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Molecular Analysis of Complete SSU to LSU rDNA Sequence in the Harmful Dinoflagellate *Alexandrium tamarense* (Korean Isolate, HY970328M)

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Abstract – New PCR primers (N=18) were designed for the isolation of complete SSU to LSU rDNA sequences from the dinoflagellate *Alexandrium tamarense*. Standard PCR, employing each primer set selected for amplifications of less than 1.5 kb, successfully amplified the expected rDNA regions of *A. tamarense* (Korean isolate, HY970328M). Complete SSU, LSU rDNAs and ITS sequences, including 5.8S rDNA, were recorded at 1,800 bp, 520 bp and 3,393 bp, respectively. The LSU rDNA sequence was the first report in *Alexandrium* genus. No intron was found in the LSU rRNA coding region. Twelve D-domains within the LSU rDNA were put together into 1,879 bp (44.4% G+C), and cores into 1514 bp (42.8% G+C). The core sequence was significantly different (0.0867 of genetic distance, 91% sequence similarity) in comparison with *Prorocentrum micans* (GenBank access. no. X16108). The D2 region was the longest in length (300 bp) and highly variable among the 12 D-domains. In a phylogenetic analysis using complete LSU rDNA sequences of a variety of phytoplankton, *A. tamarense* was clearly separated with high resolution against other species. The result suggests that the sequence may resolve the taxonomic ambiguities of *Alexandrium* genus, particularly of the tamarensis complex.

Key words – *Alexandrium tamarense*, large-subunit rDNA, phylogenetic analysis, D-domain

1. Introduction

Toxic dinoflagellates *Alexandrium* are arguably the most important harmful algal bloom (HAB) species, based on the number of species involved in toxic algal blooms and their extensive geographical distribution. In addition, these species are responsible for paralytic shellfish poisoning along the northeastern coasts of the United States and Canada and other temperate coastal waters throughout the world. These organisms pose an important problem in population biology and taxonomy as well as a serious economic and public health concern (Anderson 1989; Han *et al.* 1993; Han and Terazaki 1993; Kim *et al.* 2002; Lee *et al.* 2003).

Correct and rapid detection of HAB species is important in monitoring their dispersion throughout the world and minimizing fishery damage. The identification is mostly based on microscopic examination, which requires considerable taxonomic experience, and is sometimes laborious and time-consuming. Recently, many molecular techniques, such as alternative methods, have been developed to discriminate between these morphological resemblances. These methods include isozyme patterns (Cembella *et al.* 1988; Hayhome *et al.* 1989; Sako *et al.* 1990), immunological properties (Sako *et al.* 1993), toxin profiles (Cembella *et al.* 1987; Anderson *et al.* 1994) and more recently genetic makeup (Destombe *et al.* 1992; Adachi *et al.* 1994; Scholin *et al.* 1994; Scholin and Anderson 1996; Hirashita *et al.* 2000; Sako 2000; Usup *et al.* 2002). Furthermore, with recent advances in DNA sequencing technology, many DNA sequences are currently revealed and easily available in the public database. With this knowledge, DNA sequence-based genotyping is a promising tool for the identification of HAB species (*e.g.* Ki *et al.* 2004). It is, thus, not surprising that many studies have been carried out on the taxonomy, phylogeny and genetic diversity of these species.

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Ribosomal DNA (rDNA) is the region of the genome coding for the RNA component of ribosomes. In eukaryotes, rDNA sequences may be found in the nucleus and organelles. Eukaryotic nuclear rDNA is tandemly organized, with copy numbers up to the order of 10,000. Each repeat unit consists of the gene coding for the small subunit (SSU), large subunit (LSU) and the 5.8S nuclear rDNA. These coding regions separated from each other in the primary transcript by internal transcribed spacers (ITS). The rDNA coding regions have remained relatively constant within the same taxa, and this ubiquity and conservation have made them important tools for reconstructing phylogenetic relationships. The rDNA gene sequences are now commonly used in taxonomic and phylogenetic studies on the marine dinoflagellates. In addition, the rDNA gene has different degrees of sequence variability among the different subunits and regions, in which these sequences may have varying suitability for comparison at the inter-genetic level or inter-species level (Adachi *et al.* 1996).

Many rDNA sequences from dinoflagellates (*e.g.* the genera *Akashiwo, Alexandrium, Gymnodinium, Karenia, Pfiesteria*) have been deposited in the public database (*i.e.* EMBL/DDBJ/GenBank), in which all the sequences are estimated as approximately 20,000 strands until June 30, 2005. The complete sequences of both 18S rDNA and ITS regions have been revealed from the representatives of marine dinonoflagellates, and are available in the GenBank.

The LSU rDNA, which is the largest rDNA coding region, has also been sequenced and used for the inference of evolutionary history of marine microorganisms. The main advantage of using the LSU rDNA is that it contains relatively well-conserved core segments considered essential for proper ribosome function. In addition, it contains 12 hyper variable domains (Higman *et al.* 2001; Usup *et al.* 2002), often designated as divergent (D) domains or expansion segments, which are significantly larger compared with the homologous regions in prokaryotes. Although there is a significant variation in the size and sequence of the D domains among highly divergent eukaryotes, the secondary structures of some domains have remained relatively well conserved. The sequence variability in the D domains makes them useful for the reconstruction of relatively recent evolutionary events, whereas the core segments have evolved at a slower rate, permitting phylogenies to be constructed for organisms, which diverged a long time ago. In addition, the variability of LSU rDNA may allow us various options for design primers, which can prove to be a suitable target for deriving primers and probes for identification of these harmful species.

