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Mitochondrial DNA control region variation in Sanfratellano horse and two other Sicilian autochthonous breeds

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ABSTRACT - Mitochondrial D-loop hypervariable region was analysed in 20 Sanfratellano and two other Sicilian autochthonous horse breeds (20 Sicilian Oriental Purebred and 20 Sicilian Indigenous) in order to investigate matrilineal genetic diversity. A total of 20 different haplotypes were identified sequencing a fragment of 397 bp; overall, haplotypes showed 31 polymorphic sites (7.8%). High diversity was detected in Sanfratellano (11 haplotypes) and Sicilian Indigenous (13 haplotypes), whereas only one haplotype was found in Sicilian Oriental Purebred. Sanfratellano sequences were compared with those belonging to the other Sicilian autochthonous horses and 118 sequences selected from the GenBank database in order to calculate the statistics of molecular diversity. Six haplotypes were exclusive of Sanfratellano which shares haplotype C, D, H, and O with the Sicilian Indigenous and haplotype U with the Sicilian Oriental Purebred; not significant differentiation was found between Sanfratellano and Sicilian Indigenous. BLAST search showed Sicilian haplotypes overlap with the database sequences but for three. Phylogenetic analysis did not show monophyletic group for Sanfratellano samples or the other breeds included in this analysis.

Key words: Horse breeds, mtDNA, D-Loop region, Sequence variation.

Introduction – Sanfratellano breed is mainly reared in the Natural Park of Nebrodi (Sicily) and represents a rare example of semi-feral autochthonous horse. The origin of this breed is uncertain due to the loss of clear historical information and many hypotheses were performed. One of the most credible suppositions is that horses were carried to Sicily by knights coming back from the Near East at the Crusades time; subsequently these horses were crossed with the ancient Sicilian autochthonous breed and found their natural environment in the mountainous forest area of Nebrodi (Hendricks, 2007). Two other autochthonous horses are historically bred in Sicily: Sicilian Indigenous and Sicilian Oriental Purebred. The first one is an heterogeneous population originating from ancient local mares and sires crossed during ages with uncertain breeds; historical information suggests that is probably linked to the Persano breed reared in Sicily in the "Borbon Real Casa di Ficuzza" until 1834. Sicilian Oriental Purebred instead, is a genetic type of Arab-Oriental matrix belonging to the Italian Stood Book since 1875; it represents a Sicilian nucleus of Oriental horses imported from Syria and Mesopotamia since 1864 (Balbo, 1995). Mitochondrial DNA has been widely used, during the last ten years, as a molecular tool in phylogenetic studies. Vilà *et al.* (2001) suggested the multiple matrilines origin of the modern horse; Jansen *et al.* (2002) accomplished a phylogenetic network among Oriental and European horses showing mtDNA haplotypes grouped into 17 distinct phylogenetic clusters, some of them corresponding to group of breeds and/or geographic areas. mtDNA was even combined with historical information, in order to examine genetic relationships among European breeds (Aberle *et al.*, 2007; Pérez-Gutiérrez *et al.*, 2008).

The aim of this study was to examine the matrilineal genetic diversity of Sanfratellano horse com-

pared to the other Sicilian autochthonous, cosmopolitan and geographically distinct breeds for a better understanding of historical relationships among Sicilian autochthonous horses.

