Development of a DNA-Based Method Aimed at Identifying the Fish Species Present in Food Products

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Analysis of restriction fragment length polymorphism (RFLP) profiles of a 464 bp amplicon obtained from the mitochondrial cytochrome *b* gene was used to differentiate between several different fish species. The method was tested by a collaborative study in which 12 European laboratories participated to ascertain whether the method was reproducible. Each laboratory was required to identify 10 unknown samples by comparison with RFLP profiles from authentic species. From a total of 120 tests performed, unknown samples were correctly identified in 96% of cases. Further work attempting to use the method to analyze mixed and processed fish samples was also performed. In all cases the species contained within mixed samples were correctly identified, indicating the efficacy of the method for detecting fraudulent substitution of fish species in food products.

Keywords: *Species identification; PCR-RFLP; cytochrome b; interlaboratory study*

INTRODUCTION

The ability to differentiate between fish species is an important commercial issue, especially as once external characteristics such as fins, skin, and head have been removed, many fish species can look identical. Although several techniques are available for the identification of raw untreated fish samples (*1*, *2*), the techniques are not applicable to products that have undergone processing by the food industry. The development of DNA-based techniques such as restriction fragment length polymorphism (RFLP) has allowed the identification of fish products following heavy processing (*3*−*7*), although most methods to date have investigated only one particular group of fish, for example, tuna species (*4*) and salmon species (*7*).

Techniques for performing species identification can be classified on the basis of the nature of their targets: first, techniques that target multiple gene locations simultaneously to produce fingerprint-like patterns such as random amplification of polymorphic DNA (RAPDs) and, second, techniques that target single or very few gene locations such as RFLP and single-strand conformation polymorphism (SSCP). The distinct advantage of the second set of techniques is not only their ability to identify fish species from both heavily processed and therefore degraded samples but also their ability to easily identify multiple species within a single product (*4*, *8*).

The main aim of this study was to obtain DNA from a wide range of fish species that was suitable for PCR-RFLP analysis. The intention was to use PCR-RFLP not only to analyze both closely related samples but also to develop a single protocol for analyzing phylogenetically distinct fish species that could be adopted by the food industry.

MATERIALS AND METHODS

The following laboratories participated in the collaborative study: Norwegian Institute of Fisheries and Aquaculture, Tromsø, Norway; Chemische Landesuntersuchungsanstalt, Freiburg im Breisgau, Germany; Hanse Analytik GmbH, Bremen, Germany; Laboratory of the Government Chemist, Teddington, U.K.; Instituto de Investigaciones Marinas, Vigo, Spain; Universidad de Santiago de Compostela, Spain; Institute of Biochemistry and Technology, Federal Research Centre for Fisheries, Hamburg, Germany; Instituto de Investigacao das Pescas do Mar, Lisbon, Portugal; Rowett Research Institute, Aberdeen, U.K.; National Food Administration, Uppsala, Sweden; Departamento de Biotechnologia, UTPAM, Lisbon, Portugal; Lagoas-Marcosende, Vigo, Spain.

Origin and Preparation of Samples. Specimens of 36 fish species comprising 5 distinct families (Table 1) were provided by the Instituto de Investigaciones Marinas (Vigo, Spain), the Rowett Research Institute (Aberdeen, U.K.), and the Federal Research Center for Fisheries (Hamburg, Germany) and used as reference species for RFLP profiles.

DNA Extraction. DNA was extracted following the CTAB method of Rogers and Bendich (*9*), as detailed previously in Russell et al. (*7*).

PCR Amplification. Primers used for amplification were those originally described by Burgener (*10*). The primers were designated L14735 5′-AAA AAC CAC CGT TGT TAT TCA ACT

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Table 1. Authentic Fish Species

