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Identification of eleven species of the Pleuronectidae family using DNA-based techniques

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Abstract

Flatfish are one of the largest families in the order Pleuronectiformes and are economically important edible marine fish species. However, they have similar morphological characteristics leading to challenges in classifying correctly, which may result in mislabeling and illegal sales, such as fraudulent labeling of processed food. Therefore, accurate identification is important to ensure the quality and safety of domestic markets in Korea. Species-specific primers were prepared from the mainly consumed eleven species of the order Pleuronectiformes. To rapidly identify the 11 flatfish species, a highly efficient, rapid, multiplex polymerase chain reaction (PCR) with species-specific primers was developed. Species-specific primer sets were designed for the mitochondrial DNA cytochrome c oxidase subunit I gene. Species-specific multiplex PCR (MSS-PCR) either specifically amplified a PCR product of a unique size or failed. This MSS-PCR analysis is easy to perform and yields reliable results in less time than the previous Sanger sequencing methods. This technique could be a powerful tool for the identification of the 11 species b the family Pleuronectidae and can contribute to the prevention of falsified labeling and protection of consumer rights.

Keywords: Pleuronectidae family, Mitochondrial DNA, Cytochrome c oxidase subunit I gene (COI) identification, Polymerase chain reaction (PCR), Multiplex species-specific

Introduction

Pleuronectidae, commonly known as the flatfish family, comprises 40 genera and 103 species worldwide. Japan has 17 genera and 33 species, whereas South Korea has 18 genera and 25 species reported (Lee et al., 2019). Among the 25 species reported in South Korea, 13, including the yellowfin sole, have either limited records in the literature or very low occurrence frequencies. The remaining 12 species, *Pleuronectes yokohamae*, *Pleuronectes herzensteini*, *Hippoglossoides pinetorum*, *Hippoglossoides dubius*,

Kareius bicoloratus, Pleuronichthys cornutus, Clidoderma asperrimum, Eopsetta grigorjewi, Glyptocephalus stelleri, Platichthys stellatus, Tanakius kitaharae (=Glyptocephalus kitaharae), and Microstomus achne are considered important edible fishery resources. However, these 12 species are difficult to classify morphologically due to their similar appearances. Except for the Greenland halibut, the catch quantities are not recorded by species but grouped under the generic term "flatfish."

Recently, various seafood products have been imported and distributed throughout South Korea. According to the Fishery

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This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/bync/4.0/) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited. Copyright © 2023 The Korean Society of Fisheries and Aquatic Science Information Portal System (www.fips.go.kr) of the Ministry of Oceans and Fisheries, the import volume of flatfish has more than doubled since 2017, reaching over 3,500 tons. Owing to the wide variety of species and their similar appearance, flatfish are susceptible to economic fraud, where cheaper species are misrepresented and sold as more expensive ones, leading to frequent occurrences of economically motivated adulteration in the domestic seafood market. Given the high potential for disputes regarding the identification of species and their origins in the flatfish family, further research is required to obtain genetic information through genetic characteristic analyses and establish a reliable classification system.

Previous studies on the flatfish family have mainly focused on the morphological characteristics and distribution of species, such as *Verasper variegatus*, *P. cornutus*, *P. yokohamae*, *G. stelleri*, and *T. kitaharae*. Additionally, there have been molecular phylogenetic studies on the Korean flatfish family. However, methods of species identification of the flatfish family using genetic analysis remains unreported.

The species classification of aquatic organisms serves as a fundamental unit for the management of biological resources. There is a growing awareness of the importance of species classification, given its importance in ecosystem research to ensure food safety through the prevention of misrepresentation of species, and combat counterfeit and substandard food products.

Among the various methods currently used for species identification, DNA-based species identification techniques that utilize molecular biology approaches offer higher reliability in analyzing processed samples, including those subjected to high temperatures and drying during the manufacturing process, owing to the stability of their physicochemical compositions (Axayácatl & Juan, 2008). Among these methods, the multiplex species-specific polymerase chain reaction (MSS-PCR) analysis allows for species identification by only performing PCR amplification followed by electrophoretic analysis without the need for pretreatments with restriction enzymes. MSS-PCR is simpler and exhibits higher reproducibility compared to methods such as restriction fragment length polymorphism (RFLP) and amplified fragment length polymorphism (Acar et al., 2017; Kang et al., 2015; Kim et al., 2014; Noh et al., 2017; Rasmussen & Morrissey, 2008). Furthermore, by analyzing intraand interspecies polymorphisms using nucleotide sequences and designing primers, it is possible to mitigate false-positive and false-negative results resulting from differences in genetic variation (Axayácatl & Juan, 2008; Hsieh & Hwang, 2004; Hsieh et al., 2010).

In this study, we focused on a variety of flounder species within the family Pleuronectidae that are challenging to differentiate owing to their similar morphologies. Specifically, we analyzed the genetic characteristics of 11 species from 10 genera: G. stelleri, P. herzensteini, T. kitaharae, C. asperrimum, K. bicoloratus, M. achne, P. yokohamae, E. grigorjewi, P. stellatus, Cleisthenes pinetorum, and Limanda aspera. Through genetic analysis, we gained insights into their genetic characteristics. We analyzed intra-and interspecies genetic variations using the cytochrome c oxidase subunit I (COI) gene region of mitochondrial DNA (mtDNA), which has been proposed as the species identification barcode region by the Consortium for the Barcode of Life. Additionally, we aimed to establish MSS-PCR conditions, enabling the identification of 11 flounder species from the Pleuronectidae family in a single PCR reaction by exploring species-specific positions with distinct inter-species variations (Ward et al., 2009).

Our findings will contribute to the systematic resource management and food safety of imported and domestic flounder species within the Pleuronectidae family by establishing an MSS-PCR analysis method using DNA-based molecular biology techniques.

Materials and Methods

Sample selection and genomic DNA extraction

Overall, 322 samples from 11 species belonging to 10 genera of flounder were obtained from *G. stelleri*, *P. herzensteini*, *T. kitaharae*, *C. asperrimum*, *K. bicoloratus*, *M. achne*, *P. yokohamae*, *E. grigorjewi*, *P. stellatus*, *C. pinetorum*, and *L. aspera* (Table 1). These samples were procured from domestic fish markets between January and June 2019 or were provided by the Marine and Fishery Resource Donation and Preservation Institute at Pukyong National University. Of the 322 samples, 77 were used for primer design, and the remaining 245 for primer validation.

