GENETIC DIVERSITY IN A POPULATION OF LUSITANO STALLIONS BORN OVER THREE DECADES

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Abstract

The Lusitano Horse (LH) is a Portuguese equine breed, with special economic relevance in the national and international scene and one of the most ancients in the world. A group of 2699 males of the LH breed, representatives of all possible paternal lineages of the breed, born over three decades (between 1985 and 2010) and registered as stallions in the Studbook, were genotyped with the current and the additional panel of microsatellite markers recommended for horse genotyping by the International Society for Animal Genetics.

All loci were polymorphic and informative. The genetic diversity, quantified by the expected heterozygosity, was 70 %, which is above the average estimated for the genetic diversity for American and other European breeds. The mean allelic richness was 8.19 for the whole group and the mean inbreeding index value was 3.4 %, which is within the average values for other equine breeds. All loci but two showed deficit in heterozygotes and only four were in Hardy-Weinberg Equilibrium.

Monitoring the genetic diversity of males along the years could contribute to informed decisions in the management of paternal lineages and for LH breeding and conservation strategies.

Keywords: *genetic diversity, parentage control, STR markers, Portuguese native equine breed, Lusitano Horse*

1. INTRODUCTION

The Lusitano Horse (LH) is, besides the Garrano, the Sorraia and the Pónei da Terceira, economically, the most important Portuguese autochthonous equine breed and one of the most ancients in the word. The LH has its origins in wild and domesticated animals from the Iberian Peninsula intermixed with animals brought by several migration events that occurred in the past (Oom 1992; Lopes et al. 2005). The horses from this gene pool contributed to the development of many other modern European horses and were later introduced and dispersed throughout the Americas, where they were the founders of numerous New World breeds (Gonzaga 2004; Luís et al. 2006). According to the DAD-IS Database (2022), the actual population of LH is 5200 individuals registered in the studbook of which 1500 are breeding males and 3500 breeding females distributed over 500 studs.

The official studbook of the LH in Portugal was established in 1967 and is managed by the Associação Portuguesa de Criadores do Cavalo Puro Sangue Lusitano (APSL). Since 1992, the regulations demand parentage testing to confirm the declared genealogies. These tests were initially performed by blood group analysis and proteins polymorphisms but since 1998, they are carried out exclusively

based on microsatellite markers (STRs) by the Laboratório de Genética Molecular de Alter of Instituto Nacional de Investigação Agrária e Veterinária (INIAV, IP).

The breed is reared in many countries, with Brazil recording the second largest population (da Silva Faria et al. 2018) followed by France and Spain (Vicente et al. 2012).

Sires enter reproduction at seven years and are trained and used in equestrian activities for several years, that implies that horses delay their long reproductive career of breeding individuals and leads to high generation interval, close to 11 years (Vicente et al. 2012) and consequently to the possibility of an increase in inbreeding (Cozzi et al. 2022). Thus, is very important to monitor the genetic diversity and the results could be compared with those of other breeds.

In this study we analyzed a group of 2699 stallions of the LH breed, born between 1985 and 2010 and registered in the Studbook ("Livro de Adultos", Book of Breeders section) providing information on the genetic variation and giving an indication of the levels of intra-breed variability. This group of stallions contains representatives of all possible paternal lineages of the Lusitano breed born over three decades.

2. MATERIALS AND METHODS

2.1. Population sampling

From the 41126 Lusitano samples stored at the Laboratório de Genética Molecular de Alter, between August 1998 and December 2012, we selected all males, alive (at the time of the study), not castrated, registered in the Book of Breeders and born between 1985 and 2010, making a total of 2699 animals. These animals were genotyped in a routine setting in paternity tests.

2.2. DNA extraction

Genomic DNA was extracted from whole blood samples, collected in 10 ml tubes with EDTA anti-coagulant, using the saline method (Montgomery and Sise 1990), resuspended in TE (pH 8.0) and preserved at 4 °C. The DNA was quantified by spectrophotometry and diluted to a final concentration of 3 ng/µl.

