Fish Aqua Sci 12(3), 227-235, 2009

KOSFAS FISHERIES AND AQUATIC SCIENCES
The Korean Society of Fisheries and Aquatic Science

Abundance of the Toxic Dinoflagellate *Alexandrium catenella* in Jinhae Bay, Korea as Measured by Specific Real-time PCR Probe

Tae-Gyu PARK*, Yang-Soon KANG and Young-Tae PARK
Ecology and Oceanography Division, National Fisheries Research and Development Institute (NFRDI), Busan 619-705, Korea

The marine toxic dinoflagellate *Alexandrium catenella* has been implicated in numerous paralytic shellfish poisoning (PSP) events in many countries. Due to difficulties in rapidly identifying *A. catenella*, field-based study of this species has been problematic. The present study developed a TaqMan format *A. catenella*-specific probe for real-time PCR assay (specific to Korean genotype) based on LSU rDNA sequence information for studying geographic and temporal distribution of the species in surface sediments and water columns of Jinhae Bay, Korea. The field survey from 2007 to 2008 revealed that *A. catenella* occurred in most seasons at low densities, mostly below 1 cell mL^{-1}, and was more abundant in spring (maximum cell density of 2 cells mL^{-1}) when shellfish exceed the quarantine toxin level for PSP toxins in Jinhae Bay.

Key words: *Alexandrium catenella*, Dinoflagellate, PSP toxins, Real-time PCR probe

Introduction

The marine dinoflagellate *Alexandrium catenella* (Whedon et Kofoid) Balech produces paralytic shellfish poisoning (PSP) toxins and is responsible for a wide variety of environmental and public health problems (Hallegraeff, 1993; 2003). The genus *Alexandrium* Halim includes about 30 species, and the majority of toxic blooms have been caused by *A. tamarense* complex (*A. catenella*, *Alexandrium tamarense* (Lebour) Balech and *Alexandrium fundyense* Balech) and/or *Alexandrium minutum* Halim (Cembella, 1998; Scholin et al., 1995; Hallegraeff, 2003). *A. catenella* is distributed worldwide, mainly in coastal areas, and often co-occurs with other *Alexandrium* species (Faust and Gulledge, 2002; Hallegraeff, 2002; Lundholm and Moestrup, 2006). The first known bloom of *Alexandrium* species and PSP event in Korean waters occurred in Jinhae Bay in 1978 (Cho, 1978). Since then, blooms and PSP events have recurred, especially along the southern coasts of Korea (Kim and Kim, 2004). Korean *A. catenella* has been known to produce PSP toxins and their components are species-specific in Korean *A. catenella* (Kim 2000).

The life cycles of the genus *Alexandrium* consist of asexual (vegetative cells) and sexual (resting cysts) reproduction (Anderson, 1980). Vegetative cells of *Alexandrium* species are morphologically defined by their distinct thecal plate tabulation and cell shape, whereas *Alexandrium* cysts do not have species-specific morphological characteristics (Balech, 1995). Morphological identification of *Alexandrium* species requires light microscopy and/or scanning electron microscopy (SEM) analyses on laboratory-cultured or field-derived cells. For identification of cysts, viable cysts from field samples are incubated in culture medium and germinated cells are then examined for species identification under a microscope. These conventional identification methods are labor-intensive and not suitable for rapid sample processing. To overcome these difficulties, real-time quantitative PCR assay, offering highly sensitive and rapid analysis, has been used for the detection and quantification of a number of dinoflagellates, including *Karlodinium veneficum* (Ballantine) J. Larsen, *Pfiesteria piscicida* Steidinger et Burkholder, *Pfiesteria shumwayae* Glasgow et Burkholder, and *Cryptoperidiniopsis brodyi* Steidinger et Litaker (Bowers et al., 2000; Zhang and Lin, 2005; Park et al., 2007, 2009a, b; Handy et al., 2008).

Due to the difficulties of rapid identification of *A. catenella* in field samples, the species' geographic and temporal distributions are poorly understood. The present study developed an *A. catenella*-specific real-time PCR assay (specific to Korean genotype) using a

*Corresponding author: taegyupark@gmail.com

TaqMan probe based on large subunit (LSU) rDNA sequence information. An oligonucleotide probe was used to investigate geographical and temporal distributions of *A. catenella* in Korean coastal waters and sediments. These results will contribute to the understanding of the occurrence of *A. catenella* in environmental samples.

Materials and Methods

Cultures

Cultures were obtained from NFRDI's collection of microalgae, Seoul National University, University of Tasmania, and CCMP (Provasoli-Guillard National Center for Culture of Marine Phytoplankton) (Table 1). Mixotrophic/phototrophic cultures were maintained in an f/2 medium of 28‰ salinity (Guillard and Ryther, 1962) without sodium silicate at 24°C, with cool white fluorescent lamps of 100 μmol photons m^{-2} s^{-1} on a 12:12-h light : dark cycle. Heterotrophic dinoflagellates were grown in an f/2 medium of 15‰ salinity at 24°C in the dark, and *Rhodomonas salina* (Wislouch) Hill et Wetherbee was supplied as food.

