A peer-reviewed version of this preprint was published in PeerJ on 31 January 2018.

View the peer-reviewed version (peerj.com/articles/4198), which is the preferred citable publication unless you specifically need to cite this preprint.

Csizmár N, Mihók S, Jávor A, Kusza S. 2018. Genetic analysis of the Hungarian draft horse population using partial mitochondrial DNA D-loop sequencing. PeerJ 6:e4198 https://doi.org/10.7717/peerj.4198

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Background. Hungarian draft is a horse breed with a recent mixed ancestry. The interest in their conservation and characterization has increased over the last few years. It was developed in the 1920s by crossing local mares with draught horses imported from France and Belgium. The aim of this work is to contribute to the characterization of the endangered Hungarian heavy draft horse populations in order to obtain useful information to implement conservation strategies for these genetic stocks.

Methods. To genetically characterize the breed and to set up the basis for a conservation programme, in this present study a hypervariable region of the mithocrondial DNA (D-loop) was used to assess genetic diversity in Hungarian draft horses. Two hundred and eighty five sequences obtained in our laboratory and 419 downloaded sequences available from Genbank were analyzed.

Results. One hundred and sixty-four haplotypes were revealed. Thirty-six polymorphic sites were observed. High haplotype and nucleotide diversity values (H_d=0.954±0.004; π=0.028±0.0004) were identified in Hungarian populations, although they were higher within than among the total number of breeds (H_d =0.972±0.002; π=0.03097±0.002). Fourteen of the previously observed seventeen haplogroups were detected.

Discussion. Our samples showed a large intra- and interbreed variation. There were no clear clustering on the median joining tree. The overall information given in this work led us to consider that the genetic scenario of this breed is more likely to be due to 'ancestrally' different genetic backrounds. This study could contribute to the development of a detailed breeding plan of Hungarian draft horse and help to formulate its genetic conservation plan, with the aim of increasing the population size, but avoiding inbreeding while, on the other hand, also facilitating genetic exchange among the populations.

Genetic analysis of the Hungarian draft horse population using partial mitochondrial DNA D-loop sequencing

Nikolett Csizmár1– Sándor Mihók1–András Jávor1–Szilvia Kusza 1*

[1] Institute of Animal Husbandry, Biotechnology and Nature Conservation, University of Debrecen, Hungary

Corresponding author:

Szilvia Kusza, PhD

4032 Debrecen, Böszörményi str. 138.Hungary

Email address: kusza@agr.unideb.hu

Abstract

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Keywords: *Equus caballus,* genetic diversity, mtDNA, D-loop region, Hungarian Draft horse

Introduction

In recent decades, various types of animal species have been investigated with special emphasis on improving the efficiency of selection programs. Thanks to this, the use of modern molecular genetic methods has increased considerably. As a consequence of the development of feeding technologies and the acceleration of transport an communication, local native breeds have been worldwide replaced by modern, high-productivity varieties. However, the genetic value of gene conservation divided the fate of varieties: those having excellent secondary traits and rare alleles resulting in considerable diversity contribute to both the current and future preservation of desirable properties (Notter, 1999; Bruford et al., 2003; Toro, Fernandez & Caballero, 2009). It is generally accepted that detailed molecular genetic data describing inter- and intraspecies diversity are essential for the effective management of genetic resources among economic animal varieties (Weitzman, 1993; Hall & Bradley, 1995; Barker, 1999; Ruane, 2000; Bruford et al., 2003; Simianer, 2005; Toro & Caballero, 2005; Toro, Fernandez & Caballero, 2009). These data as well as the continuous development of technology offer many new opportunities for researchers. In molecular genetic studies serving gene conservation, varieties are the basic units (Groeneveld et al., 2010).

In phylogenetic studies of mammalian sepcies/groups, mitochondrial DNA is a widely used molecular genetic device. The entire horse (*Equus caballus*) mitochondrial genome sequence has been available since 1994 (Xu & Arnason, 1994). The species is represented worldwide by more than 58 million animals (FAOSTAT, 2010). Modern age horses can be traced back to the domestication diversification process, which began 5000-6000 years ago in the Eurasian steppe region (Lippold et al., 2011; Ludwig et al., 2009; Outram et al., 2009). A significant

portion of the observed diversity of modern maternal lines was also observed at the time of domestication (Keyser-Tracqui et al., 2005). Due to these evolutionary processes, modern horses today form really close populations, whose individuals carry unique bloodlines and / or phenotypes (Petersen et al., 2013). After the Second World War various horse populations declined Europe-wide, leading to the loss of rare/specific genetic material and a reduction of genetic diversity. However, in recent years the issue of the preservation of genetic diversity has gained special emphasis on the international level, and one of the main considerations in this area of scientific research activities is to preserve the biodiversity of local varieties (Georgescu & Costache, 2012). The import of heavy horses to Hungary was started in the second half of the 19th century. These were mainly stallions of the Belgian, Percheron, Breton and Ardens breeds. Until after World War II, no organized breeding of heavy horses existed in Hungary in any particular sense. After World War II, there was a great need for horses to be used in field work on farms and also in transportation in Hungary. The foundation stock of this breed initially were native Hungarian mares which were bred with various other breeds such as Noriker, Percheron and Ardennes, and also with the available native Hungarian stallions. As a result of breeding work, a few local types (Muraközi and Pinkafői) were developed. The Hungarian Draft Horse Breeders National Association has records of approximately 800 mares today. The maternal side of certain individuals of the currect stock contains unknowns in 3^{rd} – 4^{th} ancient lines, since brand-marking and pedigree registration was obligatory only from 1993. In the winger maternal side of the Hungarian cold-blooded horse breeding stock, original pedigree documentation is missing and the founding stallions of the breed are unknown. Therefore it is especially important to explore the genetic background of the remaining stock and to map the different possible relatives in order to get a first insight into the Hungarian population, because up to now no such study has been done on this breed.

