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Molecular Phylogenetic Relationships Within the Genus *Alexandrium* (Dinophyceae) Based on the Nuclear-Encoded SSU and LSU rDNA D1-D2 Sequences

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LSU rDNA D1-D2 and SSU rDNA genes of 23 strains in seven *Alexandrium* (Halim) species, *A. tamarense* (Lebour) Balech, *A. catenella* (Whedon et Kofoid), *A. fraterculus* (Balech) Balech, *A. affine* (Inoue et Fukuyo) Balech, *A. insuetum* Balech, *A. pseudogonyaulax* (Biecheler) Horiguchi ex Yuki et Fukuyo and *A. tamiyavanichii* Balech, were sequenced and the data were used for molecular phylogenetic analysis. The sequence data revealed 11 and 7 ribotypes in the LSU rDNA D1-D2 region and 4 and 17 ribotypes in the SSU rDNA region of *A. catenella* and *A. tamarense*, respectively. Other *Alexandrium* species had also 1 to 5 ribotypes in the two regions. With the exception of CMC2 and CMC3 of *A. catenella*, all *A. tamarense* and *A. catenella* strains had a common ribotype, a functionally expressed rRNA gene (here termed type A), in both gene regions. In addition to the functionally expressed gene, several pseudogenes were obtained that were found to be good tools to analyze the population designation of regional isolates by grouping them according to shared ribotypes. From the phylogenetic analysis of the sequence data determined in this study and retrieved from GenBank, the genus *Alexandrium* was divided into 14 groups: 1) *A. tamarense*, 2) *A. excavatum*, 3) *A. catenella*, 4) Tasmanian *A. tamarense*, 5) *A. affine* (and/or *A. concavum*), 6) Thai *A. tamarense*, 7) *A. tamiyavanichii*, 8) *A. fraterculus*, 9) *A. margalefii*, 10) *A. andersonii*, 11) *A. ostenfeldii*, 12) *A. minutum* (or *A. lusitanicum*), 13) *A. insuetum*, and 14) *A. pseudogonyaulax*. The SSU rDNA gene sequence of *A. fundyense* was so similar to those of *A. tamarense* used in this study that the two species were difficult to discriminate each other. *A. tamiyavanichii* was closest to the *A. tamarense* strain isolated in Thailand and close to the long chain-forming species of *A. affine* and *A. fraterculus*. The phylogenetic tree showed that *A. margalefii*, *A. andersonii*, *A. ostenfeldii*, *A. minutum* and *A. insuetum* constituted the basal relative complex, and that *A. pseudogonyaulax* is an ancestral taxon in the genus *Alexandrium*.

Key words: *Alexandrium*, LSU rDNA (Large Subunit Ribosomal DNA), SSU rDNA (Small Subunit Ribosomal DNA), Phylogenetic tree, NJ, ML, MP Methods

INTRODUCTION

The marine dinoflagellate genus *Alexandrium* is the major toxic phytoplankton responsible for paralytic shellfish poisoning (PSP) throughout the world. PSP toxin producers within the genus *Alexandrium*, particularly *A. tamarense* and *A. catenella*, have caused high-level intoxication of fishery products and human fatalities. In recent years, these deleterious species have apparently been increasing globally (Hallegraeff, 1993).

Studies on *Alexandrium* have mainly focused on the designation of morphotypic species and the detection of toxin profiles including toxin composition and productivity in relation to taxonomy and field monitoring. Morphological identification of the more than 20 species of *Alexandrium* based on criteria that usually involve microtabulation of fine thecal plates is not clear-cut, however, because of the occurrence of very closely related species complexes, especially within the toxic species of *A. tamarense*, *A. fundyense*

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and *A. catenella*, and the existence of morphologically intermediate species (Taylor, 1984).

Though toxin productivity has been considered as a variable biochemical character, depending on environmental features such as physical and chemical traits of marine habitats rather than species (Prakash, 1967; Proctor *et al.*, 1975; Shimizu, 1978; Alan *et al.*, 1979; Hall, 1982; Maranda *et al.*, 1985; Boyer *et al.*, 1986; Ogata *et al.*, 1987; Anderson, 1990; Sako *et al.*, 1993; Anderson, 1997), we recently found that the toxin components produced by *A. tamarense* and *A. catenella* of Korean isolates were clearly species-specific (Kim, 2000). Those results should provide one criterion in species or population designation, at least for Korean *Alexandrium* species. Although PSP toxin components are species-specific in *Alexandrium*, toxin analysis is too time-consuming to be used to distinguish the species systematically.

Since the late 1980s, a number of molecular biological approaches have been applied to detect the species-specific signatures in the genus *Alexandrium*. These include enzyme electrophoretic pattern analysis (Cembella and Taylor, 1986; Cembella *et al.*, 1988; Hayhome *et al.*, 1989: Sako *et al.*, 1990; Bolch *et al.*, 1999), RFLP (restriction fragment length polymorphism) analysis (Boczar *et al.*, 1991; Adachi *et al.*, 1994: Scholin *et al.*, 1994: Scholin and Anderson, 1994), immunological analysis (Adachi *et al.*, 1993a; Vrieling *et al.*, 1993: Mendoza *et al.*, 1995; Penna and Magnani, 2000; Costas and Lopez-Rodas, 1994), and RAPD (random amplified polymorphic DNA) (Bolch *et al.*, 1998; Adachi *et al.*, 1997). The construction of systematic molecular phylogenetic relationships within the genus *Alexandrium* has been considerably advanced by the works of Scholin *et al.* (1994) and Adachi *et al.* (1996). Scholin *et al.* (1994) analyzed LSU rDNA D1-D2 sequences and clarified the taxonomical confusion. They divided the 'tamarensis species complex' into five types: North American, Western European, Temperate Asian, Tasmanian, and tropical Asian. They also subdivided the North American type into Western, Eastern and Alternate, and the Temperate Asian type into Korean and Japanese populations, and suggested that these regional populations were distinct and that morphological description of the 'tamarensis species complex' provides less clear species-identification criteria than the LSU rDNA gene. They showed that *A. affine*, *A. minutum*, *A. lusitanicum* and *A. andersonii* were genetically distinct from the 'tamarensis species complex'. An interesting result was that the intermediate non-toxic Western European cluster included Japanese WKS-1. From their result, the genus *Alexandrium* investigated was classified phylogenetically into eight distinct species: 1) North American *A. tamarense* complex (including *A. fundyense* and *A. catenella*), 2) Western European *A. tamarense*, 3) Temperate Asian *A. catenella* (including *A. tamarense*), 4) Tasmanian *A. tamarense*, 5) Tropical Asian *A. tamarense*, 6) *A. affine*, 7) *A. minutum* (and *A. lusitanicum*), 8) *A. andersonii*. Adachi *et al.* (1996) built phylogenetic relationships among *Alexandrium* using *A. tamarense*, *A. fundyense* and *A. catenella* together with *A. insuetum*, *A. pseudogonyaulax* and *A. affine* by analyzing 5.8S rDNA and flanking internal transcribed spacers 1 and 2 (ITS1 and ITS2). Their results were similar to those of Scholin *et al.* (1994), showing that *A. tamarense* type, WKS-1 type, *A. catenella* type, and Thai type could be separated from each other. The above two reports showed that genetic comparison gave more objective resolution than morphological criteria in the taxonomy of the *Alexandrium* species complex. Scholin *et al.* (1993) examined two distinct SSU rRNA genes, a functionally expressed rRNA gene and a pseudogene (or pseudogene-like sequence), from *A. fundyense*, and Scholin *et al.* (1995) discussed on putative evolution of the *Alexandrium* based on the LSU and SSU rRNA genes.

