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Development of a PCR method for detection of *Pseudoalteromonas marina* associated with green spot disease in *Pyropia yezoensis**

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Abstract *Pseudoalteromonas marina* is one of the potential pathogens that cause green spot disease (GSD) in *Pyropia yezoensis*. To prevent GSD from development and spread, an effective method to detect the pathogen at early GSD infection stages need to be established. In this research, PCR methods were established targeting the *dnaA* gene (encoding chromosome replication initiator protein) and the *dnaN* gene (encoding *β* sliding clamp of DNA polymerase III protein) to detect *P. marina* with three primer pairs pws-*dnaA*2 (Forward, 5′-ACCGCATTAACGAACTACTCGTG-3′; Reverse, 5′-TGCCATTACCTACAGCATGG-3′), pcs-*dnaN*2 (Forward, 5′-CTTACAACGTTATCAGCGGC-3′; Reverse, 5′-GTTGAGTATTAAGTGATTGAGTAAGC-3′) or pws-*dnaN*3 (Forward, 5′-ACTTACAA-CGTTATCAGCGGC-3′; Reverse, 5′-ACTGCTGTTTGAGTCTGCTAAC-3′). Three PCR methods corresponding to the three primer pairs sufficiently distinguished *P. marina* from 22 bacterial species, thus resulting in detection limits of 4 to 4×10^2 CFU cells or 2.37×10^1 to 2.37×10^3 fg of *P. marina* DNA per PCR reaction. In an artificial infection experiment of *P. yezoensis* infected with *P. marina*, all established PCRs successfully detected *P. marina* at early GSD infection stages. The results show that the established PCRs are specific and sensitive, and are potential for applications in early diagnosis of GSD in *Pyropia*.

Keyword: *Pyropia yezoensis*; green spot disease (GSD); *Pseudoalteromonas marina*; PCR detection; early diagnosis

1 INTRODUCTION

Pyropia (Bangiales, Rhodophyta) is a popular food consumed worldwide because it is rich in vitamins, minerals, antioxidants and dietary fiber (Cao et al., 2016). There are approximately 134 species in the *Pyropia* genus distributed throughout the world, some of which have been extensively cultured in Asia, including *Pyropia yezoensis*, *P. haitanensis*, *P. tenera*, *P. dentata* and *P. seriata*. Among these species, *P. yezoensis* has the highest value, and the annual gross production is worth more than US $800 million in China (Lu et al., 2018). Over the past decade, cultivation of *Pyropia* has been hampered by several diseases, thus leading to an annual loss of approximate 10% of the production in Japan and Korea and 25%–

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30% of that in China (Gachon et al., 2010). In the year of 2005, 2006, and 2008, production of *P. haitanensis* in Fujian Province, South China suffered from a loss of 26.7%, 20%, and 28% respectively, due to the disease, and even 80% in some areas (Lai, 2009; Liu et al., 2012). Inappropriate culture conditions, such as high culture density, inadequate water exchange, and lack of air-drying, may cause severe physiological diseases of *Pyropia* (Ding, 2008). Infectious diseases caused by microorganisms cannot be ignored. *Pyropia* can be colonized by diverse microorganisms including oomycetes, fungi, bacteria, viruses and protists, which cause various diseases such as red rot disease (Arasaki, 1947; Fujita and Zenitani, 1977; Kerwin et al., 1992; Ma, 1996; Ding and Ma, 2005; Park et al., 2006; Lee et al., 2017), *Olpidiopsis* disease (Sekimoto et al., 2009; Klochkova et al., 2012; Kwak et al., 2017), green spot disease (GSD) (Fujita, 1990; Sunairi et al., 1995; Kim et al., 2016), cyanobacterial felt disease and diatom felt disease (Lee et al., 2012; Kim et al., 2014). Red rot disease and *Olpidiopsis* disease, caused by two oomycete pathogens, *Pythium* sp. and *Olpidiopsis* sp., are the most common diseases in *Pyropia* cultivation, and they greatly decrease the production of *Pyropia*. However, in some *Pyropia* cultivation seasons, GSD causes damage almost as severe as that caused by oomycete diseases. For example, during 2012–2013, an outbreak of GSD from Seocheon sea farms in South Korea resulted in losses valued at approximately US $1.1 million, comparable to the *Olpidiopsis* disease loss of US $1.6 million (Kim et al., 2014).

