JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Genetic Identification of Horse Mackerel and Related Species in Seafood Products by Means of Forensically Informative Nucleotide Sequencing Methodology

Fátima C. Lago, Beatriz Herrero, Juan M. Vieites, and Montserrat Espiñeira*

Area of Molecular Biology and Biotechnology, ANFACO-CECOPESCA, Vigo, 36310 Pontevedra, Spain

ABSTRACT: In the present study, a methodology based on the amplification of a fragment of mitochondrial *cytochrome b* and subsequent phylogenetic analysis (FINS: forensically informative nucleotide sequencing) to genetically identify horse mackerels have been developed. This methodology makes possible the identification of more than 20 species belonging to the families Carangidae, Mullidae, and Scombridae. The main novelty of this work lies in the longest number of different horse mackerel species included and in the applicability of the developed methods to all kinds of processed products that can be found by consumers in markets around the world, including those that have undergone intensive processes of transformation, as for instance canned foods. Finally, the methods were applied to 15 commercial samples, all of them canned products. Therefore, these methods are useful for checking the fulfillment of labeling regulations for horse mackerels and horse mackerel products, verifying the correct traceability in commercial trade, and fisheries control.

KEYWORDS: Horse mackerel, *Trachurus*, Carangidae, genetic identification, FINS, PCR

INTRODUCTION

Horse mackerels belong to the family Carangidae. This group of small pelagic fish, which includes about 30 genera and approximately 140 species, is well represented in all tropical and subtropical seas. The main characteristics of this taxon are its aerodynamic shape, laterally compressed body, slender tail base, and a strongly forked caudal fin.

The properties of their meat, which is tasty but not overly juicy and has a high nutritional value, makes most horse mackerels highly valued for human consumption, which positions it as a very important pelagic resource in artisanal and industrial fisheries around the world. Preferably, they are sold whole, fresh, chilled, or frozen, but they also can be found for sale gutted or beheaded, filleted, and ready to cook skinless and boneless. They are also sold dried, salted, and smoked, and small size mackerels are intended for the canning industry, mainly in oil or pickled. When they are sold whole, their identification at the species level can be possible through their morphological features; in other cases, the mentioned characteristics are not present, hindering and preventing their identification.

On the other hand, because of high demand of this resource, horse mackerel fisheries are continually threatened by overexploitation. In the case of Chilean jack mackerel (*Trachurus murphyi*), the large number of captures along with the high rate of migration and the seasonality of the species has made this resource undergo a critical situation marked by a decline in its stock by 80% in the last 15 years.[1,2] This requires the existence of appropriate management of this resource, which takes into account the minimum size [3] and catch volumes, with the need to update existing legislation to date that regulates the catch of this pelagic species.[4,5]

Into a globalized market, both the high demand for horse mackerels and the severe shortages that cross some of the species require the need to create a methodology for rapid and accurate identification. Molecular techniques arise in response to this need, allowing the authentication of the final product, avoiding unintended substitutions or fraud in the labeling and rigorous control of the species caught.

In this sense, molecular techniques based on DNA analysis are the best option as they provide more genetic information. In addition, mitochondrial DNA has certain characteristics (high mutation rate, small genome size, maternal inheritance, lack of recombination, and high number of copies per cell) that will provide numerous advantages over nuclear DNA, making it an excellent marker for this type of analysis. In turn, *cytochrome b* (*cyt b*) mitochondrial also presents optimal characteristics (slow evolution, conserved sequence, low intraspecific variation, and high interspecific variation) that has led to its use as a molecular marker in numerous studies of species belonging to the Carangidae family [6−8] and other studies of genetic identification of different taxonomic groups.[9−15] To date, there have been many works based on molecular techniques to identify different species of horse mackerels. Most of them are based on analysis of mitochondrial DNA fragments through polymerase chain reaction−restriction fragment length polymorphism (PCR-RFLP)[16−18] or SNP.[19] They all have great disadvantages that include few species. All of these studies only include the Mediterranean species (*Trachurus mediterraneus*, *Trachurus trachurus*, and *Trachurus picturatus*) and, in some cases, included two species of the genus *Mullus*.[19] For these reasons, in this paper has been developed a methodology based on the amplification of a fragment of mitochondrial *cyt b* by means of PCR and subsequent phylogenetic

Received: November 23, 2010
Accepted: January 5, 2011
Revised: December 29, 2010
Published: February 18, 2011

