Genetic structure and admixture between the Posavina and Croatian Coldblood in contrast to Lipizzan horse from Croatia

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ABSTRACT: The Posavina and Croatian Coldblood are Croatian autochthonous horse breeds with interwoven breeding histories for which studbooks have only recently been established. The Lipizzan breed has the oldest formalized breeding and no record of recent genetic introgression from other breeds in Croatia. We analyzed the genetic structure, interbreeding, and breed characteristics by genotyping nine dinucleotide microsatellite loci for 53 Posavina, 37 Croatian Coldblood, and 33 Lipizzan horses and showed that differing breeding schemes and histories have had a strong and measurable impact on the population genetic structure within and between the three breeds. A Bayesian clustering method demonstrated that two population clusters best explain the genetic structure. Samples from the pre-defined breeds of the Posavina and Croatian Coldblood were assigned to a separate genetic cluster, while Lipizzan specimens were assigned to another distinct genetic group. Twelve samples of the Posavina/Croatian Coldblood cluster (13%) showed admixed ancestry with Lipizzan horses. A test for heterozygosity excess, allele frequency distribution mode-shift, and M-ratio test were used to detect genetic evidence of recent population bottlenecks, none of which provided evidence for bottlenecks in the Posavina and Croatian Coldblood populations. In contrast, although somewhat ambiguous, evidence suggests a genetic bottleneck in the Lipizzan population in Croatia.

Keywords: autochthonous horse breed; microsatellite; bottleneck

Domesticated animals are recognized as an important part of biodiversity. Unfortunately, the global biodiversity crisis extends to domestic breeds too, especially to the autochthonous, "unimproved" localized breeds. An understanding of the evolutionary history of different local domestic breeds and quantitative data on their genetic relationships can provide critically important inputs for the conservation and breed management (Rege and Gibson, 2003). Modern molecular tools are used to assess genetic variation, structure, and demographic history within and between breeds. Genetic variation at nuclear microsatellite loci differs significantly between breeds and may reflect sex-biased dispersal and breeding (Vilà et al., 2001). Breeds may differ in the number and origin of founder animals, proportion, and origin of admixture from other breeds, inbreeding, and historic demographic population bottlenecks.

The Posavina and Croatian Coldblood are indigenous Croatian horse breeds. The Posavina was primarily used for agriculture and transportation

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but suffered a substantial decrease in population size due to mechanization and recent war in Croatia. The breed is very well adapted to harsh conditions, especially flooded areas, and is reported to be resistant to infectious diseases (Kovač, 1994). The creation of an organized studbook is relatively recent, suggesting genetic introgression from other breeds may have been frequent. The history of the Posavina is tightly interwoven with the history of the Croatian Coldblood. During the 1990s the Coldblood breeding was heavily influenced by the Posavina as only these breeders could receive annual premiums. In 1991, the Croatian Livestock Centre created studbooks for the Posavina and Croatian Coldblood. The Posavina studbook was closed in 2004 (Čačić et al., 2004), while the Croatian Coldblood studbook was closed in 2008 (Čačić et al., 2008). In contrast, the Lipizzan studbook was introduced in 1580 in the Austro-Hungarian Empire; breeding in Croatia started in 1806 (Mandić and Rastija, 1997). There is no reported influence of any other breed on the Lipizzans in Croatia. However, whether there has been admixture between the Lipizzans and local autochthonous horse breeds in Croatia remains unknown.

In a study of Austrian, Croatian, and German draught horses, the Croatian Coldblood and Posavina showed an admixture pattern between two distinct clusters that were closely related (Druml et al., 2007). Ivanković et al. (2009) investigated genetic structure of the Posavina and Croatian Coldblood, in comparison with the Murinsulaner horse being the third Croatian autochthonous breed and found that the Posavina and the Croatian Coldblood were the most closely related populations. Genetic diversity and differentiation of Lipizzan horses from seven European countries including Croatia were revealed by genetic distance and principal component analysis (Achmann et al., 2004). Moderate genetic differentiation between subpopulations was found indicating that the genetic results were consistent with the known breeding history.

