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Genetic diversity in Bulgarian Thoroughbred using microsatellite DNA markers

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Abstract. *Data set of 161 mares and stallions in age of 3 and more years of Bulgarian Thoroughbred horse race were investigated by 12 microsatellite loci. The high levels of polymorphism were detected with microsatellite loci within the population. In horse breeding this technology has the potential to be of great use in monitoring levels of genetic variation within stocks as well as for parentage and relatedness purposes. The middle realization of the observed and possible genotypes, depending of established alleles per locus, is 72.3 % which are the implications of very high level of genetic diversity, the prerequisite of accuracy of parentage verification. Maintaining pedigree information on all individuals in the breeding program and using this information to arrange matings will ensure only minimum increases in inbreeding every generation.*

Keywords: Bulgarian thoroughbred, microsatellite loci, diversity, parentage verification

Introduction

One of the difficulties in implementing a selective breeding program in horse stocks is maintaining pedigree information. 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. Another difficulty in managing a selective breeding program is loss of genetic variability and increases in inbreeding as a result of the inadvertent mating of related individuals (Curik et al., 2003). The effects of inbreeding in horses will result in a decrease in genetic diversity, which will limit the potential for genetic gain from artificial selection. Once reliable pedigree information is available, matings can be arranged in order to minimize inbreeding.

Parentage verification validates the horse pedigrees that make up the studbooks and thus is an important function of breed registries. Microsatellite markers are widely adopted to quantify genetic variation within and among breeds and to assign individuals to reference populations (Cunningham et al., 2001; Bruford et al., 2003;). Microsatellite markers are evenly distributed across genome and highly polymorphic. This technology provides a sensitive method for parentage verification and individual identification. It can also be used to screen for markers linked to performance traits or genetic disorders. PCR have the attractive features: can be highly automated, require only small amounts of biological samples, are not restricted to fresh blood samples and is simpler and cheaper than other techniques. Microsatellites are highly polymorphic genetic markers with codominantly inherited alleles that are relatively easy to score. They are tandem repeats of short motifs ranging from di- to tetranucleotides with variability in the number of repeats. A number of studies with different sets of microsatellite markers and their usefulness for parentage testing have been reported for different horse breeds (Breen et al., 1994; Gralak et al., 1994; Binns et al., 1995; Canon et al., 2000; Lui's et al., 2002; Tozaki et al., 2003; Juras et al., 2004; Vega-Pla et al., 2006). The laboratories using routine genotyping to check pedigree records use appropriate sets of primers to respective species and breeds that they test.

The main objective of this study is to analyze genetic structure of Bulgarian thoroughbred using set of 12 microsatellite markers.

Material and methods

Sample preparation. Genomic DNA was prepared from hair roots of 161 mares and stallions in age of 3 and more years. DNA was isolated using JETQUICK kits (Genomed GmbH, Germany), following the instructions of manufacturer.

*Microsatellite analysis.*A total of 12 microsatellite markers with known genomic assignments are included in this study. The set of markers, chromosome localizations and primers are given at Table 1.

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 microsatellite 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 microsatellite alleles were determined. Allele frequencies, the observed number of alleles, observed heterozigosity, observed and expected genotypes were calculated.

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Table 1. Microstellite markers used in the research.

Microsatellite markers	Primers (sequencing)	Chromosome №
AHT4	AACCGCCTGAGCAAGGAAGT CCCAGAGAGTTTACCCT	24
AHT5	ACGGACACATCCCTGCCTGC GCAGGCTAAGGGGGCTCAGC	8
ASB2	CCTTCCTGTAGTTTAAGCTTCTG CACAACTGAGTTCTCTGATAGG	15
HMS2	ACGGTGGCAACTGCCAAGGAAG CTTGCAGTCGATGTGTATTAAATG	10
HMS3	CCAACTCTTGTGCACATAACAAGA CCATCCTCACTTTTTCACTTTGTT	9
HMS6	GAAGCTGCCAGTATTCAACCATTG CTCCATCTTGTGAAGTGTAACTCA	4
HMS7	CAGGAAACTCATGTTGATACCATC TGTTGTTGAAACATACCTTGACTGT	1
HTG4	CTATCTCAGTCTTGATTGCAGGAC CTCCCTCCCTCCCTCTGTTCTC	9
HTG6	CCTGCTTGGAGGCTGTGATAAGAT GTTCACTGAATGTCAAATTCTGCT	15
HTG7	CCTGAAGCAGAACATCCCTCCTTG ATAAAGTGTCTGGGCAGAGCTGCT	4
HTG10	CAATTCCCGCCCCACCCCCGGCA TTTTTATTCTGATCTGTCACATTT	21
VHL20	CAAGTCCTCTTACTTGAAGACTAG AACTCAGGGAGAATCTTCCTCAG	30

The Euclidean distances were calculated among progeny groups of stallions, after that the classification were made, using software package Statistica v.4.0 of StatSoft Int.

