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ARTICLE

Genetic characterization of Egyptian and Italian sheep breeds using mitochondrial DNA

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Abstract A 721-bp fragment from 15,541 to 16,261 bp (NC_001941.1) of the mtDNA control region from different Egyptian and Italian sheep breeds was amplified. The PCR products were purified and sequenced. From the amplified fragment of 721-bp, a region of 423 bp after excluding a central region rich in tandem repeats was analyzed.

Within all tested breeds, the haplotype diversity and average number of pairwise differences were 0.97571 and 7.01484, respectively. The genetic distances (D) and the average number of pairwise differences (Dxy) between breeds were estimated. The lowest distance was observed between Laticauda and Italian Muflon followed by distance between Sarda and Italian Muflon while the highest distance was observed between Barki and Sarda followed by distance between Barki and Laticauda.

Phylogenetic analysis showed the presence of three haplogroups – HapA, HapB and HapC – in the examined samples with the absence of other two haplogroups HapD and HapE. All Italian samples cluster with B haplogroup and also in the Egyptian breeds the most dominant haplogroup was B (62 out of 67 analyzed samples). In Egyptian Barki breed one individual clusters with A haplogroup and another individual with C haplogroup. In Ossimi breed two individuals cluster with C haplogroup and in Rahmani there is one sample belonging to A haplogroup.

The matrix of pairwise differences among breeds was used to perform a Principal Component Analysis (PCA). This analysis showed that the Italian breeds are clearly separated from the Egyptian breeds; moreover the Egyptian Barki breed is separated from Ossimi and Rahmani.

1. Introduction

The earliest archaeozoological evidence for domestic sheep comes from a restricted area of southwestern Asia: modern Iran, Turkey and Cyprus [38]. A pioneering genetic study examining the karyotypes of the various species of extant wild sheep [3,5,26] showed that Italian domestic sheep was derived

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from the Asiatic mouflon (*Ovis orientalis*) of Anatolia and southwest Iran. On the other hand, Egyptian sheep was derived from North Africa in the coastal Mediterranean zone, Northern Syria and Southern Turkey [9].

Mitochondrial sequencing has been used to explain the origins of many modern domestic livestock species. Sheep data are beginning to match the pattern observed in other domestic species. Previous studies in goat [8,21,31], cattle [2,20], and pig [17] have revealed additional maternal clades. The existence of multiple mtDNA lineages and their mixing within breeds [4,22,24,27,28] could be due to multiple domestication events and subsequent human selection or to introgression between domestic and wild species.

The lineages identified in sheep were two [14,15,40], three [12,27] and then five [22]. The main haplotypes A and B are both found in Asia, while B dominates in Europe. Haplotype C has been found in Portugal, Turkey, the Caucasus and China [36]. Haplotype D, present in Rumanian Karachai and Caucasian animals, is possibly related to the A haplotype. Haplotype E has been discovered in the Middle East; Syria, Israel and Turkey.

It is likely that a high number of breeds are being, and will be lost in the near future, before their characteristics can be studied and their potential evaluated. This is particularly worrying in the present scenario because of uncertainties due to rapid climate change, increasing and differentiating market demand and human demographic expansion [7]. In these conditions it is more strategically important than ever to preserve as much the farm animal diversity as possible, to ensure a prompt and proper response to the needs of future generations. In this study, we used mtDNA to analyze genetic characterization and biodiversity between Egyptian and Italian sheep breeds.

2. Materials and methods

2.1. Animals

Blood samples were collected from different sheep breeds reared in Egypt and Italy. In Egypt, three main sheep breeds were studied; *Barki* (20 animals), *Ossimi* (22 animals) and *Rahmani* (25 animals) whereas in Italy this study analyzed samples of *Sarda* (22 animals) and *Laticauda* (19 animals) breeds in addition to samples of the *Italian Muflon* (*Ovis musimon*) (8 animals).

The selected animals were collected from different local farms and only 2–3 individuals were collected from one herd to minimize the likelihood of any close genetic relationships.

