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Discrimination of Three *Trachurus* Species Using Both Mitochondrial- and Nuclear-Based DNA Approaches

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A double-DNA approach was developed to discriminate the three *Trachurus* species that abide in European waters: *T. trachurus*, *T. mediterraneus*, and *T. picturatus*. The analysis aimed at both mitochondrial and nuclear loci. Polymerase Chain Reaction (PCR) amplification of the cytochrome *b* gene of mtDNA was followed by restriction analysis with three species-specific enzymes: *Nla*III, *Nci*I, and *Bsm*AI. Digestion with these endonucleases yielded species-specific electrophoretic profiles. The universality of the results was verified by screening a large number of individuals from 12 geographical regions covering most of the distribution of the species. Additionally, the nuclear multicopy 5S rRNA gene was selected as an alternative candidate for the discrimination of the three *Trachurus* species. A simple agarose gel electrophoretic analysis of the amplicons proved to be capable of leading to unambiguous identification of the three *Trachurus* species. Thus, the double-DNA methodology presented here allows the accurate discrimination of *Trachurus* fish species and the detection of commercial fraud.

KEYWORDS: Genetic identification; PCR-RFLP; cytochrome *b*; 5S rDNA; *Trachurus*

INTRODUCTION

Three species of the genus *Trachurus*, *T. trachurus*, *T. mediterraneus*, and *T. picturatus*, are found in European waters (*1*−*3*). These species have overlapping habitats and are commercially exploited, especially in the northeastern Atlantic waters (International Council for the Exploration of the Seas, 2001). The observation of morphological features provides a good means for adult *Trachurus* species identification. However, similarities of specific life-history traits and sympatric occurrence make their identification difficult, especially in the case of young juveniles, as morphological features cannot always be easily distinguished. This results in unintentional or fraudulent mislabeling of fish. For this reason, new analytical methods must be developed to be used for the identification of mislabeled *Trachurus* products in order to detect and eliminate commercial fraud. These new approaches could also provide a new method of egg identification as all three species have eggs similar to those of horse mackerel, and they cannot be properly identified with the current methods. Furthermore, the development of genetic markers for fish species identification will increasingly be required for legislation designed to regulate regional fisheries and products in Europe.

Many analytical methods have been developed for fish species identification and/or discrimination. Most rely mainly on the analysis of proteins, high-performance liquid chromatography, and immunoassays (*4*−*6*). However, advances in molecular biology techniques have allowed the development of DNA-based methods (*7*, *8*). Most of the genetic approaches to the determination of species identity are based on the amplification of a region of mitochondrial DNA by the Polymerase Chain Reaction (PCR) followed either by direct sequence analysis of the amplified fragment or by restriction fragment length polymorphism (RFLP) analysis (see, e.g., refs *5* and *9*−*11*). MtDNA is a broadly used genetic tool, and one of its advantages is the high copy numbers of the mitochondrial genome (*12*) compared to nuclear genome within a cell. Thus, mtDNA-based methods can be applied to small amounts of tissue, such as eggs or processed samples. The mitochondrial-encoded cytochrome *b* gene is a useful genetic marker for species identification because of its interspecific heterogeneity capable of producing species-specific restriction fragment patterns (*13*, *14*).

Apart from mtDNA, nuclear genes such as 5S ribosomal DNA (5S rDNA) could also be a suitable candidate for the genetic discrimination of related species, because in higher eukaryotes the 5S rDNA comprises a 120 bp highly conserved coding sequence and a variable nontranscribed spacer (NTS). This unit is tandemly repeated, usually arranged head to tail, and is species specific (*15*, *16*). The objective of this work was to develop a simple DNA method for the discrimination of three *Trachurus* species using both PCR-RFLP analysis of a 370 bp fragment of cytochrome *b* of mtDNA and a PCR-based agarose gel electrophoresis of the nuclear 5S ribosomal DNA.

