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Mitochondrial DNA variation and genetic relationships in Spanish donkey breeds (*Equus asinus*)

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Summary

In this study, the mitochondrial DNA diversity of six Spanish donkey breeds and two African donkey populations (one from Morocco and the other from Zimbabwe) was analysed. A total of 79 animals were sequenced for 313 bp of the cytochrome *b* gene, and 91 individuals for 383 bp of the D-loop region or control-region. Sequence comparisons and phylogenetic analyses of both Spanish and African populations revealed low diversity. Only six and seven haplotypes respectively were found in cytochrome *b* and the D-loop region. Relatively low nucleotide diversity (π) values were detected in these populations. The π values, from the D-loop region, ranged from 0.0006 to 0.0169 for the Catalana and Andaluza breeds, respectively. The obtained results seem to confirm the existence of two divergent maternal lineages of African origin (*Equus asinus africanus* and *E. a. somaliensis*). In this paper the genetic relationships between these breeds are analysed and compared with those obtained in other European populations. Also, the data on the genetic relationships between the populations, obtained from nuclear DNA (microsatellites) and mitochondrial DNA (cytochrome *b* and D-loop region) is argued and interpreted.

Zusammenfassung

Variation mitochondrialer DNA und genetische Beziehungen zwischen spanischen Eselrassen (Equus asinus)

In dieser Studie wurde die Diversität mitochondrialer DNA von sechs spanischen Eselrassen und zwei afrikanischen Eselpopulationen (eine aus Marokko und die andere aus Zimbabwe) analysiert. Es wurden insgesamt 79 Tiere für 313 bp des Cytochrom b-Gens sequenziert und 91 Individuen für 383 bp der D-Loop Region oder der Kontrollregion. Sequenzvergleiche und phylogenetische Analysen zeigten geringe Diversität. In der Cytochrom b und der D-Loop Region wurden nur sechs beziehungsweise sieben Haplotypen gefunden. Es wurden relativ geringe Nukleotiddiversitätswerte (π) entdeckt. Die π-Werte lagen zwischen 0,0006 und 0,0169 für die katalanischen bzw. die andalusischen Rassen. Die Ergebnisse scheinen die Existenz von zwei verschiedenen maternalen Linien afrikanischen Ursprungs (*Equus asinus africans* und *E. a. somaliensis*) zu bestätigen. In dieser Veröffentlichung werden die genetischen Beziehungen zwischen diesen Rassen analysiert und mit denen verglichen, die für andere europäische Populationen erhalten wurden. Darüber hinaus werden die Daten der genetischen Beziehungen zwischen den Populationen, die durch nukleäre DNA (Mikrosatelliten) und mitchondriale DNA (Cytochrom b und D-Loop Region) erhalten wurden, erörtert und interpretiert.

Introduction

The origin and evolution of the modern Spanish domestic donkey (*Equus asinus*) is uncertain. Several wild donkey species or subspecies, the taxonomies of which are

confused, have been proposed as ancestors of modern domestic donkeys. The number of animals currently recorded for these breeds is very small, and they are included in the FAO list of domestic animal breeds to be conserved.

Darwin (cited by Salvans and Torrens 1959) admits the theory of the origins of the donkey, from a single and common African trunk. Nevertheless, other authors (Adametz 1943; Epstein 1984; Clutton-Brock 1987; Camac 1989) who indicate that today's domestic asses, including the Spanish breeds, seem to be derived from two ancestral sources: the Nubian ass (*Equus asinus africanus*), a native of the Nile Basin which gave rise to the North African breeds, as well as to the Andaluza (from South of the Iberian Peninsula) and Majorera (from the Canary Islands) breeds, both of grey-brown coat (Aparicio 1960; García Dory et al. 1990; Yanes 1999; Jordana and Avellanet 2002), and the Somalian ass (*E. a. somaliensis*) which subsequently gave rise to the donkeys of Southwest Asia and probably also to the majority of European breeds, including the Catalana, Mallorquina, Encartaciones and Zamorano-Leonesa breeds (Epstein 1984), the four of black coat (from North of the Iberian Peninsula).

