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Genetic variability in local Brazilian horse lines using microsatellite markers

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ABSTRACT. Genetic variability at 11 microsatellite markers was analyzed in five naturalized/local Brazilian horse breeds or genetic groups. Blood samples were collected from 328 animals of the breeds Campeira (Santa Catarina State), Lavradeira (Roraima State), Pantaneira (Pantanal Mato-Grossense), Mangalarga Marchador (Minas Gerais State), as well as the genetic group Baixadeiro (Maranhão State), and the exotic breeds English Thoroughbred and Arab. We found significant genetic variability within evaluated microsatellite loci, with observed heterozygosis varying between 0.426 and 0.768 and polymorphism information content values of 0.751 to 0.914. All breeds showed high inbreeding coefficients and were not in Hardy-Weinberg equilibrium. The smallest genetic distance was seen between the Pantaneira and Arab breeds. The principal component

analyzes and Bayesian approach demonstrated that the exotic breeds have had a significant influence on the genetic formation of the local breeds, with introgression of English Throroughbred in Pantaneira and Lavradeira, as well as genetic proximity between the Arab, Pantaneira and Mangalarga Marchador populations. This study shows the need to conserve traits acquired by naturalized horse breeds over centuries of natural selection in Brazil due to the genetic uniqueness of each group, suggesting a reduced gene flow between them. These results reinforce the need to include these herds in animal genetic resource conservation programs to maximize the genetic variability and conserve useful allele combinations.

Key words: Animal genetic resources; Conservation genetics; *Equus caballus*; Livestock; Molecular markers

INTRODUCTION

The first horses were brought to Brazil by colonizers in the sixteenth century. Over the centuries these animals evolved and adapted to local environmental, sanitary and management conditions in the different habitats found in the country (Braga, 2000), giving origin to naturalized or local Brazilian breeds, also known as "creole" or "local adapted". These naturalized breeds have adapted to widely different natural environments such as the southern highlands (Campeira in Santa Catarina State), tropical northern Brazil (Lavradeira in Roraima and the genetic group Baixadeiro in Maranhão), the Pantanal in the central west (Pantaneira in the Pantanal Mato-Grossense), and southeast (Mangalarga Machador in Minas Gerais). Most of these breeds are used to manage cattle in native pastures (McManus et al., 2005, 2008) and changes in their genetic composition may affect their usefulness in production systems. The populations of these breeds decreased due to crossing, usually with English-Arabian, Arabian and English Thoroughbred, to improve conformation and increase stature (Beck, 1985), although the occurrence of diseases such as tripanosomiasis and equine infectious anemia increased.

The conservation of these breeds, which have been mischaracterized and have had their germplasm diluted, is justifiable due to their importance in their respective regions, as well as the maintenance of genes that are unique due to centuries of natural selection under Brazilian environmental conditions. As such, genetic characterization is an important tool for orientation of conservation strategies. While some breeds have highly active Breeder's Associations (the Pantaneira breed, for example), others are maintained in conservation nuclei by EMBRAPA. Some information is available on morphological data on these breeds (Cabral et al., 2004a,b; McManus et al., 2005, 2008) or from low information content RAPD markers that do not allow comparative analyses across independent studies (Egito et al., 2007).

Genetic characterization using microsatellite molecular markers is an important tool in the identification of genetic diversity due to their high degree of polymorphism and high occurrence frequency in the genome (Takezaki and Nei, 1996), thereby contributing to the orientation of conservation strategies.

The aim of this study was to estimate genetic variability in five breeds and/or genetic groups of naturalized horses in Brazil, comparing them with two exotic commercial breeds using 11 microsatellite markers to aid in the inclusion of these breeds in the genetic conservation program.

MATERIAL AND METHODS

Blood was collected from five breeds and genetic groups distributed throughout Brazil: Campeira (Santa Catarina State), Lavradeira (Roraima), Pantaneira (Pantanal Mato-Grossense), Mangalarga Marchador (Minas Gerais), and Baixadeiro (Maranhão). English Thoroughbred and Arab horses were also included as outgroups in the analysis. In all, 328 horses were analyzed (Table 1). The samples were deposited in the DNA and Tissue Bank at the Animal Genetics Laboratory of Embrapa Recursos Genéticos e Biotecnologia, CENARGEN, Brasilia, DF, Brazil.

Table 1. Description of samples collected per breed to analyze genetic diversity in Brazilian breeds.

