doi: 10.17221/51/2017-CJAS

Genetic Diversity and Admixture in Three Native Draught Horse Breeds Assessed Using Microsatellite Markers

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ABSTRACT

Vostrá-Vydrová H., Vostrý L., Hofmanová B., Moravčíková N., Veselá Z., Vrtková I., Novotná A., Kasarda R. (2018): **Genetic diversity and admixture in three native draught horse breeds assessed using microsatellite markers**. Czech J. Anim. Sci., 63, 85–93.

In this study, we aimed to estimate and compare genetic diversity of two native draught horse breeds and check the possible influence of Noriker breed population on these native breeds. Genetic analyses of relationships and admixture were performed in two native endangered draught horse populations (Silesian Noriker and Czech-Moravian Belgian horses) and one open breed (Noriker). Totally 104 alleles from 13 microsatellite loci were detected in 1298 horses. The average number of alleles per locus was the highest in the Czech-Moravian Belgian horse (7.62) and the lowest in the Silesian Noriker (7.31), the differences were non-significant, whereas the observed and expected heterozygosities per breed ranged from 0.680 (Czech-Moravian Belgian) to 0.719 (Noriker) and from 0.678 (Silesian Noriker) to 0.714 (Noriker). The estimates of Wright's F_{ST} between each pair of breeds indicated a low level of genetic segregation. At the individual level across the analyzed population, formation of two clusters was observed with respect to historical breed development. Moreover, the membership probability outputs showed that the frequencies of alleles varied across the two main regions represented by the Czech-Moravian Belgian and other analyzed breeds. Our results indicated high genetic variability, low inbreeding, and low genetic differentiation, especially between Silesian Noriker and Noriker, which is caused by the high level of admixture. This high level of admixture was in accordance with geographical location, history, and breeding practices of the analyzed breeds. The Silesian Noriker and Noriker breeds seem to be the most genetically related and the decision to consider them as the same population is thus highly supported. The study provides data and information utilizable in the management of conservation programs planned to reduce inbreeding and to minimize loss of genetic variability.

Keywords: population structure; endangered breeds; gene flow; genetic distances

Supported by the Ministry of Agriculture of the Czech Republic (Project No. QJ1510141), by the Slovak Research and Development Agency (grant No. APVV-14-0054), and by the institutional support for the development of Mendel University in Brno.

doi: 10.17221/51/2017-CJAS

Genetic diversity studies in domestic animals focus on evaluating genetic variation within and across breeds mainly for conservation purposes. It is difficult to say which breed-specific gene combinations may be valuable for agriculture in the future (Bjornstad et al. 2000). The evaluation of genetic diversity between livestock breeds is an important prerequisite for developing effective and meaningful breed conservation programs. An effective management of farm animal resources requires comprehensive knowledge of the breeds' characteristics including data on population size and structure and within and between breed genetic diversity. The population of the autochthonous Czech-Moravian Belgian (CMB) horse was created in the last 120 years based on imports of mostly original Belgian stallions, a lower number of Walloon stallions, and several original Belgian mares. The second autochthonous breed – Silesian Noriker (SN) was created in the last 100 years by crossing domestic warm-blood and draught mares with original Austrian Noriker horses. Initially, the absorptive crossing of mares of domestic origin had been realized in both breeds, and later, the mares with the known Belgian or Noriker ancestors in their first and second generation of pedigree were subjected to *inter se* breeding. Therefore, two breeds of draught horses were created that are adapted to existing living conditions and that are different from the other types of foreign breeds of draught horses. The CMB horse was bred as a working horse for forestry works, while the SN was bred as a working horse for field works. The Noriker (N) is one of the oldest mountain draft horses in Europe and is geographically and historically related to other autochthonous European horse breeds such as Black forest horse and South German Coldblood (Druml et al. 2007). The N has been continually bred on the territory of the Czech Republic for approximately 100 years.