The present study was prompted by our interest in the molecular phylogenetic positions and relationships of the toxic and non-toxic *Alexandrium* occurring in Korean coastal waters, the massive occurrence of toxic *A. tamiyavanichii* in Japan, and the paucity of sequence data of the SSU rDNA of *Alexandrium*.

Here, we aimed to construct the molecular phylogenetic relationships by sequencing the LSU rDNA D1-D2 region of 16 morphotypic species (including 3 newly sequenced morphotypic species, *A. tamiyavanichii*, *A. fraterculus* and *A. pseudogonyaulax*), and the SSU rDNA region of 12 morphotypic species (including 6 new14 sequenced morphotypic species, *A. catenella*, *A. tamiyavanichii*, *A. fraterculus*, *A. affine*, *A. insuetum* and *A. pseudogonyaueax*). The sequence data were also used for comparative analysis of regional populations using functionally expressed rDNA genes and pseudogenes.

MATERIALS AND METHODS

Strains and culture condition

The unialgal cultures used in this study were clonally isolated from Korean and Japanese coastal waters

Table 1. *Alexandrium* strains used in sequence determination of the LSU rDNA D1-D2 region and SSU rDNA full length for molecular phylogenetic analysis

Morphotypic species	Strains	Origin
Alexandrium affine	AFF37	Harima Nada, Japan
A. catenella	ACY12	Harima Nada, Japan
A. catenella	CMC2	Tongyoung, Korea
A. catenella	CMC3	Tongyoung, Korea
A. catenella	DPC7	Pusan Tadaepo, Korea
A. catenella	DPC8	Pusan Tadaepo, Korea
A. catenella	YSC9811	Yosu, Korea
A. fraterculus	DPW9709	Pusan Tadaepo, Korea
A. fraterculus	SJW9709	Chinhae Bay, Korea
A. insuetum	AI104	Utiumi Bay (Shoudo shima), Japan
A. pseudogonyaulax	AP391	Harima Nada, Japan
A. tamarense	HAT4	Hiroshima Bay, Japan
A. tamarense	HI12	Hiroshima Bay, Japan
A. tamarense	KJC97111	Kojedo, Korea
A. tamarense	SJC9516	Chinhae Bay, Korea
A. tamarense	SJC9522	Chinhae Bay, Korea
A. tamarense	SJW97042	Chinhae Bay, Korea
A. tamarense	SJW97046	Chinhae Bay, Korea
A. tamarense	UL7	Ulsan, Korea
A. tamarense	ULW9903	Ulsan, Korea
A. tamiyavanichii	TAMI2201	Harima Nada, Japan
A. tamiyavanichii	TAMI2207	Harima Nada, Japan
A. tamiyavanichii	TAMI22012	Harima Nada, Japan

(Table 1). The strain AFF37, AI104, TAMI2201, TAMI2207, TAMI22012 and AP391 were kindly donated by Dr. Yoshimatsu. All cultures were maintained under a 14:10 L:D regime at 100 $\mu Em^{-2}S^{-1}$ with cool white bolbs at 15°C for *A. tamarense* and 20°C for all the others and grown on SW II medium (Sako *et al.* 1990) in 2.8-L Fernbach flasks. All cultures were identified morphologically by criteria of Fukuyo (1985).

Total DNA/RNA extraction and purification, and cDNA synthesis

Cells were harvested at the growth stage between the late exponential and the early stationary phase by centrifugation for 5 min at 2000 rpm at room temperature. Pelleted cells were stored at −80°C and completely grounded in liquid nitrogen using a pestle and mortar. Total DNA was extracted using Sepa-Gene kit (Sanko Junyaku Co., Ltd., Tokyo, Japan), and digested using RNase A. Finally extracted total DNA was purified with ethanol precipitation and then dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Total RNA was extracted from representative *Alexandrium* isolates (KJC97111, ULW9903, HAT4, DPC7 and ACY12 for LSU rDNA, and KJC97111, ULW9903, HAT4, DPC7 and SJC9516 for SSU rDNA) at the exponential growth phase. Harvested cells were rapidly frozen in liquid nitrogen and grounded as described at total DNA extraction. Total RNA extraction was carried out by acid phenol method. cDNA synthesis was performed by RT-PCR (reverse-transcriptase polymerase chain reaction) using cDNA synthesis kit (GIBCOBRL, Rockville, MD, USA) with random oligonucleotide primers (6 nt) according to the manufacturer's instructions

Cloning and sequencing

Approximately 700 nt of the LSU rDNA/rRNA D1-D2 and 1800 nt of SSU rDNA/rRNA were amplified by PCR (Saiki *et al.*, 1988) with the primers designed by Scholin *et al.* (1994) and Bergquist and Reeves (1995, unpublished), respectively. PCR amplification reactions for both regions consisted of denaturation at 96°C for 3 min as an initial step, then 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 1 min, and extension at 72°C for 2 min. Extension

Table 2. Oligonucleotide inner-primers used in sequencing LSU DNA D1-D2 and SSU rDNA full length from seven *Alexandrium* species. Sequences are listed 5' to 3'