Materials and Methods

Ethics Statement

DNA sampling was limited to the collection of hairs pulled from the mane or tail by the horse owner or researcher. All animal work was conducted in accordance with the international and Hungarian national governing bodies (The Hungarian Animals Breeders Association –HABA, and Department of Operative Techniques and Surgical Research in Debrecen). All horses in this study were client-owned, and no harmful invasive procedure was performed on them; and there was no animal experimentation according to the legal definitions in Europe (Subject 5f of Article 1, Chapter I of the Directive 2010/63/UE of the European Parliament and of the Council), and in Hungary (40/2013. (II. 14.) Government Decree on animal research, this way no ethical approval is required.

Samples

Two hundred eighty five samples from registered mares from all over Hungary representing 35.63% of the Hungarian draft horse population were used. For the analyses hair samples from the tail complete with follicles were used -no invasive procedure was performed on our animals, for this reason no ethical approval is required- and were stored airtight till examinations at the laboratory at room temperature. Samples were examined in the Laboratory of Animal Genetics at the University of Debrecen. Genomic DNA was isolated from the stored hair samples (FAO/IAEA, 2004), and was carried out based on the published Chelex-based protocol (Walsh, Metzger & Higuchi, 2013).

Genbank sequences

We downloaded 419 available Genbank sequences from 52 different breeds. We considered the founding ancestors of Hungarian draft (KY512807 - KY513091) and also used populations with distant origin. Genbank Accession numbers: AB329597, AF064632-

AF431969, AJ413825- AJ413900, AY246186- AY575139, DQ324048, EF014970-EF014989, EF494073- EF494083, EF495133- EF495151, EU093045- EU093073, EU256571- EU256622, GQ119632- GQ119636, GU339390, GU563634, GU563651, GU563669- GU563711, HQ439455- HQ593058, HQ848967- HQ848977, JN398377-JN398457, KC847166, KF192350- KF192499, KF849272- KF849290, KT757760-KT757761.

mtDNA amplification, sequencing and analysis

Primers from published horse mtDNA sequence (Xu & Arnason, 1994) were designed for amplifying a 398-bp fragment from the most variable segment of horse mtDNA between positions 15531 and 15752: Forward 5'-CCCCCACATAACACCATACC-3', Reverse 5'-AGACAGGCATCCCCCTAGAT-3'. Necessary ingredients for the starting amplification mixture were as follows: 5µl isolated genomic DNA, 8.8µl dNTP (25mM)/Fermentas, 1µl GoTaq Flexi Buffer Promega, 8.2µl $MgCl_2$ (25mM) Promega, 1µl forward and 1µl reverse primer (10 pmol/µl) Sigma, 5µl dH_2O. After every failed reaction only two components were changed at a time. The reaction mixture was heated to 95°C for 10min, followed by 35 cycles each consisting of 20sec denaturation at 95°C, 30sec annealing at 62°C, 30sec of extension at 70°C and then a final 10min extension at 72°C. Samples were sent to and sequencing was done by the Macrogen Company (The Netherlands, Amsterdam). The correct reading of nucleotides and the comparison of sequences were done with the CodonCodeAlignerV.6.0.2. program, whereas statistical analysis was performed with two versions of Mega (Mega6 (Tamura et al., 2013) and Mega7.0 (Kumar, Stecher & Tamura, 2016), with DnaSP5.1. (Librado & Rozas, 2009) and Network 5.0. (Bandelt, Forster & Rohl, 1999). The DnaSP5.1. software was used for calculating the number of haplotypes, and haplotype and nucleotide diversities. Genetic distances among different mtDNA haplotypes were calculated by the two-parameter method of Kimura (Kimura, 1980). We used Arlequin 3.5.2.2. (Excoffier &

Lischer, 2010) software for calculating pairwise F_{ST} values and detecting shared haplotypes among populations. Median joining networks were constructed using NETWORK version 5.0.0.1 (Bandelt, Forster & Rohl, 1999).

Results

Indices of the genetic diversity of the Hungarian population

Analysis of the mitochondrial control region sequence (part of the mitochondrial HVR I) from 285 individuals identified 55 haplotypes based on 33 variable nucleotide sites (212-bp sequence). Thirty-six polymorphic sites were detected, which represented 16.98% of the total mtDNA sequence analyzed (212 bp). The average ratio of the four nucleotides A, T, C, G was 32.7%, 28.1%, 27.4%, and 11.8%, respectively. We observed high haplotype and nucleotide diversity values (H_d=0.954±0.004; π=0.028±0.0004). The average number of pairwise differences was k=5.77497. During sequence analysis we considered only 204 from the included 212 positions, after excluding sites with gaps. By these values Among the 55 haplotypes identified in these 204 sequences, we observed 22 unique ones (i.e. found only in a single animal), whereas the most frequent haplotype was #34 identified in thirty individuals. Eight haplotypes contain 55.78% of the total amount of analyzed sequences, with the remaining 47 haplotypes including less than ten individuals in each group. The haplotypes obtained were compared with an *Equus caballus* reference sequence available from Genbank and also used by (Hill et al., 2002). The details of the variable positions in basepairs 15531–15752 are given in Table 1.

