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Molecular methods for the differentiation of species used in production of cod-fish can detect commercial frauds

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Abstract

A molecular approach was used to differentiate eight species commonly used in the production of cod-fish. Since visual identification can only be applied easily on whole fish, we used the PCR method to obtain a short fragment of the *cytochrome B* (*cytB*) gene that was then analyzed by RFLP, SSCP and DGGE. While RFLP and SSCP resulted in differentiation of only some of the species tested, DGGE was able to produce patterns that made possible the identification of the species considered. The application of molecular methods to the identification of the species in this study was found to be useful, fast and reliable.

Keywords: Identification; Cod-fish; PCR-based techniques; *cytB* gene

1. Introduction

In the last few years, consumers have become more and more demanding in the choice of foodstuff. Important parameters that drive this selection are the safety and the correspondence of the ingredients with what has been declared on the label. This fact calls for the availability of reliable and rapid methods to assess the hygienic quality of food and to identify food components. Especially 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 foodstuff is an important aspect to consider when allergic problems towards specific species, or ethical/religious issues are taken into account. Moreover the definition of the species contained in a specific preparation is a crucial step in the food quality control to avoid possible commercial frauds. In this last case, it is very important to assess that species of high commercial value are not sold, partially or entirely substituted with other species of lower commercial value.

An increasing number of studies and techniques have become available to identify different animal species in food. Histological analysis of tissues, fatty acids composition, antigen–antibody gel diffusion (Kangethe, Gathuma, & Lindqvist, 1986), SDS-PAGE (Zerifi, Labie, & Bernard, 1991), ELISA-assays (Andrews, Berger, Mageau, Schwab, & Johnston, 1992; Martin, Wardale, Jones, Hernandez, & Patterson, 1991) and Isoelectricfocusing (IEF) (King, 1984; Renon, Colombo, Colombo, Biondi, & Malandra, 2001) are only some of the numerous techniques available for the species identification.

Recently, molecular methods based on nucleic acids amplification (PCR) have been developed and employed to reach the goal of the species differentiation. Usually PCR is coupled with other techniques able to detect differences in the sequence of the products obtained by PCR amplification. So far restriction fragment length polymorphism (RFLP) (Meyer, Hofelein, Luthy, & Candrian, 1995) and single strand conformation polymorphism (SSCP) (Rehbein, Kress, & Schmidt, 1997) have been the techniques most frequently used for this purpose.

Regarding fish-species identification, methods such as IEF (AOAC, 1998), liquid chromatography (Osman,

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Asoor, & Marsh, 1987), immuno-diffusion (Carrera et al., 1996) and molecular methods (Barlet & Davidson, 1991; Céspedes et al., 1998; Cocolin, D'Agaro, Manzano, Lanari, & Comi, 2000; O'Reilly & Wright, 1995) have been used.

In this study we used different PCR-based methods to differentiate the fish species normally used for the production of cod-fish. RFLP, SSCP, denaturing gradient gel electrophoresis (DGGE) and direct sequencing of a small portion of the *cytB* gene were employed.

2. Materials and methods

2.1. Fish species

The species studied in this paper are the following (in parenthesis is reported the number of the samples for each species considered in the study): *Gadus morhua* (2), *G. macrocephalus* (1), *G. ogac* (1), *Molva molva* (2), *Melanogrammus aeglefinus* (1), *Brosmi brosme* (1), *Pollachius virens* (3) and *Theragra calchograma* (1). The samples of the species were collected from local markets and identified based on their morphological characteristics.

2.2. DNA extraction

Extraction of DNA was performed using the GenElute Mammalian Genomic DNA Kit (Sigma, Milan, Italy) starting from about 0.1 g of fish muscle. DNA was re-suspended in 50 μl of sterile water and used for PCR amplification.