DNA was extracted from samples using a DNeasy[®] Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany). Approximately 20 mg of tissue from each sample was used for genomic DNA (gDNA) extraction, following the instructions provided in the manual accompanying the DNeasy[®] Blood & Tissue Kit. The extracted gDNA was quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and stored at -20 °C until further genetic analysis.

Scientific name	Sample site	Collection dates	Sample number (n)		From
			Design (n = 77)	Verification (n = 245)	
Glyptocephalus stelleri	Daejinhang Jumunjinhang Jukdosijang Phohang	2019.01.	22	56	Fishery market
T. kitaharae	Yeosu	2019.01.–03.	10	20	PKNU
Clidoderma asperrimum	Jumunjinhang Wando	2019.01.	4	11	Fishery market
Eopsetta grigorjewi	Mokpo Boryeong	2019.03.	5	28	PKNU
Cleisthenes pinetorum	Jumunjinhang Phohang	2019.01.	6	30	Fishery market
Pleuronectes herzensteini	Jumunjinhang Daejinhang Phohang	2019.01.	7	9	Fishery market
Microstomus achne	Hupohang Phohang	2019.01.	1	11	Fishery market
Platichthys stellatus	Jumunjinhang Phohang	2019.01.	5	5	Fishery market
Kareius bicoloratus	Jumunjinhang Daejinhang Phohang	2019.01.	4	19	Fishery market
Pleuronectes yokohamae	Hupohang Phohang	2019.01.	2	43	Fishery market
Limanda aspera	China	2019.01.	11	18	Fishery market

Table 1. Information of specimens used in this study

PKNU, Pukyong National University.

Sequencing

To explore inter- and intra-species variations and identify conserved regions within the COI gene region of mtDNA, we aligned mtDNA nucleotide sequences from 11 flounder species (*G. stelleri*, MH032428.1; *P. herzensteini*, MH032530.1; *Glyptocephalus kitaharae*, MH032424.1; *C. asperrimum*, MH032407.1; *K. bicoloratus*, MH032487.1; *M. achne*, MH032470.1; *P. yokohamae*, KT878309.1; *E. grigorjewi*, MH032414.1; *P. stellatus*, EF424428.1; *C. pinetorum*, MH032404.1; *L. aspera*, MH032456.1) registered in the National Center for Biotechnology Information (NCBI) GenBank. We used BioEdit (http://mbio. ncsu. edu/BioEdit/biodeit.html) to align the nucleotide sequences. Based on the conserved regions identified in the alignment, we designed a specific forward primer (GJM-F128) and reverse primer (GJM-R1370) to amplify the COI gene region of these 11 species (Fig. 1 and Table 2).

To amplify of the COI gene region, the following reaction

mixture was prepared:1 μ L of extracted gDNA, 2 μ L of 10 × Taq buffer, 0.4 μ L of 2.5 mM dNTP mixture, 0.8 μ L of 10 pmol forward primer, 0.8 μ L of 10 pmol reverse primer, and 0.5 units of HS-Taq polymerase (Anti-HS Taq, TNT Research, Jeonju, Korea), making a total volume of 20 μ L. The PCR conditions were set using ABI Verity Fast Thermal Cyclers (Applied Biosystems, Foster, CA, USA), with an initial pre-denaturation step at 95 °C for 10 min, followed by denaturation at 95 °C for 40 s, annealing at 54 °C for 40 s, and extension at 72 °C for 50 s, repeated for 35 cycles. The final extension step was performed at 72 °C for 7 min. The amplified PCR products were analyzed using an automated DNA sequencing instrument with an ABI 3730XL DNA analyzer (Applied Biosystems).

Species-specific primer design

The bidirectional nucleotide sequences of the COI gene region from 11 species of the Pleuronectidae family were aligned using

