MARINE BIOTECHNOLOGY

Species-Specific Detection and Quantification of Toxic Marine Dinoflagellates *Alexandrium tamarense* and *A. catenella* by Real-Time PCR Assay

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Abstract

A Real-time polymerase chain reaction (PCR) assay was designed and evaluated for rapid detection and quantification of the toxic dinoflagellates *Alexandrium catenella* and *A. tamarense*, which cause paralytic shellfish poisoning. Two sets of PCR primers and fluorogenic probes targeting these two species were derived from the sequence of 28S ribosomal DNA. PCR specificity was examined in closely related *Alexandrium* spp. and many other microalgae. *A. catenella*–specific primers and probe detected the PCR amplification only from *A. catenella* strains, and nonspecific signals were not detected from any microalgae. Also, *A. tamarense–specific* primers and probe also detected the targeted species, suggesting the strict species specificity of each PCR. This assay could detect one cell of each species, showing its high sensitivity. Moreover, using the developed standard curves, *A. tamarense* and *A. catenella* could be quantified in agreement with the quantification by optical microscopy. The performance characteristics of species specificity, sensitivity, and rapidity suggest that this method is applicable to the monitoring of the toxic *A. tamarense* and *A. catenella*.

Key words: toxic dinoflagellate — *Alexandrium* — paralytic shellfish poisoning (PSP) — real-time PCR — species-specific identification —quantification

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Introduction

Several marine dinoflagellates produce potent neurotoxins that accumulate in filter-feeding shellfish, causing paralytic shellfish poisoning (PSP) in humans and other mammals. Most of the toxic species belong to the genus *Alexandrium* Halim, and 10 *Alexandrium* spp. are known to produce these toxins (Schantz et al., 1966; Balech, 1995). In Japan PSP is often caused by *A. catenella* (Whedon and Kofoid) and *Alexandrium tamarense* (Lebor) Balech, and has a negative impact on human health and the economy (Shumway, 1990).

To better understand the population dynamics and the occurrence of PSP, it is essential to identify and quantify toxic *Alexandrium* species, such as *A. tamarense* and *A. catenella*, rapidly and accurately. However, it has been problematic to discriminate the toxic species from nontoxic *Alexandrium* species, including 20 morphologically similar species (Balech, 1995). Therefore considerable efforts have been concentrated on the development of identification techniques for these dinoflagellates. Molecular assays that use a genetic marker as a criterion for classification have been utilized to identify and quantify harmful algal bloom (HAB) species including *Alexandrium* spp. (Lenaers et al., 1991; Scholin et al., 1994; Adachi et al., 1996; Sako et al., 2004)

One of these molecular assays employs the polymerase chain reaction (PCR), which is an important tool in several fields, including diagnostic and forensic medicine and molecular biology (Muller et al., 1995; Mauchline et al., 2002; Zhang and Lin, 2002). In microbiology this tool is often applied to identify or detect microscopic cells whose morphologic identification is impossible, and, for example, is often used to detect pathogenic bacteria such as *Salmonella* in food (Cocolin et al., 1998; Lofstrom et al., 2004). The PCR assay has been also investi-

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gated for HAB species (Heley et al., 1999; Penna and Magnani, 1999, 2000). These methods developed for HAB species, however, require complicated operations to obtain high sensitivity and reproducibility; because the detection of PCR product depends on electrophoretic analysis. Moreover, the product is analyzed after the amplification process, so that quantification by PCR is difficult because the amplification reaches a plateau.

Real-time PCR based on the Taq Man probe and 5′–3′-exonuclease activity of the *Taq* polymerase has been developed (Holland et al., 1991). This technique uses not only two primers but also a fluorogenic oligonucleotide probe designed to hybridize within the sequence targeted by the primers. This probe is labeled at the 5′ end with a reporter dye such as 6-carboxy-fluorescein (FAM) and at the 3′ end with a quencher dye such as 6-carboxy-tetramethyl-rhodamine (TAMRA). Although the quencher dye suppresses the fluorescent emission of the reporter dye when the two are in close proximity, the 5′–3′-exonuclease activity of *Tag* polymerase nicks the labeled probe, and the PCR product is detected simultaneously and automatically by the emission of the reporter, which is separated from the quencher (Lee et al., 1993). As the emission by quencher dye is derived only from a targeted PCR product, the specificity of detection is higher than that with the usual PCR requiring electrophoretic analysis. Moreover, with simultaneous and automatic analysis by fluorometer in real time, it is possible to quantify many products correctly, rapidly, and simply without the problem of the amplification plateau (Oberst et al., 1998; Shin et al., 1999; Sharma and Carlson, 2000; Guiver et al., 2001).

