DOI:10.1021/jf103241y

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Interlaboratory Evaluation of a Real-Time Multiplex Polymerase Chain Reaction Method for Identification of Salmon and Trout Species in Commercial Products

Rosalee S. Rasmussen Hellberg,*,† Amanda M. Naaum,§ Sara M. Handy,# Robert H. Hanner,§ Jonathan R. Deeds,# Haile F. Yancy,⊥ and Michael T. Morrissey†

†Food Innovation Center, Oregon State University, 1207 N.W. Naito Parkway, Portland, Oregon 97211, United States , §Department of Integrative Biology, University of Guelph, 50 Stone Road East, Guelph, Ontario N1G 2W1, Canada, #Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, 5100 Paint Branch Parkway, College Park, Maryland 20740, United States, and ⊥Office of Research, Center for Veterinary Medicine, U.S. Food and Drug Administration, 8401 Muirkirk Road, Laurel, Maryland 20708, United States

This interlaboratory study evaluated a real-time multiplex polymerase chain reaction (PCR) method for identification of salmon and trout species in a range of commercial products in North America. Eighty salmon and trout products were tested with this method by three independent laboratories. Samples were collected in the United States and Canada, and only the collecting institution was aware of the species declaration. Following analysis with real-time PCR, all three laboratories were able to identify species in 79 of the 80 products, with 100% agreement on species assignment. A low level of fraud was detected, with only four products (5%) found to be substituted or mixtures of two species. The results for two of the fraudulent products were confirmed with alternate methods, but the other two products were heavily processed and could not be verified with methods other than real-time PCR. Overall, the results of this study show the usefulness and versatility of this real-time PCR method for the identification of commercial salmon and trout species.

KEYWORDS: Real-time PCR; interlaboratory study; salmon; trout; species identification; multiplex PCR

INTRODUCTION

There are seven commercially important species of salmon and trout in North America from the genera *Oncorhynchus* and *Salmo*: Atlantic salmon (*Salmo salar*), rainbow (steelhead) trout (*Oncorhynchus mykiss*), Chinook salmon (*Oncorhynchus tshawytscha*), sockeye salmon (*Oncorhynchus nerka*), coho salmon (*Oncorhynchus kisutch*), chum salmon (*Oncorhynchus keta*), and pink salmon (*Oncorhynchus gorbuscha*). Atlantic salmon and rainbow trout are farm-raised, whereas the remaining five species are primarily wild-caught and are collectively referred to as Pacific salmon. The United States is a significant producer of Pacific salmon, harvesting about 300,000 t in 2008 (*1*), whereas Canada produces a high proportion of farmed salmonids, with 110,000 t for the same year (*2*). Although the seven commercial salmon and trout species are similar in appearance, they are sold at markedly different prices due to variations in quality, supply and demand, and marketing, as well as competition between wild-caught and farm-raised salmon (*3*). These price differentials increase the potential for species substitution, which is prohibited in the United States under the Federal Food Drug and Cosmetic Act Section 403: Misbranded Food, and can lead to economic fraud and food safety risks (*4*). Currently, National Marine Fisheries (NMFS) inspects about one-third of U.S. commercial seafood as part of a voluntary, fee-for-service program (*5*), and surveys of North American seafood have reported 25−34% of commercial products to be mislabeled (*6*, *7*). With regard to salmon, the U.S. Food and Drug Administration (USFDA) has reported substitution of pink salmon for chum salmon, farm-raised salmon for wild-caught salmon, and steelhead trout for salmon (*8*).

The official method for seafood species identification is based on protein analysis with isoelectric focusing (AOAC Official Method 980.16), which is highly sensitive to sampling and processing conditions and exhibits complex banding patterns that are difficult to interpret, much less replicate among laboratories (*9*, *10*). Furthermore, analysis of heat-treated foods and closely related species is often problematic with protein-based methods (*11*, *12*). An alternative method that shows considerable promise for detecting commercial seafood substitution is DNA barcoding (*7*, *13*, *14*). This method involves sequencing a 650 bp 5′ region of the cytochrome *c* oxidase subunit I (COI) gene, termed the "DNA barcode", for an unknown sample and comparing that sequence to a comprehensive reference sequence database (*15*, *16*). This database is being developed through the International Barcode of Life project (www.ibolproject.org) and already includes DNA barcodes from over 900 individuals representing the commercially important salmon and trout species in North

*Corresponding author [e-mail rosalee.hellberg@gmail.com; phone (503) 872-6650; fax (503) 872-6648].

 Published on Web 01/07/2011

America (*17*−*19*). The USFDA is also considering the use of DNA barcoding as an official regulatory method and is currently incorporating DNA barcodes into the *Regulatory Fish Encyclopedia* (*13*). Although DNA barcoding has been shown to be effective in differentiating the commercially important species of salmon and trout in North America (*17*), it remains relatively time-consuming and requires costly equipment. Perhaps even more problematic is the fact that barcode sequencing is not amenable to the detection of mixtures without the addition of an even more time-consuming, labor-intensive, and costly cloning step, which is necessary to evaluate the diverse PCR amplification products that would be obtained from a multispecies mixture. Moreover, barcoding cannot be reliably used to identify species in heavily processed food products.

Real-time PCR is a rapid method of species identification that can be used with mixed-species and heavily processed products. Real-time PCR assays have been developed for the identification of several fish species, including flatfish (*20*), eel (*21*), tuna (*22*), cod (*23*), and snappers and drum (*24*). Recently, a species-specific real-time multiplex PCR method was developed to differentiate the seven commercially important species of salmon and trout (*25*). The assay is based on diagnostic nucleotides in the DNA barcode region and targets small DNA fragments (< 220 bp), enabling species identification in a broad range of products. A small-scale test of this method with six commercial salmon products showed promising results; however, a more extensive investigation is warranted to determine if this method can be used for routine and reliable species identification with commercial salmon products and to verify the use of previously suggested detection cutoff values. Therefore, the objective of this study was to evaluate the effectiveness of the multiplex real-time PCR method developed previously for identifying salmon and trout species in a range of commercial products sold in North America and to test the method in multiple laboratories using different real-time PCR platforms.

MATERIALS AND METHODS

Interlaboratory Study Participants. Three laboratories participated in this study: (1) Seafood Research and Education Center, Department of Food Science and Technology, Oregon State University, Astoria, OR; (2) Department of Integrative Biology, University of Guelph, Guelph, ON, Canada; and (3) Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, College Park, MD. These laboratories are hereafter referred to as participants 1, 2, and 3, respectively.

Sample Collection and Preparation. A total of 80 commercial salmon and trout products representing a variety of species and processing methods were obtained from retail outlets in Canada and the United States in November 2009. Forty products were collected by participant 1 from 9 locations in Oregon and Washington (USA), and the other 40 products were collected by participant 2 from 8 locations in Ontario (Canada). Each product was assigned a random three-digit number, and only the collecting laboratory was aware of the species declaration linked to each product. Tissue samples from each product were subsampled into three sets of 2 mL tubes and preserved in 95% ethanol. Products with increased susceptibility to species mixing (i.e., canned and retort-packed salmon, salmon burgers, and frozen salmon dinners) were individually blended with up to 30 mL of sterile water for at least 2 min prior to subsampling. Three sets of tubes containing subsamples of all products were then distributed to all three study participants for interlaboratory testing. Each laboratory also received reference DNA and expected cycle threshold values (Ct) for each of the target species, as determined previously (*25*), to optimize the baseline and threshold settings for each instrument. Participants were also provided with reference tissue samples of the seven commercial salmon and trout species as positive controls for DNA extraction and real-time PCR. These reference samples were verified in a previous study with DNA barcoding (*17*).