Due to a lot of recent sequencing efforts, the number of complete eukaryotic LSU rRNA sequences, including SSU rDNA and ITS regions, have increased significantly (Ben Ali *et al.* 1999, 2001), and currently approximately 157 sequences of complete LSU rDNA are available for organisms belonging to the so-called crown eukaryots (see The European Ribosomal RNA database, http://www.psb.ugent.be/rRNA/). In dinoflagellates, Lenaers *et al.* (1989) firstly reported the complete LSU rDNA sequence of the dinoflagellate *Prorocentrum micans* (GenBank accession no. X16108). Recently, another sequence of *P. donghaiense* (accession no. AY822610) has been deposited in GenBank. Most DNA sequences concerned with the LSU rDNA of dinoflagellates have been focused on the region spanning the D1 and D2 hyper variable domains (Scholin *et al.* 1994; Higman *et al.* 2001; Usup *et al.* 2002). None of the complete LSU rDNA sequences from the dinoflgaellate *Alexandrium* have been found in the literature in mind.

In this study, we presented a long rDNA sequence comprising complete SSU to LSU rDNAs from *Alexandrium tamarense*. To achieve this work, we had newly designed PCR and sequencing primers and evaluated their usability to other harmful algae. In addition, we discussed the characteristics of each complete rDNA segment, based on the sequence's length and G+C contents.

2. Materials and Methods

Isolation of *Alexandrium* cells

Single *Alexandrium tamarense* cells were isolated by capillary method from the field samples collected from Masan Bay in Korea, at Mar. 28, 1997. The isolate, HY970328M, was grown in silicon-stopper sealed flasks for gas exchange, and maintained in a f/2 medium, pH 8.2 (Guillard and Ryther 1962), at 15°C, 12:12-h light:dark cycle, and a photon flux density of ca. 65 μmol photons $\cdot m^{-2} \cdot s^{-1}$.

Cell harvest and DNA purification

Approximately 200 mL of clonal cultures, reaching mid-logarithmic phase, were filtered through a 20-μm

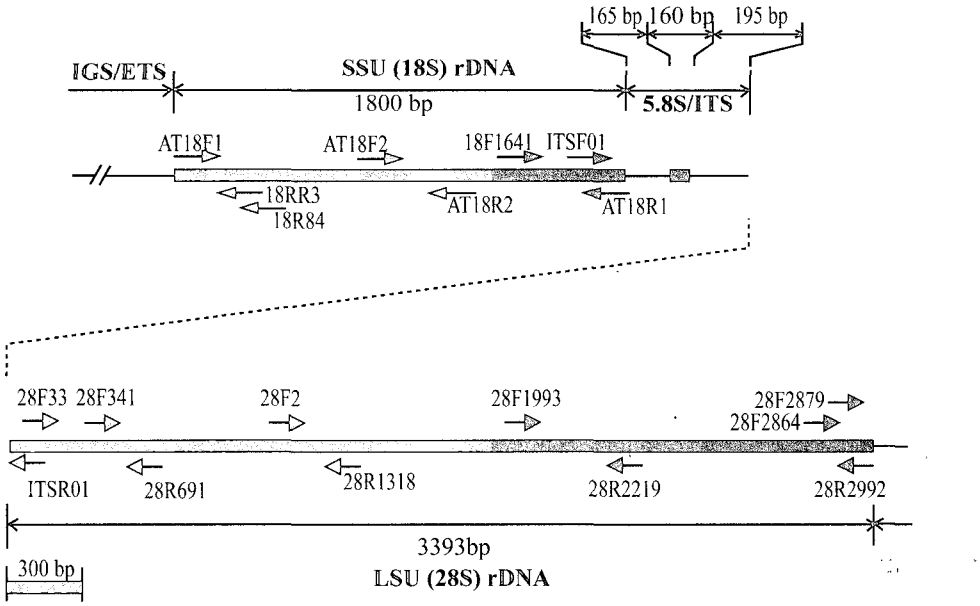

Fig. 1. A schematic diagram of *Alexandrium tamarense* rDNA genes. Primer positions correspond to the deposited sequence for *A. tamarense* (HY970328M). Darkened boxes indicate the ribosomal genes, and thin lines represent ITS or intergenic spacer (IGS). Arrows represent locations of primers used for the amplification of the rDNA from dinoflagellates.

pore size nylon mesh and gently washed several times with a fresh medium to facilitate the removal of possible bacterial contaminants. The cells concentrated on the mesh were transferred to a 1.5-mL micro tube. 100 μL of 1 x TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA) were added, and the tube was stored at –20°C until DNA extraction. Genomic DNA was isolated from the stored cells using the DNeasy Plant mini kit (Qiagen, Valencia, CA) according to the manufacture's instructions.

Isolation of entire SSU to LSU rDNA

Based on conserved sequences among related dinoflagellate species, new PCR primers were designed for the amplification of the complete SSU to LSU rDNA and ITS regions (Table 1). Particularly, the primers for isolation of the complete LSU rDNA were designed based on the conserved sequences of five algal LSU rDNAs (*e.g. Chattonella subsalsa*, AF409126; *Heterosigma akashiwo*, AF409124; *Prorocentum micans*, X16108; *Rhizosolenia setigera*, AF289048; *Sarcocystis moulei*, AF012884). A schematic drawing represents a map of the rDNA of *A. tamarense* (HY970328M) and the positions of primer binding sites (Fig. 1). All of the primers used in this study were synthesized on a PolyGen 10-column DNA synthesizer (PolyGen, Hamburg, Germany).