Table 1. Species Included in the Present Work

family	scientific name	common name	samples[a]	location[b]	NCBI
Carangidae	*Trachurus capensis*	Cape horse mackerel	3	AT, ZAF	AY526536
	Trachurus declivis	Jack mackerel	4	AUS, NZ, TAS	AY526542
	Trachurus delagoa	African scad	2	ZAF	
	Trachurus indicus	Arabian scad	2	IND	
	Trachurus japonicus	Jack mackerel	3	JAP	AB018994
	Trachurus lathami	Rough scad	2	CAN, USA	AF363748
	Trachurus mediterraneus	Mediterranean horse mackerel	3	ESP, GRE	EU224037
	Trachurus murphyi	Inca scad	7	PER, CHI, ARG, AUS	
	Trachurus novaezelandiae	Yellowtail horse mackerel	2	AUS	AY526545
	Trachurus picturatus	Blue jack mackerel	3	MOR, AT, M	EF439614
	Trachurus symmetricus	Pacific jack mackerel	3	USA, MEX	AY526541
	Trachurus trachurus	Atlantic horse mackerel	10	ESP, M, POR	EU224040
	Trachurus trecae	Cunene horse mackerel	2	NAM, MOR	AY050740
	Caranx caballus	Green jack	2	USA	AY050721
	Caranx crysos	Blue runner	2	AT, M	EF392575
	Caranx hippos	Crevalle jack	3	AT, M	AY050720
	Caranx sexfasciatus	Bigeye trevally	2	IND	
	Carangoides ferdau	Blue kingfish	2	IND	
	Decapterus macrosoma	Scad	3	IND, PER	
	Pseudocaranx dentex	Silver travally	3	ESP	EF392607
	Selar crumenophthalmus	Bigeye scad	2	PER	
	Uraspis secunda	Cottonmouth jack	2	ZAF	
Mullidae	*Mullus barbatus*	Red mullet	2	UK, ESP	EU036452
Scombridae	*Rastrelliger kanagurta*	Indian mackerel	3	IND	
	Rastrelliger faughni	Island mackerel	2	IND	

[a] Between 3 and 10 individuals were studied by each sample. [b] Location abbreviations: ARG, Argentina; AT, Atlantic; AUS, Australia; CAN, Canada; CHI, China; ESP, Spain; GRE, Greece; IND, India; JAP, Japan; M, Mediterranean Sea; MEX, Mexico; MOR, Morocco; NAM, Namibia; NZ, New Zealand; PER, Peru; POR, Portugal; TAS, Tasmania; UK, United Kingdom; USA, United States; and ZAF, South Africa.

analysis. Unlike the studies published to date, this technique allows us to genetically identify over 20 species of horse mackerels that can be found by consumers in markets around the world in its different forms of marketing. Besides the advantages already mentioned, the development of these methodologies has the final aim of improving fisheries policy, ensuring compliance with current legislation as far as catch and size, and preserving endangered stocks.

MATERIALS AND METHODS

Sample Collection and Storage. Samples of different horse mackerel and related species were collected from several locations around the world (Table 1). When it was possible, the individuals were identified according to morphological characteristics.[20,21] In other cases, ethanol-preserved fish tissues were provided by universities and research centers located around the world. Once identified, samples were labeled and preserved at −80 °C. Moreover, 15 canned products labeled as horse mackerel were provided by import industries or purchased in supermarkets and shops from Europe, to apply the developed methodology to commercial samples (Table 2).

DNA Extraction. Genomic DNA was extracted from 30 mg of muscle in fresh and frozen samples, according to the method described by Roger and Bendich with slight modifications.[22] The obtained DNA was diluted in 100 μL of 1× Tris−EDTA (TE) buffer (Sigma). In the case of products used for the methodological validation and commercial samples from amounts of tissue comprising between 100 and 300 mg, the extraction of DNA was carried out by the CTAB method previously described and subsequent purification by means of the Nucleospin Extract II kit (Macherey-Nagel) following the supplier's protocol.

The quality and quantity were determined by measuring the absorbance at 260 nm and the 260/280 nm and 234/260 ratios [23] using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). DNA extractions were appropriately labeled and stored at −80 °C for subsequent tasks.

Primers Design, PCR Amplification, and DNA Sequencing. Sequences of the *cyt b* gene were downloaded from the National Center for Biotechnology Information (NCBI) (Table 1). These were aligned with BioEdit 7.0,[24] and for them, a degenerate primer set was designed by hand. The name and sequence of the forward and reverse primers are, respectively, JUREL F, 5′-CAC GAA ACM GGV TCC AAC AA-3′, and JUREL R, 5′-ATG GCR TAK GCA AAB AGG AA-3′ .

