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CICLO XX

Genetic traceability: a tool for the authentication and valorisation of animal products

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Riassunto

La tracciabilità, ottenuta con l'impiego di analisi molecolari, si propone come un valido strumento per l'autenticazione e la valorizzazione dei prodotti di origine animale.

Lo scopo di questa tesi è stato quello di verificare la possibilità di utilizzare metodologie molecolari per un sistema di tracciabilità genetica individuale e di razza in prodotti animali di origine bovina. Tra i marcatori molecolari oggi esistenti sono stati scelti i microsatelliti; essi sono probabilmente i più impiegati grazie alla loro facilità d'impiego e all'elevato polimorfismo che li rende altamente informativi.

Il primo contributo rappresenta una recensione degli studi finora svolti nell'ambito della tracciabilità genetica individuale, di razza e di specie paragonando l'utilizzo di diversi marcatori molecolari e approcci statistici.

Il secondo contributo sperimentale riguarda la possibilità di utilizzare un set di dodici microsatelliti per tracciare individualmente gli animali appartenenti a sei razze bovine tra le più largamente diffuse in Italia: Frisona, Bruna, Chianina, Marchigiana, Romagnola e Piemontese. E' stata dunque calcolata la probabilità di trovare, per caso, due individui che mostrino lo stesso profilo genetico ai loci studiati, considerando un numero decrescente di microsatelliti. Lo studio ha messo in luce come, usando solamente i cinque marcatori più polimorfici in ciascuna razza, la possibilità di trovare due animali identici sia di cinque su un milione. Tuttavia, per poter snellire le analisi in laboratorio e per ridurne i costi, è stato individuato un set composto da otto microsatelliti in grado di dare risultati soddisfacenti in tutte le razze studiate. L'impiego di questa metodologia potrebbe rivelarsi utile come strumento di verifica e garanzia delle informazioni già presenti obbligatoriamente in etichetta per tutelare la filiera e scoprire eventuali frodi. Inoltre questa tecnica potrebbe risultare utile quando si renda necessario il ritiro di tutti i tagli provenienti dallo stesso animale per identificarli con certezza.

Il terzo contributo sperimentale è invece improntato sull'utilizzo di ventuno microsatelliti per la definizione di un sistema di tracciabilità di razza in quattro razze bovine italiane da carne: Chianina, Marchigiana, Romagnola e Piemontese. Infatti queste razze, grazie alle loro spiccate caratteristiche qualitative, riescono a spuntare un prezzo maggiore sul mercato. Inoltre, le tre razze dell'Italia centrale, essendo autoctone e allevate principalmente in aree specifiche, sono protette dal marchio europeo IGP (Indicazione Geografica Protetta). Dunque, un sistema di tracciabilità genetica potrebbe essere uno

strumento di verifica e di valorizzazione di queste produzioni. Dei due approcci statistici testati, quello basato su un algoritmo di tipo Bayesiano è risultato essere il migliore riuscendo ad attribuire correttamente la razza al 90% circa degli individui con l'analisi di sei microsatelliti. Tuttavia, considerando una soglia di assegnazione del 90%, e utilizzando l'informazione relativa a ventuno marcatori, il 52% dei genotipi è stato attribuito correttamente. Tale risultato sembra dipendere dalla bassa differenziazione genetica stimata tra le razze studiate (FST = 0,049). I risultati ottenuti suggeriscono la ricerca di microsatelliti che presentino alleli razza-specifici per poter migliorare il potere discriminante.

L'ultimo contributo si prefigge lo scopo di caratterizzare geneticamente la razza Burlina, una razza bovina autoctona italiana allevata in Veneto che attualmente conta circa 350 animali. La Burlina è un animale di piccola taglia che veniva allevato nelle zone montane delle province di Treviso e Vicenza dove era apprezzata per le sue doti di pascolatrice. E' stata gradualmente sostituita da razze più produttive quali la Bruna e la Frisona e ha rischiato di scomparire prima di essere sottoposta a un piano di conservazione. In questo studio, la Burlina è stata caratterizzata geneticamente e confrontata, tramite l'uso di dodici microsatelliti, con le razze Bruna e Frisona. Le analisi condotte hanno dimostrato la diversità genetica della Burlina rispetto alle altre due razze studiate anche se è risultata essere geneticamente molto vicina alla Frisona. Inoltre, l'analisi per l'assegnazione di razza ha attribuito una considerevole percentuale di individui di razza Burlina alla Frisona, a causa degli incroci avvenuti in passato. Rispetto alle altre due razze la Burlina ha evidenziato una maggiore variabilità genetica e un basso livello di inbreeding. Tali risultati contribuiscono alla pianificazione di un piano di conservazione che avvii alla riproduzione solamente gli animali "più Burlini". La possibilità di mettere a punto un sistema di tracciabilità genetica per i prodotti ottenuti dalla razza Burlina, (come il formaggio tipico Morlacco), è di sicuro interesse anche se i risultati ottenuti dalla genotipizzazione dei dodici microsatelliti non sono al momento sufficienti.

Summary

Traceability, obtained by molecular analysis, is a reliable proposal for the authentication and valorisation of animal products.

Aim of this thesis was to verify the possibility to use molecular methodologies for the assessment of a genetic traceability system able to discriminate among individuals and breeds in bovine animal products. Among the molecular markers available today, microsatellites were considered the best suiting this scope. They are widely employed due to their easy use and to their elevated polymorphism that provides a high degree of information.

The first contribution, proposes a synthesis of the major advances in individual, breed, and species genetic identification in the recent years, comparing different molecular markers and statistical approaches.

The second experimental contribution, concerns the possibility to use a set of twelve microsatellites for the individual identification of animals belonging to six bovine breeds widely reared in Italy: Holstein Friesian, Brown Swiss, Chianina, Marchigiana, Romagnola and Piemontese. The probability to find, by chance, two animals sharing the same genetic profile at the analyzed loci, was estimated considering a decreasing number of markers. The study evidenced that, investigating only the five most polymorphic markers per breed, the probability to find two identical individuals was approximately five in one million. Moreover, to reduce the laboratory work and costs, a set composed by eight markers, giving satisfactory results in all considered breeds, was assessed. Use of this technology could be useful as verification and guarantee of the information compulsorily present on the product label, to safeguard the production chain and detect possible frauds; moreover, when a more powerful and secure identification is required, for example, in the recall of all animal cuts in case of health's risks.

The third experimental contribution, deals with the investigation of twenty-one microsatellites to define a breed traceability system in four Italian beef cattle breeds: Chianina, Marchigiana, Romagnola and Piemontese. Such discrimination technique could be an important tool for product verification and valorisation. In fact, due to the high quality of their beef, these animals show a higher market price. Moreover, beef from Chianina, Marchigiana and Romagnola, is protected by the European label PGI (Protected Geographic Indication) because of its peculiarities and the limited area in

which the animals are reared. Two statistical approaches were tested, the one based on a Bayesian algorithm gave best results. In fact, it achieved a correct assignment rate of 90% of tested individuals using six microsatellites. However, considering a threshold probability of 90% and using the information derived from twenty-one microsatellites, only 52 % of the genotypes were correctly allocated. Such results were mainly due to the low genetic differentiation estimated among breeds (F_{ST} = 0.049). Results suggest that, to improve the discrimination power of markers, microsatellites showing private alleles should be sought.

The last experimental contribution, aims to characterize genetically the Burlina cattle breed. Burlina is an indigenous Italian cattle breed from the Veneto region (North-East Italy); it is a small sized animal mainly reared in the mountain area of Treviso and Vicenza provinces where it was appreciated for its good grazing characteristics. Burlina has been gradually substituted by more productive breeds such as Holstein Friesian and Brown Swiss and it was seriously endangered before the beginning of a conservation plan. Nowadays about 350 animals are registered in the Italian Herd Book . In this study Burlina was genetically characterize investigating twelve microsatellites and it was compared with Holstein Friesian and Brown Swiss. The obtained results evidenced the genetic diversity and distinctiveness of Burlina population, even if the estimation of genetic distances showed a moderate/high similarity with Holstein. Moreover, the assignment of a moderate percentage of Burlina animals to Holstein Friesian, witnessed that crosses between them took place in the past. Compared to the other breeds, Burlina showed a higher genetic variability and a lower inbreeding. These results contribute to the assessment of a conservation plan aiming to conserve animals where the contribution from foreign breeds is as small as possible. The implementation of a genetic traceability system for products derived from Burlina breed (such as the typical Morlacco cheese) is of certain interest; anyway results obtained from the genotyping of the twelve microsatellites are not sufficient to achieve this goal.

General introduction

The best explanation of the word "traceability" is found in the European Regulation 178/2002 which defines it as "the ability to trace and follow a food, feed, food producing animal or ingredients, through all stages of production and distribution". The mentioned regulation is the fundamental law on food safety in Europe and, since its application (1st January 2005), the definition of a traceability system for the whole food sector, has become mandatory in all member countries. Reasons for the implementation of this system are many, they can be resumed in two main points: protect public health and answer to consumers' demand of transparency, quality and safety.

Genetic traceability is based on product identification through DNA analysis by use of molecular markers. Use of molecular methods, allows for individual, breed and species identification and is a secure tool as DNA is inalterable and present in every tissue.

Aim of the present thesis was to investigate the use of microsatellite markers for individual and breed genetic traceability in some Italian cattle breeds, to verify if this methodology could actually be applied with satisfactory results to the Italian livestock situation. The thesis is composed of four contributions dealing with different aspects of genetic traceability as is briefly described in the following paragraphs.

The first contribution focuses on the state of the art of genetic traceability, proposing a synthesis of the major advances in individual, breed and species identification during the last twenty years. Indeed, since the introduction of polymerase chain reaction (PCR), in 1989, several molecular markers have been discovered and used for these goals. The knowledge acquired from previous studies performed on this topic, was the starting point for the development of the other experimental contributions included in this thesis. In fact, on the basis of earlier research, it was decided to concentrate on microsatellite markers as they have already proved to be particularly suitable for studies on genetic characterization in many different animal species. In addition they are easy to use and provide a high level of information.

The second contribution, deals with the possibility to use a set of twelve STR markers for individual identification in six cattle breeds. The analysed samples were collected from both beef and dairy breeds widely reared in Italy. The main objective of this study was to asses the most adequate number of STR markers able to well discriminate among animals without showing exaggerate costs.

In the third contribution, authors investigated the opportunity to utilize twenty-one STR markers for breed genetic traceability in the four most reared Italian beef cattle breeds: Chianina, Marchigiana, Romagnola and Piemontese. In this case, traceability is proposed as a tool for product certification and valorisation. Indeed, beef obtained from these white Italian breeds can be sold at higher prices due to its quality. For this reason fraud and falsification of the label information is a real threat and could be profitable. Genetic traceability could become a tool for the verification of product origin.

Finally, the last contribution focused on the genetic characterization of a local Italian dairy cattle breed called Burlina, nowadays endangered, counting only about 350 animals. In this case, twelve STR were used to compare Burlina with Holstein Friesian and Brown Swiss, the breeds that actually substituted Burlina in its natural mountain environment. Aim of this study was, first of all, the genetic characterization of Burlina that has never been investigated before, to verify its genetic uniqueness. Secondarily, the possibility to assess a conservation program was evaluated on the basis of the collected molecular data. Once the uniqueness and the possibility to safeguard the Burlina population is ascertained, also the setting up of a traceability system for its products could be studied. In fact, from Burlina milk, a typical cheese called Morlacco has always been prepared which represents an interesting niche product for the Veneto region where Burlina is mainly reared.

First contribution:

Genetic traceability of livestock products. A review

Genetic traceability of livestock products. A review

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Abstract

Traceability is the ability to maintain the identification of animal or animal products all along the production chain. It represents an essential tool to safeguard public and animal health and to valorize typical production systems. European food legislation is particularly strict and traceability systems, based on product labeling, have become mandatory in all European countries. However, the implementation of this system does not ensure consumers against fraud. Paper documents can be counterfeit so researchers have focused on the study of genetic traceability systems based on products identification through DNA analysis. In fact DNA is inalterable, detectable in every cell, resistant to heat treatments and allows for individual, breed or species identification. Even if results are promising, these techniques are too expensive to be converted in routine tests but they could be a trusted tool for verification of suspected fraud. The present review proposes a synthesis of the major advances made in individual, breed and species genetic identification in the last years focusing on advantages and disadvantages and on their real future applications for animal productions.

Keywords: Traceability; Molecular markers; DNA; Livestock products; Meat

Traceability: What and Why

Traceability is defined as a system able to maintain a credible custody of identification for animals or animal products through various steps within the food chain, from the farm to the retailer (McKean, 2001). In particular, this term was defined by the European Regulation (ER) 178/2002 as "the ability to trace and follow a food, feed, food producing animal or ingredients, through all stages of production and distribution". While, following the ISO 8402 standard norms, traceability is defined as "the capacity of establishing a product's origin process history, use and provenance by reference to written records" (ISO, 1994). However, like other traceability definitions, ISO 8402 does not define which parameters have to be measured or how history or origin should be determined. As proposed by Golan, Krissof, Kuchler, Nelson and Price (2004) a traceability system might be characterized by: its breadth, depth and precision. The breadth depends on the amount of information recorded (e.g. feed regime, pedigree

information or details of animal's veterinary care), the depth consists on how far, back or forward, the system tracks (to a grain elevator, farm or field), in many cases, the depth is determined by the breadth or attributes of interest. Finally, the precision is the degree of assurance with which the system can pinpoint the movement of a particular product, and is described with reference to an acceptable error rate.

In the last few years traceability issues have grown in importance due to the consumers' increasing attention to food quality matters. The consumers' lack of confidence, in particular towards food of animal origin, is due to several reasons including both food safety and socio-economic changes. Bovine Spongiform Encephalopathy (BSE) has certainly been the most serious food safety problem of the last years causing a drastic reduction of beef consumption in all Europe. It was then followed by the dioxin crisis and the avian influenza in the poultry sector (Ciampolini, Leveziel, Mozzanti, Grohs, & Cianci, 2000; Goffaux, China, Dams, Clinquart, & Daube, 2005). Furthermore, the incidence of food borne diseases due to microbial contamination of processed food, has increased in the last decade leading to additional food scares in the buyers (Opara & Mazaud, 2001).

Besides these "food scandals" socio-economical reasons have also contributed to increase people's interest in what they eat and in how and where it is produced. For example, it is worth mentioning that the main reasons for the negative trend on meat consumption are not due only to the negative impact of the food scandals involving meat products, but also to the new food habits of the younger generation and the progressive decline of the organoleptic meat properties (Cozzi & Ragno, 2003). In fact, for read meat, a loss of taste and flavor has been observed, probably related to the reduced marbling (Kerry & Ledward, 2002). In the same way, the reduction of intramuscular fat depositionseems to have a negative effect on meat tenderness (Seideman, Koohmaraie, & Crouse, 1987). At present, consumers are more aware than some years ago of ecological and environmental matters and the demand for organic food and for products obtained in an eco-sustainable systems has increased (Opara & Mazaud, 2001), nevertheless the industrialization processes, as well as the market globalization, have made difficult for people to keep a check on food processing methods (Ajmone-Marsan, Milanesi, & Negrini, 2004).

All these reasons have contributed to the need of finding a system to trace food products. Traceability is the answer to the consumers' demand of transparency and it is becoming synonymous with safe and high quality food. Authorities and scientists are still debating on how the perfect trace back system should work and several authors have compared, in their publications, the efficacy of different traceability methods (Stanford, Stitt, Kellar, & McAllister, 2001; Barcos, 2001; Marchant, 2002; Meuwissen, Velthuis, Hogeveen, & Huirne, 2003). Barcos (2001), focusing on animal identification. They stated that a good system should be convenient, easy to use and read, durable, respecting animal and public health and able to avoid fraud. Several identification methods have been studied including different kind of tags, ruminal bolus or retinal analysis. At present policymakers have implemented mandatory methods based on tags or labels as will be described in the next chapter, such methodologies are easy to use but often cannot prevent from fraud (Barcos, 2001; Stanford et al., 2001). Debates on food safety issues and on traceability matters involves not only policymakers and scientists but also economists as implementation of traceability systems, is strongly related to cost. Realization of any kind of system results in costs and benefits for both industries and consumers; in particular for food companies it is a tool to counterattack liability claims and to improve recall efficacy but, on the other one hand, consumers willingness to pay for this service must be studied (Meuwissen et al., 2003).

Though, traceability issues concerns many different aspects tied not only to food safety and policymakers decisions but involving economical aspects and consumers' decision making behavior, implementation of such systems must necessary cover all these aspects.

The European Legislation on Traceability

The European Union (EU) has always paid great attention to food safety, first of all because the agro-alimentary sector on its whole is very important for European economy. The EU is the biggest producer of food products and beverages in the world (European Commission, 2000) with a food and beverages industries production of 15% of the total EU manufacturing output, corresponding to 600 billions Euro. The second reason can be found in the Treaty of Rome (1957) instituting the EU, and stating that one of its aims is the "achievement of a high level of health protection" and "the

strengthen of consumers' protection". So, food safety measures have always been present in the EU legislation but, in the last years, in particular after the first BSE outspread in 1997, the legislation had been implemented in order to be faithful to its aims regarding health protection and to gain consumers' trust.

The three most important EU documents regarding food safety are the Green Paper on the general principles of food law in the European Union (1997), the White Paper on Food Safety (2000) and the ER 178/2002 (applied from 1st January 2005); in particular with the latter a traceability system has been introduced in the food sector, even if for the beef industry such system already existed thanks to the ER 1760/2000 and 1825/2000 issued soon after the BSE crisis.

The Green Paper on the principles of EU legislation about food products (1997), is made up of six parts regarding different aspects of food safety such as: the actual legislation of member countries, the need to simplify EU legislation and, above all, the need to implement it for better protecting consumers' health, a must after the BSE outbreaks.

The White Paper on Food Safety (2000) followed few years later containing strategies for updating the actual legislation; among the given suggestions there were: the institution of an independent European Alimentary Authority, the risk analysis as main instrument for food safety, the application of the precaution principle, the need of controls on food products and the consumers' information. In addition, for the first time, it introduced the concept of traceability for feed and animal products "from farm to fork" and transparency was the *leit motiv* of the entire document.

The White Paper was the base for ER 178/2002 applied from the 1st January 2005. This regulation has stressed the importance of a traceability system declaring that "the experience demonstrated how the impossibility to reconstruct the trail of a food could be a danger for the market of such product" while, a traceability system able to keep all the information regarding food production can help to proceed to its recall in case of danger without damaging the entire sector. So, since 2005, the regulation has become mandatory for all member countries which must define a traceability system for the whole food sector; in addition it permits to achieve an agreement among the different member countries legislations in which several differences were present leading to problems regarding the free exchange of food among them. Though ER 178/2002 is the

fundamental law regarding food safety, it has been followed by several other regulations; for animal products the most important are 852/2004, 853/2004, 854/2004 and 882/2004, all of them corroborate the importance of a traceability system and the need to control them by authorities.

Not only EU has such a strict legislation on food products traceability; in fact traceability systems based on animal identification have been implemented in several countries. In Canada, Australia and New Zealand a trace back system based on tagging was established in 2001, in Japan as well strict rules have been established in the same year, and in Brazil and Argentina traceability systems are in use even if with different depth. In the United States (USA) a trace back system was proposed even if it would not be mandatory or providing comprehensive information but still on voluntary basis (Marchant, 2002; Smith, Tatum, Belk, Scanga, Grandin, & Sofos, 2005). Moreover, in the last few years the discussion on the identification and registration of genetically modified organisms (GMOs), between the EU and the USA, contributed to increase the traceability requirements and transparency in food chains. Labeling of GMOs is obligatory in the USA only if the product differs essentially from the "original", e.g. if the nutritional value differs, or if the product contains an allergen that it is not present in the original. The EU demands that all GMO products, with a GMO contamination of > 0.9%, must be labeled as such.

Conventional and Geographical Traceability

Traceability systems are mandatory in all EU member countries and, as described before, they are particularly important for livestock and animal products. Anyway there are several types of traceability depending on how it is obtained and on which information it furnishes.

The so-called conventional traceability consists on the labeling system such as in the beef sector and on the management of processed food by batches. It is extremely useful for keeping individual information of each animal and it is less expensive and easier to achieve than other methods. For example in the beef industry, legislation requires the identification of each animal through ear tags with a specific code given from the Veterinary Services, a passport accompanying the animal in all its movements, and a central database collecting all the information; the identification code must be

maintained also after slaughtering in the carcass and in every meat cut. As mentioned this method present several advantages, ear tags are quite durable, easy to apply and to read allowing for fast data transmission, anyway they are also easy to remove even if they cannot be easily used again (Barcos, 2001), furthermore being based on papery documents, it could be counterfeit (Cunningham & Meghen, 2001). Anyway the General Food Law refers not only to meat but to every food and feed products, this has several implications for producers; in fact the source of all ingredients must be traced and processors must be able to prove that their suppliers can provide food traceability as well. Conventional traceability applies to everything contributing to food safety, including packaging, closures, seals, jars, etc. and covers everything that happens to the products before, during and after the manufacturing, packaging, and distribution; all these information must be stored resulting in an enormous collection of data that must be accurate, easy accessible and maintained for extended period of time (Schwägele, 2005).

Geographic traceability instead does not aim to identify an individual or a batch but the geographic origin of a product through the study of "track elements" such as volatile compounds, microbial flora, stable isotopes and infrared spectroscopy (Mauriello, Moio, Genovese, & Ercolini, 2003; Pillonel et al., 2003; Franke, Gremaud, Hadorn, & Kreuzer, 2005; Schwägele, 2005). It is particularly useful for typical foods labeled with the Protected Designation of Origin (PDO) or Protected Geographical Indication (PGI) European labels which are used for foodstuffs produced, processed and prepared in a given geographical area using recognized methodology the previous and for products whose geographical link must cover only one production stage the latter. In Europe and in the world Italy is leader in these productions with 145 labels between PDO and PGI, it is followed by France, Portugal, Spain and Greece. Considering their economical value, which represent an interesting way for the development of livestock systems located in less competitive area, and embody typical added values like tradition and high quality, it is easy to understand why researchers are focusing on geographic traceability topics.

Genetic Traceability

Genetic traceability, according to its name, is based on the identification of both animals and their products through the study of DNA. It is based on some DNA characteristic the first one is being enormously variable among individuals (expect for monozygotic twins and clones) (Mackie et al., 1999, Cunningham & Meghen, 2001): a-DNA is inalterable during the all animal life; b-DNA is stable to the different treatments of processed food; c-DNA is present in every cell of the organism.

Once the DNA is extracted from the chosen matrix (it can either be animal tissue, blood, muscle, hair, sperm, faeces or even a processed food such as cheese or canned meat) it is analyzed by molecular markers to obtain a fingerprinting (Figure 1) or specific allelic frequencies allowing for individual, breed or species identification. Since the introduction of Polymerase Chain Reaction (PCR) in 1989, many different markers have been discovered and studied, at present the most widely used are microsatellites also known as Short Tandem Repeats (STR) and Single Nucleotide Polymorphism (SNP) (Mariani et al., 2005). As already mentioned DNA analysis furnishes different level of identification: the individual one is of great interest for the verification of meat cut and it is strictly linked to food safety, while breed and species discrimination are interesting to detect frauds and to protect and valorize typical productions. The use of these technologies in animals and their productions is just an extension of techniques already in use for human testing and routinely applied for forensic caseworks (Cunningham & Meghen, 2001).