In this paper we contrast three horse breeds in Croatia with differing breeding schemes and histories: the Posavina and Croatian Coldblood with interwoven breeding histories and only recent studbook establishment vs. the Lipizzan with the oldest formalized breeding structure and no record of recent genetic introgression. We investigate their genetic population structures, genetic admixtures, and demographic history.

MATERIAL AND METHODS

Sample collection

Blood samples from 37 Croatian Coldblood and 53 Posavina horses were collected across central Croatia in 1999 and 2001. Blood samples from 33 Lipizzan horses were collected in Djakovo stables (Croatia) in 2000.

Molecular analyses

DNA was extracted from 500 ml of whole blood after digestion with proteinase K, separation with NaCl and chlorophorm, and precipitation with isopropanol. DNA was dissolved in 150 ml of sterile distilled water. The nine horse-specific, ISAG-FAO recommended microsatellite loci (FAO, 2011) were utilized: HTG4 (Ellegren et al., 1992), HTG7, HTG10 (Marklund et al., 1994), HMS2, HMS3, HMS6 (Guérin et al., 1994), VHL20 (van Haeringen et al., 1994), ASB2 (Breen et al., 1994), and AHT5 (Binns et al., 1995). PCR amplification was carried out in a 12 ml reaction containing 10mM Tris-HCl, 200mM $(NH_4)_2SO_4$, 50mM each dNTP, 1.5mM $MgCl_2$, 5 ng of BSA, 0.1 U Amplitaq® Gold DNA polymerase (PerkinElmer, Waltham, USA), and between 0.5mM and 0.75mM fluorescent primer and non-fluorescent primer, and 2ml DNA extract. PCR cycling profile was 1 min at 94°C, then 30 cycles of 15 s at 94°C, 30–60 s at 45–48°C, 60 s at 72°C, followed by a final extension at 72°C for 2 min. All PCR products were electrophoretically separated using an ABI PRISM™ 377 DNA sequencer (Applied Biosystems, Carlsbad, USA). Allele sizes were scored against the size standard GS350 Tamra (PerkinElmer, Waltham, USA) using GeneScan™ Analysis 2.1 and Genotyper™ 2.1 software.

Statistical methods

Number of alleles, expected and observed heterozygosities, and deviations from Hardy-Weinberg equilibrium for each breed are reported in Galov et al. (2005). In order to detect genotyping errors due to null alleles we used Micro-Checker v. 2.2.3 (Van Oosterhout et al., 2004). Because the test indicated null alleles at the HMS6 locus in Croatian Coldblood ($P < 0.001$) we excluded this locus from population structure analyses. Population structure

was investigated by Bayesian clustering implemented in STRUCTURE software v. 2.3.3 (Pritchard et al., 2000), which probabilistically assigns the individual animals to populations based on their multi-locus genotypes. Loosely speaking, animals are assigned to populations in such a way so as to achieve Hardy-Weinberg and linkage equilibrium. We applied the model allowing for correlated allele frequencies and an admixture model, which permits mixed ancestry within animals. We ran the simulations both without and with the breed assignment as prior information (LOCPRIOR model) (Hubisz et al., 2009). A total of 1 000 000 Markov Chain Monte Carlo iterations, after a burn-in period of 100 000 iterations, were run for each number of genetic clusters (K, ranging 1–5). For each value of K we repeated the analysis ten times and estimated the posterior probability of clustering from the average logarithmic probability of data across repeats. The most probable number of clusters was selected by calculating the ΔK value, which is based on the rate of change in the log probability of data between successive K values (Evanno et al., 2005) using the program STRUCTURE HARVESTER v. 0.6 (Earl and vonHoldt, 2012).

