Mitochondrial D-loop sequence variation among autochthonous horse breeds in Croatia

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ABSTRACT: Genetic variation in three Croatian coldblood horse populations was analysed using a sequence analysis of the proximal part (nt 15 498–15 821) of the D-loop region of mtDNA. Twenty unrelated horses were chosen from the Posavina horse and the Croatian Coldblood breeds and fifteen horses from the Murinsulaner horse population. Sequencing of the proximal part of the mtDNA D-loop region revealed 26 polymorphic sites representing thirty haplotypes which were clustered into eight haplogroups. A wide variety of mitochondrial haplotypes of the analysed horse breeds were clustered into eight different haplogroups. Two haplogroups (D and F) were specific to Posavina horse, five haplogroups were shared among the Croatian Coldblood and Murinsulaner horse, the fact that can be explained by selection strategy at the beginning of the 20th century and possible gene flow between the two populations. These results indicate the presence of many ancient maternal lineages with high diversity in mtDNA. The genetic information based on mtDNA typing has a great importance for the future breed conservation strategy, especially for the critically endangered breed such as Murinsulaner horse.

Keywords: mtDNA; genetic diversity; horse; conservation genetics

In the framework of breed conservation, genetic characterisation is an important step allowing preservation of the breed integrity and is a prerequisite for efficient management of genetic resources (Bjørnstad and Røed, 2002). Managing genetic diversity is one of the primary goals in conservation programmes (Toro et al., 2003). Molecular analysis provides a reliable tool which can be used together with the quantitative approach and traditional breeding strategies for an efficient design of preservation strategy (Dovc et al., 2006). Genetic distances can also be used to determine the population structure and genetic distinctiveness of a population or breed (MacHugh et al., 1998). The control region of the mitochondrial DNA (mtDNA) is, due to its high mutation rate, lack of recombinations and maternal inheritance, a very useful marker system for population and evolutionary biology. The mitochondrial DNA studies in horses have proved to be useful to characterize intra- and inter-breed relationships (Ishida et al., 1994; Ishida et al., 1995; Kavar et al., 1999; Kim et al., 1999; Vilà et al., 2001; Jansen et al., 2002; Hill et al., 2002; Mirol et al., 2002; Cozzi et al., 2004; Lopes et al., 2005; Royo et al., 2005; McGahern et al., 2006).

Horse breeds are a part of the entire regional and global genetic heritage. A total of 181 horse breeds in the world (23.03%) are classified as being 'at risk' and 11.07% of horse breeds already became extinct (FAO, 2007). In Europe, the au-

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tochthonous coldblood horse breeds are particularly endangered, primarily due to the loss of their traditional use as working animals. In Croatia, three autochthonous horse breeds have survived (Posavina horse, Croatian Coldblood, Murinsulaner horse) and they are characterised by their exterior and biochemical markers (Ivanković and Caput, 2004a,b). These breeds were bred in geographically narrow areas, using different breeding and selection approaches. Posavina horse was formed according to earlier studies by introducing the Arab genome, the Croatian Coldblood was under a significant influence of the English Thoroughbred and Belgian Coldblood, while Murinsulaner horse was affected by the Noric and Percheron 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 can be found in Slovenia and Bosnia and Herzegovina. The Croatian Coldblood is bred in Central and Northwestern Croatia. Until the mid-20th century, the Murinsulaner population was distributed in the territories of Croatia, Slovenia, Hungary and Austria, but the present population is limited to the area of Međimurje (Figure 1). During the last four decades, the number of animals belonging to the three autochthonous horse populations has drastically decreased. Populations of Posavina horse and Croatian Coldblood are potentially endangered, while the Murinsulaner horse is critically endangered (HSC, 2008). According to the population size and the characteristics of the population, a constant genetic loss in each generation can be expected in all populations (Bodó, 1992). In the framework of this study, we analysed mtDNA of the three autochthonous coldblood horse breeds in order to support phenotypic and biochemical data with molecular genetic analyses, and to provide a genetically based tool for effective conservation programmes.

MATERIAL AND METHODS

Blood samples were collected from 20 Posavina horses (PH), 20 Croatian Coldbloods (CC) and 15 Murinsulaner horses (MH). Our sampling included individuals from several locations per population in the breeding area (Figure 1). A smaller number of individuals of the Murinsulaner population included in the analysis is a consequence of the reduced size of the total population and distribution in a narrow geographical area. Mitochondrial