Breed	Symbol	N	Number of localities sampled	Brazilian State
Campeira	EC	48	7	Santa Catarina
Lavradeira	EL	48	2	Roraima
Pantaneira	EP	48	6	Mato Grosso and Mato Grosso Sul
Baixadeiro	EB	45	3	Maranhão
Mangalarga Marchador	EM	43	3	Federal District, Goiás
Arab	EA	48	3	Federal District, Goiás
English Thoroughbred	ET	48	3	Federal District
Total	7	328	27	

N = number of animals sampled for each breed.

Genomic DNA was extracted from leukocytes following the adapted protocol of Miller et al. (1988). The material was collected in 10-mL vacuum tubes containing EDTA and kept refrigerated (5°C) until processing for lymfocyte separation. The post-separation steps were as follows: 1) centrifugation at 3000 rpm/10 min; 2) removal of layer of leukocytes with a Pasteur pipette and deposited in a polyethylene tube; 3) storage in a freezer (-20°C) for later extraction.

A total of 11 microsatellite markers, recommended by the Equine Genetics and Thoroughbred Parentage Testing Standardization Committee of the International Society for Animal Genetics (ISAG) (Hoffmann et al., 2004), were used in this study (Table 2).

Table 2. Microsatellites typified for characterization of breeds and genetic groups of horses in Brazil.

Loci	Chromosome	Sequence (5'-3')	Size (bp)	Reference
*HMS03**	9	ccaactctttgtcacataacaaga ccatcctcacttttttcactttgtt	151-185	Hoffmann et al., 2004
*HMS07**	1	caggaaactcatgttgataccatc tgttgttgaaacatacctttgactgt	161-195	Hoffmann et al., 2004
*HMS02**	10	acggtggcaatgtgtattaaatg ccaactctttgtcacataacaaga	209-247	Hoffmann et al., 2004
*HTG06**	15	cctgcttggaggctgtgataagat gttcactgaatgtcaaattctgct	85-103	Hoffmann et al., 2004
*HTG07**	4	cctgaagcagaacatccctccttg ataaagtgtctgggcagagctgct	111-127	Hoffmann et al., 2004
*VHL20**	30	caagtcctcttacttgaagactag aactcagggagaatcttcctcag	85-107	Hoffmann et al., 2004
LEX005	27	aaggcaatgcttatcaaatgc ttacccgcagtgacttctatt	225-293	Guérin et al., 2003
UMO11	20	tgaaagtagaaagggatgtgg tctcagagcagaagtccctg	157-191	Meyer et al., 1997
*HTG08**	9	caggccgtagatgactaccaatga ttttcagagttaattggtatcaca	167-201	Hoffmann et al., 2004
NHEQ100	1	ccaaagcagaacatgtgaagtt tggcatagatgttagctcagtga	189-223	Guérin et al., 2003
*HMS06**	4	gaagctgccagtattcaaccattg ctccatcttgtgaagtgtaactca	153-177	Hoffmann et al., 2004

Amplification using PCR was carried out in a final volume of 23 µL [9 ng genomic DNA, 10% Tris-HCL, pH 8.4, 1.0-2.0 µM $MgCl_2$ (depending on the locus used), 200 µM DNTP (8% of the final reaction volume), 2.5 mg/mL BSA (8% of the final reaction volume) and 1.5 IU Taq DNA polymerase as well as 1 µL of the primer (5 ng/µL)]. The amplifications were carried out in thermocyclers, with the initial denaturation of 5 min at 94°C, followed by 35 to 40 cycles of 1 min at 94°C, 52°-61°C (depending on the locus used) for 1 min and a final extension at 72°C for 30 min to reduce stutter bands.

The amplification products were visualized on a 6% polyacrylamide denaturing gel. Alleles were identified across gels by their appearance and position on the gel, according to a regression function (Schaffer and Sederoff, 1981) relative to 10-bp ladder (Invitrogen) and internal control (samples of known allele size) per locus on each gel.

Allelic frequency was estimated by direct counting. The FSTAT program (Goudet, 2002) was used to calculate allelic richness standardized for variations in population sample size. Breed differentiation was estimated using Wright's statistics (F_{IT}, F_{IS} and F_{ST}) using the methodology proposed by Weir and Cockerhan (1984) and the indicative P value adjusted using Bonferroni's procedure in the same program.

