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Research Article

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

Min-Ji Park[1], Han-Cheol Lee[1], Ji-Young Yang[2], Jung-Beom Kim[1]*

[1]*Department of Food Science and Technology, Suncheon National University, Suncheon 57922, Korea*
[2]*Department 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/μL. 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.

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

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***Corresponding author**
Jung-Beom Kim
Tel: +82-61-750-3259
E-mail: okjbkim@scnu.ac.kr

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.,

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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℃, 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 μL) was prepared by mixing 5 μL Rapi:Detect^{TM}Master Mix (Genesystem, Korea), 1 μL (10 ng/μL) genomic DNA, 1 μL (10 pmol/μL) primer, and 2 μL 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/μL 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/μL 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 (Tm) value of 52-66℃ (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℃, and the primer binding time was divided into 15 and 25 s (Table 2). The bonding temperature was set on the basis of the Tm values of the primers developed in this

Table 1. Primer sequence designed in this study

Species		Sequences	Product size (bp)	Tm (℃)	Target gene
Oncorhynchus keta	Forward	5'-TCT AGG GGA TGA CCA GAT CTA CA -3'	184	59.3	*CO I*[1)]
	Reverse	5'-GGA CGG AGG TAG GAG TCA GA -3'		59.7	
Oncorhynchus mykiss	Forward	5'-CCC TAA TAA TCG GAG CCC CT -3'	133	58.3	*CO I*
	Reverse	5'-ACT GTT CAT CCA GTA CCC GC -3'		59.7	

[1)] *CO I*, cytochrome C oxidase subunit I.

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

Primer name	Sample	58℃		59℃		60℃	
		15 s	25 s	15 s	25 s	15 s	25 s
Oncorhynchus keta	Negative	–[1)]	–	–	–	–	–
	Oncorhynchus keta	19.32	19.04	19.27	19.15	19.78	19.12
	Oncorhynchus mykiss	23.02	23.18	23.07	23.30	23.95	24.11
Oncorhynchus mykiss	Negative	–[2)]	–	–	–	–	–
	Oncorhynchus keta	19.76	19.85	20.22	20.42	20.70	21.01
	Oncorhynchus mykiss	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 Tm value is the temperature at which the DNA double strand is separated into single strands, and the Tm value of the primer represents the optimal PCR reaction temperature in the range of 52-66℃ (Buck et al., 1999). The annealing temperature range was set from 59 to ±1℃. 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℃ for 30 sec, followed by 5 minutes at 95℃, annealing at 60℃ for 25 s, and extension at 72℃ 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)]
Oncorhynchus keta	Negative	–[2)]	–	–	–	–
	Oncorhynchus keta	19.11	19.13	19.13	19.12	0.01
	Oncorhynchus mykiss	–	–	–	–	–
	Oncorhynchus nerka	–	–	–	–	–
	Oncorhynchus tshawytscha	–	–	–	–	–
	Oncorhynchus kisutch	–	–	–	–	–
	Salmo salar	–	–	–	–	–
	Oncorhynchus masou	–	–	–	–	–

[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)]
Oncorhynchus mykiss	Negative	–[2)]	–	–	–	–
	Oncorhynchus keta	–	–	–	–	
	Oncorhynchus mykiss	15.25	15.74	15.05	15.35	0.35
	Oncorhynchus nerka	–	–	–	–	–
	Oncorhynchus tshawytscha	–	–	–	–	–
	Oncorhynchus kisutch	–	–	–	–	–
	Salmo salar	–	–	–	–	–
	Oncorhynchus masou	–	–	–	–	–

[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

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

Species	Concentration (ng/μL)	Ct value			Mean	SD[1)]
		Repeat 1	Repeat 2	Repeat 3		
Oncorhynchus keta	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)]	-	-	-	-
Oncorhynchus mykiss	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		
Oncorhynchus keta	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
Oncorhynchus mykiss	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 ≤ slope ≤

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
Oncorhynchus keta	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
Oncorhynchus mykiss	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 ≤ % ≤ 110), and linearity (R^2 ≥ 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% ≤ accuracy ≤ 125%) and repeatability (RSDr ≤ 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/μL and the LOQ of rainbow trout was 0.8 ng/μL. The acceptance standard for LOQ (95% ≤ 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

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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
	Oncorhynchus keta	*Oncorhynchus mykiss*		
M1	+	-	*Oncorhynchus keta*	*Oncorhynchus keta*
M2	+	-	*Oncorhynchus keta*	*Oncorhynchus keta*
M3	+	-	*Oncorhynchus keta*	*Oncorhynchus keta*
M4	+	-	*Oncorhynchus keta*	*Oncorhynchus keta*
M5	+	-	*Oncorhynchus keta*	*Oncorhynchus keta*
M6	+	-	*Oncorhynchus keta*	*Oncorhynchus keta*
M7	+	-	*Oncorhynchus keta*	*Oncorhynchus keta*
M8	+	-	*Oncorhynchus keta*	*Oncorhynchus keta*
M9	-	+	*Oncorhynchus mykiss*	*Oncorhynchus mykiss*
M10	-	+	*Oncorhynchus mykiss*	*Oncorhynchus mykiss*
M11	-	+	*Oncorhynchus mykiss*	*Oncorhynchus mykiss*
M12	-	+	*Oncorhynchus mykiss*	*Oncorhynchus mykiss*
M13	-	+	*Oncorhynchus mykiss*	*Oncorhynchus mykiss*
M14	-	+	*Oncorhynchus mykiss*	*Oncorhynchus mykiss*

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/μL. 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.

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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

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