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Original article

Preliminary Application of Molecular Monitoring of the Pacific Herring (*Clupea pallasii*) Based on Real-time PCR Assay Utilization on Environmental Water Samples

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Abstract Pacific herring, *Clupea pallasii*, a keystone species with significant ecological and commercial importance, is declining globally throughout much of its range. While traditional fishing equipment methods remain limited, new sensitive and rapid detection methods should be developed to monitor fisheries resources. To monitor the presence and quantity of *C. pallasii* from environmental DNA (eDNA) extracted from seawater samples, a pair of primers and a TaqMan® probe specific to this fish based on mitochondrial cytochrome *b* (COB) sequences were designed for the real-time PCR (qPCR) assay. The combination of our molecular markers showed high specificity in the qPCR assay, which affirmed the success of presenting a positive signal only in the *C. pallasii* specimens. The markers also showed a high sensitivity for detecting *C. pallasii* genomic DNA in the range of 1 pg∼100 ng rxn^{-1} and its DNA plasmid containing COB amplicon in the range of 1∼100,000 copies rxn^{-1}, which produced linear standard calibration curves (r^2 = 0.99). We performed a qPCR assay for environmental water samples obtained from 29 sampling stations in the southeastern coastal regions of South Korea using molecular markers. The assay successfully detected the *C. pallasii* eDNA from 14 stations (48.2%), with the highest mean concentration in Jinhae Bay with a value of 76.09 ± 18.39 pg L^{-1} (246.20 ± 58.58 copies L^{-1}). Our preliminary application of molecular monitoring of *C. pallasii* will provide essential information for efficient ecological control and management of this valuable fisheries resource.

Key words: *Clupea pallasii*, environmental DNA, molecular marker, molecular monitoring, real-time PCR

INTRODUCTION

The Pacific herring *Clupea pallasii* Valenciennes 1847 (Teleostei; Clupeiformes; Clupeidae), a low trophic-level fish or forage fish, is abundantly distributed along coastal

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waters and marginal seas and has important cultural, economic, and ecological impacts in many countries around the North Pacific (Hay *et al.*, 2008; Okouchi *et al.*, 2008; McKechnie *et al.*, 2014). According to statistics published in 1985 by the Food and Agriculture Organization of the United States (FAO), the total catch of *C. pallasii* was 176,342 tons, of which approximately 65% was exploited from the western coasts of the North Pacific and 35% from the eastern coast. The catch started to increase in the 2000s in both Korea and Japan, but dramatically decreased to a level of only 1∼2% (several thousand tons) of their peak harvest in the early 20th century in Japan, mainly due to the collapse of spawning stocks (Morita, 1985; Kitada *et al.*, 2017; Tomiyasu *et al.*, 2018). Moon *et al.* (2019) reported that the spawning season of *C. pallasii* begins in January and ends in February, and that the spawning beds are located in Jinhae Bay, South Korea. However, there is little information on the spawning characteristics, locality, and homing migration, owing to the difficulty in sampling. This information would allow us to establish an effective ecological management plan for this important fish resource and to designate new marine protected areas for conservation of its spawning grounds (Shirafuji *et al.*, 2018).

Recent studies have revealed that there are significant variations in the morphology and phylogeography of *C. pallasii* populations in the North Pacific coastlines (Hay *et al.*, 2008; Liu *et al.*, 2011; Grant *et al.*, 2012). In South Korea, this species is commonly distributed around the southern and eastern coasts of the Korean peninsula, but information is lacking on its phylogeography and demography. A population genetic study on the *C. pallasii* populations in South Korea was conducted and it was concluded that there was no significant genetic differentiation, despite the recent active population expansion (Gwak and Roy, 2021).

Traditional net and acoustic survey methods have generally been used to gather information on the distribution and abundance of fish eggs and larvae to estimate their spawning biomass, season, and locality. However, such methods are time-consuming, expensive, and laborious to apply to the large field sampling spatial scales (Plough *et al.*, 2018). The morphological identification of ichthyoplankton is also difficult when the samples are collected using nets (Harada *et al.*, 2015). Therefore, more recently, molecular techniques have been used to identify various marine organisms whose morphologies have not been well determined, particularly during their early life stages (Watanabe *et al.*, 2004; Kochzius *et al.*, 2010).

Real-time PCR (qPCR) has revolutionized the detection and quantification methods of target gene(s) or species and is an ideal choice for scientists because of its high sensitivity and specificity. This technique has been applied in a variety of biological areas such as pathogen diagnostics, gene expression, and genotyping, as well as in various fields including medical science, virology, bacteriology, food microbiology and safety, and environmental biology (Espy *et al.*, 2006; Deepak *et al.*, 2007). Its advantages have also been recognized in the detection and estimation of the abundance of a target species from the genetic materials collected from its surrounding environment (Plough *et al.*, 2018). Using real-time PCR on environmental samples has been proven to be a powerful tool for monitoring aquatic organisms, especially fish species (Watanabe *et al.*, 2004; Harada *et al.*, 2015; Minamoto *et al.*, 2019).

