JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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# Novel Real-Time PCR Method Based on Growth Hormone Gene for Identification of *Salmonidae* Ingredient in Food

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**ABSTRACT:** To avoid fraudulent substitutions in fish markets, the proper methods are needed to test the authenticity of the ingredients. As a preferable methodology, a quantitative real-time polymerase chain reaction (qPCR) method was used in this study to identify species from the *Salmonidae* family based on the salmon growth hormone gene. Fish samples of six genera from the *Salmonidae* family were tested to identify the specificity, sensitivity, and applicability of the established method. Results showed that the method was highly specific for salmonid detection. Ct values were obtained only from 31 *Salmonidae* fish species samples. The relative and absolute limits of detection were 0.01% and 25 pg of genomic DNA, respectively, which could meet with the requirements of routine detections. To test the applicability of the method, the content of salmonid ingredients in 16 commercial food products was quantified from standard curves constructed from DNA of two *Salmonidae* species. The results revealed that the salmonid ingredient was detected in 12 samples, indicating that 25% of the labels are inauthentic. These results demonstrate that the developed qPCR method is suitable for identification of *Salmonidae* ingredients.

**KEYWORDS:** *Salmonidae, real-time PCR, growth hormone gene, species identification, food*

## INTRODUCTION

Salmonids (*Salmonidae*), the third largest cultured fish in the world, [1] have substantial commercial value and health benefits. Several *Salmonidae* species, commonly referred to as "salmon", are very popular in aquaculture markets and restaurants (e.g., Atlantic salmon, Chinook salmon, etc.). Because salmon is rich in omega-3 fatty acids, proteins, and vitamins, consumption of this particular fish has increased on a global basis over the years. In 2009, the global salmon and trout production reached 3.664 million tons in Europe, Australia, Asia, South America, and North America; 0.22 million tons were imported into our country.[2,3]

According to their taxonomy, *Salmonidae* belongs to the order *Salmoniformes* of the class *Osteichthyes*. *Salmonidae* is divided into three subfamilies: *Salmoninae*, *Coregoninae*, and *Thymallinae*.[1] The subfamily *Salmoninae* contains five genera (*Oncorhynchus*, *Salvelinus*, *Hucho*, *Brachymystax*, and *Salmo*), which includes the most familiar salmonid species: *S. salar* (Atlantic salmon), *O. tshawytscha* (Chinook salmon), *O. masou* (Masu salmon), *O. keta* (Chum salmon), *O. kisutch* (Coho salmon), *O. gorbuscha* (Pink salmon), *O. nerka* (Sockeye salmon), and *H. Taimen* (Danube salmon). The subfamilies *Coregoninae* and *Thymallinae* only have one genus each.[1,4]

Salmonids, which are delicious, nutritious, and contaminant free, have been processed by various methods (i.e., freshly sliced on ice, smoked, sauced, filleted, pickled, fried, frozen, and canned). Salmonid fish has been added to baby foods and health foods, resulting in an increase in the price of the final products. Compared to the whole fish, which can be identified by morphological traits, fish-derived products are difficult to identify in terms of genus and species. Therefore, fraudulent substitutions of expensive fish species for cheaper ones are common in fish markets around the world.[5,6] Because the ingredients in a processed food are difficult to discern and considering the cost of the salmonid fish, it is necessary to establish a method that allows correct identification of *Salmonidae* fish ingredients in processed foods.

In the past 20 years, several techniques based on proteins and nucleic acids were developed to identify fish species. Using a protein-based technique, Sharyn et al. (1992) identified several fish species by HPLC.[7] Moreover, SDS-PAGE and 2-D electrophoresis methods have also been used during fish authentication.[8,9] However, proteins are easily denatured during heat treatments and food pressurization processes. Moreover, closely related species have been difficult to identify using only protein-based techniques.[10] In the past years, polymerase chain reaction (PCR) methods, which are nucleic acid based and possess a much better specificity, reliability, and sensitivity than protein-based techniques, were developed and used for identification and authentication of plant, animal, and microorganism species.[11−18] For fish species identification, techniques based on RLFP, RAPD, SSCP, AFLP, sequencing, and microarrays have been established.[19] Compared to these methods, quantitative real-time PCR (qPCR) is fast, economic, easy to use, specific, reproducible, and sensitive.[20,21] In addition, the whole amplification process can be tracked. Thus, qPCR has been widely used in many fields including gene expression analysis and diagnostic microbiology and for fish species identification (e.g., Atlantic bluefin tuna, Atlantic cod, Atlantic salmon, European hake).[15−18]

Received: February 19, 2013
Revised: April 20, 2013
Accepted: April 21, 2013
Published: April 22, 2013

  

In the present study, a qPCR method specific for salmonid growth hormone gene was developed to identify fish of *Salmonidae* family and its derived products. The method was also verified in routine analysis of *Salmonidae* ingredient in commercial foods.

