Development of a Method for the Genetic Identification of Flatfish Species on the Basis of Mitochondrial DNA Sequences

MONTSERRAT ESPIÑEIRA, NEREA GONZÁLEZ-LAVÍN, JUAN M. VIEITES, AND
FRANCISCO J. SANTACLARA*

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

In the present study a method for genetic identification of flatfish species was developed. The technique is based on DNA sequencing of amplified DNA by PCR and subsequent phylogenetic analysis (*FINS*). A phylogenetic tree using the cytochrome oxidase subunit I (COI) was constructed and the bootstrap values calculated. The mentioned technique allows the genetic identification of more than 50 flatfish species in fresh, frozen, and precooked products. This analytical system was validated and subsequently applied to 30 commercial samples, obtaining 13 that were incorrectly labeled (43%). Four of the mislabeled samples were whole fish (31%), and nine were fillets (69%). The species with the higher rate of incorrect labeling were *Pleuronectes platessa* (17%) and *Solea solea* (10%). Other species incorrectly labeled were *Hipoglossus hipoglossus* (7%), *Reinharditus hippoglossoides*, *Limanda ferruginea*, and *Microstomus kitt* (3% each species). Therefore, this molecular tool is appropriate to clarify questions related with the correct labeling of commercial products, the traceability of raw materials, and the control of imported flatfish, and also can be applied to questions linked to the control of fisheries.

INTRODUCTION

The order Pleuronectiformes includes a lot of fish species known as flatfish, characterized by a bilaterally asymmetrical and greatly compressed body. The main feature of this group is their taxonomic complexity since it includes more than 600 species distributed in 11 families. Many of them have excellent organoleptic or sensory properties and nutritive value. For these reasons, they are much demanded in the international market.

From a commercial point of view, the identification of flatfish based on morphological characters cannot be carried out in processed products because the fish lack head and skin, as is the case in frozen fillets or slices; the main format for these products are commercialized. However, there exists a large number of normative regulations about the labeling of fishery and aquaculture products legally defined at regional, national, and international levels because this is an important issue linked to the protection of the consumer rights.

An irregular practice is to replace one species by other similar species, but with different organoleptic characteristics and, usually, a smaller economic value. For example, it is possible to find a lot of flatfish identified as sole in the market, which

fraudulently replace *Solea solea*. The majority of these species are halibut, flounder, turbot, and sole species different from the European sole, coming from anywhere in the world. Sometimes, these substitutions are not deliberate because of the difficulty in identifying some different species fished together (in the same fishery) on the basis of the morphological characters. In other cases, this happens deliberately, and as consequence of that, the consumer cannot assign the slices or fillets to a specific species. The substitution of a fish species for others with less value represents fraud and violates the consumer's rights because it impedes their choice of one fixed product versus others based on the information of the label since this is not correct. Moreover, the fishing and transformation industry could be affected by means of unfair competition. To avoid these irregular situations, different analysis techniques that permit the identification of species may be used to protect consumers' rights and at the same time also allow a loyal and honest competition in the fishing industry. Specifically, the molecular biology techniques provide a valuable tool to detect labeling mistakes in fishing products. In this context, several mitochondrial genes were studied extensively in many fish, even some mitochondrial genomes were completely sequenced (*1–4*). In the field of genetic identification of species, specifically flatfish, several studies have been carried out to date, but all of them have some drawbacks. For instance, they do not cover most of the species

