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## RESEARCH ARTICLE




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# Sequence analysis of the mitochondrial D-loop region throws a new light on the origin of Hungarian Nonius, Danubian Horse and Serbian Nonius

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## ABSTRACT

The objective of our study was to investigate the genetic structure of yet uninvestigated populations of three closely related horse breeds – the Danubian Horse, the Hungarian Nonius and the Serbian Nonius – in order to clarify their origin and genetic diversity. A 640-bp-long fragment of the mtDNA D-loop region was amplified and sequenced. The results showed that the investigated breeds have different genetic profiles although they share some common characteristics. We identified nine of the 17 haplogroups described in modern horses. Most of the obtained sequences fall into the M, L, G, and O'P lineages, which is indicative of the genetic profile of the ancestral mares that had probably been used at the initial stages of the formation of the breeds. The population of the Danubian Horse is characterised by a high prevalence of the Anatolian specific haplogroup G (45%), followed by the Western Eurasian specific haplogroups L and M (both about 21%). In the Hungarian Nonius breed we found the highest frequency of the Western Eurasian haplogroup M (44%), followed by the Middle Eastern O'P (26%) and the Central Asian specific E (13%) and G (13%). The Serbian Nonius showed a distinct genetic profile, characterised by a high prevalence of the rare European haplogroup D (67%), followed by the Central Asian specific haplogroup G (17%). The high percentage of haplogroups shared especially between the Danubian and the Hungarian Nonius indicates the possibility of a common origin of the two breeds. In contrast, the Serbian Nonius showed a specific genetic profile, which can be explained by a different and independent origin.

## KEYWORDS

D-loop region, Hungarian Nonius horse, horse mitochondrial haplogroups, genetic diversity

## INTRODUCTION

Horse breeding has been an important facet of Bulgarian culture, largely due to the country's geographic location and open plains. The role of the horse in everyday life used to be so important that it has become a symbol of prosperity and power and hence a symbol of cultural traditions and beliefs. As a predominantly agrarian country in the early 20th century,

Bulgaria was facing the problem of creating more massive, strong and durable horses for agricultural needs. In this direction, the Hungarian breed Nonius provided a favourable opportunity in terms of breeding and improvement of native horse breeds.

Nonius, or Noniusz, is one of the most famous Hungarian horse breeds. The main goal in the formation of this breed was to create a light draught and utility horse for Hungary's military. The beginning of the creation of the breed took place in 1816, when a stallion by the name of Nonius Senior (foundation sire) was captured from the Rosières-aux-Salines, a stud farm in France, and brought to the Mezőhegyes stud farm (Moravčíková et al., 2016). After that, the founder stallion sired very good foals out of Spanish mares, the advantageous traits of which could be fixed by inbreeding, and his female descendants were bred with Thoroughbred and Arabian stallions to correct the balance and elegance of the breed (Mihók et al., 2009). The creation of the breed also involved mares of Arabian, Lipizzaner, Thoroughbred, Spanish, and Norman origin. Independently of the breeding process in the Mezőhegyes stud farm, in 1948 another stud – Mata stud farm (about 3 km north of Hortobágy village), located in Debrecen (Hungary), began to form the breed in its own way (Toth et al., 2006). Currently, the Hungarian Nonius horse population consists of about 450 mares and 80 stallions, and the largest number of animals are bred in the Mezőhegyes stud farm (Moravčíková et al., 2016). Today, the breed is mainly used for riding, competitive driving sports, and equestrian tourism, and to a less extent in agriculture (Sandor and Adam, 2019).

The Nonius horse breed was at the basis of the creation of the Danubian Horse in Bulgaria, officially recognised as a breed in 1951 (Barzev et al., 2005). The Danubian Horse was created in the Clementina stud farm in the village of Pobeda (Pleven region). The first import was made by Hungary in 1893, when the following breeds were exported: Hungarian Nonius, Lipizzaner, Trotters, Anglo-Arabian, Shagya Arabian, etc., after which unsystematic crossbreeding was carried out for about 25 years. The basis of the tribal nucleus was constituted by stallions and mares of the breed Hungarian Nonius, imported from Hungary (from the Mezőhegyes stud farm) and from the former Republic of Yugoslavia in 1956 (Barzev et al., 2005). From the beginning, purebred breeding of the imported stallions and mares from the Nonius breed was applied (Karaivanov et al., 1989). Simultaneously, there was also crossbreeding with Hungarian Nonius stallions and partial reproductive crossbreeding, the so-called 'grading-up', i.e. sires of the Hungarian Nonius breed were continually backcrossed with females of the previous generation of local and improved mares (Barzev et al., 2005). The application of crossbreeding and partially reproducible crossbreeding involved mares of different origin – Anglo-Arabian, half-bred English, and native mares (Karaivanov et al., 1989). These three breeding methods, along with the strict selection, have contributed to the creation of a specific kind of Bulgarian Nonius, which differs from the Nonius breeds in Hungary, Romania, and the former Republic of Yugoslavia. According to data published by the