DNA Extraction and PCR Preparation. DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) with overnight lysis and elution in 60−100 μL of AE buffer. A reagent blank was included in each DNA extraction and subsequent PCR as a negative control. Nucleic acid concentrations were determined using either a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA) or a BioPhotometer plus (Eppendorf, Brinkman Instruments, Inc., Westbury, NY) combined with either UVettes (Eppendorf) or a Hellma Traycell (Hellma GmbH & Co. KG, Müllheim, Germany). Extracted DNA samples were adjusted to 20−30 ng/μL for use in real-time PCR.

TaqMan minor groove binder (MGB) probes and PCR primers used in this study are described in Rasmussen Hellberg et al. (*25*). These primers and probes target species-specific regions of the DNA barcode in all seven commercially important salmon and trout species in North America, as well as a universal region of cytochrome *b*. All probes and degenerate primers were synthesized by Applied Biosystems (Foster City, CA), and nondegenerate primers were synthesized by TriLink Biotechnologies (San Diego, CA). Template DNA, primers, and probes were diluted for use in PCR using Tris-EDTA (TE) buffer (pH 8.0) containing 0.2 M trehalose as a preservation agent (*26*). Each set of PCR primers and probe was prepared by participant 1 as a 20× working solution and then distributed to participants 2 and 3.

Real-Time Multiplex PCR. Real-time PCR was conducted on three different platforms: a 7500 Real-Time PCR System (Applied Biosystems; participant 1), a StepOnePlus Real-Time PCR System (Applied Biosystems; participant 2), and a SmartCycler II (Cepheid, Sunnyvale, CA; participant 3). All reactions contained 12.5 μL of 2× QuantiTect Multiplex PCR NoROX Master Mix (Qiagen), 0.10−0.60 μM final concentration of primers, 0.10−0.60 μM final concentration of TaqMan MGB probes (Applied Biosystems), 40−60 ng of template DNA, and sterile water for a total reaction volume of 25 μL (*25*). Each sample was tested against all seven species-specific primers and probes, along with the universal primers and probe set. The species-specific primers and probes were combined into three multiplex sets (one triplex set targeting *S. salar*, *O. keta*, and *O. tshawytscha*; one duplex set targeting *O. nerka* and *O. kisutch*; and one duplex set targeting *O. gorbuscha* and *O. mykiss*) for use with Applied Biosystems instruments, and the universal set was run separately. Cycling conditions, primer and probe details, and multiplex sets are described in Rasmussen Hellberg et al. (*25*), with some modifications for use with the SmartCycler II. This PCR platform required an alternate TaqMan probe dye set (FAM and TET) compared to the dye set compatible with Applied Biosystems instruments (FAM, VIC, and NED), which limited the multiplexing capability. Therefore, instead of the triplex set used previously to simultaneously detect *S. salar*, *O. keta*, and *O. tshawytscha*, a duplex reaction was carried out with the SmartCycler II targeting *S. salar* and *O. keta*, and a separate singleplex reaction was conducted targeting *O. tshawytscha*. The two other duplex arrangements were the same as previously described (*25*). The PCR cycling conditions for the universal set were also adjusted for use with the SmartCycler II to include an extension step of 72 °C for 60 s, based on the manufacturer's recommendations.

All real-time PCR settings were optimized for species identification using reference DNA from each of the target species. Participant 1 utilized previously established settings (baseline = 3−15 cycles, threshold = 4.0×10^4 (FAM), 2.0×10^4 (VIC), and 1.8×10^4 (NED) fluorescent units) (*25*); participant 2 utilized a baseline setting of 3−15 cycles and thresholds of 5.5×10^3 (FAM), 3.5×10^3 (VIC), and 1.5×10^3 (NED) fluorescent units; and participant 3 utilized the default program settings for the SmartCycler II (baseline = 3−15 cycles and threshold = 30 fluorescent units). The cycle threshold (Ct) value, defined as the cycle at which the fluorescence signal crosses the threshold level, was recorded for each sample. A Ct value below 25 in fresh/frozen (uncooked) or heat-treated (partially or fully cooked) samples or a Ct value below 30 in heat-sterilized (canned or retort-packed) samples was used to identify species in food products, as recommended previously (*25*). A nontemplate PCR control was included alongside all reactions.

Any samples found to contain substituted species or exhibiting secondary signals of Ct < 30 were examined further to verify the results. Fresh/frozen or heat-treated samples that exhibited a Ct value between 25 and 30 were subjected to repeat DNA extraction and real-time PCR. These tests were carried out in duplicate, and a Q-test was used for the rejection of outliers, as described in Smith et al. (*27*). The original DNA sample was also tested with previously published methods for salmon species identification: PCR-restriction fragment length polymorphism

(RFLP) analysis (*28*) and DNA barcoding (*17*), as described below. In cases when species substitution or a secondary signal below the Ct cutoff value (i.e., mixed-species sample) was found for a particular sample by all three laboratories, the sample was also tested with PCR-RFLP (*28*) and DNA barcoding (*17*). Mixed-species samples were further tested with conventional species-specific PCR (*25*) and sequencing of the species-specific amplicon, as described below.

DNA Barcoding. DNA barcoding was carried out as described in Rasmussen et al. (*17*). Sequences were edited and aligned with CodonCode Aligner version 3.5.6 and MEGA version 4.0 (*29*). Species were determined using the Barcode of Life Database (BOLD) online identification system (http://www.boldsystems.org/views/idrequest.php) with the public record database search option, which includes all published COI records from BOLD and GenBank with a minimum sequence length of 500 bp. The sequences were also examined for any previously undetected nucleotide mismatches that might explain cross-specific primer binding and real-time amplification signals below 30 PCR cycles.

PCR-RFLP. PCR-RFLP was carried out for a 146 bp fragment of the cytochrome *b* gene, as described in Espiñeira et al. (*28*) with some modifications. The reaction mixture consisted of 0.2 mM of each dNTP, 1× PCR buffer, 2 mM $MgCl_2$, 1.25 units of AmpliTaq DNA polymerase (Applied Biosystems), 0.6 μM of each primer, 100−300 ng of DNA template, and sterile water for a final volume of 50 μL. PCR was carried out with a MyCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) under the following cycling conditions: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 52 °C for 60 s, and 72 °C for 60 s, and then a final extension step at 72 °C for 7 min. A nontemplate PCR control was included alongside all reactions. PCR amplicons were digested for 2 h at 55 °C with the restriction enzyme *Fau*I (New England Biolabs, Ipswich, MA) and at 37 °C with the restriction enzymes *Mnl* (New England Biolabs) and *Rsa*I (Promega, Madison, WI), an isochizomer of *Afa*I. Restriction digests contained 3.5−5 units of enzyme and 12.0 μL of PCR product in a total volume of 25 μL. PCR amplicons were verified using the Lonza FlashGel DNA System (Basel, Switzerland) with 2.2% agarose FlashGel DNA cassettes run at 275 V for 6 min alongside a 50 bp FlashGel DNA marker, and restriction digest results were separated using 3.0% NuSieve 3:1 agarose gels (Lonza) run at 140 V for 50 min alongside a 20 bp EZ Load molecular ruler (Bio-Rad Laboratories). The results were scanned and visualized with GelDoc XR and Quantity One software version 4.5.2 (Bio-Rad Laboratories), and band sizes were recorded for each sample.

Conventional Species-Specific PCR and Sequencing. Conventional species-specific PCR was carried out as described in Rasmussen Hellberg et al. (*25*), with the exception that only one set of species-specific primers was utilized per PCR tube (singleplex reaction). Each mixed-species sample was tested with species-specific primers targeting the primary and secondary species detected during real-time PCR. All primers, reaction conditions, and gel electrophoresis methods are described in Rasmussen Hellberg et al. (*25*). The PCR products for the secondary species were cleaned with ExoSAP-IT (USB Corp., Cleveland OH) based on the manufacturer's instructions and then sequenced bidirectionally at Genewiz (South Plainfield, NJ) with an ABI 3730*xl* DNA Analyzer (Applied Biosystems). All ABI files were imported into Sequencher 4.9 (Gene Codes Corp., Ann Arbor, MI) for analysis. Because the sequences were too short for analysis with the BOLD identification system, they were instead examined using a nucleotide diagnostic or character attribute approach (*30*, *31*). Diagnostic nucleotides were determined on the basis of a set of over 350 reference sequences obtained previously (*17*, *18*) representing the primary and secondary species detected during real-time PCR (*O. tshawytscha*, *O. keta*, and *O. gorbuscha*).

