Determination of PCR-RFLP Profiles for Fish Species Using the Agilent 2100 Bioanalyzer

Application

Food Safety

Authors

Steve Garrett and John Dooley
Molecular Biology Group, Dept. of Chemistry and Biochemistry
Campden & Chorleywoood Food Research Association, Chipping Campden
Gloucestershire, GL55 6LD
UK

Abstract

This application note shows how the Agilent 2100 Bioanalyzer was used in polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) fragment analysis for fish species identification. A 464bp cytochrome-b target sequence, found in all vertebrate fish, was first amplified and then digested with restriction enzymes. The fragments were resolved on the DNA 500 LabChip®, allowing simple comparison with authentic species profiles due to the accuracy of fragment size determination. Use of the Bioanalyzer offered significant benefits over traditional gel electrophoresis and DNA staining techniques for PCR-RFLP fragment analysis.

Introduction

The diversity of fish products available to consumers has increased significantly in recent years. Products can range from premium grade fish steaks to low cost fish fingers. As fish are caught, processed, and distributed by a global network of operators, there is a need to ensure the authenticity and the origin of fish used in the products.

There is, therefore, a need to have reliable and simple species identification methods to support enforcement and compliance with labelling legislation (EC Council Regulation No. 104/2000 and EC Commission Regulation No. 2065/2001).

Methods of fish species identification based on morphological characteristics are suited to whole fish; however, fish species identification becomes more problematic once it is processed. Protein profiling is used for fish identification; however, this method requires the analysis of species reference materials alongside the samples and is less reliable when applied to processed food products as the proteins become denatured. DNA based methods offer an alternative approach to species identification as DNA remains detectable in all but the most heavily processed samples.

Direct sequencing is the most definitive method of identification; however, it cannot easily be applied to samples suspected or known to contain more than one species. Alternative techniques, using polymerase chain reaction (PCR), were developed to identify fish species based on DNA fingerprint patterns. Methods used include RAPDs (random amplified polymorphic DNA), SSCP (single strand conformation polymorphism) and PCR-RFLPs.

A PCR-RFLP technique, which involved digesting an amplified 464bp region of the cytochrome b gene with restriction enzymes to generate DNA profiles, was previously developed for the identification of salmon species [1, 2].

The aim of this work was to improve the method for identification of salmon and other species by replacing conventional gel electrophoresis and staining steps with the Agilent 2100 Bioanalyzer. The generation of species-specific PCR-RFLP profiles on the 2100 Bioanalyzer combined with accurate sizing and quantification of individual DNA fragments, offered significant advantage over gel-based approaches in terms of ease-of-use, speed, and accuracy of identification.

Materials and Methods

All chemicals used for this work, unless otherwise stated, were supplied by Sigma-Aldrich and were of molecular biology grade or equivalent. PCR primers were supplied by MWG-Biotech UK Ltd. PCR-RFLP profiles were generated using a DNA500 LabChip and the Agilent 2100 Bioanalyzer. AmpliTaq® Gold DNA polymerase from Applied Biosystems was used in all PCR reactions. Restriction enzymes were obtained from New England Biolabs and used per the manufacturer's instructions.

Fish Samples

Commercially important salmon and white fish species samples were obtained from appropriate fishery research laboratories in the UK, Canada, Alaska, New Zealand, and Japan. Five individuals were used to minimize the effects of polymorphic variation within the population. Additional samples of each fish species were obtained from local UK fishmongers and retailers.

