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EVALUATION OF THE 17-PLEX STR KIT FOR PARENTAGE TESTING OF POLISH COLDBLOOD AND HUCUL HORSES*

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Abstract

In this study, 17 microsatellite markers were evaluated for parentage testing in a group of Hucul and Polish Coldblood horses, whose parents were excluded in single loci. Despite the high polymorphism of the majority of 17 microsatellites an increase in homozygosity and deviations from H-W proportions were observed in some loci in the investigated populations. Deviations were observed mainly in loci where single exclusions were found. DNA typing problems of some microsatellite sequences, sex linkage of markers and the occurrence of null alleles are the basis for erroneous parentage exclusion. The relationship between these issues and population statistics and their impact on efficiency of parentage testing is discussed.

Key words: horses, microsatellites, parentage testing, single-locus exclusion

Short Tandem Repeat (STR) loci, i.e. microsatellites, are a class of nuclear DNA markers consisting of tandemly repeated sequence motifs of two to seven base pairs in length. Alleles of STR loci vary by the number of times a given sequence motif is repeated. STR alleles are detected using Polymerase Chain Reaction (PCR) and by separating the amplification products using electrophoresis. Due to their high level of polymorphism (informativeness) and Mendelian inheritance, microsatellites have become the markers of choice for parentage testing and individual identification. In Poland, the polymorphism of these markers has been proved to be useful in purebred Arabian (Gralak et al., 1998) and Anglo-Arabian (Ząbek et al., 2006), Thoroughbred (Gralak et al., 1998; Niemczewski and Żórkowski, 2000; Ząbek et al., 2003) and Silesian (Ząbek et al., 2003), Polish Primitive (Gralak et al., 2001), Polish Heavy (Iwańczyk et al., 2006) and Biłgoraj horses (Ząbek et al., 2005). Combining a set of more than 7 highly informative microsatelite markers in one multiplex reaction can exclude wrongly determined parentage with probability greater than 99% (Ząbek, 2006). Some problems occur during application of STR systems in routine parent-

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age testing. Proper designation of STR alleles is disturbed by nonspecific addition of adenine to the 3' end of the PCR products (plus A product) and by mutations in the primer-binding site, which prevent the amplification of one of the alleles in a microsatellite locus (null allele) (Eggleston-Stott et al., 1997). Cases when only one locus excludes the alleged parentage are the common signs of related STR typing problems. Therefore it was assumed that exclusion is validated if inconsistency in genetic basis between offspring and alleged parent will be discovered in at least two microsatellite loci.

In this work we discuss the use of 17 microsatellite markers for parentage testing in a group of Hucul (hc) and Polish Coldblood (pcb) horses, which represent cases where single-locus exclusions of one of their parents were detected. Population of individuals sampled from such difficult parentage cases was considered to be appropriate for finding the reasons of single exclusions at particular loci. The STR panel encompasses the following equine loci: *VHL20*, *HTG4*, *AHT4*, *HMS7*, *HTG6*, *AHT5*, *HMS6*, *ASB23*, *ASB2*, *HTG10*, *HTG7*, *HMS3*, *HMS2*, *ASB17*, *LEX3*, *HMS1* and *CA425*. These include nine loci recommended by the Equine Genetics and Thoroughbred Parentage Testing Standardization Committee of the International Society for Animal Genetics (ISAG) and eight additional loci commonly used for horse parentage testing and identification worldwide (Table 1). Evaluation was made of the quality of DNA profiles of the investigated microsatellite loci, deviations from Hardy-Weinberg proportions in relation to the differences between observed and expected heterozygosity for particular marker loci, and the frequency of null alleles.

Table 1. The list of microsatellite markers and chromosomal location

Locus	Chromosome	Literature
AHT4*	24	Binns et al. (1995)
AHT5	8	Binns et al. (1995)
ASB17	2	Breen et al. (1997)
ASB2	15	Breen et al. (1997)
ASB23	3	Irvin et al. (1998)
HMS1	15	Guerin et al. (1994)
HMS2	10	Guerin et al. (1994)
HMS3	9	Guerin et al. (1994)
HMS6	4	Guerin et al. (1994)
HMS7	1	Guerin et al. (1994)
HTG10	21	Marklund et al. (1994)
HTG4	9	Ellegren et al. (1992)
HTG6	15	Ellegren et al. (1992)
HTG7	4	Marklund et al. (1994)
LEX3	X	Coogle et al. (1996)
UCDEQ425	28	Eggleston-Stott et al. (1997a)
VHL20	30	Van Haeringen et al. (1994)

*ISAG recommended markers for parentage testing are in bold.

