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APPLICATION OF MOLECULAR MARKERS FOR GENETIC TRACEABILITY OF SICILIAN AUTOCHTHONOUS BREEDS AND TYPICAL DAIRY PRODUCTS

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Ringrazio con tutto il mio cuore la mia famiglia:
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Abstract

The Sicilian cattle, sheep and goat breeds and their dairy products are an important source for the economy of livestock sector. Some of these dairy products are “mono-breed” and are important elements for the conservation and valorization of animal populations, territories and traditions.

Valorization, authentication, and traceability of dairy products require wide knowledge on breeds genetic structure, morphological and attitudinal traits, geographic distribution/environment, and genetic diversity within and among breeds.

Molecular traceability is a useful tool for preservation and valorization of typical products that can lead to develop marginal areas, to promote the conservation of biodiversity and, therefore, the protection of local breeds.

The application of molecular markers, such as microsatellites and mitochondrial DNA, is a very important tool to study the genetic diversity of caprine, bovine and ovine Sicilian autochthonous breeds and for characterization and valorization of their dairy products.

In this thesis, microsatellite markers were used for analysis of Girgentana goat breed individuals and dairy products. Moreover, mitochondrial DNA was considered for its species specificity in order to develop molecular protocols for authentication and traceability of mono-species Sicilian dairy products.

The overall aim of this thesis was the application of molecular technologies for the characterization and valorization of Sicilian autochthonous breeds for genetic traceability of typical dairy products.

In Chapter 2, a total of 388 individual samples of Girgentana, Maltese and Derivata di Siria Sicilian autochthonous goat breeds were analyzed by 20 microsatellite markers in order to identify breed specific microsatellite markers that can be used for traceability of Girgentana dairy products. Private alleles were found in each analyzed breed but we focused our attention mainly on alleles present at the same time in Maltese and Derivata di Siria and absent in Girgentana. Only eight microsatellite markers within the analyzed panel showed these alleles and, therefore, they were tested on DNA pools of each breed. Three markers, FCB20, SRCRSP5, and TGLA122 presented alleles useful for traceability purpose of Girgentana dairy

products, and they were tested on DNA samples extracted from cheeses. Considering our results, these microsatellite markers could be applied in a genetic traceability system of Girgentana dairy products in order to detect adulteration due to Maltese and Derivata di Siria goat milk.

In Chapter 3, species-specific multiplex-PCR protocols for identification of cattle's, sheep's and goat's milk in mono-species Sicilian dairy products were reported. DNA from blood and experimental cheeses of Sicilian autochthonous breeds was extracted to amplify the *12S* and *16S rRNA* genes of mitochondrial DNA. Fragments of different length were obtained using specific primers for ovine, bovine and caprine species (172 bp, 256 bp, and 326 bp, respectively). In next step, multiplex-PCR protocols were applied on mixtures of DNA pools and results showed a sensitive threshold of 0.1% for bovine/caprine and ovine/caprine DNA mixtures, and 0.5% for bovine/ovine and ovine/bovine DNA mixtures. Finally, the same assay was applied to bovine/ovine experimental cheeses to detect the minimum threshold of milk adulteration. The results showed a sensitive threshold of 0.1% for bovine/ovine cheeses and 0.5% for ovine/bovine ones. The proposed assay represents a rapid and straightforward method for the detections of adulteration in mono-species dairy products.

Riassunto

In Sicilia, le razze bovine, ovine e caprine e le loro produzioni lattiero-casearie rappresentano una risorsa importante per l'economia del settore zootecnico. Alcuni di questi prodotti di origine animale sono "prodotti monorazza" e rappresentano elementi importanti per la conservazione e lo sviluppo di queste popolazioni, dei territori e delle tradizioni locali.

Il processo di valorizzazione, autenticazione e tracciabilità delle produzioni lattiero-casearie richiede una conoscenza approfondita sulla struttura genetica delle razze, sulle caratteristiche morfologiche e attitudinali, sulla distribuzione geografica/ambiente di produzione e sulla diversità genetica entro e tra razze.

Uno strumento utile per la tutela e la valorizzazione dei prodotti tipici, che possono portare allo sviluppo di aree marginali, favorendo la conservazione della biodiversità e di conseguenza la tutela delle razze locali, è rappresentato dalla tracciabilità molecolare.

L'applicazione di marcatori molecolari, quali i microsatelliti e il DNA mitocondriale, è uno strumento importante per studiare la diversità genetica delle razze caprine, bovine e ovine autoctone siciliane e per la caratterizzazione e la valorizzazione delle loro produzioni lattiero-casearie.

In questa tesi, sono stati utilizzati i marcatori microsatelliti per analizzare non solo gli individui di razza caprina Girgentana ma anche le produzioni lattiero-casearie da essa derivate. Inoltre, è stato preso in considerazione il DNA mitocondriale, grazie alla sua specificità, per sviluppare protocolli molecolari utili per autenticare e tracciare le produzioni lattiero-casearie siciliane mono-specie.

L'obiettivo generale di questa tesi è l'applicazione di tecnologie molecolari per la caratterizzazione e valorizzazione delle razze autoctone siciliane e per la tracciabilità genetica delle produzioni lattiero-casearie tipiche siciliane da esse derivate.

Nel capitolo 2, sono stati analizzati 20 marcatori microsatelliti in 388 campioni individuali appartenenti alle razze caprine autoctone Siciliane: Girgentana, Maltese e Derivata di Siria al fine di tracciare i prodotti lattiero-caseari di razza Girgentana. In ogni razza analizzata sono stati rilevati alleli privati, ma la nostra attenzione si è focalizzata principalmente sugli alleli presenti contemporaneamente nelle razze caprine Maltese e

Derivata di Siria e assenti nella razza Girgentana. All'interno del pannello analizzato, solo 8 marcatori microsatelliti hanno mostrato questi alleli e, pertanto, sono stati testati su pool di DNA delle tre razze. In particolare, 3 marcatori (FCB20, SRCRSP5 e TGLA122) presentavano alleli utili per il sistema di tracciabilità dei prodotti lattiero-casearie pertanto sono stati testati su campioni di DNA estratti da formaggio. Considerando i risultati ottenuti, questi marcatori microsatelliti potrebbero essere applicati in un sistema di tracciabilità genetica dei prodotti lattiero-caseari di razza Girgentana al fine di rilevare eventuali adulterazioni causate dall'aggiunta di latte caprino di razza Maltese e Derivata di Siria.

Nel capitolo 3, sono stati riportati i protocolli di multiplex-PCR per identificare la presenza di latte di individui appartenenti alle specie bovina, ovina e caprina in prodotti lattiero-caseari Siciliani mono-specie. Dal sangue di individui appartenenti alle principali razze autoctone Siciliane e dai formaggi sperimentali è stato estratto il DNA per l'amplificazione dei geni *12S* e *16S rRNA* del DNA mitocondriale. L'uso di primers specie-specifici ha permesso l'amplificazione di frammenti di diversa lunghezza per le specie ovina, bovina e caprina, rispettivamente di 172 bp, 256 bp e 326 bp. Nella fase successiva, il protocollo di multiplex-PCR è stato applicato a pools di DNA ed i risultati hanno evidenziato una soglia di detezione dello 0,1% per le miscele di pools di DNA bovino/caprino e ovino/caprino, mentre una soglia di detezione dello 0,5% per le miscele di pools di DNA bovino/ovino e ovino/bovino. Infine lo stesso protocollo è stato applicato ai campioni di DNA estratto dai formaggi sperimentali al fine di rilevare la soglia minima di adulterazione nel latte. I risultati fin qui ottenuti hanno mostrato una soglia di detezione dello 0,1% di latte ovino in formaggi bovini e dello 0,5% di latte bovino in formaggi ovini. Il test proposto rappresenta un metodo rapido e semplice per rilevare eventuali adulterazioni dei prodotti lattiero-caseari mono-specie.

Chapter 1

General Introduction

1.1 Traceability

In recent years, the "traceability" has become the keyword of the agri-food scene, since its objective is to ensure food security. According to Caswell (1998) food security is an attribute which defines the quality of a product showing a logical distinction between safety and quality concepts.

The "traceability" 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 was 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.

In particular, in the livestock sector, traceability is the ability to maintain control of the origin of products and the identity of animals through various steps within the food chain, from the farmer to retailer (McKean, 2001).

The importance of traceability of animals and animal products has grown as food production and marketing have been industrialized and globalized, thus making direct consumer check on food processing methods difficult (Ajmone-Marsan *et al.*, 2004; Mariani *et al.*, 2005).

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. It was then followed by the dioxin crisis and the avian influenza in the poultry sector (Ciampolini *et al.*, 2000; Goffaux *et al.*, 2005; Bánáti *et al.*, 2011). Furthermore, the incidence of food borne diseases due to microbial contamination of processed food has increased 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.

All these reasons have contributed to the need of finding a system to trace food products. Therefore, traceability is the answer to the consumers' demand of transparency and it is becoming synonymous of safe and high quality food (Dalvit *et al.*, 2007).

Furthermore, the possibility of verifying the origin of animal products would allow not only the assessment of product quality but also the valorization of the local products of specific breeds (Mariani *et al.*, 2005).

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 (Barcos, 2001; Marchant, 2002; Stanford *et al.*, 2001; Bottero *et al.*, 2011; Zachar *et al.*, 2011) focusing on animal identification.

1.2 EU legislation on traceability

The EU has always paid great attention to food safety, first of all because the agro-alimentary sector on the whole is very important for the European economy, and then to achieve of a high level of health protection and the strengthening of consumers' protection after the BSE outbreak.

The three most important EU documents regarding food safety are the Green Paper on the general principles of food law in the EU (1997), The White Paper on Food Safety (2000) and the ER 178/2002 (applied from 1st January 2005).

The Green Paper on the principles of EU legislation about food products (1997) is made up of six parts regarding different aspects of food.

The White Paper on Food Safety (2000) contained strategies for updating the actual legislation. In particular, 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 main aim of EU policy is found in first article of ER 178/2002, which incorporates the Chapter 2 of the White Paper, and it is "ensuring a high level of protection of human health and consumer interests in relation to food".

The General Food Law covers the entire supply chain [Regulation (EC) 178 (2002), Article 18, paragraph 1] and it was followed by several other regulations. For animal products the most important regulations 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. Methodologies for the analyses of the food and feed materials combined with information technology systems are essential to delivering a working tracking and tracing system (Schwägele *et al.*, 2005).

Nevertheless, an appropriate set of analytical tools, for the verification of the declaration of origin of animal products, are necessary. Reliable analytical tools would not only protect consumers but would allow producers of traditional regional specialties (often protected labels) to get their products clearly differentiated from imitations (Franke *et al.*, 2005).

1.3 Conventional and geographical traceability

Conventional traceability system involves the labeling of beef and processed food in batches. It is extremely useful to keep the information of each individual animal, is less expensive and easier to reach than other methods, but, being based on tags and paper documents is subject to substantial risk of accidental mislabeling at certain points of the chain production easy falsification (Cunningham & Meghen, 2001; Capoferri *et al.*, 2005).

Geographic traceability 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 or microbial flora (Mauriello *et al.*, 2003; Pillonel *et al.*, 2003; Franke *et al.*, 2005; Schwägele, 2005). It is particularly useful for typical local foods labeled with the protected designation of origin (PDO) or protected geographical indication (PGI).

These designations constitute an element for the protection of the biodiversity of a territory (geological, pedological, climatical), plants, animals and microbes, practices and production systems, linked to the history and the local culture. On the other hand they assure suitable income for the rural population, protecting the products with a denomination against a competition based on low prices. International rules are opportune in order to protect the

typical products, their specific characteristics and their biodiversity, encouraging the permanent residence of the population in the rural areas, especially in the less favorite areas (Scintu *et al.*, 2007).

