A Multiplex PCR Method for the Identification of Commercially Important Salmon and Trout Species (*Oncorhynchus* and *Salmo*) in North America

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Abstract: The purpose of this study was to develop a species-specific multiplex polymerase chain reaction (PCR) method that allows for the detection of salmon species substitution on the commercial market. Species-specific primers and TaqMan® probes were developed based on a comprehensive collection of mitochondrial 5′ cytochrome *c* oxidase subunit I (COI) deoxyribonucleic acid (DNA) "barcode" sequences. Primers and probes were combined into multiplex assays and tested for specificity against 112 reference samples representing 25 species. Sensitivity and linearity tests were conducted using 10-fold serial dilutions of target DNA (single-species samples) and DNA admixtures containing the target species at levels of 10%, 1.0%, and 0.1% mixed with a secondary species. The specificity tests showed positive signals for the target DNA in both real-time and conventional PCR systems. Nonspecific amplification in both systems was minimal; however, false positives were detected at low levels (1.2% to 8.3%) in conventional PCR. Detection levels were similar for admixtures and single-species samples based on a 30 PCR cycle cut-off, with limits of 0.25 to 2.5 ng (1% to 10%) in conventional PCR and 0.05 to 5.0 ng (0.1% to 10%) in real-time PCR. A small-scale test with food samples showed promising results, with species identification possible even in heavily processed food items. Overall, this study presents a rapid, specific, and sensitive method for salmon species identification that can be applied to mixed-species and heavily processed samples in either conventional or real-time PCR formats.

Keywords: multiplex PCR, real-time PCR, salmon, species identification, trout

Practical Application: This study provides a newly developed method for salmon and trout species identification that will assist both industry and regulatory agencies in the detection and prevention of species substitution. This multiplex PCR method allows for rapid, high-throughput species identification even in heavily processed and mixed-species samples. An inter-laboratory study is currently being carried out to assess the ability of this method to identify species in a variety of commercial salmon and trout products.

Introduction

The North American commercial salmon and trout industry includes 7 species from the genera *Oncorhynchus* and *Salmo*: chum salmon (*O. keta*), Chinook salmon (*O. tshawytscha*), pink salmon (*O. gorbuscha*), rainbow (steelhead) trout (*O. mykiss*), sockeye salmon (*O. nerka*), coho salmon (*O. kisutch*), and Atlantic salmon (*S. salar*). These species are similar in appearance but command dramatically different prices, making them susceptible to market substitution (Knapp and others 2007; USFDA 2009). Previous DNA-based methods for the identification of salmon and trout species have relied on multiple post-polymerase chain reaction (PCR) steps, such as restriction fragment length polymorphism (RFLP) analysis (McKay and others 1997; Withler and others 1997; Russell and others 2000; Horstkotte and Rehbein 2003; Purcell and others 2004; Espiñeira and others 2009) or single-stranded conformational polymorphism (SSCP) analysis (Rehbein 2005). While these methods are useful, they do exhibit several disadvantages for application in the food industry, where speed of analysis is of critical importance (Rasmussen and Morrissey 2008). Furthermore, most studies have utilized DNA targets that are too long to be recovered from canned products, where fragments are typically degraded to ≤ 300 to 350 bp (Pardo and Perez-Villareal 2004; Chapela and others 2007; Hsieh and others 2007).

A species-specific multiplex PCR method for salmon and trout species identification would have several advantages over previous methods. This method allows for the simultaneous and rapid identification of multiple species in a single PCR tube, including mixed-species and heavily processed samples, and can be applied to both conventional and real-time PCR (Rasmussen and Morrissey 2008). Multiplex PCR assays have been successfully developed for the authentication of numerous fish species (Rocha-Olivares

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1998; Pank and others 2001; Taylor and others 2002; Trotta and others 2005; Michelini and others 2007; Rocha-Olivares and Chávez-González 2008; Mendonça and others 2009), including 3 salmonids (*O. mykiss*, *O. tshawytscha*, and *O. kisutch*) (Greig and others 2002). While the salmonid assay showed promising results, it did not consider identification of the 4 additional commercial salmon species in North America and it targeted nuclear DNA (nDNA). For species identification in food systems, mitochondrial DNA (mtDNA) has been reported to be preferable over nDNA, in part because it exhibits a high copy number and is easier to extract from processed samples (Civera 2003; Rasmussen and Morrissey 2008).

The mtDNA gene coding for cytochrome *c* oxidase subunit I (COI) is a good candidate for species identification because it exhibits substantial divergence between species, but is relatively conserved within species (Hebert and others 2003). A comprehensive reference sequence library is currently being assembled for a 650 bp region of this gene (the "DNA barcode") for all fish species under the Fish Barcode of Life campaign (Ward and others 2009), and while the reference library is only partially complete, it has already demonstrated broad utility for detecting market substitution in North American seafood (Wong and Hanner 2008). A thorough investigation of DNA barcode sequences ($n = 865$) from commercially important salmon and trout species in North America has demonstrated that each species possesses a unique cluster of closely related barcode haplotypes that do not overlap with any of their congeners (Rasmussen and others 2009). This compilation of DNA barcode sequence information has provided an excellent platform for the design of a robust, species-specific multiplex PCR assay, which would facilitate species identification of commercial salmon products in a more timely and cost-effective manner than probing their barcode sequences through traditional Sanger sequencing methods. Perhaps more importantly, this approach also facilitates the detection of species mixtures, which is a challenge for sequence-based approaches to detection unless a costly and time-consuming cloning step is included.

The objective of this study was to develop a species-specific multiplex PCR method based on COI DNA barcode sequences for the identification of the 7 commercially important salmon and trout species (genera *Oncorhynchus* and *Salmo*) in North America. The assay was developed for use in either conventional PCR with gel electrophoresis or real-time PCR with species-specific fluorescent probes.

Materials and Methods

Multiplex PCR assay design and optimization

Species-specific primers and TaqMan® (Roche Molecular Systems, Inc., Pleasanton, Calif., U.S.A.) minor groove binder (MGB™) probes (Epoch Biosciences, Bothell, Wash., U.S.A.) were developed for *S. salar, O. keta, O. tshawytscha, O. gorbuscha, O. mykiss, O. nerka*, and *O. kisutch* based on 915 COI DNA barcode sequences (Table 1). The sequences were derived from specimens representing a wide geographic range in North America, including Canada (British Columbia, Quebec, and Ontario), the United States (California, Oregon, Washington, Alaska, and Idaho), and Chile. Sequences for the following background salmonid species were also screened against all primers and probes to ensure specificity: *Oncorhynchus clarkii*, *Oncorhynchus masou*, *Salmo trutta*, *Salvelinus alpinus*, *Salvelinus confluentus*, *Salvelinus fontinalis*, *Salvelinus malma*, and *Salvelinus namaycush* (Table 1). In addition to the species-specific primers and probes, a set of universal primers and probe was developed as a PCR amplification control. The universal set was designed based on mitochondrial cytochrome *b* nucleotide sequences ($n = 46$) for the 7 target species (Table 1). All sequences were collapsed into representative haplotypes based on sequence identity matrices generated in BioEdit version 7.0.9.0 (Hall 1999) for primer and probe development.

Primers and probes were designed using AlleleID 7.0 (Premier Biosoft Intl., Palo Alto, Calif., U.S.A.) and further modifications were carried out by the authors based on laboratory results. Premier Biosoft's online tools NetPrimer and Beacon Designer were also utilized to assess primer characteristics and multiplexing

Table 1– GenBank accession numbers for all sequences used to design PCR primers and probes in this study. Species-specific primers and probes were designed for all target species based on diagnostic nucleotides within the COI DNA barcode sequence and a set of universal primers and probes was designed for all target species based on conserved nucleotides within the cytochrome *b* gene.

Assay design	Gene target	Sequences (*n*)	Species[ab]	GenBank accession numbers	References
Universal	Cytochrome *b*	46	Target	AF312563, AF312574, AF165077-AF165079, AF165083, AF202032, AJ314561-AJ314564, AJ314566-AJ314568, AY032629-AY032632, DQ449932-DQ449933, DQ449936, L29771, AB049024, AF053591, AF125208-AF125209, AF125212, AF133701, AF172395, AF392054, AY587167-AY587172, BT044011, D58401, EF055889, EF077658, EF105341, EF126369, EF455489, EU492280-EU492281, U12143	(Zardoya and others 1995; Phillips and others 2000; Russell and others 2000; Wolf and others 2000; Brown and Thorgaard 2002; Docker and Heath 2003; Kyle and Wilson 2007)
Species-specific	COI DNA barcode	915	Target	FJ998665-FJ998742, FJ998744-FJ998759, FJ998761-FJ999106, FJ999108-FJ999276, FJ999279-FJ999493, FJ999495-FJ999507, FJ999509-FJ999526, FJ999530-FJ999539, FJ164927-FJ164936, EU524202-EU524234, EU524349-EU524353, EU525056-EU525057.	(Hubert and others 2008; Rasmussen and others 2009; Steinke and others 2009)
Species-specific (background screening)	COI DNA barcode	120	Background	FJ998606-FJ998664, EU522398-EU522425, EU524190-EU524201, EU524354-EU524367, DQ533707, DQ642056, DQ656543, DQ858456, DQ864464-DQ864465	(Hubert and others 2008; Rasmussen and others 2009)

