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Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2020 17(SI-1): 90-99. **OPEN ACCESS**

Development of Species-Specific Primer Sets for Australian Redclaw Crayfish (*Cherax quadricarinatus*) Detection from Water Environmental DNA (eDNA)

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The Australian redclaw crayfish, *Cherax quadricarinatus,* is currently an invasive alien species in Malaysia. It was initially introduced into Malaysia for aquaculture purposes and aquarium trade but has been released into the wild, causing a significant impact on the native freshwater ecosystem. Environmental DNA monitoring is an attractive molecular tool for species detection and monitoring in aquatic habitat due to its higher sensitivity over the traditional method. In this study, we design the first species-specific primer sets that is capable of detecting *C. quadricarinatus* from the water sample. Two species-specific primer sets (CQCOI_F1_R1 and CQCOI_F2_R3) have been designed based on the mitochondrial COI gene region. Primer specificity was tested and confirmed on both tissue and water samples of targeted species (*C. quadricarinatus*) and non-targeted species (*Cherax destructor, Procambarus clarkii, Macrobrachium rosenbergii*). Due to the longer primer length that can presumably confer higher observed PCR specificity, the CQCOI_F2_R3 primer pair was recommended for future field testing. The designed primers can contribute to aquatic biodiversity conservation in Malaysia by enabling the early detection, control and management of *C. quadricarinatus* population.

Keywords: *Cherax quadricarinatus*, biodiversity conservation, eDNA, invasive species, species-specific primer

INTRODUCTION

Non-native crayfish species were introduced globally as a result of anthropogenic factors. The negative impacts from the introduction include suppression of native species, habitat alteration which has been reported extensively (Olden et al. 2006; Coughran et al. 2009; Holdich et al. 2009; Kawai et al. 2009; Buřič et al. 2011; Chucholl, 2016; Patoka et al. 2016). Australian redclaw crayfish, *Cherax quadricarinatus* (Von Martens, 1868) is among the non-native crayfish that become invasive into the Malaysian freshwater system. The species native ecosystem ranged between the freshwater bodies of Northern Queensland and Southern New Guinea (Patoka et al. 2016). Their biological features are capable of tolerating a wide range of environmental parameters such as pH, dissolved oxygen, and temperature along with a flexible diet which might also help them establish a population in the wild

(Bortolini et al. 2007; Patoka et al. 2016).

The introduction of the crayfish in Malaysia was due to aquaculture purposes begins in the early 1980s for aquarium trade (Bella et al. 2011; Coughran and Leckie, 2007; Faulkes, 2015; Naqiuddin et al. 2016). It is believed that the crayfish might accidentally escape into the wild from the hatchery, or deliberately released by the hobbyists (Belle and Yeo, 2010; Gozlan, 2010; Belle et al. 2011; Naqiuddin et al. 2016). The presence of *C*. *quadricarinatus* in the native water bodies might cause several impacts such as disease spreading, preying on native species and habitat alteration. According to Ahyong and Yeo (2007), Longshaw (2011) and Saoud et al. (2013), *Cherax quadricarinatus* is a potential host to several parasites and diseases including the *Aphanomyces* sp., fungus, bacilliform virus, parvovirus and the gram-negative bacteria like *Rickettsia* sp. In addition, James et al. (2014) reported that invasive crayfish have the potential to affect the growth rate of the native organisms and the biomass of algae. This urges the detection and monitoring of this invasive crayfish species to avoid further damages to the native ecosystem (Hulme, 2012; Lodge et al. 2006, 2016; Vander Zanden et al. 2010). Species detection using traditional survey methods such as trapping and netting has several limitations such as high cost and laborious with a very low detection rate especially when the target species is low in abundance (Rees et al. 2014; Schmidt et al. 2013; Gu and Swihart, 2004). Some of the measures suggested to be taken include the usage of effective and highly sensitive technology for invasive alien species surveillance, practicing proper management of commercial pathways to reduce the introduction of alien species.

Environmental DNA (eDNA) is a new and still advancing method used as a monitoring tool in the management of aquatic organisms, especially invasive ones (Dejean et al. 2012; Lodge et al. 2012; Goldberg et al. 2013; Piaggio et al. 2013; Rees et al. 2014; Takahara et al. 2015; Dougherty et al. 2016; Cai et al. 2017). A DNA sample extracted from the environmental samples such as water, soil, and air without extracting physical tissue from the actual target organisms is referred to an eDNA (Ficetola et al. 2008; Dougherty et al. 2016). However, to detect a specific target species from these environmental samples, it is necessary to acquire a species-specific primer. To date, there has been no report on the development of *C. quadricarinatus* species-specific primer that can be used to detect the target species from the water samples in Malaysia.

