

# Genetic Diversity of Sheep Breeds from Albania, Greece, and Italy Assessed by Mitochondrial DNA and Nuclear Polymorphisms (SNPs)

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We employed mtDNA and nuclear SNPs to investigate the genetic diversity of sheep breeds of three countries of the Mediterranean basin: Albania, Greece, and Italy. In total, 154 unique mtDNA haplotypes were detected by means of D-loop sequence analysis. The major nucleotide diversity was observed in Albania. We identified haplogroups, A, B, and C in Albanian and Greek samples, while Italian individuals clustered in groups A and B. In general, the data show a pattern reflecting old migrations that occurred in postneolithic and historical times. PCA analysis on SNP data differentiated breeds with good correspondence to geographical locations. This could reflect geographical isolation, selection operated by local sheep farmers, and different flock management and breed admixture that occurred in the last centuries.

**KEYWORDS:** mtDNA, sheep, SNPs, Mediterranean, domestication

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## 1. INTRODUCTION

The earliest archaeozoological evidence of domestic sheep comes from a restricted area of south-western Asia: modern Iran, Turkey, and Cyprus [1]. A pioneering genetic study examining the karyotypes of the various species of extant wild sheep [2, 3] showed that domestic sheep derive from the Asiatic mouflon (*Ovis orientalis*) of Anatolia, western Iran, and southwest Iran. Afterwards, a probable migration of the Neolithic farmers occurred out of the Near East and across Europe following two main routes, through the continental heartland up the Danube valley or along the Mediterranean coast [4, 5] crossing the sea to the major islands. Archaeological data and radiocarbon dates on seeds or bones provide support for an earlier arrival in Western Europe via the Mediterranean route rather than the “Danubian” route [6].

Both archaeozoological evidence and genetic evidence indicate that the domestication of wild sheep occurred 8000–9000 years ago. The first appearance of the remains of domestic sheep in the western part of Mediterranean Europe, dating approximately 5400 BC, is believed to reflect a rapid spread by sea [7, 8]. The Mediterranean Sea also had a key role in the history of livestock in postneolithic times, when peoples like Phoenicians, Greeks, Romans, and Berbers probably introduced new species of animals and new breeds of livestock into southwest Europe by sea. Some settlers may have improved local livestock by importing stock from overseas [8], which explained the unexpectedly high within-breed diversity in domestic goats [9, 10], the differential cattle migration along the Mediterranean coast [11], and the close genetic relationship between Tuscan and Near Eastern cattle breeds [12]. The role of the Mediterranean Sea as a natural corridor connecting the South West Europe to the Near East and North Africa is particularly plausible for domestic sheep and goats that were adaptable to various environments and easy to transport due to their size [8]. Subsequently, sheep breeds developed after selective breeding for desirable traits (wool, milk, and meat production) and environmental tolerance. Since domestication, sheep have established a wide geographic range due to their adaptability to poor diets and extreme climatic conditions as well as their manageable size.

The genetic history of sheep has been investigated using three major sources of genomic variation: autosomes, Y chromosome, and mitochondrial genome. Analysis of the nonrecombining region of the Y chromosome has revealed patterns of male-mediated introgression during breed development [13, 14]. Recent surveys have tested collections of animals from southern and northern Europe [15] or Europe and the Middle East [16] using microsatellites and enabled the analysis of genetic partitioning at a continental scale. Interestingly, southern European breeds displayed increased genetic diversity and decreased genetic differentiation compared to their northern European counterparts. This is consistent with the expectation that genetic diversity will be maintained high in populations close to the centre of domestication but decreases with increasing geographic distances. Kijas et al. [17] used a SNP panel to analyse sheep nuclear genome, providing the indication that breeds cluster into large groups based on geographic origin and that SNPs can successfully identify population substructures within individual breeds.

A recent study on retrovirus integrations [18] has provided additional information on the introduction of sheep into Europe, indicating an early arrival of the primitive sheep populations (European mouflons, North-Atlantic Island breeds) and a subsequent advent of wool producing sheep.

However, most of the information about history and domestication of the species have been gathered using mtDNA. The existence of multiple mtDNA lineages and their admixture within breeds [8, 19–22] could be due to multiple domestication events and subsequent human selection or introgression by domestic and wild species.

Mitochondrial DNA analyses in sheep identified an increasing number of maternal lineages: two [23–25], three [20, 26], and then five [22]. The main haplogroups A and B are both found in Asia, while B dominates in Europe. Haplogroup C has been found in Portugal, Turkey, the Caucasus, and China [7]. Haplogroup D, present in Rumanian Karachai and Caucasian animals, is possibly related to the haplogroup A. In contrast to taurine cattle, the sheep haplogroups hardly correlate with geography.

Because of their mode of inheritance, mitochondrial markers are more likely to lead to biased estimates of species phylogeny [27]. Combining nuclear and mitochondrial markers may help in avoiding

**TABLE 1:** Country of origin, breeds, and acronyms used in computations.

Country	Breed	Acronym
Albania	Bardhoka	BAR
	Ruda	RUD
	Shkordane	SHK
Greece	Kalarritiko	KAL
	Orino	ORI
	Pilioritiko	PIL
	Kefalleneas	KEF
	Lesvos	LES
	Kymi	KIM
	Karagouniko	KAR
	Skopelos	SKO
	Anogeiano	ANO
Sfakia	SFA	
Italy	Bergamasca	BER
	Delle Langhe	LAN
	Laticauda	LAT
	Altamura	ALT
	Gentile di Puglia	GDP

this problem. Nuclear genome evolves five-to-ten times slower than mtDNA; it is contributed by both parents and its variability is less affected by demographic forces such as bottleneck. Therefore, nuclear markers can detect more recent genetic events that influence the extant divergence of domestic breeds. Several studies have demonstrated that the combination of nuclear and mtDNA markers can increase the information obtained [27–30]. The use of both markers might provide a more accurate and comprehensive understanding of a species' history [31]. SNP markers could help in understanding the recent evolutionary history of domestic animals [10, 32].

