

Final Technical Report for Food Standards Agency Project Q01069

**Application of a Chip-based Capillary Electrophoresis System to
Enable Simple PCR-RFLP identification of Fish Species**

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Executive Summary

The diversity of fresh, frozen and fish-based products available to the consumer 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. This is especially true in the European Union (EU) where stringent fish catch quotas have been introduced in an attempt to limit the decline of native fish stocks. There is, therefore, a need to have reliable species identification methods to support enforcement and compliance with labelling legislation (EC Council Regulation No. 104/2000 and EC Commission Regulation No. 2065/2001).

Fish identification can be achieved using morphological characteristics or protein profiles; however, this becomes more difficult once the fish has been processed. DNA based methods, especially PCR-RFLP profiling, have become the most appropriate approach to species identification. PCR-RFLPs require the use of acrylamide gel electrophoresis and staining for fragment visualisation, which means the approach is unlikely to be adopted by many routine quality assurance or enforcement laboratories, who require methods that are rapid, simple, cost effective and accurate. A recent innovation that combines fragment separation and detection in an easy-to-use chip-based format is likely to make the PCR-RFLP method more appealing to analytical laboratories.

The aims of this study were to improve an existing PCR-RFLP approach to salmon species identification developed by Russell *et al.* (2000) by simplifying the final fingerprint detection stage. The objective of the new method was to replace the conventional gel-electrophoretic steps for fragment separation, detection and analysis, by employing the Agilent 2100 Bioanalyser to analyse the PCR-RFLP fingerprints. Additional aims of the study were to expand the new method to include a wider range of white fish species which could be readily detected using this approach and to complete a ring-trial of the method with the assistance of routine

analytical laboratories, including public analysts, to assess the transferability of the method.

Analysis of salmon and trout species was performed following the protocol described by Russell *et al.* (2000) but the analysis of fragments by gel electrophoresis was replaced by analysis on the 2100 Bioanalyser. Using this approach it was possible to identify all species tested by comparing fragment sizes obtained from the 2100 Bioanalyser with those reported and expected (based on analysis of sequence data). This analysis showed that fragment detection on the 2100 Bioanalyser was accurate and sensitive, allowing detection of small fragments that were not observed using the conventional gel-based method, but which were expected from sequence data. The power of this capillary electrophoretic approach also resolved a doublet (two fragments of about 6bp size difference) in some species, which was not reported by Russell *et al.* (2000). This doublet was caused by the introduction of a DdeI restriction site into PCR products, by primer H15149, during DNA amplification. This was consistently resolved and did not affect species identification. Additionally it was noted that two fragments generated by restriction digestion with enzyme NlaIII and expected to be around 180 and 195bp were co-migrating as a single fragment of around 185bp. Again this observation was made consistently and did not affect the identification of these species.

Experimental repeatability, for the determination of fragment sizes of replicate analyses of the same sample carried out on different occasions, was examined using salmon and trout samples. The overall variation was found to be less than 3%, which permitted species identification without the need to run reference materials with every sample. This suggested that this method was applicable to the detection of any fish species so long as profile data was generated using authentic materials.

To expand the method to include additional white fish species, authentic samples from five individuals of ten species were obtained. Sequence data from all five individuals was generated and used to determine expected PCR-RFLP profiles with a number of enzymes. Using the expected profiles three restriction enzymes, DdeI, HaeIII and NlaIII, were identified as potentially able to differentiate the ten species. This was confirmed experimentally. Results showed that all but two of the ten

species could be differentiated using Ddel alone and that use of the additional enzymes allowed these two species to be distinguished.

Admixtures of DNA or fresh or freeze-dried fish materials were analysed to determine a limit of detection (LOD) for the method. A LOD of between 2 and 5% was determined, depending on the species present in the admixtures. The method was also used to confirm the authenticity of commercial products (fish fingers and fishcakes) prepared from white fish materials before being tried in different laboratories.

The method was assessed by five UK-based analytical laboratories, including public analysts. None of the labs had experience of PCR-RFLP profiling for fish identification and only one had experience of using the 2100 Bioanalyser. After a training event held at CCFRA, participants were provided with 19 unknown samples (both single species and admixtures) and the necessary equipment and reagents to perform the analysis. The results obtained were generally very good with one laboratory identifying all species correctly in the 19 samples. Two laboratories failed to identify Pacific cod in an admixture containing 10% Pacific cod and 90% coley but correctly identified all the other samples. One laboratory also failed to identify haddock in an admixture containing 2% haddock in cod. However, one laboratory failed to identify six samples correctly due to problems associated with DNA contamination and profile generation on the 2100 Bioanalyser.

Overall observations showed that generation of PCR-RFLP profiles on the 2100 Bioanalyser was rapid and easy to perform. Generation of profiles, from post-restriction digestion material, for 12 samples was achieved within 40 minutes. Additionally the small volume (1 μ L) required for this analysis allowed significant savings in the reaction volumes for PCR (20%) and restriction digestion (50%) to be made. This in turn resulted in significant cost savings due to the reduced amount of expensive enzymes required for these smaller reaction volumes. These savings, along with the relatively low cost and ease of use of the instrument, should make this method suitable for use in a wide range of analytical laboratories involved with species identification.

Contents

1	INTRODUCTION	11
1.1	The need for species identification and detection methods	11
1.2	Protein-based methods of identification	11
1.3	DNA-based methods of detection	12
1.3.1	General approaches for species identification	12
1.3.2	PCR-RFLP approaches to fish species identification	13
1.3.3	Methods of fragment detection	14
1.4	Aims of the project	15
2.	EXPERIMENTAL PROCEDURES	16
2.1	Procurement and preparation of fish samples	16
2.1.1	Authentic fish samples	16
2.1.2	Additional fish samples	17
	Table 1a: Details of authentic fish samples used in this study.	19
	Table 1b: Details of admixture samples used in this study.	20
	Table 1c: Details of commercial products used in this study.	21
2.2	Extraction of DNA from fish materials	21
2.2.1	CTAB DNA extraction method	22
2.2.2	Tepnel DNA extraction method	22
2.3	Preparation of DNA admixtures	23
2.4	Methods of DNA analysis	23
2.4.1	DNA amplification	23
	Table 2 : Details of PCR primers used in this study.	24
2.4.2	Restriction digestion	24
2.4.3	PCR-RFLP profiling	24
2.5	Assay Development	25
2.5.1	DNA sequencing	25
2.5.2	Sequence analysis	25
3.	RESULTS AND DISCUSSION	26
3.1	Collection of authentic fish materials	26
3.2	Evaluation of PCR-RFLP profiles generated on the 2100 Bioanalyser for species identification	27
3.2.1	Analysis of PCR amplified products	27

Table 3 : Amplified DNA fragment size variations observed following separation of DNA on different DNA500 LabChips.	28
3.2.2 Analysis of PCR-RFLP profiles	28
Table 4: Expected and observed PCR-RFLP fragment sizes obtained with five restriction enzymes and four salmonid species.	30
3.2.3 Salmon and trout profiles	31
Table 5 : PCR-RFLP fragment sizes obtained following separation of DNA cleaved with Ddel on four different DNA500 LabChips.	33
3.2.4 Application of method to other salmonid species	35
3.3 Expansion of the method to include species other than salmonids	36
3.3.1 Production and analysis of sequence data	36
Table 6: Average PCR-RFLP fragment sizes for salmonid species.	37
3.3.2 Generation of PCR-RFLP profiles	41
3.3.3 Method optimisation	41
3.3.4 Expected and observed PCR-RFLP profiles	42
Table 7: Expected and observed PCR-RFLP fragment sizes generated with the three enzymes Ddel, HaeIII and NlaIII from the ten white fish species.	45
3.4 Validation of the PCR-RFLP method	46
3.4.1 DNA admixtures	46
3.4.2 Fish-meat admixtures	46
3.4.3 Freeze-dried admixtures	47
3.4.4 White fish-based products	48
Table 8: Results obtained following analysis of commercial products using the PCR-RFLP method.	49
3.5 Analysis of method application by ring-trial	51
3.5.1 Participants and samples used in the ring-trial	51
Table 9: Summary of results obtained from analysis of fish samples by participants in ring-trial.	52
3.5.2 Results from the ring-trial	54
3.5.3 Results from laboratory 3	57
3.5.4 Comments about the method	59
3.5.5 Summary of ring-trial results	60
4. CONCLUSIONS	61
5. ACKNOWLEDGEMENTS	63
6. REFERENCES	65
7. FIGURES	69
Figure 1: PCR-RFLP patterns obtained from salmon and trout with enzymes Ddel and HaeIII.	69
Figure 2: Sequence of the 464bp amplified fragment (L strand) from salmon and trout.	70
Figure 3: PCR-RFLP patterns generated from salmon and trout admixtures with enzyme Ddel.	71

Figure 4: PCR-RFLP patterns generated from salmon and trout admixtures with enzyme Sau3AI.	72
Figure 5: Alignment of cytochrome b gene sequence data generated from authentic fish samples used in this study.	73
Figure 6: PCR-RFLP profiles from the ten white fish species used in this study.	77
Figure 7: PCR-RFLP profiles generated from white fish DNA admixtures.	78
Figure 8: PCR-RFLP profiles generated from DNA admixtures of Atlantic cod and haddock.	79
Figure 9: PCR-RFLP profiles generated from fish-meat admixtures of Atlantic cod and haddock.	80
Figure 10: PCR-RFLP profiles generated from freeze-dried admixtures of Atlantic cod and haddock.	81
8. APPENDICES	82
8.1 Letters of authenticity supplied with authentic fish samples used in this work	82
8.2 PCR-RFLP fragments obtained following analysis of admixtures prepared from fish-meat.	91
8.3 Protocol and recording sheets provided for participants in ring-trial	94
8.4 Individual results returned by participants in ring-trial following analysis of 19 freeze-dried fish samples	113

Glossary

2100 Bioanalyser : A small-scale capillary electrophoretic system using lab-on-a-chip technology and microfluidics for the specific separation of DNA or protein fragments.

ATP : Adenosine triphosphate.

Capillary electrophoresis (CE) : A method of fragment separation using small diameter capillary tubes. Fragments are separated based on size and charge and are detected using a variety of methods, in this case laser-induced fluorescence (LIF).

Copy number : The number of copies of a gene per cell. Some genes are multi-copy, i.e. there is more than one copy on the genome. Mitochondrial and chloroplast genes are multi-copy by virtue of the fact that cells contain more than one of these organelles. Some chloroplast genes, including the rRNA5S gene, are also multi-copy within the chloroplast genome.

Cytochrome b (cyt b) : A mitochondrial gene widely used for species identification and population studies.

DNA : Deoxyribonucleic acid. This molecule comprises strings of the four bases (G, A, T, C) forming genes.

DNA500 LabChip : see LabChip.

dNTP : Deoxynucleotide-triphosphate. An abbreviation for any of the four bases (see specific bases) forming DNA.

Fluorescent Units (FU) : A measure of fragment intensity used by the 2100 Bioanalyser. The default fragment detection limit is 4 FUs.

Gel electrophoresis : A method used to separate proteins or DNA fragments on acrylamide or agarose gel matrices. Fragments migrate on the basis of size and charge when an electric current is applied. The gel matrix acts as a sieve to separate the fragments based on size.

Gene : An ordered series of bases which code for a specific protein.

Genome : The total DNA content of an individual or organelle.

LabChip : Small (3cm²), disposable, single-use plastic and glass units containing etched capillaries attached directly to twelve sample loading wells. DNA fragments up to 500bp can be separated on the chip by the 2100 Bioanalyser.

Mitochondria/mitochondrion : Intercellular bodies found in most cells. Each mitochondrion contains its own genome.

PCR : Polymerase Chain Reaction – a method of amplifying a specific gene or region of DNA to produce millions of copies.

PCR-RFLP : A combination of PCR and RFLP analysis used to generate simplified fingerprints useful for the identification of organisms at the species level or above.

Primer : A short oligonucleotide designed to anneal to specific regions of DNA in order to facilitate the PCR. Primers are designed to complement regions of DNA bounding the gene of interest.

Restriction enzyme : Natural bacterial enzymes used to cleave DNA molecules at specific positions (recognition sites), e.g. EcoRI site is GAATTC. These enzymes are widely used by molecular biologists to generate RFLP fingerprints.

Restriction Fragment Length Polymorphisms (RFLP) : A type of DNA fingerprint generated by cleaving larger DNA fragments into a series of smaller fragments. Generally fragments are resolved by gel electrophoresis. This method is useful for the identification of organisms at the individual level or above.

RNA : Ribonucleic acid. A single stranded structure which forms the link between expression of DNA and the formation of proteins.

SDW : Sterile distilled water of molecular biology grade.

Taq polymerase : A specific, heat-stable DNA polymerase used to replicate DNA targets during PCR.

1 Introduction

1.1 The need for species identification and detection methods

The diversity of fresh, frozen and fish-based products available to the consumer has increased significantly in recent years. Products can range from premium grade fish steaks to low cost minced fish products such as fish fingers. As fish are caught, processed and distributed via a global network of companies there is a need to ensure the authenticity and origin of fish used in the products. This is especially true in the European Union (EU) where stringent fish catch quotas have been introduced in an attempt to limit the decline of native white fish stocks. There is, therefore, a need to have reliable species identification methods to support enforcement of labelling legislation (EC Council Regulation No. 104/2000 and EC Commission Regulation No. 2065/2001) and for companies to ensure the quality and authenticity of their products.

Methods of fish species identification based on morphological characteristics are suited to whole or lightly processed fish; however, the identification of fish species becomes more difficult once it has been processed and the skin removed.

1.2 Protein-based methods of identification

Protein-based methods are routinely used for the identification of fish species in raw products such as fillets, as they are relatively quick and inexpensive. Identification is achieved by generating unique species-specific profiles (Colombo *et al.*, 2000; Tepedino *et al.*, 2001). Profiles are compared to profiles generated from suitable reference material to confirm species identity. These methods; however, have limited use on processed food products as the proteins become denatured. Also they cannot be reliably applied to products containing more than one fish species as profiles become too complex to interpret.

In terms of simplicity and speed, antibody based methods would be most appropriate. However, only a limited number of immunoassays have been developed and none are available for wide scale commercial use (Cespedes *et al.*, 1999a; Asensio *et al.*, 2003). Further immunoassays are being developed as part of FSA project Q01068.

Due to the lack immunoassay methods and the instability of proteins during processing, DNA based methods have become the favoured approach to species identification as DNA remains detectable in all but the most heavily processed samples.

1.3 DNA-based methods of detection

1.3.1 General approaches for species identification

Although direct DNA sequencing is the most definitive method of identification, it is a lengthy and expensive process which does not readily lend itself to routine analysis. It is also unsuited to samples containing more than one species. Alternative techniques, using the polymerase chain reaction (PCR), have been applied to variable regions of DNA such as the cytochrome b or 5S rDNA genes. Amplification of species-specific PCR products from the 5S rDNA gene has been used to differentiate sole and Greenland halibut species (Cespedes *et al.*, 1999b); however, its use is limited to these two species only. Other methods, which can be applied to a wider range of species, include SSCP (single strand conformation polymorphism) (Cespedes *et al.*, 1999c), PCR-RFLP (restriction fragment length polymorphism [Cespedes *et al.*, 1998; Russell *et al.*, 2000; Hold *et al.*, 2001; Sotelo *et al.*, 2001; Sanjuan & Comesana, 2002]) or RAPD (random amplified polymorphic DNA) fingerprinting (Partis & Wells, 1996; Bielawski & Pumo, 1997; Asensio *et al.*, 2002). SSCP patterns can be used in situations where the species are very closely related. They have been used successfully to generate species-specific patterns for sole, European plaice, flounder and Greenland halibut (Cespedes *et al.*, 1999c). Partis & Wells (1996) used RAPDs to confirm the authenticity of fish fillets reported to be species of dory, perch, oreo or barramundi. Although RAPDs are easy to produce

and require no prior knowledge of the DNA sequence of the species under investigation, the reproducibility of the technique is questionable. Jonas *et al.* (2000) report a low correlation (54%) between fingerprints from the same *Legionella* strain analysed on four separate occasions. They concluded that, in order to compare a further new sample, it would be necessary to repeat all samples. Inter-laboratory variation is also well recognised and as such RAPDs are not widely used.

1.3.2 PCR-RFLP approaches to fish species identification

A PCR-RFLP approach to species identification has been used widely to study a variety of species including fish (Cespedes *et al.*, 1998; Quinteiro *et al.*, 1998, 2001; Russell *et al.*, 2000; Hold *et al.*, 2001; Sotelo *et al.*, 2001; Sanjuan & Comesana, 2002; Jerome *et al.*, 2003). This technique involves amplifying a relatively short piece of DNA using PCR amplification. This amplified DNA is then divided into several aliquots before each aliquot is cleaved using restriction enzymes to produce a series of fingerprint patterns. The advantage of this approach is that the PCR step allows detection of low abundance species because target DNA is amplified to produce a large amount of test material. The PCR-RFLP patterns are also relatively simple and are far less complex than standard RFLP patterns. This makes it more feasible to identify a single species within an admixture.

The majority of studies use mitochondrial genes for identification purposes as the structure of this genome is both well documented and multi-copy. By far the most widely used gene target is the cytochrome b gene; however, other genes have also been used, including the mitochondrial DNA control region. Quinteiro *et al.* (2001) used a 156bp fragment of this control region to differentiate eleven hake species of the *Merluccius* genus by generating PCR-RFLP profiles with four different restriction enzymes.

Since the work of Kocher *et al.* (1989) the cytochrome b gene has been a favourite choice for population and speciation studies, including studies on fish species. A fish identification assay, based on PCR-RFLP profiles from a 464bp region of this gene, was used in a validation study by five EU laboratories for the identification of salmon species in food products (Hold *et al.*, 2001). In a further study the same method was

applied to hake, salmon, sardine, eel and flatfish species (G. Hold, personal communication). A similar method was used to differentiate 21 species of flatfish by variations in their cytochrome b gene (Sotelo *et al.*, 2001).

For analysis of canned products smaller amplicons are preferable. Quinteiro *et al.* (1998) developed a PCR-RFLP assay based on a small (126bp) fragment of the cytochrome b gene. This target was useful for the identification of canned tuna species. More recently Jerome *et al.* (2003) reported the use of PCR-RFLPs to differentiate the sardine species *Sardina pilchardus*, *S. aurita*, *S. sprattus* and *S. melasnoctictus* from a total of nine sardine type species. This differentiation was performed using a 142bp amplified fragment of the cytochrome b gene, which was specifically designed to be used with canned sardines.

1.3.3 Methods of fragment detection

1.3.3.1 Conventional gel electrophoresis

Although the DNA methods described above are useful for identification purposes, they are less reliable for samples containing a mixture of different species and are not able to quantify fish species present in a product. For the most part they use gel electrophoresis and staining for endpoint detection. Fragment resolution is best performed on acrylamide gels, which by necessity are large (over 30cm²) and thin (<2mm). 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.

1.3.3.2 Novel separation technologies

Other technologies offer more affordable ways of PCR–endpoint detection and are capable of multiple target analysis. The Agilent 2100 Bioanalyser uses advances in microfluidic-based lab-on-a-chip technology. This system incorporates conventional capillary electrophoresis (CE) technology (analyte separation and detection) into an easy-to-use chip-based (LabChip[®]) format. The LabChips used by the system are small (3cm²), disposable, single-use units containing etched capillaries attached

directly to sample loading wells. DNA fragments are separated by CE and detected using laser-induced fluorescence by the 2100 Bioanalyser. This enables accurate sizing and quantification of individual DNA fragments, which gives the system a significant advantage over conventional gel-based approaches in terms of ease of use, speed and safety. This makes the 2100 Bioanalyser ideally suited to analysis of multiple small DNA fragments. This technology is likely to be more accessible to a wider range of laboratories as the cost of the instrumentation and disposable chips are relatively low compared to that for real-time PCR analysis. The use of the 2100 Bioanalyser for food analysis, specifically semi-quantitative GM soya analysis (McDowell *et al.*, 2001), olive oil authenticity (Dooley *et al.*, 2003) and in the development of meat species assays (Dooley & Garrett, 2001), has been reported.