Regions	Primers[a]	*A. affine*	*A. catenella*	*A. fraterculus*	*A. insuetum*
LSU	in1F	TGAGGGTGAGAATCCTGTTT (183-202)	GCCATTTGAAAGAAAGCATC (161-180)	AAGAAAGTGTCAATGAAGGT (170-190)	TGAGGGTGAGATTCCTTTTT (183-202)
	in1R	GCACCAGACACATGTATCAA (596-577)	CCGCAGGAAAATTACCATTC (540-521)	CAAATAACTAGCACACACAG (497-478)	ATCATTGCAAACACATGCAT (570-551)
SSU	In1F	TGCAACTAAACCTAACTTTG (171-190)	GGTTGTGGTCGTTGGTTGCA (197-216)	TGTGTCAACACCTGACTTGGG (171-191)	GCATCAAAACCTGACTTCTG (173-192)
	in1R	CTAGGAATTCCTCGTTTAAG 1589-1570)	CACAAGCTGATGACTCAAGC (1592-1611)	GGGCAGGGACATAATCAGCAC (1612-1632)	GACGGGCGGTGTGTACAAAG (1632-1651)
	in2F	ATTACCCAATCCTAGCACAG (441-460)	TAAGGAAGGCAGCAGGCGCG (418-437)	GGCACAGGGAGGTAGTGACA (454-473)	GGCTACCACATCTAAGGAAG (406-425)
	in2R	GAATTAACCAGACAAATCAC (1319-1300)	CACACAAAGTCCCTCTAAGA (1491-13820	CCAGACAAATCACTCCACCA (1312-1293)	CATCTAAGGGCATCACAGAC (1453-1434)
	in3F	GCACAACTGCACTTGACTGT (707-726)	CAGCCTGAGCATTTATCTTG (684-703)	ACTGCACTTGACTGTGTGGT (712-731)	GGGTGAGTATCTGGCACAGC (668-687)
	in3R	CACTTTGATTTCTCATAGGG (1099-1080)	GACCATACTCCCCCCGGAAC (1123-1104)	GGTATCTGATCGTCTTCGAT (1006-987)	GGTAAGTTTCCCCGTGTTGA (1213-1094)
Regions	Primers	*A. pseudogonyaulax*	*A. tamarense*	*A. taniyavanichii*	
LSU	in1F	AGCGGAGGTACAGTTGCAAG (143-162)	TCCTGTTTGTCATGTGCAGCC (195-215)	GCTGGCACACAAAGCAAGAATC (171-192)	
	in1R	ATGCAATCTAACACCCGCAA (550-531)	CATGTGCAAAGGTAATCAGAT (521-501)	GCTGGCACACAAAGCAAGAATC (488-467)	
SSU	In1F	CTTACATGGATAACTGTGGT (134-153)	GGGGAAAGGTTGTGGTCGTT (189-208)	AGAAAGCGTCAATGAGGGTGAG (190-208)	
	in1R	ACTCAATCGGTAGGAGCGAC (1666-1647)	GCTGATGACTCAAGCTTACT (1605-1586)	GGGAAAGGTTGTGGTCGTT (1603-1584)	
	in2F	AATGGCTACCACATCTAAGG (404-423)	AAGGCAGCAGGCGCGCAAAT (421-440)	GGGAAAGGTTGTGGTCGTT (458-477)	
	in2R	TTGCCCACATAGCCGGAGTT (1360-1379)	CACAAAGTCCCTCTAAGAAG (1396-1377)	CAGGGAGGTAGTGACAAGAA (1400-1381)	
	in3F	TGGGTGAGTATCTGGCACAA (668-687)	CTGCACTTGACTGTGTGGTG (711-730)	CACAAAGTCCCTCTAAGAAG (714-733)	
	in3R	GTTTCAGCCTTGCGACCATA (1136-1117)	CCCCCGGAACCCAAACACT (1113-1094)	TGCACTTGACTGTGTGGTGT (1094-1075)	

[a]F and R indicate forward and reverse direction, respectively.

at 72°C was performed for 7 min as a final step. PCR products were subjected to electrophoresis on 1.2% agarose gel and stained with ethidium bromide. The DNA fragments were sliced from the gel, transferred to 1.5-ml microtubes, eluted from gel slices, and purified with phenol and PCI (phenol:chloroform:isoamylalcohol=25:24:1) extraction reagents. Finally, purified PCR products were dissolved in TE buffer, ligated into a vector (pGEM®-T Easy Vector, Promega) according to the manufacturer's instructions and tranformed into *Escherichia coli* competent cells (INVαF'). Ten to 20 clones were selected for each *Alexandrium* isolate, and plasmids containing the insert were purified with Miniprep kit (Bio-Rad). Six to 12 clones were sequenced using a Taq Dye Deoxy Terminator Cycle Sequencing Ready Reaction Mixture kit (Amersham Biosciences). Sequence reactions were run on an ABI 373A automated sequencer (Applied Biosystems). Oligonucleotides and sites of the inner primers used in this study were listed in Table 2. Nucleotide sequences obtained from each clone were analyzed using the software DNASIS-Mac (Hitachi Software Engineering Co, Ltd.). All new sequences were deposited in the EMBL/DDBJ/GenBank (Accession number AB088225 to AB088342).

Molecular phylogenetic analysis

Sequence data obtained in this study were compared with those retrieved from GenBank (Table 3) to determine the phylogenetic relationships and taxonomic positions. Sequences were aligned using ClustalX (Thompson *et al.*, 1997), revised by removal or correction of uncertain sites manually to increase alignment similarity, and transferred to PHYLIP Format. Phylogenetic analyses were carried out using the PHYLIP package (Version 3.572c, Felsenstein, 1995). Bootstrapping confidences (Felsenstein, 1985) were generated from SEQBOOT, and distance matrix was calculated from DNADIST using Kimura's two-parameter collection (Kimura, 1980) and converted into evolutionary phylogenetic trees inferred from NJ (Saitou and Nei, 1987) with NEIGHBOR. Phylogenetic trees inferred from MP (maximum parsimony) and ML (maximum likelihood) algorithms (Felsenstein, 1981) were subjected to DNAMP and DNAML using F84 DISTANCE MODEL (Felsenstein, 1984), respectively. The addition of taxa were jumbled, ts/tv ratios of 2.9 were selected, and data sets of 100 were conducted using empirical base frequencies with one category of substitution rate. Consensus reconstruction trees were inferred from CONSENSE. Bootstrap reliability of robustness of topologies was carried out with 1000, 100 and 100 replications for NJ, ML and MP, respectively.

RESULTS

Sequence data of LSU rDNA D1-D2 and the phylogenies inferred from our data

Sequences of the LSU rDNA D1-D2 region were determined for 23 strains in seven *Alexandrium* species: *A. tamrense*, *A. catenella*, *A. fraterculus*, *A. affine*, *A. insuetum*, *A. pseudogonyaulax* and *A. tamiyavanichii*. The results are summarized in Table 4. From the sequence data, *A. tamarense* strains horbored 7 ribotypes (A to G), and *A. catenella* strains horbored 11 ribotypes (A to K). Functionally expressed rRNA examined in all *A. tamarense* strains was the same type, which had no sequence variations and so was that of *A. catenella*. Each strain of *A. catenella* and *A. tamarense* harbored a common rRNA type (functionally expressed, type A), with the exception of strains CMC2 and CMC3 of *A. catenella*.