Individual Genetic Traceability

Animal individual identification is useful for safeguarding public and animal health providing safe products for both domestic and export consumption. In addition also national diseases monitoring and eradication programs depend heavily on correct animal identification (Cunningham & Meghen, 2001); though, after the BSE outbreaks in the EU and the foot and mouth disease in the United Kingdom, trace back systems have become an issue of international concern (Barcos, 2001; Stanford et al., 2001). As already mentioned, the beef sector suffered a serious crisis after BSE outbreaks, and, since then, consumers are worried about meat quality, its origin and integrity all through food chain until consumption; as consequence the EU has regulated the beef

labeling system with ER 1825/2000 (Arana, Soret, Lasa, & Alfonso, 2002) that is substantially based on papery documents and tags. Typing of DNA has been proposed as a future implementation of individual identification method due to its precision, durability and possibility to overcome limits of conventional traceability systems. Several studies have been conducted on many different cattle breeds, their aim was to assess a panel of molecular markers able to discriminate one individual from another. To test the panel efficacy the so called match probability (MP) is calculated, it is defined as the probability to find, by chance, two individuals sharing the same genotypic profile at the studied *loci* (Weir, 1996). For example, if the frequency of all alleles detected at all analyzed *loci* is the same and equal to 0,25 the cumulative probability (%) of a chance match is 0.125^n x100, where *n* is the number of *loci*.

The most widely used markers are microsatellites (Peelman et al., 1998, Sancristobal-Gaudy, Renand, Amigues, Boscher, Leveziel, & Bibé, 2000; Arana et al., 2002, Vazquez, Pérez, Ureña, Gudín, Albornoz, & Domínguez, 2004, Herraeza, Schafer, Mosner, Fries, & Wink, 2005; Dalvit, Targhetta, Gervaso, De Marchi, Mantovani, & Cassandro, 2006; Orrù, Napolitano, Catillo, & Moioli, 2006) and the most recent SNP (Heaton et al., 2002, Heaton et al., 2005, Herraeza et al., 2005). In Table 1 are shown the results of these researches, the type of utilized markers and the studied breeds. The presented studies revealed the efficacy of both markers for individual traceability with different results depending on the type, number and level of polymorphism of chosen markers. No author obtained MP values higher than one over one million evidencing a good power of discrimination of the method, anyway to choose which is the best MP discrimination threshold the population size has to be considered; for a population of four thousands of animal MP values in the order of 10^{-6} are adequate but if several millions of animals are breed such value does not ensure a good level of discrimination.

It is worth mentioning an important aspect when choosing the markers and the breeds to analyze; Orrù et al. (2006) in their study on four cattle breeds put in evidence that the informative content of each microsatellite varied from one breed to another depending on the typical breed allelic frequencies and on the presence of private alleles; though when implementing a genetic trace back system it would be interesting to choose different panels for each breed or, to contain costs, to choose a panel

permitting to achieve good efficacy in all breeds; in both cases preliminary analyses on all breeds are needed to determine the genetic structure of each population.

The mentioned studies concern the identification of a single meat cut but also tracing of individual animals in mixtures should be ensured considering that ground meat, sausages and potted meat present a greater health risk than carcasses and meat cuts (Barcos, 2001). For this purpose Shackell, Mathias, Cave, and Dodds (2005) recently published an article on the possibility to use microsatellites for tracing ground beef mixtures. In this case microsatellites markers were used to analyze samples containing a mixture of individuals making impossible, looking at the corresponding electropherogram, the differentiation among "stutter", true alleles and their interaction; though instead of assigning alleles, a DNA "signal" profile was created for each marker and sample including the area of every observed peak. They achieved a good success rate distinguishing individuals among mixtures containing meat from up to five different animals, while when considering more individuals results were not satisfactory. Anyway such technique could be the appropriate tool to verify for example that the correct batch has been recalled, as suggested by the authors.

Even if beef sector is obviously the most involved Goffaux et al. (2005) highlighted that such system could be applied in Belgium to porcine channel where traceability stops at the slaughter-houses making impossible to link a piece of meat to an animal. They proposed the use of twenty-one SNP markers giving a MP of 7×10^{-9}, such test was considered sufficiently significant as the total Belgian pig population is of about 7×10^{-6}. Concluding, effective genetic meat traceability could be possible but it must face two problems: the high costs of analyses and the management of the collected individual samples. The second one is particularly tricky, in fact if research could define the most appropriate markers reducing the number and consequently the costs, a new organization of the beef chain is required; the national herd has to be sampled, possibly by the Veterinary Services when applying ear tags, and samples must be conserved to be analyzed in case of need; this will necessary lead to the creation of "banks" in which samples, like hairs, could be easily stored. As suggested by Cunningham and Meghen (2001) DNA analyses should be required only in same cases, for particular investigations and on a random basis, in this way the integrity of the ear tag could be guaranteed; such system has already been implemented by an Irish supermarket chain.

In addition as suggested by Barcos (2001) a harmonization and standardization of individual trace back systems in all countries should be advisable as world trade of animal and animal products has grown and public health as to be ensure, it is worth mentioning that some European governments are actually considering this possibility (Cunningham and Meghen, 2001).

Breed Genetic Traceability

Breed genetic traceability permits to assign or exclude the breed of origin to a product; such ability has become more and more important as today many typical products, some protected by the European labels PDO or PGI, are prepared by one breed only or cannot be made with some breeds. Some examples are the Italian PDO cheese Parmigiano Reggiano "Vacche Rosse" produced only with milk obtained by the Reggiana dairy cows (Gandini & Oldenbroek, 1999) while for the meat industry both Italy and Spain obtained the PGI label for beef from some native breeds: Chianina, Marchigiana, Romagnola, Podolica and Maremmana for Italy and Pirenaica for Spain (Arana et al. 2002). Not only cattle breeds are involved in such productions, the Spanish PDO Jamon Iberico made with Iberian pig breeds only (Garcìa et al., 2006) is a good example. The list could be long and it is essentially made up of products typical of the Mediterranean countries such as France, Italy and Spain (Pancaldi et al., 2005) and most of the studies are performed in such nations. It is important to underline that these products are usually very ancient and their preservation consist also in the protection of old traditions and cultures, the utilized breeds are often small sized and endangered, which only chance to survive is their use for the production of typical and high quality products. Though researches regarding breed genetic traceability are often linked with studies on breed characterization (Óvilo, Cervera, Castellanos, & Martínez-Zapater, 2000; Ciampolini et al., 2000; Maudet, Luikart, & Taberlet, 2002; Carriòn et al., 2003; De Marchi, Targhetta, Contiero, & Cassandro, 2003) and, sometimes, also conservation through the use of molecular markers methods (Alderson & Plastow, 2004; De Marchi, Dalvit, Targhetta, & Cassandro, 2006). If individual traceability is an instrument to ensure food safety, breed traceability is a mean to defend and valorize particular food products.

To assign an individual or a product to a breed two approaches are possible, as reported by Ajmone-Marsan et al. (2004): a-deterministic: consisting on finding molecular markers with different allelic variants fixed in different breeds, though it will be possible to develop simple analyses protocols without the need of statistical inference; b-probabilistic: consisting on utilizing a set of markers with typical allelic frequencies in different breeds. Breed assignment is obtained by statistical methods based on maximum likelihood functions (Paetkau, Clivert, Stirling, & Strobeck, 1995), Bayesian methods (Rannala & Mountain, 1997) and genetic distances methods (Cornuet, Piry, Luikart, Estoup, & Solignac, 1999).

Deterministic Approach

In the last years, researches have focused on both approaches, the deterministic one is mainly based on the study of genes coding for coat color, the principal character allowing for breed differentiation and under human selection in European cattle breeds (Maudet & Taberlet, 2002). In Table 2 is shown a classification of the most important identified *loci* based on their known functions coding for coat color. Interest in these studies is mainly based on the possibility to determine the breed of origin of cheese finding molecular markers that are specific to each cow breed and developing a technique to detect these markers in cheese (a mixture of milk from several individuals). In cattle the pigmentation is determined by the distribution of two pigments: eu- and pheomelanin, producing brown or black and red to yellow pigmentation respectively. Tyrosinase, the rate-limiting enzyme involved in the synthesis of both melanins, is regulated by the melanocyte stimulating hormone (MSH). This hormone and several other melanotropic peptides, stimulate melanin formation in melanocytes by binding to the melanocortin-1-receptor (MC1R), a G-protein-coupled receptor encoded by the *Extension* gene (Robbins et al., 1993). In addition, the amounts of eu- and pheomelanin in the melanocyte are controlled by the agouti gene encoding the Agouti Signal Protein (ASP), that acts as an antagonist of MSH signaling through the MC1R, even if its mechanism of action is controversial (Furumura, Sakai, Potter, Vieira, Barsh, & Hearing, 1998). The MC1R gene has been analyzed in different species (Crepaldi, Fornarelli, & Marelli, 2005) in cattle population many mutations have been observed and three main alleles have been detected (Klungland, Vage, Gomez-

Raya, Adalsteinsson, & Lien, 1995): the E^+ so called "wild type" encoding the normal functional receptor, the dominant E^D caused by a T/C substitution changing the 99^{th} amino acid to proline with a consequent high level of eumelanin, the e containing a G-deletion giving rise to a non-functional receptor resulting in pheomelanin production giving red color in homozygotes. In addition other four alleles have been detected: Rouzaud et al. (2000) and Maudet and Taberlet (2002) discover a new allele named E^1 in the Aubrac, Gasconne and Tarentaise breeds in a study considering different French cow breeds while Graphodatskaya, Joerg, and Stranzinger (2002) detected two new alleles in the Brown Swiss breed (E^{d1} and E^{d2}) and one in the Simmental breed (e^f). In Table 3 are shown the polymorphisms detected in different cattle breeds, studies have been performed also on beef breeds, in fact the beef sector as well encounters commercial problems in recognizing and protecting meat of high quality derived from specialized breeds from others, resulting in economic losses for the farmers (Ciampolini et al., 2000). It is worth mentioning that in all the Italian beef cattle breeds analyzed (Chianina, Marchigiana, Piemontese and Maremmana) a new mutation has been detected consisting in a base substitution (C/T) in the 667-bp position resulting in an amino acid change (Arg to Trp) (Maudet & Taberlet, 2002), Crepaldi, Fornarelli, and Marilli (2003) in a study concerning Italian beef cattle breeds confirmed the presence of such mutation also in their samples.

These results are promising for establishing a traceability method based on coat color markers giving the possibility to distinguish among some breeds, additional studies on other coat color genes are anyway needed to complete the information and increase the discriminating power of such markers; analyses on *spotting* gene, affecting spotting extension, for example, could in fact increase the informative power derived from *extension* alleles. However studies on coat color genes are utilized also for assessing genetic diversity to maintain traditional color types for the preservation of the cultural and historical value of endangered native breeds (Kantanen et al., 2000).

Dairy and beef sectors are not the only involved in genetic traceability systems, more and more studies are carried out in the swine sector. In fact different pig breeds have been developed to satisfy particular market requirements; for example in the United Kingdom (UK) the Large White and the Landrace breeds were selected for bacon production while Berkshire is a pork pig, on the other one hand the Spanish market

aims to valorize and protect hams obtained by Iberian pig breeds selected for outdoor rearing and production of specialist ham (Alderson & Plastow, 2003; Carrión et al., 2003), in both cases breed differentiation is an important tool for the protection of typical quality products. In the UK efforts have been made for the discrimination of Berkshire and Tamworth breeds through the use of the MC1R and KIT polymorphisms which control much of the variation of coat color in swine; such tools might be used as part of Quality Assurance scheme for the Traditional Breeds Meat Marketing Scheme as already happens for the British Wild Boar Association (Alderson & Plastow, 2003; Carrión et al., 2003). In fact, in the case of wild boar, discrimination is easy due to a variant of the MC1R *locus* not found in commercial pigs, Tamworth and Berkshire differentiation instead requires the use of both MC1R and KIT *loci* analyses as reported by Alderson and Plastow (2003).

Spanish market aims to trace Iberian pig products that have been differentiated in Spain as a component of a sustainable system supporting biodiversity and delivering outputs of the highest quality with special sensory properties. The Iberian Cured Ham has acquired an excellent reputation and can cost up to ten times more than a normal cured ham, this led to an indiscriminate use of the term "Iberico" (Carrión et al., 2003; Garcìa et al., 2006). Anyway for the production of Iberian Cured ham the Spanish legislation allows up to 50% Duroc origin in the animals, hams obtained by pure Iberian pigs are instead called Pure Iberian Ham. Studies on coat color genes have been performed for these breeds as well, but the variation of coat color in such breeds (from blonde to black) and the allowance for using Duroc crossbreeds, made discrimination, through the only use of these genes, difficult. Carrión et al. (2003) in their study collected samples from Iberian hams in some markets and analyzed them through the use of both MC1R and KIT *loci*, they found a selection of MC1R alleles and evidence of a new haplotype that could be the origin of the red-chestnut type but they could not identify discrimination test; Fernandez, Fabuel, Alves, Rodriguez, Silió, and Óvilo (2004) solved the problem implementing results obtained from the analysis of MC1R with those obtained from the analysis of four microsatellite *loci*, of the *pink-eyed dilution* gene and of nine amplified fragment length polymorphism (AFLP) being able to discriminate also between pure Iberian and Duroc crossbreeds genotypes. AFLP procedure was applied also by Alves, Castellanos, Óvilo, Silió, and Rodríguez (2002) which highlighted the

presence of nine fragments detected in the Duroc breed only while three polymorphisms were found only in Iberian pigs, use of such technology would allow the detection of crossbred animals with a whole probability of exclusion of a pure Iberian origin of 0.97 and 0.71 for the 50% and 25% Duroc crossbreeding. Further studies conducted in Iberian strains only evidenced the presence of strain-specific AFLP markers, in this case such information appear important for management and conservation of highly inbred Iberian strains as differentiation among strains is not required for high quality production (Óvilo et al., 2000). Studies on conservation and management of small populations often use AFLP markers allowing for the detection of breed specific markers, for example De Marchi et al. (2006) in a study investigating the genetic variation of four Italian indigenous chicken breeds found specific markers in every breed analyzed which could enable their differentiation on the market. Anyway even if the exposed results are promising and could enhance the use of AFLP markers for breed traceability methods, it must be said that researchers should be extended to a greater number of individual samples to verify the exclusiveness of detected markers; use of pooled samples in fact could be useful to highlight the presence of such markers but these differences may be due to simple differences in the allelic frequencies of the population as demonstrated by Negrini et al. (2003) in their research on some Italian cattle breeds.

Concluding, the use of AFLP markers for breed genetic traceability is suggested by several authors (Alves et al., 2002; Negrini et al., 2003; De Marchi et al., 2006;), Óvilo et al. (2000) affirmed that using microsatellites detection of any strain-specific allele fixed in the population was impossible, evidencing a lower discrimination power than AFLP, at least for closely related individuals. Anyway all authors agreed with the opinion that AFLP technique is complex, expensive and not easy to apply to routine tests, to overcome these disadvantages it is advisable to convert AFLP markers in simpler PCR-based tests (Óvilo et al., 2000; Alves et al., 2002,) as Sasazaki et al. (2004) did in their study aimed to distinguish the Japanese Black cattle from a cross of Japanese Black and Holstein Friesian.

Probabilistic Approach

Assigning individuals to populations have a wide range of applications both in population genetics, for example for evaluating population differentiation in polar bears (Paetkau et al., 1995) or for classifying individual fish (Taylor, Beacham, & Kaeriyama, 1994) or honey bees (Cornuet, Aulagnier, Lek, Franck, & Solignac, 1996) and in forensics as for verifying the authenticity of a labeled food product. The utilized methodology, based on analyses of individual multilocus genotypes, relies on the fact that individuals will have genotypes more similar when they come from the same population (Cornuet et al., 1999); these "genetic methods" are based on the likelihood that the genotype of the individual to be assigned occurs in each of two or more candidate populations (Paetkau et al., 1995; Rannala & Mountain, 1997) or on genetic distances between the individual and a population (Cornuet et al., 1999). These statistical tools could be used for the assessment of a breed traceability system, anyway, according to Cornuet et al. (1999) maximum likelihood methods, in particular the one based on a Bayesian approach, permits to obtain the best results but population must be in Hardy-Weinberg and linkage equilibrium; distance based methodologies permits to overcome this problem and could be more appropriated if these two assumptions are not fulfilled. There are other aspects affecting correct assignment such as the number of scored *loci* and animals, the *loci* variability, the population differentiation and their significance has been investigated by Bjørnstad and Røed (2002). According to them both genetic differentiation and number of scored *loci* are highly important, for very differentiated breeds ($0.200 < Fst < 0.259$) only three *loci* could be sufficient to have an assignment precision of 95%; *loci* having an intermediate to high variability within and across populations yield higher assignment precision while breed sample size is not critical as long as more than 20 animals per breed are analyzed. Several studies on different species confirm the efficacy of Bayesian approach if an appropriate number of markers is scored (Bjørnstad & Røed, 2001; Negrini et al., 2003; Vega-Pla, Martínez, Cabello, Rodríguez-Gallardo, & Delgado, 2003; Ciampolini et al., 2006; Dalvit et al., 2006; Filippini et al. 2006; García et al., 2006). The major problem for the effective applicability of these methods are the choice of the *loci* to be analyzed and the creation of a pooled database collecting the allele frequencies of all possible alternative breed of origin weighted by the population size in

order to allow to answer the critical question "what is the probability that this animal is actually from this breed?" (Ciampolini et al., 2000; 2006); collection of samples from all alternative breeds is essential in fact maximum likelihood methods test if the analyzed sample belongs to one of the reference population and the result may be inexact if this condition is not met (Baudouin, Piry, & Cornuet, 2004). Baudouin et al. (2004) as well stressed the importance of the quality of the reference population set from which derives the quality of obtained results; information about genetic diversity of the populations, their equilibrium and a adequate number of samples (collected avoiding closely related individuals) are essential.

The main critics on the use of such methodologies lies on the difficulties to use the necessary statistical tools that often need a high know-how making difficult to use them as routine tests (Baudouin et al., 2004, García et al., 2006), to try to overcome this situation some authors proposed user-friendly software, available for free on the internet, permitting to compute the necessary calculations, the most widely used are *Structure* (Pritchard, 2000) and GeneClass2 (Baudouin et al., 2004), both are based on a Bayesian approach but the assignment procedure is different.

Species Genetic Traceability

Species identification in meat products has always been important for consumers because of social, religious, health and economic implications; nowadays carcasses and whole fish are rarely displayed while either fresh or frozen cuts, processed and ready to eat food are increasingly available making species identification difficult. For this reason fraudulent adulteration could take place substituting the declared meat or fish species with others of lower commercial value (Hunt, Parkes, & Lumley, 1997; Martinez & Malmheden Yman, 1998), such falsification is actually very common in game meat products resulting in a great profit due to the higher prices of these species than beef or pork (Blackett & Keim, 1992; Wolf, Rentsch, & Hübner, 1999). Fish industry also is involved in species counterfeits especially in the case of canned fish such as tuna whose *genus* is made up of many different species characterized by different quality (Unseld, Beyermann, Brandt, & Hiesel, 1995). Dairy sector is subject of frauds regarding milk and above all cheese species of origin, in fact the greater availability and the lower cost of cow milk rather than goat, ewe or buffalo milk lead to fraudulent substitutions in

cheese manufacturing (Maudet and Taberlet, 2001). To understand the importance of these frauds it is worth mentioning than in Italy the addition of undeclared bovine milk to water buffalo milk for making cheese is the most frequent fraud reported by the Central Inspectorate for Repression of Frauds of the Italian Ministry of Agricultural and Forestry Policy in 1998 and 1999 for all foods of animal origin; in 1998 and 1999 approximately 13% of cheeses tested contained undeclared non-water buffalo milk (Rea, Chikuni, Branciari, Sangamayya, Ranucci, & Avellini, 2001).

First approaches for species identification were based on protein analyses and immunological assay (Berger, Mageau, Schwab, & Johnston, 1988; Patterson & Jones, 1990), these methodologies presents two main disadvantages: protein expression is tissue dependant and proteins may be denaturized during processing and heating (Hunt et al., 1997, Martinez and Malmheden Yman, 1998) though the legislation still recognize such methods as official, in fact the reference technique for the detection of cows' milk is based on isoelectric focusing of β-casein (European Commission, 1996); anyway researchers focused their attention on the study of DNA that is present in every cell and is relatively stable to food processing being detectable even in ripened cheese (Plath, Krause, & Einspanier, 1997). DNA based analytical approaches were investigated for the first time at the end of 1980's and beginning of 1990's employing simple slot/blot assay using total genomic species DNA as probe being able to clearly identify species such pork and chicken but not among ruminant species (Bauer, Teifel-Greding, & Liebhardt, 1987; Winterø, Thomson, & Davies, 1990; Chikuni, Ozutsumi, Koishikawa, & Kato, 1990; Ebbehøj & Thomsen, 1991), Hunt et al. (1997) set up a method for the detection of several different species by the use of species-specific oligonucleotide probes obtaining satisfactory results until the minimum admixture level of 2.5% without the use of PCR amplification that, in such period, was still considered too sensitive and associated with many technical problems while Janssen, Hägele, Buntjer, and Lenstra (1998) utilized PCR generated probes. Instead, few years later, PCR based technique overwhelmed other methods being used and studied also today; RFLP technique was investigated by several authors on both genomic and mitochondrial DNA (Ram, Ram, & Baidoun, 1996; Plath et al., 1997; Quintero et al., 1998; Wolf et al., 1999; Montiel-Sosa, Ruiz-Pesini, Montoya, Roncalés, López-Pérez, & Pérez-Martos, 2000; Bania, Ugorski, Polanowski, & Adamczyk, 2001) but finally the most recent techniques are based on the

amplification of primers designed to give different length fragments from different species as suggested by Matsunaga et al. (1999). Mitochondrial DNA in particular, presents several advantages if compared to genomic, it is present in thousands of copies per cell improving the possibility to amplify template molecules of adequate size, the vast knowledge on its organization and the availability of reported sequences in many species makes the design of specific primers easier and its large variability allows reliable identification of precise species in mixtures (Mackie et al., 1999; Montiel-Sosa et al., 2000; Maudet & Taberlet, 2001). Cytochrome b gene has been widely investigated allowing for easy and clear species differentiation both in tuna and salmon species (Bartelett & Davidson; 1991, Unseld et al.; 1995, Quintero et al., 1998; Russel et al., 2000; Rehbein, 2005) in meat (Matsunaga et al., 1999) and in dairy products (Bania et al., 2001; Rea et al., 2001), in this last case also the study of the β-casein gene has been proposed by Plath et al. (1997) while Maudet and Taberlet (2001) suggested the use of primers designed on the control region also called D-loop that was already investigated for meat differentiation by Fei, Okayama, Yamanoue, Nishikawa, Mannen, and Tsuji (1996). A recent study conducted by Bellis, Ashton, Freney, Blair, and Griffiths (2003) suggested instead the amplification of a variable intron within the highly conserved *TP53* tumor suppressor gene which produces fragments of different sizes among species. AFLP technique could also be employed for species differentiation as evidenced by Cassandro, Targhetta, De Marchi, Dalvit, Barcaccia, and Bittante (2005) who utilized these markers to distinguish among avian species, anyway such method has already been widely used for species differentiation in plants (Cervera et al., 2000).
Concluding it is important to underline that most of these studies utilized commercial samples collected in supermarket or butcheries to verify their techniques and evidenced several cases of counterfeits and contamination suggesting that controls should be more strict and appropriate in order to preserve consumers against frauds.