We aimed at investigating the geographic distribution of the genetic diversity of sheep breeds in Albania, Greece, and Italy and to gather information on the migration history of the species. To accomplish that, we employed sequence data from the mitochondrial D-loop and 27 nuclear loci (SNPs).

## 2. MATERIALS AND METHODS

### 2.1. Sampling and DNA Extraction

We focused on sheep breeds of Albania, Greece, and Italy. Samples of the European mouflon were also included. About twenty unrelated samples per breed were selected. Three animals per flock from 11 farms spread over the traditional rearing area were sampled. A total of 313 animals from 18 sheep breeds were analyzed. Breeds, acronyms used, and country of origin of each breed are reported in Table 1. Part of the samples were obtained from a previous project (Econogene, <http://www.econogene.eu/>). Blood samples were collected in EDTA tubes and frozen at  $-20^{\circ}\text{C}$  until extraction. Genomic DNA was isolated using standard procedures, checked for DNA quality on agarose gel and quantified using a DTX microplate reader (Beckman Coulter) after staining with PicoGreen (Invitrogen).

### 2.2. Amplification and Sequencing of the Mitochondrial D-Loop

To amplify the partial D-loop of 721 bp, primers described by Tapio et al. [7] were used from 15,541 to 16,261 of the complete sequence described by Hiendleder et al. [33] available in GenBank (NC\_001941.1).

Polymerase chain reaction (PCR) was performed in a total volume of 50  $\mu$ L containing 20 ng of genomic DNA, 40 pMol of each primer (Sigma-Aldrich), 200  $\mu$ M dNTPs, 5X PCR buffer, and 5 units of *Taq* DNA polymerase (Promega) on a PCR Thermo Cycler (MJ Research). A 5 minutes denaturation step at 95°C was followed by 14 cycles of denaturation at 95°C for 30 sec, annealing for 30 sec starting at 62°C and decreasing 0.5°C per cycle and extension at 72°C for 120 sec, then by 20 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 120 sec; the final extension step was carried out at 72°C for 5 minutes. PCR products were purified through ExoSap-IT (USB Corporation) to remove residual primers and dNTPs and used as templates for forward and reverse sequencing reactions.

Sequencing was performed using the primers described by Tapio et al. [7] with a CEQ 8800 sequencer using DTCS QuickStart Kit and purifying with Agencourt CleanSEQ 96 (Beckman Coulter), according to the manufacturer's instructions. After the optimization of the sequencing protocol, sequencing was outsourced to Macrogen (<http://www.macrogen.com/>). The sequences of D-loop were submitted to GenBank (accession numbers: JN184789–JN184999).

### 2.3. Mitochondrial Sequence Analysis

A fragment of 435 bp, running from 15,541 to 16,261 bp (NC\_001904.1), was selected excluding a central region rich in tandem repeats (from 15,644 to 15,932 bp). mtDNA variations were identified on a total of 313 sequences of 18 breeds analyzed and aligned with BioEdit software [34]. DnaSP 5.00 software [35] was used to calculate haplotype, sequence variation, average number of nucleotide differences (D), and average number of nucleotide substitutions (Dxy) per site between breeds. Neighbour-joining tree for all haplotypes was constructed using Mega version 5 [36]. Analysis of molecular variance (AMOVA) was performed with Arlequin version 3.11 [37]. Sequences of the same D-loop fragment in wild sheep, published by Hiendleder et al. [33], were obtained from GenBank, *Ovis vignei arkal* (AY091489.1), *Ovis vignei bochariensis* (AY091490.1, AY091491.1, and AF039580.1), *Ovis ammon collium* (AY091492.1), *Ovis ammon nigrimontana* (AY091493.1 and AY091494.1), and used as outgroups in phylogenetic analysis.

Geographic distribution of eigenvectors was performed to investigate population genetic differences on the basis of their geographic distances. This approach permitted the generation of a synthetic configuration of locations based on the pairwise genetic distances that matched the real geographic configuration. Principal component analysis (PCA) scores for the first two components, obtained using Nei's 1973 genetic distance, were plotted on a geographic map. As breeds are scattered among several farms, a virtual geographic entity representing the centroid of each breed on geographic maps was created using WGS84 geographical coordinates [38]. For a given component, it is a measure of the variance accounted for by that component. On thematic maps produced with the geographic information system (GIS) Manifold software package (Manifold System, Version 7, Manifold Net Ltd., Carson City, USA, <http://www.manifold.net/>), all breeds are thus represented according to a geometric distribution (see Figures 3(a) and 3(b)). Breeds showing high eigenvectors contribute sensibly to the explanation of the variance related to the component displayed. Classes were elaborated on the basis of the criterion of the natural breaks (Jenks optimization method). This algorithm reduces the variance within classes and maximizes the variance between classes. Colour classes were chosen in order to support the distinction between the different categories of behaviours observed: green: positive contribution; yellow: intermediary values; red: negative contribution to the component displayed.

