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Enhancing fish species identification using novel markers and emerging technologies

Angers Alexandre
Ballin Nicolai Zederkopff
Hofherr Johann
Kagkli Dafni M.
Lievens Antoon
Maquet Alain
Martinsohn Jann T.
Paracchini Valentina
Petrillo Mauro
Puertas-Gallardo Antonio

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Abstract

Establishing an efficient traceability framework for fish products is crucial for consumer protection and fisheries management and conservation. This is well reflected in the EU legislation. The EU general food law emphasizes strongly that European citizens must have access to safe and wholesome food of the highest standard. Consumer protection is supported by a stringent traceability concept as stipulated in Regulation (EC) 178/2002. This notion is also expressed in the Common Fisheries Policy (CFP) basic regulation (EU) 1380/2013, according to which fishing and aquaculture must be environmentally, economically and socially sustainable while providing a source of healthy food for all EU citizens. Under the CFP the need for traceability is not exclusively raised in the context of consumer protection, but also as a necessary component for fisheries control and enforcement in Regulation (EU) 1224/2009 and in the context of the EU's ambitious strategy to fight Illegal, Unreported and Unregulated (IUU) fishing under the remit of Regulation (EC) 1005/2008.

Recent scientific advances, particularly in the fields of genetics and genomics, have led to the development of novel and improved technologies, and efforts are under way to harness their potential for the species identification of unknown fish samples or products. This report reviews these efforts, describing the technologies and the early results obtained for fish product traceability. Each of these technologies have the potential to fill some specific existing gaps, although they come with their own individual set of disadvantages. Understanding those and monitoring progress is thus crucial for their proper integration in existing traceability frameworks.

1 Introduction

The ongoing global population growth, tied to a general increase of income and urbanization, has led to a steady increase of fish consumption. According to the 2016 edition of "The EU Fish Market", the seafood supply in the EU grew by almost 650.000 tonnes between 2013 and 2014 (+4,5%), and *per capita* fish consumption per year increased to 25,5 kg, meaning that EU consumers ate, on average, 1 kg of fish more than in 2013 (Directorate-General for Maritime Affairs and Fisheries of the European Commission, European Market Observatory for Fisheries and Aquaculture Products (EUMOFA), 2016).

In this context, the potential for fraud, in particular the substitution of cheaper fish for more expensive species without appropriate labelling, is increased. Numerous studies have highlighted the extent of this problem for fish (see, recently, (Kappel and Schröder, 2016; Khaksar et al., 2015; Vandamme et al., 2016)). Generally it is difficult for the consumer to identify fish species and for processed products this also constitutes a challenge for control and enforcement authorities; indeed, the meat of many species of fish share the same taste or texture and the sale of the fishes as fillets or other processed products renders visual identification of the exact species difficult if not impossible.

Establishing an efficient seafood traceability framework is thus crucial for the protection of the consumer and the management of sustainable fisheries. Acknowledging the need of progress for the development of powerful independent control technologies and approaches, a number of international projects were funded under the European Union Framework Programme scheme in order to address the development and implementation of readily available methods for species identification. A short overview of some of these efforts concerned with fish products and seafood are depicted in the following table:

Table 1: A list of scientific projects funded under the European Union Framework Programme which explored DNA-based technologies for fish species identification, also in processed products.

Project name	FP	Duration	Description and Outcome
DNAIS	4	1997-2000	This study explored the best identification methodology to be applied in different types of fish products (smoked, canned, heated, *etc*.)
FishTrace	5	2003-2006	FishTrace established a genetic catalogue of a large, representative number of European marine fish species regularly commercialised in the European markets. A publicly accessible database was established, compiling new standardised data (taxonomy, molecular genetics, and reference collections) that was complemented with existing data from other sources.
DNACHIP	6	2004-2006	This project aimed at implementing DNA chip array hybridization for identification of fish species, their eggs and larvae.
FISH AND CHIPS	6	2004-2007	The aim of the project was the development of DNA microarrays for the identification of marine organisms in European Seas as a cost effective,

			reliable and efficient technology in biodiversity and ecosystem science.
EUROFISHCODE	6	2006-2007	EUROFISHCODE was aimed at implementing DNA barcoding as an identification tool for fish and shellfish species from 'catch to fork', at the European level.
LusoAquaBarcode	7	2008-2011	LusoAquaBarcode aimed at implementing DNA barcoding into aquatic biodiversity research in Portugal, and Europe, and priming the development of new macrobenthos monitoring tools. The project contributed to the creation and development of a reference library of DNA barcodes for over 300 species of marine invertebrates of Portugal, with emphasis on three main groups: annelids, crustaceans and molluscs.

Results having emerged from the projects depicted in Table 1, and other studies show that DNA-based methods are the most powerful approach to verify and support traceability, due to their flexibility, rapidity, and cost-effectiveness. DNA is thermostable and generally relatively stable, and genetic markers allow the identification of species, also in processed products, at a higher resolution and with higher reliability, than proteins. These methods include species-specific real-time polymerase chain reaction (RT-PCR), detection of single-nucleotide polymorphisms (SNPs), and DNA barcoding (fore recent examples, see (Espiñeira and Vieites, 2016; Ferrito et al., 2016; Galal-Khallaf et al., 2017; Di Pinto et al., 2016)).

Protein-based methods also exist, such as protein isoelectric focusing (IEF) of soluble muscle proteins, that was used for a time as the standard method for seafood species identification (Lundstrom, 1977). This technique remains popular, sometimes coupled with Enzyme Linked Immunosorbent Assay (ELISA), another protein-based technique (Wang and Hsieh, 2016).

A full description of these methods, including recommended protocols, have been recently published by the JRC (Maretto and Maquet, 2015) as a background document for the Coordinated Control Plan Fish Species Substitution held in 2015[1]

In recent years, a number of technologies have been developed to overcome some of the shortcomings inherent to former approaches. In this report, we review a number of these technologies, focussing on those that were developed as applications underpinning fish species identification. Some, like RT-PCR, are based on the amplification of species-specific regions in the genome; others are variations of existing barcoding techniques, linked to emerging sequencing technologies. Some, not being based on DNA analysis, rely on protein, chemical and even visual characteristics of the tested product.

