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PhD Thesis

Application of molecular markers to investigate genetic diversity in Sicilian livestock

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Contents

Abstract

The genetic diversity of farm animal species and breeds is an important resource in all livestock systems. To evaluate genetic diversity in livestock populations several methods have been developed based on pedigree information or on molecular data. The increasing availability of molecular markers for most farm animal species and the development of techniques to analyze molecular variation are widening the capacity to characterize breeds genetic variation. Moreover, little is known about the genetic diversity in Sicilian livestock breeds and populations. The aim of this thesis was to explore the genetic diversity of the Sicilian autochthonous breeds and populations using molecular markers.

In chapter 2, the promoter region of ovine *β-lactoglobulin* (*BLG*) gene was sequenced in order to identify polymorphisms, infer and analyze haplotypes, and phylogenetic relationship among the Valle del Belice sheep breed and other three breeds considered as ancestors. Sequencing analysis and alignment of the obtained sequences showed the presence of 36 single nucleotide polymorphisms (SNPs) and one deletion. We found four binding sites for milk protein binding factors (MPBFs) and five binding sites for nuclear factor-I (NF-I). The number of identified polymorphisms showed high variability within breeds. A total of 22 haplotypes found in best reconstruction were inferred considering all 37 polymorphic sites. Haplotypes were used for the reconstruction of a phylogenetic tree using the Neighbor-Joining algorithm. Analysis of genetic diversity indexes showed that the Sarda sheep breed presented the lowest nucleotide diversity, whereas the Comisana sheep breed presented the highest one. Comparing the nucleotide diversity among breeds, the highest value was obtained between Valle del Belice and Pinzirita sheep

breeds, whereas the lowest one was between Valle del Belice and Sarda sheep breeds. Considering that polymorphisms in the promoter region of *BLG* gene could have a functional role associated with milk composition, the lowest value of nucleotide diversity between Valle del Belice and Sarda sheep breeds may be related to a higher similarity of milk composition of these two breeds compared to the others.

In Chapter 3, microsatellite markers were used to explore the genetic structure of the four Sicilian autochthonous and Sarda sheep breeds, and to determine their genetic relationship. A total of 259 alleles were observed with average polymorphic information content (PIC) equal to 0.76, showing that the used microsatellites panel was highly informative. The low value of genetic differentiation among breeds (F_{st}=0.049) may indicate that these breeds have a low differentiation level probably due to common history and breeding practices. The low F_{is} and F_{it} values indicated low level of inbreeding within and among breeds. The Unrooted Neighbor-Joining dendrogram obtained from the Reynold's genetic distances, and factorial correspondence analysis revealed a separation between Barbaresca and the other sheep breeds. The Bayesian assignment test showed that Barbaresca and Sarda sheep breeds had more defined genetic structure, whereas the lowest assignment value was found in the Pinzirita sheep breed. Our results indicated high genetic variability, low inbreeding and low genetic differentiation, except for Barbaresca sheep breed, and were in accordance with geographical location, history, and breeding practices. The low robustness of the assignment test makes it unfeasible for traceability purposes, due to the high level of admixture, in particular for Sicilian dairy sheep breeds.

In Chapter 4 were reported for the first time the estimates of population structure, the levels of inbreeding (F) and coancestry (*f*), and the linkage disequilibrium (LD) in two Sicilian autochthonous cattle breeds, the Cinisara and the Modicana, using the Illumina Bovine SNP50K v2 BeadChip. Principal Components Analysis and Bayesian clustering algorithm showed that animals from the two Sicilian breeds formed non-overlapping clusters and are clearly separated populations, even from the Holstein control population. The average molecular F and *f* coefficients were moderately high, and the current estimates of N_e were low in both breeds. These values indicated a low genetic variability. The average r^2 was notably lower than the values observed in other cattle breeds. The highest r^2 values were found in chromosome 14, where causative mutations affecting variation in milk production traits have been reported. The low value of LD indicated that the present chip is not an optimum, and that a denser SNP array is needed to capture more LD information. The levels of inbreeding and N_e reported in this study point out the need to establish a conservation program for these autochthonous cattle breeds.

In Chapter 5 genome wide levels of LD, the number of haplotype blocks for each chromosome in each breed and the genetic diversity was assessed in the Sicilian dairy sheep breeds, using the Illumina Ovine SNP50K v1 BeadChip. The LD declined as a function of distance and average r^2 was notably lower than the value observed in other sheep breeds. Few and small haplotype blocks were observed in Comisana and Pinzirita sheep breeds, which contained just two SNPs. The number of haplotype blocks reported in our study for the Sicilian dairy sheep breeds were extremely lower than those reported in other livestock species. PCA showed that while Valle del Belice and Pinzirita sheep breeds formed a unique cluster,

the Comisana sheep breed showed the presence of substructure. PCA using a subset of SNPs showed lack of ability to discriminate among the breeds. The Pinzirita sheep breed displayed the highest genetic diversity, whereas the lowest value was found in the Valle del Belice sheep breed. The information generated from this study has important implications for the design and applications of association studies as well as for the development of selection breeding programs.

Riassunto

La diversità genetica delle specie e razze di interesse zootecnico, rappresenta un'importante risorsa in tutti i sistemi di allevamento . Per lo studio della diversità genetica, nel corso dei decenni sono stati sviluppati diversi metodi che si basano su informazioni del pedigree o su dati molecolari (microsatelliti e SNPs, *Single Nucleotide Polymorphisms*). Con l'aumento della disponibilità di marcatori molecolari per la maggior parte delle specie di interesse zootecnico, e con lo sviluppo di sofisticate tecniche analitiche, sta crescendo la capacità di caratterizzare la variabilità genetica delle razze. Inoltre, ad oggi, poche sono le informazioni sulla diversità genetica delle razze e delle popolazioni autoctone siciliane. L'obiettivo di questa tesi è stato quello di studiare la diversità e la struttura genetica nelle razze e popolazioni zootecniche autoctone siciliane mediante l'uso di marcatori molecolari.

Nel capitolo 2 è stata sequenziata la regione *promoter* del gene della β-lattoglobulina (BLG) ovina al fine di individuare i polimorfismi, calcolare e analizzare gli aplotipi e studiare le relazione filogenetiche tra la razza Valle del Belice e le altre razze considerate sue progenitrici. L'allineamento e l'analisi delle sequenze ottenute hanno evidenziato la presenza di 36 SNPs e una delezione, sottolineando un'elevata variabilità all'interno delle razze. Sono stati individuati quattro siti di legame per *Milk Protein Binding Factors* (MPBFs) e cinque siti di legame *Nuclear Factor-I* (NF-I). Utilizzando i siti polimorfici identificati, sono stati calcolati 22 aplotipi, usati per la ricostruzione di un albero filogenetico tramite l'algoritmo Neighbor-Joining. L'analisi degli indici di diversità genetica ha mostrato valori più bassi di diversità nucleotidica per la razza Sarda, mentre la razza Comisana ha presentato i valori più alti.

Confrontando la diversità nucleotidica tra le razze, il valore più alto è stato ottenuto tra le razze Valle del Belice e Pinzirita, mentre quello più basso è stato ottenuto tra la razza Valle del Belice e la razza Sarda. Considerando che i polimorfismi nella regione *promoter* del gene della BLG potrebbero avere un ruolo funzionale associato alla composizione del latte, il valore più basso della diversità nucleotidica tra le razze Valle del Belice e Sarda, potrebbe essere correlato ad una maggiore somiglianza nella produzione qualitativa del latte di queste due razze rispetto alle altre. Nel capitolo 3, un pannello di 20 marcatori microsatelliti è stato utilizzato per studiare e caratterizzare la struttura genetica delle quattro razze ovine autoctone siciliane (Barbaresca, Comisana, Pinzirita e Valle del Belice) e della razza Sarda, e per determinare le relazioni filogenetiche che intercorrono tra esse. Il numero di alleli trovati (259) ed il contenuto di informazione polimorfica (PIC) indicano che il pannello utilizzato è altamente informativo (0,76). I valori di F_{st}, F_{is} e F_{it} hanno mostrato una bassa differenziazione genetica e bassi livelli di consanguineità all'interno delle razze e tra esse. Il dendrogramma *Neighbor-Joining*, ottenuto sulla base delle distanze genetiche di Reynold, e l'analisi delle corrispondenti fattoriali, hanno evidenziato una marcata separazione tra la razza Barbaresca e le altre razze ovine. Il test di assegnazione bayesiano ha mostrato una struttura genetica più omogenea per le razze Barbaresca e Sarda, mentre il più basso valore di assegnazione è stato trovato nella razza Pinzirita. I nostri risultati indicano la presenza di una elevata variabilità, bassa consanguineità e bassa differenziazione genetica, fatta eccezione per la razza Barbaresca, in accordo con la posizione geografica, i possibili flussi genici e le pratiche di allevamento. La bassa robustezza del test di assegnazione rende inutilizzabile l'uso dei marcatori microsatelliti ai fini della tracciabilità delle produzioni lattiero casearie, a causa dell'elevata promiscuità e del flusso genico, in particolare per le razze ovine da latte autoctone siciliane. Nel capitolo 4 vengono riportate per la prima volta, le stime riguardanti la struttura genetica, i livelli di inbreeding (*F*) e coancestry (*f*) e il linkage disequilibrium(LD) nelle razze bovine autoctone siciliane Cinisara e Modicana, utilizzando l'Illumina Bovine SNP50K v2 BeadChip. L'Analisi delle Componenti Principali (PCA) e l'algoritmo di assegnazione basato sulla statistica bayesiana, hanno mostrato che gli animali delle due razze formano due *clusters* distinti. I coefficienti *F* e *f* erano moderatamente elevati, mentre le attuali stime sulla effettiva dimensione della popolazione (N_e) erano basse in entrambe le razze, sottolineando una bassa variabilità genetica. Il valore medio del LD calcolato con r^2 è risultato notevolmente inferiore rispetto ai valori medi riportati in letteratura per le altre razze bovine. I più alti valori di r^2 sono stati trovati nel cromosoma 14, all'interno del quale sono state descritte diverse mutazioni che influenzano la produzione quanti-qualitativa del latte. Il basso valore di LD suggerisce che il presente chip non è ottimale, e che un pannello a più alta densità è necessario per acquisire le informazioni riguardanti i livelli di LD, mentre i parametri riguardanti la consanguineità e N_e, indicano la necessità di avviare programmi di conservazione per il recupero di queste razze bovine autoctone. Il capitolo 5 riporta i risultati relativi al calcolo del LD, alla stima del numero di blocchi di aplotipi per cromosoma e alla diversità genetica nelle razze ovine autoctone siciliane allevate per la produzione di latte, utilizzando gli Ovine Beadchip SNP50K v1 BeadChip. I valori del LD diminuivano in funzione della distanza e la media del coefficiente r^2 era notevolmente inferiore al valore osservato nelle altre razze ovine.

Sono stati trovati un ridotto numero di blocchi di aplotipi e un ridotto numero di SNPs per blocco, in particolare nelle razze Comisana e Pinzirita. La PCA ha mostrato che, mentre la Valle del Belice e la Pinzirita formano due gruppi omogenei e distinti per razza, la Comisana evidenzia la presenza di sottostrutture. La PCA condotta con un subset di SNPs ha mostrato una scarsa capacità di discriminare le razze tra loro. La razza Pinzirita ha evidenziato i più alti livelli di diversità genetica, mentre i valori più bassi sono stati riscontrati nella razza Valle del Belice. Le informazioni generate da questo studio potrebbero essere utilizzate per gli studi di associazione, nonché per lo sviluppo di programmi di selezione e miglioramento genetico.

1

General Introduction

1.1 Introduction

Livestock breeds have been formed by centuries of human and natural selection. Breeds have been selected to fit a wide range of environmental conditions and human needs [1]. The definition of a breed, as applied by the Food and Agriculture Organisation of the United Nations, is based on the homogeneity of external characteristics, or on a generally accepted identity of animals of a geographically or culturally separated group [2].

Studies of genetic diversity in domestic animals are based on evaluation of the genetic variation within breeds and genetic relationships among them, since the breed is the management unit for which factors such as inbreeding are controlled [3].

Interest in the conservation of local livestock types has increased over the last 25 years in response to the expansion of highly productive livestock breeds at the expense of local populations [4]. In fact, the selection of a few highly productive breeds has caused the decline of numerous other breeds. The need of conservation comes from the potential rate of decrease of genetic variation. The loss of genetic variation within and between breeds is detrimental not only from the perspectives of culture and conservation but also utility since lost genes may be of future economic interest. Within breeds, high rates of loss of genetic variation leads to reduced chances of breed survival due to decreased fitness through inbreeding depression. These are all due to small effective population sizes, or, equivalently, high rates of inbreeding [5]. In fact, the loss of gene pool may lead to an increase of the frequency of unfavorable genes resulting in a further increase of the risk of extinction. The genetic diversity found in local breeds allows farmers to develop new characteristics in response to changes in environment, diseases, or market conditions [6]. Indigenous breeds often possess gene combinations and special adaptations not found in other breeds. These adaptive traits include tolerance to various diseases, fluctuations in feed quality, extreme climatic conditions and the ability to survive and reproduce for long periods of time. Moreover, genetic diversity is essential for the sustainability of livestock species; in fact, genetic diversity within breeds is needed for long-term genetic improvement of livestock breeds, for selection of new traits or traits in a changing environment, and to preserve low performance due to inbreeding.

The conservation of farm animal resources is important for coping with future breeding needs and for facilitating the sustainable use of marginal areas. Therefore, for all aspects mentioned above, it is important to prevent further loss of breeds and of diversity within breeds.

1.2 Genetic diversity

Genetic diversity can be defined as the additive genetic variance within and between breeds and populations [7]. To evaluate genetic diversity in livestock populations, several methods have been developed [8]. These methods are based on pedigree information or on molecular data when pedigree information is not available. Moreover, genetic diversity can be estimated by combining pedigree and molecular data [9]. In several situations, use of SNP markers instead of pedigree information for genetic diversity estimation can be helpful, for example, for situations with poor or absent pedigree information. Moreover, SNP markers can be used for a more precise estimation of genetic diversity than pedigree information, even when pedigree data is available and accurate, in case the density of the SNP data is high enough. The SNP data allows to estimate the absolute genetic diversity, without relying on an arbitrary base population [10]. Furthermore, missing pedigree data and pedigree errors can result in a low estimated inbreeding in a population that is highly inbred, resulting in a negative effect on conservation [11].

The increasing availability of molecular markers for most farm animal species and the development of techniques to analyze molecular variation are widening the capacity to characterize breeds genetic variation. Molecular markers, revealing polymorphisms at DNA level, are now key players in animal genetics. However, due to the existence of various molecular biology techniques to produce them, and to the various biological implications some can have, a large variety exists, from which choices will have to be made according to purposes [12].

The development of tools for DNA analysis, that has taken place in the last few decades, has increased enormously the capacity to characterize variation within and between breeds. The restricted traditional characterization by means of phenotypic attributes can now be complemented by an increasing available number of molecular markers and the development of sophisticated statistical techniques for their analysis [13].

1.3 Genetic markers systems used in livestock populations

Commonly considered DNA markers are Microsatellites and Single Nucleotide Polymorphisms (SNPs).

Microsatellites, also known as Simple Sequence Repeats (SSRs) or Short Tandem Repeats (STRs), are repeated sequences of 2-6 base pairs of DNA. They generally occur in non-coding regions of the genome. Microsatellites have been the marker system of choice in population genetic studies for the major part of the last 25 years, due to extremely informative polymorphic nature, abundance in the genome, and neutrality with respect to selection. The popularity of those markers stems from the possibility to genotype individuals with high polymorphic and co-dominant genetic marker [14] at reasonable cost. The usefulness of microsatellite markers for the estimation of genetic diversity and relationships among livestock breeds has been documented in numerous studies (e.g., [15-16]). However, several disadvantages have been associated to microsatellites. In fact, recurrent mutation may lead to homoplastic alleles that are identical by state but not by descendent [17].

Single Nucleotide Polymorphisms (**SNPs**) have become the marker of choice for many studies in animal genetics and genomics, and SNP identification has benefited greatly from the rapid development in next generation sequencing technologies [18]. SNPs are bi-allelic genetic markers, and they are easy to evaluate and interpret, and are widely distributed within genomes. SNP genotyping allows the simultaneous high-throughput interrogation of hundreds of thousands of loci with high precision at an affordable cost that enables large-scale studies [19]. In addition, this approach considers selective variation that classical neutral markers (e.g. microsatellites) ignore. With proper coverage and density over the whole-genome, SNPs could capture the linkage disequilibrium (LD) information embedded in the genome, are now the markers of choice in QTL analysis and genomic selection and, already, several studies used SNP data for genetic diversity estimation in livestock breeds (e.g., [20-21]). The high-density SNP array has also been useful in understanding the phylogenetic relationships among domestic animals

[22], to predict the copy number variations [23], for paternity testing [24] and tracing the geographic origins of animal products [25].

1.4 Aim of the thesis

Potentially, there is much unrecognized beneficial genetic variability in local breeds and populations, which supposes important reservoirs of non-exploited genetic resources. In Sicily, dairy production represents an important resource for hilly and mountain areas economy, in which other economic activities are limited, and the dairy products are the link among product-territory, territory-breed and breed-product. Nowadays, several local breeds and populations, belonging to livestock species (cattle, sheep and goat), are reared in extensive traditional systems.

These breeds present differences in both morphology and production traits, show excellent adaptability to the local environments and are not subject to breeding programs. In fact, the development of breeding programs for local breeds is too costly for breeding organizations, and the absence of pedigree records is a threat for the existence of these breeds. Their milk is used for the production of traditional raw milk cheeses, sometimes protected designation of origin (PDO) cheeses. In some cases, the quality of these products is linked to a specific breed. Assignment of individuals to a specific breed, especially when the phenotypic differentiation between breeds is difficult, is therefore of great importance both for biodiversity purposes and dairy products traceability. The socio-economic and ecological values, the historical, cultural and genetic heritage of these breeds/populations are unquestionable. However, all aspects mentioned above, which creates income for a small group of farmers, justify the establishment of conservation programs.

The availability of molecular markers has resulted in new opportunities to estimate genetic diversity within livestock breeds and populations in deep details, and to improve prioritization of animals for conservation of genetic diversity [10].

The overall objective of this thesis was to explore the genetic structure of the Sicilian autochthonous breeds and populations, through the analysis of the genetic diversity within and among breeds, and to determine their genetic relationship, using molecular markers.

The first aim was to sequence the full-length promoter region of *β-lactoglobulin* (*BLG*) gene in four sheep breeds reared in Sicily, in order to: identify polymorphisms, infer and analyze haplotypes and analyze phylogenetic relationship among the Valle del Belice breed and the other three breeds considered as ancestors (Chapter 2). The next step was to explore the genetic structure of the four Sicilian autochthonous sheep breeds, through the analysis of the genetic diversity within and among breeds, and to determine their genetic relationship, using microsatellite markers. Moreover, microsatellite markers were used for the proper assignment of an individual to a specific breed (Chapter 3).

In Chapter 4, were reported for the first time the estimates of population structure, levels of inbreeding and coancestry, and linkage disequilibrium (LD) from a genome wide perspective in Cinisara and Modicana cattle breeds, using the Illumina Bovine SNP50K v2 BeadChip.

Finally, in Chapter 5, the genome wide levels of LD, the number of haplotype blocks for each chromosome in each breed, and the genetic diversity was assessed in the Sicilian dairy sheep breeds, using high density genotyping arrays.

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2

Study of polymorphisms in the promoter region of ovine *β-lactoglobulin* gene and phylogenetic analysis among the Valle del Belice breed and other sheep breeds considered as ancestors

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Abstract

The aim of this work was to sequence the promoter region of *β-lactoglobulin* (*BLG*) gene in four sheep breeds, in order to identify polymorphisms, infer and analyze haplotypes, and phylogenetic relationship among the Valle del Belice breed and the other three breeds considered as ancestors. Sequencing analysis and alignment of the obtained sequences showed the presence of 36 single nucleotide polymorphisms (SNPs) and one deletion. A total of 22 haplotypes found in "best" reconstruction were inferred considering the 37 polymorphic sites identified. Haplotypes were used for the reconstruction of a phylogenetic tree using the Neighbor-Joining algorithm. The number of polymorphisms identified showed high variability within breeds. Analysis of genetic diversity indexes showed that the Sarda breed presented the lowest nucleotide diversity, whereas the Comisana breed presented the highest one. Comparing the nucleotide diversity among breeds, the highest value was obtained between Valle del Belice and Pinzirita breeds, whereas the lowest one was between Valle del Belice and Sarda breeds. Considering that polymorphisms in the promoter region of *BLG* gene could have a functional role associated with milk composition, the lowest value of nucleotide diversity between Valle del Belice and Sarda breeds may be related to a higher similarity of milk composition of these two breeds compared to the others. Further analyses will be conducted in order to evaluate the possible correlation between the genetic diversity indexes and the BLG content in milk of our breeds.

Keywords: β-lactoglobulin, polymorphisms, sheep breeds, phylogenetic analysis

2.1 Introduction

β-lactoglobulin (*BLG*) is synthesized by secreting cells of mammary gland and it is the major whey protein in the milk of ruminants. It is also found in the milk of different mammalian species including cats [1], dogs and dolphins [2] but it is lacking in humans [3, 4], rodents, and lagomorphs [5]. It is a globular protein, belonging to the family of lipocalins, small proteins with some peculiarities, such as the ability to bind hydrophobic molecules [6]. Although no clear physiological functions have been defined for *BLG*, a role in the transport of retinol and fatty acids has been suggested [6, 7]. However, the general affinity of *BLG* with these hydrophobic molecules did not allow ascribing a specific role [7, 8].

The *BLG* encoding gene has been sequenced in sheep [9], cattle [10], and goats [11], and mapped on chromosome 3 in sheep and chromosome 11 in goats and cattle [12].

