Journal of Food Protection, Vol. 65, No. 6, 2002, Pages 1016–1023

Molecular Identification of Nine Commercial Flatfish Species by Polymerase Chain Reaction–Restriction Fragment Length Polymorphism Analysis of a Segment of the Cytochrome *b* Region

ANDRÉS SANJUAN* AND ANGEL S. COMESAÑA

Xenética Evolutiva Molecular, Facultade de Ciencias-Bioloxía, Universidade de Vigo, E-36200 Vigo, Spain

MS 01-179: Received 18 May 2001/Accepted 8 October 2001

ABSTRACT

Commercial refrigerated or frozen flatfish fillets are sometimes mislabeled, and identification of these mislabeled products is necessary to prevent fraudulent substitution. Identification of nine commercial flatfish species (order *Pleuronectiformes*), *Hippoglossus hippoglossus* (halibut), *Lepidorhombus boscii* (four-spotted scaldfish), *Lepidorhombus whiffiagonis* (megrin), *Platichthys flesus* (flounder), *Pleuronectes platessa* (European plaice), *Reinhardtius hippoglossoides* (Greenland halibut), *Scophthalmus maximus* (turbot), *Scophthalmus rhombus* (brill), and *Solea vulgaris* (=*Solea solea*) (sole), was carried out on the basis of the amplification of a 486-bp segment of the mitochondrial genome ($tRNA^{Glu}$/cytochrome *b*) by using the polymerase chain reaction (PCR) and universal primers. Sequences of PCR-amplified DNA from the flatfish species were used to select eight restriction enzymes (REs). The PCR products were cut with each RE, resulting in species-specific restriction fragment length polymorphism. Seven species groups could be identified by application of the single RE *Dde*I and six species groups by using *Hae*III, *Hin*fI, *Mae*I, or *Mbo*I. Different combinations of only a couple of these REs could unambiguously identify the nine flatfish species. Genetic polymorphisms of the target sequence were examined by comparison with previously published DNA sequences, and the results of this comparison confirmed the usefulness of this technique in distinguishing and genetically characterizing refrigerated or frozen pieces of these nine flatfish species.

The market for processed fishery products is steadily growing, and the identification of commercial fish species becomes a problem when the distinguishing features of fish are removed at the processing stage. Commercial refrigerated or frozen flatfish fillets are sometimes mislabeled. Several products purporting to contain expensive and popular flatfish species are highly susceptible to fraudulent or unintentional substitution of cheaper fish species. Identification of these products is necessary to prevent fraudulent substitution.

Numerous analytical methods for fish species authentication have been developed. Most of these methods rely on the analysis of proteins and involve mainly electrophoretic techniques, high-performance liquid chromatography, and immunoassays *(1, 15, 18, 19, 29* and references therein*)*. Advances in molecular biology techniques have allowed the development of DNA-based methods *(13, 29)*. DNA carries an organism's genetic information, which is the same in all cell types, and it is a very stable and long-lived biological molecule. Most of the genetic approaches to the determination of species identity are based on the amplification of a region of mitochondrial or nuclear DNA by the polymerase chain reaction (PCR) with conserved primers. Applications of the PCR to fish species identification have proliferated because of the simplicity, specificity, and sensitivity of this technique *(24)*. One of the PCR-based methods used for fish species identification has been direct sequence analysis of the amplified fragments *(2, 3, 13, 18, 25, 26* and references therein*)*. Restriction fragment length polymorphism (RFLP) of PCR products constitutes an alternative for the identification of species that is simpler than sequencing *(6–13, 18, 20, 25–27, 32* and references therein*)*.

The mitochondrial encoded cytochrome *b* (cyt *b*) gene is a useful genetic marker for the identification of the species origin of a fish sample (for details, see Bartlett and Davidson *(3)* and Davidson *(13)*). The cyt *b* gene is intraspecifically conserved, but it contains enough interspecific heterogeneity to produce species-specific restriction fragment patterns *(5, 21)*. Cyt *b* is among the most extensively sequenced genes in vertebrates *(16)*. Cyt *b* sequences can be found in international data banks and can be used to compare sequences and to obtain restriction patterns.

Different cyt *b* gene sequences have been studied to identify several flatfish species (order *Pleuronectiformes*). A 359-bp amplified fragment was digested with three restriction endonucleases (REs) to distinguish *Platichthys flesus, Pleuronectes platessa,* and *Solea vulgaris* (=*Solea solea*) *(11)*. This amplicon was not detected in *Reinhardtius hippoglossoides,* but a 201-bp fragment was subsequently amplified and digested with four REs to identify these four flatfish species *(12)*. Moreover, other flatfish species, including *Scophthalmus rhombus,* have also been characterized by PCR-RFLP of the 5′ end of the cyt *b* fragment *(9, 32)*.

The objective of this work was to develop a simple

* Author for correspondence. Tel: 34 986 812569; Fax: 34 986 812556; E-mail: asanjuan@uvigo.es.

TABLE 1. *Studied commercial flatfish species (order* Pleuronectiformes*) from the North Atlantic, along with the numbers of sequenced and RE-analyzed individuals*

Species	Sequenced individuals	RE-analyzed individuals
Family *Scophthalmidae*		
Lepidorhombus boscii (four-spotted scaldfish)	2	15
L. whiffiagonis (megrin)	3	15
Scophthalmus maximus (turbot)	3	15
S. rhombus (brill)	2	15
Family *Pleuronectidae*		
Hippoglossus hippoglossus (halibut)	3	10
Platichthys flesus (flounder)	2	15
Pleuronectes platessa (European plaice)	2	15
Reinhardtius hippoglossoides (Greenland halibut)	3	15
Family *Soleidae*		
Solea vulgaris (=*S. solea*) (sole)	2	15
Total	22	130

method for the identification of refrigerated and frozen fillets from nine commercial flatfish species by PCR-RFLP analysis of a 486-bp fragment in the mitochondrial $tRNA^{Glu}$/cyt *b* region. This method can be applied to the detection of fraudulent or unintentional mislabeling of processed products containing these species.