An analysis of Hardy-Weinberg equilibrium (HWE) was obtained using GENEPOP version 3.3 (Raymond and Rousset, 1995) and the principal component analysis was carried out using the PCA software (Goudet, 1999), comparing the values of F_{ST}.

The average exclusion probability given genetic information of both parents (Jamieson, 1994) was calculated from observed allele frequencies, using the Cervus 1.0 software (Marshall et al., 1998). The observed heterozygosity (H_O), expected heterozygosity (H_E) and the polymorphic information content (PIC) were also calculated with the Cervus 1.0 software.

A hierarquical analysis of molecular variance (AMOVA) was carried out using ARLEQUIN (Schneider et al., 2000). Based on genotypes at the 11 marker loci, individual animals were clustered into a given number of populations and assigned probabilistically to clusters inferred with a Bayesian approach implemented by the STRUCTURE software (Pritchard et al., 2000). The tests were performed based on an admixture model where the allelic frequencies were correlated applying a burn-in period of 40,000 and 80,000 iterations for data collection. Two to twelve inferred clusters were performed with five independent runs each. Results were entered into the DISTRUCT program (Rosenberg, 2004) to provide a graphic display. In order to find the best K-value that explained the pattern of genetic variation, a method proposed by Evanno et al. (2005) was employed.

RESULTS AND DISCUSSION

Farm animal genetic diversity is required to meet current production needs in various environments, to allow sustained genetic improvement and to facilitate rapid adaptation to changing breeding objectives (Notter, 1999). Genetic characterization is the first step in breed conservation and may have implications for future breeding strategies (Solis et al., 2005). The importance of naturalized horse breeds in Brazil has been shown in several studies. Silva et al. (2005) found that the Pantaneira horse was better adapted to its environment than exotic breeds in daily management of cattle, and Egito et al. (2007) conducted the first study with RAPD markers in herds of the Pantaneira horse.

An average of 14.36 alleles/locus was found (Table 3), similar to results found by other authors, Cunningham et al. (2001) and Tozaki et al. (2001), both with Thoroughbred

horses. This variability is higher than that found in RAPD studies. For example Bailey and Lear (1994) studying Arabian and Thoroughbred breeds found an average of 3.6 polymorphic bands/primer. In Brazil, Martins (1996), studying three Brazilian breeds (Lavradeira, Crioulo and Campolina) found 2.9 bands/primer, using 29 markers. Apostolidis et al. (2001) found 10.2 bands/primer and 51 polymorphic bands in Greek horses (Thessalian, Skyros Pony, Pinia, Cretan, and Andravida). These authors did not find any specific markers, while Bailey and Lear (1994) and Martins (1996) did find breed-specific RAPD markers for Arabian/Thoroughbred and Lavradeira horses, respectively. Microsatellite studies in horses also showed lower number of alleles per locus in Italian (Pieragostini et al., 2005), Portuguese (Luis et al., 2007) and Asian (Shahsavarani and Rahimi-Mianji, 2010) horses. This may be a reflection of a lack of structured breeding programs for these breeds as well as markers used and population structure. Allelic richness was the highest for HMS07 (15.248) and the lowest for HTG07 (7.86) with a mean of 12.48.

Table 3. Analysis of genetic structure per locus.

Locus	N_A	Mean N_A	PIC	H_O	H_E	F_{IS}[1]	PE (1)	PE (2)
HMS03	16	14.059	0.910	0.712	0.917	0.154	0.708	0.829
HMS07	17	15.248	0.914	0.678	0.921	0.204	0.722	0.838
HMS06	11	9.167	0.823	0.631	0.841	0.202	0.523	0.691
VHL20	12	11.062	0.886	0.759	0.897	0.126	0.648	0.788
HMS02	14	12.87	0.882	0.568	0.893	0.269	0.641	0.782
HTG08	18	13.828	0.887	0.635	0.897	0.251	0.656	0.792
HTG06	10	9.346	0.826	0.636	0.845	0.157	0.527	0.693
HTG07	10	7.86	0.751	0.678	0.782	0,070	0.407	0.586
LEX05	19	14.314	0.842	0.426	0.855	0.430	0.567	0.725
UMO11	15	11.611	0.864	0.768	0.877	0.102	0.599	0.751
NHEQ100	16	13.167	0.893	0.538	0.903	0.284	0.666	0.800
Mean	14.36	12.48	0.862	0.639	0.875	0.204	99.99%[2]	99.99%[2]

N_A = number of alleles per locus; PIC = polymorphic information content; H_O = observed heterozygosity; H_E = expected heterozygosity; F_{IS} = inbreeding coefficient; PE = exclusion probability knowing neither PE (1) or one PE (2) parent. [1]Significantly different ($P < 0.05$); [2]Overall probabilities of all loci.

