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Fish Species Identification in Surimi-Based Products

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Whole fish morphologically identified as belonging to *Theragra chalcogramma*, *Merluccius merluccius*, *Merluccius hubbsi*, and *Merluccius capensis* and 19 fish products commercialized as surimi with different commercial brands and labeled as *T. chalcogramma* were analyzed by direct sequence analysis of the cytochrome *b* gene. A phylogenetic analysis of surimi products was performed as well. Results demonstrated that mislabeling is a large-scale phenomenon, since 84.2% of surimi-based fish products sold as *T. chalcogramma* (16/19) were prepared with species different from the one declared. In fact, only three samples (samples 15−17) were found to belong to *T. chalcogramma*. In the remaining samples, *Merluccidae* (samples 4−14), *Gadidae* (samples 18 and 19), *Sparidae* (sample 1), and *Pomacentridae* (samples 2 and 3) families were detected. A phylogenetic tree was constructed, and the bootstrap value was calculated. According to this methodology, 11 samples were grouped in the same clade as *Merluccius* spp.

KEYWORDS: Surimi; *Gadiformes*; cytochrome b gene; PCR; direct sequencing; *Theragra chalcogramma*

INTRODUCTION

During the last decades, food produced from fish meat has been widely used. One of the most typical fish products is surimi, an intermediate product composed of boneless, minced, water-washed, lean white fish, which is mixed with food-grade cryoprotectants and stored frozen until used for the production of textured/flavored products (*1*), which are prepared in different shapes. The physical properties of the fish meat gel are dependent on myosin, which is the most abundant myofibrillar component in some fish species such as Alaska pollack and is responsible for the texture and color, the most important aspects of the product quality (*2*). Surimi-based products are prepared in different textured and flavored ways and are gaining increasing attention worldwide, as a consequence of the diffusion of Japanese restaurants and culinary traditions in North America, Europe, and elsewhere. The resources of Alaska pollack are becoming depleted, and new species that were previously underutilized are being used. Substitution of fish products is very common, due to the profits resulting from the use of less expensive fish species. Mislabeling not only defrauds consumers but could also adversely affect estimates of fish stock sizes if it influences the reporting of catch data that are used in fisheries management (*3*). EU Commission Regulation no. 2065/2001 of 22 October 2001 has established detailed rules for consumer information about fish and fish products. The consumers have become more and more demanding in food quality and are looking for safety and the correspondence of the ingredients with what has been declared on the label. This fact requires the availability of rapid and reliable methods to assess the quality of food and to identify food components. In particular, for meat- or fish-based foods, methods able to differentiate and identify species commonly used must be developed. The necessity to identify different species in foodstuffs is an important aspect to consider when allergic problems toward specific species or ethical issues are taken into account. The identification of the fish species used in a specific preparation is a crucial step in food quality control to avoid possible commercial fraud. It is very important to control species of high commercial value so that they are not sold mixed with other species of lower commercial value. Because the external features allowing the morphological identification of whole fish are not apparent after the preparation of surimi, analytical methods are needed to detect mislabeling. Traditional methods of fish species identification such as isoelectric focusing (IEF) of proteins (*4*) or high-performance liquid chromatography (*5*, *6*) are applicable only to raw fish. Other electrophoretic methods, such as sodium dodecyl sulfate−polyacrylamide gel electrophoresis or urea IEF (*7*−*9*) can be used to identify processed fish (cooked, smoked) but cannot be applied when proteins have been highly denatured. In fact, these methods often fail in species detection when heated meat mixtures and other food products with complex mixtures are used (*10*). Therefore, other methods, based on molecules that are heat-stable and long-lived, such as the DNA molecule, are required (*11*). The application of molecular biology has been widely used for fish species identification (*12*−*14*) and mis-