A. tamarense strains had a common rDNA segment of 706 nt in length with the exception of HAT4 (705 nt, type F) harboring indel at three positions, 126 (T insertion), 130 (A deletion) and 167 (G deletion). Type F was thus the most heterogeneous among the *A. tamarense* ribotypes (sequence data not shown). Strains ULW9903 and UL7 of *A. tamarense* shared type B, which was not found in other strains of this species.

The gene length of *A. catenella* (709 nt) was longer than that of *A. tamarense* by three nucleotides indel at two sites, 574 and 596-597. Gene clones from all *A. catenella* strains had the same length without any indel, despite a number of substitutions within the ribotypes. *A. catenella* strains DPC7, DPC8 and YSC shared common gene types A, E, I and H, and DPC8 and YSC shared type B. CMC2 (ribotypes C, F, J and K), CMC3 (D) and ACY12 (G) did not share any common types.

Strain AFF37 of *A. affine* harbored two ribotypes, A and B, each of 711 nt, which had only one difference in sequence. Three strains of *A. tamiyavanichii*, TAMI2201, TAMI2207 and TAMI22012, harbored ribotype A of 710 nt, and TAMI2207 also had B and C ribotypes. Two strains of *A. fraterculus*, DPW9709 and SJW9709, harbored only one ribotype of 717 nt, the longest among the *Alexandrium* examined in this

Table 3. Taxa sampled from GenBank for molecular phylogenetic analysis of the genus *Alexandrium* inferred from LSU rDNA D1-D2 and SSU rDNA sequences

Taxon	Strains	Origin	GenBank acce. no	Gene regions[a]
A. affine	CU1	Gulf of Thailand	U44935	L
A. affine	Pa4V	Concarneau Bay of France	AF318229	L
A. affine	X21	Spain.	L38630	L
A. andersonii	TCO2	Eastham, MA, U.S.A.	U44937	L
A. catenella	11bis	France	AF318220	L
A. catenella	X11	France	AF318219	L
A. catenella	A3	Denmark	AF200667	L
A. catenella	K-0270	Denmark	AF200666	L
A. catenella	HK1998	Hong Kong, China	AF118547	L
A. catenella	HK1989	Hong Kong, China	AF118546	L
A. catenella	ACBOPNZ	New Zealand	AF019408	L
A. catenella	K-0270	Denmark	AF200667	L
A. catenella	ACQH01	Sweden	AY056823	L
A. cohorticular	-	Malaysia	AF174614	L
A. cohorticular	ACMS01	Malaysia	AF113935	S
A. concavum	-	North Island, New Zealand	AF032348	L
A. excavatum	PEV1	Sweden	AY056824	L
A. excavatum	Ge1V	Spain	L38632	L
A. fundyense	-	USA	U09048	S
A. insuetum	X6	Corsica, France	AF318233	L
A. lusitanicum	Al18V	Spain	L386231	L
A. margalefii	-	Bream Bay, New Zealand	AF033531	L
A. margalefii	-	New Zealand	U27498	S
A. minutum	X10	France	AF318221	L
A. minutum	Al1V	Spain	L38626	L
A. minutum	-	New Zealand	U27499	S
A. ostenfeldii	-	New Zealand	AF033533	L
A. ostenfeldii	-	New Zealand	U27500	S
A. tamarense	UW4-1	UK	AJ303447	L
A. tamarense	UW4-2	UK	AJ303448	L
A. tamarense	Alex61-1	UK	AJ303446	L
A. tamarense	Alex61-1	UK	AJ303445	L
A. tamarense	AlexW12	UK	AJ303443	L
A. tamarense	AlexW7	UK	AJ303440	L
A. tamarense	K-0055	Denmark	AF200668	L
A. tamarense	-	Plymouth, U.K	AF033534	L
A. tamarense	ATBB01	Bell Bay, Tasmania, Australia	U44933	L
A. tamarense	CU13	The Gulf of Thailand, Thailand	U44934	L
A. tamarense	MUCC99	Canada	AF022191	S
A. tamarense	-	Canada	X5494	S

[a]L and S indicate LSU and SSU rDNA.

study. *A. insuetum* harbored two ribotypes of 716 nt. *A. pseudogonyaulax* also harbored two ribotypes of 701 nt, the shortest found, which differed in having substitutions at two sites.

Fifty-five sequence data newly obtained in present study were subjected to pylogenetic analysis. The results using MP, ML and NJ methods revealed that these strains distributed into 7 distinct groups of *A. tamarense* clade, *A. catenella* clade, *A. affine* clade, *A. tamiyavanichii* clade, *A. fraterculus* clade, and *A.*

Table 4. rDNA (and rRNA) sequence types and its length examined from 7 *Alexandrium* 23 strains isolated from Korea and Japan

Species	Strain	LSU D1/D2		SSU	
		Gene type[a]	Length	Gene type	Length
A. affine	AFF37	A	711	A	1802
		B	711		
A. catenella	ACY12	rRNA	709	rRNA	1801
		A	709	A	1801
		G	709		
	DPC7	rRNA	709	rRNA	1801
		A	709	A	1801
		E	709		
		H	709		
		I	709		
	DPC8	A	709	A	1801
		B	709	C	1801
		E	709		
		H	709		
		I	709		
	CMC2	C	709		
		F	709		
		J	709		
		K	709		
	CMC3	D	709		
	YSC9811	A	709	A	1801
		B	709	B	1801
		E	709		
		H	709		
A. fraterculus	DPW9709	A	717	A	1801
	SJW9709	A	717	A	1801
A. insuetum	AI104	A	716	A	1804
		B	716		
A. pseudogonyaulax	AP391	A	701	A	1802
		B	701		
A. tamarense	HAT4	rRNA	706	rRNA	1800
		A	706	A	1800
		F	705	B	1800
				C	1800
				I	1799
	HI28	A	706	A	1800
				Q	1800
	KJC97111	rRNA	706	rRNA	1800
		A	706	A	1800
		E	706	O	1798
	SJC9516	A	706	rRNA	1800
		C	706	A	1800
				F	1800
				G	1802
				H	1797
				M	1800
				N	1798
	SJC9522	A	706	A	1800
A. tamarense	SJW97043	A	706	A	1800
		D	706	O	1798
	SJW97046	A	706	A	1800
		G	706	J	1798
	UL7	A	706	A	1800
		B	706		
	ULW9903	rRNA	706	rRNA	1800
		A	706	A	1800
		B	706	D	1800
				E	1800
				K	1798
				L	1801
				P	1798
A. tamiyavanichii	TAMI2201	A	710	A	1800
	TAMI2207	A	710	A	1800
		B	710	B	1800
		C	710	C	1800
				D	1800
				G	1800
				H	1800
	TAMI22012	A	710	A	1800
				E	1800
				F	1801
				F	1801

[a]Gene type was determined from the approximate DNA distance based on the rRNA types (type A) functionally expressed in *A. tamarense* and *A. catenella*, and on the common rDNA type (type A) in the other *Alexandrium* species.