A large number of variants have been reported for bovine and ovine *BLG* protein. Three co-dominant alleles (A, B, and C) have been reported in sheep that differ by one or more amino acid changes. *BLG* variant A differs from *BLG* variant B in the amino acid sequence at position 20 ($Tyr_A \rightarrow His_B$) [13, 14], whereas it differs from *BLG* variant C at position 148 ($Arg_A \rightarrow Gln_C$) [15]. Variants A and B are the most common and have been reported in several breeds [13, 14], whereas variant C has been reported only in milk from Merinoland, Hungarian Merino, Pleven [16], and Carranzana and Lacha [17] breeds. Many studies on the effect of ovine *BLG* polymorphisms on milk production traits have been carried out, but results are still conflicting. Some authors reported the positive effect of variant B on milk production and quality and whey protein content [18-22], whereas others reported the positive effect of variant A on fat and protein content and enzymatic properties [23-25]. However, other studies reported no direct association between genotypes at this locus and milk characteristics [26-29].

Several potential binding sites for specific mammary gland transcription factors (TFs) were found by Watson et al. [30] within the ovine *BLG* promoter region. Since they have been identified in the 5'-flanking region of many expressed milk protein genes in different species [30-32], it has been suggested that these factors are important regulators of milk protein gene expression. Therefore, the presence of polymorphisms in the *BLG* promoter region could influence the binding affinity of TFs and could affect both the expression level and the content of BLG in milk [33].

In Sicily, dairy sheep production represents an important resource for the economy of hill and mountain areas, in which other economic activities are difficult to develop [34]. The main breeds reared are Valle del Belice, Comisana, Pinzirita, and Sarda, which are genetically connected among them. Based on historical, geographical, and morphological information, it seems indeed that the Valle del Belice breed derives from the Pinzirita, to which is similar for the horned trait in males, crossed with the Comisana, to which is similar for the coat color (i.e. white with red head) and for the high milk production. Subsequently, the cross between these two breeds was likely crossed with the Sarda breed [35]. Nowadays, the Valle del Belice breed is the most appreciated dairy sheep breed reared on the island. The aim of this work was to sequence the full-length promoter region of *BLG* gene in four sheep breeds reared in Sicily, in order to: i) identify polymorphisms; ii) infer and analyze haplotypes; and iii) analyze phylogenetic relationship among the Valle del Belice breed and the other three breeds.

2.2 Materials and methods

Amplification of sheep BLG promoter region

A total of 50 randomly chosen unrelated (i.e. without common parents) animals from several farms located in different areas of Sicily and belonging to the four breeds (Valle del Belice n=20; Comisana n=10; Pinzirita n=10; and Sarda n=10) were analyzed. Genomic DNA was extracted from blood buffy coats of nucleated cells using a salting out method [36]. Primers BLG-F1 and BLG-R1 (Table 2.1) were used to amplify a fragment of 2255 bp, containing 2138 bp of the promoter region and 117 bp of exon 1 of *BLG* gene, as reported by Sardina et al. [37] in goat gene (GenBank Acc. No. Z33881). PCR reaction was performed in a final volume of 25 µl using 2X PCR Master Mix (Fermentas), 10 µM of each primer, and approximately 75 ng of genomic DNA. The thermal cycling conditions were: 95°C for 5 min, 30 cycles of 95°C for 30 s, 59°C for 1 min and 72°C for 1 min 30 s, and a final extension of 72°C for 5 min. The PCR products were checked by electrophoresis on 1% agarose gel stained with ethidium bromide.

DNA sequencing reaction

PCR products were purified using 10 U of Exonuclease I and 1 U of Shrimp Alkaline Phosphatase (Fermentas). The resulting PCR products did not need additional purification before sequencing. Primers BLG-F1 and BLG-R1 and other eight internal primers (Table 2.1) were used for sequencing reaction with BigDye Terminator v3.1 Cycle Sequencing Kit in an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems).

Table 2.1 Primers used to amplify and sequence the promoter region of sheep *β-lactoglobulin* gene, as reported by Sardina et al. [37] in goat gene (GenBank Acc. No Z33881).

Forward Primers	**Sequence**
BLG-F1	5'-AGG CCA GAG GTG CTT TAT TTC CGT-3'
BLG-F2	5'-TAG TCT CTG CCT CCG TGT TCA CAT-3'
BLG-F3	5'-AAC CTC CAA CCA AGA TGC TGA CCA-3'
BLG-F4	5'-AGG GTC AGG TCA CTT TCC CGT-3'
BLG-F5	5'-AGA AGG CCT CCT ATT GTC CTC GTA GA-3'
Reverse Primers	**Sequence**
BLG-R1	5'-TCC ATG GTC TGG GTG ACG ATG ATG-3'
BLG-R2	5'-TTC CCG GAA TCC TAC TTG GCT CAT-3'
BLG-R3	5'-ACC AGC TCC TCC AAA CCA TGT GA-3'
BLG-R4	5'-AGT GAC TAA ACC ACT CAT CAC AGG G-3'
BLG-R5	5'-CAA CAA GGA ACT TCA GGT TGG AAT-3'

Statistical analysis

SeqScape v3.1 software (Applied Biosystems) was used to analyze the nucleotide sequences, whereas Clustal W software [38] was used to align the sequences (GenBank Acc. No. FR821261-FR821310). TESS software [39] was used to predict TFs binding sites, using information collected in TRANSFAC database [40]. Genetic diversity indexes, such as number of polymorphic sites, nucleotide diversity (π), average number of nucleotide differences (k), number of haplotypes (h), and haplotype diversity (Hd) within and among breeds were estimated with the DnaSP v5.10.01 software [41]. PHASE v2.1.1 software [42, 43] (with -MR0 -d1 options), included in DNAsp v5.10.01 software package [41], was used to infer haplotypes within the whole sample analyzed. Finally, MEGA v4.0 software [44] was used for the reconstruction of a phylogenetic tree using the Neighbor-Joining (NJ) algorithm with nucleotide substitution model and 1,000 bootstrap replications.

2.3 Results and discussion

Identification of polymorphisms and genetic diversity indexes

Sequencing analysis and alignment of the obtained sequences showed the presence of 37 polymorphic sites in the *BLG* promoter region: 36 single nucleotide polymorphisms (SNPs) and one deletion (Table 2.2), which equates to about one polymorphism per approximately 60 bp. The number of polymorphisms identified in our breeds showed high variability of the *BLG* promoter region as reported by Sardina et al. [37] in goat and by Ganai et al. [45] in cattle. Valle del Belice and Comisana breeds have all point mutations (36 SNPs and the deletion) in common, whereas the Pinzirita breed presented 34 SNPs and the deletion, and the Sarda breed 29 SNPs and the deletion. Using the TRANSFAC database [40], we found four binding sites for milk protein binding factor (MPBF) and five binding sites for nuclear factor-I (NF-I) within the promoter region of sheep *BLG* gene, as reported by Watson et al. [30]. Since at least five NF-I have been identified, these authors suggested that these factors could play a regulatory role in *BLG* transcription. The polymorphic site -246 A/T, we found in our breeds, lies within a region of sheep *BLG* promoter, in which a NF-I binding site is involved (-253/-240) (TESS - TRANSFAC Site Record R03872), causing the loss of the latter.

Promoter	-1981	-1935	-1913	-1911	-1909	-1815	-1791	-1780	-1770	-1733	-1631	-1448	-1437	-1245	-1230	-983	-966	-941	-919	-903	-764	-722	-696	-654	-575	-545	-528	-496	-477	-447	-438	-246	-163	-134	-117	-46	-42
X68105	**A**	**T**	**C**	**C**	**T**	**G**	**T**	**T**	**C**	**A**	**G**	**G**	**G**	**C**	**A**	**G**	**C**	**A**	**A**	**T**	**G**	**G**	**T**	**G**	**G**	**C**	**A**	**C**	**T**	**A**	**C**	**A**	**T**	**G**	**G**	**T**	**T**
VdB-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	Y	R	R	Y	-	-	-	-	Y	W	Y	K	R	K	Y
VdB-2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	Y	R	R	Y	-	-	-	-	Y	W	Y	K	R	K	Y
VdB-3	R	Y	Y	Y	K	K	Y	K	Y	R	R	S	R	Y	R	-	-	R	R	-	R	-	-	-	-	-	R	-	Y	M	-	W	Y	K	R	K	Y
VdB-4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VdB-5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VdB-6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VdB-7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VdB-8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VdB-9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VdB-10	R	Y	Y	Y	K	K	Y	K	Y	R	R	S	R	Y	R	R	-	R	-	-	R	-	-	-	-	-	R	S	Y	M	-	W	Y	K	R	K	Y
VdB-11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VdB-12	R	Y	Y	Y	K	K	Y	K	Y	R	R	S	R	Y	R	R	-	-	-	-	R	-	-	-	-	-	R	-	Y	M	-	W	Y	K	R	K	Y
VdB-13	G	C	T	T	G	T	C	G	T	G	A	C	A	T	G	A	del	G	G	C	A	-	-	-	-	-	G	-	C	C	-	T	C	T	A	G	C
VdB-14	G	C	T	T	G	T	C	G	T	G	A	C	A	T	G	A	del	G	-	-	-	-	-	-	-	-	G	-	C	C	-	T	C	T	A	G	C
VdB-15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VdB-16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VdB-17	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VdB-18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VdB-19	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
VdB-20	R	Y	Y	Y	K	K	Y	K	Y	R	R	S	R	Y	R	R	-	-	-	-	R	-	-	-	-	-	R	-	Y	M	-	W	Y	K	R	K	Y
COM-1	R	Y	Y	Y	K	K	Y	K	Y	R	R	S	R	Y	R	-	-	-	-	-	R	-	-	-	-	-	R	-	Y	M	-	W	Y	K	R	K	Y
COM-2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R	Y	R	R	Y	-	-	-	-	Y	W	Y	K	R	K	Y
COM-3	R	Y	Y	Y	K	K	Y	K	Y	R	R	S	R	Y	R	-	-	R	-	Y	R	-	-	-	-	-	R	-	Y	M	-	W	Y	K	R	K	Y
COM-4	R	Y	Y	Y	K	K	Y	K	Y	R	R	S	R	Y	R	-	-	-	-	-	R	-	-	-	-	-	R	S	Y	M	-	W	Y	K	R	K	Y
COM-5	G	C	T	T	G	T	C	G	T	G	A	C	A	T	G	A	del	G	G	C	A	-	-	-	-	-	G	-	C	C	-	T	C	T	A	G	C
COM-6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
COM-7	R	Y	Y	Y	K	K	Y	K	Y	R	R	S	R	Y	R	R	-	-	-	-	R	-	-	-	-	-	R	-	Y	M	-	W	Y	K	R	K	Y
COM-8	R	Y	Y	Y	K	K	Y	K	Y	R	R	S	R	Y	R	R	-	-	-	-	R	-	-	-	-	-	R	-	Y	M	-	W	Y	K	R	K	Y
COM-9	R	Y	Y	Y	K	K	Y	K	Y	R	R	S	R	Y	R	-	-	R	-	Y	R	-	-	-	-	-	R	S	Y	M	-	W	Y	K	R	K	Y
COM-10	R	Y	Y	Y	K	K	Y	K	Y	R	R	S	R	Y	R	R	-	-	-	-	R	-	-	-	-	-	R	-	Y	M	-	W	Y	K	R	K	Y
PIN-1	G	C	T	T	G	T	C	G	T	G	A	C	A	T	G	A	del	G	G	C	A	-	-	-	-	-	G	-	C	C	-	T	C	T	A	G	C
PIN-2	R	Y	Y	Y	K	K	Y	K	Y	R	R	S	R	Y	R	R	-	R	-	Y	R	-	-	-	-	-	R	S	Y	M	-	W	Y	K	R	K	Y
PIN-3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PIN-4	R	Y	Y	Y	-	-	Y	K	Y	-	-	S	R	Y	-	R	-	-	-	-	-	-	-	-	-	-	R	-	Y	M	-	W	Y	K	R	K	Y
PIN-5	R	Y	Y	Y	K	K	Y	K	Y	R	R	S	R	Y	R	R	-	-	-	-	-	-	-	-	-	-	R	-	Y	M	-	W	Y	K	R	K	Y
PIN-6	R	Y	Y	Y	K	K	Y	K	Y	R	R	S	R	Y	R	R	-	-	-	-	-	-	-	R	R	Y	R	-	Y	M	Y	T	C	T	A	G	C
PIN-7	R	Y	Y	Y	K	K	Y	K	Y	R	R	S	R	Y	R	R	-	-	-	-	R	-	-	R	R	Y	R	-	Y	M	Y	T	C	T	A	G	C
PIN-8	R	Y	Y	Y	K	K	Y	K	Y	R	R	S	R	Y	R	R	-	-	-	-	R	-	-	-	-	-	R	-	Y	M	-	W	Y	K	R	K	Y
PIN-9	R	Y	Y	Y	K	K	Y	K	Y	R	R	S	R	Y	R	R	-	-	-	-	-	-	-	-	-	-	R	-	Y	M	Y	T	C	T	A	G	C
PIN-10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SAR-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SAR-2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SAR-3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SAR-4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SAR-5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SAR-6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SAR-7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SAR-8	G	C	T	T	G	T	C	G	T	G	A	C	A	T	G	A	-	G	-	C	A	-	-	-	-	-	G	G	C	C	-	T	C	T	A	G	C
SAR-9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SAR-10	R	Y	Y	Y	K	K	Y	K	Y	R	R	S	R	Y	R	R	del	R	-	-	R	-	-	-	-	-	R	-	Y	M	-	W	Y	K	R	K	Y

Table 2.2 Polymorphic sites identified in the *β-lg* promoter region (GenBank Acc. No X68105) of sheep breeds. (VdB=Valle del Belice; COM=Comisana; PIN=Pinzirita; SAR=Sarda)

Analysis of genetic diversity indexes (Table 2.3) showed that the Sarda breed presented the lowest nucleotide diversity, which results in a reduced number of haplotypes with a consequent low haplotype diversity. The low nucleotide and haplotype diversity within the Sarda breed was due to the presence of low proportion of animals showing polymorphisms (Table 2.2). Although Comisana and Valle del Belice breeds present the same number of SNPs, the former was characterized by the highest variability, presenting the highest nucleotide diversity, which results in the highest number of haplotypes with a consequent high haplotype diversity (Table 2.3), whereas the Valle del Belice breed showed lower nucleotide diversity, lower number of haplotypes, and consequently lower haplotype diversity.

Table 2.3 Genetic diversity indexes in the four sheep breeds. Nucleotide diversity (**π**), number of haplotype (**h**), Haplotype diversity (**Hd**) and standard deviation (s.d.).

Breed	**Polymorphic site**	**π±s.d**	**h**	**Hd ±s.d**
Valle del Belice	36	0.00459±0.00097	8	0.438±0.098
Comisana	36	0.00703±0.00055	10	0.837±0.076
Pinzirita	34	0.00695±0.00047	9	0.826±0.073
Sarda	29	0.00355±0.00151	3	0.279±0.123

These results may be explained considering the higher proportion of polymorphic animals in the Comisana breed (90%) compared to the Valle del Belice breed (40%) (Table 2.2). Moreover, the lower number of haplotypes in the Valle del Belice breed compared to the Comisana breed can be explained by the fact that some positions that are in homozygous condition in the Valle del Belice breed are in heterozygous condition in the Comisana breed. The Pinzirita breed, which presents a lower number of SNPs compared to the Valle del Belice breed, presented higher nucleotide diversity, higher number of haplotypes, and higher haplotype diversity (Table 2.3), probably due to a higher proportion of polymorphic individuals in the Pinzirita breed (80%) compared to the Valle del Belice breed. Moreover, the heterozygous condition for some positions within the Pinzirita breed, compared to the Valle del Belice breed, led to a higher number of haplotypes.

It is interesting to highlight that among the four breeds, those characterized by lower proportion of polymorphic individuals were Valle del Belice and Sarda breeds. It is possible to hypothesize that this is influenced by the selection pressure. However, in the Sicilian farming system, natural mating is the common practice and the exchange of rams among flocks is quite unusual. This leads to an increase of inbreeding within the population and a consequent decrease of variability (heterozygous condition).

Table 2.4 shows the nucleotide diversity and the average number of nucleotide differences estimated between the Valle del Belice breed and the other three breeds. The highest value of nucleotide diversity was obtained between Valle del Belice and Pinzirita breeds, due to a higher presence of mutated sites in homozygous condition in the Pinzirita breed than in the Valle del Belice breed (Table 2.2). The lowest value of nucleotide diversity between breeds was obtained between Valle del Belice and Sarda breeds, due to a lower presence of mutated sites in homozygous condition in the Sarda breed (Table 2.2). These results were confirmed by the average number of nucleotide differences between breeds (Table 2.4). Valle del Belice and Pinzirita breeds presented the highest average number of nucleotide difference, whereas the lowest value was found in Valle del Belice and Sarda breeds. A previous study conducted on the genetic structure and relationship between the Valle del Belice breed and the other sheep breeds considered as ancestors, using the genetic polymorphisms of seven protein systems, has reported the lowest genetic distance between Valle del Belice and Pinzirita breeds and the highest one between Valle del Belice and Sarda breeds [46], which is not in agreement with our results. Considering that polymorphisms in the promoter region of *BLG* gene could have a functional role associated with milk composition, the lowest value of nucleotide diversity between Valle del Belice and Sarda breeds may be related to a higher similarity of milk composition of these two breeds compared to the others.

Table 2.4 Nucleotide diversity (π) and average number of nucleotide differences (k) between Valle del Belice breed and the other three breeds.

Breed	**π**	**k**
Valle del Belice-Comisana	0.00564	12.104
Valle del Belice-Pinzirita	0.00566	12.138
Valle del Belice-Sarda	0.00421	9.027

Identification of haplotypes and phylogenetic analysis

On a total of 36 possible haplotypes, 22 haplotypes in "best" reconstruction were inferred considering the 37 polymorphic sites identified (Table 2.5). Of the 22 haplotypes, seven were specific for the Pinzirita breed, four for the Comisana breed, three for the Sarda breed, and two for the Valle del Belice breed (Table 2.5). Among the analyzed breeds only Valle del Belice and Comisana breeds shared four haplotypes. Haplotype H1 showed the highest frequency (0.617) and was found in all breeds, followed by haplotype H22 with a frequency of 0.060. In particular, haplotype H22 was the only one shared among Valle del Belice, Pinzirita, and Comisana breeds and it was specific of animals presenting the deletion at position -966.

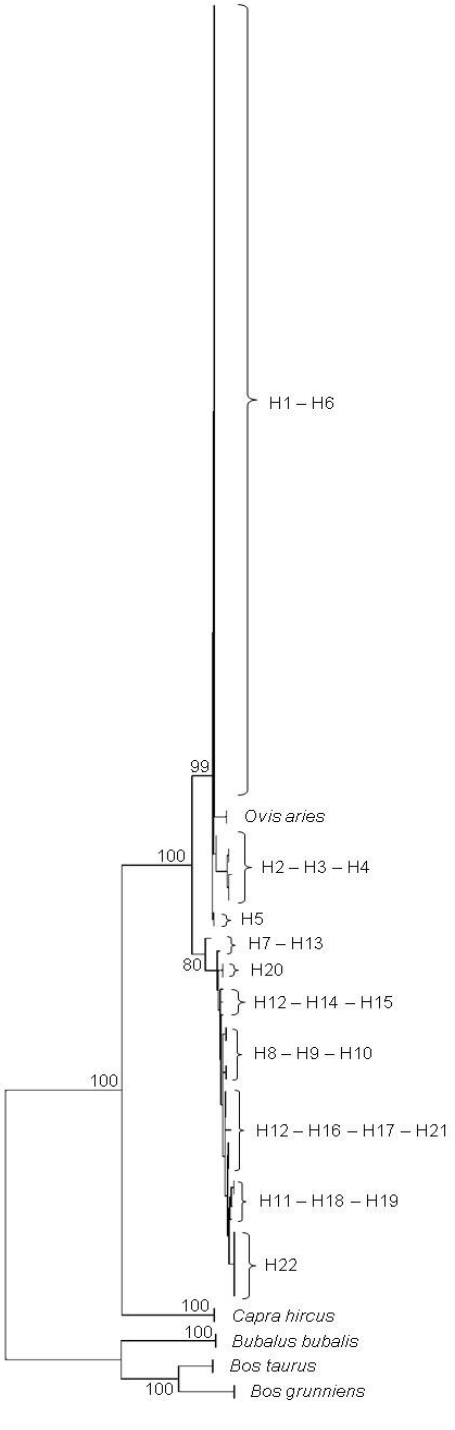
Table 2.5 Haplotypes identified in the four sheep breeds, frequencies (Freq.) and standard error (S.E.).