	fish species	family name	study code	location
1	***Merluccius merluccius*/European hake**[a]	**Merlucciidae**	**Mmer**	**Northeast Atlantic (NEA), Spain**
2	*Merluccius hubbsi*/Argentine hake	Merlucciidae	Mhub	Southwest Atlantic (SWA), Argentina
3	*Merluccius polli*/Benguela hake	Merlucciidae	Mpol	Southeast Atlantic (SEA), Mauritania
4	*Merluccius senegalensis*/Senegalese hake	Merlucciidae	Msen	SEA, Senegal
5	*Merluccius gayi*/South Pacific hake	Merlucciidae	Mgay	Southeast Pacific (SEP), Chile
6	*Merluccius australis*/southern hake	Merlucciidae	Maus	SEP, Chile
7	*Merluccius bilinearis*/silver hake	Merlucciidae	Mbil	Northwest Atlantic (NWA), U.S.A.
8	*Merluccius albidus*/offshore hake	Merlucciidae	Malb	NWA, USA
9	*Merluccius productus*/North Pacific hake	Merlucciidae	Mpro	Northeast Pacific (NEP), U.S.A.
10	***Anguilla rostrata*/American eel**	**Anguillidae**	**Aros**	**Canada**
11	***Anguilla australis*/Australian eel**	**Anguillidae**	**Aaus**	**Australia or New Zealand**
12	*Anguilla anguilla*/European eel	Anguillidae	Aang	Baltic Sea
13	***Sardina pilchardus*/European pilchard**	**Clupeidae**	**Spil**	**Portuguese coast**
14	*Sardinella aurita*/Spanish sardine	Clupeidae	Saur	Mediterranean Sea
15	*Sardinops sagax*/Pacific sardine	Clupeidae	Ssag	Mediterranean Sea
16	***Salmo salar*/Atlantic salmon**	**Salmonidae**	**Ssal**	**Scotland, U.K.**
17	***Oncorhynchus keta*/Keta/chum salmon**	**Salmonidae**	**Oket**	**Canada**
18	*Oncorhynchus kisutch*/Coho/silver salmon	Salmonidae	Okis	Canada
19	*Oncorhynchus gorbuscha*/pink salmon	Salmonidae	Ogor	Canada
20	***Oncorhynchus nerka*/red/sockeye salmon**	**Salmonidae**	**Oner**	**Canada**
21	*Oncorhynchus tshawytscha*/Spring/King/Chinook salmon	Salmonidae	Otsh	Canada
22	*Oncorhynchus mykiss*/rainbow trout	Salmonidae	Omyk	Scotland, U.K.
23	*Salvelinus alpinus*/Arctic char	Salmonidae	Salp	Norway
24	*Salvelinus fontinalis*/Brook Trout	Salmonidae	Sfon	Germany
25	*Salmo trutta*/brown trout	Salmonidae	Stru	Scotland, U.K.
26	*Solea solea*/sole	Soleidae	Ssol	Spain
27	*Glyptocephalus cynoglossus*/witch flounder	Pleuronectidae	Gcyn	Spain
28	*Pleuronectes platessa*/European plaice	Pleuronectidae	Ppla	Spain
29	***Scophthalmus maximus*/turbot**	**Scophthalmidae**	**Smax**	**Spain**
30	***Lepidorhombus boscii*/four spotted megrim**	**Scophthalmidae**	**Lbos**	**Spain**
31	*Lepidorhombus whiffiagonis*/megrim	Scophthalmidae	Lwhi	Spain
32	***Scophthalmus rhombus*/brill**	**Scophthalmidae**	**Srho**	**Spain**
33	*Platichthys flesus*/flounder	Pleuronectidae	Pfle	Spain
34	*Hippoglossoides elassodon*/flathead sole	Soleidae	Hela	Spain
35	*Limanda ferruginea*/yellowtail flounder	Pleuronectidae	Lfer	Spain
36	*Limanda limanda*/Dab	Pleuronectidae	Llim	Spain

[a] Samples highlighted in bold denote selection for collaborative study.

A-3′ and H15149ad 5′-GCI CCT CAR AAT GAY ATT TGT CCT CA-3′, which amplified a fragment of ~464 bp from the mitochondrial cytochrome *b* gene.

Reactions were prepared as follows: 2.0 mM $MgCl_2$, 200 μM each dNTP (Bioline), 1.25 units Taq DNA polymerase (Bioline), 25 pM each primer, and 50−100 ng of template DNA in a 50 μL reaction volume. Reactions were overlayed with mineral oil, and PCR was carried out using a Perkin-Elmer DNA Thermal Cycler 480 as follows: preheating step, 96 °C for 3 min; cycling parameters, 96 °C for 30 s, 63 °C for 60 s, 72 °C for 60 s, × 40 cycles; final extension step, 72 °C for 3 min.

RFLP. Restriction digests were performed as follows using the enzymes *Aci*I, *Dde*I, *Hae*III, *Hin*cII, *Hin*fI, *Msp*I, and *Nla*III (New England Biolabs; Boehringer).