[1] http://ec.europa.eu/food/safety/official_controls/food_fraud/fish_substitution/tests_en

2 Amplification-based approaches

The Polymerase chain reaction (PCR) is a method that allows exponential amplification of short DNA sequences (usually 100 to 600 bases). PCR entails the use of a pair of primers, each about 20 nucleotides in length, that are complementary to a defined sequence on each of the two strands of the DNA. This provides the possibility of species-specific amplification by selecting primer annealing sequences found only in the genomes of the species to be detected. Most of these assays, developed for the identification of major commercial species, have recently been reviewed recently (Maretto and Maquet, 2015).

2.1 LAMP

Loop-mediated isothermal amplification (LAMP) of DNA was first described by Notomi et al (Notomi et al., 2000). For this approach, a DNA polymerase with a high strand displacement activity and two primer pairs are required. The two primer pairs recognize six distinct genomic targets (labelled F1c, F2c, F3c, B2c, B1c, and B3c), which allows a high specificity. The primers consist of two outer primers called F3 and B3, and two inner primers are called FIP and BIP (forward inner primer and backward inner primer). The F3 and B3 primers target regions denoted F3c and B3c, respectively. The FIP primers target regions denoted F1c and F2c, and the BIP primers target the regions B1c and B2c. The inner primers have a dual function targeting the sense and antisense strand of which one target is used for extension and one for self-priming. The polymerase initiates the three step amplification.

The first step produces the starting material, the second step is the cycling amplification, and the third step elongates and recycles. Both primer pairs are used in the first step, but only the two inner primers are required during the second and third step. Consequently, the target sequence is tripled every half cycle. The speed of the exponential amplification can be increased with the addition of loop primers, which can decreases the time of analysis to less than 30 min. Loop primers should target the sequences located between the F2c F1c and the B1c B2c regions but are not required for the LAMP assay. During the LAMP amplification, magnesium pyrophosphate is formed. This generates turbidity that can be detected through visual inspection or through a turbidimeter. However, specific dyes can also be used for detection. The LAMP assay has already been described in several applications for clinical diagnostics in humans and animals. More recently, applications in meat, plant, and fish authentication have been published.

A study on ostrich targeting the mitochondrial cytochrome b gene obtained a LOD of 0.01% (w/w) in a binary mixture of raw meat (Abdulmawjood et al., 2014). Loop primers were successfully used to decrease the time of detection to less than 20 min. A study on ovine and bovine targeted the cytochrome oxidase subunit 1 and the cytochrome b, respectively (Ma et al., 2016). The limit of detection (LOD) in binary mixtures of heat treated meat ranged from 0.01% (w/w) for bovine to 0.001% (w/w) for ovine. Loop primers reduced efficiency and were therefore not used. A larger study that included seven species, targeted different mitochondrial regions and obtained LODs in binary mixtures of heat treated meat in the range between 0.01% horse (w/w) and 0.0001% lamb (w/w) (Cho et al., 2014). Loop primers were successfully used to decrease the time of detection to less than 30 min. In the above studies, the mitochondrial regions used for primer design and the resulting LOD's are similar to real time PCR methods (Ballin et al., 2009).

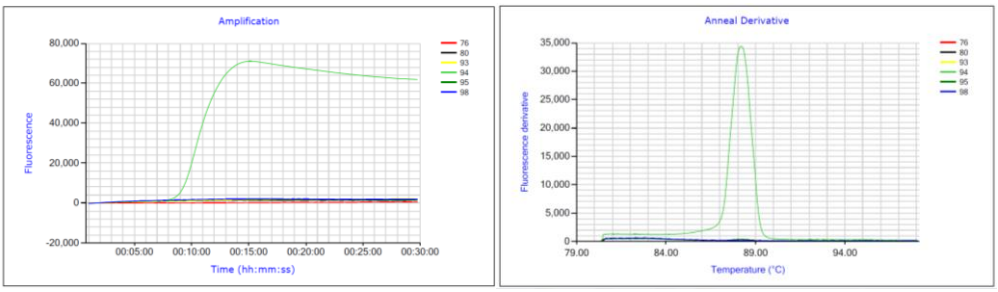
Focke et al. (2013) successfully developed a method that detected several different plant species including caraway, cumin, and black and white mustard (Focke et al., 2013). Target sequences in the regions coding for the 18S ribosomal RNA were used for LAMP primer design and a comparison to real time PCR was performed. Detection limits for all species were determined in baked bread to be 0.0016% (w/w), which is comparable to

earlier results from real time PCR (Focke et al., 2011). The possibility to use the LAMP method for quantification was also demonstrated. Designed loop primers successfully decreased the time of analysis, but increased the variation of the LAMP results, and were therefore excluded. The total time needed for the analysis depended on the experiment, but was in average about 120 min.

LAMP primers that targeted the mitochondrial cytochrome b gene was used in fish speciation to determine Atlantic cod (*Gadus morhua*) in processed fish products (Saull et al., 2016). The detection limit was 0.1% (w/w) *G. morhua* in a binary mixture of raw fish. Loop primers were not designed. A lack of amplification of closely related species such as Pacific cod (*Gadus macrocephalus*) and Alaska Pollock (*Gadus chalcogrammus*, formerly known as *Theragra chalcogramma*) showed high specificity. The total time of analysis was 60 min.

The LAMP assay is highly specific, cheap, fast, and simple. In addition, kits and portable machines are commercially available, which offers the possibility for methods to be applied on site, which is a great asset for inspection and control that depends on swift response times. These interesting potentials in food authentication together with the limited number of available methods prompted the JRC to develop LAMP based protocols for fish species identification. So far, JRC has focused on the species Pollock (*Pollachius virens), G. chalcogrammus,* and Atlantic herring *(Clupea harengus)*. Primers for the *P. virens* and *G. chalcogrammus* species were designed to target the cytochrome b sequence. Primers for the *C. harengus* species targeted a conserved region in the Zic family member 5 (zic5) gene. In each case, a primer blast and practical experiments demonstrated no cross reactivity. The cross reactivity experiment with *C. harengus* shows the amplification and anneal derivative for six species (Figure 1). Additional close related species should be included in cross reactivity studies if a full validation is performed. Detection limits were 0.1% for fresh and 10% for cooked *G. chalcogrammus*, 0.1% for fresh and 1% for cooked *C. harengus* in binary mixtures of fish. Detection limit was not established for the species of *P. virens*. Loop primers were successfully designed in each assay, which ensured a time of analysis of less than 40 min (manuscript in preparation).