In this study we developed and evaluated real-time PCR for the detection and quantification of the toxic species *A. catenella* and *A. tamarense*. First, the specificity, sensitivity, objectivity, and reproductibility of primers and probes were examined after setting the thermal cycling conditions. Next, the quantification of these species was attempted using standard curves obtained by PCR of serial dilutions of cells.

Materials and Methods

Cultures. Clonal strains of *Alexandrium* and the other microalgae employed in this study are shown in Table 1. All clonal strains of *Alexandrium* except for *A. pseudogonyaulax* Biecheler and *A. tamiyavanichii* Balech were grown in SWIIm medium (Sako et al., 1990) at 15°C under previously described conditions (14:10-hour light-dark cycle, 100 μmol photons· m^{-2}· s^{-1}; Adachi et al., 1994). *A. pseudogonyaulax* and *A. tamiyavanichii* were grown at 20°C under the same conditions. Other strains were grown in SWM3 (Chen et al., 1969) at 20°C under the same conditions. All cells were harvested in mid-exponential growth phase and collected by centrifugation (1000*g* for 1 minute) in a 1.5-ml microcentrifuge tube.

Seawater Sample. Seawater used to obtain the standard curve for field investigation was collected from Maizuru Bay. It was checked by optical microscope to confirm that it did not contain *Alexandrium* cells. The sample was stored at 4°C until the examination.

Field samples for analysis were collected at several locations in Japan (Kure Bay, Tokuyama Bay, and Kitsuki Bay on the Seto Inland Sea, and the Tsuo harbor at Yuya-cho on the Sea of Japan) from 1999 to 2002 (Table 2). To estimate the number of organisms present, the samples were examined using an optical microscope. After being counted, cells in these samples were collected by centrifugation (1000*g* for 1 minute) in polypropylene centrifuge tubes and preserved at −30°C until DNA extraction.

Preparation of DNA. Collected cells were suspended by addition of 150 μl of TE (10 mM Tris-HCl, pH 7.5; 1 mM EDTA, pH 8.0) and boiled at 100°C. Then 150 μl of phenol–chloroform–isoamyl alcohol (25:24:1) was added, and suspended cells were shaken at room temperature. After centrifugation supernatant was transferred to a new tube. Then 15 μl of 3 M sodium acetate (pH 5.2) and 400 μl of 99.5% ethanol (−20°C) were added. After centrifugation DNA was pelleted, rinsed with 70% ethanol, then dried and dissolved in 10 μl of TE.

PCR Amplification. Taq Man probes and primers were designed for specific identification of dinoflagellates within the toxic North American *Alexandrium* species (*A. tamarense/fundyense/catenella*) including *A. tamarense* occurring typically in Japan (Scholin et al. 1994) and within the toxic Temperate Asian *Alexandrium* group including *A. catenella* occurring typically in Japan (Scholin et al., 1994), by comparison of the sequences of the D1/D2 region in 28S ribosomal DNA (rDNA) from *A. tamarense, A. catenella, A. tamiyavanichii, A. insuetum* Balech, *A. minutum* Halim, *A. pseudogonyaulax, A. affine* (Inoue et Fukuyo), *A. concavum* Fukuyo, *A. ostenfeldii* (Paulsen) Balech et Tangen, and *A. andersonii* Balech (Figure. 1). For PCR of *A. tamarense*, the Taq Man probe, named Taq man tam, was 5′FAM-AGAGCTTTGGGCTGTGGGTGTA-TAMRA 3′, and the forward and reverse primers were tamF (5′-TGCTTGGTGGGAGTGTTGCA- 3′) and tamR (5′-

Table 1. Strains Used and Specificity of PCR with Species-Selective Primers and Taq Man Probes

