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Effectiveness of nine polymorphic microsatellite markers in parentage testing in Posavina, Croatian Coldblood and Lipizzaner horse breeds in Croatia

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Abstract

The global biodiversity crisis extends to autochthonous local breeds of livestock. There is an increasing danger that these rare breeds become extinct and with them their locally adapted gene pool. Modern molecular tools such as parentage testing using microsatellite genotyping are powerful in guiding management and conservation. We tested nine microsatellite markers in three Croatian horse breeds and obtained high exclusion probabilities (EPs) for the most common test scenario 'one parent and offspring known and the other parent tested' (99.9% in Posavina and Croatian Coldblood and 99.3% in Lipizzaner), despite that Lipizzaner has an overall lower genetic variability at microsatellite loci. To become a useful tool in breed management in countries with developing economies, genetic screening systems must be designed to be statistically powerful yet economically viable. Therefore, a suite of six markers that can be run in two multiplex systems and which still gives high exclusion probabilities (99.5% in Posavina and Croatian Coldblood and 98% in Lipizzaner) was chosen.

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1. Introduction

An important aspect of animal breeding is accurate validation of relatedness and efficient management of pedigrees. With the emergence of the Polymerase Chain Reaction (PCR) and discovery of microsatellites, relatedness analysis, paternity testing and pedigree control in general have adopted the new technologies which have advantages over previously used methods such as blood typing (Binns et al., 1995; Bowling et al., 1997). Paternity analysis generally follows either an exclusion approach (Jamieson and Taylor, 1997), a maximum likelihood approach (Marshall et al., 1998) or a combination of both (e.g. Goossens et al., 2002). Molecular screening is relatively expensive. Especially for countries with emergent economies, the development of a robust and cost-effective marker system is essential if this powerful technology is to be made widely accessible for breeding or conservation (Goossens et al., 2002). Efficient marker systems require easily scored markers that can be multiplexed.

The Posavina and Croatian Coldblood are indigenous horse breeds of Croatia. Although their histories are interwoven to a great extent by interbreeding, the Posavina horse breed has been recognized as a Croatian autochthonous breed with unique features and breeding area, which makes it distinct from the Croatian Coldblood. The breeding area of the Posavina is on the flood plains of the river Sava and its tributaries. From early spring until late autumn, the mares with foals live unrestrained in open fields and are stabled only during floods and snowfalls. Mares often foal in the fields as well and only stallions are kept stabled all year round. The breed is very well adapted to harsh environmental conditions, it is resistant to disease, modest and tractable (Kovač, 1994). The creation of organised studbooks for the Posavina and Croatian Coldblood is recent. The Posavina studbook was started in the early 1990s and finally established in 1999, but work is still in progress for Croatian Coldblood studbook. Constructing a good pedigree is of vital importance for both breeds in order to preserve these native breeds as part of world livestock biodiversity heritage. In contrast to the other two breeds, the Lipizzaner has a long history of organised breeding. The studbook for Lipizzaner was established in 1806 in Croatia. The number of

horses of all three breeds varied substantially in the past, but the latest official report states that there were 1600 Posavina, 1322 Croatian Coldblood and 691 Lipizzaner registered in their respective studbooks in 2002 (Anonymous, 2003).

Here, we evaluate nine microsatellite loci in parentage testing in three Croatian horse breeds (Posavina, Croatian Coldblood and Lipizzaner) and design a marker system for future low-cost genotyping, which will give high combined exclusion probabilities (EPs), but at the same time will balance the statistical power of parentage testing with the effort required.