To a large extent, the SN and CMB breeds have been geographically separated. World War II negatively impacted breeding programmes because there was a steep decrease in the number of horses. The SN, N, and CMB breeds were registered as separate breeds until the 1960s, but because of their decreasing numbers, all animals were merged into a single studbook "synthetic breed" called the Czech Draught Horse. At that time, stallions and mares were used across the original breeds and all offspring, including the crossbreeds, were considered as "purebred individuals" of this new synthetic population. These genetically crossbred animals were also included in the breeding process. This administrative decision facilitated the use of stallions regardless of their breed, resulting in a decrease in the number of stallions needed for breeding, leading to both a bottleneck and increased variability in the number of mating per stallion. However, in 1989, draught horses in the Czech Republic were reassigned into the original three populations (SN, N, and CMB) based on morphological analysis. During that period, although the analyzed breeds were separated, these animals were maintained as open populations and "crossbreeding" was conducted between them. After 1996 and 1999, the populations of SN and CMB horses were certified as rare and endangered breeds (Genetic Resources), respectively, and their studbooks were closed. Although SN stallions were still used for breeding with N mares, their offspring, with more than 50% of the SN breed's genes, were included in the studbook, and these animals were regularly included in the SN breed. Based on the above-mentioned historical development, these three analyzed draught breeds can genetically be considered as a single breed with three subpopulations.

In animal breeding, the knowledge of genetic characterization and genetic structure is the first step in breed conservation and may have implications for future breeding strategies and management plans. The analysis of the genetic structure of a population can be carried out using genealogical or molecular information. In the case of missing or incomplete pedigree, it would be better to use molecular information to characterize a population; moreover, the molecular information indicates the additional relatedness between animals appearing as founders in the pedigree (Delgado et al. 2014). This may be applicable to the studied breeds when the founders of the SN and N breeds may be relatives. A primary genealogical analysis based on pedigree information (Vostra-Vydrova et al. 2016) suggested high genetic similarity between the above-mentioned draught breeds. The aim of this study was to explore the genetic structure of three draught horse breeds through the analysis of genetic diversity within and between breeds in order to investigate the extent of genetic variation characterizing these native horses and to determine their genetic relationship using microsatellite markers.

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MATERIAL AND METHODS

Animal data collection. In this study, 1298 individuals from three historically close breeds were used: 349 SN, 397 N, and 552 CMB. Data were provided by the Association of Horse Breeder Unions as an umbrella organization of individual breeders. In the analysis, 146 males and 203 females of the SN breed were included, with the oldest individual born in 1983, and the youngest in 2016. In the N breed, 169 males and 228 females born between 1989 and 2016 were analyzed, and in CMB, 307 males and 228 females born between 1989 and 2016 were subjected to analysis. The total set of 13 microsatellite markers (*AHT4*, *AHT5*, *ASB2*, *HMS1*, *HMS2*, *HMS3*, *HMS6*, *HMS7*, *HTG4*, *HTG6*, *HTG7*, *HTG10*, and *VHL20*) recommended for parentage testing by the International Society for Animal Genetics (ISAG) and Equine Genetics Standing Committee was used for the analysis.

Genetic diversity. Genetic variability within populations was characterized as allele frequency, mean number of alleles, observed heterozygosity (H_O), genetic diversity, which is often called expected heterozygosity (Weir 1996), and polymorphism information content (PIC) (Botstein et al. 1980). Testing of the Hardy–Weinberg equilibrium was done using the PowerMarker program (Liu and Muse 2005). The Wright's fixation indices: F_{IS} – reduction in heterozygosity of an individual due to nonrandom mating within its subpopulation, F_{ST} – reduction in heterozygosity of subpopulation due to random genetic drift (fixation index), and F_{IT} – reduction in heterozygosity of an individual due to non-random mating and population subdivision relative to total population (overall inbreeding coefficient) were evaluated by the Weir and Cockerham (1984) method using the FSTAT program (Goudet 2001). The analysis of molecular variance was done using the PEGAS package (Paradis 2010). Genetic differences among individuals and between populations were evaluated by Nei's distances (D_A) (Nei et al. 1983), which assume differences caused by mutations and genetic drift. These indices provide more reliable results specifically for microsatellite data.