Typical symptoms of GSD are lesions with green borders scattered on infected blades in late disease stages, although different colors of spots have been observed in different disease cases. This disease was first named in Japan in 1968 and was formerly known as perforating disease (Suto and Umebayashi, 1954; Saito et al., 1972). Several bacterial species have been identified to be associated with GSD, such as *Micrococcus* sp., *Vibrio* sp. and *Pseudomonas* sp. These bacteria cause small green or black spots (Suto et al., 1954; Nakao et al., 1972), whereas *Flavobacterium* sp. LAD-1 causes pinholes at the centers of thalli (Sunairi et al., 1995). Beyond the bacterial pathogens, a chloroplast virus has been reported to be a pathogen of GSD, leading to whole blade breakdown within several days (Kim et al., 2016). These results imply to that a specific disease phenotype in the marine environment may be mediated by different pathogens (Kumar et al., 2016).

In China, GSD usually occurs in November to December, in some cases causing whole farm loss within 1 week (Mou, 2012). Two bacterial species, *Pseudoaltermonas* sp. and *Vibrio* sp., have been identified to be potential pathogens causing GSD symptoms *in vitro*, and *Pseudoalteromonas* appears to be the most common agent in GSD cases (Yan et al., 2002; Ding, 2008; Han et al., 2015; Li et al., 2018). Although certain members of *Pseudoalteromonas* have been reported to be opportunistic pathogens of marine animals (Sandaa et al., 2008; Wang et al., 2013), several studies have found that *Pseudoalteromonas* is associated with holle-rotten disease and red spot disease in kelp, *Laminaria japonica* (Sawabe et al., 1998; Gachon et al., 2010). At present, no effective methods are available for the treatment and control of GSD. To minimize losses, cold storage and air-drying of algae are common useful methods to prevent spreading of GSD in early infection stages (Yan et al., 2002; Ding, 2008). However, at early infection stages, GSD disease are always be neglected under field conditions, owing to the symptoms of needle size spots are not easily observable with the naked eye. The commonly found symptoms such as bleaching, spotting or rotting are features of late stages of disease (Egan and Gardiner, 2016). Thus, it is essential to establish an effective method to monitor the pathogen at early infection stages to allow ample time to prevent disease development or spread.

Previously, we isolated and identified a *P. marina* strain causing GSD symptoms in *P. yezoensis* (Li et al., 2018). Here, we established a specific and sensitive PCR method for detecting *P. marina*. This PCR method effectively detected *P. marina* at early infection stages of GSD. To the best of our knowledge, this is the first report of detection of a GSD pathogen.

2 MATERIAL AND METHOD

2.1 Bacteria, *Pyropia* and growth conditions

The bacterial strains used in this study are listed in Table 1. All strains were cultured in Zobell 2216E medium at 20°C. Healthy *P. yezoensis* thalli were incubated in Provasoli's enriched seawater (PES) (Provasoli, 1968). Routine culture conditions for thalli were used, at 15°C, salinity 30 and 62.5 μmol photons/(m^2·s) on a 12 h light:12 h dark light cycle (12L:12D) with continuous aeration, and the seawater was replaced every 3 days.

Table 1 Bacterial strains used in this study

Specie	Strain	Source
Pseudoalteromonas	*P. marina* tbzcY1	This laboratory (Li et al., 2018)
	P. phenolica PHCGY4s-1 (2)	This laboratory
	P. virvidis PHCYZ1-1w-6 (2)	This laboratory
	P. peptidolytica PHCDT1-1w-2 (2)	This laboratory
	P. piscida PHCGY7-1w-1 (2)	This laboratory
	P. luteoviolacea PHCGY8-1w-8 (2)	This laboratory
	P. rubra PHCGY7-1s-11 (2)-1	This laboratory
	P. flavipulchra CDM8	This laboratory
Vibrio	*V. anguillarum* ATCC 43305	ATCC
	V. anguillarum ATCC 43307	ATCC
	V. parahemolyticus YMD1	This laboratory
	V. harveyi YMD2	This laboratory
	V. vulnificus YMD3	This laboratory
	V. fluvialis 1.1608	CGMCC
	V. alginolyticus 1.1607	CGMCC
Others	*Aeromonas salmonicida* LZST-4	This laboratory
	Alteromonas macleodii PHCRD2-102ww-1 (2)	This laboratory
	Edwardsiella tarda ATCC15947	ATCC
	Pseudomonas libanensis PHCYZ1-4w-9 (2)	This laboratory
	Maribactor goseongensis PHCRD2-102ws-12 (2)	This laboratory
	Staphylococcus aureus YPC1	This laboratory
	Escherichia coli YPC2	This laboratory