In all cases, the PCR reactions were carried out in a total volume of 50 μL with the following composition: 100−300 ng of DNA template was added to a PCR mix consisting of 0.8 mM dNTP mix (Bioline), 5 μL of 10× buffer, 2 mM $MgCl_2$, 0.75 units of BioTaq DNA polymerase (Bioline), 0.8 μL of a 10 μM solution of each primer (Sigma Genosys), and molecular biology grade water (Eppendorf) to adjust to the final volume.

Polymerase chain reactions were carried out in a MyCycler thermocycler (BIO-RAD). Conditions of cycling were as follows: a preheating step at 94 °C for 5 min, 35 cycles of amplification (95 °C for 20 s, 58 °C for 20 s, and 72 °C for 20 s), and a final extension step of 72 °C for 7 min. PCR amplicons were visualized on 2% agarose gels (Sigma) in 1× TBE buffer (Sigma) with 0.3 μg/mL of ethidium bromide (Sigma). DNA fragments were visualized using the Molecular Imager Gel Doc XR System transilluminator and the software Quantity One_ v 4.5.2 (BIO-RAD).

Table 2. Commercial Samples Analyzed with the Methodology Developed

canned products	samples	labeled species	identified species	sample code[a]
		natural		
natural in water	1	Horse Mackerel/Chinchards	*T. murphyi*	S1
		oils		
olive oil	1	Horse Mackerel/Chinchards	*T. murphyi*	S2
	3	Horse Mackerel/Chinchards	*T. trachurus*	S3−S5
sunflower oil	2	Horse Mackerel/Chinchards	*T. murphyi*	S6−S7
	2	Horse Mackerel/Chinchards	*T. trachurus*	S8−S9
soya oil	1	Horse Mackerel/Chinchards	*T. trachurus*	S10
		sauces		
tomato sauce	1	Horse Mackerel/Chinchards	*T. murphyi*	S11
	1	Horse Mackerel/Chinchards	*T. trachurus*	S12
curry sauce	1	Horse Mackerel/Chinchards	*T. trachurus*	S13
pickled sauce	2	Horse Mackerel/Chinchards	*T. trachurus*	S14−S15

[a] Code shown in Figure 2 that locates the commercial samples in the phylogenetic tree of the studied species.

The 50 Base Pair Ladder DNA marker (Tracklt, Invitrogen) was used to estimate the size of the amplicons.

Next, double-stranded DNA was purified using the Nucleospin 96 Extract II (Macherey-Nagel) according to the manufacturer's instructions. The concentration and purity were measured by means of the Nano-DropTM ND-1000 spectrophotometer (Thermo Scientific) as described for DNA extraction. Subsequently, PCR products were sequenced with the primers used for amplification in an automatic DNA Genetic Analyzer (ABI Prism 3130 Genetic Analyzer) using the BigDye Terminator cycle sequencing kit v.1.1 (Applied Biosystems) following the manufacturer's recommendations. Raw data were analyzed using the Sequence Analysis software v.5.3.1. (Applied Biosystems).

The sequences were analyzed with the Chromas 1.45 software[25] and aligned with Clustal W[26] available in the program BioEdit 7.0.[24] The nucleotide sequences obtained were submitted to the GeneBank database of the National Centre for Biotechnology Information (NCBI).

Development of FINS Methodology. Phylogenetic analyses were carried out using the software Mega 4.0 using the Tamura−Nei model to calculate the genetic distances between sequences.[27] The inference of the phylogenetic tree was carried out with the neighbor-joining method.[28] The reliability of the clades formed at the species level in the tree was evaluated by means of a bootstrap test with 2000 replications. Also, the MEGABLAST search available at NCBI was assessed to assign the sequences to a particular species.[29]

Methodological Validation. Individuals from different species were authenticated on the basis of their morphological traits, and main commercial treatment types were applied to them. Treatments included canning, fresh, and frozen for each one of the different kinds of sauces, and condiments were used.

The treatment applied to canned samples involved 121 °C temperature and 1.2 bar of overpressure, and the time varied depending on the size of the can. All of these treatments were carried out in the pilot plant of CECOPESCA (Spanish National Centre of Fish Processing Technology). Next, products were analyzed with the methodology developed in the present work. The coincidence percentage between identified species on the basis of morphological traits and the genetic methodology developed was calculated to establish the specificity of the method.