Six to 10 μL of each purified PCR product was digested with 10 units of each enzyme in a final volume of 25 μL overnight at the manufacturers' recommended temperature. The reaction was stopped by the addition of loading buffer (0.05% w/v bromophenol blue; 40% w/v sucrose; 0.1 M EDTA; 0.5% w/v SDS). DNA restriction fragments were resolved using Cleangel 48S (Pharmacia Biotech) for native PAGE, following the manufacturers' instructions, with bands visualized using the Pharmacia Plus One Silver DNA staining kit.

Preparation of Samples for Collaborative Study. From the collection of 36 reference species, 10 samples were selected to give a representative set of unknown samples (Table 1). These samples were supplied to participating laboratories as raw tissue preserved in ethanol, which was removed before further analysis. A protocol detailing the procedure for analysis of samples (as described above) was also provided along with the samples.

Preparation of Mixed Samples. Samples containing two or three different fish species selected from the 10 unknown samples were also prepared and analyzed as "blind" samples by the Rowett Research Institute following the protocol provided for analysis of samples. Five samples were prepared by mixing PCR amplification products, and the other five samples were prepared by mixing tissues and then performing the protocol as described previously.

Preparation of Processed Samples. Samples of the 10 fish selected for the collaborative study were also subjected to heat treatment (100 °C, 15 min) to produce cooked products. Samples were heat treated as individual species and also in the mixes described above. These samples were then DNA extracted and PCR amplified as described previously to confirm whether thermal processing affected the ability to generate PCR amplification products from the samples. Mixed samples were then subjected to RFLP analysis to confirm that all species remained detectable.

RESULTS

Differentiation of Fish Species by RFLP Analysis. The restriction endonucleases *Aci*I, *Dde*I, *Hae*III, *Hin*cII, *Hin*fI, *Msp*I, and *Nla*III were selected for this study, as comparison of the profiles generated with these enzymes using both computer-simulated restriction site mapping of sequences present within Genbank and laboratory investigation (Table 2) allowed almost all 36 fish species to be differentiated. Only the hake species *M. merluccius* and *M. senegalensis* remained undifferentiated following computer analysis. These seven enzymes were also selected on the basis of cost and incubation temperatures, which being identical (37 °C) allowed all RFLP analyses to be performed at the same time. Figure 1 shows the RFLP profiles generated from the 36 different fish species when treated with the enzyme *Hin*fI. Several different profiles were observed (some of which did not match those predicted by the

Table 2. Computer-Calculated RFLP Profiles from Fish Sequences Held within Genbank and from Laboratory Investigations (See Footnotes for Database Accession Numbers)