Notwithstanding, some other authors (Dechambre and Sanson, cited by Aparicio 1960; Sotillo and Serrano 1985; Lorenzo 1997) support the theory of two different ancestral sources: one for the *E. a. africanus*, originating in Northeast Africa, and the other for the *E. a. europeus*, originating in the Mediterranean Basin, and especially the Balearic Isles, giving rise to the majority of the European donkey breeds, including the four black-coated Spanish breeds mentioned above.

In previous papers (Aranguren-Méndez et al. 2001, 2002), the results from an analysis of 15 microsatellite loci were presented. The relationships between these breeds, using D_A and Reynolds' genetic distances, showed that the four black-coated donkey breeds (Catalana, Encartaciones, Mallorquina and Zamorana-Leonesa) formed one cluster, meanwhile the Andaluza breed formed another closed cluster with the Moroccan ass (*E. a. africanus*). This supports the theory of this breed's African origin. However, the low bootstrap value observed (43% support) reflected the instability of the topology.

According to mtDNA analysis, the difference between the donkey and the horse suggests that the evolutionary separation of the two species occurred ~9 million years ago (Xu et al. 1996). This is distinctly earlier than the paleontological data of 3–5 MYA (Lindsay et al. 1980). *Equus asinus* was domesticated approximately 6000 years ago, probably in either Egypt or Mesopotamia (Littauer and Crouwel 1979).

Mammalian mitochondrial DNA (mtDNA) is maternally inherited, as originally demonstrated in horse–donkey hybrids (Hutchison et al. 1974), and this could be very valuable in phylogenetic studies, particularly of donkey populations, as reflected in the previous work by Ivankovic et al. (2002), on Croatian donkey populations.

The main goal of this study was to determine the genetic variability and relationships of Spanish donkey breeds based on the analysis of the cytochrome *b* and D-loop region sequences. The genetic variability of Spanish donkeys was compared with that of African donkeys, which provided insights into the origin of the geographic groups and their diversification. Finally, the genetic information on Spanish donkeys was integrated in order to identify populations with low levels of genetic variation and to define evolutionary and management units for conservation.

Materials and methods

Population samples and preparation of DNA

Total DNA (genomic and mitochondrial) was extracted from blood random samples. Seventy-one Spanish donkeys from six breeds [Andaluza (AND), Catalana (CAT), Encartaciones (ENC), Majorera (MAJ), Mallorquina (MALL) and Zamorano-Leonesa

(ZAM)] were used to obtain mtDNA D-loop sequences. Samples were taken from 61 animals for the cytochrome *b* sequences. Methods of extraction and treatment were as described previously by Aranguren-Méndez et al. (2001). Moreover, blood samples from 20 African donkeys, from two different locations – Morocco (MOR) n = 9; and Zimbabwe (ZIM), n = 11, were included, as, presumably, genuine members of *E. a. africanus* and *E. a. somaliensis*, respectively.

PCR amplification of mtDNA cytochrome *b* and D-loop regions

PCR primers for the hypervariable region of the D-loop, between $tRNA^{Pro}$ and the large central conserved sequence block were designed based on the donkey sequence (Xu et al. 1996). The primer sequence amplified a 383 bp fragment between sites 15387 and 15769. Two compatible oligonucleotide primers were designed from the donkey mitochondrial D-loop sequence, i.e. Donk-F (5′-CCC AAG GAC TAT CAA GGA AG-3′) and Donk-R (5′-TTG GAG GGA TTG CTG ATT TC-3′). The partial (313 bp) cytochrome *b* region between nucleotides 14391 and 14703 was amplified by PCR and was sequenced using the primers CYTB-F (5′-CTG CCG AGA CGT TAA CTA C-3′) and CYTB-R (5′-GGC TTT GTC TAC TGA GAA TC-3′).

PCR was carried out in a 20 μl reaction, with 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 20 μM dNTPs, 10 pmol of each primer, 0.2 U AmpliTaq gold (PE Applied Biosytems, MA, USA) and 50 ng of template DNA. Thermal cycling was performed on a PE 9700 thermal cycler. Thermocycling was performed as follows: a 94°C 'hot star' for 10 min, followed by 25 cycles each consisting of 94°C for 45 s, 60°C for 45 s and 72 for 45 s, final extension at 72°C for 20 min and holding at 4°C.

