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Short communication

Current methods for seafood authenticity testing in Europe: Is there a need for harmonisation?

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ABSTRACT

Mislabelling of food products has recently received a great deal of public scrutiny, but it remains unclear exactly what methods are being utilised in laboratories testing the authenticity of foods. In order to gain insight into the specific area of the analysis of seafood, a questionnaire focussing on the taxonomic groups typically analysed and the techniques utilised was sent to over one hundred accredited laboratories across the UK, Ireland, Spain, Portugal, France and Germany. Forty-five responded positively, demonstrating significant differences in both the species analysed and methods utilised among the countries included in the survey. Indeed, a diversity of methods was employed across laboratories and efforts to harmonise and/or standardise testing were evident only at national scale. This contrasts with the EU wide scale of regulation on seafood labelling, and may lead to inconsistencies in the results produced in countries.

1. Introduction

Mislabelling of food products, so that the description or labelling does not accurately reflect the purveyed food, has recently received a large amount of public attention. This came to great prominence during the 2013 "horse meat scandal" in Europe, where a range of supposedly beef products were found to contain horse flesh (FSAI, 2013). What makes this discovery surprising is that it took place despite the clear set of European Union (EU) regulations relating to food traceability and labelling, which require a complex system of checks to ensure that food remains authentic and traceable (Schröder, 2008). In fact, it was only through the use of DNA based methodologies for identifying species that this food fraud was detected.

This case clearly demonstrates the utility of DNA based authenticity techniques as a tool in food control, which have been shown to be particularly useful in the specific case of testing seafood, particularly fish, due to astounding biological diversity that underpins this complex market. The global trade of seafood products over the last 40 years increased from 0.8 million metric tons worth $1.3 billion in 1975 to 2.4 million metric tons worth $16.5 billion in 2012 (NOAA, 2013). This has also been accompanied by greater complexity in commodity flows, with some products crossing multiple national boundaries during the supply chain, including movements into territories without stringent traceability requirements (D'Amico et al., 2014). There is also a huge diversity of species and products available on the global seafood market, such that the U.S. Food and Drug administration includes approximately 1700 species of commercial finfish and shellfish in its Seafood list (FDA, 2013).

Whilst species identification can usually be made from morphological characteristics from fish or shellfish in their whole form, seafood is often processed before reaching the consumer. This

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potentially creates a situation where substitution of species, particularly for economic gain (i.e. where a low value product is substituted for a higher value one) may occur, but is difficult, even impossible, to identify without authenticity testing. Indeed, numerous genetic studies have now been published that demonstrate high levels of substitution and mislabelling across a variety of seafood products (e.g. Griffiths et al. 2013; Rehbein & Oliveira, 2012; Miller & Mariani, 2010; Wong & Hanner, 2008).

These studies have clearly demonstrated that seafood mislabelling is a widespread phenomenon, but they also highlight the huge diversity of methodologies that have been developed for identifying/distinguishing between species. The exhaustive recent reviews by Rasmussen and Morrissey (2008), Teletchea (2009) and Lago, Alonso, Vietes, and Espineira (2013) emphasise the fact that many traditional and official methods used in species identification are based on the biochemical analysis of specific proteins, e.g. isoelectric focussing (IEF), high performance chromatography or immunoassay. These approaches have a number of disadvantages, the most significant being that many can only be applied to fresh samples, and they cannot be used on highly processed (i.e. cooked or canned products) because the proteins become denatured upon heating. In comparison, DNA is a more thermostable molecule and although it may become degraded during processing, short fragments are generally recoverable and can form the basis of authenticity tests in processed foods (Mackie et al., 1999; Quinteiro et al., 1998). Furthermore, detailed surveys of the scientific literature on seafood authenticity reveal it to be a continuously evolving field, with over 150 peer-reviewed papers on the topic in the period 1995–2008 (Rasmussen & Morrissey, 2009; Teletchea, 2009).

