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RESEARCH ARTICLE

# Mitochondrial DNA genetic diversity in six Italian donkey breeds (*Equus asinus*)

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**ABSTRACT**

Donkeys have played an important role in agricultural land practices and in human historical periods of recent past and, still today, are used as a working power in several world areas. The objective of this study was to identify genetic variability in six Italian donkey breeds using mtDNA D-loop. Fifteen haplotypes, grouped in three haplogroups, were identified. The genetic indices were informative and showed a high population genetic variability. The results of AMOVA analyses based on geographic structuring of Italian populations highlighted that the majority of the observed variance is due to differences among samples within breeds. Comparison among Italian haplotypes and mtDNA D-loop sequences belonging to European domestic and Ethiopian donkeys and wild asses, clearly define two clades referred to Nubian lineage. The results can be useful to complement safeguard planes for donkey breeds that are considered to extinction endangered.

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## Introduction

The domestication of animals largely have contributed to the improvement of the living conditions of the primitive communities and had fundamental effects on human societies. Particularly, the history and origins of the donkey are of strong interest given that, like the horse, the donkey played an important role in agricultural culture and it is still used as a working power in several world rural areas.

The family of the *Equidae*, including the horses, donkeys or asses, and zebras, was extensively studied during the past two centuries. According to currently accepted International Union for the Conservation of Nature and Natural Resources IUCN taxonomy (Moehlman 2002), modern equids are represented by the eight extant species of the *Equus genus*: domestic horse (*Equus caballus*), Przewalski's horse (*Equus przewalskii*), kiang (*Equus kiang*), Asiatic wild ass (*Equus hemionus*), African wild ass (*Equus africanus*), mountain zebra (*Equus zebra*), plains zebra (*Equus quagga*) and Grevy's zebra (*Equus grevyi*). The domestic donkey (*Equus africanus asinus*) therefore is considered a sub-specie of African wild ass and many authors suggested that its domestication occurred in the arid tropical and subtropical territories of North East Africa (Blench 2000; Beja-Pereira et al. 2004). Despite that, other studies keep open the debate on the domestication and evolutionary history of the donkeys. In fact, Rossel et al. (2008) analyzing archeological remains excavated in Egypt, strongly supported the African origin of the domestic donkeys.; Marshall (2007) proposed on the other hand that early northeast African pastoralists domesticated the donkey under conditions of increased aridity 7000–6500 BP in the Sahara desert.

Beja-Pereira et al. (2004) in a worldwide genetic study on modern donkeys using mitochondrial DNA identified two distinct wild African ass sub-species: the Nubian wild ass (*Equus africanus africanus*) and the Somali wild ass (*Equus africanus somalicus*). According to this study, the authors found two main clade: the first (clade 1) included the domestic donkeys related to the Nubian wild ass: the second one (clade 2) involved donkeys descending from the Somali wild ass. The authors described high levels of genetic diversities in both the lineages of Northeast Africa territories and suggested that this area is one of the primary centres of donkey domestication. However, a study by Kimura et al. (2011) reported that the mtDNA sequences obtained from the extant Somali wild ass were classified together with the previously identified Somali wild ass specimens, but failed to show any sequence similarity with domestic donkeys of both clades. The study from Kimura et al. (2011) suggested the existence in Northeast Africa territories of another ancestor of the domestic donkeys of clade 2, belonging to an additional yet unrecognized extinct wild population. In a recent study by Kefena et al. (2014) based on genetic and on linguistic and zooarchaeological evidences (Marshall 2007), confirmed that Ethiopia could be one of the major hotspots of donkey diversity and domestication.

Despite its uniquely maternal origin, nowadays, mtDNA nucleotide sequences are a recognized tool for resolving phylogenetic relationships at different evolutionary level,

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because of its special properties such as: more copies than nuclear DNA, well-known gene structure, lack of introns, high-mutation rate, absence of recombination events (Xu et al. 1996). As such previous studies on donkey mitochondrial DNA D-loop (control region) sequences well depicted the genetic relationships and origin of worldwide modern donkey breeds (Oakenfull et al. 2000; Ivankovic et al. 2002; Aranguren-Mendez et al. 2004; Beja-Pereira et al. 2004; Lopez et al. 2005; Chen et al. 2006; Lei et al. 2007; Han et al. 2014; Kefena et al. 2014; Pérez-Pardal et al. 2014; Cinar Kul et al. 2016).

In Italy donkeys have been bred for centuries for various purposes. Historical and archeological evidence suggest that this livestock specie was present and already exploited by humans from around 2000 BC (Kugler et al. 2007–2008). However, during the last century donkey populations size, have suffered a decrease caused by the increased mechanization of agriculture and the depopulation of rural districts that reduced the need for donkeys and mules as draft animals. According to FAO (http://dad.fao.org/), some Italian autochthonous donkey breeds are extinct. In particular, today in Italy eight donkey breeds classified as critically endangered (Asinara, Pantesco, Grigio Siciliano and Romagnolo), or endangered (Amiata, Sardo, Martina Franca and Ragusano) are still bred. However, genetic studies on the biodiversity Italian donkey breeds are limited, and mainly focused on the donkey genetic variability performed using protein markers and microsatellites (Cristofalo et al. 1994; Cosseddu et al. 2001; Ciampolini et al. 2007; Guastella et al. 2007; Matassino et al. 2014; Bordonaro et al. 2012; Colli et al. 2013). Recently Bertolini et al. (2015) used a whole genome sequencing approach to study the evolutionary aspects of Italian donkey populations and to highlight divergence with horse genome.

In the present study, we analyzed the genetic variability at mitochondrial DNA level of six Italian autochthonous breeds. In particular, we considered two breeds living in Sardinia, Asinara and Sardo donkey, two breeds living in North Central Italy, Amiata donkey bred on the slopes of the Amiata mountain (Central Italy) and Romagnolo donkey bred in Emilia Romagna region (North Italy), and two breeds bred in South Italy, Martina Franca donkey and Ragusano donkey autochthonous of Puglia and Sicily respectively.

