Detection of Fish Killing Dinoflagellates *Cochlodinium polykrikoides* and *Karodinium veneficum* (Dinophyceae) in the East China Sea by Real-time PCR

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The rDNAs of fish-killing dinoflagellates *Cochlodinium polykrikoides* and *Karodinium veneficum* were detected from the East China Sea by species-specific real-time PCR probes. Sequence analyses using the partial ITS sequences from the real-time PCR products showed identical sequences with *C. polykrikoides* and *K. veneficum*, respectively and low expectation values (E-value) of less than 1e-5 suggesting the presence of these organisms in the East China Sea shelf water that flows into the Tsushima Strait and the Yellow Sea.

**Key Words:** *Cochlodinium polykrikoides*, East China Sea, *Karodinium veneficum*, real-time PCR, red tide

INTRODUCTION

Recurring blooms of the marine mixotrophic dinoflagellate *Cochlodinium polykrikoides* Margalef have caused massive fish kills in the South Sea of Korea with losses of US $5-20 million per year in 2000-2007 (NFRDI 2007). *C. polykrikoides* blooms and its related economic losses have been also reported in other countries such as Japan, China, Malaysia, and Philippines (e.g. Azanza et al. 2008). *Karodinium veneficum* (Ballantine) J. Larsen producing water soluble toxins that kill fish through gill disruption has been implicated in numerous fish kill events around the world since 1950 (Place et al. 2008). Low to medium abundances (approximately 1,000 cells mL^-1^) of *K. veneficum* have been also reported from Korean waters and its wide distribution in Korean sediments has been revealed by real-time PCR methods although this species-related fish kills have not been recorded in Korea (Park et al. 2009a, b). The East China Sea is an epicontinental sea bounded by the Ryukyu archipelago, Japan, Korea, China, and Taiwan. It receives a rich supply of nutrients from Changjiang and other rivers as well as from the Kuroshio (Gong et al. 1996), and it has high concentrations of Chl a, high primary productivity, high concentrations of particulate organic matter, and abundant ichthyoplankton (e.g. Liu et al. 1995). The East China Sea is considered to consist of three main currents including the Kuroshio, the Tsushima Warm Current, and the Yellow Sea Warm Current (Ichikawa and Beardsley, 2002). The Tsushima Warm Current flows northwardly from the East China Sea into the East Sea of Korea through the Tsushima Strait located between Korea (South Sea) and Japan (Kyushu), and it has sources of the Taiwan Warm Current and the Kuroshio Branch Currents, which transport a mixture of the Kuroshio Water and the coastal water diluted by river discharge mainly from the Chinese continent (Ichikawa and Beardsley 2002). A number of studies reported potential dispersal of marine organisms through the Kuroshio Current (Heath et al. 1998; Cho and Matsuoka 2001). For example, the Kuroshio Current is likely to have a large effect on the dispersal of sardine eggs and larvae (Heath et al. 1998). Wide distribution of cysts of toxic dinoflagellate *Alexandrium* species has been reported from the East China Sea indicating potential influence of the "seed bed" on *Alexandrium* blooms in coastal areas by dispersal of *Alexandrium* species through currents (Cho and Matsuoka 2001). There have been several hypotheses on sources of *C. polykrikoides* blooms in the South Sea of Korea. One possibility is that this species has been transferred from the open sea (the East China Sea) to southern coasts of Korea through the Kuroshio and the Tsushima Warm Current. However, occurrences

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of *C. polykrikoides* in the East China Sea have not been reported yet.

Real-time PCR is a technique that offers highly sensitive, accurate, and quantitative analysis (Coyne et al. 2006; Park et al. 2007a). TaqMan based real-time PCR probes targeting rDNA have been successfully used for specific detection of dinoflagellates in water samples (Coyne et al. 2006; Park et al. 2007a). In this study, surface water samples from the East China Sea were analyzed by real-time PCR probes specific to *C. polykrikoides* and *K. veneficum*. Positive real-time PCR amplicons were sequenced and their sequence similarities were examined.