Collection of water column and sediment samples

Water column samples of 250 mL were collected from 21 stations in Jinhae Bay, South Korea at one month intervals from April to November 2007 and February to September 2008 (Fig. 1). Water samples were not collected from December 2007 to January 2008, as this was the winter season in Korea. Water samples were filtered onto a 1.2 μm pore-size glass microfibre GF/C filter (25 mm diameter; Whatman, Ltd. Maidstone, England). Filtered samples were placed in 2 mL microcentrifuge tubes and stored at -70°C until DNA extraction. To prevent the degradation of target DNA, filtered samples were processed on a research vessel.

Sediment samples were collected from 79 stations in Korean coastal waters. Fifty-four, ten, and fifteen samples were obtained from sediments of the South, East, and Yellow Seas, respectively. Of the 54 South Sea samples, 21 were collected in Jinhae Bay from the same sampling stations as the water samples (Fig. 1). These samples were collected in September 2007 using a Lafonte grab. The surface layer of the sediment (approximately the top 2 cm) was transferred into a 50 mL conical tube and stored at 4°C until DNA extraction.

DNA extraction from cultures and environmental samples

A phenol-chloroform extraction protocol was used for the extraction of genomic DNA from the water column samples and laboratory cultures, following Hosoi-Tanabe and Sako (2005). Filter samples were suspended in 900 μL of TE buffer (10 mM Tris-HCl,

Table 1. Cultures used in this study

Culture code	Date of isolation	Locality	Identification
NFFACA1	April, 2000	Japan	*Alexandrium catenella*
CCMP1911	September, 1998	Sequim Bay, USA	*Alexandrium catenella*
CCMP116	June, 1984	Ria de Vigo, Spain	*Alexandrium tamarense*
CCMP112	November, 1985	Ria de Vigo, Spain	*Alexandrium affine*
CCMP113	September, 1987	Ria de Vigo, Spain	*Alexandrium minutum*
CCMP1718	January, 1987	Massachusetts, USA	*Alexandrium andersoni*
CCMP1719	November, 1985	New Hampshire, USA	*Alexandrium fundyense*
CCMP1773	1986	Fjorden, Denmark	*Alexandrium ostenfeldii*
CCMP1888	1962	Laguna Obidos, Portugal	*Alexandrium lusitanicum*
CCMP2082	June, 1985	Uchiumi Bay, Japan	*Alexandrium insuetum*
CCMP2215	January, 1984	Kanagawa, Japan	*Alexandrium hiranoi*
NFFCPO1	September, 2003	Gijang, South Korea	*Cochlodinium polykrikoides*
NFFCPO2	August, 2005	Yeosu, South Korea	*Cochlodinium polykrikoides*
NFFGIM1	May, 1998	Narodo, South Korea	*Gymnodinium impudicum*
CCMP1678	1993	East Victoria, Australia	*Gymnodinium impudicum*
Sa-1 YD	June, 2007	South Korea	*Stoeckeria algicida*
PPSB25	May, 2003	Surabaya, Indonesia, Ballast water	*Pfiesteria piscicida*
CCMP2807	December, 2002	North Carolina, USA	*Pfiesteria shumwayae*
CBWA12	February, 2003	Brunswick River, Western Australia	*Cryptoperidiniopsis brodyi*
CBHU1	February, 2003	Huon River, Tasmania, Australia	*Cryptoperidiniopsis brodyi*
CCMP2229	August, 2001	Florida, USA	*Karenia brevis*
CCMP1974	1995	Chesapeake Bay, USA	*Karlodinium veneficum*
CCMP415	1976	Norway	*Karlodinium veneficum*
CCMP1589	September, 1992	Rhode Island, USA	*Prorocentrum micans*
CCMP1170	February, 1982	Victoria, Australia	*Rhodomonas salina*

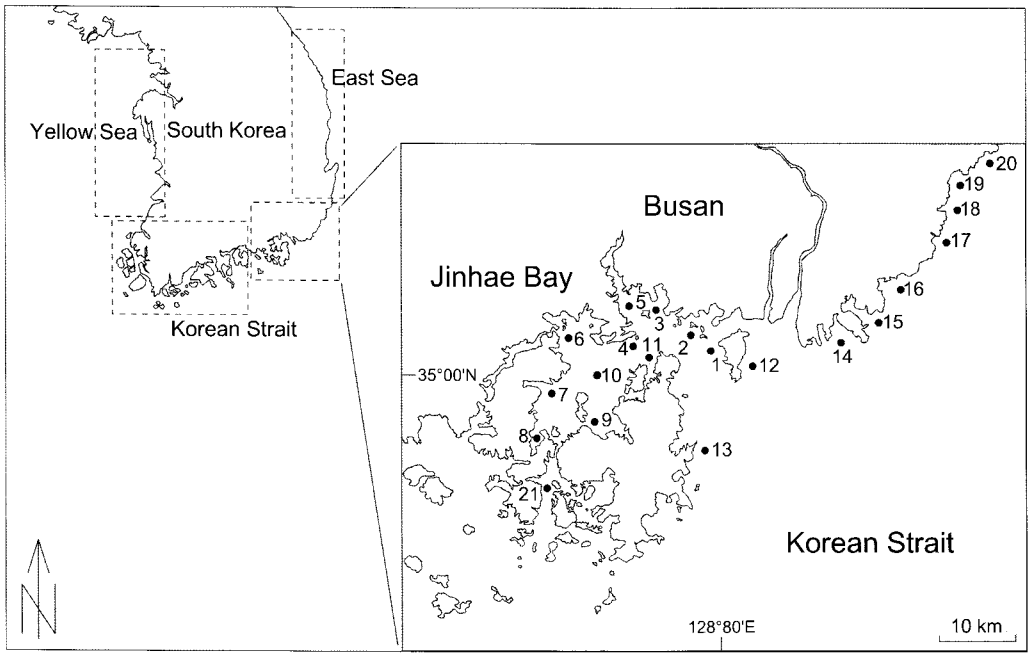

Fig. 1. Locations where water and sediment samples were collected for this study. Surface waters were sampled from 21 stations in Jinhae Bay and surface sediments were sampled from 79 stations in East, South, and Yellow Seas. Dashed lines indicate where the sediment samples were collected.