In this study, we present the species-specific primer that can specifically be used in the DNA amplification and positive detection of *C. quadricarinatus* from environmental water sample.

MATERIALS AND METHODS

Tissue Sampling

A total number of ten samples were obtained from aquarium shop in Besut, Terengganu which include four *Cherax quadricarinatus* (Australian redclaw crayfish), two *Cherax destructor* (common yabby), two *Procambarus clarkii* (red swamp crayfish) and two *Macrobrachium rosenbergii* (giant freshwater prawn). Muscle tissue were collected from the claw part of each sample and kept separately into 1.5 mL microcentrifuge tubes containing 95% ethanol.

DNA Extraction from Tissue Samples

Approximately 25 mg of tissue of each sample were used for the DNA extraction process using FavorPrep™ Tissue Genomic DNA Extraction Kit (Favorgen Biotech Corp, Taiwan) following the kit protocol. Then, 50 µL of elution buffer was used to elute the DNA to provide a better DNA yield and concentration. The eluted DNA concentration was evaluated by running on 1% agarose gel and visualized using FlourChem E. (Protein Simple, California, USA).

DNA Extraction from Water Samples

Four water samples were used for eDNA extraction. (1) water sources which consisted of *C*. *quadricarinatus*, (2) tap water, (3) water sources which consisted of other species like tilapia, and (4) ddH_2O as a negative control. Each water sample was filtered separately using 0.22 µm PES membrane (JET Biofil, China). The membrane trapped the genetic component from the water and was used for DNA extraction. The membrane is ground in 1.5 mL microcentrifuge tubes and proceeded with FavorPrep™ Tissue Genomic DNA Extraction Kit (Favorgen Biotech Corp, Taiwan) following the kit protocol for DNA collection.

Amplification and Sanger Sequencing

To obtain a sequence that can be used to design species-specific primer, a universal primer set of Cytochrome Oxidase subunit I (COI) for invertebrates designed by Folmer et al. (1994) was used with slight modification in annealing temperature. Both forward primer LCO1490 (5'-GGTC AACA AATCA TAAA GATA TTGG-3') and

reverse primer HCO2198 (5'-TAAA CTTC AGGG TGAC CAAA AAATCA-3') are capable of amplifying a sequence of 710 base pair (bp) of COI gene fragment. PCR amplification was performed using 25 µL PCR mixture which consisted of 8.5 µL ddH_2O, 1.0 µL 10 µM of primers, 12.5 µL exTEN 2xPCR Master-Mix (Axil Scientific, Singapore) and 2.0 µL DNA template (1-50 ng/µL) on Veriti 96-Well Thermal Cycler (Applied Biosystems, California, USA). The PCR condition for the optimization process were as follow: Initial denaturation 95 °C for 5 minutes, 35 cycles of denaturation at 95 °C for 30 sec, three replicates annealing at 50 °C, 54 °C and 58 °C respectively for 30 sec, and extension at 72 °C for 45 sec, and final extension at 72 °C for 10 minutes. The PCR products were run and visualised on 2% agarose gel and FlourChem E. (Protein Simple, California, USA) respectively. Only four successful tissue PCR products of *C. quadricarinatus* were sent for sanger sequencing at Apical Scientific Sdn Bhd using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and proceeded by 3730xl Genetic Analyzer (Applied Biosystems).

Sequence Analysis of Tissue Samples

Successful sequences received from the company were aligned using Clustal W in MEGA v7 (Kumar et al. 2016). The sequences identities were confirmed using Basic Local Alignment Search Tool (BLAST) against National Centre for Biotechnology Information (NCBI). Within this sequence, the conserve region was detected and used for the species-specific primer design and development of *C. quadricarinatus*.