### 2.4. Nuclear Polymorphism Analysis

The same 313 sheep belonging to 18 breeds sequenced at D-loop were genotyped with 37 previously described SNPs [39]. SNP ascertainment bias was minimised by sequencing target DNA in at least 8 individuals from different populations. Large-scale genotyping of all animals was performed by outsourcing to a commercial genotyping company (<http://www.Kbioscience.co.uk/>).

**TABLE 2:** Sample size per country ( $n$ ), number of haplotypes observed (Haplotypes), number of polymorphic sites, mean number of pairwise differences among sequences (pairwise diff.), haplotype diversity ( $h$ ), and nucleotide diversity ( $\pi$ ) are shown.

Country	$n$	Haplotypes	Polymorphic sites	Pairwise diff.	$h$	$\pi$
Italy	93	62	58	4.180	0.978	0.01007
Greece	167	83	73	5.934	0.934	0.01469
Albania	53	37	57	8.704	0.979	0.02107

Allele frequencies, Nei's estimation of observed and expected heterozygosities ( $H_o$  and  $H_e$ , resp.), were calculated using Fstat 2.93 [40]. Weir and Cockerham's [41] estimates of  $F_{is}$  per population,  $F_{st}$  per locus, and population pairs were calculated for each locus using Genalex 4.0 [42]. The same software was used to test deviations from Hardy-Weinberg equilibrium (HWE) for each locus and population and for locus over all populations; test for conformity with HWE expectations was assessed by calculating the Chi-squared value.

Correlation between geographic and Nei's 1973 pairwise genetic distances was tested using Mantel tests (999 permutation) implemented in Genalex 4.0 software [42].

A PCA was performed on the covariance matrix of SNP frequency data to investigate spatial patterns of genetic variation using GENETIX software [43].

Nei [44] and Reynolds [45] genetic distances between population pairs were calculated using PowerMarker v3.25 [46].

Geographic distribution of eigenvectors was performed as described above using pairwise genetic distances [47] calculated on the basis of the selected SNP markers.

### 3. RESULTS

#### 3.1. Mitochondrial Haplotypes

Ninety-three polymorphic sites and 154 haplotypes were identified from 313 sequences. Relatively high haplotype diversity was found in all three sampled geographic regions; the largest nucleotide diversity is present in Albania (0.02107) while the highest number of haplotypes observed is recorded in Greece (83) (Table 2).

The average number of nucleotide differences and the average number of nucleotide substitutions per site were used to calculate the genetic distance between breeds. The lowest distance was observed between Laticauda and Anogeiano (D: 2.357—Dxy: 0.006), while the highest distance was observed between Bardhoka and Kymi (D: 12.450—Dxy: 0.03) (Table 3).

AMOVA revealed that mitochondrial diversity is mainly distributed within breeds (95.04%) and only in part among regions (0.90%); low variability was also found among breeds/within regions (4.06%) (Table 4).

#### 3.2. Phylogenetic Analysis and Haplogroups

The NJ tree obtained from mtDNA haplotypes and wild sheep sequences, used as out-group, revealed three of the five haplogroups described in the literature: A, B, and C (Figure 1). Haplogroup B is the most frequent among the analyzed samples (89%), while A and C are less common (8% and 3%, resp.). Greek and Albanian breeds are present in all three haplogroups, while Italian breeds are present only in haplogroups B and A (Table 5). This is shown also in Figure 2, representing the percentage of occurrence of each haplogroup in Albania, Greece, and Italy.

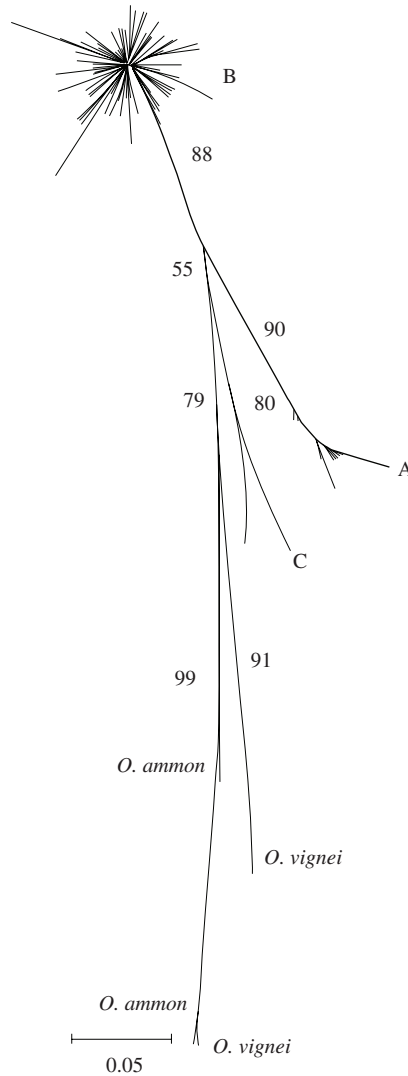
**TABLE 3:** Average number of nucleotide differences, D (below), and average number of nucleotide substitutions per site between populations, Dxy [48] (above).