In Europe, Italy is leader in these productions with 266 labels between PDO and PGI (UE Regulation n. 1151/2012); it is followed by France, Portugal, Spain and Greece.

1.4 Genetic traceability

Considering the limits of conventional traceability systems, it is necessary to develop reliable methods which allow a fast and unequivocal identification of animal species (Schwägele *et al.*, 2005). In this context, the DNA identification technology offers a powerful tool to control conventional animal identification systems (Capoferri *et al.*, 2005).

Genetic traceability is based on the identification of both animals and their products through the analysis of DNA. The recent development of molecular biology techniques, with special regard to the direct analysis of DNA, have made possible to identify differences in the DNA of individuals from different populations, species or breeds that allow their identification and classification (Mackie *et al.*, 1999; Cunningham & Meghen, 2001). These different DNA sequences have been called genetic markers (Mariani *et al.*, 2005).

So a genetic marker is any characteristic of the organisms which varies within populations and which is determined by genes and not by environment, thus being inheritable. DNA is therefore a permanent and unalterable label (Mackie *et al.*, 1999; Cunningham & Meghen, 2001) which offers the possibility of identification at three different levels: individual (neutral markers polymorphic between individuals), breed (breed-neutral markers specific allelic variants of candidate genes) and species (neutral AFLP markers, mitochondrial DNA, sequences interspersed repeated).

1.4.1 Individual traceability

Traceability on individual level found its best application on the beef sector. Animal individual identification is useful for safeguarding public and animal health providing safe products for both domestic and export consumption. 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 *et al.*, 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.

The most widely used markers are microsatellites (Ciampolini *et al.*, 2000; Peelman *et al.*, 1998; Sancristobal-Gaudy *et al.*, 2000; Arana *et al.*, 2002; Vazquez *et al.*, 2004; Herraeza *et al.*, 2005; Shackell *et al.*, 2005; Dalvit *et al.*, 2006, 2008; Orrù *et al.*, 2006; Felmer *et al.*, 2008; Putnova *et al.*, 2011; Fernandez *et al.*, 2013; Oh *et al.*, 2014) and SNPs (Single Nucleotide Polymorphisms) (Heaton *et al.*, 2002, 2005, 2014; Capoferri *et al.*, 2005; Herraeza *et al.*, 2005; Negrini *et al.*, 2008; Karniol *et al.*, 2009; Orrù *et al.*, 2009; Ramos *et al.*, 2011; Fernandez *et al.*, 2013). All these researches revealed the efficacy of both markers for individual traceability with different results depending on type, number of chosen markers and level of polymorphism.

Microsatellites, also called SSR (Simple Sequence Repeats), are short tandem repeat units of 1-6 nucleotides and occupy regions usually not larger than 100 bp; are ubiquitous in the genomes of eukaryotes and are present, though rarely, in small bacterial genomes. In mammals, it is possible to find a microsatellite length of at least 20 bp each 6 Kb. These markers are hypervariable, highly informative and in livestock species are also used, for paternity analysis.

The SNPs, which identify individual mutations of nucleotides, are present throughout the genome both in non-coding regions than in coding regions. SNPs within the coding regions of candidate genes are of particular interest as may facilitate the identification of breed specific markers. SNPs are bi-allelic markers suitable for automation. The identification of a panel of SNP markers used for individual, breed or species traceability represents an innovative tool in terms of cost and accuracy.

1.4.2 Breed traceability

While the problem of individual traceability is essentially resolved through the markers described above and the techniques used in the future will depend exclusively on the costs of diagnostic assays, the current challenge is the development of diagnostic protocols for breed traceability.

Breed traceability allows assigning or excluding breed of origin to a product. This is of great importance, especially for local and typical products, some of them protected by the European label PDO (Protected Designation of Origin) or PGI (Protected Geographical Identification). Given the importance of these products, as mentioned above, and their strong link with territory, traditions and autochthonous breeds their production becomes important for conservation of the breeds and then reevaluation of production area from economical point of view.

So the researches on breed genetic traceability are often linked with studies on characterization of the breed (Ovilo *et al.*, 2000; Ciampolini *et al.*, 2000; Maudet *et al.*, 2002; Carrión *et al.*, 2003; De Marchi *et al.*, 2003; Fernandez *et al.*, 2004; Russo *et al.*, 2004, 2009; Crepaldi *et al.*, 2005; D'Alessandro *et al.*, 2007) and, sometimes, even conservation through the use of methods based on molecular markers (Alderson *et al.*, 2004; De Marchi *et al.*, 2006; Dalvit *et al.*, 2008; Orrù *et al.*, 2009).

The assignment of an individual to a breed using molecular methods can be performed, from the theoretical point of view, primarily through two strategies, as reported by Ajmone-Marsan *et al.* (2004):

- *DETERMINISTIC APPROACH* establishes the origin of a product by analyzing molecular markers with different alleles fixed in different breeds. These markers, actively sought in several species, would allow the development of simple diagnostic protocols that do not require any statistical inference (Milanesi *et al.*, 2003). The deterministic approach was mainly based on the study of genes coding for coat color, the principal character allowing breed differentiation (Maudet & Taberlet, 2002). Many studies concerning the MC1R gene, responsible of coat color in many species (Klungland *et al.*, 1995; Rouzaud *et al.*, 2000; Kriegesmann *et al.*, 2001; Maudet *et al.*, 2002; Carrion *et al.*, 2003; Crepaldi *et al.*,

2003, 2005; Russo *et al.*, 2004, 2007; D'Alessandro *et al.*, 2007; Fontanesi *et al.* 2009), and the search for breed specific AFLP markers (Alves *et al.*, 2002; Negrini *et al.*, 2003).

- *PROBABILISTIC APPROACH* uses panels of markers with allele frequency characteristics of each breed (Milanesi *et al.*, 2003). Assigning individuals to populations is of great importance to verifying the authenticity of labeled food products. The utilized methodology, based on analyses of individual multilocus genotypes, relies on the fact that individuals will have more similar genotypes when they come from the same population (Cornuet *et al.*, 1999); these "*genetic methods*" are based on the likelihood that genotype of 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 population (Cornuet *et al.*, 1999). These statistical tools could be used for the assessment of breed traceability system. Several studies on different species confirm the efficacy of the Bayesian approach if an appropriate number of markers is scored (Bjørnstad & Røed, 2001; Negrini *et al.*, 2003; Vega-Pla *et al.*, 2003; Ciampolini *et al.*, 2006; Dalvit *et al.*, 2006, 2008; Filippini *et al.*, 2006; García *et al.*, 2006).

1.4.3 Species traceability

The species genetic traceability in animal products has received great attention in recent years (Bottero *et al.*, 2003; Mafra *et al.*, 2004, 2007; Murugaiah *et al.*, 2009; Wang *et al.*, 2010 ; Zachar *et al.*, 2011; Dalmasso *et al.*, 2011). It was important for several reasons related to social, religious, economic and public health implications. In particular, in the dairy industry it is important to certify milk and, especially, the origin of the cheeses because these dairy products are subject of frauds. According to Maudet & Taberlet (2001) in dairy sector the greater availability and the lower cost of cow's milk rather than goat, ewe or buffalo milk leads to fraudulent substitution in cheese manufacturing.

Among the most common frauds it's possible to found adulteration (due to the replacement of part of the product with another of different origin or lower economic cost); sophistication (the addition of substances not covered by production rules); fakes (substitution

of one product with another; it's the most serious fraud in food industry); counterfeits (misuse of trademarks of local products).

There are several studies on species traceability because labeled "fraudulent" can also lead to serious consequences for human health, especially to consumers bearers of allergies and/or intolerances (Bottero *et al.*, 2003; Mafra *et al.*, 2004, 2007; Lopez-Calleja *et al.*, 2004, 2005; Darwish *et al.*, 2009). The EU Council Regulation 1086/1996 established a reference method for the detection of cow' milk-based isoelectro-focusing of γ-casein, however, the chemical methods may fail in species identification after excessive proteolysis or heat-induced denaturation of proteins indicator. Instead, molecular methods may be the solution as the DNA persists even in the ripened cheese (Plath *et al.*, 1997).

The molecular markers used for species traceability have been AFLP (Amplified Fragment Length Polymorphism); RFLP (Restriction Fragment Length Polymorphism); mtDNA (mitochondrial DNA).

The AFLP markers are genomic fragments detected after selective polymerase chain reaction (PCR) amplification which provide a number of appealing features in the fingerprinting of genomes of different complexity (Herbergs *et al.*, 1999; Ransom & Zon 1999; Ajmone-Marsan *et al.*, 1997, 1999, 2001; Buntjer *et al.* 1997; Alves *et al.*, 2002; Negrini *et al.*, 2003). It's a technology adopted for investigation of biodiversity in a wide variety of microbial, plant and animal species (Zhu *et al.*, 1998; Lazzi *et al.*, 2009). The advantage of this technology lies in its efficiency in generating large numbers of markers in any species, regardless the availability of sequencing information (Mariani *et al.*, 2005).

The RFLP is a difference in homologous DNA sequences that can be detected by the presence of fragments of different lengths after digestion with endonucleases. RFLP markers are co-dominant and highly locus-specific. After electrophoresis separation, a polymorphic pattern may be distinguished. The RFLP technique was investigated by several authors on both genomic and mitochondrial DNA (Plath *et al.*, 1997; Quinteiro *et al.*, 1998; Ram & Baidoun, 1996; Wolf *et al.*, 1999; Bania *et al.*, 2001; Montiel-Sosa *et al.*, 2000; Verkaar *et al.*, 2002; Bottero *et al.*, 2003; Abdel-Rahman *et al.*, 2007) 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 presents several advantages if compared to genomic one. 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 within mixtures (Mackie *et al.*, 1999; Maudet & Taberlet, 2001; Montiel-Sosa *et al.*, 2000; Bottero *et al.*, 2003; Lopez-Calleja *et al.*, 2004, 2005; Mafra *et al.*, 2004, 2007; Feligini *et al.*, 2005; Abdel-Rahman *et al.*, 2007; Dalmasso *et al.*, 2011).

With this in mind, the development of an efficient traceability system for livestock products through molecular analysis will provide added value by ensuring a "biological signature" to products of animal origin, helping to promote the expansion of market.

The aims of this thesis are presented in two works:

1. Application of microsatellite markers as potential tool for traceability of Girgentana dairy products.
2. Developed of multiplex-PCR protocol to amplify *12s* and *16s rRNA* genes of mtDNA for traceability of typical mono-species cheeses.

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Chapter 2

Application of microsatellite markers as potential tool for traceability of dairy products

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Abstract

With the goal of developing a genetic traceability system for dairy products, the aim of this study was to identify specific microsatellite markers able to discriminate among the most important Sicilian dairy goat breeds, in order to detect possible adulteration in Girgentana dairy products. A total of 20 microsatellite markers were analyzed on 338 individual samples from Girgentana, Maltese and derivate di Siria goat breeds. The first step was to identify breed specific microsatellite markers that can be used for the traceability of dairy products. Presence of private alleles was evidenced in each breed. We have focused our attention mainly on the alleles present at the same time in Maltese and Derivata di Siria and absent in Girgentana. Only eight microsatellite markers showed these alleles and, therefore they were tested on DNA pools of the three breeds and subsequently on DNA samples extracted from cheeses. Only three markers (FCB20, SRCRSP5, and TGLA122) presented alleles useful for traceability purpose of Girgentana dairy products. Considering our results, these microsatellite markers could be applied in a genetic traceability system of Girgentana dairy products in order to detect adulteration due to Maltese and Derivata di Siria goat breeds.

