Use of Restriction Fragment Length Polymorphism To Distinguish between Salmon Species

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Identification of 10 salmon species using DNA-based methodology was investigated. Amplification of DNA was carried out using a primer set which amplified a region of the mitochondrial cytochrome *b* gene. Sequences of PCR-amplified DNA from the salmon species were used to select six restriction enzymes allowing species to be uniquely classified. RFLP patterns generated following analysis with each enzyme were resolved using polyacrylamide gel electrophoresis and visualized by silver staining. Results indicate that it is possible to differentiate between all 10 salmon species and that the technique could be easily adopted by the food industry for analysis of processed salmon products.

Keywords: *Salmon; species identification; PCR−RFLP*

INTRODUCTION

The ability to identify salmon species following the removal of external characteristics by processing such as canning and smoking, although problematic, is of great commercial importance. The most commonly used methods for identifying raw fish, such as isoelectric focusing (IEF) of water-soluble proteins (LeBlanc and LeBlanc, 1994; Gallardo et al., 1995), are not applicable to thermally treated products, due to severe protein denaturation. This has led to alternative identification approaches being investigated (Mackie, 1990; Rehbein, 1990; Sotelo et al., 1993; Quinteiro et al., 1998).

Techniques based on the analysis of nucleic acids, such as mitochondrial or genomic DNA, present advantages over protein-based techniques, as they are not dependent on tissue source, age of the individual, or sample damage. The use of DNA analysis techniques for differentiating between closely related species, particularly sturgeon and tuna species, has already been reported (Rehbein et al., 1995; Borgo et al., 1996; DeSalle and Birstein, 1996; Bossier, 1999). Rehbein et al. showed species-specific DNA profiles for several tuna species following amplification of short mitochondrial DNA sequences and subsequent single-strand conformation polymorphism (SSCP) analysis. However, the amplified sequence was only 123 base pairs, as difficulties were experienced when amplifying larger fragments from canned tuna samples. The use of polymerase chain reaction−restriction fragment length polymorphism (PCR−RFLP) has also been used to authenticate canned fish products (Ram et al., 1996; Quinteiro et al., 1998). Results indicate that the method provides a simple, cost-efficient alternative to sequencing for species identification (Carrera et al., 1998, 1999a,b).

Table 1. Authentic Salmon Species

sample no.	salmon species	location
1	*Salmo salar*/Atlantic salmon	Scotland, U.K.
2	*Oncorhynchus keta*/keta/chum	Canada
3	*O. kisutch*/coho/silver	Canada
4	*O. gorbuscha*/ pink salmon	Canada
5	*O. nerka*/red/sockeye salmon	Canada
6	*O. tschawytscha*/spring/king/chinook	Canada
7	*O. mykiss*/rainbow trout	Scotland, U.K.; Spain
8	*Salvelinus alpinus*/Arctic char	Norway
9	*Salvelinus fontinalis*/brook trout	Germany
10	*Salmo trutta*/brown trout	Scotland, U.K.

The main objective of this study was to obtain DNA from authentic salmon species and produce an amplified fragment suitable for sequencing from both raw and heat-treated samples. This sequence information would then be used to identify restriction enzymes, which allowed discrimination between species, and provide a simple, cost-effective method for identifying salmon species present in food products.

MATERIALS AND METHODS

Preparation of Samples. Specimens of 10 salmon species (Table 1) were morphologically identified and authenticated, although *Oncorhynchus kisutch* was only available as a processed product. At least three individuals from each species were examined with the exception of *Salvelinus fontinalis*, for which only one sample was available. Raw samples were then subjected to heat treatment by placing aliquots of each fish

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species in microcentrifuge tubes and placing in a boiling water bath for 15 min.

Extraction of DNA. DNA was extracted following the CTAB method of Rogers and Bendich (1985), as detailed below.

Tissue samples from muscle and fin clips (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 mL^{-1} were added. The mixture was incubated in a water bath at 65 °C for 1 h, 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 were 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 heated at 65 °C for 10 min. A total of 400 μL of 2-propanol (100%) was 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%) and centrifuged again, and the pellet was resuspended in 100 μL of buffer 4 (10 mM Tris, 1 mM EDTA, pH 8.0).

PCR Amplification. Primers used for amplification were those described by Burgener (1997). 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 by using the following: 2.0 mM $MgCl_2$, 250 μM each dNTP (Promega), 1 unit (U) of Taq DNA polymerase (Biogene), 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, 94 °C for 5 min; cycling parameters, 94 °C for 40 s, 50 °C for 80 s, and 72 °C for 80 s, ×35 cycles; final extension step, 72 °C for 7 min. PCR products were purified using the Wizard DNA purification kit (Promega).

