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Resolving the intra-specific succession within *Cochlodinium polykrikoides* populations in southern Korean coastal waters via use of quantitative PCR assays

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ABSTRACT

While the toxic dinoflagellate *Cochlodinium polykrikoides* is known to form blooms that are maintained for extended periods, the genetic differentiation of these blooms are currently unknown. To assess this, we developed a real-time PCR assay to quantify *C. polykrikoides* at the intra-specific level, and applied this assay to field samples collected in Korean coastal waters from summer through fall. Assays were successfully developed to target the large-subunit ribosomal RNA region of the three major ribotypes of *C. polykrikoides*: Philippines, East Asian, and American/Malaysian. Significant linear relationships ($r^2 \geq 0.995$) were established between C_t and the log of the copy number for each ribotype qPCR assay. Using these assays, *C. polykrikoides* blooms in Korean coastal waters were found to be comprised of Philippines and East Asian ribotypes but not the American/Malaysian ribotype. The Philippines ribotype was found to be highly abundant during summer bloom initiation and peak, whereas the East Asian ribotype became the dominant ribotype in the fall. As such, this newly developed qPCR assay can be used to quantify the cryptic ecological succession of sub-populations of *C. polykrikoides* during blooms that light microscopy and previously developed qPCR assays cannot resolve.

1. Introduction

The ichthyotoxic unarmored dinoflagellate *Cochlodinium polykrikoides* a taxa of harmful algal blooms (HAB) responsible for substantial mortality to both wild and farmed fish (Kudela and Gobler, 2012). The presence of *C. polykrikoides* has been reported in tropical, subtropical and temperate waters, such as British Columbia, Canada (Whyte et al., 2001), the US east coast (Gobler et al., 2008), Mexico in the eastern Pacific, the coastal waters of Costa Rica (Vargas-Montero et al., 2004, 2006), Japan (Yuki and Yoshimatsu, 1989), China (Qi et al., 1993), and Korea (Kim, 1998a; Cho and Costas, 2004). In recent decades, harmful algal blooms caused by *C. polykrikoides* Margalef have exhibited an apparent increase in harmful impacts worldwide (Kudela and Gobler, 2012). At the same time, the economic damage to fisheries and aquaculture due to massive mortality has also increased sharply, particularly in Korea (Kim et al., 2001, 2007). In 1995, a particularly severe and widespread *C. polykrikoides* bloom persisted for nearly eight weeks along the entire south coast of Korea, ultimately resulting in economic losses of up to 95 million US dollars (Kim, 1998b). Since then, harmful algal blooms of this species have been an annual feature along southern Korean coastal waters. Therefore, intensive investigation, including studies of growth characteristics and vertical migration, have been conducted to obtain a better understanding of the factors influencing the formation of *C. polykrikoides* blooms (Park et al., 2001; Kim et al., 2004; Jeong et al., 2004). In addition, various studies have been undertaken to clarify the mechanisms of *C. polykrikoides* bloom formation, such as overwintering strategies, hyaline cysts (temporary cyst), and resting cysts (Matsuoka and Fukuyo, 2000; Kim et al., 2002; Tang and Gobler, 2012), and the current-driven movement of *C. polykrikoides* blooms in the Andaman Sea, the East China Sea, and the East Sea/the Sea of Japan have been evaluated using ocean-color satellite imagery (Azanza and Baula, 2005; Miyahara et al.,

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2005; Ahn et al., 2006; Kim et al., 2007). However, the bloom formation mechanism of this species is not yet fully understood. In particular, it is unclear how *C. polykrikoides* blooms are maintained for such long periods (≥8 weeks; Kim et al., 2001; Gobler et al., 2008). Many researchers have suggested that genetic differentiation within *C. polykrikoides* may be one explanation for long-term bloom maintenance (Kudela and Gobler, 2012), as genetic differentiation may lead to variations in physiological and ecological characteristics. Similarly to *C. polykrikoides* bloom, the toxic dinoflagellate *Alexandrium fundyense* bloom occurred during 8 weeks (May to July, 2005) and affected over 700 km of coastline on the U.S. Northeast Coast (Anderson et al., 2005). According to Erdner et al. (2011), *A. fundyense* bloom in the northeastern U.S. harbor composed by at least two genetically distinct sub-populations. Moreover, two sub-populations of *A. fundyense* are responsible for early- and late bloom and originated from the northern and southern areas of the bloom, respectively. Therefore, clarification of genetic differentiation of sub-populations could allow for improved understanding of the mechanisms of *C. polykrikoides* blooms.

