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Simultaneous detection of harmful algae by multiple polymerase chain reaction coupled with reverse dot blot hybridization

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ABSTRACT

Harmful algal blooms (HABs) caused by microscopic algae present a threat to human health, ecosystem, fishery, tourism, and aquaculture worldwide. HAB warning and monitoring projects require a simple and rapid method for accurate parallel identification of causative algae. This study presents a useful method for simultaneous detection of harmful algae by multiple PCR coupled with reverse dot blot hybridization (MPCRDBH). A variety of probes, including positive, negative, and specific, were first developed by sequencing and consequent sequence analysis of large subunit rDNA D1–D2 from target species and used for specificity test by blot hybridization. The MPCRDBH assay mainly included five steps: (1) microalgal DNA isolation; (2) amplification and labeling of target DNA by multiple PCR; (3) probe tailing and fixation onto positively charged nylon membrane; (4) reverse dot blot hybridization; and (5) hybridization signal recognition by naked eyes. The reverse dot blot hybridization conditions were optimized, and the appropriate parameters were as follows: ultraviolet cross-linking time, 0.5 min; probe density, 2 μM; Dig-labeled PCR product density, 200 ng; hybridization time and temperature, 2 h and 42 °C; and washing time and temperature, 2 × 5 min and 47 °C. Sensitivity tests showed that MPCRDBH demonstrated a detection limit of 0.6 cell. MPCRDBH recovered all target species and was not affected by background DNA. MPCRDBH also demonstrated a stable detection performance for fixative (acidic Lugol's solution)-preserved samples over 30 d using simulated field samples. MPCRDBH applicability was assessed and proven effective for parallel detection of target microalgae in the field samples. The developed MPCRDBH exhibited a simple membrane-based DNA array preparation and hybridization signal recognition compared with other current DNA arrays. The assay presented in this study is specific and sensitive for parallel detection of microalgae, with stable performance. Therefore, this assay is promising for field monitoring of natural samples.

1. Introduction

Harmful algal blooms (HABs) are natural events that have become increasingly serious in recent years. HABs occur in many regions of the world. Some bloom-forming algae are toxic and can cause serious disease and death in humans, fish, seabirds, and other marine life through the food web (Hallegraeff, 1993). HABs are destructive to marine ecosystems and can cause great economic losses to fisheries, tourism, and aquaculture (Hoagland et al., 2002; Hallegraeff, 2003).

In generally, most HABs are caused by microalgae, which are often difficult to distinguish from their related species. Traditional methods of species identification by morphological characteristics rely on light, epifluorescence, and even electron microscopy, which are time consuming, laborious, and costly. In particular, routine monitoring in coastal areas with high incidence of potential HABs is challenging for traditional methods because it involves large-scale sample analysis. Therefore, developing a rapid and accurate alternative or supplementary approach for morphological examination of HABs is currently needed.

Numerous studies associated with detection protocols for harmful algae have been published over the past decade. The established methods can be generally categorized into four groups

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according to their target molecules, namely, toxin (Cembella et al., 1987), proteins (Gas et al., 2009), carbohydrates (Aguilera and Gonzaláz-gil, 2001), and nucleic acids, including RNA (Chen et al., 2013) and DNA (Yuan et al., 2012). Molecular methods targeting nucleic acid are the most widely used techniques because of the relatively stable RNA and DNA expression compared with the other target molecules. To date, several methods have been developed, including fluorescence *in situ* hybridization (Chen et al., 2011, 2013), quantitative PCR (He and Wu, 2007; Park and Park, 2010), sandwich hybridization (Mikulski et al., 2008; Zhen et al., 2009), loop-mediated isothermal amplification technology (Zhang et al., 2009), and nucleic acid sequence-based amplification technique (Casper et al., 2004; Ulrich et al., 2010).

The established alternative tools for microscopic examination could certainly satisfy the need for specific and rapid detection of harmful algae (Blair et al., 2009), indicating the presence of particular microalgae in water samples. However, most of these methods can only perform single species-detection in water samples at a time. Causative organisms of HABs are diverse, and more than 260 species among approximately 4000 established microalgae have been recorded to form blooms (Zhou et al., 2001). More than 140 HABs are distributed along the Chinese coast, and more than 40 of which have formed algal blooms (Zhou et al., 2001). In recent years, an increasing number of microalgal species has been found to form algal blooms (Wang et al., 2012). Therefore, overall recognition of potential HAB in the samples collected from areas with high incidence of algal blooms is required. However, using methods that detect single species results in delayed warning of potentially imminent HABs. Researchers should focus on developing a novel method for parallel detection of several microalgae.

The DNA microarray is a novel technology that can analyze large-scale genes with high speed, specificity, and sensitivity. To date, this technology is mainly applied to gene expression analysis (Sherlock, 2000; Takahashi et al., 2011) and pathogenic microorganism detection (Hwang et al., 2011). Coupled with species-specific probes (the so-called taxonomic probes), DNA microarrays or phylochips are also used to detect environmental microbes. These conditions have led to the introduction of the DNA microarray to rapidly analyze samples containing more than one target. The application of the DNA microarray for the identification of marine organisms is a relatively new and innovative research. Ki and Han (2006) first developed a low-density array capable of distinguishing 10 harmful microalgae, including *Alexandrium* spp., *Akashiwo sanguinea*, *Cochlodinium polykrikoides*, *Chattonella marina*, and *Gymnodinium* spp. Gescher et al. (2008) designed and tested a low-density oligonucleotide array. Their method can successfully detect and distinguish various species of the genus *Alexandrium* that exhibit similar morphologies that are difficult to distinguish with standard microscopy. Galluzzi et al. (2011) developed an oligonucleotide microarray for 20 marine dinoflagellates for field monitoring. A series of microarrays have recently been established for the detection of toxic algae with a remarkable characteristic of quantifying target cells (Dittami et al., 2013a,b; Edvardsen et al., 2013; Kegel et al., 2013; McCoy et al., 2013; Taylor et al., 2013). Various kinds of DNA arrays use fiber optic (Ahn et al., 2006), electrode (Diercks et al., 2008), and bead as probe carrier (Scorzetti et al., 2009), which are different from the common DNA microarray immobilizing taxonomic probes onto a glass slide.

