Hyperbranched rolling circle amplification as a novel method for rapid and sensitive detection of Amphidinium carterae

Guofu Chen, Panpan Cai, Chunyun Zhang, Yuanyuan Wang, Shibeif Zhang, Changlu Guo, Dou Ding Lu

Abstract

High quality of coastal water is critical to marine ecosystems, marine fisheries, public health, and aquatic environment. Specially, bio-toxin derived from toxic microalgae is currently threatening many coastal countries. Therefore, development of rapid and sensitive methods for the detection of toxin-producing microalgae is necessary for warning of water quality. In this paper, we established a novel method for rapid and sensitive detection of Amphidinium carterae by hyperbranched rolling circle amplification (HRCA). The partial large subunit rDNA (LSU D1–D2) of A. carterae was sequenced to design species-specific padlock probe (PLP). The PLP-coupled with two amplification primers were employed for HRCA. The optimized HRCA conditions were as follows: padlock concentration, 20 pm; ligation temperature, 65 °C; ligation time, 15 min; amplification temperature, 61 °C; and amplification time, 15 min. The developed HRCA was confirmed to be specific for A. carterae by tests with other algae. The sensitivity of HRCA was 100-fold higher than regular PCR, exhibiting a detection limit of 1 fg/μL representing 283 copies for the recombinant plasmid containing the target LSU D1–D2, and 1 cell for target species. Finally, a simplified protocol was applied to the simulated field and environmental materials, and exhibited a good performance. The whole detection could be completed within 1.5 h, displaying a repeated detection limit of 1 cell. The positive HRCA results could be visualized through coloration reaction by adding the fluorescent dye SYBR Green 1 to the amplification products. The HRCA provides a useful tool to quickly screen large sample sets for A. carterae, as well as other toxic species.

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1. Introduction

Security of coastal water is closely linked with human and ecosystem health, as well as with fishing, aquaculture, and tourism industries. Microalgae, existing either in unicellular forms or in colonies, are invisible organisms playing a key role in the aquatic ecosystems, mainly because they as producers of organic material form the base of the aquatic food chain. However, due to the eutrophication of seawater accompanied with more and more intensive industrial and agricultural activities, the frequency, duration, and geographic extent of harmful algal blooms due to over proliferation of microalgae appear to be increasing. Of the about 4000 described microalgal species (Sournia et al., 1991), a few more than 100 have been found to be toxic (Sournia, 1995). Therefore, seawater contamination by bio-toxin mainly from toxic algae has posed a potential threat on many coastal countries and negatively impacts large geographic areas.

The unarmoured dinoflagellate, Amphidinium carterae Hulburt, belongs to family Gymnodiniaeae of the order Gymnodiniiales of the class Dinophyceae (Droop, 1953). This species is well known to be one of the most common harmful algae associated with ciguatera fish poisoning (Zingone, 2010), which is the most frequently reported marine toxin-derived disease worldwide and causes illness for more than 50 000 people each year (Botana, 2008). A. carterae also could form red tide reaching a maximum density of 1.2 × 10^4 cells/ml, and resulted in fish mass mortality (Yasumoto, 1990; Baig et al., 2006). The toxic effects of A. carterae have been widely studied (Anderson and Lobel, 1987; Yasumoto et al., 1987; Lewis, 1991; Baig et al., 2006; Pagliara and Caroppo, 2012), and its role in fish mortalities is thought to be related to the hemolysins, low molecular weight compounds produced by this dinoflagellate. A. carterae is a cosmopolitan species, but mainly habits in tropical and subtropical waters. In China, this dinoflagellate has first been reported to occur in the South China Sea and...
Sanyan sea area of Hainan Province (Lin et al., 2001), but is currently found to have a more wide distribution along the Chinese coast (Han et al., 2004). Therefore, it is necessary to develop highly effective detection methods for monitoring of this harmful algae. 

The traditional methods for the identification and detection of harmful algae mainly rely on morphological features. However, morphological examine is time-consuming and labor-intensive. Moreover, harmful algae are difficult to distinguish because of their cryptic morphological diversity under different environmental conditions. For example, *Amphidinium carterae* has a length varying between 12 and 20 μm, and a width between 8 and 10 μm (Baig et al., 2006; Pagliara and Caroppo, 2012). More importantly, target species is likely to be neglected by microscopy examine when it is a minor component in the natural samples. 

