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Research Note

Mitochondrial Cytochrome *b* DNA Sequence Variations: An Approach to Fish Species Identification in Processed Fish Products

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ABSTRACT

The identification of fish species in food products is problematic because morphological features of the fish are partially or completely lost during processing. It is important to determine fish origin because of the increasing international seafood trade and because European Community Regulation 104/2000 requires that the products be labeled correctly. Sequence analysis of PCR products from a conserved region of the cytochrome *b* gene was used to identity fish species belonging to the families *Gadidae* and *Merluccidae* in 18 different processed fish products. This method allowed the identification of fish species in all samples. Fish in all of the examined products belonged to these two families, with the exception of one sample of smoked baccalà (salt cod), which was not included in the *Gadidae* cluster.

The increasing global diffusion of fish products for human consumption requires accurate sanitary and quality controls. Fish species identification has become a problem in some products because the morphological characters of the fish are partially or completely lost during processing, and fraudulent substitutions with less valuable fish are possible. Protein electrophoresis is a well-established procedure for identification of raw fish *(29, 35)*. Fish species have also been identified using immunological methods *(1, 11, 20, 21, 38, 40)*. However, these methods utilize the water-soluble protein fraction, which is often modified by heat processing. The improvement of molecular biological techniques may solve problems related to fish identification by enabling direct analysis of nucleotide sequences of nuclear or mitochondrial DNA (mtDNA). The application of PCR methods to food analysis has increased because of the simplicity, specificity, and sensitivity of the technique *(33)*. Kocher et al. *(28)* used PCR techniques to amplify homologous segments of mtDNA from more than 100 animal species, including fish, to study their phylogenetic relationships and variability. PCR assays for fish identification are currently used extensively *(3, 4, 6–8, 12–14, 16, 22, 25)*. Analysis of the restriction enzyme pattern of PCR-amplified fragments also has been a useful way to identify heat-processed fish fillets *(11, 24, 34)*. However, this method is unsatisfactory for identifying closely related species because similar fragments are produced by the restriction endonucleases *(17, 26)*. The development of suitable analytical methods for fish species identification in prepared and transformed fish products is of great interest to enforcement agencies involved with labeling regulations and the authentication of fish in various products to prevent the substitution of fish species *(30, 31)*. One objective of the present study was the development of a method to identify fish species in prepared and transformed fish products by a PCR method and sequence analysis of a 359-bp fragment of the mitochondrial cytochrome *b* (*cytb*) gene. The examined products included fish species differing in commercial value and belonging to the families *Merluccidae* and *Gadidae*. Another objective was evaluation of sequence variation in a *cytb* mitochondrial gene fragment in different *Merluccius* species to increase the relatively sparse database for this family *(32, 36)*.

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MATERIALS AND METHODS

Mitochondrial DNA sequences from GenBank. To facilitate comparison, the following *Gadidae, Merluccidae,* and *Batrachoidiformes cytb* sequences were obtained from GenBank: *Merluccidae*: *Merluccius merluccius,* accession AF120096; *Gadidae*: *Gadus morhua,* accession AF081682; *Gadus morhua* island haplotype, accession M98569; *Gadus ogac,* accession AY237811; *Gadus macrocephalus,* accession AY237802; *Theragra chalcogramma,* accession AY237810; *Batrachoidiformes*: *Halobatrachus didactylus,* accession AF165351; *Opsanus pardus,* accession AF165347 (Table 1).

Fish samples. The partial *cytb* sequences derived from whole fish (Food Veterinary Office, Campania, Italy) morphologically identified as belonging to the *Merluccidae* family (Table 1) and from 18 different processed fish samples belonging to the *Gadidae*

TABLE 1. *Fish species for which DNA sequences were obtained from GenBank or from whole fish samples*

Species	GenBank accession number/origin
Sequences	
Merluccius merluccius	AF120096
Gadus morhua	AF081682
G. morhua island haplotype	M98569
Theragra chalcogramma	AY237810
Gadus macrocephalus	AY237802
Gadus ogac	AY237811
Halobactrachus didactylus	AF165351
Opsanus pardus	AF165347
Whole fish	
Merluccius hubbsi	Food Veterinary Office, Campania, Italy
Merluccius capensis	Food Veterinary Office, Campania, Italy
M. merluccius	Food Veterinary Office, Campania, Italy
M. merluccius	Food Veterinary Office, Campania, Italy

and *Merluccidae* families (Table 2) were examined. The samples were bought retail in Campania and rapidly brought to the laboratory, where they were refrigerated or kept at the temperature indicated on the package until analyzed.