National Association of Horse Breeding, in 2019 the number of horses controlled by the Association registered in the Stud Book and the Register of the breed was 250 in total. The low population of the breed threatens it with decay due to inbreeding. This fact imposes the following prerequisites for the development of the breed: (1) Use Thoroughbred stallions for the production of horses suitable for equestrian sports; (2) Lighten up the type of horses and the production of horses for driving championships through the use of stallions of the Hungarian Nonius breed (Barzev et al., 2005).

The creation of the Serbian Nonius was started at the Karadjordjevo stud farm in 1885, using mainly horses of the Nonius breed from the Mezőhegyes stud farm in Hungary. During the breeding, Thoroughbred horses were mostly used. Depending on the extent of the impact of Thoroughbred horse, two types of Nonius were created: a lighter type (higher impact), finer skeletons and lively temperament, and a heavier type (less influence), which was much larger and stronger, and intended primarily for agricultural work. Currently, the largest number of animals of the breed are concentrated on the territory of Vojvodina, mostly represented in the Karadjordjevo horse farm (50 horses), while the rest of the horses are owned by a relatively small number of individual breeders. The breeding strategy has been to preserve the strong-harness Nonius horse, with distinctive race characteristics of exterior, correct movement for the harness and the riding, which is usable for traditional agricultural work and equestrian tourism, therapeutic riding, recreation, etc.

Despite the widespread popularity of the Nonius horse, so far there have been no data on the genetic structure of the breed population. There is also no information about the genetic profile of the Danubian Horse and the Serbian Nonius, which have been created on its basis. This circumstance has determined the purpose of the present study.

## MATERIALS AND METHODS

### Animal welfare/ethical statement

All experimental procedures were reviewed and approved by the Animal Research Ethics Committee of the Bulgarian Food Safety Agency (BFSA) (Ar. 154 of the Law on Veterinary Activity) in accordance with Directive 86/609 of the European Union.

### Sample collection

Hair samples from mane and/or tail were collected from 93 animals belonging to three horse populations: 23 Hungarian Nonius horses from Mata stud farm (Debrecen, Hungary), 58 Danubian Horses mainly from private tribal horse farms in Bulgaria and the former Klementina stud farm in the village of Pobeda (Pleven region, Bulgaria), and 12 Serbian Nonius from the Karadjordjevo stud farm (Karadjordjevo, Autonomous Province of Vojvodina). The samples were collected in a way ensuring that each extracted hair contained a follicle and had sufficient length for the shaft samples.



For the all three breeds – Danubian Horse, Hungarian Nonius and Serbian Nonius – analysed in this study, there are genealogical data recorded in studbooks. Genealogical information from pedigree documentation was taken into account in order to select unrelated animals, while some samples from the Danubian Horse were randomly sampled from private tribal horse farms.

### DNA extractions, PCR amplification and sequencing

Total DNA was extracted from the hair follicle and hair shaft samples using a GeneMATRIX Tissue DNA purification kit (Cat. no. E3550, EURx Ltd., Gdańsk, Poland), according to the manufacturer's instruction. Briefly, 1–3 hair follicles or 3 pieces of hair shaft approximately 0.5 cm in length were cut and placed in an Eppendorf tube. After that, they were mixed with 350 µL of buffer Lyse T, 20 µL of 1 M DTT and 20 µL of Proteinase K and incubated overnight at 56 °C with shaking. The quality and quantity of the isolated DNA were checked by 1% agarose gel electrophoresis and then visualised under UV trans-illuminator gel documentation systems after staining with SimpliSafe™ (Cat. no. E4600; EURx Ltd., Gdańsk, Poland). The isolated DNA was stored at –20 °C before analysis.

Based on the horse mtDNA sequence X79547 (Xiufeng and Arnason, 1994), a 665-bp fragment (from np 15,434 to np 16,098) of the mtDNA D-loop region (hypervariable region I, HVR1) was amplified using the following primers: F15453 5'-CACCCAAAGCTGAAATTCTAC-3' and R16078 5'-ATAACACCTTATGGTTGCTG-3' (Hristov et al., 2017). All PCR reactions were performed with 10 ng template DNA in a final volume of 50 µL (NZYTaQ II 2×Colourless Master Mix, Cat. no. MB354; NZYTech, Lda. – Genes and Enzymes, Lisbon, Portugal). The PCR conditions were as follows: initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s, primers hybridisation at 50 °C for 30 s, elongation at 72 °C for 1 min, and final elongation at 72 °C for 10 min. The successfully amplified products were purified with a GeneMATRIX PCR/DNA Clean-Up Purification Kit (Cat. no. E3520; EURx Ltd., Gdańsk, Poland) and sequenced in both directions using a PlateSeq kit (Eurofins Genomics Ebersberg, Germany).