2.2. Genomic DNA extraction

Blood was collected into EDTA tubes from unrelated animals of each sheep breed. DNA was extracted from the blood samples according to established protocol [25] with minor modifications. Briefly, blood samples were mixed with cold 2× sucrose–Triton and centrifuged at 5000 rpm for 15 min at 4 °C. The nuclear pellet was suspended in lysis buffer, sodium dodecyl sulfate and proteinase K and incubated overnight in a shaking water bath at 37 °C. Nucleic acids were extracted with saturated NaCl solution. The DNA was picked up and washed in 70% ethanol. The DNA was dissolved in 1× TE buffer. DNA concentration was determined, using Nano Drop1000 Thermo Scientific spectrophotometer, and then diluted to the working concentration of 50 ng/μl, which is suitable for polymerase chain reaction.

2.2.1. Polymerase chain reaction (PCR)

Primers for the amplification of the D-loop region were designed using the software PolyPrimers [37], starting from the complete mtDNA sequence of *Ovis aries* in Genebank (NC_001941.1).

Primer OA_15346F: GGAGAACAACCAACCTCCCTA
Primer OA_157R: TGATTCGAAGGGCGTTACTC

A PCR cocktail consists of 4 μl of each oligonucleotide primer (10 pM/μl), 5 μl of dNTPs (2 mM), 10 μl of 5× PCR buffer, 1 μl of Taq polymerase (5 units/μl) and 25 μl of sterile water. This PCR cocktail was added to 50 ng of genomic DNA. The reaction mixture was preheated at 95 °C for 5 min followed by 35 cycles; 30 s at 95 °C, 30 s at 62 °C and 2 min at 72 °C; followed by final extension at 72 °C for 2 min.

2.3. Sequencing analysis of mtDNA

The forward primer (OA_15346F, GGAGAACAACCAAC CTCCCTA) and an internal primer (OA_16032F, ATGCGTA TCCTGTCCATTAG) were used for sequencing. The sequencing protocol was developed using the sequencer Ceq8800, after purification of the fragments with ExoSap-IT (USB Corporation) to remove residual primers and dNTPs. Sequencing was performed in Macrogen Incorporation (Seoul, Korea).

2.4. Data analysis

* D-loop sequences were aligned using the BioEdit software [13] in order to identify and trace individual haplotype mutations.
* Haplotype structure, sequence variation, average number of nucleotide differences (D) and average number of nucleotide substitutions (Dxy) per site between breeds were calculated using the DnaSP 5.00 software [19].
* Neighbor-joining (NJ) tree for all samples was constructed using the Mega version 5.0 software [35].
* Principal Component Analysis (PCA) was performed on the covariance matrix of pairwise differences [34].

3. Results and discussion

Threats to biodiversity are increasing, whether measured in terms of extinction rate, destruction of ecosystems and habitat or loss of genetic diversity within the species utilized in agriculture. The formulation of the modern breed concept during mid-1800s has caused remarkable changes in the livestock sector: large-scale production expanded [30] and its application to breeding and husbandry practices led to the formation of well-defined breeds, exposed to intense anthropogenic selection. As a consequence, farmers progressively substituted the less productive, locally adapted, native breeds with highly productive cosmopolitan breeds and progressively abandoned marginal areas [33]. Therefore a significant number of cattle, sheep,

and goat breeds already disappeared and many are presently endangered. According to FAO [6], 20% of the breeds world-wide are classified as being critically endangered, critical-maintained, endangered, or endangered-maintained. Currently, more than 1300 different breeds of sheep are known for more than 1.1 billion animals. However, 181 breeds are now extinct, more than 12% of identified breeds and many other breeds are threatened. In these conditions it is more strategically important than ever to preserve as much the farm animal diversity as possible, to ensure a prompt and proper response to the needs of future generations.

Recent surveys have tested collections of animals from Southern and Northern Europe or Europe and the Middle East [18] 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 with their Northern European counterparts. A SNP panel to analyze sheep nuclear genome, providing the indication that breeds cluster into large groups based on geographic origin, and that of SNPs can successfully identify population substructure within individual breeds [16].

The studies on the structure and function of mtDNA become highlights in the research area of molecular evolution, classification, population genetic analysis, relative identification, forensic judgment, aging, disease diagnosis, apoptosis and quantitative traits loci (QTL) [1,10,23,32,39]. In this study, we used mtDNA to analyze genetic characterization and biodiversity between Egyptian and Italian sheep breeds.