pH 7.5; 1 mM EDTA, pH 8.0) and ground with wooden applicator sticks. Samples were boiled at 100°C for 5 min, after which 900 µL of phenol: chloroform: isoamyl alcohol (25:24:1) was added. Samples were then mixed thoroughly and centrifuged at 14,000 rpm for 10 min. The supernatant (300 µL) was transferred to a new tube, and 30 µL of 3 M sodium acetate (pH 5.2) and 700 µL of 99.5% of ethanol (-20°C) were added, followed by incubation of the sample at -20°C for 30 min. The DNA samples were centrifuged at 14,000 rpm for 20 min at 4°C and rinsed twice with 70% ethanol. They were then air-dried and dissolved in 100 µL of TE buffer.

Genomic DNA from sediment samples was extracted using the UltraClean Soil DNA Kit (MoBio Laboratories, Inc., Carlsbad, CA, USA). Five mL of 0.3 M NaCl was added to each 50 mL conical tube containing approximately 60 g of sediment. Samples were mixed thoroughly by vortexing and centrifuged at 2,000 rpm for 20 min. After the supernatant was decanted, a sterile spatula was used to transfer approximately 1 g of the top layer of the wet sediment to a tube containing beads and lysis buffer supplied with the kit. For chemical and mechanical lysis of cysts, samples were vortexed at maximum speed for 10 min. DNA was extracted from the samples following the manufacturer's protocol and stored at -20 or -70°C.

Design of the *A. catenella*-specific TaqMan probe for real-time PCR assay

A total of 87 LSU rDNA sequences of *A. catenella* were available from GenBank. These sequences, from various countries such as Korea, Japan, Australia, New Zealand, Hong Kong, and France were aligned with sequences of other organisms (more than 100 species of dinoflagellate) available from GenBank using the program ClustalX (Thompson et al., 1997). Because *Alexandrium tamarense* (Lebour) Balech is the species most closely related to *A. catenella*, *A. catenella* sequences were also aligned with all *A. tamarense* sequences available from GenBank. Unique sequences were manually searched for from these alignments to design a TaqMan-based species-specific probe for real-time PCR assay. The *A. catenella*-specific probe (169 bp amplicon in size) was designed on the D2 region of LSU rDNA because this region was highly variable between species but was conserved within Korean genotype of *A. catenella* (Table 2). Primer sequences had at least 12 bp mismatches with sequences of closely related organisms, and the primer-probe combination had at least 16 bp mismatches. The sequence specificity of the probe and primers was also checked against published sequences in GenBank by BLAST homology search. The optimal melting temperature and secondary structure of the primer and probe

Table 2. Primers and TaqMan probes for species-specific real-time PCR. The binding sites of the primers and probes are the LSU rDNA and the ITS2 rDNA for *A. catenella* and *C. brodyi*, respectively. The sequences of field derived PCR product amplified by ACLSUF-ACLSUR primers are identical to *A. catenella* sequences (GenBank accession number: AY082048, LSU D2 rDNA)

Dinoflagellate	Forward/ Reverse/ Probe	Name	Sequence (5'→3')	Reference
Alexandrium catenella	Forward	ACLSUF	TGGCTGCAAGTGCAATAATTC	This study
	Reverse	ACLSUR	CAAGGACAAGGACACAAACAAATAC	
	Probe	ACLSUP	FAM–CCCGCAGGAAAATTACCATTCATGTG–BHQ1	
Cryptoperidiniopsis brodyi	Forward	CBITSF	TTGACACGTTGAAGTGAWGGA	Park et al. 2007
	Reverse	CBITSR	ACAGCCAATGAAAGAGTKATGACAA	
	Probe	CBITSP	FAM–CATCTCATCGCTCGCCGTCGAT–BHQ1	

Table 3. The C_T values of TaqMan-based *A. catenella* specific real-time PCR by different primer and probe concentrations ranging from 100 to 300 nM and 100 to 200 nM, respectively. Only 100 nM of probe concentration is shown. [a] Mean ± standard deviation of triplicate wells

Forward / Reverse primers / Probe concentrations	C_T values[a]	Cells mL^{-1} [a]
100/100/100 nM	21.10 ± 1.22	7,191 ± 5,849
100/200/100 nM	19.48 ± 0.62	18,460 ± 8,026
100/300/100 nM	20.37 ± 0.81	10,322 ± 4,973
200/100/100 nM	22.18 ± 0.84	3,039 ± 1,786
200/200/100 nM	20.44 ± 0.82	9,856 ± 4,692
200/300/100 nM	18.60 ± 0.23	32,161 ± 5,479
300/100/100 nM	19.32 ± 1.20	23,548 ± 15,095
300/200/100 nM	18.14 ± 0.12	44,029 ± 3,609
300/300/100 nM	18.04 ± 0.16	47,260 ± 5,381

sequences were predicted by Primer 3 (Whitehead Institute and Howard Hughes Medical Institute, Chevy Chase, MD, USA) and Oligo analyzer 3 (Integrated DNA Technologies, Inc., Coralville, Iowa, USA) software. The TaqMan probe was dual-labeled with FAM (fluorescent dye, 6-carboxyfluorescein) and BHQ1 (non-fluorescent black-hole quencher 1) at the 5'- and 3'-ends, respectively. The primer-probe set was synthesized by Sigma-Proligo (Paris, France).

