Genetic structure of three Croatian horse breeds: implications for their conservation strategy

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ABSTRACT

The genetic variability for a sample of 107 animals from three autochthonous Croatian horse breeds was estimated using 20 microsatellites. The average number of alleles per locus (6.3) and proportion of heterozygosity (0.732) indicated a moderate variability. The expected heterozygosity was similar among all breeds and ranged between 0.724 in the Posavina horse, and 0.737 in the Croatian Coldblood and Murinsulaner horse. The inbreeding coefficient F_{IS} was low and non-significant over the three populations. The genetic differentiation among the three populations was low (F_{ST}=0.026), suggesting that only 2.6% of the total genetic variability was due to differences between the breeds, and 97% to individual differences. The results of pairwise genetic differentiation suggest that the Posavina horse and the Croatian Coldblood were the most closely related populations (F_{ST}=0.016). These results are confirmed by Nei's genetic distances with the highest value observed between the Posavina horse and the Murinsulaner (0.082) and the lowest between the Posavina horse and the Croatian Coldblood (0.044). An assignment test correctly assigned 82% of individuals to the correct breed. Strategies for preserving the original native genes in the Croatian native horse breeds should be considered in order to prevent these breeds from becoming extinct and include them in the future breeding programmes.

Key words: Genetic diversity, Horse breed, Microsatellite, Conservation.

RIASSUNTO

STRUTTURA GENETICA DI RAZZE EQUINE CROATE: IMPLICAZIONI E STRATEGIE DI CONSERVAZIONE

Nel presente studio è stata stimata la variabilità genetica di tre razze autoctone croate attraverso lo studio di 20 loci microsatelliti in un campione rappresentato da 107 animali. Il numero medio di alleli per locus (6,3) e la proporzione di eterozigosi (0,732) hanno indicato una variabilità moderata. L'eterozigosi attesa è stata simile tra le tre razze: da 0,724 (Posavina) a 0,737 (Croatian Coldblood e, Murinsulaner). Il Coefficiente di consanguineità F_{IS} è risultato basso e non significativo nelle tre popolazioni. La bassa

differenziazione genetica fra le tre popolazioni (F_{ST}=0,026) suggerisce che solo il 2,6% della variabilità genetica totale è risultata imputabile a differenze tra le razze, e il 97% a differenze individuali. I risultati della differenziazione genetica tra coppie suggeriscono che la razza Posavina e la razza Croatian Coldblood siano le popolazioni geneticamente più vicine (F_{ST}=0,016). Questi risultati sono stati confermati dalle distanze genetiche di Nei, con il valore più alto tra la razza Posavina e la razza Murinsulaner (0,082) e il valore più basso tra la razza Posavina e la razza Croatian Coldblood (0,044). Un test di attribuzione ha assegnato correttamente l'82% di individui alla razza giusta. Si conclude che dovrebbero essere messe in atto strategie atte a preservare il patrimonio genetico delle popolazioni equine autoctone della Croazia al fine di prevenirne l'estinzione e con l'obiettivo di includerle, nel futuro, in programmi di miglioramento genetico.

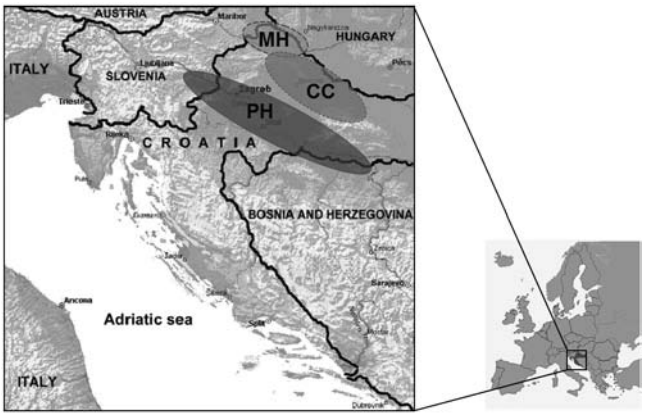
Parole chiave: *Variabilità genetica, Razze equine, Microsatelliti, Conservazione.*

