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Microsatellites in Portuguese autochthonous horse breeds: usefulness for parentage testing

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Abstract

The use of DNA technology for parentage testing is increasing every day. Most laboratories have improved their exclusion probabilities (PE) by the addition of DNA microsatellite loci to standard blood-typing results. The efficacy of each *locus* depends on the number of alleles detected and their frequencies in the breeds tested. Here we analyzed the usefulness of six microsatellites for routine parentage testing in three Portuguese autochthonous horse breeds: Lusitano, Sorraia and Garrano. The DNA loci analyzed - ASB2, HMS3, HMS7, HTG4, HTG10, and VHL20 - were chosen based upon the polymorphism detected in other breeds with Iberian horse influence. The estimated probability of exclusion of wrongly named parents (PE) was high, with values ranging from 88.5% to 99.6%.

Key words: microsatellites, DNA, Portuguese horse breeds, parentage testing.

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Introduction

Breed registry authorities have adopted parentage testing programs worldwide to assure horse pedigree integrity, which is essential for breeding management plans. The most efficient genetic markers for such use are co-dominant and inherited by mendelian principles. Traditionally, in most laboratories, a battery of 14 to 17 systems (7 blood groups and 10 protein polymorphisms) has been used (*e.g.* Bowling, 1996). DNA testing techniques, now available and progressively adopted by most responsible laboratories, can greatly improve the success of parentage tests, providing an excellent alternative to traditional methods. They have the attractive features that they can be highly automated, require only small amounts of biological samples, are not restricted to fresh blood samples and the techniques involved are simpler and cheaper.

Microsatellites (SSR or STR) are highly polymorphic genetic markers with co dominantly inherited alleles that are relatively easy to score. They consist of strings of tandem repeats of short motifs ranging from mono- to pentanucleotides (Schlötterer and Pemberton, 1998) with variability in the number of repeats. Highly variable STR loci are common in mammalian genomes and can readily be typed by PCR (Polymerase Chain Reaction) followed by electrophoresis.

A number of STR markers are now in use for parentage testing in several horse breeds (*e.g.* Binns *et al.*, 1995; Bowling, 1996; Bowling *et al.*, 1997; Meriaux *et al.*, 1998; Gralack *et al.*, 1998; Vega-Pla *et al.*, 1998; Wimmers *et al.*, 1998), and other breed registries are now in the process of converting to a DNA based technology. Since 1998, parentage testing for Lusitano horses born in Portugal and Brazil, as well as for all horse breeds with officially recorded pedigrees (Studbook), is performed based exclusively on DNA technology, at the Laboratório de Genética Molecular of the National Stud Service/Portuguese Ministry of Agriculture (Coudelaria de Alter).

The designation and number of microsatellites that should be used in parentage testing is yet a matter of discussion and depends on the characteristics of each locus and on the variability of the breed under study (Double *et al.*, 1997). At the referred Laboratory, 6 horse microsatellites, chosen for being highly polymorphic in other Iberian breeds, are being used. Here we present preliminary results obtained for the three Portuguese autochthonous horse breeds and the efficacy that can be accomplished using this set of microsatellite markers.

Material and Methods

Blood samples were collected in EDTA-K Venoject tubes from 227 Lusitano, 35 Sorraia and 88 Garrano horses.

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Genotyping was performed by the analysis of 6 dinucleotide horse microsatellites: ASB2 (Breen *et al.*, 1997), HMS3, HMS7 (Guérin *et al.*, 1994), HTG4 (Ellegren *et al.*, 1992), HTG10 (Marklund *et al.*, 1994), and VHL20 (Van Haeringen *et al.*, 1994). Whenever an exclusion was observed based upon only one of the above marker *loci*, three additional *loci* were tested: HMS2 (Guérin *et al.*, 1994), HTG6 (Ellegren *et al.*, 1992) and LEX23 (Coogle *et al.*, 1996).

DNA was extracted from whole blood samples by the high salt extraction procedure (Montgomery and Sise, 1990), suspended in TE pH 8.0 and stored at 4 °C.

PCR amplifications were performed in 0.2 mL tubes in a MJ-Research PTC-100 thermocycler, using 5 cycles with denaturation at 95 ºC (30 s), annealing at 58 °C (30 s) and extension at 72 °C (30 s) followed by 35 cycles with denaturation at 95 °C (30 s), annealing at 57 °C (30 s) and extension at 72 °C (30 s). The first denaturation step was performed at 95 °C for 5 min and the last extension was prolonged to 10 min at 72 °C.

