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SHORT COMMUNICATION

Development of a rapid assay to detect the jellyfish *Cyanea nozakii* using a loop-mediated isothermal amplification method

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Abstract

Blooms of the harmful jellyfish *Cyanea nozakii*, which are a severe nuisance to fisheries and tourisms, frequently occur in the northern East China Sea, Yellow Sea, and Bohai Sea. To provide early warning of this species, a simple and effective molecular method for identifying *C. nozakii* was developed using the loop-mediated isothermal amplification method (LAMP). The LAMP assay is highly specific and uses a set of four primers that target six different regions on the mitochondrial cytochrome c oxidase subunit I (*COI*) gene of *C. nozakii*. The amplification conditions, including the dNTP and betaine concentrations, the inner primer to outer primer concentration ratio, reaction time and temperature, were optimized. The LAMP assay amplified DNA extracted from tissue samples of *C. nozakii* but did not amplify DNA from other common scyphozoans and hydrozoans collected in the same region. In addition, the LAMP assay was more sensitive than conventional PCR. Therefore, the established LAMP assay is a sensitive, specific, fast, and easily performed method for detection of *C. nozakii* at different stages in their life cycle.

Keywords

Cyanea nozakii, cytochrome c oxidase subunit I, jellyfish blooms, LAMP, mitochondrial DNA

History

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Introduction

The harmful jellyfish *Cyanea nozakii* is distributed widely in the northern East China Sea, Yellow Sea, and Bohai Sea (Hong & Lin, 2010). Since the late 1990s, blooms of *C. nozakii* frequently occurred in the fishing grounds (e.g. Lvsi, Dasha, Yangtze Estuary, and Liaodong Bay) (Chen et al., 2007; Dong et al., 2010; Ge & He, 2004; Xian et al., 2005). These has caused serious damages to local fisheries, including splitting and ruining fishing nets, preying on and killing juvenile fish, crabs and mollusks, and producing toxins that are dangerous to people and marine animals (Feng et al., 2010; Zhong et al., 2003; Zhou & Huang, 1956). For example, in the summer of 2004, the bloom of *C. nozakii* in Liaodong Bay in the Bohai Sea caused a nearly 80% decline in the edible jellyfish *Rhopilema esculentum* and economic losses of approximately US $70 million (Ge & He, 2004).

The life cycle of *C. nozakii* is highly complex and diverse, with combinations of planulae, benthic polyps, ephyra, and pelagic medusa (Dong et al., 2006; Lucas, 2012). Compared with pelagic medusa (Dong et al., 2005; Lu et al., 2003; Wang et al., 2014; Zhong et al., 2003; Zhou & Huang, 1956), limited work has considered the larval and juvenile stages of *C. nozakii* due to their small and cryptic nature (Dong et al., 2006). Currently, adult *Cyanea* species are usually identified through traditional morphological identification, which is time consuming and requires specialized taxonomic knowledge and experience (Dong et al., 2005). However, it is difficult to identify *C. nozakii* in their larval and juvenile stages. For example, the planulae of *C. nozakii* vary from slipper-shaped to irregularly oval with 90–180 μm in length and 60–95 μm in width, and the benthic scyphistomae vary in size and shape (Dong et al., 2006). Molecular data can provide useful information for species identification of *C. nozakii* at different stages of their life cycle, because the genome remains the same at different life stages (Miranda et al., 2010).

As an alternative to morphological identification, molecular techniques have been used to identify the medusozoa (Bayaha & Graham, 2009; Bucklin et al., 2011; Daryanabard & Dawson, 2008; Ki et al., 2008, 2010; Laakmann & Holst, 2014; Ortman et al. 2010). In these studies, mitochondrial cytochrome c oxidase subunit I (*COI*) gene was widely used as the DNA barcode for marine Metazoa and Medusozoa (Bucklin et al., 2011; Daryanabard & Dawson, 2008; Ki et al., 2008; Laakmann & Holst, 2014; Ortman et al. 2010). Various detection methods using genetic markers have been developed to detect jellyfish, including DNA sequencing (Daryanabard & Dawson, 2008; Ki et al., 2008; Laakmann & Holst, 2014), real-time PCR (Bayaha & Graham, 2009) and DNA microarray (Ki et al., 2010). These molecular techniques are highly sensitive, specific, and efficient for detecting harmful jellyfish at different stages of their life cycle. However, these techniques also require specialized and expensive instruments, which may preclude practical application of these methods. Therefore, a simple and effective method for identifying and detecting the harmful jellyfish *C. nozakii* needs to be developed.