Application to Commercial Products. After the validation of the methods developed in the present work, these were applied to 15 canned products labeled as horse mackerel (Table 2). These products were acquired in supermarkets from Europe. The purpose of these analyses was to evaluate the situation regarding the labeling of these products on the market.

RESULTS AND DISCUSSION

PCR Amplification. Obtained amplification and sequencing PCR products and DNA amplification with the primers JUREL F/R generated an amplicon of 239 bp in all of the species included in the present study (Table 1) (accession numbers HQ593670−HQ593727). The main objective of the design of primers by hand is the fact that in this way it is possible to make a thorough analysis of conserved mitochondrial DNA regions in all species under study. In this way, we can design primers that allow one to differentiate all of these species.

In the case of fish that have undergone different treatments such as canned, it is not possible to amplify PCR products of a large size, because the thermal treatment generates DNA fragmentation. This was the case for the canned products, where fragments of little sizes were amplified. Quinteiro et al. established a maximum fragment size in canned products of 176 bp to ensure the amplification.[30] Other authors, under certain conditions, amplified fragments higher than 250 bp from canned products.[11,31−34] However, we consider that it is important to have at one's disposal a method that can be used routinely, allowing the easily amplification of DNA and providing a reliable species identification. Herein, the amplification of a small fragment is proposed that contains enough information to enable the differentiation of all of the studied species.

The presence of additives used in the alimentary industry as spices or sauces attenuates or inclusively inhibits the DNA amplification. Moreover, the different kinds of sauces added produce differences in the quantity and quality of the extracted DNA as this molecule is very sensitive to acid and alkaline agents. In this sense, it is worth highlighting the pickled products, in which the low pH produces higher DNA degradation. For this reason, the strategy of amplification proposed facilitates the successful PCR amplification in any seafood product.[11]

Development of Forensically Informative Nucleotide Sequencing (FINS) Methodology. The FINS technique described by Bartlett and Davidson[35] was used in the present study to develop an identification method for horse mackerel and related species. Sequences of unknown samples are compared with sequences belonging to pattern specimens on the basis of this technique.

