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ORIGINAL ARTICLE

D-loop sequence mitochondrial DNA variability of Sarda goat and other goat breeds and populations reared in the Mediterranean area

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Summary

To provide useful knowledge on goat breed origin and history, we studied the mitochondrial DNA (mtDNA) of 69 goats from five different breeds, Camosciata delle Alpi, Maltese, Nubian, Saanen and Sarda, and one population, the Tunisian. All goats analysed displayed a moderate haplotype and nucleotide diversity. The highest was in the Sarda – the autochthonous breed reared in Sardinia. On the basis of mtDNA control region sequences, animals showed a high genetic haplotype diversity, 35 haplotypes were each represented by a single sequence and only a few haplotypes were shared among the animals. New haplotypes of goats reared in the Mediterranean area were identified and the majority of Italian goats belonged to haplogroup A. This result confirmed worldwide distribution and diversity of haplogroup A.

Introduction

Goat breeding is an important resource in developing countries where small ruminants are a source of human nutrition and an integral part of rural farming. This species has good productive performances and an excellent ability to adapt to a wide range of climatic conditions (McDowell & Woodward 1982; Devendra 1999). Many breeds originated in European and Mediterranean countries: Alpine and Saanen in the Alps, Maltese in the south of Italy and Tunisian and Nubian in North-African areas. The Sarda goat – a local breed from Sardinia – is included in the Mediterranean group and is a medium-sized goat with a comparative high milk yield for its size. The Sarda breed was only recognized in 1985, and today there are about 250 000 Sarda goats but only 10 100 animals are registered in the official herd book (AssoNaPa 2008). It is the most numerous breed reared in Sardinia, the leading region in Italian goat breeding, which possesses 291 000 heads (IZS 2008) and produces about 20% of Italian goat milk, which is mainly transformed into cheese. The Arbi is the autochthonous goat from Tunisia and belongs to the same indigenous population that lives throughout North Africa. It is a long haired and a small-sized goat and reared in extensive mixed farming systems, together with sheep and cows, or semi-intensive oasis systems. The breed produces mainly meat, but it shows a high genetic potential for milk production (Vacca *et al.* 2009). National projects for development of the small ruminant sector and biodiversity conservation strategies are currently developed in Tunisia for the native goat (FAO 2007). Goat milk can be used as food for people with cow-milk allergy and cheeses are appreciated by consumers (Boyazoglu *et al.* 2005). Furthermore, meat of suckling kids is a delicacy and prices paid to farmers are constantly higher than that of lamb meat. Goat milk–derived products are an important source of profit in France and Greece, as these countries have started to exploit the value of their typical products. Indeed, under well-organized management, goat farming is a profitable way of marketing marginal natural

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resources without endangering the environment. The study of autochthonous breeds can play an important role in the preservation of natural resources and the rural environment and landscape, in particular the protection of biodiversity. To extend the knowledge of goats reared in the Mediterranean area, we studied a particular region of mitochondrial DNA (mtDNA), the D-loop region. To date, sequences from many species are known and the complete sequence of goat mitochondrial genome (Accession number: GenBank AF533441) was deposited in 2003 (Parma *et al.* 2003). Many studies used mtDNA as an important means of population studies. Luikart *et al.* made the first important research in 2001; Naderi *et al.*, using a large mtDNA analysis, identified six haplogroups mtDNA in 2007, and Amills *et al.* analysed the genetic diversity of South and Central American goats in 2009. These studies confirmed a weak phylogeographic structure in goat species, when compared to cattle. This result has been explained by some authors (Luikart *et al.* 2001; Amills *et al.* 2009) because goat, owing to its moderate size and ability to adapt to different environments, well-suited to the intercontinental transportation in ancient times. The aim of this research was to study genetic diversity based on the analysis of mtDNA D-loop of local goat breeds and populations reared in the Mediterranean area and the relationships with other cosmopolitan breeds.

