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Genetic diversity of the Hungarian draft horse assessed by mitochondrial DNA

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SUMMARY

Hungarian draft is a horse breed with a recent mixed ancestry. It was developed in the 1920s by crossing local mares with draught horses imported from France and Belgium. To genetically characterize the breed and to set up the basis for a conservation programme, we have employed a molecular marker: a 256-bp D-loop mitochondrial DNA fragment. We analyzed 124 horses representing Hungarian draft horses to assess the maternal phylogeography of the breed. Sequence analysis of a 256-bp segment revealed a total of 34 haplotypes with thirty-four polymorphic sites. High haplotype and nucleotide diversity values (Hd=0.953±0.001; π=0.024±0.001) were detected. The average number of pairwise differences were k=5.998. This breed counts 800 mares today, and only survive due to breeding programmes, this way each haplotype frequency depends on the extent to which mares are involved into the breeding. The reduced number of surviving maternal lineages emphasizes the importance of establishing a conservation plan for this endangered breed. Due to the revealed 34 polymorphic sites we could presuppose twelve maternal linages, which could be a first step for making a breeding programme.

Keywords: *Hungarian draft, draught horse, genetic diversity, mitochondrial DNA, maternal lineages, control region*

ÖSSZEFOGLALÁS

A magyar hidegvérű alapvetően vegyes származású lófajta. Hazai kancáink, belga valamint francia hidegvérű ménekkel való átkeresztezése során az 1920-as években kialakult fajta. Genetikai hátterének feltérképezéséhez, továbbá kiindulási alapként egy konzervációs program létrehozásához egy 256 bázispárból álló molekuláris markert alkalmaztunk a mitokondriális DNS D-loop régióján belül. A kancacsaládok előzetes meghatározásához 124 lovat vizsgáltunk, reprezentálva a magyar állományt. A 256 bázispár hosszú szakasz szekvencia analízise során 34 haplotípust különítettünk el, mely harmincnégy polimorfikus helyet határozott meg. Magas haplotípus, valamint nuklaotid diverzitást (Hd=0,953±0,001; π=0,024±0,001) tapasztaltunk. A páronkénti átlagos eltérések k=5,998 voltak. A fajta ma hozzávetőleg 800 nőivarú egyedet számlál és döntően a tenyésztési programoknak köszönheti fennmaradását. Ennek okán a haplotípusok gyakorisága attól függ, hogy a különböző kancák milyen súlyban szerepelnek a tenyésztésben. A megmaradt kancacsaládok számának csökkenése okán előtérbe kerül génmegőrzés fontossága. A vizsgálataink során kapott 34 polimorfikus hely analízisét követően tizenkét lehetséges kancacsaládot különítettünk el, mely első lépésként szolgálhat egy tenyésztési program megtervezésében.

Kulcsszavak: *Magyar hidegvérű ló, hidegvérű, genetikai diverzitás, mitokondriális DNS, kancacsalád, kontroll régió*

INTRODUCTION

Understanding the genetic diversity of equids and classifying their populations is essential for an appropriate conservation plan to be developed (Oakenfull et al., 2000). The cold-blooded stud holding heavy draft horse breeding in Hungary began in 1897. The origin of this horse breed dates back to horse Nora and Pinzgau. The foundation stock of this breed was initially the native Hungarian mares which were made to breed with other various breeds like the Noriker, Percheron, Ardennes and also with the native Hungarian stallions. Two distinct types emerged: the Pinkafő or Pinkafeld which is a large, massive type and the Muraközi which was smaller and more agile. During World War II. the breed's numbers decreased seriously, then the Ardennes breed/blood was involved in the breeding. The Hungarian Draft Horse Breeders National Association records approximately 800 mares today. Maternal side of the currents stock's certain individuals contains unknowns in 3rd–4th ancient lines, since the brand-marking and pedigree registration was obligatory only from 1993. Since the regime transition the Hungarian draft horse population passed almost entirely into private ownership and the purchase of imported cold-blooded animals became easy. The unreasonable import adversely affected the composition of native cold-blooded horses' population. For these reasons it is necessary to know the genetic background of the breed and its relation to other European populations. The organized breeding began only in 1913. The existing population was crossed with Belgian draught horses, thus improving the defects in the appearance of the breed. The Herd-book registration started in 1922, when purposefully selected Belgian import stallions are also used for breeding. After the Second World War French and Belgian Ardennes stallions were purposefully used for converter crosses, in this way till 1953 the Hungarian draft became a state-recognized breed (Bodó et al., 2000). From the 1970s the breeding of draft horses is increased, and since the need to hauling of heavy loads decreased dramatically,the meat production came into focus during the 1980s. That was the reason why most of the stock were crossed with the Percheron breed. Whenever the population of a breed decreased to a few individuals, and the population is going to be increased, the preservation of the remaining genetic diversity should have a priority (Mihók and Ernst, 2015). Since we are lacking of written documentation of previous breeding stock of the Hungarian draft horse, it is important to investigate the genetic diversity of the existing original mare groups, in order to be able to use the original maternal founders for breeding (Pongrácz, 2005).

