COMPREHENSIVE REVIEWS IN FOOD SCIENCE AND FOOD SAFETY

DNA-Based Methods for the Identification of Commercial Fish and Seafood Species

Rosalee S. Rasmussen and Michael T. Morrissey

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. 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 article, the major DNA-based methods currently employed in the authentication of commercial fish and seafood species are discussed and future trends are highlighted. Examples of commercial applications and the use of online database resources are also considered.

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 1; Civera 2003; Martinez and others 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 (Moretti and others 2003; Martinez and others 2005). 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 2006). 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://vm.cfsan.fda.gov/~frf/seaintro.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, 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 and others 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 and others 2005; Teletchea and others 2005). Furthermore, to enforce laws against poaching and trade of overexploited species, reliable methods for species diagnosis are essential (Baker and others 2000; Kyle and Wilson 2007).

Research into methods for the identification of fish and seafood species presents several challenges that must be overcome. For

MS 20080188 Submitted 3/12/2008, Accepted 4/13/2008. Author Rasmussen is with Dept. of Food Science and Technology, OSU Seafood Laboratory, Oregon State Univ., 2001 Marine Dr., Room 253, Astoria, OR 97103, U.S.A. Author Morrissey is with Oregon State University Food Innovation Center, 1207 NW Naito Parkway, Portland, OR 97209, U.S.A. Direct inquiries to author Morrissey (E-mail: michael.morrissey@oregonstate.edu).

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example, it has been estimated that more than 20000 species of fish and seafood are utilized worldwide for human consumption (Martinez and others 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 (Mackie and others 1999; Chapela and others 2002). A number of compounds present in processed foods may also serve as inhibitors of DNA amplification during the polymerase chain reaction (PCR) (Teletchea and others 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 and others 2007). This review discusses 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.

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 (Sotelo and others 1993; Civera 2003; Moretti and others 2003). Some common methods include isoelectric focusing (IEF), capillary electrophoresis (CE), high-performance liquid chromatography (HPLC), and immunoassay 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-mentioned methods impractical (Mackie and others 1999; Akasaki and others 2006). 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 and others 1999; Moretti and others 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 (Carrera and others 1997; Asensio and others 2003). However, immunoassays 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 and others 1993; Woolfe and Primrose 2004).

Table 1—Examples of seafood substitution (provided online by the USFDA: http://www.cfsan.fda.gov/~frf/rfeecon.html). 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 Fisheries of the United States 2006 (Natl. Marine Fisheries Service, Fisheries Statistics Div., Silver Spring, Md., U.S.A.). 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 to 6.00 per kilogram
Yellowtail	Mahi mahi	NA
Mako shark	Swordfish	NA
Alaska pollock	Cod	$0.62 to 3.35 per kilogram
Sea bass	Halibut	$0.71 to 1.79 per kilogram
Arrowtooth flounder	Dover sole	$0.66 per kilogram
Paddlefish and other fish roe	Caviar (sturgeon species)	>$1000 per kilogram[a]
Steelhead trout	Salmon	up to $3.02 per kilogram
Farm-raised salmon	Wild salmon	up to $1.74 per kilogram
Pink salmon	Chum salmon	$0.37 per kilogram
Imported crabmeat	Blue crabmeat	NA

[a]According to 2008 retail prices.

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 and others 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 and others 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 and others 2006; Moran and Garcia-Vazquez 2006), flatfish (Sanjuan and Comesana 2002; Comesana and others 2003), salmonids (Dooley and others 2005a; Zhang and Cai 2006), scombroids (Hsieh and others 2007; Lin and Hwang 2007), sardines and anchovies (Jerome and others 2003; Santaclara and others 2006), eels (Lin and others 2002), mollusks (Rego and others 2002; Klinbunga and others 2003), and many more.

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). 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 describes the above-mentioned steps in greater detail, with a focus on the analysis of PCR fragments for species determination.

DNA extraction

Although the basic steps in the isolation of DNA from tissue are fairly constant (Figure 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 and others 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 and others (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 and others 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 and others 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 and others 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 and others 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 researchers 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 and others 2007).

