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Sensitive, Accurate PCR Assays for Detecting Harmful Dinoflagellate *Cochlodinium polykrikoides* Using a Specific Oligonucleotide Primer Set

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Harmful *Cochlodinium polykrikoides* is a notorious harmful algal bloom (HAB) species that is causing mass mortality of farmed fish along the Korean coast with increasing frequency. We analyzed the sequence of the large subunit (LSU) rDNA D1-D3 region of *C. polykrikoides* and conducted phylogenetic analyses using Bayesian inference of phylogeny and the maximum likelihood method. The molecular phylogeny showed that *C. polykrikoides* had the genetic relationship to *Amphidinium* and *Gymnodinium* species supported only by the relatively high posterior probabilities of Bayesian inference. Based on the LSU rDNA sequence data of diverse dinoflagellate taxa, we designed the *C. polykrikoides*-specific PCR primer set, CPOLY01 and CPOLY02 and developed PCR detection assays for its sensitive, accurate HAB monitoring. CPOLY01 and CPOLY02 specifically amplified *C. polykrikoides* and did not cross-react with any dinoflagellates tested in this study or environmental water samples. The effective annealing temperature (T_p) of CPOLY01 and CPOLY02 was 67℃. At this temperature, the conventional and nested PCR assays were sensitive over a wide range of *C. polykrikoides* cell numbers with detection limits of 0.05 and 0.0001 cells/reaction, respectively.

Key words: *Cochlodinium polykrikoides*, Harmful algal bloom (HAB), PCR detection assay, Species-specific PCR primer set

Introduction

Since its first report of a harmful algal bloom (HAB) in the Korean coast in 1982, *Cochlodinium polykrikoides* Margalef has bloomed and spread every summer, causing great economic damage to local fisheries industry around the Korean coast in the last decade (Kim, 1997; Kim et al., 2001b). Furthermore, HABs involving *Cochlodinium* Schütt species have recently been reported worldwide (Yuki and Yoshimatsu, 1989; Whyte et al., 2001). In Korea, this unarmored dinoflagellate has been the subject of a series of comprehensive studies on its ecology (Suh et al., 2000; Kim et al., 2002; Lim et al., 2002), molecular characterization (Park and Park, 1999; Cho et al., 2000, 2001; Kim et al., 2004), and physiology (Kim et al., 1999, 2000; Kim et al., 2001a; Lee et al., 2001).

The rapid and accurate identification and enumeration of the target species of interest areprerequisites for HAB monitoring programs. However, the conventional HAB monitoring of *C. polykrikoides* relies on optical microscopic observation, which is time-consuming and laborious, and is seriously hampered by distortion of the cell morphology that occurs after fixation, the lack of morphologically informative characters for unequivocal species identification, an incomplete knowledge of the life-cycle, and the vulnerability of the cell wall. To overcome such problems, molecular probes for HAB species have recently been developed, including lectins, antibodies, and oligonucleotide probes, which allow HAB monitoring without sophisticated taxonomic expertise and skill (Scholin et al., 2003).

The nuclear-encoded large subunit (LSU) ribosomal DNA (rDNA) molecules that contain both conserved and divergent domains have been used to resolve phylogenetic relationships of dinoflagellates in broad taxonomic levels (Scholin et al., 1994; Zardoya et al., 1995; Daugbjerg et al., 2000; Rehnstam-Holm et al., 2002; Edvardsen et al., 2003;

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John et al., 2003b). Especially, the LSU rDNA D1-D2 region is a hot spot that has accumulated great sequence variations during evolution (Wuyts et al., 2001), allowing the design of highly species-specific oligonucleotide primers or probes. In practice, this region has been used as the target molecule to detect harmful microalgae (Scholin et al., 1997; Miller and Scholin, 1998; Puel et al., 1998; Rehnstam-Holm et al., 2002; John et al., 2003a). Moreover, the rDNA operon is generally suitable for the genetic characterization of target organisms because of its unusually high copy number per genome and the huge database for comparative analyses.

In this study, we analyzed the sequence of the LSU rDNA D1-D3 region of *C. polykrikoides* and conducted phylogenetic analyses to understand its genetic relationship to other dinoflagellate taxa. We also designed a *C. polykrikoides*-specific PCR primer set using information for the LSU rDNA sequence and described PCR detection assays for its sensitive, accurate HAB monitoring.

