compared our molecular data with GenBank-registered sequences of several species of *Alexandrium* (including various geographic samples of *A. tamarense*) originating from several areas of the world.

Materials and Methods

Cultures

Five *A. tamarense* isolates (AT-2, AT-6, AT-10, AT-A and AT-B) were isolated from red tide waters in the Jinhae Bay, Korea, on February, 1997. All isolates were grown in f/2-Si[17] and were incubated in the Harmful Algal Culture Room, National Fisheries Research and Development Institute at 20℃ and 14:10 h LD (light: dark) photoperiod.

Isolation of genomic DNA

Cultures were harvested during the exponential phase by centrifugation. Pellets were immediately preserved at -20℃ until ready to use. Approximately 0.05g of algal pellets were suspended in 500 L of extraction buffer (100 mM Tris-HCl, pH 8.0, 40 mM EDTA), and 150 L of 10% (w/v) sodium dodecyl sulphate (SDS), and incubated at 55℃ for 30 min. The supernatant was extracted twice with phenol:chloroform:isoamylalchol (25:24:1, v/v/v) prior to ethanol precipitation.

Amplification and sequencing

The ITS1, ITS2 and 5.8S rDNA regions of *Alexandrium* species were amplified by polymerase chain reaction (PCR) using primers[1] were derived from the conserved regions of small sub-unit (SSU) and large sub-unit (LSU) rDNA, respectively[2]. PCR amplifications were carried out with Perkin-Elmer 2400 Thermocycler. PCR reactions

ypically contained a 50 L mixture: 1.25 unit *Taq* DNA polymerase (*Ex Taq*, TaKaRa Co. JAPAN); 1 *Ex Taq* buffer (TaKaRa Co. JAPAN); 0.2 mM dNTP; 20~100 ng otal genomic DNA; and 100 pmol of each primer. The hermocycling profile included an initial denaturation tep of 95℃ for 30 sec, followed by 35 cycles of 30 sec t 95℃, primer annealing for 1 min at 50~53℃, and xtenstion for 5 min at 72℃. Amplification products were separated electrophoretically on ethidium bromide-tained 1.5% agarose 1 TBE gels to check the yield, urity and length of the amplified products. Following a urification step with QIAGEN gel elution kit (Qiagen, Wartworth CA), all PCR products were sequenced directly on the Perkin-Elmer Applied Biosystems (ABI) 377A DNA sequencer using a ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer), according to the manufacture's protocol.

Moecular data analysis

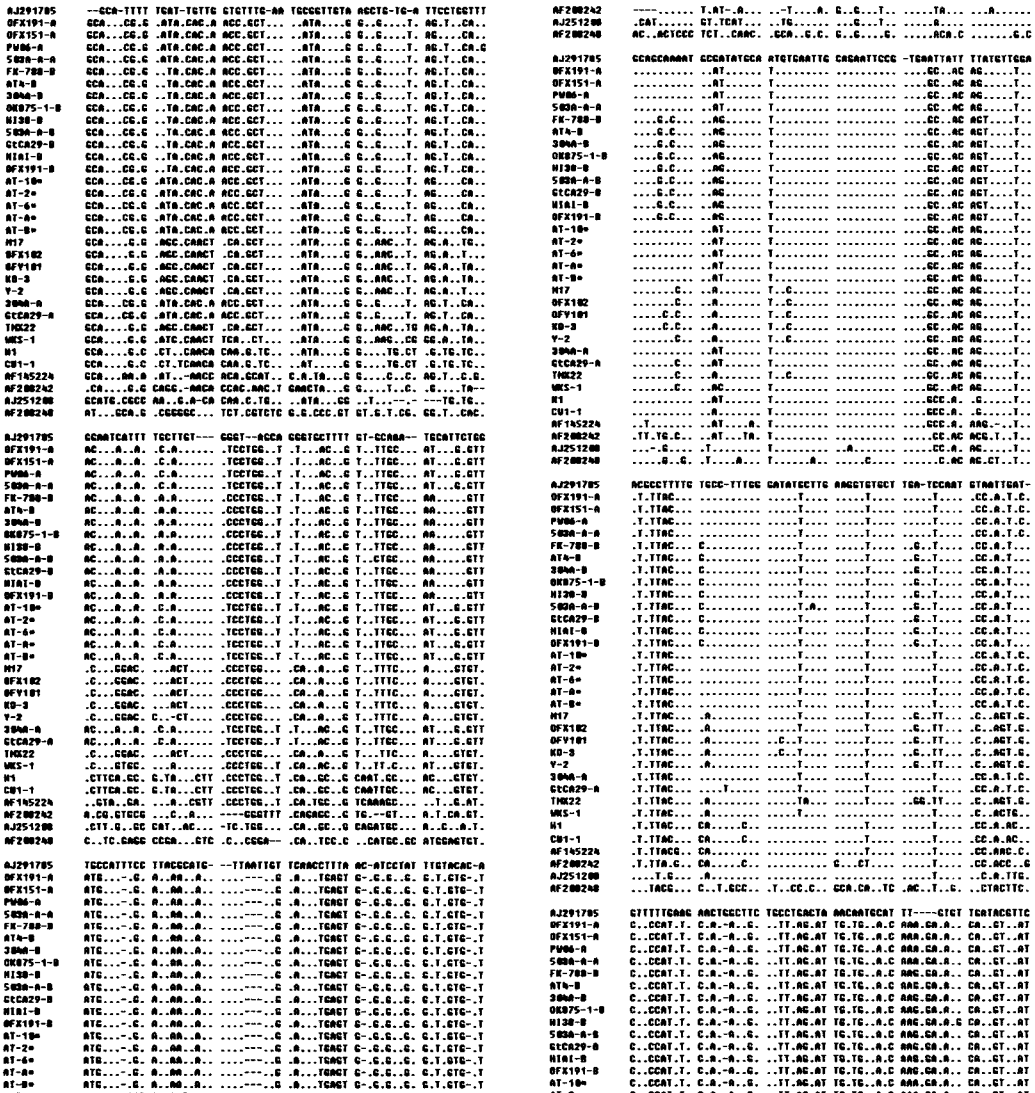
The determined ribosomal DNA sequences have been deposited in the European Molecular Biology Laboratory (EMBL) data library (Heidelberg, FRG) and accession numbers were indicated as follows: AT-A (AF374224), AT-B (AF374225), AT-10 (AF374226), AT-2 (AF374227) and AT-6 (AF374228). Sequence data were aligned using IBI MacVector version 4.1.1 and phylogenetic analyses were performed using both distance and parsimony methods. PAUP (version 3.1)[32] was used to infer relationships among Korean isolates of *A. tamarense* from this study and related strains such as *A. tamarense*, *A. catenella*, *A. affine*, *A. fundyense*[2], *A. frateculus* (GenBank accession number AF208242), *A. cohorticular* (GenBank accession number AF145224), *A. taylori* (Gen Bank accession numbers AJ201785, AJ251653, AJ251654 and AJ300451), *A. margalefi* (GenBank accession number AJ251208). The multiple alignment was subjected to parsimony analysis conducted with PAUP (version 3.1) [32]. Deletion/insertion was weighted equally to transition and transversion, and heuristic searches were performed. As an alternative to the parsimony analysis, the aligned data set was also subjected to Neighbor-Joining (NJ) and Maximum Likehood (ML) methods of PHYLIP (Phylogeny Inference Package, version 3.5c)[14]. The NJ algorithm was applied to a distance matrix obtained using the Kimura two-parameter correction[23]. As an indication of confidence in the branching order, a bootstrap analysis (100 replications) was completed for both distance and parsimony methods. The sequence from the dinoflagellate *Cochlodinium polykrikoides* (GenBank accession number AF208243) was used as an outgroup.