2. Materials and methods

Blood samples were collected from 53 Posavina, 37 Croatian Coldblood and 33 Lipizzaner in Croatia, in vials with EDTA (K₃). The samples were taken from unrelated animals and included both females and males. Samples of Posavina and Croatian Coldblood horses were taken from several different locations, and for Lipizzaner, they were taken from Djakovo stables. DNA was extracted from 500 µL of whole blood after digestion with proteinase K, separation with NaCl and chloroform and precipitation with isopropanol. The DNA pellet was dissolved in 150 µL of sterile distilled water (Gomerčić, 2000). We utilised nine horse-specific microsatellite loci: HTG4 (Ellegren et al., 1992); HTG7, HTG10 (Marklund et al., 1994); HMS2, HMS3, HMS6 (Guérin et al., 1994); VHL20 (Van Haeringen et al., 1994); ASB2; AHT5 contained in the Equine Paternity PCR Typing Kit, StockMarks for Horses (PE Applied Biosystems). PCR amplification was carried out in a 12 µL reaction containing 10 mM Tris-HCl, 200 mM (NH₄)₂SO₄, 50 µM each dNTP, 1.5 mM MgCl₂, 5 ng of BSA, 0.1 U Amplitaq® Gold DNA polymerase (Perkin Elmer), and between 0.5 and 0.75 µM fluorescent primer and non-fluorescent primer, and 2 µL DNA. Thirty PCR cycles were used (initial denaturation 94 °C for 1 min, 94 °C for 15 s, between 45 and 48 °C for 30 to 60 s, 72 °C for 60 s, followed by a final extension at 72 °C for 2 min) using the Perkin Elmer Gene Amp PCR System 9700. All PCR products were electrophoretically separated using an ABI PRISM™ 377 DNA sequencer (Perkin Elmer). Allele sizes were scored against the size standard GS350 ROX

(Perkin Elmer) using GeneScan™ Analysis 2.1 and Genotyper™ 2.1 software.

Allelic diversity (number of alleles), observed (H_o) and expected (H_e) heterozygosities and F_{is} values (Weir and Cockerham, 1984) were obtained using GENEPOP 3.1b (Raymond and Rousset, 1995). We calculated mean allelic diversities using 1000 simulation sampling of $n=33$ individuals, which is the smallest sample of breeds, in order to correct for sampling bias in the calculation of total allelic diversity using POPASSIGN (Goossens et al., 2002). In order to quantify the average capability of the microsatellites used for the parentage analyses, we calculated exclusion probabilities for three most likely scenarios arising in pedigree control testing (Jamieson and Taylor, 1997). First scenario: one parent and offspring are known and the other parent is tested (one-parent exclusion probability, formula 1a in Jamieson and Taylor, 1997). Second scenario: one parental genotype is unavailable, the offspring is known and the other parent is tested (missing-parent exclusion probability, formula 2a in Jamieson and Taylor, 1997). Third scenario: both parents are known and offspring is tested if it is falsely attributed to those two parents (two-parent exclusion probability, formula 3a in Jamieson and Taylor, (1997)). The exclusion probabilities are based on observed allele frequency distributions assuming Hardy–Weinberg equilibrium (HWE). Deviations from HWE were evaluated for all locus–population combinations by calculating HWE probabilities with the program GENEPOP 3.1b (Raymond and Rousset, 1995) using complete enumeration for loci up to four alleles and a Markov chain method for loci with more than four alleles. P -values were corrected for multiple statistical tests by the Bonferroni method. Exclusion probabilities were calculated using POPASSIGN (Goossens et al., 2002).

3. Results

All tested loci were highly polymorphic (Table 1). Allelic diversity in Lipizzaner was 4.78 ± 0.40 S.E. across loci. Posavina and Croatian Coldblood have higher allelic diversities (approximately 7.0 across loci) even after correction for higher sample sizes by

simulation sampling (Table 1). Number of alleles ranges from 5 to 10 in Posavina, from 4 to 9 in Croatian Coldblood and from 3 to 6 in Lipizzaner (Table 1).

The inbreeding index F_{is} indicates no inbreeding within the three breeds, but F_{is} for locus HMS6 was high in Coldblood horses (Table 1). Only 1 of 27 tests for deviation from Hardy–Weinberg equilibrium was significant after a table-wide sequential Bonferroni correction for multiple tests (Table 1, HMS6 Coldblood horses, $P < 0.05$). Significant deviation from HWE combined with substantial heterozygote deficit at locus HMS6 is likely to indicate locus-specific genotyping problems due to null alleles.

The Posavina and Croatian Coldblood have identical values for combined exclusion probabilities and they range from 98.6% to 99.9995%, whereas exclusion probabilities for the Lipizzaner are lower (they range from 93% to 99.97%), as expected from the lower allelic diversity and heterozygosity in this breed (Table 1). The individual exclusion probabilities range from 5.8% (locus HTG4, missing parent scenario, Lipizzaner) to 90.1% (locus VHL20, two-parent scenario, Croatian Coldblood). All the individual and combined exclusion probabilities can be found in Table 2.

