Comparison of loop-mediated isothermal amplification with hyperbranched rolling circle amplification as a simple detection method for *Heterosigma akashiwo*

Chunyun Zhang[a], Yuanyuan Wang[a], Changlu Guo[a], Guofu Chen[a,*], Guangfeng Kan[a], Panpan Cai[a], Jin Zhou[b]

[a] *College of Oceanology, Harbin Institute of Technology (Weihai), Weihai 264209, PR China*
[b] *Division of Ocean Science and Technology, Graduate School at Shenzhen, Tsinghua University, Shenzhen 518055, PR China*

ARTICLE INFO

ABSTRACT

The fish-killing alga *Heterosigma akashiwo* is a globally distributed, toxic, and bloom-forming raphidophyte that has caused great losses to the fishing industry in many coastal countries. Therefore, rapid and sensitive detection methods should be developed to present timely warning of harmful algal blooms. In this study, hyperbranched rolling circle amplification (HRCA) was established for the detection of *H. akashiwo* and compared with loop-mediated isothermal amplification (LAMP) in terms of specificity and sensitivity. The partial D1–D2 sequence of the large subunit (LSU) of rDNA of *H. akashiwo* was used to design a specific padlock probe for HRCA and two pairs of specific primers for LAMP. The parameters for HRCA were optimized. Cross-reactivity tests showed that the specificity of the developed HRCA for *H. akashiwo* was greater than that of LAMP in this study. The sensitivities of HRCA and LAMP were comparable and were 10-fold higher than that of regular PCR. These methods also yielded a detection limit of $20\,fg/\mu L$ for the recombinant plasmid containing the target LSU D1–D2 and 1 cell for target species. The test with the simulated field samples indicated that the developed HRCA obtained a detection limit of $5\,cells\,mL^{-1}$, which was lower than the warning cell density ($100\,cells\,mL^{-1}$) of *H. akashiwo*. The visual detection of positive HRCA could be achieved via coloration reaction with the addition of fluorescent SYBR Green I dye to the amplification products. The developed HRCA was also efficient for field samples with target cell densities ranging from $10\,cells\,mL^{-1}$ to $1000\,cells\,mL^{-1}$. Therefore, the proposed HRCA detection protocols are possibly applicable to the field monitoring of *H. akashiwo*.

© 2018 Published by Elsevier B.V.

1. Introduction

Harmful algal blooms (HABs), especially caused by toxic species, have negatively affected fishery, human health, tourism, and aquatic ecosystems. The golden-brown marine alga *Heterosigma akashiwo* (Hada) Hada (Chromophyta: Raphidophyceae) is a well-known eurythermal and euryhaline flagellate that is distributed worldwide. This harmful alga has been associated with fish-killing blooms in the coasts of several countries, including China (Tseng et al., 1993), Japan (Honjo, 1992), New Zealand (Chang et al., 1990), South Africa (Bates et al., 2004), and the United States

(Kempton et al., 2008; Rensel et al., 2010). *H. akashiwo* also induces sub-lethal effects on the southeastern oyster *Crassostrea virginica* (Keppler et al., 2005). Thus, *H. akashiwo* monitoring is necessary to relay a warning regarding possible HABs and avoid or reduce fishery losses.

Difficulties in identifying *H. akashiwo* have been encountered in morphological examinations under light microscopy (LM), which is currently the most commonly used method to monitor harmful algae. *H. akashiwo* cells are small (8–25 μm in length and 6–15 μm in width), fragile, and morphologically variable; furthermore, the morphological characteristics of these cells often change under different water conditions (Guo, 2004; Bowers et al., 2006). The use of fixatives may alter their morphological characteristics, size, and color (Woelfl and Whitton, 2000; Chen et al., 2008). As such, species delimitation becomes challenging. The taxonomic classification of this species has been the subject of debates and revisions;

for instance, this species has been given several names, including *Olisthodiscus carterae*, *Entomosigma akashiwo*, *Heterosigma akashiwo*, *Heterosigma inlandica*, and *Heterosigma carterae* (Connell, 2000). Moreover, the designation of the genus *Heterosigma* with regard to a high taxonomic rank has been uncertain (Connell, 2000). Therefore, experience and professional skills are required to identify *Heterosigma* species microscopically in natural populations.

Current methods for the identification and detection of harmful algae are based on microscopic examinations of morphological features under LM. However, this technique is limited due to low efficiency and inaccuracy. Consequently, novel methods that enable rapid and accurate detection of harmful algae have been developed. Molecular approaches show potential for HAB sample analysis because of their highly efficient, rapid, and accurate detection of specific organisms regardless of their morphological characteristics. Thus, several molecular methods, including fluorescence *in situ* hybridization (FISH) (Scholin et al., 1996; Chen et al., 2013a, 2013b), sandwich hybridization assay (Mikulski et al., 2008; Zhen et al., 2009), quantitative real-time PCR (QRT-PCR) (Eckford-Soper and Daugbjerg, 2015; Zhang et al., 2016), and DNA array (Ki and Han, 2006; Zhang et al., 2015; Nagai et al., 2016), have been established to detect harmful algae. However, most of these methods are highly dependent on special instruments and require professional operation skills, which restrict their practical applications.

