Molecular Identification of the Toxic *Alexandrium tamiyavanichii* (Dinophyceae) by the Whole-cell FISH Method

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The dinoflagellate *Alexandrium tamiyavanichii* Balech, a producer of toxins causing paralytic shellfish poisoning (PSP), has recently been considered as one of main organisms responsible for toxication of shellfish in Japan. In this study, *A. tamiyavanichii* was subjected to a molecular phylogenetic analysis inferred from 28S rDNA D1-D2 sequences and a species-specific LSU rRNA-targeted oligonucleotide DNA probe was designed to identify *A. tamiyavanichii* using the whole cell-FISH (fluorescence *in situ* hybridization). The sequences of the 28S rDNA D1-D2 region of *A. tamiyavanichii* showed no difference from *A. cohorticular* AF174614 (present name *A. tamiyavanichii*) and formed a distinct clade from the 'tamarensis species complex'. The probe, TAMID2, reacted specifically with *A. tamiyavanichii* cultured cells, without any cross-reaction with other species belonging to the same genus, including *A. tamarense*, *A. catenella*, *A. affine*, *A. fraterculus*, *A. insuetum* and *A. pseudogonyaulax*. In a test of cross-reactivity with a field sample, TAMID2 reacted consistently with only *A. tamiyavanichii*, indicating that the present protocol involving the TAMID2 probe might be useful for detecting toxic *A. tamiyavanichii* in a simple and rapid manner.

Key words: Whole cell-FISH (fluorescence *in situ* hybridization), DNA probe, *Alexandrium tamiyavanichii*

Introduction

Recently, the morphologically and molecular-phylogenetically poorly-defined toxic species *A. tamiyavanichii* has caused PSP toxication of shellfish. Since its first confirmation in 1999 in Harima Nada, Japan, PSP toxication by this species has spread, and consequently caused economic and sanitary problems (Hashimoto et al., 2002).

A. tamiyavanichii (*Protogonyaulax tamiyavani- chii*) found in the Gulf of Thailand as a toxic strain was reported by Kodama et al. (1988) and Hashimoto et al. (2002) demonstrated that wild vegetative cells contained GTX1-5, STX and C1-2 and bivalves feeding on this alga were intoxicated with GTX1-5,

STX, (dcSTX), (neoSTX), and C1, 2, (3), (4), showing strong toxicity and diversity of toxin components.

Difficulties in field monitoring for *A. tamiyavanichii* are derived not only by its long- chain-forming features shared by *A. fraterculus* (Balech) and *A. affine* (Inoue and Fukuyo), but also from the overlapping occurrence of their vegetative cells in the same water column. In particular, the introduction of the toxic *Alexandrium* species into a water column with low cell density is likely to be beyond the limits of traditional monitoring methods. Therefore, a rapid and reliable identification method for the toxic *Alexandrium* is required.

DNA probing using fluorescent-labeling has been used recently as an alternative to conventional identification methods; targeted probing to either rRNA

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or rDNA has been given much attention as a powerful culture-independent tool (Giovannoni et al., 1988; Amann et al., 1990; DeLong and Shah, 1990; Distel et al., 1991; Worden et al., 2000). Despite the extensive literature on *Alexandrium* and other species causing harmful algal blooms (HABs), the application of DNA probing for species identification and detection is restricted to a few reports (Adachi et al., 1996a; Scholin et al., 1996; Miller and Scholin et al., 1996; Parson et al., 1999). Development of reliable molecular biological identification methods is a prerequisite for tackling the problems of toxic *Alexandrium*.

This study described the morphological fine features of *A. tamiyavanichii* isolated in Japan and constructed the molecular phylogenetic relationships of the species with long chain-forming *A. affine* and *A. fraterculus* inferred from sequences of the 28S rDNA D1-D2 domain. Also, we designed a species-specific oligonucleotides DNA probe for identification and detection of the species based on the whole-cell FISH (fluorescence *in situ* hybridization) method.