### Statistical analyses

All 93 obtained DNA sequences were manually edited and aligned with the MEGA7 program (Kumar et al., 2016), using the horse reference mtDNA sequence X79547 (Xiufeng and Arnason, 1994). The obtained sequences (about 640 bp covered tRNA-Pro genes and the beginning of the D-loop region, HVR1) were deposited in the National Center for Biotechnology Information (NCBI) GenBank database under accession numbers MG420898–MG420955 (Danubian Horse), MG420956–MG420978 (Hungarian Nonius) and MG420979–MG420990 (Serbian Nonius). Sequences were analysed by polymorphic single-nucleotide polymorphism position, and haplogroups were determined according to Achilli et al. (2012). In order to have a more

complete picture of the three investigated breeds, we included in the analyses data from two Bulgarian plain horse breeds: East Bulgarian Horse ( $n = 39$ , GenBank Acc. no. MK465388–MK465426) and Pleven Horse ( $n = 11$ , Acc. no. MK465427–MK465437).

The phylogenetic relationships among haplotypes were visualised through the construction of two median-joining trees using Network 10.0 ([www.fluxus-engineering.com](http://www.fluxus-engineering.com)).

In order to graphically display (and summarise) the mitochondrial relationships among the analysed samples, the principal component analysis (PCA) was applied, using Excel software implemented by XLSTAT (Achilli et al., 2007).

Numbers of polymorphic sites (S), haplotypes (H), haplotype diversity (Hd), nucleotide diversity ( $\pi$ ), and average number of pair-wise nucleotide differences within a population (K) were estimated using DnaSP6.0 (Rozas et al., 2017). Values of non-synonymous (dN) and synonymous (dS) substitutions were estimated and compared using the Z test ( $P < 0.05$  was considered significant) based on the method of Nei and Gojobori (1986) with Jukes and Cantor correction. Tajima's D value (Tajima, 1989) and Fu and Li's D and F values (Fu and Li, 1993) were analysed using DnaSP6.0 to evaluate the neutral theory of evolution (Rozas et al., 2017).

## RESULTS

### mtDNA sequence polymorphism and tests of neutrality

The analysis of 640 bp from the mitochondrial D-loop sequences showed a high average haplotype diversity ( $H_d = 0.886$ ) with 12 haplotypes in Hungarian Nonius and Serbian Nonius, and 13 haplotypes in Danubian Horses (Table 1). Overall, we identified from one to six haplotypes in the investigated breeds from a total of 51 distinct haplotypes (Supplementary Table 1). Only one haplotype was common between Hungarian Nonius and Serbian Nonius as well as between the Danubian Horse and the Serbian Nonius. The haplotype with the highest frequency among our samples was Hap34, found in six sequences out of 93, 6.45%, followed by Hap5 and Hap28 (found in five sequences, 5.38%), etc. (Supplementary Table 1). The number of polymorphic sites (S, excluding gaps and ambiguous sites) was detected to be the highest in Nonius horses (53) and the lowest in Danubian Horses (16). These results confirmed a different genetic structure in all investigated horse breeds. The highest nucleotide differences were found in the Serbian Nonius ( $K = 19.121$ ), whereas the lowest in the Danubian Horse ( $K = 2.445$ ). Haplotype diversity was higher for the Serbian Nonius ( $1.000 \pm 0.034$ ) than for the Hungarian Nonius ( $0.909 \pm 0.036$ ) or the Danubian Horse ( $0.692 \pm 0.020$ ) (Table 1). The  $\pi$  value was the highest in the Serbian Nonius ( $0.03119 \pm 0.00298$ ), suggesting that the most nucleotide diversity was concentrated in this horse breed. The estimated Tajima's D value and Fu and Li's D and L tests were negative in the Hungarian Nonius and the Danubian Horses, suggesting population size expansion (e.g., after a

Table 1. mtDNA sequence polymorphism and tests of neutrality in Hungarian Nonius, Serbian Nonius and Danubian horses

