



Research Article

Development and validation of ultra-fast quantitative real-time PCR method to differentiate between *Oncorhynchus keta* and *Oncorhynchus mykiss*

Min-Ji Park¹, Han-Cheol Lee¹, Ji-Young Yang², Jung-Beom Kim^{1*}

¹Department of Food Science and Technology, Suncheon National University, Suncheon 57922, Korea

²Department of Food Science and Technology, Pukyong National University, Busan 48513, Korea

Abstract The ultra-fast quantitative real-time polymerase chain reaction (qPCR) assay was developed and validated to differentiate the morphologically similar ones, *Oncorhynchus keta* and *Oncorhynchus mykiss*. Species-specific primers were designed for the *COI* genes of mtDNA. The species-specific primers designed for *O. keta* and *O. mykiss* were selectively amplified by *O. keta* and *O. mykiss* DNA, respectively. The sensitivity of *O. keta* and *O. mykiss* primers was 1 ng/ μ L. Quantitative testing showed that the results met the 'Guidelines on Standard Procedures for Preparing Analysis Method such as Food' proposed by the Ministry of Food and Drug Safety. The qPCR method developed and validated in this study for identifying *O. keta* and *O. mykiss* has advantages such as speed and field applicability. Therefore, this method is expected to help control forgery and alteration of raw materials in the seafood industry.



OPEN ACCESS

Citation: Park MJ, Lee HC, Yang JY, Kim JB. Development and validation of ultra-fast quantitative real-time PCR method to differentiate between *Oncorhynchus keta* and *Oncorhynchus mykiss*. Korean J Food Preserv, 30(3), 383-394 (2023)

Received: April 19, 2023

Revised: May 18, 2023

Accepted: May 21, 2023

***Corresponding author**

Jung-Beom Kim

Tel: +82-61-750-3259

E-mail: okjbkim@scnu.ac.kr

Copyright © 2023 The Korean Society of Food Preservation. This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/4.0>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Keywords *Oncorhynchus keta*, *Oncorhynchus mykiss*, ultra-fast PCR, validation, monitoring

1. Introduction

Salmon (*Oncorhynchus keta*), rainbow trout (*O. mykiss*), and masu salmon (*O. masou*), which belong to the genus *Oncorhynchus*, inhabit the coast of South Korea (Jun et al., 2020; Kang et al., 2007). Salmon contains high-quality protein and omega-3 fatty acids such as eicosapentaenoic acid, docosahexaenoic acid, and vitamin A (Cha et al., 2020). Therefore, salmon was declared as one of the world's top 10 super-foods in the United States in 2002 (Cha et al., 2020; Horowitz, 2002). Since salmon was selected as one of the world's top 10 super-foods, attention to and consumption of salmon have been rising rapidly and globally. In addition, salmon consumption in South Korea has more than doubled from 12,000 tons in 2010 to 34,000 tons in 2015 (Horowitz, 2002; MOF, 2016). However, the prices of salmon hit an all-time high in 2015 owing to the repeated failure of salmon farming. Some people have suggested rainbow trout as an alternative to salmon due to the unstable prices and production of salmon (Heu et al., 2015; Paek

and Park, 2016). Rainbow trout has characteristics similar to salmon, with a bright red flesh. Since the 1960s, rainbow trout has been successfully cultured mainly in Gangwon-do, Korea, with production reaching more than 3,000 tons since 2011 (Heu et al., 2015; Kim, 2018; Kim et al., 2015d; Lee et al., 2021; Park et al., 2017).

For salmon and rainbow trout sold in the market, it is difficult to morphologically distinguish the original product as these are processed in the form of sashimi or smoked products after filleting the inedible frames (Heu et al., 2008; Kang et al., 2006; Kang et al., 2014; Kim et al., 2019a; Ko et al., 2016). Accordingly, cases of fake food (economically motivated adulteration, EMA) to gain economic benefits, such as counterfeiting and selling relatively inexpensive rainbow trout as salmon, have been reported. Therefore, scientific methods are required to differentiate salmon from rainbow trout. Fake food refers to food that is intentionally mixed with low-priced raw materials or mis-labelled as expensive raw materials for processed seafood, and is often difficult to identify using morphological or sensory methods (Park et al., 2013; Warner et al., 2019). Recently, the manufacture and distribution of fake foods have been increasing rapidly. In particular, cases of fake seafood have been reported through the media, resulting in an increase in anxiety among customers of seafood (Kim et al., 2015b; Park et al., 2012a; Park et al., 2012b). Accordingly, there is a need to develop scientific technologies that can accurately classify and identify species to ensure food safety and eradicate fake food.