Molecular methods targeting ribosomal RNA (rRNA) genes are promising alternatives to the traditional morphological identification. Recently, various molecular methods, including fluorescence in situ hybridization (FISH) (Scholin et al., 1996; Chen et al., 2013a,b), sandwhich hybridization assay (Mikulski et al., 2008; Zhen et al., 2009), real-time PCR (Yuan et al., 2012; Penna and Galluzzi, 2013), loop-mediated isothermal amplification (LAMP) (Nagai et al., 2012; Nagal, 2013), multiplex PCR assay (Nagal, 2011), and DNA array (Ki and Han, 2006; Smith et al., 2012), have been applied to the detection of harmful algae. 

Despite their high efficiency in species delimitation compared with the microscopy-based methods, specific instruments are involved in these methods except LAMP. Epifluorescence microscopes, microplate readers, real time PCR thermocyclers, and microarray scanners are required by FISH, SHA, real-time PCR, and DNA array. In addition, these detection methods still require technical expertise. These shortcomings mentioned as above consequently preclude their practical application. Hence, alternative methods are needed to provide rapid and reliable detection and confirmation. 

Recently, we successfully introduce LAMP into rapid and sensitive detection of harmful algae (Chen et al., 2013a,b). In the current study, we focus on another isothermal amplification method – hyperbranched rolling circle amplification (HRCA). HRCA is a combination of specific molecular recognition and universal amplification, which offers an alternative platform that can be handled by individuals without specialized training to detect target species quickly and accurately (Dean et al., 2001). This technique mainly relies on a kind of special nucleotide nucleic acid sequence – padlock probe (PLP), which is single-stranded and linear containing about 100 bases. Typical PLP consists of two terminal fragments of 15–25 bases complementary to the 5′- and 3′-terminal of the target sequence and a linker region (LR) of 50–80 bases. Typical detection of target sequences by HRCA comprises three steps. First, both terminal sequences hybridize to the target in a head-to-tail fashion, resulting in a closed and circular molecule with DNA ligase. The key point is that the ligation can only take place when both terminal segments recognize their target sequences correctly. Secondly, non-circularized probes are removed by exonuclease treatment, reducing subsequent ligation-independent amplification (Kaechoaren et al., 2008). Finally, rolling circle amplification is carried out using one primer with the same sequence as a segment of PLP, and another complementary to part of PLP, with DNA polymerase. HRCA is a highly efficient amplification method, able to synthesize 10^9 amplicons from single copy of target sequence within 1 h (Nilsson, 2006).

Because of its obvious advantages over other nucleic amplification methods, HRCA has been widely used for SNP genotyping, as well as for the detection of a number of human pathogenic microorganisms, including viruses (Wang et al., 2005a,b; Steain et al., 2009), bacteria (Tong et al., 2007), and fungi (Kaocharoen et al., 2008; Zhou et al., 2008; Tsui et al., 2010; Liu et al., 2014). However, there is still no report about applying HRCA to the detection of harmful algae in the field samples. Therefore, in this study, we further developed and explored the practical application of HRCA for rapid detection of harmful algae by using *Amphidinium carterae* as a test species.

2. Materials and methods

2.1. Microalgal cultures

The algal strains used in this study are listed in Table 1. All clonal cultures were purchased, kindly donated, or established by micropipette isolation. The algal cultures were maintained in 250 mL flasks supplied with 100 mL of sterile-filtered f/2 or f/2 + Si seawater medium (Guillard, 1975) at a salinity of 36 psu. All cultures were grown at 20–22 °C under a 12 h light:dark cycle with a light intensity of 50–100 μmol photons m⁻² s⁻¹. The cultures were stirred manually daily, with the nutrient medium replaced weekly or monthly.

2.2. PCR, cloning, and sequencing

Genomic DNA of *Amphidinium carterae* was extracted from algal culture (about 1.0 × 10^6 cells) during log phase with Plant Genomic DNA Rapid Extraction Kit (Sangon Biotech, Shanghai, China). DNA purity and concentration were determined spectrophotometrically by measuring the absorbance at 260 nm and A260/A280 ratio using the Nanodrop 2000 microspectrophotometer (Thermo Fisher