Breed	S	Eta	K	H	Hd ± SD	$\pi \pm SD$	Fu and Li's D test	Fu and Li's F test	Tajima's D
Hungarian Nonius	55	58	13.557	12	0.909 ± 0.036	0.02282 ± 0.00302	-1.14089 ( $P > 0.10$ ) <sup>a</sup>	-1.11830 ( $P > 0.10$ ) <sup>a</sup>	-0.54020 ( $P > 0.10$ ) <sup>a</sup>
Serbian Nonius	45	49	19.121	12	1.000 ± 0.034	0.03119 ± 0.00298	0.80420 ( $P > 0.10$ ) <sup>a</sup>	0.92111 ( $P > 0.10$ ) <sup>a</sup>	0.81844 ( $P > 0.10$ ) <sup>a</sup>
Danubian horse	16	16	2.445	13	0.748 ± 0.047	0.01519 ± 0.00179	-1.59001 ( $P > 0.10$ ) <sup>a</sup>	-1.59565 ( $P > 0.05$ ) <sup>a</sup>	-0.88460 ( $P > 0.10$ ) <sup>a</sup>

S = number of polymorphic sites. Eta = total number of mutations. K = average number of nucleotide differences. H = number of haplotypes. Hd = number of haplotypes.  $\pi$  = observed average pairwise nucleotide diversity. <sup>a</sup>Not significant.

bottleneck or a selective sweep) and/or purifying selection, i.e. a rare or previously non-existing allele rapidly increasing in frequency due to natural selection (genetic hitchhiking). In contrast to the Hungarian Nonius and the Danubian Horse, in the Serbian Nonius we observed positive Tajima's D value and Fu and Li's D and L tests, which is an indicator for a decrease in population size and/or balancing selection, since balancing selection maintains mutations at intermediate frequencies.

### Phylogenetic analyses and haplogroup classification

The analysis of diagnostic mutational motifs showed that all obtained haplotypes belonged to nine haplogroups (D, O'P, G, A, L, C, Q, E and M) (Table 1 and Fig. 1). The majority of the samples – 31 out of 93 (33.3%) – fell in haplogroup G, and the highest frequency of this group was observed in the Danubian Horse (45.0%, 26/58). The second most common haplogroup was M (24.7%, 23/98) with the highest values in the Hungarian Nonius (43.5%) and the Danubian Horse (21.8%). This haplogroup was not found in the Serbian Nonius. Haplogroup O'P (9.7%, 9/93) was another represented group which was found with different frequencies in the Hungarian Nonius (26.1%), the Danubian Horse (3.5%) and the Serbian Nonius (8.3%). In addition to these haplogroups, there are those that are specific to each breed (Table 1 and Fig. 1). For example, in the Serbian Nonius we found a high prevalence of the rare haplogroup D (66.7%, 8/12), which was not established in the other two breeds. Haplogroup L is specific for the population of the Danubian Horse (21.0%, 12/58), while the other rare haplogroup E was found only in the Hungarian Nonius horse population (13.0%, 3/23).

When comparing the mitochondrial profiles of the studied breeds with two other Bulgarian plain populations of horses (the East Bulgarian Horse and the Pleven Horse), significant differences were found regarding the Hungarian Nonius and the Serbian Nonius, but there were also certain similarities with the Danubian Horse (Fig. 1). The population of the East Bulgarian Horse was characterised by the highest frequency of haplogroup Q (35.90%, 14/39), followed by haplogroup L (33.33% 13/39), and haplogroup A (10.26%, 4/39). In the same way as in the East Bulgarian Horse, in the Pleven Horse breed haplogroup L (45.45% 5/13) was found to be predominant, followed by haplogroup C (27.27% 3/11). In the East Bulgarian and the Pleven Horse breeds the very rare haplogroup N was observed (7.69% and 9.09%, respectively). The obtained results showed that Bulgarian plain horse breeds (Danubian, East Bulgarian and Pleven Horses) shared the common haplogroup L, which was observed with a high frequency in all three breeds.

The network analysis of the part of the D-loop region in three Bulgarian plain horses, Hungarian Nonius and Serbian Nonius showed high haplotype diversity (Fig. 2). Among all 60 identified haplotypes, the most represented ones belonged to haplogroups G (13 Hap, 21.7%), M (10 Hap, 16.7%), L (9 Hap, 15.0%), and Q (6 Hap, 10.0%) (Supplementary Table 1).