2.3. PCR amplification and Genotyping

The laboratory used six STRs (HMS3, HMS7, HTG4, HTG10, VHL20 and ASB2) until 2001, since they are extremely polymorphic, especially in breeds with Iberian influence (Luís et al. 2002). In 2001, three additional markers (AHT4, AHT5 and HMS6) were added from a panel recommended by the International Society for Animal Genetics (ISAG), making a total of nine markers that were systematically used until 2009.

From 2009 on, routinely the laboratory used 17 markers, of which 12 belong to the core panel recommended by ISAG (AHT4, AHT5, ASB2, HMS2, HMS3, HMS6, HMS7, HTG10, HTG4, VHL20, ASB17 and ASB23) and 5 to the additional ISAG STR panel (HTG6, HTG7, UCDEQ425, HMS1 and LEX3) (Table 2). From the additional panel of *loci* analyzed, LEX3 was excluded, since this marker is heterosomic, connected to the X-chromosome, meaning that it is homozygous in males, causing significant deviations to the Hardy-Weinberg Equilibrium (HWE) and high differences between expected and observed heterozygosity values (Zabek and Fornal 2009).

2.4. For 6 and 9 STRs

PCR amplification was performed with a 10 µL final solution containing 1 µL of genomic DNA, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, fluorescent and non-fluorescent primers with a concentration ranging from 0.5 pM to 3 pM, 0.5 U of Taq DNA polymerase and 1X reaction buffer. The amplification of the fragments was performed in a MJ-Research PTC-100TM thermal cycler (MJ Research Inc, Watertown. MA, USA), initiated with a denaturation step at 95 ºC (5 min), followed by 5 cycles with denaturation at 95 ºC (30 sec), hybridization at 58 ºC (30 sec) and extension at 72 ºC (30 sec) and 35 cycles with denaturation at 95 ºC (30 sec), hybridization at 57 ºC (30 sec) and extension at 71 ºC (30

sec) the last extension was extended for 10 min at 72 ºC. The *loci* HMS7, HMS3, HTG4 and VHL20, were amplified in tetraplex, the *loci* ASB2, HMS6 and HTG10 in triplex and the *loci* AHT4 and AHT5, in singleplex. PCR products were separated on denaturing 6 % polyacrylamide gels, 0.25 mm wide, using LI-COR 4000 and 4200 automated fluorescence sequencers (Li-Cor Biosciences, Lincoln, NE, USA). The resulting images were analysed with RFLPscan v.3.1 software (Scanalytics, CPS Inc., USA). The size of the DNA fragments was identified based on the 50-350 bp Licor internal size standard.

2.5. For 17 STRs

PCR amplification was performed using a commercial kit from Applied Biosystems (Applera Europe, Germany) with 17 primer pairs from which the forward primers are fluorescently labelled, following the manufacturer's instructions. Amplification was carried out in a MJ-Research PTC-100 ™ thermal cycler (MJ Research Inc., Watertown, MA, USA), starting with 95 ^{0}C denaturation for 10 min followed by 30 cycles with denaturation at 95 ^{0}C (30 sec), hybridization at 60 ^{0}C (30 sec) and extension at 72 ^{0}C (30 sec) with a last extension of 60 min at 72 ^{0}C, finishing at 4 ^{0}C. The amplified products were separated in an ABI 3130 Capillary Sequencer, with detection capacity for 5 fluorescent markers, 6-FAM, VIC, NED and PET. Each sample was run with the LIZ 500 marker and the G5 filter. The sizes of the resulting fragments for the 17 microsatellites were analyzed with Genemapper 4.0. This method was regularly checked and validated by the ISAG horse parentage comparison tests.