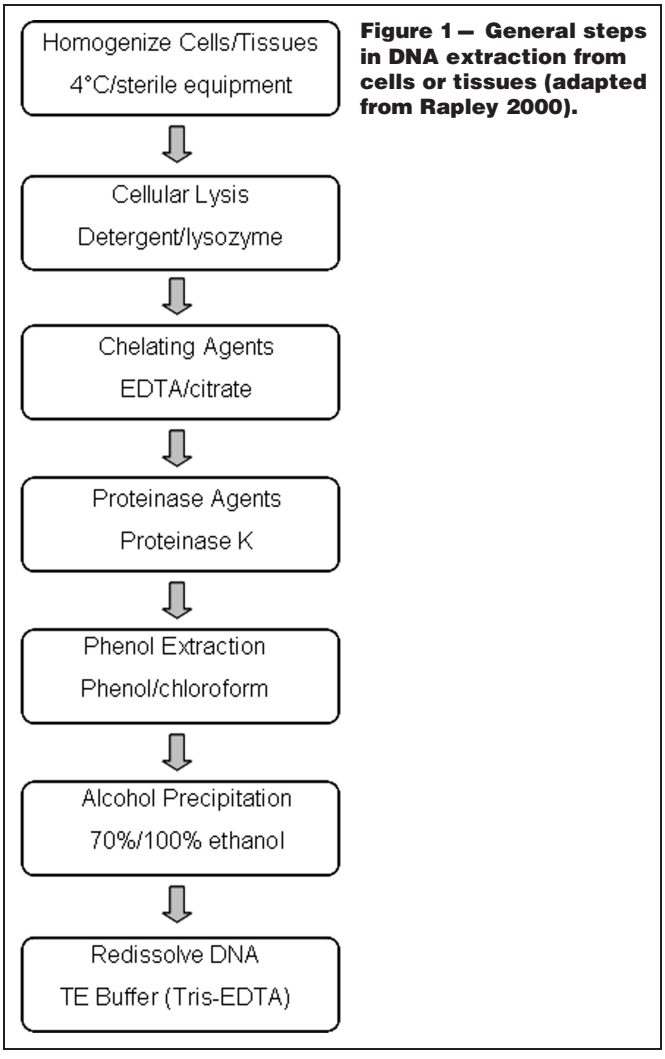

Figure 1 — General steps in DNA extraction from cells or tissues (adapted from Rapley 2000).

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), which requires much less starting material and exhibits greater versatility and sensitivity (Lenstra 2003; Gil 2007). Amplification of genetic material with PCR requires a thermostable DNA polymerase, 2 oligonucleotide primers, 4 deoxynucleotide triphosphates (dNTPs), and magnesium ions (Marmiroli and others 2003). PCR involves numerous cycles of 3 reaction steps carried out at different temperatures: denaturation (approximately 95 °C), annealing (50 to 60 °C), and extension (approximately 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 to 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 and others 2003). The possibility of using quantitative PCR techniques in fish and seafood authentication is 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 and others 2000). Determination of fish and seafood species can be carried out using either nuclear DNA (nDNA) or mitochondrial DNA (mtDNA) (Martinez and others 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" (Rego and others 2002; Ramella and others 2005; Zhang and Cai 2006). These techniques do not require prior knowledge of the DNA sequence and are 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 and others 2000). Some major advantages of mtDNA over nDNA are that (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 (Cespedes and others 2000; Civera 2003; Aranishi and others 2005a). 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 and others 2000b; Martinez and others 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 and others 2000a; Comesana and others 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 which 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 to increase the validity of the method (Teletchea and others 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 may be 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 (Ram and others 1996; Quinteiro and others 1998; Jerome and others 2003; Perez and others 2004; Chapela and others 2007). 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 and others 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 (Quinteiro and others 1998; Rehbein and others 1999b; Pardo and Perez-Villareal 2004; Lin and Hwang 2007).