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Different mitochondrial DNA (mtDNA)-based studies have been conducted previously on other native horse breeds such as Gidran and Hucul (Mihók, 2011). There are many equine mtDNA-based genetic relatedness conditions mapping publication in literature (Oakenfull and Ryder, 1998; Kavar et al., 1999; Kim et al., 1999; Bowling et al., 2000; Oakenfull et al., 2000; Vilà et al., 2001; Iwańczyk et al., 2006; Pérez-Gutiérrez et al., 2008; Ivanković et al., 2009). The mtDNA D-loop has been widely used in evolutionary or phylogenetic studies because of its higher mutation rates compared with other regions of mtDNA or nuclear DNA. We have cloned the equine D-loop region by PCR and determined its nucleotide sequence, then clarified the characteristic features of the D-loop sequence for determining the different diversity values among the sampled population and separate haplotypes into presupposed groups by the calculated genetic distance values.

MATERIAL AND METHODS

Preparation of the samples

In order to protect the genetic resource of this native horse breed, the genetic diversity of mitochondrial DNA D-loop was investigated, which is known to be more variable than other parts of the mitochondrial genome. We had 124 samples representing the Hungarian draft horse population. For the analyses hair samples from the tail or from mane with their follicles were used. We examined them in the Laboratory of Animal Genetics at the University of Debrecen. Genomic DNA was isolated from the stored hair samples (FAO/IAEA, 2004). We added 100 µl Hair Buffer on the top of hair follicles. The mixture contained: 250 µl Tween 20, 5 ml from puffer of 10 mM which used for PCR reactions and do not contain $MgCl_2$, 5 ml $MgCl_2$ (25 mM). 1 µl proteinase K to each sample was added to the reaction mixture for facilitating. Subsequently, the samples for 60 min at 37 °C and 20 min at 80 °C were incubated in a water bath. In order to evaluate the success of the isolation, concentration measurements were performed using NanoDrop® ND-1000. We diluted sample's concentration falling between 10 and 100 mg l^{-1} for the preparation of the PCR reaction.

Optimization of the PCR reaction

The main moment of the optimization was to determine the PCR conditions and the necessary reaction mixture. Necessary ingredients for the starting amplification mixture were as follows: 5 µl isolated genomic DNA, 8,8 µl dNTP (25 mM)/Fermentas, 1 µl GoTaq Flexi Buffer Promega, 8.2 µl $MgCl_2$ (25 mM) Promega, 1 µl forward and 1 µl reverse primer (10 pmol μl^{-1}) Sigma, 5 µl dH_2O. At the same time, after every failed reaction only two-component was changed. The PCR reaction starting condition shown in *Table 1*.

The primers connection is changed according to the annealing temperature, which was previously determined by gradient PCR. The success of PCR products are checked with a 2% agarose gel electrophoresis prepared in advance. The gel composition was as follows: 2% Seakemagarose, 1x TAE (Tris-acid-EDTA) puffer, then the products stained with GelRed (Biotium, USA) DNA paint. We applied 3 µl from each sample at the agarose gel, then it run at 100 voltage till the marking paint has reached the bottom of the gel.

Table 1.

Conditions of PCR reactions

Initial denaturation	95 °C	10 min	
Denaturation	95 °C	30 sec	35 cycles
Annealing	62 °C	30 sec	
Extention	70 °C	30 sec	
Final extention	72 °C	10 min	
	10 °C	∞	

Analyzation

Samples were sent, and sequencing method were done by Macrogen Company (The Netherlands, Amsterdam). 256 bp long mtDNA D-loop sequences were analyzed between 15531–752 bp region The correct reading of nucleotides and the comparison of sequences were done with CodonCodeAlignerV.6.0.2. program, while the statistical analysis were performed with two version of Mega (Mega6 (Tamura et al., 2013) and Mega7.0 (Kumar et al., 2016), with DnaSP5.1. (Librado and Rozas, 2009) and Network 5.0. (Bandelt et al., 1999). We revealed 34 polymorphic sites representing thirty-four haplotypes. DnaSP5.1. software was used for calculate number of haplotypes, haplotype and nucleotide diversities.