Polymorphic sites in the control region of the Hungarian draft horse population sequenced Nucleotide positions 15531–15752 as compared to GenBank reference sequence X79547 [11].

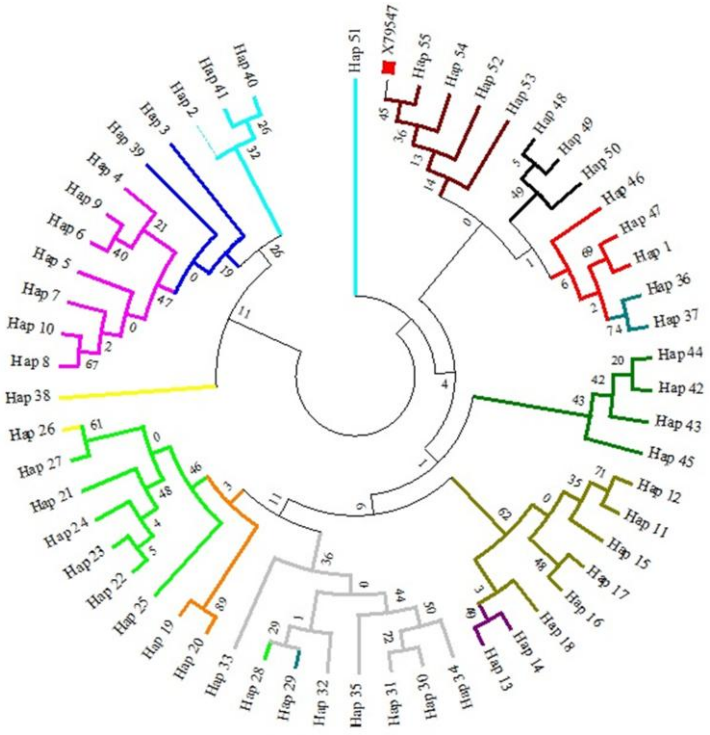
	Reference sequence	Accession number: X79547.1	15533	15534	15536	15538	15542	15566	15567	15596	15597	15598	15600	15601	15602	15603	15604	15615	15616	15617	15632	15635	15641	15649	15650	15659	15666	15667	15672	15685	15703	15709	15718	15720	15726	15737	15740
Haplogroups	**Haplotypes**	**Number of mares**	**A**	**C**	**T**	**A**	**C**	**T**	**T**	**A**	**A**	**T**	**G**	**T**	**C**	**T**	**G**	**A**	**A**	**T**	**T**	**C**	**T**	**A**	**A**	**T**	**G**	**A**	**G**	**A**	**T**	**C**	**C**	**G**	**G**	**T**	**A**
C2	1	1	.	.	.	.	.	.	.	.	.	.	.	C	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
A6*	2	1	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G		A	.	.	.	.	.	.	A	.	.	.
A2	3	4	.	.	.	.	T	.	.	.	G	.	.	.	T	.	.	.	.	.	.	.	.	.	G	.	A		.	.		.	.	A	.	.	.
A1	4	1	.	.	.	G	T	C	.	.	.	.	.	.	T	.	.	.	.	.	.	T	.	.	G	.	A	.	.	.	C	.	.	A	.	.	.
A1	5	1	.	.	.	.	.	.	.	.	G	.	.	.	T	.	.	G	.	.	.	T	.	.	G	.	A	.	.	.	C	.	.	A	.	.	.
A1	6	2	.	.	.	.	T	.	.	.	G	.	A	.	T	.	.	.	.	.	.	T	.	.	G		A	.	.	.	C	.	.	A	.	.	.
A1	7	1	.	.	.	.	T	.	.	.	G	.	.	.	T	.	.	.	.	.	.	T	.	.	.	.	A	.	.	.	C	.	.	A	.	.	.
A1	8	4	.	.	.	.	T	.	.	.	G	.	.	.	T	.	.	.	.	.	C	T	.	.	G	.	A	.	.	.	C	.	.	A	.	.	.
A1	9	19	.	.	.	.	T	.	.	.	G	.	.	.	T	.	.	.	.	.	.	T	.	.	G	.	A	.	.	.	C	.	.	A	.	.	.
A1	10	3	.	.	.	.	T	.	.	.	G	.	.	.	T	.	.	.	.	.	.	T	.	.	G		A	.	.	.	C	.	.	A	.	.	.
D2	11	1	.	T	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	G	.	.	.	.	.	G	.	.	.	A	.	.	.
D2	12	12	.	T	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	A	.	.	.
D3	13	1	.	T	.	.	.	.	.	.	.	.	.	T	C	A	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	A	.	.	.
D3	14	24	.	T	.	.	.	.	C	.	.	.	.	T	C	A	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	A	.	.	.
D2	15	3	.	T	.	.	.	.	.	.	.	.	.	T	C	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	A	.	.	.
D2	16	1	.	.	.	.	.	C	.	.	.	.	.	T	C	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	A	.	.	.
D2	17	8	.	T	.	.	.	C	.	.	.	.	.	T	C	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	A	.	.	.
D2	18	16	.	T	.	.	.	.	.	.	.	.	.	T	C	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	A	.	.	.
G	19	1	.	.	.	.	.	.	.	.	.	C	.	T	.	.	G	G	.	.	.	.	.	G	.	C	.	.	.	.	C	.	.	A	.	.	.
G	20	3	.	.	.	.	.	.	.	.	.	.	.	T	.	.	G	G	.	.	.	.	.	.	.	C	.	.	.	.	C	.	.	A	.	.	.
F2	21	1	C	.	C	.	.	.	.	.	.	.	.	T	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	A	A	.	G
F2	22	5	.	.	.	.	.	.	.	.	.	.	.	T	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	A	A	.	G
F2	23	1	.	.	.	.	.	.	.	.	.	.	.	T	.		.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	A	A	.	G
F2	24	2	.	.	.	.	.	.	.	.	.	.	.	T	.	A	.	.	.	.	.	.	.	.	.	.	.	.	A		C	.	.	A	A	.	G
F2	25	1	.	.	.	.	.	.	.	.	.	.	.	T	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	A	.	.	G
B1	26	2	.	.	.	.	.	.	.	G	.	.	.	T	.	A	.	.	.	.	.	.	.	.	G	.	.	.	.	.	C	.	.	A	.	.	G
F2	27	1	.	.	.	.	.	.	.	.	.	.	.	T	.	A	.	.	.	.	.	.	.	.	G	.	.	.	.	.	C	.	.	A	.	.	G
F2	28	1	.	.	.	.	.	.	.	.	.	.	.	T	C	A	.	.	.	.	.	.	.	.	.	.	.	.	G	.	C	.	.	A	.	.	.
B2	29	1	.	.	.	G	.	.	.	.	.	.	.	T	C	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	C	.	.	A	.	.	.
F1	30	2	.	.	.	.	.	.	.	G	.	.	.	T	.	A	.	.	.	.	.	.	.	.	.	.	.	.	G	.	C	.	.	A	.	.	.
F1	31	1	.	.	.	.	.	.	.	G	.	.	.	T	.	A	.	.	.	.	.	.	.	.	.	.	.	.	G	.	C	.	.	A	.	.	.
F1	32	1	.	.	.	.	.	.	.	.	.	.	.	T	.		.	.	.	.	.	.	.	.	.	.	.	.	G	.	C	.	T	A	.	.	.
F1	33	5	.	.	.	.	.	.	.	.	.	.	.	T	.		.	.	.	.	.	.	.	.	.	.	.	.	G	.	C	.	.	A	.	.	.
F1	34	30	.	.	.	.	.	.	.	G	.	.	.	T	.	A	.	.	.	.	.	.	.	.	.	.	.	.	G	.	C	.	.	A	.	.	.
F1	35	6	.	.	.	.	.	.	.	G	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	C	.	.	A	.	.	.
B2	36	22	.	.	.	G	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	T	.	A	.	.	.
B2	37	1	.	.	.	G	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	A	.	.	.
B1	38	2	.	.	.	.	.	.	.	G	.	.	.	T	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	A	.	.	.
A2	39	3	.	.	.	.	T	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	G	.	A	.	.	.	.	.	.	A	.	.	.
A6	40	5	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	A	.	.	.	.	.	.	A	.	.	.
A6	41	9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	A	.	.	.	.	.	.	A	.	.	.
C1	42	2	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	.	.	.	C	.	.	.	.	.	.	.	A	.	.	.
C1	43	1	.	.	.	.	.	.	.	G	.	.	.	T	.	.	.	.	.	C	.	.	.	.	.	C	.	.	.	.	.	.	.	A	.	.	.
C1	44	21	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	.	.	.	C	.	.	.	.	.	.	.	A	.	.	.
C1	45	6	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
C2	46	2	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
C2	47	15	.	.	.	.	.	.	.	.	.	.	C	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
E	48	1	.	.	.	.	.	.	.	G	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	C	.
E	49	5	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	C	.
E	50	2	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	C	.
A6	51	2	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	A	.	.	.
A5*	52	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.
A5*	53	1	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A5*	54	8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.
A5*	55	9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Sequence identity is indicated by '.', gaps by '-'.