Materials and Methods

Cultures

Clonal cultures of seven *Alexandrium* species, *A. affine*, *A. catenella*, *A. fraterculus*, *A. insuetum*, *A. pseudogonyaulax*, *A. tamarense* and *A. tamiya-vanichii*, and other dinoflagellates, *Prorocentrum micans*, *P. minimum*, *Heterocapsa circularisquma*, *H. triquetra*, *Gymnodinium catenatum*, *Chattonella marina* and *Heterosigma akashiwo*, were established from an isolated vegetative cell in natural sea waters (Table 1). All the isolates were incubated in a SW Ⅱ medium (Sako et al., 1990) at 20 ℃, on a 14:10 L:D regime at 100 μEm^{-2}S^{-1} using cool white bulbs. Morphological taxonomy followed the criteria of Fukuyo (1985).

Molecular phylogenetic analysis

Three isolates of *A. tamiyavanichii* and one isolate each of *A. affine* and *A. fraterculus* listed in Table 1 were subjected to sequence-determination of the 28S rDNA D1-D2 domain. DNA preparation and sequencing procedures were conducted according to

Table 1. Strains list of phytoplankton used in whole-cell FISH for test of cross-reactivity of *Alexandrium tamiyavanichii*-specific DNA probe TAMID2

Species	Strain	Origin	Toxicity	Probe reactivity
A. tamarense	ULW9903	Ulsan, Korea	Yes[a]	-
A. tamarense	KJC97111	Koje, Korea	Yes[a]	-
A. tamarense	SJW97043	Jinhae Bay, Korea	Yes[a]	-
A. tamarense	HAT4	Hiroshima Bay, Japan	Yes[b]	-
A. catenella	DPC7	Dadaepo, Busan	Yes[a]	-
A. catenella	CMC2	Thongyong, Korea	Yes[a]	-
A. catenella	YSC9802	Yosu, Korea	Yes[a]	-
A. catenella	ACY12	Harima Nada, Japan	Yes[b]	-
A. affine	AFF37	Harima Nada, Japan	No[c]	-
A. fraterculus	DPW9709	Jinhae Bay, Korea	No[a]	-
A. fraterculus	SJW9709	Jinhae Bay, Korea	No[a]	-
A. insuetum	AI104	Utiumi Bay, Japan	No[c]	-
A. pseudogonyaulax	AP391	Harima Nada, Japan	No[c]	-
A. tamiyavanichii	TAMI2201	Harima Nada, Japan	Yes[d]	+
A. tamiyavanichii	TAMI2207	Harima Nada, Japan	Yes[d]	+
A. tamiyavanichii	TAMI22012	Harima Nada, Japan	Yes[d]	+
Prorocentrum micans	PM981	Jinhae Bay, Korea	No[a]	-
Prorocentrum minimum	PM982	Jinhae Bay, Korea	No[a]	-
Heterocapsa circularisquma	HC991	Jinhae Bay, Korea	No[a]	-
Heterocapsa triquetra	HT001	Jinhae Bay, Korea	No[a]	-
Gymnodinium catenatum	GC981	Jinhae Bay, Korea	No[a]	-
Chattonella marina	NIES121	Kagoshima Bay, Japan	No[c]	-
Heterosigma akashiwo	OS11	Osaka Bay, Japan	No[c]	-

[a,b,c,d]PSP toxicity was determined by C.J. Kim (unpublished data); Yoshida et al., 2001; Sako et al., 1999 and S.A. Yoshimatsu (personal communication), respectively.
[−]The isolates labeled with the probe TAMID2.
[+]The isolates not labled with the probe TAMID2.