ATCC: American Type Culture Collection; CGMCC: China General Microbiological Culture Collection Center.

Table 2 Primer sequences used in this study

Gene	Primer name	Sequence (5′→3′)	Amplification length (bp)
dnaN	pws-*dnaN-for*	ACTTACAACGTTATCAGCGGC	721
	pws-*dnaN-rev*3	ACTGCTGTTTGAGTCTGCTAAC	
dnaA	pws-*dnaA-for*	ACCGCATTAACGAACTACTCGTG	386
	pws-*dnaA-rev*2	TGCCATTACCTACAGCATGG	
dnaN	pcs-*dnaN-for*	CTTACAACGTTATCAGCGGC	253
	pcs-*dnaN-rev*2	GTTGAGTATTAAGTGATTGAGTAAGC	

2.2 PCR primers and amplification

Pseudoalteromonas marina tbzcY1, previously isolated and characterized from GSD *Pyropia* (Li et al., 2018), was used in PCR development. Two genes, *dnaA* (GenBank accession No. MH681053) and *dnaN* (GenBank accession No. MH681054), encoding a chromosomal replication initiation protein and *β* sliding clamp of DNA polymerase III in prokaryotes, were used as gene targets for PCR primer design. BioEdit 7.2 (Hall, 2011) was used to identify the hypervariable regions of *dnaA* and *dnaN* through alignment of the nucleotide sequences of *dnaA* and *dnaN* of *Pseudoalteromonas* sequence deposited in NCBI (Figs.S1 and S2). Three primer pairs, pws-*dnaN*3, pws-*dnaA*2 and pcs-*dnaN*2, were designed using Primer v5.0 (Table 2). The genomic DNA of *P. marina* tbzcY1 was extracted with a Bacterial DNA Kit (Omega Biotek, Doraville, Ga., USA) and quantified with a NanoDrop-2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). PCR was carried out in 25 μL mixtures containing 0.5 μmol/L of each primer, 10 ng of genomic DNA and 12.5 μL 2× Hieff™ PCR Master Mix (Yeasen Biotech, Beijing, China). Gradient PCR was performed to determine the optimal annealing

temperature. Aliquots of 5 μL PCR products were evaluated by 1.5% agarose gel electrophoresis. PCR products were sequenced by Shanghai Personal Biotech Company to confirm proper amplification.

2.3 PCR specificity and sensitivity

For determination of PCR specificity, the 22 bacterial strains listed in Table 1 were used in the experiment. DNA templates were prepared by boiling 1×10^5 CFU bacteria at 100°C for 5 min, and 1 μL of supernatant from the boiled cells was added in the PCR reaction system. For determination of PCR sensitivity, overnight cultures of *P. marina* tbzcY1 were serially diluted 10-fold with sterilized seawater to obtain concentrations of 10^3–10^8 CFU/mL. The diluted bacterial suspensions were boiled at 100°C for 5 min, and 1 μL supernatant of boiled cells was used in PCR to determine the cell detection limit. In addition, for determination of the DNA detection limit, pure genomic DNA of *P. marina* tbzcY1 was serially diluted 10-fold with sterilized seawater to achieve a content of 10^0–10^6 fg/μL, and 1 μL DNA solution from each dilution was used in PCR amplification.