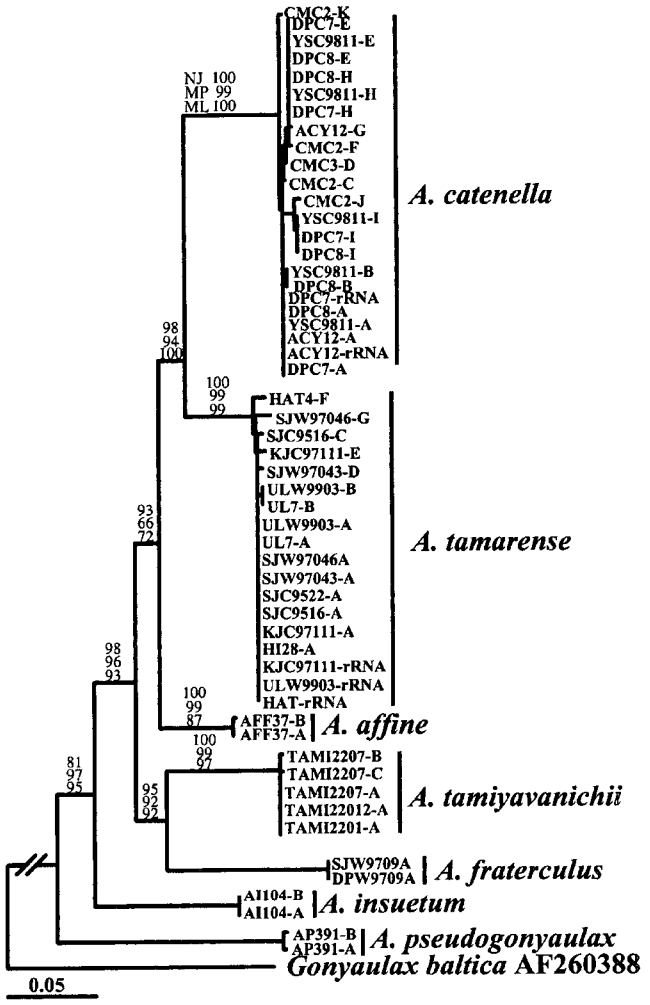

Fig. 1. Putative molecular phylogentic tree inferred from LSU rDNA sequences of regional strains of the genus *Alexandrium* determined in this study. The tree topology was constructed by the N-J method. Bootstrap values are shown for NJ, MP and ML at the internodes. *Gonyaulax baltica* was used as an outgroup taxon. Scale bar indicates 5% divergence.

pseudogonyaulax clade (Fig. 1). The clades of *A. tamarense* and *A. catenella* were clearly divided as distinct sister groups. *A. fraterculus* and *A. tamiyavanichii* were also sister groups, and they were diverged prior to the *A. affine*, *A. catenella* and *A. tamarense*. *A. pseudogonyaulax* was the ancestral taxon among the *Alexandrium* species examined, with high bootstrap values (NJ:MP:ML:81:97:95).

Twenty-nine sequences determined in present study and 35 sequences retrieved from GenBank (Table 3) were used to analyze the phylogenetic relationships within the genus *Alexandrium*. Phylogenetic analysis using the NJ method showed that they were divided into 14 groups: *A. tamarense*, *A. excavatum*, *A. catenella*, Tasmanian *A. tamaremse*, *A. affine* (and/or *A. concavum*), Thai *A. tamarense*, *A. tamiyavanichii*, *A. fraterculus*, *A. margalefii*, *A. andersonii*, *A. ostenfeldii*, *A. minutum* (and/or *A. lusitanicum*), *A. insuetum* and *A. pseudogonyaulax* (Fig. 2). *A. tamarense* U.K. AJ303445, U.K. AJ303448, New Zealand AF033534 and ribotype F of HAT4 formed a sub-group in the *A. tamarense* cluster. *A. tamarense* U.K AJ303447 and U.K AJ303446 were distinguished from other types by a long branch within this cluster. Denmark AF00668 was the same sequences with ribotypes A and C.

In the *A. catenella* cluster, only Chinese AF118546 was a significantly distant type from the main cluster. French AF318219 was identical with ribotypes A, B and I, and French AF318220, Chinese AF118547 and New Zealand AF019408 were identical with D, E, F, G and H. Ribotypes from CMC2 were unique among those from all *A. catenella* strains. Australian *A. tamarense* was positioned as a sister group of the *A. catenella* cluster and was independent another taxon with 0.05 distance by at least 47 substitutions and one indel compared with Chinese AF118546 (data not shown). *A. excavatum* cluster contained some strains of *A. tamarense* and was intermediate between the clusters of *A. tamarense* and *A. catenella*.

The *A. affine* cluster containing *A. concavum* was positioned as a sister group of the 'tamarensis species complex'. Two types of AFF37 formed a cluster with Thailand U44935, Spain L38630 and France AF318229, but they do not have completely identical sequences. *A. fraterculus* and *A. tamiyavanichii* occupied the outer group of the 'tamarensis species complex'. Malaysian *A. cohorticula* AF174614 had the same sequence with that of *A. tamiyavanichii*. The Thai *A. tamarense* was positioned as a sister taxon of the *A. tamiyavanichii* clade with ca. 0.04 distance (distance data not shown). *A. margalefii*, *A. andersonii*, *A. ostenfeldii*, *A. minutum* (containing *A. lusitaniucm*) and *A. insuetum* formed a cluster or clade complex (MAOMI complex). The MAOMI complex was positioned as a near-basal member that diverged after *A. pseudogonyaulax*, which was the earliest diverged or emerged ancestral taxon within the *Alexandrium* species examined, though bootstrapping support was relatively low at 58%. *A. insuetum* AI104 formed a cluster with French AF318233.