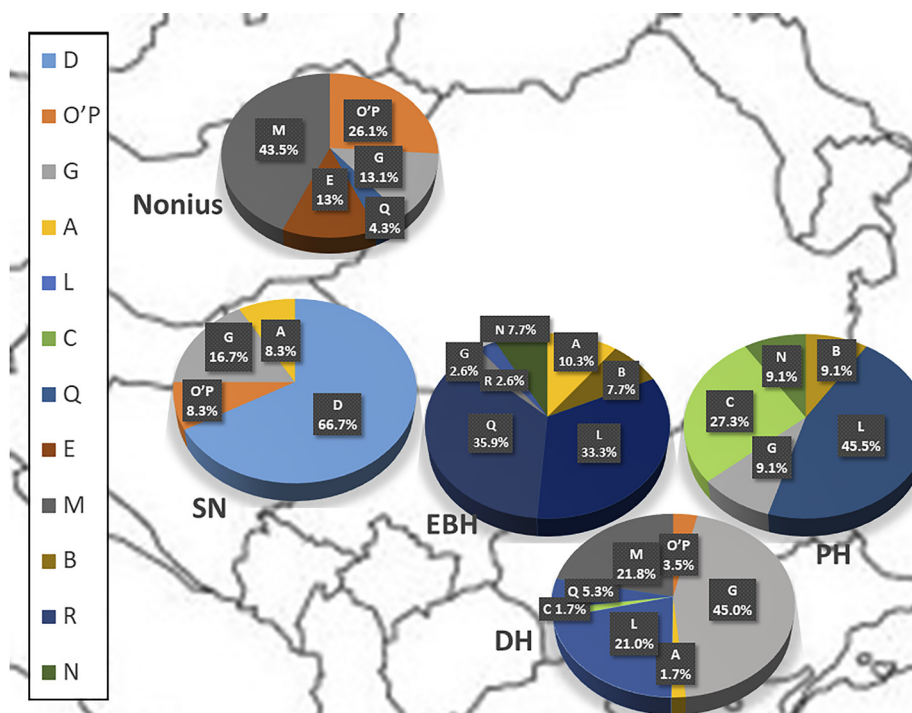


Fig. 1. A map showing sampling location and haplogroup diversity. Pie charts are proportional to sample size (for details see Supplementary Table S1). Abbreviations: SN – Serbian Nonius; DH – Danubian Horse; EBH – East Bulgarian horse; PH – Pleven Horse

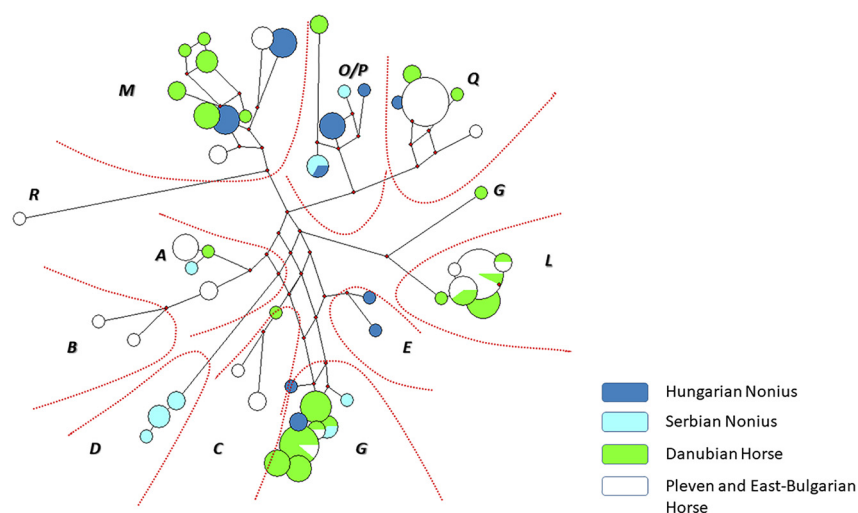


Fig. 2. Median-Joining Network based on D-loop control-region sequences of three plain Bulgarian horse breeds, Nonius and Serbian Nonius. Median joining network was constructed based on 26 haplotypes in 139 mtDNA sequences. The sequence variations are from Supplementary Table S1. Circle areas are proportional to haplotype frequencies

We also noticed some common haplotypes among the investigated samples. Hap23 was shared between the Danubian Horse and the Serbian Nonius, Hap32 and Hap39 were detected in the Danubian and the Pleven Horse breeds, Hap36 was found in the Danubian and the East Bulgarian Horse, etc. (Supplementary Table 1). These haplotypes encompass only Bulgarian horse breeds, which suggests a common maternal ancestry in the formation of modern Bulgarian plain horses.