## MATERIALS AND METHODS

**Sample Collection and Preparation.** Salmonid fresh samples were obtained from the main cultured and captured area around the world. *Salmonindae* samples included *Oncorhynchus mykiss* from the United States, Australia, Norway, Chile, and China, *O. keta* from Russia, Japan, and China, *O. masou* from Japan, *Salvelinus alpinus* and *S. leucomaenis* from Japan, *Hucho taimen* from China, *Brachymystax lenok* from China, and *Salmo salar* from Norway, Canada, Australia, United Kingdom, Faroe Islands, and China. The *Coregoninae* samples were *Coregonus ussuriensis Berg*, *C. Peled*, *C. nasus*, *C. tugun*, and *C. muksun* from China. The *Thymallidae* samples consisted of *Thymallus thymallus*, *T. arcticus yaluensis*, *T. arcticus grubei*, *T. arcticus arcticus*, and *T. brevirostris* from China. For each species at least five specimens were collected. Samples were identified based on morphological traits. The scientific name of fish species and geographic location of collection are provided in Table 1.

**Table 1. *Salmonidae* Species Collected**

| subfamily | genus | species | location |
|---|---|---|---|
| *Salmonindae* | *Oncorhynchus* | *O. mykiss* | United States; Norway; Australia; Chile; Qinghai and Beijing (China) |
| | | *O. masou* | Japan |
| | | *O. keta* | Japan; Russia; Heilongjiang (China) |
| | *Salvelinus* | *S. alpinus* | Japan |
| | | *S. leucomaenis* | Japan |
| | *Hucho* | *H. taimen* | Heilongjiang |
| | *Brachymystax* | *B. lenok* | Heilongjiang |
| | *Salmo* | *S. salar* | Norway; Canada; Australia; United Kingdom; Faroe Islands; Qinghai |
| *Coregoninae* | *Coregonus* | *C. ussuriensis Berg* | Heilongjiang |
| | | *C. Peled* | Xinjiang and Qinghai (China) |
| | | *C. nasus* | Qinghai |
| | | *C. tugun* | Qinghai |
| | | *C. muksun* | Qinghai |
| *Thymallidae* | *Thymallus* | *T. thymallus* | Heilongjiang |
| | | *T. arcticus yaluensis* | Heilongjiang |
| | | *T. arcticus grubei* | Heilongjiang |
| | | *T. arcticus arcticus* | Heilongjiang |
| | | *T. brevirostris* | Heilongjiang |

To evaluate the specificity of the developed qPCR method, samples of several fish species from other related families and order were also prepared; these samples include *Esox reicherti*, *Esox lucius*, *Hemisalanx prognathus Regan*, *Nibea albiflora*, *Argyrosomus argentatus*, *Atrobucca alcocki*, *Pleuronectes platessa*, *Hippoglossus hippoglossus*, *Pangasius bocourti*, *Nemipterus virgatus*, *Thunnus Maccoyii*, *Thunnus albacares*, *Sebastiscus marmoratus*, *Pneumatophorus japonicus*, *Histiophorus orientalis*, *Tenualosa reevesii*, *Hypophthalmichthys molitrix*, *Carassius auratus*, *Helostoma temminckii*, *Gadus morhua*, *Oreochromis aureus*, and *Scomberomorus niphonius*. All samples were stored at −80 °C.

Sixteen commercial products, which according to the label contained salmon ingredients, were purchased from supermarkets and retail stores in Shanghai, China (Table 2). Products were ice fresh, smoked, or salt-treated and used in salads, cheese or noodle mixtures, or with vegetables and sausage.

**Table 2. Commercial Products Tested Using the qPCR Methodology Based on GH Gene**

| code | name | treatment |
|---|---|---|
| 1 | salmon sausage 1 | frozen |
| 2 | lactoferrin nutritional cereal | rice noodles |
| 3 | vegetable fish | mashed |
| 4 | salmon nutritional noodle | noodles |
| 5 | salmon and fibrous vegetables cereal | rice noodles |
| 6 | salmon nutritional noodle | noodles |
| 7 | lecithin-enriched noodle | noodles |
| 8 | red salmon salad | salad |
| 9 | salmon crispy meat | cooked food |
| 10 | salmon with lentinus noodles | noodle |
| 11 | honeydew salmon | cooked food |
| 12 | salmon and tomato paste | mashed |
| 13 | salmon caviar | frozen |
| 14 | salmon cheese | piece |
| 15 | salmon sausage 2 | frozen |
| 16 | smoked salmon | slice |

To evaluate the sensitivity of the developed qPCR method, nine groups of food matrix-salmonid samples (at six different percentages) were prepared. Three food matrices (maize, crucian, and chicken) were mixed with the flesh of *S. salar*, *S. alpinus*, and *T. thymallus*. Before mixing, the matrix and salmon flesh were thoroughly frozen dried and powdered by a Freezer Mill (Spex SamplePrep, Metuchen, NJ, USA). Dry maize (*Zea mays L.*) powder (13.50 g) was mixed with *S. salar* powder (1.50 g) using the Freezer Mill, resulting in a 10% (w/w) sample (labeled Aa1). Then 13.50 g of maize powder was mixed with 1.50 g of Aa1, resulting in a 1.0% sample (Aa2). The 0.1% (Aa3), 0.01% (Aa4), 0.001% (Aa5), and 0.0001% (Aa6) (w/w) samples were prepared in the same manner. The maize powder with *S. alpinus* (Ba1−6) and *T. thymallus* (Ca1−6) were similarly prepared. The other food matrices consisting of crucian (*Carassius niphonius*) and chicken (*Gallus gallus domesticus*) mixture powders with three salmonid species (Ab1−6, Bb1−6, Cb1−6, Ac1−6, Bc1−6, Cc1−6) were also prepared in the same way. All mixtures were kept at −20 °C.