Protein and physicochemical analysis methods have been used to determine the species of marine products in the past (Chun et al., 2014; Kim et al., 2014a; Kim et al., 2019a; Noh et al., 2017; Park et al., 2012a; Park et al., 2012b). The protein analysis

method can not distinguish between the morphologically similar species, and the accuracy of the analysis results decreases because of changes in the protein structure during processing, such as heating and drying (Chung et al., 2017; Esposti et al., 1993; Kim et al., 2014b). These methods are not suitable for determining the raw materials of processed foods because of the limitations in using complex experimental methods and expensive equipment (Kim et al., 2018). Therefore, molecular analysis methods using genes have recently been developed (Kim et al., 2015c). Genetic analysis has high specificity and sensitivity, and can be applied to processed foods that undergo heat treatment. Therefore, it is suitable as an analytical method for determining raw materials for processed marine products (Axayacatl and Juan, 2008; Chung et al., 2017; Hold et al., 2001). Representative genetic analysis methods include polymerase chain reaction (PCR), which includes multiplex PCR, real-time PCR, and ultra-fast quantitative real-time PCR (qPCR; Chung et al., 2017; Kim et al., 2019a).

Multiplex PCR can detect two or more samples in one experiment, but takes a long time to conduct electrophoresis for analyzing the results (Chung et al., 2017; Koh et al., 2011). Additionally, in order to accurately determine the electrophoresis result with the naked eye, the separation distance of each PCR product size needs to be 100 bp or more. Therefore, strict conditions are required when designing the primer (Kim et al., 2019a; Kim et al., 2019b; Koh et al., 2011). Real-time PCR has high sensitivity and is capable of qualitative and quantitative experiments, but requires expensive reagents and equipment and takes a long time (Koh et al., 2011). The qPCR can detect and record fluorescence expressed during the gene amplification process to observe PCR reactions in real time (Wang et al.,

2016). Since PCR chips have been used, heat transfer has become faster than that in real-time PCR using PCR tubes, and PCR reactions can proceed quickly (Kim et al., 2007; Lee et al., 2007). Furthermore, it can conduct both qualitative and quantitative experiments, has a portable design, and can perform PCR on the spot. It has been reported that genetic analysis for identifying salmon and rainbow trout has been conducted using DNA chips (Hwang et al., 2007). The DNA chip method can simultaneously compare a large number of genes. However, producing DNA chips is a time-consuming and expensive process and requires expensive chip scanner equipment to decode the results (Im et al., 2012; Kim et al., 2008; Lee et al., 2000).

As salmon and rainbow trout are sold in the form of fillets, quantitative analysis can be used to determine whether mixing, and a genetic analysis method is needed to rapidly determine multiple samples in one experiment. Therefore, in this study, an qPCR method that can conduct rapid qualitative and quantitative experiments on salmon and rainbow trout, which are difficult to distinguish morphologically, was developed and validated, and field applicability was confirmed through monitoring.

2. Materials and methods

2.1. Samples

The seven reference samples used in this study were provided by Pukyong National University: salmon (*Oncorhynchus keta*), rainbow trout (*O. mykiss*), sockeye salmon (*O. nerka*), king salmon (*O. tshawytscha*), coho salmon (*O. kisutch*), masu salmon (*O. masou*), and Atlantic salmon (*Salmo salar*). These reference species showed similarity of 98% or more in the sequence of the cytochrome C oxidase subunit I (*COI*) region, confirming that they were

the same species. A total of 14 samples (8 salmon and 6 rainbow trout) were purchased from the market and used for monitoring.

2.2. Species-specific primer design

Species-specific primers were designed for the *COI* genes of salmon and rainbow trout mitochondrial DNA (mtDNA) registered in the National Center for Biotechnology Information (NCBI) database. The Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo>) program was used for comparison and analysis of the DNA sequences, and the Primer 3 program was used for species-specific primer design.

2.3. Ultra-fast quantitative real-time PCR optimum reaction conditions

To establish the optimum reaction conditions for ultra-fast PCR, the experiment was conducted by varying the binding temperature and time. The annealing temperatures were 58, 59, and 60°C, and the annealing times were 15 and 25 s. The qPCR reaction was repeated thrice using the UF-150 GENECHECKER Ultra-Fast PCR system (Genesystem, Korea). The PCR reaction was checked in real time using the GeneRecorder.exe-Shortcut (Genesystem, Korea) program. The PCR reaction mixture (10 μ L) was prepared by mixing 5 μ L Rapi:Detect™ Master Mix (Genesystem, Korea), 1 μ L (10 ng/ μ L) genomic DNA, 1 μ L (10 pmol/ μ L) primer, and 2 μ L sterile distilled water.

2.4. Validation of ultra-fast quantitative real-time PCR

To test the specificity of the developed species-specific primer, genomic DNA of five species (*O. nerka*, *O. tshawytscha*, *O. kisutch*, *O. masou* and *Salmo salar*) was extracted, and the extracted genomic DNA concentration was diluted to 10 ng/ μ L for use as template DNA for PCR. Sterile distilled

water was used as a negative control. The specificity experiment was repeated thrice. In order to test the sensitivity of the developed species-specific primer, the genomic DNA concentrations of the salmon and rainbow trout reference samples were diluted to 10, 5, 2.5, 1, and 0.1 ng/ μ L and used as a template DNA in PCRs. The sensitivity experiment was repeated thrice. Calibrators were manufactured by mixing salmon and rainbow trout genomic DNA at 100%, 70%, 50%, 30%, and 10% to confirm the slope, amplification efficiency, and linearity of the developed species-specific primers. The calibration experiment was repeated thrice. Unknown samples were prepared by mixing salmon and rainbow trout genomic DNA at 80%, 50%, and 20% to confirm the accuracy and relative standard deviation (RSDr) of the developed species-specific primers. The unknown sample test was repeated thrice. To calculate the limit of quantitation (LOQ), salmon genomic DNA was diluted to 10%, 9%, and 8% to produce unknown samples. Genomic DNA from rainbow trout was diluted to 10%, 9%, 8%, 7%, and 6% to produce unknown samples, and the quantitative limit experiment was repeated 20 times.

