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Microsatellites in Lithuanian native horse breeds: usefulness for parentage testing

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DNA technology is now the standard tool for equine parentage testing. Here we analyze two twelve microsatellite panels for the routine parentage testing in three Lithuanian horse breeds: Þemaitukai, Heavy-type Þemaitukai and Lithuanian Heavy Draught. The DNA loci analyzed were *VHL20, HTG4, AHT4, HMS7, HTG6, AHT5, HMS6, ASB2, HTG10, HTG7, HMS, HMS2, ASB17, ASB23* and *LEX33.* The estimated probability of exclusion of wrongly named parents (*PE*) was high, with the values ranging from 99.91% for Þemaitukai and 99.99% for Heavy-type Þemaitukai and Lithuanian Heavy Draught.

Key words*:* microsatellites, DNA, horses, parentage testing, exclusion probability

INTRODUCTION

A correct pedigree is important for any domestic horse breed whether rare or not. For breeds that are common, an incorrect pedigree can frustrate breeding plans for selective improvement of the breed. For rare breeds, correct pedigrees are important for developing breeding strategies that minimize inbreeding. Parentage verification validates the horse pedigrees that make up the studbooks and thus is an important function of breed registries. In most laboratories providing horse blood typing service, a battery of about 15 systems of blood markers has been used. The efficiency of the test in revealing an erroneously assigned stallion (or mare) is in the range 90–97% [1–3] depending on breed and the composition of the test. The average capability of any marker system to exclude any given relationship is conditioned by the genotypes of the reported relatives, by the frequency occurrence of marker allelomorphs in a particular breed and by the number of independent marker systems tested. Three general formulae to calibrate the average capability of marker systems to dispute falsely reported pedigree records recently have been described by Jamieson and Taylor [4]. Most laboratories have improved their exclusion probabilities (*PE*) by the addition of DNA microsatellite loci to standard blood typing results or completely replacing them by DNA analysis. Microsatellites are 2–4 bp simple sequence repeats interspread through the genome. DNA testing techniques have several advantages over the traditional parentage testing methods in terms of ability to use a range of easily obtained sample tissues including hair, and in ease of laboratory analysis with commonly available molecular reagents. Also, the technique is highly automated, microsatellites are relatively easy to score, and often they are highly polymorphic. A number of studies with different sets of microsatellite markers and their usefulness for parentage testing have been reported for common [5, 6] and indigenous horse breeds [7–9].

Until recently, conservation efforts have focused on wild species, but now domesticated animals are recognized as an important part of biodiversity and more efforts to save rare breeds are made. The conservation of the various breeds in their tradition forms allows them to serve as reminders of the history and culture of various human groups. Loss of the breeds will result in lost choices for future generations. Since different populations hold different genetic variants, a useful tool in assessing and evaluating genetic information is blood typing or DNA-typing. The laboratories using routine genotyping to check pedigree records use appropriate sets of primers to respective species and breeds that they test. Here we present results for three Lithuanian horse breeds and the efficiency that can be accomplished using two different sets of microsatellite markers.

MATERIALS AND METHODS

Fresh blood was collected in a preserving buffer EDTA from 31 Þemaitukai, 30 Þemaitukai heavy type and 24 Lithuanian Heavy Draught horses. DNA

Table 1. **Allele frequency for Þemaitukai (ZO), heavy type Þemaitukai (ZH) and Lithuanian heavy draught (LHD)**