PIC values here varied from 0.751 to 0.914, therefore all were informative. H_O averaged 0.637 (ranging from 0.426 for LEX05 to 0.768 for UMO11) while H_E had a mean of 0.875 (ranging from 0.782 for HTG07 to 0.921 for HMS07). This was higher than what was found by Bjornstad and Roed (2002) in the English Thoroughbred breed and by Luis et al. (2007) for the Sorraia breed but was similar to the findings of Shahsavarani and Rahimi-Mianji (2010) for the Caspian horse and Avdi and Banos (2008) for the Greek Skyros breed. Several markers displayed a significant deficit of heterozygotes due to within-population inbreeding in both subspecies and in the combined analysis. Such result has been commonly observed in surveys of horse breeds in other countries. The lower average H_O compared to the H_E may reflect the narrow genetic base of the current population of these breeds.

The breeds were not in HWE ($P < 0.001$). This can probably be attributed to small population size, nonrandom mating, inbreeding or genetic drift. The possible occurrence of null alleles could have led to false observation of homozygotes, which could account for more deviations from HWE (Shahsavarani and Rahimi-Mianji, 2010).

F_{IS} ranged from 0.070 (HTG07) to 0.430 (LEX05) with a mean of 0.204. Positive F_{IS} value suggested inbreeding to be one of the main causes for the shortage of heterozygotes in these populations. F_{IS} values were significant for excess homozygotes in all breeds, after a

Bonferroni's correction for multiple tests ($P < 0.05$). These results may reflect the effect of the use a small number of stallions in each generation or mating among closely related animals, especially in populations where there is no control over reproduction. The heterozygote deficiency could also be explained as a Wahlund effect if population subdivision is occurring, linkage with loci under selection (genetic hitchhiking), population heterogeneity, null alleles (non-amplifying alleles), or inbreeding.

The panel of loci studied was efficient for the exclusion of paternity, where all loci showed 99.99% exclusion probability when one or neither parent was known. These values are identical to those found by Tozaki et al. (2001) and higher than the ones found by Luis et al. (2002) (88.5 and 99.6% knowing one or neither parent, respectively). In the breed analysis, Pantaneira showed the best results, with an exclusion probability of 99.91% knowing a single parent, and Campeira the worst results with a probability of 99.59%.

AMOVA showed that 12.37% ($P < 0.001$) of the total genetic variance was between breeds (Table 4), similar to those found by Paiva et al. (2005) for sheep breeds and Egito et al. (2007) for cattle breeds in Brazil. These results showed that a reasonable genetic variability exists among the studied breeds when compared with other authors (Jordana, et al., 1999; Zabek et al., 2005). Careful selection of animals for breeding and conservation should be carried out to ensure that variability is maintained within these populations.

Table 4. Analysis of molecular variance for seven genetic groups of equines based on allele frequencies from 11 microsatellite markers.

Source of variation	d.f.	Sum of squares	% Variation	P values
Between breeds	6	332.424	12.37	0.00001*
Within breeds	649	2527.562	87.63	
Total	655	2859.986		

d.f. = degrees of freedom.

The data previously obtained are corroborated by the values of F_{ST} (Table 5) which had overall significance of $P < 0.0003$. The lowest genetic difference analysis by AMOVA was between the Pantaneira and Arab breeds, with a value of 7.4%, and the highest was between the English Thoroughbred and the genetic group Baixadeiro (16%). Baixadeiro was classified as the most distant in relation to the other breeds, emphasizing the need for conservation of this population. Management strategies need to be intensified to minimize inbreeding effects in these breeds. The highest distances were found with the Campeira horse, maybe due to its geographical isolation in the south of the country.

Table 5. Percentage of estimated genetic distances from F_{ST} index between naturalized and commercial breeds of horses in Brazil.

Breed	EC	EL	EP	ET	EA	EB
EL	0.11644					
EP	0.13137	0.10712				
ET	0.14040	0.12443	0.10607			
EA	0.14682	0.11749	0.07438	0.13135		
EB	0.13980	0.10688	0.11260	0.16666	0.11827	
EM	0.14940	0.12423	0.11900	0.12873	0.10780	0.12751

EP = Pantaneira; EA = Arab; EM = Mangalarga Marchador; ET = English Thoroughbred; EC = Campeira; EL = Lavradeira; EB = Baixadeiro. All results were significant ($P < 0.01$).