1.4 Aims of the project

The initial aim of this study was to improve an existing PCR-RFLP approach to salmon species identification developed by Russell *et al.* (2000) by simplifying the final fingerprint detection stage. The objective was to replace the gel-electrophoretic steps for fragment separation, detection and analysis, by employing the Agilent Bioanalyser to analyse PCR-RFLP fingerprints.

A second aim of the study was to expand the method to enable detection of a wider range of fish species associated with white fish products.

The final aim of the study was to complete a ring-trial of the method with the assistance of a selection of analytical laboratories, including public analysts, to assess the transferability of the method.

2. Experimental Procedures

All chemicals used for this work, unless otherwise stated, were supplied by Sigma-Aldrich (Poole, Dorset, UK) and were of molecular biology grade or equivalent. Primers for PCR were supplied by MWG-Biotech UK Ltd (Milton Keynes, UK) and were of high-pure salt-free (HPSF) grade. PCR amplification was performed using either a PE9600 or PE2400 PCR machine (Applied Biosystems, Warrington, Cheshire, UK). PCR-RFLP profiles were generated using the Agilent 2100 Bioanalyser (Agilent Technologies UK Ltd, Stockport, Cheshire, UK). All DNA sequencing was performed by Lark Technologies (Takeley, Essex, UK) using the BigDye Terminator protocol (Applied Biosystems).

2.1 Procurement and preparation of fish samples

2.1.1 Authentic fish samples

Authentic samples from up to five individuals of each fish species were obtained from various sources. Samples provided were either (i) whole frozen fish, (ii) portions of fish-meat either frozen or preserved in ethanol or (iii) dried dorsal fin clips. All samples were provided with letters of authenticity or known to be of authentic origin. Sample sizes (of frozen or preserved fish) were between 3g and 5g. All samples were stored at -20°C upon receipt. Details of samples and sources are shown in Table 1a. Samples (200mg) for DNA extraction were removed from each frozen fish sample using a fresh, sterile scalpel. The work area was cleaned with 80% ethanol between each sample, from a single species, and with hypochlorite solution (available Chlorine = 5000ppm) and 80% ethanol between each species. Fresh gloves were worn for each sample.

2.1.2 Additional fish samples

2.1.2.1 *Fish fillets*

Fish fillets were obtained from local fishmongers and authenticated using PCR-RFLP profiles. Samples were blended to produce an homogeneous mixture suitable for DNA extraction. Samples were stored, for a maximum of 2 days, at 4°C until processed or at -20°C for long-term storage.

2.1.2.2 *Fish admixtures (fish-meat)*

Fillets of species to be mixed were cut into cubes of roughly 2cm³ before they were blended in a food processor to produce an homogeneous stock of fish muscle (fish-meat) of each species. Admixtures were prepared by blending together small, equal amounts of each fish-meat stock for 30 seconds. An equal weight of additional fish-meat was added and the whole re-blended for 30 seconds. This process was repeated until the correct blend had been achieved. The final amount of fish-meat admixture was blended for a further 30 seconds. Details of admixtures prepared are shown in Table 1b. Fish-meat admixtures were aliquoted into plastic screw-top containers containing approximately 20g and frozen at -20°C until required. Samples (2g) for DNA extraction were removed from a single pot as required.

2.1.2.3 *Fish admixtures (freeze-dried)*

Samples of fish fillet from single fish species were cut into cubes (~2cm³). Cubes (1 or 2) were placed into a clean 50ml falcon tube. The tops of the tubes were sealed with parafilm and 3 small holes were punctured into this. Cubed fish material was freeze-dried using an Edwards Freeze Dryer Modulyo. Dried material was ground into a coarse powder using a glass rod. Portions of the powdered fish material were finely ground using a pestle and mortar cooled in liquid nitrogen before being mixed together to produce admixtures. Freeze-dried admixtures containing 2%, 5% or 10% of one species in another were produced. Details of admixtures are shown in Table 1b. Portions (200mg) of the freeze-dried admixtures, suitable for DNA extraction, were aliquoted into 2ml screw-top micro-tubes. All freeze-dried materials were stored dried at -20°C until required.

2.1.2.4 *Fish-based products*

Fish products, as detailed in Table 1c, were obtained from local retailers and stored at -20°C until required. Products were thawed overnight at 4°C prior to extraction. Where products (e.g. fish fingers, fishcakes etc) had coatings (e.g. breadcrumbs) these were removed as far as possible before the fish part was blended to produce an homogeneous sample. DNA was extracted from portions (2g) of the homogenised fish sample. Products composed of fish in a sauce were blended whole to ensure homogeneity prior to DNA extraction.

Table 1a: Details of authentic fish samples used in this study.

Common name	Latin name	Source
White Fish species		
Atlantic Cod	<i>Gadus morhua</i>	IFR, Norwich
Pacific Cod	<i>Gadus macrocephalus</i>	IFR, Norwich
Coley (Saithe)	<i>Pollachius virens</i>	IFR, Norwich
Haddock	<i>Melanogrammus aeglefinus</i>	IFR, Norwich
European Hake	<i>Merluccius merluccius</i>	IFR, Norwich
South African Hake	<i>Merluccius paradoxus</i>	IFR, Norwich
European Plaice	<i>Pleuronectes platessa</i>	IFR, Norwich
Whiting	<i>Merlangus merlangus</i>	IFR, Norwich
Alaskan (Walleye) Pollock	<i>Theragra chalcogramma</i>	AFSC, Alaska
Hoki	<i>Macruronus novaezelandiae</i>	NIWA, New Zealand
Salmonid species		
Atlantic Salmon	<i>Salmo salar</i>	IAS, Scotland
Red / Sockeye Salmon	<i>Oncorhynchus nerka</i>	FOC, Canada
Pink / Humpback Salmon	<i>Oncorhynchus gorbuscha</i>	FOC, Canada
Chinook Salmon	<i>Oncorhynchus tshawytscha</i>	FOC, Canada
Coho / Silver Salmon	<i>Oncorhynchus kisutch</i>	FOC, Canada
Keta / Chum Salmon	<i>Oncorhynchus keta</i>	FOC, Canada
Cut-throat Trout	<i>Oncorhynchus clarki clarki</i>	FOC, Canada
Dolly Varden	<i>Salvelinus malma malma</i>	FOC, Canada
Cherry Salmon	<i>Oncorhynchus masou masou</i>	NSRC, Japan

IFR : Institute of Food Research, Norwich. These samples originally came from The Centre for Environment Fisheries and Aquaculture Science (CEFAS), Lowestoft where they had been authenticated.

NIWA : National Institute of Water & Atmospheric Research, New Zealand.

FOC : Fisheries and Oceans Canada, Canada.

NSRC : National Salmon Resources Center, Japan.

IAS : Institute of Aquaculture, University of Stirling, Scotland.

Table 1b: Details of admixture samples used in this study.

Species 1	Species 2	Other components
DNA admixtures		
5% Coley	95% Atlantic Cod	
5% Hoki	95% Atlantic Cod	
5% Whiting	95% Haddock	
5% SA Hake	95% Haddock	
5% Coley	95% Pacific Cod	
5% Hoki	95% Haddock	
5% SA Hake	95% Atlantic Cod	
5% Whiting	95% Pacific Cod	
5% Hoki	95% Coley	
5% Whiting	95% Atlantic Cod	
5% A. Pollock	95% Atlantic Cod	
5% Hoki	95% Pacific Cod	
5% Coley	95% Pacific Cod	41% total soya
5% Whiting	95% Haddock	41% total soya
5% SA Hake	95% Atlantic Cod	41% total soya
10% Coley	90% Pacific Cod	41% total soya
Fish-meat		
1 – 25% Atlantic Cod	75 – 99% Haddock	
1 – 25% Haddock	75 – 99% Atlantic Cod	
Freeze-dried materials		
Single species samples of each of the 10 white fish species as shown above		
2 – 10% Atlantic Cod	90 – 98% Haddock	
2 – 10% Haddock	90 – 98% Atlantic Cod	
10% Whiting	90% Coley	
10% Pacific Cod	90% Coley	
5% Hoki	95% Atlantic Cod	

Table 1c: Details of commercial products used in this study.

Sample name	Declared species
Haddock portion in oven crisp crumb	Haddock (54%)
100% hoki fish fingers	Hoki fillet (64%)
Cod fish cakes	Minced cod (45%)
Salmon cakes in crunch crumb	Salmon (37%), white fish (37%, pollock, haddock, cod)
Fish fingers	Minced white fish (62%)

2.2 Extraction of DNA from fish materials

Approaches to DNA extraction were not considered as part of this study; however, a reliable DNA extraction method is vital to the success of any detection method. It is known that extraction of good quality DNA from processed products is more difficult than extraction from raw materials as the DNA is degraded during the processing stages. Two approaches to DNA extraction (a CTAB based method and a magnetic-bead based method) were used in this study.

The CTAB method is routinely used by many labs for the extraction of DNA from different samples. An on-going FSA sponsored project (Q01084) has confirmed that the CTAB method is very well suited to DNA extraction from raw and processed meat products. More recently, kits aimed at extracting DNA from difficult matrices such as foods have become available. These kits use different extraction protocols, such as resins, filters or magnetic bead technologies, to separate pure DNA (which is suitable for further downstream applications, e.g. PCR) from extraneous cellular materials. Some of these methods have the advantage that they can be adapted for use with robotic extraction techniques for processing of larger sample numbers.

2.2.1 CTAB DNA extraction method

DNA was extracted using a CTAB–Proteinase K method (Murray and Thompson, 1980). Samples (2g) were suspended in 5ml of CTAB buffer (2% CTAB [hexadecyltrimethylammonium bromide], 100mM Tris-HCl, 20mM EDTA, 1.4M NaCl, pH 8.0) and 40µl of Proteinase K solution (20mg/ml) was added. Samples were vortexed vigorously and then incubated overnight at 60°C. After incubation, 1ml of supernatant was removed to a 2.0ml Eppendorf tube, cooled to room temperature and centrifuged at 13,000g for 10 minutes. The clear supernatant was recovered and an equal volume of chloroform added. The solution was vortexed vigorously and then centrifuged at 16,000g for 15 minutes before the upper aqueous layer was removed to a clean 1.5ml Eppendorf tube. An equal volume of isopropanol was added and the DNA precipitated at room temperature 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 room temperature. The DNA pellet was resuspended in 100µl of SDW and purified using Promega's Wizard® Purification Resin as per the manufacturer's protocol. DNA extracts were recovered in 50µl of 1xTE (10mM Tris-Cl, pH 7.4, 1mM EDTA, pH 8.0) buffer. Final DNA concentrations (ng/µl) were determined using a GeneQuant pro DNA calculator (Pharmacia).

2.2.2 Tepnel DNA extraction method

DNA was extracted from freeze-dried material using the Tepnel Biokits DNA Extraction kit as per the manufacturer's instructions. The protocol for DNA extraction from Raw Meat Samples was used with the following modifications: the volume of Tissue Extraction Solution was doubled to 800µL and the volume of RNA solution doubled to 40µL to compensate for the use of dried material. DNA was quantified using the GeneQuant pro DNA calculator.

2.3 Preparation of DNA admixtures

DNA extracts (10ng/μL) from several authentic samples was pooled and used to prepare DNA admixtures. Admixtures of 1, 5, 10 or 25% (v/v) one fish species in a second fish species were prepared from pooled DNA stocks of each species. Appropriate volumes of DNA were mixed thoroughly in 1.5ml Eppendorf tubes to produce a minimum volume of 500μl of each DNA admixture. Admixtures were then aliquoted into smaller volumes (50–100μl) and stored at -20°C until required.

Additional DNA admixtures containing two fish species and soya DNA were also prepared as described above. These admixtures contained a total of 59% (v/v) fish DNA and 41% (v/v) soya DNA. The proportions of the two fish species relative to each other were 5% (v/v) or 10% (v/v) one fish in the second. Details of all DNA admixtures used are shown in Table 1b.

2.4 Methods of DNA analysis

2.4.1 DNA amplification

PCR products (464bp of the cytochrome b gene) were produced by amplification of DNA extracts (50ng) in 20μL reactions containing 1x Amplitaq Gold PCR buffer (Applied Biosystems), 300nM of each primer (Table 2), 200nM dNTPs, 5mM MgCl₂ and 0.05U/μl of Amplitaq Gold (Applied Biosystems). Amplification profiles (94°C for 5 minutes [denaturation]; 40 cycles of: 94°C for 40s, 50°C for 80s, 72°C for 80s [amplification]; 72°C for 7 minutes [final extension]) were applied using GeneAmp PE2400 or PE9600 PCR machines (Applied Biosystems). Unpurified PCR products (1μl) were applied to the Bioanalyser to confirm amplification.

Table 2 : Details of PCR primers used in this study.
 Primers are as described in Russell *et al.*, 2000 .

Primer	Sequence (5' – 3')
L14735	AAA AAC CAC CGT TGT TAT TCA ACT A
H15149*	GCI CCT CAR AAT GAY ATT TGT CCT CA

* Primer sequence contains mismatches Y (C or T), R (A or G) and Inosine (I)

2.4.2 Restriction digestion

Restriction enzymes were obtained from New England Biolabs and used as per the manufacturer's instructions. Unpurified PCR product (2.5µl) was digested overnight at 37°C with 2–5 units of enzyme in a total volume of 5µl. Reactions were terminated by incubation at 65°C for 10 minutes.

2.4.3 PCR-RFLP profiling

Reagents and DNA500 LabChips were prepared following manufacturers' instructions. Batches (~500µl) of gel matrix (used to fill LabChip capillaries) were prepared as required or at 4 weekly intervals. All reagents were stored at 4°C when not in use and allowed to reach room temperature for 1 hour before use. Digested PCR products (5µL) were mixed with 5µL of 20mM EDTA, to achieve a final concentration of 10mM EDTA, prior to loading on to LabChips. Aliquots (1µL) of the reaction mix were loaded on to the LabChip, as per manufacturers' instructions, and analysed on the 2100 Bioanalyser.

2.5 Assay Development

In order that the effects of any variation observed in a single individual did not unduly affect the results, all analyses for assay development were performed using the five individuals of each authentic species.

2.5.1 DNA sequencing

Sequencing PCRs were performed in 25 μ L reaction volumes using the same conditions as shown above (section 2.4.1). Amplification was confirmed by running 1 μ L on the 2100 Bioanalyser. The remaining 24 μ L of PCR product was sent to Lark Technologies for sequencing. PCR products were cleaned using a Qiagen PCR purification kit before being sequenced in both directions. Sequence data was provided to CCFRA as a text file and graphical sequence output file.

2.5.2 Sequence analysis

2.5.2.1 *Generation of consensus sequence*

Full DNA sequences for each PCR product were generated by aligning forward and reverse contigs using the SeqMan module of the LaserGene software suite (Ver 5.05; DNASTAR Inc, Madison, USA). Discrepancies were corrected manually using information in the graphical sequence file. Complete sequence data for each individual from a single species was aligned using the MegAlign module of the LaserGene package and a consensus sequence produced.

2.5.2.2 *Predicted PCR-RFLP profiles*

Consensus sequences from all species were used to predict PCR-RFLP patterns. These were produced using the AnnHyb software (Ver 4-17; <http://annhyb.free.fr>) package and all commercially available enzymes in the REBASE (file gcgenz.304; <http://rebase.neb.com/>) database of restriction enzymes.

3. Results and discussion

3.1 Collection of authentic fish materials

Authentic fish samples (five individual fish per species) were obtained from various sources as detailed below and summarised in Table 1a. A letter of authenticity with details of fish species, catch location and date were provided where possible. These are shown in Appendix 8.1.

A whole frozen authentic Atlantic salmon was obtained from the Institute of Aquaculture, Stirling University. Cherry salmon samples were obtained from the National Salmon Resources Center, Sapporo, Japan. These samples were provided as muscle samples fixed in ethyl alcohol. All other salmon samples were obtained from Fisheries and Oceans Canada, Nanaimo, Canada. These samples had been dried at 60°C and then ground to a coarse powder before being stored frozen (below -20°C).

Most of the European white fish species were provided as frozen 3-5g samples by The Institute of Food Research (IFR), Norwich. These samples were taken from authentic fish samples originally supplied to IFR by The Centre for Environment, Fisheries and Aquaculture Science (CEFAS), Lowestoft. Samples of lyophilised DNA and dried fin clips of Alaskan pollock (also known as Walleye pollock) (*Theragra chalcogramma*) were obtained from the Alaska Fisheries Science Center (AFSC), Seattle, USA. New Zealand hoki (*Macruronus novaezelandiae*) samples (~5g) were obtained from the National Institute of Water & Atmospheric Research Ltd (NIWA), Wellington, New Zealand as fish-meat preserved in ethanol.

3.2 Evaluation of PCR-RFLP profiles generated on the 2100 Bioanalyser for species identification

An initial evaluation of the PCR-RFLP method reported by Russell *et al.* (2000) was performed using salmon and trout DNA. This work was performed to determine if it was feasible to use the 2100 Bioanalyser to replace the gel electrophoresis stage of the method. During this stage of the study the protocol as described by Russell *et al.* (2000) was followed, i.e. DNA amplification was performed in 25µl reactions and PCRs were cleaned using a Qiagen PCR cleanup kit (Qiagen, Crawley, West Sussex) as per the manufacturer's instructions prior to restriction digestion.

3.2.1 Analysis of PCR amplified products

The primers used in this study (Table 2) were those used by Russell *et al.* (2000) and enabled the amplification of a 464bp region of the cytochrome b gene from the mitochondria of all fish species. Following PCR, amplified DNA fragments were separated on the Agilent 2100 Bioanalyser using DNA500 LabChips to confirm the presence of the expected 464bp fragment.

Analysis was performed on nine separate LabChips using aliquots from different PCRs generated on different occasions. Results showed that determination of the 464bp fragment size had a relative standard deviation of about 2% (Table 3). Mean observed product sizes obtained from salmon and trout were 461bp (SD = 8.6) and 455bp (SD = 9.6) respectively (Table 3). The mean estimated size for the amplified salmon DNA fragment was about 6bp larger than the size of the trout DNA (Table 3). Whilst the difference in fragment size between salmon and trout is not statistically significant, on all occasions that the two were determined, the fragment for salmon was consistently larger than that for trout. Amplified DNA was sequenced to confirm the actual fragment size from both species. Sequenced fragments from both species were found to be 464bp. An analysis of the total molecular weight of each fragment (derived from the numbers of each base, A, C, G, T, in the sequence) indicated that the expected difference in molecular weight (259.2 Daltons) is less than the weight of an average base (308 Daltons). This difference couldn't account for the apparent

size difference observed on the 2100 Bioanalyser. Differences in DNA folding offer a possible reason for the apparent difference in fragment sizes, determined by the 2100 Bioanalyser. Linear DNA molecules are known to fold into more globular shapes under the right conditions. It is, therefore, possible that the fragments from the two species have folded differently due to sequence variations and that this has affected the final shape and ultimately migration rates of the two products.

Table 3 : Amplified DNA fragment size variations observed following separation of DNA on different DNA500 LabChips.