Optimization of primer-probe concentrations

A dilution series of primer and probe concentrations was tested by real-time PCR to achieve optimal assay performance. The testing primer and probe concentrations ranged from 100 to 300 nM (100, 200, 300 nM) and 100 to 200 nM (100, 150, 200 nM; final concentrations), respectively. The lowest C_T values and the highest calculated cell numbers were produced at 300 nM of forward primer, 300 nM of reverse primer, and 100 nM of probe concentrations (Table 3). Accordingly, these concentrations were used for all assays, including standard curves and field samples.

Specificity of the real-time PCR probe

Assay specificity was tested against 19 other dinoflagellates and *R. salina* (total of 25 cultures, Table 1). Prior to TaqMan-based real-time PCR, the specificity of the forward and reverse primers was tested using SYTO9-based real-time PCR, which does not require a fluorogenic probe. The absence of nonspecific reactions was checked by inspecting the melting curves and PCR amplicons of the target DNA (169 bp) in a 2% agarose gel stained with ethidium bromide. Subsequently, the specificity of the TaqMan-based probe was tested using related species.

Real-time PCR conditions

When tested by the Primer 3 computer program, primers had similar melting temperatures (Tm) of approximately 60°C, and the probe had a Tm 10°C higher than that of the primers. Subsequently, 60°C was chosen as the annealing temperature. The following reagents were added for the TaqMan-based real-time PCR assay: 5 μL of platinum quantitative PCR supermix-UDG (Invitrogen, Eugene, Oregon, USA), primers at a final concentration of 0.3 μM, fluorogenic probe at a final concentration of 0.1 μM, 1 μL of template DNA, and PCR-grade water to a final volume of 10 μL. For the SYTO9-based real-time PCR assay, the following components were added: SYTO9 dye (Molecular Probes, Eugene, Oregon, USA) at a final concentration of 2.0 μM, platinum quantitative PCR supermix-UDG, primers, template DNA, and PCR-grade water as described above. The thermal cycling conditions consisted of 2

min at 50°C (uracil DNA glycosylase incubation for prevention of the reamplification of carryover PCR products) and 2 min at 95°C, followed by 45 cycles (50 cycles for SYTO9) of 10 s at 95°C and 45 s (40 s for SYTO9) at 60°C. Both the SYTO9 and TaqMan (FAM)-based assays were analyzed in the FAM channel of the Rotor Gene instrument, as they have similar excitation/emission maxima (470-510 nm). Fluorescence data were collected at the end of each cycle by the real-time PCR instrument (Rotor Gene 6000, Corbett Research, Sydney, Australia). For the SYTO9-based assay, DNA melting curves were monitored from 70°C to 85°C in 0.2°C increments using a 20-s hold at each step on the FAM channel, and these were analyzed using the Rotor Gene software (v1.7 build 61).

Standard curves for quantification of cell numbers

For the construction of a standard curve using laboratory-cultured *A. catenella*, cell numbers were estimated by light microscopy before cell harvesting (total 19,050 cells). Genomic DNA was extracted, and 10-fold serial dilutions of the DNA extracts were used to construct the standard curve (triplicate measurements from real-time PCR). The numbers of cells of *A. catenella* in surface water samples were calculated as C_T values and were measured by comparison with the standard curve. The accuracy of the standard curves was evaluated using known concentrations of *A. catenella* (12,800, 2,560, and 512 cells; $n=3$) spiked into sterile-filtered field samples (0.2-μm membrane filter; MFS, California). After DNA extraction, cell numbers estimated using real-time PCR were compared to the cell numbers estimated using light microscopy.

For quantification of *A. catenella* DNA copy numbers in sediments, *A. catenella* genomic DNA was amplified with primers ITS2-LSUB (Litaker et al., 2003; Galluzzi et al., 2004). The PCR product was cloned using a TOPO TA Cloning kit (Invitrogen) and insert-containing plasmids were isolated from overnight bacterial cultures using a plasmid purification kit (Promega, Madison, WI, USA). Plasmid DNA yield was determined using the PicoGreen double-stranded DNA (dsDNA) quantification kit following the manufacturer's protocol (Molecular Probes). A standard curve was constructed with 10-fold serial dilutions of plasmid DNA (10^2-10^7 copies) containing the ITS-LSU rDNA. The rDNA copy numbers of *A. catenella* in sediment samples were then measured by comparison with the plasmid standard curve.