In this study, we newly designed a pair of oligonucleotide primers and a TaqMan® probe highly specific to *C. pallasii* and applied the qPCR assay for the first time to field environmental seawater samples from its spawning ground after homing migration on the southeastern coast of South Korea.

MATERIALS AND METHODS

1. Sampling of fish specimens and sequencing

Specimens of *C. pallasii* ($n = 96$) were collected during an R/V *Tamgu* cruise by the National Institute of Fisheries Science in 2018, and taxonomically-related species of the family Clupeidae along with common seafood fish species were purchased from local fisheries markets in the same year. A piece of pelvic fins was excised to extract genomic DNA (gDNA) from the clupeid specimens according to Asahida *et al.* (1996). The gDNA was resuspended in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). Its quantity and quality were checked using a DS-11 spectrophotometer (DeNovix Inc., Wilmington, DE, USA).

To amplify the mitochondrial cytochrome *b* gene (COB), a PCR run was conducted with a 20-μL reaction volume using the *AccuPower*® PCR PreMix (Bioneer, Daejeon, South Korea) containing 0.2 μM of the forward and reverse primers newly designed in this study [CLU-14342f (5′-CCACCGTTGTYATTCAACTA-3′) and CLU-15609r

(5′-TAGCTTTGGGAGTTAGRGGT-3′)] and 1 μL of gDNA (20 ng μL^{-1}). PCR was performed using the following cycling conditions in a ProFlex PCR System (Thermo Fisher Scientific, Waltham, MA USA): an initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 2 min. The reaction was completed with a final extension step at 72°C for 5 min. The PCR product was purified using the *AccuPrep*® PCR Purification Kit (Bioneer) and directly sequenced on the Applied Biosystems® 3730*xl* DNA Analyzer (Thermo Fisher Scientific).

2. Design of primers and probe

To design a pair of primers and a TaqMan® probe for the qPCR assay, we retrieved COB sequences from the all clupeiform species from a public database, GenBank (https://www.ncbi.nlm.nih.gov/) and aligned them with those analyzed in this study using ClustalW in BioEdit 7.2 (Hall, 2011). After comparing the aligned nucleotide matrix, the species-specific positions of *C. pallasii* were selected and designed for the forward and reverse primers, and the TaqMan® probe for qPCR assay. Their melting temperatures (T_m) and secondary structures were predicted using Primer Express 3.0 (Thermo Fisher Scientific) and optimized prior to oligonucleotide synthesis.

3. Seawater sampling and eDNA extraction

Environmental seawater (1 L) was sampled in a sterile sampling bottle at a 10-m depth from the surface at 29 stations on the southeastern coasts of South Korea: eight stations in Gamak Bay, six in Jaran Bay, eight in Jinhae Bay, and seven on the Tongyeong coast, on March 8∼9, 2018 (Fig. 1). The seawater sample was immediately vacuum-filtered through a glass microfiber filter (Grade GF/F circles, 47 mm, Whatman, Marlborough, MA, USA) on board. The filter was inserted into a 1.5 mL microtube after folding in half four times using uncontaminated forceps. The tube was placed in a cooled ice-

