Identification of Flatfish (*Pleuronectiforme*) Species Using DNA-Based Techniques

Carmen G. Sotelo,*,† Pilar Calo-Mata,† Mª Jose Chapela,† Ricardo I. Pérez-Martín,† Harmut Rehbein,‡ Georgina L. Hold,§ Valerie J. Russell,§ Susan Pryde,§ Javier Quinteiro,‖ Mónica Izquierdo,‖ Manuel Rey-Méndez,‖ Carla Rosa,⊥ and Ana T. Santos⊥

Instituto de Investigaciones Marinas, Consejo Superior de Investigaciones Cientificas, Eduardo Cabello 6, 36208 Vigo, Spain, Bundesforschungsanstalt für Fischerei, Institut für Biochemie und Technologie, Palmaille 9, 22769 Hamburg, Germany, Rowett Research Institute, Greenburn Road, Aberdeen AB21 9SB, Scotland, U.K., Departamento de Bioquímica y Biología Molecular, Facultad de Biología, Universidad de Santiago de Compostela, Santiago de Compostela, Spain, and Instituto de Investigaçao das Pescas e do Mar, Av. Brasilia, 1400 Lisboa, Portugal

Identification of flatfish species using a DNA-based methodology was studied. The polymerase chain reaction was employed to obtain a 464 bp amplicon from mitochondrial cytochrome b gene. The sequences from this fragment belonging to 24 species were analyzed using a genetic distance method, and polymorphic sites were determined. The fragment was found to be highly polymorphic (231 sites), and this permitted the differentiation of most of the species. Phylogenetic tree construction was employed to allow the identification of flatfish species. As a result, each species was grouped in a well-differentiated clade, except for two pairs: *Limanda ferruginea* and *L. limanda*, and *Solea impar* and *S. lascaris*, which could not be differentiated. On the basis of the sequences obtained, restriction enzymes were selected to provide specific restriction profiles, which allow the differentiation of 21 species of flatfish in a faster and less expensive manner than sequencing. This polymerase chain reaction−restriction fragment length polymorphism methodology (PCR−RFLP) was tested using commercial samples.

Keywords: *Polymerase chain reaction; authentication; flatfish; mitochondrial DNA; cytochrome b gene; sequencing; restriction endonucleases*

INTRODUCTION

Flatfish comprise a large number of species distributed in 14 families (*1*, *2*); among them, those most commonly found in food markets are Pleuronectidae, Scophthalmidae, and Soleidae. Flatfishes are sold in European markets in different ways, mostly as frozen fillets, but there are a number of other products, such as battered fillets or baby foods that may be labeled as containing some flatfish species. The more expensive species in the European market are sole (*Solea solea)*, turbot (*Scophthalmus maximus*), European plaice (*Pleuronectes platessa*), and flounder (*Platichtys flesus*). Legislation in different countries across Europe establishes a specific label for each of these species. There are other species with less commercial value, such as dab (*Limanda limanda*), Greenland halibut (*Reinhardtius hippoglossoides*), megrim (*Lepidorhombus whiffiagonis*), and sand soles (*Solea impar* and other *Solea* spp.) which might also be labeled specifically (i.e., sole fillet - Greenland Halibut).

Identification of flatfish is of interest not only to consumers, but also to the fish industry that buys the raw fish for use in the manufacture of convenience products. The use of biochemical characteristics for species identification purposes has been reported widely (*3*, *4*, *5)*; among these characteristics, isoelectric focusing profiles of water-soluble proteins or electrophoretic mobility have applications when analyzing refrigerated or frozen seafood products (*6*). However, proteins cannot always be used as biochemical markers for species identification because they become denatured when food products are subjected to thermal treatment such as smoking, cooking, or sterilization during processing. DNA molecules stand those treatments, at least partially, allowing their extraction and posterior analysis (*7*). Also, protein analysis requires the use of authentic species protein extracts which have to be analyzed together with the unknown samples. When a reference is not available the analyst can give only a negative answer to the question "which species is this?" (i.e., unknown sample "x" is not a *Solea solea*).

The identification of species in a group with a very high number of species, such as flatfish, requires that most commercial species be subjected to study. In previous works, DNA has been used as a biochemical marker (*8*−*11*), cytochrome b fragments have been used in some of these works to differentiate sole (*Solea solea*), European plaice (*Pleuronectes platessa*), flounder (*Platichthys flesus*), and Greenland halibut (*Reinhardtius hippoglossoides*)(*8*, *9*), and to differentiate sole (*Solea solea*) and *Microchirus acevia* (*10*). These works used polymerase chain reaction−restriction fragment length

* To whom correspondence should be addressed. Phone: +34-986-214471. Fax: +34-986-292762. E-mail: carmen@iim.csic.es.

† Consejo Superior de Investigaciones Cientificas.

‡ Institut für Biochemie und Technologie.

§ Rowett Research Institute.

‖ Universidad de Santiago de Compostela.

⊥ Instituto de Investigaçao das Pescas e do Mar.