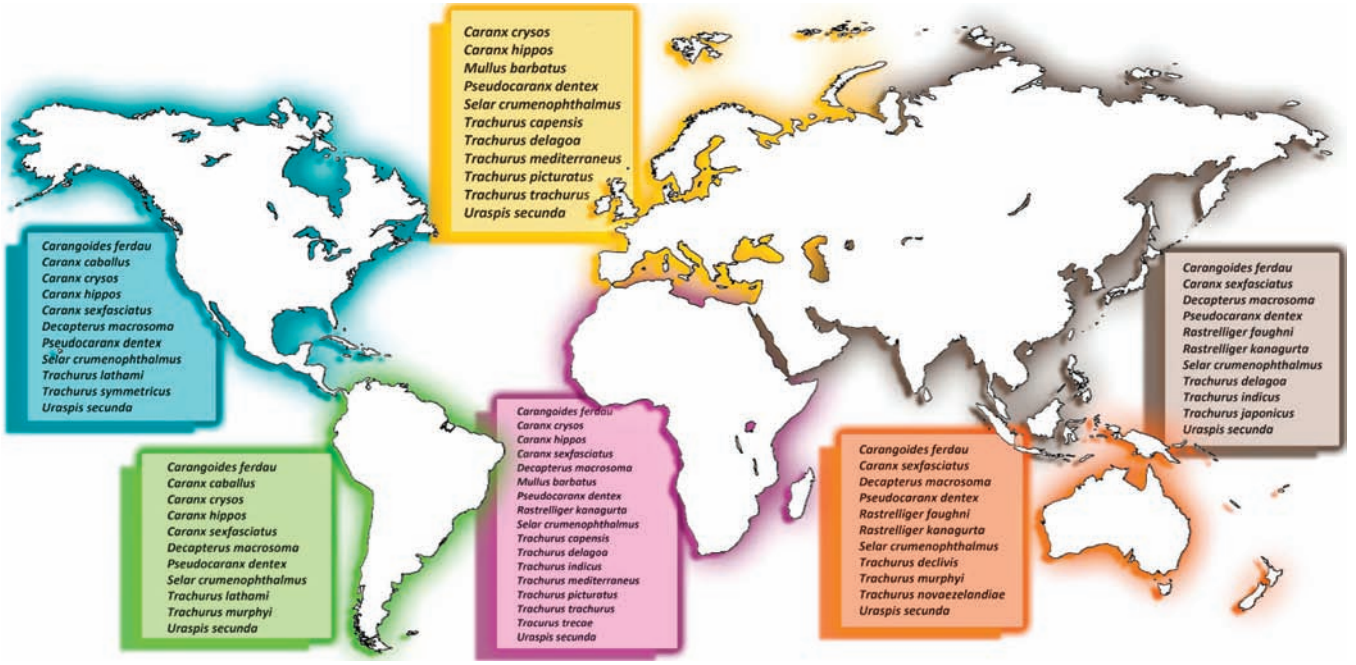

Figure 1. Distribution map of the horse mackerel and related species included in the present study.

The genetic distances between the *cyt b* gene sequences of all of the studied species were estimated using the Tamura−Nei method.[36] The phylogenetic analysis of the amplified fragment (199 bp without primers) was carried out, allowing establishment of the relationships among species by means of the construction of phylogeny using the data set. In the tree obtained, all of the sequences belonging to individuals of the same species were grouped in the same cluster, allowing their identification.

The phylogenetic tree contains species from three families belonging to order Perciformes: Carangidae, Mullidae, and Scombridae. In the Carangidae family, it is worth highlighting the clade of genus *Trachurus* that is a strongly supported clade with a bootstrap value of 99 (Figure 2). The results obtained in this clade agree with the studios carried out by Bektas et al. and Karaiskou et al.[7,8] Both coincide in the fact that genetic distances between the species indicated that *T. mediterraneus* and *T. picturatus* are more closely related than *T. trachurus*. The bootstrap method can be used to obtain the support of the different groups obtained in the phylogenetic tree. It has been calculated that bootstrap values higher or equal to 70 usually correspond to a probability higher or equal to 95% that the corresponding cluster is real,[37] giving a quantitative measurement of the certainty of the assignment of a sample to a particular species. The phylogenetic tree constructed from 199 bp sequences shows that all of the sequences belonging to individuals of the same species are grouped in the same cluster (Figure 2). All clusters are strongly supported, with bootstrap values higher than 70, allowing the reliable assignation of each individual to a particular species. Therefore, the proposed strategy of amplification allows the amplification of a DNA fragment sufficiently long to discriminate successfully all of the horse mackerel and related species of commercial importance, even in canned products and others formats in which the DNA is highly degraded.

BLAST analysis is a suitable technique to find regions of local similarity between sequences and can even be a suitable technique to identify species. Specifically, the MEGABLAST search available at NCBI was assessed to assign any horse mackerel and related species DNA sequences to a particular species. The phylogenetic assignments generated by the proposed FINS technique were compared to the results obtained by BLAST, and the same species assignments were obtained (data not shown). It is worth highlighting that for many species found that their sequences included into NCBI had not been properly assigned with the correct species. Therefore, these two techniques could be used to identify horse mackerels and related species herein studied.

Methodological Validation. The aim of the methodological validation was to check whether the manufacturing process by which the processed foods had undergone had not influenced the identification of these species. Different products were prepared in the pilot plant of CECOPESCA, simulating the conditions used in the food industry (Table 2). This approach is useful to assess the function and optimization of the conditions of the developed methodology. The standard individuals were subjected to several transformation processes, allowing evaluation of the influence of different variables on the genetic methods herein proposed.

Identified species in these samples by means of the method herein developed were in agreement with those based on morphological characters. Therefore, the methodology shows a specificity of 100%.

Application to Commercial Products. The developed methodology was applied to 15 canned products labeled as horse mackerel. This application allowed knowledge of the degree of fulfillment of the labeling regulations in these seafood products. It is worth highlighting that any of tested cans were labeled with the scientific name of the species. All were labeled with the commercial denomination of the species as Horse Mackerel or Chinchard. This is a major problem in all products made from horse mackerel, as this family includes a large number of very similar morphologically species but not all have the same commercial value. Obtained results showed that the most