	Haplotypes	Freq.	S.E.
H1[a,b,c,d]	ATCCTGTTCAGGGCAGCAATGGTGGCACTACATGGTT	0.617	0.008
H2[c]	ATCCTGTTCAGGGCAGCAATGGTGGCACTATTCTAGC	0.007	0.004
H3 [a,b]	ATCCTGTTCAGGGCAGCAATGACAATACTATTCTAGC	0.030	0.000
H4 [c]	ATCCTGTTCAGGGCAGCAATAGTAATACTATTCTAGC	0.020	0.000
H5 [b]	ATCCTGTTCAGGGCAGCGATGGTGGCACTACATGGTT	0.017	0.006
H6 [d]	ATCCTGTTCAGGGCAG5AATGGTGGCACTACATGGTT	0.010	0.000
H7 [c]	GCTTTGCGTAGCATAACAATAGTGGCGCCCCTCTAGC	0.010	0.000
H8 [b]	GCTTGTCGTGACATGGCAATAGTGGCGCCCCTCTAGC	0.012	0.003
H9 [b]	GCTTGTCGTGACATGGCAATAGTGGCGGCCCTCTAGC	0.014	0.007
H10 [a,b]	GCTTGTCGTGACATGGCAGTAGTGGCGCCCCTCTAGC	0.020	0.000
H11[a]	GCTTGTCGTGACATGGCGACAGTGGCGGCCCTCTAGC	0.009	0.003
H12 [c]	GCTTGTCGTGACATGACAATGGTGGCGCCCCTCTAGC	0.030	0.000
H13 [c]	GCTTGTCGTGACATGACAATAGTGGCACTCCTTTAGC	0.007	0.004
H14 [c]	GCTTGTCGTGACATGACAATAGTGGCGCTCCTCTAGC	0.009	0.002
H15 [b]	GCTTGTCGTGACATGACAATAGTGGCGCCACTCTAGC	0.010	0.000
H16 [a,b]	GCTTGTCGTGACATGACAATAGTGGCGCCCCTCTAGC	0.020	0.002
H17 [a,b]	GCTTGTCGTGACATGACGATAGTGGCGCCCCTCTAGC	0.019	0.002
H18 [c]	GCTTGTCGTGACATGACGATAGTGGCGGCCCTCTGGC	0.007	0.004
H19 [d]	GCTTGTCGTGACATGACGACAGTGGCGGCCCTCTAGC	0.020	0.000
H20 [a]	GCTTGTCGTGACATGA5GATGGTGGCACCCCTCTAGC	0.020	0.000
H21 [d]	GCTTGTCGTGACATGA5GATAGTGGCGCCCCTCTAGC	0.010	0.000
H22[a,b,c]	GCTTGTCGTGACATGA5GGCAGTGGCGCCCCTCTAGC	0.060	0.000

Haplotypes identified in Valle del Belice (a), Comisana (b), Pinzirita (c) and Sarda (d) sheep breeds

Haplotypes were used for the reconstruction of a phylogenetic tree, using *BLG* promoter region of *Capra hircus* (GenBank Acc. No Z33881), *Bos taurus* (GenBank Acc. No. Z48305), *Bos grunniens* (GenBank Acc. No. AF194981), and *Bubalus bubalis* (GenBank Acc. No. AM238696) as outliers. The NJ tree (Figure 2.1) showed the presence of some haplotypes closely related to the consensus *BLG* promoter region of *Ovis aries* and in particular haplotypes H1 and H6, identical to the former except for the deletion at position -966. On the same branch are haplotypes H2, H3, H4, and H5 that showed polymorphisms in the proximal promoter region and in particular in the region between position -764 and position -42. The other haplotypes (H7-H22) were placed in a different branch and among them, haplotype H22 was the closest to the outlier sequences branch due to presence of all polymorphic sites in mutated homozygous condition compared to *Ovis aries* consensus.

Figure 2.1 Phylogenetic tree obtained using the Neighbor-Joining algorithm with nucleotide substitution model and 1,000 bootstrap replications.



2.4 Conclusion

Results showed high genetic variability in the *BLG* promoter region within our breeds. The presence of the polymorphic site -246 A/T could influence the binding affinity of NF-I in the region -253/-240 of the *BLG* promoter. Analysis of genetic diversity of the promoter region of *BLG* gene revealed the highest value of genetic diversity between Valle del Belice and Pinzirita breeds and the lowest one between Valle del Belice and Sarda breeds. The lowest value of genetic diversity between Valle del Belice and Sarda breeds may be related to a higher similarity of milk composition of these two breeds compared to the others. However, at present literature does not present any evidence about that. Further analyses will be conducted on a wider sample in order to estimate the possible effect that the loss of TF could have on *BLG* gene expression level and to evaluate the possible correlation between the genetic diversity indexes and the BLG content in milk of our breeds.

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3

Genetic diversity and population structure of Sicilian sheep breeds using microsatellite markers

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Abstract

Genetic diversity studies in domestic animals aim at evaluating genetic variation within and across breeds mainly for conservation purposes. In Sicily, dairy sheep production represents an important resource for hilly and mountain areas economy. Their milk is used for the production of traditional raw milk cheeses, sometimes Protected Designation of Origin (PDO) cheeses. In some cases, the quality of these products is linked to a specific breed, i.e. mono-breed labelled cheeses and it is therefore important to be able to distinguish the milk of a breed from that of others, in order to guarantee both the consumer and the breed itself. In order to investigate the genetic structure and to perform an assignment test, a total of 331 individuals (Barbaresca, BAR n=57, Comisana, COM n=65, Pinzirita, PIN n=75, Sarda, SAR n=64, and Valle del Belice, VDB n=70) were analysed using a panel of 20 microsatellite markers. A total of 259 alleles were observed with average polymorphic information content equal to 0.76, showing that the microsatellites panel used was highly informative. Estimates of observed heterozygosity ranged from 0.65 in the BAR breed to 0.75 in the COM breed. The low value of genetic differentiation among breeds (F_{st} = 0.049) may indicate that these breeds are little differentiated probably due to common history and breeding practices. The low F_{is} and F_{it} values indicated low level of inbreeding within and among breeds. The Unrooted Neighbor-Joining dendrogram obtained from the Reynold's genetic distances, and factorial correspondence analysis revealed a separation between BAR and the other sheep breeds. Recent migration rates were estimated, showing that four out of the five breeds have not received a significant proportion of migrants. Only for the PIN breed a recent introgression rate from the

VDB breed (7.2%) was observed. The Bayesian assignment test showed that BAR and SAR breeds had a more definite genetic structure (proportion of assignment of 92% and 86.6%, respectively), whereas the lowest assignment value was found in the PIN breed (67.1%). Our results indicated high genetic variability, low inbreeding and low genetic differentiation, except for BAR breed, and were in accordance with geographical location, history, and breeding practices. The low robustness of the assignment test makes it unfeasible for traceability purposes, due to the high level of admixture, in particular for COM, PIN and VDB.

Keywords: Sicilian sheep breed, microsatellite markers, genetic diversity, population structure

3.1 Introduction

In the past years, selection programs have mainly put high emphasis on production traits, which led to an increased specialization for traits such as milk yield and quality, meat, wool, etc. This happened sometimes even by crossbreeding the local breeds with exotic ones, to generate populations with the desired phenotypes. This hybridization process, however, has resulted in an increased reliance on a small number of breeds to meet the local's food requirements, which could lead to the disappearance of local breeds. Nevertheless, this aspect has received greater interest in the last years, based on the awareness that indigenous and locally developed sheep breeds are an important asset, because of the unique combinations of adaptive traits developed to respond effectively to the pressures of the local environment [1]. From these considerations and given the importance of the local genetic resources, it is easily understandable the considerable interest given nowadays to genetic diversity studies in domestic animals in general and, recently, in small ruminants [2]. Genetic diversity studies in domestic animals aim at evaluating genetic variation within and across breeds, since the breed is the management unit for which factors such as inbreeding are controlled [3]. However, the definition of a breed, as applied by FAO, frequently does not reflect the underlying genetic population structure. Therefore, a molecular genetics study of the population diversity and structure may improve the understanding of the actual genetic resources.

In Sicily, dairy sheep production represents an important resource for hilly and mountain areas economy, in which other economic activities are limited [4]. Their milk is mainly used for the production of traditional raw milk cheeses, sometimes Protected Designation of Origin (PDO) cheeses as laid down in the European Union legislation. In some cases, the quality of these products is linked to a specific breed, i.e. mono-breed labelled cheeses and it is therefore important to be able to distinguish the milk of a breed from that of others, in order to guarantee both the consumer and the breed itself. Assignment of individuals to a specific breed, especially when the phenotypic differentiation between breeds is difficult, is therefore of great importance both for biodiversity purposes and dairy products traceability.

Nowadays only four native sheep breeds are reared in Sicily: Barbaresca (BAR), Comisana (COM), Pinzirita (PIN), and Valle del Belice (VDB). These breeds present differences in both morphology and production traits and show excellent adaptability to the local environments. In particular, BAR and PIN breeds, due to their good adaptive traits and hardiness are raised on farms located in marginal areas, representing therefore an important genetic resource for present and future needs. In addition to these autochthonous breeds, the Sarda (SAR) breed, reared mainly in Sardinia, was considered due to its likely contribution to the origin of the VDB breed. Based on historical, geographical, and morphological information, it is likely that the VDB breed derives from the PIN breed, to which is similar for the horned trait in the males, crossed with the COM breed, to which is similar for coat colour (i.e. white with red head) and milk production. Subsequently, the cross between these two breeds was likely crossed with the SAR breed [5]. In addition, the BAR breed derives from crosses between Tunisian Barbary breed from North Africa and the PIN breed, and posterior selection for growth performance [6]. In recent years, several microsatellite studies on sheep genetic diversity, population structure, genetic differentiation, and phylogenetic reconstruction aiming at identifying endangered populations as well as developing genetic conservation strategies have been published [7-9]. Aim of this study was to explore the genetic structure of the four Sicilian autochthonous sheep and one Italian breed, through the analysis of the genetic diversity within and across breeds, and determine their genetic relationship, using microsatellite markers. Moreover, microsatellite markers were used for the proper assignment of an individual to a specific breed.

3.2 Materials and Methods

Sampling and DNA extraction

A total of 331 blood samples were used for the analysis, 267 of which belonging to the four Sicilian breeds (BAR (n=57), COM (n=65), PIN (n=75) and VDB (n=70)); the remaining 64 samples for the SAR breed were provided by the AGRIS Sardegna. All samples were collected from randomly chosen unrelated individuals, in several farms of different areas of the two Islands, in order to get samples as representative as possible. Genomic DNA was extracted from blood buffy coats of nucleated cells using a salting out method [10].

Microsatellites Amplifications and Analysis

A total of 20 microsatellite markers (Table S1) were selected as suggested by ISAG and FAO (http://www.fao.org/dad-is/). Moreover, some loci (*i.e.*, DU323541, DU223896 and DU194351*)* were selected based on location and DNA sequence information available at NCBI website (http://www.ncbi.nlm.nih.gov/genome/guide/sheep/).

Genotypes for all 20 microsatellite markers were determined by means of four multiplex fluorescent PCR reactions and fragment lengths determined in a single semi-automated multiplex electrophoresis run by using an AB3130 Genetic Analyzer and GeneMapper version 4.0 with recommended protocols (Applied Biosystems). Each reaction was performed in a total volume of 20 µl containing 50 ng template DNA, 1X Qiagen Multiplex PCR Master Mix, 1X PCR Master Mix, primer mix, and nuclease-free water. For the four multiplex reactions, the PCR program was: initial denaturation at 95°C for 15 min; 32 cycles of 95°C for 45 s, 58°C for 1 min 50 s, and 72°C for 1 min 20 s; and final extension at 60°C for 30 min. Amplification was carried out using the GeneAmp PCR system 9700. A total of 3.7 µl of Multiplex PCR was mixed with 0.3 µl of LIZ 500 Size Standard and 6.0 µl of Hi-Di Formamide. The multiplex PCR/LIZ/Formamide mixture was denatured for 5 min at 95°C and then immediately snap cooled on ice for 3 min before performing a capillary electrophoresis on an AB3130 Sequencer (Applied Biosystems).

Statistical analysis

Total number of observed alleles per locus (TNA), the mean number of alleles observed in a population over all loci genotyped (MNA) and its standard deviation, allelic richness, a measure of the number of alleles independent of sample size, (AR), observed and expected heterozygosity (H_o and H_S, respectively) per locus and overall loci in the whole sample [11], and Wright's fixation index (F_{is}, F_{it} and F_{st}) [12] were estimated using FSTAT 2.9.3 software [13]. Moreover, FSTAT was used to estimate observed and expected heterozygosity (H_o and H_e respectively) within breed. Polymorphism information content (PIC) per locus was estimated with Cervus 2.0 software [14] and the deviation from Hardy-Weinberg equilibrium (heterozygote deficiency) with the GENEPOP package version 4.0.11 [15] using a Markov Chain method (dememorization 10,000, batches 100, and iterations per batch 5,000). In addition, GENEPOP software was used to assess genotypic linkage disequilibrium (LD) between each pair of loci in the whole sample. Nei's minimum distance [11] and Reynold's distance [16], both recommended for populations with short divergence time [17], were used to estimate pair-wise genetic distances among breeds. The Reynold's genetic distance was used to reconstruct a neighbour-joining consensus tree using the Phylip package version 3.69 [18], and the dendrogram was depicted using the software TreeView version 1.6.6 [19]. Tree robustness was evaluated by bootstrapping over loci (1000 replicates). Moreover, a factorial correspondence analysis (FCA) was performed based on the individual multilocus genotype using the GENETIX version 4.03 [20]. In order to investigate the occurrence of recent migration among the breeds considered, the program BayesAss+ [21] was used, with 3x10^6 iterations, a burn-in of 999,999, and thin 2,000. This program simultaneously estimates the probability distribution of allelic frequencies for each locus, migration rates among populations (*m*) and inbreeding coefficient for each population (*F*).

STRUCTURE version 2.3.1 [22] was used to analyse the genetic structure and identify the true number of populations (clusters) and assign the individuals to each cluster. The program estimates the natural logarithm of the probability that a given genotype (*G*) is part of a given population (*K*). The model used was based on an assumption of admixture and correlated allele frequencies as suggested by several authors [22-24]. The

Ln Pr(*G*|*K*) was calculated for *K* ranging from 1 to 9, without prior information on the breed of origin, to estimate the most likely number of clusters in the dataset, with 50 independent runs for each *K*. All runs consisted of a burn-in period of 100,000 steps, followed by 100,000 Markov Chain Monte Carlo (MCMC) iterations.

3.3 Results

In total, 259 alleles were observed for the 20 loci surveyed. The TNA per locus ranged from seven for OarFCB128 and APPO10 to 24 for OarCP49 with an average of 12.95±4.32 (Table 3.1). The PIC considering all loci was equal to 0.76±0.10, showing that the microsatellites panel used was highly informative. DU194351 was found to be the least informative marker (0.50), whereas DU216028 the most informative one (0.89) (Table 3.1). The number of alleles per locus observed in each breed ranged from 3 to 18. The BAR breed showed the lowest number of observed alleles (3) and the lowest overall mean number of alleles (6.15±1.74). The highest number of observed alleles per locus was found in the PIN breed (18) with a mean number of alleles equal to 11.05±3.43 (Table 3.1). Private alleles, *i.e.* alleles unique for a single population, were evidenced in all breeds with low frequency (<3%). However, two private alleles of the microsatellite INRA132 (161 and 179 bp alleles) were found with higher frequency (6.2% and 10.2%, respectively) in the SAR breed. Hardy-Weinberg equilibrium was consistently rejected for DU223896 in all breeds (Table S2) and this marker was not further considered. The average observed (H_o) and expected (H_s) heterozygosities and the overall genetic diversity (H_t) for the 19 microsatellites are given in Table S3.

Table 3.1 Total number of alleles per locus (TNA), number of observed alleles per locus and per breed, and polymorphism information content (PIC).

MARKER	TNA	BAR	COM	PIN	SAR	VDB	PIC
OarFCB128	7	5	6	6	7	7	0.68
IDVGA45	13	3	8	11	10	12	0.79
BM827B	8	6	7	7	6	7	0.73
ILSTS011	8	5	6	7	6	8	0.72
DU323541	17	7	11	17	10	12	0.86
OarCP49	24	10	16	18	17	13	0.87
INRA063A	17	8	14	15	13	14	0.83
SPS115	8	5	6	8	7	6	0.74
LSCV36B	14	7	10	13	12	12	0.84
MAF209	12	6	10	12	8	10	0.76
BRN	10	5	7	8	7	8	0.58
McM527	10	6	8	9	6	10	0.80
ILSTS005	11	6	8	9	10	9	0.63
TCRBV6	15	5	12	11	11	11	0.75
DU223896	17	7	8	14	10	11	0.82
APPO10	7	3	6	7	7	7	0.70
INRA132	15	7	11	13	12	11	0.84
CSRD247	16	7	12	12	10	10	0.80
DU194351	17	5	10	12	9	9	0.50
DU216028	13	9	12	13	11	12	0.89
Overall mean	12.95	6.15	9.40	11.05	9.45	9.95	0.76
S.D.	4.32	1.74	2.87	3.43	2.82	2.26	0.10

BAR = Barbaresca; COM = Comisana; PIN = Pinzirita; SAR = Sarda; VDB = Valle del Belice

Considering the whole sample, H_o ranged from 0.430 to 0.876, H_s from 0.504 to 0.866, and H_t from 0.517 to 0.899. DU194351 marker showed the lowest H_S (0.504), whereas DU216028 the highest one (0.866). The H_o was always higher than 0.50, except for the marker DU194351. However, up to seven markers were found to be not in Hardy-Weinberg equilibrium across breeds; in fact for these markers the observed heterozygosity was lower than the expected one. Heterozygote deficiency (F_{is}) analysis revealed that all five breeds exhibited deviations from HWE at some loci (Table S3). Considering the whole sample, the number of markers showing a significant deviation from Hardy-Weinberg equilibrium ranged from 2 (BAR) to 7 (COM) (Table S2). Significant linkage disequilibrium (P<0.001) was found between BM827B and DU323541, INRA063A and LSCV36B, and OarFCB128 and DU216028.

The MNA and AR for each breed are showed in Table 3.2. MNA ranged between 6.05±1.78 to 10.95±3.45 for BAR and PIN breeds, respectively, whereas AR ranged between 5.66±1.57 to 9.44±2.84 for the same breed.

Table 3.2 Mean number of alleles (MNA), allelic richness (AR), observed (H_o) and expected (H_e) heterozygosity and standard deviation (s.d).

Breed	MNA± s.d.	AR± s.d.	H_o ± s.d.	H_e ± s.d.
BAR	6.05 ± 1.78	5.66 ± 1.57	0.65 ± 0.165	0.67 ± 0.094
COM	9.47 ± 2.93	8.73 ± 2.71	0.75 ± 0.131	0.77 ± 0.095
PIN	10.95 ± 3.45	9.44 ± 2.84	0.72 ± 0.141	0.78 ± 0.092
SAR	9.42 ± 2.89	8.39 ± 2.32	0.71 ± 0.108	0.75 ± 0.102
VDB	9.89 ± 2.31	8.64 ± 1.95	0.71 ± 0.123	0.77 ± 0.097

BAR = Barbaresca; COM = Comisana; PIN = Pinzirita; SAR = Sarda; VDB = Valle del Belice

Table S3 also reports the population differentiation examined by fixation indices for each locus and across all loci and the mean estimates of *F*-statistic obtained were: F_{it} = 0.080, F_{is} = 0.032 and F_{st} = 0.049. An overview of the genetic diversity parameters for each breed is given in Table 3.2. Estimates of H_o ranged from 0.65 in BAR to 0.75 in COM, whereas estimates of H_e from 0.67 in BAR to 0.78 in PIN. Table 3.3 shows the Nei and Reynolds genetic distance estimates. The lowest values were observed between COM and PIN breeds for both Reynolds (0.025) and Nei-minimum (0.020), whereas the highest ones between BAR and SAR breeds (0.110 and 0.087 for Reynolds and Nei-minimum, respectively). The BAR breed showed the highest genetic distance in relation to the other four breeds.

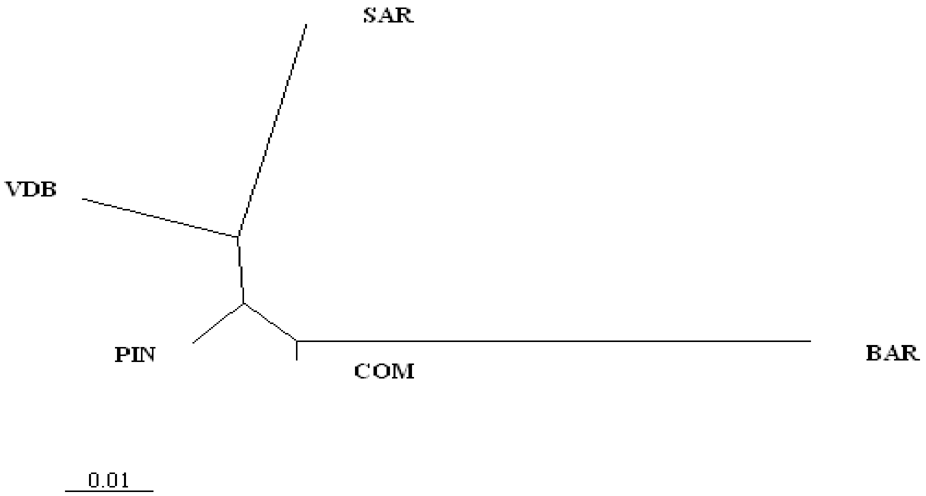
Table 3.3 Reynolds (above the diagonal) and Nei-minimum (below the diagonal) genetic distance per pair of breeds.

Breed	**BAR**	**COM**	**PIN**	**SAR**	**VDB**
BAR	-	0.077	0.075	0.110	0.095
COM	0.061	-	0.025	0.057	0.036
PIN	0.058	0.020	-	0.050	0.036
SAR	0.087	0.045	0.040	-	0.054
VDB	0.075	0.028	0.028	0.043	-

BAR = Barbaresca; COM = Comisana; PIN = Pinzirita; SAR = Sarda; VDB = Valle del Belice

The Reynold’s genetic distances were also used to reconstruct the Unrooted Neighbor-Joining dendrogram (Figure 3.1), showing two clear clusters: VDB/SAR and PIN/COM, confirming that the BAR breed appears again to be more distant from the other breeds.

Figure 3.1 Unrooted Neighbor-Joining dendrogram showing the genetic relationships among sheep breeds using Reynolds genetic distance. (BAR = Barbaresca; COM = Comisana; PIN = Pinzirita; SAR = Sarda; VDB = Valle del Belice)



The factorial correspondence analysis was performed including all breeds and loci using the corresponding allele frequencies (Figure 3.2). The first three components explained the 86.46% of the total variation, 38.52% of which explained by Axis 1 that clearly separates the BAR breed from the other breeds; 28.49% explained by Axis 2 that separates the SAR breed; and 19.45% explained by Axis 3 that separates the VDB breed.

Estimates of the recent migration rates (*m*) (up to the second generation of migrants) and inbreeding coefficients per breed are presented in Table 3.4. Values on the diagonal are defined as the proportion of individuals in each generation that are not migrants. All breeds but PIN have not received a significant proportion of migrants. Nevertheless, recent introgression was observed for the PIN breed from the VDB breed at a rate of 7.2%. The posterior distribution of inbreeding coefficients ranged from 0.03 (COM) to 0.08 (PIN).