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Table 1. Origin and Number of Individuals of the Three *Trachurus* Species Digested with the Restriction Enzymes for Cytochrome *b* Analysis

species	geographic areas	RFLP analysis
T. mediterraneus	northern Aegean Sea	11
	southern Aegean Sea	13
	Ionian Sea	13
	western Italy	6
	eastern Spain	10
	Portugal	14
T. trachurus	northern Aegean Sea	13
	southern Aegean Sea	13
	Ionian Sea	16
	western Italy	8
	Portugal	12
	northwestern Spain (VIIIb)	16
	northern Spain (VIIIc)	11
	northwestern France	6
	England	7
	western Norway	9
	southern Africa	7
T. picturatus	northern Aegean Sea	16
	southern Aegean Sea	12
	Ionian Sea	12
	western Italy	4
	Portugal	10
total		239

MATERIALS AND METHODS

Samples Collection and DNA Extraction. Sets of 239 individuals for cytochrome *b* and 100 individuals for 5S rRNA gene belonging to three different species of the genus *Trachurus* were analyzed (**Table 1**). Sampling areas covered most of the distribution of the species in European waters including *Trachurus* specimens from South Africa. White muscle tissue was taken from each individual and either stored at −30 °C or kept in 90% ethanol. Two different DNA extraction methods have been assayed to obtain high molecular weight DNA suitable for PCR amplifications.

Method 1. Total DNA was extracted from frozen fish tissue muscles according to the method described by Taggart et al. (*17*). Approximately 50 mg of muscle tissue was lysed with 375 μL of extraction buffer (0.2 M EDTA and 0.5% sarcosyl) and 10 μL of 20 μg/mL proteinase K. The mixture was incubated overnight at 55 °C. After 1 h of incubation with 10 μL of 20 μg/mL RNase, DNA was extracted once with 400 μL of phenol and once with 400 μL of chlorophorm/isoamyl alcohol (24:1) and was then centrifuged for 2−3 min at 12000*g*. The top aqueous layer was transferred to a new tube, and DNA was precipitated with 1 mL of 95% ice-cold ethanol for 4 h at 4 °C. Absolute ethanol was removed, and DNA was washed with 1 mL of 70% ethanol overnight. The centrifugation pellets were dried at room temperature (1 h) and finally resuspended in 150 μL of TE buffer (10 mM Tris and 1 mM EDTA, pH 8) and stored at −20 °C.

Method 2. For ethanol-preserved fish tissue, total DNA was extracted according to the CTAB method described by Hillis et al. (*18*). Approximately 50 mg of muscle tissue was digested with 500 μL of 2×CTAB buffer (0.1 M Tris-HCl, 0.2 M EDTA, 1.4 M NaCl, and 2% CTAB) and 10 μL of 20 μg/mL proteinase K. The mixture was incubated overnight at 55 °C. DNA was extracted once with 500 μL of phenol/chloroform/isoamyl alcohol in a 25:24:1 ratio and once with 500 μL of chloroform, followed by centrifugation for 3 min at 12000*g* and transfer of the top layer each time. The aqueous phase was carefully transferred to a new tube, and DNA was precipitated following the steps in method 1.

PCR Amplification. PCR was employed to amplify a segment of ∼370 bp of the mitochondrial cytochrome *b* gene. PCR amplification of cytochrome *b* was performed using the universal primers H_{15149} and L_{14841} described by Kocher et al. (*19*). PCR conditions were described in Karaiskou et al. (*20*).

The set of primers used for the PCR amplification of the 5S rDNA gene was designated as follows: 5SA (5′-TACGCCCGATCTCGTC-CGATC-3′) (forward primer) and 5SB (5′-CAGGCTGGTATGGCCG-TAAGC-3′) (reverse primer). These oligonucleotides correspond to primers designed by Pendas et al. (*15*) for the amplification of one unit of any tandemly arranged 5S rDNA (including both coding and NTS sequences) in salmon and rainbow and brown trout.

Double-stranded DNA amplification was performed in 25 μL reaction volumes containing 1.5 units of Taq polymerase (Gibco-BRL), 5 μL of 10× reaction buffer, 2 mM $MgCl_2$, 0.5 mM dNTPs, 20 pmol of each primer, and ∼50−100 ng of template DNA. Thermal cycling amplification conditions were as follows: initial denaturation at 95 °C for 4 min, followed by 30 cycles of strand denaturation at 95 °C for 20 s, annealing at 67 °C for 50 s, primer extension at 72 °C for 30 s, and a final 7-min elongation time at 72 °C. Electrophoresis of 5 μL of the amplified product was performed for 1 h at 100 V in 1.5% agarose gel, run in 1×TBE buffer, and the gel was stained in a solution containing 0.5 μg/mL ethidium bromide. The size of the PCR products was checked against a 100-bp DNA ladder (Gibco BRL). The resulting DNA fragments were visualized by UV transillumination and photographed.