Materials and Methods

Algal culture and genomic DNA extraction

Cochlodinium polykrikoides BSW0109 was established from Busan, Korea and cultured in GPM medium (Loeblich, 1975) at 20℃, ca. 50 μmoL photons/m^2/s and 14L:10D photocycle. Cells in the exponential growth phase were harvested, and genomic DNA was extracted after RNase treatment (Hong et al., 1995). Total DNA was eluted in 100 μL of ddH$_2$O, quantified in triplicate using Gene Quant Pro (Biochrom Co.), and used as a stock preparation.

PCR and sequencing

A general PCR was carried out in a 50 μL-volume in a thermal cycler (My Cycler™, Bio-Rad) after adding the following reagents: 1×*Ex Taq*™ Buffer, 200 μM dNTPs, 0.2 μM primers (D1R: 5'- ACCCG CTGAATTTAAGCATA-3'; D3B: 5'-TCGGAAGG AACCAGCTACTA-3') (Scholin et al., 1994; Nunn et al., 1996), 1 μL template DNA (50 ng/μL; ca. 1,340 cells), and 1.25 units *TaKaRa Ex Taq*™ (TaKaRa, Japan). PCR cycle was consisted of an initial denaturation at 94℃ for 5 min, 25 cycles of 94℃ for 30 sec, 55℃ for 1 min and 72℃ for 1 min, and a final elongation at 72℃ for 7 min. The PCR visualized under UV light after electrophoresing products were on a 1% agarose gel in TAE (40 mM Tris-acetate, 1 mM EDTA [pH 8.0]) and staining it in EtBr (0.5 μg/mL).

After gel purification of the appropriate PCR band using a QIAquick™ Gel Extraction Kit (Qiagen, Germany), we performed a cycle-sequencing reaction with the PCR primer set and internal sequencing primers using an ABI PRISM BigDye™ Terminator v3.0 Cycle Sequencing Kit (Applied Biosystems, Perkin Elmer) according to the manufacturer's instructions. The sequencing reaction was run on an ABI 3100 Sequencer (Applied Biosystems). The LSU rDNA sequence of *C. polykrikoides* determined in this study was submitted to GenBank under accession number AY725423.

Phylogenetic analyses

The LSU rDNA sequence data of diverse dinoflagellates were downloaded from GenBank (http://ncbi.nlm.nih.gov) for reconstructing phylogeny. Sequences of the LSU rDNA D1-D3 region were aligned using Clustal W (Thompson et al., 1994) with inference to the secondary structure information of *Toxoplasma gondii* (Wuyts et al., 2001). The hypervariable LSU rDNA D2 domain, of which the secondary structure was hard to predict, and PCR primer regions were removed prior to final phylogenetic analyses. *T. gondii* (GenBank accession number: X75453) was used as the outgroup.

The phylogenetic tree of Bayesian inference was constructed in MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001; Huelsenbeck et al., 2001). Markov chain Monte Carlo (MCMC) from a random starting tree was initiated using six rate classes of nucleotide changes (the GTR+I+G model). The analysis was run for 1,000,000 generations in four chains, sampling trees every 100 generations. The likelihood scores had reached stationarity by 10,000 generations, and so the first 100 sampled trees were discarded ("burnin" =100). A 50% majority-rule consensus tree was obtained from the last MCMC trees after convergence.

Hierarchical likelihood ratio tests (hLRTs) in Modeltest Version 3.06 (Posada and Crandall, 1998) were used to determine the best-fit evolutionary model for the maximum likelihood (ML) analysis. The ML tree was constructed with the TrN+I+G model in PAUP* 4.0b10 (Swofford, 2002) with the following likelihood settings, determined from Modeltest: base frequencies of A=0.2834, C=0.1707, G=0.2587, T=0.2872; base substitution rates of AC=1.0000, AG=2.6971, AT=1.0000, CG=0.0000, CT=7.2050, GT=1.0000; assumed proportion of invariable sites=0.2109; gamma shape parameter=0.8090. The ML

analysis was performed using the heuristic search option with random addition of sequences (10 replicates) and tree-bisection-reconnection (TBR) branch swapping. The robustness of tree topologies was evaluated by the bootstrap analysis with 1,000 replicates (Felsenstein, 1985).

Design of specific oligonucleotide primers

Forward and reverse *C. polykrikoides*-specific PCR primers nested within the LSU rDNA D1-D3 region were developed from an alignment of 95 LSU rDNA sequences for a broad range of dinoflagellate taxa retrieved from GenBank and our unpublished data following the general rules of the primer design of Sharrocks et al. (1994): CPOLY01 (5'-GTACACG-GCTTGCACTTGCA-3') and CPOLY02 (5'-TGGTCG-TAGACGTGTGTCAG-3'). The standardized probe names were L-S-C.poly-0243(*Prorocentrum micans*) -a-S-20 and L-S-C.poly-0684(*P. micans*)-a-A-20, respectively (Alm et al., 1996).