Sequencing the PCR products

The PCR products from each sample were gel-purified prior to sequencing analysis using a Q/Aquick Gel Extraction Kit (QIAGEN). DNA was sequenced by single-strand PCR using the ABI PRISM™ Dye Terminater Cycle Sequencing kit (Applied Biosystems Division, Perkin-Elmer Cetus, Emeryville, CA, USA) following protocols recommended by the manufacturer. Sequences were determined using an ABI prism 310 DNA sequencer and analysed with sequencer software (Perkin-Elmer, Applied Biosystems).

Statistical analyses

All sequences of the mtDNA D-loop and cytochrome *b* regions were aligned using Clustal-X multiple alignment software (Thompson et al. 1997), and all haplotypes and their frequencies were calculated. The multiple alignments of these sequences were edited manually.

Haplotype diversity (h), nucleotide diversity (π) (Nei and Tajima 1981; Nei 1987), and their standard deviation (SD) for Spanish and African donkey haplotypes were estimated using the program in the ARLEQUIN 2.0 package (Schneider et al. 2000).

The relationships between different mtDNA haplotypes were inferred in two ways. Firstly, by genetic distance, estimated using the Tamura–Nei distance in the cytochrome *b* and D-loop sequences (Tamura and Nei 1993), and calculated on the basis of an equal substitution rate per site using MEGA software (Kumar et al. 2001). They were then used to generate a neighbour-joining (NJ) tree (Saitou and Nei 1987). The statistical confidence of each node in the NJ analysis was estimated by 1000 bootstrap replicates. Secondly, a minimum-spanning tree (Prim 1957), based on the number of nucleotide differences between each haplotype was produced using the MST program in the NTSYS package (Rohlf 2001).

The nucleotide divergence between populations (d_A; Nei 1987) was estimated using the REAP computer package (McElroy et al. 1991), and the NJ tree thereby generated using MEGA. Nucleotide divergence (d_A) was estimated by $d_A = d_{XY} - (d_X + d_Y)/2$, where d_{XY} is the average number of nucleotide substitutions for a randomly chosen pair of haplotypes in each population. However, Nei (1985) indicated that the pattern of population differentiation may be inferred using d_{XY}, which has a smaller variation coefficient than d_A. Thus d_{XY} estimates (also from REAP) were used in MEGA to generate a NJ tree.

The estimates of nucleotide divergence between the populations (d_A) and the assumed rates of nucleotides substitution per site per year (λ) for the D-loop region were used to estimate the time of divergence (t, in years) between the donkey breeds, and between the donkey and horse populations, where $d_A = 2\lambda t$ (Nei 1987). The mtDNA sequence for the horse (*E. caballus*) (H1; accession no. X79547; Ishida et al. 1994) was used as an outgroup equine taxon. Population differentiation also was assessed using θ_{ST} estimated from an entirely pair-wise population comparison by amova (using 1000 randomizations) in the arlequin software.

Results

Cytochrome *b*

The 79 sequences of a 313-bp portion of cytochrome *b* revealed only six haplotypes (Table 1). A total of 19 nucleotide differences, compared to the reference sequence of the horse, were found. However, only four nucleotide positions within donkey populations were polymorphic (Table 2).

The haplotype SPAN-2 was identical to the haplotype reported by Xu et al. (1996), which is the most common haplotype in both Spanish and African breeds. The most ancestral haplotypes of cytochrome *b* were SPAN-1, SPAN-2 and SPAN-3, especially the first two. The two African populations only showed SPAN-2, whereas the only populations that showed SPAN-1 and SPAN-3 were the Andaluza and Majorera breeds.

The derived haplotypes, SPAN-5 and SPAN-6, were found only in individual two and one of the Mallorquina breed, respectively (Table 1). A neighbour-joining tree of the six haplotypes is shown in Fig. 1(a), and a relationship is also shown in the minimum-spanning tree (Fig. 2a).

