Identification of Anchovy (*Engraulis encrasicholus* L.) and Gilt Sardine (*Sardinella aurita*) by Polymerase Chain Reaction, Sequence of Their Mitochondrial Cytochrome b Gene, and Restriction Analysis of Polymerase Chain Reaction Products in Semipreserves

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A method of authenticating anchovy (*Engraulis encrasicholus* L.) and gilt sardine (*Sardinella aurita*) semipreserves (salt-cured and fillets in oil) has been developed by polymerase chain reaction (PCR) followed by sequence and restriction site analysis. The amplification of a fragment of the cytochrome b gene by universal primers produced a 376 base pairs (bp) fragment in all samples analyzed. Digestion of PCR products with XhoI, TaqI, AluI, and HinfI endonucleases yelded species-specific profiles distinguishing anchovy from gilt sardine. Therefore, the restriction length fragment polymorphism (RLFP) technique can be used to determine the species identity of anchovy and gilt sardine in semipreserves.

Keywords: *Polymerase chain reaction; species; fish; Engraulis encrasicholus L.; Sardinella aurita; mitochondrial DNA; cytochrome b gene*

INTRODUCTION

The identification of processed food is mandatory for its correct labeling and value assessment. Generally, the consumer is protected by a number of rules governing this matter. As far as anchovy semipreserves are concerned, Italian law (RDL no. 1548, 1927) states that the word "Anchovy" defines only the species *Engraulis encrasicholus L*.

Anchovy semipreserves as a salt-cured product or fillets in oil are particularly appreciated in Italy. In this country, the anchovy catch in 1995 was 41 102 tons, the majority of which was destined for processing, in particular, 11 000 tons for the production of salt-cured anchovies and 13 000 tons for fillets in oil. Domestic production, however, is not sufficient to meet market demand; therefore, considerable amounts of salt-cured anchovies (ca. 5500 tons) and fillets in oil (ca. 2500 tons) are imported from other countries, especially from Spain, Greece, Argentina, and Turkey (*1*).

The European anchovy (*Engaulis encrasicholus* L.) has a characteristic taste, slightly bitter, and a muscular texture. Because of its high popularity, high demand, and poor catch, anchovy semipreserves are susceptible to substitution using closely related fish species such as gilt sardine (*Sardinella aurita*), sprat (*Clupea sprattus* L.), and sardine (*Sardina pilchardus*). In particular, anchovies are often substituted with gilt sardine. Even though anchovy semipreserves look and taste like those less-valued products from other closely related species, substitution of anchovy (*Engraulis encrasicholus* L.) with other fish species is fraudulent; so, specific identification of anchovy is required for detection of inappropriately labeled products. Although observation of morphological features provides a ready means of species identification, these characteristics are often lost during processing, and the species are no longer recognizable. In the case of anchovy and gilt sardine semipreserves, conventional processing involves beheading, gutting, salting, and filleting, so distinguishing features are completly removed and the identity of the fish cannot be established on the basis of morphological features.

As an alternative to morphological analysis, many analytical methods have been developed for fish species identification. Most of them rely on the analysis of proteins by electrophoretic techniques such as isoelectric focusing (IEF) and capillary electrophoresis (CE), or high-performance liquid chromatography or immunoassay techniques such as the precipitation test or enzyme-linked immunoassay (Elisa) techniques (*2*).

Often, protein-based assays are not suitable for species identification, especially in the case of preserved foods. In fact, proteins lose their biological activity soon after the fish has died; their presence and characteristics depend on the examined cell type and many of them are heat-labile (*3*, *4*). For species identification it would be preferable to analyze DNA rather than proteins. DNA is contained in every kind of cell and is the same in all cell types of an organism; therefore, the same DNA analysis can be applied independently of the tissue or organ processed, whereas proteins vary from tissue to

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Figure 1. Electrophoretic analysis of PCR products of 376 bp from cytochrome b gene on 7% acrylamide gel, stained with ethidium bromide: (from left to right) molecular weight marker (8÷587 bp) (M); *Engraulis encrasicholus* L., fresh (lane 1),; salt-cured (lane 3), in oil (lane 5); *Sardinella aurita* fresh (lane 2), salt-cured (lane 4), in oil (lane 6).

tissue. Moreover, DNA is less affected by food-processing technologies than proteins are.