PCR detection assays

Before performing PCR detection assays, the effective annealing temperature (T_p) of CPOLY01 and CPOLY02 was determined by gradually increasing the annealing temperature starting from 55℃, to optimize the PCR condition. The optimal temperature determined was used for subsequent PCR assays.

The specificity of CPOLY01 and CPOLY02 was tested against the genomic DNAs of fifteen dino flagellates, which were maintained in the Laboratory of Aquaculture and the Environment, Pukyong National University, and heterogeneous environmental DNA. An environmental water sample (1 L) containing diverse microorganisms was collected from Jinhae Bay, Korea at July 2003 and concentrated by centrifugation at 1,500×*g*. The environmental DNA was extracted as mentioned above.

For the nested PCR assay, the primary PCR was carried out with an universal primer set, D1R and D3B (external primers) followed by the secondary PCR with CPOLY01 and CPOLY02 (internal primers) using 1 μL of the PCR product as template under the same condition as the first round except that annealing occurred at 67℃ instead of 55℃.

The detection limits were investigated by the serial dilution of a template DNA stock preparation equivalent to 10,000 to 0.001 cell(s) in the conventional PCR assay and 50 to 0.0001 cell(s) in the nested PCR assay.

Results and Discussion

In both Bayesian and ML trees, *Cochlodinium polykrikoides* showed genetic relationship to *Amphidinium* Claparède and Lachmann and *Gymnodinium* (Stein) G. Hansen and Moestrup species (Fig. 1). However, this phylogenetic relationship was only supported by the relatively high posterior probabilities of Bayesian inference, and poorly supported by the low bootstrap values of the ML analysis. *C. polykrikoides* is morphologically well characterized by 1.8-2.0 cingular rotation. Its close phylogenetic relationship to *Amphidinium* species, which has the short epitheca less than one-third of the body length, was peculiar and unexpected. Recently, several research groups conducted taxonomic redefinitions of *Gymnodinium* sensu lato and *Gyrodinium* sensu lato mainly based on the detailed cell morphology and the rDNA sequence data (Daugbjerg et al., 2000; Takano and Horiguchi, 2004; Hansen and Daugbjerg, 2004). They recognized the shape of the apical groove as the most distinct external morphological characteristics for the classification of unarmored dinoflagellate taxa. Morphologically *Gymnodinium* sensu G. Hansen and Moestrup is newly characterized by a photosynthetic unarmored dinoflagellate group with the horseshoe-shaped apical groove running in an anticlockwise direction (Daugbjerg et al., 2000). Several *Cochlodinium* species were known to possess apical grooves (Kofoid and Swezy, 1921; Takayama, 1985), but their fine structure has not yet been elucidated. The phylogenetic resolution of *C. polykrikoides* may require the detailed observation of the apical grooves and the molecular data of additional related taxa such as *C. miniatum* Kofoid and Swezy, *C. strangulatum* Shütt, and *C. vinctum* Kofoid and Swezy.

Two oligonucleotide PCR primers specific for *C. polykrikoides* (CPOLY01 and CPOLY02) were designed from sequence information for the LSU rDNA D1-D3 region. The PCR using the species-specific primers produced a single expected amplicon of 475 base pairs (Fig. 2).

The effective annealing temperature (T_p) of CPOLY01 and CPOLY02 was investigated by gradually increasing the annealing temperature starting from 55℃. The yield of the PCR product did not diminish significantly until the annealing temperature exceeded 67℃ (Fig. 2). T_p is higher than the melting temperatures (T_m) of the individual primers (both 62℃) calculated using the formula T_m=