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Table 1. Authentic Species Used in the Present Study

key	common name	scientific name	specimens[a]
		Pleuronectidae	
WF	witch flounder	Glyptocephalus cynoglossus	1, 2, 3, 38, 128, 129, I, GB
LS	lemon sole	Microstomus kitt	44, 45, GB
HIPP	halibut	Hippoglossus hippoglossus	I
GH	Greenland halibut	Reinharditius hippoglossoides	1, 2, 3, I, GB
DB	dab	Limanda limanda	2, I
YF	yellowtail flounder	Limanda ferruginea	3
HPE	flathead sole	Hippoglossoides elassodon	1
HPLA	long rough dab	Hippoglossoides platessoides	I
EP	European plaice	Pleuronectes platessa	6*, 7, 69, I*, GB*
FL	flounder	Platichthys flesus	1, 2, 5, 134, GB, I
		Scophthalmidae	
T	turbot	Scophthalmus maximus	1, 16, 72, I, GB
B	brill	Scophthalmus rhombus	1, 2, GB
M	megrim	Lepidorhombus wiffiagonis.	1, 2, 3
FSM	four spotted megrim	Lepidorhombus boscii	1, 2, 3, I
		Soleidae	
WS	wedge sole	Dicologoglosa cuneata	1, 2, 3, 4
SL	sand sole	Solea lascaris	2, 3
S	sole	Solea solea	1, 15, 116, 117, I
SI	sand sole	Solea impar	GB
SK	Klein's sole	Solea kleini	GB
SSE	Senegalese sole	Solea senegalensis	GB
MO	eyed sole	Monochirus ocellatus	GB
MV	wedge sole	Microchirus variegatus	GB
MH	whiskered sole	Monochirus hispidus	GB
BL	little sole	Buglossidium luteum	GB

[a] Specimens column indicates each of the individuals used for performing the analysis. *, fresh/frozen samples obtained at the local market or from the fish and food industry (Pez Austral, Hero S. A.). Arabic numeration indicates different specimens obtained fresh or frozen in Spain. Roman numeration indicates different specimens obtained fresh or frozen in Germany. *GB, obtained from the GeneBank for comparison.

polymorphism methodology (PCR−RFLP) as a means of differentiating and identifying these species. However, the usefulness of these methodologies is questionable when a limited number of species is studied. The main problem is that there is no guarantee that a nonstudied species will produce a specific RFLP pattern. This means that it can give exactly the same pattern as other studied species. In fact, in previous experimental work by ourselves, we have find that using one of the PCR−RFLP methods developed for the identification of four flatfish species was not longer able to differentiate the four species if twelve flatfish species were analyzed. Such a situation can lead to misidentification of unknown samples. The only way to overcome this problem is to use as many species as possible, which could be present in commercial products, when developing a particular identification technique, but especially when developing PCR−RFLP methods.

The present study will expand the number of flatfish species involved in the development of identification techniques, including those species which are likely present in European markets (24 species). The final objective is to develop identification techniques based on DNA analysis which provide a realistic tool for flatfish species identification.

MATERIALS AND METHODS

Authentic Flatfish Species and Commercial Samples. Some samples of authentic species were obtained fresh in the local fish market, whereas others were obtained frozen from the fish and food industry (Pez Austral, Hero España S. A.) (Table 1). Both types of samples were stored frozen until analyzed.

Frozen commercial samples were obtained in local markets; the samples were either frozen fillets or whole frozen specimen.

Extraction of DNA. DNA extraction from previously thawed frozen muscle was carried out using the standard Wizard DNA Cleanup System (Promega). A 150-mg sample of tissue was placed in a plastic micro tube and suspended in 860 μL of lysis buffer containing 2 mM EDTA, 150 mM NaCl, 1% SDS, and 10 mM Tris−HCl pH 8. To this, 120 μL of guanidium thiocyanate (Sigma Chemicals) and 40 μL of proteinase K solution (20 mg/mL) (Gibco) were added and incubated in a waterbath at 56 °C. After 2 h, extra proteinase K (40 μL) was added to the solution, and it was left overnight in the waterbath at 56 °C. The resulting homogenate was then centrifuged, and the supernatant was collected.

For isolation of the DNA, 500 μL of the collected solution was placed in a syringe barrel attached to a Wizard Minicolumn, to which 1 mL of Wizard DNA Cleanup Resin (Promega) was added. The vacuum was applied to draw the solution through the minicolumn, and the column was washed with 2 mL of 80% 2-propanol before re-applying the vacuum. The column was then transferred to a clean microfuge tube and spun for 2 min. DNA was eluted from the column with 50 μL of prewarmed water (70 °C) and centrifuging, after 1 min, at 10000*g* for 20 s. The DNA solution was collected and stored at −20 °C.

Quantitation of DNA. DNA content in the extracts was measured by a fluorescence assay based on the dye Hoechst 33258 (Molecular Probes) (*12*) in a LS-3B fluorescence spectrometer (Perkin-Elmer), with calf thymus DNA (Sigma) used as a standard.

PCR Amplification of DNA Samples. The primers used amplify a region of 464 bp of the cytochrome b (*13*).