Sequence data of SSU rDNA and the phylogeny

Sequences of SSU rDNA determined are listed in Table 4. *A. tamarense* harbored 17 different ribotypes (A to Q) which varied in length from 1798 to 1802 nt. Each *A. tamarense* strain harbored a common

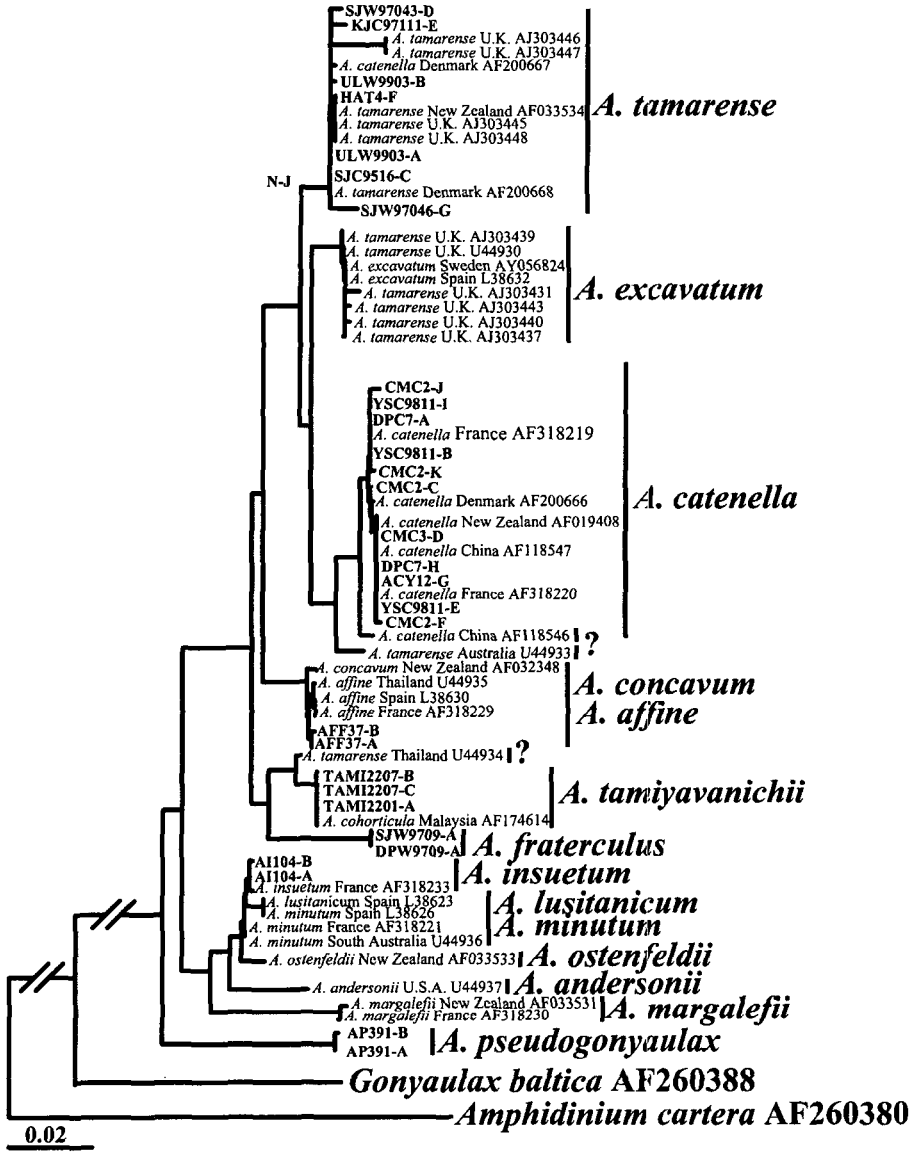

Fig. 2. Putative molecular phylogenetic tree inferred from LSU rDNA D1-D2 sequences of the genus *Alexandrium*. The tree topology was derived by the N-J method. Sequences determined in this study are shown in bold characters. *Gonyaulax baltica* and *Amphidinium cartera* were used as outgroups. Scale bar indicates 2% divergence.

ribotype A of rRNA. In addition to the common ribotype A, type O was shared in SJW97043 and KJC97111. Unlike *A. tamarense*, *A. catenella* had only 4 different ribotypes (A to D). All strains of *A. catenella* harbored a common ribotype A. Three strains of *A. tamiyavanichii* harbored all 8 different ribotypes that had substituions at no more than 3 sites. The other species, *A. affine*, *A. insuetum*, *A. fraterculus* and *A. pseudogonyaulax*, harbored only one ribotype. Fifty-six sequences newly determined in this study and 7 sequences of SSU rDNA retrieved from GenBank were used to analyze phylogentic relationships. Fig. 3 shows that phylogenetic tree inferred from SSU rDNA sequences is almost identical to that from the LSU rDNA. The phylogeny revealed 11 clusters or clades: *A. tamarense* (including *A. fundyense*), *A. tamarense* (composed of two Canadian strains), *A. catenella*, *A. affine*, *A. tamiyavanichii* (including *A. cohorticula*), *A. fraterculus*, *A. margalefii*, *A. minutum*, *A. ostenfeldii*, *A. insuetum* and *A. pseudogonyaulax*. Canadian *A. tamarense* X5494 and AF022192 were located between *A. tamarense* and the *A. catenella* cluster. The sister clusters of *A. tamiyavanichii* and *A. fraterculus* were independent of *A. affine* and the MAOMI cluster complex (*A. andersonii* was not available from GenBank). The MAOMI cluster complex occupied the basal position, but none of the positions was robust due to their relatively close genetic distance (ca. 0.008-0.028, data not shown) and low bootstrapping values (<60%). Though *A. pseudogonyaulax* was placed as the basal taxon, the relationships between this clade and the MAOMI cluster

