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Main Lecture

Genetic authentication and traceability of food products of animal origin: new developments and perspectives

Luca Fontanesi

DIPROVAL, Sezione di Allevamenti Zootecnici, Università di Bologna, Italy

Corresponding author: Luca Fontanesi. DIPROVAL, Sezione di Allevamenti Zootecnici, Facoltà di Agraria, Università di Bologna. Via F.lli Rosselli 107, 42100 Reggio Emilia, Italy - Tel. +39 0522 290516 - Fax: +39 0522 290523 - Email: luca.fontanesi@unibo.it

Abstract - In recent years, both the demand and the supply for food of animal origin have experienced important changes making of fundamental importance the implementation of traceability systems. DNA analysis has the potential to overcome the limits of the conventional authentication and traceability procedures. Different levels can be considered: species identification, breed traceability, individual traceability, sex determination, and identification of genetically modified animals. DNA analysis for these levels makes use of endogenous DNA, i.e. DNA of animal origin that constitutes the fingerprinting of the animal itself or of its derived products. However, another source of DNA that can be analysed for authentication or traceability purposes is exogenous DNA, i.e. DNA added to the products that is not derived from the animals from which the products are obtained. Using exogenous DNA, other levels could be considered for traceability: year of production, consortium, farm, processing industry, etc. New technologies and innovative approaches are changing the way to consider and apply genetic authentication and traceability of food of animal origin. The advantages will be for both the consumers and producers creating added values for the animal production sector.

Key words: Animal genomics, Authentication, Coat colour genetics, Traceability.

Introduction - In recent years, both the demand and the supply for food of animal origin have experienced important changes. From the consumers’ side, food products, in general, but in particular those of animal origin are facing a loss of confidence in the food supply chain derived from recent food scares. Bovine spongiform encephalopathy (BSE) crisis of the beef sector, avian influenza of the poultry sector, dioxin contamination of both chicken and pork productions, and the incidence of food borne disease of microbial origin and of other contaminants derived from the food processing steps or from the feeding or treatments of the animals have largely contributed to raise consumers’ health concerns and to ask for regulations and safety procedures. However, the decrease of consumers’ confidence cannot be attributed only to these health crises but is also caused by the “distance” between production and consumption, i.e. consumers do not feel a direct contact/link or do not understand the industrialized processes and the global market of the food productions. The supply side had to adapt the production chains to follow the consumers’ requests and the new regulations that have been adopted. New marketing strategies have been developed including product differentiation based on quality assurance, breed and geographical origin and specific labels and brands, creating added value for a large number of animal products. These superior-quality products, in turn, can risk more frequently to be targeted by fraudsters causing a detrimental economic loss for the production sector.
chain and cheat of consumers. Food frauds, in general, are big problems that the Italian agro-food industry, well known around the world for its superior brands, has to face with loss of billion of euros per year.

Therefore, together with appropriate political actions and directives, both consumers and producers can benefit from the application of precise and secure systems and methodologies having as objective the authentication and/or traceability of food of animal origin (i.e., Verbeke and Ward, 2006; van Rijswijk et al., 2008). These terms have similar general meaning but concern different routes to guarantee food quality and safety. In this context, authentication can be defined as the act of establishing or confirming something (food of animal origin) as authentic, that is, that claims made by or about the subject are true. This might involve confirming the identity of a product, its origins, or assuring that a product is a trusted one. Traceability can be defined according to the European Regulation n. 178/2002 as the ability to trace and follow a food, feed, food producing animal or ingredients, through all stages of production and distribution.

