JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Genetic Identification of Squids (Families *Ommastrephidae* and *Loliginidae*) by PCR–RFLP and FINS Methodologies

FRANCISCO J. SANTACLARA,* MONTSERRAT ESPIÑEIRA, AND JUAN M. VIEITES

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

Cephalopods are a taxonomic group that contains a great number of families, genera and species, with many of them very important at the commercial level. The existence of very similar species in this class added up to the transformation process applied to them makes it difficult or even impossible for species identification based on morphological characterization. Moreover, the global commerce makes it possible that one determined species can be marketed in its antipodes. These questions suggest the necessity of molecular techniques to solve this situation. In the present work, a genetic method was developed on the basis of the polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) and forensically informative nucleotide sequencing (FINS) technique and makes possible the identification of more than 20 species belonging to the families *Ommastrephidae* and *Loliginidae*, as well as some octopus and sepia species. The PCR was employed to amplify 651 and 208 bp fragments of the mitochondrial cytochrome *b* gene. These molecular systems were applied to fresh, frozen, precooked, even canned cephalopods, allowing for the identification of the species included in these products. Therefore, these molecular tools could be applied in questions related to correct labeling, traceability, and importation controls of squids, sepias, and octopuses.

KEYWORDS: Squids; genetic identification; *Cephalopoda*; PCR–RFLP; FINS

INTRODUCTION

Cephalopods are marine organisms belonging to the *Cephalopoda* class, characterized by a great number of families (specifically 21) that, in turn, contain great species diversity, many of them with high commercial importance. From these species, industries manufacture their products that entail different transformation processes.

The great morphologic similarity among different cephalopod species, particularly the shortfin (family *Ommastrephidae*) and longfin squids (family *Loliginidae*), makes it very difficult to identify them. Moreover, in many cases, the raw material is commercialized in different transformation levels, impeding or making it difficult for specific assignation by means of morphological-based methods.

Moreover, the volume of cephalopods commercialized in the world during 2004 was 3775161 Tm (including squids, sepias, and octopuses) (data from FISHTAT, FAO). Most of this raw material is destined to be consumed after a processing treatment, and this fact makes possible the substitution of species into this taxonomic group.

All of these factors pointed out the need for analytical methods that allow for the determination of the authenticity of the raw materials included in these products.

* To whom correspondence should be addressed. Telephone: (34) 986-469-301. Fax: (34) 986-469-269. E-mail: fransanta@anfaco.es.

Several previous works studied the genetic identification of some cephalopod species using molecular techniques. The main disadvantage of these studies is the low number of species studied. An outstanding work carried out by Colombo et al. designed a polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method to identify the families *Loliginidae* and *Ommastrephidae*. In that work, five species belonging to *Illex*, *Todarodes*, *Todaropsis*, and *Loligo* genera (*1*) were studied.

Other studies focused on this taxonomic group were those carried out by Chapela et al., who designed two methods for genetic identification that include eight cephalopod species belonging to six genera and two families, using the PCR–RFLP and forensically informative nucleotide sequencing (FINS) techniques (*2, 3*).

The PCR–RFLP is usually used in the laboratories devoted to the identification and authentication of species, because of its simplicity and reliability (*3–10*). However, some authors criticized the reliability of these methods, especially when there are many species in a determined taxonomic group, because they could produce the same restriction profile in a studied and not studied species. This fact could generate an incorrect identification. As a consequence, some authors advise the use of techniques based on sequencing and phylogenetic analysis (*3,9,11*), because of their high power of diagnosis, which minimizes the mistakes in assignations (species that were not studied probably

10.1021/jf0707177 CCC: $37.00
Published on Web 11/03/2007

Table 1. Cephalopods Species Included in This Work

family	species	code	number of samples	seq[a]	RFLP[b]	GC %[c]	NCBI accession number
Loliginidae	*Loligo gahi*	Lg	4	8	3	0.303	EF423116–EF423123
	Loligo vulgaris	Lv	3	6	5	0.306	EF423060–EF423065
	Loliolus japonica	Lj	2	4	2	0.288	EF423074–EF423077
	Uroteuthis chinensis	Uc	4	8	3	0.278	EF423128–EF423135
	Loligo opalescens	Lo	2	4	3	0.311	EF423158–EF423161
	Loligo pealei	Lp	3	6	3	0.312	EF423104–EF423109
	Loligo forbesi	Lf	4	8	3	0.287	EF423052–EF423059
	Loligo reynaudi	Lr	2	4	2	0.302	EF423066–EF423069
	Loligo bleekeri	Lb	2	4	3	0.400	EF423148–EF423151
	Alloteuthis subulata	As	3	6	3	0.282	EF423110–EF423115
Ommastrephidae	*Todarodes pacificus*	Tp	3	6	3	0.274	EF423142–EF423147
	Illex argentinus	Ia	4	8	3	0.373	EF423096–EF423103
	Illex illecebrosus	Ii	2	4	2	0.270	EF423048–EF423051
	Illex coindetii	Ic	8	16	3	0.270	EF423032–EF423047
	Todaropsis eblanae	Te	4	8	3	0.291	EF423088–EF423095
	Todarodes sagittatus	Ts	2	4	3	0.281	EF423011–EF423014
	Todarodes filippovae	Tf	2	4	3	0.299	EF423124–EF423127
	Nototodarus sloani	Ns	2	4	3	0.309	EF423070–EF423073
	Dosidicus gigas	Dg	3	6	3	0.323	EF423082–EF423087
	Ommastrephes bartrami	Ob	3	6	3	0.367	EF423136–EF423141
Sepiidae	*Sepia officinalis*	So	2	4	2	0.301	EF423078–EF423081
Octopodidae	*Octopus vulgaris*	Ov	10	16	3	0.291	EF423015–EF423031
	Eledone cirrhosa	Ec	3	6	3	0.267	EF423152–EF423157