The high cost of instruments required for current phylochips limits their application. For example, a DNA microarrayer is required for a common DNA array to spot probes on a glass slide during DNA array preparation. Hybridization signal detection is performed using special instruments, such as a fluorescence scanner (microarray scanner). These special and costly instruments used in the DNA array assay must greatly compromise their practical use in coastal area monitoring, during which numerous samples are usually examined.

Based on the detection principle of the DNA array, we introduce multiple PCR-coupled reverse dot blot hybridization (MPCRDBH) for parallel detection of harmful algae. This assay, which includes DNA array preparation and hybridization signal detection, can be performed without any special instrument. The detailed parameters for membrane-based DNA array and hybridization protocol were optimized. The detection limit, sensitivity, and stability of MPCRBH were also tested. The applicability of MPCRBH was further assessed using simulated and field samples.

2. Materials and methods

2.1. Algal cultures

All microalgae employed in this study are shown in Table 1. Clonal cultures were established by chains of cells, sequentially through droplets of sterile seawater. The cultures were maintained in f/2 medium (Guillard, 1975) at pH 8.2 from 20 °C to 22 °C and a light intensity of 100 $\mu E\ m^{-2}\ s^{-1}$ provided by cool-white fluorescent bulbs on a 12:12 photoperiod. For field studies, surface seawater samples were collected from Golden Bay Coast, Weihai in October 2012 and the East China Sea in July 2012.

2.2. Sequence amplification, cloning, and sequencing

Genomic DNA of target microalgae, including *Heterosigma akashiwo*, *Prorocentrum donghaiense*, *Prorocentrum micans*, *Nitzschia closterium*, *Skeletonema costatum*, and *Chaetoceros debilis*,

Table 1
Microalgal species used in this study.

Species	Taxonomy	Geographic origin
Heterosigma akashiwo	Raphidophyceae	South China Sea
Prorocentrum donghaiense	Dinophyceae	Zhejiang, East China Sea
Prorocentrum micans	Dinophyceae	Zhejiang, East China Sea
Nitzschia closterium	Dinophyceae	Weihai Bay, Yellow Sea
Skeletonema costatum	Bacillariophyceae	Weihai Bay, Yellow Sea
Chaetoceros debilis	Bacillariophyceae	Weihai Bay, Yellow Sea
Chattonella marina	Raphidophyceae	Daya Bay, South China Sea
Chaetoceros affinis	Bacillariophyceae	Weihai Bay, Yellow Sea
Chaetoceros curvisetus	Bacillariophyceae	Weihai Bay, Yellow Sea
Chaetoceros didymus	Bacillariophyceae	Weihai Bay, Yellow Sea
Prorocentrum minimum	Dinophyceae	East China Sea
Prorocentrum triestinum	Dinophyceae	East China Sea
Prorocentrum lima	Dinophyceae	Hongkong, East China Sea
Nitzschia longissima	Bacillariophyceae	Weihai Bay, Yellow Sea
Nitzschia angularis	Bacillariophyceae	Weihai Bay, Yellow Sea
Skeletonema pseudocostatum	Bacillariophyceae	Weihai Bay, Yellow Sea
Skeletonema marinoi	Bacillariophyceae	Weihai Bay, Yellow Sea
Alexandrium tamarense	Dinophyceae	East China Sea
Alexandrium minutum	Dinophyceae	Hongkong, East China Sea
Karlodinium veneficum	Dinophyceae	East China Sea
Thalassiosira pseudonana	Bacillariophyceae	East China Sea
Karenia sp.1	Dinophyceae	Wenzhou, East China Sea
Karenia sp.2	Dinophyceae	Wenzhou, East China Sea
Symbiodinium sp	Dinophyceae	Hangzhou, East China Sea
Dierateria zhanjiangensis	Prymnesiophyte	Xiamen, East China Sea
Emiliania huxleyi	Prymnesiophyceae	South China Sea
Takayama puchellum	Dinophyceae	Xiamen Bay, East China Sea
Thalassiosira weissflogii	Bacillariophyceae	Jiangshu, East China Sea
Phaeocystis globosa	Prymnesiophyte	South China Sea
Nannochloropsis oceanica	Eustigmatophyceae	Xiamen Bay, East China Sea
Amphidinium carterae	Dinophyceae	USA
Gymnodinium sanguineum	Dinophyceae	Xiamen Bay, East China Sea
Gymnodinium impudicum	Dinophyceae	Bohai Sea
Gyrodinium instriatum	Dinophyceae	Lianyun Port, East China Sea
Pseudo-nitzschia pungens	Bacillariophyceae	Zhujiang Estuary, East China Sea