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Development and field application of rRNA-targeted probes for the detection of *Cochlodinium polykrikoides* Margalef in Korean coastal waters using whole cell and sandwich hybridization formats☆

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Abstract

The dinoflagellate, *Cochlodinium polykrikoides* Margalef, has been responsible for mass mortalities of both wild and farmed fish along the Korean coast on virtually an annual basis since 1982. Economic impacts to the fishing and aquaculture industries are extensive, with a loss of USD $95 million reported in 1995 alone. The use of taxon-specific molecular probes for harmful algal species is recognized as a promising approach for the early detection of bloom formation and as part of an effective mitigation strategy. We have developed and successfully applied large subunit ribosomal RNA (LSU rRNA)-targeted probes in both whole cell and sandwich hybridization assay (SHA) formats for the species-specific detection of *C. polykrikoides* in Korean coastal waters. Sequences of the D1–D3 variable regions used to design probes were identical between five Korean and one Hong Kong *C. polykrikoides* isolates, while sequences for several N. American *Cochlodinium* isolates differed to varying degrees from the former. The automated SHA detected *C. polykrikoides* at levels as low as ~1–3 cells/L in the field, demonstrating its suitability for detecting the target species at pre-bloom concentrations. This method should thus prove valuable to existing monitoring programs aimed at providing aquaculture interests with an early warning of frequently devastating bloom events.
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Keywords: *Cochlodinium polykrikoides*; Harmful algal blooms; Korea; LSU rRNA; Molecular probes; Sandwich hybridization

1. Introduction

The sale of fishery products on both domestic and foreign markets represents an important source of income to the Korean economy. However, recurring blooms of the dinoflagellate, *Cochlodinium polykrikoides* Margalef, first recorded off of the Korean coast in 1982, have resulted in widespread mass mortalities of wild and farmed fish, and were designated by the government as natural disasters in 1990 (Kim, 1998). Losses attributed to these now virtually annual events reached a peak in 1995 of an estimated USD $95 million (Kim, 1998). Blooms of *Cochlodinium* species and their associated impacts to finfish aquaculture have since been reported from a number of other countries (see Kudela et al., 2008). For example, in 1999, a bloom of *Cochlodinium* sp. along the west coast of N. America in British Columbia, Canada, caused losses to the farmed salmon industry of approximately CDN $2 million (Whyte et al., 2001).

Due to the economic impacts of *C. polykrikoides* blooms, the Korean government currently supports a vigorous effort to control HABs and mitigate their devastating effects. In addition to direct control measures involving the application of clays to remove cells from the water column (Kim, 2006), a promising approach to mitigation has been the monitoring of phytoplankton assemblages for the presence and increasing

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abundance of harmful species. Early detection of bloom formation along with attempts to circumvent losses by moving net pens or early harvesting are critical aims of the aquaculture industry in Korea and throughout the world's coastal waters (Kim, 1998).

Detection of harmful algal species has traditionally been performed by light microscopic observation. More recently, the application of molecular probe-based approaches to organism detection has resulted in improved speed and efficiency of monitoring various HAB taxa (Tyrrell et al., 2001; Anderson et al., 2005). The use of LSU rRNA-targeted molecular probes in both a whole cell (WC) format using fluorescence in-situ hybridization (FISH) and a sandwich hybridization assay (SHA) using a semi-automated format have been demonstrated as effective means to detect the presence of harmful algae (Scholin et al., 1996, 1999; Tyrrell et al., 2002; John et al., 2005; Kim et al., 2005; Mikulski et al., 2005). The WC method involves fluorescently labeling intact cells with species-specific oligonucleotide probes and visualizing target cells using epifluorescence microscopy. This method increases the speed of cell enumeration and permits visual discrimination between morphologically similar species (Rhodes et al., 2004; Mikulski et al., 2005; John et al., 2005). The SHA method detects the presence of target rRNA in a sample lysate by employing a ''sandwich'' of probes, one that is species-specific and captures the target molecules on a solid support and a second (often less specific; e.g., genus level) 'signal' probe that binds to another site on the same rRNA molecule and facilitates quantification based on colorimetric detection (Scholin et al., 1996; Anderson et al., 2005). This latter method circumvents the need for time-consuming microscopic evaluation of material and enables detection of target species at very low 'pre-bloom' cell densities by concentrating large volumes of water prior to preparing the sample lysate. Oligonucleotide probes applied using WC and/or SHA have been employed successfully for HAB monitoring and extensive field data sets are available (Rhodes et al., 1998; Scholin et al., 1999; Matweyou et al., 2004; Anderson et al., 2005; Ayers et al., 2005; O'Halloran et al., 2006). The SHA has also been modified by inclusion of a nuclease protection assay for detecting species of the toxic dinoflagellate genus *Prorocentrum*, when the traditional SHA approach was unsuccessful (Cai et al., 2006).