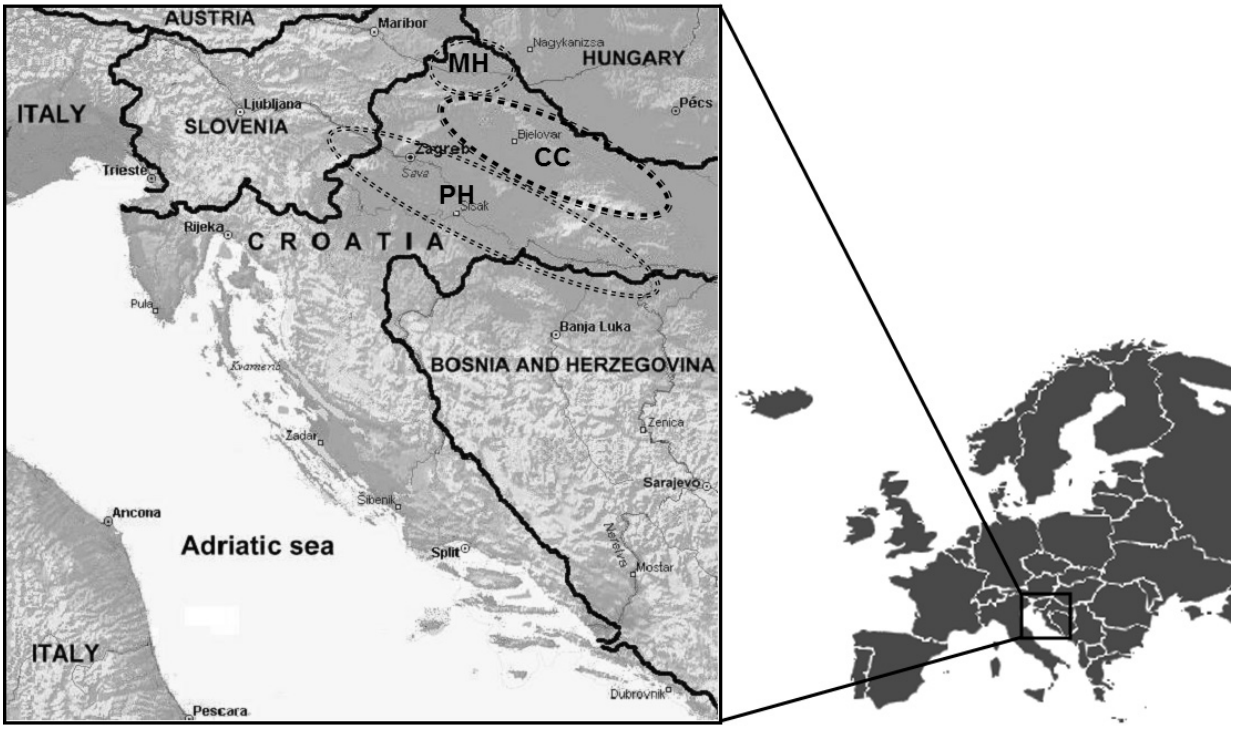

Figure 1. Geographical locations of the three sampled autochthonous coldblood horse breeds (PH – Posavina horses, CC – Croatian Coldbloods, MH – Murinsulaner horses)

Table 1. The haplotypes and nucleotide substitutions identified relative to the reference sequence GenBank X79547. In the column "sample", each breed corresponds to the following GenBank accession numbers: PH = EF495133-43; CC = EF494073-83; MH = EF495144-51

Haplotype	Sample	Nucleotide differences from the reference sequence (X79547)																											
		15 811	15 806	15 776	15 770	15 765	15 763	15 748	15 740	15 720	15 718	15 703	15 667	15 659	15 650	15 649	15 616	15 615	15 604	15 603	15 602	15 597a	15 585	15 583	15 540	15 534	15 526
X79547		T	C	A	A	G	-	C	T	G	A	A	A	A	T	A	T	C	G	A	A	A	C	C	A	C	C
CP1	PH1	.	.	.	.	A	-	T	.	A	.	.	G	.	.	.	C	.	A	G	.	.	G	T	.	T	G
CP2	PH2. PH6. PH10.	.	.	.	.		A	-	.	C	A	.	.	.	.	.	.	.	.	A		.	.	G		.	T
CP3	PH14	.	.	.	.	A	-	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	G	T	.	T	G
CP4	PH3. PH18. PH9	.	.	.	.	A	-	T	.	A	.	.	G	.	.	.	C	.	A	G	.	G	.	T	G		. G	
CP5	PH4. PH19. PH17	.	.	.	.	A	-	T	.	A	.	.	.	.	.	.	C	.	A	G	.	G	.	T	G	T	G
CP6	PH5	.	T		.	.	A	-	.	C	A	.	.	.	.	.	.	.	.	A	.	.	G	.	T	.	T
CP7	PH16. CC6	.	.	.	.	A	.	T	.	A	.	.	.	.	.	.	C	.	A	G	.	G	.	T	G	.	G
CP8	PH7. PH11	.	.	.	.	A	-	T	.	A	.	.	G	.	.	.	C	.	A	G	.	G	.	T	G	T	G
CP9	PH8	.	.	.	.	A	-	.	C	A	.	.	.	.	.	.	.	.	A	.	G	G	.	T	.	T	.
CP10	PH12	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	G	T	.	T	G
CP11	PH13. PH20	.	.	.	.	A	-	.	C	A	.	.	.	.	.	.	.	.	A	G	.	G	.	T	.	T	G
CC1	PH15. CC8	C	.	G	.	A	-	T	.	.	.	.	G	.	.	.	C	T	A	.	.	G	.	T	G	.	G
CC2	CC1	C	.	G	.	.	-	T	.	.	.	.	G	.	C	.	.	.	A	.	.	.	G	T	.	T	.
CC3	CC2. CC13. CC19	.	T	.	.	.	-	T	.	A	.	.	G	.	C	.	C	.	A	.	.	.	.	T	.	T	G
CC4	CC3	C	.	G	.	A	-	T	.	.	.	.	G	.	C	.	C	T	A	.	.	G	.	T	.	.	.
CC5	CC4. CC20	.	T	.	.	.	G	T	.	.	C	C	G	.	.	.	C	T	A	G	.	.	G	T	G	.	G
CC	CC5. CC14	C	.	G	.	.	-	T	.	.	.	.	.	.	C	.	.	.	A	.	.	.	G	T	.	T	G
CC7	CC15. MH10	.	.	.	.	.	-	.	.	.	.	.	G	G	.	.	.	.	.	.	.	.	.	T	.	.	G
CC8	CC11. CC17. MH3	.	T	.	.	.	-	T	.	.	.	.	G	.	C	.	.	.	A	.	.	.	.	T	.	T	G
CC9	CC7. CC18. MH14	C	.	G	.	A	-	T	.	.	.	.	G	.	.	.	C	T	A	.	.	G	.	T	.	.	.
CC10	CC9	.	T	.	.	.	-	T	.	.	.	.	G	.	C	.	.	.	A	G	.	.	.	T	.	T	.
CC11	CC10	.	.	.	.	.	-	T	.	.	.	.	G	.	C	.	.	.	A	.	.	.	.	T	.	T	G
CM1	CC12	.	.	.	T	A	-	.	C	A	.	.	G	.	.	.	.	.	A	.	.	G	.	T	.	T	.
CM2	MH1	.	T	.	.	.	G	T	.	.	C	C	.	.	.	G	C	.	A	G	.	.	G	T	.	.	G
CM3	CC16. MH2	C	T	G	.	.	-	.	.	.	.	.	G	.	C	.	.	.	A	.	.	.	G	T	.	T	.