* To whom correspondence should be addressed. Tel: (34) 986 469 301. Fax: (34) 986 469 269. E-mail: fran-santa@hotmail.com.
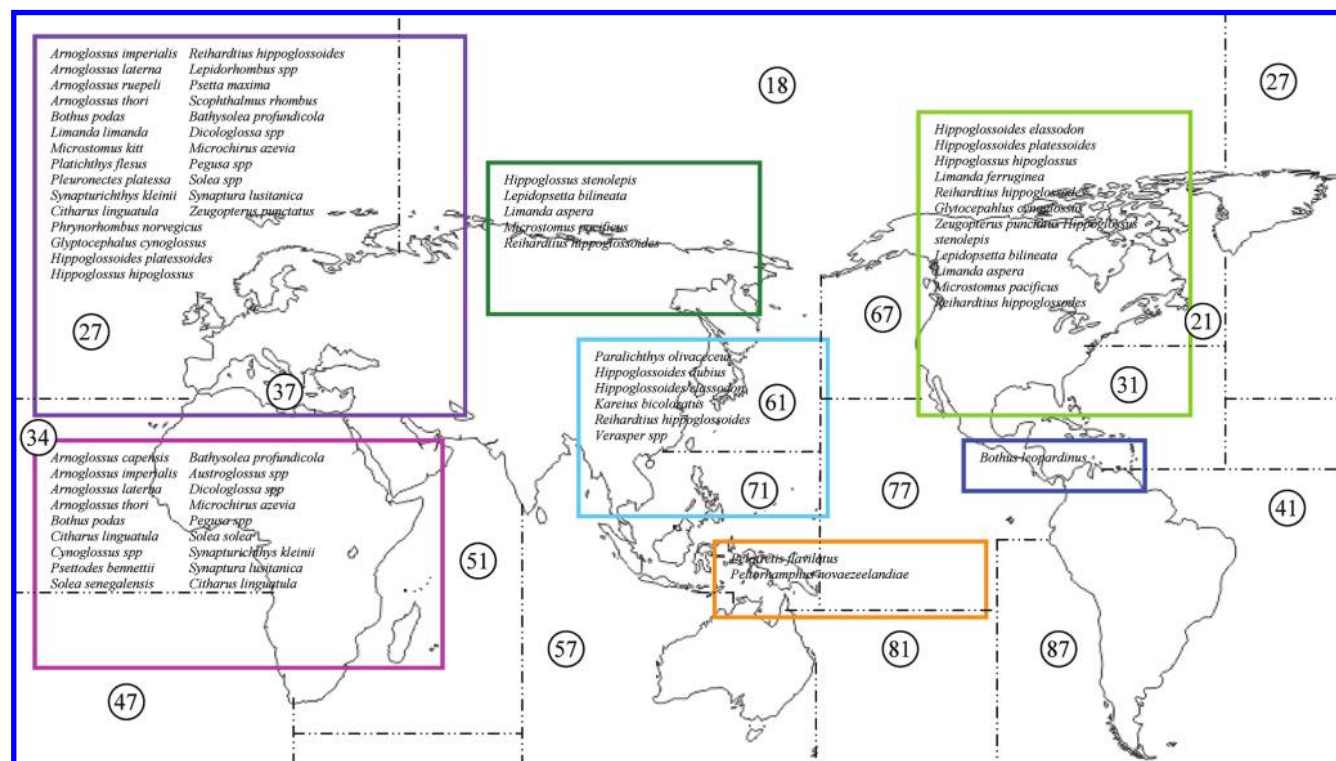
Method for the Genetic Identification of Flatfish Species

J. Agric. Food Chem., Vol. 56, No. 19, 2008 **8955**

Figure 1. Distribution map of the flatfish species included in the present study. Circled numbers indicate the different FAO areas.

present in the actual international market (*5–7*); or few individuals were included so that intraspecific variability without study can exist (*8*).

Because of that, in the present work the genetic variability of more than 50 flatfish species with great commercial interest was studied. The selected molecular markers were two protein-coding mitochondrial genes, the *cytochrome b* (*cyt b*) and the *cytochrome oxidase* subunit I (COI).

The main objective was to develop a genetic method that allows one to evaluate the correct labeling of the analyzed products and therefore the labeling situation of flatfish in the market.

MATERIALS AND METHODS

1. Sampling and DNA Extraction. Authentic flatfish samples were collected from different marine locations around world (**Figure 1**). The species, number of specimens, the location of samples, and other data are shown in **Table 1**. Samples were labeled after arriving at the laboratory and preserved at −80 °C until DNA extraction. When it was possible, the specimens were identified on the basis of morphological traits according to different bibliographic references (*9–12*).

Total genomic DNA was extracted from 30 mg of muscle tissues according to the standard CTAB phenol−chloroform protocol described by Roger and Bendich with slight modifications (*13*) and subsequently was visualized in agarose gels (*Sigma*) at 1% in TBE buffer with 5 μg/mL of ethidium bromide (*Sigma*) under ultraviolet light using a Molecular Imager Gel Doc XR System transiluminator and the software Quantity One v 4.5.2 (Bio-Rad).

Quality and quantity of the obtained DNA was measured by spectrophotometric analysis at 234, 260, and 280 nm by means of a UV−vis spectrophotometer (Biophotometer Eppendorf) (*14*).

2. Amplification of the PCR Products. Two mitochondrial gene fragments encoding *cyt b* and COI were amplified. Amplifications of the two partial *cyt b* gene fragments were carried out using the primers L14735/H15149AD (*15*) and L14735/*TRUCCYTB-R* (*16*). Amplification of the COI gene fragment was performed using the primers *COIF-ALT/COIR-ALT* (*17*) (**Table 2** and **Figure 2**).

All these amplifications were carried out in a final volume of 50 μL containing 100 ng of DNA template, 5 μL of 10× buffer, 2 mM MgCl₂,

0.4 μL of 100 mM dNTP, 0.8 μM solution of each primer, and 1 unit of T*aq*-polymerase (Bioline). All reactions were performed using a Bio-Rad MyCycler thermocycler. The program of cycling for primer sets *COIF-ALT/COIR-ALT* and L14735/*TRUCCYTB-R* was the following: a preheating step of 3 min at 95 °C, then 35 cycles (30 s at 95 °C, 30 s at 54 °C, and 30 s at 72 °C), and a final extension step of 3 min at 72 °C. Conditions for amplification with the primer set L14735/H15149AD were as follows: a preheating step of 3 min at 96 °C, then 40 cycles (20 s at 96 °C, 15 s at 50 °C, and 2 min at 60 °C).