Figure 3.2 Spatial representation of the breeds as defined by the Factorial Correspondence Analysis. (BAR = Barbaresca; COM = Comisana; PIN = Pinzirita; SAR = Sarda; VDB = Valle del Belice)

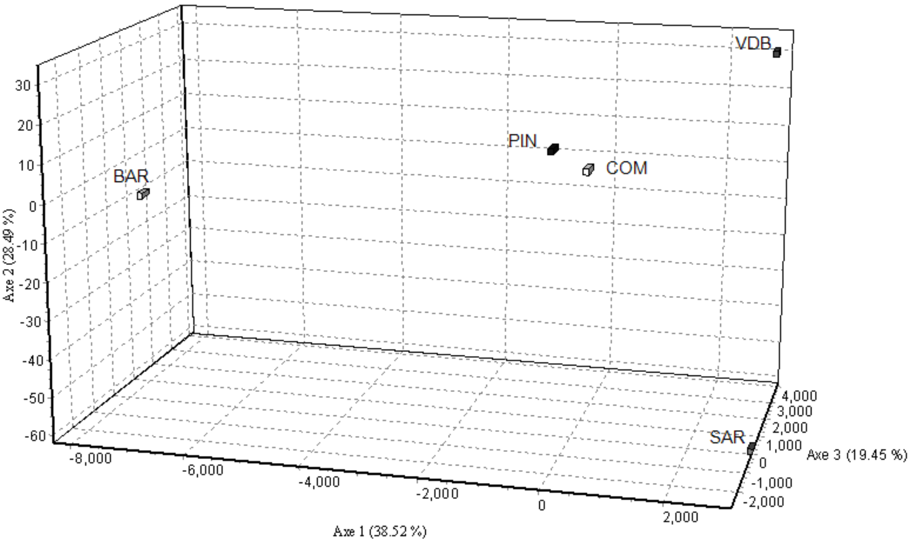


Table 3.4 Means of the posterior distribution of the migration rates and means and standard deviations (s.d.) of the posterior distribution of the inbreeding coefficient (*F*) for each population.

	BAR	**COM**	**PIN**	**SAR**	**VDB**	***F* ± s.d.**
BAR	0.9848	0.0239	0.0031	0.0024	0.0025	0.03 ± 0.015
COM	0.0023	0.9392	0.0221	0.0185	0.0051	0.03 ± 0.013
PIN	0.0053	0.0156	0.8991	0.0031	0.0266	0.08 ± 0.017
SAR	0.0053	0.0062	0.0036	0.9720	0.0110	0.04 ± 0.014
VDB	0.0024	0.0151	0.0721	0.0040	0.9548	0.07 ± 0.014

BAR = Barbaresca; COM = Comisana; PIN = Pinzirita; SAR = Sarda; VDB = Valle del Belice

Assignment test was performed using the program STRUCTURE with the number of expected population (K) ranging from 1 to 9. The Ln Pr($G|K$) increased from $K = 2$ to $K = 5$, reached a "plateau" at $K = 5$, while did not show a significant fluctuation from $K = 5$ to $K = 8$ and then decreased for $K = 9$ (Figure S1). Therefore it was assumed that $K = 5$ is the most likely number of clusters. For $K = 2$ the BAR separates from other breeds, while for $K = 3$ it is the SAR that appears isolated and the Sicilian dairy breeds remained in the same cluster; at $K = 4$ the VDB separates from the other Sicilian dairy breeds (COM and PIN) and, finally, for $K = 5$ each breed tends to have their own distinct cluster (Figure 3.3).

Figure 3.3 Estimated population structure of the 5 sheep breeds for *K* ranging from 2 to 5. (BAR = Barbaresca; COM = Comisana; PIN = Pinzirita; SAR = Sarda; VDB = Valle del Belice)

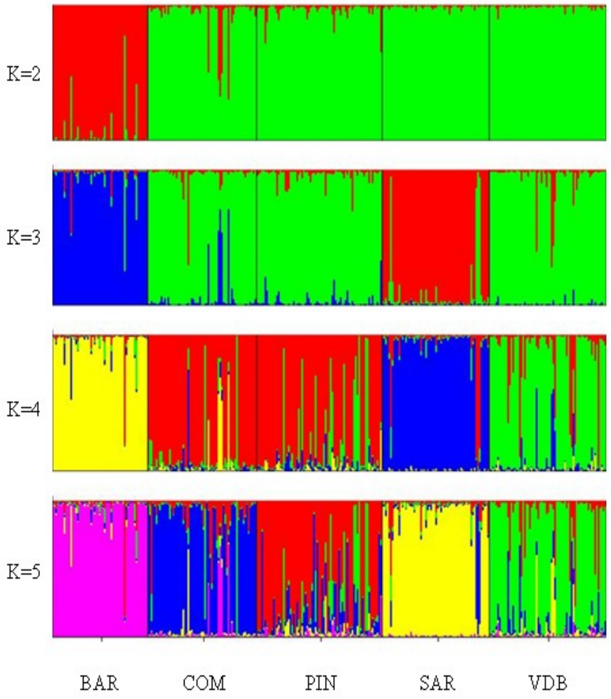


Table 3.5 shows the assignment proportion of each breed to the eight most likely clusters inferred, choosing the iteration with the minimum variance.

Table 3.5 Number of individuals (N) per breed and proportion of membership of each breed in each of the 5 clusters inferred in the most likely run of the program STRUCTURE.

	Inferred clusters					
Breed	**1**	**2**	**3**	**4**	**5**	**N**
BAR	0.031	0.016	0.014	0.020	0.919	57
COM	0.094	0.054	0.769	0.027	0.056	65
PIN	0.675	0.124	0.151	0.027	0.023	75
SAR	0.042	0.023	0.059	0.867	0.009	64
VDB	0.100	0.761	0.074	0.049	0.015	70

BAR = Barbaresca; COM = Comisana; PIN = Pinzirita; SAR = Sarda; VDB = Valle del Belice

Clusters five and four included the BAR and the SAR individuals with 91.9% and 86.7%, respectively, showing a significant proportion of assignment for these breeds; cluster three and two included the COM and the VDB individuals with a proportion of assignment of 76.9% and 76.1%, respectively. The lowest value of assignment was showed in the PIN breed with a proportion of assignment of 67.5% in the cluster one.

3.4 Discussion

In this study we investigated the genetic variability detected through microsatellite markers in the four native sheep breeds reared in Sicily and in the SAR breed. The majority of the markers were highly polymorphic and generally in Hardy-Weinberg equilibrium except for the marker DU223896 that showed the largest difference between observed and expected heterozygosity. The significant deviation observed for the marker DU223896 may be explained by unobserved null alleles leading to high within-breed F_{is} values, ranging from 0.3139 to 0.6415. Genetic linkage disequilibrium was found for some microsatellite markers and it can be due to a variety of factors, including physical linkage, epistatic selection, and genetic hitchhiking [25]. However, given that these markers have been mapped to different chromosomes, physical linkage was excluded.

Overall mean heterozygosity reflects a notably high variability, characteristic of microsatellites derived from a greater mutation level than other genetic markers, which makes them a valuable tool for genetic diversity analyses [26]. The high mean heterozygosity values could be attributed to low inbreeding levels found, low selection pressure, gene flow among Sicilian sheep breeds, and the large number of alleles present in all breeds. Another parameter indicative of the genetic variation is the PIC estimate, which was higher than 0.50 and therefore highly informative [27]. Estimates of observed heterozygosity over all loci confirmed the remarkable level of genetic variability in these breeds.

Most of the breeds considered in this study have never been genetically characterized before; therefore, it was not always possible to compare our results with what is reported in literature. Previous studies presented similar H_e estimates for the SAR breed [28-29], but lower H_o (0.60 and 0.66, respectively); whereas our H_e and H_o estimates for the COM breed were higher than those reported by Lawson Handley et al. [29]. However, it has to be considered that differences in the estimates can be also attributed to the different number of markers and/or sampling scheme used. The similarities among breeds for the average number of alleles across loci and H_e, except for BAR, can be explained by the phylogenetic relationships; in fact as reported above, COM, PIN, SAR, and VDB seem to be genetically related among them. The lower number of alleles observed in the BAR breed is probably due to a reduced effective population size. This breed is, indeed, reared in a very restricted area of Sicily and nowadays about 1,200 animals are enrolled in the herd book [30]. The number of private alleles found in the SAR breed was probably due to geographic isolation, i.e. determining a reduced or absent gene flow with the Sicilian breeds.

The low F_{is} (within population inbreeding estimate) and F_{it} (total inbreeding estimate) values indicate low inbreeding level within and among breeds. The F_{st} (measurement of population differentiation) value may indicate that these breeds are not differentiated enough and that they may have a common history and breeding practices. To confirm this hypothesis another analysis was performed, by removing the SAR breed from the whole sample and F_{st} value decreased from 0.049 to 0.046, underlying the low differentiation among these breeds. Thus, a large part of the total genetic diversity can be explained by the variation within breeds (0.951) and to a smaller extent by the variation among breeds (0.049). Lack of differentiation between the Sicilian breeds could be due to geographic proximity, similarities in environment and breeding practices, but most likely due past gene flow among them. These estimates of genetic differentiation are comparable to those reported by other authors for indigenous Ethiopian (F_{st} = 0.046) [31], Portuguese (F_{st} = 0.049) [32], and Alpine (F_{st} = 0.057) [7] sheep breeds and/or populations, but lower than those reported by Baumung et al. [33] in Austrian sheep breeds and Arora et al. [11] in Indian sheep breeds (0.080 and 0.111, respectively). Moreover, Lawson Handley et al. [29] in a study on European sheep breeds, including the COM and the SAR, reported that the Southern breeds are characterized by higher within-breed diversity, lower genetic differentiation, and higher level of heterozygosity than Northern breeds.

In PIN and VDB breeds the higher genetic diversity (H_e and average number of alleles per locus), but at the same time the highest coefficient of inbreeding suggested that whereas PIN and VDB have a wide genetic base, individuals belonging to these two breeds were more inbred than those in the other breeds. The rather high level of genetic variability in PIN and VDB breeds is not surprising, as these are the most numerous sheep breeds reared in Sicily. The highest level of inbreeding could be affected by the farming system. In Sicily rams are reared together with ewes, therefore mating with close relatives can be quite frequent. Moreover, the reduced or absent exchange of rams between different flocks of the same breed may have induced the development of a substructure within the populations. The results of genetic distances are in agreement with a previous study conducted on the genetic structure and relationship between the VDB breed and the other sheep breeds considered as ancestors, using the genetic polymorphisms of seven protein systems [34]. The Unrooted Neighbor-Joining dendrogram showed two clear clusters in accordance with the phylogenetic relationships among breeds. Moreover, the FCA analysis showed a relationship between COM and PIN breeds. The genetic closeness between these two breeds might be explained considering that these breeds are characterized by a common breeding system and geographical husbandry area, which might have led to genetic exchange between them. This seems to be partially confirmed by the structure analysis, showing that COM and PIN breeds shared a cluster.

The FCA results corroborate the findings based on the Nei and Reynolds genetic distances, which also indicated the isolation and a greater genetic distance of the BAR breed. In fact, the least variable breeds are usually the most distinct ones [35]. It is important to highlight that this breed was mainly reared for meat production, unlike the other breeds reared exclusively for milk production; therefore, there was less gene exchange between the former and the other breeds.

We investigated population structure by varying K from one to nine. Although the analysis showed the highest probability of forming eight clusters, K = 5 was chosen as the best value to describe the genetic structure of our breeds, since the increase of Ln Pr($G|K$) from K = 5 to K = 8 was low if compared with the increase from K = 1 to K = 5. However, as suggested by Pritchard et al. [22], the inferred clusters are not necessarily the corresponding real ancestral population and they can be determined by sampling schemes. Assuming K = 3 the BAR and the SAR formed two distinct groups suggesting that admixture was nearly zero for these two breeds. The high average percentage of assignment of individuals for BAR and SAR breeds pointed out the existence of clear genetic differences compared to other breeds and this result is also confirmed by the geographic distribution of these two breeds as well as the different breeding system (*i.e.*, meat production) for the former. The COM, PIN and VDB breeds exhibited the presence of admixture, in fact the COM and the PIN clustered together up to $K = 4$, confirming the genetic closeness between these breeds. For $K = 5$, COM, PIN and VDB breeds formed three distinct clusters but with proportion of membership split in two or more clusters. This result could be probably due to the phylogenetic relationships among these breeds and/or to the migration of individuals among the several farms present in the area.

3.5 Conclusions

This study allowed exploring the genetic structure of the Sicilian autochthonous sheep breeds. Our results indicated that significant amounts of genetic variation are still maintained in these sheep breeds. The results are in accordance with their geographical location, origin, and breeding practices. The identification of genetic relationship and gene flow among livestock breeds/populations is important for breeders and conservationists. In order to maintain the existing genetic diversity, breeding strategies (i.e. oriented seedstock exchange) aiming at maintaining effective population size, minimizing inbreeding and genetic drift should be implemented for the different breeds, especially for the BAR that could be considered the most differentiated and endangered breed. In the case of BAR, besides the strategies suggested before, a conservation program is recommended and an *in vitro* (e.g. germplasm bank) and *in situ* (e.g. conservation population) approaches should be considered for future purposes. The robustness of assignment test depends on the genetic differentiation but, unfortunately, it isn´t the case for COM, PIN and VDB, and resulted in an unsatisfactory assignment performance and thus it cannot be used for traceability purposes.

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Table S1 Microsatellite marker panel information.

MARKER	Chr	cM	Size range	locus
OarFCB128	2	96.1	98-132	UniSTS:250719
IDVGA45	18	44.2	162-188	UniSTS:251455
BM827B	3	161.6	205-217	UniSTS:250735
ILSTS011	9	40.1	264-290	UniSTS:250863
DU323541	8	-	365-421	GenBank:DU323541
OarCP49	17	29.9	80-108	UniSTS:251389
INRA063A	14	65.1	157-179	UniSTS:251139
SPS115	15	34.8	230-250	UniSTS:279634
LSCV36B	11	72.6	445-469	UniSTS:45189
MAF209	17	49.4	103-129	UniSTS:251175
BRN	7	47.2	138-148	UniSTS:251060
McM527	5	127.4	167-183	UniSTS:251416
ILSTS005	7	134.4	186-216	UniSTS:250860
TCRBV6	4	136.0	226-270	UniSTS:279515
DU223896	12	-	338-364	GenBank:DU223896
APPO10	1	149.6	107-125	UniSTS:279440
INRA132	20	9.5	145-179	UniSTS:251168
CSRD247	14	25.5	215-251	UniSTS:253556
DU194351	13	-	354-386	GenBank:DU194351
DU216028	23	-	472-510	GenBank:DU216028

Chr=chromosome; cM=centimorgan

Table S2 Heterozygote deficiency (HW testing) within population (F_{is}) values per marker and breed.

MARKER	BAR	COM	PIN	SAR	VDB
OarFCB128	-0.0359	-0.0452	0.0409	0.0267	-0.0209
IDVGA45	-0.0213	0.0198	-0.0098	-0.0462	0.0923
*BM827B****	0.0410	0.2482*	0.3095***	0.1452*	0.0989
ILSTS011	-0.0608	-0.0640	0.0323	0.0512	0.1042
DU323541	-0.0667	-0.0416	0.0590	-0.0144	-0.0404
*OarCP49**	-0.0500	0.0167*	0.0371	0.0810	0.0226
*INRA063A**	-0.1345	0.0525*	0.0309	0.0132	0.0374*
SPS115	0.0989	-0.0710	-0.0634	0.0773*	0.0207
*LSCV36B****	0.0875	-0.0125	0.0821*	0.1839**	-0.0339*
MAF209	0.0117	0.0163	0.0365	-0.0732	-0.0142
BRN	-0.1680	0.0240	0.1974	-0.0137	0.0072
McM527	-0.0112	-0.0080	0.0343	0.0106	0.0822
ILSTS005	-0.0170	-0.1070	0.0683	-0.0705	0.0666
*TCRBV6**	0.0478	0.0142*	0.0537	-0.0212	0.2230*
*DU223896****	0.6415***	0.3139***	0.4362***	0.3157**	0.4206***
*APPO10**	0.0006	0.1124*	0.0642	0.0325	0.1530
INRA132	-0.0255	-0.0608	-0.0589	0.0827	0.0786
CSRD247	-0.0337	0.0365	-0.0242	0.0289	0.0607**
*DU194351****	0.4166**	0.1356**	0.1659*	-0.0211	0.0139
DU216028	-0.1048	-0.0709	-0.0276	0.1169	0.0147

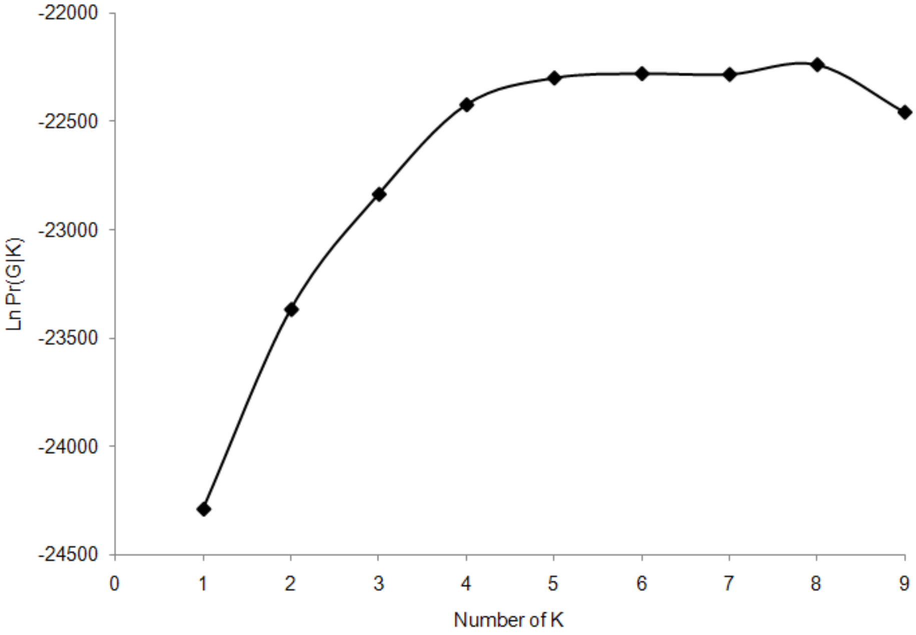
One, two and three asterisks mean, respectively, a significant deviation from Hardy-Weinberg equilibrium for $P < 0.05$, $P < 0.01$, and $P < 0.001$

Table S3 Nei's estimation of heterozygosity and Wright's fixation index of each microsatellite considering the whole sample.

MARKER	H_o	H_s	H_t	F_{it}	F_{st}	F_{is}
OarFCB128	0.695	0.691	0.713	0.035	0.038	-0.003
IDVGA45	0.764	0.770	0.809	0.066	0.057	0.010
BM827B	0.618	0.747	0.764	0.199	0.025	0.179
ILSTS011	0.723	0.731	0.761	0.071	0.046	0.026
DU323541	0.840	0.825	0.869	0.044	0.059	-0.016
OarCP49	0.832	0.851	0.876	0.059	0.035	0.024
INRA063A	0.797	0.797	0.847	0.074	0.068	0.006
SPS115	0.716	0.722	0.778	0.087	0.085	0.003
LSCV36B	0.755	0.805	0.858	0.123	0.071	0.056
MAF209	0.765	0.761	0.788	0.038	0.043	-0.005
BRN	0.611	0.620	0.642	0.063	0.039	0.025
McM527	0.762	0.780	0.819	0.079	0.055	0.026
ILSTS005	0.657	0.648	0.662	0.017	0.027	-0.010
TCRBV6	0.695	0.742	0.772	0.111	0.048	0.066
APPO10	0.664	0.719	0.741	0.112	0.036	0.079
INRA132	0.826	0.828	0.854	0.039	0.036	0.003
CSRD247	0.741	0.754	0.817	0.103	0.087	0.017
DU194351	0.430	0.504	0.517	0.161	0.027	0.138
DU216028	0.876	0.866	0.899	0.033	0.043	-0.010
Overall	0.724	0.745	0.778	0.080	0.049	0.032

Observed heterozygosity (Ho), Expected heterozygosity (Hs), Total heterozygosity (Ht), Total inbreeding estimate (Fit), Measurement of population differentiation (Fst),Within population inbreeding estimate (Fis)

Figure S1 Plot of estimated posterior probabilities of the data LnPr(G|K) for different number of inferred clusters (K=1 to 9).



4

Genome wide structure in indigenous Sicilian cattle breeds

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Abstract

Background: In recent years, there has been great interest in recovering and preserving local breeds. Genomic technologies, such as high-throughput genotyping based on SNP arrays, provide background information concerning genome structure in domestic animals. The aim of this work was to investigate the genetic structure, the genome-wide estimates of inbreeding, coancestry and effective population size (N_e), and the patterns of linkage disequilibrium (LD) in two Sicilian local cattle breeds, Cinisara and Modicana, using the Illumina Bovine SNP50K BeadChip.

Results: Principal Components Analysis and Bayesian clustering algorithm showed that animals from the two Sicilian breeds formed non-overlapping clusters and are clearly separated populations, even from the Holstein control population. Between the Sicilian cattle breeds, the Modicana was the most differentiated population, whereas the Cinisara animals showed a lowest value of assignment, the presence of substructure and genetic links occurred between both breeds. The average molecular inbreeding and coancestry coefficients were moderately high, and the current estimates of N_e were low in both breeds. These values indicated a low genetic variability. Average r^2 was notably lower than the values observed in other cattle breeds. The highest r^2 values were found in chromosome 14, where causative mutations affecting variation in milk production traits have been reported.