The results obtained in the pairwise F_{ST} values were confirmed using principal component analysis (Figure 1) highlighting the distribution of breeds and/or groups. The Arab, Pantaneira and Mangalarga Marchador are closest to each other compared to the rest of the breeds studied and the Baixadeiro and English Thoroughbred were the most divergent. The first and second components explained 23.34 and 22.33%, respectively, of the total variation observed between breeds.

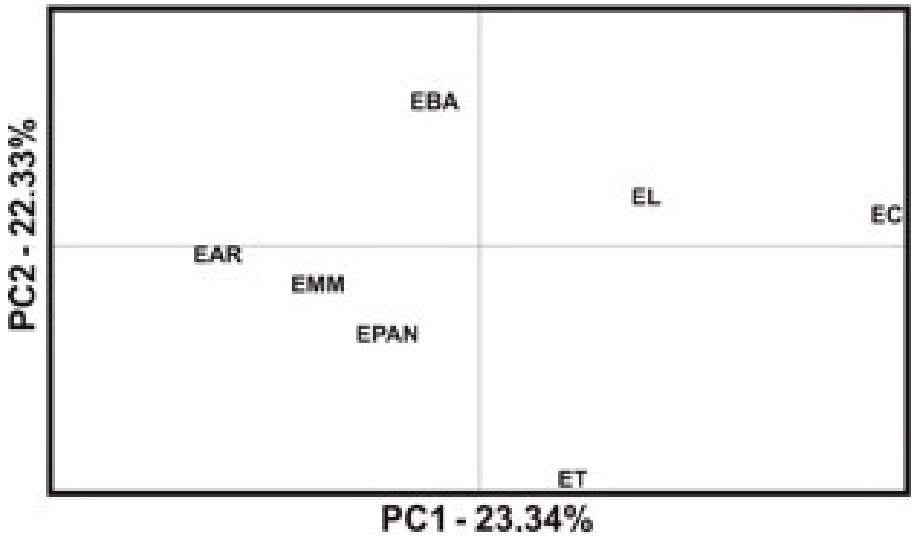

Figure 1. Analysis of principal components for five naturalized Brazilian horse breeds and two exotic breeds based on F_{ST} values from 11 microssatellite loci. EPAN = Pantaneira breed; EA = Arab; EMM = Mangalarga Marchador; ET = English Thoroughbred; EC = Campeira; EL = Lavradeira; EBA = Baixadeiro group.

Using the delta K-test, the highest signs were for K = 4 and K = 7 (Figure 2). With K = 4 it can be suggested that there are at least four sources of diversity for Brazilian horses (Figure 3). The first is formed by the Campeira, the second by Pantaneira and Arab, the third by English Thoroughbred and Mangalarga Marchador, and the last by Baixadeiro. The Lavradeira had alleles similar to the Campeira and Baixadeiro, in agreement with Figure 1. The closeness between the Baixadeiro and Lavradeira (K = 3) was also expected due to their geographical closeness in the north of the country. Another clear pattern was the importance of the Arab for the formation of the Pantaneira (K = 4, 5, 6) and the introgression of English Throroughbred in both Pantaneira and Lavradeira possibly indicating recent crossing events (Figure 3). With K = 7, all seven genetic groups are correctly identified. Here the genetic uniqueness of each group is seen, which suggested a reduced gene flow between them, unlike other studies with conservation animals in Brazil (ex., Egito et al., 2007; Sollero et al., 2009). Based on this analysis, all breeds were well-defined genetic entities meaning that their entrance in the conservation program is vital for the continued maintenance of valuable genetic variability for traits linked to disease and heat resistance and tolerance.