**DNA Extraction.** DNA was extracted and purified from approximately 100 mg of either fresh salmon or processed samples using a DNeasy Blood & Tissue Kit (QIAGEN, Düsseldorf, Germany). The quantity and quality of the extracted DNA were assessed by calculating the absorbance ratio at $A_{260}$ and $A_{280}$ obtained from a UV spectrophotometer (Eppendorf Co., Germany) and by running a 0.8% agarose gel electrophoresis. DNA samples were stored at −20 °C.

**Primer and Probe Design.** Growth hormone (GH) gene from salmonid fish species was selected for setup using the qPCR method. Complete and partial sequences from different genera and species of the *Salmonidae* family were retrieved from the GenBank database with the following accession numbers: EU090916, X17594, X59762, U28360, U28359, NM001124690, U28156, U28157, U14551, U28362, AY614010, JN241634, J03797, AY219408, U29954, and AY498872. To obtain a highly conserved region, sequences were aligned using the Clustal X 2.1 software (the Conway Institute UCD, Dublin, Ireland).[22] The primer and probe design was performed using the Primer Express 3.0 software (Applied Biosystems, Foster City, CA, USA). A set of two forward primers GH-FP-1, 5′CAC AGG GGA GCC AGG ATG 3′, and GH-FP-2, 5′ CAC AGG GGA GCC AGG AMG 3′, and the reverse primer GH-RP, 5′ CAG GTT CTG GTA GTA GTT CC CGT AG 3′, were used. The TaqMan probe was labeled on the 5′ end with a fluorescent reporter dye (6-carboxyfluorescein; FAM) and on the 3′ end with a black hole quencher-1 (BHQ1): 5′ FAM-TGA GCC TGG ATG ACA ATG ACT CTC AG-BHQ1 3′. The specificity of the primers and probe was

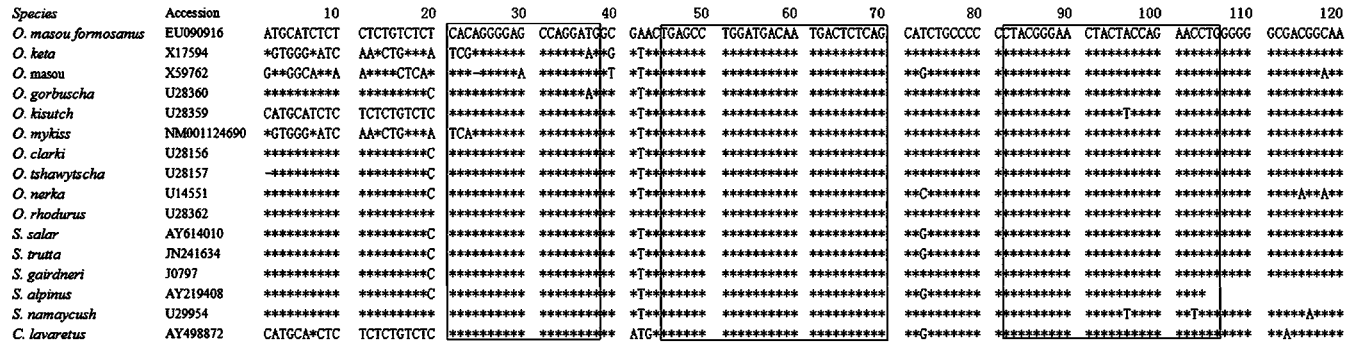


**Figure 1.** DNA fragment sequences of 14 *Salmonidae* species showing the locations of the designed primers and probe.

evaluated by BLASTN from NCBI. To evaluate the efficacy of DNA extraction, two housekeeping genes, specific for *18S rRNA* and *12S rRNA*, respectively, were used with the following primers sequence: *18S* rRNA1 (5′-TCT GCC CTA TCA ACT TTC GAT GGT A-3′) and *18S* rRNA2 (5′-AAT TTG CGC GCC TGC TGC CTT CCT T-3′)[23] and *12S*-Fish-1F (5′-TAA GAG GGC CGG TAA AAC TC-3′) and *12S*-Fish-2R (5′-GTG GGG TAT CTA ATC CCA G-3′).[11] The primers and fluorescent probe were synthesized and purified by Takara Biotechnology Co., Ltd. (Dalian, China) and Shanghai Huirui Biotechnology Co., Ltd. (Shanghai, China).

**Real-Time PCR Conditions.** Real-time PCR was performed in an ABI Prism 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using 25 μL of mixture volume per well. Each reaction consisted of 5 μL of DNA template, 12.5 μL of 2× Hot Start qPCR Master Mix I (Shanghai Ruicheng Biotechnology Co., Ltd., China), 500 nM final primer concentration (GH-FP and GH-RP), and 250 nM final probe concentration. PCR reactions were carried out using the following thermal cycling protocol: 95 °C for 10 min, then 45 cycles at 95 °C for 15 s, and 60 °C for 1 min. Fluorescence was monitored during the annealing and extension step (60 °C) of the PCR cycle.

