



Review Article

Y-chromosomal insights into the breeding history and sire line genealogies of two traditional Baroque horse breeds: Lipizzaner and Kladruber

L. Radovic^{a,b}, V. Remer^a, D. Rigler^a, S. Felkel^{a,b,c}, G. Brem^a, B. Wallner^{a,*}^a Department of Biomedical Sciences and Pathobiology, Animal breeding and genetics, University of Veterinary Medicine Vienna, Veterinärplatz 1, 1210 Vienna, Austria^b Graduate School of Population Genetics Vienna, University of Veterinary Medicine Vienna, Veterinärplatz 1, 1210 Vienna, Austria^c Department of Medical Biochemistry and Microbiology, Uppsala University, P.O. Box 256, SE-751 05, Uppsala, Sweden

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ABSTRACT

The paternally inherited, male-specific part of the Y chromosome (MSY) is an ideal marker for studying the origin, genealogies, and historical connections of horse patriline. Here, we performed fine-scaled MSY haplotype (HT) analysis in two Baroque horse breeds, the Lipizzaner and the Kladruber, both known for their long-standing tradition of sire line breeding and interconnected genealogies. We genotyped 95 MSY markers using KASP™ technology in 90 stallions representing all patriline of both breeds. We identified 14 HTs across eight Lipizzaner sire lines and six HTs in eight Kladruber lines. Y-chromosomal analysis confirmed the presumed Arabian and Iberian origins of two Lipizzaner and two Kladruber foundation sires. Interestingly, six Lipizzaner sire lines clustered into the MSY haplogroup (HG) daC.Tb, a HG previously associated with the Turkoman horse, suggesting a Turkish origin of several Lipizzaner foundation sires, contrary to documented records. We also found evidence for an undocumented Arabian founder in the Lipizzaner breed. Furthermore, three private HTs were detected in Kladruber horses, highlighting the uniqueness of their foundation sires. HTs in shared patriline between Lipizzaner and Kladruber were consistent, while three Lipizzaner sublines showed discrepancies between MSY results and recorded pedigree data. In conclusion, MSY haplotyping validated historical breed documentation, revealed new insights into the origins and distribution of sire lines, and proved effective in resolving parentage issues across generations.

1. Introduction

Horses have a long-standing tradition of stallion-centered breeding, with a few foundation sires dominating the paternal lineages in many breeds [1]. The male-specific part of the Y chromosome (MSY) is inherited without recombination from father to sons, resulting in all members of a sire line carrying the same MSY haplotype (HT) [2]. This makes MSY HT analysis an ideal method for reconstructing the male demographics of horse breeds. Robust MSY HT analysis using biallelic markers has been established recently [3,4]. Most modern horse breeds cluster into a haplogroup (HG) that emerged only around 1,500 years ago (ya) [3–10], with the worldwide dominance of the so-called ‘Crown HG’ (‘daC’) attributed to HTs of influential foundation sires, such as the English Thoroughbred [4] and Arabian [9]. Their HTs within the Crown have been delineated, and their over-regional impact demonstrated [4, 11,12]. MSY haplotyping has also been used to resolve historical paternity disputes in Thoroughbreds and Arabians [3,9].

Here, we investigate MSY patterns in Lipizzaner and Kladruber

horses for the first time. Both breeds were established in the 16th century within the Habsburg Monarchy and symbolize the Baroque period of European history [13,14]. The Lipizzaner, renowned for its performances at the Vienna Spanish Riding School, was created in 1580 as a noble horse for shows and parades. Concurrently, the Kladruber was established as a heavy carriage and riding horse [13,14]. The foundation stock for both breeds include horses from the Slovenian Karst region, Iberian, Neapolitan, Arabian and German and Danish breeds [13,15].

For nearly four centuries, Lipizzaner have been bred at state-owned stud farms across Slovenia, Austria, Croatia, Hungary, Italy, Romania, and Slovakia under differing breeding goals, witnessed by deep pedigree records [15,16]. Line breeding is a strong tradition, mirrored in the naming system: similar to surnames in humans, a male Lipizzaner foal is named after its sire line (first) and dam line (second) [15,17,18]. Out of the 89 foundation sires, only six classical and two additional sire lines are known today. These include the classical sire lines after the foundation sires Conversano, 1767, Favory Senior, 1779, Maestoso, 1739, Neapolitano, 1790, Pluto, 1765, and Siglavy, 1810, along with two

* Corresponding author.

