A review of current and emerging DNA-based methodologies for the determination of the geographical point of origin of food stuffs

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Aim

The aim of this report is to provide a current review on the use of existing and emerging DNA technologies with respect to their application for determining the geographical point of origin of food stuffs. This review evaluates the performance of DNA based techniques which could be utilised for the traceability of food products and as an aid in enforcement of EU regulation 1169/2011 for food labelling compliance. Example applications of country of origin labelling are provided, as well as the potential advantages and disadvantages of each of the DNA based technologies.

Executive summary

Recent changes in EU legislation have highlighted the requirement for the traceability of food produce. The recently implemented EU Regulation 1169/2011 stipulates that all fresh and frozen meat and fish products must be clearly labelled with their geographic origin as of December 2014. Although both elemental and isotopic based measurements have provided the basis for much of the information pertaining to the point of origin of food products during the last decade, alternative rapid and cost effective methodologies will be required in order to assist the relevant authorities with enforcement of the new labelling requirements (e.g. for Government Chemist referee cases). Molecular biology approaches focusing on DNA-based techniques are increasingly being evaluated for use in traceability studies, as is

evident from the number of national and international traceability projects now in existence.

DNA based approaches for country of origin labelling include Cleaved Amplified Polymorphisms (CAPS), microsatellites, species specific PCR amplification, Single Nucleotide Polymorphisms (SNPs), and DNA sequencing (e.g. targeting the nuclear genome or the metagenome). CAPS often require prior DNA sequence knowledge of the target species genome, and incomplete amplicon restriction can result in the generation of ambiguous fingerprints. Microsatellites have been shown to be highly reproducible and informative, but somewhat limited in their throughput and can be resource intensive. Species specific PCR amplification can be rapid, robust and cost effective, but again is dependent upon the availability of prior accurate DNA sequence information of the species of interest. SNPs have a high frequency of occurrence, are highly reproducible and the analysis can be automated. With the advent of modern DNA sequencing approaches, barcoding mitochondrial targets can be cost effective, and there is broad acceptance for the use of this approach for speciation analysis by the general scientific community. Metagenomics (sequencing the population of microbial genomes associated with a sample) may have the greatest potential for country of origin labelling, but requires a large investment of resources in databasing, bioinformatics and dedicated IT expertise in order to support this new and evolving area.

As with those approaches based on chemical and isotopic composition of a sample, the accuracy of many of these DNA based methods will only be as good as the accuracy of available databases detailing the correct information. Chemical and isotopic measurement techniques are likely to remain the definitive methods of choice with respect to determining the geographical point of origin of a broad spectrum of biological and non-biological substances, but DNA based methods can serve as a means for either screening a sample of interest, or as an adjunct to conventional analytical methods.

Introduction

In today's modern society, consumers demand clear and accurate information about the food they consume in order to enable them to make informed decisions regarding their diet and the nature of the food they purchase. The reasons for this are numerous, but decreased public confidence in food quality and safety, particularly for those products which are produced outside of the UK or EU, rank highly. Many of these concerns have been shown to be as a result of the number of food associated health scares that have recently come to the public attention, which have included concerns about bovine spongiform encephalopathy (BSE) [1], the UK 2007 foot and mouth disease outbreak [2], and the 2013 EU horse meat issue [3, 4].

Prior to 2015, UK food regulation was legislated for by the 1996 food regulation act, [5] and EU directive 2000/13/EC [6] which required that the food supplied to a consumer must be exactly what was stated on the product label, or in its description. As of December 2014 this legislation was extended with the implementation of EU council regulation 1169/2011 EU which made it obligatory that all fresh and frozen meat, as well as fish produce, be clearly labelled with the point of origin.

In the majority of cases, the existence of conventional paper traceability and tagging systems are likely to assist in improving consumer confidence. However, due to modern widespread transportation of food products across international borders, numerous opportunities now exist for the unscrupulous relabelling of products by the criminal fraternity. As a consequence, there exists a growing requirement from the enforcement agencies, for access to rapid and reliable analytical methods that can verify the validity of source of origin labelling. This requirement will provide a significant driver to the scientific community to assist with the development of new techniques and assays which will enhance current capability in the determination of point of origin for many food products.