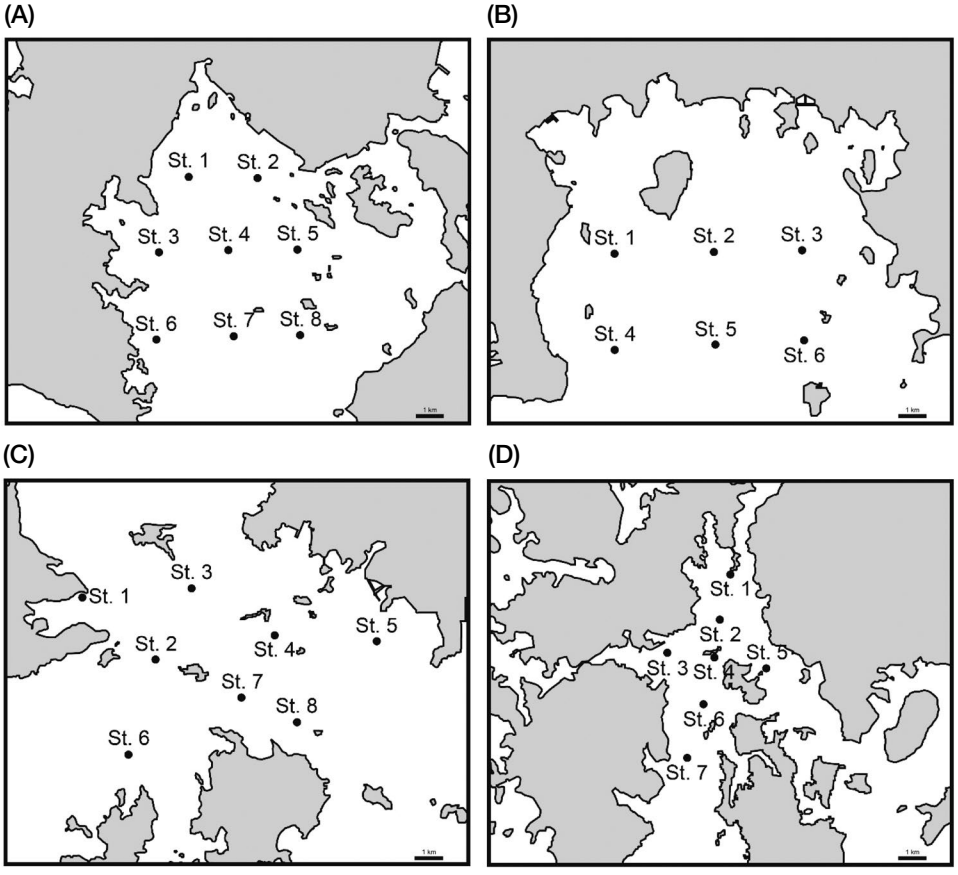

Fig. 1. Sampling areas of field environmental samples for the Pacific herring, *Clupea pallasii* in the southeastern coasts of the Korean peninsula. (A) Gamak Bay, (B) Jaran Bay, (C) Jinhae Bay, and (D) Tongyeong coast.