Standard PCR reactions were carried out in 1 x PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin), to which < 0.1 mg genomic DNA template, 200 mM each of four dNTPs, 0.5 mM each of primers, and 0.2 Units *Taq* polymerase (Promega, Madison, WI) per 10-mL reaction were added. Using a UNO-II Thermoblock (Biometra, Göttingen, Germany), PCR thermocycling parameters were as follows: initially 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 55°C for 30 s and extension at 72°C for 60 s. After the cycles, final extension was completed at 72°C for 5 min. The PCR products (2 mL) were analyzed by 1.5% agarose gel electrophoresis according to standard methods (Sambrook *et al.* 1989).

Isolation of ETS regions adjacent to the SSU and LSU rDNAs

The upstream and downstream regions of SSU to LSU rDNA were isolated by ETS walking PCR following a Ki and Han's method (Ki and Han 2005). Each unknown ETS region, which is the boundary to the SSU or LSU rDNA, was primarily amplified from genomic DNA using a conserved universal primer (*e.g.* 18R84, 28F2864) and 4 walking primers linked to a universal M13F sequence. The PCR reactions were performed with the same mixtures

of standard PCR, except for primers and thermocyclings. PCR thermocycling parameters were as follows: initially 95°C for 4 min, followed by 5 cycles (denaturation at 95°C for 20 s, annealing at 60°C for 30 s and extension at 72°C for 1 minute), 2 cycles (denaturation at 95°C for 20 s, annealing at 36°C for 30 s, ramping to 72°C about 0.5°C s^{-1}, and extension at 72°C for 1 min), and finally 30 cycles (denaturation at 95°C for 20 s, annealing at 55°C for 30 s and extension at 72°C for 1 min). After the cycles, final extension was completed at 72°C for 5 min. The PCR fragments were diluted with 1:100 ratio of 1 x TE buffer for the next amplification without checking on the agarose gel. The 3' ETS walking PCR had the same protocol, except for the two conserved primers.

The diluted fragments were used as the DNA template in the second amplification using the nested PCR and universal M13F primers. The PCRs were followed by the same standard conditions. After completion of the cycles, the PCR products (3 mL) were thoroughly mixed with 3 μL of D.W. and 2 μL of 6 x blue/orange loading dye (Promega), followed by heating at 95°C for 5 min for separation and quenching on ice. After 5 min, the samples were carefully loaded onto a 1.5% agarose gel in 1 x TBE buffer and electro-separated under 8 $V \cdot cm^{-1}$ (running for about 1-2 h). Agarose gels were stained with ethidium bromide and destained with D.W. for 30 min. The electro-separated DNA was visualized at 254 nm UV, and then picked up the core band with an aerosol barrier tip as quickly as possible. We soak the tip into a 1.5-mL micro tube containing 100 mL of D.W. and then separated the gel from the tip by using a pipette, and heated it at 95°C for 5 min and immediately vortexed the tube to release the DNA fragment. The purified eluate was re-amplified with the same PCR primers under identical conditions. After the completion of the cycles, the third PCR product was analyzed under the previously described method.

Direct DNA sequencing

DNA cycle sequencing reactions were performed directly, using the PCR products as the template, without purification of the PCR amplified fragments (see Ki *et al.* 2004). The PCR products (3-6 μL) were subjected to DNA cycle sequencing using a ThermoSequenase™ version 2.0 Cycle Sequencing Kit (USB, Cleveland, OH) in the presence of 1.5 picomoles of nested sequencing primers (Table 1). All the primers were labeled with near infrared dye (IRD) at the 5' end. DNA cycle sequencing reactions were followed by a previous work (Ki *et al.* 2004), and the DNA fragments were separated using a Dual Dye Automated Sequencer (LONG READIR 4200, Li-cor, NE).

Sequence analysis

For sequence comparison, alignment was performed with each rDNA sequence revealed from the present study and retrieved from DDBJ/EMBL/GenBank, using the Clustal W with the default settings for gap inclusion and extension. Various regions were further aligned manually using the BioEdit 5.09 (North Carolina State University, NC), and regions that could not be aligned unambiguously were excluded from the analysis. Genetic distance values were calculated by using the aligned DNA sequences according to the Kimura 2-parameter model. Phylogenetic tree was inferred by use of the neighbor-joining (NJ) algorithm in MEGA3 (Kumar *et al.* 2004). All characters were weighted equally and gaps were treated as missing data. The strength of the internal branches from the resulting trees was statistically tested by bootstrap analysis from 1,000 bootstrap replications.

3. Results

PCR and 3' ETS walking

Normal PCR, employing 14 primers designed newly, was applied for the isolation of nearly complete SSU to LSU rDNA region of *Alexandrium tamarense* (HY970328M). Each primer set was chosen for the amplification of up to 1.5 kb in length from rDNA coding regions. The PCRs successfully amplified the targeted rDNA, including the entire ITS regions, from *A. tamarense* (data not shown). In addition, ETS walkings followed by nested PCR could successfully isolate the 5' and 3' ETS regions adjacent to the rDNA. After an expected DNA band was picked up from the agarose gel by using an aerosol barrier tip, the fragment was re-amplified with the same primers to render a single of DNA band as a sequencing grade of DNA template (data not shown).