Primer Design

Previous protocol of Miya et al. (2015) on primer design and validation was followed in this study with slight modification. Species-specific primer was designed using Primer3 v0.4.0 software (Kõressaar and Remm, 2007; Untergasser et al. 2012; Kõressaar et al. 2018). The conserved region sequence of the *C. quadricarinatus* from the analysis was inserted into the primer design software and few sets of primer sets were suggested with the amplified regions for each primer. The GC ratio was also considered in order to find the most suitable species-specific primer to be used. Two sets of primers were selected **(1):** CQCOI_F1 and CQCOI_R2 which are (5'-AGC CCC TGA TAT AGC CTT CC-3') and (5'– CTG TCC CGA CAC CTC TCT CT-3'); and **(2):** CQCOI_F2 and CQCOI_R3 which are (5'– AGC CCC TGA TAT AGC CTT CCC TCG AAT AAA-3') and (5'– GCC TAG GTC GAC TGA TGC TCC TGC A-3') respectively. The primer sets were then synthesized at Apical Scientific Sdn Bhd.

Primer Validation

The primer sets specificity was confirmed both using in-Silico and in Vitro test. The in-Silico test was conducted by analysing the amplified regions of the selected primer using BLAST tool in the NCBI to validate the significance of the suggested regions with other species. In vitro test was conducted using the same volume and reagents of PCR mixture used during the universal primer amplification. The only difference here is the newly designed species-specific primer sets were used. The thermal condition used for the new primer was also similar as the universal COI primer and the amplification was performed on Veriti 96-Well Thermal Cycler (Applied Biosystems, California, USA) but three annealing temperature used were 60°C, 64°C and 68°C to evaluate the optimum temperature that capable producing a bright single band on 2% agarose gel.

Primer Workability Test

Two Primer workability tests were conducted in this study (1) test on 10 DNA template extracted from tissue and (2) test on 4 DNA template extracted from water. The species-specific primers designed were used in the amplification of all 14 samples. The PCR mixture of 25µL which consisted of 8.5µL ddH_2O, 1.0 µL 10 µM of designed species-specific primers, 12.5 µL exTEN 2X PCR Master-Mix (Axil Scientific, Singapore) and 2.0 µL DNA template (1-50 ng/µL) on Veriti 96-Well Thermal Cycler (Applied Biosystems, California, USA). The thermal condition was as follow: initial denaturation 95 °C for 5 minutes, 35 cycles of denaturation at 95 °C for 30 sec, and extension at 72 °C for 45 sec and final extension at 72 °C for 10 minutes. The annealing temperature used for the primer was based on the optimised annealing temperature in primer validation process. The PCR product were run on 2% agarose gel to confirm the amplified product size.

RESULTS

COI Gene Amplification from Tissue Samples

Using an annealing temperature of 54°C, a single band at approximately 710 bp was observed across all samples (Figure 1), except for sample UG2 whereby a faint band at around

1,000 bp could be observed.

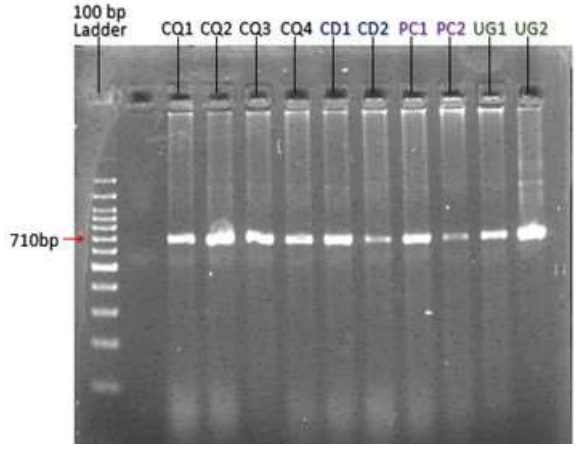

Figure 1: PCR amplification using universal COI primer amplified at optimal temperature of 54°C. CQ *C. quadricarinatus*; CD *C. destructor*; PC *P. clarkii*; UG *M. rosenbergii*

Species-Specific Primer Design and Validity Testing

Two primer sets were designed using Primer3 v0.4.0 software. The primer set CQCOI_F1_R2 and CQCOI_F2_R3 produced the amplicon size of 119bp and 175bp respectively. Each primer sets GC content were 55%. The information about the two primer sets is summarised in Table 1 below. In-silico test indicated both primer sets were theoretically species enough to detect only the target species *C. quadricarinatus* after BLAST in the NCBI database. This is because the primer sequence has index of similarity between 93.37% to 100% with the *C. quadricarinatus* sequences with the Genbank. which indicate very high accuracy. Also, the sequences from newly designed primer sets do not match with the closely related species of *C. quadricarinatus* used in this study.