2.4 Detection of *P. marina* in early *P. yezoensis* infection

To obtain early infection samples of *Pyropia*, an artificial infection experiment was established under laboratory conditions. Overnight cultures of *P. marina* tbzcY1 were centrifuged at 5 000×*g* at 4°C, and the cell pellets were collected and resuspended in sterilized seawater. *P. yezoensis* thalli, 3–4 cm wide and 10–12 cm long, were immersed in 0.7% potassium iodide solution for 5 min to avoid bacterial contamination, then washed with sterilized seawater three times. After being dried with sterilized gauze, 0.2 g lavers (approximately 20 pieces of thallus) were immersed in 200 mL sterilized PES containing 1×10^7 CFU/mL *P. marina* cells. Lavers without addition of *P. marina* were used as a control group. After infection, all groups were cultured under routine conditions without aeration, and growth and disease symptoms were observed each day under a microscope. When the early GSD symptoms appeared microscopically on the infected thalli with enlarged intercellular gaps, concentrated or pyknotic cytoplasm and cell necrosis, the thalli were collected from the infected group together with the control group. All groups of thalli were rinsed three times with sterilized seawater and dried with sterilized gauze. Thalli collected from a cultivation farm with no occurrence of GSD were included in this experiment as a negative control. Aliquots of 100 mg laver were ground with liquid nitrogen and subjected to DNA extraction with a Plant Genomic DNA Kit (TianGen, Beijing, China). Aliquots of 1 μL of the extracted DNA were used as templates in PCR detection. Every detection reaction was carried out in triplicate.

3 RESULT

3.1 Establishment of PCR detection of *P. marina* tbzcY1

Three pairs of primers, pws-*dnaA*2, pcs-*dnaN*2 and pws-*dnaN*3, were designed for PCR detection of *P. marina* by targeting the hypervariable regions of *dnaA* and *dnaN* of *P. marina* tbzcY1 (Figs.S1 and S2). As shown in Fig.S3, all primer pairs successfully amplified the expected DNA from boiled cells of *P. marina* tbzcY1, generating fragments of 386 bp, 253 bp and 721 bp. The optimal PCR program was found to be 5 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 57°C, and 20 s (for primer pairs pws-*dnaA*2 and pcs-*dnaN*2) or 48 s (for pws-*dnaN*3) at 72°C, and 7 min at 72°C.

3.2 Specificity of PCR detection

A total of 22 strains, including eight *Pseudoalteromonas* sp., seven *Vibrio* sp. and seven other bacterial species, were used to determine the specificity of PCR detection established with the primer pairs pws-*dnaA*2, pcs-*dnaN*2 and pws-*dnaN*3. As shown in Fig.1, the expected DNA bands were amplified from *P. marina* tbzcY1 DNA by all primer pairs but not from DNA from other bacterial strains. These amplicons were confirmed to be the DNA target by DNA sequencing. Thus, this result indicated that the established PCR with the three primer pairs specifically differentiated *P. marina* from other bacterial species.

3.3 Sensitivity of PCR detection

The boiled bacterial DNA at a serial dilution of 10^3–10^8 CFU/mL and the pure DNA at a serial dilution of 10^0–10^6 fg/μL were used as DNA templates to determine the sensitivity of the PCR detection. When the boiled bacterial DNA was used as a template, a detection limit of four CFU per reaction was achieved with primer pair pws-*dnaA*2; the detection limits were 4×10^2 CFU per reaction with primer pairs pcs-*dnaN*2 and pws-*dnaN*3 (Fig.2a). When pure DNA was used