the methods described by Adachi et al. (1996b). Approximately 700 nt of the LSU rDNA D1-D2 was amplified by PCR using the primers designed by Scholin et al. (1994). PCR amplification was performed by denaturing at 96 ℃ for 3 min as an initial step, followed by 30 cycles of denaturing at 94 ℃ for 30 sec, annealing at 55 ℃ for 1 min, and extension at 72 ℃ for 2 min. The Final extension was performed at 72 ℃ for 7 min. The positions and sequences of the primers in1F and in1R in the 5' -3' direction for sequencing of each species were as follows: 170-190, AAGAAAGTGTCAATGAAGGT and 497-478, CAAATAACTAGCACACACAG for *A. fraterculus*, 183-202, TGAGGGTGAGAATCCTGTTT and 596-577, GCACCAGACACATGTATCAA for *A. affine*, and 171-192, GCTGGCACACAAAGCAAGAATC and 488-467, GCTGGCACACAAAGCAAGAATC for *A. tamiyavanichii*. Sequence data obtained in this study and retrieved from GenBank (accession numbers shown in Fig. 2) were used for phylogenetic analysis using ClustalX (Thomson et al., 1997). The alignment generated was revised manually to increase alignment similarity, and converted to a PHYLIP format. Phylogenetic analyses were carried out using the PHYLIP package (Felsenstein, 1995). Bootstrapping confidences (Felsenstein, 1985) with 100 replications were generated from SEQBOOT, and a distance matrix was calculated with DNADIST with 100 multiple data sets using the F84 distance model (Felsenstein, 1984). Phylogenetic trees were constructed using a neighborjoining (NJ) option with 100 multiple data (Saitou and Nei, 1987). A transition/transversion ratio of 2.0 was selected and data sets of 100 were conducted using empirical base frequencies with one category of substitution rate. CONSENSE of PHYLIP and TREE VIEW (Page, 1996) were used for the consensus tree and tree reconstruction, respectively.

DNA probe design

The 28S rDNA D1-D2 sequences of *Alexandrium* from GenBank and this study were aligned to screen species-specific regions for designing an *A. tamiyavanichii*-specific DNA probe. Oligonucleotide DNA probe TAMID2 specific for *A. tamiyavanichii*, composed of 20 sequences (445-5'TGTACTTAGACCA-CACCATA3'-426, complementary to coding strand), was designed. The 5' end of the sequence was labeled with FITC (fluorescein isothiocyanate) to visualize the hybridized intact cells under an epifluorescence microscope. The fluorescent DNA probe was syn-

thesized commercially and purified through a cartridge (Amersham Pharmacia Biotech, Tokyo, Japan).

Screening of the optimum hybridization and washing temperatures

To determine the optimum hybridization and washing temperatures, TAMID2 was tested with combinations of five hybridization temperatures from 35 to 55 ℃ and seven washing temperatures from 35 to 65 ℃ using *A. tamiyavanichii* (TAMI2201) cultured cells. Hybridized cells were counted with a Coulter EPICS Elite Flow Cytometer (Coulter) equipped with a single 6-W argon ion laser (excitation, 488 nm of 250 Mw). FITC was detected at 520-530 nm with FS (forward scatter) and SS (side scatter) as parameters. Detection sensitivity of the flow cytometer was calibrated with fluorescently labeled beads (Flow-Check^{TM} Fluorospheres, Backman Coulter).

The DNA probe TAMID2 showed the highest relative fluorescent intensity with a hybridization and washing temperature of 35 ℃ and the second highest with hybridization at 35 ℃ and washing at 40 ℃ (data not shown). To raise probe specificity, we adapted the second best hybridization: washing temperature set at 35:40 ℃ for this whole cellFISH method.

Labeling-reactivity and specificity using whole-cell FISH

To test the reactivity and specificity of labeling by the DNA probe TAMID2, cultured cells of the *Alexandrium* species and representative red-tide forming dinoflagellates (see Table 1) were harvested in the exponential growth phase. Pelleted cells were dehydrated with an ethanol series of 50% for 1 min and 99% for 30 min at room temperature with still state to ease for DNA probes to hybridize and to remove autofluorescence from chlorophylls without fixation procedure. The dehydrated cells were air-dried completely, then subjected to hybridization by adding 200 μL of a hybridization buffer (40% formamide/5×SSC; 83 mM sodium nitrate and 83 mM sodium citrate, pH 7.0) containing the DNA probe (denatured at 100 ℃ for 5 min, chilled on ice and adjusted to a final concentration of 0.5 pmol). Cells were hybridized with the fluorescence probe at 35 ℃ for 5 min in a water bath. Hybridized cells were incubated in a 5×SSC washing buffer at 40 ℃ for 5 min twice to remove the remained unbound DNA probes and non-specifically hybridized probes. The remaining 10-20 μL of buffer containing the cell pellet

