Application Note

Fish species identification using PCR-RFLP and the QIAxcel® Advanced

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Introduction

Fishing and aquaculture play a considerable economic role in the European Union (1), accounting for 24% of the worldwide fish market (52.2 billion € for 12.3 million tons in 2011). In the EU, aquaculture is regulated through the definition of feed allowed in aquaculture production, including clear restrictions regarding the use of PAP (processed animal proteins), by-products from healthy animals developed to feed other animals (2). Intra-species feeding or cannibalism is prohibited, which means that feeding salmon with Salmonidae species, for example, is forbidden. These regulations are crucial in controlling fish feed composition and ensuring a supply of correctly labelled, high-quality fish to consumers.

Despite a long history of regulations to maintain a healthy fish industry, fraudulent practices still exist. One of the most common is the mislabeling of fish species. An initial study in the US between 2010 and 2012 showed that 33% of fish products were mislabeled. The study covered 1200 seafood products originating from 674 retailers and revealed fraud involving mainly red snapper and tuna (3). A similar study in France, based on 371 samples, showed that 3.5% of fish were mislabeled. Fraud involved mainly tuna and cod (4, 5). These studies attest to the importance of controlling all stages of fish distribution.

Various techniques are used for fish species identification. The most commonly used is PCR-RFLP (restriction fragment length polymorphism), but other methods, including qPCR, sequencing, and species-specific PCR, are also used (Table 1).

PCR-RFLP is a well-documented, easily mastered and inexpensive technique. However, RFLP can be time-consuming and requires special equipment, which makes it a poor candidate for standardized workflows. Furthermore, mutagenic and hazardous products, such as ethidium bromide, are often used in the visualization of DNA sequences. Results from gel electrophoresis can also be complex and interpretation may require specific software, although misidentification can be minimized by maintaining a database of possible profiles. To overcome these problems, we tested the QIAxcel native capillary electrophoresis system as an alternative to conventional gel electrophoresis. QIAxcel Advanced has numerous advantages: analysis is fast (96 samples in 1 h 30 m), inexpensive, and does not require handling ethidium bromide. Additionally, the QIAxcel ScreenGel® software enables semi-automated interpretation. The software calculates size of the analyzed fragments, which can then be interpreted with the ScreenGel software and the Excel® spreadsheets it produces. ▷

Table 1. Most common methods used for identification of fish species

Analytical method	Advantages	Inconveniences	References
PCR-RFLP	• Enables admixture analysis • Reproducible, sensitive and specific results • Enables analysis of processed samples	• Point mutations can induce false positives or negatives • Identification relies on a profiles database • Without automation, analysis is time-consuming	6, 7
qPCR	• Produces quantitative results • Enables analysis of processed samples • Detects trace amounts • Reproducible, sensitive and specific results	• Detects only prespecified species • Expensive when examining several fish species (requires 1 analysis per species)	8
PCR sequencing (Sanger Method)	• No prior knowledge required (internet database) • Delivers high-quality information • Reproducible and specific results	• Does not enable admixture analysis • High analysis costs	9
PCR sequencing (NGS)	• Enables admixture analysis • Produces quantitative results • No prior knowledge required (internet database) • Delivers high-quality information • Reproducible and specific results	• Time-intensive (1 week for results) • High analysis costs	10, 11
Species-specific PCR	• Simple method • Enables analysis of processed samples • Reproducible, sensitive and specific results	• Detects only prespecified species • Expensive when examining several fish species (requires 1 analysis per species)	12

Materials and methods

The analysis of 8 samples, from sample grinding to identification, was performed in less than 6 h. Samples were ground for homogenization and then lysed via chemical (Buffer ATL), thermal (up to 65°C) or mechanical (1400 rpm agitation) treatment for up to 1 h. DNA was then extracted and purified using the QIAsymphony® DSP DNA Mini Kit on the QIAsymphony. Cytochrome B was amplified with TopTaq Master Mix to yield a fragment of 470 bp. The fragment was digested with a panel of suitable enzymes (AluI, HaeIII, HinfI, DdeI, and TaqI) and the resulting fragments were separated on the QIAxcel Advanced using the QIAxcel DNA High Resolution Kit, the OM500 method, QX Alignment Marker 15 bp/600 bp and QX DNA Size Marker 25–500 bp. Fragment sizes were estimated with the QIAxcel ScreenGel software and results were interpreted from the resulting Excel spreadsheets by comparison to a database of possible profiles. The database contained matching internal and published data. The internal data were validated by analyzing fresh fish with all enzymes at 3 different times, and the published data were validated by testing fish samples with our method.

