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Mitochondrial variability in the D-loop of four equine breeds shown by PCR-SSCP analysis

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Abstract

A fragment of 466 base pairs from a highly variable peripheral region of the mitochondrial D-loop of horses was amplified and analyzed by single stranded conformational polymorphism (SSCP). Fourteen distinct SSCP variants were detected in 100 horses belonging to four breeds (Arabian, ARB; Thoroughbred, TB; Argentinian Creole, ARC; and Peruvian Paso from Argentina, PPA). Each breed showed four to eight SSCP variants, many of which were shared between two or three of the studied breeds. Arabian horses were the most variable (eigth variants), with three variants unique to the breed. PPA and ARC showed two and one characteristic SSCP variants, respectively, while TB shared all its variants with at least one of the other breeds. An analysis based on the presence/absence of the variants revealed a closer relationship between PPA and TB, which was not completely unexpected considering the mixed ancestry of the PPA mares. The results also confirm the efficiency of SSCP to detect variability in horse mitochondrial DNA.

Key words: MTDNA, equine, PCR.

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Introduction

Mammalian mitochondrial DNA (mtDNA) is maternally inherited, although rare events of paternal leakage have been reported (Kondo *et al.*, 1990; Gyllensten *et al.*, 1991; Avise, 1991; Kaneda *et al.*, 1995). The rate of nucleotide substitution is 5 to 10 times higher in mtDNA compared to nuclear DNA (Brown *et al.*, 1979). In humans, the substitution rate of the non-coding control region (D-loop) has been estimated to be 2.8 to 5 times higher than the rate for the rest of the mitochondrial genome (Aquadro and Greenberg, 1982); and within this region, the two peripheral domains (hypervariable region) evolve even faster (Pesole *et al*., 1999). Given the maternal transmission, the reported absence of recombination (Brown, 1985; Hagelberg *et al*., 1999) and the high substitution rate, mtDNA has proven to be a powerful tool in the analysis of intra- and interspecific variation, population structure and phylogeny. Recent reports have described mitochondrial D-loop variation in horses (Ishida *et al*., 1994a,b; 1995; Marklund *et al*., 1994; Dovc *et al.*, 1996; Bowling *et al*., 1998; Kavar *et al*., 1999). In this study we used SSCP analysis (Orita *et al*., 1989) of a peripheral region of the D-loop to estimate the variability and relationship among four horse breeds, Argentine Creole (ARC), Peruvian Paso from Argentina (PPA), Arabian (ARB) and Thoroughbred (TB). The Arabian horse is an ancient breed which originated on the Arabian Peninsula (Bailey and Lear, 1994; Andrade, 1954). The Thoroughbred breed originated in the United Kingdom from the crossbreeding of English mares of Tarpan ancestry with Arabian, Spanish, Turkish and Barb horses (Bailey and Lear, 1994). The origin of the Peruvian Paso can be traced to the 16th century, from Barb and Andalusian horses brought to Peru by Spanish conquerors. The Argentinean Creole horse constitutes a direct descendent of the Iberian horses of the 1500s, especially Andalusian, Spanish Pure Breed, Barb and Arabian (Cabrera, 1945).

In this report we analyze the variability of a fragment of the mitochondrial D-loop from four horse breeds to establish a preliminary pattern of variability prior to investigating sequence variation.

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Material and Methods

The ARB, TB and ARC horses used in this study were traced back three maternal generations, and the PPA two generations. Total horse DNA was extracted from 500 µL of blood with DNAzol (Gibco BRL) following the