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

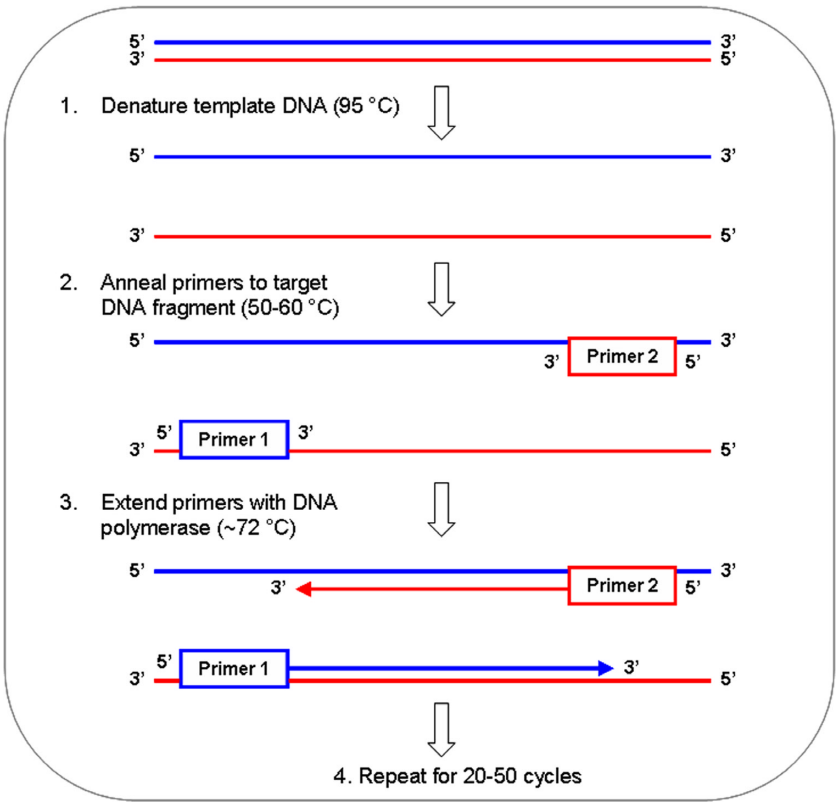

Figure 2—Main steps in the amplification of a target DNA fragment with the polymerase chain reaction. The DNA double helix is first denatured at a high temperature into complementary single strands; then, the temperature is reduced to allow primers to anneal to both ends of the target DNA sequence; and next, DNA polymerase extends the primers using a mixture of 4 dNTPs (dCTP, dGTP, dATP, and dTTP). These 3 steps are usually repeated for 20 to 50 cycles, resulting in the production of millions of copies of the target DNA fragment.

(Sotelo and others 2001; Rehbein and others 2002; Calo-Mata and others 2003; Chow and others 2003; Pepe and others 2005; Teletchea and others 2005; Santaclara and others 2006). 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 (Mackie and others 1999; Aranishi and others 2005a). 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 (Wolf and others 1999, 2000; Sanjuan and Comesana 2002; Akasaki and others 2006).

Some other common mtDNA targets in species identification research are the small 12S rRNA gene (819 to 975 bp in vertebrates) and the larger 16S rRNA gene (1571 to 1640 bp in vertebrates), which have been used to identify flatfish, eel, cardinalfish, cephalopods, mackerel, hairtail species, crab, and several others (Cespedes and others 2000; Chapela and others 2002; Comesana and others 2003; Karaiskou and others 2003; Mabuchi and others 2003; Imai and others 2004; Chakraborty and others 2005; Itoi and others 2005). 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 and others 2000). This gene experiences less degeneracy than the mitochondrial protein-coding genes; however, it does contain sufficient variation for interspecies differentiation (Comesana and others 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 and others 2001); the gene coding for cytochrome oxidase subunit III (COSIII), which has been used to differentiate rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) (Carrera and others 1999a); and the flanking region between COSIII and the ATPase genes (termed *ATCO*), used to differentiate various species of scombroids (Takeyama and others 2001; Chow and others 2003).

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 (Carrera and others 2000a; Aranishi 2005; Clarke and others 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 (Withler and others 1997; Carrera and others 2000b; Fernandez and others 2000; Shivji and others 2002; Klinbunga and others 2003). 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 and others 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 and others 2002), and variations in the gene coding for 18S rRNA allowed for identification of 4 species of abalone (Klinbunga and others 2003). The highly conserved α-actin gene was reported to be useful in the detection of 3 species of clams (Fernandez and others 2000), while an exon and an adjacent intron of the MHC class II β1 gene were used to identify several species of salmonids (Withler and others 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 to 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 and others 2003). 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 (Verkaar and others 2001; Nijman and others 2002, 2003), 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 and others 2000, 2004), smelt (Beacham and others 2005), channel catfish (*Ictalurus punctatus*) (Waldbieser and others 2001), sun-catfish (*Horabagrus brachysoma*) (Gopalakrishnan and others 2006), carp (Lal and others 2004), salmonids (Greig and others 2003; Bucklin and others 2007), 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 and others 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 and others 2003). The researchers 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 and others 2004). VNTRs have also been developed to differentiate 4 similar eel species (Maes and others 2006); to identify the sturgeon species *Acipenser stellatus*, a producer of highly prized black caviar (Jenneckens and

