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APPLICATION OF 7 STR MARKERS FOR PARENTAGE TESTING AND GENETIC DISTANCE STUDY OF EQUIDAE*

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Abstract

The genotyping efficiency and polymorphism of 7 microsatellite markers (AHT084, COR006, COR017, COR018, COR040, COR055, COR088) was evaluated in order to apply them to parentage testing among a number of warm-blooded, cold-blooded and primitive horse breeds and to illustrate genetic differences between the breeds investigated. The amplification and sequence structure of these STR markers was also verified in other Equidae like zebra, kulan, donkey and Przewalski horse. Microsatellite allelic differentiation was similar to the allele numbers reported, with an extremely wide allelic range observed at AHT084 locus. However, due to genotyping difficulties AHT084 is not a suitable marker for parentage testing. The use of the other 6 STR markers among most of the horse breeds studied allows excluding wrongly assigned parentage with a probability of 0.99. Fragment analysis and sequencing of STR alleles confirmed the presence of investigated tandem repeats in other Equidae species. Clustering of investigated horse breeds on the tree of Fst distance was consistent with their breeding history, clearly separating breeds into 3 horse types mentioned above.

Key words: Equidae, parentage testing, STR

Microsatellite sequences are one of the most polymorphic markers frequently occurring in the mammalian genomes. They are tandemly repeated sequences of mostly 2 to 4 base pairs in length (short tandem repeats – STR) which can be quite well genotyped through multiplex PCR assays combined with automated fragment analysis on capillary electrophoresis systems. Even in the time of wide genome scanning with the use of SNP microarrays, microsatellites are still used in construction of linkage maps, when narrowing down the regions of QTLs (Dierks et al., 2010). Microsatellite sequences are still the markers of choice in equine parentage testing and individual identification, unlike SNP markers being proposed for parentage

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verification in other species like cattle (http://www.isag.us/Docs/EquineGeneticsParentage_CT.pdf). In Poland, the polymorphism of these markers has been proved to be useful in purebred Arabian (Gralak et al., 1998), Anglo-Arabian (Ząbek, 2006), Thoroughbred (Gralak et al., 1998; Niemczewski and Żórkowski, 2000; Ząbek et al., 2003), Silesian (Ząbek et al., 2003), Polish Primitive (Gralak et al., 2001) and Polish Heavy horses (Iwańczyk et al., 2006). Recently a set of 17 commonly used microsatellite markers for horse parentage control was proposed as a forensic panel for individual identification in horses (van de Goor et al., 2010). Application of the above set of microsatellites for horse parentage testing was also described in Polish horse breeds (Ząbek and Fornal, 2009). Microsatellite polymorphism still plays an important role in the assessment of genetic diversity of livestock. A number of recent population studies in Equidae species focused on detection of inbreeding levels in a number of different breeds using both pedigree and DNA microsatellite typing data (Ząbek et al., 2006; Janssens et al., 2010).

The present work discusses the application of 7 autosomal microsatellites to parentage testing and diversity study among a number of horse populations of different origin and also STR genotyping efficiency in other Equidae species. The study used the AHT084 marker investigated by Swinburne et al. (2003), COR006, COR017 and COR018 loci studied by Hopman et al. (1999), COR040 studied by Murphie et al. (1999), COR055 studied by Ruth et al. (1999) and COR088 investigated by Tallmadge et al. (1999). Microsatellite polymorphism was described in a number of warm-blooded horse breeds, two populations of cold-blooded horses and one of population of the primitive horse. Genotyping and analysis of sequence homology of the above-mentioned tandem repeats were also performed in four other Equidae species.