DNA sequencing reactions were performed using IRD-labeled primers (see Table 1) and the PCR fragments without further purification of the products and cloning. Reliable and accurate base identification was obtained for all the PCR fragments obtained here (data not shown). The partial sequences were completely assembled with

Table 1. Primers used for PCR amplification of 5' ETS, 18S, ITS, and complete LSU rDNA from the genus *Alexandrium*, and for sequencing of their products. Bases have been given the appropriate IUPAC code

Target rDNA	Primer[a]	Nucleotide sequence 5' to 3'	Position[b] (in SSU or LSU rDNA)	Purpose
SSU	AT18F02	AGAACGAAAGTTAAGGGATCGAAGACG	972-998*	PCR
SSU	AT18R01	RMWTGATCCTTCYGCAGGTTCACC	1777-1800*	PCR
SSU	AT18F01	YACCTGGTTGATCCTGCCAGTAG	1-23*	PCR
SSU	AT18R02	GTTTCAGCCTTGCGACCATACTCC	1112-1135*	PCR
SSU	18F1641	TCCCTGCCCTTTGTACACAC	1620-1639*	PCR
SSU	ITSR01	WCCRCTTACTTATATGCTTAAAYTCAGC	56-29**	PCR
SSU	ITSF01	GAGGAAGGAGAAGTCGTAACAAGG	1745-1768*	PCR
LSU	28R691	CTTGGTCCGTGTTTCAAGAC	729-710**	PCR
LSU	28F341	AGCACACAAGTACCATGAGG	431-360**	PCR
LSU	28R1318	TCGGCAGGTGAGTTGTTACACAC	1341-1319**	PCR
LSU	28F2	AGGCTCGTAGCGATACTGACGTGC	916-939**	PCR
LSU	28R2219	CAGAGCACTGGGCAGAAATCAC	2239-2218**	PCR
LSU	28F1993	TTGGGGGATTGGCTCTGAGG	1973-1992**	PCR
LSU	28R2992	AAACTAACCTGTCTCACGACGGTC	3012-2990**	PCR
5' ETS	18RR3	GCTTATACTRAGACATGCATG	50-70*	PCR
5' ETS	18R84	TTAATGAGCCATTYGCAGTT	84-103*	PCR
3' ETS	28F2864	TTACCACAGGGATAACTGGCTTG	2839-2861**	PCR
3' ETS	28F2879	CTTGTGGCAGCCAAGCGTTC	2858-2877**	PCR
SSU	SATITSF01	TAACAAGGTTTCCGTAGGTG	1761-1780*	Sequencing
SSU	SAT18F01	CCAGTAGTCATATGCTTGTC	17-36 *	Sequencing
SSU	SAT18R02	AGCCTTGCGACCATACTCC	1130-1112*	Sequencing
SSU	SAT18F02	GATCAGATACCGTCCTAGTC	998-1017*	Sequencing
SSU	SAT18R01	GTTACGACTTCTCCTTCCTC	1764-1745*	Sequencing
SSU	SATITSF01	TAACAAGGTTTCCGTAGGTG	1761-1780*	Sequencing
ITS	SGSITSR01	CTTATATGCTTAAATTCAGC	48-29**	Sequencing
SSU	SAX18F3	TGATCCGGTGAATAATTTGG	1664-1683*	Sequencing
SSU	S18F1-1	GTAATTCCAGCTCCAATAGC	576-595*	Sequencing
SSU	S18R2-1	CTATTGGAGCTGGAATTACCG	574-594*	Sequencing
LSU	SPM28F01	AAGCATATAAGTAAGCGGAG	38-57**	Sequencing
LSU	S28F341	AGCACACAAGTACCATGAGG	341-360**	Sequencing
LSU	S28-R328	TTCATATTTCCCTCATGGTACTTG	370-347**	Sequencing
LSU	S28F2-700	ACTGACGTGCAAATCGTTCG	930-949**	Sequencing
LSU	S28-F1499	TAGTGCAGATCTTGGTGGTAG	1478-1498**	Sequencing
LSU	SPM28-R1310	TGAGTTGTTACACACTCCTTAGC	1333-1311**	Sequencing
LSU	S28-R1483	TGCTACTACCACCAAGATCTGC	1503-1482**	Sequencing
LSU	SPM28-R2214	CTGGGCAGAAATCACATTGC	2232-2213**	Sequencing
LSU	SPM28-F2456	GACCCTGTTGAGCTTGACTC	2436-2455**	Sequencing
LSU	SPM28-F1997	GGATTGGCTCTGAGGGTTG	1978-1996**	Sequencing
LSU	SPM28-R2986	GTCTCACGACGGTCTAAACC	3003-2984**	Sequencing
5' ETS	S18R3-700	TATACTGAGACATGCATGGC	67-48*	Sequencing
5' ETS	SPM28-F2887	AGCCAAGCGTTCATAGCGAC	2866-2885**	Sequencing

[a]F, forward primer, R, reverse primer, S, sequencing primer.
[b]Corresponding to the numbering in the *A. tamarense* sequence (HY970328M) for 18S rDNA (*) and for LSU rDNA (**), respectively.

Table 2. Sequence length and G+C content (%) measured from *Alexandrium* species.

Locus	Content (%)				Complete length (bp)
	T (U)	C	A	G	
SSU rDNA	29.9	17.4	27.6	25.1	1,800
ITS1	33.9	16.4	24.2	25.5	165
5.8S	35.0	12.5	30.0	22.5	160
ITS2	32.8	16.9	25.1	25.1	195
LSU rDNA*	29.3	17.3	27.0	26.5	3393

Asterisk (*) represents the first reveled DNA sequence in *Alexandrium* genus

the Sequencher 3.0 software (Gene Codes, Ann Arbor, MI). Complete SSU, LSU rDNAs and ITS sequences including 5.8S rDNA were recorded at 1,800 bp, 520 bp and 3,393 bp, respectively. The SSU to LSU rDNA sequence has been deposited in GenBank as an accession no. AY831406.

