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Analysis of genetic diversity among certain horse breeds from Bosnia and Herzegovina

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Abstract
In the present study, for the first time we investigated the genetic diversity among horse breeds from Bosnia and Herzegovina: potential Bosnian and Herzegovinian mountain horse, Arabian horse, Thoroughbred horse and crossbreeds, with special emphasis on the gene pool of potential Bosnian and Herzegovinian mountain horse. In total, 138 animals were genotyped for 17 microsatellite loci. Compared to the other breeds, potential Bosnian and Herzegovinian mountain horse showed quite a high genetic variability. The mean number of alleles was 14.1765. The average observed heterozygosity was 0.6589 and the expected heterozygosity was 0.8451. The mean value of polymorphic information content was 0.8286. The results of AMOVA test showed 8.44% of genetic variation among populations. The highest genetic variation within population was showed by potential Bosnian and Herzegovinian mountain horse (27.13). The same breed showed the highest individual variation (17.35). Overall F_{ST} value showed high level of the genetic differentiation among breeds (8.87%), and the pairwise F_{ST} values were all significant. Highest inter-group genetic differentiation was observed among Arabian horse and Thoroughbred horse (groups of pure breeds) and potential Bosnian and Herzegovinian mountain horse.
The results show that the potential Bosnian and Herzegovinian mountain horse has a high within breed variability, more than could be expected. In the gene pool of potential Bosnian and Herzegovinian mountain horse is present a part of the gene pool of other breeds. Also, these results show that there are very good preconditions for the revitalization of the gene pool of potential Bosnian and Herzegovinian mountain horse.

Keywords
Horse — genetic diversity — microsatellites

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Introduction

Horses are members of the *Equidae* family, large land mammals notable for its speed, strength and endurance. The horses influence on human history and civilization make them one of the most important domestic animals (13). Genetic characterization of a breed is the first step in the conservation of breeds, determination of future breeding strategies, and is important to protect breed integrity (2, 19).

Identification of genetic variation among various horses requires the development of genetic markers. Among available genetic markers, microsatellites have useful properties for research on genetic diversity (8). In the recent years, microsatellite markers are markers of choice in livestock genetic characterization studies and have frequently been used to evaluate genetic distances, characterise local breeds, for equine population studies as well as parentage control (6, 10, 21). Microsatellites are highly polymorphic genetics markers, considered especially suitable for biodiversity evaluation, owing to their co-dominant inheritance, high heterozygosity, ubiquitous presence through the genome, easy and reliable scoring, and high degree of polymorphism (4, 8, 16).

The aim of this study was to analyze genetic diversity among certain groups of different breeds of horses with special emphasis on the gene pool of potential Bosnian and Herzegovinian mountain horse by using 17 microsatellite loci recommended by the International Society for Animal Genetics (ISAG).

Material and Methods

The study was carried out on 138 animals divided into the following groups: BOS–62 individuals (potential Bosnian and Herzegovinian mountain horse); ARA-20 individuals (Purebred Arabian horse); ENG-36 individuals (Thoroughbred horse); KBA-7 individuals (crosses between Bosnian and Herzegovinian mountain horse and Arabian horse); KBR-9 individuals (crosses between Bosnian and Herzegovinian mountain and Belgian horses, crosses between Bosnian and Herzegovinian mountain horses and Holstein, crosses between Bosnian and Herzegovinian mountain and

Lipizzaner horses); KBN-4 individuals (crosses between Bosnian and Herzegovinian mountain horse with an unknown other parent). All horses were raised in Bosnia and Herzegovina.