Locus	Breed	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V
	ZO				0.113			0.016	0.403	0.065	0.016	0.387						
VHL20	ZH				0.067	0.033		0.05	0.150	0.050	0.517	0.083	0.050					
	LHD				0.104			0.125	0.021	0.188	0.146	0.271	0.062	0.083				
	ZO						0.081	0.307	0.274		0.193			0.145				
HTG10	ZH				0.033		0.218	0.150	0.133	0.083	0.350			0.033				
	LHD						0.042	0.062	0.125	0.083	0.271		0.021	0.375	0.021			
	ZO						0.048	0.048	0.452	0.145	0.065	0.242						
HTG4	ZH						0.083	0.016	0.617	0.167		0.117						
	LHD						0.104	0.104	0.312		0.292	0.188						
	ZO					0.226	0.096	0.032	0.032	0.307	0.307							
AHT5	ZH				0.033	0.300	0.167	0.050	0.083	0.234	0.133							
	LHD				0.041	0.25	0.125	0.021	0.146	0.188	0.188	0.041						
	ZO			0.258		0.226				0.032	0.436	0.048						
AHT4	ZH			0.217	0.067	0.217	0.117	0.083		0.067	0.150	0.083						
	LHD			0.208	0.125	0.271	0.062	0.042			0.271	0.021						
	ZO								0.467	0.113	0.065	0.236	0.129					
HMS3	ZH								0.068	0.150	0.033	0.333	0.033	0.333	0.050			
	LHD				0.042					0.062	0.062	0.438	0.292	0.104				
	ZO							0.742	0.194		0.048	0.016						
HMS6	ZH						0.233	0.100	0.267		0.150	0.250						
	LHD						0.062	0.397	0.062		0.125	0.333	0.021					
	ZO							0.177		0.500	0.323							
HMS7	ZH					0.067		0.233	0.350	0.050	0.283		0.017					
	LHD					0.167	0.021	0.208	0.188	0.333	0.083							
	ZO				0.129	0.016	0.387			0.371	0.032	0.065						
ASB2	ZH				0.017		0.100		0.366	0.150	0.017		0.350					
	LHD				0.438		0.062	0.062	0.146	0.167		0.021	0.104					
	ZO			0.145		0.016	0.209			0.097		0.468	0.065					
HTG6	ZH			0.183	0.017	0.150	0.200					0.450						
	LHD			0.021		0.021	0.104				0.062	0.792						
	ZO							0.468		0.339	0.032	0.161						
HTG7	ZH							0.217			0.167	0.616						
	LHD							0.177		0.229	0.187	0.417						
	ZO			0.016	0.774				0.098	0.032			0.032		0.048			
HMS2	ZH				0.317		0.017	0.416	0.217						0.033			
	LHD				0.479	0.042	0.188	0.125	0.104	0.041					0.021			
	ZO	0.050			0.067	0.417	0.033			0.283	0.05			0.033	0.067			
ASB17	ZH	0.050				0.017	0.050		0.100	0.400	0.067		0.100	0.100	0.117			
	LHD	0.187					0.083	0.021	0.125	0.167	0.021	0.208	0.042	0.146				
	ZO				0.017	0.250	0.033	0.033							0.083		0.583	
ASB23	ZH				0.117	0.150	0.283	0.100							0.067		0.283	
	LHD				0.042		0.187	0.062					0.021		0.187	0.125	0.354	0.021
	ZO						0.083	0.117	0.200		0.383		0.030	0.183				
LEX33	ZH		0.050					0.267	0.450			0.017		0.133	0.083			
	LHD		0.167					0.104	0.271	0.021		0.083	0.021	0.208	0.104		0.021	

was extracted from whole blood using Puregene DNA extraction Kit (Gentra).

Two typing panels were tested. The first panel consisted of the microsatellites *VHL20*, *HTG4*, *AHT4*, *HMS7*, *HTG6*, *AHT5*, *HMS6*, *ASB2*, *HTG10*, *HTG7*, *HMS3* and *HMS2* [5, 10–14]. Amplification of microsatellites in multiple PCR reactions was performed in 25 µl total volume reactions containing 50 ng of genomic DNA, from 0.07 to 0.8 pmol of primers, 1xPCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs and l U AmpliTaq. For the second panel HMS2, HTG6 and HTG7 were replaced with ASB17, ASB23 and LEX33 [15, 16].

For microsattelite amplification a hot start procedure was used, in which DNA template and primers were combined and heated at 95 °C for 10 min. The temperature was then lowered and held at 85 °C for 10 min for addition of the remaining reagents. Thirty 1-minute cycles at 95 °C, 30 s at 56 °C, 30 s at 72 °C for multiplex reaction with four primers, AHT5, ASB2, HTG10 and HMS3, was used. For the other multiplex group, 30 1-minute cycles at 95 °C, 30 s at 60 °C and 30 s at 74 °C were used. The cycling was completed with a final extension at 72 °C for 30 min for both multiplex groups.