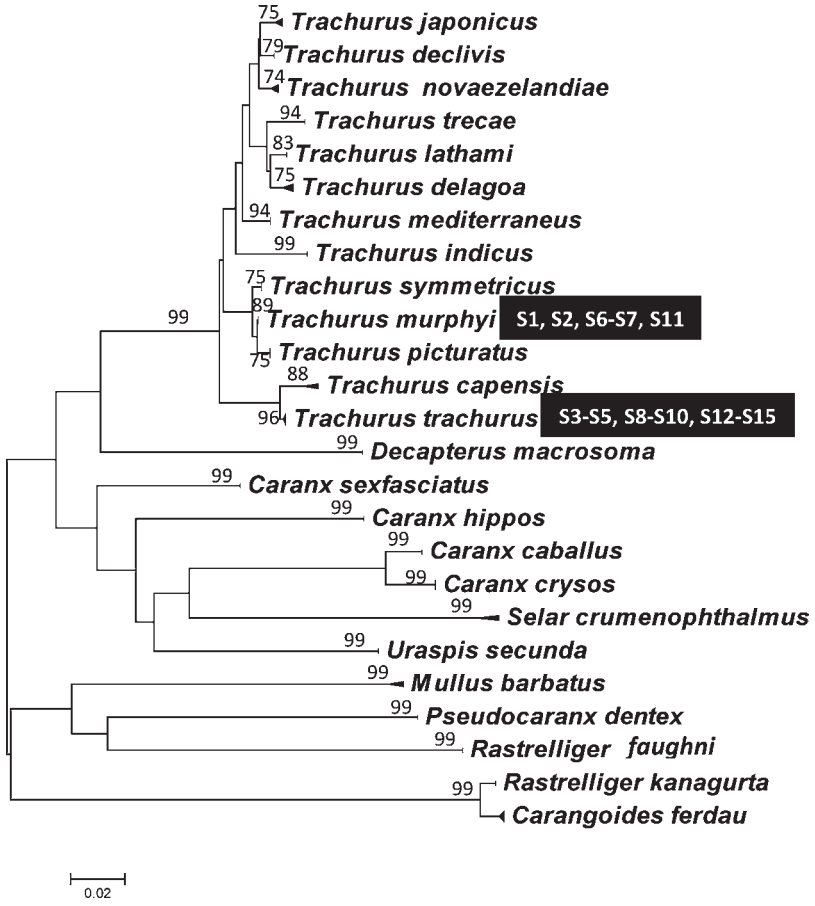

Figure 2. Neighbor-joining tree showing the relationships among the studied species, carried from the alignment of 239 bp of the *cyt b* gene (fragment of 199 bp without primers).The S1−S15 codes belong to the commercial samples analyzed.

common species used in canning are *T. murphyi* and *T. trachurus* (Table 2).

In conclusion, in the present work, one DNA-based method that allowed the genetic identification of the most important commercialized species of horse mackerel and related species has been developed. The main advantage of this method as compared to the ones published up to date is that besides including a high number of species (25 species), it is based on the use of a fragment with a size below 250 bp and can be applied to all kinds of processed products, including those that have undergone intensive transformation processes. The possible applications of this method are the following: normative control of raw and processed products, particularly the authenticity of imported species; verification of the traceability of different fishing batches along the commercial chain; correct labeling; protection of the consumer's rights; fair competence among fishing operators; and fisheries' control.

AUTHOR INFORMATION

Corresponding Author

*Tel: (34)986 469 301. Fax: (34)986 469 269. E-mail: montse@anfaco.es.

ACKNOWLEDGMENT

We thank Rod Asher (Marine Biology Cawthron Institute, New Zealand), Vaseeharan Baskaralingam (Alagappa University, India), Jose A. González (ICCM, Spain), Sean Fennessy (Oceanographic Research Institute, South Africa), Kim Smith (Estuarine & Inshore Fisheries Australia), Tracey Fairweather (Marine and Coastal Management, South Africa), Konstantinos Triantafyllidis (University of Thessaloniki, Greece), Aland Connell (South Africa), Jorge Castillo Pizarro (Instituto de Fomento Pesquero, Chile), Dianne J. Bray (Museum Victoria, Australia), H. J. Walker Jr. (Scripps Institution of Oceanography, United States), Katherine Maslenikov (University of Washington Fish Collection, United States), Sahar Mehanna (National Institute of Oceanography and Fisheries, Egypt), and Grigorius Krey (Fisheries Research Institute, Greece) for kindly supplying the horse mackerel and related species samples. This work was funded by the European Fisheries Fund (EFF) and the Ministerio de Madio Ambiente y Medio Rural y Marino (Secretaría General del Mar) under the order ARM/1193/2009 of the 6th of May.

REFERENCES

(1) IFOP. Instrumento Financiero de Orientación de la Pesca (IFOP). Informe Final: Investigación Evaluación de Stock y CTP Jurel, 2010.