Determination of the point of origin of food and food products is an analytically challenging problem, and one that is currently the focus of global scientific attention, particularly in Europe and the United States. A number of different techniques have been evaluated for the purpose of addressing this question, of which the majority have been focused on the use of mass spectrometry based techniques. These fall into three broad categories including those which employ the analysis of elemental

composition, particularly methods based on inductively coupled plasma atomic emission spectroscopy (ICPMS) [7], those which analyse stable isotope ratio's, such as isotope ratio mass spectrometry (IRMS) [8], or those based on a combination of the two approaches [9].

Recent publications in the field have highlighted a number of studies that have examined a range of alternative approaches. These have included methods based on the technique of nuclear magnetic resonance spectroscopy (NMR) [10], near and infrared spectroscopy (NIR)[11-13], multispectral imaging (MSI) ([14]) and nucleic acid sequence composition [15, 16].

DNA based technologies

Although conventional analytical approaches such as IRMS and ICPMS are capable of providing a good indication as to the likely geographical origin of a sample, instrumentation and running costs, plus the requirement for highly trained analysts, and a protracted workflow, make their use for routine sample analysis difficult. With lower cost implications, and an increasing number of nucleic acid based assays becoming available for food authenticity testing, DNA-based molecular methods have experienced a rapid uptake by many enforcement agencies. Recent technical advances with nucleic acid based marker systems have made the exploitation of genetic variation, where present, possible, and which can be used to provide an indication as to where a product may have originated. A number of techniques have been, or are currently in the process of being evaluated for this purpose [17-19].

Three characteristics of DNA make it an extremely useful tool for food authenticity testing. Firstly it is an extremely stable and durable bio-molecule that can be recovered from a variety of biological materials including those that have been subject to non-optimal storage conditions. Secondly, it is found in all biological tissues or fluids that contain nucleated cells (or non-nucleated with respect to mitochondria or plastids). And thirdly, DNA can provide more information than other bio-molecules owing to the degeneracy of the genetic code and the presence of significant stretches of non-coding regions.

Numerous technical advances within the field of molecular genetics have enabled the routine analysis of genetic variation to be performed in many food testing

accredited laboratories. Current molecular methods are quick, precise and reliable, and as a result the analysis of genetic variation has rapidly become the method of choice for a number of applications including that of food authenticity. Many of the DNA-based approaches used for authenticity testing have the potential to be applied to determining a sample's point of origin.

The choice of DNA technology is principally governed by sample DNA quality and integrity. For example, Quinteiro *et al* [20] found that attempts to detect gene specific fragments above 299 bp were unsuccessful when analysing DNA isolated from canned processed tuna. DNA degradation in raw meat, as verified by comet assay [21], has been reported to progress at relatively slow rates at temperatures below $4^{o}C$ [22]. However, on exposure to high temperatures and pressure as would be experienced during the canning process, DNA tends to degrade rapidly. For example, the high temperature and pressures used during sterilization procedures have been shown to degrade DNA into fragments of 500 bp or less [7]. In addition, treatment with heat or acid can also result in the depurination of DNA [23] which would preclude its use with many amplification, or ligation based technologies [24].

Cleaved Amplified Polymorphic Sequences (CAPS)

The cleaved amplified polymorphic sequence method (CAPS), is an extension of the restriction fragment length polymorphism (RFLP) approach. RFLPs are based on a technique which exploits previously characterised DNA sequence variation existing between two samples, and which has led to the modification of a DNA restriction enzyme recognition sites at, or close to, a locus of interest. The technique involves the fragmentation of a DNA sample using a restriction enzyme or enzymes, followed by the size separation of the resulting DNA fragments using agarose gel or capillary electrophoresis. An RFLP occurs when the length of a detected fragment varies between two individuals, and the resulting pattern of fragments detected can be used as a means of identification [25]. CAPS works on the principle that genetic differences between individuals can create or abolish restriction endonuclease restriction sites, and that these differences can be detected in the resulting DNA fragment length following digestion of the amplicon with an appropriate endonuclease restriction enzyme. The method offers several key advantages over the original RFLP method, including: simplified workflow, high through-put capacity, and robust data generation [26]. The technique has been widely applied for the

analysis of plant [27, 28] meat [18, 29], and fish [20, 30] based samples, and has been reported as being used for identification of the possible geographical source of numerous species, including: beef [31], swordfish (*Xiphias gladius*) [32], papaya [33], and coffee [34]. However, the technique requires some prior sequence knowledge of the amplicon or target species genome, and suffers from problems associated with incomplete amplicon restriction which can result in the generation of ambiguous fingerprints. Despite these limitations this method is one of the most frequently used molecular approaches for point of origin analyses [35].

