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Recent advances in DNA and other techniques for identifying seafood provenance in the supply chain

This fact sheet outlines some of the techniques currently used to accurately identify seafood and seafood products, and reviews some of the benefits and limitations of the latest DNA and other techniques used for identification purposes within commercial, enforcement and fisheries science.

There have been several recent reports in the media concerning mislabelled fish, both in the national and international press. Some reports reveal obvious fraud where an expensive species is substituted with a cheaper one such as pangasius being sold as cod, or inadvertent substitution of species, where it is equally possible that the expensive species is sold as the cheap species, and vice versa [1], [2].

A report produced by the Joint Research Centre of the European Commission in May 2011 *Deterring Illegal Activities in the Fisheries Sector* [3] examined the use of various genetic and chemical techniques for forensic examination of fish to identify the species, origin of the sample (geographical area or stock) and distinguish between wild caught and farmed fish. The intention is to deter illegal activities such as mislabelling of fish, and for use in monitoring, control and enforcement.

What does the supply chain need to know?

The seafood industry needs to be able to accurately determine the species and origin of seafood for traceability (European Regulation 178/2002) and labelling purposes (European Regulation 104/2000). Such data would be useful to operators and enforcers in order to ensure compliance with fisheries management regulations, and hence detect the presence of IUU[1] fish. European Regulation 1224/2009 provides for inspections and traceability all along the production chain with genetic analysis explicitly mentioned as a tool to aid traceability. In particular the supply chain needs to be able to determine:

i) **The species of a fish;** to enable assurance of compliance with food labelling regulations and trades descriptions and to estimate levels of infringement.

[1] Illegal Unreported Unregulated

ii) **The stock the fish originates from;** the management 'stock' is the unit of management used in fisheries. Because different stocks can be in a different condition in relation to sustainability, tracing the fish to a particular stock may be important in relation to sustainability claims. This also relates to enforcement; Total Allowable Catches and quotas relate to specific stocks so there may be a requirement to assess the origin of a catch in the case of suspected misreporting.

iii) **The production method; wild caught or aquaculture**; labelling regulations require the production method; whether wild caught or aquaculture to be stated on the label. Also fish escaping from farms and influencing the ecology of wild stocks is an important issue in some locations.

Techniques that are currently available to identify species, origin and production method

The JRC report *Deterring Illegal Activities in the Fisheries Sector* [3] outlines four generic techniques for identifying species, origin and production method, not all of which are DNA based. The four are:

1. **Genetic markers; electrophoretic protein separation techniques**. These techniques; (see BOX 1), rely on there being variation in protein composition due to genetic differences between species. In its most widely used form the proteins are drawn through a polyacrylamide gel using an electric potential and separated according to their mobility in the gel [4]. Comparisons are made against reference samples for identification of species. These techniques are widely used in regulation and commerce, to identify fresh or frozen fish. Because of their ease of use these techniques are likely to be used routinely by both industry and regulators.

2. **Genetic markers using DNA based methods.** There are a wide range of methods based on analysis of genetic variations between different species' DNA (See BOXES 2, 3 and 4). Ward [5] found that by analysing genetic differences on a specific gene[2] taken from 1088 fish species, 97.5 – 97.9% of fish species were distinguishable. However, most of these techniques are currently only available for use in specialist laboratories by specialist staff.

   The Food Standards Agency commissioned Campden BRI [6] to develop a routine method suitable for uptake by local authority food control laboratories. Several UK laboratories, including Campden BRI (www.campden.co.uk), offer this method for commercial and regulatory use (see BOXES 3 and 4 for a description of this method). The method was also used in a FSA survey on fish species used by the catering sector in 2008 [1]. The Campden BRI method is suitable for identification of the most commonly used species, however modifications are required for identification of species in canned salmon and tuna. There have also been advances in the availability of genetic data from online databases [7] [8] [9], which potentially enable the use of these techniques for identification of many fish species.