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Table 1. Sequences Taken from GenBank and Sequences Obtained from Whole Fish Samples

species	GenBank accession no.	family
M. merluccius	AF469623	*Merluccidae*
M. productus	DQ174065	*Merluccidae*
M. australis	AB248669	*Merluccidae*
M. gayi	AB248667	*Merluccidae*
G. morhua	AF081682	*Gadidae*
G. morhua from island	M98549	*Gadidae*
G. macrocephalus	AF081683	*Gadidae*
G. ogac	AF081684	*Gadidae*
T. chalcogramma	AF081685	*Gadidae*
M. dipterigia	AF469625	*Lotidae*
M. molva	AF469626	*Lotidae*
B. brosme	AY237808	*Lotidae*
M. aeglefinus	AY237809	*Gadidae*
P. pollachius	AY237806	*Gadidae*
P. virens	AY237807	*Gadidae*
M. poutassou	AJ517491	*Gadidae*
Parargyrops edita	AY208605	*Sparidae*
Pomacentrus philippinus	AY208605	*Pomacentridae*

whole fish	country
M. capensis	Food Veterinary Office, Campania, Italy
M. hubbsi	Food Veterinary Office, Campania, Italy
M. merluccius	Food Veterinary Office, Campania, Italy
T. chalcogramma	Food Veterinary Office, Campania, Italy

labeling detection (*15*, *16*). Among these molecular techniques, polymerase chain reaction (PCR) and DNA sequencing have proven to be useful tools (*17*−*22*). The integrity of DNA present in food must be retained during production and storage to allow the detection of specific base pair (bp) sequences, which are characteristic for a species. The DNA quality is related to the average molecular weight of the DNA, which is the length of the individual double strands expressed as the number of bps. DNA degradation results in a decrease of the length of the molecules, usually without changes in the base pair sequence. Ebbehøj and Thomsen (*23*) investigated the quality of DNA from meat during storage and heat treatment. Heating of the meat before DNA extraction led to a degradation of DNA, which exhibited a length of 300 bp when the meat was heated for 10 min at 121 °C. From surimi products, it was difficult to obtain fragments larger than 400−500 bp, due the severe treatment during the product preparations. The nucleotide sequence of the *cytochrome b* (*cyt b*) gene contains species-specific information and has been used in phylogeny as well as in forensic investigations in a number of studies (*24*−*32*). Furthermore, *cyt b* is located on the mitochondrial genome, taking advantage of the sensitivity of PCR-based mtDNA typing in the forensic context (*33*−*35*). The aim of this work was to identify the fish species in surimi-based products labeled as *Theragra chalcogramma* by PCR and direct sequencing analysis of the *cyt b* gene. A phylogenetic analysis of surimi products was performed as well, to better characterize the species employed in surimi-based fish preparation.

MATERIALS AND METHODS

Samples Collection. The partial *cyt b* sequences of four whole fish kindly provided by the Food Veterinary Office and morphologically identified as belonging to *T. chalcogramma*, *Merluccius merluccius*, *Merluccius hubbsi*, and *Merluccius capensis* (**Table 1**) and 19 samples other differently processed fish products commercialized as surimi with different commercial brands and labeled as *T. chalcogramma* were analyzed. The 19 samples, shaped as crab sticks (samples 2−4, 10, 11, 14, and 16), crab flakes (samples 5−7, 12, 13, 18, and 19), and crab legs (samples 1, 8, 9, 15, and 17) were bought at retail in Naples (Italy) and rapidly brought in insulated boxes to the laboratory where they were kept refrigerated or at the temperatures indicated on the package until DNA analysis.

Isolation, Amplification, and Sequencing. Total cellular DNA was extracted from fish muscle according to the DNA extraction method of Doyle and Doyle (*36*). One gram pieces of tissue were dipped in liquid nitrogen and homogenized in 20 mL of CTAB 2× buffer in a sterile tube (2× CTAB, 100 mM Tris HCl at pH 8.0, 1.4 M NaCl, 20 mM EDTA at pH 8.0, and 0.2% β-mercaptoethanol). The tube was incubated at 65 °C for 60 min. DNA was subsequently extracted from lysate one time with phenol−chloroform and twice with chloroform and then precipitated with isopropilic alcohol. The pellet was washed twice with 70% ethanol and dried under a laminar flow. The DNA was suspended with 50 μL of sterile distilled water. The primers used for amplification were universal primers: CYTB1 5′-CCATCCAACATCTCAGCATGATGAAA-3′ and CYTB2 5′-GCCCCTCAGAATGATATTTGTCCTCA-3′ (*27*).