E-mail address: barbara.wallner@vetmeduni.ac.at (B. Wallner).<https://doi.org/10.1016/j.jevs.2024.105252>

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additional lines after Incitato, 1802, and Tulipan, 1860 [19] (Table 1).

The Kladruber population, bred at the Kladruber nad Labem National Stud, includes five classic foundation lines - after Generale, 1787, Generalissimus, 1797, Sacramoso, 1800, Solo, 1927, and Favory Senior, 1779 - as well as three non-classical lines: after Siglavy Pakra, 1946, Romke, 1966, and Rudolfo, 1968 (<https://www.nhkladruby.cz/en/>; last access 27.09.2024). Although the initial population exhibited various colours, only grey and black varieties have persisted since the late 18th century. The breed has faced numerous bottlenecks due to shifts in breeding goals, wars, and other historical events, nearly leading to extinction [20]. Great efforts have been made to preserve the breed, including the introduction of non-classic lines featuring Lipizzaner, Lusitano and Friesian stallions [13,20].

To date, only autosomal and maternally inherited mitochondrial DNA (mtDNA) variation has been utilized to study the breeds' genetic diversity and population structure [17,21–28]. By studying MSY patterns in Lipizzaner and Kladruber horses, we aim to i) unravel the origin of sire lines, ii) illustrate the connection between the breeds and iii) uncover possible incongruences in pedigree records that occurred generations ago.

2. Materials and methods

2.1. Ethical approval

The study was discussed and approved by the institutional ethics and welfare committee of the University of Veterinary Medicine Vienna in concordance with Good Scientific Practice guidelines and national legislation (ETK-10/05/2016). The biosamples for Lipizzaner, and most Kladruber horses were collected within the scope of the EU-INCO Kopernikusproject: 'Biotechnical methods in the maintenance of the genetic diversity in the Lipizzan horse breed' (Project No IC15CT96-0904) in the 1990s and archived at the University of Veterinary Medicine Vienna. Permission for the scientific use of samples and corresponding pedigree data was granted by all involved horse owners and stud farms. All included samples are coded to ensure the confidentiality of individuals; details on samples are provided in Supplementary Table S1.

2.2. MSY haplotyping to decode paternal histories

2.2.1. The concept of tracing patriline

A combined analysis of pedigree information and MSY HTs was conducted to gain accurate insights into male ancestry over time. Pedigrees capture historical relationships among family members, documenting both paternal and maternal ancestors (Fig. 1a). The MSY and mtDNA serve as genetic mirrors of patrilineal and matrilineal descent (sire line and dam lines, respectively), as illustrated in Fig. 1b [9,29]

Genetic variation in the non-recombining region of the Y chromosome defines an individual's MSY HT, while the MSY HTs within a breed reflect the history of its male lineages. The relationships among MSY HTs are typically represented as a phylogenetic tree (Fig. 1c). The MSY phylogeny used here is based on a comprehensive sequencing study [3] and includes data from 170 horses, comprising 14 Lipizzaner and three Kladruber horses.

The MSY tree follows a hierarchical structure, with branches reflecting genetic relationships among HTs. The hierarchy of the tree begins at the root, representing the most recent common ancestor (MRCA) of all HTs in the phylogeny. It branches out at internal nodes ('*HTs') and culminates in terminal nodes that represent HTs in current lineages. A group of related HTs that share a common ancestor is categorized into haplogroups (HGs). The hierarchical structure of the MSY phylogeny is also reflected in the HT nomenclature, where major HGs are assigned primary labels, and further divergences receive additional suffixes (e.g., daC_Ao-aA1 - daC_Ao-aA1a - daC_Ao-aA1a1, daC_Ao-aA1a2, daC_Ao-aA1a3, etc.). This naming convention follows established human guidelines [30] and is detailed by Remer et al. [9].

MSY HTs are stable across generations, contributing to a robust MSY HT phylogeny in which a single mutation per branch can reliably identify an individual's HT (HT determining variant). Over time, some HTs have been lost, and their allelic combinations cannot be retrieved from extant samples, while certain HGs have persisted and represent extant patriline. Additionally, new (*de novo*) mutations can arise within the timeframe of pedigree records, resulting in sublines of a patriline that carry slightly different, derived HTs [3,9].

