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Quantitative real-time PCR detection of a harmful unarmoured dinoflagellate, *Karlodinium australe* (Dinophyceae)

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SUMMARY

We investigated a harmful algal bloom (HAB) associated with the massive fish kills in Johor Strait, Malaysia, which recurred a year after the first incident in 2014. This incident has urged for the need to have a rapid and precise method in HAB monitoring. In this study, we develop a SYBR green-based real-time PCR (qPCR) to detect the culpable dinoflagellate species, *Karlodinium australe*. Species-specific qPCR primers were designed in the gene region of the second internal transcribed spacer of the ribosomal RNA gene (rDNA). The species specificity of the primers designed was evaluated by screening on the non-target species (*Karlodinium veneficum*, *Takayama* spp., and *Karenia* spp.) and no cross-detection was observed. The extractable gene copies per cell of *K. australe* determined in this study were 19 998 $\pm$ 505 ($P < 0.0001$). Estimation of cell densities by q*PCR* in the experimental spiked samples showed high correlation with data determined microscopically ($R^2 = 0.93$). Using the q*PCR* assay developed in this study, we successfully detected the 2015 bloom species as *K. australe*. Single-cell PCR and rDNA sequencing from the field samples further confirmed the finding. With the sensitivity as low as five cells, the qPCR assay developed in this study could effectively and rapidly detect cells of *K. australe* in the environmental samples for monitoring purpose.

Key words: fish kill, Malaysia, ribosomal RNA gene, second internal transcribed spacer.

INTRODUCTION

Rapidly expanding aquaculture activities and industrialization in coastal regions may have contributed to an increase in nutrient loadings, and resulted in localized eutrophication. Together with favorable physical conditions, this will promote rapid growth of phytoplankton, and algal bloom is likely to occur (Hall *et al.* 2008). Some of these algal blooms are known to cause harm by producing biotoxins that can intoxicate humans or kill marine organisms. Thus, rapid harmful algal detection tools are needed for regular monitoring and early prediction of these harmful algal blooms (HABs).

Johor Strait, located between the southern Peninsular Malaysia and Singapore, is an important site of mariculture industries for both countries. Fish farms and the number of cages in the Strait have been reported to have increased compared to a decade ago, with the production of milkfish (*Chanos chanos*) at US $ 6755/metric tons for Singapore, the second highest fish mariculture production among Southeast Asian countries in 2014 (SEAFDEC 2017). This is accompanied by the increasingly reported cases of HABs in the strait. The first HAB event was recorded in 2002, where a massive bloom of *Prorocentrum minimum* (Pavillard) Schiller occurred in the strait (Usup *et al.* 2003). In a field survey along the strait, Tan *et al.* (2013) encountered a low cell abundance of *Karlodinium* species, identified as *K. veneficum* (Ballantine) Larsen at that time based on the morphological observations. The presence of *Karlodinium* species in the strait has gained serious attention as some species from the genus are known to kill fish (e.g. Deeds *et al.* 2002; Kempton *et al.* 2002). The genus *Karlodinium* was raised from polyphyletic *Gymnodinium* sensu *lato* after several taxonomic amendments based on the morphological ultrastructure features, the composition of photosynthetic pigments and the nuclear-encoded large subunit ribosomal RNA gene (LSU rDNA) sequences (Daugbjerg *et al.* 2000; Bergholtz *et al.* 2005). Before 2008, the genus comprised of four species: *K. armiger* Bergholtz, Daugbjerg & Moestrup, *K. australe* de Salas, Bolch & Hallegraeff, *K. veneficum* and *K. vitiligo* (Ballantine) Larsen (Bergholtz *et al.* 2005; de Salas *et al.* 2005). Meanwhile, *K. micrum* has been synonymized as *K. veneficum* (Bergholtz *et al.* 2005). The number of described *Karlodinium* species has grown to 11, with the addition of species: *K. antarcticum* de Sales, *K. ballantinum* de Salas, *K. conicum* de Salas, *K. corrugatum* de Salas, *K. decipiens* de Salas & Laza-Martinez (de Salas *et al.* 2008), *K. corsicum* (Paulmier) Siano & Zingone, and *K. gentienii* Nézan, Chomérat & Siano (Siano *et al.* 2009). Species of *Karlodinium* have a broad global distribution and have been reported from both the Pacific

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and Atlantic oceans, spanning from the cold temperate waters to the warm tropical waters (summarized in Appendix S1 in the Supporting Information, with references herein).