was gently pipetted, moved onto the slidegalss and observed under an epifluorescent microscope (Nikon ECLIPSE E800) equipped with a fluorescein band pass filter set for FITC and counter stain (TRITC. PI) (excitation 450-490 nm; DM, 505 nm; BA, 520 nm) at 400 times magnification. Fluorescence and light images of cells were taken using a color chilled 3 CCD camera (Hamamatsu C5810) with an exposure time of 0.4 sec.

To test the cross-reactivity of the DNA probe, natural sea water samples were collected by netting (20 μm-pore size) on August 29, 2002 in Maizuru Bay, Kyoto, Japan. Concentrated samples were prepared in polypropylene bottles without fixation, maintained in an icebox (ca. 5-10℃) during transport to the laboratory and subjected to the whole-cell FISH within 5 h after sampling. In the laboratory, field samples were concentrated further with a sieve (20 μm-pore size) and placed in 50-mL polypropylene disposable centrifuge tubes at room temperature. One mL of cultured *A. tamiyavanichii* cells (ca. 1,000 cells/mL) was added to 1 Ml of the natural seawater samples to test cross-reactivity of the DNA probe. Hybridization and washing temperatures were at 35: 40℃. After treatment, the cells were resuspended and observed as described above.

Results

Morphological description of *A. tamiyavanichii*

The diameter of the *A. tamiyavanichii* vegetative cell (Fig. 1) ranged from 40 to 60 μm with a slightly slender shape. As expected, the cells formed long chains (Fig. 1A). The apical pore complex (APC) had a relatively large apical attachment pore (a.a.p.) (Fig. 1D) and connected directly to the 1' plate inscribed with a ventral pore (v.p.) (Fig. 1C). The anterior sulcal plate (s.a.) (Fig. 1B) assumed a unique form with a precingular part (p.pr.) (Fig. 1E) with a dome-like shape, which overlapped the bottom angle of 1' and unciform apophysis (a.u.) of the hooked end and chopped posterior sinus (s.) were characterized in Fig. 1E. The posterior attachment pore (s.p.) was nearly central in the posterior plate (Fig. 1F).

Molecular phylogenetic relationships of *A. tamiyavanichii* within the genus *Alexandrium*

Sequences of the 28S rDNA D1-D2 region of three *A. tamiyavanichii* strains, TAMI2201, TAMI2207 and TAMI22012, were 710 nt in length without insertion or deletion. *A. affine* (AFF37) and *A. fraterculus* (DPW9709) displayed sequences of 711 and 717 nt in length. Sequences of *Alexandrium* determined in
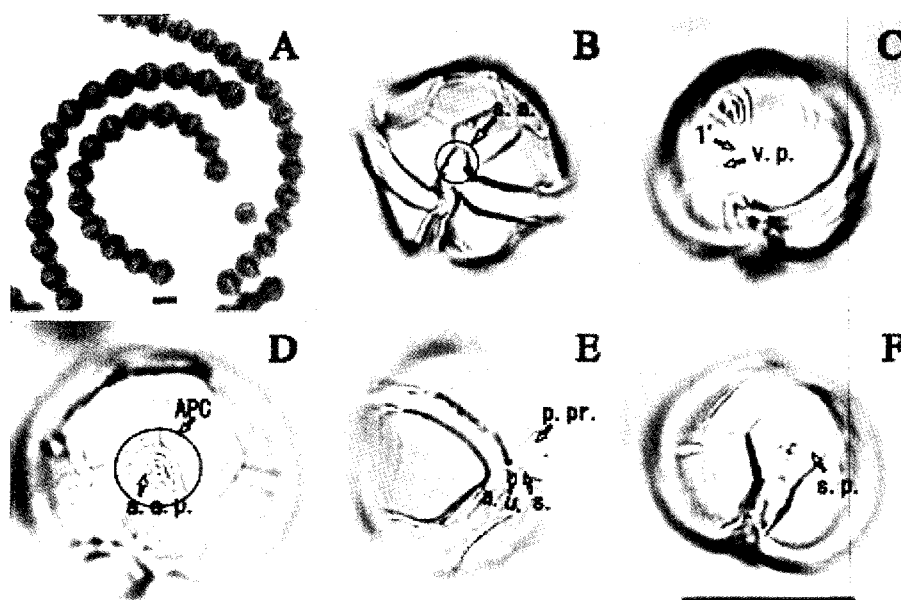