2.2.2. Sampleset

Documentation of paternal ancestry from studbooks and pedigree records was considered to design the dataset. We first reconstructed paternal male tail lineages, encompassing the names, birth years, birthplaces, and breed affiliations of ancestors, which were stored in string format as previously described [9,31]. Based on this information, we selected 66 Lipizzaner stallions from seven state studs: Austria=19, Croatia=5, Hungary=7, Italy=9, Romania=14, Slovakia=3, and Slovenia=9, representing six classical and two additional sire lines. The 24 Kladruber horses chosen from the Kladruber nad Labem state stud farm encompassed all existing patriline, including both color varieties of the breed. Blood or hair root samples were collected from all horses, and information on samples - including breed, biosample, sampling location, patriline and reconstructed pedigree records - is provided in Supplementary Table 1.

2.2.3. DNA isolation

Genomic DNA was extracted using the nexttec™ DNA Isolation Systems (Hilgertshausen, Germany) from hair roots or blood, following the standard protocol. DNA was diluted to a standardized DNA concentration of 5 ng/μl using TE buffer.

2.2.4. Genotyping

We used the most recent horse MSY phylogeny, which included 14 Lipizzaner and three Kladruber horses [3,9], to construct a downscaled topology based on 95 HT-determining 'key' variants (89 SNPs, five short indels, and one microsatellite; Supplementary Table S2 and Figure S1). This structure defines 94 MSY HTs and serves as the backbone for our HT analysis. The determination of variant allelic states was conducted following the hierarchy of the downscaled HT structure up to the terminal branches, as previously described [9]. Genotyping was performed via competitive allele-specific PCR genotyping method (KASP™, lgcgroup.com) on a CFX96 Touch® BioRad Real-Time PCR machine, using the standard KASP™ genotyping protocol (lgcgroup.com). Each reaction included samples with known allelic states as positive controls, while DNA from females and water served as negative controls. Raw data were obtained using Bio-Rad CFX Manager 3.1® software (BioRad).

2.3. Data analysis

Genotyping results were catenated into a single file, and allelic states of untested variants inferred from the HT structure [3], were manually imputed, as previously described [10]. A median-joining haplotype network was constructed using Network 10.2 [32] and the output was converted into an HT frequency plot using Canva Pro (Canva, <https://www.canva.com/pro/>, last accessed on September 27, 2024). HTs assigned to internal branching points are indicated with an asterisk (*).

3. Results

3.1. MSY HT spectra in Lipizzaner and Kladruber sire lines

We determined MSY HTs in 66 Lipizzaner and 24 Kladruber stallions according to a published HT phylogeny [3], which included the expected HTs for both breeds [3,9]. Pedigree-based sampling was conducted to ensure representation of all active sire lines, including their sublines, with the dataset (Table 1).

Table 1

Representation of Lipizzaner and Kladruher sire lines in the dataset, including information of the foundation sire, the number of stallions in the dataset representing each line (n), the common ancestor of the stallions in the dataset and the HTs detected. Details are given in Table S1.