Based on the determined variable positions we classified the resulting haplotypes into haplogoups previously defined by (Jansen et al, 2002), (Fig 1). We used the same reference sequence as in the analysis of variable positions (availability in Genbank is X79547).

A maximum likelihood tree represents the phylogenetic relationship among 285 partial mtDNA D-loop sequences from members of the genus Equus including haplotypes of the Hungarian draft and reference sequence.

The phylogenetic tree was based on the Tamura-Nei model of evolution with gamma distribution of rates and 1,000 bootstrap replicates (Tamura & Nei, 1993). Different colors represent different haplogroups differentiated by sequence motifs of the mtDNA clusters by [25]: A1 (light purple), A2 (dark blue), A5 (brown), A6 (light blue), B1 (yellow), B2 (turquoise), C1 (dark green), C2 (red), D2 (mustard yellow), D3 (dark purple), E (black), F1 (grey), F2 (light green), G (orange). The red square represents the reference sequence.



We detected fourteen of the seventeen haplogroups previously observed. 15.79% of the examined population (45 mares) belonged to haplogroup F1; these mares belong to haplotypes 30–35. D2 proved to be a very common haplogroup: it included 42 individuals and six haplotypes (14.74%). B1 and G were rare haplogroups represented only by 2 haplotypes (4 individuals) each. However, it is important to note that two haplotypes with four mares

showed variable locations within the G haplogroup, which is fairly rare. During the process of comparing Genbank sequences to our sequences no new mutations were found, individuals do not possess unknown motifs. The number of nucleotide differences and Kimura two-parameter distances were calculated among fifty-five mtDNA haplotypes. The Kimura two-parameter distances among haplotypes ranged from 0.005 to 0.063.