Materials and methods

Animals and DNA analysis

The hypervariable region 1 (HVR1) of mtDNA was analysed in 69 goats of five pure breeds (10 Camosciata delle Alpi goats, 10 Maltese, 10 Nubian, 10 Saanen and 19 Sarda) and one local population (10 Tunisian Arbi) to study the genetic diversity and phylogeographic structure. All the goats were selected according to the herd book and the phenotypic standard for these breeds. They came from several farms located in different areas of Sardinia and Tunisia. Blood samples were taken by one puncture from the jugular vein of each animal, using sample tubes containing EDTA (Becton Dickinson, Plymouth, UK). DNA was isolated from whole blood (300 μl) using a commercial kit (Puregene Blood Core Kit B; QIAGEN Sciences, MD, USA). The D-loop fragment of 778 bp (from position 15711 to 16489) was amplified with primers FW 5′-CGCTCGCCTACACACAAATA-3′ and REV 5′-AATGCCCATGCCTACCATTA-3′, and PCR contained 100 μM of dNTPs, 0.5 μM of each primer, 1.5 mM $MgCl_2$ as described by Amills *et al.* (2004); Taq DNA polymerase was reduced to 0.625 U in a final 50 μl volume because Taq Platinum was used (INVITROGEN Srl, S. Giuliano Milanese, Italy), which is highly efficient. The thermal profile was 32 cycles of 94°C for 1 min, 64°C for 1 min and 72°C for 1 min. PCR products were checked in 1% agarose gel stained with ethidium bromide to confirm PCR and no specific amplification. Amplified products were purified by Charge Switch PCR clean up kit and Magnarack (INVITROGEN Srl). PCR products were sequenced in both forward and reverse direction by means of a DNA sequencer (Applied Biosystems 3100, Applied Biosystems, Foster City, CA, USA). DNA sequences were edited using CROMAS LITE 2.01 (http://www.technelysium.com.au).

Data analyses

To obtain a more complete report, data was analysed by means of different methods. All the 69 sequences of mtDNA HVR1 region were aligned and compared with the mtDNA complete sequence of *Capra hircus* (GenBank AF533441) using CLUSTALX v. 1.81. software (Thompson *et al.* 1997). The table of haplotypes was built using MEGA software package version 4.0 (Kumar *et al.* 2004; Tamura *et al.* 2007). Haplotype diversity (hd), nucleotide diversity (π), average number of nucleotide differences (k) and Fu's *Fs* statistic were calculated using DnaSP software version 4.2 (Rozas *et al.* 2003). To include all the data from Italian goat breeds, a total of 232 sequences were aligned, using CLUSTAL X v1.81. Forty-nine sequences (GenBank FJ571522–41, FJ571552–61, FJ571572–73, FJ571575–87, FJ571590–93) were from our study; 67 sequences (GenBank DQ241305–71) were Sicilian goats included in the report by Sardina *et al.* (2006); 104 sequences by Naderi *et al.* (2007) (GenBank EF618088–100, EF618102–105, EF618108–140, EF618142, EF618144–145, EF618147–157, EF618159–160, EF618163–166, EF6 18168–169, EF618173–183, EF618185, EF618187–190); 11 by Luikart *et al.* (2001) (GenBank AJ317674–78, AJ317680–85) and, as the reference sequence, the only available *C. hircus* mithocondrion complete genome (GenBank AF533441, Parma *et al.* 2003). All the sequences are reported in Table S1. In this comparison, we analysed a segment of 477 bp from position 15711 to 16187, to include all the sequences from the other studies. The alignments were imported in MEGA v4.0, and a neighbour-joining haplotype tree (NJ) was constructed using

Kimura 2-parameter distance model with 1000 bootstrap replications.

Median-joining network (Bandelt *et al.* 1999) and mismatch analysis were calculated to investigate haplotype relationship, using NETWORK 4.5.0.1 (http://www.fluxus-engineering.com). Positions were weighted according to their mutational frequency, the relative weight of transversions versus transitions was set to 3 according to Bandelt (2007), whereas the value of ε was set to 0. The parameter, ε, specifies a weighted genetic distance among the known sequences in the data set, within which potential median vectors may be constructed (Bandelt 2007). The maximum parsimony (MP) calculation option (Polzin & Daneschmand 2003) was used to identify the unnecessary median vectors and links which can be switched off in the results display.

