PCR-SSCP: A Simple Method for the Authentication of Grouper (*Epinephelus guaza*), Wreck Fish (*Polyprion americanus*), and Nile Perch (*Lates niloticus*) Fillets

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A method of DNA analysis has been developed to verify the authenticity of grouper (*Epinephelus guaza*), wreck fish (*Polyprion americanus*), and Nile perch (*Lates niloticus*) fillets. A short fragment (208 bp) of the mitochondrial 12S rRNA gene was amplified by the polymerase chain reaction and analyzed by single-strand conformation polymorphism to get species-specific patterns of single-stranded DNA (ssDNA). DNA strands were separated by native polyacrylamide gel electrophoresis and visualized by silver staining. Discrimination among the three fish species studied was possible, because each one expressed a specific ssDNA pattern.

Keywords: *Fish species identification; PCR-SSCP; 12S rRNA gene; Epinephelus guaza; Polyprion americanus; Lates niloticus*

INTRODUCTION

Identification of fish species in the marketplace can be uncertain whenever the usual external characteristics such as skin pigmentation, shape, size, and appearance are removed on processing and only a portion of flesh is available. For this reason, when the whole fish is transformed into fillets, opportunities for substitution or adulteration increase (*1*).

Nile perch (*Lates niloticus*) fillets are frequently labeled and marketed either as grouper (*Epinephelus guaza*) or as wreck fish (*Polyprion americanus*) because of the higher popularity and quality of the two latter species. Additionally, grouper and wreck fish are closely related species (family Serranidae) that may be misidentified in the market and are commonly sold as grouper, which is more demanded by consumers. The development of analytical methods for fish species identification is therefore necessary for preventing willful as well as unintentional substitution of different fish species (*2*).

In recent years, various protein-based techniques including immunological, electrophoretic, and chromatographic methods have been used for fish species identification. Among these, immunological techniques have offered an alternative means for the identification of fish species and proved to be suitable for routine analysis of a large number of samples (*3*−*5*). Electrophoretic techniques and methods such as liquid chromatography and high-performance liquid chromatography (HPLC) have also been reported, allowing identification of processed fish products (*6*−*9*). However, these methods are laborious and require substantial equipment, and a large data bank of various seafood species is needed for effective protein profile comparison.

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 (*1*). DNA can be analyzed 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 (*10*).

PCR-based methods commonly used for fish species identification include random amplified polymorphic DNA (RAPD) (*11*, *12*), PCR restriction fragment length polymorphism (PCR-RFLP) (*13*−*15*), and PCR single-strand conformational polymorphism (PCR-SSCP) (*16*−*18*).

RAPD, also known as arbitrary primed PCR (AP-PCR), is a very fast method that provides a great number of polymorphisms, but its major drawback is poor reproducibility of the results. PCR-RFLP has quickly gained acceptance among fish species identification techniques. However, this technique detects differences in DNA sequences only when the differences are present in the specific recognition site for the corresponding endonuclease. This limitation has been overcome by the PCR-SSCP technique, which is based on the relationship between the electrophoretic mobility of a single-stranded DNA (ssDNA) and its folded conformation, which in turn reflects the nucleotide sequence (*19*). In the PCR-SSCP technique, the amplified product is denatured to a single-stranded form and electrophoresed on a non-denaturing polyacrylamide gel. Any difference in the sequences causes a shift in the mobility of the analyzed molecule, which is visualized at the end of the process (*20*).

In our study, amplification of a short fragment of the mitochondrial 12S rRNA gene by PCR, followed by SSCP analysis of the amplicons, was used for the

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10.1021/jf001185w CCC: $20.00
Published on Web 03/06/2001

specific identification of grouper (*E. guaza*), wreck fish (*P. americanus*), and Nile perch (*L. niloticus*) fillets.

MATERIALS AND METHODS

Sample Selection and DNA Extraction. Nile perch, grouper, and wreck fish were obtained from MercaMadrid (Madrid, Spain) and other local markets. Every specimen was morphologically identified according to the keys of Bauchot and Pras (*21*). Fifteen individuals of each species were analyzed. Genomic DNA was extracted from fish muscle, according to a previously described procedure (*22*).

PCR Amplification. The primers used for PCR amplification of the 12S rRNA gene (Cruachem Ltd., Glasgow, U.K.) were oligonucleotide 12SA (forward primer), 5′-AAACTAGGATTAGATACCCTATTAT-3′ (25-mer), designed by Simon et al. (*23*), and oligonucleotide 12SB (reverse primer), 5′-GACGACGGCGGTATATAGGC-3′ (20-mer), selected for the amplification of a 208 bp region of the 12S rRNA from Nile perch, grouper, and wreck fish. Primer 12SB was designed after comparison of previously reported 12S rRNA sequences from a 436 bp amplified fragment on these three fish species (*15*).