Several conventional systems, compulsory or not, are usually used to authentify and trace animal products “from the farm to consumer’s fork” and back. These systems make use of records and identification supports that follow the animals and their products from farms to slaughterhouses, processing and packaging plants, retailers and, at the end, to consumers’ tables. However, the large number of passages, the nature of the information transmitted and the supports used in these transitions increase the possibility of errors and the risk of counterfeits. The use of genetic systems based on the analysis of the DNA has the potential to overcome the limits of the conventional authentication and traceability procedures because DNA i) is present in all animal cells and tissues and, consequently, in all animal products, ii) is unique for each animal (only true twins have identical DNA), iii) is stable for long periods and to physical treatments, and iv) can be easily isolated and analysed. From these characteristics, it seems almost obvious that the DNA considered for investigation is the endogenous DNA, i.e. DNA of animal origin that constitutes the fingerprinting of the animal itself or of its derived products. However, another source of DNA that can be analysed for authentication or traceability purposes is exogenous DNA, i.e. DNA added to the products that is not derived from the animals from which the products are obtained (Fontanesi et al., 2007). The latter approach represents an innovative methodology that can overcome some limits of the endogenous DNA. In addition, other innovative genetic systems for animal product authentication could be based on the analysis of RNA and proteins but in these cases several problems, related to the nature of these molecules, should be evaluated and considered (Fontanesi et al., 2008a). Recent advances in genomic technologies and the development of high throughput platforms for DNA analysis as well as the complete sequencing of the genome of some important livestock and other species have opened new

Figure 1. Different levels of genetic authentication and traceability of animal products.
perspectives for genetic authentication and traceability of animal products, even if the cost of the analyses and of their applications at different levels still remain the limiting factor.

**Different levels of genetic authentication and traceability** - There are different levels for which genetic authentication and traceability systems can be applied, according to the needs and reasons that prompted the questions that should be answered and the information that are expected to be obtained (Figure 1). These systems are traditionally based on the analysis of endogenous DNA. New approaches are based on the use of exogenous DNA.

*Species identification* - The higher level is for species identification. This is an important issue for species conservation and protection of biodiversity, public health, economic, religious, and legal reasons and for forensic applications. Food labelling regulations require that all ingredients in foodstuffs must be declared. However, as in food preparations the species of origin cannot be directly detected by visual or sensorial evaluation, substitution of high value products with others of lower commercial value can be frequent, especially in the dairy and fish industry and for game meat. A large number of methods have been developed (reviewed in Bellis *et al.*, 2003; Mafra *et al.*, 2008). These methods rely on PCR-based analysis of DNA fragments containing sequence information that can discriminate the species of origin. Mitochondrial DNA is usually preferred to genomic DNA because it is present in several to thousands of copies in each cell, making the possibility to amplify DNA fragments easier, even from processed food and specimens of different status of preservation. In general, the proposed methods can distinguish among a restricted number of species. Therefore, several protocols should be applied when it is not clear *at priori* which could be the range of species involved in the adulteration. To overcome this problem, recently, a commercial microarray-based tool has been developed (Chisholm *et al.*, 2008). This system allows screening of samples for 33 species of fish, birds, and mammals in one test. It utilises a reverse dot hybridisation technique on a DNA-microarray that analyses the vertebrate mitochondrial cytochrome b gene. The recent international initiative devoted to developing DNA barcoding as a global standard for the identification of biological species is producing a large number of information that can also be applied for species identification of animal products of different origin (Consortium for the Barcode of Life, http://www.barcoding.si.edu/).

*Breed traceability* - Breed traceability wants to assure the quality of the products that is linked to the consumers’ identification of a particular breed-originated production, represented by mono-breed labelled lines of meat as well as dairy products (Table 1). In several cases, these products have obtained the protected denomination of origin (PDO), the protected geographical indication (PGI) or the traditional speciality guaranteed (TSG). This interest derives from the fact that a marketing link between breed and their originated products can contribute to improve breed profitability and, in turn, sustainability of such farm animal productions with important impacts on rural economy of particular geographic areas and on breed conservation and biodiversity (i.e., de Roest and Menghi, 2000; Gandini and Villa, 2003).

A classical example on this issue is the recovery of the Reggiana breed through the production and valorisation of Parmigiano Reggiano cheese obtained from this breed only. *This mono-breed cheese is sold at about the double the market prize of undifferentiated Parmigiano Reggiano cheese* (Russo *et al.*, 2007). Breed traceability is particularly challenging. The classic definition of breed is “animals that, through selection and
Table 1. Examples of cattle and pig mono-breed or restricted-breed products.