To test for evidence of recent bottleneck events, we used three different approaches. The first approach assumes that, in a recently reduced population, the gene diversity will be higher than that expected at equilibrium. When a population has been recently reduced in size, there will be a deficit of rare alleles relative to the number expected in a population at equilibrium, because rare alleles are generally lost first through genetic drift. Since rare alleles contribute relatively little to expected heterozygosity, there will be an excess of observed heterozygosity when compared with a population at equilibrium with an equivalent number of alleles (Luikart et al., 1998). Gene diversity was estimated under two models of molecular evolution: the stepwise mutation model (SMM) and the two-phase model (TPM). The TPM has been shown to be the most appropriate for microsatellite DNA data (DiRienzo et al., 1994). We used TPM with 95% of single-step mutations and 5% of multiple-step mutations, and a variance among multiple steps of 12, as recommended by Piry et al. (1999). Ten thousand iterations were used for each mutation model. The probability of significant heterozygosity excess was determined using Wilcoxon signed-rank test. Second, we tested the distribution of allele frequencies for a bottleneck-induced mode shift. Mode shift is a transient distortion in the distribution of allele frequencies such that the frequency of alleles at low frequency becomes lower than the frequency of alleles in an intermediate allele frequency class (Luikart et al., 1998). For these analyses we used the program Bottleneck, v. 1.2.02. (Piry et al., 1999). Third, the M-ratio test is based on the ratio of the observed number of microsatellite alleles to the range of allele sizes. Because alleles are randomly lost as a result of genetic drift, the M-ratio is expected to decrease in bottlenecked populations (Garza and Williamson, 2001). This analysis was carried out with Critical-M and M-P-Val programs from Garza and Williamson (2001). The parameters for the M ratio test were set as follows: proportion of one-step mutations (p_s) = 0.9 and average size of non one-step mutations (Δg) = 3.5, as suggested by the authors (Garza and Williamson, 2001). We tested two values of θ parameter (1 and 2), which is based on effective population size, prior to the bottleneck and mutation rate.

RESULTS

Cluster analysis (STRUCTURE software used) excluding sample group information identified K = 2 as the most likely number of clusters. The maximum value of mean log-likelihood was observed at K = 2 and estimates of ΔK revealed the largest increase in the likelihood of the number of clusters also at K = 2 (Figure 1). Samples of the pre-defined Posavina and Croatian Coldblood breeds were assigned to one genetic cluster, while the Lipizzan samples were assigned to another (Figure 2). We also ran STRUCTURE software simulations using sample group information (LOCPRIOR model) to assist the inference of possible weak population structure between the Posavina and Croatian Coldblood, but it did not alter the results. Likewise, the inclusion of the locus HMS6 did not alter the results. Estimated membership coefficients (Q) for the majority of animals in the Posavina/Croatian Coldblood cluster were high, with Q > 0.8. However, 12 samples (13.33%) showed admixed ancestry with the Lipizzan cluster at a Q > 0.8 level, among them, one sample of the Croatian Coldblood and Posavina had an estimated membership of the Lipizzan cluster of > 0.6 (Figure 2, upper graph).

The Lipizzan population cluster showed negligible admixture from the Posavina/Croatian Coldblood group (Figure 2).

