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Genetic diversity of Thoroughbred horse population from Bosnia and Herzegovina based on 17 microsatellite markers

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Abstract

The focus of this study was on genetic diversity of TB horse population raised in B&H. Genomic DNA was genotyped by using 17 microsatellite markers. A total of 103 alleles were detected. The average number of alleles per locus was 6.059 and effective number of alleles was 3.293. Means of observed and expected heterozygosity were calculated 0.645 and 0.696, respectively. The average PIC values was 0.649 and inbreeding coefficient was 0.090. Based on all observed parameters, ASB2 locus showed the highest genetic diversity while locus HMS2 was the least diverse. These results suggest that the population of TB horses from B&H is not affected by substantial loss of genetic diversity, indicating the presence of reasonably high level of genetic variability.

Key Words: Genetic diversity, Molecular markers, Thoroughbred horse

The Thoroughbred (TB) horse is an important horse breed best known as a racehorse. This breed is a favorite for use in the horse racing industry and has exceptional physiological

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traits[12,13]. TB horse is one of the oldest breeds of domestic animals with pedigree records spanning three centuries. The origin of TB horse was made by crossing imported eastern stallions (Arabian, Barb or Turk) with mares of English native horse, and then by the artificial selection for horse racing from early 1700's. Nowadays, the TB breed is widespread in many countries around the world[17,24,26].

During the war period in Bosnia and Herzegovina (B&H) (1992–1995) the total number of farm animals, including horses, has been more than halved. The number of horses declined by 65 per cent compared to 1991. Unfortunately, available statistical data indicate that the number of horses, including TB horses, remains in decline. B&H still has no Stud Book of TB horses, and official information about the number of administrated TB horses, the number of annually born TB horses and the number of registered stallions in B&H are almost non-existent[33]. Such dynamic population changes clearly call for the investigation of genetic information of remaining TB in B&H.

According to the requirements of the International Society for Animal Genetics (ISAG) and the International Stud Book Committee (ISBC) from 2001, genetic laboratories are obliged to conduct testing of TB horses using microsatellite loci of DNA. The microsatellites are characterized by the codominant character of inheritance and a high level of polymorphism. These markers are an important tool not only for parentage control, but also in genetic and population analyses[24].

Genetic diversity of TB horse from India[1], Romania[6], Slovakia[11], Egypt[17], Canada[21] and Korea[26] has been investigated based on microsatellite data. However, genetic diversity of TB horse population from B&H has not been studied yet. In this paper we report the results of the first genetic analysis of TB horse population from B&H using 17 microsatellite markers currently recommended by International Society for Animal Genetics (ISAG)[9].

The study included 30 whole-blood samples from Thoroughbred horses raised in the Western and Central Bosnia and Herzegovina (16 stallions and 14 mares), from 2 to 10 years of age. All sampled horses were born in B&H and unrelated to each other. Blood samples (3 ml) were collected from *v. jugularis* using sterile venipuncture needles and EDTA vacuum containers. Genomic DNA was isolated according to the modified protocol (3 ml of blood; 10 ml of Lysis buffer; 4 ml of PBS; 4 ml of Kern-lysis buffer; 150 µl of 20% SDS; 100 µl of protease and 0,5 ml 6 M NaCl) for the isolation of DNA from human blood by salting-out method[17]. The concentration of isolated DNA was determined by spectrophotometry, using UV mini - 1240 (*Shimadzu*) spectrophotometer. Improved StockMarks® Equine Genotyping Kit (*Applied Biosystems*), designed for simultaneous amplification of 17 horse microsatellite markers, was used for the analysis of nuclear DNA polymorphism. PCR was performed according to the manufacturer's protocol. PCR products were analyzed on an ABI Prism™ 310 Genetic Analyzer. Sizing of the amplified fragments was performed using GeneMapper ID v3.2 software. Allele size range, major allele frequency (f_M), number of different alleles (A_N), polymorphic information content (PIC)[3], observed heterozygosity (H_o), expected heterozygosity (H_E)[19], inbreeding coefficient (F)[31] and deviation from *Hardy-Weinberg* equilibrium (HWE)[8] were calculated using POWERMARKER 3.25[15]. The number of effective alleles (A_E) was estimated using GenAlEx software[20]. Simple ratio between the number of effective alleles and the number of observed alleles (A_E/A_N), as well as major allele frequency index (If_M) were calculated as suggested by Pojskic[22]. This ratio indicates whether there is a disproportion between the effective number of alleles and the number detected by direct counting. Major allele frequency index shows how much is the larger frequency greater than expected assuming equal frequencies of all detected alleles at a given locus. It is expressed as $f_M/(1/A_N)$ where f_M represents the major allele

frequency and A_N is the number of detected alleles at a given locus.