Table 1. to be continued

Haplotype	Sample	Nucleotide differences from the reference sequence (X79547)																									
		15 811	15 806	15 776	15 770	15 765	15 763	15 748	15 740	15 720	15 718	15 703	15 667	15 659	15 650	15 649	15 616	15 615	15 604	15 603	15 602	15 597a	15 585	15 583	15 540	15 534	15 526
X79547		T	C	A	A	G	-	C	T	G	A	A	A	A	T	A	T	C	G	A	A	A	C	C	A	C	C
CM4	MH15	·	T	·	·	·	G	T	·	·	C	C	G	·	·	G	C	T	A	·	·	·	G	T	G	·	G
CM5	MH4. MH8	C	·	G	·	A	-	T	·	·	·	·	·	·	C	·	C	T	A	·	·	G	·	T	·	·	G
CM6	MH11 MH12	C	T	G	·	·	-	·	·	·	·	·	G	·	C	·	·	·	·	·	·	·	G	T	·	T	·
CM7	MH5	C	·	G	·	A	-	T	·	·	·	·	G	·	C	·	C	T	A	·	·	G	·	T	·	·	G
CM8	MH6. MH13	·	T	·	·	·	-	·	·	·	·	·	G	G	·	·	·	·	·	·	·	·	·	T	·	·	G
	MH7																										
	MH9																										

DNA was isolated from blood samples as described by White and Densmore (1992). The proximal part of the D-loop region was PCR amplified using primers P28 (5'-GTAAAACGACGGCCAGT CTCAC-CATCAACCCCCAAAGC-3') and HF (5'-CCTGAAGTAGGAACCAGATG-3') amplifying a fragment of the D-loop region, between the $tRNA^{Pro}$ gene and the central conserved sequence block (GenBank Acc, No. X79547, nt 15 498–15 821). The PCR reaction mixture (20 µl) contained template of DNA (50 ng), 10 pmol of each primer, reaction buffer (10mM Tris-HCl pH 8.3; 50mM KCl; 5mM $MgCl_2$; 20 µM dNTPs) and 0.4 U *Taq* Polymerase (PE Applied Biosystems, MA). The PCR reaction was performed on MJ Research PTC-100 thermal cycler starting with initial denaturation at 95°C for 5 min, followed by 32 cycles of 94°C (60 s), 52°C (30 s), 72°C (60 s) and final extension at 72°C (5 min). PCR fragments were sequenced using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit and ABI PRISM^{TM} 310 Genetic Analyzer (PE Applied Biosystems, MA). Multiple alignment of mtDNA sequences was performed using Clustal-W programme (Thompson et al., 1994) and used for further analysis by the MEGA3 (Kumar et al., 2004) and Phylip programme package (Felsenstein, 1993). Partitioning of genetic diversity using the analysis of molecular variance (AMOVA) and linearized Φ_{ST} values (Slatkin, 1995) was performed with the Arlequin package version 3.0 (Excoffier et al., 2005). Genetic distances among different mtDNA haplotypes were calculated by the two-parameter method of Kimura (Kimura, 1980) and an unrooted tree was drawn using the Neighbour-Joining method (Saitou and Nei, 1987). Mitochondrial DNA haplogroups were predicted on the basis of a distance matrix of the observed haplotypes (limit *d* value = 0.015). Bootstrap analyses (1 000 data sets) were used to assess confidence in the branching order and values higher than 20 were entered into the dendrogram. To estimate the inter- and intra-species sequence divergence in the mtDNA control region, data from this study were compared with 22 nucleotide sequences of the mtDNA control region (nt15 498 to 15 821) of two species from the genus *Equus*.