Endonuclease Digestion and Restriction Fragment Length Analysis of Cytochrome *b*. The Bioedit (*21*) program was used to search all restriction sites present in cytochrome *b* sequences obtained after the analysis of 84 individuals of all three *Trachurus* species (*20*). A set of enzymes was chosen on the basis of the predicted specific patterns they would produce and which, in theory, would enable species identification. Another important requirement is that any restriction enzyme selected should have a target sequence with the lowest intraspecific variability. For this purpose, a high number of individuals were screened.

PCR products (3 μL) were subjected to restriction endonuclease digestions with 10 units of each enzyme in a final reaction volume of 6 μL. Incubation temperature and duration of reaction were chosen according to the manufacturer's protocol (New England Biolabs, Hertfordshire, U.K.). Digested samples were electrophoresed in 10% acrylamide gel (29:1 ratio of acrylamide/bisacrylamide) for 6 h at 260 V using 1×TBE buffer. The sizes of the resulting DNA fragments were estimated by comparison with a commercial φχ174/*Hae*III ladder (New England Biolabs, Hertfordshire, U.K.). DNA restriction fragments were visualized by a silver-staining DNA method. The glass plate with the bound gel is placed in a tray, where it is washed twice for 3 min with 200 mL of fixing solution (10% ethanol and 0.5% acetic acid). The gel was impregnated with 200 mL of 0.1% $AgNO_3$ for 10 min, washed with 200 mL of distilled water, and then developed by applying 200 mL of developing solution [1.5% NaOH, 0.8 mL of 37% formaldehyde, and 0.1 g of $Na(BH)_4$] until the bands appeared.

RESULTS AND DISCUSSION

The first step in developing genetic markers is to obtain a good quality of DNA, suitable for use in PCR amplification. The first protocol used to obtain DNA from frozen tissue (*17*) gave a good quality of total DNA, and no problem was encountered in the amplification of both mitochondrial and nuclear genes. However, when this method was tested in ethanol-preserved tissue, degraded DNA was obtained, unsuitable for PCR amplification. For this reason, the CTAB methodology was tested (*18*). In the CTAB extraction procedure, a strong cationic detergent is used as lysis buffer followed by an organic solvent extraction step to remove protein and PCR inhibitors that might be included in sample preservatives. The results revealed that the DNA recovery rate and extracted DNA quality were better when the CTAB method was used to obtain DNA from the ethanol-preserved *Trachurus* samples.

Discrimination of *Trachurus* Species by RFLP Analysis of Cytochrome *b*. A 370 bp fragment of cytochrome *b* was successfully amplified in all fresh and ethanol-preserved tissue of all three species. Sequence analysis of 239 bp of cytochrome *b* was previously obtained for 84 individuals of the three *Trachurus* species (Genbank AF489403-24) (*20*) and revealed