H15149AD: 5′-GCICCTCARAATGAYATTTGTCCTCA-3′ (26 mers)

L14735: 5′-AAAAACCACCGTTGTTATTCAACTA-3′ (25 mers)

PCR reactions were performed in volumes of 25 μL using Ready-to-Go PCR beads (Amersham Pharmacia Biotech) which contained, when reconstituted, 200 μM of each dNTP in 50 mM KCl, 1.5−2 mM $MgCl_2$, 10 mM Tris−HCl pH 9 at room temperature, and 1.5 U of Taq polymerase. To the reaction, 2 μL of each primer (10 μM) was added, and 125 ng of the template DNA was added. Amplifications were carried out in a GeneAmp 2400 PCR system (Perkin-Elmer), with a preheat-

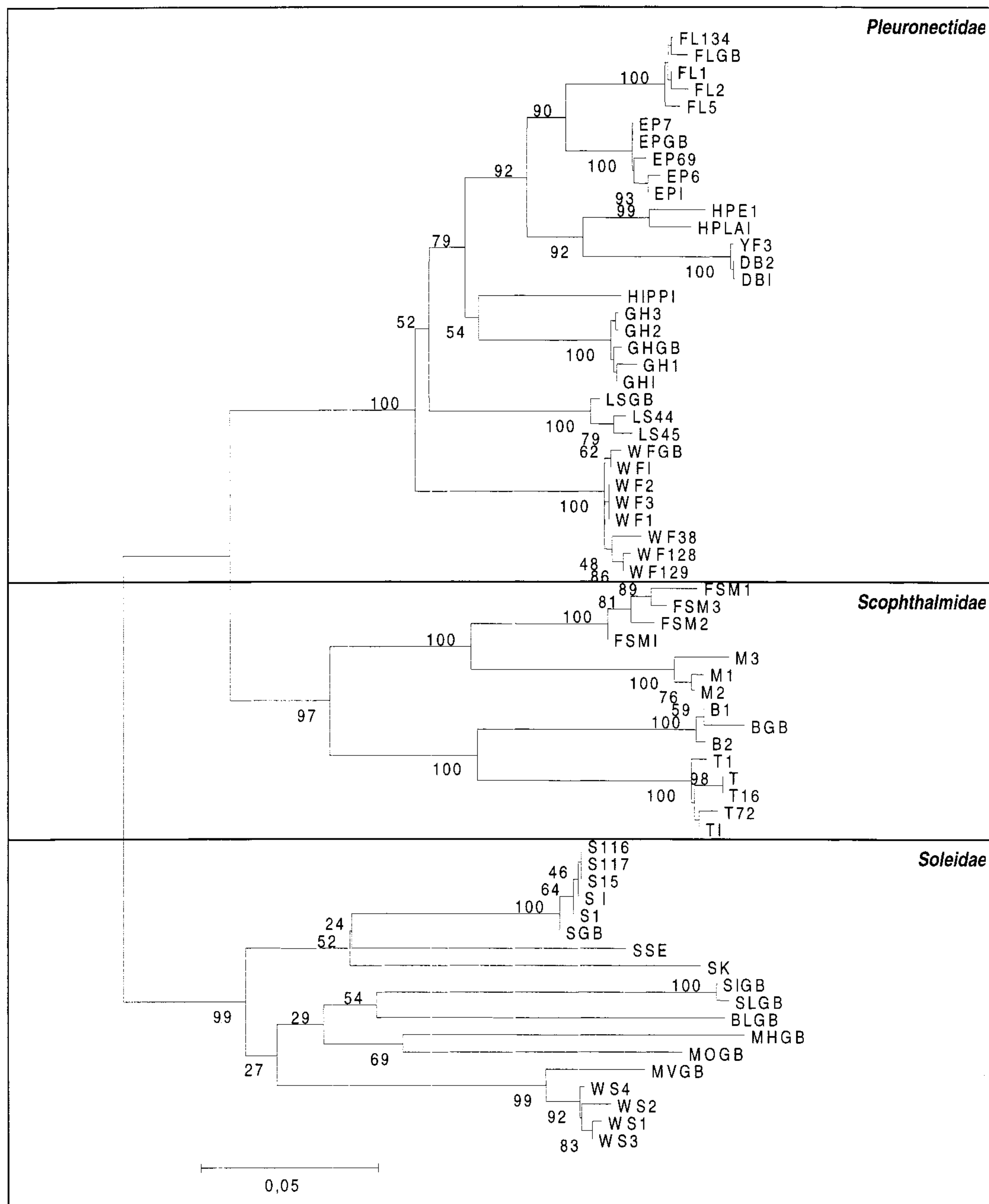

Figure 1. Phylogenetic tree of Burgener fragment sequences from 24 flatfish species. See keys for species in Table 1.

ing step of 5 min at 94 °C, then 35 cycles of 40 s at 94 °C, 80 s at 50 °C, 80 s at 72 °C, and a final extension step of 7 min at 72 °C.

Sequencing of PCR Fragments. Aliquots of 20 μL of PCR product were treated with 2 μL of Exonuclease I and 2 μL of shrimp alkaline phosphatase (Amersham Pharmacia Biotech). The mixture was incubated at 37 °C for 30 min and then at 80 °C for another 15 min. The purified PCR products were quantified with the Hoechst 33258 method described above.

Sequencing reactions were prepared with the ABI Prism dRhodamine Terminator cycle sequencing ready reaction kit (Applied Biosystems). To 4 μL of Terminator mix from the aforementioned kit, 90 to 200 ng of cleaned PCR product, 6.4 pmol of the corresponding primer, and distilled water up to 10 μL were added. The components were mixed, and the tube was loaded in the thermal cycler. The conditions of the sequencing reaction were 25 cycles at 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 4 min. The extension products were purified using an ethanol/magnesium chloride precipitation procedure for removal of the nonincorporated dye terminators. The pellet was dried at 30 °C with a centrifuge with a vacuum device and stored at −20 °C.