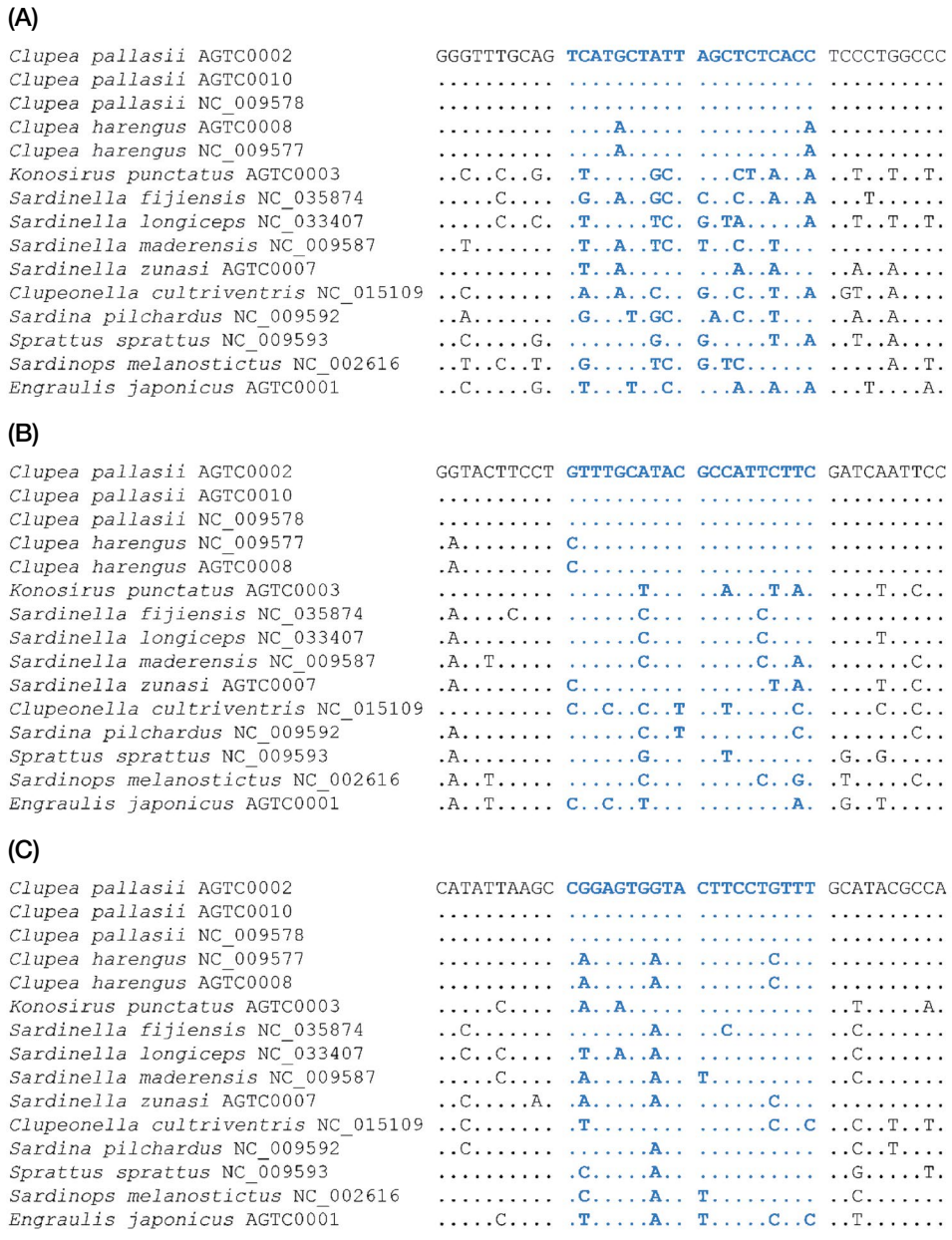

Fig. 2. Partial sequence alignments of mitochondrial cytochrome *b* region of the Pacific herring, *Clupea pallasii* with other species of the order Clupeiformes usually found along Korean coastal waters. Oligonucleotides of forward (A) and reverse (B) primers and TaqMan® probe (C) specific to the *C. pallasii* sequence was highlighted in bold and blue. Their sequences were newly analyzed in this study or retrieved from the public database, GenBank in NCBI and indicated with voucher numbers or GenBank accession numbers.

box with polythene bags containing ice, away from light exposure, that was moved directly to the laboratory. The filter was broken up using a bead beater, Bead Ruptor 12 (OMNI International, Kennesaw, GA, USA), and the environmental DNA (eDNA) was extracted using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and eluted in 20 µL of sterile distilled water.

4. qPCR analysis

qPCR amplification was conducted with a 20-µL reaction volume using GoTaq® Probe qPCR Master Mix (Promega, Madison, WI, USA) containing 1 µL of eDNA as a template, 0.2 µM of the forward and reverse primers [Cpa-COB-701f (5′-TCATGCTATTAGCTCTCACC-3′) and Cpa-COB-825r

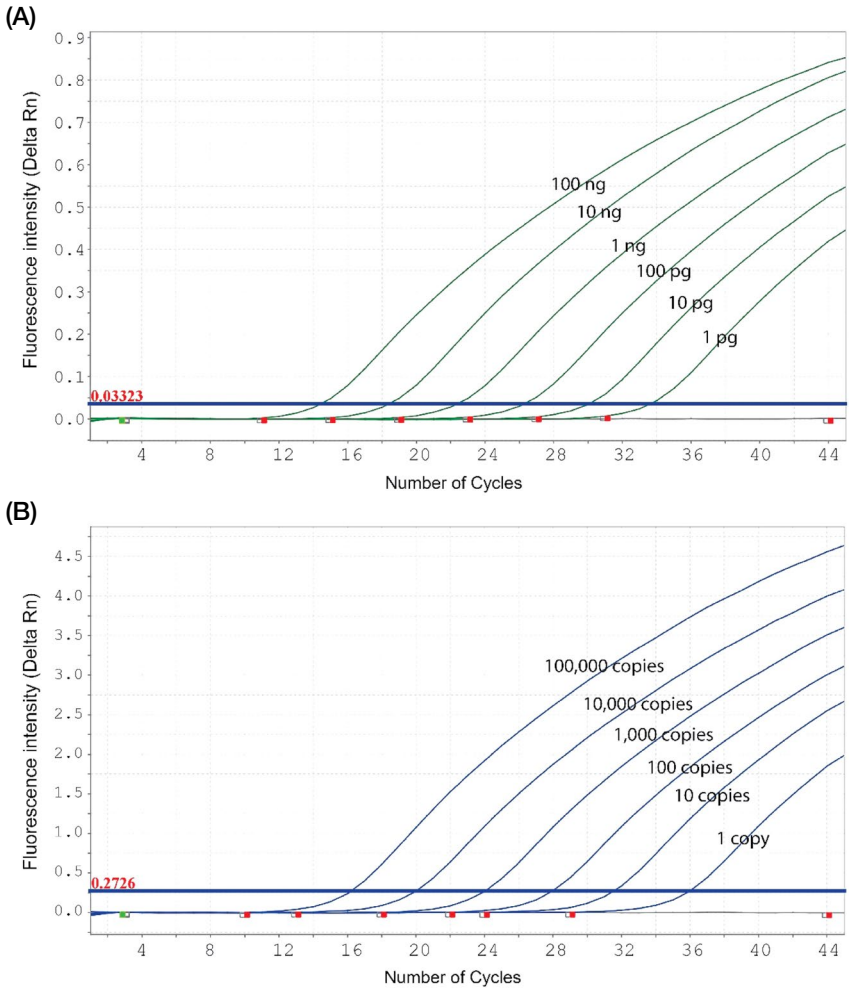

Fig. 3. Amplification curves of real-time PCR analyses using the specific primers and TaqMan® probe on the Pacific herring, *Clupea pallasii* developed in this study to test for sensitivity. Serially diluted concentrations of the genomic DNA (gDNA) (1 pg∼100 ng rxn^{-1}) with 14.35∼33.54 C_T values (A); a serial dilution of the plasmid DNA (1∼100,000 copies rxn^{-1}) with 16.18∼35.99 C_T values (B). Each showed a detection limit as low as 1 pg rxn^{-1} of the gDNA concentration with a C_T value of 33.54, and a detection limit as low as 1 copy rxn^{-1} of the plasmid DNA with a C_T value of 35.99.