Material and methods

The studied populations of horses comprised 195 males and 29 females of hc horses and 70 males and 6 females of pcb horses. All individuals represent cases where inconsistency between parental and offspring genotype was only detected in a single locus.

DNA was prepared from 300 µl of blood using Wizard kit (Promega) according to the manufacturer's protocol. The final DNA concentration was in the range of 20 to 60 ηg/µl. 2 µl of DNA were amplified in one multiplex reaction using a commercial kit from Finnzymes Diagnostic (Finnland), which includes the Phusion™ hot start DNA polymerase with proofreading activity and fluorescently labelled primers specific for 17 microsatellite loci (Table 1). PCR products were separated in a capillary electrophoresis system on a 3130xl genetic analyser (Applied Biosystems). Each sample was run with size standard LIZ 500, applied for automated DNA fragment analysis with the use of five fluorescent dyes. Fragment analysis and verification of DNA profiles for 17 STR loci were performed in the Genemapper 4.0 software.

The calculation of allele frequency, observed (Ho) and expected heterozygosity (He) (Nei, 1978), polymorphism information content (PIC) (Botstein et al., 1987) and probability of exclusion (PE) of wrongly assigned parentage when one and both parental genotypes are known (Jamieson and Taylor, 1997) were performed using the CERVUS 2.0 program. This package was also used to perform the Chi square test for the presence of Hardy-Weinberg equilibrium (H-W). The frequency of null alleles was estimated for each marker locus, using an iterative algorithm based on the difference between the observed and expected frequency of homozygotes (Summers and Amos, 1997) (CERVUS 2.0 package).

Results

One to 3 horses of both breeds came from cases where exclusion was only detected in one of the loci *AHT5*, *ASB2*, *CA425*, *HMS3*, *HMS7* or *VHL20* (Table 2). In loci *ASB17* and *HTG10*, the number of such exclusions concerned 4 (pcb) to 10 individuals (hc). The greatest number of single exclusions was characteristic of marker LEX3 in both horse populations (Table 2).

Table 2. Number of hc and pcb males and females, one of whose parents was excluded in a single locus

Excluding marker	Hc horses	Pcb horses
AHT5	1♀	
ASB17	4♂ 6♀	
ASB2	1♂ 1♀	1♀
CA425		1♀
HMS3	1♂ 1♀	1♀
HMS7	1♀	
HTG10	2♂ 3♀	3♂ 1♀
LEX3	187♂ 17♀	67♂ 1♀
VHL20		1♂

Well-resolved DNA profiles were obtained after simultaneous amplification in the majority of 17 microsatellite loci. Lower amplification yield was visible for marker *AHT5* and *HMS6* (Figure 1).

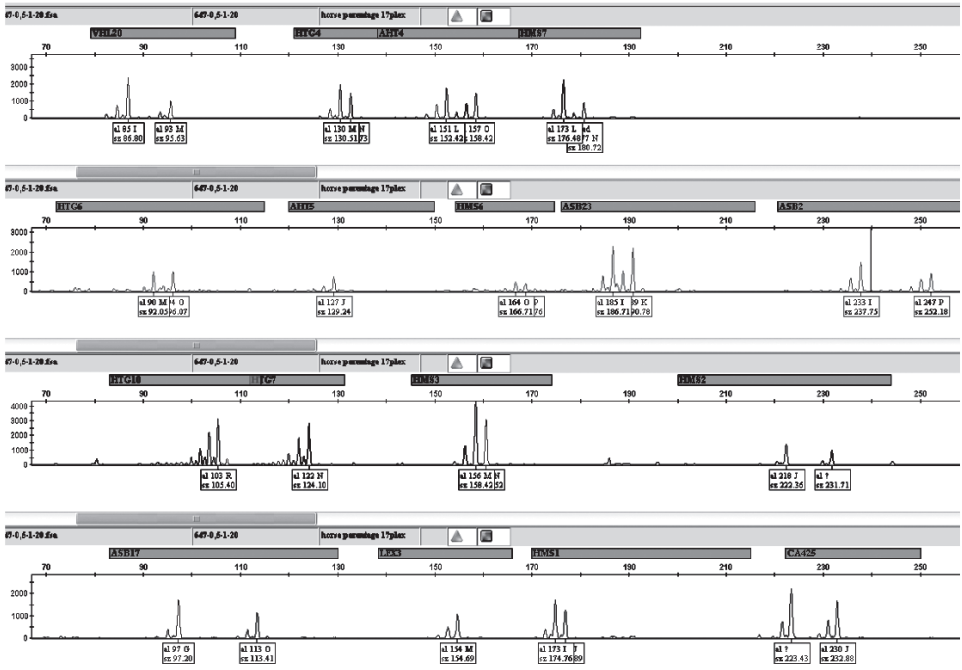

Figure 1. DNA profile in loci of 17 equine STR markers

Unbalanced amplification of alleles was observed for marker *HMS1* (Figure 2).