[a]Target species: *S. salar, O. keta, O. tshawytscha, O. gorbuscha, O. mykiss, O. nerka, and O. kisutch*.
[b]Background species: *O. clarkii, O. masou, S. trutta, S. alpinus, S. confluentus, S. fontinalis, S. malma, and S. namaycush*.

capabilities. Primers were designed so that the species-specific point mutation(s) was as close to the 3′-end as possible. TaqMan probes were designed based on guidelines provided by Premier Biosoft Intl. and Applied Biosystems, Inc. (Foster City, Calif., U.S.A.). An MGB group was conjugated to the 3′-end of each probe to improve specificity and increase melting temperatures (Kutyavin and others 2000). Conventional PCR products within each multiplex set were designed to have at least a 30 bp difference in size to allow for species diagnosis with a 3% agarose gel (Henegariu and others 1997). In some cases, the primers used in conventional PCR were modified from the real-time primers to meet this requirement and to reduce cross-reactivity in multiplex sets. All PCR products were designed to be less than 250 bp to allow for species diagnosis in heavily processed products. All primers and probes were tested against the Basic Local Alignment Search Tool (BLAST) to ensure specificity in both singleplex and multiplex reactions. PCR assays were optimized for cycling conditions, primer and probe concentrations, and template DNA concentration, as outlined by Edwards and Logan (2009) and Henegariu and others (1997). Primer and probe sets were optimized in singleplex reactions before being combined into a multiplex format. The species-specific and universal primers and probes that were developed in this study, along with their optimized reaction concentrations, amplicon sizes, diagnostic nucleotide sites, and final multiplex tube assignments, are given in Table 2. In a few cases, the specificity of a real-time PCR primer had to be reduced as compared to the conventional PCR primer to allow for optimal probe location and design.

Table 2–Species-specific and universal PCR primers and probes developed for real-time and conventional PCR assays. Diagnostic nucleotide sites utilized in combination to provide specificity are underlined, and single nucleotide sites showing specificity against all target and background species are underlined and in boldface type.

Target species	PCR system	Primer/probe[a]	Primer/probe sequence (5′-3′)	Optimal concentration in PCR (μM)	Amplicon size (bp)	Multiplex set[b]
Universal set	Real-time	F	CCAGCACCHTCTAAYATYTCAGT	0.60	205 bp	U
		R	AAGAAAGATGCYCCGTTRGC	0.60		
		P	6FAM-CTDACATCTCGGCA-MGB	0.60		
	Conventional	F	Same as real-time	0.60	205 bp	GKU
		R	Same as real-time	0.60		
Atlantic salmon (*Salmo salar*)	Real-time	F	AGCAGAACTCAGCCAGCCT	0.10	214 bp	STKe
		R	AAAGGAGGGAGGGAGAAGTCAA	0.20		
		P	6FAM-CCTTCTGGGAGATGACC-MGB	0.14		
	Conventional	F	Same as real-time	0.13	219 bp	STN
		R	AGAAGAAAGGAGGGAGGGAGA	0.13		
Chum salmon (*Oncorhynchus keta*)	Real-time	F	TTGTCTGAGCTGTACTAATCACTG	0.20	104 bp	STKe
		R	AAGTGGTGTTTAAATTTCGATC	0.20		
		P	VIC-CAACATAGTAATACCTGCTG-MGB	0.10		
	Conventional	F	Same as real-time	0.15	104 bp	MKe
		R	Same as real-time	0.15		
Chinook salmon (*Oncorhynchus tshawytscha*)	Real-time	F	GATAGTAGGCACCGCCCTTAGT	0.20	183 bp	STKe
		R	CCGATCATTAGGGGAATTAATCAGT	0.20		
		P	NED-TCATAATCGGCATAACTAT-MGB	0.10		
	Conventional	F	GGAGCCTCAGTTGATCTRAC**G**	0.60	103 bp	STN
		R	GGGGTTTTATGTTAATAATGGT**A**G	0.60		
Pink salmon (*Oncorhynchus gorbuscha*)	Real-time	F	TACGACCATTATCAACATAAAACC**A**	0.30	143 bp	GM
		R	GGTCCGTGAGCAACATAGT**G**	0.20		
		P	6FAM-CGGC**A**ATCTCTCAGT-MGB	0.13		
	Conventional	F	Same as real-time	0.13	143 bp	GKU
		R	Same as real-time	0.13		
Rainbow (steelhead) trout (*Oncorhynchus mykiss*)	Real-time	F	ACCATTATTAACATAAAACCTCCAG	0.20	121 bp	GM
		R	GTAATGCCTGCTGCCAGGA	0.30		
		P	VIC-CGTTTGAGCCGTGCTA-MGB	0.13		
	Conventional	F	Same as real-time	0.20	73 bp	MKe
		R	TAACTAGCACGGCTCAAAC**G**	0.20		
Sockeye salmon (*Oncorhynchus nerka*)	Real-time	F	GGAAACCTTGCCCACGC**G**	0.20	152 bp	NK
		R	AAAAGTGGGGT**C**TGGTACTGAG	0.30		
		P	6FAM-CTCTGTTGACTTAACCATC-MGB	0.13		
	Conventional	F	CCAGCCATCTCTCAGTACCA**G**A	0.08	183 bp	STN
		R	GAGGTGTTGGTATAAAATCGGAT	0.08		
Coho salmon (*Oncorhynchus kisutch*)	Real-time	F	CGCTCTTCTAGGGGATGA**T**C	0.30	95 bp	NK
		R	CTCCGATCATAATCGGCATG	0.30		
		P	VIC-ATTTACAACGTAATCGTC-MGB	0.13		
	Conventional	F	Same as real-time	0.20	95 bp	GKU
		R	Same as real-time	0.20		

[a]F = forward primer; R = reverse primer; P = TaqMan MGB probe.
[b]Multiplex sets for real-time PCR: U = universal set/positive control; STKe = *S. salar*, *O. tshawytscha*, and *O. keta*; GM = *O. gorbuscha* and *O. mykiss*; and NK = *O. nerka* and *O. kisutch*; multiplex sets for conventional PCR: GKU = *O. gorbuscha*, *O. kisutch*, and universal set; STN = *S. salar*, *O. tshawytscha*, and *O. nerka*; and MKe = *O. mykiss* and *O. keta*.

Sample collection

Authenticated reference samples were collected for the salmonids *O. tshawytscha* ($n = 12$), *O. nerka* ($n = 10$), *O. kisutch* ($n = 10$), *O. keta* ($n = 10$), *O. gorbuscha* ($n = 10$), *O. mykiss* ($n = 11$), *S. salar* ($n = 10$), and *O. clarkii* ($n = 10$), and for the nonsalmonids *Hypsopsetta guttulata* ($n = 1$), *Psettichthys melanostictus* ($n = 1$), *Citharichthys sordidus* ($n = 1$), *Microstomus pacificus* ($n = 1$), *Parophrys vetulus* ($n = 1$), *Eopsetta jordani* ($n = 1$), *Sebastolobus alascanus* ($n = 1$), *Sebastes helvomaculatus* ($n = 1$), *Thunnus albacares* ($n = 1$), *Thunnus alalunga* ($n = 1$), and *Sardinops sagax* ($n = 1$). Samples were obtained from the following donors: Alaska Dept. of Fish and Game Gene Conservation Laboratory, American Gold Seafoods, Clear Springs Foods, Creative Salmon, Idaho Dept. of Fish and Game, Marine Fisheries Genetics Laboratory at Hatfield Marine Science Center (Oregon State Univ.), Marine Harvest Canada, Oregon Dept. of Fish and Wildlife, Pacific Seafood, Salmon of the Americas, Washington Dept. of Fish and Wildlife Molecular Genetics Lab, Pacific Salmon Treaty, and the Washington State General Fund. Samples were in the form of fin clips, axillary process clips, scales, heart tissue, muscle tissue, and liver tissue, and were stored frozen, preserved in ethanol, or dried. DNA extracts were also accessed from select background salmonid samples collected in a previous barcoding study (Hubert and others 2008): *S. trutta* ($n = 3$), *S. alpinus* ($n = 3$), *S. confluentus* ($n = 3$), *S. fontinalis* ($n = 3$), *S. malma* ($n = 3$), and *S. namaycush* ($n = 3$). In total, 112 reference samples were collected for use in this study, encompassing 25 species from multiple geographic locations in the United States (Alaska, Idaho, Oregon, and Washington), Canada (British Columbia, Quebec, and New Brunswick), and Chile. For small-scale testing with food samples, 1 fresh salmon fillet and 2 smoked salmon products were purchased from local retailers, and 3 canned salmon products were donated by the Seafood Products Assn.

DNA extraction and PCR preparation

DNA extraction was carried out with the DNeasy Blood and Tissue Kit (Qiagen, Valencia, Calif., U.S.A.). A reagent blank was included in each DNA extraction and subsequent PCR as a negative control. Nucleic acid concentrations were determined with a BioPhotometer plus (Eppendorf, Brinkman Instruments, Inc., Westbury, N.Y., U.S.A.) combined with either UVettes (Eppendorf) or a Hellma® Traycell (Hellma GmbH & Co. KG, Müllheim, Germany). Template DNA, primers, and probes were diluted for use in PCR using TE buffer containing 0.2 M trehalose as a preservation agent (Smith and others 2005). All DNA extracts were adjusted to 25 ng/μL and primers and probes were diluted to final PCR concentrations of 0.05 to 1.0 μM for optimization. TaqMan MGB probes and degenerate primers were purchased from Applied Biosystems and nondegenerate primers were purchased from TriLink Biotechnologies (San Diego, Calif., U.S.A.).