DNA extraction. Total cellular DNA was extracted from fish muscle according to the DNA extraction method of Doyle and Doyle *(18)*. Pieces of tissue (1 g) were dipped in liquid nitrogen, homogenized in 20 ml of 2× CTAB buffer (2× CTAB: 100 mM Tris HCl, pH 8.0; 1.4 M NaCl; 20 mM EDTA, pH 8.0; and 0.2% β-mercaptoethanol), and incubated at 65°C for 60 min. DNA was subsequently extracted from lysate once with phenol-chloroform and twice with chloroform and then precipitated with isopropyl alcohol. The pellets were washed twice with 70% ethanol and dried under a laminar flow. The DNA was suspended with 50 μl of sterile distilled water.

Amplification and sequencing. The primers used were universal primers: CYTB1, 5′-CCATCCAACTCTCAGCATGATGAAA-3′; and CYTB2, 5′-GCCCCTCAGAATGATATTTGTCCTCA-3′ *(9)*. These primers amplify a 359-bp region, which represents about 2% of the fish mtDNA genome consisting of 16.5 kilobases *(2, 27)*. Amplifications of DNA were carried out in a final volume of 100 μl containing 1× PCR Buffer Plus (Gibco, Invitrogen, Carlsbad, Calif.), 1 to 10 ng of DNA, 2.0 mM $MgCl_2$, 200 mM of each dNTP, 20 to 25 pM of each primers, 1.0 μl of *Taq* DNA polymerase, and sterile distilled water. A positive control (DNA from *M. merluccius* whole fish) and a negative control (no DNA added) were included in parallel with each amplification set. A Robocycler 96 (Stratagene, La Jolla, Calif.) was used to perform a preliminary denaturation at 95°C for 5 min, 30 amplification cycles of denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, and primer extension at 72°C for 1 min, and a final extension at 72°C for 7 min.

The PCR amplification products were analyzed by agarose gel electrophoresis on a 1.5% gel containing 1 μg/ml ethidium bromide. Electrophoresis separation was performed at 60 V for 1 h. The resulting DNA fragments were visualized by UV transillumination (Fig. 1). The amplification products were purified with

TABLE 2. *Fish products examined using the PCR sequencing technique*

Sample no.	Processed fish product	Declared species	Treatment
1	Baccalà	*Gadus morhua*	Salted and rehydrated
2	Baccalà	*G. morhua*	Salted and rehydrated
3	Fillet	Merluzzo from Argentina[a]	Frozen
4	Caciucco (Italian fish soup)	Atlantic merluzzo	Cooked and frozen
5	Fish sticks	Atlantic merluzzo	Frozen
6	Fillet	Atlantic merluzzo	Frozen
7	Fillet	Atlantic merluzzo	Frozen
8	Fish soup	Atlantic merluzzo	Cooked and frozen
9	Fillet	Atlantic merluzzo	Frozen
10	Panaree	Atlantic merluzzo	Cooked and frozen
11	Panaree	Atlantic merluzzo	Cooked and frozen
12	Fish sticks	Atlantic merluzzo	Frozen
13	Fillet	Atlantic merluzzo	Frozen
14	Fillet	Atlantic merluzzo	Frozen
15	Fillet	Atlantic merluzzo	Frozen and experimentally cooked
16	Smoked baccalà	*G. morhua*	Smoked and rehydrated
17	Fillet	Atlantic merluzzo	Frozen
18	Fillet alla livornese	*Merluccius capensis*	Cooked and frozen

[a] *Merluzzo* is the Italian official denomination used for fish belonging to the *Gadidae* or *Merluccidae* families.