(5′-GAAGAATGGCGTATGCAAAC-3′)] and 0.2 μM of the TaqMan® probe [Cpa-COB-809p (5′-FAM-CGGAGTGG TACTTCCTGTTT-MGB-Eclipse-3′)] that were synthesized by Macrogen, Inc. (Seoul, South Korea). The following standard cycling conditions were performed in a QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific): activation at 95°C for 2 min, 40 cycles of denaturation at 95°C for 15 s, and annealing and extension at 63°C for 30 s. The *C. pallasii* gDNA (20 ng μL^{-1}) was used as a positive control, and sterile distilled water as a negative control to trace careless contamination during filtering, eDNA extraction, and qPCR analysis.

The sensitivity of oligonucleotide primers and TaqMan® probe newly designed in this study was tested against the serially diluted gDNA (1 pg∼100 ng rxn^{-1}) of *C. pallasii* and plasmid DNA (100,000∼1 copies rxn^{-1}), in which its COB amplicon was inserted. Both results were used to produce standard calibration curves to calculate the *C. pallasii* eDNA quantities. Their specificity was also checked against the clupeid species, *C. harengus*, *Konosirus punctatus*, and *Sardinella zunasi*, along with eight *C. pallasii* specimens and 13 seafood fish species: *Conger myriaster*, *Dasyatis zugei*, *Engraulis japonicus*, *Gadus chalcogrammus*, *G. macrocephalus*, *Larimichthys polyactis*, *Lophiomus setigerus*, *Psenopsis anomala*, *Salmo salar*, *Scomberomorus niphonius*, *Syngnathus schlegeli*, *Thunnus thynnus*, and *Trachurus japon-*

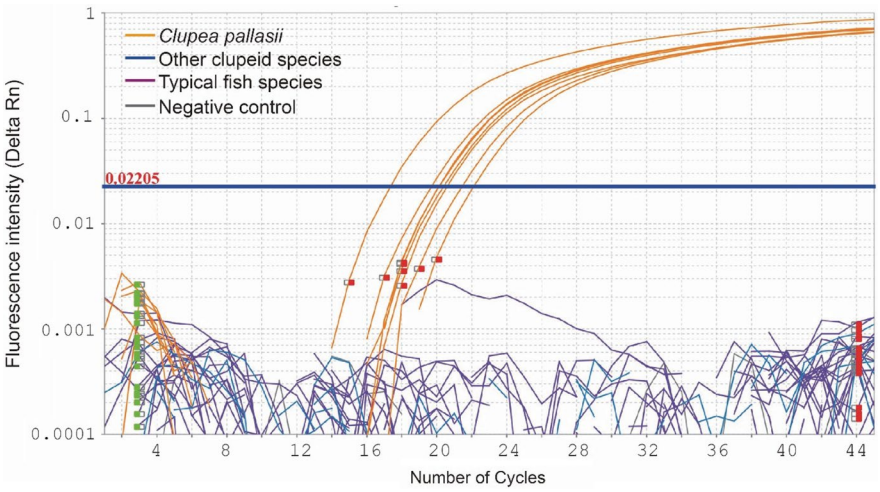

Fig. 4. An amplification curve of real-time PCR analyses using the specific primers and TaqMan® probe on the Pacific herring, *Clupea pallasii* developed in this study to test their specificity. The test was carried out against four clupeid species, *C. harengus*, *Konosirus punctatus*, and *Sardinella zunasi*, including the eight *C. pallasii* specimens, and 13 common seafood fish species, *Conger myriaster*, *Dasyatis zugei*, *Engraulis japonicus*, *Gadus chalcogrammus*, *G. macrocephalus*, *Larimichthys polyactis*, *Lophiomus setigerus*, *Psenopsis anomala*, *Salmo salar*, *Scomberomorus niphonius*, *Syngnathus schlegeli*, *Thunnus thynnus*, and *Trachurus japonicus*, which are easily available at local markets. Only the eight specimens of *C. pallasii* produced a positive signal, while the other clupeid species as well as common seafood fish species produced no signals.

icus, which were easily available at local markets in South Korea.

RESULTS

1. Design of oligonucleotide primers and probe and qPCR analysis

In this study, we designed a pair of primers and a TaqMan® probe specific to *C. pallasii* based on the COB sequences of 94 specimens collected along the southeastern coasts of South Korea, as well as those of 11 clupeiform species newly analyzed in this study or available from a public database (GenBank in NCBI) after alignment and comparison (Fig. 2). The consensus sequence was generated to increase the representation of the primers and probe for all *C. pallasii* specimens. The forward and reverse primers (Cpa-COB-701f and Cpa-COB-825r) each consisting of a 20-mer oligonucleotide, produced a 144-bp amplicon that was predicted and confirmed by conventional PCR amplification, and the TaqMan® probe (Cpa-COB-809p) was composed of a 20-mer oligonucleotide with a fluorophore, fluorescein (FAM) covalently attached to the 5′-end and a quencher, MGB-Eclipse at the 3′-end. The combination of molecular markers was unique to *C. pallasii* and was not found in the other clupeiform species (Fig. 2).