Introduction

The conservation of biodiversity still remains an important concern. Despite worldwide efforts, about one third of all livestock breeds are threatened by extinction (FAO, 2007). A total of 181 horse breeds in the world (23.03%) are classified as being 'at risk' and 11.07% horse breeds became extinct (FAO, 2007). Having lost their primary aspect of utilization (work), the autochthonous coldblood horse breeds in Europe became endangered. In Croatia, three autochthonous horse breeds have survived (Posavina horse, Croatian Coldblood and Murinsulaner horse). Until now, they have been characterized for morphological traits, biochemical markers (Ivanković and Caput, 2004a, 2004b) and microsatellites (Druml *et al.*, 2007). They originated in geographically close areas (Figure 1), although by different breeding and selection strategies. The Posavina horse was formed by earlier steady infusion of the Arab horse genome; the Croatian Coldblood was under a significant influence of the English Thoroughbred and the Belgian Coldblood, while the Murinsulaner horse was influenced by the Noric and Percheron coldblood breeds (Romić, 1975; Ivanković and Caput, 2004a). The majority of the Posavina horse population is located in the territory of Croatia, while a minor part may be found in Slovenia, Bosnia and Herzegovina. The Croatian Coldblood is bred in central and north-western Croatia. Until the mid-twentieth century, the Murinsulaner population had been distributed in the territories of Croatia, Slovenia, Hungary and Austria, but the present population is found exclusively in the area of Medimurje.

Since the number of animals has decreased drastically in the last fifteen years, the government introduced subsidies in order to prevent the extinction of these breeds. An annual report (CLC, 2007) shows the Posavina horse population size at approximately 3500 individuals, the Croatian Coldblood at 4900, and the Murinsulaner horse at 36 individuals. According to the population size and the characteristics of the populations, and as indicated by FAO standards, a constant genetic loss in each generation can be expected in all populations. So far, the breeding strategy of autochthonous horse breeds has been based on phenotypic merit and poor pedigree reports, without genetic diversity knowledge within and between the breeds. Molecular characterization is an essential prerequisite for the development of an effective and meaningful conservation programme. Among the array of molecular markers, microsatellites are considered especially suitable for biodiversity evaluation, owing to their codominant inheritance, high heterozygosity, ease and reliability of scoring, ubiquitous presence throughout the ge-

Figure 1. Geographical locations of the three sampled autochthonous coldblood horse breeds (PH Posavina horse, CC Croatian Coldblood, MH Murinsulaner horse).



nome, and a high degree of polymorphism (Takezaki and Nei, 1996). Microsatellite analysis is now a widespread technique for the designated genetic variability (Bjørnstad and Røed, 2001; Bjørnstad *et al.*, 2003; Juras *et al.*, 2003; Aberle *et al.*, 2004; Krüger *et al.*, 2005; Solis *et al.*, 2005; Druml *et al.*, 2007; Luis *et al.*, 2007b). In this study, we analyzed three autochthonous coldblood horse breeds with the aim to ascertain the levels of genetic variability and estimate genetic distances between them. Furthermore, the results will provide information that can be useful as a basis for an effective conservation program, especially for the critically endangered Murinsulaner horse.

Material and methods

Blood samples were taken from 45 individuals Posavina horses (PH), 40 Croatian Coldblood individuals (CC), and 22 individual Murinsulan horses (MH). The animals were chosen from wide, boundless geographical areas, sampling four to five individuals per herd (10 to 12 herds overall) at several locations. Twenty microsatellite markers were used HMS1, HMS2, HMS3, HMS6, HMS7 (Guérin *et al.*, 1994), HTG4, HTG6 (Ellegren *et al.*, 1992), HTG7, HTG10, HTG15 (Marklund *et al.*, 1994), UCDEQ405 (Eggleston-Stott *et al.*, 1997), VHL20 (Van Haeringen *et al.*, 1994), AHT21 (Swinburne *et al.*, 1997), AHT4, AHT5 (Binns *et al.*, 1995), ASB2 (Breen *et al.*, 1997), LEX003 (Coogle *et al.*, 1996a), LEX033 (Coogle *et al.*, 1996b), TKY19 (Kakoi *et al.*, 1999) and TKY321 (Tozaki *et al.*, 2000). Thirteen of them are recommended by the International Society for Animal Genetics (ISAG) for analysis of the genetic diversity in horses. An appropriate amount of PCR products (0.5-1.6 µl) was mixed with 12 µl formamide and 1µl of Genescan-350 ROX standard and analyzed on ABI PRISMTM 310 Genetic Analyzer (PE Applied Biosystems, MA).