MATERIALS AND METHODS

Samples and DNA extraction. Refrigerated or frozen samples of nine commercial North Atlantic flatfish species (order *Pleuronectiformes*), *Lepidorhombus boscii* (four-spotted scaldfish), *Lepidorhombus whiffiagonis* (megrin), *Scophthalmus maximus* (turbot), *S. rhombus* (brill), *Hippoglossus hippoglossus* (halibut), *P. flesus* (flounder), *P. platessa* (European plaice), *Reinhardtius hippoglossoides* (Greenland halibut), and *S. vulgaris* (=*S. solea*) (sole), were collected (Table 1). For most species, whole individuals were obtained at fish markets in northwest Spain (Cangas, Ferrol, and Vigo). Frozen whole *R. hippoglossoides* individuals were obtained directly from a scientific expedition in the northwest Atlantic waters. Frozen *H. hippoglossus* adults and juveniles were obtained from a manufacturer in Vigo, Spain, and from Newfoundland, Canada, waters, along with caudal pieces of previously identified individuals from Norwegian fish markets. Every specimen was morphologically identified *(28, 31)*.

Total DNA was extracted from the muscle tissue of each individual *(14)*. Approximately 100 mg of muscle tissue was digested with 500 μl of extraction buffer (50 mM Tris, 25 mM EDTA, 1% SDS) and 10 μl of 20 mg/ml proteinase K (Roche Diagnostic GmbH, Mannheim, Germany). The mixture was incubated overnight at 37°C. DNA was extracted once with 600 μl of phenol/chloroform/isoamylalcohol (25:24:1) and once with 550 μl of chloroform and was then precipitated overnight with 1 ml of 95% ethanol at −20°C. The tubes were centrifuged at 6,000 × *g* for 10 min, and then the ethanol was discarded and the pellets were dried in a vacuum concentrator system (SpeedVac plus, Savant) for 10 min. The dried pellets were resuspended in 200 μl of TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]) and stored at −20°C.

PCR. A 486-bp portion of the mitochondrial genome in the $tRNA^{Glu}$/cyt *b* region was amplified by PCR using the universal primers L14724 (5′-CGA AGC TTG ATA TGA AAA ACC ATC GTT G-3′ *(23)*) and H15149 (5′-AAA CTG CAG CCC CTC AGA ATG ATA TTT GTC CTC A-3′ *(17)*), which work well for many fish taxa *(22)*. The primers were synthesized by Amersham Pharmacia Biotech (Buckinghamshire, UK). Double-stranded amplifications were carried out with a final volume of 50 to 100 μl containing 9 mM Tris HCl (pH 9.0), 2 mM $MgCl_2$, 45 mM KCl, 0.09% Triton X-100, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTP (Amersham Pharmacia Biotech), 0.06 μM each primer; 1.5 U of *Taq* DNA polymerase (Promega, Southampton, UK), and 3 μl of the above extracted genomic DNA as a template. A negative control reaction (without DNA) was also included for each PCR set to test for possible contamination. The cycling conditions on a Master Gradient thermal cycler (Eppendorf, Hamburg, Germany) consisted of denaturation at 95°C for 5 min, amplification with 30 cycles of 94°C for 60 s, 50°C for 60 s, and 72°C for 120 s, and a final extension at 72°C for 10 min.

PCR products were examined by electrophoresis through a 3.0% SeaKem LE and Nusieve GTG agarose (2:1) gel (FMC BioProducts, Rockland, Maine) in TBE buffer (89 mM Tris base, 89 mM boric acid, 2.5 mM ethylenediaminetetra-acetic acid [pH 7.5]) and stained by ethidium bromide. As a size reference, a 100- or 50-bp ladder (Amersham Pharmacia Biotech) was used.

DNA sequencing. Double-stranded DNA from PCR reactions with sufficient amounts of amplified DNA were cleaned using the Qiaquick PCR purification kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The DNA was eluted in 30 to 40 μl of the EB buffer provided with the kit. The concentration of the purified PCR product was estimated by agarose gel electrophoresis with a standard (Mass Ruler, BioRad Laboratories, Richmond, Calif.) as a reference marker.

Purified PCR products were directly sequenced in a Beckman CEQ2000 sequencer (Beckman Instruments, Bucks, UK) at the Facultade de Ciencias, Universidade de Vigo, Spain. DNA sequencing was accomplished with fluorescence dye labeled dideoxynucleotides and reagents provided with the CEQ Dye Terminator Cycle Sequencing Kit (Beckman Instruments). For each sequencing reaction, approximately 30 ng (about 100 fmol) of purified PCR product was added. To substantiate correct DNA sequences, restriction site locations, and fragment sizes, direct and reverse sequences of PCR fragments for each of the primers used in initial PCR amplification were obtained. Sequences were analyzed and prepared for publication with the help of the ESEE computer program *(4)*. All sequences are given as their non-template-strand equivalents (Fig. 1) and have been deposited in GenBank (accession numbers AF413798 to AF413810).

Restriction site analysis of PCR products. For the restriction site analysis of the $tRNA^{Glu}$/cyt *b* region, DNA sequences were compared with other sequences previously reported or included in international databases. Several REs were selected for species-specific restriction patterns with the DNASIS computer program (Hitachi Software Engineering Europe, Ardon, France).

