MARINE BIOTECHNOLOGY

Development of Molecular Identification Method for Genus *Alexandrium* (Dinophyceae) Using Whole-Cell FISH

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Abstract

We have developed a method to identify species in the genus *Alexandrium* using whole-cell fluorescent in situ hybridization with FITC-labeled oligonucleotide probes that target large subunit ribosomal rRNA molecules. The probes were designed based on the sequence of the rDNA D1-D2 region of *Alexandrium* species. DNA probes specific for toxic *A. tamarense* and *A. catenella* and nontoxic *A. affine*, *A. fraterculus*, *A. insuetum*, and *A. pseudogonyaulax*, respectively, were applied to vegetative cells of all above *Alexandrium* species to test the sensitivity of the probes. Each DNA probe hybridized specifically with vegetative cells of the corresponding *Alexandrium* species and showed no cross-reactivity to noncorresponding *Alexandrium* species. In addition, no cross-reactivity of the probes was observed in experiments using concentrated natural seawater samples. The TAMAD2 probe, which is highly specific to *A. tamarense*, a common toxic species in Korean coastal waters, provides a simple and reliable molecular tool for identification of toxic *Alexandrium* species.

Key words: DNA probe — *Alexandrium* — whole-cell FISH

Introduction

Harmful algal blooms (HABs) have become a worldwide problem, causing immense economic losses in aquaculture and much human suffering as a result of accidental exposure. These blooms are not a temporary natural phenomenon, but rather an anthropologic problem caused by the disposal of untreated domestic and industrial waste, and by improvements in aquaculture (Lam and Ho, 1989; Okaichi, 1989; Hallegraeff, 1993).

Certain toxic dinoflagellate species of the genus *Alexandrium* (Halim) are lethal to humans, owing to their production of paralytic shellfish poisoning (PSP) toxins, which block sodium channels in animal nerve cells (Schantz et al., 1966; Baden and Trainer, 1993; Kao, 1993;). The *Alexandrium* species are planktonic-sized marine thecate and photosynthetic dinoflagellates that belong to the phylum Dinophyta, class Dinophyceae, order Gonyaulacales, and family Gonyaulacaceae. The species *A. tamarense* (Lebour) Balech, *A. catenella* (Whedon and Kofoid) Balech, *A. acatenella* (Whedon and Kofoid) Balech, *A. cohorticula* (Balech) Balech, *A. fundyense* Balech, *A. ostenfeldii* (Paulsen) Balech and Tangen, *A. minutum* Halim, *A. tamiyavanichii* Balech, and some related species (Balech, 1995) have caused many PSP-related events worldwide. Human ingestion of aquaculture products that contain bivalves contaminated with these species can result in poisoning episodes, paralytic diseases, and even death. Rapid detection of the presence of toxic dinoflagellates, identification of the species, and characterization of their abundance and distribution in algal blooms would aid in the protection of sensitive aquaculture areas and the prevention of human illness.

In addition to the production of PSP toxin by these species, another long-term problem with the *Alexandrium* genus is confusion in the species nomenclature. There are 28 species in this genus, with 3 equivocal *Alexandrium* spp. in addition to *A. depressum* Gaarder (Balech, 1995). Morphologic identification of *Alexandrium* species is usually based on the microtabulation in the fine thecal

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plates, chain formation, cell size, and overall shape. Strong morphologic similarities between *Alexandrium* species have led to much taxonomic confusion. The worldwide dispersion of the genus (Shumway, 1990; Hallegraeff, 1995) also highlights the need for the development of exact species identification and population analysis methods.