**Specificity and Sensitivity of Real-Time PCR.** The primer and probe specificity was tested by amplifying the genomic DNA of *Salmoninae*, *Coregoninae*, *Thymallidae*, and other fish families and orders. Genomic DNA from fish samples ($n$ = 53) was used: salmonid fish ($n$ = 31) and nonsalmonid fish ($n$ = 22). Relative and absolute sensitivities were determined by the sample percentages and genomic DNA dilutions, respectively. Sensitivity was measured by the limit of detection (LOD) value, which is the lowest treatment concentration with a fluorescent signal that is significantly different from the negative control. To evaluate the absolute sensitivity, DNA samples from seven representative salmonid species (*O. mykiss, H. taimen, S. alpinus, B. lenok, S. salar, C. ussuriensis Berg,* and *T. thymallus*) were prepared; a 10-fold serial dilution (from 100 to 0.001 ng/μL) and a dilution of 0.005 ng/μL were performed with the DNA samples. Five PCR reactions were performed in parallel and repeated four times. Positive amplification times were calculated. To determine the relative LOD of the real-time PCR method, three salmonid species (*S. salar*, *S. alpinus*, and *T. thymallus*) in frozen-dry powder form were thoroughly mixed with maize, crucian, or chicken dry powders, resulting in 1.0%, 0.1%, 0.01%, 0.001%, and 0.0001% final salmonid content (w/w). The three food matrices simulated food matrices in the market. Nine groups of the different percentage mixtures were amplified 20 times per sample, and positive amplification times were calculated.

**Standard Curves.** To evaluate the applicability of the qPCR method, standard curves were constructed using seven concentrations of pure salmonid genomic DNA: 100, 20, 10, 2, 1, 0.2, and 0.1 ng/μL diluted in 0.1× TE buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). DNA dilutions of six representative species (*O. mykiss, S. alpinus, B. lenok, S. salar, C. ussuriensis Berg,* and *T. thymallus*) were used. Dilutions were performed on ice to protect the DNA. Five PCR reactions were performed in parallel, and the whole experiment was repeated three times. The standard curve was constructed by plotting the Ct values (from the seven DNA concentrations) against the logarithm of the genomic DNA concentration. The PCR efficiency ($E = [10^{(-1/\text{slope})} - 1] \times 100\%$) and the square regression coefficient ($R^2$) of the curves were calculated.

**Analyses and Quantification of the Salmonidae Ingredient in Commercial Products by qPCR.** Sixteen commercial food products, labeled as containing salmon, were analyzed (Table 2). These products, purchased from supermarkets and retail stores in Shanghai, were processed by different treatments (oil immersion, fumed, ground, pickled, dried, and powdered, mixed with salads or cheese, among others). Three similar samples in parallel were removed from each product.

Forty-eight DNA samples were first amplified by the *18S rRNA* and *12S rRNA* primer sets to assess the extraction efficacy. PCR reactions consisted of 25 μL volumes with 1× Premix Ex *Taq* HS (Takara Biotechnology Co., Ltd., Dalian, China), 400 nM of each primer, and 5 μL of genomic DNA as template. Amplifications were carried out in a Mastercycler ProThermal Cycler (Eppendorf Co., Hamburg, Germany) with the following program: 95 °C for 5 min; 36 cycles at 95 °C for 30 s; 56 (for *18S rRNA*) or 55 °C (for *12S rRNA*) for 30 s; 72 °C for 30 s; and 72 °C for 5 min. Amplicons were analyzed by electrophoresis in 2.0% (w/v) agarose gels (previously stained with ethidium bromide) at 120 V for approximately 30 min.

Using qPCR, the *Salmonidae* ingredients of 16 food products were analyzed. Ct values of each sample were converted to DNA quantity according to the standard curve formulas constructed for *S. salar* and *O. mykiss*. For quantification, three parallel repeats were carried out for each sample. After the calculation, the original mean DNA concentrations of the 16 samples were plotted.

## RESULTS AND DISCUSSION

**Development of the Real-Time PCR Method.** Reliable detection methods depend on the use of appropriate target genes. For the probe and primer design, candidate genes should be highly conserved and with a high interspecific variability. Several mitochondrial (mt) genes, such as *Cyt b*, *ITS1*, COI, p53 gene, *18S rRNA*, *12S rRNA*, and *5S rRNA*, have been used to identify fish species.[11,17,23−27] Compared with mt genes, nuclear genes have more constant copy number in different tissues that is considered to be more feasible in quantification. Therefore, in the present study, a nuclear gene, the salmon growth hormone (GH) gene, which has one to two copies in a haploid genome and which promotes growth, was selected as the candidate gene. The GH gene has been previously used as a positive control to detect genetically modified coho salmon.[28] As a result of the conservation in the *Salmonidae* species and the high variability between *Salmonidae* and the related families, the GH gene was suitable for identification of the *Salmonidae* fish family.

