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Development of a real-time PCR method for *Thalassiosira rotula* rapid detection

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Abstract

Gene specific primers and DNA probe were designed based on the sequence of 18S rDNA cloned from the red tide alga *Thalassiosira rotula*. A real-time fluorescent quantitative PCR (RFQ-PCR) method was developed for quantitative detection of *T. rotula*. The RFQ-PCR assay data showed that the results obtained with the RFQ-PCR quite good agreement with those with the light microscope (LM) counting method, which suggested that the RFQ-PCR could be a useful method for red tide alga detection.

Key words: red tide, *Thalassiosira rotula*, fluorescent quantitative PCR, 18S rDNA

1 Introduction

The occurrence of red tide events in sea waters along temperate coasts throughout the world appears to be an enlarging threat against human health, fishery resources, and tourism industry (Anderson, 1989; Smayda, 1990).

Thalassiosira rotula, which belongs to planktonic diatom, is a cosmopolitan harmful alga. It frequently appears along the coasts of China, and is classified into the red tide species catalogue in China (Qi, 2003). This alga produces some short-chain aldehyde that proves to restrain the growth of copepod spawn (Adrianna et al., 2004), thus the expanding red tide resulted from *T. rotula* could affect the population structure of copepod, further threatened the coastal fishery industry and ecological environment. So, the investigation on *T. rotula* has become a focus of the marine ecological research.

Monitor of the alga species is the key to predicting and preventing the red tide. In the early phase of red tide development, the cell density is relatively low and the cell size is usually small. While the conventional micro-phytoplankton detection was largely based on morphological features under microscopy, it is time consuming, labor tedious, and difficult for long-term monitoring. Furthermore, in some cases, the subjective prejudice should not be neglected (Rehnstam-Holm et al., 2002). Therefore, more sensitive and rapid methods are required to detect this alga and study the related dynamic variation. On the basis of the genetic diversity of different genera and species (LaJeunesse, 2001; Moon-van et al., 2001), molecular biological technologies could be

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introduced into the detection of a microorganism in both seawater and sediment (Guillou et al, 2002; Saito et al, 2002; Scholin et al, 1995). The polymerase chain reaction (PCR) has been successfully applied to the detection of various toxic dinoflagellates in seawater samples (Bowers et al, 2000; Godhe et al, 2001; Rollo et al, 1995), and the real-time fluorescent quantitative PCR (RFQ-PCR) is applied widely because of many advantages, such as high sensitivity, high specificity, and good repeatability. It has been used in pathogen (Bell and Ranford-Cartwright, 2002) and virus detection (Luo et al, 2004; Zhan et al, 2002), cancer diagnosis (Xu and Miller, 2004; Schnütch et al, 1998), gene expression (Cherry et al, 2004), immunology research (Lang et al, 1997), genetic mutation and polymorphism analysis (Walburger et al, 2001), and marine environmental bacteria detection (Du et al, 2006).

Here, we reported the development of a RFQ-PCR approach for *T. rotula* qualitative and quantitative detection in seawater. The RFQ-PCR assay was validated with the microscope counting method. It suggested that the RFQ-PCR can be a useful tool for red tide alga detection.

2 Materials and methods

2.1 Plants and cultures

Algae used in experiments are presented in Table 1. All of the 15 species are familiar in the Chinese sea area, and ten species of them are diatoms, five species are dinoflagellates. All species were identified and single cell samples were from the College of Marine Life Science, Ocean University of China. The algae were cultured to the exponential stage in the f/2 medium at 22～25 ℃ and exposed under the illumination approximately 4 000 lx under a 12h∶12h (time ratio of light to dark).

Table 1. Algae and the cell densities in their exponential stage

Species	Cell concentration/cm^{-3}	Source
Chaetoceros curvisetus	4.8×10^{7}	Jiaozhou Bay, China
Chaetoceros debilis	3.2×10^{6}	Jiaozhou Bay, China
Chaetoceros gracile	6.8×10^{6}	Jiaozhou Bay, China
Chaetoceros minimum	3.4×10^{7}	Jiaozhou Bay, China
Navicula membranacea	4.4×10^{5}	Jiaozhou Bay, China
Nitzschia closterium	1.2×10^{6}	Jiaozhou Bay, China
Gymnodinium sp.	5.1×10^{5}	Jiaozhou Bay, China
Gymnodinium mikimoto	1.5×10^{6}	Jiaozhou Bay, China
Melosira sp.	1.4×10^{5}	Jiaozhou Bay, China
Pseudonitzschia pungens	1.6×10^{4}	Jiaozhou Bay, China
Skeletonema costatum	3.8×10^{6}	Jiaozhou Bay, China
Alexandrium sp.	1.8×10^{4}	Dapeng Bay, China
Heterosigma akashiwo	2.3×10^{4}	Dalian Bay, China
Prorocentrem minimun	1.1×10^{3}	Bohai Gulf, China
Thalassiosira rotula	2.2×10^{5}	Jiaozhou Bay, China