DNA analysis, compared to protein analysis, is more advantageous because it allows direct examination of the nucleotide sequences of DNA either inside the nucleus (nDNA) or in the mitochondria (mtDNA) and examination of DNA regions with different degrees of mutation rate. Moreover, it is able to detect polymorphisms that do not change the amino acid sequence because of the degeneracy of the genetic code (*4*−*6*).

Most genetic approaches to determine fish species identity are based on targeted amplification of conserved mtDNA regions by PCR technique, followed by sequencing of the amplified fragments (*3, 7*).

As to species identification, mtDNA was preferred to nDNA for a number of reasons: it is much smaller than nDNA (approximately 5 orders of magnitude), there are several copies of mtDNA inside a cell, and introns are absent (*6*). The use of the universal primers, designed by Kocher et al. (*8*), made it possible to amplify and sequence the conserved region of cytochrome b gene in more than 100 animal species, including mammals (*9*), birds and fish (*10, 11*), anphibians, and some invertebrates. Direct sequence analysis of PCR products obtained with the primers designed by Kocher et al. (*8*), or with slightly modified ones, was used to assess inter- and intraspecific differentiation of some fish species: Atlantic cod (*Gadus mohrua*) (*11, 12*), some salmonid species (*13*−*15*), and some tuna species (*10*), presenting interspecific variations that may be useful for their identification.

This paper deals with a simple, inexpensive method that is useful for routine analysis to distinguish anchovy from gilt sardine. The authors demonstrate that amplification of the conserved region of mitochondria cytochrome b by PCR technique and following digestion of restriction enzyme can be applied for this purpose.

As GeneBank and other databases essential to sequences of cytochrome b gene of *Engraulis encrasicholus* L. (anchovy) and *Sardinella aurita* (gilt sardine) were not available, part of the mitochondrial cytochrome b gene has been cloned and sequenced to search the distinguishing species-specific restriction sites.

RLFP analysis, submitted to digestions by restriction enzymes and determined on the basis of a known sequence of target DNA, allows detection of genetic variations between and within species (*16*) because the base substitution in the restriction target sequence causes the destruction or the creation of a restriction site.

This method can be applied to fresh and semipreserved samples (salt-cured and fillets in oil) in order to discover fraudulent or unintentional mislabeling of *Engraulis encrasicholus* L. (anchovy) and *Sardinella aurita* (gilt sardine) in the preserved food market.

MATERIALS AND METHODS

Sample Selection and DNA Extraction. Raw *Engraulis encrasicholus* L. and *Sardinella aurita* were collected, during the year, in different fish-markets of Cefalù (Palermo), Italy, and the semipreserved samples (salt-cured and fillets in oil) were obtained from local salters. Every specimen was morphologically identified following the keys of chard proposed by the FAO (*17*). Ten individuals of each species were analyzed.

Nuclear and mitochondrial DNA were extracted from fish muscle samples (raw and semipreserved) according to the modified method described by DeSalle et al. (*18*). Muscle tissue (1 g) of each sample was homogenized by Ultra-Turrax in 10 mL of extraction buffer (0.01 M NaCl; 0.01 M Na_2EDTA; and 0.01M Tris−HCl pH 8); 100 μL of proteinase k (20 mg/mL) and SDS at final concentration of 1% were added. The samples were incubated overnight at 37 °C. After incubation, DNA was extracted twice with an equal volume of Tris−HCl saturated phenol and once with an equal volume of chloroform. Then DNA was precipitated by adding 1/10 of volume of sodium acetate pH 5.3 and 2.5 volumes of ice-cold 70% ethanol. The tubes were centrifuged at 2000 rpm for 30 min at 4 °C. The pellet was allowed to dry at room temperature and resuspended in 500 μL of sterile distilled water. The concentration of DNA was estimated by UV absorbance at 260 nm.

