Environmental DNA and Specific Primers for Detecting the Invasive Species *Ectopleura crocea* (Hydrozoa: Anthoathecata) in Seawater Samples

Philjae Kim 1, Tae Joong Yoon 2 and Sook Shin 2,3,*

1 Research & Development Division, National Science Museum, Daejeon 34143, Korea; swubio@naver.com
2 Institute of Marine Life Resources, Sahmyook University, Seoul 01795, Korea; redyoon@gmail.com
3 Department of Animal Biotechnology & Resource, Sahmyook University, Seoul 01795, Korea
* Correspondence: shins@syu.ac.kr; Tel.: +82-2-3399-1717

Received: 31 January 2020; Accepted: 16 March 2020; Published: 18 March 2020

Abstract: In marine environments, environmental DNA (eDNA) can be effectively detected and possibly quantified when combined with molecular techniques, as demonstrated by several recent studies. In this study, we developed a species-specific primer set and a probe to detect the distribution and biomass of an invasive hydrozoan in South Korea, *Ectopleura crocea*. These molecular markers were designed to amplify a 187 bp region based on mitochondrial cytochrome c oxidase subunit I (COI) of *E. crocea* and were tested on seawater samples from 35 Korean harbors in 2017. Of the 35 sites we investigated, only nine harbors returned positive detections when using traditional survey methods, while surveys based on the use of eDNA techniques detected *E. crocea* DNA in all seawater samples. These results suggest that eDNA surveys based on molecular techniques are more effective at identifying species distribution and estimating biomass than traditional surveys based on visual assessment of morphology.

Keywords: *Ectopleura crocea*; environmental DNA; invasive species; monitoring; species-specific markers; quantitative PCR

1. Introduction

Environmental DNA (eDNA) refers to DNA that can be extracted from environmental samples such as soil, sediments, water, or snow [1], and species detection methods using eDNA have become new monitoring tools for the study and management of organisms in various ecosystems [2–4]. Organisms shed small amounts of DNA into their habitat (for example, in feces, skin cells, saliva, or other secretions); therefore, eDNA sampling methods provide an option for the rapid detection of species without physical capture or visual confirmation based on morphology [5]. This method, therefore, has frequently been applied to the detection and monitoring of invasive, rare, and endangered species [6–10]. In marine ecosystems, in which it is difficult to detect organisms visually, the use of eDNA for genetic monitoring has been tested in several recent studies, which suggest that eDNA is distributed within seawater and can be effectively used for detecting and possibly quantifying species presence [3,11].

Invasive species change the ecosystems that they invade and are a threat to native communities [12]. In marine environments, the uncontrolled spread (e.g., via ballast water, floating debris, and attachment to ship hulls and so on) of invasive species afflicts marine resources, such as fisheries, aquaculture, tourism, and marine infrastructure [13]. Once an invasive species is widespread, its management can become unfeasible owing to the costs of control or eradication, which often increase exponentially [14]. Therefore, early detection can be critical for effective control and management of invasive species.
However, traditional detection methods have limited utility: (1) misidentification of target species [15], (2) detection of populations at low densities [7,16], (3) observation of particular life stages of species [17], and (4) detection of early stages of introduction, especially in aquatic environments with low visibility [18,19]. These limitations of traditional methods have led many researchers to develop new approaches, including methods involving the analysis of eDNA using species-specific molecular markers for monitoring invasive species [15,20–22]. These eDNA methods have been applied to various invasive species, for example, amphibians [23], fish [7], reptiles [24,25], arthropods [3], gastropods [26], bivalves [9,27], tunicates [28] and bryozoans [29].

*E. crocea* is a hydrozoan species belonging to the class Hydrozoa, order Anthoathecata, family Tubulariidae, and a biofouling species alongside other taxa such as bryozoans, tunicates, sponges, and macroalgae [30–32]. Many biofouling species cause problems for the aquaculture industry by covering the infrastructure used in farming, for example, ropes, pipes, and cages and by attaching to the shells of the farmed organisms [33–35]. *E. crocea* is native to the northern Atlantic Ocean and spread to the Pacific Ocean via attachment to the hulls of ships [36]. Presently, it has a global distribution, including Australia; New Zealand; the Pacific and Atlantic coasts of the USA; Europe, including the Mediterranean; and Japan [37]. It was previously found that biofouling by *E. crocea* caused a reduction in mussel growth and reduced their food consumption through competitive interference [38,39]. In Korea, *E. crocea* was first reported on Daheuksan Island in 1966 [40] (Figure 1) and is now distributed nationwide.

![Figure 1. Sites of *E. crocea* detection by field survey with quantitative polymerase chain reaction (qPCR) analysis. The sites at which *E. crocea* was detected using both field survey and qPCR are marked as ●; ○ indicates the sites at which *E. crocea* was detected using qPCR analysis alone. The star (*) indicates the sampling sites for the molecular analysis.](image)

In this study, we developed species-specific primers and a probe for detecting the distribution and abundance of the invasive hydrozoan *E. crocea*, in South Korea. The aim was to apply an eDNA-based monitoring method with quantitative polymerase chain reaction (qPCR) analysis to this marine invasive species. Our study tested whether eDNA can be used to successfully detect invasive benthic hydroids...
from marine environments and used a qPCR method that could quantify the number of detected target species based upon DNA to estimate the biomass of organisms, rather than the conventional PCR method that only establishes the presence or absence of DNA. We also compared the eDNA method to the traditional visual surveying method based on morphological assessment. Additionally, we examined the spatial distribution of E. crocea along the Korean coast, and the Ct value of qPCR for the estimation of its DNA concentration was measured using the eDNA approach.

2. Materials and Methods

2.1. Sample Collection

Korean E. crocea colonies were collected in September 2016 from shallow water at depths of 0.5–5.0 m from Geojin (38°26′53.06″N, 128°27′46.04″E), Mulchi (38°09′21.08″N, 128°36′32.01″E), and Jeju (33°31′17.05″N, 126°32′12.01″E) harbors in South Korea (Figure 1). The collected samples were immediately fixed with 95% ethanol and stored at room temperature until DNA extraction.

2.2. Molecular Identification of E. crocea

2.2.1. DNA Extraction, Amplification, and Sequencing

Total genomic DNA was isolated from each of the three samples of E. crocea colonies using a DNeasy Blood & Tissue DNA isolation kit (QIAGEN, Hilden, Germany), following the manufacturer’s instructions. The quality and concentration of the isolated genomic DNA was measured using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). All genomic DNA samples were stored at −20 °C until use.

The amplification of the barcode region of the mitochondrial cytochrome c oxidase subunit I (COI) was performed using the universal primers LCO1490 and HCO2198 [41] (Table 1) in a total volume of 25 μL, containing 2.5 μL of 10X Ex Taq Buffer containing 20 mM MgCl₂ (Clontech, California, USA), 1 μL of 2.5 mM dNTPs (Clontech, California, USA), 1 μL of each primer at 10 pmol, 1.5 μL of 150–250 ng/μL of template DNA, 0.3 μL of 5 U/μL of Taq polymerase (Clontech, California, USA), and 17.7 μL of distilled water. The PCR conditions were as follows: an initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 sec, annealing at 50 °C for 90 sec, extension at 72 °C for 90 sec, and a final extension at 72 °C for 7 min. The PCR products were separated using electrophoresis on 1% agarose gel stained with ethidium bromide using 1X TAE buffer (Bioneer, Daejeon, Korea), and 100-bp DNA ladders (Elpis Biotech, Daejeon, Korea) were loaded at 2 μL to confirm the size.

| Primer & Probe | Sequence | Source |
|---------------|----------|--------|
| LCO1490       | 5'-GGTCAACAAATCATAAAGATATTGG-3' | Folmer et al. (1994) [41] |
| HCO2198       | 5'-TAA ACTTCAGGGTGACCAAAAAATCA-3' | In this study |
| EcCr_SF       | 5'-TCTTTACTAGGAGATGACCATCTC-3' | |
| EcCr_SR       | 5'-AGTCAGAAAACCTAARTTATTAAGT-3' | |
| EcCr_Probe    | 5'-6-Fam-TGCCAGTTTTAATTGGGTGATTTGG-BHQ-1-3' | |