Results and discussion

Genetic structure of Bulgarian thoroughbred are given in Table 2. The most polymorphic are loci ASB2 and HTG10 with 32 and 22 different genotypes. The loci HMS3 and HMS7 have 16 genotypes and HMS2 has 15 different genotypes. The diversity of the other loci are between 7 – of the HTG7 till 12 – of the VHL20. The levels of homo- and heterozigosity (Table 3) show that from 61 % till 83 % of genotypes are heterozigosis. Our results are similar to those of Georgescu et al., (2005) in Romanian thoroughbred.

The average values of observed heterozygosity indicate sufficient heterogenity between animals. The higher value was detected in ASB2 (0.83) and lowest in HTG4 (0.60) system.

Allele frequencies at the microsatellite loci are given in Table 4. The number of allele per each locus ranged from 3 (HMS1) to 9 (ASB2). . The most polymorhic is the ASB2 locus with 9 alleles. The rest of loci have between 5 and 7 alleles. Allele K is absent in 4 loci – HMS1, HMS3, HTG6 and VHL20. Allele G is presented only in 2 loci – HTG6 and HTG 7. The allele numbers and heterozygosity levels observed across the studied loci indicate presence of reasonably high level of genetic variability in Bulgarian thoroughbred.

The observed and possible genotypes, depending of established alleles per locus are given at Table 5. There are only 2 loci with all possible genotypes. In the rest of them the realization vary between 47.61 % till 86.67 %. The middle realization is 72.3 %

Table 2. Genotype frequencies of the microstellite loci

AHT4		AHT5		ASB2				HMS2	
Genotypes	Frequencies	Genotypes	Frequencies	Genotypes	Frequencies	Genotypes	Frequencies	Genotypes	Frequencies
O/O	0.1750	J/J	0.0559	B/K	0.0062	O/O	0.0248	H/H	0.0186
K/K	0.0375	K/K	0.1429	B/N	0.0062	O/P	0.0186	H/J	0.0062
J/J	0.0125	M/M	0.0373	B/O	0.0062	O/Q	0.0124	H/K	0.0683
K/O	0.2938	N/N	0.0186	I/Q	0.0062	O/R	0.0124	H/L	0.1491
J/O	0.1188	J/K	0.2050	K/K	0.0870	P/P	0.0124	H/M	0.0124
H/J	0.1000	J/M	0.1056	K/M	0.0870	P/Q	0.0186	J/K	0.0621
H/O	0.0813	J/N	0.0683	K/N	0.0435	P/R	0.0186	J/L	0.0621
J/K	0.0875	J/O	0.0186	K/O	0.0745	Q/Q	0.0621	K/K	0.0435
H/K	0.0750	K/M	0.1366	K/P	0.0745	Q/R	0.0311	K/L	0.1801
I/K	0.0125	K/N	0.1242	K/Q	0.1118	R/R	0.0124	K/M	0.0248
H/H	0.0063	M/N	0.0683	K/R	0.0683			K/O	0.0062
		M/O	0.0124	M/M	0.0124			L/L	0.3043
		N/O	0.0062	M/N	0.0373			L/M	0.0497
				M/O	0.0311			L/R	0.0062
				M/P	0.0186			M/M	0.0062
				M/Q	0.0186				
				M/R	0.0062				
				N/N	0.0248				
				N/O	0.0124				
				N/P	0.0124				
				N/Q	0.0248				
				N/R	0.0062				

Continuation of Table 2

HMS3		HMS6		HMS7		HTG4	
Genotypes	Frequencies	Genotypes	Frequencies	Genotypes	Frequencies	Genotypes	Frequencies
I/I	0.1615	K/K	0.0186	J/J	0.0186	I/I	0.0062
I/M	0.1615	K/L	0.0248	J/L	0.0497	K/K	0.1801
I/N	0.0373	K/M	0.0559	J/M	0.0435	K/L	0.0248
I/O	0.0807	K/P	0.0932	J/N	0.0621	K/M	0.4286
I/P	0.2236	L/L	0.0062	J/O	0.0621	K/N	0.0311
M/M	0.0248	L/M	0.0311	K/O	0.0062	K/P	0.0248
M/N	0.0062	L/P	0.0807	L/L	0.0311	L/M	0.0062
M/O	0.0683	M/M	0.0994	L/M	0.0807	M/M	0.2174
M/P	0.0683	M/P	0.3043	L/N	0.1180	M/N	0.0311
N/N	0.0062	O/P	0.0248	L/O	0.0559	M/P	0.0497
N/O	0.0248	P/P	0.2609	M/M	0.0311		
N/P	0.0124			M/N	0.0807		
O/O	0.0124			M/O	0.0807		
O/P	0.0559			N/N	0.1056		
P/P	0.0559			N/O	0.1118		
				O/O	0.0621		