Results

ITS DNA sequences

Electrophoresis of the PCR reactions conducted on the five *A. tamarense* isolates collected from Jinhae Bay (AT-10, AT-2, AT-6, AT-A and AT-B) showed that a single fragment was amplified in each reaction. Direct sequencing of these products confirmed their ribosomal nature and revealed a complete similarity with the sequence of strain *A. tamarense* OFX151-A collected from Ofunato Bay (Japan). Thus, OFX151-A was regarded as the representative of these five clones in the rest of this study.

The multiple sequence alignment of 28 isolates distributed among the species *A. tamarense*, *A. fundyense*, *A. affine*, *A. catenella*, *A. taylori*, *A. fraterculus*, *A. cohorticula*, and *A. margalefi* was presented in Fig. 1 and included the sequence of *C. polykrikoides* as an outgroup. It reveals considerable nucleotide variation in the ITS1 and ITS2 regions, but little variation in the 5.8S rDNA region. The sizes of the ITS1, 5.8S rDNA and ITS2 portions of 42 different clones of *Alexandrium*, including those examined in Fig. 1, were also are compared (Table 1). These data show that the size of the ITS + 5.8S rDNA region vary from 481 bp (in *A. margalefi*) to 528 bp (in *A. affine*). Strains of *A. tamarense*, *A. fundyense* and *A. frateculus*, however, appeared very similar in size (519 bp 520 bp). Table 1 also indicated that these length variations



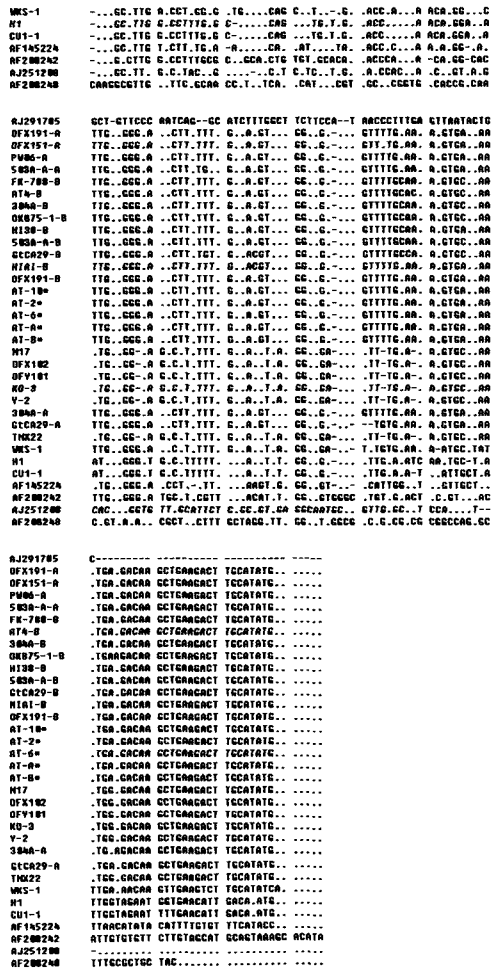


Fig. 1. Multiple alignment of 5.8S rDNA region and flanking internal transcribed spacers ITS1 and ITS2 sequences of 28 isolates of *Alexandrium*.
A hyphen and a period correspond to a gap and a base identical to that of the top sequence, respectively. ITS1 length spans from 1 to 177 bp; the 5.8S coding region varied from 178 to 339 bp; whereas ITS2 is from 340 to 600 bp. Korean isolates of *A. tamarense* (AT-A, AT-B, AT-2, AT-6 and AT-10) have the same sequence of the Japanese *A. tamarense* (OFX151-A). AJ291785 is identical sequence of AJ300451, AJ251654 and AJ251653 (Genbank accession number). An isolate of *A. tamarense* (OFX151-A) is identical sequence of AT-4-A, OK875-1-A, FK-788-A, HIAI-A and HI38-A. M17 is identical sequence of TNY11. The source of each sequence are as follows: OFX191-A, OFX151-A, PW06-A, 503A-A-A, FK-788-B, AT4-B, 304A-B, OK875-1-B, HI38-B, 503A-A-B, HIAI-B, OFX191-B, 304A-A, WKS-1 for A. tamarense 2; GtCA29-B, GtCA29-A for *A. fundyense* 2; M17, OFX102, OFY101, KO-3, TNX22 for *A. catenella* 2; HI, CU1-1 for *A. affine* 2; AH291785 for *A. taylori;* AF145224 for *A. cohorticular;* AF208242 for *A. frateculus;* AJ251208 for *A. margalefi;* AF20824 for *C. polykrikoides* as an outgroup.