Conclusions: This study has reported for the first time in these local populations, estimates of population structure, levels of coancestry and inbreeding, and linkage disequilibrium from a genome-wide perspective. The levels of inbreeding and N_e showed in this study point out the necessity of establishing a conservation program in these autochthonous breeds. The control of molecular inbreeding and coancestry would restrict inbreeding depression, the probability of losing beneficial rare alleles, and therefore the risk of extinction. The extreme low values of LD would indicate that the present chip cannot pick up the real LD existing in these two breeds, and that a high density SNP array would be better to capture the LD information. The results generated from this study have important implications for the design and applications of association studies as well as for the development of conservation and/or selection breeding programs.

Keywords: Genetic diversity, linkage disequilibrium, sicilian cattle breeds, single nucleotide polymorphism

4.1 Introduction

The global decline in livestock genetic diversity is mainly the result of the massive use of a small number of selected breeds. However, in recent years, an increasing interest in recovering and preserving local breeds and/or populations has taken place [1]. Potentially, there is much unrecognized beneficial genetic variability in local breeds and populations, which supposes important reservoirs of non-exploited genetic resources. This genetic variability guarantees its selection response to productive and adaptation traits improvement, to cope with new environmental conditions, changes in market demands, husbandry practices and disease challenges [2]. Maintaining the highest levels of genetic diversity and limiting the increase in inbreeding is the premise of most conservation programs [3]. Traditionally, inbreeding (F) and coancestry (f) have been estimated on the basis of pedigree information [4]. The effective population size (N_e), a general indicator of the risk of genetic erosion, contains relevant information for the monitoring of the genetic diversity and helps to explain how populations evolved [5]. Managing the rate of inbreeding and coancestry, or equivalently, the N_e, provides a general framework to control the loss of variability avoiding or alleviating the reductions in viability and fertility; i.e., inbreeding depression [6]. F and f estimates depend on the completeness and accuracy of the available pedigree records which is in most cases inexistent or wrong in local breeds. Currently, with the availability of high-density single nucleotide polymorphism (SNP) chips, these coefficients can be estimated accurately in the absence of pedigree information [7,8]. SNP genotyping allows the simultaneous high-throughput interrogation of hundreds of thousands of loci with high precision at an affordable cost that enables large-scale studies [9]. In addition, this approach considers selective variation that classical neutral markers (e.g. microsatellites) ignore. Moreover, with the application of genome-wide SNP genotyping, the study in livestock population of the extent of linkage disequilibrium (LD), the non-random association of alleles at different loci, has gained more attention. The extent of LD is often used to determine the optimal number of markers required for fine mapping of quantitative trait loci (QTL) [10], genomic selection [11] and increasing the understanding of genomic architecture and the evolutionary history of the populations [12].

In Sicily, dairy production represents an important resource for hilly and mountain areas economy, in which other economic activities are limited, and the dairy products are the link among product-territory, territory-breed and breed-product. An interesting situation is represented by two local cattle breeds farmed in extensive traditional systems in this area. The Cinisara breed is characterized by a uniform black coat and less frequently by black spotted coat, and the Modicana breed is characterized by a solid red coat. Both breeds are farmed in Sicily and their economic importance lies on the traditional production systems of two typical 'pasta filata' cheeses: 'Caciocavallo Palermitano' and Ragusano P.D.O. (Protected Designation of Origin), respectively. These two breeds are well adapted to the harshness of marginal mountain areas of Sicily, due to their good grazing characteristics, and resistance to environmental conditions. Therefore, the socio-economic and ecological values, the historical, cultural and genetic heritage of these two breeds are unquestionable. The Cinisara and Modicana breeds are not subject to breeding programs. In fact, the development of breeding programs for local breeds is too costly for breeding organizations, and the absence of pedigree records is a threat for the existence of these breeds. However, all aspects mentioned above, which creates income for a small group of farmers, justifies the establishment of a conservation program [13]. In the last 50 years these local breeds have undergone a progressive reduction in size, mainly due to the mechanization of agriculture and to the introduction of more specialized and productive cosmopolitan breeds. The risk of population extinction states the necessity of exploring the genetic characteristics of these two breeds, never studied before from a genetic perspective.

The aim of this study was to investigate these breeds from a genetic perspective, including the analysis of: i) genetic structure, ii) genome-wide estimates of F and f and $N_{e,}$ and iii) the patterns of LD for the two Sicilian local cattle breeds, Cinisara and Modicana.

4.2 Materials and Methods

DNA sampling and genotyping

A total of 144 animals from 14 farms were used for the analysis. Samples consisted of 72 Cinisara (CIN) (68 cows and 4 bulls) and 72 Modicana (MOD) (69 cows and 3 bulls) animals born between 1999 and 2010 and chosen on the basis of their phenotypic profiles (morphological traits as coat color) and information supplied by the farmers (year of birth). For these cattle breeds pedigree data are not available. About 10 ml of blood was collected from caudal vein using tubes with EDTA as anticoagulant. Genomic DNA was extracted from buffy coats of nucleated cells using the Salting Out method [14]. The concentration of extracted DNA was assessed with the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

The CIN and MOD animals were genotyped for 54 609 SNPs, using the Illumina Bovine SNP50K v2 BeadChip, and following the standard operating procedures recommended by the manufacturer. Genotyping data were initially tested for quality using the Illumina Genome Studio Genotyping Module v1.0 software. Only the SNPs located on the autosomes were considered in further analyses. SNPs unmapped were discarded. The markers were filtered according to quality criteria that included: (i) Call Frequency (proportion of samples with genotype at each locus) ($\geq$ 0.98), (ii) Gen Train Score (quality of the probe that determines the shape and separation of clusters) (> 0.70), (iii) AB R Mean (genotype signal intensity) (> 0.35), (iv) Minor Allele Frequency (MAF) ($\geq$ 0.05), and (v) Hardy-Weinberg equilibrium (HWE) (p-value 0.001). SNPs that did not satisfy these quality criteria were excluded.

Genetic structure

STRUCTURE version 2.3.1 [15] was used to analyze the genetic structure, to identify the true number of populations (clusters) and to assign the individuals to each cluster. Genotypes from 96 animals of Holstein Friesian (HOL) cattle breed, tested with the same quality criteria, were included in this analysis and used to investigate the relationship among breeds. Unlinked SNPs were selected using the --indep option of the PLINK program [16] with the following parameters: 50 SNPs per window, a shift of 5 SNPs between windows and a variation inflation factor's threshold of 2. From these markers, a random set of 10 000 SNPs were used. Analysis was performed considering both the admixture model and the correlated allele frequencies between populations [15]. The length of the burn-in and MCMC (Monte Carlo Markov chain) were 50 000 steps and 100 000 iterations, respectively. The Ln Pr(G|K) was calculated for each K ranging from 1 to 6 without prior information on the breed and farm origins, to estimate the most likely number of clusters in the dataset, running 10 independent replicates for each K.

The genetic relationship between individuals was also estimated by Principal Components Analysis (PCA) of genetic distance. These values were calculated using PLINK program [16] through the use of commands --cluster and --distance matrix. PCA of the genetic distance (D) matrix was performed using the multidimensional scaling (MDS) option within PLINK. It should be noted that when MDS is applied to D, it is numerically identical to PCA [16]. The graphical representation was depicted using the statistical *R* software [17]. The same software was used to visualize the IBS matrices using the Heatmap function.

Inbreeding and coancestry molecular coefficients, rates of inbreeding and coancestry and effective population size

Following Malécot [18], molecular *f* coefficient between pairs of individuals is defined as the probability that two alleles taken at random, one from each individual, are identical in state. Similarly, molecular *F* coefficient for each individual is defined as the probability that the two alleles of a locus are identical in state. The molecular *f* coefficient between individuals *i* and *j* was calculated as:

$$f_{ij} = (1/L)\sum_{l=1}^{L}\left[\left(\sum_{k=1}^{2}\sum_{m=1}^{2} I_{lk(i)m(j)}\right)/4\right]$$

where L is the number of markers and $I_{lk(i)m(j)}$ is the identity of the k^{th} allele from individual i with the m^{th} allele from the animal j at locus l, that takes a value of 1 if alleles are identical and 0 if they are not. The molecular F coefficient for individual i was calculated as $F = 2f_{ii} - 1$ (i.e. f_{ii} is the molecular self-coancestry). Thus, F was estimated as the proportion of homozygous genotypes.

Rate of molecular inbreeding per year (ΔF) was computed by regressing the natural logarithm of $(1-F)$ on year of birth. Rate of molecular coancestry per year (Δf) was estimated in the same way, i.e. by regressing the natural logarithm of $(1-f)$ for each pair of individuals on year of birth. The N_e was estimated from the rates of inbreeding ($N_{eF} = 1/2L\Delta F$) and coancestry ($N_{ef} = 1/2L\Delta f$) per generation, assuming a generation interval L of six years.

Linkage Disequilibrium

A standard descriptive Linkage Disequilibrium parameter, the squared correlation coefficient of allele frequencies at a pair of loci (r^2), was obtained using PLINK [16]. For each SNP, pairwise LD between adjacent SNPs was calculated on each chromosome. Also r^2 was estimated for all pairwise combinations of SNPs using Haploview v4.2 software [19]. For each chromosome, pairwise r^2 was calculated for SNPs between 5 kb and 50 Mb apart. To visualize the LD pattern per chromosome, r^2 values were stacked and plotted as a function of inter-marker distance categories.

4.3 Results and discussion

A descriptive summary of chromosomes and SNPs is shown in Table 4.1. The number of SNPs per chromosome ranged from 2513 (chromosome

BTA1) to 711 (BTA27) in CIN and from 2366 (BTA1) to 690 (BTA28) in MOD, and the average density of SNPs per Mb were 15 for CIN and 14 for MOD. The overall mean MAF was 0.248±0.016 for CIN and 0.240±0.013 for MOD being these values in agreement with those reported by Matukumalli et al. [9] in a study on development and characterization of a high density SNP genotyping assay for several cattle breeds. A high proportion of monomorphic SNPs was expected in both breeds given its reduced population size across years. In particular, the MOD breed presented a higher number of fixed alleles (2412 SNPs) respect to CIN breed (461 SNPs). Among the 54 609 SNPs included in the chip only 52 886 mapping to bovine autosomes were considered for further analysis. After screening, the final number of samples and SNPs were 71 and 38 502 for CIN and 69 and 36 311 for MOD cattle breeds.

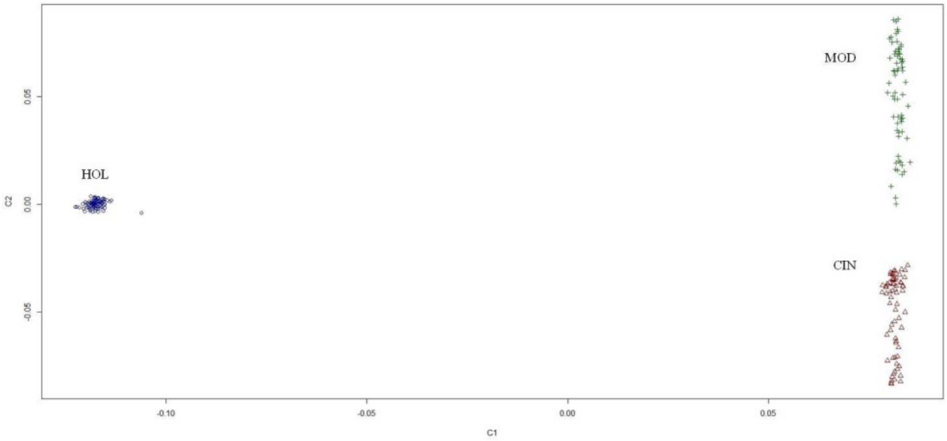
Table 4.1 Descriptive summary of chromosomes and SNPs. Total number of single nucleotide polymorphisms (nº SNPs), number of SNPs after filtered and average minimum allele frequency (MAF) in Cinisara (CIN) and Modicana (MOD) cattle breeds and length of the covered chromosome.

		CIN		MOD	
Chr	nº SNPs	SNP after filtered	Average MAF	SNP after filtered	Average MAF
1	3430	2513	0.250	2366	0.238
2	2829	2044	0.276	1956	0.264
3	2549	1881	0.278	1781	0.261
4	2570	1851	0.252	1703	0.243
5	2271	1584	0.250	1427	0.241
6	2575	1932	0.248	1750	0.242
7	2352	1653	0.247	1613	0.235
8	2429	1796	0.247	1671	0.234
9	2095	1510	0.252	1384	0.246
10	2206	1623	0.243	1513	0.237
11	2295	1690	0.233	1607	0.223
12	1773	1238	0.237	1171	0.228
13	1850	1341	0.235	1270	0.230
14	1831	1385	0.228	1294	0.223
15	1762	1263	0.237	1191	0.224
16	1726	1215	0.234	1178	0.228
17	1600	1176	0.238	1115	0.230
18	1376	1015	0.237	947	0.233
19	1420	1055	0.225	1008	0.229
20	1568	1169	0.213	1123	0.211
21	1483	1053	0.247	1000	0.239
22	1324	954	0.264	906	0.252
23	1093	815	0.262	766	0.247
24	1312	967	0.271	920	0.258
25	1004	738	0.260	722	0.256
26	1116	808	0.258	781	0.251
27	981	711	0.271	693	0.254
28	980	712	0.276	690	0.260
29	1086	810	0.266	765	0.247
Total	52886	38502	0.249±0.013	36311	0.240±0.016

Genetic structure

Principal components analysis (PCA) was used to cluster animals and explore the relationship among individuals and populations. As mentioned before, a sample of Holstein was included in this analysis. The PCA showed that animals from the three breeds formed non-overlapping clusters and are clearly separated populations. This indicated a clear genetic division existing among Holstein and Sicilian cattle breeds (Figure 4.1).

Figure 4.1 Principal Components Analysis among Holstein (HOL), Cinisara (CIN) and Modicana (MOD) cattle breeds



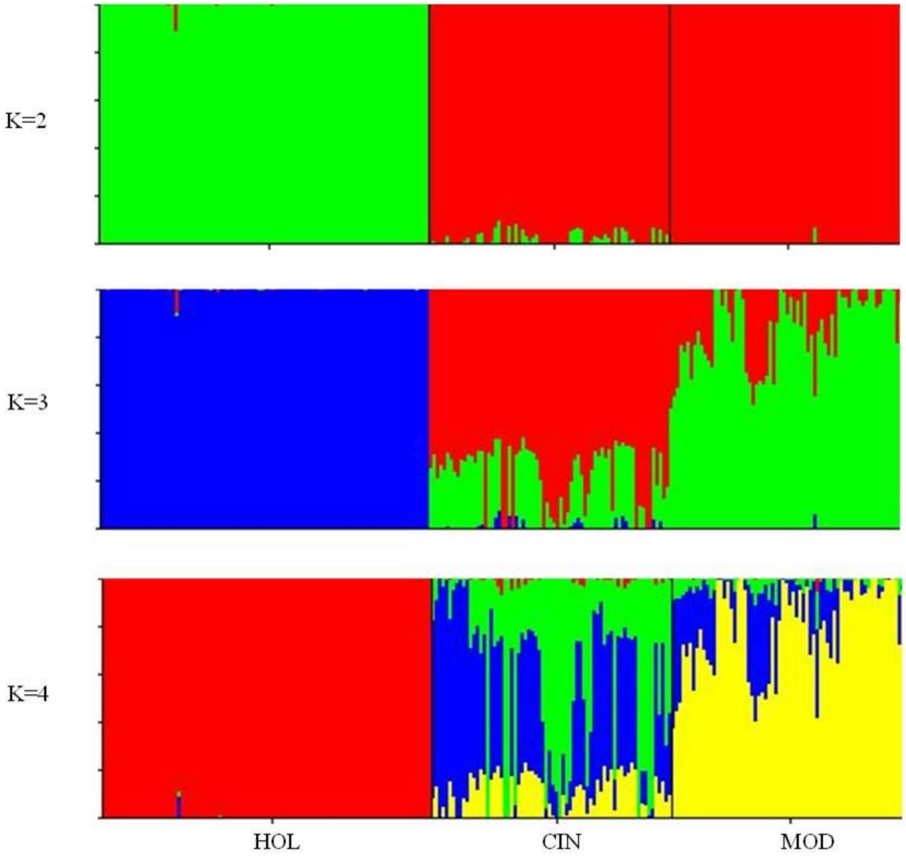
The function Heatmap of genetic similarity corroborated the findings obtained with the PCA and showed that individuals from HOL and MOD were closer to individuals from their same population respect to individuals of the CIN breed, that showed the presence of substructure [See Additional file 1: Figure S1].

Genotype data was also analyzed using a Bayesian clustering algorithm to search for admixture between populations and to infer population structure. Results from analysis of admixture considering a range of 1 to 6 potential clusters (K) pointed out that the highest average likelihood value with the smallest variance among replicates were obtained for K = 4. Ln Pr(G|K) increased from K = 1 to K = 4, reaching a plateau at K = 4, while did not show a significant fluctuation from K = 4 to K = 5, and then rapidly decreased for K = 6 [See Additional file 2: Figure S2].

For K = 2 the HOL is separated from Sicilian breeds and for K = 3 each breed tends to have its own distinct cluster, suggesting that admixture was nearly absent among HOL and Sicilian cattle breeds. A graphic representation of the estimated membership coefficients to the four clusters for each individual is shown in Figure 4.2.

The HOL is the most differentiated breed with 99.8% of the individuals assigned to cluster 1, whereas the CIN animals showed a lowest value of assignment with a proportion of 46.9% of the individuals assigned to cluster 3 and 41.6% assigned to cluster 2. Cluster 4 included the MOD individuals with 79.7%. Furthermore, 17.2% of the MOD individuals were assigned to the cluster 3 and 10.8% of the CIN individuals to the cluster 4 [See Additional file 3: Table S1]. Again, the results evidenced that for the Sicilian cattle breeds, the MOD represented a well-supported group, with some degree of introgression with CIN genes, while CIN was split in two subgroups. Therefore, model based clustering suggested that admixture has occurred and genetic links exist between CIN and MOD breeds.

Figure 4.2 Estimated population structure of Holstein (HOL), Cinisara (CIN) and Modicana (MOD) cattle breeds for K ranging from 2 to 4



These results may be explained considering that CIN and MOD are two ancient cattle breeds reared in the same area, with possible (natural or artificial) gene flow between them, having a common history and breeding practices. The CIN herd book was founded in 1996 and before this date, the individuals of the CIN breed were registered in the MOD herd book, which was founded in 1975. Furthermore, for CIN, the clustering analysis suggested that this breed does not constitute a homogenous population, but a mixture of two populations. However, according to the herd book and morphological traits recorded, it does not seem to be crossbred individuals among the samples used in this work; hence, the genetic structure detected for CIN breed could be due to possible introgression with genes from other breeds. In the past this breed was probably crossed with Holstein individuals to improve milk production [20]. Crossbreeding is a common practice among breeders and it is used to modify the characteristics of breeds according to the needs and also to expand the size of a population when it is becoming too small. Depending on the situation, crossbreeding can be considered as a positive or negative process for the management of populations [21]. This practice can offset the loss of genetic diversity and to preserve from excessive inbreeding, but it could be detrimental when small-endangered populations are concerned [3]. Another explanation may be the geographical isolation of some farms of CIN breed and the sampling of CIN animals from independent farms. This would generate a population subdivision as consequence of genetic drift (e.g. Wahlund effect). A PCA conducted only for the CIN animals [See Additional file 4: Figure S3] considering the herd of origin, showed that individuals which clustered together belonged to farms located in the same area. In fact, as suggested by Pritchard et al., [15], the inferred clusters are not necessarily the corresponding real ancestral populations and they can be determined by sampling schemes. Moreover, the presence of substructure could evoke concerns about the generation of false positive results when using LD mapping as the only means to locate genes underlying complex traits [22]. Therefore, the genetic structure observed is in agreement with the demographic history that occurred during the formation of the two breeds.

Inbreeding and coancestry molecular coefficients, rate of inbreeding and coancestry per year and effective population size

The average molecular *F* and *f* coefficients were 0.68±0.024 and 0.67±0.03 in CIN and 0.69±0.020 and 0.70±0.03 in MOD cattle breeds, respectively (Table 4.2). Similar results were reported by Saura et al. [23] in a study on genome-wide estimates of *F* and *f* in an endangered strain of Iberian pigs. High values of *F* and *f* in local breeds, with low population size, as CIN and MOD, can compromise the viability of the populations.

Table 4.2 Estimates of genetic diversity for Cinisara and Modicana cattle breeds. Inbreeding (*F*) and coancestry (*f*) coefficients, rate of inbreeding (Δ*F*) and coancestry (Δ*f*) per year, standard deviation (s.d) and effective population size estimated from the rate of inbreeding (N_eF) and the rate of coancestry (N_ef).

Breed	***F*±s.d**	***f*± s.d**	**Δ*F***	**Δ*f*±s.d**	**N_eF**	**N_ef**
Cinisara	0.68±0.024	0.67±0.03	0.004	0.022±0.07	19.38	3.77
Modicana	0.69±0.020	0.70±0.03	0.007	0.010±0.07	11.90	7.94

In fact, in terms of genetic variability, the endangered populations were less diverse, probably due to the reduced number of animals. Estimation of molecular *f* was slightly higher than those reported by other authors in local cattle breeds and populations characterized by reduction in their population sizes [24-26]. Bozzi et al. [27], in a study for conservation in Tuscan cattle breeds using microsatellite markers, obtained maximum value for *f* of 0.48. However, the results for CIN and MOD breeds were not unexpected considering the reduced number of reared animals and the farming system. In Sicily, natural mating is the common practice and the exchange of bulls among flocks is quite unusual; therefore, mating with close relatives can be quite frequent. Rates of molecular inbreeding (ΔF) and coancestry (Δf) per year were 0.004 and 0.022 in CIN and 0.007 and 0.010 in MOD cattle breeds, respectively (Figures 4.3 and 4.4). Estimates of N_e from ΔF and Δf are shown in Table 4.2. N_e values estimated from the ΔF were about 19 animals in CIN and 12 animals in MOD, and those calculated from the Δf, were about 4 and 8 animals in CIN and MOD cattle breeds, respectively. The current estimates of N_e were low, probably due to the intra-population genetic structuring. In animal breeding, the recommendation is to maintain a N_e of at least 50 to 100 [28]. The control of F and f would restrict inbreeding depression, the probability of losing beneficial rare alleles, and therefore the risk of extinction [3]. Therefore, monitoring N_e and possible control of resultant F may be crucial to implementing genetic improvement programs.

