Identification of Nile Perch (*Lates niloticus*), Grouper (*Epinephelus guaza*), and Wreck Fish (*Polyprion americanus*) by Polymerase Chain Reaction–Restriction Fragment Length Polymorphism of a 12S rRNA Gene Fragment

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**ABSTRACT**

Restriction site analysis of polymerase chain reaction (PCR) products from a conserved region of the 12S rRNA gene has been used for the specific identification of Nile perch (*Lates niloticus*), grouper (*Epinephelus guaza*), and wreck fish (*Polyprion americanus*). Amplification of DNA isolated from muscle samples was carried out using a set of primers flanking a region of 436 bp from the mitochondrial 12S rRNA gene. Digestions of the PCR products with *Rsa*I and *Sau*96I endonucleases, followed by agarose gel electrophoresis of the digested PCR products, yielded specific profiles that enabled direct identification of each species analyzed.

Knowledge of the species of fish destined for human consumption is necessary for both nutritional and economic reasons, including the adherence to fair pricing policies and the meeting of quality control requirements and legal regulations. However, the identification of fish species becomes a problem when the morphological characters are removed on processing and only a portion of flesh is available (12). Consequently, there is a risk of willful or unintentional substitution of lower valued fish species for high valued fish in seafood products.

Nile perch (*Lates niloticus*) fillets are many times labeled and marketed either as grouper (*Epinephelus guaza*) or as wreck fish (*Polyprion americanus*). Although the three fish species fillets are similar in texture, the quality attributes and price are higher for the two latter ones. Additionally, grouper and wreck fish are closely related species that may be misidentified in the marketplace and are commonly sold as grouper, which is more in demand by consumers. The development of suitable analytical methods for fish species identification are therefore of great interest to enforce labeling regulations in the authentication of fish species (9, 11).

Over the last few years, several molecular methods have been developed for fish species identification, including electrophoretic, chromatographic, and immunological techniques (1, 7, 10, 18, 20). Although most of these methods are of considerable value in certain instances, they are not convenient for routine sample analysis because they are relatively costly, time consuming, and complex to perform. Moreover, they have the disadvantage of relying on the analysis of proteins, many of which are heat labile. As an alternative to protein analysis, a number of DNA-based identification techniques have been developed. The DNA molecule offers a number of advantages when compared to proteins: all cell types of an individual contain identical genetic information independent of the origin of the sample, the DNA molecule is stable at high temperature, and the information content of DNA is greater than that of proteins (5).

In recent years, the polymerase chain reaction (PCR), followed by nucleotide sequencing or restriction fragment length polymorphism (RFLP) analysis, has permitted the identification of different fish species (6, 8, 16). According to this, we report in this paper a method for the specific identification of Nile perch (*L. niloticus*), grouper (*E. guaza*), and wreck fish (*P. americanus*) based on PCR-RFLP analysis of a conserved region in the mitochondrial 12S rRNA gene. This method can be applied to the detection of fraudulent or unintentional mislabeling of these species in the processed product market.

**MATERIALS AND METHODS**

Sample selection and DNA extraction. Nile perch, grouper, and wreck fish samples were obtained from Mercamadrid (Madrid, Spain) and other local markets. Every specimen was morphologically identified following the keys of Bauchot and Pras (3). Total cellular DNA was extracted from fish muscle according to a previously described procedure (8). Fifteen individuals of each species were analyzed.

PCR amplification of a 436-bp fragment from the 12S rRNA gene. The set of primers used for PCR amplification of the 12S rRNA gene were 12S1 and 12S2 oligonucleotides (17):
Double-stranded amplifications were carried out in a final volume of 50 μl, containing 10 mM Tris-HCl, pH 8.8, 1.5 mM MgCl₂, 50 mM KCl, 0.1% Triton X-100, 0.2 mM each of dATP, dGTP, and dCTP, 50 pmol of each primer, 750 ng of template DNA, and 2 U of DynaZyme II DNA polymerase (Finzymes Oy, Espoo, Finland). PCR was carried out in a Progene thermal cycler (Techne Ltd., Cambridge, UK) programmed to perform a denaturation step of 93°C for 2 min, followed by 35 cycles consisting of 30 s at 93°C, 30 s at 50°C, and 45 s at 72°C. The last extension step was 5 min longer.