In order to eliminate the confusion in the *Alexandrium* taxonomy, molecular phylogenetic analysis has been used to establish more concrete criteria and to provide basal genetic information (Destombe et al., 1992; Scholin and Anderson, 1994; Scholin et al., 1994; Zardoya et al., 1995; Adachi et al., 1996a; Usup et al., 2002). We conducted phylogenetic analysis based on comparison of the newly determined sequences of the large subunit (LSU) ribosomal DNA D1-D2 and small subunit (SSU) rRNA regions from *A. affine*, *A. catenella*, *A. fraterculus*, *A. insuetum*, *A. pseudogonyaulax*, *A. tamiyavanichii*, and *A. tamarense* with sequences from functionally expressed rRNA and those retrieved from the database. These analyses divided the genus *Alexandrium* into 14 groups: (1) *A. tamarense*, (2) *A. excavatum*, (3) *A. catenella*, (4) Tasmanian *A. tamarense*, (5) *A. affine* (and/or *A. concavum*), 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*. Ribosomal DNA and RNA sequence information can be used to design molecular probes for the detection, identification, and enumeration of these groups (Giovannoni et al, 1988; Amann et al., 1990, 1991, 1995; DeLong and Shah, 1990; Distel et al., 1991; Worden et al., 2000). The use of DNA probes has been applied to only a limited number of HAB species (Adachi et al., 1996b; Scholin et al., 1996, 1997, 1999; Miller and Scholin, 1996; Parson et al., 1999). It is a matter of great interest to develop methods to identify *Alexandrium* species and other PSP producers exactly and rapidly during field monitoring. However, near real-time monitoring of *Alexandrium* species has not been achieved because of difficulties resulting from the highly similar morphologies of the species and a lack of the extensive genetic information required for molecular approaches. In particular, difficulties arise from the so-called tamarensis species complex; this refers to the overlap of the morphologic characteristics of some species with those of others. For example, *A. excavatum* and *A. tamarense*, as well as *A. tamiyavanichii*, share characteristics with *A. affine* and *A. fraterculus* that are related to chain formation and occurrence in natural seawater columns; this overlap confounds morphologic identification.

Adachi et al. (1996b) used molecular techniques to identify *Alexandrium* species, targeting the genomic rRNA gene (rDNA) to identify *A. tamarense* and *A. catenella*; however, the hybridization used in this technique is time-consuming and produces a weak labeling signal.

To develop a molecular identification technique for the toxic *A. tamiyavanichii*, we designed a cellular-rRNA-targeted DNA probe for use in whole-cell fluorescent in situ hybridization (FISH) (Kim, 2003). The *A. tamiyavanichii*–specific DNA probe TAMID2, with a design based on the LSU rDNA regions D1-D2, successfully hybridized with the targeted vegetative cells and showed no cross-reactivity either with *Alexandrium* congeners such as *A. tamarense*, *A. catenella*, *A. affine*, *A. fraterculus*, *A. insuetum*, and *A. pseudogonyaulax* or with concentrated natural seawater samples. Simple and rapid procedures were also established for the use of FISH with whole cells of these species.

In the present study we aimed to design species-specific DNA probes for 6 *Alexandrium* species, *A. tamarense*, *A. catenella*, *A. affine*, *A. fraterculus*, *A. insuetum*, and *A. pseudogonyaulax*, for use in molecular identification with the whole-cell FISH technique previously developed by Kim (2003). The resulting probes were tested for cross-reactivity to *Alexandrium* congeners and concentrated natural seawater samples and used in the detection and identification of toxic *A. tamarense* species in Korean coastal waters.

Materials and Methods

Cultivation and Identification of* Alexandrium *Species for Testing with Whole-Cell FISH. Clonal cultures of *Alexandrium* were established from cultures collected in Korea and Japan (Table 1). *A. tamarense* was incubated at 15°C, and the other *Alexandrium* species were maintained at 20°C in SWIIm medium (Sako et al., 1990) under a 14 hours–10 hours light-dark regimen with 100 μE $m^{-2}s^{-1}$ provided by cool white bulbs. All *Alexandrium* species were identified ribotypically through determination of the sequences of the LSU rDNA Dl-D2 and SSU rDNA regions (Table 1).

Probe Design. Sequences corresponding to the LSU rDNA D1-D2 regions of *Alexandrium* species obtained from GenBank (Table 1) were aligned using CLUSTAL X (Thomson et al., 1997). A species-specific region was identified for use in the design of oligonucleotide DNA probes for 6 *Alexandrium* species: *A. tamarense*, *A. catenella*, *A. affine*, *A. fraterculus*, *A. insuetum*, and *A. pseudogonyaulax*.