Genetic differentiation of different horse breeds based on mtDNA D-loop sequence

Our purpose was to explore the mitochondrial genetic relationships between different European horse breeds (especially cold-blooded ones) based on the sequences determined in our 285 individuals and a total of 419 sequences downloaded from the Genbank database. These eventually added up to 704 different sequences, representing 52 different breeds, including our 285 individual Hungarian cold-blooded/Hungarian draft horses. The breeds represent a wide geographic area as well as different horse types. We tried to select cold-blooded varieties, which play an important role in developing the breeds like Breton, Noriker, Belgian cold-blooded or Percheron, and also included other common horse breeds like Akhal Teke, Shetland Pony and Przewalskii. In order to analyse these sequences, we matched them up and cut out from them the above-mentioned 212-bp sequences in the HVR I region. Altogether 168 polymorphic sites were identified in the 212-bp mtDNA D-loop fragment in all horse populations (13 indels), representing a total of 164 different haplotypes. Thus the average percentage of polymorphic sites was 79.24% for all DNA sequences analyzed. High diversity values were observed among the total number of breeds. (H_d=0.972±0.002; π=0.03097±0.002). The average number of pairwise differences was k=6.164. This represents a large intra- and interbreed variation. The counts of haplotypes and polymorphic sites are shown in Table 2.

200

201 **Number of sequenced individuals (n), total number of haplotypes and polymorphic sites**

202 **with their dispersion within 52 different horse populations**

Population	n	Polymorphic sites	Pi	Haplotypes	Transitions	Transversions	Indels	Nucleotide diversity	Sd
Akhal Teke	16	19	5.643	14	19			0.032063	0.018182
American Paint horse	1			1					
Andalusian	2	6	6.214	2	6			0.035309	0.038044
Arabian	10	20	7.375	9	20			0.041903	0.024226
Belgian	13	17	4.719	8	14		3	0.026361	0.015505
Breton	58	35	6.235	29	25	12		0.035427	0.018931
Caspanian Pony	5	13	5.795	5	13			0.032929	0.022161
Chincoteague pony	1			1					
Cleveland bay horse	11	6	1.935	3	6			0.010996	0.007613
Clydesdale	17	169		9	42	127	11	0.011143	0.07582
Croatian heavy draft	11	14	4.473	9	11	2	1	0.025273	0.015209
Exmoor Pony	1			1					
Fell horse	2	8	8.261	2	4	4		0.046939	0.049699
Finn horse	2	3	3.052	2	3			0.017343	0.019983
Giara horse	2	1	1.006	2	1			0.005714	0.008070
Gotland	3	6	4.098	3	6			0.023284	0.019733
Hanovarian	3	11	7.668	3	11			0.043566	0.034921
Holstein	2	6	6.214	2	6			0.035309	0.038044
Hucul	10	10	4.282	4	10			0.024328	0.014881
Hungarian draft	285	36	6.012	55	34	1	3	0.034159	0.018063
Icelandic Horse	2	1	1.006	2	1			0.005714	0.008070
Iranian	14	22	5.902	14	22			0.033534	0.019124
Italian	3	11	7.686	3	11			0.043669	0.034998
Italian heavy draft	27	26	5.627	22	26			0.031969	0.017628
Lithuanian Heavy	3	11	7.713	3	11			0.043823	0.035113
Maremanno	15	22	5.777	12	22			0.032826	0.018658
Murinsulaner	8	17	7.152	8	13	3	1	0.040404	0.024160
Noriker	10	15	5.219	6	15			0.029654	0.017720
Norvegian Fjord	2	4	4.094	2	4			0.023260	0.025946
Oldenburg	1			1					
Percheron	3	6	4.110	3	6			0.023355	0.019786
Polish Heavy	3	9	6.219	3	9			0.035334	0.028765
Polish Primitiv	3	7	4.822	3	7			0.027396	0.022819
Posavina	20	18	4.431	12	18			0.025173	0.014472
Przewalksii	3			1					
Pura Raza Espanola	17	15	4.833	14	15			0.027462	0.015777
Rhineland Heavy	25	22	6.064	16	22			0.034453	0.018915
Romanian Draft	1			1					
Saddlebred	1			1					
Scottish Highland	2	6	6.214	2	6			0.035309	0.038044
Shetland Pony	12	13	4.922	5	12	1		0.027963	0.016489
Shire	10	15	5.065	8	15			0.028778	0.017253
Silesian	1			1					
Suffolk Punch	1			1					
Syrian	5	8	3.703	5	8			0.021041	0.014903
Thoroughbred	1			1					
Trakehner	4	14	7.497	4	14			0.042595	0.030120
Turkoman Akhal Tek	19	18	4.932	13	18			0.028020	0.015949
Vladimir Draft	21	24	5.975	14	24			0.033947	0.018821
Westfalian	1			1					
Wielkopolski	3	7	4.805	3	7			0.027300	0.022748
Zemaitukai Heavy	7	14	5.619	6	14			0.031928	0.019971