To compare the relationships among Italian and Mediterranean goat breeds, 885 mtDNA sequences of *C. hircus* were included. The accession number of the sequences was from this study and other reports (Luikart *et al.* 2001; Parma *et al.* 2003; Pereira *et al.* 2005; Sardina *et al.* 2006; Naderi *et al.* 2007). In this last comparison, we analysed a segment of 442 bp from position 15746 to 16187. Phylogenetic analysis was performed with MEGA v4.0, and the NJ tree was constructed using Kimura 2-parameter genetic distances with 1000 bootstrap replications.

Results

The analysis of the 704 bp HVR1 sequences revealed 89 variable sites; no insertion/deletion was found in the 69 sequences in the HVR1 of the control region. We identified 27 singleton sites and 62 parsimony informative sites. A singleton site contains at least two types of nucleotides (or amino acids) with, at most, one occurring multiple times. DNAsp identifies a site as a singleton site if at least three sequences contain unambiguous nucleotides or amino acids. A site is parsimony-informative if it contains at least two types of nucleotides (or amino acids), and at least two of them occur with a minimum frequency of two (http://www.megasoftware.net). The number of polymorphic sites ranged from 26 in Nubian to 36 in Tunisian (Table 1). The comparison of the 69 sequences revealed 52 haplotypes (Table 1 and Figure 1). The number of haplotypes detected in each breed ranged from 8 (Camosciata delle Alpi) to 14 (Sarda). Thirty-five haplotypes were each represented by a single sequence, and only few haplotypes were shared among different breeds. Haplotype 1 was shared between Maltese and Saanen; haplotype 12 was shared between Nubian and Saanen; haplotype 31 was shared between Sarda and Maltese (Figure 1). Haplotypes diversity (Hd) values were moderate, supporting the findings of other researchers (Sardina *et al.* 2006), ranging from 0.93 ± 0.077 in the Maltese to 0.98 ± 0.054 in the Tunisian and Saanen (Table 2). The nucleotide diversity (π) ranged from 0.013 ± 0.0013 (Saanen) to 0.015 ± 0.0014 (Tunisian). The average number of nucleotide differences (k) was quite relevant and the highest was in the Sarda breed (10.80) (Table 2). The mismatch distribution analysis performed in our study showed a unimodal bell-shaped distribution. Pairwise differences ranged from 1 to 19. The major peak was at 11 mutational differences (Figure 2a). To evaluate the relationships among the Italian goat breeds, in Table S1, we compared our sequences to 67 mtDNA sequences of Sicilian goat breeds reported by Sardina *et al.* (2006), 104 reported by Naderi *et al.* (2007) and 11 reported by Luikart *et al.* (2001).

Table 1 Haplotypes, parsimony informative sites, singleton and polymorphic sites for each breed and population

Breed/ population	n	Haplotypes	Parsimony informative sites	Singleton sites	Polymorphic sites
Camosciata delle Alpi	10	8	18	10	28
Maltese	10	8	12	21	33
Nubian	10	8	21	5	26
Saanen	10	9	13	18	31
Sarda	19	14	26	9	35
Tunisian	10	9	10	26	36
All	69	52	62	27	89

The alignment of the 477 bp of 232 mtDNA control region sequences (nps 15711–16187) revealed 133 variable sites, 48 singleton variable sites and, 85 parsimony informative sites and 154 haplotypes (Table S1). The analysis of the 232 sequences revealed a high haplotype diversity ($Hd = 0.99 \pm 0.0012$) in accordance with those reported for South-European and North-African goats by Naderi *et al.* (2007). Nucleotide diversity (π) was 0.02 ± 0.0013 and average number of nucleotide differences (k) was 9.85. The Fu and Li F-statistic was a negative and non-significant value of -3.20 ($p = 0.060$). The mismatch analysis of pairwise distribution for goats reared in Italy revealed a bimodal distribution with a major peak at nine mutational differences and a secondary one at 47 mutational differences (Figure 2b). Many surveys on the genetic structure of domestic goat mtDNA carried out on a global level