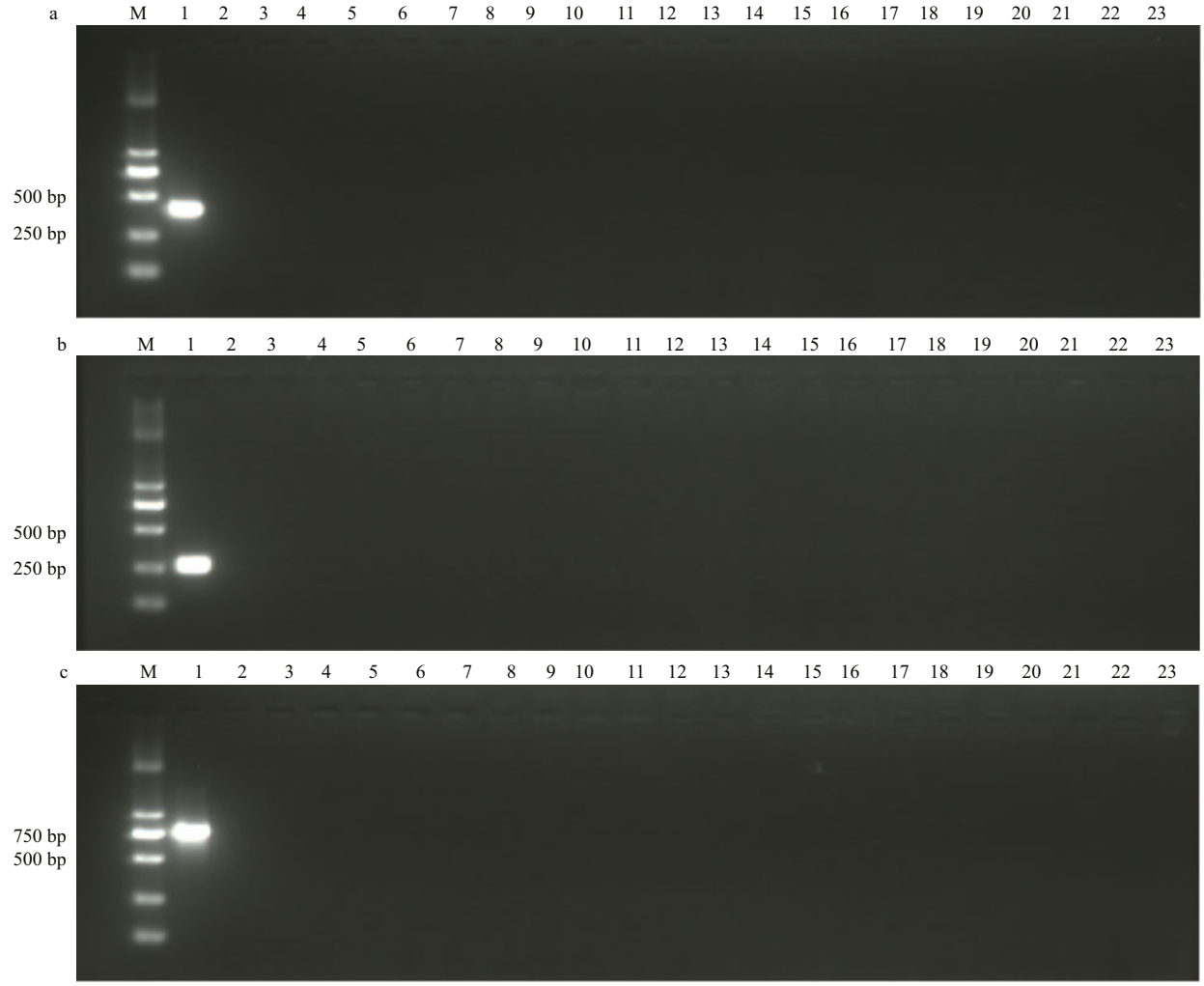

Fig.1 Specificity of PCR detection of *P. marina* tbzcY1 with primers pws-*dnaA*2 (a), pcs-*dnaN*2 (b) and pws-*dnaN*3 (c)

M: DL 2000 marker; 1 to 22: amplification with DNA template of *P. marina*, *P. phenolica*, *P. virvidis*, *P. peptidolytica*, *P. piscida*, *P. luteoviolacea*, *P. rubra*, *P. flavipulchra*, *V. anguillarum* ATCC 43305, *V. anguillarum* 43307, *V. parahemolyticus*, *V. harveyi*, *V. vulnificus*, *V. fluvialis*, *V. alginolyticus*, *Ed. tarda*, *A. salmonicida*, *Ps. libanensis*, *Al. macleodii*, *M. goseongensis*, *S. aureus* and *E. coli*, respectively; 23, negative control without bacterial DNA.