DNA for genotyping analysis was extracted from the whole blood. Blood samples (3 ml) were collected from *V. jugularis* using sterile venipuncture needles and EDTA vacuum containers. Genomic DNA was isolated by the original protocol for isolation of DNA from human blood by method of salting out (15), which was modified and adjusted to horses blood and our laboratory conditions. The concentration of isolated DNA was determined by spectrophotometry, using a spectrophotometer UV mini - 1240 (*Shimadzu*). For polymorphism analysis of nuclear DNA, improved StockMarks© Equine Genotyping Kit (*Applied Biosystems*) designed for simultaneous genotyping 17 horse microsatellite markers, was used. Selected microsatellite regions were amplified with the PCR. The size of the amplified microsatellite fragments was analyzed using a genetic analyzer ABI Prism^{TM} 310 *Genetic Analyzer*. Determination of the size of the amplified fragments was performed using GeneMapper ID v3.2 software. Biostatistic and population-genetic analysis of molecular markers were done by the following software packages: POWERMARKER 3.25 (11) (estimated number of alleles (Allele No), Polymorphic Information Content (PIC), observed (H_O) and expected heterozygosity (H_E), the fixation index (F_{ST}) and pairwise F_{ST} (pF_{ST}), analysis of molecular variance (AMOVA test), and MEGA4 (20) (Neighbor-Joining (NJ) dendrogram).

Results

In this paper we reported the results of the first analysis of genetic diversity among certain horse breeds from Bosnia and Herzegovina with special emphasis on the gene pool of potential Bosnian and Herzegovinian mountain horse. Results for the number of alleles (Allele N_O), observed heterozygosity (H_O), expected heterozygosity (H_E) and polymorphic information content (PIC) for all groups are given in Table 1. The highest mean Allele No was observed for the group BOS (14.1765) and the lowest for the groups ARA (5.5822) and KBR (5.2353). The same groups showed the highest and the lowest mean values of H_E (0.8451, 0.7017 and 0.7002 respectively) and PIC values (0.8286, 0.6530 and 0.6622 respectively). The highest mean value of H_O was showed by the groups KBR (0.7255) and KBA (0.7213) and the lowest group ARA (0.6261).

Analysis of molecular variance (AMOVA test) showed 8.44% of genetic variation among populations (Table 2). The highest genetic variation within population showed group BOS (27.13). The same group showed the highest within individuals variation (17.35).

The fixation index, measure of population differentiation (F_{ST}) showed high level of genetic differentiation among breeds (8.87%). The results of pairwise differences (pF_{ST}) for all groups are presented in Table 3. The pFST values were all significant. Group BOS showed the greatest differentiation compared to the group ENG (9.37%) and group ARA (8.83%). Highest inter-group genetic differentiation was observed among groups of pure breeds and group BOS.

Table 1. Summary statistics for all tested groups showing number of alleles (Allele No), expected heterozygosity (H_E), observed heterozygosity (H_O) and polymorphic information content (PIC) for 17 microsatellite loci

Group	Allele No	HE	HO	PIC
BOS	14.1765	0.8451	0.6589	0.8286
ENG	8.5294	0.7233	0.6487	0.6856
ARA	5.5822	0.7017	0.6261	0.653
KBR	5.2353	0.7002	0.7255	0.6622
KBA	6.8824	0.7867	0.7213	0.7592
KBN	7.1765	0.8007	0.6471	0.7768

Table 2. Results of analysis of molecular variance (AMOVA test)

Source	Sum Of Square	Percentage
Among Populations	336.5905	0.0844
Within Population (ARA)	262.0611	0.0657
Within Population (BOS)	1082.2161	0.2713
Within Population (ENG)	488.2778	0.1224
Within Population (KBA)	100.5714	0.0252
Within Population (KBN)	113.5714	0.0285
Within Population (KBR)	68.8333	0.0173
Within Individuals (ARA)	212	0.0531
Within Individuals (BOS)	692	0.1735
Within Individuals (ENG)	379	0.0995
Within Individuals (KBA)	85	0.0213
Within Individuals (KBN)	77	0.0193
Within Individuals (KBR)	74	0.0186
Total	3989.1216	1

