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Population genetic study over 32,000 equines from Uruguay using seventeen forensically informative STR loci

Dear Editor,

It is known that horses (*Equus caballus*) had an important role in many activities that influenced the development of various civilizations around the world. For many reasons, men set the selection of animals according to ability, skills and disposition desired, giving rise of specific groups of horses which have distinctive characteristics transmitted to offspring consistently, the breeds. It is estimated that there are currently about 300 breeds of horses [1]. Due to this ancient and close interaction between horses and humans, relevant forensically cases such as theft horses, identity fraud and, more recently, the sale of adulterated or counterfeited semen doses, and doping control are the most observed crimes. Therefore, it is recommended that forensic analysis techniques become more efficient as possible with increasingly improved accuracy.

Uruguay, a small country in the southeastern region of South America bordered by Argentina and Brazil, is characterized by the strength of the agricultural sector and livestock production. As the most recent data collected by OIE (World Organization for Animal Health), it is estimated that Uruguay has about 400,000 horses in its territory [2]. The Rural Association of Uruguay, the oldest institution of this country in agricultural matters, and the Uruguayan Stud Book, responsible for the registration and identification of all Thoroughbreds born in the country or imported, are pioneering institutions in Latin America in establishing typification of horses through DNA analysis [3,4]. For this reason, both institutions have grouped into their records thousands of genotypes of horses of various breeds representing significantly the horse population of Uruguay.

The aim of this paper is to present a genetic study of forensic interest of a total group up to 32,053 purebred horse of five different breeds: Appaloosa (n = 109), Arabian (n = 1661), Uruguayan Criollo (n = 5906), Thoroughbred (n = 23,203) and Quarter Horse (n = 1174), using the seventeen STR loci recommended by International Society for Animal Genetics (ISAG) for genotyping horses (http://www.isag.us/Docs/EquineGenParentage2014.pdf). All samples were obtained from animals subjected to pedigree registration on Rural Association of Uruguay or Uruguayan Stud Book. DNA was isolated from hair root or semen straws samples using proteinase K in two types of extraction buffer: Buffer 1 to hair root samples ($MgCl_2$, PCR Buffer and Tween 20) and Buffer 2 to semen straws samples (EDTA, SDS, Tris, NaCl and DTT). Final concentration after DNA extraction: 50–100 ng/μL of equine genomic DNA. The seventeen STR loci were amplified in a single PCR multiplex performed on Veriti™ Thermal Cycler (Applied Biosystems, Foster City, CA, USA) or GeneAmp 9700 PCR System (Applied Biosystems) using fluorescent labeled primers which the sequences are available on http://www.cstl.nist.gov/strbase/horseSTRs.htm. The other reagents for PCR amplification (PCR Buffer, AmpliTaq Gold DNA Polymerase, dNTP mix) were carried out using the StockMarks® for Horses Genotyping Kit (Applied Biosystems). The amount of equine genomic DNA was 25–50 ng/μL per sample. Thermocycling conditions were: pre-incubation for 10 min at 95 °C, followed by thirty cycles of 30 s at 95 °C, 30 s at 60 °C and 60 s at 72 °C, with a final incubation for 60 min at 72 °C. The electrophoresis and typing were performed on an ABI 3500 Genetic Analyzer (Applied Biosystems) using GeneScan™ 500 LIZ® Size Standard as internal lane standards according to the manufacturer's protocols. The data was collected by Data Collection v1.0 software (Applied Biosystems) and analyzed using GeneMapper ID-X v1.3 software (Applied Biosystems).

Calculations of allele frequencies, observed (Ho) and expected (He) heterozygosity, polymorphism information content (PIC) and p-values of the Hardy-Weinberg equilibrium (HWE) test for all seventeen loci, were assessed using CERVUS version 3.0.3 [5]. Bonferroni's correction was used for HWE test, which assumes that a 0.05 significance level obtained for seventeen tests (one per locus) yields an actual significance threshold of 0.0029 [6]. Power of discrimination (PD), power of exclusion (PE) and probability of identity (P_{ID}) were estimated with PowerStats (Promega Corporation) [7]. Estimated coefficients of inbreeding (F_{IS}) within breeds, fixation indices (F_{ST}) among breeds, and total inbreeding (F_{IT}) using an analysis of molecular variance (AMOVA) were performed with ARLEQUIN version 3.1 [8] and GENEPOP version 4.5.1 [9]. UPGMA trees were built from Nei's genetic distance matrix using the PHYLIP software package version 3.69 [10] and visualized with TreeView software version 1.6.6 [11]. The allele frequencies and forensically informative parameters are available in Supplementary data in the online version DOI: 10.1016/j.fsigen.2016.10.011 (Tables S1–S5).