Population structure and genetic relationship. Subsequently, to determine genetic structure and to infer genetic admixtures, a discriminant analysis of principal components (DAPC) implemented in the Adegenet R package (Jombart and Ahmed 2011) was used for microsatellite data. The DAPC approach proposes an optimum distribution of individuals into predefined groups in relation to the discriminant function of principal components. An optimum number of clusters was defined by the K-averaging algorithm that makes use of the Bayesian information criterion. In addition, the DAPC was used to assign individuals and to obtain the membership probability which presents the overall genetic background of an individual. A trade-off between the power of discriminant analysis and overfitting of the given analysis was assessed by the α-score (Jombart and Ahmed 2011). The population structure was further evaluated using a Bayesian clustering approach proposed by Pritchard et al. (2000), implemented in the Structure 2.1 program. The analysis was performed by replications based on the Monte Carlo method using a model with admixtures and correlated allele frequencies when 10^6 iterations were used with a 10^5 burn-in period. The run of each tested K-value and the number of clusters (1–10) was repeated 10 times. The most likely K-value in the dataset was identified according to Evanno et al. (2005) using the STRUCTURE HARVESTER (Version 0.6.8) (Earl and von Holdt 2012). Further, the occurrence of recent migration among populations (from one to three generations) was evaluated by the Bayesian MCMC method implemented in the BAYESASS program (Wilson and Rannala 2003). For estimation, 10^6 iterations with a burn-in of 10^5 iterations were used.

RESULTS

Genetic diversity. Each of the analyzed loci appeared as polymorphic, and their alleles were present in or shared by all studied populations. The total number of alleles, average number of alleles, and PCI value found on 13 microsatellite markers across all breeds and overall information about differences and total statistics are shown in Table 1. Statistically significant deviations from the Hardy–Weinberg equilibrium were found in more than two thirds of the microsatellite loci. The levels of genetic diversity estimated within the studied populations are documented in Table 2 and Supplementary Table S1 in Supplementary Online Material (SOM). The parameters of genetic diversity (H_O, genetic diversity, allelic richness

doi: 10.17221/51/2017-CJAS

Table 1. Characteristics of 13 microsatellite loci analyzed in three horse populations (n = 1298). Number of successfully genotyped individuals (N), number of alleles (N_A), polymorphism information content (PIC), observed heterozygosity (H_O), gene diversity, Wright's F_{ST} and Hardy–Weinberg equilibrium (HWE) statistics

Locus	N	N_A	PIC	H_O	Gene diversity	F_{IT}	F_{ST}	F_{IS}	HWE
AHT4	1 297	10	0.796	0.810	0.819	0.019	0.022	–0.004	*
AHT5	1 285	9	0.756	0.778	0.787	0.024	0.034	–0.012	
ASB2	1 290	11	0.702	0.718	0.739	0.050	0.060	–0.011	
HMS1	1 292	8	0.521	0.536	0.594	0.142	0.141	–0.001	**
HMS2	1 289	10	0.670	0.697	0.704	0.025	0.040	–0.015	**
HMS3	1 230	8	0.670	0.720	0.695	0.025	0.022	–0.053	**
HMS6	1 296	6	0.648	0.710	0.701	0.003	0.043	–0.042	***
HMS7	1 278	6	0.739	0.703	0.774	0.102	0.032	0.076	***
HTG10	1 263	11	0.709	0.713	0.735	0.038	0.024	0.015	***
HTG4	1 295	7	0.654	0.686	0.707	0.040	0.031	0.009	**
HTG6	1 295	7	0.422	0.456	0.460	0.020	0.033	–0.014	
HTG7	1 280	6	0.671	0.703	0.721	0.051	0.075	–0.026	***
VHL20	1 297	10	0.811	0.798	0.832	0.065	0.073	–0.009	***

*P < 0.05, **P < 0.01, ***P < 0.001

(MNA), and Wright's F_{IS}) exhibited minimum differences. Observed heterozygosity (0.661–0.719) and the inbreeding coefficient evaluated as Wright's F_{IS} index (from –0.002 to –0.014), as the most commonly used indicators of genetic diversity, indicate a sufficient proportion of heterozygosity across all breeds.