These primers amplify a 359 base pair region, which represents about 2% of fish mtDNA genome consisting of 16.5 kilobases (*37*, *38*). Amplifications of DNA were carried out in a final volume of 100 μL containing 1× PCR Buffer Plus (GIBCO), 1−10 ng of DNA, 2.0 mM $MgCl_2$, 200 mM each dNTP, 20−25 pM each primers, 1.0 μL of Taq DNA polymerase, and distilled sterile water. PCR was carried out both on samples of surimi-based fish DNA and on a negative control, without DNA. A Robocycler 96 (Stratagene) was used to perform a preliminary denaturation at 95 °C for 5 min and 30 amplification cycles with the following step-cycle profile: denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s, primer extension at 72 °C for 1 min, and final extension at 72 °C for 7 min. PCR amplification products (5 μL) were analyzed by electrophoretic separation in 1.5% agarose gel stained with ethidium bromide in TAE buffer (Tris, acetic acid, and EDTA, pH 8). Electrophoresis separation was performed at 60 V for 1 h. The resulting DNA fragments were visualized by UV transillumination. The amplification products were purified with the Microcon G-100 column according to the instructions of the manufacturer. The DNA was eluted in 25 μL of sterile distilled water. Purified PCR products were sequenced by a Cycle Sequencing Ready Reaction Kit (Perkin- Elmer) in an ABI Prism model 310 DNA Sequencer (Perkin-Elmer).

Genetic Distance and Phylogenetic Analysis. The study of the sequence alignments of mtDNA fragments was performed using the Clustal X program and Software BioEdit version 5.0.6. To facilitate comparison, the following *cyt b* sequences were taken from GenBank: *M. merluccius* (AF469623), *Merluccius productus* (DQ174065), *Merluccius gayi* (AB248667), and *Merluccius australis* (AB248669) for the *Merluccidae* family; *Gadus morhua* (AF081682), *G. morhua* from island (M98549), *Gadus ogac* (AF081684), *Gadus macrocephalus* (AF081683), *Melanogrammus aeglefinus* (AY237809), *Pollachius pollachius* (AY237806), *Pollachius virens* (AY237807), *T. chalcogramma* (AF081685), and *Micromesistius poutassou* (AJ517491) for the *Gadidae* family; and *Brosme brosme* (AY237808), *Molva molva* (AF469626), and *Molva dipterigia* (AF469625) for the *Lotidae* family. Sequences from samples taxonomically identified as belonging to the *Merluccidae* family (*M. capensis*, *M. merluccius*, and *M. hubbsi*) and *Gadidae* family (*T. chalcogramma*) (**Table 1**) were used for the construction of the neighbor-joining tree analysis (**Figure 1**). The data set resulting from the sequenced samples and the GenBank sequences was analyzed in PAUP 4 (*39*). The neighbor-joining analyses of *cyt b* sequences used the best-fit model of nucleotide substitution. The model was selected according to the Akaike information criterion (AIC) and calculated using Modeltest version 3.4 (HKY85 model) (*40*). Phylogenetic relationships of the sequences with the species that showed a higher homology, and species variability were calculated.