Once the extension products were purified, electrophoresis was carried out in an ABI PRISM 377 DNA Sequencer (Applied Biosystems) using 6% polyacrylamide gels for fluorescent DNA sequencing.

Prior to sample loading, the pooled and dried reaction products were suspended in loading buffer (Applied Biosystems), containing five parts of deionized formamide to one part of 25 mM EDTA pH 8.0 and 50 mg/mL dextran blue (Applied Biosystems), and the gel was electrophoresed for 5 h at 50 °C and 32 W. The collected data were processed using the software CHROMAS and VISED with CLUSTAL used to align the sequences (*14*), and MEGA to construct phylogenetic trees (*15*)

Restriction Fragment Length Polymorphism (RFLP) Analysis of the Fragment. A search for restriction sites was done using the sequences obtained from the fragment, choosing a set of enzymes on the basis of the predictable specific pattern

Table 2. Fragments Obtained with the Restriction Enzymes HincII, HinfI, and NlaIII for the Species Studied

	HincII[a]	E1	HinfI[a]	E2	NlaIII[a]	E3
			Pleuronectidae			
WF	93, 162, 209	A	149, 117, 198	A	286, 88, 90	A
LS	93, 162, 209	A	149, 117, 198	A	195, 91, 88, 90	B
HIPP	93, 162, 209	A	149, 315	B	286, 88, 90	A
GH	93, 162, 209	A	149, 234, 81*[1]	C	286, 88, 90	A
DB	255, 209	B	149, 315*[2]	B	286, 88, 90	A
YF	255, 209	B	149, 315	B	286, 88, 90	A
HPE	93, 162, 209	A	149, 315	B	286, 88, 90	A
HPLA	93, 162, 209	A	149, 315	B	286, 88, 90	A
EP	255, 209	B	149, 13, 302	D	195, 91, 88, 90	B
FL	255, 10, 199	C	149, 117, 198	A	286, 88, 90	A
			Scophthalmidae			
T	NC	0	179, 285	E	NC	0
B	265, 199	D	NC	0	195, 269*[3]	C
M	255, 209*[4]	B	162, 302	F	286, 38, 140	D
FSM	93, 162, 10, 199	E	NC*[5]	0	286, 178*[6]	E
			Soleidae			
WS	78, 15, 371	F	266, 198	G	125, 70, 108, 161	F
SL	360, 104	G	179, 285	E	NC	0
S	360, 104	G	179, 285	E	125, 339	G
SI	265, 199	D	396, 68	H	NC	0
SK	NC	0	NC	0	399, 65	H
SSE	360, 104	G	NC	0	374, 90	J
MO	255, 209	B	266, 198	G	195, 245, 24	K
MV	NC	0	266, 198	G	195, 108, 161	L
MH	NC	0	NC	0	NC	0
BL	255, 209	B	NC	0	286, 74, 80, 24	M

[a] The following designations are used in the restriction enzyme columns: *[1], one specimen out of 5 gave the fragmentation 149, 189, 45, 81; *[2], one specimen out of 2 gave the fragmentation 149, 234, 81; *[3], one specimen out of 2 gave the fragmentation 408, 56; *[4], one specimen out of 3 gave the fragmentation: 93, 162, 202; *[5], one specimen out of 4 gave the fragmentation 381, 81; *[6], one specimen out of 4 gave the fragmentation 286, 63, 115.

they would produce, and which, in theory, would enable species identification.

Two PCR reactions from each sample were concentrated to a volume of 10 μL using a Microcon-30 microconcentrator (Millipore). Aliquots from concentrated PCR amplicons were digested separately with Hinc II (New England Biolabs Inc.), Hinf I (Amersham Pharmacia Biotech), and Nla III (New England Biolabs Inc.). The volumes of each component of the reactions were 2.5 μL of PCR product, 1 μL of buffer 10x (supplied by the manufacturer with each enzyme), and 10 U of the enzyme. All digestions were carried out at 37 °C for 6 h in 10 μL volumes using reaction conditions specified by the manufacturer.

Separation of DNA fragments was carried out in a GeneGel Excel 12.5 (T 12.5%, C 2%) (Amersham Pharmacia Biotech), loading 6.5 μL of the digestion products on the gels. Anode buffer was 0.4% SDS and 0.45 M Tris Acetate pH 8.3; cathode buffer was 0.6% SDS and 0.08 M Tricine. The electrophoresis was carried out on a GenePhor (Amersham Pharmacia Biotech) with a temperature of the cooling plate of 15 °C, and a voltage of 200 V. The run was stopped when the tracking dye reached the anode edge of the gel. DNA restriction fragments were visualized by silver staining using the method of Heuskeshoven and Dermick (*16*).