Table 1. Properties of species-specific primer sets designed using Primer3 software

Property	CQCOI_F1_R2	CQCOI_F2_R3
PL	F1, 20 bp R2, 20 bp	F2, 30 bp R3, 25 bp
Tm	F1, 59.54°C R2, 59.95°C	F2, 61.9 °C R3, 64.1 °C
PPS	F1, 7.0 R2, 4.0	F2, 4.0 R3, 6.0
AS	119 bp	175 bp
GC%	55	55

PL Primer Length; **AS** Amplicon Size; **PPS** Primer Pair Score

Species-Specific Primer Performance Test

The result of PCR amplification of both primer CQCOI_F1_R2 and CQCOI_F2_R3 at annealing temperature of 60 °C showed non-specificity of the primers (Figure 2). At this level of temperature on former set of primer, another two species (*P. clarkii* and *M. rosenbergii*) were also amplified (Figure 2a). While on the latter primer set, non-specificity was also observed by the amplification of *P. clarkii* on the same temperature (Figure 2b).

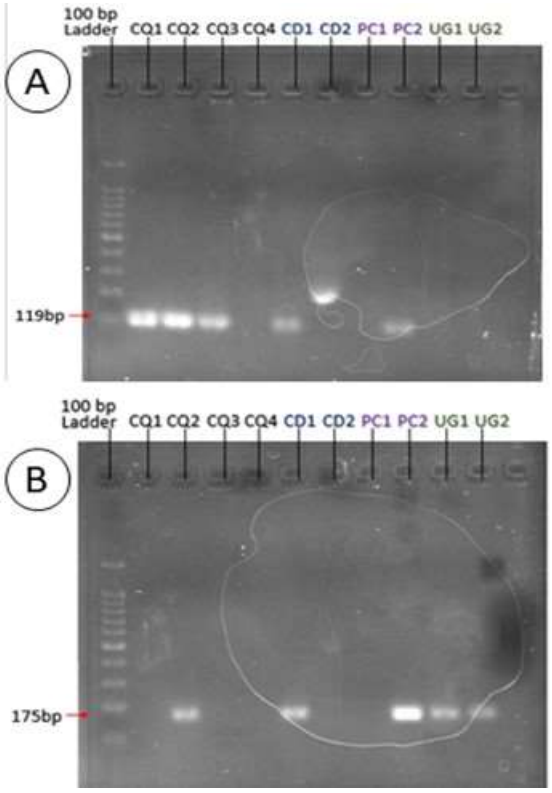

Figure 2: PCR amplification for species-specific primer at annealing temperature 60°C (a)CQCOI_F1_R2 (b) CQCOI_F2_R3. CQ *C. quadricarinatus*; CD *C. destructor*; PC *P. clarkii*; UG *M. rosenbergii*

After adjusting the annealing temperature to 64 °C and run, only *C. quadricarinatus* samples were successfully amplified for both specific primer sets designed (Figure 3). The result showed the clear bands at approximately 119bp and 175bp for CQCOI_F1_R2 and CQCOI_F2_R3 primers respectively. Thus, CQCOI_F2_R3 primer set band is more specific and clearer compared to the CQCOI_F1_R2 primer bands (Figure 3a and 3b). Hence, 64 °C revealed to be a specific temperature towards *C. quadricarinatus* for both primer sets.

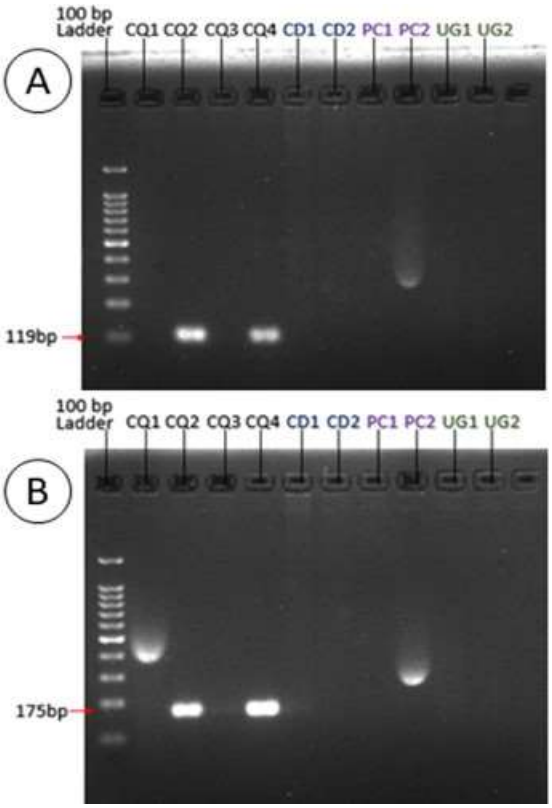

Figure 3: PCR amplification for species-specific primer at annealing temperature 64°C (a) CQCOI_F1_R2 (b) CQCOI_F2_R3. CQ *C. quadricarinatus*; CD *C. destructor*; PC *P. clarkii*; UG *M. rosenbergii*