Conventional multiplex PCR

Conventional PCR primers were combined into 3 different multiplex sets: STN, containing primers targeting *S. salar*, *O. tshawytscha*, and *O. nerka*; MKe, containing primers targeting *O. mykiss* and *O. keta*; and GKU, containing primers targeting *O. gorbuscha* and *O. kisutch*, as well as the universal primer set. Multiplex PCR was carried out in 25 μL volumes containing 12.5 μL 2X Multiplex PCR Master Mix (Qiagen), 0.08 to 0.60 μM final concentration of primers (Table 2), 1 μL template DNA (25 ng/μL), and sterile water, under the following optimized PCR cycling conditions: 95 °C for 15 min to activate the HotStarTaq DNA polymerase, followed by 30 cycles of 94 °C for 30 s, 63 °C for 60 s, and 72 °C for 90 s, with a final extension step of 72 °C for 10 min on a MyCycler™ Thermal Cycler (Bio-Rad Laboratories, Hercules, Calif., U.S.A.). All reactions included a nontemplate control (NTC) and reagent blanks from DNA extraction. PCR products were analyzed with 10 μL loading volumes in 3% NuSieve 3:1 agarose gels (Lonza Group Ltd., Basel, Switzerland) containing 0.5 μg/mL ethidium bromide and run at 140 volts for 50 min. The results were scanned and visualized using GelDoc™ XR and Quantity One® Software (version 4.5.2, Bio-Rad Laboratories, 2004). Each food sample was tested against all 3 multiplex sets and species were identified based on the occurrence of a species-specific band in the agarose gel.

Real-time multiplex PCR

Species-specific primers and probes were combined into the following multiplex sets for real-time PCR: STKe, targeting *S. salar*, *O. tshawytscha*, and *O. keta*; GM, targeting *O. gorbuscha* and *O. mykiss*; and NK, targeting *O. nerka* and *O. kisutch*. The universal set (U) was run separately. Real-time multiplex PCR was carried out in 25 μL volumes containing 12.5 μL 2X QuantiTect Multiplex PCR NoROX Master Mix (Qiagen), 0.10 to 0.60 μM final concentration of primers (Table 2), 0.10 to 0.60 μM final concentration of TaqMan MGB probes (Table 2), 2 μL template DNA (25 ng/μL), and sterile water. PCR cycling conditions for species-specific multiplex sets involved an initial step at 15 min at 95 °C to activate the HotStarTaq DNA polymerase, followed by 40 cycles of 94 °C for 60 s and 63 °C for 60 s. The universal set performed optimally under the same conditions, except the annealing temperature (T_a) was lowered to 53 °C. Data collection for all samples took place at the annealing step of each cycle, and PCR was carried out for 40 cycles to quantify cross-reactivity with background species. All reactions included an NTC and reagent blanks from DNA extraction. Initial tests were carried out using a SmartCycler II (Cepheid, Sunnyvale, Calif., U.S.A.) with the default program settings (baseline 3 to 15 cycles; threshold 30 fluorescent units) and the fluorescent reporter dyes 6-carboxy-fluorescein (FAM™, Life Technology Corp., Carlsbad, Calif., U.S.A.) and tetrachloro-6-carboxyfluorescein (TET™, Life Technology Corp.). Finalized multiplex sets listed in Table 2 were tested for specificity, linearity, and sensitivity with a 7500 Real-Time PCR System (Applied Biosystems) using a baseline of 3 to 15 cycles and threshold settings of 4.0×10^4, 2.0×10^4, and 1.8×10^4 fluorescent units for probes containing the reporter dyes FAM, VIC® (Life Technology Corp.) and NED™ (Life Technology Corp.), respectively. The cycle threshold (Ct) value for each sample was determined based on the point at which the fluorescence generated within a reaction exceeded the threshold limit. Each food sample was tested with the 7500 Real-Time PCR System against all 3 multiplex sets and the universal control. Species in these samples were identified based on the occurrence of a Ct value below 25 (fresh or lightly processed samples) or 30 (canned samples) with a species-specific probe.

Specificity tests

All finalized multiplex sets were tested against 10 to 12 individuals from each of the target species, with a total of 73 samples representing 52 different DNA barcode haplotypes (3 to 11 haplotypes per species) previously identified (Rasmussen and others 2009). All multiplex sets were also screened against *O. clarkii* ($n = 10$) and 1 individual per species of the following nonsalmonids: *H. guttulata*, *P. melanostictus*, *C. sordidus*, *M. pacificus*, *P. vetulus*, *E. jordani*, *S. alascanus*, *S. helvomaculatus*, *T. albacares*, *T. alalunga*, and

S. sagax. All samples listed here are described under sample collection. Percent cross-reactivity was determined by comparing the Ct values obtained for the target DNA to Ct values obtained for background species, where a difference of 1 Ct represents a cross-reactivity of 50% (Dooley and others 2004). These values were corrected for PCR reaction efficiencies (E, described below), so that percent cross-reactivity = $100 * [(0.50 \div E)\wedge(Ct_{background} - Ct_{target})]$. During the initial specificity testing and prior to finalization of multiplex tube assignments, real-time PCR primers and probes were screened against the following salmonid species (3 individuals per species): S. *trutta*, *S. alpinus*, *S. confluentus*, *S. fontinalis*, *S. malma*, and *S. namaycush*. Because the results of these tests reflect only the specificity of individual primer-probe sets and do not show the potential for cross-reactivity in the finalized multiplex reactions, the results are discussed in the text, but not included in the data analysis for multiplex specificity tests (Table 3).

Sensitivity and linearity tests

Multiplex sets were tested for sensitivity and linearity according to a standard curve with 10-fold serial dilutions of the target DNA at 0.025 to 250 ng/μL. Ct values obtained for each dilution were then plotted against the logarithm of the template DNA amount (ng), and the slope (m) was calculated by linear regression. Reaction efficiency (E) for real-time PCR was then calculated as described in Raymaekers and others (2009), where $E = 10^{-1/m} - 1$. To provide a representative range of sensitivity and linearity levels, the universal set was tested against 3 target species (*S. salar*, *O. keta*, and *O. kisutch*) that showed a range of Ct values at 50 ng. DNA admixtures were prepared containing levels of 0%, 0.1%, 1%, and 10.0% of the target species mixed with a secondary species (Table 4) for a total DNA concentration of 25 ng/μL. Admixtures and standard curve dilutions were tested in triplicate using volumes of 2 μL DNA for real-time PCR and 1 μL DNA for conventional PCR. Real-time PCR Ct values for DNA admixtures and equivalent amounts of DNA in single-species samples were analyzed for significant differences using a paired-samples t-test, with a predetermined significance level of $P < 0.05$ (2-tailed). Statistical analysis was carried out with SPSS 13.0 for Windows software (SPSS Inc., Chicago, Ill., U.S.A.). Theoretical limits of detection for single-species samples using a cut-off of Ct < 30 were also calculated based on the average Ct values for 50 ng of target DNA and with the assumption that for every 50% reduction in DNA, there is a corresponding increase of 1 cycle in the Ct value (Dooley and others 2004). Percent reduction values were adjusted according to the PCR efficiencies calculated previously (that is, adjusted percent reduction = $100 * 0.50 \div E$).

Results and Discussion

As shown in Table 2, species-specific and universal primers and probes were developed for all 7 target species for application in both real-time and conventional PCR systems. Following singleplex optimization, primers and probes were combined into multiplex groupings targeting 2 to 3 species each (Table 2). These multiplex sets were modified for use in each PCR system to minimize nonspecific amplification and allow for amplicons of diagnosable sizes in conventional PCR.

Specificity tests

Real-time multiplex PCR results. As shown in Table 3 and Figure 1, all species-specific real-time PCR assays showed positive signals for the target species, with average Ct values ranging from 17.67 ± 1.15 (*O. mykiss* assay) to 21.02 ± 3.54 (*O. keta* assay). Average cross-reactivity with background species was extremely low, ranging from 0.0001% (Ct = 39.42 ± 1.66; *O. mykiss* assay) to 0.0007% (Ct = 39.05 ± 2.26; *O. keta* assay). The maximum cross-reactivity observed ranged from 0.0042% (Ct = 35.43) for an *O. keta* sample in the *O. gorbuscha* assay to 0.2723% (Ct = 30.00) for an *O. clarkii* sample in the *O. keta* assay. These values are

Table 3–Specificity of the real-time and conventional PCR assays with 50 and 25 ng template DNA, respectively. A Ct value of 40 was recorded if no amplification signal could be detected after 40 cycles. Percent cross-reactivity was calculated as explained in Dooley and others (2004), where a difference of 1 Ct represents a cross-reactivity of 50%, with corrections made for PCR efficiency.