Figure 1. A cross reactivity experiment with *Clupea harengus* primers showing the amplification and anneal derivative.



Number codes: 76 *Triglia licerna*, 80 *Microstomus kitt*, 93 *Raja clavata*, 94 *Clupea harengus*, 95 *Mullus surmuletus*, 98 *Xiphias gladius*.

2.2 High resolution melting curves

High resolution melting analysis (HRM) foresees amplification of the target of interest with subsequent melting of the amplicon; it is performed in the presence of a saturation dye which allows the discrimination of small sequence variations among target sequences. The melting temperature (Tm) of a DNA molecule depends on the length of the sequence as well as on the number of its nucleotides guanine and cytosine (GC content). The main advantage is that HRM is performed in real-time, in a closed tube, thus allowing for fast detection of even single-base variants and small insertions or

deletions; the exact difference at sequence level is not determined, but if necessary, the amplicon can be subsequently sequenced and the difference identified.

Thanks to its potential, HRM has been used to characterise pathogens, spoilage microorganisms, to discriminate among genetically modified organisms (Druml and Cichna-Markl, 2014) and to detect food allergens (Costa et al., 2012). In addition, HRM is also applicable in the field of adulteration and authenticity and has gained special interest, as the economic impact of adulteration is very high.

In the case of codfish adulteration, a species frequently substituted, Tomás et al. managed to discriminate the economically more valuable Atlantic cod (*Gadus morhua*) related but commercially less valuable species Pacific cod (*Gadus macrocephalus*) and Alaska or walleye pollock (*Gadus chalcogrammus*), using short amplicon HRM analysis (Tomás et al., 2017). Similar successful approaches have been used not only for fish (Dawnay et al., 2016) but also for plant species and their substitution and/or adulteration with closely related ones, in some cases also putatively toxic species (Ganopoulos et al., 2012; Jaakola et al., 2010; Villa et al., 2016) as well as wine (Pereira et al., 2017). In addition, HRM has also been successfully applied to safeguard the quality designations of the European Union, e.g., the "protected designation of origin (PDO) or the "protected geographical indication" (PGI) (Ganopoulos et al., 2012).

DNA barcodes, Single Nucleotide Polymorphisms (SNPs) and HRM analysis have been coupled to result in a powerful tool to screen and discriminate among closely related species. In case reference control samples are used in the run, HRM can provide accurate results thanks to its being fast, reliable, cheap, and of high-throughput even when degraded DNA is used. In cases of mixtures instead, HRM cannot provide a response in terms of identification but can elucidate differences among samples; the most accurate response in case of mixtures, or complex samples can probably only be given by Next Generation Sequencing (NGS) which seems to be the analytical solution of the future applicable also in the field of food fraud.

3 Sequencing approaches

3.1 Metabarcoding

DNA barcoding involves the amplification of specific DNA regions by the polymerase chain reaction (PCR), followed by sequencing of the produced amplicon (Hebert et al., 2003a, 2003b). The species of the tested sample is identified at the sequencing step by comparing the sequence to a database of reference sequences, determined *a priori* for the species of interest. For fish, the FISH-Barcoding of Life (Fish-BOL) campaign (Ward et al., 2009) has already compiled the sequence of a specific region of the COI gene for more than 100 000 specimens representing more than 10 000 species, and barcoding is a popular strategy to detect fraud and mislabelling (Maretto and Maquet, 2015).

The main caveat of DNA barcoding is the traditional use of Sanger sequencing, which does not produce a useful output when the sequencing reaction contains a mixture of different DNAs. For this reason, different sequencing strategies are needed to co-identify multiple species in a complex sample, with studies going as far as cloning individual PCR fragments for separate Sanger sequencing (Galal-Khallaf et al., 2016a; Muñoz-Colmenero et al., 2017).

Another possibility is the use of Next Generation Sequencing technologies, that can sequence hundreds of thousands DNA strands in parallel. This gives these techniques a remarkable sensitivity, allowing an evaluation of the fish biodiversity in different environments by collecting, amplifying and sequencing environmental DNA (eDNA) (Civade et al., 2016; Evans et al., 2016; Valentini et al., 2016)

More specifically applied to the control along the fish product supply chain, a variety of techniques were recently proposed, using adaptations of the mitochondrial targets used for traditional barcoding (Armani et al., 2015; Galal-Khallaf et al., 2016b; Shokralla et al., 2015). An important adaption includes shortening the length of the amplified region used as a barcode, in order to accommodate one of the limitations of NGS, which is the much shorter sequence length they can read compared to Sanger (Kircher and Kelso, 2010). This can result in a loss of discriminating power, specially for closely related species (Armani et al., 2015); additional barcode candidates, in regions with different evolution histories, are needed to broaden the specificity of barcoding strategies (Deagle et al., 2014). Using available whole genome sequences, recent work from the JRC identified a set of DNA barcodes candidates in the nuclear genome sequences (Paracchini et al., submitted). Specific primers pairs were shown to efficiently identify fish species of the flatfish family, a choice driven by an analysis on the frequency of documented fraud cases, summarized from various publications (eg. according to Marine Stewardship Council 2012[2], 2013[3] and consumer reports[4]), along with the availability of validated vouchers in the database FishTrace. A recent report by Oceana which also looked at seafood fraud in EU Institutions, showed that flatfish mislabelling appears to be particularly frequent[5].

[2] https://www.msc.org/documents/chain-of-custody-documents/dna-testing-methodology-and-results-2012/view

[3] https://www.msc.org/documents/chain-of-custody-documents/dna-test-summary-report-2013/view

[4] http://www.consumerreports.org/cro/magazine-archive/2011/december/food/fake-fish/overview/index.htm

[5] http://eu.oceana.org/sites/default/files/421/oceana_factsheet_seafood_fraud_brussels_eng.pdf

3.2 Nanopore sequencing

Due to its small size and low equipment cost, the Oxford Nanopore MinION sequencer is drawing extensive interest in the genomics community, particularly for pathogen surveillance and clinical diagnostic applications, because of the real-time nature of this sequencing platform.

