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

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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 Daeheuksan Island in 1966 [40] (Figure 1) and is now distributed nationwide.

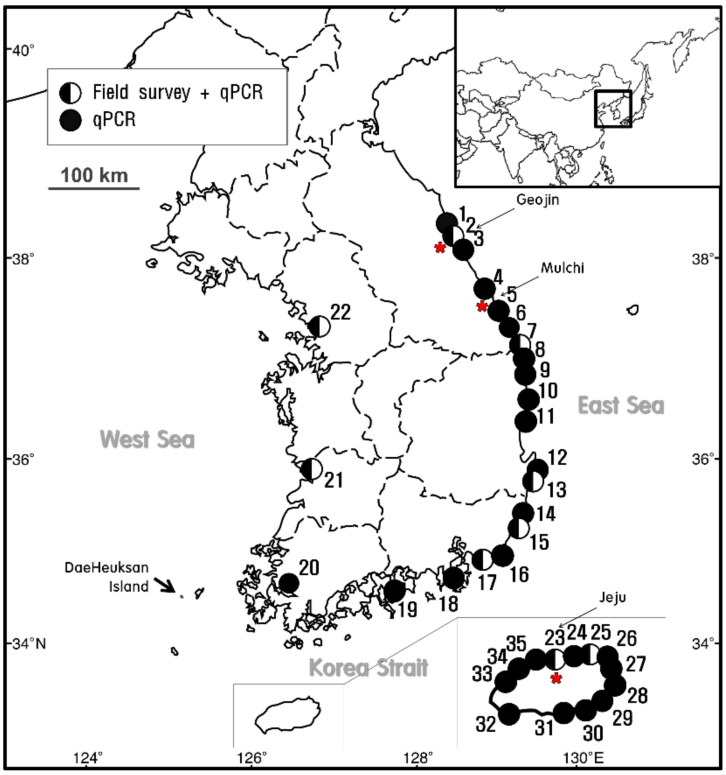

Figure 1. Sites of *E. crocea* detection by field survey with quantitative polymerase chain reaction (qPCR) analysis. The sites at which *E. crocea* was detected using both field survey and qPCR are marked as ◐; ● indicates the sites at which *E. crocea* was detected using qPCR analysis alone. The star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

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 $MgCl_2$ (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.

Table 1. Sequences of the universal primers used in the amplification of cytochrome *c* oxidase subunit I (COI) and the *E. crocea*-specific primers and probe designed in this study.

Primer & Probe	Sequence	Source
LCO1490	5′-GGTCAACAAATCATAAAGATATTGG-3′	Folmer et al. (1994) [41]
HCO2198	5′-TAA ACTTCAGGGTGACCAAAAAATCA-3′	
EcCr_SF	5′-TCTTTACTAGGAGATGACCATCTC-3′	In this study
EcCr_SR	5′-AGTCAGAAACTTAARTTATTAAGT-3′	
EcCr Probe	5′-6-Fam-TGCCAGTTTTAATTGGTGGATTTGG-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 phylogenic 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: Solanderiidae) 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 Ascidiacea (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 Ascidiacea, 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	-	-
	2016	Geojin
Ectopleura crocea	2016	Mulchi
	2016	Jeju
Bougainvillia ramosa	2016	Incheon
Campanularia hincksii	2016	Yeosu
Obelia dichotoma	2016	Gunsan
Obelia longissimi	2016	Mokpo
Laomedea calceolifera	2017	Busan
Phylum CHORDATA	-	-
Class Ascidiacea	-	-
Ascidiella 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 Asteroidea	-	-
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
Protankyra 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_2O 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		Gonghyunjin	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		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		Chuksan	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: PCR efficiency = $(10^{(-1/\text{slope})}) - 1$ [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).