A 721-bp fragment from 15,541 to 16,261 bp (NC_001941.1) of the mtDNA control region was amplified using specific primers and the polymerase chain reaction was performed. The PCR products were purified and sequenced. From the amplified fragment of 721-bp, a region of 423 bp after excluding a central region rich in tandem repeats was analyzed.

The analyzed samples in this study were 116 samples belonging to 6 sheep breeds in Egypt and Italy: Italian Laticauda breed (ITLAT, 19 sequences), Italian Sarda breed (ITSAR, 22 sequences), Italian Muflon (ITMUS, 8 sequences), Egyptian Barki breed (EGBAR, 20 sequences), Egyptian Ossimi breed (EGOSS, 22 sequences) and Egyptian Rahmani breed (EGRAH, 25 sequences). The alignment of all 116 analyzed samples was done using the BioEdit software. The DnaSP 5.00 software was used to identify the sequence variation and polymorphic sites in the aligned sequences.

The result showed the presence of 77 polymorphic sites leading to the formation of 59 haplotypes (Fig. 1). The most common haplotype appears in 10 samples. All these 10 samples belong to Italian breeds, 8 of them from Sarda, one from Laticauda and the last one from Italian Muflon. So this haplotype is considered a specific haplotype for Italian breeds especially Sarda. Another 2 haplotypes are specific also for Italian breeds and present in 5 and 4 samples, respectively.

There is a haplotype which is considered a specific haplotype for Egyptian breeds and present in 9 samples; 4 from each Rahmani and Ossimi and one from Barki breeds. Another 2 haplotypes are specific for Egyptian breeds which are present in 6 and 5 samples, respectively. In addition there are also 4 specific haplotypes for Egyptian breeds, each of which appears in 4 samples. The remaining haplotypes are present in samples with numbers ranging from 3 to one sample.

These specific haplotypes for Italian as well as for Egyptian sheep breeds is an indication for the clear separation between sheep breeds in both countries where there is no haplotype being shared between Egyptian and Italian breeds. On the other hand, the sharing of different Italian breeds in specific Italian haplotypes and the same thing for Egyptian breeds showed the presence of low genetic differentiation and high gene flow between the breeds in both countries.

The statistical analysis of genetic diversity within the 6 tested breeds (Table 1) using the DnaSP 5.00 software showed that the highest number of haplotypes (17 haplotypes) was found in Italian Laticauda breed where its 19 animals possessed 23 polymorphic sites. Whereas, the Egyptian breeds have a number of haplotypes ranging from 15 in Barki breed (46 polymorphic sites in its 20 animals) to 11 in Ossimi breeds (33 polymorphic sites in its 22 animals) and 8 in Rahmani breed (31 polymorphic sites in its 25 animals). The lowest haplotype number was in Italian Muflon where its 8 animals possessed 3 polymorphic sites forming 3 haplotypes whereas the number of haplotypes was 14 in Italian Sarda (26 polymorphic sites in its 22 animals). The total number of polymorphic sites in all 116 tested animals was 77 resulting in 59 haplotypes.

The haplotype diversity in Italian breeds ranged from 0.98830 in Laticauda (with average number of pairwise differences K: 3.85965) to 0.87446 in Sarda (with *K*: 2.92208). Due to the small tested number of animals from Italian Muflon, the haplotype diversity within its animals was 0.67857 with K = 1.67857. On the other hand, the haplotype diversity and average number of pairwise differences in Egyptian breeds were 0.96316 and 7.64211 in Barki, 0.90909 and 6.09524 in Ossimi and 0.88333 and 4.13333 in Rahmani breed, respectively. Within all tested breeds, the haplotype diversity and average number of pairwise differences were 0.97571 and 7.01484, respectively.

The result showed the Italian breeds possess low nucleotide diversity than Egyptian breeds where it ranged from 0.00681 in Sarda to 0.00630 in Laticauda and 0.00403 in Muflon and from 0.01815 in Barki to 0.01448 in Ossimi and 0.00982 in Rahmani Egyptian breeds with total nucleotide diversity of 0.0150 for 6 tested breeds (Table 1).