Table 3. Results of pairwise differences (pF_{ST}) for all tested groups

	ARA	BOS	ENG	KBA	KBN	KBR
ARA	0	0.0883	0.1292	0.1431	0.0958	0.2129
BOS	0.0883	0	0.0937	0.0207	0.002	0.0679
ENG	0.1292	0.0937	0	0.107	0.0703	0.1762
KBA	0.1431	0.0207	0.107	0	-0.0094	0.0227
KBN	0.0958	0.002	0.0703	-0.0094	0	0.0494
KBR	0.2129	0.0679	0.1762	0.0227	0.0494	0

Constructed Neighbor-Joining (NJ) tree based on (pF_{ST}) shows a clear position of groups ARA and ENG in a separate cluster. Groups of crossbreeds formed a separate cluster, too. Position of the group BOS is between groups of the pure breeds and crossbreeds, and formed the third cluster (Figure 1).

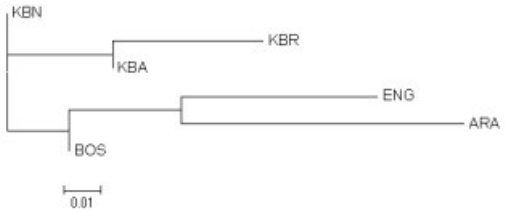

Figure 1. Neighbor-Joining tree based on (pF_{ST}) among all tested groups

Discussion and conclusion

During the research described in this paper we carried out the genetic analysis of the certain horse breeds from Bosnia and Herzegovina using microsatellite markers. First data of molecular genetic structure and mutual population relations of potential Bosnian and Herzegovinian mountain horse compared to pure breeds and crossbreeds, were obtained. The number of alleles per loci is a simple and common measure of genetic diversity and, in some cases, may be more informative than genetic heterozygosity (17). The mean number of alleles for group BOS in our study was higher compared to the other tested groups, and those reported in the previous studies (from 4.3 to 9.58) (9, 18, 19). The differences among breeds and their mean number of alleles could depend on the analyzed number of alleles, number of samples as well as the population structure. Our results of the mean number of alleles for other groups included in this study were consistent with the results from the previous studies.

The average levels of H_O and H_O reported in the literature for other horse breeds mostly ranged from 0.302 to 0.78 for H_O, and from 0.305 to 0.82 for H_E (1, 3, 5, 7, 12, 14, 17, 18, 19, 22, 23). Our data for H_O and H_E for all tested groups are consistent with data from the previous studies. Only group BOS showed higher values of expected heterozygosity. The mean allele number and heterozygosity levels observed in our study indicate the presence of reasonably high level of genetic variability in group BOS.

Genetic markers showing PIC values higher than 0.5 are normally considered as informative in population genetic analysis (18). All mean PIC values in our study were above this level and we can validate the high polymorphism of chosen markers and high degree of informativeness of chosen markers in evaluation of genetic diversity.

The results of AMOVA test indicate a clear differentiation among the groups of horses. The highest genetic variation within population, and within individuals observed for group BOS indicate that this group cannot be treated as a completely pure breed, and confirm the presence of the gene pool of other breeds. The analysis of F_{ST} showed that genetic differences between the breeds accounted for 8.87 of the total variation (F_{ST} = 8.87; p = 0.001). This estimate is similar to those reported in Spanish Celtic breeds and western Mediterranean breeds, but lower than the F_{ST} values obtained in other studies on European breeds (1, 3, 5, 14). The values of the genetic differentiation of 10% are typical for differentiation between geographically isolated populations of horses as well as within the different breeds (23). Our results of AMOVA test and F_{ST} were similar, and demonstrate clear differentiation among the tested groups.

The pairwise F_{ST} values were all significant. Highest inter-group genetic differentiation, observed among the groups of pure breeds (ARA and ENG) and group BOS, indicates that the group BOS, whatever the variable was, is differentiated in relation to the groups of pure breeds. It is noteworthy that the group BOS showed the greatest genetic variability, probably because of its wide genetic base and heterogeneity. Our results indicate that in the gene pool of group BOS, present is a part of the gene pool of other breeds, and that this group cannot be treated as a complete purebread. The position of the group BOS on phylogenetic tree, except suggesting that this group in gene pool contains the genes of other breeds, provides confirmation that there are very good preconditions for the revitalization of the gene pool of the group BOS.