PCR Amplification of a Fragment of the Mitochondrial Cytochrome b Gene. PCR was employed to amplify a segment of 376 base pairs (bp) of mitochondrial cytochrome b gene. PCR amplification reactions were carried out in a total volume of 50 μL of a solution containing 100 ng of DNA template, PCR buffer (Tris−HCl 10 mM pH 8.3, KCl 50 mM); 50 pmol of each primer; 200 μM of each dNTP; 2 mM of $MgCl_2$; and 0.8 U of Ampli Taq DNA Polymerase (Perkin-Elmer).

The primers used for PCR amplification L 14841 (5′-AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA-3′) (forward primer) and H 15149 (5′-AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3′) (reverse primer) were designed by Kocher et al. (*8*) to amplify a conservated region of cytochrome b gene.

PCR was carried out in a GeneAmp PCR System 9700 (PE Applied Biosystem, Foster City, CA). The following PCR conditions were used: a denaturation step at 95 °C for 5 min, followed by 35 cycles consisting of 95 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 7 min.

The reaction products of PCR (5 μL) were analyzed by 7% polyacrylamide gel in TBE buffer (0.045 M Tris−borate, 0.001 M EDTA pH 8). Electrophoretic separation was performed at 150 V for 1 h and then gel was stained in a solution containing 0.5 μg/mL ethidium bromide. The size of the resulting DNA fragments was compared with a commercial 500 bp ladder (8÷587 bp) (Marker V, Roche Molecular System, Inc., Branchburg, NJ). The resulting DNA fragments were visualized by UV transillumination and photographed.

Cloning and Sequencing of PCR Products. PCR products were purified and concentrated by a volume of 10 μL by using Microcon 100 Centrifugal Filter Devices (Millipore Corporation, Bedford, MA) with a nucleotide cutoff of 120 bp of double-stranded DNA. The purified and concentrate PCR products were cloned by using TOPO TA cloning kit (Invitrogen, Carlsbad, CA) and recombinant plasmids were recovered from cells grown in LB medium by using a Miniprep kit (Qiagen GmbH, Hilden, Germany), according to the manufac-

		10	20	30	40	50	
Sardinella aurita	1	AAAAAGCTTC	CATCCAACAT	CTCAGCATGA	TGAAATTTTG	GGTCACTTCT	50
Engraulis encrasicholus L.	1	AAAAAGCTTC	CATCCAACAT	CTCAGCATGA	TGAAATTTTG	GATCCCTTTT	50
		60	70	80	90	AluI 100	
Sardinella aurita	51	AGGACTGTGT	TTAGCGACAC	AAATCCTAAC	AGGTCTGTTT	TTAGCTATAC	100
Engraulis encrasicholus L.	51	AGGACTATGC	TTGGCCACAC	AAATCCTTAC	AGGACTTTTC	CTAGCCATAC	100
		110	120	130	140	150	
Sardinella aurita	101	ATTATACCTC	AGACATTGCC	ACCGCCTTCT	CCTCCGTTGC	CCACATTTGC	150
Engraulis encrasicholus L.	101	ACTACACCTC	AGACATCGCT	ACCGCTTTCT	CGTCAGTGGC	CCACATTTGT TaqI	150
		160	170	HinfI TaqI 180	190	200	
Sardinella aurita	151	CGTGACGTCA	ACTATGGATG	ACTGATTCGA	AGCATGCACG	CAAACGGAGC	200
Engraulis encrasicholus L.	151	CGAGACGTAA	ATTACGGGTG	ACTAATCCGG	AATATGCATG	CAAACGGAGC	200
		210	220	230	240	HinfI 250	
Sardinella aurita	201	ATCTTTCTTC	TTCATTTGCA	TTTACGCCCA	CATTGGACGA	GGACTCTACT	250
Engraulis encrasicholus L.	201	CTCATTCTTC	TTCATCTGCA	TCTATGCACA	CATTGCTCGA XhoI TaqI	GGACTGTACT	250
		260	270	280	290	300	
Sardinella aurita	251	ACGGGTCTTA	CCTCTATAAG	GAAACCTGAA	ATATTGGGGT	CGTTCTCCTT	300
Engraulis encrasicholus L.	251	ACGGTTCTTA	TCTTTACATA	GAAACTTGAA	ACATCGGAGT	AGTACTACTT	300
		310	320	330	340	350	
Sardinella aurita	301	CTCCTAGTCA	TGATGACCGC	CTTCGTAGGC	TACGTCCTTC	CATGAGGACA	350
Engraulis encrasicholus L.	301	CTTTTAGTTA	TGATGACTGC	CTTCGTTGGG	TACGTACTAC	CCTGAGGACA	350
		360	370	380	390	400	
Sardinella aurita	351	AATATCATTC	TGAGGGGCTG	CAGTTT			400
Engraulis encrasicholus L.	351	AATATCATTC	TGAGGGGCTG	CAGTTT			400