RESULTS AND DISCUSSION

One hundred and twenty-four 256 bp long mtDNA D-loop sequences were analyzed and revealed 34 polymorphic sites representing thirty-four haplotypes (h=34). Among the 34 haplotypes there were 14 unique ones (containing a single individual), while the most common haplotype contained 12 animals. The average number of pairwise differences were k=5.998. High haplotype and nucleotide diversity values (Hd=0.953± 0.001; π=0.024±0.001) were detected, it was higher than in Iranian horses (0.838±0.056) reported by Moridi et al. (2012) and quite similar to the result of Pérez-Gutiérrez (2008) who reported 0.975 and to a Chinese native horse having 0.977 (Zhang et al., 2012).

After the determination of haplotypes, we calculated the pairwise genetic distance between them. The Mega software was used for calculating the P-distance and the Tamura-Nei distance among haplotypes where the resulting values ranged from 0.004 to 0.051. We got the same results for grouping the individuals into haplotypes with DnaSp and Network software as well. We separated the haplotypes into different groups by these values, in this way we have determined 12 presupposed maternal lineages (*Figure 1*).

The Network program applied Reduced Median-Joining algorithm and drawing a network from using the samples which contains haplotypes. Colors indicate the presumed mare groups determined by genetic distance between haplotypes. The size of laps is proportional to the number of individuals they contain. The lines indicate the distance between different haplotypes depending on number of mutations. In some cases the groups cannot separated easily.

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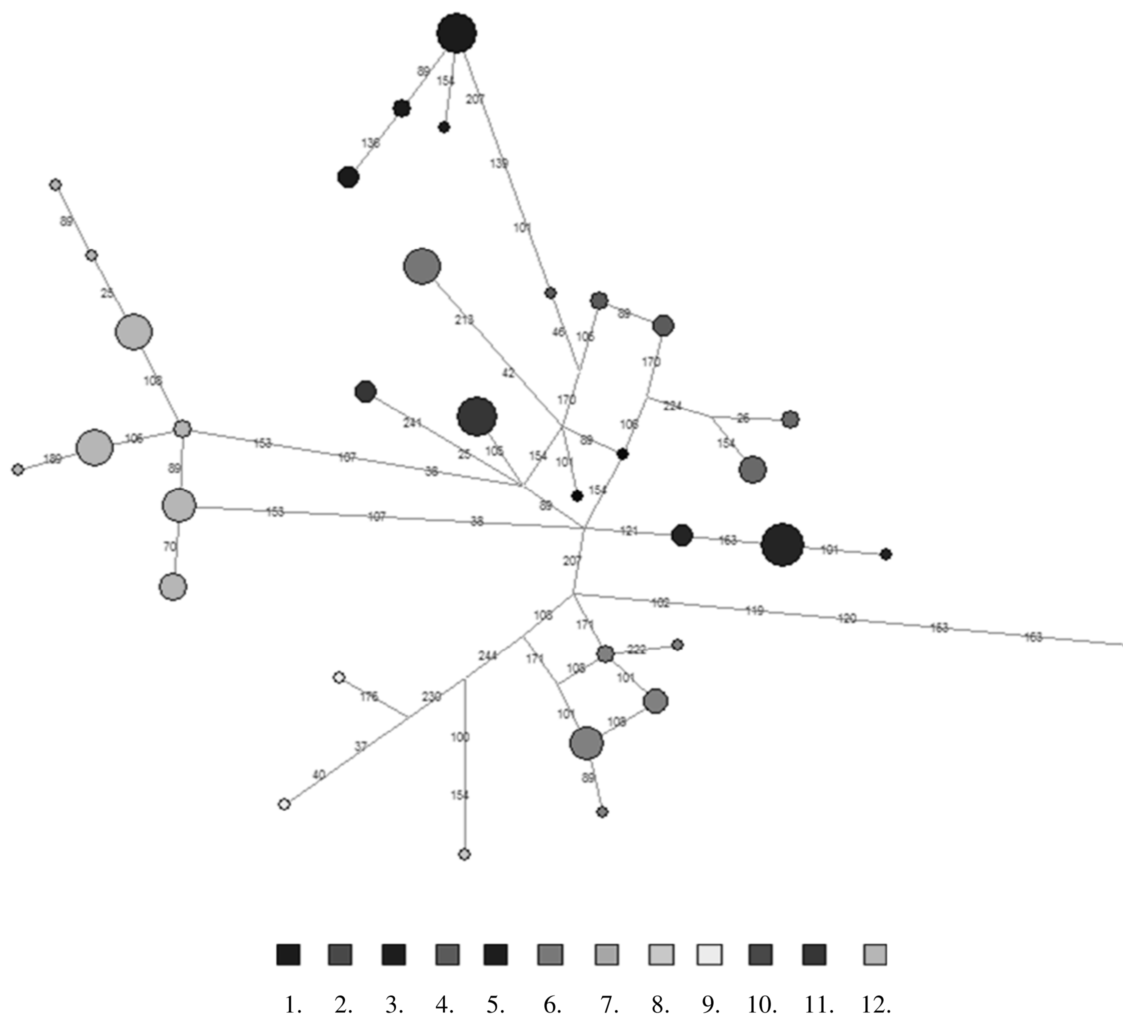
Figure 1: **Network figure of candidate mare families**