Figure 4.3 Rate of inbreeding per year in Cinisara (a) and Modicana (b) cattle breeds.

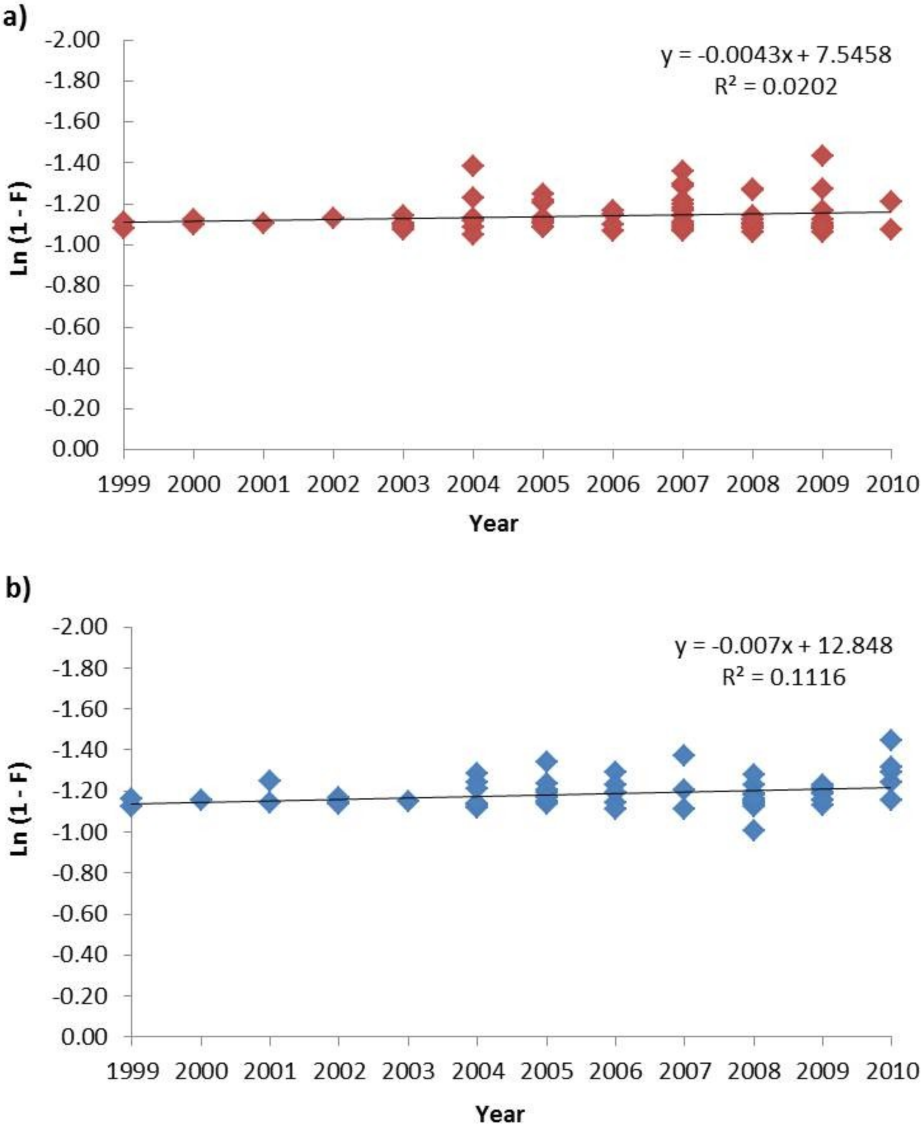
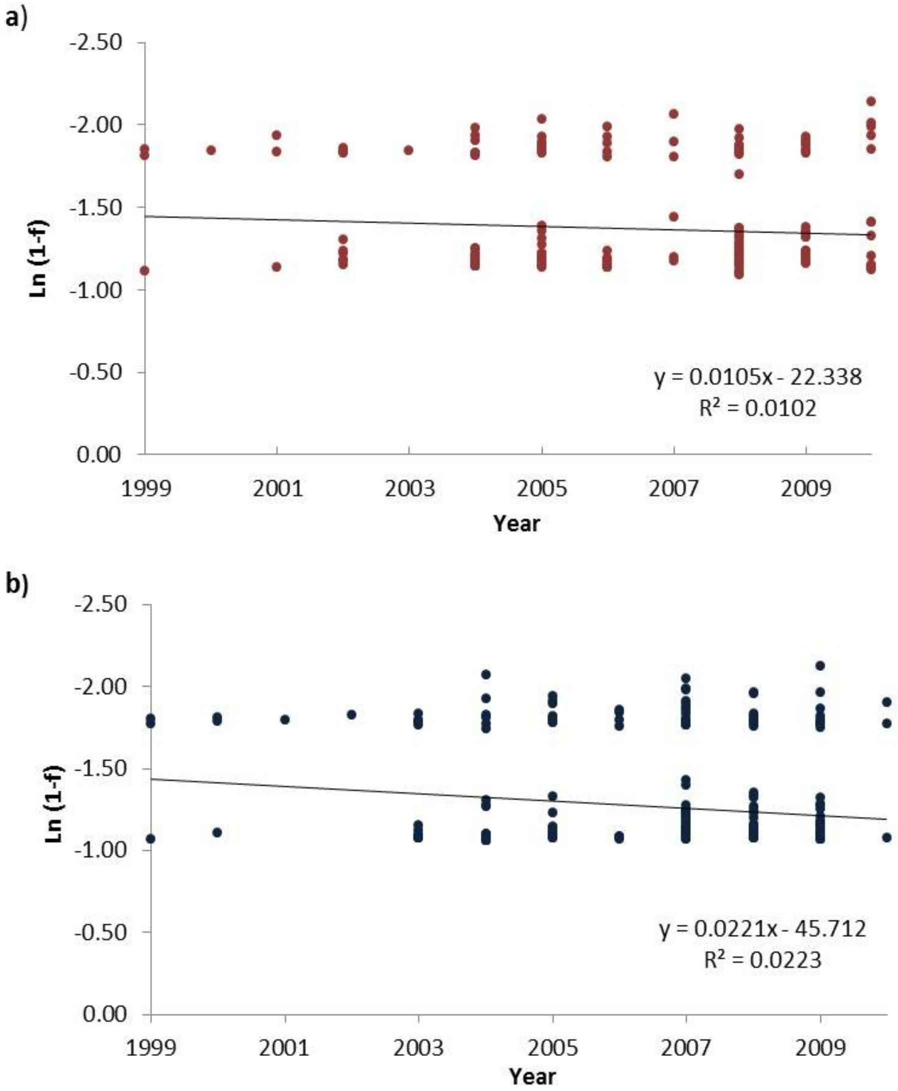


Figure 4.4 Rate of coancestry per year in Cinisara (a) and Modicana (b) cattle breeds.



Linkage Disequilibrium (LD)

The extent of linkage disequilibrium was first evaluated for each adjacent syntenic SNPs pair. The average distance between adjacent SNPs pairs for the entire autosomal genome were about 57 and 60 kb for CIN and MOD cattle breeds, respectively (Table 4.3). Average LD between adjacent SNPs was 0.034±0.025 for CIN and 0.039±0.033 for MOD cattle breeds, and some variations in the LD value across chromosomes in both populations were observed. The r^2 ranged from 0.018±0.026 for BTA5 to 0.106±0.199 for BTA14 in CIN, and from 0.019±0.027 for BTA2 to 0.126±0.221 for BTA14 in MOD (Table 4.3). Chromosomes 14, 15, 16 and 17 showed the highest average LD values in both breeds, whereas small differences were observed among other chromosomes (Table 4.3). Differences in LD among chromosomes have already been reported in Holstein cattle, and these can be attributed to recombination rate, heterozygosity, genetic drift and effect of selection [29]. Other authors reported an average LD between adjacent SNP pairs over the 29 different chromosomes relatively similar [22,30,31]. The comparison of LD levels obtained in different studies is not straightforward, because of differences in several factors as sample size, type of LD measures (*D'* or r^2), marker types (microsatellite or SNP), marker density and distribution, and population demography [29]. Moreover, so far, studies of the extent of LD have been reported mostly for dairy and beef cattle breeds under selection, and there is little knowledge about the degree of genome-wide LD in local cattle breeds and populations.

Table 4.3 Average distance (bp), Linkage Disequilibrium (r^2) and standard deviation (s.d.) between adjacent single nucleotide polymorphisms (SNPs) on each chromosome (Chr) in Cinisara (CIN) and Modicana (MOD) breeds.

Chr	**Average spacing between adjacent SNP (bp)**	**r^2 ± s.d. (CIN)**	**Average spacing between adjacent SNP (bp)**	**r^2 ± s.d. (MOD)**
1	55628	0.022±0.044	58098	0.025±0.055
2	58719	0.019±0.026	61716	0.019±0.027
3	56769	0.020±0.027	60062	0.021±0.029
4	57437	0.020±0.032	60710	0.020±0.036
5	66710	0.018±0.026	71942	0.020±0.028
6	55571	0.020±0.027	58351	0.021±0.037
7	59701	0.019±0.033	62042	0.021±0.030
8	55361	0.020±0.030	58098	0.020±0.029
9	60997	0.019±0.031	65751	0.021±0.028
10	56076	0.022±0.058	58692	0.022±0.053
11	55735	0.036±0.091	58802	0.038±0.091
12	64363	0.045±0.109	67516	0.062±0.132
13	55137	0.069±0.156	56511	0.077±0.166
14	55141	**0.106±0.199**	57189	**0.126±0.221**
15	58366	0.083±0.171	61566	0.113±0.204
16	58791	0.085±0.178	61228	0.105±0.198
17	55595	0.083±0.182	59340	0.106±0.218
18	58446	0.046±0.127	61525	0.055±0.148
19	53940	0.023±0.056	56431	0.023±0.054
20	54156	0.021±0.044	55933	0.025±0.051
21	59796	0.019±0.027	62641	0.021±0.030
22	56682	0.020±0.027	58749	0.023±0.036
23	57327	0.022±0.038	59823	0.024±0.039
24	57034	0.019±0.027	59955	0.025±0.051
25	51814	0.021±0.029	52970	0.025±0.038
26	56033	0.021±0.029	57705	0.025±0.038
27	56524	0.021±0.040	57675	0.023±0.045
28	58092	0.019±0.026	60056	0.020±0.029
29	56266	0.019±0.026	58993	0.021±0.029
Mean	57318±3003	0.034±0.025	60002±3677	0.039±0.033

Considering the levels of LD between adjacent markers, the average r^2 in these local Sicilian cattle breeds was surprisingly much lower than those reported for indigenous Swiss Eriger (r^2=0.24) [31], Blonde d'Aquitaine (r^2=0.20) [22] and Chinese and Nordic Holsteins (r^2=0.20 and 0.21) [32]. In fact, r^2 values between adjacent SNPs for Sicilian cattle breeds are similar to that reported for non-syntenic SNPs in Blonde d'Aquitaine cattle breed [22]. Other factors, as MAF and sample size, may be affecting the extent of LD within a genome. In both breeds, more than 67% of SNPs had a MAF larger than 0.2, suggesting that the effect of low MAF on the overall LD estimated should be small. Moreover, Khatkar et al. [33] pointed out that sample size near 70 individuals was sufficient for unbiased and accurate estimates of LD (r^2) across marker intervals spanning < 1 kb to > 50 Mb.

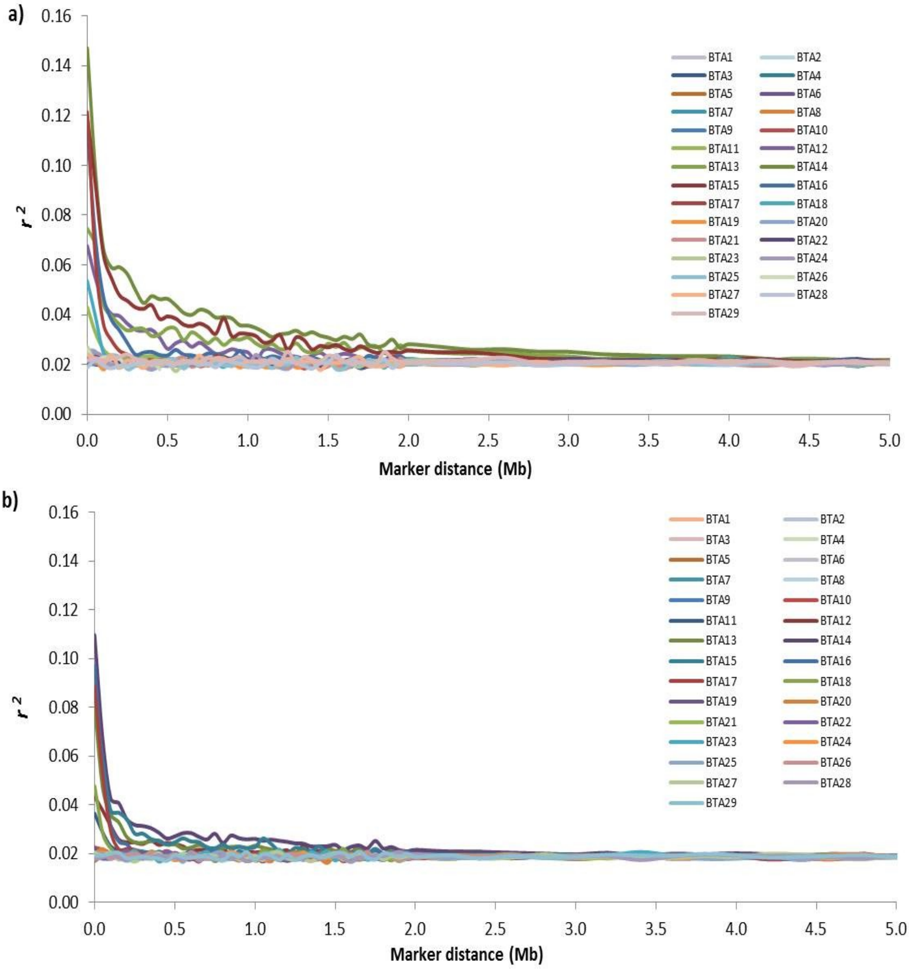
Table S2 [See Additional file 5: Table S2] shows the average values of r^2 estimated for all pairwise combinations of SNPs on each chromosome and breed. The mean values of r^2 pooled over all the autosomes were of 0.019±0.0008 for CIN and 0.021±0.0011 for MOD cattle breeds. García-Gámez et al. [34], in a study on LD for Spanish Churra sheep, reported an average r^2 estimated for all pairwise combinations per chromosomes that ranged from a 0.006 in OAR1 to 0.015 in OAR20. In some chromosomes, the level of pairwise LD decreases quickly with physical distance between SNPs; in particular, the highest values were observed for chromosomes 13, 14 and 15 and for SNPs located in close proximity (Figure 4.5). For SNPs up to 50 kb apart, the average r^2 was 0.034 and 0.040 in CIN and MOD cattle breeds, respectively; for SNPs separated by 200-500 kb the average r^2 was 0.020 and 0.024, and when SNPs are separated by more than 500 kb, the average r^2 was 0.019 and 0.022 (CIN and MOD, respectively) (Table 4.4).

Table 4.4 Mean Linkage Disequilibrium (r^2) among syntenic SNPs over different map distances in Cinisara (CIN) and Modicana (MOD) breeds.

Distance range (kb)	r^2 (CIN)	r^2 (MOD)
<50	0.034	0.040
50-100	0.027	0.033
100-200	0.022	0.027
200-500	0.020	0.024
>500	0.019	0.022

The surprising result was the low values of r^2 observed for most chromosomes, in which no change in r^2 with the physical distance was observed (Figure 4.5). The decay of LD in a genome determines the power of QTL detection in association mapping studies and helps determine the number of markers that are required for successful association mapping and genomic selection. Meuwissen et al. [11], in a simulation to predict genomic breeding values from dense markers across the whole genome with accuracies up to 0.85, found a required r^2 level of 0.2. Qanbari et al. [29] considered a threshold of 0.25 as a useful LD for association studies. Therefore, these results support the use of more dense SNP panels for a high power association mapping and genomic selection efficiency in future breeding programs per CIN and MOD cattle breeds.

Figure 4.5 Distribution of r^2 between syntenic SNP pairs on each autosome as a function of physical distance in Modicana (a) and Cinisara (b) cattle breeds.



The highest value of r^2 estimated for each adjacent syntenic SNPs pair and all pairwise combinations of SNPs was on chromosome 14 in both breeds, which is in agreement with previous works in the species. Different studies have identified causative mutations affecting variation in milk production traits on bovine chromosome 14 [35]. Mai et al. [36]

detected 33 genome-wide QTL on BTA 14 for milk production traits; Jiang et al. [37] showed 86 significant SNPs affecting milk production traits, located within QTL regions on BTA14. Pryce et al. [38], in a genome-wide association study for milk production traits on two dairy cattle breeds, showed the largest effect on chromosome 14 where important genes for milk production as *DGAT1* and *CYP11B1* are mapped. The mutations in these genes have been found to have an effect on milk production traits [36,39]. From this fact, it is possible to hypothesize that the highest value of r^2 found for BTA 14 in both breeds, may be due to a selection made directly by the farmers to increase milk production traits in an empirical way. In fact, as mentioned above, the extent of LD can be affected by different factors, including the directional selection.

The low levels of LD detected in CIN and MOD cattle breeds could be explained by an effect of sampling method, given that individuals from different farms have been included in each population, or ascertainment bias, given that CIN and MOD cattle breeds did not participate in the design of the chip. In addition, the possibility of crossbreeding with other breeds in the past could have broken the patterns, showing lower levels of LD than expected considering the values of inbreeding detected in these populations.

4.4 Conclusion

This study has reported for the first time estimates of population structure, levels of inbreeding and coancestry, and linkage disequilibrium from a genome-wide perspective in Cinisara and Modicana cattle breeds. Our results indicate that animals from the two breeds formed two different clusters with some degree of gene exchange between them; the CIN breed showing also certain introgression with Holstein genes. The high levels of inbreeding and coancestry as well as the low N_e obtained in this study, can compromise the viability of the populations and states the necessity of implementing conservation programs to preserve these breeds. Avoiding mating among relatives, i.e. minimize coancestry, is the strategy to control the increase in inbreeding, and it is responsibility of all breeders to participate in pedigree recording to perform the appropriate matings. Regarding the results derived from LD analyses, a more in depth investigation increasing sample size and performing technical replication would be required if the aim is to perform QTL mapping studies, in order to state the suitability of the Illumina Bovine SNP50K v2 BeadChip for this task.

The information generated in this study has important implications from an economic and scientific perspective, highlighting the necessity of implementing a conservation program for these endangered autochthonous breeds.

Competing interests

The authors declare that they have no competing interests.

Author's contributions

SM, MS, BP and MSe conceived and designed the experiments. SM drafted the manuscript. MS and MSe revised and helped to draft the manuscript. SM, RDG and MTS carried out DNA extraction, purification and analyses. SM, MT, MS and JS analyzed the data and performed the statistical analysis. BP and MSe were the principal investigators that organized the project. All authors contributed in refining the manuscript and approved the final manuscript.