Allelic frequencies and number of alleles per locus were estimated by direct counting. The expected (H_e) and observed (H_o) heterozygosities were calculated according to Saito and Nei (1987) and deviations from Hardy–Weinberg equilibrium (HWE) were tested using Fisher's exact test, using the null hypothesis H_0=no heterozygote deficiency,

with the level of significance determined by a Markov-chain randomization by Genepop v3.4 (Raymond and Rousset, 1995).

The value for the fixation index (F_{ST}) and migration rate (*Nm*) was calculated according to Nei (1973) and Slatkin and Barton (1989), and allelic richness (AR), F_{IT} and F_{IS} index using FSTAT v2.9.3 computer program (Goudet, 1995).

Nei's unbiased D_A-distance (Nei *et al.*, 1983) and the proportion of shared alleles D_{PS}=-ln(PS) were calculated (Bowcock *et al.*, 1994). The neighbour-joining trees of the D_A-distance and the individual D_{PS}-distances were calculated by the program Neighbor from the Phylip program package (Felsenstein, 1993) and plotted by the program Treeview (Page, 1996). To test the stability of the D_A-distance tree, 1000 distance-matrices were produced by bootstrapping over loci (Felsenstein, 1985). The resulting consensus tree was generated using the program Consensus from the Phylip program package (Felsenstein, 1993).

An assignment test was carried out with the program Doh (Brzustowski, 2002). The program Doh implements the multilocus genotype from several populations and determines from which population each individual is most likely to have come, by using the assignment index procedure, the highest probability of an individual's genotype in any of the populations. Calculations are described by Paetkau *et al.* (1995).

The bottleneck hypothesis was investigated using Bottleneck 1.2.02. (Cornuet and Luikart, 1996). Three different tests were performed using allele frequency data, Standardized differences test, Wilcoxon sign test and a qualitative test of mode shift, to find out whether the population was in mutation drift equilibrium. All three proposed models of microsatellite mutation, Stepwise (SMM), Infinite Allele (IAM) and Two-Phased Model (TPM), were used.

Results

All amplified loci were polymorphic in the analyzed breeds, with a total of 126 different alleles detected across loci. The measures of variability of twenty microsatellite markers in PH, CC, and MH are given in Table 1. The PIC values found in 20 loci ranged from 0.3409 in HMS1 to 0.6517 in ASB2 with a mean value of 0.512. According to Botstein *et al.* (1980), thirteen loci are highly informative (PIC>0.5), whereas seven are reasonably informative (0.5>PIC>0.25). The average F_{IT} across loci was positive and significant (0.025, P<0.01). Markers ASB2 (F_{IT}=0.118, P<0.01), HTG10 and LEX033 (both F_{IT}=0.093, P<0.01) contribute the most to the observed deficit of heterozygotes (Table 1).

The lowest mean number of alleles (MNA) was found in MH (5.55) and equal values in PH and CC (5.9). The number of private alleles in each breed was two (VHL20 and ASB2 in PH, UCDQ405 and ATH5 in CC, LEX033 and HMS2 in MH). Most of the private alleles were in very low frequencies and below 5%, except the allele at locus ASB2 in PH, which had a higher frequency (17%). Regarding the microsatellite loci tested across populations, the lowest observed and expected heterozygosity was found in PH (Ho=0.71; He=0.724), the highest observed heterozygosity in MH (0.741), while MH and CC have the same value for the expected heterozygosity (He=0.737) (Table 2). The exact test for the presence of HWE showed deviations in PH at three loci (HMS2, LEX003, P<0.01; HTG10, P<0.05), in CC at two loci (HTG6, LEX033, P<0.01) and in MH at three loci (HTG10, TKY321 and VHL20, P<0.05), respectively. In addition, HW disequilibrium was statistically significant over all the breeds and loci (P<0.05).

The mean value of within-population in-

Table 1. Summary statistics 20 microsatellite loci analyzed in the three autochthonous coldblood horse breeds; number of alleles (nA), observed and expected heterozygosity (Ho and He), polymorphism information content (PIC), Wright F-statistics (F_{ST}, F_{IS}, F_{IT}) and migration rate (*Nm*).

