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Maternal origin of Medjimurje horse

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ABSTRACT

Maternal origin of highly endangered population of Medjimurje horse was analysed with intention to establish maternal lineages, to characterise its position among other horse breeds, to compare Croatian and Hungarian subpopulations and to compare Medjimurje horse with related breeds. To achieve those goals we sequenced fragment of mitochondrial control region (mtDNA D-loop) of 58 Medjimurje horses (27 from Croatian and 24 from Hungarian sub-population) and 7 Norikers from Austria (Carinthia region). We also analysed additional 95 sequences taken from the GeneBank database, representing large number of breeds and representative haplotypes, with particular stress on Arab Horse (17) and Renish German Draft Horse (24). The analyses have shown that out of 51 MH sequences there were 19 different maternal lineages (haplotypes). Out of 153 analysed sequences, nine haplotypes were unique for MH (six for MH-CRO and three for MH-HUN). It is interesting that among those unique haplotypes one was characteristic for nine MH-CRO horses and one for five MH-HUN horses. The phylogenetic analysis has illustrated that, although, maternal lineages of MH horse are present over distinct phylogenetic clusters there is some tendency of grouping around specific haplotypes and the similar pattern was noted between haplotypes of two MH subpopulations. Here, we have not found strong reasons why not to make common breeding program for MH. However, we should be aware that mtDNA analysis reflects remote origin and before proceeding with practical conservation decisions it would be necessary to analyse those two subpopulations based on microsatellite or SNP polymorphism.
(Keywords: Medjimurje horse, haplotypes, phylogenetic analysis)

INTRODUCTION

Medjimurje horse is autochthonous breed named after northern region of Croatia that is separated by two rivers, Mura and Drava. The history of this draft horse is linked to the Austro-Hungarian Empire where it was spread over the region that today is Austria, Croatia, Hungary and Slovenia. So, the breed is also recognized under names Muraközi ló (in hungarian language), Murinsulaner (in german language) or Medjimurski konj (in croatian language). The origin of the breed is linked to the Noric horse, Arab horse and cold blooded horses from Belgium, Croatia and Germany. Today, there is only 38 horses registered as purebred and the breed is critically endangered in Croatia, while found in traces in Hungary, Slovenia, and Poland. As a part of revitalization plan of Medjimurje horse, we have performed mtDNA analysis. The objective of this study was; a) to

establish maternal lines for future pedigree controle and mating policy, b) to analyse maternal position of Medjimurje horse among world horses, c) to compare maternal origin of Medjimurje horse between Croatian (MH-CRO) and Hungarian (MH-HUN) sub-population, and d) to compare maternal origin of Medjimurje horse (MH) with Noriker (NO), Rhenish German Draught Horse (RD) and Arab horse (AR) as breeds that have influenced its formation.

MATERIALS AND METHODS

Blood samples of 51 Medjimurje horses (MH) were collected from Medjimurje region in Croatia (27) and from breeding nucleus situated in Őrség National Park in Hungary (24) while blood samples from seven Norikers were collected in Carinthia region in Austria.
All DNA was isolated using Sigma blood kit (Sigma-Aldrich, Germany) according to manufacturer's recommendations. Sequencing was done following the procedure described in *Aberle et al.* (2007). Thus, a 1260-bp fragment of the horse D-loop mtDNA, including parts of the tRNA-Thr, tRNA-Pro and tRNAPhe regions between nucleotides 15402 and 22, was amplified using the forward (5'-AACGTTTCCCAAGGACT-3') and reverse (5'-GCATTTTCAGTGCCTTGCTT-3') (Invitrogen) primers. The polymerase chain reaction (PCR) protocol was performed in a 20 µl reaction mix containing approximately 50 ng of total DNA, 0.2 µM of each forward and reverse primer and Master mix (Qiagen). The PCR was carried out in a iCycler (Biorad, USA) thermocycler and consisted of: an initial denaturation step at 95 ^{o}C for 15 min followed by 34 cycles at 94 ^{o}C, for 45 s, annealing at 62 ^{o}C for 45 s, and elongation at 72 ^{o}C for 80 s with a final elongation step of 10 min at 72 ^{o}C. PCR products were purified using ExoSAPIT (USB, Cleveland, OH) following the manufacturer's recommendations. DNA sequencing was preformed from the PCR product on an ABI 3130 DNA automated sequencer (Applied Biosystems, USA) using the ABI Prism Big Dye Terminator 3.1 Sequencing Kit (Applied Biosystems, USA). Obtained mtDNA sequences were aligned using the program CLUSTAL as implemented in the software MEGA 4.1 Beta 3 (*Tamura et al.,* 2007; available at http://www.megasoftware.net/mega41.html). Aligned sequences were truncated to the length of 276 bp (nucleotide positions from 15469 to 15745 according to *Xu and Arnason,* 1994; accession number X79547) to enable comparison with systematically known haplotypes and well known horse breeds (*Jansen et al.,* 2002).