Genetic relationship and population subdivision. The application of molecular variability analysis to the hierarchic population structure indicated that the subdivision of the studied population into particular breeds explained 4.79% of total genetic variability of the set. Within individuals across the whole population 94.45% of variability was distributed and the rest of variability (0.76%) was explained by differences between individuals within breeds (data not shown). Genetic differences between and within populations were tested for a better description of genetic relationships between individuals by the pairwise F_{ST} coefficients and Nei's genetic distances (D_A) (Table 3). The largest distance was determined between SN and CMB breeds and the smallest between SN and N. Genetic distances estimated at the level of individuals showed clear genetic separation of the first cluster (CMB) and the second cluster (SN and N) (Figure 2A).

Genetic structure and level of admixture. To infer the population genetic structure and to assess the level of admixture, DAPC was applied to genotyping data. The distribution of individuals according to the Bayesian information criterion (BIC) analysis showed that inferred clusters do not correspond to actual groups (Figure 1). For this reason the clusters in DAPC were inferred in line with the prior assumption of the population distribution (K = 3). The agreement between prior and posterior assignment was 83.40%. Based on the α-score (Jombart and Collins 2015), which indicates the number of principal components

Table 2. Genetic diversity across three horse populations based on 13 microsatellite loci. Observed heterozygosity (H_O), gene diversity, mean number of alleles (MNA), and Wright's F_{IS} index with confidence intervals (95%)

Population	H_O	Gene diversity	MNA	F_{IS} (CI 95%)
SN	0.691 ± 0.028	0.680 ± 0.027	7.308 ± 0.485	–0.014 (–0.058–0.031)
N	0.719 ± 0.021	0.714 ± 0.020	7.154 ± 0.421	–0.005 (–0.037–0.027)
CMB	0.680 ± 0.038	0.678 ± 0.038	7.615 ± 0.560	–0.002 (–0.041–0.037)

SN = Silesian Noriker, N = Noriker, CMB = Czech-Moravian Belgian, CI = confidence interval

doi: 10.17221/51/2017-CJAS

Table 3. Wright's F_{ST} (above the diagonal) and Nei's minimum genetic distance (below the diagonal) per pair of breeds

Population	SN	N	CMB
SN		0.0069	0.0665
N	0.0634		0.0573
CMB	0.6297	0.5481	

SN = Silesian Noriker, N = Noriker, CMB = Czech-Moravian Belgian

(PCs) adjusted for the successful repeated assignment of individuals, 26 PCA axes were left in DAPC. These axes correspond to more than 85% of variability. The two discriminant functions obtained correspond to 100% of variance. The first discriminant function clearly detected only two genetic clusters corresponding to CMB and to the remaining Noriker breeds (SN and N) (Figure 1B). Subsequently, using the first and the second discriminant function, a very close relationship was determined between the SN and N (Figure 1A). As expected, a certain level of admixture was revealed in all studied breeds (Figure 2B). The occurrence of individuals with a probability of admixtures of other breeds higher than 90% was determined in all breeds. The highest admixtures in the SN breed were shown by an individual with 99% of the genetic endowment of the CMB breed. In the N breed, it was an individual with 99% of the CMB breed, and in the CMB breed, it was an individual with 88% of the SN breed and 11% of the N breed. The highest occurrence of individuals with a probability of admixtures of other breeds higher than 80% was in the N breed – 11% of individuals (8% in SN and 4% in CMB).