Species	*Strain*	*Toxicity*[i]	*Isolation location*	*PCR for A. tamarense*[j]	*PCR for A. catenella*[j]
Alexandrium tamarense	OF151	Yes	Ofunato Bay, Japan	+	–
A. tamarense	HI28[a]	Yes	Hiroshima Bay, Japan	+	–
A. tamarense	At503[b]	Yes	Mikawa Bay, Japan	+	–
A. tamarense	HAT18	Yes	Hiroshima Bay, Japan	+	–
A. tamarense	SHO18	Yes	Hiroshima Bay, Japan	+	–
Alexandrium catenella	OF71	Yes	Ofunato Bay, Japan	–	+
A. catenella	OF72	Yes	Ofunato Bay, Japan	–	+
A. catenella	TN11	Yes	Tanabe Bay, Japan	–	+
A. catenella	Acko5	Yes	Uranouchi Bay, Japan	–	+
A. catenella	AcY9	Yes	Yamakawa Bay, Japan	–	+
A. catenella	DPC8[c]	Yes	Pusan Tdaepo, Korea	–	+
Alexandrium tamiyavanihiii	TAMI220-1 [d]	Yes	Harima-nada, Japan	–	–
A. tamiyavanichii	TAMI220-7[d]	Yes	Harima-nada, Japan	–	–
A. tamiyavanichii	TAMI220-12[d]	Yes	Harima-nada, Japan	–	–
Alexandrium affine	O1[d]	No	Osaka Bay, Japan	–	–
A. affine	AFF37-1[d]	No	Harima-nada, Japan	–	–
A. affine	T1	No	Tachibana Bay, Japan	–	–
Alexandrium ostenfeldii	K-0287	Yes	Denmark	–	–
Alexandrium fraterculus	SJASW-9709-1[c]	No	Chinhae Bay, Korea	–	–
Alexandrium insuetum	Ai140-1[d]	No	Harima-nada, Japan	–	–
Alexandrium pseudogonyaulax[e]		No	Japan	–	–
Gymnodinium mikimotoi	G303[a]	No	Suo-nada, Japan	–	–
Gymnodinium catenatum	GC21V[f]	Yes	Vigo, Spain	–	–
G. catenatum	MZ13	Yes	Miyazu Bay	–	–
Amphidimium carterae	NIES-331	No	Iriomote Is1., Japan	–	–
Heterocapsa triquetra	NIES-7	No	Osaka Bay, Japan	–	–
Heterocapsa circularisquama	HA92-1[g]	No	Ago Bay, Japan	–	–
Heterosigma akashiwo	893[h]	No	Hiroshima Bay, Japan	–	–
H. akashiwo	NIES-293	No	Onagawa Bay, Japan	–	–
Prorocentrum micans	NIES-12	No	Osaka Bay, Japan	–	–
Chattonella antiqua	NIES-1	No	Harima-nada, Japan	–	–
Chattonella marine	NIES-559	No	Maizuru Bay, Japan	–	–
Chattonella ovata	NIES-603	No	Harima Nada, Japan	–	–
Chattonella verrculosa	NIES-670	No	Harima Nada, Japan	–	–

[a–g]Donors of strains. [a]Dr. M. Yamaguchi; [b]Dr. M. Ishida; [c]Dr. C.H. Kim; [d]Dr. S. Yoshimatsu; [e]Dr. M. Yoshida; [f]Dr. B. Reguera; [g]Dr. T. Uchida; [h]Dr. I. Imai.
[i]PSP toxicity was assayed by HPLC- fluorometric method (Sako et al., 1992).
[j]"+" indicates PCR positive; "–", PCR negative.

TAAGTCCAAGGAAGGAAGCATC-3′), respectively. PCR of *A. catenella* used Taq man cat (5′-FAMAT-GGGTTTTGGCTGCAAGTGCATAMRA-3′), and catF (5′-CCTCAGTGAGATTGTAGTGC-3′) and catR (5′-GTGCAAA GGTAATCAAATGTCC-3′). The length of targeted fragment was 160 bp on PCR for *A. catenella* and 230 bp on PCR for *A. tamarense*. The primers and probe were synthesized by Hokkaido System Science Co., Ltd., and received in a lyophilized form. Thermal cycling and detection of the amplicon were performed with a Smart Cycler System (Cepheid Inc.) in 25-μl Smart Cycler reaction tubes in triplicate. PCR

Table 2. Cell Density Calculated by Optical Microscopy and Real-Time PCR

			A. tamarense (cells/L)		*A. catenella (cells/L)*	
Sample	*Collection site*	*Collection date*	*Optical microscopy*	*Real-time PCR*	*Optical microscopy*	*Real-time PCR*
1	Kure Bay, Japan	April 1999	332 ± 39	266 ± 89	None	Neg.
2[a]	Tokuyama Bay, Japan	May 1999	None	Neg.	344,250 ± 43,459	301,219 ± 8,297
3[a]	Tokuyama Bay, Japan	June 2000	None	Neg.	129,435 ± 17,500	97,231 ± 10,945
4	Kitsuki Bay, Japan	April 2001	41 ± 23	21 ± 29	None	Neg.
5[a]	Tsuo Harbor, Japan	June 2002	None	Neg.	3,864,895 ± 19,562	4,136,844 ± 28,533

[a]Samples 2 and 3 formed bloom by other microalgae: sample 2 contained 1.9×10^6 cells/L of *Heterosigma akashiwo* and 1.2×10^5 cells/L of *Prorocentrum dentatum;* samples 3 contained 1.5×10^5 cells/ml of *H. akashiwo* and 4.8×10^4 cells/L of *P. triestinum. Alexandrium* sp. in sample 5 formed the bloom.