RESULTS

DNA Sequences and Genetic Distance of Flatfish Species. Burgener fragment DNA sequences of the species studied in the laboratory were obtained (*13*); this fragment is part of the mitochondrial cytochrome b gene. In this study we used some sequences from the EMBL database for the same fragment for comparison purposes and genetic distance measurement. Analysis of the aligned sequences showed that there are 231 variable sites (data not presented), therefore there are 182 monomorphic nucleotides out of 413. Among the 231 polymorphic sites, 136 have only 2 variants, 55 have 3 variants, and 40 have 4 variants. These sequences were used for genetic distance measurement using the Tamura−Nei algorithm; on the basis of these distances, a phylogenetic tree was constructed using the Neibourgh-joining method (Figure 1), and the significance of each branch is indicated by the bootstrap test. Each studied family is grouped in a different branch of the unrooted tree (Pleuronectidae, Scophthalmidae, and Soleidae), and the 24 species studied are well-differentiated in different clades except *Limanda ferruginea* (YF) and *Limanda limanda* (DB), and *Solea impar* (SI) and *Solea lascaris* (SL). The number of polymorphic sites in this fragment is quite high; among these polymorphic sites several were selected with diagnostic value. The identification of most of the 24 species is possible with this sequence fragment by using forensincally informative nucleotide sequencing (FINS).

RFLP Patterns. Sequences obtained for the specimens and species analyzed in this study, together with those sequences obtained from EMBL, were subjected to a search for restriction enzyme targets. Three enzymes were selected which gave a different fragment for most of the species studied: Hinc II, Hinf I, and Nla III. Another requirement is that any restriction enzyme selected should have a target sequence with the lowest intraspecific variability. Table 2 presents the theoretical fragmentation of the 24 species studied with these three selected restriction enzymes. The intraspecific variability was rather low, and only in some cases the same species can present two patterns for one of the enzymes. As it can be observed in this table, six species produced two different digestion patterns with some of the enzymes. The fragmentation produced equal patterns for HIPP, HPE, and HPLA, and for DB and YF, meaning that those species could not be differentiated using these enzymes.

A set of eleven reference species was used to test the patterns predicted by the sequences. Figures 2, 3, and 4 show the appearance of the RFLP fragments obtained

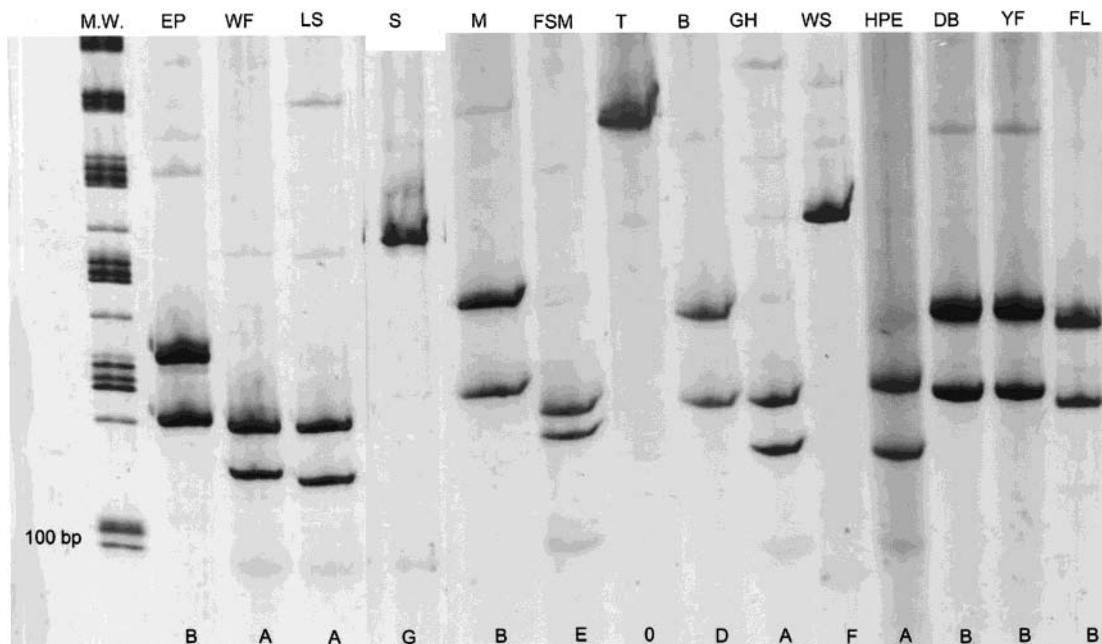

Figure 2. PCR−RFLP patterns of flatfish species following digestion with restriction enzyme Hinc II. Haplotypes corresponding to Table 3 are indicated at the bottom of each line.

