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SPATIAL STRUCTURE OF THE LIPIZZAN HORSE GENE POOL BASED ON MICROSATELLITE VARIATIONS ANALYSIS

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ABSTRACT

The aim of this study was to determine the current state of genetic diversity and to assess the substructure and spatial structure at individual level based on analysis of microsatellite variations within Lipizzan horse population. The genomic DNA samples were obtained from totally 418 horses, originating from Slovenian (357) and Slovak (61) studs. A set of 13 microsatellite markers (AHT4, AHT5, ASB2, HMS1, HMS2, HMS3, HMS6, HMS7, HTG10, HTG4, HTG6, HTG7, and VHL20) have been used for analysis of genetic variability. Across all microsatellite loci the average number of alleles 6.65 and effective allele number at level 3.37 were found. The obtained Shannon's information index (I=1.37) indicated high degree within population genetic diversity. The prevalence of heterozygous genotype in sample confirmed also the average value of observed heterozygosity (Ho=0.67) and FIS index (-0.026). The most of the genetic variation in sample was conserved within individuals (95%) and the subdivision of horse populations explained only 4%. Similarly, the obtained pairwise values of FST index (0.02) and Nei's genetic identity (0.90) reflected mainly common ancestors used in breeding history of both population. But the principal coordinate analysis showed the division of individuals into the two separate clusters according to the studs where they come from. The membership probability resulted from spatial structure analysis suggested that the frequencies of alleles varied across the two regions that indicated the evidence of strong distinction in relation to the current breeding status of analysed populations and strategy of studs.

Keywords: *diversity, genetic markers, Lipizzan, population substructure.*

INTRODUCTION

In the last years the issue of conserving the genetic diversity as a component of the conservation of the environment has been raised at an international level. In this respect, one of main aspects of scientific research activities is conserving the biodiversity of local genetic resources, especially those of economic and cultural interest (Georgescu and Costache, 2012). Worldwide, the populations of numerous domestic animals, especially horses, are in steady decline, with some already extinct, thereby affecting both inter and intra-breed diversities (Bömcke et al.,

2011). Among many genetic issues involved in the conservation of such populations, a crucial question is how much gene flow should be maintained between populations (Mahrous et al., 2011).

Despite lower production and abilities to compete with high-productive breeds, local breeds are still important for countries as their heritage. Out of 27 horse breeds kept in the Slovak Republic 8 could be considered as endangered. Within them the population of Lipizzan horses belong based on the population size to critically endangered groups. Its effective management requires comprehensive knowledge of the population characteristics, including data on effective population size andstructure, geographical distribution, the production environment, and within and between-breed genetic diversity.Integration of these types of data will result in themost complete representation possible of biological diversityand will thus facilitate the effective preservation of breed (Groeneveld et al., 2010).

Generally, the Lipizzan as one of the oldest European horse breed deserve special attention because they represent an important gene pool, containing genetic material from several important historical breeds, some of which are almost extinct (Dovc et al., 2006; Barcaccia et al., 2013). The original herd in Lipica has been formed by stallions and mares imported from Spain. Later, imports of Andalusians, Barbs, and Italian horses at the beginning of 19th century, Arab horses contributed to the formation of the Lipizzan gene pool (Barcaccia et al., 2013). Currently, the base population of Lipizzan horses is divided into a number of rather large, mostly state-owned, studs with limited exchange of horses over the last few decades. The breeding goals of the studs are partly changing over time. Whereas the primary goal of the Austrian stud at Piber is still to provide horses for classic dressage at the Spanish Riding School Vienna, the Hungarian stud in Szilvásvárad has specialised in breeding of top horses for coach driving. The Slovenian, Slovakian and Croatian studs are breeding riding horses while the Romanian studs are providing stallions for improvement of the local farm horse population. Such differences in breeding may be reflected in the morphology of the horses and lead to a separation of the genetic pools of the breed (Zechner et al., 2001; Zechner et al., 2002).

