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# Mitochondrial DNA analysis and phylogenetic tree construction of maternal lines in the Lipizzan horses

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## Abstract

The Lipizzan horse breed comprises 62 mare family lines. Mitochondrial DNA (mtDNA), especially the D-loop region, plays a vital role in tracing maternal lineage and studying the origin of these family lines. The primary aim of this study is to construct phylogenetic trees using the Neighbour-Joining and Median-Joining methods, thereby analysing the genetic diversity and evolutionary relationships among these mare family lines. This study included 13 horses from Serbia that belong to different mare family lines including Thais (Rebecca-Thais) (n=2), Drava-Dubovina (Djebrin) (n=2), Wera (Theodorosta) (n=4), Batosta (Africa) (n=3), and Zora (n=2). The D-loop region was sequenced, and the sequences were subjected to bioinformatics analyses. Multiple alignments of all obtained sequences and those downloaded from the GenBank were performed with the reference mtDNA sequence downloaded from the GenBank using the MEGA 11 software, and phylogenetic trees were constructed using the PopART 1.7 and iTOL v6 online tools for visualization. Results of this study indicate that the Zora family line, though not officially recognized as a Lipizzan mare family line by the LIF (Lipizzan International Federation), forms a cluster with classical Lipizzan mare lines, including Monteaura, Betalka, Wera, and Allegra. The Neighbour-Joining tree provided a hierarchical representation of genetic distances, while the Median-Joining network revealed multiple evolutionary pathways, illustrating intraspecific genetic variability.

***Key words:*** mtDNA, Mare family lines, Horses, Neighbour-Joining method, Median-Joining method.

# Introduction

Sequencing of the entire mitochondrial genome showed a circle of double-stranded DNA, whose length is 16.6 kb, containing a non-coding control region, 13 protein coding genes, two rRNA coding genes, and 22 tRNA coding genes (Schaefer et al., 2001; Guo et al., 2019). The sequence of the horse (Equus caballus) mitochondrial DNA was sequenced by Xu and Árnason (1994), NCBI Reference Sequence: NC_001640.1. The animal mtDNA is maternally inherited, non-recombining, and has a higher mutation rate compared to the nuclear DNA (Ladoukakis and Zouros, 2017). The gene content and order, including 22 tRNA, 2 rRNA, and 13 protein coding genes, are highly coding different subunits of the mitochondrial respiratory chain. The control region in the majority of mtDNA molecules in the mitochondrion is characterized by a typical structure, called displacement loop (D-loop). Due to the high mutation rate in the D-loop region and strictly maternal mode of inheritance, sequencing of the control region has become a widely used tool for tracing mtDNA sequence diversity among founder mares in pedigreed populations (Kavar et al., 1999; Bowling et al., 2000; Hill et al., 2002; Kavar et al., 2002). The Lipizzan horse breed was established in Lipica in 1580. The population was formed from nine stallions and 24 mares imported from Spain, along with mares from the local white Karst horses. Later imports of Andalusians, Barbs, Neapolitan, and Arab horses significantly contributed to the breeding of the Lipizzan horse (Clutton-Brock, 1999). Lipizzan stallion lines were developed from very outstanding males which succeeded to form their own lineages during more than a century. There are eight Lipizzan stallion lines: six belong to the classical Lipizzan stallion lines (Pluto, Neapolitano, Favory, Conversano, Maestoso, and Siglavy), whereas two are considered non-classical (Tulipan and Incitato). Similar to the formation of stallion lines, the establishment of mare family lines was inevitable for the development of the Lipizzan breed. Within the Lipizzan breed, 62 mares established dominant families which are recognized mare family lines today (Dovč et al., 2006).

