AN ABSTRACT OF THE DISSERTATION OF

Rosalee S. Rasmussen for the degree of Doctor of Philosophy in Food Science and Technology presented on December 14, 2009.

Title: DNA-based Identification of Commercially Important Salmon and Trout Species (Genera *Oncorhynchus* and *Salmo*) in North America

Abstract approved:

Michael T. Morrissey

There are seven commercially important species of salmon and trout (genera *Oncorhynchus* and *Salmo*) in North America, many of which are closely related but command markedly different prices. The purpose of this research was to provide improved and novel methods for the detection of salmon species substitution on the commercial market. This work took place in three parts: first an existing method was optimized and improved upon, then a comprehensive collection of reference salmon sequences was built for use in species identification with DNA barcoding, and finally, based on these sequences, a novel species-specific multiplex polymerase chain reaction (PCR) assay was developed.

In the first study, a PCR-restriction fragment length polymorphism (RFLP) method for salmon species identification was optimized for use with U.S. commercial products. The restriction digest was shortened to 1 h rather than overnight and the method was successful with lightly processed products. However, heavily processed samples could not be identified. Next, DNA barcoding was examined as a method for salmon species identification. Sequence information was collected for 924 reference samples from a wide geographic range. Sequences showed low intraspecies divergences (mean 0.26%), and the mean congeneric divergence was 32-fold greater, at 8.22%. The minimum interspecies divergence was always greater than the

maximum intraspecies divergence, indicating that these species can be differentiated using DNA barcodes. In the next study, species-specific primers and probes were developed based on DNA barcode sequence information to diagnose salmon species in both real-time and conventional PCR systems. The primers and probes were combined into multiplex assays and tested for specificity against 94-112 reference samples representing 19-25 species. Strong signals were detected for the target species in both systems, and nonspecific amplification was minimal. Both assays showed high sensitivity, with detection levels of 0.05 to 5.0 ng (0.1 to 10% in DNA admixtures). 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 a conventional or real-time format.

DNA-based Identification of Commercially Important Salmon and Trout Species (Genera *Oncorhynchus* and *Salmo*) in North America

by
Rosalee S. Rasmussen

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Rosalee S. Rasmussen, Author

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Jessica Walsh was involved with the laboratory portion and editing of Chapter 3. Dr. Paul D.N. Hebert assisted with the reviewing and editing of Chapter 4.

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CHAPTER 1

INTRODUCTION

Salmon are an important part of the North American fish and seafood market, contributing strongly to production in both commercial fisheries and aquaculture. In the United States, salmon was ranked among the four highest species groups in terms of the amount (300,000 mt) and value (U.S. $395 million) of domestic landings in 2008 (Voorhees, 2009). Although landings of commercial salmon in Canada only amounted to about 2% of U.S. harvests, salmon and trout aquaculture production was very strong, with 110,000 mt valued at U.S. $620 million (DFO, 2009). North American salmon and trout production includes seven important species, each commanding a different market price. The commercial fisheries include Chinook salmon (*Oncorhynchus tshawytscha*), sockeye salmon (*Oncorhynchus nerka*), coho salmon (*Oncorhynchus kisutch*), chum salmon (*Oncorhynchus keta*), and pink salmon (*Oncorhynchus gorbuscha*), while aquaculture production is primarily focused on Atlantic salmon (*Salmo salar*) and rainbow (steelhead) trout (*Oncorhynchus mykiss*). After harvest, these fish are generally processed into fresh/frozen fillets, smoked, or canned foods. Because most of these species are closely related and similar in appearance, they are very difficult to differentiate after their morphological identifiers have been removed. The combination of these factors, along with the range of prices commanded by different species, makes salmon and trout susceptible to market substitution for the purpose of economic gains. The U.S. Food and Drug

Administration (FDA) has identified several cases of salmon and trout mislabeling on the commercial market, including the substitution of pink salmon for chum salmon; steelhead trout for salmon; and farmed salmon for wild-caught salmon (USFDA, 2009). Several DNA-based methods have been developed for the identification of salmon and trout species (Espiñeira et al., 2009; Horstkotte and Rehbein, 2003; McKay et al., 1997; Purcell et al., 2004; Rehbein, 2005; Russell et al., 2000; Withler et al., 1997); however, these methods are not ideal for use in the food industry, where analysis must be rapid, readily adapted for high-throughput situations, and applicable to heavily processed and mixed-species samples. A species-specific multiplex PCR assay, which combines multiple primer sets into one PCR tube, has the potential to meet these requirements. An earlier study reported differentiation of three salmon and trout species with this method for conservation purposes (Greig et al., 2002); however, a multiplex PCR assay that enables differentiation of all seven commercially important salmon and trout species listed above has yet to be developed.

The overall goal of this project was to provide improved and novel methods for the detection of salmon and trout species substitution on the North American commercial market. The underlying objectives were (1) to test and improve upon a current method for salmon and trout species identification based on polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) analysis; (2) to investigate the use of DNA barcoding for salmon and trout species identification through a comprehensive sequencing effort involving many individuals from a wide geographic range, and (3) to use the results of DNA barcode sequencing as the basis for the design of a novel species-specific multiplex PCR assay. An important criterion for this assay is that it can rapidly identify all seven commercial salmon and trout species in a high-throughput manner, even in heavily processed and mixed-species samples. Improved methods for salmon and trout species identification will enhance the ability of both private and regulatory agencies to detect and prevent economic fraud in the North American commercial fish and seafood market.

CHAPTER 2

DNA-BASED METHODS FOR THE IDENTIFICATION OF COMMERCIAL FISH AND SEAFOOD SPECIES

Rosalee S. Rasmussen and Michael T. Morrissey

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2.1 ABSTRACT

The detection of species substitution has become an important topic within the food industry and there is a growing need for rapid, reliable, and reproducible tests to verify species in commercial fish and seafood products. Increases in international trade and global seafood consumption, along with fluctuations in the supply and demand of different fish and seafood species, have resulted in intentional product mislabeling. The effects of species substitution are far-reaching and include economic fraud, health hazards, and illegal trade of protected species. In order to improve detection of commercial seafood fraud, a variety of DNA-based techniques have been developed, including Multiplex PCR, FINS, PCR-RFLP, PCR-RAPD, PCR-AFLP, and PCR-SSCP, which are all based on polymorphisms in the genetic codes of different species. These techniques have been applied in the differentiation of many types of fish and seafood species, such as gadoids, salmonids, scombroids, and bivalves. Some emerging technologies in this field include the use of real-time PCR, lab-on-a-chip, and DNA microarray chips. In this review paper, the major DNA-based methods currently employed in the authentication of commercial fish and seafood species will be discussed and future trends will be highlighted. Examples of commercial applications and the use of online database resources will also be considered.

2.2 INTRODUCTION

The authentication of fish and seafood species has become an important issue within the seafood industry. Increases in international trade, rising worldwide fish and seafood consumption, and varying levels of supply and demand of certain species have led to cases of economic fraud, in which one seafood species is illegally substituted for another (Table 2.1) (Civera, 2003; Martinez et al., 2005). Regulatory organizations, such as the European Union, have established labeling laws for fish and aquaculture products requiring traceability information such as species identification, origin of fish, and production method (Martinez et al., 2005; Moretti et al., 2003). Seafood substitution has been prohibited in the United States according to the Federal Food Drug and Cosmetic Act Section 403(b): Misbranded Food, which declares "a food shall be deemed to be misbranded if it is offered for sale under the name of another food" (United States Code, Title 21, Chapter 9, Subchapter IV, Section 343) (USFDA, 2009). In order to promote correct labeling of fish and seafood, the U.S. Food and Drug Administration (USFDA) Center for Food Safety and Applied Nutrition (CFSAN) has compiled an online Seafood List that gives the acceptable market names for imported and domestically available seafood species (http://www.cfsan.fda.gov/guidance.html).

Enforcement of labeling regulations becomes complicated in processed foods, such as frozen fillets and precooked seafoods, because the original identifying morphological characteristics are absent (Moran and Garcia-Vazquez, 2006). Therefore, in order to enforce labeling regulations and prevent product substitution, there is a need for sensitive analytical methods that can be used to determine the species of a seafood product with no detectable external features (Gil, 2007; Mafra et al., 2007). In addition to the detrimental effects that seafood adulteration can have on the commercial market, it can also put consumers at risk of purchasing potentially harmful and mislabeled products and reduce the effectiveness of marine conservation and management programs that help protect ocean habitats and endangered species (Civera, 2003; Martinez et al., 2005; Teletchea et al., 2005). Furthermore, in order to

enforce laws against poaching and trade of overexploited species, reliable methods for species diagnosis are essential (Baker et al., 2000; Kyle and Wilson, 2007).

Research into methods for the identification of fish and seafood species presents several challenges that must be overcome. For example, it has been estimated that more than 20,000 species of fish and seafood are utilized worldwide for human consumption (Martinez et al., 2005). Current methods for species recognition are based on the discovery of polymorphism in protein or deoxyribonucleic acid (DNA) characteristics that are unique to each species. Therefore, the analytical techniques used to establish the unique fingerprint must first be optimized for the specific product under investigation and then they must be able to provide undeniable and repeatable results that prove species identification (Woolfe and Primrose, 2004). Complications can arise when a number of species have similar fingerprints or when individuals from the same species show different fingerprints due to intraspecies variation. Additionally, certain processing steps are known to denature proteins and partially degrade DNA, making analysis of processed seafood products especially demanding (Chapela et al., 2002; Mackie et al., 1999). A number of compounds present in processed foods may also serve as inhibitors of DNA amplification during the polymerase chain reaction (PCR) (Teletchea et al., 2005). Therefore, a number of diagnostic techniques have been developed and optimized for the differentiation of fish and seafood species in a variety of product types (Gil, 2007; Mafra et al., 2007). This review will discuss the use of DNA-based techniques in the authentication of fish and seafood species; commercial applications of these techniques; online resources that provide support for fish and seafood species identification; and future trends in this field.

2.3 COMPARISON OF PROTEIN AND DNA-BASED METHODS

Analytical diagnosis of fish and seafood has traditionally been based on species-specific electrophoretic, chromatographic, or immunological characteristics of proteins (Civera, 2003; Moretti et al., 2003; Sotelo et al., 1993). Some common methods include isoelectric focusing (IEF), capillary electrophoresis (CE), high-performance liquid chromatography (HPLC), and immuno-assay systems. While

these methods are generally reliable for use with fresh or frozen tissue, intense heat-processing or drying can destroy the biochemical properties and structural integrity of proteins, making analysis with some of the above methods impractical (Akasaki et al., 2006; Mackie et al., 1999). Although proteins in some cooked fish products have been analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and urea IEF, these methods are not effective when the tissue has been heat-sterilized (Mackie et al., 1999; Moretti et al., 2003). One protein-based method that may prove to be useful, even in heat-sterilized products, is enzyme-linked immunosorbent assay (ELISA), which has been used for the identification of several fish species (Asensio et al., 2003; Carrera et al., 1997). However, immuno-assays can be ineffective at differentiating closely related species and require the development of an antibody against the specific protein of interest (Bartlett and Davidson, 1992; Sotelo et al., 1993; Woolfe and Primrose, 2004).

The use of DNA-based methods for species detection presents a number of advantages over protein-based methods, including increased specificity, sensitivity, and reliable performance with highly processed samples (Lenstra, 2003). Although DNA molecules can degrade during processing, they are more thermostable than proteins: DNA fragments as long as 300 bp can still be recovered following sterilization (Chapela et al., 2007). Also, DNA has the potential to provide a greater amount of information due to the degeneracy of the genetic code and the existence of noncoding regions (Lockley and Bardsley, 2000). Whereas proteins vary with tissue type, age, and status, DNA is largely independent of these factors and is present in all cell types (Bossier, 1999; Civera, 2003). Since analytical methods based on DNA have been shown to have several advantages over those based on proteins, numerous genetic methods are currently being investigated that allow for identification of certain fish and seafood species (Civera, 2003; Gil, 2007; Mafra et al., 2007). Some methods include the use of PCR along with restriction fragment length polymorphism (RFLP), forensically informative nucleotide sequencing (FINS), amplified fragment length polymorphism (AFLP), or single-stranded conformational polymorphism (SSCP). The aforementioned techniques have been applied to the identification of numerous

species of fish and seafood, including gadoids (Akasaki et al., 2006; Moran and Garcia-Vazquez, 2006), flatfish (Comesana et al., 2003; Sanjuan and Comesana, 2002), salmonids (Dooley et al., 2005a; Zhang and Cai, 2006), scombroids (Hsieh et al., 2007; Lin and Hwang, 2007), sardines and anchovies (Jerome et al., 2003; Santaclara et al., 2006), eels (Lin et al., 2002), mollusks (Klinbunga et al., 2003; Rego et al., 2002), and many more.

2.4 DNA-BASED METHODS FOR SEAFOOD SPECIES IDENTIFICATION

Genetic species identification is based on the principle of DNA polymorphisms, or genetic variations that take place as a result of naturally-occurring mutations in the genetic code (Liu and Cordes, 2004). In order to detect species-specific genetic polymorphisms, DNA is first extracted from the target organism and then the DNA fragment(s) of interest is amplified using PCR. The resulting PCR amplicons are then analyzed to reveal the characteristic polymorphisms under study. This section will describe the above steps in greater detail, with a focus on the analysis of PCR fragments for species determination.

2.4.1 DNA extraction

Although the basic steps in the isolation of DNA from tissue are fairly constant (Figure 2.1), a variety of modifications exist for DNA extraction from aquatic species, including numerous commercially available kits. Oftentimes, the choice of DNA extraction method is dependent on the status of the starting material, and factors such as tissue type and DNA integrity are taken into account. DNA can be damaged by events such as heat exposure, low pH, and nucleases that cause enzymatic degradation, depurination, and hydrolysis (Marmiroli et al., 2003). DNA found in processed seafoods may have undergone significant damage, with the result being reduced quality and shorter target sequences than those found in a freshly harvested sample. Therefore, a common challenge in the application of genetic methods to the authentication of commercial fish and seafood products is to obtain DNA of sufficient quality and quantity for downstream analysis.

One of the more common methods for extraction of DNA from seafood has been the proteinase K-SDS digestion method, reported by Quinteiro et al. (1998) to be

effective at extracting DNA from both raw and canned samples. In this method, tissue lysis is carried out using proteinase K and SDS; the proteins are removed with phenol/chloroform; and then the DNA is precipitated with addition of alcohol. Although this method previously involved an overnight lysis, recent improvements involving the use of urea in the extraction buffer allowed for a reduced lysis period of just 1 h when DNA was extracted from frozen fish muscle tissue and cod roe (Aranishi and Okimoto, 2004; Aranishi et al., 2005a). Furthermore, a recent study on DNA extraction from caviar reported the possibility of extracting sufficient DNA for PCR amplification in less than 15 min (Aranishi et al., 2006). In this method, termed the urea-Chelex protocol, samples are mixed with an extraction buffer that contains a chelating resin, and then placed in boiling water for 8 min, thereby eliminating the need for an incubation step.

A study was recently conducted to determine the optimal DNA extraction methods suitable for species identification in a variety of canned tuna products (Chapela et al., 2007). Four different methods were considered: Wizard DNA Clean Up with prior digestion with proteinase K, Nucleospin (Clontech), Genomic Prep (Amersham Pharmacia Biotech), and the cetyl-trimethylammonium bromide (CTAB) precipitation method (Chapela et al., 2007). Several packing materials used in canned tuna products (for example, brine, oil, vinegar, and tomato sauce) were also examined in terms of effect on DNA quality and quantity. The study was focused on extraction of DNA from canned light tuna containing yellowfin (*Thunnus albacares*). For all procedures, an attempt was made to amplify 5 different fragments of the mitochondrial cytochrome *b* (mt cyt *b*) gene ranging in size from 100 to 300 bp. Fragments above 250 bp could not be amplified for DNA from tuna stored in brine or vinegar; however, for DNA from tuna stored in oil or tomato sauce, fragments up to 300 bp in length were successfully amplified. The Wizard DNA Clean Up procedure showed the greatest performance in terms of fragment size range and DNA quality from tuna stored in different packing materials. However, the authors reported that the optimal procedure varies with packing media, where the CTAB method was recommended for tuna canned in oil or vinegar; the Wizard method was recommended for tuna canned

in brine; and the Genomic Prep method was suggested to be best for tuna canned in tomato sauce (Chapela et al., 2007).

2.4.2 DNA amplification

Although early DNA-based identification tests utilized species-specific DNA hybridization probes, the major assays currently used in food inspection are based on PCR amplification (Figure 2.2), which requires much less starting material and exhibits greater versatility and sensitivity (Gil, 2007; Lenstra, 2003). Amplification of genetic material with PCR requires a thermostable DNA polymerase, 2 oligonucleotide primers, 4 deoxynucleotide triphosphates (dNTPs), and magnesium ions (Marmiroli et al., 2003). PCR involves numerous cycles of 3 reaction steps carried out at different temperatures: denaturation (~95 °C), annealing (50-60 °C), and extension (~72 °C). During these 3 steps, the template DNA is first separated into 2 single strands by heat denaturation, then the oligonucleotide primers anneal to complementary sequences on opposing ends of a particular fragment of the template DNA, and next a thermostable DNA polymerase uses the 4 dNTPs to synthesize copies of the target DNA fragment. Generally about 20-50 cycles of denaturation, annealing, and extension are performed, and the DNA fragment is amplified into millions of copies. The amplified DNA fragment, called an amplicon, is then present in sufficient amounts for analysis by a variety of PCR-based techniques, including sequencing or RFLP. A major drawback to conventional PCR, however, is that the DNA is not amplified in a constant manner and, therefore, accurate quantitative information cannot be obtained (Marmiroli et al., 2003). The possibility of using quantitative PCR techniques in fish and seafood authentication will be discussed in subsequent sections.

Selection of genetic material. Given that most genetic techniques currently used in species identification require the ability to amplify target DNA using PCR, properties such as the integrity and origin of the DNA can become important determining factors in choosing target DNA fragments (Bossier, 1999). Additional factors that must be considered include mutation rate and sequence length (Cespedes et al., 2000). Determination of fish and seafood species can be carried out using either nuclear DNA

(nDNA) or mitochondrial DNA (mtDNA) (Martinez et al., 2005). As an alternative to the amplification and analysis of a specific fragment, some current methods are reliant on random amplification of part of the genomic DNA to produce a genetic "fingerprint" (Ramella et al., 2005; Rego et al., 2002; Zhang and Cai, 2006). These techniques do not require prior knowledge of the DNA sequence and will be discussed in detail in subsequent sections.

Mitochondrial DNA. Animal mtDNA contains 1 major noncoding region, 13 protein-coding genes, 22 genes coding for transfer ribonucleic acid (tRNA), and 2 genes coding for ribosomal RNA (rRNA) (Cespedes et al., 2000). Some major advantages of mtDNA over nDNA are: (1) it is relatively simple and small compared to nDNA because it lacks features such as large noncoding sequences (introns), pseudogenes, repetitive DNA, and transposable elements; (2) it is relatively easy to extract; (3) it does not undergo genetic rearrangements such as recombination; and (4) sequence ambiguities resulting from heterozygous genotypes are avoided (Aranishi et al., 2005a; Cespedes et al., 2000; Civera, 2003). Further, mtDNA, which is maternally inherited, exhibits a higher copy number and a faster rate of mutation, making it generally more appropriate in the study of evolutionary genetics and inter- and intraspecies variability (Carrera et al., 2000b; Martinez et al., 2005). Due to the widespread use of mtDNA in genetic research, many universal primers have already been designed, thus facilitating the amplification of mtDNA fragments for fish and seafood species diagnosis (Carrera et al., 2000a; Comesana et al., 2003). However, high intraspecies variation observed in a target DNA sequence can become a disadvantage to species diagnostic methods that rely on stretches of DNA that are assumed to be conserved within a species (Civera, 2003). Therefore, it has been recommended that several individuals, representing the full range of distribution, are collected and tested for each species in order to increase the validity of the method (Teletchea et al., 2005). An additional factor to consider is that the maternal inheritance pattern of mtDNA may produce misleading results in the event of species hybridization, in which case analysis of nuclear DNA is preferable (Lenstra, 2003).

Whether mtDNA or nDNA is employed may also depend on the integrity of the target DNA fragment. When DNA undergoes thermal treatment, it can be degraded into fragments ranging from less than 100 bp up to about 500 bp (Chapela et al., 2007; Jerome et al., 2003; Perez et al., 2004; Quinteiro et al., 1998; Ram et al., 1996). In this case, mtDNA is generally preferred due to its relative abundance compared to nDNA and the theory that the circular structure of mtDNA gives it greater resistance to heat-induced degradation (Borgo et al., 1996; Bossier, 1999; Civera, 2003). Indeed, mtDNA has been used for species identification even in products containing severely degraded genetic material, such as canned tuna (Lin and Hwang, 2007; Pardo and Perez-Villareal, 2004b; Quinteiro et al., 1998; Rehbein et al., 1999b).

The most common mtDNA gene exploited in species identification research has been mt cyt *b*, which has been used to identify flatfish, gadoids, anchovies, eels, scombroids, and many others (Calo-Mata et al., 2003; Chow et al., 2003; Pepe et al., 2005; Rehbein et al., 2002; Santaclara et al., 2006; Sotelo et al., 2001; Teletchea et al., 2005). Due to its relatively high interspecies variation and low intraspecies variation, the cyt *b* sequence shows considerable variation and allows for the differentiation of even closely related species (Aranishi et al., 2005a; Mackie et al., 1999). Several studies have also targeted a region of mtDNA coding for both mt cyt *b* and a neighboring tRNA sequence (mt $tRNA^{Glu}$-cyt *b*) for the detection of species such as flatfish, codfish, sturgeon, salmonids, gadoids, and scombroids (Akasaki et al., 2006; Sanjuan and Comesana, 2002; Wolf et al., 2000; Wolf et al., 1999).

Some other common mtDNA targets in species identification research are the small 12S rRNA gene (819-975 bp in vertebrates) and the larger 16S rRNA gene (1571-1640 bp in vertebrates), which have been used to identify flatfish, eel, cardinalfish, cephalopods, mackerel, hairtail species, crab, and several others (Cespedes et al., 2000; Chakraborty et al., 2005; Chapela et al., 2002; Comesana et al., 2003; Imai et al., 2004; Itoi et al., 2005; Karaiskou et al., 2003; Mabuchi et al., 2003). The mitochondrial gene coding for 12S rRNA has been reported to be a good candidate for authentication of fish and seafood due to its acceptable length, mutation

rate, and availability of sequence information in databases (Cespedes et al., 2000). This gene experiences less degeneracy than the mitochondrial protein-coding genes; however, it does contain sufficient variation for interspecies differentiation (Comesana et al., 2003). In addition to the cyt *b* and rRNA sequences, there exist several additional mtDNA targets that have experienced limited use in fish and seafood species identification. These include the mt control region, used to identify hake (*Merluccius*) species (Quinteiro et al., 2001); the gene coding for cytochrome *c* oxidase subunit III (COSIII), which has been used to differentiate rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) (Carrera et al., 1999a); and the flanking region between COSIII and the ATPase genes (termed *ATCO*), used to differentiate various species of scombroids (Chow et al., 2003; Takeyama et al., 2001).

Nuclear DNA. Despite the advantages of mtDNA in species identification research, a number of nDNA targets have also proven to be successful in the differentiation of fish and seafood species. For example, the nuclear 5S rRNA gene has been used to identify mackerel, gadoids, salmonids, sharks, and others (Aranishi, 2005; Carrera et al., 2000a; Clarke et al., 2006; Moran and Garcia-Vazquez, 2006). This gene consists of a small 120 bp conserved region coding for 5S rRNA and a variable region of noncoding DNA termed the nontranscribed spacer (NTS) that has a species-specific length and sequence (Aranishi, 2005). Due to the rapid mutation rate of the NTS region, 5S rRNA amplicons can often be differentiated by species simply by visualizing the fragment length using gel electrophoresis, without the need for further analysis such as sequencing or RFLP (Moran and Garcia-Vazquez, 2006). This method has been reported to be useful for species recognition in a variety of samples, including larvae, eggs, and frozen or canned foods, and it is simple enough that it can be used in a classroom setting for students investigating molecular methods for fish species authentication, as outlined by Moran and Garcia-Vazquez (2006). Additional nDNA markers that have been used in species identification include the *p53* gene, the nuclear ribosomal internal transcribed spacer 2 (*ITS2*) locus, the 18S rRNA gene, the gene coding for α-actin, and a major histocompatibility complex (MHC) class II gene (Carrera et al., 2000b; Fernandez et al., 2000; Klinbunga et al., 2003; Shivji et al.,

2002; Withler et al., 1997). Studies on species diagnosis using these genes have been based on species-specific variations in DNA sequence. A 2-exon fragment of the *p53* gene was employed for the differentiation of Atlantic salmon and rainbow trout (Carrera et al., 2000b). The *ITS2* locus, which is located between the 5.8S rDNA and 28S rDNA coding regions, has been used to differentiate 6 common species of shark (Shivji et al., 2002), and variations in the gene coding for 18S rRNA allowed for identification of 4 species of abalone (Klinbunga et al., 2003). The highly conserved α-actin gene was reported to be useful in the detection of 3 species of clams (Fernandez et al., 2000), while an exon and an adjacent intron of the MHC class II β1 gene were used to identify several species of salmonids (Withler et al., 2004).

Satellite DNA. In addition to the above gene targets, nDNA also contains tandemly repeated segments of DNA that occur throughout the genome and exhibit a high degree of polymorphism. These regions of DNA are either rich in adenine and thymine or in guanine and cytosine and can be classified into 3 categories, based on the length and location of their repeat sequences: satellites, which have long repeat units (hundreds to thousands of nucleotides in length) and are often clustered in the centromeres; minisatellites, which have smaller repeat sequences (9-65 nt) and are dispersed throughout the nuclear DNA; and microsatellites, also referred to as simple sequence repeats (SSRs), that are tandem arrays of 2 to 8 base pairs and are also dispersed throughout the genome (Brown and Epifanio, 2003). Polymorphisms in the number of repeated segments (up to 100 repeats) at a given locus allow for differentiation of individuals (Imsiridou et al., 2003). In order to carry out satellite-based research, primers are developed to amplify a specific locus and variations in tandem repeats between individuals can be revealed by size separation using gel electrophoresis. In satellite fragment length polymorphism (SFLP), the amplified satellite DNA undergoes a restriction digest, and the resulting ratio of repeat units with and without restriction sites allows for differentiation of species and hybrids (Lenstra, 2003). While use of SFLPs has been reported in the identification of several terrestrial animal hybrids (Nijman et al., 2002; Nijman et al., 2003; Verkaar et al., 2001), a

literature search for SFLP implementation in fish and seafood did not show any published studies in this area.

Thanks to their high levels of degeneracy and variability, mini- and microsatellites, also referred to as variable number of tandem repeats (VNTR), have proven to be very useful in studies on population genetics (Brown and Epifanio, 2003). For example, microsatellite markers have been developed for phylogenetic analyses with numerous marine species, including rainbow trout (Beacham et al., 2004; Beacham et al., 2000), smelt (Beacham et al., 2005), channel catfish (*Ictalurus punctatus*) (Waldbieser et al., 2001), sun-catfish (*Horabagrus brachysoma*) (Gopalakrishnan et al., 2006), carp (Lal et al., 2004), salmonids (Bucklin et al., 2007; Greig et al., 2003), and many more (Liu and Cordes, 2004). VNTR-based methods may prove to be advantageous for fish species identification due to their sensitivity, speed (variants at 2 loci can be identified simultaneously), and ability to identify commercially processed samples (Castillo et al., 2003). Indeed, a study on Atlantic hakes reported the ability to use microsatellite markers that had previously been developed for population studies in fish species authentication (Castillo et al., 2003). The authors reported that only 2 microsatellite loci were necessary to differentiate all hake samples and they emphasized the usefulness of the method on a commercial scale for fish labeling, authentication, and inspection programs. More recently, the use of microsatellite technology was reported to help convict or exonerate individuals in Canada suspected of fish fraud involving salmonids (Withler et al., 2004). VNTRs have also been developed to differentiate 4 similar eel species (Maes et al., 2006); to identify the sturgeon species *Acipenser stellatus*, a producer of highly-prized black caviar (Jenneckens et al., 2001); to differentiate wild and hatchery-raised red drum (*Sciaenops ocellatus*) (Renshaw et al., 2006); and to identify 3 Pacific salmonid species (Greig et al., 2002). Despite the potential advantages of microsatellites, they have not been widely used in fish and seafood species authentication studies. This may be partially due to the high level of cost and effort involved in the initial research that must be carried out to develop appropriate markers and primers (Liu and Cordes, 2004).

Multigene families. In addition to microsatellites, multigene families represent another case in which genetic analysis is based on polymorphisms in repeated DNA sequences (Moretti et al., 2003). One example is the actin multigene family, which has been used for the identification of a number of vertebrate species (Martinez et al., 2005). Actin genes contain sequences that code for different molecular forms of the actin protein, along with noncoding stretches of DNA (introns) that vary considerably in length and number. In order to use these genetic polymorphisms to identify species, universal primers are designed to amplify the variable regions and produce a species-specific genetic fingerprint. Although actin multigene families have not been exploited for fish and seafood species identification, they represent yet another potentially valuable genetic marker.

Selection of PCR primers. PCR primers, which can be either universal or species-specific, are responsible for binding specific regions of target DNA to define the PCR fragment to be amplified. Therefore, selection of the appropriate primers for DNA amplification is an important factor to consider for the successful identification of fish and seafood species.

Universal primers. Universal primers are designed to anneal to regions of DNA that are generally conserved across species groups and amplify a DNA fragment that exhibits interspecies variation (Carrera et al., 2000b). To facilitate universal amplification, these primers are often degenerate at certain nucleotide positions that are known to vary with species. Universal primers are useful for the amplification of a DNA fragment for sequencing and subsequent design of species-specific primers, as in the case of cephalopod species differentiation with a fragment of the mt 16S rRNA gene (Chapela et al., 2002). In other cases, universal primers are utilized to amplify the target DNA and then species-specific differences in sequence are analyzed by RFLP (Akasaki et al., 2006; Sanjuan and Comesana, 2002; Santaclara et al., 2006). For example, a pair of universal degenerate primers (H15149AD, L14735) has been used to amplify a fragment of the mitochondrial gene cytochrome *b* in over 40 species of fish, which could subsequently be identified at the species level using restriction enzymes (Calo-Mata et al., 2003; Russell et al., 2000; Sotelo et al., 2001). An

alternative to using a single primer pair with degenerate sites for the amplification of a universal gene fragment is the application of a cocktail of primers associated with the gene target. For example, the use of primer cocktails was reported in the amplification and sequencing of segments of the cytochrome *c* oxidase subunit I (COI) gene for use in DNA barcoding (Ivanova et al., 2007).

Species-specific primers and multiplex PCR. Species-specific primers are designed on the basis of diagnostic nucleotide sites to anneal only to DNA from a given species (Lockley and Bardsley, 2000). Although this method requires detailed knowledge of the DNA sequences from target species, this information is becoming increasingly available with the use of genetic databases. Also, the use of species-specific primers allows for simple detection of species by the presence or absence of the PCR amplicon on an agarose gel, with no need for traditional analytical procedures such as sequencing, RFLP, or SSCP. In multiplex PCR, multiple species can be analyzed in a single run by using a combination of species-specific primers and universal primers, resulting in DNA fragment lengths that vary with species (Apte and Daniel, 2003). The length of the fragments can be predicted if the complete sequence is known and a given species can be identified by the appearance of an amplicon of appropriate size on an agarose gel. Multiplex PCR with the nuclear ribosomal *ITS2* locus and the mt cyt *b* gene has been used for species diagnosis of a variety of pelagic sharks, such as great white (*Carcharodon carcharias*), hammerhead (order Carcharhiniformes), basking shark (*Cetorhinus maximus*), and mako (*Isurus paucus* and *Isurus oxyrinchus*), whose fins are commonly sold on the global shark fin market (Abercrombie et al., 2005; Clarke et al., 2006; Magnussen et al., 2007; Shivji et al., 2002). Multiplex PCR assays have also been developed to identify swordfish (*Xiphias gladius*) in processed products (Hsieh et al., 2004); to differentiate sole (*Solea solea*) and Greenland halibut (*Reinhardtius hippoglossoides*) (Cespedes et al., 1999); to identify 3 species of Pacific salmonids (Greig et al., 2002); and to differentiate fillets of Nile perch (*Lates niloticus*), grouper (*Epinephelus guaza*), and wreck fish (*Polyprion americanus*) (Asensio, 2007; Asensio et al., 2001). A further advantage of multiplex PCR is that real-time PCR probes, such as TaqMan™, can also be applied,

which allows for a rapid, quantitative analysis that does not require the use of gel electrophoresis (Marmiroli et al., 2003). For example, Trotta et al. (2005) reported the development of a multiplex PCR assay that allowed for the discrimination of grouper from commonly substituted species based on analysis with either conventional gel electrophoresis or a real-time system. Use of real-time PCR will be discussed further in the Future Trends section of this paper.

2.5 POST-PCR ANALYSIS METHODS

Following DNA extraction and PCR amplification, the resulting DNA fragments must be properly analyzed in order to verify the presence or absence of species-specific genetic markers. As shown in Figure 2.3, a variety of methods are available for this purpose. Selection of the most appropriate analytical method is a crucial step in species recognition and involves the consideration of several factors, such as the quality of the starting material and the type and number of species to be differentiated (Table 2.2). For routine use in species identification, these techniques must have a relatively low cost of operation and should be reproducible, quick, and dependable (Bossier, 1999). As mentioned previously, when species-specific or multiplex PCR primers are utilized, analysis may be as simple as visualization of the amplicons with gel electrophoresis. However, in many cases, such as with the analysis of RFLPs, SSCPs, random amplified polymorphic DNA (RAPD), and AFLPs, additional procedures are necessary. Despite the wide range of available techniques, the majority of DNA-based fish and seafood identification studies to date have been carried out using either RFLP or sequencing analysis of PCR-amplified fragments of mtDNA (especially cyt *b*). This is fairly consistent with general trends in this field: a literature search of food and forensic molecular identification methods revealed that over 90% of published studies used either RFLP, species-specific PCR or FINS (Teletchea et al., 2005). This section of the review will discuss the basic principles, suitable applications, and advantages/disadvantages of the major post-PCR analytical methods currently being employed in fish and seafood species identification research.

2.5.1 Forensically informative nucleotide sequencing (FINS)

FINS is a DNA-based procedure first described by Bartlett and Davidson (1992). In order to identify a species using FINS, a specific DNA fragment is amplified by PCR, its nucleotide sequence is determined, and the sequence is then compared to related sequences in a database using phylogenetic analysis. The sequence with the lowest genetic distance, or number of nucleotide substitutions, from the target fragment represents the species group to which the original sample belongs (Bartlett and Davidson, 1992). A combination of 2 mathematical modeling systems are generally employed to carry out the phylogenetic analysis: the Tamura-Nei method, to calculate the genetic distances among sequences (Tamura and Nei, 1993), and the Neighbor-Joining method, to construct a phylogenetic tree based on these genetic differences (Saitou and Nei, 1987).

Since FINS is based on nucleotide sequence substitutions, it is important to select a fragment that exhibits high interspecies variability, but low intraspecies variability in order to avoid ambiguities in the determination of species (Bossier, 1999). A common choice for use in FINS is the mt cyt *b* gene. This method has been used to successfully identify a number of fish samples, including canned salmon, salted cod, partially cooked battered cod, and pickled herring (Bartlett and Davidson, 1992); fresh, frozen, or salted gadoid species (Calo-Mata et al., 2003); frozen or canned sardines and sardine-type products (Jerome et al., 2003); fresh/frozen anchovy species (Santaclara et al., 2006); and fresh/frozen or canned cephalopods and "squid rings" products (Chapela et al., 2003). Extensive phylogenetic research with the mt cyt *b* gene has resulted in the accumulation of a great amount of sequence data that can be used to properly identify species origin, as in the above studies (Lockley and Bardsley, 2000). Another DNA fragment that has been analyzed with FINS is the mt 16S rRNA gene, which was used to differentiate between a variety of fresh, frozen, or processed (squid rings) cephalopod species (Chapela et al., 2002).

Although sequencing has proven to be the most direct and reliable way to obtain information from PCR fragments, it is also time-consuming and expensive, making it impractical for routine use in many laboratories (Chapela et al., 2002; Dooley et al., 2005a; Lockley and Bardsley, 2000). Additionally, sequencing is not

appropriate for the analysis of samples containing multiple species (Lenstra, 2003). Therefore, even though sequence analysis with FINS is a valuable technique in phylogenetic and population studies, it may prove to be inappropriate for some applications of species identification in commercial fish and seafood products (Carrera et al., 2000b). On the other hand, numerous studies have shown successful diagnosis of species using FINS, and ongoing technological advances have led to the development of protocols that are simpler and easier than they once were, thus increasing the feasibility of sequencing for species identification (Chapela et al., 2003).

2.5.2 Restriction fragment length polymorphism (RFLP)

A popular alternative to FINS is PCR-RFLP, which is based on polymorphisms in the lengths of particular restriction fragments of genetic code. As mentioned earlier, species-specific variations in the lengths of particular fragments can sometimes be analyzed simply by PCR amplification and visualization on an agarose gel. However, when the variations are too small to be detected in this way (<100 bp difference), PCR amplicons can be digested with restriction enzymes (endo-nucleases) and then analyzed using gel electrophoresis to develop species-specific restriction profiles (Liu and Cordes, 2004). In order to establish a protocol for species identification using PCR-RFLP, the target DNA fragment must initially be amplified by PCR and then sequenced to identify polymorphisms among the species of interest. Next, appropriate restriction enzymes are chosen that will be able to recognize and cut specific sequences of DNA, resulting in a pattern of restriction fragments that varies with species (Liu and Cordes, 2004). Once the sequence of the fragment has been established, the initial sequencing step is no longer necessary, as the PCR amplicon of interest is simply digested with the pre-selected restriction enzymes and then its restriction pattern is compared with reference samples for species identification. This procedure has been widely used in fish and seafood authentication research due to a number of advantages that it offers over other techniques. To begin with, it is less costly, simpler, and more suitable for routine laboratory analysis than techniques, such as FINS, that are based on nucleotide sequencing analysis (Aranishi, 2005; Carrera et

al., 1999a; Cespedes et al., 2000). Additionally, PCR-RFLP is a relatively rapid, reproducible, and robust laboratory technique that does not require expensive equipment (Aranishi, 2005). Due to its many advantages, PCR-RFLP may be a good candidate for large-scale studies involving fish species detection, such as those that might be used by food inspection agencies to enforce labeling regulations (Aranishi, 2005; Cespedes et al., 2000).

PCR-RFLP is one of the most common methods used in fish and seafood species identification and has been carried out with a variety of DNA fragments. As with FINS, the most widely used DNA fragment is mt cyt *b*, which has been used to identify fish and seafood such as scombroids (Chow et al., 2003; Horstkotte and Rehbein, 2003; Quinteiro et al., 1998; Ram et al., 1996), flatfish (Cespedes et al., 1998a; Cespedes et al., 1998b; Sotelo et al., 2001), gadoids (Aranishi et al., 2005a; Aranishi et al., 2005b; Calo-Mata et al., 2003; Pepe et al., 2005; Perez et al., 2004), salmonids (Russell et al., 2000), and a number of others. Additional DNA fragments that have been analyzed by PCR-RFLP for species identification include (but are not limited to): nuclear 5S rRNA to differentiate mackerel species (Aranishi, 2005), *p53*, mt 16S rRNA, and COSIII to differentiate Atlantic salmon from rainbow trout (Carrera et al., 2000b; Carrera et al., 1999a; Carrera et al., 1999b), mt 16S rRNA to identify various species of clams and hairtails (Chakraborty et al., 2005; Fernandez et al., 2002), *ATCO* to differentiate scombroid species (Chow et al., 2003; Takeyama et al., 2001), and mt 12S rRNA to differentiate sole from Greenland halibut and to identify various flatfish species (Cespedes et al., 2000; Comesana et al., 2003). The results of these studies have shown that PCR-RFLP is suitable for analysis of closely related species, samples containing mixed species, and samples that have undergone various levels of processing, including heat sterilization.

While PCR-RFLP has become a prominent method in the field of species identification, it continues to contain a number of drawbacks. A major disadvantage of PCR-RFLP is the possibility for intraspecies variation, in which individuals from the same species exhibit different restriction patterns due to degeneracy in the DNA fragment being analyzed (Akasaki et al., 2006; Lockley and Bardsley, 2000; Mackie et

al., 1999). Therefore, in order to avoid false negatives, numerous individuals from the same species must be analyzed to verify a lack of intraspecies polymorphisms at the target sites. An additional complication is that there is no guarantee that all species will give unique restriction patterns. Consequently, an unknown sample containing a species that has not yet been analyzed with PCR-RFLP could be falsely identified if its restriction profile matches that of a previously studied species (Sotelo et al., 2001). Due to the these limitations, it has been recommended that species identification with PCR-RFLP is carried out with caution if there is not substantial information available concerning sequence polymorphisms within and between species groups (Mackie et al., 1999; Sotelo et al., 2001). One approach for minimizing the identification errors caused by the above complications is the use of at least 2 diagnostic restriction sites (Lenstra, 2003).

Lab-on-a-chip capillary electrophoresis. A recently investigated development in PCR-RFLP has been the replacement of the gel electrophoresis step with microfluidic, lab-on-a-chip technology, which utilizes CE to analyze DNA fragments (Dooley et al., 2005a; Dooley et al., 2005b). Lab-on-a-chip CE is considered an improvement to the traditional PCR-RFLP procedure because it is easy to use, and it has been reported to exhibit increased sensitivity, speed, reliability, and safety compared to gel-based methods. Following a typical restriction digest with a PCR-amplified DNA fragment, the resulting restriction fragments are loaded into a microchip (3 cm^2), separated using CE, and then detected and quantified using laser-induced fluorescence (Dooley et al., 2005a; Dooley et al., 2005b). The microchips are single-use units that contain etched capillaries attached directly to sample loading wells. Recently, lab-on-a-chip was demonstrated to be effective in fish authentication studies, including the differentiation of rainbow trout and Atlantic salmon (Dooley et al., 2005a) and identification of a number of whitefish species (Dooley et al., 2005b). This technology has also been utilized in the authentication of genetically modified soy (McDowell et al., 2001), olive oil (Dooley et al., 2003), and a variety of meat species (Dooley and Garrett, 2001). The high level of sensitivity displayed by lab-on-a-chip allows for the detection of DNA fragments that may be too small for visualization using gel

electrophoresis. Also, fish species that are present at a level of just 5% in a fish admixture have been detected by lab-on-a-chip analysis (Dooley et al., 2005b). Despite the many advantages that lab-on-a-chip offers in the field of DNA-based species identification, it continues to possess some of the drawbacks mentioned above for PCR-RFLP, including the need for predetermined RFLP profiles for species determination.

2.5.3 Single-stranded conformational polymorphism (SSCP)

SSCP is an alternative to methods such as FINS or RFLP for the detection of interspecies polymorphisms, especially when closely related species are being analyzed (Bossier, 1999). Although RFLP has been reported to be simpler and more robust, SSCP is a highly sensitive technique that is less problematic than RFLP or RAPD in regards to intraspecies variation (Akasaki et al., 2006; Mackie et al., 1999; Rehbein et al., 1997). Analysis with SSCP begins with PCR amplification of a specific DNA fragment in all species being examined (Lockley and Bardsley, 2000). The resulting amplicon is then denatured into a fragment of single-stranded DNA that has a secondary structure dependent on its sequence. Variations in sequence, which may be as small as a single nucleotide, can be detected by differences in electrophoretic mobility with PAGE. SSCP patterns are visualized by silver staining and then compared to the profiles of authentic species in order to correctly identify an unknown sample (Mackie et al., 1999). SSCP has been reported to be capable of both analyzing small DNA fragments (~100 bp) and detecting species in mixed samples (Mackie et al., 1999; Rehbein et al., 1999b).

In general, SSCP analysis has been based on variations in the sequence of the mt cyt *b* gene. Although not as widely used as PCR-RFLP or sequencing methods, PCR-SSCP has been utilized to identify a variety of fish species, including salmonids, sardines, herring, eel, tuna, bonito, and sturgeon (Rehbein et al., 1999a; Rehbein et al., 1997; Rehbein et al., 1999b; Rehbein et al., 2002). Despite its success, SSCP analysis is more demanding than RFLP and continues to have a number of setbacks. For example, the high sensitivity of PCR-SSCP also commands a high level of reproducibility, with no differences in the conditions from one analysis to the next

(Lockley and Bardsley, 2000). Also, reference samples must always be run on the same gel as the unknown, and the level of information obtained from SSCP is much less than that obtained through sequencing (Rehbein et al., 1997).

2.5.4 Random amplified polymorphic DNA (RAPD)

Unlike the above methods, RAPD does not target predetermined DNA fragments. Instead, an arbitrary primer is designed without previous knowledge of the target DNA sequence, and during PCR this primer randomly amplifies segments of DNA (Williams et al., 1990). Due to variations in the genetic code, RAPD analysis on different species results in unique patterns of DNA fragments. In order to carry out RAPD, a short primer around 10 nt in length is constructed and then added to a PCR reaction with the target DNA. Next, the PCR amplicons are analyzed using gel electrophoresis and, if the resulting band patterns are species-specific, the DNA fingerprint for that species is established. When an unknown sample is analyzed using the same primer, its band pattern can be compared to that for known samples in order to verify the species.

RAPD has the potential to be used as an accurate, rapid tool for exposing commercial fraud (Ramella et al., 2005). The method is relatively cheap, fast, and simple; it does not require prior knowledge of the genome sequence; and primers are commercially available (Liu and Cordes, 2004; Lockley and Bardsley, 2000; Rego et al., 2002). Additionally, RAPD requires minimal DNA and allows for both intra- and interspecies differentiation (Ramella et al., 2005). Compared to other available methods, such as RFLP and AFLP, RAPD has been suggested to be the least expensive and the most reliable for species identification when there is no prior knowledge of the genome sequence (Liu and Cordes, 2004). RAPD protocols have been developed for both agricultural animals (Lockley and Bardsley, 2000) and marine organisms, including catfish (Liu et al., 1998b), tilapia (Ahmed et al., 2004), mussels (Rego et al., 2002), Asian arowana (dragonfish: *Scleropages formosus*) (Yue et al., 2002), and blackfin goosefish (*Lophius gastrophysus*) (Ramella et al., 2005). However, most fish research with PCR-RAPD has been focused on mapping out

population genetics rather than revealing commercial fraud through species identification (Ali et al., 2004).

Despite its advantages, PCR-RAPD has a number of disadvantages. A major concern is reproducibility of the method, especially when the target DNA is limited or slightly degraded (Lockley and Bardsley, 2000; Rego et al., 2002). For example, if the template DNA is of poor quality, some of the larger fragments common to specific fingerprints might be absent. Also, reaction conditions must be constant and stringent, in order to ensure that the DNA fingerprints produced accurately reflect the corresponding species. An additional complication is the possibility of false matches occurring when different DNA regions from 2 different species produce PCR fragments of similar length (Liu and Cordes, 2004).

2.5.5 Amplified fragment length polymorphism (AFLP)

First described by Vos et al. (1995), AFLP is a novel fingerprinting technique that draws upon aspects of both RFLP and RAPD (Bensch and Akesson, 2005). AFLP analysis begins with digestion of whole genomic DNA with 2 restriction enzymes, one that has a shorter sequence and cuts more frequently and another that has a slightly longer sequence and cuts less frequently. The most commonly used enzymes in AFLP are *Mse*I (4 bp recognition sequence) and *Eco*RI (6 bp recognition sequence) (Liu and Cordes, 2004). Adaptor molecules that recognize the restriction sequences are then ligated to the DNA restriction fragments and then PCR amplification is carried out with primers that anneal to the adaptor molecules (Blears et al., 1998). These primers contain an additional base at the 3'-end and, therefore, amplify only a subset (1/16) of the available DNA fragments (Bensch and Akesson, 2005). The resulting amplicons are then used as template DNA for a second, more selective, PCR amplification that involves primers containing 2 additional overhanging bases. This PCR step further reduces the number of available DNA fragments by 1/256, resulting in a total of about 100 fragments. These fragments are separated by size using gel electrophoresis and detected by a fluorescent or radioactive label on the *Eco*RI adaptor-specific primer (Bensch and Akesson, 2005; Bossier,

1999). The overall result is a specific DNA fingerprint, where inter- and intraspecies polymorphisms are revealed by the presence or absence of specific fragments.

AFLP has a number of advantages that make it an attractive tool for species diagnosis. The method can be carried out independently of the source or complexity of the target DNA, and AFLP banding patterns are highly complex and information-rich (Blears et al., 1998; Bossier, 1999). Although it is similar to RAPD in that it does not require prior knowledge of the DNA sequence, AFLP analysis shows greater levels of reproducibility and polymorphism (Bossier, 1999; Liu and Cordes, 2004). Since there is no need for sequencing, AFLP has relatively low start-up costs and time requirements. This allows for the examination of many loci (>1000) at a moderate cost, compared to other species identification techniques, such as single nucleotide polymorphisms (SNPs), microsatellites, and multigene sequencing, that are generally restricted to <50 loci due to high costs and long start-up times (Bensch and Akesson, 2005). Although AFLP analysis results in numerous informative markers and complex banding patterns, information on individual DNA fragments is not as specific as with other techniques. This may be considered a drawback when genetic information is desired on a per-locus basis (for example, differentiating recessive from dominant genotypes) rather than an overall fingerprint. Furthermore, the development of AFLP markers is fairly labor-intensive and requires DNA of high quality and high molecular weight.

Even though AFLP analysis has been extensively utilized for genetic research involving plants, fungi, and bacteria, it has experienced limited use in the field of animal research (Bensch and Akesson, 2005). AFLP markers have been developed for a few aquatic species, including catfish (Liu et al., 1998a), oysters (Li and Guo, 2004; Yu and Guo, 2003), trout (Young et al., 1998), bass, and tuna (Han and Ely, 2002). However, the majority of studies have focused on the use of AFLP for constructing genetic linkage maps rather than species differentiation in commercially available food products. According to Zhang and Cai (2006), AFLP has yet to be exploited in fish fraud research because it is relatively time-consuming and has not been adapted for large-scale applications. In order to overcome these setbacks, the authors used

AFLP analysis on rainbow trout to develop a species-specific AFLP marker. Primers were designed that would amplify a segment of this marker termed the sequence characterized amplified region (SCAR). Use of the AFLP-derived SCAR allowed for differentiation of rainbow trout from Atlantic salmon and was reported to increase the overall speed, reliability, and ease of the method for applications in commercial fraud detection (Zhang and Cai, 2006).

2.5.6 Others

Expressed sequence tags (ESTs). ESTs are short stretches of transcribed nucleotide sequences that can be used to identify gene transcripts and analyze SNPs (Nagaraj et al., 2007). ESTs with polymorphisms are currently valuable in genome mapping (Liu and Cordes, 2004), and EST sequencing projects are being carried out for numerous organisms (Nagaraj et al., 2007). For example, a recent study used ESTs to identify microsatellite regions in channel catfish that were reported to be useful for genetic linkage mapping (Serapion et al., 2004). However, there has been very little research into ESTs for commercial species identification, and aquaculture genetics in general, most likely due to a need for greater bioinformatics capabilities (Liu and Cordes, 2004). In particular, the large volume of data generated in EST research has proven challenging to organize and analyze efficiently (Nagaraj et al., 2007).