Species	Expected fragment size (bp)	Number of chips	Mean observed fragment size (bp)	SD	%RSD	Min. size observed (bp)	Max. size observed (bp)
Salmon	464bp	9	461	8.6	1.87	448	474
Trout	464bp	8	455	9.6	2.10	445	468

3.2.2 Analysis of PCR-RFLP profiles

Following cleavage of the amplified DNA fragment with restriction enzymes, species-specific PCR-RFLP patterns were resolved on the 2100 Bioanalyser. An example of a PCR-RFLP pattern is shown in Figure 1, which shows PCR-RFLP profiles for salmon and trout samples generated with enzymes DdeI and HaeIII. Observed and expected fragment sizes for a selection of five enzymes and four salmonids are shown in Table 4. As can be seen, patterns were similar to those reported previously (Russell *et al.*, 2000; Hold *et al.*, 2001). Actual fragment sizes obtained in this study generally matched those predicted by the AnnHyb software, which confirmed that use of the 2100 Bioanalyser did not affect PCR-RFLP profiles. 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 (25–100bp), which had not been reported previously by Russell *et al.* (2000) were observed in some digests. 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 primer H15149 and is explained in more detail later.

From Table 4 it can be seen that *O. gorbuscha* and *O. mykiss* profiles generated with NlaIII have only two fragments when three are expected. This phenomenon has also been observed in some of the white fish species (see later). This is believed to be due to the co-migration 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 shown in Table 4. From the sequence data there is 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 co-migration is due to molecular weight similarities between the two fragments. The co-migration 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 Bioanalyser matched those expected or previously reported (Russell *et al.*, 2000; Hold *et al.*, 2001), 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.

Table 4: Expected and observed PCR-RFLP fragment sizes obtained with five restriction enzymes and four salmonid species.

Species		Expected ¹ (E) and observed (O) fragment sizes for each enzyme (bp)				
		DdeI ²	Bsp1286I	HaeIII	NlaIII	Sau3AI
<i>O. nerka</i> (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
<i>O. gorbuscha</i> (pink salmon)	E	360, 130	U/C ³	U/C	210, 190, 100	390, 120
	O	349, 343, 112	464	421	181, 92	338, 115
<i>S. salar</i> (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
<i>O. mykiss</i> (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

¹ Sizes as reported by Russell *et al.* (2000).

² Extra fragments in observed DdeI profiles are due to restriction site introduced by primer H15149. See text for full explanation.

³ U/C – uncut with enzyme

3.2.3 Salmon and trout profiles

Digestion of Atlantic salmon and rainbow trout DNA with HaeIII produced three bands in these two species (Table 4); however, only two fragments of 350bp and 130bp were reported in both species by Russell *et al.* (2000). Due to sizing variations the two larger bands (318bp in salmon and 313bp in trout) observed here are believed to correspond to the 350bp fragment reported previously. It is likely that the smaller (~34bp) fragment was not resolved using conventional gel electrophoresis, hence it was not reported as part of the PCR-RFLP pattern for these species. This shows that the extended range over which CE can resolve closely sized DNA fragments is superior to that of gel-based methods. This means that the 2100 Bioanalyser, with its improved detection of smaller fragments, could be useful for improving species identification by providing a more complete species-specific PCR-RFLP fingerprint.

From the work of Russell *et al.* (2000) it was expected that fragments of 350bp and 130bp in salmon and 360bp and 130bp in trout would be observed following digestion with DdeI; however, a band of around 110bp was observed in both species along with two larger bands of around 321bp and 312bp (salmon) and 348bp and 339bp (trout). These larger doublets were consistently differentiated by the 2100 Bioanalyser, which suggested that they were not artefacts; however, they were not reported by Russell *et al.* (2000) or Hold *et al.* (2001). This suggested that either the doublets were peculiar to samples used during this work or that the resolving power of the 2100 Bioanalyser enabled detection of two bands at this position, whereas the conventional gel-based method was only able to differentiate a single band. To confirm that two fragments were expected, predicted DdeI restriction patterns for salmon and trout were produced using AnnHyb software and DNA sequences produced previously. DNA sequence, primer binding positions and predicted DdeI restriction sites for salmon and trout are shown in Figure 2. Predictions revealed a cleavage site in salmon and trout at around position 456, which is around 7bp from the end of the selected sequence. Cleavage at this site results in a 7bp fragment (which is not detected by the 2100 Bioanalyser) and a 456bp fragment. Cleavage of the 456bp fragment, from salmon, at positions 144 and 117 would produce fragments of 25bp, 117bp and 312bp, while cleavage of the trout 456bp fragment at

position 116 would produce fragments of 116bp and 339bp. Allowing for size variations of fragments on the 2100 Bioanalyser, this would account for the bands observed at around 312bp in salmon and 339bp in trout. The second, larger, band of the doublet was determined to be the result of non-cleavage of some of the amplified fragments at position 456. During PCR, primer H15149 is incorporated into half the amplified fragments. This primer (Table 2) was designed with several redundant bases in order to make it universal, so it is not an exact match for the salmon and trout sequences. One of the redundant bases (R) in H15149 either incorporates a DdeI site if a 'G' base is present or disrupts the DdeI restriction site (if an 'A' is incorporated); therefore, cleavage does not occur. The occurrence of the doublet in salmon and trout is, therefore, not due to incomplete digestion but to the incorporation of different bases found in primer H15149 during PCR.

3.2.3.1 *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 LabChip 3 but using a second, fresh batch of gel matrix (matrix B). Overall variation (encompassing variation due to LabChip-to-LabChip, PCR and gel matrix) is shown in Table 5, 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, i.e. 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).

Table 5 : PCR-RFLP fragment sizes obtained following separation of DNA cleaved with DdeI on four different DNA500 LabChips.

Expected band size (bp)	Observed fragment sizes on each LabChip (bp) ²					Mean	%RSD
	LabChip 1 (fresh matrix A)	LabChip 2 (fresh matrix A)	LabChip 3 (week old matrix A)	LabChip 4 (fresh matrix B)			
Salmon analysis ¹							
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	

¹ An expected 27bp fragment from salmon is not detected by the 2100 Bioanalyser.

² Fragment sizes shown in Table 5 differ slightly from those in Tables 4 and 6 because a smaller sample set was used to generate Table 5. Data from Table 5 has subsequently been incorporated into Tables 4 and 6.

3.2.3.2 Determination of sensitivity

Detection sensitivity was determined using DNA admixtures of salmon and trout. Amplification of DNA admixtures resulted in a single PCR product. Restriction digests were produced using DdeI and Sau3AI. These enzymes were selected to allow a comparison between profiles produced using an enzyme which cleaves both species and one which cleaves a single species only. Sau3AI was chosen as it does not cleave the trout amplicon; therefore, PCR-RFLP fingerprints from admixtures were unlikely to be as complex as those produced by DdeI. Examples of PCR-RFLP patterns produced using these enzymes are shown in Figures 3 and 4. Figures show fingerprints obtained from each single species and DNA admixtures of the two species.

Results from amplification and digestion of DNA admixtures prepared from salmon and trout DNA using DdeI are shown in Figure 3, which shows that patterns from both species can be readily identified in admixtures containing as little as 5% salmon DNA in trout DNA. In this admixture (lane 4, Figure 3) a fragment of 110bp can be seen, which is a combined product from both salmon and trout. Also visible are two sets of fragment doublets at around 312 and 320bp, and 339 and 348bp, which are the result of digestion of salmon and trout respectively. The presence of the smaller doublet clearly indicates the presence of salmon in the admixture. Figure 3 (lanes 6 – 9) shows the results of analysis of DNA admixtures containing 1 – 25% trout DNA in salmon DNA. From this analysis it appears that it is not possible to detect trout DNA when it is present at less than 25% of the total DNA.

Figure 4 shows that Sau3AI does not digest trout DNA; however, salmon DNA is cut once to produce two fragments of around 88bp and 368bp (expected 94bp and 370bp). The salmon digestion pattern is useful for detecting the presence of salmon DNA and, as can be seen (Figure 4), permits detection down to a level of 5% salmon DNA in trout DNA. Admixtures containing greater than 75% salmon DNA (Figure 4, lanes 8 – 11) produced distinct salmon patterns, indicating the presence of salmon; however, it is not possible to readily detect the presence of trout DNA in these DNA admixtures.

These results (detection of low levels of salmon DNA in trout DNA but poor detection of trout DNA in salmon DNA) suggest that salmon DNA is being preferentially amplified relative to trout DNA. This means that the final 464bp PCR fragment is predominantly salmon and so after cleavage with enzymes there is insufficient trout DNA present for detection. This is, therefore, having an effect on the level of detection for these species using this method. Subsequent work with admixtures of white fish (see later) lends support to the belief that preferential DNA amplification may be occurring.

Work to determine the exact cause of this preferential amplification was not performed; however, it may be due to differences in the quality of the template DNA from each species. The quality of template DNA could have been affected by DNA degradation, which occurred prior to DNA extraction. It has been shown that DNA degradation can greatly affect PCR but that some of this effect can be overcome by

the use of smaller PCR targets (Garrett *et al.*, 2001). Differences in the treatment of the samples prior to analysis may explain any differences in DNA quality. It is known that cycles of freeze-thawing lead to DNA degradation and it may be that this has occurred to a greater extent in the trout samples analysed here. Also the action of freezing and thawing leads to cellular degradation with the subsequent release of enzymes, which accelerate the rate of DNA degradation. If the trout DNA had become degraded in this manner it would result in smaller DNA fragments available as template DNA for PCR. As the amplicon target is relatively large (464bp) any degradation would limit the amount of PCR target available and so reduce the amplification efficiency. It is possible that using a smaller (<200bp) PCR target or increasing the number of PCR cycles may improve the detection limit for trout by increasing the final yield of trout derived PCR product.

3.2.4 Application of method to other salmonid species

3.2.4.1 *Sequence data*

Sequence data for the five authentic cherry salmon samples (for which there was no or little available data in GenBank) was generated by amplification of the 464bp fragment of the cytochrome b gene using the two primers shown in Table 2. Data from the five individuals was aligned and used to generate a cherry salmon consensus sequence, which is shown in Figure 5 (C. Salmon).

PCR-RFLP profiles were generated experimentally from the authentic salmonid samples (including cherry salmon), for which sequence data was available or fragment sizes were reported by Hold *et al.* (2001). These profiles were compared to those reported, by Hold, to confirm that expected fragment sizes were obtained. The results of this analysis are shown in Table 6, which shows the observed fragment sizes for each species with each of the three enzymes. Also shown are the observed fragment sizes obtained from the Atlantic salmon and rainbow trout samples described above. Analysis of this data indicates that it is possible to use enzyme DdeI to specifically differentiate Chinook, Coho, Atlantic and cherry salmon; however, the remaining salmonids have similar profiles with this enzyme which does

not allow the individual species to be identified. By using a combination of profiles generated with all three enzymes, DdeI, HaeIII and NlaIII, it is possible to differentiate all the salmonid species.

3.3 Expansion of the method to include species other than salmonids

3.3.1 Production and analysis of sequence data

3.3.1.1 Individual sequence data

DNA extracts from each individual of each non-salmonid species (Table 1a) were amplified in readiness for DNA sequencing. Amplification of expected 464bp PCR products was confirmed before the remainder of each PCR was sent for sequencing. All PCR clean-up and sequencing was performed by Lark Technologies who provided CCFRA with sequence data. Data from forward and reverse sequencing reactions from each individual fish were aligned to produce a complete sequence for each fish sample. Owing to the degeneracy of primer H15149, exact matching of this end of the PCR products was not practicable. This resulted in a small variation (± 2 bp) in the length of PCR products.

Table 6: Average PCR-RFLP fragment sizes for salmonid species.

Species	Number of analysis ¹	Enzyme	Average observed sizes (bp)	Range of observed sizes (bp)		
Red / Sockeye salmon (<i>Oncorhynchus nerka</i>)	5	DdeI	114	113 – 114		
			346	345 – 348		
			353	352 – 355		
		Hae III	35	35 – 35		
			102	101 – 102		
			320	318 – 321		
			421	421		
			272	271 – 274		
		Pink / Humpback salmon (<i>Oncorhynchus gorbuscha</i>)	5	DdeI	112	112 – 112
					343	342 – 343
349	349 – 350					
Hae III	421			420 – 422		
Nla III	92			92 – 93		
	181	180 – 182				
Chinook salmon (<i>Oncorhynchus tshawytscha</i>)	5	DdeI	177	174 – 178		
			273	270 – 274		
			280	279 – 281		
		Hae III	99	98 – 100		
			318	312 – 321		
			Nla III	439	436 – 441	

(continued)

¹ This is a minimum number of analyses performed. In some species more analyses have been performed with some enzymes.

Coho / Silver salmon (<i>Oncorhynchus kisutch</i>)	7		65	64 – 66
		Ddel	113	112 – 113
			274	271 – 276
			281	278 – 283
		Ddel	472	472 – 475
		Hae III	100	99 – 101
			319	316 – 320
Keta / Chum salmon (<i>Oncorhynchus keta</i>)	5		112	112 – 113
		Ddel	341	340 – 342
			349	347 – 351
		Hae III	420	419 – 423
		Nla III	181	180 – 181
Cherry salmon (<i>Oncorhynchus masou masou</i>)	5		270	269 – 271
			180	179 – 180
		Ddel	255	253 – 258
			262	261 – 265
		Hae III	35	34 – 37
			100	99 – 100
			318	316 – 321
Nla III	93	93 – 93		
	159	158 – 162		
	184	183 – 185		

(continued)

Cut-throat trout (<i>Oncorhynchus clarki clarki</i>) ^ψ	2		112	112
		Ddel	343	343
			351	351
		Hae III	99	98 – 99
			316	315 – 317
		Nla III	93	92 – 93
		183	183 – 184	
Dolly varden (<i>Salvelinus malma malma</i>) ^ψ	2	Ddel	Not tested	~
		Hae III	102	101 – 102
			311	310 – 311
		Nla III	450	448 – 452
Atlantic salmon (<i>Salmo salar</i>)	5		110	107 – 111
		Ddel	312	305 – 317
			321	314 – 326
		Hae III	35	34 – 36
			98	97 – 99
			318	312 – 323
		Nla III	438	430 – 448
Rainbow trout (<i>Oncorhynchus mykiss</i>)	4		111	110 – 111
		Ddel	339	338 – 343
			348	347 – 352
		Hae III	33	33 – 35
			100	99 – 101
			313	307 – 317
Nla III	92	91 – 92		
		185	184 – 186	

3.3.1.2 *Consensus sequence data*

Sequences from the five individuals of each species were aligned and a species-specific consensus sequence for each species was produced. These alignments also highlighted point mutation variations between the individuals of each species, which is expected in large fish populations.

The consensus sequences from each of the ten white fish species were aligned in order to confirm that sufficient sequence variation existed between the species for the PCR-RFLP method to be useful. Aligned consensus sequences for the ten species are shown in Figure 5. Interestingly this alignment revealed the presence of an additional 5bp in the hoki (*Macruronus novaezelandiae*) sequence, which was not present in any other species. From an evolutionary point this suggests a divergence of the hoki species from the other fish species used in this study. This longer (469bp) PCR product has been consistently amplified in all hoki samples subsequently analysed. This indicates that this insert is not an artefact.

3.3.1.3 *Predicted PCR-RFLP profiles*

Consensus sequences for each species were also used to predict expected PCR-RFLP patterns for each of the white fish species. Profiles were generated for commonly available enzymes with recognition sites of >4bp. Expected patterns were visualised graphically using an Excel spreadsheet. Enzymes which produced the greatest pattern variability across the species were selected for further analysis. From over 120 enzymes examined, DdeI was predicted to show the greatest variability across the ten species. This enzyme was predicted to produce nine distinct profiles from the ten fish species, which would allow the differentiation of all species except Alaskan pollock (*Theragra chalcogramma*) and Pacific cod (*Gadus macrocephalus*). Two additional enzymes, HaeIII and NlaIII, were selected on the basis that they were predicted to differentiate between Alaskan pollock and Pacific cod.

3.3.2 Generation of PCR-RFLP profiles

PCR-RFLP patterns for the white fish species were produced experimentally by amplifying DNA extracts from the authentic fish and then digesting the PCR products with the three enzymes, DdeI, HaeIII and NlaIII. Examples of the PCR-RFLP profiles generated using a single sample from all ten species and the three enzymes are shown in Figure 6. This clearly shows that, as predicted, most species can be differentiated using DdeI alone. Figure 6 also shows that profiles generated by the two additional enzymes (HaeIII and NlaIII) complement profiles generated by enzyme DdeI when identifying the different fish species.

3.3.3 Method optimisation

3.3.3.1 *Reduction of reaction volumes*

Initially analysis was performed using 25µL PCR reactions. PCR products were purified using the QIAquick PCR purification kit (Qiagen, Crawley, W. Sussex) before being cleaved with restriction enzymes in a total restriction digest reaction volume of 10µL. As only 1µL is required for analysis on the 2100 Bioanalyser the potential of reducing the reaction volumes was investigated. Restriction digest volumes were optimised in a total volume of 5µL, which means five replicate analyses could be performed on the 2100 Bioanalyser from each sample. Further reduction of this volume was not possible as results became too inconsistent. This is likely to be the result of the large relative errors introduced when small volumes of each specific component (enzyme, buffer etc) were being pipetted. PCR volumes were also reduced to 20µL from 25µL. Although PCR could be performed in smaller volumes it was found that this did not always leave enough sample for repeating the analysis if required.

3.3.3.2 *Elimination of PCR clean-up*

Removal of the PCR clean-up step was also investigated. Restriction digestion was performed on amplified DNA products with and without PCR clean-up. No difference in fragment numbers or sizes was observed between purified and unpurified PCR samples; however, in most cases analysis without the PCR clean-up step produced

more intense fragment peaks on the 2100 Bioanalyser. This made the detection of some fragments easier.

3.3.3.3 *Effects of restriction enzyme buffers*

Following modification of the method to eliminate the PCR clean-up step it was noticed that problems with loss of the upper size marker on the DNA500 LabChip were occurring in samples cleaved with HaeIII. The size markers used by Agilent are DNA fragments and are liable to DNA cleavage by restriction enzymes during chip preparation and running. It is likely, therefore, that the size markers contain a HaeIII restriction site but not DdeI or NlaIII sites. Even though a heat inactivation step was used after restriction digestion was performed, it appears that some enzyme remained active. The addition of EDTA (to all restriction digests) to achieve a final concentration of 10mM eliminated this problem by chelating metal ions required by the enzyme.

A particular problem of retarded DNA fragment migration rates, which resulted in the loss of the upper marker, was observed with some batches of DdeI enzyme only. This was determined to be due to the high salt concentration in the DdeI enzyme reaction buffer. Loss of the upper marker made it impossible to size DNA fragments correctly or to identify species present in a sample. The addition of an equal volume (5 μ L) of 20mM EDTA overcame this problem by reducing the salt concentration to within the tolerance of the DNA LabChip while maintaining the 10mM EDTA required for enzyme inactivation.

Overall the protocol outlined above using reduced reaction volumes (restriction digests and PCR), omitting the PCR clean-up step and diluting the final restriction digest, had little effect on the final PCR-RFLP profiles obtained, but did result in a faster analysis time and reduced costs for PCR and restriction digests compared to the conventional gel-based method described by Russell *et al.* (2000).