The qPCR amplification produced a positive signal with a C_T value of 17.17 and no background signal using the molecular markers against a standard concentration of gDNA (20 ng μL^{-1}) extracted from *C. pallasii*. The sensitivity test against serially diluted concentrations of the *C. pallasii* gDNA (1 pg∼100 ng rxn^{-1}) showed an inverse proportion with C_T values and a detection limit as low as 1 pg rxn^{-1} of the gDNA with a C_T value of 33.54 (Fig. 3A). This correlation was used as the standard calibration curve that produced a linear regression, $y = -3.935x + 35.778$ ($r^2 = 0.99$, efficiency = 79.52%).

In addition, the sensitivity test against a serial dilution of plasmid DNA (1∼100,000 copies rxn^{-1}) in which the COB amplicon of *C. pallasii* was inserted, also showed an inverse proportion with C_T values and a detection limit as low as 1 copy rxn^{-1} of the plasmid DNA with a C_T value of 35.99 (Fig. 3B). Thus, the C_T value of 36.00 was set as the lowest detection limit to yield an acceptable level of precision and accuracy for our qPCR assay. This correlation was used as the standard calibration curve that produced a linear regression, $y = -3.85x + 22.178$ ($r^2 = 0.99$, efficiency = 81.85%). Both linear regressions were used to detect and quantify *C. pallasii* eDNA from the field environmental samples.

To confirm the specificity of the molecular markers for *C. pallasii*, we carried out qPCR amplification against four

Table 1. Quantification of environmental DNA (eDNA) of the field environmental samples from the southeastern coasts of South Korea by real-time PCR analysis using the specific primers and probe on the Pacific herring, *Clupea pallasii*.

Area	Station	C_T Value*	eDNA Conc. (pg rxn^{-1})	eDNA Conc. (pg L^{-1})	No. copies rxn^{-1}	No. copies L^{-1}
Gamak Bay	St. 1	32.15	2.57	77.01	8.34	250.20
Gamak Bay	St. 2	35.08	0.45	13.42	1.51	45.26
Gamak Bay	St. 3	ND	–	–	–	–
Gamak Bay	St. 4	ND	–	–	–	–
Gamak Bay	St. 5	ND	–	–	–	–
Gamak Bay	St. 6	ND	–	–	–	–
Gamak Bay	St. 7	ND	–	–	–	–
Gamak Bay	St. 8	34.52	0.62	18.73	2.09	62.73
Jaran Bay	St. 1	ND	–	–	–	–
Jaran Bay	St. 2	35.61	0.33	9.75	1.10	33.11
Jaran Bay	St. 3	ND	–	–	–	–
Jaran Bay	St. 4	ND	–	–	–	–
Jaran Bay	St. 5	34.44	0.65	19.60	2.19	65.59
Jaran Bay	St. 6	ND	–	–	–	–
Jinhae Bay	St. 1	32.95	1.59	47.68	5.22	156.49
Jinhae Bay	St. 2	35.72	0.30	9.12	1.03	31.02
Jinhae Bay	St. 3	ND	–	–	–	–
Jinhae Bay	St. 4	ND	–	–	–	–
Jinhae Bay	St. 5	32.70	1.85	55.54	6.06	181.69
Jinhae Bay	St. 6	31.03	5.03	150.95	16.11	483.27
Jinhae Bay	St. 7	31.80	3.16	94.84	10.22	306.70
Jinhae Bay	St. 8	31.74	3.28	98.42	10.60	318.01
Tongyeong coast	St. 1	31.97	2.86	85.82	9.27	278.11
Tongyeong coast	St. 2	32.62	1.95	58.41	6.36	190.88
Tongyeong coast	St. 3	ND	–	–	–	–
Tongyeong coast	St. 4	ND	–	–	–	–
Tongyeong coast	St. 5	ND	–	–	–	–
Tongyeong coast	St. 6	ND	–	–	–	–
Tongyeong coast	St. 7	34.32	0.70	21.01	2.34	70.20

*ND: not detected

clupeid species, and the results showed that only the eight *C. pallasii* specimens successfully produced a positive signal, while the other clupeids and the seafood fish species failed to produce signals (Fig. 4).

2. Application to field environmental samples

Our molecular markers specific to *C. pallasii* were preliminarily applied to the eDNA extracted from the environmental samples ($n = 29$) collected from the surface at the southeastern coast of South Korea. When detected, the qPCR assay showed C_T values from 31.03 to 35.72 corresponding to gDNA concentrations from 9.12 pg L^{-1} to 150.95 pg L^{-1} and the plasmid copy numbers from 31.02 copies L^{-1} to 483.27 copies L^{-1} (Table 1). The highest values of over 50 ng L^{-1} of gDNA or 164 copies L^{-1} of plasmid DNA were only found at stations 5, 6, 7, and 8 in Jinhae Bay, stations 1 and 2 on the Tongyeong coast, and station 1 in Gamak Bay.