The genetic distances (D) and the average number of pairwise differences (Dxy) between breeds were estimated. The lowest distance was observed between Italian Laticauda and Muflon (D: 2.612 and Dxy: 0.00626) followed by distance between Italian Sarda and Muflon (D: 2.682 and Dxy: 0.00636) then distance between Italian Laticauda and Sarda (D: 2.732 and Dxy: 0.00657) while the highest distance was observed between Egyptian Barki and Italian Sarda (D: 7.923 and Dxy: 0.01886) followed by distance between Egyptian Barki and Italian Laticauda (D: 7.826 and Dxy: 0.01877) then distance between Egyptian Barki and Italian Muflon (D: 7.750 and Dxy: 0.01841 (Table 2).

Neighbor-joining (Phylogeny) tree was constructed using the Mega 5.0 software (Fig. 2). The sequences of the 116 analyzed samples were aligned with reference sequences of different haplogroups to define the haplogroups to which the analyzed samples belonged. Reference sequences used for defining haplogroups were: DQ852286 (A1) and DQ852287 (A2) for A haplogroup; DQ852285 (B1), DQ852282 (B2) and AF039579 (B3) for B haplogroup; DQ097460 (C1), DQ097462 (C2) and DQ852283 (C3) for C haplogroup;

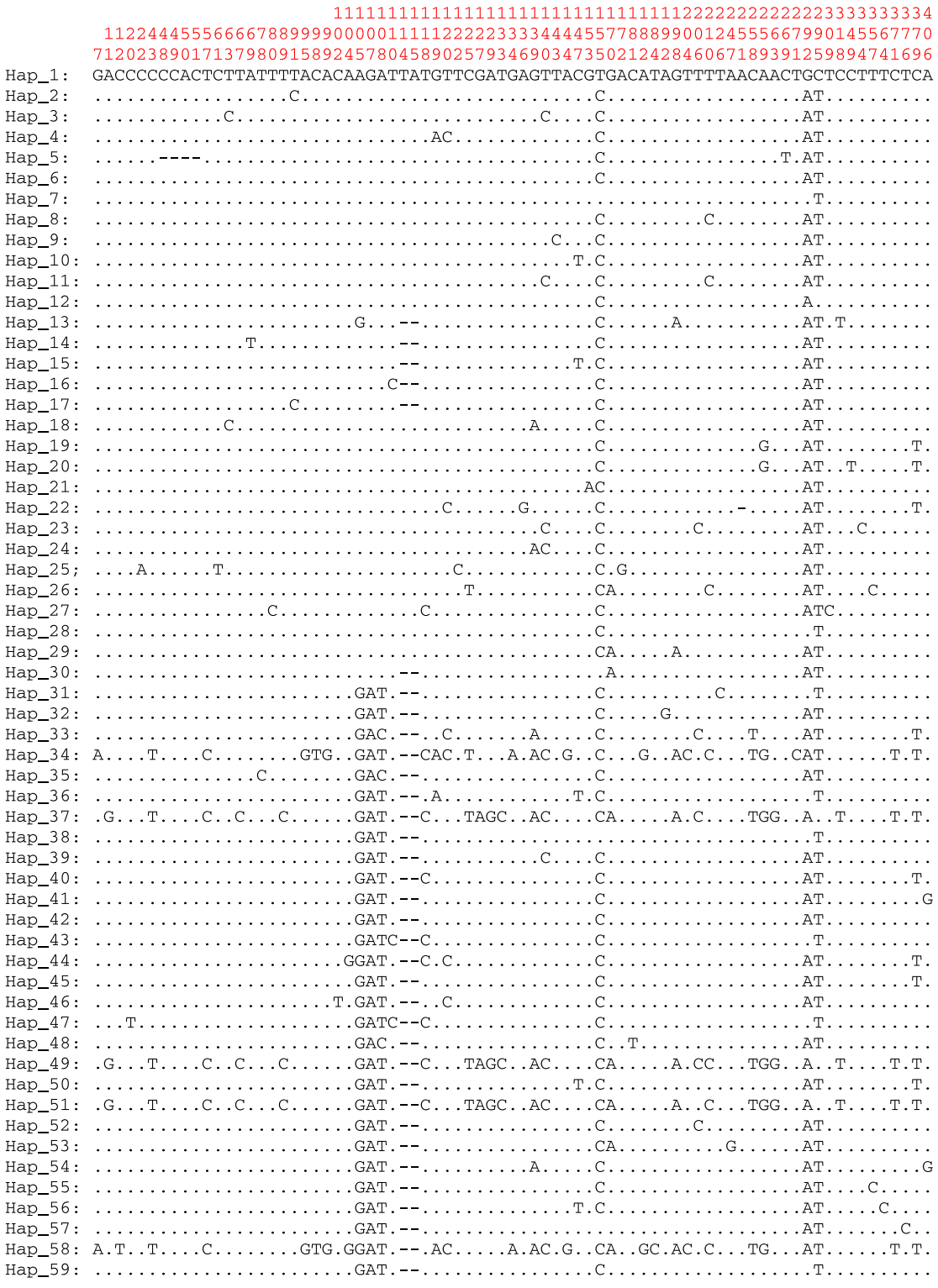

Figure 1 Sequences of 59 haplotype sequences. The variable site No. in red.

DQ852288 (D1) and DQ852289 (D2) for D haplogroup and DQ852280 (E1) and DQ852281 (E2) for E haplogroup.

The phylogeny result showed the presence of three haplogroups (HapA, HapB and HapC) in the 116 examined samples. The other two haplogroups described in literatures (HapD and HapE) were not found. The result showed that 111 out of 116 tested animals cluster with haplogroup B (95.69%) whereas three tested animals cluster with haplogroup C (2.59%) and two animals cluster with haplogroup A (1.72%) (Table 3).

Table 1 The genetic diversity data.

Population	No. of sequences (N)	No. of polymorphic sites (S)	No. of haplotypes (H)	Haplotype diversity (HD)	Average number of pairwise differences (K)	Nucleotide diversity (π)
ITLAT	19	23	17	0.98830	3.85965	0.00630
ITSAR	22	26	14	0.87446	2.92208	0.00681
ITMUS	8	3	3	0.67857	1.67857	0.00403
EGBAR	20	46	15	0.96316	7.64211	0.01815