The primary aim of the current study was to develop species-specific, LSU rRNA-targeted molecular probes for the detection of *C. polykrikoides* in Korean coastal waters. Our immediate objectives were to incorporate these probes into a rapid, reliable SHA assay capable of identifying this species while still a minor component of the phytoplankton assemblage and to develop a corresponding WC method for visual confirmation. Ultimately, these assays will be implemented by both surveillance and research programs to provide valuable near-real time data on the abundance and distribution of *C. polykrikoides*. This information will support efforts to monitor and model bloom populations, thereby providing the advanced warning needed for an effective management/mitigation strategy.

2. Materials and methods

2.1. Culture growth and maintenance

Isolates of *C. polykrikoides* originating from different locations along the Korean coast and obtained in different years were provided by the Korean National Fisheries Research and Development Institute (Busan, Republic of Korea) (Table 1). Three *C. polykrikoides* isolates from North America and one from Hong Kong were obtained from the Colección de Dinoflagelados Marinos (CODIMAR, La Paz, BCS, Mexico; isolate CPPV-1), the Woods Hole Oceanographic Institution (Woods Hole, MA, USA; isolates CPCB10 and HK), and the Dept. of Fisheries and Oceans Canada (Naniamo, BC, Canada; isolate CS01) for phylogenetic comparisons, probe design, and cross-reactivity testing. All cultures were maintained in 25 mL batch cultures in 50 mL borosilicate glass tubes on a 16 h:8 h light:dark cycle with a photon flux density of $\sim$75 μmol m^{-2} s^{-1} (model QSL; Biospherical Instruments, San Diego, CA, USA). The five Korean isolates were maintained at 20 °C in seawater (30 psu) amended with *f*/2 nutrients (−Si) and trace metals with 0.01 μM selenium added (Guillard, 1975). Growth medium for the remaining isolates was amended with L1 nutrients (Guillard and Hargraves, 1993) and these cultures were maintained at appropriate temperatures – CS01/15 °C, CPPV-1/25 °C, HK and CPCB10/20 °C. Cultures of additional taxa used for cross-reactivity testing were maintained as described for the Korean *C. polykrikoides* isolates; silicate (104 μM) was added to diatom cultures.

2.2. DNA extraction, PCR amplification, and sequencing

Algal DNA was extracted according to a modified CTAB/PCI/CI protocol (Scholin et al., 1994b) described previously by Mikulski et al. (2005). Hypervariable regions D1–D3 of the LSU rDNA were amplified by polymerase chain reaction (PCR) using primers D1R and D3Ca (Lenaers et al., 1989). Details of the PCR protocol are given in Mikulski et al. (2005). Amplification products were purified (Wizard® PCR prep kit, Promega Corp., Madison, WI, USA) and sequenced in both directions using four internal primers (D2Ra, D2C, D1C, Scholin et al., 1994b; D3Rf2, Mikulski et al., 2005), in addition to the above PCR primers. Sequencing was performed on an ABI Prism 3730xl sequencer (Applied Biosystems, Foster City,

Table 1
Details of *Cochlodinium* isolates employed and sequenced in this study

Strain	Date isolated	Location
C. poly	Sept. 2000	Namhae, Republic of Korea
PP-3	Sept. 2001	Tongyeong, Republic of Korea
PP-6	Sept. 2001	Busan, Republic of Korea
CP 2002	Aug. 2002	Busan, Republic of Korea
CP 2002-1	Aug. 2002	Namhae, Republic of Korea
CS01	Sept. 1999	Coal Harbour, BC, Canada
HK	Unknown	Hong Kong
CPCB10	Sept. 2001	Cotuit Bay, MA, USA
CPPV-1	Unknown	Bahía de La Paz, B.C.S., México