Sequencing of PCR Products. Direct sequencing of PCR products from a representative of each salmon species was carried out using an ABI Prism BigDye terminator cycle sequencing ready reaction kit and analyzed using ABI-seqed-675 software, utilizing the BBSRC computer facilities at Daresbury. The "map" program was used to examine DNA sequences, and out of a total of 226 restriction endonucleases, a combination of six was selected which gave theoretical differentiation between most salmon species.

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

A total of 6−10 μL of each purified PCR product was 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 (Pharmacia Biotech) for native PAGE, following the manufacturer's instructions, with bands visualized using the Pharmacia Plus One silver DNA staining kit.

RESULTS

Amplification of Samples and Sequence Analysis of PCR Products. PCR amplification generated the expected size product from all samples whether raw or heat treated. Comparison of the DNA sequences from all salmon species indicated that, from a 438 base pair fragment, 348 bases were monomorphic while 90 were polymorphic (Table 2). The map analysis search for restriction endonucleases whose cut sites were capable of discriminating between all 10 salmon species indicated six enzymes that effectively identified eight of the species, although the two remaining species, *Salvelinus alpinus* and *S. fontinalis*, remained undifferentiated. PCR products digested using the six restriction enzymes were resolved using PAGE. Figures1 and 2 show the RFLP profiles generated from a single digest of each salmon species, with all other individuals from each salmon species behaving as shown. The results indicate that differences in RFLP patterns were detected from salmon species which had been expected to generate identical profiles when digested with *Dde*I and *Sau*3AI (see Figure 1, salmon species 1 and 2 restricted using *Dde*I). Results from map analysis indicated authentic species 1, 2, 4, 5, 7, 8, 9, and 10 would generate identical RFLP profiles following restriction with *Dde*I. However, on analysis, samples 1, 8, and 10 generated a profile different from that of samples 2, 4, 5, 7, and 9. Differences in RFLP profile were also noted when *Sau*3AI was used to restrict samples, with authentic species 1 and 10 subsequently separated from species 2, 4, and 5.

The detection of the differing RFLP patterns allowed *S. alpinus* and *S. fontinalis* to be differentiated, which was not previously thought possible, allowing all 10 salmon species to be uniquely classified, with the size of restriction fragments generated following digestion with the six enzymes presented in Table 3.

Classification of certain species was possible from a single enzyme digest. For example, *Oncorhynchus keta* was identified following *Eco*RII restriction as no other species generated the same RFLP pattern. A unique profile was also detected for *Oncorhynchus kisutch*, when profiles from *Dde*I were resolved, and *Oncorhynchus nerka* and *Oncorhynchus tschawytscha* when restricted with *Nla*III. However, identification of the six other species required a combination of RFLP profiles to be analyzed in order to confirm the species present. For instance, *Salmo salar* and *Salvelinus alpinus* generated identical profiles from the first three enzymes (Table 3, Figure 1), with only results from *Bsp*1286I and *Sau*3AI allowing differentiation.

DISCUSSION

The ability to distinguish between different salmon species from tissues when external characteristics have been removed is of great commercial importance. This study presents the first DNA-based method which is capable of identifying between 10 different salmon species without sequencing and subsequent phylogenetic analysis. The method also utilizes a DNA fragment of a size that can be amplified from processed products and is therefore a technique which could easily be adopted by the food industry and food control.

Alternative RFLP-based methods have been published for identification of salmon species. However, these methods only investigate two or three raw salmonid species, and the methods applicability to food product analysis is still undetermined (Carrera et al., 1999b). Attempts to address the usability of our technique for the analysis of commercial food products have indicated that the method can be applied to a wide range of products including products containing more than one salmon species (Hold et al., in preparation). However,

Table 2. Sequence Alignment of the 464 bp Amplicon (L Strand) from the 10 Salmon Species