Figure 2. Alignment of DNA sequences of amplified fragment of cytochrome b gene. Restriction sites for *Engraulis encrasicholus* L. and *Sardinella aurita* are shown with a shadow.

		10	20	30	40	50	
Sardinella aurita	1	KKLPSNISAW	WNFGSLLGLC	LATQILTGLF	LAMHYTSDIA	TAFSSVAHIC	50
Engraulis encrasicholus L.	1	KKLPSNISAW	WNFGSLLGLC	LATQILTGLF	LAMHYTSDIA	TAFSSVAHIC	50
		60	70	80	90	100	
Sardinella aurita	51	RDVNYGWLIR	SMHANGASFF	FICIYAHIGR	GLYYGSYLYK	ETWNIGVVLL	100
Engraulis encrasicholus L.	51	RDVNYGWLIR	NMHANGASFF	FICIYAHIAR	GLYYGSYLYM	ETWNIGVVLL	100
		110	120	130	140	150	
Sardinella aurita	101	LLVMMTAFVG	YVLPWGQMSF	WGAAV			150
Engraulis encrasicholus L.	101						150

Figure 3. Alignment of amino acidic sequences of the proteins.

turer's instructions. At least ten recombinant plasmids for each species were sequenced in a ABI PRISM 310 automated sequencer (PE Applied Biosystems, Foster City, CA). Sequence analysis was performed by DNA Sequencing Analysis and Factura Software (PE Applied Biosystems) to remove ambiguous bases according to IUB standard code. The sequences were therefore aligned by Sequence Navigator Software (PE Applied Biosystems) and fitted in frame with the amino acid sequence of the proteins.

Restriction Site Analysis of PCR Products. The search for restriction endonuclease site was performed on the sequences of cytochrome b gene by McDNAse software (Hitachi Software Engineering America Ltd., San Bruno, CA).

PCR products were concentrated and purified by using Microcon 100 Centrifugal Filter Devices. Four enzymes were tested: XhoI, target sequence (C↓TCGAG); AluI, target sequence (AG↓CT); HinfI, target sequence (G↓ANTC); and TaqI, target sequence (T↓CGA) (New England BioLabs, Beverly, MA). Digests were performed in 10-μL volumes with 1 μL of amplified DNA and 10 U of enzyme, according to the reaction conditions specified by the manufacturer. Digestion reactions were incubated for 1 h at 37 °C (XhoI; AluI; HinfI) or 65 °C

(TaqI). The resulting fragments were separated by electrophoresis on 7% polyacrylamide gel in TBE buffer as previously described.