### Table 1

<table>
<thead>
<tr>
<th>Species</th>
<th>Taxonomy</th>
<th>Geographic origin</th>
<th>HRCA test*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Amphidinium carterae</em></td>
<td>Dinophyceae</td>
<td>South China Sea</td>
<td>*</td>
</tr>
<tr>
<td>Procentrum donghaiense</td>
<td>Dinophyceae</td>
<td>Zhejiang, East China Sea</td>
<td>–</td>
</tr>
<tr>
<td>Procentrum triestinum</td>
<td>Dinophyceae</td>
<td>East China Sea</td>
<td>–</td>
</tr>
<tr>
<td>Procentrum lima</td>
<td>Dinophyceae</td>
<td>Daya Bay, East China Sea</td>
<td>–</td>
</tr>
<tr>
<td>Procentrum minimum</td>
<td>Dinophyceae</td>
<td>Daya Bay, East China Sea</td>
<td>–</td>
</tr>
<tr>
<td>Alexandrium tamarense</td>
<td>Dinophyceae</td>
<td>East China Sea</td>
<td>–</td>
</tr>
<tr>
<td>Gymnodinium sanguineum</td>
<td>Dinophyceae</td>
<td>Xiamen, East China Sea</td>
<td>–</td>
</tr>
<tr>
<td>Chaetoceros debilis</td>
<td>Bacillariophyceae</td>
<td>Weihai, Yellow Sea</td>
<td>–</td>
</tr>
<tr>
<td>Thalassiosira weissflogi</td>
<td>Bacillariophyceae</td>
<td>South China Sea</td>
<td>–</td>
</tr>
<tr>
<td>Pseudo-nitzschia pungens</td>
<td>Bacillariophyceae</td>
<td>Zhoushan, East China Sea</td>
<td>–</td>
</tr>
<tr>
<td>Nitzschia closterium</td>
<td>Bacillariophyceae</td>
<td>Weihai, Yellow Sea</td>
<td>–</td>
</tr>
<tr>
<td>Dieraterea zhanjiangensis</td>
<td>Prynnesiophyceae</td>
<td>Xiamen, East China Sea</td>
<td>–</td>
</tr>
<tr>
<td>Prymnesium parvum</td>
<td>Prynnesiophyceae</td>
<td>Bohai Sea</td>
<td>–</td>
</tr>
<tr>
<td>Tetraselmis Chui</td>
<td>Chlorophyceae</td>
<td>Shenzhen, East China Sea</td>
<td>–</td>
</tr>
<tr>
<td>Heterosigma akashiwo</td>
<td>Raphidophyceae</td>
<td>South China Sea</td>
<td>–</td>
</tr>
<tr>
<td>Nanochloropsis oceanica</td>
<td>Eustigmatophyceae</td>
<td>Xiamen, East China Sea</td>
<td>–</td>
</tr>
</tbody>
</table>

*The positive and negative HRCA results were represented by “+” and “−”, respectively.*
Table 2
Summary of primers and PLP used in this study.

<table>
<thead>
<tr>
<th>Primer/probe name</th>
<th>Sequence (5'–3')</th>
<th>Amplification type</th>
<th>Target species</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>ACCCGCTGAATTIAACATA</td>
<td>PCR</td>
<td>Eukaryotic algae</td>
<td>Scholin et al. (1994)</td>
</tr>
<tr>
<td>D2</td>
<td>CCTTGGCTCGCTTCTAAAGACGA</td>
<td>PCR</td>
<td>Eukaryotic algae</td>
<td>Scholin et al. (1994)</td>
</tr>
<tr>
<td>Q-Ac-f</td>
<td>CCTACCTGCTTGGACATGCC</td>
<td>PCR</td>
<td>Amphidinium carterae</td>
<td>This study</td>
</tr>
<tr>
<td>Q-Ac-r</td>
<td>GTCAAGGTAGGCTGTCCGGC</td>
<td>PCR</td>
<td>Amphidinium carterae</td>
<td>This study</td>
</tr>
<tr>
<td>PLP</td>
<td>GCCAGTCCTACAAAGTAGACATCAGCA</td>
<td>Cyclization</td>
<td>Amphidinium carterae</td>
<td>This study</td>
</tr>
<tr>
<td>P1</td>
<td>GCGGTGTGCTGCTTGGGCTTCCAATTAGGCACTACCTGG</td>
<td>HRCA</td>
<td>–</td>
<td>Szemes et al. (2005)</td>
</tr>
<tr>
<td>P2</td>
<td>GCGATGTTCCTTGGTATAGGCAACCTG</td>
<td>HRCA</td>
<td>–</td>
<td>Szemes et al. (2005)</td>
</tr>
</tbody>
</table>

Note: The lowercase letters of PLP denote the connection parts of both ends, while the capital letters denote the both terminal regions recognizing the target sequence.

Scientific, MA, USA). The isolated genomic DNA was applied to PCR-amplify the D1–D2 region of the 28S rDNA gene with the universal primers D1 and D2 (Table 2) (Scholin et al., 1994). The PCR products were purified by Sanprep Type DNA Gel Extraction Kit (Sangon Biotech., Shanghai, China). The purified PCR products were ligated with pMD™ 18-T Vector (TaKaRa, Dalian, China) and transformed into competent Escherichia coli. The positive clones were screened by colony PCR for sequencing at the Beijing Nuosi Genome Research Center Co., Ltd. (Beijing, China).