### Principal component analysis

In order to graphically display and summarise the information concerning haplogroup frequencies, we performed a principal component analysis (PCA). The PCA was carried out by considering only our samples (Supplementary Table 2 and Fig. 3). After reducing the haplogroups to PCs (principal components), we analysed the co-ordinates of the observations for the Hungarian Nonius and Serbian Nonius, as well

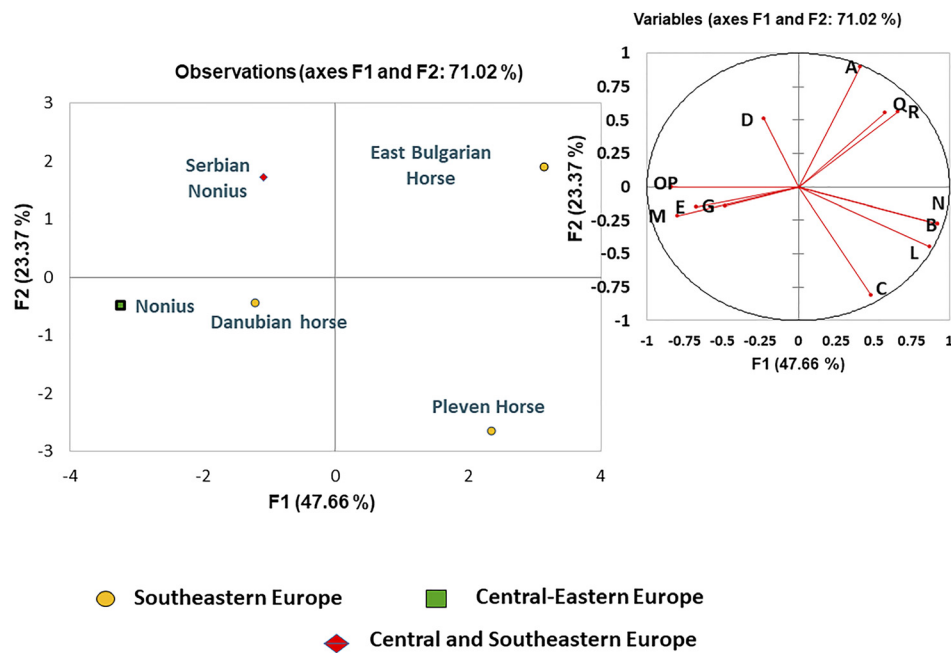


Fig. 3. Principal component analysis (PCA) plot representing the genetic landscape of three modern Bulgarian plain horse breeds, Hungarian Nonius and Serbian Nonius, based on haplogroup frequencies from D-loop region data. The right upper plot represents the contribution of each haplogroup to the first and second PC (projections of the axes of the original variables). Diamonds represent the centroids of breeds from the same macrogeographic area

Table 2. Haplogroup frequencies in Danubian Horse (DH), Hungarian Nonius (HN) and Serbian Nonius (SN) populations

Haplogroup	HN (n)	%	SN (n)	%	DH (n)	%	Total	%
D	0	0.0	8	66.7	0	0.0	8	8.6
O'P	6	26.1	1	8.3	2	3.5	9	9.7
G	3	13.1	2	16.7	26	45.0	31	33.3
A	0	0.0	1	8.3	1	1.7	2	2.2
L	0	0.0	0	0.0	12	21.0	12	12.9
C	0	0.0	0	0.0	1	1.7	1	1.1
Q	1	4.3	0	0.0	3	5.3	4	4.3
E	3	13.0	0	0.0	0	0.0	3	3.2
M	10	43.5	0	0.0	13	21.8	23	24.7

The affiliation of haplogroups is according to Achilli et al. (2012).

as for three plain Bulgarian horse breeds – the Danubian Horse, the East Bulgarian and the Plevan Horse in a two-dimensional graph based on haplogroup frequencies from the mitochondrial D-loop region. The outlier position of the Plevan horse was confirmed particularly along the first PCA, while the second PCA distinguished the Serbian Nonius from the other breeds. We observed that the Hungarian Nonius horse was in the same quarter as the Danubian Horse due to their sharing the same haplogroups – M, G, and O'P, as shown by the centroids of the two breeds (Supplementary Table 2 and Fig. 3). The PCA analysis revealed a specific localisation of the East Bulgarian and the Plevan Horse breeds in contrast to the Danubian Horse. Obviously, all Bulgarian plain horse breeds have an original maternal inheritance. The position of the Serbian Nonius on the PCA plot and its clear separation from the Hungarian Nonius and the Danubian Horse is an interesting finding. It

suggests a different concept regarding the formation of the breed and the possible participation of ancestral mares with a specific genetic profile that were used in the initial stages of breeding selections in contrast to the Hungarian Nonius and the Danubian Horse.

## DISCUSSION

### Tracing of the genetic roots of the Hungarian Nonius horse

The analysis of the genetic diversity in the Hungarian Nonius horse showed an unusually high frequency of haplogroup M – 43.5% from all 23 investigated samples (Fig. 1 and Table 2). Haplogroup M is present with a frequency of about 7% in the modern and about 17% in the ancient

European horse population (Achilli et al., 2012). It is a haplogroup of typical European origin as it was established even during the Mesolithic/Neolithic era (5,200–4,900 BC) and later during the Neolithic/Bronze age in the Iberian Peninsula (Cieslak et al., 2010; Lira et al., 2010). The contribution of native Iberian horses to the Iron Age Spanish horse suggests that the genetic influence of these horses continued to persist in Iberia until at least the 7th century BC in a domestic context (Seco-Morais et al., 2007; Fages et al., 2019). Taking into account that Spanish mares were involved in the creation of the Nonius horse breed and native Spanish horses were the direct ancestors of the Hungarian ones back in the Early Bronze Age, it is not surprising why haplogroup M is present with such a high frequency in the Nonius population (Lopes et al., 2005; Royo et al., 2005).