Table 1. *Alexandrium* Species Tested with Whole-Cell FISH

Species	*Strain*	*Origin*
A. affine	AFF37	Harima Nada, Japan
A. catenella	DPC7	Pusan, Korea
	ACY12	Harima Nada, Japan
A. fraterculus	DPW9709	Pusan, Korea
A. insuetum	AI104	Utiumi Bay, Japan
A. tamarense	KJC9711	Kojedo, Korea
	HAT4	Hiroshima Bay, Japan
A. pseudogonyaulax	AP391	Harima Nada, Japan

The sequences complementary to the rDNA coding strand, and the sites and melting temperatures of the probes, are shown in Table 2. Portions of the 5′ end of each probe were labeled with fiuorescein isothiocyanate (FITC). Each DNA probe was synthesized commercially and cartridge-purified (Amersham Pharmacia Biotech). Working stocks of the DNA probes were prepared by resuspending the powder containing the DNA oligonucleotides in 10 mM Tris HCl (pH 7.4) to a final concentration of 100 pmol μl^{-1} of probe.

Reactivity of DNA Probes with* Alexandrium *Cells. Cells of each of the 6 cultured *Alexandrium* species were harvested by centrifugation, and the supernatant was removed. The pelleted cells were dehydrated, and autofluorescence originating from chlorophyll was removed using an ethanol series of 50% for 1 minute and 100% for 30 minutes at room temperature. The dehydrated cells were air-dried completely and then subjected to hybridization for 5 minutes at 35°C, in a water bath, in 200 μl of a hybridization buffer containing 40% formamide (Sigma), 5× SSC (83.3 mM NaCl, 83.3 mM trisodium citrate, pH 7.0), and DNA probe (final concentration, 0.5 pmol). Hybridized cells were then washed twice in 5× SSC at 45°C for 5 minutes to remove unhybridized DNA probe. Afterward, the pellets, which contained 10 to 20 μl of cells, were gently resuspended by pipetting and observed at 400× magnification under an epifluorescence microscope (Nikon ECLIPSE E800) equipped with a fluorescein band pass filter set for FITC and counterstain (TRITC. PI) (excitation, 450–490 nm; DM, 505 nm; BA, 520 nm). Fluorescence and light images of the cells were taken using a color-chilled 3-CCD camera (Hamamatsu C5810) with a fixed exposure time of 0.4 seconds.

Reactivity of DNA Probes with Nontargeted* Alexandrium *Congeners and Natural Seawater Samples. Each DNA probe was tested for cross-reaction to cultured vegetative cells of the *Alexandrium* congeners. Whole-cell FISH was performed as described above. Natural seawater samples were collected in netting with a pore size of 20 μm on August 29, 2002, at Maizuru, Kyoto, Japan. Concentrated samples were prepared in polypropylene bottles and not treated for fixation; they were maintained in an icebox at 5° to 10°C during transportation to the laboratory, and then subjected to whole-cell FISH with each DNA probe, within 5 hours after sampling. In the laboratory, field samples were concentrated using a sieve with 20-μm pores and transferred to 50-ml polypropylene disposable centrifuge tubes at room temperature. One milliliter of cultured *Alexandrium* cells (approx. 5000–10,000 cells/ml) was added to 1 ml of the wild samples to test for cross-reaction. The hybridization and washing temperatures were set at the relatively high 40° and 45°C, respectively, to improve the specificity of the probes. After the treatments the cells were resuspended and observed under an epifluorescence microscope (Nikon ECLIPSE E800) under the same conditions as those described above.

Identification of* Alexandrium *Species Isolated from Korea and Japan, Using DNA Probes. Forty-three *Alexandrium* strains from Korea and Japan were identified using 6 *Alexandrium*-species-specific DNA probes. Whole-cell FISH was performed, as described above, and the strains tested are listed in Table 1. The results of the molecular identification were reconfirmed by comparison with ribotypically determined *Alexandrium* strains.

In order to detect and identify the *Alexandrium* species in natural seawater, field sampling was conducted at Sujongri in Chinhae Bay, Korea, in April 2003, during monitoring for *A. tamarense* and

Table 2. *Alexandrium*-Species-Specific DNA Probes with Designs Based on Sequences of LSU D1-D2 Region of rDNA

Species	*Probe*	*Sequences*[a] *(site)*	T_m(°C)
A. affine	AFFD2	TAGGTTTTGATTGTGGGTGT (445–464)	52.0
A. catenella	CATED2	TGGGTTTTGGCTGCAAGTGC (444–463)	70.4
A. fraterculus	FRAD2	TCGATGTGAATTGCAAATGC (447–466)	64.6
A. insuetum	INSUD2	TATATGTTGGATGTGGGTGC (447–466)	60.6
A. tamarense	TAMAD2	GAGCTTTGGGCTGTGGGTGT (444–463)	68.6
A. pseudogonyaulax	PSEDOD2	GTGTKAATTGTATGAGCYTT (444–463)	51.5

[a]Sequences are listed 5′ to 3′ and include primer regions.