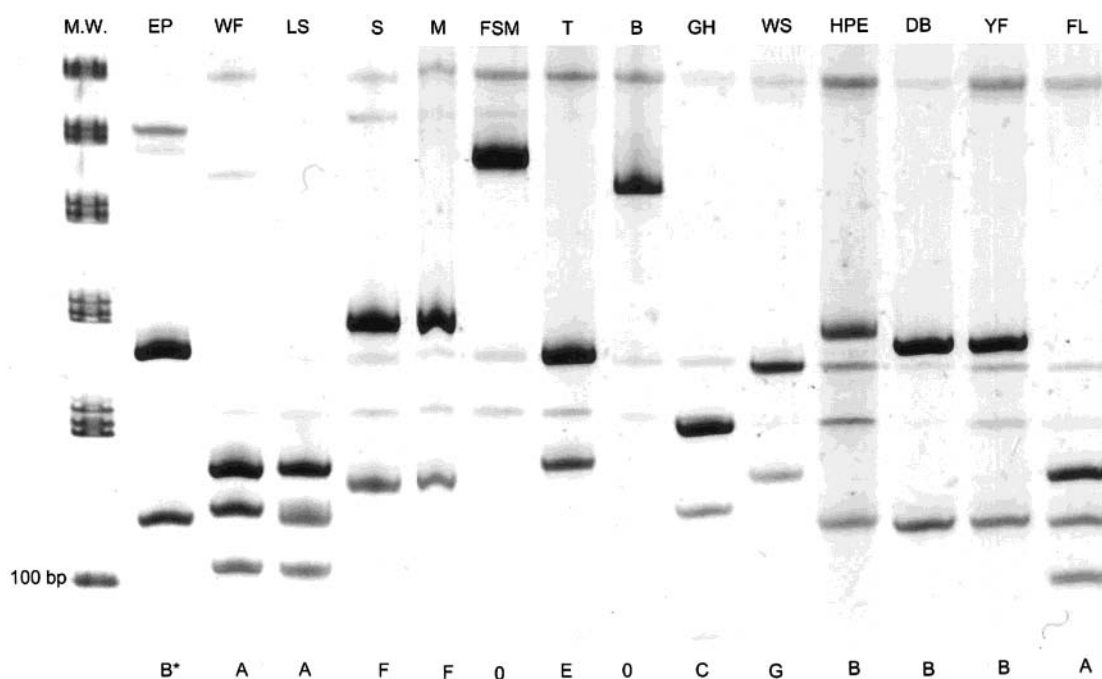

Figure 3. PCR−RFLP patterns of flatfish species following digestion with restriction enzyme Hinf I. Haplotypes corresponding to Table 3 are indicated at the bottom of each line.

by digesting the PCR product with three restriction enzymes: Nla III, Hinf I, and Hinc II.

Commercial Samples. A set of 14 frozen commercial samples was analyzed by PCR−RFLP using the restriction enzymes selected. The identification of these samples was made by comparing the size of the fragments obtained with the PCR product obtained from these samples with those from the authentic samples. Frozen commercial samples were of two types: whole frozen specimens labeled as sole and frozen fillets labeled also as sole. Table 3 shows the approximate fragment size of the commercial samples obtained with the three enzymes. Identification of samples was possible in all cases with the exception of CFS12 and CFS13. The identification of the commercial samples is presented in Table 4. The identification of the samples was possible in most of the cases, but the CFS14 gave a pattern not found in the reference sample for Hinf I; on the basis of the pattern of Hinc II and Nla III the species must belong to the Pleuronectidae family (WF, HIPP, GH, HPE, or HPLA). CFS12 and CFS13 must be Soleidae species because Nla III patterns are characteristic of Soleidae. As it can be observed in Table 4, most of the commercial samples were identified as GH (*Reinhardtius hippoglossoides*); this species is often labeled and marketed as sole. All commercial samples were sequenced, and the sequences were analyzed by FINS (data not shown). The results confirmed the

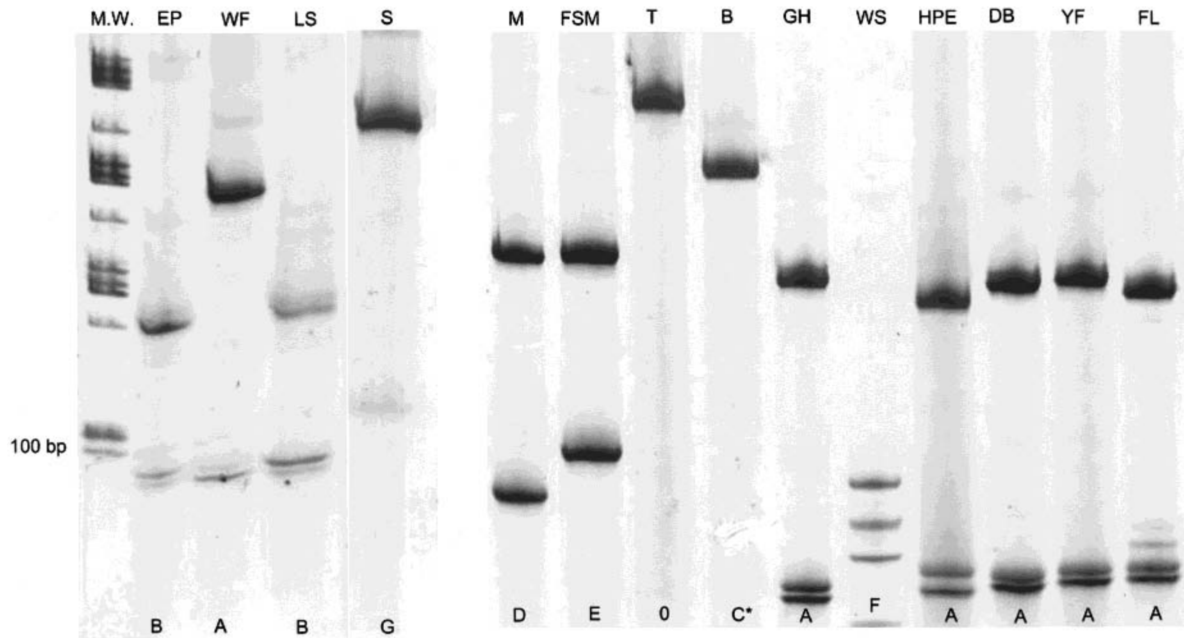

Figure 4. PCR−RFLP patterns of flatfish species following digestion with restriction enzyme Nla III. Haplotypes corresponding to Table 3 are indicated at the bottom of each line.