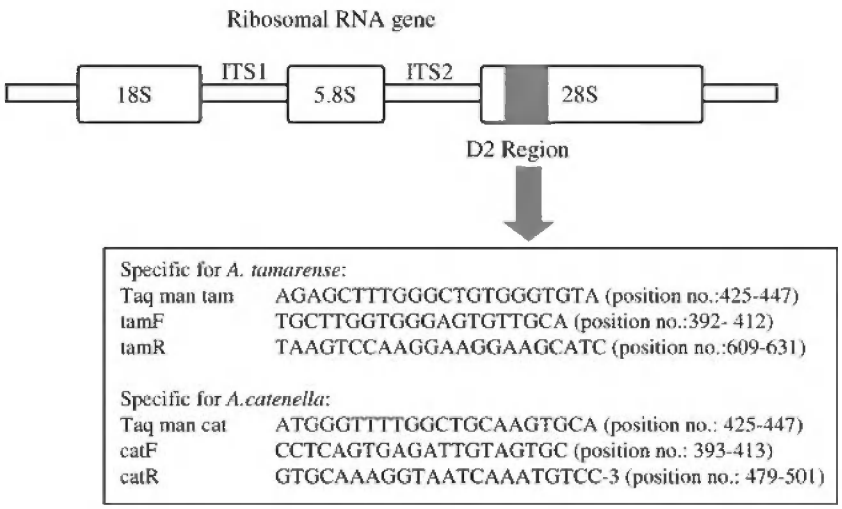

Fig. 1. Position and sequence of primers and probes. Probes and primers were designed to the D2 region of the 28S rRNA gene that was defined by Scholin et al. (1994). Sequences of primers and probes are shown in 5′ → 3′. Position number corresponds to alignment position indicated by Scholin et al. (1994).

was carried out in 25-µl volumes containing 1 × PCR *EX Taq* buffer to which were added 20 mM Mg^{2+}, 200 uM dATP, dTTP, dGTP, and dCTP, 0.3 µM each primer, 0.2 µM fluorogenic probe, and 1.25 U *Taq* DNA polymerase (*Takara Ex Taq*, TaKaRa Bio Inc.). The cycling conditions were as follows: one cycle of heating at 95°C for 3 minutes, then 40 cycles of 94°C for 15 seconds, 56°C for 30 seconds, and 72°C for 30 seconds.

Results

Specificity of Taq Man Probe and Primer. Taq Man probes and primers were designed for specific identification of the toxic *A. tamarense* within the North American *Alexandrium* species (*A. tamarense/fundyense/catenella)* and the toxic *A. catenella* within the Temperate Asian *Alexandrium* group (Scholin et al., 1994), by comparison of the sequences of the D1/D2 region in 28S rDNA in 10 *Alexandrium* species containing our sequence data: *A. tamarense, A. catenella, A. tamiyavanichii, A. insuetum, A. minutum, A. pseudogonyaulax, A. affine, A. concavum, A. ostenfeldii,* and *A. andersonii* (Figure 1). When the PCR assay using the probes and primer targeted to *A. catenella* was applied to 8 species of *Alexandrium* and other HAB species, amplification was detected only with the *A. catenella* strains, which were collected from different regions in Japan and Korea (Table 1). Nonspecific signals were not detected from any other *Alexandrium* species (Figure 2) or any other HAB species. The PCR assay using tamF and tamR PCR primers and *Taq Man* probe detected only *A. tamarense* strains (data not shown), and no nonspecific amplification was detected. Controls containing no template DNA were negative with both sets of species-selective primers and probe.

Next, to examine the effect of nontargeted DNA on the PCR assay, the targeted *A. catenella* DNA was assayed in the presence of various nontargeted DNAs. First, 50 ng of *A. catenella* DNA was used as a positive control (Figure. 3, A). Second, 50 ng of *A. catenella* DNA was mixed with a DNA mixture composed of 100 ng of *A. tamarense*, 100 ng of *A. tamiyavanichii*, 20 ng of *A. insuetum*, 20 ng of *A. minutum*, 20 ng of *A. pseudogonyaulax*, 20 ng of *A. affine*, and 20 ng of *A. ostenfeldii* (Figure 3 B). Third, 50 ng of *A. catenella* DNA was mixed with 200 ng of a DNA mixture of equal amounts of DNAs of 5 HAB species: *Gymnodinium catenatum, Heterocapsa circularisquama* Horiguchi, *Heterosigma akashiwo* (Hada) Hara et Chihara, *Chattonella antiqua* (Hada) Ono, and *Gymnodinium mikimotoi* Miyake et Kominami ex Oda (Figure 3, C). Fourth, 50 ng of *A.*