2.5. Monitoring

A total of 14 cases (8 salmon and 6 rainbow trout) were purchased from the market and monitored to confirm the field applicability of the salmon and rainbow trout qPCR method developed in this study.

3. Results and discussion

3.1. Species-specific primer design

Primers for salmon and rainbow trout were designed for the *COI* gene of the mtDNA registered in the NCBI database. As mtDNA is inherited from the mother, there is no gene recombination. Therefore, it is suitable for analyzing genetic changes within and between species (Kang et al., 2010b; Kim et al., 2015a). In particular, the *COI* gene of mtDNA is widely used to identify species (Hebert et al., 2003; Kang et al., 2010a; Seo et al., 2010). Species-specific primers were designed using the Crustal Omega program, and the DNA sequences of the developed primers are shown in Table 1. The salmon and rainbow trout primers used in this study were designed to have a product size of 200 bp for quick and accurate determination in the field (Kim et al., 2015b). Each primer was designed in consideration of 18-24 bp, GC content ratio of 40-60%, and melting temperature (T_m) value of 52-66°C (Buck et al., 1999).

3.2. Optimum reaction conditions for ultra-fast quantitative real-time PCR

To establish the optimal reaction conditions for ultra-fast PCR, the primer binding temperature was divided into 58, 59, and 60°C, and the primer binding time was divided into 15 and 25 s (Table 2). The bonding temperature was set on the basis of the T_m values of the primers developed in this

Table 1. Primer sequence designed in this study

Species		Sequences	Product size (bp)	T_m (°C)	Target gene
<i>Oncorhynchus keta</i>	Forward	5'-TCT AGG GGA TGA CCA GAT CTA CA -3'	184	59.3	<i>COI</i> ¹⁾
	Reverse	5'-GGA CGG AGG TAG GAG TCA GA -3'		59.7	
<i>Oncorhynchus mykiss</i>	Forward	5'-CCC TAA TAA TCG GAG CCC CT -3'	133	58.3	<i>COI</i>
	Reverse	5'-ACT GTT CAT CCA GTA CCC GC -3'		59.7	

¹⁾*COI*, cytochrome C oxidase subunit I.

Table 2. Threshold cycle (Ct) values under different annealing temperatures and times

Primer name	Sample	58°C		59°C		60°C	
		15 s	25 s	15 s	25 s	15 s	25 s
<i>Oncorhynchus keta</i>	Negative	- ¹⁾	-	-	-	-	-
	<i>Oncorhynchus keta</i>	19.32	19.04	19.27	19.15	19.78	19.12
	<i>Oncorhynchus mykiss</i>	23.02	23.18	23.07	23.30	23.95	24.11
<i>Oncorhynchus mykiss</i>	Negative	- ²⁾	-	-	-	-	-
	<i>Oncorhynchus keta</i>	19.76	19.85	20.22	20.42	20.70	21.01
	<i>Oncorhynchus mykiss</i>	15.48	15.61	15.43	15.56	15.17	15.35

¹⁾-, Ct value of 24 or more.

²⁾-, Ct value of 20 or more.

study. The T_m value is the temperature at which the DNA double strand is separated into single strands, and the T_m value of the primer represents the optimal PCR reaction temperature in the range of 52-66°C (Buck et al., 1999). The annealing temperature range was set from 59 to $\pm 1^\circ\text{C}$. In the optimal reaction condition setting experiment, DNA extracted from salmon and rainbow trout reference samples was used as template DNA, and sterile distilled water was used as the negative control. As a result of repeating the experiments thrice, all samples except for the negative control were amplified under the conditions set to the temperature of the third step and the time of the second step. Therefore, the condition with the highest discrimination in the threshold cycle (Ct) value of salmon and rainbow trout was selected. The qPCR optimal reaction condition was thus established to perform pre-denaturation at 95°C for 30 sec, followed by 5 minutes at 95°C, annealing at 60°C for 25 s, and extension at 72°C for 5 s.