Among the *Karlodinium* species, *K. veneficum* is known to produce ichthyotoxins, karlotoxins (KmTx), a family of amphidinol-like compound (reviewed in Wagoner *et al.* 2010). The species *K. australe* has never been related to any fish kill event until February 2014, where a bloom of *K. australe* occurred in the West Johor Strait, Malaysia and caused massive fish kills (Lim *et al.* 2014); this was the first report where the species killed fish. In the subsequent year, the aquaculture operators in the West Johor Strait had been struck again with the losses of hundreds of tons of caged fishes (Teng *et al.* 2016). It had been suspected that the bloom was attributed to the same species *K. australe*. A field investigation has been undertaken and the bloom samples were used for the evaluation of the quantitative real-time PCR (qPCR) assay developed in the present study.

In this study, a species-specific primer pair targeting *K. australe* was designed to target the second internal transcribed spacer (ITS2) of rDNA for the qPCR detection. Assay specificity and amplification efficiency (AE) were evaluated, and further validated using the spiked samples for quantification of cell number. The qPCR assay was successfully applied to the 2015 bloom samples and positively detected the species. The bloom species was also confirmed by single-cell PCR and rDNA sequencing as *K. australe*.

MATERIALS AND METHODS

Algal cultures

Clonal cultures of *Karlodinium australe* were established from the western (site A; 1.38°N, 103.64°E) and central parts of Johor Strait (site B; 1.46°N, 103.75°E) during a massive bloom of *K. australe* in 2015 (Appendix S2 in the Supporting Information). The species identity was confirmed by microscopic observations and DNA sequence verification of the D1–D3 LSU and ITS rDNA (Lim *et al.* 2014). The cultures were grown in ES-DK medium (Kokinos & Anderson 1995) with a pH of 7.8–8.0, and a salinity of 30. Cultures were maintained at 25 ± 0.5°C under a 12 : 12 h LD photoperiod in a MLR-352 versatile environmental test chamber (Panasonic, Gunma, Japan), illuminated by cool-white fluorescent bulbs with light intensity of ~100 μmol photons m^{-2} s^{-1}. Six strains of *K. australe* (KaJB05, KaJB08, KaJB10, KaJB11, KaJB12 and KaJB15) and other dinoflagellate strains were used to evaluate the specificity of the assay (Table 1).

Design of species-specific primers

The development of qPCR assay in this study was conformed to the MIQE guidelines (Minimum Information for Publication of qPCR Experiments; Bustin *et al.* 2009). Nucleotide sequences of the ITS rDNA of 51 related taxa including *Karlodinium veneficum*, *K. armiger*, *Karenia* sp., *Takayama* spp. and *Gymnodinium* sp. were retrieved from GenBank nucleotide database (Appendix S3 in the Supporting Information). The sequences were aligned with ITS rDNA sequences of *K. australe* (GenBank accession: KJ670418, KJ670419, KJ670420, KJ670421) using Clustal X (Thompson *et al.* 1997), and edited by BioEdit Sequence Alignment Editor v7.0.9.0 (Hall 1999). Multiple sequence alignment was performed to search for variable sites for potential species-specific primer regions.

A phylogenetic analysis was performed for ITS2 rDNA of *Karlodinium* species inferred by sequence-structure information. A total of 44 ITS2 secondary structures of *Karlodinium* species (Appendix S3 in the Supporting Information) were modeled following the procedures as detailed in Lim *et al.* (2014). Three outgroup taxa of *Takayama* species were chosen. Multiple sequence-structure alignment was generated in 4SALE v1.7 using a 12 × 12 specific scoring matrix (Seibel *et al.* 2006, 2008; Wolf *et al.* 2014). The aligned dataset was saved in the format of one-letter encoded for maximum

Table 1. Dinoflagellate strains used in this study

Species	Strain	Locality	Date of collection
Alexandrium affine	AaPa	PulauAman, Penang, Malaysia	May 2002
A. andersonii	AnPa01	PulauAman, Penang, Malaysia	—
A. tamutum	AuKa01	Pulau Abai, Sabah, Malaysia	—
A. minutum	AmKB02	Tumpat, Kelantan, Malaysia	September 2001
A. tamiyavanichii	AcSM01	SamariangBatu, Sarawak, Malaysia	March 2011
A. cf. *tamarense*	AtPA01	PulauAman, Penang, Malaysia	May 2002
Coolia malayensis	CmKd01	Kota Kinabalu, Sabah, Malaysia	September 2010
Karlodinium australe	KaJB05	Johor Strait, Malaysia	March 2015
K. australe	KaJB08	Johor Strait, Malaysia	March 2015
K. australe	KaJB10	Johor Strait, Malaysia	March 2015
K. australe	KaJB11	Johor Strait, Malaysia	March 2015
K. australe	KaJB12	Johor Strait, Malaysia	March 2015
K. australe	KaJB15	Johor Strait, Malaysia	March 2015
K. veneficum	CCMA144	East China Sea, China	September 2015
K. veneficum	LAMB090611	East China Sea, China	June 2009
K. veneficum	LAMB090601	North Sea, Germany	June 2009
Karenia brevis	CCMA165	USA	—
Karenia mikimotoi	CCMA83	East China Sea, China	July 2012
Protoceratium sp.	GgSM01	Samariang Batu, Sarawak, Malaysia	August 2010
Takayama xiamenensis	CCMA186	East China Sea, China	July 2003