			Real-time PCR results			Conventional PCR results	
Target species	Target individuals tested (*n*)	Background individuals tested for cross-reactivity (n)	Average Ct ± SD for target species	Average Ct ± SD for background species (% cross-reactivity)	Minimum Ct observed in background species (% cross-reactivity)	Target amplicon detected (% of target samples)	Cross-reactivity of background species (% of samples giving false signal)
Universal	83 (salmonids)	11 (non-salmonids)	20.23 ± 1.97	35.38 ± 5.56 (0.0151%)	23.48 with *C. sordidus* (15.14%)	100%	0%
S. salar	10	84	19.03 ± 0.77	39.76 ± 1.36 (0.0002%)	30.84 with *O. tshawytscha* (0.0623%)	100%	0%
O. keta	10	84	21.02 ± 3.54	39.05 ± 2.26 (0.0007%)	30.00 with *O. clarkii* (0.2723%)	100%[a]	0%
O. tshawytscha	12	82	20.50 ± 2.15	38.74 ± 2.45 (0.0005%)	31.75 with *O. keta* (0.0513%)	100%[a]	0%
O. gorbuscha	10	84	19.32 ± 1.05	39.86 ± 0.62 (0.0003%)	35.43 with *O. keta* (0.0042%)	100%	1.2% (false positive faint band with 1 *O. clarkii* sample)
O. mykiss	11	83	17.67 ± 1.15	39.42 ± 1.66 (0.0001%)	31.82 with *O. nerka* (0.0091%)	100%	0%
O. nerka	10	84	19.56 ± 1.06	38.59 ± 2.84 (0.0005%)	30.42 with *O. keta* (0.0943%)	100%	8.3% (false positives with 7 *O. mykiss* samples)
O. kisutch	10	84	19.37 ± 1.56	39.24 ± 1.86 (0.0002%)	31.70 with *O. gorbuscha* (0.0303%)	100%	0%

[a]For the *O. tshawytscha* and *O. keta* assays, 3 reference samples showed target bands on an agarose gel that were visible but faint compared to the bands for other target samples.

fairly similar to previous reports of TaqMan probe cross-reactivity in species-specific meat assays, where values generally ranged from 0.000% to 0.098%, with the exception of a high value of 16.5% (Brodman and Moor 2003; Dooley and others 2004). As shown in Figure 1, nonspecific amplification in species-specific assays did not occur until very late in the reactions (≥30 cycles), around the same time that the target amplification curve reached the plateau phase. When PCR protocols are carried out for an excessive number of thermocycles (that is, after the plateau phase has been reached), nonspecific and incomplete products are often generated (van Pelt-Verkuil and others 2008). Because the identification of target species is generally achieved after 25 cycles, in practice this assay would be stopped by 30 cycles and interference from nonspecific amplification would be unlikely.

The universal assay showed a positive signal for target species (Ct = 20.23 ± 1.97), with low average cross-reactivity (0.0151%). However, since the universal set was not specifically designed to discriminate the target salmonid species from nonsalmonids, Ct signals below 30 were detected with 2 of the nonsalmonids tested: *C. sordidus* (Ct = 23.48) and *S. alascanus* (Ct = 27.81). These results indicate that the universal set may be used to support data obtained from species-specific assays in terms of DNA quality, absence of the target DNA, and PCR amplification success, but a positive result with the universal set cannot be used as firm evidence for the presence of the target species.

Most of the assays showed low standard deviations (<±2.0) for the average Ct values generated with target species. However, the standard deviations for the *O. tshawytscha* (±2.15) and *O. keta* (±3.54) assays exceeded this level. The elevated variation may be explained by 3 specific reference samples that gave relatively high Ct values (24.05 to 29.84) with the target probe. These samples also showed elevated Ct values (24.81 to 31.50) when screened against the universal set, suggesting that the reduced signal was not due to COI specificity problems, but rather to problems with PCR inhibition and/or DNA template quality. DNA extracts from these samples appeared as light smears on an agarose gel, indicating DNA degradation (van Pelt-Verkuil and others 2008). When these samples were removed from the data set, the average Ct values were reduced to 20.05 ± 1.55, 19.53 ± 1.06, and 20.04 ± 1.44 for the *O. tshawytscha*, *O. keta*, and universal assays, respectively. Overall, 97% of reference samples gave target Ct values below 25 in the species-specific assays, while 2 degraded samples exhibited Ct values between 25 and 30. These results indicate that acceptable cut-off values for the detection of target species would be approximately Ct < 25 for intact samples and Ct < 30 for degraded samples.

In initial tests, the real-time PCR primers also showed good specificity against the background salmonids *S. trutta*, *S. alpinus*, *S. confluentus*, *S. fontinalis*, *S. malma*, and *S. namaycush*. Since the tests were carried out with different multiplex arrangements and, in the case of *S. salar*, at a lower annealing temperature (53 °C), these results cannot be directly compared to the results in Table 2, but they do provide a good indicator of the specificity of the individual primer-probe sets. All average cross-reactivity values for

Table 4–Results of sensitivity tests for target DNA in admixtures and single-species samples for both real-time and conventional multiplex PCR assays. The total amount of DNA in real-time PCR admixtures was 50 ng and the total amount of DNA in conventional PCR admixtures was 25 ng. Conventional PCR results are reported as visible (+), faint/barely visible (±), or not visible (−) for the target band in a 3% agarose gel.

			Real-time PCR results		Conventional PCR results	
Target species	Spike level of target species	Mixer species	Ct ± SD for target species in admixture	Ct ± SD in single-species sample	Band visibility for target species in admixture	Band visibility in single-species sample
S. salar	0.0%	*O. tshawytscha*	40.00 ± 0.00	n/a	−	n/a
	0.1%		31.84 ± 0.17[a]	31.72 ± 0.12[a]	−	−
	1.0%		27.33 ± 0.02[a]	28.03 ± 0.18[b]	+	+
	10%		24.13 ± 0.07[a]	24.71 ± 0.19[b]	+	+
O. keta	0.0%	*O. tshawytscha*	39.58 ± 0.72	n/a	−	n/a
	0.1%		31.05 ± 0.05[a]	30.80 ± 0.34[a]	−	−
	1.0%		27.20 ± 0.08[a]	27.45 ± 0.12[a]	+	±
	10%		23.96 ± 0.13[a]	23.70 ± 0.05[a]	+	+
O. tshawytscha	0.0%	*S. salar*	40.00 ± 0.00	n/a	−	n/a
	0.1%		33.68 ± 0.31[a]	32.56 ± 0.21[b]	−	−
	1.0%		30.25 ± 0.45[a]	29.27 ± 0.09[a]	±	±
	10%		25.39 ± 0.19[a]	25.82 ± 0.14[a]	+	+
O. gorbuscha	0.0%	*O. keta*	38.41 ± 1.37	n/a	−	n/a
	0.1%		29.57 ± 0.16[a]	29.74 ± 0.14[a]	±/-	−
	1.0%		26.41 ± 0.65[a]	26.38 ± 0.09[a]	+	±
	10%		22.84 ± 0.32[a]	22.93 ± 0.10[a]	+	+
O. mykiss	0.0%	*S. salar*	40.00 ± 0.00	n/a	−	n/a
	0.1%		28.73 ± 1.24[a]	28.68 ± 0.21[a]	−	−
	1.0%		25.50 ± 0.09[a]	25.25 ± 0.07[a]	+	±
	10%		22.10 ± 0.06[a]	21.69 ± 0.36[a]	+	+
O. nerka	0.0%	*O. tshawytscha*	40.00 ± 0.00	n/a	−	n/a
	0.1%		30.80 ± 0.30[a]	30.91 ± 0.24[a]	−	−
	1.0%		27.76 ± 0.34[a]	27.50 ± 0.22[a]	+	±
	10%		24.49 ± 0.17[a]	23.91 ± 0.13[a]	+	+
O. kisutch	0.0%	*O. tshawytscha*	40.00 ± 0.00	n/a	−	n/a
	0.1%		30.67 ± 0.26[a]	29.26 ± 0.10[b]	±	±
	1.0%		25.14 ± 0.58[a]	25.39 ± 0.59[a]	+	+
	10%		21.83 ± 0.13[a]	22.20 ± 0.10[a]	+	+

[ab]Values in the same row with a different superscript letter are significantly different, according to a paired samples *t*-test ($P < 0.05$).