The GH genomic DNA sequences of different species were aligned and obtained from the NCBI Web site. On the basis of the conserved sequence of the exon region, a probe and a pair

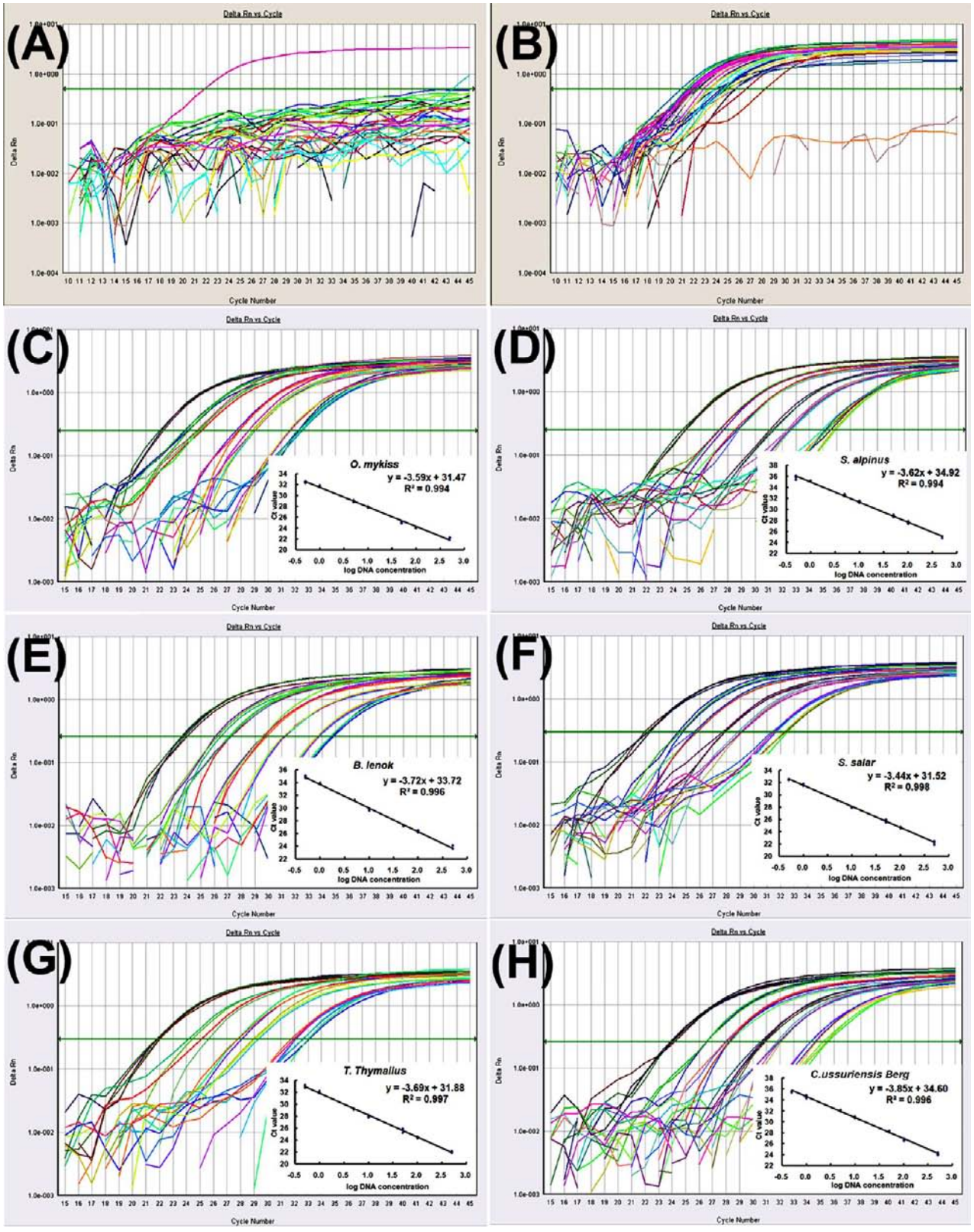


**Figure 2.** Specificity tests of the developed qPCR method and standard curves constructed from samples of six salmonid species. (A and B) Specificity tests using 22 nonsalmonid samples (A) and 31 salmonid samples as templates (B). Positive signal in A was obtained from a *S. salar* DNA sample (positive control). (C−H) Amplification plots and standard curves from seven genomic DNA concentrations of *O. mykiss* (C), *S.alpinus* (D), *B. lenok* (E), *S. salar* (F), *T. thymallus* (G), and *C. ussuriensis Berg* (H).

of specific primers were designed. Results of the sequence alignment and the primer and probe locations are shown in Figure 1.

To obtain the reaction curve with the lowest Ct value and the highest final fluorescence, real-time PCR conditions were optimized. The primer and probe concentrations are one of the


dx.doi.org/10.1021/jf400769y | J. Agric. Food Chem. 2013, 61, 5170−5177

**Table 3. Absolute LOD Values from Real-Time PCR**

| | concentration (ng/μL) | | | | | | |
|---|---|---|---|---|---|---|---|
| sample species | 100 | 10 | 1 | 0.1 | 0.01 | 0.005 | 0.001 |
| *S. salar* | 20/20 | 20/20 | 20/20 | 20/20 | 20/20 | 20/20 | 16/20[a] |
| *O. mykiss* | 20/20 | 20/20 | 20/20 | 20/20 | 20/20 | 19/20 | 12/20 |
| *S. alpinus* | 20/20 | 20/20 | 20/20 | 20/20 | 20/20 | 20/20 | 15/20 |
| *H. taimen* | 20/20 | 20/20 | 20/20 | 20/20 | 20/20 | 19/20 | 10/20 |
| *B. lenok* | 20/20 | 20/20 | 20/20 | 20/20 | 20/20 | 19/20 | 11/20 |
| *C. ussuriensis Berg* | 20/20 | 20/20 | 20/20 | 20/20 | 20/20 | 20/20 | 12/20 |
| *T. thymallus* | 20/20 | 20/20 | 20/20 | 20/20 | 20/20 | 20/20 | 9/20 |

[a]The species was detected 16 out of 20 times.