Statistical Analysis. The Ct values obtained during real-time PCR testing were compared among the three laboratories and among the three processing groups using one-way analysis of variance (ANOVA) and Tukey's honestly significant different (HSD) test, with significance set at $p < 0.05$. The Ct values of universal and species-specific assays were compared using a paired samples *t* test, with significance set at $p < 0.05$, two tailed. All statistical tests were conducted with SPSS 13.0 for Windows (SPSS Inc., an IBM company, Chicago, IL).

RESULTS AND DISCUSSION

Interlaboratory Analysis of Reference Samples. As shown in **Table 1**, the real-time multiplex PCR method allowed for detection of each target species on all three PCR platforms, based on reference sample testing. The use of reference DNA samples was found to be essential for the initial optimization of threshold and baseline settings, likely due to variations in fluorescence excitation and emission detection among the different PCR platforms (*32*). Although the default settings were appropriate for the SmartCycler II, the Applied Biosystems instruments required manual determination of settings. The finalized settings determined for the three platforms resulted in Ct values for each reference DNA sample that had standard deviations within about one cycle of the mean, on average.

Table 1. Average Ct Values Obtained with Reference DNA and Reference Tissue Samples Following Determination of Real-Time PCR Settings[a]

species	assay	reference DNA Ct ± SD	reference tissue Ct ± SD
O. tshawytscha	species-specific	20.9 ± 1.0	20.5 ± 0.9
	universal	n/a	19.5 ± 1.3
O. nerka	species-specific	19.9 ± 1.0	18.0 ± 0.8
	universal	n/a	17.5 ± 0.5
O. keta	species-specific	20.2 ± 0.7	24.1 ± 5.6
	universal	n/a	25.6 ± 2.9
O. kisutch	species-specific	20.1 ± 0.9	21.2 ± 1.1
	universal	n/a	20.4 ± 0.8
O. gorbuscha	species-specific	20.3 ± 1.3	17.5 ± 0.1
	universal	n/a	18.6 ± 0.8
O. mykiss	species-specific	18.7 ± 1.0	19.4 ± 1.4
	universal	n/a	20.1 ± 1.3
S. salar	species-specific	18.7 ± 1.4	17.7 ± 0.2
	universal	n/a	17.2 ± 0.4

[a] The expected Ct values for the reference DNA were determined by participant 1 during method development (*25*), and participants 2 and 3 adjusted real-time PCR settings accordingly to allow for similar Ct values and no background signals at Ct < 30. The Ct values in the table are reported as the average and standard deviation of combined test results obtained by the three study participants.

Reference tissue samples that were provided as positive controls for DNA extraction and real-time multiplex PCR were also detected by all three participants, with most of the standard deviations within about one cycle of the mean, similar to the results found with reference DNA testing. However, one exception was the *O. keta* reference sample, which showed elevated Ct values when tested by participants 1 (Ct = 26.7) and 2 (Ct = 27.7). The universal Ct values for this sample were also high for these two laboratories (27.3−27.4). Elevated Ct values (Ct = 25−30) were previously found more frequently for reference tissue samples of *O. keta* compared to other species (*25*), indicating that the *O. keta* primers and probe set may be more sensitive to DNA degradation.

Interlaboratory Analysis of Commercial Products. As shown in **Table 2**, a total of 80 salmon and trout products were tested by all three study participants with real-time multiplex PCR. These items represent many of the product types available in North America, including fresh/frozen fillets, frozen salmon dinners and burgers, smoked salmon and trout, salmon jerky, canned salmon, and retort-packed smoked salmon. Overall, there were 28 fresh/frozen (uncooked) products, 29 heat-treated (partially or fully cooked) products, and 23 heat-sterilized (canned or retort-packed) products. On the basis of the results of real-time multiplex PCR, all three laboratories were able to identify species in 79 of the 80 products (98.8%), with 100% agreement on the species assignment.

Table 2. Real-Time PCR Results for All Commercial Salmon and Trout Products[a]