DNA Extraction

DNA extraction was performed using a modification of the CTAB method. Samples (2 g wet weight) were suspended in 5 mL of CTAB buffer (2% CTAB [hexadecyltrimethylammonium bromide], 100-mM Tris-HCl, 20-mM EDTA, 1.4-M NaCl, pH 8.0) and 40 µL of Proteinase K solution (20 mg/mL) was added. Samples were mixed thoroughly and then incubated overnight at 60 °C. After incubation, 1 mL of supernatant was transferred to a 2.0-mL Eppendorf tube, cooled to room temperature (RT), and centrifuged at 13,000g for 10 minutes. The clear supernatant was recovered and an equal volume of chloroform was added. The solution was vortexed and then centrifuged at 16,000g for 15 minutes before the upper aqueous layer was transferred to a clean 1.5-mL Eppendorf tube. An equal volume of isopropanol was added and the DNA precipitated at RT for 30 minutes. DNA was pelleted by centrifugation at 16,000g for 15 minutes, washed in 70% ethanol and air dried for 30 minutes at RT. The DNA pellet was resuspended in 100 µL of sterile distilled water (SDW) and purified using Promega's Wizard® Purification Resin per the manufacturer's protocol. DNA extracts were recovered in 50 µL of 1×TE (10-mM Tris-HCl, pH 7.4, 1-mM EDTA, pH 8.0) buffer. Final DNA concentrations (ng/µL) were determined using a GeneQuant pro DNA calculator (Pharmacia).

DNA Amplification

PCR products (464bp target from the cytochrome b gene) were produced by amplification of DNA extracts (50 ng) in 20-µL reactions containing 1× AmpliTaq Gold PCR buffer (Applied Biosystems), 300 nM of each primer (L14735: 5'-AAA AAC CAC CGT TGT TAT TCA ACT A-3' and H15149: 5'-GCI CCT CAR AAT GAY ATT TGT CCT CA-3'), 200-nM dNTPs, 5-mM $MgCl_2$ and 0.05-U/µL of AmpliTaq Gold (Applied Biosystems). Amplification profiles (94 °C for 5 minutes [denaturation]; 40 cycles of: 94 °C for 40 s, 50 °C for 80 s, 72 °C for 80 s [amplification]; 72 °C for 7 minutes [final extension] were applied using a PE9600 PCR machine (Applied Biosystems). Unpurified PCR products (1 µL) were applied to the 2100 Bioanalyzer to confirm amplification.

PCR-RFLP Profiling

Unpurified PCR product (2.5 µL) was digested for 3 or more hours with two to five units of enzyme in a total volume of 5 µL. Reactions were terminated by incubation at 65 °C for 10 minutes. Digested PCR products (5 µL) were mixed with 5 µL of 20-mM EDTA, to achieve a final concentration of 10-mM EDTA, prior to loading on to DNA500 LabChips. Aliquots (1 µL) of the reaction mix were analyzed on the 2100 Bioanalyzer, per manufacturers' instructions.

Results

Evaluation of PCR-RFLP Profiles Generated on the 2100 Bioanalyzer for Species Identification

An initial evaluation of the PCR-RFLP method was performed using salmonid species.

Following cleavage of the amplified DNA fragment with restriction enzymes, species-specific PCR-RFLP patterns were resolved on the 2100 Bioanalyzer. An example of a PCR-RFLP pattern is given in Figure 1, which shows PCR-RFLP profiles for salmon and trout samples generated with enzymes DdeI and HaeIII.