EGOSS	22	33	11	0.90909	6.09524	0.01448
EGRAH	25	31	8	0.88333	4.13333	0.00982
Total	116	77	59	0.97571	7.01484	0.0150

Table 2 Average pairwise differences between populations.

	ITLAT	ITSAR	ITMUS	EGBAR	EGOSS	EGRAH
ITLAT	–	0.00657	0.00626	0.01877	0.01785	0.01528
ITSAR	2.732	–	0.00636	0.01886	0.01790	0.01558
ITMUS	2.612	2.682	–	0.01841	0.01749	0.01538
EGBAR	7.826	7.923	7.750	–	0.01608	0.01435
EGOSS	7.443	7.517	7.364	6.770	–	0.01276
EGRAH	6.373	6.542	6.475	6.040	5.371	–

Average number of nucleotide difference between populations D (below).
Average number of nucleotide substitution per site between populations, Dxy (above).

All 49 examined Italian animals of the three breeds cluster with haplogroup B without any animal cluster with any other recorded haplogroups. In Egyptian breeds the most dominant haplogroup was B (62 out of 67 analyzed samples), however, the remaining 5 Egyptian animals cluster with A or C haplogroups. In Egyptian Barki breed, one individual clusters with haplogroup A and another individual clusters with haplogroup C. In Ossimi breed, two individuals cluster with haplogroup C whereas in Rahmani breed there is one sample belonging to haplogroup A.

The covariance matrix of pairwise differences among breeds was used to perform a PCA analysis for investigating the spatial patterns of genetic variation (Fig. 3).

The PCA analysis showed a clear separation between Italian and Egyptian breeds. The Egyptian Barki breed is separated from other two Egyptian breeds Ossimi and Rahmani whereas the three Italian breeds are closed together without separation between them as shown in Fig. 3.

MtDNA variations in European, Caucasian, and Asian areas were detected by sequencing of a highly variable segment of mtDNA in 406 unrelated animals from 48 breeds [36]. Three haplotype groups; A, B, and C were found and group B has been observed primarily in European domestic sheep and the European mouflon carries this haplotype group. This result agreed with our finding in this work where all Italian tested breeds belonged to haplogroup B including Italian Muflon which is derived from European Muflon.