Conclusions

Traceability of livestock products is an essential tool to safeguard public and animal health and to valorize typical foods, for this reason the European Union has applied since 2005 a strict legislation on labeling systems. Anyway it has been demonstrated

that traceability methods based only on batch codes or papery documents are not always trustful being easy to be counterfeit.

At present DNA based techniques seemed to be the most secure tool of verification for products of animal origin and researches have highlighted enormous improvements in the last few years, nevertheless these techniques are already used for human testing in forensic caseworks. The major problem for their effective applicability are the high costs, unsustainable if such methods are meant to be employed as routine test, but affordable if they are needed just as verification in particular occasions (e.g. when recall of a batch is required). Anyway any type of traceability method is related with increasing costs for food companies but it is essential to analyze which part of these additional costs could be translated in benefits; for example methods ensuring an efficient recall, such DNA technologies, could prevent from recalling safe batches. Also consumers' willing to pay for safer food should be better studied, in general consumers agree on paying extra price for food safety issues especially in developed countries (Henson, 1996; Unnevher, 2000) even if some authors think that actual buying decision are mostly based on economic convenience than on the presence of label or certification (Blend & van Ravenswaay, 1999). As stated by Gellynck, Januszewska, Verbeke, Viaene (2005) in their study on consumers' attitude towards meat products, consumers' behavior towards traceability differs from country to country and it is also dependant on the perception of quality of meat. For example Gellynck et al. (2005) divided the Belgian meat consumers among "enthusiasts" and "pessimists" evidencing different perceptions among these groups. In spite of these differences authors highlighted that functional attributes as efficient products recall, possibility to identify individual responsibility and a complete traceability of the meat chain can be regarded as minimum requirements of a traceability systems for all consumers (Gellynck et al.,2005; Meuwissen et al., 2003); keeping this aspects in mind, the potential of DNA technologies appears straightforward.

The second problem to overcome is the attainment of an agreement on markers and approaches to be utilized, in fact, as witnessed by all the studies cited in the present review, the scientific community is still debating on many different approaches and guidelines are needed. A first step has been done by the International Society for Animal Genetics and the Food and Agriculture Organization Standing Committee which

proposed sets of microsatellite markers in different species for the study of animal genetic diversity and for conservation purposes. Actually more and more researches are carried out following these suggestions giving the opportunity to compare different results.

Among the three different level of identification the individual one regarding meat cut identification, appears the easiest to be implemented due the few number of needed markers, translated in low costs, and to the univocal and simpler authors' statistical approach; the main problem is maybe the organization of hair samples collection from every animal at birth. Breeds and species traceability is as well needed but application of genetic methods is tricky, deterministic approach seemed to be simpler because statistical inference is not necessary but at present such techniques are not able to ensure satisfactory level of discrimination at least for breed determination; on the other one hand probabilistic approaches are promising but the difficulties of statistical calculations have to be overcome. Concluding genetic traceability is a useful and trustful tool for products identification and could be the solution to consumers' lack of confidence as people strongly rely on DNA analysis, but to be really applicable more cooperation among researchers and among people involved in the food production chain is necessary in order to find the costless and simpler organization solutions.

Tables

Table 1. Match probability values obtained in recent studies of individual genetic traceability in cattle.

Type and Number of Markers	Match Probability	Breeds	References
STR[a]– 12	$1.9*10^{-11}$	Piemontese, Chianina, Marchigiana, Romagnola	Dalvit et al., 2006
STR– 10	$2.4*10^{-8}$	Galloway	Herraeza et al., 2005
STR – 14	$2.3*10^{-11}$	Galloway	Herraeza et al., 2005
STR – 17	$1.4*10^{-13}$	Galloway	Herraeza et al., 2005
SNP[b] - 43	$5.3*10^{-11}$	Galloway	Herraeza et al., 2005
SNP – 20	$4.3*10^{-8}$	Holstein Friesian and others	Heaton et al., 2005
SNP – 32	$2.0*10^{-13}$	American Angus	Heaton et al., 2002
STR - 10	$>10^{-7}$	Pirenaica	Arana et al., 2002
STR – 13	$>10^{-15}$	Piemontese, Chinina, Holstein Friesian, Italian Simmental	Orrù et al., 2006
STR - 11	$5*10^{-12}$	Charolaise	Sancristobal-Gaudy et al., 2000
STR - 10	$1*10^{-10}$	Belgium beef cattle	Peelman et al., 1998

[a]STR: Short Tandem Repeats; [b]SNP: Single Nucleotide Polymorphism

Table 2: Genes coding for coat colour (Searle, 1968; Olson, 1999)

Locus	Symbol	Function	Coding Molecule	Effects
Extension	E	Involved in the melanogenesis regulation	melanocortin receptor 1 (MC1R)	Controls the proportion of the two melanin types
Agouti	A	Involved in the melanogenesis regulation	agouti signaling protein (ASIP)	Controls the proportion of the two melanin types
Spotted or White Spotting	S W	Involved in the melanocytes development and migration during embryogenesis	KIT	Affects spotting extension and pigmentation intensity
Roan	R	Involved in the melanocytes development and migration during embryogenesis	mast cell growth factor (MGF)	Determines the roan color in the Shorthorn and Blue Belgian breeds.
Slaty		Involved in the melanin biosynthesis	Tyrosinase-related protein 2 (TYRP2)	Controls dilution of coat color
Albino	C	Involved in the melanin biosynthesis	tyrosinase (TYR)	Controls dilution of coat color
Brown	B	Involved in the melanin biosynthesis	tyrosinase-related protein 1 (TYRP1)	Controls dilution of coat color determining brown color
Dilute	D	Involved in melanocytes morphology	myosin type V (MYO5A)	Controls dilution of coat color
Silver	PMEL17	Involved in melanosome structure and functions	transmembrane melanosome protein	Determines the grey coat color

Table 3: Polymorphisms of the extension *locus* in cattle breeds

Allele	Breed	Breed origin	References
E^D	Italian, French and Finnish Holstein Friesian, Vosgienne, Jutland Breed, Danish Black-Pied, Northern Finncattle, Western Finncattle, Icelandic Cattle, Blacksided Troender and Norland Cattle, Western Fjord cattle, Doela Cattle, Norwegian Cattle, Swedish Mountain Cattle, Swedish Black and White	Italy, France, Denmark, Finland, Sweden, Iceland, Norway	Kantanen et al., 2000; Rouzad et al., 2000; Maudet & Taberlet, 2002; Russo et al., 2003; Crepaldi et al., 2003
e	Italian, French and Finnish Holstein Friesian, Italian and French Simmental, Brown Swiss, Reggiana, Chianina, Romagnola, Limousine, Blonde d'Aquitaine, Charolais, Salers, Abondance, Montbéliarde, Maine Anjou, Villard de Lans, Danish Shorthorn, Red Danish, Western Finncattle, Eastern Finncattle, Finnish Ayrshire, Icelandic Cattle, Blacksided Troender and Norland Cattle, Western Fjord cattle, Doela Cattle, Eastern Red Polled, Telemark Cattle, Western Red Polled, Norwegian Cattle, Swedish Mountain Cattle, Swedish Red Polled Cattle, Swedish Red and White	Italy, France, Finland, Switzerland, Denmark, Finland, Iceland, Norway, Sweden	Kantanen et al., 2000; Rouzad et al., 2000; Graphodatskaya et al., 2002; Maudet & Taberlet, 2002; Crepaldi et al., 2003; Russo et al., 2003
E^+	Chianina, Marchigiana, Piemontese, Romagnola, Cabannina, Rendena, Aubrac, Gasconne, Normande, Tarentaise, Blanc Bleu, Danish Jersey, Jutlan Breed, Eastern and Western Finncattle, Icelandic Cattle, Blacksided Troender and Norland Cattle, Western Fjord cattle, Doela Cattle, Western Red Polled, Swedish Mountain Cattle	Italy, France, Denmark, Finland, Iceland, Norway, Sweden	Kantanen et al., 2000; Rouzad et al., 2000; Maudet & Taberlet 2002; Crepaldi et al., 2003
E^1	Aubrac, Gasconne, Tarentaise,	France	Rouzad et al., 2000; Maudet & Taberlet 2002
E^{d1}, E^{d2}	Brown Swiss	Switzerland	Graphodatskaya et al., 2002
e^f	Italian and French Simmental	Italy, France	Graphodatskaya et al., 2002
Unknown	Chianina, Marchigiana, Piemontese, Maremmana, Valdostana Pezzata Rossa, Flamande	Italy, France	Maudet & Taberlet 2002; Crepaldi et al., 2003

References

Alderson, G.L.H., & Plastow, G.S. (2004). Use of DNA markers to assist with product traceability and pedigree analysis and their role in breed conservation. *Animal Genetic Resource Information, 35*, 1-7.

Alves, E., Castellanos, C., Ovilo, C., Silió, L., & Rodríguez, C. (2002). Differentiation of the raw material of the Iberian pig meat industry based on the use of amplified fragment length polymorphism. *Meat Science, 61*, 157-162.

Ajmone-Marsan, P., Milanesi, E., & Negrini, R. (2004). Breed traceability using molecular methods. In *Proceedings of the 7th World Conference of the Brown Swiss cattle breeders* (pp. 101-104), 3-7 March 2004, Verona, Italy.

Arana, A., Soret, B, Lasa, I., & Alfonso, L. (2002). Meat traceability using DNA markers: application to the beef industry. *Meat Science, 61*, 367-373.

Bania, J., Ugorski, M., Polanowski, A., & Adamczyk, E. (2001). Application of polymerase chain reaction for detection of goats' milk adulteration by milk of cow. *Journal of Dairy Research, 68*, 333-336.

Barcos, L.O. (2001). Recent developments in animal identification and the traceability of animal products in international trade. *Revue scientifique et technique (International Office of Epizootics), 20*(2), 640-651.

Bartlett, S.E., & Davidson, W.S. (1991). Identification of Thunnus tuna species by the Polymerase Chain Reaction and direct sequence analysis of their mitochondrial cytochrome b genes. *Canadian Journal of Fisheries and Aquatic Sciences, 48*, 309-317.

Baudouin, L., Piry, S., & Cornuet, J.M. (2004). Analytical Bayesian approach for assigning individuals to populations. *Journal of Heredity, 95*(3), 217-224.

Bauer, C., Teifel-Greding, J., & Liebhardt, E. (1987). Species identification of heat denaturized meat samples by DNA analysis. *Archiv für Lebensmittelhygiene, 38*, 149-176.

Bellis, C., Ashton, K.J., Freney, L., Blair, B, & Griffiths, L.R. (2003). A molecular genetic approach for forensic animal species identification. *Forensic Science International, 134*, 99-108.

Berger, R, Mageau, K., Schwab, B., & Johnston, R. (1988). Detection of poultry and pork in cooked and canned meat foods by enzyme-linked immunosorbent assays. *Journal - Association of Official Analytical Chemists, 71*, 406-409.

Blackett, R.S., & Keim, P. (1992). Big game species identification by deoxyribonucleic acid (DNA) probes. *Journal of Forensic Sciences, 37*(2), 590-596.

Blend, J.R., & van Ravenswaay, E.O. (1999). Measuring consumer demand for eco-labeled apples. *American Journal of Agricultural Economics, 81*, 1072-1077.

Bjørnstad, G., & Røed, K.H. (2001). Breed demarcation and potential for breed allocation of horses assessed by microsatellites markers. *Animal Genetics, 32,* 59-65.

Bjørnstad, G., & Røed, K.H. (2002). Evaluation of factors affecting individual assignment precision using microsatellite data from horse breeds and simulated breed crosses. *Animal Genetics, 33*, 264-270.

Carrión, D., Day, A., Evans, G., Mitsuhashi, T., Archibald, A., Haley, C., Andersson, L., & Plastow, G. (2003). The use of *MC1R* and *KIT* genotypes for breed characterisation. *Archivos de Zootecnia, 52*, 237-244.

Cassandro, M., Targhetta, C., De Marchi, M., Dalvit, C., Barcaccia, G., & Bittante, G. (2005). DNA fingerprinting characterization of 4 Italian poultry species. In *Proceedings of the XIII International Conference on the Status of Plant & Animal Genome Research* (pp. 210), 15-19 January 2005, San Diego, California, USA.

Cervera, M.T., Remington, D., Frigerio J.M., Storme, V., Ivens, B., Boerjan, W., & Plomion, C. (2000). Improved AFLP analysis of tree species. *Canadian Journal of Forest Research, 30*(10), 1608-1616.

Ciampolini, R., Leveziel, H., Mozzanti, E., Grohs, C., & Cianci, D. (2000). Genomic identification of an individual or its tissue. *Meat Science, 54*, 35-40.

Ciampolini, R., Cetica, V., Ciani, E., Mozzanti, E., Fosella, X., Marroni, F., Biagetti, M., Sebastiani, C., Papa, P., Filippini, G., Cianci, D., & Presciuttini, S. (2006). Statistical analysis of individual assignment tests among four cattle breeds using fifteen STR loci. *Journal of Animal Science, 84*, 11-19.

Chikuni, K., Ozutsumi, K., Koishikawa, T., & Kato, S. (1990). Species identification of cooked meats by DNA hybridisation assay. *Meat Science, 27*, 119-128.

Cornuet, J.M., Aulagnier, S., Lek, S., Franck, P., & Solignac, M. (1996). Classifying individuals among infra-specific taxa using microsatellite data and neural networks. *Comptes rendus de l'Académie des sciences. Série III, Sciences de la vie, 319*, 1167-1177.

Cornuet, J.M., Piry, S., Luikart, G., Estoup, A., & Solignac, M. (1999). New methods employing multilocus genotypes to select or exclude populations as origins of individuals. *Genetics, 153*, 1989-2000.

Cozzi, G., Ragno, E. (2003). Meat production and market in Italy. *Agriculturae conspectus scientificus, 68,*(2)*,* 1331-7768*.*

Crepaldi, P., Fornarelli, F., & Marilli, M. (2005). MC1R gene: comparison between different farm animal species. *Italian Journal of Animal Science, 4*(2), 43-45.

Crepaldi, P., Marilli, M., Gorni, C., Meggiolaro, D., & Cicogna, M. (2003). Preliminary study on MC1R polymorphism in some cattle breeds raised in Italy. *Italian Journal of Animal Science, 2*(1), 13-15.

Cunningham, E.P., & Meghen, C.M. (2001). Biological identification systems: genetic markers. *Revue scientifique et technique (International Office of Epizootics), 20*(2), 491-499.

Dalvit, C., Targhetta, C., Gervaso, M., De Marchi, M., Mantovani, R., & Cassandro, M. (2006). Application of a panel of microsatellite markers for the genetic traceability of bovine origin products. In *Proceedings 57th Annual Meeting of the European Association for Animal Production* (pp.26), 17-21 September 2006, Antalya, Turkey.

De Marchi, M., Targhetta, C., Contiero, B., & Cassandro, M. (2003). Genetic traceability of chicken breeds. *Agriculturae conspectus scientificus, 68*(4), 255-259.

De Marchi, M., Dalvit, C., Targhetta, C., & Cassandro, M. (2006). Assesing genetic diversity in indigenous Veneto chicken breeds using AFLP markers. *Animal Genetics, 37*(2), 101-105.

Ebbehøj, K.F., & Thomsen, P.D. (1991). Species differentiation of heated meat products by DNA hybridisation. *Meat Science, 30*, 221-234.

European Economic Community, (1957). Treaty Establishing the European Economic Community and connected documents. *Publishing Services of the European Communities,* 5-83.

European Commission, (1996). Commission Regulation (EEC) No. 1081/96 of 14 June 1996. Reference Method for the detection of cows' milk and caseinate in cheeses from ewes' milk, goats' milk and buffalos' milk. *Official Journal of the European Communities, L142*, 15-25.

European Commission, (1997). Green Paper on the general principles of food law in the European Union.

European Commission, (2000). Council Regulation (EC) No. 1760/2000 of 17 July 2000 establishing a system for the identification and registration of bovine animals and regarding the labeling of beef and beef products and repealing Council Regulation (EC) No 820/97. *Official Journal of the European Communities, L 204*, 1-10.

European Commission, (2000). Commission Regulation (EC) No. 1825/2000 of 25 August 2000 laying down detailed rules for the application of Regulation (EC) No 1760/2000 of the European Parliament and of the Council as regards the labelling of beef and beef products. *Official Journal of the European Communities, L 216*, 8-12.

European Commission, (2000). White Paper on Food Safety.

European Commission, (2002). Council Regulation (EC) No 178/2002 of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. *Official Journal of the European Communities, L 31*, 1-24.

Food and Agricultural Organization (FAO), (2004). *Secondary Guidelines for Development of National Farm Animal Genetic Resources Management Plans*. Measurement of Domestic Animal Diversity (MoDAD): Recommended Microsatellite Markers.

Fei, S., Okayama, T., Yamanoue, M., Nishikawa, I., Mannen, H., & Tsuji, S. (1996). Species identification of meats and meat products by PCR. *Animal Science and Technology* (Jpn), *67*, 900-905.

Fernández, A., Fabuel, E., Alves, E., Rodriguez, C., Silió, L., & Óvilo, C. (2004). DNA tests based on coat colour genes for authentication of the raw material of meat products from Iberian pigs. *Journal of the Science of Food and Agriculture 84*, 1855-1860.

Filippini, G., Cetica, V., Ciampolini, R., Biagetti, M., Cecchi, F., Mazzanti, E., Ciani, E., Sebastiani, C., & Venditti, G. (2006). Beef traceability using molecular methodologies. *Veterinary Research Communications, 30*(1), 375-377.

Franke, B.M., Gremaud, G., Hadorn, R., & Kreuzer, M. (2005). Geographic origin of meat-elements of an analytical approach to its authentication. *European Food Research and Technology, 221*, 493-503.

Furumura, M., Sakai, C., Potter, S.B., Vieira, W.D., Barsh, G.S., & Hearing, V.J. (1998). Characterization of genes modulated during pheomelanogenesis using differential display. *Proceedings of the National Academy of Sciences of the United States of America, 95*, 7374-7378.

Gandini, G.C., & Oldenbroek, J.K. (1999). Choosing the conservation strategy. In J.K. Oldenbroek, *Genebanks and conservation of farm animal genetic resources* (pp. 11-31), Lelystad, The Netherlands: DLO Institute for Animal Science and Health.

García, D., Martínez, A., Dunner, S., Vega-Pla, J.L., Fernández, C., Delgado, J.V., & Cañón, J. (2006). Estimation of the genetic admixture composition of Iberian dry-cured ham samples using DNA multilocus genotypes. *Meat Science, 72*, 560-566.

Gellynck, X., Januszewska, R., Verbeke, W., Viaene, J. (2005). Firm's costs of traceability confronted with consumer requirements. *Proceedings 92nd Seminar on Quality Management and Quality Assurance in Food Chains of the European Association of Agricultural Economists*, 2-4 March 2005, Gottingen, Germany. http://www.eaae.uni-goettingen.de/

Goffaux, F., China, B., Dams, L., Clinquart, A., & Daube, G. (2005). Development of a genetic traceability test in pig based on single nucleotide polymorphism detection. *Forensic Science International, 151*, 239-247.

Golan, E., Krissof, B., Kuchler, F., Nelson, K., & Price, G. (2004). Traceability in the US food supply: economic theory and industry studies. *Agricultural Economic report no. AER830* (pp. 56). Eshington, DC: Economic research service of the United States department of Agriculture.

Graphodatskaya, D., Joerg, H., & Stranzinger, G. (2002). Molecular and pharmacological characterization of the MSH-R alleles in Swiss cattle breeds. *Journal of receptor and signal transduction research, 22*, 421-430.

Heaton, M.P., Harhay, G.P., Bennett, G.L., Stone, R.T., Grosse, W.M., Casas, E., Keele, J.W., Smith, T.P., Chitko-McKown, C.G., & Laegreid, W.W. (2002). Selection and use of SNP markers for animal identification and paternity analysis in U.S. beef cattle. *Mammalian Genome, 13*(5), 272-281.

Heaton, M.P., Keen, J.E., Clawson, M.L., Harhay, G.P., Bauer, N., Schultz, C., Green, B.T., Durso, L., Chitko-McKown, C.G., & Laegreid, W.W. (2005). Use of bovine single

nucleotide polymorphism markers to verify sample tracking in beef processing. *Journal of American Veterinary Medical Association, 226*(8), 1311-1314.

Henson, S. (1996). Consumer willingness to pay for reductions in the risk of food poisoning in the UK. *Journal of Agricultural Economics*, *47*, 403-420.

Herraeza, D.L., Schafer, H., Mosner, J., Fries, H.R., & Wink, M. (2005). Comparison of microsatellite and single nucleotide polymorphism markers for the genetic analysis of a Galloway cattle population. *Zeitschrift Naturforschung, C 60*(7-8), 637-643.

Hunt, D.J., Parkes, H.C., & Lumley, I.D. (1997). Identification of the species of origin of raw and cooked meat products using oligonucleotide probes. *Food Chemistry*, *60*(3), 437-442.

International Organization for Standardization (ISO), (1994) ISO 8402: Quality management and quality assurance – vocabulary. ISO, Geneva.

Janssen, F.W., Hägele, G.H., Buntjer, J.B., and Lenstra, J.A. (1998). Species identification in meat by using PCR-generated satellite probes. *Journal of Industrial Microbiology & Biotechnology, 21*, 115-120.

Kantanen, J., Olsaker, I., Brusgaard, K., Eythorsdottir, E., Holm, L., Lien, S., Danell, B., & Adalsteinsson, S. (2000). Frequencies of genes for coat colour and horns in Nordic cattle breeds. *Genetic Selection Evolution, 32*, 561-576.

Klungland, H., Vage, D.I., Gomez-Raya, L., Adalsteinsson, S., & Lien, S. (1995). The role of melanocyte-stimulating hormone (MSH) receptor in bovine coat colour determination. *Mammalian Genome, 6*, 636-639.

Mackie, I.M., Pryde, S.E., Gonzales-Sotelo, C., Medina, I., Pérez-Martin, R., Quinteiro, J., Rey-Méndez, M. & Rehbein, H. (1999). Challenges in the identification of species of canned fish. *Trends in Food Science and Technology 10*, 9-14.

Marchant, J. (2002). Secure animal identification and source verification. JM Communications, UK. Copyright Optibrand Ltd., LLC.

Mariani, P., Panzitta F., Nardelli Costa J., Lazzari B., Crepaldi P., Marilli M., Fornarelli F., Fusi M., Milanesi E., Negrini R., Silveri R., Filippini F., & Ajmone Marsan, P. (2005). Metodi molecolari per la tracciabilità dei prodotti di origine animale. In *Proceedings of the 4th World Italian Beef Cattle Congress* (pp.297-302), 29 April – 1 May 2005, Gubbio, Italy.

Martinez, I., & Malmheden Yman, I. (1998). Species identification in meat products by RAPD analysis. *Food Research International, 31*, 459-466.

Matsunaga, T., Chikuni, K., Tanabe, R., Muroya, S., Shibata, K., Yamada, J., & Shinmura, Y. (1999). A quick and simple method for the identification of meat species and meat products by PCR assay. *Meat Science, 51*, 143-148.

Maudet, C., & Taberlet, P. (2001). Detection of cows' milk in goats' cheeses inferred from mitochoandrial DNA polymorphism. *Journal of Dairy Research, 68*, 229-235.