Breed	Sire lines and details on foundation sire of the line	n	Common ancestor of stallions in the dataset	MSY HT
Lipizzaner	Pluto, 1765; gray Spanish stallion from the Royal Danish Stud Frederiksborg; classical line	7	Pluto Fantasca 10, 1895, Fagaras	daC_Tb-oB4b (n = 7)
	Conversano, 1767; black original Neapolitan; classical line	12	Conversano SlatinaIII, 1887, Lipica	daC_Hs-aS2* (n = 10) daC_Hs-aS2b (n = 2)
	Maestoso, 1739; grey, crossbred Neapolitan-Spanish stallion from the court stud farm in Kladrub; classical line	12	Maestoso Perletta, 1854, Lipica	daC_Tb-oB4* (n = 7) daC_Ao-aA1a2a (n = 5)
	Favory Senior, 1779; dun Kladruher stallion from the court stud farm in Kladrub; classical line	12	Favory Moscovita, 1822, Lipica	daC_Tb-oL2* (n = 2) daC_Tb-oL2a (n = 3) daC_Tb-oL2b (n = 3) daC_Ao-aA1a2+ (n = 4)
	Neapolitano, 1790; original Neapolitan, from Lipica; classical line	7	Neapolitano Mahonia, 1868, Lipica	daC_Tb-oL* (n = 6) daC_Tb-oL3 (n = 1)
	Siglavý, 1810; original Arabian imported from Syria; classical line	11	Siglavý Slavina III, 1893, Lipica	daC_Ao-aA1a2* (n = 5) daC_Ao-aA1a2a (n = 2) daC_Tb-oB4b (n = 3) daC_Tb-oL2b (n = 1)
	Tulipan, 1860; youngest sire line, originating from Terezovac Croatia; non-classical line	4	Tulipan Akarhogy, 1898, Terezovac	daC_Tb-dW3 (n = 4)
	Incitato, 1802; from a Transylvanian private stud transferred to the Hungarian stud farm Mezöhegyes; non-classical line	1	Incitato XIII-2, 1991, Szilvássvár	daC_Tb-oB* (n = 1)
	Generale, 1787; Kladruher from Slovakia (Kopčany), grey variety; classical line	3	Generale XLV, 1975	daC_T2k (n = 3)
	Generalissimus, 1797; Kladruher from Slovakia (Kopčany), grey variety; stems from Generale, 1787; classical line	3	Generalissimus Amadeus XXX, 1978	daC_T2k (n = 3)
Kladruher	Sacramoso, 1800; Kladruher from Moravia (Kroměříž), black variety; classical line	6	Sacramoso XXX, 1927	daC_Ao-nM1b (n = 6)
	Solo, 1927; Kladruher from Kladruby nad Labem National Stud, black variety; stems from Sacramoso, 1800; classical line	3	Solo Magnifica VII, 1975	daC_Ao-nM1b (n = 3)
	Favory Senior, 1779; Kladruher from Kladruby nad Labem National Stud, dun; classical line	3	Favory XVIII, 1933, Babolna	daC_Tb-oL2b (n = 3)
	Siglavý Pakra, 1946; Lipizzaner from Croatia (Đakovo), black variety; non-classical line	2	Siglavý Pakra Barbara III, 1984	daC_Ao-aA1a2a (n = 2)
	Romke, 1966; Friesian horse from Netherlands, black variety; traces back to Nemo, 1885; non-classical line	2	Romke Eleona II, 1980	daC_Ad-bN (n = 2)
	Rudolfo, 1968; Lusitano originating from Portugal, grey variety; non-classical line	2	Rudolfo I, 1984	daC_Ao-nM1a (n = 2)

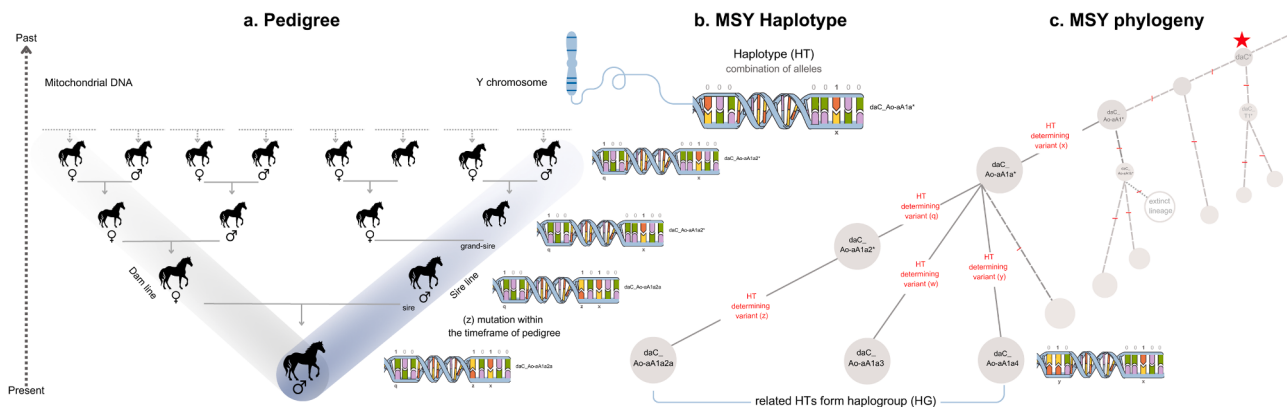


Figure 1. The concept of patriline tracing with pedigree and MSY information. a) The panel illustrates the pedigree, highlighting the maternal (dam) line traced through mitochondrial DNA and paternal (sire) line traced through the Y chromosome. b) An MSY haplotype (HT) is the combination of alleles (0/1) at polymorphic markers (here for example q, w, x, y, z). An example of a HT in a male tail lineage is shown, including the emergence of a new HT through a *de novo* mutation in the timeframe of the pedigree and corresponding HT names. c) A simplified representation of the horse MSY HT tree is shown, with the most recent common ancestor (MRCA) of the Crown indicated by a red star. HT determining variants are indicated in red on the branches. Internal nodes (*HTs) and the HTs of extant lineages are labeled on the terminal nodes. As illustrated at the bottom, closely related HTs are grouped into haplogroups (HG).