Single nucleotide polymorphisms (SNPs). SNPs are variations in a single base pair and represent the most common polymorphism that occurs in organisms. They have gained popularity in genetic research because they can reveal differences between individuals that would not be detected using other genetic markers; they are abundant and evenly distributed throughout the genome; and they are adaptable to automation (He et al., 2003; Liu and Cordes, 2004). The most accurate and commonly used technique to analyze SNPs is direct DNA sequencing; however, SNPs can be analyzed using SSCP or heteroduplex analysis. SNPs were recently identified in catfish by comparative analysis of 849 ESTs in blue catfish (*Ictalurus furcatus*) and >11,000 ESTs from channel catfish (He et al., 2003). The authors reported ESTs to be a rich source of SNPs, which could then be used in genetic linkage mapping.

Although SNPs have proven valuable to the field of genomics, their discovery is quite challenging and can be very costly, with the need for specialized equipment (Liu and Cordes, 2004). Despite these drawbacks, analysis of SNPs with TaqMan probes was recently employed to successfully differentiate 2 eel species (Itoi et al., 2005). The TaqMan probes were designed to be species-specific based on SNPs, and PCR with these probes revealed differences in fluorescence intensity levels that could be used to verify the presence or absence of species. This method was reported to be a rapid, powerful tool for species identification using either fresh or processed samples.

2.6 COMMERCIAL APPLICATIONS

Some of the DNA-based methods discussed above for the identification of fish and seafood species have been utilized by various companies to provide food testing services or products. One example is the U.S.-based molecular diagnostics company Applied Food Technologies (http://www.appliedfoodtechnologies.com/), which uses AUTHENTI-KIT^{SM} DNA technology to identify animal species in food products, including the following fish and seafood species: channel catfish (*Ictalurus punctatus*), basa (*Pangasius bocourti*), tra (*Pangasius hypophthalmus*), Atlantic blue crab (*Callinectes sapidus*), and Asian blue swimming crab (*Portunis pelagicus*). Applied Food Technologies is currently working in collaboration with the USFDA and the Fish Barcoding of Life Initiative (FISH-BOL, discussed further in the following section), in order to standardize DNA sequencing methods for the identification of fish and seafood species (Applewhite and Bennett, 2008). Another species identification company that offers testing services for fish and seafood products is Therion International, LLC (http://www.theriondna.com/). With analyses such as mtDNA sequencing and amplification of species-specific microsatellite loci, Therion International is able to identify commonly substituted species in food products, including grouper, red snapper, mahi mahi, tuna, Chilean seabass, walleye, and zander (no scientific names given).

On the other hand, a number of companies offer commercial test kits that can be purchased for the purpose of fish species identification. For example, the biotechnology company Bionostra (http://www.bionostra.net/), located in Madrid,

Spain, offers the Fish ID Kit, which is a fish species identification kit based on amplification and analysis of mtDNA. Another Spanish biotechnology company, Biotools (www.biotools.net), offers 2 kits based on genetic markers for the detection of fish species in fresh and processed samples: (1) the BIOFISH Cod Kit, which utilizes RFLP analysis to identify cod (*Gadus morhua*), Alaska cod (*Gadus macrocephalus*), *Pollachius virens*, pollack (*Pollachius pollachius*), and Arctic cod (*Arctogadus glacialis*), and (2) the BIOFISH Salmon Kit, which allows for identification of Atlantic salmon and two trout species (*Oncorhynchus mykiss* and *Salmo trutta*). Biotools also offers a series of BIOFISH SEQ kits, which allow for species identification based on DNA sequencing for the following groups of fish: flatfish (7 species), sardines (7 species), hake (10 species), and tuna (10 species). The U.K.-based company Tepnel Life Sciences (www.tepnel.com) also offers a series of fish species identification kits that allow for the detection of cod, hake, coley, haddock, pollock, whiting, trout, and salmon (no scientific names given) in most raw and processed products. Tepnel utilizes magnetic bead technology for DNA extraction, followed by a multiplex PCR and analysis of the results with gel electrophoresis. In addition to the above diagnostic methods, a DNA microarray chip has also been utilized commercially for fish species identification by the European company bioMerieux. This DNA chip, called the FoodExpert-ID®, will be discussed further in the section dealing with current challenges and future trends.

2.7 ONLINE RESOURCES

The majority of food authentication studies have relied on the DNA database GenBank as a source of sequence information. GenBank is an expansive collection of all publicly available DNA sequences for genes in a multitude of species. This database is produced by the National Center for Biotechnology Information (NCBI) and can be accessed online at the NCBI website (http://www.ncbi.nlm.nih.gov). However, while GenBank is freely accessible and provides sequence information for many species, this database has been criticized for its susceptibility to misidentification of species or population, missing information, and inconsistent terminology. In recent years, several online resources have been developed for

specific use in the field of DNA-based identification of fish and seafood species (Table 2.3). Examples of these databases will be described in this section.

In an attempt to catalogue all life forms in DNA terms, the Consortium for the Barcoding of Life (CBOL; http://www.barcoding.si.edu/) was established. This initiative is focused on sequencing the mt COI gene in all biological species. The sector of the project focused on fish species identification is FISH-BOL (http://www.fishbol.org/), which has established barcodes for a growing number of marine and freshwater species (currently over 4500). Although data from this project may prove useful in species detection for prevention of commercial fraud, there is currently less information on COI than on the molecular marker mt cyt *b*, which is supported by more sequence data from a greater number of species (Dawnay et al., 2007). Moreover, a literature search for species identification studies using the combined databases Academic Search Premier and Agricola resulted in 288 hits with the search terms "species identification and cytochrome b or cyt b gene" and only 142 hits using the search terms "species identification and cytochrome c oxidase subunit I or COI gene." Standardizing the identification approach to be limited to COI could potentially be a major source of controversy, as it has become in the field of taxonomy (DeSalle et al., 2005). On the other hand, the compilation of sequence information for a specific gene in all species could greatly improve genetic identification techniques and provide a focused effort for fraud prevention. To this effect, USFDA researchers have recently been investigating the possibility of incorporating DNA COI Barcodes in the *Regulatory Fish Encyclopedia* (RFE) (Yancy et al., 2008).

The RFE was developed by CFSAN in an attempt to assist government officials and purchasers of seafood in the correct identification of species and detection of species substitution and economic fraud. This database can be found online (USFDA, 2009), and it currently includes detailed information on 94 commercially important fish species in the United States (Tenge et al., 1997). Specific characteristics of each fish species are readily available, including high-resolution images of the whole and filleted fish; geographic, taxonomic, and nomenclature information; and expected IEF protein patterns and analysis toolkits. In addition to

protein patterns, the organization is currently working to post the species-specific DNA patterns and sequence information for these fish. Yancy et al. (2008) recently reported the development of DNA COI Barcodes for 72 species of fish that may be used as an additional identification resource available in the RFE. The accuracy of this method was also tested for use with commercial samples. A blind study was carried out with 60 unknown fish species that were all identified correctly using the online identification engine BOLD, which is provided by the Barcode of Life data system. The supplementation of the RFE with results from the Barcode of Life project might help to provide a focused, nationwide effort for the development of species differentiation methods. Additionally, the availability of DNA Barcodes in a publicly accessible format could greatly facilitate efforts to enforce regulatory labeling laws for fish and seafood species. A recently published study reported the use of DNA barcoding to identify species in a variety of smoked fish products (Smith et al., 2008). An approximately 600-bp fragment of the COI gene was amplified from each sample, sequenced, and then matched against reference COI sequences from BOLD and GenBank. This method allowed for species identification in products representing fish species spanning 10 families and 4 orders, and it was predicted to become a standard tool for identification of fish species in food products.

Another project that has been focused on sequence information for specific genes is the FishTrace Consortium (http://www.fishtrace.org), which is comprised of 53 members from several European institutions (Sevilla et al., 2007). The FishTrace Database provides detailed information on a number of fish species common to Europe, along with DNA barcoding data for the genes mt cyt *b* and nuclear rhodopsin. The sequence data have been obtained from referenced FishTrace specimens and the database provides online tools that can be used to predict restriction enzyme cutting sites, carry out BLAST searches, and construct phylogenetic trees. The barcoding information used by FishTrace includes a longer DNA sequence than that used in COI studies, and it has been argued that the use of DNA barcodes longer in length will allow for increased efficiency of identification labels (Sevilla et al., 2007). Also, the combination of 2 genes that exhibit different genomic positions and rates of evolution,

such as mt cyt *b* and rhodopsin, was reported to be valuable for the efficiency of DNA barcoding.

A promising resource for mitochondrial sequence information of commercially important fish species in Europe is a database launched by AZTI-Tecnalia (http://www.azti.es/dna_database). This DNA database was produced in association with the traceability research sector of SEAFOODplus, an integrated seafood research project. The AZTI-Tecnalia database allows for rapid access to sequence information for fish species from 5 different families: Engraulidae, Gadidae, Merlucciidae, Scombridae, and Zeidae. More than 700 mitochondrial DNA sequences are available from different regions, including cyt b, D-loop, 16S RNA, 12S RNA, tRNA-Val, along with sequence information for 1 nuclear DNA site (tropomyosine). In addition to offering sequence information, AZTI-Technalia and SEAFOODplus are currently developing plasmidic standards to help with the validation of DNA methodologies for identifying fish and seafood species.

Another genetic database is being created by a group in Ontario, Canada, for the purpose of enforcing laws that protect endangered and exploited aquatic species (Kyle and Wilson, 2007). This database, which is not yet available online, aims to compile sequence information for a 500-bp portion of the mt cyt *b* gene in a variety of fish species. Molecular identification of species can be achieved through sequence comparisons utilizing phylogenetic analysis and a BLAST search algorithm. In order to initiate development of the database, the gene fragment was sequenced for 26 fish taxa harvested in Ontario, including fish from the families Salmonidae, Centrarchidae, Percidae, Esocidae, Acipenseridae, and Gadidae (Kyle and Wilson, 2007). This method was reported to be a highly effective tool for discrimination of harvested fish species, with great potential in the field of fisheries enforcement. In order to increase the value of information in the database, a validation system was suggested. Under this system, sequences entered for reference specimens would have to be verified by repeated analyses in an independent laboratory before they could be relied upon in forensic work.

Fish and seafood species authentication could also benefit from the development of a database that incorporates information on reference materials generated from a variety of DNA techniques. For example, a compilation of the results of RFLP analyses on scombroid species could show genes of interest, recommended restriction enzymes, and the expected restriction profiles for reference species. The chance of misidentification due to intraspecies variation would be reduced by allowing multiple laboratories to enter results from studies on scombroids from a variety of geographic locations. To this effect, a prototype database termed Genetics for Identification of Fish Origin was developed that allows for the diagnosis of fish stocks based on a variety of DNA-based methodologies, including RFLP, DNA sequencing, DNA microsatellites, and allozyme electrophoresis (Imsiridou et al., 2003). The database was created by the Joint Research Center of the European Commission, with the primary motivation being the ability to determine place of origin for commercial fish in order to prevent illegal harvests (http://fishgen.jrc.it/welcome.php3). The database includes information on genetic identification studies for 11 different species, including Atlantic cod (*Gadus morhua*), European hake (*M. merluccius*), Chinook salmon (*Oncorhynchus tshawytscha*), and Atlantic salmon.

2.8 CURRENT CHALLENGES AND EMERGING TRENDS

Some of the major challenges facing genetic food authentication research are the recovery of DNA in highly processed or complex matrices; development of methods that are more simple, rapid, and inexpensive for routine use in a regulatory setting; simultaneous identification of a wide range of species in a food; and quantification of a species in a mixed sample (Mackie et al., 1999; Martinez et al., 2005; Teletchea et al., 2005; Woolfe and Primrose, 2004). Currently, several genetic authentication methods are being investigated to meet these challenges. For example, the use of multiplex PCR with species-specific primers can increase the speed and simplicity of analysis because it does not require additional steps, such as a restriction digest, and it allows for the simultaneous detection of multiple species. Some feasible approaches that may eliminate the need for gel electrophoresis include the use of lab-

on-a-chip technology with capillary electrophoresis (Dooley et al., 2005a) and HPLC (Horstkotte and Rehbein, 2003). Another option for reducing time spent in post-PCR procedures is offered by Lonza Group Ltd. (http://www.lonzabioscience.com/prod.flash). This company has developed the FlashGel® DNA System, which uses a precast agarose gel run at high voltage to separate DNA in just 2-7 minutes. It also allows for DNA migration to be observed in real-time and does not require UV light.

2.8.1 DNA chips

DNA chips (also known as DNA microarrays or DNA macroarrays) may prove to be a valuable tool in the coming years because they have the potential to simultaneously identify up to hundreds or thousands of species (Teletchea et al., 2005). On a smaller scale, a DNA chip was developed that allowed for differentiation of 6 animal species commonly consumed in Europe (Peter et al., 2004). Universal primers were used to amplify a 377-bp fragment of the mt cyt *b* gene, and the resulting fragments could then be identified in a microarray with species-specific oligonucleotide probes. This DNA chip was able to detect species present at only 0.1% in an admixture and could identify up to 4 different species simultaneously in mixed commercial food samples. Interestingly, a commercial DNA chip-based product called the FoodExpert-ID® was launched in France in 2004 by the biological diagnostics company bioMerieux (http://www.biomerieux.com). According to the company, this product contained the first high-density DNA chip for use with species identification in food and animal feeds, and it was able to detect 33 different species of vertebrates, including 15 species of fish. However, the company does not have plans to launch the product in the U.S. and may actually discontinue the product line, as it has not yet found a strong market. Despite their potential advantages, array-based methods have not yet been heavily exploited for species identification in foods; they are still fairly inaccessible due to high costs and long start-up times. Despite these setbacks, research in this direction has continued, and a DNA microarray was recently developed to differentiate 11 commercially important fish species based on a 600-bp fragment of the 16S rDNA gene (Kochzius et al., 2008). Based on these results, a

"Fish Chip" for identification of approximately 50 species found in European Seas is currently being developed for authentication and research purposes in the fisheries industry.

2.8.2 Quantitative PCR

PCR-based techniques that allow for the quantification of target DNA include quantitative competitive PCR (QC-PCR) and real-time PCR. In QC-PCR, the same primers are used for the co-amplification of the target DNA along with an internal standard (the competitor), which differs by either having a small intron or a mutated restriction site (Gilliland et al., 1990). The relative amount of each product can then be determined based on the density of the PCR bands on an ethidium bromide-stained gel. QC-PCR has been reported to be useful in the detection and quantification of genetically modified soybean and maize in food products (Hubner et al., 1999) and porcine DNA in meat products (Wolf and Luthy, 2001). Although QC-PCR has been widely used in other fields, very few studies have utilized this technology for the detection and quantification of animal species in food products, and no published studies were found regarding QC-PCR protocols for the detection of commercial fish and seafood species.

Another way to quantitatively measure DNA is through real-time PCR methods, which use fluorescent probes to obtain results during the reaction and do not require gel electrophoresis (Figure 2.4). A number of fluorescence-based methodologies have been outlined, including the use of primers with fluorescent tags (Amplifluor™), a probe with a reporter fluorophore at one end and a quencher fluorophore at the other end (TaqMan™), 'molecular beacons' that fluoresce when bound to a specific amplicon, Scorpion™ primers, and LightCycler™ technology (Lockley and Bardsley, 2000; Marras et al., 2006). These methods are advantageous not only in their speed and simplicity, but also in the ability to quantify targeted genetic material. In fact, TaqMan probes have been investigated for their ability to detect and quantify DNA from fish species (Hird et al., 2005; Sotelo et al., 2003) and canned meat products (Laube et al., 2007a). Hird et al. (2005) reported the first successful development of a real-time PCR assay with TaqMan probes for the

quantification of whitefish. This method could be used to detect haddock in a complex food matrix containing other fish species. The methodology was optimized specifically for haddock and was able to quantify samples to within 7% of the true percentage of haddock. Because of DNA degradation during processing, this method was only reported to be useful with raw or lightly processed food products. The application of real-time PCR to multiplex assays has been reported to be effective for the differentiation of 3 species of gadoids (Taylor et al., 2002), 2 eel species (Itoi et al., 2005), and 2 tuna species (Lopez and Pardo, 2005). Real-time PCR was recently utilized in the development and design of a 'ready-to-use' reaction plate for the detection of small fragments ($\leq$ 212 bp) of DNA from 7 different animal species commonly found in processed foods (Laube et al., 2007b). Despite the advantages of real-time PCR, some limitations remain. For example, multiplex real-time reactions are generally restricted to 4 fluorogenic probe colors per tube; the size of PCR products cannot be monitored in a closed system; and some systems are not compatible with the chemical properties of fluorogenic probes (Arya et al., 2005).

2.8.3 Electrochemical DNA sensors

An innovative method for the detection of PCR products was recently described by Lai et al. (2006). This method was based on the use of electrochemical DNA (E-DNA) sensors to detect *Salmonella* Typhimurium. An advantage of E-DNA technology is its potential for use in a field-portable, hand-held species identification device. This application is not as feasible in other emerging techniques, such as lab-on-a-chip CE and fluorescence-based methodologies due to analytical needs such as power-intensive laser light sources, high numerical aperture optics, and use of relatively high voltages. Despite the potential for the use of E-DNA sensors in the detection of mislabeled fish and seafood products, analytical protocols for this purpose have not yet been developed.

2.9 CONCLUSIONS

The illegal mislabeling of fish and seafood species can have detrimental effects on both the industry and the consumer. To prevent these effects, which include economic fraud and health hazards, a research priority has been the development of

species authentication techniques that are rapid, reliable, and reproducible. These include methods based on either species-specific/multiplex PCR or post-PCR analysis methods, such as DNA sequencing, RFLP, SSCP, RAPD, and AFLP. Numerous nuclear and mitochondrial genetic markers have also been examined, with the most prominent being the mitochondrial gene cytochrome *b*. While genetic differentiation techniques have been extensively researched among certain fish groups, including the gadoids, salmonids, and scombroids, many challenges still remain. These include the optimization of methods that use smaller fragments, which can be analyzed in both raw and processed products, and the identification and quantification of species in mixed samples. In response to these challenges, future trends point to the use of technologies such as DNA microarray chips and quantitative real-time PCR methods. Furthermore, the use of databases has become increasingly important in this field by providing a compilation of genetic information on a variety of fish and seafood species.

Table 2.1 Examples of seafood substitution (USFDA, 2009). Potential economic gain represents the difference in average ex-vessel prices (U.S. landings 2006) between the 2 species groups listed. Average ex-vessel prices were obtained from Voorhees (2007). Economic gain may be higher in some circumstances, such as in the case of substitution for a particularly expensive product or when comparing prices between final products.

True identity	Mislabeled as	Potential economic gain
Rockfish	Red snapper	$5.42-6.00/kg
Yellowtail	Mahi mahi	n/a
Mako shark	Swordfish	n/a
Alaska pollock	Cod	$0.62-3.35/kg
Sea bass	Halibut	$0.71-1.79/kg
Arrowtooth flounder	Dover sole	$0.66/kg
Paddlefish and other fish roe	Caviar (sturgeon species)	>$1,000/kg[a]
Steelhead trout	Salmon	up to $3.02/kg
Farm-raised salmon	Wild salmon	up to $1.74/kg
Pink salmon	Chum salmon	$0.37/kg
Imported crabmeat	Blue crabmeat	n/a

[a]According to 2008 retail prices

Table 2.2 Comparison of major DNA-based methods used in fish and seafood species identification for the prevention of commercial fraud. This table is adapted from earlier versions by Bossier (1999) and Liu and Cordes (2004).

DNA-based method	Abbreviation	Requires prior DNA sequence information?	Quantity of loci analyzed	Robustness to DNA degradation	Potential for inter-laboratory reproducibility	Cost	Potential for database construction	Potential for intraspecies variation errors	Examples of fish and seafood species identified with method
Species-specific primers and multiplex PCR	n/a	Yes	Single	Med.-High	High	Med.	High	Med.	Flatfish, gadiformes, salmonids, scombroids, percoids, sturgeon, eels, sharks, mollusks
DNA sequencing + phylogenetic mapping	FINS	Yes	Single	Med.-High	High	High	High	Low	Cephalopods, gadiformes, mollusks
Restriction fragment length polymorphism	RFLP	Yes	Single	Med.-High	High	Med.	Med.-High	Med.	Flatfish, gadiformes, salmonids, scombroids, percoids, sturgeon, eels, mollusks
Single-stranded conformational polymorphism	SSCP	Yes	Single	Med.-High	Med.	Med.	Med.-High	Low- Med.	Salmonids, scombroids, sturgeon, eels

Table 2.2 (Continued)

Random amplified polymorphic DNA	RAPD	No	Multiple	Low-Med.	Low-Med.	Med.	Med.-High	Low-Med.	Percoids, goosefish, mollusks
Amplified fragment length polymorphism	AFLP	No	Multiple	Low-Med.	Med.-High	Med. to high	Med.-High	Low-Med.	Salmonids, scombroids

Table 2.3 Examples of online resources dedicated to DNA-based fish and seafood species identification.

Online resource	Description	Target DNA	Organization	URL
Fish Barcode of Life (FISH-BOL)	Part of the Barcoding of Life project; focused on fish species identification based on DNA sequences.	mt COI gene	Consortium for the Barcoding of Life (CBOL)	http://www.fishbol.org/
Regulatory Fish Encyclopedia (RFE)	Plans to post species-specific sequence information based on results of FISHBOL	mt COI gene	Center for Food Safety and Applied Nutrition (CFSAN)	http://www.fda.gov/Food/FoodSafety/Product-SpecificInformation/Seafood/RegulatoryFishEncyclopediaRFE/default.htm
FishTrace Database	Provides species identification tools for fish species common to Europe	mt cyt *b* and rhodopsin genes	FishTrace Consortium	http://www.fishtrace.org
AZTI-Tecnalia Database	Contains over 700 mitochondrial DNA sequences for commercially important fish species in Europe	mt DNA (cyt *b*, D-loop, 16S RNA, 12S RNA, tRNA-Val) and nuclear DNA (tropomysine)	AZTI-Tecnalia in association with SEAFOODplus	http://www.azti.es/dna_database
Genetics for Identification of Fish Origin	Focused on determining place of origin of commercial fish species; provides results of a variety of species ID methods for 11 fish species	RFLPs, DNA sequencing, DNA microsatellites and allozyme electrophoresis	Joint Research Center of the European Commission	http://fishgen.jrc.it/welcome.php3

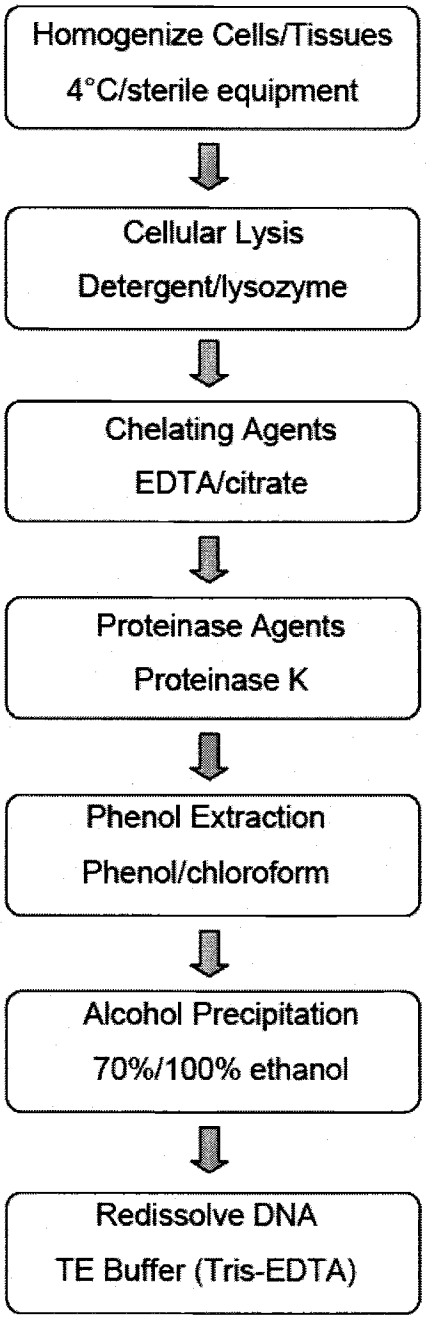

Figure 2.1 General steps in DNA extraction from cells or tissue (adapted from Rapley, 2000).

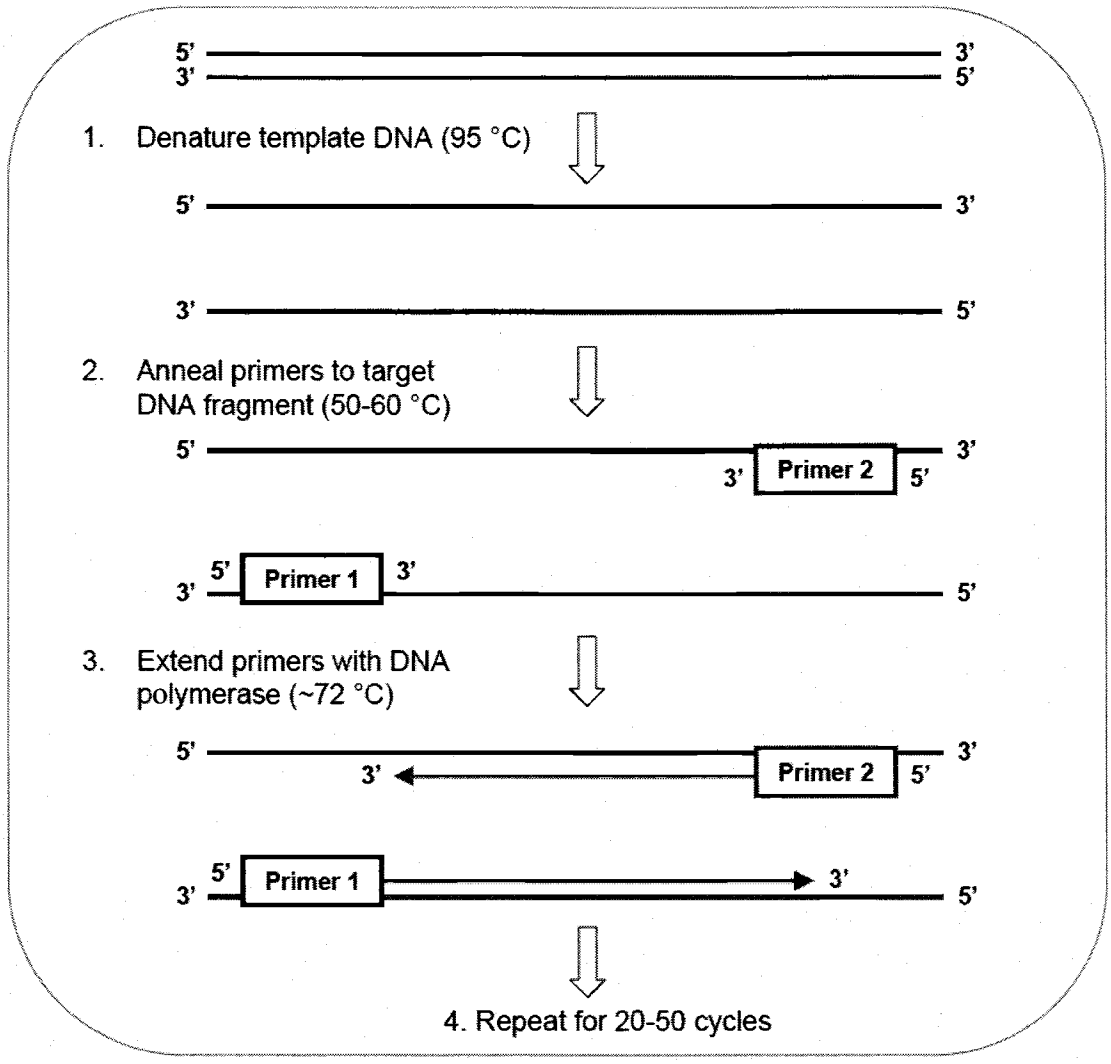

Figure 2.2 Main steps in the amplification of a target DNA fragment with the polymerase chain reaction. The DNA double helix is first denatured at a high temperature into complementary single strands; then, the temperature is reduced to allow primers to anneal to both ends of the target DNA sequence; and next, DNA polymerase extends the primers using a mixture of 4 dNTPs (dCTP, dGTP, dATP and dTTP). These three steps are usually repeated for 20-50 cycles, resulting in the production of millions of copies of the target DNA fragment.

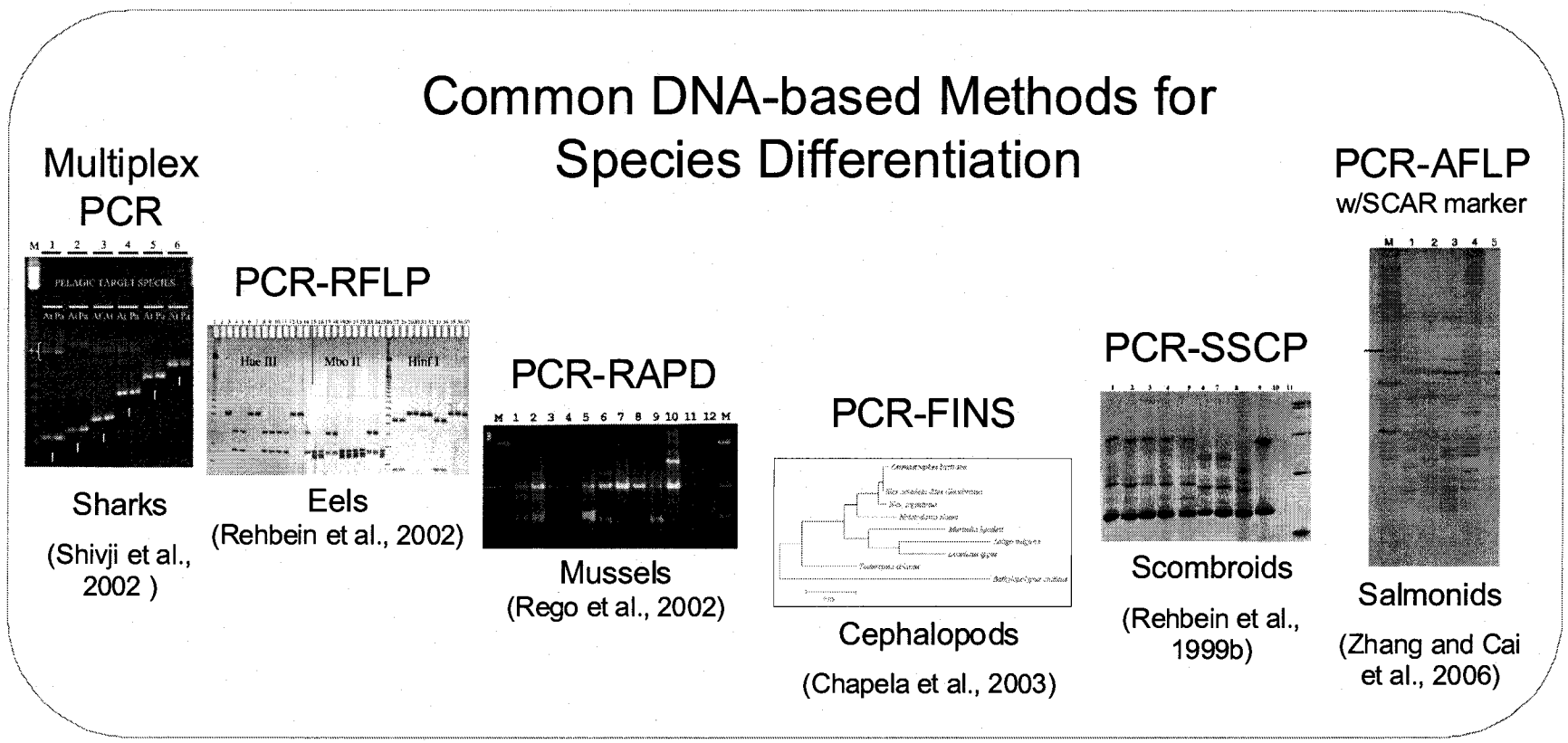

Figure 2.3. Examples of common DNA-based diagnostic methods that have been utilized for the identification of fish and seafood species.

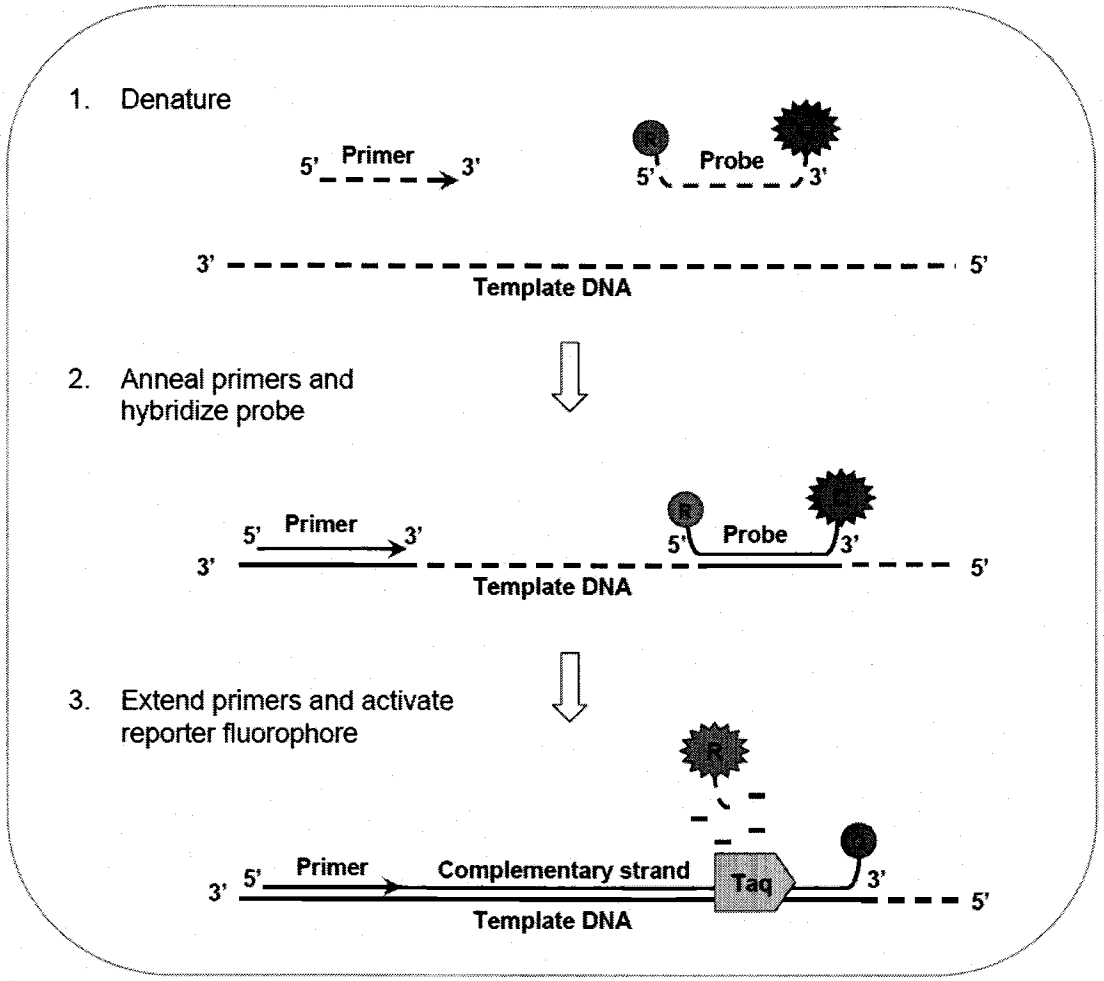

Figure 2.4 Real-time PCR using TaqMan™ probes. The probe contains a reporter (R) fluorophore and a quencher (Q) fluorophore. When the probe is intact, the quencher fluorophore prevents the reporter fluorophore from emitting fluorescence. Probes are designed to hybridize with a complementary sequence on the target DNA fragment. Following DNA denaturation, the TaqMan probe hybridizes to the target DNA and then during primer extension, *Taq* polymerase separates the reporter fluorophore from the quencher. The result is emission of a specific fluorescent signal that can be detected and quantified.

CHAPTER 3

APPLICATION OF A PCR-RFLP METHOD TO IDENTIFY SALMON SPECIES IN U.S. COMMERCIAL PRODUCTS

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3.1 ABSTRACT

A polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method for salmon species identification was optimized for use with U.S. commercial products. Reference specimens of 6 salmonid species were collected and morphologically verified. A 463-464 bp fragment of the mitochondrial $tRNA^{Glu}$/cytochrome *b* gene was PCR-amplified, digested with two restriction enzymes (Sau3AI and NlaIII), and analyzed with agarose gel electrophoresis. All 6 species were successfully differentiated with this method and the restriction digest was shortened to 1 h rather than overnight. A decision-making flowchart was developed based on these results that allows for species diagnosis within 2-3 steps. After the method was optimized, it was tested with a variety of commercial salmon products (n = 29), including canned, smoked, jerky and fresh fillet samples. Salmon species identification was successful for all 14 smoked and fresh/frozen fillet products, with the possibility of same-day species diagnosis. Species identification was also achieved for 2 out of 3 jerky products, but required overnight lysis. The remainder of the samples could not be diagnosed, including canned salmon, pouch-sterilized salmon, and canned paté. Overall, this method showed high potential for use in same-day species authentication with lightly processed seafood, but heavily processed products will require alternate methods.

3.2 INTRODUCTION

The Pacific salmon commercial fishery is an important economic staple in the United States and was valued at over US $380 million in 2007 (Voorhees, 2008). However, due to the wide variation in prices between Pacific salmon species and competition with farm-raised salmon, there is increasing concern regarding the occurrence of illegal salmon species substitution. Some examples of salmon species substitution given by the U.S. Food and Drug Administration's (FDA) *Regulatory Fish Encyclopedia* are the substitution of wild salmon with farmed salmon; substitution of chum salmon (*Oncorhynchus keta*) with pink salmon (*Oncorhynchus gorbuscha*); and substitution of salmon with steelhead trout (*Oncorhynchus mykiss*). In order to enforce labeling regulations and prevent product substitution, there is a need for reliable and accurate methods that can be used to determine the species of a seafood product with no detectable external features.

Russell et al. (2000) previously reported the ability to differentiate 10 salmonid species based on polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis of a 463-464 bp portion of the mitochondrial genome ($tRNA^{Glu}$/cytochrome *b*). Hold et al. (2001a) tested this method with 70 commercial salmon samples purchased in the United Kingdom, including smoked, pickled, and fish cake products, with successful species diagnosis in most cases. Variations of this method have also been developed for differentiation of an additional 24 species of fish, including sardines, eel, flatfish, and hakes (Hold et al., 2001b). Although this method shows promise for use in the detection of salmonid species substitution, it continues to have several limitations. For example, previous studies have called for an overnight restriction digest with 6 enzymes, eliminating the possibility of same-day species diagnosis. Indeed, Russell et al. (2000) indicated that results may take as long as 2 days to obtain. Further, while the method shows potential for use in the United States, it remains to be optimized with U.S. commercial products and species types. All commercial products examined previously by Hold et al. (2001a) were purchased in the United Kingdom, and consisted primarily of smoked Atlantic salmon, with no canned products tested. On the other hand, in the United States, a significant portion

of salmon (20%) is consumed as a canned product and Pacific salmon species occupy a considerable share of the market (~30%) (Knapp et al., 2007). Some of the salmonid species tested in previous studies are not prominent in the U.S. commercial market, such as brook trout (*Salvelinus fontinalis*) and brown trout (*Salmo trutta*), and, therefore, these species are of low concern when testing U.S. products.

The objective of this study was to apply the PCR-RFLP method developed previously (Russell et al., 2000) to species identification of commercial salmon products sold in the United States. In the first part of this study, the method was verified and optimized using reference salmon samples. Next, the optimized method was tested with a variety of commercial salmon products purchased in the United States. The 7 target salmonid species used in this study were those commonly found in U.S. commercial products: 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*).

3.3 MATERIALS AND METHODS

3.3.1 Sample collection and preparation

Reference specimens of 6 salmonid species (*S. salar*, *O. mykiss*, *O. tshawytscha*, *O. nerka*, *O. keta*, and *O. kisutch*) were obtained fresh/frozen during July and August of 2007 (Table 3.1). Each species was represented by one specimen. All specimens were morphologically identified using information from FishBase (http://www.fishbase.org) and the Washington Department of Fish and Wildlife's Pacific Salmon Identification Sheet (http://wdfw.wa.gov/fish/identification/pac_salmon_id.pdf). Each fish specimen was filleted and approximately 100 g of muscle tissue was homogenized for use in DNA extraction, PCR, and restriction digests, as described in the following sections. A whole *O. gorbuscha* reference specimen was not available at the time of this study, so identification of this species in commercial samples was based on computer-predicted restriction fragment sizes using a partial sequence of the mitochondrial genome

(GenBank accession no. AF165077) previously published for *O. gorbuscha* (Wolf et al., 2000).

Following method verification and optimization with reference samples, 29 commercial salmon products were purchased from 2 locations in Astoria, OR. The products represented 7 different species, 8 different companies, and a variety of product types, including fresh fillet, smoked salmon, canned salmon, salmon/trout paté (canned), retort pouch-sterilized salmon, and salmon jerky. A representative sample was taken from each product for use in DNA extraction, PCR, and restriction digests, as described below.

3.3.2 DNA extraction

DNA was extracted from salmonid samples using the DNeasy® Blood & Tissue Kit, Purification of Total DNA from Animal Tissues, Spin-Column Protocol (Qiagen®, Valencia, CA). All materials were sterilized prior to contact with the samples or use in the DNA extraction procedure. Cell lysis of fresh and smoked samples required 2 h, whereas canned, jerky, and paté products required an overnight lysis. A reagent blank was run with each extraction as a negative control. For DNA extraction with commercial samples, the *O. keta* reference specimen was used as a positive control.

3.3.3 PCR amplification

PCR amplification was carried out on a 463-464 bp region of $tRNA^{Glu}$/cytochrome *b*, using a pair of universal primers: L14735 (5'-AAA AAC CAC CGT TGT TAT TCA ACT A-3') and H15149ad (5'-GCI CCT CAR AAT GAY ATT TGT CCT CA-3') (Russell et al., 2000; Wolf et al., 2000). When DNA from the fresh/frozen reference specimens and from lightly processed commercial samples (i.e., fresh fillet and smoked salmon) was amplified, PCR was carried out in 50 µl volumes with the following components: 20 µl sterile nanopure water, 1.5 µl of each 10µM primer solution, 25 µl EconoTaq™ Plus Green 2X Master Mix (Lucigen® Corporation, Middleton, WI) and 2 µl template DNA. When DNA from the more heavily processed commercial products (i.e., canned salmon, canned salmon paté, pouch-sterilized salmon, and salmon jerky) was amplified, the components of the PCR

mixture remained constant, except that the volume of sterile nanopure water was reduced to 18 µl and the volume of purified DNA was increased to 4 µl. PCR was carried out with a Gene Cycler™ (Bio-Rad Laboratories, Hercules, CA) under the following conditions: an initial denaturation step carried out at 94 °C for 5 min; 35 cycles of 94 °C for 40 s, 50 °C for 80 s, and 72 °C for 80 s; and a final extension step at 72 °C for 7 min. All PCR assays were accompanied by 2 negative controls: (1) the reagent blank from DNA extraction and (2) a no-template blank, in which sterile nanopure water was used in place of template DNA. For PCR assays with commercial samples, the purified DNA from the *O. keta* salmon reference specimen was used as a positive control.

3.3.4 Restriction site analysis

Based on the results of previous studies (Russell et al., 2000; Wolf et al., 2000), the restriction enzymes NlaIII and Sau3AI were chosen for differentiation of the 7 commercial salmonid species examined in this study (*S. salar*, *O. mykiss*, *O. tshawytscha*, *O. nerka*, *O. keta*, *O. kisutch*, and *O. gorbuscha*). Reactions were carried out in a total volume of 25 µl and restriction enzymes were purchased from New England BioLabs® (Ipswich, MA). The NlaIII digest contained 4 µl sterile nanopure water, 2.5 µl 10X New England Buffer 4, 2.5 µl 10X BSA, 8 µl PCR product, and 8 µl NlaIII (1U/µl). The Sau3AI digest contained 2 µl sterile nanopure water, 2.5 µl 10X New England Buffer 4, 2.5 µl 10X BSA, 8 µl PCR product, and 10 µl Sau3AI or its isochizomer BfuCI (1U/µl). Restriction digests were carried out in separate tubes in a 37 °C water bath for one hour and the results were analyzed with agarose gel electrophoresis.

3.3.5 Gel electrophoresis

The results of the DNA extraction, PCR amplification, and restriction site analyses were visualized using agarose gel electrophoresis. Ultra Pure DNA Grade Agarose (Bio-Rad Laboratories) was dissolved in 1X Tris/Boric Acid/EDTA (TBE) Buffer (pH 8.4, Bio-Rad Laboratories) at concentrations of 1.5% (w/v) for the products of DNA extraction and PCR amplification and 2.0% (w/v) for the products of the restriction digests. A gel loading volume of 15 µl was utilized for the products of

the DNA extraction and restriction digests, while a gel loading volume of 5 µl was employed for the products of PCR amplification. A 100 bp EZ Load™ Molecular Ruler (Bio-Rad Laboratories) was also loaded into all gels for verification of DNA sizes. All gels were run at 140 V for 40 min, followed by a 15 min stain in 0.5 µg/ml ethidium bromide (Bio-Rad Laboratories), and a 15 min de-stain. The results were scanned and visualized using GelDoc™ XR and Quantity One® Software (Version 4.5.2, Bio-Rad Laboratories, 2004). A log curve was developed based on the migration distances of the molecular ruler, and this was used to calculate DNA fragment sizes for the restriction digest products. After the expected band patterns were determined for each species with the reference specimens and using computer-predicted values in the case of *O. gorbuscha*, a decision-making flowchart was developed to allow for rapid species diagnosis in commercial products (Fig. 3.1). All expected fragment sizes were rounded to 2 significant figures to account for experimental variation. This chart was utilized for species identification of the commercial samples analyzed in this study. Once an initial species diagnosis was determined with the chart, the expected fragments for that species were matched against the established reference band patterns to confirm the diagnosis.

3.4 RESULTS AND DISCUSSION

3.4.1 Reference samples

DNA extraction and amplification of the 463-464 bp fragment of $tRNA^{Glu}$/cytochrome *b* was successful with all reference samples listed in Table 3.1. As shown in Figure 3.2, the reference samples could be differentiated according to species following digestion with the restriction enzymes Sau3AI and NlaIII. Due to similarity in NlaIII band patterns for *O. kisutch*, *O. keta*, and *O. nerka* (outlined with boxes in Fig. 3.2), it was determined that a positive reference control containing *O. keta* should be run in each diagnostic gel to facilitate identification of these 3 species. In contrast with Russell et al. (2000), who called for an overnight restriction digest, results in the present study could be obtained following just a 1 h restriction digest, allowing for same-day species diagnosis.

As shown in Table 3.2, the restriction digest band sizes were slightly different from those previously reported by Russell et al. (2000), with a general trend for observed bands to be 10-40 bp smaller in the present study. However, the band sizes observed in the present study were generally closer in value to the predicted sizes calculated from DNA sequences in GenBank for these 7 salmonid species. The differences in band sizes are likely due to the use of different gel electrophoresis systems: Russell et al. (2000) utilized polyacrylamide gel electrophoresis (PAGE), while the present study employed agarose gel electrophoresis. Agarose gels are easier to use and relatively low-cost; however, they have lower resolution level than PAGE (Wilson and Walker, 2000). Despite these differences, the overall species-specific band patterns for both studies were in agreement and neither gel type was able to detect the 24 bp fragments produced by NlaIII digestion of PCR products from *S. salar*, *O. nerka*, *O. tshawytscha*, and *O. keta*. The lower resolution power of agarose gels was only a concern in the case of the *O. mykiss* NlaIII digestion, in which the 2 fragments that were close to 200 bp were clearly separated with PAGE (Russell et al., 2000), but appeared as a smear using agarose gel electrophoresis (see Fig. 3.2). However, due to the unique combination of NlaIII and Sau3AI band patterns for the *O. mykiss* DNA fragment, species diagnosis based on agarose gel electrophoresis was still possible.

3.4.2 Commercial samples

Fresh and smoked samples. As shown in Table 3.3, species diagnosis with PCR-RFLP was successful for all 14 fresh and smoked salmon products tested. Species were identified first with the decision-making flowchart (Fig. 3.1) and then the diagnosis was confirmed by comparison with the reference band patterns. This flowchart proved to be an effective tool for interpreting the results of the restriction digests, allowing for a species diagnosis within 2-3 basic steps. Of the commercial products in which salmon species was declared on the label, the laboratory diagnosis confirmed the declaration in all cases. In the one smoked product with no species declaration (no. 2), the species was determined to be *O. keta*. The use of a 1 h restriction digest greatly reduced the time required for species identification in

commercial products. Same-day species diagnosis was possible in all 14 fresh and smoked products. Figure 3.3 gives an example of the restriction digest results from analysis of several commercially smoked products (nos. 7-14). As can be observed in a comparison of Figures 3.2 and 3.3, the band quality of the lightly processed samples was slightly inferior to the band quality of the fresh/frozen reference specimens, indicating the effects of processing on DNA integrity. However, the difference in band quality did not interfere with species diagnosis in the lightly processed samples. Although the diagnostic bands for *O. nerka* (150 bp and 280 bp) were clearly present in the NlaIII digest, there was also a substantial amount of PCR product around 460 bp. The results of the Sau3AI digest for this product show only the 2 bands distinctive for *O. nerka*, indicating that the product did not contain additional species, but rather that the PCR product was not completely digested by NlaIII. Despite the incomplete digestion observed with this product, species diagnosis was still possible due to the comparison of NlaIII and Sau3AI results.

Salmon jerky. As shown in Table 3.3, species diagnosis was successful for 2 out of 3 salmon jerky products examined (nos. 15 and 16). Both jerky products, which had no species declaration, were found to contain *O. keta*. This diagnosis was confirmed by a subsequent communication with the manufacturer. Although fresh and smoked products could undergo same-day species diagnosis, the jerky products required an overnight lysis step to allow for successful PCR. The more extensive lysis required in the case of salmon jerky was likely due to the higher degree of processing involved in jerky preparation, which calls for a combination of curing, smoking and drying steps. Also, smoked salmon is known to contain PCR inhibitors, including organic and phenolic compounds and Maillard reaction products (Rossen et al., 1992; Simon et al., 1996). The more extensive smoking involved in the production of salmon jerky may have led to increased levels of these inhibitors.

Species identification in the wine-maple salmon jerky (no. 17) was not possible, even following an overnight lysis. This may have been due to the additional ingredients present in this product (i.e., sugar, soy sauce, cooking wine, and additional spices). For example, soy sauce is known to contain several compounds that induce

DNA strand breaks, including 4-hydroxy-5-methyl-3(*2H*)-furanone (HMF) along with 2,5-dimethyl-4-hydroxy-3(*2H*)-furanone (DMHF), both of which are mainly generated as a result of heating during the Maillard reaction (Colin Slaughter, 1999; Hiramoto et al., 1996; Li et al., 1998). Therefore, use of this method to diagnose species in the wine-maple jerky may require additional laboratory steps to enhance PCR and/or remove inhibitors, such as nested primer PCR (Pardo and Perez-Villareal, 2004b), treatment with hydroxyl radical scavengers (Hiramoto et al., 1996; Li et al., 1998), and ether extraction or column purification (Simon et al., 1996).