The PCR mix, with a final volume of 10 µL, consisted of 1X PCR reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs mixture, 0.2 to 2 pmol of fluorescence labelled and unlabelled primers and 1U of *Taq* DNA Polymerase (Promega). HMS7 and HTG4 were amplified in a multiplex reaction.

PCR products were separated by electrophoresis in 6% Long Ranger denaturing gels (0.25 mm thick) for 1-2 h using an automated fluorescence 4000S LI-COR sequencer.

Gel image results were analyzed with RFLPScan 3.1 (Scanalytics, CPS Inc., USA). The DNA fragments were sized by reference to the Li-Cor STR marker. Allele identifi-

Table I - Allele frequencies, observed heterozygosity (Ho), expected heterozygosity (He) and polymorphic information content (PIC) for Lusitano (Lus), Sorraia (Sor) and Garrano (Garra) horse breeds. The exclusive alleles are underlined and the most frequent allele, for each breed, appears in bold.

		Alleles and Frequencies													
Locus	Breed	C	I	J	K	L	M (244)[1]	N	O	P	Q	R	Ho	He	PIC
ASB2	Lus	0.002	0.040	0.002	0.123	0.009	0.156	0.101	0.112	0.015	**0.370**	0.068	0.775	0.796	0.771
	Sor	-	0.114	-	0.129	-	-	0.014	**0.443**	-	0.300	-	0.686	0.694	0.631
	Garra	-	0.034	-	0.188	0.006	**0.364**	0.125	0.068	0.017	0.148	0.051	0.739	0.791	0.760
				G	I	J	M (160)	N	O	P	Q	R			
HMS3	Lus			0.013	0.247	-	0.108	**0.236**	0.093	0.141	0.090	0.053	0.780	0.832	0.808
	Sor			-	-	-	-	-	-	**0.786**	-	0.214	0.371	0.342	0.280
	Garra			-	0.023	0.023	0.119	0.011	0.210	**0.523**	0.068	0.023	0.625	0.666	0.625
				J	K	L	M (179)	N	O	P	Q				
HMS7	Lus			0.033	0.071	0.291	0.106	0.128	**0.357**	0.002	0.013		0.709	0.756	0.718
	Sor			-	0.157	0.086	**0.757**	-	-	-	-		0.400	0.400	0.358
	Garra			-	0.091	**0.301**	0.193	0.068	0.222	0.063	0.063		0.852	0.807	0.775
					K	L	M (133)	N	O	P	Q				
HTG4	Lus				0.289	0.093	**0.423**	0.128	0.042	0.011	0.015		0.714	0.712	0.667
	Sor				**0.400**	0.171	0.300	-	0.129	-	-		0.686	0.714	0.651
	Garra				0.091	0.085	**0.534**	0.176	-	0.097	0.017		0.739	0.662	0.625
				I	K	L	M (101)	N	O	P	Q	R			
HTG10	Lus			0.053	0.082	0.093	0.093	0.004	**0.476**	0.009	0.073	0.119	0.678	0.729	0.705
	Sor			-	-	-	-	-	0.100	-	-	**0.900**	0.086	0.183	0.164
	Garra			0.040	0.040	0.119	0.080	0.085	**0.290**	0.040	0.063	0.244	0.886	0.825	0.799
			I	J	K	L	M (97)	N	O	P	Q	R			
VHL20	Lus[2]		0.148	0.015	0.002	0.033	0.181	0.167	0.176	0.029	0.060	**0.189**	0.753	0.847	0.826
	Sor		0.029	-	-	-	0.129	**0.543**	-	-	0.157	0.143	0.686	0.652	0.603
	Garra[3]		0.097	0.040	0.011	0.034	**0.301**	0.102	0.085	0.125	0.034	0.171	0.886	0.838	0.815
											Mean	Lusitano	0.735	0.779	0.749
												Sorraia	0.486	0.498	0.448
												Garrano	0.788	0.765	0.733

[1]Base pair size of M allele in parenthesis. [2]χ^2 = 4.510; p = 0.033, 1 d.f. [3]χ^2 = 3.868; p = 0.049, 1 d.f.

cation was based on their base-pair size and alphabetically designation was in accordance with the results of ISAG Horse Comparison test 1999/2000 (Minnesota, USA). Data were stored in RFLPDataBase 3.1 (Scanalytics, CPS Inc., USA) and then transferred to an internal database.