Electrophoresis of a 10-μl portion of the amplification product was performed for 45 min at 100 V in a 1.5% LM2 agarose gel. Each DNA fragment was excised from the agarose gel using a sterile scalpel. The gel slice was purified using the Qiaquick gel extraction kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer’s instructions. The DNA was eluted in 20 μl of sterile distilled water. The concentration of the purified PCR product was estimated by agarose gel electrophoresis using a standard ladder (Gibco BRL).

Cleanup and sequencing of the PCR products. Electrophoresis of 80 μl of PCR products from each species was performed for 45 min at 100 V in a 1% LM2 agarose gel containing ethidium bromide (1 μg/ml) in Tris-acetate buffer (0.004 M Tris-acetate, 0.001 M EDTA, pH 8.0). DNA fragments were visualized by UV transillumination and analyzed using a Geldoc 1000 UV Fluorescent Gel Documentation System–PC (Bio-Rad Laboratories, Hercules, Ga.).

Restriction site mapping and enzymatic digestion of PCR products. Restriction maps of 12S rRNA gene sequences from Nile perch, grouper, and wreck fish were obtained using the Wisconsin Package, version 9.0. Two endonucleases were then selected for restriction analysis of the amplified PCR products: Rsal and Sau961. Digests were performed in 20 μl volumes with 100 ng amplified DNA, 10 U enzyme, and a 1 to 10 dilution of the manufacturer’s recommended 10× digestion buffer. Digestions were incubated for 15 h at 37°C. The resulting fragments were separated by electrophoresis in a 3.5% MS8 agarose gel (Hispanlab) containing 1 μg/ml ethidium bromide for 1 h at 80 V. The sizes of the DNA fragments were estimated by comparison with a commercial 1-kb plus DNA ladder (Gibco BRL).

RESULTS

Primers 12S1 and 12S2 consistently amplified a fragment of 436 bp from the 12S rRNA gene of Nile perch, grouper, and wreck fish (Fig. 1). PCR products from at least three individuals of each species were sequenced. After detailed comparison of the restriction maps of 12S rRNA sequences, two restriction endonucleases, Rsal and Sau961, were found to be potentially useful for the specific identification of Nile perch, grouper, and wreck fish (Fig. 2).

Results following digestion of the PCR products from the three species analyzed showed that band sizes obtained by electrophoresis in 3.5% MS8 agarase gel were in agreement with the expected sizes for the restriction fragments inferred from the sequence analysis: Rsal cleaved the analyzed 12S rRNA segment of wreck fish into two fragments of 346 and 90 bp; grouper into three fragments of 189, 173, and 74 bp; and Nile perch into two fragments of 234 and 202 bp (Fig. 3). Similarly, digestions performed with Sau961 resulted in two DNA fragments of 402 and 34 bp in wreck fish PCR products; three fragments of 224, 178, and 34 bp were obtained in grouper; and, finally, the two Sau961 restriction sites present in Nile perch sequences yielded two DNA fragments of 224 and 212 bp (Fig. 4). Results obtained with 15 different individuals from each species did not show intraspecific polymorphism for the restriction sites tested.

DISCUSSION

In view of the increases in international seafood trade and the existing laws requiring that products be correctly labeled, there is a growing need for techniques that enable authentication of fish and seafood products (13). In search for fast and simple genetic techniques, PCR-RFLP has gained acceptance among fish species identification methods, because it is much easier to perform and less costly than conventional DNA sequencing and nucleotide sequence analysis (14). In this work we have developed a method for the specific discrimination between Nile perch, grouper, and wreck fish based on RFLP analysis of PCR-amplified products from the mitochondrial 12S rRNA gene.