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                1                                                  50
S.salar  .......... CCGTACCTTA ATGGCCAACC TCCGAAAAAC TCACCCGCTC
O.keta   .......... .......C.. .......... .......... C..T..T...
O.kisutch .......... .......C.. .......... .......... C..T..T...
O.gorbuscha .......... .......C.. .......... .......... C..T..T...
O.nerka  .......... .......C.. .......... .......... C..T..T...
O.tschawytscha .......... .......C.. .......... .......... C..T..T...
O.mykiss .......... .......C.. .......... .......... C.....T...
S.alpinus .......... .......... .......... .......... ..........
S.fontinalis .......... .......... .......... .......... ..........
S.trutta .......... .......C.. .......... .......... ......C...
               51                                                 100
S.salar  CTAAAAATTG CTAATGACGC ACTAGTCGAT CTCCCAGCAC CATCTAACAT
O.keta   ........C. .......... .........C .......... ..........
O.kisutch ........C. .......... .........C .......... .C........
O.gorbuscha ........C. .......... .........C .......... ..........
O.nerka  ........C. .......... .........C .......... ..........
O.tschawytscha ........C. .......... .........C .......... .C........
O.mykiss ........C. .......... .........C .......... .T.....T..
S.alpinus .......... .......... .........C .....T..C. .C.....T..
S.fontinalis .......... .......... .........C .....TA.C. .C.....T..
S.trutta .......... .......... .......... .......... ..........
              101                                                150
S.salar  CTCAGTTTGA TGAAACTTTG GCTCACTCTT AGGCCTATGT CTAGCCACCC
O.keta   ......C... .......... .T......C. G...T....C ..........
O.kisutch ......C... .......... ........C. .........N T.........
O.gorbuscha ......C... .......... ........C. ....T....C ..........
O.nerka  ......C... .......... .......AC. .......... T.........
O.tschawytscha T.....C... .......... ........C. .......... T......CCC
O.mykiss ......C... .......... .......AC. .......... T....T....
S.alpinus ......C... .......... .T........ .......... T.G.......
S.fontinalis ......C... .......... .T......C. .......... T.........
S.trutta .......... .......... .......... ....T..... ........
               151                                                200
S.salar  AAATCCTTAC CGGGCTCTTC CTAGCCATAC ACTACACCTC CGATATCTCA
O.keta   ....T..... .......... ........G. .......... ...C..T...
O.kisutch ....T..... .......... T.......G. ....T..... ...C..T...
O.gorbuscha ....T..... ......A... .........G. .......T.. ......T...
O.nerka  ....T..... .......... .......... ....T..... ...C..T...
O.tschawytscha ....T..... .......... T..N...... ....T....N ...C..T...
O.mykiss ....T..... .......... ........G. ....T..... ...C..T...
S.alpinus ....T..... ...A...... .......... .......... ......T...
S.fontinalis ....T..... ...A...... .......... .......... ......T..G
               201                                                250
S.salar  ACAGCTTTTT CCTCTGTTTG CCACATTTGC CGAGATGTTA GCTATGGCTG
O.keta   .......... .......C.. ......C... .........C. ....C.....
O.kisutch .......... .......C.. ...T..C... .......... .T..C.....
O.gorbuscha .......... .......C.. ......C... .........C. ....C.....
O.nerka  .......... ....C..C.. ......C... .......... .T..C.....
O.tschawytscha .......... .......C.. .......... ...N...... .T..C.....
O.mykiss .........C. .......... ......C... .......... .T..C.....
S.alpinus .......... .......G.. ...T..C... .........A. .T..C.....
S.fontinalis .......... .......A.. .........T .........A. .T..C.....
S.trutta .....C.... .......... .......... .......... ....C.....