Recently, a novel Loop-mediated isothermal amplification (LAMP) diagnostic method was developed and performed by the Bst DNA polymerase under isothermal conditions (Notomi et al., 2000). The LAMP assay presents some advantages over traditional PCR-based methods. For example, the LAMP reaction does not require expensive laboratory equipment such as a

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thermal cycler; the result is obtained within 1 h or less; the result shows specificity because 4–6 primers are required that hybridize against 6–8 distinct sequences in the target DNA; and the amplified products are visualized simply through an increase in turbidity or change in color detected through the use of an intercalating fluorescent dye such as SYBR Green I (Mori et al., 2001; Notomi et al., 2000). LAMP-based assays have been used for rapid and sensitive detection of microorganisms and harmful microalgae (Chen & Cui, 2009; Chen et al., 2013; Nagai et al., 2012; Picon-Camacho et al., 2013; Woźniakowski et al., 2012; Zhang et al., 2013, 2014). The aim of the present study was to develop a sensitive, specific, and cost-effective LAMP-based approach for the detection of the harmful jellyfish *C. nozakii*.

Materials and methods

Sample collection

A total of 22 *C. nozakii* specimens used in this study were collected in the Jiaozhou Bay during a local jellyfish blooming time in 2013. To test the specificity of the method, 15 species of scyphozoan and hydrozoan jellyfish that are commonly distributed in the Yellow and Bohai Seas were also collected, including *Nemopilema nomurai*, *Rhopilema esculentum*, *Aurelia aureta*, *Sarsia tubulosa*, *Clytia gracilis*, *C. hemisphaerica*, *C. xiamenensis*, *Corymorpha bigelowi*, *Eirene ceylonensis*, *Malagazzia taeniogonia*, *Parisotoma notabilis*, *Sugiura chengshanense*, *Turritopsis nutricula*, *Obelia* sp., and *Rathkea octopunctata* (Tables 1 and 4). Medusae tissue extracted from the bell margin or oral arms was preserved in 95% ethanol for DNA extraction.

DNA extraction, PCR, and sequencing

The total genomic DNA was extracted using a TIANamp Marine Animals DNA Kit (Tiangen, Beijing, China). Approximately 50 mg tissues of each individual were weighed and used for DNA extraction. The concentration of the extracted genomic DNA was determined and adjusted with a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). DNA was dissolved in 100 μL of TE buffer and stored at −20 °C until use.

Table 1. Information sources on the mitochondrial cytochrome c oxidase subunit I (*COI*) gene of *Cyanea* species used for LAMP primer design for *Cyanea nozakii*.

No.	Species	Isolation source	Accession number
1	*Cyanea nozakii*	Jiaozhou Bay, China	This study
2	*C. purpurea*	Yangtze River Estuary, China	JQ353737
3	*C. capillata*	Baltic Sea	JX995346
4	*C. rosea*	Merimbula Lake, Australia	AY902922
5	*C. lamarckii*	North Sea	JX995362
6	*C.annaskala*	Port Phillip Bay, Australia	AY902923

The mitochondrial COI fragments from *C. nozakii* were amplified using the universal primers LCO1490 and HCO2198 under previously described PCR conditions (Folmer et al., 1994). The PCR reactions were carried out in a volume of 50 μL that consisted of 50–100 ng genomic DNA, 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.25 mM primers, and 2.5 U Taq DNA polymerase (Tiangen, Beijing, China). The temperature profile was defined as follows: 94 °C for 3 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at 54.5 °C for 30 s, and extension at 72 °C for 60 s; followed by a final extension at 72 °C for 5 min. The PCR products were analyzed by 1% agarose gel electrophoresis according to the standard method. PCR-amplified DNA fragments were purified and sequenced with an ABI 3730 automatic DNA sequencer at Sangon Biotech Co., Ltd (Shanghai, China) using the same primers described above. All PCR products were sequenced in both directions to obtain accurate sequences.

Design and synthesis of the primers for LAMP

Three sets of LAMP primers targeting the mitochondrial cytochrome c oxidase subunit I gene (COI) of *C. nozakii* were designed based on the gene sequences obtained from this study (Table 1) using the program PrimerExplorer V4 (http://primerexplorer.jp/elamp4.0.0/index.html). Then, a BLAST search was used to confirm the specificity of the designed primers against a broad range of organisms. CN-FIP and CN-BIP were inner primers, and CN-F3 and CN-B3 were out primers (Table 2). Primers were synthesized by Sangon Biotech Co., Ltd (Shanghai, China).