PCR-inhibitor removal from environmental samples and template DNA dilution

The dilution method was used for the removal of PCR inhibitors and/or adjustment of template DNA concentration in environmental samples. A real-time PCR probe specific for *C. brodyi* (Table 2) was used for checking the inhibitors, because *C. brodyi* has not been reported in Korean waters. Water samples collected in August (the warmer season in Korea) and sediment samples from Jinhae Bay (two samples each) were used for the inhibitor test. Serial DNA dilutions of field samples (non-dilution, 10-fold, and 50-fold dilutions) spiked with *C. brodyi* DNA (CBWA12; 1.5 ng μL^{-1}) were amplified using real-time PCR, and their C_T values were compared. *C. brodyi* DNA without the addition of field DNA was used as a positive control. The absence of inhibitors was confirmed in 10-fold and 50-fold DNA dilutions. Twenty other field samples were randomly chosen and were tested to confirm the absence of the inhibitors. Subsequently, all field DNA samples were 10-fold diluted, and the results of dilution were calculated into the cell estimates for the quantitative component of the real-time PCR assay.

Verification of assay specificity by environmental sample sequencing

One of the positive field samples was chosen for sequence analyses. The rDNA (169 bp in size) of a positive sample was amplified with primers ACITS and ACITSR in a standard PCR platform using Takara *EX Taq* DNA polymerase (Takara Mirus Bio, Madison, WI, USA) with 39 cycles of 94°C for 1 min, 60°C for 1 min 30 s, and 72°C for 2 min. A positive DNA band was visualized on a 2% agarose gel stained with ethidium bromide, the PCR product was cloned, and the insert-containing plasmid DNA was purified. Two clones of the purified product were selected for sequencing, and their analyses were conducted with pUC/M13 primers (Promega) by Macrogen (Seoul, Korea). Expectation values (E-values) and sequence similarities of the field sequences were estimated using a nucleotide BLAST program.

Results

Specificity of the real-time PCR assay for *A. catenella*

Specificity of forward and reverse primers was tested using SYTO9 format real-time PCR, which does not require a fluorogenic probe. The result of the SYTO9 real-time PCR visualized on an agarose gel

showed only one amplicon of the expected size (169 bp) for the target species and no non-specific reactions against the related species listed in Table 1 (part of results shown in Fig. 2). Assay specificity was then tested in TaqMan format real-time PCR against related species. The primer-probe set was specific to *A. catenella* and did not react with other species (Fig. 2). For further confirmation of assay specificity, a positive real-time PCR product derived from a surface water sample taken at St. 6 (May 2008) was sequenced. The partial LSU rDNA sequence (169 bp) was identical to previously reported *A. catenella* sequences (GenBank accession numbers: AY082048 etc. a total of 40 sequences from GenBank were identical to the sequence), and low expectation values (E-value) of less than 1e-5 were estimated by a nucleotide BLAST program, in-dicating that the real-time PCR probe produces *A. catenella*-specific reactions in Jinhae Bay. Although the real-time PCR probe was specific to Korean isolates, the sequences of the probe were identical to some *A. tamarense* isolates from the U.S.A. (GenBank accession numbers: EU165300, AY268612, AY338749; Lilly et al., 2007), indicating that this DNA probe may not offer specific detection when applied to other countries.

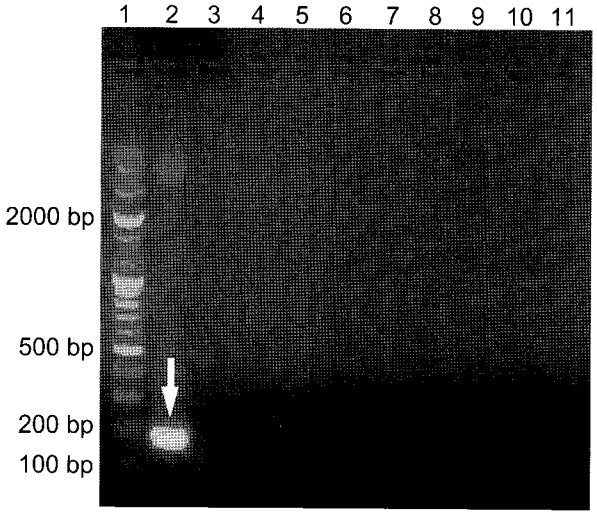

Fig. 2. An agarose gel showing *A. catenella*-selective real-time PCR product (169bp). Assay specificity was tested against related species. Lanes: 1, 2 kb-ladder molecular size marker; 2, *A. catenella*; 3, *A. tamarense*; 4, *A. affine*; 5, *A. minutum*; 6, *A. andersoni*; 7, *A. fundyense*; 8, *A. ostenfeldii*; 9, *A. lusitanicum*; 10, *A. insuetum*; 11, no-template control. Other related species tested (Table 1) were not shown.

Assay sensitivity and standard curve

Ten-fold serial dilutions of DNA extracted from *A. catenella* culture and plasmid DNA containing *A. catenella* LSU rDNA were used for the construction of standard curves. A strong linear correlation between log (plasmid DNA copy number or known cell number) and C_T value was found for the assay (correlation coefficient R^2 of 0.99 for plasmid DNA and extracted cell DNA). The *A. catenella*-specific assay gave a detection limit of 1.9 cells per reaction within the dynamic range of DNA copy numbers in the linear relationship. When DNA extracts from known cell numbers of *A. catenella* (12,800, 2,560, and 512 cells) spiked with environmental samples were compared to the cell standard curve, cell numbers of the 10-fold diluted spiked cells (1,780± 530, 412±102, and 40±25 cells, respectively; n=3; P>0.05 by Student's t-test) did not significantly differ from those of the standard curve (1,280, 256, and 51 cells, respectively).

Geographical and temporal distributions of *A. catenella*.

