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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

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Lophius represents the most important genus of the family Lophiidae from a commercial point of view. The main marketing formats of the species included in this genus are tails and cheeks, making impossible the species identification on the basis of their morphological characters. In the present study, two methods based on the polymerase chain reaction−restriction fragment length polymorphism (PCR−RFLP) and phylogenetic analysis of DNA sequences [forensically informative nucleotide sequencing (FINS)] were developed to differentiate the seven species contained in the genus *Lophius*. In both cases, the molecular marker studied was the cytochrome oxidase subunit I gene (COI). The RFLP analysis of the PCR products digested with the endonuclease M*bo* I generated species-specific restriction profiles, and the phylogenetic analysis showing a neighbor-joining tree with independent nodes was strongly supported for all of the studied species. These methods were applied to 40 commercial samples, allowing us to detect the samples incorrectly labeled. The fraudulent labeling ratio was higher in processed products (68.75%) than whole fish (31.25%). The species subjected to mislabeling were *L. budegassa* (68.75%), *L. vomerinus* (18.75%), and *L. piscatorius* (12.5%). Therefore, both methodologies can be independently used to authenticate the species belonging to the genus *Lophius*, being useful to check the fulfillment of labeling regulations of seafood products and to verify the correct traceability of commercial trade and the control of fisheries.

KEYWORDS: *Lophius*; anglerfish; genetic identification; PCR−RFLP; FINS; COI

1. INTRODUCTION

Anglerfish constitute a group of fish species that belong to the family Lophiidae, which includes 4 genera (*Sladenia*, *Lophiodes*, *Lophiomus*, and *Lophius*) and 25 species (*1*). The main characteristic of this taxonomic group is a depressed and broad head, while the posterior portion of the body tapers up to the tail. *Lophius*, the most important genus of the family Lophiidae at a commercial level, includes the following 7 species: *L. budegassa* (black-bellied angler) and *L. piscatorius* (white-bellied angler), both inhabiting the Northeastern Atlantic, *L. americanus* (American angler) and *L. gastrophysus* (blackfin goosefish), which are distributed in the Atlantic coast of America, *L. vaillanti* (shortspin African angler), which is found in the African Eastern Atlantic coast, *L. vomerinus* (cape monk), which occurs in the Namibian and South African coast, and *L. litulon* (yellow goosefish), which is distributed in the Northwest Pacific (**Figure 1**).

The total world catch of anglerfish in 2005 was approximately 116 000 tons [Food and Agriculture Organization (FAO)], and half of them correspond to *L. piscatorius*. The import of anglerfish by European countries went up 3818% in 2005, with regard to 1985 (*2*). These data reflect the great increase in trade flow of this group of species in the latter years and their importance as a resource for human consumption. To avoid the excessive exploitation of these resources in the future as a result of the catch rates, the European Union (EU) has established total allowable catches (TAC) for anglerfish since 1993. However, the illegal, unregulated, and unreported fishing (IUU) could make it difficult to check the fulfilment of the established TAC.

Concerning the labeling of fish products, the European Commission Regulation 104/2000 and 2065/2001 (*3*) establish dispositions relative to the information of consumers about fish products and the traceability control. The EU only allows commercializing fish products if they contain the appropriate