parsimony (MP) and maximum likelihood (ML) analyses, while another aligned dataset was saved in sequence-only for Bayesian inference (BI). The MP was performed in PAUP v4.0b.10 (Swofford 2002) and bootstrap support was estimated based on 1000 bootstrap replicates. ML tree was constructed using phangorn (Schliep 2011) implemented in R (R Core Team 2011), with 500 replicates for bootstrap support. BI analysis was performed using MrBayes v3.2.5 (Huelsenbeck & Ronquist 2001), and posterior probabilities were estimated with 25% burn-in from four simultaneous MCMC chains of 5 000 000 generations.

The primer sites were selected within the ITS2 region, with the amplicon length limited to <150 bp. The potential primer sequences were then analyzed by using IDT OligoAnalyzer 3.1 (http://sg.idtdna.com/calc/analyzer). The primers for *K. australe* were designed based on the same criteria as in Kon *et al.* (2015). The primer specificity was analyzed in silico by using Primer-BLAST (Ye *et al.* 2012) to determine potential cross-reactivity with non-target *Karlodinium* species. The specific *K. australe* primers that designed in this study were Karloits2F (5′-ACATATGCCAAGTGTTTTCGCGCTTG-3′, GC content of 46.2%, T_m = 60.5°C) and Karloits2R (5′-GCAACGAAAGAGA-CACACAAGGTGC-3′, GC content of 52.0%, T_m = 60.6°C) with an amplicon size of 143 bp. The primers were then synthesized for further qPCR assay (First Base, Selangor, Malaysia).

Evaluation of primers specificity

Primer cross-reactivity test was performed by qPCR using the genomic DNAs (gDNAs) from cultures of *K. australe* and other closely related species (*K. veneficum*, *Karenia* spp. and *Takayama xiamenensis* Gu) (Table 1). Positive gDNAs were confirmed by amplifying the LSU rDNA using the primer pair D1R (Lenaers *et al.* 1989) and D3Ca (Scholin & Anderson 1996). PCR was performed on a peqSTAR 96X Universal Gradient thermocycler (Peqlab, Erlangen, Germany) as described in Kon *et al.* (2015). The PCR products were visualized on a 2% agarose gel stained with SYBR® Safe DNA gel stain (Invitrogen, Life Technologies, Carlsbab, CA, USA), and captured using an Infinity ST5 Gel Imaging System (Vilber Lourmat, Marne-la-Vallée, France).

Primers' specificity was also tested against gDNAs of other non-target phytoplankton species by conventional PCR (*Alexandrium tamiyavanichii* Balech, *A. affine* (Inoue & Fukuyo) Balech, *A. minutum* Halim, *A. tamutum* Montresor, Beran & John, *A. andersonii* Balech, *A.* cf. *tamarense* (Lebour) Balech, *Protoceratium* sp. and *Coolia malayensis* Leaw, P.T. Lim & Usup; Table 1). Conventional PCR and cross-reactivity test by qPCR were performed as described above using Karloits2F and Karloits2R.

qPCR assay and amplification efficiency

The qPCR assay was performed on an Applied Biosystems 7500 Fast Real-time PCR System (Applied Biosystems, Life Technologies, Austin, TX, USA). The 20 μL-reaction contained 10 μL of SYBR Select Master Mix (Applied Biosystems) and 2 μL of DNA template. The primer concentrations were optimized by qPCR (the range of 250–400 nM were tested) and incorporated an optimum primer concentration of 300 nM in each qPCR reaction. No template control (NTC) and positive control using the synthetic gene fragments were included in each qPCR run. The qPCR cycling conditions are: a hold stage at 95°C for 20 s, followed by 40 cycles of 95°C for 3 s, and 60°C for 30 s. A melting curve analysis (MCA) was performed following the amplification cycles.

gBlocks gene fragment of the ITS2 rDNA of *K. australe* flanked by both forward and reversed primers, with an extra ten base pair (bp) long at both ends, was synthesized (Integrated DNA Technologies, Coralville, IA, USA). The synthetic gene fragments were used as a standard DNA for calibration curve construction. The standard DNA was included in every assay of the environmental samples, for assessing AE.