The PCR products were directly sequenced in both directions, with the primers used for amplification (Cosmogenetech, Seoul, South Korea). The sequences obtained were edited using BioEdit [42] to assess the quality of sequencing and also compared with international databases employing the BLASTn program within the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) to confirm the species identification. Three E. crocea sequences were deposited in the GenBank of the NCBI (accession numbers: MH809674–MH809676).
2.2.2. Sequence and Phylogenetic Analyses

We obtained the sequences registered in NCBI for phylogenetic analysis using the COI barcode region used for species identification. The 13 sequences of different lengths were aligned using Clustal X [43], and 436 bp of the COI sequences, which is the overlap of all the sequences, were selected. The 436 bp of the COI sequences were aligned using Clustal X [43] and the genetic distance was calculated using MEGA 7.0 [44]. Furthermore, we investigated the genetic distances and phylogenetic relationships of three Korean *E. crocea* with two other *E. crocea* and eight sequences from other *Ectopleura* species registered in the NCBI: *E. crocea* (JX121589.1 from Switzerland; JX898187.1 from Brazil), *E. marina* (JX121592.1 from Switzerland), *E. larynx* (GU812435.1, JN109193.1, and JX121591.1 from Switzerland; and KT601631.1 from the USA), *E. dumortierii* (JX121590.1 from Switzerland; and KC440096.1 from Germany), *E. wrighti* (JX121593.1 from Switzerland). Genetic distances were calculated according to the Kimura 2-parameter (K2P) model [45,46], and bootstrap analysis was conducted with 1000 replicates. Phylogenetic analysis was performed using the neighbor-joining method with MEGA 7.0 [44]. The hydrozoan *Solanderia secunda* (JX121599.1) (Hydrozoa: Anthoathecata: Solandertiidae) was chosen as outgroup for the phylogenetic analysis.

2.3. Species-Specific Primers and eDNA

2.3.1. *E. crocea*-Specific Primers and Probe Design

We designed species-specific primers for amplifying short target regions of the COI DNA of *E. crocea* (Table 1). The COI DNA sequences of marine species belong to various taxonomic classes, such as Demospongiae (Porifera); Hydrozoa, Scyphozoa, and Anthozoa (Cnidaria); Gymnolaemata (Bryozoa); Hexanauplia (Arthropoda); Crinoidea, Asteroidea, Ophiuroidea, Echinoidea, and Holothuroidea (Echinodermata); and Ascidiae (Chordata) were obtained from GenBank (available in Appendix A, Table A1). To design the new primers, sequences were aligned using Clustal X [43] and analyzed to determine regions that were conserved for *E. crocea* but sufficiently variable in related species. The probe was designed as a nucleotide sequence with high GC content and a high probability of binding (a conserved region in *E. crocea* showing low intraspecific variation) in the sequence amplified with species-specific primers.

For determination of primer specificity, we used the genomic DNA of related and co-occurring marine benthic invertebrates belonging to the classes Ascidiae, Asteroidea, Crinoidea, Echinoidea, Gymnolaemata, Holothuroidea, Hydrozoa, and Ophiuroidea (Table 2). The *E. crocea*-specific region was amplified using the same PCR mixture and thermal cycling conditions as the COI amplification except for the primers used, which were the *E. crocea*-specific primers developed in this study, and the annealing temperature (62 °C). The PCR products were separated using electrophoresis on 1% agarose gel stained with ethidium bromide (Bioneer, Daejeon, Korea) and 100-bp DNA ladders (Elpis Biotech, Daejeon, Korea) were loaded at 2 µL to confirm the size.

To confirm whether the *E. crocea*-specific primers accurately amplified the COI region we expected the PCR products were directly sequenced in both directions with the primers used for amplification (Cosmogenetech, Seoul, Korea), and the sequences obtained were confirmed using BioEdit [42].
Table 2. List of species used for the determination of the specificity of *E. crocea*-specific primers.

| Taxon | Date | Location |
|-------|------|----------|
| Phylum CNIDARIA | - | - |
| Class Hydrozoa | - | - |
| *Ectopleura crocea* | 2016 | Geojin |
| *Bougainvillia ramosa* | 2016 | Incheon |
| *Campanularia hincksii* | 2016 | Yeosu |
| *Obelia dichotoma* | 2016 | Gunsan |
| *Obelia longissima* | 2016 | Mokpo |
| *Laomedea calceolifera* | 2017 | Busan |
| Phylum CHORDATA | - | - |
| Class Ascidiacea | - | - |
| *Ascidella aspersa* | 2017 | Chuksan |
| *Herdmania momus* | 2016 | Seongsanpo |
| Phylum BRYOZOA | - | - |
| Class Gymnolaemata | - | - |
| *Bugula neritina* | 2016 | Anmok |
| *Tricellaria occidentalis* | 2016 | Ulsan |
| *Watersipora subtorquata* | 2016 | Jongdal |
| Phylum ECHINODERMATA | - | - |
| Class Crinoidea | - | - |
| *Antedon serrata* | 2009 | Busan |
| *Heliometra glacialis* | 2016 | Daejin |
| Class Asteroida | - | - |
| *Patiria pectinifera* | 2016 | Juckbyeon |
| *Asterias amurensis* | 2016 | Dadaepo |
| Class Ophiuroidea | - | - |
| *Ophiactis savignyi* | 2016 | Dodu |
| *Ophiopholis mirabilis* | 2016 | Gampo |
| Class Echinoidea | - | - |
| *Temnopleurus hardwickii* | 2016 | Mipo |
| *Prionocidaris japonica* | 2012 | Aewol |
| Class Holothuroidea | - | - |
| *Eupentacta chronhjelmi* | 2016 | Tongyeong |
| *Protanksia bidentata* | 2015 | Incheon |

2.3.2. Collection and Isolation of eDNA

To confirm whether *E. crocea*-specific DNA fragments could be successfully amplified from environmental samples, we collected 8 L of seawater using a plastic beaker (2 L) at a depth of 0.5–1.0 m from each of the 35 harbors or ports investigated in 2016 (Table 3, Figure 1). A large clog was removed from the collected seawater using 300 µm nylon mesh, and the seawater was then filtered using a 3.0 µm nitrocellulose membrane (Merck Millipore, Darmstadt, Germany) [47,48]. The collected seawater samples were vacuum filtered using filtration bottles. The filtration discs were soaked in distilled water (5 min) and washed three times with DNA-free distilled water after each filtering [47].

The filtered membranes were stored in sample tubes, which were maintained in an icebox that contained dry ice (−70 °C) and transported to the laboratory for extraction of eDNA. In each experimental step, we wore latex gloves and used clean equipment (e.g., beakers and filtering tools) for each sample to avoid contamination.

eDNA was isolated from the membrane using a Genomic DNA Extraction Kit (Bioneer, Daejeon, Korea), following the manufacturer’s instructions with modifications (i.e., 400 µL ddH$_2$O was used in the elution instead of the elution buffer supplied with the kit). The quality and concentration of eDNA were measured using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA), and then all eDNA samples were stored at −20°C until use.
Table 3. Seawater sampling sites in the field survey.