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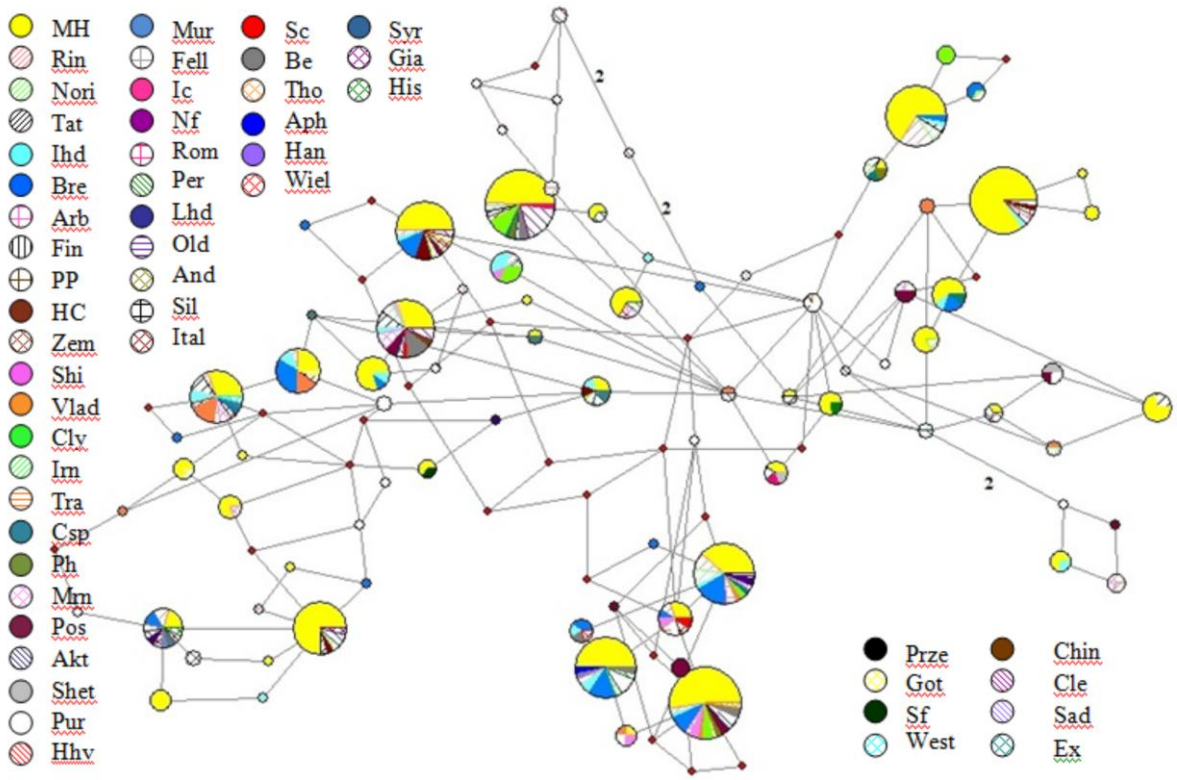
The lowest nucleotide diversities were found in Giara and Icelandic Horse, whereas the highest values were in Fell horse. We observed the most polymorphic sites in Clydesdale, where the highest numbers of insertion/deletion positions and transitions as well as transversions occurred. The Hungarian draft breed shared its haplotypes with twenty other populations in our study. It is noteworthy that Exmoor Pony, a breed known to have originated in the UK also shared haplotypes with two heavy horses, Italian Heavy and Rhineland Heavy, but not with the two other native UK cold-blooded breeds, Clydesdale and Shire. On the other hand, shared haplotypes among populations are indicators of common founder lineages. A network of 25 oriental and European breeds was drawn up on the basis of mtDNA sequences (Jansen et al., 2002), which showed that from the total amount of haplotypes (93) only nine included draft horses. The concensus Neighbor-joining tree and the Median-joining network (Fig. 2) showed that individuals from different populations share identical haplotypes. This indicates possible common ancestry.

Median network of horse haplotypes.

Included are those of the 285 Hungarian draft individuals analysed in this study, plus sequences of European breeds available in the Genbank nucleotide database. Sectors are proportional to the frequency of each haplotype. Horizontal bars represent the mutational steps. MH, Hungarian draft, our samples (number of samples: 285); Rin, Rhineland Heavy draft (25); Nori, Noriker (10); Tat, Turkoman Akhal Teke (19); Ital, Italian heavy draught (27); Bre, Breton (58); Arb, Arabian (10); Fin, Finn horse (2); PP, Polish primitiv (3); HC, Hucul (10); Zem, Zemaitukai heavy type (7); Shi, Shire (10); Vlad, Vladimir draught horse (21); Cly, Clydesdale (17); Irn, Iranian (14); Tra, Trakehner (4); Csp, Caspian Pony (5); Ph, Polish heavy (3); Mrn, Maremmano (15); Pos, Posavina (20); Akt, Akhal teke (16); Shet,