Fig. 1. Morphological designation of *A. tamiyavanichii*: A, figure showing long chain-forming vegetative cells; B, ventral view; C, apical view; D, structure of apical pore complex; E, ventral view showing anterior sulcal plate (s.a.) with a precingular part (p.pr.); F, antapical view. Scale bar=30 μm. Abbreviations by Balech (1995) and Yoshida (2000) were used.

the present study (*A. affine* AFF37, AB088227; *A. fraterculus* DPW9709, AB088244; *A. tamiyavanichii* TAMI2201, AB088264; *A. tamiyavanichii* TAMI-2207, AB088265; *A. tamiyavanichii* TAMI22012, AB088263) and retrieved from GenBank (see Fig. 2) were subjected to a molecular phylogenetic analysis. Fig. 2 shows the molecular phylogenetic relationships inferred from the sequences of 28S rDNA D1-D2 region of the genus *Alexandrium*. *A. tamiyavanichii* strain TAMI2201 and *A. cohorticular* AF-174614 showed no differences in sequence, suggesting that they are synonymous. The *A. tamiyavanichii* clade is supposed to have diverged prior to the 'tamarensis species complex' and latter than the basal clades composed of *A. andersonii, A. margalefii, A. ostenfeldii, A. minutum* and *A. insuetum* (Fig. 2). The *A. tamiyavanichii* clade had relatively close sister relationships with *A. fraterculus* as well as *A. affine*, but these two clades were clearly distinct from each other.
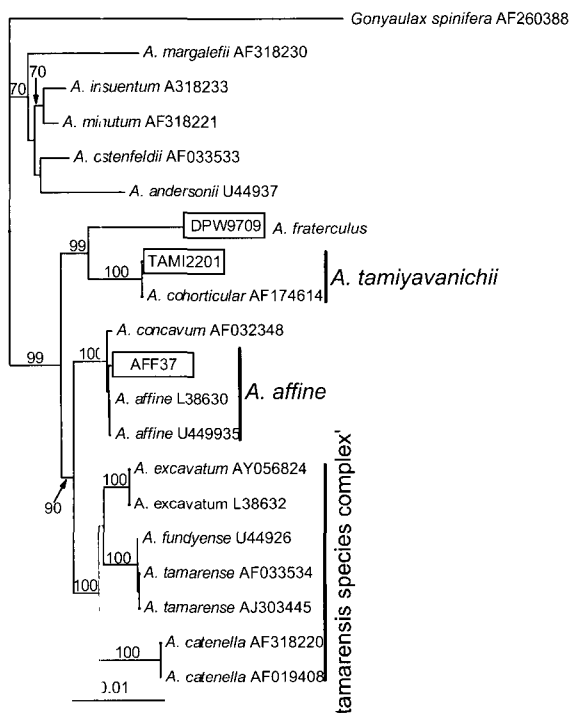

Fig. 2. Putative molecular phylogenetic tree inferred from 28S rDNA D1-D2 sequences of the genus *Alexandrium*. The tree topology was derived by the N-J method using ca. 570 nt. Bootstrap values over 60% are shown at the internodes. *Alexandrium affine*, *A. fraterculus* and *A. tamiyavanichii* isolates sequenced in the present study are boxed and the sequence data of their 28S rDNA D1-D2 regions have been deposited in the EMBL/DDBJ/GenBank. *Gonyaulax spinifera* was used to root as an outgroup taxon.

