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Validation of a PCR-RFLP based method for the identification of salmon species in food products

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Abstract The use of a PCR-RFLP based method for the identification of salmon species in food products was investigated. The reliability and practicality of the method was tested by a collaborative study in which five European laboratories participated. Two unknown samples (a commercial product of known species composition and a mix of two salmon species) required identification by comparison with authentic reference species. From a total of 50 cases, 100% of authentic species were correctly assigned, with all unknown samples also correctly identified. A larger scale analysis of UK commercial products was also performed spanning the whole range of salmon products available. In almost all cases the salmon species declared was confirmed, although, a trout species was detected in one product declaring only the presence of salmon. The investigation confirms the reproducibility of the method in different laboratories, and its applicability for commercial product analysis.

Keywords Salmon · Species identification · PCR-RFLP · Inter-laboratory study

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Introduction

Identification of the salmon species present in food products is becoming increasingly important as tighter labeling legislation is enforced in EU member countries. The traditional methods for identification of raw fish such as isoelectric focusing (IEF) of proteins [1–3] are not applicable to processed products due to the denaturation of proteins during processing, especially thermal treatment. Therefore, the use of DNA-based identification techniques such as single strand conformation polymorphism (SSCP) and restriction fragment length polymorphism (RFLP) for authenticating fish species is becoming more popular [2, 4–9]. Of these techniques RFLP allows relatively cheap and simple identification of unknown samples by reference to authentic species.

The use of RFLP to uniquely identify salmon species has been reported [9–13]. The method described by Russell et al. amplified a 464 bp fragment from the mitochondrial cytochrome b gene with subsequent RFLP analysis to allow identification. Unique profiles were generated for all salmon species when the different enzyme profiles were performed, with the method also shown to be successful with heat-treated samples.

The aim of this study was to test the reproducibility of the method of Russell et al., by performing the method in different European laboratories, with the aim of identifying unknown samples by reference to authentic species. The method's applicability for analysis of food products, especially heavily processed foods, was also investigated by analysis of 70 UK food products containing salmon.

The analysis of numerous commercial products had the aim of validating the method with different products including smoked, cooked, and pickled types. This will assess the applicability of the method to all types of food products containing salmon, whilst the inter-laboratory trial will investigate the reproducibility of the method in different laboratories. The results of both studies are discussed below.

Materials and methods

The following laboratories participated in the inter-laboratory study:

1. Rowett Research Institute, Greenburn Road, Aberdeen, AB 21 9SB, Scotland, UK,
2. Bundesforschungsanstalt für Fischerei, Institut für Biochemie und Technologie, Palmaille 9, 22769 Hamburg, Germany,
3. Departamento de Bioquimica y Biologia Molecular, Facultad de Biologia, Universidad de Santiago de Compostela, 15706 Santiago de Compostela, Spain,
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5. Instituto de Investigação das Pescas e do Mar, Avenida de Brasìlia, Lisbon, Portugal.

Preparation of samples for inter-laboratory trial

Samples of the ten authentic salmon samples (Table 1) were morphologically identified, and supplied to participating laboratories as either raw tissue preserved in ethanol, or as a DNA preparation by laboratory 1. Unknown samples for identification (unknown samples 1 and 2; Table 1), were prepared by laboratory 1, before distribution to participating partners, although without the knowledge of the analyst participating in the study.

Selection of commercial products

A selection of 70 commercial products comprising a wide range of products available in retail outlets in Britain were selected for identification. The types of products included both cheaper and the more expensive items, with smoked, pickled, and heat-treated products analyzed.

DNA extraction and PCR conditions

The extraction of DNA was performed following the method described in [14], as detailed below.

Tissue samples (50–100 mg wet weight) were cut into small pieces, and homogenized with 0.5 mL of buffer 1A [1.2% w/v hexadecyltrimethylammonium bromide (CTAB); 60 mM Tris; 10 mM EDTA; 0.8 M NaCl; pH 8.0]. Directly before use, MDP (3-mercapto-1, 2-propanediol) to a final concentration of 0.1% v/v and proteinase K to a final concentration of 0.5 mg $\times$ ml^{-1} were added. The mixture was incubated in a water-bath at 65 °C for 1 hour, cooled to room temperature, and centrifuged for 10 min in a microcentrifuge, at maximum speed, without refrigeration. The supernatant was mixed with 500 μL of chloroform for 30 s, and the two phases separated by centrifugation. The supernatant was retained and washed again with chloroform, before being mixed with two volumes of buffer 2 (1% w/v CTAB; 50 mM Tris; 10 mM EDTA, pH 8.0) and centrifuged to pellet the DNA/ CTAB complex. The pellet was subsequently solubilized by the addition of 400 μL of buffer 3 (1 M NaCl; 10 mM tris; 1 mM EDTA; pH adjusted to 8.0 using HCl) and heating at 65 °C for 10 min. 400 μL of isopropanol (100%) were then added to the dissolved pellet, and the mixture was allowed to stand at room temperature for 10 min before brief centrifugation at maximum speed. The precipitate was washed with 500 μL of ethanol (70%), centrifuged again, and the pellet suspended in 100 μL of buffer 4 (10 mM Tris; 1 mM EDTA; pH 8.0).