An assignment test was performed using the STRUCTURE program with the number of expected populations from K = 1 to K = 6. The value lnP(K) increases from K = 1 to K = 6 while the highest increase is just from K = 1 to K = 2, and a further increase is slower (Supplementary Table S2 in SOM). The results from the approach proposed by Evanno et al. (2005) also indicated identical conclusions. The highlighted values for K = 2 (Supplementary Table S2 in SOM) represent the most suitable number of genetic groups identified by the two methods, i.e., two clusters correspond-

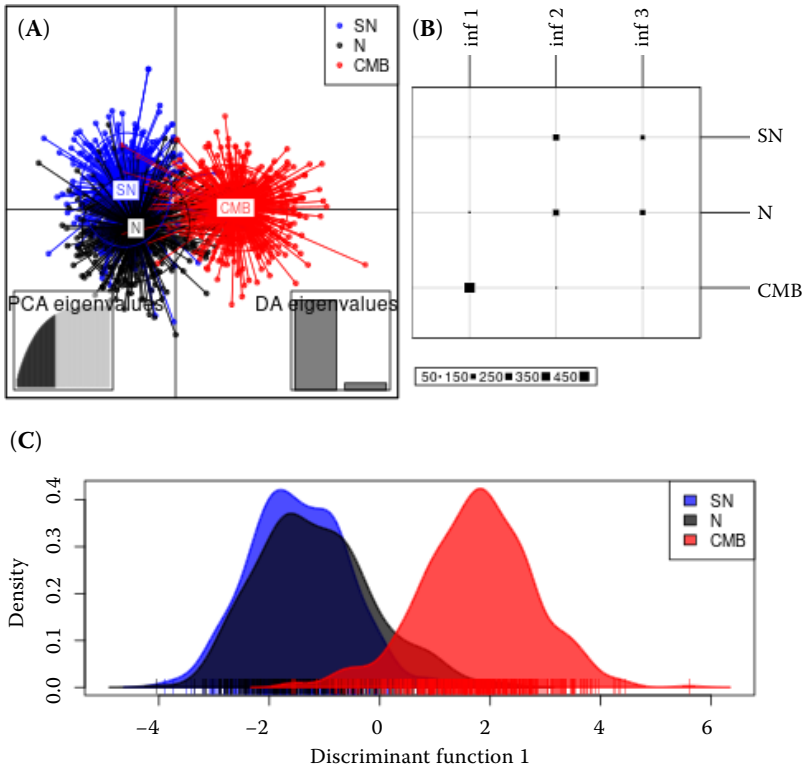

Figure 1. Genetic clusters determined using discriminant analysis of principal components (**A**), based on the first discriminant function (**B**), and the Bayesian information criterion (BIC) statistic results referring to differentiation between inferred and original clusters (**C**) for the Czech-Moravian Belgian (CMB), Noriker (N), and Silesian Noriker (SN) breeds

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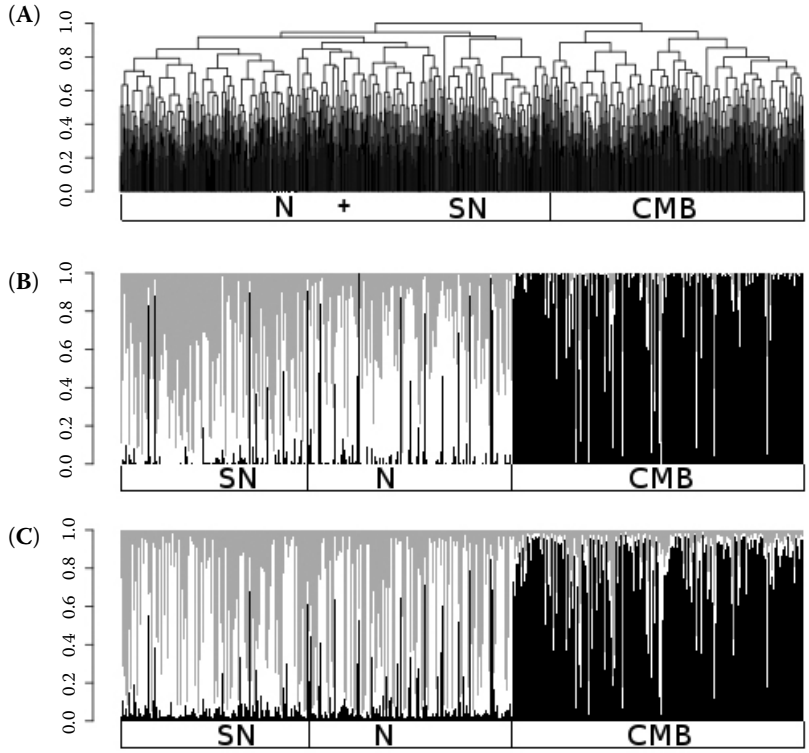