Archaeological evidences showed that sheep domestication began in the central part of the Fertile Crescent and from this region; the migration was happened [29]. The main haplotypes A and B are both found in Asia, while B dominates in Europe [22]. A high frequency of A in New Zealand resulted from early imports of Indian animals into Australia [14]. Haplotype C is less frequent, but has been found in Portugal, Turkey, the Caucasus and China [36]. Haplotype D, present in Rumanian Karachai and Caucasian animals, is possibly related to the A haplotype. Haplotype E, which is an intermediate between A and C, is also rare and has only been found in two Turkish animals [11]. These reports support our findings about the belonging of five tested animals to haplogroups C and A. It is a logical result because these two haplogroups are dominant in Asia [11] and in the steppe and semi-desert regions where the fat-tailed sheep are distributed [36].

Same results for the highest frequency of haplogroup B followed by haplogroups A and C with rare haplogroups D and E were reported in nine breeds of sheep from modern-day Turkey and Israel [22]. Only 6 individuals out of examined 197 sheep were clustered separately from the three predominant ovine mtDNA lineages (A, B, and C) in two additional mtDNA lineages denoted D and E.

4. Conclusion

The specific haplotypes for Italian as well as for Egyptian sheep breeds is an indication for the clear separation between sheep breeds in both countries where there is no haplotype being shared between Egyptian and Italian breeds. On the other hand, the sharing of different Italian breeds in specific Italian haplotypes and the same thing for Egyptian breeds showed the presence of low genetic differentiation and high gene flow within the breeds in both countries.

The lower genetic distance between Italian breeds than Egyptian breeds is an indication for the increasing of inbreeding resulting from modern methods of selection exclusively oriented high productivity in Italian breeds than in Egyptian breeds. The recorded high genetic distance between Egyptian Barki breed and three Italian breeds is reasonable and