3.3. Validation of ultra-fast quantitative real-time PCR qualitative testing

Specificity refers to the ability to specifically and accurately measure a substance to be analyzed

(CAC, 2019; EPA, 2016). The Ct value of qPCR for determining salmon was set to 24, which represents a point of distinction from rainbow trout, and was determined to be positive if the Ct value was less than 24 and negative if the Ct value was 24 or higher. The Ct value of the qPCR for determining rainbow trout was set to 20, which is a section representing distinction from salmon, and was determined to be positive if the Ct value was less than 20 and negative if the Ct value was 20 or higher. The salmon primer showed that only the salmon template DNA was positive with a Ct value of less than 24, and the template DNA extracted from five other species, including rainbow trout, was negative with a Ct value of 24 or more (Table 3). The rainbow trout primer was determined to be positive with only rainbow trout template DNA with a Ct value of less than 20, and template DNA extracted from five species other than salmon was determined to be negative with a Ct value of 20 or more (Table 4). In this study, species-specific amplification was confirmed for salmon and rainbow trout primers, and a non-specific reaction did not occur. Therefore, they showed a specificity of 100%.

Sensitivity is the ratio of changes in measured values according to changes in the amount or

Table 3. Threshold cycle (Ct) values of specificity in ultra-fast quantitative real-time PCR for *Oncorhynchus keta*

Primer name	Sample	Ct value				
		Repeat 1	Repeat 2	Repeat 3	Mean	SD ¹⁾
<i>Oncorhynchus keta</i>	Negative	– ²⁾	–	–	–	–
	<i>Oncorhynchus keta</i>	19.11	19.13	19.13	19.12	0.01
	<i>Oncorhynchus mykiss</i>	–	–	–	–	–
	<i>Oncorhynchus nerka</i>	–	–	–	–	–
	<i>Oncorhynchus tshawytscha</i>	–	–	–	–	–
	<i>Oncorhynchus kisutch</i>	–	–	–	–	–
	<i>Salmo salar</i>	–	–	–	–	–
	<i>Oncorhynchus masou</i>	–	–	–	–	–

¹⁾SD, standard deviation.

²⁾–, Ct value of 24 or more.

Table 4. Threshold cycle (Ct) values of specificity in ultra-fast quantitative real-time PCR for *Oncorhynchus mykiss*

Primer name	Sample	Ct value				
		Repeat 1	Repeat 2	Repeat 3	Mean	SD ¹⁾
<i>Oncorhynchus mykiss</i>	Negative	– ²⁾	–	–	–	–
	<i>Oncorhynchus keta</i>	–	–	–	–	–
	<i>Oncorhynchus mykiss</i>	15.25	15.74	15.05	15.35	0.35
	<i>Oncorhynchus nerka</i>	–	–	–	–	–
	<i>Oncorhynchus tshawytscha</i>	–	–	–	–	–
	<i>Oncorhynchus kisutch</i>	–	–	–	–	–
	<i>Salmo salar</i>	–	–	–	–	–
	<i>Oncorhynchus masou</i>	–	–	–	–	–

¹⁾SD, standard deviation.

²⁾–, Ct value of 20 or more.

concentration of the material to be analyzed, and represents the limit of detection (LOD) (CAC, 2019; EPA, 2016). LOD refers to the minimum amount or minimum concentration that can qualitatively detect a substance to be analyzed. The sensitivities of the salmon and rainbow trout primers are shown in Table 5. To determine the sensitivity of salmon and rainbow trout primers, each template DNA was diluted to concentrations of 10, 5, 2.5, 1, and 0.1 ng/μL. Both salmon and rainbow trout primers could be amplified up to 1 ng/μL. These results confirmed that the general rule meets the condition

of setting the template DNA concentration to 100 ng/μL or less (Kim et al., 2014b; Kim et al., 2015c; Park et al., 2013; Song et al., 2008).

3.4. Validation of ultra-fast quantitative real-time PCR quantitative testing

Genomic DNA from salmon and rainbow trout was diluted to 100%, 70%, 50%, 30%, and 10%, and standard curves were generated. Table 6 shows the Ct values for the calibration of salmon and rainbow trout and the mean and standard deviation for the Ct values. The salmon calibrator showed a slope of

Table 5. Threshold cycle (Ct) values of sensitivity in ultra-fast quantitative real-time PCR for *Oncorhynchus keta* and *Oncorhynchus mykiss*

Species	Concentration (ng/ μ L)	Ct value			Mean	SD ¹⁾
		Repeat 1	Repeat 2	Repeat 3		
<i>Oncorhynchus keta</i>	10	19.28	19.16	19.17	19.20	0.05
	5	20.13	20.16	20.27	20.19	0.06
	2.5	21.11	21.17	21.26	21.18	0.06
	1	22.69	23.19	23.29	23.06	0.26
	0.1	24.83	-	-	24.83	-
	Negative	- ²⁾	-	-	-	-
<i>Oncorhynchus mykiss</i>	10	15.67	16.17	16.17	16.00	0.24
	5	16.72	16.20	16.84	16.59	0.28
	2.5	17.58	17.20	16.97	17.25	0.25
	1	19.11	19.13	18.97	19.07	0.07
	0.1	22.15	22.86	22.83	22.61	0.33
	Negative	- ³⁾	-	-	-	-

¹⁾SD, standard deviation.²⁾-, Ct value of 24 or more.³⁾-, Ct value of 20 or more.**Table 6.** Threshold cycle (Ct) values of calibrator in ultra-fast quantitative real-time PCR for *Oncorhynchus keta* and *Oncorhynchus mykiss*