In order to ensure the proper working of PCR amplification, PCR products were loaded in agarose gels (Sigma) at 2% in TBE buffer and 5 μg/mL of ethidium bromide (Sigma) allowing band detection. DNA fragments were visualized using the Molecular Imager Gel Doc XR System transiluminator and the software Quantity One v 4.5.2 (Bio-Rad). Size of amplified fragments was estimated from the molecular marker *pGEM* (Promega). Double-stranded PCR products were purified before sequencing reaction using Nucleospin Extract II (Macherey-Nagel) according to the manufacturer's protocol. The concentration and purity were estimated by spectrophotometric measurement at 260 and 280 nm by means of an UV−vis spectrophotometer (Biophotometer Eppendorf).

3. Sequencing of the PCR Products. PCR products were sequenced in both directions to avoid sequencing errors using the same primers of PCR amplification. Both strands were sequenced on an ABI Prism 310 DNA Genetic Analyzer (Applied Biosystems) using BigDye Terminator Cycle Sequencing Ready Reaction Kit v1.1 (Applied Biosystems) following the manufacturer's instructions. Nucleotide sequences obtained were corrected with Chromas 1.45 (*18*) and subsequently aligned with the program BioEdit 7.0 (*19*). The alignments were corrected by hand, and the primer sequences were excluded from the sequencing data. Moreover, DNA sequences from different databases were included in the alignment (**Table 1**). From this alignment, a polymorphism analysis was carried out using DnaSP 4.0 (*20*).

4. Development of *FINS* (Forensically Informative Nucleotide Sequencing) Methodology. The phylogenetic analyses were carried out with Mega 3.0 (*21*). The genetic distances among the obtained sequences (COI and *cyt b*) and those obtained from the GenBank database were estimated using the Tamura and Nei substitution model, and the inference of the phylogenetic tree was carried out

Table 1. Samples Included in This Work and the Location of Collection[a]

family	scientific name	common name	samples	location	Seq COI and *cyt b*
Bothidae	*Arnoglossus capensis*	Cape flounder	2	ZAF	3
	Arnoglossus imperialis	Imperial scaldfish	2	AEC, M	3
	Arnoglossus laterna	Scaldfish	2	PRT	3
	Arnoglossus rueppelii	Rüppell's scaldback	2	AN	3
	Arnoglossus thori	Thor's scaldfish	2	ITA	3
	Bothus leopardinus	Pacific leopard flounder	2	MEX	3
	Bothus podas	Wide-eyed flounder	2	PRT	3
Citharidae	*Citharus linguatula*	Atlantic spotted flounder	2	PRT	3
Cynoglossidae	*Cynoglossus browni*	Nigerian tonguesole	2	ZAF	3
	Cynoglossus canariensis	Canary tonguesole	2	SEN	3
	Cynoglossus senegalensis	Senegalese tonguesole	2	SEN	3
	Cynoglossus zanzibarensis	Zanzibar tonguesole	2	ZAF	4
Paralichthyidae	*Paralichthys olivaceus*	Olive flounder	2	NC002386, AB028664	
Pleuronectidae	*Glyptocephalus cynoglossus*	Witch	4	ANW, ANE	3
	Hippoglossoides dubius	Flathead flounder	2	PNW	3
	Hippoglossoides elassodon	Flathead sole	2	USA	5
	Hippoglossoides platessoides	American plaice	3	NOR	3
	Hippoglossus hipoglossus	Atlantic halibut	4	CAN, NOR, USA	3
	Hippoglossus stenolepis	Pacific halibut	2	USA	3
	Kareius bicoloratus	Stone flounder		NC003176, AP002951	3
	Lepidopsetta bilineata	Rock sole	3	USA	3
	Limanda aspera	Yellowfin sole	2	USA	3
	Limanda ferruginea	Yellowtail flounder	4	USA, CAN	3
	Limanda limanda	Dab	2	NOR	4
	Microstomus kitt	Lemon sole	2	NOR	3
	Microstomus pacificus	Dover sole	2	USA	3
	Platichthys flesus	Flounder	3	ANE, AEC	3
	Pleuronectes platessa	European plaice	2	NOR, CAN	3
	Reinhardtius hippoglossoides	Greenland halibut	9	CAN	3
	Verasper moseri	Barfin flounder		NC008461, EF025506	
	Verasper variegatus	Spotted halibut		NC007939, DQ403797	
Psettodidae	*Psettodes bennettii*	Spiny turbot	2	SEN	3
Rhombosoleidae	*Pelotretis flavilatus*	Southern lemon sole	2	NZL, BOLD[b]	5
	Peltorhamphus novaezeelandiae	New Zealand sole	2	NZL, BOLD[b]	3
Scophthalmidae	*Lepidorhombus boscii*	Fourspotted megrim	3	CAN, PRT	3
	Lepidorhombus whiffiagonis	Megrim	2	PRT	3
	Phrynorthombus norvegicus	Norwegian topknot	2	NOR	3
	Psetta maxima	Turbot	4	NOR	3
	Scophthalmus rhombus	Brill	2	PRT	3
	Zeugopterus punctatus	Topknot	2	PRT	3
Soleidae	*Austroglossus pectoralis*	Mud sole	2	ZAF	3
	Austroglossus microlepis	West coast sole	2	ZAF	3
	Bathysolea profundicola	Deepwater sole	2	ANE	3
	Buglossidium luteum	Solenette	2	PRT	3
	Dicologlossa cuneata	Wedge sole	3	ANE, AEC	3
	Dicologlossa hexophthalma	Ocellated wedge sole	2	ANE	3
	Microchirus azevia	Bastard sole	2	PRT	3
	Pegusa lascaris	Sand sole	2	PRT	3
	Pegusa impar	Adriatic sole	2	GRE	3
	Solea senegalensis	Senegalese sole	4	ANE, AEC, ASE	3
	Solea aegyptiaca	Egyptian sole	2	EGY	3
	Solea solea	Common sole	6	ESP, IRL, ITA	5
	Synapturichthys kleinii	Klein's sole	2	PRT	3
	Synaptura lusitanica	Portuguese sole	2	ANE, AEC	3