The PCR products were analyzed by polyacrylamide gel electrophoresis followed by automated multicolor fluorescence technology for fragment analysis. The DNA separation and analysis was done using the ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA), fragment sizes of microsattlite alleles were determined using the STRand computer software [17]. Alphanumerical nomenclature was used for allele size designa-

tion in accordance with the International Society for Animal Genetics.

Allele frequencies, the observed number of alleles (*Na*), the effective number of alleles (*Ne*), observed heterozigosity (*Ho*) and expected heterozigosity (*He*) were calculated using the Popgene package version 1.31 [18]. We used Cervus 2.0 software [19] to calculate the polymorphic information content (*PIC*) and the average exclusion probability (*PE*).

Table 2. **Individual and cumulative values of observed heterozigosity (*Ho*), expected heterozigosity (*He*), polymorphic information content (*PIC*) and average exclusion probability (*PE*), observed number of alleles (*Na*) and effective number of alleles (*Ne*)**

Locus	Breed	Na	Ne	Ho	He	PIC	PE
	ZO	6	3.031	0.774	0.681	0.61	0.412
VHL20	ZH	8	3.232	0.733	0.702	0.667	0.498
	LHD	8	5.969	0.958	0.850	0.812	0.669
	ZO	5	4.271	0.935	0.778	0.727	0.544
HTG10	ZH	7	4.569	0.800	0.794	0.751	0.583
	LHD	8	4.114	0.833	0.773	0.723	0.550
	ZO	6	3.419	0.806	0.719	0.668	0.482
HTG4	ZH	5	2.332	0.400	0.581	0.533	0.349
	LHD	5	4.174	0.833	0.777	0.721	0.537
	ZO	6	3.996	0.742	0.762	0.707	0.520
AHT5	ZH	7	4.986	0.733	0.813	0.771	0.609
	LHD	8	5.760	0.917	0.844	0.803	0.654
	ZO	5	3.219	0.806	0.701	0.636	0.435
AHT4	ZH	8	6.545	0.967	0.862	0.829	0.694
	LHD	7	4.721	0.792	0.805	0.756	0.586
	ZO	5	3.297	0.710	0.708	0.656	0.467
HMS3	ZH	7	3.939	0.700	0.759	0.707	0.526
	LHD	6	3.368	0.667	0.718	0.659	0.472
	ZO	4	1.693	0.516	0.416	0.365	0.207
HMS6	ZH	5	4.534	0.867	0.793	0.743	0.563
	LHD	6	3.429	0.708	0.723	0.660	0.470
	ZO	3	2.594	0.613	0.625	0.540	0.330
HMS7	ZH	6	3.781	0.700	0.748	0.690	0.498
	LHD	6	4.448	0.750	0.792	0.741	0.564
	ZO	6	3.230	0.645	0.702	0.636	0.440
ASB2	ZH	6	3.448	0.767	0.722	0.660	0.464
	LHD	7	3.853	0.708	0.756	0.711	0.539
	ZO	6	3.360	0.645	0.717	0.669	0.485
HTG6	ZH	5	3.346	0.900	0.713	0.656	0.461
	LHD	5	1.557	0.333	0.365	0.338	0.199
	ZO	4	2.773	0.677	0.650	0.571	0.364
HTG7	ZH	3	2.198	0.667	0.554	0.486	0.291
	LHD	4	3.459	0.792	0.726	0.662	0.462
	ZO	6	1.630	0.387	0.393	0.370	0.226
HMS2	ZH	5	3.103	0.767	0.689	0.616	0.409
	LHD	7	3.388	0.833	0.720	0.672	0.494
			Cumulative 1				
	ZO	**5.167**	**3.043**	**0.688**	**0.654**	**0.596**	**0.9985**
	ZH	**6.000**	**3.834**	**0.750**	**0.727**	**0.676**	**0.9998**
	LHD	**6.417**	**4.020**	**0.760**	**0.737**	**0.688**	**0.9999**
	ZO	8	3.791	0.833	0.742	0.694	0.518
ASB17	ZH	9	4.687	0.867	0.800	0.767	0.617
	LHD	9	6.545	0.667	0.865	0.829	0.692
	ZO	6	2.455	0.567	0.598	0.538	0.350
ASB23	ZH	6	4.737	0.867	0.802	0.758	0.589
	LHD	8	4.589	0.833	0.799	0.753	0.587
	ZO	6	4.081	0.767	0.771	0.724	0.546
LEX33	ZH	6	3.321	0.633	0.711	0.655	0.465
	LHD	9	5.731	0.833	0.843	0.803	0.656
			Cumulative 2				
	ZO	**5.500**	**3.235**	**0.722**	**0.683**	**0.624**	**0.9991**
	ZH	**6.667**	**4.176**	**0.752**	**0.757**	**0.711**	**0.9999**
	LHD	**7.25**	**4.725**	**0.792**	**0.795**	**0.748**	**0.9999**