2.3. Design of PLP and primers

The obtained sequence from Amphidinium carterae was searched using BLASTn for the most similar sequences from other related species in public database GenBank. Relevant nucleic acid sequences derived from GenBank and from the above sequencing study were aligned by using ClustalW implemented with the program Bioedit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), through which a fragment of 43 bp specific for A. carterae were identified to be potential PLP target complementary region. Both the 5' and 3' arm regions of PLP were manually designed following the rules put forward by Szemes et al. (2005). The PLP arm sequences were combined with the universal primer sites (P1 and P2), and a ZipCode sequence which is not necessary for this study, but might be used in the future array study. MFold (http://www.bioinfo.rpi.edu/applications/mfold/) was used to predict the secondary structure of the designed PLP. When necessary, PLP arm sequences were adjusted to avoid strong secondary structures that might interfere with efficient ligation. Specific primers (Q-Ac-r and Q-Ac-f) for conventional PCR detection of A. carterae were designed from LSU D1–D2 using Primer Premier 5.0. The detailed sequence information of PLP and primers is summarized in Table 2.

2.4. HRCA reaction

Three separate steps, including ligation, exonuclease treatment, and nucleic acid amplification were involved in a typical HRCA detection. Cycled ligation of PLP was performed in a 10 μL reaction mixture, containing 1× ligation buffer, 20 ng genomic DNA, 2.4 U Taq ligase (New England Biolabs, UK) and 20 pmol PLP. Reaction mixtures were prepared on ice and transferred rapidly onto a thermal cycler. The ligation reactions were carried out according to the following programs: 94 °C for 4 min, 15 cycles of 94 °C for 30 s, 65 °C for 5 min, and a final inactivation step at 95 °C for 15 min. After ligation, 10 μL of exonuclease mixture containing 2× Exonuclease I buffer and 15 U exonuclease I (Fermentas) was added to each reaction, and the samples were then incubated at 37 °C for 2.5 h, followed by inactivation at 80 °C for 15 min. HRCA reactions were performed in a 50 μL volume containing 4 U of Bst DNA polymerase (New England Biolabs, UK), 0.3 mM of dNTPs mix, 0.4 μM of universal primers (P1 and P2), and 1× Bst DNA polymerase buffer. The amplification was achieved by incubation at 65 °C for 15 min.

2.5. Detection of HRCA products

The HRCA products can be confirmed by electrophoresis analysis and visual coloration reaction. For electrophoresis analysis, the HRCA products are examined by 2.0% agarose gel electrophoresis against known standards, stained with ethidium bromide, and visualized on an imaging analyzer. The positive signals were visualized as a ladder of bands starting at one unit circle length and extending in discrete increments to several thousands of nucleotides. For coloration reaction, 1% SYBR Green I (Biov, Xiamen Baiweixin Biotech Co., Ltd.) was directly added into the HRCA reactions, which would turn green when the HRCA products were present.

2.6. Specificity confirmation of HRCA reaction

A total of 16 microalgal species were used to test the specificity of HRCA reaction. The genomic DNA of all the test species were extracted with Plant Genomic DNA Rapid Extraction Kit and then used as templates to carry out HRCA and conventional PCR with the primers D1 and D2. The DNA isolation, HRCA, and PCR were performed as described in Sections 2.2 and 2.4, respectively. The specificity of HRCA amplification was further validated via digestion of the HRCA products with the restriction endonuclease to form products with the expected length. The sequence of PLP was used to search for restriction endonuclease sites through the Enzyme tool implemented in the program Primer Premier 5.0. A restriction endonuclease site of Asu I located in the linkage fragment of PLP was used as the specific site to identify HRCA products consisting of one or several repeats of singular PLP.