Among 66 Lipizzaner males descending from eight foundation sires, we detected 14 distinct Crown HTs, while six HTs were observed in 24 Kladruher horses tracing back to eight founders (Fig. 2, Table 1 and Table S1).

In Lipizzaner, all stallions from the Conversano line clustered into HG daC.Hs-aS2, carrying either daC.Hs-aS2* ($n = 10$) or the derived HT daC.Hs-aS2b ($n = 2$). Other than in Lipizzaner, the HG daC.Hs-aS has so far only been detected in North African, South American and Iberian breeds [3,4,6,10]. Thus, the MSY HT results align with the presumed Iberian origin of the foundation sire of the line. The majority of Lipizzaner stallions from the Siglavy line ($n = 5$) clustered into HG daC.Ao-aA1a2*. This HT was identified as an Arabian signature and has been previously described in the Siglavy sire line [9]. Two stallions tracing back to Siglavy Capriola IV, 1931, carried the derived sub-line HT daC.Ao-aA1a2a, which emerged within the timeframe of pedigree documentation, as previously described [9].

A remarkable pattern was observed on the branch of HT daC.Ao-aA1a2a, determined by two variants (qEZ and qDK) according to [9], (see Figure S1 and Supplementary Table S2). Four stallions from the Favory line, descending from Favory Montedora, 1841, exhibited the derived allele for the qEZ variant but surprisingly carried the ancestral allele for qDK, contrary to the expected uniform result from the MSY phylogeny. Consequently, these stallions introduced a previously undescribed intermediate HT, defined here as daC.Ao-aA1a2+ (see Fig. 2 and Figure S1).

From the remaining six Lipizzaner sire lines (Favory, Maestoso, Incitato, Neapolitano, Pluto, Tulipan), 38 stallions grouped prominently into the HG daC.Tb. This HG has been previously reported to include various breeds, such as Thoroughbreds, Warmbloods, Akhal Teke, Turkoman, and Arabian horses [3,4,6,9,10].

Considering the diverse origins of the six foundation sires (Table 1), we expected them to cluster on different branches of the Crown topology. However, we observed a joint grouping of males from the Neapolitano ($n = 7$) and Favory ($n = 8$) lines within the HG daC.Tb-oL, as well as a clustering of the Maestoso and Pluto lines in HG daC.Tb-oB4 ($n = 7$ for each). Notably, all representatives of the Favory line in our dataset, descending from Favory Moscovita, 1822, carried HTs that are downstream to the Neapolitano HTs (daC.Tb-oL* and daC.Tb-oL3): two males in daC.Tb-oL2*, three in daC.Tb-oL2a, and three in daC.Tb-oL2b. Similarly, the five stallions from the Pluto line carried daC.Tb-oB4b, which is downstream of the HT found in the Maestoso line (daC.Tb-oB4*).

The four males tracing back to Tulipan, the founder of a non-classical line, formed a distinct subbranch, daC.Tb-dW3. The HG daC.Tb-dW is

attributed to the Thoroughbred lineage originating from the stallion Eclipse, 1764 [4]. The single stallion from the Incitato line in our dataset was located at the internal branching point daC.Tb-oB*. Interestingly, previous sequencing efforts did not reveal any unique HT determining markers for this line [9].

In Kladruher horses, the most prominent HT was daC.Ao-nM1b, as observed in nine males from two sire lines, followed by daC.T2k, found in six individuals from two sire lines. At the sire line level, the six males carrying the HT daC.T2k belong to the classical lines of Generale and Generalissimus, with three samples from each patriline. This shared HT is expected since the founder of the Generalissimus line in our dataset is the son of a Generale stallion (Generalissimus Amadeus XXX, 1978), ensuring a direct paternal lineage. An analogous situation applies to the HT daC.Ao-nM1b, which was reported in six individuals of the Sacramento line and three from its successor sire line - Solo, both tracing back to Sacramento XXXI, 1927.

All analyzed males from the Favory patriline in Kladruher horses carried the HT daC.Tb-oL2b, consistent with findings from the Lipizzaner data. This congruence is expected, as the foundation sire of the Kladruher stallions in our dataset was the Lipizzaner stallion Favory XVIII, 1933 from Babolna (Supplementary Table S1).

Among the non-classical Kladruher sire lines, the two males descended from Rudolfo carried the HT daC.Ao-nM1a. This HG was previously reported in several breeds, including Hafflingers, Norikers, Arab-Barbs, and Lusitano horses [3,4,6,9,10]. Since the foundation sire Rudolfo, 1968 was a Lusitano itself, the MSY pattern aligns with the Iberian origin of this founder [25].

