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Review

PCR-based methods for fish and fishery products authentication

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This work intends to provide an updated and extensive overview on the PCR-based methods for fish and fishery products authentication. Various techniques such as PCR-sequencing, Multiplex-PCR, PCR-RFLP, PCR-SSCP, RAPD, Real-Time PCR and PCR lab-on-a-chip are described and discussed. Moreover, commercial PCR kits for fish species identification are provided in this review. These methods could allow consumers protection against fraudulent practices in the fishery industry and enforce national and trans-national laws and regulations.

Introduction

Over the last years, there has been a tremendous growth in fish consumption due to changes in consumer attitudes towards health and nutrition. Fish species can be identified by knowledgeable fishermen, wholesalers restaurateurs and consumers while the specimen remains in its whole state. However, once fillets are prepared from the fish, speciation becomes more difficult. The matter becomes further complicated as fish is processed where mincing, battering, crumbing and frying operations take place. Consequently, there is a risk of wilful or unintentional substitution of low valued fish species for high valued fish in fish and fishery products (Mackie, 1996).

European Council (EC, 1999) Regulation Nr 104/2000 of Dec.17. 1999, on the common organization of the markets in fishery and aquaculture products, specifies that these products may not be offered for retail use unless they are labeled with the commercial name of the species, the production method and the capture zone. This regulation has enforced all the member states to make and publish a list including the common and scientific names of all species commercially available. Therefore, the development of analytical methods for species identification is necessary to detect and avoid wilful, as well as unintentional substitution of different fish and shellfish species and to enforce labeling regulations (Mackie *et al*., 1999).

Numerous analytical techniques which rely on protein analysis have been developed for fish species identification: electrophoretic techniques such as isoelectric focusing or SDS-PAGE (Ataman, Celik, & Rehbein, 2006; Mackie *et al*., 2000); chromatographic techniques (Horstkotte & Rehbein, 2003; Knuutinen & Harjula, 1998) and immunological techniques such as immunodiffusion and ELISA (Fernández *et al*., 2002a; Ochiai, Ochiai, Hashimoto, & Watabe, 2001). Although most of these methods are of considerable value in certain instances, they are not suitable for routine sample analysis because proteins lose their biological activity after animal death, and their presence and characteristics depend on the cell types. Furthermore, most of them are heat labile. Thus, for fish species identification in heat-processed matrices, a DNA method rather than protein analysis is preferable (Lockley & Bardsley, 2000a).

Advances in DNA technology have led to rapid development of genetic methods for fish species identification. DNA offers advantages over proteins, including stability at high temperature, presence in all tissue types, and greater variation with genetic code (Mackie, 1996). DNA can be analysed using techniques such as sequencing, DNA–DNA hybridization and the polymerase chain reaction (PCR), which are based on the detection of species-specific DNA sequences in food products. In particular, PCR-based techniques have a high potential because of their rapidity, increased sensitivity and specificity (Lockley & Bardsley, 2000a).

The majority of work related to exploiting DNA analysis has focused on using PCR to amplify the specific fragments of DNA of interest. On the use of this methodology, short synthetic oligonucleotide primers anneal to complementary strands of the DNA duplex and direct the synthesis of millions of copies of the fragment of DNA they have been chosen to flank (Saiki *et al*., 1988). The amplification products or amplicons can then be analysed further by a variety of

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methods such as PCR-sequencing, multiplex-PCR, PCR-restriction fragment length polymorphism (PCR-RFLP), PCR-single strand conformation polymorphism (PCR-SSCP), Random Amplified Polymorphic DNA (RAPD), Real-Time PCR and PCR lab-on-a-chip.

Tests based on PCR can be undertaken with much lower amounts of starting material than other DNA-based methods such as DNA hybridization, due to the amplification that the method provides. Highly purified DNA is not generally required, although samples should be free from contaminants that may inhibit the reaction (Rossen, Norskov, Holmstom, & Rasmussen, 1992). The sensitivity of the method does mean that it is susceptible to contamination, although this can be minimized. Appropriate laboratory practices, such as having different areas for pre- and post-PCR sample manipulation, are routinely adopted in laboratories where large amounts of work are undertaken (Lockley & Bardsley, 2000a).