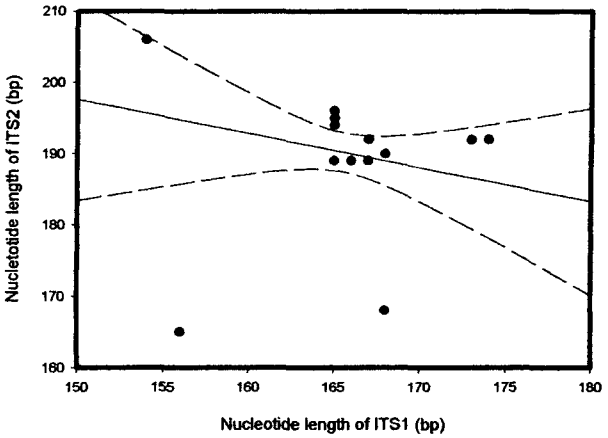


Fig. 2. The relationship between ITS1 and ITS2 nucleotide length. Long dash lines represent 95% confidence intervals.

primarily concern spacers ITS1 and ITS2, rather than the 5.8S rDNA portion. For example, the size of ITS1 fragment ranged from 165 bp (in *A. tamarense* OK875-1-A) to 174 bp (in *A. affine* CU1-1), whereas the length of the corresponding 5.8S rDNA fragments only varied from 160 bp to 162 bp. Moreover, the relationship between the nucleotide length of ITS1 and that of ITS2 was negative, as shown in Fig. 2, whereas the relationship between their G+C content was found to be positive (Fig. 3).

The result of the pairwise sequence comparison of the 28 isolates of *Alexandrium* examined in this study is

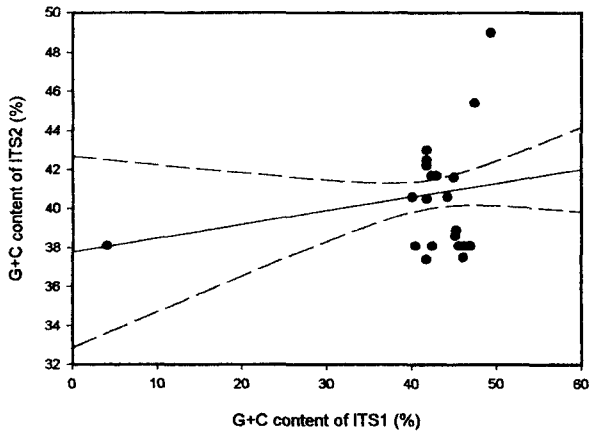


Fig. 3. The relationship between ITS1 and ITS2 G+C content. Long dash lines represent 95% confidence intervals.

Table 1. Nucleotide length of ITS1, ITS2 and 5.8S rDNA sequences

Species (Locality)	ITS1	5.8S rDNA	ITS2	Total
A. tamarense OK875-1-A (Okirai Bay, Japan)[2]	165	160	194	519
A. tamarense FK-788-A (Funka Bay, Japan)[2]	165	160	194	519
A. tamarense OFX191-A (Ofunato Bay, Japan)[2]	165	160	194	519
A. tamarense 304A-A (Mikawa Bay, Japan)[2]	165	160	194	519
A. tamarense AT4-A (Harima Nada, Japan)[2]	165	160	194	519
A. tamarense HIAI-A (Hiroshima Bay, Japan)[2]	165	160	194	519
A. tamarense HI38-A (Hiroshima Bay, Japan)[2]	165	160	194	519
A. fundyense GtCA29-A (Cape Ann, USA)[2]	165	160	194	519
A. tamarense OFX151-A (Ofunato Bay, Japan)[2]	165	160	194	519
A. tamarense PW06-A (Port Benny, USA)[2]	165	160	194	519
A. tamarense 503A-A-A (Mikawa Bay, Japan)[2]	165	160	194	519
A. taylori AJ291785 (Vulcano, Italy)	168	160	168	496
A. taylori AJ300451 (Vulcano, Italy)	168	160	168	496
A. margalefi AJ251208 (Sicily, Italy)	156	160	165	481
A. taylori AJ251654 (Spain)	168	160	168	496
A. taylori AJ251653 (Sicily, Italy)	168	160	168	496
A. cohorticular AF145224 (Malaysia)	168	159	190	517
A. tamarense FK-788-B (Funka Bay, Japan)[2]	165	160	196	521
A. tamarense OK8751-B (Okirai Bay, Japan)[2]	165	160	195	520
A. tamarense AT4-B (Harima Nada, Jpan)[2]	165	160	195	520
A. fundyense GtCA29-B (Cape Ann, USA)[2]	165	160	195	520
A. tamarense HIAI-B (Hiroshima Bay, Japan)[2]	165	160	195	520
A. tamarense OFX191-B (Ofunato Bay, Japan)[2]	165	160	195	520
A. tamarense HI38-B (Hiroshima Bay, Japan)[2]	165	160	195	520
A. tamarense 304A-B (Mikawa Bay, Japan)[2]	165	160	195	520
A. tamarense 503A-A-B (Mikawa Bay, Japan)[2]	165	160	195	514
A. catenella M17 (Harima Nada, Japan)[2]	167	160	189	516
A. catenella TNY11 (Tanabe Bay, Japan)[2]	167	160	189	516
A. catenella OFX102 (Ofunato Bay, Japan)[2]	167	160	189	517
A. catenella Y-2 (Yamakawa, Japan)[2]	166	161	189	516
A. catenella OFY101 (Ofunato Bay, Japan)[2]	167	161	189	517
A. catenella KO-3 (Uranouchi Inlet, Japan)[2]	167	161	189	517
A. catenella TNX22 (Tanabe Bay, Japan)[2]	166	162	189	517
A. tamarense WKS-1 (Kushimoto, Japan)[2]	167	161	192	520
A. affine H1 (Harima Nada, Japan)[2]	173	162	192	527
A. affine CU1-1 (Gulf of Thailand, Thailand)[2]	174	162	192	528
A. tamarense AT-A* (Chinhae, Korea) AF374224	165	160	194	519
A. tamarense AT-B* (Chinhae, Korea) AF374225	165	160	194	519
A. tamarense AT-6* (Chinhae, Korea) AF374228	165	160	194	519
A. tamarense AT-2* (Chinhae, Korea) AF374227	165	160	194	519
A. tamarense AT-10* (Chinhae, Korea) AF374226	165	160	194	519
A. frateculus AF208242 (Korea)	154	160	206	520
C. polykrikoides AF208248-out[1]	243	160	182	585

Note: Superscript letters represent the strain used in our study. [1]Species was reported in Cho *et al.* as molecular analysis of morphologically similar dinoflagellates *Cochlodinium polykrikoides, Gyrodinium impudicum* and *Gymnodinium catenatum* based on Internal Transcribed Spacer and 5.8S rDNA genes 12. [2]Data from Adachi *et al.* 2.

presented in Table 2 and reveals a sequence divergence ranging from 0.3% (1 bp) to 53% (305 bp). Among the 12 isolates of *A. tamarense*, it varied from 0.3 to 4.0% (1-20 bp), the maximum divergence (4%) being observed between strains 503A-A-A and FK-788-B. Interestingly, the degree of heterogeneity between *A. tamarense* and *A. fundyense* strains appeared rather small, i.e. of 1.2~2.3% (6-12 bp), when compared to the divergence noted within the *A. tamarense* group of isolates.