RESULTS AND DISCUSSION

Allele frequencies at the microsatellite loci of Lithuanian horse breeds are given in Table 1. Observed heterozigosity (*Ho*), expected heterozigosity (*He*), the observed number of alleles (*Na*), the effective number of alleles (*Ne*), polymorphic information content (*PIC*) and the average exclusion probabilities (*PE*) are given in Table 2.

The total number of alleles at all 15 loci found was 82 in Þemaitukai horses, 93 in Þemaitukai heavy type and 103 in Lithuanian Heavy Draught. Only 3 alleles were found in Þemaitukai horses at *HMS7* and 4 alleles at *HTG7* and *HMS6*. Þemaitukai heavy type had three alleles in the *HTG7* locus. The least number of alleles in all breeds was observed in the *HTG7* locus. The microsatellite loci *ASB17* and *VHL20* were the most polymorphic in Lithuanian horse breeds. The heterozygosity was highest in Lithuanian Heavy Draught (0.76) and lowest in Þemaitukai horses (0.688) for the first set of primers and for the second set 0.79 and 0.72, respectively. The *PIC* values were lowest at the loci *HMS6* and *HMS2* in Þemaitukai horses, at *HTG7* in Þemaitukai heavy type and at *HTG6* in Lithuanian Heavy Draught. The first set of markers is highly efficient in Þemaitukai, Þemaitukai heavy type and Lithuanian Heavy Draught horses, with *PE* values 0.9985, 0.9998 and 0.9999, respectively. The second set of markers was even more efficient, with the probability of excluding wrongly named parents ranging from 99.91% for Þemaitukai to 99.99% for heavy-type Þemaitukai and Lithuanian Heavy Draught.

DNA-based typing has replaced blood group and protein marker typing in most laboratories due to its

high efficacy. In common breeds such as Thorouhgbreds, Arabians as well as in indigenous breeds such as Sorraia, Noric horse and many others, *PE* values exceding 0.99 have been reported [6–8]. A similar efficacy is observed in the Lithuanian breeds. DNA testing will have an important place in the conservation of rare breeds by ensuring that pedigrees are correct. This will allow a precise management of inbreeding levels and preservation of specific lineages. For routine horse parentage testing in Lithuania we would recommend to use the second set of microsatellites, which is more efficient than the first set.

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LIETUVOS VIETINIØ VEISLIØ ARKLIØ KILMËS TYRIMAS PAGAL DNR MIKROSATELITØ ÞYMENØ POLIMORFIZMÀ

S a n t r a u k a

DNR technologija paremti tyrimai, kaip ir kraujo grupiø bei baltymø polimorfizmo tyrimai, pripaþinti arkliø kilmës patikrinimo standartine priemone. Ðiame straipsnyje mes apþvelgiame du dvylikos mikrosatelitø rinkinius ir jø patikimumà tikrinant Lietuvos vietiniø arkliø veisliø (Þemaitukø, Stambiøjø Þemaitukø ir Lietuvos sunkiøjø) kilmæ.

Buvo tirti DNR lokusai: *VHL20, HTG4, AHT4, HMS7, HTG6, AHT5, HMS6, ASB2, HTG10, HTG7, HMS, HMS2, ASB17, ASB23* ir *LEX33*. Kilmës nustatymo patikimumas, naudojant DNR mikrosatelitiniø þymekliø polimorfizmo tyrimus, svyravo nuo 99,91% Þemaitukø iki 99,99% Stambiøjø Þemaitukø ir Lietuvos sunkiøjø arkliø veislëse.

Raktaþodþiai: mikrosatelitai, DNR, arkliai, kilmës patikrinimas, metodo patikimumas