Of the different DNA markers used for fish species identification, mitochondrial DNA (mtDNA) possesses several advantages over nuclear DNA for studies of speciation in fish products. It is relatively more abundant in total nucleic acid preparations than nuclear DNA, with the copy number of the mitochondrial genome exceeding that of the nuclear genome several fold (Alberts *et al*., 1994). Mitochondrial DNA tends to be maternally inherited so that individuals normally possess only one allele and thus sequence ambiguities from heterozygous genotypes are generally avoided. The relatively high mutation rate compared to nuclear genes has tended to result in the accumulation of enough point mutations to allow the discrimination of even closely related species. It should however be noted that mitochondrial DNA also exhibits a degree of intraspecific variability and so care has to be taken when studying differences between organisms based on single base polymorphisms (Chow & Inogue, 1993). However, the use of nuclear markers may be useful for fish species discrimination because of the existence of introns of different sizes which allow sometimes the amplification of species-specific DNA fragments (Ferguson *et al*., 1995).

Moreover, in the last years, advances in PCR technology have led to rapid development of different commercial kits for fish species differentiation. The simplicity of these tests and the short time required for the analysis make them suitable for fish screening tests of a large number of samples. Furthermore, they can be used by regulatory agencies to detect fish species adulteration and to enforce national and trans-national laws and regulations.

On the basis of this information, this work reports the PCR-based methods applicability on fish authenticity in the last few years. The use of PCR-sequencing, multiplex-PCR, PCR-RFLP, PCR-SSCP, RAPD, Real-Time PCR and PCR lab-on-a-chip for fish species identification is described and discussed. Moreover, PCR commercial kits for fish species discrimination are also discussed in this work.

PCR-sequencing

The most direct means of obtaining information from PCR products is by sequencing. Information thus obtained has been used to identify various fish (Jérôme, Lemaire, Bautista, Fleurence, & Etienne, 2003; Lin, Poh, & Tzeng, 2001; Murgia, Tola, Archer, Vallerga, & Hirano, 2002). Such works have tended to concentrate on the amplification of mitochondrial DNA sequences, generally the cytochrome *b*.

In the last recent years, sequencing and phylogenetic mtDNA analysis have been used to check for mislabelling of red snapper (Marko *et al*., 2004). Moreover, Pepe *et al*. (2005) identified fish of families *Gadidae* and *Merlucciidae* in 18 differently processed fish products by sequencing of PCR products from a conserved region of the cytochrome *b* gene.

Also, fragments of nuclear genes such as alpha-actine, 5S ribosomal DNA, p-53, growth hormone-encoding, among others, have been sequenced for the discrimination of various fish species (Canapa, Barucca, MarinellI, & Olmo, 2000; Pelliccia, Barzotti, Volpi, Bucciarelli, & Rocchi, 1998; Venkatesh & Brenner, 1997; Watabe, Hirayama, Imai, Kikuchi, & Yamashita, 1995).

Sequencing is time consuming and technically demanding but will produce a relatively large amount of information, requiring good data handling capacity. These data can be used in other PCR-based methods such as PCR-RFLP to fish species identification (Quinteiro *et al*., 1998; Ram, Ram, & Baidoun, 1996; Sebastio, Zanelli, & Neri-Tauro, 2001).

Species-specific PCR primers

Detailed sequence information has become available for many species and consequently phylogenetically informative single base polymorphisms may be identified that enable species-specific primers to be designed. Under suitably stringent reaction conditions, such primers generate a product only in the presence of DNA from a given species. Complete sequence information permits the size of the product to be predicted, so that identification is confirmed if an appropriately sized amplicon is seen on a gel. By pairing species-specific primers with a non-selective counterpart, it is possible to test for the presence of more than one species simultaneously. The non-selective primer tends to be based on a sequence that is common to all species under study in a given system; its precise location in the gene can be used to dictate the size of the amplification products that will be generated. Using such a regime, multiplex reactions have described for fish species identification (Asensio, González, Fernández, Céspedes,*et al*., 2001; Céspedes *et al*., 1999a; DeSalle & Birstein, 1996; Hsieh, Chai, Cheng, Hsieh, & Hwang, 2004; Lockley & Bardsley, 2000b; Taylor, Fox, Rico, & Rico, 2002).