[a] Location abbreviations: AT, Atlantic; ASE, Atlantic Southeast; AN, Atlantic North; ANE, Atlantic Northeast; ANW, Atlantic Northwest; AEC, Atlantic Eastern Central; CAN, Canada; EGY, Egypt; SP, Spain; GRE, Greece; ITA, Italy; M, Mediterranean Sea; MEX, Mexico; NOR, Norway; NZL, New Zealand; PRT, Portugal; PNW, Pacific Northwest; SEN, Senegal; USA, United States; ZAF, South Africa; IRL, Ireland. [b] Barcode of life (BOLD) sequences supplied by Dr. Steinke.

with the Neighbor-Joining method. The reliability of the clades formed at the species level in the tree was evaluated by means of the bootstrap test with 2000 replications.

5. Methodological Validation. Individuals of the different species were authenticated on the basis of their morphological traits. Subsequently, from these were prepared fried fillets and slices, and they were frozen at −80 °C. All these treatments were carried out

in the pilot plant of CECOPESCA (Spanish National Centre of Fish Processing Technology). Subsequently, the samples were analyzed with the methodology developed in the present work.

Results of the species assignment on the basis of morphology and genetic probes were compared. The coincidence percentage between the species identified on the basis of morphological traits

Method for the Genetic Identification of Flatfish Species

J. Agric. Food Chem., Vol. 56, No. 19, 2008 **8957**

Table 2. Primers Used in This Work

name	sequence 5′–3′	size (bp)	described
COIF-ALT	ACA AAT CAY AAR GAY ATY GG	699	Mikkelsen, P. M. et al. (2006)
COIR-ALT	TTC AGG RTG NCC RAA RAA YCA		Mikkelsen, P. M. et al. (2006)
L14735	AAA AAC CAC CGT TGT TAT TCA ACT A	1226	Burgener et al. (1997)
TRUCCYTB-R	CCG ACT TCC GGA TTA CAA GAC CG		Sevilla, R. et al. (2007)
L14735	AAA AAC CAC CGT TGT TAT TCA ACT A	463–465	Burgener et al. (1997)
H15149AD	CCI CCT CAR AAT GAY ATT TGT CCT CA		Burgener et al. (1997)

and the genetic methodology developed was calculated to establish the specificity of the method.

Also, the identity of PCR products was confirmed by the Basic Local Alignment Search Tool (BLAST) (*22*) in the database of the National Center for Biotechnology Information (NCBI).

6. Application to Commercial Samples. Once the methods previously described were validated, these were applied to 30 fresh and frozen flatfish products purchased in stores and supermarkets in Spain with the aim of evaluating their correct labeling.

RESULTS AND DISCUSSION

In the present study, the genetic variability of two molecular markers (*cyt b* and COI) were studied in 54 flatfish species. These two mitochondrial genes were used in many previous works of genetic identification of species (*23–25*). Therefore, they seem good candidates for this purpose. All the data shown in this work refer to the COI marker because it offers many advantages as compared to the *cyt b* gene, as described in the following sections.

Amplification and Sequencing of PCR Products. The amplification of partial regions of the *cyt b* and COI genes was obtained for all of the samples included in this study (**Table 1**). The primers used for the PCR amplification of COI (*COIF-ALT/COIR-ALT*) and *cyt b* (L14735/*TRUCCYTB-R* and L14735/H15149AD) generated fragments of 699, 1226, and 463–465 bp, respectively.