Mitochondrial DNA has been widely used as a marker
1 N.perch1 NNACTAGGAT TAGATACCT ATTATCCTAG CCCTAANCAT TGAATTTACA
N.perch2 ................................. ................................. CTAG CCCTAAACAT TGAATTTACA
grouper1 ................................. ................................. ................................. CTNCCATG TGAATCTTTTT
grouper2 ................................. ................................. CTTAGGAT TGAATTTTTTT
wreck fish1 ................................. ................................. ................................. CAAGAGATT CAGATGGNGGA
wreck fish2 ................................. ................................. ................................. CTTAGCAGAT CAGATGGNGGA

51 N.perch1 TTACACCACTA CATCAGGCA CAGGTATTAG AGGCCAGGCT TAAACCCCAA
N.perch2 TTACACCACTA CATCAGGCA CAGGTATTAG AGGCCAGGCT TAAACCCCAC
grouper1 TTACACCACTA CATCAGGCA CAGGTATTAG AGGCCAGGCT TAAACCCCAA
grouper2 TTACACCACTA CATCAGGCA CAGGTATTAG AGGCCAGGCT TAAACCCCAA
wreck fish1 CTACACCACTA CATCAGGCA CAGGTATTAG AGGCCAGGCT TAAACCCCAA
wreck fish2 CTACACCACTA CATCAGGCA CAGGTATTAG AGGCCAGGCT TAAACCCCAA

101 N.perch1 AGGACTTGCC GTGCTTAGT TAGCCATCAT TAGCCATCAT TCTATTAATGG
N.perch2 AGGACTTGCC GTGCTTAGT TAGCCATCAT TAGCCATCAT TCTATTAATGG
grouper1 AGGACTTGCC GTGCTTAGT TAGCCATCAT TAGCCATCAT TCTATTAATGG
grouper2 AGGACTTGCC GTGCTTAGT TAGCCATCAT TAGCCATCAT TCTATTAATGG
wreck fish1 AGGACTTGCC GTGCTTAGT TAGCCATCAT TAGCCATCAT TCTATTAATGG
wreck fish2 AGGACTTGCC GTGCTTAGT TAGCCATCAT TAGCCATCAT TCTATTAATGG

151 N.perch1 ATTAACCGGC TTTAACCTTG CCTTCTCTCT TCTTTTCGCC TTAATATCAG
N.perch2 ATTAACCGGC TTTAACCTTG CCTTCTCTCT TCTTTTCGCC TTAATATCAG
grouper1 ATTAACCGGC TTTAACCTTG CCTTCTCTCT TCTTTTCGCC TTAATATCAG
grouper2 ATTAACCGGC TTTAACCTTG CCTTCTCTCT TCTTTTCGCC TTAATATCAG
wreck fish1 ATTAACCGGC TTTAACCTTG CCTTCTCTCT TCTTTTCGCC TTAATATCAG
wreck fish2 ATTAACCGGC TTTAACCTTG CCTTCTCTCT TCTTTTCGCC TTAATATCAG

201 N.perch1 CGGTGTCAG CCTACCTTTG GAGGCCAGA TGGTACCGA GACCCCGAC
N.perch2 CGGTGTCAG CCTACCTTTG GAGGCCAGA TGGTACCGA GACCCCGAC
grouper1 CGGTGTCAG CCTACCTTTG GAGGCCAGA TGGTACCGA GACCCCGAC
grouper2 CGGTGTCAG CCTACCTTTG GAGGCCAGA TGGTACCGA GACCCCGAC
wreck fish1 CGGTGTCAG CCTACCTTTG GAGGCCAGA TGGTACCGA GACCCCGAC
wreck fish2 CGGTGTCAG CCTACCTTTG GAGGCCAGA TGGTACCGA GACCCCGAC

251 N.perch1 GCCCAAACCG TCCAGTTGAG TGTACCGGA TAGGAGAGGA AAGAAATGGG
N.perch2 GCCCAAACCG TCCAGTTGAG TGTACCGGA TAGGAGAGGA AAGAAATGGG
grouper1 GCCCAAACCG TCCAGTTGAG TGTACCGGA TAGGAGAGGA AAGAAATGGG
grouper2 GCCCAAACCG TCCAGTTGAG TGTACCGGA TAGGAGAGGA AAGAAATGGG
wreck fish1 GCCCAAACCG TCCAGTTGAG TGTACCGGA TAGGAGAGGA AAGAAATGGG
wreck fish2 GCCCAAACCG TCCAGTTGAG TGTACCGGA TAGGAGAGGA AAGAAATGGG