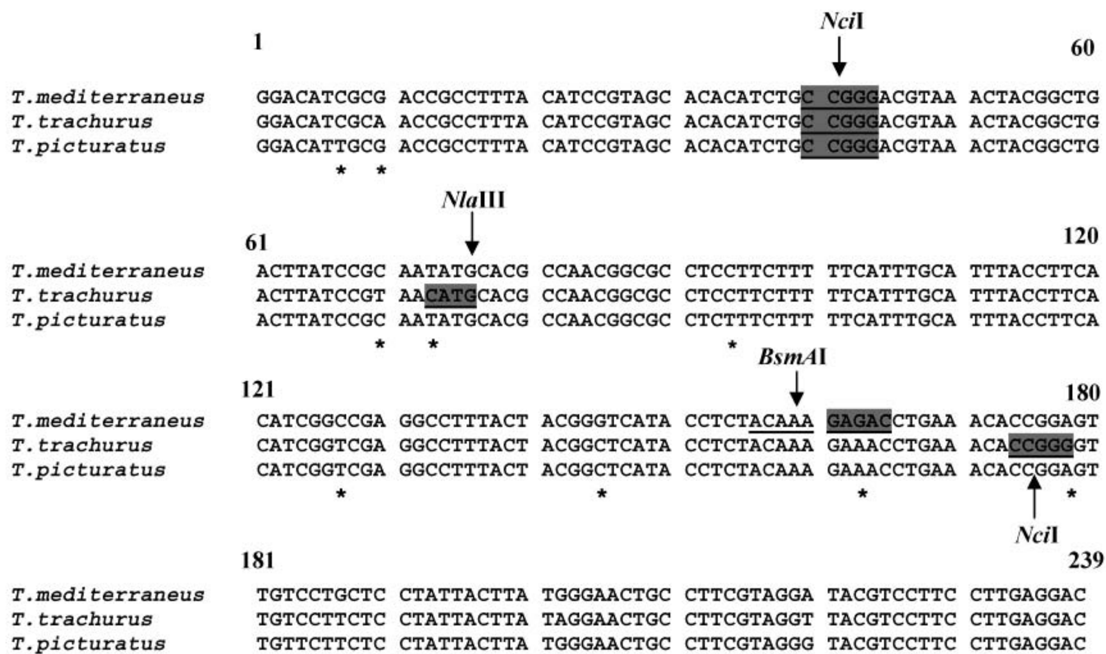

Figure 1. Alignment of cytochrome *b* sequences (239 bp) of the three *Trachurus* species obtained by Genbank (AF48994, AF448982, AF449002 for *T. mediterraneus*, *T. trachurus*, and *T. picturatus*, respectively). Restriction sites for *Nla*III, *Nci*I, and *Bsm*AI are shaded. Asterisks refer to polymorphic sites between the three sequences.

fixed interspecific variations. Specifically, eight and six species-specific positions distinguished *T. trachurus* from *T. mediterraneus* and *T. picturatus*, respectively, and four nucleotide sites distinguished *T. mediterraneus* from *T. picturatus*. The validity of these positions was verified by the relatively broad geographical range of the analyzed samples. In the search for appropriate endonucleases to distinguish and identify PCR products of three *Trachurus* species, the previous sequences were compared to define different restriction sites that could generate species-specific restriction patterns. Three restriction enzymes were found to be potentially useful and not expensive for this identification: *Nla*III (CATG↓), *Nci*I (CC↓$^{C}_{G}$GG), and *Bsm*AI [GTCTC(N)$_1$↓] (**Figure 1**). Sequence comparison revealed the presence of one *Nla*III restriction site present only in *T. trachurus* and one *Bsm*AI site present only in *T. mediterraneus*. Concerning the *Nci*I restriction enzyme, there are two restriction sites in *T. trachurus*, whereas *T. mediterraneus* and *T. picturatus* have only one.

The results of the RFLP analysis of the three *Trachurus* species with the three suitable restriction enzymes are illustrated in **Figure 2**. For *Nla*III digestion, *T. trachurus* gives a pattern of two fragments of around 175 and 195 bp, as expected from sequence analysis, and does not cleave the PCR products from *T. mediterraneus* and *T. picturatus*. Additionally, *T. trachurus* can be discriminated from the other two *Trachurus* species by two *Nci*I restriction sites that produce patterns of around 145, 135, and 90 bp fragments, whereas the unique *Nci*I restriction site in *T. mediterraneus* and *T. picturatus* yields two DNA fragments of around 225 and 145 bp. Finally, the single restriction site for *Bsm*AI present only in *T. mediterraneus* yields two fragments of around 260 and 110 bp but does not cleave *T. trachurus* and *T. picturatus*. Thus, there are two useful endonucleases (*Nla*III and *Nci*I) for the discrimination of *T. trachurus* from the other two species and one enzyme (*Bsm*AI) for the discrimination of *T. mediterraneus*. No enzyme was found for the direct identification of *T. picturatus*.

One of the problems that can eliminate the efficiency of the method is the existence of intraspecific variability as possible mutations can lead to gain or loss of restriction recognition sites. Consequently, any restriction enzyme selected should have a target sequence with the lowest intraspecific polymorphism. The absence of intraspecific variability was confirmed by analyzing 118 individuals of *Trachurus trachurus,* 67 individuals of *T. mediterraneus*, and 54 individuals of *T. picturatus* across the geographical distribution of the species (**Table 1**). The patterns obtained after samples had been screened with the two (*Nla*III and *Nci*I) restriction enzymes showed band sizes that were in agreement with the expected sizes for the restriction fragments inferred from the sequence analysis, revealing the universality of the PCR-RFLP method. As far as the *Bsm*AI restriction enzyme is concerned, the efficiency of the method was slightly lower as two individuals of *T. mediterraneus* (from Portugal) of 67 did not present the *Bsm*AI recognition site. Nevertheless, PCR-RFLP analysis seems to be a suitable method for the accurate identification of three *Trachurus* species with success ranging from 100% for *Nla*III and *Nci*I to 97% for *Bsm*AI.