Phylogenetic analyses

To investigate the phylogenetic relationships among these 28 *Alexandrium* isolates, a PAUP analysis was conducted on their aligned data set. Although the degree of confidence in the branching order of the parsimony tree in Fig. 4 appeared relatively low, some groups were characterized by high bootstrap values, for instance the HI and CU1-1 group and the OFY101 and KO-3 group (100% and 91%, respectively). *A. cohorticula* and *A. frateculus* also tended to form an independent molecular group (bootstrap value of 87%). Surprisingly, strain *A. tamarense* WKS-1 was found to be more closely related to *A. catenella* isolates than to other *A. tamarense* strains (bootstrap value of 85%). Another interesting result concerned the *A. tamarense* strains collected from identical localities in Japan (e.g. Mikawa Bay and Ofunato Bay), which were found to cluster into two different subgroups (i.e. OFX191-A, 503A-A-A and 304A-A in group I, versus OFX191-B, 503A-A-B and 304A-A-B in

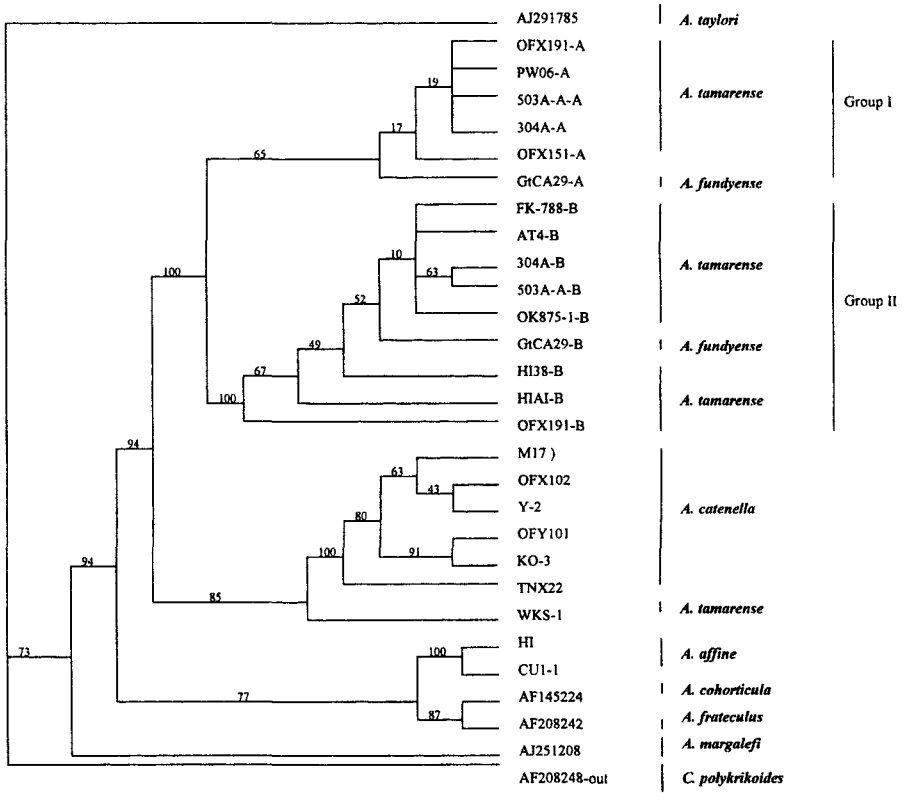


Fig. 4. Phylogenetic analysis of relationships among 28 isolates of *Alexandrium* performed on ITS regions including 5.8S rDNA sequences, using a parsimony method.
Outgroup species was *C. polykrikoides*. The tree was constructed using a PAUP analysis. The topology represents the consensus tree from an heuristic search yielding two equally most parsimonious trees (tree length = 1287, consistency index (CI) = 0.730, retention index (RI) = 0.771). Bootstrap values (100 replications) are given above the internal nodes.

Table 2. Pairwise comparisons among 28 isolates of *Alexandrium* species and *Cochlodinium polykrikoides* as an outgroup obtained from the sequences of ITS regions