2.2 DNA isolation

DNA extraction was carried out by using the modified method of Rajeshwar et al (2001). Briefly, algal cells in the exponential growth phase were counted in the solution (Olympus, Hamburg, Germany), and harvested by centrifugation at 10 000 r/min for 5 min and resuspended in 500 μL TE buffer (10 mmol/dm^3 Tris-HCl, pH 8.0 and 1 mmol/dm^3 EDTA) (Sangon Corporation, Shanghai, China). One millilitres extraction buffer [pre-warmed to 55 ℃, 3% (ratio of mass to volume) CTAB, 1% (ratio of mass to volume) sarkosyl, 20 mmol/dm^3 EDTA, 1.4 mol/dm^3 NaCl, 0.1 mol/dm^3 Tris-HCl pH 8.0, volume fraction 1% 2-mercaptoethanol (Sangon)] was added and the mixture was incubated at 55 ℃ for 1 h with a gentle inversion every 10 min. The resulting suspension was allowed to cool at 4 ℃ for 3～5 min. A total of 2 mL of chloroform/isoamyl alcohol (volume ratio is 24∶1) (Sangon) was added

and mixed by gentle inversion (about 25～30 times) until an emulsion was formed. After centrifugation (10 000 r/min for 5 min at 4 ℃), the supernatant was extracted with chloroform/isoamyl alcohol (volume ratio is 24∶1) one more time. The DNA was ethanol precipitated at -20 ℃ for 2 h followed by a centrifugation 10 000 r/min for 10 min at 4 ℃. The precipitate was briefly rinsed twice with ice-cold 70% ethanol, dried and rehydrated at room temperature in 50 μL TE buffer and stored at -20 ℃.

2.3 18S rDNA cloning

The isolated *T. rotula* genome DNA was used as template for the first PCR amplification with 18S rDNA universal primers (forward, 5′-gct cgn mwy war grt taa gcc atg c-3′; reverse, 5′-cct tgg tcc gtg ttt caa ga-3′). The amplified fragment was purified and used as template for the second PCR (forward, 5′-cct ttg tac aca ccg ccc-3′; reverse, 5′-cac ggt act tgt wyr cta tcg gt-3′). The amplified fragment was subcloned into the pMD18-T vector (TaKaRa) then sequenced.

2.4 RFQ-PCR primers

The *T. rotula* 18S rDNA sequence and the homologue retrieved from GenBank were aligned by ClustalW method using the program MegAlign (DNA Star 5.0), and *T. rotula*-specific Primer and TaqMan probe were designed by the software Primer Premier 5.0, the intended amplified fragment was 83 bp. The specificity of primers was tested by amplification of genome DNA from various microalgae. The 50 μL PCR reaction mixture includes 0.2 μmol/dm^3 primer, 1 ×*Taq* polymerase buffer, 1.5 mmol/dm^3 $MgCl_2$, 200 μmol/dm^3 dNTP; and 0.5U *Taq* DNA polymerase. Thermal program was carried out as follows: 3 min at 94 ℃, 30 cycles at 94 ℃ for 30 s, 59 ℃ for 30 s, and 72 ℃ for 30 s, and a final extension at 72 ℃ for 5 min.

2.5 Development of RFQ-PCR method for *T. rotula* detection

Taqman probe was synthesized and labeled with FAM at the 5′ terminal and TAMRA at the 3′ terminal. Real-time PCR was performed on a Bioer Linegene FQD-33A (Bioer, Japan). The 50 μL real-time PCR reaction mixture includes 0.4 μmol/dm^3 of each *T. rotula* forward and reverse primer, TaqMan probe *T. rotula* concentration of 0.16 μmol/dm^3, 1 ×real-time PCR buffer, 4.0 mmol/dm^3 $MgCl_2$, 300μmol/dm^3 dNTP; 1 μL template DNA and 0.5U *Taq* HS polymerase. Amplification was conducted by using the following program: 5 min at 95 ℃, followed by 40 cycles of 15 s at 94 ℃, 50 s at 60 ℃.

DNA extracted from *T. rotula* was diluted into a 10-fold serial, 1 μL of each diluted sample (total six diluted samples) was used in the real-time PCR assay with triplet. Calibration curve was constructed with the cell number against the cycle threshold value.

3 Results

3.1 18S rDNA sequence and primer design

The amplified fragment of 1 887 bp was confirmed to share 99% identity with the *T. rotula* sequence in GenBank (AF374480).