Species	Test level (%)	Ct value			Mean	SD ¹⁾
		Repeat 1	Repeat 2	Repeat 3		
<i>Oncorhynchus keta</i>	100	19.10	19.80	19.34	19.41	0.36
	70	20.16	20.02	20.92	20.37	0.48
	50	21.01	20.97	20.15	20.71	0.49
	30	21.12	21.95	22.02	21.70	0.50
	10	22.81	22.92	23.02	22.92	0.11
<i>Oncorhynchus mykiss</i>	100	15.52	15.74	15.42	15.56	0.16
	70	17.10	16.42	16.28	16.60	0.44
	50	17.11	17.36	17.20	17.22	0.13
	30	18.07	18.26	18.10	18.14	0.10
	10	19.10	19.28	19.01	19.13	0.14

¹⁾SD, standard deviation.

-3.4, an amplification efficiency of approximately 97%, and a linearity of 0.99. The rainbow trout calibrator showed a slope of -3.4, an amplification

efficiency of approximately 95%, and a linearity of 0.99 (Table 7). We take this as evidence that the acceptance criteria for the slope ($-3.6 \leq \text{slope} \leq$

Table 7. Calibration results in ultra-fast quantitative real-time PCR for *Oncorhynchus keta* and *Oncorhynchus mykiss*

Species	Parameter	Repeat 1	Repeat 2	Repeat 3	Mean
<i>Oncorhynchus keta</i>	Slope	-3.4	-3.3	-3.5	-3.4
	PCR efficiency (%)	96.53	93.67	101.10	97.10
	Linearity (R ²)	0.99	0.99	0.99	0.99
<i>Oncorhynchus mykiss</i>	Slope	-3.23	-3.5	-3.5	-3.4
	PCR efficiency (%)	103.96	92.14	91.21	95.77
	Linearity (R ²)	0.99	0.99	0.99	0.99

-3.1), amplification efficiency ($89 \leq \% \leq 110$), and linearity ($R^2 \geq 0.98$) met the values recommended by the ‘Guidelines on Standard Procedures for Preparing Analysis Method Such as Food’, proposed by the Ministry of Food and Drug Safety (MFDS, 2016).

Genomic DNA of salmon and rainbow trout were diluted to 80%, 50%, and 20%, and the quantitative values of unknown samples were calculated. The values for mean, standard deviation, and relative standard deviation for the unknown samples of salmon and rainbow trout are analyzed. In this study, 80% of unknown salmon samples showed 86.79% accuracy and 2% repeatability, 50% of the sample showed 75% accuracy and 8.72% repeatability, and 20% of the sample showed 94.85% accuracy and 2% repeatability. In the case of the unknown rainbow trout samples, 80% showed 86.63% accuracy and 9.32% repeatability, 50% showed 83.86% accuracy and 5.3% repeatability, and 20% showed 109.45% accuracy and 15.04% repeatability. These results confirmed that the acceptance criteria for the accuracy ($75\% \leq \text{accuracy} \leq 125\%$) and repeatability ($RSDr \leq 25\%$) of the ‘Guidelines on Standard Procedures for Preparing Analysis Method Such as Food’ proposed by the Ministry of Food and Drug Safety were met (MFDS, 2016).

The genomic DNA of salmon was diluted to 10%, 9%, and 8%, and the genomic DNA of rainbow trout

was diluted to 10%, 9%, 8%, 7%, and 6%, and then tested to calculate the positive rate of unknown samples. For the unknown salmon samples, it was confirmed that 10% of the samples were amplified 20 times out of 20, and 9% of the samples were amplified 5 times out of 20. For the unknown samples of rainbow trout, it was confirmed that 10%, 9%, and 8% of samples were amplified 20 times out of 20, and 7% of samples were amplified 9 times out of 20. The LOQ of salmon was found to be 1 ng/ μ L and the LOQ of rainbow trout was 0.8 ng/ μ L. The acceptance standard for LOQ ($95\% \leq \text{detection}$) of the ‘Guidelines on Standard Procedures for Preparing Analysis Method Such as Food’ proposed by the Ministry of Food and Drug Safety (MFDS, 2016) was also confirmed.

3.5. Monitoring

Using the salmon and rainbow trout qPCR determination method designed in this study, 14 salmon and rainbow trout in circulation were purchased, and the status of forgery was confirmed. As a result of this study, eight salmon and six rainbow trout cases were determined, confirming that the raw material name and the experimental results were 100% identical, and there were no cases of salmon and rainbow trout forgery (Table 8). Our findings confirm that the qPCR determination method is suitable for the identification of salmon

Table 8. Confirming results of monitoring samples by using the ultra-fast quantitative real-time PCR developed in this study