The purified PCR products were separately digested with eight REs (Roche Diagnostic; New England BioLabs, Beverly, Mass.) with 4- or 5-bp recognition sequences (Table 2): *Alu*I, *Dde*I, *Hae*III, *Hap*II, *Hin*fI, *Mae*I, *Mbo*I, and *Nci*I. Satisfactory results were also obtained without previous purification of the amplified DNA fragments. A 10-μl aliquot of PCR product with about 100 to 150 ng of amplified DNA was directly digested through incubation at 37°C overnight with 5 U of an RE. The resulting fragments were separated by electrophoresis on a 3.0%

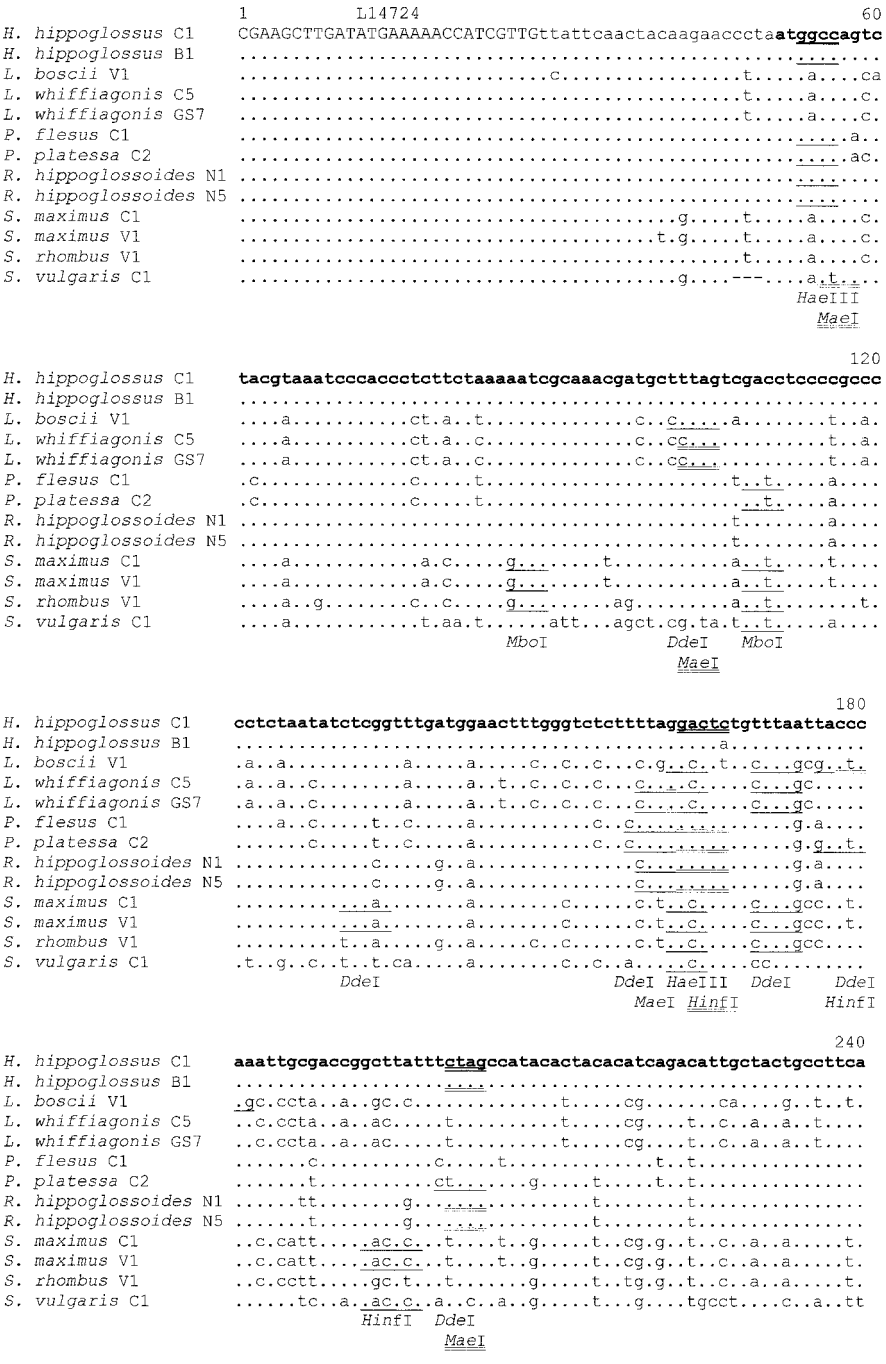

FIGURE 1. *Aligned DNA sequences of the amplified tRNA^{Glu}/cyt* b *region for nine flatfish species. Codes for each sequence include the species name, one or two letters for the area of origin (B, Bergen, Norway; C, commercial from the northwest Iberian waters, except* H. hippoglossus *C1, which is of unknown origin; GS, Grand Sole bank; N, Newfoundland, Canada, waters; V, Ría de Vigo, Spain) and the number of the individual. In the first DNA sequence (that of* H. hippoglossus *C1), the corresponding coding sequence of the cyt* b *gene is in boldface type, and the positions of primers L14724 and H15149, used for PCR amplification and sequencing, are indicated in capital letters. A dot indicates identity with the first DNA sequence, and a dash indicates a deletion. Restriction recognition sites for REs with diagnostic capability are underlined (single or double).*

SeaKem LE and Nusieve GTG agarose (2:1) gel (FMC BioProducts) containing ethidium bromide in TBE buffer at 10 V/cm for 60 or 90 min, depending on the lengths of the fragments. Gels were sometimes incubated in an ethidium bromide solution (1 μg/ml) for 10 min after electrophoresis. The restriction fragments were visualized by UV transillumination and photographed with a Polaroid camera, and their sizes were determined by comparison with a 50- or 100-bp ladder (Amersham Pharmacia Biotech) with 1D Manager DNA analysis software (TDI, Alcobendas, Madrid, Spain). Only fragments larger than 70 bp were considered in the RFLP analysis, as smaller fragments were not always clearly visible.

RESULTS AND DISCUSSION

DNA sequence analysis. Twenty-two PCR products of the tRNA^{Glu}/cyt *b* region for nine flatfish species were directly sequenced for both senses with universal primers L14724 *(23)* and H15149 *(17)* (Table 1). Alignment of the 486-bp sequences showed 13 different sequences for all species combined (Fig. 1). The four species for which three individuals were sequenced (*H. hippoglossus, L. whiffiagonis, R. hippoglossoides,* and *S. maximus*; Table 1) showed two different DNA sequences each. A 3-bp deletion before the coding sequence of cyt *b* gene was found for *S. vulgaris*. Published cyt *b* sequences for *P. flesus, P. platessa, R. hippoglossoides, S. rhombus,* and *S. vulgaris (11, 30, 32)* were compared with sequences obtained in this work, and no large differences were found, as the inferred polypeptide sequences were very close for each species.