The PCR product obtained with L14735/H15149AD primers was included in the fragment of 1226 bp (**Figures 2** and **3**). The length polymorphism between species in these PCR products was caused by indels in the glutamic acid tRNA, located at 5′ of the *cyt b* gene. Some problems were detected to amplify the *cyt b* gene fragments. Both primers sets L14735/TRUCCYTB-R and L14735/H15149AD were applied to all of the taxonomic groups included in this study, and some species did not amplify the awaited PCR product with the mentioned

primers (for instance *Arnoglossus capensis*, *Arnoglossus thori*, or *Bothus podas*). This lack of amplification is an important drawback of using this molecular marker in the system of genetic identification because the species not amplified cannot be assigned to a particular species with the proposed technique.
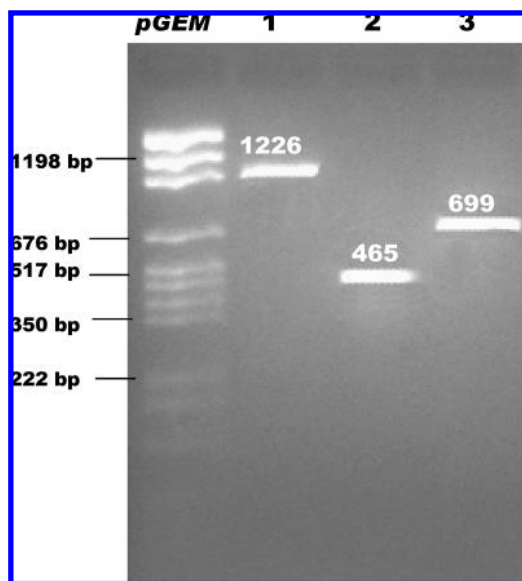

Figure 3. PCR products and size obtained from flatfish species amplified in this work. Lane *pGEM*, molecular marker *pGEM* (*Promega*); lane 1, PCR product obtained with primers L14735/TRUCCYTB-R; lane 2, PCR product obtained with primers L14735/H15149AD; lane 3, PCR product obtained with primers COIF-ALT/COIR-ALT.
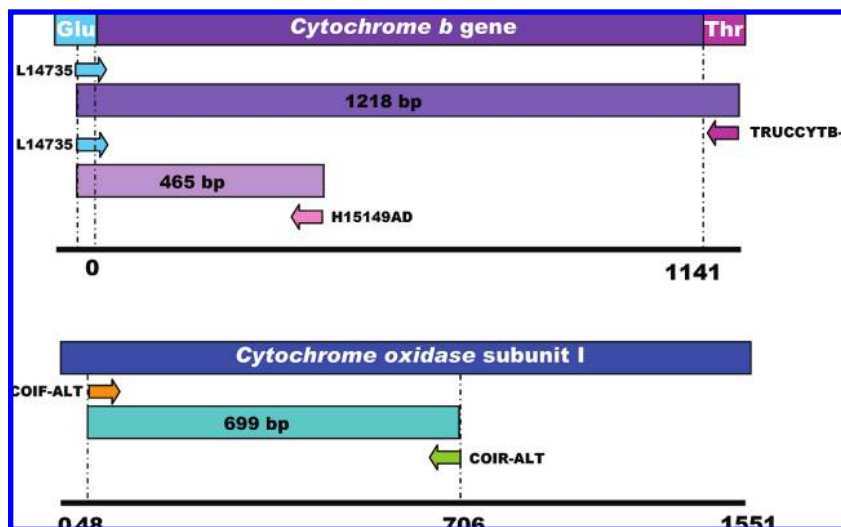