Characteristics of SSU to LSU rDNA sequence

Each rDNA sequence was characterized as the G+C composition and length (Table 2). *Alexandrium tamarense* was measured at 29.9% of dT, 17.4% of dC, 27.6% of dT and 25.1% of dG within the complete SSU rDNA. The complete ITS1, 5.8S rDNA and ITS2 sequences were recored as 165 bp, 160 bp and 195 bp, respectively. The complete LSU rDNA sequence of *A. tamarense* was defined, as judged by the pre-revealed ITS 2 and inferred LSU rDNA secondary structure (Data not shown). The LSU rDNA nucleotide compositions were recorded as nearly identical to those of SSU rDNA, which were measured as 29.3% (dT), 17.3% (dC), 27.0% (dA), 25.1% (dG), respectively. Core sequences within the LSU rDNA were extracted from the complete sequence, and compared with the previously reported sequence of *Prorocentrum micans* (GenBank accession no. X16108), as shown in Fig. 2. The sequences were relatively variable (0.0867 of genetic distance, 91% sequence similarity) between them. In contrast, twelve D domains, including the core region, within the LSU rDNA from *A. tamarense* were characterized based on the nucleotide compositions and sequence length, as summarized in Table 3. Nucleotide of all the cores was at 1,879 bp and 44.3 of G+C contents, and that of 12 D domains showed a content of 1,514 bp and 42.8 of G+C, respectively. Of these D domains, the D2 domain was the longest region in length, which was at 300 bp and 43.6% of G+C content.

Phylogenetic relationship within *Alexandrium* genus, based on the complete SSU rDNA

A data matrix comprising 22 complete SSU rDNA sequences from *Alexandrium* genus was compiled in order to elucidate the phylogenetic relationships among the *Alexandrium* isolates. In a rooted NJ tree, the branch topology showed that the sequences of *Alexandrium* species were generally divided into three divergent groups (Fig. 3): group 1 (*A. affine, A. catenella, A. cohorticula, A. fundyense, A. tamarense, A. tamiyavanichi*), group 2 (*A. insuetum, A. margaelefi, A. minutum, A. ostenfeldii*) and group 3 (*A. pseudogonyaulax, A. taylori*), supported strongly in bootstrap analyses. Particularly within group 1, it was divided into three subclusters: *Affine* clade, *tamarensis* clade, *cohorticula* clade. *A. tamarense* could not be grouped clearly into the monophyletic clade.

Phylogenetic position of *A. tamarense*, inferred from complete LSU rDNAs

A phylogetic tree was inferred from the representative phytoplankton, including apicomplexa and ciliates, using their entire LSU rDNA sequences (37 strands and a new LSU rDNA sequence of *Alexandrium tamarense*) obtained form GenBank. In view of a broad phylogeny, the LSU rDNA sequences were clearly divided into three groups: alveolate, stramenopiles, and haptopyte+cryptomonad (Fig. 4). The Korean *A. tamarense* isolate was grouped into a dinoflagellate clade, which formed a monophyletic lineage. In mid-point phylogeny, the tree topology showed that the dinoflagellates were clustered with apicomplea rather than with ciliates, supporting a bootstrap value of 89%.

4. Discussion

The complete SSU or LSU rDNA of *Alexandrium tamarense* could be isolated by PCR amplification with specially developed primers, which were designed from the two end sides of both eukaryotic SSU or LSU rDNA sequence. To access complete 18S rDNA isolation, Medlin *et al.* (1988) used firstly two universal primers and isolated the complete 18S rDNA of *Alexandrium*, after which a lot of researchers have been using them to obtain PCR fragments of complete 18S rDNA from the harmful algae. Although they are valuable in PCR amplification of the complete SSU rDNA regions, the complementary nucleotides corresponding to the primer