| Species of origin | Countries | Breeds                          | Products          | Brands/Labels            | DNA based authentication systems\(^1\) (Reference)\(^2\) |
|-------------------|-----------|---------------------------------|-------------------|--------------------------|----------------------------------------------------------|
| Cattle            | Italy     | Reggiana                        | Cheese            | Parmigiano Reggiano      | R (Russo et al., 2007)                                    |
|                   |           | Bruna Italiana                  | Dairy products/cheese | disolabruna              | R, P (Crepaldi et al., 2003; Russo et al., 2007)         |
|                   |           | Pezzata Rossa Italiana          | Cheese/beef       | Solo di Pezzata Rossa Italiana | P (Crepaldi et al., 2003; Russo et al., 2007)         |
|                   |           | Valdostana                      | Cheese            | Fontina Valdostana       | NA                                                       |
|                   |           | Rendena                         | Cheese            | Formaggio Ranza Rendena  | S                                                        |
|                   |           | Modenese                        | Cheese            | Parmigiano Reggiano      | S                                                        |
|                   |           | Burlina                         | Cheese            | Morlacco di Solo Burlina | S                                                        |
|                   |           | Jersey                           | Dairy products    | Private brands           | NA                                                       |
|                   |           | Piemontese                      | Beef              | Razza Piemontese         | R, P                                                     |
|                   |           | Romagnola, Marchigiana, Chianina, Maremmana, Podolica | Beef            | Vitellone Bianco         | P (Negrini et al., 2008)                                  |
|                   |           |                                  |                   | Appennino Centrale (5R)  |                                                          |
|                   | France    | Abondance, Tarentaise           | Cheese            | Beaufort                 | P (Maudet and Taberlet, 2002)                            |
|                   |           | Abondance, Montbéliarde, Tarentaise | Cheese               | Abondance                 | P (Maudet and Taberlet, 2002)                            |
|                   |           | Abondance, Montbéliarde, Tarentaise | Cheese               | Reblochon de Savoie       | P (Maudet and Taberlet, 2002)                            |
|                   |           | Charolais                        | Beef              | Charolaise Bourbonnais, Tendre Charolais Label Rouge | R (Oulmouden et al., 2005)                                |
|                   |           | Limousine                        | Beef              | Blason Prestige Bœuf Limousin, Limousin Junior | P                                                        |
|                   |           | Blonde d’Aquitaine               | Beef              | Bœuf Blonde d’Aquitaine  | NA                                                       |
|                   |           | Belgian Blue, Charolais          | Beef              | Bœuf Belle Bleue         | NA                                                       |
|                   |           | Bazadaise, Blonde d’Aquitaine, Limousine | Beef               | Bœuf de Bazas            | NA                                                       |
|                   |           | Limousine, Blonde d’Aquitaine, Bazadaise | Beef              | Bœuf de Chalosse         | P (Negrini et al., 2008)                                  |
|                   |           | Blonde d’Aquitaine               | Beef              | Bœuf Excellence, Bœuf Blonde d’Aquitaine | NA                                                       |
|                   |           | Aubrac                           | Beef              | Bœuf Fermier Aubrac      | NA                                                       |

continued >>
breeding, have come to resemble one another and pass those traits uniformly to their offspring”. Therefore, the identification of DNA markers that can discriminate different breeds could simply rely on the genomic signature that derived from these selection processes. However, the constitution of the modern breeds originated starting from complex relationships across populations and interchanging of genetic materials creating complicated patterns of variability and similarities among breeds. In addition, as breeds are not separated by biological reproductive barriers, separation of genetic pools is not absolute, further complicating the identification of breed specific DNA markers

| Table 1. | Continuation |
|----------|--------------|
|          | Gascon Beef  | Bœuf Gasconne | NA |
|          | Salers Beef  | Salers Label Rouge | NA |
|          | Parthenaise and crosses Beef | La Parthenaise | NA |
| Ireland  | Hereford Beef | Irish Hereford Prime Beef | NA |
| United Kingdom | Highland Beef | Guaranteed Pure Highland Beef | P (Negrini et al., 2008) |
| United States | Angus Beef | Premium Gold Angus Beef, Certified Angus Beef (other private brands) | R |
|          | Hereford Beef | Certified Hereford Beef | NA |
|          | Wagyu Beef | Several private brands | NA |
| Australia | Angus Beef | Certified Australian Angus Beef | NA |
| New Zealand | Hereford Beef | Hereford Prime | NA |
| Pig Italy | Cinta Senese Cured products/pork | Suino Cinto Toscano | P, S (Fontanesi et al., 2005; Fontanesi et al., manuscript in preparation) |
|          | Nero Siciliano Cured products/pork | Suino Nero dei Nebrodi | P, S (D’Alessandro et al., 2007; Fontanesi et al., manuscript in preparation) |
|          | Mora Romagnola Cured products/pork | Suino Mora Romagnola | NA |
| Spain | Iberian Cured products/pork | Cerdo Iberico | P (Fernández et al., 2004) |
| United States | Berkshire Pork | Private brands | P |