3.3.4 Expected and observed PCR-RFLP profiles

Expected and observed PCR-RFLP fragment sizes are shown in Table 7. In the majority of cases the experimental PCR-RFLP patterns matched those predicted

using AnnHyb software; however, some differences (which are detailed below) were seen. Observed and expected profiles were defined as matching if the predicted and experimental fragment sizes were within 5% of each other. A variation of 5% was chosen as the 2100 Bioanalyser is reported to size fragments with variations of ± 5 bp for fragments of 25-100bp and $\pm 5\%$ for fragments of 100-500bp. The largest actual observed fragment variation in all profiles generated was 3.7%, which is within the variation limits claimed for the LabChip. The main differences between predicted and experimental profiles were observed when enzymes DdeI and NlaIII were used to generate profiles as described below.

3.3.4.1 Analysis with DdeI

Some species (e.g. hoki and plaice), when digested with enzyme DdeI, produced a DNA fragment doublet, i.e. two fragments with a size difference of about 10bp. This observation (see Figure 1) has been reported previously following analysis of salmon and trout (Dooley *et al.*, 2004) and is due to the introduction (into some PCR products) of a DdeI restriction site by the primer H15149. This doublet was also observed in whiting, but it was not consistently resolved (as a separate peak) by the 2100 Bioanalyser. Where the two peaks were not fully resolved a shoulder, corresponding to the smaller 341bp fragment, was always seen on the 347bp fragment peak on the electropherogram.

Although some natural genetic variation within each fish population was revealed as point mutations in the sequence data from the five individuals of each species (data not shown) this did not manifest itself as differences in the PCR-RFLP profiles between the individuals of a single species, except in the hoki species. Analysis of the five hoki individuals with DdeI produced three distinctly different PCR-RFLP profiles. The most common profile (35bp, 175bp, 255bp & 262bp, shown in Table 7) was observed in three individuals. The other two individuals each produced different profiles comprising (a) 206bp, 254bp & 261bp or (b) 35bp & 447bp. Re-examination of sequences for each individual hoki indicated that these profiles were due to the loss of DdeI restriction sites in these individuals. These restriction site losses had been masked during the original profile predictions due to the use of the hoki consensus sequence. No variation between the five individuals of hoki was observed when PCR-RFLP profiles were generated using the other two enzymes, HaeIII and

NlaIII. The hoki species can be identified by using either HaeIII or NlaIII. As such the occurrence of different DdeI profiles does not detract from identifying this species in a sample. The generation of multiple PCR-RFLP profiles with any of the three enzymes with individuals from the other species used in this study was not noted.

3.3.4.2 *Analysis with NlaIII*

Haddock and whiting PCR-RFLP profiles generated with enzyme NlaIII produced some unexpected results. These species were predicted to produce identical profiles, each containing three DNA fragments of 91bp, 180bp and 193bp with this enzyme. Experimental results indicated that haddock contained two fragments of 94bp and 183bp and whiting contained two fragments of 100bp and 186bp. The smaller observed fragments correspond to the predicted 91bp fragment; however, the larger fragment observed in both species is part way between the predicted 180bp and 193bp fragments. Sequence data from all ten individuals of these two species was checked for predicted digestion patterns. This confirmed that the predicted three fragment PCR-RFLP profiles were correct. It is believed that the two larger predicted fragments of 180bp and 193bp are co-migrating as a single band, as observed experimentally.

3.3.4.3 *Detection of small fragments*

During analysis of samples with all three enzymes, some smaller fragments of between 25bp and 42bp were inconsistently detected by the 2100 Bioanalyser. This was probably because they were too close to the sizing limits for the DNA500 LabChip (25–500bp) or did not fluoresce sufficiently to be detected. Poor fluorescence is likely to be due to the incorporation of only small amounts of dye in these small, low concentration fragments. It should be noted that not detecting these fragments did not affect the ability to identify individual species.

Table 7: Expected and observed PCR-RFLP fragment sizes generated with the three enzymes Ddel, HaeIII and NlaIII from the ten white fish species.

White fish species	Ddel fragments		HaeIII fragment		NlaIII fragments	
	expected	observed ¹	expected	observed ¹	expected	observed ¹
A. Cod	(25) ² , 87, 118, 234	84, 115, 234	41, 109, 316	37, 102, 321	88, 92, 284	89, 100, 280
P. Cod	(24), 205, 234	198, 235	41, 109, 316	37, 102, 320	88, 92, 284	89, 100, 279
Coley	(25), 118, 321	117, 328	41, 109, 316	37, 103, 323	89, 375	101, 372
Haddock	(25), 439	433	41, 423	37, 429	91, 180, 193	94, 183
E. Hake	158, 306	155, 307, 314	42, 109, 124, 189	38, 101, 127, 185	Uncut	477 [uncut]
SA Hake	25, 158, 282	155, 285	42, 109, 124, 189	38, 101, 128, 185	Uncut	473 [uncut]
E. Plaice	42, 145, 270, 277	[32] ³ , 138, 266, 273	41, 131, 292	37, 129, 286	88, 89, 91, 196	86, 100, 187
Whiting	117, 339, 347	115, 344 ⁴ , 355	41, 71, 109, 242	37, 101, 326	89, 179, 196	100, 186
A. Pollock	(24), 205, 234	198, 232	41, 109, 316	38, 67, 101, 243	38, 50, 88, 287	38, 47, 98, 278
Hoki ⁵	32, 176, 252, 261	[35], 175, 255, 262	30, 46, 109, 285	[23], 44, 101, 288	45, 58, 90, 263	44, 67, 105, 264

¹ Observed sizes are the mean sizes observed from analysis performed on at least 3 different occasions.

² Numbers in round brackets, (), are predicted fragments which are not always detected by the 2100 Bioanalyser.

³ Numbers in square brackets, [], are small fragments detected intermittently by the 2100 Bioanalyser

⁴ This doublet in whiting is not consistently resolved by the 2100 Bioanalyser; however, the smaller product can be observed as a shoulder on the larger fragment peak.

⁵ Point mutations in the authentic hoki samples produced three PCR-RFLP profiles for Ddel. Alternative PCR-RFLP profiles contain fragment sizes of (a) 206, 254, & 261 or (b) 35 & 447 bp.

3.4 Validation of the PCR-RFLP method

3.4.1 DNA admixtures

The application of this method for detecting fish species in samples containing more than one species was initially investigated using DNA admixtures. An example of the profiles obtained following analysis with all three enzymes and the first four DNA admixtures 1–4 (see Table 1b) is shown in Figure 7. This shows that complex patterns comprising profiles for both species are detected on the 2100 Bioanalyser. It can also be seen that the profiles from the species present at 5% are not as strong (i.e. fragment intensities are less) as the profiles for the species present at 95%. This is as expected as there is a 20 fold difference in the proportions of each fish; however, this does not detract from the ability to detect the fish species present at the lower amount. Using this approach both species in all DNA admixtures shown in Table 1b were detected, even in those admixtures containing soya DNA where the actual DNA content for the lower concentration species is below 3%.

An additional analysis using haddock and Atlantic cod DNA admixtures down to a level of 1% was performed. The limit at which both species could be detected in the admixtures was 5%; however, in some replicates it was possible to detect 1% cod in haddock or 2% haddock in cod. Figure 8 shows the results from the analysis of admixtures containing 2% haddock or 2% cod with enzyme Ddel. As can be seen in Figures 8b and 8c the peak heights of the fragments corresponding to the species present at the lowest concentration are low; however, they can be detected sufficiently to determine the presence of the species. These observations suggested that this method should be suitable for the sensitive detection of a specific fish species present in a DNA admixture at around 5%.

3.4.2 Fish-meat admixtures

To confirm the application of this approach to the detection of fish species in products the method was applied to fish admixtures prepared from minced fillets of haddock and cod. Results from this analysis are shown in Figure 9, which shows electropherograms of the PCR-RFLP profiles obtained with each enzyme from

admixtures prepared from 5% of each species (green line), 100% cod (red line) or 100% haddock (blue line). Note that only the 5% admixtures are shown for clarity, but that in some replicates it was possible to detect species-specific fragments in admixtures containing less than 5% of the species. Full details of the expected and observed fragment sizes for all samples (admixtures and single species) are tabulated in Appendix 8.2.

From Figure 9 it is possible to see peaks in the admixtures which correspond to peaks from each of the two individual species. Peak heights of cod specific fragments in admixtures containing 5% cod (top row) are less than four fluorescent units (FU) on the Y-axis, which is the default minimum fluorescence used by the 2100 Bioanalyser for automatic fragment detection. This means manual intervention is required in order to confirm the presence of this species in the admixtures. In comparison peak heights for haddock fragments in the 5% haddock samples (bottom row) are generally above five FUs (except Ddel which is about three FU), which makes automatic detection of fragments specific to this species possible.

These observations suggest that there may have been preferential amplification of the haddock DNA target compared to the cod DNA target in the PCRs, which has affected the intensities (and hence detection limit) in the final PCR-RFLP profiles. Possible reasons for this have been outlined earlier (section 3.2.3.2) when similar results were obtained with salmon and trout admixtures.

3.4.3 Freeze-dried admixtures

Further analysis was performed on admixtures prepared from freeze-dried samples of fish fillets as shown in Table 1b. These samples were prepared especially for the ring-trial of this method (see below). Analysis was performed using freeze-dried samples in order to overcome possible problems of sample deterioration due to cycles of freeze-thawing during preparation and distribution. The results of analysis performed on the haddock and cod admixtures are shown in Figure 10, which shows PCR-RFLP patterns obtained from samples prepared from 100% haddock or Atlantic cod and admixtures containing between 1 and 5% of these two species. From Figure

10 it can be seen that it is possible to detect both species when present in the admixtures at a level of 5%. In some replicates it was also possible to detect cod or haddock at 2% in the admixtures, which is comparable to the level of detection achieved using fish-meat admixtures. The detection of these species at this low level has also been reported by participants in the ring-trial (see section 3.5).

3.4.4 White fish-based products

A final application of the method to fish based products purchased locally was performed. For those products where particular species were declared it was relatively easy to check for the presence of the expected fragment sizes corresponding to the declared species and hence confirm its presence in the sample. Additional species were also readily identified by the presence of extra DNA fragments in the three enzyme profiles. Some products, however, were described as containing “white fish” and in this case it was not possible to check the expected fragment sizes to confirm the presence of a particular species. A prediction of possible species in these types of products was made by matching fragment sizes obtained to those expected for the ten species. A summary of the results is shown in Table 8, which contains expected and observed PCR-RFLP profiles for five different fish products as detailed in Table 1c. The expected fragments shown are the average fragment sizes previously obtained from authentic materials rather than the predicted fragment sizes obtained from sequence data.

From Table 8 it can be seen that none of the five products tested contained undeclared species. The first three products, which contained a single species of either haddock, hoki or cod, were readily identified from their respective PCR-RFLP profiles generated with all three enzymes.

Analysis of the salmon fish cakes clearly indicated the presence of Atlantic salmon; however, smaller peaks corresponding to Alaskan pollock were also observed, suggesting the presence of this species. Alaskan pollock was declared on the package as one of three possible white fish species forming 37% of the product. There was no evidence of the other two species (haddock or cod) in this sample.

Table 8: Results obtained following analysis of commercial products using the PCR-RFLP method.

Sample ¹	Enzyme	Expected fragment sizes (bp)	Observed fragment sizes (bp)	Potential species identified
Haddock portions in oven crisp crumb (haddock)	Ddel	433	441	Haddock
	HaeIII	429, 37	419, 36	Haddock
	NlaIII	183, 94	179, 92	Haddock
100% hoki fish fingers (hoki)	Ddel	262, (255), 175, 35	258, 250, 170, 26	Hoki
	HaeIII	288, 101, 44, 23	286, 101, 42, 22	Hoki
	NlaIII	264, 105, 67, 44	264, 103, 67, 42	Hoki
Cod fish cakes (cod)	Ddel	234, 115, 84	238, 115, 86	Atlantic cod
	HaeIII	321, 102, 37	318, 101, 36	Atlantic cod
	NlaIII	280, 100, 89	277, 100, 88	Atlantic cod
Salmon cakes in crunch crumb (salmon & white fish [pollock, haddock, cod])	Ddel	321, 312, 110 & <u>232</u> , <u>198</u>	319, 310, <u>229</u> , <u>195</u> , 110	Atlantic salmon, Alaskan pollock
	HaeIII	318, 98, 35 & <u>243</u> , <u>101</u> , <u>67</u> , <u>38</u>	319, <u>241</u> , 99 ² , <u>67</u> , 35 ²	Atlantic salmon, Alaskan pollock
	NlaIII	438 & <u>278</u> , <u>98</u> , <u>47</u> , <u>38</u>	435, <u>276</u>	Atlantic salmon, Alaskan pollock
Fish fingers (white fish)	Ddel	n/a	229 ² , 194 ²	Pacific cod, Alaskan pollock
	HaeIII	n/a	<u>324</u> , 242, 100 ² , 66, 37 ²	Pacific cod, Alaskan pollock, Atlantic cod, coley, whiting
	NlaIII	n/a	275, 98, 47, 38	Alaskan pollock

¹ species in brackets were those declared on product

² fragments derived from both species

The final fish finger product was declared to contain white fish only with no named species. Examination of PCR-RFLP profiles suggested that this product contained Atlantic cod and Alaskan pollock as profiles similar to these two species were detected by both enzymes DdeI and HaeIII. However, only an Alaskan pollock profile was detected with NlaIII, which may indicate that the majority of this product is Alaskan pollock with a smaller amount of Atlantic cod. No further analysis of these samples was undertaken to confirm this result.

These results indicate that this method is well suited to confirming the presence of a declared species in fish-based products and that it is also possible to determine some of the species present in a product described as containing white fish. It should be noted, however, that the determination of species in a product where no specific species has been declared is limited to those species for which authenticated profiles are available. To improve the reliability of this approach (i.e. to identify undeclared fish species) it would be useful to expand the number of fish species for which PCR-RFLP profiles are available. Despite the need for predetermined profiles, for species identification, this is still a very useful method for rapidly confirming the presence of fish species in fish products and for recognising the presence of additional species which may or should not be present.

3.5 Analysis of method application by ring-trial

3.5.1 Participants and samples used in the ring-trial

A ring-trial of this method involving five independent laboratories, including several UK public analysts, was carried out to determine the ease of use of the method. Of the five laboratories none had previous experience of this PCR-RFLP approach for fish identification and only one had previous experience of using the 2100 Bioanalyser. Four of the participants shared two 2100 Bioanalysers, kindly loaned by Agilent Technologies for this trial, and the fifth used their own 2100 Bioanalyser. Participants were provided with 20 freeze-dried samples (Table 9), a protocol and results recording sheets (see Appendix 8.3), a Tepnel Magnetic DNA extraction kit, all solutions needed to perform the analysis (e.g. PCR mastermix and restriction enzymes) and a list of fragment sizes expected for each species with each enzyme. Participants were also provided with a CD-R to return their raw data files from the 2100 Bioanalyser to CCFRA for reviewing.

The 20 samples provided consisted of about 20–40mg of 19 unknowns and an Atlantic cod positive control, which was used to ensure that each stage of the protocol had proceeded correctly. Unknown samples contained either a single species or admixtures of two species, one of which was present at 1, 2 or 5%. Because PCR-RFLP profiles generated on the 2100 Bioanalyser are accurate and repeatable it is not necessary to run standards (positive controls) with samples so these were not provided except for the cod control sample.

Participants attended a training day at CCFRA where they were shown the operation of the Agilent 2100 Bioanalyser. For the trial the participants were asked to extract DNA from all the samples, perform PCR amplification on the extracted DNA and then produce PCR-RFLP profiles from the amplified DNA. Samples were identified by comparing PCR-RFLP patterns obtained with each sample to PCR-RFLP patterns provided (and previously obtained at CCFRA from authentic materials).

Table 9: Summary of results obtained from analysis of fish samples by participants in ring-trial.

Sample ID code	Species present in sample	Comments about sample ¹	Percentage of labs correctly identifying main species ²	Percentage of labs correctly identifying minor species ²	Percentage of labs fully identifying sample correctly ²
1	Whiting (10%) Coley (90%)	admixture	100	100 ³	100
2	Haddock	confirmed sample	100	n/a	100
3	Haddock (10%) A. Cod (90%)	admixture	100	100	100
4	Hoki	confirmed sample	100	n/a	100
5 ⁴	SA Hake	authentic sample	100	n/a	100
6 ⁴	Pacific Cod (10%) Coley (90%)	admixture	100	25	25
7 ⁴	A. Cod (10%) Haddock (90%)	admixture	100	100	100
8 ⁴	E. Plaice	authentic sample	100	n/a	100
9 ⁴	Whiting	authentic sample	100	n/a	100
10	Haddock (5%) A. Cod (95%)	admixture	100	100	100
11	A. Cod (2%) Haddock (98%)	admixture	100	100	100
12	A. Cod (5%) Haddock (95%)	admixture	100	100 ⁵	100

(continued)

13	Alaskan Pollock	confirmed sample	100	n/a	100
14	E. Hake	authentic sample	100	n/a	100
15	Haddock (2%) A. Cod (98%)	admixture	100	80 ⁶	80
16	Hoki (5%) A. Cod (95%)	admixture	100	100	100
17	Pacific Cod	authentic sample	100	n/a	100
18	Coley	confirmed sample	100	n/a	100
19	Alaskan Pollock	confirmed sample	100	n/a	100
COD ⁷	Atlantic Cod	confirmed sample	100	n/a	100

¹ Samples supplied to participants were prepared from either authentic material (authentic sample) or from shop bought fillets which had been checked for species at CCFRA (confirmed sample). Admixtures were prepared from confirmed samples.

² A total of five labs participated in the trial and all five provided results contributing to percentages shown here.

³ One lab reported whiting as a possible species due to presence of 185bp fragments in NlaIII digests.

⁴ Results for these five samples are based on data from four labs only.

⁵ One lab reported the presence of hoki rather than Atlantic cod; however, analysis of the data files indicated only Atlantic cod and haddock were present in this sample. Values have been adjusted to reflect this correction.

⁶ Haddock was not reported by one lab.

⁷ This was a positive control sample used to confirm progress at each stage of the analysis. Although not reported by two labs, a check of the PCR-RFLP profiles produced with this sample indicated that Atlantic cod had been correctly identified.

An initial problem with the Ddel digests was reported by one of the labs; this was found to be due to the high salt concentration in the enzyme buffer (100mM \pm 3% NaCl). This concentration (100mM) is at the limit for the 2100 Bioanalyser and it was concluded that this was affecting the migration rates of the DNA in the capillaries of the DNA500 LabChip. The initial method was, therefore, modified to include a dilution step as shown in the method (Version 2) in Appendix 8.3. A summary of the modification is provided below:

- The original method had stated to add 1 μ L of 60mM EDTA to the 5 μ L digest. This resulted in a final concentration of 10mM EDTA. An aliquot (1 μ L) was then added to the DNA500 LabChip to generate PCR-RFLP profiles.
- The modification replaced these steps with the addition of 5 μ L of 20mM EDTA solution. This provided a 1 in 2 dilution of the DNA sample with a concurrent dilution of the salt concentration and also provided a final 10mM EDTA concentration. Again 1 μ L of this diluted DNA digest solution was added to the 2100 Bioanalyser.

3.5.2 Results from the ring-trial

Results obtained from the participants in the ring-trial are summarised in Table 9. Full results returned from each lab are shown in Appendix 8.4. In order to maintain anonymity each lab was assigned a random number. From the results returned to CCFRA it was clear that one of the labs (lab 3) had a problem during the analysis of some of the samples (samples 5–9). This was revealed by the presence of multiple PCR-RFLP fragments in the profiles, some of which did not correspond to fragments expected with any of the test fish species. This suggested that contamination of these samples had occurred during the analysis, which affected the detection of species in the samples. From an examination of data obtained following amplification of the 464bp PCR fragment it was observed that samples 6, 7, 8 and 12 contained two or three extraneous DNA fragments, most of which were between 438bp and 709bp. Sample 5; however, contained eight such fragments ranging from 129bp to 685bp. Sample 9 contained no extraneous PCR products.