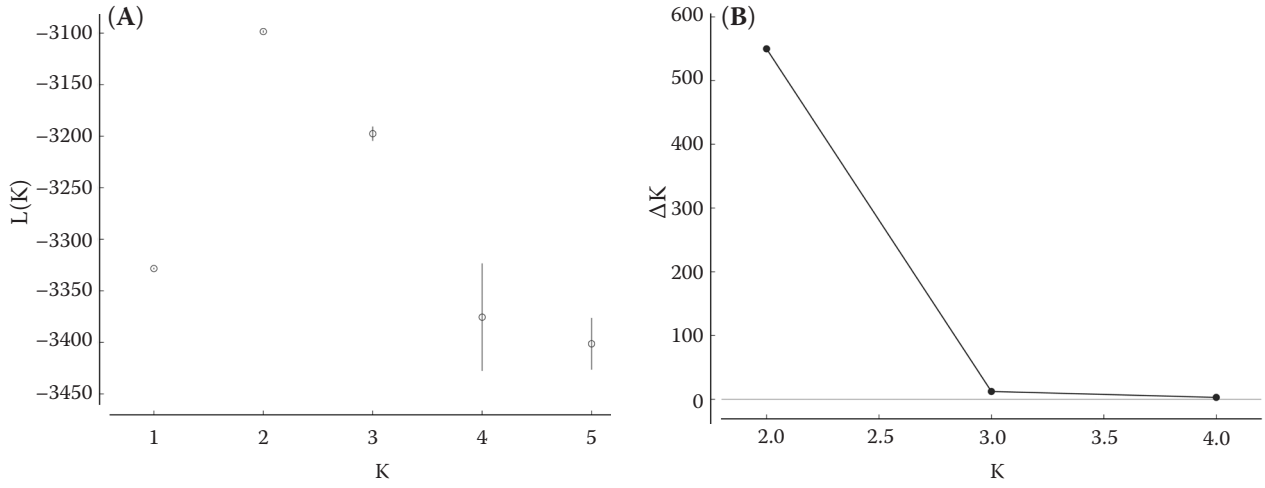

Figure 1. Mean log likelyhood (L(K)) and standard deviation (SD) that display likelyhood variance per K value (**A**), and the rate of change in the log probability of data between successive values of K (ΔK) (**B**) as a function of the number of genetic clusters (K) averaged over 10 independent STRUCTURE software runs for each K

For the Bottleneck analysis, Wilcoxon signed-rank tests were not significant for any breed under two mutational models: TPM with 95% single-step mutations (P = 0.248 for the Posavina, 0.179 for the Croatian Coldblood, and 0.179 for the Lipizzan) and SMM (P = 0.589, 0.367, and 0.455, respectively). In addition, allele frequency distributions for the Posavina and Croatian Coldblood were normally L-shaped, as expected for a stable population under mutation-drift equilibrium (Figure 3). However,