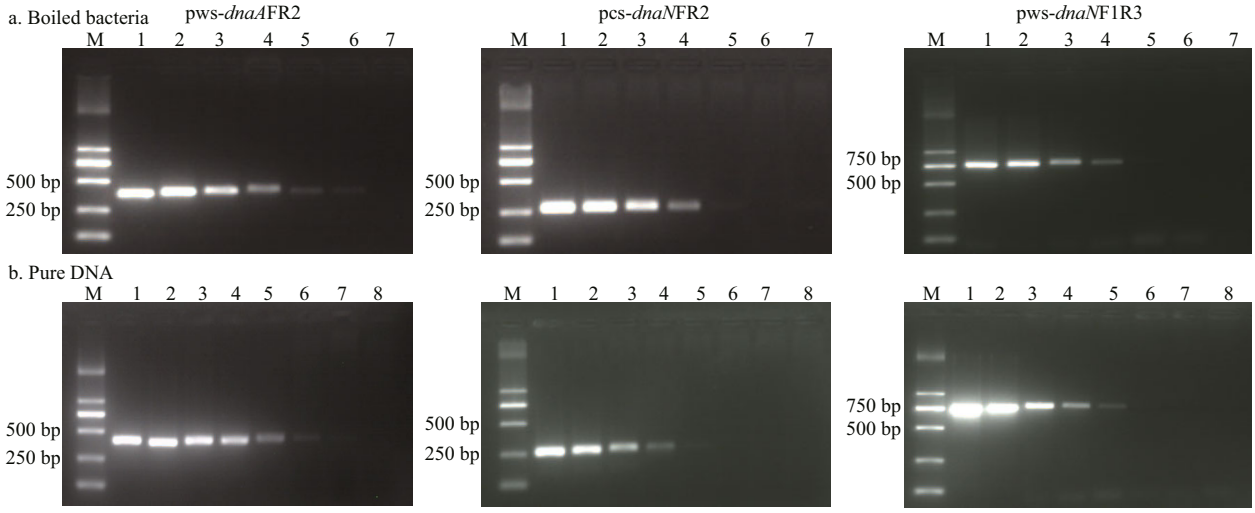

Fig.2 Sensitivity of PCR detection of *P. marina* with boiled bacteria (a) and pure DNA (b) as templates

M: DL 2000 marker. In (a), 1 to 6: amplification with boiled *P. marina* at 4×10^5 CFU, 4×10^4 CFU, 4×10^3 CFU, 4×10^2 CFU, 4×10^1 CFU and 4×10^0 CFU for each reaction; 7: negative control without bacterial cells; In (b), 1 to 7: amplification with pure DNA of *P. marina* at 2.37×10^6 fg, 2.37×10^5 fg, 2.37×10^4 fg, 2.37×10^3 fg, 2.37×10^2 fg, 2.37×10^1 fg and 2.37×10^0 fg for each reaction; 8: negative control without bacterial DNA.