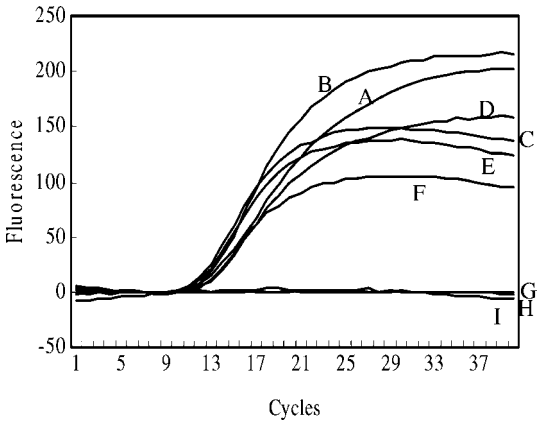

Fig. 2. Specificity of *A. catenella* primers and probe. A–F were *A. catenella* strains: OF72 (A); TN11 (B); AcY9 (C); Acko5 (D); DPC8 (E); and OF71 (F). G: *A. tamarense* HAT18. H: *A. tamiyavanichii* TAMI220-1. I: Negative control not containing DNA. Concentration of DNA from all strains was 50 ng per reaction.

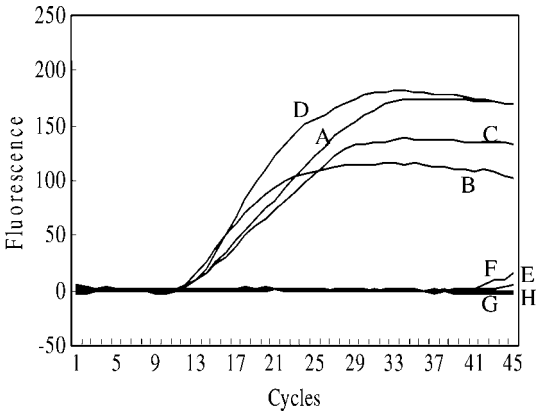

Fig. 3. Effect of background DNA on PCR assay with *A. catenella* primers and probe. A: *A. catenella* DNA, 50 ng. B: Seven species of *Alexandrium* spp. DNA, 300 ng (100 ng of *A. tamarense*, 100 ng of *A. tamiyavanichii*, 20 ng of *A. insuetum*, 20 ng of *A. minutum*, 20 ng of *A. pseudogonyaulax*, 20 ng of *A. affine*, and 20 ng of *A. ostenfeldii*), and *A. catenella* DNA, 50 ng. C: Five HAB species DNA, 200 ng (equal amounts of DNAs of *Gymnodinium catenatum*, *Heterocapsa circularisquama*, *Heterosigma akashiwo*, *Chattonella antiqua*, and *Gymnodinium mikimotoi*), and *A. catenella* DNA, 50 ng. D: DNA, 200 ng, extracted from seawater, and *A. catenella* DNA, 50 ng. E: *A. tamarense* DNA, 200 ng. F: *A. tamiyavanichii* DNA, 200 ng. G: Seven species of *Alexandrium* spp. (same as B) DNA, 300 ng. H: Negative control not containing DNA.

catenella DNA was mixed with DNA extracted from natural seawater collected from Maizuru Bay (Figure 3 D). As a result, the targeted *A. catenella* DNA in Figure 3(A–D) was amplified at almost the same number of cycles, suggesting that the assay was not affected by a large amount of nontargeted DNA (Figure 3). Nonspecific signals were not detected from any other *Alexandrium* species (Figure 3E, F) or any other *Alexandrium* species mixture (G). Also controls containing no template DNA were negative with both sets of species-selective primers and probe. When the same examinations were performed on *A. tamarense*, the targeted DNA was amplified regardless of nontargeted DNA and all primers and probe sets showed high specificity to the targeted species (data not shown).

Quantification and Sensitivity of PCR Assay. Quantifiability of the PCR assay was confirmed by plotting the threshold cycle against serial dilutions (50,000 cells to 1 cell) of *A. catenella* cells (Figure 4 A-a). Discrimination of threshold cycle fluorescence from background fluorescence was performed automatically using Smart Cycler software. As a result, the plots showed high linearity (R^2 = 0.97) (Figure 4, A-b). This analysis also showed that this PCR assay could detect a single cell of *A. catenella* (Figure 4, A-a), which was impossible to detect by the conventional PCR using electrophoresis before the amplification reached a plateau. The high linearity and sensitivity to detect a single cell was also found with *A. tamarense* (Figure 4 B).