3.2 The specificity test of *T. rotula* primers

The primers *T. rotula* (forward, 5′-ttg tgg ctt ggc tcc ttc att-3′; reverse, 5′-ttg tta cga ctt cac ctt cct cta a-3′) and Taqman probe *T. rotula* (5′-ggc ctg acc gcg aga act tgt ccg-3′) were located in the 18s rDNA region of *T. rotula*. The specificity of the primers was tested by amplification of 14 species of microalgae collection in the laboratory. The PCR examination detected an 83 bp amplification signal only

from *T. rotula* genome DNA (Fig. 1, Lanes 3, 14), and all the other 14 algae sepecies got no amplification fragment, so the primer is specific for *T. rotula*.

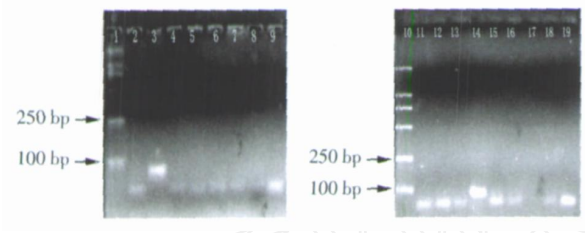

Fig. 1. Test of the specificity of the primer *T. rotula* to *T. rotula*. The primers used are *T. rotula* primer 1 and *T. rotula* primer 2. The DNA templates used are as follows: Lanes 1, 10. 2 000 bp DNA ladder plus (from the top to down, the size of each bank is 2 000, 1 000, 750, 500, 250, 100 bp). Lanes 3, 14. *T. rotula*. Lane 2. *C. curvisetus*. Lane 4. *C. debilis*. Lane 5. *C. minimum*. Lane 6. *C. gracile*. Lane 7. *G. mikimoto*. Lane 8. *G.* sp.. Lane 9. *N. closterium*. Lane 11. *A. tamarens*. Lane 12. *P. pungens*. Lane 13. *S. costatum*. Lane 15. *Melosira* sp.. Lane 16. *H. akashiwo*. Lane 17. *P. minimun*. Lane18. *N. membranacea*. Lane 19. H_2O.

3.3 Development of RFQ - PCR calibration curve for testing *T. rotula*

DNA extracted from different concentrations of *T. rotula* was tested in FRQ - PCR with *T. rotula* primers and *T. rotula Taqman* probe, each dilution was tested in triplicates and the corresponding cylce threshold values were measured (Fig. 2). Further, a regression curve was delineated according to the development of the fluorescent densities in the RFQ - PCR with the increasing number of *T. rotula* cells (Fig. 3). The regression equation was $y = -3.485\,7x + 40.174$, in which x indicates the logarithm of cell number, and y indicates the average values of cycle threshold, with $r = 0.998$.

3.4 Validation of RFQ - PCR method

Samples of *T. rotula* were counted by light microscope (LM) and spiked into 1 mL solution to form four different cell densities. DNA extraction was tested by RFQ - PCR, and the cell number was calculated with the regression equation of calibration curve (Table 2). The data showed that two methods were comparable in all four samples. In fact, analysis of variance showed that, with a P value of 0.05, there was no significant difference between the means of counting by the LM and the RFQ - PCR method for each sample (see Table 3), thus the RFQ - PCR method for *T. rotula* was reliable.

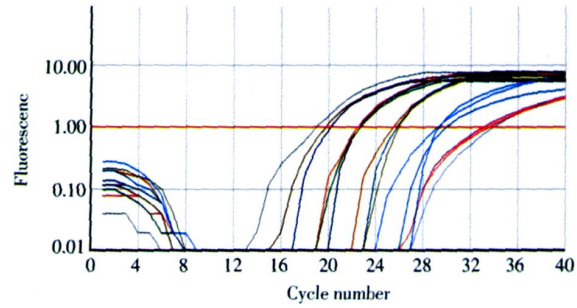

Fig. 2. The RFQ - PCR of diluted DNA solutions. Each dilution is tested for three times.

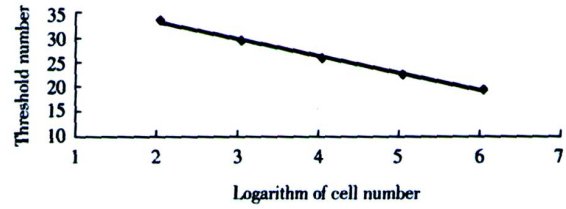

Fig. 3. The calibration curve for *T. rotula* by RFQ - PCR. The corresponding *T. rotula* cell number (cell number per microlitre DNA) of abscissa (5 dilutions) is 1.1×10^6, 1.1×10^5, 1.1×10^4, 1.1×10^3, 1.1×10^2. The relative standard deviation of each data point is 0.6%～2.1%.