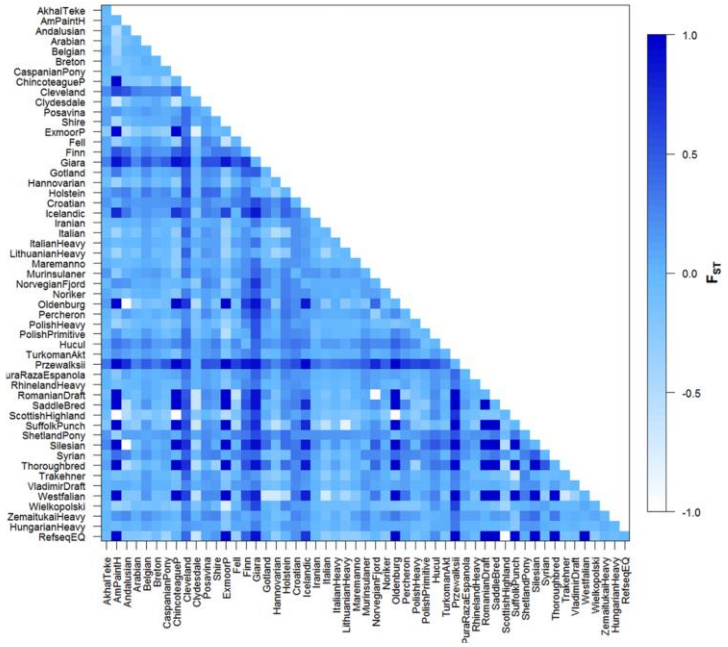
Shetland pony (12); Pur, Pura Raza Espanola (17); Hhv, Croatian heavy draft (11); Mur, Murinsulaner (8); Fell, Fell (2); Ic, Icelandic Horse (2); Nf, Norwegian Fjord (2); Rom, Romanian draft horse (1); Per, Percheron (3); Lhd, Lithuanian heavy drought (3); Old, Oldenburg (1); And, Andalusian (2); Sil, Silesian (1); Ital, Italian (3); Sc, Scottish Highland (2); Be, Belgian (13); Tho, Thoroughbred horse (1); Aph, American Paint horse (1); Han, Hanovarian (3); Wiel, Wielkopolski (3); Syr, Syrian (5); Gia, Giara horse (2); His, Holstein (2); Prze, Przewalskii (3); Got, Gotland (3); Sf, Suffolk Punch (1); West, Westfalian (1); Chin, Chincoteague pony (1); Cle, Cleveland bay horse (11); Sad, Saddlebred (1); Ex, Exmoor pony (1).



The pairwise F_{ST} values are shown in Fig 3. In the course of the analysis, 53 pairwise F_{ST} comparison values were recorded. In some cases negative values were recorded and these equate to zero F_{ST} values. Our FST values fall into a wide range, 0.00–1.00. The F_{ST} comparison values obtained were significant in 492 pairwise calculations.

Matrix of pairwase F_{ST} values.

Significance level=0.05.



Eleven populations that did not show any difference from other horse breeds were the following: American Paint Horse, Chincoteague Pony, Exmoor Pony, Norvegian Fjord, Oldenburg, Romanian Draft, SaddleBred, Suffolk Punch, Silesian, Thoroughbred, Westfalian. As expected, Przewalskii could be differentiated from domesticated horses. Four breeds could be separated from only one other population on any level of significance, these were: Italian-Cleveland Bay horse F_{ST}=0.4461, Syrian-Polish Heavy Horse F_{ST}=0.2217, Scottish Highland-Hucul F_{ST}=0.2373, Wielkopolski-Finn horse F_{ST}=0.3062. Croatian Heavy draft was significantly different from 34 other horse populations; on the other hand, this breed also has only a recent mixed ancestry, which in this case means relationship with 18 other breeds, mainly other cold-blooded horses. The Hungarian draft studied was significantly separable from 12 other populations, namely: ShetlandPony, Przewalski, Hucul, Murinsulaner, Croatian Heavy draft, Giara horse, Belgian, Breton, Cleveland, Clydesdale, Posavina, Shire.

Surprisingly, a significant difference was observed between Belgian horses and Hungarian draft, even though the import of cold-blooded Belgian Heavy horses started as early as before the First World War and led to the establishment of a cold-blooded flock in Hungary at that time (Becze, Lukáts & Zilahy, 1957).

Discussion

Basically, not all horse breeds have history, and it is quite rare that they are clearly separated genetically from other populations. Evolution has left its mark in the pedigrees of our domesticated horses. There is no other farm animal species that exhibits a similar level of mitochondrial DNA variation (Cieslak et al., 2010). No genetic studies have been done on endangered Hungarian cold-blooded horses, therefore the purpose of this work was to contribute to the characterization of the endangered Hungarian heavy draft horse populations in order to obtain useful information to implement conservation strategies for these genetic stocks. Above all it can be said that there is a high genetic variability in the small population of the Hungarian cold-blooded horse. MtDNA analysis revealed multiple maternal origins, the absence of a population structure, and inbreeding. The reasons for the presence of such a large amount of genetic variation could have several explanations: multiple origins, large-scale introgression of local lineages into the domestic stock, or an enormous number of female founders (Cieslak et al., 2010).