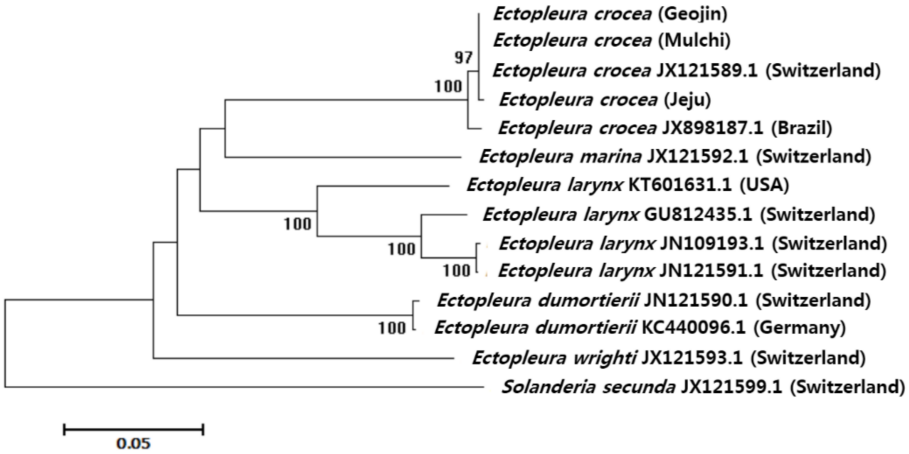

Figure 2. Neighbor-joining tree of the aligned 436 bp partial COI mt-DNA sequence for *E. crocea* and other *Ectopleura* species (obtained from GenBank). Sampling locations and GenBank accession numbers of the species are provided. Bootstrap resampling values were supported at ≥70. The scale bar represents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

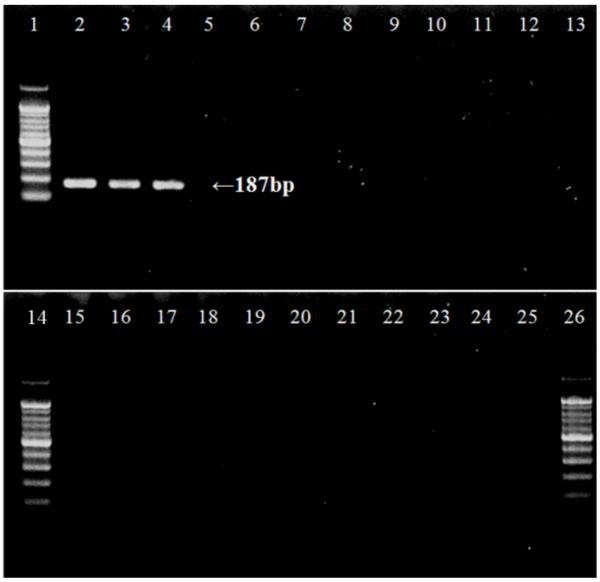

Figure 3. Determination of the specificity of *E. crocea*-specific primers using agarose gel electrophoresis of PCR products using species-specific primers for target and outgroup species. 1) DNA size marker 100 bp ladder, 2–4) *E. crocea*, 5) *Bougainvillia muscus*, 6) *Campanularia hincksii*, 7) *Obelia dichotoma*, 8) *Obelia longissima*, 9) *Laomedea calceolifera*, 10) *Ascidiella 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) *Temnopleurus 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.

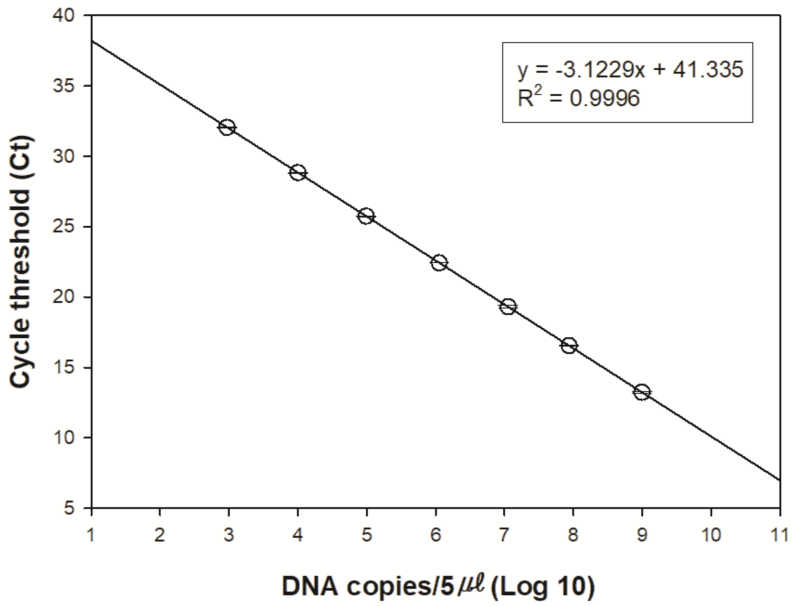

Figure 4. Quantitative PCR assay for *E. crocea*. The template employed 10-fold serial diluted COI mt-DNA cloned plasmid DNA and Ct values were plotted over log DNA concentrations. The error bars indicate the standard deviations.

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.