The obtained results can be of great help to farmers, offering a broad and essential base of information by providing guidelines for the cultivation and establishment of breeding programs. These data can be very useful for the development of conservation programs and management strategies in order to protect and preserve our breed.

The research is a contribution to the study and conservation of animal genetic resources. Contributes to knowledge about the population structure and assess the existing genetic diversity of Bosnian and Herzegovinian mountain horse. Also, it opens the way and gives the scientific basis and guidelines for further researches in order to complete the information on the origin and evolution of our breed. Such researches would help in preserving Bosnian and Herzegovinian mountain horse, which would certainly contribute to the overall conservation of biodiversity at the global level.

References

1. Aberle K.S., Hamann H., Drogemuller C., Distl O. (2004): Genetic diversity in German draught horse breeds compared with a group of primitive, riding and wild horses by means of microsatellite DNA markers. Anim Genet, 35: 270-77.
2. Burocziova M., Riha J. (2009): Horse breed discrimination using machine learning methods. J Appl Genet, 50(4): 375-77.
3. Canon J., Checa M.L., Vega-Pla J.L., Vallejo M., Dunner S. (2000): The genetic structure of Spanish Celtic horse breeds inferred from microsatellite data. Anim Genet, 31: 39-48.
4. Chauhan M., Gupta A.K., Dhillon S. (2004): Genetic characterization of Indian Spiti horses. J Genet, 83(3): 291-95.
5. Di Stasio L., Perrotta G., Blasi M., Lisa C. (2008): Genetic characterization of the Bardigiano horse using microsatellite markers. Ital. J Anim Sci, 7: 243-250.
6. Fornal A., Radko A., Piestrzynska-Kajtoch A. (2013): Genetic polymorphism of Hucul horse population based on 17 microsatellite loci. Acta Biochem Pol, 60(4): 761-5.
7. Iwanczyk E., Juras R., Cholewinski G., Cothran E.G. (2006): Genetic structure and phylogenetic relationships of the Polish heavy horse. J Appl Genet, 47(4): 353-59.
8. Jungwoo E., Jeong-An g., Bong-Hwan C., Kyoung-Tag D., Byung-Wook C., Heui-Soo k. (2014) Genetic profiling of thoroughbred racehorses by microsatellite marker analysis. Genes Genom, 36:119-123.
9. Kusza C., Priskin K., Ivankovic A., Jedrzejewska B., Podgorski T., Javor A., Mihok S. (2013): Genetic characterization and population bottleneck in the Hu-