Phylogenetics is the study of the evolutionary histories of living organisms, and represents the evolutionary divergences by finite directed (weighted) graphs, or directed (weighted) trees, known as phylogeny (Peng, 2007). Any biological information that can be used to infer the evolutionary relationship among the taxa is known as a phylogenetic information marker. It can be anything such as DNA sequence, RNA sequence, protein, RFLP, AFLP, ISSR, allozymes, and conserved intronic positions, etc. Identification of conserved genetic loci (coding- or non-coding) is the first step in analysing the phylogenetic relationship (Reddy, 2011). There are two types of phylogenetic methods, character-based methods, which are based on discrete characters from molecular sequences from individual taxa, and those that are based on the distance, the degrees of differences between pairs of sequences. In distance-based methods there are two different algorithms, the cluster-based (UPMGA - Unweighted Pair Group Method using Arithmetic average, and Neighbour-Joining) and the optimality-based (Fitch-Margoliash and Minimum Evolution) (Peng, 2007). We distinguish between two types of phylogenetic trees, rooted and unrooted trees. A rooted tree has a node identified as the root from which ultimately all other nodes descend, hence a rooted tree has direction. This direction corresponds to evolutionary time; the closer a node is to the root of the tree, the older it is in time. Unrooted trees lack a root, and hence do not specify evolutionary relationships in quite the same way, and they do not refer to ancestors and descendants (Page and Holmes, 1998). There is a large number of studies on horse populations that use phylogenetics as an analysis of diversity between populations as well as within a population (Ishida et al., 1995; Jansen et al., 2002; Dovč et al., 2006; Khanshour and Cothran, 2013; Yunzhou et al., 2016; Cieslak et al., 2017; Sheikh, 2023; Zorc et al., 2023).

In this paper, the focus will be on constructing phylogenetic trees based on mitochondrial DNA sequence data from horses of the Lipizzan breed. Lipizzaner is one of the most popular horse breeds in Serbia. According to the data from the Lipizzan International Federation for 2024 (https://www.lipizzan-online.com), the total population in Serbia was 964 purebred horses. At the Karađorđevo Stud Farm only 16 purebred Lipizzaner horses are bred, but a certain number of horses whose maternal lines are not recognized internationally also are bred there. One of these lines of mares is named Zora and, to our knowledge, there is one study (Zorc et al., 2023) conducted on the mtDNA characterization of this mare family line which used the Median-Joining method to construct a phylogenetic tree.

This paper includes two types of trees using different algorithms, the Neighbour-Joining method and the Median-Joining method. The Neighbour-Joining method constructs bifurcating phylogenetic trees, offering a single hierarchical solution that minimizes branch lengths but cannot handle complex evolutionary events like recombination. In contrast, the Median-Joining method builds networks that can include loops and multiple evolutionary paths, making it more suitable for analysing intraspecific data with recombination or genetic variability (Saitou and Nei, 1987; Bandelt et al., 1999). The main aim of this study is to present phylogenetic trees based on sequencing results.

## Material and Methods

### Sampling and DNA extraction

This study included 13 horses from different mare family lines including Thais (Rebecca-Thais) (n=2), Drava-Dubovina (Djebrin) (n=2), Wera (Theodorosta) (n=4), Batosta (Africa) (n=3), and Zora (n=2), which is currently not recognized as a purebred Lipizzan mare family line by the LIF (Lipizzan International Federation - https://www.lipizzan-online.com).

Hair samples were collected from horses in Serbia. The DNeasy Blood & Tissue Kit was used for DNA extraction, following the manufacturer's protocol. The quantity and quality of the extracted DNA samples was assessed using the NanoPhotometer® N60 and gel electrophoresis.

### Amplification and mitochondrial DNA sequencing

Fragments of the mtDNA control region (D-loop) were amplified using the forward primer 5'-CAC CCA AAG CTG AAA TTC TAC-3' and the reverse primer 5'-ATA ACA CCT TAT GGT TGC TG-3' (Hristov et al., 2016). The length of the amplified fragments was 665bp and was visualized on a 1% agarose gel. The PCR reaction conditions are shown in Table 1.