Locus	nA	Ho	He	PIC	F_{ST}	F_{IS}	F_{IT}	*Nm*
AHT4	6	0.722	0.738	0.535	0.042**	0.022	0.065	5.66
ASB2	8	0.752	0.806	0.652	0.036**	0.068*	0.118**	6.64
HMS1	5	0.651	0.626	0.341	0.010*	-0.040	-0.073	23.95
HMS2	10	0.706	0.713	0.501	0.042**	0.009	0.048	5.69
HMS3	6	0.722	0.754	0.536	0.026**	0.044	0.082	9.50
AHT21	6	0.770	0.733	0.502	0.042**	-0.052	-0.013	5.73
AHT5	7	0.747	0.784	0.577	0.019**	0.049	0.071	13.10
HMS6	6	0.705	0.669	0.436	0.023	-0.055	-0.015	10.54
HMS7	6	0.700	0.720	0.501	0.021*	0.028	0.037	11.51
HTG10	8	0.766	0.806	0.635	0.023**	0.051	0.093*	10.50
HTG15	4	0.690	0.661	0.368	0.016	-0.045	-0.056	15.50
HTG4	5	0.721	0.654	0.406	0.033	-0.104	-0.065	7.31
HTG6	4	0.727	0.677	0.401	0.017*	-0.075	-0.031	14.34
HTG7	4	0.695	0.715	0.458	0.010	0.028	0.040	24.92
LEX003	7	0.774	0.757	0.565	0.029**	-0.023	0.020	8.49
LEX033	7	0.723	0.789	0.592	0.025**	0.086	0.093*	9.83
TKY19	5	0.775	0.752	0.524	0.018**	-0.032	-0.019	13.39
TKY321	7	0.740	0.779	0.591	0.038**	0.051	0.072	6.26
UCDEQ405	6	0.713	0.724	0.489	0.030**	0.015	0.008	7.96
VHL20	9	0.783	0.795	0.624	0.019	0.008	0.025	12.87
Mean	6.3	0.729	0.732	0.512	0.026**	0.004	0.025**	9.25

*Statistical significance: *P<0.05; **P<0.01.*

breeding estimates (F_{IS}=0.004) indicated a low level of inbreeding in the population (Table 1) and was not significant in any of the studied horse populations. Table 2 shows a comparison of within-population inbreeding.

To investigate the population subdivision and the average number of migrants per generation, we estimated the F_{ST} and *Nm* values, respectively (Table 1). The F_{ST} values indicate that 2.6% of the total genetic variations are explained by differences between the breeds, with the remaining 97% corresponding to differences among individuals. The average number of migrants per generation (*Nm*), with the mean value over loci 9.25, pointed out that a large number of gametes are exchanged per generation. The contribution of the microsatellite markers for breed differentiation was estimated

Table 2. Number of alleles (nA), allelic richness (AR), observed and expected heterozygosity (Ho and He), inbreeding estimates within population (FIS) in Posavina horse (PH), Croatian Coldblood (CC) and Murinsulaner Horse (MH).

Locus	nA			AR			Ho			He			F_{IS}		
	PH	CC	MH	PH	CC	MH	PH	CC	MH	PH	CC	MH	PH	CC	MH
AHT21	6	6	6	3.49	3.80	3.69	0.67	0.83	0.82	0.66	0.73	0.81	-0.018	-0.13	-0.009
AHT4	6	6	5	8.75	7.70	8.21	0.69	0.75	0.73	0.73	0.75	0.74	0.055	-0.01	0.018
AHT5	5	7	5	4.87	5.10	5.16	0.67	0.80	0.77	0.74	0.83	0.78	0.102	0.037	0.011
ASB2	8	7	6	5.72	6.35	6.21	0.69	0.75	0.82	0.83	0.79	0.80	0.175*	0.047	-0.026
HMS1	5	4	5	4.81	5.00	4.96	0.71	0.65	0.59	0.60	0.62	0.66	-0.198	-0.05	0.112
HMS2	8	9	7	4.99	5.91	6.07	0.67	0.73	0.73	0.71	0.71	0.72	0.058	-0.02	-0.006
HMS3	6	6	6	5.93	6.81	6.56	0.67	0.73	0.77	0.71	0.78	0.77	0.063	0.071	-0.001
HMS6	6	6	5	4.00	3.98	3.99	0.69	0.70	0.73	0.71	0.69	0.61	0.031	-0.01	-0.206
HMS7	6	5	6	3.99	3.00	3.91	0.69	0.78	0.64	0.72	0.77	0.67	0.037	-0.01	0.056
HTG10	8	7	7	4.68	4.91	4.77	0.73	0.70	0.86	0.84	0.77	0.81	0.126*	0.092	-0.067
HTG15	4	4	4	5.71	5.74	5.73	0.69	0.70	0.68	0.61	0.66	0.71	-0.124	-0.06	0.037
HTG4	5	5	5	7.79	6.86	7.51	0.71	0.73	0.73	0.66	0.68	0.62	-0.079	-0.07	-0.175
HTG6	4	3	4	5.71	5.00	5.48	0.67	0.65	0.86	0.68	0.65	0.71	0.017	-0.01	-0.228
HTG7	4	4	4	5.80	5.68	5.57	0.73	0.63	0.73	0.75	0.71	0.69	0.019	0.122	-0.062
LEX003	6	7	6	5.55	5.79	5.59	0.78	0.73	0.82	0.77	0.77	0.73	-0.007	0.057	-0.125
LEX033	5	6	7	7.34	7.40	7.37	0.71	0.78	0.68	0.77	0.78	0.81	0.081	0.012	0.162*
TKY19	5	5	5	3.97	3.96	4.60	0.78	0.78	0.77	0.70	0.79	0.76	-0.106	0.015	-0.011
TKY321	7	7	5	7.75	6.71	7.66	0.69	0.85	0.68	0.76	0.79	0.80	0.09	-0.08	0.145
UCDEQ405	5	6	5	4.49	6.53	5.94	0.78	0.73	0.64	0.71	0.70	0.76	-0.094	-0.04	0.171
VHL20	9	8	8	5.89	5.90	5.76	0.80	0.78	0.77	0.82	0.78	0.78	0.004	0.01	0.01
Mean	5.9	5.9	5.5	5.56	5.61	5.74	0.71	0.74	0.74	0.72	0.74	0.74	0.018	0.001	-0.005