mtDNA analysis

The contents of A+T was richer in the mtDNA D-loop region. It was in accordance with other studies, where A+T was 55.8%, whereas C+G was 44.2% (Zhang et al., 2012), and also matched the requirement with the order of nucleotide composition of A>C>T>G with more A+T than G+C base pairs (Ji et al., 2008). Only three of the 36 detected polymorphisms

showed insertions/deletions of single base pairs; there were 34 transitions and one transversion, which shows a shift towards transitions (Kim et al., 2009). The observed high haplotype and nucleotide diversity values proved to be more than the values detected by (Moridi et al., 2013) in Iranian horses, and less but quite similar to the diversity data of 0.975 and 0.977 reported in (Pérez-Gutiérrez, De la Peña & Arana, 2008) and (Zhang et al., 2012), respectively. Direct comparisons with other studies have to be carefully considered, because different and partly different markers were used in other reports. Fifty-five haplotypes were identified in our Hungarian cold-blooded samples. This number is quite similar to other findings reported in (Kavar et al., 1999) and (Bowling A, Del Valle & Bowling M, 2000). In the course of the analysis of haplogroups, we detected in our samples fourteen of those defined previously by (Jansen et al., 2002). Four of our Hungarian draft horses belonged to haplogroup G, which is really rare. Comparative research (McGahern et al., 2006) processing 962 sequences found just one archaic, 25 Europian, two Middle Eastern and two Far Eastern equines that contained variable positions which can be classified into haplogroup G. In general, the highest within-breed diversity was observed in breeds that are recently drived, as mentioned also by (Petersen et al., 2013). We searched for shared haplotypes between the mentioned 52 different breeds and our samples. Shared haplotypes between Hungarian draft and 44 other breeds were observed. The foundation stock of Hungarian draft 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. Ten haplotypes were shared with each of Breton, Rhineland Heavy horse and Akhal Teke, and fourteen with Italian heavy horse. In the case of the cold-blooded horses examined, the 27th hapoltype was often shared. Our samples have three shared haplotypes with Hannoverian horses (3 haplotypes), not unexpectedly, as this breed is known to be an outbred population, influenced by many different breeds from different regions (Aberle et al., 2007). Similar results were reported in

mtDNA (Vilà et al., 2001). This indicates possible gene flow among those horse populations, or common ancestry. The genetic clustering analysis did not show any clear pattern of differentiation among all populations. Haplotypes inside a population were observed in separate haplogroups. Also, haplotypes from the same breed frequently clustered in separate groups that included breeds of completely different origins and breed types. This is typical of mtDNA results (Vilà et al., 2001). F_{ST} analysis supports this unclear pattern of differentiation showing high rates of mtDNA sharing between populations. This state is also confirmed by the observation that the median-joining network does not have a start-like structure, suggesting that a large number of founders could have produced the Hungarian cold-blooded breed. The neighbor-joining tree with 419 Genbank sequences and 55 haplotypes from the present study could be divided into three clusters and contains haplogroups A-G. Haplotypes of Hungarian cold-blooded horses were distributed across the whole tree, in haplogroups. Mitochondrial lineage diversity changed over time by breeding or hybridization and introgression; as a result, a breed is not necessarily isolated from other populations (Cieslak et al., 2010). The Kimura two-parameter distances among haplotypes ranged from 0.005 to 0.063. This is a rather wider interval than those observed in the case of other Hungarian horses like Hucul, where these distances ranged from 0.004 to 0.054 (Kusza et al., 2013). Also, these values indicate higher within-breed variation than the one observed by (Cothran, Juras & Macijauskiene, 2005). Nucleotide sequence diversity ranged from 0.47% to 6.1%. Since variability in domestic horse breeds is very high, they cannot be sharply distinguished from each other. Due to frequent recrossings, the history of the various breeds of horses is different from the process of the formation of natural populations (Priskin, 2010). Understanding the genetic diversity of equids and classifying their populations is essential for an appropriate conservation plan to be developed (Oakenfull, Lim & Ryder, 2000). In the course of analysis there was no sign of the genetic signature of a bottleneck, but (Keller et al.,

2001) reported that immigration – even at a low level – can erase bottleneck signatures within a few generations of reduction. Genetic diversity within and among breeds can also influence decisions affecting the breeds or species to be preserved, although it is really hard to determine criteria such as appearance or relevancy in different generations (Thaon d'Arnoldi, Foulley & Ollivier, 1998). In another study greater mtDNA diversity was found in old Iberian breeds than in American breeds, although they have a recent mixed ancestry (Lira et al., 2010). Therefore, on the one hand, these F_{ST} values and data are informative; on the other hand they show that our varieties have recently been developed from numerous national mares and only from a few stallions, with breeding programs determining the participation of individuals in breeding. For this reason, these data in themselves cannot be used to explain the development of different varieties. It has been stated that the maintenance of small isolated groups is the choice management strategy to preserve variability (Toro & Caballero, 2005), which also needs scientific planning and a breeding plan. In this study the results obtained with mitochondrial markers are consistent with and prove the recent hybrid origin of the breed. The high variability levels emphasize the importance of the conservation of this breed, as it can be an important reservoir of genetic biodiversity.

Conclusion

Hungarian heavy draft counts 800 mares today, and only survives due to breeding programs; in this way each haplotype frequency depends on the extent to which mares are involved in the breeding. Since breeders lack written documentation, the maternal side of the current stock's certain individuals contains unknowns in the 3rd–4th ancient lines. However, we confirmed the multiple origins in the maternal lineage of domestic horse breeds reported by other researchers (Hill et al., 2002). We present high nucleotide and haplotype diversity

values, no haplotypes clearly separable from other populations, and in this case no clear clustering on the median joining tree. Both heterozygosity and diversity levels were found to be high in this breed. Almost 40% of the Hungarian population were sampled, but in is unclear whether further increases in sample size would add to the differentiating ability of the methodology. It is important that good management practices continue to ensure the survival of this breed of economic significance. The results presented here could be regarded as a genetic portrait of the Hungarian cold-blooded horse population.

Acknowledgments

The study was supported by the Hungarian Draft Horse Breeders Association. Nikolett Sziszkosz is thanked for her help in laboratory work.

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