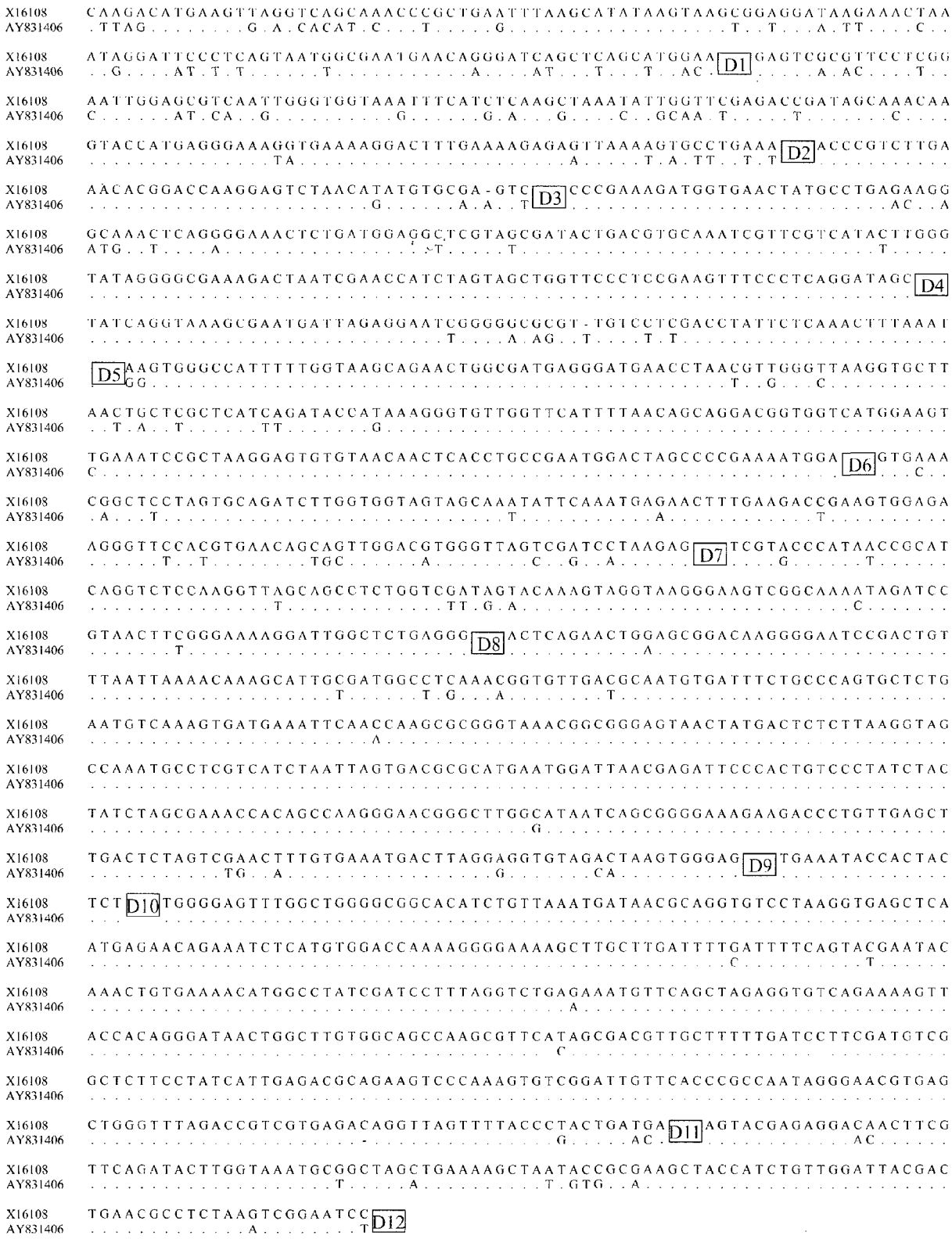

Fig. 2. An alignment of complete LSU rDNA conserved core sequences. A sequence for *Prorocentum micans* (GenBanK accession no. X16108) was as a reference (Lenaers *et al.* 1989). A period (.) represents a base identical to sequence located below the nucleotide of each alignment set, and a dash (-) denotes an alignment gap. The 12 variable domains and their positions are represented as boxes.

Table 3. Lengths (bp) and A, T, G, C contents (%) of different segments in the LSU rDNA of *Alexandrium tamarense* (HY970328M)

Locus	Content (%)				Complete length (bp)
	T (U)	C	A	G	
Core	25.6	18.1	30	26.3	1879
D domains					
Total D	33.9	16.2	23.2	26.6	1514
D1	31.7	15.5	25.4	27.5	142
D2	36.3	17.3	20	26.3	300
D3	28.0	15	28	29	107
D4	43.8	12.5	12.5	31.3	16
D5	34.6	7.7	30.8	26.9	52
D6	23.8	20.8	26.7	28.7	101
D7	32.3	19.3	22.8	25.6	285
D8	29.3	20	19.3	31.4	140
D9	41.7	16.7	8.3	33.3	12
D10	42.9	9.3	23.8	23.8	105
D11	30.8	11.5	42.3	15.4	26
D12	39.0	13.6	22.8	24.6	228

binding sites could not be adequately sequenced, since they were originally from the oligonucleotide sequences rather than from the template DNA of the genome. Thus, DNA sequencing could not detect variations in primer binding sites. Ki and Han (2005) have recently developed a simple ETS walking PCR method, followed by the subsequent nested PCR. With this approach, the unknown regions adjacent to the known rDNA could be easily amplified and sequenced. Thus, we have applied this method to *A. tamarense* and could obtain approximately 400 nucleotides individually from both the upstream 5' ETS of SSU rDNA and the downstream 3' ETS of LSU rDNA.

As noted previously, the complete SSU rDNA, including ITS regions, and the partial LSU rDNA from the harmful dinoflagellates have already been sequenced, and many are now available and accessible easily at public databases (*e.g.* EMBL/DDBJ/GenBank). In fact, many complete SSU rDNA and ITS regions from *Alexandrium* genus have been revealed and their sequences have been deposited in GenBank. However, the LSU rDNA sequences from the *Alexandrium* have been focused mostly on their D1 and D2 regions; while none of the complete sequences have been reported in the *Alexandrium* genus.