This method, called multiplex-PCR, has the potential to produce considerable savings of time and effort within the laboratory without compromising test utility. In addition, qualitative admixture detection may also be possible.

Obviously, prior sequence knowledge is required in order to design primers and appropriate controls should be included to preclude the possibility of false positive or negative results being obtained (Edwards & Gibbs, 1994).

PCR-RFLP

When PCR is undertaken without species-specific primers, some form of secondary discriminatory technique is required such as RFLP. RFLP analysis of PCR products has been extensively used for species discrimination and a single primer pair can produce a fragment that can be used for the identification of multiple species with judicious choice of restriction enzymes.

In search for fast and simple genetic techniques, PCR-RFLP has gained acceptance among fish species identification methods, since it is much easier to perform and less costly than conventional DNA sequencing and nucleotide sequence analysis (Meyer, Höfelein, Lüthy, & Candrian, 1995). This method has been used for the discrimination of mackerel species (Arahishi, 2005), commercial canned tuna species (Lin & Hwang, 2007; Pardo & Pérez-Villareal, 2004), eel species (Rehbein *et al*., 2002), flat fish species (Céspedes *et al*., 1998; Comesaña, Abella, & Sanjuan, 2003), cephalopod mollusks (Colombo *et al*., 2002), or different processed fish products (Akasaki, Yanagimoto, Yamakami, Tomonaga, & Sato, 2006; Chakraborty, Aranishi, & Iwatsuki, 2007; Hsieh, Chai, & Hwang, 2007).

The method has the drawback that incomplete digestion may occasionally occur and intraspecific variation could delete or create additional restriction sites (Lockley & Bardsley, 2000a). Furthermore, as heat processing may reduce the fragment size of DNA, analyses of canned fish have tended to rely on the amplification of relatively short regions of DNA (Meyer *et al*., 1995). It is theoretically possible that, although inter-specific variation may be contained within these amplicons, it may not actually be associated with restriction enzyme recognition sequences, although this does not yet appear to have presented as a problem in practice.

On the other hand, fishery products such as smoked tuna or canned fish for example, are supplemented with spices and sauces. The presence of these additives, used in the food industry, as well as in the canning process, where DNA is usually severely degraded, itself might exert an inhibitory effect on the PCR. Ram *et al*. (1996) were not able to identify some of their canned tuna samples due to the inhibitory effect of additives. To avoid these inhibitory effects, amplification of DNA fragments using nested primers in two consecutive PCRs (Nested Primer PCR) has been used for fish species identification because it allows the amplification of fragments from low DNA concentrations with high sensitivity and efficiency (Pardo & Pérez-Villareal, 2004; Zhang, Huang, Cai, & Huang, 2006). This process adds another level of specificity, meaning that all products non-specifically amplified in the first round will not be amplified in the second either. Thus, this methodology increases the PCR sensitivity, since two pairs of primers are required to amplify the target sequence for a final product to be generated.

PCR-SSCP

This technique is based in the relationship between the electrophoretic mobility of a single-stranded DNA and its folded conformation, which in turn reflects the nucleotide sequence (Orita, Suzuki, Sekiya, & Hayashi, 1989). The amplified product is denatured to a single-stranded form and electrophoresed on a non-denaturing polyacrilamide gel. Any difference in the sequences causes a shift in the mobility of the analysed molecule, which is visualized at the end of the process (Hayashi, 1991).

PCR-SSCP has proved successful for the identification of fishery products such as salmon, trout, eel and sturgeon (Rehbein, Kress, & Schmidt, 1997), canned tuna species (Rehbein *et al*., 1999; Weder, Rehbein, & Kaiser, 2004), flat fish species (Céspedes *et al*., 1999b), grouper, Nile perch and wreck fish fillets (Asensio, González, Fernández, Rodríguez, *et al*., 2001), clam species (Fernández *et al*., 2002b) and cod fish (Comi, Iacumin, Rantsiou, Cantoni, & Cocolin, 2005), among others.