**MATERIALS AND METHODS**

**Cultures and field samples**

One *C. polykrikoides* strain (NFFCPO1) from Korea was used as a positive control for *C. polykrikoides*-specific real-time PCR assay. *K. veneficum* strain (CCMP1974 from USA) was obtained from CCMP (Provasoli-Guillard National Center for Culture of Marine Phytoplankton) and was used for a positive control of real-time PCR. *Cryptoperidiniopsis brodyi* Steidinger et Litaker (CBWA12 from Australia) obtained from University of Tasmania was used for an internal control to confirm the absence of PCR inhibitors in environmental samples. Cultures were maintained in 1/2 medium of 28% of salinity (Guillard and Ryther 1962) without sodium silicate at 24°C, with cool white fluorescent lamps on a 12 : 12-h light : dark cycle. Surface water samples were collected from 12 stations in the East China Sea from 15th to 17th May 2008 (Table 1; Fig. 1). Sixty liter water samples were collected from the surface with 10 liter buckets, poured through phytoplankton net (10 μm mesh size), and concentrated in a 50 mL cod end container. The water samples were filtered onto a 1.2 μm pore-size, 25 mm diameter glass microfibre GF/C filter (Whatman, Ltd., Maidstone, England). The filtered sample was placed in a 2 mL microcentrifuge tube, and stored at −70°C until DNA extraction. Subsamples of the concentrated waters were also observed by light microscopy.

**Real-time PCR assay**

A phenol-chloroform extraction protocol was used for genomic DNA extraction from field samples (Park et al. 2009a). Briefly, filtered samples were suspended in TE buffer and were boiled at 100°C for 5 min, followed by adding phenol : chloroform : isoamyl alcohol (25 : 24 : 1). The samples were precipitated with ethanol and dissolved in TE buffer. The presence of PCR inhibitors and/or excessive DNA amounts in the field samples was checked using a real-time PCR probe (Table 2) specific for *C. brodyi* that has not been reported in Chinese, Japanese, and Korean waters. Serial DNA dilutions of field water samples (non-dilution, 20-fold, and 200-fold dilutions) spiked with *C. brodyi* DNA (CBWA12; 1.5 ng μL⁻¹) were amplified using real-time PCR, and their Ct values were calculated and compared. The absence of inhibitors was confirmed in 20-fold and 200-fold DNA dilutions. Accordingly, all field samples were 20-fold diluted for real-time PCR assay. For cell quantification, numbers of *C. polykrikoides* and *K. veneficum* were estimated by light microscopy using a hemocytometer (Blau
Table 2. Primers and TaqMan probes for conventional PCR and real-time PCR assays

<table>
<thead>
<tr>
<th>Dinoflagellate</th>
<th>Forward/Reverse/Probe</th>
<th>Code</th>
<th>Sequence (5' → 3')</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cochloldinium polykrikoides</em></td>
<td>Forward</td>
<td>CPTISF</td>
<td>CCGCAACCTTTGTCAAACA</td>
<td>Park et al. 2009c</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CPTISR2</td>
<td>GGTITGCTGATCATACATCTGTCT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>CPTISP</td>
<td>FAM-CAACCGTGATACCCGCTAGCTTTGC-TAMRA</td>
<td></td>
</tr>
<tr>
<td><em>Karoldinium veneficum</em></td>
<td>Forward</td>
<td>KVTISF3</td>
<td>CTGTGAACATTCTTTGAGCTCTT</td>
<td>Park et al. 2009a</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>KVTISR3</td>
<td>TACCAGTAGCTTGCCAGACA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>KVTISP3</td>
<td>FAM-AGGTTGAATCCCAATGCTGGTCCCTA-TAMRA</td>
<td></td>
</tr>
<tr>
<td><em>Cryptoperidiniopsis brodii</em></td>
<td>Forward</td>
<td>CBTISF</td>
<td>TTGACACGTGAAAGTGAGA</td>
<td>Park et al. 2007a</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CBTISR</td>
<td>ACAGCCAATGAAAGTGATGAGCAA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Probe</td>
<td>CBTISP</td>
<td>FAM-CATCTCAGGTGCTGCCGCTGAT-TAMRA</td>
<td></td>
</tr>
</tbody>
</table>