[2] Mitochondrial cytochrome oxidase I (COI) gene

3. **Fatty acid analysis.** Fatty acids are important structural elements and sources of energy for all living organisms. The composition of fatty acids in the body is determined by genetic and environmental conditions, particularly relating to diet and food supply. Fish of a given species, from a given location, are likely to have a characteristic fatty acid profile. This method has been used to verify the origin of a catch of herring by a Norwegian purse seiner, and to distinguish between farmed and wild caught salmon.

4. **Microchemistry.** Trace elements[3] are taken up from the surrounding waters and deposited in hard tissues, such as otoliths. Different geographical areas have different combinations of trace elements. Therefore, fish which have lived in different areas are likely to have different micro-chemical fingerprints. These methods, along with other techniques, has been used to distinguish populations of sole, cod and blue fin tuna. A useful feature of this technique is that hard tissues like otoliths do not degrade after death. However, to use otolith analysis, fish heads have to be retained.

Techniques that are currently available to assess provenance of seafood

There are a number of different speciation techniques. Since the 1970s electrophoretic protein separation techniques have been the main method used for speciation of fresh and frozen fish in the supply chain. The novel DNA and other techniques are of value when tracing the geographical provenance of seafood when this is required, and for the speciation of fish in processed and canned product.

There have been advances in routine analytical tools available for non specialist laboratories. The most important in the UK is the PCR- RFLP Lab-on-a-chip technique developed at Campden BRI [6] (described in BOXES 3 and 4). This enables the analysis of a wide range of fish products. Recently, Agilent Technologies [10] have produced a commercial kit based on the Campden BRI method.

How these techniques have been used

DNA techniques have added to the methods available for the detection of mislabelling, both by public authorities and private businesses. These and other techniques for determining the speciation and origin of fish and fishery products have been used for the following purposes:

1. Enforcement

The above techniques have already been used in enforcement of trading standards and fisheries management regulations. Some examples from the JRC [3] report include:

i) There have been several cases, in the UK, Europe and North America, where genetic techniques have been successfully used to establish the correct species of fish in legal

[3] Such as (among others) Strontium, Barium, Molybdenum, Iron and Lead.

cases, some of these involving high values of fish product (up to $US 15 million), and some involving protected species, such as certain species of shark and abalone.

ii) The use of genetic analysis, with other evidence, has been used to identify origins of cod and sprat, which was used as evidence of misreporting in Denmark. It has also been used successfully in tracing the origins of escaped farmed Atlantic salmon in Norway, and for tracing wild Pacific salmon origins in Canada.

Whilst analysis using these techniques can assign fish to a particular geographical location, there is a need for baseline data on the genetic make up of the fish in the location to which it is to be assigned. Often the allocation is based on the probability or 'likelihood' of a particular origin. This has implications for use in forensic analysis; it is always useful to have other evidence available as well. However, a common effect of the presence of DNA evidence is that defendants tend to be more likely to admit their guilt prior to going to court, thereby saving on court costs.

2. Fisheries science

There are many scientific uses for these techniques. For example Bui et al [11] used DNA methods to determine the species of eggs in plankton samples, in order to map spawning grounds of cod off eastern Canada. Fish stocks are assessed in given geographical areas described as 'management stocks'. However, these do not always coincide with the underlying biological populations. There can be sub populations within the overall management stocks, or fish can migrate between management stocks. Thus methods such as those described above, which can improve our understanding of the genetic variation in relation to the geographical origin of fish, is of value in improving fisheries management.

3. Industrial and commercial

These techniques can be used by processors to improve assurance that the raw materials are the species described. Secondary processors and retailers can also use them to check on part processed and processed product. Users should consider which technique will be most cost effective for their purpose. There is a requirement to decide on a sampling strategy that addresses the areas of highest risk in their supply chain and building a sampling programme which will address this risk. Simple observations of fish skin, flesh appearance, and sensory assessment, form a part of this process.

Developing a relationship with an appropriate supplier of analytical services is a key element of this strategy. Such a supplier should be able to advise on costs and benefits, especially where a routine programme is being considered. Samples taken on an ad hoc basis are almost certain to cost more per sample than routine work, whichever technique is chosen.