All performed analyses were, further, based on the 276 bp truncated fragment. DnaSP v5.10 (*Librado and Rozas,* 2009; available at http://www.ub.edu/dnasp/) was used to estimate measures of DNA sequence variation within and between populations. The construction of the neighbour-joining tree was done using Kimura two-parameter model with complete deletion option (*Kimura,* 1980) by means of a MEGA 4.1 Beta 3 software. The tree was based on 58 target sequences (MH and NO) and 95 sequences additional 95 sequences taken from the GeneBank database, representing large number of breeds and representative haplotypes, with particular stress on Arab Horse (17) and Renish German Draft Horse (24).

RESULTS AND DISCUSSION

In the 276-bp mtDNA D-loop fragment of 51 analysed MH sequences there were 26 polymorphic sites (9.4% for the total DNA sequence analysed), representing a total of 19 different haplotypes i.e. maternal lineages from the horse breeding approach , among which nine and seven were present only within MH-CRO and MH-HUN sub-

populations, respectively, while three haplotypes were present in, both, sub-populations (*Table 1*). Out of 153 analysed sequences, nine haplotypes were unique for MH (six for MH-CRO and three for MH-HUN). It is interesting that among those unique haplotypes one was characteristic for nine MH-CRO horses and one for five MH-HUN horses, see *Table 1*. The presence of a large number of horses within specific haplotypes is, most likely, a consequence of the high degree of relatedness caused by the small population size. From the NOk population, only three haplotypes where unique out of 153 sequences while each of seven analysed horses had different haplotype which is a consequence of the genetic population broadness of NO (*Table 1*). MH horses have shared haplotypes with RD (11), AR (5), NO (3) and Lipizzan (3) horses as well as with Islandic (1) and Shetland (1) ponies and Kazah horse (1).

Table 1

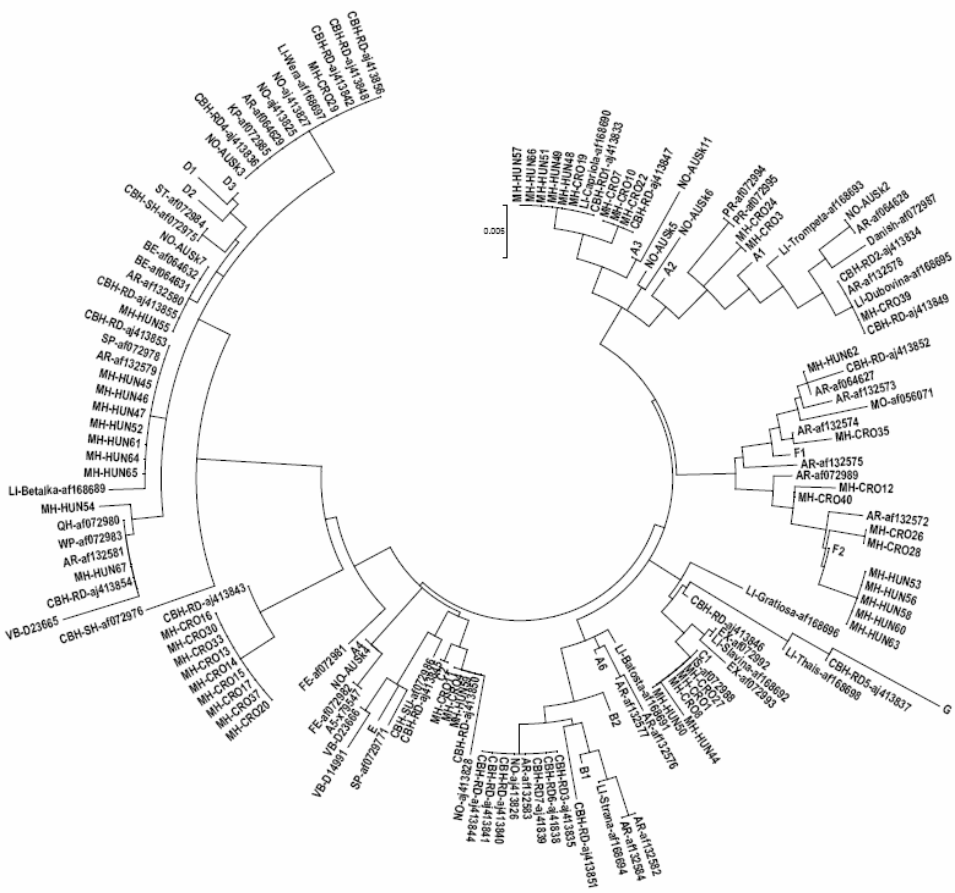
Variable nucleotides in a fragment of DNA D-loop of the Medjimurje horse from Croatia (MH-CRO, 27 horses) and Hungary (MH-HUN, 24 horses) and Noriker horse from Austrian region Carinthia (NOk, 7 horses) haplotypes compared with reference sequence (*Xu and Arnason,* 1994; accession number X79547, in bold)