Identification of flatfish species by RFLP. The restriction maps of the sequences were compared to deter-

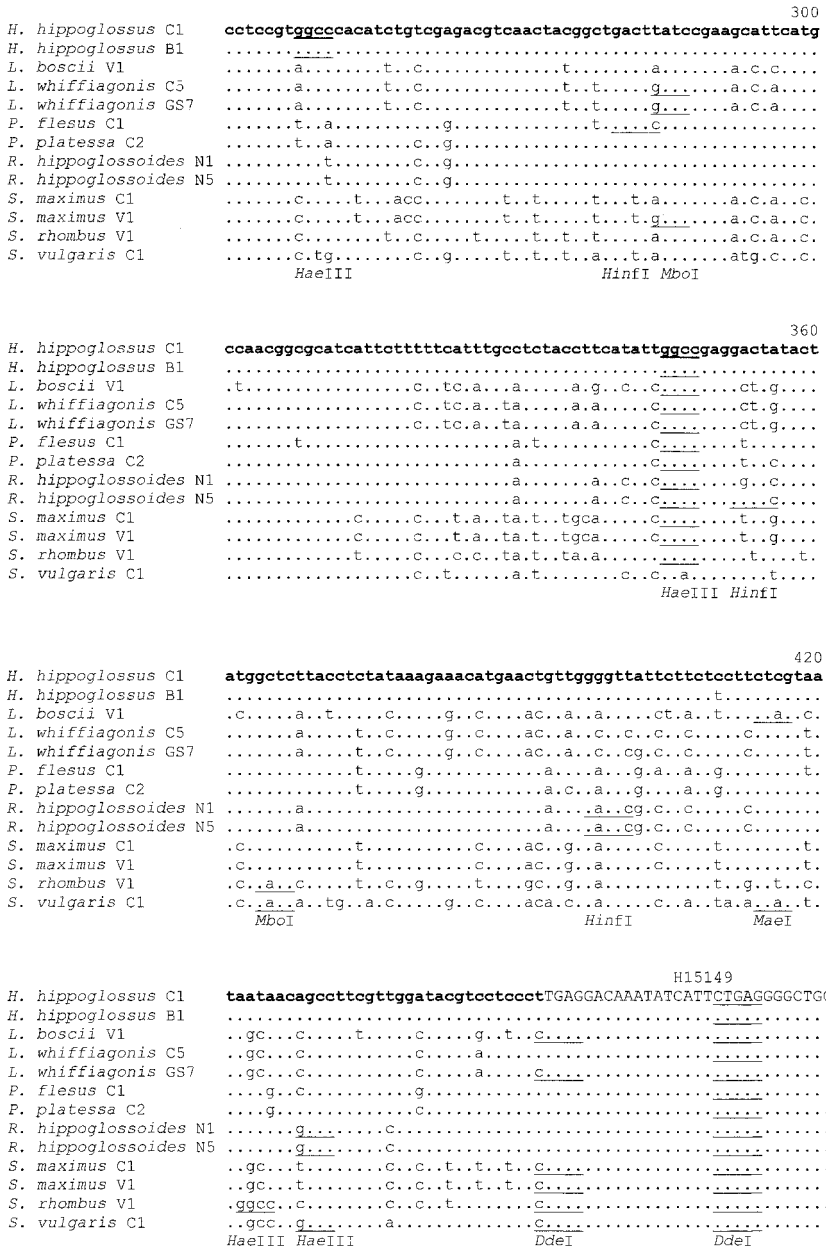

FIGURE 1. *Continued.*

mine which REs could distinguish and identify the PCR products of each flatfish species. Eight REs with 4- or 5-bp recognition sequences were selected for screening, and different electrophoretic restriction patterns were detected for each RE (Table 2).

Agarose electrophoresis following digestion of PCR products from 130 individuals of the nine flatfish species showed band sizes that were in agreement with the expected sizes for the restriction fragments inferred from the sequence analysis. To visualize and clearly distinguish fragments of similar lengths for our electrophoretic conditions, agarose electrophoresis was carried out for 90 min, e.g., for the 470-bp fragment (haplotype A) and the 449-bp fragment (haplotype B) with *Dde*I, or for the long fragment of haplotype B (323 bp) and the 310-bp fragment of haplotype E with *Hin*fI. Each RE yielded different restriction patterns, depending on the studied flatfish species (Table 3). Intraspecific restriction polymorphism was found only with *Dde*I for *L. whiffiagonis,* with *Hin*fI for *H. hippoglossus* and *R. hippoglossoides,* and with *Mbo*I for *S. maximus* (Table 3).

The RE with the strongest diagnostic capability was *Dde*I, followed by *Hin*fI, *Hae*III, *Mae*I, and *Mbo*I (Table 3 and Fig. 2). *Dde*I produced eight different restriction patterns (haplotypes) for all species (Table 3). Five of the nine studied species (*L. boscii, P flesus, P. platessa, S. maximus,* and *S. vulgaris*) and two species groups (one group included *H. hippoglossus* and *R. hippoglossoides,* and the other included *S. rhombus* and several *L. whiffiagonis* individuals) showed different haplotypes. Several *L. whiffiagonis* individuals yielded haplotype E instead of haplotype F according to the expected sizes by the absence of a restriction site in position 453 (300 bp = 282 bp + 18 bp; see Fig. 1 and Table 2). Consequently, the numbers of species groups and species identified with only *Dde*I can be considered 7 and 5, respectively (Table 3). The enzyme *Hin*fI

TABLE 2. *Haplotypes (restriction patterns) for the cytochrome* b *segment of nine flatfish species for eight 4- and 5-bp cutting REs with the expected length of the fragments shown in the 5′-to-3′ order of the DNA sequence*[a]