TAMID2 reactivity to targeted *A. tamiyavanichii* cells and its cross-reactivity to non-targeted *Alexandrium* and other dinoflagellates

The sequence of the TAMID2 had many nucleotide mismatches with the corresponding region in *A. affine, A, andersonii, A. catenella, A. excavatum, A. fraterculus, A. insuetum, A. margalefii, A. minutum, A. ostenfeldii* and *A. tamarense* as shown in Table 2.

Fig. 3 shows the reactivity and specificity of the oligonucleotide probe TAMID2 to targeted *A. tamiyavanichii* and non-targeted toxic *A. tamarense, A. catenella* and non-toxic *A. affine* and *A. fraterculus* cultured cells. TAMID2 reacted with *A. tamiyavanichii* (TAMI2201) cultured cells in the whole-cell FISH, and most cells were clearly visualized with strong fluorescent intensity against a dark background. *A. tamiyavanichii* cultured cells subjected to the whole-cell FISH without the probe TAMID2 showed only pyrenoid-like spots (Fig. 3 B and B'). The probe showed no traces of cross-reaction with various other *Alexandrium* species, including toxic *A. tamarense* and *A. catenella*, and non-toxic *A. affine, A. fraterculus, A. insuetum* and *A. pseudogonyaulax* or with cultured cells of other genera, *Prorocentrium micans, Prorocentrum minimum, Heterocapsa circulariscuma, Heterocapsa triquetra, Gymnodinium catenatum, Chattonella marina* and *Heterosigma akashiwo* (Table 1). Autofluorescence in photosynthetic organisms, which has become a major problem in whole-cell FISH, was completely eliminated by ethanol dehydration in all species tested in the present study, therefore, it did not act as an obstacle to effective hybridization.

Detection of the targeted *A. tamiyavanichii* cultured cells and cross-reactivity in natural field samples

The DNA probe TAMID2 reacted with only *A. tamiyavanichii* cultured cells without any cross-reactivity to other components of a natural field sample composed of various diatoms, dinoflagellates and zooplanktons as shown in Fig. 4. The fluorescent intensity of *A. tamiyavanichii* cultured cells was strong, and each cell was completely covered with the signal. Nonspecific staining of detritus-like amorphous clusters was occasionally found in low frequencies but could be removed by size-fraction of filtration. Autofluorescence in the field sample was not completely eliminated, as typified by that

Table 2. *Alexandrium* species-specific region of LSU rDNA D1-D2 domain

Species[a]	Sequences[b]	Account no.
A. affine	TAGGTTTTGA---TTGTGGGTGTG	AB088227
A. andersonii	GCA---------------ACTTGGGCGCA	AB088244
A. catenella	TGGGTTTTGG---CTGCAAGTGCA	AF318220
A. excavatum	TAGGTTTTGG---GTGTGGGTGAA	AY056824
A. fraterculus	TCGATGTGAA---TTGCAAATGCA	AB088244
A. insuetum	TTATATGTTG--GATGTGGGTGCG	AF318234
A. margalefii	TTATTTGTGTTGCATCAGGGTGCA	AF318230
A. minutum	TTA--TATGGTTGATGTGGGTGCG	AF318222
A. ostenfeldii	---------------------TGTGGGCGCA	AF033533
A. tamarense	GAGCTTTGGG---CTGTGGGTGTA	U44929
A. tamiyavanichii	TATGGTGTG-----GTCTAAGTACA	AB088265