(2) Cárdenas, L.; Silva, A. X.; Magoulas, A.; Cabezas, J.; Poulin, E.; Ojeda, F. P. Genetic population structure in the Chilean jack mackerel, *Trachurus murphyi* (Nichols) across the South-eastern Pacific Ocean. *Fish. Res.* **2009**, *100* (2), 109–115.

(3) Reglamento (UE) No. 53/2010 del Consejo de 14 de enero de 2010 por el que se establecen, para 2010, las posibilidades de pesca para determinadas poblaciones y grupos de poblaciones de peces, aplicables en aguas de la UE y, en el caso de los buques de la UE, en las demás aguas

donde sea necessario establecer limitaciones de capturas y se modifican los Reglamentos (CE) No. 1359/2008, (CE) No. 754/2009, (CE) No. 1226/2009 y (CE) No. 1287/2009.

(4) Reglamento (CE) No. 1542/2007 de la Comisión de 20 de diciembre de 2007 sobre métodos de desembarque y pesaje del arenque, la caballa y el jurel.

(5) Reglamento (UE) No. 607/2010 de la Comisión de 9 de julio de 2010 por el que se modifica el Reglamento (CE) No. 1542/2007 sobre métodos de desembarque y pesaje del arenque, la caballa y el jurel.

(6) Reed, D. L.; Carpenter, K. E.; deGravelle, M. J. Molecular systematics of the Jacks (Perciformes: Carangidae) based on mitochondrial cytochrome b sequences using parsimony, likelihood, and Bayesian approaches Mol. *Phylogenet. Evol.* **2002**, *23* (3), 513–524.

(7) Karaiskou, N.; Apostolidis, A. P.; Triantafyllidis, A.; Kouvatsi, A.; Triantaphyllidis, C. Genetic Identification and Phylogeny of Three Species of the Genus Trachurus Based on Mitochondrial DNA Analysis. *Mar. Biotechnol.* **2010**, *5*, 493–504.

(8) Bektas, Y.; Belduz, A. O. Molecular phylogeny of Turkish Trachurus species (Perciformes: Carangidae) inferred from mitochondrial DNA analyses. *J. Fish Biol.* **2008**, *73* (5), 1228–1248.

(9) Espiñeira, M.; Gonzalez-Lavin, N.; Vieites, J. M.; Santaclara, F. J. Authentication of Anglerfish Species (Lophius spp) by Means of Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) and Forensically Informative Nucleotide Sequencing (FINS) Methodologies. *J. Agric. Food Chem.* **2008**, *56* (22), 10594–10599.

(10) Espiñeira, M.; Gonzalez-Lavin, N.; Vieites, J. M.; Santaclara, F. J. Development of a method for the genetic identification of flatfish species on the basis of mitochondrial DNA sequences. *J. Agric. Food Chem.* **2008**, *56* (19), 8954–8961.

(11) Espiñeira, M.; Gonzalez-Lavin, N.; Vieites, J. M.; Santaclara, F. J. Development of a method for the identification of scombroid and common substitute species in seafood products by FINS. *Food Chem.* **2009**, *117* (4), 698–704.

(12) Espiñeira, M.; Vieites, J. M.; Santaclara, F. J. Development of a genetic method for the identification of salmon, trout, and bream in seafood products by means of PCR-RFLP and FINS methodologies. *Eur. Food Res. Technol.* **2009**, *229* (5), 785–793.

(13) Espiñeira, M.; Vieites, J. M.; Santaclara, F. J. Species authentication of octopus, cuttlefish, bobtail and bottle squids (families Octopodidae, Sepiidae and Sepiolidae) by FINS methodology in seafoods. *Food Chem.* **2010**, *121* (2), 527–532.

(14) Santaclara, F. J.; Cabado, A. G.; Vieites, J. M. Development of a method for genetic identification of four species of anchovies: *E. encrasicolus*, *E. anchoita*, *E. ringens*, and *E. japonicus*. *Eur. Food Res. Technol.* **2006**, *223* (5), 609–614.

(15) Santaclara, F. J.; Espiñeira, M.; Vieites, J. M. Genetic identification of squids (Families Ommastrephidae and Loliginidae) by PCR-RFLP and FINS methodologies. *J. Agric. Food Chem.* **2007**, *55* (24), 9913–9920.