Double-stranded amplification reactions contained the following: 10 mM Tris-HCl (pH 8.8); 1.5 mM $MgCl_2$; 50 mM KCl; 0.1% Triton X-100; 0.2 mM each dATP, dTTP, dGTP, and dCTP; 50 pmol of each primer; 500 ng of template DNA; and 2 units of DynaZyme II DNA polymerase (Finnzymes Oy, Espoo, Finland). Reaction volumes were 50 μL, and no mineral oil was used to overlay reactions. Positive and negative controls were routinely introduced in every PCR performed.

A Progene thermal cycler (Techne Ltd., Cambridge, U.K.) was used to carry out a preliminary denaturation at 93 °C for 2 min and 35 cycles of amplification with the following step-cycle profile: strand denaturation at 93 °C for 30 s; primer annealing at 50 °C for 30 s; and primer extension at 72 °C for 45 s. The last extension step was 5 min longer.

Purification of PCR Products. Electrophoresis of 80 μL of PCR product from each fish species was performed for 40 min at 100 V in a 1.5% LM-2 (Hispanlab, Madrid, Spain) agarose gel containing ethidium bromide (1 μg/mL) in Tris−acetate buffer. The DNA fragment was excised from the agarose gel with a sterile scalpel, and the gel slice was purified with the Qiaquick gel extraction kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. The DNA was eluted in 15 μL of sterile distilled water. The concentration of the purified PCR product was estimated by agarose gel electrophoresis using a standard (Mass Ruler, Bio-Rad Laboratories, Hercules, CA) as reference marker. A Geldoc 1000 System-PC (Bio-Rad) was used for that purpose.

Preparation of ssDNA. ssDNA was prepared following the PCR-SSCP PhastSystem separation technique (Pharmacia Biotech AB, Uppsala, Sweden) (*24*). Briefly, after purification of PCR products, 1 μL of diluted sample (containing 5 ng of DNA) was mixed with 3 μL of denaturing solution (98% formamide, 0.05% bromphenol blue, 0.05% xylene cyanol, and 2% glycerol), heated for 5 min at 95 °C, and placed immediately on ice for 5 min. The samples were loaded onto the polyacrylamide gel without delay.

Polyacrylamide Gel Electrophoresis (PAGE). Phastgel homogeneous 12.5 and Phastgel DNA buffer strips (Pharmacia) were used for native PAGE using the Pharmacia PhastSystem. Running conditions were as follows: (1) prerun, 400 V, 10.0 mA, 2.5 W, 15 °C, 100 Vh; (2) sample application, 400 V, 1.0 mA, 2.5 W, 15 °C, 2 Vh; (3) run, 400 V, 10.0 mA, 2.5 W, 15 °C, 100 Vh.

DNA bands were visualized by silver staining in the development unit of the PhastSystem, using the Phastgel DNA silver staining kit (Pharmacia) and following the operating instructions given by the manufacturer. Total electrophoresis and staining time was ∼2 h.

RESULTS AND DISCUSSION

The use of PCR-based techniques has opened a wide range of possibilities for fish species identification studies. In particular, the PCR-based method used in this work, PCR-SSCP of mtDNA, appears to be promising for this purpose because it has proved to be successful for the identification of fishery products such as salmon, trout, eel, and sturgeon (*25*), canned tuna species (*18*), and flatfish species (*17*), among others (*2*, *16*).

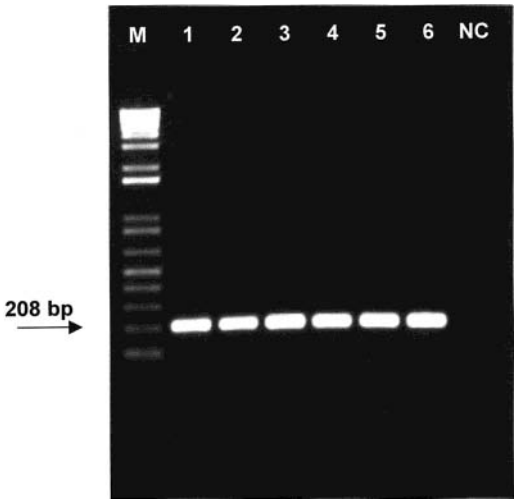

Figure 1. Electrophoretic analysis of the 208 bp fragment of the 12S rRNA gene amplified with 12SA and 12SB primers from (lanes 1 and 2) grouper (*E. guaza*), (lanes 3 and 4) wreck fish (*P. americanus*), and (lanes 5 and 6) Nile perch (*L. niloticus*) samples. M indicates 1 kb plus DNA ladder for molecular weight marker, and NC indicates negative control.