PCR amplification

Primers used for amplification, were those described in [15]. The primers were designated L14735 5′-AAA AAC CAC CGT TGT TAT TCA ACT A-3′ and H15149ad 5′-GCI CCT CAR AAT GAY ATT TGT CCT CA-3′.

Reactions were prepared as follows: 2.0 mM $MgCl_2$, 250 μM each dNTP (Promega), 1 unit (U) Taq DNA polymerase (Biogene), 25 ρM 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; 94 °C for 5 min; Cycling Parameters: 94 °C for 40 s, 50 °C for 80 s, 72 °C for 80 s, $\times$ 35 cycles; Final extension step: 72 °C for 7 min. PCR products were purified using Wizard DNA purification kit (Promega).

Restriction digests

Restriction digests were performed as follows using the enzymes: *Dde* I; *Nla* III; *Hae* III; *Bsp* 1286I; *Eco* RII; *Sau* 3AI (New England Biolabs; Boehringer).

Table 1 Authentic salmon species and unknown samples included within the inter-laboratory trial

Sample number	Salmon species
1	*Salmo salar*/Atlantic salmon
2	*Oncorhynchus keta*/Keta/Chum salmon
3	*Oncorhynchus kisutch*/Coho/Silver salmon
4	*Oncorhynchus gorbuscha*/Pink salmon
5	*Oncorhynchus nerka*/Red salmon
6	*Oncorhynchus tschawytscha*/Spring/King/ Chinook salmon
7	*Oncorhynchus mykiss*/Rainbow trout
8	*Salvelinus alpinus*/Arctic char
9	*Salvelinus fontinalis*/ Brook trout
10	*Salmo trutta*/Brown trout
Unknown samples	
1	*O. keta* and *O. gorbuscha*
2	*S. salar* (commercial product)

Table 2 Confirmation of authentic species from participating laboratories[a]

Lab.	Authentic species									
	1	2	3	4	5	6	7	8	9	10
RRI	+	+	+	+	+	+	+	+	+	+
IBF	+	+	+	+	+	+	+	+	+	+
U. de S.	+	+	+	+	+	+	+	+	+	+
IIM	+	+	+	+	+	+	+	+	+	+
IPIMAR	+	+	+	+	+	+	+	+	+	+

[a] + = Confirmation of predicted RFLP profile

Table 3 Identification of unknown samples by participating laboratories

Laboratory	Species present in unknown sample 1	Species present in unknown sample 2
RRI	*O. keta* and *O. gorbuscha*	*S. salar*
IBF	*O. keta* and *O. gorbuscha*	*S. salar*
U. de S.	*O. keta* and *O. gorbuscha*	*S. salar*
IIM	*O. keta* and *O. gorbuscha*	*S. salar*
IPIMAR	*O. keta* and *O. gorbuscha*	*S. salar*

Table 4 RFLP profiles of authentic salmon species depicted as unique codes

Salmon species	Enzyme cut code					
	Dde I	*Nla* III	*Hae* III	*Bsp* 1286I	*Eco* RII	*Sau* 3AI
1 *S. salar*	A[b]	0[a]	A	A	0	A
2 *O. keta*	B[b]	A	0	A	A	B
3 *O. kisutch*	C[b]	B	A	A	0	0
4 *O. gorbuscha*	B	C	0	0	B	B
5 *O. nerka*	B	D[b]	A	A	0	B
6 *O. tschawytscha*	C	0	A	A	0	0
7 *O. mykiss*	B	E[b]	A	A	B	0
8 *S. alpinus*	A	0	A	0	0	0
9 *S. fontinalis*	B	0	A	0	0	0
10 *S. trutta*	A	0	0	0	0	A

[a] 0 denotes PCR product unaffected by restriction enzyme
[b] Letters A–E denote different restriction profiles generated by a particular enzyme. For example, if only one profile was expressed, it was classified as pattern A, however, if multiple profiles were detected, these were identified by consecutive lettering