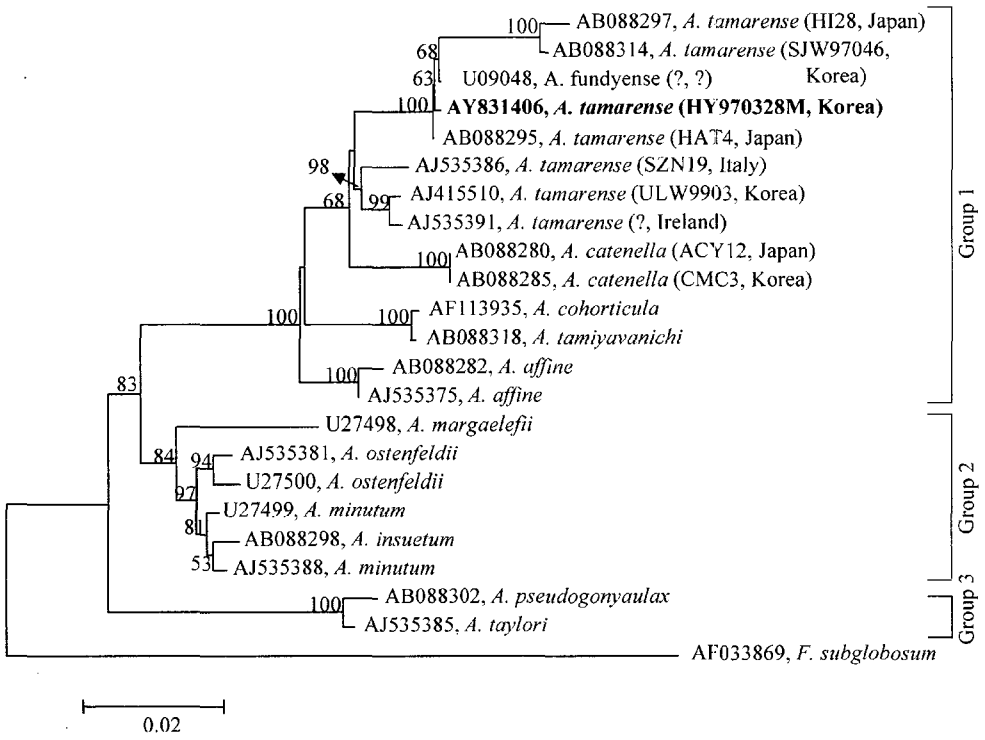

Fig. 3. Phylogenetic relationship of *Alexandrium* species, particularly focusing on *A. tamarense*, based on the completely aligned 18S rDNA sequences (22 strands). DNA sequence determined in this study is in bold. The NJ tree was constructed using the Kimura 2-parameter method (Kimura *et al.* 1980) and 1,000 bootstrapped replicates in MEGA3. *Fragilidium subglobosum* was included as the outgroup. Bootstrap values of less than 50% are not shown. Branch lengths represent genetic distance among the taxa.

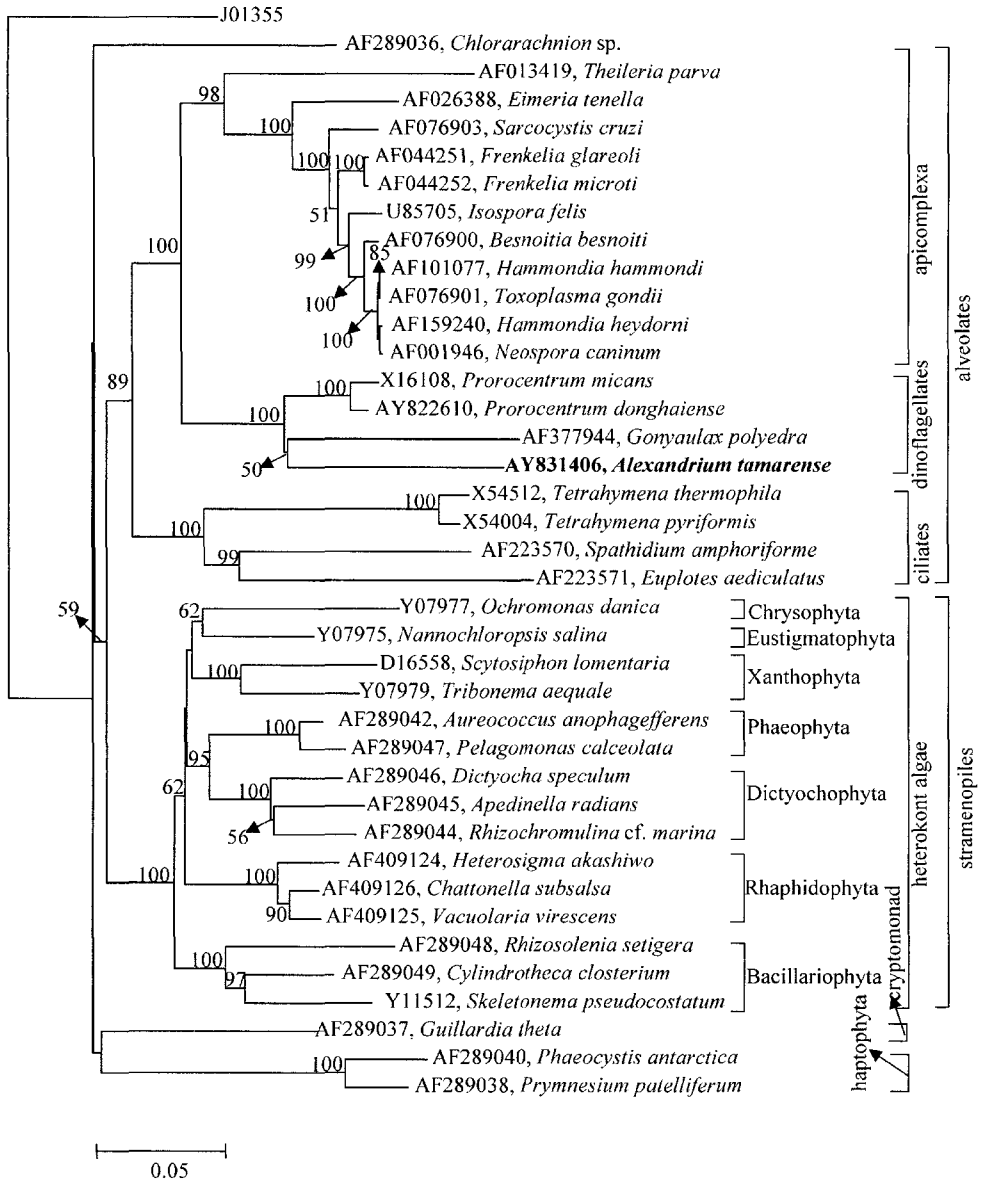

Fig. 4. Phylogenetic position of the genus *Alexandrium tamarense,* based on nearly complete LSU rDNA sequences inferred using neighbor-joining method in MEGA3. The yeast *Saccharomyces cerevisiae* was included as the outgroup. The numbers above internal branches represent the values of bootstrap support (higher than 50%) in NJ (Kimura 2-parameter) analysis. Branch lengths represent genetic distance among the taxa. That which appears in bold represents the sequence determined in this study.