Figure 2. Hierarchical plot representing the inferred relationship between individuals (**A**), membership probability resulting from discriminant analysis of principal components (K = 3) (**B**), and representative results from Bayesian assignment analysis implemented in the program STRUCTURE (K = 3) (**C**). Czech-Moravian Belgian (black), Noriker (white), and Silesian Noriker (grey)

ing to a difference between the CMB and Noriker breeds. For a better comparison of the analyses, particularly in relation to admixtures, only results at the K-level equalling 3 were visualized (Figure 2C). Table 4 documents the proportional assignment of each breed to three clusters. Cluster 3 includes the CMB breed with 77%. Cluster 1 includes the breeds SN with 41% and N with 40%, and cluster 2 involves SN with 52% and N with 48%. The estimate of a recent migration rate (to the third generation) is shown in Table 5. High proportions of individuals within each population were derived from the same population (migration rates m = 0.71–0.94) and relatively low levels of migration were detected between populations (m = 0.003–0.05). The only exception to the overall low levels of migration was the apparent migration from N to SN (m = 0.26).

Table 4. Number of individuals (*n*) per breed and proportion of membership of each breed in each of the 3 clusters inferred in the most likely run of the program STRUCTURE

Breed	Inferred cluster			
	1	2	3	*n*
SN	0.4083	0.5198	0.0719	349
N	0.4032	0.4756	0.1211	396
CMB	0.1126	0.1110	0.7764	553

SN = Silesian Noriker, N = Noriker, CMB = Czech-Moravian Belgian

Table 5. Means of posterior distributions of the migration rate into each population are shown, with the standard deviation in brackets. The populations each individual was sampled from are listed in the rows, and columns represent the populations they migrated from. Values along the diagonal are the proportions of individuals within a population derived from that population

Sample location	Putative population of origin		
	SN	N	CMB
SN	0.94 (0.01)	0.05 (0.01)	0.003 (0.004)
N	0.26 (0.02)	0.71 (0.02)	0.03 (0.008)
CMB	0.02 (0.008)	0.01 (0.003)	0.97 (0.01)