All the equine microsatellite markers, reported in the study, were amplified successfully. Results for allele size, major allele frequency (f_M), major allele frequency index (If_M), number of different alleles (A_N), number of effective alleles (A_E), A_E/A_N ratio, observed heterozygosity (H_O), expected heterozygosity (H_E), deviation from *Hardy-Weinberg* equilibrium (HWE), polymorphic information content (PIC) and inbreeding coefficient (F) are given in Table 1. A total of 103 alleles were detected across 17 microsatellite markers in TB horse population. PCR product size range varied from 77–101 bp at HTG6 locus to 219–253 bp at ASB2 locus. The number of observed alleles at individual loci was variable, ranging from 2 (HTG4) to 10 (ASB2) with a mean value of 6.059. The range of effective number of alleles spanned from 1.946 (HTG4) to 6.406 (ASB2) with a mean value of 3.293. The highest ratio between number of effective alleles and number of different alleles was detected for HTG4 and the lowest for CA425 locus. The mean value for this ratio was 0.544. The largest value of major allele frequency index was detected for CA425 (3.600) and the lowest for HTG4 (1.167) with a mean value of 2.542. The observed heterozygosity values across the 17 polymorphic marker loci ranged from 0.400 (LEX3) to 0.800 (ASB17) with a mean of 0.645. The expected heterozygosity varied from 0.486 (HTG4) to 0.844 (ASB2) with a mean of 0.696. The PIC values varied from 0.368 at locus HTG4 to 0.826 at locus ASB2, with a mean value of 0.649. Of the 17 markers, HMS7, ASB23, ASB2, HTG10, HMS3, ASB17 and LEX3 loci have relatively high PIC value (>0.7), whereas the other loci used in this study (except HTG4) showed PIC values higher than 0.5. The inbreeding coefficient fluctuated from -0.009 (AHT4) to 0.507 (LEX3), with a mean of 0.090. Statistically significant deviation ($P < 0.05$) from HWE was observed for ASB23, HMS3 and LEX3 loci.

These preliminary results are the first genetic diversity survey of TB horse population raised in Bosnia and Herzegovina. Our detected allelic sizes (spanning between 77 and 253 bp) in general concur with previously described range[28]. Even though some discrepancies in allele sizes between this study and previous reports can be attributed to the difference in the instrument used to analyze the samples, we observed alleles that fall outside of expected size range for loci HTG4, HTG7, HMS1, HMS6, ASB2, ASB17 and ASB23. Our results of the average number of effective alleles per locus were lower than that previously reported for TB horses from India[1] (3.85), Poland[10], (3.589), Lithuania[14] (3.662), Egypt[17] (4.853) and Canada[21] (3.87). The most polymorphic locus in our study was ASB2 with 10 allelic variants. The highest number of alleles for ASB2 locus was described in TB racehorses from Slovakia[11], Korea[13] and Egypt[17]. The least polymorphic locus was HTG4 with 2 allelic variants. The same locus showed the lowest number of alleles in TB horses in Slovakia[11]. Our data for the mean number of alleles were similar to the results of Iwanczyk *et al.*[10] (6.167), Luis *et al.*[16] (6.25), Mahrous *et al.*[17] (6.600) and Sun-yong and Gil-jae[26] (6.36). Lower mean number of alleles for investigated TB horse populations were reported in other previous studies (4.2–5.73)[1,2,4,7,21,25,27,29,30]. Generally, the differences in average number of alleles may be attributed to different set of chosen microsatellite markers, number of analyzed markers, number of individuals in study, as well as population structure. The high level of AE/AN ratio indicates that large portion of detected alleles actually have major participation in genetic diversity of TB horse population raised in B&H, which could provide a solid start in perspective revitalization of TB horse population in B&H.