others 2001); to differentiate wild and hatchery-raised red drum (*Sciaenops ocellatus*) (Renshaw and others 2006); and to identify 3 Pacific salmonid species (Greig and others 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 and others 2003). One example is the actin multigene family, which has been used for the identification of a number of vertebrate species (Martinez and others 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. 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 and others 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 and others 2002). In other cases, universal primers are utilized to amplify the target DNA and then species-specific differences in sequence are analyzed by RFLP (Sanjuan and Comesana 2002; Akasaki and others 2006; Santaclara and others 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 (Russell and others 2000; Sotelo and others 2001; Calo-Mata and others 2003). 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 and others 2007).

Species-specific primers and multiplex PCR. Species-specific primers are designed on the basis of single nucleotide polymorphisms (SNPs) 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 (Shivji and others 2002; Abercrombie and others 2005; Clarke and others 2006; Magnussen and others 2007). Multiplex PCR assays have also been developed to identify swordfish (*Xiphias gladius*) in processed products (Hsieh and others 2004); to differentiate sole (*Solea solea*) and Greenland halibut (*Reinhardtius hippoglossoides*) (Cespedes and others 1999); to identify 3 species of Pacific salmonids (Greig and others 2002); and to differentiate fillets of Nile perch (*Lates niloticus*), grouper (*Epinephelus guaza*), and wreck fish (*Polyprion americanus*) (Asensio and others 2001; Asensio 2007). 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 and others 2003). For example, Trotta and others (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 is discussed further in the Emerging Trends section of this article.

Post-PCR analysis methods

Following DNA extraction and PCR amplification, the resulting DNA fragments must be properly analyzed to verify the presence or absence of species-specific genetic markers. As shown in Figure 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). 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 and others 2005). This section of the review discusses 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.

Forensically informative nucleotide sequencing (FINS). FINS is a DNA-based procedure first described by Bartlett and Davidson (1992). 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 is 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 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 and others 2003); frozen or canned sardines and sardine-type products (Jerome and others 2003); fresh/frozen anchovy species (Santaclara and others 2006); and fresh/frozen or canned cephalopods and "squid rings" products (Chapela and others 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-mentioned 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 and others 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 (Lockley and Bardsley 2000; Chapela and others 2002; Dooley and others 2005a). 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 use in species identification, especially when a large number of samples are involved (Carrera and others 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 and others 2003).

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 previously, 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 (endonucleases) and then analyzed using gel electrophoresis to develop species-specific restriction profiles (Liu and Cordes 2004). 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, because the PCR amplicon of interest is simply digested with the preselected 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 (Carrera and others 1999a; Cespedes and others 2000; Aranishi 2005). 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 (Cespedes and others 2000; Aranishi 2005).

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 (Ram and others 1996; Quinteiro and others 1998; Chow and others 2003; Horstkotte and Rehbein 2003), flatfish (Cespedes and others 1998a, 1998b; Sotelo and others 2001), gadoids (Calo-Mata and others 2003; Perez and others 2004; Aranishi and others 2005a, 2005b; Pepe and others 2005), salmonids (Russell and others 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 and others 1999a, 1999b,

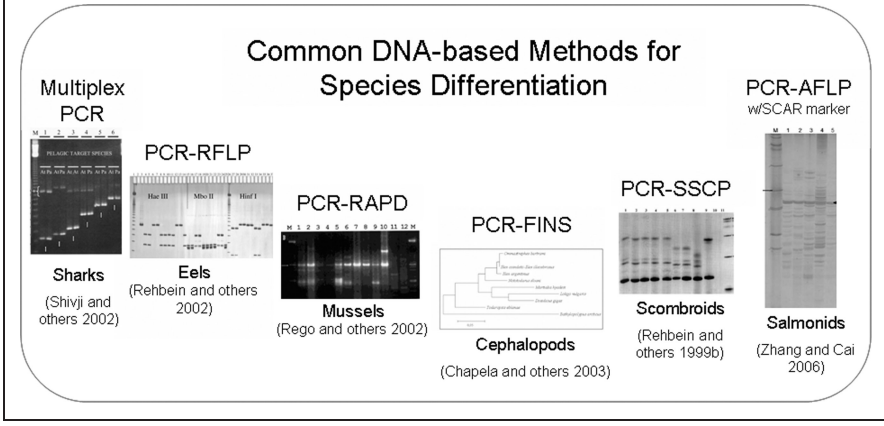

Figure 3—Examples of common DNA-based diagnostic methods that have been utilized for the identification of fish and seafood species.