The lyophilized, synthetic gBlocks gene fragment was first re-suspended in 200 μL TE buffer (Tris 1 M, EDTA 0.5 M, pH 8) to obtain a stock concentration of 1 ng μL^{-1}. The gene fragment copy numbers in 1 ng μL^{-1} was then calculated as described in Kon *et al.* (2015). The stock solution was diluted to 1×10^8 gene copies, followed by 10-fold serial dilutions in triplicate, ranging from 1×10^2–10^7 copies. The serially diluted synthetic gene fragments were used as template in qPCR reactions in triplicate for the calibration curve construction. Calibration curve was constructed by plotting triplicate C_q values against the log-transformed gene copy numbers using GraphPad Prism v. 5.03 (GraphPad Inc., La Jolla, CA, USA). Linear regression analysis was performed on the calibration curve to determine R^2 value and the slope. The AE was calculated as AE = $[10^{(-1/\text{slope})} - 1] \times 100\%$.

Determination of extractable gene copies per cell

The extractable ITS2 gene copy numbers per cell of *K. australe* was determined from samples containing spiked cultured cells of *K. australe* ranging from 20–300 cells mL^{-1}. One milliliter of cultured cells was spiked into the environmental sample. The environment sample was collected by a 20-μm plankton net from Melawi, Malaysia (6.00°N, 102.42°E). We confirmed no *Karlodinium* cells in the sample microscopically, and rDNA amplification and sequencing using the ITS primer pair, ITS1F and ITS1R (Leaw *et al.* 2001). Spiked samples were then concentrated by gentle sieving through a 5 μm-mesh sieve, and then transferred to a microfuge tube. The cell density of *Karlodinium* in each tube was counted in triplicate using a Sedgewick-Rafter counting slide under a microscope. The gDNAs of the samples were extracted by using a commercial DNA extraction kit, DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and eluted in 30 μL elution buffer. The samples were then qPCR analyzed in triplicate as described above. The gene copies of each sample determined from qPCR based on the calibration curve were then plotted against the cell densities determined microscopically. The regression slope was defined as extractable gene copies per cell.

Assay sensitivity and accuracy

Culture strains KaJB11 and KaJB15 at exponential growth phase were used in these experiments. The assay sensitivity in terms of cell numbers was tested with minimum number of cells. Samples containing one, five, and ten cells per reaction were prepared by singly isolation using micropipetting

technique. The gDNAs were extracted using the same DNA extraction kit as mentioned above, and qPCR analyzed using the same method as described above.

Random-spiked cell samples were used to test the assay accuracy in quantifying the target cells by comparing the actual cell densities that were based on microscopic cell counts and that of those based on qPCR cell estimates. Spiked cell samples were prepared by sieving as described above. The cell densities in each sample range from 5 to 600 cells mL^{-1} (5, 14, 28, 56, 74, 139, 278 and 556 cells mL^{-1}) were confirmed by microscopic cell counts in one or two replicates. The gDNA extraction was performed as mentioned above and then qPCR was performed in triplicate. The potential cell densities estimated by qPCR assay were then compared with the actual cell densities.

Sample collection during the 2015 bloom event

In March 2015, a recurrent algal bloom in the Johor Strait has caused massive fish kill and severe aquaculture losses to the operators. A field investigation was conducted to identify the species responsible for the event. Two-liter surface water samples were collected from the bloom site at the central part of the Johor Strait (site B; Appendix S2 in the Supporting Information). The samples were then preserved with 2% formalin and kept at –20°C.

Aliquots of 20, 100, and 200 μL preserved samples (in triplicate) were sieved through a 5 μm-mesh sieve and rinsed with TE buffer (Tris 1 M, EDTA 0.5 M, pH 8) to wash off the formalin preservative prior to DNA extraction. The environmental DNA (eDNA) was extracted using the same DNA extraction kit as above. The eDNA was then analyzed by SYBR Green I-based qPCR assay. MCA was performed following the SYBR Green-based qPCR amplification. Positive controls using the gBlock gene fragments were included in each run to assess if C_q delayed.

RESULTS AND DISCUSSION

Primer specificity

The ITS rDNA has been widely used in phylogenetic studies owing to its sequence heterogeneity at lower taxonomic levels. The ITS2 phylogenetic tree inferred from the sequence-structure information based on MP, ML, and BI yielded identical tree topologies; only BI tree is shown with MP/ML bootstrap supports and BI clade credibility values presented (Appendix S4 in the Supporting Information). The trees revealed a highly supported sister clade comprised of *K. autrale* and *K. armiger* (MP/ML/BI, 92/100/1.00), which is congruent to that of the LSU tree in Lim *et al.* (2014). *Karlodinium veneficum* formed a highly supported clade, consisted of two subclades: subclade I comprised of *K. veneficum* strains from Balearic Sea of the North eastern Atlantic Ocean, while subclade II were found widely distributed in the north western and north eastern Atlantic and Western Pacific (Appendix S4 in the Supporting Information). Lim *et al.* (2014) has demonstrated the presence of four hemi-compensatory base changes (HCBCs) in the ITS2 transcript when compared to that of *K. armiger*; 3–7 HCBCs when compared to *K. veneficum*.