RESULTS AND DISCUSSION

Primers L 14841 and H 15149 consistently amplified a conserved fragment of 376 bp (including size of primers) of the cytochrome b gene in all fresh and semipreserved (salt-cured and fillets in oil) anchovy and gilt sardine samples analyzed (Figure 1). PCR products from 5 different anchovies and 5 different gilt sardines were cloned and sequenced (Figure 2); the amino acidic sequence is shown in Figure 3.

The sequence of the 376 bp fragment of the cytochrome b gene in *Engraulis encrasicholus* L. and *Sardinella aurita* was different for each studied species. The nucleotidic sequences revealed interspecific variations. The sequences differed from each other for 64 positions; 38 substitutions were transitions (i.e., the interchange of pyrimidines, C ↔ T, or purines A ↔ G); 14 substitutions were transversions (i.e., a change from a purine to a pyrimidine or vice versa) according to Kocher et al (*8*) who showed that within a particular species, and also between closely related species, transitions are more common than tranversions are. There were fewer amino acid substitutions than nucleotidic ones. So, amino acidic sequences of proteins differed from each other only for three amino acids: serine/asparagine for anchovy, and glycine/alanine and lysine/methionine for gilt sardine, according to the degeneracy of the genetic code.

In searching for appropriate endonucleases distinguishing and identifying PCR products of anchovy from those of gilt sardine, the two sequences were compared by using suitable software to find different restriction sites that could generate species-specific restriction profiles. On the basis of these studies, four restriction enzymes potentially useful for this purpose were found: XhoI, AluI, HinfI, and TaqI (Figure 2). The analysis of the sequences shows the presence of HinfI and AluI sites in gilt sardine and their lack in anchovy. On the other hand only one XhoI site is present in anchovy and not in gilt sardine. Finally, TaqI sites were found in both species but with different restriction patterns for the two different species. The results from restriction site analysis of PCR products were in agreement with those expected from sequence analysis. In Figures 4, 5, 6, and 7 restriction profiles obtained after digestion of PCR products of fresh and semipreserved anchovy and gilt sardine are shown.

As illustrated in Figure 4, anchovy has an XhoI restriction site that gives two fragments of 139 and 237 bp (including primer regions), as expected from sequence analysis and does not cleave PCR products from gilt sardines. Two TaqI restriction sites yielding three DNA fragments of 95, 129, and 152 bp are also present in anchovy PCR products, whereas a single TaqI restriction site is present in gilt sardine PCR products yelding two fragments of 178 and 198 bp (Figure 5).

A single restriction site for AluI is present in gilt sardine PCR products yielding two expected fragments of 95 and 281 bp (Figure 6) but does not cleave anchovy. Finally, two HinfI restriction sites give three fragments of 77, 124, and 175 bp (Figure 7) only in gilt sardine, as expected.

The restriction patterns from all anchovy and gilt sardine samples did not show intraspecific polymorphism for the four restriction endonucleases tested. It should be stressed that, although it is likely that hypervariable sequences of mtDNA molecule will prove to be more useful in studies of intraspecific variations (*4, 19, 20*), relatively conserved sequences may be ideally suitable for interspecific comparison (*14*).