Results for the average level of H_O detected in our work were similar to results on TB horses reported by Glowatzki-Mullis *et al.*[7] (0.65), Solis *et al.*[25] (0.633) and Tozaki *et al.*[27] (0.63). Behl *et al.*[1] reported lower Ho values for TB horses from India (0.53). In other studies on TB horse

Table 1. Allele size range, major allele frequency (f_M), major allele frequency index (If_M), number of different alleles (A_N), number of effective alleles (A_E), A_E/A_N ratio, observed heterozygosity (H_O), expected heterozygosity (H_E), deviation from *Hardy-Weinberg* equilibrium (HWE), polymorphic information content (PIC) and inbreeding coefficient (F) at 17 microsatellite loci in Thoroughbred horse population from Bosnia and Herzegovina

Locus	Allele size range (bp)	f_M	If_M	A_N	A_E	A_E/A_N	H_O	H_E	HWE p-value	PIC	F
VHL20	84–96	0.350	1.750	5	3.846	0.769	0.767	0.740	0.963	0.694	−0.019
HTG4	126–130	0.583	1.167	2	1.946	0.973	0.500	0.486	1.000	0.368	−0.012
AHT4	144–158	0.383	1.533	4	3.502	0.876	0.733	0.714	0.462	0.662	−0.009
HMS7	171–181	0.383	2.300	6	4.206	0.701	0.700	0.762	0.233	0.730	0.098
HTG6	77–101	0.500	3.000	6	2.609	0.435	0.767	0.617	0.071	0.547	−0.227
AHT5	128–140	0.583	3.500	6	2.476	0.413	0.633	0.596	0.587	0.551	−0.046
HMS6	156–188	0.433	3.033	7	2.990	0.427	0.633	0.666	0.686	0.607	0.065
ASB23	176–218	0.283	2.550	9	5.128	0.570	0.633	0.805	0.009	0.779	0.229
ASB2	219–253	0.250	2.500	10	6.406	0.641	0.733	0.844	0.320	0.826	0.148
HTG10	85–105	0.350	2.450	7	4.265	0.609	0.733	0.766	0.886	0.731	0.059
HTG7	112–126	0.417	2.500	6	3.371	0.562	0.533	0.703	0.090	0.654	0.258
HMS3	148–164	0.400	2.400	6	4.082	0.680	0.500	0.755	0.004	0.723	0.353
HMS2	216–226	0.600	3.000	5	2.323	0.465	0.500	0.569	0.736	0.518	0.139
ASB17	96–120	0.283	1.983	7	5.143	0.735	0.800	0.806	0.117	0.778	0.024
LEX3	139–161	0.267	1.867	7	4.800	0.686	0.400	0.792	0.000	0.760	0.507
HMS1	172–182	0.467	1.867	4	2.757	0.689	0.767	0.637	0.572	0.567	−0.187
CA425	228-240	0.600	3.600	6	2.381	0.397	0.633	0.580	0.904	0.536	−0.075
Mean	—	0.420	2.542	6.059	3.293	0.544	0.645	0.696	—	0.649	0.090

populations the average values of Ho were higher than values found in our study (0.663–0.880)[2,4,6,10,13,14,16,17,21,26,29,30]. The average level of H_E reported in the literature for TB horse populations mostly ranged from 0.65 to 0.829[1,2,4,6,7,10,14,16,17,21,25,26,29,30]. Our data for H_E are consistent with data from previous studies. Comparing our results of mean number of alleles and observed heterozygosity with results previously reported for European TB horse population[27] we can conclude that the mean number of alleles in TB horse population from B&H was above the mean for European TB horses (6.059 compared to 4.2). Observed heterozygosity was similar to the results observed for European TB horses (0.645 compared to 0.63). Average number of effective alleles detected in our study was similar to results for domestic horse breeds[14] (3.293 compared to 3.531). Observed heterozygosity was below the mean for domestic horse breeds (0.645 compared to 0.697), while expected heterozygosity was higher than the mean for domestic horse breeds (0.696 compared to 0.674). The level of observed heterozygosity for loci VHL20, HTG4, AHT4, HTG6, AHT5, HMS1 and CA425 was higher than expected, while for the rest of employed loci observed heterozygosity was lower. According to Berber *et al.*[2] larger disproportion between observed and expected heterozygosity could be an indicator of within-population inbreeding or conversely population subdivision reduction. In our study, the greatest difference between H_O and H_E was observed for LEX3 locus. The same difference between H_O and H_E for this locus was observed in Fornal *et al.*[5] and Zabek *et al.*[32] as well. Fornal *et al.*[5] explained that this difference probably occurs because of the linkage of LEX3 locus to X-chromosome. The allele numbers and

heterozygosity levels observed across the studied loci indicate presence of reasonably high level of genetic variability in TB horse from B&H. Given the size of overall population, such high values can be interpreted by the fact that, after the war, stallions of TB horses from other countries were imported in B&H, which certainly affected the genetic pool of the existing population. Observed results could also be conditioned by the experiment design (the samples were collected in two locations and all sampled horses were not related to each other).