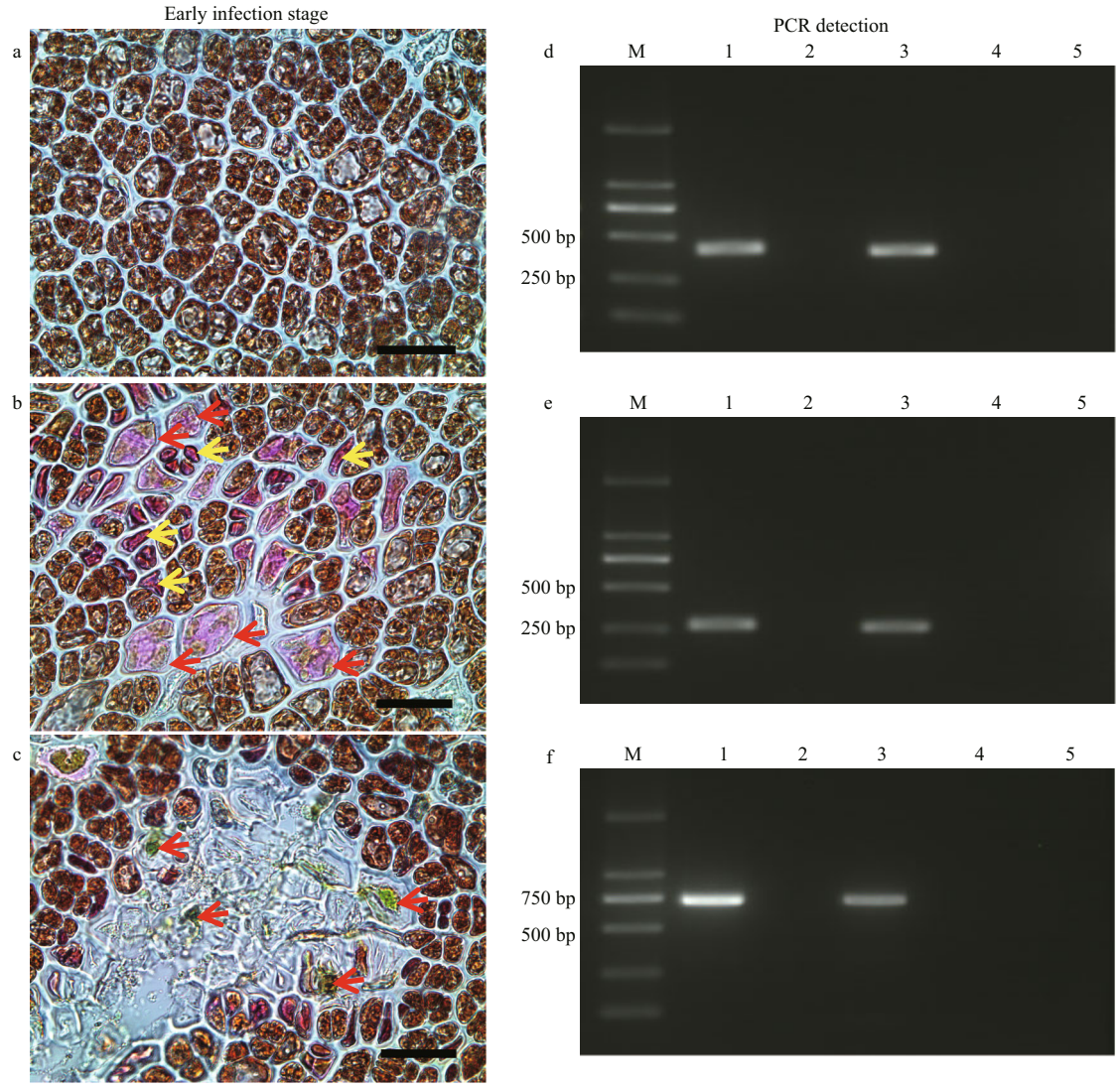

Fig.3 PCR detection to GSD of an early stage

Microscopic GSD symptoms of an early infection stage in *P. yezoensis* infected with *P. marina* tbzcY1. a. thallus from control group; b. infected thallus showing cells with concentrated (yellow arrow) or pinkish cytoplasm (red arrow); c. infected thallus showing necrotic greenish (arrow). Scale bars in a, b & c: 50 μm. PCR detection with primers pws-*dnaA*2 (d), pcs-*dnaN*2 (e), and pws- *dnaN*3 (f). M: DL 2000 marker; 1–4: the extracted DNA from *P. marina* tbzcY1, control laver, infected laver and farmed laver; 5: blank control.

as a template, the detection limits were 2.37×10^{1} fg, 2.37×10^{2} fg, and 2.37×10^{3} fg with primer pair pws-*dnaA*2, pws-*dnaN*3 and pcs-*dnaN*2, respectively (Fig.2b). These results indicated that primer pair pws-*dnaA*2 was most sensitive in PCR detection.

3.4 Detection of *P. marina* tbzcY1 in early stages of *P. yezoensis* infection

After 10 days of infection with *P. marina* tbzcY1, no symptoms were visible on infected thalli by the naked eye, but under a microscope, abnormal cells with concentrated or pinkish cytoplasm (Fig.3b) and a necrotic greenish area (Fig.3c) were observed. As a comparison, the control thalli did not show microscopic symptoms (Fig.3a). When PCRs were performed with the total DNA extracted from the infected and control thalli, specific DNA fragments were detected in the infected group by all three primer-pairs, whereas no DNA fragments were detected in the control group and the healthy farmed lavers (Fig.3d–f). These results indicate that the established PCR was able to detect *P. marina* in early stages of infection of *P. yezoensis*.

