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SHORT COMMUNICATION

AUTHENTICATION OF FISH SPECIES USING A SIMPLE PCR-RFLP METHOD

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Abstract

A polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was developed as a tool to prevent commercial frauds in fish products. The PCR was used to amplify the cytochrome b gene, part of the mitochondrial genome. The PCR products were digested with different restriction endonucleases (AluI, HaeIII, HinfI, Hsp92, TaqI) to identify five fish species - *Mugil cephalus*, *Pomatomus saltator*, *Belone belone*, *Merlangius merlangus,* and *Oncorhynchus mykiss*. None of the tested enzymes, alone, was able to distinguish between the five fish species, but by combining the results of two digestions, all five species could be differentiated. Thus, this method can be used to expose fraudulent substitutions with less valuable fish.

Introduction

Authentication of fish species is necessary to be able to assure consumers of accurate labeling and maintain confidence in the quality and safety of food (Ram et al., 1996; Rehbein et al., 1997; Klossa-Kilia et al., 2002). But it is difficult to detect substitution of cheaper fish for more expensive species when the usual identifying characteristics are removed by processing and only a portion of the flesh is available for inspection (Céspedes et al., 2000).

Recently, a number of methods have been developed for identifying fish species, including electrophoresis, isoelectric focusing, liquid chromatography, and immunoassays (Sotelo et al., 1993; Gallardo et al., 1995). With these

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techniques, specific protein patterns have been obtained for many species and a number of products. However, closely related fish species or canned fish cannot be differentiated by these methods because the differences between proteins are too small or the proteins have been destroyed by heating (Rehbein, 1990).

DNA-based techniques can be used to identify fish species. The advantages of such techniques are not only their ability to identify closely related or heavily processed fish species but also their ability to easily identify multiple species within a single product (Mackie et al., 1999). Among DNA-based techniques, restriction fragment length polymorphism (RFLP) combines simplicity, speed, resolving power, and low cost and is an interesting approach to fish species identification (Wolf et al., 1999).

The main aim of this study was to determine how to differentiate among frozen fillets of *Mugil cephalus*, *Pomatomus saltator*, *Belone belone*, *Merlangius merlangus*, and *Oncorhynchus mykiss* by amplifying selected regions of the mitochondrial cytochrome b gene, digesting them with restriction endonucleases, and analyzing their RFLP.

Materials and Methods

Collection of samples. Five individuals of each species (*Mugil cephalus*, *Pomatomus saltator*, *Belone belone*, *Merlangius merlangus*, *Oncorhynchus mykiss*) were acquired from local commercial fishers. All specimens were morphologically identified and stored at -20°C until the DNA was extracted.

DNA extraction. A 50-mg frozen muscle sample was minced with a sterile surgical blade and transferred to a 5-ml sterile tube. An extraction buffer of 2 ml of 400mM Tris-HCL (pH 8.0), 150 mM NaCl, 60 mM EDTA, 1% (w/v) sodium dodecyl sulfate (SDS), and 5 µl of 20 mg/ml proteinase K was added. The mixture was incubated at 50°C for 12 h. After digestion, 2 ml phenol-chloroform-isoamyl alcohol (25:24:1) was added. The samples were vortexed for 5 min and centrifuged at 3000 x g for 5 min at 4°C. Two ml of the clear aqueous supernatant was treated with 200 µl sodium acetate and 5 ml of 100% ethanol to allow precipitation of the DNA, which was collected by centrifugation at 14,000 x g for 10 min at 4°C. The pellet was washed twice with 70% ethanol and dried under vacuum. The DNA was resuspended in 50 µl sterile distilled water and used directly in the PCR.

PCR reaction. DNA amplification was carried out in a final volume of 25 µl containing a PCR buffer of 100mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, and 0.01% gelatin, plus 2 µl $MgCl_2$, 0.25 µl of each dNTP (0.2 mM), 1 µl of each primer (20 pmol), 0.5 µl Taq-polymerase (5 IU), and 3 µl of the extracted DNA. The two primers used for amplification were designated cyt b1 (5'-cca tcc aac atc tca gca tga tga aa-3') and cyt b2 (5'-ccc ctc aga atg ata ttt gtc ctc a-3'; Barlett and Davidson, 1991). PCR amplification conditions were as follows: one preliminary denaturation step at 94°C for 3 min followed by 35 PCR cycles, standard denaturation at 94°C for 1 min, annealing at 53°C for 1 min, primer extension at 72°C for 2 min, and a final extension at 72°C for 5 min. PCR products were examined by electrophoresis through a 1% agarose gel (Agarose, Sigma) in 1x TBE (Tris-Borate-EDTA) buffer (0.89 M Tris borate, pH 8.3, containing 0.02 M EDTA), stained with ethidium bromide. A wide range (50-10,000 bp) DNA marker (Sigma) was used for size reference,