Additionally this lab appeared to have had a problem with some samples analysed with HaeIII where the upper marker was not visible. It is likely that the loss of the upper marker was due to the EDTA not being fully mixed into these samples prior to loading them onto the 2100 Bioanalyser. In these samples the fragments detected were incorrectly sized and so it would not be possible to identify the species present using this enzyme.

Considering the problems outlined above, the five samples (numbers 5, 6, 7, 8 & 9) for this lab have been omitted from the general analysis of all results as described below; however, they have been considered separately at the end of this section.

3.5.2.1 Samples comprising a single species

From Table 9 it can be seen that of the eleven samples containing a single species, all were correctly identified by all labs (100%). This should be expected if the method is to be used for species identification. This result verifies that species can be identified using this approach without the need to run authentic samples along with the unknowns. Again this is an advantage of this method over the conventional protein identification method routinely used by many labs.

3.5.2.2 Cod and haddock admixtures

Admixtures of haddock and cod at, below and above the expected limit of detection (LOD) for this method were provided to participants in order that the 5% LOD could be verified. It was expected that all samples of 5% or 10% would be correctly identified by all labs but that some of the admixtures containing 2% haddock in cod or 2% cod in haddock may not be correctly identified as these are below the LOD.

Analysis of admixtures containing cod or haddock at 5 or 10% indicated that all labs correctly identified the presence of the major component in the admixture. When haddock comprised the minor species in these admixtures it was correctly identified by all labs; however, in the sample prepared from 5% Atlantic cod in haddock (sample 12), one lab mis-classified the Atlantic cod as hoki. From an examination of the raw data for this lab it is unclear how hoki was identified in this sample. It was, however, noted that the concentration of DNA fragments in some enzyme profiles

was low, which resulted in a low peak height on the electropherogram. This meant it was necessary to manually select some peaks in these profiles. Including these low concentration DNA fragments in the profiles indicated the presence of Atlantic cod and haddock only in this sample and not hoki. Also noted in one of the replicates of this sample was the presence of three DNA fragments of 284, 289 and 296bp, the middle one of which produced an intense peak on the electropherogram. It could be that these are the result of cleavage of a larger product (568bp) observed in the amplified PCR sample. However, these three fragments do not indicate the presence of hoki in this sample.

Of the five participants only one lab failed to identify the presence of 2% haddock in cod; however, all participants correctly identified the presence of 2% cod in haddock. Also the main species present at 98% was identified correctly by all participants. Examination of these revealed that fragments corresponding to the lower (2%) proportion fish species were visible on the electropherograms in all cases, including those where the lab did not formally identify the second species. However, the heights of the peaks (for the lower proportion species) were below the default threshold of the 2100 Bioanalyser and so were not recorded by the analyst. As the limit of detection for the method was defined as 5% it was not expected that species present below this level would be detected using the method. However, in these samples it was possible, by manually adjusting the peak detection threshold, to identify the fish species present at 2%.

3.5.2.3 *Admixtures of other fish species*

Three other admixtures (samples 1, 6 and 16) containing different fish species were also included in the trial. Of these admixtures sample 16, which contained 5% hoki and 95% Atlantic cod, was correctly identified by all 5 labs (100%). Sample 1 (10% whiting in 90% coley) was also correctly identified by all labs, although one lab reported this sample as possibly containing whiting due to the presence of the 186bp (reported 185bp and 187bp in replicates) fragment in the NlaIII digest pattern.

The final admixture (sample 6) contained 10% Pacific cod in 90% coley. All labs (100%) correctly identified the presence of coley in this sample; however, only one (lab 4) identified the presence of 10% Pacific cod in the sample. From an

examination of the data files it was possible to identify small peaks (especially the 235bp and 198bp fragments of Ddel) that indicate the presence of Pacific cod in these samples; however, the height of these peaks was below the default detection threshold (4 FUs) of the 2100 Bioanalyser.

It is likely that with more experience of this approach and in the interpretation of data from the 2100 Bioanalyser all labs will be able to identify the presence of fish species found at low levels in fish admixtures or products. A change to the method sent to participants in the trial to indicate that they should perform this manual adjustment is also recommended.

3.5.3 Results from laboratory 3

Results reported and data files from lab 3 indicated that this lab may have had problems with samples 5 – 9. These samples for this lab were excluded from the analysis above and are described in more detail here. Note that DNA fragment sizes reported below have been automatically detected by the 2100 Bioanalyser (as stated in the method) and have not been manually selected. A problem with HaeIII profiles, due to the loss of the upper marker, meant it would not have been possible for lab 3 to identify any species using this enzyme. It is not clear if this HaeIII problem was obvious to lab 3 who did not report any problems with the method to CCFRA. Re-analysis of the data from lab 3 by CCFRA staff is described below. Data for HaeIII was not used for this re-analysis.

3.5.3.1 *Sample 5 – SA hake*

Initial investigations of the check on PCR amplification indicated that this lab had produced extraneous PCR products in some of these samples, the worst being the presence of eight PCR products of between 129bp and 685bp in sample 5 (South African hake). Some of these PCR products were later detected in the PCR-RFLP profiles for this sample and were reported as part of the profile. This appears to have caused problems in the identification of SA hake and led to the belief that more than one species was present in this sample. From an examination of the raw data files for this lab it was seen that Ddel digests of this sample produced fragments of about 158bp and 288bp. This alone would indicate the presence of SA hake only in this

sample. Enzyme NlaIII does not cut SA hake and a large (477bp) fragment, with a high fluorescence, was easily detected on the profile for both replicates. Additional fragments of 129, 550 & ~566bp were also noted; however, these were observed following the check of the PCR and so it is suspected they were carried over from this reaction. None of the extra DNA fragments observed match those for any fish species, so it is not clear how Alaskan pollock was also reported for this sample.

3.5.3.2 *Sample 6 – Pacific cod (10%) and coley (90%)*

Lab 3 reported this sample as containing European hake, Atlantic cod and Alaskan pollock. As for sample 5, results for profiles generated using enzyme DdeI indicate the presence of coley, Pacific cod or Alaskan pollock only. Results from using enzyme NlaIII indicate the presence of coley and Pacific cod only and not Alaskan pollock; however, some extra fragments of over ~415bp might suggest the presence of either of the hake species. By combining the DdeI and NlaIII results the presence of only coley and Pacific cod should have been reported.

3.5.3.3 *Sample 7 – Atlantic cod (10%) and haddock (90%)*

This sample was reported as containing both Atlantic cod and haddock but in addition coley was identified. Again starting with results of analysis with DdeI it was found that one replicate had the correct fragments for the identification of Atlantic cod and haddock; however, the second replicate was reported to have fragment sizes of 284, 139 & 103bp. It was noted that the upper marker in this replicate had been incorrectly selected and that when adjusted correctly fragments of size 442, 236, 116 & 87bp were observed, which are as expected with cod and haddock. Data from NlaIII also supports the presence of haddock and either of the cod species. Although a couple of extra bands were seen in these replicates they do not correspond with fragments that would indicate the presence of coley. These products are possibly derived from extra fragments amplified during PCR.

3.5.3.4 *Sample 8 – E. plaice*

Although this sample contains only European plaice it was reported to contain plaice along with haddock, coley and whiting. From looking at the reported fragment sizes and data files for the DdeI profiles, it could be seen that one replicate had lost the upper marker, so fragment sizing was incorrect. The second replicate, however,

produced fragments indicative of *E. plaice* only. The reported fragments obtained with *NlaIII* supports the presence of *E. plaice*, although the presence of the 367bp fragment could also indicate the presence of *coley*. A combination of *DdeI* and *NlaIII* data indicates only the presence of *E. plaice* in this sample.

3.5.3.5 *Sample 9 – Whiting*

The *DdeI* fragments reported for this sample by lab 3 indicate the presence of whiting only. In fact this lab has achieved a very good separation of the 344bp and 355 bp fragments (reported as 354bp and 360bp). The *NlaIII* profiles suggest the presence of whiting or haddock in this sample, so by a combination of these two profiles only whiting should be identified.

3.5.4 Comments about the method

Participants in the trial were invited to make comments about the method. In general, from discussions with the participants, most seemed impressed with the method and the speed with which analysis could be achieved. A few additional specific comments were provided by some labs and are summarised below:

- Instead of running all PCR products on chips, which is wasteful, it would be better to run the controls and a random selection of samples
- Some condensation of samples occurred in overnight digests so a centrifugation step was required to recover solutions prior to loading on 2100 Bioanalyser.
- Some problems were reported with a loss of communication between the 2100 Bioanalyser and the laptop computer or the computer crashing during runs.
- Initial problems with *DdeI* samples were overcome by a 1:1 dilution of the digests prior to addition to the LabChip.

Note that this was performed before the method was modified to version 2.

- Some extra peaks were noted in some samples.

A particular 55bp fragment was noted in some samples. This is believed to be a PCR primer-dimer; however, its size does not correspond with any known fragments and it is not cleaved by any of the enzymes so does not detract from species identification.

- In some situations where fragments were visible on the electropherogram and gel image they were not automatically detected by the 2100 Bioanalyser. Manual intervention was required to select these fragments if they corresponded with fragments of known species.

This is the manual intervention noted above and is subject to user interpretation; however, given more experience users should be able to apply their judgement more reliably.

3.5.5 Summary of ring-trial results

Overall the results from this trial were very good, especially considering the lack of experience of the participants in the use and interpretation of data from the 2100 Bioanalyser. This suggests that this PCR-RFLP method is easy-to-perform, robust and should be readily transferable with a minimum of training to analytical laboratories wishing to perform this type of analysis. The only real problem observed in the method was the loss of the upper marker with enzyme HaeIII for five samples analysed by lab 3. This was not deemed to be a problem with the method but rather a problem with the experimental procedures of this lab as all other labs reported correct results for these samples.

Given that this method is not specific but offers a useful general screen to identify a wide range of fish species, if results similar to some of those reported above (i.e. difficulty in detecting a species at a low concentration, <5–10%) were obtained under normal conditions they would allow the analyst to perform a second, more specific check using a species-specific assay. This shows the advantage of this assay over the use of specific tests, where a wider range of specific assays would be needed to identify a limited number of species.

4. Conclusions

The results of this work show that use of the Agilent 2100 Bioanalyser for fish species identification is feasible and in some cases provides an improvement on PCR-RFLP fragment resolution and detection in comparison to conventional gel-based methods. Use of this system allowed the simultaneous post-digest analysis of 12 samples in under 40 minutes, which is a considerable time reduction on conventional gel-based methods. The small volumes (1 μ l) required by the system also allow savings to be made by reducing the overall PCR and restriction digest volumes. Further advantages of the 2100 Bioanalyser are the relatively small amount of space required for the system, compared to that required to run and stain acrylamide gels, as well as with the great reduction in the volume of potentially toxic DNA staining material used for detection by both systems. Handling of LabChips, which are of plastic and glass construction and only 3cm x 3cm, is considerably easier than handling acrylamide gels. LabChip manipulation is also negligible compared to conventional acrylamide gels which require handling during the DNA staining and detection stages. These advantages, i.e. speed, accuracy, ease of handling and reduced costs of consumables, make this system ideal for the further development of fish (or other species) identification methods based on PCR-RFLP or other DNA profiling techniques.

The limit of detection (LOD) of this method was determined to be around 5%; however, this was determined in only a limited number of fish admixtures and further work will be required to confirm that this limit is applicable to other fish species. There is also a question over this limit in respect of prior treatment (e.g. freezing, drying etc) of fish samples, which is beyond the control of the analyst, and how this may affect this limit. The method is, however, offered as a qualitative test for screening a sample for the presence or absence of fish species. As such this method is extremely useful as it has the potential to detect a wide range of fish species following the development of suitable PCR-RFLP profiles. The expansion of this method to include a wider range of fish species can be readily achieved, assuming that authentic fish materials can be obtained with which to generate profiles. It is

envisaged that this approach would be useful as an initial screen of a sample, after which a more accurate quantitative measurement could be applied if required.

The transfer of this method to other analytical labs with little or no experience of this type of analysis has shown that this approach offers a quick-to-learn and easy-to-use approach for the analysis of fish products. Analysts were also able to identify species present without the need to run reference materials alongside the samples, which is an added bonus as obtaining reference material for the development and checking of assays is time consuming and sometimes difficult to achieve. Analysis of unknowns without the need for reference material also means it is possible to process more samples per analytical run as space does not need to be reserved for the references themselves.

The only major draw-back of the method described here is the use of the relatively large 464bp PCR target which is not ideally suited to heavily processed products such as canned salmon, tunas or sardines. As such it would be prudent to develop a smaller (ideally <200bp) PCR target which would be better suited to the identification of canned fish species. It is likely that this approach would require the development of several genus-specific targets specifically designed for analysis of, for example, canned salmon, canned tuna or canned sardines.

5. Acknowledgements

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CCFRA is also indebted to the following people who have helped in securing authentic fish samples for this project.

Name	Company/Institute	Species supplied
Dr. Mike Canino	Alaska Fisheries Science Center, Alaska	Alaskan (Walleye) pollock
Dr. Andy Shinn	Institute of Aquaculture, Stirling University, Scotland	Atlantic salmon
Dr. Mark Trudel	Fisheries and Oceans Canada, Canada	Chinook, Coho, Chum, Sockeye & Pink salmon; Dolly varden & Cut-throat trout
Dr. Shigehiko Urawa	National Salmon Resources Center, Japan	Cherry salmon
Dr. Vicky Webb	National Institute of Water & Atmospheric Research, New Zealand	Hoki
Dr. Mark Winterbone	Institute of Food Research, UK	Haddock, Whiting, Coley, Atlantic & Pacific cod, E & SA Hake, Plaice

CCFRA is also indebted to Dr. Rainer Nitsche and Dr. Scott Harrison, from Agilent Technologies Ltd, who provided 2100 Bioanalyser instruments and technical expertise without which it would not have been possible to run the ring-trial, and to Dr. Jacqui Coutts, from Tepnel Biosystems, who provided DNA extraction kits for participants in the ring-trial.

Final thanks are offered to (in alphabetical order) Dr. Jacqui Coutts (Tepnel Biosciences), Dr. Hez Hird (Central Science Laboratory), Mrs. Hazel Pitman (Casella GMSS), Mrs. Carol Stevens (Worcestershire Scientific Services) and Dr. Hernan Valdivia (LGC), and all their staff who kindly participated in the ring-trial of the fish identification method.

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7. Figures

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).

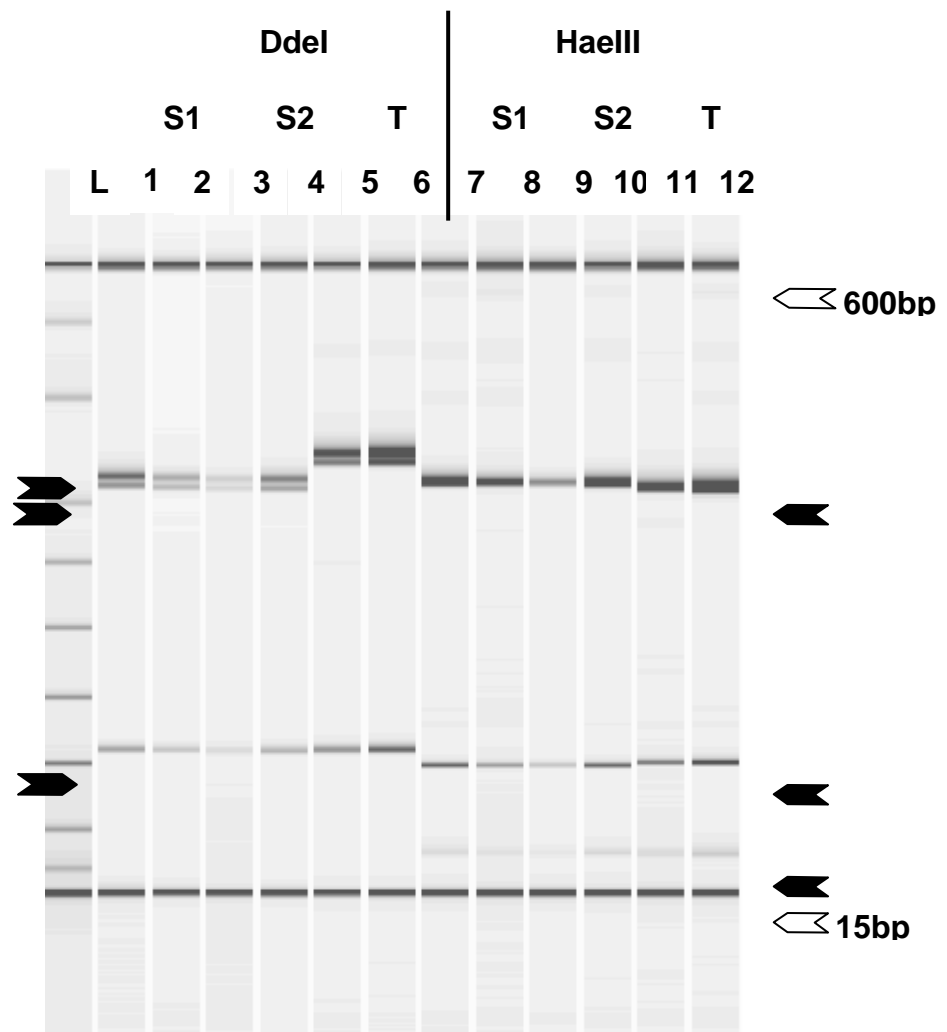


Figure 2: Sequence of the 464bp amplified fragment (L strand) from salmon and trout.

The Ddel restriction sites (CTNAG, underlined) and primer binding sites (bold italics) are shown. Cleavage in the H primer region occurs only when primers with 'C' bases are incorporated at position marked Y, otherwise no cleavage occurs when a 'T' base is incorporated at this position.

Salmon

AAAAACCACC **GTTGTTATTC** **AACTACAAGA** ACCTTAATGG CCAACCTCCG
 AAAAACTCAC CCGCTCCTAA AAATTGCTAA TGACGCACTA GTCGATCTCC
 CAGCACCATC TAACATCTCA GTTTGATGAA ACTTTGGCTC ACTCTTAGGC
 CTATGTCTAG CCACCCAAAT CCTTACCGGG CTCTTCCTAG CCATACACTA
 CACCTCCGAT ATCTCAACAG CTTTTTCCTC TGTTTGCCAC ATTTGCCGAG
 ATGTTAGCTA TGGCTGACTC ATCCGTAACA TTCACGCTAA CGGAGCATCT
 TTCTTCTTTA TCTGTATTTA TATACACATC GCCCGAGGAC TTTATTATGG
 TTCCTATCTA TATAAAGAAA CCTGAAATAT CGGAGTTGTA CTTCTACTTC
 TCACTATAAT AACTGCCTTC GTAGGCTACG TTCTTCCATG **AGGACAAATR**
TCCTTYTGAG **GAGC**

Trout

AAAAACCACC **GTTGTTATTC** **AACTACAAGA** ACCTAATGGC CAACCTCCGA
 AAAACCCACC CTCTCCTAAA AATCGCTAAT GACGCACTAG TCGACCTCCC
 AGCACCTTCT AATATCTCAG TCTGATGAAA CTTTGGCTCA CTACTAGGCC
 TATGTTTAGC TACCCTAATT CTTACCGGGC TCTTCCTAGC CATGCACTAT
 ACCTCCGACA TTTCAACAGC TTTCTCCTCT GTTTGCCACA TCTGCCGAGA
 TGTTAGTTAC GGCTGGCTCA TTCGAAACAT CCATGCCAAC GGAGCATCTT
 TCTTTTTTAT CTGTATTTAT ATACATATCG CCCGAGGACT TTRACTACGGC
 TCGTACCTCT ACAAAGAAAC CTGGAATATC GGAGTTGTAC TTTTACTTCT
 CACTATAATA ACTGCCTTTG TAGGCTACGT CCTCCCGTGA **GGACAAATRT**
CATTYTGAGG **ACGC**

Figure 3: PCR-RFLP patterns generated from salmon and trout admixtures with enzyme DdeI.