               251                                                300
S.salar  ACTCATCCGT AACATTCACG CTAACGGAGC ATCTTTCTTC TTTATCTGTA
O.keta   ...A..T..G .....C.... .......... .........T .....T....
O.kisutch ......T..A .....C. T. .C........ .........T .....TN.C.
O.gorbuscha ...A..T... .....C..T. .......... .........T .....T....
O.nerka  ......T..A .....C..T. .C........ .........T .....T....
O.tschawytscha ......T..A ..T..C.... .C.....G.. .........T .....TN.C.
O.mykiss G.....T..A .....C..T. .C........ .........T ..........
S.alpinus .........G ..T..C.... .......... .......... ..........
S.fontinalis G........A ..T..C.... .......... .......... ..........
S.trutta .........A .......... .......... .......... ..........
               301                                                350
S.salar  TTTATATACA CATCGCCCGA GGACTTTATT ATGGTTCCTA TCTATATAAA
O.keta   .......... T........G .......... .C..A..... C..G..C...
O.kisutch .......... T......... .......... .C..C..A.. C..C..C...
O.gorbuscha .......G.. T........G .......... ....A..A.. C.....C...
O.nerka  .......G.. .......... .......... ....A..A.. C..G..C...
O.tschawytscha .......... T......... .......... ....C..TN. C..C..C...
O.mykiss .......... T......... ........C. .C..C..G.. C..C..C...
S.alpinus ....C..... T......... .....C..C. .C..G..... C.........
S.fontinalis .......G.. T......... .....A..C. .C..G..... C.........
S.trutta .......... T......... .....C..C. .......... C.........
               351                                                400
S.salar  GAAACCTGAA ATATCGGAGT TGTACTTCTA CTTCTCACTA TAATAACTGC
O.keta   ........G. .......... .......T.. .......... ..........
O.kisutch .......... .......G.. G......... .......... ..........
O.gorbuscha ........G. G......... .......T.. .......... ..........
O.nerka  .......... .......... ......CT.. .......... ..........
O.tschawytscha .......... ....T..G.. G......... .......... .......C..
O.mykiss ........G. .......... .......T.. .......... ..........
S.alpinus .......... ....T..... A...T.A... .....A.... ....G.....
S.fontinalis .......... ....T..G.. A...T.AT.. .......... ....G..A..
S.trutta .......... .......... C.....G... .......... .......C..
               401                                                450
S.salar  CTTCGTAGGC TACGTTCTTC CATGAGGACA AATNTCATTC TG
O.keta   A.....G... .....C..C. .G........ .........T G.
O.kisutch ...T..G..A ....NC..C. .......... .......... ..
O.gorbuscha A......... .....C..C. .G........ .......... ..
O.nerka  A......... .....C..C. .......... .........T G.
O.tschawytscha ...T..N... ....NC..C. .......... .......... ..
O.mykiss ...T...... .....C..C. .G........ ...A...... ..
S.alpinus ...T...... .......... .......... .......... ..
S.fontinalis T..T...... ........C. .......... .......... ..
S.trutta ......G... .......... .......... .......... ..
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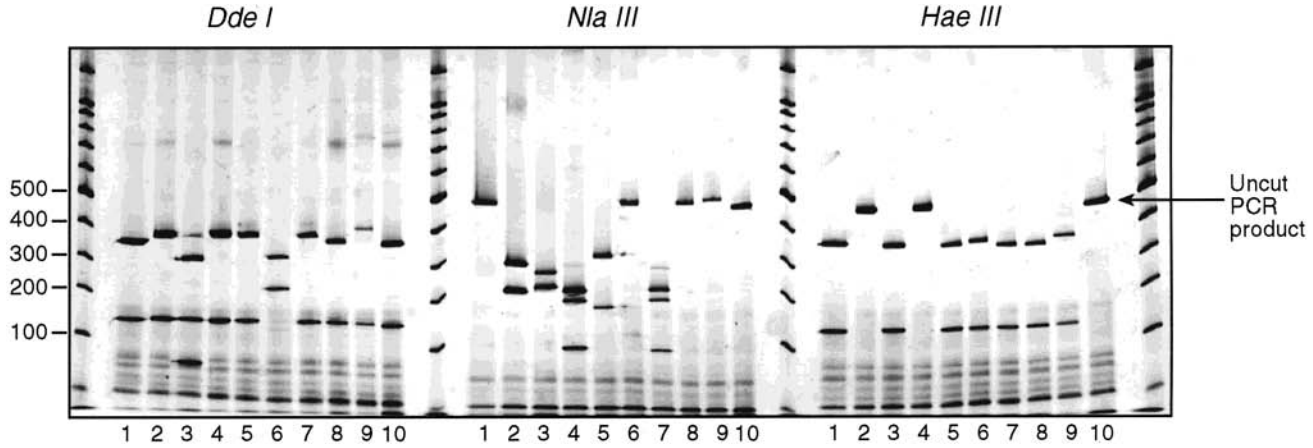

Figure 1. RFLP patterns of 10 salmon species following digestion with restriction enzymes *Dde*I, *Nla*III, and *Hae*III. Preparation of RFLP digests and rehydration, running, and staining of CleanGel 10% 48S were performed exactly as described in Materials and Methods. Lanes 1−10 refer to authentic salmon samples 1−10 detailed in Table 1, digested with *Dde*I, *Nla*III, and *Hae*III, respectively, with 100 bp DNA ladder lanes included as reference.