[a] Number of sequenced individuals. [b] Number of individuals analyzed by the RFLP technique. [c] Percentage of guanine plus cytosine in the sequenced 605 bp fragment.

had a characteristic sequence, which places this far away from the sequences of the other species studied). Therefore, FINS analysis is more appropriate than RFLP, because this method uses more information than RFLP to assign one sample to a determined species (*2*, *3*, *7*, *11*, *12*). Moreover, more laboratories have genetic analysers, indispensable to obtain the DNA sequences. The genetic distance among different sequences can be calculated. These distance values can be used to construct a phylogenetic tree, where each species is located in a node, allowing for the identification of blind samples, because these are grouped with the reference sequences belonging to one determined species.

The previous works on cephalopods carried out to date can not be applied to canned products, because of the large size of the PCR product amplified. The thermal treatment produces the DNA fragmentation, and this fact prevents the DNA amplification by PCR. In this sense, Quinteiro et al. determined that the maximum size of the DNA fragment amplified in canned products is around 170 bp. Other authors achieved amplification of fragments with higher sizes (*7*, *12*).

In the present study, the more important squid species with commercial interest were studied using the methodological approximations cited previously: PCR–RFLP and FINS. The main objective was to develop a simple methodological analysis to identify different squids species and, in this manner, its origin. These methods were applied to all types of products, from fresh to canned products elaborated in our Technological Center, using as raw material genuine individuals previously studied based on their morphological traits. Thus, the methodology herein developed can be very useful in the normative control of those products, particularly in the authenticity of imported species, the verification of the traceability of different fishing batches along the commercial chain, the correct labeling, and the protection of the rights of the consumer.

MATERIALS AND METHODS

1. Sampling and DNA Extraction. Authentic cephalopod samples were collected from different locations around the world. The number of samples by species ranged from 2 to 10 (**Table 1**). These samples were preserved in 70% ethanol at −80 °C until being processed for DNA extraction. Some commercial samples were provided by importer industries, and others were obtained in local markets. When it was possible, the specimens that composed the samples were identified on the basis of morphological traits according to different bibliographic references (*13*–*15*).

Genomic DNA of all of the samples previously described (**Table 1**) was extracted from a piece of 30 mg of muscle tissue according to the standard CTAB phenol-chloroform protocol described by Roger and Bendich with slight modifications (*16*). In the case of the products elaborated for us to validate the methods and others purchased in the local market, DNA was extracted from a piece of 200 mg of tissue. When the products included oil (samples used in the validation method and market study), this was removed by submerging the piece of cephalopod in a solution of methanol/chloroform/water (2:1:0.8) for 2 h, prior to DNA extraction.

The extracted DNA was visualized in agarose gels (Sigma) at 1% in TBE buffer with 5 μg/mL of ethidium bromide (Sigma). The quality and quantity of the DNA obtained was measured with an espectrophotometer (Eppendorf Biophotometer).

2. Amplification and Sequencing of the PCR Products. Three sequences of the cytochrome *b* gene were downloaded from the National Center for Biotechnology Information (NCBI) (*Octopus vulgaris*, *Todarodes pacificus*, and *Loligo bleekeri* with accession numbers AB158363, AB158364, and AB029616, respectively). These were aligned with BioEdit 7.0 (*17*), and for them, a degenerate primer set was designed by hand. The name and sequence of the forward and reverse primers are, respectively, CEF H, 5′-TTA TGG KTG RGT RYT DCG TTA T-3′ and CEF L, 5′-TAC HCC YCC WAR TTT WYT AGG AAT-3′ (**Table 2**). These primer sets were used both in the PCR amplification and in the sequencing, allowing us to obtain the nucleotidic sequences of all studied species in the conditions described below. An internal fragment was selected and amplified with the primers CEF H and H15149AD described by Burgener (*18*) (**Figure 1**).

The amplifications were carried out in a final volume of 50 μL containing 100 ng of DNA template, 5 μL of 10× buffer, 2 mM $MgCl_2$, 0.4 μL of 100 mM dNTP, 4 μL of a 10 μM solution of each primer, and 1 unit of Taq-polymerase (Bioline). PCR of the samples that undergo thermal treatment were carried out with 400 ng of DNA. PCR was performed in a thermocycler Uno II (Biometra). The cycles program was the following: a preheating step of 3 min at 95 °C, then 35 cycles of 30 s at 95 °C, 1 min at 50 °C, 1 min and 30 s at 72 °C, and a final extension step of 7 min at 72 °C.

To ensure the proper working of PCR amplification, PCR products were loaded in agarose gels (Sigma) at 2% in TBE buffer and

Table 2. Alignment of *L. bleekeri*, *T. pacificus*, and *O. vulgaris*, Showing the Position of the Primers CEF H and CEF L Designed in This Study

Species	Accession number	Sequence
Primer *CEF H*		5′ TTATGGKTGR GTRYTDCGTT AT 3′
Loligo bleekeri	AB029616	......T..A ..GT.A.... ..
Todarodes pacificus	AB158364	......G..G ..AC.T.... ..
Octopus vulgaris	AB158363	......G..A ..AT.G.... ..
Primer *CEF L*		5′ TACHCCYCCW ARTTTWYTAG GAAT 3′
Loligo bleekeri	AB029616	...A..C..T .A...TC... ....
Todarodes pacificus	AB158364	...C..T..A .G...TC... ....
Octopus vulgaris	AB158363	...T..C..T .A...AT... ....