Maudet, C., Luikart, G., & Taberlet, P. (2002). Genetic diversity and assignment tests among seven French cattle breeds based on microsatellite DNA analysis. *Journal of Animal Science, 80*, 942-950.

Maudet, C., & Taberlet, P. (2002). Holstein's milk detection in cheeses inferred from melanocortin receptor 1 (*MC1R*) gene polymorphism. *Journal of Dairy Science, 85*, 707-715.

Mauriello, G., Moio, L., Genovese, A., & Ercolini, D. (2003). Relationship between flavouring capabilities, bacterial composition, and geographical origin of natural whey cultures used for traditional water-buffalo mozzarella cheese manufacture. *Journal of Dairy Science, 86*, 486,497.

McKean, J.D. (2001). The importance of traceability for public health and consumer protection. *Revue scientifique et technique (International Office of Epizootics), 20* (2), 363-378.

Montiel-Sosa, J.F., Ruiz-Pesini, E., Montoya, J., Roncalés, P., López-Pérez, M.J., & Pérez-Martos, A. (2000). Direct and highly species-specific detection of pork meat and fat in meat products by PCR amplification of mitochondrial DNA. *Journal of Agriculture and Food Chemistry, 48*, 2829-2832.

Meuwissen, M.P.M., Velthuis, A.G.J., Hogeveen, H., & Huirne, R.B.M. (2003). Traceability and certification in meat supply chains. *Journal of Agribusinness*, *21*(2), 167-181.

Negrini, R., Milanesi, E., Chegdani, F., Bernardi, J., Filippini, F., Valentini, A., & Ajmone-Marsan, P. (2003). Biallelic markers as a tool for the traceability of bovine breeds. In *Proceedings of the 54th Annual Meeting of European Association for Animal Production* (pp.91), 31st August – 3rd September 2003, Rome, Italy.

Olson, T. (1999). Genetics of color variation. In *The Genetics of Cattle* (pp.33-54). London, United Kingdom: R .Fries and A. Ruvinsky.

Opara, L., & Mazaud, F. (2001). Food traceability from field to plate. *Outlook on agriculture, 30*(4), 239-247.

Orrù, L., Napolitano, F., Catillo, G., & Moioli, B. (2006). Meat molecular traceability: How to choose the best set of microsatellites? *Meat Scence, 72*, 312-317.

Óvilo, C., Cervera, M.T., Castellanos, C., & Martínez-Zapater, J.M. (2000). Characterisation of Iberian pig genotypes using AFLP markers. *Animal Genetics, 31*, 117-122.

Paetkau, D., Clivert, W., Stirling, I., & Strobeck, C. (1995). Microsatellite analysis of population structure in Canadian polar bears. *Molecular Ecology, 4*, 347-354.

Pancaldi, M., Carboni, E., Paganelli, A., Righini, G., Salvi, A., Fontanesi, L., Russo, V., Davoli, R., Beretti, F., & Ravaglia, S. (2005). Come garantire l'autenticità dei prodotti agroalimentari tipici. Utilizzo combinato di traccianti biologici e analisi del DNA. *Industrie Alimentari*, *44*(452), 1127-1133.

Patterson, R.L.S., & Jones, S.J. (1990). Review of current techniques for the verification of the species origin of meat. *Analyst, 115*, 501-506.

Peelman, L.J., Mortiaux, F., Van Zeveren, A., Dansercoer, A., Mommens, G., Coopman, F., Bouquet, Y., Burny, A., Renaville, R., & Portetelle, D. (1998). Evaluation of the genetic variability of 23 bovine microsatellite markers in four Belgian cattle breeds. *Animal Genetics, 29*, 161-167.

Pillonel, L., Badertscher, R., Froidevaux, P., Haberhauer, G., Hölzl Horn, P., Jakob, A., Pfammatter, E., Piantini, U., Rossmann, A., Tabacchi, R., & Bosset, J.O. (2003). Stable isotope ratios, major, trace and radioactive elements in Emmental cheeses of different origins. *Lebensmittel-Wissenschaft + Tecnologie*, *36*, 615-623.

Plath, A., Krause, I., & Einspanier, R. (1997). Species identification in dairy products by three different DNA-based techniques. *Zeitschrift für Lebensmittel Untersuchung und Forschung, 205*, 437-441.

Pritchard, J.K. (2000). Inference of population structure using multilocus genotype data. *Genetics, 155*, 945-959.

Quinteiro, J., Sotelo, C.G., Rehbein, H., Pryde, S.E., Medina, I., Pérez-Martín, R.I., Rey-Méndez, M., & Mackie, I.M. (1998). Use of mtDNA direct polymerase chain reaction

(PCR) sequencing and PCR-Restriction Fragment Length Polymorphism methodologies in species identification of canned tuna. *Journal of Agricultural and Food Chemistry, 46*, 1662-1669.

Ram, J.L., Ram, M.L., & Baidoun, F.F. (1996). Authentication of canned tuna and bonito by sequence and restriction site analysis of polymerase chain reaction products of mitochondrial DNA. *Journal of Agricultural and Food Chemistry, 44*, 2460-2467.

Rannala, B., & Mountain, J.L. (1997). Detecting immigration by using multilocus genotypes. In *Proceeding of the National Academy of Science (USA)*, *94*, 9197-9221.

Rea, S., Chikuni, K., Branciari, R, Sangamayya, R.S., Ranucci, D., & Avellini, P. (2001). Use of duplex polymerase chain reaction (duplex-PCR) technique to identify bovine and water buffalo milk used in making mozzarella cheese. *Journal of Dairy Research,* 68, 689-698.

Rehbein, H. (2005). Identification of the fish species of raw or cold-smoked salmon and salmon caviar by single-strand conformation polymorphism (SSCP) analysis. *European Food Research and Technology, 220*, 625-632.

Robbins, L.S., Nadeau, J.H., Johnson, K.R., Kelly, M.A., Roselli-Rehfuss, L., Baack, E., Mountjoy, K.G., & Cone, R.D. (1993). Pigmentation phenotypes of variant extension locus alleles result from point mutations that alter MSH receptor function. *Cell, 72*, 827-834.

Rouzaud, F., Martin, J., Gallet, P.F., Delourme, D., Goulemot-Leger, V., Amigues, Y., Ménissier, F., Levéziel, H., Julien, R., & Oulmouden, A. (2000). A first genotyping assay of French cattle breeds based on a new allele of the *extension* gene encoding the melanocortin-1 receptor (MC1R). *Genetic Selection Evolution, 32*, 511-520.

Russel, V.J., Hold, G.L., Pryde, S.E., Rehbein, H., Quinteiro, J., Rey-Méndez, M., Sotelo, C.G., Pérez-Martín, R.I., Santos, A.T., & Rosa, C. (2000). Use of restriction fragment length polymorphism to distinguish between Salmon species. *Journal of Agricultural and Food Chemistry, 48*, 2184-2188.

Russo V., & Fontanesi L. (2004). Coat colour gene analysis and breed traceability. In *Proceedings of the 7th World Conference of the Brown Swiss cattle breeders (pp. 95-100)*, 3-7 March 2004, Verona, Italy.

Sancristobal-Gaudy, M., Renand, G., Amigues, Y., Boscher, M.Y., Leveziel, H., & Bibé, B. (2000). Traçabilté individuelle des viandes bovine à l'aide de marqueurs génétiques. *INRA Production Animale, 13*(4), 269-276.

Sasazaki, S., Itoh, K., Arimitsu, S., Imada, T., Takasuga, A., Nagaishi, H., Takano, S., Mannen, H., & Tsuji, S. (2004). Development of breed identification markers derived from AFLP in beef cattle. *Meat Science, 67*, 275-280.

Schwägele, F. (2005). Traceability from a European perspective. *Meat Science, 71*, 164-173.

Searle, A.G. (1968). *Comparative genetics of coat colour in mammals*. London, United Kingdom: Logo Press Limited.

Smith, G.C., Tatum, J.D., Belk, K.E., Scanga, J.A., Grandin, T., & Sofos, J.N. (2005). Traceability from a U.S. Perspective. *Meat Science, 71*, 174-193.

Shackell, G.H., Mathias, H.C., Cave, V.M., & Dodds, K.G. (2005). Evaluation of microsatellites as a potential tool for product tracing of ground beef mixtures. *Meat Science, 70*, 337-345.

Stanford, K, Stitt, J., Kellar, J.A., & McAllister, T.A. (2001). Traceability in cattle and small ruminants in Canada. *Revue scientifique et technique (International Office of Epizootics), 20*(2), 510-522.

Taylor, E.B., Beacham, T.D., & Kaeriyama, M. (1994). Population structure and identification of North Pacific Ocean chum salmon (*Oncorhynchus keta*) revealed by an analysis of minisatellite DNA variation. *Canadian journal of fisheries and aquatic sciences, 51*, 1430-1442.

Unnevehr, L.J. (2000). Food safety issues and fresh food product exports from LDCs. *Agricultural Economics*, 23, 231-241.

Unseld, M., Beyermann, B., Brandt, P., & Hiesel, R. (1995). Identification of the species origin of highly processed meat products by mitochondrial DNA sequences. *PCR Methods and Applications, 4*, 241-243.

Vázquez, J.F., Pérez, T., Ureña, F., Gudín, E., Albornoz, J., & Domínguez, A. (2004). Practical application of DNA fingerprinting to trace beef. *Journal of Food Protection, 67*(5), 972-979.

Vega-Pla, J.L., Martínez, A.M., Cabello, A., Rodríguez-Gallardo, P.P., & Delgado, J.V. (2003). Preliminary study of individual assignment of Iberian pigs using DNA genetic markers. *Archivos de Zootecnia, 52*, 225-230.

Weir, B.S. (1996). Genetic data analysis II. Methods for discrete population genetic data. Sunderland, Massachusetts: Sinauer Associates, Inc. Publishers.

Winterø, A.K., Thomson, P.D., & Davies, W. (1990). A comparison of DNA-Hybridisation, Immunodiffusion countercurrent Immunoelectrophoresis and isoelectric focusing for detecting the admixture of pork to beef. *Meat Science, 27*, 75-85.

Wolf, C., Rentsch, J., & Hübner, P. (1999). PCR-RFLP analysis of mitochondrial DNA: a reliable method for species identification. *Journal of Agricult$ure and Food Chemistry, 47*, 1350-1355.

Second contribution:

Genetic traceability of meat using microsatellite markers

Genetic traceability of meat using microsatellite markers

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Abstract

Traceability systems have become necessary, especially for beef products, to protect consumers' health. Aims of this study were to validate and to test a set of twelve microsatellite (STR) markers for the assessment of a genetic traceability system in six cattle breeds. The probability to find, by chance, two individuals sharing the same profile at the studied loci, was computed considering different number of STR, pooling the alleles in each breed, in the total population and in the dairy and beef population separately. Best results were then obtained considering match probabilities per breed. In this study, genotyping the five most polymorphic loci, the probability of finding two identical animals was approximately five in one million. Match probability values increased when the pooled marker sets were used, but were still satisfactory; moreover, use of the pooled marker sets will reduce the cost of analyses.

Keywords: Genetic traceability; Meat; Individual identification; Match probability; Cattle breeds; Microsatellite

Introduction

After the Bovine Spongiform Encephalopathy outbreak, which caused a decrease in beef consumption in many European countries (Ciampolini, Leveziel, Mozzanti, Grohs & Cianci, 2000), the development of a traceability system has become necessary to protect public health and ensure food safety. The European Union implemented, through regulations EC 1760/2000 and 1825/2000, a mandatory labeling system for beef and beef products. According to the mentioned legislation, every beef cut must show a label carrying the following information: an identification code referring to an animal or to a group of animals, and the country where the animal was born, fattened, slaughtered, and sectioned. However, as pointed out by several authors (Sancristobal-Gaudy, Renand, Amigues, Boscher, Leveziel & Bibé, 2000; Cunningham & Meghen, 2001; Orrù, Napolitano, Catillo, & Moioli, 2006), frauds and mistakes along the production chain cannot be fully avoided by this system. Animal identification using DNA based techniques could address this problem, as DNA is inalterable during all animal life and it is present in every tissue. Microsatellite (STR) markers, due to the high polymorphism, had already been widely investigated for many applications such as

paternity analysis (Jobling & Gill, 2004) and breed assignment tests in many species (Bjørnstad & Røed 2001; Koskinen, 2003; Ciampolini et al., 2006); they were also investigated in studies on individual identification highlighting promising results (Sancristobal-Gaudy et al., 2000; Vázquez, Pérez, Ureña, Gudín, Albornoz, & Domínguez, 2004; Orrù et al., 2006).

The main objective of the present study was to set up a panel of STRs to implement a genetic traceability system in six cattle breeds. This goal consisted of two major tasks: to validate twelve STR markers on the basis of their genetic variation among and within cattle breeds, and to set up an efficient set of STR markers for individual identification of the studied breeds considering the genetic differentiation among them.

Materials and Methods

Animal Sampling and Genotyping

The dataset consisted of 183 animals belonging to six cattle breeds, four of them are indigenous Italian beef breeds: Chianina (CH, n = 27), Marchigiana (MA, n = 27), Romagnola (RO, n = 23), and Piemontese (PI, n = 33) while two are cosmopolitan dairy breeds: Holstein Friesian (HF, n = 29) and Brown Swiss (BS, n = 44). Samples from beef breeds were collected in performance stations, samples derived from dairy breeds, in herds located in different geographical area. No pedigree data were recorded and samples were collected randomly in order to reproduce the market situation. Samples of the PI breed derived from animals of the ANABORAPI breeder association located in Cuneo (North-West Italy), the CH, MA, and RO breed samples were collected from the ANABIC breeder association located in Perugia (Central Italy). Samples belonging to the HF and BS breeds were collected in six different farms of the Trentino Alto Adige region (North-East Italy). Blood samples were collected from each animal in 5 ml vacutainar tubes containing sodium citrate as anticoagulant, and stored at -20°C until analyses were performed. DNA extraction was carried out with the use of the "Gentra System PUREGENE DNA purification kit" starting from 300 µl of whole blood. DNA samples were then amplified by PCR in correspondence of the following twelve STR loci: BM1818, ETH185, MM12, TGLA126, BM203, TGLA122, RM12, ILST008, SPS115, BL42, ETH3 and TGLA53 (Table 1). The investigated loci were chosen in

accordance to ISAG/FAO Standing Committee Recommendations (2004) and consulting previous studies (Kemp, Brezinsky & Teale, 1993; Bishop et al., 1994; Barendse et al. 1997; Grosz, Solinas-Toldo, Stone, Kappes, Fries & Beattie, 1997), in order to have highly polymorphic markers located all over the genome. For the amplification, 25 ng of DNA were added to a reaction mix containing: 1 pmol/µl of primer forward and reverse, 1X PCR buffer (16 mM $(NH_4)_2SO_4$, 67 mM Tris HCl pH 8,8, 0,01% Tween 20), 0,26 mM of every dNTPs, 2,5 mM of $MgCl_2$ and 0,8 U of *Taq* DNA polymerase, in a final volume of 20 µl. The twelve STRs were individually analyzed by a PX2 Thermohybaid thermal cycle at the following conditions: initial denaturation step of 5 min at 95°C, 40 cycles of 30 s at 94°C, 1 min at the primer specific annealing temperature and 1 min at 72°C, followed by a final extension of 10 min at 72°C. Allele size was determined with a Perkin Elmer ABI Prism 3730XL Genetic Analyzer, using GeneScan 2.0 and Genotyper 3.7 software (Perkin Elmer).

Statistical Analysis

Genetic variability of markers and breeds was analyzed aiming to validate the chosen STR set. Allelic frequencies and observed and expected heterozygosity, in the whole population and per breed, were calculated with Genetix 4.03 (Belkhir et al., 1998). The Fstat 2.9.3 program (Goudet, 1995) was used in calculations of mean number of alleles, allelic richness, and *F*-statistics estimates (Weir and Cockerham, 1984) per locus, in the total sample and in each breed. Polymorphism Information Content (PIC) per each locus was calculated with the software Cervus 2.0 (Marshall, 1998). Test for population differentiation was performed by GENEPOP 3.4 (Raymond and Rousset, 1995), for each locus an unbiased estimate of the Fisher's exact test was performed to verify if the allelic distribution was different among breeds.

The efficacy of the marker set was tested to be used for individual identification. Values of Match Probability (MP), defined as the probability of finding two individuals sharing, by chance, the same genotypic profile, were calculated according to Weir (1996) and Arana, Soret, Lasa, and Alfonso (2002). Match probability values were computed overall loci and for smaller marker sets to verify if a satisfactory level of identification could be achieved with fewer than twelve STRs. Moreover, calculations were performed in the

whole population, in each breed, and in dairy and beef breeds separately to verify if differences in population variability affected individual assignment tests.

Results and discussion

Genetic Variation

In Table 2, the number of detected alleles, the observed and expected heterozygosity, and the PIC for the twelve analyzed loci are shown. A total of 118 alleles have been detected in the six breeds; observed number of alleles per locus varied between 3 (RM12) and 18 (TGLA122) with an average of 9.8 and a standard deviation of 4.9; the expected heterozygosity ranged between 0.326 (RM12) and 0.666 (ETH 185). The PIC all over loci was equal to 0.638, revealing the satisfactory information content carried by the chosen markers. Ciampolini et al. (1995), analyzing the same beef cattle breeds but with different STRs, obtained similar results. RM12 was found to be the least informative marker while TGLA53 and ETH185 the most (0.867 and 0.828, respectively).

The number of alleles varied among breeds. The allelic richness - an estimation of the number of alleles per locus weighted by population size - revealed that BS presented the lowest number of alleles per locus (4.8) while PI presented the highest (6.0). The same variation was observed for expected heterozygosity, indicating that BS is the breed showing the lowest genetic variation (0.572) while PI the highest (0.686) (data not shown). In their study, Ciampolini et al. (1995) also found PI to be the breed with the highest number of alleles per locus, followed by MA as shown in this research. Moreover, Moioli, Napolitano, and Catillo (2004) obtained comparable results for the PI breed. These findings seemed to confirm PI and MA as the beef cattle breeds with the highest genetic variation. Presence of private alleles was evidenced in each breed (Table 3). In particular, almost 23% of detected alleles belonged to one breed only - HF presented 7 private alleles, two of them showing a frequency of about 10% (allele 162 and 170 of TGLA122). Relatively high frequencies were found also for allele 156 of TGLA122 in the PI breed (21%) and for allele 229 of BM203 in BS (24%). It should also be noted that a high proportion (50%) of TGLA122 alleles were found to be private.

Finally, analyses of Wright's *F*-statistics over all loci and per breed were performed; results are shown in Table 4. Estimates revealed an average value of homozygote excess of 9.2% in the total sample due, for a large part, to the variation of gene frequencies among breeds (F_{ST} = 8.4%) and to a much lesser extent to homozygote excess within breeds (F_{IS} = 1%). In Table 4, estimates of the F_{IS} index in each breed and at each locus are shown. This index was called f_{IT} and revealed a low homozygote excess in the studied breeds over all loci, ranging from 0.001 in CH to 0.029 in BS. The exposed findings permitted to retain all loci for further analysis. Moreover, estimates of Wright's *F*-statistics are comparable with what found in a recent study by Ciampolini et al. (2006) on four Italian cattle breeds including CH and HF, although their estimations of homozygote excesses were higher in both breeds, possibly due to the use of different markers. Estimates of F_{ST} found in the present study showing that the genetic variability in the total sample accounted for only 8.4% to differences among breeds and for 91.6% to individual variability, were as well in accordance with what found in several studies on cattle breeds (Schmid, Saitbekova, Gaillard & Dolf, 1999; Kantanen et al., 2000; Cañón et al., 2001; Jordana et al., 2003). Differences in allelic frequencies resulted highly significant ($P < 0.0001$) among breeds as expected; the genetic differentiation (F_{ST}) between pair of breeds are shown in Table 5. The BS was the most differentiated breed while MA-PI (0.0326) and MA–CH (0.0332) couples were the most similar. Beef breeds are more similar among them than among dairy breeds, with the exception of the PI–HF pair; these results fell in the same range of values reported by Orrù et al. (2006) for some cattle breeds including PI, HF and CH.

Animal identification

Values of MP calculated using different marker sets are shown in Table 6 and in Table 7 for beef and dairy breeds respectively. In each studied breed MP values were computed considering firstly all twelve STRs, and then smaller sets, until finally considering only two markers. These sets were built choosing different numbers of the most polymorphic STRs in each breed.

Choosing only the five most polymorphic markers in each breed, the probability to find two animals sharing the same profile was, on average, five in one million. In the PI breed it was even lower, at approximately two in one million, while in BS this probability

was higher at almost ten in one million (Table 6 and 7). Such differences seemed to be due to the different allelic frequencies observed in the studied breeds which led to different population structure. In particular, it has already been mentioned that the genetic variation of BS was the lowest while that of PI the highest. In consideration that only about 50,000 MA and CH, 16,000 RO, and 200,000 PI animals were reared in Italy in 2005 (ANABIC, 2006; ANABORAPI, 2006), using only the four more polymorphic markers in each breed are sufficient to obtain a reliable individual genetic traceability system. It should be considered that ease of use must be achieved in order for such a genetic tracing system to be used as a routine procedure. For this reason, it would be beneficial to set up a STR set giving satisfactory results in all breeds as discussed, which would simplify the laboratory work and reduce the analyses costs. In Table 6 and 7, MP values obtained pooling the alleles in the total population are shown. These values were always higher than those considering the different breeds using 5 and 2 markers, with the exception of the PI breed which showed the same values using 5 STRs. In some cases, the pooled values were almost ten times higher; this was always true for the BS breed, which could be explained by the fact that BS is the most differentiated breed, as illustrated in Table 4 by F_{ST} estimates. Similar considerations arose also in the research performed by Orrù et al. (2006) using different STR sets. Additional sets of markers were built pooling the alleles in the dairy and beef populations. No significant differences were observed in the discrimination power of these sets for the beef breeds while the pooled dairy set gave satisfactory results, especially in BS. Once again these results are related to the population characterization; BS is more distant than HF from the beef breeds.

It is important to consider the number of slaughtered animals when choosing the adequate number of markers to analyze. In 2005, a total of about 4,1 millions animals were slaughtered in Italy: 2,5 million beef cattle and about other 1,6 million cows, bulls and veal, including dairy breed animals (ISTAT, 2006). Our findings showed that in these circumstances, a reliable animal identification is achieved using the 5 more polymorphic STR in each breed. Therefore, when a laboratory is asked to verify the exactness of the label information, only 5 loci are critically necessary for investigation if the breed of origin is known. This system requires the definition of different marker sets, one for each breed. A unique marker set must be identified to avoid the costs due

to the setting up of many different marker sets, and to achieve a greater ease in the laboratory work. This study showed that reliable results are obtained with a STR set consisting of 8 markers, found by pooling the alleles in the entire population (Table 6 and 7).

Conclusions

Two aspects are relevant when studying this topic: the knowledge of population variability for the breeds involved in the traceability system, which has been shown to affect markers discrimination; and the choice of the most adequate marker set, that must be done after analysis of markers' genetic variation and polymorphism. A practical application of such system should be complementary to the conventional traceability based on meat cut labeling. It could be useful when more powerful and secure identification is required, for example, in the recall of all animal cuts in case of health's risks. Finally, it is worth mentioning the relatively high economic costs of this tool which presently do not permit its use as a routine for all slaughtered animals.