Furthermore, few sequence data comprising the entire SSU to LSU rDNA sequences from the same strains or isolates have been reported. To address these issues, we designed new PCR primers for the complete isolation of SSU to LSU rDNA from the dinoflagellates, and had applied these primers for the PCR-isolation of complete SSU to LSU rDNA from *A. tamarense*, as a model organism, and could reveal its entire sequence.

The set of primers designed here was selected to amplify the genomic DNA for those less than 1.5 kb in length, although the size of PCR fragments varied from about 1 kb to maximum 7 kb with adjusting forward and reverse primer pairs. Of the various sizes of PCR products, a length under 1.5 kb was considered suitable for PCR amplification based on various quality DNA templates. Actually, the PCRs in this study have been used for DNA templates without phenol/chlroroform purification of the samples. Thus, the PCR fragments must partially overlap

each other and assemble to form almost the entire SSU to LSU rDNA sequence. In like manner, the PCRs, employing the new primers, could also amplify the complete rDNA in phytoplankton. For example, we could obtain the PCR fragments of rDNA from the gnomic DNA isolated from other genera *Akashiwo, Gymnodinium, Gyrodinium, Chattonella, Peridinium* and *Heterosigma* (data not shown). This result showed that all of primers used here could generally be applicable for PCR amplification of the rDNA from HAB species.

The complete SSU rDNA sequence from a Korean strain HY970328M was recorded as being 1,800 bp in length, and none of introns was found in the rDNA sequence. The complete sequence of *Alexandrium tamarense* ranged from 1,753 to 1,800 bp in length, in which the variation occurred according to the isolated origins (*e.g.* 1,800 bp for Asian strains, around 1,770 bp at European strains). In the phylogenetic tree of the complete SSU rDNA sequence, *A. tamarense* was clearly clustered into the same species. However, the G+C content in the HY970328M strain was measured at 42.5%, which was in good agreement with those obtained by calculation of *A. tamarense* sequences retrieved from GenBank. In a previous study, Scholin and Anderson (1996) reported that variations of SSU rDNA sequence had occurred according to the geographically segregated cells, despite the 18S rDNA sequences being highly conserved within the same species. So far, many studies concerning with each *Alexandrium* relationship have been performed by using molecular tools (Adachi *et al.* 1994; Scholin *et al.* 1994; Scholin and Anderson 1996; Hirashita *et al.* 2000; Sako 2000; Usup *et al.* 2002; Kim and Kim 2004; Kim *et al.* 2004). The results, however, have not been clearly resolved so far, and further studies are therefore needed, particularly based on the morphological observations together with molecular analyses of the same strain.

The LSU rDNA has been, alternatively, considered as useful regions for revealing the relationship of *Alexandrium* based on keeping the geographical origins of the cells in mind. Most studies, however, have been concerned with the D1 and D2 domains rather than the complete sequence, while the LSU rDNA has 12 D-domains within there. We first sequenced the complete LSU rDNA of *A. tamarense*. It was recorded as being 3,393 bp in length, which was slightly shorter than that of the dinoflagelate *Prorocentrum micans* (accession no. X16108; 3408 bp), whereas it was longer than that of *P. donghaiense* (accession no. AY822610; 3376 bp). In addition, it was generally longer than that of a diatom *Rhizosolenia setigera* (accession no. AF289048; 3331 bp), but somewhat shorter than that of other protist LSU rDNAs such as the apicomplexa *Toxoplasma gondii* (accession no. AF076901; 3212 bp) and *Frenkelia microti* (accession no. AF044252; 3283 bp), and the ciliate *Tetrahymena thermophila* (accession no.: X54512; 3347 bp).

The actual G+C content of *A. tamarense* LSU rDNA gene was 43.8%, which was smaller than those estimated from other dinoflagellates *Crypthecodinium cohnii* (46.5%; Rae 1970), *Prorocentrum micans* (accession no. X16108; 47.6%) and *Peridinium cinctum* (Gressel *et al.* 1975; 49.6%), about 44.5% at three apicomplexa (*Eimeria tenella*, accession no. AF026388; *Plasmodium falciparum*, accession no. U21939; *Toxoplasma gondii*, accession no. X75429), and the ciliate *T. thermophila* (accession no. X54512; 44.8%). It was significantly lower than the protists *Crithidia fasciculata* (accession no. Y00055; 51.2%), *Physarum polycephalum* (accession no. V01159; 53.5%), and vertebrates (up to 69.1% in *Homo sapiens*). In a broad comparison of the representative phytoplankton (38 strands in Fig. 4 dataset), including apicomplexa and ciliates, the general pattern for length was not found in the complete LSU rDNA, whereas the G+C content was similar to those of the two alveolates, apicomplexan and ciliate. In phylogenetic analysis, the individual broad-taxonomic groups (*e.g.* apicomplexan, ciliate, dinoflagellate, heterokont algae) were sufficiently clustered around each other as in the previous study (Ben Ali *et al.* 2001). Particularly, *Alexandrium tamarense* was separated at a high resolution compared with other species. However, we could not investigate the *Alexandrium* relationship here, because the DNA sequence data of *Alexandrium* is very limited. To reveal their phylogenetic relationships clearly, further studies are therefore needed to determine the nucleotide sequences of the rRNA and/or genes of many organisms including a wide range of taxa.

In summary, this study had applied new PCR primers for the isolation of complete SSU to LSU rDNA regions, and reported here for the first time the complete LSU rDNA sequence from the dinoflagellate *Alexandrium tamarense*. The new primer pairs developed here can generally be applicable for the isolation of complete SSU to LSU rDNA from harmful algae. Phylogenetic inference

showed that the complete LSU sequences were considered as an alternative region in the broad phytoplankton relationships due to high resolution. We will expect this region to resolve clearly the relationships of *Alexandrium* genus in the future.

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