D-loop

D-loop region revealed more genetic diversity among donkey breeds (Table 3). Based on sequence data analysis, there are seven haplotypes, and including the sequences from the donkey (Xu et al. 1996) and the horse (Ishida et al. 1994), there are 40 variable sites in the

Table 1. **Frequencies of the six cytochrome *b* haplotypes observed in six Spanish donkey breeds and two African populations**

Haplotype	AND (18)[1]	CAT (12)	ENC (7)	MAJ (10)	MALL (7)	ZAM (7)	MOR (7)	ZIM (11)	Total (79)
SPAN-1	0.33	–	–	–	–	–	–	–	0.076
SPAN-2	0.56	1.00	0.86	0.60	0.29	1.00	0.86	1.00	0.759
SPAN-3	0.11	–	–	0.40	–	–	–	–	0.076
SPAN-4	–	–	0.14	–	0.29	–	0.14	–	0.051
SPAN-5	–	–	–	–	0.29	–	–	–	0.025
SPAN-6	–	–	–	–	0.13	–	–	–	0.013

[1]Number of animals sampled from each population.

Table 2. **Polymorphic nucleotide sites for six cytochrome *b* haplotypes, observed in 79 Spanish and African donkeys analysed**

	Position																		
Haplotype	14418	14421	14448	14457	14469	14472	14481	14496	14502	14517	14535	14553	14559	14571	14592	14604	14616	14622	14629
SPAN-1	C	C	C	C	T	C	G	T	C	A	T	A	C	A	A	C	G	T	C
SPAN-2[1]	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
SPAN-3	.	T	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.
SPAN-4	.	T	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.
SPAN-5	.	T	.	.	C	.	.	.	.	.	.	.	.	.	C	.	.	.	.
SPAN-6	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.
HORSE[2]	T	.	A	T	.	T	A	C	T	G	C	T	T	G	C	T	A	C	G

[1]Concordance with the X97337 sequence of *E. asinus*.
[2]Horse sequence (Genbank, accession number X79547).

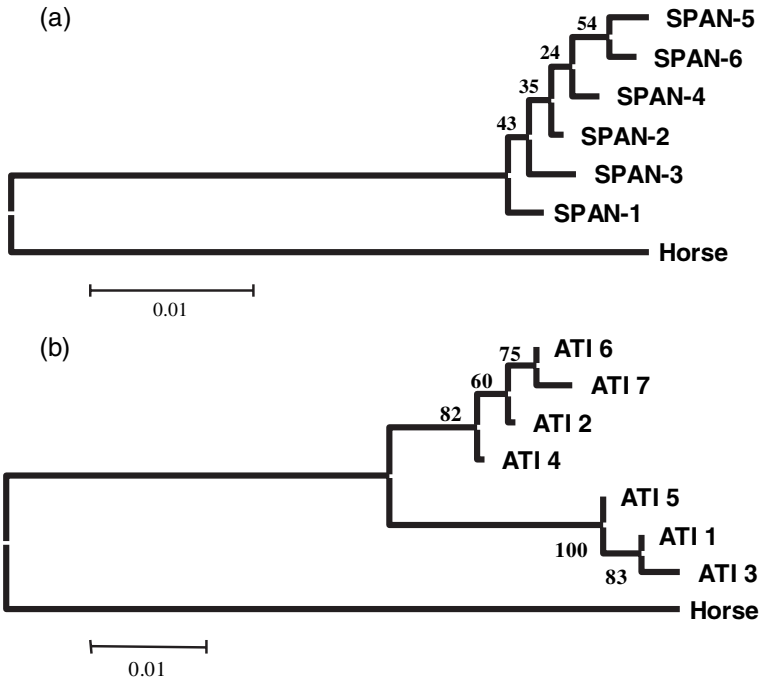

Fig. 1. Neighbour joining trees (NJ) for the six cytochrome *b* (a) and seven D-loop (b) donkey haplotypes, with the horse sequence as an outgroup. The numbers at the nodes are the percentages for 1000 bootstrap resamplings

total of 383 nucleotides. In the donkey, 13 sites were polymorphic; while 27 additional sites, including the horse insertion at position 15540 (assigned as 15540b), differed between the donkey and the horse.

The frequencies of each haplotype in each population and overall frequencies are shown in Table 4. It should be emphasized that four of the seven haplotypes were present only in one or two breeds, and that none was present in all breeds at the same time. The most frequent haplotypes, in both the Spanish and African breeds were the ATI-1 and ATI-3, present in seven and five of the eight total populations, respectively.