2.7. Comparison of the sensitivities of HRCA and PCR

The sensitivities of HRCA and PCR were compared by tests with plasmid containing LSU D1–D2 sequence of Amphidinium carterae and crude DNA extraction of A. carterae cells. The plasmid was extracted from cultures of positive colony obtained in Section 2.2 with commercial kit (ca. SK8191) provided by Sangon Biotech. (Shanghai, China). The obtained plasmid solution was adjusted to a concentration of 100 ng/μL, and then 10-fold diluted with deionized water. The resulting plasmid solutions at various dilutions were used to conduct HRCA and PCR in parallel, respectively. For the cell tests, cell density of target species was first determined. In brief, A. carterae cultures in the exponential stage were swirled gently to ensure a homogenous cell distribution, and a subsample was fixed with Lugol’s iodine at a final concentration of 2%. The fixed sample was observed directly under light microscopy (LM) using a hemocytometer. Three independent
counts were performed and the average was used as the final cell density. According to the cell density, approximately 10⁶ cells of *A. carterae* in the exponential phase were harvested and then rinsed twice with deionized water by centrifugation (4000 \( \times \) g for 15 min). The algal pellets were used to make up crude DNA extract with a commercial kit (Cat. SK8411, Sangon Biotech) following the manufacturer's instructions. The obtained original crude extract was 10-fold diluted with deionized water. Then, the resulting crude DNA extract solutions representing different cell densities were used to perform HRCA and PCR, respectively.

2.8. HRCA analysis of simulated and environmental samples

The ability of HRCA to detect *Amphidinium carterae* was assessed using the simulated and field samples. The simplified HRCA protocol used for the simulative and environmental samples was as follows: cycled ligation of PLP at 94 °C for 4 min, 65 °C for 15 min, 95 °C for 15 min; incubation on ice for 5 min; amplification at 61 °C for 15 min. For the simulative test, the environmental seawater samples were collected from the Weihai Bay (Weihai, Shandong Province), which were tested negative for the presence of *A. carterae* under the HRCA assay. Briefly, 10 mL aliquot of the field samples was spiked with 10⁵ cells of *A. carterae* to prepare an original simulative field sample. Then, the original simulative field sample was 10-fold diluted with seawater to make up a series of samples with final concentrations of *A. carterae* ranging from 10 000 to 0.01 cells mL⁻¹. The environmental samples were obtained from different sites in the South China Sea from August to September 2013. The presence or absence of *A. carterae* was confirmed by LM examine, and the cell density was determined via direct counting using the methods described in Section 2.7. All the simulated samples and aliquots of environmental samples were used to collect target cells by centrifugation at 5000 \( \times \) g for 10 min. The obtained cell pellets were resuspended in the lysis solution provided by the commercial kit (Cat. SK8411, Sangon Biotech) to make up crude DNA extracts following the manufacturer’s instructions. The crude DNA extracts from all the simulative samples were used to perform PCR with the specific primers (Q-Ac-r and Q-Ac-f) for *A. carterae* and HRCA, respectively, whereas only HRCA was performed on the environmental samples.

3. Results and discussion

3.1. Optimization of HRCA conditions

To confirm the cyclized PLP was the initiator of HRCA, four cycled ligation reactions with all normal reaction components including DNA template, PLP, and *Taq* DNA ligase, and without PLP, *Taq* DNA ligase, or DNA template, respectively, were established. The results of HRCA with these cyclization products are shown in Fig. 1. HRCA could occur when all of normal components were included in the cycled ligation reaction. By contrast, successful amplification was not detected for the cycled ligation reactions without PLP, *Taq* ligase, or DNA template. These results ascertain that HRCA can happen only when the linear PLP is cyclized with ligase.

Self-cyclization may happen to PLP even when no DNA ligase is present, which would result in false positive results interring with the judgment of presence of target DNA. Therefore, HRCA coupled with no-template cycled ligation reactions containing a PLP concentration ranging from 200 to 0.2 pM was investigated (Fig. 2). Ideally, no subsequent HRCA should occur when no DNA template is present in the cycled ligation reaction. However, positive HRCA could be detected for cycled ligation with a PLP concentration of 200 pM. Fortunately, when PLP concentrations were or less than 20 pM, HRCA could not occur. Moreover, successful HRCA could be detected when DNA template is added to the cycled ligation reaction (data not shown). Therefore, 20 pM of PLP was finally used in the following tests.