2.3. PCR protocol

PCR was performed in a final volume of 50 μl using a Mini Cycler machine (Genenco, Florence, Italy). The reaction mix contained 10 mM TrisHCl, pH 8, 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM dNTPs each (dATP, dCTP, dGTP, dTTP), 0.2 μM of each primer, 1.25 U *Taq*-polymerase (Applied Biosynthesis, Milan, Italy) and 2 μl of the extracted DNA. The primers used for the amplification were *cytB*1 (5′-CCA TCC AAC ATC TCA GCA TGA TGA AA-3′) and *cytB*2 (5′-CCC CTC AGA ATG ATA TTT GTC CTC-3′) described by Barlet and Davidson (1991). When PCR products were subjected to DGGE analysis, a GC-clamped (5′-GCC AGC GGC CCG GCG CGG GCC CGG CGG CGG GGG CCG CGG C-3′) *cytB*1 primer was used in the amplification reaction to increase the sensitivity of the method for the detection of point mutations, as previously described (Sheffield, Cox, Lerman, & Myers, 1989). A step-down PCR was carried out. Primer annealing for the first 15 cycles of amplification was performed at 48 °C for 1 min and for the remaining 20 at 38 °C for the same time. Denaturation was at 95 °C for 1 min and extension at 68 °C for 1 min. An initial denaturation at 95 °C for 5 min and a final extension at 68 °C for 7 min completed the cycle. Five μl of the PCR product were analyzed in a 2% agarose gel (Roche, Milan, Italy) in 0.5X TBE (1X is 45 mM Tris–borate, 1 mM EDTA, pH 8), containing 0.5 μg/ml ethidium bromide. After the electrophoretic run, DNA molecules were visualized under UV light and analyzed by using the BioImaging System GeneGenius (SynGene, Cambridge, United Kingdom).

2.4. Restriction analysis

Five μl of the PCR product were subjected to restriction analysis using the following restriction endonucleases: *Nla*III, *Nla*IV, *Rsa*I, *Taq*I, *Fok*I, *Alu*I and *Eco*RV (Roche, Milan, Italy) following the instructions of the manufacturers. Enzymes were chosen on the basis of the results obtained by using the molecular biology software "DNA Strider". Fragments were run in 3% agarose gel in 0.5X TBE, containing 0.5 μg/ml ethidium bromide. Restriction patterns were detected under UV light and analyzed using the BioImaging System GeneGenius. Analyses were performed at least three times.

2.5. SSCP analysis

After DNA amplification, 3 μl of PCR product were mixed with 26 μl of a denaturing solution containing 95% (vol/vol) formamide (Sigma, Milan, Italy), 0.2 M NaOH, 0.5 g/l bromophenol blue (Sigma, Milan, Italy) and 0.5 g/l xylene cyanol, and 1 μl of hydroxy-methyl–mercury (Prodotti Gianni, Milan, Italy). Tubes were treated at 95 °C for 5 min and cooled down immediately in an ice-bath. A total of 7 μl were immediately loaded in a CleanGel 10% 48S (Pharmacia Biotech, Milan, Italy) and run for 60 min at 600 V in the Multiphor II apparatus (Pharmacia Biotech, Milan, Italy) at 15 °C. SSCP patters were stained using the Silver Staining DNA kit (Pharmacia Biotech, Milan, Italy) and analyzed using the BioImaging System GeneGenius. Analyses were performed at least three times.

2.6. DGGE analysis

The Dcode Universal Mutation Detection System™ (BioRad, Hercules, CA) was used for the sequence-specific separation of the PCR products. Electrophoresis was performed in a 0.8 mm polyacrylamide gel (8% [wt/vol] acrylamide:bisacrylamide 37.5:1) containing a 30–50% urea–formamide denaturing gradient (100% corresponds to 7 M urea and 40% [wt/vol] formamide) in TAE 1.25X (1X is 40 mM Tris–acetate, 1 mM EDTA, pH 8) increasing in the direction of the electrophoretic run. The gels were subjected to a constant voltage of 85 V for 15 h at 60 °C. After electrophoresis, they were