RESULTS AND DISCUSSION

A short fragment (359 bp) of the *cyt b* gene was chosen for species identification as this fragment has the widest taxonomic

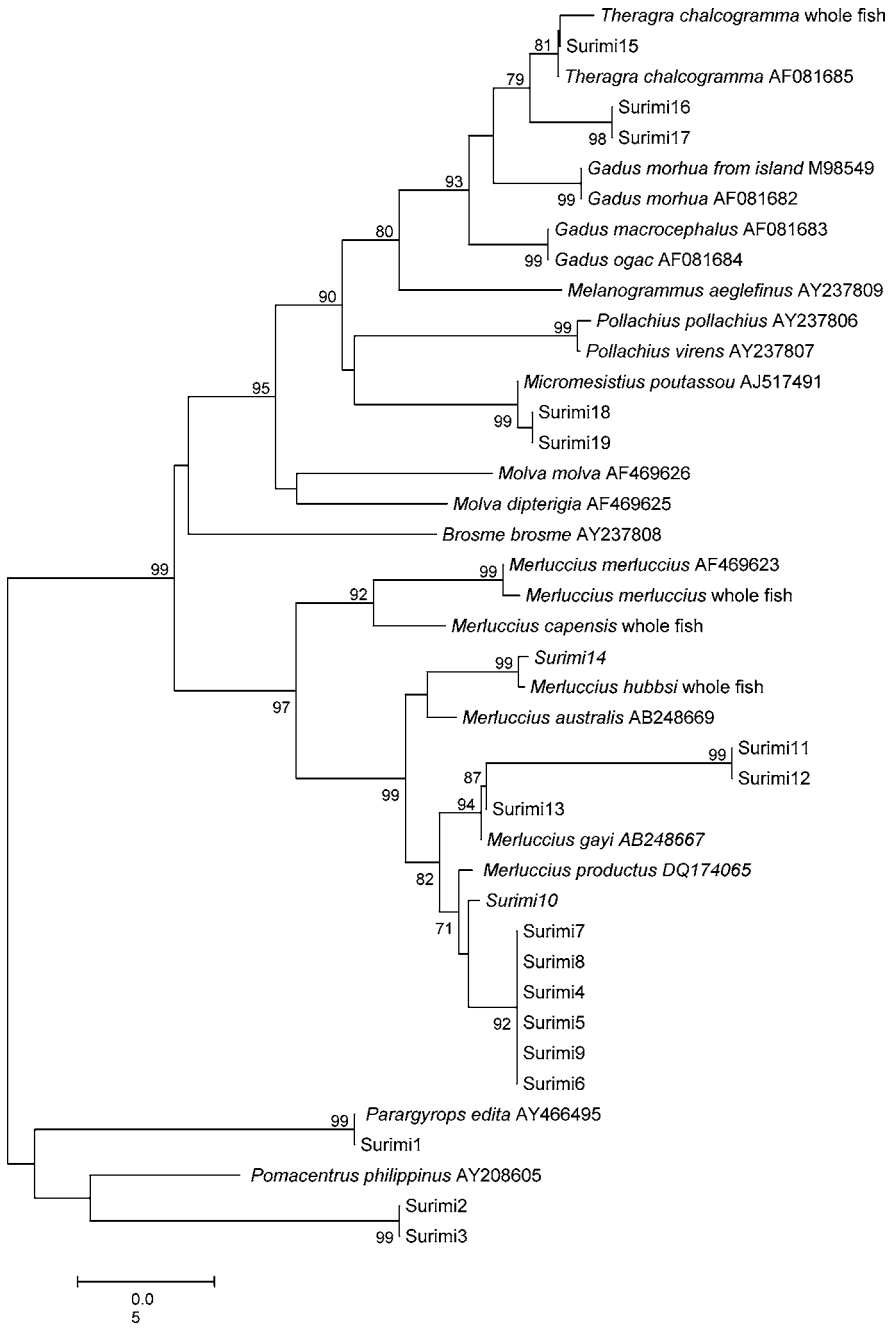

Figure 1. Neighbor-joining tree based on partial *cyt b* DNA sequences from retail surimi-based fish products and reference sequences (GenBank accession number). Bootstrap values at nodes greater than 70 are shown (1000 replicates) using the HKY85 model (*18*, *40*).