Optimization of LAMP conditions

The LAMP reaction was conducted following the method described in Notomi et al. (2000). Various parameters, including the concentrations of dNTP and betaine, the concentration ratio of inner primer to outer primer, the reaction time, and the temperature, were tested to optimize the LAMP reactions. LAMP was performed using 0–0.8 mM dNTPs and 0.2–1.4 M betaine, and the ratios of inner primer concentration to outer primer concentration ranged from 1:1 to 16:1; the other conditions were unchanged. The reaction temperature was tested at 57, 59, 61, 63, and 65 °C at a predetermined time of 60 min followed by 80 °C for 5 min for the termination. To determine the optimum incubation time for the LAMP assay, the mixture was tested at 62 °C for 10, 20, 30, 40, 50, 60, 70, and 80 min. The optimized LAMP conditions are summarized in Table 3 and were used in subsequent studies. The LAMP reactions were carried out in a volume of 25 μL that consisted of 1.6 μM each of inner primer, 0.2 μM each of outer primer, 0.6 M betaine, 0.4 mM dNTPs, 1 × thermopol buffer (20 mM Tris-HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 6 mM $MgSO_4$, 0.1% Triton X-100), 8 U Bst DNA polymerase large fragment (New England Biolabs Inc., Ipswich, MA), and specified amounts of DNA. The mixture was incubated at 63 °C for 30 min and then heated at 80 °C for 10 min to terminate the reaction.

Table 2. Primer sequences for LAMP detection in *Cyanea nozakii*. Mitochondrial cytochrome c oxidase subunit I (COI) gene was used for the LAMP detection.

Primer name	Primer sequence	Length
CN-F3	ATGCCTGTTCTTATAGGAGG	20
CN-B3	CCATGTCTACAGATCCTCC	19
CN-FIP	TGTTAAGTCTGGGGAAAGCCATATTTGGTAACTGACTTATACCTTTG	47
CN-BIP	ATCAGCTTTAATAGAACAAGGTGCATGAATAGATGCTAAAGTGGGATA	48

The LAMP products were analyzed by 1% agarose gel electrophoresis and photographed with a Gel Doc™ XR + imaging system transilluminator (Bio-Rad, Hercules, CA). In addition, the result could be directly observed through the white magnesium pyrophosphate precipitate or the green color generated by adding 1% SYBR Green I.

Sensitivity of the LAMP assay

To determine the sensitivity of the LAMP assay, 10-fold serial dilutions (from 100 ng μL^{-1} to 10^{-5} ng μL^{-1}) of DNA extracted from *C. nozakii* were used as templates for LAMP under the optimized conditions. In addition, a COI PCR test was performed as described in DNA extraction, PCR, and sequencing section. Products obtained were analyzed by 1% agarose gel electrophoresis and visualized with a Gel Doc™ XR + imaging system transilluminator (Bio-Rad, Hercules, CA).

Table 3. Optimized LAMP conditions, including reaction time and temperature, the dNTP and betaine concentrations, and the inner primer to outer primer concentration ratio.

Reaction time	Reaction temperature	dNTP concentrations	Betaine concentrations	Ratio of inner primer to outer primer
30 min	63 °C	0.4 mM	0.6 M	8:1

Species-specific detection by LAMP

To confirm the species-specific detection of *C. nozakii* by the LAMP method, amplification of the target and non-target species was conducted (Table 4). In the case of *C. nozakii*, 22 individuals of *C. nozakii* were tested for amplification of the target species, and 15 species of non-target species were also tested for amplification. DNA concentrations of all tested samples were adjusted to 10–50 ng μL^{-1}.

Results and discussion

Three primer sets specific to mt COI were created using the automatic search function of Primer Explorer V4. Then, the best primer sets were selected through further BLAST search against a wide range of organisms. Finally, one specific primer set for the target DNA was selected for primer screening using LAMP (Table 2). The LAMP assay was carried out using *C. nozakii* DNA to establish the optimal conditions. The results of all the tests are shown in Figure 1. The optimized LAMP conditions are summarized in Table 3. The results indicated that the target gene could not be amplified when the concentration of betaine was higher than 1.2 M, and optimal amplification was achieved at

Table 4. Species-specific detection of *Cyanea nozakii* by the LAMP method.