Clupea pallasii eDNA was detected at 14 stations, three from Gamak Bay, two from Jaran Bay, six from Jinhae Bay, and three from the Tongyeong coast, with detection rates of 37.5%, 33.3%, 75.0%, and 42.9%, respectively (Table 2). The mean quantities were the highest in Jinhae Bay (76.09 ± 18.39 pg L^{-1}) and the lowest in Jaran Bay (14.68 ± 3.48 pg L^{-1}), with values corresponding to 246.20 ± 58.58 copies L^{-1} and 49.35 ± 11.48 copies L^{-1}, respectively.

Table 2. Detection rate (%), concentration, and number of copies of the environmental DNA (eDNA) from the positive field environmental samples at the southeastern coasts of South Korea by real-time PCR analysis using the specific primers and probe on the Pacific herring, *Clupea pallasii*.

Area	Detected stations/total stations (detection rate)	eDNA concentration (mean ± SE) (pg L^{-1})	eDNA copies no. L^{-1} (mean ± SE)
Gamak Bay	3/8 (37.5%)	13.42~77.01 (36.39 ± 16.63)	45.26~250.20 (119.40 ± 53.56)
Jaran Bay	2/6 (33.3%)	9.75~19.60 (14.68 ± 3.48)	33.11~65.59 (49.35 ± 11.48)
Jinhae Bay	6/8 (75.0%)	9.12~150.95 (76.09 ± 18.39)	31.02~483.27 (246.20 ± 58.58)
Tongyeong coast	3/7 (42.9%)	21.01~85.82 (55.08 ± 15.34)	70.20~278.11 (179.73 ± 49.22)

DISCUSSION

Molecular markers from mitochondrial DNA as opposed to nuclear DNA have been widely used to detect and monitor fish resources owing to their high cellular copy number, functional neutrality, maternal inheritance, and high mutation rate. The COB region, which accumulates the large sequence database of vertebrates, is considered one of the most commonly used markers for molecular identification, phylogenetics, and molecular ecology (Li *et al.*, 2018). Liu *et al.* (2011) carried out a phylogeographic study of *C. pallasii* populations along the North Pacific coastlines of Asia and North America using the COB region. This suggests that this mitochondrial DNA region is suitable for use in designing specific oligonucleotide markers for diagnostic, phylogenetic, and molecular ecological studies of diverse fish species. In the last decade, many studies have been successfully performed by applying this region to monitor fish spawning activity (Harada *et al.*, 2015; Takeuchi *et al.*, 2019a) and detect endangered or invasive fish species (Bylemans *et al.*, 2017; Plough *et al.*, 2018; Minamoto *et al.*, 2019).

In this study, we newly designed a pair of primers and a TaqMan® probe for *C. pallasii*, which is an important fisheries resource worldwide (Li *et al.*, 2020). The successful production of robust and consistent amplification signals from the eight *C. pallasii* samples without any cross-reactivity with the cluster of examined species suggested that the specifically designed molecular markers worked well in detecting and distinguishing *C. pallasii* from the other non-target species. The molecular markers also showed high sensitivity with a detection limit as low as 1 pg rxn^{-1} of the *C. pallasii* gDNA and as low as 1 copy rxn^{-1} of the *C. pallasii* plasmid DNA that contained its COB fragment as an insert. Compared to a previous study, these results indicated that the real-time PCR assay in this study is more applicable and effective in the eDNA-based analysis when the eDNA concentration is usually confined and restricted (Takahara *et al.*, 2012; Katano *et al.*, 2017).

Environmental DNA (eDNA) assay is an important technological and scientific breakthrough in various fields, including molecular biology, ecology, and environmental science, during the past decade (Thomsen and Willerskev, 2015). Using a miniscule concentration of genetic materials from substances such as urine, mucus, skin, and feces released from living or dead organisms into their surrounding habitats (soil, sediment, water, air, etc.), the target species could potentially be detected and traced without visual, auditory or other contact (Ficetola *et al.*, 2008; Stewart, 2019). However, eDNA concentration varies tremendously among environments, since eDNA is susceptible to decay when directly exposed to certain environmental conditions. Collins *et al.* (2018) observed the persistence of eDNA in marine waters and concluded that in the inshore environment, eDNA degrades 1.6 times faster than the offshore area, whereas there is no significant variation in decay rates between summer and winter. They also added that salinity index varies more between locations than pH and is likely a better predictor of eDNA decay, while pH varies more according to seasons. Monitoring eDNA facilitates the building of a comprehensive sampling plan with the expectation of achieving precise data for efficient monitoring and conservation of target species.