The molecular genetic studies of diversity regarding the horse populations are largely based upon microsatellite markers which offer advantages that are particularly appropriate for conservation projects (Bordonaro et al., 2012). During the last years many studies of horse populations have described their usefulness for estimation of genetic relationship on both intra- and interbreed level as well as for determination of genetic diversity among and within populations (Achmann et al., 2004; Dovc et al., 2006; Bordonaro et al., 2012). Nowadays, microsatellites are being progressively replaced by SNP markers, but in small populations or breeds genotyping with high density SNP chips turns out to be very expensive, thereby limiting the availability of such data (Conant et al., 2011).

The aim of this study was to determine the current state of genetic diversity in the Slovak nucleus of the Lipizzan horse breed in comparison to the Lipizzan population originating from Slovenian studs based on microsatellite markers. The analyses of substructure and spatial structure of populations have been prepared to

provide information of potentially separation of both populations from each other that could result due to the differences of breeding strategy within each stud.

MATERIALS AND METHODS

The genotyping data have been collected from a total of 418 individuals with Slovak (61) and Slovenian (357) origin. The collected sample of 61 Slovak animals represents the nucleus of Lipizzan horses kept in Slovakia. Across and within populations, the variations of thirteen microsatellite markers primarily recommended to paternity testing (*AHT4*, *AHT5*, *ASB2*, *HMS1*, *HMS2*, *HMS3*, *HMS6*, *HMS7*, *HTG10*, *HTG4*, *HTG6*, *HTG7*, and *VHL20*) have been analysed to evaluate the Lipizzan horse gene pool.

The genetic diversity within and across both Lipizzan populations was measured as the mean number of alleles (MNA), observed heterozygosity (H_o), gene diversity expressed as expected heterozygosity (H_e), effective allele number (N_e) and Shannon's information index (I) using the Genalex version 6.1 (Peakall and Smouse, 2012). The significance of differences between observed and expected genotype frequencies that reflects the departure of HWE (Hardy-Weinberg equilibrium) has been tested by Chi-square test. The genetic differentiation (F_{ST}) and amount of inbreeding-like effect across (F_{IT}) and within (F_{IS}) Lipizzan populations were assessed based on Wright's F-statistic according to Weir and Cockerham (1984).

The molecular variance analysis (AMOVA) that estimates the genetic structure indices based on information about allelic content of haplotypes, as well as their frequencies stored enter as a matrix of Euclidean squared distances was performed based on 10,000 permutations using the Arlequin v3.5 (Excoffier et al., 2005). The genetic relationships among analysed individuals arising from the microsatellite variations were evaluated on individual level using Nei's distance and on population level based on Wright's F_{ST} index. Subsequently, the population genetic structure was tested based on the principal coordinate analysis (PCoA) using Genalex version 6.1 and discriminant analysis of principal components (DAPC) implemented in R package *adegenet* 1.3-0 (Jombart and Ahmed, 2011). The spatial Bayesian clustering algorithm adopted in Tess 2.3.1 (Chen et al., 2007) was then used to assign individuals into the clusters and evaluate their membership probability. In the analysis assuming admixture both the CAR and the BYM models was used to define the spatial prior for admixture proportions. Twenty runs were simulated from K=2 to K=10. The run with lowest DIC value was considered as the best and provided information about the hard clustering assignment of individuals and their neighbourhood system. The results from analyses were visualized by R software v 3.2.2 (R Core Team, 2013).

RESULTS AND DISCUSSION

Across thirteen analysed microsatellites totally 114 alleles were identified. The number of alleles per locus ranged from 5 (*AHT5*) to 12 (*HTG10*). The effective allele numbers per locus was in average 3.27±0.18. The value of Shannon's