Microsatellites

Microsatellites, or simple sequence repeats (SSRs), are tandemly repeated units of short nucleotide motifs. They are typically co-dominant and have been routinely used for kinship, population and other speciation based studies. However, they can also be used for studies of gene duplication or deletion, with marker assisted breeding selection, or for genetic fingerprinting. Historically, microsatellites were first used using DNA hybridisation based techniques. However, with the development of PCR technology the marker system is now primarily performed with use of amplification technology. By designing primers to the regions flanking the microsatellite locus, an amplicon can be generated which reflects the number of repeats present at a locus of interest. Variation in amplicon size can be visualised with use of gel or capillary electrophoresis which can aid with maximising sample throughput. Because the regions flanking microsatellite loci can provide specificity at both the genus and species level [36, 37], their use can be used to provide an indication as to point of origin.

Microsatellite analysis has been shown to be an effective tool for individual assignment as well as genetic variation analysis [38, 39], and have been used to identify the point of origin of numerous commercially important species. For example, Yuan *et al*, [40] have reported on the use of a panel of 12 Dendrobium derived microsatellite markers to differentiate between Dendrobium species (Orchidaceae) originating from different geographical locations. In Traditional Chinese Medicine (TCM), an important class of medicine is produced from the stems of various Dendrobium species (Orchidacea), and medicinal practitioners hold the consensus that herbs grown in different geographical locations vary in their curative effects [41-43]. Selecting an appropriate population of medicinal herbs is therefore a key factor

in the modernisation of TCM [43] and Yuan *et al* conclude that availability of marker data will assist in the authentication of Dendrobium TCM products. Microsatellites have also been reported in the literature as being used to identify: cultivars of apple [44, 45], as well as breeds of goat [46, 47], cattle[48, 49], geese [50, 51], and sheep [52, 53]. Microsatellite analysis has been shown to be highly reproducible and informative, but the throughput can be limiting compared to more modern DNA approaches, as well as sometimes being technically challenging and needing a large number of consumables in the laboratory environment.

Species-specific PCR primers

With the availability of high quality sequence information for an increasing number of plant, animal and microbial species, it is now possible to identify phylogenetically informative molecular markers for a number of important species including Atlantic sturgeons (*Acipenser sturio*, *Acipenser oxyrinchus*) [54], truffle (*Tuber magnatum* Pico) [55], and Barley (*Hordeum.vulgare* L) [56] .The availability of such comprehensive sequence information can enable the PCR assays to be designed that under suitable reaction conditions will lead to the synthesis of an amplicon only in the presence of DNA from the target species. This approach has been applied to point of origin studies for a number of plant, animal and fish species, including: wheat and barley [57], chicken, turkey, pig, cow and sheep [58], and oyster (*Crassostrea ariakensis*) [59]. The approach is rapid, robust and cost effective, and has the added advantage that it can be adapted to provide quantitative estimates of target abundance when utilising quantitative real-time PCR (qPCR). These attributes have collectively resulted in qPCR becoming the gold standard method for species determination and quantification.

Single Nucleotide Polymorphisms (SNPs)

SNPs have been reported as being the most frequently occurring form of DNA sequence polymorphism [60]. Due to their frequency of occurrence, comparatively dense and uniform distribution in genomes, SNPs provide an important source of variability for use in a number of applications, including the identification of species and cultivars [61, 62]. Although individual SNPs are less informative than many other marker types because of their biallelic nature, they have several key advantages which include: high frequency of occurrence, associated ease of automation, low-

scoring error rates and high levels of SNP assay reproducibility both within and between laboratories [63]. Initial development of SNP based assays were based on microarray hybridisation technology [64]. This is still the case where high throughput genome wide association studies are required to be performed [64], however in many instances where small subsets of SNPs are required the use of hybridisations has been replaced with amplification based assays. This format has been applied for traceability studies to: beef samples [65-67], fish [68, 69], and olive oil [70].