sample	sample code	processing group	product description	species declared	species identified	species-specific Ct ± SD	universal Ct ± SD
1	383	fresh/frozen	Alaskan salmon fillet, Santa Fe style	salmon	*O. gorbuscha*	19.4 ± 0.9	19.2 ± 1.2
2	117		boneless trout fillet	*O. mykiss*	*O. mykiss*	21.0 ± 1.7	21.5 ± 0.6
3	501		frozen dinner (uncooked), salmon fisherman's pie	*S. salar*	*S. salar*	20.9 ± 2.7	20.5 ± 1.5
4	424		frozen dinner (uncooked), wild salmon with basil	*O. keta* or *O. gorbuscha*	*O. gorbuscha*	18.4 ± 0.9	18.1 ± 0.1
5	142		maple salmon skewers	*O. keta* or *O. gorbuscha*	*O. keta*	18.1 ± 1.0	18.7 ± 0.3
6	583		salmon fillet	*O. keta*	*O. keta*	18.6 ± 0.9	18.7 ± 0.7
7	715		salmon fillet	*O. kisutch*	*O. kisutch*	19.9 ± 1.5	18.2 ± 0.8
8	922		salmon fillet	*O. mykiss*	*O. mykiss*	20.1 ± 1.2	20.5 ± 0.5
9	609		salmon fillet	*S. salar*	*S. salar*	19.5 ± 0.7	18.5 ± 1.3
10	484		salmon fillet	*S. salar*	*S. salar*	20.2 ± 0.2	20.7 ± 0.3
11	415		salmon fillet	*S. salar*	*S. salar*	19.5 ± 0.3	19.6 ± 0.2
12	842		salmon fillet	*O. keta* or *O. gorbuscha*	*O. keta*	18.6 ± 0.9	18.9 ± 0.6
13	725		salmon steaks	*S. salar*	*S. salar*	19.8 ± 0.8	19.8 ± 0.6
14	517		skinless boneless salmon portions	*S. salar*	*S. salar*	17.9 ± 0.9	18.8 ± 1.2
15	855		Thai chili salmon fillet	*O. keta*	*O. keta*	18.9 ± 1.2	18.7 ± 0.5
16	578		trout fillet	*O. mykiss*	*O. mykiss*	19.3 ± 2.0	20.6 ± 1.4
17	903		whole dressed trout	*O. mykiss*	*O. mykiss*	19.6 ± 0.9	20.8 ± 0.4
18	373		wild Pacific salmon	*O. keta*	*O. keta*	17.8 ± 1.7	17.7 ± 0.3
19	673		wild Pacific salmon breaded fillet, lemon pepper	*O. keta* or *O. gorbuscha*	*O. gorbuscha*	18.9 ± 1.6	18.8 ± 1.3
20	192		wild Pacific salmon fillet	*O. gorbuscha*	*O. gorbuscha*	19.1 ± 1.1	19.1 ± 0.3
21	363		wild Pacific salmon fillet	*O. keta* or *O. gorbuscha*	*O. gorbuscha*	18.2 ± 1.8	17.7 ± 0.7
22	445		wild Pacific salmon fillet	*O. keta* or *O. gorbuscha*	*O. gorbuscha*	19.1 ± 1.0	18.6 ± 0.6
23	127		wild Pacific salmon fillet	*O. keta* or *O. gorbuscha*	*O. keta*	18.7 ± 1.2	19.3 ± 0.5
24	255		wild Pacific salmon fillet	*O. keta* or *O. gorbuscha*	*O. gorbuscha*	17.6 ± 1.0	17.1 ± 0.2
25	147		wild salmon fillet	*O. kisutch*	*O. kisutch*	22.6 ± 1.3	21.1 ± 0.4
26	168		wild salmon fillet	*O. nerka*	*O. nerka*	18.5 ± 0.7	17.5 ± 0.2
27	717		wild salmon portions, vacuum-packed	*O. nerka*	*O. nerka*	18.2 ± 0.4	17.7 ± 0.8
28	426		wild salmon skin-on fillet	*O. nerka*	*O. nerka*	18.8 ± 0.4	18.8 ± 0.9
29	856	heat-treated	cold-smoked nova-style Alaskan wild salmon lox	salmon	*O. keta*	18.5 ± 1.2	19.8 ± 0.8
30	958		cold-smoked salmon	*O. tshawytscha* (verbal declaration)	*S. salar*	19.1 ± 1.3	19.7 ± 1.0
31	888		cold-smoked salmon	*S. salar*	*S. salar*	18.9 ± 0.2	19.5 ± 0.5
32	422		cold-smoked salmon pinwheels	salmon	*S. salar*	22.7 ± 0.9	22.6 ± 0.9
33	279		cold-smoked wild Alaskan salmon	*O. nerka*	*O. nerka*	19.4 ± 0.3	19.7 ± 0.6
34	266		cold-smoked wild nova salmon	salmon	*O. keta* and *O. tshawytscha*	18.4 ± 1.1; 23.8 ± 0.9	18.7 ± 0.5
35	260		cold-smoked wild Pacific salmon	*O. nerka*	*O. nerka*	19.1 ± 0.7	19.1 ± 0.3
36	980		frozen grilled salmon fillet	salmon	*O. gorbuscha*	19.0 ± 1.5	18.4 ± 0.8
37	948		frozen parfried salmon burger (from whole fillet)	*O. keta* or *O. gorbuscha*	*O. gorbuscha*	19.1 ± 1.4	20.1 ± 3.2
38	833		hot-smoked farm-raised salmon	*O. kisutch*	*O. kisutch*	21.9 ± 2.3	21.0 ± 1.0
39	978		hot-smoked farm-raised salmon, cracked pepper	salmon	*O. kisutch*	21.7 ± 1.7	21.0 ± 0.8
40	808		hot-smoked premium wild salmon	salmon	*O. keta*	19.0 ± 0.9	20.1 ± 1.0
41	836		hot-smoked salmon	*O. keta*	*O. keta*	20.2 ± 1.2	21.1 ± 0.5
42	244		hot-smoked salmon, cracked pepper	*S. salar*	*S. salar*	19.4 ± 0.2	20.1 ± 0.3
43	843		hot-smoked wild Alaskan salmon	*O. nerka*	*O. nerka*	19.5 ± 0.5	19.4 ± 0.8
44	911		hot-smoked wild Pacific salmon	salmon	*O. keta*	19.1 ± 2.2	19.7 ± 1.3
45	822		hot-smoked wild salmon	*O. tshawytscha*	*O. tshawytscha*	21.5 ± 2.3	19.1 ± 0.6
46	636		jerky chew, shredded salmon	salmon	*O. keta*	19.4 ± 1.9	20.6 ± 1.8
47	568		jerky, wild salmon, regular flavor, cherry and alder smoked	*O. tshawytscha*	*O. tshawytscha*	17.9 ± 0.5	17.3 ± 0.6
48	873		jerky, wild salmon, teriyaki flavor, cherry and alder smoked	*O. tshawytscha*	*O. tshawytscha*	19.8 ± 0.6	18.9 ± 1.0
49	894		jerky, wild salmon, wine-maple smoked	salmon	*O. keta*	20.1 ± 1.4	21.5 ± 1.0
50	789		oven-smoked salmon, fully cooked	*S. salar*	*S. salar*	18.9 ± 1.0	19.5 ± 1.0
51	176		smoked salmon	*O. nerka*	*O. nerka*	19.4 ± 0.5	19.1 ± 0.4
52	212		smoked salmon ring, sliced	*O. kisutch*	*O. kisutch*	19.2 ± 1.2	18.4 ± 0.1
53	384		smoked salmon, peppered	*S. salar*	*S. salar*	20.2 ± 0.5	20.4 ± 0.7
54	338		smoked salmon, peppered	*S. salar*	*S. salar*	19.5 ± 0.9	19.7 ± 0.8
55	871		smoked wild Pacific salmon	*O. kisutch*	*O. kisutch*	22.2 ± 1.1	21.2 ± 0.8
56	890		smoked wild salmon	*O. nerka*	*O. nerka*	18.5 ± 0.1	18.0 ± 0.5
57	536		smoked wild salmon	*O. nerka*	*O. nerka*	18.5 ± 0.4	18.8 ± 0.5
58	104	heat-sterilized	canned Pacific salmon	*O. gorbuscha*	*O. gorbuscha*	21.3 ± 0.9	23.9 ± 0.5
59	829		canned Pacific salmon	*O. gorbuscha*	*O. gorbuscha*	21.1 ± 1.1	23.0 ± 0.1
60	590		canned Pacific salmon	*O. nerka*	*O. nerka*	21.7 ± 0.7	23.3 ± 0.3
61	276		canned Pacific salmon	*O. nerka*	*O. nerka*	20.3 ± 0.6	21.7 ± 0.5
62	803		canned Pacific salmon Thai salad	*O. gorbuscha*	*O. gorbuscha*	27.3 ± 2.1	29.8 ± 2.9
63	345		canned salmon	*O. gorbuscha*	*O. gorbuscha*	20.0 ± 0.6	21.3 ± 0.3
64	380		canned salmon	*O. nerka*	*O. nerka*	20.6 ± 0.5	21.9 ± 1.0
65	408		canned salmon	*S. salar*	*S. salar*	25.3 ± 1.1	24.9 ± 2.1

Table 2. Continued

sample	sample code	processing group	product description	species declared	species identified	species-specific Ct ± SD	universal Ct ± SD
66	733		canned salmon chunk style	*O. gorbuscha*	*O. gorbuscha* and *O. keta*	21.8 ± 0.7; 27.8 ± 1.2	23.4 ± 0.4
67	412		canned salmon, natural flavor	*S. salar*	*S. salar*	22.6 ± 0.5	22.7 ± 0.2
68	718		canned wild Alaskan salmon	*O. nerka*	*O. nerka*	23.8 ± 0.3	26.3 ± 0.5
69	382		canned wild Columbia River royal salmon	*O. tshawytscha*	*O. tshawytscha*	26.7 ± 0.2	26.8 ± 0.9
70	624		canned wild Pacific salmon	*O. gorbuscha*	*O. gorbuscha*	20.5 ± 0.6	21.8 ± 0.2
71	589		canned wild Pacific salmon	*O. gorbuscha*	*O. gorbuscha*	19.5 ± 0.5	20.2 ± 0.3
72	904		canned wild Pacific salmon	*O. gorbuscha*	*O. gorbuscha*	21.0 ± 0.9	23.3 ± 0.1
73	741		canned wild Pacific salmon	*O. nerka*	*O. nerka*	21.2 ± 0.2	23.1 ± 0.3
74	537		canned wild Pacific salmon	*O. nerka*	*O. nerka*	20.7 ± 0.1	22.2 ± 0.5
75	407		canned wild salmon	*O. gorbuscha*	*O. gorbuscha*	23.1 ± 0.6	25.9 ± 1.1
76	135		canned wild salmon	*O. nerka*	*O. nerka*	22.5 ± 1.0	23.6 ± 0.4
77	991		hot-smoked and retort-packed Alaskan salmon	*O. gorbuscha*	not identified	>30.0	>30.0
78	218		hot-smoked and retort-packed salmon	*O. nerka*	*O. nerka*	25.1 ± 1.3	29.6 ± 2.6
79	196		retort-packed grilled wild salmon steak, mandarin orange glaze	*O. gorbuscha*	*O. gorbuscha*	22.4 ± 0.7	24.8 ± 0.4
80	752		smoked and canned Alaskan salmon	*O. kisutch*	*O. keta*	25.4 ± 1.9	>30.0

[a] Each product was linked to a random, three-digit sample code so that only the collecting institution was aware of the species declaration. The Ct values determined for each sample are reported as the average and standard deviation of combined test results obtained by the three study participants. Species in fresh/frozen and heat-treated products were identified on the basis of a Ct value below 25.0, and species in heat-sterilized samples were identified on the basis of a Ct value below 30.0. Only the signals detected within these Ct cutoff values are reported.