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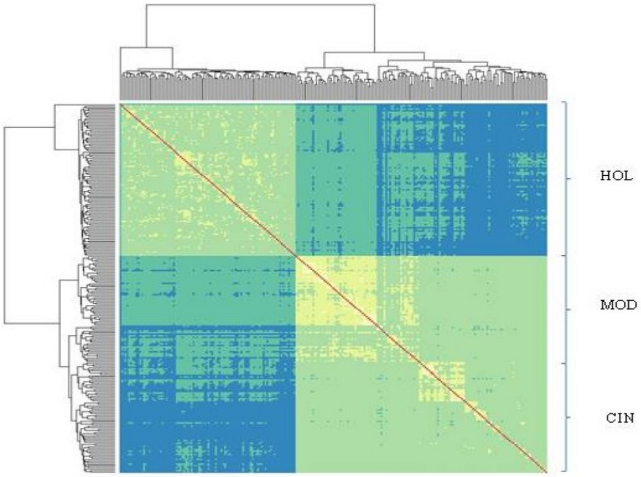
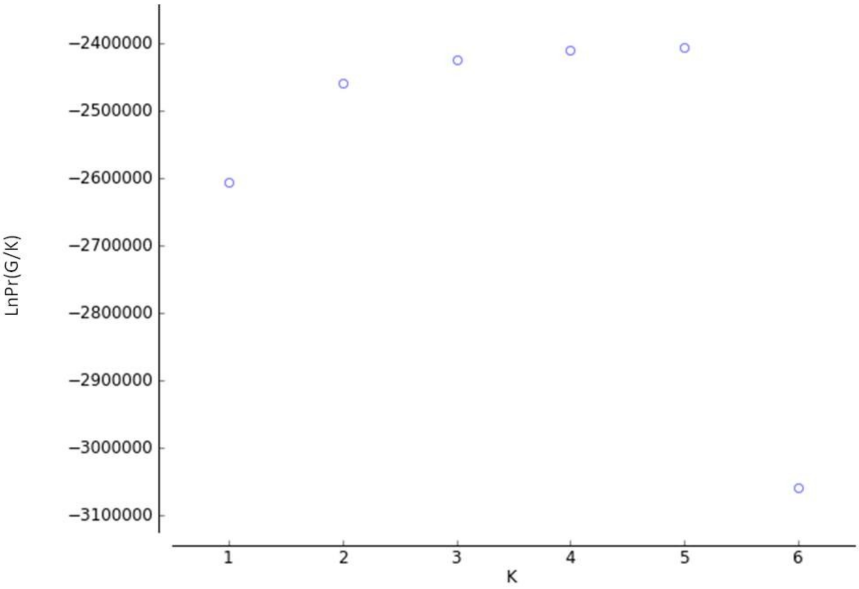
Additional file 1 Figure S1 Heatmap of genetic similarity among Holstein (HOL), Cinisara (CIN) and Modicana (MOD) cattle breeds



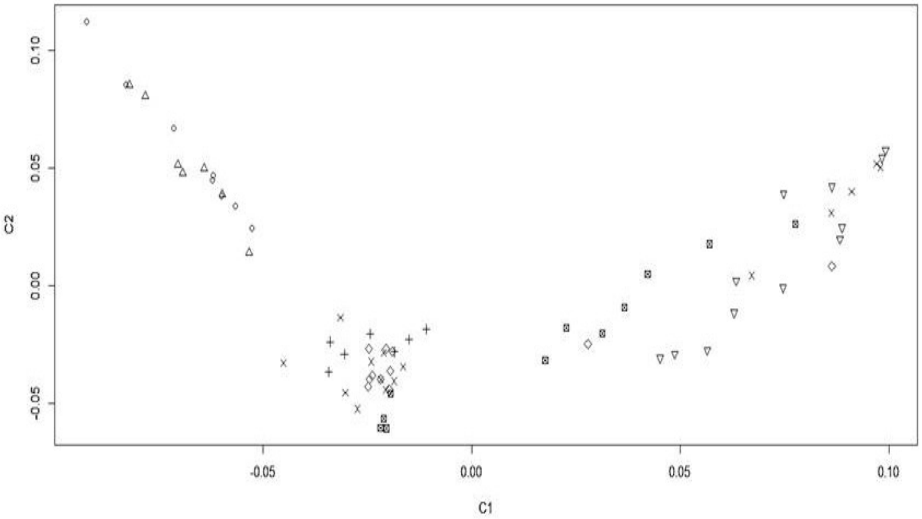
Additional file 2 Figure S2 Plot of estimated posterior probabilities of the data LnPr(G|K) for different number of inferred clusters (K=1 to 6)



Additional file 3 Table S1 Proportion of membership of three breeds in each of 4 clusters inferred in the most likely run of the STRUCTURE software

Breed	**K1**	**K2**	**K3**	**K4**
Holstein	**0.998**	0.001	0.001	0.000
Cinisara	0.007	**0.416**	**0.469**	0.108
Modicana	0.001	0.030	0.172	**0.797**

Additional file 4 Figure S3 Principal Components Analysis for farms in Cinisara cattle breed



Additional file 5 Table S2 Average Linkage Disequilibrium (*r^2*) for all pairwise combinations of SNPs that were less than 50 Mb, on each chromosome (Chr) in Cinisara (CIN) and Modicana (MOD) cattle breeds

Chr	***r^2* ± SD (CIN)**	***r^2* ± SD (MOD)**
1	0.019±0.0005	0.021±0.0009
2	0.019±0.0004	0.021±0.0005
3	0.019±0.0005	0.020±0.0007
4	0.019±0.0005	0.021±0.0005
5	0.018±0.0005	0.021±0.0007
6	0.019±0.0005	0.021±0.0006
7	0.019±0.0007	0.021±0.0008
8	0.018±0.0005	0.021±0.0006
9	0.019±0.0006	0.021±0.0007
10	0.019±0.0009	0.021±0.0009
11	0.020±0.0025	0.022±0.0032
12	0.021±0.0042	0.026±0.0085
13	0.023±0.0084	0.028±0.0092
14	**0.026**±0.0129	**0.035**±0.0197
15	0.024±0.0102	0.032±0.0164
16	0.021±0.0107	0.025±0.0134
17	0.021±0.0097	0.024±0.0136
18	0.020±0.0039	0.022±0.0048
19	0.019±0.0008	0.020±0.0011
20	0.019±0.0009	0.021±0.0013
21	0.019±0.0006	0.020±0.0009
22	0.019±0.0008	0.021±0.0009
23	0.019±0.0009	0.021±0.0012
24	0.019±0.0008	0.021±0.0011
25	0.019±0.0008	0.021±0.0012
26	0.019±0.0008	0.022±0.0023
27	0.019±0.0010	0.021±0.0011
28	0.019±0.0008	0.020±0.0011
29	0.019±0.0008	0.021±0.0011

5

Genome wide linkage disequilibrium and genetic diversity in three autochthonous Sicilian dairy sheep breeds

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Abstract

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

Results: Average r^2 between adjacent SNPs across all chromosomes was 0.052±0.006 for COM, 0.033±0.002 for PIN and 0.075±0.015 for VDB sheep breeds, and some variations in the LD value across chromosomes were observed, in particular for VDB and COM. For markers separated by more than 1,000 kb, the average r^2 was 0.035, 0.029 and 0.023 (for VDB, COM and PIN, respectively). The LD declined as a function of distance and average r^2 was notably lower than the value observed in other sheep breeds. A very few and small haplotype blocks were observed in the COM and PIN sheep breeds, which contained just two SNPs. The number of haplotype blocks reported in our study for the Sicilian dairy sheep breeds were extremely lower than those reported in other livestock species. The Principal Component Analysis (PCA) showed that while VDB and PIN sheep breeds formed a unique cluster, the COM sheep breed showed the presence of substructure. PCA using a subset of SNPs showed lack of ability to discriminate among the breeds. The PIN sheep breed displayed the highest genetic diversity, whereas the lowest value was found in the VDB sheep 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. We found a lower value of LD and this level indicates that the Illumina Ovine SNP50K genotyping array is not an optimum, and that a denser SNP array is needed to capture more LD information. Our results indicate that breeds formed non-overlapping clusters and are clearly separated populations and that the COM 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 the development of selection breeding programs.

Keywords: OvineSNP50K BeadChip, Sicilian dairy sheep breeds, Linkage Disequilibrium, genetic diversity

5.1 Background

The application of recently developed genomic technology, 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]. Species with extensive LD will require fewer markers than those with low levels of LD. Moreover, for high-resolution association mapping, it is necessary to identify block-like structures of haplotypes and a minimal set of SNPs that capture the most common haplotypes of each block [6]. Construction of haplotype blocks and identification of tag SNPs have been found to be quite informative in identification of specific markers for association mapping in humans [7]. 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 [8]. Moreover, knowledge concerning the extent of genetic diversity, as the level of inbreeding and population structure is critical for each of these applications [9]. In local breeds, maintaining genetic variability is an important requirement for animal breeding strategies; this guarantees selection response to productive and adaptive traits improvement, to cope with new environmental conditions, changes in market demands, husbandry practices and disease challenges [10]. Currently, with the availability of high-density SNP arrays, genetic diversity can be estimated accurately in the absence of pedigree information [11]. In Sicily, dairy sheep production represents an important resource for hilly and mountain areas economy, in which other economic activities are limited [12]. Sheep milk is mainly used for the production of traditional raw milk cheeses, sometimes protected designation of origin (PDO) cheeses as laid down in the European Union legislation. In some cases, the quality of these dairy products is linked to a specific breed, i.e. mono-breed labeled cheeses and therefore, it is important to be able to distinguish the milk of a breed from that of others, in order to guarantee the consumers and to safeguard the breed itself [13]. Nowadays, three native dairy sheep breeds are reared in Sicily: Comisana (COM), Pinzirita (PIN), and Valle del Belice (VDB). These breeds present differences both in morphology and production traits, showing excellent adaptability to local environments. The aim of this study was to estimate genome wide levels of LD and the genetic diversity in three Sicilian dairy sheep breeds using high density genotyping arrays.

5.2 Results and discussion

In the present study, we used the OvineSNP50K Genotyping BeadChip to characterize LD and to analyze genetic diversity in the Sicilian dairy sheep breeds.

Out of a total of 54,241 SNPs genotyped in this study, 378 were unmapped and 1,450 were located on sex chromosomes. Thus, 52,413 SNPs mapped onto the 26 sheep autosomes were used in the following described analyses. After screening, the final number of samples and

SNPs were 47 and 43,891 for VDB, 47 and 44,425 for COM and 53 and 45,362 for PIN sheep breeds. The distribution of these SNPs per chromosome and breed is reported in Additional file 1.

Linkage Disequilibrium (LD)

The extent of linkage disequilibrium was first evaluated for each adjacent syntenic SNPs pair. We choose in our study r^2 as a measure of LD, because is the most suitable measure of LD for biallelic markers [14] and to avoid the influence of small sample size [15]. The average distance between adjacent SNPs pairs for the entire autosomal genome were about 62, 61, and 60 kb for VDB, COM and PIN sheep breeds, respectively (Table 5.1). The r^2 ranged from 0.045±0.094 for OAR24 to 0.102±0.167 for OAR6 in VDB, and from 0.038±0.058 for OAR1 to 0.062±0.084 for OAR22 in COM, whereas small differences among chromosomes were observed in PIN (Table 5.1). These results can be attributed to recombination rate varying between and within chromosomes, differences in chromosome length, heterozygosity, genetic drift, effect of selection [16]. The effect of selection on LD is dependent upon the direction, intensity, duration and consistency of selection over time. In fact, the PIN breed is not subject to breeding programs, while the COM and VDB breeds are characterized by low selection pressure.

Table 5.1 Average space (bp), Linkage Disequilibrium (r^2) and standard deviation (s.d.) between adjacent single nucleotide polymorphisms (SNPs) on each chromosome (OAR) in the Sicilian sheep breeds. Valle del Belice (VDB), Comisana (COM) and Pinzirita (PIN) sheep breeds

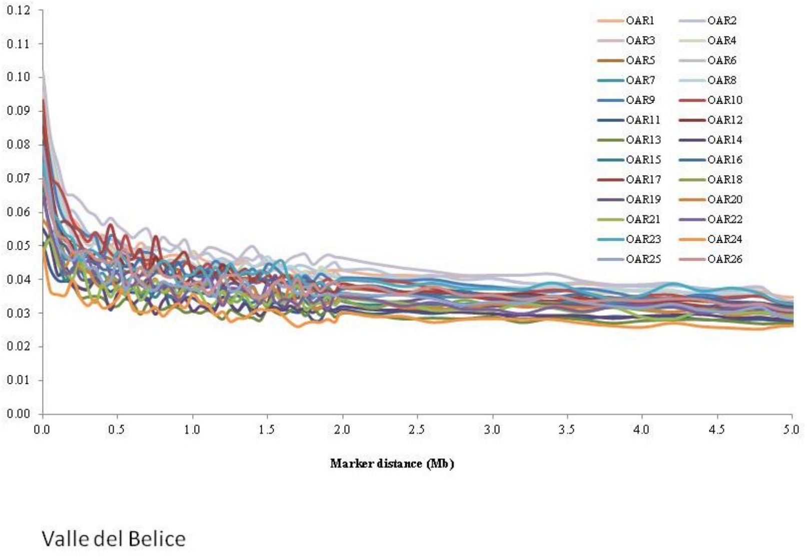
	VDB		COM		PIN	
OAR	**average space**	**r^2 ± s.d.**	**average space**	**r^2 ± s.d.**	**average space**	**r^2 ± s.d.**
1	61045	0.090±0.156	60320	0.038±0.058	58978	0.027±0.037
2	57122	0.094±0.167	57098	0.039±0.057	55426	0.028±0.041
3	57820	0.093±0.165	57112	0.040±0.061	55798	0.029±0.043
4	56244	0.098±0.169	54601	0.046±0.066	53792	0.032±0.046
5	58447	0.086±0.154	57320	0.049±0.075	56290	0.029±0.043
6	59426	**0.102±0.167**	58669	0.047±0.067	56709	0.031±0.046
7	57467	0.074±0.141	56925	0.051±0.071	56423	0.032±0.045
8	55652	0.085±0.148	54474	0.055±0.076	54293	0.032±0.048
9	55790	0.087±0.155	54938	0.055±0.082	53841	0.033±0.044
10	61582	0.090±0.161	60396	0.050±0.068	59820	0.032±0.045
11	68931	0.054±0.103	67065	0.055±0.077	65169	0.032±0.047
12	60458	0.073±0.131	59087	0.056±0.076	58012	0.034±0.049
13	62054	0.054±0.107	60864	0.052±0.079	60409	0.033±0.047
14	69806	0.054±0.104	69025	0.055±0.075	69025	0.035±0.061
15	63309	0.065±0.126	62516	0.055±0.081	61532	0.032±0.047
16	58145	0.082±0.145	58498	0.052±0.071	56566	0.033±0.051
17	66522	0.078±0.147	64139	0.058±0.082	62653	0.034±0.047
18	60872	0.070±0.130	59776	0.056±0.078	59136	0.034±0.049
19	60840	0.070±0.146	61012	0.057±0.078	59884	0.034±0.056
20	58821	0.065±0.133	58713	0.057±0.079	58097	0.035±0.052
21	73615	0.051±0.091	71376	0.052±0.071	71794	0.033±0.043
22	60279	0.064±0.122	60393	**0.062±0.084**	58592	0.037±0.056
23	70131	0.071±0.126	68570	0.054±0.078	67301	0.036±0.055
24	71810	0.045±0.094	71232	0.048±0.063	70663	0.037±0.056
25	55956	0.076±0.149	56817	0.059±0.081	55121	0.035±0.055
26	64515	0.070±0.131	64202	0.050±0.069	61966	0.031±0.044
mean	61795	0.075±0.015	60966	0.052±0.006	59895	0.033±0.002

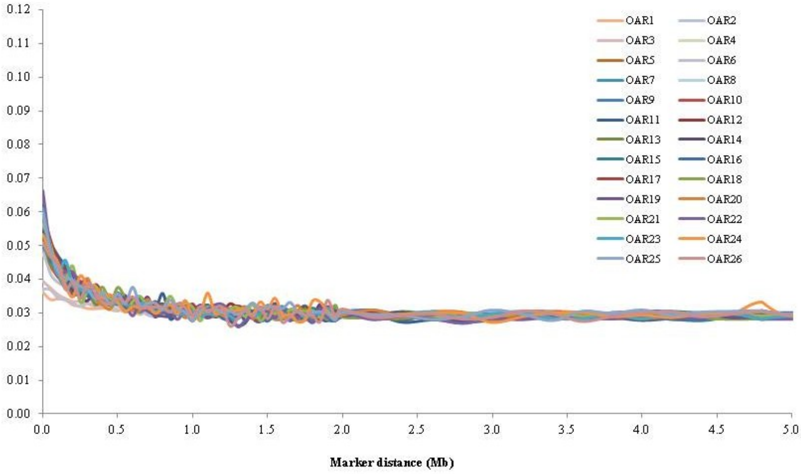
The mean values of r^2 estimated for all pairwise combinations of SNPs pooled over all the autosomes were 0.031±0.003 for COM, 0.025±0.003 for PIN and 0.038±0.004 for VDB sheep breeds. As reported in Figure 5.1 (a, b and c) and Table 5.2, the level of pairwise LD decreased with increasing distance between SNPs.

Table 5.2 Mean Linkage Disequilibrium (r^2) among syntenic SNPs over different map distances in Sicilian sheep breeds. Valle del Belice (VDB), Comisana (COM) and Pinzirita (PIN) sheep breeds

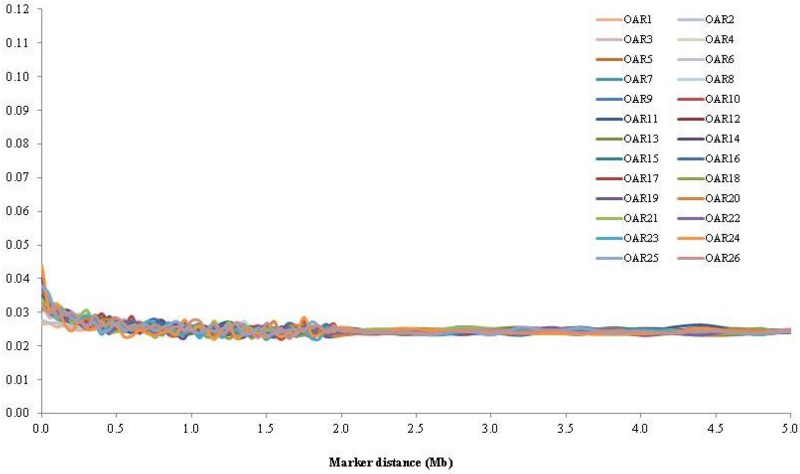
Distance range (kb)	r^2 (VDB)	r^2 (COM)	r^2 (PIN)
<50	0.075	0.054	0.035
50-100	0.063	0.046	0.032
100-200	0.052	0.041	0.029
200-500	0.046	0.035	0.027
500-1000	0.042	0.032	0.025
>1000	0.035	0.029	0.023

Figures 5.1 Distribution of r^2 between syntenic SNP pairs on each autosome as a function of physical distance in Valle del Belice (1a), Comisana (1b) and Pinzirita (1c) sheep breeds.





Comisana



Pinzirita

For SNPs up to 50 kb apart, the average r^2 was 0.075, 0.054, and 0.035, for SNPs separated by 200-500 kb the average r^2 was 0.046, 0.035, and 0.027, and when SNPs were separated by more than 1,000 kb, the average r^2 was 0.035, 0.029 and 0.023 in VDB, COM and PIN sheep breeds, respectively. The comparison of LD levels obtained in different studies is not straightforward, because of differences in several factors as sample size, type of LD measures (D' or r^2), marker types (microsatellite or SNP), marker density and distribution, and population demography [16]. Moreover, so far, results of the extent of LD have been reported for sheep breeds under selection, and there is little knowledge about the degree of genome-wide LD in local sheep breeds. García-Gámez et al. [1] in a study on LD in Spanish Churra sheep reported an average r^2 estimated for all pairwise combinations per chromosomes that ranged from 0.006 in OAR1 to 0.015 in OAR20. Usai et al. [17] in a study of LD in a sample of Sarda rams showed higher value, with an average r^2 value over 1,000 kb of 0.072. Previous studies in five populations of domestic sheep based on microsatellite markers [8] and for wild sheep based on dense panel of SNPs [18] showed LD extend over long distance. The level of r^2 for adjacent syntenic SNPs pair and all pairwise combinations of SNPs in the Sicilian sheep breeds was notably lower than the values observed in other livestock species as pig [19,20], cattle [16,21], and horse [22,23]. This can be explained considering the intensive artificial selection to which commercial animal breeding populations have been subjected for many generations and the ensuing reduction in effective population size.

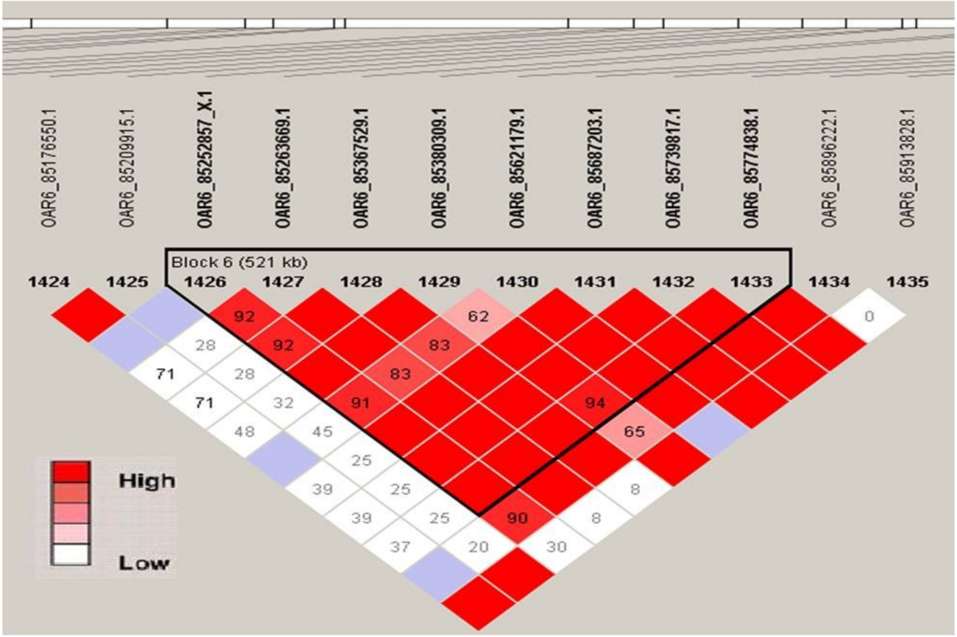
The number of haplotype blocks and the number of SNPs captured by the blocks for each chromosome in each breed are shown in Table 5.3. Our results for average LD within chromosome and breed are in agreement with the block structure across the genome. In fact, VDB sheep breed had the highest number of blocks, whereas the PIN sheep breed had the lowest one. The major goal of using haplotype block is to capture most of genetic variation with a small number of SNPs characterizing the block.

Table 5.3 Number of blocks and number of SNPs per block on each chromosome (OAR) in the Sicilian sheep breeds. Valle del Belice (VDB), Comisana (COM) and Pinzirita (PIN) sheep breeds

	VDB		COM		PIN	
OAR	**Number of blocks**	**Number of SNPs in blocks**	**Number of blocks**	**Number of SNPs in blocks**	**Number of blocks**	**Number of SNPs in blocks**
1	22	51	2	4	--	--
2	14	32	--	--	1	2
3	16	38	--	--	1	2
4	10	24	--	--	--	--
5	4	8	3	6	--	--
6	9	24	1	2	--	--
7	6	12	--	--	--	--
8	2	4	--	--	1	2
9	11	24	2	4	--	--
10	7	23	--	--	--	--
11	1	2	--	--	--	--
12	8	16	--	--	--	--
13	--	--	--	--	--	--
14	1	2	--	--	1	3
15	2	4	2	4	1	2
16	4	11	--	--	--	--
17	6	19	1	2	--	--
18	--	--	--	--	--	--
19	2	6	1	2	--	--
20	1	2	--	--	--	--
21	--	--	1	2	--	--
22	1	2	--	--	--	--
23	1	2	2	4	1	2
24	1	2	--	--	--	--
25	2	4	--	--	--	--
26	1	2	--	--	--	--
Tot	132	314	15	30	6	13

In addition, the use of low density panels for genomic selection is of interest. A very few and small blocks were observed in the COM and PIN sheep breeds, which contained just two SNPs. The number of haplotype blocks reported in our study for the Sicilian dairy sheep breeds (132 for VDB, 15 for COM and 6 for PIN breeds) (Table 5.3) were extremely lower than those reported in Churra sheep breed [1], in German Holstein cattle breed [16] and in commercial pig lines [19]. The highest value of r^2 estimated for each adjacent syntenic SNPs pair and all pairwise combinations of SNPs was found for OAR6 in VDB sheep breed. Chromosomes showing higher LD also have more and longer blocks than chromosomes with lower average LD. In fact, the region of OAR6 in VDB sheep breed contained 9 haplotype blocks and the longest one involved 8 SNPs and covered 521 kb (Figure 5.2).