Tab. 1 - Polymerase chain reaction programme for the amplification of the mtDNA control region

| Phase | | Temperature | Time |
|---|---|---|---|
| Initial denaturation | | 95 °C | 3 min |
| 35 cycles | Denaturation | 95 °C | 30 s |
| | Annealing | 46 °C | 30 s |
| | Extension | 72 °C | 40 s |
| Final extension | | 72 °C | 5 min |
| Hold | | 4 °C | ∞ |

The amplified fragments were sequenced using the Sanger sequencing with the reverse primer 5'-ATA ACA CCT TAT GGT TGC TG-3' (Hristov et al., 2016) on an Applied Biosystems 3500 Genetic Analyzer. The sequencing results were then processed using bioinformatics software and online tools.

### Bioinformatics analysis

Sequencing chromatograms were manually reviewed and processed using the MEGA 11 software (Tamura et al., 2021). The mitochondrial DNA sequences, specifically the control region (D-loop), from 13 Lipizzan horses were aligned using the same software. Additionally, all sequences obtained were aligned with sequences (385bp) from the mare family lines downloaded from the GenBank (Benson et al., 2013). The sequences (385bp) representing 45 different mare family lines downloaded from the GenBank, along with their accession numbers, are shown in Table 2. Multiple alignments of all sequences obtained and downloaded were performed with the reference mtDNA sequence downloaded from the GenBank (X79547 – Xu and Árnason [1994]) using the MEGA 11 software.

Tab. 2 - Mare family lines along with their accession numbers (GenBank)

| Haplotype | Accession Number | Haplotype | Accession Number | Haplotype | Accession Number | Haplotype | Accession Number |
|---|---|---|---|---|---|---|---|
| Allegra | AY057413.1 | Gaetana | AY057433.1 | MH2L | KP731356.1 | Thais | AF168698.1 |
| A | AY057420.1 | Gratiosa | AF168696.1 | MH3L | KP731357.1 | Trompeta | AF168693.1 |
| Batosta | AF168691.1 | G | AY057426.1 | MH4L | KP731358.1 | T | AY057412.1 |
| Betalka | AF168689.1 | H | AY057419.1 | MH5L | KP731359.1 | U | AY057410.1 |
| B | AY057421.1 | I | AY057427.1 | N | AY057434.1 | V | AY057411.1 |
| Boka | DQ233731.1 | J | AY057428.1 | O | AY057415.1 | Wera | AF168697.1 |
| Capriola | AF168690.1 | K | AY057432.1 | P | AY057414.1 | X | AY057418.1 |
| C | AY057422.1 | L | AY057430.1 | Q | AY057417.1 | Z | AY057429.1 |
| Dubovina | AF168695.1 | Monteaura | AY057416.1 | R | AY057409.1 | Y | DQ233732.1 |
| D | AY057423.1 | M | AY057431.1 | Slavina | AF168692.1 | | |
| E | AY057424.1 | MH1aL | KP731354.1 | Strana | AF168694.1 | | |
| F | AY057425.1 | MH1bL | KP731355.1 | S | AY057408.1 | | |

The multiple alignment was exported to the NEXUS format and converted to a PHY file, suitable for importing into PopART 1.7 (Leigh and Bryant, 2015). In this programme, a Median-Joining network was constructed from the aligned sequences. The MEGA 11 (Tamura et al., 2021) programme was used to build the second type of tree, the Neighbour-Joining tree, from which it was exported to the text format. The iTOL v6. (Interactive Tree of Life [Letunic and Bork, 2024] - https://itol.embl.de/ ) online tool was used for the aesthetic correction of this tree. In order to have a better insight into the distances between the mare family lines analysed, a heat map with numerical values was constructed based on the alignment.

## Results and Discussion

After obtaining the results and analysing 13 sequences of mitochondrial DNA from three classical mare family lines of Lipizzan horses (Drava - Dubovina (n=2), Wera (n=4), Batosta (n=3)), one Croatian mare family line (Thais (n=2)), and the Zora family line from Serbia (n=2), which is currently not recognized as purebred Lipizzan, two different phylogenetic trees were formed. As a result of aligning the sequences and those downloaded from the GenBank, all sequences corresponded to a specific mare family line (Figures 1 and 2). Also, these two figures show that the Zora family line formed a cluster along with several classical mare family line haplotypes of the Lipizzan horses (Monteaura, Betalka, Wera, Allegra).