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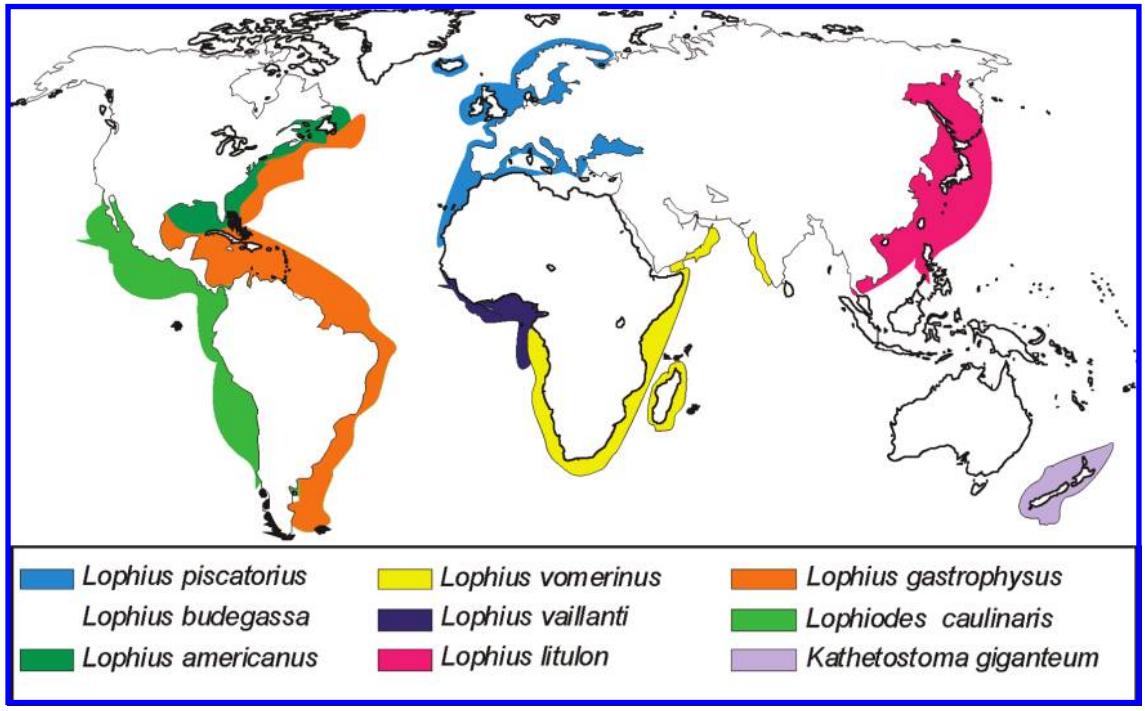

Figure 1. Distribution map of all of the species belonging to the genus *Lophius, Lophiodes caulinaris,* and *Kathetostoma giganteum.*

Table 1. Samples Included in This Work[a]

scientific name	common name	samples	location	RFLP[b]	seq[c]	size fragments	H[d]
Lophius americanus	American angler	4	USA, CAN	30	10	699	A
Lophius budegassa	black-bellied angler	6	GBR, PRT	30	10	128, 102, 375, 93	B
Lophius gastrophysus	blackfin goosefish	3	USA, BRA	30	10	455, 244	C
Lophius litulon	anglerfish	7	JPN, KOR, CHN	30	10	194, 36, 204, 199, 66	D
Lophius piscatorius	black-bellied angler	6	GBR, NOR	30	10	230, 204, 66, 109, 93	E
Lophius vaillanti	shortspin African angler	6	NAM	30	10	529, 77, 56, 37	F
Lophius vomerinus	cape monk	6	NAM, ZAF	30	16	230, 376, 93 40, 190, 376, 93	G H
Lophiodes caulinaris	spottedtail angler	4	MEX, PER	8	8	633, 66	I
Kathestostoma giganteum	New Zealand monkfish	3	NZL	30	10	633, 66	J

[a] Locations abbreviations: CAN, Canada; CHN, China; GBR, United Kingdom; KOR, Korea; MEX, Mexico; NAM, Namibia; NOR, Norway; NZL, New Zealand; PER, Peru; PRT, Portugal; USA, United States; ZAF, South Africa. [b] Number of individuals sequenced. [c] Number of individuals analyzed by the RFLP technique. [d] Restriction haplotype.

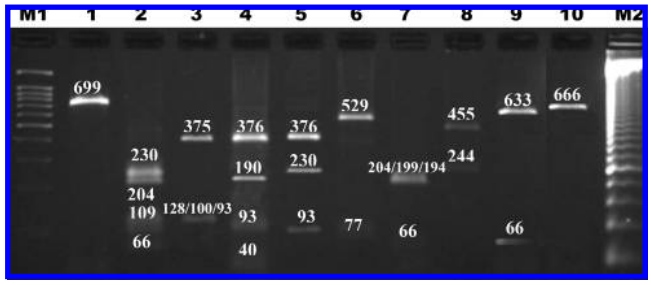

Figure 2. RFLP analysis with M*bo* I of PCR products obtained with COIF-ALT and COIR-ALT primers in all species included in this work. Lane 1, PCR products and *L. americanus*; lane 2, *L. piscatorius*; lane 3, *L. budegassa*; lanes 4 and 5, *L. vomerinus*; lane 6, *L. vaillanti*; lane 7, *L. litulon*; lane 8, *L. gastrophysus*; lane 9, *K. giganteum*; lane 10, *L. caulinaris*; lane M1, molecular size marker 100−1500 bp (Dominion-MBL); and lane M2, molecular marker 50 bp DNA ladder (GE Healthcare).

information about the commercial denomination and scientific name of the species.