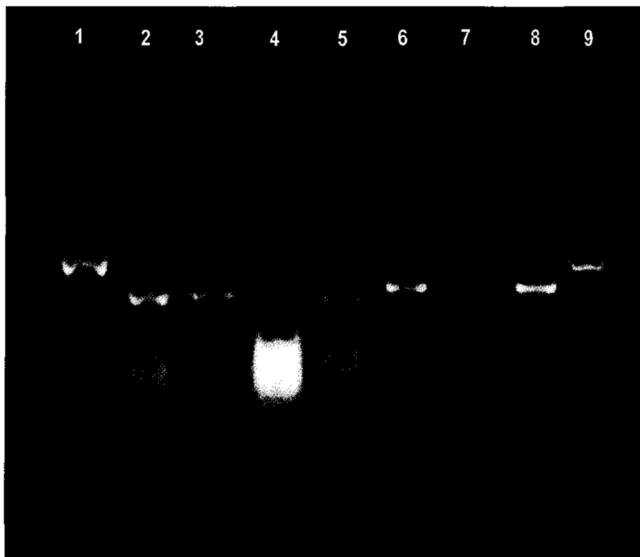

FIGURE 1. *Electrophoretic analysis of PCR products of the cytochrome* b *fragments. Lanes 1 and 9, molecular weight marker 100-bp ladder; lanes 2, 3, 5, and 6, PCR products for fish samples; lane 4, negative control; lane 8, positive control.*

the G-100 column (Microcon, Bangalore, India) according to the manufacturer's instructions. The DNA was eluted in 25 μl of sterile distilled water. Purified PCR products were sequenced using a Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Norwalk, Conn.) in an ABI Prism model 310 DNA Sequencer (Perkin-Elmer).

Construction of neighbor-joining trees and bootstrap analysis were performed using PAUP version 4 *(37)*. Sequence alignments of mtDNA fragments were analyzed using BioEdit version 5.0.6 (Ibis Therapeutics, Carlsbad, Calif.). For the construction of the neighbor-joining tree, the sequenced fragments were compared with GenBank data for the cytb fragments belonging to the families *Gadidae* (AF011682, M98569, AY237810, AY237802, and AY237811) and *Merluccidae* (AF120096). *Halobatrachus didactylus* and *Opsanus pardus* (AF165351 and AF165347, respectively) were used as taxonomic outgroups belonging to the order *Batrachoidiformes,* which is considered the sister group for *Gadiformes (23)* (Table 2). The HKY85 *(23)* correction was used to convert pairwise sequence similarities into evolutionary distances, and starting trees were obtained via neighbor joining *(20).*

RESULTS AND DISCUSSION

Amplification of DNA, using a 359-bp fragment of the mitochondrial *cytb* gene, was carried out for all the samples of prepared and processed fish (frozen, smoked, cooked, salted, and dried) (Table 2). The sequence tree constructed from the data contained two main groups (Fig. 2).

The first group included the sequences of *Gadidae* species taken from GenBank and the sequences obtained from the two samples of baccalà (salt cod, salted and rehydrated) labeled *G. morhua.* The second group included the *M. merluccius* sequence (the only representative of the *Merluccidae* in GenBank) and the four whole fish identified by morphological characters as belonging to the genus *Merluccius* (*M. hubbsi, M. capensis,* and *M. merluccius*). This group includes also all the sequences of the samples labeled merluzzo (official Italian name for fish belonging to families *Gadidae* and *Merluccidae*) (samples 3 through 15 and 17) and sample 16 labeled *G. morhua* (Table 2). Sample 16, a rehydrated and smoked baccalà labeled *G. morhua,* was distant from samples 1 and 2, which also were labeled *G. morhua,* and was outside the *Gadidae* cluster.