	110	120	130	140 150
G. stelleri (MH032428.1) G. kitaharae (MH032424.1) C. asperrimum (MH032407.1) E. grigorjewi (MH032414.1) C. pinetorum (MH032404.1) P. herzensteini (KF386365.1) M. achne (MH032473.1) P. stellatus (EF424428.1) K. bicoloratus (MH032487.1) P. yokohamae (KT878309.1) L. aspera (MH032456.1)	GACCAAATTTATAACG GACCAAATTTATAACG GACCAAATTTATAACG GACCAAATTTATAACG GACCAAATTTATAACG GACCAAATTTATAACG GACCAAATTTATAACG GACCAAATTTATAACG GACCAAATTTATAACG GACCAAATTTATAACG GACCAAATTTATAACG	TAATCGTCACC TAATCGTCACC TAATCGTCACC TAATCGTCACC TGATCGTCACC TAATCGTCACC TAATCGTCACC TAATCGTCACC TAATCGTCACC TAATCGTCACC TAATCGTCACC	GCACACGCCT GCACACGCCT GCACACGCCT GCACACGCCT GCACACGCCT GCACACGCCT GCACACGCCT GCACACGCCT GCACACGCCT GCACACGCCT GCACACGCCT	TTGTAATAATCTT TTGTAATAATCTT TTGTAATAATCTT TTGTAATAATCTT TTGTAATAATCTT TTGTAATAATCTT TTGTAATAATCTT TTGTAATAATCTT TTGTAATAATCTT TTGTAATAATCTT TTGTAATAATCTT TTGTAATAATCTT
G. stelleri (MH032428.1) G. kitaharae (MH032424.1) C. asperrimum (MH032407.1) E. grigorjewi (MH032404.1) P. herzensteini (KF386365.1) M. achne (MH032473.1) P. stellatus (EF424428.1) K. bicoloratus (MH032487.1) P. yokohamae (KT878309.1) L. aspera (MH032456.1)	310 TGTTGAAGCCGGGGGCT GGTTGAAGCCGGGGGCA CGTTGAAGCCGGGGCA CGTTGAAGCCGGGGCA CGTCGAAGCCGGGGCG TGTTGAAGCCGGGGCCA TGTTGAAGCCGGGGCCA TGTTGAAGCCGGGGCCA CGTAGAAGCCGGGGCA CGTAGAAGCCGGGGCA	320 GGTACTGGGTG GGGACTGGGTG GGTACGGGTG GGGACTGGGTG GGGACTGGCTG GGAACAGGGTG GGAACAGGGTG GGAACAGGGTG GGAACAGGGTG GGAACTGGGTG	AACCGTTTAC AACCGTTTAC AACCGTATAT AACCGTATAT AACCGTATAT AACCGTATAT AACCGTATAT AACCGTATAT AACCGTGTAT AACCGTGTAT AACCGTGTAT	340 340 CCTCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC
G. stelleri (MH032428.1) G. kitaharae (MH032424.1) C. asperrimum (MH032407.1) E. grigorjewi (MH032404.1) C. pinetorum (MH032404.1) P. herzensteini (KF386365.1) M. achne (MH032473.1) P. stellatus (EF424428.1) K. bicoloratus (MH032487.1) P. yokohamae (KT878309.1) L. aspera (MH032456.1)	360 GTAATCTGGCGCACGC GTAATCTAGCCCACGC GCAATCTAGCCCACGC GAATCTGGCCCACGC GAAATCTAGCACACGC GCAACCTAGCACACGC GCAACCTAGCACACGC GAAACCTAGCACACGC GAAATCTAGCACACGC GAAATCTAGCACACGC	370 CGGAGCATCCC CGGAGCATCCC CGGAGCGTCCC CGGAGCCTCCC CGGAGCCTCCC CGGAGCCTCCC CGGAGCCTCCC CGGGCATCCC CGGGCATCCC CGGGCATCCC CGGAGCATCCC	380 T A G A C C T A A C T A G A C C T A A C T A G A C C T A A C T A G A C C T A A C T A G A C C T C A C T A G A C C T C A C T A G A C C T C A C T A G A C C T C A C T A G A C C T C A C T A G A C C T C A C T A G A C C T A A C	390 400 AATCTTCTCGCTT AATCTTCTCACTA AATTTTCTCACTT AATTTTCTCACTT AATTTTCTCCCTT GATTTTCTCCCTT GATTTTTCCCCTG AATCTTTCCCTT GATCTTTTCCCTT CATTTTCCCTT CATTTTCCCCTT CATTTTCCCTT
G. stelleri (MH032428.1) G. kitaharae (MH032424.1) C. asperrimum (MH032407.1) E. grigorjewi (MH032414.1) C. pinetorum (MH032404.1) P. herzensteini (KF386365.1) M. achne (MH032473.1) P. stellatus (EF424428.1) K. bicoloratus (MH032487.1) P. yokohamae (KT878309.1) L. aspera (MH032456.1)	410 CACCTCGCGGGTATCT CACCTCGCGGGTATCT CACCTTGCAGGGATTT CACCTTGCAGGGATTT CACCTTGCCGGGATTT CACCTCGCGGAATTT CACCTCGCGGAATTT CACCTTGCCGGAATTT CACCTTGCCGGAATTT CACCTTGCCGGAATTT CACCTTGCCGGAATTT CACCTTGCCGGGATTT CACCTTGCCGGGATTT (b)PH-F403	420 CATCAATTCTA CATCGATCCTC CATCAATCCTC CATCAATCCTG CATCAATCCTG CTTCAATTCTA CCTCAATTCTA CATCAATTCTA CATCAATTCTA CATCAATTCTA CATCAATTCTA	430 GGGGCTATTA GGGGCCAATTA GGGGCAATTA GGGGCAATTA GGGGCAATCA GGGGCAATCA GGGGCAATCA GGGCCAATCA GGGCCAATCA GGGCCAATCA GGGCCAATCA	440 450 ACTTTATCACTAC ACTTTATTACTAC ATTTTATTACTAC ACTTTATTACTAC ACTTTATTACTAC ACTTTATTACCAC ACTTTATTACCAC ACTTTATTACCAC ACTTTATTACCAC ACTTTATTACCAC ACTTTATTACCAC ACTTTATTACCAC
G. stelleri (MH032428.1) G. kitaharae (MH032424.1) C. asperrimum (MH032407.1) E. grigorjewi (MH032414.1) C. pinetorum (MH032404.1) P. herzensteini (KF386365.1) M. achne (MH032473.1) P. stellatus (EF424428.1) K. bicoloratus (MH032487.1) P. yokohamae (KT878309.1) L. aspera (MH032456.1)	480 CATCATCAACATAAAA CATCATCAACATAAAA CATCATCAACATGAAA TATCATCAACATGAAA CATTATTAACATGAAA CATTATCAACATGAAA CATTATCAACATAAAA CATTATCAACATGAAA CATCATCAACATGAAA CATCATCAACATGAAA CATCATCAACATGAAA	470 CCT A C A A C A G T CCG A C A A C A G T CCG A C A A C A G T CCT C A G C A G T CCT A C A G C A G T CC A A C A G C A G T CC A A C A G C A G T CC A A C A G C A G T CC A A C A G C A G T CC A A C A G C A G T CC A A C A G C A G T CC A C A G C A G T CC A C A G C A G T (c)GK-F475	480 	490 500 1 1 CAAATCCCATTAT CAAATCCCATTAT CAAATCCCATTAT CAAATCCCATTAT CAAATCCCATTAT CAAATCCCACTAT CAAATCCCACTAT CAAATCCCACTAT

Fig. 1. Common primer information for COI region designed from mitochondrial sequences of *Glyptocephalus stelleri* (MH032428.1), *Glyptocephalus kitaharae* (MH032424.1), *Clidoderma asperrimum* (MH032407.1), *Eopsetta grigorjewi* (MH032414.1), *Cleisthenes pinetorum* (MH032404.1), *Pleuronectes herzensteini* (KF386365.1), *Microstomus achne* (MH032473.1), *Platichthys stellatus* (EF424428.1), *Kareius bicoloratus* (MH032487.1), *Pleuronectes yokohamae* (KT878309.1) and *Limanda aspera* (MH032456.1). COI, cytochrome c oxidase subunit I.