Table 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 interlaboratory 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	NA	Yes	Single	Medium-high	High	Medium	High	Medium	Flatfish, gadiformes, salmonids, scombroids, percoids, sturgeon, eels, sharks, mollusks
DNA sequencing + phylogenetic mapping	FINS	Yes	Single	Medium-high	High	High	High	Low	Cephalopods, gadiformes, mollusks
Restriction fragment length polymorphism	RFLP	Yes	Single	Medium-high	High	Medium	Medium-high	Medium	Flatfish, gadiformes, salmonids, scombroids, percoids, sturgeon, eels, mollusks
Single-stranded conformational polymorphism	SSCP	Yes	Single	Medium-high	Medium	Medium	Medium-high	Low-medium	Salmonids, scombroids, sturgeon, eels
Random amplified polymorphic DNA	RAPD	No	Multiple	Low-medium	Low-medium	Medium	Medium-high	Low-medium	Percoids, goosefish, mollusks
Amplified fragment length polymorphism	AFLP	No	Multiple	Low-medium	Medium-high	Medium to high	Medium-high	Low-medium	Salmonids, scombroids

2000b); mt 16S rRNA to identify various species of clams and hairtails (Fernandez and others 2002; Chakraborty and others 2005); *ATCO* to differentiate scombroid species (Takeyama and others 2001; Chow and others 2003); and mt 12S rRNA to differentiate sole from Greenland halibut and to identify various flatfish species (Cespedes and others 2000; Comesana and others 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 (Mackie and others 1999; Lockley and Bardsley 2000; Akasaki and others 2006). Therefore, 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 and others 2001). Due to these limitations, it has been recommended that species identification with PCR-RFLP be carried out with caution if there is no substantial information available concerning sequence polymorphisms within and between species groups (Mackie and others 1999; Sotelo and others 2001). One approach for minimizing the identification errors caused by the above-mentioned 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 and others 2005a, 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 and others 2005a, 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 and others 2005a) and identification of a number of whitefish species (Dooley and others 2005b). This technology has also been utilized in the authentication of genetically modified soy (McDowell and others 2001), olive oil (Dooley and others 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 and others 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 previously for PCR-RFLP, including the need for predetermined RFLP profiles for species determination.

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 regard to intraspecies variation (Rehbein and others 1997; Mackie and others 1999; Akasaki and others 2006). 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 to correctly identify an unknown sample (Mackie and others 1999). SSCP has been reported to be capable of both analyzing small DNA fragments (approximately 100 bp) and detecting species in mixed samples (Mackie and others 1999; Rehbein and others 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 and others 1997, 1999a, 1999b, 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 and others 1997).

Random amplified polymorphic DNA (RAPD). Unlike the above-mentioned 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 and others 1990). Due to variations in the genetic code, RAPD analysis on different species results in unique patterns of DNA fragments. To carry out RAPD, a short primer about 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 to verify the species.

RAPD has the potential to be used as an accurate, rapid tool for exposing commercial fraud (Ramella and others 2005). The method is relatively cheap, fast, and simple; it does not require prior knowledge of the genome sequence; and primers are commercially available (Lockley and Bardsley 2000; Rego and others 2002; Liu and Cordes 2004). Additionally, RAPD requires minimal DNA and allows for both intra- and interspecies differentiation (Ramella and others 2005). Compared to other available methods such as RFLP and AFLP, RAPD has been suggested to be the least expensive and 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 and others 1998b), tilapia (Ahmed and others 2004), mussels (Rego and others 2002), Asian arowana (dragonfish: *Scleropages formosus*) (Yue and others 2002), and blackfin goosefish (*Lophius gastrophysus*) (Ramella and others 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 and others 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 and others 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 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).

Amplified fragment length polymorphism (AFLP). First described by Vos and others (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 and others 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 2nd, 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 (Bossier 1999; Bensch and Akesson 2005). 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 and others 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 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 and others 1998a), oysters (Yu and Guo 2003; Li and Guo 2004), trout (Young and others

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. To overcome these setbacks, the researchers 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).

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 and others 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 and others 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 and others 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 and others 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 and others 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 >11000 ESTs from channel catfish (He and others 2003). The researchers 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 and others 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.