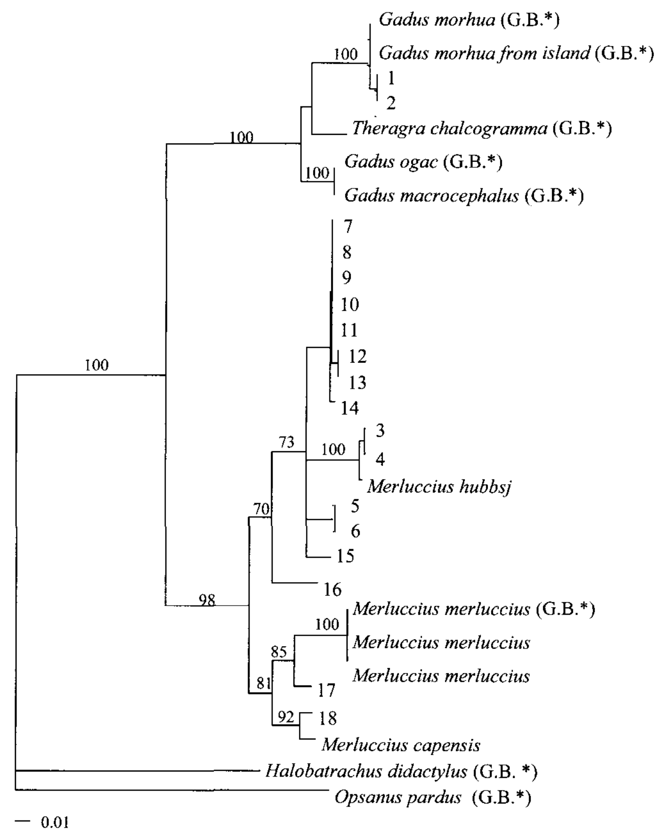

FIGURE 2. *Neighbor-joining tree based on partial cytochrome* b *DNA sequences from retail* "Merluccius" *and reference sequences (GenBank accession numbers). Bootstrap values greater than 70 (1,000 replicates) based on the HKY85 model* (20, 24) *are shown at nodes.*

The *Merluccidae* cluster was composed of two subgroups, the first comprising the sequences of *M. hubbsi* (whole fish) and 14 fish products (samples 3 through 16) and the second comprising the sequences of *M. merluccius* (whole fish), *M. capensis* (whole fish), *M. merluccius* (GenBank), and two fish products (samples 17 and 18), which were labeled Atlantic merluzzo and *M. capensis,* respectively.

The analysis of the sequences from the two *M. merluccius* whole fish revealed 100% similarity with the sequence of *M. merluccius* taken from GenBank.

The direct analysis of the sequences of two samples of rehydrated baccalà labeled *G. morhua* revealed 99.6% similarity with the sequence of *G. morhua* taken from GenBank, which suggests the presence of *G. morhua* haplotypes *(9, 10).*

The sequence analysis of sample 3, a merluzzo fillet sample labeled as merluzzo from Argentina, revealed 95.3% similarity with the sequence of *M. hubbsi* (whole fish) and 85.6% similarity with *M. merluccius* (GenBank).

The direct analysis of the sequence of sample 18 (a cooked and frozen fillet alla livornese labeled *M. capensis*) revealed 94.2% similarity with the sequence of *M. capensis*

(whole fish) and 88.4% similarity with the sequence of *M. merluccius* (GenBank).

There is an increasing need for techniques to allow species identification in processed fish products to meet growing consumer demand for assurances of food quality and safety. Mitochondrial DNA has been widely used for species identification *(5, 34, 39)*. In particular, the mitochondrial gene for *cytb* satisfies most of the requirements for differentiation of closely related species.

The mtDNA primers CYTB1 and CYTB2 used in this work allowed the amplification of a 359-bp conserved region of *cytb* from all the prepared and processed fish products. The amplification of *cytb* for fish identification has been used for other processed fish products *(4, 15)*. These analyses revealed that all the fish in the examined products belonged to the families *Gadidae* or *Merluccidae*. No sample contained fish species of lower commercial value. However, the sample of smoked baccalà labeled *G. morhua* was outside the *Gadidae* cluster on the sequence tree. Italian Decreto Ministeriale 28 October 1997 of the Ministry of Agricultural Political (GU 20 December 1997, no. 296) established that only *G. morhua, G. ogac, G. macrocephalus,* and *Gadus callarias* may be used to produce baccalà.

The *cytb* gene sequence analysis of samples labeled *G. morhua* revealed only a few base differences when compared with the sequence of the same species taken from GenBank. The few data in GenBank for the *cytb* fragment of *Merluccius* spp. prevented a more precise species identification of some products. Further studies on this family are needed.

For samples labeled as merluzzo from Argentina and *M. hubbsi*, an examination of nucleotide variability within this species in a representative number of individuals is needed to confirm the identification.

Because of the increases in the international seafood trade and the existing laws requiring that products be correctly labeled, as specified by European Community Regulation 104/2000 *(19)*, it is important to be able to determine what fish species are included in various fish products. Some species have point mutations in different areas of the genome, and sequence analysis can be used to distinguish different haplotypes of the same species.

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