Against this backdrop of increasing regulatory complexity and technological development, it is unclear what methods are actually being utilised in public and private authenticity laboratories. The EU regulations (numbers 104/2000, 2065/2001, 01224/2009 and 404/2011) concerning food labelling and traceability contain little/no guidance on what techniques should be applied and no recognition of an "official" method. While the plethora of DNA techniques available offers undeniable advantages in biological identification, their ability to distinguish particular groups of species may vary considerably (Teletchea, 2009; Viñas & Tudela, 2009). Therefore, the aim of this study was to survey public and private laboratories that conduct seafood authenticity testing across Europe to investigate how products, particularly finfish products, are analysed. This knowledge is vital in a regulatory and legal context as it remains key to accurately testing food and providing robust evidence for prosecuting those that break the law. It also represents a fundamental step towards the establishment of an efficient, validated, standardised transnational procedure for monitoring authenticity in the seafood market.

2. Materials & methods

2.1. Survey development

In order to maximise the number of responses, a relatively simple questionnaire was constructed, which included eight brief questions and avoided asking for commercially sensitive information that could potentially have prevented response from commercial or private laboratories (Supplementary material 1). The questions posed that generated the most insight were:

- Are you a public or private testing facility?
- What species are you most commonly asked to check for mislabelling?
- What biochemical or molecular method(s) do you use for distinguishing between species in the analysis of sea-foods?
- In any molecular genetic methods, what region of the DNA do you use (nuclear vs mitochondrial, specific protein coding genes or non-coding regions; can you tell us which you use for each taxonomic group)?
- Are you developing any novel approaches that you could tell us about in broad terms?

The remaining questions were related to the methodologies employed and generally concerned the capabilities of the laboratories i.e. in terms of equipment, specificity of methods and the extent of reference data collected.

The questionnaire was translated and e-mailed to 101 authenticity laboratories involved in the testing of seafood in the United Kingdom (UK), Republic of Ireland (ROI), France, Germany, Spain and Portugal, which include five of the top ten countries in Europe in terms of total supply of fisheries products (FAO, 2009). The questionnaires were initially sent out in November 2012, with efforts to elicit responses continuing until March 2013. How laboratories were short-listed in each country varied, but efforts were made to contact both public and private authenticity facilities that have some degree of officially recognised accreditation (e.g. from the United Kingdom Accreditation Service, Portuguese Institute for Accreditation, National Association of German Chemists, Spanish National Entity for Accreditation or French Committee of Accreditation). However, given the survey's authors' experience in the field, questionnaires were also distributed through previously established networks of contacts.

2.2. Statistical analysis

Given that the amount of detail provided by different laboratories to many questions varied considerably, efforts were made to standardise answers by grouping specific responses into broader categories, prior to statistical analyses. Additionally, as single responses were gathered from Portugal and ROI, these were combined with those from their geographically and culturally most proximate neighbours; Spain and UK, respectively, for statistical testing.

To explore general patterns in the data, principal component analysis (PCA) was conducted in PRIMER-6 (Clarke & Warwick, 2001), with each testing laboratory representing an individual data point in the ordination. The software was also used to conduct a non-parametric analysis of similarity (ANOSIM), utilising the Bray–Curtis distance measure. Specifically, the ANOSIM was used to test if there were significant differences between countries in terms of the types of products/species laboratories commonly test, and also for differences in the authenticity methods employed. The hypothesis being that different countries will have cultural differences in the seafood products they consume, leading to significant differences in the types of products analysed and the authenticity methodologies tailored to them.

3. Results

The results for the responses to each of the questions are examined below, one at a time.

3.1. Are you a public or private testing facility?

Of the 101 laboratories contacted, 45 responded positively; a response rate of 44.6%. Across all the countries included, 66.7% of completed questionnaires originated from public laboratories and 33.3% from private facilities (summarised in Supplementary material 2).