Current use of Nanopore for barcoding/species identification includes:

- Metagenomics analysis of complex samples including environmental or biological samples, e.g. Australia's pink lake, infection control (or pathogen detection), microbiomes and more
- DNA/RNA analysis in remote locations
- Species identification and molecular evolution with environmental impact e.g. new nitrogen-fixing bacteria ID
- Species taxonomy and relative abundance studies e.g. WIMP (WIMP -What's in my pot- is a quantitative analysis tool for real-time species identification for bacteria, fungi, archaea and viruses) or 16S, and COI.

The use of next-generation sequencing for detecting viruses, bacteria, and parasites in clinical samples and in a hospital environment is well described (Greninger et al., 2015; Kilianski et al., 2015; Pallen, 2014; Wang et al., 2015). These pathogen sequences enable the identification and surveillance of host-pathogen interactions, diagnostic targets, response to vaccines, and pathogen evolution (Quick et al., 2016). Nanopore MinION is a new tool in this area that provides substantial advantages in read length, portability, and time to pathogen identification. Clinical applications to date include studies of chikungunya virus (Greninger et al., 2015), hepatitis virus C (Greninger et al., 2015), *Salmonella enterica* (Quick et al., 2015), and *Salmonella typhimurium* (Ashton et al., 2015), as well as work on antibiotic resistance genes (Judge et al., 2015).

The most inspired clinical use of the MinION so far involved teams of African and European scientists who analysed Ebola samples on-site in West Africa (Hoenen et al., 2016; Quick et al., 2016), applying MinION sequencing technology for real-time on-site monitoring of an epidemic.

Similarly, the ZiBRA project was established, aiming at performing real-time genomic surveillance of Zika virus in Brazil.

Despite the large use of Nanopore sequencing technology for identification of bacterial or virus species, its applicability to eukaryotic species is not so well explored.

One of the limitations of the Nanopore technology is that the data produced currently have single read accuracy around 92% (Ip et al., 2015), and existing algorithms are not equipped to handle long sequence data with error rates above a few percent. Goodwin et al reported a method to correct long read sequences produced by Nanopore (Goodwin et al., 2015). They demonstrated how single-molecule, long-read data generated by the Oxford MinION can be successfully used to compliment short-read data to create highly contiguous genome assemblies in case of small eukaryotic genomes (*Saccharomyces cerevisiae*) (Goodwin et al., 2015).

Improvement in read-length, base-call accuracies, base modification detection and throughput for Nanopore sequencers has rapidly evolved and is likely to continue.

4 Other approaches

4.1 Proteomics approaches

Much like DNA-based methods, protein-based approaches for species identification focus on sequence differences, albeit Amino Acid sequence differences between similar proteins of different species. Traditional proteomics approaches for visualising such differences (and by comparison to a reference, identifying a sample) include 2D gel electrophoresis and iso-electric focussing (IEF). More recently, mass spectrometry (MS) has gained popularity as small differences in amino acid sequences can lead to large differences in peptide masses (Etienne et al., 2000; Mazzeo and Siciliano, 2016).

Essentially, a mass spectrometer measures the masses of the molecules within a sample. The technique relies on magnetic & electric fields which exert forces on charged particles (ions) in vacuum in order to determine their mass-charge ratio. Therefore, the sample must be ionized prior to be analysed. In addition, the ions must be introduced into the system as a gas so that the molecules are individualized allowing the determination of their single masses. However, proteins are non-volatile, and thermally unstable and require special 'soft' ionization methods that do not cause extensive degradation. Beside the ionization source, a mass spectrometer also consist of a mass analyser, a detector and the data processing electronics.

The workflow usually consists of breaking down the protein in smaller parts (peptides) which yields a spectrum of masses (a peptide mass fingerprint) which is used as input for a search in a database that holds the predicted masses for each protein. Complex samples can hold an overwhelming number of components that yield spectrums that are very difficult to interpret. Therefore, it is usually necessary to enrich, purify, or separate the proteins prior to MS analysis (eg. via HPLC or upstream MS).

Traditional targets for identifying fish include sarcoplasmatic proteins and parvalbumin, but advances in proteomics have paved the way for the discovery of many new biomarkers that can establish the identity of fish species in food samples (Balog et al., 2016; Ortea et al., 2012; Rehbein et al., 2000; Tedesco et al., 2014).

Mass spectrometry is a versatile tool for protein analysis, able to see very small differences between proteins and not limited to any specific protein. Unfortunately, a mass spectrometer is an expensive, non-portable laboratory equipment that may require extended sample preparation, and relies on the availability of reference baseline databases for the identification of proteins.

4.2 Chromatography/Chemical profiling

Chromatography is an analytical technique commonly used for separating a mixture of chemical substances into its individual components, so that the individual components can be thoroughly analysed. This analytical approach aims at defining chemical, physical, sensory characteristics, adulteration and contamination of a food, in order to ensure its quality and safety for the consumers, supporting and implementing the laws and regulations of the field (Gallo and Ferranti, 2016).

There are many types of chromatography: liquid chromatography (LC) was revolutionized by the introduction of high performance LC (HPLC) (McDonald and Patrick, 2008) and more recently by ultra high performance LC (UPLC). HPLC made possible for the first time the high resolution separation of complex mixtures of non-volatile analytes in much lower times (a few minutes) compared to classical LC analyses. The introduction of gas chromatography allowed the separation, identification and determination of chemical compounds in complex mixtures and the control of the purity of volatile or volatilized compounds. The combination of these methods with powerful identification techniques, such as mass spectrometry, infrared and UV spectroscopy and ion spectroscopic techniques such as nuclear magnetic resonance, has made possible the

identification of most of the constituents of complex mixture and their quantitative variations and alterations in a food matrix.

Chromatography has been used for the identification of fish species (Ashoor and Knox, 1985; Knuutinen and Harjula, 1998). In a recent publication chromatography is applied for the analysis of proximate composition of fish, nutritional quality (minerals, fatty acids and free amino acids content) and presence of contaminants, with the aim of identifying variations from the natural composition (Manthey-Karl et al., 2016).