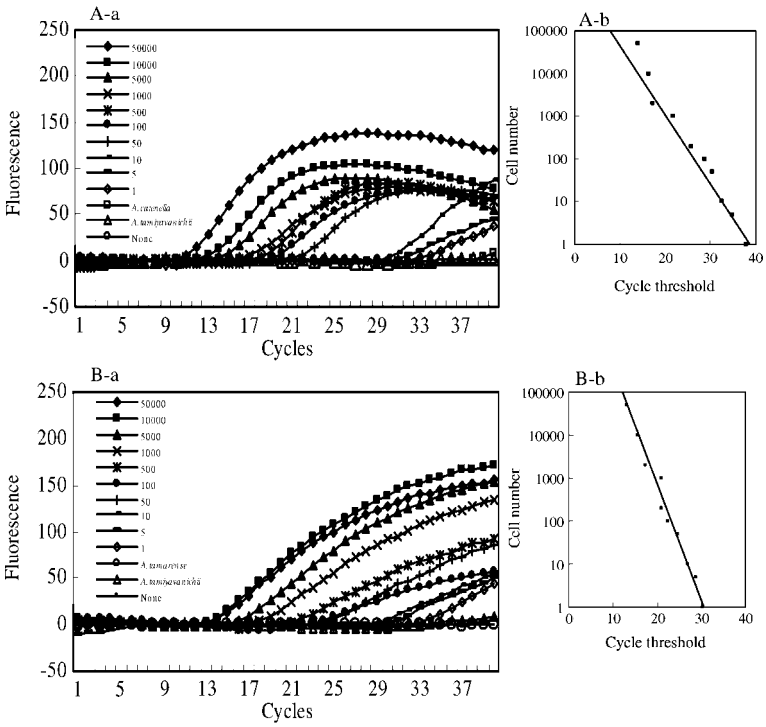

Fig. 4. PCR assay using *A. catenella* (A) and *A. tamarense* (B) primers and probes. A-a, B-a: Serial dilutions of cells (numbers indicate cell number of targeted species in one reaction for each curve). Concentration of control DNA was 50 ng per reaction. A-b, B-b: Cycle threshold plotted against the cell number.

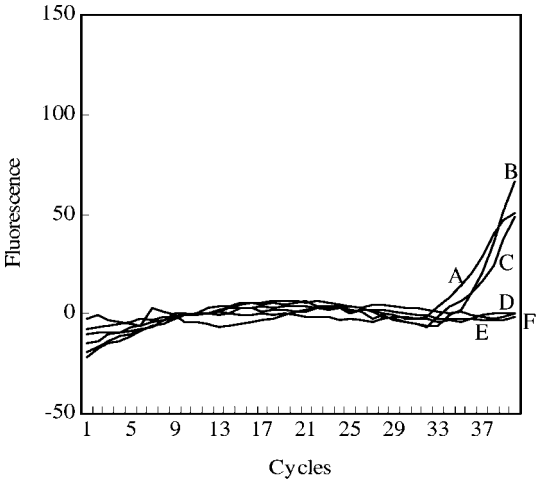

Fig. 5. PCR assay with single cells of *A. catenella*. A: Single cells were picked up with a Pasteur pipette, washed with TE buffer 3 times, and placed directly into a PCR tube. B: Single cells were treated in the same way as in A, then incubated at 100°C for 10 minutes before thermal cycling. C: DNA was extracted from a single cell and placed into a PCR tube. Concentration of DNA of *A. tamarense* HAT18 (D) and *A. tamiyavanichii* TAMI 220-1 (E) was 50 ng per reaction. F: Negative control without DNA.

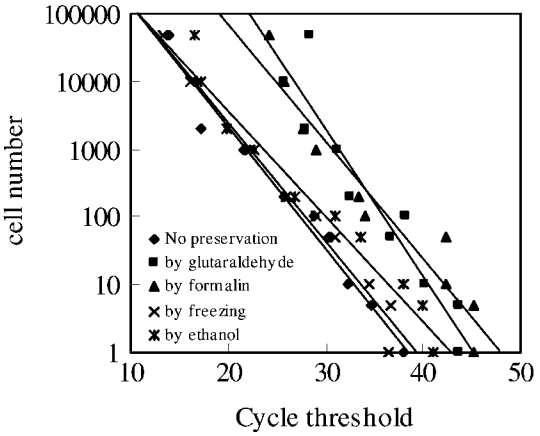

Fig. 6. Effect of various fixation and preservation methods (1 week) on PCR assay of *A. catenella* cells. Cycle threshold is plotted against the cell number. See text for details.

To confirm the sensitivity of detection and the loss of DNA in the process of DNA extraction, single cells of *A. catenella* were treated by 3 different methods and applied to PCR: (A) a single cell was picked up with a Pasteur pipette, washed with TE buffer 3 times, and placed directly into a PCR tube; (B) a single cell was treated in the same way as in the first treatment, and then incubated at 100°C for 10 minutes before thermal cycling; (C) DNA was extracted from a single cell and placed into a PCR tube. The specific amplification was distinguished from background regardless of the way in which the cells were treated (Figure 5). Slightly higher sensitivity was observed when the cell was placed directly into the tube. Similar results were obtained with *A. tamarense*, showing the high sensitivity of designed primers and probes for two species (data not shown).