First, the Median-Joining network has been formed, including loops and multiple evolutionary paths, which displays the topological position of haplotypes as well as the differences in nucleotides between binder haplotypes represented by dashes which tell a lot about the distances between the mare family lines (Figure 1).

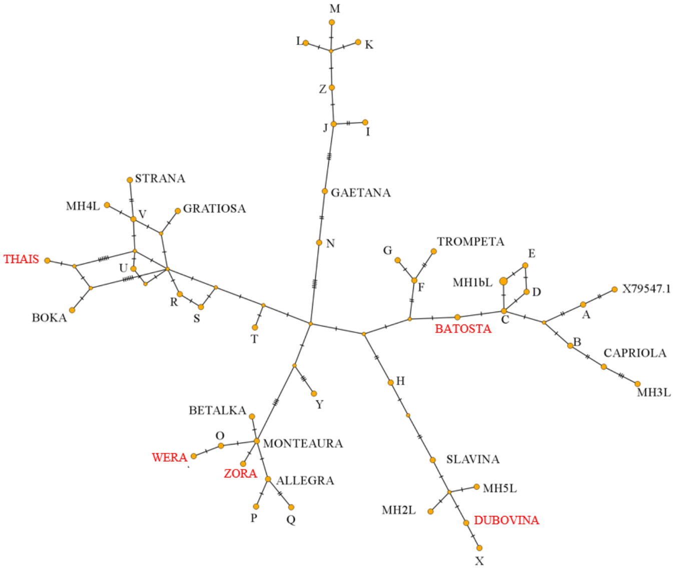

Fig. 1 - The Median-Joining network (A)

The second type of a tree, the Neighbour-Joining tree, was constructed and visualised with colour-coded clusters, that is, groups of haplotypes. The clusters are highlighted in colours, which is significant for visualization and easier recognition of clusters during the construction and presentation of the phylogenetic trees. Also, on this tree, individuals of the Zora family line formed a cluster along with other classical mare family lines (Monteaura, Betalka, Wera, Allegra), highlighted in grey-blue (Figure 2).

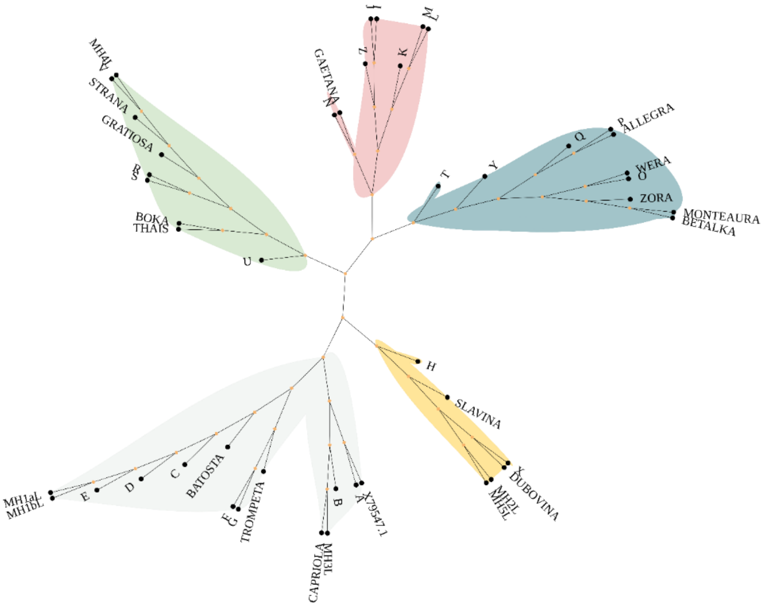

Fig. 2 - The Neighbour-Joining tree (B) constructed for the Lipizzan mare families under study