Another haplogroup found with a high frequency in this breed is O'P (26.1%, 6/23) (Table 1). In contrast to haplogroup M, haplogroup O'P is a typical Middle Eastern haplogroup with the highest frequency (cca. 8%) in Middle Eastern modern horses (Achilli et al., 2012) and about 10% in ancient Asian samples (Fages et al., 2019). This haplogroup emerged for the first time in prehistoric Anatolia during the late Pleistocene and early Holocene; after that, during the Bronze Age (around 2,000 BC) the frequency of O'P decreased significantly (Guimaraes et al., 2020). It is possible that haplogroup O'P was incorporated into Oriental horse breeds from Bronze Age Anatolian horses. Perhaps the most famous breed among oriental horses and in the world, created in the Middle East, and, in particular, in the Arabian Peninsula (over the last 3,000 years) by the nomadic Bedouin people, is the Arabian horse (Głazewska, 2010). For more than 100 years Arabian horses have been used to improve many horse breeds (Knorr, 1912). This improvement reached its peak in the 19th and 20th century with the creation of the English Thoroughbred racing horse (Bower et al., 2011). In addition, the contribution of Arabian mares to Hungarian Nonius horse formation may explain the presence of haplogroup O'P in its population.

It is also interesting to note that haplogroup G (13.1%, 3/23) is present in the Hungarian Nonius population (Fig. 1 and Table 2). This haplogroup is of typical Central Asiatic origin and is present with a frequency of around 16.0% and 22.0% in modern and ancient horse populations, respectively (Priskin et al., 2010; Achilli et al., 2012).

Another typical Central Asiatic haplogroup that is observed in the Hungarian Nonius horse is the rare haplogroup E, which is present in around 2% of the modern horse population (Achilli et al., 2012). These lineages, i.e. haplogroups G and E, may have arrived in this region after the appearance of the Yamnaya people (with already domesticated horses) in the central and eastern part of the Carpathian Basin in the Early Bronze Age and/or with the arrival of ancient Hungarians from the Eastern European steppes to the Carpathian Basin at the end of the 9th century AD (Fóthi et al., 2020).

The Tajima's D as well as Fu and Li's D and F statistics are negative in the population of Hungarian Nonius horse (Table 1). These values may result from population expansion, i.e. rapid increases in the frequency of some favourable neutral allele due to natural selection (genetic hitchhiking) (François et al., 2010). It can be assumed that haplogroup G has undergone a selective sweep in the population of the Nonius horse because the ancient Hungarians have appreciated the excellent exterior, speed and stamina of the Turkoman horses.

### Genetic diversity of the Danubian Horse

There are three modern plain horse breeds in Bulgaria – the Danubian Horse, the Pleven Horse and the East Bulgarian Horse. Mitochondrial analysis of the Danubian Horse breed has revealed almost 45% (26/58) presence of haplogroup G (Fig. 1 and Table 2). The presence of this haplogroup in the Danubian Horse population seems logical, considering the contribution of Nonius mares to the creation of the breed. The high frequency of haplogroup G in the Danubian Horse may be explained with population bottlenecks and/or founder events. Most likely, the mares imported from the Mezőhegyes stud farm had an identical genetic profile, and they constituted the tribal nucleus for the creation of the Danubian Horse. This assumption is supported by the so-called 'grading-up' process, i.e. the use of purebred animals over a series of generations to provide a 'nearly purebred' result, which was used in the creation of the Danubian Horse breed (Barzev et al., 2005). The calculated negative value of Fu and Li's D and F tests and Tajima's D also confirms the above suggestion (Table 1).

Another haplogroup with a relatively high frequency is haplogroup M (21.8%, 13/58) (Fig. 1 and Table 2). The origin and distribution of this haplogroup have already been reviewed regarding the Hungarian Nonius horse, and, logically, it is present in the Danubian Horse population.