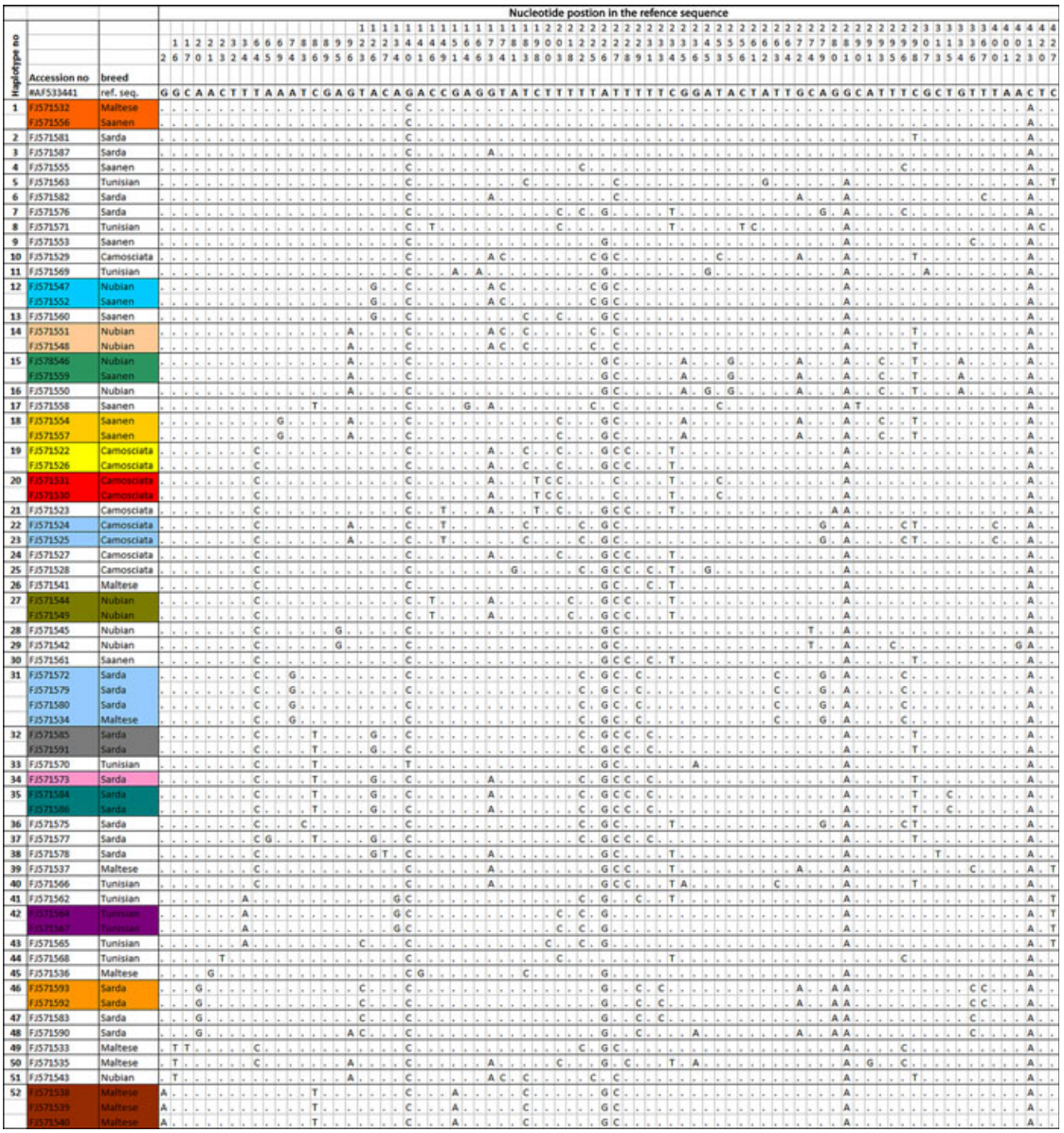

Figure 1 Sequence variations of 69 sequences of the Sarda, Maltese, Nubian, Camosciata delle Alpi, Saanen and Tunisian goats (from position 15746 to 16450, 704 bp). The number of individuals sharing the same haplotype were listed in the second column, with the same colour. Haplotypes are identified after the comparison with the reference sequence AF533441 (Parma *et al.* 2003).