cul horse based on microsatellite and mitochondrial data. Biological Journal of the Linnean Society, 109: 54-65.
10. Leroy G., Calleda L., Verrier E., Meriaux C., Ricard A., Danchin-Burge C., Rognon X. (2009): Genetic diversity of a large set of horse breeds raised in France assessed by microsatellite polymorphism. Genet Sel Evol, 41(1): 31.
11. Liu K., Muse S. (2005): Powermarker: Integrated analysis enviroment for genetic marker data. Bioinformatics, 21(9): 2128-9.
12. Luis C., Juras R., Oom M.M., Cothran E.G. (2007): Genetic diversity and relationships of Portuguese and other horse breeds based on protein and microsatellite loci variation. Anim Genet, 38, 20-7.
13. Mahrous K.F., Hassanane M., Abdel Mordy M., Shafey H.I., Hassan N. (2011): Genetic variations in horse using microsatellite markers. Journal of Genetic Engineering and Biotechnology, 9: 103-9.
14. Marletta D., Tupac-Yupanqui I., Bordonaro S., Garcia D., Guastella A.M., Criscione A., Canon J., Dunner S. (2006): Analysis of genetic diversity and the determination of relationships among western Mediterranean horse breeds using microsatellite markers. J Anim Breed Genet, 123: 315-25.
15. Miller S.A., Dykes D.D., Polesky H.F. (1988): A simple salting-out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res, 16: 1215.
16. Moshkelani S., Rabiee S., Javaheri - Koupaei M. (2011) : DNA fingerprinting of Iranian Arab horse using fourteen microsatellites marker. Res J Biol Sci, 6(8): 402-5.
17. Plante Y., Vega-Pla J.L., Lucas Z., Colling D., de-March B., Buchanan F. (2007): Genetic diversity in a feral horse population from Sable Island, Canada. J Heredity, 98(6): 594-602.
18. Shasavarani H., Rahimi-Mianji G. (2010): Analysis of genetic diversity and estimation of inbreeding coefficient within Caspian horse population using microsatellite markers. Afr J Biotechnol, 9(3): 293-99.
19. Solis A., Jugo B.M., Meriaux J.C., Iriondo M., Mazon L.I., Aguirre A.I., Vicario A., Estomba A. (2005): Genetic diversity within and among four South European native horse breeds based on microsatellite DNA analysis: Implications for conservation. J Heredity, 96(6): 670-78.
20. Tamura k., Dudley J., Nei M., Kumar S. (2007): Mega4: Molecular Evolutionary Genetics Analysis software version 4.0. Molecular Biology and Evolution, 24: 1596-9.
21. Teneva A. (2009): Molecular markers in animal genome analysis. Biotechnol Animal Husbandry, 25(5-6): 1267-1284.
22. Viluma A., Jonkus D. (2010): Parentage determination by 17 microsatelitte markers of Latvian warmblood horse. LLU, RAKSTI 24(319): 31-7.
23. Zabek T., Nogaj A., Radko A., Nogaj J., Slota E. (2005): Genetic variation of Polish endangered Bilgoraj horses and two common horse breeds in microsatellite loci. J Appl Genet, 46(3): 299-305.

Analiza genetičke raznolikosti između nekih pasmina konja iz Bosne i Hercegovine

Sažetak

Uvod

Konji predstavljaju važan animalni genetički resurs. U ljudskoj historiji, niti jedna domaća životinja nije odigrala tako značajnu ulogu u socijalnom progresu i političkom razvoju kao konj, što ga čini jednom od najvažnijih domaćih životinja. Identifikacija genetičke raznolikosti između različitih konja zahtijevala je razvoj mikrosatelitnih markera, koji su se poka-zali kao idealan izbor za studije genetičke raznolikosti. Mikro-sateliti su visokopolimorfni genetički markeri, podjednako distri-buirani kroz genom, s kodominantnim nasljeđivanjem i lako se analiziraju.

Cilj istraživanja je bila analiza genetičke raznolikosti između pojedinih pasmina konja iz Bosne i Hercegovine, s posebnim akcentom na ispitivanje genetičke osnove bosanskohercegovačkog brdskog konja, korištenjem seta od 17 mikrosatelitnih markera preporučenih od strane ISAG.