The utility of cytochrome *b* as a suitable candidate for fish species identification and/or discrimination was investigated in previous studies. It has been used to differentiate between sole (*Solea solea*), European plaice (*Pleuronectes platessa*), flounder (*Platichthys flesus*), and Greenland halibut (*Reinhardtius hippoglossoides*) (*22*) and also to differentiate sole (*Solea solea*) from *Microchirus acevia* (*23*) using the PCR-RFLP methodology. The present results reinforce the results of previous studies, as digestion of cytochrome *b* with specific enzymes and evaluation of the results in large numbers of individuals make it possible to produce species-specific patterns suitable to discriminate the three *Trachurus* species.

Discrimination of *Trachurus* Species by the 5S rRNA Multicopy Gene. The fact that the organization of 5S rDNA presents no intraspecific polymorphism and at the same time high interspecific variability makes it a very good candidate for comparison of closely related species. Interspecific size differences in the 5S rDNA were sufficient to distinguish subspecies of mice (*24*), coregonid fish species (*25*), sole species (*26*), and Atlantic salmon and brown trout (*27*) and even for

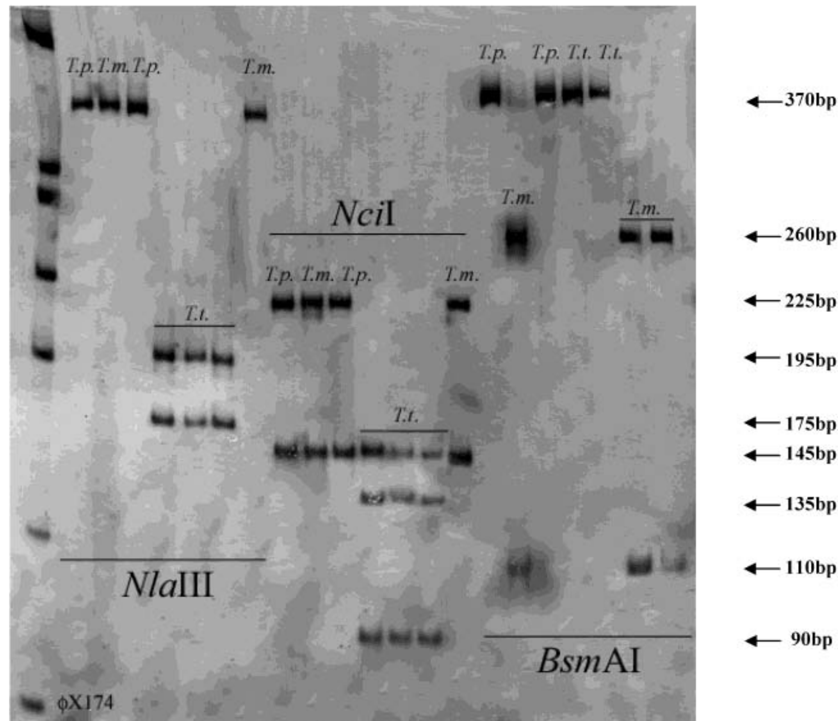

Figure 2. PCR-RFLP patterns observed in the three *Trachurus* species after digestion of cytochrome *b* with three restriction enzymes, *Nla*III, *Nci*I, and *Bsm*AI, and separation in 10% acrylamide gel electrophoresis. T.m. = *T. mediterraneus*; T.t. = *T. trachurus*; T.p. = *T. picturatus*.