Table 5 RFLP profiles generated by commercial products

Enzyme cut code									
Product number	*Dde* I	*Nla* III	*Hae* III	*Bsp* 1286I	*Eco* RII	*Sau* 3AI	Identification	Species declared	Product description
1	A	0	A	A	0	A	*S. salar*	*S. salar*	Smoked salmon
2	A	0	A	A	0	A	*S. salar*	*S. salar*	Sm. Salmon
3	A	0	A	A	0	A	*S. salar*	*S. salar*	Sm. Salmon
4	A	0	A	A	0	A	*S. salar*	Salmon	Sm. Salmon mousse
5	A	0	A	A	0	A	*S. salar*	Salmon	Sm. Salmon
6	A	0	A	A	0	A	*S. salar*	Salmon	Sm. Salmon
7	A	0	A	A	0	A	*S. salar*	Salmon	Sm. Salmon
8	A	0	A	A	0	A	*S. salar*	*S. salar*	Sm. Salmon
9	A	0	A	A	0	A	*S. salar*	*S. salar*	Sm. Salmon
10	A	0	A	A	0	A	*S. salar*	Salmon	Sm. Salmon
11	A	0	A	A	0	A	*S. salar*	*S. salar*	Sm. Salmon
12	A	0	A	A	0	A	*S. salar*	*S. salar*	Sm. Salmon
13	A	0	A	A	0	A	*S. salar*	Salmon	Sm. Salmon
14	A	0	A	A	0	A	*S. salar*	Salmon	Sm. Salmon paté
15	A	0	A	A	0	A	*S. salar*	Salmon	Sm. Salmon paté
16	A	0	A	A	0	A	*S. salar*	Salmon	Sm. Salmon mousse
17	A	0	A	A	0	A	*S. salar*	*S. salar*	Salmon
18	A	0	A	A	0	A	*S. salar*	*S. salar*	Sm. Salmon
19	A	0	A	A	0	A	*S. salar*	Salmon	Sm. Salmon mousse
20	A	0	A	A	0	A	*S. salar*	Salmon	Sm. Salmon paté
21	A	0	A	A	0	A	*S. salar*	*S. salar*	Sm. Salmon
22	A	0	A	A	0	A	*S. salar*	Salmon	Sm. Salmon parcels
23	A	0	A	A	0	A	*S. salar*	Salmon	Sm. Salmon paté
24	A	0	A	A	0	A	*S. salar*	Salmon	Sm. Salmon paté
25	A	0	A	A	0	A	*S. salar*	*S. salar*	Sm. Salmon
26	A	0	A	A	0	A	*S. salar*	*S. salar*	Sm. Salmon
27	A	0	A	A	0	A	*S. salar*	*S. salar*	Sm. Salmon
28	A	0	A	A	0	A	*S. salar*	Salmon	Sm. Salmon
29	A	0	A	A	0	A	*S. salar*	*S. salar*	Sm. Salmon
30	A	0	A	A	0	A	*S. salar*	Salmon	Sm. Salmon parcels
31	A	0	A	A	0	A	*S. salar*	Salmon	Sm. Salmon
32	A	0	A	A	0	A	*S. salar*	Salmon	Sm. Salmon parcels
33	A	0	A	A	0	A	*S. salar*	Salmon	Sm. Salmon paté
34	B	A	0	A	A	B	*O. keta*	Salmon	Salmon fillets
35	B	A	0	A	A	B	*O. keta*	Salmon	Salmon
36	B	C	0	0	B	B	*O. gorbuscha*	Salmon	Salmon mornay
37	B	A	0	A	A	B	*O. keta*	*O. gorbuscha*	Pink Salmon and Pollack fish cakes