Stability of PCR Assay. The stability of the PCR assay was examined using cells cultured under different conditions. First, the effect of cultivation temperature on PCR assay was examined. DNAs extracted from 100 cells of *A. catenella* that were cultured at 4 different temperatures (10°, 15°, 20°, and 25°C) for 10 days were assayed in the same run, and amplifications were detected at the same threshold cycle regardless of cultivation temperature (data not shown). The same result was obtained with *A. tamarense* (data not shown).

Next, the influence of lack of nutrients in culture medium on the PCR assay was examined. DNAs from 100 cells of *A. catenella* that had been cultured in 3 different media (SWIIm, SWIIm lacking nitrogen source, and SWIIm lacking phosphorus source) for 10 days were assayed in the same run. No difference in threshold cycle was observed between limited nutrient medium and the optimal medium (data not shown). The assay of *A. tamarense* was also not affected by the lack of nutrients (data not shown).

Finally, the effect of fixation method on the PCR assay was examined because samples must be preserved by fixation when they cannot be assayed immediately. Four fixation methods were examined: with formalin (final concentration, 1%) at 4°C, with glutaraldehyde (final concentration, 1%) at 4°C, with ethanol (final concentration 50%) at 4°C, and by freezing at −20°C. *A. catenella* cells were preserved for 1 week by each fixation method and assayed in the same run with control DNA that was immediately extracted from cultured cells without preservation. Freezing at −20°C showed almost the same cycle threshold as control, which was the highest efficiency in the 4 methods. Ethanol fixation slightly decreased efficiency compared with control. Formalin and glutaraldehyde fixation decreased the sensitivity: the threshold cycle increased by about 8 cycles with formalin and with glutaraldehyde (Figure 6).

PCR Assay of Field Samples. To obtain a curve for quantification of field samples, a serial dilution (50,000 cells to 1 cell) of cultured *A. catenella* cells was added to natural seawater not containing *Alexandrium* cells and assayed by PCR. The plots of

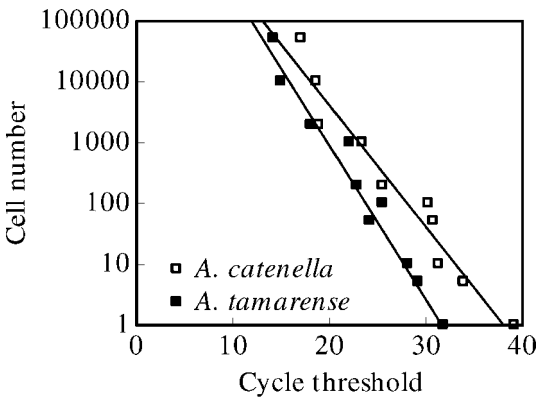

Fig. 7. PCR assay on *A. catenella* and *A. tamarense* cells added to seawater. Cycle threshold is plotted against the cell number.

detected cells versus the number of threshold cycles were almost same as those obtained with cultured cells, showing a highly linearity; R^2 value was 0.97 (Figure 7), which was thought to be useful for quantification of the targeted cells in a filed sample. The plots for *A. tamarense* in seawater also showed high linearity (R^2 = 0.93).

Five field samples collected at several locations in Japan were assayed using two sets of primers and probe for *A. tamarense* and *A. catenella* (Table 2). Cell numbers calculated from the standard curves in each assay were compared with counts made by optical microscopy. Overall, the cell densities estimated from the standard curve and by optical microscopy were similar. Although two samples from Tokuyama Bay in 1999 and 2000 represented a bloom of other microalgae (sample 2 contained 1900 cells/ml of *Heterosigma akashiwo* and 125 cells/ml of *Prorocentrum dentatum*; sample 3 contained 150 cells/ml of *Heterosigma akashiwo* and 48 cells/ml of *Prorocentrum triestinum*), the targeted *A. catenella* cells were detected accurately, without being affected by the large amount of other microalgal DNA and several factors in the field sample that might inhibit or promote the amplification of PCR.