Points to consider when selecting a speciation method:

- Electrophoretic protein separation techniques (BOX 1) can be used for routine monitoring of fresh or frozen fish, whereas the DNA techniques, particularly those which can analyse mixtures and processed fish, such as the RFLP technique described in BOX 4, are also able to analyse processed, canned and/or mixed samples. However, the technique chosen will depend on the relative time, costs and facilities available.

- In order to ensure that the results are meaningful it is important to ensure that samples are handled according to approved protocols. When collecting the evidence – and this is especially true for material which will ultimately serve for DNA analysis – contamination control and prevention of cross-contamination at the point of collection are essential. It is this part of the procedure that is more likely to be disputed than the laboratory evidence. The presence of other constituents, such as breadcrumbs and other materials, may interfere with the analysis. Separation of this material from the flesh to be analysed is important. The best approach is to take advice from the analyst responsible for doing the analysis.

- Analytical laboratories offering commercial services should be able to demonstrate their competence. ISO 17025 is the standard appropriate to testing and calibration laboratories. Laboratories should be accredited for the methods offered. Laboratories are accredited to ISO 17025 by a third party Accreditation Body which audits their compliance.

- It is important to give the laboratory a list of which species are suspected in the samples. The laboratory will therefore be able to choose the techniques appropriately. There may be some closely related species for which the genetic markers do not give sufficient discrimination. The analyst carrying out the analysis should be aware of any species they cannot distinguish.

- For samples containing mixed species, DNA techniques should properly be described as presence/absence techniques, rather than quantitative analysis of the percentage of each species. The sensitivity of the technique should be discussed with the laboratory. There is evidence that some species' DNA may amplify at different rates during the PCR step (BOX 3). DNA from an initially minor species in a mixture may increase during the PCR step.

- In the interpretation of the results it is important to bear in mind the sensitivity of the technique – if a mixture of species is found from processed fish samples it is possible that some of the material may have originated through cross-over with fish previously used on the production line.

Next steps

The JRC report [3] calls for an EU-wide approach to integrate these techniques into the Common Fisheries Policy, and to provide evidence of general traceability. An EU-wide approach is necessary because there needs to be consistency between Member States in choosing the appropriate techniques and associated protocols. Also, most techniques depend on reference databases for the reliable interpretation of the results. DNA reference databases for fish species are well established [7], [8] [9], but for many species data designed to help define their geographical origin would require more work. Such databases will have to be maintained and updated according to a set of protocols (on data management and accessibility) ideally approved by the EU. The JRC report makes the point that such developments need to be worked out in consultation with stakeholders to ensure that the introduction of the new methods is not a new financial or administrative burden, but a measure that improves good working practices, reassures consumers and identifies bad practice.

The EU-wide approach advocated in the report will be necessary, especially since standardisation (of sampling protocols, reference databases etc) will lead to transparency and consistency in the way the techniques are used and the results interpreted. Until this EU-wide approach is adopted, results will have to be interpreted with some care, but private businesses should be encouraged to consider the full range of techniques when developing control plans.

In more detail

BOX 1. Electrophoretic separation techniques [4]

Electrophoretic separation techniques are widely used in biochemical analysis to separate electrically charged molecules in an electric field. When a voltage is applied between positive and negative electrodes the molecules will move towards one or other electrode. The speed at which each molecule moves is dependent on its size and electrical charge. For proteins, is this dependent on its amino acid composition, which varies due to genetic differences between species. For fish identification using protein separation techniques, water soluble proteins are obtained by mixing a sample of fish flesh with water, which is then centrifuged. Then a small amount of the liquid fraction is applied to the end of a column of polyacrylamide gel or jelly. An electrical potential is set up along the column, which draws the protein through the gel for a set period of time, before the fastest fraction has reached the other end of the gel (see top figure). Proteins from different species will stop in different locations on the gel. The proteins are then stained and the resulting distribution patterns compared between unknown samples and standards; it takes a skilled eye to read the gels for identification. A typical result is shown in the figure below:

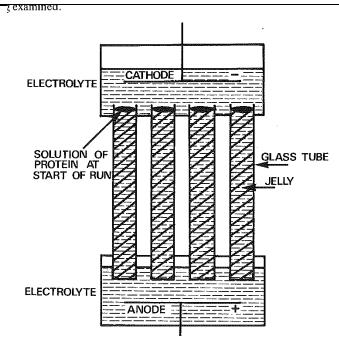
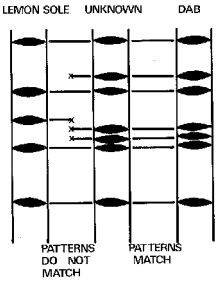


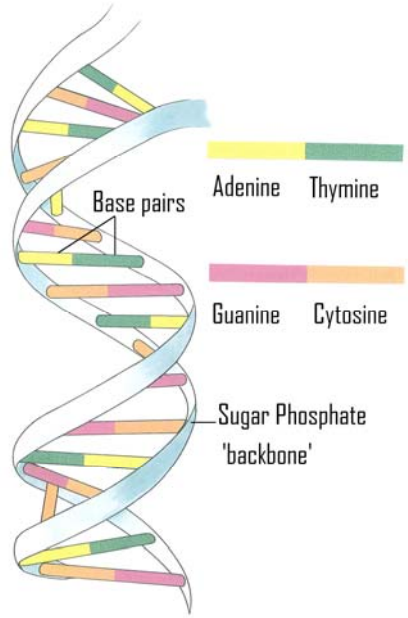
Diagram of basic electrophoresis apparatus from [4]



Identification an unknown species by matching the pattern from [4]

How do DNA tests work?

BOX 2. Structure of DNA



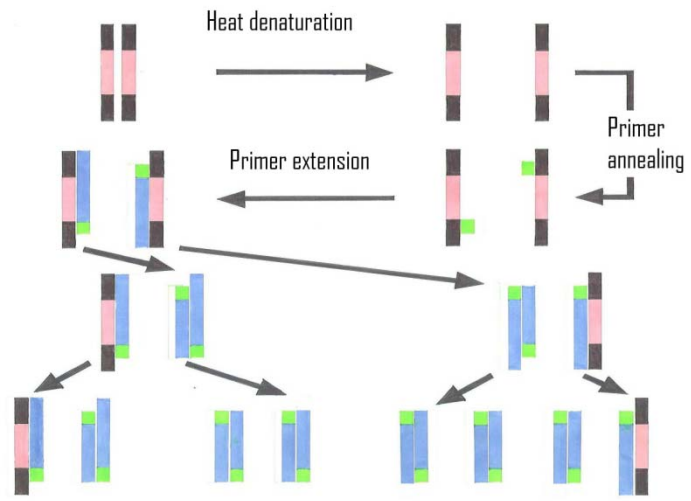
Deoxyribonucleic acid or DNA is a chemical that stores genetic information in the cells of all living organisms. The information is stored as sequences of molecules known as 'base pairs' – these form the genetic code (genome) of an individual. It is the sequence of base pairs, consisting of two on opposite sides of the double helix which are usually known by their initial letters;

Adenine = A
Thymine = T
Guanine = G
Cytosine = C

which codes the characteristics of the living organism. They always form pairs as shown on the figure; A pairs with T and G with C.

The majority of DNA is in the nucleus of the cell, but DNA is also present in the mitochondria, which are structures inside the cell which carry out aerobic respiration. The characteristics of mitochondrial DNA is widely used in species identification [5].

BOX 3. Amplification of DNA; polymerase chain reaction (PCR)



Cycle 1	Splits the DNA into two - exposing the two sides of the molecule. **Primers** attach to specific sections of the DNA, and it is induced to extend new strands of DNA starting at the primers.
Cycle 2	Many copies of the **Amplified DNA;** specific section of DNA between the two primers are produced.
Cycle 3	