The PIC values, detected in our study, suggested that 94,12% markers were quite informative (PIC $>$ 0.5) in terms of their suitability for genetic diversity studies while remaining locus was reasonably informative. Values below 50% can be considered uninformative[23], although in the present case this only applies to HTG4 locus.

Most loci indicate no major inbreeding in TB horse population from B&H, while overall value shows moderate characteristics. The value for LEX3 locus is probably encumbered with error because of its linkage to X-chromosome.

Results of our study indicate presence of reasonably high level of genetic variability in TB horse from B&H. However, assuming values of H_O, H_E and PIC around 0.7, we consider loci VHL20, HMS7, ASB2, HTG10 and ASB17 as the most polymorphic. When all observed parameters are taken into account, it can be concluded that population of TB horse raised in B&H is not affected by substantial loss of genetic diversity which is very important in the process of revitalization. The present work is a contribution to the knowledge of population structure and assessment of genetic diversity of TB horse population from B&H, offering basic information that may be helpful to horse breeders in designing and managing future breeding strategies.

References

1) Behl, R., Behl, J., Gupta, N. and Gupta, S. C. 2007. Genetic relationships of five Indian horse breeds using microsatellite markers. *Animal*, **1**: 483–488.
2) Berber, N., Gaouar, S., Leroy, G., Kididi, S., Tabet Aouel, N. and Saidi Mehtar, N. 2014. Molecular characterization and differentiation of five horse breeds raised in Algeria using polymorphic microsatellite markers. *J. Anim. Breed. Genet*., **131**: 387–394.
3) Botstein, D., White, R. L., Skolnick, M. and Davis, R. W. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am. J. Hum. Genet.,* **32**: 314–331.
4) Canon, J., Checa, M. L., Vega-Pla, J. L., Vallejo, M. and Dunner, S. 2000. The genetic structure of Spanish Celtic horse breeds inferred from microsatellite data. *Anim. Genet*., **31**: 39–48.
5) Fornal, A., Radko, A. and Piestrzynska-Kajtoch, A. 2013. Genetic polymorphism of Hucul horse population based on 17 microsatellite loci. *Acta Biochim. Pol.*, **60**: 761–765.
6) Georgescu, S. E., Manea, M. A. and Costache, M. 2008. The genetic structure of indigenous Romanian Hucul horse breed inferred from microsatellite data. *Rom. Biotech. Lett.*, **13**: 4030–4036.
7) Glowatzki-Mullis, M. L., Muntwyler, J., Pfister, W., Marti, E., Rieder, S., Poncet, S. A. and Gaillard, C. 2005. Genetic diversity among horse populations with a special focus on the Franches-Montagnes breed. *Anim. Genet*., **37**: 33–39.
8) Guo, S. W. and Thompson, E. A. 1992. Performing the exact test of Hardy-Weinberg proportion for multiple alleles. *Biometrics*, **48**: 361–372.
9) ISAG, 2014. ISAG 2014 Workshop Report. (http://www.isag.us/committees.asp), Accessed: February 17, 2016.
10) Iwanczyk, E., Juras, R. Cholewinski, G. and Cothran, E. G. 2006. Genetic structure and phylogenetic relationships of the Polish heavy horse. *J. Appl. Genet*., **47**: 353–359.
11) Jakabova, D., Trandžik, J., Chrastina, J., Hudecova, L., Zetochova, E., Bulla, J., Bugarsky, A., Jakab, F. and Kozlik, P. 2002. Effectiveness of six highly polymorphic microsatellite markers in resolving paternity cases in Thoroughbred horses in Slovakia.