Commercial Applications

Some of the DNA-based methods discussed previously 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℠ 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) 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 Intl., LLC (http://www.theriondna.com/). With analyses such as mtDNA sequencing and amplification of species-specific microsatellite loci, Therion Intl. 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 2 trout species (*Oncorhynchus mykiss* and *Salmo trutta*). Biotools also offers a series of BIOFISH SEQ kits that 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-mentioned 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®, is discussed further in the section dealing with current challenges and future trends.

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 Natl. 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 3). Examples of these databases are 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

Table 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 (FISHBOL)	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.cfsan.fda.gov/~frf/rfe0.html
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

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 and others 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 and others 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 and others 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 at http://www.cfsan.fda.gov/~frf/rfe0.html, and it currently includes detailed information on 94 commercially important fish species in the United States (Tenge and others 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 and others (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 and others 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 comprises 53 members from several European institutions (Sevilla and others 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 and others 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, and 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. 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. 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 and others 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 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.

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 and others 1999; Woolfe and Primrose 2004; Martinez and others 2005; Teletchea and others 2005). 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 and others 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 to 7 min. It also allows for DNA migration to be observed in real time and does not require UV light.

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 and others 2005). On a smaller scale, a DNA chip was developed that allowed for differentiation of 6 animal species commonly consumed in Europe (Peter and others 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 1st 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 United States and may actually discontinue the product line, because 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 and others 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.

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 having either a small intron or

a mutated restriction site (Gilliland and others 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 and others 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 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 and others 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 (Sotelo and others 2003; Hird and others 2005) and canned meat products (Laube and others 2007a). Hird and others (2005) reported the 1st 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 reported to be useful only 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 and others 2002), 2 eel species (Itoi and others 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 (≤212 bp) of DNA from 7 different animal species commonly found in processed foods (Laube and others 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 and others 2005).

Electrochemical DNA sensors

An innovative method for the detection of PCR products was recently described by Lai and others (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.

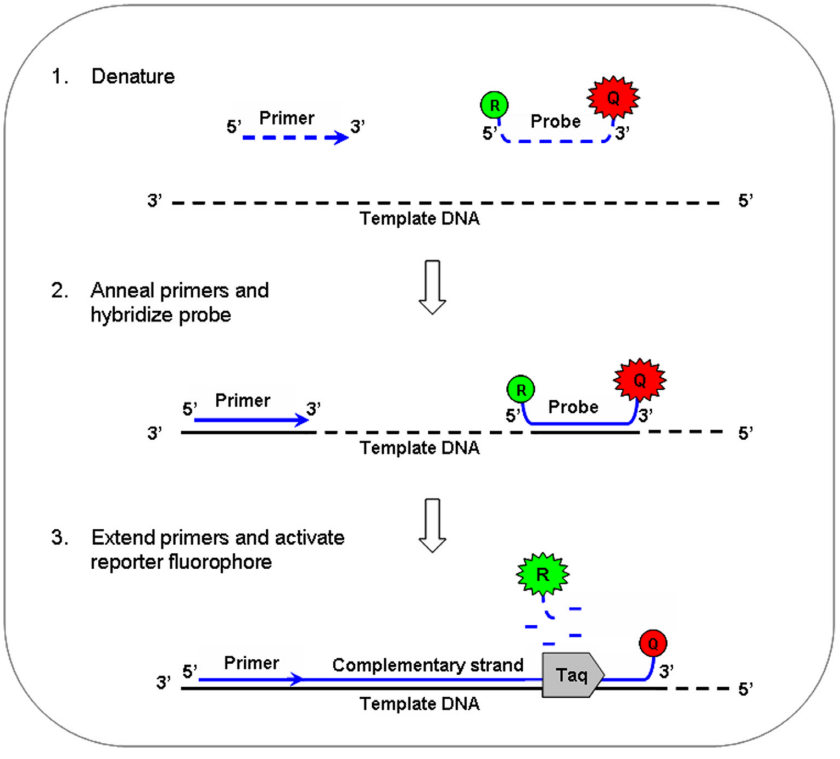

Figure 4—Real-time PCR using TaqMan probes. The probe contains a reporter (R) fluorophore and a quencher (Q) fluorophore. When the probe is intact, the quencher fluorophore prevents the reporter fluorophore from emitting fluorescence. Probes are designed to hybridize with a complementary sequence on 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.

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.

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