have identified six haplogroups named A, B, C, D, F and G. Nowadays, they exemplify the mitochondrial polymorphism of different maternal origins of the domestic goat. The term haplogroup has been proposed instead of the ambiguous 'maternal lineage' (Naderi *et al.* 2007). Unweighted mean pairwise differences are 9.17, indicating a strong differentiation between haplogroups A and F. The value for Fu's *Fs* statistic was −34.32, and the p-value was 0. We used two different analyses to investigate the relationships among Italian goats: a median-joining network and a NJ tree based on Kimura two-parameter distances (Kimura 1980) with 1000 bootstrap replications (Figure 3). Both analyses confirmed that the haplogroup F of Sicilian goats was considerably different from other Italian goats, with a difference of 44 single mutations.

To perform a more complete analysis of sequences used in our research, 885 mtDNA sequences of *C. hircus* from Mediterranean areas were added; the majority of haplotypes belonged to haplogroup A, but Mediterranean goats also belong to haplogroups

Table 2 Values of haplotypes diversity (hd), nucleotide diversity (π) and average number of nucleotide differences (k) for each breed and population

Breed/population	hd ± SD	π ± SD	k
Camosciata delle Alpi	0.96 ± 0.059	0.014 ± 0.0019	9.73
Maltese	0.93 ± 0.077	0.014 ± 0.0017	9.51
Nubian	0.96 ± 0.059	0.014 ± 0.0142	9.91
Saanen	0.98 ± 0.054	0.013 ± 0.0013	9.18
Sarda	0.97 ± 0.028	0.015 ± 0.0010	10.80
Tunisian	0.98 ± 0.054	0.015 ± 0.0014	10.18
All	0.99 ± 0.004	0.016 ± 0.0005	10.95

B (Greece), C (France, Portugal and Spain), G (Egypt) and F (Sicily). All the 69 sequences in our study were clustered in haplogroup A (Figure 4).

Discussion

The Sarda goat is the most important Italian autochthonous goat because of the high number of animals reared. It is also interesting for its perfect environmental adaptation. Goats reared in Sardinia are mainly represented by three different genetic bases (Sarda, Maltese and crossbred) which can be identified using an empirical approach (morphology