The aims of this study were: (i) to analyze genetic variability between six Italian donkey breeds, by means of mitochondrial DNA (mtDNA) D-loop sequences, (ii) to evaluate the presence of genetic breed sub-structuring and (iii) to compare mtDNA of Italian breeds with Modern European donkey breeds, African wild asses and Ethiopian donkey to understand their genetic relationships.

## Materials and methods

### Sample collection, PCR conditions, amplification, and sequencing protocols

A total of 104 donkeys blood samples belonging to six Italian breeds (Amiata donkey AMD = 8; Asinara donkey ASD = 28; Ragusano donkey RAD = 6; Romagnolo donkey ROD = 8; Martina Franca donkey MFD = 5 and Sardo donkey SAD = 49) were available in an existing historical bio-bank at the Department of Veterinary Medicine of the University of Milan.

The mtDNA was isolated from whole blood samples (collected in 0.5 M EDTA and stored at −20 °C) using the NucleoSpin® Blood kit (Macherey-Nagel) following the manufacturer's instructions.

According to the complete donkey mtDNA sequence GenBank X97337 (Xu et al. 1996) two pairs of primers were designed to amplify a 478 bp fragment of the donkey mtDNA comprised between sites 15,386 to 15,863, using Primer3web version 4.0.0 (http://bioinfo.ut.ee/primer3) (F_DONK: CCCCAAGGACTATCAA for forward primer and R_DONK: GTTCTTTCTTCAGGGCCATT for reverse primer).

PCR were carried out using a MJ Research PTC-200 following conditions previously reported in Cozzi et al. (2004). PCR products were purified using SEPHADEX® G-50 (SIGMA) and sequenced for both strands using Big Dye® Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems™) according to the manufacturer's instructions. The PCR products were analyzed by ABI PRISM® 310 Genetic Analyser and the nucleotide composition was determined using specific software. The raw sequence trace files were checked for the presence of ambiguous bases using software Chromas v. 2.5.1 (http://www.technelysium.com.au/). The donkey mtDNA sequences obtained were deposited in the GenBank (GenBank ID: KX622700-KX622727).

### Data analyses

All the obtained sequences were aligned with the complete donkey mtDNA sequence using ClustalW algorithm implemented in BioEdit (Hall 1999) and MEGA v.7.0.14 software (Kumar et al. 2016).

### Italian donkey population analyses

The maximum composite likelihood estimate of the nucleotide substitution pattern was calculated using MEGA v.7.0.14 software.

Number of polymorphic sites (S), parsimony informative ($S_{PI}$) and singleton site ($S_S$), number of haplotypes (NH), private ($H_P$) and shared haplotypes ($H_S$), haplotype diversity (hd), nucleotide diversity ($\pi$) and average number of nucleotide differences (k) were calculated according to Tajima (1983) and Nei (1987) using DnaSP5 v.5.10.01 software (Librado & Rozas 2009). Pairwise population genetic differentiation ($F_{ST}$) and the average number of pairwise differences within and between populations were calculated using Arlequin v.3.5.2.2 (Excoffier & Lischer 2010).

The MEGA v.7.0.14 software was used to analyze the relationships among the haplotypes identified in Italian donkey populations and those identified in wild asses from GenBank (*Equus asinus somalicus, Equus asinus africanus* and *Equus hemionus luteus*) (GenBank ID are listed in the Supplementary Table S1).

Genetic relationships among breeds were reconstructed using Median-Joining Networks (MJN) by Network v.4.6 software (Bandelt et al. 1999). A Neighbour-joining (NJ) tree (Saitou & Nei 1987) was constructed based on the

Kimura-2-parameter model distances (Kimura 1980) calculated among the haplotypes using MEGA v.7.0.14 software. The bootstrap analysis, running 1000 bootstrap replicates, was applied in order to check the robustness of the resulting dendrogram. In addition, the Arlequin v.3.5.2.2 software was used to perform an Analysis of MOlecular VAriance (AMOVA).

***Italian donkey breeds vs. other donkey populations*** The Italian mtDNA sequences were compared with 172 publically available domestic donkey's mtDNA D-loop sequences deposited in GenBank (http://www.ncbi.nlm.nih.gov/), comprising European domestic donkeys, wild asses and Ethiopian donkeys (GenBank ID are listed in the Supplementary Table S1).

All the 172 D-loop sequences were trimmed (236 bp overlapping positions) in order to allow the alignment and the comparison with Italian donkey breed sequences.

The DnaSP5 v.5.10.01 software was used in order to obtain haplotypes from all final D-loop sequences dataset. Based on the haplotypes a MJN by Network v.4.6 software (Bandelt et al. 1999) was constructed. Finally, the AMOVA analysis was performed using the final D-loop sequences dataset.

## Results and discussion

### *Italian donkey population analyses*

The mtDNA D-loop sequences were obtained for all analyzed samples. Twenty parsimony informative polymorphic sites were identified in the 104 Italian donkey mtDNA D-loop sequences 478 bp. According to the literature (Kumar et al. 2016) the parsimony informative sites are defined as mutations that have a minimum of two nucleotides that are present at least twice in the sampled population, whereas non-informative sites are singleton sites. All the polymorphic site were transition. The maximum composite likelihood estimate of the nucleotide substitution pattern, identified for the 104 sequences, was 30.55% (A), 27.1% (T), 29.1% (C), and 13.2% (G). Positions containing gaps and missing data were eliminated.

At population level, all the breeds (except for ROD) showed one or more singleton sites. As reported in Table 1 the MFD breed counted the highest number of non-informative singleton sites ($S_S$), as well as the lowest number of polymorphic sites (S) was identified in RAD breed.

In addition, the haplotypes identified in the analyzed breeds ranged from 3 (MFD) to 6 (AMD and SAD).