The mean proportion of typed individuals and mean heterozygosity values are similar when calculated for each horse group. Assuming the presence of both parental genotypes, the combined probability of exclusion (CPE) was greater than 0.99999 in both horse populations. When only one parental genotype is known, CPE value is lower but still exceeds 0.999 (Table 3).

Table 3. Measures of amplification efficiency, genetic variability (mean He) and exclusion efficiency of wrongly determined parentage (CPE)

	hc	pcb
Mean proportion of individuals typed	0.996	0.995
Mean expected heterozygosity (He)	0.726	0.723
(CPE) (one parent)	0.999486	0.99943
(CPE) (both parents)	0.999998	0.999997

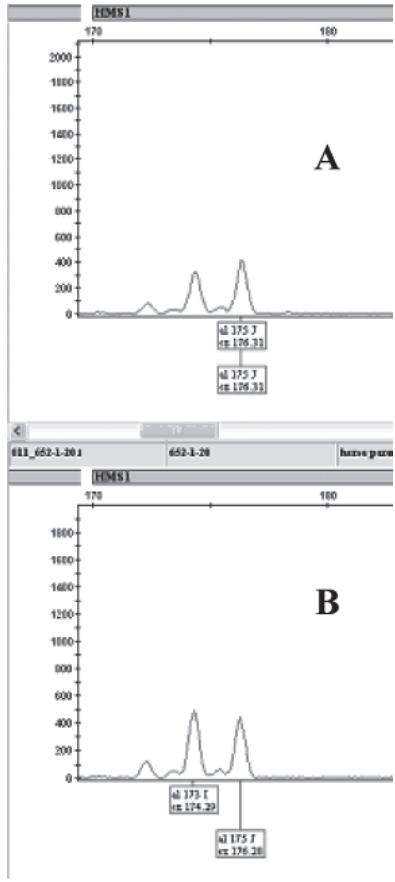

Figure 2. Results of unbalanced amplification of *HMS1* alleles in one sample. A: true amplification product *HMS1-J*. B: Falsely detected allele *HMS1-I* being the 2-bp stutter for allele *J*

In the whole set of 17 microsatellite loci 140 alleles were identified in hc horses and 128 alleles in pcb horses in the range of 4 to 12 alleles in different loci. A greater number of homozygous genotypes in relation to the heterozygous ones is characteristic of loci *HTG6* and *LEX3* in hc and pcb horses (Table 4). In the hc population deviations from HW proportions revealed by the Chi square test were significant for marker *AHT4* and highly significant for *HMS1* and *LEX3*. In the pcb population deviations from HW proportions were significant for markers *HMS6* and *HTG6* and highly significant for *LEX3*. The greatest differences between Ho and He values were characteristic of locus *LEX3* in the hc population and markers *ASB17*, *HMS6*, *HTG10* and *LEX3* in the pcb population. PIC values were greater than 0.5 for single markers from the set, except for the PIC calculated for *HMS1* in pcb population and PIC for *HTG6* in hc and pcb horses. The frequency of null alleles greater than 0.05 was characteristic of markers *ASB17*, *HMS6* and *HTG10* in pcb horses and *LEX3* in hc and pcb population (Table 4).

Table 4. Marker characteristics in the studied populations of horses (number of heterozygotes, Hets; number of homozygotes, Homs; observed heterozygosity, Ho; expected heterozygosity, He; polymorphic information content, PIC), results of the Chi square test for H-W proportions and null allele frequency