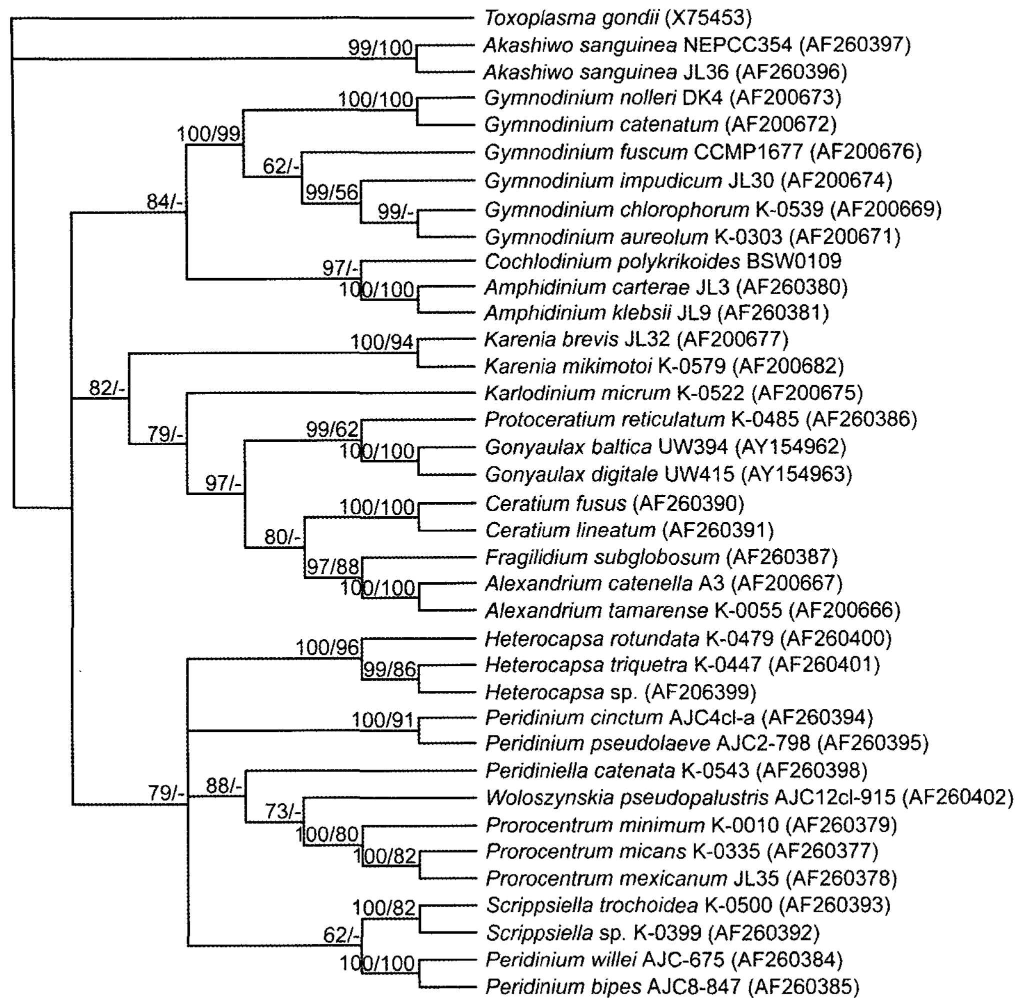

Fig. 1. The molecular phylogeny of the diverse dinoflagellate taxa based on their sequences of the LSU rDNA D1 and D3 domains. *Toxoplasma gondii* was used as the outgroup. The phylogenetic tree of Bayesian inference was constructed in MrBayes 3.0b4. Markov chain Monte Carlo (MCMC) from a random starting tree was initiated using six rate classes of nucleotide changes (the GTR+I+G model). A 50% majority-rule consensus tree was obtained from the last MCMC trees after convergence. Posterior probability of Bayesian inference and the bootstrap value of the maximum likelihood analysis above 50% were indicated at each node of branches. The LSU rDNA sequence of *Cochlodinium polykrikoides* was investigated in this study.

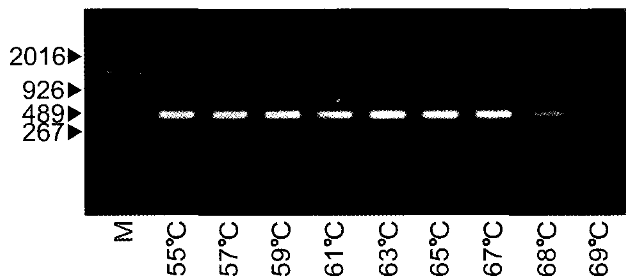

Fig. 2. The effective annealing temperature (T_p) of the *Cochlodinium polykrikoides*-specific PCR primer set, CPOLY01 and CPOLY02. M=size marker.

[2(A+T)+4(G+C)]℃ (Sharrocks, 1994). Annealing at higher temperatures is highly desirable for the PCR detection assays because it decreases non-specific PCR reactions and the total running time (Wu et al., 1991; Saiki, 1994).