Figure 2. Location and size of the DNA fragments (cytochrome *b* and cytochrome oxidase subunit I) amplified in this work and the position of the primer sets used.
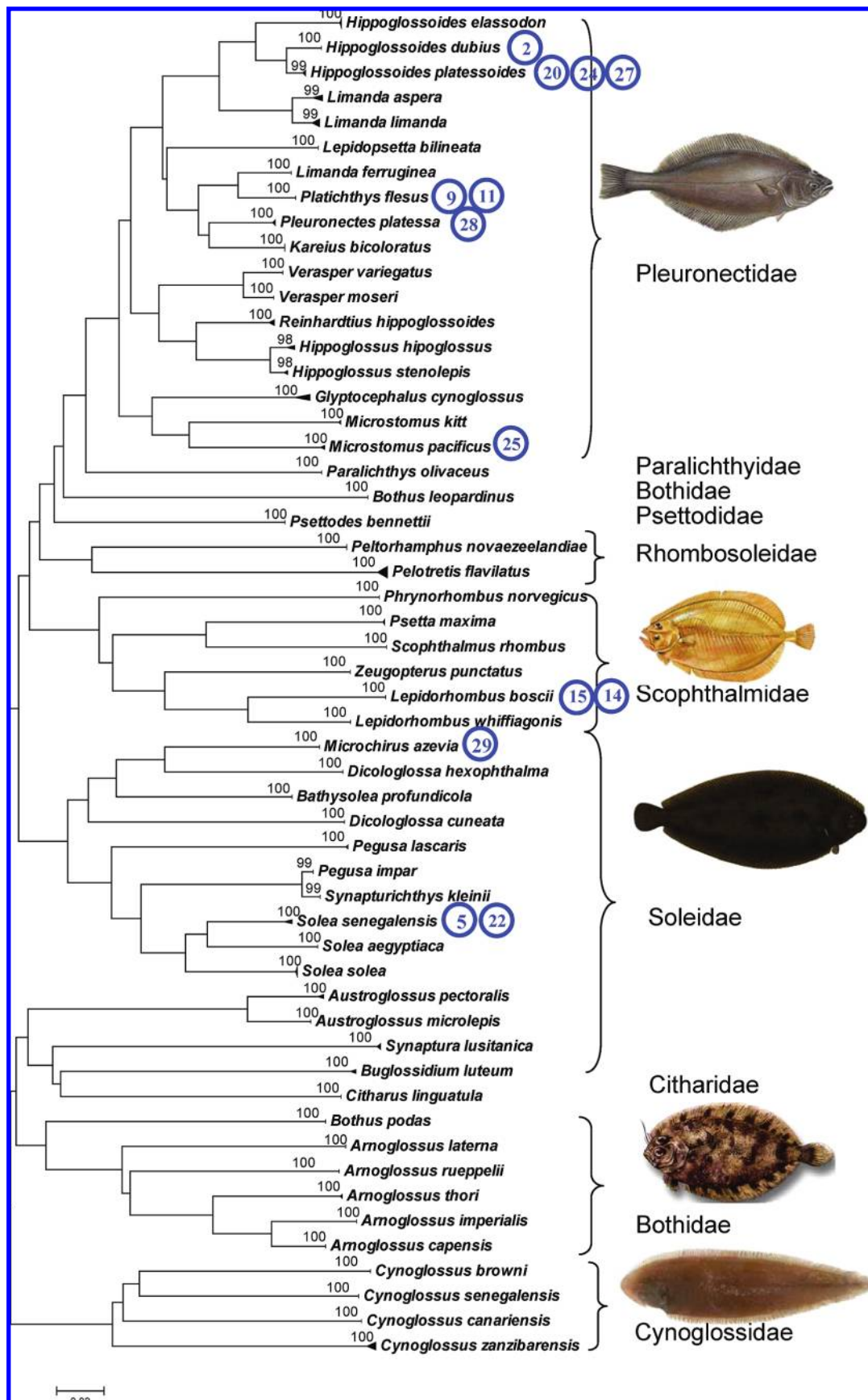
8958 *J. Agric. Food Chem.,* Vol. 56, No. 19, 2008

Espiñeira et al.

Figure 4. Phylogenetic tree showing the relationships among the studied flatfish species, carried from the alignment of 658 bp of the COI gene (fragment of 699 bp without primers). Circled numbers belong to the commercial samples analyzed where a mislabeling was detected.

In contrast, the COI fragment was amplified and sequenced in all of the studied species without exceptions. For this reason, we suggest the use of the COI fragment in order to carry out the genetic identification of flatfish.

Method for the Genetic Identification of Flatfish Species

J. Agric. Food Chem., Vol. 56, No. 19, 2008 **8959**

Table 3. Commercial Samples Analyzed with the Method Developed

products	species labeled	species identified	N samples[a]	N nt differences[b]
		Incorrect Labeled		
whole fish[c]	*Solea solea*	*Solea senegalensis*	2	60
	Solea solea	*Microchirus azevia*	1	119
	Pleuronectes platessa	*Platichthys flesus*	1	42
frozen fillets	*Hippoglossus hipoglossus*	*Lepidorhombus boscii*	2	146
	Pleuronectes platessa	*Hippoglossoides platessoides*	3	69
	Pleuronectes platessa	*Hippoglossoides dubius*	1	73
	Reinhardtius hippoglossoides	*Pleuronectes platessa*	1	76
	Limanda ferruginea	*Platichthys flesus*	1	30
	Microstomus kitt	*Microstomus pacificus*	1	73
		Correctly Labeled		
whole fish[c]	*Dicologlossa cuneata*	*Dicologlossa cuneata*	2	0
	Dicologlossa hexophthalma	*Dicologlossa hexophthalma*	1	0
	Solea solea	*Solea solea*	1	0
	Microchirus azevia	*Microchirus azevia*	2	0
	Synaptura lusitanica	*Synaptura lusitanica*	2	0
frozen fillets	*Lepidorhombus spp*	*Lepidorhombus whiffiagonis*	1	0
	Glyptocephalus cynoglossus	*Glyptocephalus cynoglossus*	1	0
	Psetta maxima	*Psetta maxima*	3	0
	Scophthalmus rhombus	*Scophthalmus rhombus*	2	0
	Hippoglossoides elassodon	*Hippoglossoides elassodon*	1	0
	Limanda ferruginea	*Limanda ferruginea*	1	0

[a] The number of samples included in this study. [b] The number of nucleotidic differences among the labeled species and the detected one. [c] Whole fish includes the fresh fish and frozen fish.

The obtained sequences in the frame of this study were deposited in GenBank with the following accession numbers: EU513601−EU513755 (COI) and EU513756−EU513883 (*cyt b*).