SN = Silesian Noriker, N = Noriker, CMB = Czech-Moravian Belgian

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DISCUSSION

Genetic variability, determined by means of microsatellite markers in two Czech native draught horse breeds (SN and CMB) and Noriker breed, was analyzed in this study. Until now, the autochthonous breeds included in this analysis have been published in only a few studies with a different focus, so our results cannot directly be compared with results published in the literature. Results found in Noriker breed are in a good agreement with findings of a previous study on this breed (Druml et al. 2007). All studied microsatellite markers showed high variability, and only the markers *AHT4*, *ASB2*, *HMS2*, *HTG4*, and *HTG6* deviated from the Hardy–Weinberg equilibrium. Generally, the genetic diversity of microsatellite loci can be affected by many factors, including genetic drift, impact of selective breeding, effect of individual stallions, and random effects (Petersen et al. 2013). Estimated parameters of genetic diversity exhibited non-significant differences between breeds (Table 2), which fully corresponds to their historical development. Genetic diversities within the studied breeds reached similar values to those in the Old Kladruber horse (Vostry et al. 2011; Kasarda et al. 2016), the breed that also belongs to genetic resources of the Czech Republic. The determined values of genetic diversity were lower than e.g., those in Polish cold-blood horses (Iwanczyk et al. 2006) or Polish Konik (Szwaczkowski et al. 2016). The observed lower proportion of genetic diversity distributed within autochthonous breeds can be explained mainly by the fact that the local breeds generally show higher degrees of diversity than breeds with more limited numbers of stallions and mares in the breeding scheme. The MNA (allelic richness) assesses genetic diversity with regard to the degree of bottleneck (Greenbaum et al. 2014). Observed heterozygosity and the coefficient of inbreeding measured by Wright's F_{IS} index, which are used as general indicators of the genetic diversity level, indicate a sufficient level of heterozygosity in the studied populations. These results also correspond to the values of inbreeding coefficient estimated by the pedigree analysis for these analyzed breeds (Vostra-Vydrova et al. 2016). The maintained level of genetic variability within the analyzed populations was comparable with values of observed heterozygosity in German draught horses (Aberle et al. 2004). Our results show slightly lower values of observed heterozygosity than other horse populations, including Anglo-Arab, Arabian, Thoroughbred, and French Trotter horses (Berber et al. 2014).

The low F_{IS} value (within population inbreeding estimate) indicates a low inbreeding level within breeds. The low F_{ST} value (measurement of population differentiation) and the value in a genetic distance matrix (D_A) show a considerable gene flow between the analyzed breeds. These values also indicate that the breeds are not differentiated enough and that they may have a common history and breeding practices. The estimated F_{ST} values are lower than those determined between two colour varieties within the Old Kladruber horse breed (F_{ST} = 0.082) (Kasarda et al. 2016). These results are fully consistent with the values of average inbreeding coefficients and F_{ST} estimated by Vostra-Vydrova et al. (2016) by means of pedigree analysis. The low values of F_{ST} and genetic distances between the analyzed breeds result from the crossing between individuals from different breeds between 1960 and 1990. In addition, mares born from the crossing of SN stallions and N mares were included in the SN studbook based on the common historical development of both breeds. This is in accord with the high value of gene flow from the N breed to the SN breed determined by the migration rate (Table 5).

A comparison of the DAPC and STRUCTURE results shows that the STRUCTURE program revealed a higher level of admixture. However, both results confirm that the structure of the studied breeds was not sufficiently differentiated and that there was a high level of admixture between the SN and N breeds. An admixture between the CMB breed and the SN or N breeds was also proven. According to the admixture plots some animals from the CMB breed belong to SN or N population and *vice versa*. Breeding associations are not interested in reclassification of these animals, however.

The reassignment of these individuals will depend on the decision of the breeding association. The chosen methods were not able to correctly separate the SN and N breeds due to their close genetic relationship. When the history of a breed is complex, as in the case of draught horses involved in this study, it is difficult or even impossible to define breeds as clearly distinctive units. In a such situation, this study might provide the

doi: 10.17221/51/2017-CJAS

first insight into further conservation strategies that should be considered. With respect to DAPC and STRUCTURE results, SN and N might be considered as one breeding group consisting of two subpopulations.

CONCLUSION

In conclusion, this study gives an insight into the genetic structure and diversity of the three most numerous, most used, and historically most important draught horse breeds kept in the Czech Republic. Although the analyzed breeds are diverse, our data suggest a low level of differentiation as well as a high gene flow between them, as indicated by the tests of genetic differentiation and assignment of individuals to populations. The low F_{ST} values between the SN and N breeds can be explained by the crossing of Noriker breeds. The migration rate indicates a continuous gene flow between the SN and N breeds. The analysis demonstrates the genetically wrong inclusion of many individuals in different breeds. Hence, these individuals should be reassigned to appropriate studbooks. The results of this study should be applied to the conservation of gene resources of draught horses in the Czech Republic. These methods should take into account the restriction of gene flow between the SN and N breeds by preventing crosses between them, and the maintenance of a high level of within-breed genetic diversity.

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Received: 2017–05–17
Accepted after corrections: 2017–12–04