A. catenella. Two-liter water samples were collected from the surface layer, and the samples were transported to the laboratory under cool conditions without applying fixation methods. Ten-milliliter samples were placed into a 2-cm-diameter filter apparatus that contained a 20-μm-pore-size nylon sieve. Whole-cell FISH was performed as described above, except that the dehydrated samples were divided into 2 identical volumes and subjected to hybridization with the DNA probes TAMAD2 and CATED2, which are specific to *A. tamarense* and *A. catenella*, respectively. After 2 washes in 5× SSC buffer, the filtrates were mounted onto a glass slide for observation under an epifluorescence microscope, and images were taken as described above.

Results

Labeling of Six* Alexandrium *Species with Specific DNA Probes. Figure 1 shows the results of whole-cell FISH using 6 oligonucleotide DNA probes specific for each targeted *Alexandrium* species. All DNA probes reacted with the targeted cultured *Alexandrium* vegetative cells, as indicated by a fluorescent green color (bright green). Specifically hybridized *Alexandrium* cells were easily identified owing to the retention of their original cell shapes.

Specificity of DNA Probes to* Alexandrium *and Wild Samples. To confirm the species specificity of the DNA probes, each probe was tested for cross-reaction to cultured cells of *A. tamarense*, *A. catenella*, *A. fraterculus*, *A. affine*, *A. insuetum*, *A. tamiyavanichii*, and *A. pseudogonyaulox*. All of the conditions and whole-cell FISH procedures were as described above, except that the hybridization and washing temperatures used were relatively low at 35°C and 40°C, respectively, in order to detect very weak cross-reactivity.

None of the 6 DNA probes reacted with non-targeted *Alexandrium* species, indicating that all of the DNA probes are highly specific (Table 3). None of the probes showed cross-reactivity to field samples composed of various diatoms, dinoflagellates, and zooplanktons, and all of the targeted *Alexandrium* species were detectable with each probe, although the fluorescence intensity was variable (Figure 2). This detection can be attributed to the poor condition of some *Alexandrium* cells in the field samples. Occasionally, detritus-like amorphous clusters were labeled by the DNA probes, a phenomenon that could probably be avoided by size fractionation of the samples using a filter apparatus.