1R: Routine application; P: proposed; S: studies under way; NA: information not available.
2References describing applications or including useful data for the application/development of a DNA based test are reported (when appropriate).

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useful for breed traceability. Different approaches have been proposed for breed traceability (reviewed in Dalvit et al., 2007): i) a probabilistic approach mainly based on the use of microsatellites, AFLP, or, more recently, single nucleotide polymorphisms (SNPs) combined with different computational analyses, that assign individuals to a particular breed with a certain probability (i.e., Blott et al., 1999; Ciampolini et al., 2000; Maudet et al., 2002; Negrini et al., 2007; 2008); ii) a deterministic approach based on the use of few breed specific or exclusive markers whose informativeness is due only to their presence or absence in the analysed products (i.e., Maudet and Taberlet, 2002; Russo et al., 2007). The former needs the constitution of databases of allele or genotype frequencies for each considered breed. It was proposed for breed traceability of individual meat cuts. The allocation probability, in several cases is not high. However, the use of high throughput genotyping technologies based on the analysis of commercial chips including thousands of SNPs will increase this probability. The main drawback of this approach is that it cannot be used to assign the breed of origin for products constituted by mixtures of several/many animals, like most dairy products are. The deterministic approach, as relies on the identification of markers that are present or absent in all (or most) animals of a particular breed, can be applied to mixture of products obtained from more animals. Useful markers for this approach can be identified looking at mutations in genes affecting the main traits that differentiate the breeds, such as coat colour (Russo and Fontanesi, 2004; Russo et al., 2007).

An example of routinely applied breed traceability using a deterministic approach is the analysis of mutations in the MC1R gene for authentication of Parmigiano Reggiano cheese, produced only with milk of Reggiana dairy cattle breed (Russo et al., 2007). This breed is characterized by solid red coat colour and is fixed for a mutation causing the recessive e allele at the Extension/MC1R locus. Therefore, from the analysis of this gene it is possible to exclude frauds derived by mixture of milk obtained from other breeds in which the e allele is not fixed, such as Holstein-Friesian (with high frequency of the dominant black determining allele, E^D) or Brown-Swiss (almost fixed for the E^D allele at the Extension/MC1R locus). Cheese obtained with milk of Simmental cows cannot be distinguished from that obtained from Reggiana milk because the high frequency of the e allele in the Red Pied breed. However, the identification of markers causing the spotted patterns of the Simmental breed could make the detection of its milk possible. Other studies have identified mutations in a candidate gene for the Spotted locus in cattle (Fontanesi et al., manuscript in preparation), with potential applications for this aim. Coat colour genetics can be also applied for breed traceability of products obtained from several other livestock species including, for example, pigs (Fontanesi et al., manuscript in preparation), goats (Fontanesi et al., 2009), and rabbit (Fontanesi et al., 2006). However, in most cases, discrimination of different breeds based on markers in genes affecting coat colours is not absolute. Similar coat colours might be derived from different mutations in several genes or caused by epistatic effects, complicating the identification of useful DNA markers for this approach.