In this study, a 143-bp length sequence signature of *K. australe* in the ITS2 rDNA were selected; the region was verified in silico to be uniquely distinctive to *K. australe* when compared to the ITS2 transcripts of other *Karlodinium* species. Furthermore, the *K. australe* species-specific primer sites (Karloits2F and Karloits2R) are readily variable from its closely related species, *Karlodinium* spp., *Karenia* spp. and *Takayama* sp. Testing of primers specificity in silico showed that the primers only hit on sequences of *K. australe* in the blastn search, but no match to *K. amiger* and *K. veneficum* in the GenBank nucleotide database (NCBI). The primers demonstrated 100% identity to sequences of *K. australe* in a 100% query coverage when blasted in NCBI GenBank nucleotide database; the blastn search hit all ITS sequences of *K. australe* (accessions: KJ670418, KJ670419, KJ670420 and KJ670421).

The in situ specificity of the primer pair was evaluated using gDNAs of the closely related species, *K. veneficum*, *Karenia* spp. and *Takayama xiamenensis* (Table 1). The gDNAs were first confirmed by showing positive LSU rDNA amplification in the conventional PCR (Fig. 1a). The assay specificity evaluated by conventional PCR using various gDNAs from other non-target species showed that only gDNA from *K. australe* (KaJB15) produced positive amplification, but other dinoflagellates (*Alexandrium tamiyavanichii*, *A. affine*, *A. minutum*, *A. tamutum*, *A. andersonii*, *A. tamarense*, *Protoceratium* cf. *reticulatum* and *Coolia malayensis*) did not (Appendix S5 in the Supporting Information). The qPCR specificity test performed using the *K. australe*-specific primer pair also showed no positive amplification other than *K. australe* (KaJB05) (Fig. 1b,c), confirming no cross reactivity to the non-target species.

qPCR assay specificity

The gene fragment calibration curve constructed with the optimized primer concentration of 300 nM showed AE of 94.6%. Figure 2 shows the calibration curve with a linear dynamic range over a six-order of magnitude (10^2–10^7 copies, R^2 = 1.00, slope = –3.46). The standard DNAs (positive controls) using gBlock gene fragments amplified together with the unknown samples showed AEs >90%. The NTC included in each qPCR run showed no amplification throughout the study.

The qPCR assay in this study had a limit of detection of as low as 10^2 gene copies, as shown in the linear detection range (Fig. 2). The assay tested using samples containing only one target cell showed positive amplification which produced positive melt peak in the MCA; however, the C_q obtained for single cells was inconsistent, probably due to variable gene copy number in one cell. It has been reported that different species has different number of gene copies, and even vary slightly at different growth phases between cells of the same species (e.g. Galluzzi *et al.* 2010). Alternative possible explanation in this deviation is the efficiency of commercial DNA extraction kit in recovering the DNA. Nishimura *et al.* (2016) has demonstrated that the DNA recovery efficiency (RE) of DNeasy Plant Mini Kit (Qiagen) for *Gambierdiscus* spp. could range from 6 to 88%. Furthermore, Kon *et al.* (2015) has shown that RE of the same kit on *Alexandrium tamiyavanichii*