| Number | Region       | Location      | Latitude   | Longitude  |
|--------|--------------|---------------|------------|-----------|
| 1      | East Sea     | Daejin        | 38°29′56.61″N | 128°25′34.18″E |
| 2      |              | Geojin        | 38°26′53.14″N | 128°27′46.37″E |
| 3      |              | Ganghyunjin   | 38°21′22.38″N | 128°30′43.26″E |
| 4      |              | Jangsa        | 38°13′35.24″N | 128°35′16.66″E |
| 5      |              | Jumunjin      | 37°53′31.41″N | 128°49′47.61″E |
| 6      |              | Sacheon       | 37°50′11.44″N | 128°52′42.30″E |
| 7      | Korea Strait | Mukho         | 37°33′15.38″N | 129°07′06.64″E |
| 8      |              | Donghae       | 37°29′21.30″N | 129°07′23.35″E |
| 9      |              | Imwon         | 37°13′46.03″N | 129°20′45.80″E |
| 10     |              | Jukbyeon      | 37°03′17.32″N | 129°25′26.30″E |
| 11     |              | Chusun        | 36°30′33.25″N | 129°26′54.35″E |
| 12     |              | Guryongpo     | 35°59′22.58″N | 129°33′19.97″E |
| 13     |              | Yangpo        | 35°52′56.34″N | 129°31′35.13″E |
| 14     | Korea Strait | Ulsan         | 35°31′09.47″N | 129°22′24.47″E |
| 15     |              | Bangeojin     | 35°29′05.79″N | 129°25′44.76″E |
| 16     |              | Daebyeon      | 35°13′29.29″N | 129°13′41.95″E |
| 17     |              | Busan         | 35°07′02.60″N | 129°02′55.49″E |
| 18     |              | Kwangyang     | 34°54′55.32″N | 127°40′52.40″E |
| 19     |              | Tongyeong     | 34°50′23.05″N | 128°25′12.58″E |
| 20     |              | Mokpo         | 34°46′51.50″N | 126°22′59.76″E |
| 21     | Yellow Sea   | Bieung        | 35°56′11.21″N | 126°31′38.01″E |
| 22     |              | Incheon       | 37°27′34.05″N | 126°37′32.32″E |
| 23     | Jeju Island  | Jeju          | 33°31′13.94″N | 126°32′11.91″E |
| 24     |              | Jocheon       | 33°32′26.53″N | 126°38′08.00″E |
| 25     |              | Bukchon       | 33°33′10.65″N | 126°41′55.92″E |
| 26     |              | Gimnyeong     | 33°33′30.54″N | 126°44′11.94″E |
| 27     |              | Jongdal       | 33°28′23.88″N | 126°56′00.78″E |
| 28     |              | Seongsanpo    | 33°28′22.41″N | 126°56′04.58″E |
| 29     |              | Pyoseon       | 33°19′30.34″N | 126°50′47.13″E |
| 30     |              | Wimi          | 33°16′18.15″N | 126°39′48.39″E |
| 31     |              | Seogwipo      | 33°14′23.44″N | 126°33′52.28″E |
| 32     |              | Moseulpo      | 33°12′58.15″N | 126°15′01.93″E |
| 33     |              | Hallim        | 33°24′44.97″N | 126°15′20.51″E |
| 34     |              | Aewol         | 33°28′54.67″N | 126°19′58.14″E |
| 35     |              | Dodu          | 33°30′27.14″N | 126°27′55.53″E |

2.3.3. eDNA Amplification

We used qPCR to confirm the presence and abundance of *E. crocea* DNA in our seawater samples with species-specific primers and a probe that we developed in this study. qPCR was performed in a total volume of 20 µL of a buffer solution consisting of 10 µL of qPCR BIO Probe Mix Hi-ROX (PCR Biosystems, London, UK), 5 µL of template eDNA, 1 µL of TaqMan probe at 5 pmol (Metabion, Martinsried, Germany), 1 µL of each primer at 10 pmol (Cosmogenetech, Seoul, Korea), and 2 µL of diethylpyrocarbonate (DEPC)-treated water. The cycling protocol, with optimum temperature, was as follows: 95 °C for 3 min, followed by 45 cycles at 95 °C for 5 sec (denaturation) and 60 °C for 30 sec (annealing/extension), using an Applied Biosystems thermal cycler (Applied Biosystems, California, USA). For confirmed contamination, we used the distilled water as template in the experiment.

For quantification of the DNA copies, standards of known amounts of the template were created using DNA cloning (GNC Bio, Daejeon, Korea). We amplified the PCR product using species-specific primers we developed (EcCr_SF and EcCr_SR) and cloned into the pGEM-T Easy vector (Promega, USA). Cloning DNA, which calculated the number of copies [49], was serially diluted 10-fold and used as a template for the standard curve. We estimated the quantity of *E. crocea* DNA in 8 L of seawater using a formula obtained from the y-intercept and slope, with which the number of detected DNA
copies could be calculated. The number of copies of *E. crocea* DNA was estimated by substituting the Ct value in the formula to estimate species biomass. PCR efficiency could be calculated by substituting the slope value in the following formula: $\text{PCR efficiency} = (10^{-\text{slope}}) - 1$ [50]. Also, we defined the limit of detection (LOD) and limit of quantification (LOQ) for the *E. crocea* eDNA analysis as the lowest values of the linear range covered by the standard curve we obtained. A cut-off value was determined at Ct 41, which increase the probability of false detection at a high Ct value. The Ct values detected below 41 were decided to be a reliable value.

All work, including the molecular techniques, was performed in the Molecular Phylogenetic Laboratory at Sahmyook University, South Korea.

### 3. Results

The barcode region of the COI gene (658 bp) in the *E. crocea* specimens collected from Geojin, Mulchi, and Jeju harbors was amplified and sequenced successfully using universal primers. Genetic variation among the three *E. crocea* sequences was very low at 0–0.2%. Based on the sequences (436 bp) of *E. crocea* that we obtained from GenBank, intraspecific variation within *E. crocea* was 0.0–1.1%; interspecific variation among species of the same genus (*Ectopleura*) was 17.6–23.2% and was 31.8–35.0% among species of the same class (Hydrozoa) (data available in Appendix A, Table A2). In the neighbor-joining tree, the *E. crocea* from South Korea formed a clade with *E. crocea* from Switzerland and Brazil (Figure 2).

![Figure 2.](image)

**Figure 2.** Neighbor-joining tree of the aligned 436 bp partial COI mt-DNA sequence for *E. crocea* and other *Ectopleura* species (obtained from GenBank). Sampling locations and GenBank accession numbers of the species are provided. Bootstrap resampling values were supported at ≥70. The scale bar represents the genetic distance.

The *E. crocea*-specific primers (EcCr_SF (forward) and EcCr_SR (reverse)) were designed based on the barcode region of the COI gene of *E. crocea* species with related species and co-occurring marine invertebrates, including species in Arthropoda, Bryozoa, Chordata, Cnidaria, Echinodermata, and Porifera (Tables 1 and A2). The alignment with 223 COI sequences of related marine invertebrate species showed differences at the sequence level, and even for closely related hydrozoan species, there are apparent differences in the *E. crocea*-specific primer binding regions. The primer specificity was verified using PCR amplification for the different species, including native and non-native species inhabiting the Korean coasts (Table 2). Subsequently, the species-specific primer pair successfully produced a single, clear 187 bp band for three *E. crocea* DNA samples (confirmed using sequencing), and these primers did not amplify any non-target species at 62 °C (Figure 3).
Figure 3. Determination of the specificity of *E. crocea*-specific primers using agarose gel electrophoresis of PCR products using species-specific primers for target and outgroup species. 1) DNA size marker 100 bp ladder, 2–4) *E. crocea*, 5) *Bougainvillia muscus*, 6) *Campanularia hincksii*, 7) *Obelia dichotoma*, 8) *Obelia longissima*, 9) *Laomedea calceolifera*, 10) *Asciella aspersa*, 11) *Ciona robusta*, 12) *Bugula neritina*, 13) *Tricellaria occidentalis*, 14) DNA size marker 100 bp ladder, 15) *Watersipora subtorquata*, 16) *Antedon serrata*, 17) *Heliometra glacialis*, 18) *Patiria pectinifera*, 19) *Asterias amurensis*, 20) *Ophiactis savignyi*, 21) *Ophiopholis mirabilis*, 22) *Tennopleurus hardwickii*, 23) *Phalacrocidaris japonica*, 24) *Eupentacta chronhjelmi*, 25) *Protankyra bidentata*, 26) DNA size marker 100 bp ladder.

We developed the applicable molecular markers for the development of a simple species detection tool with increased accuracy and efficiency, which can be used with seawater samples and applied to invasive species. We calculated the number of *E. crocea* DNA copies using a standard curve through the y-intercept and slope by substituting the Ct value into the formula of the standard curve (Figure 4) [51]. The amplification efficiency calculated from the slope (−3.1229) of the regression line was 109% (Figure 4).