2.6. Statistical analysis

The analysis of the genetic diversity of the 2699 breeding males was estimated using the CERVUS 3.0.7 program (Marshall et al. 1998) by determining the total and mean number of alleles, their frequencies of observed (Ho) and of expected heterozygosity (He) (Nei 1978), the polymorphic information content (PIC) (Botstein et al. 1980) and the estimated frequency for the occurrence of null alleles (Summers and Amos 1997). Deviations from HWE with Fisher's exact test (Guo and Thompson 1992) were calculated by using Genepop 3.4 (Raymond and Rousset 1995). This test indicates the probability of error in rejecting the null hypothesis (H0) that if the population is in HWE (Raymond and Rousset 1995). The deficit of heterozygosity *per locus* (F_{IS}) and the mean value for all *loci* were estimated according to Weir and Cockerham (1984).

The Bayesian approach of STRUCTURE (Pritchard et al. 2000) was used to infer the genetic structure of the population. A 20.000 initial burn-in was used to minimize the effect of the starting configurations, followed by 100.000 MC iterations, as recommended by Falush et al. (2007) with 10 independent replicates each. Several sets of inferred clusters where tested to determine the most appropriate number of clusters for modeling the data. All runs used an admixture model with correlated frequencies and the parameter of individual admixture alpha set to be the same for all clusters and with a uniform prior.

The most likely number of clusters (K) was determined by calculating ΔK according to the Evanno method (Evanno et al. 2005) in Structure Harvester (Earl and von Holdt 2012).

3. RESULTS

For For the total of 2699 male Lusitano animals 131 alleles were obtained for the 16 *loci* analyzed, with a mean number of alleles of 8.19±2.40 *per locus*. The number of alleles *per locus* varied between 3 (HTG7) and 13 (ASB17), Ho ranged from 0.191 (HTG7) to 0.819 (VHL20), and He varied between 0.207 (HTG7) and 0.828 (ASB17) (**Table 1**). These values indicate that for the group of animals analyzed, the least polymorphic *locus* is HTG7 and the most polymorphic one is ASB17. The observed heterozygosity was lower than the expected one for all *loci*, with mean values of 0.678±0.151 and 0.700±0.155, respectively, except for UCDEQ425.

Locus	N	Fragments Size (bp)	n	HO	HE	PIC
AHT4[C]	2157	146-162	9	0.758	0.789	0.762
AHT5[C]	2100	128-146	8	0.759	0.792	0.759
ASB2[C]	2675	222-254	12	0.774	0.788	0.762
HMS2[C]	844	218-238	6	0.743	0.768	0.731
HMS3[C]	2689	146-168	8	0.793	0.824	0.803
HMS6[C]	2168	159-169	7	0.597	0.606	0.570
HMS7[C]	2688	173-187	8	0.657	0.697	0.644
HTG10[C]	2689	91-111	10	0.691	0.722	0.695
HTG7[A]	2697	129-141	7	0.642	0.648	0.592
HTG4[C]	2692	89-107	10	0.819	0.827	0.803
VHL20[C]	761	97-123	13	0.774	0.828	0.809
ASB17[C]	900	184-212	8	0.781	0.797	0.765
ASB23[C]	993	82-100	6	0.656	0.682	0.625
HTG6[A]	893	120-128	3	0.191	0.207	0.194
UCDEQ425[A]	713	224-244	9	0.658	0.656	0.594
HMS1[A]	847	176-192	7	0.551	0.571	0.499
Mean ± SE	-	-	8.19 ±2.40	0.678±0.151	0.700±0.155	0.678±0.158

Table 1. Genetic variability of the analyzed population for 16 *loci*.

Source: No. of alleles per *locus* (n); number of individuals analyzed (N); Heterozygosity observed (HO); Heterozygosity expected (HE); Polymorphic information content (PIC); ([C]) Markers from the ISAG core STR panel; ([A]) Marker from the ISAG additional STR panel.

All *loci* are very informative, with the PIC higher than 0.5, except for HTG7. Values ranged from 0.194 (HTG7) to 0.809 (ASB17).