Although inefficient HRCA might not affect detection via electrophoresis, much higher yield of amplified products is required for the visual detection by coloration with addition of...
fluorescent dye, because the yield of amplification products directly determines the detection limit. However, previous studies have indicated that the efficiency of HRCA is not affected by the amounts of Taq DNA ligase, dNTPs, and amplification primes (Tao et al., 2003; Wang et al., 2005a,b). Therefore, the optimization of HRCA conditions mainly focused on PLP ligation temperature, ligation time, amplification temperature, and amplification time. The HRCA results with different ligation temperatures ranging from 53 to 65°C are shown in Fig. S-A. Ligation temperature has little effect on the HRCA performance, since no visible difference was confirmed from the electrophoresis pattern with amplification products under different ligation temperatures. However, the highest ligation temperature of 65°C was used in the following HRCA to avoid the possible non-specific ligation in the practical use. Effects of ligation time on HRCA are shown in Fig. S-B. The HRCA products could be detected when the ligation time was increased from 5 to 10 min. Moreover, the yield of amplification products remarkably increased when the ligation time was increased from 10 to 15 min. The increase in the yield of amplification products was not significant when the ligation time was longer than 15 min. Therefore, a ligation for 15 min was considered to be long enough for the subsequent HRCA. The HRCA results under amplification temperatures ranging from 58 to 64°C are shown in Fig. S-C. The yield of HRCA was positively related with amplification temperature in a range from 58 to 61°C. However, the HRCA yield decreased sharply when the amplification temperature was increased from 62 to 64°C. Therefore, 61°C was set as the optimal amplification temperature. Finally, the HRCA performance with an amplification time range from 5 to 90 min was compared. Successful HRCA occurred when the amplification time was increased from 5 to 15 min (Fig. S-D). Moreover, no significant increase in the yield of amplified products was observed when the amplification time was prolonged. To reduce detection time, the final amplification time was set as 15 min. Based on the rough quantitative analysis by electrophoresis of amplification products as above, the optimized HRCA conditions were used in the subsequent HRCA tests.

Supplementary figure related to this article can be found, in the online version, at doi:10.1016/j.jhal.2015.05.012.

It should be pointed out that optimization of HRCA conditions is served as an intermediate step for the final establishment of detection protocol for field detection. We did not compare the amplification efficiency by using a more sensitive approach, such as a plate reader that can read colorimetric reactions, or using a highly sensitive turbidity meter. By contrast, we simply determine the amplification efficiency by naked-eye observation of gel electrophoresis pattern of HRCA products, the strategy of which has also been adopted in previous study (Zhao et al., 2014). Moreover, gel electrophoresis analysis of HRCA products is more straightforward than the readers, and it does not affect the final application of HRCA to field detection of target species.

3.2. Specificity of HRCA for A. carterae

The specificity of probes is extremely important for the molecular methods for detection of target harmful algae, since the marine environmental samples are usually composed of various kinds of microorganisms, including bacteria, protozoan, and microalgae. For the three sequences (PLP and both amplification primers) used in the HRCA detection, a specific PLP capable of recognizing target sequence distinguishing the target cells from other microorganisms is the key to the specific detection, whereas the primers involved in the HRCA is universal. In the methodology, HRCA based on PLP is highly specific for the target sequence because the cyclization of PLP catalyzed by Taq DNA ligase would occur only when the terminal sequences are completely complementary to the target sequence. In fact, HRCA is capable of recognizing singular base difference, the characteristic of which has been applied to SNP genotyping research (Pickering et al., 2002). Therefore, this method is especially suitable for the differentiation among the related species with nearly identical rRNA gene sequences. It is a great advantage over other current nucleic acid amplification methods requiring several primers to accomplish amplification, including PCR (Mullis and Faloona, 1987), nucleic acid sequence-based amplification (Compton, 1991), strand displacement amplification (Walker et al., 1992), signal mediated amplification of RNA technology (Wharam et al., 2001), and single chimeric primer for amplification (Kurn et al., 2005).

To experimentally confirm the specificity of the designed PLP, clonal cultures of 16 microalgal species, which are widely distributed along the Chinese coast and are more likely to coexist with the target species and interfere with the detection in the practical application, were used to perform HRCA tests. These microalgae belong to five classes, including Dinophyceae (7), Bacillariophyceae (4), Prymnesiophyceae (2), Chlorophyceae (1), Raphidophyceae (1), and Eustigmatophyceae (1). The results of the HRCA tests are summarized in Table 1, and the results of electrophoresis analysis and coloration reaction by adding fluorescence dye are shown in Fig. 3. Positive HRCA reactions did not occur with other test algae except Amphidinium carterae (Fig. 3, Table 1). However, the closely related species, like Amphidinium incoloratum, Amphidinium herdmani, Amphidinium trullu, and so on, were not included in our cross-reactivity testing because of the lack of living isolates. Therefore, the possible cross-reactivity with these species also could not be excluded.