Development of a *FINS* Method for the Genetic Identification of Flatfish Species. The application of the *FINS* technique makes it possible to develop a method to identify all the studied flatfish. This technique was described by Bartlett and Davidson (*26*), who proposed the genetic identification of species using phylogenetic analysis of DNA sequences. For the application of this method, it was necessary to obtain the pattern of sequences belonging to flatfish. The analysis is developed by means of comparisons of unknown species (the sample object of study) and pattern sequences (belonging to reference individuals of each species).

Phylogenetic analysis based on partial COI and *cyt b* genes sequences were carried out separately, allowing the establishment of the relationships among species of flatfish by means of the construction of phylogenies using this two data sets.

The genetic distances between the obtained sequences of COI were related with the degree of divergence between species. The intraspecific distance mean was 0.00324 ± 0.0023. Interspecific distances were, in general, 2 orders of magnitude higher than the intraspecific ones. The mean value for these was 0.249 ± 0.05.

From the distance matrix of COI, one phylogenetic tree was constructed using the Neighbor-Joining method. Samples belonging to the same species were grouped into the same clade. Bootstrap values were higher than 96% using the COI matrix (**Figure 4**). These bootstrap values reflect the robustness of the nodes obtained from the original data sets.

The *cyt b* gene offers less powerful species assigment than COI. Another advantage of this second molecular marker is that it allows amplification of the PCR product in all the studied species, unlike the *cyt b* gene that shows problems in amplification. For this reason, the COI gene is proposed for the genetic identification of flatfish in this study. Other primer sets could be evaluated in these cases of amplification problems, for instance, using the primers cited in

the work of Sevilla et al., where 12 primers are described for amplifying and sequencing the complete *cyt b* gene in more than 200 marine fish species (*16*).

BLAST Analysis for Genetic Identification of Flatfish Species. *BLAST* analysis is a suitable technique to find regions of local similarity between sequences and can even be a suitable technique to identify species. This method is similar to the *FINS* since it uses DNA sequences and a database. Specifically, the *MEGABLAST* search available at NCBI was assessed to assign any flatfish DNA sequence to a particular species. The phylogenetic assignments generated by the proposed *FINS* technique were compared to the results obtained by *BLAST*. The same results of the species assignment were obtained (data not shown). Therefore, these two techniques could be used to identify the flatfish species herein studied.

Differentiation of *Limanda limanda−L. ferruginea* and *Pegusa lascaris−P. impar*. In previous work, Sotelo et al. pointed out the impossibility of differentiating these two pairs of species (*Limanda limanda−L. ferruginea* and *Pegusa lascaris−P. impar*) using the 463−465 bp fragment of the *cyt b* gene (*8*). The results emanated from the present work disagree with those previously obtained by Sotelo et al. since these two pairs of species can be perfectly identified, both with the *cyt b* gene and COI (**Figure 4**). Our findings are in accordance with the work of Pardo et al., who using a fragment of about 644 bp of 16S rDNA (a marker with lower substitution rate than *cyt b* and COI) differentiated *P. lascaris* and *P. impar*. (*27*).

Methodological Validation. The species assignment of the processed products prepared in the pilot plant of CECOPESCA by the *FINS* and *BLAST* approximations herein described were in agreement with those based on morphological characters. Therefore, the techniques showed a specificity of 100% when applied to flatfish products.

Application to Commercial Samples. The method developed in the present study was applied to 30 commercial samples, allowing us to verify the fulfillment of the labeling rules of the flatfish. The *FINS* and *BLAST* methods herein developed were

applied to these commercial products, which were identified as some species from those included in this work. Thirteen analyzed samples contained a different species from those indicated in the label, meaning 43% of the samples were incorrectly labeled. These species were *Pleuronectes platessa* (17%), *Solea solea* (10%), *Hipoglossus hipoglossus* (7%), *Reinharditus hippoglossoides* (3%), *Limanda ferruginea* (3%), and *Microstomus kitt* (3%) (**Table 3**). The fraudulent labeling ratio was higher in processed samples (69%) than in whole fish (31%) because morphological assignment to a particular species is not possible in processed products.

The phylogenetic analysis described previously allowed us to assign all the analyzed samples to a particular species with bootstrap values higher than 96%. All the commercial samples showed a nucleotide sequence equal to some standard specimen. The **Table 3** includes the number of nucleotide differences between the labeled and the detected species. These differences were very high in all cases, with a mean of 53 positions in the 30 analyzed samples.

In conclusion, this article describes a DNA-based method that allows the genetic identification of flatfish species in fresh and frozen forms, and those that have not undergone an intensive process of transformation. It is worth highlighting that this method is the most completely developed one to date in terms of the number of species included (more than 50). The developed tool is based on the amplification and sequencing of DNA following phylogenetic analysis and can be very useful in the normative control of raw and processed products produced from flatfish, particularly in the authenticity of imported species, the verification of the traceability of different fishing batches along the commercial chain, correct labeling, the protection of the consumer's rights, the fair competence among fishing operators, and also for fisheries control.