Results and discussion

This validation study examined PCR-RFLP for routine identification of fish species, generating results within 8 h. Several fish species, fish mixtures and processed samples were used to validate the method. Our assessment took into consideration that the validation may be limited by either polymorphisms or point mutations causing false negatives and false positives. We also accounted for low-quality food samples, given that overly degraded DNA, even from barely processed food, cannot be analyzed. Table 2 lists the 34 fish species (salmons, tuna, trout, hake) that could be identified with this method based on our database.

Figure 1 presents an analysis of DNA from crude fish, both a fish mixture and individual fish species. The HinfI digest (top right) can be used as an example to illustrate the principle of the RFLP approach. The fish mixture (lane 1) revealed 5 bands corresponding to different fish species comprising the mixture. These bands appear also in lanes 2–4 containing DNA from the individual fish species. The bands at 55 and 221 bp were also visible in lane 2 for *Scomber scombrus*.

Table 2. List of fish species that could be identified.

Scientific species names			
Anguilla anguilla	*Merluccius merluccius*	*Scomber scombrus*	*Boops boops*
Microstomus kitt	*Scophthalmus rhombus*	*Coryphaenoides rupestris*	*Mulus surmuletus*
Sparus aurata	*Cynoglossus senegalensis*	*Oncorhynchus gorbuscha*	*Stizostedion luciperca; Sander lucioperca*
Dicentrarchus labrax	*Oncorhynchus keta*	*Theragra chalcogramma*	*Epinephelus sp.*
Oncorhynchus kisutch	*Thunnus albacares*	*Gadus morhua*	*Oncorhynchus mykiss or Salmo gaidneri*
Thunnus thynnus	*Glyptocephalus cynoglossus*	*Perca fluviatilis*	*Trachurus trachurus*
Lates niloticus	*Pleuronectes platessa*	*Xiphias gladius*	*Lophius sp.*
Pollachius virens	*Zeus faber*	*Melanogrammus aeglefinus*	*Salmo salar*
Merlanguis merlanguis	*Sardina pilchardus*		