The Hardy-Weinberg equilibrium test showed significant deviation ($P < 0.05$) in various loci in all five horse breeds of this study. Even after applying Bonferroni's correction using the total number of loci analyzed ($P < 0.0029$), the differences observed were also statistically significant in many loci on four of the five breeds, except Appaloosa, as seen in Table S1 in Supplementary data in the online version DOI: 10.1016/j.fsigen.2016.10.011. Regarding heterozygosity, in general, the observed (Ho) were lower than the expected (He) in almost all loci of five breeds. The loci with lower polymorphism information content (PIC) was HTG7 (Appaloosa, Arabian and Uruguayan Criollo) and HTG4 (Thoroughbred and Quarter Horse). The loci with higher PIC was ASB17 (Appaloosa and Uruguayan Criollo), VHL20 (Arabian), ASB2 (Thoroughbred) and LEX3 (Quarter Horse). The probability of

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Table 1
F_{IS}, F_{ST} and F_{IT} coefficients for five horse breeds to each of the seventeen loci and the overall.

Marker	F_{IS}	F_{ST}	F_{IT}
VHL20	0.0041	0.0448	0.0487
HTG4	0.0024	0.1416	0.1437
AHT4	0.0036	0.0251	0.0286
HMS7	0.0029	0.0404	0.0432
HTG6	0.0063	0.1665	0.1717
AHT5	0.0112	0.0938	0.1039
HMS6	−0.0011	0.0414	0.0403
ASB23	0.0085	0.0738	0.0817
ASB2	0.0198	0.0548	0.0735
HTG10	0.1141	0.0744	0.1800
HTG7	0.0106	0.1173	0.1267
HMS3	0.0226	0.1007	0.1210
HMS2	0.0103	0.2353	0.2431
ASB17	0.0021	0.0598	0.0618
LEX3	0.3653	0.0979	0.4274
HMS1	−0.0077	0.0314	0.0240
CA425	0.0217	0.0406	0.0614
All	0.0368	0.0845	0.1182

Table 2
Inbreeding coefficient (F_{IS}) estimated for five horse breeds.

Breed	F_{IS}
Appaloosa	0.0183
Arabian	0.0240
U. Criollo	0.0332
Thoroughbred	0.0374
Quarter Horse	0.0491

Table 3
Genetic distances (F_{ST} analysis) among five breeds of horses from Uruguay.

Breed	Appaloosa	Arabian	U. Criollo	Thoroughbred	Quarter Horse
Appaloosa	*	0.0677	0.0321	0.0607	0.0165
Arabian	0.0677	*	0.0765	0.1047	0.0720
U. Criollo	0.0321	0.0765	*	0.0876	0.0414
Thoroughbred	0.0607	0.1047	0.0876	*	0.0612
Quarter Horse	0.0165	0.0720	0.0414	0.0612	*

identity (P_{ID}), that indicates probability of two individuals within the population sharing the same genotype, showed the following overall values for each breed: 2.15×10^{-17} (Appaloosa), 2.41×10^{-15} (Arabian), 1.15×10^{-18} (Uruguayan Criollo), 2.28×10^{-15} (Thoroughbred) and 5.95×10^{-18} (Quarter Horse). The values obtained for combined Power of Discrimination (PD) and combined Power of Exclusion (PE) for seventeen markers were, respectively: 0.99999999999999999 and 0.99999 in Appaloosa breed; 0.999999999999999 and 0.9999 in Arabian breed; 0.99999999999999999 and 0.99999 in Uruguayan Criollo breed; 0.999999999999999 and 0.9999 in Thoroughbred breed; 0.99999999999999999 and 0.99999 in Quarter Horse breed.

In Table 1 the values of F_{IS}, F_{ST} and F_{IT} for each of the seventeen loci and the overall can be found. The positive values of F_{IS} (Table 2) indicate heterozygote deficiency and sub-structuration in all five