Anglerfish are valued for the high quality of their meat, but this depends upon the species, which have different commercial value. For example, with regard to European species, *L. budegassa* and *L. piscatorius*, the first has greater commercial value than the last one, and the same happens with the African species, because *L. vomerinus* has greater commercial value than *L. vaillanti* (*4*). Furthermore, aspects linked to the fish quality can affect in a different way to different species, for instance, the strictness of hygienic sanitary conditions of transformation plants, which are worse in third-world countries than in developed ones, or the amount of heavy metals accumulated (*5*).

The main way of marketing these species are whole specimens, tails, cheeks, and livers. The species identification is possible in whole individuals based on morphological characteristics but is impossible in processed products.

The increase of processed products and international trade can cause the deliberate or unintentional substitution of species. This fact can be overcome by the application of non-morphological analytical methods, for instance DNA-based methods developed for the genetic identification of cephalopods (*6*), bivalves (*7*), anchovies (*8*), gadoids (*9*), tuna (*10*), and flatfish (*11*).

Concerning the genetic studies of anglerfish species, it is worth highlighting the work of Sanjuan et al. (*12*), who identified two species (*L. budegassa* and *L. piscatorius*) by polymerase chain reaction−restriction fragment length polymorphism (PCR−RFLP) of a fragment of cytochrome *b*, a gene belonging to the mitochondrial genome.

However, the existence of a global market entails the necessity of identification techniques that take into account the maximum number of species that are susceptible to be marketed under the same commercial denomination.

In the present work, two methods for the genetic identification of species belonging to the genus *Lophius* were designed. The first of them is based on the PCR−RFLP technique, and the second one is based on the phylogenetic analysis. In both cases, the selected molecular marker was a fragment of the cytochrome oxidase subunit I (COI) gene. The importance of the present work lies in the fact that up to now there is not any work about the genetic identification of the seven species of the genus *Lophius*. *Lophiodes caulinaris* (Family Lophiidae) and *Kathetostoma giganteum* (family Uranoscopidae) were included as outgroup because seafood products elaborated with it are labeled using the commercial denomination "Spotted tail angler" and "New Zealand Monkfish", respectively. This fact can produce confusion with the *Lophius* species. Moreover, the existence of genetic identification methods, such as that proposed in this work, are necessary to improve the quality of fish products because they allow for a guarantee of their correct traceability and authenticity.

2. MATERIALS AND METHODS

2.1. Sample Collection and DNA Extraction. Authentic specimens of all of the species of the genus *Lophius, L. caulinaris;*-and *K. giganteum* were collected from different locations around the world (**Figure 1**). The number of samples of each species, the common name, and the capture location are shown in the **Table 1**. The entire specimens were morphologically characterized according to different bibliographic references (*1, 13, 14*). Samples were stored at −80 °C until DNA extraction, which was performed from 30 mg of muscle tissue using the cetyl trimethyl ammonium bromide (CTAB) phenol−chloroform protocol described by Roger and Bendich (*15*), with slight modifications. DNA extractions were loaded in 1% agarose gels (Sigma) containing 5 μg/mL ethidium bromide (Sigma), and the electrophoresis was run in TBE buffer at 70 V for 50 min. The gels were visualized under ultraviolet light using the Molecular Imager Gel Doc XR system transilluminator and analyzed with the software Quantity One, version 4.5.2 (Bio-Rad).

2.2. Amplification and Sequencing of the PCR Products. A fragment of the COI gene was amplified using the primers COIF-ALT (5′-ACA AAT CAY AAR GAY ATY GG-3′) and COIR-ALT (5′-TGR TTY TTY GGN CAY CCT GAA-3′), described by Mikkelsen et al. (*7*). PCR reactions were performed in a final volume of 50 μL containing 100 ng of DNA template, 5 μL 10× buffer, 2 mM $MgCl_2$, 0.4 μL of 100 mM dNTP, 0.8 μM solution of each primer, and 1 unit of T*aq* DNA polymerase (Bioline). The PCR amplifications were carried out in a Bio-Rad MyCycler thermocycler, and the cycling conditions were an initial 3 min preheating step at 95 °C, followed by 35 cycles (30 s at 95 °C, 1 min at 54 °C, and 1 min at 72 °C), and a final extension step of 3 min at 72 °C.