Note: squares indicates the color of presupposed mare groups which can be seen on the network figure.

We have to note that the 124 individual sampled in this study is only 16% of the whole Hungarian draft horse population. Due to the high variability we merged two haplotypes into one group if p≤0.009, and not more than two mutations can be found between them. Two of them has only a single haplotype, like 8 and 10, while the most diverse group was the 12th group which contains 8 haplotypes and 35 individuals.

The genetic distance estimation is most often used in the formula of Tamura-Nei (Kumar et al., 2015). We calculated pairwise genetic distance values between the mare families, so determined the Tajima-Nei distance (Tajima and Nei, 1984), and the p distance (Kim et al., 1999). In *Figure 2* the values on the diagonal shows the Tamura-Nei (Tamura et al., 2013) distances, the substitution rate based on equality. Values above the diagonal shows the absolute distances of the complete mitochondrial DNA D-loop region taking into account the nucleotide differences between them. To demonstrate that the 12 candidate mare families could be clearly separated, we also calculated p distance and Tamura-Nei distance between the presupposed mare groups. Estimates of evolutionary divergence over sequence pairs between groups were done with MEGA7. The number of base differences per site from averaging over all sequence pairs between groups are shown. The analysis involved 124 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 255 positions in the final dataset. We detected p=0.012 distance as minimum between the 3 and 11 presupposed maternal groups, and p=0.045 distance as maximum between 1 and 10 suspected mare groups. Overall, it should be noted that since the variation within the groups, in some cases greater than that between the groups, in this way the presupposed maternal lineages are considered hypothetical, it would be necessary to perform another analysis with other markers and with more animals for more precise results.

CONCLUSIONS

This breed counts 800 mares today, and only survive due to breeding programmes, this way each haplotype frequency depends on the extent to which mares are involved into the breeding.

The study involved a total population of mares about 16% of the total, among which there are certainly individuals that carry the marks of the original type, so that breed conservation organization can provide valuable data in this study.

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Figure 2: **Genetic distances between candidate mare families**

	1	2	3	4	5	6	7	8	9	10	11	12
1	-	0.022	0.021	0.030	0.035	0.036	0.027	0.032	0.040	0.045	0.031	0.043
2	0.021	-	0.012	0.018	0.022	0.015	0.028	0.026	0.035	0.039	0.019	0.028
3	0.021	0.012	-	0.012	0.015	0.016	0.019	0.020	0.028	0.033	0.013	0.023
4	0.030	0.018	0.012	-	0.024	0.024	0.030	0.028	0.037	0.041	0.017	0.029
5	0.034	0.021	0.015	0.023	-	0.018	0.021	0.028	0.028	0.025	0.016	0.026
6	0.035	0.015	0.016	0.024	0.017	-	0.024	0.028	0.031	0.035	0.019	0.026
7	0.026	0.027	0.018	0.029	0.021	0.024	-	0.022	0.022	0.030	0.023	0.031
8	0.031	0.025	0.020	0.027	0.027	0.027	0.021	-	0.016	0.037	0.029	0.036
9	0.039	0.033	0.027	0.035	0.027	0.030	0.021	0.016	-	0.037	0.029	0.036
10	0.042	0.037	0.031	0.039	0.025	0.034	0.029	0.035	0.035	-	0.033	0.034
11	0.030	0.019	0.013	0.017	0.016	0.019	0.022	0.028	0.028	0.032	-	0.022
12	0.041	0.027	0.023	0.028	0.025	0.025	0.030	0.035	0.035	0.033	0.021	-

* Belowdiagonal: absolute distances (p-distance), above diagonal Tamura-Nei distance.

Overall, we determined 12 presupposed maternal lineages from 124 individuals which reveals 34 haplotypes. Our ongoing research showed – as it was expected – high genetic diversity for the Hungarian draft horse. In our future work we intend to extend the study for more animals, and perform comparison study including other European cold blooded horses as well.

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