5 μg/mL of ethidium bromide to allow for band detection. The size of the amplified fragments was estimated from molecular marker pGEM (Promega).

PCR products were cleaned before the sequencing reaction using NucleoSpin Extract II (Macherey–Nagel) according to the protocol of the manufacturer.

Both DNA strands were sequenced on an ABI Prism 310 DNA Genetic Analyzer (Applied Biosystems), with the primers described previously (CEF H and CEF L) and using BigDye Terminator Cycle Sequencing Ready Reaction Kit version 1.1 (Applied Biosystems) according to the recommendations of the manufacturer. Nucleotidic sequences obtained were corrected with Chromas 1.45 (*19*) and subsequently aligned with BioEdit 7.0 (*17*). From this alignment, a polymorphism analysis was carried out using DnaSP 4.0 (*20*). The following parameters were estimated for each species and for the total of the sequences: number of haplotypes, haplotype diversity, variable and invariable sites, number of mutations, synonymous and replacement changes, and the percentage of guanine plus cytosine.

3. Development of the PCR–RFLP Method. The nucleotidic sequences obtained were used to generate the restriction map of each species with the assistance of Webcutter 2.0 (*21*). Several enzymes were selected because they generated a restriction profile exclusive and easily differentiable in agarose gels for each species.

About 100 ng of crude PCR product per species was digested separately with the selected restrictases. Digestions were carried out overnight at the temperature indicated by the supplier, adding 2 units of enzyme to the PCR products without purification.

Restriction fragments were visualized in low melting agarose gels at 3% (Pronadisa) in TBE buffer. The etidium bromide (Sigma) was included in the gel for DNA detection (final concentration of 5 μg/mL). The electrophoresis conditions were 70 V and 90 min. The gels were observed in an image analyzer Gel Doc XR (BIO-RAD). Size fragments were estimated from the ladder of 50 bp (Amersham Biosciences) and pGEM (Promega).

4. Development of the FINS Methodology. All of the sequences obtained were aligned with BioEdit 7.0 (*17*). The phylogenetic analyses were carried out with Mega 3.0 (*22*). The genetic distances among sequences were calculated using the nucleotide model of Tamura–Nei and the inference of the phylogenetic tree with the de Neighbour–Joining method. The reliability of the tree was evaluated by means of a bootstrap test with 2000 replications.

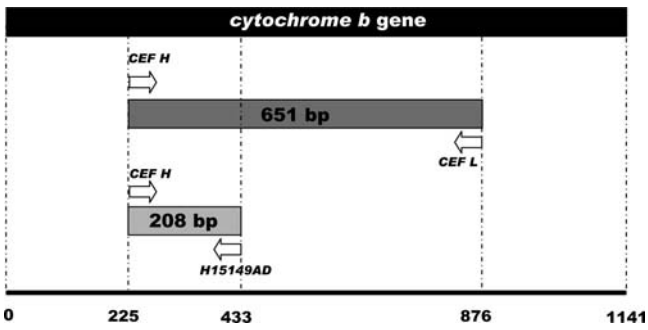

Figure 1. Location and size of the DNA fragments of the cytochrome *b* gene amplified in this work and position of the designed primer sets.

The internal fragment delimited by CEF H and H15149AD primers was used to identify the species included in canned products. The calculation of distances and construction of the tree was performed as in the previous case.

5. Methodological Validation. A total of 46 samples (2 individuals of each considered species) were prepared as described below: whole individuals were classified in species based on their morphological traits. The origin zone was taken into account for the species assignation. After these two considerations, the 46 individuals were assigned to one determined species. Different products, among them: rings, tubes, and arms in different preparations (fried, frozen, coated in butter, canned, etc.) were elaborated from these individuals. The most extreme treatment that was applied to the samples was the sterilization in a horizontal retort steel-air, at 115 °C for 50 min, with 1.2 bars of overpressure.

PCR–RFLP and FINS methodologies developed in this work and described in the precedent paragraphs were applied to these samples to evaluate the correct operation of these analytical methods.

Results of the species assignation based on morphology and genetic probes were compared. The coincidence percentage between the species identified on the basis of morphological traits and the genetic methodology developed was calculated to establish the specificity of the method.

6. Market Study. Once the methods previously described were validated, these were applied to 15 commercial samples, basically fresh, frozen, and canned cephalopod products. These samples were purchased in different formats and processing levels in stores and supermarkets of Pontevedra, Spain.

RESULTS AND DISCUSSION

1. Amplification and Sequencing of PCR Products. The suitability of the mitochondrial cytochrome *b* gene to develop a DNA-based method for the genetic identification of cephalopod species was assessed in this study. The main characteristics of this molecular marker that convert it for genetic identification of species are that it is a mitochondrial region; therefore, it has a mutation rate higher than the nuclear genome, allowing for the differentiation of close species, it is highly conserved with regard to other mitocondrial regions, it is present multiple times in a cell (this fact makes it easy for amplification when transformation processes are applied to the raw material), and nucleotidic substitutions in the mitochondrial genome are higher than in the nuclear genome, allowing for the accumulation of nucleotidic differences faster in this molecule. These nucleo-