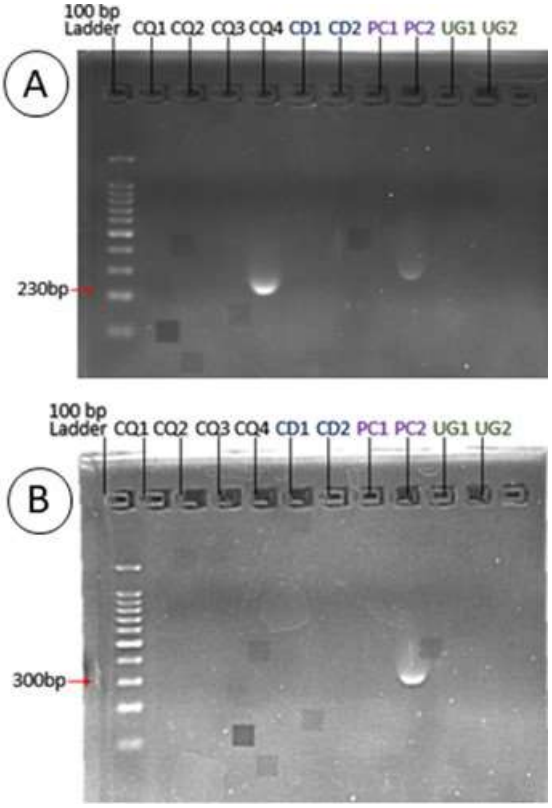

Figure 4: PCR amplification for species-specific primer at annealing temperature 68°C (a) CQCOI_F1_R2 (b) CQCOI_F2_R3. CQ *C. quadricarinatus*; CD *C. destructor*; PC *P. clarkii*; UG *M. rosenbergii*

PCR optimisation was conducted again on the specific primer sets using annealing temperature of 68°C. This is for further confirmation and authentication that the designed primers can only work on *C. quadricarinatus*. However, only one band of *C. quadricarinatus* was visualised on the gel with an unclear smeared band of *P. clarkii* (Figure 4a). Figure 4b further showed that only *P. clarkii* was amplified using this temperature value.

Primer Test on Water Samples

Although, both primer sets were proven to be species specific on *C. quadricarinatus* at 64 °C. But, only CQCOI_F2_R3 was selected because it possessed a longer primer length which can increase its specificity. Also, brighter bands were produced when tested with tissue samples as shown earlier in Figure 3b. In total of four DNA extracted from water samples, only sample of *C. quadricarinatus* were successfully amplified showing a bright single band, visible at 175 bp (Figure 5).

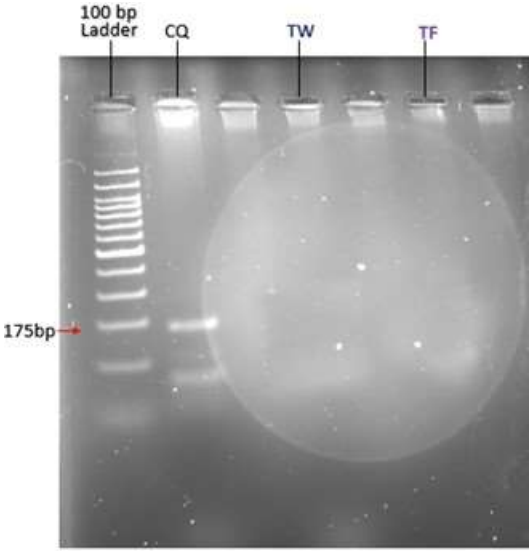

Figure 5: PCR amplification for CQCOI_F2_R3 primer at annealing temperature 64°C using eDNA. CQ *C. quadricarinatus*; TW Tap Water; TF Tilapia Fish

DISCUSSION

The current study presents a newly-designed species-specific primers capable of detecting the presence of invasive *C. quadricarinatus* from environmental samples, even at low concentrations or quality of the species' DNA. This is possible due to the high abundance of mtDNA copies in the mitochondria (Benecke and Wells, 2001; Ahmad-Syazni et al. 2017; Ha et al. 2017; Khaleel et al. 2019, 2020). Also, the COI region of mtDNA is highly conserved with low intra-species mutation rate, which qualifies the region to be useful in the design of species-specific and universal primers (Otranto and Stevens, 2002; Ardura et al. 2017; Crane et al. 2018).