Of the different DNA markers used for fish species identification, the mitochondrial genome has become a very popular tool because of its high copy number, ease of isolation from the nuclear genome, small size, and rapid accumulation of mutations (*26*−*28*). For these reasons, the mitochondrial 12S rRNA gene was selected for the development of the PCR-SSCP technique described in this work. 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 (*29*), although fragment size and sequence context (the sequence of the adjacent DNA) can have unpredictable effects on mobility shifts associated with particular base changes (*20*, *30*). According to these considerations, a 208 bp fragment (primers included) of the mitochondrial 12S rRNA gene was selected for PCR amplification and SSCP analysis. In a previous work, a primer pair designed by Simon et al. (*23*) was used for amplification and RFLP analysis of a 436 bp fragment from Nile perch, grouper, and wreck fish 12S rRNA (*15*). Comparison of 12S rRNA sequences from these species allowed the design of the primer 12SB used in this work. This primer, together with Simon et al.'s forward oligonucleotide (12SA), successfully amplified the short 208 bp targeted fragment in the grouper, wreck fish, and Nile perch species analyzed in this study (Figure 1).

Once the target sequence is amplified, it is important to check the concentration and purity of the PCR product. Free oligonucleotides can anneal to PCR product strand and alter their mobility, even at concentrations as low as 150−600 nM. At high concentration,

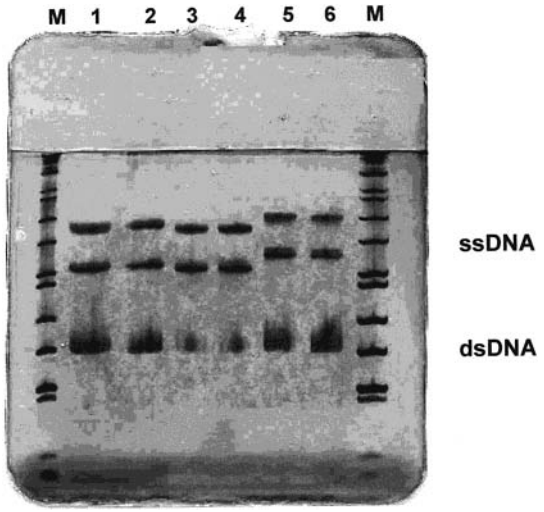

Figure 2. SSCP using primers 12SA and 12SB for PCR amplification. Samples are (lanes 1 and 2) grouper (*E. guaza*), (lanes 3 and 4) wreck fish (*P. americanus*), and (lanes 5 and 6) Nile perch (*L. niloticus*). M indicates 1 kb plus DNA ladder for molecular weight marker.

denatured PCR products may reanneal quickly, complicating the SSCP pattern. These problems can be avoided by removing primers prior to SSCP analysis or by diluting PCR products (*31*). Therefore, amplified PCR products were purified, and their DNA concentration was determined. The amount of DNA was then adjusted to 5 ng/μL by diluting the samples with sterile water.

The SSCP patterns obtained from the 208 bp segment of the 12S rRNA gene from grouper, wreck fish, and Nile perch were species-specific and allowed identification of all species studied (Figure 2). As is generally expected for haploid mitochondrial genes, two bands of ssDNA were obtained for each amplicon. Fifteen individuals of grouper, wreck fish, and Nile perch were analyzed, and results did not show intraspecific polymorphisms.

The advantages of PCR-SSCP analysis over other PCR-based techniques include the following: (1) Even single base changes in a sequence are likely to result in different conformations of 12S rRNA, which can be separated by native gel electrophoresis (*16*). Highly closely related species, such as the grouper and wreck fish samples analyzed in this study, may therefore be accurately discriminated by the SSCP technique. (2) Analysis of degraded DNA is possible because short fragments are well suited for SSCP analysis (*18*). (3) In contrast to RAPD or RFLP, only a few bands need to be examined with this technique. (4) Although the technique is very sensitive in the detection of base changes, intraspecific variation of patterns is generally less problematic than that obtained by other methods such as RFLP or RAPD (*11*). (5) The PhastSystem (Pharmacia) electrophoretic protocol provides the SSCP technique with a fast and simple approach. The total electrophoresis and staining time is ~2 h, making this procedure suitable for routine analysis in food control laboratories.

Despite 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 (*20*, *30*). Moreover, 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 (*32*). For these reasons, the necessity to perform the method under carefully controlled conditions to obtain reproducible DNA patterns is considered to be the most important limitation of the SSCP technique (*17*, *25*).

The results obtained in this study demostrate that the PCR-SSCP using a short fragment of the 12S rRNA gene is a reliable method for identifying grouper, wreck fish, and Nile perch fillets. The technique is simple, fast, and straightforward and may therefore be recommended for use in food control laboratories.

ACKNOWLEDGMENT

We are indebted to Ángel Mendizábal (S.V.O., MercaMadrid) for kindly supplying fish samples.

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Received for review September 27, 2000. Revised manuscript received January 25, 2001. Accepted January 25, 2001. This work was supported by the Comunidad Autónoma de Madrid (Project 07G/0027/2000). A.F. and L.A. are recipients of fellowships from the Ministerio de Educación y Ciencia.

JF001185W