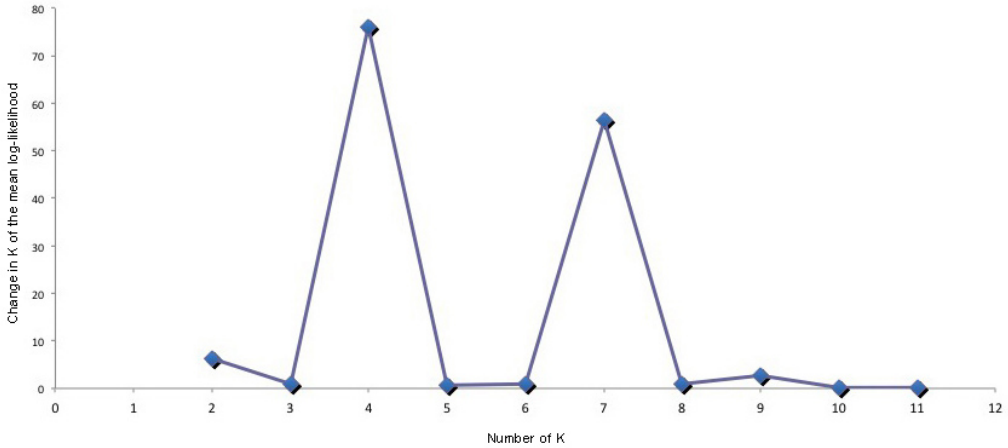

Figure 2. Delta K values from the mean log-likelihood probabilities from STRUCTURE runs where inferred clusters (K) ranged from 1 to 12.

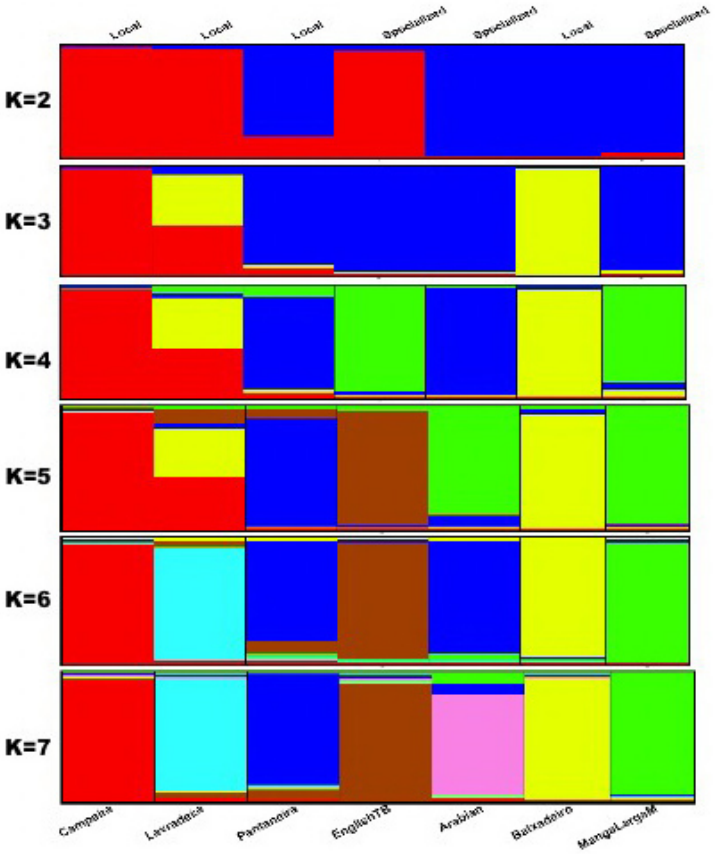

Figure 3. Graph of 328 individual matrices obtained from the STRUCTURE software analyses for K = 2 to K = 7 for Brazilian Horse Breeds.

To the best of our knowledge this is the most comprehensive report on the genetic structure and diversity of horse breeds in Brazil. Brazil has one of the largest horse populations in the world and widely different ecosystems to which native breeds are adapted. Silva Filho et al. (2007) studied the horses from Marajo Island in northern Brazil with commercial breeds (English Thoroughbred, Mangalarga Marchador, Brazilian Showjumper, and Quarterhorse) but only three microsatellites (VHL20, HTG04 and HMS07). Their results also showed a narrow genetic base for these breeds but with lower diversity due to the lower number of breeds.

Our results demonstrate that a loss of genetic variability in the five naturalized breeds and, consequently, the potential loss of the characteristics acquired for the breeds during the process of adaptation to the Brazilian environment. The limitation of their gene pool in microsatellite loci was a result of inbreeding events occurring in small populations and the use of exotic breeds (such as English Thoroughbred and Arab). However, significant variability seen using variance analysis classified the breeds studied as distinct groups. More studies are needed to increase the knowledge about naturalized horse breeds in Brazil. These results reinforce the need to include these herds in animal genetic resource conservation programs to maximize the genetic variability and conserve useful genes.

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