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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*	EU237474
		Aplysina fulva	EU237476
		Callyspongia plicifera	EU237477
		Chondrilla nucula	EU237478
		Halisarca dujardini	EU237483
		Hippospongia lachne	EU237484
		Igernella notabilis	EU237485
		Iotrochota birotulata	EU237486
		Plakinastrella onkodes	EU237487
		Topsentia ophiraphidites	EU237482
		Vaceletia sp.	EU237489
		Xestospongia muta	EU237490
Cnidaria	Hydrozoa	*Clava multicornis*	JN700935, NC 016465
		Craspedacusta sowerbyi	JN593332, NC 018537
		Cubaia aphrodite	JN700942, NC 016467
		Hydra magnipapillata	NC 011221
		Hydra oligactis	EU237491, NC 010214
		Hydra sinensis	JX089978, NC 021406
		Hydra vulgaris	HM369413
		Hydra vulgaris	HM369414
		Laomedea flexuosa	JN700945, NC 016463
		Turritopsis dohrnii	KT020766, KT899097, NC 031213
	Scyphozoa	*Aurelia aurita*	DQ787873, HQ694729, NC 008446
		Aurelia sp.	LC005413, LC005414
		Cassiopea frondosa	JN700936, NC 016466
		Chrysaora quinquecirrha	HQ694730, NC 020459
		Craspedacusta sowerbyi	JN593332
		Haliclystus antarcticus	KU947038, NC 030337
	Anthozoa	*Alveopora allingi*	AB907079
		Alveopora catalai	AB907081
		Alveopora excelsa	AB907085
		Alveopora japonica	AB907087
		Alveopora sp.	KJ634271
		Alveopora spongiosa	AB907093
		Alveopora tizardi	AB907096
		Alveopora verrilliana	AB907097

Table A1. *Cont.*

Phylum	Class	Species	Accession Number
Arthropoda	Hexanauplia	*Acasta sulcata*	KJ754818, NC 029168
		Amphibalanus amphitrite	KF588709, NC 024525
		Armatobalanus allium	KJ754817, NC 029167
		Balanus balanus	KM660676, NC 026466
		Capitulum mitella	AB167462
		Chelonibia testudinaria	KJ754819, NC 029169
		Chthamalus antennatus	KP294312, NC 026730
		Epopella plicata	KM008743, NC 033393
		Lepas anserifera	KP294311, NC 026576
		Lepas australis	KM017964, NC 025295
		Megabalanus ajax	KF501046, NC 024636
		Megabalanus volcano	AB167539, NC 006293
		Pollicipes mitella	AY514042
		Pollicipes polymerus	AY456188, NC 005936
		Striatobalanus amaryllis	KF493890, NC 024526
		Tetraclita japonica	AB126701, NC 008974
		Tetraclita serrata	KJ434948, NC 029154
		Tetraclitella divisa	KJ754822, NC 029170
Bryozoa	Gymnolaemata	*Bugula dentata*	KC129718
		Bugula flabellata	AY061749
		Bugula fulva	KC129719
		Bugula migottoi	KC129720
		Bugula neritina	AY690838, KC129722, KC129735, KC129735, KC129754, KC129822
		Bugula stolonifera	KC129849
		Bugula turrita	KC129850
		Celleporella hyalina	JQ839275, JQ839276, NC 018344
		Flustra foliacea	JQ061319, NC 016722
		Flustrellidra hispida	DQ157889, NC 008192
		Membranipora grandicella	NC 018355
		Tubulipora flabellaris	EU563937
		Watersipora subtorquata	EU365892, NC 011820
Echinodermata	Crinoidea	*Antedon mediterranea*	AM404181, NC 010692
		Florometra serratissima	NC 001878
		Neogymnocrinus richeri	DQ068951, NC 007689
		Phanogenia gracilis	DQ068952, NC 007690
	Asteroidea	*Acanthaster brevispinus*	AB231476, NC 007789
		Acanthaster planci	AB231475, NC 007788
		Aphelasterias japonica	NC 025766
		Asterias amurensis	AB183559, NC 006665
		Astropecten polyacanthus	AB183560, NC 006666
		Luidia quinalia	AB183558
		Patiria pectinifera	D16387
	Ophiuroidea	*Amphipholis squamata*	FN562578, NC 013876
		Astrospartus mediterraneus	FN562580, NC 013878
		Astrospartus mediterraneus	NC 013878
		Ophiacantha linea	NC 023254
		Ophiocomina nigra	FN562577, NC 013874
		Ophiopholis aculeata	AF314589, NC 005334
		Ophiura albida	AM404180, NC 010691
		Ophiura lutkeni	AY184223, NC 005930
	Echinoidea	*Arbacia lixula*	NC 001770
		Echinocardium cordatum	NC 013881
		Heliocidaris crassispina	NC 023774
		Hemicentrotus pulcherrimus	NC 023771
		Loxechinus albus	JX888466
		Mesocentrotus franciscanus	NC 024177
		Mesocentrotus nudus	NC 020771
		Nacospatangus alta	NC 023255
		Paracentrotus lividus	J04815
		Pseudocentrotus depressus	KC490913, NC 023773
		Sterechinus neumayeri	NC 027063
		Strongylocentrotus droebachiensis	EU054306, NC 009940
		Strongylocentrotus intermedius	KC490912, NC 023772
		Strongylocentrotus pallidus	NC 009941
		Strongylocentrotus purpuratus	NC 001453
		Temnopleurus hardwickii	NC 026200
	Holothuroidea	*Apostichopus japonicus*	EU294194
		Balanoglossus clavigerus	NC 013877
		Cucumaria miniata	AY182376
		Holothuria forskali	NC 013884
		Holothuria scabra	NC 027086
		Parastichopus californicus	NC 026727
		Parastichopus nigripunctatus	NC 013432
		Parastichopus parvimensis	NC 029699
		Peniagone sp.	KF915304
		Stichopus horrens	HQ000092, NC 014454