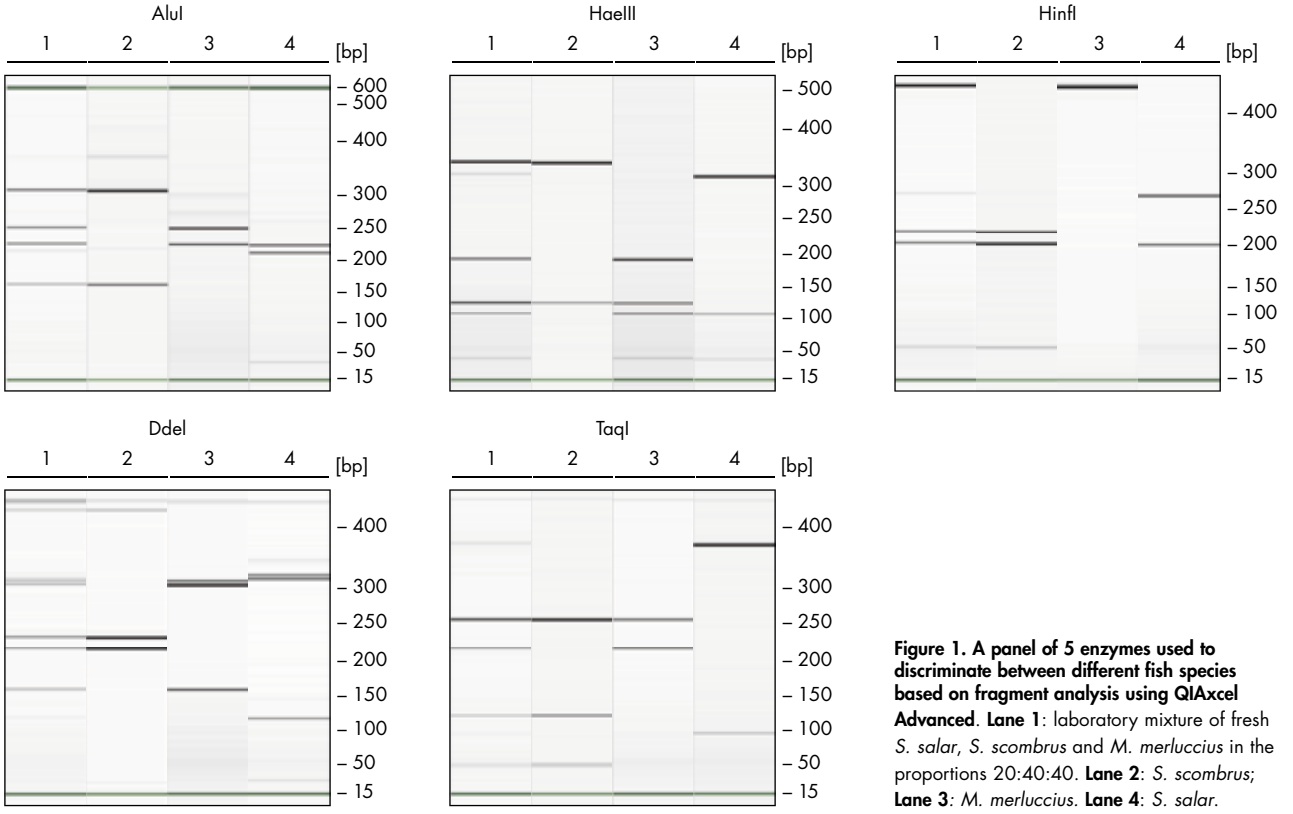

Figure 1. A panel of 5 enzymes used to discriminate between different fish species based on fragment analysis using QIAxcel Advanced. **Lane 1**: laboratory mixture of fresh *S. salar*, *S. scombrus* and *M. merluccius* in the proportions 20:40:40. **Lane 2**: *S. scombrus*; **Lane 3**: *M. merluccius*. **Lane 4**: *S. salar*.

The band at 204 bp was also present in lane 2 for *S. scombrus*, and in lane 4 for *Salmo salar*. The band at 273 bp appeared in lane 4 for *S. salar*, and finally, the band at 466 bp was visible in lane 3 for *Merluccius merluccius*.

Table 3 compares sizes of the observed HinfI restriction fragments with the theoretically expected fragments. The deviation average between theoretical and observed data was 4 bp. Experiments with the other enzymes generated ▷

similar results. To assess utility of the method for processed commercial foodsamples, salmon spinach lasagna was digested with 4 enzymes, of which 1 enzyme was added for result confirmation (data not shown). Three different salmon species were identified in the lasagna, *S. salar*, *Oncorhynchus gorbuscha* and *O. keta* (Figure 2). The AluI digest was uninformative as it gave rise to the same band pattern for all 3 species, however, fragments from HaeIII, HinfI and DdeI digestion enabled discrimination of *S. salar* from *O. keta* and *O. gorbusha*. For example, the HaeIII restriction profile consisted of 4 bands, each coming from the band patterns of different salmons. The band at 41 bp was a fragment common to all three species, the bands at 109 bp and 309 bp came from *S. salar*, and the band at 419 bp came from *O. keta/O. gorbusha*. The two *Oncorhynchus* species were then identified using a fifth enzyme (data not shown).

The PCR-RFLP method proved successful in fish species identification. Each fish species had a unique profile when using the set of enzymes described above. Correlation between theoretical and observed data was good for both fresh crude and frozen fish (data no shown). Moreover, the method enabled discrimination of mixtures containing up to 3 species. During the validation, we observed that some fish, like *Theragra chalcogramma*, had a polymorphic profile or point mutations. Such samples may give rise to false negatives or false positives, which must be taken into consideration for interpretation. The method also works well for several processed foods, such as salmon spinach lasagna, crab sticks or salmon parmentier, as long as the extracted DNA is not overly degraded. Only severly processed foods (e.g., canned rillettes) contain excessively degraded DNA that cannot be analyzed.

Table 3. Comparison of theoretical and observed band sizes arising from HinfI restriction digests

Fish species	Theoretical band sizes (bp)	Observed band sizes (bp)	Differences
S. scombrus	56	55	–1
	201	204	+3
	214	221	+7
M. merluccius	464	466	+2
S. salar	198	204	+6
	266	273	+7

Conclusions

- The tested fish species generate unique digestion profiles and can be readily identified based on comparison to known profiles in a database. Up to 3 different species can be identified in mixtures.
- Commercial samples of varying processing degree can be analyzed, as long as the DNA has not been overly degraded.
- The QIAxcel Advanced facilitates the identification of fish species based on PCR-RFLP and provides results in less than 8 h.
- Using the QIAxcel Advanced, the method is inexpensive and reliable, making it a good candidate for routine use in fish species identification.