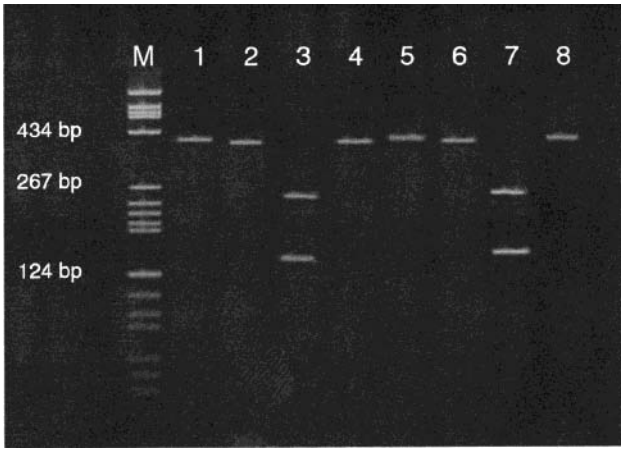

Figure 4. RLFP patterns of *Engraulis encrasicholus* L. and *Sardinella aurita* digested with XhoI on 7% acrylamide gel, stained with ethidium bromide: (from left to right) molecular weight marker (8÷587 bp) (M); fresh, undigested *Engraulis encrasicholus* L. (lane 1) and *Sardinella aurita* (lane 2); fresh, digested *Engraulis encrasicholus* L. (lane 3) and *Sardinella aurita* (lane 4); in oil, undigested *Engraulis encrasicholus* L. (lane 5) and *Sardinella aurita* (lane 6); in oil, digested *Engraulis encrasicholus* L. (lane 7) and *Sardinella aurita* (lane 8).

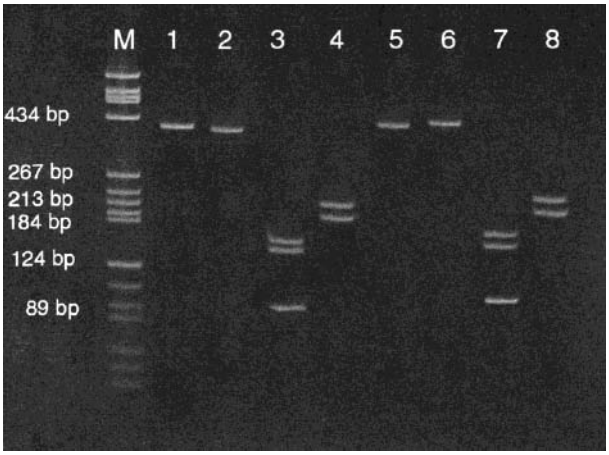

Figure 5. RLFP patterns of *Engraulis encrasicholus* L. and *Sardinella aurita* digested with Taq I on 7% acrylamide gel, stained with ethidium bromide: (from left to right) molecular weight marker (8÷587 bp) (M); fresh, undigested *Engraulis encrasicholus* L. (lane 1) and *Sardinella aurita* (lane 2); fresh, digested *Engraulis encrasicholus* L. (lane 3) and *Sardinella aurita* (lane 4); in oil, undigested *Engraulis encrasicholus* L. (lane 5) and *Sardinella aurita* (lane 6); in oil, digested *Engraulis encrasicholus* L. (lane 7) and *Sardinella aurita* (lane 8).

The extraction method used in the present work allowed us to obtain sufficient amounts of mtDNA and amplification products from all the samples tested (fresh and semipreserved) showing that preserving conditions (salt-curing or filleting and packaging in oil) did not affect the quality of mtDNA.

The same result was obtained with fresh and semipreserved samples of anchovy and gilt sardine, so the mtDNA RFLP method based on the sequencing of a fragment of cytochrome b gene can be applied with good