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Tables

Table 1. Investigated STRs, primer sequence, location, annealing temperature and size of the amplified fragments.

Locus	Primer sequence	Chromosome	Annealing t°	Fragment size (bp)
BM1818	FW: AGCTGGGAATATAACCAAAGG RW: AGTGCTTTCAAGGTCCATGC	23	58°	257 - 279
ETH185	FW: TGCATGGACAGAGCAGCCTGGC RW: GCACCCCAACGAAAGCTCCCAG	17	64°	216 - 242
MM12	FW: CAAGACAGGTGTTTCAATCT RW: ATCGACTCTGGGGATGATGT	9	58°	108 - 134
TGLA126	FW: CTAATTTAGAATGAGAGAGGCTTCT RW: TTGGTCTCTATTCTCTGAATATTCC	20	58°	118 - 130
BM203	FW: GGGTGTGACATTTTGTTCCC RW: CTGCTCGCCACTAGTCCTTC	27	58°	207 - 237
TGLA122	FW: CCCTCCTCCAGGTAAATCAGC RW: AATCACATGGCAAATAAGTACATAC	21	58°	136 - 182
RM12	FW: CTGAGCTCAGGGGTTTTTGCT RW: ACTGGGAACCAAGGACTGTCA	7	58°	103 - 107
ILST008	FW: GAATCATGGATTTTCTGGGG RW: TAGCAGTGAGTGAGGTTGGC	14	60°	173 - 178
SPS115	FW: AAAGTGACACAACAGCTTCTCCAG RW: AACGAGTGTCCTAGTTTGGCTGTG	15	64°	247 - 261
BL42	FW: CAAGGTCAAGTCCAAATGCC RW: GCATTTTTGTGTTAATTTCATGC	13	58°	231 - 237
ETH3	FW: GAACCTGCCTCTCCTGCATTGG RW: ACTCTGCCTGTGGCCAAGTAGG	19	62°	98 - 126
TGLA53	FW: GCTTTCAGAAATAGTTTGCATTCA RW: ATCTTCACATGATATTACAGCAGA	16	62°	151 - 183

Table 2. Number of observed alleles per locus (N_a), observed (Ho) and expected (He) heterozigosity, and Polymorphism Information Content (PIC) for the twelve investigated STR loci.

Locus	N_a	Ho	He	PIC
BM1818	8	0.637	0.557	0.655
ETH185	14	0.738	0.666	0.828
MM12	12	0.754	0.605	0.73
TGLA126	7	0.736	0.596	0.695
BM203	14	0.663	0.563	0.713
TGLA122	18	0.698	0.631	0.759
RM12	3	0.411	0.326	0.326
ILST008	4	0.478	0.416	0.386
SPS115	8	0.649	0.531	0.641
BL42	4	0.439	0.373	0.426
ETH3	10	0.615	0.528	0.628
TGLA53	16	0.777	0.663	0.867
Mean ± SD[1]	9.8 ± 4.9	0.633 ± 0.126	0.538 ± 0.112	0.638 ± 0.172

[1]Standard Deviation

Table 3. Private alleles (frequencies in brackets), in the six cattle breeds: Chianina (CH), Marchigiana (MA), Romagnola (RO), Piemontese (PI), Holstein Friesian (HF), and Brown Swiss (BS). Alleles in bold presented frequencies higher than 0.10.

Locus	Breed					
	CH	MA	RO	PI	HF	BS
BM1818						279 (0.023)
ETH185		226 (0.019)		218 (0.045)	216 (0.019) 235 (0.093)	
MM12	122 (0.019)					
BM203					209 (0.034)	**229 (0.244)** 237 (0.081)
TGLA122	168 (0.054) 174 (0.036)		158 (0.022)	**156 (0.212)** 180 (0.030)	**162 (0.138)** **170 (0.103)** 182 (0.052)	154 (0.023)
RM12		107 (0.019)				
ILST008			173 (0.023) 178 (0.045)			
ETH3	106 (0.056)			98 (0.016) 120 (0.016)		
TGLA53	177 (0.019)			181 (0.016)	155 (0.034)	

Table 4. Wright's *F*-statistics computed for the six cattle breeds: Chianina (CH), Marchigiana (MA), Romagnola (RO), Piemontese (PI), Holstein Friesian (HF), and Brown Swiss (BS)

Locus				CH	MA	RO	PI	HF	BS
	F_{IT}	F_{ST}	F_{IS}	f_{IT}	f_{IT}	f_{IT}	f_{IT}	f_{IT}	f_{IT}
BM1818	0.069	0.037	0.033	-0.040	0.006	0.207	0.030	0.044	0.020
ETH185	0.144_b**	0.091	0.058	-0.068	0.136	0.151_b**	-0.012	0.162	0.022
MM12	0.011	0.049	-0.040	0.027	-0.047	-0.184	-0.020	-0.151	0.022
TGLA126	0.019	0.077	-0.063	-0.138	0.100	0.198_b**	0.033	-0.359_a**	-0.189
BM203	0.080	0.068	0.013	0.174	-0.206_a*	-0.011	0.034	0.062	0.022
TGLA122	0.139_b*	0.054	0.090	0.094_b*	0.044	-0.059	0.066	-0.017	0.287_b**
RM12	0.085	0.129	-0.050	-0.066	-0.063	-0.182	-0.054	-0.042	0.234
ILST008	0.064	0.035	0.029	0.245	-0.117	0.033	-0.030	0.017	0.040
SPS115	0.042	0.072	-0.032	-0.051	0.087	-0.016	-0.141	-0.085	0.010
BL42	0.176	0.175	0.001	-0.177	0.185	0.078	0.171	-0.155	-0.135
ETH3	0.101	0.083	0.019	0.013	-0.028	-0.011	0.088	0.062	-0.029
TGLA53	0.163	0.149	0.016	0.001	0.030	-0.037	0.062	-0.001	0.029_b*
Averages	0.092	0.084	0.009	0.001	0.013	0.020	0.022	-0.032	0.029

$_a$Test for heterozygote excess (*P < 0.05; **P < 0.01).

$_b$Test for heterozygote deficiency (*P < 0.05; **P < 0.01).

Table 5. F_{ST} estimates between the analyzed breeds: Chianina (CH), Marchigiana (MA), Romagnola (RO), Piemontese (PI), Holstein Friesian (HF), and Brown Swiss (BS)

Breed	CH	MA	RO	PI	HF
CH	-	-	-	-	-
MA	0.0332	-	-	-	-
RO	0.0483	0.0619	-	-	-
PI	0.0629	0.0326	0.0538	-	-
HF	0.0988	0.0652	0.0694	0.0508	-
BS	0.1432	0.1133	0.1285	0.1073	0.1009

Table 6. Match probability values considering different marker sets in the Chianina (CH), Marchigiana (MA), Romagnola (RO), and Piemontese (PI) beef breeds

N of loci	Breed											
	CH			MA			RO			PI		
	A[1]	B[2]	C[3]	A	B	C	A	B	C	A	B	C
12	3.576E-10	3.576E-10	3.576E-10	1.477E-10	1.477E-10	1.477E-10	1.321E-09	1.321E-09	1.321E-09	1.980E-11	1.980E-11	1.980E-11
8	2.346E-08	3.587E-08	2.346E-08	1.008E-08	1.219E-08	1.561E-08	6.430E-08	1.192E-07	1.573E-07	3.270E-09	3.270E-09	3.515E-09
5	5.482E-06	1.212E-05	7.845E-06	2.476E-06	6.504E-06	5.531E-06	5.011E-06	3.669E-05	7.711E-06	1.912E-06	1.912E-06	2.379E-06
2	4.853E-03	4.853E-03	4.853E-03	3.345E-03	3.719E-03	3.719E-03	3.448E-03	3.448E-03	3.448E-03	2.109E-03	3.873E-03	3.873E-03

[1]Markers were chosen according to their match probability values in the considered breed

[2]Markers were chosen according to their match probability values in the all population

[3]Markers were chosen according to their match probability values in the beef breed population

Table 7. Match probability values considering different marker sets in the Holstein Friesian (HF) and Brown Swiss (BS) dairy breeds.

N of loci	Breed					
	HF			BS		
	A[1]	B[2]	C[3]	A	B	C
12	9.093E-10	9.093E-10	9.093E-10	1.478E-08	1.478E-08	1.478E-08
8	4.198E-08	7.154E-08	5.943E-08	1.839E-07	2.875E-07	3.279E-07
5	3.737E-06	4.744E-06	4.744E-06	9.900E-06	1.200E-05	1.200E-05
2	1.933E-03	3.083E-03	7.064E-03	3.596E-03	2.763E-02	6.520E-03

[1]Markers were chosen according to their match probability values in the considered breed

[2]Markers were chosen according to their match probability values in the all population

[3]Markers were chosen according to their match probability values in the dairy breed population

References

ANABIC. 2006. Available: http://www.anabic.it. Accessed Aug. 22, 2007.

ANABORAPI. 2006. Available: http://www.anaborapi.it/att_lg-1.htm. Accessed Aug. 22, 2007.

Arana, A., Soret, B, Lasa, I., & Alfonso, L. (2002). Meat traceability using DNA markers: application to the beef industry. *Meat Science, 61*, 367-373.

Barendse, W., Vaiman, D., Kemp, S.J. et al. (1997) A medium-density genetic linkage map of the bovine genome. *Nature Genet.*, *6*, 227-235.

Belkhir, K., P. Borsa, J. Goudet, L. Chikri, & Bonhomme, F. (1998). GENETIX, logiciel sous Windows TM pour la génétique des populations. Laboratoire Génome et Populations, CNRS UPR 9060, Université de Montpellier II, Montpellier, France. Available: http//www.genetix.univ-montp2.fr/genetix.htm. Accessed Aug. 22, 2007.

Bishop, M.D., Kappes, S.M., Keele, J.W., Stone, R.T., Sunden, S.L.F., Hawkins, G.A., Toldo, S.S., Fries, R., Grosz, M.D., Yoo, J. & Beattie, C.W. (1994). A genetic linkage map for cattle. *Genetics*, *136*, 619-639.

Bjørnstad, G., & Røed, K.H. (2001). Breed demarcation and potential for breed allocation of horses assessed by microsatellites markers. *Animal Genetics, 32*, 59-65.

Cañón, J., Alexandrino, P., Bessa, I., Carleos, C., Carretero, Y., Dunner, S., Ferron, N., Garcia, D., Jordana, J., Laloë, D., Pereira, A., Sanchez, A. & Moazami-Goudarzi, K. (2001). Genetic diversity measures of local European beef cattle breeds for conservation purposes. *Genetic Selection Evolution, 33*, 311-332.

Ciampolini, R., Moazami-Goudarzi, K., Vaiman, D., Dillmann, C., Mozzanti, E., Foulley, J.L., Leveziel, H. & Cianci, D. (1995). Individual multilocus genotypes using microsatellite polymorphisms to permit the analysis of the genetic variability within and between Italian beef cattle breeds. *Journal of Animal Science, 73*, 3259-3268.

Ciampolini, R., Leveziel, H., Mozzanti, E., Grohs, C. & Cianci, D. (2000). Genomic identification of an individual or its tissue. *Meat Science, 54*, 35-40.

Ciampolini, R., Cetica, V., Ciani, E., Mozzanti, E., Fosella, X., Marroni, F., Biagetti, M., Sebastiani, C., Papa, P., Filippini, G., Cianci, D. & Presciuttini, S. (2006). Statistical analysis of individual assignment tests among four cattle breeds using fifteen STR loci. *Journal Animal Science, 84*, 11-19.

Cunningham, E.P., & Meghen, C.M. (2001). Biological identification systems: genetic markers. *Revue Scientifique et Technique (International Office of Epizootics, 20(2)*, 491-499.

European Commission (2000). Commission Regulation (EC) No. 1825/2000 of 25 August 2000 laying down detailed rules for the application of Regulation (EC) No 1760/2000 of the European Parliament and of the Council as regards the labelling of beef and beef products. *Official Journal of the European Communities, L216*, 8-12.

European Commission (2000). Council Regulation (EC) No. 1760/2000 of 17 July 2000 establishing a system for the identification and registration of bovine animals and regarding the labeling of beef and beef products and repealing Council Regulation (EC) No 820/97. *Official Journal of the European Communities, L204*, 1-10.

Goudet, J. (1995). FSTAT (version 1.2): a computer program to calculate F statistics. *Journal of Heredity, 86(5)*, 485-486.

Grosz, M.D., Solinas-Toldo, S., Stone, R.T., Kappes, S.M., Fries, R. & Beattie, C.W. (1997). Chromosomal localization of six bovine microsatellite markers. *Animal Genet*ics, *28*, 39-40*.*

ISAG/FAO Standing Committee (2004). Secondary guidelines for development of national farm animal genetic resources management plans. Measurement of domestic animal diversity (MoDAD): Recommended Microsatellite markers. Available: http://dad.fao.org/en/refer/library/guideline/marker.pdf. Accessed Aug. 22, 2007.

ISTAT (2006). Available: http://www.istat.it/agricoltura/datiagri/carnirosse/. Accessed Aug. 22, 2007.

Jobling, M.A., & Gill, P. (2004). Encoded evidence: DNA in forensic analysis. *Nature Reviews Genetics, 10*, 739-751.

Jordana, J., Alexandrino, P., Beja-Pereira, A., Bessa, I., Cañón, J., Carrettero, Y., Dunner, S., Laloë, D., Moazami-Goudarzi, K., Sanchez, A. & Ferrand, N. (2003). Genetic structure of eighteen local south European beef cattle breeds by comparative *F*-statistics analysis. *Journal of Animal Breeding and Genetics, 120*, 73-87.

Kantanen, J., Olsaker, I., Holm, L.E., Lien, S., Vilkki, J., Brusgaard, K., Eythorsdottir, E., Danell, B. & Adalsteinsson, S. (2000). Genetic diversity and population structure of 20 North European cattle breeds. *Journal of Heredity, 91,* 446-457.

Kemp, S.J., Brezinsky, L. & Teale, A.J. (1993). ILSTS008: a polymorphic bovine microsatellite. *Animal Genet*ics, *24*, 74.

Koskinen, M.T. (2003). Individual assignment using microsatellite DNA reveals unambiguous breed identification in the domestic dog. *Animal Genetics, 34*, 297-301.

Marshall, T.C., Slate, J., Kruuk, L.E.B. & Pemberton, J.M. (1998). Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology, 7*, 639-655.

Moioli, B., Napolitano, F. & Cavillo, G. (2004). Genetic diversity between Piedmontese, Maremmana, and Podolica cattle breeds. *Journal of Heredity, 95*, 250-256.

Orrù, L., Napolitano, F., Catillo, G. & Moioli, B. (2006). Meat molecular traceability: How to choose the best set of microsatellites? *Meat Science, 72*, 312-317.

Raymond, M. & Rousset, F. (1995). GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity, 86*, 248-249.

SanCristobal-Gaudy, M., Renand, G., Amigues, Y., Boscher, M.Y., Leveziel, H. & Bibé, B. (2000). Traçabilté individuelle des viandes bovine à l'aide de marqueurs génétiques. *INRA Production Animale, 13(4)*, 269-276.

Schmid, M., Saitbekova, N., Gaillard, C. & Dolf, G. (1999). Genetic diversity in Swiss cattle breeds. *Journal of Animal Breeding and Genetics, 116*, 1-8.

Vázquez, J.F., Pérez, T., Ureña, F., Gudín, E., Albornoz, J. & Domínguez, A. (2004). Practical application of DNA fingerprinting to trace beef. *Journal of Food Protection, 67(5)*, 972-979.

Weir, B.S. (1996). Genetic Data Analysis II. Sinauer, Sunderland, MA.

Weir, B.S. & Cockerham, C.C. (1984) Estimating *F*-statistics with special regard to system of mating. *Evolution, 38*, 1358-1370.

Third contribution

Breed assignment test in four Italian beef cattle breeds

Breed assignment test in four Italian beef cattle breeds

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Abstract

The assessment of a method able to assign individuals to the breed of origin is outstanding to certify origin and quality of livestock products. A set of twenty-one microsatellites was tested for breed identification in four native Italian beef breeds: Chianina, Marchigiana, Romagnola, and Piemontese. Two statistical approaches, based on maximum likelihood and on a Bayesian method, were evaluated. Different marker sets, chosen in order of the highest gene diversity and F_{ST} estimates were also tested. The Bayesian method performed always better, achieving a correct assignment rate of about 90% even with six microsatellites. The marker sets with the highest gene diversity showed to perform best. Considering a threshold probability of 90%, only 52.5% of the genotypes were correctly allocated. Such results are mainly due to the low genetic differentiation estimates among breeds (F_{ST} = 0.049). These findings suggest that markers with high gene diversity and presence of private alleles should be investigated and the Bayesian method should be used.

Keywords: beef breeds; assignment test; microsatellites

Introduction

Traceability is defined as a system able to maintain a credible custody of identification for animals or animal products through various steps within the food chain, from the farm to the retailer (McKean, 2001). Since 2005, traceability systems have become mandatory in the European Union countries, as useful tool to improve food safety and prevent frauds (E.R. 178/2002). In livestock production, such systems are needed to assign a product to the individual or the breed it belongs to. In fact, nowadays, breed names are increasingly used as brand names (Narrod & Fuglie, 2000) and many dairy and meat products are obtained from one breed only.

Assignment tests, based on molecular data, could be a trustful tool to verify the origin information, as suggested by Ciampolini et al. (2006) and Negrini et al. (2007). In particular, microsatellite markers (STR) have already been widely used for assignment tests in different species (Cañon et al., 2001; Álvarez, Royo, Fernández, Gutiérrez, Gómez & Goyache, 2004; Glowatzki-Mullis et al., 2005) evidencing their potential.

However, assignment tests can be performed by mean of a number of statistical tools. Cornuet, Piry, Luikart, Estoup and Solignac (1999) clustered statistical methods in two groups: the first, based on genetic distances, and the second, based on differences in allelic frequencies among the considered breeds. Many authors agree with the superiority of the second group where best results are usually obtained implementing a Bayesian approach (Cornuet et al., 1999; Talle, Fimland, Syrstad, Meuwissen & Klungland, 2005; Negrini et al., 2007).

This study aimed to test the efficacy of different subsets of STR markers for breed assignment in four Italian beef cattle breeds. Firstly, to test the markers, the reference population was analyzed. Secondly, pure breed individual genotypes were simulated and assignment tests were performed without including any prior information on their origins in the used algorithms. Several marker subsets were tested according to different criteria of variability with two assignment methods based on allelic frequencies to identify the most suitable.

Materials and Methods

Breeds

One hundred and seven individual blood samples were collected randomly in performance stations, including: Chianina (CHI, n = 28), Marchigiana (MAR, n = 27), Romagnola (ROM, n = 23), and Piemontese (PIE, n = 29). Samples of CHI, MAR, and ROM were collected from the ANABIC breeder association (Perugia, central Italy), while samples of PIE derived from the ANABORAPI breeder association (Cuneo, north-west Italy). The studied breeds are the most important Italian beef cattle breeds. Piemontese is the most reared, with more than 200,000 animals enrolled in the Italian Herd Book (ANABORAPI, 2006). It is mainly found in north-west Italy (Piemonte region) were the majority of the herds are located. It is highly specialized for beef production due to double-muscling, inducted by a specific mutation of myostatin gene (Grobet et al., 1998). Chianina, MAR, and ROM originated in central Italy were they are reared also nowadays. Their consistency ranges from about 17,000 individuals for ROM to almost 50,000 for MAR (ANABIC, 2006) and their meat is protected by the PGI European label "Vitellone bianco dell'Appenino Centrale". Among them the MAR breed is the "youngest"

as in the past it was crossed with both CHI and ROM to improve its productive characteristics and only since 1928 outcross was stopped and its own selection has started (ANABIC, 2007).

Microsatellite analysis

Individual blood samples were collected in 5 ml vacutainer tubes containing sodium citrate, and stored at -20°C. DNA extraction was carried out employing the "Gentra System PUREGENE DNA purification kit" starting from 300 µl of whole blood. DNA samples were amplified by PCR in correspondence of the following 21 STR loci: BM1818, ETH185, MM12, TGLA126, BM203, TGLA122, RM12, ILST008, SPS115, BL42, ETH3, TGLA53, INRA006, INRA64, INRA016, ETH152, CSSM14, BM1824, TGLA57, ETH10, and ETH225. STR were chosen in accordance to ISAG/FAO Standing Committee Recommendations (12 loci are listed among the recommended for cattle), and consulting previous studies, in order to have high polymorphic markers spread all over the genome. Markers were analyzed following different procedures. The first group was composed by BM1818, ETH185, MM12, TGLA126, BM203, TGLA122, RM12, ILST008, SPS115, BL42, ETH3, TGLA53. For the amplification, 25 ng of DNA were added to a reaction mix containing: 1 pmol/µl of primer forward and reverse, 1X PCR buffer (16 mM $(NH_4)_2SO_4$, 67 mM Tris HCl pH 8.8, 0.01% Tween 20), 0.26 mM of every dNTPs, 2.5 mM of $MgCl_2$ and 0.8 U of *Taq* DNA polymerase, in a final volume of 20 µl. For the amplification of STR in the second group, 50 ng of DNA were added to a reaction mix containing: 1 pmol/µl of primer forward and reverse, 1X PCR buffer (16 mM $(NH_4)_2SO_4$, 67 mM Tris HCl pH 8.8, 0.01% Tween 20), 0.17 mM of every dNTPs, 1.75 mM of $MgCl_2$ and 1.5 U of *Taq* DNA polymerase, in a final volume of 25 µl. The 21 STR were individually analyzed by a PX2 Thermohybaid thermal cycle at the following conditions: initial denaturation step of 5 min at 95°C, 40 cycles of 30 s at 94°C, 1 min at the primer specific annealing temperature and 1 min at 72°C followed by a final extension of 10 min at 72°C, for STR of the first group and, an initial denaturation step of 5 min at 94°C, 30 cycles of 45 s at 94°C, 45 s at the primer annealing temperature and 30 s at 72°C followed by a final extension of 10 min at 72°C, for the second group. Allele size was determined with a Perkin Elmer ABI Prism 3730XL Genetic Analyzer, using

GeneScan 2.0 and Genotyper 3.7 software (Perkin Elmer) and with a CEQ™ 8000 Genetic Analysis System (Beckman Coulter).

Statistical analysis

Genetic variability of markers and breeds was analyzed using the following software packages. The Fstat 2.9.3 program (Goudet, 1995) was employed in calculations of allelic frequencies, mean number of alleles, gene diversity (Nei, 1987), and *F*-statistics estimates (Weir & Cockerham, 1984) per locus, per breed, and in the total sample. Tests for deviation from Hardy-Weinberg (H-W) equilibrium and for population differentiation were performed by the GENEPOP 3.4 software (Raymond & Rousset, 1995). For the H-W equilibrium estimation, per locus and per breed, the exact test of Guo and Thompson (1992) was performed. Once a deviation at some loci was evidenced a more powerful test was applied to evaluate heterozygote deficiency and excess following the suggestions of Rousset and Raymond (1995). Test for population differentiation was computed as suggested by Raymond and Rousset (1995); for each locus an unbiased estimate of the Fisher's exact test was performed to verify if the allelic distribution was different among breeds.