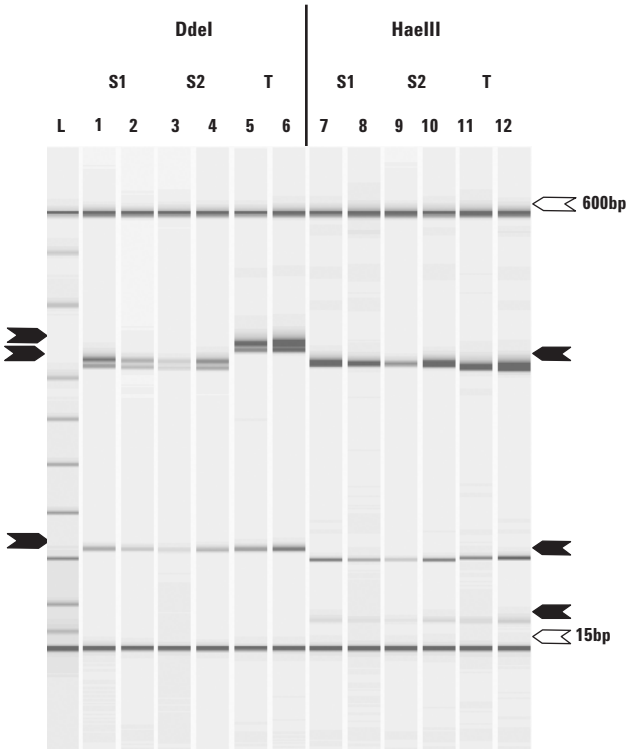

Figure 1. PCR-RFLP patterns obtained from salmon and trout with enzymes DdeI and HaeIII. PCR-RFLP patterns obtained when amplified DNA from two salmon (S1, S2) and one trout (T) samples were digested with DdeI (lanes 1–6) or HaeIII (lanes 7–12). A 15bp–600bp ladder (L) is shown. All wells contain 15bp and 600bp size markers. DNA fragments of note are indicated (arrows).

Observed and expected fragment sizes for a selection of five enzymes and four salmonids are shown in Table 1. As can be seen, patterns were similar to those reported previously [1, 2].

Expected DNA fragments of greater than about 25bp were readily detected; however, some smaller fragments were not detected because they could not be distinguished from the lower 15bp size marker or were outside the detection range (25bp–500bp) of the LabChip. Small DNA fragments (25bp–100bp), which were not reported previously, were observed in some digests [2]. This highlights the improved band resolution of this method in comparison to gel electrophoretic methods. This improved resolution is also highlighted by profiles generated with DdeI, where all four species have an extra fragment that is about 9bp smaller than the expected larger fragment. This is due to an extra DdeI site situated in primer H15149

Table 1 shows that *O. gorbuscha* and *O. mykiss* profiles generated with NlaIII have only two fragments when three are expected. This is believed to be due to the comigration of the two larger fragments. An analysis of the sequence of the *O. mykiss* 464bp amplicon indicated that cleavage of the amplicon with NlaIII should produce fragments of 192bp, 180bp and 91bp. These respectively equate to the 210bp, 190bp and 100bp fragments reported by Russell *et al.* (2000) and appear in Table 1. From the sequence data there is

Table 1. Expected and Observed PCR-RFLP Fragment Sizes Obtained with Five Restriction Enzymes and Four Salmonid Species

		Expected* (E) and observed (O) fragment sizes for each enzyme (bp)				
Species		**DdeI****	**Bsp1286I**	**HaeIII**	**NlaIII**	**Sau3AI**
O. nerka						
(Red salmon)	E	360, 130	300, 200	350, 130	310, 180	390, 120
	O	353, 346, 114	289, 172	320, 102, 35 or 421	272, 160	340, 115
O. gorbuscha						
(Pink salmon)	E	360, 130	U/C***	U/C	210, 190, 100	390, 120
	O	349, 343, 112	464	421	181, 92	338, 115
S. salar						
(Atlantic salmon)	E	350, 130	300, 200	350, 130	U/C	410, 110
	O	321, 312, 110	287, 172	318, 98, 35	438	370, 88
O. mykiss						
(Rainbow trout)	E	360, 130	300, 200	350, 130	210, 190, 100	U/C
	O	348, 339, 111	279, 174	313, 100, 33	185, 92	451

*Sizes as reported by Russell *et al.* (2000).

**Extra fragments in observed DdeI profiles are due to restriction site introduced by primer H15149.

***U/C Uncut with enzyme.

no evidence that the smaller 180bp fragment contains a higher proportion of heavier A or G bases or the larger 192bp fragment a higher proportion of lighter C or T bases, which could cause their respective molecular weights to converge. The calculated molecular weight difference (3277 Daltons) between the two fragments is equivalent to the difference in the number of bases. This makes it unlikely that comigration is due to molecular weight similarities between the two fragments. The comigration of these two fragments as a single fragment is consistently observed and does not detract from the identification of these species.