However, several factors should be considered before a specific DNA fragment is selected for amplification and SSCP analysis. Short amplicons (under 300 bp) have two advantages over longer ones: (i) differences in the nucleotide sequence mostly result in significant changes of conformation and (ii) the intraspecific variability of the DNA patterns is expected to be lower (Rehbein *et al*., 1997), although, fragment size and sequence context (the sequence of the adjacent DNA) can have unpredictable effects on mobility shifts associated with particular base changes (Fujita & Silver, 1994; Hayashi, 1991).

The advantages of PCR-SSCP analysis over other PCR-based techniques include (1) even single base changes in a sequence are likely to result in different conformations, which can be separated by native gel electrophoresis (Oohara, 1997). Highly close-related species may therefore be accurately discriminated by the SSCP technique; (2) analysis of degraded DNA is possible, since short fragments are well suited for SSCP analysis (Rehbein *et al*., 1999); (3) in contrast to RAPD or RFLP only a few bands need to be examined with this technique; and (4) although the technique is very sensitive in detection of base changes, intraspecific variation of patterns is generally less problematic than that obtained by other methods such as RFLP or RAPD (Bardakci & Skibinski, 1994).

In spite of the mentioned advantages, it is important to consider that SSCP analysis is affected by a number of conditions tested, such as temperature, glycerol concentration, gel concentration, acrylamide/bis (-acrylamide) ratio, type of denaturing solution, buffer concentration, and addition of different compounds to the gel matrix (Fujita & Silver, 1994; Hayashi, 1991). In addition, the efficiency of SSCP is very sequence dependent and may therefore vary significantly from one fragment to another. Changes in the

mobility of shorter fragments at approximately the same base composition indicate that the efficiency of SSCP is also dependent on fragment size (Glavac & Dean, 1993). For these reasons, the necessity to perform the method under carefully controlled conditions to obtain reproducible DNA patterns is considered as the most important limitation of the SSCP technique (Hayashi, 1991).

RAPD

The RAPD technique involves PCR amplification with a single primer to generate a collection of DNA fragments or fingerprint, which is expected to be consistent for the same primer, DNA and conditions used. The choice of the primer is random and the information generated can range from differentiation at an individual or species level. Moreover, the method is relatively simple to set up, as no prior knowledge of the genetic makeup of the fish is required (Williams, Kubelik, Livak, Rafalski, & Tingey, 1990).

This technique has been used for the discrimination of populations of Hilsa shad (Dahle, Rahman, & Eriksen, 1997), species of Anguilla (Takagi & Taniguchi, 1995), tilapia fish species and subspecies (Bardakci & Skibinski, 1994), species of the genus *Barbus* (Callejas & Ochando, 2001), grouper, Nile perch and wreck fish (Asensio *et al*., 2002), salmonids (Jin *et al*., 2006; Yamazaki, Shimada, & Tago, 2005), among others (Dinesh, Lim, Chua, Chan, & Phang, 1993; Partis & Wells, 1996).

The main advantages of RAPD are (i) it does not require previous knowledge of DNA sequences of the species under study and (ii) it targets many sequences in the DNA of the sample, producing DNA patterns that allow comparison of many loci simultaneously (Caetano-Anollés, Bassam, & Gresshoff, 1991). The simplicity, relatively low cost of the technique and requirement of only nanograms of template provide additional advantages in the use of RAPD for species identification. In contrast, other DNA-based fingerprinting methods, such as restriction fragment length polymorphism (RFLP) and single strand conformation polymorphism (SSCP): (i) require larger quantities of relatively pure DNA, (ii) target one or a few loci and (iii) some previous knowledge of specific DNA sequences is needed for designing specific primers. Additionally, most DNA-based methods are more laborious and time consuming than RAPD making them less suitable for large population based studies (Partis & Wells, 1996).