Species	Genbank accession number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. *A. taylori*[1]	AJ291785	-	0.450	0.477	0.452	0.449	0.457	0.457	0.457	0.459	0.461	0.461	0.459	0.457	0.457	0.473
2. *A. tamarense* OFX191-A[2]		259	-	0.003	0.002	0.003	0.037	0.038	0.038	0.038	0.038	0.043	0.042	0.035	0.031	0.184
3. *A. tamarense* OFX151-A[3]		257	2	-	0.005	0.007	0.040	0.042	0.042	0.042	0.042	0.047	0.045	0.038	0.035	0.184
4. *A. tamarense* PWO6-A		260	1	3	-	0.005	0.038	0.040	0.040	0.040	0.040	0.045	0.043	0.037	0.033	0.186
5. *A. tamarense* 503A-A-A		258	2	4	3	-	0.040	0.042	0.042	0.042	0.042	0.047	0.042	0.038	0.035	0.188
6. *A. tamarense* FK-788-B	AB006994	263	21	23	22	23	-	0.002	0.003	0.002	0.005	0.007	0.005	0.005	0.005	0.200
7. *A. tamarense* AT4-B		263	22	24	23	24	1	-	0.005	0.003	0.007	0.009	0.007	0.007	0.007	0.200
8. *A. tamarense* 304A-B		263	22	24	23	24	2	3	-	0.005	0.009	0.007	0.009	0.009	0.009	0.200
9. *A. tamarense* OK875-1-B		264	22	24	23	24	1	2	3	-	0.007	0.009	0.007	0.007	0.007	0.202
10. *A. tamarense* H138-B		265	22	24	23	24	3	4	5	4	-	0.012	0.010	0.007	0.007	0.205
11. *A. tamarense* 503A-A-B		265	25	27	26	27	4	5	4	5	7	-	0.012	0.012	0.012	0.205
12. *A. fundyense* GtCA29-B		264	24	26	25	24	3	4	5	4	6	7	-	0.007	0.010	0.205
13. *A. tamarense* HIAI-B		263	20	22	21	22	3	4	5	4	4	7	4	-	0.003	0.202
14. *A. tamarense* OFX191-B		263	18	20	19	20	3	4	5	4	4	7	6	2	-	0.198
15. *A. catenella* M17[4]	AB006990	272	106	106	107	108	115	115	115	116	118	118	118	116	114	-
16. *A. catenella* OFX102		271	106	106	107	108	115	115	115	116	118	118	118	116	114	1
17. *A. catenella* OFY101		275	109	109	110	111	118	118	118	119	121	121	121	119	117	5
18. *A. catenella* KO-3		274	108	108	109	110	117	117	117	118	120	120	120	118	116	4
19. *A. catenella* Y-2		272	108	108	109	110	117	117	117	118	120	120	120	118	116	3
20. *A. tamarense* 304A-A		259	2	4	3	4	23	24	24	22	24	27	26	22	20	107
21. *A. fundyense* GtCA29-A		262	11	12	12	13	32	33	33	33	33	36	35	31	29	111
22. *A. catenella* TNX22		272	108	108	109	110	117	117	117	118	120	119	120	118	116	11
23. *A. tamarense* WKS-1	AB006991	275	98	98	99	100	110	111	111	111	113	114	113	111	109	91
24. *A. affine* H1	AB006995	288	155	156	156	157	164	164	164	164	167	167	167	165	163	167
25. *A. affine* CU1-1		287	159	160	160	161	168	168	168	168	170	171	171	168	166	169
26. *A. cohorticular*	AF145224	289	180	182	181	182	188	188	189	188	190	190	189	188	187	194
27. *A. frateculus*	AF208242	333	236	236	236	236	243	242	242	243	244	244	242	241	242	234
28. *A. margalefi*	AJ251208	257	234	235	235	235	240	240	241	241	242	243	242	242	242	245
29. *C. polykrikoides* -out	AF20824	334	311	312	312	313	315	314	314	315	315	315	316	314	315	335

group II). The same observation applied to *A fundyense* strains GtCA29-A and GtCA29-B, both collected at Cape Ann (USA). This result is in marked contrast with the one obtained for the 5 Korean isolates of *A. tamarense* obtained from Jinhae Bay, which showed completely identical sequences.

The phylogenetic trees inferred from the distance analysis of the aligned data set (NJ and ML methods) are shown in Figs. 5 and 6, respectively. Their topologies were quite similar to the one obtained with the parsimony method. In particular, group I and II clearly formed two separate clusters, with strong to moderate support (98% and 56%, respectively). They were grouped in the form of polytomy in the distance analysis, compared with the parsimony phylogenies. This observation also applies to *A. catenella* isolates and strain *A. tamarense* WKS-1, which formed a unique molecular group (bootstrap values of 98% and 96%, respectively).

Discussion

Scholin *et al.* and Scholin and Anderson reported first to exhibit two distinct genes (A gene and B gene) in the SSU rDNA [28, 29]. Likely, Adachi *et al.* also proposed to the existence of 1 gene and 2 gene in the ITS regions, indicating this region was a useful molecular marker for the determination of population in *Alexandrium* complex [2]. Consequently, five strains of *A. tamarense* from Jinhae Bay had clear distinct two genes in the ITS regions that

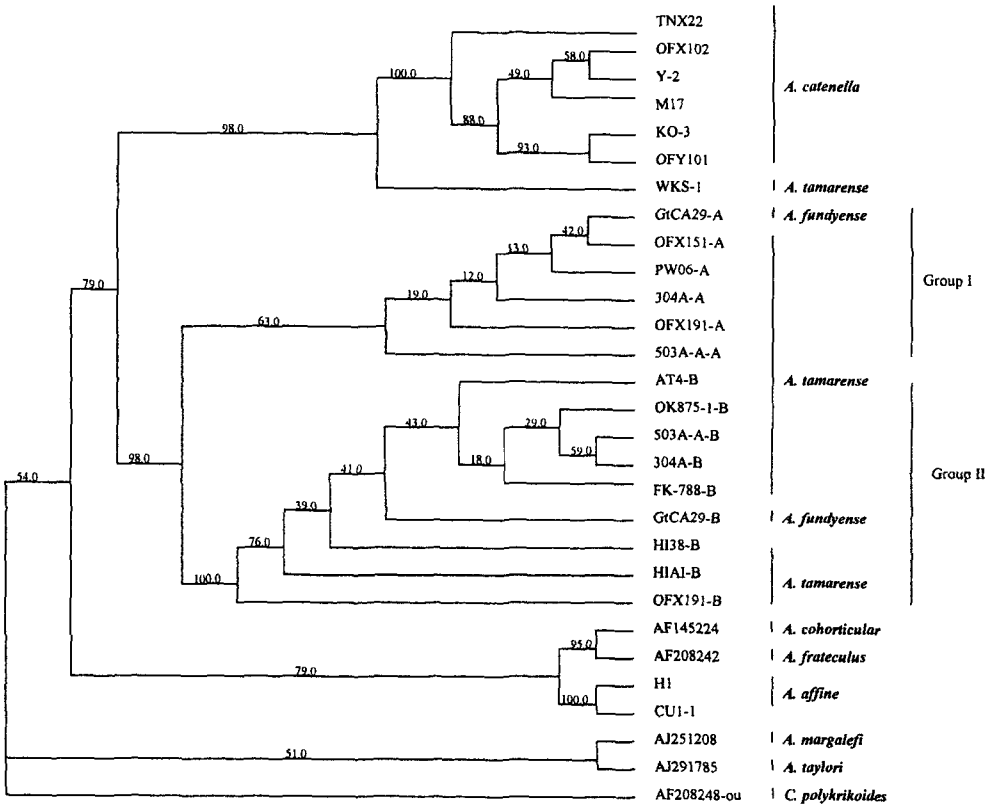


Fig. 5. Phylogenetic analysis of relationships among 28 isolates of *Alexandrium* performed on ITS regions including 5.8S rDNA sequences, using a distance method.

Outgroup species was C. polykrikoides. The tree was obtained using the subprogram NEIGHBOR incorporated in the PHYLIP package with the option of Kimuras 2-parameter method 23. Bootstrap values (100 replications) are given above the internal nodes.