4 DISCUSSION

PCR is a highly specific and sensitive method for the identification and detection of various organisms. Many housekeeping genes have been used as gene

targets to identify various bacteria by PCR, including 16S rRNA, *recA*, *ropB*, *gyrB* and *grpEL* (Mohkam et al., 2016; Wei et al., 2018). At present, at least 40 species of *Pseudoalteromonas* have been found (https://en.wikipedia.org/wiki/Pseudoalteromonas). A real-time quantitative PCR assay has been established to assess the abundance of *Pseudoalteromonas* species in marine samples (Skovhus et al., 2004), but this method cannot identify these *Pseudoalteromonas* strains at the species level. No available rapid approach was previously established for differentiation of *Pseudoalteromonas* strains at the species level. Our previous study showed that *P. marina* strain tbzcY1 is associated with GSD in *P. yezoensis* (Li et al., 2018). Consequently, in this present work we sought to find suitable gene markers for establishing an effective means of identifying and distinguishing these bacteria, to monitor and control the spread of this pathogen.

Among the housekeeping genes in prokaryotes, *dnaA* and *dnaN*, contain hypervariable regions that provide useful loci for the differentiation of bacteria strains at the species level. For one example, *dnaN* was shown a useful tool for rapid classification and identification of *Leuconostoc mesenteroides* species and its related species (Feng et al., 2016). For another example, a rapid real-time PCR method that targets the specific insertion in the *dnaA-dnaN* genome region was used for differentiation of epidemiologically and clinically significant *Mycobacterium tuberculosis* strains from geographically and genetically diverse collection representing areas (Mokrousov et al., 2014). By analyzing *dnaA* and *dnaN* gene sequences from different *Pseudoalteromonas* species deposited in GenBank, we found many variable sites, especially in the *dnaA* interspecies hypervariable region spanning 125 bp (Figs.S1 and S2). Therefore, three primer pairs were designed in this study according to the *dnaA* and *dnaN* sequences. These primer pairs effectively amplified the expected *dnaA* and *dnaN* fragments from *P. marina* tbzcY1 DNA in optimal PCR conditions (Fig.S3). Moreover, these primer pairs distinguished *P. marina* tbzcY1 from different species of *Pseudoalteromonas*, *Vibrio* and seven other bacterial species (Fig.1), thus suggesting that the PCR assay was specific for the detection of *P. marina*.

During the sea culture of *Pyropia*, it is difficult to identify and determine early GSD infection with the naked eye. By the time apparent GSD symptoms appear, the disease has entered late infection stages leading to irretrievable losses. Therefore, a sensitive system able to detect *P. marina* before obvious GSD symptoms occur is required. According to results obtained under laboratory conditions, the established PCR with three primer pairs was able to detect 4 to 4×10^2 CFU cells or 2.37×10^1 to 2.37×10^3 fg DNA of *P. marina* in one PCR reaction, thus indicating highly sensitive detection of *Pseudoalteromonas*, as compared with the real-time quantitative PCR method established by Skovhus (2004). Moreover, the established PCR with three primer pairs successfully detected *P. marina* from early stages of GSD infection in *P. yezoensis*. Thus, in this study, we established a specific and sensitive PCR method to detect the potentially pathogenic *P. marina* associated with GSD in *P. yezoensis*, and we demonstrated that this method has potential applications in early diagnosis of GSD in farmed *Pyropia*.

Next, we plan to find effective approaches to identify other potential pathogens associated with *Pyropia* GSD, including members of *Micrococcus*, *Vibrio*, *Pseudomonas* and *Flavobacterium*. On the basis of these results, we will attempt to establish a multiplex PCR to detect these pathogens, and/or to develop rapid and sensitive detection kits for field diagnosis of GSD.

5 CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

6 DATA AVAILABILITY STATEMENT

All the sequence using in this study have been deposited in GenBank, with accession Nos. MH681053 and MH681054. Other data that support the findings of this study are available from the corresponding author upon reasonable request.

7 ETHICAL APPROVAL

This article does not contain any studies with animals performed by any of the authors.

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Electronic supplementary material

Supplementary material (Supplementary Figs.S1–S3) is available in the online version of this article at https://doi.org/10.1007/s00343-019-9045-5.