Keywords: microsatellite markers, traceability, dairy products, Girgentana goat breed

2.1. Introduction

Traceability has been 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). Traceability of farm animals to their source breed is becoming an important issue for the authentication of their products, as there is an increasing interest in marketing mono-breed labelled lines of meat as well as dairy products, which in some cases have obtained the protected designation of origin (PDO). This interest derives from the fact that a marketing link between breed and their originated products can contribute to improve breed profitability and sustainability of such farm animal production with significant impact on the rural economy of particular geographic areas and on breed conservation and biodiversity (Russo *et al.*, 2007).

Genetic traceability is based on the identification of both animal and their products through the study of DNA. Typing of DNA has been proposed as the most suitable identification method due to its precision, durability and possibility to overcome limits of conventional traceability systems. In fact, DNA molecules have the features to be inalterable during animal life, are stable to different treatments of processed foods and are present in every organism. Several different markers have been discovered, studied and used in agriculture and livestock; at present, the most widely used for traceability purpose are microsatellites and Single Nucleotide Polymorphisms (SNPs), for their high level of polymorphism and high reproducibility (Kumar *et al.*, 2009; Galimberti *et al.*, 2013). Microsatellite markers had been widely investigated for many applications such as genetic identification, assessment of parentage, breed assignment tests and traceability (Heaton *et al.*, 2002; Orrù *et al.*, 2006; Dalvit *et al.*, 2008; Tolone *et al.*, 2012; Fernàndez *et al.*, 2013; Rosa *et al.*, 2013). DNA analysis furnished different levels of identification: the individual one is of great interest because it is strictly linked to food safety, while breed and species discrimination are interesting to detect fraud and to protect and valorize typical products (Dalvit *et al.*, 2007; Bottero *et al.*, 2011). Breed genetic traceability allowed the assignment or exclusion of the breed of origin to a product. Some examples are the Italian PDO cheese Parmiggiano Reggiano produced only with milk obtained from the Reggiana dairy cows

(Gandini *et al.*, 1999) and the Spanish PDO Jamon Iberico made with Iberian pig breeds (Garcìa *et al.*, 2006). These are all typical products of the Mediterranean areas (Pancaldi *et al.*, 2005). It is important to underline that these products are usually ancient and their preservation is linked with the protection of traditions and cultures. The herds of the utilized breeds are often small and endangered, and their chance of survival is their use for the production of typical and high quality products. This means that breed traceability is important both to defend and valorize particular food products and livestock breeds.

An interesting situation is represented by the Girgentana goat, an ancient breed reared in a restricted area of Sicily for its good dairy production. Due to sanitary policies, the size of the Girgentana population decreased almost 90% in 20 years. In 1983, the population consisted of 30,000 Girgentana goats, nowadays only 374 heads are enrolled in the Herd Book (ASSONAPA, 2013) and it was listed by FAO with endangered risk status. Over recent years this breed has become almost extinct, in part as a consequence of marked decrease in fresh goat milk consumption. Therefore, it could be interesting to evaluate the possibility of revitalizing interest in milk produced by this breed in order to regain an important economic role in the production of drinking milk (such as milk for infants) and niche dairy products (Mastrangelo *et al.*, 2013). Recently emerging interests in this breed have resulted in the production of dairy products obtained with only Girgentana milk.

With the goal of developing a genetic traceability system for dairy products, the aim of this study was to identify specific microsatellite markers able to discriminate among the most important Sicilian dairy goat breeds, in order to detect possible adulteration in Girgentana dairy products. For this purpose we have focused our attention mainly on the three most important local goat breeds reared in Sicilia, Girgentana, Maltese and Derivata di Siria.

2.2 Materials and Methods

2.2.1 Blood sampling and DNA extraction

For this preliminary study, a total of 338 individual samples, belonging to Girgentana (264), Maltese (41) and Derivata di Siria (33) goat breeds were collected. Animals were randomly

sampled from different flocks located in Sicily provinces. About 10 ml of blood was collected from jugular vein using Vacutainer tubes containing EDTA as anticoagulant. Genomic DNA was extracted from buffy coats of nucleated cells using a salting out method (Miller *et al.*, 1988). The concentration of extracted DNA was checked using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and samples were stored at 4°C until use.

2.2.2 Cheese sampling and DNA extraction

A total of three cheese samples of Girgentana goat breed were collected from Sicilian local dairy farms and stored at -20°C until use. Farmers declared that milk from other goat breeds was not used to produce these cheeses. As the milk contains somatic cells that are included as component in cheese and in other processed dairy products, the DNA from these cells represents the trace of the milk producer animals. For DNA extraction the CTAB method of ISO 21571:2005(E) was used, making some changes to the protocol. Three samples from each cheese were collected for DNA extraction and used as technical and biological replicates. The concentration of extracted DNA was checked using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and samples were stored at 4°C until use.

2.2.3 Experimental DNA pools preparation

Several DNA pools were prepared mixing DNA from Girgentana, Maltese and Derivata di Siria goat breeds in different proportion (Table 1).
The DNA pools were created considering the different alleles present in the three goat breeds in order to assess the detection power of microsatellite markers.

2.2.4 Microsatellite markers amplification and analysis

A total of 20 microsatellite markers were amplified in five multiplex-PCR reactions (Table 2).

Markers were chosen according to the International Society for Animal Genetics (ISAG) and the Food and Agriculture Organization (FAO) guidelines, in order to be polymorphic and located all over the genome. Each PCR reaction was performed in a total volume of 10 µl containing 100 ng of genomic DNA, 1X PCR buffer with $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.8 mM dNTPs, primer mix and 1 U of *Taq* DNA polymerase.

The thermal cycling condition were initial denaturation at 95°C for 10 min, 35 cycles 95°C for 1 min, 56°C for 1 min, and 72°C for 2 min, followed by final extension at 60°C for 30 min. Capillary electrophoresis was performed using ABI PRISM 3130*xl* Genetic Analyzer (Applied Biosystems) and allele size was assigned using GeneMapper v4.0 software.

The DNA pools and all extracted DNA samples from cheese were checked for microsatellite markers which showed alleles present in Maltese and Derivata di Siria and absent in Girgentana goat breed.

2.2.5 Statistical analysis

Allele frequencies, mean number of alleles (MNA), allelic richness (AR), observed (Ho) and expected (He) heterozygosity, Polymorphic Information Content (PIC), and exact P-value associated with the null hypothesis of Hardy Weinberg equilibrium (HWE) for all loci were estimated using CERVUS 3.0.3 (Marshall *et al.*, 1998), FSTAT 2.9.3.2 (Goudet, 1995), ARLEQUIN 3.5.1.2 (Excoffier *et al.*, 2010), and GENEPOP 4.0.11 (Rousset, 2008) software.

2.3. Results and discussion

All 338 individual samples were genotyped for 20 microsatellite markers. Of the 20 microsatellite markers used in this study, STA5B* marker was monomorphic in all breeds and, therefore, it was excluded from the statistical analyses. In Table 3, the number of detected alleles, Ho and He, and PIC for the 19 analyzed loci are shown.

A total of 159 alleles have been identified in the Sicilian goat breeds. Observed number of alleles per locus ranged from 3 (INRA104 and ETH225) to 12 (BRN) with an average of value of 8.386 ± 2.692. The PIC all over loci was equal to 0.610 ± 0.188, and considering that this value was higher than 0.50, the microsatellites panel was highly

informative (Botstein, White, Skolnick, & Davis, 1980). The microsatellite set gave satisfactory results in all breeds, with the mean proportion of individuals typed of 0.963, and this simplified the laboratory work and reduced the analyses costs.

Moreover, this set showed good variability considering the mean values of Ho and He (0.528 and 0.647, respectively). Only two microsatellite markers (CSRD247 and FCB11) were not in HWE in Girgentana, Maltese and Derivata di Siria goat breeds (data not shown). The first step was to identify breed specific microsatellite markers that can be used for the traceability of dairy products. Presence of private alleles was evidenced in each breed. In particular, 17 private alleles were found in Girgentana, 16 in Maltese, and 5 in Derivata di Siria goat breeds (Table 4).

Considering the alleles distribution within the three breeds, it is possible to note some differences that can be used to identify or exclude the breed of origin of dairy product. For this purpose, we have focused our attention mainly on the alleles present at the same time in Maltese and Derivata di Siria and absent in Girgentana. In fact, the deterministic approach is based on the identification and use of few breed specific or exclusive markers that are present or absent in all animals of a particular breed, and that can be applied to mixture of products obtained from more animals.

Only eight microsatellite markers showed these alleles as reported in Table 5, therefore they were tested on DNA pools of Girgentana, Maltese and Derivata di Siria breeds and subsequently on DNA samples extracted from cheeses. Considering that DNA pools were constructed with genotyped individuals of the three goat breeds, we assigned alleles to any of the observed peaks in electropherograms.

When microsatellite markers are analyzed, small amounts of fragments smaller/greater than the “real” allele are also amplified. This phenomenon is routinely referred to as “stutter” and, when present, it could be difficult to distinguish low peaks due to “stutter” from their interaction with true alleles (Shackell *et al.*, 2005). The analyzed microsatellite markers presented real allele peaks and “stutter” 2 and/or 4 base-pairs smaller and/or greater than the respective alleles (Fig. 1A). When analysis is performed on single individuals was easy to distinguish between alleles and “stutter” even if the analyzed samples were heterozygous (Fig. 1B). Otherwise, when DNA pools contained mixture of DNA from different individuals, the

electropherograms showed peaks that are combination of true alleles and the "stutter" from these alleles (Fig. 1C). Therefore, depending on the shape of microsatellite it could be difficult to distinguish between low peaks due to "stutter" and alleles of individuals making a minor contribution to the pool.

To test the eight microsatellites, we first analyzed each of them on pools of DNA of single breed constituted by mixing an increasing number of individuals with known genotypes (from 2 to 50). We obtained good results for the three separated breeds; in Figure 2 we reported, as example, the FCB48 microsatellite marker of 3 DNA pools contained 50 individuals.

We also conducted the same analysis for different DNA pools (Table 1) of the three breeds with the same good results, especially because, as reported in Figure 3, alleles with low frequency within Maltese and Derivata di Siria (e.g. 156 bp) were successfully detected when DNA pools containing the three goat breeds.

From the visual analysis of electropherograms of DNA pools containing the three goat breeds for each of the eight microsatellites, it was possible to detect that only three markers (FCB20, SRCRSP5, and TGLA122) presented alleles useful for traceability purpose of Girgentana dairy products (data not shown). In fact, these three microsatellites presented the smaller or the greater allele in Maltese and Derivata di Siria breeds and, therefore, it was not possible to confuse the real allele peaks with the "stutter" when unknown alleles are present within the samples. We analyzed FCB20, SRCRSP5, and TGLA122 markers in DNA samples extracted from cheeses (n=3) and we repeated the analysis on replicates (9 samples in total). Moreover, we compared the electropherograms with those obtained from DNA pool with 9:1 ratio (Table 1) and we did not detect specific alleles of Maltese and Derivata di Siria breeds (Figure 4, 5, 6).

Considering our results, these microsatellite markers could be applied in a genetic traceability system of Girgentana dairy products in order to detect adulteration due to Maltese and Derivata di Siria goat breeds. Concerning other goat breeds reared in Sicily (Argentata dell'Etna, Messinese, Saanen and Camosciata delle Alpi), it should be improbable that the Girgentana products are obtained with mixtures of milk from these breeds, especially because they are not reared in the same geographical area of Girgentana goat.