The breed genetic diversity (Table 1) evaluated by the nucleotide diversity value ($\pi$) ranged from 0.005 (RAD) to 0.025 (MFD) with an average value of 0.018. The haplotype diversity value (*hd*) ranged from 0.638 (ASD) and 0.893 (AMD) with an average value of 0.862. In addition, the average number of nucleotide differences (*k*) ranged from 1.2 (MFD) to 9.750 (ROD) with an average value of 8.412.

The MFD breed showed three haplotypes, the highest $\pi$ value (0.02) and the highest number of parsimony sites ($S_S = 11$), but the haplotypes with the lowest nucleotide diversity ($k = 1.2$). On the contrary RAD have the lowest $\pi$ (0.005) and K ($k = 2.2$) value, but haplotypes have a high hd value (0.867). The high *k* value (9.750) for the ROD breed is due to the high difference among the five haplotypes identified in the population.

Despite the low number of samples, a high *hd* value, with two private haplotypes (H6 and H11) for the AMD breed was found (Table 2). The two Sardinian breeds showed the presence of mitochondrial genetic variability, with similar *hd*, *k* and $\pi$ values among them. These results confirm the findings obtained by Cosseddu et al. (2001) who used microsatellites markers to evaluate genetic variability between the two Sardinian breeds.

As reported in Table 1, all the average molecular indices show the presence of genetic variability among the Italian breeds. These results are similar to those observed in Croatian and Balkan donkeys by Ivankovic et al. (2002) and Pérez-Pardal et al. (2014), in Chinese donkeys by Lei et al. (2007) and Han et al. (2014), Ethiopian donkey by Kefena et al. (2014) and in Anatolian and Cypriot donkey populations by Cinar Kul et al. (2016).

Among these, the haplotype H14, identified in four donkeys, showed 18 nucleotide substitutions compared to the sequence X97337, whereas the haplotype H1 was identical to the reference sequence (Table 2).

The fifteen haplotypes found comparing our 104 donkey sequences with the donkey reference (accession number X97337) are shown in Table 2. All the haplotypes were shared by at least two breeds, except for the H6 (AMD), H8 (SAD), H10 (MFD), H11 (AMD) and H15 (SAD) haplotypes that are private ones (Table 2). Table 2 also reports the absolute and the relative haplotype frequencies. The most frequent haplotype found among the breeds was H3 (25%) followed by H2 (19.2%) and then H13 (17.3%), whereas H6 and H11 (1%) were the less frequent ones.

The matrix of pairwise fixation indexes ($F_{ST}$) is shown in Figure 1. The $F_{ST}$ values range from 0.001 (SAD vs. ROD) to 0.822 (RAD vs. MFD). Except for AMD, the larger differences in $F_{ST}$ values were found between the MFD breed and the

**Table 1.** D-loop nucleotide polymorphisms and molecular diversity indices of the six Italian donkey breeds.

| Breed | *n* | S | $S_{PI}$ | $S_S$ | NH | $P_H$ | $S_H$ | $\pi \pm$ s.d. | hd ± s.d. | *k* |
|---|---|---|---|---|---|---|---|---|---|---|
| AMD | 8 | 13 | 12 | 1 | 6 | 2 | 4 | 0.013 ± 0.0026 | 0.893 ± 0.012 | 6.143 |
| ASD | 28 | 18 | 17 | 1 | 4 | 0 | 4 | 0.015 ± 0.0025 | 0.638 ± 0.069 | 7.024 |
| MFD | 5 | 13 | 2 | 11 | 3 | 1 | 2 | 0.025 ± 0.0010 | 0.700 ± 0.048 | 1.2 |
| RAD | 6 | 5 | 4 | 1 | 4 | 0 | 4 | 0.005 ± 0.0009 | 0.867 ± 0.017 | 2.2 |
| ROD | 8 | 18 | 18 | 0 | 5 | 0 | 5 | 0.020 ± 0.0032 | 0.786 ± 0.151 | 9.75 |
| SAD | 49 | 20 | 19 | 1 | 6 | 2 | 4 | 0.019 ± 0.0010 | 0.711 ± 0.048 | 8.961 |

Sample size (*n*), total polymorphic sites (S), parsimony informative ($S_{PI}$) and singleton site ($S_S$), number of haplotypes (NH), private haplotype ($P_H$), shared haplotype ($S_H$), nucleotide diversity ($\pi$), haplotype diversity (*hd*) with their standard deviations (s.d.) and average number of nucleotide differences (*k*) within and across the six populations.

**Table 2.** Polymorphic sites of the donkey mtDNA D-loop sequence nucleotide position 15,385 to 15,862, according to the reference sequence X97337 and absolute and relative haplotype frequencies.