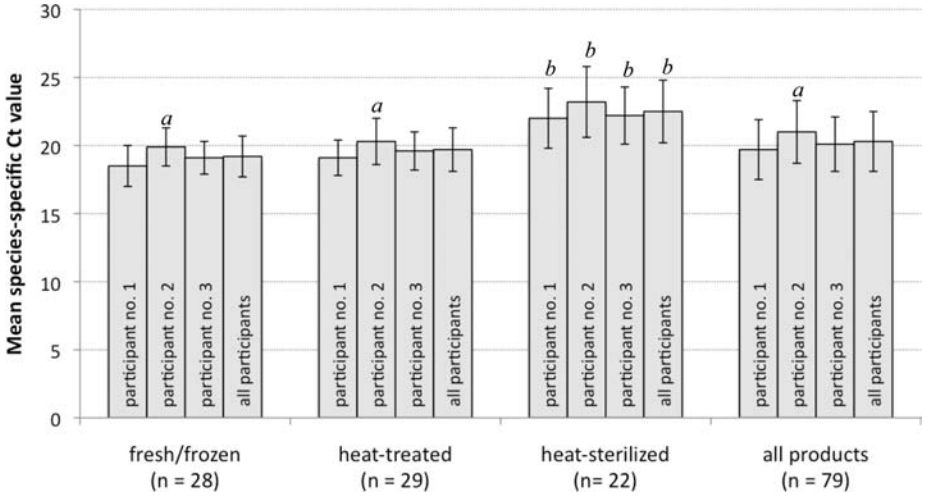

Figure 1. Comparison of real-time multiplex PCR results for species-specific assays according to processing group (fresh/frozen, heat-treated, or heat-sterilized) and study participant (1, 2, or 3). The species-specific Ct values among processing groups and study participants were statistically analyzed for the 79 products in which species were detected. Error bars represent the standard deviation within each group. *a*, Mean species-specific Ct value is significantly different ($p < 0.05$) from the mean species-specific Ct value of participant 1 for the same processing group, according to ANOVA, Tukey's HSD test ($p < 0.05$). *b*, Mean Ct value of the heat-sterilized group is significantly different ($p < 0.05$) from the mean Ct values obtained by the same study participant for other processing groups, according to ANOVA, Tukey's HSD test ($p < 0.05$).

The one product that could not be identified was hot-smoked and retort-packed Alaskan salmon (sample 77). None of the three study participants was able to obtain a Ct value below 30 for this product with any of the species-specific assays, and the universal Ct value for this product was also above 30, indicating that the sample was heavily degraded. All three study participants detected two cases of species substitution (samples 30 and 80) and two cases of mixed-species samples (samples 34 and 66). These products are discussed in detail in later sections. The majority (75%) of fresh/frozen products labeled "*O. keta* or *O. gorbuscha*" was identified as *O. gorbuscha*, and the remaining products were identified as *O. keta*. There were 10 fresh/frozen or heat-treated products purchased in the United States that were labeled "salmon" with no species declaration, and testing with real-time multiplex PCR indicated that 80% contained either *O. keta* or *O. gorbuscha*. *O. keta* and *O. gorbuscha* have the lowest values among the commercial salmon species, with average 2008 ex-vessel prices of U.S. $1.17 and U.S. $0.64 per kg, respectively (*1*), and it is not surprising that they are the main species present in generically labeled salmon products.

When the results among study participants were compared, the means of the species-specific Ct values obtained for all commercial products were within about 1 cycle of each other, ranging from 19.7 ± 2.2 (participant 1) to 21.0 ± 2.3 (participant 2) (**Figure 1**). Although the Ct values were in general agreement among the three laboratories, there was a significant difference ($p < 0.05$) between participants 1 and 2 when the species-specific results for fresh/frozen, heat-treated, and combined products were compared. This is likely due to slight variations in the real-time PCR platforms and settings used. However, these differences did not affect the ability of either laboratory to identify species in commercial products. Overall, the results were very similar among the three laboratories, despite the potential for variation during the DNA extraction and real-time PCR steps carried out

independently by each study participant. There were no significant differences among the mean species-specific Ct values reported by the three laboratories for the heat-sterilized group, due to the wide variation in detection levels for these samples. The means of the universal Ct values obtained for all commercial products by the three study participants were within 0.4 cycle of each other, ranging from 20.7 ± 3.2 (participant 1) to 21.0 ± 3.3 (participant 3) (results not shown). These values showed greater variability than the species-specific values, and there were no significant differences among the three laboratories for the universal Ct values found within any of the processing groups.

Effects of Processing on Real-Time PCR Signal Detection. As shown in **Figure 1**, all study participants reported significantly higher ($p < 0.05$) Ct values for the heat-sterilized products compared to the fresh/frozen and heat-treated products. The combined mean species-specific Ct value for the heat-sterilized products was 22.5 ± 2.3, compared to 19.2 ± 1.5 and 19.7 ± 1.6 for the fresh/frozen and heat-treated products, respectively. The combined mean Ct values for the universal assay were also significantly higher ($p < 0.05$) in the heat-sterilized group (24.6 ± 3.8) compared to the fresh/frozen (19.1 ± 1.3) and heat-treated (19.7 ± 1.4) groupings (results not shown). Elevated Ct values are generally expected with heavily processed products due to the high level of DNA degradation that takes place during heat sterilization. Previous studies have also reported Ct values of heat-sterilized products to be 3−4 cycles higher than those of raw or lightly processed samples (*25*, *33*, *34*). Despite the later signals for the heat-sterilized products, 22 of the 23 samples tested were successfully identified by all study participants on the basis of the previously suggested species-specific Ct cutoff value of <30. These products showed wide variations in DNA quality and signal detection, with Ct values ranging from 19.0 to 29.8 for species-specific assays. Most universal Ct values obtained with these 22 products were also within the 20−30 cycle range; however, universal Ct values above 30 were recorded by one or more of the study participants for samples 80 (smoked and canned Alaskan salmon), 62 (canned Pacific salmon Thai salad), and 78 (hot-smoked and retort-packed salmon).

In the case of the heat-sterilized group, the combined mean Ct values of the species-specific (22.5 ± 2.3) and universal (24.6 ± 3.8) assays were significantly different from each other ($p < 0.05$, paired samples t test), but there was no significant difference between these two assays for the other two processing groups. The universal set previously showed decreased efficiency and sensitivity compared to the species-specific assays, likely due to the multiple degenerate sites found within the universal primers and probe (*25*). Furthermore, the universal target DNA fragment is relatively long (205 bp) compared to most of the species-specific assays, with which six of the seven target DNA fragments are <200 bp in length, and it is therefore more sensitive to the effects of DNA degradation. Overall, the results indicate that the previously suggested species-specific cutoff values of Ct < 30 for species identification in heat-sterilized products and Ct < 25 in fresh and lightly processed products enable species identification in most cases (98.8%).