4.3 DNA arrays

Microarray technology is not very often used for species identification and only few examples are reported in literature, like when specimens are in poor condition or comprise very limited material. Marine organisms and especially their diverse developmental stages are difficult to identify by morphological characters and DNA-based identification methods offer an analytically powerful addition or even an alternative. DNA microarrays have been investigated as potential technology for the identification of fish species, including the applicability of the three mitochondrial genes 16S rRNA (16S), cytochrome b (cyt b), and cytochrome oxidase subunit I (COI) for the identification of fish species by combining techniques of "DNA barcoding" and microarrays (Kochzius et al., 2008). It has been demonstrated that the mainly 16S rDNA gene is suitable for designing oligonucleotide probes (Kochzius et al., 2010).

A very recent application is the development of DNA macroarray, a less expensive modification of the microarray. The technology is similar, but the spots are larger (about 1 mm as compared to 250µ). Macroarrays can be visually interpreted without a microscope, and that radioisotopes as well as fluorescent labels can be used. They are considered a valid alternative to 2-dimensional electrophoresis, isoelectric focussing, protein capillary electrophoresis, HPLC or ELISA. In fact, these protein-based analytical tools used in the field of species identification in food are susceptible to protein denaturation due to physical or chemical industrial treatments. Moreover, compared to other non-protein biomarker-based methods like peptide biomarker mass spectrometry and lipid-based biomarkers, low cost–low density (LCD) macroarrays shows higher sensitivity and are not affected by cooking steps (Cottenet et al., 2016).

In the field of food safety LCD macroarrays were a first approach with rapid and successful implementation at the operational level in Quality Assurance laboratories. The elevated risk of cross-contamination of materials, mainly on shared lines and/or equipment, may require the development and implementation of effective protocols that define cleaning and verification procedures to avoid contamination. In the case of DNA-based methods, one can in many cases identify well below 1% of "foreign" DNA, but a constraint may be that some multi-methods simply allow the identification of foreign DNA irrespective of the amount. This may be useful for cases where zero tolerance is expected. However, for substitution of fish species, the aim is to go one step further toward untargeted approaches such as those using Next Generation Sequencing, described in section 3.

4.4 Automated visual identification with deep learning

Recent studies have shown the potential of Machine Learning algorithms to scan and learn from image and video collections in order to automatically identify the species when presented with the images of unknown specimens. These take advantage of ongoing efforts in the development of cross-language information retrieval tools, including imageCLEF and LifeCLEF (Clough et al., 2004; Joly et al., 2016).

Most of these methods are not intrusive, requiring only the availability of media and internet connection to analyse images of whole, eventually live fishes. These include the work of Salman et al., showing the recognition of fish species from underwater video sequences, even in very bad light and water conditions (Salman et al., 2016). The accuracy of this method was shown to be between 90% and 95%. The researchers

proposed further improvements and the possibility to improve the performance with colour information and more sophisticated algorithms and, for example, better and accurate loss functions. In the same vein, a recent study presented a technique for image recognition of underwater images, based on Faster R-CNN (Region-based Convolutional Neural Network) running on a state of the art Hardware GPU NVIDIA Tesla K20 (Li et al., 2016). The results were impressive on speed and accuracy.

Other applications include the possibility to identify fish out of the sea, such as in fish markets or inside fishing vessels. A recent study describes an app that could be installed on smartphones in order to take pictures of the fish and send them to a cloud server for processing and recognition (Rossi et al., 2016). The answer would then arrive directly to the smart device. Further improvements involve the possibility of recognising the image using a local database, without the need to send a request to the cloud servers.

Other approaches are more intrusive, classifying for example the fish species based on the morphological characteristics of the otolith outline contour which are part of the inner ear of fishes (Salimi et al., 2016). The method for the classification uses deep learning techniques where the algorithm learns the unique shape of different species and, based on the training and testing image sets, achieves the identification of different species and fishes. The accuracy of this fully automated method is between 92% and 96%, which is expected to increase with bigger training sets.

5 Conclusion

This report summarised different novel technologies, for which various groups have recently published their efforts in harnessing their possibilities in a framework of fish species identification. . Each of these have their individual sets of advantages and disadvantages. Some, like automated visual identification, require only a cell phone able to take pictures and connect to the internet; however, they are unable to accommodate any type of processing that would disrupt the appearance of the whole specimen. On the other side of the spectrum, genomics and proteomics techniques are extremely informative and resilient to most processing; however, they require extensive (and expensive) laboratory equipment, as well as a proper bioinformatics infrastructure to handle and process the data. Techniques like LAMP can be performed cheap, fast, and on-site; however, they require *a priori* expectations of the species present in the sample to be tested, and need different assays to be developed and validated for each species of interest.

The European Union recently amended its Common Organisation of the Markets (COM) in fishery and aquaculture products (FAPs) (EU, 2013). Although the amendment resulted in major improvements through the introduction of new requirements for the labelling of fisheries and aquaculture products, some of the provisions are still seem to be burdened with shortcomings. D'Amico et al. (2016) highlighted the exclusion of processed FAPs from the scope of article 35, that provides specific rules for consumer mandatory information for prepacked and non-prepacked FAPs. FAPs are the most traded commodity worldwide and the complexity of their trade flows renders them particularly prone to fraudulent replacements. Incidentally, some FAPs also belong to the most challenging products with respect to species identification. These observations along with the needs emerging from EU food law and the Common Fisheries Policy, which are outlined in the introduction of this report, show that new, powerful, cost-efficient species identification approaches are needed, ideally with short response-times and the possibility to perform on-the-spot analysis. This is why efforts are necessary to properly foster the uptake and integration of the methods discussed in this report in existing traceability and food control schemes.

References

Abdulmawjood, A., Grabowski, N., Fohler, S., Kittler, S., Nagengast, H., and Klein, G. (2014). Development of Loop-Mediated Isothermal Amplification (LAMP) Assay for Rapid and Sensitive Identification of Ostrich Meat. PLoS ONE *9*.

Armani, A., Guardone, L., Castigliego, L., D'Amico, P., Messina, A., Malandra, R., Gianfaldoni, D., and Guidi, A. (2015). DNA and Mini-DNA barcoding for the identification of Porgies species (family Sparidae) of commercial interest on the international market. Food Control *50*, 589–596.

Ashoor, S.H., and Knox, M.J. (1985). Identification of fish speceis by high-performance liquid chromatography. J. Chromatogr. A *324*, 199–202.