	BER	ALT	LAT	LAN	GDP	BAR	RUD	SHK	KAL	ORI	PIL	KEF	LES	KIM	KAR	SKO	ANO	SFA
BER	*	0.009	0.009	0.012	0.011	0.018	0.011	0.021	0.009	0.017	0.009	0.018	0.018	0.025	0.012	0.011	0.008	0.009
ALT	3.908	*	0.007	0.011	0.009	0.017	0.009	0.02	0.007	0.016	0.008	0.016	0.017	0.024	0.011	0.009	0.006	0.008
LAT	3.728	2.93	*	0.01	0.009	0.016	0.009	0.02	0.007	0.015	0.007	0.016	0.017	0.025	0.01	0.008	0.006	0.007
LAN	5.079	4.425	4.279	*	0.012	0.019	0.013	0.022	0.01	0.018	0.01	0.019	0.019	0.026	0.013	0.012	0.01	0.01
GDP	4.632	3.906	3.756	5.134	*	0.018	0.011	0.021	0.009	0.017	0.01	0.017	0.018	0.024	0.012	0.011	0.009	0.009
BAR	7.447	6.933	6.856	8.107	7.579	*	0.018	0.026	0.016	0.023	0.017	0.023	0.024	0.03	0.02	0.018	0.016	0.016
RUD	4.612	3.95	3.7	5.245	4.711	7.647	*	0.021	0.009	0.017	0.01	0.018	0.018	0.025	0.012	0.011	0.008	0.009
SHK	8.778	8.272	8.345	9.269	8.696	10.567	8.789	*	0.02	0.025	0.02	0.025	0.025	0.029	0.023	0.021	0.02	0.02
KAL	3.712	3	2.708	4.183	3.754	6.741	3.778	8.241	*	0.015	0.007	0.016	0.017	0.024	0.01	0.008	0.006	0.007
ORI	7.108	6.488	6.395	7.536	7.132	9.4	7.122	10.395	6.352	*	0.016	0.023	0.023	0.029	0.018	0.017	0.015	0.015
PIL	3.91	3.289	3.011	4.5	4.021	7.049	4.067	8.419	2.978	6.556	*	0.017	0.017	0.025	0.011	0.009	0.007	0.007
KEF	7.349	6.861	6.832	7.853	7.319	9.804	7.475	10.306	6.757	9.486	6.971	*	0.022	0.026	0.019	0.018	0.016	0.016
LES	7.515	7.042	6.941	8.053	7.507	10.1	7.588	10.396	6.896	9.576	7.125	9.367	*	0.027	0.019	0.018	0.016	0.016
KIM	10.426	10.083	10.276	10.994	10.211	12.45	10.438	11.931	10.194	12.028	10.383	11.141	11.375	*	0.026	0.026	0.024	0.024
KAR	5.144	4.478	4.197	5.595	5.139	8.217	5.185	9.378	4.294	7.65	4.503	7.894	7.975	10.975	*	0.012	0.01	0.01
SKO	4.497	3.852	3.538	4.989	4.544	7.585	4.611	8.883	3.472	7.102	3.719	7.455	7.618	10.813	4.936	*	0.008	0.009
ANO	3.514	2.678	2.357	4.053	3.526	6.614	3.505	8.123	2.439	6.132	2.712	6.655	6.73	10.079	4.003	3.301	*	0.006
SFA	3.768	3.164	2.878	4.266	3.801	6.698	3.834	8.015	2.906	6.266	3.182	6.658	6.77	9.757	4.284	3.596	2.643	*



**TABLE 4:** Hierarchical analysis of molecular variance (AMOVA) with 10,000 permutations.

Source of variation	Variation (%)	Fixation indices [49]	P value
Among regions	0.90	FSC: 0.0495	<0.01
Among breeds/within regions	4.06	$F_{st}$ : 0.04960	<0.01
Within breeds	95.04	FCT: 0.00903	<0.5

**FIGURE 1:** Neighbour-joining tree based on the 154 mtDNA haplotypes, showing differences among haplogroups and wild sheep (outgroup). Numbers indicate the percentage bootstrap support (10,000 resamplings).

### 3.3. SNP Analysis

A total of 37 SNPs identified as polymorphic on eighteen sheep breeds selected throughout Europe [39] were applied to genotype 313 individuals of three Albanian, ten Greek, and five Italian sheep breeds. After removing those found monomorphic in the selected breeds, 27 SNPs were used for the analysis.

The frequencies of the major alleles ranged from 0.99 for the locus *LEP1* to 0.538 for the locus *IL2\_1*. Except for *CAST\_1*, *LEP1*, *LEP2*, *GDF8*, and *PRNP\_1*, which show frequencies of the rare alleles of 0.035,

**TABLE 5:** Sequences analysed, number of variable sites (# Var), number of haplotypes (Hap), and percentage of haplotypes in the haplogroups A, B, or C per breed.

Breed	Sequences	# Var	Hap	A	B	C
BAR	15	43	14	0.07	0.79	0.14
RUD	20	35	15	0.07	0.93	0
SHK	18	42	11	0.09	0.73	0.18
ANO	19	12	7	0	1	0
KAL	18	18	11	0	1	0
KAR	20	38	14	0.07	0.93	0
KEF	16	31	11	0.18	0.82	0
KIM	8	27	6	0.33	0.77	0
LES	16	38	13	0.15	0.85	0
ORI	18	43	13	0.08	0.84	0.08
PIL	15	17	11	0	1	0
SFA	19	15	11	0	1	0
SKO	18	27	13	0	1	0
ALT	18	19	11	0	1	0
BER	17	30	12	0.08	0.92	0
GDP	19	32	16	0.06	0.94	0
LAN	20	41	17	0.06	0.94	0
LAT	19	17	13	0	1	0

0.010, 0.020, 0.021, and 0.036, respectively, the remaining SNPs have rare allele frequency of greater than 5%. Observed heterozygosity of all loci ranged from 0.019 (*LEP1*) to 0.489 (*IL2\_1*), with a mean of 0.250. Expected heterozygosity of the loci ranged from 0.018 (*LEP1*) to 0.474 (*IL2\_1*), with a mean of 0.259.