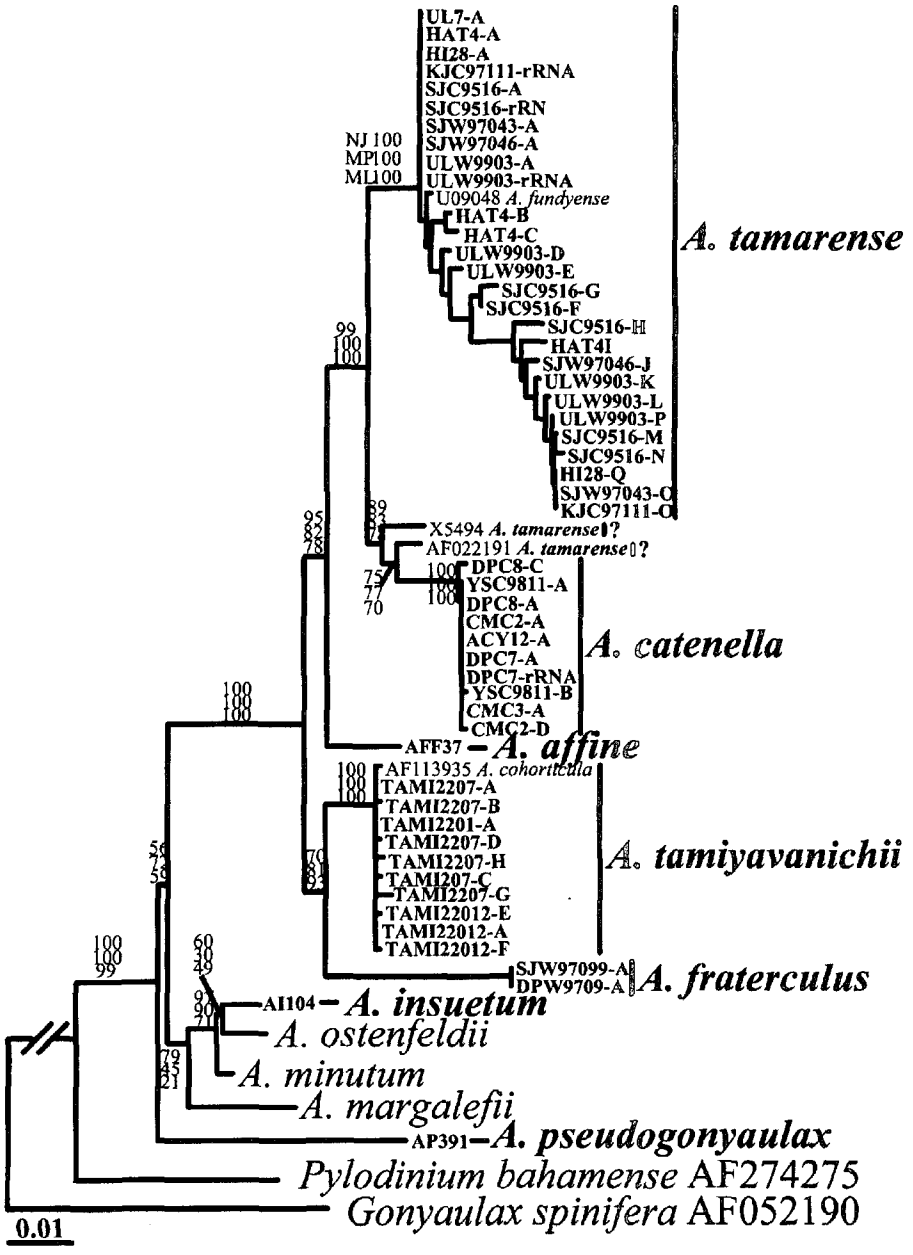

Fig. 3. Putative molecular phylogenetic tree infereed from SSU rDNA sequences of the genus *Alexandirum*. The tree topology was constructed by the N-J method. Bootstrap values are shown for N-J, MP and ML at the internodes. Sequences determined in this study are indicated in bold type. *Pylodinium bahamense* and *Gonyaulax spinifera* were used as outgroups. Scale bar indicates 1% divergence.

complex were not clear because of the relatively low bootstrapping values (NJ:MP:ML:56:72:59).

DISCUSSION

Species designations and phylogenetic lineages

Phylogenetic analysis inferred from the LSU rDNA D1-D2 and SSU rDNA sequences clearly showed the genetically divergent lineage of at least 14 *Alexandrium* groups: *A. tamarense* (and/or *A. fundyense*), *A. excavatum* (and/or *A. tamarense*), *A. catenella*, Tasmanian *A. tamarense*, *A. affine* (and/or *A. concavum*), Thai *A. tamarense*, *A. tamiyavanichii* (or *A. cohorticula*), *A. fraterculus*, *A. margalefii*, *A. andersonii*, *A. ostenfeldii*, *A. minutum* (and/or *A. lusitanicum*), *A. insuetum*, and *A. pseudogonyaulax* clade.

A. tamarense and *A. catenella*, members of the 'tamarensis species complex', were clearly distinct from each other. Scholin *et al.* (1994) divided *A. tamarense/fundyese* species complex into three regional populations, Western, Eastern and Alternate of North America, without distinction between *A. tamarense* and *A. fundyense*, but strain AFNFA3.1 did not follow this population trend. They reported that sequences TGTGG at 106-110 (exclusive primer regions) and

deletion of G at position 148 from Eastern types, GtCA29, AFNF3.2, AFNFA4, GTMP SHER and 172/24W, were different from Western types, PW06, PI32, BGtl, and equivocally positioned AFNF3.1, which had GTGGA at 106-110 and no deletion at position 148. Northern American Eastern strains other than AFNFA3.1 and Alternate strains (OF041 and OF051) were clearly characterized by two continuous nucleotides deletions at 590-591. Most *A. tamarense* examined in this study were generally close or almost identical to the North American Alternate type, and *A. catenella* was identical to the Temperate Asian type. Interestingly, we found that ribotype F of strain HAT4 (*A. tamarense*) displayed TGTGG at 106-110 and deletions G at 148 and T and G at 590-591, just like the LSU rDNA sequence of the American Eastern type.

In the SSU rDNA sequence analysis, it became clear that *A. fundyense* was similar to *A. tamarense* from Korea and Japan (Fig. 3). These genetic result suggests that it is difficult to discriminate *A. fundyense* from *A. tamarense*, as described by Adachi *et al.* (1996), who also showed that *A. fundyense* GtCA29 from U.S.A. was close to *A. tamarense* in its 5.8S rDNA sequences. It is not possible, however, to draw a final conclusion about their relationship, because there are a massive number of gene copies of rDNA.