RE name and recognition site	Haplotypes							
	A	B	C	D	E	F	G	H
*Alu*I, AG!CT	5	5	5	5	5			
	481	89	177	199	169			
		389	304	282	60			
					252			
*Dde*I, C!TNAG	470	449	158	158	170	170	131	101
	16	18	312	42	300	282	39	69
		16	16	270	16	18	282	8
				16		16	18	274
							16	18
								16
*Hae*III, GG!CC	54	54	54	163	163	160		
	292	195	292	183	183	266		
	140	97	83	140	77	57		
		140	57		63			
*Hap*II, C!CGG	486	190	257	190				
		296	226	70				
				226				
*Hin*fI, G!ANTC	486	163	176	193	163	163	163	163
		323	310	293	13	117	234	189
					310	206	89	45
								89
*Mae*I, C!TAG	486	201	159	201	102	52		
		285	42	213	57	359		
			285	72	327	72		
*Mbo*I = *Nde*II, !GATC	486	107	283	85	85	104	85	
		379	203	22	22	256	22	
				379	176	123	256	
					203		123	
*Nci*I, CC!SGG	486	191	261					
		295	225					

[a] The PCR product for *Solea vulgaris* is 483 bp instead of 486 bp because of a 3-bp deletion at the 5′ end, and the first fragments of haplotypes D for *Hin*fI and C for *Nci*I, as well as other fragments of *S. vulgaris*–specific haplotypes for other REs, are 3 bp short.

TABLE 3. *Restriction patterns (haplotypes) of eight 4- and 5-bp cutting REs for the nine studied flatfish species*[a]

RE	Flatfish species									No. of haplotypes	No. of species groups identified	No. of species identified
	H. hippoglossus	*L. boscii*	*L. whiffiagonis*	*P. flesus*	*P. platessa*	*R. hippoglossoides*	*S. maximus*	*S. rhombus*	*S. vulgaris*[b]			
*Alu*I	A	**C**	**E**	*D*	A	A	*D*	A	**B**	5	5	3
*Dde*I	*A*	**H**	**E**, *F*	**C**	**D**	*A*	**G**	*F*	**B**	8	7	5
*Hae*III	**B**	D	D	*A*	*A*	**C**	D	**E**	**F**	6	6	4
*Hap*II	B	*A*	*A*	B	*D*	*D*	B	B	**C**	4	4	1
*Hin*fI	A, **B**	**C**	A	**F**	**E**	**G, H**	*D*	A	*D*	8	6	4
*Mae*I	*B*	**D**	**E**	*B*	A	**C**	A	A	**F**	6	6	4
*Mbo*I = *Nde*II	A	A	**C**	*B*	*B*	A	**D, E**	**G**	**F**	7	6	4
*Nci*I	A	A	A	A	C	C	A	**B**	C	3	3	1
No. of species-specific haplotypes	2	4	4	2	2	4	3	3	6			

[a] Bold letters represent haplotypes characteristic of a single species, and italic letters represent haplotypes that are shared by two species.
[b] The PCR product is 483 bp instead of 486 bp because of a 3-bp deletion.