CPOLY01 and CPOLY02 amplified only *C. polykrikoides* and did not cross-react with any dinoflagellate taxa across wide taxonomic levels (Table 1) and the heterogeneous DNA extracted from an environmental water sample containing diverse microorganisms, such as copepods, diatoms, dinoflagellates, raphidophytes, and tintinids (Fig. 3). In the

Table 1. List of dinoflagellate strains used in the specificity test of the *Cochlodinium polykrikoides*-specific primer set, CPOLY01 and CPOLY02

Species	Strain code	Sampling site	Sampling date
Akashiwo sanguinea	GSW0207	Gosung	July 2002
Alexandrium affine	YOC10	Saemangeum	May 2002
Alexandrium catenella	JHW9706	Jinhae Bay	June 1997
Alexandrium fraterculus	JHW0108-1	Jinhae Bay	Aug. 2001
Alexandrium tamarense	JHW0004-12	Jinhae Bay	Apr. 2000
Cochlodinium polykrikoides	BSW0109	Busan	Sep. 2001
Gymnodinium catenatum	JHW9910	Jinhae Bay	Oct. 1999
Gymnodinium sp.	KG-03	?	?
Gyrodinium instriatum	JHW0007-2	Jinhae Bay	July 2000
Heterocapsa triquetra	GSW0206-2	Gosung	June 2002
Lingulodinium polyedrum	DRW0108	Deukryang Bay	Aug. 2001
Pheopolykrikos hartmannii	JHC0203	Jinhae Bay	Mar. 2002
Prorocentrum micans	GSW0208	Gosung	Aug. 2002
Protoceratium reticulatum	JHW0007-6	Jinhae Bay	July 2000
Pyrophacus steinii	JHW0007-3	Jinhae Bay	July 2000
Scrippsiella trochoidea	GSW9808	Gunsan	Aug. 1998

?, data not known

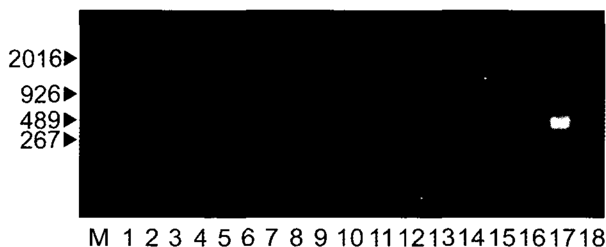

Fig. 3. The specificity of the *Cochlodinium polykrikoides*-specific primer set, CPOLY01 and CPOLY02. M= size marker, 1=*Akashiwo sanguinea*, 2=*Alexandrium affine*, 3=*Al. catenella*, 4=*Al. fraterculus*, 5=*Al. tamarense*, 6=*Gymnodinium catenatum*, 7=*Gymnodinium* sp., 8=*Gyrodinium instriatum*, 9=*Heterocapsa triquetra*, 10= *Lingulodinium polyedrum*, 11=*Pheopolykrikos hartmannii*, 12=*Prorocentrum micans*, 13=*Protoceratium reticulatum*, 14=*Pyrophacus steinii*, 15=*Scrippsiella trochoidea*, 16=the heterogeneous environmental DNA, 17=*Cochlodinium polykrikoides*, 18=a negative control.

phylogenies inferred from the LSU rDNA sequence data, *C. polykrikoides* formed an independent branch separated from morphologically closely related *G. catenatum* Graham and *G. impudicum* (Fraga and Bravo) G. Hansen and Moestrup (Lee et al., 2001) (Fig. 1), verifying its divergent genetic nature. The use of two species-specific primers appeared to improve the specificity significantly.

The serial dilution of the template DNA stock preparation revealed that the conventional PCR assay was capable of detecting as few as 0.05 cells/reaction after 25 cycles (Fig. 3). This is far lower than the *C. polykrikoides* concentration at the red tide attention stage (>300 cells/mL) (http://www.nfrdi.re.kr/korea/tech/environment07.htm). The nested PCR assay increased the detection limit to 0.0001 cells/reaction, which is at least 100-fold more sensitive than the former. Lee et al. (1998) and Kho et al. (2000) also reported this superior sensitivity of the nested PCR.