Among the 35 sites monitored, *E. crocea* colonies were observed in only nine sites using traditional survey methods; however, eDNA from *E. crocea* was successfully amplified from all of the seawater samples we collected (Figure 1). All qPCR reactions showed target amplification from 33 to 39 Ct, and negative controls had no amplification response. The Ct values of sites 3, 4, 8, 9, and 11 from the East Sea coast; site 20 from the West Sea coast; and site 30 from Jeju Islands were included in the range from 33 to 35 and concentrations of *E. crocea* DNA were calculated as 5049–31,346 copies/L. In the case of site 1 from the East Sea coast and sites 23, 24, 25, 29, 31, and 33 from Jeju Island, Ct values were obtained in the range of 39–40 and the number of observed DNA copies was 258–400 copies/L. As a result, the survey sites at which the concentration of *E. crocea* DNA was relatively high appeared mainly along the east coast, whereas the survey sites in which the DNA concentration was relatively low tended to be found on Jeju Island. In addition, comparatively high *E. crocea* DNA concentrations were detected at sites 3, 4, 8, 9, 11, 20, and 30, at which no colonies of *E. crocea* were detected during the field survey.
Figure 4. Quantitative PCR assay for *E. crocea*. The template employed 10-fold serial diluted COI mt-DNA cloned plasmid DNA and Ct values were plotted over log DNA concentrations. The error bars indicate the standard deviations.

4. Discussion

A short DNA sequence (<1,000 bp) from the barcode region for species identification has been used in the development of molecular markers for target species detection in mixed samples of unknown species DNA as an alternative to morphological identification [52]. Candidate regions for barcoding must exhibit low intraspecific sequence variation but sufficiently high interspecific variation to unambiguously differentiate species; so, barcoding regions are chosen based on observable patterns of molecular evolution [53,54]. In many marine invertebrates, COI has been used for the DNA barcode region, for example, bivalves [55], bryozoans [56], cnidarians [57], echinoderms [58], and gastropods [59]. In our study, we amplified and compared the mitochondrial COI sequences of hydrozoan species belonging to the genus *Ectopleura* for phylogenetic analysis (Figure 2, Table A2). Our results showed that intraspecific variation was small (0.0–1.1%), while interspecific variation was extensive (17.6–23.2%). This finding suggests that COI could be a suitable region for molecular identification of hydrozoan species, and for designing species-specific markers for *E. crocea*.

The species-specific markers were designed based on specific nucleotide regions of target species so that hybridization can occur in the target species. Such an approach has clear advantages when reliable differentiation of specimens based on morphological characteristics is difficult, or when species are required to be rapidly identified without taxonomic expertise [60]. This molecular technique is also applicable to other studies; for example, the sampling and monitoring of target species in field surveys have previously depended on the taxonomic expertise or phylogenetic knowledge of those involved. However, a morphological determination may be hampered by damage to key characteristics for classification due to invasive trapping [61], and subsequent misidentification can occur [18]. Moreover, not all life stages are equally identifiable during traditional surveys (particularly when identifying larval specimens to the species-level) [15], but the use of species-specific markers enables the detection of cryptic life stages of the target species [7]. However, it should be noted that this may be a disadvantage in research that requires distinctions between juvenile and adult stages of the target species.

We attempted to design the species-specific primers to have the shortest possible amplified template lengths to increase the likelihood of target DNA detection in the eDNA method. This finding is significant for early species detection because short DNA fragments can persist in the environment.
for a relatively long time [9]. High degradation of extracellular DNA in environmental sources is caused by several factors such as water, UV radiation, enzymes, and activity of bacteria and fungi in the environment [62]. It was suggested that the rapid degradation of eDNA by various environmental factors negatively affects eDNA detection, especially in marine environments [63]. Primer pairs amplifying short fragments, therefore, are more appropriate for the detection of target DNA when the DNA of the target species is subject to degradation. The species-specific primers that amplified short DNA fragments (~100 bp) are appropriate for the detection of target nucleotides in degraded DNA samples [20]. We attempted to produce the shortest primers possible, and we designed the primers that amplify a 187 bp fragment of the *E. crocea* COI gene (Figure 3).

The advantages of eDNA surveying were revealed when compared with traditional monitoring techniques. The eDNA techniques are cost-effective, non-invasive, and independent of weather conditions. Furthermore, eDNA techniques also have a higher probability of detection and minimize handling time, errors, and sample amounts when monitoring species, such as when estimating population size, organism biomass, and predicting novel distribution ranges [64,65]. Our results suggest that eDNA techniques are efficient for detecting target species at more sites than field survey methods (Figure 1). It was considered that the high detection rate of short fragments acted on the efficacy of the eDNA methods. In addition, previous studies have reported that eDNA methods can be used to identify the relationship between eDNA concentration and the biomass of organisms [47,66]. For the qPCR assay, the amplification efficiency and coefficient of determination ($r^2$) of the individual detection systems have the minimum acceptance criteria of 90–110% and $>0.98$, respectively [67]. In our results, the PCR efficiency of the standard curve was 109% (Figure 4). We calculated the *E. crocea* DNA concentration in seawater samples using a standard curve and Ct value from qPCR data. At Jeju Island, all of the sites we investigated except one (site 30) have lower *E. crocea* DNA concentrations than other survey sites on the Korean peninsula. Site 30 showed extremely high DNA concentrations, but there was no organism present. While DNA concentrations were low at sites 23 and 25, *E. crocea* was detected using a visual survey. This finding suggests that the distribution of *E. crocea* species is difficult to predict directly from visual surveys, suggesting that eDNA analysis is appropriate for examining the distribution of aquatic organisms and applies to the study of the marine environment. With the eDNA method, we could indirectly estimate the biomass of *E. crocea* in distribution sites without the observation of organisms. We considered that eDNA concentration is a reasonable representation of the biomass. However, this is not in the case when estimating organism density. The following additional data are required for measuring density: 1) the amount of eDNA captured on the filter and the proportion of this eDNA subsequently obtained from DNA extraction, 2) the affinity of the primer for the target sequences, 3) the probability of PCR or sequencing, 4) errors during experimental steps and 5) comparing the area covered by an organism and the number of environmental sources sampled [68,69].

In our results, DNA was detected at nine sites (2, 7, 13, 15, 17, 21, 22, 23, and 25); non-detection of colonies is described in Figure 2. As we mentioned in a previous study, the results of eDNA studies could be interpreted in various ways, especially concerning the discordance between eDNA detection and visual survey methods [29]. There may have been contamination issues during the experimental procedures for sites from which identifications were obtained using eDNA methods with qPCR, despite no visual evidence of the presence of the organism during the field survey [18]. However, there was no evidence of contamination in the negative control. The positive results of eDNA methods can be interpreted as a result of the possibility of a correlation between an organism’s biomass and eDNA concentration, or the limitations of visual surveys based on traditional methods, because our target species is a marine organism and frequently hidden in its aquatic environment. Furthermore, it can be challenging to detect, especially at the larval stage. Moreover, for designing more effective species, specific markers in eDNA-based study and the nucleotide sequences of target species and relative species must be known. These limitations, such as contaminations and essential background data, explain why eDNA methods can not be independently used without traditional survey.
Herein, we designed and tested species-specific molecular markers using qPCR assays for the
detection of the invasive hydrozoan species *E. crocea*, which is widely known as a biofouling species in
South Korea. The molecular markers we designed successfully amplified its DNA from eDNA samples
even when mixed with the DNA of other species. In conclusion, we suggest the use of eDNA-based
species-specific markers for detecting target species DNA as a complement to traditional monitoring
tools. Furthermore, the eDNA-based methods, coupled with seawater sampling, may help to improve
the management of invasive species through efficient early detection.

**Author Contributions:** Conceptualization: T.J.Y.; Data curation: P.K.; Funding acquisition: S.S.; Investigation:
P.K.; Project administration: T.J.Y.; Supervision: S.S.; Writing—original draft: P.K.; Writing—review & editing:
T.J.Y. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was a part of the project titled ‘Improvement of management strategies on marine
disturbing and harmful organisms’, funded by the Ministry of Oceans and Fisheries, Korea. The grant number
is 1525009457.