Recently, Iwataki et al. (2008) reported that *Cochlodinium polykrikoides* had significant genetic differentiation in the large ribosomal subunit, and could therefore be separated into three distinct sub-clades in the phylogenetic tree generated in that study. These clades or "ribotypes" can be phylogenetically diverse groups of *C. polykrikoides* differentiated based on their large-subunit (LSU) ribosomal RNA gene sequences. The three ribotypes are the East Asian (Hong Kong, Japanese, and Korean), Philippines, and American/Malaysian ribotypes. Monitoring the dynamics of these ribotypes in field during blooms would significantly advance our understanding of *C. polykrikoides* blooms. However, light microscopy is incapable of discriminating between these ribotypes due to nearly identical morphologies (Iwataki et al., 2008). Even current quantitative real-time PCR assays (Park and Park, 2010) cannot distinguish among the three ribotypes as the molecular markers used are optimized for species-, not clonal-level, distinction. Hence, the aim of this study was to develop a sensitive and accurate assay for the quantitative analysis of all three *C. polykrikoides* ribotypes and examine the suitability of these qPCR assays for tracking the dynamics of these ribotypes during blooms in Korean waters.

2. Materials and methods

2.1. Algal cultures

Algal strains were obtained from the CCMP (Provasoli-Guillard National Center for Marine Algae and Microbiota, ME, USA), the National Research Laboratory for Water Environmental Ecology and Restoration of Hanyang University (Seoul, South Korea), the KIOST (Korea Institute of Ocean Science and Technology, Ansan, South Korea), the NIES (National Institute for Environmental Studies, Tsukuba, Japan), and the Gobler laboratory (School of Marine and Atmospheric Sciences, Stony Brook University, NY, USA) (Table 1). All strains were cultured at 20 °C in f/2 growth medium (Guillard, 1975) or GSe medium (Doblin et al., 1999) with a salinity of 31–33 under cool-white fluorescent lamps (photon flux of 100 μE m^{-2} s^{-1}) on a 12-h light:12-h dark photoperiod. *Cryptoperidiniopsis brodyi* (CCMP 2781, 2782), *Luciella masanensis* (CCMP 1835, 1873), *Pfiesteria piscicida* (CCMP1830, 1831), and *Pseudopfiesteria shumwayae* (CCMP 2089, 2807) were maintained in f/2 medium with a salinity of 15 at 20 °C and fed *Rhodomonas* sp. (CCMP 768). Table 1 shows the list of the strains used in this study.

2.2. Collection and processing of environmental samples

Field sampling was performed from August to November 2009 on Geum-o Island (St. Y) near Yeosu in Jeollanam-do and Mi-jo Harbor (St. M) in Gyeongsangnam-do, which are both located in the southern part of Korea (Fig. 1). The water depths were as follows: (i) St. Y, 29–31 m, and (ii) St. M, 3–4 m. Water samples were collected at six water depths at St. Y (0, 3, 5, 10, 20, and 30 m) and two water depths at St. M (0 and 3 m), in this sequence, using a 4.2 l Van Dorn water sampler (Wildlife supply company, MI, USA). One liter of each water sample was fixed with 1% Lugol's solution. After gentle mixing, the preserved field samples were counted

Table 1
Specificity of the primer sets used in qPCR with EvaGreen.

Species	Strain	CPSF2 CPSR3	PhiCPSF PhiCPSR	AMCPSF AMCPSR	Species	Strain	CPSF2 CPSR3	PhiCPSF PhiCPSR	AMCPSF AMCPSR
Alexandrium sp.	HY981028M	n.d	n.d	n.d	*Fibrocapsa japonica*	D-133 /CCMP 1661	n.d	n.d	n.d
Akashiwo sanguinea	0806-HYPH-AS10	n.d	n.d	n.d	*Gymnodinium catenatum*	GnCt-K01	n.d	n.d	n.d
Amphidinium sp.	CCMP 1684	n.d	n.d	n.d	*Gymnodinium impudicum*	NF-F-GIM-1	n.d	n.d	n.d
Chattonella antiqua	CCMP 2050 /YSIP0806	n.d	n.d	n.d	*Heterocapsa triquetra*	HtTq_K01	n.d	n.d	n.d
Chattonella marina	CCMP 2049 /CMGM	n.d	n.d	n.d	*Heterosigma akashiwo*	CCMP 452 /NIES 298	n.d	n.d	n.d
Chattonella ovata	JH0805	n.d	n.d	n.d	*Prorocentrum micans*	KMCC D-086	n.d	n.d	n.d
Chattonella subsalsa	CCMP 217	n.d	n.d	n.d	*Cryptoperidiniopsis* ribotype	CCMP 1828	n.d	n.d	n.d
Cochlodinium polykrikoides					*Pfiesteria piscicida*	CCMP 1830 /CCMP 1831	n.d	n.d	n.d
East Asian ribotype	EA-CP 01 /KORDI-CP /Regular-CP	Pos[†]	n.d	n.d	*Luciella masanensis*	CCMP 1835 /CCMP 1873	n.d	n.d	n.d
Philippines ribotype	HYID1108-CP	n.d	Pos	n.d	*Pfiesteria shumwayae*	CCMP 2089 /CCMP 2807	n.d	n.d	n.d
American/ Malaysian ribotype	CPMHC-4/ CPMC-40C/ CP 01/CPOFP-11	n.d	n.d	Pos	*Cryptoperidiniopsis brodyi*	CCMP 2781 /CCMP 2782	n.d	n.d	n.d

[*] n.d: Not detected.
[†] Pos: Positive.