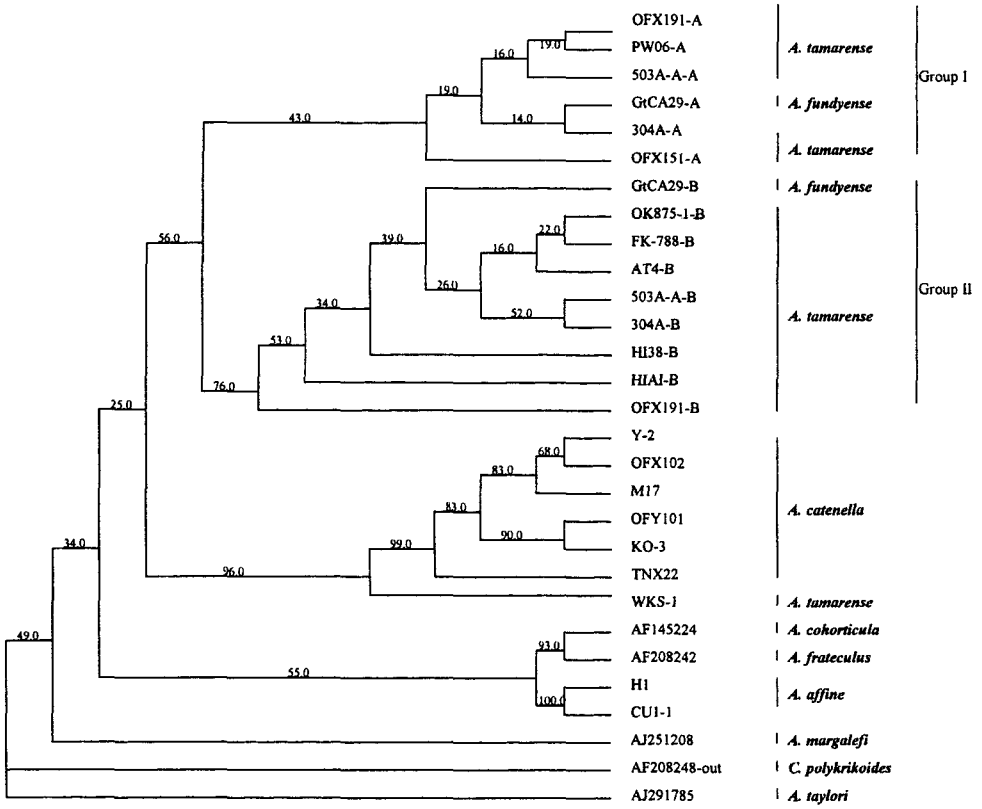


Fig. 6. Phylogenetic analysis of relationships among 28 isolates of *Alexandrium* performed on ITS regions including 5.8S rDNA sequences, using a distance method.
Outgroup species was *C. polykrikoides*. The unrooted tree was inferred using the subprogram DNAML incoporated in the PHYLIP package with the option of Kimuras 2-parameter method 23. Bootstrap values (100 replications) are given above the internal nodes.

were closer to A gene than to B gene. This is indicated that a test of genetic population structure allows our which is the *A. tamarense* in Korean waters formed a large genetic group or not, although geographically close populations roughly formed immediate clusters. We extend to sampling sites in the future.

Korean isolates of *A. tamarense* (AT-A, AT-B, AT-2, AT-6 and AT-10) collected at the same site, Jinhae Bay, are characterized by similar thecal plate morphology based on their apical pore complex (APC), first apical plate and posterior sulcal plate[10]. In contrast, their toxin composition and content was found to vary significantly, AT-6 being regarded as the most toxic isolate[10]. Environmental causes have been suggested to explain spatial and temporal heterogeneity, since several isolates are known to exhibit the variability in toxin concentration under different laboratory conditions[20,21]. However, since toxicity has been found to change not only between different strains but also among different clones of the same isolate, the factors influencing the formation of toxic *A. tamarense* can not only be environmental, and genetic differences seem to play an important role on the variation of toxin concentration. Consequently, characterization of genetic variability in toxin production of *A. tamarense* populations from Jinhae Bay is an important area for further study and understanding.

In the present study, it was shown that they share the

same ITS+5.8S rDNA sequence with OFX151-A, a Japanese isolate. Although *A. tamarense* fuse two gametes (female and male) from vegetative cells which lead to an enlarged, thick-walled zygote with a new combination of genetic material[4-6], it appears to be related to no genetic divergence and phylogenetic relationships in Korean *A. tamarense* even during the course of sexual cross-breeding over generations. Previously, the use of DNA sequences targeting ITS regions to determine the relatedness among harmful dinoflagellate isolates isolated from Korean coastal waters showed the same nucleotide sequence as Korean *A. tamarense* in this study[11-13,22]. Thus, one possible explanation is likely that ITS DNA genotypes would show the equivalent result of population in Korean harmful dinoflagellates. On the other hand, samples of *A. tamarense* collected at various sites in Japanese waters possessed a low genetic diversity (Table 2), with almost identical nucleotide length of ITS and 5.8S rDNA with Korean strains (Table 1). Additionally, all of them produce toxins responsible for PSP contaminated mussels and oysters[2].

Minor and variable morphometric parameters may have led to the identification of *A. tamarense*, *A. catenella* and *A. fundyense*, whose separation from *A. tamarense* is difficult to justify based on the ITS gene analyses. *Alexandrium taylori*, *A. cohorticular*, *A. affine* and *A. frateculus* are non-toxic and have considerably different morphological features than the other *Alexandrium* included in this study (Table 1). The clustering of *A. tamarense*, *A. catenella*, *A. fundyense* and *A. affine* has been supported by the genetic relationships within the genus *Alexandrium*. The SSU gene data also support the apparently branching of species with the major *Alexandrium* cluster[28,29]. However, the genera of *A. taylori*, *A. cohorticular* and *A. frateculus* may deserve in dependant status. Consequently, the genus *Alexandrium* may not be described within molecular taxonomic limits.

Surprisingly, WKS-1 among Japanese *A. tamarense* did not present any of toxin profile possessed by the high-performance liquid chromatography-fluorometric analysis [2], and also was high in genetic divergence and paced in different phylogenetic group in our study. The current taxonomic status are not attributed to discriminate them on the basis of fine-scale structures. In microorganisms, possible mechanisms for exchange of genetic material is explained that transformation is so far the only known biological mechanism for exchange of chromosomal DNA in bacteria[24]. That is to say that homologous recombination is a ubiquitous mechanisms for incorporating DNA into the genome. Consequently, stable exchange of genetic solidarity within closely related groups can be explained by homologous recombination frequency. However, there are also other possible mechanisms for genetic exchange within genetically clustered groups. This is introduced by conjugation, simultaneous competence and recognition sequence[24]. The exchange of genetic information could not be a general mechanism. The observed heterogeneous distribution of the toxic and non-toxic strains may be result of gene change at a number of times thoughout evolution. Further studies addressing these mechanisms of heterogeneous strain of *A. tamarense* are necessary. However, this study clearly suggest that these ITS "genotypes" could be useful markers in discriminating toxic from non-toxic *A. tamarense* isolates as a species identification.