Significant deviations from Hardy-Weinberg equilibrium (HWE) for each locus and population ( $P$  value  $< 0.01$ ) were observed for nine loci. Locus *BMPR* was not in HWE in Skopelos breed. Locus *ACVR2B\_2* was not in HWE in Bardhoka population, and locus *SERPINA3* was not in HWE in Karagouniko population. Loci *ZP2* and *KRTAP6* were not in HWE in Altamura. *PRNP\_1* was not in HWE in four Italian breeds: Bergamasca, Delle Langhe, Gentile di Puglia, and Laticauda. *CSN3* was not in HWE in Ruda, Skopelos, and Bergamasca populations; *KRT1* in Delle Langhe, Laticauda, and Karagouniko; *GHR* in Karagouniko and Laticauda.

$F_{is}$  per population ranged from  $-0.102$  (Shkordane) to  $0.276$  (Altamura) with a mean of  $0.033$  ( $P$  value =  $0.00010$  on 9720 randomisations).

The analysis of population differentiation revealed an overall  $F_{st}$  of  $0.048$ , that is, 4.8% of allelic variation accounted across breeds and 95.2% within breeds. Weir and Cockerham's [41] estimate of  $F_{st}$  per locus ranged from  $0.0132$  (*IL2\_2*) to  $0.098$  (*DESMIN\_2*), with a mean of  $0.047$ .

Both Nei's [44] and Reynolds' [45] genetic distances were calculated (Table 6). The distances range from  $0.022$  (Ruda-Karagouniko) to  $0.253$  (Sfakia-Gentile di Puglia) using Reynolds' distances and from  $0.008$  (Ruda-Karagouniko) to  $0.096$  (Gentile di Puglia-Anogeiano) using Nei's distances. Both indices indicate Ruda and Karagouniko as the breeds with the minimum pairwise distance and show the maximum distances between the Italian breed Gentile di Puglia and Anogeiano, Karagouniko, Kefalleneas, Kymi, Lesvos, Pilioritiko, Sfakia, Skopelos, and Bergamasca breeds. The Mantel test showed correspondence between geographic and genetic distances with a  $P = 0.04$ .



**TABLE 6:** Pairwise population matrix of Nei's [44], below, and Reynolds' [45], above, genetic distances. Breed codes are as in Table 1.

BAR	RUD	SHK	ANO	KAL	KAR	KEF	KIM	LES	ORI	PIL	SFA	SKO	ALT	BER	GDP	LAN	LAT	
BAR	*	0.060	0.046	0.065	0.052	0.054	0.055	0.104	0.110	0.117	0.083	0.061	0.070	0.103	0.167	0.121	0.055	
RUD		*	0.036	0.072	0.082	0.022	0.046	0.067	0.059	0.148	0.104	0.062	0.062	0.049	0.203	0.100	0.051	
SHK			*	0.072	0.072	0.039	0.058	0.078	0.087	0.112	0.063	0.080	0.042	0.062	0.174	0.123	0.058	
ANO				*	0.086	0.055	0.075	0.109	0.096	0.147	0.103	0.045	0.098	0.085	0.247	0.122	0.073	
KAL					*	0.078	0.071	0.127	0.124	0.076	0.062	0.107	0.112	0.104	0.159	0.119	0.092	
KAR						*	0.047	0.071	0.072	0.142	0.103	0.057	0.060	0.048	0.216	0.102	0.054	
KEF							*	0.095	0.091	0.141	0.106	0.074	0.076	0.091	0.248	0.124	0.058	
KIM								*	0.081	0.154	0.134	0.107	0.115	0.100	0.241	0.157	0.075	
LES									*	0.180	0.148	0.095	0.110	0.086	0.249	0.134	0.070	
ORI										*	0.090	0.169	0.161	0.168	0.152	0.163	0.140	
PIL											*	0.124	0.104	0.125	0.152	0.161	0.107	
SFA												*	0.098	0.097	0.253	0.122	0.067	
SKO													*	0.083	0.232	0.152	0.098	
ALT														*	0.226	0.132	0.073	
BER															*	0.233	0.070	0.083
GDP																*	0.078	0.099
LAN																	*	0.048
LAT																		*

### 3.4. PCA on SNPs

Genetic relationships were also explored by means of PCA. To examine the overall pattern of population differentiation, we considered the first three axes, which cumulatively explained 48.87% of the total inertia contained in the data set (Figure 4). From PCA, it can be seen that the some breeds are quite differentiated, with good correspondence to geographical locations, even if SNPs were few (4 of the 5 Italian breeds are well separated from the main cluster). Particularly, a differentiation between northern Italian (Bergamasca and Delle Langhe) and southern Italian (Gentile di Puglia, Altamura, and Laticauda) breeds can be seen. The projection of loci in the space formed by the first three principal components (data not shown) shows that the differentiation of outlying breeds is caused by a small number of SNPs: the Delle Langhe population is mainly affected by alleles in the *MSTN* gene along the second component.; the Skopelos breed position in the graph is affected by alleles in the *PRP* gene and the Laticauda breed (lesser) differentiation is mainly due to alleles in the *CALPA* and *LEP* genes.