Material and methods – Blood samples were obtained from 20 Sanfratellano (SAN) horses and two other Sicilian autochthonous breeds, 20 Sicilian Oriental Purebred (SOP) and 20 Sicilian Indigenous (SIC). Sampling was achieved choosing maternally unrelated horses by using pedigree information when available. DNA was extracted following standard methods. Mitochondrial control region (D-loop) was amplified by PCR using primers designed according to the published horse sequence (X79547). The amplicon of 397 bp (15382-15788) was purified and sequenced using the BigDye Terminator Kit (Applied Biosystems) by an ABI PRISM® 310 Genetic Analyzer. The aligned sequences were edited in MEGA 4 (Tamura *et al.*, 2007) in order to identify the polymorphic sites and to accomplish the BLAST search. AMOVA was performed within the sample of Sicilian autochthonous horses by using Arlequin 3.1 (Excoffier *et al.*, 2006); the same program was used to evaluate haplotype diversity (h), nucleotide diversity (π_n) and the mean number of pairwise differences (π) among Sicilian and 118 other sequences available in literature, selected from the GenBank database and belonging to Thoroughbred (THO), Arab (ARA), Barb (BAR) and Andalusian (AND) breeds. The interbreed differentiation was even assesses by computing the pairwise Φ_{ST} and the linearized Φ_{ST} used as genetic distance indexes. Phylogenetic analysis of Sicilian autochthonous haplotypes was accomplished by MEGA 4 using the neighbour-joining algorithm on K2-parameter distance. Moreover, in order to collect more information, phylogenetic relationships were assessed by NETWORK 4.5.0.1 including haplotypes belonging to cosmopolitan and geographically distant horse breeds.

Results and conclusions – A total of 31 polymorphic sites, with an average percentage of 7.8%, were identified in 20 detected haplotypes (Table 1). The GenBank Acc. no. for the obtained sequences are: EU750716-EU750731, EU569297, EU604815-EU604817.

Table 1. Nucleotide substitutions for 20 Sicilian autochthonous horse haplotypes relative to the reference sequence X79547.

	Position of nucleotide substitutions relative to GenBank X79547																														
Haplotype	15494	15495	15496	15526	15534	15538	15540	15542	15574	15585	15596	15597	15598	15601	15602	15603	15604	15615	15616	15617	15635	15649	15650	15659	15666	15698	15703	15709	15718	15720	15726	15740
X79547	T	T	A	T	C	A	A	C	G	G	A	A	T	T	C	T	G	A	A	T	C	A	A	T	G	T	T	C	C	G	G	A
A	.	C	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	C	.	.	.	C	.	.	.	.	.	A	.	.
B	.	C	.	.	.	.	.	.	.	.	.	.	.	.	T	.	A	.	.	.	.	.	.	C	.	.	C	.	.	A	A	G
C	.	C	.	.	.	.	.	.	.	.	.	.	.	C	T	.	.	.	.	.	.	.	G	.	.	.	.	.	.	A	.	.
D	.	C	.	.	.	.	.	T	.	A	.	G	.	.	T	.	.	.	.	.	T	.	G	.	A	.	C	.	.	A	.	.
E	.	C	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	G	.	A	.	.	.	.	A	.	.
G	.	C	.	.	.	G	.	.	.	.	G	.	.	.	T	.	.	.	.	.	.	.	G	.	.	.	.	T	.	A	.	.
H	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	A	.	.	.	.	A	.	.
I	.	C	.	.	.	.	.	.	.	.	.	.	C	.	T	.	.	G	G	.	.	.	.	.	.	.	C	.	.	A	.	.
K	.	C	.	.	.	.	.	.	.	A	.	.	.	.	T	.	.	.	.	.	.	.	G	.	.	.	.	.	.	A	.	.
L	.	C	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	A	C	.	.	A	.	.
M	.	C	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	G	.	.	.	.	.	.	A	.	.
N	.	C	.	.	.	.	.	.	.	A	.	.	.	.	T	.	.	.	.	C	.	.	.	C	.	.	.	.	.	A	.	.
O	.	C	.	C	.	.	G	.	.	A	.	.	.	.	T	.	.	.	.	.	.	G	.	.	.	.	.	.	T	A	.	.
P	.	C	.	C	.	.	G	.	.	A	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	T	A	.	.
R	C	C	G	.	T	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	G	.	.	.	.	.	.	.	A	.	.
S	C	C	G	.	T	.	.	.	A	A	.	.	.	.	T	C	A	.	.	.	.	G	.	.	.	.	.	.	.	A	.	.
T	C	C	G	.	T	.	.	.	.	.	.	.	.	.	T	C	.	.	.	.	.	G	.	.	.	.	.	.	.	A	.	.
U	C	C	G	.	T	.	.	.	.	A	.	.	.	.	T	C	A	.	.	.	.	G	.	.	.	.	.	.	.	A	.	.
V	C	C	G	.	T	.	.	.	.	A	.	.	.	.	T	C	.	.	.	.	.	G	.	.	.	.	.	.	.	A	.	.
Z	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Table 2. Haplotype (h) and nucleotide diversity (π_n), mean number of pairwise differences (π) and polymorphic sites (p.s.). Haplotypes distribution in Sicilian horses and GenBank sequences.