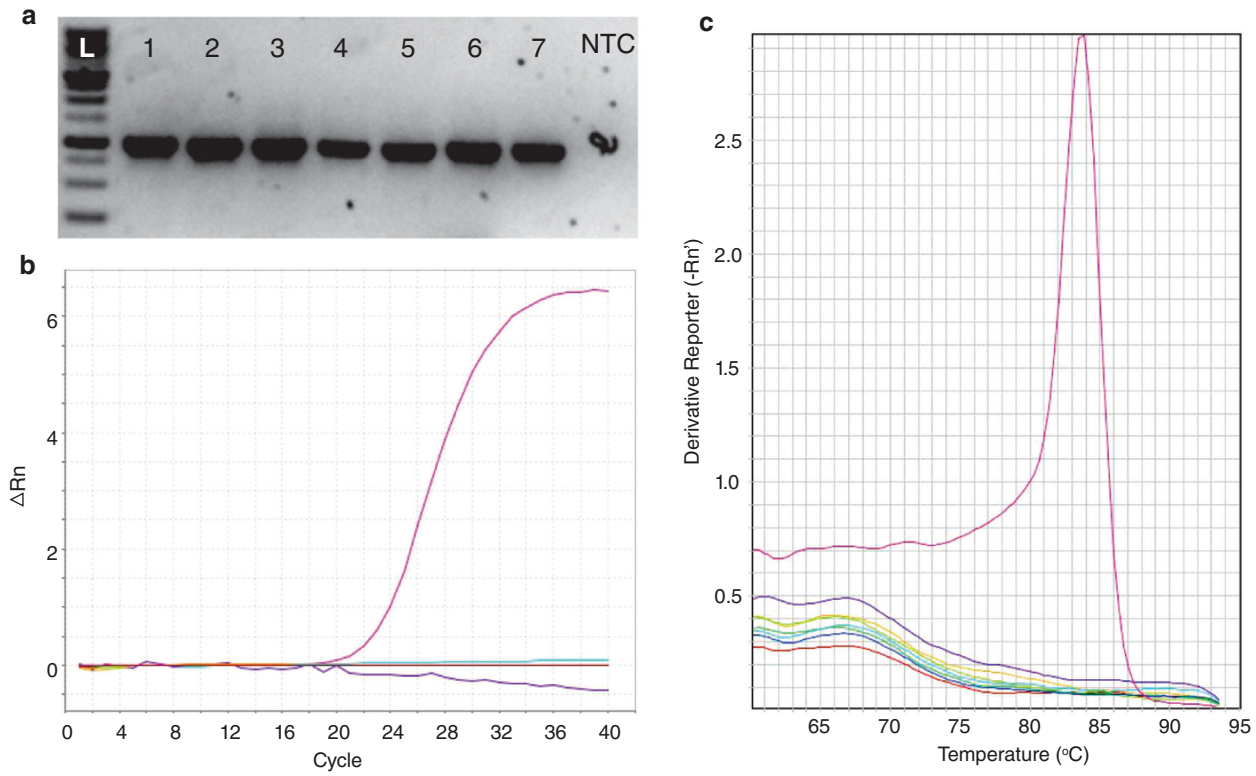

Fig. 1. qPCR cross-reactivity test of the *Karlodinium australe* species-specific primers performed using gDNAs of closely related species. (a) Gel image showing positive LSU rDNA amplification in the conventional PCR using gDNAs of: 1, *K. veneficum* LAMB090611; 2, *K. veneficum* LAMB090601; 3, *K. veneficum* CCMA144; 4, *Karenia mikimotoi* CCMA83; 5, *K. brevis* CCMA165; 6, *Takayama xiamenensis* CCMA186; 7, *K. australe* KaJB05; NTC, no template control; and L, 1kb DNA ladder. (b) The corresponding amplification curves. (c) Melting curves showing only templates from *K. australe* produced positive amplification.

was as low as 8.0 ± 2.9% (mean ± SD) with the range values between 5.6 and 11.0%. Samples containing five and 10 target cells, however, were consistently amplified and yielded constant C_q values for a reliable quantification. As such, the minimum number of cells for a reliable quantification was determined to be five cells per reaction.

The value of copy numbers per cell has been used to estimate the cell abundance in an unknown sample. The extractable gene copies per cell of *K. australe* determined in this study was 19 998 ± 505 (slope ± SD) ($R^2 = 0.98$, $P < 0.0001$; Fig. 3). Estimation of cell densities in random-spiked cell samples using this copy numbers per cell showed a positive correlation with cell densities determined microscopically ($R^2 = 0.93$, $P < 0.0001$; Fig. 4). Comparison of qPCR cell estimates with the actual cell densities that were based on microscopic cell count revealed reliable quantification of the qPCR assay developed in this study.

Species detection during the bloom

The plankton samples collected during the recurrent bloom in 2015 that caused massive fish kills in the Strait showed extremely high density of *Karlodinium*-like monospecific dinoflagellate, with maximum cell densities up to 2×10^8 cells L^{-1} based on microscopic cell counts. Several studies have demonstrated that the main fish-killing *Karlodinium* species, *K. veneficum*, has been associated with numerous fish kill events globally (reviewed in Place *et al.* 2012) after its first bloom in Walvis Bay, Namibia causing massive fish kills (Bergholtz *et al.* 2005). The killing agent, KmTx, has been discovered (Kempton *et al.* 2002). Recently, *K. australe* has been attributed to massive fish mortality in Johor Strait, Malaysia (Lim *et al.* 2014); however, whether the killing agent was due to the same mechanism of KmTx has not yet been identified.

Morphological discrimination between species of *Karlodinium* as well its closely related taxa, i.e. *Karenia* and *Takayama*, is challenging as the morphological features are ambiguous, particularly in fixed samples (Bergholtz *et al.* 2005). Fixation of unarmored dinoflagellates can be sometimes problematic and make traditional microscopic identification difficult. Improper fixation for unarmored dinoflagellates that lack the protective theca may cause cell shape distortion and shrinkage (Sherr & Sherr 1993; McCoy *et al.* 2014). The morphological characteristics such as cell size, position of nucleus, and the arrangement of chloroplasts that used to identify species of *Karlodinium* are also subtle and often misleading. Hence, molecular approach becomes an alternative diagnostic tool in detecting and identifying the species.