RFLP. Five restriction endonucleases were used to identify the fish species: AluI, HaeIII, HinfI, Hsp92 and TaqI. Ten µl of the PCR mixture was digested using 10 units of the restriction enzyme at 37°C for 12 h. Restriction fragments were separated on a 1.5% agarose gel for 1.5 h at 90 V. DNA fragments were observed and photographed under UV illumination. The sizes of the DNA bands were compared to DNA markers run on the same gel.

Results

Results of the RFLP analyses are shown in Fig. 1. All the enzymes used in the RFLP digested the amplification products, but none, alone, was able to differentiate among all five fish species. When the results from two endonucleases were combined, it was found

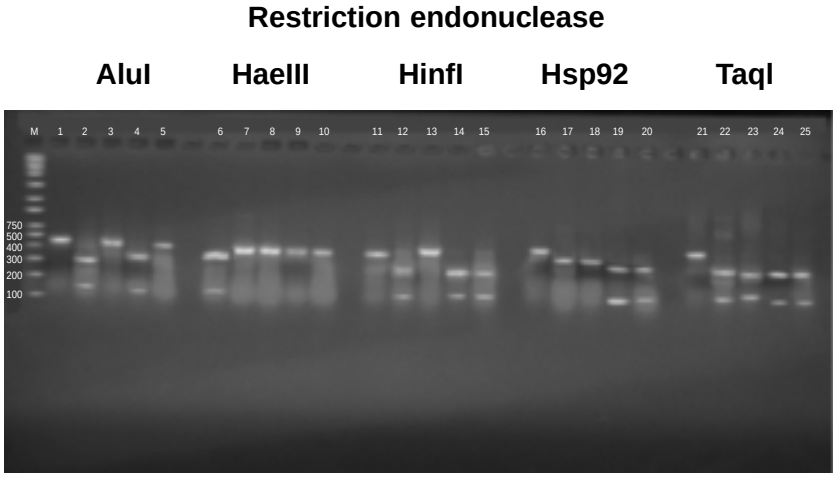

Fig. 1. Restriction patterns resulting from enzyme analysis of cytochrome b PCR products obtained from five fish species: Lanes 1, 6, 11, 16, 21 *Mugil cephalus*, lanes 2, 7, 12, 17, 22 *Belone belone*, lanes 3, 8, 13, 18, 23 *Merlangius merlangus*, lanes 4, 9, 14, 19, 24 *Oncorhynchus mykiss*, and lanes 5, 10, 15, 20, 25 *Pomatomus saltator*. M = molecular weight marker.

that the AluI and Hsp92 restriction enzymes could be used together to identify all the species.

Discussion

Some of the commercial fish species used in our study can easily be identified when sold as a whole fish by traditional taxonomic classification methods. However, another technique is needed to identify processed fish products and prevent fraud through species substitution. The protocol used in this study was based on specific amplification of PCR products from the gene encoding cytochrome b (a specific part of the mitochondrial DNA) and restriction enzyme analysis with endonucleases.

Mitochondrial DNA has been widely used as a marker in species identification. It has major advantages: (a) it is easy to isolate, (b) it has a simple genetic structure, (c) it exhibits a straight-forward mode of genetic transmission, and (d) mitochondrial genes in vertebrates have a high mutation rate (Unseld et al., 1995). PCR-RFLP analysis of the mitochondrial DNA was selected due to its simplicity, speed, resolving power, and low cost (Céspedes et al., 2000). The primers used in this study were previously used to identify tuna species (Barlett and Davidson, 1991) and to distinguish among flatfish (Céspedes et al., 1998).

In conclusion, this study demonstrates that PCR-RFLP is a sensitive technique for the identification of frozen fish fillets and can be used in food control laboratories as an accurate and rapid tool for exposing commercial frauds. Further investigation is needed to learn more about other Mediterranean species.

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