15491	15495	15496	15497	15504	15527	15535	15541	15542	15543	15575	15587	15599	15603	15604	15605	15606	15637	15651	15652	15661	15668	15669	15674	15707	15713	15724	15730	15740	15742	15744	15745	MH-CRO	MH-HUN	NOk
C	**T**	**T**	**A**	**T**	**T**	**C**	**A**	**C**	**C**	**-**	**G**	**A**	**T**	**C**	**T**	**G**	**C**	**A**	**A**	**T**	**G**	**A**	**G**	**T**	**C**	**G**	**G**	**-**	**T**	**A**	**A**			
.	.	C	.	.	.	.	.	.	.	-	.	.	.	T	.	A	.	.	.	.	A	.	.	.	.	A	.	-	.	.	.	0	0	**1**
.	.	C	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	-	.	.	.	0	0	1
.	C	C	G	.	.	T	.	.	.	-	A	.	.	.	C	.	.	G	.	.	.	.	.	.	.	A	.	-	.	.	.	1	0	1
.	.	C	.	.	.	.	.	.	T	-	A	G	.	T	.	.	T	.	G	.	A	.	.	C	.	.	.	-	.	.	.	0	0	1
T	.	C	.	A	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	A	.	-	.	.	.	0	0	**1**
.	C	C	G	.	.	T	.	.	.	-	.	.	.	T	C	.	.	G	.	.	.	.	.	.	T	A	.	-	.	.	.	0	1	1
.	.	C	.	.	.	.	.	.	.	-	.	.	.	.	.	A	.	.	.	.	A	.	.	C	T	A	.	-	.	.	.	0	0	**1**
.	.	C	.	.	.	.	.	.	.	-	.	.	.	.	.	.	.	.	G	.	A	.	.	.	.	A	.	-	.	.	.	3	0	0
.	.	C	.	.	.	.	.	.	.	-	A	.	C	T	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.	2	1	0
.	.	C	.	.	.	.	.	.	.	-	.	.	.	T	.	A	.	.	.	.	.	.	.	C	.	A	.	-	.	.	.	**1**	0	0
.	.	C	.	.	C	.	G	.	.	-	A	.	.	T	.	.	.	G	.	.	.	.	.	.	.	A	.	-	.	.	.	**9**	0	0
.	.	C	.	.	.	.	.	.	T	-	A	G	.	T	.	.	.	.	G	.	A	.	.	.	.	A	.	-	C	.	.	**2**	0	0
.	.	C	.	.	.	.	.	.	.	-	.	.	.	T	.	A	.	.	.	C	.	.	.	C	.	A	.	-	.	.	.	**2**	0	0
.	.	C	.	.	.	.	.	.	.	-	.	.	.	T	.	.	.	.	.	C	.	.	.	.	.	A	A	-	.	.	G	3	1	0
.	.	C	.	.	.	.	.	.	.	-	A	.	.	.	.	.	.	.	G	.	A	.	.	.	.	A	.	-	.	.	.	1	5	0
.	.	C	.	.	.	.	.	.	.	-	.	G	.	T	.	.	.	.	.	.	.	G	.	C	.	A	.	-	.	.	.	**1**	0	0
.	.	C	.	.	.	.	.	.	T	-	.	G	.	T	.	.	T	.	G	.	A	.	.	C	T	A	.	-	.	.	.	1	0	0
.	.	C	.	.	.	.	.	.	.	-	.	.	.	T	.	A	.	.	.	.	.	.	.	C	.	A	.	-	.	.	.	**1**	0	0
.	.	C	.	.	.	.	T	.	.	-	.	.	.	T	.	.	.	.	.	C	.	.	.	.	.	A	.	-	.	.	.	0	**1**	0
.	C	C	G	.	.	T	.	.	.	-	A	.	.	T	C	.	.	G	.	.	.	.	.	.	.	A	.	-	.	.	.	0	7	0
.	.	C	.	.	.	.	.	.	.	-	.	.	.	T	.	A	.	.	.	.	.	.	A	C	T	A	.	-	.	.	.	0	**5**	0
.	C	C	G	.	.	T	.	.	.	-	A	.	.	.	C	A	.	G	.	.	.	.	.	.	.	.	.	-	.	.	.	0	**1**	0
.	.	C	.	.	.	.	.	.	.	-	.	G	.	T	.	A	.	.	.	.	.	G	.	C	.	A	.	-	.	.	.	0	1	0
.	C	C	G	.	.	T	.	.	.	-	A	.	.	T	C	A	.	G	.	.	.	.	.	.	.	A	A	-	.	.	G	0	1	0