Locus	Breed	Allele No.	Allelic range (bp)	Hets	Homs	Ho	He	Difference between Ho and He	PIC	Significance level of HW test	Null freq.
AHT4	hc	9	148–164	189	35	0.844	0.788	0.056	0.758	<0.05	–0.0361
	pcb	8	148–164	58	18	0.763	0.788	0.025	0.754	ns**	0.0077
AHT5	hc	7	157–167	159	65	0.71	0.682	0.028	0.645	ns	–0.0268
	pcb	6	157–167	57	19	0.75	0.773	0.023	0.733	ns	0.0122
ASB17	hc	15	95–119	187	35	0.842	0.84	0.002	0.82	ns	–0.0045
	pcb	11	93–123	61	14	0.813	0.853	0.04	0.831	ns	0.0229
ASB2	hc	9	222–254	182	42	0.813	0.812	0.001	0.785	ns	–0.0011
	pcb	10	236–252	58	18	0.763	0.717	0.046	0.68	ns	–0.0354
ASB23	hc	10	185–209	156	66	0.703	0.68	0.023	0.639	ns	–0.0173
	pcb	7	185–209	45	30	0.6	0.678	0.078	0.627	ns	0.0562
CA425	hc	11	222–240	188	34	0.847	0.815	0.032	0.789	ns	–0.022
	pcb	9	222–240	52	23	0.693	0.732	0.039	0.687	ns	0.0277
HMS1	hc	7	173–189	184	38	0.829	0.783	0.046	0.751	<0.01	–0.0335
	pcb	7	175–183	39	36	0.52	0.527	0.007	0.473	ns	–0.0013
HMS2	hc	8	220–240	160	64	0.714	0.745	0.031	0.715	ns	0.0271
	pcb	7	220–240	49	27	0.645	0.662	0.017	0.616	ns	0.0142
HMS3	hc	7	152–170	175	49	0.781	0.802	0.021	0.772	ns	0.0133
	pcb	7	152–170	58	18	0.763	0.752	0.011	0.716	ns	–0.0152
HMS6	hc	6	134–144	154	70	0.688	0.724	0.036	0.681	ns	0.0301
	pcb	6	134–144	50	26	0.658	0.745	0.087	0.702	<0.05	0.0655
HMS7	hc	6	173–181	142	82	0.634	0.627	0.007	0.586	ns	–0.0086
	pcb	7	175–183	58	18	0.763	0.723	0.04	0.67	ns	–0.0338
HTG10	hc	9	93–113	176	48	0.786	0.765	0.021	0.738	ns	–0.011
	pcb	9	93–113	55	21	0.724	0.806	0.082	0.773	ns	0.051
HTG4	hc	6	129–139	152	72	0.679	0.693	0.014	0.638	ns	0.0107
	pcb	5	131–139	54	22	0.711	0.702	0.009	0.656	ns	–0.0019
HTG6	hc	6	84–100	94	130	0.42	0.407	0.013	0.385	ns	–0.0074
	pcb	5	84–100	25	51	0.329	0.378	0.049	0.349	<0.05	0.0789
HTG7	hc	4	120–128	136	88	0.607	0.585	0.022	0.524	ns	–0.0179
	pcb	5	120–128	62	14	0.816	0.75	0.066	0.699	ns	–0.0478
LEX3	hc	10	140–162	15	201	0.069	0.752	0.683	0.718	<0.01	0.8315
	pcb	10	140–160	4	69	0.055	0.883	0.828	0.864	<0.01	0.8823
VHL20	hc	10	125–145	187	37	0.835	0.844	0.009	0.824	ns	0.0051
	pcb	9	125–143	61	15	0.803	0.83	0.027	0.801	ns	0.0124

*Possible presence of null allele if frequency is greater than 0.05; ** Not significant.

Discussion

Almost all samples were successfully amplified in all STR loci and any lack of amplification was rather due to poor DNA quality and possible mutations in the primer binding sites of the sequences of PCR templates. The use of DNA polymerase of proofreading activity included in the PCR kit resulted in a substantial reduction of falsely determined alleles because of amplification difficulties like +A products, normally observed when using Taq DNA polymerase. In this study stutter peaks of the microsatellite alleles were usually reduced in height, which facilitated the determination of true alleles. One exception is comparable amplification yield of stutter peaks and true alleles at *HMS1* locus.

Both horse populations are characterized by a great total number of alleles in loci of 17 STR markers and mean heterozygosity values being similar to those reported in many other horse populations such as American Quarter horses (Bowling et al., 1997), Warmblood horses from the Czech Republic (Hamanova et al., 2001), Anglo Arabian horses from Poland (Ząbek et al., 2006), Polish Heavy horses (Iwańczyk et al., 2006), Polish Primitive horses (Gralak et al., 2001) and some of the native Spanish horse populations (Canon et al., 2000). The reason for visible great variation in microsatellite loci is the polymorphic nature of tandem repeats in contrast to other types of genetic markers such as SNP markers or previously used blood group systems (Gralak et al., 1998). PIC values greater than 0.5 for the majority of 17 microsatellite markers in hc and pcb horses result in high power of exclusion (CPE value) for this set of 17 microsatellites. All these properties make this panel of markers theoretically very useful in resolving parentage of hc and pcb horses.