PCR products were loaded and visualized in 2% agarosa gels as described above for DNA extractions, using a 100−1500 bp DNA ladder (Dominion-MBL) as the molecular-weight standard. Double-stranded DNA products were purified using the Nucleospin 96 Extract II (Macherey-Nagel) according to the instructions of the manufacturer. The concentration and purity were measured by means of an ultraviolet−visible spectrophotometer (Biophotometer Eppendorf). Subsequently, sequencing reactions were carried out in a final volume of 10 μL with the CEQ Dye Terminator cycle sequencing Quick Start kit (Beckman Coulter). The thermal cycle sequencing reaction and the subsequent sequencing products cleanup by ethanol precipitation were carried out in accordance with the instructions of the manufacturer (Beckman Coulter), and the sequences were obtained on a CEQ 8800 genetic analysis system (Beckman Coulter). Next, these sequences were analyzed with the Chromas 1.45 software (*16*) and aligned with Clustal W (*17*), available in the program BioEdit 7.0 (*18*). The sequences obtained were submitted to the GeneBank database of the National Centre for Biotechnology Information (NCBI).

The intra- and interspecific polymorphism analysis was performed with DnaSP 4.0 software (*19*) from the resulting alignment.

2.3. Development of the PCR−RFLP Methodology. Restriction maps of the DNA sequences obtained were generated using the software Webcutter 2.0 (*20*). The enzyme selection was determined by their ability to generate characteristic restriction profiles for each species with band sizes easily distinguishable on agarose gels. About 100 ng of PCR product was digested with 2 units of the selected enzyme. Digestive reactions were incubated at 37 °C for 2 h. The results of PCR−RFLP analysis were read as described above, except that the electrophoresis was performed on 3% low melting agarose gels (Pronadisa) at 70 V for 110 min. Size fragments were estimated from 100−1500 bp (Dominion-MBL) and 50 bp (GE Healthcare) DNA ladders.

2.4. Development of the Forensically Informative Nucleotide Sequencing (FINS) Methodology. Phylogenetic analyses were carried out using the software Mega 3.0 (*21*). The genetic distances among the obtained sequences were estimated using the Tamura and Nei substitution model (*22*), and the inference of the phylogenetic tree was carried out with the neighbor-joining method (*23*). The species *L. caulinaris* and *K. giganteum* were included as outgroup. The degree of confidence assigned to the nodes in the phylogenetic trees was estimated by bootstrapping (*24*) with 2000 replicates (*25*).

2.5. Methodological Validation. The treatments applied to samples were performed in the pilot plant of CECOPESCA (Spanish National Centre of Fish Processing Technology). From specimens of the different species of the genus *Lophius* previously authenticated on the basis of their morphological features, different kinds of processed products were prepared. Fillets, tails, and cheeks were frozen at −24 °C, fried, and precooked in different sauces at 100 °C.

These products were analyzed in the same way as the standard species used to develop the methodology described in this work.

The results obtained using morphological features were compared to those obtained by means of the application of the methodology developed to determine the specificity of the method.

2.6. Application to Commercial Samples. After the validation of the methods developed in the present work, these were applied to 40 fresh and frozen products labeled as some anglerfish species. These products were acquired in supermarkets from Spain, and the purpose of these analyses was to evaluate the situation regarding the labeling of these species in the Spanish market.

3. RESULTS AND DISCUSSION

3.1. Amplification and Sequencing of PCR Products. The Cytochrome *b* (cyt *b*) and COI genes have been widely used as molecular markers in the genetic identification of a great number of species belonging to different taxa (*6−11, 26−28*). The selection of a mitochondrial molecular marker is due to its numerous advantages with regard to the nuclear ones. Among them, mitochondrial molecular marker generally evolves much faster than nuclear DNA, and there are several copies inside a cell (*29*). Moreover, the high abundance of mitochondrial DNA in total cellular nucleic acid preparations allows for more effective PCR amplifications in comparison to nuclear DNA.

In this work, we evaluate the COI gene to identify all of the species contained in the genus *Lophius*. DNA amplification with the COIF-ALT and COIR-ALT primers generated a 699 bp fragment in all of the samples included in the present study (**Table 1**).

All of the sequences obtained in this study were deposited in the NCBI database with accession numbers EU660676−EU660716, EU683975−EU683999, EU854573–EU854575, and FJ263274–FJ263277.