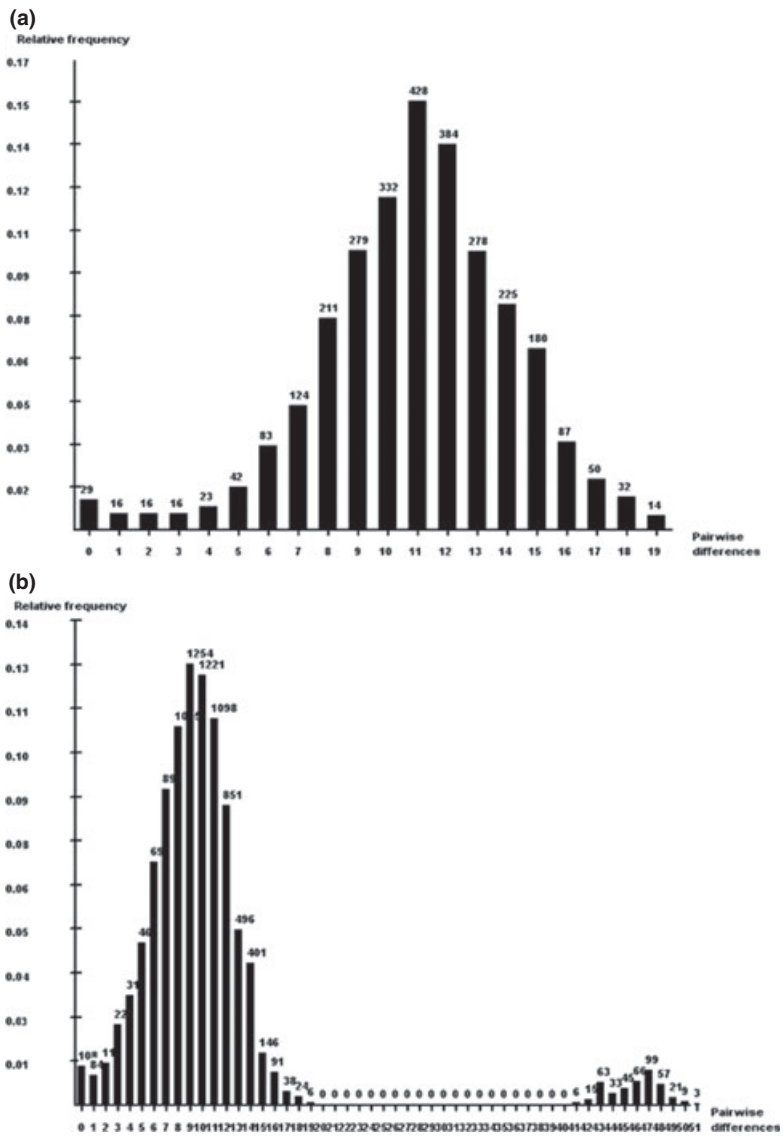

Figure 2 Mismatch distribution for mitochondrial DNA sequences of goats from our study (a) and for Italian breeds (b).

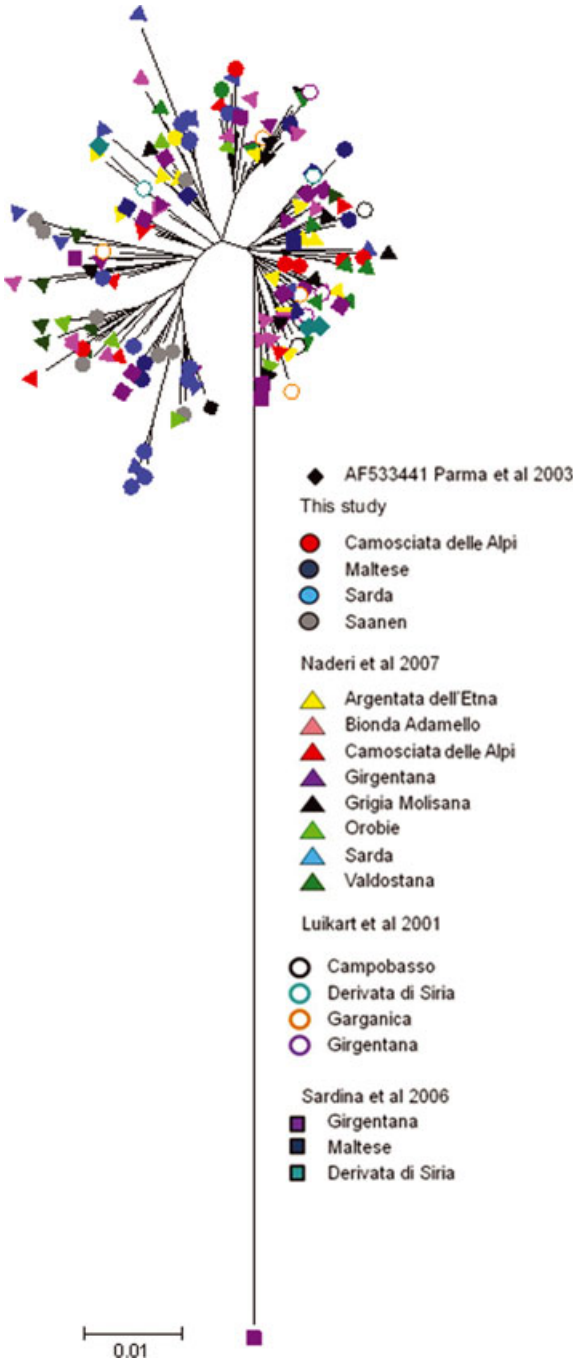

Figure 3 Neighbour-joining tree constructed using Kimura 2-parameter genetic distances between haplotypes found in Italian goat breeds.