Ashton, P.M., Nair, S., Dallman, T., Rubino, S., Rabsch, W., Mwaigwisya, S., Wain, J., and O'Grady, J. (2015). MinION nanopore sequencing identifies the position and structure of a bacterial antibiotic resistance island. Nat. Biotechnol. *33*, 296–300.

Ballin, N.Z., Vogensen, F.K., and Karlsson, A.H. (2009). Species determination – Can we detect and quantify meat adulteration? Meat Sci. *83*, 165–174.

Balog, J., Perenyi, D., Guallar-Hoyas, C., Egri, A., Pringle, S.D., Stead, S., Chevallier, O.P., Elliott, C.T., and Takats, Z. (2016). Identification of the Species of Origin for Meat Products by Rapid Evaporative Ionization Mass Spectrometry. J. Agric. Food Chem. *64*, 4793–4800.

Cho, A.-R., Dong, H.-J., and Cho, S. (2014). Meat Species Identification using Loop-mediated Isothermal Amplification Assay Targeting Species-specific Mitochondrial DNA. Korean J. Food Sci. Anim. Resour. *34*, 799–807.

Civade, R., Dejean, T., Valentini, A., Roset, N., Raymond, J.-C., Bonin, A., Taberlet, P., and Pont, D. (2016). Spatial Representativeness of Environmental DNA Metabarcoding Signal for Fish Biodiversity Assessment in a Natural Freshwater System. PLOS ONE *11*, e0157366.

Clough, P., Sanderson, M., and Müller, H. (2004). The CLEF Cross Language Image Retrieval Track (ImageCLEF) 2004. In Image and Video Retrieval, P. Enser, Y. Kompatsiaris, N.E. O'Connor, A.F. Smeaton, and A.W.M. Smeulders, eds. (Springer Berlin Heidelberg), pp. 243–251.

Costa, J., Mafra, I., and Oliveira, M.B.P.P. (2012). High resolution melting analysis as a new approach to detect almond DNA encoding for Pru du 5 allergen in foods. Food Chem. *133*, 1062–1069.

Cottenet, G., Sonnard, V., Blancpain, C., Ho, H.Z., Leong, H.L., and Chuah, P.F. (2016). A DNA macro-array to simultaneously identify 32 meat species in food samples. Food Control *67*, 135–143.

D'Amico, P., Armani, A., Gianfaldoni, D., and Guidi, A. (2016). New provisions for the labelling of fishery and aquaculture products: Difficulties in the implementation of Regulation (EU) n. 1379/2013. Mar. Policy *71*, 147–156.

Dawnay, N., Hughes, R., Court, D.S., and Duxbury, N. (2016). Species detection using HyBeacon® probe technology: Working towards rapid onsite testing in non-human forensic and food authentication applications. Forensic Sci. Int. Genet. *20*, 103–111.

Deagle, B.E., Jarman, S.N., Coissac, E., Pompanon, F., and Taberlet, P. (2014). DNA metabarcoding and the cytochrome c oxidase subunit I marker: not a perfect match. Biol. Lett. *10*, 20140562.

Directorate-General for Maritime Affairs and Fisheries of the European Commission, European Market Observatory for Fisheries and Aquaculture Products (EUMOFA) (2016). EUMOFA The EU Fish Market - 2016 Edition.

Druml, B., and Cichna-Markl, M. (2014). High resolution melting (HRM) analysis of DNA – Its role and potential in food analysis. Food Chem. *158*, 245–254.

Espiñeira, M., and Vieites, J.M. (2016). FAST Real Time PCR for control of intra-species recycling in aquaculture feed, focused to the most relevant fish species farmed in Europe. Food Chem. *204*, 352–357.

Etienne, M., Jérôme, M., Fleurence, J., Rehbein, H., Kündiger, R., Mendes, R., Costa, H., Pérez-Martín, R., Piñeiro-González, C., Craig, A., et al. (2000). Identification of Fish Species after Cooking by SDS−PAGE and Urea IEF: A Collaborative Study. J. Agric. Food Chem. *48*, 2653–2658.

EU (2013). Regulation (EU) No 1379/2013.

Evans, N.T., Olds, B.P., Renshaw, M.A., Turner, C.R., Li, Y., Jerde, C.L., Mahon, A.R., Pfrender, M.E., Lamberti, G.A., and Lodge, D.M. (2016). Quantification of mesocosm fish and amphibian species diversity via environmental DNA metabarcoding. Mol. Ecol. Resour. *16*, 29–41.

Ferrito, V., Bertolino, V., and Pappalardo, A.M. (2016). White fish authentication by COIBar-RFLP: Toward a common strategy for the rapid identification of species in convenience seafood. Food Control *70*, 130–137.

Focke, F., Haase, I., and Fischer, M. (2011). Determination of plant contaminants in food using real-time-PCR. Dtsch. Lebensm.-Rundsch. *107*, 221–224.

Focke, F., Haase, I., and Fischer, M. (2013). Loop-Mediated Isothermal Amplification (LAMP): Methods for Plant Species Identification in Food. J. Agric. Food Chem. *61*, 2943–2949.

Galal-Khallaf, A., Ardura, A., Borrell, Y.J., and Garcia-Vazquez, E. (2016a). Towards more sustainable surimi? PCR-cloning approach for DNA barcoding reveals the use of species of low trophic level and aquaculture in Asian surimi. Food Control *61*, 62–69.

Galal-Khallaf, A., Osman, A.G.M., Carleos, C.E., Garcia-Vazquez, E., and Borrell, Y.J. (2016b). A case study for assessing fish traceability in Egyptian aquafeed formulations using pyrosequencing and metabarcoding. Fish. Res. *174*, 143–150.

Galal-Khallaf, A., Mohammed-Geba, K., Osman, A.G.M., AbouelFadl, K.Y., Borrell, Y.J., and Garcia-Vazquez, E. (2017). SNP-based PCR-RFLP, T-RFLP and FINS methodologies for the identification of commercial fish species in Egypt. Fish. Res. *185*, 34–42.

Gallo, M., and Ferranti, P. (2016). The evolution of analytical chemistry methods in foodomics. J. Chromatogr. A *1428*, 3–15.