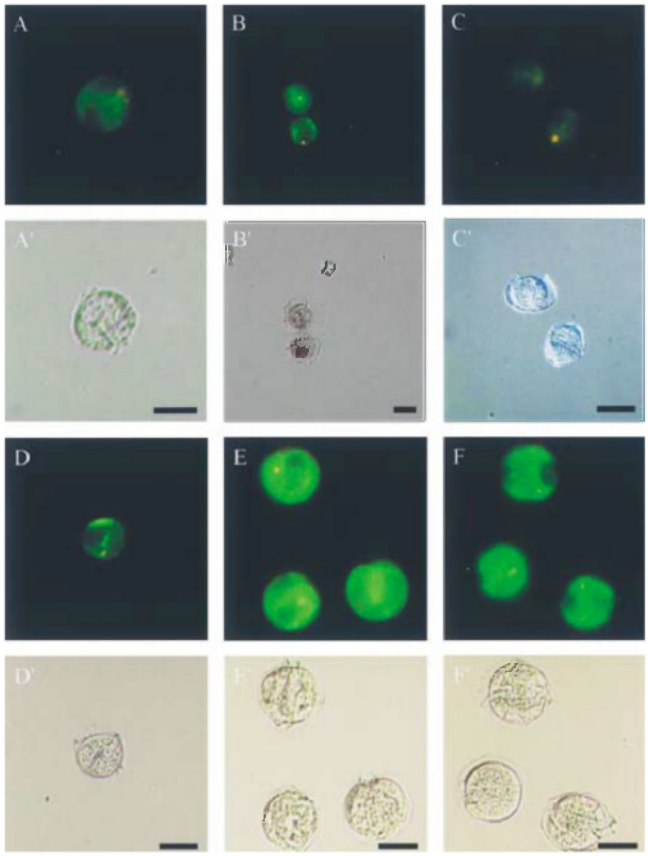

Fig. 1. Epifluorescence (A to F) and light (A′ to F′) micrographs of the 6 *Alexandrium* species *A. affine* (**A** and **A′**), *A. fraterculus* (**B** and **B′**), *A. insuetum* (**C** and **C′**), *A. pseudogonyaulax* (**D** and **D′**), *A. catenella* (**E** and **E′**), and *A. tamarense* (**F** and **F′**), hybridized with the following DNA probes: AFFD2 specific for *A. affine* (**A** and **A′**), FRAD2 specific for *A. fraterculus* (**B** and **B′**), INSUD2 specific for *A. insuetum* (**C** and **C′**), PSEUDOD2 specific for *A. pseudogonyaulax* (**D** and **D′**), CATED2 specific for *A. catenella* (**E** and **E′**), and TAMAD2 specific for *A. tamarense* (**F** and **F′**). Scale bar = 30 μm.

Identification of* Alexandrium *Species Using DNA Probes. Analysis of specimens of 7 *Alexandrium* species and 18 strains using the 6 DNA probes resulted in the same speciation pattern that had been determined using the rDNA D1-D2 sequences summarized in Table 2. The Japanese strains ACY12 (*A. catenella*) and HAT4 (*A. tamarense*) reacted with the *A. catenella*–and *A. tamarense*–specific DNA probes, as did the Korean strains. The ribotypically undetermined *Alexandrium* species SMG0205 was identified as *A. affine*; SOC2, SOC6, and SOC9, as *A. catenella*; and SOC3, YOC2, and YOC3 as *A. tamarense*.

Considerable quantities of *Alexandrium* were observed in the natural seawater samples collected from Chinhae Bay, Korea. In whole-cell FISH, all of the *Alexandrium* cells collected reacted only with

Table 3. Summary of Molecular Identification of *Alexandrium* Species Using Six DNA Probes

Strain (ribotypic species)	*Origin*	*Acc. no.*[a]	*DNA probe*[b]					
			AFFD2	*CATED2*	*FRAD2*	*INSUD2*	*PSEUDOD2*	*TAMAD2*
AFF37 (*A. affine*)	Harima Nada, Japan	AB088227	+	–	–	–	–	–
SMG0205	Saemangeum (Yellow Sea), Korea	Unidentified	+	–	–	–	–	–
DPC7 (*A. catenella*)	Busan Dadaepo, Korea	AB088234	–	+	–	–	–	–
DPC8 (*A. catenella*)	Busan Dadaepo, Korea	AB088239	–	+	–	–	–	–
CMC2 (*A. catenella*)	Thongyong, Korea	AB088229	–	+	–	–	–	–
CMC3 (*A. catenella*)	Thongyong, Korea	AB088233	–	+	–	–	–	–
YSC9811 (*A. catenella*)	Yosu, Korea	AB088273	–	+	–	–	–	–
SOC2	Yosu open sea, Korea	Unidentified	–	+	–	–	–	–
SOC6	Yosu open sea, Korea	Unidentified	–	+	–	–	–	–
SOC9	Kojedo open sea, Korea	Unidentified	–	+	–	–	–	–
ACY12 (*A. catenella*)	Harima Nada, Japan	AB088225	–	+	–	–	–	–
DPW9709 (*A. fraterculus*)	Busan Dadaepo, Korea	AB088244	–	–	+	–	–	–
SPW9709 (*A. fraterculus*)	Chinhae Bay, Korea	AB088262	–	–	+	–	–	–
AI104 (*A. insuetum*)	Utiumi Bay, Japan	AB088248	–	–	–	+	–	–
AP391 (*A. pseudogonyaulax*)	Harima Nada, Japan	AB088279	–	–	–	–	+	–
KJC971 (*A. tamarense*)	Kojedo, Korea	AB088250	–	–	–	–	–	+
SJW97043 (*A. tamarense*)	Chinhae Bae, Korea	AB088258	–	–	–	–	–	+
SJW9516 (*A. tamarense*)	Chinhae Bae, Korea	AB088255	–	–	–	–	–	+
ULW9903 (*A. tamarense*)	Ulsan, Korea	AB088270	–	–	–	–	–	+
SOC3	Busan open sea, Korea	Unidentified	–	–	–	–	–	+
YOC2	Saemangeum (Yellow Sea), Korea	Unidentified	–	–	–	–	–	+
YOC3	Saemangeum (Yellow Sea), Korea	Unidentified	–	–	–	–	–	+
TAMI2201 (*A. tamiyavanichii*)	Harima Nada. Janan	AB088264	–	–	–	–	–	–

[a]Sequences of rDNA D1-D2 region.
[b]AFFD2, CATED2, FRAD2, INSUD2, PSEUDOD2, and TAMAD2 are specific to *A. affine*, *A. catenella*, *A. fraterculus*, *A. insuetum*, *A. pseudogonyaulax*, and *A. tamarense*–specific DNA probe, respectively.
+ and – indicate positive and negative reaction to each DNA probes, respectively.