Canned and pouch-sterilized salmon product. Species identification was not possible with any of the canned (n = 11) or pouch-sterilized salmon products (n = 1) examined in this study (Table 3.3). Although an overnight lysis step did allow for successful DNA extraction from most of these heavily processed samples, the 463-464 bp fragment of $tRNA^{Glu}$/cytochrome *b* could not be readily amplified for routine species diagnosis. In the few cases where PCR fragments were obtained from these samples, the results were not easily repeated and the amplicons were too faint for reliable species identification following a restriction digest. While previous studies (Hold et al., 2001a; Hold et al., 2001b; Russell et al., 2000) reported the ability to amplify the 463-464 bp fragment of $tRNA^{Glu}$/cytochrome *b* in heat-treated samples, none of these samples represented the extreme heat treatment involved in canning salmon. The heat-treated samples tested by Hold et al. (2000) consisted of cooked fish, such as salmon fish cakes, and the heat-treatment used in 2 other studies (Hold et al., 2001b; Russell et al., 2000) consisted of placing samples in boiling water for 15 min.

In addition to the extensive heat processing used during canning, the canned paté products also contained a number of known PCR inhibitors, including milk proteins, polysaccharides, and fats, which could have contributed to the problems encountered with these products (Wilson, 1997). Furthermore, both paté products contained small amounts of salmon or trout dispersed in a complex food matrix, making it challenging to obtain a concentrated tissue sample sufficient for DNA analysis. The results of the present study were consistent with previous literature

concerning species identification in processed foods, in which DNA fragments above 350 bp have not been successfully amplified from canned fish (Bartlett and Davidson, 1992; Chapela et al., 2007; Hsieh et al., 2007; Pardo and Perez-Villareal, 2004b; Quinteiro et al., 1998; Unseld et al., 1995).

3.4.3 Current and future challenges

PCR-RFLP is generally known to be a low-cost and convenient method for species detection; however, there are a number of limitations that must be addressed in order to improve its suitability for routine laboratory species identification of commercial salmon products (Rasmussen and Morrissey, 2008). As reported in the current study and previous literature, a fragment size of 463-464 bp is not likely to survive the extreme heat treatment used for canning, and therefore a smaller-sized fragment and/or an alternative method may be necessary for analysis of heavily processed products. Also, although same-day species diagnosis was possible in the present study, gel electrophoresis remains fairly time-consuming, requiring about 70 min until the results can be analyzed. Alternatively, the use of pre-cast agarose gels or chip-based capillary electrophoresis (CE) would allow for a more rapid analysis time. Indeed, a recent study into the differentiation of *S. salar* and *O. mykiss* with PCR-RFLP reported the ability to complete analysis in a shorter time period (40 min) and detect smaller fragments (25-100 bp) using a CE system as opposed to traditional gel electrophoresis systems (Dooley et al., 2005a). These advantages will likely facilitate future species identification with this PCR-RFLP method by allowing for a wider range of detection sizes, along with a more rapid and reproducible species diagnosis.

Another challenge this method faces is the reliable analysis of a product containing multiple fish species. Although PCR-RFLP on the $tRNA^{Glu}$/cytochrome *b* fragment has been reported to be successful for species differentiation in mixtures containing up to 3 different fish species, the samples were digested with 7 different restriction enzymes and the results then had to be compared with the RFLP profiles from 36 different reference specimens (Hold et al., 2001b). In these cases, an alternative method, such as species-specific PCR, may prove to be more effective and

straightforward for routine species diagnosis (Rasmussen and Morrissey, 2008; Wolf et al., 2000).

A remaining challenge that needs to be addressed is the vulnerability of this method to intraspecies variation. Intraspecies variation can become a problem when individuals from the same species demonstrate variability at restriction enzyme cutting sites, which would result in multiple restriction digest patterns and interfere with species diagnosis. In order to improve the reliability of the PCR-RFLP method for salmonid species differentiation, the 463-464 bp fragment of $tRNA^{Glu}$/cytochrome *b* must be analyzed in numerous individuals from each species representing a wide geographic range (Russell et al., 2000; Wolf et al., 2000).

3.4.4 Conclusions

The results of this study, combined with previous publications, indicate that PCR-RFLP on the 463-464 bp fragment of $tRNA^{Glu}$/cytochrome *b* shows good potential for use in salmonid species authentication with lightly processed seafood, such as fresh fillets and commercially smoked products. The number of restriction enzymes and the restriction digest time were both reduced, allowing for same-day species diagnosis of U.S. commercial salmon. While species authentication with this method was successful in all fresh and smoked products, it was only somewhat successful with salmon jerky and it was not possible in the more heavily processed samples. These more extensively processed products may require additional treatments to enhance PCR amplification and/or the development of a different diagnostic method that targets a smaller DNA fragment. The next steps in this research will include the development of improved methods for salmonid species identification in heavily processed products and in mixed-species products.

Table 3.1 Reference salmonid specimens used in this study.

Species name	**Common name**	**Wild or farm-raised**	**Harvest region**
Oncorhynchus nerka	Sockeye salmon	Wild-caught	Juneau, Alaska
Oncorhynchus keta	Chum salmon	Wild-caught	Juneau, Alaska
Salmo salar	Atlantic salmon	Farm-raised	Campbell River, British Columbia, Canada
Oncorhynchus mykiss	Rainbow trout	Farm-raised	Idaho
Oncorhynchus kisutch	Coho salmon	Wild-caught	Youngs Bay, Oregon
Oncorhynchus tshawytscha	Chinook salmon	Wild-caught	Clatskanie River, Oregon

Table 3.2 Predicted and observed fragment sizes following digestion of the 463-464 bp segment of the cytochrome *b* gene with the restriction enzymes NlaIII and Sau3AI.

Species	**NlaIII fragment size (bp)**			**Sau3AI fragment size (bp)**		
	Observed	Previously reported (Russell et al., 2000)	Computer-calculated[a]	Observed	Previously reported (Russell et al., 2000)	Computer-calculated
S. salar	450	Uncut	24, 440	100, 380	110, 410	93, 371
O. nerka	150, 280	180, 310	24, 154, 285	120, 340	120, 390	115, 348
O. mykiss	100, 180-200[b]	100, 190, 210	91, 178, 194	460	Uncut	463
O. keta	190, 260	210, 300	194, 269	110, 330	120, 390	115, 348
O. tshawytscha	450	Uncut	24, 439	460	Uncut	463
O. kisutch	190, 240	220, 260	24, 194, 245	450	Uncut	463
O. gorbuscha	ND[c]	100, 190, 210	91, 178, 194	ND	120, 390	115, 348

[a]Based on GenBank accession numbers: AF165083 and U12143 (*S. salar*), NC_008615 (*O. nerka*), L29771 (*O. mykiss*), AF165078 (*O. keta*), AF392054 (*O. tshawytscha*), AF165079 (*O. kisutch*), AF165077 (*O. gorbuscha*)
[b]Bands appeared on the gel as a smear from 180 to 200 bp.
[c]ND = not determined

Table 3.3 Commercial products analyzed in this study and results of species diagnosis with PCR-RFLP.

Product no.	Product description	Commercial brand[a]	Country of production	Species declared	Lab diagnosis
1	Fresh trout fillet (farm-raised)	A	USA	*O. mykiss*	*O. mykiss*
2	Sliced-smoked Nova salmon (wild)	B	USA	No species given	*O. keta*
3	Smoked wild Alaskan salmon	C	USA	*O. nerka*	*O. nerka*
4	Smoked Cajun farmed salmon	C	USA	*S. salar*	*S. salar*
5	Smoked wild salmon	C	USA	*O. nerka*	*O. nerka*
6	Smoked wild salmon	D	USA	*O. tshawytscha*	*O. tshawytscha*
7	Smoked wild summer salmon	E	USA	*O. tshawytscha*	*O. tshawytscha*
8	Smoked wild fall salmon	E	USA	*O. tshawytscha*	*O. tshawytscha*
9	Smoked wild BBQ fall salmon	E	USA	*O. tshawytscha*	*O. tshawytscha*
10	Smoked wild salmon	E	USA	*O. kisutch*	*O. kisutch*
11	Smoked wild salmon	E	USA	*O. nerka*	*O. nerka*
12	Smoked pepper blend salmon	E	USA	*S. salar*	*S. salar*
13	Smoked garlic pepper salmon	E	USA	*S. salar*	*S. salar*
14	Smoked wine-maple salmon	E	USA	*S. salar*	*S. salar*
15	Smoked peppered salmon jerky (wild)	E	USA	No species given	*O. keta*
16	Jane's hot smoked salmon jerky	E	USA	No species given	*O. keta*
17	Smoked wine-maple salmon jerky	E	USA	No species given	ND[b]
18	Smoked salmon paté (canned)	F	Canada	No species given	ND
19	Trout paté (canned)	F	Canada	*O. mykiss*	ND
20	Pouch-sterilized wild Alaskan salmon	G	Thailand	*O. gorbuscha*	ND
21	Canned wild Alaskan salmon	A	USA	*O. gorbuscha*	ND
22	Canned salmon	G	Thailand	*O. gorbuscha*	ND
23	Canned wild salmon	H	Thailand	*O. gorbuscha*	ND
24	Canned Alaskan salmon	H	USA	*O. nerka*	ND
25	Canned salmon (farm-raised)	E	USA	*S. salar*	ND
26	Canned salmon	E	USA	*O. tshawytscha*	ND
27	Canned salmon	E	USA	*O. nerka*	ND
28	Canned salmon	E	USA	*O. keta*	ND
29	Canned Alaska salmon	G	USA	*O. gorbuscha*	ND

[a]Letters A-H represent the 8 different brand names of these commercial products (actual names have been omitted).
[b]ND = not determined

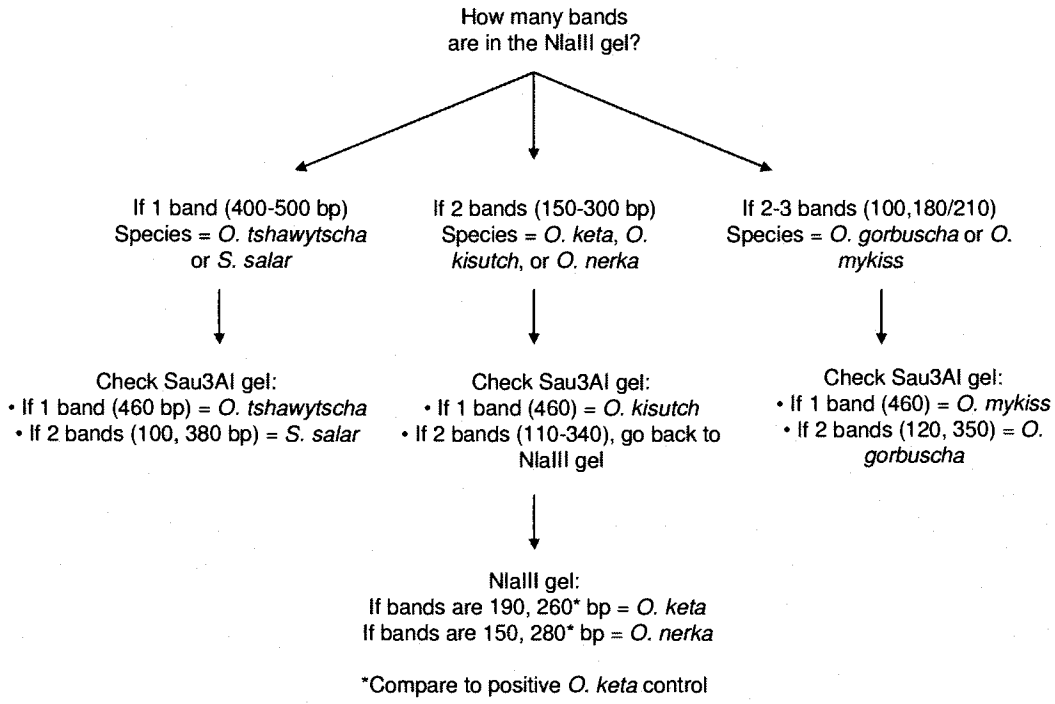

Figure 3.1 Decision-making flowchart used for salmonid species identification in commercial samples, based on the results of digestion with the restriction enzymes NlaIII and Sau3AI.

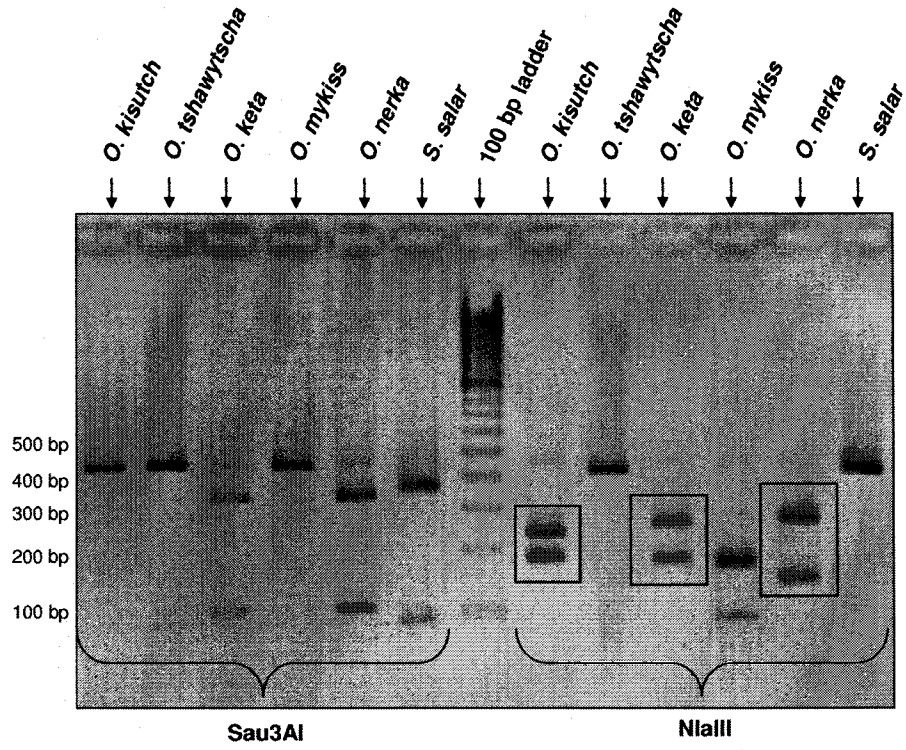

Figure 3.2 Agarose gel showing the results of restriction digests carried out with Sau3AI and NlaIII on the reference specimens described in Table 3.1. Boxed outlines indicate samples with similar-sized restriction fragments.

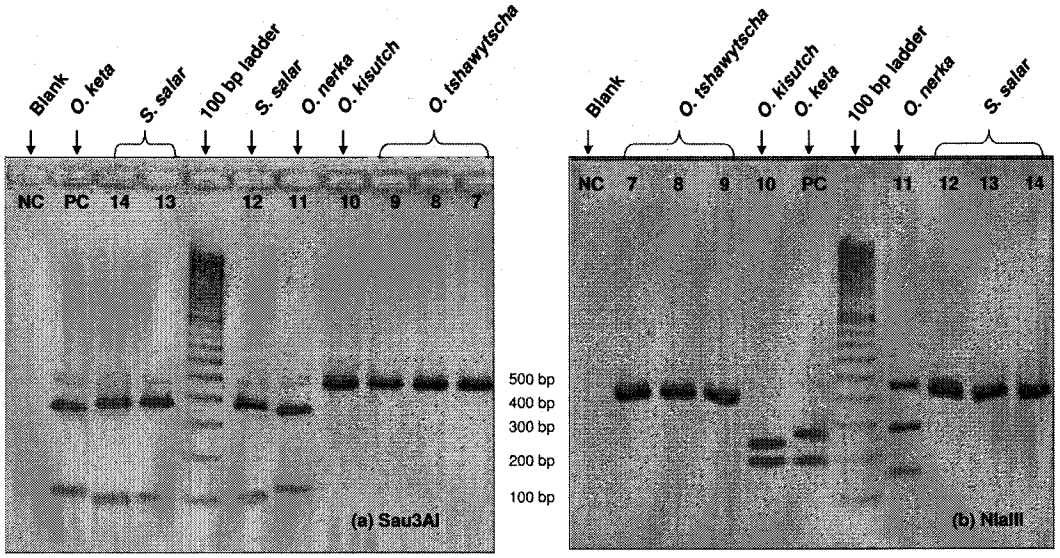

Figure 3.3 Agarose gels showing the results of restriction digests carried out with (a) Sau3AI and (b) NlaIII on the commercial products (nos. 7-14) described in Table 3.3. Lane numbers correspond to sample numbers, NC = negative control, and PC = positive control.

CHAPTER 4

DNA BARCODING OF COMMERCIALLY IMPORTANT SALMON AND TROUT SPECIES (*ONCORHYNCHUS* AND *SALMO)* FROM NORTH AMERICA

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4.1 ABSTRACT

The present study investigated the ability of DNA barcoding to reliably identify the seven commercially important salmon and trout species (genera *Oncorhynchus* and *Salmo*) in North America. More than 1000 salmonid reference samples were collected from a wide geographic range. DNA extracts from these samples were sequenced for the standard 650 bp barcode region of the cytochrome *c* oxidase subunit I gene (COI). DNA barcodes showed low intraspecies divergences (mean, 0.26%; range, 0.04-1.09%), and the mean congeneric divergence was 32-fold greater, at 8.22% (range, 3.42-12.67%). The minimum interspecies divergence was always greater than the maximum intraspecies divergence, indicating that these species can be reliably differentiated using DNA barcodes. Furthermore, several shorter barcode regions (109-218 bp), termed "mini-barcodes", were identified *in silico* that can differentiate all eight species, providing a potential means for species identification in heavily processed products.

4.2 INTRODUCTION

There are seven commercially important salmon and trout species in North America belonging to the genera *Oncorhynchus* and *Salmo*. Chinook salmon (*Oncorhynchus tshawytscha*), sockeye salmon (*Oncorhynchus nerka*), coho salmon (*Oncorhynchus kisutch*), chum salmon (*Oncorhynchus keta*), and pink salmon (*Oncorhynchus gorbuscha*) are primarily wild harvested, whereas rainbow (steelhead) trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) are sold only as farm-raised products. The wide variation in quality and availability of these salmonid species leads to substantial market differentials, with average ex-vessel/ex-farm prices per kilogram ranging from U.S. $0.29 for *O. gorbuscha* to U.S. $5.71 for *O. tshawytscha* (Johnson, 2007). In fact, prices for the highly valued spring chinook reached U.S. $22/kg (whole fish weight) in early 2009 (Fishhawk Fisheries, Inc., personal communication). After processing, species identification of salmonids becomes difficult because of the similar appearance of fillets from different species. Not surprisingly, given these value differences, the U.S. Food and Drug Administration (FDA) has detected cases of fraud involving the substitution of *O. keta* with *O. gorbuscha*, the substitution of salmon with *O. mykiss* and the substitution of wild salmon with farmed salmon (USFDA, 2009).

To advance its capacity to detect such substitutions in the marketplace, the FDA is considering the adoption of DNA barcoding as an official regulatory method, a shift that will see the incorporation of DNA barcodes into the *Regulatory Fish Encyclopedia* (Yancy et al., 2008). DNA barcoding is a method for species identification that is based on the surveillance of sequence diversity in a 650 bp region of the mitochondrial gene coding for cytochrome *c* oxidase I (COI) (Hebert et al., 2003). This gene region generally shows little variation within a species but substantial divergence between species, allowing for species differentiation. To use this approach for species identification, the DNA barcode of an unknown sample is screened against a reference sequence library and a species assignment is made when the query sequence matches just one of the species in the reference library. A reference library of DNA barcodes for all fish species is currently under assembly by

the Fish Barcode of Life campaign (FISH-BOL) (Ward et al., 2009). With records now in place for more than 6500 species, barcodes have proven to unambiguously discriminate about 93% of freshwater species and 98% of marine species.

Despite the high potential of DNA barcoding for fish identification, some salmonids may lack the diagnostic sites required for species differentiation. They are a closely related group of anadromous and non-anadromous species with marked intraspecific diversity (Waples et al., 2001), suggesting the possibility of overlap between intra- and interspecific divergences. Furthermore, while rates of nucleotide substitution in mitochondrial (mt) DNA are typically about 2% per million years, mtDNA seems to evolve more slowly in salmonids, at about 1% per million years (Smith, 1992). Perhaps as a consequence, recent studies have reported between-species divergence values that are exceptionally low (<1.0%) for some salmonids (Hubert et al., 2008; Schlei et al., 2008). Hubert et al. (2008) did obtain promising results for the seven commercially important salmonid species mentioned above, as all interspecies divergences were greater than 3%, while intraspecific divergences were below 1%; however, their sample sizes were small (2-12 per species), and all specimens were derived from Canadian waters. Moreover, another study reported very high intraspecies divergence (7.3%) in *O. mykiss* (n = 8), with one cluster showing greater similarity to *O. kisutch*, raising concerns in relation to the diagnosability of these species through DNA barcoding (Yancy et al., 2008). To determine if the DNA barcode region can reliably differentiate commercially important salmon and trout species, a thorough examination of barcode divergence within and between these species is required, including individuals from a wide geographic range.

This study involves the comprehensive analysis of DNA barcode divergences within and among key salmon and trout species (*Oncorhynchus* and *Salmo*). It examines the extent of geographic variation in barcode sequences and the clarity of the barcode gap needed for species identification. In addition, the prospects of delivering species identifications through a smaller segment of the barcode region for use in the case of heavily processed foods were explored *in silico*.

4.3 MATERIALS AND METHODS

4.3.1 Sample collection and preparation

The primary target species were *O. tshawytscha*, *O. nerka*, *O. kisutch*, *O. keta*, *O. gorbuscha*, *O. mykiss*, and *S. salar*. As well, four subspecies of cutthroat trout (*Oncorhynchus clarkii clarkii, O. c. bouvierii, O. c. utah, O. c. lewisii*) were screened because of their close relationship with the other taxa and reported hybridization with *O. mykiss* (Behnke, 1992). Reference tissue and DNA samples were obtained for 1035 specimens from the Alaska Department of Fish and Game Gene Conservation Laboratory, American Gold Seafoods, Casitas Municipal Water District, Clear Springs Foods, Creative Salmon, Idaho Department of Fish and Game, Marine Harvest Canada, National Marine Fisheries Southwest Fisheries Science Center, Oregon Department of Fish and Wildlife, Marine Fisheries Genetics Laboratory at Hatfield Marine Science Center (Oregon State University), Salmon of the Americas, Washington Department of Fish and Wildlife Molecular Genetics Lab, Pacific Salmon Treaty, and the Washington State General Fund. Samples consisted of fin clips, axillary process clips, scales, heart tissue, muscle tissue, liver tissue, and purified DNA. The purified DNA samples (n = 71) were extracted from salmonid specimens using the Qiagen DNeasy Blood and Tissue kit (Valencia, CA) and stored in AE buffer. All other samples were stored frozen, preserved in ethanol, dried, or in lysis buffer. A total of 838 samples from both wild and hatchery stocks were collected from locations in Alaska, Washington, Oregon, Idaho, Utah, and California (Fig. 4.1), representing 89 water bodies (i.e., rivers, creeks, lakes, and bays) and 143 specific sites, with an average of 5.9 individuals collected per site. In addition to the sampling locations shown in Fig. 4.1, tissue samples (n = 197) of *O. mykiss, S. salar*, and *O. tshawytscha* were acquired from aquaculture facilities in the United States (Washington and Idaho), Canada (British Columbia), and Chile. After completion of the sample collection, molecular analysis of all samples was carried out at the Canadian Center for DNA Barcoding (CCDB) at the University of Guelph, Ontario, Canada.

4.3.2 DNA extraction

DNA was extracted from tissue samples using a silica-based automated protocol, as described in Ivanova et al. (2006). DNA from 94 scale samples was eluted in 30 µl sterile ddH_2O, while DNA from all other sample types was eluted in 60 µl sterile ddH_2O. In an attempt to maximize recovery of DNA from salmon scales, an additional 94 scale samples were subjected to a semi-automated, plant-based DNA extraction protocol (Ivanova et al., 2008). The lysis step was modified to include an overnight incubation at 56 °C with 50 µl of cetyltrimethylammonium bromide (CTAB) buffer and proteinase K (20 mg/ml) instead of tissue disruption with carbide beads. DNA obtained from this protocol was eluted in 50 µl sterile ddH_2O.

4.3.3 PCR amplification

Polymerase chain reactions (PCRs) were carried out using a Mastercycler EP Gradient (Eppendorf, Brinkman Instruments, Inc., Westbury, NY). The total reaction volume was 12.5 µl and included the following components: 6.25 µl of 10% trehalose, 2.0 µl of ddH_2O, 1.25 µl of 10X PCR buffer [10 mM KCl, 10mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.8), 2 mM $MgSO_4$, 0.1% Triton X-100], 0.625 µl $MgCl_2$ (50 mM), 0.125 µl of each primer cocktail (0.01 mM), 0.0625 µl dNTPs (10 mM), 0.0625 µl *Taq* DNA polymerase (New England Biolabs, Ipswich, MA), and 2.0 µl template DNA. A set of fish primer cocktails (C_FishF1t1 and C_FishR1t1) with M13 tails was used under the following reaction conditions: 94 °C for 2 min; 35 cycles of 94 °C for 30 s, 52 °C for 40 s, and 72 °C for 1 min; and a final extension step of 72 °C for 10 min (Ivanova et al., 2007). In cases where C_FishF1t1 and C_FishR1t1 failed to generate an amplicon, an additional primer cocktail (C_VF1LFt1 and C_VR1LRt1) was used in combination with M13 tails under the following reaction conditions: 94 °C for 1 min; five cycles of 94 °C for 30 s, 50 °C for 40 s and 72 °C for 1 min; 35 cycles of 94 °C for 30 s, 54 °C for 40 s and 72 °C for 1 min; and a final extension step of 72 °C for 10 min (Ivanova et al., 2007). All primer cocktails are described in Ivanova et al. (2007). PCR products were separated on 2% agarose gels using an E-Gel96 pre-cast agarose electrophoresis system (Invitrogen, Carlsbad, CA). Images were photographed under UV light with an AlphaImager 3400 imaging system (Alpha Innotech Corp., San Leandro, CA) and processed with Invitrogen E-editor software.

4.3.4 Sequencing

PCR products were sequenced bidirectionally with BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Inc., Foster City, CA) on an ABI 3730XL DNA analyzer capillary sequencer (Applied Biosystems, Inc.). Contiguous read lengths and trace scores were generated for all sequences using Applied Biosystems sequence scanner software, version 1.0. Sequences were assembled and edited using CodonCode Aligner, version 2.0.6. All sequences were aligned in MEGA, version 3.1 (Kumar et al., 2008) before uploading to the Barcode of Life Data System (BOLD; Ratnasingham and Hebert, 2007).

4.3.5 Mini-barcodes *in silico* test

A total of 11 mini-barcode regions (107-218 bp) were analyzed *in silico* based on previously identified segments of the full-length barcode (Hajibabaei et al., 2006; Meusnier et al., 2008). Barcode sequences that were obtained in the current study were selected for mini-barcode analysis according to the following criteria: (1) original barcode sequence greater than 500 bp and (2) no gaps in the mini-barcode region. All suitable barcode sequences were examined for genetic distances in the mini-barcode region, as described in Data analysis.

4.3.6 Data analysis

The sampling locations for wild and hatchery specimens examined in this study were mapped with ESRI ArcMap 9.2 software (Environmental Systems Research Institute, Inc., Redlands, CA). Genetic distances among barcode and mini-barcode sequences were quantified using the Kimura two-parameter (K2P) distance model (Kimura, 1980) through the BOLD online interface (www.barcodinglife.org). Barcode haplotypes were identified using sequence identity matrices generated in BioEdit Sequence Alignment Editor version 7.0.9 (Hall, 1999). Neighbor-joining trees (Saitou and Nei, 1987) were generated in MEGA version 4.0 (Tamura et al., 2007) using the K2P distance model for all representative haplotypes of the full data set. All codon positions were included, and all positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons. Branch support was assessed with bootstrap analysis (1000 replicates) with sequences from *S.*

salar used to root the tree. In cases where only one individual displayed a specific haplotype, the trace files for that sequence were double-checked to ensure that no errors were made in base-calling. Regression analyses were carried out with SPSS 13.0 for Windows to determine the relationships between the number of individuals sampled per species and (1) the number of haplotypes, (2) the mean intraspecies divergence, and (3) the maximum intraspecies divergence. Significance levels were set at $p < 0.05$

4.4 RESULTS AND DISCUSSION

4.4.1 Barcode recovery

Partial or full barcode sequences (302-652 bp) were obtained from 934 of the 1035 individuals (GenBank accession nos. FJ998606-FJ999539; Appendix Table A.1). Sequences greater than 500 bp in length were recovered from 924 individuals (89%), and barcodes greater than 600 bp were recovered from 874 individuals (84%). Amplification and sequencing failures may be due to factors such as the presence of PCR inhibitors, primer mismatches, or DNA degradation (*13*). Many of the unsuccessful samples in this study consisted of degraded tissue or scales, which contain known PCR inhibitors (i.e., mucopolysaccharides). The number of sequences greater than 500 bp recovered per species ranged from 47 (*O. gorbuscha*) to 216 (*O. mykiss*), with an average of 132 individuals per species (Table 4.1). No insertions, deletions, or stop codons were observed in these sequences, indicating that all barcodes represent the functional mitochondrial COI sequence.

4.4.2 Barcode divergences and haplotypes

Genetic divergences were calculated for all COI barcodes with a sequence length greater than 500 bp, the minimum required length for formal barcode status, and less than 1% uncertain base calls (Ratnasingham and Hebert, 2007). Regression analyses indicated no significant relationships between the number of individuals analyzed per species and the mean within-species sequence divergence ($R^2 = 0.171, p = 0.154$), the maximum within-species divergence ($R^2 = 0.131, p = 0.189$), or the number of haplotypes ($R^2 = 0.38, p = 0.051$), indicating that sampling efforts on each species were sufficiently comprehensive to provide a good understanding of variation.

The average intraspecies variation (Table 4.1) ranged from a low of 0.04% in *O. keta* (maximum of 0.47%) to a high of 1.09% in *O. clarkii* (maximum of 1.96%). The restricted genetic divergence in *O. keta* supports a prior report of very low mtDNA diversity in this species from 42 populations [(n = 788) (Park et al., 1993)]. Relatively high levels of genetic divergence among cutthroat trout have also been found previously (Waples et al., 2001). An analysis of data from the "Barcoding of Canadian freshwater fishes" project on BOLD (Hubert et al., 2008) also revealed higher divergence among 12 individuals of *O. clarkii* from Canada (mean intraspecies divergence of 0.97%, maximum of 1.87%) than for other *Oncorhynchus* species.

When *O. clarkii* was excluded from the dataset, the highest mean intraspecies divergence for the seven target salmonid species dropped to 0.40% (*O. nerka*), indicating that the COI barcode region is highly conserved among these species. This conclusion agrees with Hubert et al. (2008) whose data show mean intraspecies divergence values ranging from 0% in *O. keta* (n = 2) and *O. gorbuscha* (n = 8) to 0.57% in *O. nerka* (n = 4). In contrast, a previous study examining the potential use of DNA barcodes for regulatory purposes reported a maximum of 7.3% intraspecies divergence in *O. mykiss*, an exceptionally high value (Yancy et al., 2008). The authors suggested that this case may have been due to the mislabeling of a tissue sample from *O. kisutch* as *O. mykiss*. The mean intraspecies divergence for *O. mykiss* (n = 216) in the current study was very low, at 0.14% (maximum of 0.62%), supporting the suggestion that the deeply divergent sequence in the previous study was not derived from *O. mykiss*.

Each COI barcode haplotype (n = 78) encountered in this study was restricted to a single species (Fig. 4.2), but the number of haplotypes per species ranged from a low of 3 (*S. salar*) to a high of 16 (*O. mykiss*), with an average of 10. While some haplotypes were widespread throughout the sampling range, many were restricted to a particular region. For example, 7 of the 11 haplotypes in *O. gorbuscha* were unique to Alaska, 2 were unique to Washington State, and the remaining 2 were detected in individuals from both states. For most species, the majority of individuals belonged to one or two haplotypes, while the remaining haplotypes were rare. For example, 74

individuals of *O. keta* belonged to one haplotype (HAP22), while the other 7 haplotypes for this species were observed in only 1-6 individuals. Similarly, almost half of the individuals of *O. mykiss* shared a haplotype (HAP54) that was detected in all collection states, while 11 haplotypes were unique to 1-9 individuals in Washington, Oregon, Idaho, or California. Interestingly, some fish from aquaculture broodstocks of *O. mykiss* exhibited haplotypes (HAP52, HAP57, HAP58, and HAP59) that were not detected in the wild, but other aquaculture fish from the same source shared haplotypes with wild stocks. Barcodes for *O. tshawytscha* showed a slightly different trend, with 8 haplotypes that contained more than 10 individuals each. Two of those haplotypes were unique to Oregon (HAP06 and HAP08), while one was unique to Alaska (HAP05). *O. nerka* showed a similar trend, with most samples distributed among 3 haplotypes (HAP15, HAP16, and HAP17). HAP15 (n = 17) and 2 other haplotypes were unique to Alaska, whereas 2 haplotypes were unique to Oregon, and 1 was unique to Idaho. Among the 10 haplotypes for *O. kisutch*, 6 were unique to Alaska, Washington, Oregon, or California. A previous study based on restriction site variation reported 3 COI/COII haplotypes for *O. kisutch* (n = 70) in Alaska (Gharrett et al., 2001), but this study revealed 5 haplotypes in this state. Each of the four subspecies of *O. clarkii* included in the present analysis (*O. c. clarkii*, *O. c. bouvierii*, *O. c. utah*, and *O. c. lewisii*) had at least one haplotype that was not present in the other subspecies. The only shared haplotype among *O. clarkii* subspecies was HAP73, found in both *O. c. bouvierii* and *O. c. utah* (collected from the Bear River drainage). Previous reports also have indicated that populations of *O. c. utah* from the Bear River drainage are more closely related genetically to *O. c. bouvierii* than to other populations of *O. c. utah* (Campbell et al., 2007; Martin et al., 1985).

As shown in Table 4.2, the mean divergence between species within the same genus was 8.22% (range, 3.42-12.67%), a value 32-fold greater than the mean intraspecies divergence (0.26%) for the species examined in this study. The mean intraspecies divergence found in this study was slightly lower than previous fish barcoding studies, which have reported mean conspecific divergences of 0.30% (range, 0-7.42%), 0.39% (range, 0-14.08%) and 0.99% (0.19% when possible

misidentifications were omitted) for 194 Canadian fish species (Hubert et al., 2008), 207 Australian fish species (Ward et al., 2005), and 72 U.S. commercial fish species (Yancy et al., 2008), respectively. The mean congeneric divergence between species was similar to previous studies, which have reported values of 8.29-9.93% (25 to 27-fold greater than the conspecific divergences) (Hubert et al., 2008; Ward and Holmes, 2007; Ward et al., 2005). The mean divergence between the *Oncorhynchus* and *Salmo* genera (15.65%) was also in agreement with previous values of mean divergence between fish genera within the same family (15.38-15.46%) (Hubert et al., 2008; Ward et al., 2005).

As indicated in the K2P neighbor-joining tree (Fig. 4.2), there was clear separation between species (99-100% bootstrap values) with no shared or overlapping barcodes. The nearest neighbor distances (i.e., minimum divergence between species) for the eight salmonids in this study ranged from 3.42% between *O. tshawytscha* and *O. kisutch* to 13.45% between *S. salar* and *O. nerka*. The barcode data from *S. salar* was also compared to the closely related *Salmo trutta* using samples from the Canadian freshwater fishes project. There was no overlap between the two species, and the minimum divergence was 7.28%. Within the genus *Oncorhynchus*, all nearest neighbor values were under 5.0%, with the exception of *O. nerka*, whose nearest neighbor (*O. kisutch*) was 8.13% away. These values mirror those found for the same species from Canadian waters (range within *Oncorhynchus*, 3.8-8.36%; 14.35% between *S. salar* and *O. nerka*) (Hubert et al., 2008). A neighbor-joining tree illustrating the combined data from these two projects is available as Fig. A.1 in the Appendix. Most of the nearest neighbor distances within the genus *Oncorhynchus* were lower than the average value (7.5%) reported for 194 Canadian freshwater fish species (Hubert et al., 2008). Despite the low divergences of the *Oncorhynchus* species, the high ratio of congeneric to conspecific divergence (>30-fold) ensured effective barcode-based species differentiation. Overall, the intra- and interspecific nucleotide divergence values found here are similar to those found in previous studies investigating mtDNA divergence among the Pacific salmonids (Cronin et al., 1993; McVeigh and Davidson, 1991; Park et al., 2000; Thomas and Beckenbach, 1989;

Thomas et al., 1986; Wilson et al., 1985; Wilson et al., 1987). For example, Thomas et al. (1986) found relatively low intraspecific divergence (<1%) and slightly higher interspecific divergences, ranging from 2.46% for *O. kisutch* and *O. tshawytscha* up to 6.68% for *O. kisutch* and *O. keta*, in an analysis of mtDNA restriction site cleavage for six *Oncorhynchus* species.

The interspecies divergence values found in this study can be used to estimate the divergence rate of the barcode region among the Pacific salmonids. Speciation of *O. keta, O. nerka,* and *O. gorbuscha* and speciation of *O. tshawytscha* from *O. kisutch* is believed to have occurred at least six million years ago (Augerot et al., 2005; Smith, 1992). The average interspecies divergence values within these groups were 8.27 and 4.31%, respectively. If the estimated speciation times are correct, the average barcode sequence divergence rates are 1.38% per million years for *O. keta, O. nerka,* and *O. gorbuscha* and 0.72% per million years for *O. tshawytscha* and *O. kisutch*. These rates are in general agreement with the previously estimated mtDNA divergence rate of approximately 1% per million years for some Pacific salmonids (Smith, 1992).

4.4.3 Barcode gaps

To determine if barcode gaps are present between the salmonid species examined in this study, the relationships between inter- and intraspecies divergences were compared for each species. A graphic representation was created by plotting the minimum interspecies divergence on the *y*-axis and the maximum intraspecies divergence on the *x*-axis (Fig. 4.3). The line on the graph represents cases of a 1:1 ratio between these two values. Data points above the line represent species that may be differentiated through DNA barcoding, while those falling below it represent species that cannot be differentiated through DNA barcoding. As shown in Fig. 4.3a, all salmonid species examined in this study fell above the line, indicating that they can be differentiated using DNA barcodes.

4.4.4 Mini-barcodes

Full-length DNA barcodes have been used to successfully identify fish species in a variety of commercial fish products, including fresh, smoked, and cooked fish (Smith et al., 2008; Wong and Hanner, 2008). However, it is often impossible to

recover a full-length DNA barcode from heavily processed products, such as canned fish, because of DNA degradation (Rasmussen and Morrissey, 2009). The use of shorter barcode sequences, "mini-barcodes", has been proposed as a way to enable DNA barcode analysis of degraded samples (Hajibabaei et al., 2006; Meusnier et al., 2008). Previously identified mini-barcode regions were examined for their ability to differentiate commercially important salmon and trout species (Table 4.3). Among the ~100 bp mini-barcodes, barcode gaps were present for 4-8 of the salmonid species. The mini-barcodes 109-4 and 109-5 had the ability to differentiate all eight salmonid species, with 109-5 providing slightly greater diagnostic power (Fig. 4.3b). Because of their diagnostic capabilities, these two 109 bp mini-barcodes were combined as a 218 bp region for comparison to previously identified 218 bp mini-barcodes (Hajibabaei et al., 2006). Among the 218 bp regions examined, 218-2, 218-3, and 109-4 + 109-5 showed barcode gaps for all eight species, whereas 218-1 produced barcode gaps for all species except *O. clarkii*. In a comparison of barcode gap charts for the 218 bp regions, both 218-3 and 109-4 + 109-5 exhibited the strongest species resolution. Interestingly, the mini-barcode gaps produced by the analysis of 109-5 were comparable in diagnostic strength to the 218 bp mini-barcodes, indicating that a 109 bp mini-barcode region is sufficient for species differentiation in this case. Overall, the mini-barcodes 109-5, 218-3, and 109-4 + 109-5 show the best diagnostic capabilities for the reliable identification of all eight salmon and trout species examined in this study.

4.4.5 Summary and conclusions

A comprehensive analysis of DNA barcode sequence divergences in commercially important species of North American salmon and trout species revealed mean within-species divergences that were all below 1%. No cases of shared haplotypes were detected, indicating an absence of species hybridization. The barcode region exhibited 32-fold greater divergence for congeneric species (8.22%) compared to conspecific individuals (0.26%), and all species demonstrated a barcode gap when full-length sequences were analyzed. These results indicate that DNA barcodes can reliably identify salmon and trout species in the North American commercial market.

Furthermore, three mini-barcode regions were identified to have strong diagnostic power among the salmonids, enabling differentiation of all species in this study. Future research efforts may be directed toward the development of appropriate mini-barcode primers and validation of this method in heavily processed products. Work will also be undertaken to develop a species-specific multiplex PCR assay to enable the rapid identification of salmon species in commercial food products. On a larger scale, the development of a COI barcode oligonucleotide microarray for high-throughput identification of commercial fish species is another potential area of research in this field.

Table 4.1 Salmonid species collected and sequenced for the DNA barcode region. Intraspecies genetic divergences, based on the K2P model, are reported in terms of mean ± standard deviation for barcodes greater than 500 bp (n = 924).

Species	**Number of individuals**		**Mean intraspecies divergence (%) ± SD**
	Collected	Sequenced (>500 bp)	
Oncorhynchus tshawytscha	229	212	0.38 ± 0.23
Oncorhynchus nerka	81	67	0.40 ± 0.34
Oncorhynchus keta	119	90	0.04 ± 0.08
Oncorhynchus kisutch	156	146	0.19 ± 0.17
Oncorhynchus gorbuscha	50	47	0.31 ± 0.22
Oncorhynchus mykiss	219	216	0.14 ± 0.12
Salmo salar	116	87	0.29 ± 0.29
Oncorhynchus clarkii subspp.[a]	65	59	1.09 ± 0.72

[a]*O. clarkii clarkii, O. clarkii bouvierii, O. clarkii utah, O. clarkii lewisii*

Table 4.2 Summary of the K2P genetic distances for all barcodes obtained in this study greater than 500 bp. Data are from 924 individuals representing 8 salmonid species and 2 genera (*Salmo* and *Oncorhynchus*).

Comparisons within	**Number of comparisons**	**Mean**	**Minimum**	**Maximum**	**SE**
Species	68920	0.263	0	1.955	0.001
Genus, between species	284687	8.224	3.419	12.67	0.004
Family, between genus	72819	15.65	13.45	19.72	0.003

Table 4.3 Mini-barcode regions examined in this study and salmonid species exhibiting barcode gaps in these regions. The mini-barcode regions selected for analysis were originally described in Hajibabaei et al. (2006) and Meusnier et al. (2008).

Mini-barcode	Position[a]	Salmonid sequences analyzed (n)	Salmonid species with barcode gap[b]
Universal mini-barcode	nt 1-127	822	OKi, OG, OKe, ON, SS
109-1	nt 1-109	822	OT, OKi, OKe, ON, SS
109-2	nt 110-218	921	OT, OKi, OM, ON, SS
109-3	nt 219-327	924	OG, OKe, OM, ON, SS, OC
109-4	nt 328-436	924	OT, OKi, OG, OKe, OM, ON, SS, OC
109-5	nt 437-545	923	OT, OKi, OG, OKe, OM, ON, SS, OC
109-6	nt 546-652	807	OG, OKe, ON, SS
109-4 + 109-5	nt 328-545	923	OT, OKi, OG, OKe, OM, ON, SS, OC
218-1	nt 1-218	822	OT, OKi, OG, OKe, OM, ON, SS
218-2	nt 219-436	924	OT, OKi, OG, OKe, OM, ON, SS, OC
218-3	nt 437-652	807	OT, OKi, OG, OKe, OM, ON, SS, OC

[a]Relative to the 5' end of the full-length barcode region.
[b]OT, *Oncorhynchus tshawytscha*; Oki, *Oncorhynchus kisutch*; OG, *Oncorhynchus gorbuscha*; OKe, *Oncorhynchus keta*; OM, *Oncorhynchus mykiss*; ON, *Oncorhynchus nerka*; SS, *Salmo salar*; OC, *Oncorhynchus clarkii* subspp.

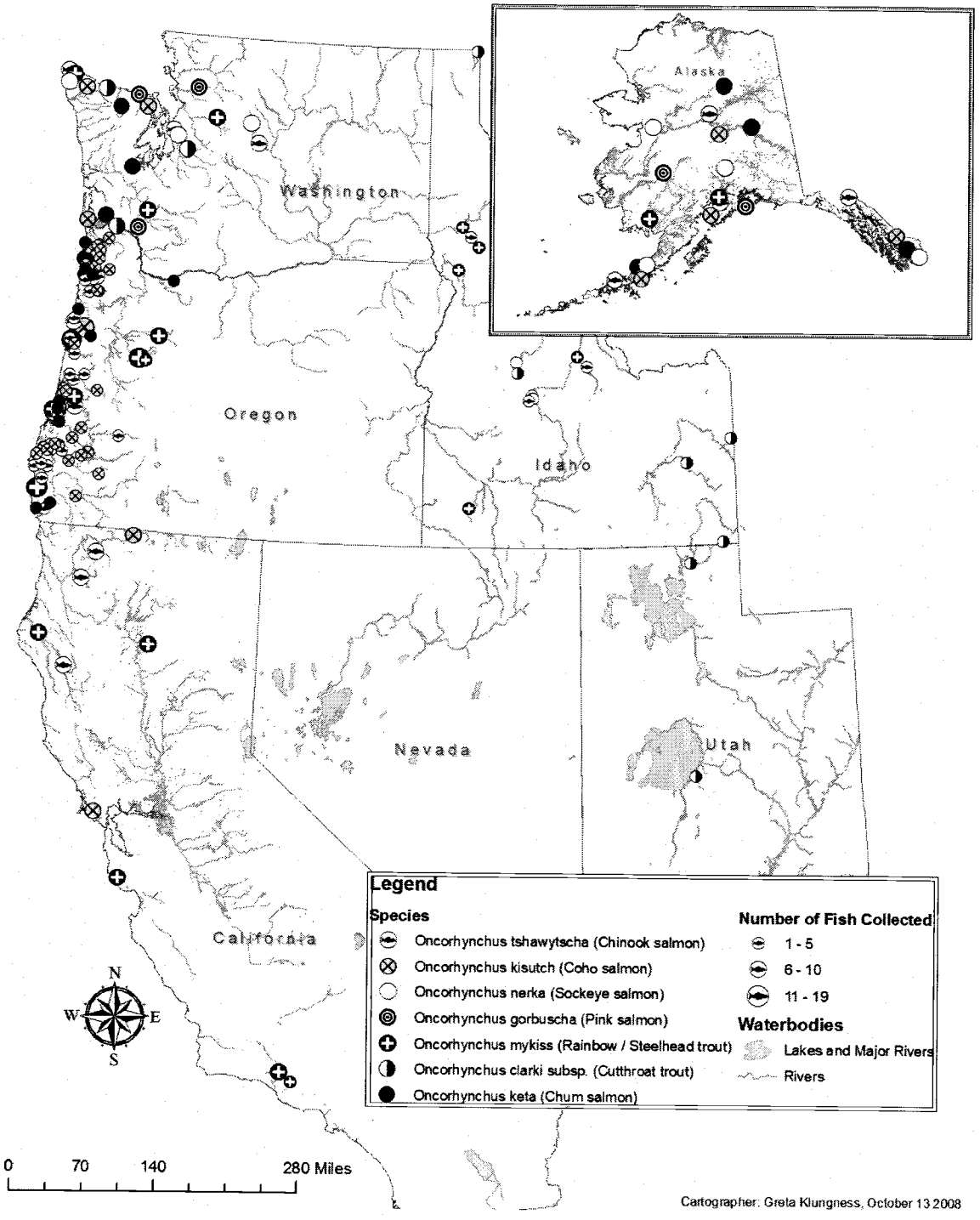


Figure 4.1 Geographic origins of reference salmonid tissues obtained in this study from wild and hatchery stocks (n = 838). Salmonids from farmed locations are not shown (n = 197). Icons are representative of the collection regions but, in some cases, do not reflect the exact site.

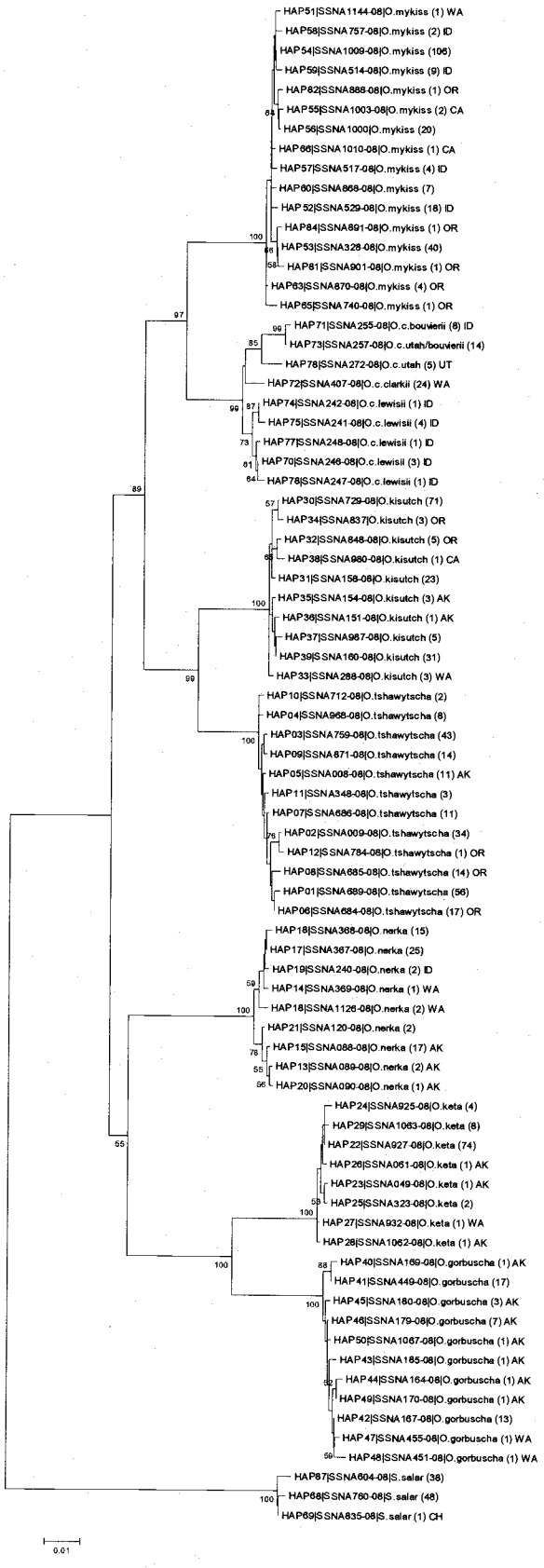
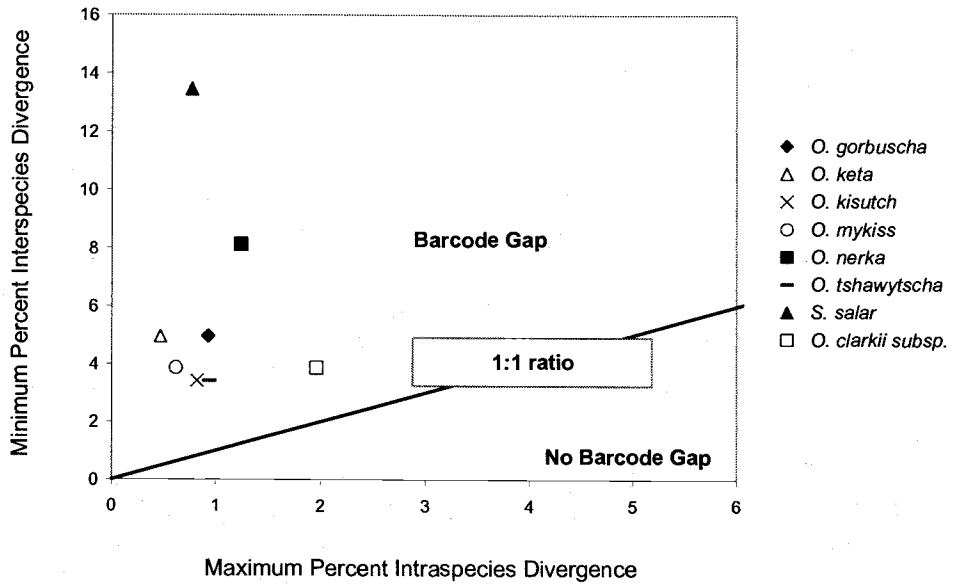


Figure 4.2 K2P neighbor joining consensus tree of all salmonid COI barcode haplotypes (n = 78) identified in this study. Bootstrap values greater than 50 are shown (1000 replicates). The tree is drawn to scale and units are the number of base substitutions per site. Branch labels include haplotype number, BOLD sample number, species, and number of individuals with this haplotype. In cases where the haplotype was found to be unique to one geographic region, the abbreviation for that region is also given (AK, Alaska; WA, Washington; OR, Oregon; CA, California; ID, Idaho; UT, Utah; CH, Chile).