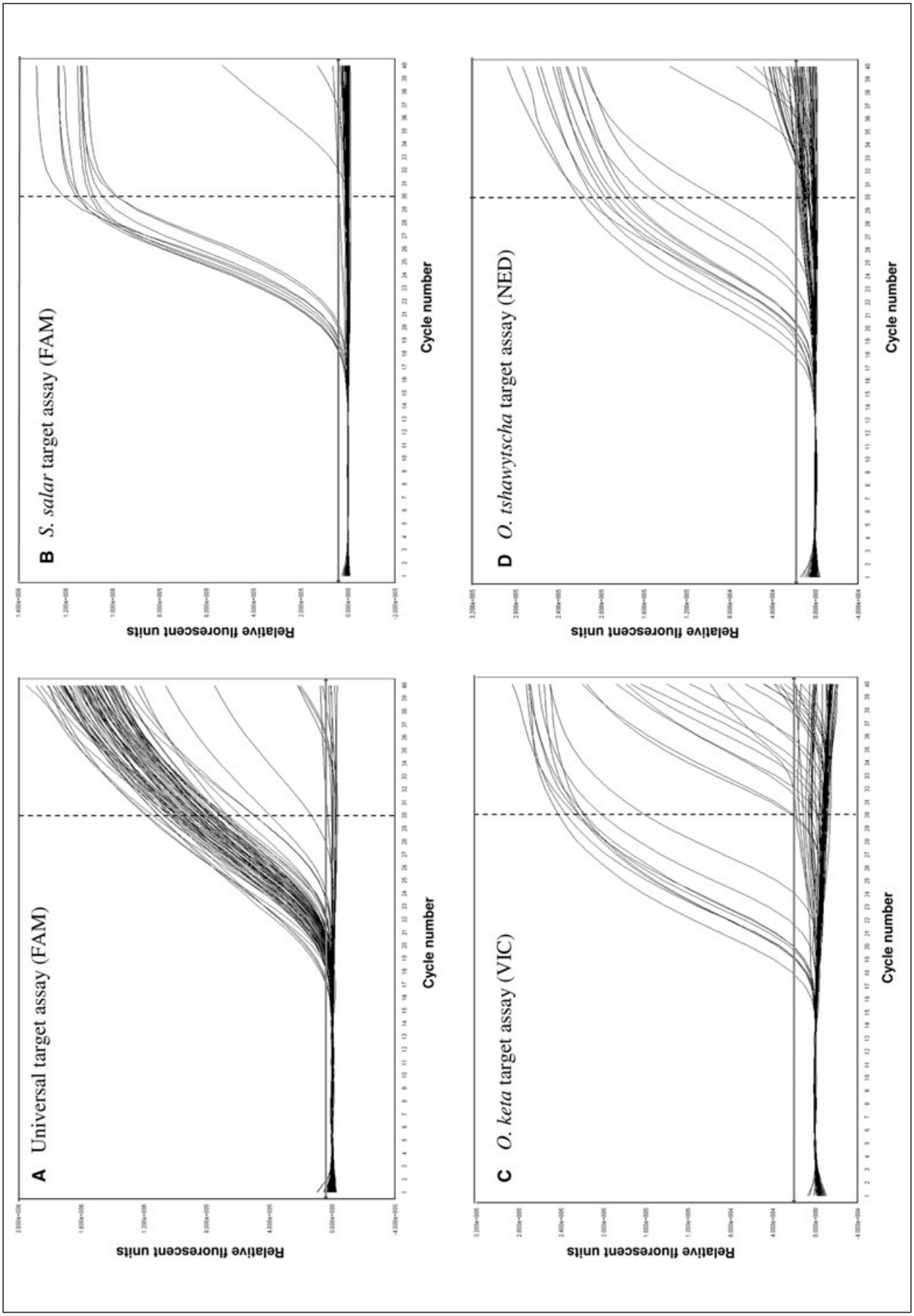


Figure 1–Subset of real-time PCR results for reference samples tested with (A) universal primers and probe and (B–D) a species-specific multiplex set (primers and probes targeting *S. salar*, *O. keta*, and *O. tshawytscha*). All lines crossing the threshold before cycle nr 30 (indicated by a vertical dashed line) correspond to individuals of the target species in each species-specific assay; lines crossing the threshold after 30 cycles are a result of nonspecific amplification/cross-reactivity with background species. The results of the remaining species-specific real-time PCR multiplex sets are shown in the appendix.

the assays carried out at 63 °C were ≤0.001%, with a minimum overall Ct value of 33.56 for an *S. malma* sample tested against the *O. nerka* probe. In the case of the *S. salar* reaction carried out in a duplex with the universal set, no signals were detected for any of the background salmonids; however, the Ct value for the target species was higher (33.96) than in the T_a = 63 °C reaction. The universal set also had a delayed signal in the duplex reaction for the 7 species targeted in this study, with an average Ct value of 27.89 ± 1.77. Signals were detected with the universal set for *S. namaycush* (36.62 ± 5.85), *S. malma* (30.08 ± 1.40), *S. alpinus* (31.21 ± 1.92), and *S. confluentus* (34.19 ± 1.56), with a minimum Ct value of 28.74 for a sample of *S. malma*. As discussed previously, the universal set is not specific for the target salmon and trout species of this study, and it is not surprising that signals were detected with additional salmonid species.

Conventional multiplex PCR results. As shown in Table 3, the conventional multiplex PCR assays also exhibited consistent identification of the target species, with 100% of target samples showing the expected PCR product in gel electrophoresis. Five of the 7 species-specific primer sets showed 0% cross-reactivity with background salmonids and all of the primer sets showed 0% cross-reactivity with nonsalmonids. Figure 2 gives an example of the agarose gel results with analysis of 1 sample per species against all 3 multiplex sets. As shown, target bands within each multiplex set could be differentiated with a 3.0% agarose gel, and the universal primers were able to amplify a common band in all samples. The degraded reference samples of *O. keta* and *O. tshawytscha* that exhibited elevated Ct values in real-time PCR also showed reduced amplification in conventional PCR (results not shown). The amplicon bands for these samples were visible, but faint compared to other reference samples. Amplification with the universal set also showed reduced band visibility with these samples, further indicating the poor quality of the template DNA. The universal set exhibited heightened specificity (0% cross-reactivity) in conventional PCR as compared with real-time PCR, likely due to the use of a higher annealing temperature.

There were a few cases of nonspecific amplification (that is, unexpected products, extremely faint or "ghost" bands, and cross-reactivity) detected in conventional PCR. Two of the 10 *O. keta* samples tested against the MKe multiplex set exhibited a very faint band at 160 bp in addition to the target band, corresponding to the expected PCR product (156 bp) that would result from a combination of the *O. mykiss* forward primer and the *O. keta* reverse primer. However, this result should not interfere with the ability to detect the target species, as the species-specific bands are 104 bp (*O. keta*) and 73 bp (*O. mykiss*). Faint false positive bands were detected at levels of 1.2% to 8.3% for 2 out of 7 species-specific primer sets in conventional PCR. The *O. nerka*-specific primers repeatedly showed a faint *O. nerka* target band when tested against 7 *O. mykiss* reference samples. These results indicate that the *O. nerka* primers are exhibiting low levels of cross-reactivity with *O. mykiss* DNA. Therefore, in cases where a sample gives a strong positive band when tested against the *O. mykiss* primers *and* a faint positive band when tested against the *O. nerka* primers, it is most likely a sample of *O. mykiss* and will need to undergo further testing (for example, with the real-time PCR method described previously or with direct sequencing of the COI barcode amplicon) to verify species. Barely visible or "ghost" bands were observed in agarose gels in 5 instances during reference sample screening. PCR was repeated in duplicate for the DNA extracts of these samples and the only case of a recurring band was found with 1 *O. clarkii* sample screened against the GKU multiplex set. This sample repeatedly gave an extremely faint band at the expected size for *O. gorbuscha* (143 bp). Although this result is a concern, it would be unlikely to cause a false positive result due to the extreme faintness of the band in the gel. Previous studies have also reported the occurrence of false positives and PCR artifact bands in conventional multiplex PCR assays, with false positives occurring at levels of 4.2% to 7.2% for background samples tested against species-specific primer sets (Rocha-Olivares 1998; Hare and others 2000; Hill and others 2001). While their occurrence is undesirable, the false positives detected in this study appeared as very faint bands in gel electrophoresis and should not cause strong interferences with species diagnosis.

Sensitivity and linearity tests

Table 4 shows the results of admixture and single-species sensitivity tests for both real-time and conventional multiplex PCR assays, and an example of the admixture test results in both systems is given in Figure 3, using the *S. salar* in *O. tshawytscha* admixture. In most cases, similar sensitivity levels were observed for target DNA in admixtures compared to single-species samples in both conventional and real-time PCR. There were only 4 instances in which a significant difference ($P < 0.05$) was found between Ct values for equivalent amounts of DNA in single-species samples and admixtures, and the type of sample that showed greater sensitivity was variable. These results indicate that sensitivity is generally not reduced for the target species when combined with another species in a DNA admixture.

Real-time multiplex PCR detection limits and linearity results. Because target sensitivity values detected after 30 cycles would not be readily discriminated from nonspecific amplification, a cut-off of Ct < 30 was used to determine detection levels. The admixture detection limits for real-time PCR were 0.1% (0.05 ng) for *O. mykiss* and *O. gorbuscha*, 1.0% (0.5 ng) for *S. salar, O. keta, O. nerka,* and *O. kisutch*, and 10% (5.0 ng) for

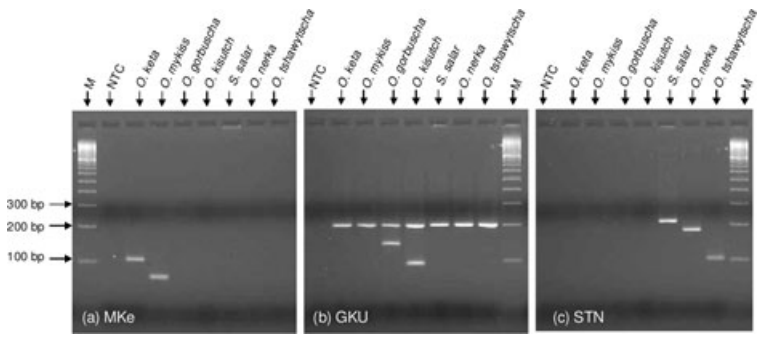


Figure 2–Results of conventional multiplex PCR specificity testing as visualized with agarose gel electrophoresis. The three agarose gel photos illustrate the species-specific and universal bands occurring for the seven target salmon and trout species (25 ng DNA) tested against the three multiplex sets developed here: (a) MKe, containing primers that target *O. keta* (104 bp) and *O. mykiss* (73 bp); (b) GKU, containing primers that target *O. gorbuscha* (143 bp) and *O. kisutch* (95 bp), as well as the universal primer set (205 bp); and (c) STN, containing primers that target *S. salar* (219 bp), *O. nerka* (183 bp), and *O. tshawytscha* (103 bp). In addition to the target samples, each gel contains an EZ Load (Bio-Rad) 100 bp molecular ruler (M) and a non-template control (NTC).