RESULTS

The 324-bp fragment of the mtDNA D-loop region was sequenced. Out of the 26 polymorphic sites, 25 were caused by transitions or transver-

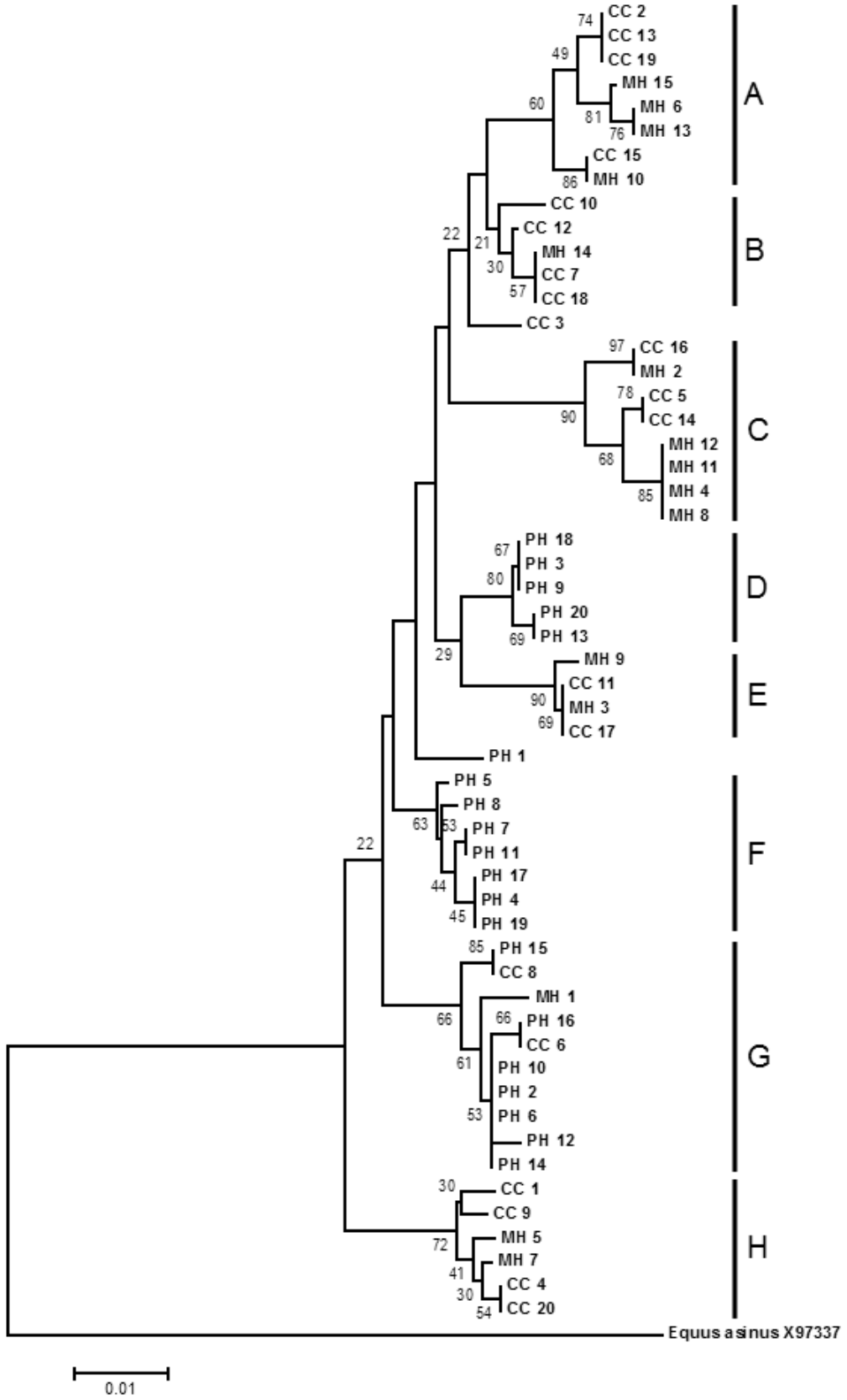

Figure 2. The neighbour-joining tree of horse haplotypes based on the D-loop region mtDNA (nt 15 498–15 821) sequences along with a donkey sequence as outgroup (GenBank Acc No. X97337). The values on the branch are bootstrap support based on 1 000 replications

Table 2. Number of nucleotide differences (below the diagonal) and Kimura two-parameter distances (above diagonal) among eight mtDNA haplogroups and other horse breeds (Belgian horse – Acc No. AF064631, AF064632; Thoroughbred horse – Acc No. D14991, D23666, AF072975; Arabian horse – Acc No. AF132568, AF132577, AF132590; Mongolian horse – Acc No. AF014415, AF056071)

Haplo-groups	A	B	C	D	E	F	G	H	Belgian horse	Thor. horse	Arabian horse	Mong. horse
A		0.0167	0.0390	0.0250	0.0281	*0.0418*	*0.0345*	*0.0266*	*0.0247*	*0.0438*	*0.0277*	*0.0413*
B	5.30		*0.0299*	*0.0235*	*0.0192*	*0.0287*	*0.0281*	*0.0294*	*0.0132*	*0.0364*	*0.0205*	*0.0331*
C	12.13	9.40		*0.0357*	*0.0344*	*0.0319*	*0.0494*	*0.0374*	*0.0286*	*0.0520*	*0.0341*	*0.0327*
D	7.85	7.40	11.10		0.0185	0.0277	*0.0238*	*0.0406*	*0.0182*	*0.0423*	*0.0263*	*0.0423*
E	8.81	6.05	10.75	5.85		*0.0321*	*0.0303*	*0.0346*	*0.0307*	*0.0379*	*0.0264*	*0.0346*
F	12.93	8.94	10.00	8.69	9.96		*0.0231*	*0.0247*	*0.0233*	*0.0467*	*0.0286*	*0.0248*
G	10.75	8.78	15.25	7.50	9.45	7.27		*0.0344*	*0.0311*	*0.0436*	*0.0252*	*0.0365*
H	8.33	9.17	11.67	12.57	10.75	7.74	10.70		*0.0305*	*0.0478*	*0.0338*	*0.0371*
Belgian	7.75	4.20	9.00	9.60	5.75	9.71	7.30	9.50		*0.0305*	*0.0212*	*0.0272*
Thoroug.	13.50	11.30	16.00	13.10	11.75	14.36	13.40	14.67	9.50		*0.0327*	*0.0240*
Arabian	8.67	6.47	10.67	8.27	8.25	8.95	7.90	10.50	6.67	10.17		*0.0389*
Mongol.	12.75	10.30	10.25	13.10	10.75	7.79	11.30	11.50	8.60	12.00	7.50	