| | Position of nucleotide substitutions | | | | | | | | | | | | | | | | | Number of samples/breed | | | | | | % Of haplotype frequency |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Haplotypes | 15,484 | 15,490 | 15,503 | 15,569 | 15,580 | 15,590 | 15,597 | 15,598 | 15,599 | 15,621 | 15,644 | 15,652 | 15,662 | 15,698 | 15,770 | 15,801 | 15,806 | 15,821 | 15,822 | 15,829 | AMD | ASD | MFD | RAD | ROD | SAD | |
| **X97337** | G | C | T | A | A | A | T | C | A | A | G | C | A | C | T | C | C | C | G | G | | | | | | | |
| H1 | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | 1 | | | | 1 | | 1.9 |
| H2 | . | . | . | . | . | . | . | . | G | . | . | . | . | . | . | . | . | . | . | . | | 15 | | 1 | 1 | 3 | 19.2 |
| H3 | . | . | . | . | . | . | . | . | G | . | . | . | . | . | . | . | . | . | A | . | | 3 | | | | 23 | 25.0 |
| H4 | . | . | . | . | . | . | C | . | G | . | . | . | . | . | . | . | . | . | . | . | | 2 | | 2 | | | 4.8 |
| H5 | . | . | . | . | . | . | . | T | G | . | . | . | . | . | . | . | . | . | . | . | | | | 1 | 1 | | 1.9 |
| H6 | . | . | . | G | . | . | . | . | . | . | . | . | . | . | . | T | . | . | . | . | 1 | | | | | | 1.0 |
| H7 | . | . | . | . | . | . | . | T | G | . | A | . | . | . | . | T | . | . | . | . | | | | 1 | 2 | | 2.9 |
| H8 | . | . | . | G | . | . | . | T | G | . | A | . | . | . | . | T | . | . | . | . | | | | | | 3 | 2.9 |
| H9 | . | . | C | G | G | G | . | T | G | G | . | G | . | T | . | T | . | . | A | A | 3 | | 1 | | | | 3.8 |
| H10 | . | . | C | G | G | G | . | T | G | G | A | T | G | T | . | T | . | T | A | A | | | 3 | | | | 2.9 |
| H11 | . | . | C | G | G | G | . | T | G | G | A | . | G | T | . | T | . | T | A | A | 1 | | | | | | 1.0 |
| H12 | . | . | C | G | G | G | . | T | G | G | A | T | G | T | . | T | . | T | A | . | 1 | | 1 | | | | 1.9 |
| H13 | A | T | C | G | . | G | . | T | G | G | A | T | G | T | C | T | T | T | A | A | | 8 | | | | 10 | 17.3 |
| H14 | A | T | C | G | G | G | . | T | G | G | A | T | G | T | C | T | T | T | A | A | | | | | 4 | 1 | 4.8 |
| H15 | A | T | C | G | G | G | . | T | G | . | A | T | G | T | C | T | T | T | A | A | | | | | | 9 | 8.7 |

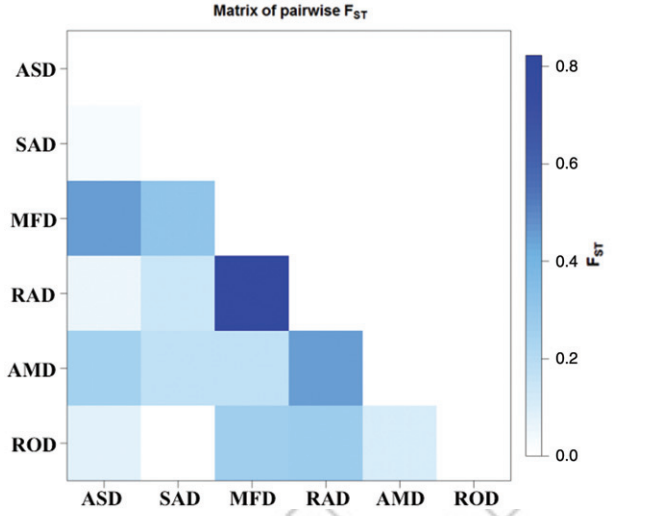


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**Figure 1.** Matrix of pairwise fixation indexes ($F_{ST}$) calculated for the six Italian donkey breeds.

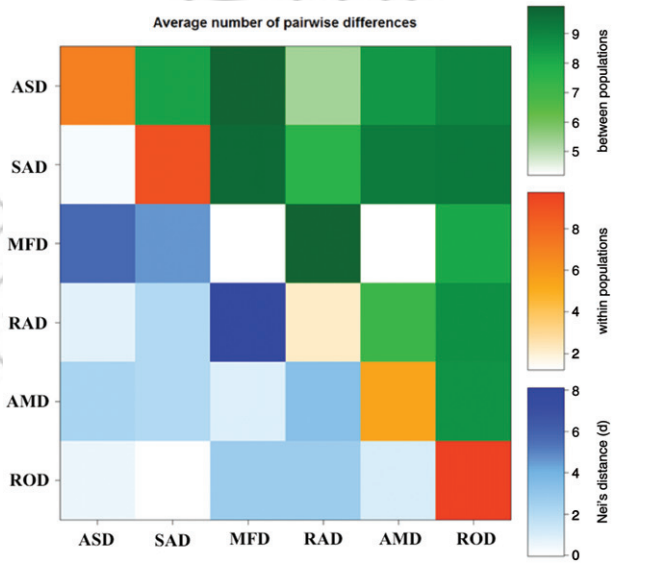


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**Figure 2.** Nei's average number of pairwise differences ($\pi$) within and between the six Italian donkey breeds. On diagonal, the $\pi$ within sampled populations in orange; above the diagonal, the $\pi_{XY}$ between pairs of populations in green; below the diagonal, the Nei DA distance or the net number of nucleotide differences between the populations in blue.

Q6

other populations, ranging from 0.257 (MFD vs. ROD) to 0.822 (MFD vs. RAD).

The Nei's average number of pairwise differences within and between the populations calculated for the six Italian donkey breeds is shown in Figure 2.

MFD appears more separated than the other breeds showing the minor number of differences within samples in the population (the white box in orange diagonal in Figure 2). This may be due to conservation programs undergoing for a long time in this breed aiming in maintaining the breed peculiarities without crossing with non MFD animals (Rizzi et al. 2011).

The two Sardinian breeds (ASD and SAD) showed a high within population nucleotide variability (Figure 2). The low $F_{ST}$ value (Figure 1) found between the two Sardinian populations (ASD vs. SAD = 0.031) highlights their closely genetic

relationship at mtDNA level, despite the evident differences in morphological characteristics (i.e. coat colour) of these breeds. In fact, one of the hypotheses on the origin of ASD is based on the possibility that the albino mutation occurred in a 'grey' haplotype, present in the Sardinia donkey. It is highly probable that the isolation of the ASD donkey population and its high-inbreeding level have raised the frequency of the 'albino pattern' in this population (Utzeri et al. 2016). The closely relationship between the two populations was found by Colli et al. (2013) who reported a similar $F_{ST}$ value (0.041) using a panel of 16 microsatellites. The same result holds using the Nei's distance as shown in Figure 2.