PCR-RFLP patterns obtained following cleavage of salmon (S) or trout (T) DNA alone (lanes 1 and 2) or as admixtures (lanes 3 – 9) with enzyme DdeI. A 15bp – 600bp ladder (L) is shown. All wells contain 15bp and 600bp size markers. DNA fragments of note are indicated (arrows).

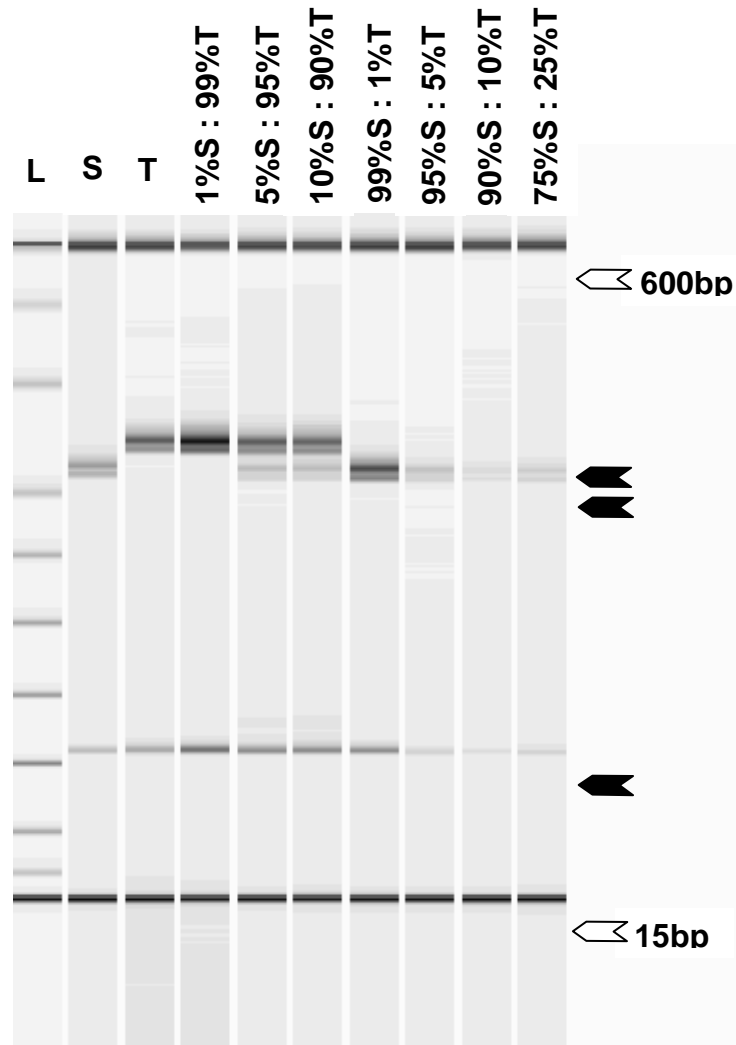
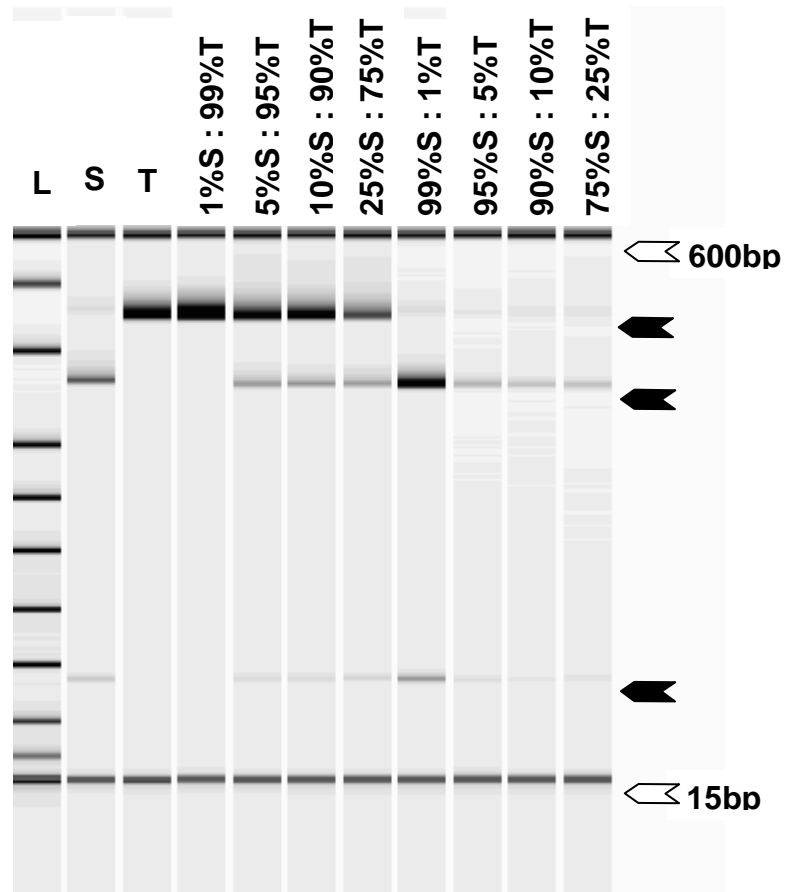


Figure 4: PCR-RFLP patterns generated from salmon and trout admixtures with enzyme *Sau3A*I. PCR-RFLP patterns obtained following cleavage of salmon (S) or trout (T) DNA alone (lanes 1 and 2) or as admixtures (lanes 3 – 10) with enzyme *Sau3A*I. A 15bp – 600bp ladder (L) is shown. All wells contain 15bp and 600bp size markers. DNA fragments of note are indicated (arrows).



T C T C A G T A T G A T G A A A T T T T G G C T C T C T T C T A G G C C T T T G C T T A A T T A C T C A A C T T T C T A A Majority

	120	130	140	150	160	170	
121	. T . . C . . T A T	Hoki
116	. T	A. Pollock
116	A. Cod
116	. T	P. Cod
116	Coley
117	E. Hake
117	SA. Hake
116	. T	Haddock
116	Plaice
116	Whiting
118	C. Salmon

C A G G A C T A T T T C T A G C C A T A C A C T A T A C C T C A G A C A T C G A G A C A G C C T T C T C A T C C G T A G Majority

	180	190	200	210	220	230	
181 C . . C . . C	Hoki
176	A. Pollock
176	A. Cod
176	P. Cod
176	Coley
177	E. Hake
177	SA. Hake
176	Haddock
176	. C . . C T C T	Plaice
176	Whiting
178	. C C . . C T	C. Salmon

T C C A C A T C T G C C G T G A T G T A A A T T A C G G C T G A C T A A T T C G A A A T A T A C A T G C C A A C G G T G Majority

	240	250	260	270	280	290	
241 G C T G C T A Hoki
236	. T T G T T A. Pollock
236 T C G T T A. Cod
236 T T G T P. Cod
236	. T . . T T G C T Coley
237	. A C C A C C C E. Hake
237	. A C G C C C SA. Hake
236	. T . . T T C C T Haddock
236	C A G C C T G C T C Plaice
236	. A . . T T C C T Whiting
238	G A T G T C C A C. Salmon

C C T C T T T C T T C T T C A T T T G T C T T T A T A T A C A T A T T G C C C G A G G T C T C T A T T A T G G C T C T T Majority

	300	310	320	330	340	350	
301 A . G C C T . A C C . T C A C T . A . . C . . C . . A . . A . . Hoki
296 T C G C C A. Pollock
296 T G C T A. Cod
296 T C P. Cod
296	. T T C G T A T C Coley
297	. T C C C C C A C A E. Hake
297	. T C C C C . C C A C A SA. Hake
296	. T T C T T Haddock
296	. A . . A T C A . C C C . T C . G C C Plaice
296 T C C Whiting
298	. A T A C G A T A C. Salmon

A C C T T T T G T A G A A A C A T G A A A C A T C G G A G T T G T T C T T T T C C T T T T A G T A A T A A T A A C C T Majority

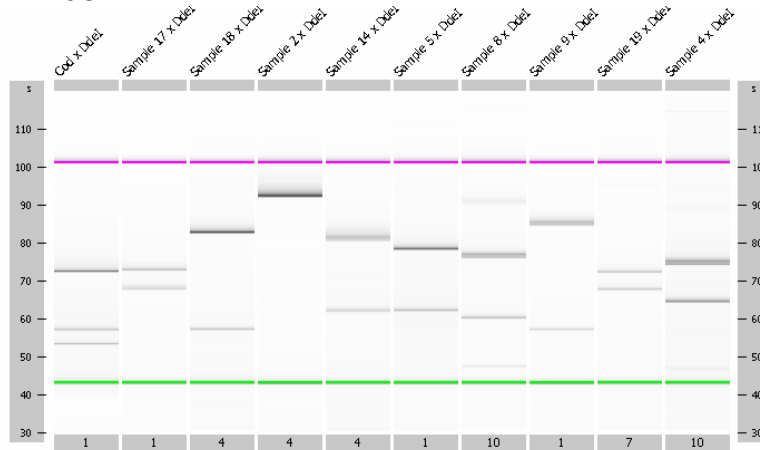
	360	370	380	390	400	410	
361	T . A . G . A C C . T	T	T . . A . . A T . G	C	T	T G Hoki
356	. T G	A. Pollock
356	. T G G C	A. Cod
356	. T C	P. Cod
356	. T . C T G	Coley
357	. . T . A . . C A G T A . A	G . . . G E. Hake
357	. . T . A . . . A C A . A	C C G SA. Hake
356	. T G G C G Haddock
356 A . A A G C T A C . G . . . C . C G . . A G Plaice
356 C G Whiting
358 G . A C A A C T A A C . C A C T G . . T G C. Salmon

C T T T C G T A G G C T A C G T C C T C C C T G A G G A C A A A T G T C A T T X T G A G G G C - G C Majority

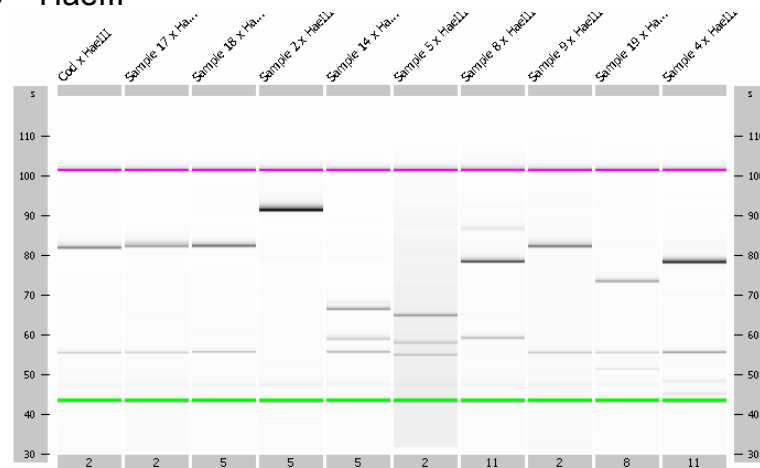
	420	430	440	450	460	
421	. C T G . . N . C . . Hoki
416 T . . T N - A. Pollock
416 T G . . N . - A. Cod
416 T - - P. Cod
416 T - Coley
417	. C T - E. Hake
417	. C T . . T - SA. Hake
416 T . . T G - Haddock
416	. C T T - Plaice
416 T . . T . .	. T G - Whiting
418	. A T T . . A N C C. Salmon

Figure 6: PCR-RFLP profiles from the ten 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.

A – DdeI



B – HaeIII



C – NlaIII



Figure 7: PCR-RFLP profiles generated from white fish DNA admixtures. Profiles were generated using enzymes DdeI, HaeIII or NlaIII and DNA amplified from the first four DNA admixtures (1 – 4) shown in Table 1b.

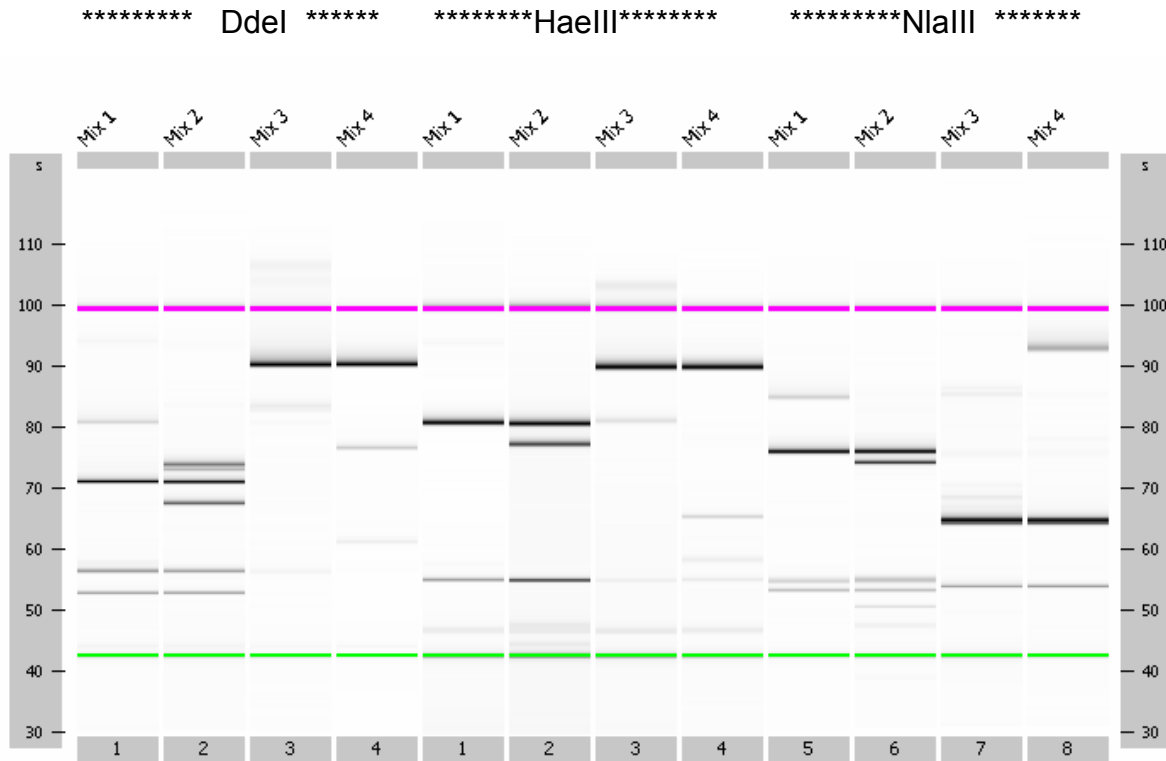
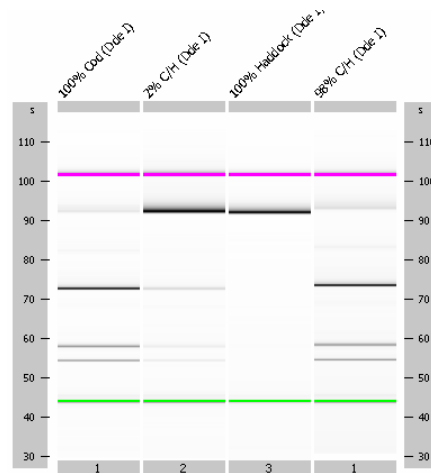


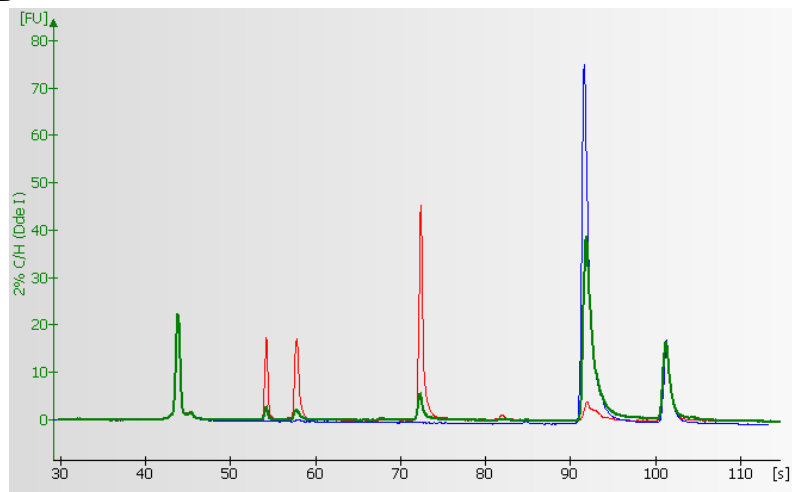
Figure 8: PCR-RFLP profiles generated from DNA admixtures of Atlantic cod and haddock.

Profiles were generated from DNA admixtures of 2% Atlantic cod in haddock and 2% haddock in cod using enzyme DdeI. Gel-like images of 100% cod and haddock and the 2% admixtures are shown in A. Electropherograms show the presence of species defining peaks for cod (red line) or haddock (blue line) when traces for 2% cod in haddock (B) or 2% haddock in cod (C) are superimposed (green line).

A



B



C

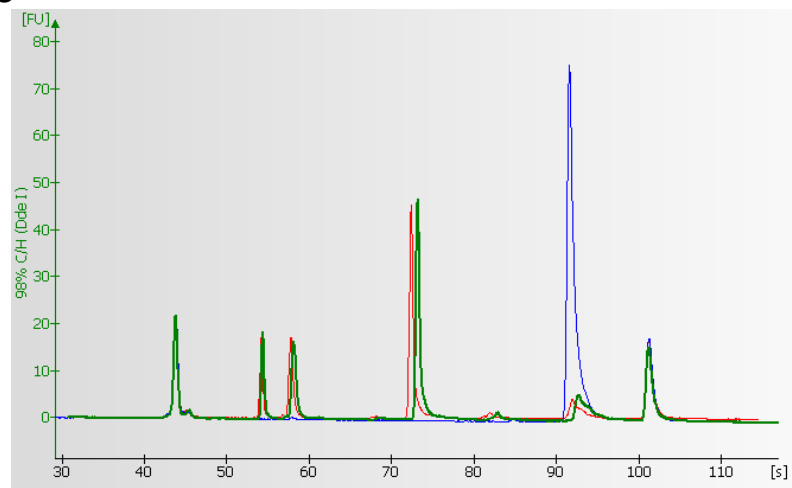


Figure 9: PCR-RFLP profiles generated from fish-meat admixtures of Atlantic cod and haddock. Profiles were generated from fish-meat admixtures of 5% Atlantic cod in haddock (top) and 5% haddock in cod (bottom) using enzymes DdeI, HaeIII and NlaIII. Electropherograms show the presence of species defining peaks for cod (red line) or haddock (blue line) when traces for 5% cod in haddock (B) or 5% haddock in cod (C) are superimposed (green line).