Overall, the profiles generated by the 2100 Bioanalyzer matched those expected or previously reported [1, 2], which supports the use of this approach for the identification of fish species. Further work was performed using Atlantic salmon and trout and a smaller number of enzymes to confirm the application of this approach.

Experimental Repeatability

In order to determine the experimental repeatability (LabChip-to-LabChip variability) of the complete assay, duplicate PCRs were produced from two salmon and one trout sample. Amplified fragments were cleaved with DdeI and digests stored at 4 °C until required. PCR-RFLP patterns were separated on four occasions using different DNA500 LabChips primed with two different batches of gel matrix, A and B. Two LabChips were run using a freshly prepared gel matrix (matrix A) while a third LabChip was run when gel matrix A was 1-week old. The fourth LabChip was run on the same date as the third LabChip but using a second, fresh batch of gel matrix (matrix B). Overall variation (encompassing variation due to LabChip-to-LabChip, PCR and gel matrix) appears in Table 2, which shows the results of analysis with the four LabChips following digestion with enzyme DdeI. Results are the mean fragment sizes observed on each LabChip from two PCR replicates of each species. Absolute fragment size variations within a single LabChip, that is, for PCR replicates of the same sample or between the two salmon samples, were less than 2bp and were only observed between the larger (>300bp) fragments. The overall absolute size variation for each fragment, which included variation due to different LabChips, PCRs and gel matrices, was slightly greater; the biggest variation was 6bp for the 320bp fragment in salmon (321bp to 327bp).

Fish Species Identification

Using sequence data generated from 10 different white fish species, theoretical PCR-RFLP profiles were generated from a range of restriction enzymes. Closer examination of these theoretical profiles indicated that only three enzymes would be needed to differentiate all the white fish species. Experimental profiles were generated to confirm that species identification was possible using just these three enzymes. Results of this analysis are shown in Figure 2, which shows that 9 of the 10 species could be identified based on profiles generated with only one enzyme. The remaining two species were differentiated using the other two enzymes.

Table 2. PCR-RFLP Fragment Sizes Obtained Following Separation of DNA Cleaved with DdeI on Four Different DNA500 LabChips

	Observed fragment sizes on each LabChip (bp)					
Expected band size (bp)	LabChip 1 (Fresh matrix A)	LabChip 2 (Fresh matrix A)	LabChip 3 (Week old matrix A)	LabChip 4 (Fresh matrix B)	Mean	%RSD
Salmon analysis*						
117	111	111	110	111	111	0.34
312	314	316	314	317	315	0.43
320	323	325	323	326	324	0.51
Trout analysis						
116	111	111	110	111	111	0.45
339	338	341	338	343	340	0.72
348	347	349	347	352	349	0.61

*An expected 27bp fragment from salmon is not detected by the 2100 Bioanalyzer.