sions, and one was a consequence of an insertion (Table 1). The nucleotide sequence diversity among haplotypes ranges from 0.31% to 5.26%, and Kimura two-parameter distances among haplotypes range from 0.017 to 0.049. The average sequence diversity within Posavina horse is smaller (5.579; 1.72%) than within the Croatian Coldblood (8.176; 2.52%) and Murinsulaner horse (9.124; 2.82%). The mean sequence distance within Posavina horse is smaller (0.0176) than within the Croatian Coldblood (0.0259) and Murinsulaner horse (0.0290).

Thirty different mtDNA haplotypes were subdivided into eight haplogroups (Figure 2). The mean distance within haplotypes ranges from 0.0015 (haplogroup E) to 0.0073 (haplogroup A). Haplogroup A comprises horses belonging to haplotypes CC2, CC6, CM3 and CM6. The mean nucleotide diversity within haplogroup A is 2.357. Haplogroup B is represented by haplotypes CC8, CC10 and CC11, whereas haplogroup C comprises haplotypes CC5, CM2 and CM4. Haplogroup C has a characteristic insertion at position 15 597Ş (Table 1). Haplotypes CP3 and CP10 are clustered in haplogroup D and the mean nucleotide diversity within this haplogroup is 0.501. Haplogroup E comprises haplotypes CC7 and CM8. Haplogroup F comprises haplotypes CP4, CP5, CP7 and CP8. The mean nucleotide diversity within haplogroup F is 1.048. Haplotypes CP11, CM1, CP6, CP2 and CP9 are present in haplogroup G. Haplogroup H is represented by haplotypes CC1, CC4, CC9, CM5 and CM7. Haplotypes CC3 and PH1 show higher distances than other haplotypes and they are separated in the neighbour-joining tree (Figure 2).

The nucleotide sequence diversity among haplogroups ranges from 1.81% (D–E) to 4.71% (C–G), and Kimura two-parameter distances among haplotypes range from 0.0185 to 0.0494 (Table 2). The AMOVA analysis showed substantial subdivision between breeds (F_{ST} = 0.2004 ± 0.042 , P < 0.001), but with a large fraction of variation found within the populations. Partitioning levels of genetic diversity within and among the populations/groups revealed that 73.79% of the total genetic variance existed within the populations, and 25.70% between the Posavina horse population and the Croatian Coldblood/Murinsulaner horse population group (Table 3). Linearized Φ_{ST} values were significant for PH-CC pairs (0.327; P < 0.05) and PH-MH pairs (0.458; P < 0.05). In the Posavina horse population, the dominant haplogroups are D and F, followed by haplogroup G (0.70). The frequencies of the six hap-