A phenomenon in the G+C content of ITS regions known as the GC balance was documented in a wide range of organisms by Torres *et al.*[35]. *Alexandrium* species showed a G+C content in their total sequences lower than in any other harmful dinoflagellates including *C. polykrikoides* (approximately 40% vs. 57.4%, respectively)[12]. Bernardi *et al.* and Salinas *et al.* suggested that temperature was an important selection factor of GC bias in the genomes of plants and warm-blooded animals[8,27]. For example, the genomes of warm-blooded vertebrates have higher G+C content than those of cool-blooded vertebrates[27]. Subsequently, Takamatsu *et al.* proposed that the relatively low G+C content of

fungus might reflect the low optimum temperature of the fungus[33]. However, this conclusion has not been generalized to harmful dinoflagellates yet. In Korean waters, the blooms involving *C. polykrikoides* generally occur during the summer season[13], whereas *Alexandrium* species are reported from mid-March to early July[10]. These observations tend to indicate that the reason for different blooming seasons, depending on the species of harmful dinoflagellates, is likely to be associated with their G+C content. Further study is needed to determine the effect of G+C content on the ecological and physiological characteristics of harmful dinoflagellates.

Acknowledgements

This work was supported by a grant-in-aid from the Maritime Affairs and Fisheries Ministry of Korea.

References

1. Adachi, M., Y. Sako and Y. Ishida. 1994. Restriction fragment length polymorphism of ribosomal DNA internal transcribed spacer and 5.8S regions in Japanese *Alexandrium* species (Dinophyceae). *J. Phycol.* **30,** 857-863.
2. Adachi, M., Y. Sako and Y. Ishida. 1996. Analysis of *Alexandrium* (Dinophyceae) species using sequences of the 5.8S ribosomal DNA and internal transcribed spacer regions. *J. Phycol.* **32,** 424-432.
3. Adachi, M., Y. Sako and Y. Ishida. 1997. Analysis of *Gymnodinium catenatum* (Dinophyceae) using sequences of the 5.8S rDNA-ITS regions and random amplified polymorphic DNA. *Fish. Sci.* **63,** 701-707.
4. Anderson, D. M and N. L. Lindquist. 1985. Time-course measurements of phosphorus depletion and cyst formation in the dinoflagellate *Gonyaulax tamarensis*. *J. Exp. Mar. Biol. Ecol.* **86,** 1-13.
5. Anderson, D. M., S. W. Chisholm and C. J. Watras. 1983. Importance of life cycle events in the population dynamics of *Gonyaulax tamarensis*. *Mar. Bio.* **76,** 179-189.
6. Anderson, D. M., D. M. Kulis and B. J. Binder. 1984. Sexuality and cyst formation in the dinoflagellate *Gonyaulax tamarensis*: cyst yield in batch cultures. *J. Phycol.* **20,** 418-425.
7. Balech, E. 1985. The genus *Alexandrium* or *Gonyaulax* of the tamarense group. In: Anderson, D. M., A. W. White and D. G. Baden D.G. (eds), *Toxic Dinoflagellates*, Elsevier, New York, pp. 33-38.
8. Bernardi, G., B. Olofsson, J. Filipsik, M. Zerial, J. Salinas, G. Cuny, M. Meunier and F. Rodier. 1985. The mosaic genome of warm-blooded vertebrates. *Science* **228,** 953-958.
9. Chang, F. H., D. M. Anderson, D. M. Kulis and D. G. Till. 1997. Toxin producton of *Alexandrium minutum* (Dinophyceae) from the Bay of plenty, New Zealand. *Toxicon* **35,** 393-409.
10. Cho, E. S. and H. J. Lee. 2001c. Thecal plates, toxin content and growth of five clones of *Alexandrium tamarense* (Dinophyceae) isolated from the Jinhae Bay, Korea. *Phycologia* **40,** 435-439.
11. Cho, E. S., G. Y. Kim and Y. C. Cho. 2001a. Molecular analysis of morphologically similar dinoflagellates *Cochlodinium polykrikoides*, *Gyrodinium impudicum* and *Gymnodinium catenatum* based internal transcribed spacer and 5.8S rDNA regions. *Algae* **16,** 53-57.
12. Cho, E. S., G. Y. Kim, H. S. Park, B. H. Nam and J. D. Lee. 2001b. Phylogenetic relationship among several Korean coastal red tide dinoflagellates based on their rDNA internal transcribed spacer sequences. *Kor. J. Lif. Sci.* **11,** 74-80.
13. Cho, E. S., H. G. Kim and Y. C. Cho. 2000. Sequence analysis of *Cochlodinium polykrikoides* isolated from Korean coastal waters using sequences of Internal Transcribed Spacers and 5.8S rDNA. *J. Kor. Soc. Oceano.* **35,** 158-160.
14. Felsenstein, J. 1993. PHYLIP (Phylogeny Inference Package) Version 3.5c. Department of Genetics, University of Washington, Seattle.
15. Fukuyo, Y. 1985. Morphology of *Protogonyaulax tamarensis* (Lebour) Taylor and *Protogonyaulax catenella* (Whedon and Kofoid) Taylor from Japanese coastal waters. *Bull. Mar. Sci.* **37,** 529-537.
16. Furlong, J. C., J. Forbes, M. Robertson and B. E. H. Madden. 1983. The external transcribed spacer and preceding region of *Xenopus borealis* rDNA: comparison with the corresponding region of *Xenopus*

laevis rDNA. *Nucelic Acids Res.* **11,** 8183-8196.