Species Substitution. Species substitution was detected in 2 of the 79 samples (2.5%) that could be identified at the species level. One product was a cold-smoked item sold at a seafood counter (sample 30) that did not have a species label but was verbally declared to be farmed *O. tshawytscha*. This product was determined to be *S. salar* by all three laboratories, on the basis of the results of real-time multiplex PCR. The other substituted product (sample 80) was a smoked and canned item labeled *O. kisutch* that was determined to be *O. keta* by all three laboratories. Both of these cases represent substitution of a lower value species for a higher value species. For example, in 2008 the average ex-vessel price for *O. tshawytscha* was U.S. $7.19/kg compared to an average import price for *S. salar* of U.S. $5.57/kg, and the average ex-vessel price of *O. kisutch* (U.S. $2.66/kg) was more than twice that for *O. keta* (*1*, *35*). Additional testing of these products with DNA barcoding and PCR-RFLP confirmed the species diagnosis of *S. salar* for sample 30, with a 100% barcode sequence identity match. However, sample 80 was a heavily processed product that contained highly degraded DNA and/or PCR inhibitors (universal Ct > 30) and could not be identified with DNA barcoding or PCR-RFLP. As a follow-up, three additional cans of this product were also collected from the same retail store for testing with real-time multiplex PCR. These products were also found to be heavily degraded and/or contained additional PCR inhibitors due to smoking (universal Ct > 30) and did not show positive signals for any species below 30 cycles. The lowest signals observed in these products were for *O. kisutch*, the species listed on the label, with Ct values ranging from 30.9 to 34.95, but it should be noted that these additional cans were from a different lot than sample 80.

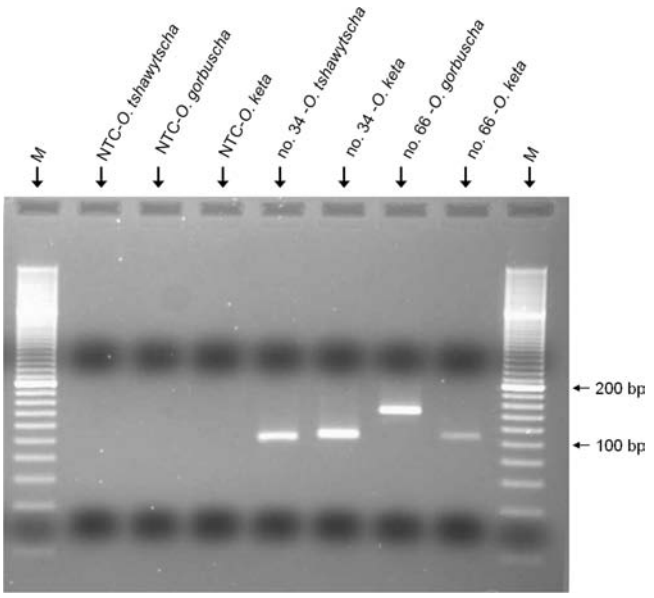

Figure 2. 3% agarose gel showing the results of conventional species-specific PCR for samples 34 and 66. Sample 34 was tested in singleplex reactions with the *O. tshawytscha* assay (103 bp) and the *O. keta* assay (104 bp). Sample 66 was tested in singleplex reactions with the *O. gorbuscha* assay (143 bp) and the *O. keta* assay. All species-specific assays included a nontemplate control (NTC), and the gel was run with a 20 bp molecular ruler (M).

Mixed-Species Samples. Two products were found to contain multiple species on the basis of the real-time multiplex PCR results of all three study participants. Sample 34, a cold-smoked product labeled "salmon", was found to contain *O. keta* as the primary species and *O. tshawytscha* as the secondary species, whereas sample 66, a canned product labeled *O. gorbuscha*, was found to contain *O. gorbuscha* as the primary species and *O. keta* as the secondary species. In a verbal communication with the salmon production department for sample 34, it was revealed that this product primarily contains *O. keta* and occasionally contains *O. tshawytscha*. Therefore, it is possible that *O. tshawytscha* was detected as the secondary species in real-time PCR as a result of processing operations at the facility. Additional testing of these two products with DNA barcoding and PCR-RFLP confirmed the primary species in both cases, with 100% barcode sequence identity matches. The results of PCR-RFLP also showed a very faint secondary band pattern in an agarose gel corresponding to the secondary species (*O. tshawytscha*) in sample 34; however,

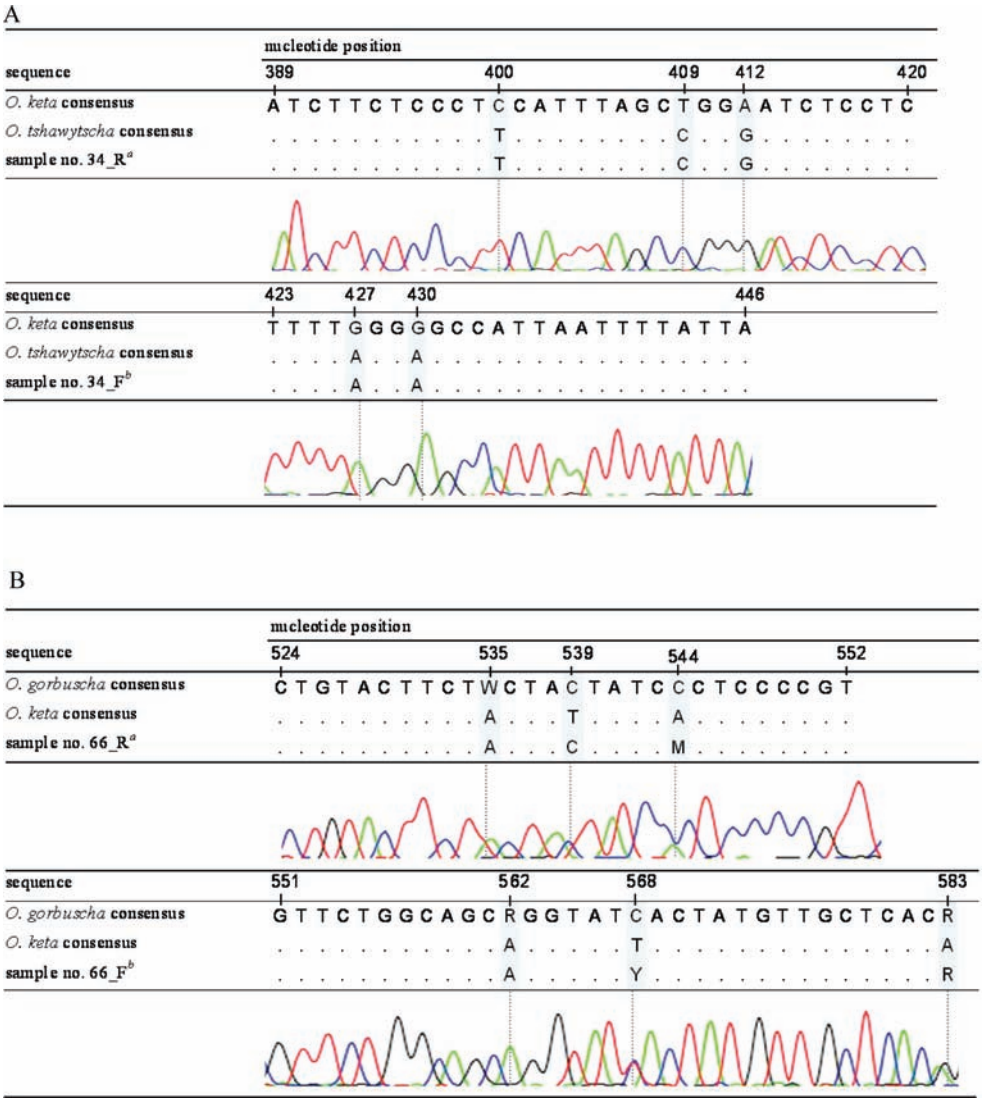

Figure 3. Diagnostic nucleotide analysis showing consensus sequences from over 350 reference samples aligned with the base calls and trace file peaks obtained for (**A**) sample 34, sequenced with *O. tshawytscha*-specific primers, and (**B**) sample 66, sequenced with *O. keta*-specific primers. Sequence fragments shown do not include the primer-binding regions. The nucleotide position is relative to the 5′-end of the 652 bp DNA barcode, and the shaded regions indicate diagnostic nucleotide sites. [a] Sequence obtained with reverse primer. [b] Sequence obtained with forward primer. A, adenine, green; T, thymine, red; C, cytosine, blue; G, guanine, black; W, A or T; Y, C or T; M, A or C; R, A or G.

no secondary band pattern was observed for sample 66. The DNA barcode sequences obtained for these products did not show any unexpected nucleotide substitutions in the primer or probe binding regions that would have led to cross-reactivity.