		560	570	580	590 600
G. stelleri (MH032428.1) G. kitaharae (MH032424.1)	GTCTTAGCO	GCTGGGATT GCTGGGATT	ACAATGCTCCT ACAATGCTCTT		CAACCTAAACAC CAACCTAAACAC
C. asperrimum (MH032407.1) F. ariaoriewi (MH032414.1)	GTCTTAGCO	GCTGGAATC	ACCATGCTAC1	TAACAGATCG	CAATCTTAACAC TAACCTTAACAC
C. pinetorum (MH032404.1)	GTCTTAGCO	GCTGGCATC	ACAATGCTGCI	CACAGACCG	TAACCTAAACAC
P. herzensteini (KF386365.1) M. achne (MH032473.1)	GTTCTGGCC	GCTGGCATC	ACAATGCTACI ACGATGTTGCI	TACAGACCG	CAACCTAAACAC AAATCTAAACAC
P. stellatus (EF424428.1)	GTCTTAGCO	GCTGGCATT	ACAATGCTACT	AACAGACCG	CAACCTGAACAC
K. bicoloratus (MH032487.1) P. vokobamae (KT878309.1)	GTCCTAGCO	GCTGGCATT	ACAATGCTACI		
L. aspera (MH032456.1)	GTCTTAGCO	GCTGGCATC	ACAATGCTACT	GACAGACCG	CAACCTAAACAC
				(d)	KB-F594
		660 · · · · · · · ·	670 .	680 .	690 700 .
G. stelleri (MH032428.1)	TATTCTGAT	TCTTTGGTC	ACCCAGAGGT	CTACATCTTA	ATCCTTCCAGGC
C. asperrimum (MH032407.1)	TATTCTGG	TCTTTGGTC	ACCCAGAAGT	ATACATTCTT	ATTCTCCCAGGT
E. grigorjewi (MH032414.1)	TATTCTGAT	TCTTTGGTC	ACCCTGAGGT	ATACATTCTT	ATCCTCCCAGGC
C. pinetorum (MH032404.1) P. herzensteini (KF386365.1)	TATTCTGA	TCTTTGGCC	ACCCAGAGGT	CTACATTTTA	ATTCTCCCAGGC
M. achne (MH032473.1)	TATTCTGG	TCTTTGGCC	ACCCAGAAGT	ATATATTCTT	ATTCTTCCGGGC
P. stellatus (EF424428.1) K. bicoloratus (MH022497.1)	TGTTCTGAT	TCTTTGGCC	ACCCAGAGGT	ATACATTTTA	ATTCTTCCAGGC
P. yokohamae (KT878309.1)	TATTCTGG	TCTTTGGTC	ATCCAGAAGT	TTACATTTTA	ATTCTTCCAGGC
L. aspera (MH032456.1)	TATTCTGAT	TCTTTGGCC	ACCCAGAGGT	АТАТАТТТА	ATTCTTCCAGGC
		700			(e)CA-F700
			.	/80 · · · · · · · ·	.
G. stelleri (MH032428.1) G. kitabarao (MH032424.1)	CTTTGGCT	CATGGGAAT	AGTCTGAGCT	A T G A T G G C T A	TTGGACTCCTAG
C. asperrimum (MH032407.1)	TTTTGGCT	CATGGGAAT	AGTCTGGGCT	ATAATAGCCA	TCGGACTCCTCG
E. grigorjewi (MH032414.1)	TTTTGGCT	TATGGGGAT	AGTCTGAGCT	ATGATGGCCA	TCGGGCTCCTAG
C. pinetorum (MH032404.1) P. herzensteini (KF386365.1)	CTTTGGCTA	CATGGGCAT	GGTCTGGGCT/	A I G A I A G C I A A T A A T G G C T A	TCGGACTGCTGG
M. achne (MH032473.1)	CTTTGGCT	CATGGGGAT	AGTCTGGGCT	ATGATGGCTA	TCGGCCTCCTAG
P. stellatus (EF424428.1) K. bicoloratus (MH022497.1)	CTTTGGCT	CATGGGCAT	GGTCTGAGCT	A T G A T G G C T A	TTGGACTCCTGG
P. yokohamae (KT878309.1)	CTTTGGTT	CATAGGAAT	AGTCTGGGCT	ATGATGGCTA	TCGGACTCCTGG
L. aspera (MH032456.1)	CTTTGGCT	TATAGGAAT	AGTCTGAGCT	ATGATGGCTA (f)	TTGGACTCCTGG MA-F793
		810 .	820 .	830	840 850 .
G. stelleri (MH032428.1)	GATTCATCO	GTCTGAGCCC	ACCACATGTT	CACGGTCGGA	ATAGACGTAGAC
G. Kitanarae (MH032424.1) C. asperrimum (MH032407.1)	GGTTCATC	GTCTGGGCCC	ATCACATGTT	TACGGTCGGA	ATGGATGTAGAC
E. grigorjewi (MH032414.1)	GGTTCATT	GTCTGGGC <mark>CC</mark>	ACCACATGTT	C A C G G T A <mark>G G A</mark>	A T A G A T G T A G A C
C. pinetorum (MH032404.1) P. herzensteini (KE386365.1)	GCTTCATCO	GTATGGGCCC	ATCACATGTT	TACGGTTGGA	ATAGATGTGGAC
M. achne (MH032473.1)	GGTTCATC	GTCTGGGCCC	ATCACATGTT	CACAGTCGGA	ATAGACGTAGAC
P. stellatus (EF424428.1)	GCTTCATCO	GT A T G G G C C C	ATCACATGTT	TACAGTCGGA	ATAGACGTAGAC
K. bicoloratus (MH032487.1) P. vokohamae (KT878309.1)	GCTTCATC	GTATGGGCCC	ACCACATGIT	TACAGTIGGA	ATAGACGTAGAC
L. aspera (MH032456.1)	GCTTCATCO	GT A T G G G <mark>C T C</mark>	ATCACATGTT	TACAGTTGGA	A T A G A C G T G G A C
				(g)EG-F835	
		860	870	880	890 900
G. stelleri (MH032428.1)	ACACGAGCO	TACTTTACA	TCTGCCACAAT	TAATCATTGC	GATCCCAACCGG
G. kitaharae (MH032424.1)	ACACGAGCO	TACTTTACA	TCTGCCACAAT	TAATTATTGC.	AATCCCAACCGG
E. grigorjewi (MH032407.1)	ACACGAGGC	TACTTTACC	GCTGCCACAA	TAATCATIGC	GATCCCAACCGG
C. pinetorum (MH032404.1)	ACACGAGCO	TACTTTACT	TCTGCCACAA	I G A T T A T C G C	CATCCCAACAGG
P. herzensteini (KF386365.1) M. achne (MH032473.1)	ACACGAGGC		TCTGCCACAA1	TAATCATTGC	
P. stellatus (EF424428.1)	ACACGAGCI	TACTTTACC	TCAGCCACAAI	TAATTATTGC	CATCCCAACCGG
K. bicoloratus (MH032487.1)	ACACGAGCI	TACTTTACC	TCAGCCACAAT	TAATCATTGC	CATCCCAACCGG
r. yokonamae (K1878309.1) L. aspera (MH032456.1)	ACACGAGCO	TACTTTACC	TCTGCCACAA	I GATTATIGC	CATCCCAACTGG