Clupea pallasii migrates from the open ocean in the East

Sea to coastal areas of the southeastern Korean peninsula to spawn (Lee *et al.*, 2017; Moon *et al.*, 2019). After hatching, the larvae and juveniles continuously stay and mature in Jinhae Bay from January to March, gradually moving toward the offshore area of Jinhae Bay with the seasonal rise in water temperature, and eventually leaving the bay (Lee *et al.*, 2014; Moon *et al.*, 2018). Our environmental samples were collected during March 8∼9, 2018 on the southeastern coast (Gamak Bay, Jaran Bay, Jinhae Bay, and the Tongyeong coast) of South Korea, and our sampling sites included the spawning beds of *C. pallasii* (Jinhae Bay and the Tongyeong coast). The molecular monitoring based on qPCR assay with specific molecular markers showed that *C. pallasii* eDNA quantities were the highest in Jinhae Bay among the four sampled regions, and six out of eight stations in this region contained *C. pallasii* eDNA, which was the greatest of all sampled areas. This result indicates that Jinhae Bay is the most important spawning bed of *C. pallasii* on the southeastern coast of South Korea. Takeuchi *et al.* (2019b) evaluated the effect of spawning on the eDNA concentration in Japanese eels and showed a significant increase in eDNA concentration after spawning. Apart from Jinhae Bay, lower quantities of *C. pallasii* eDNA were detected from only two to three stations in the other sampling regions.

Lee *et al.* (2017) observed fertilized eggs of *C. pallasii* on seaweed beds in an inner area of the Tongyeong coast by SCUBA diving in January 2014. According to Lee *et al.* (2017) and Moon *et al.* (2019), large quantities of fertilized eggs were also observed by SCUBA diving in Jinhae Bay during the winter season, suggesting that the spawning beds of *C. pallasii* mainly occur in the rocky shores of the subtidal zone where seaweed is abundant. Furthermore, Haegele and Schweigert (1985) reported that *C. pallasii* spawns on vegetation or bottom substrata in shallow nearshore habitats. Our results revealed that the sampling stations with higher eDNA quantities in Jinhae Bay and the Tongyeong coast were located near coastlines, where various algal species bloom annually. Lee *et al.* (2014) and Moon *et al.* (2018), based on the traditional netting method, reported that *C. pallasii* is the dominant larval fish species, especially around the Jam and Chilcheon islands in Jinhae Bay in the winter season. The *C. pallasii* eDNA quantities were the highest at stations 6, 7, and 8 located on the two previously mentioned islands, aligning our results with those of the traditional sampling method. In a future study, molecular monitoring should be targeted at these detailed habitats, as Yamamoto *et al.* (2016) concluded that the spatial scale of the association between eDNA and fish biomass in a coastal bay was relatively small (10∼150 m). Port *et al.* (2016) also claimed that eDNA can distinguish vertebrate community assemblages within 60 m in a kelp forest ecosystem.

According to Lee *et al.* (2017) and Moon *et al.* (2019), the spawning season of *C. pallasii* occurs from January to mid-February in Jinhae Bay. Our sampling of seawater environmental samples was conducted on March 8∼9, 2018, shortly after the spawning season. The time for eDNA to decay below the detection limit investigated in aquarium experiments was estimated to be 0.9 days for the European flounder (*Platichthys flesus*) and 6.7 days for the three-spined stickleback (*Gasterosteus aculeatus*) (Thomsen *et al.*, 2012). Sassoubre *et al.* (2016) reported that the eDNA of three marine fishes degraded within 3∼4 days in seawater mesocosm experiments. Ji *et al.* (2015) noted that *C. pallasii* larvae began to hatch naturally 10 days after fertilization at 9°C in coastal waters. Therefore, the *C. pallasii* eDNA detected in our study likely originated from the trace of organic residues of parental fish, newly hatched larvae, or juveniles from its spawning events.

The qPCR assay using the oligonucleotide markers specific to *C. pallasii* applied in this study will provide a useful tool for future ecological studies of this species, such as seasonal migration, spatio-temporal distribution, and spawning behavior. Further research on the relationship between eDNA concentrations and spawning biomass is needed to accurately measure the geographic ranges of spawning grounds (Yamamoto *et al.*, 2016).

Clupea pallasii has important cultural, economic, and ecological impacts in many countries around the North Pacific. Using qPCR on environmental samples has been proven to be a powerful tool for monitoring aquatic organisms, especially fish species. In this study, we newly designed a pair of oligonucleotide primers and a TaqMan® probe highly specific to *C. pallasii* and applied the qPCR assay for the first time to field environmental seawater samples from its spawning ground after homing migration on the southeastern coast of South Korea. The molecular markers showed high specificity and sensitivity in the qPCR assay. The qPCR assay using the markers successfully monitored the presence and quantity of *C. pallasii* in environmental water samples. Our preliminary application of molecular monitoring of *C. pallasii* will pro-

vide fundamental information for efficient ecological control and management of this valuable fisheries resource.

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