3.2. Development of a Method for Genetic Identification of Species Belonging to the Genus *Lophius*. *3.2.1. Development of the PCR−RFLP Technique.* A genetic identification

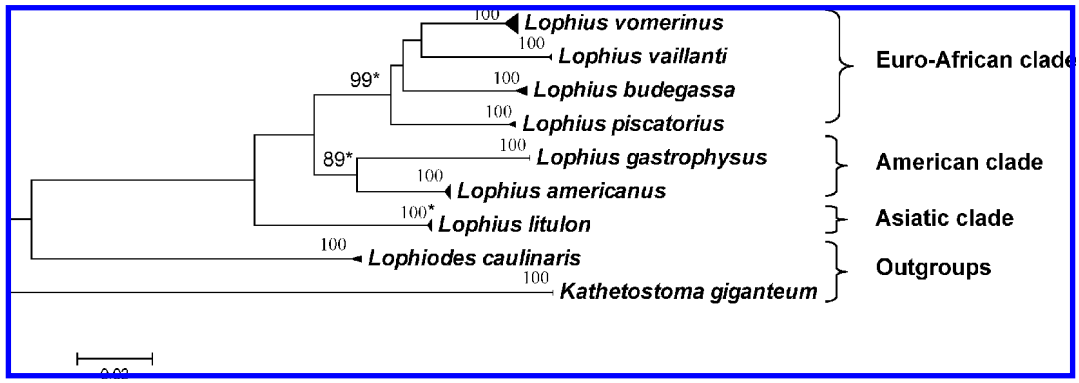

Figure 3. Neighbor-joining tree of genetic relationships among studied *Lophius* species. (*) Bootstrap values of branches that conduct the main clades (Euro-African, Asiatic, and American clade).

Table 2. Commercial Samples Analyzed with the Methods Developed

products			species labeled	species identified	*N* of samples[a]	*N* nt differences[b]
incorrectly labeled	whole fish[c]		*L. budegassa*	*L. piscatorius*	5	42
	processed fish	tails	*L. budegassa*	*L. vaillanti*	6	48
			L. vomerinus	*L. vaillanti*	2	37
		cheeks	*L. vomerinus*	*L. vaillanti*	1	37
			L. piscatorius	*L. litulon*	2	66
correctly labeled	whole fish[c]		*L. budegassa*	*L. budegassa*	9	0
			L. piscatorius	*L. piscatorius*	6	0
	processed fish	tails	*L. vomerinus*	*L. vomerinus*	2	0
			L. vaillanti	*L. vaillanti*	2	0
			L. litulon	*L. litulon*	3	0
		cheeks	*L. vaillanti*	*L. vaillanti*	1	0
			L.vomerinus	*L.vomerinus*	1	0

[a] Number of samples included in this study. [b] Number of nucleotide differences among the labeled species and the detected one. [c] Whole fish includes only fresh fish.

method based on the PCR−RFLP technique was developed. This technique has been widely used for several reasons. It is simpler and faster than sequencing and with a reduced cost. Moreover, it is especially useful for routine analysis when sequencing is not an affordable option. The studies based on the identification of marine species using PCR−RFLP analysis are very numerous, in both invertebrate (*6, 28, 30*) and vertebrate (*9, 10, 31*) species.

After the analysis of the 699 bp sequences obtained using Webcutter 2.0 software, the restrictase M*bo* I (New England BioLabs) was selected as the most suitable enzyme to distinguish all of the species of the genus *Lophius, L. caulinaris*,-and *K. giganteum*. A characteristic restriction profile was expected for each species, except for *L. vomerinus* that presents two restriction haplotypes (**Table 1** and **Figure 2**). Results following digestion of the PCR products showed that the band sizes obtained were in agreement with the expected sizes for the restriction fragments inferred from the sequence analysis (**Figure 2**). The developed methodology was applied to 30 standard individuals of each species, and changes in the expected restriction profiles were not detected.

The PCR−RFLP method developed by Sanjuan et al. (*12*) only takes into account the species *L. budegassa* and *L. piscatorius*. In that case, the analysis of a sample belonging to a not studied species of the genus *Lophius* could generate an unexpected restriction profile, and the sample could not be identified. Also, the restriction profile of a species that was not taken into account in the design of the methodology could be equal to that obtained for the studied species, generating an incorrect identification. On the contrary, the method developed in the present work allows us to identify all of the species of the genus *Lophius, L. caulinaris*, and *K. giganteum* using the restrictase M*bo* I. This enzyme is the most suitable to distinguish these eight species, because it has a simple recognition sequence (GATC), which reduces the risk of mis-identification in contrast to other enzymes with a more complex restriction target.