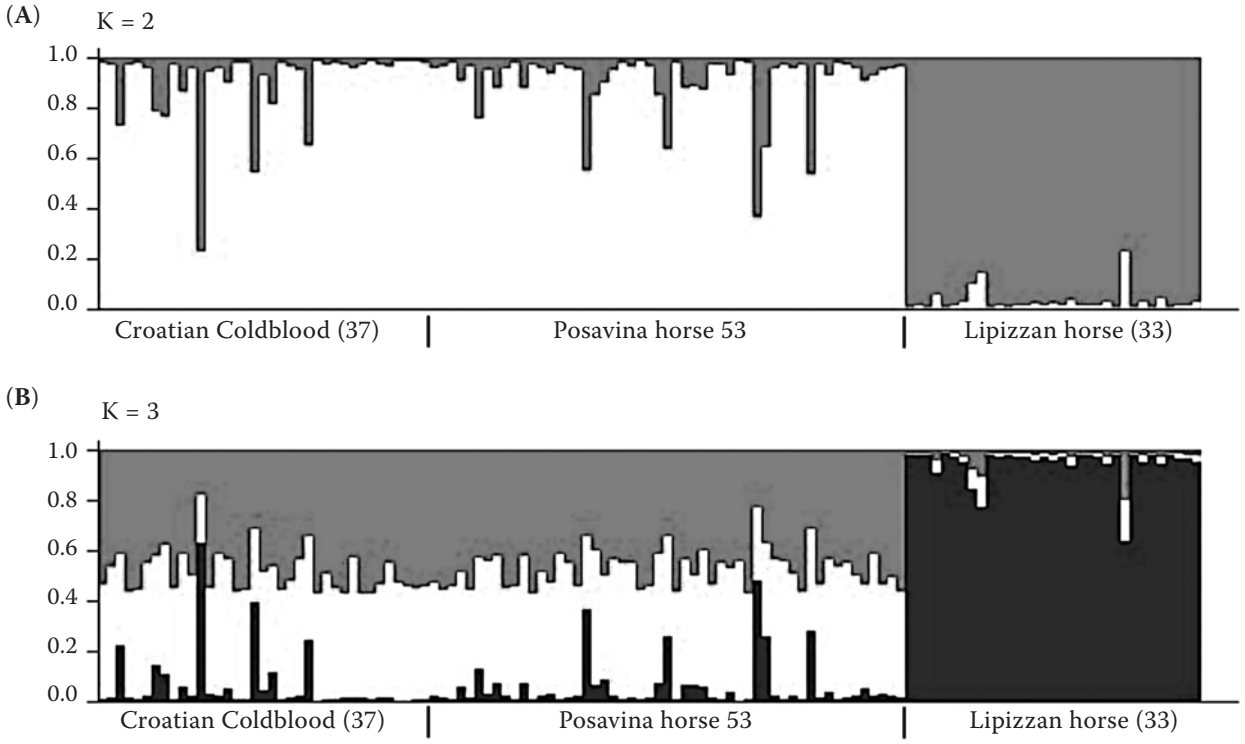

Figure 2. Probabilistic assignment of Croatian Coldblood, Posavina, and Lipizzan horses to the genetic clusters inferred by Bayesian analysis performed using STRUCTURE software, with the number of genetic clusters (K) 2 (**A**) and 3 (**B**). The most likely number of genetic clusters in the data set was identified as two. Each animal is represented by a vertical column partitioned into K segments that represent the animal's estimated membership fractions in K clusters. Numbers in parentheses indicate sample sizes