The $F_{ST}$ values (Figure 1) show the closely relationship between SAD and ROD (SAD vs. ROD = 0.001) and SAD and RAD (SAD vs. RAD = 0.152), but they well differentiated SAD from MFD (SAD vs. MFD = 0.319) as well as the Nei's distances (Figure 2) do. In fact, in the last century, SAD probably received some genetic contributions from other populations including MFD, RAD and ROD, in order to increase its structural dimension even if, in the last decade the Dipartimento di Ricerca per l'Incremento Ippico (AGRIS-Sardegna) has implemented the programs for the SAD breed conservation (Cherchi 2005).

The ROD breed is close to RAD (ROD vs. RAD = 0.279), MFD (ROD vs. MFD = 0.257) and AMD (ROD vs. AMD = 0.109), confirming the historical information indicating that these three breeds contributed to the genetic makeup of the ROD gene pool (Beretti et al. 2005; Salza 2006; Kugler et al. 2007–2008) (Figure 1). In fact, ROD showed the highest genetic variability within population (in diagonal the darkest orange box in Figure 2).

The AMD breed, characterized by morphological ancestral features referable to wild asses (stripped limbs, the dark dorsal strip and mouse coat colour), appeared closely related to all the populations (Figure 1) except for RAD (AMD vs. RAD = 0.458) and showed a medium nucleotide variability value within breed (in diagonal the orange box in Figure 2). Unlike what reported by Colli et al. (2013), our data highlighted a high haplotypes variability of the AMD mtDNA, showing four shared haplotypes and two private haplotypes.

Also the NJ dendrogram in Figure 3, constructed using our haplotypes and mtDNA sequences from *Equus asinus africanus* (Nubian wild ass), *Equus asinus somalicus* (Somali wild ass), and *Equus hemionus luteus* sequence (used as outgroup), did not confirm the similarity between the mtDNA sequences of AMD donkeys and *Equus asinus somalicus*, as reported by Colli et al. (2013). In fact, in the dendrogram the *Equus asinus somalicus* cluster is clearly separated from the one including both ours and *Equus asinus africanus* sequences. The bootstrap values at the third nodes (95), indicating the robustness of the dendrogram, support the clustering structure outcome in Figure 3.

Regarding the RAD we found four haplotypes with high similarity among them resulting in a low within population variability (in diagonal the light orange box in Figure 2). These findings do not agree with those found by Bordonaro et al. (2012) using 14 microsatellites markers.

The Figure 4(a) shows the MJN constructed based on the 104 Italian donkey mtDNA sequences and the reference sequence X97337. Three haplogroups (A, B and C), separated from 5 nucleotide substitutions each, were found. In detail, the three haplogroups include haplotypes that differ each other by few mutations: 1 to 5 mutations for haplogroup A; 1 to 3 mutations for haplogroup B; 2 to 3 mutations for haplogroup C. In addition, another haplotypes NJ tree, based on Kimura-2-parameter model distances, allowed us to confirm the haplogroups clustering distribution (Figure 4(b)). These haplogroups comprise haplotypes for which common origins are assumed since they share a characteristic pattern of mutations.

The AMOVA analysis is a useful tool to check how the genetic diversity is distributed among populations, whose structure is quantified by $F_{ST}$. We examined different possible structures by creating and comparing different population groups. We ran the analysis under two hypotheses:

Hypothesis (1) three groups according to the geographical prevalence, i.e. group 1, ASD and SAD (Sardinian populations), group 2, AMD and ROD (North Central Italy) and group 3, MFD and RAD (South Italy);

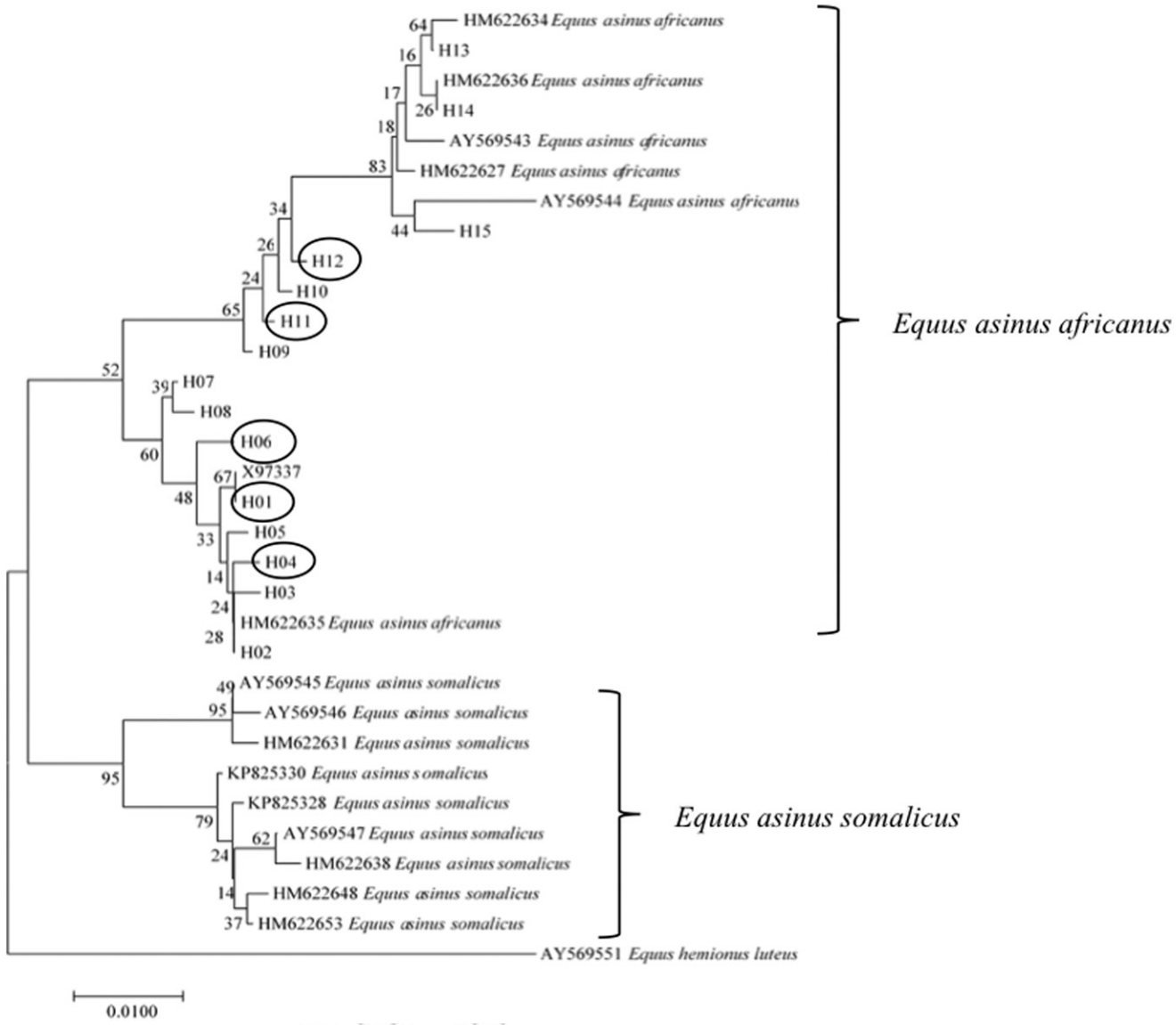
Hypothesis 2) three groups according to the donkey height at the withers, i.e. group 1, ASD and SAD (height at the withers low), group 2, MFD, ROD and RAD (height at the withers high) and group 3, AMD (height at the withers intermediate).