PCA scores calculated on mtDNA marker for the first 2 components were plotted on a geographic map, using the centroids of the sampling area of each breed (Figures 3(a) and 3(b)). The highest eigenvector contribution (coloured in green) was observed for Albanian and Greek breeds, as expected, even if four breeds show unexpectedly low diversity.

As for mtDNA analyses, the results of the PCA based on SNPs data were also used for making inferences about population genetic differences on the basis of their geographic distances. PCA scores for the first two principal components were plotted on a geographic map, using the centroids of the sampling area of each breed (Figures 3(c) and 3(d)). The line separating the map in two regions shows the isoline for an eigenvalue of 0. RUDA breed is an exception showing an eigenvalue above 0 but located in an area where all other breeds show lower eigenvalues. In accordance with the domestication history of the species, genetic diversity was higher in south-eastern populations than in north-western populations. The first component of Figure 3(c), in fact, shows a regular loss of genetic diversity towards North West.

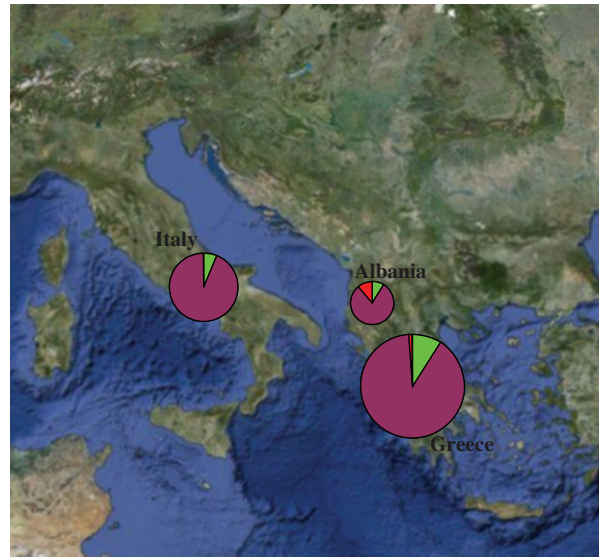
## 4. DISCUSSION

Agriculture arose mainly within the distribution range of the wild ancestors of the most valuable livestock, such as the Fertile Crescent of southwest Asia, where early farmers were able to outcompete local hunter-gatherers. Once livestock slowly spread northwest across Europe, farming also shifted northwest from the Fertile Crescent to areas where farming had never arisen independently—first to Greece, then to Italy, and finally to northwest Europe [50]. Therefore, today the most productive farming zones do not correspond to those most productive in the past. Then, founder effect, genetic drift, and natural or artificial selection led to the formation of distinct breeds or varieties [51].

We therefore focused on the analysis of sheep of three countries aligned on this route, to evidence signs of migration. Geographical isolation, natural and artificial selection for physical or productive characters, genetic drift, mutations, and interpopulation gene flows have altered gene frequencies over many generations. The genetic diversity within and across breeds and species forms the basis of our current animal genetic resources for food production and other purposes.

The nature of the markers used for the analysis can affect the detection of geographical structuring, as suggested by Naderi et al. [52]. In fact, mtDNA informativeness is limited because it does not detect male-mediated gene flow and does not predict the nuclear genomic diversity [53]. Moreover, results may be affected by phenomena such as homoplasy, incomplete lineage sorting, effective population sizes, and sex-biased dispersal [27]. By combining markers with different modes of inheritance and rates of evolution this bias can be minimized [54].

Our mtDNA analysis shows higher levels of sheep nucleotide diversity in the South-East, which is congruent with data reported in the literature [22] and congruent with the proximity to the domestication centre. This is confirmed by eigenvector analysis, which showed high contribution to variance by Albanian



**FIGURE 2:** Geographical distribution of haplogroups, circles are proportional to the number of samples. (green: haplogroup A; purple: haplogroup B; red: haplogroup C).

and Greek breeds, even if four breeds show unexpectedly low diversity. However, this behaviour can be explained by recent isolation or selection for some traits that reduced the overall genetic diversity through bottleneck.

Very high haplotypes diversity was found in all three regions analysed (greater than 0.9), in agreement with previously published works on Portuguese breeds [9], Indian breeds [55], and Balkan sheep [56]. The major mitochondrial variation is distributed within breeds (95.04%), while it is lower among regions (0.90%) and among breeds within regions (4.06%). Phylogenetic methods were employed to examine the evolutionary history of the 18 breeds. Neighbour-joining and median-joining network revealed three of the described haplogroups, A, B, and C. The mouflon shares a haplotype with domestic sheep, as previously reported by Hiendleder et al. [33].

The SNP analysis revealed a rare allele frequency  $<5\%$  for *LEP1* and *LEP2* loci, in agreement with those observed on a different European breed panel by Pariset et al. [39]. Observed and expected mean heterozygosity also showed similar values to those reported in the same paper. Expected heterozygosity values, which can indicate response to selection, are higher than observed heterozygosity values ( $H_s$  0.063, 0.07, and 0.042;  $H_o$  0.052, 0.06, and 0.038 in *CALPA*, *PRNP-1*, and *GDF8*, resp.).

Among the breeds tested, Altamura showed the highest  $F_{is}$  value suggesting the inbreeding in this population.