[a]Sequences of *A. affine* (AFF37), *A. fraterculus* (DPW9709) and *A. tamiyavanichii* (TAMI2201) were determined in the present study.
[b]Sequences are listed 5' to 3' and the sites are from 426 to 441 (see Scholin et al., 1994).
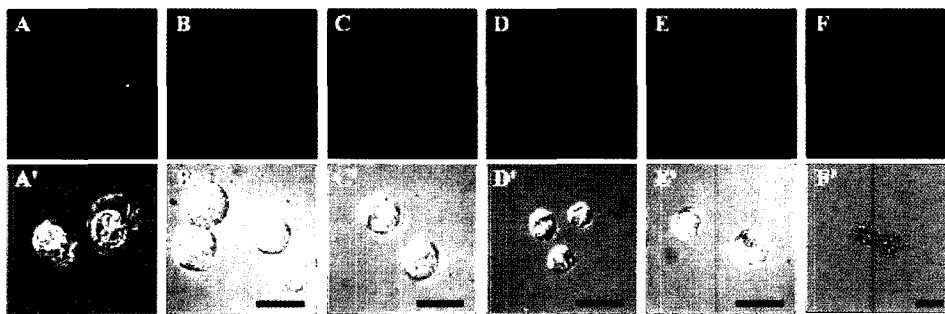

Fig. 3. Epifluorescence microscopic (A to F) and light microscopic images (A' to F') of cultured *Alexandrium* cells hybridized with DNA probe TAMID2 or treated without the probe (B and B'): A and A' and B and B', *A. tamiyavanichii* (TAMI2201); C and C', *A. tamarense* (KJC97111); D and D', *A. catenella* (DPC8); E and E', *A. affine* (AFF37); F and F', *A. fraterculus* (DPW9709). Scale bars=30 μm.
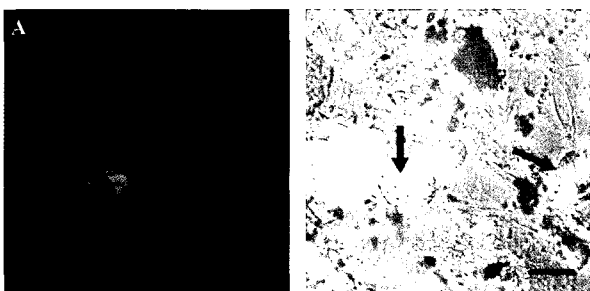

Fig. 4. Epifluorescence microscopic (A) and light microscopic images (A') of concentrated natural seawater sample hybridized with TAMID2. Arrows indicate targeted *A. tamiyavanichii* (TAMI2201) cells and scale bar=30 μm.

of *Dinophysis* spp., but its red color was clearly distinguished from the green of FITC with which the DNA probe was labeled.

Discussion

The DNA probing methods for field organisms should be simple and rapid, especially if the target organisms are near death and decomposition of their rDNA and rRNA. The DNA probe sequences should be designed with high specificity to distinguish the target organism not only from the related species but also from a great number of other bio-communities in the field.

In this FISH protocol, we omitted the CTAB (cetyltrimethyl ammonium bromide) process usually used for puncturing the cell wall. Still, the DNA probes were well hybridized with rRNA in the cytoplasm of most intact *A. tamiyavanichii* cells. Formamide was used at the concentration of 40% in a hybridization buffer of 5 X SSC, though there was a report that high concentration of formamide (>20%)

inhibited the whole cell hybridization (Dedysh et al., 2001). We also skipped the step of cell fixation, based on our empirical observation that most cellular rRNAs exposed to glutaraldehyde leaked from cells, during transportation of a field sample to the laboratory at a low temperature of 5-10 °C. A similar phenomenon was pointed out by Scholin et al. (1996), who reported that *Pseudo-nitzschia* species often exuded their cell contents when exposed to formalin and other alde-hyde-type preservatives. To prevent cell degradation during preservation, they recommended the use of an ethanol/saline-based solution for fixation-preservation. Worden et al. (2000) compared the effect of the fixation agents glutaraldehyde and parafor-maldehyde on labeling intensity, and stated that glutaraldehyde (0.1% final concentration) caused higher fluorescence background and lower signal strength in cultured *Prochlorococcus* than a parafor-maldehyde fixation. A possible explanation for this is that fixation may cause cell contraction or expansion, resulting in damage to the cell wall or even the nuclear membrane and consequent leakage of cell components. Such damage would be accelerated by agitating the natural seawater samples during the transportation. Damage was more remarkable when relatively high concentration of fixatives, ethanol and acetone, were added to the samples. We know em-pirically that nucleic acid is decomposed during the cell fixation with aldehyde-like agents. Therefore, it is considered that field samples should be con-centrated on a filter, and then treated with a series of ethanol solution to remove autofluorescence and effect dehydration. Filtered dry samples should be transported. These treatments can prevent the collapse and loss of cell, which are significant obstacles in quantitative enumeration. Additionally, temperature control is essential to accomplish a stable hybridiza-tion system.