Material and methods

This study investigated DNA samples prepared from blood or hair bulbs collected from 37 Half-bred Anglo-Arabian horses (AA), 17 Belgian Draft horses (BDH), 27 Hucul horses (Hc), 30 Polish Heavy horses (Z), 28 Wielkopolski horses (wlkp), 55 Thoroughbreds (TH) and a group of 32 horses of different origin (mixed panel) including Quarter horses, Hanoverian Warm-blooded horses and Silesian horses. Genotyping was also performed in two donkeys (*Equus asinus asinus*), two Chapman zebras (*Equus quagga chapmanni*), two kulans (*Equus hemionus kulan*) and three Przewalski horses (*Equus przewalskii*). DNA samples amplified in multiplex PCR with the use of fluorescently labelled primers flanking tandem repeats of the 7 investigated microsatellites PCR products were electrophoresed on an ABI 3130xl genetic analyser together with internal lane size standard (Applied Biosystems) dedicated for automated fragment analysis. DNA fragment sizing in base pairs and genotyping were conducted with the use of GeneMapper 4.0 software. Amplification products of homozygous individuals of zebra, kulan, donkey and Przewalski horse were sequenced with the use of unlabelled PCR primers and BigDye Terminator

1.1 Cycle sequencing kit (Applied Biosystems). Products of cycle sequencing were electrophoresed on an ABI 3130xl genetic analyser. Sequencing reads were aligned to the reference sequences of investigated microsatellite alleles in the Genedoc software (Nicholas et al., 1997).

Based on microsatellite typing data, allele frequencies, observed heterozygosity (Ho) and expected heterozygosity (He), polymorphism information content (PIC) and probability of exclusion when both parental genotypes are known (CPE) were calculated using CERVUS software (Marshall et al., 1998). Pairwise Wright's fixation index values (Fst) were calculated using Arlequin software (Excoffier, 2010), which were used as a measure of genetic distance (Wright, 1969). Using distance matrices the UPGMA tree was generated using the Phylip 3.2 package (Felsenstein, 1989) and phylogenetic dendrograms were constructed using TreeView 1.6 software (Page, 1996).

Results

After amplification DNA fragments were obtained in the range of 68 to 290 bp and their profile was typical of microsatellite alleles (Table 1, Figure 1). Depending on the population, from 8 (COR006 and COR017) to 25 alleles (AHT084) were detected at marker loci (Table 2). More than 2 allele peaks were detected in DNA profiles of some individuals when amplifying AHT084 marker (Figure 1). Due to difficulties in proper genotype determination this microsatellite was excluded from analysis of heterozygosity, polymorphism rate and from evaluation for parentage testing. This marker was used for genetic distance analysis because of its contribution to proper topology of the phylogenetic tree. Amplification yield of microsatellite alleles expressed as percent of determined genotypes was the highest for AHT084 and COR055 (88% and 77.4%) and the lowest for COR017 and COR088 (65%). The amplification efficiency for the rest of markers was above 85%.

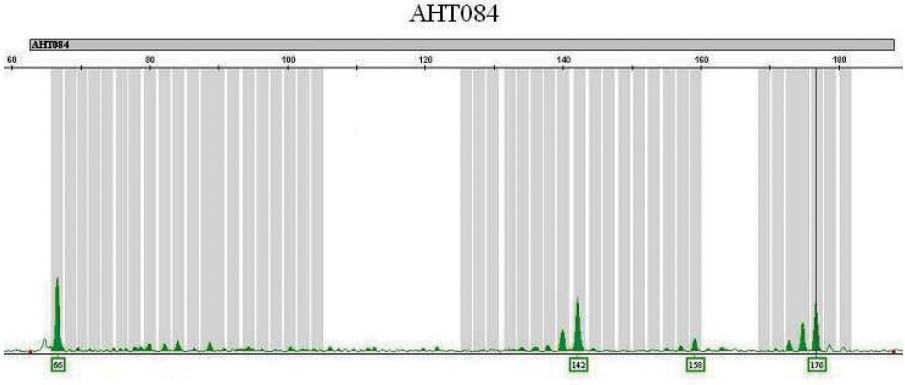


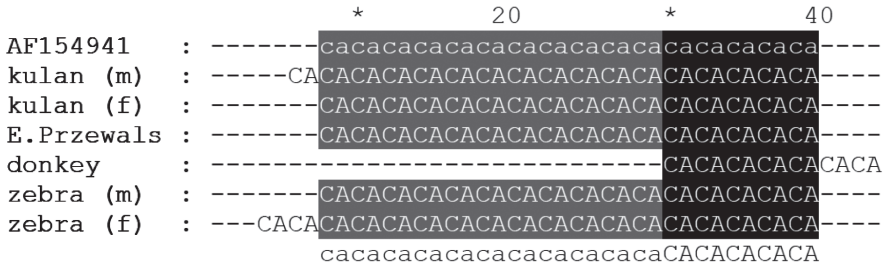
Figure 1. Allelic bins of AHT084 with DNA profile of 4 allele peaks of one individual