(h)PY-F892

Fig. 1. Continued.

		1110		1120	1130	1140	1150
G. stelleri (MH032428.1)	GGGCIGI	ATIC	GCAAICO	STIGCCG	CCTTCGTAC	ACIGATICCCGC	
G. Kitaharae (MH032424.1)	GGGCIGI	ATIC	GCAAICO	STIGCCG	CCTTCGTAC	ACIGATICCCGI	IAIII
C. asperrimum (MH032407.1)	GAGCIGI	ATTT	GCAATCO	GTIGCIG	CCTTIGICC	ACTGATTCCCAT	IGIII
E. grigorjewi (MH032414.1)	GGGCTGT	GTTT	GCAATCO	STTGCCG	CCTTCGTCC	ACTGATTCCCCC	TGTTT
C. pinetorum (MH032404.1)	GTGCTGT	GTTT	GCAATCO	STTGCCG	CCTTCGTAC	ACTGATTCCCCC	ттттт
P. herzensteini (KF386365.1)	GGGCTGT	CTTT	GCGATCO	GTTGCCG	CCTTCGTAC	ACTGATTCCCAC	TATTC
M. achne (MH032473.1)	GTGCTGT	CTTT	GCAATCO	STTGCCG	CCTTCGTCC	ACTGATTCCCCC	TATTT
P. stellatus (EF424428.1)	GAGCTGT	ATTT	GCAATCO	STTGCCG	CCTTTGTGC	ACTGATTCCCCC	TATTT
K. bicoloratus (MH032487.1)	GAGCTGT	ATTT	GCAATCO	STTGCCG	CCTTTGTAC	ACTGATTTCCCC	TATTT
P. yokohamae (KT878309.1)	GGGCTGT	ATTT	GCAATCO	STTG <mark>CC</mark> G	CTTTCGTAC	ACTGATTCCCCC	TATTT
L. aspera (MH032456.1)	GTGCTGT	GTTT	GCAATCO	STTGCCG	CCTTCGTAC	ACTGATTCCCCC	TATTT
						(j)CP-F11	47
		1210		1220	1230	1240	1250
		.					
G. stelleri (MH032428.1)	GTTTATT	GGCG	TCAACT	GACGII	TTTCCCTCA	ACACTTCCTTGG	CCTGG
G. kitaharae (MH032424.1)	GTTTATT	GGCG	TCAACT	AACGTT	TTTCCCCCA	ACATTTCCTTGG	CCTGG
C. asperrimum (MH032407.1)	ATTTATT	GGGG	TTAATT	FAACATT	CTTCCCTCA	ACATTTTCTGGG	CCTGG
E. grigorjewi (MH032414.1)	ATTTATT	GGGG	TCAATC	FGACATT	CTTCCCCCA	ACACTTCCTCGG	TCTGG
C. pinetorum (MH032404.1)	ATTTGTA	GGAG	TTAATC	ГТАСАТТ	CTTCCCCCA	GCACTTCCTTGG	CCTGG
P. herzensteini (KF386365.1)	GTTTGTG	GGGGG	TCAACCI	FCACATT	CTTCCCCCA	ACATTTTCTGGG	TCTCG
M. achne (MH032473.1)	GTTTATT	GGGG	TTAACCI	Г А А <mark>С</mark> G T T	CTTCCCACA	ACACTTCCTTGG	CCTGG
P. stellatus (EF424428.1)	GTTTGTC	GGAG	TCAATTI	ГААСАТТ	CTTCCCCCA	ACACTTCCTCGG	TCTAG
K. bicoloratus (MH032487.1)	GTTTGTA	GGGG	TTAATTI	ГААСАТТ	CTTCCCCCA	ACACTTCCTGGG	CCTAG
P. yokohamae (KT878309.1)	GTTTGTA	GGGG	TTAACCI	FCACATT	CTTCCCCCA	ACATTTCCTGGG	TCTCG
L. aspera (MH032456.1)	GTTTGTA	GGTG	TAAATCI	FCACATT	CTTCCCCCA	ACATTTTCTTGG	CCTTG
						(k)LA	-F1249
		1360		1370	1380	1390	1400
		· · I ·					· · · · I
G. stelleri (MH032428.1)	ATTCTTA	TTTA	TTATTT	GAGAAGC	ATTTACAGO	CAAACGAGAGGT	TGGAG
G. kitaharae (MH032424.1)	ATTCTTA	TTTA	TTATTI	GAGAAGC	ATTTACAGO	CAAACGAGAAGT	TGGGG
C. asperrimum (MH032407.1)	ATTTTA	TTCA	TTATCTO	GAGAAGC	ATTTACAGO	CAAACGAGAAGT	CGGGG
E. grigorjewi (MH032414.1)	ATTCTTA	TTCA	TTATTT	G A G A A G C	ATTTACAGO	CAAACGAGAGGT	CGGCG
C. pinetorum (MH032404.1)	ATTTT	TTTA	TTATTT	GAGAAGC	ATTCACAGO	CAAACGAGAAGT	CGGAG
P. herzensteini (KF386365.1)	ATTTT	TTTA	TTATTT	3 A G A A G C	ATTTACGGC	CAAACGAGAAGT	CGGCG
M. achne (MH032473.1)	ATTCTTA	TTTA	TTATTT	3 A G A A G C	ATTTACAGO	CAAACGAGAAGT	TGGTG
P. stellatus (EF424428.1)	ATTTTA	TTTA	TTATTT	3 A G A A G C	ATTTACTGC	CAAACGAGAAGT	CGGGG
K. bicoloratus (MH032487.1)	ATTTTA	TTTA	TTATTT	G A G A A G C	ATTTACTGC	CAAACGAGAAGT	CGGCG
P. yokohamae (KT878309.1)	ATTTT	STTTA	TTATTT	G A G A A G C	ATTTACGGC	CAAACGAGAAGT	CGGGG
L. aspera (MH032456.1)	ATTTT	TTTA	TTATTT	G A G A A G C	ATTTACAGO	CAAACGAGAAGT	CGGGG
					G	JM_1370-R	

Fig. 1. Continued.