The relationships between the seven haplotypes (Fig. 1b) show two major clusters. One of four haplotypes (cluster B) is found only in Spanish breeds. The remaining three haplotypes are found in Spanish (ATI-5; only in Andaluza and Majorera breeds), and in both African and Spanish donkey populations (ATI-1 and ATI-3). A relationship is also shown in the minimum-spanning tree (Fig. 2b). A clear separation of these groups is shown, given the high bootstrap values (82 and 100%). The second cluster (ATI-1, ATI-3 and ATI-5) reflected a lot more polymorphic width than the first group, with this larger dispersion attributable to these haplotypes possibly being the most ancestral of the asinine species (Nei 1987).

Intra-population haplotype diversity (h) oscillated between 0.182 and 0.737, for the Zimbabwe and Andaluza donkey populations, respectively. Nucleotide diversity (π) ranged from 0.0006 to 0.0169 for Catalana and Andaluza breeds, respectively. On average, the haplotype and nucleotide diversities were 0.421 and 0.007, respectively (data not shown).

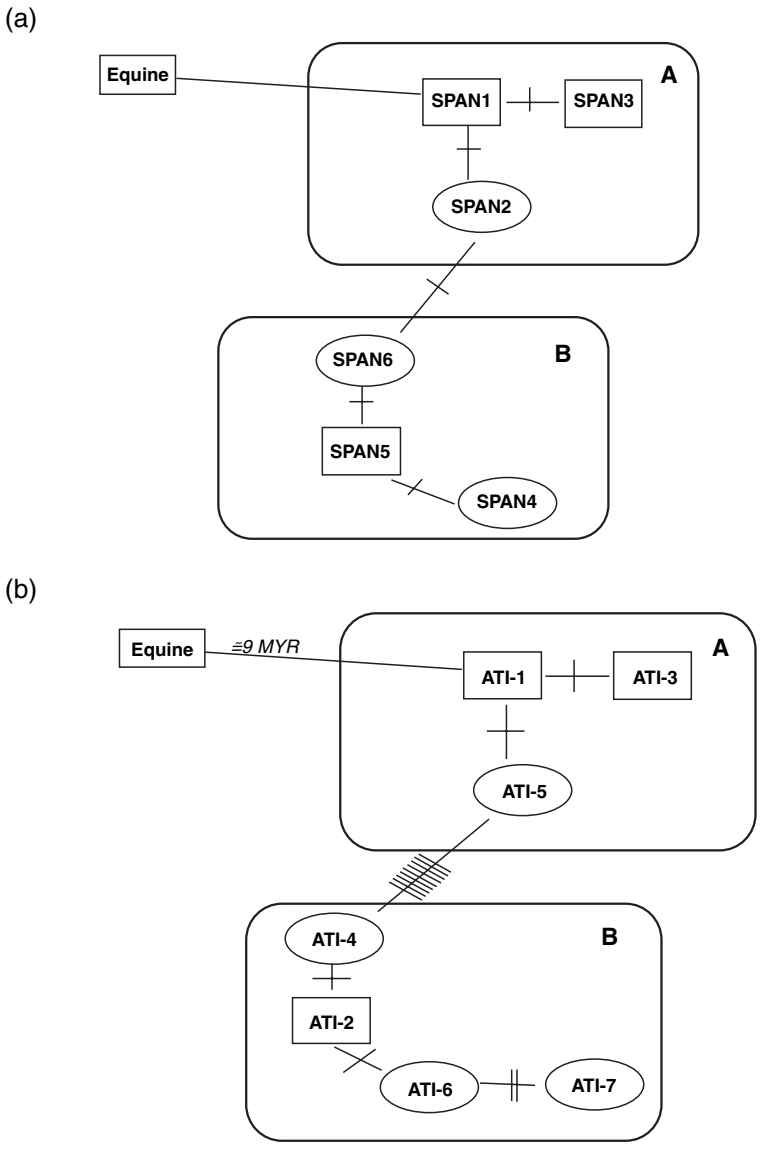

Fig. 2. Minimum spanning trees showing the network of interrelationships between the six cytochrome *b* (a) and the seven D-loop (b) mtDNA haplotypes. The capital letters (A and B) identify the clusters referred to in the text. The ticks indicate the inferred number of mutational steps between pairs of haplotypes