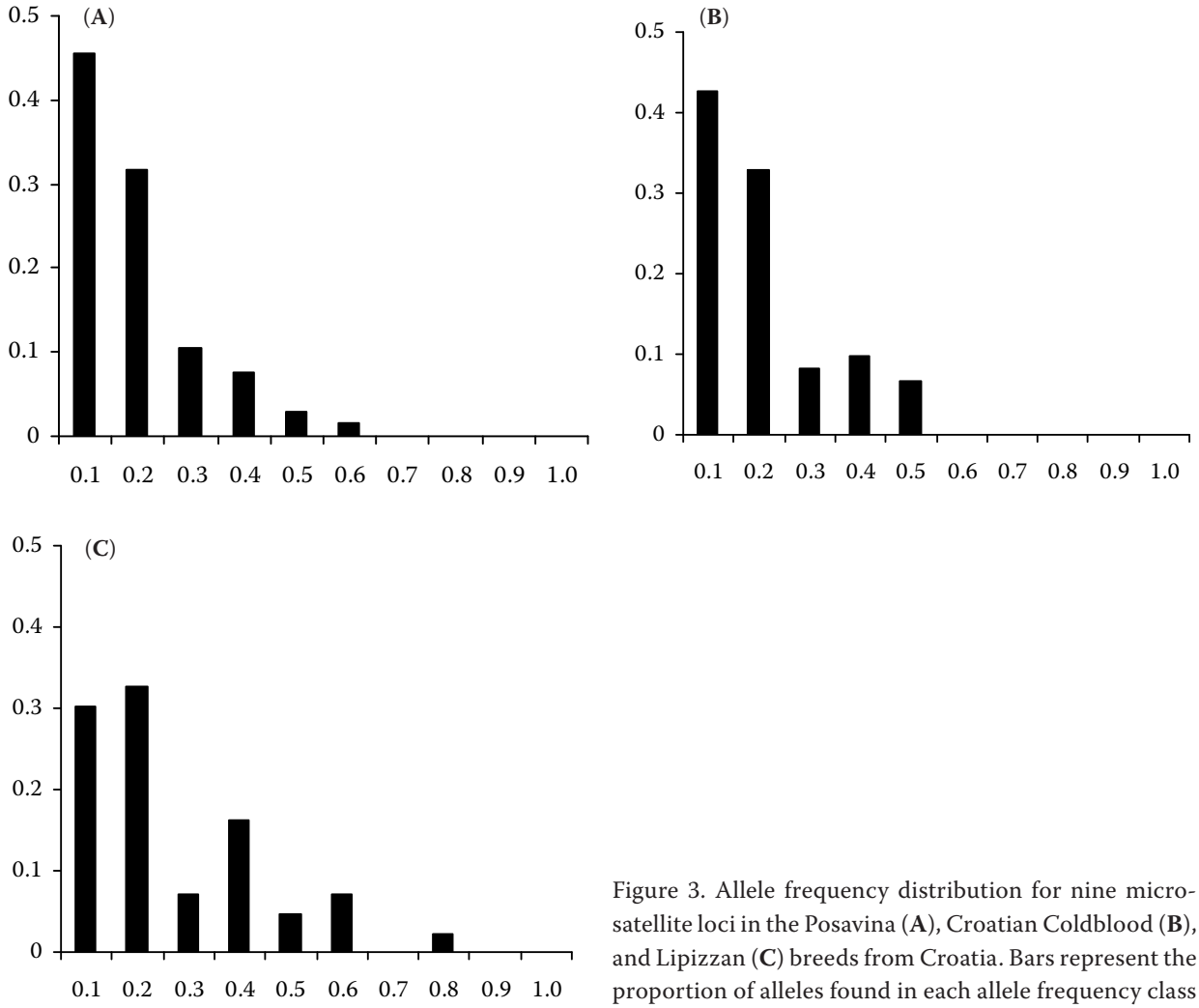

Figure 3. Allele frequency distribution for nine microsatellite loci in the Posavina (**A**), Croatian Coldblood (**B**), and Lipizzan (**C**) breeds from Croatia. Bars represent the proportion of alleles found in each allele frequency class

there was a mode shift in the distribution of allele frequencies for the Lipizzans, which might have been bottleneck-induced (Figure 3).

The M ratio test did not support the existence of a historical bottleneck in the Posavina and Croatian Coldblood. *P*-values were 0.122 for $\theta = 1$ and 0.242 for $\theta = 2$ in the Posavina population and 0.177 and 0.335 respectively in the Croatian Coldblood. However, it provided evidence that the Lipizzan population had gone through a population bottleneck (*P* values 0.004 for $\theta = 1$ and 0.010 for $\theta = 2$).

DISCUSSION

Differing breeding schemes and histories of horses in Croatia showed a strong and measurable impact on the population genetic structure within and between the Croatian Coldblood, Posavina, and Lipizzan. We observed no genetic differentiation between the Croatian Coldblood and Posavina. The clustering of animals from what was formally assigned as Croatian Coldblood and Posavina to a single genetic cluster is not surprising considering the breed histories. This could be a consequence of the recent founding of the breeds in separate studbooks, which has not provided enough time for genetic drift and artificial selection to impact the genetic structure of either breed, or a consequence of high levels of gene flow between the two breeds before the formal introduction of studbooks, or both. A substantial number of pedigree records and breeding animals were lost or changed identity due to the recent war in Croatia. Studbooks were re-established, assigning breed membership according to phenotypic appearance (Druml et al., 2007). Likewise, the studbooks have only recently been closed, in 2004 for the Posavina (Čačić et al., 2004) and in 2008 for the Croatian Coldblood (Čačić et al., 2008). It should be emphasized here

that the samples for this research were collected in 1999–2000, the period that predated formal closures of studbooks for either breed. In the Croatian Coldblood and Posavina group Druml et al. (2007) showed an admixture pattern between two distinct clusters of horses, although the significance of differentiation is unclear since the clusters did not match a prior classification of breeds. This is in contrast to our results, which do not show clustering among those two breeds. The discrepancy could be due to the larger number of microsatellite markers used for genetic differentiation by Druml et al. (2007). However, genetic divergence between the Lipizzan and Posavina/Croatian Coldblood clusters in this investigation suggests that even as few as eight highly informative microsatellite loci are sufficient to differentiate between the Lipizzan and the other two breeds, despite the historical crossing of the Posavina with the Lipizzan as recently as the mid 20th century (Benčević, 1950). There are no records that the Lipizzan has had a substantial influence on the Posavina or Croatian Coldblood, although evident admixture of 13% of animals both from the Posavina and Croatian Coldblood with the Lipizzan (Figure 2) indicates that crossing with the Lipizzan does occur sporadically.