Table 2. The comparison of data by LM and RFQ - PCR

Sample	Cell number by RFQ - PCR method	Cell number counted by LM
1	$(1.55 \pm 0.20) \times 10^6$	$(1.97 \pm 0.11) \times 10^6$
2	$(3.77 \pm 0.78) \times 10^5$	$(3.13 \pm 0.20) \times 10^5$
3	$(1.59 \pm 0.08) \times 10^5$	$(1.42 \pm 0.04) \times 10^5$
4	$(4.94 \pm 0.85) \times 10^3$	$(3.07 \pm 0.38) \times 10^3$

4 Discussion

Marine phytoplankton is very important in marine ecosystem, and the ecological dynamics of the marine phytoplankton has been widely researched (Chen et al., 2005; Huang et al., 2006; Sun et al., 2000). However, the traditional identification methods such as LM and scanning election microscopy (SEM) based on morphology have obvious disadvantages, such as time consuming, laborious and furthermore, they are subjective and erroneous in some cases. Recently, analyzing by consensus DNA sequence and developing rapid and quantitative monitoring methods have been the focus in the field of environmental microbe research. The rDNA genes provide the candidate sequences to develop various molecular assays for their highly conserved characteristics (Medina et al., 2001; Medlin et al., 1988). Different rDNA blocks have been selected as targets of PCR amplification. These regions include the small subunit, the large subunit, the 5.8S region and two internal transcribed spacers (ITS1 and ITS2). The choice of a particular region as the target was based mostly on the level of variability of each region within a particular species of interest and the requirement of assay specificity and sensitivity (Connell, 2002; Giacobbe et al., 2000; Marin et al., 2001). In addition, the copy number of ribosome gene in chromosome is stationary, so the number of DNA gene in chromosome is linearly correlative with the cell number of samples. Naturally, it is valuable to delimitate the species much similar in morphology or vary largely in shape during the lifecycle. A molecular method has become an indispensable supplement to the traditional morphological classification (Bowers et al., 2000).

RFQ - PCR includes relatively and absolutely quantitative analysis. The relatively quantitative method is usually used in analyzing the gene expression, while the absolutely quantitative method could be used to quantify the individual amount of organisms. When the absolutely quantitative method was used, a standard is necessary, that is, we should compare the RFQ - PCR method with the traditional quantitative method, so that the reliability of the RFQ - PCR method can be ensured; furthermore, a standard curve for quantitative detection can be established. In this study, the sequence of 18S rDNA of *T. rotula* was used as target region, and a RFQ - PCR method for rapid detection of *T. rotula* was developed. The linearity of the standard curve is 0.998, so the standard curve can be used in the quantitative analysis. Besides, when the absolutely quantitative analysis was carried out, the reliability and the quality of the nucleic acid extraction was very important, while it was difficult to directly analyze the extraction reliability and the nucleic acid quality. In this study, we set up several samples, and the cell number in samples was repeatedly calculated through using both LM counting method and RFQ - PCR method. The results showed that there was no significant difference between the two methods [see Table 2: analysis of variance for RFQ - PCR and LM: $t = 1.15 \sim 4.13$, the critical is 4.30 ($P = 0.05$, $n = 3$)], so the RFQ - PCR method for *T. rotula* indirectly proved reliable.

Besides the specificity of the designed PQR - PCR primers, the probe sequence was blasted with the Genbank sequences, the result showed that the most similar sequence with it (not include the species blonging to the same genus as *T. rotula*) was *Leptocylindrus danicus* (CCMP469), and their similarity was only 70%, so the probe could satisfy distinguishing the *T. rotula* from the other algae, at least the *Thalassiosira* genus from the other algae. Here we did not study the specificity for the species which belonged to the same genus, and this problem should be noticed in the further study.

When *T. rotula* forms a red tide, the normal

cell density of *T. rotula* is 10^4～10^6 cm^{-3}. This RFQ - PCR method was able to detect 10^2～10^6 cells equivalent of algal lysate (template copy number being greater than or equal to 10^2), it is available for detecting *T. rotula* species. For the samples of low density (< 100 cm^{-3}), improvements should be made to increase assay sensitivity: such as amending the means of DNA extraction, and reducing the loss of DNA during extraction, which includes the reduction of extraction process, and the increase of the level of cell splitting. Previous research found that the RFQ - PCR results with DNA template extracted according to different extraction protocols were largely different (Foulds et al., 2001), the PCR inhibition factors should be removed as far as possible; The PCR reaction system should be optimized, typically by modifying the sequences of primers and Taqman probe, and the thermal cycles program.

Quantitatively and qualitatively, the RFQ - PCR method can rapidly detect the *T. rotula* at the molecular level. The whole process from sample collection to target cell data acquirement could be finished within 4 h, which makes it possible to make high output treatment. The new RFQ - PCR detection method will prove to be a simple and useful approach for the long-term monitoring and investigation on the red tide alga along the sea coast.

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