(a)



(b)

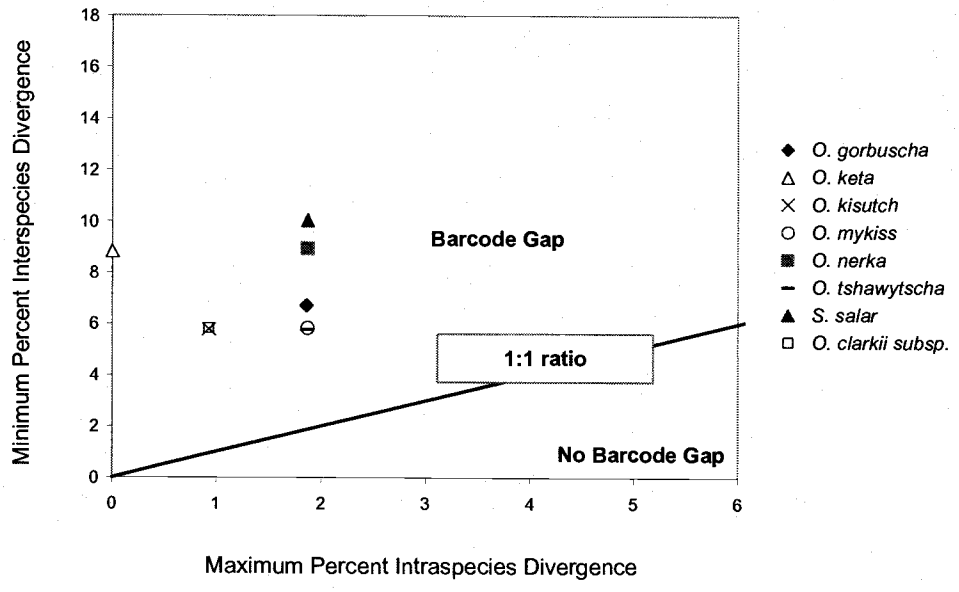


Figure 4.3 DNA barcode gaps for salmonid sequences obtained in this study with (a) COI barcodes greater than 500 bp (n = 924) and (b) COI mini-barcode 109-5 (n = 923). A data point above the 1:1 ratio line represents a species with a barcode gap (i.e., the species can be identified through DNA barcoding).

Chapter 5

A MULTIPLEX PCR ASSAY FOR THE DETECTION OF COMMERCIALLY IMPORTANT SALMON AND TROUT SPECIES (*ONCORHYNCHUS* AND *SALMO*) IN NORTH AMERICA

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5.1 ABSTRACT

The purpose of this study was to develop a species-specific multiplex polymerase chain reaction (PCR) assay 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 cytochrome *c* oxidase subunit I (COI) DNA barcode sequences. Primers and probes were combined into multiplex assays and tested for specificity against 94-112 reference samples representing 19-25 species. Sensitivity and linearity tests were conducted using 10-fold dilutions of target DNA (single-species mixtures) and three DNA admixtures containing the target species at 10%, 1.0%, and 0.1%. The specificity tests showed strong 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 (1.2-8.3%) in conventional PCR. Detection levels were similar for admixtures and single-species mixtures based on a 30 PCR cycle cut-off, with limits of 0.25 to 2.5 ng (1.0 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 a conventional or real-time format.

5.2 INTRODUCTION

The commercial salmon and trout industry in North America includes seven species from the genera *Oncorhynchus* and *Salmo*: Chinook salmon (*Oncorhynchus tshawytscha*), sockeye salmon (*Oncorhynchus nerka*), coho salmon (*Oncorhynchus kisutch*), chum salmon (*Oncorhynchus keta*), pink salmon (*Oncorhynchus gorbuscha*), rainbow (steelhead) trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*). Although these fish are closely-related, they command dramatically different prices, largely due to differences in quality, marketing, consumer demand, and competition between wild- and farm-raised salmon (Knapp et al., 2007). In general *O. tshawytscha* has been the most highly valued species, followed by *S. salar*, then *O. nerka, O. kisutch* and *O. mykiss* in the mid-range, and finally *O. keta* and *O. gorbuscha* with the lowest value. These differences have led to illegal species substitution, where a lower-value species is substituted for a higher-value species for the purpose of economic gain (USFDA, 2009). Although whole salmon and trout may be identified at the species level based on morphological factors, diagnosis becomes difficult to impossible following processing of these closely-related fish, especially with smoked or canned products.

Previous methods for the differentiation of commercially important salmon and trout species call for multiple post-polymerase chain reaction (PCR) steps, such as analysis of restriction fragment length polymorphism (RFLP) (Espiñeira et al., 2009; Horstkotte and Rehbein, 2003; McKay et al., 1997; Purcell et al., 2004; Russell et al., 2000; Withler et al., 1997) or single-stranded conformational polymorphism (SSCP) (Rehbein, 2005). While these methods are useful for species identification, they do exhibit several disadvantages that would be problematic for use in the food industry, where speed of analysis is of critical importance. Furthermore, they are susceptible to cross-contamination due to the reliance on universal primers and post-PCR procedures and are not ideal for high-throughput situations. All but the most recent of these methods are based on genetic targets of 370-3000 base pairs (bp) in length. However, these fragment sizes are not practical for use in canned products, where amplifiable

DNA fragments are generally no longer than 300-350 bp in length (Chapela et al., 2007; Hsieh et al., 2007; Pardo and Perez-Villareal, 2004a).

Species-specific multiplex PCR, which combines multiple primer sets into one tube, allows for rapid detection of species substitution in commercial fish products, including mixed-species and heavily processed samples (Rasmussen and Morrissey, 2008). Numerous species-specific multiplex PCR assays have already been developed for the authentication of fish species, such as grouper (Trotta et al., 2005), tuna (Michelini et al., 2007), rockfish (Rocha-Olivares, 1998), dolphinfish (Rocha-Olivares and Chávez-González, 2008), snappers (Bayha et al., 2008), gadoids (Taylor et al., 2002) and sharks (Pank et al., 2001; Shivji et al., 2002). With multiplex PCR, several gene targets can be amplified simultaneously and the products can be visualized following PCR with gel electrophoresis or in real time using fluorescent probes. However, species-specific primer and probe design requires detailed sequence information, including many individuals from a wide geographic range, to determine reliable diagnostic nucleotide sites. With regard to salmon species, a previous study reported the development of a multiplex PCR assay utilizing a nuclear DNA target for the identification of *O. mykiss*, *O. tshawytscha*, and *O. kisutch* (Greig et al., 2002). Although the results of this study showed successful differentiation of these three species, it did not consider identification of the four additional salmon species that are commonly sold in the North American marketplace. Also, the use of mitochondrial DNA (mtDNA) has been reported to be preferable for applications involving species identification in food systems, in part because it exhibits a high copy number and is generally easier to extract from processed samples (Civera, 2003; Rasmussen and Morrissey, 2008).

A promising mtDNA gene candidate for use in the detection of fish and seafood fraud is the cytochrome *c* oxidase subunit I (COI) gene. Previous studies have successfully utilized the COI gene for species-specific multiplex PCR detection of seafood, including sharks (Mendonça et al., 2009), bivalves (Hare et al., 2000), scallops (Marshall et al., 2007), and oysters (Wang and Guo, 2008). A comprehensive reference sequence library is currently being assembled for a 650 bp region of this

gene (the 'DNA barcode') in all fish species by the Fish Barcode of Life campaign (FISH-BOL) (Ward et al., 2009). Recently, a thorough investigation of DNA barcodes in commercially important salmon and trout species in North America was conducted, with barcode sequences obtained for 865 reference samples of the target species (Rasmussen et al., 2009). While DNA barcoding is itself a reliable method for species identification, the method requires costly equipment, is not applicable for mixed-species samples, is relatively time-consuming, and the full-length barcode is not applicable to heavily processed products. However, the compilation of DNA barcode sequence information for the commercial salmon and trout species along with over 7000 additional species already catalogued in FISH-BOL (http://www.fishbol.org/) has provided an excellent platform for the design of a robust species-specific multiplex PCR assay.

The objective of this study was to develop a species-specific multiplex PCR assay based on COI DNA barcode sequence information for the identification of the seven 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 TaqMan™ minor groove binder (MGB) probes.

5.3 MATERIALS AND METHODS

5.3.1 Multiplex PCR assay design

Species-specific primers and TaqMan MGB probes were developed for *O. tshawytscha*, *O. nerka*, *O. kisutch*, *O. keta*, *O. gorbuscha*, *O. mykiss*, and *S. salar* based on COI DNA barcode sequences with GenBank accession numbers FJ998665-FJ998742, FJ998744-FJ998759, FJ998761-FJ999106, FJ999108-FJ999276, FJ999279-FJ999493, FJ999495-FJ999507, FJ999509-FJ999526 and FJ999530-FJ999539 (Rasmussen et al., 2009); FJ164927-FJ164936 (Steinke et al., 2009); EU524202-EU524234, EU524349-EU524353, and EU525056-EU525057 (Hubert et al., 2008). A total of 915 sequences were examined for the target species, with an average of 131 sequences per species (range, 55-223). The sequences were derived from specimens representing a wide geographic range in North America, including

states/provinces in Canada (British Columbia, Quebec and Ontario) and the United States (California, Oregon, Washington, Alaska, and Idaho), as well as farmed salmonids from Chile. Sequences representing the following background salmonid species were also screened against all primers and probes to ensure specificity: *Oncorhynchus clarkii* (n = 71), *Oncorhynchus masou* (n = 5), *Salmo trutta* (n = 4), *Salvelinus alpinus* (n = 7), *Salvelinus confluentus* (n = 8), *Salvelinus fontinalis* (n = 8), *Salvelinus malma* (n = 8), and *Salvelinus namaycush* (n = 8) based on COI sequences with GenBank accession numbers FJ998606-FJ998664 (Rasmussen et al., 2009); EU522398-EU522425, EU524190-EU524201, and EU524354-EU524367 (Hubert et al., 2008); DQ533707, DQ642056, DQ656543, DQ858456, and DQ864464-DQ864465. In addition to the species-specific primers and probes, a set of universal primers and probe was developed as a control for false negatives. The universal set was designed based on mitochondrial cytochrome *b* nucleotide sequences (n = 46) for the seven target species, with GenBank accession numbers AF312563 and AF312574 (Docker and Heath, 2003); AF165077-AF165079 and AF165083 (Wolf et al., 2000); AF202032 (Phillips et al., 2000); AJ314561-AJ314564 and AJ314566-AJ314568 (Russell et al., 2000); AY032629-AY032632 (Brown and Thorgaard, 2002); DQ449932-DQ449933 and DQ449936 (Kyle and Wilson, 2007); L29771 (Zardoya et al., 1995); AB049024, AF053591, AF125208-AF125209, AF125212, AF133701, AF172395, AF392054, AY587167-AY587172, BT044011, D58401, EF055889, EF077658, EF105341, EF126369, EF455489, EU492280-EU492281, and U12143. 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 International, Palo Alto, CA) 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 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 International and Applied Biosystems, Inc. (Foster City, CA). An MGB group was conjugated to the 3'-end of each probe in order to improve specificity and increase melting temperatures (Kutyavin et al., 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 3.0% agarose gel (Henegariu et al., 1997). In some cases the primers used in conventional PCR were modified from the real-time primers in order to meet this requirement and to reduce cross-reactivity in multiplex sets. All PCR products were designed to be less than 250 bp in order 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 et al. (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 size, and final multiplex tube assignments, are given in Table 5.1.

5.3.2 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 non-salmonids *Hypsopsetta guttulata* (n = 1), *Psettichthys melanostictus* (n = 1), *Citharichtys 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 Department of Fish and Game Gene Conservation Laboratory, American Gold Seafoods, Clear Springs Foods, Creative Salmon, Idaho Department of Fish and Game, Marine Fisheries Genetics Laboratory at Hatfield Marine Science Center (Oregon State University), Marine Harvest Canada, Oregon Department of Fish and

Wildlife, Pacific Seafood, Salmon of the Americas, Washington Department 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 samples collected in a previous barcoding study (Hubert et al., 2008). These included the following background salmonids: *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 used in this study for optimization, specificity, linearity, and sensitivity tests, encompassing 25 species from multiple geographic locations in the United States (Alaska, Idaho, Oregon, Washington), Canada (British Columbia, Quebec, New Brunswick), and Chile. For small-scale testing with food samples, one fresh salmon fillet (declared species *O. kisutch*) and two smoked salmon products (one declared as *O. keta* and the other declared as *O. nerka*) were purchased from local retailers, and three canned salmon products containing *O. tshawytscha*, *O. nerka*, and *O. gorbuscha* were donated by the Seafood Products Association.

5.3.3 DNA extraction and PCR preparation

DNA extraction was carried out with the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). Samples were lysed overnight and DNA was eluted in 60-100 µl AE buffer. 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, NY) 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 et al., 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. All TaqMan MGB probes and degenerate primers were purchased from Applied Biosystems and non-degenerate primers were purchased from TriLink Biotechnologies (San Diego, CA).

5.3.4 Conventional multiplex PCR

Conventional PCR primers were combined into three 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. Primers were optimized to concentrations at which uniform amplification signals were obtained for the target fragment, without interference from nonspecific amplification. All specificity, linearity and sensitivity tests were conducted with the finalized multiplex sets and primer concentrations listed in Table 5.1. Multiplex PCR was carried out in 25 µl volumes containing 12.5 µl 2X Multiplex PCR Master Mix (Qiagen), 0.08-0.60 µM final concentration of primers (Table 5.1), 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, CA). All reactions included a no template PCR blank and reagent blanks from DNA extraction as negative controls. PCR products were analyzed using 10 µl loading volumes in 3.0% 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. All gels included an EZ Load 100 bp molecular ruler (Bio-Rad). The results were scanned and visualized using GelDoc™ XR and Quantity One® Software (Version 4.5.2, Bio-Rad Laboratories, 2004).

5.3.5 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*. Final primer and probe concentrations were determined using the same parameters described for conventional PCR. Real-time multiplex PCR was carried out in 25 µl volumes containing 12.5 µl 2X QuantiTect® Multiplex PCR NoROX Master Mix (Qiagen), 0.10-0.60 µM final concentration of primers (Table 5.1), 0.10-0.6 µM

final concentration of TaqMan MGB probes (Table 5.1), 2 µl template DNA (25 ng/µl), and sterile water. PCR cycling conditions for species-specific multiplex sets began with 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 primers and probe set (U) 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. Although signals for all target species were obtained before cycle 30, PCR was carried out for 40 cycles in order to quantify cross-reactivity with background species. All reactions included a no template PCR blank and reagent blanks from DNA extraction as negative controls. Initial tests were carried out using a SmartCycler II (Cepheid, Sunnyvale, CA) with the default program settings (baseline 3-15 cycles; threshold 30 fluorescent units) and the fluorescent reporter dyes 6-carboxy-fluorescein (FAM) and tetrachloro-6-carboxyfluorescein (TET). Finalized multiplex sets listed in Table 5.1 were tested for specificity, linearity, and sensitivity with a 7500 Real-Time PCR System (Applied Biosystems) using a baseline of 3-15 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 and NED, 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.

5.3.6 Specificity tests

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 (three individuals/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 5.2). All finalized multiplex sets were tested against 10-12 individuals from each of the target species, with a total of 73 samples representing 52 different DNA barcode haplotypes (3-11 haplotypes per species) previously identified (Rasmussen et al., 2009). All

multiplex sets were also screened against *Oncorhynchus clarkii* (n = 10) and one individual per species of the following non-salmonids: *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.

5.3.7 Sensitivity and linearity tests

Sensitivity and linearity values were determined according to a standard curve with five 10-fold dilutions ranging from 250 to 0.025 ng/µl. Reaction efficiency for real-time PCR was calculated as described in Raymaekers et al. (2009). In order to provide a representative range of detection levels, the universal set was tested for linearity against three target species (*S. salar*, *O. keta*, and *O. kisutch*) that showed a range of Ct values at 50 ng. DNA admixtures containing the target species were mixed at levels of 0, 0.1, 1.0, and 10.0% with a secondary species (Table 5.3) for a total DNA concentration of 25 ng/µl. In most cases the admixtures contained a species of lesser value mixed with a higher-value species. For real-time PCR, 2 µl of each admixture and standard curve dilution was used and 1 µl of each admixture and standard curve dilution was tested with conventional PCR. All tests were carried out in triplicate. Real-time PCR Ct values for DNA admixtures and equivalent amounts of DNA in single-species mixtures were analyzed for significant differences using a paired-samples t-test, with a pre-determined significance level of $p < 0.05$ (two-tailed). Statistical analysis was carried out with SPSS 13.0 for Windows software. Theoretical limits of detection for single-species mixtures 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 one cycle in the Ct value (Dooley et al., 2004).

5.3.8 Food samples

Following optimization and specificity tests, the newly developed primers and probes were tested against six food samples (described under sample collection). The samples underwent DNA extraction and PCR amplification as described in previous

subsections. Results for conventional PCR were recorded as band sizes on a 3.0% agarose gel and real-time PCR results were recorded as Ct values.

5.4 RESULTS AND DISCUSSION

5.4.1 Multiplex PCR assay design

The purpose of this work was to develop both a conventional and real-time multiplex PCR assay to discriminate seven commercially important salmon and trout species. As shown in Table 5.1, species-specific primers and probes were developed for all seven target species in both PCR systems, as well as a set of universal primers and probe for the detection of false-negatives, which may occur due to reagent failure, presence of inhibitors, or failures in the PCR cycling process (Rossen et al., 1992; Wilson, 1997). All species-specific primers and probes were designed to target short (< 250 bp) regions of the COI DNA barcode, whereas the universal set targets a 205 bp fragment of the cytochrome *b* gene. The universal set was designed to target a different gene region to avoid the chance of nonspecific amplification of the COI regions (e.g., a universal primer combining with a species-specific primer to amplify an additional DNA fragment). Cytochrome *b* was chosen as the gene target because, like COI, it is a relatively conserved protein-coding mitochondrial gene that has been extensively studied (Rasmussen and Morrissey, 2009) and sequence information for the target species is readily available in GenBank.

Primers ranged in length from 19 to 25 nucleotides (nt) and TaqMan MGB probes ranged from 14 to 20 nt, in accordance with previous recommendations (Henegariu et al., 1997; Kutyavin et al., 2000). The highest primer specificity is obtained by placing the diagnostic nucleotide site(s) as close to the 3'-end as possible and the highest probe specificity is obtained by placing the diagnostic site(s) in the central third of the probe, avoiding the second and third nucleotides from the 3'-end. The nucleotide sites in the primers and probes that provide specificity for each target species in this study are underlined in Table 5.1. In several cases, a combination of two or three nucleotide sites was used to provide specificity against all background species; however, for some of the primers and probes, a single diagnostic nucleotide site allowed for discrimination of the target species from all background species.

These nucleotides are underlined and in boldface type in Table 5.1. In a few cases, the specificity of a real-time PCR primer had to be reduced as compared to the conventional PCR primer in order to allow for optimal probe location and design.

As shown in Table 5.1, the optimized concentrations for real-time PCR ranged from 0.10 to 0.30 μM for primers and probes without degenerate sites and to 0.60 μM for degenerate oligonucleotides. Because degenerate oligonucleotides are actually a mixture of different sequences, only a fraction of the molecules are complementary to the template DNA and a higher concentration is generally required for amplification. The species-specific primer concentrations were reduced slightly for conventional PCR in order to avoid non-specific amplification, which began to occur as concentration was increased. These concentrations ranged from 0.08 μM to 0.20 μM for primer sets without degenerate sites and to 0.60 μM for degenerate primer sets. Overall, the optimal concentrations determined here are consistent with previous multiplex PCR optimization results (Henegariu et al., 1997).

Following singleplex optimization, primers and probes were combined into multiplex groupings targeting 2-3 species each (Table 5.1). These multiplex sets were modified for use in each PCR system to minimize non-specific amplification and allow for amplicons of diagnosable sizes in conventional PCR. Three multiplex sets were developed for use in conventional PCR and all reactions were optimized under the same PCR cycling conditions. In the case of real-time PCR, three multiplex sets were optimized at the same PCR cycling conditions, and an additional run was required for testing with the universal set. Whereas all species-specific primers and probes showed optimal results at $T_a = 63°C$, the degenerate universal set required a lower annealing temperature ($T_a = 53°C$). Single base mismatches are known to significantly reduce the melting temperature between a probe and its target (Kwok et al., 1994), and degenerate oligonucleotides generally require reduced annealing temperatures (10-15°C lower) due to these mismatches (van Pelt-Verkuil et al., 2008). Real-time PCR requires that the TaqMan probe bind the target sequence prior to primer extension and it is likely that the degeneracy of the universal probe reduced the efficiency of the reaction, thus resulting in a reduced T_a.

5.4.2 Specificity tests

Real-time multiplex PCR results. As shown in Table 5.2 and Figure 5.1, all species-specific real-time PCR assays showed strong 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 0.0000% (Ct = 39.42 ± 1.66; *O. mykiss* assay) to 0.0004% (Ct = 39.05 ± 2.26; *O. keta* assay). The maximum cross-reactivity observed ranged from 0.0014% (Ct = 35.43) for an *O. keta* sample in the *O. gorbuscha* assay to 0.1970% (Ct = 30.00) for an *O. clarkii* sample in the *O. keta* assay. These values are 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 et al., 2004). The universal assay showed a strong signal for target species (Ct = 20.23 ± 1.97), with low average cross-reactivity (0.0027%). However, since the universal set was designed to act as a control for false negatives, but not to specifically discriminate the target salmonid species from non-salmonids, Ct signals below 30 were detected with two of the non-salmonids 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.

The differences in specificity for some primers in terms of the position of diagnostic nucleotide sites did not appear to influence the Ct values determined empirically for background species, due to the strong specificity of the target probes. As shown in Fig. 5.1a-h, 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 (i.e., after the plateau phase has been reached), non-specific and incomplete products are often generated (van Pelt-Verkuil et al., 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 highly unlikely.

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 three specific reference DNA samples that gave relatively high Ct values (24.05-29.84) with the target probe. These samples also showed elevated Ct values (24.81-31.50) 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 et al., 2008). When these samples were removed from the dataset, 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 two degraded samples exhibited Ct values below 30. These results indicate that acceptable cut-off values for the detection of target species would be approximately Ct < 25 for fresh 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 5.1, but they do provide a good indicator of the specificity of the individual primer-probe sets. All average cross-reactivity values for the assays carried out at 63°C were ≤ 0.0004%, 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 Ta = 63°C reaction and therefore, the cross-reactivity was calculated to be 1.152% when samples giving

no signal were assigned a Ct value of 40. The universal set also had a delayed signal in the duplex reaction for the seven species targeted in this study, with an average Ct value of 27.89 ± 1.77, and an average cross reactivity with background salmonids of 0.5689%. 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 above, 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 5.2, the conventional multiplex PCR assays also exhibited consistent detection for the target species, with 100% of target samples showing the expected PCR product in gel electrophoresis. Five of the seven species-specific primer sets showed 0% cross-reactivity with background salmonids and all of the primer sets showed 0% cross-reactivity with non-salmonids. The positioning of diagnostic sites in the conventional PCR primers did not result in any obvious differences in cross-reactivity or specificity. Figure 5.2 gives an example of the agarose gel results with analysis of one sample per species against all three multiplex sets. All target bands could be differentiated in 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. The amplicon bands for these samples were visible, but faint compared to other reference samples.

Although the universal set did show reactivity in the real-time assay with two of the non-salmonids tested, there were no visible bands in the agarose gel for any of these species following the conventional PCR assay. This is probably due to the differences in annealing temperature used for the universal set in real-time PCR (53°C) versus conventional PCR (63°C), since a lower annealing temperature is known to reduce primer specificity.

Non-specific amplification. Despite the positive results found for most of the samples tested in the conventional multiplex PCR assay, there were a few cases of non-specific

amplification (that is, unexpected products, ghost bands, and cross-reactivity). Non-specific PCR products are typically easy to detect, as they produce smears or products of incorrect length in gel electrophoresis (van Pelt-Verkuil et al., 2008). The occurrence of an unexpected product due to multiplexing was observed in two of the ten *O. keta* samples tested against the MKe multiplex set, which exhibited a very faint band around 160 bp in addition to the target band (Fig. 5.3d). This is believed to be the result of the *O. mykiss* forward primer and the *O. keta* reverse primer binding to *O. keta* template DNA to produce a PCR product (predicted size 156 bp). Although this result is undesirable, it should not interfere with the ability to detect the target species with this multiplex set, as the expected species-specific bands are 104 bp (*O. keta*) and 73 bp (*O. mykiss*).

In a few cases (n = 5) during reference sample screening, an unexpected barely visible or 'ghost band' was observed in the agarose gel. PCR was repeated in duplicate for the DNA extracts of these samples and only in one case was the ghost band found to be recurring. An *O. clarkii* sample screened against the GKU multiplex set repeatedly gave a ghost band at the expected size for *O. gorbuscha* (143 bp) (Fig. 5.3c). The DNA template sequence for this sample was screened against the *O. gorbuscha* primers and no additional nucleotide matches were found. It is possible that the *O. clarkii* DNA became contaminated with a very small amount of *O. gorbuscha* DNA. Additional tissue was not available for this sample, so further testing could not be conducted. Although this ghost band is of concern, it would be unlikely to cause a false positive result due to its extreme faintness in the gel.

As reported in Table 5.1, false positive bands were detected in 8.3% of samples tested against the *O. nerka* primers. These bands were relatively faint compared to the bands from *O. nerka* samples and all corresponded to reference samples of *O. mykiss* (Fig. 5.3a). The *O. nerka* primer sequences do show a closer match to the template DNA of the *O. mykiss* samples than to the template DNA of other species, but each primer still contains a diagnostic site near the 3'-end and would not be expected to amplify *O. mykiss* DNA. The DNA extraction step was repeated for four of the samples that had additional tissue available and these samples were

then run through PCR and gel electrophoresis. These samples showed positive bands with the *O. mykiss* primers and extremely faint false positive bands with the *O. nerka* primers (Fig. 5.3b). Based on these results, it appears that the *O. nerka* primers may be exhibiting low levels of cross-reactivity with *O. mykiss* DNA, resulting in faint false positive bands for some samples. 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 (e.g., with the real-time PCR method described above) to verify species. Despite the cross-reactivity detected with *O. mykiss*, the *O. nerka* primers were still able to differentiate the target species from all other commercially important salmon species tested here and will still be useful in species identification.

Overall, faint false positive bands were detected at levels of 1.2-8.3% for two out of seven species-specific primer sets. 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 (Hare et al., 2000; Hill et al., 2001; Rocha-Olivares, 1998). 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.

5.4.3 Sensitivity and linearity tests

Table 5.3 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 Fig. 5.4, using the *S. salar* in *O. tshawytscha* admixture. In most cases, individual assays showed similar sensitivity levels for target DNA in admixtures compared to single-species mixtures, with no significant differences ($p < 0.05$) between Ct values. These results indicate that sensitivity is generally not reduced for the target species when combined with another species. There were only four instances in which a significant difference ($p < 0.05$) was found and there was no apparent trend for detection of the target species in one

mixture to be greater than the detection in the other mixture. In two cases, the detection of the target species in an admixture was reduced compared to detection in single-species mixtures (*S. salar* at 1.0% and 10%) and in the other two cases the detection of the target species in admixtures was greater than the detection in single-species mixtures (*O. tshawytscha* at 0.1% and *O. kisutch* at 0.1%).

Because target sensitivity values detected after 30 cycles would not be readily discriminated from non-specific amplification, a cut-off of Ct < 30 was used to determine detection levels. Real-time PCR Ct values for target species in admixtures at 0.1% (0.05 ng) were above 30.00 for all species except *O. mykiss* (28.73 ± 1.24) and *O. gorbuscha* (29.57 ± 29.57). At 1.0% (0.5 ng) admixture levels, Ct values dropped below 30.00 for *S. salar, O. keta, O. nerka,* and *O. kisutch*. The *O. tshawytscha* real-time assay showed the least sensitive detection levels, with Ct values of 30.25 ± 0.45 at 1.0% and 25.39 ± 0.19 at 10% (5.0 ng). For the rest of the target species, the average Ct value at 10% admixture levels ranged from 22.10 ± 0.06 (*O. mykiss*) to 24.49 ± 0.17 (*O. nerka*). The single-species detection limits at Ct < 30 were 0.05 ng for *O. gorbuscha, O. mykiss,* and *O. kisutch*, and 0.5 ng for the remaining species. These results generally corresponded with or were slightly higher than the theoretical detection limits for Ct < 30 (calculated amount of DNA at Ct = 29.99 based on average Ct for 50 ng of target species), which ranged from 0.01 ng for *O. mykiss* to 0.10 ng for *O. keta*. The universal set showed detection of target DNA at levels of 0.5 to 5.0 ng in single-species mixtures (results not shown), with Ct < 30. The empirically determined detection level for the universal set was much higher than the theoretical limit (0.06 ng), probably due to a reduced ability of the degenerate primers and probe to anneal to low amounts of template DNA. Overall, the empirically determined sensitivity levels for real-time PCR (Ct < 30) ranged from 0.1 to 10% in admixtures and 0.05 to 0.5 ng in single-species mixtures. These results are similar to previous studies investigating real-time PCR detection in meat systems, which have shown empirically determined admixture detection limits of 0.1-0.5% in 50 ng DNA (Dooley et al., 2004) and a single-species limit of 2 ng DNA (Brodman and Moor, 2003) for Ct < 30.

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 mixtures (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 mixtures (Matsunaga et al., 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 1-5% (0.5-5 ng) in DNA admixtures and 2-5% (1-5 ng) in freeze-dried salmon fillet admixtures (Dooley et al., 2005b).

As shown in Fig. 5.5, the results of the standard curves in species-specific real-time PCR assays showed strong 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 is likely due 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 et al. (2009), who stated that efficiency should be 90-110% and the R^2 value should be 0.99-0.999.

5.4.4 Food samples

A small-scale test with six commercial salmon products was carried out with both the real-time and multiplex PCR assays developed in this investigation (Table 5.4). Both assays allowed for a positive species diagnosis, based either on a Ct value

below 25-30 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 5.2), 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. 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 universal Ct values for the food samples in Table 5.4 tended to be similar to or higher than the average Ct values found with reference samples. The universal Ct value was higher than average for all canned samples, especially in the case of *O. tshawytscha*. As discussed in the specificity section, a cut-off value of Ct < 30 should be considered for degraded samples, such as canned products. A larger-scale test of these methods with commercial products will be necessary to examine species identification and recommended cut-off values for processed products.

5.4.5 Conclusions and summary

In this project, a multiplex PCR assay was developed for the identification of seven commercially important salmon and trout species in both real-time and conventional formats. Both systems were able to successfully identify the target species, even in heavily processed food products. The conventional multiplex PCR assay showed 0% non-specific amplification for five of the species-specific primer sets, and faint false positive bands detected at low levels (1.2-8.3% of reference samples) for two of the primer sets. The real-time multiplex PCR assay also showed minimal levels of cross-reactivity (0.0000% to 0.1970%), with no non-specific amplification prior to 30 cycles. Both assays showed good sensitivity for the target DNA in admixtures and single-species mixtures, with detection as low as 0.1% (0.05

ng) in real-time PCR (for Ct < 30) and 1.0% (0.25 ng) in conventional PCR. These assays allow for rapid species diagnosis following DNA extraction, requiring ~4 h with conventional PCR and ~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, which contain pre-mixed and aliquoted PCR mixes and may be stored frozen for up to three months (Ivanova et al., 2005). 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. The use of a lower reaction volume (e.g., 12.5 µl) could further reduce the cost per reaction. The next step in this research 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.

Table 5.1 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. All species-specific primers and probes target regions of the COI DNA barcode, whereas the universal set targets a fragment of the cytochrome *b* gene.

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	AGCAGAACTCAGCCA**G**CC**T**	0.10	214 bp	STKe
		R	AAAGGAGGGAGGGAG**A**AGTCA**A**	0.20		
		P	6FAM-CCTTCT**G**GG**A**GATGACC-MGB	0.14		
	Conventional	F	same as real-time	0.13	219 bp	STN
		R	AGAAGAAAGGAGGGAGGGAG**A**	0.13		
Chum salmon (*Oncorhynchus keta*)	Real-time	F	TTGTCTGAGCTGT**A**CTA**A**T**C**ACTG	0.20	104 bp	STKe
		R	AAGTGGTGTTTAAATTTCG**A**TC	0.20		
		P	VIC-CAACATAGT**A**AT**A**CCTGCTG-MGB	0.10		
	Conventional	F	same as real-time	0.15	104 bp	MKe
		R	same as real-time	0.15		

Table 5.1 (Continued)

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	GGAGCCTCAGTTGATCTRACG	0.60	103 bp	STN
		R	GGGGTTTTATGTTAATAATGGTAG	0.60		
Pink salmon (*Oncorhynchus gorbuscha*)	Real-time	F	TACGACCATTATCAACATAAAACCA	0.30	143 bp	GM
		R	GGTCCGTGAGCAACATAGTG	0.20		
		P	6FAM-CGGCAATCTCTCAGT-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	TAACTAGCACGGCTCAAACG	0.20		
Sockeye salmon (*Oncorhynchus nerka*)	Real-time	F	GGAAACCTTGCCCACGCG	0.20	152 bp	NK
		R	AAAAGTGGGGTCTGGTACTGAG	0.30		
		P	6FAM-CTCTGTTGACTTAACCATC-MGB	0.13		
	Conventional	F	CCAGCCATCTCTCAGTACCAGA	0.08	183 bp	STN
		R	GAGGTGTTGGTATAAAATCGGAT	0.08		
Coho salmon (*Oncorhynchus kisutch*)	Real-time	F	CGCTCTTCTAGGGGATGATC	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: STKe = *S. salar*, *O. tshawytscha*, and *O. keta*; GM = *O. gorbuscha* and *O. mykiss*; NK = *O. nerka* and *O. kisutch*; U = universal set/positive control. Multiplex sets for conventional PCR: STN = *S. salar*, *O. tshawytscha*, and *O. nerka*; MKe = *O. mykiss* and *O. keta*; GKU = *O. gorbuscha*, *O. kisutch*, and universal set.

Table 5.2 Specificity of the real-time and conventional PCR assays with 50 ng 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 et al. (2004), where a difference of 1 Ct represents a cross-reactivity of 50%.

Target species	**Target individuals tested (n)**	**Background individuals tested for cross-reactivity (n)**	**Real-time PCR results**			**Conventional PCR results**	
			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 (0.0027%)	23.48 with *C. sordidus* (10.50%)	100%	0%
S. salar	10	84	19.03 ± 0.77	39.76 ± 1.36 (0.0001%)	30.84 with *O. tshawytscha* (0.0280%)	100%	0%
O. keta	10	84	21.02 ± 3.54	39.05 ± 2.26 (0.0004%)	30.00 with *O. clarkii* (0.1970%)	100%[a]	0%
O. tshawytscha	12	82	20.50 ± 2.15	38.74 ± 2.45 (0.0003%)	31.75 with *O. keta* (0.0411%)	100%[a]	0%
O. gorbuscha	10	84	19.32 ± 1.05	39.86 ± 0.62 (0.0001%)	35.43 with *O. keta* (0.0014%)	100%	1.2% (false positive ghost band with one *O. clarkii* sample)

Table 5.2 (Continued)

O. mykiss	11	83	17.67 ± 1.15	39.42 ± 1.66 (0.0000%)	31.82 with *O. nerka* (0.0055%)	100%	0%
O. nerka	10	84	19.56 ± 1.06	38.59 ± 2.84 (0.0002%)	30.42 with *O. keta* (0.0536%)	100%	8.3% (false positives with 7 *O. mykiss* samples)
O. kisutch	10	84	19.37 ± 1.56	39.24 ± 1.86 (0.0001%)	31.70 with *O. gorbuscha* (0.0194%)	100%	0%

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

Table 5.3 Results of sensitivity tests for target DNA in admixtures and single-species mixtures 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.

Target species	Spike level of target species	Mixer species	Real-time PCR results		Conventional PCR results	
			Ct ± SD for target species in admixture	Ct ± SD in single-species mixture	Band visibility for target species in admixture	Band visibility in single-species mixture
S. salar	0.0%	***O. tshawytscha***	40.00 ± 0.00	n/a	Not visible	n/a
	0.1%		31.84 ± 0.17[a]	31.72 ± 0.12[a]	Very faint/not visible	Not visible
	1.0%		27.33 ± 0.02[a]	28.03 ± 0.18[b]	Visible	Visible
	10%		24.13 ± 0.07[a]	24.71 ± 0.19[b]	Visible	Visible
O. keta	0.0%	***O. tshawytscha***	39.58 ± 0.72	n/a	Not visible	n/a
	0.1%		31.05 ± 0.05[a]	30.80 ± 0.34[a]	Not visible	Not visible
	1.0%		27.20 ± 0.08[a]	27.45 ± 0.12[a]	Visible	Faint
	10%		23.96 ± 0.13[a]	23.70 ± 0.05[a]	Visible	Visible
O. tshawytscha	0.0%	***S. salar***	40.00 ± 0.00	n/a	Not visible	n/a
	0.1%		33.68 ± 0.31[a]	32.56 ± 0.21[b]	Not visible	Not visible
	1.0%		30.25 ± 0.45[a]	29.27 ± 0.09[a]	Very faint/not visible	Very faint
	10%		25.39 ± 0.19[a]	25.82 ± 0.14[a]	Visible	Visible

Table 5.3 (Continued)

O. gorbuscha	0.0%	***O. keta***	38.41 ± 1.37	n/a	Not visible	n/a
	0.1%		29.57 ± 0.16[a]	29.74 ± 0.14[a]	Very faint	Not visible
	1.0%		26.41 ± 0.65[a]	26.38 ± 0.09[a]	Visible	Faint
	10%		22.84 ± 0.32[a]	22.93 ± 0.10[a]	Visible	Visible
O. mykiss	0.0%	***S. salar***	40.00 ± 0.00	n/a	Not visible	n/a
	0.1%		28.73 ± 1.24[a]	28.68 ± 0.21[a]	Not visible	Not visible
	1.0%		25.50 ± 0.09[a]	25.25 ± 0.07[a]	Visible	Faint
	10%		22.10 ± 0.06[a]	21.69 ± 0.36[a]	Visible	Visible
O. nerka	0.0%	***O. tshawytscha***	40.00 ± 0.00	n/a	Not visible	n/a
	0.1%		30.80 ± 0.30[a]	30.91 ± 0.24[a]	Not visible	Not visible
	1.0%		27.76 ± 0.34[a]	27.50 ± 0.22[a]	Visible	Faint
	10%		24.49 ± 0.17[a]	23.91 ± 0.13[a]	Visible	Visible
O. kisutch	0.0%	***O. tshawytscha***	40.00 ± 0.00	n/a	Not visible	n/a
	0.1%		30.67 ± 0.26[a]	29.26 ± 0.10[b]	Faint	Faint
	1.0%		25.14 ± 0.58[a]	25.39 ± 0.59[a]	Visible	Visible
	10%		21.83 ± 0.13[a]	22.20 ± 0.10[a]	Visible	Visible

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

Table 5.4 Real-time and conventional PCR results of small-scale testing with commercial salmon products.

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*

Figure 5.1 Results of real-time PCR specificity tests with (a) universal and (b-h) species-specific multiplex assays. Relative fluorescent units are plotted on the *y*-axis and cycle number is plotted on the *x*-axis. All lines crossing the threshold before cycle number 30 (indicated by a vertical dashed line) correspond to the target species in each species-specific assay; lines crossing the threshold after 30 cycles are a result of nonspecific amplification.

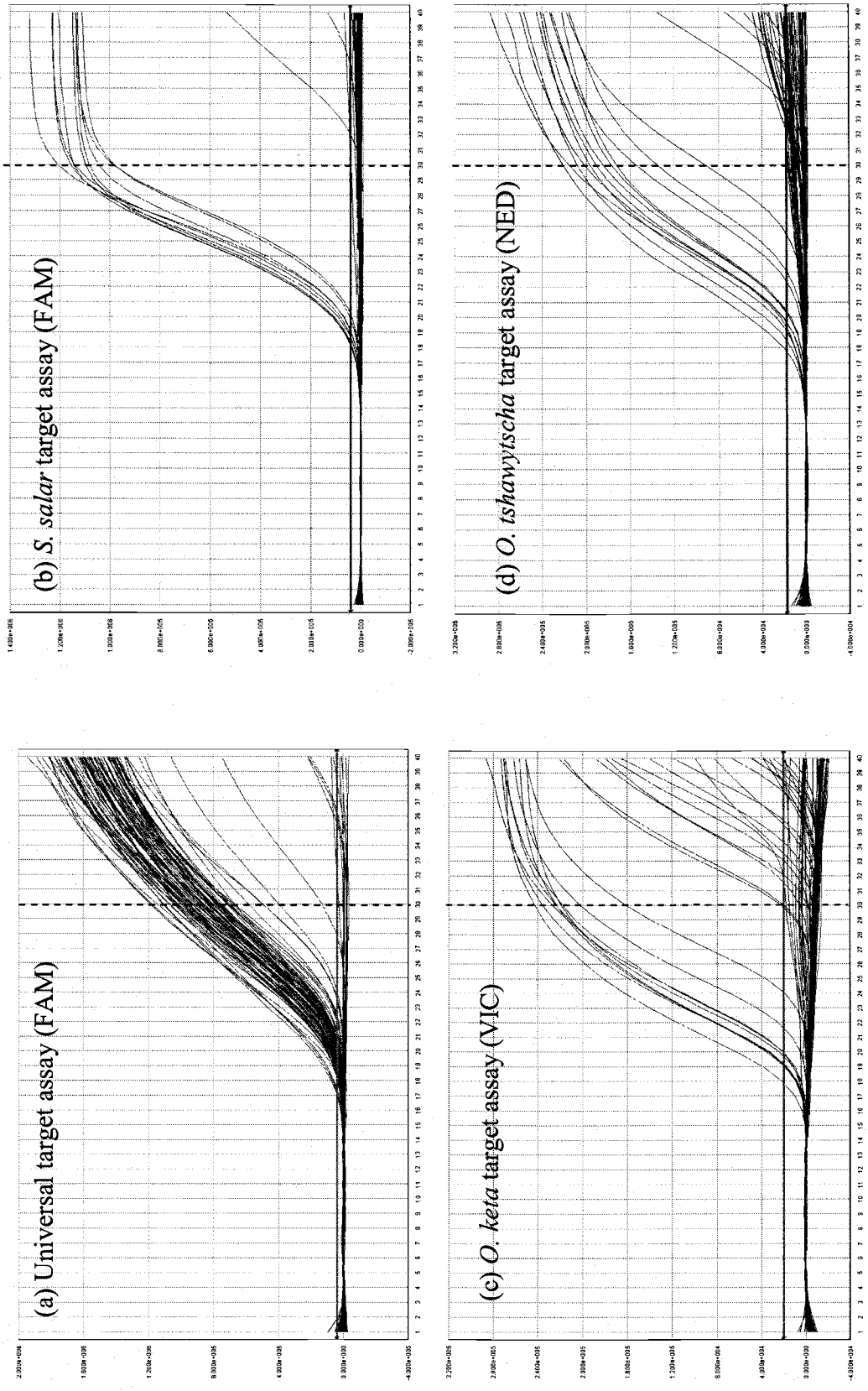

(a) Universal target assay (FAM)
(b) S. salar target assay (FAM)
(c) O. keta target assay (VIC)
(d) O. tshawytscha target assay (NED)

Figure 5.1

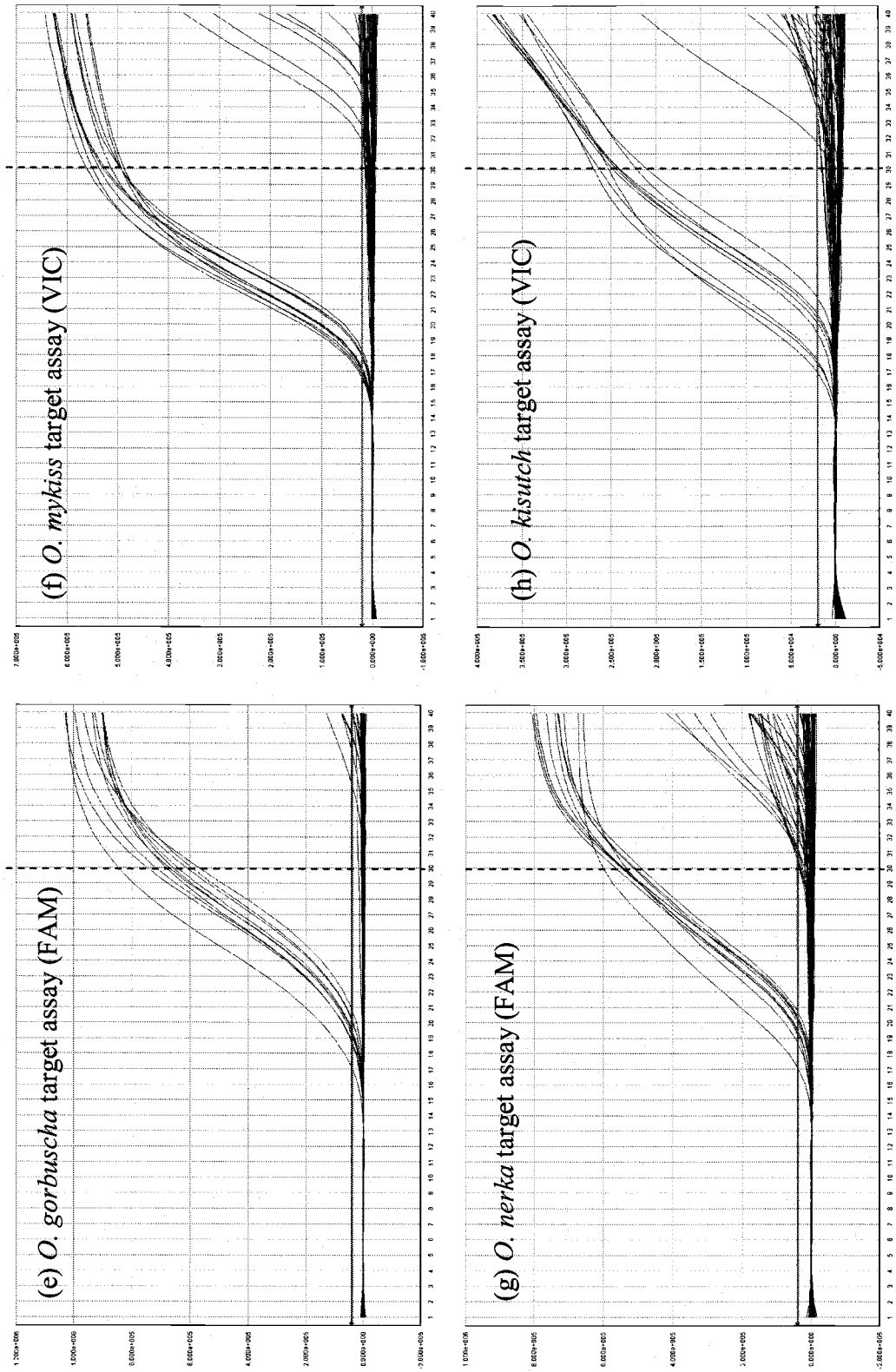


Figure 5.1 (Continued)

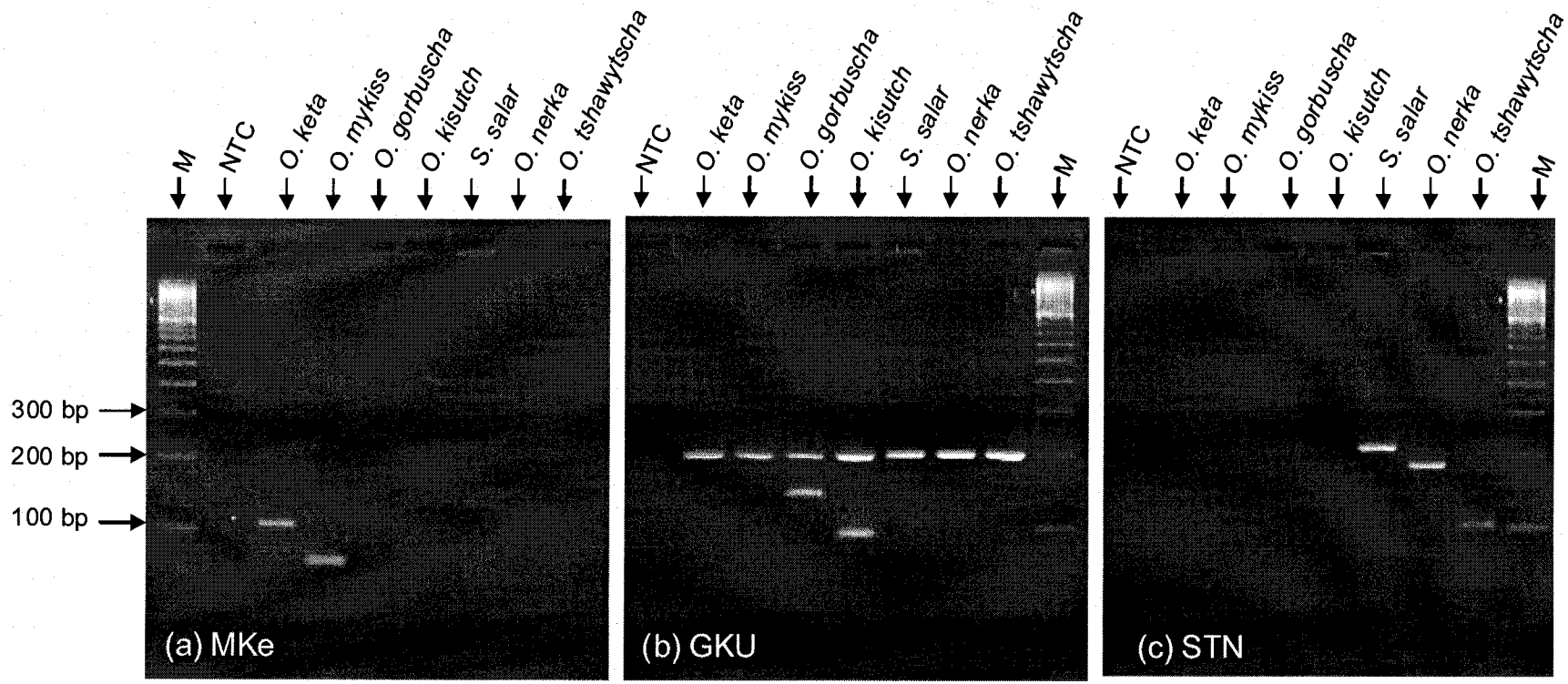


Figure 5.2 Results of conventional multiplex PCR specificity testing as visualized with agarose gel electrophoresis. The three agarose gel photos illustrate the species-specific and universal bands occurring for the seven target salmon and trout species (25 ng DNA) tested against the three multiplex 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 a 100 bp molecular ruler (M) and a no-template control (NTC).