3.2. What species are you most commonly asked to check for mislabelling?

A total of 38 laboratories provided information on the species they typically test and the level of detail provided varied considerably, some facilities listed Latin names, whilst others included much broader commercial designations. Therefore, the responses were classified into wider taxonomic groups, which also included species that are commonly used as substitutes e.g. the gadoid classification includes any responses of: "cod", "haddock", "gadoids", "white fish" and "pangasius" or "panga", as species from the tropical catfish Pangasiidae have widely been used as a substitute for gadoids. A total of 18 classes were constructed (of which nine included only one or two records, see Supplementary material 3). By far the most commonly tested species groups were: gadoids, flat fish, tunas and salmonids. Global comparisons were highly significant ($R = 0.447$, p-value $= 0.001$, Table 1), suggesting big differences between the countries in terms of the products and species commonly tested. A simplified version of the dataset, with the species classes that only incorporating one or two records removed, was analysed via PCA in order to reduce the number of variable vectors and make the figure clearer (Fig. 1, the PCA with all vectors is also included in Supplementary materials 4). It clearly demonstrates how testing in the UK, ROI and France is dominated by gadoids and salmonids, whilst flat fish are more predominant in Germany and a combination of hakes, clupeids and tunas are important in Spain and Portugal.

3.3. What biochemical or molecular method(s) do you use for distinguishing between species in the analysis of seafood?

All 45 laboratories answered this question. Similar to above, the responses were grouped into 12 broader methodological classes (Supplementary material 5). So for example, any sequencing based identification methods, regardless of the gene/region targeted were classed under forensically informative nucleotide sequencing (FINS). The three most widely utilised methods were FINS (in 68.9% of laboratories), restriction fragment length polymorphism (RFLP, 40.0%) and IEF (20.0%). This reflects the fact the DNA-based methods were far more prevalent, with only 10 of the laboratories including a biochemical protein-based protocol in their response. Global testing of the results demonstrated significant differences between countries (ANOSIM; $R = 0.259$ $p = 0.001$, Table 1). Further comparison of the pairwise tests between countries shows that much of this result is due to the UK & ROI group (for which all three tests against other groups were significant at the 95% confidence interval, Table 1). A simplified version of the dataset, where classes of method with a single record were removed in order to reduce the number of variable vectors, was analysed via PCA (Fig. 2, the PCA with all vectors is also included in Supplementary materials 6). It shows the importance of FINS, RFLP and IEF across Europe, but there is little evidence of different patterns in methodological application between countries surveyed.

The survey included three further questions that are related to methods of choice. First, "are your methods universal or tailored to specific groups of fish?" Thirty nine laboratories responded, 5.1% said their methods were specific to certain groups, 43.6% utilised universal methods and 51.3% used both. Second, "have you developed your own databases of reference material or baseline information to distinguish between species?" All 45 laboratories responded, 24.4% exclusively employed public databases, 35.6% utilised their own private reference data and 40.0% used both. Finally, "what key pieces of equipment do you use in distinguishing between species?" The majority of laboratories that responded to this question indicated they had access to basic molecular biology equipment e.g. PCR machines, electrophoresis kit etc.

Table 1
Results of the ANOSIM testing for differences in responses among the countries surveyed. Both global and pair-wise tests between countries are included. Results significant at the 95% confidence interval are highlighted in bold, those remain significant after sequential Bonferroni correction (initial value 0.05/7) are also marked with*.

Test	Species		Methods		DNA markers	
	R statistic	*p*-value	*R* statistic	*p*-value	*R* statistic	*p*-value
Global	**0.447**	**0.001***	**0.259**	**0.001***	0.121	0.050
UK/ROI & Spain/Portugal	0.633	0.091	**0.362**	**0.001***	**0.320**	**0.002***
UK/ROI & France	**0.229**	**0.001***	**0.366**	**0.026**	0.158	0.364
UK/ROI & Germany	**0.377**	**0.035**	**0.358**	**0.001***	−0.034	0.675
Spain/Portugal & France	**0.394**	**0.001***	0.156	0.130	0.037	0.444
Spain/Portugal & Germany	**0.526**	**0.001***	0.041	0.200	**0.180**	**0.026**
France & Germany	0.276	0.062	**0.383**	**0.011***	−0.005	0.505