**Conflicts of Interest:** The authors declare no conflicts of interest.

**Appendix A**

**Table A1.** Supplementary data about the design of *E. crocea*-specific primers in this study.

| Phylum   | Class         | Species                    | Accession Number       |
|----------|---------------|----------------------------|------------------------|
| Porifera | Demospongiae  | *Amphimedon compressa*     | EU257474               |
|          |               | *Aplysina fulva*           | EU257476               |
|          |               | *Callypogorgia plicifera*  | EU257477               |
|          |               | *Chondrilla macula*        | EU257478               |
|          |               | *Halicera deajerdini*      | EU257483               |
|          |               | *Hippopononga taehne*      | EU257484               |
|          |               | *Igernella notabilis*      | EU257485               |
|          |               | *Iotrocheta broenula*      | EU257486               |
|          |               | *Plakinastrella enkades*   | EU257487               |
|          |               | *Topsentia ophiophidites*  | EU257482               |
|          |               | *Vaceletta sp.*            | EU257489               |
|          |               | *Xestospongia muta*        | EU257490               |
|          |               | *Clava multicornis*        | JN700035, NC_014465    |
|          |               | *Craspedacuta scutellata*  | JN593332, NC_018537    |
|          |               | *Cubaia aphrodite*         | JN700042, NC_014467    |
|          |               | *Hydra magnipapillata*     | NC_011221              |
|          |               | *Hydra oligactis*          | EU237491, NC_010214    |
|          |               | *Hydra sinensis*           | JX089976, NC_021406    |
|          |               | *Hydra vulgaris*           | HM369413, HM369414     |
|          |               | *Laomedea flexuosa*        | JN700045, NC_014463    |
|          |               | *Turritopsis dohrnii*      | KT020766, KT999857, NC_031213 |
| Cnidaria | *Aurelia aurita* | DQ787873, HQ694729, NC_018446 |
|          | *Aurelia sp.*  | LC005413, LC005414         |
|          | *Cassiopea frenosa* | JN700036, NC_014666     |
|          | *Chrysaora quinquecirrha* | HQ694730, NC_020459   |
|          | *Craspedacuta scutellata* | JN93332                |
|          | *Helichythus antarcticus* | KU947038, NC_030337    |
| Anthozoa | *Alveopora allingsi* | AB007079                |
|          | *Alveopora catalina* | AB007081                |
|          | *Alveopora exscura* | AB007085                |
|          | *Alveopora japonica* | AB007087                |
|          | *Alveopora sp.*  | KJ634271                |
|          | *Alveopora spongiosa* | AB007093               |
|          | *Alveopora tirsardi* | AB007096                |
|          | *Alveopora czerilliana* | AB007097               |
| Phylum          | Class          | Species                        | Accession Number         |
|-----------------|----------------|--------------------------------|--------------------------|
| Arthropoda      | Hexanauplia    | Acasta sulcata                 | KJ754818, NC 029168      |
|                 |                | Amphipholias amphithre          | KF580709, NC 024525      |
|                 |                | Armobalanus allium             | KJ754817, NC 029167      |
|                 |                | Balanus balanus                | KM666676, NC 026466      |
|                 |                | Capitulata metella             | A167462                  |
|                 |                | Chelonibia testudinaria         | KJ754819, NC 029169      |
|                 |                | Chthamalus antennatus          | KP294312, NC 026730      |
|                 |                | Epopelina plicata              | KM008743, NC 033933      |
|                 |                | Lepas anserifera               | KP294311, NC 026576      |
|                 |                | Lepas australis                | KM017964, NC 025295      |
|                 |                | Megabalanus ajax               | KF010146, NC 024636      |
|                 |                | Megabalanus vesico             | AB167539, NC 026923      |
|                 |                | Pollicipes mitella             | A1514042                 |
|                 |                | Pollicipes polymerus           | AY456188, NC 030936      |
|                 |                | Striatobalanus amarillus       | KF493890, NC 024526      |
|                 |                | Tetraclita japonica            | AB126701, NC 018974      |
|                 |                | Tetraclita serrata             | KJ434948, NC 029315      |
|                 |                | Tetragritella divisa           | KJ754822, NC 029170      |
| Bryozoa         | Gymnolaemata   | Bugula dentata                 | KC129718                  |
|                 |                | Bugula decipiens               | AY690838, KC129722, KC129735, KC129734, KC129822 |
|                 |                | Bugula fulva                   | KY697919                  |
|                 |                | Bugula nigrota                 | KC129720                  |
|                 |                | Bugula nertina                 | AY690838, KC129722, KC129735, KC129734, KC129822 |
|                 |                | Bugula stolonifera             | KC129743                  |
|                 |                | Calliopera floridula           | J061319, NC 016722       |
|                 |                | Celleporella hyalina           | JQ258789, NC 008192      |
|                 |                | Flustra foliacea               | EU369397                  |
|                 |                | Membranipora grandicella       | NC 018585                 |
|                 |                | Tubulipora flabellaris         | EU35892, NC 011820       |
| Crinoidea       |                | Antedon mediterranea           | AM40730, NC 031989       |
|                 |                | Flavometra serrata             | NC 011828                 |
|                 |                | Neogymnocoma richeri           | DQ06995, NC 007089       |
|                 |                | Phanogorgia gracilis           | DQ06992, NC 003069       |
| Asteroidea      |                | Acanthaster brevispinus        | AB231475, NC 007789      |
|                 |                | Acanthaster planci             | AB231475, NC 007788      |
|                 |                | Apheraster japonica            | NC 025766                 |
|                 |                | Astoraster amurense            | AB183359, NC 016665      |
|                 |                | Astropecten polycanthus        | AB183360, NC 006666      |
|                 |                | Luidia quinata                 | AB183356                  |
|                 |                | Patiria pectinifera            | D16387                    |
| Ophiuroidea     |                | Amphipholis squamata           | FN62578, NC 013876       |
|                 |                | Astroastera mediterranea       | FN62580, NC 013878       |
|                 |                | Ophiura depressus              | NC 033878                 |
|                 |                | Ophiocoma nigra                | FN62577, NC 031874       |
|                 |                | Ophiocoma aculeata             | AF31839, NC 053374       |
|                 |                | Ophiura albidula               | AM404180, NC 016591      |
|                 |                | Ophiura linlerta               | AY184223, NC 003930      |
| Echinodermata   |                | Arbacia lixula                 | NC 001770                 |
|                 |                | Echinochondrus cordatus        | NC 033881                 |
|                 |                | Helicocidaris crassispina      | NC 023774                 |
|                 |                | Hemicentrotus pulcherrimus     | NC 023771                 |
|                 |                | Loxechinus albifrons           | NC 024177                 |
|                 |                | Meoanecintus franciscanus      | NC 020771                 |
|                 |                | Nacopsantangus alta            | NC 023255                 |
|                 |                | Paracentrotus lividus          | J04815                    |
|                 |                | Pseudocentrotus depressus      | KC409913, NC 023773      |
|                 |                | Sterechinus neumayeri          | NC 027063                 |
|                 |                | Strongyocephalanteres dubheincharissi | EU654306, NC 039040     |
|                 |                | Strongyocephalanteres intermedius | KC409912, NC 023772     |
|                 |                | Strongyocephalanteres pallidus | NC 02941                 |
|                 |                | Strongyocephalanteres purpuratus | NC 01453                 |
|                 |                | Teromaneus harziotickii        | NC 02509                  |
| Holothuroidea   |                | Apostichopus japonicus         | EU284194                  |
|                 |                | Balanoglossus clarigerus       | NC 013877                 |
|                 |                | Cucumaria miniata             | A133237                   |
|                 |                | Holothuria forskali            | NC 03384                  |
|                 |                | Holothuria scabra              | NC 027086                 |
|                 |                | Parantactopus californicus     | NC 02672                  |
|                 |                | Pariactopus nigricornatus      | NC 01344                  |
|                 |                | Pariactopus parvifrons         | NC 02967                  |
|                 |                | Peniagone sp.                  | K91535                    |
|                 |                | Stichopus horrens              | HQ00092, NC 01454        |
Table A1. Cont.