Discussion

Assay with Taq Man Probe. The real-time PCR assay using 5′-3′-exonuclease activity of the *Taq* polymerase was applied to cultured *A. tamarense, A. catenella,* and several other microalgae. The sets of primers and probe used in this study were proven to have high species specificity, with no cross-reaction with DNA extracts from other *Alexandrium* species or other HAB species. This result supports real-time PCR as a useful technique for discriminating among cultured *Alexandrium* spp. Several molecular biological techniques for the identification of HAB species use a genetic marker, in most cases one that depends on rRNA gene family. In this study the targeted region in 28S rDNA was shown to be adequate for accurate identification of closely related species. Moreover, the designed primers and probes targeted dinoflagellates within the North American *Alexandrium* species (*A. tamarense/fundyense/catenella*) and within the Temperate Asian *Alexandrium* group, both of which comprised toxic species (Scholin et al., 1994). Therefore, the comparison with database sequences revealed that the sets of primers and probe used in this study could specifically discriminate the toxic strains belonging to these two groups, which might be made clear by expanding the studies to include species and strains of *Alexandrium* from other regions of the globe.

Single cells were assayed after treatment in 3 different ways (details in Figure 5), and a slightly higher sensitivity was observed when the cell was placed directly into the tube, but there was no great difference among the 3 treatments. The application not of extracted DNA, but of intact cells to the PCR assay might be better for the accuracy of quantification, because some DNA could be lost in the process of extraction. However, field samples are likely to contain factors that influence PCR, so it was thought that DNA should be extracted in order to remove these factors.

Real-time PCR is known to provide accurate quantification because the analysis is performed in real time. However, application of this method to *Alexandrium* had not been reported. A conventional PCR assay using electrophoretic analysis had been presented by Penna and Magnani (1999, 2000), but their assay used radioisotope-labeled DNA probe or an enzyme-linked immunosorbent assay (ELISA) method, with troublesome quantification. Furthermore, the smallest number of detectable cells was 150, considerably higher than in the real-time PCR assay in this study. This number of toxic *Alexandrium* cells (150 cells/L) is capable of causing PSP, and thus the PCR assay developed by Penna and Magnani might not be useful for monitoring. Heley et al. (1999) also reported detection of *Alexandrium* by PCR, but their analysis does not allow accurate quantification. Recently, Galluzzi et al. (2004) developed a real-time PCR assay using the 5.8S rDNA region for the detection of *Alexandrium* species and showed that the assay allowed accurate quantification and was useful for monitoring coastal waters. However, this procedure does not allow one to identify each species and also count the nontoxic ones. Accordingly, the occurrence of toxic species sometimes included a bloom of nontoxic

Alexandrium species and toxic species like *A. tamarense* and *A. catenella* could not be strictly monitored by their PCR assay. Our species-specific PCR targeting the toxic species might make more detailed monitoring possible.

Trial of PCR for Field Samples. For application of PCR to field samples, the effect of several factors that might be present in natural samples was examined. Cells cultured under conditions of deleted nutrients were detectable in the same way as cells cultured under optimum conditions, showing that this method could be useful for field samples that inhabit variable environmental conditions. Moreover, this result indicates that the number of copies of rDNA in a cell was not influenced by the change of surroundings, and PCR targeting rDNA could be used to quantify cells. Also, the similarity of the amplification curves of several strains of *A. catenella* (Figure 2) reveals the invariability of copy number of rDNA in the population.

Although application of the PCR assay to field samples often requires their preservation, the sensitivity was considerably lower with preservation using formalin and glutaraldehyde than with no preservation (Figure 6). Bowers et al. (2000) also reported that the sensitivity of detection of *Pfiesteria piscicida* by PCR assay was greatly decreased by fixation with Lugol's solution, which might be due the difficulty of extraction of total DNA. In contrast, freezing did not affect the sensitivity, and the sensitivity with preservation using ethanol was near to that without fixation. Therefore, these two methods, freezing and ethanol, were considered to be better for cell preservation. In fact, it was possible to detect and quantify the targeted cells from field samples preserved by freezing (−20°C) for 3 years, suggesting the utility of freezing as a preservation method (Table 2).

Serial dilution of targeted *Alexandrium* cells was performed with natural seawater not containing the targeted *Alexandrium* cells, and the cell number was plotted to the cycle threshold. The curve obtained for each species was highly linear, and the quantification of field samples using these curves was mostly in agreement with the quantification by optical microscopy. This result indicates that it might be possible to apply the PCR assay to field samples and achieve accurate quantification. Moreover, although a large number of *Alexandrium* spp. cells in field samples of blooms could be examined using the real-time PCR developed previously (Galluzzi et al., 2004), our PCR detected a small number of the targeted species from field samples not forming a bloom.

The procedure of real-time PCR developed here was less time-consuming and laborious than analysis by light microscopy, and might provide a useful tool for monitoring in place of identification based on the morphologic characteristics. More data from field sample are necessary confirm this potential. Moreover, although at present it is difficult to discriminate between *A. tamarense* and *A. catenella* cysts on the basis of morphologic characteristics, the performance characteristics and procedures of this method are thought to be applicable to species-specific detection of cysts in bottom sediments, which could be clarified by more examinations of cysts.

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