Overall, our results are 10-1,000 times as sensitive as the detection limits of *Pfiesteria piscicida* Steidinger and Burkholder targeting the nuclear SSU rDNA (0.6 cells/reaction) (Bowers et al., 2000) and *Karlodinium micrum* (Leadbeater and Dodge) J. Larsen targeting the plastid SSU rDNA (0.375 cells/reaction) (Tengs et al., 2001) using real-time PCR assays, of *P. piscicida* targeting the mitochondrial cytochrome *b* gene (*cob*) (0.2 cells/reaction) using conventional PCR assay (Zhang and Lin, 2002), and of *Karenia brevis* (Davis) G. Hansen and Moestrup targeting the plastid Rubisco gene (*rbc*L) using a real-time RT-PCR assay (1 cell/reaction) (Gray et al., 2003). All of these PCR-based assays are generally more sensitive than whole-cell in situ hybridization assays using fluorescein-labeled DNA probes (Miller and Scholin, 1998; Rehnstam-Holm et al., 2002; John et al., 2003a). However, the sensitivity of our PCR assays may decrease when applied to environmental water samples that contain heterogeneous DNA and *Taq* polymerase inhibitors (Bowers et al., 2000; Saito

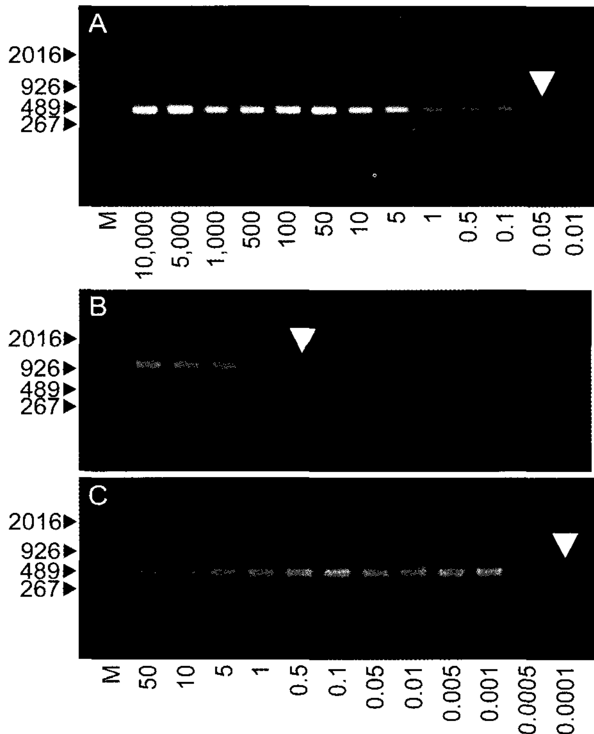

Fig. 4. The detection limit (cells/reaction) of the *Cochlodinium polykrikoides*-specific primer set, CPOLY01 and CPOLY02 (A) by the conventional PCR assay and (C) by the nested PCR assay after the amplification of the LSU rDNA D1-D3 region (B). Each arrowhead indicates the minimum detection limit. M=size marker.

et al., 2002; Litaker et al., 2003).

In this study, our PCR-based assays were proven both sensitive and accurate for detecting the harmful dinoflagellate *C. polykrikoides* over a wide range of cell numbers. The high sensitivity and specificity of the *C. polykrikoides*-specific primer set may allow detection of the target microorganism at its initial HAB stage without ambiguity. Furthermore, this technique can be applied to biogeographic mapping of the unidentified *C. polykrikoides* cyst in sediments (e.g., Godhe et al., 2002; Saito et al., 2002), which is generally recognized to play a critical role in HAB initiation (Anderson, 1998). This technique will also be widely applicable to other HAB species occurring along the Korean coast after their genetic information is uncovered and appropriate oligonucleotide primers are designed. Among the advantages of the PCR detection assay are high sample throughput, simplicity, superior sensitivity and specificity, and time- and cost-effectiveness. By contrast, the main drawback of this end-point PCR assay is the lack of quantification, which is frequently biased by various biological (e.g., genome copy number of a target molecule), chemical (e.g., activity of *Taq* DNA polymerase, the complementary nature of primers, $MgCl_2$ concentration, number of cycles, self-splicing of a primer, tertiary structure of template, use of fixative), and physical (e.g., DNA extraction efficiency, pipetting errors, sample preservation) factors.

The accurate quantification of a target microorganism of interest is essential for the risk assessment during HAB monitoring. Therefore, in the future study, the application of molecular techniques such as competitive PCR (Saito et al., 2002), real-time PCR (Bowers et al., 2000; Tengs et al., 2001; Gray et al., 2003), and sandwich-hybridization (Scholin et al., 1997; Tyrrell et al., 2002) will be required to detect and enumerate HAB species and to increase the direct applicability of the assay to environmental water samples.

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