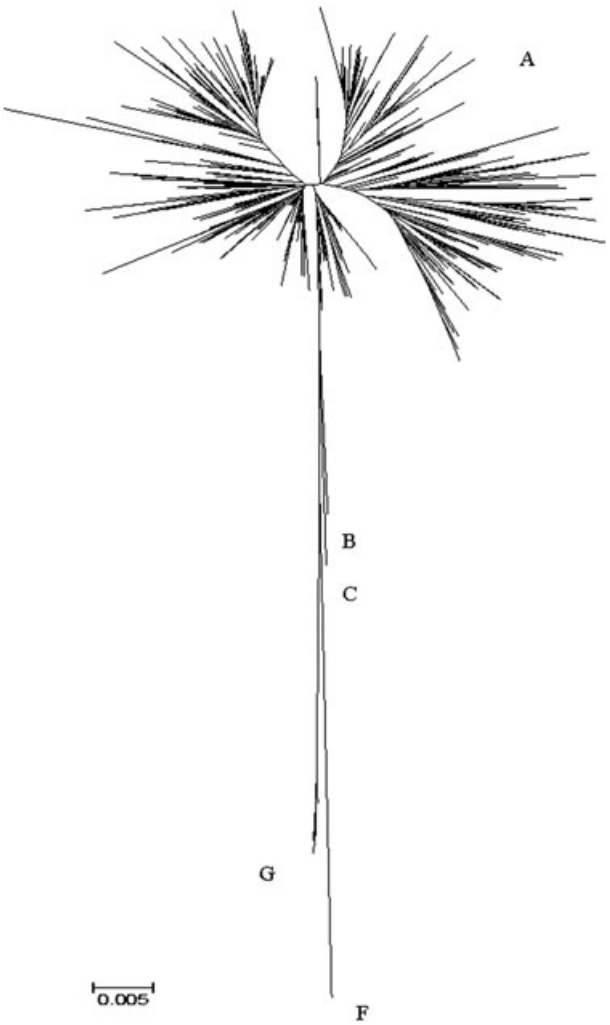

Figure 4 Neighbour-joining tree, constructed using Kimura 2-parameter genetic distances with 1000 bootstrap replications, of 885 mitochondrial DNA sequences of *Capra hircus* from Mediterranean areas.

and breeding histories) (Sechi *et al.* 2007). Although the Sarda has been crossed with specialized breed, above all the Maltese, to increase its production, goats belonging to several flocks located in different areas of Sardinia maintained their original morphological and productive traits. Haplotype diversity and nucleotide diversity of mtDNA are important indices for assessing population polymorphism and genetic differentiation. High values of haplotype and nucleotide diversity indicate high polymorphism of the population (Liu *et al.* 2006). Fifty-two haplotypes were identified in our study. The ratio between the number of haplotypes and the animals analysed, 52/69 = 0.75, is similar to those of Iberian and European population (Amills *et al.* 2009). Analysed breeds displayed a high number of unique haplotypes and only a few haplotypes were shared among the different breeds involved in our study. Sarda and Tunisian breeds showed the highest nucleotide diversity and a moderate haplotype diversity. The average number of nucleotide differences ranged from 9.51 (Maltese) to 10.80 (Sarda), revealing a