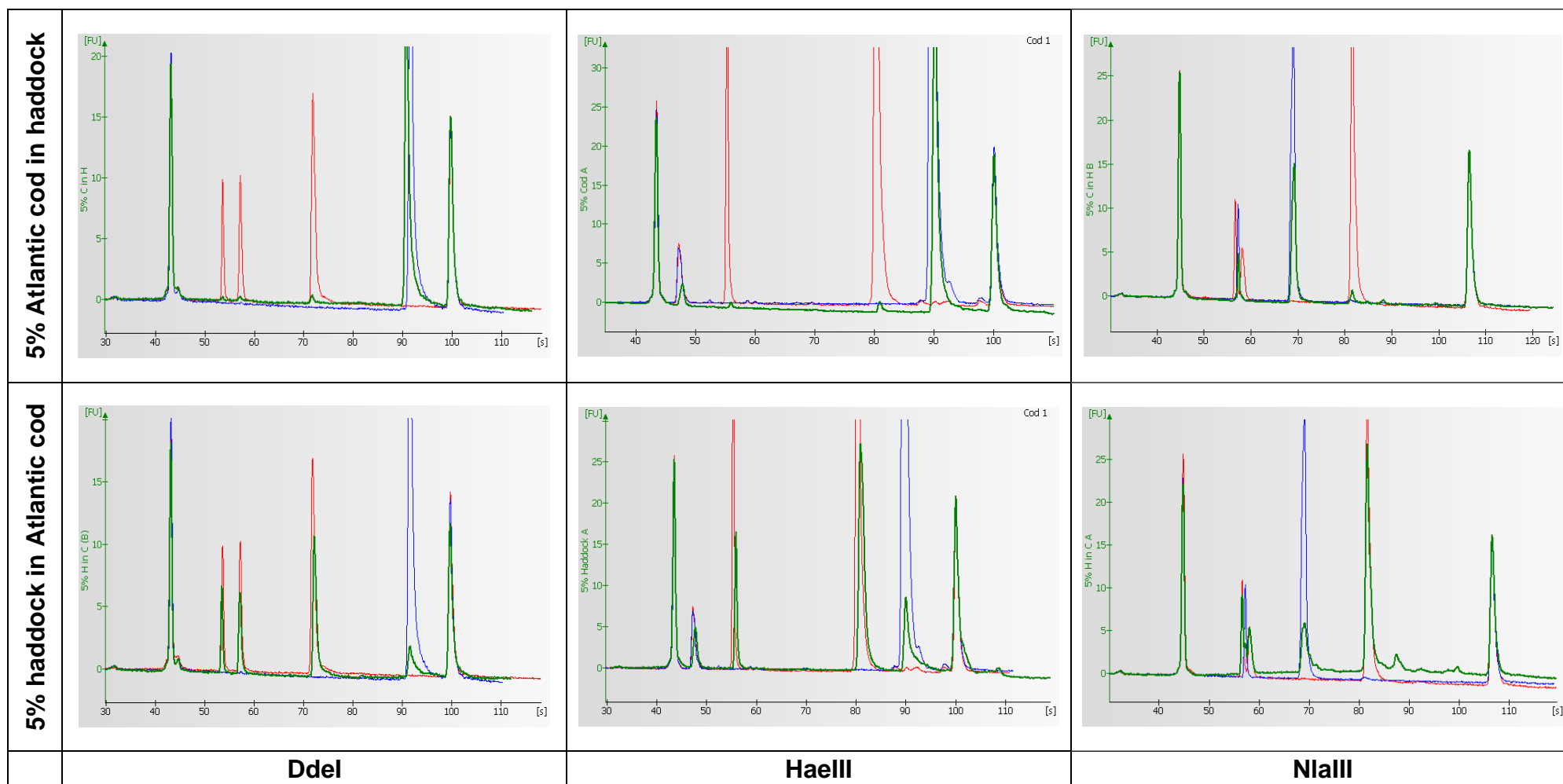
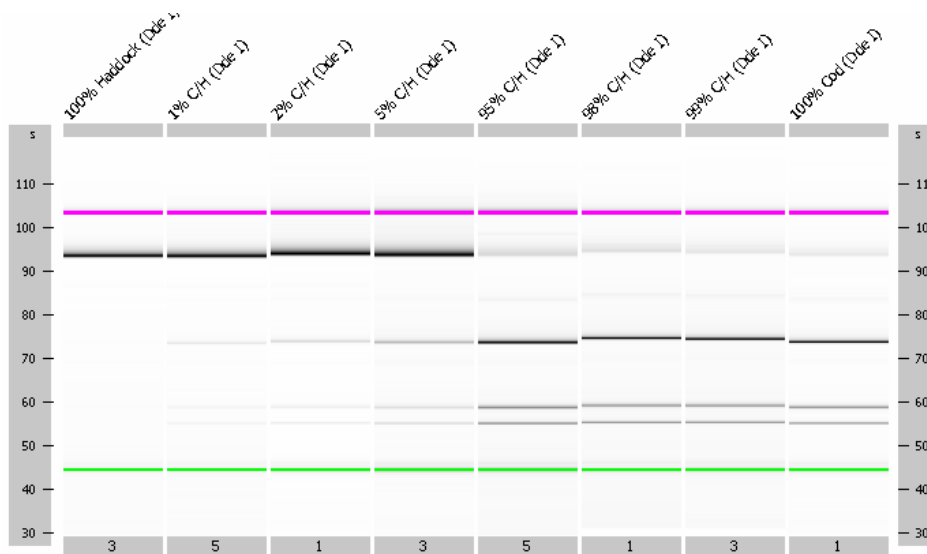


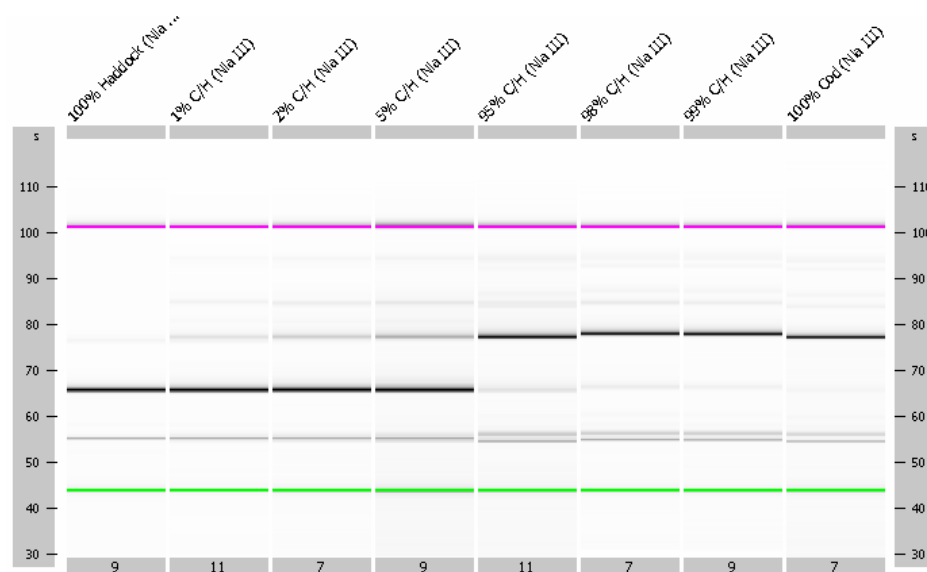
Figure 10: PCR-RFLP profiles generated from freeze-dried admixtures of Atlantic cod and haddock.

Profiles were generated from freeze-dried admixtures of 1–5% Atlantic cod in haddock and 1–5% haddock in cod using enzyme DdeI (A) or NlaIII (B). The intensity of cod or haddock fragments can be seen to increase or decrease depending on the amount of each species present in the sample. Profiles generated from 100% haddock or 100% cod are shown at the ends of each gel.

A



B



8. Appendices

8.1 Letters of authenticity supplied with authentic fish samples used in this work

8.2 PCR-RFLP fragments obtained following analysis of admixtures prepared from fish-meat.

Atlantic cod fragments are shown underlined, Haddock fragments are shown in italics. Figures in brackets were present in profiles but at a low concentration, which was not automatically detected by the 2100 Bioanalyser. Detection was based on the presence, in each sample, of species-specific PCR-RFLP profiles generated from authentic fish samples as shown in Table 7.

Analysis performed using enzyme DdeI

Admixture	PCR-RFLP fragments observed in sample	PCR-RFLP fragments expected with Atlantic cod	PCR-RFLP fragments expected with haddock	Species detected
25% haddock 75% A. cod	445, <u>238</u> , <u>117</u> , <u>87</u>	<u>234</u> , <u>115</u> , <u>84</u>	433	A. cod, Haddock
5% haddock 95% A. cod	452, <u>240</u> , <u>118</u> , <u>87</u>	<u>234</u> , <u>115</u> , <u>84</u>	433	A. cod, Haddock
2% haddock 98% A. cod	452, <u>240</u> , <u>118</u> , <u>87</u>	<u>234</u> , <u>115</u> , <u>84</u>	433	A. cod, Haddock
1% haddock 99% A. cod	451, <u>239</u> , <u>118</u> , <u>87</u>	<u>234</u> , <u>115</u> , <u>84</u>	433	A. cod, Haddock
25% A. cod 75% Haddock	443, (<u>237</u>), (<u>116</u>), (<u>86</u>)	<u>234</u> , <u>115</u> , <u>84</u>	433	A. cod, Haddock
10% A. cod 90% Haddock	441, <u>237</u> , <u>116</u> , <u>86</u>	<u>234</u> , <u>115</u> , <u>84</u>	433	A. cod, Haddock
5% A. cod 95% Haddock	441, (<u>237</u>), (<u>116</u>), (<u>86</u>)	<u>234</u> , <u>115</u> , <u>84</u>	433	A. cod, Haddock
2% A. cod 98% Haddock	441, (<u>237</u>)	<u>234</u> , <u>115</u> , <u>84</u>	433	A. cod ¹ , Haddock
1% A. cod 99% Haddock	441	<u>234</u> , <u>115</u> , <u>84</u>	433	Haddock

¹ Presence of species based on partial profile

Analysis performed using enzyme Haelll

Admixture	PCR-RFLP fragments observed in sample	PCR-RFLP fragments expected with Atlantic cod	PCR-RFLP fragments expected with haddock	Species detected
25% haddock 75% A. cod	422, <u>318</u> , <u>102</u> , <u>37</u>	<u>321</u> , <u>102</u> , <u>37</u>	429, 37	A. cod, Haddock
5% haddock 95% A. cod	423, <u>318</u> , <u>102</u> , <u>38</u>	<u>321</u> , <u>102</u> , <u>37</u>	429, 37	A. cod, Haddock
2% haddock 98% A. cod	421, <u>318</u> , <u>101</u> , <u>37</u>	<u>321</u> , <u>102</u> , <u>37</u>	429, 37	A. cod, Haddock
1% haddock 99% A. cod	Not tested	<u>321</u> , <u>102</u> , <u>37</u>	429, 37	
25% A. cod 75% Haddock	429, (<u>322</u>), (<u>39</u>)	<u>321</u> , <u>102</u> , <u>37</u>	429, 37	A. cod ¹ , Haddock
10% A. cod 90% Haddock	426, <u>320</u> , <u>103</u> , <u>39</u>	<u>321</u> , <u>102</u> , <u>37</u>	429, 37	A. cod ¹ , Haddock
5% A. cod 95% Haddock	424, (<u>318</u>), (<u>38</u>)	<u>321</u> , <u>102</u> , <u>37</u>	429, 37	A. cod ¹ , Haddock
2% A. cod 98% Haddock	425, (<u>318</u>), (<u>39</u>)	<u>321</u> , <u>102</u> , <u>37</u>	429, 37	A. cod ¹ , Haddock
1% A. cod 99% Haddock	Not tested	<u>321</u> , <u>102</u> , <u>37</u>	429, 37	

¹ Presence of species based on partial profile

Analysis performed using enzyme NlaIII

Admixture	PCR-RFLP fragments observed in sample	PCR-RFLP fragments expected with Atlantic cod	PCR-RFLP fragments expected with haddock	Species detected
25% haddock 75% A. cod	338, <u>281</u> , 185, <u>102</u> , 96, <u>91</u>	<u>280</u> , <u>100</u> , <u>89</u>	183, 94	A. cod, Haddock
5% haddock 95% A. cod	(337), <u>281</u> , 184, <u>102</u> , 96, <u>91</u>	<u>280</u> , <u>100</u> , <u>89</u>	183, 94	A. cod, Haddock
2% haddock 98% A. cod	(338), <u>281</u> , 184, <u>102</u> , 95, <u>91</u>	<u>280</u> , <u>100</u> , <u>89</u>	183, 94	A. cod, Haddock
1% haddock 99% A. cod	(346), <u>280</u> , 184, <u>102</u> , <u>91</u>	<u>280</u> , <u>100</u> , <u>89</u>	183, 94	A. cod, Haddock
25% A. cod 75% Haddock	<u>281</u> , 184, 96	<u>280</u> , <u>100</u> , <u>89</u>	183, 94	A. cod ¹ , Haddock
10% A. cod 90% Haddock	<u>282</u> , 185, 97	<u>280</u> , <u>100</u> , <u>89</u>	183, 94	A. cod ¹ , Haddock
5% A. cod 95% Haddock	<u>281</u> , 185, 97	<u>280</u> , <u>100</u> , <u>89</u>	183, 94	A. cod ¹ , Haddock
2% A. cod 98% Haddock	(<u>280</u>), 184, 96	<u>280</u> , <u>100</u> , <u>89</u>	183, 94	A. cod ¹ , Haddock
1% A. cod 99% Haddock	(<u>281</u>), 185, 96	<u>280</u> , <u>100</u> , <u>89</u>	183, 94	A. cod ¹ , Haddock

¹ Presence of species based on partial profile

8.3 Protocol and recording sheets provided for participants in ring-trial

FISH & CHIP METHOD TRIAL

**CONDUCTED BY CAMPDEN & CHORLEYWOOD FOOD
RESEARCH ASSOCIATION**

FOR FOOD STANDARDS AGENCY PROJECT Q01069

METHOD

**IDENTIFICATION OF FISH SPECIES BY PCR-RFLP
ANALYSIS USING THE AGILENT 2100 BIOANALYSER**

30/04/04

VERSION 2

1. INTRODUCTION

Fish species identification can be achieved by generating specific DNA fingerprint patterns that can be compared to those produced from authentic reference samples.

In this method, the polymerase chain reaction (PCR) is used to amplify a common fish target sequence from the mitochondrial cytochrome b gene. Species-specific fragment patterns are then produced using DNA cleaving enzymes. The fragment patterns are quickly resolved on a chip-based capillary electrophoresis instrument, the Agilent 2100 Bioanalyser.

2. SCOPE AND FIELD OF APPLICATION

This method is suitable for the qualitative determination of species present in raw, frozen and cooked products containing fish.

3. REFERENCES

Russell *et al.* (2000) *J. Agric. Food Chem.* **48**, 2184-2188.

Hold *et al.* (2001) *Eur. Food Res. Technol.* **212**, 385-89.

4. PRINCIPLE OF METHOD

The polymerase chain reaction (PCR) is used to detect DNA sequences in living organisms and in materials derived from living organisms. It relies on the binding of single-stranded DNA fragments (primers) to a specific DNA target sequence and the copying of this target in the presence of excess amounts of DNA subunits (nucleotides) and a DNA polymerase (Taq). Multiple cycles at specific temperatures result in the million-fold copying of the target sequence.

Restriction enzymes are naturally produced by bacterial strains to degrade DNA at sequence specific sites, e.g. EcoR1 only cuts the six base-pair pattern G↓AATTC between the G and first A as shown. By selecting the correct enzymes it is possible to digest total DNA from different species to produce species-specific DNA fragments. These fragments can be separated by electrophoretic methods to produce species-specific patterns known as restriction fragment length polymorphism (RFLP) fingerprints. However, the standard technique requires large amounts of purified DNA and can be affected by processing conditions applied to food.

PCR-RFLP techniques combine DNA amplification and RFLPs to produce fewer fragment patterns that are easier to interpret. They also have the advantage that only small amounts of DNA are required as the PCR step increases the amount of template DNA for restriction digests. In this method, PCR primers are used to amplify a mitochondrial cytb gene sequence that is common to all vertebrate fish species. Restriction enzymes (three in total) are used to digest amplified DNA to produce species-specific fingerprints. Species identification is achieved by separating DNA fragments by capillary electrophoresis using the Agilent 2100 Bioanalyser and a DNA500 LabChip. Individual species are identified by their unique fingerprint patterns.

5. Equipment and reagents to be provided by participant
- 5.1 **Oven with rotator or shaker for constant heating at 65°C**
- 5.2 Applied Biosystems PE9600 Thermocycler or alternative 96 well thermocycler with heated lid
- 5.3 Benchtop micro-centrifuge
- 5.4 Benchtop whirlimixer/vortex.
- 5.5 Set of Gilson pipettes or equivalent in size range P2, P10, P20, P100, P200, P1000
- 5.6 Sterile filter pipette tips to fit pipettes as shown.
- 5.7 Laminar flow hood with UV facility. If unavailable a separate, sterile area suitable for the preparation of PCR reactions.
- 5.8 Sterile Eppendorf tubes – 0.5ml, 1.5 ml and 2.0ml (preferably screw cap)
- 5.9 Sterile 0.2 ml thin walled PCR tubes

5.10 Sterile molecular biology grade water (MBGW)

5.11 Ice

6. Equipment and reagents provided via CCFRA

- 6.1 Agilent 2100 Bioanalyser and laptop computer. **(This will be provided by Agilent)**
- 6.2 DNA 500 LabChips **(provided by Agilent)**
- 6.3 Mini vortex for shaking LabChips **(provided by Agilent)**
- 6.4 Tepnel DNA extraction Kit and magnetic rack **(provided by Tepnel)**
- 6.5 Two tubes of Fish DNA Amplification Mastermix labelled "PCR MIX".
- 6.6 AmpliTaq Gold labelled "TAQ"
- 6.7 Three restriction enzymes labelled "DdeI", "HaeIII" or "NlaIII"
- 6.8 Buffers for restriction digests labelled "BUFF 2", "BUFF 3" or "BUFF 4"
- 6.9 Stock BSA (100x)
- 6.10 20mM EDTA solution labelled "EDTA"
- 6.11 Table of expected fragment sizes for authentic fish samples **(Table A)**
- 6.12 Authentic Species Master Sheet showing expected patterns for each of the authentic fish species **(Chart B)**
- 6.13 Blank Restriction Fragment Pattern Recording Sheets for recording of results for each unknown sample provided. **(Chart C)**
- 6.13 CCFRA Fish & Chip Trial Recording Sheet **(Table D)**
- 6.14 CD-R for burning result files onto

7. Unknown samples and control samples

- 7.1 One freeze dried cod sample (20-40mg) in a 2ml screw capped micro-tube labelled "COD". This is the control sample.
- 7.2 Nineteen freeze dried fish samples (20-40mg) in 2ml screw capped micro-tubes labelled with sample specific codes.

8. ANALYTICAL PROCEDURE

8.1 DNA extraction using Tepnel Biokits DNA extraction kit (Follow instructions for DNA extraction from Raw Meat Samples)

Extractions should be performed in the 2ml tubes containing samples.

An extraction blank (a tube without any sample) should also be analysed. Handle this tube last.

The following adjustments to the protocol are required when using the freeze dried samples provided.

Step 10.2.1: Add 800µL of Tissue Extraction Solution to the samples and 40µL of the Tepnel proteinase K solution, i.e. double the volumes stated in the Tepnel protocol.

Note that the samples will absorb some of the extraction buffer and form a gelatinous lump in the bottom of the tube. This should be broken up during incubation by vortexing every 15 minutes.

New step: Perform steps as detailed in Tepnel protocol 10.12.1 – 10.12.3. Do not perform step 10.12.4 at this stage but leave beads in water.