Table 2. Information of COI region common primers from mtDNA sequences of the eleven species of family Pleuronectidae

Oligo name	Sequence (5' \rightarrow 3')	Mer	Tm (°C)	GC (%)	Product size
GJM-F128	GCA CAC GCC TTT GTA ATA AT	20	59	40	1 242 bp
GJM-R1370	CGT TTG GCT GTA AAT GCT TC	20	62	45	1,242 DP

COI, cytochrome c oxidase subunit I; mtDNA, mitochondrial DNA; Tm, temperature.

the DNASTAR SeqMan software (DNASTAR, Madison, WI, USA). Finally, a 1,262 bp sequence of the COI gene region was obtained. The obtained sequences were analyzed to identify species-specific single nucleotide polymorphisms (SNPs), excluding intraspecific variations caused by point mutations. Species-specific primers were designed to differentiate each species based on the size differences of the PCR amplification products during gel electrophoresis (Fig. 2). Primers were designed based on the size of the PCR amplification products

for each species to achieve species discrimination. Primers were designed with a species-specific SNP located at the 3' end to enable the amplification of only the target species. Eleven forward primers were used in this study. Species-specific forward primers were also designed to allow amplification at similar melting temperature (Tm) values for convenience in the experimental procedures. For five species of Pleuronectidae, *G. stelleri*, *T. kitaharae*, *C. asperrimum*, *E. grigorjewi*, and *C. pinetorum*, the primers were designed at 58 °C, while for the six species, *P.*



Fig. 2. Sensitivity for detection of the eleven species of family Pleuronectidae with species specific primer sets respectively. The sensitivity analysis was determined 50 ng/ μ L to 0.01 ng/ μ L of the genomic DNA each of one individuals. Lane: (M) 100 bp DNA ladder (Dynebio); (1) 50 ng; (2) 10 ng; (3) 1 ng; (4) 0.1 ng; (5) 0.01 ng.

herzensteini, *M. achne*, *P. stellatus*, *K. bicoloratus*, *Pleuronectes yokohamae* and *L. aspera* the primers were designed at $62 \,^{\circ}$ C. We also considered factors such as GC content to prevent self-dimer formation while designing the primers (Table 3).

Multiplex species-specific (MSS)-polymerase chain reaction (PCR)

For MSS-PCR amplification, three sets of primer mixtures capable of distinguishing 11 Pleuronectidae species were prepared. The first set comprised five species: *G. stelleri*, *T. kitaharae*, *C. asperrimum*, *E. grigorjewi*, and *C. pinetorum*. The second set comprised three species, *P. herzensteini*, *M. achne* and *P. stellatus*. The third set comprised three species: *K. bicoloratus*, *P. hokohamae*, and *L. aspera*.

The reaction mixture for MSS-PCR amplification was composed of 1 μ L of gDNA extracted from each sample, 1 μ L of 10x Ex Taq buffer, 0.2 μ L (for 5 species) to 0.3 μ L (for 3 species) of a 2.5 mM dNTP mixture, 0.3 μ L (for 3 species) to 0.4 μ L (for 5 species) of 10 pmol forward primer, 0.6 μ L (for 3 species) to 0.8 μ L (for 5 species) of 10 pmol reverse primer, and 0.5 units of HS-

Taq polymerase (Anti-HS Taq) in a total volume of 10 µL.

The PCR conditions were as follows: pre-denaturation at $95 \,^{\circ}$ C for 10 min, denaturation at $95 \,^{\circ}$ C for 40 s, annealing at $58 \,^{\circ}$ C (for 5 species) to $62 \,^{\circ}$ C (for 3 species) for 40 s, and extension at $72 \,^{\circ}$ C for 50 s. These cycles were repeated 30 times, followed by a final extension at $72 \,^{\circ}$ C for 7 min. Amplification was performed using the ABI Verity Fast Thermal Cycler.

PCR was performed using an ABI Verity Fast Thermal Cycler under the same conditions described above, with different annealing temperatures for each set. Subsequently, the amplified PCR products were separated on a 2% agarose gel at 100 V for 40 min by gel electrophoresis. The gel was stained with 1X loading dye (Dynebio, Seongnam, Korea), and the PCR products were visualized using a Gel Doc image analysis system (ATTO, Tokyo, Japan).

Results

Species-specific region discovery

Based on the COI gene nucleotide sequences registered in the

NCBI GenBank database, we explored conserved regions with minimal variation and designed species-specific forward and reverse primers for 11 species of the Pleuronectidae family (Fig. 1). The designed primers were applicable to all 11 Pleuronectidae species. They were used as reference markers to confirm amplification by PCR and served as a universal primer set for the Pleuronectidae family.

Analysis of cytochrome c oxidase subunit I (COI) gene sequences in 11 species of the Pleuronectidae family

The COI gene region (1,262 bp) obtained from the 11 species was analyzed using DNA Sequence Polymorphism software (DnaSP, ver. 5.10.01) with universal primers designed to apply to all 11 species. In the analyzed sequences, excluding intraspecies genetic variations, distinct interspecies variations were observed in the form of species-specific SNPs across all 11 species (Fig. 1). Based on these observations, species-specific forward primers were designed with SNPs located at the 3' end, allowing for differentiation among the 11 species of the Pleuronectidae family with sequence differences of approximately 100 bp or more (Fig. 1 and Table 3).