species	fragment size (base pairs) with enzyme						
	*Aci*I	*Dde*I	*Hae*III	*Hin*cII	*Hin*fI	*Msp*I	*Nla*III
M. merluccius	27, 42, 43, 51, 140, 160	155, 308	39, 109, 126, 189	U[a]	U	U	U
M. hubbsi	42, 43, 51, 76, 84, 167	U	39, 109, 126, 189	U	81, 382	U	124, 339
M. polli	42, 51, 160, 210	155, 308	39, 109, 126, 189	U	U	U	124, 339
M. senegalensis	27, 42, 43, 160, 191	155, 308	39, 109, 126, 189	U	U	U	U
M. gayi	42, 51, 160, 210	U	39, 109, 315	U	U	U	124, 339
M. australis	42, 43, 51, 160, 167	U	39, 109, 126, 189	U	81, 382	U	124, 339
M. bilinaris	42, 43, 51, 159, 167	U	39, 109, 126, 189	U	U	U	124, 339
M. albidus	42, 43, 51, 160, 167	U	39, 109, 126, 189	U	81, 382	U	124, 339
M. productus	42, 51, 160, 210	U	39, 109, 315	U	81, 382	U	124, 339
A. rostrata[b]	U	227, 236	169, 294	209, 254	81, 382	U	24, 66, 88, 124, 161
A. australis[c]	217, 246	227, 236	U	209, 254	U	U	24, 66, 88, 124, 161
A. anguilla[d]	U	227, 236	169, 294	209, 254	U	U	24, 66, 88, 124, 161
S. pilchardus	58, 159, 246	185, 278	14, 46, 102, 301	92, 371	53, 102, 114, 194	99, 364	24, 32, 34, 179, 194
S. aurita[e]							
S. sagax[e]							
S. salar[f]	60, 403	27, 116, 320	39, 109, 315	U	198, 265	175, 288	24, 439
O. keta[g]	U	116, 347	39, 424	92, 371	U	131, 157, 175	194, 269
O. kisutch[h]	U	69, 116, 278	39, 109, 315	92, 371	198, 265	175, 288	24, 194, 245
O. gorbuscha[i]	U	116, 347	39, 424	92, 371	U	131, 157, 175	91, 178, 194
O. nerka	U	116, 347	39, 109, 315	92, 371	198, 265	175, 288	24, 154, 285
O. tshawytscha	60, 403	165, 276	31, 109, 315	92, 371	196, 265	175, 288	24, 439
O. mykiss[j]	U	116, 347	39, 109, 315	92, 371	U	175, 288	91, 178, 194
S. alpinus	U	27, 116, 320	11, 39, 109,304	92, 371	72, 87, 126, 178	97, 175, 191	24, 439
S. fontinalis[k]	U	116, 347	39, 109, 315	92, 371	178, 285	175, 288	24, 439
S. trutta	51, 412	27, 116, 320	39, 424	U	45, 72, 81, 87, 178	175, 288	24, 439
S. solea	196, 217	U	23, 124, 266	78, 335	154, 259	192, 221	100, 313
G. cynoglossus[l]	U	9, 131, 274	5, 97, 100, 210	68, 162, 183	117, 124, 172	U	64, 88, 261
P. platessa[m]	U	42, 119, 252	15, 106, 292	183, 230	13, 124, 276	70, 151, 192	64, 88, 91, 170
S. maximus	181, 232	39, 92, 282	106, 124, 183	U	154, 259	151, 262	U
L. boscii	22, 181, 210	131, 282	106, 124, 183	10, 68, 162, 173	55, 358	U	63, 78, 89, 183,
L. whiffiagonis	22, 181, 210	9, 62, 69, 274	106, 124, 183	183, 230	137, 276	U	38, 114, 261
S. rhombus[n]	22, 78, 88, 104, 120	130, 283	29, 39, 77, 123, 144	173, 239	U	150, 262	30, 169, 214
P. flesus[o]	22, 391	119, 294	15, 106, 292	10, 173, 230	117, 124, 172	150, 262	64, 88, 261
H. elassodon	22, 391	119, 294	15, 106, 292	68, 162, 183	124, 289	150, 262	64, 88, 261
L. ferruginea	85, 328	30, 131, 252	15, 106, 292	183, 230	124, 289	70, 151, 192	64, 88, 261
L. limanda	85, 328	30, 42, 131, 210	15, 106, 292	183, 230	124, 289	70, 151, 192	64, 88, 261

[a] U, sequences unaffected by restriction enzyme. [b] AF006716; M85080; AF006717. [c] AF006712; AF006713. [d] D84302; AF006714; AF006715. [e] Indicates no sequence data available for computer analysis. [f] AF165083; U12143. [g] AF165078; AF125212. [h] AF165079. [i] AF165077. [j] L29771; AF125208; D58401; AF125209. [k] D58399; AF154850. [l] AF165073. [m] AF165081. [n] AF165073. [o] AF113179.