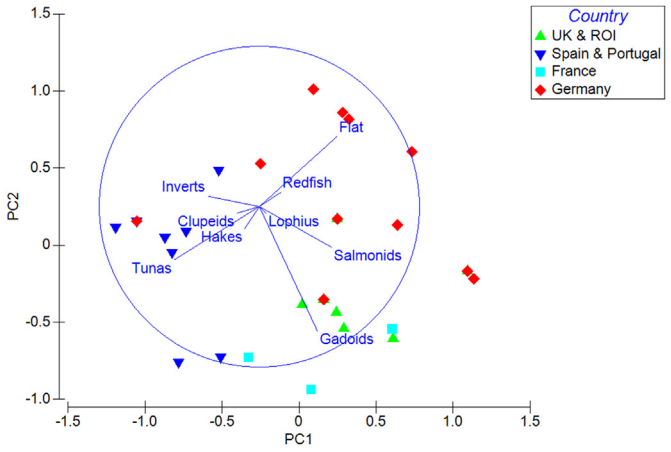

Fig. 1. PCA the species commonly tested in each authenticity laboratory. PC 1 incorporates 25.3% and PC 2 18.9% of the variation (eigenvalues = 0.382 and 0.286, respectively).

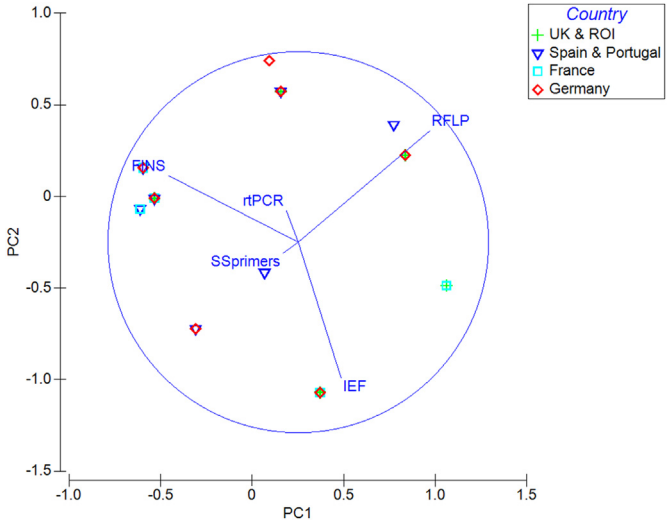

Fig. 2. PCA the methods utilised in each authenticity laboratory. PC 1 incorporates 42.5% and PC 2 27.4% of the variation (eigenvalues = 0.339 and 0.218, respectively). Methodological abbreviations; FINS = forensically informative nucleotide sequencing, RFLP = restriction fragment length polymorphism, IEF isoelectric focusing, rtPCR = real-time polymerase chain reaction & SSprimers = species specific primers.

3.4. In any molecular genetic methods, what region of the DNA do you use (nuclear vs mitochondrial, specific protein coding genes or non-coding regions; can you tell us which you use for each taxonomic group)?

40 laboratories gave some indication of the DNA they target across the methodologies they utilise. Only a quarter of these specified that at least some of their methods utilised nuclear DNA, and these involved a wide range of targets, both anonymous DNA regions e.g. microsatellites and randomly amplified polymorphic DNA (RAPD) and specific genes e.g. rhodopsin and pantophysin (Supplementary material 7). Conversely, all the laboratories positively indicated mitochondrial DNA was a focus of their authenticity testing. Three gene regions proved to be the markers of choice; Cytochrome B (cyt-b, specified in 72.5% responses), cytochrome oxidase 1 (COI, 27.5%) and 16s ribosomal DNA (16s, 17.5%). Global testing of the results failed to detect significant differences between countries, although the result was very close to the 95% confidence interval (ANOSIM; $R = 0.121$ $p = 0.050$, Table 1).

3.5. Are you developing any novel approaches that you could tell us about in broad terms?

Only two laboratories declined to answer this question, but the remaining 43 responses were generally in the negative. Interestingly, of the nine laboratories who indicated the technologies they were currently investigating, six specified "real time" PCR (rtPCR) based methods.

4. Discussion

This work represents the first effort to assess what methodologies are being applied to seafood authenticity in Europe. There are some very clear patterns that emerge from the responses: firstly, the groups of species tested across the regions varied significantly. Second, DNA based methods, particularly FINS, dominated the responses, but approaches were inconsistent between laboratories, and protein based biochemical methods are still commonly utilised. Although a total sample size of 45 laboratories does not necessarily provide enough data to investigate subtler dynamics within and between countries, some patterns are very strong and reveal a substantial lack of standardisation.