O. tshawytscha. The single-species detection limits at Ct < 30 were 0.05 ng for *O. gorbuscha, O. mykiss,* and *O. kisutch*, 0.5 ng for the remaining species, and 0.5 to 5.0 ng for the universal set. These results generally corresponded with or were slightly higher than the theoretical detection limits for Ct < 30, which ranged from 0.02 ng for *O. mykiss* to 0.17 ng for the universal set. Overall, the empirically determined sensitivity levels for real-time PCR ranged from 0.1% to 10% in admixtures and 0.05 to 5.0 ng in

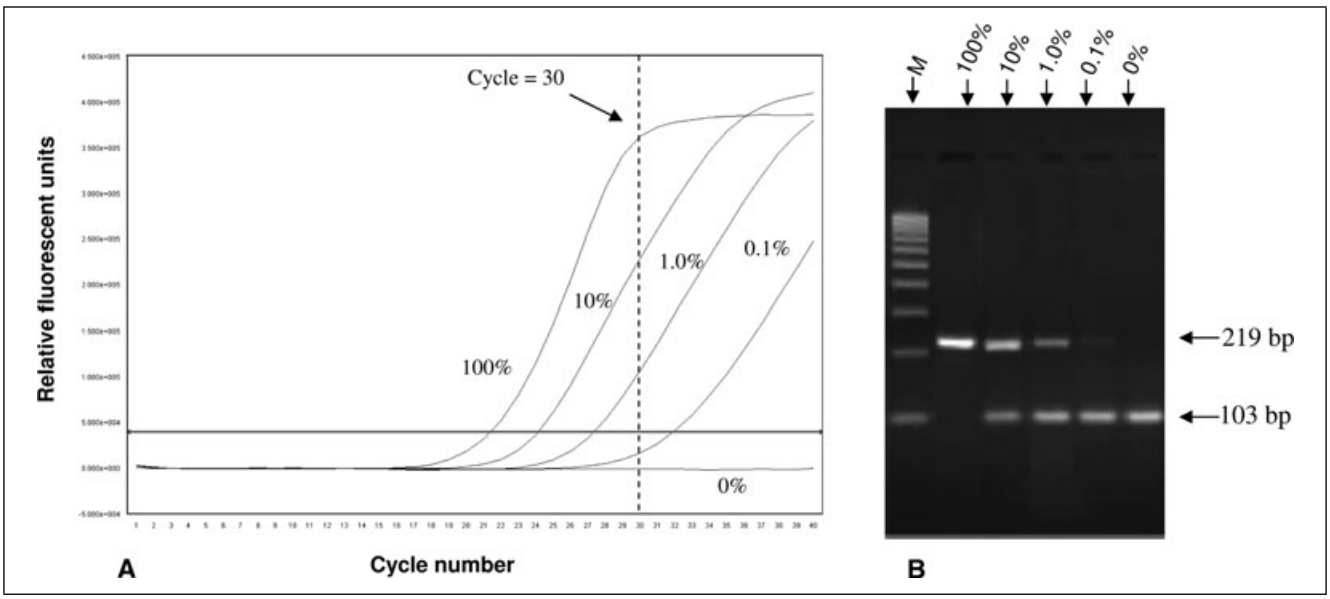
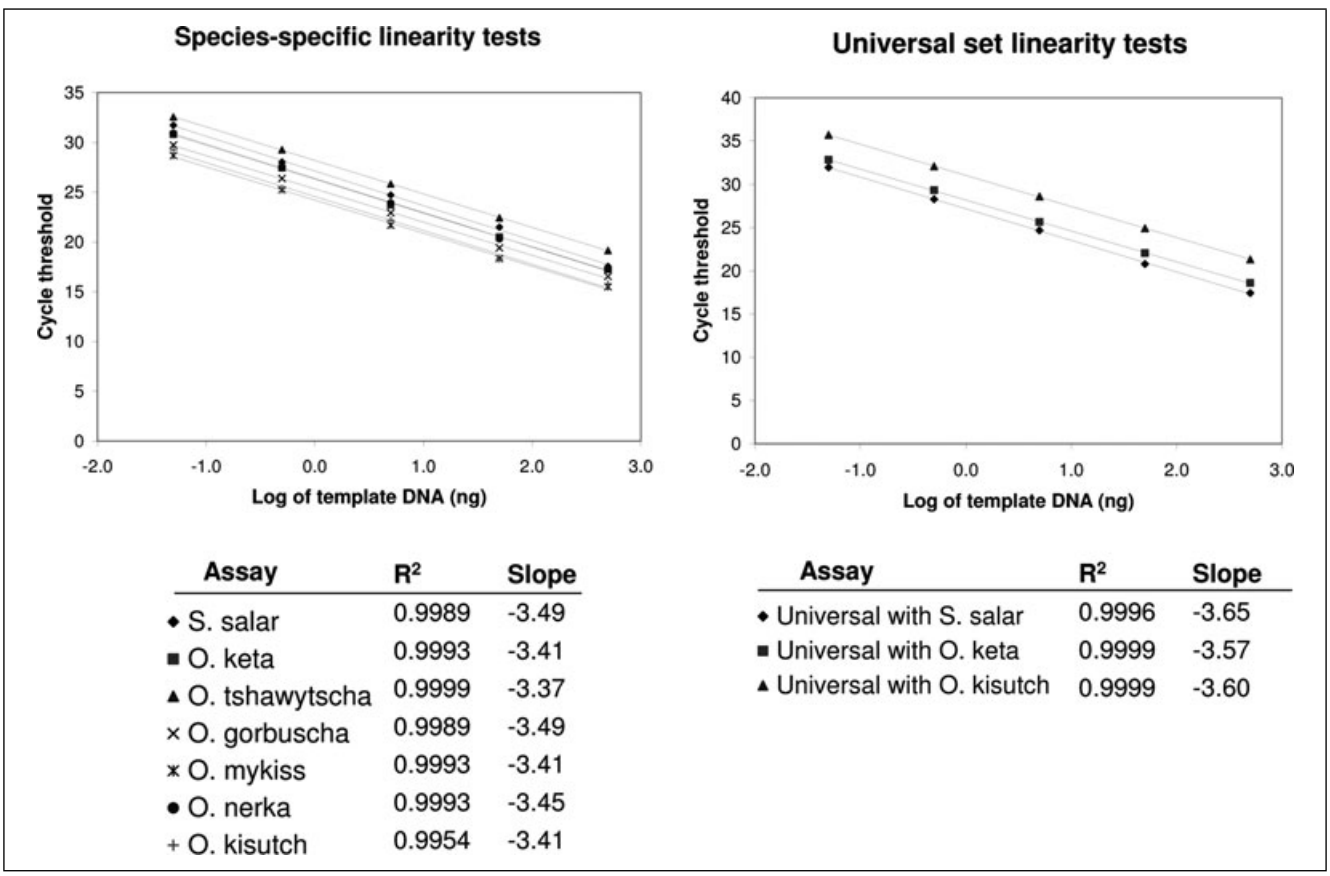


Figure 3–Example of admixture test results for *S. salar* DNA mixed with *O. tshawytscha* DNA in the (A) real-time PCR system (cycle nr 30 is marked with a vertical dashed line) and (B) conventional PCR system with an EZ Load 100 bp molecular ruler (M). For both the real-time graph and the agarose gel, the percentages given represent the level of *S. salar* reference DNA in the admixture. The real-time graph only shows the signal for *S. salar* (the *O. tshawytscha* signal is visible on a separate graph), whereas the agarose gel shows both the *S. salar* band (219 bp) and the *O. tshawytscha* band (103 bp).



Assay	R^2	Slope
◆ S. salar	0.9989	-3.49
■ O. keta	0.9993	-3.41
▲ O. tshawytscha	0.9999	-3.37
× O. gorbuscha	0.9989	-3.49
✱ O. mykiss	0.9993	-3.41
● O. nerka	0.9993	-3.45
+ O. kisutch	0.9954	-3.41

Assay	R^2	Slope
◆ Universal with S. salar	0.9996	-3.65
■ Universal with O. keta	0.9999	-3.57
▲ Universal with O. kisutch	0.9999	-3.60

Figure 4–Results of linearity tests with species-specific and universal real-time multiplex PCR assays, based on linear regression analysis. Template DNA was tested in a series of five 10-fold dilutions ranging from 500 to 0.05 ng.

single-species samples. These results are similar to previous studies investigating real-time PCR detection in meat systems, which have shown empirically determined admixture detection limits of 0.1% to 0.5% in 50 ng DNA (Dooley and others 2004) and a single-species limit of 2 ng DNA for Ct < 30 (Brodman and Moor 2003).

As shown in Figure 4, the real-time PCR species-specific standard curves showed high R^2 values, ranging from 0.9954 (*O. kisutch* assay) to 0.9999 (*O. tshawytscha* assay), with an average of 0.9987. Reaction efficiencies, calculated based on the slope of the standard curve, were also strong for the species-specific assays, ranging from 93.4% (*O. gorbuscha* and *S. salar* assays) to 98.0% (*O. tshawytscha* assay), with an average of 95.6%. The average R^2 value resulting from standard curve tests with the universal set was 0.9998 and the average efficiency was 89.4%. This efficiency is slightly lower than that found with the species-specific assays and may be attributed to the occurrence of nucleotide mismatches in the degenerate primers and probe. With the exception of the universal set efficiency, these values are within the range recommended in Raymaekers and others (2009), who stated that efficiency should be 90% to 110% and the R^2 value should be 0.99 to 0.999.