The average exclusion probability (PE) given genetic information of both parents (Jamieson, 1994) was calculated from observed allele frequencies, using the software program Cervus 1.0 (Marshall *et al.*, 1998). The observed heterozygosity (*Ho*), expected heterozygosity (*He*) and the polymorhic information content (*PIC*) also were calculated with the Cervus 1.0 software. Deviation from the Hardy-Weinberg equilibrium (HWE) was calculated for each locus, in each breed, using the χ^2-test with pooling performed in the BIOSYS-1 computer program (release 1.7) (Swofford and Selander, 1989).

Results and Discussion

For the six analyzed microsatellite loci, exclusive alleles were detected for two of the breeds: four within the Lusitano (ASB2 - C and J, HMS3 - G, HMS7 - J) and two within the Garrano (HMS3 - J) (Table I).

Lusitano and Garrano show, in general, high values for He and PIC, with the HTG4 locus being the least variable for both breeds (Table I). Both breeds presented deviation from HWE in the VHL20 locus (Table I) probably due to population sub-structuring. Nevertheless, this deviation is unlikely to be significant for the overall PE determination. The number of alleles was greatly reduced for the Sorraia horse, with only 2 alleles at the HMS3 and HTG10 loci. Those results were expected as a consequence of the high level of inbreeding in the population (Oom and Cothran 1994), so the HWE tests were not performed for these two loci. He and PIC values also were very low with the HTG4 locus being the most variable in this breed.

We compared the efficacy of traditional techniques (7 blood groups and 10 protein polymorphisms) with that of the new DNA technology in the same breeds (Table II). This set of 6 microsatellites loci proved to be very useful for paternity assignment both for Lusitano and Garrano breeds as shown by high PE values (0.996 and 0.995, respectively). As we can see, such an exclusion probability of 99% could only be accomplished using a total of 17 loci of the conventional blood-typing techniques. For the Sorraia horse, due to the reduced number of alleles and the heterogeneous distribution of allele frequencies, this set of microsatellites is not so efficient (PE = 0.885). Additional microsatellite loci will be required for an efficacious test for this breed. In fact, considering the reduced variability of this breed, the standard 17 systems of blood-typing techniques provide values of PE considerably higher than that obtained for the 7 microsatellite loci. Nevertheless, microsatellites can provide greater efficacy with fewer systems even for highly inbred populations, as suggested by Binns *et al.* (1995).

Table II - Individual and cumulative probability of exclusion values for the three horse breeds (Lusitano, Garrano, Sorraia) based on DNA methodology (6 microssatellites) and traditional blood typing methods (10 protein polymorphisms and 7 blood groups).

	DNA methodology			
	Locus	Lusitano	Sorraia	Garrano
Microsatellites	ASB2	0.617	0.433	0.600
	HMS3	0.663	0.140	0.441
	HMS7	0.540	0.203	0.615
	HTG4	0.540	0.082	0.442
	HTG10	0.478	0.449	0.654
	VHL20	0.687	0.415	0.678
	Cumulative	**0.996**	**0.885**	**0.995**
	Traditional blood typing methods[4]			
	Locus	Lusitano	Sorraia	Garrano
Protein polymorphisms	TF	0.632	0.279	0.559
	A1B	0.044	0.083	0.153
	AL	0.175	0.142	0.183
	PGD	0.070	0.016	0.032
	ES	0.206	0.308	0.255
	GC	0.010	0.000	0.100
	PGM	0.033	0.008	0.126
	GPI	0.086	0.182	0.044
	HB	0.241	0.176	0.199
	PI	0.655	0.610	0.533
	Cumulative	**0.951**	**0.900**	**0.938**
Blood groups	A	0.290	0.349	0.378
	C	0.184	0.000	0.182
	D	0.663	0.284	0.714
	K	0.002	0.031	0.000
	P	0.241	0.176	0.493
	Q	0.438	0.269	0.314
	U	0.185	0.166	0.187
	Cumulative	**0.932**	**0.773**	**0.959**
	Cumulative	**0.997**	**0.977**	**0.997**

[4] (Cothran, unpublished data) - Lusitano (N = 304), Sorraia (N = 60), Garrano (N = 88).
Protein polymorphism symbols: TF – Transferrin; A1B – A1B Glicoprotein; AL – Albumin; PGD – 6-Phosphogluconate; ES – Carboxylesterase; GC – Group-specific component; PGM – Phosphoglucomutase; GPI – Glucose phosphate isomerase; HB – Hemoglobin; PI – Protease inhibitor.

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