No.	Species		Raw materials	Result
	<i>Oncorhynchus keta</i>	<i>Oncorhynchus mykiss</i>		
M1	+	-	<i>Oncorhynchus keta</i>	<i>Oncorhynchus keta</i>
M2	+	-	<i>Oncorhynchus keta</i>	<i>Oncorhynchus keta</i>
M3	+	-	<i>Oncorhynchus keta</i>	<i>Oncorhynchus keta</i>
M4	+	-	<i>Oncorhynchus keta</i>	<i>Oncorhynchus keta</i>
M5	+	-	<i>Oncorhynchus keta</i>	<i>Oncorhynchus keta</i>
M6	+	-	<i>Oncorhynchus keta</i>	<i>Oncorhynchus keta</i>
M7	+	-	<i>Oncorhynchus keta</i>	<i>Oncorhynchus keta</i>
M8	+	-	<i>Oncorhynchus keta</i>	<i>Oncorhynchus keta</i>
M9	-	+	<i>Oncorhynchus mykiss</i>	<i>Oncorhynchus mykiss</i>
M10	-	+	<i>Oncorhynchus mykiss</i>	<i>Oncorhynchus mykiss</i>
M11	-	+	<i>Oncorhynchus mykiss</i>	<i>Oncorhynchus mykiss</i>
M12	-	+	<i>Oncorhynchus mykiss</i>	<i>Oncorhynchus mykiss</i>
M13	-	+	<i>Oncorhynchus mykiss</i>	<i>Oncorhynchus mykiss</i>
M14	-	+	<i>Oncorhynchus mykiss</i>	<i>Oncorhynchus mykiss</i>

and rainbow trout in circulation on the market.

4. Conclusions

The species-specific primers for *O. keta* and *O. mykiss* were selectively amplified by *O. keta* and *O. mykiss* DNA. The sensitivity of primers was 1 ng/ μ L. The validation test showed that the results met the 'Guidelines on Standard Procedures for Preparing Analysis Method Such as Food'. The qPCR method developed and validated in this study for identifying *O. keta* and *O. mykiss* has advantages such as speed and field applicability. Therefore, this assay is expected to help control forgery and alteration of raw materials in the seafood industry.

Acknowledgements

This research was supported by a grant (17162 MFDS 064) from Ministry of Food and Drug Safety in 2021.

Conflict of interests

The authors declare no potential conflicts of interest.

Author contributions

Conceptualization: Yang JY, Kim JB. Formal analysis: Park MJ, Lee HC. Methodology: Park MJ, Lee HC. Writing - original draft: Park MJ, Yang JY, Kim JB. Writing - review & editing: Park MJ, Yang JY, Kim JB.

Ethics approval

This article does not require IRB/IACUC approval because there are no human and animal participants.

ORCID

Min-Ji Park (First author)

<https://orcid.org/0000-0002-5107-7345>

Han-Cheol Lee

<https://orcid.org/0000-0002-1772-3964>

Ji-Young Yang

<https://orcid.org/0000-0002-4598-6542>

Jung-Beom Kim (Corresponding author)
<https://orcid.org/0000-0002-0290-2687>

References

- Axayacatl RO, Juan PCG. Molecular identification of dolphinfish species (genus *Coryphaena*) using multiplex haplotype-specific PCR of mitochondrial DNA. *Ichthyol Res*, 55, 389-393 (2008)
- Buck GA, Fox JW, Gunthorpe M, Hager KM, Naeve CW, Adams PS, Rush J. Design strategies and performance of custom DNA sequencing primers. *BioTechniques*, 27, 528-536 (1999)
- Cha JW, Yoon IS, Park SY, Kang SI, Lee JS, Heu MS, Kim JS. Taste, flavor and nutritional characteristics of fish cake made from salmon *Oncorhynchus keta* frame muscle. *Korean J Fish Aquat Sci*, 53, 281-289 (2020)
- Chun HS, Ha SD, Lee HJ, Cho SY, Kim GH, Kang KW. Current status and countermeasure for food fraud (economically motivated adulteration of food) in domestic and foreign countries. *Food Sci Ind*, 47, 3-12 (2014)
- Chung IY, Seo YB, Yang JY, Kim GD. Development and validation of real-time PCR to determine *Branchiostegus japonicus* and *B. albus* species based on mitochondrial DNA. *J Life Sci*, 27, 1331-1339 (2017)
- Codex Alimentarius Commission (CAC). Guidelines on Performance Criteria and Validation of Methods for Detection Identification and Quantification of Specific DNA Sequences and Specific Proteins in Foods. Rome, Italy, p 1-22 (2019)
- Esposti MD, Vries SD, Crimi M, Ghelli A, Patarnello T, Meyer A. Mitochondrial cytochrome b: Evolution and structure of the protein. *Biochim Biophys Acta*, 1143, 243-271 (1993)
- Hebert PDN, Cywinska A, Ball SL, Dewaard JR. Biological identifications through DNA barcodes. *Proc Biol Sci*, 270, 313-321 (2003)
- Heu MS, Choi BD, Kang SI, Kim YJ, Kim JS. Comparison on the food quality characteristics of muscles from salmonids according to species, imported country, and separated part. *Korean J Fish Aquat Sci*, 48, 16-25 (2015)
- Heu MS, Kim HJ, Yoon MS, Park DY, Park KH, Kim JS. Food component characterization of muscle from salmon frame. *J Korean Soc Food Sci Nutr*, 37, 1452-1456 (2008)
- Hold GL, Russell VJ, Pryde SE, Rehbein H, Quinteiro J, Vidal R, Rey-Mendez M, Sotelo CG, Perez-Martin RI, Santos AT, Rosa C. Development of a DNA-based method aimed at identifying the fish species present in food products. *J Agric Food Chem*, 49, 1175-1179 (2001)
- Horowitz JM. 10 Foods that pack a wallop. *Time*, 159, 76-81 (2002)
- Hwang SY, Lee YH, Kim CG, Kim GE, Jeong JW, Yoon HG, Seo HS, Jeong DG, Lee CH. Identifying method of chum salmon species or its originated stocks polynucleotide probe DNA chip and kit for identifying the same. Available from: <http://kpat.kipris.or.kr/kpat/biblioa.do?method=biblioFrame>. Accessed Dec. 10, 2021.
- Im SU, Ahn SH, Park DO, Choi YH, Song KB. Gene expression profile analysis of xylitol-sensitive and xylitol-resistant *Streptococcus mutans* in 0.5% glucosecontaining TYE media using DNA chip. *J Korean Acad Oral Health*, 36, 251-265 (2012)
- Jun KS, Kim KB, Lee MS. Quality characteristics of *Oncorhynchus masou* stock with different manufacturing methods. *Culin Sci Hosp Res*, 26, 187-199 (2020)
- Kang JH, Yu KH, Kim SK, Park JY, Kim BS, An CM. Species identification and genetic structure of *Octopus minor* from Korea and China on the basis of partial sequences of mitochondrial cytochrome oxidase I. *Korean J Malacol*, 26, 285-290 (2010a)
- Kang KT, Heu MS, Kim JS. Preparation and characteristics of fish-frame-added snacks. *J Kor Fish Soc*, 39, 261-268 (2006)