manufacturer's recommendations. The PCR primers used were 5'-AGGACTATCAAAGGAGAAGCTCTA-3' (D14991, Ishida *et al*., 1994a) and 5'-CCTGAAGTAGGAACCAGATG-3' (H16498, Meyer *et al*., 1990), which amplify a 466 bp region situated between the $tRNA^{Thr}$ (position 15397, Xu and Árnason, 1994) and the central domain of the D-loop (position 15863, Xu and Árnason, 1994). The horse D-loop contains four conserved blocks (CSB) and a region of direct eight base pair repeats (Xu and Árnason, 1994; Ishida *et al*., 1994a). The fragment amplified lies outside these blocks, and includes the most variable region of the D-loop (Ishida *et al*., 1994a). The 50 µL reaction mix contained 100 ng total horse DNA, 0.5 µM of each primer, 0.1 mM dNTPs and 2 U *Taq* polymerase (Gibco BRL) in 20 mM Tris-HCl (pH 8.4), 50 mM KCl and 2mM $MgCl_2$, under mineral oil. The PCR consisted of a first denaturation step at 96 °C for two minutes followed by 35 one-minute cycles at 94 °C, 30 s at 55 °C and one minute at 72 °C, with an elongation step of 5 min at 72 °C in the last cycle. The size of the products was estimated by 1.5% agarose gel electrophoresis with pBR322 *Msp I* Digest as size marker.

Fifteen microliters of each PCR product were added to 40 µL of LIS dye (10% sucrose, 0.01% bromophenol blue and 0.01% xylene cyanol FF; Maruya *et al*., 1996). The samples were then heated at 96 °C for 10 min, cooled on ice for at least 5 min and loaded onto a 10% polyacrylamide gel (491 acrylamide biacrylamide). Electrophoresis was carried out at 4 °C, 200V in 0.5 x TBE buffer for 18 h. The gels were subsequently silver-stained, fixed in ethanol 5%, stained with $AgNO_3$ 0.2% and revealed with 2% $CaCO_3$.

A total of 100 horses were examined (45 ARC, 18 ARB, 30 PPA and 7 TB). The PCR products were of the same length, approximately 460 base pairs, which is in agreement with the published horse sequences (Ishida *et al*., 1994a).

Results and Discussion

SSCP analysis revealed 14 variants which were consistently obtained in different runs and in different SSCP conditions (Figure 1A). Heteroplasmy was not detected. Table I shows the number of variants per breed, and indicates those patterns unique to each breed. ARC and PPA horses had five distinct SSCP patterns, TB had four and ARB horses had eight patterns. The high variability suggested by the existence of 14 different SSCP variants is also in agreement with the comparison of three sequences corresponding to the entire D-loop published by Ishida *et al*. (1994a), who found most of the substitutions (11 out of 18) within the fragment amplified in the present work. Our results are also similar to those of Marklund *et al.* (1994), who found 15 SSCP variants in a similar fragment of the D-loop corresponding to 78 horses from five Scandinavian breeds, each one of these showing between 5 and 10 variants. In a study of the same portion of the D-loop, Kavar *et al.* (1999) found three SSCP variants in 16 maternal lines of

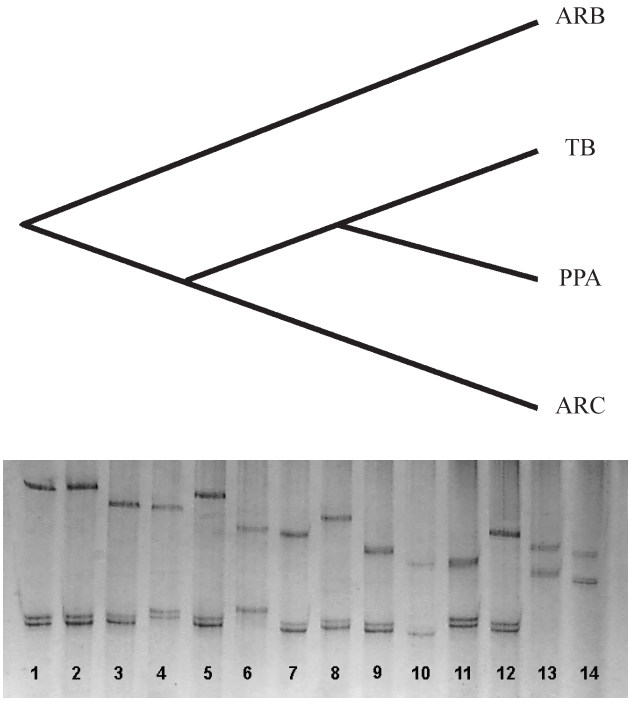

Figure 1 - A. Silver stained polyacrylamide gel showing the 14 SSCP variants detected in this study. B. Tree obtained with the Wagner parsimony method. ARC, Argentinean Creole; TB, Thoroughbred; PPA, Peruvian Paso from Argentina; ARB, Arabian.