Brand, Germany) before harvesting the cells (total 31,000 cells for *C. polykrikoides* and 59,000 cells for *K. veneficum*). After DNA extraction, 10-fold serial dilutions of the DNA extracts were used to construct the standard curve (triplicate measurements by real-time PCR). The cell number of the target species in field samples was calculated as C<sub>T</sub> values, and was measured by comparison with the standard curve. Species-specific real-time PCR assays using TaqMan probes (Table 2) were conducted using a Rotor-Gene 6000 instrument (Corbett Research, Sydney, Australia) with 1 μL of template DNA, primers and a probe at final concentration of 0.2 and 0.15 μM, 5 μL of platinum quantitative PCR supermix-UDG (Invitrogen, Eugene, Oregon, USA) and PCR grade water to a final volume of 10 μL. The thermal cycling conditions consisted of 2 min at 50°C and 2 min at 95°C following by 45 cycles of 10 s at 95°C and 45 s at 60°C.

Sequence analyses

The DNA of positive field samples was amplified in conventional PCR format 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, 72°C for 2 min. The PCR products were visualized on 2% agarose
Table 3. Sequence similarity and the expectation value (E-value) of field-derived sequences from the East China Sea measured by the nucleotide BLAST program. GenBank accession numbers of query sequences are FJ524374 and FJ524375

<table>
<thead>
<tr>
<th>Organism</th>
<th>GenBank accession number</th>
<th>Sequence similarity</th>
<th>E-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. polykrikoides</em></td>
<td>DQ779986</td>
<td>100 %</td>
<td>3e-70</td>
</tr>
<tr>
<td><em>C. polykrikoides</em></td>
<td>AY347309</td>
<td>100 %</td>
<td>3e-70</td>
</tr>
<tr>
<td><em>K. veneficum</em></td>
<td>AJ557025</td>
<td>100 %</td>
<td>8e-60</td>
</tr>
<tr>
<td><em>K. veneficum</em></td>
<td>EU038293</td>
<td>100 %</td>
<td>8e-60</td>
</tr>
</tbody>
</table>

gel stained with ethidium bromide. The products were excised from the agarose gel with a scalpel, extracted, and purified using QIAEX II Gel Extraction kit (QIAGEN, Valencia, CA, USA). The purified products were cloned (Promega, Madison, WI, USA). 5 clones of each the purified product were selected and sequenced by Macrogen (Seoul, Korea). The partial ITS sequences (147-bp for *C. polykrikoides* and 120-bp for *K. veneficum*) obtained from field samples using *C. polykrikoides*- and *K. veneficum*-specific primers (CPITSF, CPITSR2 and KVITSF3, KVITSR3; Table 2) were compared with database sequences in GenBank using a nucleotide BLAST program.

RESULTS

One of 12 surface water samples from the East China Sea showed a positive reaction to the *C. polykrikoides*-specific real-time PCR assay (Table 1). For *K. veneficum*, 11 field samples gave positive responses to the real-time PCR assay. The measured C_R values correlated well with the values calculated from the log of the starting cell number (R^2 values of 0.995 and 0.997 for *C. polykrikoides* and *K. veneficum*, respectively). Below 4 cells per liter were estimated from the field samples based on the calibrated real-time PCR assay response (Table 1). From the microscopic analysis, very low cell abundances of *Cochlodinium* and/or *Kariondinium* look-alike species were evident at below quantification level only in samples showing positives by real-time PCR (Table 1). The presence of *C. polykrikoides* and *K. veneficum* in the East China Sea samples was further confirmed by sequence analysis of field-derived PCR amplicons. The sizes of PCR products were 147-bp and 120-bp for *C. polykrikoides* and *K. veneficum*, respectively (GenBank accession nos: FJ524374, FJ524375). The partial ITS rDNA sequences were identical to documented sequences (GenBank accession nos: AY347309, DQ779984-6 from Korea for *C. polykrikoides* and AJ557026 from North Atlantic, Europe for *K. veneficum*). Low expectation values (E-value) of less than 1e-5 were estimated by a nucleotide BLAST program suggesting that the field-derived sequences are likely to be related with *C. polykrikoides* and *K. veneficum*, respectively (Table 3).