Haplotype frequencies for MH and NOk are given in the last three columns. Bold underlined haplotypes denote breed unique haplotypes out of 153 analysed sequences.

Figure 1

Neighbour-joining tree derived using the Kimura 2-parameter method with complete deletion option (*Kimura,* 1980) for 153 horse sequences (nucleotide positions from 15469 to 15745 according to *Xu and Arnason,* 1994; accession number X79547) representing phylogenetic position of Medjimurje horse in relation to worldwide horse breeds (notation taken from *Jansen et al.*, 2002). Prefix CBH stands for Coldblooded horses with exception made for the Medjimurje and Noriker horse



The phylogenetic analysis has illustrated that maternal lineages of MH horse are present over distinct phylogenetic clusters i.e. the analysis have failed to show monophyletic groups. For example, MH sequences were appearing in clusters A2, A3, D, F, E and G while not in B and, with exception of one large MH-HUN "family" and one MH-CRO horse, not in C clusters (for more detailed description of mtDNA phylogenetic clusters see *Jansen et al.,* 2002). This effect was previously noted in mtDNA studies of a large number of horses (*Jansen et al.,* 2002; *Aberle et al.,* 2007, *Royo et al.,* 2005; *Cozzi et al.,* 2004; *Iwanczyk et al.,* 2006). The similar pattern of similarity and distinction was noted between haplotypes of two MH subpopulations. Here, we have not found strong reasons

why not to make common breeding program for MH-CRO and MH-HUN sub-populations, particularly, as the effective population size of both population is extremely small. However, we should be aware that mtDNA analysis reflects remote origin and before proceeding with practical conservation decisions it would be necessary to analyse those two subpopulations based on microsatellite or SNP polymorphism (*Achmann et al.,* 2004; *Druml et al.,* 2006). In addition, the comparison of MH with other horses present in the region as an Posavina horse, Croatian Colblooded horse, Traditional Arab Horse and Bosnian Mountain horse on both mtDNA and nuclear markers would be required and this is our future goal. Furthermore, the sequence analysed here was restricted to 276-bp mtDNA D-loop fragment and we are currently analysing if larger mtDNA fragment does provide differentiation between haplotypes "occupied" with larger number of horses.

CONCLUSIONS

The number of distinctive haplotypes does offer possibilities for establishment of maternal lineages based on mtDNA analysis which might be useful in preserving genetic diversity through optimisation of specific maternal contribution. The phylogenetic analysis has illustrated that, although, maternal lineages of MH horse are present over distinct phylogenetic clusters there is some tendency of grouping around specific haplotypes and the similar pattern, was noted between haplotypes of two MH subpopulations. Overall, the results of this analysis indicated the presence of specific genetic characteristics (mtDNA haplotypes) that urge needs for improving breeding (preservation) of Medjimurje horse.

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