Table 3. Fragments Obtained with the Restriction Enzymes HincII, HinfI, and NlaIII for the Commercial Frozen Samples (CFS)

sample	Hinc II	E1	HinfI	E2	NlaIII	E3
CFS1	93, 162, 210	A	149, 234, 81	C	286, 88, 90	A
CFS2	360, 104	G	179, 285	E	125, 339	G
CFS3	93, 162, 210	A	149, 189, 45, 81	C*	286, 88, 90	A
CFS4	93, 162, 210	A	149, 189, 45, 81	C*	286, 88, 90	A
CFS5	93, 162, 210	A	149, 189, 45, 81	C*	286, 88, 90	A
CFS6	93, 162, 210	A	149, 234, 81	C	286, 88, 90	A
CFS7	93, 162, 210	A	149, 189, 45, 81	C*	286, 88, 90	A
CFS8	93, 162, 210	A	149, 189, 45, 81	C*	286, 88, 90	A
CFS9	93, 162, 210	A	149, 234, 81	C	286, 88, 90	A
CFS10	93, 162, 210	A	149, 234, 81	C	286, 88, 90	A
CFS11	93, 162, 210	A	149, 234, 81	C	286, 88, 90	A
CFS12	78, 386	?	383, 81	?	125, 339	G
CFS13	78, 386	?	149, 315	?	125, 339	G
CFS14	93, 162, 210	A	149, 117, 81	?	286, 88, 90	A

Table 4. Identification of Samples Based on PCR−RFLP with the Restriction Enzymes HincII, HinfI, and NlaIII for the Commercial Frozen Samples (CFS)

sample	haplotype	identification
CFS1	ACA	GH
CFS2	GEG	S
CFS3	AC*A	GH
CFS4	AC*A	GH
CFS5	AC*A	GH
CFS6	ACA	GH
CFS7	AC*A	GH
CFS8	AC*A	GH
CFS9	ACA	GH
CFS10	ACA	GH
CFS11	ACA	GH
CFS12	??G	Soleidae
CFS13	??G	Soleidae
CFS14	A?A	Pleuronectidae

identifications made with PCR−RFLP in all the cases. CFS14 is a GH, and the other CFS12 and CFS13 are Soleidae species not included in this work.

DISCUSSION

The identification of flatfish species has been attempted before by using proteins in our laboratory (*17*). In that case 8 species of flatfish species were differentiated using the profile generated by proteins after separation by capillary zone electrophoresis. Also in our laboratory, two-dimensional electrophoresis has been used to differentiate 9 flatfish species (*18*). The use of proteins as a tool for identifying species in commercial products has limited application. Proteins used as biochemical taxonomical markers can be used only in those cases where the product to be identified has not suffered an exhaustive thermal treatment. Refrigerated and frozen seafood products can be analyzed using the protein approach, however, when the product is heated, proteins become denatured and they are difficult to analyze, thus hindering identification of the sample. Also, when using proteins, species standards should be run with problem samples for comparison purposes.

DNA can be used as a biochemical taxonomical marker in a wide variety of process treatments because, although DNA also suffers hydrolisis (enzymes, or physical hydrolisis), in most cases it is possible to amplify a short fragment containing species diagnostic value (*19*). The identification of species in a group of species such as flatfish species requires that most commercial species should be subjected to study. In previous works, DNA has been also used as a biochemical marker (*8*−*11*); in these works different DNA fragments have been proposed for use as a diagnostic. The number of species employed for the selection of the diagnostic target has been always rather low: from as low as two species, up to four species as the highest.

FINS has been used for species identification before (*19*, *20*), in this work cytochrome b sequences from 24 species of flatfish were obtained and analyzed by phylogenetic tree construction. The phylogenetic tree (Figure 1) shows that each species is well-differentiated, and that the three major and well-supported clades (with bootstrapping values above 97%) correspond with each of the monophiletic families studied (Pleuronectidae, Scophthalmidae, and Soleidae). Unknown sample sequences from commercially relevant species can be easily identified by measuring their level of similarity against the pool of reference sequences; the lowest distance between the unknown sequence and a reference