high diversity at this level. The high mtDNA diversity was probably because of the high variability level of the mitochondrial genome (Cozzi *et al.* 2004). As suggested by Schneider & Excoffier (1999), the trace of population expansion was tested by two different methods: the Fu's *Fs* statistic (Fu 1997) and the mismatch distribution (Rogers & Harpending 1992). The value for Fu's *Fs* statistic was −34.32, and the p value was 0, indicating a population expansion and a predominant departure from neutrality. A negative value of Fu *Fs s*tatistic, with probability value p = 0.000, has been also found by Joshi *et al.* (2004) in Indian goat breed Jamunapari. Significantly negative *Fs* value is interpreted as an evidence for population expansion (Fu 1997). This result was congruent with the mismatch distribution (Figure 2a). The mismatch distribution analysis revealed a unimodal bell-shaped distribution of pairwise sequence differences (Figure 2a). The median joining network of breeds reared in Italy showed that breeds from different geographical regions intermingled and haplotypes of each breed did not cluster together. This occurrence confirmed the lack of a well-defined phylogeographic structure for the Sarda breed, as in goats from South and Central America (Amills *et al.* 2009). Some new haplotypes, other than those which were identified by Naderi *et al.* (2007) and Luikart *et al.* (2001), were recognized. They were shared among the breeds only three times, H-18, H-40, H-69 (Table S1). A Sarda (accession number Genbank EF618182, Naderi *et al.* 2007), a Maltese (Genbank FJ571532) and a Saanen goat (Genbank FJ571556) from this study shared a haplotype listed as H-18. A Camosciata delle Alpi from this study (Genbank FJ571527) and a Sarda goat (Genbank EF618186, Naderi *et al.* 2007) shared a haplotype listed as H-69. Three Sarda goats (Genbank FJ571572, FJ571579, FJ571580) and a Maltese (Genbank FJ571534) from this study shared a haplotype listed as H-40. Sarda goats of this last haplotype belonged to two different herds, located in distant geographical areas, where morphological and productive traits were similar to the ancient Sarda goat breed.

On the whole, the animals in our study showed a high genetic haplotype diversity, as 35 haplotypes were each represented by a single sequence and only a few haplotypes were shared among the animals. This last occurrence is in agreement with other authors, as it is common to find haplotypes represented by one individual or shared by only a few subjects, because mtDNA variation is a more frequent component within breeds than between them (Luikart *et al.* 2001; Naderi *et al.* 2007).

Six mitochondrial haplogroups A, B, C, D, G and F have been identified by many authors (Naderi *et al.* 2007; Royo *et al.* 2009). The most common is haplogroup A, which is observed worldwide with high frequencies (from 89% in Asia to 98% in Europe) (Pereira *et al.* 2005) and probably represents the most ancient population expansion (Luikart *et al.* 2001; Royo *et al.* 2009). Haplogroup B was found in India, Malaysia, Mongolia and Pakistan. Haplogroup C was found with a low frequency in Asia and Europe, whereas haplogroup D was found with a low frequency in Pakistan, India, China and Kyrgyzstan (Naderi *et al.* 2007). Luikart *et al.* (2001) suggest that haplogroups B and C are the result of a second domestication in Asia, which represents a relatively recent expansion. A haplogroup named G has been identified in the Middle East and North Africa (Naderi *et al.* 2007). Haplogroup F was restricted to Sicily (Sardina *et al.* 2006). To evaluate the relationships between the Sarda from this study and other Mediterranean breeds, we used 885 sequences altogether. Haplogroup A was the major haplogroup found in this comparison and in the phylogenetic tree (Figure 4). All 69 sequences analysed in our study belonged to this group.

New haplotypes of goats reared in the Mediterranean area were identified in this work, and a small contribution to the database of known sequences was provided, with a preliminary sequence characterization of mitochondrial D-loop DNA of the Sarda goat. Given that 17% of goat breeds in the world are in critical or endangered risk (FAO 2004), the study of local breeds with other genetic markers such as autosomal, Y-chromosomal and Single Nucleotide Polymorphisms (SNPs) markers could be helpful in the preservation of native breeds, which are completely adapted to the environment where they originated.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1 Sequence variations of 232 sequences of Italian goat breeds. Goats of this study are listed in red and haplotypes identified in this study and shared between breeds were evidenced in orange. Haplotypes are identified after the comparison with the reference sequence AF533441 (Parma *et al.* 2003); EF618134 is the reference number for haplotype A (Naderi *et al.* 2007).

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