Table 1. The tandem repeat structure of STR markers and allelic range (bp) among horse breeds and other Equidae species

AHT084	COR006	COR017	COR018	COR040	COR055	COR088
(AC)n ATC(AC)n AATC(AC)n TC(AC)n ACTA(AC)n	(CA)n	(CA)n	(CA)n	(CA)n	(CA)n	(CA)n
68	178	236	**246**	**270**	234	**260**
70	188	**240**	**250**	**272**	242	**274**
76	190	246	**256**	**274**	246	**276**
78	**194**	**248**	**258**	**276**	**248**	278
80	**196**	**250**	**260**	**278**	**250**	**280**
84	198	**252**	262	280	252	**282**
86	200	256	264	282	**254**	284
88	202	258	266	**284**	256	286
92[1]			**268**	286	**258**	288
94[2]			**272**	288	**260**	
96			**274**	290	**262**	
98			276		264	
102						
126						
130						
140						
142						
144						
148						
152						
158						
172						
174						
176						
178						

[1] Alleles characteristic of other Equidae species than horses are underlined.
[2] Alleles detected in horses and other Equidae species are in bold.

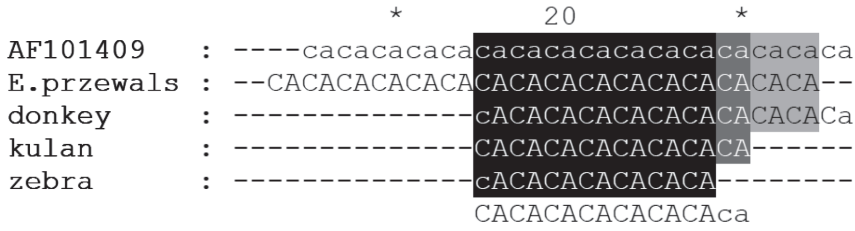
DNA amplification using equine primers for 7 microsatellite markers applied in other Equidae species revealed the presence of a 92 bp allele of AHT084 in donkey, 190 bp allele of COR006 in zebra, 236 bp allele of COR017 in kulan and zebra, and 242 bp allele of COR055 in zebra species, being outside the allelic range detected for investigated horse breeds (Table 1). Direct PCR sequencing of COR088 and COR040 alleles revealed the presence of CA dinucleotide repeats among investigated Equidae species (Figures 2 and 3). Sequencing of marker COR055 revealed GT dinucleotide repeats in other Equidae, being interrupted by a 6-bp insertion in zebra species (Figure 4).



Description:

AF154941	Genbank Accession No. of COR088 (CA)16 repeat sequence
kulan	(CA)17
Przewalski horse	(CA)16
donkey	(CA)7
zebra	(CA)18

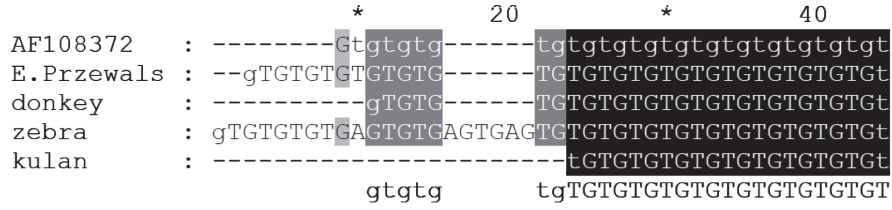
Figure 2. Sequence alignment of COR088 allele among selected Equidae species



Description:

AF101409	Genbank Accession No. of COR040 (CA)16 repeat sequence
E. Przewalski	(CA)16
donkey	(CA)10
kulan	(CA)8
zebra	(CA)7

Figure 3. Sequence alignment of COR040 allele among selected Equidae species



Description:

AF108372	Genbank Accession No. of COR055 (GT)15 repeat sequence
E. Przewalski	(GT)18
donkey	(GT)14
zebra	(GT)4 GA (GT)2 GAGTGA (GT)12
kulan	(GT)10

Figure 4. Sequence alignment of COR055 allele among selected Equidae species

Table 2. Allele number, values of observed and expected heterozygosity, and PIC among investigated horse breeds

pop	Locus	Allele No.	HObs	HExp	PIC
AA	AHT084	12	-*	-	-
BDH	AHT084	15	-	-	-
Hc	AHT084	9	-	-	-
mixed_panel	AHT084	10	-	-	-
Z	AHT084	14	-	-	-
wlkp	AHT084	10	-	-	-
TH	AHT084	9	-	-	-
Mean		**11.3**	-	-	-
AA	COR006	3	0.769	0.6	0.497
BDH	COR006	4	0.5	0.587	0.51
Hc	COR006	3	0.63	0.652	0.564
mixed_panel	COR006	6	0.71	0.658	0.591
Z	COR006	3	0.448	0.573	0.474
wlkp	COR006	4	0.64	0.7	0.621
TH	COR006	5	0.714	0.683	0.606
Mean		**4**	**0.63**	**0.636**	**0.552**
AA	COR017	6	0.769	0.662	0.568
BDH	COR017	3	0.563	0.554	0.478
Hc	COR017	4	0.741	0.687	0.614
mixed_panel	COR017	3	0.161	0.629	0.546
Z	COR017	6	0.607	0.74	0.693
wlkp	COR017	6	0.75	0.672	0.596
TH	COR017	5	0.875	0.725	0.641
Mean		**4.7**	**0.638**	**0.667**	**0.591**
AA	COR018	9	0.545	0.633	0.593
BDH	COR018	5	0.714	0.659	0.571
Hc	COR018	8	0.684	0.812	0.767
mixed_panel	COR018	9	0.429	0.698	0.659
Z	COR018	4	0.778	0.654	0.569
wlkp	COR018	9	0.65	0.76	0.716
TH	COR018	8	0.611	0.706	0.661
Mean		**7.4**	**0.63**	**0.703**	**0.648**
AA	COR040	9	0.71	0.812	0.776
BDH	COR040	5	0.857	0.758	0.657
Hc	COR040	10	0.765	0.766	0.718
mixed_panel	COR040	9	0.577	0.838	0.801
Z	COR040	5	0.444	0.739	0.65
wlkp	COR040	9	0.842	0.808	0.766
TH	COR040	7	0.722	0.801	0.763
Mean		**7.7**	**0.702**	**0.789**	**0.733**
AA	COR055	10	0.667	0.799	0.759
BDH	COR055	6	0.857	0.857	0.766
Hc	COR055	8	0.833	0.817	0.766
mixed_panel	COR055	10	0.714	0.881	0.851
Z	COR055	6	0.667	0.732	0.661
wlkp	COR055	8	0.75	0.837	0.792
TH	COR055	10	0.889	0.83	0.798
Mean		**8.3**	**0.768**	**0.822**	**0.77**
AA	COR088	7	0.429	0.684	0.612
BDH	COR088	4	0.667	0.682	0.559
Hc	COR088	4	0.333	0.743	0.663
mixed_panel	COR088	7	0.308	0.615	0.573
Z	COR088	4	0.4	0.647	0.544
wlkp	COR088	7	0.45	0.814	0.766
TH	COR088	8	0.5	0.704	0.669
Mean		**5.9**	**0.441**	**0.698**	**0.627**

* Explanation in text.

The mean values of observed and expected heterozygosity across all populations were in the range from 0.441 and 0.698 (COR088) to 0.768 and 0.822 (COR055), respectively (Table 2). Hobs calculated for COR088 was lower than 0.5 for 5 of all horse populations investigated whereas for COR055 the lowest Hobs value equals 0.667 (Table 2). Except the COR006 and COR017 loci in Z, BDH and AA horses, calculated PIC values for the investigated markers were greater than 0.5 in the rest of the populations (Table 2). The mean PIC values calculated for each marker across all investigated horse groups were highest for COR055 and COR040 (Table 2). The values of combined probability of exclusion when genotypes of both parents are known were 0.99 for the set of 6 markers in all populations studied except for lower CPE value calculated for the 17 BDH horses (Table 3).