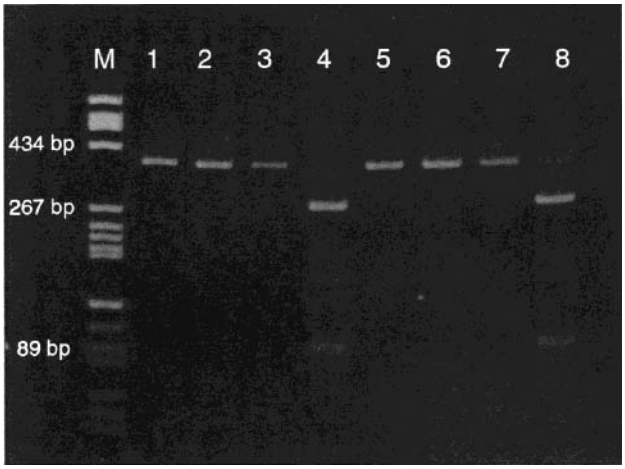

Figure 6. RLFP patterns of *Engraulis encrasicholus* L. and *Sardinella aurita* digested with AluI on 7% acrylamide gel, stained with ethidium bromide: (from left to right) molecular weight marker (8÷587 bp) (M); fresh, undigested *Engraulis encrasicholus* L. (lane 1) and *Sardinella aurita* (lane 2); fresh, digested *Engraulis encrasicholus* L. (lane 3) and *Sardinella aurita* (lane 4); in oil, undigested *Engraulis encrasicholus* L. (lane 5) and *Sardinella aurita* (lane 6); in oil, digested *Engraulis encrasicholus* L. (lane 7) and *Sardinella aurita* (lane 8).

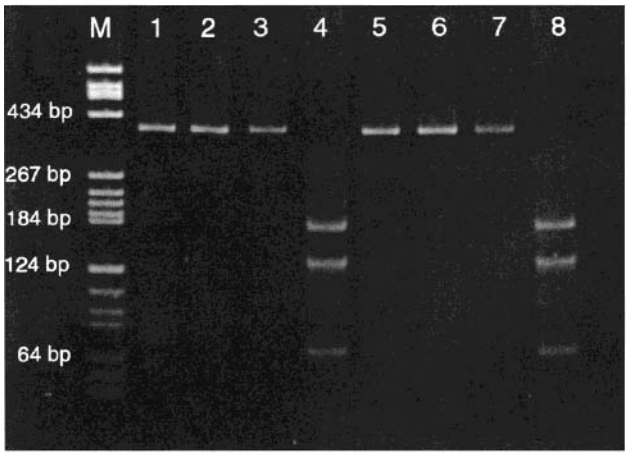

Figure 7. RLFP patterns of *Engraulis encrasicholus* L. and *Sardinella aurita* digested with HinfI on 7% acrylamide gel, stained with ethidium bromide: (from left to right) molecular weight marker (8÷587 bp) (M); fresh, undigested *Engraulis encrasicholus* L. (lane 1) and *Sardinella aurita* (lane 2); fresh, digested *Engraulis encrasicholus* L. (lane 3) and *Sardinella aurita* (lane 4); in oil, undigested *Engraulis encrasicholus* L. (lane 5) and *Sardinella aurita* (lane 6); in oil, digested *Engraulis encrasicholus* L. (lane 7) and *Sardinella aurita* (lane 8).

results to identify fresh and semipreserved samples of anchovy and gilt sardine and to detect mislabeling and substitution of species.

Moreover, this technique shows to be advantageous because it is simple, cheap, and especially useful in routine analysis of large numbers of samples.

LITERATURE CITED

(1) Sebastio, P.; Ambroggi, F.; Baldrati, G.; Gola, S.; Saccani, G.; Draisci, R.; Lucentini, L. The hygienic-sanitary risk associated with the presence of histamine in the manufacture of anchovy semipreserves (*Engraulis encrasicholus* L.). *Ind. Conserve* **1998** (2) 193−205.

(2) Sotelo, C.; Gallardo, J. M.; Pérez-Martín, R. I. Fish species identification in seafood products. *Trends Food Sci. Technol.* **1993**, *4* (12), 395−401.

(3) Barlett, S. E.; Davidson, W. S. FINS (Forensically Informative Nucleotide Sequencing): A procedure for identifying the animal origin of biological specimens. *BioTechniques* **1992**, *12*, 408−411.

(4) Ferguson, A.; Taggart, J. B.; Prodöhl, P. A.; McMeel, O.; Thompson, C.; Stone, C.; McGinnity, P.; Hynes, R. A. The application of molecular markers to the study and conservation of fish populations, with special reference to *Salmo*. *J. Fish Biol.* **1995**, *47* (Supp. A), 103−126.