Species-Specific Primer Validity

The primers designed in this study yielded an excellent output. This is achieved by observing some recommended principles such as primer length, GC content, annealing and melting temperature (Singh and Kumar, 2001). According to Wu et al. (1991), primer with a minimum length would have better efficiency and specificity. Hence, recent studies found out that primers having 17 to 34 nucleotides perform better (Ardura et al. 2017; Crane et al. 2018). This corresponds to the primer sets of the current study with minimum of 20 bp and maximum of 30 bp. Furthermore, the GC content (55%) values of designed primers fall within the recommended range values of 50-60% (Singh and Kumar, 2001) and 32-61% (Crane et al. 2018) for primer design. Higher GC contents lead to primer-dimer and reduce the chance of obtaining the targeted product. Self-complementary score indicates the possibility of the primer for binding itself and other primer pair. Untergasser et al. (2012) further explained that self-complementary score of a designed primer should be below 8, which corresponded to the result of the current study designed primers. Values above 8 could result in lower tendency for self-binding.

Annealing temperature plays a vital role in the testing and validating processes of primers sets in this study. Previous study showed that annealing temperature had significant effects on the detection rates (Doi et al. 2019) and quality of PCR products. The differences in melting temperature, as well as that in the primer sequence, such as G/C content, might have influenced the responses observed for PCR annealing temperature (Doi et al. 2019; Naqib et al. 2019). Hence, 64°C was observed to be the best for precise amplification of *C. quadricarinaus*. Before applying the newly-designed primers into the wild ecosystems, the cross-amplification tests were conducted involving the closely related indigenous species (*C. destructor*, *P. clarkii, M. rosenbergii*). The result proved that only *C. quadricarinaus* can be detected. This will provide a high probability of detecting only targeted species in the wild by new primers designed.

Species-Specific Primer Performance Test

The primers must be species-specific to ensure high-confident identification of targeted species (Díaz-Ferguson et al. 2014). Despite both primer sets showed positive results to amplify the mtDNA of *C. quadricarinaus* from its tissue samples. Only CQCOI_F2_R3 primer was chosen to be tested with eDNA extracted from the water samples. This is because, it possessed longer base pairs of 30bp (forward) and 25bp (reverse) which is longer enough for adequate specificity to bind to the mtDNA template of the targeted species. This also explained the reason for obtaining highly bright and precise bands at 64°C in Figure 3b. More so, previous study further explained that the usage of primer having 28bp to 35bp was essential in distinguishing homologous genes from different species (Singh and Kumar, 2001).

The detection level is quite important especially when the species-specific primers are needed for early detection of invasive species. Usually, at the early invasion stage, non-indigenous species are anticipated to be sparsely distributed with a very low density (Ardura et al. 2017). Here the newly-developed primers are expected to yield PCR amplification with clear amplicons at low concentrations of DNA, thus enabling the detection of targeted species from a few cells in a sample. The main benefit of the current developed primers is that they have very short target fragment (119bp and 175bp), and can be amplified from degraded DNA samples. It is previously reported that DNA fragments of approximately 400bp might survive for up to 1 week at 18°C in the lake habitat (Matsui et al. 2001).

CONCLUSION

In conclusion, two species-specific primer sets of invasive *C. quadricarinatus* (CQCOI_F1_R2 and CQCOI_F2_R3) were developed with high specificity towards its target species. We recommended the use of primer CQCOI_F2_R3 for field testing due to its more robust primer

design and performance. Since environmental water samples can contain cells and tissues of the species present in the water column, utilizing eDNA for invasive species detection could be a promising tool and can be incorporated into monitoring and management plans related to early detection, rapid response, and policy decisions for conservation.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT

We would like to offer our thank you to Malaysian government for the Fundamental Research Grant Scheme (FRGS) FRGS/1/2018/WAB13/UNISZA/ 02/2. We would also like to thank all laboratory staff in Faculty Bioresources and Food Industry, Universiti Sultan Zainal Abidin, Besut Campus for their support in laboratory work.

AUTHOR CONTRIBUTIONS

All authors have contributed their parts evenly throughout the duration of this study.

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