We obtained very good amplifications of DNA from cheese and replicates showed high reliability. Shackell *et al.* (2005) using microsatellite markers for traceability of ground beef mixtures showed that their PCR reactions were generally repeatable with low variability. Nevertheless, the method was not accurate enough when they analyzed samples consisting of more than 10 individual contributors. Several authors (Dalvit *et al.*, 2008; Fernandez *et al.*, 2013; Heaton *et al.*, 2014) reported the use of microsatellite markers and SNPs as potential tool for meat individual traceability and breed traceability on single meat cut. To the best of our knowledge, this work was the first to extend the potential use of microsatellite markers for traceability purpose on dairy products. In fact, nowadays, useful markers for this purpose were identified by looking at mutations in genes determining the most important traits that differentiate the breeds, as the coat colour (Russo *et al.*, 2007; Fontanesi *et al.*, 2011). Moreover, our results confirmed the absence of adulteration in the analyzed Girgentana dairy products and suggested that it could be possible to identify "foreign" alleles even if they are present with low frequency.

One relevant aspect when studying this topic is the knowledge of population structure and genetic relationship for the breeds involved in the traceability system (Dalvit *et al.*, 2008). The positive results obtained in this were also due to the genetic separation of Girgentana, from the other goat breeds for the differences in breeding system and origin (Siwek *et al.*, 2010).

The results can represent a first deterrent against fraud and an important tool for the valorization of Girgentana breed and for authentication of cheese obtained from Girgentana milk only.

2.5. Conclusion

Current certification system is actually based on several information flows that characterize production processes rather than product itself. The possibility of certifying origin and identity of dairy products, through their characterization, could provide the development of marginal areas as well as the conservation of Sicilian local breeds. The present study reported for the first time the potential application of microsatellite markers in a

genetic traceability system for dairy products. Considering our results, FCB20, SRCRSP5, and TGLA122 microsatellite markers could be applied in a genetic traceability system of Girgentana dairy products in order to detect adulteration due to Maltese and Derivata di Siria goat breeds.

Acknowledgements

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Table 1: Animals used to prepare DNA pools. IDs=individuals, GIR =Girgentana, MAL= Maltese, DdS= Derivata di Siria goat breeds.

DNA pools						
Total IDs	IDs GIR	IDs MAL+DdS	ratio	GIR (%)	MAL+DdS (%)	
20	10	10	1:1	50	50	
40	30	10	3:1	66.6	33.3	
60	50	10	5:1	80	20	
80	70	10	7:1	87.7	14.3	
100	90	10	9:1	90	10	

Table 2: Microsatellite markers panel information.

Locus	Primer sequence	Chromosome	Range	Multiplex-PCR
FCB48	FW: GACTCTAGAGGATCGCAAAGAACCAG RV: GAGTTAGTACAAGGATGACAAGAGGCAC	17	145-175	1
FCB20	FW: GGAAAACCCCCATATATACCTATAC RV: AAATGTGTTTAAGATTCCATACATGTG	2	85-115	
BRN	FW: CCTCCACACAGGCTTCTCTGACTT RV: CCTAACTTGCTTGAGTTATTGCCC	7	130-165	
CSRD247	FW: GGACTTGCCAGAACTCTGCAAT RV: CACTGTGGTTTGTATTAGTCAGG	14	226-246	
SRCRSP0005	FW: GGACTCTACCAACTGAGCTACAAG RV: TGAAATGAAGCTAAAGCAATGC	18	150-185	2
OLADRB	FW: CTGCCAATGCAGAGACACAAGA RV: GTCTGTCTCCTGTCTTGTCATC	20	260-300	
SRCRSP0008	FW: TGCGGTCTGGTTCTGATTTCAC RV: CCTGCATGAGAAAGTCGATGCTTAG	?	215-250	
INRA104	FW: AACATTTCAGCTGATGGTGGC RV: TTCTGTTTTGAGTGGTAAGCTG	20	135-155	
OARAE54	FW: TACTAAAGAAACATGAAGCTCCCAC RV: GGAAACATTTATTCTTATTCCTCAGTG	25	110-145	
MB099	FW: CTGGAGGTGTGTGAGCCCCATTTA RV: CTAAGAGTCGAAGGTGTGACTAGG	1	178-194	

Table 2: continued

Locus	Primer sequence	Chromosome	Range	Multiplex-PCR
BM1329	FW: TTGTTTAGGCAAGTCCAAAGTC RV: AACACCGCAGCTTCATCC	6	155-200	3
ETH225	FW: GATCACCTTGCCACTATTTCCT RV: ACATGACAGCCAGCTGCTACT	9 (Oar)	130-160	
MCM73	FW: CTCTTCATTCTGCAAAAGTTTGTCAC RV: GCTTGTGAGATGAACAATAAGTCATAGG	4	105-135	
FBC11	FW: GCAAGCAGGTTCTTTACACTAGCACC RV:GGCCTGAACTCACAAGTTGATATATCTATCAC	2	140-165	
TCRGC4	FW: AGAACAAATATCTGGAATGGTGATGCT RV: TGCTATAGGATGACATGAAGGCAAAT	4	260-320	4
STAT5B*	FW: TTGGCGGAAATGAGCTGGTGTTTC RV: TCCGTCCTGAAGTGATGTTTCCCT	AJ005638.1	260-320	
INRA023	FW: GAGTAGAGCTACAAGATAAACTTC RV: TAACTACAGGGTGTTAGATGAACTC	3 (Btau)	180-230	
SRCRSP0024	FW: AGCAAGAAGTGTCCACTGACAG RV: TCTAGGTCCATCTGTGTTATTGC	2	140-170	
TGLA122	FW: CCCTCCTCCAGGTAAATCAGC RV: AATCACATGGCAAATAAGTACATA	AC223929	125-155	5
MCM64	FW: TACAGTCCATGGGGTCACAAGAG RV: TCTGAATCTACTCCCTCCTCAGAGC	2	125-165	

Table 3: Number of alleles (k), observed (Ho) and expected (He) heterozygosity, polymorphic information content (PIC), and summary statistics for the 19 polymorphic microsatellite markers.

Locus	k	Ho	He	PIC
FCB48	8	0.777	0.793	0.763
FCB20	9	0.754	0.783	0.749
BRN	12	0.735	0.860	0.844
CSRD247	7	0.884	0.719	0.674
SP05	11	0.728	0.766	0.731
OLADRB	11	0.732	0.822	0.799
SP08	9	0.540	0.603	0.536
INRA104	3	0.430	0.459	0.369
OARAE54	9	0.467	0.565	0.540
MB099	5	0.214	0.297	0.264
MCM73	7	0.524	0.624	0.582
FCB11	8	0.563	0.827	0.802
BM1329	10	0.674	0.674	0.642
ETH225	3	0.148	0.161	0.153
INRA023	8	0.624	0.687	0.631
SP24	11	0.518	0.596	0.566
TCRGC4	6	0.705	0.706	0.653
TGLA122	11	0.485	0.535	0.504
MCM64	11	0.556	0.810	0.788
Mean±S.D.	8.368±2.692	0.582±0.187	0.647±0.185	0.610±0.188
Number of loci	19			
Total number of allele	159			
Total number of individuals	338			
Mean proportion of individuals typed	0.963			

Table 4: Private allele (frequencies in brackets) in the three goat breeds: Girgentana (GIR), Maltese (MAL), and Derivata di Siria (DdS). Alleles in bold could be used for traceability purpose for their presence in MAL and DdS and, at the same time, their absence in GIR.

	Breed		
Locus	*GIR*	*MAL*	*DdS*
FCB48	154 (0.0284)	**156 (0.0854)**	**156 (0.1250)**
FCB20		93 (0.0488)	
		105 (0.0122)	
		109 (0.0122)	**109 (0.0303)**
BRN	156 (0.1060)	166 (0.0128)	
	158 (0.0682)		
	160 (0.0076)		
CSRD247		246 (0.2927)	
SRCRSP05	159 (0.0076)	**177 (0.2195)**	**177 (0.1364)**
		181 (0.0732)	
		183 (0.0732)	
OLADRB	269 (0.0377)	**291 (0.0122)**	**291 (0.0156)**
	293 (0.0139)		
SRCRSP08		228 (0.0610)	
		230 (0.0366)	**230 (0.0606)**
			236 (0.0152)
			240 (0.0152)
OARAE54	130 (0.0511)	128 (0.0244)	
MB099		157 (0.0122)	
		187 (0.0244)	**187 (0.0781)**
MCM73	122 (0.0153)		
	132 (0.0118)		
FCB11		155 (0.1625)	
		161 (0.0125)	
BM1329		163 (0.0139)	
ETH225		**145 (0.2073)**	**145 (0.0152)**
	149 (0.0760)		
SRCRSP24	144 (0.0019)	150 (0.0854)	162 (0.0156)
			168 (0.0156)
TCRGC4			173 (0.0152)
TGLA122	133 (0.0153)	139 (0.0385)	
	145 (0.0019)		
	147 (0.0210)		
		151 (0.0769)	**151 (0.0606)**
		197 (0.0128)	
MCM64	149 (0.0344)	137 (0.0122)	
	151 (0.0496)		

Table 5: Microsatellite markers useful for traceability of Girgentana goat dairy products. MAL=Maltese, DdS=Derivata di Siria goat breed.

Locus	Allele	Frequency MAL	Frequency DdS
FCB48	156	0.0854	0.1250
FCB20	109	0.0122	0.0303
SRCRSP5	177	0.2195	0.1364
OLADRB	291	0.0122	0.0156
SRCRSP8	230	0.0366	0.0606
MB099	187	0.0244	0.0781
ETH225	145	0.2073	0.0152
TGLA122	151	0.0769	0.0606

Figure 1: Electropherograms of FCB20 (1a) and TGLA122 (1b) microsatellite markers of DNA from one heterozygous and one homozygous individual. The arrowed peaks are "stutter" 2 and 4 base-pairs smaller than the respective alleles; 1c shows electropherogram of FCB48 microsatellite marker of 4 individuals.

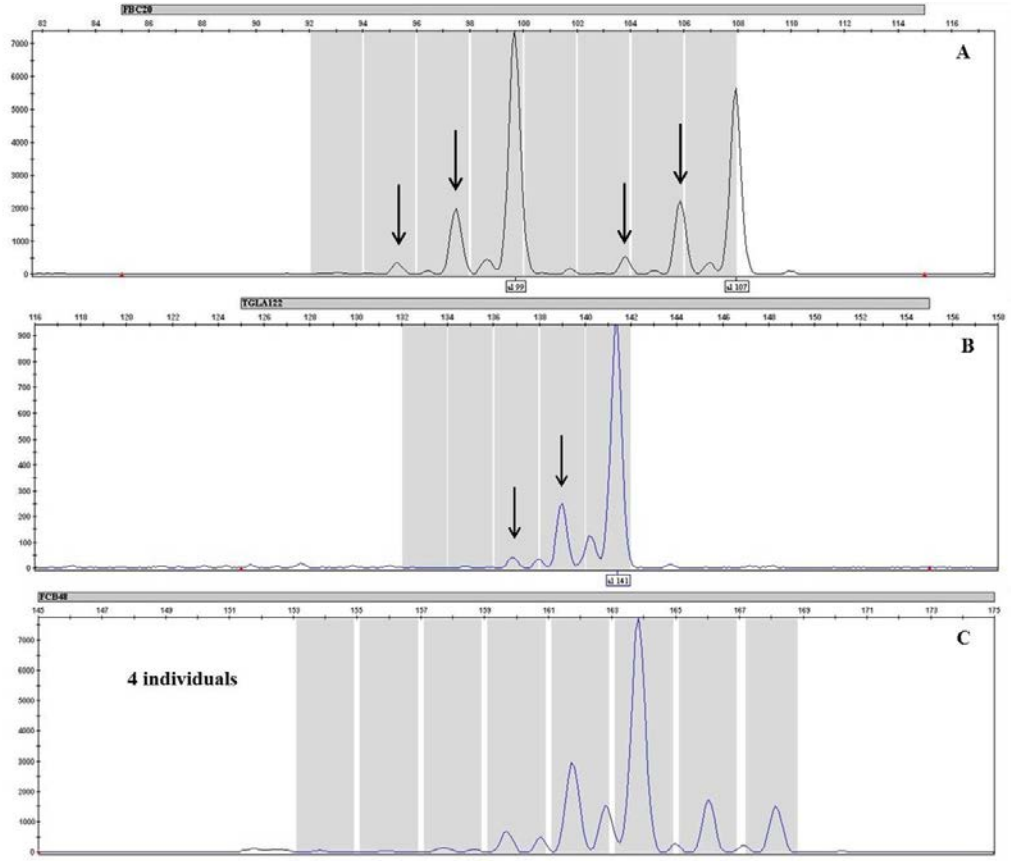


Figure 2: Electropherograms of FCB48 microsatellite marker of three DNA pools of single breed contained 50 individuals with known genotypes.