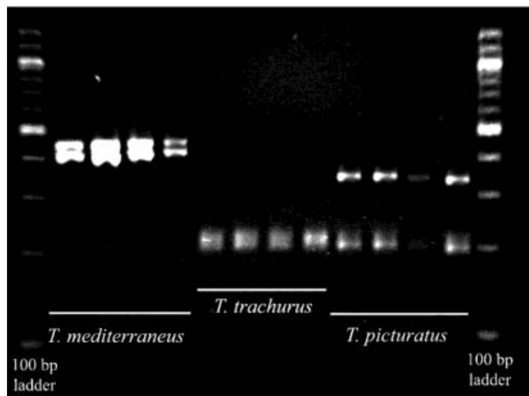

Figure 3. Agarose electrophoretic analysis after the amplification of the nuclear 5S rRNA gene in the three species of the genus *Trachurus* (*T. mediterraneus*, *T. trachurus*, and *T. picturatus*). The size of the PCR products was checked against a 100-bp DNA ladder (Gibco BRL).

the identification of underground plant parts to species (*28*). The oligonucleotides 5SA and 5SB are designed on the basis of the conserved region of the 5S rRNA gene in *Oncorhynchus mykiss* and have already been used to amplify a whole unit of the 5S rRNA gene from Atlantic salmon and brown trout (*27*), from goose and mule duck foie gras (*29*), and from sole and Greenland halibut (*26*).

On the basis of the utility of this nuclear gene as a tool for species identification, it was selected as an alternative candidate for the discrimination of the three *Trachurus* species. The 5S rRNA gene was amplified successfully in all three species, and the size of the amplified fragments was checked in agarose gel. As shown in **Figure 3**, the amplification of the three species revealed species-specific patterns as *T. trachurus* gave a double band of 210 and 230 bp, *T. mediterraneus* gave a double band of 410 and 430 bp, and *T. picturatus* gave a pattern of two bands the sizes of which were around 210 and 350 bp. The double band present in *T. trachurus* and *T. mediterraneus* could be explained as a result of a 20 bp insertion in the spacer (NTS) region of any tandemly repeated unit. Additionally, the pattern of two bands present in *T. picturatus* could be attributed to the existence in the chromosome of two basic tandemly arranged units (coding sequence + NTS) differing in the size of the spacer region or in the presence or absence of a pseudogene. Nevertheless, further sequence analysis of the 5S rRNA gene is needed to shed light on the structure of the gene and to explain the patterns obtained.

As indicated earlier (for the cytochrome *b* analysis), prior to the choice of a species-specific genetic marker for routine application, the absence of intraspecific variability must be confirmed. Analysis for the 5S rRNA gene is simpler, because it is based on the universality of the patterns obtained after a single PCR reaction. In cytochrome *b*, on the other hand, a large number of individuals are needed to check for single-site mutations that will alter the expected pattern. Therefore, for the 5S rRNA gene a smaller number of individuals were scored, representing, nevertheless, the broad geographical range of the species. In total, 35 individuals of the species *T. trachurus* were analyzed, as were 30 individuals of the species *T. picturatus* and 35 individuals of the species *T. mediterraneus*. No intraspecific polymorphism was detected as the obtained pattern and consequently the size of the amplified fragment were the same both within and between populations of the same species. Thus, the 5S rDNA with its interspecific size differences constitutes a very good species-specific nuclear marker, as its PCR amplification is probably the simplest procedure for identifying the three *Trachurus* species.

The purpose of the present study was to uncover mitochondrial and nuclear markers for the most abundant and commercially important *Trachurus* species present in European waters. However, to avoid misleading results with other closely related species, the 5S rRNA methodology was tested both in *Macrorhamphosus scolopax* and in *Trachurus trecae*. The first species, although not congeneric with the three *Trachurus* species, overlaps with them in southern European waters and

has eggs similar to horse mackerel eggs. On the basis of the fact that a further goal of the present study is to provide a new method of *Trachurus* egg identification, the obtained patterns of 5S rRNA methodology have to be different in *Trachurus* species and in *M. scolopax*. Additionally, *T. trecae* that exists from Morocco to Angola and is sometimes present in the area of Cadiz, partially overlapping with the three *Trachurus* species, was also tested to compare the obtained pattern of the amplification of the 5S rDNA gene. Both *M. scolopax* and *T. trecae* revealed totally different patterns compared to the three *Trachurus* species, and they can be perfectly discriminated in a simple agarose gel (unpublished results).