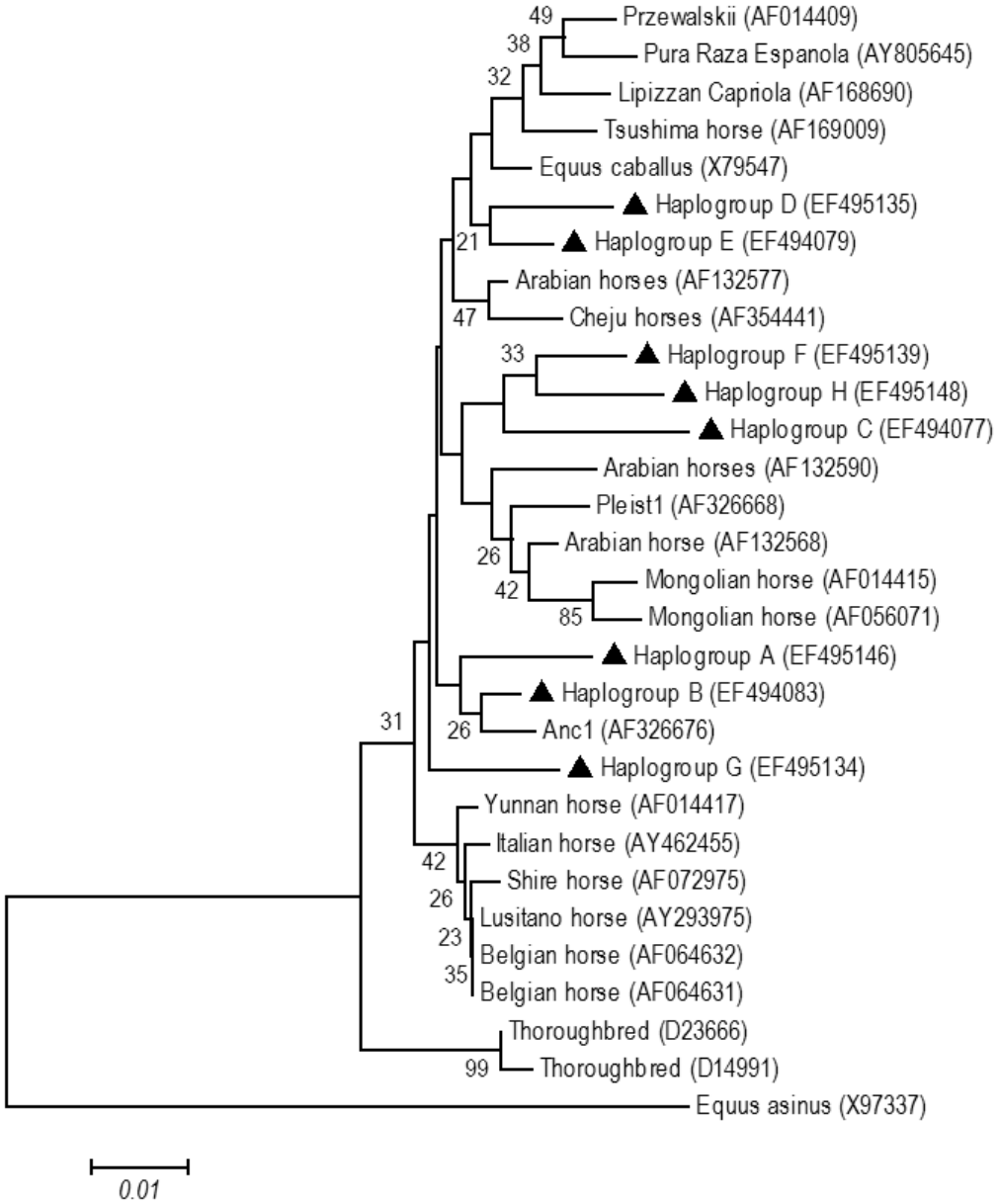

Figure 3. The neighbour-joining tree showing the relationship among twenty-five partial mtDNA D-loop sequences from members of the genus Equus including haplotypes of the Croatian autochthonous horse breeds with their sequence accession numbers (▲)

logroups A, B, C, E, G and H were 0.50, 0.80, 0.38, 0.50, 0.20 and 0.60, respectively, in the Croatian Coldblood population. In the Murinsulaner horse population, the frequencies of the six haplogroups (A, B, C, E, G, and H) ranged from 0.10 to 0.62.

Sequence data from this study were compared with twenty-two published sequences of the 324-bp fragment of the mtDNA D-loop region (nt 15 498 to 15 821) available in the GenBank database in order to determine inter- and intra-species relationships within the genus *Equus* (Figure 3). Sixty-six polymorphic sites were found and three of them were the result of insertions or deletions.

DISCUSSION

The numbers of identified haplotypes indicate a high genetic variability of the maternal component. The fact that for thousands of years the breeding

Table 3. Hierarchical analysis of molecular variance (AMOVA)

Variance component	Estimate	Variance components	Percentage of variation	Φ-statistics
Among groups	σ_a^2	*1.3113*	25.70	Φ_{CT}: 0.257
Among populations/groups	σ_b^2	*0.0260*	0.51	Φ_{SC}: 0.007
Within populations	σ_c^2	*3.7657*	73.79	Φ_{ST}: 0.262

*the Croatian Coldblood and Murinsulaner horse represent one group, while Posavina horse represents the second group

area of the analysed autochthonous horse breeds has been the crossroads of migration (conquest) routes, as evidenced by traces of horse equipment of Illyrians, Celts, Romans, Slavs, Mongols, Avars, Turks and many other peoples, was undoubtedly a major contribution factor. Through the past millennia, migrations and conquests have continually introduced the maternal component into the breeding area. A stronger selection pressure through the paternal component (stallion) has been noted only over the last two centuries, leaving no impact on the mtDNA. Two (D, F) out of eight haplogroups were specific to Posavina horse (Figure 2). The specific nature of the maternal component of Posavina horse is expected to some extent because of a more careful introduction of foreign genotypes (primarily a stallion). Posavina horse has a smaller frame, more refined head and good bone conformation; it is more resilient and less demanding in terms of forage, which are the reasons why breeders tried to preserve the maternal component of this breed. One haplogroup (G) comprises haplotypes from all three analysed horse breeds. The haplotypes within the majority of observed haplogroups (A, B, C, E, and H) belong to genotypes found in the Croatian Coldblood and the Murinsulaner horse, which indicates the relatedness of the maternal component. Earlier findings concerning the genesis of Croatian Coldblood and Murinsulaner horse justify the observed results of the mtDNA typing. Murinsulaner horse is shaped as a big-framed and powerful breed and as such, in the first half of the 20th century, it was integrated in a significant proportion maternally into breeding of the Croatian Coldblood. Our results indicate that there is a close relationship between the Croatian Coldblood and the Murinsulaner horse. The mean distance between the Posavina horse and the Croatian Coldblood on the one hand, and between the Croatian Coldblood and the Murinsulaner horse on the other, was almost equal (0.0291 and 0.0272) while the biggest mean distance was found between the Posavina and Murinsulaner horses (0.0342). The hierarchical analysis of molecular variance did support the division between the Posavina horse and the Croatian Coldblood/Murinsulaner horse populations (Φ_{CT} = 0.257; Φ_{SC} = 0.007; Table 3), which can be explained by selection strategy at the beginning of the 20th century. Historic and economic developments have significantly reflected on gene flows within and between the analysed horse breeds. By World War I, the breeding area of Murinsulaner horse was under the Hungarian administration, which tried to form a strong working horse, while the aim of Posavina horse breeding was to make a small horse, with moderate upgrading with Arab stallions (Romić, 1975). In shaping the Croatian Coldblood breed, English Thoroughbred and Belgian Coldblood stallions are used, presuming the utilisation of Murinsulaner mares as maternal components which are certified with mtDNA typing results. In the middle of the 20th century the rearing of Murinsulaner horse is neglected, with a simultaneous stronger influence of Belgian Coldblood on the Croatian Coldblood population. From the seventies of the 20th century, the use of horses of these breeds has been negligible, with remark that the breeding was interrupted by war developments in the early nineties. Such a relationship of genetic distances points to different migration of the maternal component and introduction of the paternal genetic component into the local coldblood horse breeds. The results from research on the diversity of mtDNA are supported by results of nuclear DNA typing from Croatian Coldblood and Posavina horse. On the basis of microsatellite diversity of eleven draught horse populations Druml et al. (2007) observed that Croatian Coldblood had the highest gene diversity and allele richness (0.706; 6.68), and Posavina horse had a moderate level of gene diversity and allele richness (0.684; 6.46). Concerning the moderate level of microsat-