(16) Karaiskou, N.; Triantafyllidis, A.; Alvarez, P.; Lopes, P.; Garcia-Vazquez, E.; Triantaphyllidis, C. Horse mackerel egg identification using DNA methodology. *Mar. Ecol.: Evol. Perspect.* **2007**, *28* (4), 429–434.

(17) Karaiskou, N.; Triantafyllidis, A.; Triantaphyllidis, C. Discrimination of three *Trachurus* species using both mitochondrial- and nuclear-based DNA approaches. *J. Agric. Food Chem.* **2003**, *51* (17), 4935–4940.

(18) Turan, C.; Gurlek, M.; Yaglioglu, D.; Ozturk, B. Genetic differentiation of Mediterranean horse mackerel (*Trachurus mediterraneus*) populations as revealed by mtDNA PCR-RFLP analysis. *J. Appl. Ichthyol.* **2009**, *25* (2), 142–147.

(19) Apostolidis, A. P.; Georgiadis, A.; Karaiskou, N.; Sandaltzopoulos, R. Reliable and rapid discrimination of congeneric species by mtDNA SNP analysis by multiplex PCR: Application on three *Trachurus* and two *Mullus* fish species as model cases. *Hydrobiologia* **2008**, *614* (1), 401–404.

(20) Nekrasov, V. V. *Mackerel of the World Ocean (Genus Trachurus)* **1994**, 1–228.

(21) Smith-Vaniz, W. F. Carangidae. *Fishes of the North-eastern Atlantic and the Mediterranean*; Unesco: Paris, 1986.

(22) Roger, S. O.; Bendich, A. J. Extraction of DNA from Plant Tissues. *Plant Mol. Biol. Man.* **1988**, *A6*, 1–10.

(23) Winfrey, M. R.; Rott, M. A.; Wortman, A. T. *UnraVeling DNA: Molecular Biology for the Laboratory*; Prentice Hall: New York, 1997.

(24) 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.

(25) McCarthy, C. *Chromas*, version 1.45; School of Health Science, Griffifth University: Gold Coast Campus, Queensland, Australia, 1996.

(26) Thompson, J. D.; Gibson, T. J.; Plewniak, F.; Jeanmougin, F.; Higgins, D. G. The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **1997**, *25* (24), 4876–4882.

(27) Tamura, K.; Dudley, J.; Nei, M.; Kumar, S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **2007**, *24*, 1596–1599.

(28) Saitou, N.; Nei, M. The Neighbor-Joining method—A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **1987**, *4* (4), 406–425.

(29) Wheeler, D. L. Database resources of the National Center for Biotechnology Information. *Nucleic Acids Res.* **2008**, *36*, 13–21.

(30) Quinteiro, J.; Sotelo, C. G.; Rehbein, H.; et al. Use of mtDNA direct polymerase chain reaction (PCR) sequencing and PCR-restriction fragment length polymorphism methodologies in species identification of canned tuna. *J. Agric. Food Chem.* **1998**, *46* (4), 1662–1669.

(31) Pardo, M. A.; Perez-Villareal, B. Identification of commercial canned tuna species by restriction site analysis of mitochondrial DNA products obtained by nested primer PCR. *Food Chem.* **2004**, *86* (1), 143–150.

(32) Ram, J. L.; Ram, M. L.; Baidoun, F. 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* (8), 2460–2467.

(33) Rehbein, H.; Kress, G.; Schmidt, T. Application of PCR-SSCP to species identification of fishery products. *J. Sci. Food Agric.* **1997**, *74* (1), 35–41.

(34) Unseld, M.; Beyermann, B.; Brandt, P.; Hiesel, R. Identification of the Species Origin of Highly Processed Meat-Products by Mitochondrial-DNA Sequences. *PCR—Methods Appl.* **1995**, *4* (4), 241–243.

(35) Bartlett, S. E.; Davidson, W. S. FINS (Forensically Informative Nucleotide Sequencing): A procedure for Identifying the Animal Origin of Biological Specimens. *Biotechniques* **1992**, *12* (3), 408–411.

(36) Tamura, K.; Nei, M. Estimation of the Number of Nucleotide Substitutions in the Control Region of Mitochondrial-DNA in Humans and Chimpanzees. *Mol. Biol. Evol.* **1993**, *10* (3), 512–526.

(37) Hillis, D. M.; Bull, J. J. An Empirical-Test of Bootstrapping as a Method for Assessing Confidence in Phylogenetic Analysis. *Syst. Biol.* **1993**, *42* (2), 182–192.