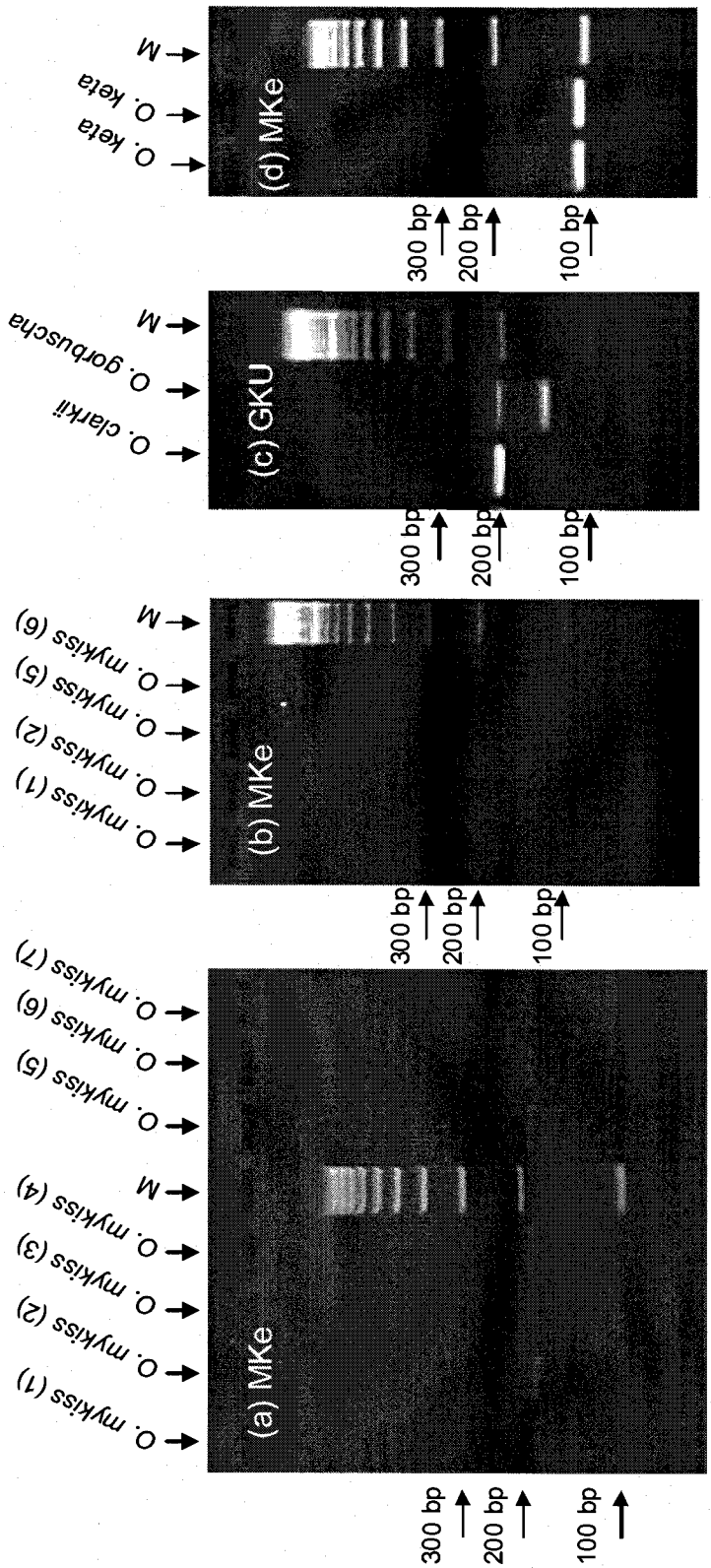


Figure 5.3 Non-specific amplification detected during conventional PCR specificity testing. Gels contain a 100 bp molecular ruler (M) and (a) *O. mykiss* samples (nos. 1-7) reacting with *O. nerka* primers to give an *O. nerka*-specific band (183 bp); (b) *O. mykiss* samples shown in gel (a) following a repeat DNA extraction and PCR; (c) *O. clarkii* sample with a universal band (205 bp) and a ghost band at the expected size for *O. gorbuscha* (143 bp), alongside an *O. gorbuscha* reference sample; and (d) *O. keta* samples showing the expected band for *O. keta* (104 bp) and an additional nonspecific band around 160 bp. Note: because all non-specific amplification bands are very faint, they may be difficult to visualize in reproduction.

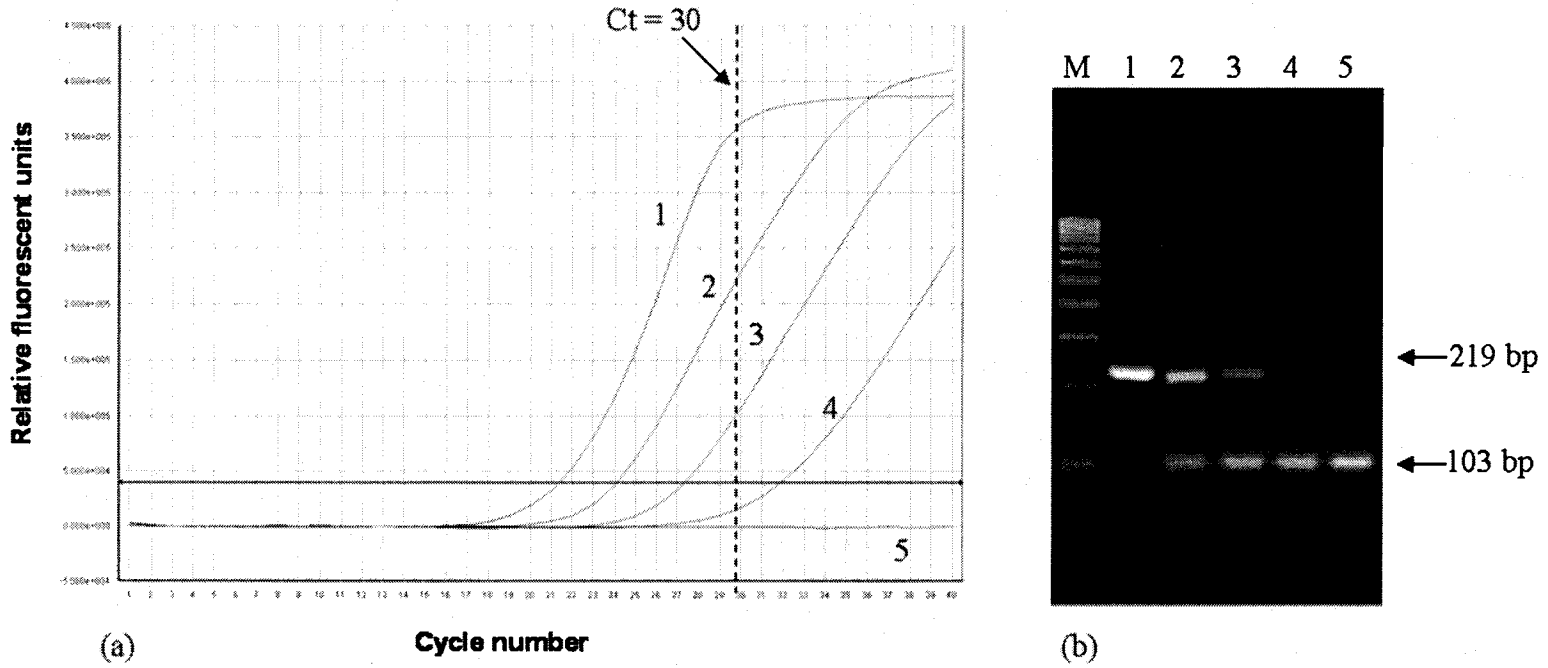
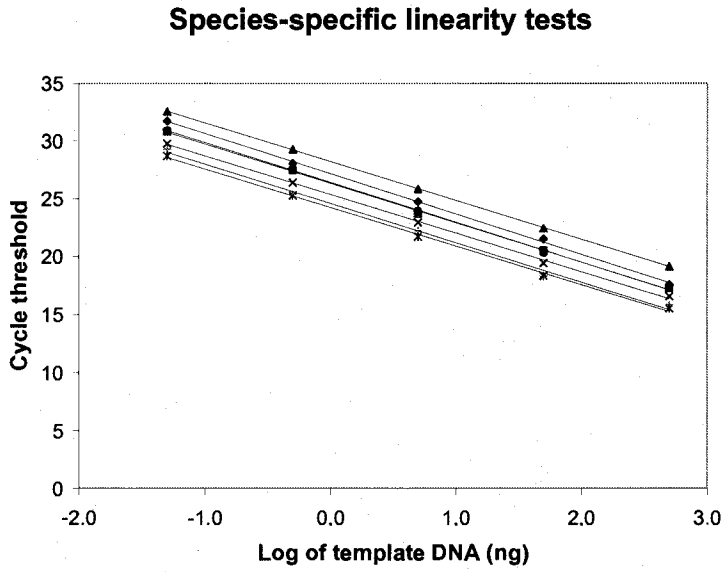
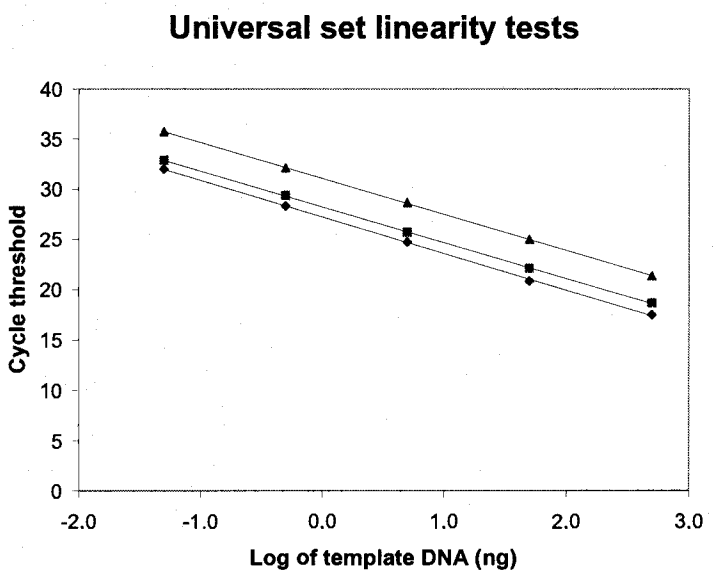


Figure 5.4 Example of admixture test results for *S. salar* in *O. tshawytscha* in the (a) real-time PCR system (cycle number 30 is marked with a dashed line) and (b) conventional PCR system with 100 bp molecular ruler (M). For both the real-time graph and the agarose gel, signals from left to right (nos. 1-5) represent samples containing the following levels of *S. salar*: 100% (1), 10% (2), 1.0% (3), 0.1% (4), and 0% (5). 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 5.5 Results of linearity tests with species-specific and universal real-time multiplex PCR assays. Template DNA was tested in a series of five 10-fold dilutions ranging from 500 ng to 0.05 ng.

CHAPTER 6

GENERAL CONCLUSIONS

The purpose of this project was to provide improved and novel methods for the DNA-based identification of commercially important salmon and trout species (genera *Oncorhynchus* and *Salmo*) in North America. In the first study, commercial salmon and trout products were tested with a previously developed method based on polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) analysis. Several improvements were made to reduce the time and materials needed for species diagnosis; however, the method still required multiple post-PCR steps and was not able to identify species in heavily processed products. Furthermore, this method was developed based on low numbers of reference sequences and was therefore vulnerable to errors arising from intraspecies variation. The second study, carried out in collaboration with the Biodiversity Institute of Ontario (Canada) and the U.S. Food and Drug Administration (FDA), consisted of a comprehensive analysis of cytochrome *c* oxidase subunit I (COI) DNA barcode sequences in commercial salmon and trout species. Analysis of 924 reference sequences showed DNA barcoding to be a reliable means for the identification of these species. DNA barcodes showed low within species variation (0.26%) and between-species variation levels that were 32-fold greater (8.22%). All full-length DNA barcodes (652 bp) and several shorter "mini-barcodes" (109-218 bp) analyzed *in silico* were able to differentiate the target species based on barcode gaps (i.e., the minimum between-species variation was always greater than the maximum within-species variation). As the FDA is currently incorporating DNA barcodes into their regulatory program, the results of this study will enhance their ability to identify salmon and trout species with this method.

In the next study, the comprehensive sequence information collected for DNA barcodes was utilized to design a species-specific multiplex PCR assay that can be used in real-time with TaqMan^{TM} minor-groove-binder (MGB) probes or in a

conventional PCR format with gel electrophoresis. This study was conducted in collaboration with the University of Guelph and the U.S. FDA. The optimized method showed strong signals with the target species, allowing for species identification in a rapid (2-4 h) and simple (presence/absence of signal) format. Furthermore, the method may be used with heavily processed products and with mixed-species samples. Species-specific multiplex PCR assays are very adaptable for high-throughput analysis with 'ready to use' plates, especially in a real-time system, where up to 96 samples can be tested simultaneously.

Overall, the results of this project represent a substantial contribution towards advancing DNA-based methods for the identification of commercial salmon and trout species in North America. These methods will benefit the food industry and regulatory agencies by providing a reliable means to detect economic fraud and ensure fair trade for a highly-valued species group that is susceptible to market substitution.

BIBLIOGRAPHY

Abercrombie, D.L., Clarke, S.C., and Shivji, M.S. 2005. Global-scale genetic identification of hammerhead sharks: Application to assessment of the international fin trade and law enforcement. Conserv. Gen. 6: 775-788.

Ahmed, M.M.M., Ali, B.A., and El-Zaeem, S. 2004. Application of RAPD markers in fish: part I - Some genera (*Tilapia*, *Sarotherodon* and *Oreochromis*) and species (*Oreochromis aureus* and *Oreochromis niloticus*) of tilapia. Int. J. Biotechnol. 6: 86-93.

Akasaki, T., Yanagimoto, T., Yamakami, K., Tomonaga, H., and Sato, S. 2006. Species identification and PCR-RFLP analysis of cytochrome *b* gene in cod fish (order *Gadiformes*) products. J. Food Sci. 71: C190-C195.

Ali, B.A., Huang, T.H., Qin, D.N., and Wang, X.M. 2004. A review of random amplified polymorphic DNA (RAPD) markers in fish research. Rev. Fish Biol. 14: 443-453.

Applewhite, L. and Bennett, S. 2008. Species identification programs for the seafood industry [abstract]. Presented at the 59th Pacific Fisheries Technologists Annual Meeting, San Francisco, CA, USA, February 3-6.

Apte, A. and Daniel, S. 2003. PCR primer design, p. 61-74. In: C.W. Dieffenbach and G.S. Dveksler (eds.). PCR Primer: A Laboratory Manual. Cold Spring Harbor, New York.

Aranishi, F. 2005. Rapid PCR-RFLP method for discrimination of imported and domestic mackerel. Mar. Biotech. 7: 571-575.

Aranishi, F. and Okimoto, T. 2004. PCR-based detection of allergenic mackerel ingredients in seafood. J. Genet. 83: 193-195.

Aranishi, F., Okimoto, T., and Izumi, S. 2005a. Identification of gadoid species (Pisces, Gadidae) by PCR-RFLP analysis. J. Appl. Genet. 46: 69-73.

Aranishi, F., Okimoto, T., and Ohkubo, M. 2006. A short-cut DNA extraction from cod caviar. J. Sci. Food Agric. 86: 425-428.

Aranishi, F., Okimoto, T., Ohkubo, M., and Izumi, S. 2005b. Molecular identification of commercial spicy pollack roe products by PCR-RFLP analysis. J. Food Sci. 70: C235-C238.

Arya, M., Shergill, I.S., Williamson, M., Gommersall, L., Arya, N., and Patel, H.R. 2005. Basic principles of real-time quantitative PCR. Expert Rev. Mol. Diagn. 5: 209-219.

Asensio, L. 2007. Application of multiplex PCR for the identification of grouper meals in the restaurant industry. Food Control. Forthcoming. doi:10.1016/j.foodcont.2007.11.002.

Asensio, L., Gonzalez, I., Fernandez, A., Cespedes, A., Rodriguez, M.A., Hernandez, P.E., Garcia, T., and Martin, R. 2001. Identification of Nile perch (*Lates niloticus*), grouper (*Epinephelus guaza*), and wreck fish (*Polyprion americanus*) fillets by PCR amplification of the 5S rDNA gene. J. AOAC International. 84: 777-781.

Asensio, L., Gonzalez, I., Rodriguez, M.A., Mayoral, B., Lopez-Calleja, I., Hernandez, P.E., Garcia, T., and Martin, R. 2003. Identification of grouper

(*Epinephelus guaza*), wreck fish (*Polyprion americanus*), and Nile perch (*Lates niloticus*) fillets by polyclonal antibody-based enzyme-linked immunosorbent assay. Journal of agricultural and food chemistry. 51: 1169-1172.

Augerot, X., Steinback, C., and Fobes, N. 2005. Atlas of Pacific Salmon. University of California Press, Berkeley, California.

Baker, C.S., Lento, G.M., Cipriano, F., and Palumbi, S.R. 2000. Predicted decline of protected whales based on molecular genetic monitoring of Japanese and Korean markets. Proc. R. Soc. Lond. B. 267: 1191-1199.

Bartlett, S.E. and Davidson, W.S. 1992. FINS (forensically informative nucleotide sequencing): A procedure for identifying the animal origin of biological specimens. BioTechniques. 12: 408-411.

Bayha, K.M., Graham, W.M., and Hernandez, F.J. 2008. 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. 4: 65-73.

Beacham, T.D., Hay, D.E., and Le, K.D. 2005. Population structure and stock identification of Eulachon (*Thaleichthys pacificus*), an anadromous smelt, in the Pacific Northwest. Mar. Biotech. 7: 363-372.

Beacham, T.D., Le, K.D., and Candy, J.R. 2004. Population structure and stock identification of steelhead trout (*Oncorhynchus mykiss*) in British Columbia and the Columbia River based on microsatellite variation. Environ. Biol. Fish. 69: 95-109.

Beacham, T.D., Pollard, S., and Le, K.D. 2000. Microsatellite DNA population structure and stock identification of steelhead trout (*Oncorhynchus mykiss*) in the Nass and Skeena rivers in northern British Columbia. Mar. Biotech. 2: 587-600.

Behnke, R.J. 1992. Native Trout of Western North America. American Fisheries Society, monograph 6. American Fisheries Society, Bethesda, MD.

Bensch, S. and Akesson, M. 2005. Ten years of AFLP in ecology and evolution: why so few animals? Mol. Ecol. 14: 2899-2914.

Blears, M.J., De Grandis, S.A., Lee, H., and Trevors, J.T. 1998. Amplified fragment length polymorphism (AFLP): a review of the procedure and its applications. J. Indust. Microbiol. Biotechnol. 21: 99-114.

Borgo, R., Souty-Grosset, C., Bouchon, D., and Gomot, L. 1996. PCR-RFLP analysis of mitochondrial DNA for identification of snail meat species. J. Food Sci. 61: 1-4.

Bossier, P. 1999. Authentication of seafood products by DNA patterns. J. Food Sci. 64: 189-193.

Brodman, P.D. and 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: 599-607.

Brown, B. and Epifanio, J. 2003. Nuclear DNA, p. 101-123. In: E.M. Hallerman (ed.). Population Genetics: Principles and Applications for Fisheries Scientists. American Fisheries Society, Bethesda, Maryland.

Brown, K.H. and Thorgaard, G.H. 2002. Mitochondrial and nuclear inheritance in an androgenetic line of rainbow trout, *Oncorhynchus mykiss*. Aquaculture. 204: 323-335.

Bucklin, K.A., Banks, M.A., and Hedgecock, D. 2007. Assessing genetic diversity of protected coho salmon (*Oncorhynchus kisutch*) populations in California. Can. J. Fish. Aquat. Sci. 64: 30-42.

Calo-Mata, P., Sotelo, C.G., Perez-Martin, R.I., Rehbein, H., Hold, G.L., Russell, V.J., Pryde, S., Quinteiro, J., Rey-Mendez, M., Rosa, C., and Santos, A.T. 2003. Identification of gadoid fish species using DNA-based techniques. Eur. Food Res. Technol. 217: 259-264.

Campbell, M., Kozfkay, C.C., Boone, A., and Teuscher, D. 2007. FY2006 Final Report: Genetic investigations of Bonneville cutthroat trout in the bear river drainage, Idaho: distribution of mitochondrial DNA diversity and rainbow trout hybridization and introgression.:1-28.

Carrera, E., Garcia, T., Cespedes, A., Gonzalez, I., Fernandez, A., Asensio, L.M., Hernandez, P.E., and Martin, R. 2000a. Differentiation of smoked *Salmo salar*, *Oncorhynchus mykiss* and *Brama raii* using the nuclear marker 5S rDNA. Int. J. Food Sci. Technol. 35: 401-406.

Carrera, E., Garcia, T., Cespedes, A., Gonzalez, I., Fernandez, A., Asensio, L.M., Hernandez, P.E., and Martin, R. 2000b. Identification of smoked Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) using PCR-restriction fragment length polymorphism of the *p53* gene. J. AOAC International. 83: 341-346.

Carrera, E., Garcia, T., Cespedes, A., Gonzalez, I., Fernandez, A., Hernandez, P., and Martin, R. 1999a. PCR-RFLP of the mitochondrial cytochrome oxidase gene: a simple method for discrimination between Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). J. Sci. Food Agric. 79: 1654-1658.

Carrera, E., Garcia, T., Cespedes, A., Gonzalez, I., Fernandez, A., Hernandez, P.E., and Martin, R. 1999b. Salmon and trout analysis by PCR-RFLP for identity authentication. J. Food Sci. 64: 410-413.

Carrera, E., Garcia, T., Cespedes, A., Gonzalez, I., Sanz, B., Hernandez, P., and Martin, R. 1997. Immunostick colorimetric ELISA assay for the identification of smoked salmon (*Salmo salar*), trout (*Oncorhynchus mykiss*) and bream (*Brama raii*). J. Sci. Food Agric. 74: 547-550.

Castillo, A.G., Martinez, J.L., and Garcia-Vazquez, E. 2003. Identification of Atlantic hake species by a simple PCR-based methodology employing microsatellite loci. J. Food Protect. 66: 2130-2134.

Cespedes, A., Garcia, T., Carrera, E., Gonzalez, I., Fernandez, A., Asensio, L., Hernandez, P., and Martin, R. 2000. Genetic differentiation between sole (*Solea solea*) and Greenland halibut (*Reinhardtius hippoglossoides*) by PCR-RFLP analysis of a 12S rRNA gene fragment. J. Sci. Food Agric. 80: 29-32.

Cespedes, A., Garcia, T., Carrera, E., Gonzalez, I., Fernandez, A., Hernandez, P.E., and Martin, R. 1999. Identification of sole (*Solea solea*) and Greenland halibut (*Reinhardtius hippoglossoides*) by PCR amplification of the 5S rDNA gene. J. Agric. Food Chem. 47: 1046-1050.

Cespedes, A., Garcia, T., Carrera, E., Gonzalez, I., Sanz, B., Hernandez, P., and Martin, R. 1998a. Identification of flatfish species using polymerase chain reaction (PCR) amplification and restriction analysis of the cytochrome b gene. J. Food Sci. 63: 206-209.

Cespedes, A., Garcia, T., Carrera, E., Gonzalez, I., Sanz, B., Hernandez, P.E., and Martin, R. 1998b. Polymerase chain reaction-restriction fragment length polymorphism analysis of a short fragment of the cytochrome *b* gene for identification of flatfish species. J. Food Protect. 61: 1684-1685.

Chakraborty, A., Aranishi, F., and Iwatsuki, Y. 2005. Molecular identification of hairtail species (Pisces: Trichiuridae) based on PCR-RFLP analysis of the mitochondrial 16S rRNA gene. J Appl. Genet. 46: 381-385.

Chapela, M.J., Sotelo, C.G., Calo-Mata, P., Perez-Martin, R.I., Rehbein, H., Hold, G.L., Quinteiro, J., Rey-Mendez, M., Rosa, C., and Santos, A.T. 2002. Identification of cephalopod species (Ommastrephidae and Loliginidae) in seafood products by forensically informative nucleotide sequencing (FINS). J. Food Sci. 67: 1672-1676.

Chapela, M.J., Sotelo, C.G., and Perez-Martin, R.I. 2003. Molecular identification of cephalopod species by FINS and PCR-RFLP of a cytochrome b gene fragment. Eur. Food Res. Technol. 217: 524-529.

Chapela, M.J., Sotelo, C.G., Perez-Martin, R.I., Pardo, M.A., Perez-Villareal, B., Gilardi, P., and Riese, J. 2007. Comparison of DNA extraction methods from muscle of canned tuna for species identification. Food Control. 18: 1211-1215.

Chow, S., Nohara, K., Tanabe, T., Itoh, T., Tsuji, S., Nishikawa, Y., Uyeyanagi, S., and Uchikawa, K. 2003. Genetic and morphological identification of larval and small juvenile tunas (Pisces: Scombridae) caught by a mid-water trawl in the western Pacific. Bull. Fish. Res. Agen. 8: 1-14.

Civera, T. 2003. Species identification and safety of fish products. Veterinary Research Communications. 27: 481-489.

Clarke, S.C., Magnussen, J.E., Abercrombie, D.L., McAllister, M.K., and Shivji, M.S. 2006. Identification of shark species composition and proportion in the Hong Kong shark fin market based on molecular genetics and trade records. Conserv. Biol. 20: 201-211.

Colin Slaughter, J. 1999. The naturally occurring furanones: formation and function from pheromone to food. Biol. Rev. Camb. Philos. Soc. 74: 259-276.

Comesana, A.S., Abella, P., and Sanjuan, A. 2003. Molecular identification of five commercial flatfish species by PCR-RFLP analysis of a 12S rRNA gene fragment. J. Sci. Food Agric. 83: 752-759.

Cronin, M.A., Spearman, W.J., Wilmot, R.I., Patton, J.C., and Bickham, J.W. 1993. Mitochondrial DNA variation in chinook and chum salmon detected by restriction enzyme analysis of PCR products. Can. J. Fish. Aquat. Sci. 50: 708-715.

Dawnay, N., Ogden, R., McEwing, R., Carvalho, G.R., and Thorpe, R.S. 2007. Validation of the barcoding gene COI for use in forensic genetic species identification. Forensic Sci. Int. 173: 1-6.

DeSalle, R., Egan, M.G., and Siddall, M. 2005. The unholy trinity: taxonomy, species delimitation and DNA barcoding. Phil. Trans. R. Soc. B. 360: 1905-1916.

DFO. 2009. Fisheries and Oceans Canada. Accessible at: http://www.dfo-mpo.gc.ca.

Docker, M.F. and Heath, D.D. 2003. Genetic comparison between sympatric anadromous steelhead and freshwater resident rainbow trout in British Columbia, Canada. Conserv. Genet. . 4: 227-231

Dooley, J.J. and Garrett, S.D. 2001. Development of meat speciation assays using the Agilent 2100 bioanalyser. Agilent technologies application note no. 5988-4069EN.

Dooley, J.J., Garrett, S.D., and Brown, H.M. 2003. Development of molecular markers suitable for the detection of olive oil adulteration with hazelnut oil. Final report for Food Standards Agency project no. Q01060.

Dooley, J.J., Paine, K.E., Garrett, S.D., and Brown, H.M. 2004. Detection of meat species using TaqMan real-time PCR assays. Meat Sci. 68: 431-438.

Dooley, J.J., Sage, H.D., Brown, H.M., and Garrett, S.D. 2005a. Improved fish species identification by use of lab-on-a-chip technology. Food Control. 16: 601-607.

Dooley, J.J., Sage, H.D., Clarke, M.A., Brown, H.M., and Garrett, S.D. 2005b. 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: 3348-3357.

Edwards, K.J. and Logan, J.M.J. 2009. Performing real-time PCR, p. 85-93. In: J.M.J. Logan, K.J. Edwards, and N. Saunders (eds.). Real-time PCR: current technology and applications. Caister Academic Press, Norfolk, UK.

Espiñeira, M., Vieites, J.M., and Santaclara, F.J. 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: 785-793.

Fernandez, A., Garcia, T., Asensio, L., Rodriguez, M.A., Gonzalez, I., Cespedes, A., Hernandez, P.E., and Martin, R. 2000. Identification of the clam species *Ruditapes decussatus* (Grooved carpet shell), *Venerupis pullastra* (Pullet carpet shell), and *Ruditapes philippinarum* (Japanese carpet shell) by PCR-RFLP. J. Agric. Food Chem. 48: 3336-3341.

Fernandez, A., Garcia, T., Gonzalez, I., Asensio, L., Rodriguez, M.A., Hernandez, P.E., and Martin, R. 2002. Polymerase chain reaction-restriction fragment length polymorphism analysis of a 16S rRNA gene fragment for authentication of four clam species. J. Food Protect. 65: 692-695.

Gharrett, A.J., Gray, A.K., and Brykov, V. 2001. Phylogeographic analysis of mitochondrial DNA variation in Alaskan coho salmon, *Oncorhynchus kisutch*. Fish. Bull. 99: 528-544.

Gil, L.A. 2007. PCR-based methods for fish and fishery products authentication. Trends Food Sci. Technol. 18: 558-566.

Gilliland, G., Perrin, S., Blanchard, K., and Bunn, H.F. 1990. Analysis of cytokine mRNA and DNA: detection and quantitation by competitive polymerase chain reaction. Proc. Natl. Acad. Sci. USA. 87: 2725-2729.

Gopalakrishnan, A., Abdul Muneer, P.M., Musammilu, K.K., Lal, K.K., Kapoor, D., and Mohindra, V. 2006. Primers from the orders Osteoglossiform and Siluriform detect polymorphic microsatellite loci in sun-catfish, *Horabagrus brachysoma* (Teleostei: Bagridae). J. Appl. Ichthyol. 22: 456-458.

Greig, C., Jacobson, D.P., and Banks, M.A. 2003. New tetranucleotide microsatellites for fine-scale discrimination among endangered chinook salmon (*Oncorhynchus tshawytscha*). Mol. Ecol. Notes. 3: 376-379.

Greig, C., Robertson, J.M., and Banks, M.A. 2002. Rapid PCR-based species tests for threatened sympatric salmonids. Conserv. Gen. 3: 83-86.

Hajibabaei, M., Smith, M.A., Janzen, D.H., Rodriguez, J.J., Whitfield, J.B., and Hebert, P.D.N. 2006. A minimalist barcode can identify a specimen whose DNA is degraded. Mol. Ecol. Notes. 6: 959-964.

Hall, T.A. 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids. Symp. Ser. 41: 95-98.

Han, K. and Ely, B. 2002. Use of AFLP analyses to assess genetic variation in *Morone* and *Thunnus* species. Mar. Biotech. 4: 141-145.

Hare, M.P., Palumbi, S.R., and Butman, C.A. 2000. Single-step species identification of bivalve larvae using multiplex polymerase chain reaction. Mar. Biol. 137: 953-961.

He, C., Chen, L., Simmons, M., Li, P., Kim, S., and Liu, Z.J. 2003. Putative SNP discovery in interspecific hybrids of catfish by comparative EST analysis. Animal Genetics. 34: 445-448.

Hebert, P.D.N., Cywinska, A., Ball, S.L., and deWaard, J.R. 2003. Biological identifications through DNA barcodes. Proc. R. Soc. London, Ser B. 270: 313-319.

Henegariu, O., Heerema, N.A., Dlouhy, S.R., Vance, G.H., and Vogt, P.H. 1997. Multiplex PCR: critical parameters and step-by-step protocol. BioTechniques. 23: 504-511.

Hill, R.S., Allen, L.D., and 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: 279-287.

Hiramoto, K., Sekiguchi, K., Ayuha, K., Aso-o, R., Moriya, N., Kato, T., and Kikugawa, K. 1996. DNA breaking activity and mutagenicity of soy sauce: characterization of the active components and identification of 4-hydroxy-5-methyl-3(*2H*)-furanone. Mutation research. 359: 119-132.

Hird, H.J., Hold, G.L., Chisholm, J., Reece, P., Russell, V.J., Brown, J., Goodier, R., and MacArthur, R. 2005. Development of a method for the quantification of haddock (*Melanogrammus aeglefinus*) in commercial products using real-time PCR. Eur. Food Res. Technol. 220: 633-637.

Hold, G.L., Russell, V.J., Pryde, S., Rehbein, H., Quinteiro, J., Rey-Mendez, M., Sotelo, C.G., Perez-Martin, R.I., Santos, A.T., and Rosa, C. 2001a. Validation of a PCR-RFLP based method for the identification of salmon species in food products. Eur. Food Res. Technol. 212: 385-389.

Hold, G.L., Russell, V.J., Pryde, S.E., Rehbein, H., Quinteiro, J., Vidal, R., Rey-Mendez, M., Sotelo, C.G., Perez-Martin, R.I., Santos, A.T., and Rosa, C. 2001b. Development of a DNA-based method aimed at identifying the fish species present in food products. J. Agric. Food Chem. 49: 1175-1179.

Horstkotte, B. and Rehbein, H. 2003. Fish species identification by means of restriction fragment length polymorphism and high-performance liquid chromatography. J. Food Sci. 68: 2658-2666.

Hsieh, H.S., Chai, T., Cheng, C.A., Hsieh, Y.W., and Hwang, D.F. 2004. Application of DNA technique for identifying the species of different processed products of swordfish meat. J. Food Sci. 69: FCT1-FCT6.

Hsieh, H.S., Chai, T.J., and Hwang, D.F. 2007. Using the PCR-RFLP method to identify the species of different processed products of billfish meats. Food Control. 18: 369-374.

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., and Bernatchez, L. 2008. Identifying Canadian freshwater fishes through DNA barcodes. PLoS ONE. 3: e2490.

Hubner, P., Studer, E., and Luthy, J. 1999. Quantitative competitive PCR for the detection of genetically modified organisms in food. Food Control. 10: 353-358.

Imai, H., Cheng, J.H., Hamasaki, K., and Numachi, K.I. 2004. Identification of four mud crab species (genus *Scylla*) using ITS-1 and 16S rDNA markers. Aquat. Living Resour. 17: 31-34.

Imsiridou, A., Hardy, H., Maudling, N., Amoutzias, G., and Zaldivar Comenges, J.M. 2003. Web database of molecular genetic data from fish stocks. J. Heredity. 94: 265-267.

Itoi, S., Nakaya, M., Kaneko, G., Kondo, H., Sezaki, K., and Watabe, S. 2005. Rapid identification of eels *Anguilla japonica* and *Anguilla anguilla* by polymerase chain reaction with single nucleotide polymorphism-based specific probes. Fisheries Sci. 71: 1356-1364.

Ivanova, N.V., deWaard, J.R., Hajibabaei, M., and Hebert, P.D.N. 2005. Protocols for high-volume DNA barcode analysis. Draft submission to: DNA working group Consortium for the Barcode of Life. Published online at http://www.dnabarcoding.ca/.

Ivanova, N.V., Dewaard, J.R., and Hebert, P.D.N. 2006. An inexpensive, automation-friendly protocol for recovering high-quality DNA. Mol. Ecol. Notes. 6: 998-1002.

Ivanova, N.V., Fazekas, A.J., and Hebert, P.D.N. 2008. Semi-automated, membrane-based protocol for DNA isolation from plants. Plant Mol. Biol. Rep. 26: 186-198.

Ivanova, N.V., Zemlak, T.S., Hanner, R.H., and Hebert, P.D.N. 2007. Universal primer cocktails for fish DNA barcoding. Mol. Ecol. Notes. 7: 544-548.

Jenneckens, I., Meyer, J.N., Horstgen-Schwark, G., May, B., Debus, M.L., Wedekind, H., and Ludwig, A. 2001. A fixed allele at microsatellite locus LS-39

exhibiting species-specificity for the black caviar producer *Acipenser stellatus*. J. Appl. Ichthyol. 17: 39-42.

Jerome, M., Lemaire, C., Verrez-Bagnis, V., and Etienne, M. 2003. Direct sequencing method for species identification of canned sardine and sardine-type products. J. Agric. Food Chem. 51: 7326-7332.

Johnson, H.M. 2007. 2006/2007 Annual Report on the United States Seafood Industry. H.M. Johnson & Associates, Jacksonville, OR.

Karaiskou, N., Apostolidis, A.P., Triantafyllidis, A., Kouvatsi, A., and Triantaphyllidis, C. 2003. Genetic identification and phylogeny of three species of the genus *Trachurus* based on mitochondrial DNA analysis. Mar. Biotech. 5: 493-504.

Kimura, M. 1980. A simple method of estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16: 111-120.

Klinbunga, S., Pripue, P., Khamnamtong, N., Puanglarp, N., Tassanakajon, A., Jarayabhand, P., Hirono, I., Aoki, T., and Menasveta, P. 2003. Genetic diversity and molecular markers of the tropical abalone (*Haliotis asinina*) in Thailand. Mar. Biotech. 5: 505-517.

Knapp, G., Roheim, C., and Anderson, J. 2007. The Great Salmon Run: Competition Between Wild and Farmed Salmon. World Wildlife Fund, Washington D.C.

Kochzius, M., Nölte, M., Weber, H., Silkenbeumer, N., Hjörleifsdottir, S., Hreggvidsson, G.O., Marteinsson, V., Kappel, K., Planes, S., Tinti, F., Magoulas, A., Garcia Vazquez, E., Turan, C., Hervet, C., Campo Falgueras, D., Antoniou, A., Landi, M., and Blohm, D. 2008. DNA microarrays for identifying fishes. Mar. Biotech. 10: 207-217.

Kumar, S., Dudley, J., Nei, M., and Tamura, K. 2008. MEGA: A biologist-centric software for evolutionary analysis of DNA and protein sequences. Briefings Bioinf. 9: 299-306.

Kutyavin, I.V., Afonina, I.A., Mills, A., Gorn, V.V., Lukhtanov, E.A., Belousov, E.S., Singer, M.J., Walburger, D.K., Lokhov, S.G., Gall, A.A., Dempcy, R., Reed, M.W., Meyer, R.B., and Hedgpeth, J. 2000. 3'-Minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. Nucleic Acids Res. 28: 655-661.

Kwok, S., Chang, S.Y., and Sninsky, J.J. 1994. A guide to the design and use of mismatched and degenerate primers. Genome Res. 3: S39-S47.

Kyle, C.J. and Wilson, C.C. 2007. Mitochondrial DNA identification of game and harvested freshwater fish species. Forensic Sci. Int. 166: 68-76.

Lai, R.Y., Lagally, E.T., Lee, S.H., Soh, H.T., Plaxco, K.W., and Heeger, A.J. 2006. Rapid, sequence-specific detection of unpurified PCR amplicons via a reusable, electrochemical sensor. PNAS. 103: 4017-4021.

Lal, K.K., Chauhan, T., Mandal, A., Singh, R.K., Khulbe, L., Ponniah, A.G., and Mohindra, V. 2004. Identification of microsatellite DNA markers for population structure analysis in Indian major carp, *Cirrhinus mrigala* (Hamilton-Buchanan, 1982). J. Appl. Ichthyol. 20: 87-91.

Laube, I., Zagon, J., and Broll, H. 2007a. Quantitative determination of commercially relevant species in foods by real-time PCR. Int. J. Food Sci. Technol. 42: 336-341.

Laube, I., Zagon, J., Spiegelberg, A., Butschke, A., Kroh, L.W., and Broll, H. 2007b. Development and design of a 'ready-to-use' reaction plate for a PCR-based simultaneous detection of animal species in foods. Int. J. Food Sci. Technol. 42: 9-17.

Lenstra, J.A. 2003. DNA methods for identifying plant and animal species in food, p. 34-53. In: M. Lees (ed.). Food Authenticity and Traceability. Woodhead Publishing Limited, Cambridge, England.

Li, L. and Guo, X. 2004. AFLP-based genetic linkage maps of the pacific oyster *Crassostrea gigas* Thunberg. Mar. Biotech. 6: 26-36.

Li, X., Hiramoto, K., Yoshida, M., Kato, T., and Kikugawa, K. 1998. Identification of 2,5-dimethyl-4-hydroxy-3(*2H*)-furanone (DMHF) and 4-hydroxy-2(or 5)-ethyl-5(or 2)-methyl-3(2H)-furanone (HEMF) with DNA breaking activity in soy sauce. Food Chem. Toxicol. 36: 305-314.

Lin, W.F. and Hwang, D.F. 2007. Application of PCR-RFLP analysis on species identification of canned tuna. Food Control. 18: 1050-1057.

Lin, Y.S., Poh, Y.P., Lin, S.M., and Tzeng, C.S. 2002. Molecular techniques to identify freshwater eels: RFLP analyses of PCR-amplified DNA fragments and allele-specific PCR from mitochondrial DNA. Zoological Studies. 41: 421-430.

Liu, Z., Nichols, A., Li, P., and Dunham, R.A. 1998a. Inheritance and usefulness of AFLP markers in channel catfish (*Ictalurus punctatus*), blue catfish (*I. furcatus*), and their F1, F2, and backcross hybrids. Mol. Gen. Genet. 258: 260-268.

Liu, Z.J. and Cordes, J.F. 2004. DNA marker technologies and their applications in aquaculture genetics. Aquaculture. 238: 1-37.

Liu, Z.J., Li, P., Argue, B., and Dunham, R. 1998b. Inheritance of RAPD markers in channel catfish (*Ictalurus punctatus*), blue catfish (*I. furcatus*), and their F1, F2 and backcross hybrids. Anim. Genet. 29: 58-62.

Lockley, A.K. and Bardsley, R.G. 2000. DNA-based methods for food authentication. Trends Food Sci. Technol. 11: 67-77.

Lopez, I. and Pardo, M.A. 2005. 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. 53: 4554-4560.

Mabuchi, K., Okuda, N., Kokita, T., and Nishida, M. 2003. Genetic comparison of two color-morphs of *Apogon properuptus* from southern Japan. Ichthyol. Res. 50: 293-296.

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

Maes, G.E., Pujolar, J.M., Raeymaekers, J.A.M., Dannewitz, J., and Volckaert, F.A.M. 2006. Microsatellite conservation and Bayesian individual assignment in four *Anguilla* species. Mar. Ecol. Prog. Ser. 319: 251-261.

Mafra, I., Ferreira, I.M., and Oliveira, M.B. 2007. Food authentication by PCR-based methods. Eur. Food Res. Technol. 227: 649-665.

Magnussen, J.E., Pikitch, E.K., Clarke, S.C., Nicholson, C., Hoelzel, A.R., and Shivji, M.S. 2007. Genetic tracking of basking shark products in international trade. Anim. Conserv. 10: 199-207.

Marmiroli, N., Peano, C., and Maestri, E. 2003. Advanced PCR techniques in identifying food components, p. 3-33. In: M. Lees (ed.). Food Authenticity and Traceability. Woodhead Publishing Limited, Cambridge, England.

Marras, S.A., Tyagi, S., and Kramer, F.R. 2006. Real-time assays with molecular beacons and other fluorescent nucleic acid hybridization probes. Clinica chimica acta. 363: 48-60.

Marshall, H.D., Johnstone, K.A., and Carr, S.M. 2007. Species-specific oligonucleotides and multiplex PCR for forensic discrimination of two species of scallops, *Placopecten magellanicus* and *Chlamys islandica*. For. Sci. Int. 167: 1-7.

Martin, M.A., Shiozawa, D.K., Loudenslager, E.J., and Jensen, J.N. 1985. Electrophoretic study of cutthroat trout populations in Utah. Great Basin Nat. 45: 677-687.

Martinez, I., James, D., and Loreal, H. 2005. Application of modern analytical techniques to ensure seafood safety and authenticity. FAO Fisheries Technical Paper. No. 455. FAO, Rome, Italy.

Matsunaga, T., Chikuni, K., Tanabe, R., Muroya, S., Shibata, K., Yamada, J., and Shinmura, Y. 1999. A quick and simple method for the identification of meat species and meat products by PCR assay. Meat Sci. 51: 143-148.

McDowell, D.G., Parkes, H.C., Archard, C.L., and Birch, L. 2001. Evaluation of LabChip technology for GMO analysis in food. Food Control. 12: 535-540.

McKay, S.J., Smith, M.J., and Devlin, R.H. 1997. Polymerase chain reaction-based species identification of salmon and coastal trout in British Columbia. Mol. Mar. Biol. Biotechnol. 6: 131-140.

McVeigh, H.P. and Davidson, W.S. 1991. A salmonid phylogeny inferred from mitochondrial cytochrome *b* gene sequences. J. Fish Biol. 39 (Supplement A): 277-282.

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

Meusnier, I., Singer, G.A.C., Landry, J.F., Hickey, D.A., Hebert, P.D.N., and Hajibabaei, M. 2008. A universal DNA mini-barcode for biodiversity analysis. BMC Genomics. 9: 214.

Michelini, E., Cevenini, L., Mezzanotte, L., Simoni, P., Baraldini, M., De Laude, L., and 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: 7638-7647.

Moran, P. and Garcia-Vazquez, E. 2006. Identification of highly prized commercial fish using a PCR-based methodology. Biochem. Mol. Biol. Educat. 34: 121-124.

Moretti, V.M., Turchini, G.M., Bellagamba, F., and Caprino, F. 2003. Traceability issues in fishery and aquaculture products. Vet. Res. Com. 27 Suppl. 1: 497-505.

Nagaraj, S.H., Gasser, R.B., and Ranganathan, S. 2007. A hitchhiker's guide to expressed sequence tag (EST) analysis. Briefings in bioinformatics. 8: 6-21.

Nijman, I.J., Hogendoorn, M.P., Gruys, E., Luikart, G., Ertugrul, O., Zagdsuren, Y., Ngere, L.O., Hasima, N., Erhardt, G., Ajmone-Marsan, P., and Lenstra, J.A. 2002. Detection of sheep-goat interspecies hybridization by analysis of satellite DNA. Online J. Vet. Res. 1: 1-6.

Nijman, I.J., Otsen, M., Verkaar, E.L., de Ruijter, C., Hanekamp, E., Ochieng, J.W., Shamshad, S., Rege, J.E., Hanotte, O., Barwegen, M.W., Sulawati, T., and Lenstra, J.A. 2003. Hybridization of banteng (*Bos javanicus*) and zebu (*Bos indicus*) revealed by mitochondrial DNA, satellite DNA, AFLP and microsatellites. Heredity. 90: 10-16.

Pank, M., Stanhope, M., Natanson, L., Kohler, N., and 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: 231-240.

Pardo, M.A. and Perez-Villareal, B. 2004a. Identification of commercial canned tuna species by restriction site analysis of mitochondrial DNA products obtained by nested primer PCR. Food Chem. 86: 143-150.

Pardo, M.A. and Perez-Villareal, B. 2004b. Identification of commercial canned tuna species by restriction site analysis of mitochondrial DNA products obtained by nested primer PCR. Food Chem. 86: 143-150.

Park, J.Y., Lee, H.J., Kim, W.J., Lee, J.H., and Min, K.S. 2000. Mitochondrial cytochrome *b* sequence variation in Korean salmonids. J. Fish Biol. 56: 1145-1154.

Park, L.K., Brainard, M.A., Dightman, D.A., and Winans, G.A. 1993. Low levels of intraspecific variation in the mitochondrial DNA of chum salmon (*Oncorhynchus keta*). Mol. Mar. Biol. Biotechnol. 2: 362-370.

Pepe, T., Trotta, M., di Marco, I., Cennamo, P., Anastasio, A., and Cortesi, M.L. 2005. Mitochondrial cytochrome *b* DNA sequence variations: an approach to fish species identification in processed fish products. J. Food Protect. 68: 421-425.

Perez, M., Alvarez, C., Balado, M., Cabado, A.G., Vieites, J.M., and Presa, P. 2004. Identification of South Atlantic hakes (*Merluccius australis* and *Merluccius hubbsi*) in processed foods by PCR-RFLPs of cytochrome *b* gene. J. Aquat. Food Prod. Technol. 13: 59-67.

Peter, C., Brunen-Nieweler, C., Cammann, K., and Borchers, T. 2004. Differentiation of animal species in food by oligonucleotide microarray hybridization. Eur. Food Res. Technol. 219: 286-293.

Phillips, R.B., Matsuoka, M.P., Konon, I., and Reed, K.M. 2000. Phylogenetic analysis of mitochondrial and nuclear sequences supports inclusion of *Acantholingua ohridana* in the genus *Salmo*. Copeia 2000. 2: 546-550.

Purcell, M., Mackey, G., LaHood, E., Huber, H., and Park, L. 2004. Molecular methods for the genetic identification of salmonid prey from Pacific harbor seal (*Phoca vitulina richardsi*) scat. Fish. Bull. 102: 213-220.

Quinteiro, J., Sotelo, C.G., Rehbein, H., Pryde, S.E., Medina, I., Perez-Martin, R.I., Rey-Mendez, M., and Mackie, I.M. 1998. Use of mtDNA direct polymerase chain reaction (PCR) sequencing and PCR-restriction fragment length polymorphism methodologies in species identification of canned tuna. J. Agric. Food Chem. 46: 1662-1669.

Quinteiro, J., Vidal, R., Izquierdo, M., Sotelo, C.G., Chapela, M.J., Perez-Martin, R.I., Rehbein, H., Hold, G.L., Russell, V.J., Pryde, S.E., Rosa, C., Santos, A.T., and Rey-Mendez, M. 2001. Identification of Hake species (*Merluccius* Genus) using sequencing and PCR-RFLP analysis of mitochondrial DNA control region sequences. J. Agric. Food Chem. 49: 5108-5114.

Ram, J.L., Ram, M.L., and Baidoun, F.F. 1996. Authentication of canned tuna and bonito by sequence and restriction site analysis of polymerase chain reaction products of mitochondrial DNA. J. Agric. Food Chem. 44: 2460-2467.