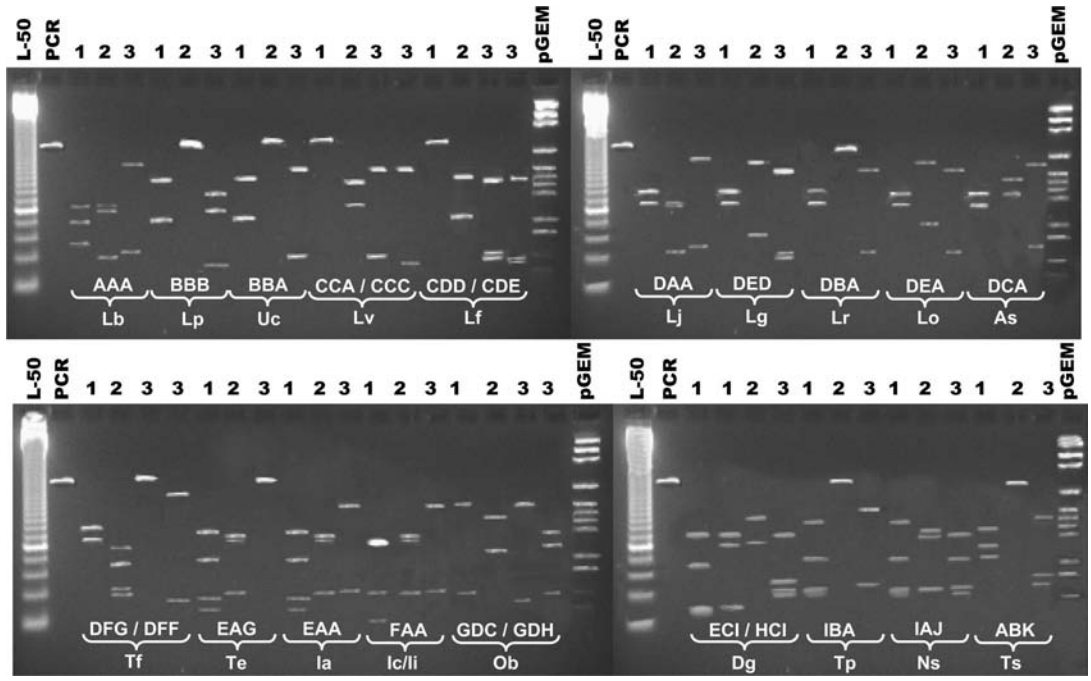

Figure 2. RFLP analysis of PCR products obtained by PCR with the CEF primer set in *Loliginidae* and *Omastrephidae* families. Lane PCR, PCR products; lane 1, PCR products after digestion with *Sfc* I; lane 2, PCR products after digestion with *Hph* I; lanes 3, PCR products after digestion with *Hinf* I; lane L-50, molecular size marker (50 bp DNA ladder, Amersham Biosciences); lanes pGEM, molecular marker pGEM (Promega). Combined and single restriction haplotipe and species codes are showed in **Table 3**.

Table 3. Restriction Haplotypes Generated after Digestion with *Sfc* I, *Hph* I, and *Hinf* I Enzymes in the Species of the Families *Loliginidae* and *Omastrephidae*

			Sfc I		*Hph* I		*Hinf* I	
species	code	CH[a]	size fragments	H[b]	size fragments	H[b]	size fragments	H[b]
L. bleekeri	Lb	AAA	285 + 163 + 203	A	252 + 282 + 117	A	533 + 118	A
L. pealei	Lp	BBB	448 + 203	B	651	B	309 + 224 + 35 + 83	B
U. chinensis	Uc	BBA	207 + 444	B			533 + 118	A
L. vulgaris	Lv	CCC			252 + 399	C	533 + 35 + 83	C
		CCA	651	C	533 + 118	A		
L. forbesi	Lf	CDD			444 + 207	D	430 + 103 + 118	D
		CDE					430 + 103 + 35 + 83	E
L. japonica	Lj	DAA			252 + 282 + 117	A	533 + 118	A
L. gahi	Lg	DED			152 + 499	E	430 + 103 + 118	D
L. reynaudi	Lr	DBA			651	B		
L. opalescens	Lo	DEA	285 + 366	D	470 + 181	E	533 + 118	A
A. subulata	As	DCA			252 + 399	C		
T. filipovae	Tf	DFF			260 + 168 + 115 + 108	F	568 + 83	F
		DFG					651	G
T. eblanae	Te	EAG	102 + 183 + 66 + 300	E			651	G
I. argentinus	Ia	EAA			252 + 282 + 117	A	533 + 118	A
I. coindetii–I. illecebrosus	Ic–Ii	FAA	102 + 249 + 51 + 249	F				
O. bartrami	Ob	GDC	102 + 549	G	444 + 207	D	533 + 23 + 95	C
		GDH					298 + 235 + 23 + 95	H
D. gigas	Dg	ECI	102 + 183 + 66 + 300	E	252 + 399	C	298 + 132 + 103 + 118	I
		HCI	102 + 249 + 300	H				
T. pacificus	Tp	IBA	102 + 183 + 366	I	651	B	533 + 118	A
N. sloani	Ns	IAJ			252 + 282 + 117	A	267 + 163 + 103 + 35 + 83	J
T. sagittatus	Ts	ABK	285 + 163 + 203	A	651	B	130 + 403 + 118	K

[a] Combined restriction haplotype. [b] Single restriction haplotype.

tidic changes will allow for the genetic identification of closely related species, for instance, species belonging to a concrete genus.

Because of these properties, many previous works in the field of genetic identification, including the present one, used this mitochondrial region (*6*, *8*, *11*, *12*, *23*).