Estimations of the nucleotide divergence (d_A) among populations (Table 5) and the N-J tree derived from them (Fig. 3), showed the populations grouped in two clusters: one formed by the Encartaciones Ass and another by the other breeds. The tree derived from nucleotide substitution (d_{XY}) between populations (Figure not shown), showed a similar distribution. These relationships are, at first, totally incongruous with the geographical distribution of the breeds. The Encartaciones breed is clearly exceptional in clustering only,

Table 3. **Polymorphic nucleotide sites for seven D-loop halotypes**

	Nucleotide position																			
	15411	15469	15484	15488	15490	15492	15503	15514	15516	15518	15521	15528	15531	15534	15540[1]	15541	15543	15544	15546	15547
ATI-1[2]	T	C	G	T	C	T	T	T	A	A	C	G	C	T	?	T	A	G	T	C
ATI-2	.	.	A	.	T	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.
ATI-3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ATI-4	.	.	A	.	T	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.
ATI-5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ATI-6	.	.	A	.	T	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.
ATI-7	C	.	A	.	T	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.
Horse	.	T	A	C	.	C	.	C	G	T	T	A	T	G	C	C	C	A	C	T

	Nucleotide position																			
	15548	15553	15559	15560	15661	15565	15569	15570	15580	15598	15599	15621	15630	15643	15644	15652	15662	15667	15698	15704
ATI-1[2]	A	A	C	T	C	T	A	C	A	C	G	A	T	G	A	C	A	A	C	C
ATI-2	.	.	.	.	.	.	G	.	G	T	.	G	.	A	.	T	G	.	T	.
ATI-3	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.
ATI-4	.	.	.	.	.	.	G	.	G	T	.	.	.	A	.	T	G	.	T	.
ATI-5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.
ATI-6	.	.	.	.	.	.	.	.	G	T	.	G	.	A	.	T	G	.	T	.
ATI-7	.	.	.	.	.	.	.	.	G	T	.	G	.	A	.	T	G	.	T	.
Horse	G	G	T	C	T	G	G	G	G	T	.	.	C	A	G	T	.	G	T	T

[1]Nucleotide insertion only present in the horse.
[2]Concordance with the X97337 sequence of *E. asinus*.

Table 4. **Frequencies of the seven D-loop mitochondrial haplotypes observed in six Spanish donkey breeds and two African populations**

Haplotype	AND (20)[1]	CAT (10)	ENC (11)	MAJ (10)	MALL (10)	ZAM (10)	MOR (9)	ZIM (11)	Total (91)
ATI-1	0.20	0.90	–	0.20	0.70	0.80	0.78	0.91	0.517
ATI-2	0.30	–	–	0.30	–	0.20	–	–	0.121
ATI-3	–	0.10	0.09	–	0.30	–	0.22	0.09	0.088
ATI-4	0.10	–	–	–	–	–	–	–	0.022
ATI-5	0.40	–	–	0.50	–	–	–	–	0.143
ATI-6	–	–	0.82	–	–	–	–	–	0.099
ATI-7	–	–	0.09	–	–	–	–	–	0.010

[1]Number of animals sampled from each population.

as is the clustering of the African populations with the Catalana and Mallorquina breeds, at least from the geographical viewpoint. In pair-wise testing of population differentiation (θ_{ST}, Table 5), 18 of the 28 combinations showed statistically significant values ($p < 0.05$).

Discussion

This study, which is one of the first of its kind, compares the mtDNA cytochrome *b* and D-loop sequences of Spanish donkeys with sequences from two African donkey populations. In a previous paper (Ivankovic et al. 2002), genetic variation of the D-loop region, in three populations of Croatian donkey breeds, was reported.

As a whole, both phylogenies, from cytochrome *b* and the D-loop, as well as the genetic relationships between the eight populations based on nucleotide divergences (d_A) and nucleotide substitutions (d_{XY}), coincided a great deal, although they did not correspond much, at first, with the relationships derived from microsatellite loci studies (Aranguren-Méndez et al. 2001, 2002).