In this study, the field samples collected from the 2015 bloom were screened with the qPCR assay developed using the *K. australe* species-specific primer pair, and the results

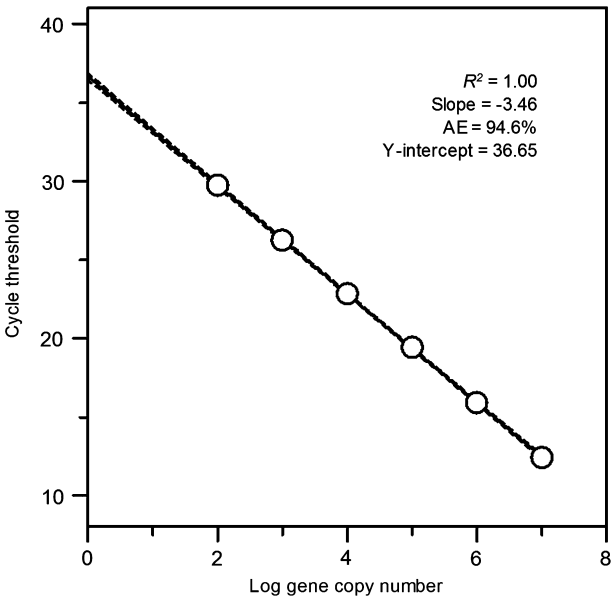

Fig. 2. Calibration curve of the cycle threshold (C_q) constructed using the *K. australe* ITS2 gBlock gene fragment. Error bars denote the standard deviation from triplicate amplifications; dashed lines represent 95% confidence limit. AE, amplification efficiency.

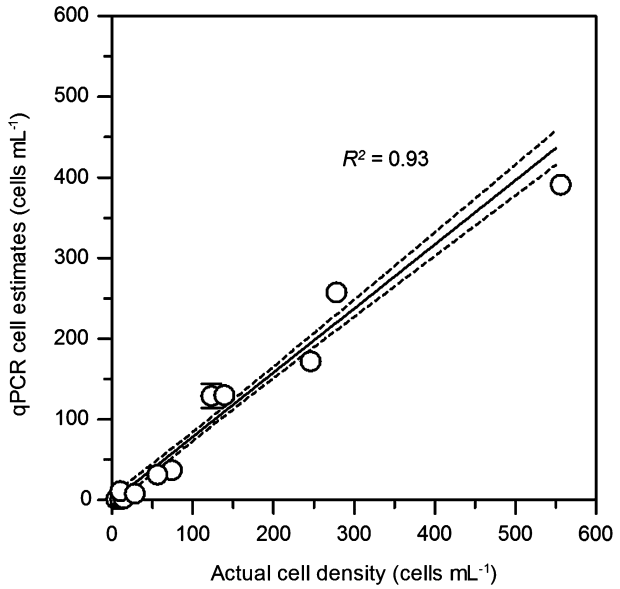

Fig. 4. Assay accuracy testing on random-spiked samples by comparing the actual cell densities and the qPCR cell estimates. The graph shows a good fit between the cell densities determined by manual cell counts and by qPCR. Dashed lines represent the 95% confidence limits.

showed positive amplification, and the MCA revealed identical melt peaks with the positive controls (Fig. 5), thus confirmed the specific amplification and species identity. Moreover, single-cell PCR and ITS rDNA sequencing from the field samples yielded identical sequences of *K. australe* in the NCBI blastn search. This study successfully detected and identified the bloom species in 2015 by our *K. australe* qPCR assay. However, the assay was not able to quantify the cell abundance during the bloom due to improper field sample preservation. The only available bloom samples collected during the time were preserved in 1% formalin preservative. qPCR quantification using the eDNA extracted from the formalin-fixed field samples had led to underestimation of cell numbers as compared to microscopic cell count. This could be accounted for the loss of cells via sieving. The formalin preserved samples used in this study have been kept for about 2 months prior to analysis; this might cause degradation of DNA in the formalin-preserved condition, or cells might burst under