Ramella, M.S., Kroth, M.A., Tagliari, C., and Arisi, A.C.M. 2005. Optimization of random amplified polymorphic DNA protocol for molecular identification of *Lophius gastrophysus*. Cienc. Tecnol. Aliment. Campinas. 25: 733-735.

Rapley, R. 2000. Molecular biology and basic techniques, p. 80-137. In: K. Wilson and J. Walker (eds.). Principles and Techniques of Practical Biochemistry. Cambridge University Press, Cambridge, United Kingdom.

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

Rasmussen, R.S. and Morrissey, M.T. 2009. Application of DNA-based methods to identify fish and seafood substitution on the commercial market. Compr. Rev. Food Sci. Food Saf. 8: 118-154.

Rasmussen, R.S., Morrissey, M.T., and Hebert, P.D.N. 2009. DNA Barcoding of Commercially Important Salmon and Trout Species (*Oncorhynchus* and *Salmo*) from North America. J. Agric. Food Chem. 57: 8379-8385.

Ratnasingham, S. and Hebert, P.D.N. 2007. BOLD: The barcode of life data system (www.barcodinglife.org). Mol. Ecol. Notes. 7: 355-364.

Raymaekers, M., Smets, R., Maes, B., and Cartuyvels, R. 2009. Checklist for optimization and validation of real-time PCR assays. J. Clin. Lab. Anal. 23: 145-151.

Rego, I., Martinez, A., Gonzalez-Tizon, A., Vieites, J., Leira, F., and Mendez, J. 2002. PCR technique for the identification of mussel species. J. Agric. Food Chem. 50: 1780-1784.

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: 625-632.

Rehbein, H., Gonzales-Sotelo, C., Perez-Martin, R.I., Quinteiro, J., Rey-Mendez, M., Pryde, S., Mackie, I.M., and Santos, A.T. 1999a. Differentiation of sturgeon caviar by single strand conformational polymorphism (PCR-SSCP) analysis. Archiv fur Lebensmittelhygiene. 50: 13-17.

Rehbein, H., Kress, G., and Schmidt, T. 1997. Application of PCR-SSCP to species identification of fishery products. J. Sci. Food Agric. 74: 35-41.

Rehbein, H., Mackie, I.M., Pryde, S., Gonzales-Sotelo, C., Medina, I., Perez-Martin, R.I., Quinteiro, J., and Rey-Mendez, M. 1999b. Fish species identification in canned tuna by PCR-SSCP: validation by a collaborative study and investigation of intra-species variability of the DNA-patterns. Food Chem. 64: 263-268.

Rehbein, H., Sotelo, C.G., Perez-Martin, R.I., Chapela-Garrido, M.J., Hold, G.L., Russell, V.J., Pryde, S., Santos, A.T., Rosa, C., Quinteiro, J., and Rey-Mendez, M. 2002. Differentiation of raw or processed eel by PCR-based techniques: restriction fragment length polymorphism analysis (RFLP) and single strand conformation polymorphism analysis (SSCP). Eur. Food Res. Technol. 214: 171-177.

Renshaw, M.A., Saillant, E., Broughton, R.E., and Gold, J.R. 2006. Application of hypervariable genetic markers to forensic identification of 'wild' from hatchery-raised red drum, *Sciaenops ocellatus*. Forensic Science International. 156: 9-15.

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: 279-290.

Rocha-Olivares, A. and Chávez-González, J.P. 2008. Molecular identification of dolphinfish species (genus *Coryphaena*) using multiplex haplotype-specific PCR of mitochondrial DNA. Ichthyol. Res. 55: 389-393.

Rossen, L., Nørskov, P., Holmstrøm, K., and Rasmussen, O.F. 1992. Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. Int. J. Food Microbiol. 17: 37-45.

Russell, V.J., Hold, G.L., Pryde, S.E., Rehbein, H., Quinteiro, J., Rey-Mendez, M., Sotelo, C.G., Perez-Martin, R.I., Santos, A.T., and Rosa, C. 2000. Use of restriction fragment length polymorphism to distinguish between salmon species. J. Agric. Food Chem. 48: 2184-2188.

Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406-425.

Sanjuan, A. and Comesana, A.S. 2002. Molecular identification of nine commercial flaffish species by polymerase chain reaction-restriction fragment length polymorphism analysis of a segment of the cytochrome *b* region. J. Food Protect. 65: 1016-1023.

Santaclara, F.J., Cabado, A.G., and Vieites, J.M. 2006. Development of a method for genetic identification of four species of anchovies: *E. encrasicolus*, *E. anchoita*, *E. ringens* and *E. japonicus*. Eur. Food Res. Technol. 223: 609-614.

Schlei, O.L., Crete-Lafreniere, A., Whiteley, A.R., Brown, R.J., Olsen, J.B., Bernatchez, L., and Wenburg, J.K. 2008. DNA barcoding of eight North American coregonine species. Mol. Ecol. Resour. 8: 1212-1218.

Serapion, J., Kucuktas, H., Feng, J., and Liu, Z. 2004. Bioinformatic mining of type I microsatellites from expressed sequence tags of channel catfish (*Ictalurus punctatus*). Mar. Biotech. 6: 364-377.

Sevilla, R.G., Diez, A., Noren, M., Mouchel, O., Jerome, M., Verrez-Bagnis, V., Van Pelt, H., Favre-Krey, L., Krey, G., FishTraceConsortium, and Bautista, J.M. 2007. Primers and polymerase chain reaction conditions for DNA barcoding teleost fish based on the mitochondrial cytochrome b and nuclear rhodopsin genes. Mol. Ecol. Notes. 7: 730-734.

Shivji, M., Clarke, S., Pank, M., Natanson, L., Kohler, N., and Stanhope, M. 2002. Genetic identification of pelagic shark body parts for conservation and trade monitoring. Conserv. Biol. 16: 1036-1047.

Simon, M.C., Gray, D.I., and Cook, N. 1996. DNA extraction and PCR methods for the detection of *Listeria monocytogenes* in cold-smoked salmon. Appl. Environ. Microbiol. 62: 822-824.

Smith, G.R. 1992. Introgression in fishes: Significance for paleontology, cladistics, and evolutionary rates. Syst. Biol. 41: 41-57.

Smith, P.J., McVeagh, S.M., and Steinke, D. 2008. DNA barcoding for the identification of smoked fish products. J. Fish Biol. 72: 464-471.

Smith, S., Hons, B.S., and Morin, P.A.M. 2005. Optimal storage conditions for highly dilute DNA samples: a role for trehalose as a preserving agent. J. Forensic Sci. 50: 1101-1108.

Sotelo, C.G., Calo-Mata, P., Chapela, M.J., Perez-Martin, R.I., Rehbein, H., Hold, G.L., Russell, V.J., Pryde, S., Quinteiro, J., Izquierdo, M., Rey-Mendez, M., Rosa, C., and Santos, A.T. 2001. Identification of flatfish (*Pleuronectiforme*) species using DNA-based techniques. J. Agric. Food Chem. 49: 4562-4569.

Sotelo, C.G., Chapela, M.J., Rey, M., and Perez-Martin, R. 2003. Development of an identification and quantitation system for cod (*Gadus morhua*) using Taqman assay. In Proceedings of the First Trans-Atlantic Fisheries Technology Conference, Reykjavik, Iceland, June 10-14. p. 195-198.

Sotelo, C.G., Pineiro, C., Gallardo, J.M., and Perez-Martin, R.I. 1993. Fish species identification in seafood products. Trends Food Sci. Technol. 4: 395-401.

Steinke, D., Zemlak, T.S., Gavin, H., and Hebert, P.D.N. 2009. DNA barcoding fishes of the Canadian Pacific. Mar. Biol. 156: 2641-2647.

Takeyama, H., Chow, S., Tsuduki, H., and Matsunaga, T. 2001. Mitochondrial DNA sequence variation within and between *Thunnus* tuna species and its application to species identification. J. Fish Biol. 58: 1646-1657.

Tamura, K., Dudley, J., Nei, M., and Kumar, S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24: 1596-1599.

Tamura, K. and Nei, M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol. Biol. Evol. 10: 512-526.

Taylor, M.I., Fox, C., Rico, I., and 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.

Teletchea, F., Maudet, C., and Hanni, C. 2005. Food and forensic molecular identification: update and challenges. Trends Biotechnol. 23: 359-366.

Tenge, B.J., Dang, N.L., Fry, F.S., Savary, W.E., Rogers, P.L., Barnett, J.D., Hill, W.E., Wiskerchen, J.E., and Wekell, M.M. 1997. Integration of computer and laboratory techniques for species identification including development of a regulatory fish encyclopedia, p. 214-226. In: R.E. Martin, R.L. Collette, and J.W. Slavin (eds.). Fish inspection, quality control, and HACCP - a global focus. Technomic Publishing, Lancaster, Pa.

Thomas, W.K. and Beckenbach, A.T. 1989. Variation in salmonid mitochondrial DNA: evolutionary constraints and mechanisms of substitution. J. Mol. Evol. 29: 233-245.

Thomas, W.K., Withler, R.E., and Beckenbach, A.T. 1986. Mitochondrial DNA analysis of Pacific salmonid evolution. Can. J. Fish. Aquat. Sci. 64: 1058-1064.

Trotta, M., Schonhuth, S., Pepe, T., Cortesi, M.L., Puyet, A., and Bautista, J.M. 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: 2039-2045.

Unseld, M., Beyermann, B., Brandt, P., and Hiesel, R. 1995. Identification of the species origin of highly processed meat products by mitochondrial DNA sequences. PCR Methods Appl. 4: 241-243.

USFDA. 2009. Regulatory Fish Encyclopedia: Seafood species substitution and economic fraud. Accessible at: http://www.fda.gov/Food/FoodSafety/Product-SpecificInformation/Seafood/RegulatoryFishEncyclopediaRFE/ucm071528.htm.

van Pelt-Verkuil, E., van Belkum, A., and Hays, J.P. 2008. Principles and Technical Aspects of PCR Amplification. Springer, The Netherlands.

Verkaar, E.L., Boutaga, K., Nijman, I.J., and Lenstra, J.A. 2001. Differentiation of bovine species in beef by PCR-RFLP of mitochondrial and satellite DNA. Meat Sci. 60: 365-369.

Voorhees, D.V. 2007. Fisheries of the United States, 2006. E.S. Pritchard (ed.). National Marine Fisheries Service, Office of Science and Technology, Silver Spring, Maryland.

Voorhees, D.V. 2008. Fisheries of the United States, 2007. E.S. Pritchard (ed.). National Marine Fisheries Service, Office of Science and Technology, Silver Spring, Maryland.

Voorhees, D.V. 2009. Fisheries of the United States, 2008. E.S. Pritchard (ed.). National Marine Fisheries Service, Office of Science and Technology, Silver Spring, Maryland.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acid. Res. 23: 4407-4414.

Waldbieser, G.C., Bosworth, B.G., Nonneman, D.J., and Wolters, W.R. 2001. A microsatellite-based genetic linkage map for channel catfish, *Ictalurus punctatus*. Genetics. 158: 727-734.

Wang, H. and Guo, X. 2008. Identification of *Crassostrea ariakensis* and related oysters by multiplex species-specific PCR. J. Shellfish Res. 27: 481-487.

Waples, R.S., Gustafson, R.G., Weitkamp, L.A., Myers, J.M., Johnson, O.W., Busby, P.J., Hard, J.J., Bryant, G.J., Waknitz, F.W., Neely, K., Teel, D., Grant, W.S., Winans, G.A., Phelps, S., Marshall, A., and Baker, B.M. 2001. Characterizing diversity in salmon from the Pacific Northwest. J. Fish Biol. 59: 1-41.

Ward, R.D., Hanner, R., and Hebert, P.D.N. 2009. The campaign to DNA barcode all fishes, FISH-BOL. J. Fish Biol. 74: 329-356.

Ward, R.D. and Holmes, B.H. 2007. An analysis of nucleotide and amino acid variability in the barcode region of cytochrome *c* oxidase I (*cox1*) in fishes. Mol. Ecol. Notes. 7: 899-907.

Ward, R.D., Zemlak, T.S., Innes, B.H., Last, P.R., and Hebert, P.D.N. 2005. DNA barcoding Australia's fish species. Phil. Trans. R. Soc. B. 360: 1847-1857.

Williams, J.G., Kubelik, A.R., Livak, K.J., Rafalski, J.A., and Tingey, S.V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acid. Res. 18: 6531-6535.

Wilson, G.M., Thomas, W.K., and Beckenbach, A.T. 1985. Intra- and inter-specific mitochondrial DNA sequence divergence in *Salmo*: Rainbow, steelhead, and cutthroat trouts. Can. J. Zool. 63: 2088-2094.

Wilson, G.M., Thomas, W.K., and Beckenbach, A.T. 1987. Mitochondrial DNA analysis of Pacific Northwest populations of *Oncorhynchus tshawytscha*. Can. J. Fish. Aquat. Sci. 44: 1301-1305.

Wilson, I.C. 1997. Inhibition and facilitation of nucleic acid amplification. Appl. Environ. Microbiol. 63: 3741-3751.

Wilson, K. and Walker, J. 2000. Principles and Techniques of Practical Biochemistry, Cambridge University Press, Cambridge, United Kingdom, pp. 784.

Withler, R., Candy, J.R., Beacham, T.D., and Miller, K.M. 2004. Forensic DNA analysis of Pacific salmonid samples for species and stock identification. Environ. Biol. Fish. 69: 275-285.

Withler, R.E., Beacham, T.D., Ming, T.J., and Miller, K.M. 1997. Species identification of Pacific salmon by means of a major histocompatibility complex gene. N. Am. J. Fish. Management. 17: 929-938.

Wolf, C., Burgener, M., Hubner, P., and Luthy, J. 2000. PCR-RFLP analysis of mitochondrial DNA: differentiation of fish species. Lebensmittel-Wissenschaft und-Technologie. 33: 144-150.

Wolf, C., Hubner, P., and Luthy, J. 1999. Differentiation of sturgeon species by PCR-RFLP. Food Res. Inter. 32: 699-705.

Wolf, C. and Luthy, J. 2001. Quantitative competitive (QC) PCR for quantification of porcine DNA. Meat Sci. 57: 161-168.

Wong, E.H.K. and Hanner, R.H. 2008. DNA barcoding detects market substitution in North American seafood. Food Res. Int. 41: 828-837.

Woolfe, M. and Primrose, S. 2004. Food forensics: using DNA technology to combat misdescription and fraud. Trends Biotechnol. 22: 222-226.

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., and Hebert, P.D.N. 2008. Potential use of DNA barcodes in regulatory science: Applications of the *Regulatory Fish Encyclopedia*. J. Food Prot. 71: 456-458.

Young, W.P., Wheeler, P.A., Coryell, V.H., Keim, P., and Thorgaard, G.H. 1998. A detailed linkage map of rainbow trout produced using doubled haploids. Genetics. 148: 839-850.

Yu, Z. and Guo, X. 2003. Genetic linkage map of the eastern oyster *Crassostrea virginica* Gmelin. Biol. Bull. 204: 327-338.

Yue, G., Li, Y., Chen, F., Cho, S., Lim, L.C., and Orban, L. 2002. Comparison of three DNA marker systems for assessing genetic diversity in Asian arowana (*Scleropages formosus*). Electrophoresis. 23: 1025-1032.

Zardoya, R., Garrido-Pertierra, A., and Bautista, J.M. 1995. The complete nucleotide sequence of the mitochondrial DNA genome of the rainbow trout, *Oncorhynchus mykiss*. J. Mol. Evol. 41: 942-951.

Zhang, J. and Cai, Z. 2006. Differentiation of the rainbow trout (*Oncorhynchus mykiss*) from Atlantic salmon (*Salmo salmar*) by the AFLP-derived SCAR. Eur. Food Res. Technol. 223: 413-417.

APPENDIX

Figure A.1 Neighbor-joining tree displaying the results of this project combined with the results of the "Barcoding of Canadian freshwater fishes" project. The tree was generated on BOLD based on the Kimura 2-parameter distance method and includes barcode sequences from a total of 979 individuals representing 9 species.

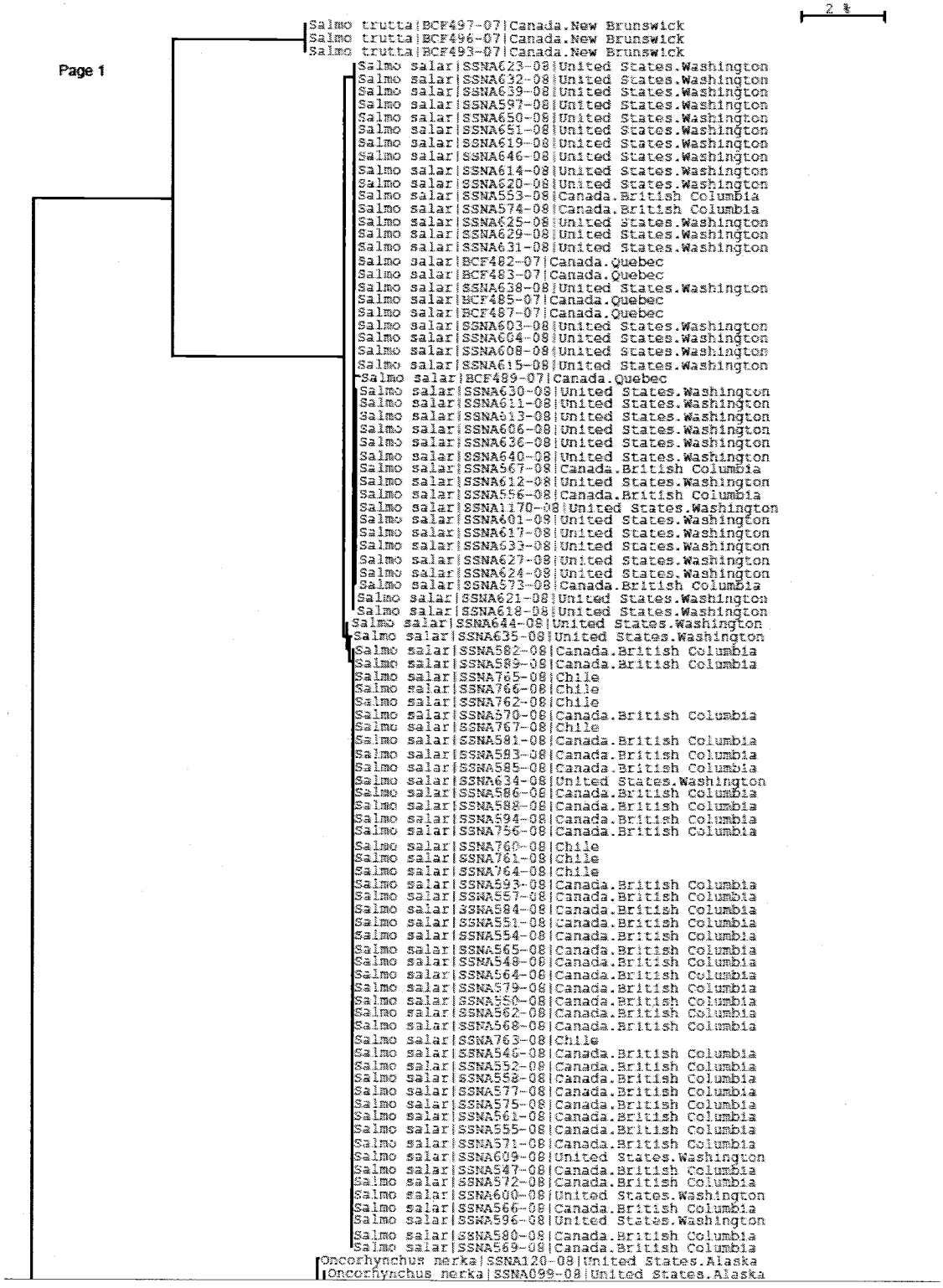
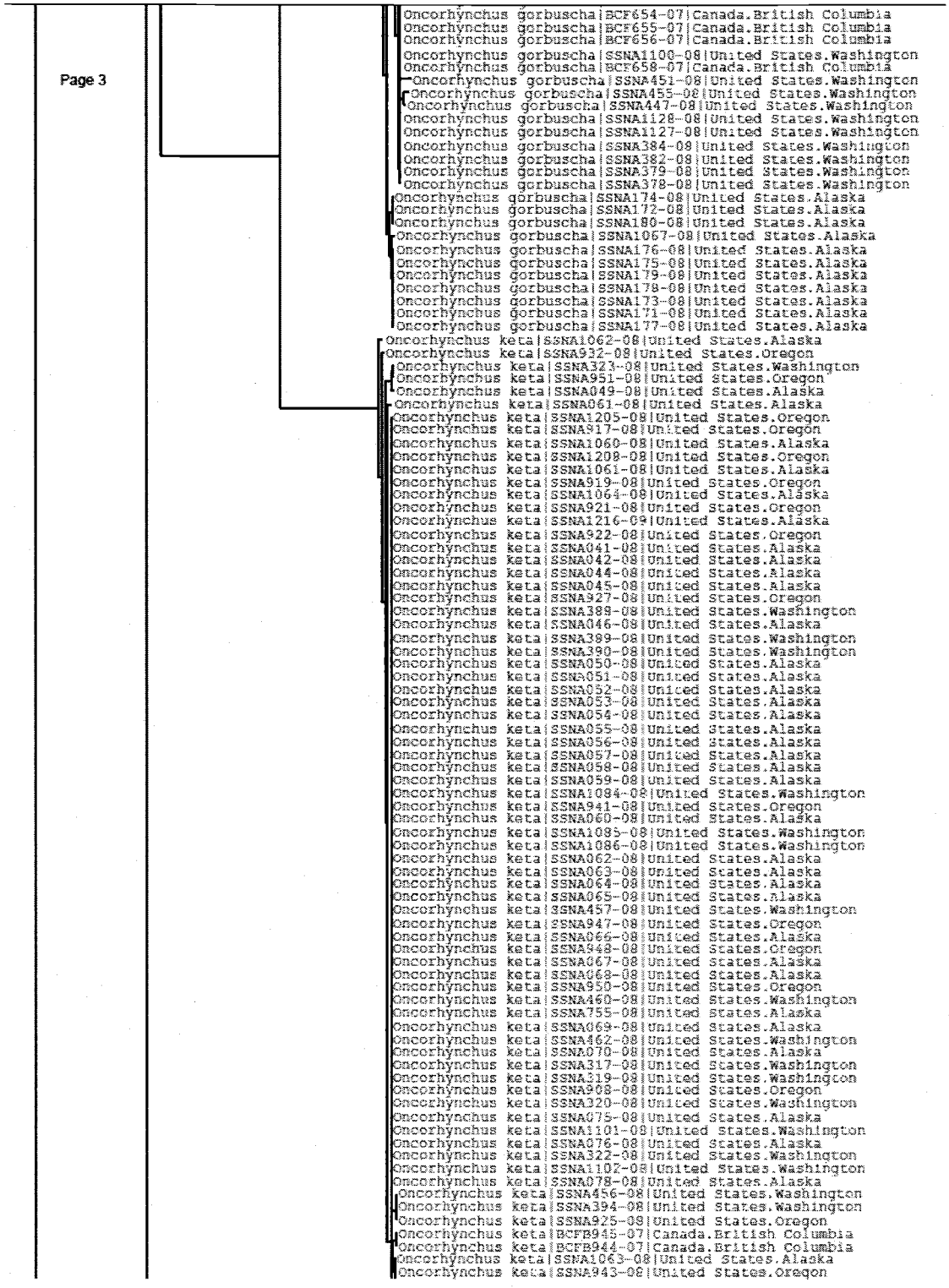

2 %
Page 1
Salmo trutta|BCF497-07|Canada.New Brunswick
Salmo trutta|BCF496-07|Canada.New Brunswick
Salmo trutta|BCF493-07|Canada.New Brunswick
Salmo salar|SSNA623-08|United States.Washington
Salmo salar|SSNA632-08|United States.Washington
Salmo salar|SSNA639-08|United States.Washington
Salmo salar|SSNA597-08|United States.Washington
Salmo salar|SSNA650-08|United States.Washington
Salmo salar|SSNA651-08|United States.Washington
Salmo salar|SSNA619-08|United States.Washington
Salmo salar|SSNA646-08|United States.Washington
Salmo salar|SSNA614-08|United States.Washington
Salmo salar|SSNA620-08|United States.Washington
Salmo salar|SSNA553-08|Canada.British Columbia
Salmo salar|SSNA574-08|Canada.British Columbia
Salmo salar|SSNA625-08|United States.Washington
Salmo salar|SSNA629-08|United States.Washington
Salmo salar|SSNA631-08|United States.Washington
Salmo salar|BCF482-07|Canada.Quebec
Salmo salar|BCF483-07|Canada.Quebec
Salmo salar|SSNA638-08|United States.Washington
Salmo salar|BCF485-07|Canada.Quebec
Salmo salar|BCF487-07|Canada.Quebec
Salmo salar|SSNA603-08|United States.Washington
Salmo salar|SSNA604-08|United States.Washington
Salmo salar|SSNA608-08|United States.Washington
Salmo salar|SSNA615-08|United States.Washington
Salmo salar|BCF489-07|Canada.Quebec
Salmo salar|SSNA630-08|United States.Washington
Salmo salar|SSNA611-08|United States.Washington
Salmo salar|SSNA613-08|United States.Washington
Salmo salar|SSNA606-08|United States.Washington
Salmo salar|SSNA636-08|United States.Washington
Salmo salar|SSNA640-08|United States.Washington
Salmo salar|SSNA567-08|Canada.British Columbia
Salmo salar|SSNA612-08|United States.Washington
Salmo salar|SSNA556-08|Canada.British Columbia
Salmo salar|SSNA1170-08|United States.Washington
Salmo salar|SSNA601-09|United States.Washington
Salmo salar|SSNA617-08|United States.Washington
Salmo salar|SSNA633-08|United States.Washington
Salmo salar|SSNA627-08|United States.Washington
Salmo salar|SSNA624-08|United States.Washington
Salmo salar|SSNA573-08|Canada.British Columbia
Salmo salar|SSNA621-08|United States.Washington
Salmo salar|SSNA618-08|United States.Washington
Salmo salar|SSNA644-08|United States.Washington
Salmo salar|SSNA635-08|United States.Washington
Salmo salar|SSNA582-08|Canada.British Columbia
Salmo salar|SSNA589-08|Canada.British Columbia
Salmo salar|SSNA765-08|Chile
Salmo salar|SSNA766-08|Chile
Salmo salar|SSNA762-08|Chile
Salmo salar|SSNA570-08|Canada.British Columbia
Salmo salar|SSNA767-08|Chile
Salmo salar|SSNA581-08|Canada.British Columbia
Salmo salar|SSNA583-08|Canada.British Columbia
Salmo salar|SSNA585-08|Canada.British Columbia
Salmo salar|SSNA634-08|United States.Washington
Salmo salar|SSNA586-08|Canada.British Columbia
Salmo salar|SSNA588-08|Canada.British Columbia
Salmo salar|SSNA594-08|Canada.British Columbia
Salmo salar|SSNA756-08|Canada.British Columbia
Salmo salar|SSNA760-08|Chile
Salmo salar|SSNA761-08|Chile
Salmo salar|SSNA764-08|Chile
Salmo salar|SSNA593-08|Canada.British Columbia
Salmo salar|SSNA557-08|Canada.British Columbia
Salmo salar|SSNA584-08|Canada.British Columbia
Salmo salar|SSNA551-08|Canada.British Columbia
Salmo salar|SSNA554-08|Canada.British Columbia
Salmo salar|SSNA565-08|Canada.British Columbia
Salmo salar|SSNA548-08|Canada.British Columbia
Salmo salar|SSNA564-08|Canada.British Columbia
Salmo salar|SSNA579-08|Canada.British Columbia
Salmo salar|SSNA550-08|Canada.British Columbia
Salmo salar|SSNA562-08|Canada.British Columbia
Salmo salar|SSNA568-08|Canada.British Columbia
Salmo salar|SSNA763-08|Chile
Salmo salar|SSNA546-08|Canada.British Columbia
Salmo salar|SSNA552-08|Canada.British Columbia
Salmo salar|SSNA558-08|Canada.British Columbia
Salmo salar|SSNA577-08|Canada.British Columbia
Salmo salar|SSNA575-08|Canada.British Columbia
Salmo salar|SSNA561-08|Canada.British Columbia
Salmo salar|SSNA555-08|Canada.British Columbia
Salmo salar|SSNA571-08|Canada.British Columbia
Salmo salar|SSNA609-08|United States.Washington
Salmo salar|SSNA547-08|Canada.British Columbia
Salmo salar|SSNA572-08|Canada.British Columbia
Salmo salar|SSNA600-08|United States.Washington
Salmo salar|SSNA566-08|Canada.British Columbia
Salmo salar|SSNA596-08|United States.Washington
Salmo salar|SSNA580-08|Canada.British Columbia
Salmo salar|SSNA569-08|Canada.British Columbia
Oncorhynchus nerka|SSNA120-08|United States.Alaska
Oncorhynchus nerka|SSNA099-08|United States.Alaska

Figure A.1

Figure A.1 (Continued)



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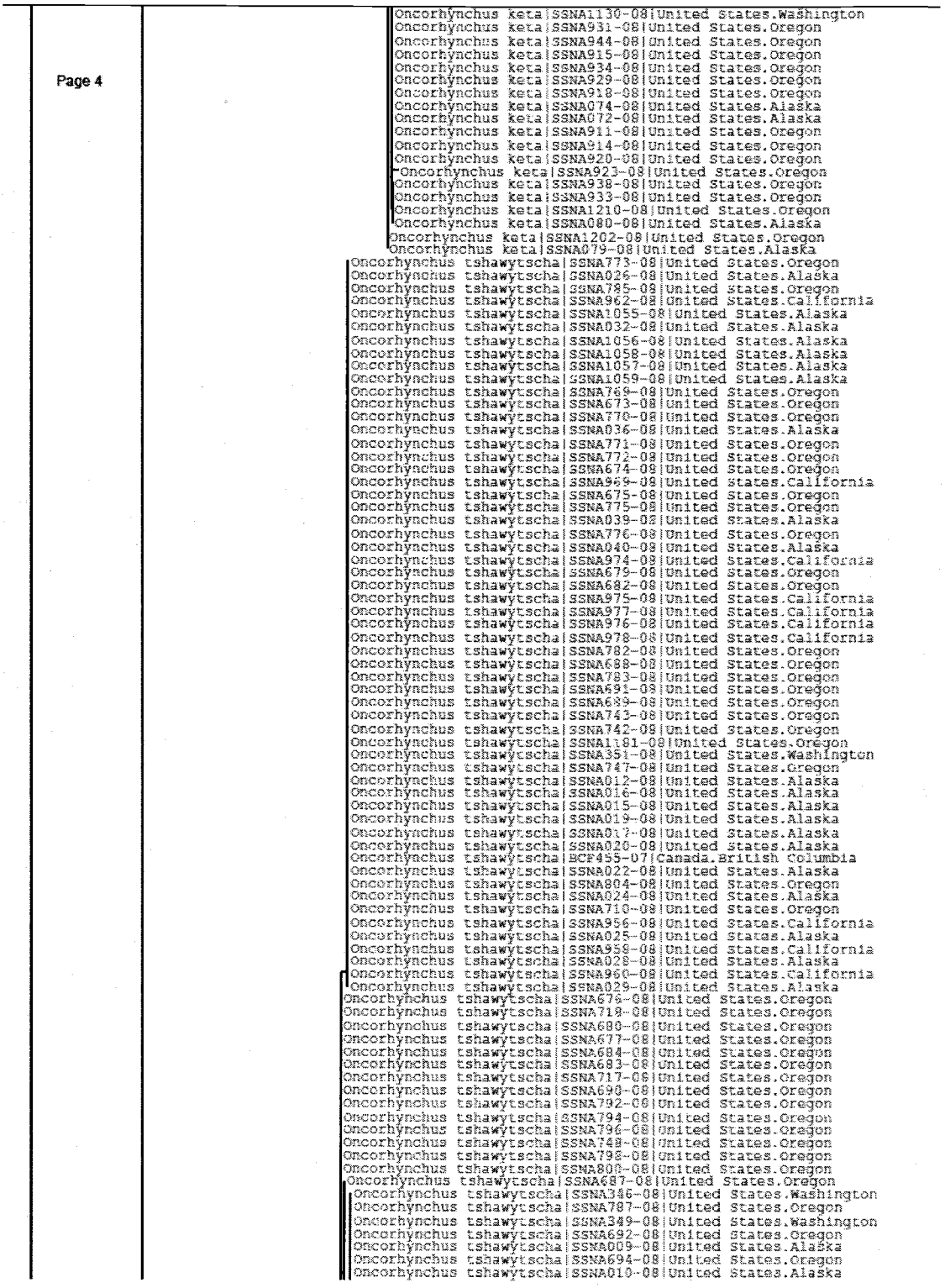

Figure A.1 (Continued)

Figure A.1 (Continued)

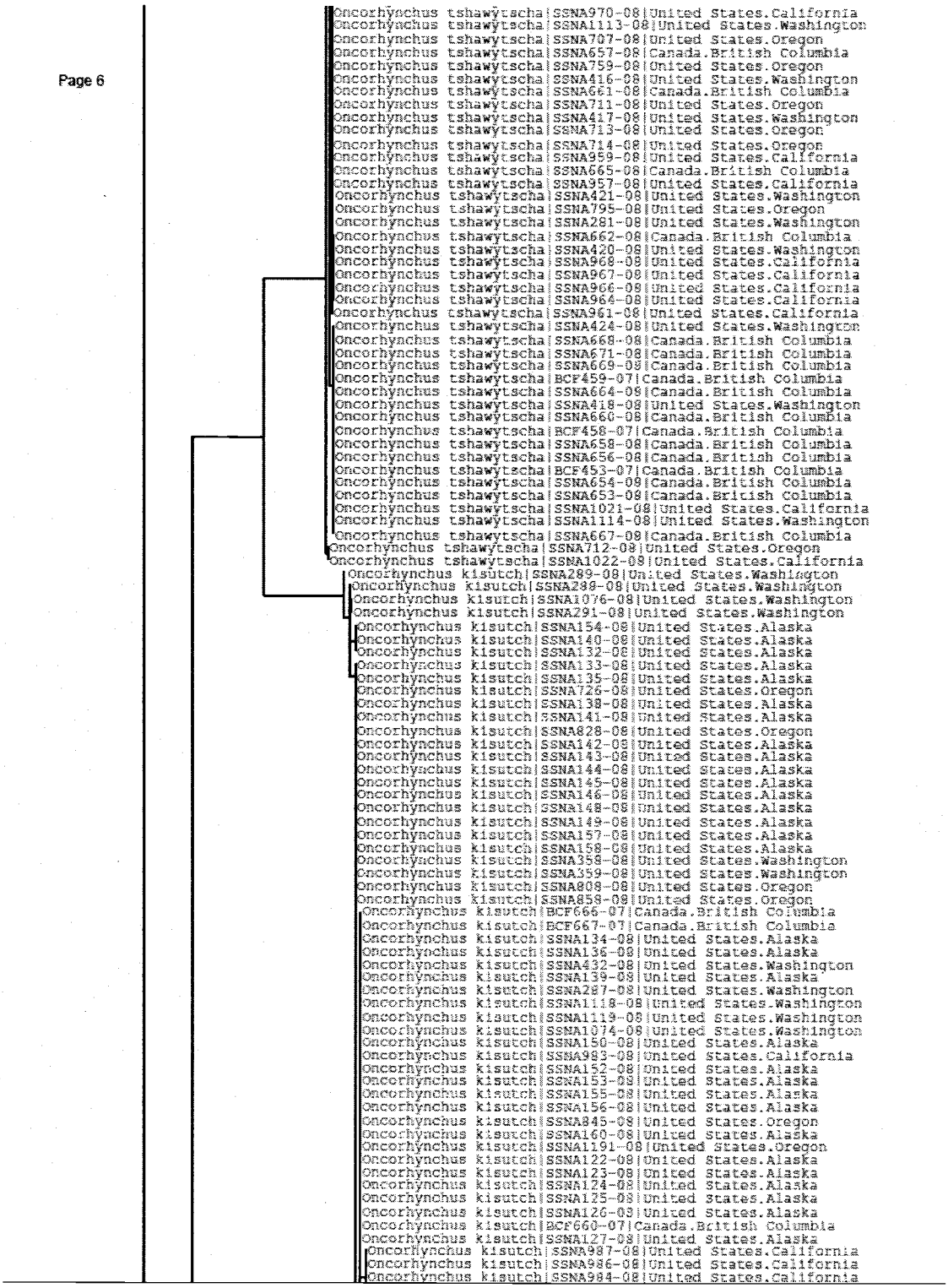

Figure A.1 (Continued)

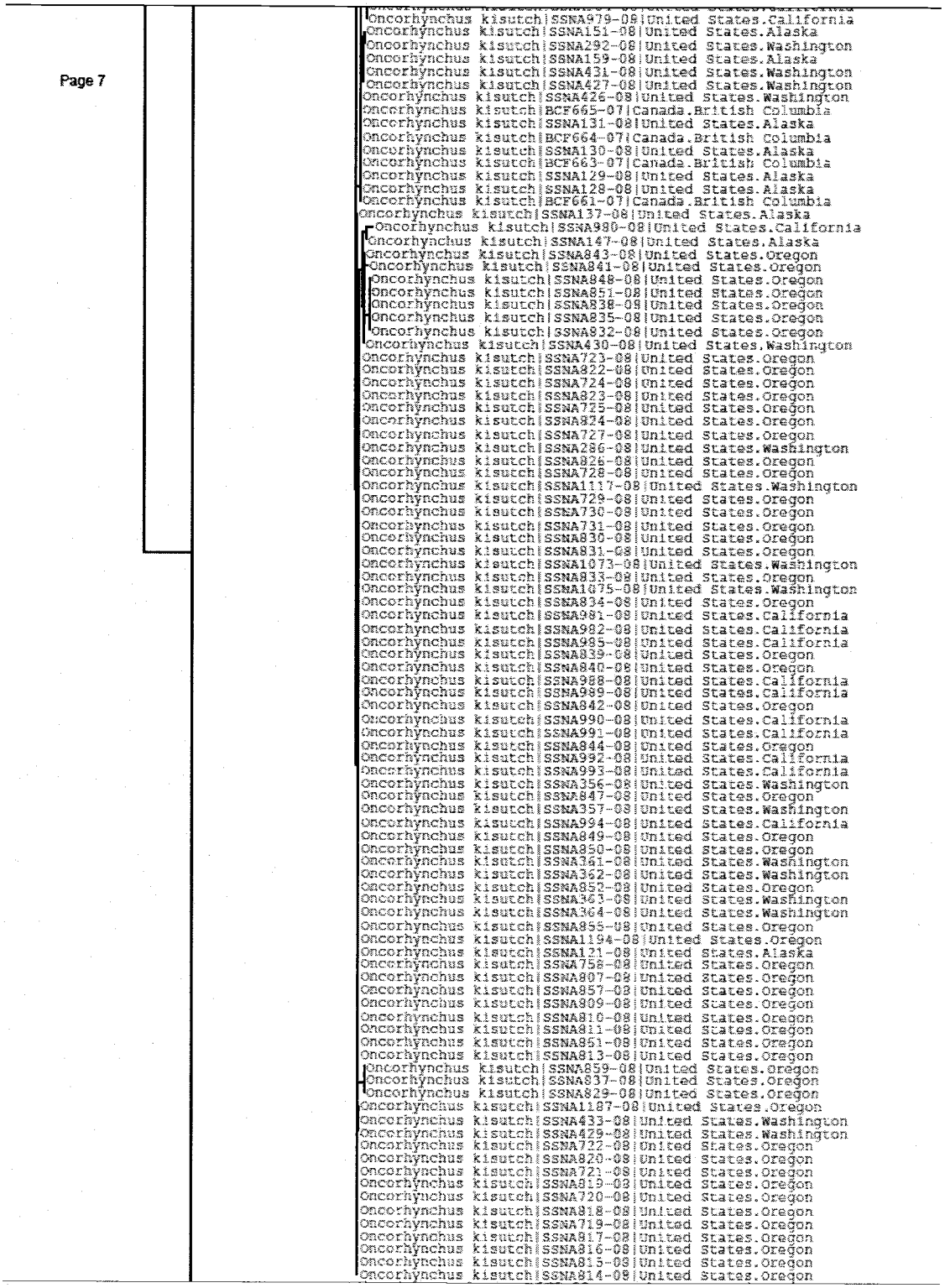

Figure A.1 (Continued)

Figure A.1 (Continued)

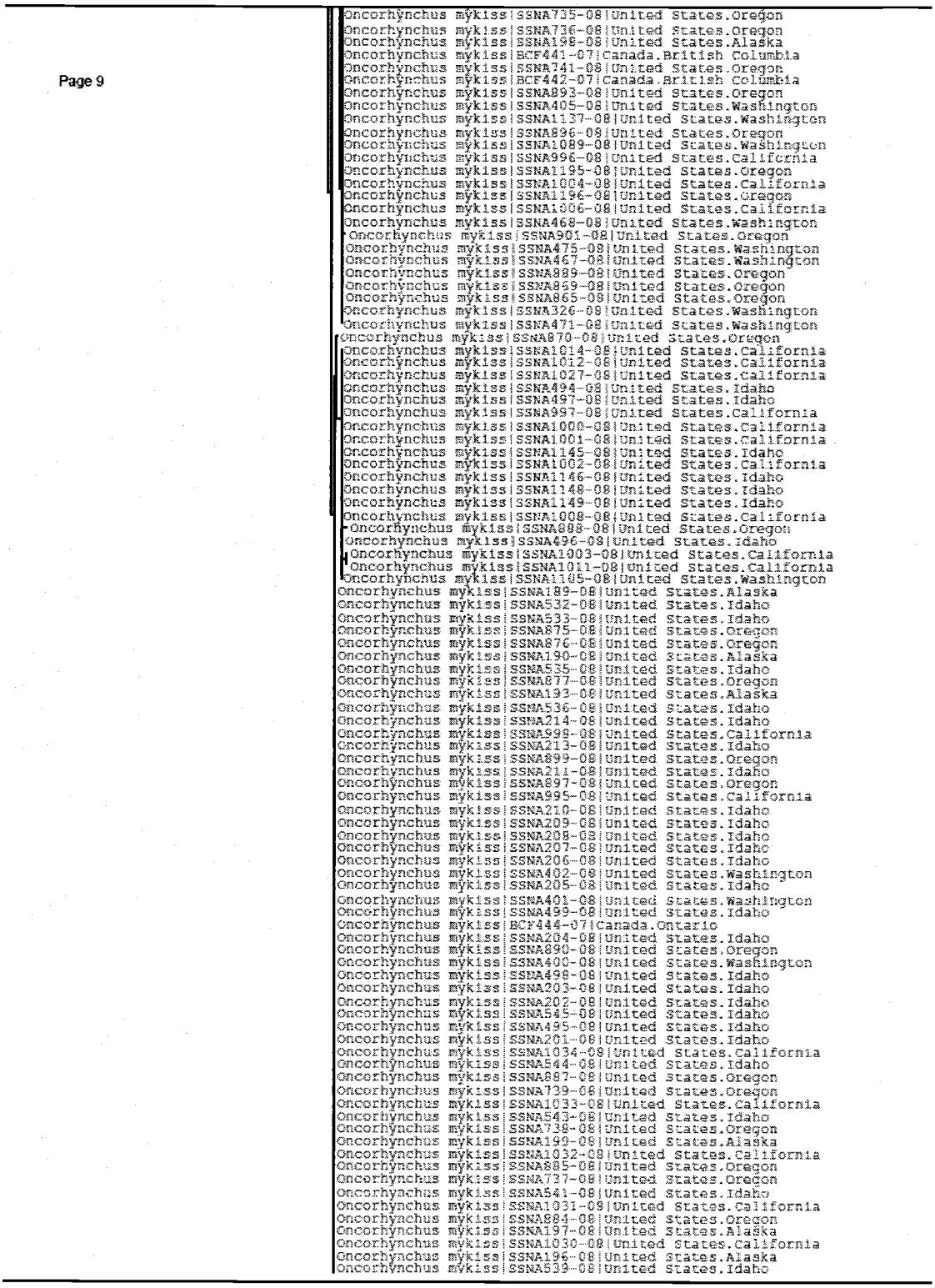

Figure A.1 (Continued)

Page 10

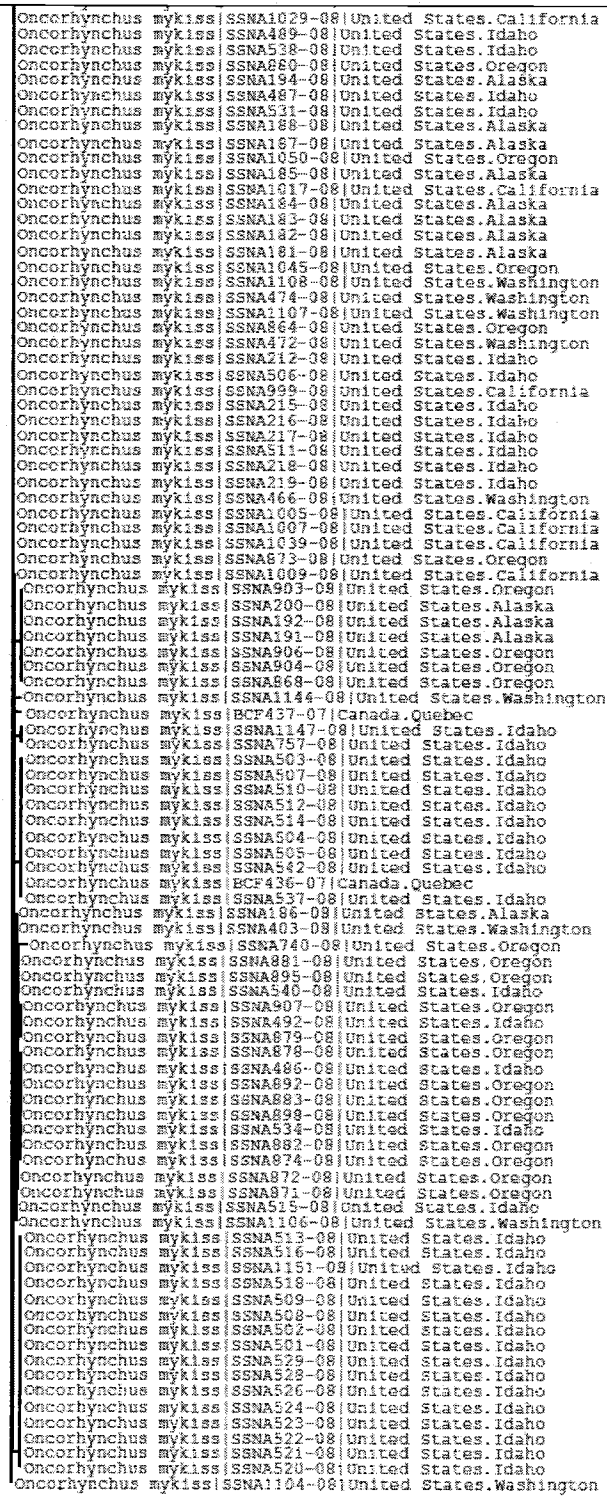

Figure A.1 (Continued)

Table A.1 Sample and sequence details for all DNA barcodes obtained in this study, including species names, GenBank accession numbers, sequence lengths, and geographic sampling regions.