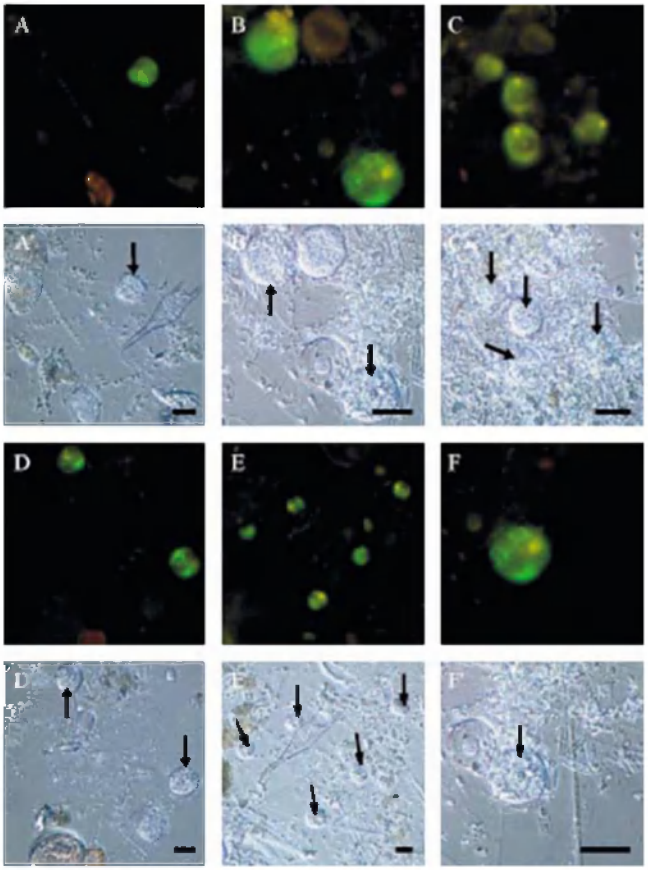

Fig. 2. Epifluorescence (A to F) and light (A′ to F′) micrographs of natural seawater samples containing the 6 added *Alexandrium* species *A. affine* (**A** and **A′**), *A. fraterculus* (**B** and **B′**), *A. insuetum* (**C** and **C′**), *A. pseudogonyaulax* (**D** and **D′**), *A. catenella* (**E** and **E′**), and *A. tamarense* (**F** and **F′**), hybridized with the following DNA probes: AFFD2 specific for *A. affine* (**A** and **A′**), FRAD2 specific for *A. fraterculus* (**B** and **B′**), INSUD2 specific for *A. insuetum* (**C** and **C′**), PSEUDOD2 specific for *A. pseudogonyaulax* (**D** and **D′**), CATED2 specific for *A. catenella* (**E** and **E′**), and TAMAD2 specific for *A. tamarense* (**F** and **F′**). Scale bar = 30 μm.