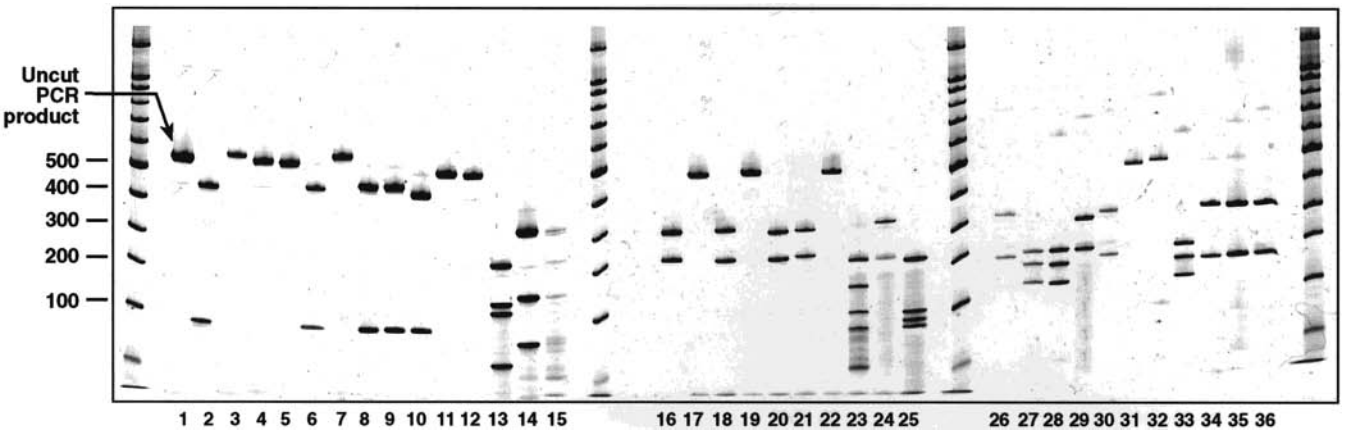

Figure 1. RFLP profiles for the 36 authentic fish species following digestion with *Hin*fI. Preparation of RFLP digests and rehydration and running and staining of Cleangel 10% 48S were performed exactly as described under Materials and Methods. Lanes 1−36 refer to authentic species detailed in Table 1, with a 100 bp DNA ladder included as reference.

computer analysis); however, when these profiles were collated with the results obtained from the other 6 enzymes, they still provided the necessary differentiation between species.

Collaborative Study. From a total of 120 cases, 7 samples were not correctly identified. However, of these 7, 2 samples failed to amplify at the PCR stage and 4, although incorrectly identified to species level, were assigned to the correct family (Table 3). In general most participants followed the protocol defined in the study, although certain deviations were noted in sample preparation. For example, certain participants opted to use DNA extraction protocols used routinely within their laboratory. In three of the four cases when samples

Table 3. Identification of Unknown Samples by Participating Laboratories

	sample									
lab	1 Oket[a]	2 Oner	3 Ssal	4 Aros	5 Spil	6 Lbos	7 Smax	8 Mmer	9 Srho	10 Aaus
1	+[b]	+	+	+	+	+	+	+	+	+
2	+	+	+	Eel[c]	ND[d]	+	+	+	ND	+
3	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+
6	+	Sal[e]	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+
9	+	+	+	+	−[f]	+	+	+	+	+
10	+	+	+	+	+	+	+	+	+	+
11	Sal	Sal	+	+	+	+	+	+	+	+
12	+	+	+	+	+	+	+	+	+	+

[a] Refer to Table 1 for sample codes. [b] +, correct identification. [c] Eel, sample identified correctly to the eel family. [d] ND, sample failed to produce a PCR product upon amplification. [e] Sal, sample identified correctly to the salmon family. [f] −, incorrect identification.

were correctly identified only to the family level, problems with restriction enzymes were identified, with participants unable to distinguish between the correct species and one other. Correct identification would have been possible if all seven restriction enzymes profiles had been available. However, it was not the same enzyme that proved to be problematic in all cases.

Analysis of Mixed-Species Products. Analysis of 10 mixed samples was also performed to determine whether the protocol could be used to identify more than one fish species within a product. Mixed samples containing fish species that could be combined within food products either intentionally or fraudulently were analyzed (Figure 2). Five of these samples (M1−M5) were prepared by mixing PCR products from single authentic species, and five were prepared by mixing tissue from species (M6−M10). This was done to determine whether all species contained within mixes prior to PCR amplification could still be determined. RFLP profiles from the mixed samples were visually examined, with the sizes of each band present in each sample recorded for all seven enzymes. Profiles from reference species were then compared to the profiles generated from the mixed samples to identify which reference species were present. All samples were correctly identified, with the profiles following digestion with the enzyme *Hin*fI shown in Figure 2. Some samples were more difficult to identify than others due to the complexity of the banding profiles generated (sample M9, Figure 2). However, by comparing the RFLP profiles generated from mixed samples with those from the 36 authentic species, by the process of elimination, even sample M9, which contained three fish species, was correctly identified.

Amplification of DNA from Processed Products. The applicability of the method to testing food products was also investigated. Authentic species and mixed samples were subjected to heat treatment prior to sample preparation. All samples whether mixed or single species produced PCR amplification products of the expected size, with only sample 6 (*L. boscii*) showing a reduced yield following thermal processing compared to the original raw sample set. Nevertheless, *L. boscii* was still detected within mixed products following thermal processing. Comparison of the RFLP profiles from mixed products following heat treatment with the original set of mixed products (shown in Figure 2) indicated no differences were detected in banding profiles, thus strongly indicating the protocol's applicability to the food industry.