- Kang SI, Kim KH, Lee JK, Kim YJ, Park SJ, Kim MW, Choi BD, Kim DS, Kim JS. Comparison of the food quality of freshwater rainbow trout *Oncorhynchus mykiss* cultured in different regions. *KFAS*, 47, 103-113 (2014)
- Kang SK, Yang H, Lee CS, Choi SH. Stomach contents of chum salmon (*Oncorhynchus keta*) fingerlings in namdae stream. *J Korean Soc Oceanogr*, 12, 86-93 (2007)
- Kang SY, Kwon ES, Moon EJ, Cho EM, Seo MS, Kim YJ, Jee SH. Usefulness of biochemical analysis for human skeletal remains assigned to the Joseon dynasty in oknam-ri site in Seocheon. *Korea J Conserv Sci*, 26, 95-107 (2010b)
- Kim BT. An analysis of the impact of FTA tariff elimination on the export price of Norwegian fresh and chilled salmon to Korea. *J Fish Bus Adm*, 49, 37-48 (2018)
- Kim EH, Lee DW, Han SH, Lim YK. Development of ultra-rapid multiplex real-time PCR for the detection of genes from avian influenza virus subtype H5N1. *Korean J Vet Res*, 47, 399-407 (2007)
- Kim EM, Kang HS, Kang JH, Kim DG, An CM, Lee HW, Park JY. Genetic relationships of sandfish (*Arctoscopus japonicas*) from five different areas of Korea and Japan based on mitochondrial DNA and microsatellite analyses. *J Life Sci*, 25, 1204-1213 (2015a)
- Kim H, Seo YB, Choi SS, Kim JH, Shin JY, Yang JY, Kim GD. Development and validation of multiplex polymerase chain reaction to determine squid species based on 16s rRNA gene. *J Food Hyg Saf*, 30, 43-50(2015b)
- Kim KH, Kim YS, Kim MR, Lee HY, Jung YK, Lee JH, Chang HS, Park YC, Kim SY, Choi JD, Jang YM. Development of species-specific primer to determine the authenticity of vegetable raw materials in food. *Food Eng Prog*, 18, 419-426 (2014a)
- Kim KH, Kim YS, Kim MR, Lee HY, Lee KH, Kim JH, Seong RS, Kang TS, Lee JH, Jang YM. Development of primer sets for the detection of *Polygonum multiflorum*, *Cynanchum wilfordii* and *C. auriculatum*. *J Food Hyg Saf*, 30, 289-294 (2015c)
- Kim KH, Lee HY, Kim YS, Kim MR, Jung YK, Lee JH, Chang HY, Park YC, KIM SY, Choi JD, Jang YM. Development of species-specific PCR to determine the animal raw material. *J Food Hyg Saf*, 29, 347-355 (2014b)
- Kim KW, Lee HK, Cho HB. Development of human papillomavirus DNA array by using lateral flow membrane assay. *Korean J Microbiol*, 44, 346-351 (2008)
- Kim NYS, Yang JY, Kim JB. Development and validation of a PCR method to discriminate between *Branchiostegus japonicus* and *Branchiostegus albus*. *J Food Sci Technol*, 51, 295-299 (2019a)
- Kim SH, Baek IK, Kim NY, Song SA, Kim S, Jeong J, Shin JH. Development and evaluation of multiplex PCR for the detection of carbapenemase-producing enterobacteriaceae. *Ann Clin Microbiol*, 22, 9-13 (2019b)
- Kim WS, Jang MS, Kim JO, Jeon YH, Oh MJ. Effect of fish pathogenic viruses on mariculture of rainbow trout (*Oncorhynchus mykiss*). *Korean J Ichthyol*, 27, 183-188 (2015d)
- Kim YS, Park HJ, Lee DH, Kim HK. Development of multiplex polymerase chain reaction assay for identification of *Angelica species*. *Korean J Medicinal Crop Sci*, 26, 26-31 (2018)
- Ko JM, Oh SW, Hong JH. Sensory drivers of sliced raw fish in Korea: Case study on flounder (*Paralichthys olivaceus*) and rockfish (*Sebastes schlegeli*). *Korean Soc Food Sci Nutr*, 45, 1192-1201 (2016)
- 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*, 34, 417-428 (2011)
- Lee DG, Bae BS, Lee JH. Development of the smart aquaculture technology and industrialization plan. *J Korean Soc Fish Mar Sci Edu*, 33,