*Statistical significance: *P<0.05.*

Table 3. F_{ST} values, above diagonal, and Nei's genetic distance (DS), below diagonal, estimated between three Croatian autochthonous horse breeds with 20 microsatellite markers.

Breed	PH	CC	MH
PH	-	0.016*	0.035*
CC	0.044	-	0.021*
MH	0.082	0.059	-

*Statistical significance: *P<0.05.*

by the significance of the F_{ST} statistics. Loci HMS6, HTG15, HTG4, HTG7 and VHL20 did not contribute to breed differentiation. All others loci contributed to breed differentiation as presented in Table 1 (12 loci, P<0.01 and 3 loci P<0.05), with the highest significant F_{ST} value at 0.0423 for ATH4. The pairwise F_{ST} coefficients ranged from 0.016 between CC and PH, to 0.035 between the MH and PH breeds, and were significant (P<0.05) among all three breeds (Table 3). The result obtained with Nei's distance comparing the three breeds showed low values with a minimum distance (0.044) between PH and CC (Table 3).

The neighbour-joining tree for individual D_{PS}-distances estimated by the proportion of shared alleles (Figure 2), shows that Croatian horses do not form defined clusters.

In 68 to 87% of cases, individuals could be assigned to a pre-defined population (Table 4). From a total of 20 individuals incorrectly assigned, eleven are allocated in the CC populations (10.28%), 5 to PH (4.67%) and 3 (2.8%) to MH.

To determine the significant number of loci with heterozygosity excess, two tests were performed under three different mutation models: Infinite Allele Model (IAM), Stepwise Mutation Model (SMM) and Two Phase Model (TPM). Using both tests under IAM, probability values were significantly different from zero (P<0.001) for all the breeds, while only the Wilcoxon test resulted significant under TPM for CC only (P=0.018, P<0.005), meaning that the three populations have undergone a recent genetic bottleneck (Table 5). The presence of the L-shaped distribution of the allele frequencies does not indicate a recent bottleneck in any of the three populations, as the alleles with the lowest frequency (0.001-0.1) were found to be abundant.

Discussion

The twenty microsatellite loci typed in this study showed a moderate mean number of alleles per locus (6.3). This value is high-

Figure 2. The neighbour-joining tree of individual allele sharing distance (PH Posavina horse, CC Croatian Coldblood, MH Murinsulaner horse).