The samples analyzed to develop the PCR−RFLP method did not show variations in the restriction recognition site for M*bo* I, although the possible existence of variation cannot be dismissed. However, the high number of samples that were taken into account give a high degree of reliability to the developed method.

3.2.2. Development of the FINS Technique. The FINS technique was described by Bartlett and Davidson (*32*) and was used to develop an identification method to identify all of the species belonging to the genus *Lophius*. The basis of this technique is the comparison of sequences of unknown samples with regard to sequences of pattern species.

The genetic distances between the COI gene sequences were estimated using the Tamura−Nei method (*22*). The interspecific distances were calculated to know the degree of divergence between species and were in general 1 order of magnitude higher than the intraspecific ones. The mean values for intra- and interspecific distances were 0.002 ± 0.000 and 0.095 ± 0.006, respectively.

From the distance matrix, a phylogenetic tree was constructed using the neighbor-joining method. All of the sequences belonging to individuals of the same species were grouped in the same cluster, allowing for the differentiation of all of the species included in the study. Moreover, bootstrap values of branches at level species were 100%, reflecting the robustness of the phylogenetic tree (**Figure 3**). Therefore, the COI fragment analyzed allows for the unequivocal identification of all of the *Lophius* species.

On the basis of the phylogenetic tree obtained, three clades strongly supported can be identified: Euro-African, American, and Asiatic, which correspond to the three large distribution

areas of these species. The phylogenetic analysis shows that *K. giganteum* and *L. caulinaris* (outgroups) form two taxa clearly differentiated from the genus *Lophius*. Therefore, also, these taxa can be identified with total reliability by means of the proposed methodology (**Figures 1** and **3**).

3.3. Methodological Validation. In the pilot plant of CECOPESCA were prepared different products emulating the conditions used by the food industry. This approach is useful to assess and optimize the conditions of the developed methodology. The standard individuals underwent several transformation processes, evaluating the influence of these variables on the genetic method herein proposed. The results obtained with the application of the PCR−RFLP and FINS methods developed to the processed products prepared in the pilot plant of CECOPESCA were in agreement with those that were based on morphological characters. Therefore, both methods show a specificity of 100% when these are applied to this kind of product.

3.4. Application to Commercial Samples. The application of the PCR−RFLP and FINS methods developed in the present study to 40 commercial samples allowed us to know the degree of fulfillment of the labeling regulations of *Lophius* species in seafood products. In 40% of the products analyzed (16 samples), the name of the species displayed in the label was not in agreement with the result of the genetic analysis (**Table 2**). These species subjected to mislabeling were *L. budegassa* (68.75%), *L. vomerinus* (18.75%), and *L. piscatorius* (12.5%). The fraudulent labeling ratio was higher in processed products (68.75%) than whole fish (31.25%). This fact can be due to the impossibility of carrying out a species identification based on morphological traits in processed products.

The PCR−RFLP technique allowed us to identify all of the samples analyzed as some *Lophius* species, because the RFLP profiles fit with the expected ones. Also, the bootstrap values obtained in the phylogenetic analyses for the branches at the species level were 100%, allowing for the reliable assignment of the samples analyzed to a particular species. The specific assignations by means of both techniques (PCR−RFLP and FINS) were in accordance in all cases.

The sequences obtained for all commercial samples were identical to those corresponding to the standard specimens. **Table 2** includes the number of nucleotide differences between the species displayed in the product label and the species contained in this one. In all cases, the number of nucleotidic differences was high, with a mean of 46 positions in the 16 samples incorrectly labeled.

In conclusion, in the present work, two DNA-based methods that allow for the genetic identification of all of the species of the genus *Lophius, L. caulinaris*, and *K. giganteum* were developed. They can be applied to all kinds of processed products available in the market for these species. The importance of this work lies in the fact that a genetic identification method to identify the seven species of the genus *Lophius* has been not developed yet. Moreover, the PCR−RFLP and FINS methods can be alternatively used depending upon the equipment available in different laboratories for carrying out the analyses. Some of the possible applications of these methods are the detection of deliberate or unintentional mislabeling of seafood products that contain these species, their traceability, as well as the verification of authenticity of imported raw material.

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