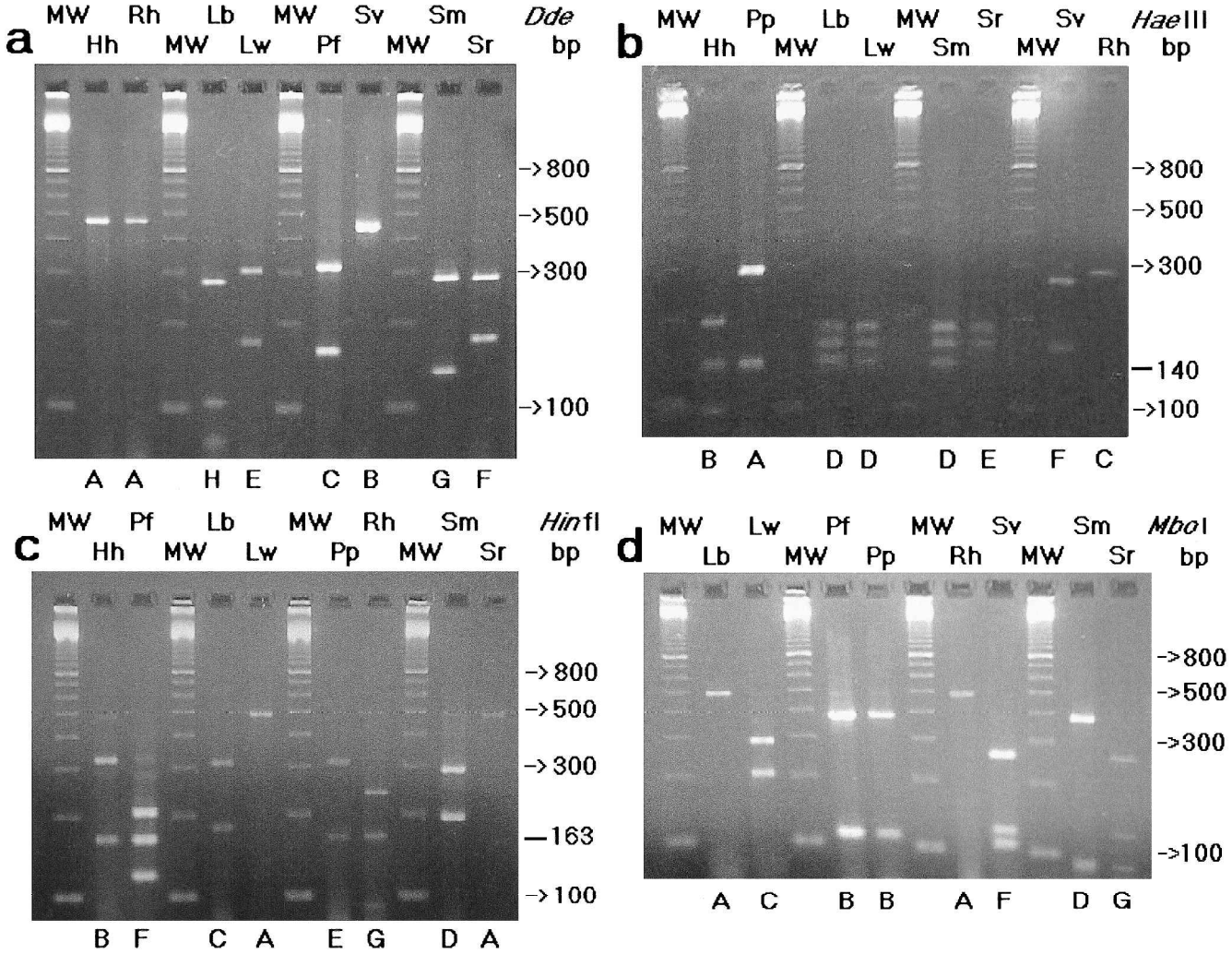

FIGURE 2. *Electrophoretic restriction patterns of the PCR products of the cyt* b *region digested with different restriction enzymes: (a)* Dde*I; (b)* Hae*III; (c)* Hin*fI; (d)* Mbo*I. MW, molecular weight marker (100-bp ladder). Species codes: Hh,* Hippoglossus hippoglossus*; Lb,* Lepidorhombus boscii*; Lw,* Lepidorhombus whiffiagonis*; Pf,* Platichthys flesus*; Pp,* Pleuronectes platessa*; Rh,* Reinhardtius hippoglossoides*; Sm,* Scophthalmus maximus*; Sr,* Scophthalmus rhombus*; Sv,* Solea vulgaris. *Haplotypes at the bottom of each figure are as in Table 2.*

distinguished four species (*L. boscii, P. flesus, P. platessa,* and *R. hippoglossoides*), two species groups (one group including *S. maximus* and the *S. vulgaris,* and the other group including *L. whiffiagonis, S. rhombus,* and several *H. hippoglossus* individuals with haplotype A), and several *H. hippoglossus* individuals with haplotype B. Consequently, the numbers of species groups and species identified with *Hin*fI can be considered 6 and 4, respectively. *Hae*III, *Mae*I, and *Mbo*I also distinguished six species groups and four species, each involving different species in the species groups and in the species identified (Table 3).

Each studied flatfish species can be identified with specific patterns of different REs (Table 3 and Fig. 2). *H. hippoglossus* was clearly identified with *Hae*III (haplotype B) (Fig. 2b); *L. boscii* was identified with *Alu*I, *Dde*I (Fig. 2a), *Hin*fI (Fig. 2c), and *Mae*I; *L. whiffiagonis* was identified with *Alu*I, *Dde*I (haplotype E) (Fig. 2a), *Mae*I, and *Mbo*I (Fig. 2d); *P. flesus* was identified with *Dde*I (Fig. 2a) and *Hin*fI (Fig. 2c); *P. platessa* was identified with *Dde*I and *Hin*fI (Fig. 2c); *R. hippoglossoides* was identified with *Hae*III (Fig. 2b), *Hin*fI (haplotypes G and H; Fig. 2c), and *Mae*I; *S. maximus* was identified with *Dde*I (Fig. 2a) and *Mbo*I (haplotypes D and E; Fig. 2d); *S. rhombus* was identified with *Hae*III (Fig. 2b), *Mbo*I (Fig. 2d), and *Nci*I; and *S. vulgaris* was identified with *Alu*I, *Dde*I (Fig. 2a), *Hae*III (Fig. 2b), *Hap*II, *Mae*I, and *Mbo*I (Fig. 2d). No single enzyme could distinguish the nine studied flatfish species, but with several combinations of only a couple of REs, tissues of these nine flatfish species can be identified. Any combination of two of the most diagnostic REs (*Dde*I, *Hin*fI, *Hae*III, *Mae*I, and *Mbo*I) except the combination of *Dde*I with *Hin*fI and *Mbo*I and the combination of *Hae*III with *Mbo*I can unambiguously identify all nine flatfish species (Table 3). Nevertheless, these results are based on a relatively small number of individuals, and the consideration of a more extensive number of individuals of each species from different origins might increase the confidence level.

These results are in agreement with those reported for digestion of a 359-bp amplified fragment of the cyt *b* gene with *Hin*fI, *Nci*I, and *Sau*3AI (isoschizomer of *Mbo*I) for

P. flesus, P. platessa, and *S. vulgaris (11).* The *Nci*I endonuclease cleaves the *P. platessa* and *S. vulgaris* PCR products in Céspedes et al.'s *(11)* position 152, which corresponds to our position 262, but it does not cleave for *P. flesus* (Fig. 1); *Sau*3AI (=*Mbo*I) cleaves the *S. vulgaris* PCR product in their position 254, which corresponds to our position 364, but it does not cleave for *P. flesus* and *P. platessa* (Fig. 1). However, in the present work, the *Mbo*I enzyme cleaved the PCR products of these three species in position 108, but this restriction recognition sequence comes before nucleotide 1 in Céspedes et al.'s *(11)* 359-bp fragments. Digestion with *Hin*fI cleaves PCR products in different sites in each species; Céspedes et al.'s *(11)* restriction position 54 (cleaving for *P. flesus* and *P. platessa* PCR products) corresponds to our position 164, their position 67 (cleaving for *P. platessa*) corresponds to our position 177, their position 84 (cleaving for *S. vulgaris*) corresponds to our position 194, and their position 171 (cleaving *P. flesus*) corresponds to our position 281 (Table 1). The absence of intraspecific polymorphism for the recognition sequences of these three REs in the report by Céspedes et al. *(11)* and in the present work, with different individuals collected in different places and years, supports the usefulness of these REs in distinguishing and genetically characterizing *P. flesus, P. platessa,* and *S. vulgaris.*