Table 5. **Matrix of d_A distances (D-loop nucleotide divergence, above diagonal), and θ_{ST} population differentiation (below diagonal), between the eight donkey populations**

	Spanish						African	
Populations	AND	CAT	ENC	MAJ	MALL	ZAM	MOR	ZIM
AND	–	0.0065	0.0076	0.0009	0.0067	0.0013	0.0065	0.0064
CAT	0.3744***	–	0.0260	0.0041	0.0000	0.0008	0.0000	0.0000
ENC	0.4281***	0.7218***	–	0.0099	0.0260	0.0152	0.0260	0.0260
MAJ	0.0000	0.4578***	0.4881***	–	0.0043	0.0000	0.0042	0.0041
MALL	0.2788*	0.0000	0.5847***	0.3282**	–	0.0010	0.0012	0.0000
ZAM	0.2672*	0.0080	0.6497***	0.3305**	0.0657	–	0.0009	0.0008
MOR	0.2992*	0.0000	0.6270***	0.3568**	0.0000	0.0153	–	0.0003
ZIM	0.3889***	0.0000	0.7342***	0.4772***	0.0408***	0.0181	0.0000	–

*p < 0.05; **p < 0.01; ***p < 0.001.
Average d_A donkey–horse = 0.5375.

Perhaps the most coherent explanations for these findings are related to the possible mixing of haplotypes among these breeds, due to repeated gene flow or migrations (interchange of breeders) that has taken place among these populations over many years. The loss of a closed phylogeographic structure between the Spanish and African populations, could be influenced by the history of these populations, especially if we consider the constant population movements of both humans and animals that have characterized these regions, and the commercial exchange that has taken place between them (Romagosa 1959).

Haplotypes rarely exist which are unique to a breed. The same is true of microsatellites, which have shown that although breeds rarely contain unique alleles, they may nevertheless be clearly differentiated by their allelic frequencies (Vila et al. 1999). However, private haplotypes in the Mallorquina breed (SPAN-5 and SPAN-6, with a frequency superior to 40%) and the Encartaciones breed (ATI-6 and ATI-7, with a frequency superior to 90%) were obtained.

The most coherent explanation for these findings could be related, firstly to its particular habitat, which is basically reproductively and geographically isolated, and secondly, to possible 'bottleneck effects', which have led to certain rare haplotypes in these populations acquiring a high frequency in the population from very few breeding animals.

The 'founder effect' would be the most reasonable explanation to understand the unexpected and marked separation between the Encartaciones breed and the other Spanish breeds. The low number of individuals analysed could be another cause explaining these discrepancies, as reported by Lau et al. (1998) in similar works concerning buffalo populations.

Our results are consistent with two maternal lineages of African origin, although with qualifications, due mainly to the ass population from Morocco, that we will now comment on.

We noted above that the most ancestral haplotypes of cytochrome *b* were SPAN-1, SPAN-2 and SPAN-3. The two African populations only show SPAN-2, whereas the only populations that show SPAN-1 and SPAN-3 are the Andaluza and Majorera breeds. Something similar in the central region of the D-loop also happens. The most ancestral haplotypes are ATI-1, ATI-3 and ATI-5. The two African populations only have ATI-1 and ATI-3, in a similar way to the Spanish peninsular breeds (with the exception of the Basque breed, Ass of the Encartaciones, that is a very particular case, and that has been commented on and argued previously). On the other hand, the only populations that

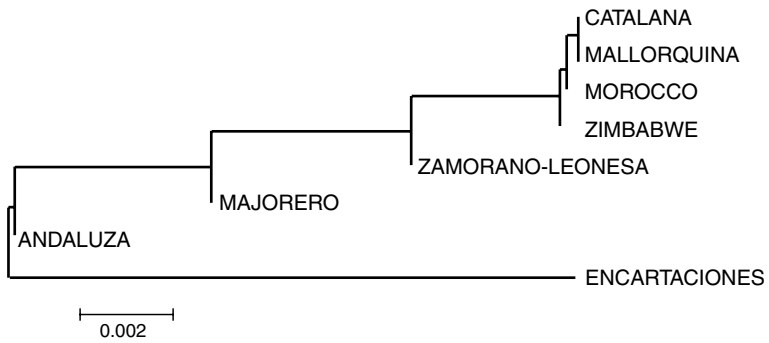

Fig. 3. Neighbour-joining tree for the eight donkey populations, based on nucleotide divergence (d_A) between populations

possess ATI-5 are again the Andaluza and the Majorera breeds. We interpreted this fact, as giving evidence that these two breeds probably have a common origin, from *E. a. africanus*.