Problems with application of microsatellite markers in parentage testing affect both the quality and yield of amplification of microsatellite alleles that are often marker specific, and improper allele sizing during automated fragment analysis. Rarely occurring inconsistency between offspring and parental genotypes in particular loci may result from the lack of amplification of one of the alleles because of polymorphism in the primer binding sites (null alleles). This may affect markers *AHT5*, *ASB2*, *HMS3*, *HMS7* and *VHL20* when interpreting the parentage of some of investigated horses. Single cases of null alleles were also reported for locus *HMS2* and *ASB2* in Lippizan horses (Achman et al., 2000). In turn, the reason for a greater number of single-locus exclusions detected for marker *ASB17* and *HTG10* was also improper allele sizing during DNA fragment analysis. For the *LEX3* marker numerous inconsistencies in this locus result from the lack of the second allele in stallions, while this microsatellite is X linked. Seven stallions from both horse groups were erroneously typed as heterozygotes in *LEX3* locus (data not shown) and amplification products of those samples had to be sequenced to indicate the true allele.

Deviations from genetic basis in single locus influenced by DNA typing errors or sex linkage have an impact on the results of population statistics. Despite high number of alleles and high polymorphic rate shown by the PIC values for particular markers, an artificial increase in homozygosity of the investigated populations is usually observed in affected loci. The presence of only one allele of *LEX3* in male individuals causes highly significant deviations from H-W proportions and large differences

between the values of observed and expected heterozygosity. The possibility of using microsatellite *LEX3* is higher when resolving parentage between female individuals than between individuals from the male lines. The evidence for this is substantially lower rate of single exclusions in *LEX3* locus of parents of female individuals (Table 2). The highest frequency of null alleles, greater than 0.05 in *LEX3* locus relates to the elevated presence of homozygous genotypes in contrast to the heterozygous ones. Similarly, an increase in homozygosity affects *HTG6* locus in hc and pcb horses because of low number of alleles in relation to other microsatellite markers. Higher null allele frequency for this marker in pcb horses might be simply the bias connected with its low polymorphism in the investigated population. Also the possible presence of null alleles and deviations from H-W proportions were detected in loci *ASB23*, *HMS6* and *HTG10* in pcb horses, which for marker *HTG10* might be related with the occurrence of factors leading to single exclusions in this locus. Deviations observed in *ASB23* and *HMS6* loci in pcb horses may also result from the population substructure of pcb horses (Wahlund effect), which form several different populations in Poland. Pcb horses are the largest horse population in Poland where French, Swedish and Belgian Coldblood horses were used in formation of different types of pcb horses in the past. In turn, highly significant deviations from H-W proportions observed in *HMS1* locus in hc horses do not correspond with null allele frequency being lower than 0.05. However, such a significant deviation from H-W proportions in *HMS1* may be the result of mistyping errors related to unbalanced amplification of alleles in this locus.

The above results show that population data may indicate the presence of null alleles in STR loci or mistyping errors that influence proper verification of parentage. Despite the high power of exclusion for any given set of STR markers, an increase of homozygosity in particular loci may be the first indicator of DNA typing problems for any given locus which has to be verified when used to resolve parentage issues. This is the case of *LEX3* marker, which should be excluded from the parentage panel of equine STR markers, because of the linkage to X-chromosome and possible occurrence of polymorphism in the primer-binding site.

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TOMASZ ZĄBEK, AGNIESZKA FORNAL

Ocena 17 markerów mikrosatelitarnych w testach kontroli rodowodów koni huculskich i polskich koni zimnokrwistych

STRESZCZENIE

Analizowano efektywność 17 markerów mikrosatelitarnych w testach kontroli rodowodów koni huculskich i polskich koni zimnokrwistych. Wybrane konie posiadają niezgodność z genotypem rodziców w loci pojedyńczych markerów. Mimo znacznego polimorfizmu większości analizowanych mikrosatelitów, w niektórych loci obserwowano wzrost homozygotyczności oraz odchylenia od stanu równowagi genetycznej H-W badanych populacji koni. Odchylenia wystąpiły głównie w loci markerów, w których stwierdzono wykluczenia rodziców badanych koni. Problemy związane z genotypowaniem sekwencji mikrosatelitarnych, sprzężeniem z płcią oraz występowaniem alleli zerowych utrudniają prawidłową analizę pochodzenia badanych koni. W tej pracy omówiono zależności między powyższymi czynnikami a wynikami badań populacyjnych oraz ich wpływ na efektywność testów weryfikujących pochodzenie.