Conventional multiplex PCR detection limits. Admixture detection limits for conventional multiplex PCR assays were similar to those found for real-time multiplex PCR. These assays generally showed faint or visible bands for target DNA at 1.0% (0.25 ng) in admixtures and all showed visible bands with 10% (2.5 ng) admixtures. As with real-time PCR, the *O. tshawytscha* assay showed the least sensitivity in DNA admixtures (10%), with bands from 1% admixtures being very faint and in some cases not visible. The universal primer set showed detection levels of 0.25 to 2.5 ng target DNA in single-species samples (results not shown). The detection levels found here are similar to a previous conventional multiplex PCR study for meat species identification, which reported a limit of 0.25 ng for single-species samples (Matsunaga and others 1999). The DNA admixture results are also comparable to a previous PCR-RFLP study using lab-on-a-chip technology to detect white fish species, which reported detection levels of 1% to 5% (0.5 to 5 ng) in DNA admixtures (Dooley and others 2005).

Food product tests

The results of the small-scale food product test are shown in Table 5. Both the conventional and real-time assays developed here allowed for a species diagnosis in all cases, based either on a Ct value below 25 or 30 or a visible species-specific band on an agarose gel. All species diagnoses corresponded to the species declaration on the product label. Ct values were close to the averages determined previously for each target species (Table 3), except in the case of the cold-smoked *O. keta* sample, which had a Ct value about 4 cycles earlier than the average, and in the case of the canned *O. tshawytscha* sample, which had a Ct value about 4 cycles later than the average. The universal Ct value observed with food samples was generally higher than the average determined with reference samples, especially in the case of canned *O. tshawytscha*. A previous study also reported delayed detection of canned meat compared to raw meat, with a difference of about 3 PCR cycles (Brodman and Moor 2003). These differences in Ct values for food samples compared to reference samples may be explained by differences in DNA quality and the presence/absence of PCR inhibitors. Foods are complex systems with many variables affecting DNA extraction and PCR success, such as tissue type, degree of processing, and additional ingredients (Brodman and Moor 2003). The Ct cut-off values of < 25 for fresh or lightly processed samples and < 30 for canned samples enabled species identification in all products tested. In addition to categorizing degraded samples in terms of processing method, amplification results with the universal set may also serve as an indicator of template DNA quality. A large-scale test of this method with commercial products will be necessary to further examine appropriate cut-off values for processed products.

Table 5–Real-time and conventional PCR results of small-scale testing with commercial salmon products. Species were diagnosed in real-time PCR based on the occurrence of Ct values below 25 (fresh or lightly processed samples) to 30 (canned samples) with species-specific probes and in conventional PCR based on the appearance of target band sizes in a 3% agarose gel.

Product	Declared species	Species detected with real-time PCR (species-specific Ct; universal Ct)	Species detected with conventional PCR
Cold-smoked salmon	*O. keta*	*O. keta* (16.84; 20.91)	*O. keta*
Hot-smoked salmon	*O. nerka*	*O. nerka* (18.93; 23.84)	*O. nerka*
Fresh/frozen salmon fillet	*O. kisutch*	*O. kisutch* (20.29; 22.24)	*O. kisutch*
Canned salmon	*O. nerka*	*O. nerka* (20.66; 24.59)	*O. nerka*
Canned salmon	*O. gorbuscha*	*O. gorbuscha* (19.34; 23.37)	*O. gorbuscha*
Canned salmon	*O. tshawytscha*	*O. tshawytscha* (24.56; 27.55)	*O. tshawytscha*

Conclusions

In this study, a multiplex PCR method was developed that allows for the identification of 7 commercially important salmon and trout species in both real-time and conventional formats. Both PCR systems showed successful differentiation of the target species, even in heavily processed food products. These assays allow for rapid species diagnosis following DNA extraction, requiring approximately 4 h with conventional PCR and approximately 2 h with real-time PCR. Furthermore, both assays could readily be adapted for high-throughput operations through the use of "ready-to-use" 96-well reaction plates, containing pre-mixed and aliquoted PCR mixes. The assays are similar in cost, with an estimated price per multiplex reaction tube of U.S. $1.50 for conventional PCR (including gel electrophoresis) and U.S. $1.85 for real-time PCR. Relative to full barcode amplification and sequencing, the techniques presented here represent a significant savings of cost and time, although the former can provide a definitive species identification in cases of ambiguity. The use of a lower reaction volume (for example, 12.5 μL) could further reduce the cost per reaction. The next step in this study will be to test the ability of these methods to identify salmon and trout species in commercial products on a larger scale using a variety of processing methods and product types.

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References

Brodman PD, Moor D. 2003. 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 65(1):599–607.

Brown KH, Thorgaard GH. 2002. Mitochondrial and nuclear inheritance in an androgenetic line of rainbow trout, *Oncorhynchus mykiss*. Aquaculture 204(3–4):323–35.

Chapela MJ, Sotelo CG, Perez-Martin RI, Pardo MA, Perez-Villareal B, Gilardi P, Riese J. 2007. Comparison of DNA extraction methods from muscle of canned tuna for species identification. Food Control 18:1211–5.

Civera T. 2003. Species identification and safety of fish products. Vet Res Commun 27:481–9.

Docker MF, Heath DD. 2003. Genetic comparison between sympatric anadromous steelhead and freshwater resident rainbow trout in British Columbia, Canada. Conserv Genet 4:227–31

Dooley JJ, Paine KE, Garrett SD, Brown HM. 2004. Detection of meat species using TaqMan real-time PCR assays. Meat Sci 68:431–8.

Dooley JJ, Sage HD, Clarke MA, Brown HM, Garrett SD. 2005. Fish species identification using PCR-RFLP analysis and lab-on-a-chip capillary electrophoresis: application to detect white fish species in food products and an interlaboratory study. J Agric Food Chem 53(9):3348–57.

Edwards KJ, Logan JMJ. 2009. Performing real-time PCR. In: Logan JMJ, Edwards KJ, Saunders N, editors. Real-time PCR: current technology and applications. Norfolk, U.K.: Caister Academic Press. p 85–93.

Espiñeira M, Vieites JM, Santaclara FJ. 2009. 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 229(5):785–93.

Greig C, Robertson JM, Banks MA. 2002. Rapid PCR-based species tests for threatened sympatric salmonids. Conserv Gen 3:83–6.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–8.

Hare MP, Palumbi SR, Butman CA. 2000. Single-step species identification of bivalve larvae using multiplex polymerase chain reaction. Mar Biol 137:953–61.

Hebert PDN, Cywinska A, Ball SL, deWaard JR. 2003. Biological identifications through DNA barcodes. Proc R Soc London, Ser B 270:313–9.

Henegariu O, Heerema NA, Dlouhy SR, Vance GH, Vogt PH. 1997. Multiplex PCR: critical parameters and step-by-step protocol. BioTechniques 23(3):504–11.

Hill RS, Allen LD, Bucklin A. 2001. Multiplexed species-specific PCR protocol to discriminate four N. Atlantic *Calanus* species, with an mtCOI gene tree for ten *Calanus* species. Mar Biol 139:279–87.

Horstkotte B, Rehbein H. 2003. Fish species identification by means of restriction fragment length polymorphism and high-performance liquid chromatography. J Food Sci 68(9):2658–66.

Hsieh HS, Chai TJ, Hwang DF. 2007. Using the PCR-RFLP method to identify the species of different processed products of billfish meats. Food Control 18:369–74.

Hubert N, Hanner R, Holm E, Mandrak NE, Taylor E, Burridge M, Watkinson D, Dumont P, Curry A, Bentzen P, Zhang J, April J, Bernatchez L. 2008. Identifying Canadian freshwater fishes through DNA barcodes. PLoS ONE 3(6):e2490.

Knapp G, Roheim C, Anderson J. 2007. The great salmon run: competition between wild and farmed salmon. Washington, D.C.: World Wildlife Fund. 302 p.

Kutyavin IV, Afonina IA, Mills A, Gorn VV, Lukhtanov EA, Belousov ES, Singer MJ, Walburger DK, Lokhov SG, Gall AA, Dempcy R, Reed MW, Meyer RB, Hedgpeth J. 2000. 3′-Minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. Nucleic Acids Res 28(2):655–61.

Kyle CJ, Wilson CC. 2007. Mitochondrial DNA identification of game and harvested freshwater fish species. Forensic Sci Int 166(1):68–76.

Matsunaga T, Chikuni K, Tanabe R, Muroya S, Shibata K, Yamada J, Shinmura Y. 1999. A quick and simple method for the identification of meat species and meat products by PCR assay. Meat Sci 51:143–8.

McKay SJ, Smith MJ, Devlin RH. 1997. Polymerase chain reaction-based species identification of salmon and coastal trout in British Columbia. Mol Mar Biol Biotechnol 6(2):131–40.

Mendonça FF, Hashimoto DT, De-Franco B, Porto-Foresti F, Gadig OBF, Oliveira C, Foresti F. 2009. Genetic identification of lamniform and carcharhiniform sharks using multiplex-PCR. Conserv Genet Resour Published online: 02 November.