Table 3. Mean observed and expected heterozygosity of 6 STR loci and combined probability of exclusion when both parental genotypes are known

Horse group	Population size	H obs.	H exp.	CPE (second parent)
AA	37	0.650	0.700	0.99
BDH	17	0.693	0.657	0.95
Hc	27	0.662	0.761	0.99
mixed breed	32	0.483	0.747	0.99
Z	30	0.558	0.735	0.99
wlkp	28	0.680	0.764	0.99
TH	55	0.718	0.680	0.99

The value of pairwise Fst distance was the largest between Thoroughbred and Hucul horses (Table 4). The population of Thoroughbreds and mixed group of horses are grouped together with Wielkopolski horses on the dendrogram constructed using pairwise Fst distance values. Belgian and Polish Heavy horses are the next two neighbouring groups on the dendrogram. Anglo-Arabians and Hucul horses form a separate branch (Figure 5).

Table 4. The pairwise Fst genetic distance

	AA	BDH	Hc	Z	wlkp	TH	mix
AA							
BDH	0.009						
Hc	0.030	0.053					
Z	0.014	0.022	0.074				
wlkp	0.043	0.031	0.086	0.052			
TH	0.063	0.08	0.118	0.099	0.038		
mix	0.017	0.039	0.092	0.039	0.019	0.015	

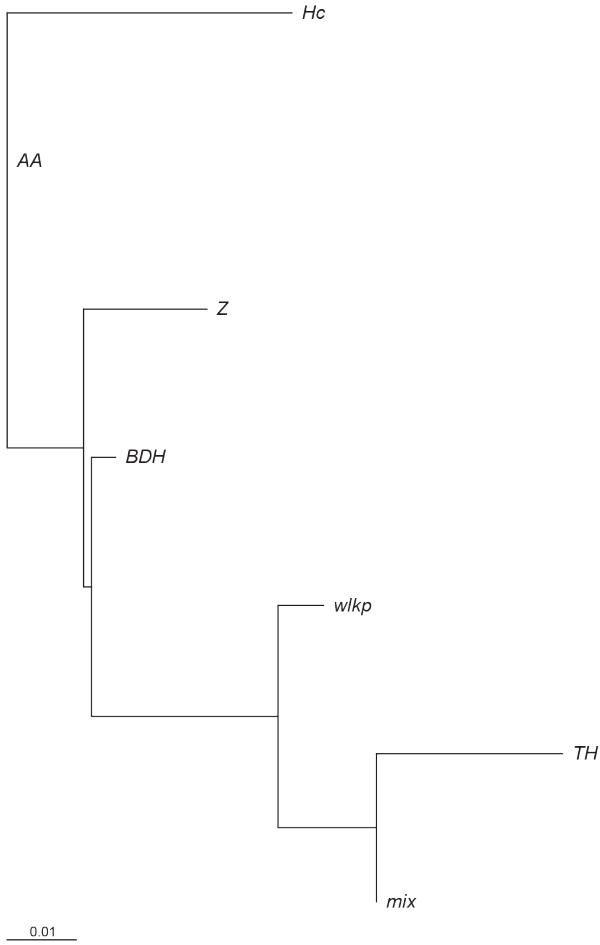


Figure 4. Dendrogram based on the Fst distance values

Discussion

A set of 7 microsatellite sequences was evaluated for its effectiveness in genetic study of a selected group of equine breeds together with their genotyping robustness in other equids. The use of the group of 226 horses of different breeds and utility types revealed a broad allelic range in selected STR loci of dinucleotide repeats. The presence of 3 or 4 allele peaks in AHT084 DNA profiles might suggest that primers designed for AHT084 region amplify two separate STR loci. As a result, almost two times higher STR alleles were identified at AHT084 in comparison to other investigated loci. A similar case of complex genotypes was described by Marklund et al. (1994) for locus HTG13. However, Swinburne et al. (2003) did not

describe such a phenomenon for AHT084 microsatellite. Also BLAST search against horse genome with the use of primer sequences applied in this work did not reveal any homology to other loci than AHT084. Therefore we cannot exclude problems with amplification of separate AHT084 alleles due to complex sequence structure of this tandem repeat. Due to genotyping difficulties this microsatellite cannot be efficiently used for parentage testing purposes.