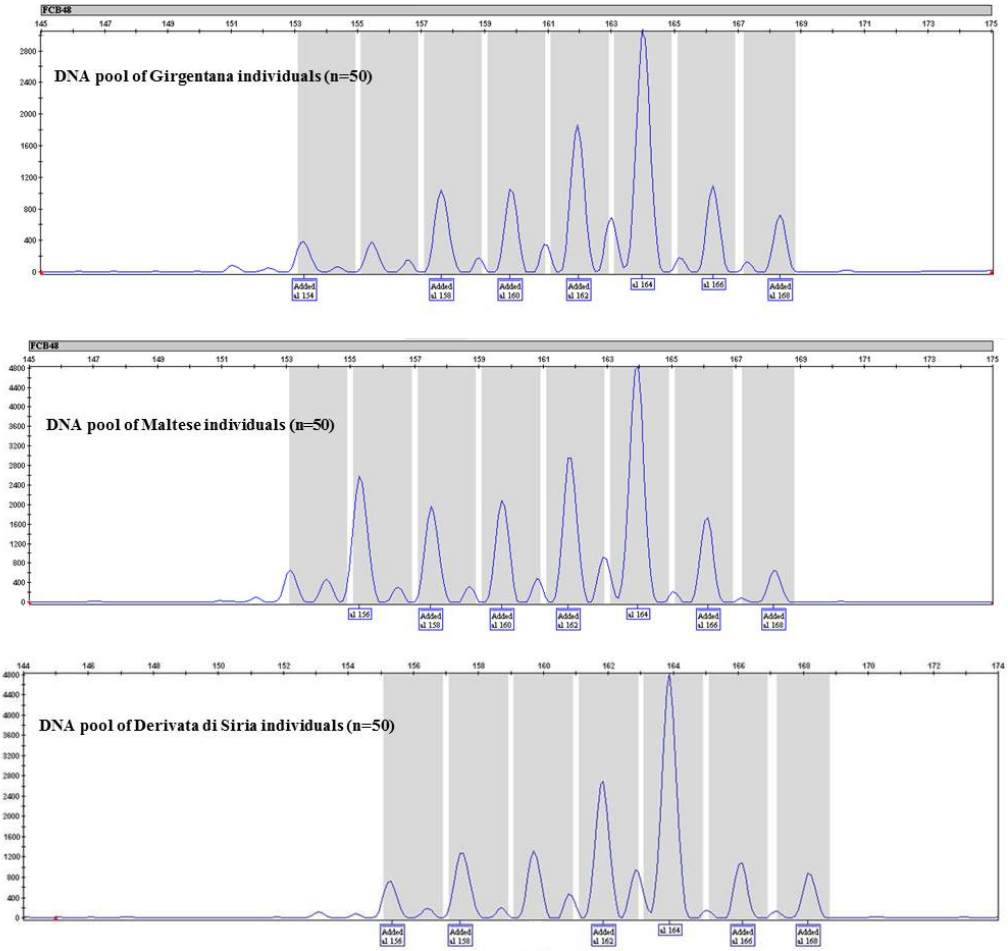


Figure 3: Electropherogram of FCB48 microsatellite marker of DNA pool of the three breeds with 5:1 ratio. The arrowed allele was present within pool with a frequency value of 0.0333.

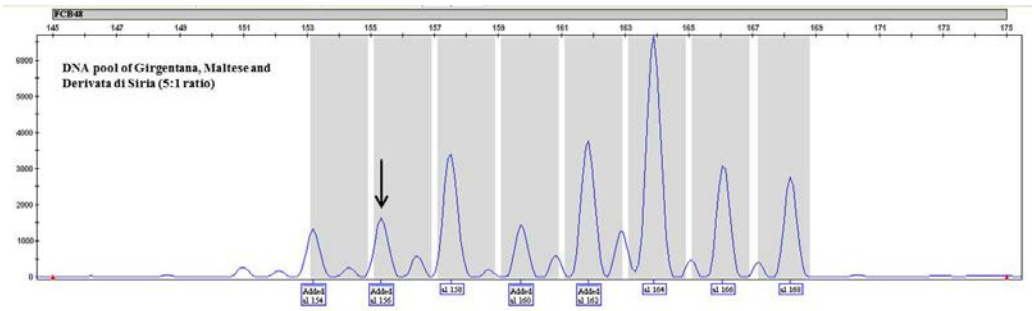


Figure 4: Electropherograms of FCB20 microsatellite marker of DNA from pool of the three breeds with 9:1 ratio (above) and from Girgentana cheese (below). The arrowed allele (109 bp) was present within the DNA pool with a frequency of 0.01 and it was useful for traceability purpose of Girgentana dairy products.

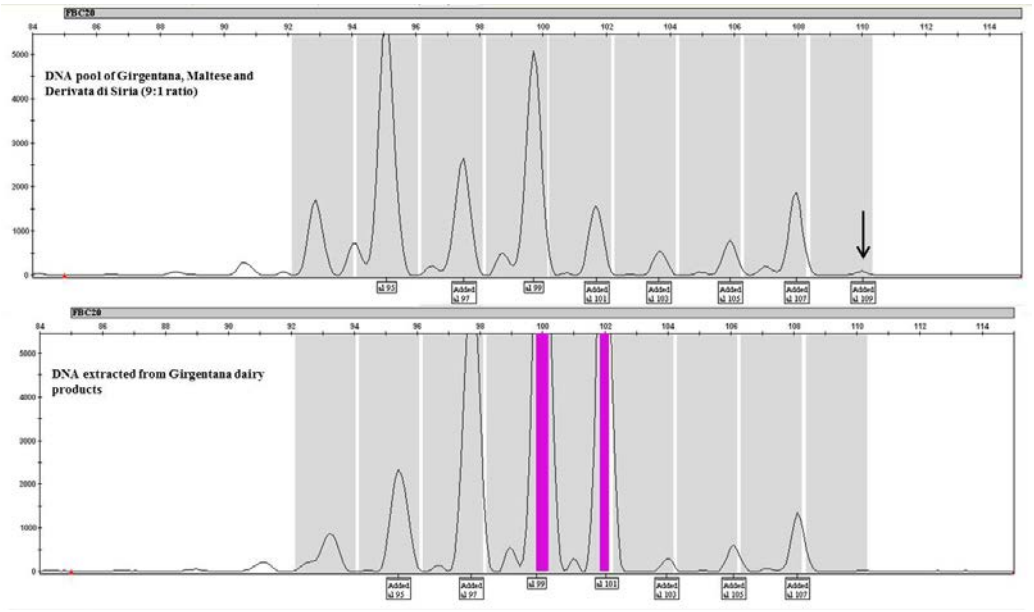


Figure 5: Electropherograms of SRCRSP5 microsatellite marker of DNA from pool of the three breeds with 9:1 ratio (above) and from Girgentana cheese (below). The arrowed allele (177 bp) was present within the DNA pool with a frequency of 0.01 and it was useful for traceability purpose of Girgentana dairy products.

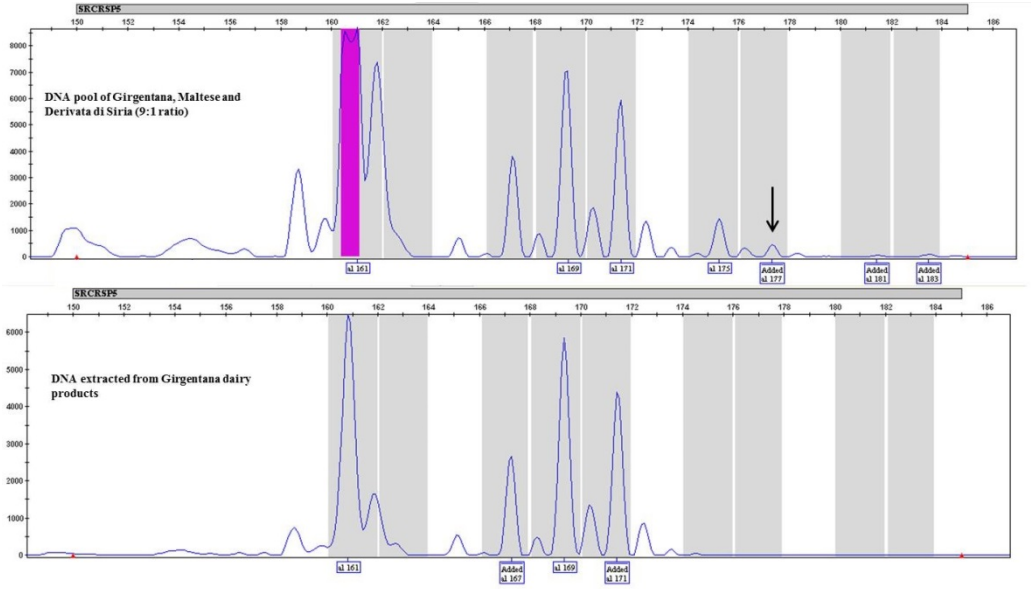
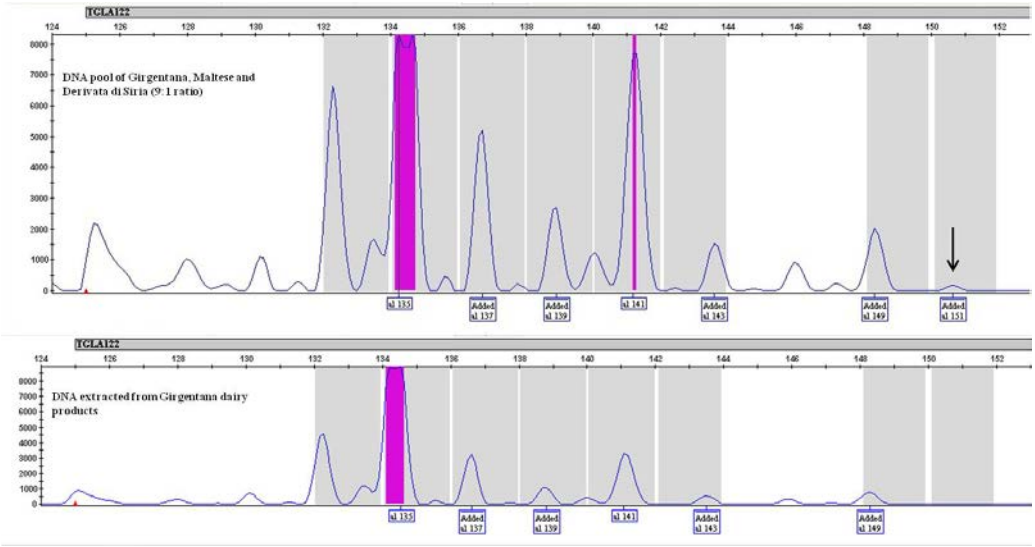


Figure 6: Electropherograms of TGLA122 microsatellite marker of DNA from pool of the three breeds with 9:1 ratio (above) and from Girgentana cheese (below). The arrowed allele (151 bp) was present within the DNA pool with a frequency of 0.01 and it was useful for traceability purpose of Girgentana dairy products.