Czech. J. Anim. Sci., **47**: 497–501.
12) Joon-Ho, L., Taeheon, L., Hak-Kyo, L., Byung-Wook, C., Dong-Hyun, S., Kyoung-Tag, D., Samsun, S., Woori, K., Hyeon Jeong, K., Heebal, K., Seoae, C. and Kyung-Do, P. 2014. Thoroughbred horse single nucleotide polymorphism and expression database: HSDB. *Asian-Australs J. Anim. Sci*., **27**: 1236–1243.
13) Jungwoo, E., Jeong-An, G., Bong-Hwan, C., Kyoung-Tag, D., Byung-Wook, C. and Heui-Soo, K. 2014. Genetic profiling of thoroughbred racehorses by microsatellite marker analysis. *Genes Genom.*, **36**: 119–123.
14) Juras, R., Cothran, E. G. and Klimas, R. 2003. Genetic Analysis of three Lithuanian native horse breeds. *Acta Agriculturae Scandinavica, Sect. A, Animal Sci*., **53**: 180–185.
15) Liu, K. and Muse, S. 2005. Powermarker: Integrated analysis enviroment for genetic marker data. *Bioinformatics*, **21**: 2128–2129.
16) Luís, C., Juras, R., Oom, M. M. and Cothran, E. G. 2007. Genetic diversity and relationships of Portuguese and other horse breeds based on protein and microsatellite loci variation. *Anim. Genet*., **38**: 20–27.
17) Mahrous, K. F., Hassanane, M., Abdel Mordy, M., Shafey, H. I. and Hassan, N. 2011. Genetic variations in horse using microsatellite markers. *J. Genet. Engineer. Biotech.*, **9**: 103–109.
18) Miller, S. A., Dykes, D. D. and Polesky, H. F. 1988. A simple salting-out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res*., **16**: 1215.
19) Nei, M. 1987. *Molecular Evolutionary Genetics*. Columbia University Press, New York.
20) Peakall, R. and Smouse, P. E. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. *Bioinformatics*, **28**: 2537–2539.
21) Plante, Y., Vega-Pla, J. L., Lucas, Z., Colling, D., deMarch, B. and Buchanan, F. 2007. Genetic diversity in a feral horse population from Sable Island, Canada. *J. Hered.*, **98**: 594–602.
22) Pojskić, N. 2005. Polymorphism of nuclear microsatellite markers in B&H populations of salmonids. PhD Diss, Faculty of Science, University of Sarajevo, COBISS.BH-ID: 16348934.
23) Reis, S. P., Goncalves, E. C., Silva, A. and Schneider, M. P. C. 2008. Genetic variability and efficiency of DNA microsatellite markers for paternity testing in horse breeds from the Brazilian Marajo archipelago. *Genet. Mol. Biol.*, **31**: 68–72.
24) Shelyov, A. V., Melnyk, O. V., Suprun, I. O., Spyrydonov, V. G., Melnychuk, S. D., Dzitsiuk, V. V. and Gopka, B. M. 2014. The comparative analysis of the allele pool of Thoroughbred horses in different countries. *Iranian J. Appl. Anim. Sci.*, **4**: 637–641
25) Solis, A., Jugo, B. M., Meriaux, J. C., Iriondo, M., Mazon, L. I., Aguirre, A. I., Vicario, A. and Estomba, A. 2005. Genetic diversity within and among four South European native horse breeds based on microsatellite DNA analysis: Implications for conservation. *J. Hered.*, **96**: 670–678.
26) Sun-young, L. and Gil-jae, C. 2006. Parentage testing of Thoroughbred horse in Korea using microsatellite DNA typing. *J. Vet. Sci*., **7**: 63–67.
27) Tozaki, T., Takezaki, N., Hasegawa, T., Ishida, N., Kurosawa, M., Tomita, M., Saitou, N. and Mukoyama, H. 2003. Microsatellite Variation in Japanese and Asian Horses and Their Phylogenetic Relationship Using a European Horse Outgroup. *J. Hered*., **94**: 374–380.
28) User Guide, StockMarks® Kits for Horses, Cattle, and Dogs. Equine, Bovine, and Canine Genotyping Kits, Applied Biosystems. Publication Number 4346135, Revision F, Chapter 3, 21–22 (2014), http://tools.lifetechnologies.com/content/sfs/manuals, Accessed: May 5, 2015.
29) van de Goor, L. H. P., van Haeringen, W. A. and Lenstra, J. A. 2011. Population studies of 17 equine STR for forensic and phylogenetic analysis. *Anim. Genet*., **42**: 627–633.
30) Vega-Pla, J. L., Calderon, J., Rodríguez-Gallardo, P. P., Martinez, A. M. and Rico, C. 2006. Saving feral horse populations: does it really matter? A case study of wild horses from Doñana National Park in southern Spain. *Anim. Genet*., **37**: 571–578.
31) Weir, B. S. 1996. *Genetic data analysis II*, Sunderland, MA: Sinauer Associates, Inc.
32) Zabek, T. and Fornal, A. 2009. Evaluation of the 17-plex STR kit for parentage testing of Polish coldblood and Hucul horses. *Ann. Anim. Sci*., **9**: 363–372.
33) Animal genetic resources in Bosnia and Herzegovina. 2003. State Veterinary Administration of Bosnia and Herzegovina. Sarajevo, 2003. ftp://ftp.fao.org. Accessed: February 14, 2016.