Ganopoulos, I., Bosmali, I., Madesis, P., and Tsaftaris, A. (2012). Microsatellite genotyping with HRM (High Resolution Melting) analysis for identification of the PGI common bean variety Plake Megalosperma Prespon. Eur. Food Res. Technol. *234*, 501–508.

Goodwin, S., Gurtowski, J., Ethe-Sayers, S., Deshpande, P., Schatz, M.C., and McCombie, W.R. (2015). Oxford Nanopore sequencing, hybrid error correction, and de novo assembly of a eukaryotic genome. Genome Res.

Greninger, A.L., Naccache, S.N., Federman, S., Yu, G., Mbala, P., Bres, V., Stryke, D., Bouquet, J., Somasekar, S., Linnen, J.M., et al. (2015). Rapid metagenomic identification of viral pathogens in clinical samples by real-time nanopore sequencing analysis. Genome Med. *7*, 99.

Hebert, P.D.N., Ratnasingham, S., and Waard, J.R. de (2003a). Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. Proc. R. Soc. Lond. B Biol. Sci. *270*, S96–S99.

Hebert, P.D.N., Cywinska, A., Ball, S.L., and deWaard, J.R. (2003b). Biological identifications through DNA barcodes. Proc. R. Soc. Lond. B Biol. Sci. *270*, 313–321.

Hoenen, T., Groseth, A., Rosenke, K., Fischer, R.J., Hoenen, A., Judson, S.D., Martellaro, C., Falzarano, D., Marzi, A., Squires, R.B., et al. (2016). Nanopore Sequencing as a Rapidly Deployable Ebola Outbreak Tool. Emerg. Infect. Dis. *22*, 331–334.

Ip, C.L.C., Loose, M., Tyson, J.R., de Cesare, M., Brown, B.L., Jain, M., Leggett, R.M., Eccles, D.A., Zalunin, V., Urban, J.M., et al. (2015). MinION Analysis and Reference Consortium: Phase 1 data release and analysis. F1000Research.

Jaakola, L., Suokas, M., and Häggman, H. (2010). Novel approaches based on DNA barcoding and high-resolution melting of amplicons for authenticity analyses of berry species. Food Chem. *123*, 494–500.

Joly, A., Goëau, H., Glotin, H., Spampinato, C., Bonnet, P., Vellinga, W.-P., Champ, J., Planqué, R., Palazzo, S., and Müller, H. (2016). LifeCLEF 2016: Multimedia Life Species Identification Challenges. In Experimental IR Meets Multilinguality, Multimodality, and Interaction, N. Fuhr, P. Quaresma, T. Gonçalves, B. Larsen, K. Balog, C. Macdonald, L. Cappellato, and N. Ferro, eds. (Springer International Publishing), pp. 286–310.

Judge, K., Harris, S.R., Reuter, S., Parkhill, J., and Peacock, S.J. (2015). Early insights into the potential of the Oxford Nanopore MinION for the detection of antimicrobial resistance genes. J. Antimicrob. Chemother. *70*, 2775–2778.

Kappel, K., and Schröder, U. (2016). Substitution of high-priced fish with low-priced species: Adulteration of common sole in German restaurants. Food Control *59*, 478–486.

Khaksar, R., Carlson, T., Schaffner, D.W., Ghorashi, M., Best, D., Jandhyala, S., Traverso, J., and Amini, S. (2015). Unmasking seafood mislabeling in U.S. markets: DNA barcoding as a unique technology for food authentication and quality control. Food Control *56*, 71–76.

Kilianski, A., Haas, J.L., Corriveau, E.J., Liem, A.T., Willis, K.L., Kadavy, D.R., Rosenzweig, C.N., and Minot, S.S. (2015). Bacterial and viral identification and differentiation by amplicon sequencing on the MinION nanopore sequencer. GigaScience *4*, 12.

Kircher, M., and Kelso, J. (2010). High-throughput DNA sequencing–concepts and limitations. Bioessays *32*, 524–536.

Knuutinen, J., and Harjula, P. (1998). Identification of fish species by reversed-phase high-performance liquid chromatography with photodiode-array detection. J. Chromatogr. B. Biomed. Sci. App. *705*, 11–21.

Kochzius, M., Nölte, M., Weber, H., Silkenbeumer, N., Hjörleifsdottir, S., Hreggvidsson, G.O., Marteinsson, V., Kappel, K., Planes, S., Tinti, F., et al. (2008). DNA Microarrays for Identifying Fishes. Mar. Biotechnol. N. Y. N *10*, 207–217.

Kochzius, M., Seidel, C., Antoniou, A., Botla, S.K., Campo, D., Cariani, A., Vazquez, E.G., Hauschild, J., Hervet, C., Hjörleifsdottir, S., et al. (2010). Identifying Fishes through DNA Barcodes and Microarrays. PLOS ONE *5*, e12620.

Li, X., Shang, M., Hao, J., and Yang, Z. (2016). Accelerating fish detection and recognition by sharing CNNs with objectness learning. In OCEANS 2016 - Shanghai, pp. 1–5.

Lundstrom, R.C. (1977). Identification of fish species by thin-layer polyacrylamide gel isoelectric focusing. Fish Bull *75*, 571.

Ma, B., Dai, M., Fang, J., Wu, Y., and Zhang, M. (2016). Visual Loop-mediated Isothermal Amplification (LAMP) Method for Identification Bovine and Ovine Gene in Animal Foodstuff. Am. J. Food Technol. *11*, 193–203.

Manthey-Karl, M., Lehmann, I., Ostermeyer, U., and Schröder, U. (2016). Natural Chemical Composition of Commercial Fish Species: Characterisation of Pangasius, Wild and Farmed Turbot and Barramundi. Foods *5*, 58.

Maretto, F., and Maquet, A. (2015). Methods for fish species identification in raw tissue and processed products - Background document for the Coordinated Control Plan Fish Species Substitution. JRC Tech. Rep.

Mazzeo, M.F., and Siciliano, R.A. (2016). Proteomics for the authentication of fish species. J. Proteomics *147*, 119–124.

McDonald, P.D., and Patrick, D. (2008). Fifty years of innovation in analysis and purification. Chem. Herit. *26*, 32–37.

Muñoz-Colmenero, M., Martínez, J.L., Roca, A., and Garcia-Vazquez, E. (2017). NGS tools for traceability in candies as high processed food products: Ion Torrent PGM versus conventional PCR-cloning. Food Chem. *214*, 631–636.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., and Hase, T. (2000). Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. *28*, e63–e63.