In the study by Kong et al. (2016), the authors review and comment on the literature in which the Median-Joining network method was used, their conclusion being that this method has almost no virtues for phylogenetic inference. On the other hand, a large number of authors use this method as adequate for phylogenetic analyses, especially those concerning genealogy (Khanshour and Cothran, 2013; Yunzhou et al., 2016; Cieslak et al., 2017; Sheikh, 2023). Also, in addition to the Median-Joining network method, the Neighbour-Joining tree construction method is often used as a different approach to phylogenetic analysis. Depending on the goal of the analysis, one of these two display methods may be preferred. In the case of the genealogy analysis of horse mare family lines, the Median-Joining network gives a more detailed picture as well as several evolutionary paths, while the Neighbour-Joining tree gives only one path of evolution and reduces the complexity of evolutionary relationships.

In the study by Zorc et al. (2023), individuals from the Zora family line formed a compact cluster with other classical mare family lines (Monteaura, Betalka, Wera, Allegra). According to this study, as well as many other studies related to phylogenetic analyses (Kavar et al., 2002; Čačić et al., 2005; Dovč et al., 2006; Cardinali et al., 2016), phylogenetic trees are often found as a means of visualizing the distances between individuals, which indicates that phylogenetic trees are an extremely important representation for this type of research.

## Conclusion

Phylogenetic trees are essential for visualizing genetic relationships and tracing evolutionary lineages. They also play a key role in verifying pedigrees, ensuring the accuracy of lineage tracking and the conservation of rare genetic lines. Results of this study indicate that the Zora family line formed a cluster with other classical mare family lines as well as that, according to the mtDNA sequence, the Zora mare family line could be distinguished from other Lipizzan mare family lines. When displaying the phylogenetic tree and network, it is important to note that the Median-Joining network provides much more information about the Lipizzan horse mare family lines and distances between them. This finding suggests that Zora could play a role in preserving genetic diversity within the Lipizzan population. This research appears to be the second study to include an mtDNA analysis of the Serbian mare family line Zora. However, in addition to the pedigree data, further studies are needed to confirm the authenticity and status of this mare family line within the Lipizzan breed.

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# Анализа митохондријске ДНК и конструкција филогенетских стабала родова кобила липицанске расе

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## Сажетак

Липицанер је раса коња која обухвата 62 рода кобила. Митохондријска ДНК (mtDNK), посебно D-loop регион, игра кључну улогу у праћењу мајчинске лозе и проучавању порекла ових родова. Примарни циљ ове студије је конструисање филогенетских стабала коришћењем метода Neighbor-Joining и Median-Joining, чиме се анализира генетска разноликост и еволутивни односи међу овим родовима. Ова студија обухвата 13 коња из Србије који припадају различитим родовима кобила, укључујући Thais (Rebecca-Thais) (n=2), Драва-Дубовина (Djebrin) (n=2), Wera (Theodorosta) (n=4), Batosta (Africa) (n=3) и Зора (n=2). Секвенциран је D-loop регион, а добијене секвенце су подвргнуте биоинформатичким анализама. Вишеструка поравнања свих добијених секвенци, као и оних преузетих са GenBank базе података извршена су помоћу референтне mtDNK sekvence preuzete sa GenBank-a, користећи софтвер MEGA 11, док су филогенетска стабла конструисана помоћу алата PopART 1.7 и iTOL v6 за визуализацију. Резултати ове студије указују на то да род Зора, иако званично није признат као род липицанера од стране LIF (Lipizzan International Federation), формира кластер са класичним родовима кобила ове расе, укључујући Monteaura, Betalka, Wera и Allegra. Neighbor-Joining стабло је пружило хијерархијски приказ генетских удаљености, док је Median-Joining мрежа открила више еволутивних путева, илуструјући унутарврсну генетску варијабилност.

***Кључне ријечи***: mtDNK, родови кобила, коњи, Neighbor-Joining метода, Median-Joining метода.

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