The Table 3 reports the results for the AMOVA analysis according to two hypotheses. The results highlight that the majority of the observed variance is due to differences among samples within breeds. The most part of the variation is observed within the populations (85.5% Hypothesis 1 and 85.2% Hypothesis 2), whereas the differences among groups represent only the 1.5% and 2.18% of the variation respectively (Table 3).

### *Italian donkey breeds vs. other donkey populations*

The Italian mtDNA sequences were compared with 172 publically available mtDNA D-loop sequences belonging to European domestic and Ethiopian donkeys and wild asses. In addition, to assess a hierarchical structure among Italian populations, European domestic donkeys, Ethiopian donkeys and wild asses, the AMOVA analysis was performed under different hypotheses grouping breeds in different clusters. The hypothesis that better defined the clustering strategy in groups resulted to be in agreement with the historical data on the of Italian donkey breeds origin. According to this hypothesis the breeds have been clustered in six groups: (1) *Equus asinus africanus* (Nubian wild ass), *Equus asinus somalicus* (Somali wild ass); (2) Ethiopian donkeys and AMD; (3) ASD and SAD; (4) Spanish donkey, MFD, RAD and ROD; (5) Balkan donkey; (6) Turkey and Cyprus donkey breeds (Table 4). As expected the variation within population of 54.02% , highlights the differences among samples within breeds (Table 4). A considerable amount of variation (37.64%) can be attributed to differences found among populations within defined groups. Instead, the variance among groups resulted low (8.34%) (Table 4).

The MJN analysis identified 62 haplotypes from 276 mtDNA D-loop sequences (172 GenBank sequences and our




Q7 **Figure 3.** Comparison among the D-loop sequences using a NJ tree based on Kimura-2-parameter model distances. The haplotypes considered are identified in *Equus asinus africanus* (Nubian wild ass), *Equus asinus somalicus* (Somali wild ass), *Equus hemionus luteus* (outgroup) and Italian donkey breeds. The bootstrap values represent the robustness of the dendrogram. In black circle, the haplotypes with Amiata donkey D-loop sequences.

104 Italian donkey breeds) showing a high variability (*Hd* = 0.931) (Supplementary Table S2). In Figure 5 two different clades are clearly defined: clade 1 includes the 61,6% of sequences, whereas clade 2 includes the 38,4% of sequences. Out of 62 haplotypes, 58 are referred to the Nubian lineage, whereas only four haplotypes belong to Somali lineage. The Somali haplotypes are separated by clade 1 and 2 by 6 mutations (15,489, 15,536, 15,541, 15,637, 15,652 and 15,704) that represent a specific 'pattern' of *Equus asinus somalicus*, except for the 15,652 mutation also present in *Equus asinus africanus* (Figure 5). Our results are consistent with those obtained by Kimura et al. (2011) who identified two distinct clades separated from the *Equus asinus somalicus* group by 12 mutations.

The analysis clearly defines four main represented haplotypes: H2 (74%), H3 (42%), H5 (32%) and H6 (61%). Despite previous published evidences, our sequences appeared to be related only with *Equus asinus africanus* (Nubian wild ass), and then we did not confirm the relationship between Amiata donkey and *Equus asinus somalicus* (Somali wild ass) as cited in works by other authors (Colli et al. 2013) as a personal communication.

The influence of Spanish donkey populations, particularly the Catalana breed on MFD, is underlined by the common haplotype H1 and the closely relationships between H10 vs. H29 and H10 vs. H30 separated by mutation 15,592 and 15,621, respectively.

The ASD samples and the most of SAD ones are grouped in three haplotypes (H2, H3 and H5). RAD and ROD shared haplotypes H11 and H12, thus confirming the closely relationships here in before discussed. These two haplotypes derived from H2 that includes the most common haplotype found in Spanish donkeys (Aranguren-Mendez et al. 2004), confirming once again the closely relationships between the two Italian breeds and the Spanish donkeys.