DNA sequencing

Technical advances in the field of molecular biology has seen some of the previously mentioned DNA based methods for species identity superseded by the introduction of more efficient DNA based sequencing approaches. The DNA sequencing of an organism's genome is theoretically the definitive means of identification. Although Next Generation Sequencing (NGS) technology has made possible the high resolution sequencing of an individual or population of organisms [71, 72], the approach is currently too expensive and time consuming for use in routine testing of food samples. However, the adoption of alternative sequencing strategies which include sequence analysis at single or multiple loci (DNA Barcoding) [73-75] or of microbial communities present on the surfaces of food samples, could prove valuable in the determination of a samples geographical point of origin.

DNA sequencing of mitochondrial targets (DNA Barcoding)

DNA barcoding has been proposed as a universal means for species determination [73] as well as for the identification of geographical origin [76]. The rational underlying the approach is that the barcode sequence should unequivocally correspond to an individual species [77]. The method is both rapid and cost effective to implement, and has been broadly accepted by the scientific community as being the definitive method for species identification as demonstrated by the existence of multiple international barcoding projects (e.g., iBOL, PolarBOL, FISHBOL and BIOTRACER).

In practice, DNA barcoding employs the sequencing of, short, standardised DNA sequences, which can then function as a molecular tag in the classification of an organism [73]. The DNA sequence of the CO1 mitochondrial gene in animals, and

rbcL or *matK* genes in plants, have been shown to exhibit sufficient sequence variation to allow accurate discrimination at the species level.

Animal DNA barcoding has recently been successfully used for the authentication of numerous types of food sample. For example, in the seafood industry, barcoding has been used to expose the fraudulent substitution of premium fish species (e.g., Red snapper, Sea bass and Alaskan halibut) with cheaper samples (e.g., Lavender jobfish, Stripped bass, Skate, and Atlantic halibut) [74, 78-80] and was sufficiently sensitive to give an indication of the likely point of geographical origin based on the natural distribution of global stocks. DNA barcoding has also been used in the meat industry where it has been used to verify the authenticity and likely point of origin of bushmeat [81], wild game [82], and domesticated pork [83].

Tillmar *et al* [72], have reported on the use of NGS for species identification of mammals from mixed samples. The sequencing of single copy nuclear genes has also been investigated as one possible approach for determining the geographical origin of plant, animal and microbial derived samples. For example, use of sequence analysis of the amylose biosynthesis gene *Waxy* (*Wx*) has been reported by Olsen and Puruggana as being used for the determination of glutinous rice samples (*Oryza sativa var. glutinosa*) [84]. Similarly, sequence variation in the *puroindoline b* gene has been reported to have been used to distinguish between European hard wheat species by Lillemo and Morris [85]. However, the technique requires extensive prior sequence knowledge of the target sequence, and selection of an appropriate target needs to be performed on a case by case basis. In the case of plant material, extensive optimisation of the primer sequence is frequently required in order to accommodate problems associated with poly and aneuploidy genomes which are likely to contain multiple orthologous copies of the target.

DNA sequencing of microbial flora (Metagenomics)

With the introduction of commercial NGS services, microbial community profiling is currently being evaluated across many fields of interest, including industry [86], clinical [87], and environmental microbiology [88]. The surfaces of most objects, including food and feedstuffs, are populated by a variety of microorganisms, or their fragments. The variety and abundance of these microbes is dependent upon a variety of environmental factors (e.g. climate, soil ecology, spoilage, insects, disease

etc.), as well as through the activities of man and animals [89], and are indicative of a sample's point of origin. Sequencing of the entire or specific regions of these microbial genomes (often referred to as metagenomics sequencing) can be used to give an indication of the likely point of origin of a sample. Microbial complement shotgun sequencing has previously been used as means of determining the geographical origin of samples for a number of studies, from determining the patterns of modern human migration [90, 91] to the determination of fruit origin [92], but also for identifying the source of Belgian sourdoughs [93] and artisanal Zlatan cheeses [94]. In contrast to shotgun sequencing approaches, a number of optional sequencing targets have been described in the literature, and have included the 26S rDNA sequence of yeast communities [92], hyper variable regions of the 18S rRNA gene [95, 96], and the 5' region of the mitochondrial cytochrome c oxidase subunit 1 [73]. However, adoption of the technique would require the establishment of a global microbial distribution database as well as extensive use of NGS technology. Both of these attributes currently preclude its use on a day to day basis.