HTG6		HTG7		HTG10		VHL20	
Genotypes	Frequencies	Genotypes	Frequencies	Genotypes	Frequencies	Genotypes	Frequencies
G/G	0.1366	G/G	0.0062	I/I	0.2174	I/I	0.0311
G/J	0.2671	K/K	0.0559	I/K	0.0497	I/L	0.0559
G/L	0.0062	K/N	0.1429	I/L	0.0807	I/M	0.2112
G/M	0.0311	K/O	0.1615	I/M	0.1677	I/N	0.0683
G/O	0.1615	N/N	0.0994	I/O	0.1180	L/L	0.0248
J/J	0.1739	N/O	0.3168	I/R	0.0745	L/M	0.1180
J/M	0.0435	O/O	0.2174	K/K	0.0124	L/N	0.0621
J/O	0.1491			K/L	0.0062	L/O	0.0062
J/R	0.0124			K/M	0.0186	M/M	0.1491
M/O	0.0124			K/O	0.0248	M/N	0.2422
O/O	0.0062			K/R	0.0062	M/R	0.0062
				L/L	0.0062	N/N	0.0248
				L/M	0.0311		
				L/O	0.0186		
				L/R	0.0186		
				M/M	0.0248		
				M/O	0.0311		
				M/R	0.0311		
				N/O	0.0062		
				O/O	0.0248		
				O/R	0.0186		
				R/R	0.0124		

which are the implications of very high level of genetic diversity, the prerequisite of accuracy of parentage verification.

More than 20 stallions have offsprings in Bulgarian thoroughbred. Only 17 of them have 3 and more offsprings. We estimated the differences between those offspring groups on the base of calculated Euclidean distances. The results are given at Figure 1. In our study dendrogram shows relatively high distances between individuals. The levels of genetic diversity observed in the Bulgarian thoroughbred is higher to the other horse breeds in comparing studies of horse populations (Achmann et al., 2004). According to dendrogram and another biodiversity data, we can resume the population of Bulgarian thoroughbred is not affected by loss of genetic diversity. Two groups are generated and connected at level 58. The first one include offspring groups of stallions Gerold, Garant, Galiano, Iskiernik and Daren. The second group is numerous and include offsprings of Aresi, Grozni, Shibor, Gagliano, Miroljub, Diuk, Shadow, Galego, Kerch, Derslaw, Dracedion and Sinop. This is very important information to prepare breeding plans for selective improvement of the breed, to develop breeding strategies and decrease inbreeding, as a result of the inadvertent mating of related individuals.

Table 3. The levels of homo and heterozigosity

Loci	Homosigosis genotypes	Heterozigosis genotypes
AHT4	0.23	0.77
AHT5	0.25	0.75
ASB2	0.17	0.83
HMS1	0.35	0.65
HMS2	0.37	0.63
HMS3	0.26	0.74
HMS6	0.39	0.61
HMS7	0.25	0.75
HTG4	0.40	0.60
HTG6	0.32	0.68
HTG7	0.38	0.62
HTG10	0.30	0.70
VHL20	0.23	0.77

Table 5. Possible and realized genotypes depending of established alleles per locus

Loci	Alleles	Possible genotypes	Realized genotypes	% realization
AHT4	5	15	11	73.33
AHT5	5	15	13	86.67
ASB2	9	45	32	71.11
HMS1	3	6	6	100
HMS2	7	28	15	53.57
HMS3	5	15	15	100
HMS6	5	15	11	73.33
HMS7	6	21	16	76.19
HTG4	6	21	10	47.61
HTG6	6	21	11	52.38
HTG7	4	10	7	70.00
HTG10	7	28	22	78.57
VHL20	6	21	12	57.14