Table I - Number and type of SSCP variants detected in each horse breed.

Breed	n	nV	SSCP variant													
			1	2	3	4	5	6	7	8	9	10	11	12	13	14
ARC	45	5	-	2	26	-	-	-	-	-	10	-	1	6	-	-
TB	7	4	-	-	2	-	-	-	-	1	-	-	-	3	-	1
PPA	30	5	-	5	-	-	12	-	-	6	-	1	-	6	-	-
ARB	18	8	1	3	-	1	-	6	3	-	1	-	-	-	1	3

ARC, Argentinean Creole; TB, Thoroughbred; PPA, Peruvian Paso from Argentina; ARB, Arabian. In bold, those variants that are unique for each breed.
n = number of individuals.
nV = number of SSCP variants.

the Lipizzan horse breed, which included 13 haplotypes. Furthermore, Bowling *et al.* (1998) found 28 base substitutions (corresponding to 22 haplotypes) in a study based on 398 bp of the D-loop from 43 Arabian horses. The results indicate a considerable degree of variation within the Arabian breed, which was found to be the most variable in our sample.

As it can be observed from Table I, Arabian and Peruvian Paso presented five and two characteristic patterns, respectively, not seen in the other breeds. Thoroughbred horses showed no characteristic pattern, and only one SSCP variant was found exclusively in Argentine Creole horses. Six SSCP variants were shared by the studied breeds, although none by the four breeds simultaneously. Each breed shared at least one variant with each one of the others. The most common variants in PPA (variant 5) or ARB (variant 6) were unique to those breeds, while the most common variants in ARC (variant 3) and TB (variant 12) were shared by TB and ARC and PPA, respectively.

A phenogram based on a discrete two-state character data matrix (0/1, 1/0; presence/absence of the SSCP variant or allele) using the Wagner parsimony method was constructed with the MIX program of the PHYLIP software package (Felsenstein, 1993). The tree (Figure 1B) shows a close relationship between PPA and TB with ARB as a more basal taxa followed by the Argentine Creole breed.

The interpretation of the results obtained is not straightforward. Arabian and TB are considered the foundation stock for many modern horse breeds (Bowling, 1994). They have probably contributed in varying degrees to the formation of American breeds. Arabian and Barbs were brought to America by the Spanish conquerors along with horses such as the Spanish Pure Breed and the Andalusian. Their ancestry to South American breeds has been suggested by blood group and polymorphic protein loci analyses (De Andrés Cara, 1982; Rodriguez-Gallardo *et al*., 1992). A correlation between some of these races based on five polymorphic protein loci (Peral-García *et al*., 1996) resulted in similar values between the ARC, Andalusian and PP from Peru. However, the Argentinean Peruvian Paso is a breed in formation and, therefore, even when stallions are brought from Peruvian Paso in Peru, the mares are frequently not pure PP. Their position on the tree in Figure 1B may be explained by the fact that the maternal history is followed through the mtDNA, which is probably of mixed ancestry. On the other hand, the position of the ARC breed, a well established breed in Argentina, in Figure 1B is not clear, although it is well differentiated from the other breeds examined. We must note here that this unclear position could be related to the missing information about the ancestral breeds of the analyzed samples.

The reliability of these results lies fundamentally on the assumption that each SSCP variant corresponds to a different haplotype. It is generally accepted that SSCP can detect even single base substitutions (Takeda *et al*., 1995; Ostellari *et al*., 1996). The resolution power of this method is crucially dependent on the fragment examined and on the conditions of the experiment (temperature, gel concentration, run length). Under different experimental conditions, we have always obtained the same patterns. If some point mutations did not produce a new variant, the variability would be underestimated and the results would represent a first approximation to the relationship and variability of the analyzed breeds. In conclusion, SSCP allowed the detection of extensive variability in PPA, ARC, TB and ARB horse breeds, which makes this technique a valuable first step in future phylogenetic studies, including sequencing techniques.

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