The haplotypes H2 and H6 comprise the most part of Balkan donkeys and the Italian donkey populations. The Anatolian and Cyprus donkey haplotypes H55, H56, H57, H59

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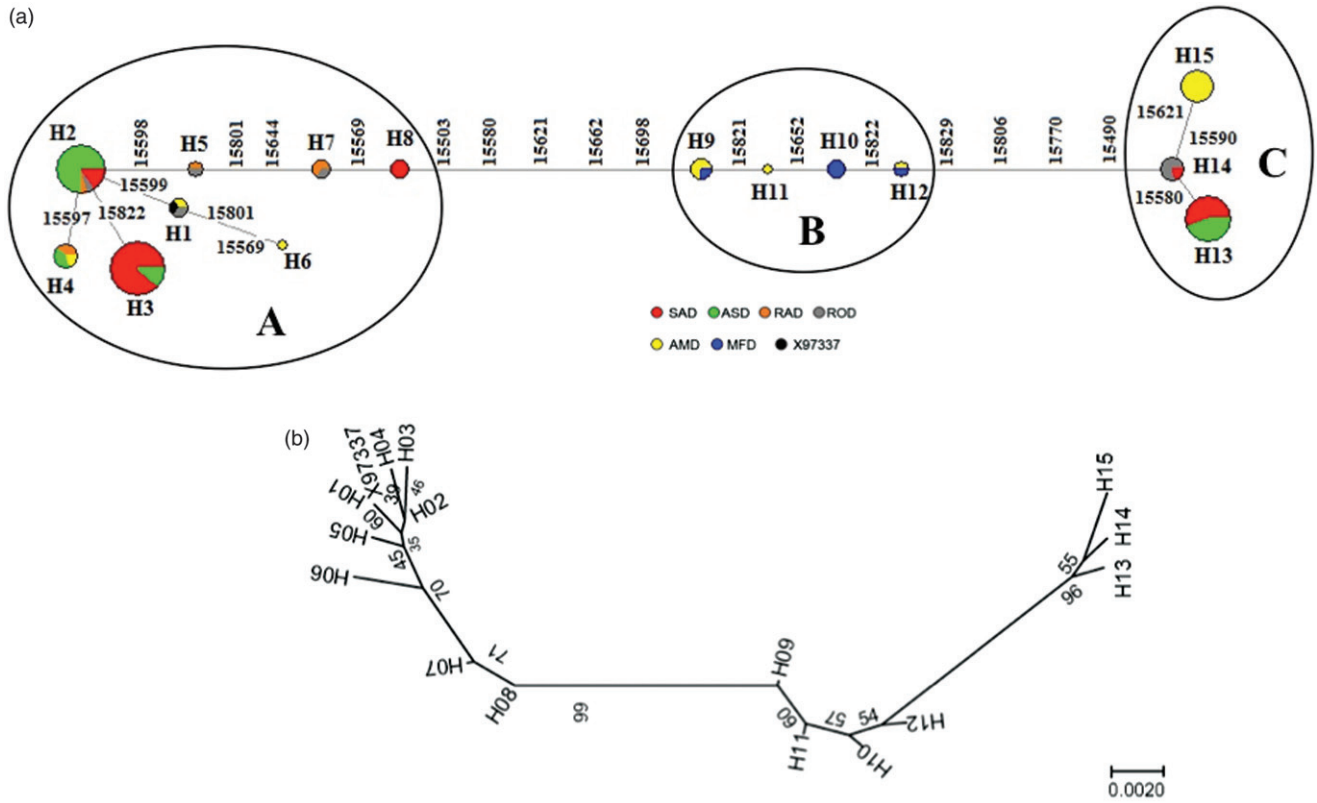


**Figure 4.** MJN and NJ tree. (a) The MJN based on the 104 donkey mtDNA sequences and the reference sequence X97337. The numbers represent the position of nucleotide substitution. Circle are proportional to the numerosity of the samples; (b) The haplotypes NJ tree based on Kimura-2-parameter model distances. The numbers represent the robustness of the dendrogram.

**Table 3.** Hierarchical AMOVA analysis among the six Italian populations.

| Source of variation | Variance component | Variance (%) | Fixation index[a] | *p*-value[b] |
|---|---|---|---|---|
| Hypothesis 1: geographical prevalence | | | | |
| Among areas | 0.066 | 1.50 | ΦCT = 0.015 | .322 |
| Among breed within areas | 0.575 | 12.97 | ΦSC = 0.132 | .004* |
| Within breeds | 3.795 | 85.53 | ΦST = 0.145 | .000* |
| Hypothesis 2: height at the withers | | | | |
| Among groups | 0.097 | 2.18 | ΦCT = 0.021 | .263 |
| Among breed within groups | 0.558 | 12.54 | ΦSC = 0.128 | .007* |
| Within breeds | 3.795 | 85.28 | ΦST = 0.147 | .003* |

[a]ΦCT: variation among groups divided by total variation; ΦSC: variation among sub-groups divided by the sum of variation among sub-groups within groups and variation within sub-groups; ΦST: the sum of variation groups divided by total variation.
[b]ns = $p > 0.05$.
*$p < .05$.
***$p < .001$.

**Table 4.** Hierarchical AMOVA analysis among the six Italian breeds, European and Ethiopian donkey populations and wild asses.

| Source of variation | Variance component | Variance (%) | Fixation index[a] | *p*-value[b] |
|---|---|---|---|---|
| Among groups | 0.371 | 8.34 | $\Phi_{CT} = 0.083$ | .458 |
| Among populations within groups | 1.676 | 37.64 | $\Phi_{SC} = 0.410$ | .000*** |
| Within populations | 2.406 | 54.02 | $\Phi_{ST} = 0.459$ | .000*** |

[a]$\Phi_{CT}$: variation among groups divided by total variation; $\Phi_{SC}$: variation among sub-groups divided by the sum of variation among sub-groups within groups and variation within sub-groups; $\Phi_{ST}$: the sum of variation groups divided by total variation.
[b]ns = $p > .05$.
*$p < .05$.
***$p < .001$.

and H60 are separated by several mutations from the Italian populations.