**Table 4. Relative LOD Values from Real-Time PCR**

| | | | percentage (w/w) | | | | | |
|---|---|---|---|---|---|---|---|---|
| sample species | matrix | code | 10% | 1% | 0.1% | 0.01% | 0.001% | 0.0001% |
| *S. salar* | maize | Aa (1−6)[a] | 20/20 | 20/20 | 20/20 | 20/20 | 19/20[b] | 6/20 |
| | crucian | Ab (1−6) | 20/20 | 20/20 | 20/20 | 20/20 | 19/20 | 5/20 |
| | chicken | Ac (1−6) | 20/20 | 20/20 | 20/20 | 20/20 | 15/20 | 5/20 |
| *S. alpinus* | maize | Ba (1−6) | 20/20 | 20/20 | 20/20 | 20/20 | 17/20 | 4/20 |
| | crucian | Bb (1−6) | 20/20 | 20/20 | 20/20 | 20/20 | 15/20 | 8/20 |
| | chicken | Bc (1−6) | 20/20 | 20/20 | 20/20 | 20/20 | 17/20 | 7/20 |
| *T. thymallus* | maize | Ca (1−6) | 20/20 | 20/20 | 20/20 | 20/20 | 18/20 | 5/20 |
| | crucian | Cb (1−6) | 20/20 | 20/20 | 20/20 | 20/20 | 17/20 | 6/20 |
| | chicken | *Cc* (1−6) | 20/20 | 20/20 | 20/20 | 20/20 | 16/20 | 4/20 |

[a]Numbers 1, 2, 3, 4, 5, and 6 represent samples of 10%, 1%, 0.1%, 0.01%, 0.001%, and 0.0001%, respectively. [b]The species was detected 19 out of 20 times.

most critical parameters in qPCR. Serial concentrations of primers and probe, ranging from 200 to 600 nM for the primers and from 150 to 300 nM for the probe, were tested. Finally, an optimal concentration of 500 nM for the primers and 250 nM for the probe were selected.

**Specificity Test.** The specificity of the designed primer set (GH-F/R) and probe (GH-P) was tested by amplifying the genomic DNA samples of 31 salmonid species and 22 other fish species (Table 1). The 31 salmonid fish samples involved all seven genera of *Salmonidae* family (*Oncorhynchus*, *Salvelinus*, *Hucho*, *Brachymystax*, *Salmo*, *Coregonus*, and *Thymallus*). The other fish samples included the related orders such as *Osmeriformes*, *Escociformes*, and other familiar edible fish species. As expected, no significant fluorescent amplification (Ct > 40) was detected in the above 22 fish samples (Figure 2 A). Fluorescence was only detected in the 31 salmonid samples with an average Ct value of approximately 23.18 ± 2.09 (Figure 2 B). There were significant differences in the Ct values between the salmonid and the other fish species, indicating that there was no cross-amplification. The results showed that the designed probe and primer set was specific for salmonid identification.

**Absolute and Relative Sensitivity Test.** The sensitivity of the method was validated by the LOD, which was assessed from the absolute sensitivity of the actual genomic DNA concentration and the relative sensitivity of the tested samples containing a certain percentage of the ingredient. To determine the absolute LOD, genomic DNA samples of representative salmonid species from seven genera (*O. mykiss*, *S. alpinus*, *B. lenok*, *H. taimen*, *S. salar*, *C. ussuriensis Berg*, and *T. thymallus*) were prepared at the following concentrations: 100, 10, 1, 0.1, 0.01, 0.005, and 0.001 ng/μL. According to the ≥95% confidence level rule, the LOD is when there are 19 or more detection times out of 20 reactions. As a result, the template concentration that could be detected for ≥19 times in seven representative salmonid species samples was 0.005 ng/μL. In other words, the lowest level that could be reliably detected was 0.025 ng of DNA (i.e., 5 μL of template) (Table 3). Twenty-five pictograms of DNA are equal to approximately 10 haploid genomic DNA contents. This level was similar to the LODs of previous studies done on other authentication assays.[17]

For the relative LOD test, samples containing six percentages of salmonid ingredients, i.e., 10%, 1.0%, 0.1%, 0.01%, 0.001%, and 0.0001% (w/w), were prepared. Taking into account the possible food processing conditions and the composition of the commercial products, three matrices, including maize (plant), crucian (fish), and chicken (animal), were used with three species of *Salmonidae*. A total of nine groups of food matrix−fish samples were tested. Results showed that the difference in the detection times in all nine groups was between 0.01% and 0.001% (Table 4). The 10%, 1.0%, 0.1%, and 0.01% samples in all nine groups could be detected in 20 reactions. Specifically, in 0.001% *S. salar* (mixed with maize or crucian), positive signals appeared in 19 out of 20 reactions, indicating that the lowest percentage that could be detected was 0.001% at a 95% confidence level. The sample containing 0.001% *S. salar* (mixed with chicken) was detected in 15 out of 20 reactions, indicating that the LOD of this group was 0.01%. In *S. alpinus* and *T. thymallus*, the 0.001% samples were detected in 15−18 out of 20 reactions. Thus, the percentage of salmonid in foods should be at least 0.01%. The absolute LOD of 25 pg of DNA and a relative LOD of 0.01% sample could meet the detection limits of food samples.