The STRUCTURE software (Pritchard, Stephens & Donnelly, 2000) was used to analyze the genetic structure of the reference population. This program implements a model-based clustering method for inferring population structure using genotype data of unlinked markers; here it was used to assign individuals to population. As suggested by Pritchard et al. (2000), analyses were performed by means of the admixture model with correlated allele frequencies. To choose the appropriate number of inferred clusters to model the data, 1 to 7 inferred clusters were performed with 3 independent runs each as suggested by Pritchard et al. (2000) and by other authors (Álvarez et al., 2004; Glowatzi-Mullis et al., 2005). All analyses used a burn-in period of 50,000 and 200,000 iterations for data collection. Once verified that the number of inferred clusters corresponded to the number of breeds included in the dataset, multilocus genotype of 30 animals for each of the 4 breeds were generated through the use of HYBRIDLAB 1.0 (Nielsen, Arve Bach & Kotlicki, 2006). The simulated genotypes were added to the reference population to estimate the correct assignment rate. Prior information on the breed of origin was added only for the reference population while no a priori

information was used for the simulated genotypes. Several marker subsets were tested, considering a decreasing number of markers. STR subsets were assessed choosing the markers according to their gene diversity and F_{ST} differentiation index estimates.
Simulated genotypes were assigned also using the frequency method based on maximum likelihood presented by Paetkau, Calvert, Stirling and Strobeck (1995) and implemented in the GeneClass2 software (Piry, Alapetite, Cornuet, Paetkau, Baudouin & Estoup 2004). No prior information was added for the simulated genotypes and the probability of each individual to belong to each population was computed with a Monte-Carlo resampling using Paetkau, Slade, Burden and Estoup (2004) algorithm and simulating 10,000 individuals. The same STR subsets used for the Bayesian method were utilized also in this case.

Results

Variability of microsatellites markers and analysis of the reference population

Analysis of the 21 STR allowed the detection of 173 alleles with an average of 8.2 alleles per locus. The greatest number of alleles per locus was found in TGLA122 and TGLA53 (14) while the lowest was evidenced in RM12 (3) (Table 1). About 23% of the observed alleles were private, their presence was detected in each breed and PIE showed the highest number (37.5%). However, frequencies of private alleles were low, with the exception of allele 8 of TGLA122 which exhibited a frequency of 0.224 in the PIE breed (data not shown). The marker gene diversity ranged from 0.214 (CSSM14) to 0.821 (TGLA53), as shown in Table 1 and the loci differentiation, measured by F_{ST}, varied between -0.004 (CSSM14) and 0.113 (TGLA57 and BL42). Table 1 shows also the two marker rankings, based on their expected heterozygosity and F_{ST} estimates. The ranking criteria lead to different results meaning that markers owing a great variability across breeds may have a little variation within them. Genetic distances, measured by pair-wise F_{ST}, are shown in Table 2. They revealed the CHI-ROM pair as the most differentiated (0.064) while the least was the MAR-PIE couple (0.035).Test for population differentiation, evidenced a significant ($P < 0.001$) difference in the allelic frequencies of each pair of breeds. Estimates of Wright's *F*-statistics revealed a moderate-low homozygote excess in the whole sample (F_{IT} = 8.8%), due in part to the

variation of allelic frequencies among breeds (F_{ST} = 4.9%) and in part to the homozygote excess within breed (F_{IS} = 4.1%) (data not shown). Analysis of homozygote excess performed at breed level, revealed a significant excess at two loci: INRA006 ($P < 0.001$) and INRA064 ($P < 0.05$) in three breeds; however, this disequilibrium did not affect the clustering and the assignment test (data not shown) so all loci were retained for further analysis. Table 3 shows the results of the analysis performed with STRUCTURE in the reference population. No information on the population of origin was used to assist clustering procedure. The software detected the presence of 4 clusters, each of them was mainly associated to one breed; CHI was the one showing the highest proportion of membership with its cluster (0.898) while MAR the lowest (0.708). When prior information on the population origin was added the proportion of membership of each pre-defined cluster increased in every breed ranging from 0.986 (MAR and ROM) to 0.994 (CHI) (data not shown).

Assignment of simulated animals with 21 microsatellites

Using the maximum likelihood method implemented in GeneClass2, 113 simulated genotypes out of 120 were correctly assigned (94.2%). The rate of correct assignment ranged from 100% in ROM and PIE to 83.3% in MAR. A percentage of 6.7 simulated CHI animals were assigned to PIE while 16.7% of MAR individuals were not assigned to any breed. No individual was correctly assigned with a certainty of 99% and only 5% of the total sample was correctly assigned considering a 95% threshold, none of them belonged to the MAR breed. The average probabilities of correct assignment per breed were quite low in all the studied breeds ranging from 59.6% in PIE to 36.5% in MAR. Use of the Bayesian algorithm implemented in the STRUCTURE software permitted to achieve better results. The 30 simulated genotypes per breed were added to the reference population dataset and no prior information on the breed of origin was given. The rate of correct assignment was 97.5% and varied from 100% (ROM and CHI) to 93.3% (MAR). In this case, two MAR simulated genotypes were wrongly assigned, one to ROM and one to PIE while, one PIE individual was considered as belonging to the CHI breed. However, also the Bayesian algorithm was not able to assign any individual with a certainty of 99% and only 5% with a probability of 95% (like GeneClass2). More satisfactory results were obtained considering a 90% threshold which permitted the

assignment of 52.5% of the entire sample (which was only 10% using the maximum likelihood approach). The average proportion of memberships of each breed to its cluster were high ranging from 89% (ROM) to 83.3% (MAR).

Assignment of simulated animals with different marker sets

Figure 1 shows the variation of the correct assignment rate using less than 21 STR. Markers were chosen according to their gene diversity and both GeneClass2 and STRUCTURE software were used. The Bayesian algorithm gave the best results being able to assign correctly more than 90% of simulated genotypes even with 6 markers. Using a lower number of microsatellites the assignment rate decreased quickly arriving to 53% analyzing only the most heterozygous marker. Figure 2 shows the correct assignment rate when marker sets were chosen according to their F_{ST} estimates. Also in this case STRUCTURE performed better than GeneClass2; however, the gene diversity ranking seemed to perform better. When using 9 or more markers results are comparable but when analyzing less, the assignment rate dropped. This trend seems to be more accentuated when applying the Bayesian method.

In assignment tests also the probability of correct assignment is important. Figure 3 shows the trend of correct assignment rate using different marker sets and applying the Bayesian algorithm with a probability of 90%. The trend is different in each breed, MAR is the breed evidencing the worst correct assignment rate while CHI and ROM the best. As expected the assignment rates decreased when using less markers, in particular when using less than 17 STR. When using only the 17 most heterozygous markers, 50% of the all simulated genotypes was correctly assigned.

Discussion

Analysis of the reference population

Breed discrimination is a topic several authors have considered owing to its relevance in conservation studies (Álvarez et al., 2004; Baumang, Cubric-Curik, Schwend, Achmann & Sölkner 2006) and to its possible use as a secure tool in traceability systems for livestock products (Ciampolini, Leveziel, Mozzanti, Grohs, & Cianci, 2000; Maudet and Taberlet, 2002; García et al., 2006; Negrini et al., 2007). This study was meant to

check the efficacy of a set of 21 STR for assignment test in a reference population composed of four beef breeds, to simulate individual genotypes and to perform assignment tests with a decreasing number of markers. In fact, as suggested by Rosenberg et al. (2001), the first step for a real and practical use of breed or population assignment methods, is to verify the suitability of collected samples to be used as reference population. To this purpose, the property of collected samples to cluster together, according to the breed designations, was checked before using the analyzed samples as reference. In the present study, the STRUCTURE software detected 4 clusters corresponding to the 4 studied breeds without using prior information. Such result allowed us to assume that the analyzed animals were a good representative sample of their breed of origin and therefore they could be used as reference population. Moreover, the good clustering ability suggested that the chosen markers were appropriate for this study evidencing a good discrimination power. In literature, most studies used a number of STR markers ranging from 20 to 27 showing expected heterozygosity estimates comparable with the present study (Bjørnstad and Røed, 2002; Talle et al., 2005; Rosenberg et al., 2001) or slightly higher (Moioli, Napolitano, & Catillo 2004; Baumung et al., 2006). However, such results depend on the studied population and on their variability. The lowest proportion of membership to its cluster, was found in the MAR breed; such finding was expected as selection on this breed has started only in 1928, and in the past it has been crossed with CHI and ROM (ANABIC, 2007). Confirmation of the existence of such crosses can be found in our analysis where a moderate proportion of MAR genome (14%) was assigned to the CHI cluster. For the same reason, simulated MAR genotypes showed the lowest correct assignment rate (Figure 3). Ciampolini et al. (2000), in a study on breed assignment tests on the same breeds, showed as well MAR to be the breed evidencing the lowest correct assignment rate, even using a different statistical approach. A better clustering could probably be achieved using more markers, even if these results seem to be more likely related to the weak differentiation among the studied breeds (F_{ST} = 0.049), than to insufficient marker information. In fact, Bjørnstad and Røed (2002), has already demonstrated that genetic differentiation affects individual assignment rate. Anyway, in a study performed by Latch, Dharmarajan, Glaubitz and Rhodes Jr. (2006) on clustering software based on a Bayesian method today available, STRUCTURE showed to be the more appropriate

even with low level of differentiation and, for this reason, it has been adopted in this study. Other researches on Italian cattle breeds evidenced higher F_{ST} level of differentiation (Moioli et al., 2004; Ciampolini et al., 2006; Negrini et al., 2007) though, they included in the dataset also dairy or foreign breeds while, the ones considered here, are all beef breeds and, except for PIE, they originated in the same area. The different origin of PIE is also demonstrated by its high proportion of private alleles. However, as private allele frequencies were low, they could depend only on the number of sampled animals and further analyses are needed to confirm such presence. Nevertheless, PIE highlighted the highest number of private alleles also in the study performed by Ciampolini et al (2000) investigating a different marker set. Indeed, as suggested by Rosenberg et al. (2001) and Bjørnstad and Røed (2002), it is likely to happen that some individuals will never be assigned with high proportion of membership because they are genetically atypical for their breeds.

Assignment of simulated genotypes

Assignment of simulated genotypes by means of STRUCTURE gave the best results even with less than 21 markers, as found in other studies (Cornuet et al., 1999; Koskinen et al., 2003; Negrini et al., 2007). The correct assignment rate using the complete STR set was slightly higher than what found by Negrini et al. (2007) in a study on breed assignment in Italian cattle breeds and comparable with the findings obtained by Ciampolini et al. (2000). Moreover, Negrini et al. (2007), also found CHI and ROM to be the breeds with the best assignment rate, according to the present study. The overall assignment rate obtained in our study is also in agreement with what found by Latch et al. (2006). In fact, they showed that in a simulated population, with F_{ST} = 0.05, 3% of individuals were misassigned and, to obtain better accuracy a higher breed differentiation is needed.

However, when applying a genetic traceability system in order to detect possible frauds, it is not enough to know which breed a sample is assigned to, but also with which probability. In fact, as shown in Figure 1 and 2, high correct assignment rate could be achieved even with small marker subsets. Investigating just 6 STR and implementing the Bayesian statistical approach, the obtained correct assignment rate was comparable with what found by Ciampolini et al. (2000) using 20 STR and considering the same

cattle breeds. Anyway, in this case the assignment probability was low. Considering a 90% threshold only 52.5% of the samples were correctly assigned, and this percentage varied according to the breed, being always higher for CHI and ROM. Such results are not satisfactory enough. Moioli et al. (2004), using 21 different STR, were able to assign correctly, with a 90% threshold, 82% of the PIE samples they analyzed, a higher percentage than what found in our study (56.7%). Anyway, such result seemed to be due to the higher breed differentiation (F_{ST} = 0.06). Maudet, Luikart and Taberlet (2002) considered individual assignment tests in seven French breed and were able to assign correctly 67% of their samples with a threshold of 95%. In their study, the genetic differentiation among the analyzed breeds was twice the one found here. The cited research performed by Ciampolini et al. (2000), did not mention any assignment threshold. In conclusions, it seemed that the low correct assignment rate when considering high threshold, owed to the weak differentiation among breeds rather than to the statistical treatment that was very powerful. Actually, the present results confirm those of Bjørnstad and Røed (2002), breed differentiation is the most critical factor for assignment precision. Anyway, it must be underlined that there is no need to be able to trace back to the breed level all the livestock products. For example, a genetic traceability system could be of practical use to distinguish between meat obtained from CHI, MAR, and ROM, from the one obtained from other breeds. In fact, CHI, MAR, and ROM beef, is protected by a PGI European label showing a higher price and, the beef industry could be interested in developing new techniques to certify the breed of origin. The STR set used in the present study, was able to discriminate among PIE and the other three breeds very well even with 15 markers. In fact only one PIE genotype was incorrectly assigned to CHI with probability lower than 60%. Analysing less markers misassignment increased and using six markers 2 CHI and 1 ROM genotypes were wrongly allocated to PIE even if with moderate low probability (46 – 53%).

Regarding the choice of STR markers, our findings showed that ranking on locus gene diversity improved the breed assignment, meaning that loci that vary highly among breeds but not within them are the most suitable, according to Rosenberg et al. (2001) and Bjørnstad and Røed (2002). Thus, the presence of private alleles are ideal for bred assignments suggesting that the most suitable STR should be monomorphic within breed but polymorphic among them.

Conclusion

The presented results evidenced that the statistical approach based on the Bayesian algorithm, together with the choice of STR showing high gene diversity, enabled the achievement of the highest correct assignment rate. These suggestions are of practical use and could be extended to every species or breed for which a discrimination system is desired. Moreover, these findings showed that the implementation of a trustful traceability system does not depend only on the choice of the most appropriate markers but, it is strongly influenced by the population one is interested to trace. Breed characterized by a weak genetic differentiation are more difficult to discriminate even using the most appropriate marker set and clustering algorithm. The marker set utilized in the present study permitted to achieve very high correct assignment rate even with 6 markers but the assignment probabilities were not satisfactory enough. Our results suggested that, when aim is to distinguish among strongly related breeds, future research should focus on the detection of diagnostic markers showing presence of private alleles.

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Tables

Table 1.: Number of alleles per locus, gene diversity, and F_{ST} estimates of the 21 STR markers and rankings of markers according to gene diversity and F_{ST}.

Locus	N° of alleles	Gene diversity	Rank based on Gene diversity	F_{ST}	Rank based on F_{ST}
INRA006	11	0.604	16	0.042	10
INRA64	6	0.532	17	0.017	19
INRA016	10	0.756	4	0.019	18
ETH152	6	0.708	8	0.100	3
CSSM14	5	0.214	21	-0.004	21
BM1824	8	0.704	9	0.057	6
TGLA57	9	0.719	6	0.113	1
ETH10	7	0.627	14	0.033	14
ETH225	7	0.686	11	0.027	16
BM1818	7	0.692	10	0.025	17
ETH185	12	0.811	2	0.048	8
MM10	10	0.717	7	0.039	12
TGLA126	7	0.765	3	0.040	11
BM203	11	0.626	15	0.037	13
TGLA122	14	0.730	5	0.052	7
RM12	3	0.461	20	0.059	5
ILST008	4	0.517	18	0.002	20
SPS115	8	0.649	13	0.031	15
BL42	4	0.468	19	0.113	2
ETH3	10	0.657	12	0.085	4
TGLA53	14	0.821	1	0.048	9

Table 2.: Genetic distances measured by pair-wise F_{ST} between the studied breeds (CHI = Chianina, MAR = Marchigiana, ROM = Romagnola, and PIE = Piemontese). All estimates differ significantly ($P < 0.001$).

	MAR	ROM	PIE
CHI	0.044	0.064	0.051
MAR		0.062	0.035
ROM			0.043

Table 3.: Proportion of membership of the studied breeds (CHI = Chianina, MAR = Marchigiana, ROM = Romagnola, PIE = Piemontese) to the four detected clusters.

	1	2	3	4
CHI	0.898	0.026	0.022	0.054
MAR	0.141	0.086	0.065	0.708
ROM	0.093	0.055	0.788	0.064
PIE	0.066	0.805	0.054	0.075

Figures

Figure 1.: Correct assignment rate as a function of the number of STR markers used. Marker sets were chosen in order of highest gene diversity.

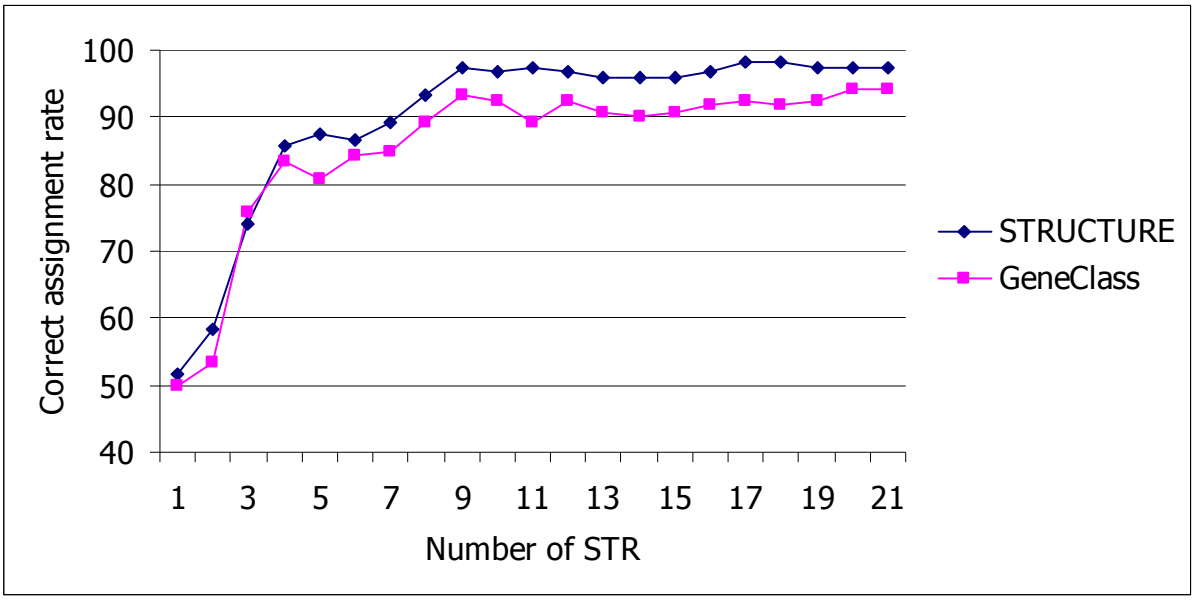


Figure 2.: Correct assignment rate as a function of the number of STR markers used. Marker sets were chosen in order of highest F_{ST} estimates.

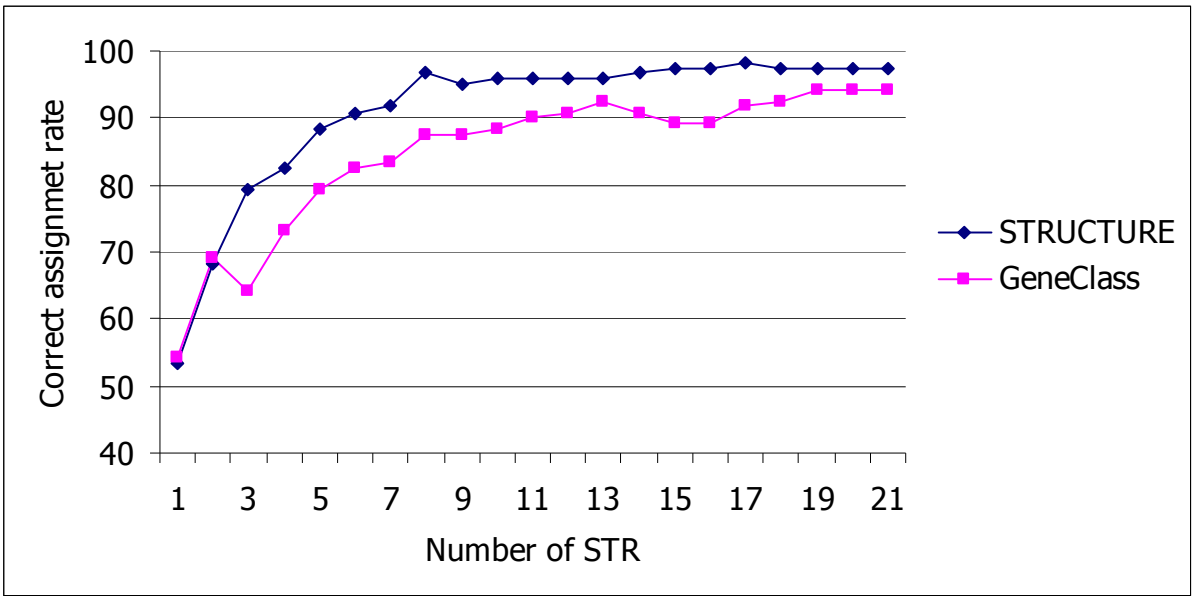
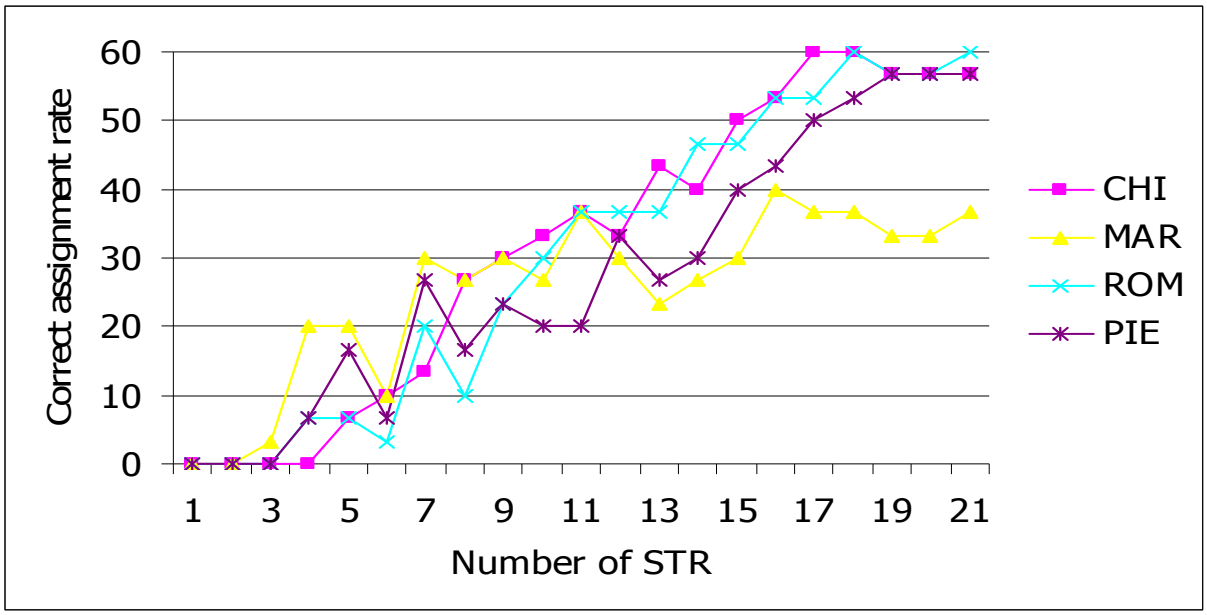


Figure 3.: Correct assignment rate as a function of the number of STR markers used with a probability higher than 90% in the 4 breeds (CHI = Chianina, MAR = Marchigiana, ROM = Romagnola, PIE = Piemontese). Markers were chosen in order of highest gene diversity and the Bayesian algorithm was applied.