However, RAPD analysis presents some disadvantages: (i) it may not be practical to identify the species of origin in products containing mixtures of species (Martínez & Malmheden Yman, 1998) and (ii) it does not seem to be adequate for analysis of severely degraded material, as in autocleaved samples (Martínez & Malmheden Yman, 1998).

Nuclear DNA markers

Several nuclear DNA (nDNA) markers have been described for fish including the preprogonadotropin-releasing hormone (GnRH) (Gross, Nilsson, & Schmitz, 1996), the growth hormone gene (Johansen, Johnsen, & Valla, 1989), the alpha-actin gene (Fernández *et al*., 2000; Watabe *et al*., 1995) and the 5S ribosomal DNA (rDNA) gene (Céspedes *et al*., 1999a). Among these genes, the 5S rDNA gene has been widely used for species identification due to its remarkable structure, allowing the direct species identification by PCR amplification without the need of later sequencing or digestion with restriction enzymes (Sastri *et al*., 1992).

In higher eukaryotes, the 5S rDNA gene comprises a 120 bp highly conserved coding sequence (5S rRNA) and a variable nontranscribed spacer (NTS), structure that make it worthy for species identification (Asensio, González, Fernández, Céspedes, *et al*., 2001; Céspedes *et al*., 1999a). These authors used the primers 5S1 and 5S2, based on the conserved region of this gene in the rainbow trout, that had already been used to amplify a whole unit of the 5S rDNA gene (coding sequence + NTS) from salmon and trout templates (Pendás, Moran, Martínez, & García-Vázquez, 1995). The length of the NTS is usually species-specific and the basic unit (5S rRNA + NTS) is tandemly repeated a variable number of times on the chromosome, depending on the species. So that, by using a simple PCR with these oligonucleotides these authors generated electrophoretic patterns consisting of amplicons of different sizes that permitted the unequivocal discrimination of the fish species analysed.

Other tandemly repeated blocks of DNA of identical or similar sequence which are dispersed throughout the genome of most, if not all, eukaryotic organisms (O'Reilly & Wright, 1995), have also used for fish species identification (Ferguson *et al*., 1995). Three different classes of this repetitive and highly polymorphic DNA have been distinguished traditionally, based on size of the repeat unit: (i) satellite DNA, which refers to long repeat units (hundreds or thousands of base pairs), (ii) minisatellite DNA or variable number of tandem repeat (VNTR) DNA (Jeffreys, Wilson, & Thein, 1985; Nakamura *et al*., 1987), which refers to genetic loci with repeats of smaller length (10–64 bp), and (iii) microsatellite DNA or simple sequence or short tandem repeat (STR) DNA (Litt & Luty, 1989), in which the repeat unit is only 2–4 bp long. The term VNTR is frequently used for both mini- and microsatellite DNA. Length variation in tandemly repeated DNA, particularly in the case of mini- and microsatellites is usually due to the changes (increases or decreases) in the copy number of repeat unit (Jeffreys, Micola, Wilson, & Wong, 1988).

These markers have been mainly used to investigate inheritance patterns of genetic markers, heterozygosity and to identify families and individuals within families in fish. For fish species identification, for instance, De la Herrán *et al*. (2004) used satellite DNAs that allowed the genetic identification of Western Mediterranean sturgeons. In other recent works, Renshaw, Saillant, Broughton, and Gold (2006) achieved the identification of 'wild' versus

hatchery-produced (cultured) red drum using hypervariable nuclear-encoded microsatellites.

The use of this class of markers is being highly expanded, mainly due to their ease of scoring through PCR, their high polymorphism and the accuracy of allele characterization. Their basic drawback remains the high cost and labor intensiveness of the first phase of the technique, i.e. the development of primers (O'Reilly & Wright, 1995). Among these markers, microsatellite DNAs have revolutionized the use of molecular genetic markers in the applications mentioned before and are being the markers destined to dominate this type of studies in the coming years.