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Chapter 3

Development of multiplex-PCR protocol to amplify 12S and 16S rRNA genes of mtDNA for traceability of Sicilian mono-species dairy products

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Abstract

For a rapid, specific and sensitive identification of cattle's, sheep's and goat's milk in mono-species Sicilian dairy products, specie-specific multiplex-PCR protocol was developed. DNA from blood and experimental cheeses of Sicilian autochthonous breeds were extracted to amplify the *12S* and *16S rRNA* genes of the mitochondrial DNA. The use of specie-specific primers for bovine, caprine and ovine species, after electrophoresis on agarose gel, yielded fragments of different length, respectively of 256 bp, 326 bp and 172 bp. In the next step, amplification by *multiplex*-PCR of binary mixtures of DNA pools showed a sensitive threshold of 0.1% for bovine/caprine and ovine/caprine DNA mixtures and 0.5% for bovine/ovine and ovine/bovine DNA mixtures. Finally, multiplex-PCR assay was applied to bovine/ovine experimental cheeses, to detect the minimum threshold of a species in a cheese formed by milk of this two species. The results of this work showed a sensitive threshold of 0.1% for bovine/ovine cheeses and 0.5% for ovine/bovine cheeses. The proposed assay represents a rapid and straightforward method for the detections of adulteration in mono-species dairy products.

Keywords: mitochondrial DNA, traceability, dairy products, autochthonous Sicilian breeds

3.1 Introduction

In recent years, many studies were performed in order to identify the species of origin in animal products (Bottero *et al.*, 2003; Ceriotti *et al.*, 2003; Mafra *et al.*, 2004, 2007; Murugaiah *et al.*, 2009; Wang *et al.*, 2010 ; Dalmasso *et al.*, 2011; Zachar *et al.*, 2011). The aim was not only to protect consumers, since the enormous consumption of these products and all food scandals that have occurred (Bánáti *et al.*, 2011), but also to protect producers. In particular, with regard to dairy products, to trace the origin of species is of fundamental importance considering the frequent allergic reactions to milk proteins and also the possible frauds to which these products are subject. Therefore, species identification in dairy sector is important not only to safeguard public health but also to verify compliance with the Production Regulations of many typical dairy products (PDO/PGI) (Maudet *et al.*, 2001; Bottero *et al.*, 2003).

The Sicilian dairy sector is characterized by several typical dairy products resulting of the links between product-territory, territory-breed/species and breed/species-product. Therefore, the development of traceability systems can lead to the promotion of these local and traditional cheeses (PDO and PGI), and thereby to the conservation and enhancement of the breeds of origin and/or local populations.

The most common fraud in dairy sector is represented by the admixture of milks from different species, resulting in mislabeling of protected designation of origin (PDO) products. To avoid the possible fraudulent substitution of milk it is necessary to develop analytical procedures able to detect frauds and protect the consumers from mislabeling (De la Fuente *et al.*, 2005; Mafra *et al.*, 2007).

Several analytical methods have been applied for species identification in milk and milk products including immunological (Hurley *et al.*, 2004; Lòpez-Calleja *et al.*, 2007; Zeleňáková *et al.*, 2008), electrophoretic (Chianese *et al.*, 1990; Molina *et al.*, 1999) and chromatographic (De Noni *et al.*, 1996; Braciari *et al.*, 2000) techniques. Some of these studies are reference points for European Community in order to detect cow's milk.

However, all these methods cannot always distinguish animal species after heat treatment of milk (López-Calleja *et al.*, 2005). On the contrary, it is possible to extract amplifiable DNA from pasteurized, filtered, and ultrafiltered milk (Branciari *et al.*, 2000).

In the last years, several PCR approaches have been developed in order to differentiate milk from different species and to identify adulteration with non-compliant milk product specifications (Plath *et al.*, 1997; Mafra *et al.*, 2004; Mayer, 2005).

Molecular traceability is able to detect adulteration of dairy products since it is based on analysis of DNA which represents an unalterable and indelible label (Bottero *et al.*, 2011). Among the genetic markers used for species traceability of dairy products, the mitochondrial DNA (mtDNA) was mainly studies due to its unique characteristics among species (Bottero *et al.*, 2002, 2003; Lopez-Calleja *et al.*, 2005).

The aim of this work was to develop a multiplex-PCR protocol to amplify *12S* and *16S rRNA* genes of mtDNA for traceability of Sicilian mono-species dairy products.

3.2 Materials and Methods

3.2.1 Blood sampling and DNA extraction

A total of 300 individuals were sampled and 10 ml of blood were collected from jugular vein, through vacutainer with EDTA. The sampled autochthonous breeds were Modicana and Cinisara cattle breeds; Comisana, Pinzirita and Valle del Belice ovine breeds, and Girgentana, Maltese and Derivata di Siria goat breeds. The individuals were chosen unrelated and from different farms located in Sicilian provinces.

Genomic DNA was extracted from blood using a *salting out* method and checked for quantity and quality NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). All samples were diluted and stored at 4°C until analysis.

3.2.2 Simplex-PCR

In a preliminary step, a simplex-PCR protocol was developed to amplify *12s* and *16s rRNA* genes in ovine, bovine and caprine species using specific primers proposed by Bottero

et al. (2003). Amplified fragments were 172 bp, 256 bp, and 326 bp, respectively, as showed in Table 1.

Amplifications were done in a final volume of 25 µl containing 1 µM of each primer, 0.8 mM of dNTP Mix, 1U of Taq DNA polymerase (Fermentas, Hanover, MD, USA), 1X PCR buffer with KCl, 3 mM $MgCl_2$, and approximately 100 ng of genomic DNA. Thermal cycling conditions were an initial denaturation at 94°C for 5 min, 35 cycles at 94°C, 53°C and 72°C for 2 min and 30 sec each, and a final extension at 72°C for 5 min. The PCR products were checked by electrophoresis on 2% agarose gel stained with ethidium bromide.

3.2.3 Multiplex-PCR and experimental DNA pools preparation

The next step involved the development of multiplex-PCR protocol to test simultaneously the species-specificity of the three primers sets (Bottero *et al.*, 2003) (Table 1), first of all on individual samples and then on DNA pools consisting of DNA of each species.

For DNA pools preparation, samples were chosen after positive amplification in simplex-PCR and in particular 10 cows, 10 goats and 10 sheep samples were used. For each sample, 10 µl of diluted DNA (50ng/µl) were used to constitute a pool with a final volume of 100 µl.

The multiplex-PCRs of individual samples and mono-species DNA pools were carried out in a final volume of 30 µl containing different concentration (from 0.5 µM to 0.8 µM) of each primers set, 0.8 mM of dNTP Mix, 1U of Taq DNA polymerase (Fermentas, Hanover, MD, USA), 1X PCR buffer with KCl, 3.5 mM $MgCl_2$, and approximately 200 ng of genomic DNA. Thermal cycling conditions were the same as simplex-PCR protocol. The amplification products were checked always by electrophoresis on 2% agarose gel stained with ethidium bromide.

After these analyses, DNA pools containing mixtures of DNA from two species were prepared as shown in Table 2. Multiplex-PCR protocol was applied to evaluate fragment amplification on agarose gel and detection limit of each mixture.

3.2.4 Reference cheeses samples and DNA extraction

Reference experimental cheeses were prepared in laboratory using mixtures of bovine and ovine raw milk from Sicilian local dairy farms. Experimental cheeses were prepared according to the classical Sicilian cheese-making procedure from raw milk starting from mixture with known concentration of different milks.

For bovine/ovine experiment, three mixtures were prepared: the first (lane 1, Fig. 4) with 0.1%, the second (lane 2, Fig. 4) with 0.5% and the third one (lane 3, Fig. 4) with 1% of ovine milk in bovine cheeses. For ovine/bovine experiment, only two mixtures were prepared: the first with (lane 1, Fig. 5) 0.5% and the second one (lane 2, Fig. 5) with 1% of bovine milk in ovine cheeses.

For DNA extraction the CTAB method of ISO 21571:2005(E) was used, making some changes to the protocol. Five samples from each cheese were collected for DNA extraction and used as technical and biological replicates. The concentration of extracted DNA was checked using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and samples were stored at 4°C until use.

3.3 Results and discussion

In this work, the mitochondrial *12S* and *16S rRNA* genes were used as a targets to apply multiplex-PCR protocol for species identification in Sicilian mono-species dairy products.

Figure 1 showed the perfect distinction and separation of three DNA fragments of ovine, bovine and caprine mtDNA genes of 172 bp, 256 bp, and 326 bp, respectively. These results were obtained from individual samples of each species. Our amplification results were in agreement with those reported by other authors on different ovine, bovine and caprine breeds (Mafra *et al.*, 2004, 2007), which used primer pairs proposed by Bottero *et al.* (2003). Good results of simplex-PCR results were also confirmed in multiplex-PCR of mono-species DNA pools created in laboratory and consisting of 10 individuals each (Figure2).

Figure 3 showed the amplification results of mixtures of DNA pools reported in Table 1 and created using different DNA percentages from each species. Our results underlined that

the detection threshold of bovine/ovine and ovine/bovine DNA pools (Figures 3a and 3b) was 0.5% while for bovine/caprine and ovine/caprine DNA pools (Figures 3c and 3d) was 0.1%.

The usefulness of multiplex-PCR protocols on species traceability of dairy products was verified on several experimental cheeses and amplification results were reported in Figures 4 and 5.

After electrophoresis on agarose gel, it was possible to assess a 0.1 % detection threshold in cheeses obtained from bovine/ovine mixture, and a 0.5% threshold in cheeses from ovine/bovine mixture.

Our results are in agreement with those reported by other authors focused on milk adulteration for cheese-making (Bottero *et al.*, 2003; Mafra *et al.*, 2004, 2007; López-Calleja *et al.*, 2005; Darwish *et al.*, 2009).

Different detection thresholds between genomic pools (0.5%) and cheeses (0.1%) in bovine/ovine experiment could be probably due to the differences between manually created DNA pools and experimental cheeses made by known amount of milk but unknown DNA percentage contribution.

Cozzolino *et al.* (2001) considered 5% detection limit as sufficient for the proof of undeclared milk components, whereas adulteration of milk by less than 5% lacks any economic effects.

3.4 Conclusions

The chosen laboratory protocol could allow us to detect milk adulterations in typical Sicilian mono-species cheeses labeled as PDO or PGI. The analysis method gave satisfactory results and this simplified laboratory work and reduced costs. Therefore, it could be useful to use this multiplex-PCR protocol in species traceability system.

The ability to detect low levels of contaminating milk could be interesting to safeguard not only mono-species dairy products protected by European labels but also allergic or intolerant subjects.

Table 1: Oligonucleotides used as PCR primers for amplification mtDNA genes

Species	Oligonucleotide primers	Amplicons
Ovis aries	FW: 5'-ATATCAACCACACGAGAGGAGAC-3' RV: 5'-TAAACTGGAGAGTGGGAGAT-3'	172 bp
Bos taurus	FW: 5'-GTACTACTAGCAACAGCTTA-3' RV: 5'-GCTTGATTCTCTTGGTGTAGAG-3'	256 bp
Capra hircus	FW: 5'-CGCCCTCCAAATCAATAAG-3' RV: 5'-AGTGTATCAGCTGCAGTAGGGTT-3'	326 bp

Table 2: Different percentage ratios for DNA pools containing mixtures of ovine (O), bovine (B) and caprine (C) DNA. Amplification results are shown in Figure 3 (Ref. Fig.3).