The degenerate primers CEF H and CEF L herein described, allowed us to obtain all of the nucleotide sequences of the studied species. This primer set amplified a 651 bp fragment, comprised between positions 225 and 876 of the 1141 bp fragment that composes this gene (**Figure 1**). These sequences were deposited in the NCBI database (accession numbers EF423011–EF423161).

The polymorphism analysis carried out with DnaSP 4.0 (*20*) showed that there were 296 variable sites and 309 monomorphic sites. Intraspecific variability was measured by means of haplotype (gene) diversity, obtaining an average value of 0.607. The average number of nucleotide differences (k) among all

studied species was 1.59. The GC % (guanine plus cytosine percentage) had a very low value in the studied cephalopod species. Other taxonomic groups (members of the family *Scombridae*, *Clupeidae*, and *Gadidae*) showed higher values than the cephalopod species (0.47, 0.50, and 0.42, respectively). The sequences used in this analysis were taken from NCBI. The low values of GC % seem to be characteristic of the cephalopod group, with average values near 0.3.

A fragment contained into the 651 bp described previously and delimited by primers CEF H and H15149AD was amplified to be used in the identification of the species included in products that undergo high temperatures and/or pressures (for instance, canned products) (**Figure 1**). This PCR had a size of 208 bp in all of the studied species. Because of the small size of this DNA fragment, it can be amplified even in canned products and allow for the identification of the species included in any food product.

2. Development of the PCR–RFLP Method. The nucleotidic sequences obtained for all of the studied species allowed us to carry out a genetic method based on the different restriction profiles generated by three restrictases. This technique, known as RFLP, is an alternative to sequencing, and it was very used in the past for different purposes, including the genetic identification of species. PCR–RFLP offers the advantages of being simplex, cheaper than other techniques, such as sequencing, and especially useful for routine analysis of a large number of samples (*24*). However, some authors advise against using RFLP to forensic identification when there are moderate levels of intraspecific variability, because this fact could make the RFLP unstable and lead to misidentifications (*3, 11*). This intraspecific variability can be due to the nature of the species or the studied markers. In this work, intraespecific variability of the cytochrome *b* fragment was studied. Available sequences from NCBI and those obtained in this work have been taken into account. The levels of intraspecific variability detected in the studied species allowed us to develop the PCR–RFLP methodology, because the restriction targets in none of case were affected, generating stables restriction profiles.

Therefore, RFLP represents a suitable technique to determinate the identity of the cephalopod species included in this work. The restrictases selected were *Sfc* I, *Hph* I, and *Hinf* I (New England Biolabs). The combined restriction profile generated by these enzymes allowed for the identification, in a univocal form, of the studied species. The enzyme *Sfc* I allow us to differentiate the families *Loliginidae* and *Omastrephidae*, except *T. filipovae* that has an identical restriction profile as some members of the *Loliginidae* family. The fact that this shortfin squid has no commercial value is an argument in favor of the use of the RFLP, which would not be affected by this inconvenience. Moreover, in some cases, this enzyme allows for the assignation at the level of species. The DNA fragments generated in the digestion were compatible with the expected on the basis of the restriction map in all cases. The DNA fragments with less than 50 bp generated in the restriction digestion were not used in identification, because the established conditions of the agarose gels did not allow us to visualize them.

The combined haplotypes of these three enzymes allowed for the identification of all of the studied species. Only *Illex coindetii* and *Illex illecebrosus* show the same restriction profile, and it is not possible differentiate between them (**Figure 2** and **Table 3**). Chapela et al. did not differentiate these same species using a 297 bp fragment of the same marker (*3*).

Moreover, the transformation process applied to the raw material makes it possible that species belonging to the families

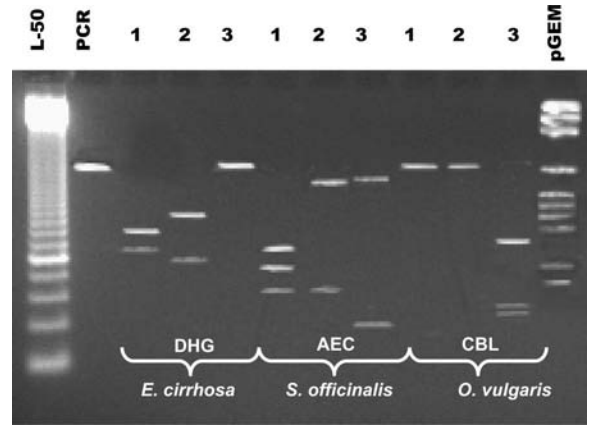

Figure 3. RFLP analysis of PCR products obtained by PCR with the *CEF* primer set in *Sepia officinalis* (family *Sepiidae*), *Octopus vulgaris* and *Eledone cirrhosa* (family Octopodidae). Lane PCR: PCR product; Lanes 1: PCR products after digestion with *Sfc* I; Lanes 2: PCR products after digestion with *Hph* I; Lanes 3: PCR products after digestion with *Hinf* I; Lane L-50: Molecular size marker 50 bp DNA ladder (*Amersham Biosciences*); Lane pGEM: Molecular marker *pGEM* (*Promega*).

Octopodidae and *Sepidae* can be labeled as one *Ommastrephidae* and *Loliginidae* species because of the fact that the specific traits to identify these species could disappear in the elaboration process. For this reason, several species of these families were included in the study to assess the specificity of the RFLP. Specifically, *Sepia officinalis* (family *Sepiidae*) and *Octopus vulgaris* and *Eledone cirrhosa* (family *Octopodidae*) were included in the RFLP analysis to verify that its restriction profiles and nucleotidic sequences do not agree with the other studied species belonging to the *Ommastrephidae* and *Loliginidae* families (**Figure 3** and **Table 4**). The genetic assignment was performed correctly by means of the RFLP technique herein proposed on the 651 bp fragment. **Figures 2** and **3** and **Tables 3** and **4** shown as these species can be identified.