In order to further confirm the failure of HRCA is not caused by the absence of DNA templates in the HRCA reactions, the parallel PCR with the universal primers (D1 and D2) were performed. As
expected, positive PCR reactions could occur with all the test algae. In addition, a restriction endonuclease reaction with Asu I was introduced to confirm the specific amplification from PLP. Because the HRCA products are mixtures consisting of nucleic acids with one or several times the length of PLP, the resulting products digested by Asu I should be 109 bp, the length of which is identical to PLP. As expected, a band of ~100 bp could be observed in the electrophoresis pattern of HRCA products digested by Asu I (Fig. 4), which is consistent with the predicted size. All of these results indicated that the developed HRCA was highly specific for *Amphidinium carterae*, and the specific PLP may be useful for the molecular identification of target species in the samples containing many different microalgae. However, the closely related species belonging to genus *Amphidinium* were excluded in our cross-reactivity test because of the lack of living species. Considering this point, the possible cross-reactivity with these species cannot be excluded.

3.3. Detection limit of HRCA

Toxin-producing algae, like *Amphidinium carterae*, are usually harmful even at a relatively low density. Therefore, detection limit is critical for a molecular detection tool for qualifying the environmental water or warning against imminent harmful blooms. An ideal detection method should be able to accurately recognize the target species even though it is a minor component in the samples. Given that the ultimate purpose of this study is to establish an effective monitoring tool for *A. carterae*, the detection limit of HRCA was tested with the target gene (LSU D1–D2)-containing plasmid and target cells, by using PCR as a control.

The results of simultaneous HRCA and PCR with a series of plasmid solutions at concentrations ranging from 100 ng/μL to 1 ag/μL are shown in Fig. 5. HRCA producing visible electrophoresis bands could occur with plasmid concentrations ranging from 100 ng/μL to 1 fg/μL, representing a detection limit of approximately 2.81 × 10^2 copies of plasmid (Fig. 5A). By contrast, successful PCR could be observed for plasmid at concentrations from 100 ng/μL to 100 fg/μL, representing a detection limit of approximately 2.81 × 10^4 copies of plasmid (Fig. 5B). Thus, detection limit tests with the plasmid indicated that the developed HRCA was 100 times more sensitive than PCR. These results were generally comparable to the similar research with other target species, despite that little difference can be found among them. For example, Cai (2009) demonstrated that HRCA with the plasmid containing target gene of *Tilletia controversa*, the causative pathogen for wheat dwarf bunt disease, showed a detection limit of 1 fg/μL, representing 223 copies of plasmid, which was 10-fold more sensitive than PCR. Likely, HRCA with the plasmid containing target gene of *Candidatus liberobacters*, the causative pathogen for Citrus Huang Long Bing, displayed a detection limit of 100 fg/μL, which was also 10-fold more sensitive than PCR (Liu, 2010). The actual difference in sensitivity of HRCA with plasmids containing different target genes among these studies may caused by several factors affecting amplification efficiency, such as the characteristics of target genes, amplification efficiency of reagents adopted in the whole HRCA procedure, and the instruments used for quantification and amplification. However, HRCA is anyhow more sensitive than the conventional PCR, the most commonly used detection method for various microorganisms in the past years (Tong et al., 2007; Steain et al., 2009; Liu et al., 2014).

Sensitivity comparison of HRCA and PCR with the crude DNA extract from *Amphidinium carterae* cells is shown in Fig. 6. As expected, HRCA and PCR displayed different amplification efficiencies. Positive results were observed when the cell number ranged from 100 000 (10-fold dilution) to 1 (10^6-fold dilution) for

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**Fig. 4.** Digestion of HRCA products with restriction endonuclease Asu I. M, DL500 DNA marker; lane 1, HRCA products; lane 2, digested HRCA products.

**Fig. 5.** Sensitivity test of HRCA (A) and PCR (B) with plasmid containing the LSU D1–D2 of *A. carterae*. M, DL500 or DL2000 DNA marker; lanes or tubes 1–11 are HRCA or PCR with plasmid solutions at concentrations ranging from 100 ng/μL to 1 ag/μL, respectively.
HRCA (Fig. 6A), and from 100,000 (10-fold dilution) to 100 (10^{-4} fold dilution) for PCR (Fig. 6B). Thus, HRCA with the crude DNA extract from _A. carterae_ also displayed a detection limit 100-fold more sensitive than PCR. Combined with the consistent data from the tests with target gene-containing plasmid, it can be concluded that the developed HRCA is actually 100-fold sensitive more than PCR, and should meet the demand for the detection of _A. carterae_ when it is a minor component of the analyzed samples.