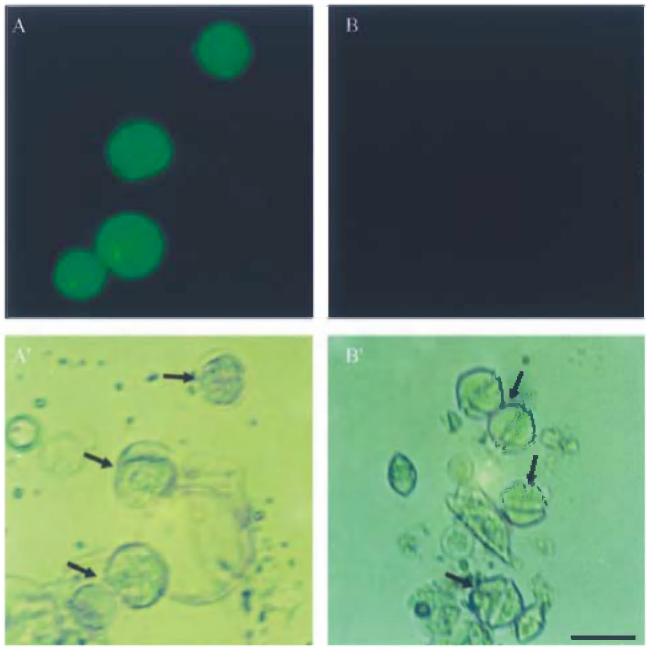

Fig. 3. Epifluorescence (A and B) and light (A′ and B′) micrographs of natural seawater samples hybridized with the DNA probes TAMAD2 (**A** and **A′**) and CATED2 (**B** and **B′**). Arrows indicate *Alexandrium* cells. Scale bar = 30 μm.

the DNA probe TAMAD2, indicating that the cells were *A. tamarense* (Figure 3). Despite the large size range of 25–50 μm of the *A. tamarense* cells in the natural seawater samples, the reactivity of the cells to the DNA probe was strong and consistent.

Discussion

Whole-cell FISH has proven a highly useful molecular tool in the detection and identification of microbes, particularly in the study of human diseases. Although the need for rapid and exact identification of HABs is pressing, to date whole-cell FISH has been used only rarely to monitor HABs.

The results of this study indicate that rapid and exact whole-cell FISH using oligonucleotide DNA probes is an extremely effective molecular tool for the identification of species in the genus *Alexandrium*. We designed 6 DNA probes targeting rRNA molecules specific to *A. tamarense*, *A. tamarense*, *A. affine*, *A. insuetum*, *A. fraterculus*, and *A. pseudogonyaulax*. The FISH methods used allowed the rapid and exact molecular identification of *Alexandrium* species. Probes exposed to concentrated natural seawater samples containing cultured *Alexandrium* vegetative cells showed high specificity and almost no cross-reactivity. Use of these probes in the field appears promising, as *A. tamarense* cells were positively detected and identified in Korean coastal waters with the specific DNA probe TAMAD2.

This whole-cell FISH approach did not require additional fixation, which can negatively affect the physical condition of the cells, nor did it require time-consuming treatment with cetyltrimethyl ammonium bromide (CTAB). The method was possible owing to the direct hybridization of a DNA probe to targeted molecules that have a planar structure; this constitutes one of the most important advantages of rapid whole-cell FISH. Another significant obstacle, interference of the fluorescence illumination of the labeling signal by the autofluorescence derived from the cells (Lee et al., 1993; Christensen et al., 1999), was almost completely removed by merely using an ethanol series for fixation.

During field trials we conducted several preliminary experiments to find ways to preserve whole cells for a longer period and to eliminate the autofluorescence originating from chlorophyll. Samples were diluted to final concentrations of 40%, 50%, and 80% ethanol and 80% acetone immediately after collection. The results were very similar to fixation with glutaraldehyde, which caused undesirable modifications in the targeted cells. Instead, we used low concentrations of ethanol: 10%, 20%, and 30%. This treatment was satisfactory for whole-cell FISH, but the fluorescence intensity was not as strong as in untreated cells. In addition, we analyzed samples that had been stored for 2 days at room temperature (15–25°C), at 4°C, or at −20°C immediately after collection. The rRNA remained in the best condition in samples that had been stored at room temperature. The samples stored at 4°C remained in relatively good condition, but there was considerable decrease in the fluorescence intensity, which may be attributable to autolysis, followed by cell death. Cells that had been frozen broke easily during the thawing process, although the level of autolysis was low. Many authors have reported decreases in the cellular rRNA content during storage in batch mode (Neidhardt and Magasanik, 1960; DeLong et al., 1989; Kemp et al., 1993; Lim et al., 1993, 1996; Poulsen et al., 1993; Ruimy et al., 1994; Zheng et al., 1996; Worden et al., 2000). We are now in search of even more effective procedures for the handling of whole cells, for use in the field without fixation, from dehydration using the ethanol series to hybridization. Nevertheless, our whole-cell FISH technique is very simple, and once hybridized, the targeted rRNA molecules will be more resistant to autolysis.

In the future whole-cell FISH systems employing multiple probes will provide powerful molecular tools for the identification of HABs caused not only by *Alexandrium*, but also by other organisms. Development of a portable system to perform these tests has strong commercial possibilities.

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