LITERATURE CITED

(1) Yamanoue, Y.; Miya, M.; Matsuura, K.; Yagishita, N.; Mabuchi, K.; Sakai, H.; Katoh, M.; Nishida, M. Phylogenetic position of tetraodontiform fishes within the higher teleosts: Bayesian infer- ences based on 44 whole mitochondrial genome sequences. *Mol. Phylogenet. Evol.* **2007**, *45* (1), 89–101.

(2) Wang, X. Z.; Wang, J.; He, S. P.; Mayden, R. L. The complete mitochondrial genome of the Chinese hook snout carp Opsari- ichthys bidens (Actinopterygii: Cyprinifonnes) and an alternative pattem of mitogenomic evolution in vertebrate. *Gene* **2007**, *399* (1), 11–19.

(3) Lavoue, S.; Miya, M.; Saitoh, K.; Ishiguro, N. B.; Nishida, M. Phylogenetic relationships among anchovies, sardines, herrings and their relatives (Clupeiformes), inferred from whole mitogenome sequences. *Mol. Phylogenet. Evol.* **2007**, *43* (3), 1096–1105.

(4) Oh, D. J.; Kim, J. Y.; Lee, J. A.; Yoon, W. J.; Park, S. Y.; Jung, Y. H. Complete mitochondrial genome of the rabbitfish Siganus fuscescens (Perciformes, Siganidae). *DNA Seguence* **2007**, *18* (4), 295–301.

(5) Cespedes, A.; Garcia, T.; Carrera, E.; Gonzalez, I.; Fernandez, A.; Asensio, L.; Hernandez, P. E.; Martin, R. Indirect enzyme-linked immunosorbent assay for the identification of sole (*Solea solea*), European plaice (*Pleuronectes platessa*), flounder (*Platichthys flesus*), and Greenland halibut (*Reinhardtius hippoglos-soides*). *J. Food Prot.* **1999**, *62* (10), 1178–1182.

(6) Cespedes, A.; Garcia, T.; Carrera, E.; Gonzalez, I.; Fernandez, A.; Asensio, L.; Hernandez, P. E.; Martin, R. Genetic differentia-tion between sole (*Solea solea*) and Greenland halibut (*Reinhard-tius hippoglossoides*) by PCR-RFLP analysis of a 12S rRNA gene fragment. *J. Sci. Food Agric.* **2000**, *80* (1), 29–32.

(7) Cespedes, A.; Garcia, T.; Carrera, E.; Gonzalez, I.; Sanz, B.; Hernandez, P. E.; Martin, R. Polymerase chain reaction restriction fragment length polymorphism analysis of a short fragment of the cytochrome b gene for identification of flatfish species. *J. Food Prot.* **1998**, *61* (12), 1684–1685.

(8) 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.

(9) Fishes of the North-eastern Atlantic and the Mediterranean 1.0. World biodiversity database series on CD-ROM; 1996.

(10) Lindberg, G.; Fedorov, V. *Fishes of Japan Sea and Nearby Parts of Okhotsk and Yellow Seas*; Part 6. Teleostomi. Osteichthys. Actinopterigii. XXXI. Pleuronectiformes; Sankt-Petersburg Uni-versity Press, 1993; p 272.

(11) Nakabo, T. *Fishes of Japan with Pictorial Keys to the Species (English Edition)*; Tokai University Press, Tokyo, 2002.

(12) Nelson, J. *Fishes of the World*; John Wiley and Sons: New York, 1994; p 624.

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

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

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

(16) Sevilla, R. G.; Diez, A.; Noren, M.; Mouchel, O.; Jerome, M.; Verrez-Bagnis, V.; van Pelt, H.; Favre-Krey, L.; Krey, G.; Bautista, J. M. Primers and polymerase chain reaction conditions for DNA barcoding teleost fish based on the mitochondrial cytochrome b and nuclear rhodopsin genes. *Mol. Ecol. Notes* **2007**, *7* (5), 730–734.

(17) Mikkelsen, P. M.; Bieler, R.; Kappner, I.; Rawlings, T. A. Phylogeny of Veneroidea (Mollusca: Bivalvia) based on morphology and molecules. *Zool. J. Linnean Soc.* **2006**, *148* (3), 439–521.

(18) Mc Carthy, C., Chromas, version 1.45; School of Health science, Griffith University, Gold Coast Campus: Queensland, Australia, 1996.

(19) 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.

Method for the Genetic Identification of Flatfish Species

J. Agric. Food Chem., Vol. 56, No. 19, 2008 **8961**

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

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

(22) Altschul, S.; Madden, T.; Schaffer, A.; Zhang, J. H.; Zhang, Z.; Miller, W.; Lipman, D. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *FASEB J.* **1998**, *12* (8), A1326−A1326.

(23) 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.

(24) 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*, 305–310.

(25) 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*, 9913–9920.

(26) 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.

(27) Pardo, B. G.; Machordom, A.; Foresti, F.; Porto-Foresti, F.; Azevedo, M. F. C.; Banon, R.; Sanchez, L.; Martinez, P. Phylogenetic analysis of flatfish (order Pleuronectiformes) based on mitochondrial 16s rDNA sequences. *Scientia Marina* **2005**, *69* (4), 531–543.