Although the three *Trachurus* species are commercially important in the European market, they are mainly consumed fresh and there are no refrigerated or frozen fish fillets or other types of processed food in local supermarkets. For this reason, misidentification and subsequent fraud were evaluated in local fisheries of two European countries (Greece and Italy) where samples of *T. meditteraneus* and *T. picturatus* were mixed and sold only as *T. mediterraneus*. Initially, the PCR-RFLP analysis of cytochrome *b* was assessed on hypothesized *T. mediterraneus* samples. Samples were found that did not present the expected *Bsm*AI pattern present only in *T. mediterraneus* (two fragments of around 260 and 110 bp) as the PCR product remained uncut. To exclude the possibility of the absence of the *Bsm*AI recognition site due to polymorphism within *T. mediterraneus* or the existence of a related species, analysis of the nuclear 5S rDNA was performed. The obtained patterns were those of *T. picturatus*, indicating that there was fraudulent or unintentional misidentification.

Consequently, the results obtained clearly support the usefulness of two different approaches for discriminating with reliability and confidence the three *Trachurus* species. PCR-RFLP analysis of the cytochrome *b* of mtDNA provides an alternative, simpler, and faster approach for species identification than sequence analysis, which is expensive and time-consuming. Simultaneously, combination of the PCR-RFLP analysis with the results obtained by a simple agarose gel electrophoretic analysis of the nuclear 5S rDNA could lead to unambiguous identification of the three *Trachurus* species.

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LITERATURE CITED

(1) Tortonese, E. *Osteichtyes (Pesci ossei)*; Calderini, Bologna, Italy, 1975; Vol. 2, p 621.

(2) Whitehead, P. J. P.; Bauchot, M. L.; Hureau, J. C.; Nielsen, J.; Tortonese, E. In *Fishes of the North-eastern Atlantic and the Mediterranean*; Unesco: Paris, France, 1986; Vol. 2, pp 517−1007.

(3) Fisher, W.; Schneider, M.; Bauchot, M. L. Fishes FAO d'indentification des espèces pour les besoins de la pêche. *Mediterraneé et Mer Noire*; Zone de pêche 37; FAO, CEE: Rome, Italy, 1987; Vol. 2, pp 761−1529.

(4) Andrews, A. T. Electrophoretic methods. In *Analytical Methods of Food Authentication*; Ashurst, P. R., Dennis, M. J., Eds.; Blackie Academic and Professional, Thomson Science: London, U.K., 1998; pp 204−239.

(5) Mackie, I. M.; Pryde, S. E.; Gonzales-Sotelo, C.; Medina, I.; Pérez-Martin, R.; Quinteiro, J.; Rey-Mendez, M.; Rehbein, H. Challenges in the identification of species of canned fish. *Trends Food Sci. Technol*. **1999**, *10*, 9−14.

(6) Etienne, M.; Jérôme, M.; Fleurence, J.; Rehbein, H.; Kundinger, R.; Mendes, R.; Costa, H.; Pérez-Martin, R.; Piñeiro-González, C.; Craig, A.; Mackie, I.; Yman, I. M.; Ferm, M.; Martinez, I.; Jessen, F.; Smelt, A.; Luten, J. B. Identification of fish species after cooking by SDS-PAGE and urea IEF: a collaborative study. *J. Agric. Food Chem.* **2000**, *48*, 2653−2658.

(7) Davidson, W. S. DNA/PCR techniques. In *Analytical Methods of Food Authentication*; Ashurst, P. R., Dennis, M. J., Eds.; Blackie Academic and Professional, Thomson Science: London, U.K., 1998; pp 182−203.

(8) Sotelo, C. G.; Piñeiro, C.; Gallado, J. M.; Pérez-Martin, R. Fish species identification in seafood products. *Trends Food Sci. Technol.* **1993**, *4*, 395−401.

(9) Bartlett, 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.

(10) Carrera, E.; García, T.; Céspedes, A.; González, I.; Sanz, B.; Hernández, P. E.; Martin, R. Identification of Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) using PCR amplification and restriction analysis of mitochondrial cytochrome *b* gene. *J. Food Prot*. **1998**, *61*, 482−486.

(11) Carrera, E.; García, T.; Céspedes, A.; González, I.; Fernández, A.; Hernández, P. E.; Martin, R. PCR-RFLP of mitochondrial cytochrome oxidase gene: a simple method for discrimination between Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). *J. Sci. Food Agric.* **1999**, *79*, 1654−1658.