Real-time PCR

A considerable number of fish products may contain muscle or other tissue from one or more fish species. Examples are cooked or sterilized fish commodities like cakes, pies, pastries, soups, pâtés, and also industrial products such as fish meal. In this field also there are forms of adulteration for economic gain such as the addition of undeclared cheaper fish in fish products that are labeled using the names of higher price and quality fish species. Consequently, to ensure compliance with labeling regulations, there is a need for suitable methods for quantitative monitoring of fish species in the market of heat-processed fishery products. As proteins may be denatured on processing and heating, also DNA methods are preferable. In this context, quantitative PCR tests such as real-time PCR have been widely used for food authentication and quantification (Brodmann & Moor, 2003; López-Calleja *et al*., 2007).

The quantitative real-time PCR (qPCR) technique is commonly based on the use of a TaqMan fluorogenic probe. The probe, labeled with a reporter and a quencher dye, binds to a target DNA between the flanking primers. During PCR amplification, the 5′ to 3′ exonuclease activity of the *Taq* DNA polymerase cleaves the probe hybridized to the template, releasing the 5′ reporter from the quenching effects of the 3′ quencher (Holland, Abramson, Watson, & Gelfand, 1991). Cleavage of the probe results in an increase of fluorescence, proportional to the amount of template DNA amplified. The use of fluorescence for detection purposes eliminates the need for post-PCR processing steps, such as gel electrophoresis and ethidium bromide staining of target DNA, easing automation of the technique and large-scale sample processing.

Compared with conventional qualitative PCR, this technique has several advantages. Fluorescence can be measured throughout the PCR, providing real-time analysis of the reaction kinetics and allowing quantification of specific DNA targets. qPCR also offers a lower potential for contamination of the PCR mixture with target DNA because the reaction tubes remain closed throughout the assay. Moreover, the threshold cycle is observed when PCR amplification is still in the exponential phase and none of the reaction components is limited (Heid, Stevens, Livak, & Williams, 1996).

The specificity and sensitivity of this technique, combined with its high speed, robustness, reliability, and the possibility of automation (Heid *et al*., 1996), contribute to the adequacy of the method for quantifying fish species in fishery products. Thus, for instance, Sotelo, Chapela, Rey, and Pérez-Martín (2003) used the TaqMan assay for the identification and quantification of cod. Trotta *et al*. (2005) used real-time PCR for the identification of fish fillets from grouper and common substitute species. Hird *et al*. (2005) used it for the detection and quantification of haddock. Presence of this fish in concentrations of up to 7% in raw or slightly heat treated products could be detected. In another work, López and Pardo (2005) applied TaqMan real-time PCR technology for the identification and quantification of albacore and yellowfin tuna.

The accuracy of this method could be affected by several factors, such as the DNA yield of the samples, which can be variable depending on the strength of the technique used to process the fish product, and by the fact that the sample material could be thermally processed in different ways. Moreover, due to its cost, it has only a remarkable interest in products with an important economic value. However, the enormous utility and possible applications of the real-time PCR will make it affordable for most laboratories in the near future.

PCR lab-on-a-chip

Over the past decade the application of microfabricated chip technology to a diversity of analytical problems has become an area of huge interest. In particular, the miniaturization of chemical and biological reaction chambers has been shown to afford gains in terms of control, speed, efficiency and functionality (Krishnan, Namasivayam, Lin, Pal, & Burns, 2001). Importantly, due to micron sized feature dimensions and closed fluidic formats, sample volumes down to the picolitre scale can be manipulated and processed with a high degree of control. Accordingly, interest in micromachining high efficiency PCR devices has been a highly visible sub-discipline within lab-on-a-chip science for the past 10 years (De Mello, 2001).

At a fundamental level the attraction of reducing sample volumes in PCR lies in improvements with respect to the rates of thermal and mass transfer. By reducing the reactor volume, the sample may be heated and cooled extremely quickly, thus negating the large time constants associated with temperature variation on the macroscale. This directly leads to improvements in both the cycle speed and the efficiency of annealing and extension (De Mello, 2001).