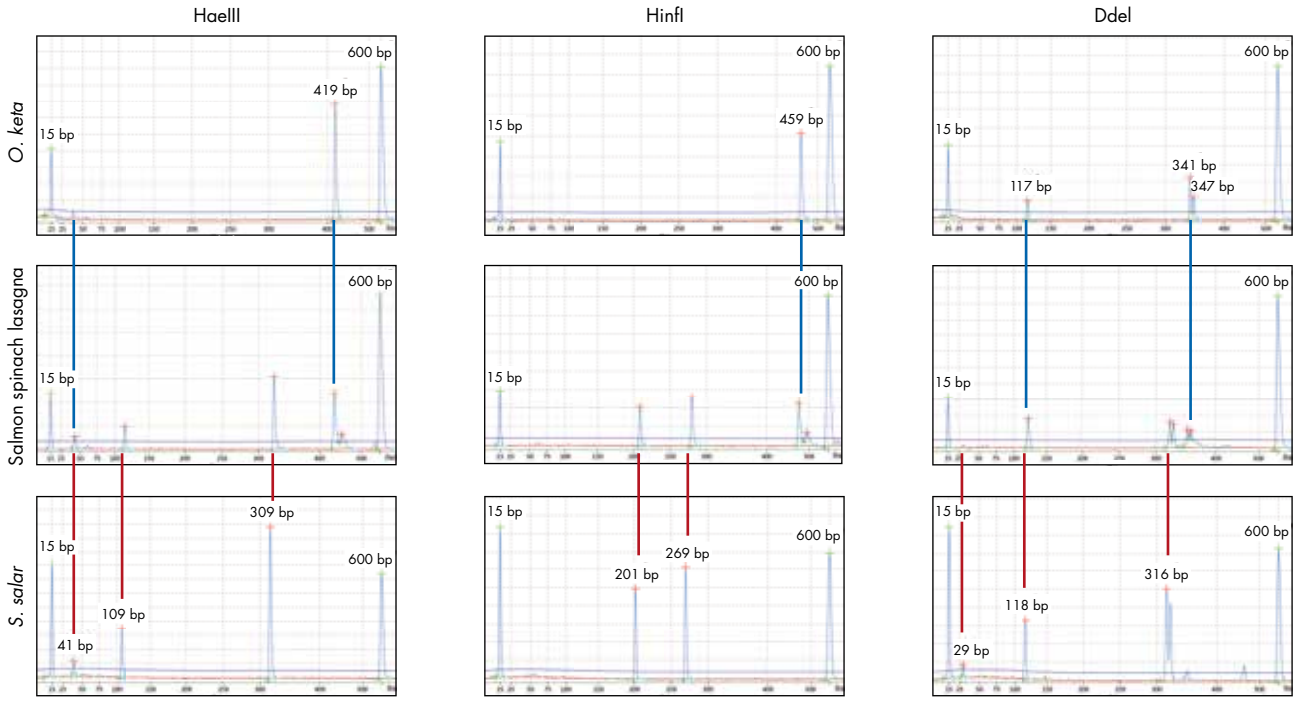

Figure 2. Analysis of a commercial food (salmon spinach lasagna) by fragment analysis using QIAxcel Advanced. *S. salar*, *O. gorbuscha* and *O. keta* were identified. HaeIII, HinfI and DdeI digestion enabled discrimination between *S. salar* and the 2 *Onchorhynchus* species. *O. gorbuscha* and *O. keta* were later identified using another enzyme (data not shown). Blue lines indicate bands in the lasagna sample corresponding to *O. keta*. Red lines indicate bands in the lasagna sample corresponding to *S. salar*.

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Ordering Information

Product	Contents	Cat. no.
QIAxcel Advanced Instrument	Capillary electrophoresis device: includes computer, QIAxcel ScreenGel software, and 1-year warranty on parts and labor	9001941
QIAxcel DNA High Resolution Kit (1200)	QIAxcel DNA High-Resolution Gel Cartridge, Buffers, Mineral Oil, QX Intensity Calibration Marker, 12-Tube Strips	929002
Buffer ATL (4 x 50 ml)	Lysis buffer use in purification of nucleic acids using QIAsymphony DSP Virus/Pathogen kits	939016
QIAsymphony DSP DNA Mini Kit (192)	For 192 preps of 200 µl each. Includes 2 reagent cartridges and enzyme racks and accessories	937236
QIAsymphony SP	QIAsymphony sample prep module: includes 1-year warranty on parts and labor	9001297
TopTaq Master Mix Kit (250)	For 200 x 50 µl reactions: 2x TopTaq Master Mix containing 250 units of TopTaq DNA Polymerase in total, 10x CoralLoad Concentrate and RNase-Free Water	200403
QX Alignment Marker 15 bp/600 bp (1.5 ml)	Alignment marker with 15 bp and 600 bp fragments	929530
QX DNA Size Marker 25–500 bp (50 µl) v2.0	DNA size marker with fragments of 25, 50, 75, 100, 150, 200, 250, 300, 400 and 500 bp; concentration 100 ng/µl	929560

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See how you can benefit from automated gel electrophoresis at **www.qiagen.com/QIAxcel**.

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