(12) Alberts, B.; Bray, D.; Lewis, J.; Raff, M.; Roberts, K.; Watson, J. D. *Molekularbiologie der Zelle*; VCH Verlagsgesellschaft: Weinheim, Germany, 1990.

(13) Carr, S. M.; Marshall, H. D. A direct approach to the measurement of genetic variation in fish populations: applications of the Polymerase Chain Reaction to studies of Atlantic cod, *Gadus morhua* L. *J. Fish Biol*. **1991**, *39* (Suppl. A), 101−107.

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

(15) Pendas, A. M.; Moran, P.; Freije, J. L.; Garcia-Vazquez, E. Chromosomal mapping and nucleotide sequence of two tandem repeats of Atlantic salmon 5S rRNA. *Cytogenet. Cell Genet*. **1994**, *67*, 31−36.

(16) Belkhiri, A.; Intengan, H.; Klassen, G. R. A tandem array of 5S ribosomal RNA genes in *Pythium irregulare. Gene* **1997**, *186*, 155−159.

(17) Taggart, J. B.; Hynes, R. A.; Prodohl, P. A.; Ferguson, A. A simplified protocol for routine total DNA isolation from salmonid fishes. *J. Fish Biol.* **1992**, *40*, 963−965.

(18) Hillis, D. M.; Moritz, C.; Mable, B. K. *Molecular Systematics*; Sinauer Associates: Sunderland, MA, 1996.

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

(20) Karaiskou, N.; Apostolidis, A.; Triantafyllidis, A.; Kouvatsi, A.; Triantaphyllidis, C. Genetic identification and phylogeny of three species of the genus *Trachurus* based on mtDNA analysis. *Mar. Biotechnol.* **2003** (in press).

(21) Hall, T. A. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids. Symp. Ser*. **1999**, *41*, 95−98.

(22) Céspedes, A.; Garcia, T.; Carrera, E.; González, I.; Sanz, B.; Hernández, P. E.; Martin, R. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism analysis of a short fragment of the cytochrome *b* gene for identification of flatfish species. *J. Food Prot.* **1998**, *61*, 1684−1685.

(23) Céspedes, A.; Garcia, T.; Carrera, E.; González, I.; Fernández, A.; Asensio, L.; Hernández, P. E.; Martin, R. Genetic discrimination among *Solea solea* and *Microchirus acevia* by RFLP analysis of PCR amplified mitochondrial DNA fragments. *Arch. Lebensmittelhyg.* **1999**, *50*, 49−72.

(24) Suzuki, H.; Moriwaki, K.; Sakurai, S. Sequences and evolutionary analysis of mouse 5S rDNA. *Mol. Biol. Evol.* **1994**, *11*, 704−710.

(25) Sajdak, S. L.; Reed, K. M.; Phillips, R. B. Intraindividual and interspecies variation in the 5S rDNA of Coregonid fish. *J. Mol. Evol*. **1998**, *46*, 680−688.

(26) Céspedes, A.; Garcia, T.; Carrera, E.; González, I.; Fernández, A.; Hernández, P. E.; Martin, R. Identification of Sole (*Solea solea*) and Greenland halibut (*Reinhardtius hippoglossoides*) by PCR amplification of the 5S rRNA gene. *J. Agric. Food Chem.* **1999**, *47*, 1046−1050.

(27) Pendas A. M.; Moran, P.; Martinez, J. L.; Garcia-Vazquez, E. Application of 5S rDNA in brown trout and in Atlantic salmon by brown trout hybrid identification. *Mol. Ecol*. **1995**, *4*, 275−276.

(28) Linder, C. R.; Moore, L. A.; Jackson, R. B. A universal molecular method for identifying underground plant parts to species. *Mol. Ecol.* **2000**, *9*, 1549−1559.

(29) Rodriguez, A.; Garcia, T.; González, I.; Asensio, L.; Fernández, A.; Lobo, E.; Hernández, P. E.; Martin, R. Identification of goose (*Anser anser*) and mule duck (*Anas platyrhynchos* × *Cairina moschata*) foie gras by multiplex polymerase chain reaction amplification of the 5S rRNA gene. *J. Agric. Food Chem.* **2001**, *48*, 2717−2721.

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