Ivanković et al. (2009) investigated genetic structure of the Posavina, Croatian Coldblood, and Murinsulaner breeds using population genetic analyses based on genetic distances and a prior assignment of samples to breeds. They found low levels of genetic differentiation among the breeds, noticeably lower than among some other ones and showed that these three breeds did not form defined clusters. Our results obtained by Bayesian clustering further emphasize lack of genetic distinctiveness between the Posavina and the Croatian Coldblood.

Lipizzan horses have been identified as a unique group, which is in agreement with the historical data – it is the oldest breed in Croatia, whose studbook was established in the 19th century, and there are no reports on the introgression from any other breeds. It further suggests that neither the recent war in Croatia nor the turmoil throughout the 20th century impacted the pedigree records and breeding of the Lipizzans at the Djakovo stables.

Mode shift in the distribution of allele frequencies and evidence for a bottleneck using M-ratio test taken together suggest historical effective population size reduction in the Lipizzans in Croatia, although the test for heterozygosity excess casts some doubt on this. If true, evidence of genetic bottleneck is in contrast with the idea that the Lipizzans from Djakovo stables are characterized by a fairly high effective number of founders (Zechner et al., 2002). In order to put our results in context, the possibility of a population bottleneck should be investigated in other Lipizzan populations. None of the analyses (test for heterozygosity excess under TPM and SMM, allele frequency distribution mode-shift, and M-ratio) provided evidence for a recent bottleneck in the Posavina or Croatian Coldblood populations. Although a dramatic demographic population decline occurred in the Posavina in recent history (Kovač, 1994), it did not cause a detectable decrease in genetic variability and there is no sign of a genetic signature of a bottleneck. Immigration, even at low levels, can have a strong effect in erasing bottleneck signatures within a few generations of reduction (Keller et al., 2001). Having studbooks only recently closed, immigration was likely in both the Posavina and Croatian Coldblood. Furthermore, the lack of evidence of a genetic bottleneck is in agreement with the fact that there are no records of a substantial population size decrease in the Croatian Coldblood in recent history. Moreover, if the two breeds used to be differentiated but have recently been joined together, evidence for a bottleneck in the joint population is not expected, even if single populations experienced bottlenecks.

Although the Posavina and Croatian Coldblood were assigned to a single genetic cluster based on the structure analysis, genetic distinctiveness is not the only criterion on which conservation decisions should be made. Conservation priority has to be goal- and context-dependent. Therefore, important considerations are the present and future economic and socio-cultural contexts in which breeds exist (Rege and Gibson, 2003). Future research should aim at gaining a more complete understanding of the molecular basis of functional diversity, which could also contribute to our insight into uniqueness of breeds, research already initiated for the Posavina and Croatian Coldblood by Arbanasic et al. (2009). The results presented here could be regarded as a genetic portrait of the Posavina and Croatian Coldblood at the time of their creation. They provide a basis for genetic monitoring of the breeds in the future which should contribute to their better management and conservation.

CONCLUSION

The results of the present study indicate that differing breeding schemes and histories of horses in Croatia showed a strong and measurable impact on the population genetic structure within and between Croatian Coldblood, Posavina, and Lipizzan breeds. The Lipizzan showed genetic differentiation from the other two breeds. We found an evident 13% admixture of animals from the Posavina/Croatian Coldblood cluster with the Lipizzan indicating that crossing of these two breeds with Lipizzans occurs sporadically. Although we observed no genetic differentiation between the Croatian Coldblood and Posavina, genetic distinctiveness is not the only criterion on the basis of which conservation decisions should be made.

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