DISCUSSION

Methodological considerations

Real-time PCR probes specific for *C. polykrikoides* and *K. veneficum* developed during previous studies were extensively tested against related organisms (Park et al. 2009a, c). These assays have been successfully used for detection of *C. polykrikoides* and *K. veneficum* in environmental samples, and the TaqMan format real-time PCR assay has been used for a number of dinoflagellates (Coyne et al. 2006; Park et al. 2007a, 2009a, c). Dilution, chemical treatments, or additional purification of template DNA have been known as valid methods for removing PCR inhibitors or adjusting template DNA concentration (Guy et al. 2003; Lin et al. 2006). In the present study, 20-fold dilution of template DNA was an effective method for PCR assays using DNA extracts from 60 L of the East China Sea water samples. When applied to field samples, there is a possibility of false conclusions caused by a cross reaction with unknown species/genotypes potentially present in environmental samples (Park et al. 2007b). Therefore, assay specificity was further confirmed by sequence analysis of real-time PCR amplicons from the East China Sea samples. The low E-value calculated by a nucleotide BLAST program suggests the presence of their vegetative cells in the East China Sea although short sequences (147-bp and 120-bp) were analyzed and were used for BLAST searches. While PCR-based assays offer highly sensitive analysis, positive PCR results must be viewed with caution due to potential contamination in samples during PCR assays. Independent check on PCR results may be desirable to prevent a false conclusion. Admittedly, it would have been preferable to have further assay specificity test using another primer set producing a larger PCR ampli-
con (> 500-bp) if specificity of the primer set can be confirmed against related organisms.

Detection of C. polykrikoides and K. veneficum from the East China Sea

C. polykrikoides is a mixotrophic dinoflagellate that is able to ingest a broad range of microbial taxa including cryptophytes and cyanobacterium Synechococcus sp. indicating a considerable grazing impact of C. polykrikoides on populations of prey species (Jeong et al. 2004, 2005). Many dinoflagellates are mixotrophic species and mixotrophy is considered to be advantageous in allowing phytoplankton to sustain population numbers either heterotrophically or photosynthetically (e.g. Jeong et al. 2004). Natural dispersals of mixotrophic dinoflagellates through advection have been suggested by previous studies (e.g. Anderson et al. 1994). For example, a scenario of continuing dispersal of Alexandrium species to the south from the north of the northern eastern U.S. is consistent with relative homogeneity of characteristics such as bioluminescence, morphology, and toxin composition (Anderson et al. 1994). A wide range of phytoplankton species (e.g. 66 species during summer in 2003) including dinoflagellates and diatoms has been reported from the surface layer of the East China Sea (e.g. Yoon et al. 2003) and some of them (Chaetoceros, Pseudonitzschia, Scripsiella, Proorocentrum species etc.) are common members of phytoplankton in coastal waters indicating that various coastal phytoplankton species can survive in the East China Sea. The East China Sea and the South Sea of Korea are linked hydrographically and there is a general south-to-north flow in these regions indicating that the East China Sea populations may be a source of C. polykrikoides blooms in the South Sea of Korea if its viable cells are successfully transferred and colonized in the South Sea. Although the number of samples was limited, our findings nevertheless showed the presence of C. polykrikoides and K. veneficum in the East China Sea. However, present results are insufficient to support the possibility of dispersal of these dinoflagellates by water currents. Ongoing efforts are needed to investigate population genetic structure and gene flow in these dinoflagellates for understanding their bloom dynamics.

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