The higher value of mono-breed products and the need to defend these productions with DNA based methods could drive breeding schemes towards the fixation of DNA markers in the breeds in which one potentially discriminatory allele is close to 100%.
**Individual traceability** - Genetic individual traceability of animals through the whole food chain is related to safety and health aspects. As each animal (excluding true twins) has its own DNA that is different from that of all other animals, in theory, individual traceability would provide all information for a complete traceability of the animals and of their products, overcoming the limits of the traditional systems based on accompanying documents and records. Indirectly, it would provide information about the breed and sex. However, implementation of full individual traceability systems based on DNA analysis in the different steps of a production chain would be prohibitive for the high costs (caused by the genotyping of all animals in all passages, creation of specific database and constitution of biorepositories) and complicated logistic organization. To capture DNA differences among animals, i) panels of microsatellite markers and, more recently, ii) panels of SNPs have been proposed (reviewed in Dalvit et al., 2008). These panels have been developed mainly for parentage testing, and used to implement individual traceability, intended as a control of the conventional procedures of animals and products traceability. However, SNPs are replacing microsatellites because the easy to automate genotyping and reading of the outputs of panels that include at least 30-50 of these markers (i.e., Heaton et al., 2002; Rohrer et al., 2007). An approximation of the individual traceability, developed to reduce the genotyping and costs, is the inference of the sire or dam or both using genotyping data on the offspring. This could be considered as a dynamic batch traceability in which sires and dams of a farm are previously genotyped making it possible to attribute parents to the meat derived from their offspring if genotyped for control purposes (Hill et al., 2008). As new technologies are reducing costs of genotyping and increasing sample processivity, individual traceability could be applied in much more cases than is currently used.

**Sex identification** - To strengthen the market position of meat producers, measures such as intervention buying and export refunds can be introduced. These schemes foresee a considerably higher subsidy for male beef meat, which is regarded to be of higher quality (Zeleny et al., 2002). For some pork productions, females are preferred for their higher quality and for the absence of boar taint. In these cases, sex identification methods are needed to avoid frauds. Another application of sexing tests can be for quick control and sample identification and matching in a production chain in which both females and males are processed. Sex determination methods have been developed by PCR amplification of Y-chromosome specific sequences in male genomic DNA or amplifying homologous fragments from both sex chromosomes. Several tests have been published in cattle and pig but the most convenient ones seem those that analyse fragment length differences between genes located on both sex chromosomes, such as AMELX and AMELY (Ennis and Gallagher, 1994; Fontanesi et al., 2008b). Sex determination tests can be implemented in parentage testing panels including sex specific markers.

**Genetic modifications** - A few genetically modified animals have been produced for food consumption even if their formal approval is still pending (Dove, 2005). The most famous example is the AquAdvantage salmon engineered with a growth hormone gene constitutive promoter that significantly booster growth rate. The EnviroPig, developed at the University of Guelph, is a transgenic pig that carries a gene for phytase driven
by a salivary gland-specific promoter that is able to cut phosphorous excretion in the animals' waste. Despite their potential controversies and the current debate about their use for human consumption, there could be the need to detect the presence of transgenic animals in the market chain. Specific tests can be easily set up using as target the inserted constructs even if some problems in detecting transgenes could be considered in chimeric animals.

Application of exogenous DNA - This approach makes use of the DNA contained in an added material to the animal originated products, establishing a link between alien DNA and the products (Fontanesi et al., 2007). Therefore, there is no need to know or sample the animals that originated the products. In this way, it is possible to operate at different levels of the supply chain. The added material is of natural origin and can be or cannot be normally part of the final transformed animal products, but in all cases it does not alter the products themselves. This system was developed and patented at the University of Bologna in collaboration with a private company. It was tested in the dry-cured ham production chain using plant originated products (wheat flour) to mark/label the legs. The wheat flours, derived from unique lines, that were previously chosen and genetically characterized with a set of specific DNA markers, were mixed to the ink commonly used to mark the green legs or to the fat preparation (whose recipe can include wheat or rice flour) used to grease the hams. The corresponding wheat DNA was identified after several months of seasoning on the hams by means of microsatellite analysis and the observed genotypes were always as expected from the wheat lines that were used to produce the tracers. This new system for product traceability and authentication revealed to be affordable and easy to apply to animal products and can be used to “label” the productions at different levels (consortium, farm of origin of the animals, year/month of production, etc.). Modifications of this system can be applied to several other food products using knowledge on microbial and plant genomes.

Conclusions - New technologies and innovative approaches are changing the way to consider and apply genetic authentication and traceability in livestock. The advantages will be for both the consumers and producers creating added values for the animal production sector. Complementing different approaches and combining genomic technologies with traditional methods it is possible to monitor, secure, and trace production chains at different levels. However, a few challenges still remain but solutions could be obtained if research investments will continue.

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