Figure 5.2 Haplotype block map of a portion of OAR6 in the form of a heat map of confidence bounds of D′ in Valle del Belice sheep breed.

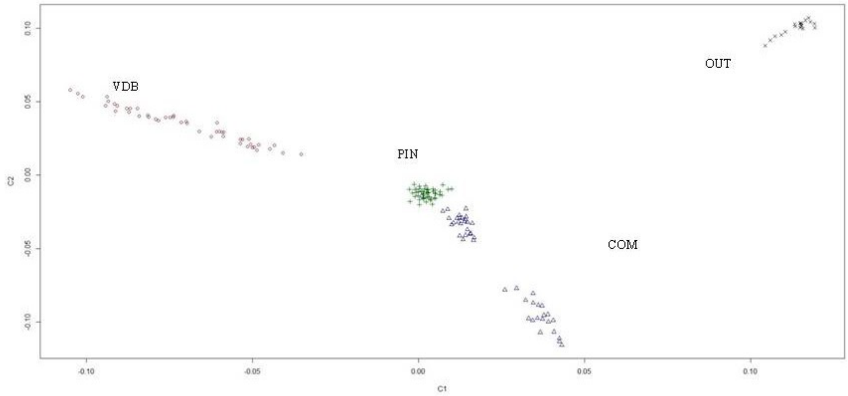


Considering the last genome assembly for *Ovis aries* (Oar_v3.1), casein genes are mapped on chromosome 6 (NC_019463.1) in a region between 85,000-85,400 kb, therefore, it could be possible to hypothesize that this haplotype block is involved within this region. Moreover, the highest value of r^2 found for OAR6 in VDB sheep breed, may be due to selection made directly by the farmers to increase milk production traits in an empirical way. In fact, as mentioned above, the extent of LD can be affected by different factors including the directional selection. The decay of LD in a genome determines the power of QTL detection in association mapping studies and helps to determine the number of markers required for successful association mapping and genomic selection. Meuwissen et al. [4], in a simulation to predict genomic breeding values from dense markers across the whole genome with accuracies up to 0.85, found a required r^2 level of 0.2. Qanbari et al. [16] considered r^2 threshold of 0.25 as a useful LD value for association studies. Therefore, these results support the need to use more dense SNP panels for high power association mapping and genomic selection efficiency in future breeding programs for Sicilian dairy sheep breeds.

Genetic diversity

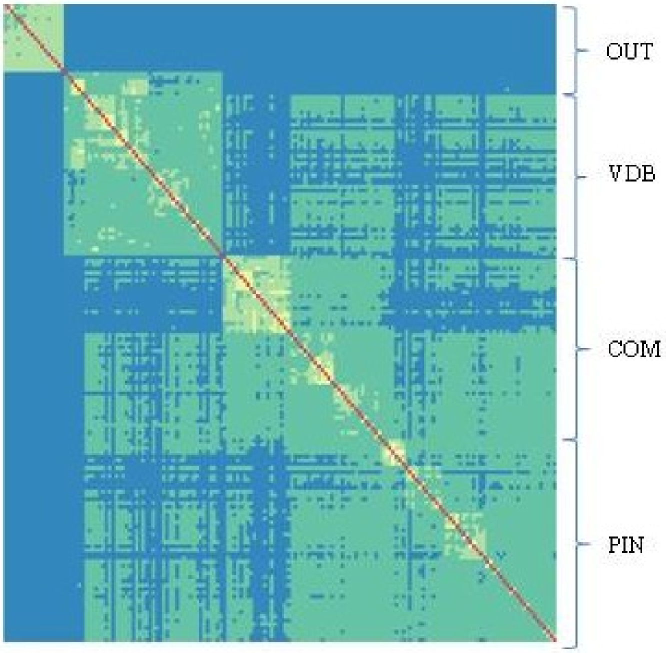
Principal Component Analysis (PCA) was used to visualize the genetic relationships among the Sicilian sheep breeds. Genotypes from additional 19 animals of another Italian sheep breed were included in the analysis and considered as outliers (OUT). A total of 44,610 SNPs shared among all breeds were analyzed. The PCA showed that the four sheep breeds formed non-overlapping clusters and are clearly separated populations. While for VDB, PIN and OUT sheep breeds, the two components (PCA1 and PCA2) clustered animals from the same breed together, the COM sheep breed showed two groups (Figure 5.3). The function Heatmap of genetic similarity corroborated the findings obtained with the PCA.

Figure 5.3 Principal components analysis among the four sheep breeds. Valle del Belice (VDB), Pinzirita (PIN), Comisana (COM) and outlier (OUT)



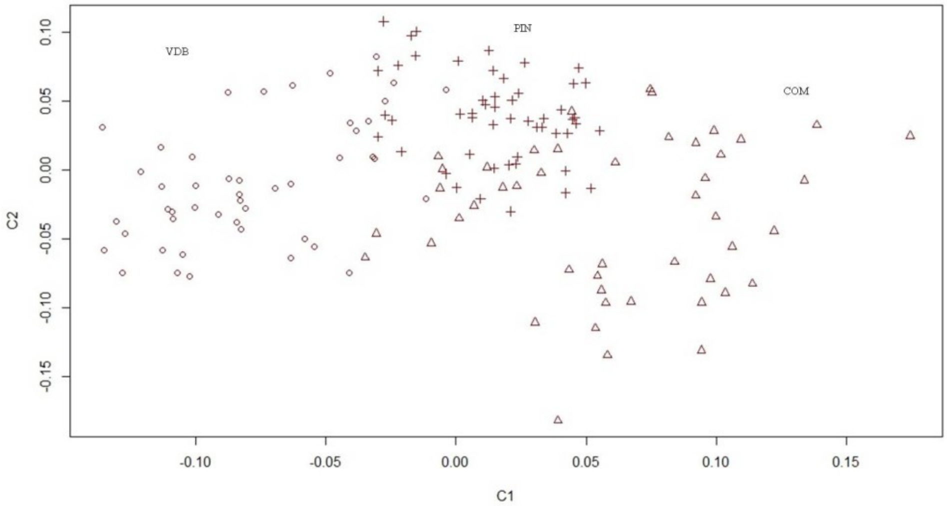
Individuals from the VDB, PIN and OUT were closer to individuals belonging to the same population while individuals from the COM showed the presence of substructure (Figure 5.4). In fact, individuals from COM occupy different areas of the cluster, indicating the presence of substructure, and this could evoke concerns about the generation of false positive results when using LD mapping as the only mean to locate genes underlying complex traits [21]. The genetic structure detected for COM sheep breed could be due to introgression of genes from other breeds or to geographical isolation of some farms or to the sampling from independent farms. This would generate a population subdivision as consequence of genetic drift (e.g. *Wahlund effect*). Moreover, some individuals of COM sheep breed were positioned near the cluster of PIN sheep breed. The genetic closeness between these two breeds might be explained considering that they are characterized by a common breeding system and geographical husbandry area, which might have led to genetic exchange between them.

Figure 5.4 Heatmap of genetic similarity among the four sheep breeds. Valle del Belice (VDB), Pinzirita (PIN), Comisana (COM) and outlier (OUT)



The relative contribution of SNP to population assignment was estimated. In fact, high throughput genotyping tools make it possible to extract interesting genetic information from animal populations that could be applied to identify useful markers for breed allocation, assignment parentage [24] and for tracing the geographic origin of animal products [9] as meat and mono-breed cheese. Among the 44,610 SNPs, a subset of 119 SNPs was used to evaluate their ability to cluster individuals belonging to the Sicilian sheep breeds. These SNPs were selected considering their informativeness in pair comparisons that means SNPs with the larger allele frequency differences between the pairs of breeds were chosen (fixed alleles in one breed and MAF > 0.25 in the other breeds). PCA using this subset of SNPs showed lack of ability to discriminate among the breeds and the presence of overlapping areas, in particular between COM and PIN sheep breeds (Figure 5.5).

Figure 5.5 Principal components analysis among Sicilian sheep breeds using marker panel contained 119 SNPs. Valle del Belice (VDB), Pinzirita (PIN) and Comisana (COM) sheep breeds



This result may be due to the close relationships among Sicilian sheep breeds, that are genetically connected among them [13,25]. Therefore, the subset of SNPs was not useful to assign individuals into discrete clusters and it was insufficient and inadequate for authentication purposes. The basic genetic diversity indices within breed were used to compare levels of heterogeneity between breeds. The overall mean MAF was 0.294 for COM, 0.301 for PIN, and 0.290 for VDB sheep breeds being these values in agreement with those reported by Kijas et al. [26] in a study on genome-wide analysis of the world's sheep breeds for the European-derived populations. Moreover, the distribution of MAF of these SNPs is approximately uniform over the genome in all breeds (Additional file 1).

The PIN sheep breed displayed the highest gene diversity (He 0.390±0.108), whereas the lowest value was found in the VDB sheep breed (He 0.379±0.155) (Table 5.4).

Table 5.4 Estimates of genetic diversity indices for Sicilian sheep breeds. Average minor allele frequency (MAF), observed heterozygosity (Ho), expected heterozygosity (He), Inbreeding coefficient (*F*), and standard deviation (s.d.)

Breeds	**MAF±s.d.**	**Ho±s.d.**	**He±s.d.**	***F*±s.d.**
Valle del Belice	0.290±0.003	0.364±0.126	0.379±0.155	0.055±0.150
Comisana	0.294±0.004	0.382±0.129	0.382±0.114	0.016±0.031
Pinzirita	0.301±0.004	0.388±0.122	0.390±0.108	0.025±0.042

These estimates of gene diversity are comparable to those reported by other authors for Southern and Mediterranean European sheep breeds [26]. Similar results for genetic diversity (Ho, He, and MAF) were reported for Sarda sheep breed [17]. We obtained some negative values for inbreeding coefficient *F,* which corresponded to animals with lower homozygosity than the average population. The highest *F*, calculated for each individual based upon observed and expected heterozygosity, was found in VDB sheep breed (0.055±0.150), whereas the lowest value in COM sheep breed. The low level of inbreeding and high genetic diversity in PIN sheep breed, but also in COM, reflect the short extent of LD.

5.3 Conclusions

This study reported for the first time the estimates of linkage disequilibrium and genetic diversity from a genome-wide perspective in Sicilian dairy sheep breeds. Knowledge concerning the behavior of LD is important for performing genomic selection and genome wide association analysis. We found lower values of LD in our breeds compared to others and this indicates that the Illumina Ovine SNP50K genotyping array is not an optimum, and that a denser SNP array is needed to capture more LD information. Our results indicated that Sicilian sheep breeds formed non-overlapping clusters and are clearly separated populations and that the COM 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 the development of conservation and/or selection breeding programs.

5.5 Methods

DNA sampling and genotyping

A total of 149 unrelated animals collected from several farms in different areas of Sicily were used for the analysis. The number of animal sampled per flock ranged from 3 to 5. Samples consisted of 48 Comisana (COM), 53 Pinzirita (PIN) and 48 Valle del Belice (VDB) animals. For these sheep breeds pedigree data are not available. About 10 ml of blood was collected from jugular vein using tubes with EDTA as anticoagulant. Genomic DNA was extracted from buffy coats of nucleated cells using salting out method [27]. The concentration of extracted DNA was assessed with the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

All animals were genotyped for 54,241 SNPs, using the Illumina OvineSNP50K Genotyping BeadChip, and following the standard operating procedures recommended by the manufacturer. Raw signal intensities were converted into genotype calls using the Illumina

GenomeStudio Genotyping Module v1.0 software (Illumina Inc., San Diego, CA) by applying a no-call threshold of 0.15. Genotyping data were initially tested for quality using the same software. The markers were filtered to exclude loci assigned to unmapped contigs. Only SNPs located on autosomes were considered in further analyses. Moreover, quality control included: Call Frequency (proportion of samples with genotype at each locus) $\geq$ 0.95, Gen Train Score (quality of the probe that determines the shape and separation of clusters) > 0.70, minor allele frequency (MAF) $\geq$ 0.05, and Hardy-Weinberg Equilibrium (HWE) *P*-value >0.001. SNPs that did not satisfy these quality criteria were discarded.

Linkage Disequilibrium

A standard descriptive Linkage Disequilibrium parameter, the squared correlation coefficient of allele frequencies at a pair of loci (r^2), was used as measure. Pairwise LD between adjacent SNPs was calculated on each chromosome using PLINK [28]. Moreover, r^2 was estimated for all pairwise combinations of SNPs using LD plot function in Haploview v4.2 software [7], exporting data to text files. For each chromosome, pairwise r^2 was calculated for SNPs between 0 and 50 Mb apart. To visualize the LD pattern per chromosome, r^2 values were stacked and plotted as a function of inter-marker distance categories. Average r^2 for SNP pairs in each interval was estimated as the arithmetic mean of all r^2. Haploview v4.2 software [7] was also used to define the haplotype blocks present in the genome. The method followed for blocks definition was previously described by Gabriel et al. [29]. This algorithm considers that a pair of

SNPs is in strong LD when the upper 95% confidence bound of *D'* is between 0.70 and 0.98.

Genetic diversity

The genetic relationship between individuals was examined by Principal Component Analysis (PCA) of genetic distance. First, the average proportion of alleles shared between animals (A_s) was calculated as IBS2+0.5*IBS1/N, where IBS1 and IBS2 are the number of loci that share either one or two alleles identical by state (IBS), respectively, and N is the number of loci tested. Genetic distance (*D*) was calculated as 1-A_s. These values were calculated using PLINK [28] through the use of commands -cluster and -distance matrix. PCA of the *D* matrix was performed using the multidimensional scaling (MDS) algorithm of pairwise genetic distance implemented in PLINK. It should be noted that when MDS is applied to *D* matrix, it is numerically identical to PCA [28]. The graphical representation was depicted using the statistical *R* software (R Development Core Team) with R-Color package. The same software was used to visualize the IBS matrix using the Heatmap function. PLINK [27] was also used to estimate the basic genetic diversity indices, including observed and expected heterozygosity (Ho and He, respectively), average MAF and the coefficient of inbreeding (*F*) for each breed. Files used for basic diversity indices (Ho, He and *F*) were pruned in PLINK considering 50 SNPs per windows, a shift of 10 SNPs between windows and a variation inflation factor's threshold of 1.5.

Competing interests

The authors have no competing to declare.

Authors' contributions

SM, MTS and BP conceived and designed the experiments. SM drafted the manuscript. RDG and LT carried out DNA extraction, purification and analyses. SM, MTS and MT analyzed the data and performed the statistical analysis. All authors contributed to editing of the article and approved the final manuscript.

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Altshuler D: **The structure of haplotype blocks in the human genome**. *Science* 2002, **296**(5576):2225-2229.

Addiotional file 1 Table S1 Average MAF (Minor Allele Frequency) per chromosome (OAR) and number of SNPs (N°) that passed quality control in the Sicilian sheep breeds. Valle del Belice (VDB), Comisana (COM) and Pinzirita (PIN) sheep breeds

	VDB		**COM**		**PIN**	
OAR	**MAF**	**N°**	**MAF**	**N°**	**MAF**	**N°**
1	0.292	4908	0.295	4967	0.299	5080
2	0.289	4607	0.291	4609	0.297	4748
3	0.286	4196	0.290	4248	0.295	4348
4	0.289	2261	0.291	2239	0.304	2364
5	0.291	1985	0.293	2024	0.297	2061
6	0.287	2117	0.291	2200	0.298	2276
7	0.298	1892	0.288	1910	0.299	1927
8	0.293	1798	0.297	1796	0.304	1802
9	0.284	1806	0.296	1834	0.307	1873
10	0.289	1529	0.291	1559	0.300	1574
11	0.286	991	0.288	998	0.301	1027
12	0.294	1424	0.284	1457	0.302	1484
13	0.291	1433	0.292	1461	0.303	1472
14	0.292	985	0.295	997	0.303	997
15	0.288	1420	0.296	1438	0.306	1461
16	0.288	1326	0.302	1318	0.303	1363
17	0.293	1179	0.297	1224	0.304	1253
18	0.290	1177	0.296	1203	0.301	1216
19	0.291	1066	0.295	1063	0.295	1083
20	0.295	937	0.295	944	0.302	954
21	0.296	750	0.291	772	0.309	769
22	0.289	912	0.294	912	0.299	940
23	0.283	945	0.298	967	0.294	986
24	0.292	617	0.292	662	0.311	627
25	0.288	859	0.297	846	0.300	872
26	0.290	771	0.302	777	0.301	805
	0.290	**43891**	**0.294**	**44425**	**0.301**	**45362**

6

General Conclusions

New technologies in molecular genetics and innovative approaches in data analysis have increased the number of available neutral markers, providing tools to assess and to estimate population genetic diversity and structure in order to improve the prioritization of animals for conservation purposes. The genetic diversity is directly and positively related with population size, and the effect of genetic drift is strong in isolated and small populations, as in certain local Sicilian breeds. This thesis reported the use of molecular markers, such as microsatellites and SNPs, for genetic characterization studies of local Sicilian sheep and cattle breeds. In fact, genetic data represent an objective tool to reliably assess genetic diversity and population structure, migration and admixture. Several methods as Bayesian analysis, genetic diversity indexes, Factorial Correspondence Analysis, Neighbor-Joining dendrogram, and Principal Component Analysis were used to assess genetic relationship and population structure in local sheep (Barbaresca, Comisana, Pinzirita, and Valle del Belice) and cattle (Cinisara and Modicana) breeds.

In Chapter 3, the genetic diversity indexes estimated with SNPs of *BLG* promoter region, revealed high genetic variability within Sicilian sheep breeds. Our results revealed the highest value of genetic diversity between Valle del Belice and Pinzirita sheep breeds and the lowest one between Valle del Belice and Sarda sheep breeds. Considering that polymorphisms in the promoter region of *BLG* gene could have a functional role associated with milk composition, the lowest value of nucleotide diversity between Valle del Belice and Sarda sheep breeds may be related to a higher similarity of milk composition of these two breeds compared to the others. However, at present literature does not present any evidence about that.

It is assumed that the availability of a higher number of genetic markers implies higher precision in genetic diversity analysis. We observed similar results between the genetic structure computed with 20 microsatellites (Chapter 3) and with ~44,000 SNPs (Chapter 5) in the Sicilian sheep breeds, in particular for genetic distance among breeds and for genetic diversity within breed. The results showed a close relationship between Comisana and Pinzirita sheep breeds and highest genetic diversity in Pinzirita sheep breed. The assignment test indicated that the molecular markers can be reliably use to assign animals to their population/breeds of origin. Moreover, this study at individual level is interesting since it makes possible to detect potential sampling errors and admixed individual. The relative contribution of SNP to population assignment, estimated using a subset of 119 SNPs, instead, showed lack of ability to discriminate among the breeds and the presence of overlapping areas, due to the close relationships among Sicilian sheep breeds, that are genetically connected among them (Chapter 5).

Linkage disequilibrium (LD) was evaluated for each adjacent syntenic SNPs pair and for all pairwise combinations of SNPs on each chromosome in Sicilian cattle and sheep breeds (Chapters 4 and 5). Our results showed a lower values of LD in Sicilian cattle and sheep breeds, compared to others breeds. Moreover, for the Sicilian sheep breeds, few and small haplotype blocks were observed. These results can be explained considering: i) the low artificial selection to which local breeds have been subjected, because the selection pressure affect the genomic variability of regions under selection; ii) ascertainment bias, given that, except for Comisana sheep breed, the Sicilian cattle and sheep breeds did not participate in the design of the chip. This indicates that the Illumina genotyping arrays are not an optimum and that a denser SNP array is needed to capture more LD information.

For the Cinisara and Modicana cattle breeds, this thesis has reported for the first time, estimates of population structure, levels of coancestry and inbreeding from a genome wide perspective. Principal Components Analysis and Bayesian clustering algorithm showed that animals from the two breeds formed non-overlapping clusters and are clearly separated populations; in particular, the Modicana was the most differentiated population, whereas the Cinisara animals showed a lowest value of assignment, the presence of substructure and genetic links occurred between them. The levels of inbreeding and N_e point out the necessity of establishing a conservation program for these autochthonous breeds. The control of molecular inbreeding and coancestry would restrict inbreeding depression, the probability of losing beneficial rare alleles, and therefore the risk of extinction.

The information generated from this thesis has important implications for the design and applications of association studies as well as for the development of conservation and/or selection breeding programs for the Sicilian cattle and sheep breeds.

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List of publications

Peer reviewed pubblications

- **S. Mastrangelo**, M.T. Sardina, V. Riggio, B. Portolano (2012). Study of polymorphisms in the promoter region of ovine b-lactoglobulin gene and phylogenetic analysis among the Valle del Belice breed and other sheep breeds considered as ancestors. *Molecular Biology Reports* 39: 745-751.
- M. Tolone, **S. Mastrangelo**, A.J.M. Rosa, B. Portolano (2012). Genetic diversity and population structure of Sicilian sheep breeds using microsatellite markers. *Small Ruminant Research* 102: 18–25.
- **S. Mastrangelo**, M.T. Sardina, M. Tolone, B. Portolano (2013). Genetic polymorphism at the *CSN1S1* gene in Girgentana dairy goat breed. *Animal Production Science* 53: 403:406.
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