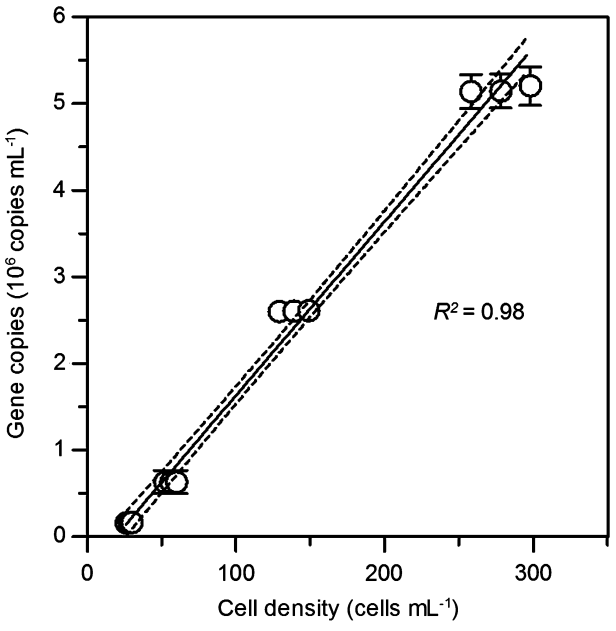

Fig. 3. Verification of the extractable gene copy number per cell determined as the slope of linear regression of cell densities enumerated microscopically vs. gene copies estimated from gene fragment calibration curve.

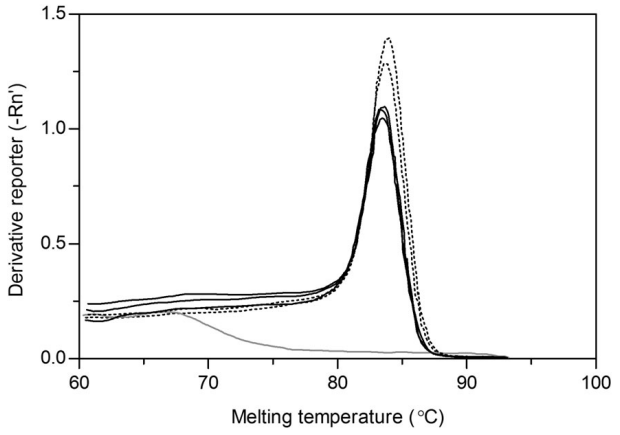

Fig. 5. Melting curve analysis showing same melt peaks between the bloom-samples (solid lines) and the gene fragment-positive controls (dash lines). NTC showed no amplification (grey line).

freezing–thawing condition. Eckford-Soper and Daugbjerg (2015) have demonstrated that qPCR amplification showed approximately seven cycles delay between the Lugol-fixed cells that were kept at –20°C for 1 and 2 months. Besides that, Godhe *et al.* (2002) showed that even formalin-treated samples could preserve cells morphology and the gDNA, and claimed that it worked well with PCR amplification, but they suggested that this preservation method might not be suitable for unarmoured dinoflagellates. Preserving *Karlodinium* in formalin preservative kept the cells observable, but the preserved cells of *K. australe* appeared smaller and transparent compared to the living cells. Saline ethanol has been demonstrated as the best preservative suggested for algae (Miller & Scholin 2000); however, *Karlodinium* cells lysed after the preservation (Kon, N.F., 2014; unpublised observation). Other alternative fixation methods of unarmored dinoflagellates have been tested and validated for qPCR-based cell quantification, for instance, Lugol's iodine was suggested as the most ideal short-term fixative (Eckford-Soper & Daugbjerg 2015), by which could be adopted in future studies. Cell densities of *K. australe* obtained from the bloom samples reported in this study was performed manually by microscopic cell count instead of qPCR quantification, until a more suitable preservation method for unarmored dinoflagellates is developed. On-field eDNA extraction and direct cold storage of the eDNA samples would be an alternative procedure to substitute sample preservation step in this purpose. It was also observed that the cells were easily broken if centrifuged under high speed. Proper sample processing steps were undertaken to avoid loss of cells. In this study, gentle sieving was used to concentrate the *Karlodinium* bloom samples rather than centrifugation, but a smaller mesh size (e.g. <5 μm-mesh) is recommended.

Conclusion

Karlodinium australe was confirmed to be responsible for the fish kill event in 2014 and 2015 in Johor Strait. There is an urgent need for routine monitoring and early warning systems to be in place to minimize the losses in aquaculture in the future. In conclusion, the *K. australe* species-specific primer pair designed in this study showed the specificity and applicability to detect *K. australe* in the environmental samples. The qPCR assay developed could be applied to the field for routine monitoring of HAB especially in Johor Strait for recurrent blooms. The qPCR assay has shown its sensitive detection of as low as five cells, which is beneficial for the detection of species at low cell numbers, especially at the initiation stage of bloom.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1. Global distribution of Karlodinium species.

Appendix S2. Map showing the Johor Strait bordering Malaysia and Singapore.

Appendix S3. ITS rDNA sequence information of Karlodinium.

Appendix S4. ITS2 Bayesian tree of Karlodinium species.

Appendix S5. Cross-reactivity test of the Karlodinium australe.