Ortea, I., Pascoal, A., Cañas, B., Gallardo, J.M., Barros-Velázquez, J., and Calo-Mata, P. (2012). Food authentication of commercially-relevant shrimp and prawn species: From classical methods to Foodomics. ELECTROPHORESIS *33*, 2201–2211.

Pallen, M.J. (2014). Diagnostic metagenomics: potential applications to bacterial, viral and parasitic infections. Parasitology *141*, 1856–1862.

Pereira, L., Gomes, S., Castro, C., Eiras-Dias, J.E., Brazão, J., Graça, A., Fernandes, J.R., and Martins-Lopes, P. (2017). High Resolution Melting (HRM) applied to wine authenticity. Food Chem. *216*, 80–86.

Di Pinto, A., Mottola, A., Marchetti, P., Bottaro, M., Terio, V., Bozzo, G., Bonerba, E., Ceci, E., and Tantillo, G. (2016). Packaged frozen fishery products: species identification, mislabeling occurrence and legislative implications. Food Chem. *194*, 279–283.

Quick, J., Ashton, P., Calus, S., Chatt, C., Gossain, S., Hawker, J., Nair, S., Neal, K., Nye, K., Peters, T., et al. (2015). Rapid draft sequencing and real-time nanopore sequencing in a hospital outbreak of Salmonella. Genome Biol. *16*, 114.

Quick, J., Loman, N.J., Duraffour, S., Simpson, J.T., Severi, E., Cowley, L., Bore, J.A., Koundouno, R., Dudas, G., Mikhail, A., et al. (2016). Real-time, portable genome sequencing for Ebola surveillance. Nature *530*, 228–232.

Rehbein, H., Kündiger, R., Pineiro, C., and Perez-Martin, R.I. (2000). Fish muscle parvalbumins as marker proteins for native and urea isoelectric focusing. Electrophoresis *21*, 1458–1463.

Rossi, F., Benso, A., Carlo, S.D., Politano, G., Savino, A., and Acutis, P.L. (2016). FishAPP: A mobile App to detect fish falsification through image processing and machine learning techniques. In 2016 IEEE International Conference on Automation, Quality and Testing, Robotics (AQTR), pp. 1–6.

Salimi, N., Loh, K.H., Dhillon, S.K., and Chong, V.C. (2016). Fully-automated identification of fish species based on otolith contour: using short-time Fourier transform and discriminant analysis (STFT-DA). PeerJ *4*, e1664.

Salman, A., Jalal, A., Shafait, F., Mian, A., Shortis, M., Seager, J., and Harvey, E. (2016). Fish species classification in unconstrained underwater environments based on deep learning. Limnol. Oceanogr. Methods *14*, 570–585.

Saull, J., Duggan, C., Hobbs, G., and Edwards, T. (2016). The detection of Atlantic cod (Gadus morhua) using loop mediated isothermal amplification in conjunction with a simplified DNA extraction process. Food Control *59*, 306–313.

Shokralla, S., Hellberg, R.S., Handy, S.M., King, I., and Hajibabaei, M. (2015). A DNA Mini-Barcoding System for Authentication of Processed Fish Products. Sci. Rep. *5*.

Stadler, R.H., Tran, L.-A., Cavin, C., Zbinden, P., and Konings, E.J.M. (2016). Analytical Approaches to Verify Food Integrity: Needs and Challenges. J. AOAC Int. *99*, 1135–1144.

Tedesco, S., Mullen, W., and Cristobal, S. (2014). High-throughput proteomics: a new tool for quality and safety in fishery products. Curr. Protein Pept. Sci. *15*, 118–133.

Tomás, C., Ferreira, I.M.P.L.V.O., and Faria, M.A. (2017). Codfish authentication by a fast Short Amplicon High Resolution Melting Analysis (SA-HRMA) method. Food Control *Complete*, 255–263.

Valentini, A., Taberlet, P., Miaud, C., Civade, R., Herder, J., Thomsen, P.F., Bellemain, E., Besnard, A., Coissac, E., Boyer, F., et al. (2016). Next-generation monitoring of aquatic biodiversity using environmental DNA metabarcoding. Mol. Ecol. *25*, 929–942.

Vandamme, S.G., Griffiths, A.M., Taylor, S.-A., Di Muri, C., Hankard, E.A., Towne, J.A., Watson, M., and Mariani, S. (2016). Sushi barcoding in the UK: another kettle of fish. PeerJ *4*, e1891.

Villa, C., Costa, J., Meira, L., Oliveira, M.B.P.P., and Mafra, I. (2016). Exploiting DNA mini-barcodes as molecular markers to authenticate saffron (Crocus sativus L.). Food Control *65*, 21–31.

Wang, D., and Hsieh, Y.-H.P. (2016). The use of imported pangasius fish in local restaurants. Food Control *65*, 136–142.

Wang, J., Moore, N.E., Deng, Y.-M., Eccles, D.A., and Hall, R.J. (2015). MinION nanopore sequencing of an influenza genome. Front. Microbiol. *6*.

Ward, R.D., Hanner, R., and Hebert, P.D.N. (2009). The campaign to DNA barcode all fishes, FISH-BOL. J. Fish Biol. *74*, 329–356.

List of abbreviations and definitions

BOL	Barcode of life
COI	Cytochrome c oxidase 1 gene
COM	Common Organization of the Markets in Fishery and Aquaculture Products Regulation
DNA	Deoxyribonucleic acid
eDNA	Environmental DNA
ELISA	Enzyme-linked immunosorbent assay
FAP	Fisheries and aquaculture product
HPLC	High performance liquid chromatography
HRM	High-Resolution Melting
IEF	Isoelectric focusing
LAMP	Loop-mediated isothermal amplification
LC	Liquid chromatography
LCD	Low cost–low density
NGS	Next-Generation Sequencing
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT-PCR	Real-Time Polymerase Chain Reaction
SNP	Single nucleotide polymorphism

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Figure 1. A cross reactivity experiment with *Clupea harengus* primers showing the amplification and anneal derivative.

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Table 1. A list of scientific projects funded under the European Union Framework Programme which explored DNA-based technologies for fish species identification, also in processed products.

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