Species	Sample area	Number	Positive	Negative
Cyanea nozakii	Yellow Sea, Qingdao, China	22	22	0
Nemopilema nomurai	Yellow Sea, Rudong, China	1	0	1
Aurelia aureta	Bohai Sea, Caofeidian, China	1	0	1
Rhopilema esculentum	Bohai Sea, Dongying, China	1	0	1
Clytia gracilis	Bohai Sea, Dongying, China	1	0	1
C. hemisphaerica	Bohai Sea, Dongying, China	1	0	1
C. xiamenensis	Bohai Sea, Dongying, China	1	0	1
Corymorpha bigelowi	Bohai Sea, Dongying, China	1	0	1
Eirene ceylonensis	Bohai Sea, Dongying, China	1	0	1
Malagazzia taeniogonia	Bohai Sea, Dongying, China	1	0	1
Obelia sp.	Yellow Sea, Rizhao, China	1	0	1
Parisotoma notabilis	Bohai Sea, Dongying, China	1	0	1
Rathkea octopunctata	Bohai Sea, Caofeidian, China	1	0	1
Sarsia tubulosa	Bohai Sea, Caofeidian, China	1	0	1
Sugiura chengshanense	Bohai Sea, Dongying, China	1	0	1
Turritopsis nutricula	Bohai Sea, Dongying, China	1	0	1

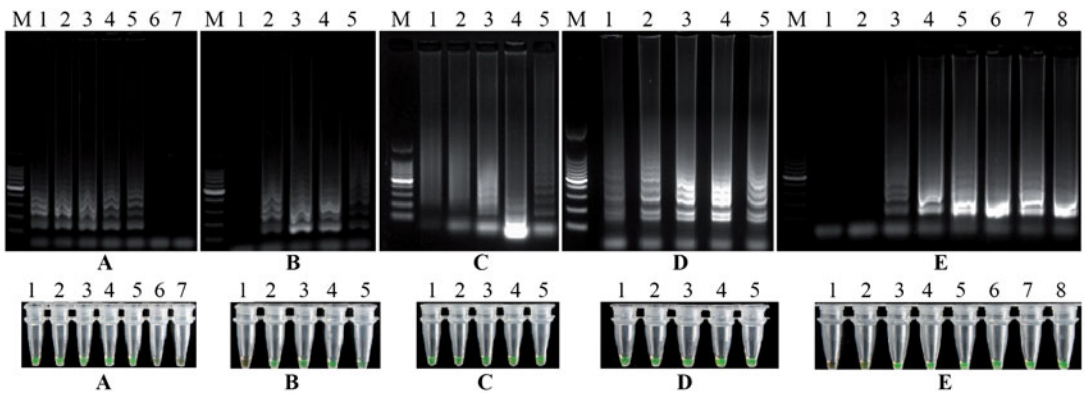

Figure 1. Optimization of LAMP conditions, observed by electrophoresis analysis and visual detection, including the betaine (A) and dNTP (B) concentrations, the inner primer to outer primer concentration ratio (C), temperature (D), and reaction time (E). M: 100 bp DNA ladder. (A) L1–7: the betaine concentration is 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, and 1.4 M; (B) L1–5: the dNTP concentration is 0, 0.2, 0.4, 0.6, and 0.8 M; (C) L1–5: the concentration ratio of inner primer to outer primer is 1:1, 2:1, 4:1, 8:1, and 16:1; (D) L1–5: the reaction temperature is 57, 59, 61, 63, and 65 °C; (E) L1–5: the reaction time is 10, 20, 30, 40, 50, 60, 70, and 80 min.