representation in the nucleotide database. DNA was extracted from all examined samples of surimi-based fish products as well as from whole fish identified on the basis of their morphological characters. The *cyt b* fragment was amplified and sequenced in order to determine the species by the information content of the derived nucleotide sequence. The good quality of DNA allowed the sequencing of both strands of the amplification products. Entrez from the National Center of Biotechnology Information (NCBI) was used for data interpretation. The comparison of sequences obtained from our samples vs the sequences available in GenBank and taken for this study (**Table 1**) revealed different levels of homology (**Figure 1**). More precise species identification was not possible since scarce data on fish species sequencing exist in GenBank.

A tree with 1000 replicates was obtained, and four clusters were observed (**Figure 1**): three distinct and major clusters, including the sequences from *Gadidae*, *Lotidae*, and *Merluccidae* fish, supported from a high bootstrap value and one cluster external to the other ones, including *Sparidae* and *Pomacentridae* species. These species were chosen because they showed the higher homology with direct sequences analyses of three samples analyzed (samples 1−3) (**Figure 1**). The *Merluccidae* cluster includes the sequences of *M. merluccius*, *M. productus*, *M. gayi*, and *M. australis* and three whole fish identified as belonging to the *Merluccidae* family (*M. hubbsi*, *M. capensis*, and *M. merluccius*) as expected. This cluster includes a large number of sequences from samples labeled as *T. chalcogramma* (samples 4−14). Sample 14 showed high homology with *M. hubbsi* forming a branch also including *M. australis*. The tree placed *M. gayi* and samples 11−13 in one branch and *M. productus* and samples from 4 to 10 in another one. The sequences of *M. merluccius* (taken from GenBank) with the sequences of *M. merluccius* (whole fish) and *M. capensis* (whole fish) were placed in another branch.

The *Gadidae* cluster groups the sequences of *Gadidae* species taken from GenBank, the sequences obtained from *T. chalcogramma* (whole fish), and the five sequences from the samples 15 to 19. *Gadidae* and *Lotidae* were recognized as monophyletic group, well-supported by bootstrap analysis. The samples 18 and 19 may be considered *Gadidae*, for their position in the cluster (**Figure 1**) is closely related to *M. poutassou*.

Results demonstrate that mislabeling is a large-scale phenomenon since 84.2% of surimi-based products sold as *T. chalcogramma* (16/19) were shown to be prepared with species different from the one declared. In fact, only three samples may be classified as belonging to *T. chalcogramma*.

The aim of this paper was the development of a molecular method to identify the species used in the production of surimi. It is important to have reliable and useful tools that can easily identify fish species used in fish processed products, as its preparations could be used fraudulently with fish species of varying commercial values.

The whole fish considered in the study can be easily classified by visual inspection using traditional taxonomic methods. In the case of processed products, as surimi-based products, other methods are required to identify fish species and to prevent species substitution frauds. In this study, PCR and sequencing analysis were used to identify the species of fish used for surimi preparation. The technique applied has the advantage that only a small amount of material is required. The method could be used for cooked fish and fish processed and stored at frozen temperatures. Surveillance to detect frauds in fish products is useful to reassure all parties within the food supply chain. This is particularly true for allergic people who cannot eat particular fish species for health problems and require assurance that they are purchasing foods conforming to their needs. The study indicates that DNA is a robust molecule that is not damaged during extended periods of fish storage or by processing and freezing. DNA can be amplified by PCR, and amplified products can be directly used for sequencing and analysis for species identification. The direct sequencing method with the use of programs for analyses comparison was applied to enforce labeling regulation in the authentication of fish and to prevent the substitution of fish species in surimi-based products. The molecular method enabled the identification of fish species substitution frauds and gave a contribution for a clear and transparent labeling of fish products, thus meeting the growing consumer's demand of correct information about food quality and safety. Further studies are being carried out to simplify the process of species identification in fish products so that it can be commonly applied at forensic laboratories.

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