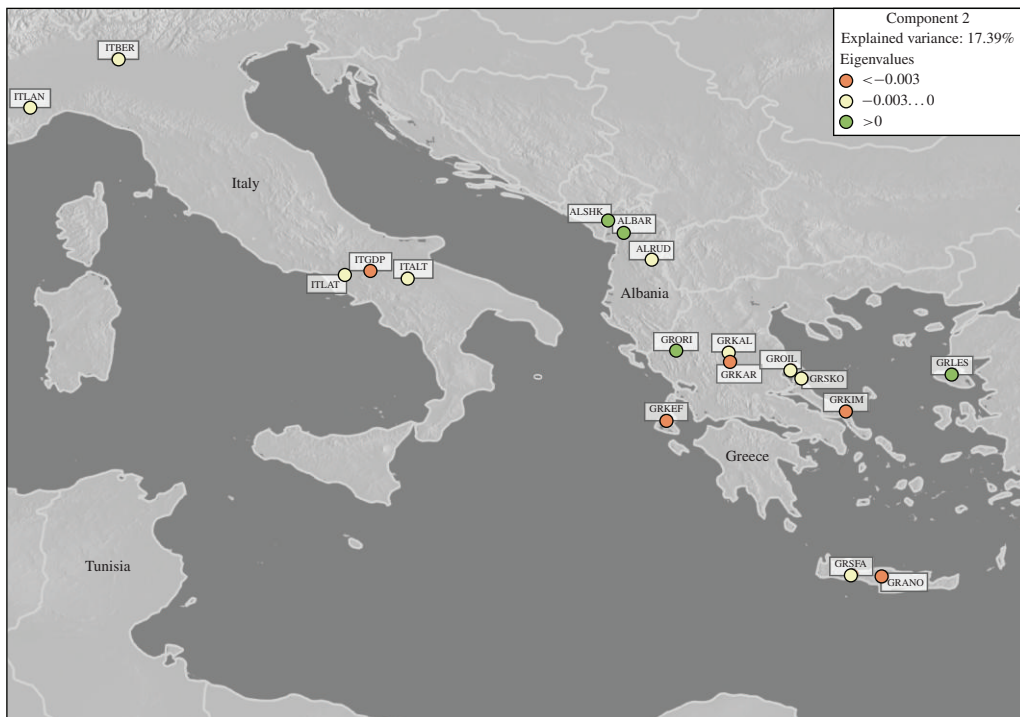
Regarding the phylogeographic structure we found that the 95.2% of variation occurred within breeds indicating the weak phylogeographic structure in sheep. These data are consistent with those previously published by Kijas et al. using a different SNP panel [17]. Sheep generally do not have a strong geographic structure and show a high genetic variability within breeds.

Anyway, Mantel test analysis using SNPs revealed a correlation between genetic and geographic distance. The possibility to assess the presence of a geographic component in genetic diversity using SNPs was already reported in previous studies on sheep [39] and goats [10, 57, 58].

In the PCA, the breeds appear differentiated with 48.9% of the variance explained by the first three principal components. Also this analysis shows a good correspondence to geographical locations: the breeds remaining separated by the main group are all Italian. PCA supports therefore a westward route to Italy that could indicate that transport of animals made by sea as already proposed for cattle [11, 12] and goats [8, 59]. This is particularly plausible because small sized species as sheep are easy to transport during migration



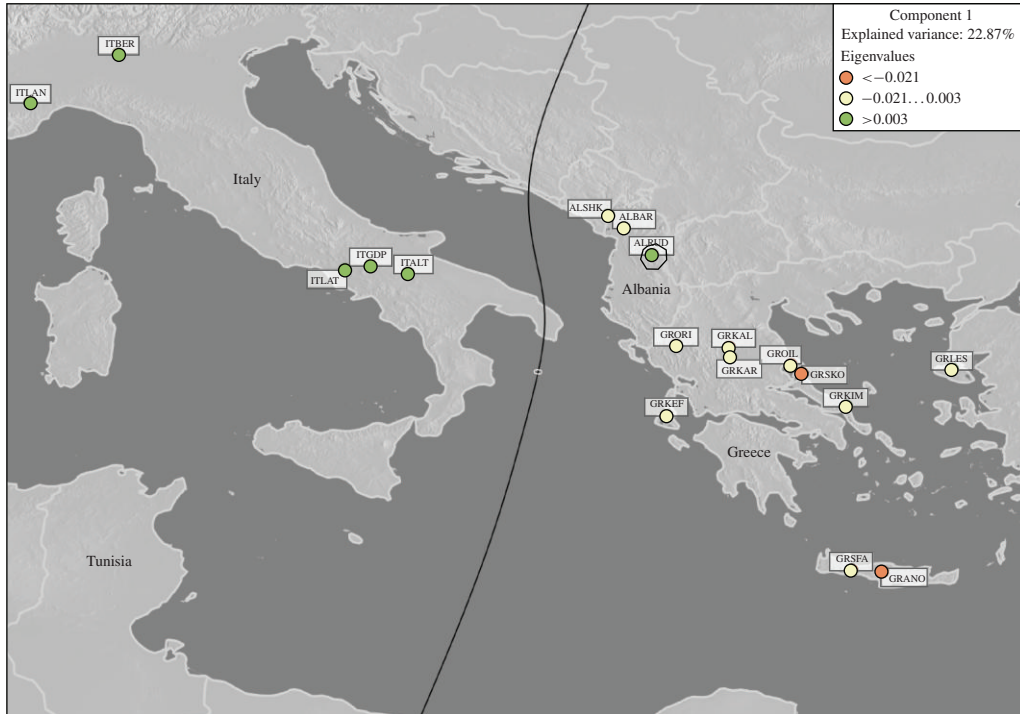
(a)



(b)

FIGURE 3: Continued.