Intraspecific restriction polymorphism. One of the problems with this technique is the existence of intraspecific variability, giving rise to the possibility of mutations and genetic variability within a species and, consequently, the loss or gain of restriction recognition sequences. Genetic database and population studies on DNA variation for each species may establish the degrees of confidence for different REs. Results of the PCR-RFLP analysis of a 464-bp fragment of cyt *b* (practically the same region used in this work but 14 and 8 bp short at the 5′ and 3′ ends, respectively) for *S. rhombus* and *P. platessa* with eight REs *(32)* are in agreement with the present results except for *S. rhombus* with *Hae*III *(32)*; a typographical mistake was found for *S. rhombus* with *Hae*III (299 bp is written instead of 199 bp). Of the five reported fragments, three have the expected lengths in the present work—the reported 5′ end fragment of 149 bp (corresponding to our 163 bp length; 149 + 14 = 163), the 3′ end fragment of 55 bp (corresponding to our 63 bp; 55 + 8 = 63), and the 77-bp fragment—but two fragments (144 and 39 bp in length) correspond to our 183-bp fragment (Tables 2 and 3). This different result is the consequence of the substitution of a G in our position 309 for a C in the reported DNA sequence (EMBL accession number AF165084) and, consequently, the addition of a recognition site for *Hae*III. Actually, there are two changes: our 5′-GC-3′ (our positions 309 and 310) corresponds to 5′-CG-3′ in the reported sequence, and the second G represents a change of a conservative amino acid. This means that more than one individual must be analyzed to obtain a high level of confidence.

On the other hand, the mtDNA sequence variation of a 401-bp portion (without primers) of the cyt *b* gene (practically the same sequence as that used in the present work) for 280 *R. hippoglossoides* individuals throughout the North Atlantic was studied *(30),* and 22 different sequences were found (GenBank accession numbers L77930 to L77951). These sequences were aligned with our sequences for a reanalysis to obtain restriction maps with the DNASIS computer program. Results of the reanalysis show that for the *Alu*I restriction enzyme, only 2.5% of 280 individuals had a restriction pattern different from that found in the present work (with a new cleavage site in our position 250); for *Hap*II, 0.4% were different (with the loss of a cleavage site in position 261); for *Hin*fI, 1.1% were different (with a new cleavage site in position 281), with 45.7% being analogous to our haplotype A and 53.2% being analogous to our haplotype B; for *Nci*I, 0.36% were different (with the loss of a cleavage site in position 262); and for *Dde*I, *Hae*III, *Mae*I, and *Mbo*I, no differences were found. Taking these results into account, at first sight, the probability that one of the restriction patterns (using a single RE) shown in this work is not present in the *R. hippoglossoides* species is very low (with the highest percentage being 2.5% with *Alu*I). However, this probability may be reduced with a couple of selected enzymes, e.g., *Dde*I and *Hae*III, *Dde*I and *Mae*I, *Hae*III and *Mae*I, or *Mae*I and *Mbo*I. Consequently, results of the reanalysis of the previously reported genetic data *(30)* clearly support the usefulness of the PCR-RFLP analysis of the cyt *b* region in identifying with strong reliability and confidence *R. hippoglossoides* in particular and any fish species in general. When two REs are used, it is enough to obtain an unambiguous identification, but often it is sufficient to show the absence of a putative species in a flatfish fillet (e.g., no sole present). Consequently, the method described here is a powerful method for the detection of mislabeling or fraudulent substitution of these species. This method provides an alternative for flatfish species identification that is simpler than sequencing. Nevertheless, because of the existence of variability that has not been previously detected, as shown above, more studies of individuals of most of these flatfish species are necessary to determine the genetic variability of each species throughout its range of distribution and, consequently, to establish a high degree of confidence.

ACKNOWLEDGMENTS

We are indebted to Franc Saborido (Instituto de Investigacións Mariñas, Vigo, Spain), Ángel Mendizábal (MercaMadrid, Madrid, Spain), Joseph Brown (Ocean Science Center, St. John's, Canada), Teresa Martínez Areal, Ana Gago, and José A. Gradín Martínez for supplying fish samples and to F. Luís Ventín Sanchez and Paz Abella for technical support. We also thank two anonymous reviewers for useful suggestions. This work was supported by grants ALI98-0535 (Dirección General de Enseñanza Superior e Investigación Científica, Ministerio de Educación y Cultura, Spain) and 64502.C847 (Universidade de Vigo, Spain) to A. Sanjuan and an equipment grant for a DNA sequencer (Xunta de Galicia, Spain). We thank the Universidade de Vigo, Spain, for the technician fellowship of F. Luís Ventín Sanchez.

REFERENCES

1. Andrews, A. T. 1998. Electrophoretic methods, p. 204–239. *In* P. R. Ashurst and M. J. Dennis (ed.), Analytical methods of food authentication. Blackie Academic and Professional, Thomson Science, London.