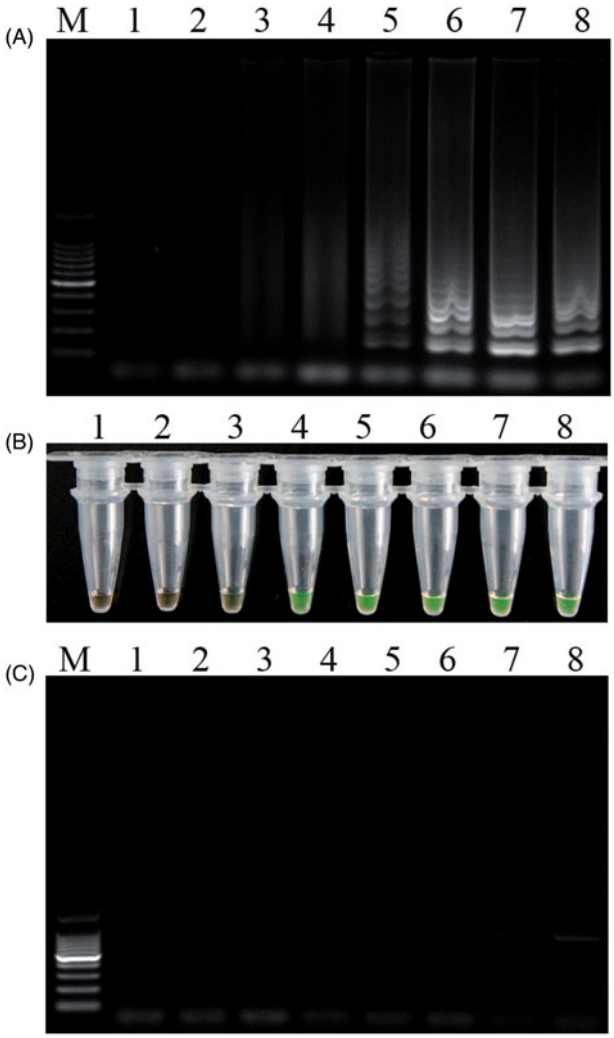

Figure 2. Comparison of the sensitivities of LAMP (A and B) and PCR (C). Lane M:100 bp DNA ladder; lanes 1–8: 10-fold dilutions of DNA corresponding to 10^{-5} ng μL^{-1}, 10^{-4} ng μL^{-1}, 10^{-3} ng μL^{-1}, 10^{-2} ng μL^{-1}, 10^{-1} ng μL^{-1}, 1 ng μL^{-1}, 10 ng μL^{-1}, and 100 ng μL^{-1}.

0.6 M (Figure 1A). Previous study suggested that betaine improved the DNA amplification by reducing the formation of secondary structure caused by GC-rich regions (Henke et al., 1997). However, the mt COI of *C. nozakii* in our study have a low GC content (34%). Lower betaine concentration may result in elevated LAMP amplification efficiency when amplifying non-GC-rich target sequences (Chen et al., 2011). The band appeared to be more clear with the addition of 0.4 mM dNTP than with other concentrations (Figure 1B). A more distinct pattern was apparent when the primer ratio was 1:8 (Figure 1C). As shown in Figure 1(D), there was amplification of the LAMP product between 57 °C and 65 °C, and the clearer bands were observed at 63 °C, suggesting that as the optimal temperature for the assay. No amplification was observed after incubation for 10 and 20 min (Figure 1E). The first LAMP products appeared after 30 min, suggesting that the minimum reaction time for the LAMP assay to detect *C. nozakii* DNA was 30 min. Based on the above results, the optimal conditions for the LAMP assay of *C. nozakii* were determined to be 63 °C for 30 min with 0.6 M betaine, 0.4 mM dNTPs, 0.2 mM each of outer primer, and 1.6 mM each of inner primer.

The detection limit for DNA extracts from *C. nozakii* in the LAMP reaction was tested. The present results showed that 0.1 ng of DNA extracted from *C. nozakii* was observed by agarose gel electrophoresis for LAMP (Figure 2A). Comparatively, a positive result was only observed when the DNA extract was 10 ng for PCR (Figure 2B). Therefore, the LAMP assay displayed a higher amplification efficiency, and LAMP was 100-fold more sensitive than PCR. The presented results are consistent with previous studies (Chen et al., 2013; Notomi et al., 2000; Picon-Camacho et al., 2013; Zhang et al., 2013). For example, Notomi et al. (2000) first demonstrated that the sensitivity of LAMP is much higher than conventional PCR. Chen et al. (2013) showed that the LAMP-based assay for detecting *Prorocentrum donghaiense* was more sensitive than PCR. Picon-Camacho et al. (2013) also confirmed that the LAMP assay for *Amyloodinium ocellatum* was exceptionally higher than conventional PCR. Zhang et al. (2013) found that the sensitivity of the LAMP method for detecting Prorocentrum was a minimum of 10 times greater than for traditional PCR.