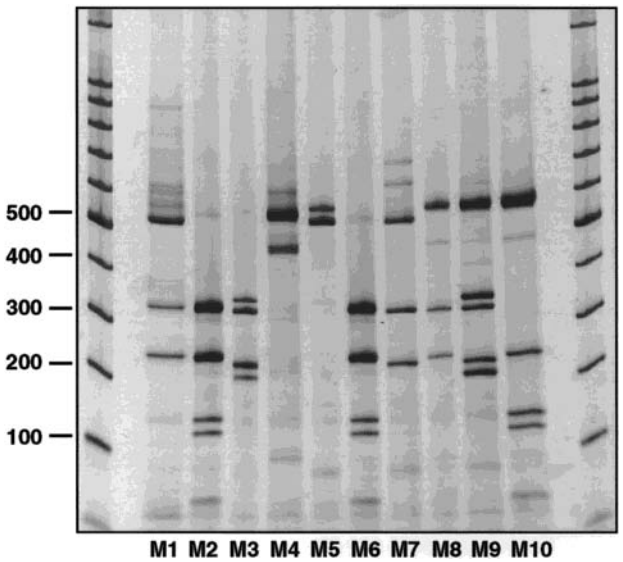

Figure 2. RFLP profiles for the 10 mixed samples digested with *Hin*fI. M1−M5 contain fish species that were mixed following PCR amplification of individual species. M6−M10 contain fish species that were mixed as tissue samples prior to analysis. M1, *Salmo salar* and *Oncorhynchis keta*; M2, *Salmo salar* and *Sardina pilchardus*; M3, *Lepidorhombus boscii* and *Scophthalmus maximus*; M4, *Anguilla rostrata* and *Anguilla australis*; M5, *Scophthalmus rhombus* and *Merluccius merluccius*; M6, *Salmo salar* and *Sardina pilchardus*; M7, *Scophthalmus rhombus* and *Scophthalmus maximus*; M8, *Merluccius merluccius* and *Oncorhynchus nerka*; M9, *Lepidorhombus boscii*, *Merluccius merluccius*, and *Scophthalmus maximus*; M10, *Sardina pilchardus* and *Merluccius merluccius*. Preparation of RFLP digests and rehydration and running and staining of Cleangel 10% 48S were performed exactly as described under Materials and Methods, with a 100 bp DNA ladder included as reference.

DISCUSSION

There are currently methods available that allow closely related fish species to be differentiated (*7*, *11*−*15*). However, few of these methods allow more than one fish group to be analyzed within a single protocol, and those that attempt to address this issue analyze only limited numbers of species from different fish groups (*15*). The aim of this study was to develop a cost-effective DNA-based technique that could identify a wider selection of fish species than has previously been attempted, with the aim of defining species present within mixed fish products and also to highlight its suitability to analyze processed products.

The methodology was designed to be performed easily, rapidly, and relatively cheaply, with restriction enzymes selected to generate distinct differences between RFLP profiles, allowing fish species to be uniquely classified. This was so that many samples could easily be identified by comparison with authentic species. The only drawback of the method for differentiating between species is the inability to distinguish between two closely related hake species. However, further work to identify an enzyme capable of differentiating these two species could be performed if required.

Results from the collaborative study indicate the ability of the method to be effectively performed by

different operators, using the facilities available within any general molecular biology laboratory. Although silver-stained acrylamide gels were recommended for use to visualize RFLP profiles, certain laboratories did not have this facility and used ethidium bromide-stained agarose gels without losing significant definition between similarly sized fragments. This was also the case when mixed samples were analyzed. The major criticism reported from the collaborative study was the difficulty in identifying unknown samples, as RFLP profiles of reference species were provided only as photographs and tables of fragment sizes. This meant that unknown sample profiles had to be cross-referenced to the protocol rather than being compared to reference samples on the same gel. This was due to the additional costs that would have been incurred by participants to analyze the 36 authentic species as well as the 10 unknown samples. It is, however, accepted that sample identification by comparison of numerous RFLP profiles is reliant on the expertise of the analyst; therefore, the use of computer programs that can perform such analyses will only improve the accuracy of results.

The current study, although limited to five fish groups, allowed the unique identification of 34 different species, with a large selection of commercially important species from each fish group investigated. However, the protocol could easily be adapted to accommodate more, or different, species by including different enzymes should the need arise. It should be noted that examination of sequence data from other eel and salmon species held within Genbank indicates that these can be resolved using the current technique; however, they were not included in this study. Such modifications to the protocol merely highlight the flexibility of the PCR-RFLP approach to fish species identification.

The main feature of the current study compared to other work is that this method can be applied to processed products, with identification of multiple species within products also shown to be easily achievable. This has huge implications in terms of detecting fraudulent substitution of cheaper fish species within products, although techniques allowing accurate quantification of species within products remain the basis of further work.

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