3. Development of the FINS Method. FINS was described by Bartlett and Davidson, who proposed the genetic identification of species using phylogenetic analysis (*25*). From the nucleotidic sequences, a matrix of distance can be calculated. The results of this analysis can be displayed in a phylogenetic tree, where sequences of the same species are grouped into clades. Also an undetermined or unknown species is allocated in the phylogenetic tree in accordance with the distance that separates it from the reference sequences of known species. This method overcomes the inconvenience of the intraspecific variability and allows for the detection of new species not studied thus far. The main inconvenience of this technique is the high cost of the genetic analysers and materials, necessary to obtain the DNA sequences.

Phylogenetic relationships among these species of cephalopods were investigated with distance methods. A Neighbor-joining phylogenetic tree based on genetic distances was constructed, showing four main groups, corresponding to the families *Loliginidae* (G1), *Ommastrephidae* (G2), *Sepiidae* (G3), and *Octopodidae* (G4) (**Figure 4**). The species included in these four groups are well-differentiated; all of the branches at the level of species have a bootstrap value higher than 85, and this values reflects the reliability of the assignation (*26*). The exceptions were *Illex coindetii* and *Illex illecebrosus* that were not differentiated by either the PCR–RFLP or FINS technique, because these two species have the same nucleotide sequence

Table 4. Haplotypes Generated after Digestion with Restriction Enzymes *Sfc* I, *Hph* I, and *Hinf* I in *Sepia officinalis* (Family *Sepiidae*) and *Octopus vulgaris* and *Eledone cirrhosa* (Family *Octopodidae*)

		Sfc I		*Hph* I		*Hinf* I	
species	CH[a]	size fragments	H[b]	size fragments	H[b]	size fragments	H[b]
Eledone cirrhosa	DGG	285 + 366	D	421 + 230	G	651	G
Sepia officinalis	AEC	285 + 163 + 203	A	149 + 502	E	533 + 35 + 85	C
Octopus vulgaris	CBL	651	C	651	B	304 + 126 + 41 + 85 + 95	L
	CBM					304 + 126 + 126 + 95	

[a] Combined restriction haplotype. [b] Single restriction haplotype.

Table 5. Commercial Samples Analyzed with the Methods Developed That Showed an Incorrect Labeling

products	species labeled	species identificated	code samples[a]
frozen squid rings	*Loligo vulgaris*	*Loligo reynaudi*	S1
cleaned, frozen squid tubes	*Todaropsis eblanae*	*Todarodes sagittatus*	S2
fresh entire squid	*Loliolus japonica*	*Alloteuthis subulata*	S3
octopus arms	*Octopus vulgaris*	*Dosidicus gigas*	S4
canned octopus	*Octopus vulgaris*	*Dosidicus gigas*	S5

[a] Code shown in **Figures 4** and **5** that locate the commercial samples in the phylogenetic tree of the studied cephalopods.

for the cytochrome *b* fragment studied. Chapela et al. found these same results using a 297 bp fragment of the same marker (*3*).

With the aim of developing a method applicable to canned products, the PCR–RFLP and FINS alternatives were assessed. Because of the high number of species included in this work and the little size of the PCR products amplified, the RFLP does not permit identify of all of these species with the fragment of 208 bp. On the other hand, the FINS technique showed the power to identify all of the studied species, showing high bootstrap values, very similar to the phylogeny of the 605 bp fragment (**Figures 4** and **5**).

4. Methodological Validation. The aim of this process was to evaluate the correct performance of the methodologies herein proposed. To verify the suitability and reliability of the proposed methods in the genetic identification of elaborated products, commercial samples were elaborated in the pilot plant of CECOPESCA (Spanish National Centre of Fish Processing Technology) from authenticated individuals based on morphological traits. Thermal treatment applied to the canned products allowed us to evaluate the correct DNA amplification in the most extreme case of degradation. In this particular case, the little fragment (208 bp) was amplified.

Subsequently, these samples were analyzed with the methodologies proposed in the present work. The analyses were performed by sequencing and phylogenetic analysis and PCR–RFLP when it was possible to amplify the 651 bp fragment. This task allowed us to verify that the whole individuals, and the elaborated products gave the same results. Therefore, the developed techniques showed a specificity of 100%, because all of the analysed samples with the genetic tools herein developed were assigned to the species determined on the basis of the morphological study.

5. Market Study. Once the methods surpassed the validation step, these were applied to 15 commercial samples, allowing us to evaluate and determine a broad outline, the labeling situation of these products in the Spanish market. All of the samples analyzed were identified as some species of those