Isothermal amplification techniques (Zhang et al., 2009; Nagai et al., 2012; Nagai, 2013; Chen et al., 2013a, 2013b; Chen et al., 2015) that can achieve DNA amplification at constant temperatures may be applied to conduct field testing, which is independent of specific instruments and professional skills. For example, hyperbranched rolling circle amplification (HRCA) is a combination of specific molecular recognition and universal amplification, which offers an alternative platform to detect target

species quickly and accurately without specialized training (Dean et al., 2001). For HRCA (Fig. 1), a special padlock probe (PLP) and a pair of universal primers are used. PLP is a linear single-stranded nucleotide with a nucleic acid sequence of about 100 bases. This sequence typically consists of two terminal fragments of 15–25 bases complementary to the 5′- and 3′-terminals of the target sequence and a linker region of 50–80 bases. PLP can hybridize to the target in a head-to-tail fashion with both terminal fragments and produce a closed and circular molecule with DNA ligase. After non-circularized probes are removed with exonuclease (Kaocharoen et al., 2008), rolling circle amplification is carried out using one primer with the same sequence as a segment of PLP and another complementary to a part of PLP with DNA polymerase. HRCA can efficiently produce 10^9 amplicons from single copy of a target sequence within 1 h (Nilsson, 2006). This technique has also been widely used for single nucleotide polymorphism (SNP) genotyping (Nilsson, 2006; Li et al., 2009), qualitative detection of DNA methylation (Zhao and Lu, 2014), and detection of genetically modified crops (Wang et al., 2015) and pathogenic microorganisms (Liu et al., 2013; Zhao et al., 2014). Loop-mediated isothermal amplification (LAMP) is another novel technique (Notomi et al., 2000) that employs four specific primers recognizing six target DNA regions and a DNA polymerase with a strand displacement activity and produces the final stem-loop DNA with several target inverted repeats and cauliflower-like structures with multiple loops. LAMP can complete within 30 min or 1 h because of its high efficiency. In LAMP, positive results can be easily confirmed by visually inspecting white magnesium pyrophosphate precipitate in the bottom of the reaction tube or viewed by adding fluorescent dyes, such as SYBR Green I. With its high efficiency, simplicity, and rapidity, LAMP has been used in diverse fields, including molecular diagnosis, identification of genetically modified organisms, food adulterations, eutrophication/cyanobacterial bloom, medicinal plants, food allergens, and pesticides, investigations on drug
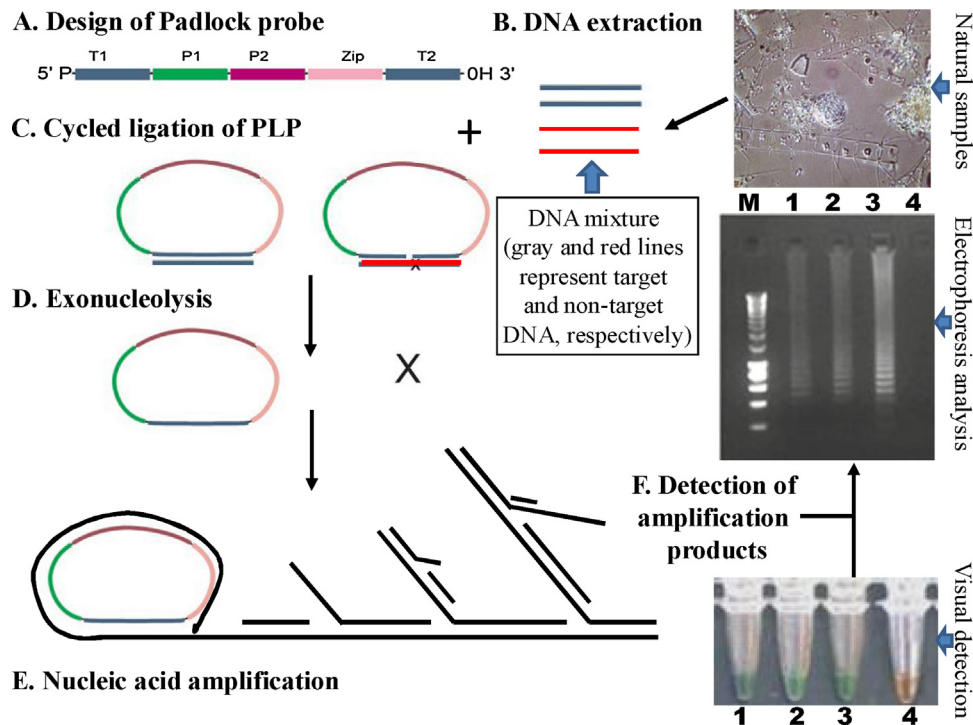

Fig. 1. Schematic representation of detection by HRCA. (A) Design of a PLP containing target-complementary sequences at the 5′ and 3′ ends (T1, T2), flanking the universal primer sites (P1, P2) and the unique identifier ZipCode sequence (Zip); (B) T1 and T2 bind to adjacent sequences on the target, and in the case of a perfect match, the probe may be circularized by a ligase. Mismatch-containing molecules are expected to be discriminated, and no ligation should occur; (C) Unreacted probes are removed by exonuclease treatment; (D) Amplification Circularized probes are amplified using two universal primers and.