According to the HWE test *per locus* with P <0.05, only 4 *loci* (HTG4, VHL20, HTG6 and HMS1) are in HWE (**Table 2**). All *loci* present deficit in heterozygotes, except for ASB2 and UCDEQ425, which corroborates the values obtained for the observed heterozygosity. For the total *loci* we observed a significant deficit of heterozygotes (F_{IS}=0.034), as confirmed by the test of χ^2, indicating that the population is not in equilibrium.

Locus	P	F_{IS}
AHT4[C]	0.0014	0.0636
AHT5[C]	0.0004	0.0347
ASB2[C]	0.0433	-0.0121
HMS2[C]	0.0375	0.0256
HMS3[C]	0.0005	0.0356
HMS6[C]	0.0119	0.0353

HMS7 [C]	0.0000	0.0382
HTG10 [C]	0.0031	0.0561
HTG7 [A]	0.0236	0.0629
HTG4 [C]	0.2324	0.0369
VHL20 [C]	0.3516	0.0402
ASB17 [C]	0.0000	0.0636
ASB23 [C]	0.0398	0.0084
HTG6 [A]	0.1658	0.0401
UCDEQ425 [A]	0.0137	-0.0208
HMS1 [A]	0.0555	0.0240
Mean ± SE	0.0612±0.1035	0.0340±0.0241

Table 2.

Source: Values obtained for 16 *loci* for the Hardy-Weinberg Equilibrium test (Genepop 3.4) with P <0.05; Probability (P); Heterozygosity Deficit (F_{IS}).

The Bayesian analysis carried out in STRUCTURE revealed that the most likely number of clusters was two, as ΔK showed a substantial increase at K=2 when K varied from 1 to 15, although some substructure was observed at K=5 and K=9 (Fig. 1).

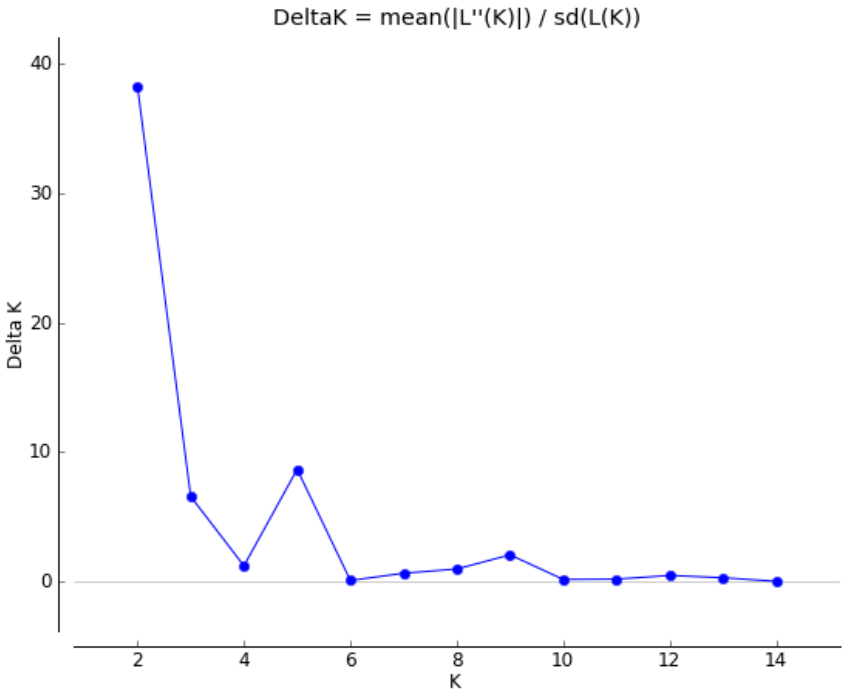


Fig. 1. Plot of ΔK obtained with STRUCTURE HARVESTER for detecting the number of genetic groups that best fit the Lusitano individuals analyzed.