Ref. Fig. 3	B+O (%)	O+B (%)	B+C (%)	O +C (%)
1	50.0+50.0	50.0+50.0	50.0+50.0	50.0+50.0
2	75.0+25.0	75.0+25.0	75.0+25.0	75.0+25.0
3	90.0+10.0	90.0+10.0	90.0+10.0	90.0+10.0
4	99.5+0.5	99.5+0.5	99.5+0.5	99.5+0.5
5	99.9+0.1	99.9+0.1	99.9+0.1	99.9+0.1

Figure 1: Amplification fragments of individual sheep (172 bp), cow (256 bp) and goat (326 bp) samples. Ladder 100 bp in the first and the last lane.

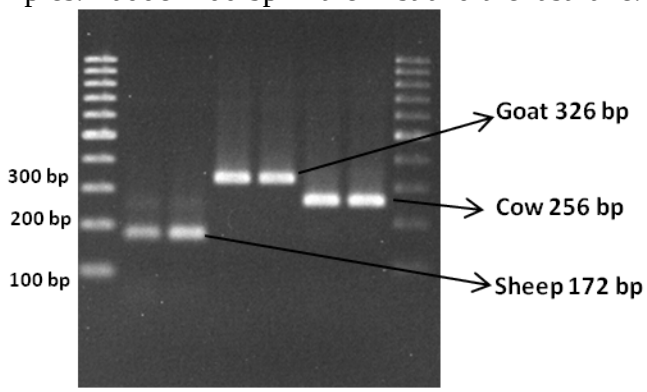


Figure 2: Amplification fragments of mono-species DNA pools consisting of 10 individual ovine (172 bp), bovine (256 bp) and caprine (326 bp) samples. Ladder 100 bp in the first lane.

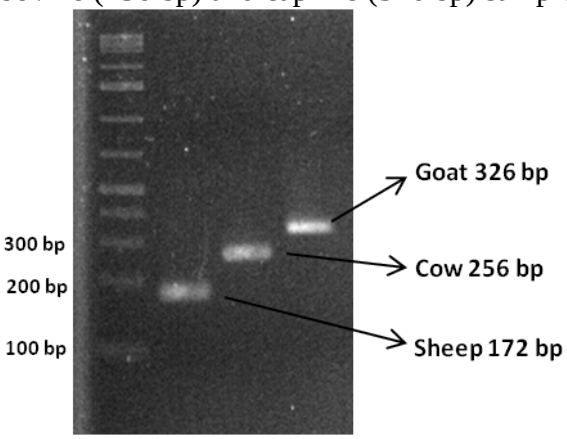


Figure 3: Amplification fragments of bovine (256 bp), ovine (172 bp) e caprine (326 bp) DNA pools obtained using different percentages of each breed (see Table 1). Ladder 100 bp in the first and the last line.

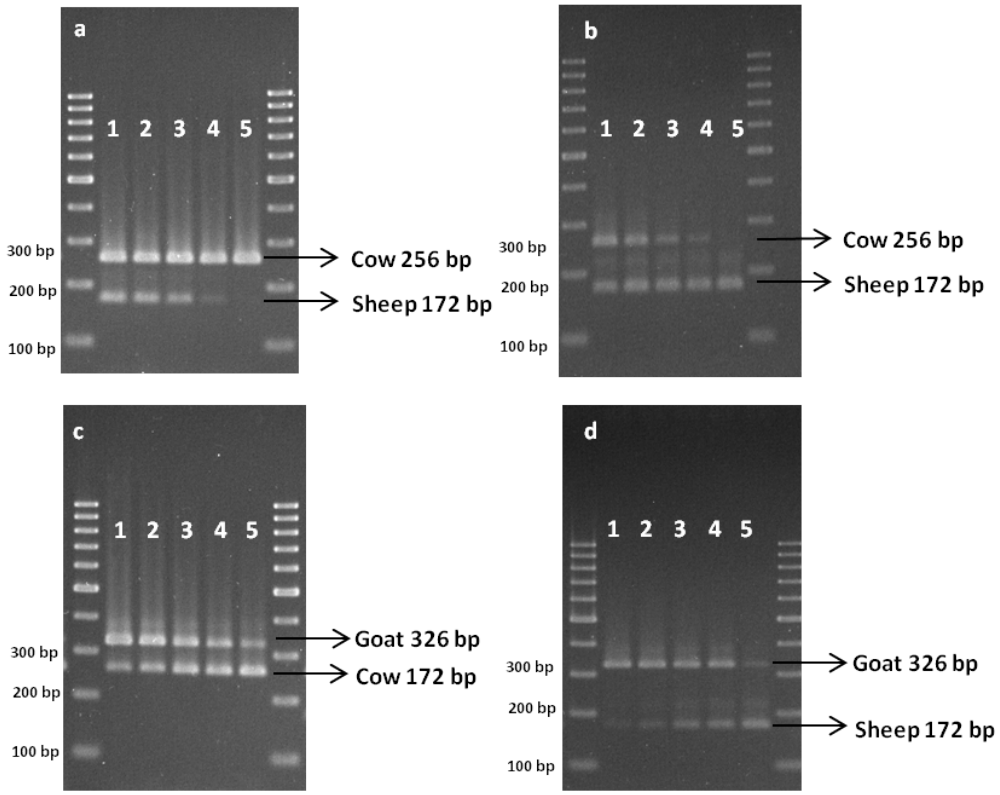


Figure 4: Amplification fragments of DNA extracted from bovine cheeses (256 bp) with 0.1% (lane 1), 0.5% (lane 2) and 1% (lane 3) of ovine milk (172 bp). Ladder 100 bp in the first lane.

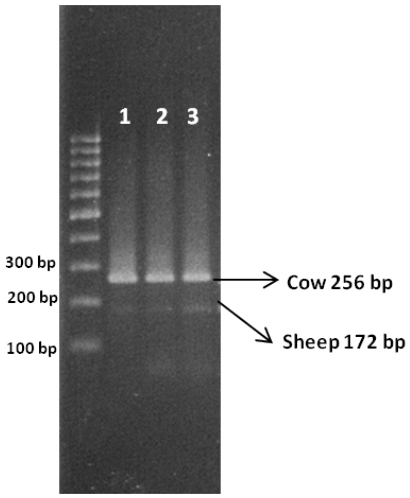
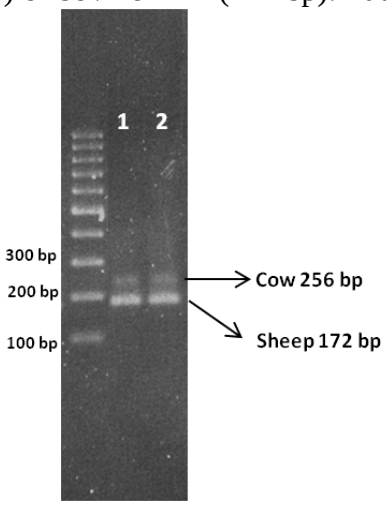


Figure 5: Amplification fragments of DNA extracted from ovine cheeses (256 bp) with 0.5% (lane 1) and 1% (lane 2) of bovine milk (172 bp). Ladder 100 bp in the first lane.



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Chapter IV

General Conclusions

In this thesis, different molecular technologies were applied for characterization and valorization of Sicilian autochthonous breeds and for genetic traceability of their typical dairy products. In particular, microsatellite markers and mitochondrial DNA were used for their specificity in order to develop molecular protocols useful for authentication and traceability of Sicilian dairy products.

Current certification system is actually based on several information flows that characterize production processes rather than product itself. The possibility of certifying origin and identity of dairy products, through their characterization, could provide the development of marginal areas as well as the conservation of Sicilian local breeds.

In Chapter 2, individual samples of Girgentana, Maltese and Derivata di Siria Sicilian autochthonous goat breeds were analyzed by 20 microsatellite markers in order to identify breed specific microsatellite markers that can be used for traceability of Girgentana dairy products.

The results of this Chapter reported for the first time the potential application of microsatellite markers in a genetic traceability system of goat dairy products. FCB20, SRCRSP5, and TGLA122 microsatellite markers could be applied in genetic traceability system of Girgentana dairy products in order to detect adulteration due to Maltese and Derivata di Siria goat milk.

In Chapter 3, species-specific multiplex-PCR protocols, based on amplification of *12S* and *16S rRNA* genes, for identification of cattle's, sheep's and goat's milk in mono-species Sicilian dairy products were reported in order to use a rapid and straightforward method for the detections of adulteration in typical Sicilian mono-species cheeses labeled as PDO or PGI.

Application of these molecular markers gave satisfactory results both on DNA extracted from blood and from complex matrices, and this simplified laboratory work and reduced costs. Therefore, it could be useful to use these markers as potential tools in breed and species traceability system.

The ability to detect low levels of contaminating milk could be interesting to safeguard not only mono-species and mono-breed dairy products protected by European labels but also allergic or intolerant subjects.

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Genome wide linkage disequilibrium and genetic structure in Sicilian dairy sheep breeds

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Abstract

Background: The recent availability of sheep genome-wide SNP panels allows providing background information concerning genome structure in domestic animals. The aim of this work was to investigate the patterns of linkage disequilibrium (LD), the genetic diversity and population structure in Valle del Belice, Comisana, and Pinzirita dairy sheep breeds using the Illumina Ovine SNP50K Genotyping array.

Results: Average r^2 between adjacent SNPs across all chromosomes was 0.155 ± 0.204 for Valle del Belice, 0.156 ± 0.208 for Comisana, and 0.128 ± 0.188 for Pinzirita breeds, and some variations in LD value across chromosomes were observed, in particular for Valle del Belice and Comisana breeds. Average values of r^2 estimated for all pairwise combinations of SNPs pooled over all autosomes were 0.058 ± 0.023 for Valle del Belice, 0.056 ± 0.021 for Comisana, and 0.037 ± 0.017 for Pinzirita breeds. The LD declined as a function of distance and average r^2 was lower than the values observed in other sheep breeds. Consistency of results among the several used approaches (Principal component analysis, Bayesian clustering, F_{ST}, Neighbor networks) showed that while Valle del Belice and Pinzirita breeds formed a unique cluster, Comisana breed showed the presence of substructure. In Valle del Belice breed, the high level of genetic differentiation within breed, the heterogeneous cluster in Admixture analysis, but at the same time the highest inbreeding coefficient, suggested that the breed had a wide genetic base with inbred individuals belonging to the same flock. The Sicilian breeds were characterized by low genetic differentiation and high level of admixture. Pinzirita breed displayed the highest genetic diversity (He, N_e) whereas the lowest value was found in Valle del Belice breed.

Conclusions: This study has reported for the first time estimates of LD and genetic diversity from a genome-wide perspective in Sicilian dairy sheep breeds. Our results indicate that breeds formed non-overlapping clusters and are clearly separated populations and that Comisana sheep breed does not constitute a homogenous population. The information generated from this study has important implications for the design and applications of association studies as well as for development of conservation and/or selection breeding programs.