DNA amplification using equine primers for 7 microsatellite markers applied in other Equidae species was successful and revealed the presence of variants being outside the allelic range detected for investigated horse breeds. Sequencing results of selected marker alleles confirmed the presence of tandem repeats among other Equidae species. The observed allelic differentiation at microsatellite loci between Przewalski horse, donkey, kulan and zebra species points to the possibility of using these markers for genetic population studies in a wider range of Equidae species. A similar study was conducted by Ząbek (2008) where a set of other autosomal STR markers was efficiently amplified in different Equidae species, revealing species-specific microsatellite alleles.

The heterozygosity and PIC values of 6 microsatellite loci are characteristic of tandem repeats being comparable with the values described for these STR loci by other authors (Hopman et al., 1999; Murphy et al., 1999; Ruth et al., 1999; Tallmadge et al., 1999). One exception is observed heterozygosity, which is substantially lower than expected heterozygosity for loci and horse groups where PCR amplification yield was smaller. The most polymorphic microsatellite markers in the majority of horse groups investigated are COR055 and COR040, which contributes the most to values of combined probability of exclusion. In this study a potential use of 6 STR markers allows excluding wrongly assigned parentage with probability of 0.99 for most populations, which is a minimum reliability requirement of DNA based parentage testing (Vankan et al., 1999). The exception is lower CPE value obtained for small BDH population in this study.

Phylogenetic relationships depicted with the use of Fst genetic distance showed appropriate placement of the majority of studied horse breeds consistent with their history and breeding directions. The illustrated relationships express a strong influence of Thoroughbreds on the formation of Wielkopolski horses and other warm-blooded horses in the mixed group studied. Small genetic differences are also observed between Polish Heavy and Belgian Draft horses which occur in the pedigrees of the former. The greater divergence of Anglo-Arabians in relation to the rest of warm-blooded horses studied in this work might underline the contribution of Purebred Arabian horses to the creation of Anglo-Arabians, unlike other warm-blooded horses being improved exclusively with the Thoroughbreds. The clear divergence between Thoroughbreds and Hucul horses expressed by greatest distance value is concordant with totally different breeding directions of both breeds and illustrates variation of breeding horse types among domesticated populations of horses worldwide.

In this work 7 microsatellite markers were evaluated for genotyping and parentage testing purposes, which were also used to reveal the genetic differences among horse populations of different origin. Exclusion probability values show that 6 of the

investigated microsatellites may be useful for horse parentage testing. AHT084 cannot be applied for routine testing due to difficulties in genotype determination. The polymorphism of the studied microsatellite markers allowed revealing the level of genetic differences between warm-blooded, cold-blooded and primitive horses. The present work also shows the potential of using the above-mentioned microsatellite sequences in study of other Equidae species.

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Zastosowanie siedmiu markerów STR do weryfikacji pochodzenia i badania dystansu genetycznego u koniowatych

STRESZCZENIE

Określono polimorfizm siedmiu sekwencji mikrosatelitarnych DNA (AHT084, COR006, COR017, COR018, COR040, COR055, COR088) w populacjach koni udomowionych reprezentujących szereg gorącokrwistych, zimnokrwistych oraz prymitywnych ras koni. Zanalizowano jakość amplifikacji oraz konserwatyzm sekwencji tych markerów u innych gatunków *E*quidae (osioł, kułan, koń Przewalskiego, zebra). Wysoki polimorfizm uzyskany dla większości badanych markerów warunkuje potencjalną przydatność tych markerów do testów weryfikacji pochodzenia badanych koni. Zsekwencjonowanie alleli trzech mikrosatelit u wybranych przedstawicieli gatunków Equidae ujawniło dwunukleotydowy motyw powtórzeń, typowy dla tych sekwencji u koni udomowionych. Wyniki analizy dystansu genetycznego między badanymi grupami koni są zgodne z historią hodowli poszczególnych ras i typów użytkowych koni badanych w tej pracy.