4. DISCUSSION

In this study, genetic analysis was carried out using STRs, more specifically microsatellites, in a group of stallions of the Lusitano breed. We used STRs, because, to date, there is no recommended panel of SNP's for equine parentage testing. Thus, the ISAG panel of STRs is universally accepted and allows the data obtained to be compared with other data from the Lusitano breed, from other Portuguese native horse breeds and from other breeds from other countries. However, the variability values detected with STRs, according to Solis et al. (2005), should always be analyzed with caution, as these microsatellites are selected according to their high polymorphism. The fact that the samples used are composed only of male individuals is not limiting, since the microsatellites studied are in the autosomal genome.

For the *loci* analyzed the genetic analysis of the male population revealed a mean He of 70.00 % and a mean Ho of 67.78 %. The value of Ho detected is above the average value of 65 % estimated in Solis et al. (2005) for the American and European breeds, and higher than the values obtained by van de Goor et al. (2011) in 24 of the 35 breeds assessed, although the value presented refers only to the genetic diversity of the males of the Lusitano breed and not to the diversity of the Lusitano breed as a whole. The value of Ho identified for the analyzed male population is, curiously, very similar to that found in previous studies of the Lusitano breed when using six STRs (e.g. Luís et al. 2007; Vicente 2015) and when using a panel of 30 STR markers (Felicetti et al. 2010), which revealed a high genetic diversity, similar to that observed in most other equine breeds.

A deficit of heterozygosity of 3.4 % for most *loci* was also observed, a figure that is within the average values (1-4 %) recorded for other equine breeds (Cañon et al. 2000; Zuccaro et al. 2008; Vdovina and Yuryeva 2021).

The result for the structure analyses, that allows investigating the population structure and eventual admixture patterns among paternal populations, suggests that there could be two genetic clusters, probably caused by a low genetic distance among the different samples analyzed. The same result of two being the most likely number of clusters was also obtained in two other independent studies, one when analyzing 47 Lusitano animals and a set of 30 other microsatellite markers (Lopes 2011) and another when 36243 Lusitano animals were analyzed with the same 16 *loci* used in this study (Lopes et al. 2015).

Thus, as already verified in previous studies, the analysis of a male population shows that the Lusitano breed does not seem to present an immediate danger in its state of conservation. However, a significant deviation from the HWE equilibrium has been detected for most of the microsatellites analyzed, most probably resulting from the fact that different breeders will most likely subject their animals to different selection strategies. This may also explain the substructure observed at K=5 and K=9.

In this study, the possibility of occurrence of null alleles, which have not been amplified in the PCR and have been analyzed as "false" homozygous, is very low because all animals have been submitted to paternity tests, where false homozygous are detected.

In future, as ISAG establishes a recommended SNP Panel, it will be important to conduct further variability studies to more accurately determine intra and inter breeds variability.

5. CONCLUSIONS

This study aimed to assess the genetic diversity of a group of 2699 males of the LH breed, born between 1985 and 2010. Our study is the first on a group of Lusitano stallions' representatives of all possible paternal lineages of the Lusitano breed born over three decades using the current ISAG recommended panel of microsatellite markers (17 *loci*).

The genetic diversity detected shows that it is similar to other equine breeds reported in the literature. However, the breeds' diversity should be monitored and taken into account in order to guide decisions on the management and conservation of the paternal lineages of the Lusitano horse.

AUTHOR CONTRIBUTIONS

DNA Extraction: M.R. and M.G.; PCR amplification and Genotyping: S.A.F.; Validation by ISAG Test: S.A.F.; Statistical analysis: S.A.F., M.S.L and C.F. Conceptualization: S.A.F. and M.S.L.; Data Curation: S.A.F., M.S.L. and N.C.; Writing-original draft: S.A.F; Writing-review and editing: M.S.L., N.C. All authors have read and agreed to the published version of the manuscript.

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