Haplotype diversity analysis revealed the following values: *C. pinetorum* 0.855, *P. herzensteini* 0.389, *P. yokohamae* 0.896, *C. asperrimum* 0.873, *K. bicoloratus* 0.495, *G. stelleri* 0.925, *M. achne* 0.889, *T. kitaharae* 0.923, *E. grigorjewi* 0.795, and *L. aspera* 0.948. However, *P. stellatus* exhibited only one haplotype, indicating an absence of genetic diversity.

Species-specific polymerase chain reaction (PCR) specificity test

To determine the maximum limit of the annealing temperature, gradient PCR reactions were performed using conditions ranging from 58 °C to 62 °C. PCR amplification was performed using forward primers specific to each species and a universal reverse primer (GJM_1370-R) specific to the Pleuronectidae family. The amplified products were visualized through electrophoresis on a 1.8% agarose gel. The optimal temperature for each primer was determined based on the temperature at which the highest species-specific amplification was observed (Table 3).

Multiplex species-specific (MSS)-polymerase chain reaction (PCR) specificity test

Upon verification of the MSS-PCR products containing equal amounts of forward primers by agarose gel electrophoresis, clear DNA amplification was observed for each species. The amplified products were of the expected size for each species: *G. stelleri* (1,064 bp), *T. kitaharae* (933 bp), *C. asperrimum* (709 bp), *P. hokohamae* (571 bp), *C. pinetorum* (259 bp), *P. herzensteini* (1,006 bp), *M. achne* (613 bp), *P. stellatus* (312 bp), *K. bicoloratus* (815 bp), *P. hokohamae* (517 bp), and *L. aspera* (159 bp). No primer dimers or nonspecific amplification was observed. Furthermore, species-specific PCR amplification was confirmed, without crossreactivity in the DNA mixtures of each primer set (Fig. 3).

Cata	Nama		Townshowssins	Tres (°C)	Due du et eine (leve)
Sets	Name	Sequence (5 \rightarrow 3)	larget species	Im(C)	Product size (bp)
Set1	Gs-F344	GGTGAACCGTTTACCCTCCC	Glyptocephalus stelleri	58	1,064
	Tk-F475	ATCAACATAAAACCGACAACC	Tanakius kitaharae		993
	Ca-F700	ATACATTCTTATTCTCCCAGGT	Clidoderma asperrimum		709
	Eg-F835	CCACCACATGTTCACGGTA	Eopsetta grigorjewi		571
	Cp-F1147	CGTACACTGATTCCCCCTT	Cleisthenes pinetorum		259
Set2	Ph-F403	CCTCACCATTTTCTCTCTTCAT	Pleuronectes herzensteini	62	1,006
	Ma-F793	GGCTATGATGGCTATCGGC	Microstomus achne		613
	Ps-F1096	CACTTCCACTATGTCCTATCA	Platichthys stellatus		312
Set3	Kb-F594	TGCTACTAACAGACCGCAACT	Kareius bicoloratus	62	815
	Py-F892	TGCCACAATAATTATTGCCATT	Pleuronectes yokohamae		517
	La-F1249	CCCAACATTTTCTTGGCCTT	Limanda aspera		159
	GJM_128-F	GCACACGCCTTTGTAATAAT	Common primers	54	1,242
	GJM 1370-R	CGTTTGGCTGTAAATGCTTC			

Table 3. Species specific primers for multiplex PCR informations

PCR, polymerase chain reaction; Tm, temperature.

(A) common primer



Fig. 3. Identification of species by multiplex PCR using common primer and species-specific primers. Samples are identified as follows: (A) common primer, (B) template mixture (1), (7), (11); 100 bp DNA ladder (Dynebio), (2) *Glyptocephalus stelleri*; (3) *Tanakius kitaharae*; (4) *Clidoderma asperrimum*; (5) *Eopsetta grigorjewi*; (6) *Cleisthenes pinetorum*; (8) *Pleuronectes herzensteini*; (9) *Microstomus achne*; (10) *Platichthys stellatus*; (12) *Kareius bicoloratus*; (13) *Pleuronectes yokohamae*; (14) *Limanda aspera*; (M) 100 bp DNA ladder (Dynebio). PCR, polymerase chain reaction.

Multiplex species-specific (MSS)- polymerase chain reaction (PCR) sensitivity analysis

To assess the sensitivity of the MSS-PCR analysis, DNA from 11 species of the Pleuronectidae family was quantified at concentrations of 50, 10, 1 ng/µL, 0.1 ng/µL, and 0.01 ng/µL for experimentation. The PCR analysis sensitivity for each species was as follows: *C. asperrimum* exhibited detection at 50 ng/µL, while *G. stelleri*, *P. herzensteini*, *P. stellatus*, *K. bicoloratus*, and *P. hokohamae* showed detection down to 0.1 ng/µL. *T. kitaharae*, *E. grigorjewi*, *C. pinetorum*, *M. achne*, and *L. aspera* demonstrated detection capabilities at 0.01 ng/µL of DNA concentration (Fig. 2).

Discussion

The diverse and morphologically similar nature of fish species within the family Pleuronectidae makes species identification based on morphological characteristics challenging. However, the application of molecular biology techniques, such as genetic analysis, has facilitated the identification of clear species (Kwun & Kim, 2010, 2016). mtDNA analysis for biological species identification offers advantages over nuclear DNA analysis because mtDNA has a higher copy number, which enables the extraction of a significant amount of DNA, even with limited sample quantities (Cohen et al., 2009; Matsui et al., 2012). Furthermore, mtDNA is maternally inherited and possesses only one allele, thereby avoiding the ambiguity of genetic sequences caused by allele heterozygosity. Due to its relatively higher mutation rate than nuclear DNA, mtDNA is commonly used in species identification and molecular phylogenetic studies involving closely related species (Matsui et al., 2012). However, the high mutation rate of mtDNA can also lead to intraspecies genetic variation, making it important to validate the accuracy of analytical methods by analyzing multiple samples in studies focusing on species identification among closely related species.