Table 5 Continued

Enzyme cut code									
Product number	*Dde* I	*Nla* III	*Hae* III	*Bsp* 1286I	*Eco* RII	*Sau* 3AI	Identification	Species declared	Product description
38	0	0	A	A	C/D	A	ND[a]	Salmon	Salmon fish cakes
39	A	0	A	A	0	A	*S. salar*	*S. salar*	Sm. Salmon trimmings
40	A	0	A	A	0	A	*S. salar*	*S. salar*	Gravadlax
41	A	0	A	A	0	A	*S. salar*	Salmon	Sm. Salmon paté
42	A	0	A	A	0	A	*S. salar*	Salmon	Sm. Salmon
43	A	0	A	A	0	A	*S. salar*	*S. salar*	Sm. Salmon
44	A	0	A	A	0	A	*S. salar*	*S. salar*	Sm. Salmon
45	A	0	A	A	0	A	*S. salar*	*S. salar*	Sm. Salmon
46	A	0	A	A	0	A	*S. salar*	*S. salar*	Sm. Salmon
47	A	0	A	A	0	A	*S. salar*	*S. salar*	Sm. Salmon
48	A	0	A	A	0	A	*S. salar*	*S. salar*	Sm. Salmon mousse
49	A	0	A	A	0	A	*S. salar*	*S. salar*	Sm. Salmon paté
50	A	0	A	A	0	A	*S. salar*	*S. salar*	Sm. Salmon mousse
51	A	0	A	A	0	A	*S. salar*	*S. salar*	Sm. Salmon paté
52	A	0	A	A	0	A	*S. salar*	Salmon	Sm. Salmon
53	A	0	A	A	0	A	*S. salar*	Salmon	Sm. Salmon bites
54	A	0	A	A	0	A	*S. salar*	*S. salar*	Sm. Salmon paté
55	A	0	A	A	0	A	*S. salar*	*S. salar*	Sm. Salmon
56	A	0	A	A	0	A	*S. salar*	*S. salar*	Sm. Salmon
57	A	0	A	A	0	A	*S. salar*	*S. salar*	Sm. Salmon
58	A	0	A	A	0	A	*S. salar*	Salmon	Sm. Salmon mousse
59	A/B	0/B	A	A	0/B	0/A	*S. salar* and *O. mykiss*	Salmon	Sm. Salmon ring
60	A	0	A	A	0	A	*S. salar*	*S. salar*	Sm. Salmon ribbons
61	A	0	A	A	0	A	*S. salar*	Salmon	Sm. Salmon paté
62	A	0	A	A	0	A	*S. salar*	Salmon	Sm. Salmon
63	A	0	A	A	0	A	*S. salar*	*S. salar*	Sm. Salmon mousse
64	A	0	A	A	0	A	*S. salar*	*S. salar*	Sm. Salmon pieces
65	A	0	A	A	0	A	*S. salar*	Salmon	Salmon fillets
66	B	A	0	A	A	B	*O. keta*	Salmon	Sm. Salmon sandwiches
67	A	0	A	A	0	A	*S. salar*	*S. salar*	Sm. Salmon
68	A	0	A	A	0	A	*S. salar*	*S. salar*	Sm. Salmon
69	A	0	A	A	0	A	*S. salar*	*S. salar*	Gravadlax
70	A	0	A	A	0	A	*S. salar*	Salmon	Sm. Salmon terrine

[a] ND = not determined

6–10 μL of each purified PCR product were digested with 10 U of each enzyme in a final volume of 25 μL overnight at the manufacturers' recommended temperature. The reaction was stopped by 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 10% (Pharmacia Biotech) for native PAGE, following the manufacturer's instructions, with bands visualized using the Pharmacia Plus One Silver DNA Staining Kit.

Results and Discussion

Inter-laboratory trial

The results of the inter-laboratory study are presented in Tables 2 and 3. RFLP profiles from all authentic species generated the expected patterns therefore indicating the method for identification of unknown species was reproducible in different laboratories (Table 2). Analysis of unknown samples was also successful, with both samples correctly identified in all cases (Table 3). Unknown sample 2 was a processed product (paté) of known composition, indicating that commercial products could also be analyzed using the method when performed in other laboratories.

Although correct in all identifications, one participant had problems with the DNA extraction protocol, and used a different method. Also, certain enzyme digests obtained from two of the laboratories did not generate profiles which could be used in the identification of unknown samples. However, sufficient information was obtained from the other enzyme profiles to allow identification.

Analysis of commercial products

In order to accurately and easily identify the salmon species present in the 70 UK commercial products, it was decided to convert the RFLP profiles generated from authentic species into unique codes. Codes were assigned depending on the effect of an enzyme on a

particular species. Uncut PCR fragments were designated 0, with digested PCR products designated A–E depending on the number of unique profiles generated by a particular enzyme. Table 4 indicates the unique RFLP codes for authentic species, which were then cross-referenced to the profiles generated by commercial samples, with species identification duly noted (Table 5).

Results showed that of the commercial products which declared the actual salmon species, the analysis method confirmed the declaration in all but one case. However, of the other products which merely declared 'salmon' as being present, all but one of the products which contained *O. mykiss* and *S. salar*, were seen to contain a single salmon species (usually *S. salar*). As the labeling indicated salmon, the detection of more than one salmon species is acceptable, however, *O. mykiss*, which is rainbow trout, although belonging to the salmon family is of much lower value than the salmon species present in the product, and is not generally accepted as a salmon species by the public. One other product produced a profile which indicated the presence of *S. salar*, although other DNA was revealed, seen by the presence of other bands in the RFLP profile. Examination of the packaging indicated that the product contained salmon and Alaska pollack (*Theragra chalcogramma*), with RFLP identifying *S. salar*, therefore the extra DNA fragment was probably due to Alaska pollack. Although, it was not possible to confirm this as the method in its current form does not allow the identification of other fish species, the ability to identify a wider range of fish by extending the authentic species included in the analysis is currently under investigation.

Other PCR-RFLP based methods have also been published which allow the identification of *Salmo salar*. However, these methods only used two salmon species within the analysis and the method's applicability to food product analysis is still undetermined [10–12].

In summary, the current study shows PCR-RFLP can be used to identify the salmon species present in commercial products, with an extensive range of products tested. The inter-laboratory trial also shows that the method can be successfully adopted by other laboratories for analysis of both mixed salmon species and commercial products.

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