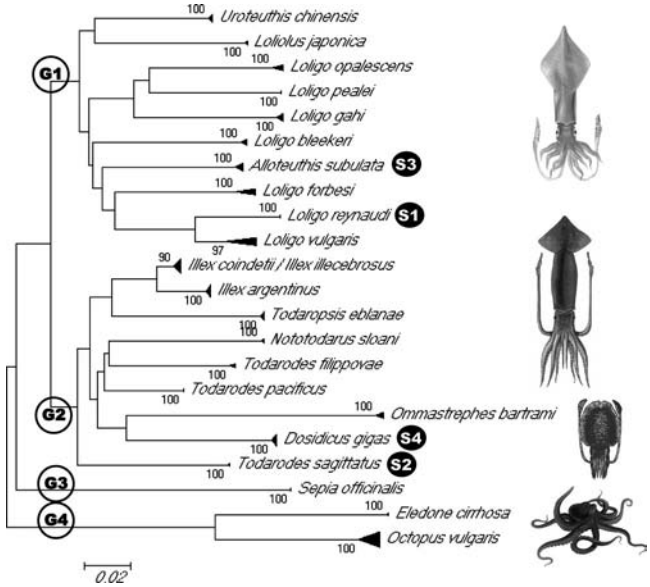

Figure 4. Neighbor-joining tree constructed from sequences of 605 bp, using the Tamura–Nei distance. Bootstap values higher than 85 are showed in each branch. The S1, S2, S3, and S4 codes belong to the commercial samples analyzed (**Table 5**), and G1, G2, G3, and G4 are the four taxonomic groups studied [families *Loliginidae* (G1), *Ommastrephidae* (G2), *Sepiidae* (G3), and *Octopodidae* (G4)].

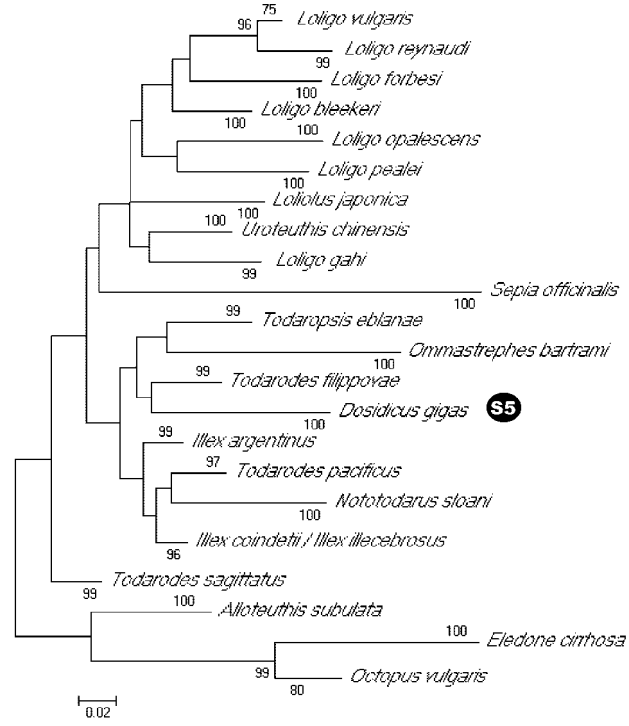

Figure 5. Neighbor-joining tree constructed from sequences of 160 bp, using the Tamura–Nei substitution model to calculate the distance matrix. Bootstrap values higher than 75 are showed in each branch. The S5 code belongs to the analyzed commercial can (**Table 5**).

included in this work. Five analysed samples contained a different species of those indicated in the label (**Figures 4** and **5** and **Table 5**).

Altogether, this paper describes two DNA-based methods that allow for the genetic identification of cephalopod species in fresh, frozen, precooked, canned, or any other foodstuffs, including those that undergo intensive thermal treatment. The lack of methods for this task makes these tools as unique available alternatives at the moment. Therefore, these methods can be useful in questions regarding the traceability, correct labeling, authenticity of imported raw material, and so on.

ACKNOWLEDGMENT

We thank Nerea Gonzalez-Lavín and Arrate Aldasoro for their helpful assistance. We also thank Elba Mora, Instituto Nacional de Pesca de Ecuador; Eileen Dillane, University College Cork, Ireland; Carol Kimbrell, Southwest Fiseries Science, La Jolla, CA; Roger T. Hanlon, Marine Biological Laboratory, Woods Hole, MA; Paco Bustamante, Université de la Rochelle, France; Richard Brings, Department of Agriculture and Rural Development, (DARDNI) U.K.; Chih-Shin Chen, Institute of Fishery Biology National, Taiwan University; Ine Moulaert, Instituut Voor Landbouw en Visserijonderzoek, Belgium; Hidetaka Furuya, Department of Biology Graduate School of Science, Osaka University, Japan; Kelly Henry, University of Rhode Island Graduate School of Oceanography, Narragansett, RI; Peter R. Teske, Rhodes University, South Africa; Joao Pereira, Instituto Nacional de Investigación Agraria y de Pesca (IPIMAR), Portugal; M. Treble, Fisheries and Oceans Canada, Freshwater Institute, Canada; Alastair Cook, CEFAS, U.K.; Stefan Hoffman, Instituut voor Landbouw, Belgium; Rebecca Marzec, Haskin Shellfish Research Laboratory, Port Norris, NJ; Barry MacDonald, Fisheries and Oceans Canada, Freshwater Institute, Canada; Bjorke Herman, Institute of Marine Research, Norway; Elisabeth Turrell and Adrian Weetman, FRS Marine Laboratory, U.K.; Nota Peristeraki, Hellenic Centre for Marine Research, Greece; Susumu Kawasaki, National Food Research Institute, Japan; and Remment ter Hofstede, Wageningen IMARES, Netherlands.

LITERATURE CITED

(1) Colombo, F.; Cerioli, M.; Colombo, M. M.; Marchisio, E.; Malandra, R.; Renon, P. A simple polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method for the differentiation of cephalopod mollusc families *Loliginidae* from *Ommastrephidae*, to avoid substitutions in fishery field. *Food Control* **2002**, *13* (3), 185–190.