These two products were further investigated with conventional species-specific PCR targeting both the primary and secondary species (*25*) and DNA sequencing of the PCR amplicons of the secondary species. The results of conventional species-specific PCR showed the presence of both the primary and secondary species detected during real-time PCR for both products (**Figure 2**). Due to the short lengths of the amplicons for the secondary species (103−104 bp), DNA sequencing resulted in read lengths of only 24−33 nucleotides (nt) after the forward and reverse primers were removed. Identification with the BOLD identification system was not possible using these short sequences, and instead the sequences were analyzed on the basis of diagnostic nucleotides (*30*, *31*) (**Figure 3**). The forward (24 nt) and reverse (32 nt) sequences recovered from sample 34 contained five diagnostic sites that differed between *O. tshawytscha* and *O. keta* (**Figure 3A**). The nucleotides occurring at each of these sites corresponded to those diagnostic for *O. tshawytscha*, supporting the results of real-time PCR. The forward (33 nt) and reverse (28 nt) sequences recovered from sample 66 overlapped at nucleotide positions 551 and 552 to form a complete fragment, representing all nucleotide positions between the forward and reverse primer-binding sites (**Figure 3B**). Within this region, there were six potentially diagnostic sites that could have allowed for differentiation of *O. keta* and *O. gorbuscha*. However, at three of these sites (positions 544, 568, and 583) the sequencing trace file contained two overlapping peaks corresponding to the nucleotides present in these two species. At two of the diagnostic sites (535 and 539) a base call was made corresponding to nucleotides in one (position 539) or both (position 535) species, but a neighboring peak for thymine (T) showed an extended slant in both cases, indicating a possible overlapping peak. The remaining diagnostic site (562) contained an adenine (A) base, which has been observed at this site in both *O. keta* and *O. gorbuscha*. Overall, these results support the real-time PCR diagnosis of a mixed-species sample, but do not provide conclusive evidence regarding the species profile of this product.

Delayed Secondary Signals. Secondary signals of Ct = 25−30 for fresh/frozen and heat-treated products were initially recorded for 15 samples, with only 1 of these samples exhibiting a secondary signal in multiple laboratories. Although the cutoff value for fresh/frozen and heat-treated samples was Ct < 25, secondary signals below 30 were not reported previously (*25*) and may be indicative of a low level of secondary species or cross-contamination. Following additional testing with real-time PCR for all 15 samples, the average Ct values were over 30 for 6 of the samples and another 5 samples were found to have DNA contamination. However, there were 4 samples (5, 18, 19, and 44) that exhibited recurring signals at Ct = 28.4−29.9 specific for *O. nerka*, even after repeat DNA extraction and real-time PCR, indicating that the tissue samples for these products may have been cross-contaminated during either processing or subsampling.

The 4 DNA samples that exhibited recurring secondary signals in real-time PCR and the 5 contaminated DNA samples were tested further with PCR-RFLP and DNA barcoding. Both tests confirmed the primary species in all cases, with barcode sequence identity matches of 99.5−100%, but a secondary species was not detected. There were no unexpected nucleotide substitutions that might have led to increased cross-reactivity in the primer and probe-binding regions of the DNA barcode sequences for these samples. Although no secondary species were observed in the PCR-RFLP test, the band patterns produced during this assay were faint even for the primary species, and a secondary species would have been difficult to detect at low levels. Overall, the results indicate that the secondary signals at Ct = 25−30 were either caused by nonspecific amplification or a low level of secondary species present at levels undetectable by other methods. Previous testing of the real-time PCR method with DNA admixtures of reference samples showed detection limits of 0.1−10% for the target species when the cutoff value was Ct < 30 and detection limits of about 10−20% for a cutoff value of Ct < 25 (*25*). Taken together, these findings suggest that a cutoff value of Ct < 25 for fresh/frozen and heat-treated samples is appropriate, allowing for species identifications that are consistent with previously established methods while minimizing false-positive signals from trace contamination.

Conclusions. The real-time multiplex PCR assay tested here is a rapid, sensitive, and reliable method for identification of salmon and trout species in commercial products. Test results among all three laboratories revealed the ability of this assay to identify species in a range of product types, including 96% of heat-sterilized products tested. The results of this study are also promising with regard to the surprisingly low level of species substitution detected in salmon and trout products (2.5%). As demonstrated here, the assay is reproducible and robust and can be carried out using a variety of real-time PCR platforms either in a single-tube format or in 96-well plates. Furthermore, the method is relatively inexpensive, with an average price per sample of U.S. $7−9 after DNA extraction, and it does not require time-consuming post-PCR processing steps. This method may be applied in regulatory settings as a rapid and high-throughput screening tool for testing commercial salmon and trout products. This study illustrates the high potential to make use of the large volume of reference sequences in the Barcode of Life database for the development of real-time PCR assays targeting commonly substituted seafood species.

ABBREVIATIONS USED

BOLD, Barcode of Life Database; COI, cytochrome *c* oxidase subunit I; Ct, cycle threshold; PCR, polymerase chain reaction; USFDA, U.S. Food and Drug Administration.

ACKNOWLEDGMENT

We thank the Oregon Department of Fish and Wildlife, Alaska Department of Fish and Game Gene Conservation Laboratory, and Salmon of the Americas for donating reference samples. We also thank Caprice Rosato and the Oregon State University Center for Genomics Research and Bioinformatics core laboratory for support and use of the Applied Biosystems 7500 real-time detection system.

LITERATURE CITED

(1) Voorhees, D. V. *Fisheries of the United States, 2008*; National Marine Fisheries Service, Office of Science and Technology: Silver Spring, MD, 2009.

(2) DFO. Fisheries and Oceans Canada; http://www.dfo-mpo.gc.ca (accessed Dec 1, 2009).

(3) Knapp, G.; Roheim, C.; Anderson, J. *The Great Salmon Run: Competition Between Wild and Farmed Salmon*; World Wildlife Fund: Washington, DC, 2007.

(4) USFDA. The Seafood List − FDA's guide to acceptable market names for seafood sold in interstate commerce; http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/Seafood/ucm113260.htm (accessed May 2010).

(5) U.S. Government Accountability Office. Seafood Fraud: FDA program changes and better collaboration among key federal agencies could improve detection and prevention. GAO-09-258, a report to the Ranking Member, Subcommittee on Oceans, Atmosphere, Fisheries, and Coast Guard, Committee on Commerce Science, and Transportation, U.S. Senate, **2009**.

(6) Tennyson, J. M.; Winters, K. S.; Powell, K. A fish by any other name: a report on species substitution. *Proceedings of the 22nd Annual Meeting of Seafood Science the Technology Society of the Americas*, Biloxi, MS, Oct 6−7, **1997**.

(7) Wong, E. H. K.; Hanner, R. H. DNA barcoding detects market substitution in North American seafood. *Food Res. Int.* **2008**, *41*, 828−837.

(8) USFDA. *Regulatory Fish Encyclopedia*; http://www.fda.gov/Food/FoodSafety/Product-SpecificInformation/Seafood/RegulatoryFish-EncyclopediaRFE/default.htm (accessed May 2010).

(9) Ludwig, A. Identification of *Acipenseriformes* species in trade. *J. Appl. Ichthyol.* **2008**, *24* (Suppl. 1), 2−19.