Table A1. *Cont.*

Phylum	Class	Species	Accession Number
Chordata	Ascidiacea	*Aplidium conicum*	FN313538, NC 013584
		Aplidium tabarquensis	HF548555
		Ascidiella aspersa	HF548561, NC 021469
		Botrylloides leachii	HF548553, HG931921, NC 024103
		Botrylloides nigrum	HF548559, NC 021467
		Botrylloides pizoni	HF548554, HG931922, NC 024104
		Botrylloides violaceus	HF548552, NC 024256
		Botryllus schlosseri	FM177702, HF548550, HF548551, HG931923, NC 021463
		Ciona intestinalis	AJ517314, NC 004447
		Ciona intestinalis type B	AM292218, NC 017929
		Ciona savignyi	AB079784, NC 004570
		Clavelina lepadiformis	AM292603, FJ839918, NC 012887
		Clavelina phlegraea	AM292604, NC 024105
		Didemnum vexillum	KM259616, KM259617, NC 026107
		Diplosoma listerianum	FN313539, NC 013556
		Halocynthia roretzi	AB024528, NC 002177
		Halocynthia spinosa	HF548558, NC 021466
		Herdmania momus	AM292602, FN296153, NC 013561
		Microcosmus sulcatus	AM292321, NC 013752
		Phallusia fumigata	NC 009834
		Phallusia mammillata	AM292320, NC 009833
		Polycarpa mytiligera	HF548556, NC 021464
		Pyura gangelion	HF548557, NC 021465
		Rhodosoma turcicum	HF548560, NC 021468
		Styela clava	HG931920
		Styela plicata	AM292601, NC 013565

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
Ectopleura crocea (Geojin)	-	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
Ectopleura crocea (Mulchi)	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
Ectopleura crocea (Jeju)	0.002	0.002	-	0.002	0.004	0.019	0.019	0.020	0.020	0.020	0.019	0.018	0.021	0.029
Ectopleura crocea JX121589.1 (Switzerland)	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
Ectopleura crocea JX898187.1 (Brazil)	0.009	0.009	0.011	0.009	-	0.019	0.019	0.020	0.021	0.021	0.019	0.018	0.021	0.029
Ectopleura dumortierii JX121590.1 (Switzerland)	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
Ectopleura dumortierii KC440096.1 (Germany)	0.187	0.187	0.190	0.187	0.185	0.003	-	0.020	0.020	0.020	0.021	0.020	0.020	0.028
Ectopleura larynx GU812435.1 (Switzerland)	0.199	0.199	0.197	0.199	0.207	0.195	0.192	-	0.009	0.008	0.014	0.020	0.021	0.028
Ectopleura larynx JN109193.1 (Switzerland)	0.204	0.204	0.202	0.204	0.211	0.192	0.195	0.038	-	0.002	0.014	0.021	0.020	0.029
Ectopleura larynx JX121591.1 (Switzerland)	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
Ectopleura larynx KT601631.1 (USA)	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
Ectopleura marina JX121592.1 (Switzerland)	0.176	0.176	0.179	0.176	0.176	0.218	0.215	0.187	0.198	0.196	0.170	-	0.022	0.027
Ectopleura wrighti JX121593.1 (Switzerland)	0.232	0.232	0.229	0.232	0.229	0.201	0.196	0.212	0.204	0.207	0.206	0.211	-	0.029
Solanderia secunda JX121599.1 (Switzerland)	0.347	0.347	0.350	0.347	0.350	0.328	0.323	0.323	0.340	0.337	0.329	0.318	0.334	-

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