| Phylum     | Class          | Species                      | Accession Number                  |
|------------|----------------|------------------------------|-----------------------------------|
| Chordata   | Ascidiacea     | Aplidium conicum            | FN313538, NC 013584               |
|            |                | Aplidium tabanquensis        | HF548555                           |
|            |                | Ascidella aspersa            | HF548561, NC 021469               |
|            |                | Botryllides leachi           | HF548553, HC031923, NC 021410     |
|            |                | Botryllides nigrem           | HF485599, NC 012467               |
|            |                | Botryllides pizoni           | HF548554, HC931922, NC 021414     |
|            |                | Botryllides violaceus        | HF48553, NC 024256                |
|            |                | Botryllus schlosseri         | FM177702, HF48550, HC485555       |
|            |                | Ciona intestinalis           | HC931923, NC 021463               |
|            |                | Ciona intestinalis type B    | AM292218, NC 037929               |
|            |                | Ciona savignyi               | AM292604, NC 024105               |
|            |                | Clavelina lepadiformis       | NC 012887                          |
|            |                | Clavelina phlegrae           | AM292604, NC 024105               |
|            |                | Diecmanum vexillum           | KM259616, KM296617, NC 021410    |
|            |                | Diploloma lizierianum        | FN313539, NC 013566               |
|            |                | Halocynthia roretzi          | AB424928, NC 021464               |
|            |                | Halocynthia spinosa          | HF485599, NC 024166               |
|            |                | Herdmania momus              | AM292602, FN296153, NC 013561    |
|            |                | Microcosmus sulcatus         | AM292321, NC 033752               |
|            |                | Phallusia fumigata           | NC 009834                          |
|            |                | Phallusia mammillata         | AM292320, NC 024105               |
|            |                | Polycarpa mytiligera         | HF485556, NC 021464               |
|            |                | Pyura gangelion              | HF485570, NC 021468               |
|            |                | Rhodosoma turcicum           | HF485560, NC 021468               |
|            |                | Styela clava                 | HG931920                          |
|            |                | Styela plicata               | AM292601, NC 031565               |

Table A2. Pairwise distances between nucleotide sequences of mitochondrial COI according to phylogenetic calculations performed using MEGA 7.0. The distances and standard errors are shown in the lower-left matrix and upper-right matrix, respectively.

|    | 1     | 2     | 3     | 4     | 5     | 6     | 7     | 8     | 9     | 10    | 11    | 12    | 13    | 14    |
|----|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| 1  |       |       |       |       |       |       |       |       |       |       |       |       |       |       |
| 2  | -     | 0.000 | 0.002 | 0.000 | 0.004 | 0.019 | 0.019 | 0.020 | 0.020 | 0.020 | 0.019 | 0.018 | 0.022 | 0.029 |
| 3  | 0.000 | -     | 0.002 | 0.000 | 0.004 | 0.019 | 0.019 | 0.020 | 0.020 | 0.020 | 0.019 | 0.018 | 0.022 | 0.029 |
| 4  | 0.002 | 0.002 | -     | 0.002 | 0.004 | 0.019 | 0.019 | 0.020 | 0.020 | 0.020 | 0.019 | 0.018 | 0.022 | 0.029 |
| 5  | 0.000 | 0.000 | 0.002 | -     | 0.004 | 0.019 | 0.019 | 0.020 | 0.020 | 0.020 | 0.019 | 0.018 | 0.022 | 0.029 |
| 6  | 0.009 | 0.009 | 0.011 | 0.009 | -     | 0.019 | 0.019 | 0.020 | 0.020 | 0.020 | 0.019 | 0.018 | 0.022 | 0.029 |
| 7  | 0.190 | 0.190 | 0.192 | 0.190 | 0.187 | -     | 0.002 | 0.020 | 0.020 | 0.020 | 0.020 | 0.020 | 0.020 | 0.028 |
| 8  | 0.199 | 0.199 | 0.197 | 0.197 | 0.201 | 0.195 | 0.192 | -     | 0.009 | 0.008 | 0.014 | 0.020 | 0.021 | 0.028 |
| 9  | 0.204 | 0.204 | 0.202 | 0.204 | 0.211 | 0.192 | 0.195 | 0.038 | -     | 0.004 | 0.014 | 0.021 | 0.020 | 0.029 |
| 10 | 0.204 | 0.204 | 0.202 | 0.204 | 0.211 | 0.192 | 0.195 | 0.036 | 0.002 | -     | 0.014 | 0.020 | 0.020 | 0.029 |
| 11 | 0.190 | 0.190 | 0.192 | 0.190 | 0.192 | 0.201 | 0.204 | 0.103 | 0.104 | 0.102 | -     | 0.018 | 0.021 | 0.028 |
| 12 | 0.176 | 0.176 | 0.176 | 0.176 | 0.216 | 0.218 | 0.125 | 0.187 | 0.198 | 0.196 | 0.170 | -     | 0.022 | 0.027 |
| 13 | 0.232 | 0.232 | 0.229 | 0.232 | 0.229 | 0.229 | 0.201 | 0.196 | 0.212 | 0.204 | 0.207 | 0.206 | 0.211 | -     |
| 14 | 0.347 | 0.347 | 0.350 | 0.347 | 0.350 | 0.328 | 0.323 | 0.323 | 0.340 | 0.337 | 0.329 | 0.318 | 0.318 | 0.334 |

References

1. Rees, H.C.; Maddison, B.C.; Middleeditch, D.J.; Patmore, J.R.; Gough, K.C. Review: The detection of aquatic animal species using environmental DNA—A review of eDNA as a survey tool in ecology. *J. Appl. Ecol.* 2014, 51, 1450–1459. [CrossRef]
2. Foote, A.D.; Thomsen, P.F.; Sveegaard, S.; Wahlberg, M.; Kielgast, J.; Kyhn, L.A.; Salling, A.B.; Galatius, A.; Orlando, L.; Gilbert, M.T.P. Investigating the potential use of environmental DNA (eDNA) for genetic monitoring of marine mammals. *PLoS ONE* 2012, 7, e41781. [CrossRef] [PubMed]
3. Thomsen, P.F.; Kielgast, J.; Iversen, L.L.; Møller, P.R.; Rasmussen, M.; Willerslev, E. Detection of a diverse marine fish fauna using environmental DNA from seawater samples. *PLoS ONE* 2012, 7, e41732. [CrossRef] [PubMed]
4. Kelly, R.P.; Port, J.A.; Yamahara, K.M.; Crowder, L.B. Using environmental DNA to census marine fishes in a large mesocosm. *PLoS ONE* 2014, 9, e86175. [CrossRef]
5. Taberlet, P.; Coissac, E.; Pompanon, F.; Brochmann, C.; Willerslev, E. Towards next-generation biodiversity assessment using DNA metabarcoding. *Mol. Ecol.* 2012, 21, 2045–2050. [CrossRef] [PubMed]
6. Jerde, C.L.; Mahon, A.R.; Chadderton, W.L.; Lodge, D.M. “Sight-unseen” detection of rare aquatic species using environmental DNA. *Conserv. Lett.* 2011, 4, 150–157. [CrossRef]
7. Dejean, T.; Valentini, A.; Miquel, C.; Taberlet, P.; Bellemain, E.; Miaud, C. Improved detection of an alien invasive species through environmental DNA barcoding: The example of the American bullfrog Lithobates catesbeianus. *J. Appl. Ecol.* 2012, 49, 953–959. [CrossRef]

8. Wilcox, T.M.; McElvee, K.S.; Young, M.K.; Jane, S.F.; Lowe, W.H.; Whiteley, A.R.; Schwartz, M.K. Robust detection of rare species using environmental DNA: The importance of primer specificity. *PLoS ONE* 2013, 8, e59520. [CrossRef] [PubMed]

9. Ardura, A.; Zaiko, A.; Martinez, J.L.; Samulioviene, A.; Semenova, A.; Garcia-Vazquez, E. eDNA and specific primers for early detection of invasive species—a case study on the bivalve *Rangia cuneata*, currently spreading in Europe. *Mar. Environ. Res.* 2015, 112, 48–55. [CrossRef]