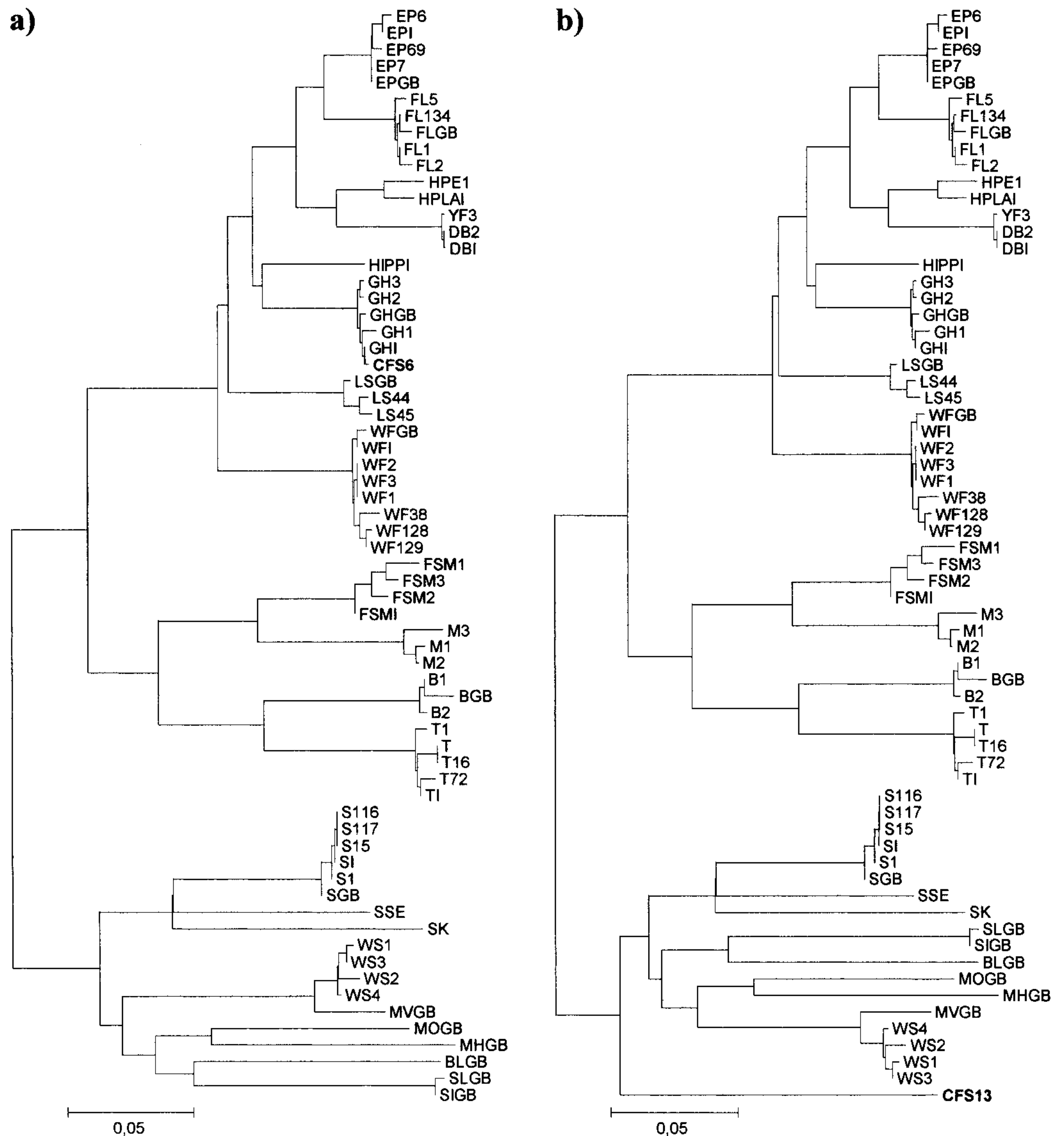

Figure 5. (a) Identification of CFS6 using FINS. CFS6 shows lowest genetic distance with GH group. (b) Identification of CFS13 using FINS. CFS13 shows the lowest distance with the Soleidae species branch.

sequence will identify the species (see Figure 5a). If the unknown sequence does not belong to any of the reference species used for the analysis, at least this method will show the family to which the sample belongs (Figure 5b).

PCR−RFLP is an inexpensive and relatively fast method that constitutes an alternative to identification methods based on sequencing (*21*). PCR−RFLP methods rely on the different location of restriction enzymes target sequences producing species-specific patterns. However, if the range of species sequences used for selecting the diagnostic restriction enzymes did not cover the range of potential species which might be used in a particular product, there is the possibility of finding that the diagnostic target selected is no longer species-specific. This is especially true when dealing with species substitution in fish products: it is common to find the substitution of a valuable species by a closely related (same family or same genus) species of a lower value (i.e., Tuna species, Hake species, etc.). The analyst who has to deal with an identification problem in food often has to answer the question of "what is the species included in this product?". If the method he or she is using is tailored for differentiating only two species, for instance Solea solea and Microchirus azevia, one of the answers may be "this is not a sole" in the case in which the unknown RFLP haplotype is different from sole. If the pattern is the same as that of sole, the honest answer will have to be "this might be sole" but there is no guarantee that it is sole because another flatfish species can give the same sole pattern but it was not considered in the study, therefore no one knows. Therefore, as stated before, these works have a limited application due to the fact that more than four flatfish species are commercialized.

The sequences of specimens analyzed in this study revealed that the fragment showed low intraspecific variability, meaning that the fragmentation pattern will be rather conserved if different individuals are tested. Identification of commercial problem samples of flatfishes by PCR−RFLP has been successfully performed in most of the cases. There have been only two samples difficult to identify (unknown patterns found) that were identified as species belonging to the Soleidae family (this fact being confirmed by FINS). PCR−RFLP methods are quite reliable, and they are a potentially valuable tool for application in quality control laboratories. Little specialized equipment is needed, i.e., a

thermalcycler for performing PCR and electrophoresis equipment. However, the problems mentioned above have to be taken into account when a definitive answer must be given to a particular identification problem.

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ABBREVIATIONS USED

DNA, deoxyribonucleic acid; SDS, sodium dodecyl sulfate; EDTA, ethylendiaminetetraacetic; FINS, forensincally informative nucleotide sequencing; RFLP, restriction fragment length polymorphism; PCR, polymerase chain reaction.

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