**Construction of Standard Curves.** To quantify the salmonid ingredient in the unknown food samples, six standard curves of six representative species were constructed using seven concentrations of genomic DNA dilutions. The linearity between the logarithm of the DNA concentration and the Ct

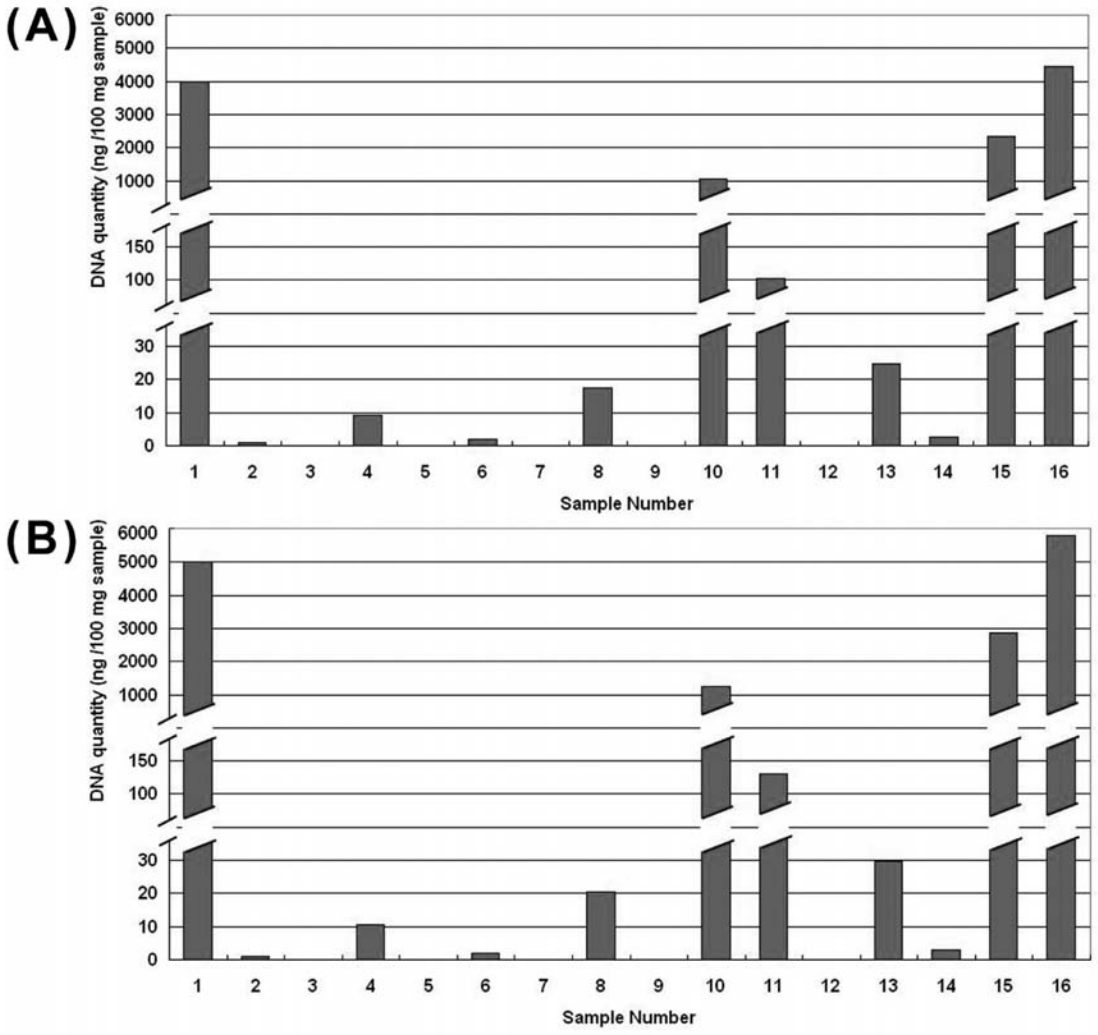


**Figure 3.** *Salmonidae* ingredient concentration in 16 lots of commercial products based on two standard curves. (A) Plot based on the standard curve of the *O. mykiss* samples. (B) Plot based on the standard curve of the *S. salar* samples.

values was assessed by $R^2$ values. Results showed that $R^2$ values were >0.99 (0.994−0.998 range), indicating that both the repeatability and the linearity of the curves were very good. The PCR efficiencies ($E$) were >80%. Specifically, $E$ was 89.91% for *O. mykiss*, 88.90% for *S. alpinus*, 85.70% for *B. lenok*, 95.30% for *S. salar*, 81.86% for *C. ussuriensis Berg*, and 86.64% for *T. thymallus* (Figure 3). The good linearity and relatively high PCR efficiency revealed that the developed qPCR method is suitable for further quantification of salmonid ingredients in food samples.