ellite diversity (F_{ST} = 0.035) and genetic purity of Croatian Coldblood and Posavina horse, Druml et al. (2007) suggested as a possibility a separate approach to conservation management and allowing substantial planned intermixing (long-term differentiation). Significant high subdivision between breeds (F_{ST}) supports the conservation strategy of a separate approach to the populations of Posavina horse and Croatian Coldblood. A high level of F_{ST} value (0.1815) was noticed in the population of South American and Spanish horse breeds (Mirol et al., 2002). In terms of conservation, the observed phylogenetic relationships should help to preserve the more original genetic groups and protect their genetic variability.

At a species level, horses possess relatively high haplotype diversity in the D-loop region of mtDNA (Kim et al., 1999; Vilà et al., 2001; Hill et al., 2002; Royo et al., 2005). The mean number of pairwise differences observed among all haplotypes was estimated at 8.81 or 2.72%. If the average rate of equid mtDNA divergence is assumed between 4.1 and 8.1% per million years (Ma; Vilŕ et al., 2001), then the average coalescence for the modern horse mtDNA sequences is estimated between 0.33 Ma (lower limit) and 0.66 Ma (upper limit) years before present (YBP). Vilŕ et al. (2001) reported that modern horse lineages coalesced at about 0.32 to 0.63 Ma, suggesting that domestic horses originated from multiple matrilines. Hill et al. (2002) estimated that the average coalescence for modern horse mtDNA sequences was between 280 000 and 560 000 YBP. A wide variety of mitochondrial haplotypes of horse breeds clustered into seven different clades with low bootstrap support, indicating a high number of ancestral matrilines of ancient origin (Vilà et al., 2001). Jansen et al. (2002) reported extensive genetic diversity summarised in 93 different mtDNA types, grouped into 17 distinct phylogenetic clusters, and found that only a few of the phylogenetic clusters corresponded to groups of breeds or geographic areas. The examination of the horse population diversity at the mtDNA level has revealed a consistent absence of geographical structure (Hill et al., 2002).

A comparison of the haplogroups observed in the analysed horse breeds with the horse mtDNA sequences available in GenBank (NCBI) revealed their significant closeness to the Belgian horse sequences (1.30–2.99%), and a greater diversity (3.49–4.94%) with respect to Thoroughbred horse (Table 2). A moderate level of nucleotide diversity was observed between the haplogroups with respect to the Arabian horse sequences (1.99–3.29%), which was expected to some extent (the breeding area of Posavina horse has been for centuries in contact with the Arabian horse population used in warfare by the Turkish Empire). The haplogroup diversity of the autochthonous horse breed in comparison with the Asian Mongolian horses is greater (2.40–4.04%). Based on these sequence comparisons, an NJ tree was constructed (Figure 3).

Our results show a relatively high genotypic diversity within and between the three analysed horse populations. The genetic diversity information based on mtDNA typing is important baseline data for the future breed conservation strategy, especially for the critically endangered Murinsulaner horse breed. The analysis of the mtDNA D-loop region complements the conclusions, pointing to the existence of multiple maternal lines in the structure of analysed autochthonous breeds. The conclusions based on the examination of genetic diversity support the opinion, based on the phenotypic traits, suggesting that Posavina horse should be bred separately from the Croatian Coldblood and Murinsulaner horse. However, given the endangered situation of the Murinsulaner horse and the earlier maternal participation of this breed in the development of the Croatian Coldblood, which could be a good donor of maternal candidate in order to increase genetic variability, decrease inbreeding, and stabilise the population size of the Murinsulaner horse. Furthermore, molecular genetic data could also be used in the selection of suitable maternal candidates from the Croatian Coldblood in order to preserve as much as possible of the recipient breed genome (Murinsulaner horse). Moderate introduction of the Croatian Coldblood genome into the critically endangered population of Murinsulaner horse would represent a good opportunity to preserve and obtain this breed.

REFERENCES

Bjørnstad G., Røed K.H. (2002): Evaluation of factors affecting individual assignment precision using microsatellite data from horse breeds and simulated breed crosses. Animal Genetics, 33, 264–270.

Bodó I. (1992): The minimum number of preserved populations. In: The management of global animal genetic resources. Food and Agriculture Organisation of the United Nations, Rome, Italy, 91–105.

Cozzi M.C., Strillacci M.G., Valiati P., Bighignoli B., Cancedda M., Zanotti M. (2004): Mitochondrial D-loop sequence variation among Italian horse breeds. Genetics Selection Evolution, 36, 663–672.