17. Guillard, R. R. L. and J. H. Ryther. 1962. Studies of marine planktonic diatoms 1. *Cyclotella nana* Hustedt, and *Detonula confervacea* (Cleve) Gran. *Can. J. Micro.* **8,** 229-239.
18. Hallegraeff, G. M. 1993. A review of harmful algal blooms and their apparent global increase. *Phycologia* **32,** 79-99.
19. Hillis, D. M. and M. T. Dixon. 1991. Ribosomal DNA: molecular evolution and phylogenetic inference. *Rev. Biol.* **66,** 411-453.
20. Kim, C. H., Y. Sako and Y. Ishida. 1993. Variation of toxin production and composition in axenic cultures of *Alexandrium catenella* and *A. tamarense*. *Nip. Sui. Gakk.* **59,** 633-639.
21. Kim C.H. 1998. Development of PSP toxigenic dinoflagellates and toxin production in Korean coastal waters. In: Kim, H. G., S. G. Lee and C. K. Lee (eds), *Harmful algal blooms in Korea and China,* Kukduk Publishing, Pusan. pp. 69-86.
22. Kim, G. Y., M. G. Ha, E. S. Cho, T. H. Lee, S. J. Lee and J. D. Lee. 1999. Molecular identification of *Gyrodinium impudicum* and *Gymnodinium sanguineum* by comparing the sequences of the internal transcribed spacers 1,2 and 5.8S ribosomal DNA. *J. Fish. Sci. Tech.* **2,** 66-77.
23. Kimura, M. 1980. A simple method for estimating evolutionary rate of base substitution through comparative studies of nucleotide sequences. *J. Mol. Evol.* **116,** 111-120.
24. Lorenz, M. G. and W. Wackernagel. 1994. Bacterial gene transfer by natural genetic transformation in the environment. *Micro. Rev.* **58,** 563-602.
25. Mackenzie, L., D. White, Y. Oshima and J. KAPA. 1996. The resting cyst and toxicity of *Alexandrium ostenfeldii* (Dinophyceae) in New Zealand. *Phycologia* **35,** 148-155.
26. Sako, Y., C. H. Kim, H. Ninomiya, M. Adachi and Y. Ishida. 1990. Isozyme and cross analysis of mating populations in the *Alexandrium catenella/tamarense* species complex. In: Graneli, E., B. Sundstrom, I. Edler and D. M. Anderson (eds), *Toxic Marine Phytoplankton,* Elsevier Science Publisher, New York. pp. 320-323.
27. Salinas, J., G. Matassi, L. M. Montero and G. Bernardi. 1988. Compositional compartmentalization and compositional patterns in the nuclear genomes of plants. *Nucleic Acids Res.* **16,** 4269-4285.
28. Scholin, C.A. and D. M. Anderson. 1994. Identification of group- and strain-specific genetic markers for globally distributed *Alexandrium* (Dinophyceae). I. RFLP analysis of SSU rRNA genes. *J. Phycol.* **30,** 744-754.
29. Scholin, C. A., D. M. Anderson and M. L. Sogin. 1993. Two distinct small-subunit ribosomal RNA genes in the North American toxic dinoflagellate *Alexandrium fundyense* (Dinophyceae). *J. Phycol.* **29,** 309-316.
30. Shumway, S. E. 1990. A review of the effects of algal blooms on shellfish and aquaculture. *J. World Aquacult. Soc.* **21,** 65-104.
31. Stediner, K. A. and O. Moestrup. 1990. The taxonomy of *Gonyaulax, Pyrodinium, Alexandrium, Gessnerium, Protogonyaulax* and *Goniodoma*. In: Graneli, E., B. Sundstrom, L. Edler and D. M. Anderson (eds), *Toxic Marine Phytoplankton,* 522-523. Elsevier, New York. pp. 522-523.
32. Swoford D.L. 1990. PAUP, version 3.1, beta 8. Sinauer and Associates, Sunderland, Mass.
33. Takamatsu, S., T. Hirata and Y. Sato. 1988. Phylogenetic analysis and predicted secondary structures of the rDNA internal transcribed spacers of the powdery mildew fungi (Erysiphaceae). *Mycoscience* **39,** 441-443.
34. Taylor, F. J. R. 1984. Toxic dinoflagellates: taxonomic and biogeographic aspects with emphasis on Protogonyaulax. In: Ragelis, E. P. (ed), *Seafood Toxins,* ACS Symposium Series 262, Amercian Chemcial Society, Washington, D.C. pp. 77-97.
35. Torres, R. A., M. Ganal and V. Hemleben. 1990. GC balance in the internal transcribed spacers ITS1 and ITS2 of nuclear ribosomal RNA genes. *J. Mol. Evol.* **30,** 171-181.

(Received August 21, 2002; Accepted December 23, 2002)

초록 : ITS 부위에 근거한 한국산 *Alexandrium tamarense* 5 클론의 계통분류학적 위치

조은섭[1] · 이삼근[1] · 김익수[2]
([1]국립수산진흥원 적조생물과, [2]농업과학기술원 잠사곤충부)

알렉산드륨 적조생물의 리보소옴 알엔에이 유전자의 ITS1, 2 및 5.8S 부위를 대상으로 종간 혹은 종내의 유전적 다양도를 조사하기 위하여 지리적으로 격리된 33 스트레인 유전자의 염기서열를 비교했다. 진해만에서 분리된 AT-2, AT-6, AT-10, AT-A, AT-B 5클론은 일본종 OFX151-A과 동일한 유전자임을 발견했다. ITS 부위에서 가장 짧은 종은 *A. margalefi*로 481 bp이며 가장 긴 종은 *A. affine*으로 528 bp로 나타났다. ITS1과 ITS2 염기서열에 대한 상호관계는 역으로 나타낸 반면에, G+C 함량에 대한 상호관계는 플러스로 나타났다. 유전적 변이율은 0.3% (1 bp)에서 53% (305 bp)였다. *A. tamarense*과 가장 적게 유전적 변이율을 보인 종은 *A. fundyense* (1.2 -2.3% =6-12 bp)인 반면에, *A. catenella*와는 큰 변이율 (19.8% = 102 bp)을 보였고, *A. catenella*와 *A. fundyense*은 19.7% 상이하였다. 알렉산드륨 적조생물의 bootstrap은 약하게 지지되는 데도 불구하고, *A. catenella* 분리종은 독립적인 그룹으로 형성하여 상호간에는 강력한 bootstrap 값은 PAUP과 NJ 분석에서 보였다. *A. cohorticula*와 *A. frateculus* 적조생물은 항상 sub-group 내에서 높은 bootstrap을 가졌다. 결론적으로 ITS 부위의 염기서열 분석은 알렉산드륨 적조생물의 집단내 혹은 집단간의 계통분류을 밝히는데 유용한 것으로 보였다.