This survey identified highly significant differences in the species commonly tested in the countries surveyed, which is entirely consistent with the cultural preferences for seafood across these regions. A range of white fish species are commonly consumed in northern France, UK and ROI, which is clearly reflected in Fig. 1. Similarly, a culture of consuming hake and clupeids in Spain and Portugal and the high value attached to sole (*Solea solea*) in Germany, are also reflected in the results. It seems very likely that if further regions of Europe were surveyed with their own traditions of consuming seafood even more complex patterns in the species tested would emerge (Armani, Castigliego, Tinacci, et al., 2012). This presents a significant issue in terms of harmonising and standardising approaches to seafood authenticity across Europe, to which the EU is generally committed, and for which all member states are governed by the same regulations regarding traceability and authenticity. It is due to the fact that laboratories in various countries are likely to be more familiar with testing for a discrete sub-set of species (and may have developed methods optimised to these groups), but any standard methodology will have to function across a much broader taxonomic range that reflects the diversity of cultural preferences in seafood consumption across the EU, and the progressively more globalised import landscape of the EU (Armani, Castigliego, & Guidi, 2012; De Silva, 2010 , pp. 7–10; Sotelo & Pérez-Martín, 2007). Nevertheless, it is important to note that there are existing efforts to harmonise testing of seafood, for example, the Food Analysis Performance Assessment Scheme (FEPAS; http://fapas.com) provides a regular fish authenticity proficiency testing scheme. This involves the analysis of "blind" samples, i.e. where the species of origin is unknown, which can be incorporated into the requirements of national accreditation bodies. In Germany, harmonisation has gone a step further; under the German Food and Feed Act (§64 Lebensmittel-und Futtermittelgesetzbuch), ring trials have been used to develop a range of officially recognised standard methods, which are coordinated by the Federal Office of Consumer Protection and Food Safety (BVL; http://www.bvl.bund.de). However, this also emphasises how harmonisation has largely been driven at a national, but not EU-wide, scale.

Despite the view that traditional and official methods used in species identification are based on the analysis of specific proteins (Rasmussen & Morrissey, 2008; Teletchea, 2009), the majority of authenticity laboratories routinely employ DNA based protocols in their analysis of seafood and have access to equipment for basic molecular genetics. This can been seen as further evidence that control laboratories, which are traditionally seen as being staffed by analytical chemists, are embracing genetic tools for distinguishing species (Woolfe, Gurung, & Walker, 2013). Nevertheless, it is important to note that IEF is still commonly utilised, remaining the third most commonly employed class of method. This probably relates to the low-cost and speed of the protocol (making it an ideal approach for an initial screening of samples), and the long period it has been the Association of Analytical Communities (AOAC) recognised method for species identification (AOAC, 1980).

The widespread use of DNA based methods does not necessarily mean that the protocols across the laboratories are standardised. The surveyed laboratories utilised a diverse set of techniques and a global test across all regions was significant, suggesting differences in the methods applied between countries. In particular, the UK and ROI group was generally identified as using a distinct set of methods to those in the rest of Europe. This reflects previous efforts of the Food Standards Agency's Food Authenticity Programme to transfer DNA methodologies to UK Official Food Control laboratories. They supported knowledge transfer activities and provided funding for a standard operating procedure (SOP) based on a RFLP protocol on a lab-on-a-chip platform (Agilent 2100 Bioanalyser) for species identification (Dooley, Garrett, Sage, Clark, & Brown, 2010; Garrett, Dooley, Brown, & Clarke, 2010). So the majority of public analyst laboratories follow the same SOP, probably making this one of the largest efforts to standardise seafood authenticity testing with a single method in Europe. However, the use of a RFLP technique in the UK contrasts with the more widespread application of FINS in the rest of Europe. The PCA scatter also illustrates how the variation in techniques used is considerable, even within most countries.