The field survey conducted in Jinhae Bay showed that *A. catenella* cell numbers ranged from 1 to 2,280 cells L^{-1} of surface water. The mean values of positives from 21 water samples at each month were shown in Fig. 3. *A. catenella* DNA was detected in most seasons expect for September and October. The maximum cell density was 2,280 cells L^{-1} in May 2008, and the highest percent of positives (47.6% of the total positives) was also found in May 2008 (Fig. 3). *A. catenella* was more abundant in spring (March to May; 4-2,280 cells L^{-1}), and high cell density was also detected in August 2008 at 1,276 cell L^{-1}, although the percent of positives was low (9.5% of the total positives). The survey of cyst distribution showed that only four samples (out of 79 samples) were positive for *A. catenella*. Two positive samples each were detected from the sediments of Jinhae Bay and Goheung, ranging from 1 to 835 rDNA copies cm^{-3} obtained from the top layer of the 60 g of sediment.

Discussion

Application of real-time PCR for quantitative detection of *A. catenella*

Due to the difficulty in rapid identification of *Alexandrium* species, molecular identification methods such as real-time PCR have been used for quantitative detection of this genus (Galluzzi et al., 2004). Real-time PCR based on a TaqMan probe can provide highly sensitive and accurate detection of target dinoflagellates (Bowers et al., 2000; Zhang and Lin, 2005; Park et al., 2007). The real-time PCR

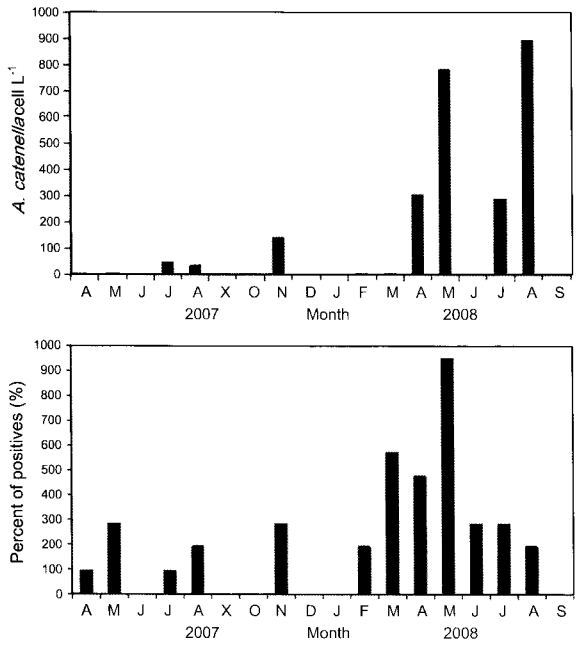

Fig. 3. Temporal variations in abundances of *A. catenella* in surface water of Jinhae Bay from April to November, 2007 and from February to September, 2008. Samples were not collected in December 2007 and January 2008. A, Abundances of *A. catenella* in water column. B, percentage of positively detected samples for *A. catenella* by real-time PCR. The values are the means of positively detected samples (by *A. catenella* specific real-time PCR) from 21 water samples at each month.

assay specific to *A. catenella* developed in this study was highly sensitive, providing a detection limit of less than 1.9 cells reaction^{-1}. An *A. catenella*-specific real-time PCR probe was previously developed based on LSU rDNA, and its specificity was extensively tested against related species (Hosoi-Tanabe and Sako, 2005). Although the existing DNA probe was designed on sequences that differed from those of closely related species by 8 bp, a primer-probe set developed in the present study differed from those of other species by 16 bp, indicating that the use of our real-time PCR probe may reduce false positives when applied to Jinhae Bay. For example, *A. tamarense* and *A. catenella* specific DNA probes previously developed for LSU rDNA were extensively tested against related species and were successfully used for field application in Japan (Kamikawa et al., 2007) but non-specific or false negative reactions were detected when applied to Korean waters (T.G. Park, unpublished). In the present study, a total 50 sequences of Korean *Alexandrium* isolates (Kim and Kim 2004; Kim et al., 2004) was used for designing the DNA probe and the sequences of the DNA probe were unique against other *Alexandrium* species from Korea. However, sequences from the real-time PCR probe used in the present study are identical to those of *A. tamarense* from some regions, including the eastern USA. Because high genetic variation exists in *Alexandrium* species by region (Kim and Kim, 2004; Lilly et al., 2007; Masseret et al., 2009), the development of an *A. catenella* DNA probe specific to isolates from various countries is problematic. Although the present real-time PCR probe may not offer species-specific detection when applied in other countries, this DNA probe may be specific to Korean isolates due to the development of the DNA probe based on the genetic characteristics of Korean isolates.

Geographical and temporal distributions of *A. catenella*.