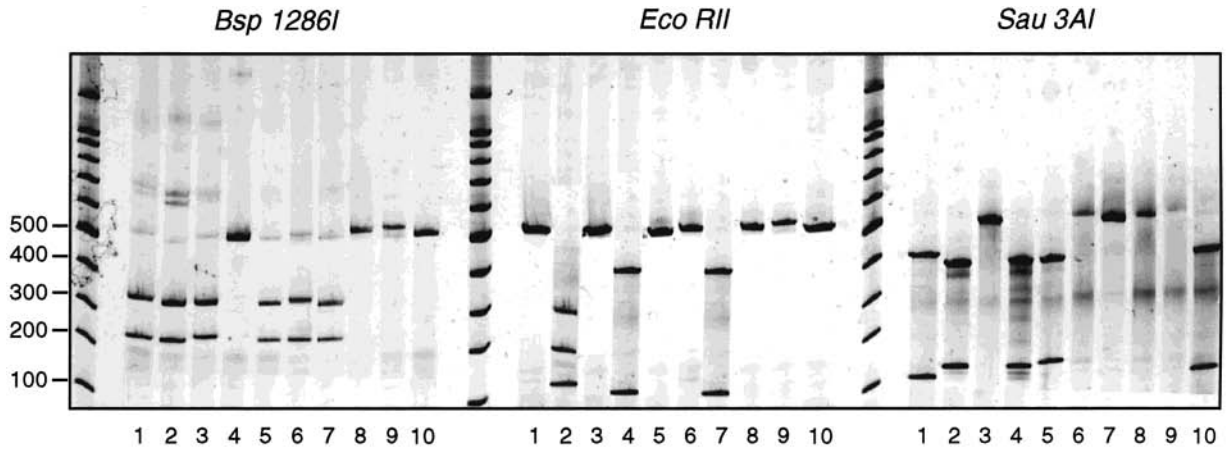

Figure 2. RFLP patterns of 10 salmon species following digestion with restriction enzymes *Bsp*1286I, *Eco*RII, and *Sau*3AI. Preparation of RFLP digests and rehydration, running, and staining of CleanGel 10% 48S were performed exactly as described in Materials and Methods. Lanes 1−10 refer to authentic salmon samples 1−10 detailed in Table 1, digested with *Bsp*1286I, *Eco*RII, and *Sau*3AI, respectively, with 100 bp DNA ladder lanes included as reference.

Table 3. Actual Size Fragments Generated from RFLP Analysis after Digestion with Six Enzymes

	enzyme cuts (base pairs)					
salmon species	*Dde*I	*Nla*III	*Hae*III	*Bsp*1286I	*Eco*RII	*Sau*3AI
1. *S. salar*	350, 130	U*[a]	350, 130	300, 200	U	410, 110
2. *O. keta*	360, 130	300, 210	U	300, 200	270, 180, 100	390, 120
3. *O. kisutch*	300, 130, 60	260, 220	350, 130	300, 200	U	U
4. *O. gorbuscha*	360, 130	210, 190, 100	U	U	400, 120	390, 120
5. *O. nerka*	360, 130	310, 180	350, 130	300, 200	U	390, 120
6. *O. tschawytscha*	300, 220	U	350, 130	300, 200	U	U
7. *O. mykiss*	360, 130	210, 190, 100	350, 130	300, 200	400, 120	U
8. *S. alpinus*	350, 130	U	350, 130	U	U	U
9. *S. fontinalis*	360, 130	U	350, 130	U	U	U
10. *S. trutta*	350, 130	U	U	U	U	410, 110

[a] U* denotes that the PCR product remained unaffected by the restriction enzyme.

the work described here does not take into account the possibility of the introduction of intraspecies variation in food products, which could be detected if fish from different geographic locations are used (Avise and Lansman, 1983; Rehbein et al., 1999). Nevertheless, this variation could easily be addressed by analyzing authentic species from different geographic locations.

Although the lack of intraspecies variation analysis in this study could potentially be considered a drawback, the presence of genetic differences due to geographic location is a factor which affects all techniques relying on DNA analysis, and this must be acknowledged when using such methodologies. However, in a recent study of over 70 commercial products, no intraspecies variation was identified (Hold et al., in preparation).

In summary, this study set out to devise a DNA-based method to allow the identification of any salmon species which may be present in food products, with the aim of applying the method to commercial products. The criteria for such analysis include cost effectiveness and rapid, relatively simple methodology which can be applied to all types of processed products. The work indicates that the method is suited to analysis of processed products, with results obtainable within 2

days. In addressing the issue of cost effectiveness, it must be remembered that the cost of all DNA-based work is significant, especially if enzymes are required, and this was one of the main criteria used in selection of suitable restriction enzymes.

For products which cannot be identified using the RFLP-based method, the option to incorporate the more costly sequence and phylogenetic analysis is still available if required.

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