Whole-cell hybridization by the simple method described above showed that *A. tamiyavanichii* cells were thoroughly labeled with TAMID2, indicating that the DNA probe reacted with the targeted rRNAs with good access to the target sites, and that the target region harboring significant substitutions bet-ween species is a relatively plane structure without folding, helices or binding with other molecules. Only the nuclear part of the cells did not permit the probe to label, ensuring an unlabeled part remained.

We chose an oligonucleotide in the variable domain D2 of 28S rDNA harboring an *Alexandrium* species-

specific region. The DNA probe TAMID2 designed for *A. tamiyavanichii* did not cross-react with any other *Alexandrium* species tested in this study. It also showed no cross-reactivity with field sample, pro-bably because of the high sequence- specificity of TAMID2 and the use of the rapid labeling method without CTAB treatment, though DNA probe capture by detritus aggregates and mucous materials was sometimes observed. For easy permeabilization of cell wall for binding of the target molecules in cytosol and the DNA probe molecules, several agents such as paraformaldehyde, diethyl ether, xylene (Arnoldi et al., 1992), hydrochloric acid, (Macnaughton et al., 1994), lipase (Davenport et al., 2000) and lysozyme (Schwiertz et al., 2000) has been compared, but even the use of only formamide of 40% in this study did not require further agents searching to obtain more effective labeling.

Direct cell counting was also conducted to assess quantification as the ratio of cell counts under epi-fluorescent and light microscope. The ratio was over 99% during the exponential growth phase and fell below 80% from the stationary growth phase, demon-strating that dead and collapsed cells were not avai-lable for probing. This confirms that labeling-intensity was predominantly dependent on the physical condi-tion of cells. We observed, however, that *A. tamarense* vegetative cells from a field sample were of various sizes, implying perhaps different physical status, but labeling-intensity was very high and even (Kim, un-published data).

Hybridization and washing temperatures for label-ing with TAMID2 were positive at 35:35, 35:40, 35:45, 40:40, 40:45, and 45 °C:45 °C, while tempera-tures of over 50 °C were not appropriate for either process because probes were denatured during both processes. Among the regimes tested, 35:35 and 35:45 were the best, illustrating that DNA probes were well hybridized under the condition when both hybridiza-tion and washing temperatures were approximately 10-15 °C and 10 °C lower, respectively, than melting temperature (T_m) (TAMID2, 52.7 °C), which is defined as the equilibrium temperature at which half of the probe-target duplexes are dissociated (Stahl and Amann, 1991). To raise probe specificity to field samples, hybridization and washing temperatures should be set at least 5 °C lower than T_m.

Fluorescent signal intensity was generally strong during the exponential growth phase. However, it declined steeply with the degradation of cells. Another drawback arose from the fast fading of the fluores-

cence signal. In order to not only diminish the fading, but also enhance the fluorescence signal, treatment of antifade agents and comparison of various fluorescence agents such as indocarbocyanine (Cy3), indodicarbocyanine (Cy5), rhodamine, Texas red, 5(6)-carboxyfluorescein-N-hydroxysuccinimide ester (FLUOS), Oregon green 538, and testing of multiple probes are necessary.

Further studies should be conducted to facilitate cell detection and quantification. We expect improvements to be achieved shortly through development of a simple system for filtration and hybridization of field samples.

Acknowledgements

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