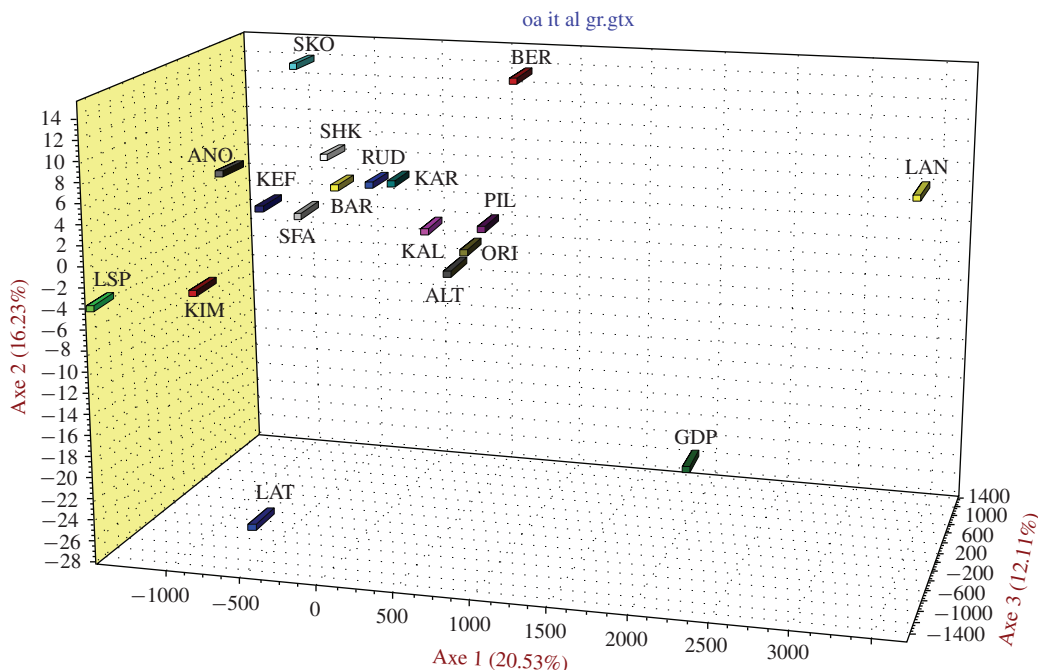


(c)



(d)

**FIGURE 3:** First (a) and second (b) components of eigenvectors spatial distribution calculated on mtDNA marker and first (c) and second (d) components calculated on SNPs markers. Background image is GTOPO30, a global digital elevation model (DEM) with a horizontal grid spacing of 30 arc sec-onds (approximately 1 kilometer) produced by the U.S. Geological Survey’s Center for Earth Resources Observation and Science (EROS). The line in the map (c) shows the isoline for an eigenvalue of 0. It appears on panel C only because this is the only geographical configuration for which a limit is so obvious. The circle around the RUDA breed shows an isoline for the eigenvalue of 0.03.



**FIGURE 4:** PCA analysis of the breeds assessed by SNP markers. The first three axes, cumulatively explaining 48.87% of the total inertia, were considered.

and commercial trade and can adapt easily to various environments [53, 60, 61]. A similar decrease in genetic diversity as well as an increase in the level of differentiation at the breed level from South-East to North-West in European sheep breeds, supporting the hypothesis of livestock migration from the Middle East towards western and northern Europe, was found by Peter et al. [16] and Lawson Handley et al. [15], using other nuclear markers.

The formulation of the modern breed concept during mid-1800s has caused remarkable changes in the livestock sector: large-scale production expanded and its application to breeding and husbandry practices led to the formation of well-defined breeds that were exposed to intense anthropogenic selection. The differentiation of three breeds observed using PCA analysis could be related to a recent selection, which appears to be linked to *CALPA* (Laticauda), *PRNP-1* (Skopelos), and *GDF8* (Delle Langhe) SNP markers. Gentile di Puglia breed seems influenced by both *CALPA* and *GDF8*. In particular, these two genes have an effect on conformation and therefore are an easy target of selection. Other SNPs related to meat traits were found potentially under the effect of selection and apparently not associated with production attitude of the breeds [58].

## 5. CONCLUSIONS

We employed mtDNA and nuclear SNPs to investigate the genetic diversity of sheep breeds of three countries of the Mediterranean basin: Albania, Greece, and Italy. Our results showed significant genetic differentiation among the sheep breeds, supported by mtDNA and by SNP. The differentiation identified by nuclear markers could indicate a reduced gene flow due to geographical isolation, associated with different flocks management, or an effect of the introduction of different stocks centuries ago (cf. Figure 3(d), showing the 2nd dimension geographic distribution of eigenvectors). In general, D-loop sequence analysis shows a pattern reflecting migrations that occurred in postneolithic and historical times, with the most divergent mtDNA lineage occurring in the southern breeds, as shown in Figure 2 and Table 5. PCA on



SNP data differentiated breeds with good correspondence to geographical locations. It is interesting that the correlation between genetic and geographic distances revealed using nuclear SNPs was not confirmed by mtDNA, for which Mantel test was not significant. Our results seem to indicate a better correlation between geographic distances and autosomal markers.

## APPENDIX

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