An exclusion probability of 99% is statistically powerful, but requires a large number of loci especially for the one-parent and missing-parent scenarios. We deemed an exclusion probability of 95% is not powerful enough and we chose a cut-off point of 97.5% for the design of a low-cost marker system. We applied three criteria for combination of loci: locus-specific exclusion probabilities, the capability of combining selected loci in the lowest number of multiplex systems possible and the exclusion of loci where significant deviations from HWE might indicate null alleles or other genotyping-related problems. Using six loci (VHL20, HTG10, ASB2, AHT5, HMS3 and HMS2, Table 2) instead of nine only reduces combined exclusion probabilities for missing-parent scenario below chosen cut-off point (Table 2). Exclusion probabilities for one-parent and two-parent scenarios stay above cut-off point in all three breeds. Loci for the two multiplexing systems for a set of six selected loci are marked in Table 2.

Table 1

Number of alleles (A_{obs} and A_{sim}), expected heterozygosity (He), observed heterozygosity (Ho), F_{is} values and the probability of the data under the assumption of the null hypothesis of Hardy–Weinberg equilibrium (P_{HWE}) for nine microsatellite loci and three Croatian horse breeds

	Locus	A_{obs}	A_{sim}	He	Ho	F_{is}	P_{HWE}
Posavina ($n=53$)	VHL20	9	8.80	0.843	0.830	0.024	0.265
	HTG10	9	8.57	0.813	0.789	0.040	0.597
	ASB2	8	7.84	0.785	0.804	-0.015	0.328
	AHT5	7	6.59	0.767	0.906	-0.171	0.327
	HTG7	5	5.00	0.769	0.750	0.034	0.542
	HMS3	6	5.94	0.749	0.789	-0.043	0.621
	HMS2	10	8.89	0.699	0.642	0.092	0.769
	HMS6	6	5.75	0.679	0.585	0.148	0.021
	HTG4	6	5.59	0.675	0.654	0.041	0.442
	mean±S.E.	7.33±0.58	7.00±0.51	0.753±0.02	0.750±0.03	-0.076	
Croatian Coldblood ($n=37$)	VHL20	9	9.00	0.875	0.892	-0.006	0.228
	HTG10	8	7.77	0.782	0.889	-0.123	0.193
	ASB2	9	8.76	0.751	0.784	-0.030	0.131
	AHT5	7	6.99	0.764	0.838	-0.083	0.939
	HTG7	4	4.00	0.734	0.703	0.056	0.210
	HMS3	6	5.90	0.749	0.784	-0.033	0.917
	HMS2	8	7.79	0.739	0.722	0.037	0.200
	HMS6	5	4.87	0.685	0.432	0.380	0.002*
	HTG4	5	5.00	0.730	0.694	0.062	0.436
	mean±S.E.	6.78±0.62	6.68±0.60	0.756±0.02	0.749±0.05	-0.048	
Lipizzaner ($n=33$)	VHL20	5	N/A	0.614	0.688	-0.104	0.434
	HTG10	6	N/A	0.653	0.727	-0.099	0.993
	ASB2	6	N/A	0.770	0.875	-0.121	0.015
	AHT5	5	N/A	0.729	0.719	0.030	0.627
	HTG7	3	N/A	0.665	0.697	-0.033	1.000
	HMS3	5	N/A	0.730	0.781	-0.055	0.838
	HMS2	6	N/A	0.734	0.781	-0.049	0.663
	HMS6	4	N/A	0.586	0.697	-0.174	0.585
	HTG4	3	N/A	0.340	0.364	-0.053	1.000
	mean±S.E.	4.78±0.40	N/A	0.647±0.04	0.703±0.05	-0.086	

Number of alleles was calculated based on all samples (A_{obs}) and based on 1000 simulation samplings based on 33 individuals only (A_{sim}).

* P_{HWE} -values are significant after Bonferroni correction at the 5% level.