information index of phenotypic diversity for molecular profiles at level of 1.35±0.06 was found. The average value of I index across all loci also reflected high degree of overall genetic variability across populations, because the Shannon's information index generally reflected the effectiveness of microsatellite markers to reveal the genetic variations. The value of observed heterozygosity ranged across microsatellites from 0.58 (*HMS1*) to 0.80 (*HMS7*), whereas the gene diversity varied from 0.55 (*HMS1*) to 0.77 (*HMS3*). Both of indices signalised sufficient proportion of genetic variability across evaluated individuals in relation to maintain of populations biodiversity. Moreover, the inbreeding-like F_{IS} index had an average negative value (-0.026), suggesting excess of heterozygous genotypes across animals. Using Chi-square test the departure from HWE ($P<0.05$) was identified up to 7 microsatellites (*VHL20*, *HTG4*, *HTG6*, *HMS6*, *ASB2*, *HTG10*, and *HMS3*).
Within the Slovak and Slovenian populations comparable state of diversity was found. A summary of analysed genetic diversity indices across all markers for both populations is given in table 1. The Wright's inbreeding coefficients F_{IS} and F_{IT} were considered as a measure of heterozygosity excess or deficiency. In accordance with identified level of heterozygosity the $F_{IS}=0.03\pm0.02$ (single animals compared to the subpopulation) and $F_{IT}=0.05\pm0.03$ (single animals compared to total population) also signalized only very low excess of homozygous loci across the Lipizzan populations. The F_{ST} index at level 0.02 indicated expected low degree of genetic differentiation between Slovak and Slovenian studs and reveals that only 2% of genetic variation was conserved between both populations.

Table 1. The descriptive statistic of analysed populations related to diversity evaluation

Population	Diversity indices					
	MNA	N_e	I	H_o	H_e	F_{IS}
Slovak Lipizzan	6.92±0.49	3.59±0.26	1.43±0.07	0.65±0.04	0.70±0.02	0.71±0.02
Slovenian Lipizzan	6.38±0.45	3.14±0.18	1.30±0.06	0.69±0.02	0.67±0.02	-0.03±0.01

To explain the proportion of differences influenced by the origin of evaluated Lipizzan horses the analysis of molecular variance was applied on genotyping data. The AMOVA showed similarly as F-statistic that most of the variance was explained by the differences conserved across individuals within separate populations (95%). The subdivision of Lipizzan horses into the subpopulation according to country of origin reflected only 4% of genetic variation and the rest of variability (1%) was divided within the individuals in whole population. In this regard the expected high level of genetic connectedness between both Lipizzan subpopulations confirmed also the value of Nei's genetic distance. Based on generally accepted criteria obtained D_A value at level 0.10 can be considered as low and the animals from Slovak and Slovenian studs only slightly genetically

differentiated. Figure 1B showed the minimum spanning network among analysed individuals constructed based on Nei's genetic distance matrix.

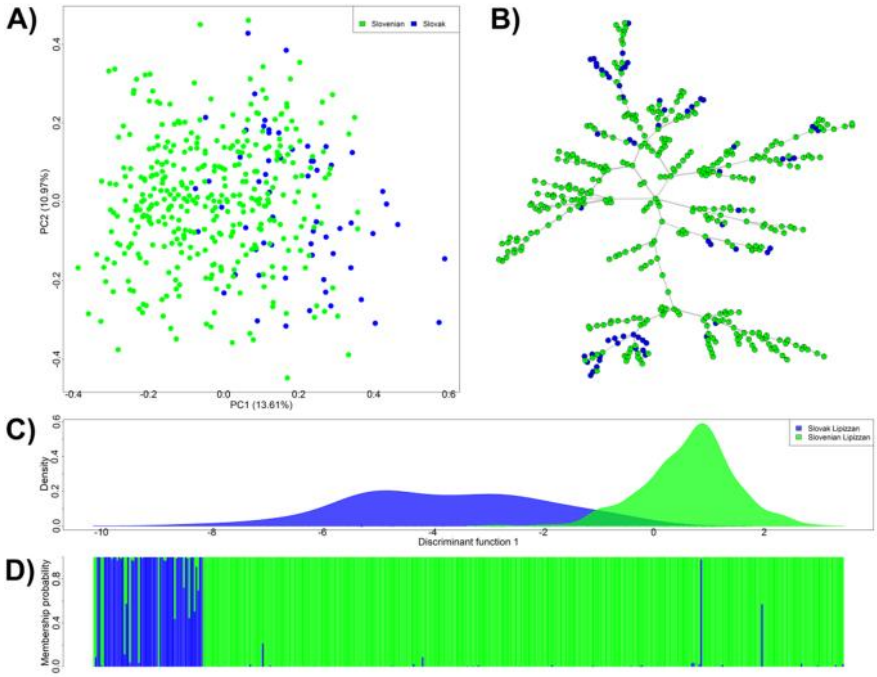

Figure 1. The structure of population based on PCoA analysis (A), the minimum spanning network representing genetic relationship between individuals based on Nei's distances (B), genetic clusters determined based on first discriminant function (C), and membership probability resulting from TESS analysis (D).