10. Ikeda, K.; Doi, H.; Tanaka, K.; Kawai, T.; Negishi, J. Using environmental DNA to detect an endangered crayfish *Cambareoides japonicas* in streams. *Conserv. Genet.* 2016, 8, 231–234. [CrossRef]

11. DiBattista, J.D.; Roberts, M.B.; Bouwmeester, J.; Bowen, B.W.; Coker, D.J.; Lozano-Cortés, D.F.; Choat, J.H.; Gaither, M.R.; Hobbs, J.A.; Khalil, M.T.; et al. A review of contemporary patterns of endemism for shallow water reef fauna in the Red Sea. *J. Biogeogr.* 2016, 43, 423–439. [CrossRef]

12. Gurevitch, J.; Padilla, D.K. Are invasive species a major cause of extinctions? *Trends Ecol. Evol.* 2004, 19, 470–474. [CrossRef] [PubMed]

13. Bax, N.; Williamson, A.; Aguero, M.; Gonzalez, E.; Geeves, W. Marine invasive alien species: A threat to global biodiversity. *Marine Policy* 2003, 27, 313–323. [CrossRef]

14. Vander Zanden, M.J.; Hansen, G.J.A.; Higgins, S.N.; Kornis, M.S. A pound of prevention, plus a pound of assessment of persistence of environmental DNA. *Mol. Ecol. Resour.* 2016, 16, 905–922. [CrossRef]

15. Gotelli, N.J.; Colwell, R.K. Quantifying biodiversity: Procedures and pitfalls in the measurement and comparison of species richness. *Ecol. Lett.* 2001, 4, 379–391. [CrossRef]

16. Darling, J.A.; Mahon, A.R. From molecules to management: Adopting DNA-based methods for monitoring biological invasions in aquatic environments. *Environ. Res.* 2011, 111, 978–988. [CrossRef]

17. DiBattista, J.D.; Roberts, M.B.; Bouwmeester, J.; Bowen, B.W.; Coker, D.J.; Lozano-Cortés, D.F.; Choat, J.H.; Gaither, M.R.; Hobbs, J.A.; Khalil, M.T.; et al. A review of contemporary patterns of endemism for shallow water reef fauna in the Red Sea. *J. Biogeogr.* 2016, 43, 423–439. [CrossRef]

18. Darling, J.A.; Mahon, A.R. From molecules to management: Adopting DNA-based methods for monitoring biological invasions in aquatic environments. *Environ. Res.* 2011, 111, 978–988. [CrossRef]

19. Golding, C.S.; Turner, C.R.; Deiner, K.; Klymus, K.E.; Thomsen, P.F.; Murphy, M.A.; Spear, S.F.; McKee, A.; Oyler-McCance, S.J.; Comman, R.S.; et al. Critical considerations for the application of environmental DNA methods to detect aquatic species. *Methods Ecol. Evol.* 2016, 7, 1299–1307. [CrossRef]

20. Hajibabaei, M.; Smith, M.A.; Janzen, D.H.; Rodrigue, J.J.; Whitfield, J.B.; Hebert, P.D.N. A minimalist barcode can identify a specimen whose DNA is degraded. *Mol. Ecol. Notes* 2006, 6, 959–964. [CrossRef] [PubMed]

21. Bohmann, K.; Evans, A.; Gilbert, M.T.P.; Carvalho, G.R.; Creer, S.; Knapp, M.; Yu, D.W.; De Bruyn, M. Environmental DNA for wildlife biology and biodiversity monitoring. *Trends. Ecol. Evol.* 2014, 29, 358–367. [CrossRef] [PubMed]

22. Dejean, T.; Valentini, A.; Miquel, C.; Taberlet, P.; Bellemain, E.; Miaud, C.; Civade, R.; Herder, J.; Thomsen, P.F.; Bellemain, E.; Besnard, A.; Coissac, E.; Boyer, F.; et al. Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. *Mol. Ecol.* 2016, 25, 929–942. [CrossRef]

23. Goldberg, C.S.; Pilliod, D.S.; Arkle, R.S.; Waits, L.P. Molecular detection of vertebrates in stream water: A demonstration using rocky mountain tailed frogs and Idaho giant salamanders. *PLoS ONE* 2011, 6, e22746. [CrossRef]

24. Piaggio, A.J.; Engeman, R.M.; Hopken, M.W.; Humphrey, J.S.; Keacher, K.L.; Bruce, W.E.; Avery, M.L. Detecting an elusive invasive species: A diagnostic PCR to detect Burmese python in Florida waters and an assessment of persistence of environmental DNA. *Mol. Ecol. Resour.* 2014, 14, 374–380. [CrossRef] [PubMed]

25. Davy, C.M.; Kidd, A.G.; Wilson, C.C. Development and validation of environmental DNA (eDNA) markers for detection of freshwater turtles. *PLoS ONE* 2015, 10, e0130965. [CrossRef] [PubMed]

26. Goldberg, C.S.; Sepulveda, A.; Ray, A.; Baumgardt, J.; Waits, L.P. Environmental DNA as a new method for early detection of New Zealand mudsnails (*Potamopyrgus antipodarum*). *Freshw. Sci.* 2013, 32, 792–800. [CrossRef]
27. Xia, Z.; Zhan, A.; Gao, Y.; Zhang, L.; Haffner, G.D.; Maclsaac, H.J. Early detection of a highly invasive bivalve based on environmental DNA (eDNA). *Biol. Invasions* 2017, 20, 437–447. [CrossRef]
28. Wood, S.A.; Pochon, X.; Ming, W.; von Ammon, U.; Woods, C.; Carter, M.; Smith, M.; Inglis, G.; Zaiko, A. Considerations for incorporating real-time PCR assays into routine marine biosecurity surveillance programmes: A case study targeting the Mediterranean fanworm (*Sabella spallanzanii*) and club tunicate (*Styela clava*). *Genome* 2019, 62, 137–146. [CrossRef]
29. Kim, P.; Kim, D.; Yoon, T.J.; Shin, S. Early detection of marine invasive species, *Bugula neritina* (Bryozoa: Cheilostomatida), using species-specific primers and environmental DNA analysis in Korea. *Mar. Environ. Res.* 2018, 139, 1–10. [CrossRef]
30. Lesser, M.P.; Shumway, S.E.; Cucci, T.; Smith, J. Impact of fouling organisms on mussel rope culture: Interspecific competition for food among suspension-feeding invertebrates. *J. Exp. Mar. Biol. Ecol.* 1992, 165, 91–102. [CrossRef]
31. De Sa, F.S.; Nalesso, R.C.; Paresgue, K. Fouling organisms on *Perna perna* mussels: Is it worth removing them? *Braz. J. Oceanogr.* 2007, 55, 155–161. [CrossRef]
32. McKindsey, C.W.; Lecuona, M.; Huot, M.; Weise, A.M. Biodeposit production and benthic loading by farmed mussels and associated tunicate epifauna in Prince Edward Island. *Aquaculture* 2009, 295, 44–51. [CrossRef]
33. Braithwaite, R.A.; McEvoy, L.A. Marine biofouling on fish farms and its remediation. *Adv. Mar. Biol.* 2005, 47, 215–252. [CrossRef] [PubMed]
34. De Nys, R.; Guenther, J. The impact and control of biofouling in marine finfish aquaculture. In *Advances in Marine Antifouling Coatings and Technologies*; Hellio, C., Yebra, D., Eds.; Woodhead: Cambridge, MA, USA, 2009; pp. 177–221. [CrossRef]
35. Dürr, S.; Watson, D.I. Biofouling and antifouling in aquaculture. In *Biofouling*; Dürr, S., Thomason, J.C., Eds.; Wiley-Blackwell: Oxford, UK, 2010; pp. 267–287.
36. Mills, C.E.; Marques, A.C.; Migotto, A.E.; Calder, D.R. Hydrozoa: Polyps, hydromedusae, and siphonophora. In *The Light and Smith Manual: Intertidal Invertebrates from Central California to Oregon*; Carlton, J.T., Ed.; University of California Press: Berkley, CA, USA, 2007; pp. 118–167.
37. Fitridge, I.; Keough, M.J. Ruinous resident: The hydroid *Ectopleura crocea* negatively affects suspended culture of the mussel *Mytilus galloprovincialis*. *Biofouling* 2013, 29, 119–131. [CrossRef]
38. Fitridge, I. The ecology of hydroids (Hydrozoa: Cnidaria) in Port Phillip Bay, Australia, and their impacts as fouling species in longline mussel culture. PhD thesis, Department of Zoology, The University of Melbourne, Melbourne, Australia, 2011.
39. Fitridge, I.; Dempster, T.; Guenther, J.; de Nys, R. The impact and control of biofouling in marine aquaculture: A review. *Biofouling* 2012, 28, 49–669. [CrossRef]
40. Rho, B.J. Studies on the marine hydroid in Korea. *J. Korean Res. Inst. Better Liv., Ewha Womans Univ.* 1969, 2, 161–174.
41. Folmer, O.; Black, M.; Hoeh, W.; Lutz, R.; Vrijenhoek, R. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol. Mar. Biol. Biotechnol.* 1994, 3, 294–299.
42. Hall, T.A. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Series* 1999, 41, 95–98.
43. Thompson, J.D.; Gibson, T.J.; Plewniak, F.; Jeanmougin, F.; Higgins, D.G. The CLUSTAL X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 1997, 25, 4876–4882. [CrossRef]
44. Kumar, S.; Stecher, G.; Tamura, K. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 2016, 33, 1870–1874. [CrossRef]
45. Kimura, M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* 1980, 16, 111–120. [CrossRef] [PubMed]
46. Collins, R.A.; Boykin, L.M.; Cruickshank, R.H.; Armstrong, K.F. Barcoding’s next top model: An evaluation of nucleotide substitution models for specimen identification. *Methods Ecol. Evol.* 2012, 3, 457–465. [CrossRef]
47. Takahara, T.; Minamoto, T.; Yamanaka, H.; Doi, H.; Kawabata, Z. Estimation of fish biomass using environmental DNA. *PLoS ONE* 2012, 7, e35868. [CrossRef] [PubMed]
48. Turner, C.R.; Barnes, M.A.; Xu, C.C.; Jones, S.E.; Jerde, C.L.; Lodge, D.M. Particle size distribution and optimal capture of aqueous macrobial eDNA. *Methods Ecol. Evol.* 2014, 5, 676–684. [CrossRef]