It may be possible to characterize populations by the divergence of pseudogenes. *A. tamarense* UL7 and ULW9903 can be recognized as population-specific regional isolates which have distinct sequences from other strains. These two strains harbored common ribotype B in LSU rDNA D1-D2, which is distinct from other types in having substitutions at positions 35 (A→G) and 400 (A→C). Ribotypes K and L of ULW9903 were also specific in harboring a substitution at position 6 (G→A) in SSU rDNA. These two results suggest that the strains from Ulsan, the southeastern coastal region of Korea, represent a discrete regional population. The sequence data of *A. catenella* DPC7, DPC8 and YSC9811 suggest that they represent the same population that shares ribotypes B, E, H and I. These population-specific differences between strains may be introduced by the presence of pseudogenes undergoing rapid evolution or mutation at the species level. Korean and Japanese *A. tamarense* and *A. catenella* strains had identical common ribotype A, but they lacked any common pseudogene in both LSU D1-D2 and SSU rDNA regions. However, the occurrence of F type of *A. tamarense*, which is very close to the American Estern type (Scholin *et al.* 1994), hampers our assertion that Korean and Japanese strains are isolated populations. It is not yet clear whether the results of Scholin *et al.* (1994) will allow regional populations to be distinguished. Little is known about the occurrence, meaning and evolution of diverse pseudogenes which may be induced in the process of meiotic division, why the pseudogenes are dominantly harbored in the representative toxic *Alexandrium* species (*A. tamarense*, *A. catenella* and *A. tamiyavanichii*), or why the diversity of pseudogenes is different in the two rDNA regions of *A. tamarense* and *A. catenella*. Pseudogenes of the *A. catenella* strains were relatively stable among regional samples, with the exception of AF1185446 from Hong Kong, China in LSU rDNA D1-D2. AF118546 was the most distant from the other types, harboring seven substitutions and one deletion comparing with the closest strain, AF118547. French AF318219 was identical with ribotypes A, B and I, and French AF318220, New Zealand AF019408 and China AF118547 were identical with gene types D, E, H and G (Fig. 2). This result suggests that *A. catenella* strains of Western Europe share a common or closely similar origin with Korean and Japanese strains, or that the species was dispersed recently. Canadian *A. tamarense* strains X5494 and AF022191 were positioned between *A. tamarense* and *A. catenella* clusters in SSU rDNA phylogeny (Fig. 3), in the position of the *A. excavatum* cluster in LSU rDNA phylogeny (Fig. 2). Balech and Tangen (1985) separated *A. excavatum* from *A. tamarense* in accordance with their morphological differences of epithecal angularity and anterior sulcal regions. This study, together with the results of Scholin *et al.* (1994) and Adachi *et al.* (1996), supports idea of Balech and Tangen (1985) from the viewpoint of rDNA sequences.

One of the remarkable taxa in the phylogenetic lineage is the Tasmanian *A. tamarense* U44933 (Fig. 2). This clade is close to the *A. catenella* cluster in a sister relation but clearly independent, indicating a possibility of a new taxon. *A. concavum* was related very closely with *A. affine*, differing by only four nucleotide substitutions in the LSU rDNA D1-D2 sequence. However, it is not yet clear whether they are same one species or not. Two ribotypes of *A. affine* AFF37 had nearly same the sequence as three other regional strains, from Thailand, Spain and France, though they had two nucleotide mismatches. *A. fraterculus*, which usually blooms in late summer in Korea, was in a sister relationship with *A. tamiyavanichii*. The dominant genetic trait of this species in LSU rDNA D1-D2 was its length heterogeneity.

The phylogenetic lineages among long chain-forming species (LCFS), *A. affine*, *A. tamiyavanichii* and *A. fraterculus*, were closely related (Fig. 1, 2 and 3) and reflected their genetic and physiological traits. *A. tamiyavanichii*, a PSP toxin producer in Japan, had more diverse ribotypes in SSU rDNA than LSU rDNA D1-D2. It shared the same ribotype A with *A. cohorticula* from Malaysia in LSU rDNA D1-D2, but did not harbor completely same ribotype in SSU rDNA. Thai *A. tamarense* was reported as a new taxon distinct from the 'tamarensis species complex' and characterized as a strain of tropical Asian origin by Scholin *et al.* (1994) and Adachi *et al.* (1996) from analyses of LSU rDNA D1-D2 and 5.8S rDNA, respectively. Our result indicated that *A. tamarense* U44934 was close to clade *A. tamiyavanichii* with 0.04 distances and a difference of 38 nucleotide substitutions and 4 deletions.

Genetic distance between Japanese and French *A. insuetum* strains was more distant than that of between *A. concvum* and *A. affine* (genetic distance matrix not shown). This genetic distance between regional isolates should be discussed through comparing their morphologies and sequences. The morphological features of *A. insuetum* appear to be well differentiated from those of other *Alexandrium* species in terms of cell size and thecal plates. In the *A. minutum* cluster, France AF318221 is considered to be separated from Spain L38626 (including L38623). Sequence comparison of *A. minutum* and *A. lusitanicum* was well described by Zardoya *et al.* (1995), who proposed that the two similar morphological species are synonymous from the supporting results of RFLP identity (Scholin and Anderson, 1994) and immunological identity (Mendoza *et al.*, 1995). The MAOMI cluster complex seems to diverge relatively early in the evolution of *Alexandrium*, and the members *A. minutum*, *A. ostenfeldii* and *A. insuetum* were close as shown in Fig. 3. *A. margalefii* may emerged or diverged relatively early in *Alexandrium* evolution, and the other members followed by divergence rather than emergence. Our reconstruction of the phylogenetic relationship suggests that *A. pseudogonyaulax* is an ancestral taxon within the genus *Alexandrium*, and it is possible that it may come from a more primitive dinoflagellate such as *Pylodinium*, *Gonyaulax* or *Protogonyaulax*. Unfortunately, insufficient data is available to merge or divide the very closely related species, such as *A. affine* and *A. concavum*, *A. tamiyavanichii* and *A. cohorticular*, *A. lugitanicum* and *A. minutum*, *A. excavatum* and *A. tamarense*, and *A. tamarense* and *A. fundyense*. It is also necessary to consider many other *Alexandirum* species, such as *A. acatenella*, *A. angustitabulatum*, *A. balechii*, *A. compressum*, *A. depressum*, *A. foedum*, *A. hiranoi*, *A. kutnerae*, *A. lee*, *A. monilatum*, *A. peruvianum*, *A. satoanum*, *A. taylorii*, and *A. tropicale*, and construct a complete *Alexandrium* phylogeny.

A. tamarense and *A. catenella*, which are thought to be close in morphology and PSP toxin productivity, were clearly distinct genetically in the phylogenetic analyses of both rDNA regions, and they were also different in terms of gene diversity (variations among diverse gene copies): *A. tamarense* was more diverse in the SSU rDNA (17 ribotypes) than in the LSU rDNA (7 ribotypes), while the reverse was true of *A. catenella*, which had 11 ribotypes in LSU and 4 in SSU (Table 4).

Molecular approaches using species-specific genetic markers hold promise as a means exact and rapid species designation. We have adopted a DNA probing method to detect PSP toxin-producers of *A. tamarense* and *A. catenella* including *A. tamiyavanichii*. We have been able to detect *A. tamarense* in natural seawater samples using a species-specific DNA probe (Kim, 2000). Whole-cell *in situ* hybridization (FISH) was also applied to toxic *A. tamiyavanichii*. The DNA probe was designed from the LSU rDNA D1-D2 region, which variable enough to allow species-specific identification. This region is suitable for designing DNA probes for detection of target species.

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