The ATI-2 haplotype also seems exclusive to the Andaluza and Majorera breeds, but the fact that it was found in two individuals of the Zamorano-Leonesa breed makes us doubt this exclusivity. In order to confirm this haplotype we should analyse more individuals of the breed, or interpret it as a possible migration of breeding females (in any moment of their history) from the Andaluza population to the Zamorana one.

Therefore, and interpreting the obtained results in this mtDNA analysis, the two African populations (MOR and ZIM) analysed, are not genuine representatives of the two ancestral commented trunks, *E. a. africanus* and *E. a. somaliensis*, respectively, but rather both could have descended from the *E. a. somaliensis* trunk.

The ass from Zimbabwe is a typical, and very pure, representative of the *E. a. somaliensis*. According to the results it is a very primitive, closed population, which has received very few influences from other populations over the centuries. For cytochrome *b*, it only possesses the SPAN-2 haplotype (the most ancestral) and for the D-loop it has, almost exclusively, ATI-1 (91%, the most ancestral) and ATI-3 (9%) also ancestral. The two trunks (*E. a. africanus* and *E. a. somaliensis*) have a common origin, ATI-1 is the common haplotype of these trunks, and ATI-3 a derived haplotype that only remained or developed in *E. a. somaliensis*.

In previous studies carried out on microsatellite DNA markers (Aranguren-Méndez et al. 2001, 2002), the Moroccan ass clustered perfectly with the Andaluza breed (the Majorera and Zimbabwe populations were not included in these works), suggesting that both have one common origin, the *E. a. africanus*. Both results (from microsatellites and from mtDNA), that at first could seem incongruous, are perfectly valid and interpretable.

With the mtDNA we are analysing a variability that is strictly inherited maternally. Therefore, the Moroccan population would have come from the *E. a. somaliensis* maternal lineage, and this would be their real origin. However, this population, over the centuries, has received a lot of influences, through repeated back-crossings, of asinine populations of the *E. a. africanus* (many breeds of this trunk inhabit the African West Coast), especially by paternal via (using or importing stallions – males – of other locations is frequent and normal). At present, the population from Morocco, genetically looks more like the members of *E. a. africanus* than those of *E. a. somaliensis*, although in their origins the first female individuals originate from *E. a. somaliensis*. These primitive maternal haplotypes have been perpetuated over the centuries, by the maternal via (great-great-grandmothers, grandmothers, mothers, daughters) and therefore are still found nowadays (it could be

interesting to analyse specific markers of the Y chromosome, paternally inherited). However, we considered that the tree based on microsatellite data gives a more approximate representation of the true genetic relationships between these breeds.

The interpretation of the existence of the two primitive maternal lineages, and of the ancestral and derived haplotypes, is very similar to work carried out previously by Ivankovic et al. (2002). The Y, W and Ws haplotypes of these authors, match the ATI-1, ATI-3 and ATI-5 haplotypes, respectively, obtained in this study. The Y haplotype would be the most ancestral, and the W and Ws derived haplotypes. The differentiation of W and Ws haplotypes from the Y haplotype occurred some 1.05–2.11 MYA.

Additional studies of mtDNA variation in other mitochondrial gene regions, in addition to fossil bone studies, as well as analysis of other African and Mediterranean breeds, could provide a better understanding of the origin and the evolutionary relationships of these Spanish donkey breeds, and of the species in general.

Accession numbers

The GenBank accession numbers for the six haplotypes of the cytochrome *b* region are: AF380130, AF380131, AF380132, AF380133, AF380134 and AF380135. The Genbank accession numbers for the seven haplotypes of the mtDNA D-loop region are: AF416593, AF416594, AF416595, AF416596, AF416597, AF416598, and AF416599, respectively.

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