Another haplogroup present in the Danubian Horse is haplogroup L (21.0%, 12/58). This haplogroup, along with M, is typically West Eurasian, present in the modern and ancient horse population with about 38.0% and 21.43% frequency, respectively (Achilli et al., 2012). In contrast to the Neolithic/Bronze Age Iberian haplogroup M, haplogroup L appeared in Spain, either due to an independent domestication event in the Iberian Peninsula or due to introgression of Barb-type horses from North Africa during the Muslim invasions in the Middle Ages (Lira et al., 2010). Quite unexpectedly, we found no presence of haplogroup L in the Nonius horse population despite its high frequency in the Iberian horses involved in the creation of the breed (Fig. 1 and Table 2). One of the possible reasons for this is related to the different process of the formation of the Nonius breed in the Mezőhegyes and the Mata stud farms. The selection process in the Mata stud farm (where our samples came from) aimed to create a heavier horse for agricultural purposes, whereas in the Mezőhegyes stud farm the goal was to create a light draft for military purposes. Moreover, while most representatives of the Nonius horse

breed are black, dark bay or brown, it may be noted that the bay individuals are more common among the Nonius horses from Hortobágy stud farm.

Danubian Horses and the other two plain Bulgarian horse breeds – Pleven and East Bulgarian – share the common haplogroup L (Fig. 1) (Hristov et al., 2020). In contrast to the Danubian Horse, the Pleven and the East Bulgarian Horse breeds showed different genetic profiles, most likely associated with the local Bulgarian breeds (Deliorman and Kamchia, respectively) which gave the start to the creation of the breeds as well as to the introgression of local horses with exotic various horse breeds (Barzev and Yuseinov, 2004).

### The enigmatic haplogroup D in the Serbian Nonius

The least data on the creation of the breed are available regarding the Serbian Nonius. Although a small number of animals were included in the analysis as a consequence of the reduced size of the total population, some conclusions can be drawn about the genetic structure of this breed. The sequence analysis of a partial D-loop region has shown the presence of the very rare haplogroup D – more than 50% (66.7%, 8/12) (Fig. 1 and Table 2). This haplogroup occurs in only about 4.5% of modern and about 10.2% of ancient samples (Achilli et al., 2012). Haplogroup D emerged for the first time in the prehistoric Anatolian horse population during the Bronze Age (around 2,000 BC). However, it is not a local, but an allochthonous lineage in Anatolia, presumably incorporated in Anatolian horses from the Pontic-Caspian steppe via the Transcaucasian route in the Early Bronze Age (the end of the third millennium BC) (Guimaraes et al., 2020). Similarly to the creation of the Hungarian Nonius and the Danubian Horse, it is very likely that the local Serbian breeds, e.g. the Serbian Mountain Pony, participated in the formation of the Serbian Nonius; however, further data would be needed to confirm this suggestion. It is interesting to note that several studies have regarded haplogroup D as an old clade characteristic of the small ponies distributed in Northern Europe (Exmoor, Fjord, Icelandic, Scottish Highland, etc.) (Winton et al., 2015, 2020). These observations represent a convenient possibility for the high frequency of haplogroup D in the Serbian Nonius.

Haplogroup G is present in the Serbian Nonius population with about 13.1% frequency (2/12) (Fig. 1 and Table 2). Like in the Danubian Horse, the presence of this haplogroup here may be explained by the influence of the Nonius breed in the creation of the Serbian Nonius.

Haplogroups O'P and A are observed with equal frequency of 8.3% in the Serbian Nonius. These haplogroups are typical representatives of Oriental horses, such as Arabian, Akhal-Teke, Barb, etc. (Jansen et al., 2002; Cieslak et al., 2010; Achilli et al., 2012). The stock of Arab and Barb horses was introduced into England as early as the 3rd century, which marked the beginning of the formation of the Thoroughbred (Mahrous et al., 2017; Cosgrove et al., 2020). The Thoroughbred horse took an active part in the

reproductive process in the creation of the Serbian Nonius, which also explains the presence of haplogroups O'P and A in the Serbian Nonius population.

In contrast to the Hungarian Nonius and the Danubian Horse, the Tajima's D as well as the Fu and Li's D and F statistics are positive in the population of the Serbian Nonius (Table 1). These data confirm that this breed was created by a balanced selection.

In conclusion, this study has demonstrated for the first time the genetic structure of the Nonius horse in Hungary and the closely related Danubian Horse and Serbian Nonius. The population of the Nonius horse expressed a highly diverse genetic structure, which can be traced back to human migration during the Early Bronze Age (around 3,000 BCE). The genetic profile of the Danubian Horse resembles that of the Hungarian Nonius horse, due to the tribal nucleus which gave rise to the breed formation. In contrast to the Nonius and the Danubian Horse, the Serbian Nonius showed a different genetic profile as a result of a balancing selection and the use of predominantly native mares in the creation of the breed.

### SUPPLEMENTARY DATA

Supplementary data to this article can be found online at <https://doi.org/10.1556/004.2021.00029>.

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