Two different approaches, including plasmid DNA and cell extract DNA standard curves, have been used for quantifying target DNA (Galluzzi et al., 2004; Zhang and Lin, 2005). The plasmid DNA standard curve uses the absolute number of gene copies in the target species. For example, 100-200 copies have been reported from a single *P. piscicida* cell (Saito et al., 2002). As no DNA copy numbers are known for *A. catenella* cells, and collecting a large amount of cysts in laboratory-cultures is difficult, the amounts of cysts in sediments were used to indicate plasmid DNA copy numbers (maximum 835 rDNA copies cm^{-3} wet sediment from the present study). Only four sediment samples out of 79 were positive for *A. catenella*. Several reasons for the low percentage of positives from the sediment samples can be proposed. First, the target DNA may not have been fully extracted from the sediments. However, this is unlikely to be the case, because the commercial soil DNA extraction kit used in this study has been successfully used for the extraction of a number of dinoflagellate cysts including *Pfiesteria* species (Bowers et al., 2006). Second, the volume of the sediment samples collected may not have been sufficient for the detection of *A. catenella* cysts, or the amount of cysts may have been very low in the sediments surveyed in this study. The use of a commercial kit with larger capacity that can process up to 10 g of sediments may be an alternative choice to improve assay sensitivity. *Alexandrium* species produce two different forms of cyst: temporary and resting cysts (Anderson, 1980). The real-time PCR method developed in this study cannot distinguish between these different types of cysts; thus, the assay may detect both temporary and resting cysts. Although this approach does not provide resting cyst-

specific quantitative detection, the amount of resistant forms of *A. catenella* in sediments can be estimated by this approach.

For the enumeration of cell numbers in surface waters, DNA extracts obtained from known cell numbers were used in this study. The quantitative results showed that *A. catenella* occurs in most seasons and is more abundant in spring. PSP events have caused the closure of shellfish harvesting grounds in Jinhae Bay when shellfish exceed the quarantine toxin level of 80 μg STXeq 100 fresh weight g^{-1}. NFRDI (2008) reported that the quarantine toxin level for *Mytilus edulis* was exceeded from March to May 2007 (maximum 879 μg STXeq 100 fresh weight g^{-1}) and from April to May 2008 (maximum 713 μg STXeq 100 fresh weight g^{-1}) in Jinhae Bay. These results suggest a positive association between higher cell densities of a causative agent in spring and PSP events during the same season, although discrepancy in these results was also shown. For example, *A. catenella* occurred at high cell densities in August, although PSP events were not recorded during that month. The low percentage of positive occurrences (9.5%) may explain the absence of PSP events in August. Another discrepancy was that lower cell densities were detected in 2007 than in 2008, although the level of PSP toxins exceeded the quarantine level during the spring season (from March to May) in 2007. One possible explanation is the co-occurrence of *A. catenella* with other toxic *Alexandrium* species during the survey period. Although the present results are insufficient to explain the discrepancies between cell densities in waters and PSP events in 2007, our results nevertheless showed that *A. catenella* might be responsible for the PSP events in spring 2008. Future studies are needed to investigate the occurrence of other *Alexandrium* species in order to understand PSP events and their bloom dynamics.

Acknowledgements

We thank Jin-Hee Choi of the National Fisheries Research and Development Institute (NFRDI) for technical support with molecular work, and Chul-Woo Park of NFRDI for providing water and sediment samples. We are also grateful to Heon-Meen Bae, Sang-Ok Chung, and Chang-Kyu Lee of NFRDI for their valuable advice and suggestions. This work was funded by a grant from NFRDI (RP-2009-ME-025).

References

Anderson, D.M. 1980. Effect of temperature condition on development and germination of *Gonyaulax tamarense* (Dinophyceae) hypnozygote. J. Phycol., 16, 166-172.

Balech, E. 1995. The genus *Alexandrium* Halim (Dinoflagellata). Sherkin Island Marine Station, Ireland.

Bowers, H.A., T. Tengs, H.B. Glasgow, J.M. Burkholder, P.A. Rublee and D.W. Oldach. 2000. Development of real-time PCR assays for rapid detection of *Pfiesteria piscicida* and related dinoflagellates. Appl. Environ. Microbiol., 66, 4641-4648.

Bowers, H.A., T.M. Trice, R.E. Magnien, D.M. Goshorn, B. Michael, E.F. Schaefer, P.A. Rublee and D.W. Oldach. 2006. Detection of *Pfiesteria* spp. by PCR in surface sediments collected from Chesapeake Bay tributaries (Maryland). Harmful Algae, 5, 342-351.

Cembella, A.D. 1998. Ecophysiology and metabolism of paralytic shellfish toxins in marine microalgae. In: Anderson, D.M., Cembella, A.D., Hallegraff, G.M. (Eds.), Physological Ecology of Harmful Algal Blooms. Springer Verlag, Berlin, Heidelberg, 381-404.

Cho, C.H. 1978. On the *Gonyaulax* red tide in Jinhae Bay. Bull. Kor. Fish. Soc., 11, 111-114.

Faust, M.A. and R.A. Gulledge. 2002. Identifying harmful marine dinoflagellates. Smithsonian Institution, Washington.

Galluzzi, L., A. Penna, E. Bertozzini, M. Vila, E. Garcés and M. Magnani. 2004. Development of a real-time PCR assay for rapid detection and quantification of *Alexandrium minutum* (a Dinoflagellate). Appl. Environ. Microbiol., 70, 1199-1206.

Guillard, R.R.L. and J.H. Ryther. 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* cleve. Can. J. Microbiol., 8, 229-239.

Hallegraeff, G.M. 1993. A review of harmful algal blooms and their apparent global increase. Phycologia, 32, 79-99.

Hallegraff, G.M. 2002. Aquaculturists' guide to harmful Australian microalgae. School of Plant Science, Hobart.

Hallegraeff, G.M. 2003. Harmful algal blooms: a global overview. In: Hallegraeff, G.M., Anderson, D.M., Cembella, A.D. (Eds.), Manual on Harmful Marine Microalgae. United Nations Educational, Scientific and Cultural Organization, Paris, 25-49.