49. Smith, C.J.; Nedwell, D.B.; Dong, L.F.; Osborn, A.M. Evaluation of quantitative polymerase chain reaction-based approaches for determining gene copy and gene transcript numbers in environmental samples. *Environ. Microbiol.* 2006, 8, 804–815. [CrossRef]

50. Zhang, A.N.; Mao, Y.; Zhang, T. Development of quantitative real-time PCR assays for different clades of “Candidatus accumulibacter”. *Sci. Rep.* 2016, 6, 23993. [CrossRef]

51. Rasmussen, R.P. Quantification on the light cycler. In *Rapid Cycle Real-time PCR*; Meuer, S., Wittwe, R.C., Nakagawara, K.I., Eds.; Springer: Berlin/Heidelberg, Germany, 2011; pp. 21–34.

52. Darling, J.A.; Blum, M.J. DNA-based methods for monitoring invasive species: A review and prospectus. *Biol. Invasions* 2007, 9, 751–765. [CrossRef]

53. Blaxter, M.L. The promise of a DNA taxonomy. *Philos. Trans.Roy. Soc. Lond. Ser. B-Biol. Sci.* 2004, 359, 669–679. [CrossRef]

54. Hebert, P.D.N.; Gregory, T.R. The promise of DNA barcoding for taxonomy. *Syst. Biol.* 2005, 54, 852–859. [CrossRef]

55. Mikkelsen, N.T.; Schander, C.; Willassen, E. Local scale DNA barcoding of bivalves (Mollusca): A case study. *Zool. Scr.* 2007, 36, 455–463. [CrossRef]

56. Go’mez, A.; Wright, P.J.; Lunt, D.H.; Cancino, J.M.; Carvalho, G.R.; Hughes, R.N. Mating trials validate the use of DNA barcoding to reveal cryptic speciation of a marine bryozoan taxon. *Proc. R. Soc. B* 2007, 274, 199–207. [CrossRef] [PubMed]

57. Moura, C.J.; Harris, D.J.; Cunha, M.R.; Rogers, A.D. DNA barcoding reveals cryptic diversity in marine hydroids (Cnidaria, Hydrozoa) from coastal and deep-sea environments. *Zool. Scr.* 2007, 37, 93–108. [CrossRef]

58. Ward, R.D.; Holmes, B.H.; O’ hara, T.D. DNA barcoding discriminates echinoderm species. *Mol. Ecol. Resour.* 2008, 8, 1202–1211. [CrossRef] [PubMed]

59. Jennings, R.M.; Bucklin, A.; Ossenbrugger, H.; Hopcroft, R.R. Species diversity of planktonic gastropods (Pteropoda and Heteropoda) from six ocean regions based on DNA barcode analysis. *Deep-Sea Res. PT. II* 2010, 57, 2199–2210. [CrossRef]

60. Van de Vossenberg, B.T.L.H.; Ibanez-Justicia, A.; Metz-Verschure, E.; van Veen, E.J.; Bruil-Dieters, M.L.; Scholte, E.J. Real-time PCR Tests in Dutch Exotic Mosquito Surveys; Implementation of *Aedes aegypti* and *Aedes albopictus* Identification Tests, and the Development of Tests for the Identification of *Aedes atropalpus* and *Aedes japonicus japonicus* (Diptera: Culicidae). *J. Med. Entomol.* 2015, 52, 336–350. [CrossRef]

61. Barnes, M.A.; Turner, C.T.; Jerde, C.L.; Renshaw, M.A.; Chadderton, W.L.; Lodge, D.M. Environmental conditions influence eDNA persistence in aquatic systems. *Environ. Sci. Technol.* 2014, 48, 1819–1827. [CrossRef]

62. Strickler, K.M.; Premier, A.K.; Goldberg, C.S. Quantifying effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms. *Biol. Conserv.* 2015, 183, 85–92. [CrossRef]

63. Thomsen, P.F.; Willems, E. Environmental DNA—An emerging tool in conservation for monitoring past and present biodiversity. *Biol. Conserv.* 2015, 183, 4–18. [CrossRef]

64. Schneider, J.; Valentini, A.; Dejean, T.; Montarsi, F.; Taberlet, P.; Glazot, O.; Fumagalli, L. Detection of invasive mosquito vectors using environmental DNA (eDNA) from water samples. *PLoS ONE* 2015, 11, e0162493. [CrossRef]

65. Inui, R.; Akamatsu, Y.; Kanno, K.; Yamanaka, H.; Takahara, T.; Minamoto, T. Environmental DNA analysis for estimating the abundance and biomass of stream fish. *Freshw. Biol.* 2017, 62, 30–39. [CrossRef]

66. Huber, I.; Block, A.; Sebah, D.; Debode, F.; Morisset, D.; Grohmann, L. Development and validation of duplication, triplex, and pentaplex real-time PCR screening assays for the detection of genetically modified organisms in food and feed. *J. Agric. Food Chem.* 2013, 61, 10293–10301. [CrossRef] [PubMed]

67. Eichmiller, J.J.; Bajer, P.G.; Sorensen, P.W. The relationship between the distribution of common carp and their environmental DNA in a small lake. *PLoS ONE* 2014, 9, e112611. [CrossRef] [PubMed]
68. Matsunaga, K.; Togashi, K. A simple method for discriminating *Bursaphelenchus xylophilus* and *B. mucronatus* by species-specific polymerase chain reaction primer pairs. *Nematology* **2004**, *6*, 273–277. [CrossRef]

69. Thomsen, P.F.; Møller, P.R.; Sigsgaard, E.E.; Knudsen, S.W.; Jørgensen, O.A.; Willerslev, E. Environmental DNA from seawater samples correlate with trawl catches of subarctic, deepwater fishes. *PLoS One* **2016**, *11*, e0165252. [CrossRef] [PubMed]

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