(2) Chapela, M. J.; Sotelo, C. G.; Calo-Mata, P.; Perez-Martin, R. I.; Rehbein, H.; Hold, G. L.; Quinteiro, J.; Rey-Mendez, M.; Rosa, C.; Santos, A. T. Identification of cephalopod species (*Ommastrephidae* and *Loliginidae*) in seafood products by forensically informative nucleotide sequencing (FINS). *J. Food Sci.* **2002**, *67* (5), 1672–1676.

(3) Chapela, M. J.; Sotelo, C. G.; Perez-Martin, R. I. Molecular identification of cephalopod species by FINS and PCR–RFLP of a cytochrome *b* gene fragment. *Eur. Food Res. Technol.* **2003**, *217* (6), 524–529.

(4) Pérez, M.; Cabado, A. G.; Vieites, J. M.; Presa, P. Experimental assessment of a new rDNA-based method for the identification of *Merluccius capensis* and *Merluccius paradoxus* in commercial products. *J. Aquat. Food Prod. Technol.* **2004**, (13), 49–57.

(5) Quinteiro, J.; Sotelo, C. G.; Rehbein, H.; Pryde, S. E.; Medina, I.; Perez-Martin, R. I.; Rey-Mendez, M.; Mackie, I. M. 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.

(6) Quinteiro, J.; Vidal, R.; Izquierdo, V.; Sotelo, C. G.; Chapela, M. J.; Perez-Martin, R. I.; Rehbein, H.; Hold, G. L.; Russell, V. J.; Pryde, S. E.; Rosa, P.; Santos, A. T.; Rey-Mendez, M. Identification of hake species (*Merluccius* genus) using sequencing and PCR–RFLP analysis of mitochondrial DNA control region sequences. *J. Agric. Food Chem.* **2001**, *49* (11), 5108–5114.

(7) Santaclara, F. J.; Espineira, M.; Cabado, A. G.; Aldasoro, A.; Gonzalez-Lavin, N.; Vieites, J. M. Development of a method for the genetic identification of mussel species belonging to *Mytilus*, *Perna*, *Aulacomya*, and other genera. *J. Agric. Food Chem.* **2006**, *54* (22), 8461–8470.

(8) Santaclara, F. J.; Espineira, M.; Cabado, A. G.; Vieites, J. M. Detection of land animal remains in fish meals by the polymerase chain reaction–restriction fragment length polymorphism technique. *J. Agric. Food Chem.* **2007**, *55* (2), 305–310.

(9) Calo-Mata, P.; Sotelo, C. G.; Perez-Martin, P. I.; Rehbein, H.; Hold, G. L.; Russell, V. J.; Pryde, S.; Quinteiro, J.; Rey-Mendez, M.; Rosa, C.; Santos, A. T. Identification of gadoid fish species using DNA-based techniques. *Eur. Food Res. Technol.* **2003**, *217* (3), 259–264.

(10) Yanagimoto, T.; Kitamura, T. Identification of three gadoid species around Hokkaido using the mitochondorial cytochrome *b* gene. *Nippon Suisan Gakkaishi* **2002**, *68* (6), 893–899.

(11) Sotelo, C. G.; Calo-Mata, P.; Chapela, M. J.; Perez-Martin, R. I.; Rehbein, H.; Hold, G. L.; Russell, V. J.; Pryde, S.; Quinteiro, J.; Izquierdo, M.; Rey-Mendez, M.; Rosa, C.; Santos, A. T. Identification of flatfish (*Pleuronectiforme*) species using DNA-based techniques. *J. Agric. Food Chem.* **2001**, *49* (10), 4562–4569.

(12) Terol, J.; Mascarell, R.; Fernandez-Pedrosa, V.; Perez-Alonso, M. Statistical validation of the identification of tuna species: Bootstrap analysis of mitochondrial DNA sequences. *J. Agric. Food Chem.* **2002**, *50* (5), 963–969.

(13) Guerra, A., *Molusca, Cephalopoda*. Museo Nacional de Ciencias Naturales. CSIC: Madrid, Spain, 1992; Vol. 1, p 327.

(14) Norman, M., *Guía de Cefalópodos del Mundo*. Grupo M&G Difusión, S.L., 2000; p 320.

(15) Roper, C. F. E.; Seewney, M. J.; Nauen, C. E. Cephalopods of the world. An annotated and illustrated catalogue of species of interest to fisheries. FAO species catalogue. 1984; Vol. 3, p 277.

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

(17) 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.

(18) Burgener, M., Molecular species differentiation of fish and mammals. Ph.D. Thesis, University of Bern, Switzerland, 1997.

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

(20) Rozas, J.; Sánchez-del Barrio, J. C.; Messeguer, X.; Rozas, R. DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics* **2003**, (19), 2496–2497.

(21) Heiman, M., Webcutter 2.0. http://www.firstmarket/cutter/cut2.html, 1997.

(22) Kumar, S.; Tamura, K.; Nei, M. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Briefings Bioinf.* **2004**, (5), 150–163.

(23) 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.

(24) Meyer, R.; Hoffelein, C.; Candrian, U. Polymerase chain reaction restriction fragment length polymorphism analysis: A simple method for species identification in food. *J. AOAC Int.* **1995**, *78* (6), 1542–1551.

(25) 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.

(26) 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.

Received for review March 12, 2007. Revised manuscript received July 2, 2007. Accepted July 13, 2007. This work was funded by Grant PGIDIT06RMA00802CT from Conselleria de Innovación e Industria de la Xunta de Galicia.

JF0707177