Handy, S.M., E. Demir, D.A. Hutchins, K.J. Portune, E.B. Whereat, C.E. Hare, J.M. Rose, M. Warner, M. Farestad, S.C. Cary and K.J. Coyne. 2008. Using quantitative real-time PCR to study competition and

community dynamics among Delaware Inland Bays harmful algae in field and laboratory studies. Harmful Algae, 7, 599-613.

Hosoi-Tanabe, S. and Y. Sako. 2005. Species-specific detection and quantification of toxic marine dinoflagellates *Alexandrium tamarense* and *A. catenella* by real-time PCR assay. Mar. Biotechnol., 7, 506-514.

Kamikawa, R., S. Nagai, S. Hosoi-Tanabe, S. Itakura, M. Yamaguchi, Y. Uchida, T. Baba and Y. Sako. 2007. Application of real-time PCR assay for detection and quantification of *Alexandrium tamarense* and *Alexandrium catenella* cysts from marine sediments. Harmful Algae, 6, 413-420.

Kim, C. J. 2000. PSP toxin production and molecular biological identification of regional isolates of the genus *Alexandrium* (Dinophyceae) occurring in Korean coastal waters. M.S. Thesis, Pukyong National University, Busan, Korea.

Kim, K.Y. and C.H. Kim. 2004. A molecular phylogenetic study on Korean *Alexandrium catenella* and *A. tamarense* isolates (Dinophyceae) based on the partial LSU rDNA sequence data. J. Kor. Soc. Oceanog., 39, 163-171.

Kim, C.J., Y. Sako, A. Uchida and C.H. Kim. 2004. Molecular phylogenetic relationships within the genus *Alexandrium* (Dinophyceae) based on the nuclear-encoded SSU and LSU rDNA D1-D2 sequences. Journal of the Korean Society of Oceanography, 39, 172-185.

Lilly, E.L., K.M. Halanych and D.M. Anderson. 2007. Species boundaries and global biogeography of the *Alexandrium tamarense* complex (Dinophyceae). J. Phycol., 43, 1329-1338.

Litaker, R.W., M.W. Vandersea, S.R. Kibler, K.S. Reece, N.A. Stokes, K.A. Steidinger, D.F. Millie, B.J. Bendis, R.J. Pigg and P.A. Tester. 2003. Identification of *Pfiesteria piscicida* (Dinophyceae) and *Pfiesteria*-like organisms using internal transcribed spacer-specific PCR assays. J. Phycol., 39, 754-761.

Lundholm, N. and Ø. Moestrup. 2006. The biogeography of harmful algae. In: Granéli, E., Turner, J.T. (Eds.), Ecology of harmful algae. Springer Verlag, Berlin, Heidelberg, 23-35.

Masseret, E., D. Grzebyk, S. Nagai, B. Genovesi, B. Lasserre, M. Laabir, Y. Collos, A. Vaquer and P. Berrebi. 2009. Unexpected genetic diversity among and within populations of the toxic dinoflagellate *Alexandrium catenella* as revealed by nuclear microsatellite markers. Appl. Environ. Microbiol., 75, 2037-2045.

NFRDI. 2008. Data on shellfish poisoning. http://www.nfrdi.re.kr (written in Korean).

Park, T.G., M.F. de Salas, C.J.S. Bolch and G.M. Hallegraeff. 2007. Development of a real-time PCR probe for quantification of the heterotrophic dinoflagellate *Cryptoperidiniopsis brodyi* (Dinophyceae) in environmental samples. Appl. Environ. Microbiol., 73, 2552-2560.

Park, T.G., Y.T. Park and Y. Lee. 2009a. Development of a SYTO9 based real-time PCR probe for detection and quantification of toxic dinoflagellate *Karlodinium veneficum* (Dinophyceae) in environmental samples. Phycologia, 48, 32-43.

Park, T.G., G.H. Park, Y.T. Park, Y.S. Kang, H.M. Bae, C.H. Kim, H.J. Jeong and Y. Lee. 2009b. Identification of the dinoflagellate community during *Cochlodinium polykrikoides* (Dinophyceae) blooms using amplified rDNA melting curve analysis and real-time PCR probes. Harmful Algae, 8, 430-440.

Saito, K., T. Drgon, J.A. Robledo, D.N. Krupatkina and G.R. Vasta. 2002. Characterization of the rRNA locus of *Pfiesteria piscicida* and development of standard and quantitative PCR-based detection assays targeted to the nontranscribed spacer. Appl. Environ. Microbiol., 68, 5394-5407.

Scholin, C.A., G.M. Hallegraeff and D.M. Anderson. 1995. Molecular evolution of the *Alexandrium tamarense* "species complex" (Dinophyceae): dispersal in the North American and West Pacific regions. Phycologia, 34, 472-485.

Thompson, J.D., T.J. Gibson, F. Plewnaik, F. Jeanmougin and D.G. Higgins. 1997. The CLUSTALX windows interface: flexible strategies for multiple sequence alignment aided by quality tools. Nucleic Acids Res., 25, 4876-4882.

Zhang H. and S. Lin. 2005. Development of a *cob*-18S rDNA gene real-time PCR assay for quantifying *Pfiesteria shumwayae* in the natural environment. Appl. Environ. Microbiol., 71, 7053-7063.

(Received 2009; Revised 2009;
Accepted 2009)