Species	BOLD Sample ID	GenBank Accession No.	Barcode Sequence Length (bp)	Country	State/Province	Region
Oncorhynchus clarkii bouvierii	SSNA1069-08	FJ998606	652	United States	Idaho	Miner Creek
Oncorhynchus clarkii clarkii	SSNA1094-08	FJ998607	618	United States	Washington	Olympic Peninsula, Lyre River
Oncorhynchus clarkii clarkii	SSNA1093-08	FJ998608	624	United States	Washington	Olympic Peninsula, Lyre River
Oncorhynchus clarkii clarkii	SSNA1091-08	FJ998609	652	United States	Washington	Olympic Peninsula, Lyre River
Oncorhynchus clarkii clarkii	SSNA1111-08	FJ998610	652	United States	Washington	Columbia River Basin, Elochoman River (Beaver Creek Hatchery)
Oncorhynchus clarkii clarkii	SSNA1109-08	FJ998611	652	United States	Washington	Columbia River Basin, Elochoman River (Beaver Creek Hatchery)
Oncorhynchus clarkii clarkii	SSNA1110-08	FJ998612	652	United States	Washington	Columbia River Basin, Elochoman River (Beaver Creek Hatchery)
Oncorhynchus clarkii clarkii	SSNA1112-08	FJ998613	652	United States	Washington	Columbia River Basin, Elochoman River (Beaver Creek Hatchery)
Oncorhynchus clarkii clarkii	SSNA1140-08	FJ998614	652	United States	Washington	Puget Sound, Cedar River
Oncorhynchus clarkii clarkii	SSNA1138-08	FJ998615	652	United States	Washington	Puget Sound, Cedar River
Oncorhynchus clarkii utah	SSNA264-08	FJ998616	652	United States	Utah	Bear Lake
Oncorhynchus clarkii clarkii	SSNA1143-08	FJ998617	652	United States	Washington	Puget Sound, Cedar River
Oncorhynchus clarkii clarkii	SSNA1142-08	FJ998618	652	United States	Washington	Puget Sound, Cedar River
Oncorhynchus clarkii utah	SSNA263-08	FJ998619	652	United States	Utah	Bear Lake
Oncorhynchus clarkii bouvierii	SSNA254-08	FJ998620	652	United States	Idaho	Corral Canyon
Oncorhynchus clarkii lewisii	SSNA241-08	FJ998621	652	United States	Idaho	Upper Elkhorn Cr.
Oncorhynchus clarkii utah	SSNA262-08	FJ998622	652	United States	Utah	Bear Lake
Oncorhynchus clarkii lewisii	SSNA249-08	FJ998623	652	United States	Idaho	Cannuck Creek
Oncorhynchus clarkii bouvierii	SSNA253-08	FJ998624	652	United States	Idaho	Corral Canyon
Oncorhynchus clarkii lewisii	SSNA248-08	FJ998625	652	United States	Idaho	Cannuck Creek
Oncorhynchus clarkii lewisii	SSNA247-08	FJ998626	652	United States	Idaho	Cannuck Creek

Table A.1 (Continued)

Oncorhynchus clarkii lewisii	SSNA243-08	FJ998627	652	United States	Idaho	Upper Elkhorn Cr.
Oncorhynchus clarkii lewisii	SSNA242-08	FJ998628	652	United States	Idaho	Upper Elkhorn Cr.
Oncorhynchus clarkii lewisii	SSNA246-08	FJ998629	652	United States	Idaho	Cannuck Creek
Oncorhynchus clarkii lewisii	SSNA244-08	FJ998630	652	United States	Idaho	Upper Elkhorn Cr.
Oncorhynchus clarkii lewisii	SSNA245-08	FJ998631	652	United States	Idaho	Upper Elkhorn Cr.
Oncorhynchus clarkii bouvierii	SSNA252-08	FJ998632	550	United States	Idaho	Corral Canyon
Oncorhynchus clarkii lewisii	SSNA250-08	FJ998633	652	United States	Idaho	Cannuck Creek
Oncorhynchus clarkii bouvierii	SSNA251-08	FJ998634	652	United States	Idaho	Corral Canyon
Oncorhynchus clarkii bouvierii	SSNA256-08	FJ998635	652	United States	Idaho	Miner Creek
Oncorhynchus clarkii utah	SSNA261-08	FJ998636	652	United States	Utah	Bear Lake
Oncorhynchus clarkii bouvierii	SSNA255-08	FJ998637	652	United States	Idaho	Corral Canyon
Oncorhynchus clarkii bouvierii	SSNA258-08	FJ998638	652	United States	Idaho	Miner Creek
Oncorhynchus clarkii bouvierii	SSNA257-08	FJ998639	652	United States	Idaho	Miner Creek
Oncorhynchus clarkii bouvierii	SSNA259-08	FJ998640	652	United States	Idaho	Miner Creek
Oncorhynchus clarkii utah	SSNA265-08	FJ998641	652	United States	Utah	Bear Lake
Oncorhynchus clarkii utah	SSNA273-08	FJ998642	652	United States	Utah	Glenwood Hatchery
Oncorhynchus clarkii utah	SSNA268-08	FJ998643	652	United States	Utah	Logan River
Oncorhynchus clarkii utah	SSNA266-08	FJ998644	652	United States	Utah	Logan River
Oncorhynchus clarkii utah	SSNA267-08	FJ998645	652	United States	Utah	Logan River
Oncorhynchus clarkii utah	SSNA272-08	FJ998646	652	United States	Utah	Glenwood Hatchery
Oncorhynchus clarkii utah	SSNA271-08	FJ998647	652	United States	Utah	Glenwood Hatchery
Oncorhynchus clarkii utah	SSNA270-08	FJ998648	652	United States	Utah	Logan River
Oncorhynchus clarkii utah	SSNA269-08	FJ998649	652	United States	Utah	Logan River
Oncorhynchus clarkii utah	SSNA274-08	FJ998650	652	United States	Utah	Glenwood Hatchery
Oncorhynchus clarkii utah	SSNA275-08	FJ998651	633	United States	Utah	Glenwood Hatchery
Oncorhynchus clarkii clarkii	SSNA344-08	FJ998652	652	United States	Washington	Olympic Peninsula, Lyre River
Oncorhynchus clarkii clarkii	SSNA338-08	FJ998653	652	United States	Washington	Olympic Peninsula, Lyre River
Oncorhynchus clarkii clarkii	SSNA339-08	FJ998654	585	United States	Washington	Olympic Peninsula, Lyre River
Oncorhynchus clarkii clarkii	SSNA342-08	FJ998655	652	United States	Washington	Olympic Peninsula, Lyre River

Table A.1 (Continued)

Oncorhynchus clarkii clarkii	SSNA341-08	FJ998656	652	United States	Washington	Olympic Peninsula, Lyre River
Oncorhynchus clarkii clarkii	SSNA408-08	FJ998657	652	United States	Washington	Columbia River Basin, Elochoman River (Beaver Creek Hatchery)
Oncorhynchus clarkii clarkii	SSNA407-08	FJ998658	652	United States	Washington	Columbia River Basin, Elochoman River (Beaver Creek Hatchery)
Oncorhynchus clarkii clarkii	SSNA414-08	FJ998659	596	United States	Washington	Columbia River Basin, Elochoman River (Beaver Creek Hatchery)
Oncorhynchus clarkii clarkii	SSNA410-08	FJ998660	652	United States	Washington	Columbia River Basin, Elochoman River (Beaver Creek Hatchery)
Oncorhynchus clarkii clarkii	SSNA411-08	FJ998661	652	United States	Washington	Columbia River Basin, Elochoman River (Beaver Creek Hatchery)
Oncorhynchus clarkii clarkii	SSNA413-08	FJ998662	652	United States	Washington	Columbia River Basin, Elochoman River (Beaver Creek Hatchery)
Oncorhynchus clarkii clarkii	SSNA478-08	FJ998663	601	United States	Washington	Puget Sound, Cedar River
Oncorhynchus clarkii clarkii	SSNA479-08	FJ998664	652	United States	Washington	Puget Sound, Cedar River
Oncorhynchus gorbuscha	SSNA180-08	FJ998665	652	United States	Alaska	George River Weir
Oncorhynchus gorbuscha	SSNA179-08	FJ998666	652	United States	Alaska	George River Weir
Oncorhynchus gorbuscha	SSNA1067-08	FJ998667	652	United States	Alaska	Totemoff Middle Tidal
Oncorhynchus gorbuscha	SSNA178-08	FJ998668	652	United States	Alaska	George River Weir
Oncorhynchus gorbuscha	SSNA177-08	FJ998669	652	United States	Alaska	George River Weir
Oncorhynchus gorbuscha	SSNA176-08	FJ998670	652	United States	Alaska	George River Weir
Oncorhynchus gorbuscha	SSNA1082-08	FJ998671	652	United States	Washington	Olympic Peninsula, Lower Dungeness River
Oncorhynchus gorbuscha	SSNA1079-08	FJ998672	652	United States	Washington	Olympic Peninsula, Lower Dungeness River
Oncorhynchus gorbuscha	SSNA1081-08	FJ998673	652	United States	Washington	Olympic Peninsula, Lower Dungeness River
Oncorhynchus gorbuscha	SSNA1100-08	FJ998674	652	United States	Washington	Columbia River Basin, Cowlitz River
Oncorhynchus gorbuscha	SSNA1127-08	FJ998675	652	United States	Washington	Puget Sound, N.F. Stilliguamish River
Oncorhynchus gorbuscha	SSNA385-08	FJ998676	652	United States	Washington	Columbia River Basin, Cowlitz River
Oncorhynchus gorbuscha	SSNA1128-08	FJ998677	652	United States	Washington	Puget Sound, N.F. Stilliguamish River
Oncorhynchus gorbuscha	SSNA306-08	FJ998678	652	United States	Washington	Olympic Peninsula, Lower Dungeness River
Oncorhynchus gorbuscha	SSNA308-08	FJ998679	652	United States	Washington	Olympic Peninsula, Lower Dungeness River
Oncorhynchus gorbuscha	SSNA314-08	FJ998680	652	United States	Washington	Olympic Peninsula, Lower Dungeness River
Oncorhynchus gorbuscha	SSNA313-08	FJ998681	630	United States	Washington	Olympic Peninsula, Lower Dungeness River

Table A.1 (Continued)

Oncorhynchus gorbuscha	SSNA315-08	FJ998682	652	United States	Washington	Olympic Peninsula, Lower Dungeness River
Oncorhynchus gorbuscha	SSNA380-08	FJ998683	652	United States	Washington	Columbia River Basin, Cowlitz River
Oncorhynchus gorbuscha	SSNA381-08	FJ998684	652	United States	Washington	Columbia River Basin, Cowlitz River
Oncorhynchus gorbuscha	SSNA379-08	FJ998685	652	United States	Washington	Columbia River Basin, Cowlitz River
Oncorhynchus gorbuscha	SSNA384-08	FJ998686	652	United States	Washington	Columbia River Basin, Cowlitz River
Oncorhynchus gorbuscha	SSNA378-08	FJ998687	652	United States	Washington	Columbia River Basin, Cowlitz River
Oncorhynchus gorbuscha	SSNA383-08	FJ998688	652	United States	Washington	Columbia River Basin, Cowlitz River
Oncorhynchus gorbuscha	SSNA382-08	FJ998689	652	United States	Washington	Columbia River Basin, Cowlitz River
Oncorhynchus gorbuscha	SSNA455-08	FJ998690	639	United States	Washington	Puget Sound, N.F. Stilliguamish River
Oncorhynchus gorbuscha	SSNA454-08	FJ998691	652	United States	Washington	Puget Sound, N.F. Stilliguamish River
Oncorhynchus gorbuscha	SSNA449-08	FJ998692	652	United States	Washington	Puget Sound, N.F. Stilliguamish River
Oncorhynchus gorbuscha	SSNA448-08	FJ998693	652	United States	Washington	Puget Sound, N.F. Stilliguamish River
Oncorhynchus gorbuscha	SSNA447-08	FJ998694	629	United States	Washington	Puget Sound, N.F. Stilliguamish River
Oncorhynchus gorbuscha	SSNA446-08	FJ998695	640	United States	Washington	Puget Sound, N.F. Stilliguamish River
Oncorhynchus gorbuscha	SSNA450-08	FJ998696	652	United States	Washington	Puget Sound, N.F. Stilliguamish River
Oncorhynchus gorbuscha	SSNA451-08	FJ998697	652	United States	Washington	Puget Sound, N.F. Stilliguamish River
Oncorhynchus gorbuscha	SSNA175-08	FJ998698	652	United States	Alaska	George River Weir
Oncorhynchus gorbuscha	SSNA174-08	FJ998699	652	United States	Alaska	George River Weir
Oncorhynchus gorbuscha	SSNA165-08	FJ998700	652	United States	Alaska	Totemoff Middle Tidal
Oncorhynchus gorbuscha	SSNA164-08	FJ998701	652	United States	Alaska	Totemoff Middle Tidal
Oncorhynchus gorbuscha	SSNA163-08	FJ998702	652	United States	Alaska	Totemoff Middle Tidal
Oncorhynchus gorbuscha	SSNA173-08	FJ998703	652	United States	Alaska	George River Weir
Oncorhynchus gorbuscha	SSNA162-08	FJ998704	652	United States	Alaska	Totemoff Middle Tidal
Oncorhynchus gorbuscha	SSNA161-08	FJ998705	652	United States	Alaska	Totemoff Middle Tidal
Oncorhynchus gorbuscha	SSNA172-08	FJ998706	652	United States	Alaska	George River Weir
Oncorhynchus gorbuscha	SSNA170-08	FJ998707	651	United States	Alaska	Totemoff Middle Tidal
Oncorhynchus gorbuscha	SSNA169-08	FJ998708	652	United States	Alaska	Totemoff Middle Tidal
Oncorhynchus gorbuscha	SSNA168-08	FJ998709	652	United States	Alaska	Totemoff Middle Tidal
Oncorhynchus gorbuscha	SSNA167-08	FJ998710	652	United States	Alaska	Totemoff Middle Tidal

Table A.1 (Continued)

Oncorhynchus gorbuscha	SSNA171-08	FJ998711	652	United States	Alaska	George River Weir
Oncorhynchus keta	SSNA1216-09	FJ998712	652	United States	Alaska	Ilnik River, Three Hills River
Oncorhynchus keta	SSNA1060-08	FJ998713	652	United States	Alaska	Neets Bay
Oncorhynchus keta	SSNA1062-08	FJ998714	652	United States	Alaska	Neets Bay
Oncorhynchus keta	SSNA1061-08	FJ998715	652	United States	Alaska	Neets Bay
Oncorhynchus keta	SSNA1064-08	FJ998716	652	United States	Alaska	Ilnik River, Three Hills River
Oncorhynchus keta	SSNA1063-08	FJ998717	639	United States	Alaska	Ilnik River, Three Hills River
Oncorhynchus keta	SSNA1086-08	FJ998718	652	United States	Washington	Olympic Peninsula, Elwah River
Oncorhynchus keta	SSNA1085-08	FJ998719	652	United States	Washington	Olympic Peninsula, Elwah River
Oncorhynchus keta	SSNA1084-08	FJ998720	652	United States	Washington	Olympic Peninsula, Elwah River
Oncorhynchus keta	SSNA1102-08	FJ998721	652	United States	Washington	Columbia River Basin, Grays River
Oncorhynchus keta	SSNA1101-08	FJ998722	652	United States	Washington	Columbia River Basin, Grays River
Oncorhynchus keta	SSNA390-08	FJ998723	652	United States	Washington	Columbia River Basin, Grays River
Oncorhynchus keta	SSNA1130-08	FJ998724	581	United States	Washington	Puget Sound, Kennedy Creek
Oncorhynchus keta	SSNA1202-08	FJ998725	652	United States	Oregon	North Coast, Wilson River
Oncorhynchus keta	SSNA1205-08	FJ998726	652	United States	Oregon	Yaquina River
Oncorhynchus keta	SSNA1210-08	FJ998727	546	United States	Oregon	Yaquina River
Oncorhynchus keta	SSNA1208-08	FJ998728	652	United States	Oregon	Yaquina River
Oncorhynchus keta	SSNA322-08	FJ998729	652	United States	Washington	Olympic Peninsula, Elwah River
Oncorhynchus keta	SSNA320-08	FJ998730	652	United States	Washington	Olympic Peninsula, Elwah River
Oncorhynchus keta	SSNA319-08	FJ998731	652	United States	Washington	Olympic Peninsula, Elwah River
Oncorhynchus keta	SSNA317-08	FJ998732	652	United States	Washington	Olympic Peninsula, Elwah River
Oncorhynchus keta	SSNA323-08	FJ998733	652	United States	Washington	Olympic Peninsula, Elwah River
Oncorhynchus keta	SSNA923-08	FJ998734	556	United States	Oregon	North Coast, Kilchis River
Oncorhynchus keta	SSNA389-08	FJ998735	652	United States	Washington	Columbia River Basin, Grays River
Oncorhynchus keta	SSNA922-08	FJ998736	652	United States	Oregon	North Coast, Kilchis River
Oncorhynchus keta	SSNA388-08	FJ998737	652	United States	Washington	Columbia River Basin, Grays River
Oncorhynchus keta	SSNA921-08	FJ998738	652	United States	Oregon	North Coast, Kilchis River
Oncorhynchus keta	SSNA914-08	FJ998739	598	United States	Oregon	North Coast, Nehalem River

Table A.1 (Continued)

Oncorhynchus keta	SSNA908-08	FJ998740	652	United States	Oregon	North Coast, Necanicum River
Oncorhynchus keta	SSNA920-08	FJ998741	570	United States	Oregon	North Coast, Kilchis River
Oncorhynchus keta	SSNA911-08	FJ998742	536	United States	Oregon	North Coast, Nehalem River
Oncorhynchus keta	SSNA913-08	FJ998743	492	United States	Oregon	North Coast, Nehalem River
Oncorhynchus keta	SSNA919-08	FJ998744	652	United States	Oregon	North Coast, Kilchis River
Oncorhynchus keta	SSNA915-08	FJ998745	588	United States	Oregon	North Coast, Nehalem River
Oncorhynchus keta	SSNA918-08	FJ998746	574	United States	Oregon	North Coast, Kilchis River
Oncorhynchus keta	SSNA917-08	FJ998747	652	United States	Oregon	North Coast, Miami River
Oncorhynchus keta	SSNA925-08	FJ998748	652	United States	Oregon	North Coast, Kilchis River
Oncorhynchus keta	SSNA929-08	FJ998749	555	United States	Oregon	Salmon River
Oncorhynchus keta	SSNA927-08	FJ998750	652	United States	Oregon	Salmon River
Oncorhynchus keta	SSNA934-08	FJ998751	595	United States	Oregon	Yaquina River
Oncorhynchus keta	SSNA931-08	FJ998752	603	United States	Oregon	Yaquina River
Oncorhynchus keta	SSNA933-08	FJ998753	523	United States	Oregon	Yaquina River
Oncorhynchus keta	SSNA932-08	FJ998754	652	United States	Oregon	Yaquina River
Oncorhynchus keta	SSNA938-08	FJ998755	580	United States	Oregon	Yaquina River
Oncorhynchus keta	SSNA947-08	FJ998756	652	United States	Oregon	Yaquina River
Oncorhynchus keta	SSNA943-08	FJ998757	606	United States	Oregon	Yaquina River
Oncorhynchus keta	SSNA941-08	FJ998758	652	United States	Oregon	Yaquina River
Oncorhynchus keta	SSNA944-08	FJ998759	628	United States	Oregon	Yaquina River
Oncorhynchus keta	SSNA953-08	FJ998760	446	United States	Oregon	South Coast, Coos River
Oncorhynchus keta	SSNA951-08	FJ998761	652	United States	Oregon	South Coast, Chetco River
Oncorhynchus keta	SSNA950-08	FJ998762	652	United States	Oregon	South Coast, Chetco River
Oncorhynchus keta	SSNA948-08	FJ998763	652	United States	Oregon	Yaquina River
Oncorhynchus keta	SSNA070-08	FJ998764	652	United States	Alaska	Salcha River
Oncorhynchus keta	SSNA394-08	FJ998765	652	United States	Washington	Columbia River Basin, Grays River
Oncorhynchus keta	SSNA755-08	FJ998766	652	United States	Alaska	
Oncorhynchus keta	SSNA456-08	FJ998767	652	United States	Washington	Puget Sound, Kennedy Creek
Oncorhynchus keta	SSNA069-08	FJ998768	652	United States	Alaska	Salcha River

Table A.1 (Continued)

Oncorhynchus keta	SSNA457-08	FJ998769	652	United States	Washington	Puget Sound, Kennedy Creek
Oncorhynchus keta	SSNA460-08	FJ998770	652	United States	Washington	Puget Sound, Kennedy Creek
Oncorhynchus keta	SSNA462-08	FJ998771	652	United States	Washington	Puget Sound, Kennedy Creek
Oncorhynchus keta	SSNA068-08	FJ998772	652	United States	Alaska	Salcha River
Oncorhynchus keta	SSNA060-08	FJ998773	652	United States	Alaska	Siwash
Oncorhynchus keta	SSNA042-08	FJ998774	652	United States	Alaska	Neets Bay
Oncorhynchus keta	SSNA041-08	FJ998775	652	United States	Alaska	Neets Bay
Oncorhynchus keta	SSNA044-08	FJ998776	652	United States	Alaska	Neets Bay
Oncorhynchus keta	SSNA046-08	FJ998777	652	United States	Alaska	Neets Bay
Oncorhynchus keta	SSNA045-08	FJ998778	652	United States	Alaska	Neets Bay
Oncorhynchus keta	SSNA059-08	FJ998779	652	United States	Alaska	Siwash
Oncorhynchus keta	SSNA049-08	FJ998780	652	United States	Alaska	Neets Bay
Oncorhynchus keta	SSNA050-08	FJ998781	652	United States	Alaska	Neets Bay
Oncorhynchus keta	SSNA058-08	FJ998782	652	United States	Alaska	Siwash
Oncorhynchus keta	SSNA052-08	FJ998783	652	United States	Alaska	Siwash
Oncorhynchus keta	SSNA051-08	FJ998784	652	United States	Alaska	Siwash
Oncorhynchus keta	SSNA057-08	FJ998785	652	United States	Alaska	Siwash
Oncorhynchus keta	SSNA056-08	FJ998786	652	United States	Alaska	Siwash
Oncorhynchus keta	SSNA054-08	FJ998787	652	United States	Alaska	Siwash
Oncorhynchus keta	SSNA053-08	FJ998788	652	United States	Alaska	Siwash
Oncorhynchus keta	SSNA055-08	FJ998789	652	United States	Alaska	Siwash
Oncorhynchus keta	SSNA061-08	FJ998790	652	United States	Alaska	Salcha River
Oncorhynchus keta	SSNA067-08	FJ998791	652	United States	Alaska	Salcha River
Oncorhynchus keta	SSNA063-08	FJ998792	652	United States	Alaska	Salcha River
Oncorhynchus keta	SSNA064-08	FJ998793	652	United States	Alaska	Salcha River
Oncorhynchus keta	SSNA062-08	FJ998794	652	United States	Alaska	Salcha River
Oncorhynchus keta	SSNA065-08	FJ998795	652	United States	Alaska	Salcha River
Oncorhynchus keta	SSNA066-08	FJ998796	652	United States	Alaska	Salcha River
Oncorhynchus keta	SSNA078-08	FJ998797	652	United States	Alaska	Ilnik River, Three Hills River

Table A.1 (Continued)

Oncorhynchus keta	SSNA080-08	FJ998798	530	United States	Alaska	Ilnik River, Three Hills River
Oncorhynchus keta	SSNA074-08	FJ998799	607	United States	Alaska	Ilnik River, Three Hills River
Oncorhynchus keta	SSNA072-08	FJ998800	564	United States	Alaska	Ilnik River, Three Hills River
Oncorhynchus keta	SSNA076-08	FJ998801	652	United States	Alaska	Ilnik River, Three Hills River
Oncorhynchus keta	SSNA075-08	FJ998802	652	United States	Alaska	Ilnik River, Three Hills River
Oncorhynchus keta	SSNA079-08	FJ998803	652	United States	Alaska	Ilnik River, Three Hills River
Oncorhynchus kisutch	SSNA1074-08	FJ998804	652	United States	Washington	Olympic Peninsula, Beaver Creek (Sol Due River tributary)
Oncorhynchus kisutch	SSNA1073-08	FJ998805	652	United States	Washington	Olympic Peninsula, Beaver Creek (Sol Due River tributary)
Oncorhynchus kisutch	SSNA980-08	FJ998806	652	United States	California	Del Norte, Klamath River
Oncorhynchus kisutch	SSNA146-08	FJ998807	652	United States	Alaska	Kantishna River
Oncorhynchus kisutch	SSNA1119-08	FJ998808	652	United States	Washington	Puget Sound, Snow Creek
Oncorhynchus kisutch	SSNA1076-08	FJ998809	652	United States	Washington	Olympic Peninsula, Beaver Creek (Sol Due River tributary)
Oncorhynchus kisutch	SSNA1075-08	FJ998810	652	United States	Washington	Olympic Peninsula, Beaver Creek (Sol Due River tributary)
Oncorhynchus kisutch	SSNA725-08	FJ998811	652	United States	Oregon	Siletz
Oncorhynchus kisutch	SSNA723-08	FJ998812	652	United States	Oregon	Siletz
Oncorhynchus kisutch	SSNA1118-08	FJ998813	652	United States	Washington	Puget Sound, Snow Creek
Oncorhynchus kisutch	SSNA1117-08	FJ998814	652	United States	Washington	Puget Sound, Snow Creek
Oncorhynchus kisutch	SSNA1191-08	FJ998815	652	United States	Oregon	South Coast, Umpqua
Oncorhynchus kisutch	SSNA1187-08	FJ998816	647	United States	Oregon	North Coast, Nestucca
Oncorhynchus kisutch	SSNA1194-08	FJ998817	652	United States	Oregon	South Coast, Rogue
Oncorhynchus kisutch	SSNA811-08	FJ998818	652	United States	Oregon	North Coast, Nehalem
Oncorhynchus kisutch	SSNA722-08	FJ998819	652	United States	Oregon	Siletz
Oncorhynchus kisutch	SSNA721-08	FJ998820	652	United States	Oregon	Siletz
Oncorhynchus kisutch	SSNA852-08	FJ998821	652	United States	Oregon	South Coast, Tenmile Lake
Oncorhynchus kisutch	SSNA720-08	FJ998822	652	United States	Oregon	Siletz
Oncorhynchus kisutch	SSNA719-08	FJ998823	652	United States	Oregon	Siletz
Oncorhynchus kisutch	SSNA289-08	FJ998824	542	United States	Washington	Olympic Peninsula, Beaver Creek (Sol Due River tributary)

Table A.1 (Continued)

Oncorhynchus kisutch	SSNA288-08	FJ998825	652	United States	Washington	Olympic Peninsula, Beaver Creek (Sol Due River tributary)
Oncorhynchus kisutch	SSNA287-08	FJ998826	652	United States	Washington	Olympic Peninsula, Beaver Creek (Sol Due River tributary)
Oncorhynchus kisutch	SSNA286-08	FJ998827	652	United States	Washington	Olympic Peninsula, Beaver Creek (Sol Due River tributary)
Oncorhynchus kisutch	SSNA292-08	FJ998828	548	United States	Washington	Olympic Peninsula, Beaver Creek (Sol Due River tributary)
Oncorhynchus kisutch	SSNA291-08	FJ998829	605	United States	Washington	Olympic Peninsula, Beaver Creek (Sol Due River tributary)
Oncorhynchus kisutch	SSNA810-08	FJ998830	652	United States	Oregon	North Coast, Nehalem
Oncorhynchus kisutch	SSNA809-08	FJ998831	652	United States	Oregon	North Coast, Nehalem
Oncorhynchus kisutch	SSNA808-08	FJ998832	652	United States	Oregon	North Coast, Necanicum
Oncorhynchus kisutch	SSNA807-08	FJ998833	652	United States	Oregon	North Coast, Necanicum
Oncorhynchus kisutch	SSNA851-08	FJ998834	652	United States	Oregon	South Coast, Tenmile Lake
Oncorhynchus kisutch	SSNA819-08	FJ998835	652	United States	Oregon	North Coast, Wilson
Oncorhynchus kisutch	SSNA816-08	FJ998836	652	United States	Oregon	North Coast, Kilchis
Oncorhynchus kisutch	SSNA724-08	FJ998837	652	United States	Oregon	Siletz
Oncorhynchus kisutch	SSNA356-08	FJ998838	652	United States	Washington	Columbia River Basin, Sea Resources Hatchery
Oncorhynchus kisutch	SSNA815-08	FJ998839	652	United States	Oregon	North Coast, Nehalem
Oncorhynchus kisutch	SSNA813-08	FJ998840	652	United States	Oregon	North Coast, Nehalem
Oncorhynchus kisutch	SSNA357-08	FJ998841	652	United States	Washington	Columbia River Basin, Sea Resources Hatchery
Oncorhynchus kisutch	SSNA358-08	FJ998842	652	United States	Washington	Columbia River Basin, Sea Resources Hatchery
Oncorhynchus kisutch	SSNA364-08	FJ998843	652	United States	Washington	Columbia River Basin, Sea Resources Hatchery
Oncorhynchus kisutch	SSNA363-08	FJ998844	652	United States	Washington	Columbia River Basin, Sea Resources Hatchery
Oncorhynchus kisutch	SSNA362-08	FJ998845	652	United States	Washington	Columbia River Basin, Sea Resources Hatchery
Oncorhynchus kisutch	SSNA361-08	FJ998846	652	United States	Washington	Columbia River Basin, Sea Resources Hatchery
Oncorhynchus kisutch	SSNA359-08	FJ998847	652	United States	Washington	Columbia River Basin, Sea Resources Hatchery
Oncorhynchus kisutch	SSNA814-08	FJ998848	652	United States	Oregon	North Coast, Nehalem
Oncorhynchus kisutch	SSNA817-08	FJ998849	652	United States	Oregon	North Coast, Kilchis
Oncorhynchus kisutch	SSNA818-08	FJ998850	652	United States	Oregon	North Coast, Wilson
Oncorhynchus kisutch	SSNA850-08	FJ998851	652	United States	Oregon	South Coast, Tenmile Lake

Table A.1 (Continued)

Oncorhynchus kisutch	SSNA829-08	FJ998852	652	United States	Oregon	Siltcoos
Oncorhynchus kisutch	SSNA820-08	FJ998853	652	United States	Oregon	North Coast, Wilson
Oncorhynchus kisutch	SSNA823-08	FJ998854	652	United States	Oregon	North Coast, Nestucca
Oncorhynchus kisutch	SSNA828-08	FJ998855	652	United States	Oregon	Siuslaw
Oncorhynchus kisutch	SSNA822-08	FJ998856	652	United States	Oregon	North Coast, Nestucca
Oncorhynchus kisutch	SSNA824-08	FJ998857	652	United States	Oregon	North Coast, Nestucca
Oncorhynchus kisutch	SSNA826-08	FJ998858	652	United States	Oregon	North Coast, Nestucca
Oncorhynchus kisutch	SSNA832-08	FJ998859	652	United States	Oregon	Tahkenitch
Oncorhynchus kisutch	SSNA849-08	FJ998860	652	United States	Oregon	South Coast, Tenmile Lake
Oncorhynchus kisutch	SSNA831-08	FJ998861	652	United States	Oregon	Tahkenitch
Oncorhynchus kisutch	SSNA830-08	FJ998862	652	United States	Oregon	Siltcoos
Oncorhynchus kisutch	SSNA835-08	FJ998863	652	United States	Oregon	Tahkenitch
Oncorhynchus kisutch	SSNA834-08	FJ998864	652	United States	Oregon	Tahkenitch
Oncorhynchus kisutch	SSNA833-08	FJ998865	652	United States	Oregon	Tahkenitch
Oncorhynchus kisutch	SSNA838-08	FJ998866	614	United States	Oregon	Tahkenitch
Oncorhynchus kisutch	SSNA840-08	FJ998867	652	United States	Oregon	Tahkenitch
Oncorhynchus kisutch	SSNA837-08	FJ998868	652	United States	Oregon	Tahkenitch
Oncorhynchus kisutch	SSNA848-08	FJ998869	652	United States	Oregon	South Coast, Tenmile Lake
Oncorhynchus kisutch	SSNA839-08	FJ998870	652	United States	Oregon	Tahkenitch
Oncorhynchus kisutch	SSNA843-08	FJ998871	623	United States	Oregon	South Coast, Umpqua
Oncorhynchus kisutch	SSNA847-08	FJ998872	652	United States	Oregon	South Coast, Umpqua
Oncorhynchus kisutch	SSNA842-08	FJ998873	652	United States	Oregon	Tahkenitch
Oncorhynchus kisutch	SSNA841-08	FJ998874	568	United States	Oregon	Tahkenitch
Oncorhynchus kisutch	SSNA845-08	FJ998875	652	United States	Oregon	South Coast, Umpqua
Oncorhynchus kisutch	SSNA844-08	FJ998876	652	United States	Oregon	South Coast, Umpqua
Oncorhynchus kisutch	SSNA979-08	FJ998877	652	United States	California	Del Norte, Klamath River
Oncorhynchus kisutch	SSNA855-08	FJ998878	652	United States	Oregon	South Coast, Coos River
Oncorhynchus kisutch	SSNA858-08	FJ998879	652	United States	Oregon	South Coast, Coquille
Oncorhynchus kisutch	SSNA857-08	FJ998880	652	United States	Oregon	South Coast, Coquille

Table A.1 (Continued)

Oncorhynchus kisutch	SSNA859-08	FJ998881	652	United States	Oregon	South Coast, Coquille
Oncorhynchus kisutch	SSNA861-08	FJ998882	652	United States	Oregon	South Coast, New
Oncorhynchus kisutch	SSNA728-08	FJ998883	652	United States	Oregon	Siletz
Oncorhynchus kisutch	SSNA726-08	FJ998884	652	United States	Oregon	Siletz
Oncorhynchus kisutch	SSNA727-08	FJ998885	652	United States	Oregon	Siletz
Oncorhynchus kisutch	SSNA731-08	FJ998886	652	United States	Oregon	Siletz
Oncorhynchus kisutch	SSNA730-08	FJ998887	652	United States	Oregon	Siletz
Oncorhynchus kisutch	SSNA729-08	FJ998888	652	United States	Oregon	Siletz
Oncorhynchus kisutch	SSNA982-08	FJ998889	652	United States	California	Del Norte, Klamath River
Oncorhynchus kisutch	SSNA992-08	FJ998890	652	United States	California	Lagunitas/Olema Creek
Oncorhynchus kisutch	SSNA981-08	FJ998891	652	United States	California	Del Norte, Klamath River
Oncorhynchus kisutch	SSNA986-08	FJ998892	652	United States	California	Del Norte, Klamath River
Oncorhynchus kisutch	SSNA990-08	FJ998893	652	United States	California	Lagunitas/Olema Creek
Oncorhynchus kisutch	SSNA991-08	FJ998894	652	United States	California	Lagunitas/Olema Creek
Oncorhynchus kisutch	SSNA985-08	FJ998895	652	United States	California	Del Norte, Klamath River
Oncorhynchus kisutch	SSNA984-08	FJ998896	652	United States	California	Del Norte, Klamath River
Oncorhynchus kisutch	SSNA983-08	FJ998897	652	United States	California	Del Norte, Klamath River
Oncorhynchus kisutch	SSNA989-08	FJ998898	652	United States	California	Lagunitas/Olema Creek
Oncorhynchus kisutch	SSNA988-08	FJ998899	652	United States	California	Lagunitas/Olema Creek
Oncorhynchus kisutch	SSNA987-08	FJ998900	652	United States	California	Lagunitas/Olema Creek
Oncorhynchus kisutch	SSNA993-08	FJ998901	652	United States	California	Lagunitas/Olema Creek
Oncorhynchus kisutch	SSNA994-08	FJ998902	652	United States	California	Lagunitas/Olema Creek
Oncorhynchus kisutch	SSNA427-08	FJ998903	630	United States	Washington	Puget Sound, Snow Creek
Oncorhynchus kisutch	SSNA426-08	FJ998904	652	United States	Washington	Puget Sound, Snow Creek
Oncorhynchus kisutch	SSNA433-08	FJ998905	644	United States	Washington	Puget Sound, Snow Creek
Oncorhynchus kisutch	SSNA432-08	FJ998906	652	United States	Washington	Puget Sound, Snow Creek
Oncorhynchus kisutch	SSNA431-08	FJ998907	629	United States	Washington	Puget Sound, Snow Creek
Oncorhynchus kisutch	SSNA430-08	FJ998908	607	United States	Washington	Puget Sound, Snow Creek
Oncorhynchus kisutch	SSNA429-08	FJ998909	645	United States	Washington	Puget Sound, Snow Creek

Table A.1 (Continued)

Oncorhynchus kisutch	SSNA135-08	FJ998910	652	United States	Alaska	Anchor River
Oncorhynchus kisutch	SSNA134-08	FJ998911	652	United States	Alaska	Anchor River
Oncorhynchus kisutch	SSNA758-08	FJ998912	652	United States	Oregon	Youngs Bay
Oncorhynchus kisutch	SSNA133-08	FJ998913	652	United States	Alaska	Anchor River
Oncorhynchus kisutch	SSNA131-08	FJ998914	652	United States	Alaska	Anchor River
Oncorhynchus kisutch	SSNA132-08	FJ998915	652	United States	Alaska	Anchor River
Oncorhynchus kisutch	SSNA121-08	FJ998916	652	United States	Alaska	Stikine River
Oncorhynchus kisutch	SSNA130-08	FJ998917	652	United States	Alaska	Stikine River
Oncorhynchus kisutch	SSNA126-08	FJ998918	652	United States	Alaska	Stikine River
Oncorhynchus kisutch	SSNA125-08	FJ998919	652	United States	Alaska	Stikine River
Oncorhynchus kisutch	SSNA124-08	FJ998920	652	United States	Alaska	Stikine River
Oncorhynchus kisutch	SSNA123-08	FJ998921	652	United States	Alaska	Stikine River
Oncorhynchus kisutch	SSNA122-08	FJ998922	652	United States	Alaska	Stikine River
Oncorhynchus kisutch	SSNA127-08	FJ998923	652	United States	Alaska	Stikine River
Oncorhynchus kisutch	SSNA129-08	FJ998924	652	United States	Alaska	Stikine River
Oncorhynchus kisutch	SSNA128-08	FJ998925	652	United States	Alaska	Stikine River
Oncorhynchus kisutch	SSNA145-08	FJ998926	652	United States	Alaska	Kantishna River
Oncorhynchus kisutch	SSNA144-08	FJ998927	652	United States	Alaska	Kantishna River
Oncorhynchus kisutch	SSNA143-08	FJ998928	652	United States	Alaska	Kantishna River
Oncorhynchus kisutch	SSNA142-08	FJ998929	652	United States	Alaska	Kantishna River
Oncorhynchus kisutch	SSNA138-08	FJ998930	652	United States	Alaska	Anchor River
Oncorhynchus kisutch	SSNA137-08	FJ998931	646	United States	Alaska	Anchor River
Oncorhynchus kisutch	SSNA136-08	FJ998932	652	United States	Alaska	Anchor River
Oncorhynchus kisutch	SSNA141-08	FJ998933	652	United States	Alaska	Kantishna River
Oncorhynchus kisutch	SSNA139-08	FJ998934	652	United States	Alaska	Anchor River
Oncorhynchus kisutch	SSNA140-08	FJ998935	652	United States	Alaska	Anchor River
Oncorhynchus kisutch	SSNA147-08	FJ998936	606	United States	Alaska	Kantishna River
Oncorhynchus kisutch	SSNA153-08	FJ998937	652	United States	Alaska	Kametolook River
Oncorhynchus kisutch	SSNA148-08	FJ998938	652	United States	Alaska	Kantishna River

Table A.1 (Continued)

Oncorhynchus kisutch	SSNA151-08	FJ998939	652	United States	Alaska	Kametolook River
Oncorhynchus kisutch	SSNA152-08	FJ998940	652	United States	Alaska	Kametolook River
Oncorhynchus kisutch	SSNA150-08	FJ998941	652	United States	Alaska	Kantishna River
Oncorhynchus kisutch	SSNA149-08	FJ998942	652	United States	Alaska	Kantishna River
Oncorhynchus kisutch	SSNA155-08	FJ998943	652	United States	Alaska	Kametolook River
Oncorhynchus kisutch	SSNA156-08	FJ998944	652	United States	Alaska	Kametolook River
Oncorhynchus kisutch	SSNA154-08	FJ998945	652	United States	Alaska	Kametolook River
Oncorhynchus kisutch	SSNA158-08	FJ998946	652	United States	Alaska	Kametolook River
Oncorhynchus kisutch	SSNA157-08	FJ998947	652	United States	Alaska	Kametolook River
Oncorhynchus kisutch	SSNA160-08	FJ998948	652	United States	Alaska	Kametolook River
Oncorhynchus kisutch	SSNA159-08	FJ998949	631	United States	Alaska	Kametolook River
Oncorhynchus mykiss	SSNA1050-08	FJ998950	652	United States	Oregon	South Coast, Smith River Falls
Oncorhynchus mykiss	SSNA1039-08	FJ998951	652	United States	California	Ventura River Basin
Oncorhynchus mykiss	SSNA1045-08	FJ998952	652	United States	Oregon	Foster Reservoir
Oncorhynchus mykiss	SSNA187-08	FJ998953	652	United States	Alaska	Agulukpak River
Oncorhynchus mykiss	SSNA757-08	FJ998954	652	United States	Idaho	Aquaculture facility
Oncorhynchus mykiss	SSNA186-08	FJ998955	650	United States	Alaska	Agulukpak River
Oncorhynchus mykiss	SSNA182-08	FJ998956	652	United States	Alaska	Agulukpak River
Oncorhynchus mykiss	SSNA496-08	FJ998957	636	United States	Idaho	Donaldson Broodstock
Oncorhynchus mykiss	SSNA181-08	FJ998958	652	United States	Alaska	Agulukpak River
Oncorhynchus mykiss	SSNA184-08	FJ998959	652	United States	Alaska	Agulukpak River
Oncorhynchus mykiss	SSNA185-08	FJ998960	652	United States	Alaska	Agulukpak River
Oncorhynchus mykiss	SSNA183-08	FJ998961	652	United States	Alaska	Agulukpak River
Oncorhynchus mykiss	SSNA1089-08	FJ998962	652	United States	Washington	Olympic Peninsula, Makak NFH (Sooes River)
Oncorhynchus mykiss	SSNA489-08	FJ998963	652	United States	Idaho	Donaldson Broodstock
Oncorhynchus mykiss	SSNA188-08	FJ998964	652	United States	Alaska	Agulukpak River
Oncorhynchus mykiss	SSNA487-08	FJ998965	652	United States	Idaho	Donaldson Broodstock
Oncorhynchus mykiss	SSNA1106-08	FJ998966	633	United States	Washington	Columbia River Basin, Cowlitz River Hatchery
Oncorhynchus mykiss	SSNA192-08	FJ998967	652	United States	Alaska	Swanson River

Table A.1 (Continued)

Oncorhynchus mykiss	SSNA201-08	FJ998968	652	United States	Idaho	Upper Rice Creek
Oncorhynchus mykiss	SSNA191-08	FJ998969	652	United States	Alaska	Swanson River
Oncorhynchus mykiss	SSNA189-08	FJ998970	652	United States	Alaska	Agulukpak River
Oncorhynchus mykiss	SSNA1105-08	FJ998971	652	United States	Washington	Columbia River Basin, Cowlitz River Hatchery
Oncorhynchus mykiss	SSNA190-08	FJ998972	652	United States	Alaska	Agulukpak River
Oncorhynchus mykiss	SSNA1104-08	FJ998973	652	United States	Washington	Columbia River Basin, Cowlitz River Hatchery
Oncorhynchus mykiss	SSNA193-08	FJ998974	652	United States	Alaska	Swanson River
Oncorhynchus mykiss	SSNA200-08	FJ998975	652	United States	Alaska	Swanson River
Oncorhynchus mykiss	SSNA199-08	FJ998976	652	United States	Alaska	Swanson River
Oncorhynchus mykiss	SSNA1108-08	FJ998977	652	United States	Washington	Columbia River Basin, Cowlitz River Hatchery
Oncorhynchus mykiss	SSNA194-08	FJ998978	652	United States	Alaska	Swanson River
Oncorhynchus mykiss	SSNA196-08	FJ998979	652	United States	Alaska	Swanson River
Oncorhynchus mykiss	SSNA1107-08	FJ998980	652	United States	Washington	Columbia River Basin, Cowlitz River Hatchery
Oncorhynchus mykiss	SSNA198-08	FJ998981	652	United States	Alaska	Swanson River
Oncorhynchus mykiss	SSNA195-08	FJ998982	652	United States	Alaska	Swanson River
Oncorhynchus mykiss	SSNA197-08	FJ998983	652	United States	Alaska	Swanson River
Oncorhynchus mykiss	SSNA206-08	FJ998984	652	United States	Idaho	Big Jacks Creek
Oncorhynchus mykiss	SSNA203-08	FJ998985	652	United States	Idaho	Upper Rice Creek
Oncorhynchus mykiss	SSNA202-08	FJ998986	652	United States	Idaho	Upper Rice Creek
Oncorhynchus mykiss	SSNA213-08	FJ998987	652	United States	Idaho	Dworshak Hatchery
Oncorhynchus mykiss	SSNA205-08	FJ998988	652	United States	Idaho	Upper Rice Creek
Oncorhynchus mykiss	SSNA1137-08	FJ998989	652	United States	Washington	Puget Sound, N.F. Skykomish River
Oncorhynchus mykiss	SSNA215-08	FJ998990	652	United States	Idaho	Dworshak Hatchery
Oncorhynchus mykiss	SSNA1145-08	FJ998991	652	United States	Idaho	Donaldson Broodstock
Oncorhynchus mykiss	SSNA1144-08	FJ998992	652	United States	Washington	Puget Sound, Cedar River
Oncorhynchus mykiss	SSNA214-08	FJ998993	652	United States	Idaho	Dworshak Hatchery
Oncorhynchus mykiss	SSNA204-08	FJ998994	652	United States	Idaho	Upper Rice Creek
Oncorhynchus mykiss	SSNA212-08	FJ998995	652	United States	Idaho	Dworshak Hatchery
Oncorhynchus mykiss	SSNA1146-08	FJ998996	652	United States	Idaho	Donaldson Broodstock

Table A.1 (Continued)

Oncorhynchus mykiss	SSNA209-08	FJ998997	652	United States	Idaho	Big Jacks Creek
Oncorhynchus mykiss	SSNA1149-08	FJ998998	652	United States	Idaho	Donaldson Broodstock
Oncorhynchus mykiss	SSNA208-08	FJ998999	652	United States	Idaho	Big Jacks Creek
Oncorhynchus mykiss	SSNA1148-08	FJ999000	652	United States	Idaho	Donaldson Broodstock
Oncorhynchus mykiss	SSNA211-08	FJ999001	652	United States	Idaho	Dworshak Hatchery
Oncorhynchus mykiss	SSNA1147-08	FJ999002	652	United States	Idaho	Donaldson Broodstock
Oncorhynchus mykiss	SSNA207-08	FJ999003	652	United States	Idaho	Big Jacks Creek
Oncorhynchus mykiss	SSNA1151-08	FJ999004	652	United States	Idaho	Troutlodge Broodstock
Oncorhynchus mykiss	SSNA210-08	FJ999005	652	United States	Idaho	Big Jacks Creek
Oncorhynchus mykiss	SSNA1150-08	FJ999006	652	United States	Idaho	Troutlodge Broodstock
Oncorhynchus mykiss	SSNA218-08	FJ999007	652	United States	Idaho	Pahsimeroi Hatchery
Oncorhynchus mykiss	SSNA217-08	FJ999008	652	United States	Idaho	Pahsimeroi Hatchery
Oncorhynchus mykiss	SSNA216-08	FJ999009	652	United States	Idaho	Pahsimeroi Hatchery
Oncorhynchus mykiss	SSNA1196-08	FJ999010	652	United States	Oregon	South Coast, Rogue
Oncorhynchus mykiss	SSNA1195-08	FJ999011	652	United States	Oregon	South Coast, Rogue
Oncorhynchus mykiss	SSNA219-08	FJ999012	652	United States	Idaho	Pahsimeroi Hatchery
Oncorhynchus mykiss	SSNA332-08	FJ999013	652	United States	Washington	Olympic Peninsula, Makak NFH (Sooes River)
Oncorhynchus mykiss	SSNA328-08	FJ999014	652	United States	Washington	Olympic Peninsula, Makak NFH (Sooes River)
Oncorhynchus mykiss	SSNA326-08	FJ999015	652	United States	Washington	Olympic Peninsula, Makak NFH (Sooes River)
Oncorhynchus mykiss	SSNA327-08	FJ999016	652	United States	Washington	Olympic Peninsula, Makak NFH (Sooes River)
Oncorhynchus mykiss	SSNA329-08	FJ999017	652	United States	Washington	Olympic Peninsula, Makak NFH (Sooes River)
Oncorhynchus mykiss	SSNA333-08	FJ999018	652	United States	Washington	Olympic Peninsula, Makak NFH (Sooes River)
Oncorhynchus mykiss	SSNA330-08	FJ999019	652	United States	Washington	Olympic Peninsula, Makak NFH (Sooes River)
Oncorhynchus mykiss	SSNA334-08	FJ999020	652	United States	Washington	Olympic Peninsula, Makak NFH (Sooes River)
Oncorhynchus mykiss	SSNA335-08	FJ999021	652	United States	Washington	Olympic Peninsula, Makak NFH (Sooes River)
Oncorhynchus mykiss	SSNA492-08	FJ999022	583	United States	Idaho	Donaldson Broodstock
Oncorhynchus mykiss	SSNA736-08	FJ999023	652	United States	Oregon	Tenmile Creek
Oncorhynchus mykiss	SSNA895-08	FJ999024	614	United States	Oregon	South Coast, Rogue
Oncorhynchus mykiss	SSNA884-08	FJ999025	652	United States	Oregon	Foster Reservoir

Table A.1 (Continued)

Oncorhynchus mykiss	SSNA495-08	FJ999026	652	United States	Idaho	Donaldson Broodstock
Oncorhynchus mykiss	SSNA883-08	FJ999027	562	United States	Oregon	Foster Reservoir
Oncorhynchus mykiss	SSNA868-08	FJ999028	652	United States	Oregon	North Coast, Nestucca
Oncorhynchus mykiss	SSNA867-08	FJ999029	652	United States	Oregon	North Coast, Nestucca
Oncorhynchus mykiss	SSNA866-08	FJ999030	652	United States	Oregon	North Coast, Nestucca
Oncorhynchus mykiss	SSNA864-08	FJ999031	652	United States	Oregon	North Coast, Nestucca
Oncorhynchus mykiss	SSNA865-08	FJ999032	626	United States	Oregon	North Coast, Nestucca
Oncorhynchus mykiss	SSNA863-08	FJ999033	652	United States	Oregon	North Coast, Nestucca
Oncorhynchus mykiss	SSNA882-08	FJ999034	530	United States	Oregon	Foster Reservoir
Oncorhynchus mykiss	SSNA876-08	FJ999035	652	United States	Oregon	N Santiam
Oncorhynchus mykiss	SSNA875-08	FJ999036	652	United States	Oregon	N Santiam
Oncorhynchus mykiss	SSNA869-08	FJ999037	607	United States	Oregon	North Coast, Nestucca
Oncorhynchus mykiss	SSNA870-08	FJ999038	615	United States	Oregon	North Coast, Nestucca
Oncorhynchus mykiss	SSNA874-08	FJ999039	528	United States	Oregon	N Santiam
Oncorhynchus mykiss	SSNA873-08	FJ999040	639	United States	Oregon	N Santiam
Oncorhynchus mykiss	SSNA872-08	FJ999041	540	United States	Oregon	N Santiam
Oncorhynchus mykiss	SSNA871-08	FJ999042	615	United States	Oregon	N Santiam
Oncorhynchus mykiss	SSNA878-08	FJ999043	602	United States	Oregon	S Santiam
Oncorhynchus mykiss	SSNA877-08	FJ999044	652	United States	Oregon	S Santiam
Oncorhynchus mykiss	SSNA879-08	FJ999045	587	United States	Oregon	S Santiam
Oncorhynchus mykiss	SSNA881-08	FJ999046	613	United States	Oregon	Foster Reservoir
Oncorhynchus mykiss	SSNA880-08	FJ999047	652	United States	Oregon	S Santiam
Oncorhynchus mykiss	SSNA735-08	FJ999048	652	United States	Oregon	Tenmile Creek
Oncorhynchus mykiss	SSNA885-08	FJ999049	652	United States	Oregon	Foster Reservoir
Oncorhynchus mykiss	SSNA888-08	FJ999050	652	United States	Oregon	South Coast, Rogue
Oncorhynchus mykiss	SSNA887-08	FJ999051	652	United States	Oregon	South Coast, Rogue
Oncorhynchus mykiss	SSNA893-08	FJ999052	652	United States	Oregon	South Coast, Rogue
Oncorhynchus mykiss	SSNA891-08	FJ999053	652	United States	Oregon	South Coast, Rogue
Oncorhynchus mykiss	SSNA734-08	FJ999054	652	United States	Oregon	Tenmile Creek

Table A.1 (Continued)

Oncorhynchus mykiss	SSNA890-08	FJ999055	652	United States	Oregon	South Coast, Rogue
Oncorhynchus mykiss	SSNA889-08	FJ999056	613	United States	Oregon	South Coast, Rogue
Oncorhynchus mykiss	SSNA733-08	FJ999057	652	United States	Oregon	Tenmile Creek
Oncorhynchus mykiss	SSNA892-08	FJ999058	613	United States	Oregon	South Coast, Rogue
Oncorhynchus mykiss	SSNA732-08	FJ999059	652	United States	Oregon	Tenmile Creek
Oncorhynchus mykiss	SSNA903-08	FJ999060	652	United States	Oregon	South Coast, Smith River Falls
Oncorhynchus mykiss	SSNA898-08	FJ999061	530	United States	Oregon	South Coast, Rogue
Oncorhynchus mykiss	SSNA897-08	FJ999062	652	United States	Oregon	South Coast, Rogue
Oncorhynchus mykiss	SSNA896-08	FJ999063	652	United States	Oregon	South Coast, Rogue
Oncorhynchus mykiss	SSNA899-08	FJ999064	652	United States	Oregon	South Coast, Rogue
Oncorhynchus mykiss	SSNA738-08	FJ999065	652	United States	Oregon	Tenmile Creek
Oncorhynchus mykiss	SSNA902-08	FJ999066	652	United States	Oregon	South Coast, Smith River Falls
Oncorhynchus mykiss	SSNA737-08	FJ999067	652	United States	Oregon	Tenmile Creek
Oncorhynchus mykiss	SSNA901-08	FJ999068	652	United States	Oregon	South Coast, Rogue
Oncorhynchus mykiss	SSNA739-08	FJ999069	652	United States	Oregon	Tenmile Creek
Oncorhynchus mykiss	SSNA741-08	FJ999070	652	United States	Oregon	Tenmile Creek
Oncorhynchus mykiss	SSNA740-08	FJ999071	652	United States	Oregon	Tenmile Creek
Oncorhynchus mykiss	SSNA907-08	FJ999072	605	United States	Oregon	South Coast, Smith River Falls
Oncorhynchus mykiss	SSNA904-08	FJ999073	652	United States	Oregon	South Coast, Smith River Falls
Oncorhynchus mykiss	SSNA906-08	FJ999074	652	United States	Oregon	South Coast, Smith River Falls
Oncorhynchus mykiss	SSNA494-08	FJ999075	652	United States	Idaho	Donaldson Broodstock
Oncorhynchus mykiss	SSNA517-08	FJ999076	652	United States	Idaho	Troutlodge Broodstock
Oncorhynchus mykiss	SSNA509-08	FJ999077	633	United States	Idaho	Black Canyon Broodstock
Oncorhynchus mykiss	SSNA1034-08	FJ999078	652	United States	California	Ventura River Basin
Oncorhynchus mykiss	SSNA540-08	FJ999079	629	United States	Idaho	Clear Springs Broodstock
Oncorhynchus mykiss	SSNA508-08	FJ999080	652	United States	Idaho	Black Canyon Broodstock
Oncorhynchus mykiss	SSNA497-08	FJ999081	652	United States	Idaho	Donaldson Broodstock
Oncorhynchus mykiss	SSNA468-08	FJ999082	652	United States	Washington	Puget Sound, N.F. Skykomish River
Oncorhynchus mykiss	SSNA507-08	FJ999083	652	United States	Idaho	Black Canyon Broodstock