This technology that uses microfluidic devices, also called PCR lab-on-a-chips, has been recently used for fish species authentication. Thus, Dooley, Sage, Brown, and Garrett (2005) used this technology to discriminate admixtures salmon/trout. These authors achieved the detection of 5% salmon DNA in trout DNA. This technology permitted an improvement on PCR-RFLP fragment resolution and

detection in comparison to conventional gel-based methods. Also, these authors utilized the same methodology in an interlaboratory study to detect white fish species in food products (Dooley, Sage, Clarke, Brown, & Garrett, 2005). For those purposes these authors used the Agilent 2100 Bioanalyser. This system incorporates conventional capillary electrophoresis (CE) technology (analyte separation and detection) into an easy-to-use chip based (LabChip®) format. The LabChips used by the system are small (3 cm^2), disposable single-use units contained etched capillaries attached directly to sample loading wells. DNA fragments were separated by capillary electrophoresis (CE) and detected using laser-induced fluorescence by the 2100 Bioanalyser. This enabled accurate sizing and quantification of individual DNA fragments, which gives the system a significant advantage over conventional gel-based approaches in terms of ease of use, speed and safety. This makes this technology ideally suited to analysis of multiple small DNA fragments. In spite of this technology is not cheap, the cost of the instrumentation and disposable chips are relatively low compared to that for real-time PCR analysis (Dooley & Garrett, 2001).

Commercial PCR kits for fish species differentiation

In the last years, advances in PCR-based methods have led to rapid development of different commercial kits for fish species identification. PCR kits contain all the necessary reagents, controls and accessories for rapid testing. Thus, for example Tepnel Biosystems company provides a DNA kit for fish speciation products. Eight species (cod, hake, coley, haddock, pollock, whiting, trout and salmon) can be detected qualitatively using a combination of magnetic particles to selectively purify DNA from sample lysates, multiplex-PCR reactions and agarose gel detection. Other company, Biotools, has developed different biofish kits: (i) biofish cod kit for the identification of cod and gadiforms species and (ii) biofish salmon kit for the differentiation of Atlantic salmon, sea trout and rainbow trout. Both kits are used in fresh and processed samples by RFLP analysis, showing unique restriction patterns corresponding to a given species. Moreover this company has developed other kits based on PCR-sequencing such as flat fish, hake, tuna and sardine kits.

Also, Bionostra company has developed other kit for fish species identification: the fishID kit. This kit is specially designed to perform a fast and specific identification of commercial fish based on mitochondrial DNA analysis. It is based on amplification of a specific DNA sequence (from the cytochrome *b* gene or control region), by use of the PCR. This kit allows the identification of a great number of fish species (more than 200 species) such as haddock, hake, lemon sole, sturgeon and albacore, among many others.

On the other hand, Biomerieux company has developed the first high density DNA Chip for the detection of animal species in food and feed. This GeneChip®, that is based on DNA microarray technology from partner Affymetrix Inc., comprises 80,000 oligonucleotide probes synthesized onto a glass surface (1 cm^2) by the process of photolithography. These probes are complementary to specific vertebrate cytochrome *b* gene sequences. The unique combination of probes enables the identification of the animal species composition of the sample. In this chip fish species such as Arctic char, Atlantic bonito, Atlantic cod, Atlantic mackerel, Atlantic salmon, brook trout, European eel, European hake, greenland cod, Japanese eel, Mozambican eel, rainbow trout, sea trout, skipjack tuna and spotted tunny can be discriminated.

All these rapid diagnostic kits could be very useful for field screening purposes in inspection programs.

Conclusions

Of the wide range of analytical methods available, PCR-based methods described in this review such as PCR-sequencing, multiplex-PCR, PCR-RFLP, PCR-SSCP, RAPD, Real-Time PCR, PCR lab-on-a-chip and including the PCR commercial kits are well suited for the determination of fish and fishery products authenticity because they are very rapid, sensitive and specific. These tests could allow consumers protection against fraudulent practices in the fishery industry and enforce national and trans-national laws and regulations.

The outlook for fish authentication indicates that research into relatively novel techniques such as PCR lab-on-a-chip and Real-Time PCR offer the greatest potential for the development of new fish discrimination applications and protocols. Furthermore, the use of nuclear markers such as microsatellite DNAs will allow to reach new applications in this food field.

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