(5) Wilson, A. C.; Cann, R. L.; Carr, S. M.; George, M.; Gyllensten, U. I.; Helm-Bychowski, K.; Higuchi, R. G.; Palumbi, S. R.; Prager, E. M.; Sage, R. D.; Stoneking, M. Mitochondrial DNA in relation to evolutionary genetics. *Biol. J. Linn. Soc.* **1985**, *26*, 375−400.

(6) Moritz, C.; Dowling, T.; Brown, W. M. Evolution of animal mitochondrial DNA: relevance for population biology and systematics. *Annu. Rev. Ecol. Syst.* **1987**, *18*, 269−292.

(7) Ram, J. L.; Ram, M. L.; Baidoun, F. Authentication of canned tuna and bonito by sequence and restriction site analysis of polymerase chain reaction products of mitochondrial DNA. *J. Agric. Food. Chem.* **1996**, *44*, 2460−2467.

(8) Kocher, T. D.; Thomas, W. K.; Meyer, A.; Edwards, S. V.; Pääbo, S.; Villabianca, F. X.; Wilson, A. C. Dynamics of motochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 6196−6200.

(9) Irwin, D. M.; Kocher, T. D.; Wilson, A. C. Evolution of the cytochrome b gene of mammals. *J. Mol. Evol.* **1991**, *32*, 128−144.

(10) Barlett, S. E.; Davidson, W. S. Identification of *Thunnus* tuna species by the Polymerase Chain Reaction and direct sequence analysis of their mitochondrial cytochrome b genes. *Can. J. Fish. Aquat. Sci.* **1991**, *48*, 309−317.

(11) Carr, S. M.; Marshall, H. D. Detection of intraspecific DNA sequence variation in the mitochondrial cytochrome b gene of Atlantic cod (*Gadus morhua*) by the polymerase chain reaction. *Can. J. Fish. Aquat. Sci.* **1991**, *48*, 48−52.

(12) Pepin, P.; Carr, S. M. Morphological, meristic and genetic analysis of stock structure in juvenile Atlantic cod (*Gadus morhua*) from the Newfoundland shelf. *Can. J. Fish. Aquat. Sci.* **1993**, *50*, 1924−1933.

(13) Hartley, S. E.; Bartley, S. E.; Davidson, W. S. Mitochondrial DNA analysis of Scottish populations of Artic Charr, *Salvelinus alpinus* L. *J. Fish Biol.* **1992**, *40*, 219−224.

(14) Lockwood, S. F.; Dillinger, J.; Birt, T. P.; Green, J. M.; Snyder, T. P. Phylogenetic relationships among members of the Coregonidae inferred from direct sequencing of PCR-amplified mitochondrial DNA. *Can. J. Fish. Aqat. Sci.* **1993**, *50*, 2112−2118.

(15) McVeigh, H. P.; Barlett, S. E.; Davidson, W. S. Polymerase chain reaction direct sequence analysis of the cytochrome b gene in *Salmo salar*. *Aquaculture* **1991**, *95*, 225−233.

(16) Avise, J. C.; Lansman, R. A.; Shade, R. O. The use of restriction endonucleases to measure mitochondrial DNA sequence, relatedness in natural populations. I. Population structure and evolution in genus *Peromyscus*. *Genetics* **1979**, *92*, 279−295.

(17) FAO. *Catalogue des noms de poissons ayant una importance commerciale en Méditerranée.* Food and Agriculture Organization of United Nations: Rome, 1960; Catalogue I.

(18) DeSalle, R.; Williams, A. K.; George, M. Isolation and characterization of animal mitochondrial DNA. *Methods Enzymol.* **1993**, *224*, 176−203.

(19) Hall, H. J.; Nawrocki, L. W. A rapid method for detecting mitochondrial DNA variation in the brown trout, *Salmo trutta*. *J. Fish Biol*. **1995**, *46*, 360−364.
(20) Hansen, M. M.; Loechcke, V. Genetic differentiation among Danish brown trout populations, as detected by RFLP anlysis of PCR amplified mitochondrial DNA segments. *J. Fish Biol*. **1996**, *48*, 422−436.

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