Table A.1 (Continued)

Oncorhynchus mykiss	SSNA506-08	FJ999084	652	United States	Idaho	Black Canyon Broodstock
Oncorhynchus mykiss	SSNA499-08	FJ999085	652	United States	Idaho	Donaldson Broodstock
Oncorhynchus mykiss	SSNA498-08	FJ999086	652	United States	Idaho	Donaldson Broodstock
Oncorhynchus mykiss	SSNA505-08	FJ999087	649	United States	Idaho	Black Canyon Broodstock
Oncorhynchus mykiss	SSNA501-08	FJ999088	652	United States	Idaho	Black Canyon Broodstock
Oncorhynchus mykiss	SSNA504-08	FJ999089	650	United States	Idaho	Black Canyon Broodstock
Oncorhynchus mykiss	SSNA502-08	FJ999090	652	United States	Idaho	Black Canyon Broodstock
Oncorhynchus mykiss	SSNA503-08	FJ999091	652	United States	Idaho	Black Canyon Broodstock
Oncorhynchus mykiss	SSNA405-08	FJ999092	652	United States	Washington	Columbia River Basin, Cowlitz River Hatchery
Oncorhynchus mykiss	SSNA515-08	FJ999093	637	United States	Idaho	Black Canyon Broodstock
Oncorhynchus mykiss	SSNA514-08	FJ999094	652	United States	Idaho	Black Canyon Broodstock
Oncorhynchus mykiss	SSNA401-08	FJ999095	652	United States	Washington	Columbia River Basin, Cowlitz River Hatchery
Oncorhynchus mykiss	SSNA400-08	FJ999096	652	United States	Washington	Columbia River Basin, Cowlitz River Hatchery
Oncorhynchus mykiss	SSNA516-08	FJ999097	652	United States	Idaho	Troutlodge Broodstock
Oncorhynchus mykiss	SSNA513-08	FJ999098	652	United States	Idaho	Black Canyon Broodstock
Oncorhynchus mykiss	SSNA512-08	FJ999099	652	United States	Idaho	Black Canyon Broodstock
Oncorhynchus mykiss	SSNA511-08	FJ999100	652	United States	Idaho	Black Canyon Broodstock
Oncorhynchus mykiss	SSNA510-08	FJ999101	652	United States	Idaho	Black Canyon Broodstock
Oncorhynchus mykiss	SSNA403-08	FJ999102	647	United States	Washington	Columbia River Basin, Cowlitz River Hatchery
Oncorhynchus mykiss	SSNA402-08	FJ999103	652	United States	Washington	Columbia River Basin, Cowlitz River Hatchery
Oncorhynchus mykiss	SSNA532-08	FJ999104	652	United States	Idaho	Clear Springs Broodstock
Oncorhynchus mykiss	SSNA1032-08	FJ999105	652	United States	California	Ventura River Basin
Oncorhynchus mykiss	SSNA1003-08	FJ999106	652	United States	California	Central Valley, Sacramento River
Oncorhynchus mykiss	SSNA1028-08	FJ999107	470	United States	California	Ventura River Basin
Oncorhynchus mykiss	SSNA1033-08	FJ999108	652	United States	California	Ventura River Basin
Oncorhynchus mykiss	SSNA539-08	FJ999109	652	United States	Idaho	Clear Springs Broodstock
Oncorhynchus mykiss	SSNA1002-08	FJ999110	652	United States	California	Eel River
Oncorhynchus mykiss	SSNA1001-08	FJ999111	652	United States	California	Eel River
Oncorhynchus mykiss	SSNA1000-08	FJ999112	652	United States	California	Eel River

Table A.1 (Continued)

Oncorhynchus mykiss	SSNA996-08	FJ999113	652	United States	California	Eel River
Oncorhynchus mykiss	SSNA995-08	FJ999114	652	United States	California	Eel River
Oncorhynchus mykiss	SSNA999-08	FJ999115	652	United States	California	Eel River
Oncorhynchus mykiss	SSNA998-08	FJ999116	652	United States	California	Eel River
Oncorhynchus mykiss	SSNA997-08	FJ999117	652	United States	California	Eel River
Oncorhynchus mykiss	SSNA1013-08	FJ999118	652	United States	California	Central coast, Scott Creek
Oncorhynchus mykiss	SSNA518-08	FJ999119	652	United States	Idaho	Troutlodge Broodstock
Oncorhynchus mykiss	SSNA1027-08	FJ999120	652	United States	California	Santa Paula River Basin
Oncorhynchus mykiss	SSNA1012-08	FJ999121	652	United States	California	Central coast, Scott Creek
Oncorhynchus mykiss	SSNA1010-08	FJ999122	652	United States	California	Central Valley, Sacramento River
Oncorhynchus mykiss	SSNA1004-08	FJ999123	652	United States	California	Central Valley, Sacramento River
Oncorhynchus mykiss	SSNA1009-08	FJ999124	652	United States	California	Central Valley, Sacramento River
Oncorhynchus mykiss	SSNA1008-08	FJ999125	652	United States	California	Central Valley, Sacramento River
Oncorhynchus mykiss	SSNA1007-08	FJ999126	652	United States	California	Central Valley, Sacramento River
Oncorhynchus mykiss	SSNA1005-08	FJ999127	652	United States	California	Central Valley, Sacramento River
Oncorhynchus mykiss	SSNA1006-08	FJ999128	652	United States	California	Central Valley, Sacramento River
Oncorhynchus mykiss	SSNA1011-08	FJ999129	652	United States	California	Central coast, Scott Creek
Oncorhynchus mykiss	SSNA1018-08	FJ999130	652	United States	California	Central coast, Scott Creek
Oncorhynchus mykiss	SSNA1017-08	FJ999131	652	United States	California	Central coast, Scott Creek
Oncorhynchus mykiss	SSNA1014-08	FJ999132	652	United States	California	Central coast, Scott Creek
Oncorhynchus mykiss	SSNA1016-08	FJ999133	652	United States	California	Central coast, Scott Creek
Oncorhynchus mykiss	SSNA1015-08	FJ999134	652	United States	California	Central coast, Scott Creek
Oncorhynchus mykiss	SSNA1030-08	FJ999135	652	United States	California	Ventura River Basin
Oncorhynchus mykiss	SSNA531-08	FJ999136	652	United States	Idaho	Clear Springs Broodstock
Oncorhynchus mykiss	SSNA1031-08	FJ999137	652	United States	California	Ventura River Basin
Oncorhynchus mykiss	SSNA1029-08	FJ999138	652	United States	California	Ventura River Basin
Oncorhynchus mykiss	SSNA521-08	FJ999139	652	United States	Idaho	Troutlodge Broodstock
Oncorhynchus mykiss	SSNA520-08	FJ999140	652	United States	Idaho	Troutlodge Broodstock
Oncorhynchus mykiss	SSNA519-08	FJ999141	652	United States	Idaho	Troutlodge Broodstock

Table A.1 (Continued)

Oncorhynchus mykiss	SSNA529-08	FJ999142	652	United States	Idaho	Troutlodge Broodstock
Oncorhynchus mykiss	SSNA525-08	FJ999143	640	United States	Idaho	Troutlodge Broodstock
Oncorhynchus mykiss	SSNA524-08	FJ999144	652	United States	Idaho	Troutlodge Broodstock
Oncorhynchus mykiss	SSNA523-08	FJ999145	652	United States	Idaho	Troutlodge Broodstock
Oncorhynchus mykiss	SSNA522-08	FJ999146	652	United States	Idaho	Troutlodge Broodstock
Oncorhynchus mykiss	SSNA528-08	FJ999147	652	United States	Idaho	Troutlodge Broodstock
Oncorhynchus mykiss	SSNA526-08	FJ999148	652	United States	Idaho	Troutlodge Broodstock
Oncorhynchus mykiss	SSNA538-08	FJ999149	652	United States	Idaho	Clear Springs Broodstock
Oncorhynchus mykiss	SSNA534-08	FJ999150	530	United States	Idaho	Clear Springs Broodstock
Oncorhynchus mykiss	SSNA533-08	FJ999151	652	United States	Idaho	Clear Springs Broodstock
Oncorhynchus mykiss	SSNA537-08	FJ999152	652	United States	Idaho	Clear Springs Broodstock
Oncorhynchus mykiss	SSNA536-08	FJ999153	652	United States	Idaho	Clear Springs Broodstock
Oncorhynchus mykiss	SSNA535-08	FJ999154	652	United States	Idaho	Clear Springs Broodstock
Oncorhynchus mykiss	SSNA541-08	FJ999155	652	United States	Idaho	Clear Springs Broodstock
Oncorhynchus mykiss	SSNA467-08	FJ999156	630	United States	Washington	Puget Sound, N.F. Skykomish River
Oncorhynchus mykiss	SSNA542-08	FJ999157	640	United States	Idaho	Clear Springs Broodstock
Oncorhynchus mykiss	SSNA466-08	FJ999158	652	United States	Washington	Puget Sound, N.F. Skykomish River
Oncorhynchus mykiss	SSNA545-08	FJ999159	652	United States	Idaho	Clear Springs Broodstock
Oncorhynchus mykiss	SSNA544-08	FJ999160	652	United States	Idaho	Clear Springs Broodstock
Oncorhynchus mykiss	SSNA543-08	FJ999161	652	United States	Idaho	Clear Springs Broodstock
Oncorhynchus mykiss	SSNA486-08	FJ999162	632	United States	Idaho	Donaldson Broodstock
Oncorhynchus mykiss	SSNA475-08	FJ999163	647	United States	Washington	Puget Sound, N.F. Skykomish River
Oncorhynchus mykiss	SSNA474-08	FJ999164	652	United States	Washington	Puget Sound, N.F. Skykomish River
Oncorhynchus mykiss	SSNA471-08	FJ999165	652	United States	Washington	Puget Sound, N.F. Skykomish River
Oncorhynchus mykiss	SSNA472-08	FJ999166	652	United States	Washington	Puget Sound, N.F. Skykomish River
Oncorhynchus nerka	SSNA1066-08	FJ999167	652	United States	Alaska	Meshik River
Oncorhynchus nerka	SSNA1065-08	FJ999168	652	United States	Alaska	Hugh Smith Lake, Bushman Creek
Oncorhynchus nerka	SSNA1077-08	FJ999169	652	United States	Washington	Olympic Peninsula, Umbrella Creek
Oncorhynchus nerka	SSNA1126-08	FJ999170	652	United States	Washington	Puget Sound, Lake Washington at Ballard Locks

Table A.1 (Continued)

Oncorhynchus nerka	SSNA238-08	FJ999171	652	United States	Idaho	Fishhook Cr.
Oncorhynchus nerka	SSNA231-08	FJ999172	652	United States	Idaho	NOAA Captive Broodstock
Oncorhynchus nerka	SSNA237-08	FJ999173	652	United States	Idaho	Fishhook Cr.
Oncorhynchus nerka	SSNA232-08	FJ999174	652	United States	Idaho	NOAA Captive Broodstock
Oncorhynchus nerka	SSNA236-08	FJ999175	652	United States	Idaho	Fishhook Cr.
Oncorhynchus nerka	SSNA235-08	FJ999176	652	United States	Idaho	NOAA Adult Release
Oncorhynchus nerka	SSNA234-08	FJ999177	652	United States	Idaho	NOAA Adult Release
Oncorhynchus nerka	SSNA233-08	FJ999178	652	United States	Idaho	NOAA Captive Broodstock
Oncorhynchus nerka	SSNA240-08	FJ999179	652	United States	Idaho	Fishhook Cr.
Oncorhynchus nerka	SSNA239-08	FJ999180	652	United States	Idaho	Fishhook Cr.
Oncorhynchus nerka	SSNA299-08	FJ999181	652	United States	Washington	Olympic Peninsula, Umbrella Creek
Oncorhynchus nerka	SSNA366-08	FJ999182	652	United States	Washington	Columbia River Basin, Lake Wenatchee/River
Oncorhynchus nerka	SSNA367-08	FJ999183	652	United States	Washington	Columbia River Basin, Lake Wenatchee/River
Oncorhynchus nerka	SSNA372-08	FJ999184	644	United States	Washington	Columbia River Basin, Lake Wenatchee/River
Oncorhynchus nerka	SSNA368-08	FJ999185	652	United States	Washington	Columbia River Basin, Lake Wenatchee/River
Oncorhynchus nerka	SSNA371-08	FJ999186	652	United States	Washington	Columbia River Basin, Lake Wenatchee/River
Oncorhynchus nerka	SSNA370-08	FJ999187	652	United States	Washington	Columbia River Basin, Lake Wenatchee/River
Oncorhynchus nerka	SSNA369-08	FJ999188	645	United States	Washington	Columbia River Basin, Lake Wenatchee/River
Oncorhynchus nerka	SSNA375-08	FJ999189	652	United States	Washington	Columbia River Basin, Lake Wenatchee/River
Oncorhynchus nerka	SSNA374-08	FJ999190	652	United States	Washington	Columbia River Basin, Lake Wenatchee/River
Oncorhynchus nerka	SSNA373-08	FJ999191	652	United States	Washington	Columbia River Basin, Lake Wenatchee/River
Oncorhynchus nerka	SSNA754-08	FJ999192	652	United States	Alaska	
Oncorhynchus nerka	SSNA110-08	FJ999193	652	United States	Alaska	Gisasa River Weir
Oncorhynchus nerka	SSNA443-08	FJ999194	652	United States	Washington	Puget Sound, Lake Washington at Ballard Locks
Oncorhynchus nerka	SSNA442-08	FJ999195	614	United States	Washington	Puget Sound, Lake Washington at Ballard Locks
Oncorhynchus nerka	SSNA440-08	FJ999196	611	United States	Washington	Puget Sound, Lake Washington at Ballard Locks
Oncorhynchus nerka	SSNA094-08	FJ999197	652	United States	Alaska	Papa Bear Lake
Oncorhynchus nerka	SSNA090-08	FJ999198	652	United States	Alaska	Hugh Smith Lake, Bushman Creek
Oncorhynchus nerka	SSNA081-08	FJ999199	652	United States	Alaska	Hugh Smith Lake, Bushman Creek

Table A.1 (Continued)

Oncorhynchus nerka	SSNA089-08	FJ999200	652	United States	Alaska	Hugh Smith Lake, Bushman Creek
Oncorhynchus nerka	SSNA088-08	FJ999201	652	United States	Alaska	Hugh Smith Lake, Bushman Creek
Oncorhynchus nerka	SSNA084-08	FJ999202	652	United States	Alaska	Hugh Smith Lake, Bushman Creek
Oncorhynchus nerka	SSNA087-08	FJ999203	652	United States	Alaska	Hugh Smith Lake, Bushman Creek
Oncorhynchus nerka	SSNA082-08	FJ999204	652	United States	Alaska	Hugh Smith Lake, Bushman Creek
Oncorhynchus nerka	SSNA086-08	FJ999205	652	United States	Alaska	Hugh Smith Lake, Bushman Creek
Oncorhynchus nerka	SSNA085-08	FJ999206	652	United States	Alaska	Hugh Smith Lake, Bushman Creek
Oncorhynchus nerka	SSNA091-08	FJ999207	652	United States	Alaska	Papa Bear Lake
Oncorhynchus nerka	SSNA093-08	FJ999208	652	United States	Alaska	Papa Bear Lake
Oncorhynchus nerka	SSNA092-08	FJ999209	652	United States	Alaska	Papa Bear Lake
Oncorhynchus nerka	SSNA106-08	FJ999210	652	United States	Alaska	Gisasa River Weir
Oncorhynchus nerka	SSNA104-08	FJ999211	652	United States	Alaska	Gisasa River Weir
Oncorhynchus nerka	SSNA102-08	FJ999212	627	United States	Alaska	Gisasa River Weir
Oncorhynchus nerka	SSNA101-08	FJ999213	652	United States	Alaska	Gisasa River Weir
Oncorhynchus nerka	SSNA100-08	FJ999214	652	United States	Alaska	Papa Bear Lake
Oncorhynchus nerka	SSNA096-08	FJ999215	652	United States	Alaska	Papa Bear Lake
Oncorhynchus nerka	SSNA095-08	FJ999216	652	United States	Alaska	Papa Bear Lake
Oncorhynchus nerka	SSNA097-08	FJ999217	652	United States	Alaska	Papa Bear Lake
Oncorhynchus nerka	SSNA099-08	FJ999218	652	United States	Alaska	Papa Bear Lake
Oncorhynchus nerka	SSNA098-08	FJ999219	652	United States	Alaska	Papa Bear Lake
Oncorhynchus nerka	SSNA103-08	FJ999220	652	United States	Alaska	Gisasa River Weir
Oncorhynchus nerka	SSNA105-08	FJ999221	652	United States	Alaska	Gisasa River Weir
Oncorhynchus nerka	SSNA109-08	FJ999222	652	United States	Alaska	Gisasa River Weir
Oncorhynchus nerka	SSNA108-08	FJ999223	652	United States	Alaska	Gisasa River Weir
Oncorhynchus nerka	SSNA112-08	FJ999224	652	United States	Alaska	Meshik River
Oncorhynchus nerka	SSNA118-08	FJ999225	648	United States	Alaska	Meshik River
Oncorhynchus nerka	SSNA117-08	FJ999226	652	United States	Alaska	Meshik River
Oncorhynchus nerka	SSNA115-08	FJ999227	652	United States	Alaska	Meshik River
Oncorhynchus nerka	SSNA114-08	FJ999228	652	United States	Alaska	Meshik River

Table A.1 (Continued)

Oncorhynchus nerka	SSNA113-08	FJ999229	652	United States	Alaska	Meshik River
Oncorhynchus nerka	SSNA116-08	FJ999230	652	United States	Alaska	Meshik River
Oncorhynchus nerka	SSNA120-08	FJ999231	652	United States	Alaska	Meshik River
Oncorhynchus nerka	SSNA119-08	FJ999232	652	United States	Alaska	Meshik River
Oncorhynchus nerka	SSNA107-08	FJ999233	652	United States	Alaska	Gisasa River Weir
Oncorhynchus tshawytscha	SSNA1057-08	FJ999234	652	United States	Alaska	Killey River
Oncorhynchus tshawytscha	SSNA1056-08	FJ999235	652	United States	Alaska	Killey River
Oncorhynchus tshawytscha	SSNA1055-08	FJ999236	652	United States	Alaska	Killey River
Oncorhynchus tshawytscha	SSNA681-08	FJ999237	652	United States	Oregon	North Coast, Necanicum
Oncorhynchus tshawytscha	SSNA1054-08	FJ999238	652	United States	Alaska	Tahini River
Oncorhynchus tshawytscha	SSNA750-08	FJ999239	652	United States	Oregon	Tenmile Creek
Oncorhynchus tshawytscha	SSNA1053-08	FJ999240	652	United States	Alaska	Tahini River
Oncorhynchus tshawytscha	SSNA788-08	FJ999241	652	United States	Oregon	Siuslaw River
Oncorhynchus tshawytscha	SSNA1059-08	FJ999242	652	United States	Alaska	Steelhead Creek
Oncorhynchus tshawytscha	SSNA659-08	FJ999243	652	Canada	British Columbia	Aquaculture facility
Oncorhynchus tshawytscha	SSNA1058-08	FJ999244	652	United States	Alaska	Killey River
Oncorhynchus tshawytscha	SSNA658-08	FJ999245	652	Canada	British Columbia	Aquaculture facility
Oncorhynchus tshawytscha	SSNA1113-08	FJ999246	652	United States	Washington	Puget Sound, University of Washington Hatchery
Oncorhynchus tshawytscha	SSNA1114-08	FJ999247	652	United States	Washington	Puget Sound, University of Washington Hatchery
Oncorhynchus tshawytscha	SSNA346-08	FJ999248	652	United States	Washington	Columbia River Basin, Tumwater Dam (Wenatchee River)
Oncorhynchus tshawytscha	SSNA787-08	FJ999249	652	United States	Oregon	Siuslaw River
Oncorhynchus tshawytscha	SSNA1181-08	FJ999250	652	United States	Oregon	North Coast, Wilson River
Oncorhynchus tshawytscha	SSNA230-08	FJ999251	652	United States	Idaho	Pahsimeroi Hatchery
Oncorhynchus tshawytscha	SSNA1177-08	FJ999252	652	Canada	British Columbia	Aquaculture facility
Oncorhynchus tshawytscha	SSNA1179-08	FJ999253	652	Canada	British Columbia	Aquaculture facility
Oncorhynchus tshawytscha	SSNA222-08	FJ999254	652	United States	Idaho	Clearwater Hatchery
Oncorhynchus tshawytscha	SSNA229-08	FJ999255	652	United States	Idaho	Pahsimeroi Hatchery
Oncorhynchus tshawytscha	SSNA221-08	FJ999256	652	United States	Idaho	Clearwater Hatchery
Oncorhynchus tshawytscha	SSNA228-08	FJ999257	652	United States	Idaho	Pahsimeroi Hatchery

Table A.1 (Continued)

Oncorhynchus tshawytscha	SSNA225-08	FJ999258	652	United States	Idaho	Clearwater Hatchery
Oncorhynchus tshawytscha	SSNA224-08	FJ999259	652	United States	Idaho	Clearwater Hatchery
Oncorhynchus tshawytscha	SSNA223-08	FJ999260	652	United States	Idaho	Clearwater Hatchery
Oncorhynchus tshawytscha	SSNA227-08	FJ999261	652	United States	Idaho	Pahsimeroi Hatchery
Oncorhynchus tshawytscha	SSNA226-08	FJ999262	652	United States	Idaho	Pahsimeroi Hatchery
Oncorhynchus tshawytscha	SSNA786-08	FJ999263	652	United States	Oregon	Siuslaw River
Oncorhynchus tshawytscha	SSNA676-08	FJ999264	652	United States	Oregon	North Coast, Kilchis
Oncorhynchus tshawytscha	SSNA790-08	FJ999265	652	United States	Oregon	South Coast, Umpqua River
Oncorhynchus tshawytscha	SSNA660-08	FJ999266	641	Canada	British Columbia	Aquaculture facility
Oncorhynchus tshawytscha	SSNA675-08	FJ999267	652	United States	Oregon	North Coast, Wilson River
Oncorhynchus tshawytscha	SSNA789-08	FJ999268	652	United States	Oregon	Siuslaw River
Oncorhynchus tshawytscha	SSNA796-08	FJ999269	652	United States	Oregon	South Coast, Sixes River
Oncorhynchus tshawytscha	SSNA792-08	FJ999270	652	United States	Oregon	South Coast, Coquille River
Oncorhynchus tshawytscha	SSNA281-08	FJ999271	613	United States	Washington	Olympic Peninsula, Makak NFH (Sooes River)
Oncorhynchus tshawytscha	SSNA276-08	FJ999272	652	United States	Washington	Olympic Peninsula, Makak NFH (Sooes River)
Oncorhynchus tshawytscha	SSNA795-08	FJ999273	607	United States	Oregon	South Coast, Coquille River
Oncorhynchus tshawytscha	SSNA278-08	FJ999274	652	United States	Washington	Olympic Peninsula, Makak NFH (Sooes River)
Oncorhynchus tshawytscha	SSNA277-08	FJ999275	652	United States	Washington	Olympic Peninsula, Makak NFH (Sooes River)
Oncorhynchus tshawytscha	SSNA794-08	FJ999276	652	United States	Oregon	South Coast, Coquille River
Oncorhynchus tshawytscha	SSNA284-08	FJ999277	478	United States	Washington	Olympic Peninsula, Makak NFH (Sooes River)
Oncorhynchus tshawytscha	SSNA285-08	FJ999278	451	United States	Washington	Olympic Peninsula, Makak NFH (Sooes River)
Oncorhynchus tshawytscha	SSNA686-08	FJ999279	652	United States	Oregon	North Coast, Alsea
Oncorhynchus tshawytscha	SSNA674-08	FJ999280	652	United States	Oregon	North Coast, Kilchis
Oncorhynchus tshawytscha	SSNA801-08	FJ999281	652	United States	Oregon	South Coast, Sixes River
Oncorhynchus tshawytscha	SSNA800-08	FJ999282	652	United States	Oregon	South Coast, Sixes River
Oncorhynchus tshawytscha	SSNA799-08	FJ999283	652	United States	Oregon	South Coast, Sixes River
Oncorhynchus tshawytscha	SSNA798-08	FJ999284	652	United States	Oregon	South Coast, Sixes River
Oncorhynchus tshawytscha	SSNA667-08	FJ999285	652	Canada	British Columbia	Aquaculture facility
Oncorhynchus tshawytscha	SSNA804-08	FJ999286	652	United States	Oregon	South Coast, Rogue River

Table A.1 (Continued)

Oncorhynchus tshawytscha	SSNA802-08	FJ999287	652	United States	Oregon	South Coast, Rogue River
Oncorhynchus tshawytscha	SSNA673-08	FJ999288	652	United States	Oregon	North Coast, Kilchis
Oncorhynchus tshawytscha	SSNA803-08	FJ999289	652	United States	Oregon	South Coast, Rogue River
Oncorhynchus tshawytscha	SSNA665-08	FJ999290	652	Canada	British Columbia	Aquaculture facility
Oncorhynchus tshawytscha	SSNA664-08	FJ999291	652	Canada	British Columbia	Aquaculture facility
Oncorhynchus tshawytscha	SSNA662-08	FJ999292	639	Canada	British Columbia	Aquaculture facility
Oncorhynchus tshawytscha	SSNA661-08	FJ999293	652	Canada	British Columbia	Aquaculture facility
Oncorhynchus tshawytscha	SSNA671-08	FJ999294	652	Canada	British Columbia	Aquaculture facility
Oncorhynchus tshawytscha	SSNA670-08	FJ999295	652	Canada	British Columbia	Aquaculture facility
Oncorhynchus tshawytscha	SSNA668-08	FJ999296	652	Canada	British Columbia	Aquaculture facility
Oncorhynchus tshawytscha	SSNA669-08	FJ999297	652	Canada	British Columbia	Aquaculture facility
Oncorhynchus tshawytscha	SSNA805-08	FJ999298	652	United States	Oregon	South Coast, Rogue River
Oncorhynchus tshawytscha	SSNA678-08	FJ999299	652	United States	Oregon	North Coast, Wilson River
Oncorhynchus tshawytscha	SSNA347-08	FJ999300	652	United States	Washington	Columbia River Basin, Tumwater Dam (Wenatchee River)
Oncorhynchus tshawytscha	SSNA679-08	FJ999301	652	United States	Oregon	North Coast, Necanicum
Oncorhynchus tshawytscha	SSNA355-08	FJ999302	652	United States	Washington	Columbia River Basin, Tumwater Dam (Wenatchee River)
Oncorhynchus tshawytscha	SSNA349-08	FJ999303	652	United States	Washington	Columbia River Basin, Tumwater Dam (Wenatchee River)
Oncorhynchus tshawytscha	SSNA354-08	FJ999304	652	United States	Washington	Columbia River Basin, Tumwater Dam (Wenatchee River)
Oncorhynchus tshawytscha	SSNA348-08	FJ999305	652	United States	Washington	Columbia River Basin, Tumwater Dam (Wenatchee River)
Oncorhynchus tshawytscha	SSNA352-08	FJ999306	652	United States	Washington	Columbia River Basin, Tumwater Dam (Wenatchee River)
Oncorhynchus tshawytscha	SSNA351-08	FJ999307	652	United States	Washington	Columbia River Basin, Tumwater Dam (Wenatchee River)
Oncorhynchus tshawytscha	SSNA677-08	FJ999308	652	United States	Oregon	North Coast, Wilson River
Oncorhynchus tshawytscha	SSNA680-08	FJ999309	652	United States	Oregon	North Coast, Necanicum
Oncorhynchus tshawytscha	SSNA749-08	FJ999310	652	United States	Oregon	Tenmile Creek
Oncorhynchus tshawytscha	SSNA685-08	FJ999311	652	United States	Oregon	North Coast, Alsea
Oncorhynchus tshawytscha	SSNA748-08	FJ999312	652	United States	Oregon	Tenmile Creek
Oncorhynchus tshawytscha	SSNA747-08	FJ999313	652	United States	Oregon	Tenmile Creek
Oncorhynchus tshawytscha	SSNA744-08	FJ999314	652	United States	Oregon	Tenmile Creek
Oncorhynchus tshawytscha	SSNA743-08	FJ999315	652	United States	Oregon	Tenmile Creek

Table A.1 (Continued)

Oncorhynchus tshawytscha	SSNA742-08	FJ999316	652	United States	Oregon	Tenmile Creek
Oncorhynchus tshawytscha	SSNA745-08	FJ999317	652	United States	Oregon	Tenmile Creek
Oncorhynchus tshawytscha	SSNA746-08	FJ999318	652	United States	Oregon	Tenmile Creek
Oncorhynchus tshawytscha	SSNA684-08	FJ999319	652	United States	Oregon	North Coast, Alsea
Oncorhynchus tshawytscha	SSNA978-08	FJ999320	652	United States	California	Mendocino, Eel River
Oncorhynchus tshawytscha	SSNA977-08	FJ999321	652	United States	California	Mendocino, Eel River
Oncorhynchus tshawytscha	SSNA683-08	FJ999322	652	United States	Oregon	North Coast, Alsea
Oncorhynchus tshawytscha	SSNA976-08	FJ999323	652	United States	California	Mendocino, Eel River
Oncorhynchus tshawytscha	SSNA682-08	FJ999324	652	United States	Oregon	North Coast, Necanicum
Oncorhynchus tshawytscha	SSNA960-08	FJ999325	652	United States	California	Del Norte, Klamath River
Oncorhynchus tshawytscha	SSNA975-08	FJ999326	652	United States	California	Mendocino, Eel River
Oncorhynchus tshawytscha	SSNA959-08	FJ999327	652	United States	California	Del Norte, Klamath River
Oncorhynchus tshawytscha	SSNA967-08	FJ999328	652	United States	California	Del Norte, Klamath River
Oncorhynchus tshawytscha	SSNA958-08	FJ999329	652	United States	California	Del Norte, Klamath River
Oncorhynchus tshawytscha	SSNA957-08	FJ999330	651	United States	California	Del Norte, Klamath River
Oncorhynchus tshawytscha	SSNA956-08	FJ999331	652	United States	California	Del Norte, Klamath River
Oncorhynchus tshawytscha	SSNA966-08	FJ999332	652	United States	California	Del Norte, Klamath River
Oncorhynchus tshawytscha	SSNA965-08	FJ999333	652	United States	California	Del Norte, Klamath River
Oncorhynchus tshawytscha	SSNA964-08	FJ999334	652	United States	California	Del Norte, Klamath River
Oncorhynchus tshawytscha	SSNA961-08	FJ999335	652	United States	California	Del Norte, Klamath River
Oncorhynchus tshawytscha	SSNA962-08	FJ999336	652	United States	California	Del Norte, Klamath River
Oncorhynchus tshawytscha	SSNA963-08	FJ999337	652	United States	California	Del Norte, Klamath River
Oncorhynchus tshawytscha	SSNA972-08	FJ999338	652	United States	California	Mendocino, Eel River
Oncorhynchus tshawytscha	SSNA974-08	FJ999339	652	United States	California	Mendocino, Eel River
Oncorhynchus tshawytscha	SSNA968-08	FJ999340	652	United States	California	Del Norte, Klamath River
Oncorhynchus tshawytscha	SSNA970-08	FJ999341	652	United States	California	Del Norte, Klamath River
Oncorhynchus tshawytscha	SSNA971-08	FJ999342	652	United States	California	Mendocino, Eel River
Oncorhynchus tshawytscha	SSNA969-08	FJ999343	652	United States	California	Del Norte, Klamath River
Oncorhynchus tshawytscha	SSNA973-08	FJ999344	652	United States	California	Mendocino, Eel River

Table A.1 (Continued)

Oncorhynchus tshawytscha	SSNA714-08	FJ999345	652	United States	Oregon	South Coast, North Umpqua
Oncorhynchus tshawytscha	SSNA751-08	FJ999346	652	United States	Oregon	Tenmile Creek
Oncorhynchus tshawytscha	SSNA715-08	FJ999347	652	United States	Oregon	South Coast, Elk River
Oncorhynchus tshawytscha	SSNA701-08	FJ999348	652	United States	Oregon	Siltetz Falls
Oncorhynchus tshawytscha	SSNA696-08	FJ999349	652	United States	Oregon	Chetco River
Oncorhynchus tshawytscha	SSNA700-08	FJ999350	652	United States	Oregon	Siltetz Falls
Oncorhynchus tshawytscha	SSNA691-08	FJ999351	652	United States	Oregon	Trask
Oncorhynchus tshawytscha	SSNA695-08	FJ999352	652	United States	Oregon	Chetco River
Oncorhynchus tshawytscha	SSNA690-08	FJ999353	652	United States	Oregon	Nestucca
Oncorhynchus tshawytscha	SSNA689-08	FJ999354	652	United States	Oregon	Nestucca
Oncorhynchus tshawytscha	SSNA688-08	FJ999355	652	United States	Oregon	Nestucca
Oncorhynchus tshawytscha	SSNA687-08	FJ999356	616	United States	Oregon	Nestucca
Oncorhynchus tshawytscha	SSNA694-08	FJ999357	652	United States	Oregon	Trask
Oncorhynchus tshawytscha	SSNA693-08	FJ999358	652	United States	Oregon	Trask
Oncorhynchus tshawytscha	SSNA692-08	FJ999359	652	United States	Oregon	Trask
Oncorhynchus tshawytscha	SSNA699-08	FJ999360	652	United States	Oregon	Siltetz Falls
Oncorhynchus tshawytscha	SSNA697-08	FJ999361	652	United States	Oregon	Chetco River
Oncorhynchus tshawytscha	SSNA698-08	FJ999362	652	United States	Oregon	Chetco River
Oncorhynchus tshawytscha	SSNA713-08	FJ999363	652	United States	Oregon	South Coast, North Umpqua
Oncorhynchus tshawytscha	SSNA718-08	FJ999364	652	United States	Oregon	South Coast, Elk River
Oncorhynchus tshawytscha	SSNA704-08	FJ999365	652	United States	Oregon	South Coast, Yaquina River
Oncorhynchus tshawytscha	SSNA703-08	FJ999366	652	United States	Oregon	South Coast, Yaquina River
Oncorhynchus tshawytscha	SSNA702-08	FJ999367	652	United States	Oregon	Siltetz Falls
Oncorhynchus tshawytscha	SSNA716-08	FJ999368	652	United States	Oregon	South Coast, Elk River
Oncorhynchus tshawytscha	SSNA711-08	FJ999369	652	United States	Oregon	South Coast, North Umpqua
Oncorhynchus tshawytscha	SSNA706-08	FJ999370	652	United States	Oregon	South Coast, Yaquina River
Oncorhynchus tshawytscha	SSNA712-08	FJ999371	652	United States	Oregon	South Coast, North Umpqua
Oncorhynchus tshawytscha	SSNA705-08	FJ999372	652	United States	Oregon	South Coast, Yaquina River
Oncorhynchus tshawytscha	SSNA710-08	FJ999373	652	United States	Oregon	South Coast, South Umpqua

Table A.1 (Continued)

Oncorhynchus tshawytscha	SSNA707-08	FJ999374	652	United States	Oregon	South Coast, South Umpqua
Oncorhynchus tshawytscha	SSNA709-08	FJ999375	652	United States	Oregon	South Coast, South Umpqua
Oncorhynchus tshawytscha	SSNA708-08	FJ999376	652	United States	Oregon	South Coast, South Umpqua
Oncorhynchus tshawytscha	SSNA752-08	FJ999377	652	United States	Oregon	Tenmile Creek
Oncorhynchus tshawytscha	SSNA753-08	FJ999378	652	United States	Oregon	Tenmile Creek
Oncorhynchus tshawytscha	SSNA417-08	FJ999379	652	United States	Washington	Puget Sound, University of Washington Hatchery
Oncorhynchus tshawytscha	SSNA416-08	FJ999380	652	United States	Washington	Puget Sound, University of Washington Hatchery
Oncorhynchus tshawytscha	SSNA418-08	FJ999381	647	United States	Washington	Puget Sound, University of Washington Hatchery
Oncorhynchus tshawytscha	SSNA1019-08	FJ999382	652	United States	California	Central Valley, Sacramento River
Oncorhynchus tshawytscha	SSNA1026-08	FJ999383	652	United States	California	Central Valley, Sacramento River
Oncorhynchus tshawytscha	SSNA1025-08	FJ999384	652	United States	California	Central Valley, Sacramento River
Oncorhynchus tshawytscha	SSNA1022-08	FJ999385	652	United States	California	Central Valley, Sacramento River
Oncorhynchus tshawytscha	SSNA420-08	FJ999386	629	United States	Washington	Puget Sound, University of Washington Hatchery
Oncorhynchus tshawytscha	SSNA1021-08	FJ999387	652	United States	California	Central Valley, Sacramento River
Oncorhynchus tshawytscha	SSNA421-08	FJ999388	572	United States	Washington	Puget Sound, University of Washington Hatchery
Oncorhynchus tshawytscha	SSNA1020-08	FJ999389	652	United States	California	Central Valley, Sacramento River
Oncorhynchus tshawytscha	SSNA1023-08	FJ999390	652	United States	California	Central Valley, Sacramento River
Oncorhynchus tshawytscha	SSNA1024-08	FJ999391	652	United States	California	Central Valley, Sacramento River
Oncorhynchus tshawytscha	SSNA424-08	FJ999392	652	United States	Washington	Puget Sound, University of Washington Hatchery
Oncorhynchus tshawytscha	SSNA005-08	FJ999393	612	United States	Alaska	Tahini River
Oncorhynchus tshawytscha	SSNA039-08	FJ999394	652	United States	Alaska	Steelhead Creek
Oncorhynchus tshawytscha	SSNA023-08	FJ999395	652	United States	Alaska	Tozitna River
Oncorhynchus tshawytscha	SSNA003-08	FJ999396	652	United States	Alaska	Tahini River
Oncorhynchus tshawytscha	SSNA002-08	FJ999397	652	United States	Alaska	Tahini River
Oncorhynchus tshawytscha	SSNA008-08	FJ999398	652	United States	Alaska	Tahini River
Oncorhynchus tshawytscha	SSNA022-08	FJ999399	652	United States	Alaska	Tozitna River
Oncorhynchus tshawytscha	SSNA006-08	FJ999400	511	United States	Alaska	Tahini River
Oncorhynchus tshawytscha	SSNA015-08	FJ999401	652	United States	Alaska	Killey River
Oncorhynchus tshawytscha	SSNA010-08	FJ999402	652	United States	Alaska	Tahini River

Table A.1 (Continued)

Oncorhynchus tshawytscha	SSNA016-08	FJ999403	652	United States	Alaska	Killey River
Oncorhynchus tshawytscha	SSNA009-08	FJ999404	652	United States	Alaska	Tahini River
Oncorhynchus tshawytscha	SSNA012-08	FJ999405	652	United States	Alaska	Killey River
Oncorhynchus tshawytscha	SSNA017-08	FJ999406	652	United States	Alaska	Killey River
Oncorhynchus tshawytscha	SSNA021-08	FJ999407	652	United States	Alaska	Tozitna River
Oncorhynchus tshawytscha	SSNA020-08	FJ999408	652	United States	Alaska	Killey River
Oncorhynchus tshawytscha	SSNA019-08	FJ999409	652	United States	Alaska	Killey River
Oncorhynchus tshawytscha	SSNA024-08	FJ999410	652	United States	Alaska	Tozitna River
Oncorhynchus tshawytscha	SSNA028-08	FJ999411	652	United States	Alaska	Tozitna River
Oncorhynchus tshawytscha	SSNA027-08	FJ999412	652	United States	Alaska	Tozitna River
Oncorhynchus tshawytscha	SSNA026-08	FJ999413	652	United States	Alaska	Tozitna River
Oncorhynchus tshawytscha	SSNA032-08	FJ999414	652	United States	Alaska	Steelhead Creek
Oncorhynchus tshawytscha	SSNA038-08	FJ999415	652	United States	Alaska	Steelhead Creek
Oncorhynchus tshawytscha	SSNA025-08	FJ999416	652	United States	Alaska	Tozitna River
Oncorhynchus tshawytscha	SSNA031-08	FJ999417	652	United States	Alaska	Steelhead Creek
Oncorhynchus tshawytscha	SSNA030-08	FJ999418	652	United States	Alaska	Tozitna River
Oncorhynchus tshawytscha	SSNA029-08	FJ999419	652	United States	Alaska	Tozitna River
Oncorhynchus tshawytscha	SSNA033-08	FJ999420	652	United States	Alaska	Steelhead Creek
Oncorhynchus tshawytscha	SSNA034-08	FJ999421	652	United States	Alaska	Steelhead Creek
Oncorhynchus tshawytscha	SSNA037-08	FJ999422	652	United States	Alaska	Steelhead Creek
Oncorhynchus tshawytscha	SSNA036-08	FJ999423	652	United States	Alaska	Steelhead Creek
Oncorhynchus tshawytscha	SSNA717-08	FJ999424	652	United States	Oregon	South Coast, Elk River
Oncorhynchus tshawytscha	SSNA040-08	FJ999425	652	United States	Alaska	Steelhead Creek
Oncorhynchus tshawytscha	SSNA657-08	FJ999426	652	Canada	British Columbia	Aquaculture facility
Oncorhynchus tshawytscha	SSNA785-08	FJ999427	581	United States	Oregon	Siuslaw River
Oncorhynchus tshawytscha	SSNA654-08	FJ999428	652	Canada	British Columbia	Aquaculture facility
Oncorhynchus tshawytscha	SSNA656-08	FJ999429	652	Canada	British Columbia	Aquaculture facility
Oncorhynchus tshawytscha	SSNA653-08	FJ999430	652	Canada	British Columbia	Aquaculture facility
Oncorhynchus tshawytscha	SSNA655-08	FJ999431	652	Canada	British Columbia	Aquaculture facility

Table A.1 (Continued)

Oncorhynchus tshawytscha	SSNA771-08	FJ999432	652	United States	Oregon	North Coast, Nehalem River
Oncorhynchus tshawytscha	SSNA784-08	FJ999433	652	United States	Oregon	Siuslaw River
Oncorhynchus tshawytscha	SSNA772-08	FJ999434	652	United States	Oregon	North Coast, Nehalem River
Oncorhynchus tshawytscha	SSNA759-08	FJ999435	652	United States	Oregon	Clatskanie River
Oncorhynchus tshawytscha	SSNA770-08	FJ999436	652	United States	Oregon	North Coast, Nehalem River
Oncorhynchus tshawytscha	SSNA769-08	FJ999437	652	United States	Oregon	North Coast, Nehalem River
Oncorhynchus tshawytscha	SSNA783-08	FJ999438	652	United States	Oregon	North Coast, Tillamook River
Oncorhynchus tshawytscha	SSNA780-08	FJ999439	652	United States	Oregon	North Coast, Wilson River
Oncorhynchus tshawytscha	SSNA778-08	FJ999440	652	United States	Oregon	North Coast, Wilson River
Oncorhynchus tshawytscha	SSNA773-08	FJ999441	617	United States	Oregon	North Coast, Nehalem River
Oncorhynchus tshawytscha	SSNA777-08	FJ999442	615	United States	Oregon	North Coast, Nehalem River
Oncorhynchus tshawytscha	SSNA776-08	FJ999443	652	United States	Oregon	North Coast, Nehalem River
Oncorhynchus tshawytscha	SSNA774-08	FJ999444	652	United States	Oregon	North Coast, Nehalem River
Oncorhynchus tshawytscha	SSNA775-08	FJ999445	652	United States	Oregon	North Coast, Nehalem River
Oncorhynchus tshawytscha	SSNA782-08	FJ999446	652	United States	Oregon	North Coast, Wilson River
Oncorhynchus tshawytscha	SSNA781-08	FJ999447	652	United States	Oregon	North Coast, Wilson River
Salmo salar	SSNA550-08	FJ999448	639	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA1170-08	FJ999449	619	United States	Washington	Aquaculture facility
Salmo salar	SSNA756-08	FJ999450	652	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA548-08	FJ999451	644	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA546-08	FJ999452	596	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA547-08	FJ999453	617	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA553-08	FJ999454	647	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA619-08	FJ999455	630	United States	Washington	Aquaculture facility
Salmo salar	SSNA552-08	FJ999456	637	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA551-08	FJ999457	647	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA558-08	FJ999458	637	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA573-08	FJ999459	643	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA557-08	FJ999460	643	Canada	British Columbia	Aquaculture facility

Table A.1 (Continued)

Salmo salar	SSNA554-08	FJ999461	638	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA555-08	FJ999462	602	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA556-08	FJ999463	625	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA564-08	FJ999464	642	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA561-08	FJ999465	633	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA572-08	FJ999466	620	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA562-08	FJ999467	640	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA565-08	FJ999468	639	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA567-08	FJ999469	641	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA571-08	FJ999470	626	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA566-08	FJ999471	627	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA570-08	FJ999472	651	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA568-08	FJ999473	641	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA569-08	FJ999474	612	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA585-08	FJ999475	652	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA574-08	FJ999476	652	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA575-08	FJ999477	635	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA579-08	FJ999478	641	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA577-08	FJ999479	581	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA580-08	FJ999480	557	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA581-08	FJ999481	652	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA582-08	FJ999482	618	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA584-08	FJ999483	650	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA583-08	FJ999484	652	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA586-08	FJ999485	652	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA589-08	FJ999486	613	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA588-08	FJ999487	652	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA597-08	FJ999488	618	United States	Washington	Aquaculture facility
Salmo salar	SSNA593-08	FJ999489	643	Canada	British Columbia	Aquaculture facility

Table A.1 (Continued)

Salmo salar	SSNA604-08	FJ999490	652	United States	Washington	Aquaculture facility
Salmo salar	SSNA618-08	FJ999491	652	United States	Washington	Aquaculture facility
Salmo salar	SSNA596-08	FJ999492	503	United States	Washington	Aquaculture facility
Salmo salar	SSNA594-08	FJ999493	652	Canada	British Columbia	Aquaculture facility
Salmo salar	SSNA602-08	FJ999494	475	United States	Washington	Aquaculture facility
Salmo salar	SSNA601-08	FJ999495	577	United States	Washington	Aquaculture facility
Salmo salar	SSNA603-08	FJ999496	652	United States	Washington	Aquaculture facility
Salmo salar	SSNA600-08	FJ999497	580	United States	Washington	Aquaculture facility
Salmo salar	SSNA617-08	FJ999498	573	United States	Washington	Aquaculture facility
Salmo salar	SSNA606-08	FJ999499	593	United States	Washington	Aquaculture facility
Salmo salar	SSNA614-08	FJ999500	612	United States	Washington	Aquaculture facility
Salmo salar	SSNA609-08	FJ999501	550	United States	Washington	Aquaculture facility
Salmo salar	SSNA613-08	FJ999502	518	United States	Washington	Aquaculture facility
Salmo salar	SSNA608-08	FJ999503	652	United States	Washington	Aquaculture facility
Salmo salar	SSNA612-08	FJ999504	580	United States	Washington	Aquaculture facility
Salmo salar	SSNA611-08	FJ999505	598	United States	Washington	Aquaculture facility
Salmo salar	SSNA615-08	FJ999506	651	United States	Washington	Aquaculture facility
Salmo salar	SSNA632-08	FJ999507	625	United States	Washington	Aquaculture facility
Salmo salar	SSNA622-08	FJ999508	427	United States	Washington	Aquaculture facility
Salmo salar	SSNA631-08	FJ999509	652	United States	Washington	Aquaculture facility
Salmo salar	SSNA621-08	FJ999510	652	United States	Washington	Aquaculture facility
Salmo salar	SSNA620-08	FJ999511	644	United States	Washington	Aquaculture facility
Salmo salar	SSNA630-08	FJ999512	649	United States	Washington	Aquaculture facility
Salmo salar	SSNA624-08	FJ999513	536	United States	Washington	Aquaculture facility
Salmo salar	SSNA629-08	FJ999514	652	United States	Washington	Aquaculture facility
Salmo salar	SSNA623-08	FJ999515	627	United States	Washington	Aquaculture facility
Salmo salar	SSNA625-08	FJ999516	652	United States	Washington	Aquaculture facility
Salmo salar	SSNA627-08	FJ999517	593	United States	Washington	Aquaculture facility
Salmo salar	SSNA633-08	FJ999518	527	United States	Washington	Aquaculture facility

Table A.1 (Continued)

Salmo salar	SSNA636-08	FJ999519	544	United States	Washington	Aquaculture facility
Salmo salar	SSNA635-08	FJ999520	616	United States	Washington	Aquaculture facility
Salmo salar	SSNA646-08	FJ999521	611	United States	Washington	Aquaculture facility
Salmo salar	SSNA634-08	FJ999522	652	United States	Washington	Aquaculture facility
Salmo salar	SSNA638-08	FJ999523	652	United States	Washington	Aquaculture facility
Salmo salar	SSNA644-08	FJ999524	523	United States	Washington	Aquaculture facility
Salmo salar	SSNA640-08	FJ999525	526	United States	Washington	Aquaculture facility
Salmo salar	SSNA639-08	FJ999526	625	United States	Washington	Aquaculture facility
Salmo salar	SSNA643-08	FJ999527	462	United States	Washington	Aquaculture facility
Salmo salar	SSNA641-08	FJ999528	302	United States	Washington	Aquaculture facility
Salmo salar	SSNA648-08	FJ999529	491	United States	Washington	Aquaculture facility
Salmo salar	SSNA650-08	FJ999530	614	United States	Washington	Aquaculture facility
Salmo salar	SSNA651-08	FJ999531	613	United States	Washington	Aquaculture facility
Salmo salar	SSNA761-08	FJ999532	652	Chile		Aquaculture facility
Salmo salar	SSNA760-08	FJ999533	652	Chile		Aquaculture facility
Salmo salar	SSNA767-08	FJ999534	652	Chile		Aquaculture facility
Salmo salar	SSNA766-08	FJ999535	645	Chile		Aquaculture facility
Salmo salar	SSNA765-08	FJ999536	639	Chile		Aquaculture facility
Salmo salar	SSNA764-08	FJ999537	652	Chile		Aquaculture facility
Salmo salar	SSNA763-08	FJ999538	532	Chile		Aquaculture facility
Salmo salar	SSNA762-08	FJ999539	648	Chile		Aquaculture facility