Michelini E, Cevenini L, Mezzanotte L, Simoni P, Baraldini M, De Laude L, Roda A. 2007. One-step triplex-polymerase chain reaction assay for the authentication of yellowfin (*Thunnus albacares*), bigeye (*Thunnus obesus*), and skipjack (*Katsuwonus pelamis*) tuna DNA from fresh, frozen, and canned tuna samples. J Agric Food Chem 55(19):7638–47.

Pank M, Stanhope M, Natanson L, Kohler N, Shivji M. 2001. Rapid and simultaneous identification of body parts from the morphologically similar sharks *Carcharhinus obscurus* and *Carcharhinus plumbeus* (Carcharhinidae) using multiplex PCR. Mar Biotech 3(3):231–40.

Pardo MA, Perez-Villareal B. 2004. Identification of commercial canned tuna species by restriction site analysis of mitochondrial DNA products obtained by nested primer PCR. Food Chem 86(1):143–50.

Phillips RB, Matsuoka MP, Konon I, Reed KM. 2000. Phylogenetic analysis of mitochondrial and nuclear sequences supports inclusion of *Acantholingua ohridana* in the genus *Salmo*. Copeia 2:546–50.

Purcell M, Mackey G, LaHood E, Huber H, Park L. 2004. Molecular methods for the genetic identification of salmonid prey from Pacific harbor seal (*Phoca vitulina richardsi*) scat. Fish Bull 102:213–20.

Rasmussen RS, Morrissey MT. 2008. DNA-based methods for the identification of commercial fish and seafood species. Compr Rev Food Sci Food Saf 7:280–95.

Rasmussen RS, Morrissey MT, Hebert PDN. 2009. DNA barcoding of commercially important salmon and trout species (*Oncorhynchus* and *Salmo*) from North America. J Agric Food Chem 57:8379–85.

Raymaekers M, Smets R, Maes B, Cartuyvels R. 2009. Checklist for optimization and validation of real-time PCR assays. J Clin Lab Anal 23:145–51.

Rehbein H. 2005. Identification of the fish species of raw or cold-smoked salmon and salmon caviar by single-stranded conformation polymorphism (SSCP) analysis. Eur Food Res Technol 220:625–32.

Rocha-Olivares A. 1998. Multiplex haplotype-specific PCR: a new approach for species identification of the early life stages of rockfishes of the species-rich genus *Sebastes* Cuvier. J Exp Mar Biol Ecol 231(2):279–90.

Rocha-Olivares A, Chávez-González JP. 2008. Molecular identification of dolphinfish species (genus *Coryphaena*) using multiplex haplotype-specific PCR of mitochondrial DNA. Ichthyol Res 55(4):389–93.

Russell VJ, Hold GL, Pryde SE, Rehbein H, Quinteiro J, Rey-Mendez M, Sotelo CG, Perez-Martin RI, Santos AT, Rosa C. 2000. Use of restriction fragment length polymorphism to distinguish between salmon species. J Agric Food Chem 48(6):2184–8.

Smith S, Hons BS, Morin PAM. 2005. Optimal storage conditions for highly dilute DNA samples: a role for trehalose as a preserving agent. J Forensic Sci 50(5):1101–8.

Steinke D, Zemlak TS, Gavin H, Hebert PDN. 2009. DNA barcoding of Pacific Canada's fishes. Mar Biol 156(12):2641–7.

Taylor MI, Fox C, Rico I, Rico C. 2002. Species-specific TaqMan probes for simultaneous identification of (*Gadus morhua* L.), haddock (*Melanogrammus aeglefinus* L.) and whiting (*Merlangius merlangus* L.). Mol Ecol Notes 2:599–601.

Trotta M, Schonhuth S, Pepe T, Cortesi ML, Puyet A, Bautista JM. 2005. Multiplex PCR method for use in real-time PCR for identification of fish fillets from grouper (*Epinephelus* and *Mycteroperca* species) and common substitute species. J Agric Food Chem 53(6):2039–45.

USFDA. 2009. *Regulatory Fish Encyclopedia*: Seafood species substitution and economic fraud. College Park, Md.: U.S. Food and Drug Administration Center for Food Safety and Applied Nutrition.

van Pelt-Verkuil E, van Belkum A, Hays JP. 2008. Principles and technical aspects of PCR amplification. The Netherlands: Springer. 325 p.

Ward RD, Hanner R, Hebert PDN. 2009. The campaign to DNA barcode all fishes, FISH-BOL. J Fish Biol 74:329–56.

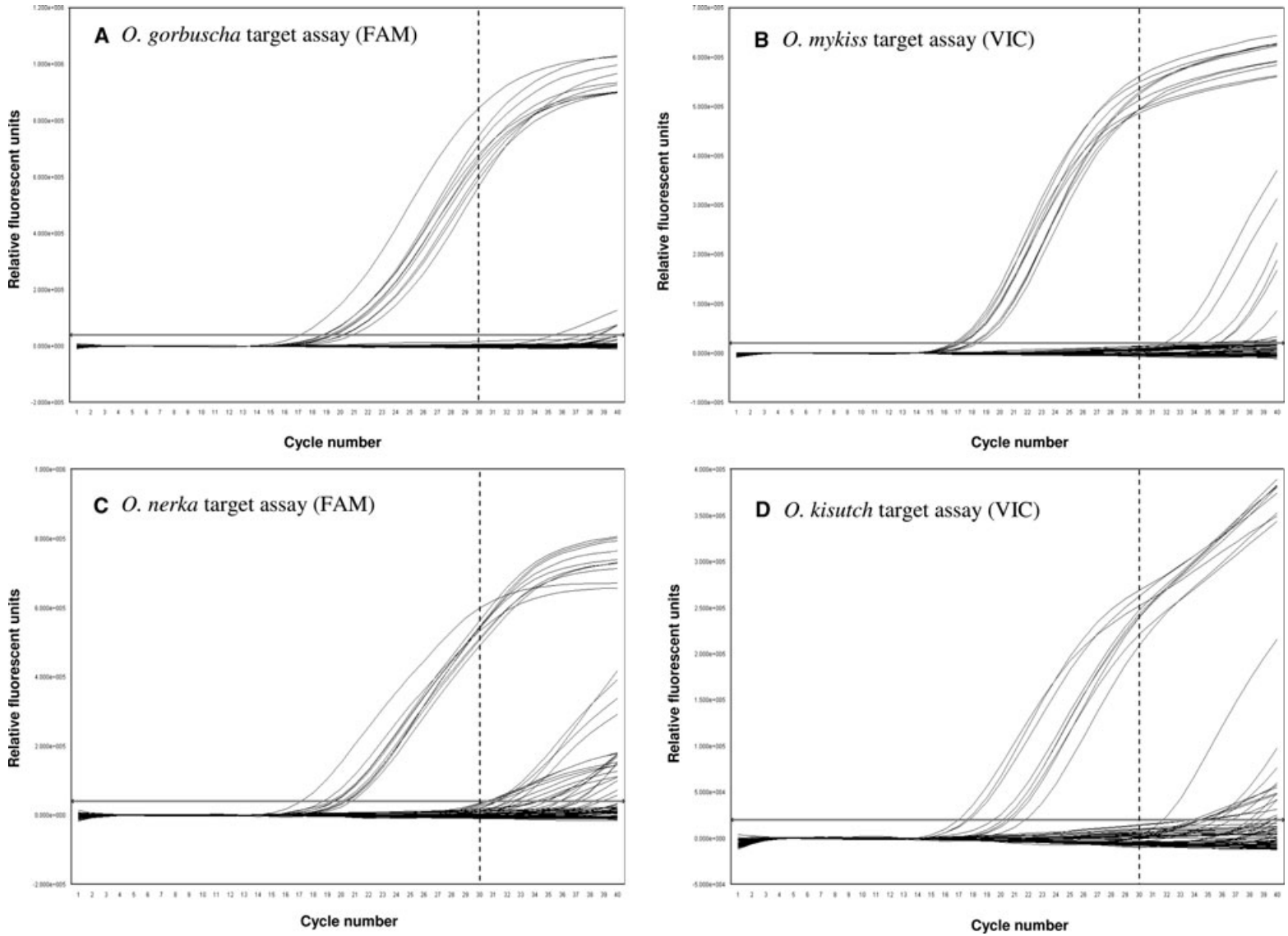
Withler RE, Beacham TD, Ming TJ, Miller KM. 1997. Species identification of Pacific salmon by means of a major histocompatibility complex gene. N Am J Fish Management 17:929–38.

Wolf C, Burgener M, Hubner P, Luthy J. 2000. PCR-RFLP analysis of mitochondrial DNA: differentiation of fish species. Lebensm Wiss Technol 33(2):144–50.

Wong EHK, Hanner RH. 2008. DNA barcoding detects market substitution in North American seafood. Food Res Int 41:828–37.

Zardoya R, Garrido-Pertierra A, Bautista JM. 1995. The complete nucleotide sequence of the mitochondrial DNA genome of the rainbow trout, *Oncorhynchus mykiss*. J Mol Evol 41(6):942–51.

Appendix



Appendix–Real-time PCR results for reference samples tested with (A–B) multiplex set specific for *O. gorbuscha* and *O. mykiss* and (C–D) multiplex set specific for *O. nerka* and *O. kisutch*. All lines crossing the threshold before cycle nr 30 (indicated by a vertical dashed line) correspond to individuals of the target species in each species-specific assay; lines crossing the threshold after 30 cycles are a result of nonspecific amplification/cross-reactivity with background species.