Conclusions

From December 2014, the European Union required that all fresh and frozen meat, as well as fish produce, be clearly labelled with their geographical point of origin (Council regulation1169/2011 EU). Although the existing paper traceability and tagging systems may help towards providing consumer confidence in the geographical origin of foods on sale in the retail market, this form of traceability has be shown to be easily subject to criminally fraudulent activity. There is a growing requirement for Enforcement Agencies to have access to reliable analytical methods that can be used to verify point of origin labelling.

Currently, traceability of the geographical origin for food and food products relies on the use of a variety of chemical and isotopic measurement techniques. These have included analysis of mineral composition, analysis of elemental isotope ratio's; nuclear magnetic resonance spectroscopy (NMR); Near and Infrared Spectroscopy (NIR); and the analysis of nucleic acid sequence composition.

Molecular biology approaches that target DNA are being increasingly used for geographical traceability, since they tend to be relatively quick, precise and reliable.

DNA based approaches for country of origin labelling include Cleaved Amplified Polymorphisms (CAPS), microsatellites, species specific PCR amplification, Single Nucleotide Polymorphisms (SNPs), and DNA sequencing (e.g. targeting the nuclear genome or the metagenome). A summary of example applications in the area of country of origin labelling, along with potential advantages and disadvantages of these approaches, is presented in Table 1. However, with use of any of these methods, DNA integrity is a critical factor in predicting the probability of a successful outcome. This limitation becomes particularly prominent when working with highly degraded DNA, such as that isolated from highly processed foods. In these instances the choice of approach and likelihood of success become progressively restrictive with increased levels of sample degradation.

The major limitation of all of the methods reviewed here, with the exception of microfloral sequencing, is that they infer geographical origin from identifying those individuals, particularly with respect to elite cultivars and breeds, most likely to be genetically suited to exist at a particular geographical location (e.g. adapted to local environmental conditions, resistant to disease etc.). This does not take into account the practicing of modern day methods where domesticated animals or plants are frequently reared at a considerable geographical distance from their natural point of origin. Exceptions to this are seen with wild game [97] and various wild medicinal plant cultivars [98], but primarily with open sea or ocean fish stocks, where SNP genotyping approaches in particular [99] have been used to identify the origins of trawler catches.

Recent developments in the field of DNA sequencing, particularly with respect to the technical advances in the field of next generation sequencing (NGS), will see the development of means for determining the diversity of microbial microflora present on the surface of a sample (the metagenome). This form of approach would most likely involve amplicon sequencing in order to reduce data complexity, and is likely to become a practical technical reality in the future. As such it would provide an alternative means for identifying the geographical point of origin for a sample. As with those approaches based on chemical and isotopic composition however, these methods will also rely on the availability of databases detailing the natural distribution of micro-organisms throughout the world.

Chemical and isotopic measurement techniques are likely to remain the definitive methods of choice with respect to determining the geographical point of origin of a broad spectrum of biological and non-biological substances. However, even these approaches are limited by the availability of appropriate databases describing the global distribution of minerals and isotopic elements. Currently therefore, DNA-based methods are likely to serve as the means of a primary screen of a sample, or as an adjunct to conventional analytical methods.

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DNA based approach	References	Examples	Advantages	Potential disadvantages
Microsatellites	[44, 100, 101] [40, 44, 102] [45, 48, 49]	Medicinal herbs Apple cultivars Wild boar Cattle breeds	High specificity High reproducibility Highly informative	Large consumable requirement Moderate throughput Limited targets Technically challenging
CAPS	[18, 28, 34] [20, 30]	Plants Animals Fish	Simple technique High specificity High reproducibility	Moderate throughput Erroneous results from partial digestion
SNPs	[65] [103] [104]	European beef Chinese pork Basmati rice	Highly informative Adaptable method	Moderate throughput Relatively expensive Technically challenging
Species specific PCR	[54] [56]	Atlantic sturgeon Barley	High reproducibility Relatively simple technique	Limited target availability Technically challenging
DNA sequencing (Barcoding)	[105]	Olive oil	Highly informative Reproducible	Requires careful primer design Moderate throughput
DNA sequencing of nuclear genes	[84, 85]	Glutinous rice Bread wheat		
DNA sequencing (Metagenome)	[94, 106]	Fermented Soya bean Zlatan Cheese	Highly informative Adaptable Reproducible	Technically challenging Current high cost Resource intensive

Table 1. Summary of example DNA-based methods applicable to the determination of geographical point of origin

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