**Identification of Commercial Products.** Sixteen processed food products purchased from supermarkets and retail stores were tested for the presence of salmonid ingredient by the developed qPCR method. When salmonid ingredient was detected in the food sample, the content was calculated from the standard curves of *S. salar* and *O. mykiss*, because the food samples were labeled as containing the "salmon" ingredient. In the market, two species (*S. salar* and *O. mykiss*) are commonly considered to be a product of "salmon".

To validate the quality of the extracted DNA, the *18S rRNA* and *12S rRNA* gene fragments were amplified to provide a positive control. A bright band (137 bp) pertaining to *18S rRNA* was obtained from all 16 samples, and a band (224 bp) pertaining to *12S rRNA* was obtained from 14 samples (samples Nos. 1, 2, 4, 6−16; data not shown), indicating that the extracted DNA samples were suitable for further analysis. After amplification, no curves were obtained from samples 3, 5, 7, and 9, indicating that the *Salmonidae* ingredient was not detected in these four samples. Combined with the amplification results of *12S rRNA*, samples 7 and 9 contained the fish ingredient but the fish species did not belong to the *Salmonidae* family, indicating that the labeled fish species was inauthentic. Inauthenticity means labeling an inexistent ingredient or including the wrong species on the label. In this study, the fish ingredient was present in 14 out of 16 samples and the salmonid ingredient was present in 12 samples, which indicates that fraudulent labeling is serious in the fish market (25% of the labels were fraudulent).

The mean Ct values of the positive samples and their three parallels are shown in Table 5. Because of the high DNA concentrations, the DNA of samples 1, 10, 15, and 16 were diluted to 1/50 using 0.1× TE buffer to include the Ct values in the standard curve range. On the basis of two standard curves, the content of the *Salmonidae* ingredient in the samples was calculated. Results from the histograms showed differences in the *Salmonidae* ingredient content per 100 mg sample (Figure 4). The highest amount was found in samples 1 and 16, with a content of 3994.63 and 4609.85 ng, respectively, based on the

**Table 5. Mean Ct Values of the 12 Positive Samples Using Real-Time PCR**

| | mean Ct value | | | | |
|---|---|---|---|---|---|
| sample code | Rep 1 | Rep 2 | Rep 3 | mean Ct | SD |
| 1[a] | 24.56 | 24.72 | 24.64 | 24.64 | 0.08 |
| 2 | 31.61 | 31.64 | 31.53 | 31.59 | 0.06 |
| 4 | 28.01 | 28.03 | 27.97 | 28.00 | 0.03 |
| 6 | 30.42 | 30.57 | 30.25 | 30.41 | 0.16 |
| 8 | 27.21 | 27.08 | 26.77 | 27.02 | 0.23 |
| 10[a] | 26.77 | 26.72 | 26.62 | 26.70 | 0.08 |
| 11 | 24.36 | 24.29 | 24.08 | 24.24 | 0.15 |
| 12 | 36.23 | 36.14 | 36.93 | 36.43 | 0.43 |
| 13 | 26.46 | 26.47 | 26.46 | 26.46 | 0.01 |
| 14 | 29.91 | 29.86 | 30.01 | 29.93 | 0.08 |
| 15[a] | 25.43 | 25.56 | 25.43 | 25.47 | 0.08 |
| 16[a] | 24.24 | 24.46 | 24.55 | 24.42 | 0.16 |

[a]Ct values were from DNA samples previously diluted (1/50 dilution).

*O. mykiss* standard curve. The *S. salar* standard curve revealed that sample 1 had a content of 5000.00 ng and sample 16 had 5806.21 ng. These results suggest that processing treatment (i.e., smoking and mashing) has little effect on DNA degradation. The lowest content was found in samples 2 and 12: 0.92 and 0.04 ng, respectively, based on the *O. mykiss* standard curve and 0.95 and 0.04 ng, respectively, based on the *S. salar* standard curve, indicating that the fish was present in the foods not as the principal component. These results suggest that the developed method was suitable for detecting traces of salmonid ingredients in foods.

Considering its high specificity, sensitivity, and applicability, the developed real-time PCR method is both reliable and suitable for detection and quantification of salmonid ingredients in food samples.

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### Funding

This work was supported by grants from the Shanghai Special Fund for Technical Barriers to Trade (12TBT011).


### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

We thank Prof. Ma Bo and Wang Bingqian of the Heilongjiang Fisheries Research Institute, Chinese Academy of Fishery Science, for their help and support and Qinghai Minze Longyangxia Ecological Fishery Co., Ltd. for providing fish samples.

## ABBREVIATIONS AND NOMENCLATURE

AFLP, amplified fragment length polymorphism; Ct, cycle threshold; LOD, limit of detection; NCBI, National Center for Biotechnology Information; PCR, polymerase chain reaction; qPCR, real-time PCR; RAPD, random amplified polymorphic DNA; RFLP, restriction fragment length polymorphism; SSCP, single-strand conformation polymorphism

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