Keywords: OvineSNP50K BeadChip, Sicilian sheep breeds, Linkage disequilibrium, Genome structure

Background

The application of recently developed genomic technology, such as high-density single nucleotide polymorphism (SNP) arrays, has great potential to increase our understanding on the genetic architecture of complex traits, to improve selection efficiency in domestic animals through genomic selection [1], and to conduct association studies [2]. However, to optimally plan whole-genome association studies, it is crucial to know the extent of linkage disequilibrium (LD), the non-random association of alleles at different loci in the genome. In fact, the extent of LD is often used to determine the optimal number of markers required for fine mapping of quantitative trait loci (QTL) [3], for genomic selection [4], and to understand the evolutionary history of the populations [5]. With this in mind, it is important to quantify the extent of LD within different breeds as this is likely to have an impact on the success of gene mapping experiments [6]. Knowledge concerning the extent of genetic diversity, as the level of inbreeding and population structure is critical for

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Development and validation of RP-HPLC method for the quantitative estimation of αs_1-genetic variants in goat milk

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ABSTRACT

A high-performance liquid chromatographic (HPLC) method was developed and validated for separation and quantification of the most common genetic variants of αs_1-casein in goat's milk, to evaluate the effect of αs_1-casein polymorphisms on casein content.

Chromatography was carried out by binary gradient technique on a reversed-phase C8 Zorbax column and the detection was made at a wavelength of 214 nm. The procedure was developed using individual raw milk samples of Girgentana goats. For calibration experiments, pure genetic variants were extracted from individual milk samples of animals with known genotypes, considering that commercial standards for goat genetic variants were not available. The data obtained for Girgentana goat breed showed that A, B, F variants were alleles associated with a content of αs_1-casein in milk of 3.2 ± 0.4, 5.4 ± 0.5 and 0.7 ± 0.1 g/L, respectively, whereas N variant was a 'null' allele associated with the absence of αs_1-casein in milk.

1. Introduction

In the milk of ruminants, more than 95% of proteins are synthesized by six structural genes, four caseins (αs_1-, β-, αs_2- and κ-caseins) and two whey proteins (α-lactalbumin and β-lactoglobulin). Among Ca-sensitive caseins (αs_1, β, and αs_2), the αs_1-casein fraction is the most extensively investigated in goat species (Martin, Szymanowska, Zwierzchowski, & Leroux, 2002; Rijnkels, 2002). The extensive polymorphism at αs_1-casein locus has been shown to affect not only the quantity of casein in goat milk, but also the structural and nutritional characteristics and technological properties of milk. In fact, polymorphism associated with a quantitative variability in casein synthesis has a significant effect on coagulation properties, micelle size and mineralsation, cheese yield, and sensory attributes (Ramunno et al., 2007). So far, at least 17 codominant alleles have been identified at DNA level, which are associated with different expression levels of αs_1-casein in milk. A first group of alleles (A, B1, B2, B3, B4, C, H, L and M) are associated with a high content of αs_1-casein (about 3.5 g/L), alleles I and E are associated with an intermediate content (about 1.1 g/L), and alleles D, F, and G with a low level (about 0.45 g/L) of this protein in milk. Alleles αs_1-casein N, 01 and 02 are 'null' alleles and have been associated with the absence of αs_1-casein in milk (Bevilacqua et al., 2002; Chianese, Ferranti, Garro, Mauriello & Addeo, 1997; Grosclaude, Mahé, Brignon, Di Stasio, & Jeunet, 1987; Martin, Ollivier-Bousquet, & Grosclaude, 1999; Ramunno et al., 2005). The presence of alleles associated with "low" and "null" content of αs_1-casein in goat milk, may be interesting considering that very low levels of αs_1-casein were found to be less allergenic than milk characterised by high level of αs_1-casein (Haenlein, 2004). Hence, the quantification of different genetic variants at αs_1-casein locus became very important for the quality of milk and also for the possible valorization of the products that are linked to a specific breed (i.e. mono-breed labeled cheeses). Nowadays, a great variety of methods have been developed to analyse milk protein fractions: alkaline urea polyacrylamide gel electrophoresis (urea-PAGE) and RP-HPLC (Reversed Phase-High Performance Liquid Chromatography) for whole caseins analysis and Cation-Exchange Chromatography (CEC) of whole casein for the fractionation of the lyophilized casein (Moatsou, Samolada, Panagiotou, & Anifantakis, 2004); Capillary Zone Electrophoresis (CZE) (Brambilla, Feligini, & Enne, 2003; Valenti, Pagano, & Avondo, 2012), RP-HPLC (Bonfatti, Grigoletto, Cecchinato, Gallo, & Carnier, 2008; Clark & Sherbon, 2000), SDS–polyacrylamide gel electrophoresis (SDS–PAGE) to identify allelic polymorphisms and Rocket Immunoelectrophoresis to estimate the contents of individual caseins (Grosclaude et al., 1987); Isoelectric focusing (IEF) and RP-HPLC/Electrospray Ionisation Mass Spectrometry (ESI-MS) to analyse the protein fractions and polymorphism of caseins of goat milk (Moatsou, Moschopoulou, Mollé, Kandarakis, & Léonil, 2008;

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SHORT COMMUNICATION

Genetic characterisation of *CSN2* gene in *Girgentana* goat breed

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Abstract

Among calcium sensitive caseins, β-casein is the most abundant in goat milk, representing up to 50% of total casein content. The goat *β-casein* locus has been widely investigated and at least ten alleles have been identified in different goat breeds. The aim of this work was to investigate the polymorphisms of *β-casein* gene in *Girgentana* dairy goat breed in order to assess the genotype distribution and evaluate how frequencies have changed during the last 10 years, as genotype is known to influence technological and nutritional milk properties. Sequencing analysis and alignment of the obtained sequences of *β-casein* exon 7, showed the presence of C, C1, and A strong alleles, and 0' null allele, with frequencies of 0.597, 0.326, 0.023, and 0.054, respectively. Seven genotypic classes were found in *Girgentana* goat breed and the most frequent genotype was CC1 (0.423) followed by CC (0.326), C1C1 (0.110), and C0' (0.096). No AA nor 0'0' homozygous individuals were found. The presence of strong alleles at *CSN2* gene in *Girgentana* goat breed could be useful for the production of milk with high protein content and good cheese-making properties. Moreover, food business operators should consider the possibility of reviving interest in *Girgentana* goat milk using weak and null genotypes at *CSN2* locus to make peculiar food products, such as drinking milk.

Introduction

Caseins are the most abundant proteins in milk of ruminants and represent about 80% of total milk proteins, while the remaining part are whey proteins (mainly β-lactoglobulin and α-lactalbumin, although other whey proteins such as immunoglobulins and lactoferrin are also present). It is well known that caseins are encoded by four linked genes which form a unique cluster including *αs1-casein* (*CNS1S1*), *β-casein* (*CNS2*), *αs2-casein* (*CNS1S2*) and *κ-caseins* (*CSN3*) genes (Grosclaude *et al*., 1978; Ferretti *et al*., 1990; Rijnkels, 2002). Goat casein genes are mapped on chromosome 6 within a region that spans about 250 Kb (Hayes *et al*., 1993; Popescu *et al*., 1996). Among caseins, the β-casein is the most abundant in milk, representing up to 50% of total casein content. The goat *CSN2* encoding gene consists of nine exons ranging in size from 24 (exon 5) to 492 bp (exon 7) (Roberts *et al*., 1992; Hayes *et al*., 1993). At least, ten alleles have been identified in goat *CSN2* gene. In particular, seven of these alleles (A, A1, C, C1, E, 0, and 0') were characterised at DNA level (Rando *et al*., 1996; Persuy *et al*., 1999; Chessa *et al*., 2005, 2008; Cosenza *et al*., 2005), whereas B and D alleles were described only at protein level (Mahé and Grosclaude, 1993; Galliano *et al*., 2004). Another variant has been found by Chianese *et al*. (2007) at protein level but it was not yet characterised. Furthermore, the genetic variants A, A1, B, C, C1, D, and E are associated with a normal β-casein content in milk (about 5 g/L per allele) (Roberts *et al*., 1992; Mahé and Grosclaude, 1993; Neveu *et al*., 2002; Galliano *et al*., 2004; Cosenza *et al*., 2005; Caroli *et al*., 2006) while the two null alleles (0 and 0'), are associated with a non-detectable amount of this protein (Ramunno *et al*., 1995; Persuy *et al*., 1999).

The *Girgentana* goat is a Sicilian goat breed reared for its good dairy production. Due to sanitary policies, the size of the *Girgentana* goat breed decreased of almost 90% in 20 years, and nowadays, only 374 heads are reared in Sicily (ASSONAPA, 2013).

The aim of this work was to investigate the genetic polymorphisms of the *β-casein* gene in the *Girgentana* dairy goat breed in order to assess the genotype distribution, as it is known that genotype influences milk properties.

Materials and methods

Samples collection

A total of 196 samples, all females, of *Girgentana* goat breed were collected. The animals belonged to 10 different herds located in Sicily, among Agrigento and Palermo provinces. Samples were collected from 15 to 25 unrelated individuals per herd. About 10 mL of blood was used for genomic DNA extraction with a salting out method (Miller *et al*., 1988). After extraction, the DNA samples were quantified, using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), then they were diluted to a final concentration of 50 ng/μL in ultrapure water and stored at 4°C until use.

Amplification protocols

Different polymerase chain reaction (PCR) protocols were used to genotype A, A1, C, C1, E, and 0' alleles of goat *CSN2* gene in *Girgentana* goat breed. The 0 allele was not genotyped because it has been identified only in Creole and Pyrenean goat breeds (Persuy *et al*., 1999). The first protocol was used to amplify a 374 bp fragment of exon 7 using primers and PCR conditions by Chessa *et al*. (2005) in order to discriminate A/A1, C/C1, E, and 0' alleles. The second protocol was used to discriminate allele C to C1 amplifying a 325 bp fragment of exon 9 using primers by Chessa *et al*. (2008) and PCR conditions by Chessa *et al*. (2005) with an annealing temperature of 56°C. Finally, the polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) protocol proposed by Cosenza *et al*. (2005) was used to discriminate allele A to A1. All PCR products were checked by electrophoresis on 2% agarose gel stained with SYBR Safe (Invitrogen, Carlsbad, CA, USA).

Sequencing protocol

The PCR products were purified using PCR Product Clean-Up protocol as suggested by Fermentas (Hanover, MD, USA) using 10 U of

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Key words: *CSN2* locus, Genetic polymorphisms, Genetic evolution, *Girgentana* goat breed.

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Molecular characterization of *κ-casein* (*CSN3*) gene in *Girgentana* dairy goat breed and identification of two new alleles

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Abstract

The κ-casein fraction plays an important role in the formation, stabilization and aggregation on casein micelles and thus affects technological and nutritional properties of milk. In this study, exon 4 of *κ-casein* (*CSN3*) gene was sequenced and analyzed in *Girgentana* goat breed. Analyses of the obtained sequences showed the presence of *A*, *B*, *D*, and *G* known alleles and two new genetic variants, named *D'* and *N*. The new *D'* allele differing from *D* in one transition $G_{284}\rightarrow A_{284}$ that did not cause amino acid change. The new *N* allele differing from *A* for five SNPs T_{245}/C_{245}, G_{284}/A_{284}, G_{309}/A_{309}, G_{471}/A_{471}, and T_{591}/C_{591}, while differing from *C* for one transition $T_{583}\rightarrow C_{583}$. Comparing the amino acid sequences of *N* and *A* alleles, the first two SNPs caused no amino acid change, whereas the other SNPs produced changes Val_{65}/Ile_{65}, Val_{119}/Ile_{119}, and Ser_{159}/Pro_{159}, respectively. Comparison of *N* allele to *C* revealed the amino acid change $Val_{156}\rightarrow Ala_{156}$. The most frequent allele was *A* (0.480) followed by *B* (0.363), *D* (0.112), and *N* (0.034). The *D'* and *G* alleles were identified only in two animals and in heterozygous conditions with a very low frequency (0.005).

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