Steps 10.6 – 10.10: Ignore these steps.

Step 10.11: Centrifuge at 10-12,000g for 10minutes.

Step 10.12.4: While samples are centrifuging (step 10.11) perform this step as detailed in the Tepnel protocol.

Step 10.13: It should be possible to transfer about 600-700µL of solution at this stage.

Step 10.29: Store DNA at 2-4°C for up to 2 weeks.

New step: The extracted DNA should be diluted 1 in 100 with MBGW water prior to PCR amplification.

8.2 PCR amplification of cytb Sequences

This method is suitable for the analysis of DNA extracts from fish products that have been diluted 1 in 100.

The method described is suitable for analysis of 10 samples, in duplicate, with associated controls, i.e PCR positive, PCR negative and extraction negative and ~2 spare samples.

Note 1: PCR should be performed in a clean, sterile area away from the DNA preparation and post PCR work areas. Ideally PCR should be performed in a dedicated PCR hood with UV light sterilisation facilities.

Note 2: Use sterile filtered pipette tips and wear disposable gloves during the procedure.

8.2.1 Remove a tube of Fish DNA Amplification Mastermix (labelled PCR MIX) from the freezer and allow to thaw completely. Once thawed vortex for 20 seconds and recover solutions by centrifuging at 16,000g for 20 seconds.

Note: One tube of mastermix (407µL) is sufficient for 25 PCR reactions @20µL final volume.

8.2.2 Label enough PCR tubes for reactions allowing two tubes per unknown sample and three additional tubes for the PCR positive, PCR negative and extraction negative controls.

8.2.3 Place all tubes in suitable rack and place into PCR hood. Tubes can also be UV sterilised if you have this facility.

8.2.4 Add 5.50µL of AmpliTaq Gold to the PCR MIX tube and mix thoroughly by vortexing for 20 seconds.

8.2.5 Centrifuge tubes at 16,000g for 30 seconds to recover solution.

8.2.6 Aliquot 15µl of PCR MIX into two replicate tubes for each sample to be tested.

PCR positive and PCR negative controls and an extraction negative control should also be prepared by aliquoting 15µl of mastermix into a tube for each control.

8.2.7 Add 5µl of diluted DNA solution into the two replicate wells for each sample. Use a fresh tip for each replicate. Cap each tube after adding DNA solution.

- 8.2.8 Repeat 8.2.7 for each unknown sample and control DNA sample (PCR positive and DNA extraction negative).
- 8.2.9 Add 5µl of sterile MBGW to the PCR negative tube. Cap the tubes after adding water.
- 8.2.10 Transfer PCR tubes to the post-PCR area.
- 8.2.11 The following steps should be performed in an area away from the DNA extraction and PCR set-up areas.
- 8.2.12 Place PCR tubes into the thermocycler.
- 8.2.13 Programme and run the thermocycler to apply the amplification profile shown in Table 1.
- 8.2.14 After the PCR programme is complete, remove tubes from thermocycler and store samples at 1°C to 6°C for up to 1 month. Alternatively, PCR products can be stored for up to six months between -15°C and -22°C.

Note: Do not return PCR products to the DNA extraction & PCR set up areas.

Table 1: PCR Amplification Conditions

	Step	Action
PCR program	95°C/5 min	Enzyme activation
	95°C/40 sec	DNA denaturation
	50°C/80 sec 40 cycles	Primer annealing
	72°C/80 sec	DNA extension
	72°C/7 min	Final extension
	4°C/hold	(optional)

8.3 Confirmation of cytb Gene Amplification

8.3.1 Before proceeding with restriction enzyme digests, run 1µl of each PCR reaction mix on an Agilent DNA500 LabChip (refer to step 8.5 and 2100 user manual for instructions).

When run is complete check results to confirm:

- A PCR fragment of about 464bp (+/- 10%) was amplified in all sample wells
- Amplified fragment concentration is in excess of 100 fluorescence units
- Good amplification occurred in the positive control (>100 fluorescence units)
- No amplification occurred in the negative controls

8.4 Restriction Digestion of PCR Products

The preparation of restriction digest reactions should be performed on ice. Restriction enzymes should only be taken from the freezer (-20°) for as short a time as possible and handled as little as possible.

Stock BSA (100x) should be diluted 1 in 30 with sterile MBGW prior to use in reaction set-up.

There is no need to perform restriction digestion on the PCR negative or extraction negative control. The PCR positive control should be used.

The method described is suitable for analysis of 10 samples, i.e. 20 PCRs (10 samples in duplicate) with a restriction digest control. For other numbers of samples adjust volumes according to Table 2, column 3 or 5.

8.4.1 Label sixty 0.2ml PCR tubes with sample name and restriction enzyme and arrange in suitable rack. Also include a tube for positive controls for each enzyme. Place all tubes on ice.

8.4.2 For each enzyme reaction prepare a restriction mastermix (REST MIX) as shown in Table 2 in a 1.5ml Eppendorf tube.

Note: BSA is required by the enzyme Nla III

8.4.3 Vortex the REST MIX thoroughly to mix. Centrifuge at 16,000g for 15 secs to recover the solution.

- 8.4.4** Aliquot 2.5µl of the REST MIX to the respective labelled PCR tubes.
- Leave the tubes on ice while performing the next steps.
- 8.4.5** Add 2.5µl of PCR product to the respective labelled PCR tubes.
- 8.4.6** Place the tubes in the PCR thermocycler and incubate samples for at least 4 hours (or overnight) at 37°C. Terminate reactions by heating samples to 65°C for 10 minutes.
- 8.4.7** Add 5µL of EDTA (20mM) to each 5µL digest and mix with pipette tip to achieve a final concentration of 10mM EDTA.
- 8.4.8** Samples can be stored at 2°C to 6°C for up to 1 week.

Table 2: Preparation Volumes for Restriction Digest Mastermix (REST MIX)

Component	Final Concentration	Digest set-up using enzymes DdeI & HaeIII		Digest set-up using enzyme NlaIII	
		Volume for 1 Digest	Volume for 21 Digests ¹	Volume for 1 Digest	Volume for 21 Digests ¹
10x Buffer ²	1x	0.5	11.55	0.5	11.55
Enzyme		0.5	11.55	0.5	11.55
BSA	1x	~	~	1.5	34.65
MBGW	~	1.5	34.65	~	~
DNA		2.5		2.5	
Total Volume	~	5		5	

1. Equivalent to 10 samples and a control.

2. See Table 3 for correct buffer to use with each enzyme.

Table 3: Restriction enzyme and buffer combinations

Enzyme	Reaction Buffer	Incubation Temperature (°C)
Dde I	Buffer 3	37

Hae III	Buffer 2	37
Nla III	Buffer 4	37

8.5 Fingerprinting samples on Agilent 2100 Bioanalyser

A DNA500 LabChip is used to generate PCR-RFLP profiles on the Agilent 2100 Bioanalyser. Individual LabChips are supplied in sealed packs and should only be opened when needed.

For detailed instructions on the use of the 2100 Bioanalyser refer to the instrument manual and the Reagent Kit Guide.

- 8.5.1 Remove tubes containing prepared gel matrix*, DNA size ladder (yellow cap) and upper and lower size markers (green cap) from 4°C and leave to warm to room temperature for 1 hour.
- 8.5.2 Prime DNA500 LabChip using prepared gel matrix.
- 8.5.3 Load 5µl of size markers into all sample wells, ensuring marker settles onto bottom of well and does not remain on sides.
- 8.5.4 Load 1µl of ladder into the well labelled with a ladder symbol, ensuring ladder settles onto bottom of well and does not remain on sides.
- 8.5.5 Aliquot DNA samples (1µl) into the sample wells (one sample per well). Ensure samples have settled onto bottom of well and have not remained on sides of well. Fill any spare wells with 1µl of size marker.

If possible put DNA samples from all three digests onto the same chip i.e. 4 samples each with 3 enzyme digests can be run on a single chip.
- 8.5.6 Use the Agilent shaker to vortex the chip for 1 minute at 2,400 rpm, then place the chip in the 2100 Bioanalyser.
- 8.5.7 Select chip assay type as DNA500 assay. Press start when chip is ready and wait for 1 – 2 minutes to ensure analyser starts and there are no problems with chip.
- 8.5.8 If chip error is reported :
 - a. stop run and remove chip.

* See Agilent “Reagent Kit Guide” for instructions on preparing gel-matrix.

- b. check chip wells to ensure samples are in bottom of wells and are not adhering to sides. If sample is on the side of the well use a pipette to move it onto the base of the well. Reload chip into analyser and restart run.
- c. If all samples are in bottom of wells invert chip and examine chip capillaries for bubbles. If chip contains bubbles discard chip and prepare a new chip
- d. If problems persist consult Agilent. You may need to run a full instrument diagnostics test.

8.5.9 During the run, enter sample and enzyme details so that each lane can be easily identified

8.5.10 After run is complete remove DNA chip from analyser and clean analyser pins with cleaning chip containing 350µl MBGW.

8.5.11 Save data file and print results of analysis, including gel image and report of fragment sizes.

8.5.12 Repeat 8.5.2 to 8.5.11 for additional chips required to run all enzymatic reactions with all samples.

9. ANALYSIS OF RESULTS

9.1 PCR Amplification

PCR products resulting from the amplification of DNA extracted from samples are separated according to size using a DNA500 LabChip.

9.1.1 The presence of a PCR product is indicated by a single, tight band on the LabChip conforming to the following criteria:

- a. the band can be aligned with appropriate PCR products from the positive controls.
- b. the PCR product should be 464* bp ± 10% in size.

* Note the hoki product is 469bp but is not readily differentiated from the 464bp product.

- c. the intensity of the PCR product band must be greater than the intensity of the size marker (15 & 600bp) bands and ideally greater than 100 fluorescence units.

If any of these criteria are not met the PCR must be repeated.

9.2 Restriction Digestion

9.2.1 Complete digestion of PCR products from samples is assumed based on comparisons to digestions of the positive control (COD) sample. The COD sample should show complete digestion with specific enzymes to produce DNA fragments of sizes shown in Table 4 or as indicated as Atlantic Cod on the Authentic Species Master Sheet provided (**Chart B**).

9.2.2 If expected fragments are not observed in positive control it is likely that complete digestion has not occurred in any of the samples. All samples will require reanalysing.

If positive control DNA shows complete digestion with a specific enzyme, it is assumed that enzymatic digestion of sample DNA has also proceeded to completion with that enzyme.

Table 4: Expected PCR-RFLP patterns for positive control (COD) sample with each enzyme

Enzyme	Expected fragment sizes (bp)	Acceptable size range (bp)
Dde I	84	76 - 92
	115	103 - 125
	234	210 - 256
Hae III	37	33 – 41
	102	92 – 112
	321	289 – 353
Nla III	89	80 – 98
	100	90 – 110
	280	252 - 308

9.3 Analysis of unknown samples

Unknown samples should be analysed for the presence of PCR-RFLP fragments which are indicative of each species..

9.3.1 Once a DNA LabChip has been run and data has been obtained select the “*Electropherogram*” tab to view the peak plots for each sample.

9.3.2 Using the Toolbar along the top of the screen select “*Electropherogram*” and scroll down and select “*View Single Sample*” to view a single sample.

9.3.3 Using the Toolbar along the top of the screen select “*Electropherogram*” and scroll down to select “*Peak Description*” and scroll down and select “*Peak Size*” to show the peak size on each peak

9.3.4 View each sample in turn and use the table of peak information (“*Peak Table*” tab) along the bottom of the screen to determine the sizes of peaks in each sample.

9.3.5 Use the fragment sizes to complete the blank Restriction Digest Pattern Recording Sheet (**Chart C**) by shading in the appropriate box corresponding to the peak sizes observed with each of the three enzymes for each sample. If there are two fragments of a similar size also put 2 in the box.

On the authentic species master sheet, the boxes are colour coded for easier pattern matching.

Red – Ddel Blue – HaeIII Green - NlaIII

When complete each sample should have three distinct patterns, one for each enzyme, corresponding to the fragment sizes observed.

9.3.6 Repeat step 9.3.4 to 9.3.5 for each sample.

9.3.7 Compare the fragment patterns from each sample with those found on Authentic Species Master Sheet provided (**Chart B**).

The patterns produced should generally be the same as those found on the authentic species master sheet, however there could be slight changes in the pattern (shifts in the shaded boxes) due to the variability in fragment size measurement on the bioanalyser.

Very small fragments (<35bp) are sometimes not detected but the patterns from the larger fragments can still be used.

A table showing the expected sizes of fragments from the authentic species is also provided (**Table A**) This can be used to double check the samples once the pattern matching has been carried out.

- 9.3.8 Also complete the CCFRA Fish & Chip Trial Result Recording Sheet (**Table D**) results sheet stating which species was found in each sample and the fragment sizes generated by the enzymes.

10. QUALITY CONTROL

10.1 Negative PCR Controls

- 10.1.1 The purpose of the negative controls are to identify if contamination has occurred during the extraction or PCR procedures.

- 10.1.2 An extraction negative must be prepared with every batch of DNA extracts. The extraction negative is usually manipulated last at each stage of the process, to pick up any possible source of contamination.

- 10.1.3 A PCR negative must be used as a method control with every set of samples amplified at the same time. For a PCR negative, 5µl sterile Milli-Q water replaces the sample DNA extract, when setting up the PCR.

The PCR negative should be manipulated last at each stage of the process, to pick up any possible source of contamination.

- 10.1.4 Both the extraction negative and PCR negative should show no PCR product present. Presence of a PCR product indicates contamination has occurred and the PCR batch is invalid and all samples must be re-amplified.

Note that occasionally low levels of PCR product are observed in these samples. This product is believed to come from residual DNA in the Taq enzyme. This result is acceptable if the fluorescence of the product is equivalent or less than that of the DNA size markers. Strong amplification is not acceptable.

- 10.1.5 An extraction negative showing a visible band of equivalent size to the positive PCR product means the PCR batch is invalid and all samples must be re-amplified. If the band persists after re-amplification, the extraction batch is invalid and all samples must be re-extracted.

10.2 Positive PCR Controls

A positive control (COD) is provided to ensure each stage has been completed successfully. The positive must be treated as an unknown sample during DNA extraction and PCR stages.

10.3 Restriction Digest Quality Control

10.3.1 The digestion positives should produce bands of sizes shown in Table 4. If digestion is incomplete, i.e. undigested DNA remains, digestion assays with that enzyme should be repeated.

10.3.2 Digested products should be in the range 15bp to 464bp ($\pm 10\%$) (largest amplicon size). If products are outside this range there is a problem with the DNA LabChip sizing. The samples should be reanalysed on a fresh LabChip.

10.4 Agilent quality control

10.4.1 See the Agilent Reagent Kit Guide, page 28 onwards for quality control measures applicable to the chips.

11 REPORTING RESULTS

The analysis of the samples and reporting of the work should be carried within the time of placement of the bioanalyser.

We would like to have all the data at CCFRA by 4th June at the latest.

The following should be completed and returned to CCFRA to report the results obtained for each lab.

- 11.1 A completed table (**Table D**) showing each sample ID code, the fragment sizes obtained with each enzyme, and the species determined to be present in that sample
- 11.2 A completed (shaded) restriction digest pattern results sheet (**Chart B**) showing the observed fragment sizes for each sample with each of the three enzymes.
- 11.3 Electronic copies of all the data files generated burned on to the CD-R supplied.
- 11.4 Any comments about the protocol, use of the analysis sheets, use of the Agilent bioanalyser, or any further comments would be welcome

Contacts during the trial

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8.4 Individual results returned by participants in ring-trial following analysis of 19 freeze-dried fish samples

Sample ID code	Species present in sample	Comments about sample ¹	Species identified by each lab ²				
			Lab 1	Lab 2	Lab 3	Lab 4	Lab 5
1	Whiting (10%) Coley (90%)	admixture	Coley Whiting	Coley Whiting	Coley Whiting	Coley Whiting	Coley Whiting ³
2	Haddock	confirmed sample	Haddock	Haddock	Haddock	Haddock	Haddock
3	Haddock (10%) A. Cod (90%)	admixture	Atlantic Cod Haddock	Atlantic Cod Haddock	Atlantic Cod Haddock	Atlantic Cod Haddock	Atlantic Cod Haddock
4	Hoki	confirmed sample	Hoki	Hoki	Hoki	Hoki	Hoki
5	SA Hake	authentic sample	SA Hake	SA Hake	SA Hake A. pollock	SA Hake	SA Hake
6	Pacific Cod (10%) Coley (90%)	admixture	Coley	Coley	Atlantic Cod A. pollock E. Hake	Coley Pacific Cod	Coley
7	A. Cod (10%) Haddock (90%)	admixture	Atlantic Cod Haddock	Atlantic Cod Haddock	Atlantic Cod Haddock Coley	Atlantic Cod Haddock	Atlantic Cod Haddock
8	E. Plaice	authentic sample	E. Plaice	E. Plaice	E. Plaice Haddock Coley Whiting	E. Plaice	E. Plaice
9	Whiting	authentic sample	Whiting	Whiting	Whiting Haddock E. Hake E. Plaice	Whiting	Whiting
10	Haddock (5%) A. Cod (95%)	admixture	Atlantic Cod Haddock	Atlantic Cod Haddock	Atlantic Cod Haddock	Atlantic Cod Haddock	Atlantic Cod Haddock

(continued)

Sample ID code	Species present in sample	Comments about sample ¹	Species identified by each lab ²				
			Lab 1	Lab 2	Lab 3	Lab 4	Lab 5
11	A. Cod (2%) Haddock (98%)	admixture	Atlantic Cod Haddock	Atlantic Cod Haddock	Atlantic Cod Haddock	Atlantic Cod Haddock	Atlantic Cod Haddock
12	A. Cod (5%) Haddock (95%)	admixture	Atlantic Cod Haddock	Atlantic Cod Haddock	Hoki Haddock	Atlantic Cod Haddock	Atlantic Cod Haddock
13	Alaskan Pollock	confirmed sample	A. Pollock	A. Pollock	A. Pollock	A. Pollock	A. Pollock
14	E. Hake	authentic sample	E. Hake	E. Hake	E. Hake	E. Hake	E. Hake
15	Haddock (2%) A. Cod (98%)	admixture	Atlantic Cod	Atlantic Cod Haddock	Atlantic Cod Haddock	Atlantic Cod Haddock	Atlantic Cod Haddock ⁴
16	Hoki (5%) A. Cod (95%)	admixture	Atlantic Cod Hoki	Atlantic Cod Hoki	Atlantic Cod Hoki	Atlantic Cod Hoki	Atlantic Cod Hoki
17	Pacific Cod	authentic sample	Pacific Cod	Pacific Cod	Pacific Cod	Pacific Cod	Pacific Cod
18	Coley	confirmed sample	Coley	Coley	Coley	Coley	Coley
19	Alaskan Pollock	confirmed sample	A. Pollock	A. Pollock	A. Pollock	A. Pollock	A. Pollock
COD ⁵	Atlantic Cod	confirmed sample	Atlantic Cod	not reported	Atlantic Cod	not reported	Atlantic Cod

¹ Samples supplied to participants were prepared from either authentic material (authentic sample) or from shop bought fillets which had been checked for species at CCFRA (confirmed sample). Admixtures were prepared from confirmed samples.

² A total of five labs participated in the trial. To maintain anonymity each was assigned a random number as shown here.

³ Sample reported as possibly containing whiting by this lab.

⁴ One replicate of this sample reported as containing haddock. The second replicate reported as possibly containing small amount of haddock. Overall sample reported as containing Atlantic cod and Haddock by this lab.

⁵ This was a positive control sample used to confirm progress at each stage of the analysis.