The MSS-PCR analysis method offers advantages over traditional non-specific primer-based methods, such as RFLP and RAPD, in terms of reproducibility, time, and cost; most importantly, it provides clear and repeatable results for species discrimination (Kim & Lee, 1990; Kim et al., 2009; Lin & Hwang, 2008; Sezaki et al., 2005). Recently, MSS-PCR analysis has been applied to the development of species analysis techniques for various aquatic products, including shrimp paste, smoked eel, mackerel, and horse mackerel (Kim et al., 2009, 2014; Noh et al., 2016). In MSS-PCR analysis, it is crucial to design primers with size differences in the PCR products to ensure clear species discrimination and establish conditions for multiplex PCR that prevent non-specific PCR reactions and maintain appropriate annealing temperatures (Koh et al., 2011; Silfvergrip, 2009).

In this study, we developed and established conditions for the MSS-PCR analysis method, which allows multiplex PCR targeting of 11 species of the Pleuronectidae family where morphological species identification is challenging. We analyzed the COI gene region of mtDNA and validated the applicability of the primers for species specificity, reproducibility, and efficiency in 11 species.

This analytical method enables accurate species identification of processed flatfish products or imported seafood, where traditional morphological identification is difficult. This can contribute to the establishment of a sound distribution system and ensure food safety. Therefore, they can be effectively utilized to enhance the safety and reliability of seafood products.

Competing interests

No potential conflict of interest relevant to this article was reported.

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Not applicable.

Availability of data and materials

Upon reasonable request, the datasets of this study can be available from the corresponding author.

Ethics approval and consent to participate

This study conformed to the guidance of animal ethical treatment for the care and use of experimental animals.

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References

Acar C, Ishizaki S, Nagashima Y. Toxicity of the lessepsian pufferfish *Lagocephalus sceleratus* from eastern Mediterranean coasts of Turkey and species identification by rapid PCR amplification. Eur Food Res Technol. 2017;243:49-57.

Axayácatl RO, Juan PCG. Molecular identification of dolphinfish species (genus *Coryphaena*) using multiplex haplotype-specific PCR of mitochondrial DNA. Ichthyol Res. 2008;55:389-93.

Cohen NJ, Deeds JR, Wong ES, Hanner RH, Yancy HF, White KD, et al. Public health response to puffer fish (tetrodotoxin) poisoning from mislabeled product. J Food Prot. 2009;72:810-7.

Hsieh CH, Chang WT, Chang HC, Hsieh HS, Chung YH, Hwang DF, et al. Puffer fish-based commercial fraud identification in a segment of cytochrome *b* region by PCR–RFLP analysis. Food Chem. 2010;121:1305-11.

Hsieh YW, Hwang DF. Molecular phylogenetic relationships of puffer fish inferred from partial sequences of cytochrome *b* gene and restriction fragment length polymorphism analysis. J Agric Food Chem. 2004;52:4159-65.

Kang JH, Noh ES, Park JY, An CM, Choi JH, Kim JK, et al. Rapid origin determination of the northern mauxia shrimp (*Acetes chinensis*) based on allele specific polymerase chain reaction of partial mitochondrial 16S rRNA gene. Asian-Australas J Anim Sci. 2015;28:568-72.

Kim IS, Lee WO. Synopsis of the suborder Tetraodontoidei (Pisces; Tetraodontiformes) from Korea. Korean J Ichthyol. 1990;2:1-27.

Kim KH, Lee HY, Kim YS, Kim MR, Jung YK, Lee JH, et al. Development of species-specific PCR to determine the animal raw material. J Food Hyg Saf. 2014;29:347-55.

Kim WJ, Kong HJ, Kim YO, Nam BH, Kim KK. Development of RAPD-SCAR and RAPD-generated PCR-RFLP markers for identification of four *Anguilla* eel species. Anim Cells Syst. 2009;13:179-86.

Koh BRD, Kim JY, Na HM, Park SD, Kim YH. Development of species-specific multiplex PCR assays of mitochondrial 12S rRNA and 16S rRNA for the identification of animal species. Korean J Vet Serv. 2011;34:417-28.

Kwun HJ, Kim JK. Validation of morphology-based identifi-

cation of two cynoglossidae larvae using mitochondrial DNA. Korean J Fish Aquat Sci. 2010;43:482-8.

- Kwun HJ, Kim JK. Re-identification of two tonguefishes (Pleuronectiformes) from Korea using morphological and molecular analyses. Korean J Fish Aquat Sci. 2016;49:208-13.
- Lee SJ, Kim JK, Ryu JH, Yu HJ, Ji HS, Im YJ, et al. Molecular identification and morphological description of larvae for ten species of the family Pleuronectidae (Pleuronectiformes, PISCES) from Korea. J Korean Soc Fish Ocean Technol. 2019;55:335-48.
- Lin WF, Hwang DF. A multiplex PCR assay for species identification of raw and cooked bonito. Food Control. 2008;19:879-85.
- Matsui S, Nakayama K, Kai Y, Yamashita Y. Genetic divergence among three morphs of *Acentrogobius pflaumii* (Gobiidae) around Japan and their identification using multiplex haplotype-specific PCR of mitochondrial DNA. Ichthyol Res. 2012;59;216-22.
- Noh ES, Kang HS, An CM, Park JY, Kim EM, Kang JH. Rapid and specific identification of genus *Cynoglossus* by multiplex PCR assays using species-specific derived from the COI region. J Life Sci. 2016;26:1007-14.
- Noh ES, Lee MN, Kim EM, Park JY, Noh JK, An CM, et al. Development of a multiplex PCR assay for rapid identification of *Larimichthys polyactis*, *L. crocea*, *Atrobucca nibe*, and *Pseudotolithus elongates*. J Life Sci. 2017;27:746-53.
- Rasmussen RS, Morrissey MT. DNA-based methods for the identification of commercial fish and seafood species. Compr Rev Food Sci Food Saf. 2008;7:280-95.
- Sezaki K, Itoi S, Watabe S. A simple method to distinguish two commercially valuable eel species in Japan *Anguilla japonica* and *A. Anguilla* using polymerase chain reaction strategy with a species-specific primer. Fish Sci. 2005;71:414-21.
- Silfvergrip AMC. CITES identification guide to the freshwater eels (Anguillidae): with focus on the European eel *Anguilla anguilla*. Stockholm: Swedish Environmental Protection Agency Press; 2009.
- Ward RD, Hanner R, Hebert PDN. The campaign to DNA barcode all fishes, FISH-BOL. J Fish Biol. 2009;74:329-56.