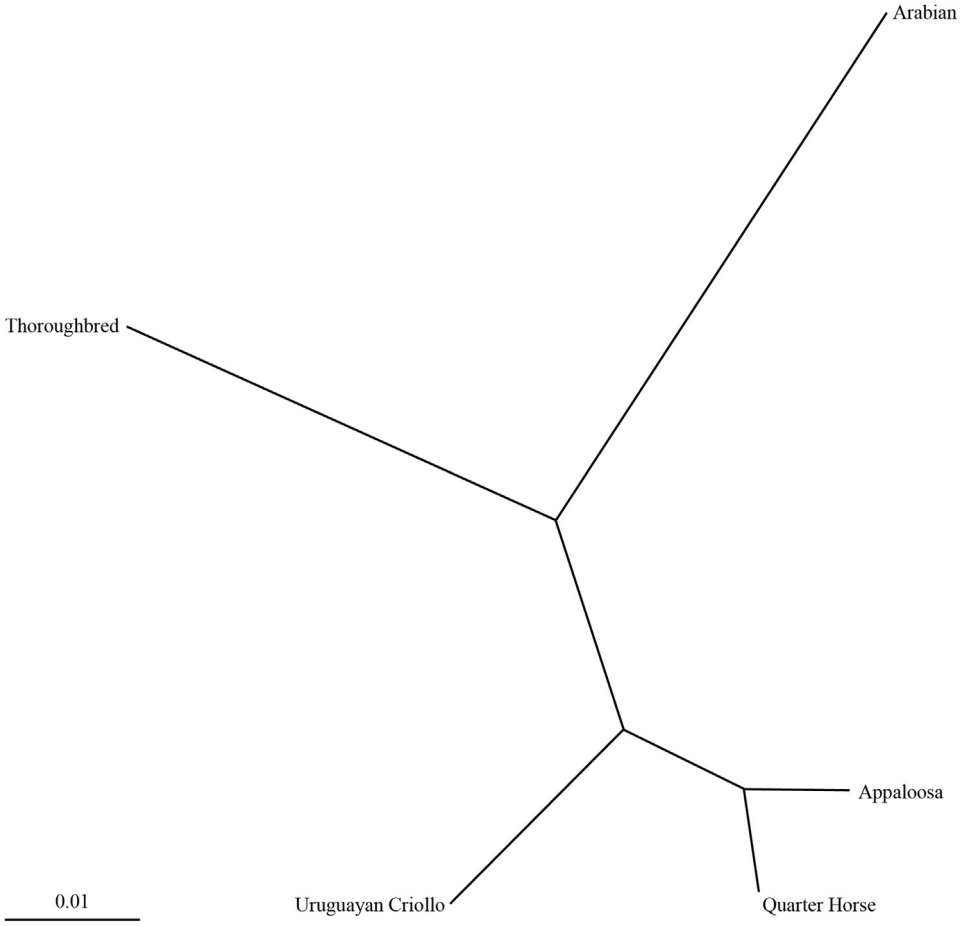

Fig. 1. Neighbour-joining tree based on pairwise Nei's genetic distances calculated between five breeds of horses from Uruguay.

horse breeds, being Quarter Horse and Thoroughbred the breeds with higher level of inbreeding. Based on genotypic frequencies of the seventeen STRs of this study, pairwise genetic distances were calculated between the five breeds, using Nei's formulas implemented in PHYLIP software (Table 3). The analysis showed a clear separation between these breeds (Fig. 1). The major genetic distance in these five breeds is between Arabian and Thoroughbred breeds, while the breeds which are closer from each other are Appaloosa and Quarter Horse, corroborating the historical data that indicate the time and the way of each horse breed arose [12–21].

Based on the largest publication and one of the most important on allelic frequencies of the seventeen STR markers present in our study [1], we compared the most frequent alleles at each locus in three horse breeds that are common in both works (Appaloosa, Arabian and Thoroughbred). In many loci, the most frequent allele of the reference publication is not the same as our study. Regarding the comparison for Uruguayan Criollo and Quarter Horse breeds, since there were no publications with this set of seventeen STR loci, it was made with studies using common genetic markers with the present study (fewer markers) [17,22–24]. In all studies, in many loci, the most frequent allele is not the same as our study.

This study presents the allele frequencies and other forensically informative parameters of seventeen STR loci that have not been published yet with horses from Uruguay. The presentation of these data, with these set of genetic markers, for Uruguayan Criollo and Quarter Horse breeds, is one of the first publications in the scientific community. However, considering the large number of animals for both breeds used in this study, and the results obtained, it is feasible to assume that these data can be used for forensic and kinship analysis, genetic identification and phylogenetic reconstruction. Finally, we consider important to determine the allelic frequencies and other parameters of forensic interest and genetic identification with animals in the same breed, in significant sample size and a certain geographic region, similar to what is recommended for human populations [25,26]. Thus, common and rare alleles of the location will be known, allowing to identify characteristics of each population as the occurrence or not of sub-structuration, linkage to traits under selection, Wahlund effect, bottleneck effect, etc. [22].

This paper follows the guidelines for publication of population data requested by the journal [27], the recommendations of International Society for Forensic Genetics (ISFG) regarding the use of non-human (animal) DNA in forensic genetic investigations [28] and the proposed allele nomenclature for seventeen equine-specific STR loci [29] as recommended by the ISFG for the nomenclature of human STRs [30].

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