- 412-420 (2021)
- Lee DW, Kim EH, Yoo MS, Han SH, Yoon BS. Ultra-rapid real-time PCR for the detection of human immunodeficiency virus (HIV). *Korean J Microbiol*, 43, 91-99 (2007)
- Lee HY, Kim SY, Woo KM. Development of labchip based realtime PCR for rapid human DNA quantification. *J Sci Crim Investig*, 10, 311-317 (2016)
- Lee SW, Lee SJ, Han EH, Sin EC, Cho KM, Kim YH. Current status on the development of molecular markers for differentiation of the origin of *Angelica* spp. *J Plant Biotechnol*, 44, 12-18 (2017)
- Lee SY, Lim G, Choi JG. DNA chip: Current status and applications. *Prospectives of Industrial Chemistry*, 3, 18-23 (2000)
- Ministry of Food and Drug Safety (MFDS). Guidelines on Standard Procedures for Preparing Analysis Method Such as Food. Cheongju, Korea, p 1-22 (2016)
- Ministry of Oceans and Fisheries (MOF). Salmon Consumption Statistics. Available from: <https://www.mofgokr/iframe/article/viewdo?articleKey=13842&boardKey=10¤tPageNo=1>. Accessed Nov. 7, 2020.
- Noh ES, Lee MN, Kim EM, Park JY, Noh JK, An CM, Kang JH. Development of a multiplex PCR assay for rapid identification of *Larimichthys polyactis*, *L. crocea*, *Atrobucca nibe*, and *Pseudotolithus elongates*. *J Life Sci*, 27, 746-753 (2017)
- Paek JY, Park KI. An economic analysis of rainbow trout (*Oncorhynchus mykiss*) aquaculture farms. *J Korean Soc Fish Mar Sci Edu*, 28, 1280-1289 (2016)
- Park KI, Baek JY, Park MJ. An analysis on perception of trouts for trout consumption. *J Korean Soc Fish Mar Sci Edu*, 30, 47-55 (2017)
- Park YC, Ahn CY, Jin SO, Lim JY, Kim KH, Lee JH, Cho TY, Lee HW, Park KS, Yoon HS. Identification of raw materials in processed meat products by PCR using species-specific primer. *J Food Hyg Saf*, 27, 68-73 (2012a)
- Park YC, Jung YH, Kim MR, Shin JH, Kim KH, Lee JH, Cho TY, Lee HJ, Lee SJ, Han SB. Development of detection method for *Nippon spinosus*, *Epinephelus bruneus*, and *Epinephelus septemfasciatus* using 16S rRNA gene. *Korean J Food Sci Technol*, 45, 1-7 (2013)
- Park YC, Lim JY, Park YE, Lim JD, Hwang CR, Kim KH, Lee JH, Cho TY, Lee HJ, Lee SJ, Han SB. Identification of faulty red pepper powder containing seasoned red-pepper sauce. *J Food Hyg Saf*, 27, 182-187 (2012b)
- Seo MW, Song JY, Kim HG. Multi-locus phylogeny analysis of Korean isolates of phytophthora species based on sequence of ribosomal and mitochondrial DNA. *Korean J Mycol*, 38, 40-47 (2010)
- Song JY, Lim JH, Nam MH, Kim HG, Kim BS. Development of PCR primers for specific identification and detection of *Botrytis cinerea* on tomato. *Korean J Mycol*, 36, 138-143 (2008)
- US Environmental Protection (EPA). Guidelines on Standard Procedures for Preparing Analysis Methods. Washington DC, USA, p 1-10 (2016)
- Wang JH, Min SH, Lim SJ, Yoon BS. The development of rapid detection method against *Ascosphaera apis* and *Aspergillus flavus* for on-site diagnosis of chalkbrood and stonebrood in honey bee. *J Apic*, 31, 31-39 (2016)
- Warner K, Roberts W, Mustain P, Lowell B, Swain M. Casting a wider net: More action needed to stop seafood fraud in the United States. Available from: <https://osf.io/preprints/marxiv/sbm8h/>. Accessed Mar. 7, 2020.