Table 2

Allele size ranges and exclusion probabilities for nine loci in the Posavina, Croatian Coldblood and Lipizzaner horse for the one-parent, missing-parent and two-parent scenarios (1, M and 2, respectively)

	Allele size range	Posavina			Croatian Coldblood			Lipizzaner		
		1	M	2	1	M	2	1	M	2
VHL20*	88–106	0.688	0.520	0.859	0.746	0.592	0.901	0.375	0.208	0.555
HTG10 ⁺	92–114	0.633	0.458	0.814	0.578	0.400	0.762	0.426	0.248	0.619
ASB2 ⁺	222–254	0.599	0.418	0.790	0.56	0.376	0.761	0.555	0.375	0.739
AHT5*	129–141	0.571	0.388	0.764	0.569	0.386	0.765	0.488	0.314	0.667
HTG7	120–130	0.544	0.366	0.723	0.485	0.311	0.660	0.369	0.221	0.517
HMS3 ⁺	152–170	0.527	0.349	0.713	0.525	0.347	0.709	0.505	0.325	0.693
HMS2*	220–240	0.500	0.313	0.707	0.533	0.350	0.729	0.506	0.327	0.691
HMS6	159–169	0.429	0.263	0.609	0.428	0.263	0.603	0.300	0.175	0.445
HTG4	128–138	0.429	0.260	0.610	0.492	0.316	0.674	0.152	0.058	0.247
Combined EP ^{9loci}		0.999	0.986	0.999995	0.999	0.986	0.999995	0.993	0.930	0.9997
Combined EP ^{6loci}		0.995	0.959	0.9999	0.995	0.960	0.9999	0.980	0.884	0.999

For efficient screening, a set of six loci was selected (multiplexing system 1 indicated by *, system 2 indicated by ⁺) to achieve maximum exclusion probability for all breeds by using the smallest possible number of loci.

4. Discussion and conclusion

The present study describes the utility of nine microsatellite markers in pedigree management in three Croatian horse breeds. It is of practical significance to estimate the probability of exclusion in different breeds (Marklund et al., 1994). We calculated exclusion probabilities for three possible scenarios in parentage testing and we received the highest values for two-parent exclusion probability and the lowest values for missing-parent exclusion probability in all three breeds, as expected (Jamieson and Taylor, 1997). Other authors have evaluated microsatellite markers in parentage testing in different horse breeds. Marklund et al. (1994) used 10 microsatellite loci, which yielded a combined exclusion probability of 96–99% in different horse breeds, whereas only nine loci used in our study yielded higher combined exclusion probabilities (>99%) for one-parent scenario, which is the most frequent scenario in parentage testing.

Our results for the Posavina and Croatian Coldblood using only six chosen loci (combined exclusion probabilities of 99.5%) are similar to those obtained by Zabek et al. (2003). They used six loci for parentage testing among Silesian and Thoroughbred horses, which yielded an exclusion probability greater than 99%, which increased to 99.9% when 12 loci were used. Luis et al. (2002) used six microsatellite loci, five of which are the same loci we used and also obtained combined exclusion probabilities over 99% for Lusitano and Garrano, but much lower (88.5%) for Sorraia horse breed. Jakabova et al. (2002) suggested using five microsatellite loci with a combined exclusion probability of 98.45% to resolve paternity cases in Thoroughbred horses in Slovakia. Current blood typing is calculated to have exclusion probabilities from 97% for paternity testing (Binns et al., 1995) to 99.7% (Luis et al., 2002). However, for conventional blood-typing techniques, a total of 17 loci are needed to accomplish the similar exclusion probability as with only six microsatellite loci. For a missing-parent scenario, combined exclusion probabilities using only six loci are below cut-off point in all three breeds and indicate less powerful parentage testing. In this case, one should consider using additional loci.

The exclusion probabilities act, however, only as guidelines for the power of parentage testing under

given scenarios of candidate parents and availability of samples. The test will be less powerful if candidate males are related or if the missing-parent scenario applies. Nevertheless, these six loci are expected to verify a putative parent or to exclude all but one male as parent in most standard applications where only a small number of candidate males exist and which are all tested, even if candidate males are related with each other. Therefore, the six loci will allow a fast and efficient screening, especially since they can be co-amplified in two PCR reactions and subsequently jointly electrophoretically scored. Only those cases where two or more males cannot be excluded as parents require the use of more than the standard six loci. Assignment of parentage using microsatellite loci is a useful tool in generating and managing reliable pedigrees, and important aspect of that usage is the total cost that can be minimized by careful selection of markers systems which often will be breed-specific. Here, we have given suggestions on how many and which microsatellite markers to use so that the whole system of parentage testing balances the power of the test with the effort and cost required. Managing pedigrees are essential for the future success and conservation of these Croatian horse breeds and other native breeds.

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