Primer set design is critical for the success of LAMP assays in detecting *C. nozakii* because samples may consist of a wide range of jellyfish species (e.g. hydrozoa and Scyphozoa). The

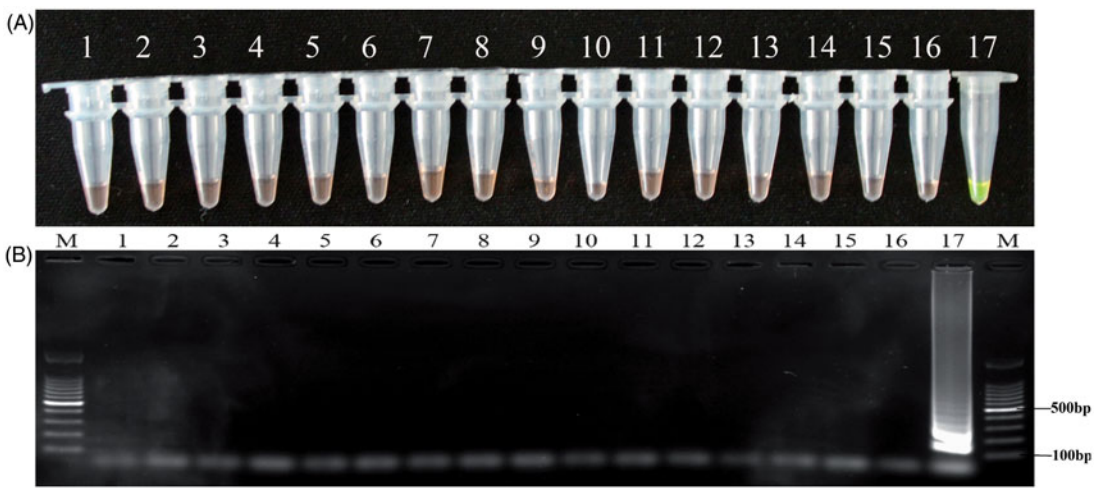

Figure 3. Specific detection of *Cyanea nozakii* by the LAMP method. (A) Amplification of LAMP products dyed with SYBR Green I was visually detected by examining color changes with the naked eye: green indicates a positive result and orange indicates a negative result. (B) Agarose gel electrophoresis. Lane M: 100 bp DNA ladder; lane 1: *Clytia gracilis*; L2, *Clytia hemisphaerica*; L3, *Clytia xiamenensis*; L4, *Corymorpha bigelowi*; L5, *Eirene ceylonensis*; L6, *Malagazzia taeniogonia*; L7, *Parisotoma notabilis*; L8, *Sugiura chengshanense*; L9, *Turritopsis nutricula*; L10, *Obelia* sp.; L11, *Rathkea octopunctata*; L12, *Sarsia tubulosa*; L13, *Aurelia aureta*; L14, *Nemopilema nomurai*; L15, *Rhopilema esculentum*; L16, negative control; L17, *Cyanea nozakii*.

specificity of primers were further determined by experimental analysis. All 22 individuals of *C. nozakii* produced positive results, while the other non-target scyphozoan and hydrozoan species produced negative results, indicating that the LAMP assay was highly specific to *C. nozakii* (Figure 3). The LAMP-based assay was highly specific for the target sequence because six independent sequences recognize the target sequence in the initial stage, and four independent sequences amplify the target sequence in the later stage of the reaction (Mori et al., 2001; Nagamine et al., 2002; Notomi et al., 2000).

In summary, the LAMP method described here is a sensitive, specific, fast, and easily performed method for the detection of the harmful jellyfish *C. nozakii*. First, the LAMP assay was performed with only a water bath or heat block for incubation under isothermal conditions. Second, the LAMP reaction can be completed in 30 min, which is suitable for analysis of a large number of samples. Third, the LAMP assay was more sensitive and specific than traditional PCR. Fourth, the amplified products are visualized simply through an increase in turbidity (Mori et al., 2001; Notomi et al., 2000) or change in color through the use of an intercalating fluorescent dye such as SYBR Green I or Calcein (Mori et al., 2001). Since the late 1990s, harmful *C. nozakii* jellyfish blooms have been frequently observed (reviewed in Dong et al., 2010). The monitoring of *C. nozakii*, especially at the larval and juvenile stages, may provide early warning of this species to avoid loss for fisheries and tourism. Therefore, the LAMP assay developed in the present study will be valuable in the detection of *C. nozakii* at different life cycle stages.

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Declaration of interest

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