Pérez-Pardal et al. (2014) reported similar results and underlined the hypothesis that the donkey mtDNA genetic background probably results from contemporaneous domestication events occurred in a limited geographical area (Northern Africa or Horn of Africa). After the first domestication event, the spreading of donkeys was, at first, very slow and scattered. The importance of the donkey's diffusion can be attributed to the evolution of long-distance trade and to the human migrations, especially occurred in Africa (Blench 2000). Afterwards, the Roman played an important role in

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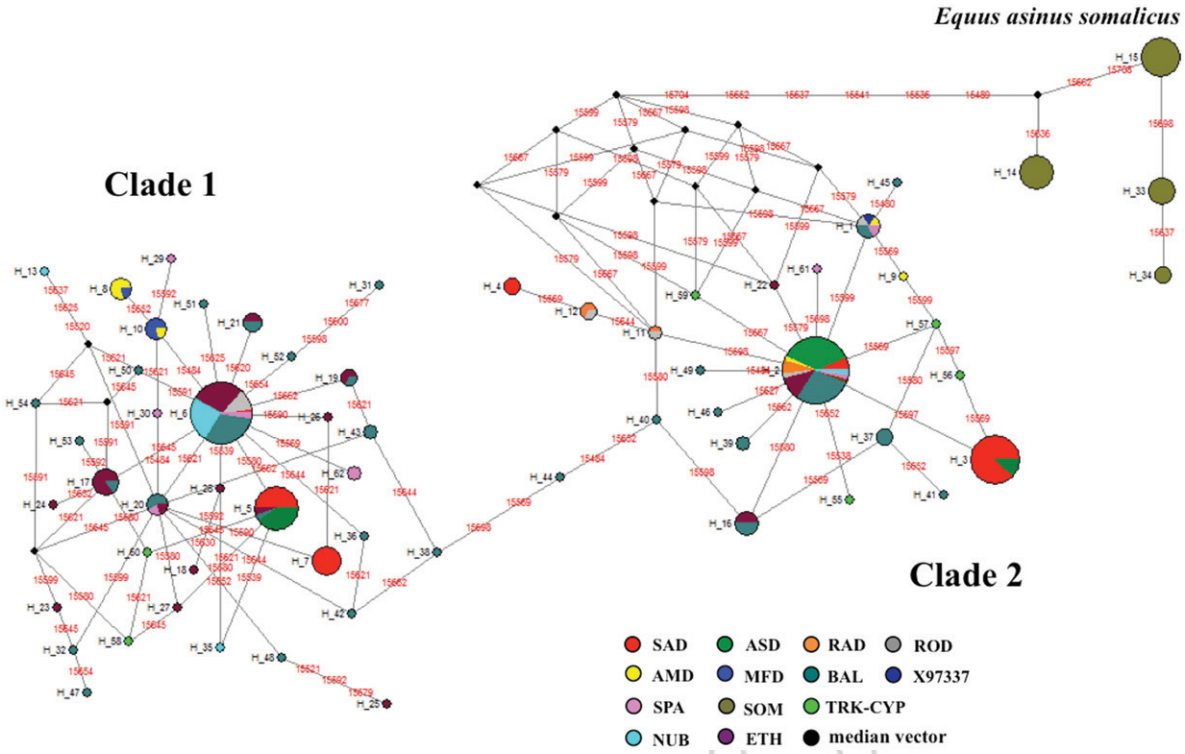


**Figure 5.** MJN based on the six Italian donkey breeds and the 172 donkey sequences from GenBank. Circle are proportional to the haplotypes frequencies; black circles are median vectors (mv) representing extant unsampled or extinct ancestral sequences; in red the number of mutation point respect to the reference sequence X97337. SAD = Sardo donkey; ASD = Asinara donkey; AMD = Amiata donkey; RAD = Ragusano donkey; ROD = Romagnolo donkey; MFD = Martina Franca donkey; BAL = Balcan donkey; SPA = Spanish donkey; SOM = *Equus asinus somalicus*; TRK-CYP = Turkey and Cyprus donkey; NUB = *Equus asinus somalicus*; ETH = Ethiopian donkey. GenBank ID are listed in the S1 supplementary table.

expansion of donkeys in neighbouring territories. In fact, the presence of donkeys belonging to clade 1 and 2 is recorded by archaeological samples from Pompei (Kimura et al. 2013).

Finally, it is interesting to note that at the beginning of the twenty-first century the trend of expansion of donkey populations, occurred in rural areas of African countries, Asia and Latin America, where human populations did not have access to mechanical power for agricultural practices. On the contrary in the industrialized countries, despite the use of donkeys for recreational purposes such as ecotourism, trekking, equestrian therapy/donkey therapy (especially with children) and for the production of milk to be used in cosmetics industry, or for human consumption in cases of cow milk allergy, the donkey populations remain relatively stable (Starkey& Starkey 2000).

## Conclusions

Despite the relatively high number of references on genetic variability of Italian donkey breeds analyzed by microsatellite markers and morphological traits, this is the first study on mtDNA D-loop genetic variability of Italian donkey breeds.

The results of our study show the existing genetic variability within and between breeds found in the six donkey populations bred in Italy. However, a geographical clustering among Italian populations was not found. The identified haplotypes were grouped in three haplogroups, each differentiated from the others by few mutations.

The relationships among our Italian donkey breed sequences and those from donkeys belonging to breeds living in Mediterranean and Balkan areas, showed the complexity of the ancestry and of the genetic makeup of the modern donkey populations.

To preserve the genetic resources represented by local populations will be one of the main goal for the future generations. The maintenance of the donkey biodiversity, nowadays at risk of loss if not of extinction, requires comprehensive knowledge of the donkey breeds characteristics, including data on population size and structure, breed geographical distribution and the within and between breed genetic diversity assessed by different marker types, including mtDNA sequences. Assessment of genetic variation as done in this study represents one of the indication that can be used in *ex situ* genetic conservation, helping the prioritization of individuals to be collected and stored in germplasm cryobanks.

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The authors declare they have no competing interests.

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