The principal coordinate analysis of population structure (Figure 1A) showed non-significant division of individuals in to the clusters according to its origin that can signalized relatively high degree of admixture between individuals originated from Slovak and Slovenian studs mainly due to the migration events during their breeding history. However, the discriminant analysis of principal components indicated the strong division among individuals into the clusters in relation to country of origin. The distinction of individuals into the groups produced by Bayesian Information Criteria (BIC) analysis showed that inferred cluster corresponded to the initial defined population membership. Based on the BIC analysis the K=2 was chosen as optimal. For sufficient reassignment of individuals 48 PCA axes were retained in DAPC that corresponded to more than 50% of variance conserved in analysed dataset. Based on the first discriminant function we were able to clearly detect two main genetic clusters in relation to the each of analysed population (Figure 1C). Similarly, the Bayesian assignment analysis

adopted in TESS that takes alongside genotyping data also the spatial distribution of samples into account showed lowest DIC value for K=2. The membership probability resulted from spatial structure analysis suggested that the frequencies of alleles varied across the two regions that indicated the evidence of strong distinction in relation to the current breeding status of analysed populations and strategy of studs (Figure 1D).

The genetic diversity of the Slovak Lipizzan horses was investigated as a part of studies included European countries representing a large fraction of the Lipizzan population (Achmann et al., 2004; Dovc et al., 2006). The analysis revealed comparable state of genetic diversity with our results within each of analysed population. Moreover, the authors similarly showed that the breeding history of the Lipizzan horses was transferred to the current genetic relationship among fragmented populations. Achmann et al. (2004) found that the Slovak Lipizzan population seems to be genetically closer to the Croatian and Hungarian than to subpopulations of Austria, Italy and Slovenia which represent the classical gene pool of Lipizzan horse breeding. One of the reason that can explain these results is mainly the fact that the Slovakian subpopulation, which was formed after the 1ST World War from the horses from Lipica, has been later due to breeding goal, geographical and socio-political barriers exchanged relatively more with horses from Croatia, Hungary and Austria than with other subpopulations.

Being among the oldest horse breeds in Europe and because of its historical connection to the Austro-Hungarian Empire, the Lipizzan horse is a living part of the European cultural heritage (Achmann et al., 2004). Within the context of horse population and breed conservation, genetic characterization is the first step in the development of proper management strategies, and molecular information is crucial for both preserving genetic diversity and preventing undesired loss of rare alleles in the Lipizzan breed (Barcaccia et al., 2013). In the connection to the pedigree data and morphological measures the genetic markers provide the basis to support the improvement of classical breeding strategies and allow the molecular genetic control of purebred status of the Lipizzan horse breed.

CONCLUSION

The present study was prepared in order to characterize current state of diversity within two Lipizzan subpopulations from Slovak and Slovenian studs based on microsatellite variability. The Slovak population represent the nucleus of Lipizzan breed that is due to the small effective population size considered in Slovakia as endangered. The results showed that both of the populations revealed sufficient level of genetic variability in the context of maintain the local genetic resources of each country. The information of the current state of molecular inbreeding and also genetic relationship between individuals within each population and among studs has important impact on maintaining of its gene pools on local level. Our study showed that the differences in breeding strategy has led to their separation from each other that indicated the evidence of strong distinction in relation to the current breeding status and strategy of studs.Due to the close genetic connection among the

Lipizzan studs resulting from the intensive animals exchange during their history the use of breeding line typical for each studs is currently prefer in order to preserve the local genetic resources of each country.

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