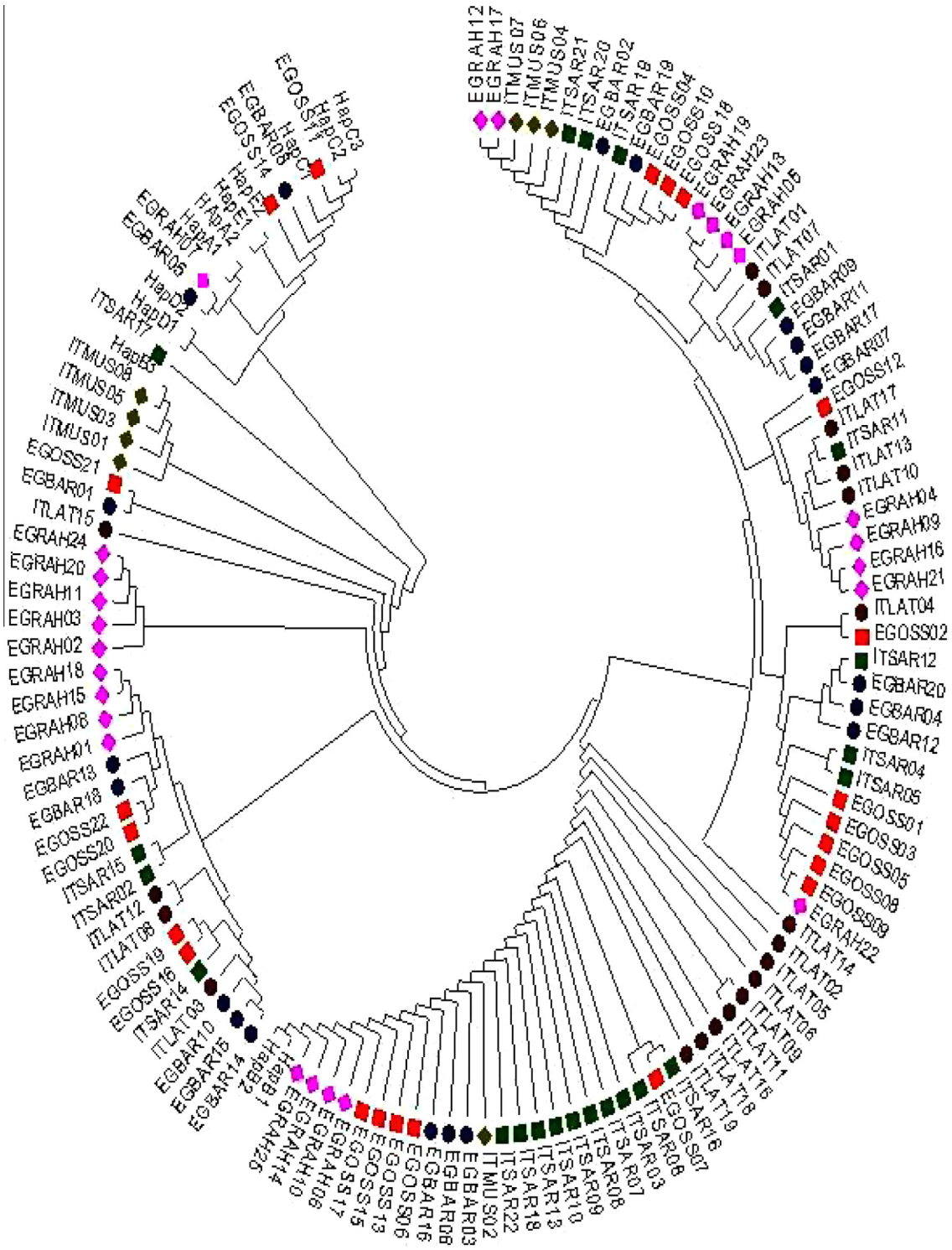

Figure 2 Neighbor-joining (NJ) tree of the tested animals as circle.

expectable because Barki breed is located in the semi-desert region and exposed to fluctuating environmental challenges.

The phylogenetic analysis showed, as expected, the B haplogroup is the most dominant haplogroup in Italian and Egyptian breeds. Moreover, the presence of the C haplotype in the Egyptian sheep breeds is in agreement with literatures which reported the presence of haplogroup C in the steppe and semi-desert region where the fat-tailed sheep are distributed.

The PCA analysis showed a clear separation between Italian and Egyptian sheep breeds. Moreover the Egyptian Barki breed is separated from Ossimi and Rahmani where Barki is well-adapted to live under desert conditions. This result is expectable because Rahmani and Ossimi breeds are located

Table 3 The different haplogroups to which the tested animals are belonging.

Breed	No. of samples	Haplogroup A		Haplogroup B		Haplogroup C	
		No.	%	No.	%	No.	%
Italian Laticauda	19	0	0	19	100	0	0
Italian Sarda	22	0	0	22	100	0	0
Italian Muflon	8	0	0	8	100	0	0
Egyptian Barki	20	1	5	18	90	1	5
Egyptian Ossimi	22	0	0	20	90.91	2	9.09
Egyptian Rahmani	25	1	4	24	96	0	0
Total	116	2	1.72	111	95.69	3	2.59

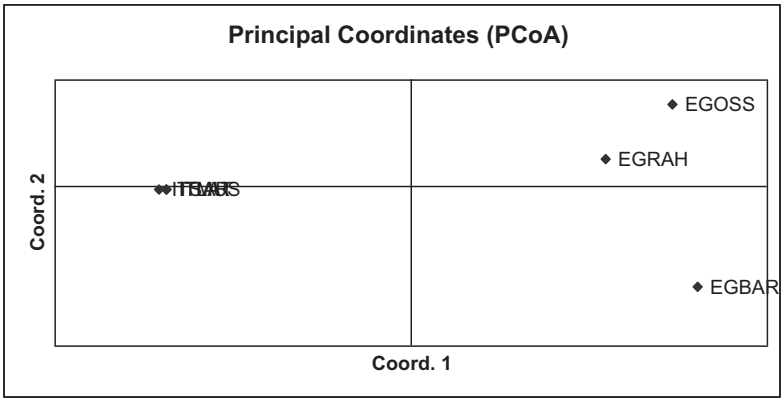

Figure 3 Principal Component Analysis (PCA).

mainly in Middle and South of Nile Delta, near to each other and away from the Barki breed which is mainly located in Mediterranean coastal strip; west of Alexandria and close to the eastern provinces of Libya.

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