Key		Section of DNA to be amplified		Primers		Amplified DNA

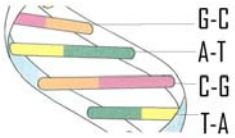
A Polymerase Chain Reaction (PCR) is used to amplify the DNA by inducing the DNA to produce many copies of itself, rapidly, in the laboratory. First the DNA is extracted from the cell. Then the PCR process (above) is carried out by cyclical heating and cooling the extracted DNA in the presence of 'primers'; short lengths single strand (only one side of the molecule) DNA. These extract specific parts of the DNA, with other constituents which enable the assembly of DNA; nucleotides and sugar phosphate backbone (BOX 2). The heating phases split the DNA down the double helix, separating the two nucleotides from each base pair apart. When cooled the new DNA is assembled on the template of the exposed strands, between the primers. This is carried out by specific DNA polymerase enzymes. These specific parts of the DNA, as extracted by the primers, reproduce many thousands of times during repeated heating and cooling cycles; this is termed amplification. Once they are amplified, the characteristics of these extracted parts of DNA can be examined.

Techniques using PCR amplification on samples where there is a mixture of species can only describe the presence and/or absence of species. This is because there is evidence that DNA from different species may amplify differently during the PCR, due to the quality of DNA from the different species; what may start as a 50:50 mixture of DNA prior to the PCR may change if the DNA of one species increases at a greater rate than the other.

BOX 4. Species identification techniques

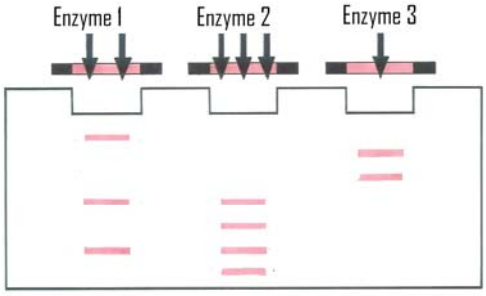
Once the DNA has been amplified as in BOX 3 it can be analysed to identify the species. Different species' DNA become distinct from each other as it mutates from generation to generation; this is termed genetic polymorphism. Methods for distinguishing species focus on finding sequences of bases on the DNA which are unique to each species, and finding the means to extract and identify them. Direct sequencing the genetic code for whole genomes, or significant parts of genomes, has now become a feasible proposition and can be used to investigate the relationships between fish populations. There are projects aiming to fully sequence Atlantic cod, Atlantic salmon, European sea bass and significant portions of the genomes of European hake, herring and common (Dover) sole [3].

Sequencing DNA



The order of the base pairs on the DNA determined for specific genes in the unknown sample. Information from data bases listing sequences of base pairs for different species are compared with the unknown. This technique requires specialized skills and apparatus

Restriction Fragment Length Polymorphism or RFLP technique [6]



In this technique sections of DNA corresponding to specific genes are obtained by PCR (BOX 3) from the unknown sample, and are treated using specific enzymes. The enzymes cut these DNA sections into several fragments. In this case enzymes 1, 2 and 3 cut the DNA into 3, 4 and 2 fragments respectively. The theoretical size of the cleaved fragments can be predicted for each species from published information [7] [8] and [9].

These fragments are then separated using electrophoretic techniques; they are drawn through a gel using electric potentials. Each fragment from each species will behave in a specific manner in the gel related to its size, and hence can be separated from the other fragments.

Recent developments have led to the miniaturisation of this process, which means separating and characterising these fragments can now be carried out using micro chip technology – 'lab-on-a chip'. The Campden BRI method uses micro chip technology and specialized machinery (Agilent 2100 Bioanalyser [10]) to separate out the fractions of DNA cleaved by three different enzymes using the RFLP technique. Each analysis is carried out on a disposable chip. The method can cope with mixtures of species in the samples, and is considered robust enough to be used in routine detection in non specialist laboratories. The LabChips used by the system are small ($3cm^2$), disposable, single-use units.

The method has been developed to detect the commonly used species in raw, frozen and the majority of processed products; however some modification is needed for the identification of species in canned fish materials. It is also able to detect mixtures of species, down to a level of 2-3% of the minor species. However, because the technique uses the PCR amplification technique (BOX 3) it less able to describe the proportions of the fish in the mixture, only the presence and/or absence.

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