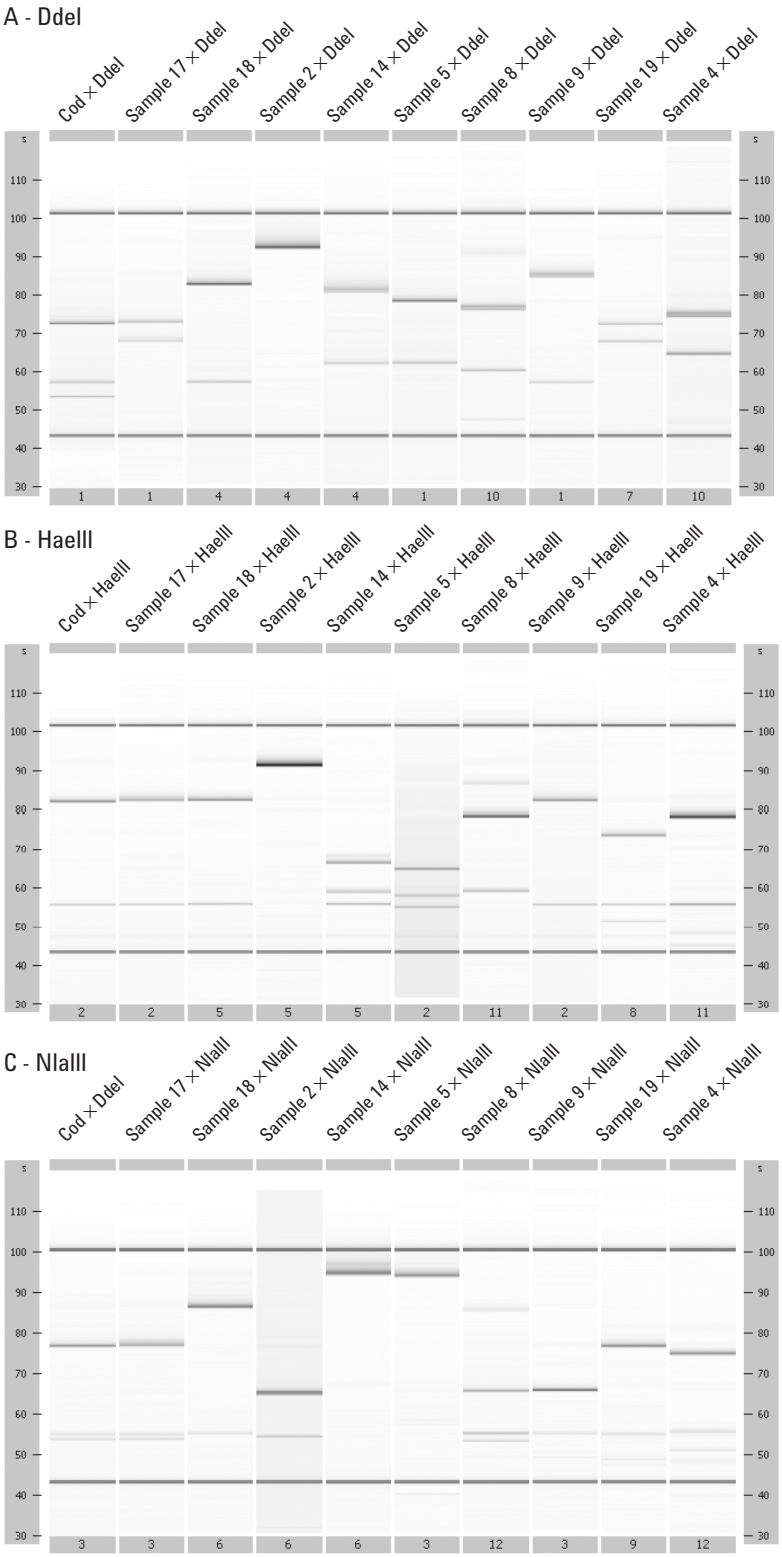

Figure 2. PCR-RFLP profiles from the 10 white fish species used in this study. Profiles were generated using enzymes DdeI (A), HaeIII (B) or NlaIII (C). Each sample number indicates a different fish species.

Analysis of a further nine salmon species was performed using these three enzymes only (data not shown). Again it was found that the salmonids could be differentiated using the three enzymes. A list of all the species studied is found in Table 3.

Table 3. Fish Species that Could be Differentiated Using PCR-RFLP with Enzymes Dde I, Hae III and NlaIII

Common name (UK)	Latin name
Atlantic cod	*Gadus morhua*
Pacific cod	*Gadus macrocephalus*
Coley (Saithe)	*Pollachius virens*
Haddock	*Melanogrammus aeglefinus*
European hake	*Merluccius merluccius*
South African hake	*Merluccius paradoxus*
European plaice	*Pleuronectes platessa*
Whiting	*Merlangus merlangus*
Alaskan(Walleye) Pollock	*Theragra chalcogramma*
Hoki	*Macruronus novaezelandiae*
Atlantic salmon	*Salmo salar*
Red / Sockeye salmon	*Oncorhynchus nerka*
Pink / Humpback salmon	*Oncorhynchus gorbuscha*
Chinook salmon	*Oncorhynchus tschawytscha*
Coho / Silver salmon	*Oncorhynchus kisutch*
Keta / Chum salmon	*Oncorhynchus keta*
Cut-throat trout	*Oncorhynchus clarki clarki*
Dolly Varden	*Salvelinus malma malma*
Cherry salmon	*Oncorhynchus masou masou*

Discussion

To identify species present in a sample when no prior knowledge of the sample is available requires a universally applicable method. PCR-RFLP profiling of a common region of the vertebrate cytochrome b gene, which is present in all fish species, enabling comparison with profiles in a database is one such universal approach.