Dovc P., Kavar T., Sölkner H., Achmann R. (2006): Development of the Lipizzan Horse Breed. Reproduction in Domestic Animals, 41, 280–285.

Druml T., Curik I., Baumung R., Aberle K., Distl O., Sölkner J. (2007): Individual-based assessment of population structure and admixture in Austrian, Croatian and German draught horses. Heredity, 98, 114–122.

Excoffier L., Laval G., Schneider S. (2005): Arlequin ver. 3.0: An integrated software package for population genetics data analysis. Evolutionary Bioinformatics Online, 1, 47–50.

FAO (2007): The State of the World's Animal Genetic Resources for Food and Agriculture. Commission on Genetic Resources for Food and Agriculture. FAO, Rome, Italy.

Felsenstein J. (1993): PHILIP (Phylogeny Inference Package), Version 3.5c. Department of Genetics, University of Washington, Seattle, USA.

HSC (2008): Croatian Livestock Center, Yearbook, Croatia.

Hill E.W., Bradley D.G., Al-Barody M., Ertugul O., Splan R.K., Zakharov I., Cunningham E.P. (2002): History and integrity of thoroughbred dam lines revealed in equine mtDNA variation. Animal Genetics, 33, 287–294.

Ishida N., Hasegawa T., Takeda K., Sakagami M., Onishi A., Inumaru S., Komatsu M., Mukoyama H. (1994): Polymorphic sequence in the D-loop region of equine mitochondrial DNA. Animal Genetics, 25, 215–221.

Ishida N., Oyunsuren T., Mashima S., Mukoyama H., Saitou N. (1995): Mitochondrial DNA Sequences of Various Species of the Genus Equus with Special Reference to the Phylogenetic Relationship Between Przewalskii's Wild Horse and Domestic Horse. Journal of Molecular Evolution, 41, 180–188.

Ivanković A., Caput P. (2004a): Exterior features of Croatian autochthonous horse breeds. Stočarstvo, 58, 15–36.

Ivanković A., Caput P. (2004b): Genetic polymorphisms blood proteins of the autochthonous horse breeds in Croatia. Stočarstvo, 58, 403–412

Jansen T., Forster P., Levine M.A., Oelke H., Hurles M., Renfrew C., Weber J., Olek K. (2002): Mitochondrial DNA and the origins of the domestic horse. Proceedings of the National Academy of Sciences of USA, 99, 10905–10910.

Kavar T., Habe F., Brem G., Dovč P. (1999): Mitochondrial D-loop sequence variation among the 16 maternal lines of the Lipizzan horse breed. Animal Genetics, 30, 423–430.

Kim K.I., Yang Y.H., Lee S.S., Park C., Ma R., Bouzat J.L., Lewin H.A. (1999): Phylogenetic relationships of Cheju horses to other horses breeds as determined by mtDNA D-loop sequence polymorphism. Animal Genetics, 30, 102–108.

Kimura M. (1980): A simple method for estimating evolutionary rate of base substitution through comparative studies of nucleotide sequences. Journal of Molecular Evolution, 16, 111–120.

Kumar S., Tamura K., Nei M. (2004): MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Briefings in Bioinformatics, 5, 150–163.

Lopes M.S., Mendonça D., Cymbron T., Valera M., da-Costa-Ferreira J., daCâmara Machado A. (2005): The Lusitano horse maternal lineage based on mitochondrial D-loop sequence variation. Animal Genetics, 36, 196–202.

McGahern A.M., Edwards C.J., Bower M.A., Heffernan A., Park S.D.E., Brophy P.O., Bradley D.G., MacHugh D.E., Hill E.W. (2006): Mitochondrial DNA sequence diversity in extant Irish horse populations and in ancient horses. Animal Genetics, 37, 498–502.

MacHugh D.E., Loftus R.T., Cunningham P., Bradley D.G. (1998): Genetic structure of seven European cattle breeds assessed using 20 microsatellite markers. Anmal Genetics, 29, 333–340.

Mirol P.M., Peral García P., Vega-Pla J.L., Dulout F.N. (2002): Phylogenetic relationships of Argentinean Creole horses and other South American and Spanish breeds inferred from mitochondrial DNA sequences. Animal Genetics, 33, 356–363.

Romić S. (1975): Capacity of growth and production characteristics of the Croatian coldblood horses. Praxis Veterinaria, 2, 87–99.

Royo L.J., Álvarez I., Beja-Pereira A., Molina A., Fernández I., Jordana J., Gómez E., Gutiérrez J.P., Goyache F. (2005): The Origins of Iberian horses assessed via mitochondrial DNA. Journal of Heredity, 96, 663–669.

Saitou N., Nei M. (1987): The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution, 4, 406–425.

Slatkin M. (1995): A measure of population subdivision based on microsatellite allele frequencies. Genetics, 139, 457–462.

Thompson J.D., Higgins D.G., Gibson T.J. (1994): Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research, 22, 4673–4680.

Toro M.A., Barragan C., Ovilo C. (2003): Estimation of genetic variability of the founder population in a con-

servation scheme using microsatellites. Animal Genetics, 34, 226–228.

White P.S., Densmore L.D. (1992): Mitochondrial DNA isolation: Molecular genetic analysis of populations. A practical approach. Oxford University Press. Oxford, UK.

Vilà C., Leonard J.A., Götherström A., Marklund S., Sandberg K., Lidén K., Wayne R.K., Ellegren H. (2001): Widespread origins of domestic horse lineages. Science, 291, 474–477.

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