301 N.perch1 CTACATTGCT CAAACACTTT CAGGAGTTAG AATAGACAGT GATGACATTG AAACATCATG
N.perch2 CTACATTGCT CAAACACTTT CAGGAGTTAG AATAGACAGT GATGACATTG AAACATCATG
grouper1 CTACATTGCT CAAACACTTT CAGGAGTTAG AATAGACAGT GATGACATTG AAACATCATG
grouper2 CTACATTGCT CAAACACTTT CAGGAGTTAG AATAGACAGT GATGACATTG AAACATCATG
wreck fish1 CTACATTGCT CAAACACTTT CAGGAGTTAG AATAGACAGT GATGACATTG AAACATCATG
wreck fish2 CTACATTGCT CAAACACTTT CAGGAGTTAG AATAGACAGT GATGACATTG AAACATCATG

351 N.perch1 CT.GAAGGAG GATTTAGTAC TAAAGAGAGA GCAGAGGATG TCCCCGAAAC
N.perch2 CT.GAAGGAG GATTTAGTAC TAAAGAGAGA GCAGAGGATG TCCCCGAAAC
grouper1 CT.GAAGGAG GATTTAGTAC TAAAGAGAGA GCAGAGGATG TCCCCGAAAC
grouper2 CT.GAAGGAG GATTTAGTAC TAAAGAGAGA GCAGAGGATG TCCCCGAAAC
wreck fish1 CT.GAAGGAG GATTTAGTAC TAAAGAGAGA GCAGAGGATG TCCCCGAAAC
wreck fish2 CT.GAAGGAG GATTTAGTAC TAAAGAGAGA GCAGAGGATG TCCCCGAAAC

401 N.perch1 CGGCTCTTAA GCCGGCAGAC ATGGCCCTTC GCCTTT
N.perch2 CGGCTCTTAA GCCGGCAGAC ATGGCCCTTC GCCTTT
grouper1 CGGCTCTTAA GCCGGCAGAC ATGGCCCTTC GCCTTT
grouper2 CGGCTCTTAA GCCGGCAGAC ATGGCCCTTC GCCTTT
wreck fish1 CGGCTCTTAA GCCGGCAGAC ATGGCCCTTC GCCTTT
wreck fish2 CGGCTCTTAA GCCGGCAGAC ATGGCCCTTC GCCTTT

Sau96I
RsaI
RsaI
RsaI
in species identification (2, 15, 19). In particular, the mitochondrial encoded gene for 12S rRNA satisfies most of the requirements for the development of species differentiation techniques: it has an acceptable length to permit detection of sequence differences between congeneric species; it accumulates point mutations quickly enough to allow, in most cases, the discrimination of even closely related species; and there are different sequences available in the databases. Additionally, the high copy number and the conserved regions of mitochondrial DNA are basic traits for the high sensitivity of PCR-based methods applied to species identification (4, 21).

The mitochondrial primers 12S1 and 12S2 (17) used in this work successfully amplified a conserved 436-bp region from the 12S rRNA gene of all Nile perch, grouper, and wreck fish individuals analyzed. The GenBank and EMBL databases were searched for sequences of the 12S rRNA gene from these species, but no such sequences were available. Thus, three PCR products from each, Nile perch, grouper, and wreck fish, were sequenced for subsequent restriction map analysis and endonuclease selection. The results obtained in this study showed that RsaI and Sau96I endonucleases independently provided specific identification of Nile perch, grouper, and wreck fish after PCR-RFLP analysis of the mitochondrial 12S rRNA fragments.

The PCR-RFLP method proposed in this work provides a powerful tool for discrimination between Nile perch, grouper, and wreck fish species. Compared to alternative techniques, such as direct sequencing of PCR products, PCR-RFLP offers the advantages of being simpler, cheaper, and especially useful for routine fish species identification in the large-scale studies such as required in inspection programs.

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