References

Álvarez, I., Royo, L.J., Fernández, J., Gutiérrez, J.P., Gómez, E., & Goyache, F., (2004). Genetic relationships and admixture among sheep breeds from Northern Spain assessed using microsatellites. *Journal of Animal Science, 82*, 2246-2252.

Associazione Nazionale Allevatori Bovini da Carne (A.N.A.B.I.C.), (2006). Available at http://www.anabic.it. Visited 09/10/2007

Associazione Nazionale Allevatori Bovini di Razza Piemontese (A.N.A.BO.RA.PI.), (2006). Available at http://www.anaborapi.it/Pan-Zootc.htm. Visited 09/10/2007

Baumung, R., Cubric-Curik, V., Schwend, K., Achmann, R., & Sölkner, J., (2006). Genetic characterisation and breed assignment in Austrian sheep breeds using microsatellite marker information. *Journal of Animal Breeding and Genetics, 123,* 265-271.

Bjørnstad, G., & Røed, K.H., (2002). Evaluation of factors affecting individual assignment precision on using microsatellite data from horse breeds and simulated breed crosses. *Animal Genetics, 33*, 264-270.

Cañón, J., Alexandrino, P., Bessa, I., Carleos, C., Carretero, Y., Dunner, S., Ferron, N., Garcia, D., Jordana, J., Laloë, D., Pereira, A., Sanchez, A., & Moazami-Goudarzi, K., (2001). Genetic diversity measures of local European beef cattle breeds for conservation purposes. *Genetics, Selection Evolution, 33,* 311-332.

Ciampolini, R., Leveziel, H., Mozzanti, E., Grohs, C., & Cianci, D. (2000). Genomic identification of the breed of an individual or its tissue. *Meat Science*, *54*, 35-40.

Ciampolini, R., Cetica, V., Ciani, E., Mozzanti, E., Fosella, X., Marroni, F., Biagetti, M., Sebastiani, C., Papa, P., Filippini, G., Cianci, D., & Presciuttini, S. (2006). Statistical analysis of individual assignment tests among four cattle breeds using fifteen STR loci. *Journal of Animal Science, 84,* 11-19.

Cornuet, J.M., Piry, S., Luikart, G., Estoup, A., & Solignac, M., (1999). New methods employing multilocus genotypes to select or exclude populations as origins of individuals. *Genetics*, 153, 1989-2000.

European Commission, (2002). Council Regulation (EC) No 178/2002 of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. *Official Journal of the European Communities, L 31*, 1-24.

García, D., Martínez, A., Dunner, S., Vega-Pla, J.L., Fernández, C., Delgado, J.V., & Cañón, J. (2006). Estimation of the genetic admixture composition of Iberian dry-cured ham samples using DNA multilocus genotypes. *Meat Science, 72*, 560-566.

Glowatzki-Mullis, M.L., Muntwyler, J., Pfister, W., Marti, E., Rieder, S., Poncet, P.A., & Gaillard, C., (2005). Genetic diversity among horse population with a special focus on the Franches- Montagnes breed. *Animal Genetics, 37,* 33-39.

Goudet, J., (1995). FSTAT (version 1.2): a computer program to calculate F statistics. *Journal of Heredity, 86(5),* 485-486.

Grobet, L., Poncelet, D., Royo, L.J., Brouwers, B., Pirottin, D., Michaux, C., Ménissier, F., Zanotti, M., Dunner, S., & Gerorges, M., (1998). Molecular definition of an allelic series of mutations disrupting the myostatin function and causing double-muscling in cattle. *Mammalian Genome, 9,* 210-213.

Guo, S.W., & Thompson, E.A., (1992). Performing the exact test of Hardy-Weinberg proportion for multiple alleles. *Biometrics 48*, 361-372.

ISAG/FAO Standing Committee, (2004). Secondary guidelines for development of national farm animal genetic resources management plans. Measurement of domestic animal diversity (MoDAD): Recommended Microsatellite markers. Available at: http://dad.fao.org/en/refer/library/guideline/marker.pdf. Visited 09/10/2007

Koskinen, M., (2003). Individual assignment using microsatellite DNA reveals unambiguous breed identification in the domestic dog. *Animal Genetics, 34,* 297-301.

Latch, E.K., Dharmarajan, G., Glaubitz, C., & Rhodes Jr., O.E., (2006). Relative performance of Bayesian clustering software for inferring population substructure and individual assignment at low levels of population differentiation. *Conservation Genetics, 7,* 295-302.

Maudet, C., Luikart, G., & Taberlet, P., (2002). Genetic diversity and assignment tests among seven French cattle breeds based on microsatellite DNA analysis. *Journal of Animal Science, 80,* 942-950.

Maudet, C., & Taberlet, P., (2002). Holstein's milk detection in cheeses inferred from melanocortin receptor 1 (*MC1R*) gene polymorphism. *Journal of Dairy Science, 85*, 707-715.

McKean, J.D. (2001). The importance of traceability for public health and consumer protection. *Revue scientifique et technique (International Office of Epizootics), 20,* 363-371.

Moioli, B., Napolitano, F., & Catillo, G., (2004). Genetic diversity between Piedmontese, Maremmana, and Podolica cattle breeds. *Journal of Heredity, 95,* 250-256.

Narrod, C.A., & Fuglie K.O. (2000). Private sector investment in livestock business. *Agribusinness, 4,* 347-354.

Negrini, R., Milanesi, E., Colli, L., Pellecchia, M., Nicoloso, L., Crepaldi, P., Lenstra, J.A., & Ajmone-Marsan, P., (2007). Breed assignment of Italian cattle using biallelic AFLP markers. *Animal Genetics, 38,* 147-153.

Nei, M., (1987). *Molecular Evolutionary Genetics*. New York: Columbia University Press.

Nielsen, E.E., Arve Bach, L., & Kotlicki, P., (2006). HYBRIDLAB (version 1.0): a program for generating simulated hybrids from population samples. *Molecular Ecology Notes, 6,* 971-973.

Paetkau, D., Calvert, W., Stirling, I., & Strobeck, C., (1995). Microsatellite analysis of population structure in Canadian polar bears. *Molecular Ecology, 4,* 347–354.

Paetkau D., Slade, R., Burden, M., & Estoup, A., (2004). Genetic assignment methods for the direct, real-time estimation of migration rate: a simulation based exploration of accuracy and power. *Molecular Ecology, 13,* 55–65.

Piry, S., Alapetite, A., Cornuet, J.M., Paetkau, D., Baudouin, L., & Estoup, A., (2004). GENECLASS2: a software for genetic assignment and first-generation migrant detection. *Journal of Heredity, 95,* 536-539.

Pritchard, J.K., Stephens, M., & Donnelly, P., (2000). Inference of population structure using multilocus genotype data. *Genetics, 155,* 945-959.

Raymond, M., & Rousset, F., (1995). GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity, 86,* 248-249.

Rosenberg N.A., Burke, T., Elo, K., Feldman, M.W., Freidlin P.J., Groenen, M.A.M., Hillel, J., Mäki-Tanila, A., Tixier-Boichard, M., Vignal, A., Wimmers, K., & Weigend, S., (2001). Empirical evaluation of genetic clustering methods using multilocus genotypes from 20 chicken breeds. *Genetics, 159,* 699-713.

Rousset, F., & Raymond, M., (1995). Testing heterozygote excess and deficiency. *Genetics 140,* 1413-1419.

Talle, S.B., Fimland, E., Syrstad, O., Meuwissen, T., & Klungland, H., (2005). Comparison of individual assignment methods and factors affecting assignment success in cattle breeds using microsatellites. *Acta Agriculturae Scandinavae, 55,* 74-79.

Weir, B.S., & Cockerham, C.C., (1984). Estimating *F*-statistics with special regard to system of mating. *Evolution 38,* 1358-1370.

Fourth contribution

Genetic characterization of the Burlina cattle breed using microsatellites markers

Genetic characterization of the Burlina cattle breed using microsatellites markers

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Summary

The present study is a contribution on the genetic characterization of the Burlina local cattle breed, and an approach to understanding the relationships between Burlina, Holstein Friesian and Brown Swiss which represent the majority of the dairy cattle reared nowadays in North-East Italy. The obtained results helped to clarify the genetic diversity and distinctiveness of Burlina population. In particular the low genetic distance between Burlina and Holstein Friesian, and the assignment of a moderate percentage of Burlina animals to Holstein Friesian, suggested that crosses between them took place in the past, while crosses with Brown Swiss seemed to be less frequent. However, analyses of marker genotypes, showed a cluster with only Burlina individuals, which demonstrates the genetic distinctness of this breed. The Burlina breed showed the highest variability among the analyzed breeds and its inbreeding coefficient was low. The data contribute to the feasibility of a conservation and selection program for this breed and the results are useful for the implementation of a conservation strategy that should aim to conserve animals where the contribution from foreign breeds is as small as possible.

Key words: Microsatellite, genetic characterization, genetic diversity, dairy cattle, Burlina.

Introduction

The Burlina is a native, Italian, dairy cattle breed reared in North-East Italy; it is a small sized animal with black spotted coat, well adapted to difficult environmental conditions as marginal mountain areas, thanks to its good grazing characteristics. The origin of Burlina is still uncertain, the most probable hypothesis is that of Chiodi (1965), who suggested that Burlina came from the Jutland peninsula and arrived in North Italy with the Cimbri population in the XIth century. Burlina spread mainly in the mountain area of Veneto region (Del Bo et al., 2001) where it has always been reared and appreciated by local farmers. Despite that, the Burlina population had almost disappeared during the First World War and later, during the 1930 – 40's, several actions were carried out to replace it with the more productive and cosmopolitan Brown Swiss (BSW) and Holstein Friesian (HFR). The number of reared animals drastically decreased from 15,000 in

1930 to 2,300 in 1972 (Santomaso, 2006). In 1980, Burlina has been enrolled in the Italian Herd Book of local breeds. Nowadays, about 350 cows are registered in the Italian Herd Book, most of them located in the Treviso and Vicenza provinces of North-East Italy (AIA, 2006). Several actions have been developed to safeguard the Burlina breed, especially in the 1980's, aiming to increase the number of reared animals and the within population variability and to minimize the crosses with other breeds.

Recently, interest in Burlina has increased because it can exploit mountain pasture better than BSW and HFR, and thus is more able to preserve pastures in the unstable and fragile mountain environment (Cozzi et al., 2004). In less productive farms, Burlina can achieve higher production than HFR which requires higher inputs to perform optimally (Bittante et al., 1992). In addition, traditionally, from Burlina milk a typical cheese called Morlacco has been produced; it is a raw whole milk cheese preferably obtained from cows at pasture, to achieve a typical herbs flavor as described by Favaro et al. (2005) for the Asiago mountain cheese. The strong link among product, breed, and environment, could be a starting point for implementing a conservation program for Burlina, especially in its native area where it can perform at its best and its breeding could become profitable and competitive. In any case, the first step towards the definition of a conservation program is the understanding of the genetic structure of Burlina in the present population.

The present study aimed to characterize the Burlina breed and to investigate its genetic distinctness compared with the two cosmopolitan dairy breeds, HFR and BSW, which, at present, are the most popular ones in North-East Italy. To this purpose, a set of 12 microsatellite markers were investigated; they are particularly suitable for these studies and have been used in many different species like pig (*Sus scrofa*, Laval et al., 2000), donkey (*Equus asinus*, Jordana et al., 2001), sheep (*Ovis aries*, Moioli et al., 2006) and cattle (*Bos taurus*, Rendo et al., 2004), thanks to the high number of alleles and variability. The comprehension of the relationships among these breeds will help both to clarify the genetic distinctness of Burlina breed and to collect information for establishing a feasible conservation scheme and of a breed traceability system for the valorization of Burlina dairy products.

Material and methods

Sampling and DNA amplification

The dataset consisted of 153 blood samples belonging to three dairy cattle breeds: Burlina (n = 80), HFR (n = 29), and BSW (n = 44). Burlina samples were collected in two farms and were provided by the breeders association of Treviso Italian province; HFR and BSW samples were collected in six farms, in order to obtain representative samples from unrelated animals. Blood samples were collected from each animal in 5 ml vacutainer tubes containing sodium citrate as anticoagulant, and stored at -20°C until analyses were performed. DNA extraction was carried out employing the "Gentra System PUREGENE DNA purification kit" (Gentra System, Minneapolis, Minnesota, USA) starting from 300 µl of whole blood. DNA samples were then amplified by PCR in correspondence of the following 12 loci: BM1818, ETH185, MM12, TGLA126, BM203, TGLA122, RM12, ILST008, SPS115, BL42, ETH3 and TGLA53. The studied loci were chosen, according to ISAG/FAO Standing Committee Recommendations (2004), and consulting previous studies (Kemp et al. 1993; Bishop et al., 1994; Barendse et al. 1997; Grosz et al., 1997), aiming to investigate high polymorphic markers spread all over the genome. For the amplification 25 ng of DNA were added to a reaction mix containing: 1 pmol/µl of primer forward and reverse, 1X PCR buffer (16 mM $(NH_4)_2SO_4$, 67 mM Tris HCl pH 8,8, 0,01% Tween 20), 0,26 mM of every dNTPs, 2,5 mM of $MgCl_2$ and 0,8 U of *Taq* DNA polymerase, in a final volume of 20 µl. The 12 microsatellites were amplified by a PX2 Thermohybaid thermal cycle at the following conditions, the X temperature being the annealing t° of each primer (annealing temperature available on request): initial denaturation step of 5 min at 95°C, 40 cycles of 30 s at 94°C, 1 min at X°C and 1 min at 72°C and a final extension of 10 min at 72°C. Allele size was determined with a Perkin Elmer ABI Prism 3730XL Genetic Analyzer, using GeneScan 2.0 and Genotyper 3.7 software (Perkin Elmer, Waltham, Massachusetts, USA).

Statistical analyses

Allelic frequencies, observed and expected heterozygosity, in the whole population and per breed, were calculated using the software Genetix 4.05.2 (Belkhir et al., 1996-2004). The Fstat 2.9.3 program (Goudet, 1995) was employed in calculations of mean number of alleles, allelic richness, gene diversity (Nei, 1987), and *F*-statistics estimates

(Weir and Cockerham, 1984) per locus, in the total sample, and in each breed. Tests for deviation from Hardy-Weinberg (H-W) equilibrium and for population differentiation were performed by the GENEPOP 3.4 software (Raymond and Rousset, 1995). For the H-W equilibrium estimation, per locus and per breed, the exact test of Guo and Thompson (1992) was performed. Once the deviation from equilibrium at some loci was evidenced, a more powerful test was applied to evaluate heterozygote deficiency and excess following the suggestions of Rousset and Raymond (1995). Global tests across loci and across breeds were assessed using the Fisher's method as implemented in GENEPOP 3.4. Test for population differentiation was performed as suggested by Raymond and Rousset (1995). For each locus an unbiased estimate of the Fisher's exact test was computed to verify if the allelic distribution was different among breeds.
The STRUCTURE software (Pritchard et al., 2000) was used to analyze the genetic structure of the studied population. This program implements a model-based clustering method for inferring population structure using genotype data of unlinked markers; here it was also used to assign individuals to the inferred populations and to identify admixture individuals. As suggested by Pritchard et al. (2000), analyses were performed firstly by means of the admixture model with correlated allele frequencies and secondarily, by the model accounting for prior population information in order to test whether any individual in the sample was misclassified. To choose the appropriate number of inferred clusters to model the data, 1 to 4 inferred clusters were performed with 3 independent runs each. All analyses used a burn-in period of 50,000 and 200,000 iterations for data collection. The correct number of inferred clusters was established as suggested by Pritchard et al. (2000) and by other authors (Álvarez et al., 2004; Glowatzki-Mullis et al., 2005) and the subsequent analyses were performed using only that value.

Results and discussion

In the whole sample, a total of 96 alleles were detected; number of alleles per locus ranged between 2 (RM12 and ILST008) and 15 (TGLA122) with an average of 8 (Table 1). Highest number of alleles per locus was found in Burlina (6.7) while BSW showed the lowest (5.4). To test if these values were just a consequence of the different number of samples per breed, the allelic richness, an estimate of the number of alleles

per locus corrected by sample size, was computed. Burlina and HFR evidenced almost the same allelic richness (6.03 and 5.99, respectively), while BSW remained the breed with the lowest locus variability (5.06). Private alleles were found in all the studied breeds: 9 were scored in BSW, 8 in Burlina and 5 in HFR (Table 1). Their frequencies were low being less than 0.1 except for allele 2 of TGLA122 detected in BUR (0.104) and allele 5 of TGLA53 in BSW (0.116). Table 1 shows also the gene diversity (Nei, 1987) per locus and per breed, on average in the three analyzed breeds it was equal to 0.624, TGLA122 showed the highest value (0.796) followed by BM203 and TGLA53 (both 0.764). Considering the results per breed, Burlina showed the highest gene diversity over all loci (0.675) followed by HFR (0.619) and BSW (0.579). These values could be considered moderately high even if they are affected by RM12 marker that presented, in all breeds, and in particular in BSW, a low gene diversity. Table 2 shows the values of observed and expected heterozygosity. Burlina showed the highest variability while BSW the lowest. Differences between observed and expected values were neither significant in any of the considered breeds nor in the whole population, meaning that the H-W equilibrium was respected. Heterozygosity estimates obtained for the Burlina breed were moderate to high and consistent to the value reported by Del Bo et al. (2001) that found an estimate of 0.68. On the other one hand, estimates observed in HFR were lower than those obtained by Peelman et al. (1998), Maudet et al. (2002) and Del Bo et al. (2001) which evidenced values of 0.68 in the first two studies and 0.69 in the last. Higher variability was observed also in BSW (0.66 in Del Bo et al., 2001). Referring to the rich literature available on genetic variability of both native and commercial cattle breeds, it can be assumed that heterozygosity estimates generally varies between 0.600 and 0.750 (Table 3). Despite the difficulties in comparing literature results, as they have been obtained with different marker sets, other authors observed a higher genetic variability in local breeds in comparison to commercial ones (Giovambattista et al., 2001; Maudet et al., 2002; Rendo et al., 2004). In particular Maudet et al. (2002), compared some native French breeds with French Holstein while Giovambattista et al. (2001) studied the indigenous Argentinean Creole Cattle in comparison with HFR. Both concluded that the strong artificial selection and the intensive use of elite sires and artificial insemination, seemed to have reduced genetic variability and effective population size in commercial widespread breeds such

as HFR. Native breeds, usually subject to local use and traditional husbandry management, seemed not to have faced these problems and have maintained a higher variability together enhancing a better adaptation to natural environment. The results obtained in the present study agree with such conclusions; in fact the Burlina breed has been enrolled in the Italian Herd Book only recently and it has always been reared by small breeders following their own breeding scheme and usually using their own sires. This could explain why Burlina variability is reasonably high even if the population size is small. The second reason could be the crossing that breeders might have carried out between Burlina and more productive breeds, causing increased heterozygosity. Maudet et al. (2002) suggested the same hypothesis to explain the moderate/high variability of a local, endangered French cattle breed (Villard de Lans). However, such variability represents an important reservoir of genetic diversity that should be conserved. Genetic diversity is in fact important to allow genetic improvement in selected breeds as well as to facilitate rapid adaptation to changed breeding goals (Notter, 1999). Table 4 shows Wright's *F*-statistics in the entire sample and per breed revealing that the homozygote excess in the whole population was moderate (F_{IT} = 8.5%), and it was only due to differences in gene frequencies among breeds, and not to a homozygote excess within them (F_{IS} = 0.00). This result is not in agreement with what has been found by other authors. In some studies on cattle (Jordana et al., 2003; Ciampolini et al., 2006) and also on sheep breeds (Álvarez et al., 2004), a significant amount of homozygote excess has always been detected. However, the higher F_{IS} values observed in the cited studies, might also be a consequence of a hidden structure in the analyzed breeds. Moreover, the results in the other studies could be explained by the deviation from H-W expectations observed in many loci, while, in our case, most of the loci were in equilibrium, both in the whole sample, and in each breed. However, analysis of heterozygote excess performed at breed level (called f_{IT} in Table 4) detected an excess at TGLA126 in both Burlina ($P < 0.01$) and HFR ($P < 0.01$). Such an excess was reflected also when considering the whole population with TGLA126 being the only marker showing a highly significant ($P < 0.001$) heterozygote excess (Table 3). A significant lack was evidenced at TGLA122 ($P < 0.01$) and TGLA53 ($P < 0.05$) loci in BSW. *F*-statistics estimations were repeated excluding TGLA126 marker. In this case the homozygote excess in the total population increased (F_{IT} = 0.104) being caused also by

a small within population (F_{IS} = 0.012) excess. Looking at the per breed F_{IS} estimates, one can notice that excluding TGLA126, they increased in all the studied breeds, in particular in BSW (0.048). Inbreeding coefficient for Burlina was still low (0.007), if compared with findings of other studies on local breeds. Moioli et al. (2004) estimate values ranging from 0.102 to 0.138 in their study on three indigenous Italian cattle breeds; Rendo et al. (2004) evidenced an F_{IS} estimate of 0.108 in the Betizu cattle, a native endangered Spanish breed showing the same population size as Burlina. Also in this case, we have to remember that the estimates were obtained analyzing different markers when we are comparing results.

Estimates of F_{ST} showed that most of the population variance was explained by individual variability while, 8.5% was explained by the presence of breeds. These findings are in agreement with literature, as Schmid et al. (1999) found estimates of 9% in their study on Swiss cattle breeds and Maudet et al. (2002) of 8% analysing seven French native breeds. Slightly lower levels of breed differentiation were observed by Cañon et al. (2001) in a study on genetic diversity of European local beef cattle breeds (7%) and by Jordana et al. (2003) in their research on 18 local South European beef breeds (6.8%). Genetic distances were measured by pair-wise F_{ST} as shown in Table 5. The BSW breed was the most differentiated one (F_{ST} = 0.103 and 0.101 with Burlina and HFR respectively) while the Burlina-HFR pair was the most similar highlighting a distance of 0.047. In their study on Alpine cattle breeds Del Bo et al. (2001) also found Burlina to be the breed showing the lowest distance from HFR. However, test for population differentiation stated that gene frequencies among breeds were highly significant ($P < 0.001$), but, per locus results, showed that in about 33% of the loci no significant difference was found between Burlina and HFR frequencies, suggesting a close relationship between them.