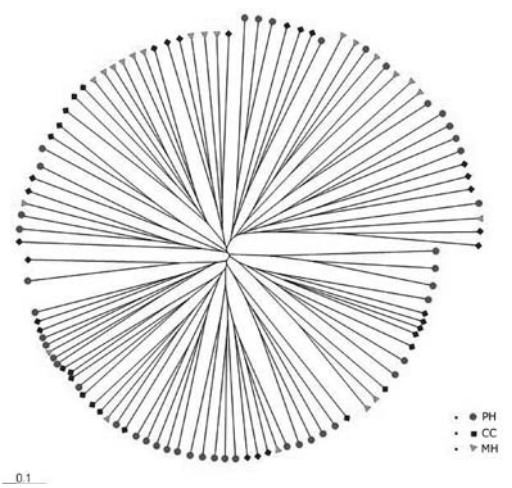


Table 4. Assignment test results for Posavina horse (PH), Croatian Coldblood (CC) and Murinsulaner horse (MH). Abbreviation as follows, number of tested animals (N), number of animals correctly assigned to their breed (in boldface) and percentage of correct assignment (N%).

Breed	N	PH	CC	MH	N %
PH	45	**39**	5	1	86.7
CC	40	4	**34**	2	85.0
MH	22	1	6	**15**	68.2

Table 5. Bottleneck analysis tested in three Croatian autochthonous horse breeds under Infinite Allele Model (IAM), Stepwise Mutation Model (SMM) and Two-Phased Model (TPM). Two different statistical procedures have been used, Standardized difference test and Wilcoxon test.

Breed	Standardized differences test			Wilcoxon test		
	IAM	SMM	TPM	IAM	SMM	TPM
PH	0.001**	0.312	0.122	0.001**	0.43	0.057
CC	0.001**	0.224	0.087	0.001**	0.062	0.018*
MH	0.001**	0.206	0.105	0.001**	0.571	0.202

*Statistical significance: *P<0.05; **P<0.01.*

er than those observed in Norwegian horse breeds (Bjørnstad *et al*., 2000), German draught horse breeds (Aberle *et al*., 2004) and the Sorraya (Louis *et al*., 2007a), but lower than in Franches-Montagnes breeds (Glowatzki-Mullis *et al*., 2006), Polish heavy horse (Iwańczyk, 2006), the Hispano-Breton (Perez-Gutierrez, 2008), and Italian breeds (Zuccaro *et al*., 2008). Higher values for MNA for CC (7.1) and PH (6.9) were reported by Druml *et al*. (2007). As expected, the degree of polymorphism exhibited by microsatellite markers was much greater than that concerning protein loci (nA=3.55; He=0.557-0.581) (Ivanković and Caput, 2004b). The low number of private alleles at all three breeds is not surprising, because at the end of the 19th century, systematic outbreeding was practiced, which led to the creation of a wide genetic base and heterogeneity (Druml *et al*., 2007). The time since the formation of the breeds as "closed populations" is still too short for a genetic drift to have had an effect. The level of AR in the native Croatian horses was similar to those previously found in western Mediterranean horse breeds (Marletta *et al*., 2006) and Danish horse breeds (Thirstrup *et al*., 2008). Among Croatian autochthonous breeds, genetic diversity (H_e) was never below 0.72 and was higher than the values reported for German draught horses (He=0.64-0.75, Aberle *et al*., 2004), Franches-Montagnes breeds (He=0.69, Glowatzki-Mullis *et al*., 2006), the Polish heavy and Belgian draft horse (He=0.39 and He=0.42, Iwańczyk, 2006), draught horses from Austria, Germany and Croatia reported by Druml *et al*.

(2007), and the Sorraya (He=0.47, Louis *et al*., 2007b). The high level of genetic variation suggests that the genome of the investigated horse breeds has diverse origins, a consequence of upgrading by different horse breeds during the previous two centuries. Also, the high levels of heterozygosity in PH, CC and MH breeds could be mainly due to the existence of a gene flow among these populations because they are geographically closely located. The F_{IT} statistics for the whole population indicates an overall decrease of 2.5% (P<0.01) in heterozygote phenotypes compared to homozygotes. The mean estimates of F_{IS} were not significant in all horse populations, indicating that they are not affected by a reduction of variation. The significant deficit (P<0.05) of heterozygotes on three loci (ASB2, HTG10 and LEX033) is possibly the result of presence of null-alleles or a greater degree of relatedness in the sampled material. Similar results concerning the ASB2 and HTG10 loci for Biłgoraj horses were obtained by Ząbek *et al.* (2005) and for Danish breeds by Thirstup *et al.* (2008). Establishment of male lineages in MH, because of the breeding system ("similar with similar mate"; Ogrizek, 1941) cannot be ruled out.