(10) Mackie, I. M.; Craig, A.; Etienne, M.; Jérôme, M.; Fleurence, J.; Jessen, F.; Smelt, A.; Kruijt, A.; Malmheden Yman, I.; Ferm, M.; Martinez, I.; Pérez-Martín, R.; Piñeiro, C.; Rehbein, H.; Kündiger, R. Species identification of smoked and gravad fish products by sodium dodecylsulphate polyacrylamide gel electrophoresis, urea isoelectric focusing and native isoelectric focusing: a collaborative study. *Food Chem.* **2000**, *71*, 1−7.

(11) Rasmussen, R. S.; Morrissey, M. T. DNA-based methods for the identification of commercial fish and seafood species. *Compr. Rev. Food Sci. Food Saf.* **2008**, *7*, 280−295.

(12) Mackie, I. M.; Pryde, S. E.; Gonzales-Sotelo, C.; Medina, I.; Perez-Martin, R. I.; Quinteiro, J.; Rey-Mendez, M.; Rehbein, H. Challenges in the identification of species of canned fish. *Trends Food Sci. Technol.* **1999**, *10*, 9−14.

(13) Yancy, H. F.; Zemlak, T. S.; Mason, J. A.; Washington, J. D.; Tenge, B. J.; Nguyen, N. T.; Barnett, J. D.; Savary, W. E.; Hill, W. E.; Moore, M. M.; Fry, F. S.; Randolph, S. C.; Rogers, P. L.; Hebert, P. D. N. Potential use of DNA barcodes in regulatory science: applications of the *Regulatory Fish Encyclopedia*. *J. Food Prot.* **2008**, *71*, 456−458.

(14) Cohen, N. J.; Deeds, J. R.; Wong, E. S.; Hanner, R. H.; Yancy, H. F.; White, K. D.; Thompson, T. M.; Wahl, M.; Pham, T.; Guichard, F. M.; Huh, I.; Austin, C.; Dizikes, G.; Gerber, S. I. Public health response to puffer fish (tetrodotoxin) poisoning from mislabeled product. *J. Food Prot.* **2009**, *72*, 810−817.

(15) Hebert, P. D. N.; Cywinska, A.; Ball, S. L.; deWaard, J. R. Biological identifications through DNA barcodes. *Proc. Biol. Sci.* **2003**, *270*, 313−321.

(16) Hebert, P. D. N.; Ratnasingham, S.; deWaard, J. R. Barcoding animal life: cytochrome *c* oxidase subunit 1 divergences among closely related species. *Proc. R. Soc. London B* **2003**, *270*, S96−S99.

(17) Rasmussen, R. S.; Morrissey, M. T.; Hebert, P. D. N. DNA barcoding of commercially important salmon and trout species (*Oncorhynchus* and *Salmo*) from North America. *J. Agric. Food Chem.* **2009**, *57*, 8379−8385.
(18) Hubert, N.; Hanner, R.; Holm, E.; Mandrak, N. E.; Taylor, E.; Burridge, M.; Watkinson, D.; Dumont, P.; Curry, A.; Bentzen, P.; Zhang, J.; April, J.; Bernatchez, L. Identifying Canadian freshwater fishes through DNA barcodes. *PLoS ONE* **2008**, *3*, e2490.
(19) Steinke, D.; Zemlak, T. S.; Gavin, H.; Hebert, P. D. N. DNA barcoding of Pacific Canada's fishes. *Mar. Biol.* **2009**, *156*, 2641−2647.
(20) Taylor, M. I.; Fox, C.; Rico, I.; Rico, C. Species-specific TaqMan probes for simultaneous identification of (*Gadus morhua* L.), haddock (*Melanogrammus aeglefinus* L.) and whiting (*Merlangius merlangus* L.). *Mol. Ecol. Notes* **2002**, *2*, 599−601.
(21) Itoi, S.; Nakaya, M.; Kaneko, G.; Kondo, H.; Sezaki, K.; Watabe, S. Rapid identification of eels *Anguilla japonica* and *Anguilla anguilla* by polymerase chain reaction with single nucleotide polymorphism-based specific probes. *Fish. Sci.* **2005**, *71*, 1356−1364.
(22) Lopez, I.; Pardo, M. A. Application of relative quantification TaqMan real-time polymerase chain reaction technology for the identification and quantification of *Thunnus alalunga* and *Thunnus albacares*. *J. Agric. Food Chem.* **2005**, *53*, 4554−4560.
(23) Herrero, B.; Madriñán, M.; Vieites, J. M.; Espiñeira, M. Authentication of Atlantic cod (*Gadus morhua*) using real time PCR. *J. Agric. Food Chem.* **2010**, *58*, 4794−4799.
(24) Bayha, K. M.; Graham, W. M.; Hernandez, F. J. Multiplex assay to identify eggs of three fish species from the northern Gulf of Mexico, using locked nucleic acid Taqman real-time PCR probes. *Aquat. Biol.* **2008**, *4*, 65−73.
(25) Rasmussen Hellberg, R. S.; Morrissey, M. T.; Hanner, R. H. A multiplex PCR method for the identification of commercially important salmon and trout species (*Oncorhynchus* and *Salmo*) in North America. *J. Food Sci.* **2010**, *75*, C595−C606.
(26) Smith, S.; Hons, B. S.; Morin, P. A. M. Optimal storage conditions for highly dilute DNA samples: a role for trehalose as a preserving agent. *J. Forensic Sci.* **2005**, *50*, 1101−1108.
(27) Smith, J. S. Evaluation of analytical data. In *Food Analysis*, 3rd ed.; Nielsen, S. S., Ed.; Kluwer Academic/Plenum Publishers: New York, 2003; pp 51−63.
(28) Espiñeira, M.; Vieites, J. M.; Santaclara, F. J. Development of a genetic method for the identification of salmon, trout, and bream in seafood products by means of PCR-RFLP and FINS methodologies. *Eur. Food Res. Technol.* **2009**, *229*, 785−793.
(29) Tamura, K.; Dudley, J.; Nei, M.; Kumar, S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **2007**, *24*, 1596−1599.
(30) Wong, E. H. K.; Shivji, M. S.; Hanner, R. H. Identifying sharks with DNA barcodes: assessing the utility of a nucleotide diagnostic approach. *Mol. Ecol. Resour.* **2009**, *9*, 243−256.
(31) Lowenstein, J. H.; Amato, G.; Kolokotronis, S. O. The real *maccoyii*: identifying tuna sushi with DNA barcodes − contrasting characteristic attributes and genetic distances. *PLoS ONE* **2009**, *4*, e7866.
(32) Logan, J. M. J.; Edwards, K. J. An overview of real-time PCR platforms. In *Real-Time PCR: An Essential Guide*; Edwards, K. J., Logan, J. M. J., Saunders, N., Eds.; Horizon Bioscience: Wymondham, U.K., 2004; pp 13−29.
(33) Brodman, P. D.; Moor, D. Sensitive and semi-quantitative TaqMan real-time polymerase chain reaction systems for the detection of beef (*Bos taurus*) and the detection of the family Mammalia in food and feed. *Meat Sci.* **2003**, *65*, 599−607.
(34) Hird, H. J.; Hold, G. L.; Chisholm, J.; Reece, P.; Russell, V. J.; Brown, J.; Goodier, R.; MacArthur, R. Development of a method for the quantification of haddock (*Melanogrammus aeglefinus*) in commercial products using real-time PCR. *Eur. Food Res. Technol.* **2005**, *220*, 633−637.
(35) NMFS. *NOAA Fisheries: Office of Science and Technology*. Imports and exports of fishery products annual summary, 2009. Current Fisheries Statistics No. 2009-2; http://www.st.nmfs.noaa.gov/st1/publications.html (accessed May 2010).

Received for review August 20, 2010. Revised manuscript received December 1, 2010. Accepted December 14, 2010. This project was funded by the Oregon Innovation Council. We also acknowledge support for A.M.N. and R.H.H. from the Advanced Foods and Materials Network (AFMNet).