The conventional PCR-RFLP fragment analysis involves gel electrophoresis, on large (over 30 cm^2), thin (<2 mm) acrylamide gels, to resolve the PCR-RFLP patterns. This makes handling and staining difficult and requires the use of large equipment and volumes of solution. All this makes these methods potentially hazardous and time consuming and can sometimes produce variable results. This type of detection is, therefore, not suited to use in enforcement and quality control laboratories where a rapid, robust detection method is required.

As an alternative, the 2100 Bioanalyzer incorporates conventional capillary electrophoresis (CE) technology into an easy-to-use chip-based format, which enables accurate sizing and quantification of individual DNA fragments. Coupled with the small (2 cm^2) size of the LabChip, this gives the system a significant advantage over conventional gel-based approaches in terms of ease-of-use, speed, and safety. This makes the 2100 Bioanalyzer ideally suited to the analysis of multiple small DNA fragments such as those found in PCR-RFLP profiles.

Using the 2100 Bioanalyzer it was possible to generate PCR-RFLP profiles that resembled those previously published for salmon species. However, generating PCR-RFLPs on the 2100 Bioanalyzer produced profiles with improved fragment resolution and detection, especially of smaller fragments that were not detected using conventional gel electrophoresis. PCR-RFLP profiles generated on the 2100 Bioanalyzer were also more consistently produced compared to gel electrophoresis.

Consequently, profiles based on three enzymes DdeI, NlaIII and HaeIII were developed for 19 commercially important species. Further studies should increase the range of species in the database, making it an important tool for the study of the authenticity of fish products.

Complete details of the development of the assays, application to fish species identification in products, and an interlaboratory study were recently published [3, 4, 5].

Acknowledgements

This work formed part of the UK's Food Standards Agency project Q01069 and the authors wish to acknowledge the FSA for their financial support.

References

1. G. L. Hold, V. J. Russell, S. E. Pryde, H. Rehbein, J. Quinteiro, M. Rey-Mendez, *et al.*, (2001), Validation of a PCR-RFLP based method for the identification of salmon species in food products. *European Food Research and Technology,* **212**, 385–389.
2. V. J. Russell, G. L. Hold, S. E. Pryde, H. Rehbein, J. Quinteiro, M. Rey-Mendez, *et al.*, (2000). Use of restriction fragment length polymorphism to distinguish between salmon species. *Journal of Agricultural and Food Chemistry*, **48** (6), 2184–2188.
3. J. J. Dooley, H. D. Sage, H. M. Brown, and S. D. Garrett, Improved fish species identification by use of lab-on-a-chip technology. (2005) *Food Control* **16** (7), 601–607.
4. J. J. Dooley, M. A. Clark, H. D. Sage, H. M. Brown, and S. D. Garrett, (2005). Fish species identification using PCR-RFLP analysis and lab-on-a-chip capillary electrophoresis: application to detect white fish species in food products and an Inter-laboratory study. *Journal of Agricultural and Food Chemistry*, **53** (**9**), 3348–3357.
5. J. J. Dooley, M. A. Clark, H. D. Sage, H. M. Brown, and S. D. Garrett, (2004) Application of a chip-based capillary electrophoresis system to enable simple PCR-RFLP identification of fish species. FSA Final Report, Q01069

For More Information

For more information on our products and services, visit our Web site at www.agilent.com/chem.

www.agilent.com/chem

Printed in the USA
July 29, 2005
5989-2982EN

Agilent Technologies