Breed	h ± s.d.	π_n ± s.d.	π	p.s.	Sicilian autochthonous haplotypes	
					n.	n. of individuals
SOP	-	-	-	-	1	$U_{(20)}$
SIC	0.93 ± 0.04	0.029 ± 0.016	7.112	28	13	$A_{(2)}$ $B_{(1)}$ $C_{(1)}$ $D_{(5)}$ $E_{(3)}$ $G_{(1)}$ $H_{(1)}$ $I_{(1)}$ $O_{(1)}$ $R_{(1)}$ $S_{(1)}$ $T_{(1)}$ $Z_{(1)}$
SAN	0.92 ± 0.04	0.026 ± 0.014	6.503	20	11	$C_{(1)}$ $D_{(1)}$ $H_{(2)}$ $K_{(1)}$ $L_{(2)}$ $M_{(1)}$ $N_{(2)}$ $O_{(1)}$ $P_{(3)}$ $U_{(5)}$ $V_{(1)}$
					GenBank sequences	
					n.	Acc. no.
THO	1.00 ± 0.02	0.026 ± 0.015	6.420	24	19	AF481305 – AF481323
ARA	0.96 ± 0.01	0.027 ± 0.014	6.657	27	63	AF132568 – AF132594
BAR	1.00 ± 0.04	0.026 ± 0.015	6.492	18	10	AJ413658 – AJ413671
AND	1.00 ± 0.01	0.027 ± 0.015	6.615	23	26	AY997165 – AY997168; AF516509 – AF516511; AY805645 – AY805664

SAN and SIC showed the highest variability with 11 and 13 haplotypes respectively; only one haplotype (U) was found in SOP thus it was excluded from the molecular diversity analysis. Six haplotypes are exclusive of SAN and nine of SIC, only three are unique of SAN whereas seven of SIC. SAN shares four haplotypes with SIC (C, D, H, and O) and one with SOP (Table 2).

The analysis of molecular diversity showed the SIC more variable than SAN in all terms (Table 2). AMOVA analysis using K2-parameter distances yielded not significant variation between SAN and SIC (Φ_{ST}=0.046; P>0.05). Genetic distance indexes revealed SAN closely related to AND and BAR haplotypes, even though an unclear genetic differentiation was noticed. BLAST search showed Sicilian haplotypes overlapped with all the GenBank sequences but for C, P, and S. Haplotype B, found in SIC, was even identified exclusively in a Bronze Age archaeological site horse (Inner Mongolia). Moreover, 2 Sanfratellano haplotypes (K and L) can be considered rare in a wide context, since were found only in some European breeds. Phylogenetic analysis revealed no defined clusters either among breeds or geographic areas. In conclusion, SAN seems differently related to the other Sicilian autochthonous breeds. The presence of haplotype U is probably due to the recent introduction of Oriental matrix mares into the Sanfratellano Herd Book; on the other hand, SIC appears more closely related to SAN because they probably derive from the same primitive strain of Sicilian autochthonous horses as historical and genetics information suggest (Fogliata, 1895; Zuccaro *et al.*, 2008).

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