The grouping of protocols into broader methodological classes to facilitate the statistical analysis actually disguises the full diversity of approaches revealed by the questionnaires. So for example, FINS was the most commonly utilised class of method, but it includes many laboratories with different target sequences, and even where laboratories utilise the same genetic region it does not necessarily imply the same primers/protocol are being applied (Burgener & Hübner, 1998; Sevilla et al., 2007). The subsequent question in the survey, regarding the regions of DNA targeted, helps clarify this issue to some degree. It demonstrates a relatively wide diversity of DNA targets, both nuclear and mitochondrial, with mitochondrial regions dominating. This reflects several advantages mitochondrial DNA presents in authenticity testing, particularly its haploid matrilineal inheritance and its high copy number within the cell (Rasmussen & Morrissey, 2008; Teletchea, 2009). It is also interesting to note that despite the global Barcode of Life initiative

promoting the sequencing of COI for identifying species (Ratnasingham & Hebert, 2007) and the US Food and Drug Administration developing this as a validated method utilised for seafood authenticity, cyt-b remains the most popular DNA target (although, since these responses refer to any DNA based method, not just FINS, there may be a systematic inflation of the role of cyt-b, as this region has been long optimised for RFLP analysis).

This survey revealed the diversity of biochemical and genetic methods that are used in laboratories across Europe, demonstrating a general lack of standardisation in testing between laboratories. This has important implications, essentially meaning that if the same sample was analysed in different laboratories, conflicting results could be generated or, more likely, the majority of specific tests for a narrow range of species/products will simply fail to identify the sample. However, inconsistency may also arise when considering the reference data that is being utilised to perform species identification. The survey specifically included a question concerning whether the laboratories employed their own private, or a publically available, database (with some using only private or public databases, and others a combination). Both kinds of databases could potentially be associated with inconsistent identifications. Public databases tend to be the most comprehensive, but may contain sequences erroneously attributed to the wrong species, which may require some interpretation. Conversely, private data collections may differ significantly between laboratories, producing an additional source of inconsistencies when comparing results. The use of reference tissues is an aspect that will play a part in future improvements of method standardization. While sequencing-based approaches rely on large amount of reference data stored in public data bases (e.g. GenBank, http://www.ncbi.nlm.nih.gov/; BOLD, www.boldsystems.org) most other techniques hinge on the existence of voucher specimens in the control labs, whose provision and exchange would represent a challenge for a robust, wide-spectrum, long-lasting standardisation initiative. Even the choice of sequence data bases in support of FINS should be based on the level of maintenance, verification and filtering of the said sequences, in order to minimize the risk of "false matches", especially for less commonly traded species. Failure to consider these constraints will have serious implications within the context of enforcement and prosecution, acting to undermine the confidence of stakeholders.

5. Conclusions

Despite the view that most traditional methods used in species identification are based on the biochemical analysis of specific proteins, DNA sequencing appears to be the most commonly applied approach (with the analysis of the cytochrome-b gene dominating). However, there is a diversity of approaches that highlights the lack of consistency in how protocols for identifying species in seafood are applied at a European level. This absence of harmonisation and standardisation could lead to inconsistencies in results generated between laboratories, which may have significant regulatory or legal implications. These results underline the need for a more rigorous standard operating procedure to be applied across the EU. Previous attempts to develop standard approaches to seafood identification at a national scale have been successful, both within the UK (Woolfe, Gurung, & Walker, 2013) and the US (Handy et al., 2011), demonstrating the feasibility of such an approach.

Authors's contributions

AMG drafted the questionnaire, coordinated the international collection of responses, contacted laboratories in the UK, analysed the data and drafted the manuscript.

CGS led the project consortium who carried out this work, provided names and contact with Spanish laboratories, provided revision and discussion of the manuscript.

RM & HAS coordinated the enquiries to the Portuguese laboratories, provided revision and discussion of the manuscript.

RIP supervised the enquires of the Spanish laboratories, provided revision and discussion of the manuscript.

US coordinated the enquiries to the German laboratories, provided revision and discussion of the manuscript.

VVB coordinated the enquiries to the French laboratories, revision and discussion of the manuscript.

MS made approaches to Irish laboratories, distributing the questionnaire to professional contacts as well as contributing to discussions of the manuscript.

SM drafted the questionnaire, contributed to data analyses and manuscript drafting.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.foodcont.2014.04.020.

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