The genetic differentiation among Croatian horse breeds is small, and 75% of loci contribute to this differentiation with F_{ST} values being low but significant (P<0.01). Most of the total genetic variation, more than 97%, corresponds to differences among individuals, whereas about 3% is due to differences among the breeds. These values of the total genetic differentiation (F_{ST}) among the breeds are noticeably lower in comparison to those found in other horse breeds: 8% in Bardigiano Horse (Di Stasio *et al.*, 2008) and in western Mediterranean breeds (Marletta *et al*., 2006), 9% in the Spanish Trotter (Azor *et al.*, 2007) and Sorraya Horse (Luis *et al.*, 2007a). These results are in accordance with those from Druml *et al*. (2007), suggesting that CC and PH are two separate, but closely related populations with a hidden admixture.

Gene flow between the three breeds was high (Nm=9.25), supported by the fact that these horses are bred in the same area (Figure 1) in the conditions of a moderate population size, no usage of artificial insemination, absence of breed improvement programs and non-defined objectives of selection. Trexler (1988) showed that if Nm>1, gene flow is enough to reduce the genetic differentiation between populations. In Figure 2, the neighbour-joining tree of the individual allele sharing distance from 107 individuals demonstrates an admixed structure as a result of a high migration rate.

In the present study, assignment test revealed a low accuracy (82%) of the overall individual assignments, demonstrating a lower average interpopulation differentiation F_{IT}=2.5%. Although the number of incorrect assigned individuals was almost the same in all three populations, a small percentage of correctly assigned individuals in MH (68.2%) can be explained with a small number of sampled individuals (n=22), i.e. the smaller size of the whole population.

Nei's genetic distance (D_S) reveals a close relationship between the two breeds, PH horse and CC (0.044), while the distance between PH and MH horse was the greatest (0.082). It is explained with a closer geographical positioning of the Posavina and Moslavina areas, than that of Posavina and Medimurje. This result is in accordance with the results of the lowest interbreed differentiation found between PH and CC (F_{ST}=0.016), consistent to the value (F_{ST}=0.018) reported by Druml *et al*. (2007).

The results of bottleneck analysis showed that all three coldblood breeds deviate from the mutation-drift equilibrium under IAM,

but since microsatellite loci have been shown to conform better to the stepwise mutation model (Shriver *et al.*, 1993) than to the infinite allele model, the detection method based on the infinite allele model was considered to be invalid (Spong and Hellborg, 2002). The Wilcoxon test appears to be suitable for this analysis because it provides a relatively high power and it can be used with as few as four polymorphic loci and any number of individuals (Cornuet and Luikart, 1996). To test for a recent bottleneck effect, the normal L-shaped distribution of a plot of allelic frequency class versus proportion of alleles reinforces the results that Croatian native horse population has not experienced any recent bottleneck. On the contrary, Wilcoxon test (TPM) for CC suggests the appearance of a bottleneck, although according to the annual report (CLC, 2007), the total number of CC is near 5000 individuals, while in the smaller population of MH (N=36), there are no such indices. Luikart *et al.* (1988) pointed out that the appearance of a genetic bottleneck in the absence of a demographic bottleneck is not unusual, and that bottleneck populations do not have model-shifted distribution. The recent war in Croatia caused a loss of pedigree records, as well as of a substantial number of individuals. The re-establishment of breeds was based on a few stallions that fecundated mares. There exists a possibility that the effective population size is small in comparison to the census size (N_e<N-census) and that sampled animals were not representative (although, sampled animals are chosen at random, with owner reference of non-relatedness). Luikart *et al.* (1988) and Pérez-Gutiérrez *et al.* (2008) show that, despite low census, the endangered breed does not seem to be in immediate risk. Although the population of MH is rather small (CLC, 2007), useful planned breeding schemes and appropriate conservation strategies might be decisive to increase the population.

Conclusions

Our results show that Croatian autochthonous horse breeds have preserved a relatively high genotypic diversity which is comparable to other European horse breeds. If the purpose is to use them in crossbreeding or introgression plans, the diversity between the populations should be prioritized. The conclusions based on the examination of genetic diversity support the opinion, based on the phenotypical features, that the Posavina horse should be bred separately from the Croatian Coldblood and Murinsulaner horse, while a moderate introduction of the Croatian Coldblood genome into the population of the Murinsulaner horse would present a good opportunity to increase genetic variability and stabilize the population size. Also, as a strategy of further conservation, we would support diversity among individuals within a breed, which contributes to diversity of the present genetic pool.

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