Results of the analyses for population structure are shown in Table 6. The most appropriate number of clusters for modeling the data was three, every cluster was associated to a breed: BSW to cluster 2, highlighting the highest proportion of membership (94%), HFR was associated to cluster 1, while most Burlina animals fell in cluster 3 with the lowest proportion of membership (66%). A significant proportion of Burlina individuals (30%) was found in the same cluster of HFR evidencing once again, the close relationship between these two breeds. It is worth mentioning that the

software inferred the clusters only on the basis of differences in the allelic frequencies, without any prior information on the breed of origin. Table 7 showed the results of the assignment test considering different thresholds. BSW performed the best results with 95% of the animals correctly assigned with a probability of more than 70%, followed by HFR (93%) and Burlina (65%). Even when no threshold was considered, about 30% of Burlina animals were not assigned to their breed of origin, instead all of them were assigned to HFR with moderate/high probability. The obtained results are the consequence of shared alleles with similar frequencies, as it was also evidenced by the test for population differentiation performed at the locus level. However, they suggested the presence of a certain degree of admixture between HFR and Burlina breeds. Presence of admixture is supported by other facts. As already cited it is likely that, in the past, crosses between these two breeds took place, probably aiming to improve Burlina production. The high amount of incorrect assignment in Burlina could be explained by this crossbreeding that caused an introgression of HFR genes into Burlina. Moreover, only since 1980 the Burlina breed has been enrolled in the Italian Herd Book of local breeds and pure breeding and conservation have started. Until 1980 breeders were pursuing their own breeding goals that not necessarily encouraged pure breeding of Burlina. Moioli et al. (2004), in their study concerning two local Italian cattle breeds, Podolica and Maremmana, found similar results and they also suggested as the reason for the high percentage of miss assignments, a recent and not well established selection program. Glowatzi-Mullis et al. (2005), in their study on genetic diversity in horse population, found that breeds not allowing crossing with other breeds were clearly unified in their own cluster. Instead, breeds who did allow could not be assigned to one cluster only and showed moderate proportion of membership to the cluster of breeds they were crossed with. Such results are in agreement with what was found in the present study. However, also the higher variability observed in Burlina could lead to difficulties in correct assignment, as observed in other local breeds (Moioli et al., 2006). Finally, one must consider that the genetic distance between Burlina and HFR was quite low (< 0.05) and, as suggested by Bjørnstad and Røed (2002), more markers should be used to differentiate between them.

As suggested by Pritchard et al. (2000), a second analysis was performed including prior information on the population of origin. As the STRUCTURE software has been

developed for study of natural population, usually the information used refers to the geographical origin of the samples. However, the model is also suitable for situations in which individuals are classified according to some characteristics other than sampling location, as in our case (Pritchard et al., 2000; Negrini et al., 2007). As expected, the percentage of assignment increased for all breeds, in particular for Burlina, but two animals were still assigned to the HFR breed, so, their genetic makeup suggested they were wrongly classified as Burlina. The assignment probability was rather low for both samples (about 55%) evidencing a real difficulty to classify these animals. Once again, the use of a higher number of markers could help to clarify this situation allowing allocating uncertain samples to breeds with higher probability.

Concluding, the presented results highlight the feasibility of a conservation program for the Burlina breed aiming to increase the number of reared animals and to reduce the percentage of admixture with HFR. This study could be extended with more markers and different breeds to better investigate the origins of the Burlina breed that, at present, are still uncertain. In addition, to improve the breed profitability, a method for the genetic traceability could be interesting in order to valorize both the breed and its dairy products.

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Tables

Table 1 Number of alleles per locus (Na), in brackets presence of private alleles, and gene diversity overall population and in each breed: Burlina (BUR), Holstein Friesian (HFR) and Brown Swiss (BSW).

Locus	Overall		BUR		HFR		BSW	
	Na	Gene diversity	Na	Gene diversity	Na	Gene diversity	Na	Gene diversity
BM1818	8	0.632	6	0.679	6 (1)	0.577	7 (1)	0.641
ETH185	10	0.744	6	0.725	9 (2)	0.796	5 (1)	0.713
MM10	8	0.721	7	0.720	7	0.629	6	0.813
TGLA126	6	0.608	6 (1)	0.629	5	0.634	4	0.561
BM203	13	0.764	11 (1)	0.799	8	0.662	9 (2)	0.832
TGLA122	15	0.796	12 (3)	0.806	9 (1)	0.848	7 (2)	0.734
RM12	2	0.284	2	0.358	2	0.343	2	0.152
ILST008	2	0.464	2	0.495	2	0.399	2	0.496
SPS115	6	0.578	6 (1)	0.538	5	0.509	4	0.688
BL42	4	0.493	4	0.692	3	0.526	4	0.26
ETH3	8	0.644	8 (2)	0.798	6	0.647	5	0.486
TGLA53	14	0.764	10	0.855	11 (1)	0.861	10 (3)	0.575
Average	8	0.624	6.7	0.675	6.1	0.619	5.4	0.579

Table 2 Number of sampled animals, observed and expected heterozygosity per breed: Burlina (BUR), Holstein Friesian (HFR) and Brown Swiss (BSW), averaged overall 12 loci.

Breed	Number of samples	Average heterozygosity	
		observed ± SD	expected ± SD
BUR	80	0.677 ± 0.155	0.670 ± 0.146
HFR	29	0.639 ± 0.169	0.609 ± 0.160
BSW	44	0.563 ± 0.202	0.572 ± 0.206

Table 3 Levels of genetic variability among European commercial and native breeds in recent studies using microsatellites.

N° of loci	Expected heterozygosity	Reference
23	Belgian Blue (0.650), Holstein Friesian (0.690), East Flamish (0.690), Red Pied (0.700)	Peelman et al. (1998)
16	Alistana (0.681), Asturiana Montaña (0.705), Asturiana Valles (0.683), Sayaguesa (0.707), Tudanca (0.651), Avileña Negra-Iberica (0.692), Bruna del Pirineus (0.672), Marucha (0.709), Pirenaica (0.628), Retinta (0.693), Alentejana (0.655), Barrosã (0.708), Maronesa (0.664), Mertolenga (0.671), Mirandesa (0.635), Aubrac (0.611), Gasconne (0.708), Salers (0.631)	Cañón et al. (2001)
17	Holstein Friesian (0.680), Original Brown Swiss (0.660), Simmental (0.620), Brown Swiss (0.660), Evolene (0.600), Herens (0.600), Aosta Red Pied (0.670), Aosta Black Pied (0.620), Aosta Chestnut (0.640), Rendena (0.650), Burlina (0.680), Oropa Red Pied (0.620), Grey Alpine (0.640)	Del Bo et al. (2001)
23	Abondance (0.650), Charolais (0.661), Limousin (0.675),Montbéliarde (0.670), Holstein Friesian (0.686), Tarentaise (0.699), Villlard de Lans (0.676)	Maudet et al. (2002)
12	Jersey (0.640)	Chikhi et al. (2004)
11	Betizu (0.715), Terrena (0.747), Monchina (0.762), Oirenaica (0.688)	Rendo et al. (2004)

Table 4 Wright's *F*-statistics computed for the cattle breeds: Burlina (BUR), Holstein Frisian (HFR) and Brown Swiss (BSW).

Locus	F_{IT}	F_{ST}	F_{IS}	BUR f_{IT}	HFR f_{IT}	BSW f_{IT}
BM1818	0.050	0.035	0.016	0.005	0.044	0.020
ETH185	0.143	0.108	0.040	0.004	0.162	0.022
MM10	0.022	0.050	-0.029	-0.024	-0.151	0.022
TGLA126	-0.158_a***	0.025	-0.187	-0.122_a**	-0.359_a**	-0.189
BM203	0.043	0.060	-0.018	-0.064	0.062	0.022
TGLA122	0.162	0.085	0.083	0.017	-0.017	0.287_b**
RM12	0.052	0.036	0.017	-0.013	-0.042	0.234
ILST00	0.108	0.073	0.038	0.043	0.017	0.040
SPS115	0.086	0.091	-0.005	0.011	-0.085	0.010
BL42	0.159	0.142	0.020	0.097	-0.155	-0.135
ETH3	0.168	0.138	0.035	0.048	0.062	-0.029
TGLA53	0.115	0.127	-0.014	-0.034	-0.001	0.029_b*
Total	0.085	0.085	0.000	-0.003	-0.032	0.029

$_a$ excess of heterozygosity

$_b$ deficit of heterozygosity

*P < 0.05, **P < 0.01, ***P < 0.001

Table 5 Genetic distances among the three cattle breeds, Burlina (BUR), Holstein Friesian (HFR) and Brown Swiss (BSW), measured by pair-wise $F_{ST.}$

Breed	HFR	BSW
BUR	0.0471	0.1027
HFR		0.1009

Table 6 Proportion of membership of each of the 3 cattle breeds, Burlina (BUR), Holstein Friesian (HFR) and Brown Swiss (BSW), in each of the 3 inferred clusters.

Breed	Inferred clusters		
	1	2	3
BUR	0.303	0.037	0.66
HFR	0.885	0.045	0.07
BSW	0.026	0.944	0.03

Table 7 Percentage of animal correctly and incorrectly assigned to their breed of origin at different probability level (P) in Burlina (BUR), Holstein Friesian (HFR) and Brown Swiss (BSW).

	Correct assignment				Incorrect assignment			
P	> 90%	> 80%	> 70%	no threshold	> 90%	> 80%	> 70%	no threshold
BUR	53.8	61.3	65.0	68.8	16.3	22.5	26.3	31.3
HFR	72.4	89.7	93.1	93.1	0.0	0.0	0.0	6.9
BSW	86.0	90.7	95.3	100.0	0.0	0.0	0.0	0.0

References

Álvarez, I., Royo, L.J., Fernández, J., Gutiérrez, J.P., Gómez, E., Goyache, F. (2004) Genetic relationships and admixture among sheep breeds from Northern Spain assessed using microsatellites. *J. Anim. Sci.,* **82,** 2246-2252.

A.I.A. (2006). *Milk recording activity: Official Statistics*. Italian Breeders Association (A.I.A.), Roma, Italy.

Barendse W., Armitage S.M., Kossarek L.M., Shalom A., Kirkpatrick B.W., Ryan A.M., Clayton D., Li L., Neibergs H.L., Zhang N., Grosse W.M., Weiss J., Creighton P., McCarthy F., Ron M., Teale A.J., Fries R., McGraw R.A., Moore S.S., Georges M., Soller M., Womack J.E., Hetzel D.J.S. (1994) A genetic linkage map of the bovine genome. *Nature Genet*., **6**, 227-235.

Belkhir, K., Borsa, P., Chikhi, L., Raufaste, N., Bonhomme, F. (1996-2004) GENETIX 4.05, logiciel sous Windows TM pour la génétique des populations. Laboratoire Génome, Populations, Interactions, CNRS UMR 5000, Université de Montpellier II, Montpellier (France). Available at: http://www.genetix.univ-montp2.fr/genetix/intro.htm.

Bishop, M.D., Kappes, S.M., Keele, J.W., Stone, R.T., Sunden, S.L.F., Hawkins, G.A., Toldo, S.S., Fries, R., Grosz, M.D., Yoo, J., Beattie, C.W. (1994) A genetic linkage map for cattle. *Genetics*, **136**, 619-639.

Bittante, G., Xiccato, G., Debattisti, P., Carnier, P. (1992) Prestazioni produttive e riproduttive di bovine di razza Burlina, Frisona e Meticce allevate in ambiente pedemontano*. Zootecnia e Nutrizione Animale* **XVIII,** – n.3-4

Bjørnstad, G., Røed, K. H. (2002) Evaluation of factors affecting individual assignment precision on using microsatellite data from horse breeds and simulated breed crosses. *Anim. Genet.,* **33**, 264-270.

Cañón, J., Alexandrino, P., Bessa, I., Carleos, C., Carretero, Y., Dunner, S., Ferron, N., Garcia, D., Jordana, J., Laloë, D., Pereira, A., Sanchez, A., Moazami-Goudarzi, K. (2001) Genetic diversity measures of local European beef cattle breeds for conservation purposes. *Genet., Select. Evol.,* **33**, 311-332.

Chikhi L., Goossens B., Treanor A., Bruford M.W. (2004) Population genetic structure of and inbreeding in an insular cattle breed, the Jersey, and its implications for genetic resource management. *Heredity*, **92**, 396-401

Chiodi, V. (1965) Un delitto zootecnico. In: Proceedings of the Italian Society for Veterinary Science **XIX**, 226-228.

Ciampolini, R., Cetica, V., Ciani, E., Mozzanti, E., Fosella, X., Marroni, F., Biagetti, M., Sebastiani, C., Papa, P., Filippini, G., Cianci, D., Presciuttini, S. (2006) Statistical analysis of individual assignment tests among four cattle breeds using fifteen STR loci. *J. Anim. Sci.,* **84**, 11-19.

Cozzi, G., Trevisan, L., Gottardo, F., Rigoni Stern, G. (2004) Un disciplinare per la gestione degli alpeggi nell'Altopiano di Asiago nel rispetto dell'ambiente e delle esigenze nutrizionali della vacca da latte. In: *Il sistema delle malghe alpine. Aspetti agro-zootecnici, paesaggistici e turistici. Quaderni SoZooAlp* **1**, 131-138.

Del Bo, L., Polli, M., Longeri, M., Cerotti, G., Looft, C., Barre-Dirie, A., Dolf, G., Zanotti, M. (2001) Genetic diversity among some cattle breeds in the Alpine area. *J. Anim. Breed. Genet.,* **118**, 317-325.

Favaro, G., Magno, F., Boaretto, A., Bailoni, L., Mantovani, R. (2005) Traceability of Asiago mountain cheese: a rapid low-cost analytical procedure for its identification based on solid-phase microextraction. *J. Anim. Sci.,* **88**, 3426-3434.

Giovambattista, G., Ripoli, M.V., Peral-Garcia, P., Bouzat, J.L. (2001) Indigenous domestic breeds as reservoirs of genetic diversity: the Argentinean Creole cattle. *Anim. Genet.* **32**, 240-247.

Glowatzki-Mullis, M. L., Muntwyler, J., Pfister, W., Marti, E., Rieder, S., Poncet, P. A., Gaillard, C. (2005) Genetic diversity among horse population with a special focus on the Franches- Montagnes breed. *Anim. Genet*., **37**, 33-39.

Goudet, J. (1995) FSTAT (version 1.2): a computer program to calculate F statistics. *J. Hered.,* **86(5)**, 485-486.

Grosz, M.D., Solinas-Toldo, S., Stone, R.T., Kappes, S.M., Fries, R., Beattie, C.W. (1997) Chromosomal localization of six bovine microsatellite markers. *Anim. Genet*., **28**, 39-40.

Guo, S.W., Thompson E.A. (1992) Performing the exact test of Hardy-Weinberg proportion for multiple alleles. *Biometrics* **48**, 361-372.

ISAG/FAO Standing Committee (2004) Secondary guidelines for development of national farm animal genetic resources management plans. Measurement of domestic animal diversity (MoDAD): Recommended Microsatellite markers. Available at:

http://dad.fao.org/cgibin/getblob.cgi?sid=c63cc827ffd7f91f27fa37ccd7139e36,50006220, visited on 16 November 2007.

Jordana, J., Alexandrino, P., Beja-Pereira, A., Bessa, I., Cañon, J., Carretero, Y., Dunner, S., Laloë, D., Moazami-Goudarzi, K., Sanchez, A., Ferrand, N. (2003) Genetic structure of eighteen local south European beef cattle breeds by comparative *F*-statistics analysis. *J. Anim. Breed. Genet.,* **120**, 73-87.

Jordana, J., Folch, P., Aranguren, J.A. (2001) Microsatellites analyses of genetic diversity in the Catalonian donkey breed. *J. Anim. Breed. Genet.,* **118,** 57-63.

Kemp, S.J., Brezinsky, L., Teale, A.J. (1993) ILSTS008: a polymorphic bovine microsatellite. *Anim. Genet.*, **24**, 74.

Laval, G., Iannuccelli, N., Legault, C., Milan, D., Groenen, M. A. M., Giuffra, E., Andersson, L., Nissen, P. E., Jørgenssen, C. B., Beeckmann, P., Geldermann, H., Foulley, J. L., Chevalet, C., Olliver, L (2000) Genetic diversity of eleven European pig breeds. *Genet. Select. Evol.,* **32**, 187-203.

Maudet, C., Luikart, G., Taberlet, P. (2002) Genetic diversity and assignment tests among seven French cattle breeds based on microsatellite DNA analysis. *J. Anim. Sci.,* **80**, 942-950.

Moioli, B., Napolitano, F., Catillo, G. (2004) Genetic diversity between Piedmontese, Maremmana, and Podolica cattle breeds. *J. Hered.,* **95**, 250-256.

Moioli, B., Napolitano, F., Orrù, L., Catillo, G. (2006) Analysis of the genetic diversity between Gentile di Puglia, Sopravissana and Sarda sheep breeds using microsatellite markers. *Ital. J. Anim. Sci.,* **5**, 73-78.

Negrini, R., Milanesi, E., Colli, L., Nicoloso, L., Crepaldi, P., Lenstra, J.A., Ajmone-Marsan, P. (2007) Breed assignment of Italian cattle using biallelic AFLP markers. *Anim. Genet.*, **38**, 147-153.

Nei, M. (1987) *Molecular Evolutionary Genetics.* Columbia University Press, New York, USA. Notter, D. R. (1999) The importance of genetic diversity in livestock populations of the future. *J. Anim. Sci.*, **80**, 1776-1785.

Peelman, L. J., Mortiaux, F., Van Zeveren, A., Dansercoer, A., Mommens, G., Coopman, F., Bouquet, Y., Burny, A., Renaville R., Portetelle, D. (1998) Evaluation of the genetic variability of 23 microsatellite markers in four Belgian cattle breeds. *Anim. Genet.*, **29**, 161-167.

Pritchard, J.K., Stephens, M., Donnelly, P. (2000) Inference of population structure using multilocus genotype data. *Genetics,* **155**, 945-959.

Raymond, M., Rousset F. (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *J. Hered.,* **86**, 248-249.

Rendo, F., Iriondo, M., Jugo, B.M., Aguirre, A., Mazón, L.I., Vicario, A., Gómez, M., Estonba, A. (2004) Analysis of the genetic structure of endangered bovine breeds from the Western Pyrenees using DNA microsatellite markers. *Biochem. Genet.,* **42**, 99-108.

Rousset, F., Raymond, M. (1995) Testing heterozygote excess and deficiency. *Genetics* **140**, 1413-1419.

Santomaso, R. (2006) Personal communication

Schmid, M., Saitbekova, N., Gaillard, C., Dolf, G. (1999) Genetic diversity in Swiss cattle breeds. *J. Anim. Breed. Genet.,* **116**, 1-8.

Weir, B.S., Cockerham, C.C. (1984) Estimating *F*-statistics with special regard to system of mating. *Evolution* **38**, 1358-1370.

General discussion

The four papers comprised in this thesis evidence, first of all, the necessity to reach a reliable traceability system to ensure food safety and quality. Consumer's demand of transparency is particularly significant for products of animal origin due to recent alarmisms (e.g. B.S.E., avian influenza). For this reason the European Union issued important regulations and directives on food label, as related in the first paper. This consumer's need of clear information, together with the development of new molecular techniques, lead to the so called "genetic traceability". In particular, the Polymerase Chain Reaction (PCR), permitted to detect different molecular markers and, already in the early 1990s, the first analytical approaches for species identification were successfully investigated.

At present, there is a rich literature on individual, breed and species identification, essentially based on a few type of molecular markers, mainly, microsatellites, SNP, AFLP. In this thesis the choice fell upon microsatellites because they are easy to use, very informative and cost effective. Indeed, nowadays, the main problem for the real applicability of a genetic traceability system is its costs, research should focus on finding few markers discriminating well among individuals and breeds. The second and the third experimental contributions aimed at this. Individual identification appeared of easier applicability investigating just eight markers. However, the study showed that population structure and differentiation are important to determine the discriminatory power of markers so, animal belonging to every slaughtered breed should be sampled and analyzed before choosing the appropriate loci to investigate. The second problem to overcome, is the sampling of the entire, in this case cattle, population reared in a country. At present, animals are identified using ear tags and passports that they must carry during the all life until slaughtering. Ear tags are affixed by the Veterinary Services; in the near future, they could also collect a sample of animal tissue in the meantime and store it until a defined period after animal death. Random checking could be carried out by the National Food Authority which could use this technique also to detect suspected frauds and to recall animal cuts in case of health risks. Nowadays, genotyping costs are decreasing, due to the improvement and the development of new technologies, so applicability of an individual genetic traceability method is feasible. Producers seem to be more an more interested on such techniques because they guarantee food safety and, consequently, they valorise their productions; for this

reason producers association and cooperatives keep a watch over researches in this field. However, an economic survey of national level, on consumer's willingness to pay for safer food should be carried out carefully, even if it has already been shown that, especially in developed countries, consumers agree to pay extra for food safety issues.

More problems arise when the goal is to discriminate among breeds, as explained in the third contribution. The applicability of a breed traceability system using microsatellite markers is actually difficult. First of all, to implement a system able to identify the product's breed of origin based on microsatellite genotyping, a reference population is necessary. The reference population must be made up of representative samples from every breed reared in the country meaning that many energies and resources must be spent on that. Universities and Research Centre have already genotyped many breeds at several loci, a strong cooperation is necessary to put all the results together and create a unique and complete database without investing other resources in vain. Secondarily, methods based on microsatellite genotyping are part of the so called "probabilistic approach" meaning that results have to be interpreted using statistical inference. Many statistical approaches are possible and have been proposed, however, despite their ability to assign a sample to the correct breed of origin, the problem is the assignment confidence that rarely is high. Moreover, the closest the breeds are among them (from a genetic point of view), the most difficult is to obtain a trusted breed designation.

For this reason, when the product we aim to assign to its breed of origin is a meat cut, obtained from one single animal, it appears much easier to trace it back to the individual it belongs to following the approach previously examined.

The real challenge is to find trustable identification methods also for processed foods deriving from different animals such as cheese or sausages which represent a DNA admixture from several individuals. This problem is extremely interesting for producers which are increasingly asking for research help. In fact, in this case, traceability is seen as a valorisation label more than a tool to guarantee food safety. In the European market in general, and in the Italian one in particular, the presence of niche products, traditionally obtained from one breed only, is very high. In this category are found mainly cheeses, some kind of sausages and ham. The utilized breeds are usually indigenous and strongly linked to one particular environment resulting in a close

product-breed-environment relationship. So, valorisation of these productions will result also in the conservation of indigenous (sometimes endangered) breeds and in the environment protection. One example of such relationship has been exposed in the last contribution of this thesis. The valorisation of Burlina productions, in particular the typical Morlacco cheese, will contribute to its safeguard and to the protection and maintenance of the mountain environment where it has always been reared.

Producers interest on the development or re-discovery of traditional foods and on their valorisation, is clearly due to consumers behaviour. Consumers are much more aware than in the past of environmental and ecological issues and they look for traditional and genuine products often identified with a particular rural environment. On the other hand, people are aware of the potential of molecular DNA based techniques due to their common use in forensics and, for this reason they trust them. Breed genetic traceability represents a combination of this two aspects, the traditional and the innovative one.

In this case a microsatellite investigation will be very hard because of the difficulties in reading the electropherogram obtained from the loci amplification. In fact, it will be composed by several peaks summing up together making impossible the allele size detection. For these products a deterministic approach based on the presence/absence of a specific marker without need of any statistical inference seems to be advisable and of easier applicability. At present, the most studied and promising loci are the ones encoding for coat colour exposed in the first contribution of this thesis. Indeed, even this approach presents some troubles, as, discrimination among few breeds, could be possible but among many represents a challenge; the investigation of many different loci together is probably the best solution.

Concluding, both producers and consumers are interested in finding a reliable method to trace back a food product to the individual or breed of origin. Genetic methods based on DNA identification have improved rapidly in recent years resulting in a constant decrease of analysis costs which have made their use feasible. Individual genetic traceability of beef using microsatellites for example, is of immediate application; the main obstacle to its implementation is the sample collection, organization and storage that should be decided by the authorities. Instead, more researches focusing on breed specific markers must be carried out before setting up a method for breed genetic traceability; in this case a deterministic approach seems to be of easier applicability. In

any case, a strong cooperation is needed among all actors involved anyhow on traceability from policymakers, universities and research institute, to producers and consumers in order to evidence the necessities, duties and responsibilities of everyone.

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