2. Bartlett, S. E., and W. S. Davidson. 1991. Identification of *Thunnus tuna* species by the polymerase chain reaction and direct sequence analysis of their mitochondrial cytochrome *b* genes. Can. J. Fish. Aquat. Sci. 48:309–317.
3. Bartlett, S. E., and W. S. Davidson. 1992. FINS (forensically informative nucleotide sequencing): a procedure for identifying the animal origin of biological specimens. BioTechniques 12:408–411.
4. Cabot, E. L., and A. T. Beckenbach. 1989. Simultaneous editing of multiple nucleic acid sequences with ESEE. Comput. Appl. BioSci. 5:233–234.
5. Carr, S. M., and H. D. Marshall. 1991. A direct approach to the measurement of genetic variation in fish populations: applications of the polymerase chain reaction to studies of Atlantic cod, *Gadus morhua* L. J. Fish Biol. 39(Suppl. A):101–107.
6. Carrera, E., T. García, A. Céspedes, I. González, A. Fernández, P. E. Hernández, and R. Martín. 1999. PCR-RFLP of the mitochondrial cytochrome oxidase gene: a simple method for discrimination between Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). J. Sci. Food Agric. 79:1654–1658.
7. Carrera, E., T. García, A. Céspedes, I. González, A. Fernández, P. E. Hernández, and R. Martín. 1999. Salmon and trout analysis by PCR-RFLP for identity authentication. J. Food Sci. 64:410–413.
8. Carrera, E., T. García, A. Céspedes, I. González, B. Sanz, P. E. Hernández, and R. Martín. 1998. Identification of Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) using PCR amplification and restriction analysis of the mitochondrial cytochrome b gene. J. Food Prot. 61:482–486.
9. Céspedes, A., T. García, E. Carrera, I. González, A. Fernández, L. Asensio, P. E. Hernández, and R. Martín. 1999. Genetic discrimination among *Solea solea* and *Microchirus azevia* by RFLP analysis of PCR amplified mitochondrial DNA fragments. Arch. Lebensmittelhyg. 50:67–70.
10. Céspedes, A., T. García, E. Carrera, I. González, A. Fernández, P. E. Hernández, and R. Martín. 1999. Identification of sole (*Solea solea*) and Greenland halibut (*Reinhardtius hippoglossoides*) by PCR amplification of the 5S rDNA gene. J. Agric. Food Chem. 47:1046–1050.
11. Céspedes, A., T. García, E. Carrera, I. González, B. Sanz, P. E. Hernández, and R. Martín. 1998. Identification of flatfish species using polymerase chain reaction (PCR) and restriction analysis of the cytochrome b gene. J. Food Sci. 63:206–209.
12. Céspedes, A., T. García, E. Carrera, I. González, B. Sanz, P. E. Hernández, and R. Martín. 1998. 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. 61:1684–1685.
13. Davidson, W. S. 1998. DNA/PCR techniques, p. 182–203. *In* P. R. Ashurst and M. J. Dennis (ed.), Analytical methods of food authentication. Blackie Academic and Professional, Thomson Science, London.
14. DeSalle, R., A. K. Williams, and M. George. 1993. Isolation and characterization of animal mitochondrial DNA. Methods Enzymol. 224:176–204.
15. Etienne, M., M. Jérôme, J. Fleurence, H. Rehbein, R. Kündinger, R. Mendes, H. Costa, R. Pérez-Martín, C. Piñeiro-González, A. Craig, I. Mackie, I. M. Yman, M. Ferm, I. Martínez, F. Jessen, A. Smelt, and J. B. Luten. 2000. Identification of fish species after cooking by SDS-PAGE and urea IEF: a collaborative study. J. Agric. Food Chem. 48:2653–2658.
16. Johns, G. C., and J. C. Avise. 1998. A comparative summary of genetic distances in the vertebrates from the mitochondrial cytochrome *b* gene. Mol. Biol. Evol. 15:1481–1490.
17. Kocher, T. D., W. K. Thomas, A. Meyer, S. V. Edwards, S. Pääbo, F. X. Villablanca, and A. C. Wilson. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proc. Natl. Acad. Sci. USA 86:6196–6200.
18. Mackie, I. M., S. E. Pryde, C. Gonzales-Sotelo, I. Medina, R. Pérez-Martín, J. Quinteiro, M. Rey-Mendez, and H. Rehbein. 1999. Challenges in the identification of species of canned fish. Trends Food Sci. Technol. 10:9–14.
19. Märtlbauer, E. 1998. Antibody techniques, p. 241–268. *In* P. R. Ashurst and M. J. Dennis (ed.), Analytical methods of food authentication. Blackie Academic and Professional, Thomson Science, London.
20. McKay, S. J., M. J. Smith, and R. H. Devlin. 1997. Polymerase chain reaction-based species identification of salmon and coastal trout in British Columbia. Mol. Mar. Biol. Biotechnol. 6:131–140.
21. McVeigh, H. P., S. E. Barlett, and W. S. Davidson. 1991. Polymerase chain reaction/direct sequence analysis of the cytochrome *b* gene in *Salmo salar.* Aquaculture 95:225–233.
22. Meyer, A. 1994. Molecular phylogenetic studies of fish, p. 219–249. *In* A. R. Beaumont (ed.), Genetics and evolution of aquatic organisms. Chapman and Hall, London.
23. Pääbo, S. 1990. Amplifying ancient DNA, p. 159–166. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR protocols. A guide to methods and applications. Academic Press, San Diego, Calif.
24. Palumbi, S. R. 1996. Nucleic acid II: the polymerase chain reaction, p. 205–247. *In* D. M. Hillis, C., Moritz, and B. K. Mable (ed.), Molecular systematics, 2nd ed. Sinauer Associates, Sunderland, Mass.
25. Quinteiro, J., C. G. Sotelo, H. Rehbein, S. E. Pryde, I. Medina, R. I. Pérez-Martín, M. Rey-Méndez, and I. M. Mackie. 1998. 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. 46:1662–1669.
26. Ram, J. L., M. L. Ram, and F. F. Baidoun. 1996. Authentication of canned tuna and bonito by sequence and restriction site analysis of polymerase chain reaction products of mitochondrial DNA. J. Agric. Food Chem. 44:2460–2467.
27. Russell, V. J., G. L. Hold, S. E. Pryde, H. Rehbein, J. Quinteiro, M. Rey-Mendez, C. G. Sotelo, R. Pérez-Martín, A. T. Santos, and C. Rosa. 2000. Use of restriction length polymorphism to distinguish between salmon species. J. Agric. Food Chem. 48:2184–2188.
28. Scott, W. B., and M. G. Scott. 1988. Atlantic fishes of Canada. Can. Bull. Fish. Aquat. Sci. 219:1–731.
29. Sotelo, C. G., C. Piñeiro, J. M. Gallardo, and R. I. Pérez-Martín. 1993. Fish species identification in seafood products. Trends Food Sci. Technol. 4:395–401.
30. Vis, M. L., S. M. Carr, W. R. Bowering, and W. S. Davidson. 1997. Greenland halibut (*Reinhardtius hippoglossoides*) in the North Atlantic are genetically homogenous. Can. J. Fish. Aquat. Sci. 54: 1813–1821.
31. Whitehead, P. J. P., M.-L. Bauchot, J.-C. Hureau, J. Nielsen, and E. Tortonese (ed.). 1986. Fishes of the north-eastern Atlantic and the Mediterranean, vol. 3. Clofnam. UNESCO, Paris.
32. Wolf, C., M. Burgener, P. Hübner, and J. Lüthy. 2000. PCR-RFLP analysis of mitochondrial DNA: differentiation of fish species. Lebensm.-Wiss. Technol. 33:144–150.