Materijal i metode

U radu su prikazani rezultati prvih istraživanja genetičke raznolikosti između sljedećih pasmina konja s područja Bosne i Hercegovine: potencijalni bosanskohercegovački brdski konj (grupa BOS–62 individue), arapski punokrvni konj (grupa ARA–20 individua), engleski punokrvni konj (grupa ENG–36 individua) te križanci u tipu bosanskohercegovačkog brdskog konja i arapskog punokrvnog konja (grupa KBA–7 individua), križanci u tipu bosanskohercegovačkog brdskog konja i belgijskog, holštajn konja i lipicanera (grupa KBR–9 individua) i križanci u tipu bosanskohercegovačkog brdskog konja, s nepoznatim drugim roditeljem (grupa KBN–4 individue). DNA za genotipizaciju je izolirana iz pune krvi 138 konja. Od svakog konja je uzeto po 3 ml krvi iz *v. jugularis* u epruvete sa EDTA. Genomska DNA je izolirana po originalnom protokolu za izolaciju DNA iz humane krvi metodom isoljavanja, koji je modificiran i prilagođen krvi konja i našim laboratorijskim uvjetima. Koncentracija izolirane DNA je utvrđena metodom spektrofotometrije UV mini - 1240 (*Shimadzu*). Za analizu polimorfizma nuklearne DNA je korišten poboljšani StockMarks© Equine Genotyping Kit (*Applied Biosystems*) dizajniran za simultanu genotipizaciju 17 mikrosatelitnih markera konja. Odabrani mikrosatelitni regioni su umnoženi PCR-om, a veličina umnoženih mikrosate-litnih markera je analizirana pomoću genetičkog analizatora ABI PrismTM 310 Genetic Analyzer. Biostatističke i popula-ciono-genetičke analize mikrosatelitnih markera su urađene softverskim paketima (GeneMapper ID v3.2 software): POWERMARKER 3.25 (procjena broja alela, test informativnosti sadržaja polimorfizma – PIC, uočena - H_O i očekivana heterozigotnost - H_E, genetička diferencijacija - F_{ST} i međugru-pna genetička diferencijacija - pF_{ST}, analiza molekularne varijance – AMOVA) i MEGA4 (Neighbor-Joining (NJ) dendrogram).

Rezultati i interpretacija

U poređenju s drugim grupama, grupa BOS je pokazala visok nivo genetičke raznolikosti. Najviše vrijednosti srednjeg broja alela (14,1765) i očekivane heterozigotnosti (0,8451) su uočene za pomenutu grupu. Vrijednosti PIC-a, uočene u našem radu, ukazuju da su primijenjeni mikrosatelitni markeri informativni, polimorfni i pogodni za ovakav tip istraživanja. Rezultati AMOVA testa pokazuju da se 8.44% genetičke varijacije odnosilo na varijaciju između populacija. Najveća unutargrupna varijacija je primijećena za grupu BOS (27,13). Ista grupa je pokazala i najveću varijaciju unutar individua (17,35). Rezultati F_{ST} testa (8,87%), koji pokazuju visok nivo genetičke diferencijacije između promatranih grupa, sukladni su s rezultatima AMOVA testa i pokazuju jasnu diferencijaciju između posmatranih grupa konja. Analizom pF_{ST} testa uočena je najveća diferencijacija između grupa "čistih pasmina" (ARA i ENG) i grupe BOS. Dobiveni rezultati su pokazali da grupa BOS ima visok nivo genetičke varijabilnosti. U genofondu grupe BOS prisutan je i dio genofonda drugih pasmina, te se ova grupa ne može tretirati kao potpuno genetički "čista". Položaj grupe BOS na filogenetičkom stablu, osim što upućuje na zaključak da ova grupa u svom genofondu sadrži primjese gena drugih pasmina, daje potvrdu da postoje dobri preduvjeti za revitalizaciju genskog fonda grupe BOS.

Dobiveni rezultati mogu biti od velike pomoći za uzgajivače, nudeći široku i bitnu bazu informacija, dajući smjernice za strategiju uzgoja te uspostavljanje programa uzgoja. Također, daju naučnu osnovu i predstavljaju vodič za buduća istraživanja u cilju zaštite i očuvanja bosanskohercegovačkog brdskog konja, što bi, svakako, doprinijelo i očuvanju biodiverziteta na globalnom nivou.

Zaključak

Rezultati rada su pokazali da potencijalni bosanskohercegovački brdski konj ima visok nivo genetičke varijabilnosti. U genofondu potencijalnog bosanskohercegovačkog brdskog konja prisutan je i dio genofonda drugih pasmina te se ova grupa ne može tretirati kao potpuno genetički "čista". Također, rezultati pokazuju da postoje vrlo dobri preduvjeti za revitalizaciju genskog fonda potencijalnog bosanskohercegovačkog brdskog konja.