Table 4. Allele frequencies at the microsatellite loci

Loci	Allele frequencies								
AHT4	O 0.4219	K 0.2719	J 0.1656	H 0.1344	I 0.0063				
AHT5	J 0.2547	K 0.3758	M 0.1988	N 0.1522	O 0.0186				
ASB2	B 0.0093	K 0.3199	N 0.0963	O 0.1087	I 0.0031	Q 0.1739	M 0.1118	P 0.0932	R 0.0839
HMS1	I 0.1335	J 0.3509	M 0.5155						
HMS2	H 0.1366	J 0.0652	K 0.2143	L 0.5280	M 0.0497	O 0.0031	R 0.0031		
HMS3	I 0.4130	O 0.1273	M 0.1770	N 0.0466	P 0.2360				
HMS6	K 0.1056	L 0.0745	M 0.2950	P 0.5124	O 0.0124				
HMS7	J 0.1273	L 0.1832	M 0.1739	N 0.2919	O 0.2205	K 0.0031			
HTG4	I 0.0062	K 0.4348	L 0.0155	M 0.4752	N 0.0311	P 0.0373			
HTG6	G 0.3696	J 0.4099	L 0.0031	M 0.0435	O 0.1677	R 0.0062			
HTG7	G 0.0062	K 0.2081	N 0.3292	O 0.4565					
HTG10	I 0.4627	O 0.1335	K 0.0652	L 0.0839	M 0.1646	R 0.0870	N 0.0031		
VHL20	I 0.1988	L 0.1460	M 0.4379	N 0.2112	O 0.0031	R 0.0031			

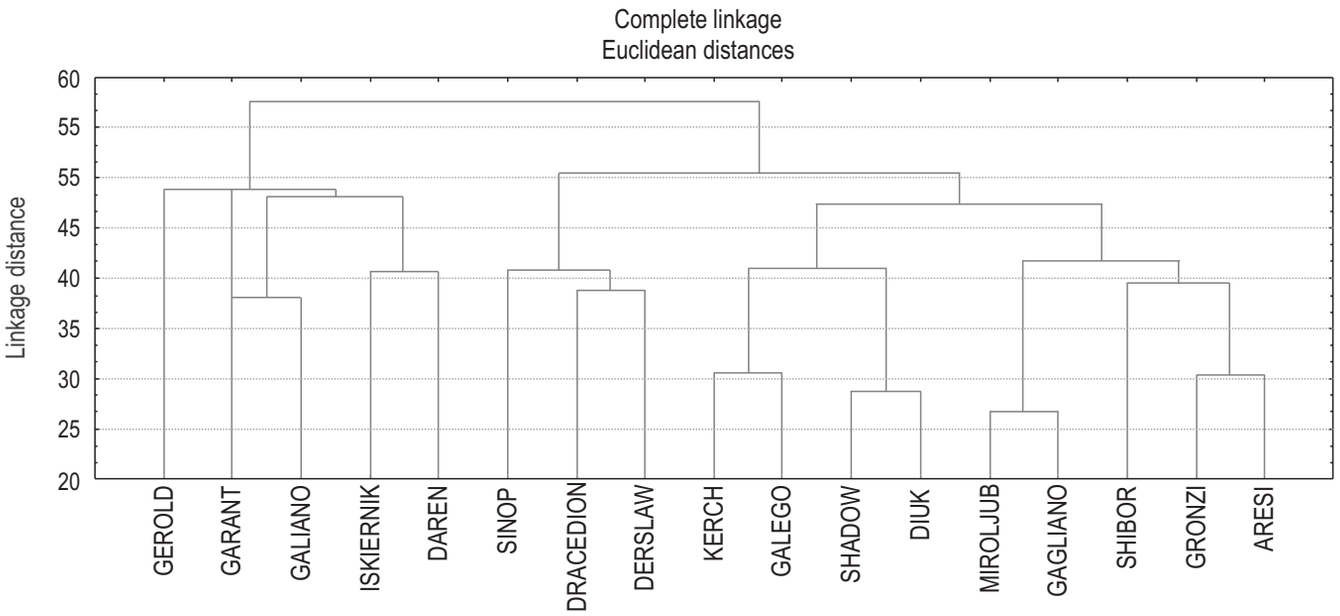

Figure 1. Euclidean distances among stalion offsping groups of Bulgarian thoroughbred

Conclusion

This study demonstrates the high levels of polymorphism detectable with microsatellite loci within the population of Bulgarian Thoroughbred horses. In horse breeding this technology has the potential to be of great use in monitoring levels of genetic variation within stocks as well as for parentage and relatedness purposes. These results also show that allelic diversity is a more sensitive measure of differences in genetic variation.

The middle realization of the observed and possible genotypes, depending of established alleles per locus, is 72.3 % which are the implications of very high level of genetic diversity, the prerequisite of accuracy of parentage verification.

Small effective population sizes and poorly managed breeding programs are generally blamed for loss of genetic diversity in farmed stocks. Maintaining pedigree information on all individuals in the breeding program and using this information to arrange matings will ensure only minimum increases in inbreeding every generation.

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Oldenbroek JK, 1999. Genebanks and the conservation of farm animal genetic resources, Second edition. DLO Institute for Animal Science and Health, Netherlands.

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