### 3.4. Application of HRCA to detect _A. carterae_ in the simulated and environmental samples

To further validate the practicability of the developed HRCA, the simulated field samples and environmental samples were introduced to HRCA and specific PCR analysis. A set of spiked field samples containing 10,000–0.001 cells of _Amphidinium carterae_ were prepared with cultures by 10-fold dilution with field seawater. The results of specific PCR, HRCA, and the subsequent coloration reactions with the fluorescent dye are shown in Fig. 7. The specific PCR resulting in an expected band of ca. 150 bp could only be observed for the simulated samples containing 10,000 and 1000 cells (Fig. 7A). The detection limit of specific PCR with the spiked samples is 10-fold higher than that of universal PCR in the sensitivity test. The reason for this could be attributed to the difference in the dilution method for the test samples. In the sensitivity test, the samples representing different amounts of target cells were made up by diluting the original crude DNA extract with de-ionized water. By contrast, the samples containing various amounts of target cells here were prepared by serially diluting the original cultures with field water. Specially, for the latter test, more and more natural inhibitors hampering nucleic acid amplification were speculated to be introduced into the crude DNA solutions when the dilution proceeded.

The HRCA results with the simulated samples are shown in Fig. 7B. Positive HRCA reactions could occur with 10,000 to 1 cells. In general, the production of HRCA deduced from the visual band brightness in the electrophoresis pattern decreased with the reduction of target cells. Specially, the band with a size of the PLP (109 bp) could not be detected when only 1 cell was contained in the simulative sample. However, other bands of the amplified products were consistent with that of typical HRCA products. Moreover, the reaction could be clearly visualized by adding fluorescent dye (Fig. 7C). Therefore, the detection limit of HRCA with the spiked samples was considered as 1 cell, equivalent to that in the previous sensitivity test with different dilution method for preparing test samples representing various amounts of the target cells. These results also indicated that HRCA might be at least less affected by the supposed interference factors affecting PCR as mentioned above. Therefore, the developed HRCA may be good enough for analysis of field samples, which are more likely to contain several materials precluding nucleic acid amplification.

For detection methods, simplicity is another point that should be carefully considered. After testing with the simulated samples, the simplified HRCA protocol was finally applied to analyze 13 field samples from the South China Sea. The electrophoresis pattern of HRCA reactions are shown in Fig. 8. The parallel HRCA reactions with de-ionized water and target gene-containing plasmid were respectively used as negative and positive control (NC and PC) to provide a reference for appraising the effectiveness of HRCA.
detected. As expected, no HRCA occurred with the NC, whereas the PC produced typical HRCA products with ladder bands. Among the 13 detected field samples, 4 were tested positive, whereas 9 were tested negative (Fig. 8A). Alternatively, the reaction solutions containing HRCA products turned green after adding SYBR Green I (Fig. 8B). The presence of the target cells in the samples was confirmed through light microscope observation according to the morphologic criteria of Pagliara and Caroppo (2012) and Baig et al. (2006), and the cell densities ranged from 0 to 100 cells ml−1. In addition, single sample could be analyzed within 1.5 h by colorimetric assay.

Compared with other harmful algae, like Alexandrium spp. and Karenia spp., few studies (Goodwin et al., 2005) have focused on identifying Amphidinium carterae through molecular method. Goodwin et al. (2005) first developed a DNA hybridization assay for the simultaneous detection of multiple species, including Karenia brevis, Karenia mikimotoi, and A. carterae, despite that this study mainly focused on the practical detection of K. brevis. However, although this method is potentially useful for the parallel analysis of several samples, it requires laborious and complicated operation, including tailing of probe, PCR, molecular hybridization, and coloration. Moreover, specific instruments, including thermal cycle apparatus and micropipette reader, are required for the detection protocol. Specially, the need for particular instruments restricts the practical application of DNA hybridization assay in the routine monitoring of water environment demanding real-time results. In comparison, HRCA is a simple procedure that requires no expensive instrument. In fact, a thermostatic equipment such as a waterbath is sufficient for HRCA. Second, HRCA is sensitive and could detect up to 1 cell, which is comparable to the DNA hybridization assay involving PCR (Goodwin et al., 2005). This sensitivity is especially suitable for the daily monitoring of water environment, wherein water samples are concentrated to collect more target cells. Finally, a great advantage of HRCA over other detection methods is that the HRCA results can be judged directly by the visual observation of colored HRCA products with addition of fluorescent dye. This remarkable feature makes HRCA suitable for the field detection of target species in the environmental samples. In conclusion, HRCA is an effective tool for simple, quick, and sensitive detection, and was therefore promising for the field monitoring of A. carterae. This study also set a good example to apply HRCA to identification and detection of harmful algae, and HRCA should be promising to be introduced to field workers for its practical and universal application in the future monitoring of toxic algae.

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