Additionally, Kladruher horses from the non-classical line founded by the Friesian stallion Romke, 1966, exhibited the HT daC.Ad-bN. Kladruher horses descended from the Lipizzaner stallion Siglavy Pakra, 1946 carried the HT daC.Ao-aA1a2*, which is characterized in Lipizzaner Siglavys.

3.2. Forensic property of MSY reveals pedigree incongruences

Comparing MSY HTs with paternal lineage documentation revealed three derived subline-specific HTs based on recent *de novo* mutations (Fig. 3). In some genealogies the MSY HTs did not accord with the paternal lineage documentation in the pedigree. Such inaccuracies become evident when MSY results signal two distinct HTs in horses descending from the same founder. We observed three apparent incongruences in classical Lipizzaner sire lines. First, among the twelve analysed stallions from Maestoso patriline, five males - descended from Maestoso IX, 1975 from Szilvasvarad - exhibited the Siglavy HT daC.Ao-

aA1a2a (Fig. 2a). Second, in the Siglavy line, three individuals - descended from Siglavy Bona II, 1929 from Lipica - displayed the Pluto HT daC_Tb-oB4b, while one stallion - descended from Siglavy Santa VIII, 1963 from Đakovo - carried the Favory HT daC_Tb-oL2b (Fig. 2b). Last, four individuals from the Favory line - descended from Favory Slava II, 1931 from Stančić - carried the herein newly defined intermediate HT daC_Ao-aA1a2+ (Fig. 2c); (see Fig. 2 and Supplementary Figure S1).

4. Discussion

Horses have been vital to human history and culture [33] and throughout history humans have shaped horses according to desired phenotypes of certain time points [34]. This was mainly conducted via stallions and therefore, resolving the sire lines in horse breeds with Y-chromosomal markers offers valuable insights into the development of breeds. Lipizzaner and Kladruber horses are the hallmarks of the Baroque era and thus hold significant historical and cultural importance [13]. By analyzing their MSY HT spectra, we verified the written ancestry of their sire lines, found some inconsistencies and drew conclusions about the breeding history that extend beyond traditional pedigree records.

Genotyping of 90 individuals in our dataset revealed 18 HTs (Fig. 2), with 14 HTs identified in the 66 Lipizzaner and six HTs in the 24 Kladruber horses analysed. The MSY HT spectrum of Lipizzaner strongly differs from that of the Kladruber horses, consistent with studies based on autosomal microsatellite markers, which also showed a clear genetic difference between the two breeds [35]. Lipizzaner and Kladruber horses shared only two MSY HTs: daC_Ao-aA1a2* and daC_Tb-oL2b, both of which originated from identical sire lines.

Our findings confirmed the Arabian and Iberian influences on the Lipizzaner breed (see Fig. 2). The use of Arabian sires to improve and constitute different breeds through history is well known [9,10,13]. The paternal Arabian influence in the Lipizzaner breed traces back to the original Arabian stallion Siglavy, 1810, imported to Europe from Syria in 1814. The Arabian origin of the foundation sire of the Siglavy line was confirmed from Y-chromosomal results, as the majority of members from this line clustered into an Arabian-defined HG [9]. The Arabian influence in the Lipizzaner breed was also reinforced from the maternal side when numerous dam lines descended from Arabian founder mares were brought to Lipica between 1830 and 1865 [15,36]. Interestingly, we detected an intermediate Arabian HT daC_Ao-aA1a2+ in the Favory lineage descended from Favory Montedora, 1841. This HT allocates basally to the HTs detected in Siglavys (Fig. 2), suggesting an undocumented introgression of the ancestral Siglavy HT into Favory lineage during the 19th century. This could have occurred between 1841 and 1890 at the Lipica stud - as both lines were active there at that time, and all Siglavys after Siglavy Slavina, 1893 already carry the derived HTs. Alternatively, it may indicate the influence of an unrecorded Arabian founder within the Lipizzaner breed. Given the current knowledge, we cannot explicitly distinguish between these two scenarios. However, the extensive screening of Arabian lineages by Remer et al. [9] did not identify this HT in any other Arabian lineage, which supports the first scenario.

In Kladruber horses, the Arabian MSY signature also traces back to the Siglavy lineage, through the Lipizzaner Siglavy Pakra, 1946, who was introduced recently to aid breed restoration. The MSY HTs align with its documented ancestry, underlining the connection between the two analyzed breeds.

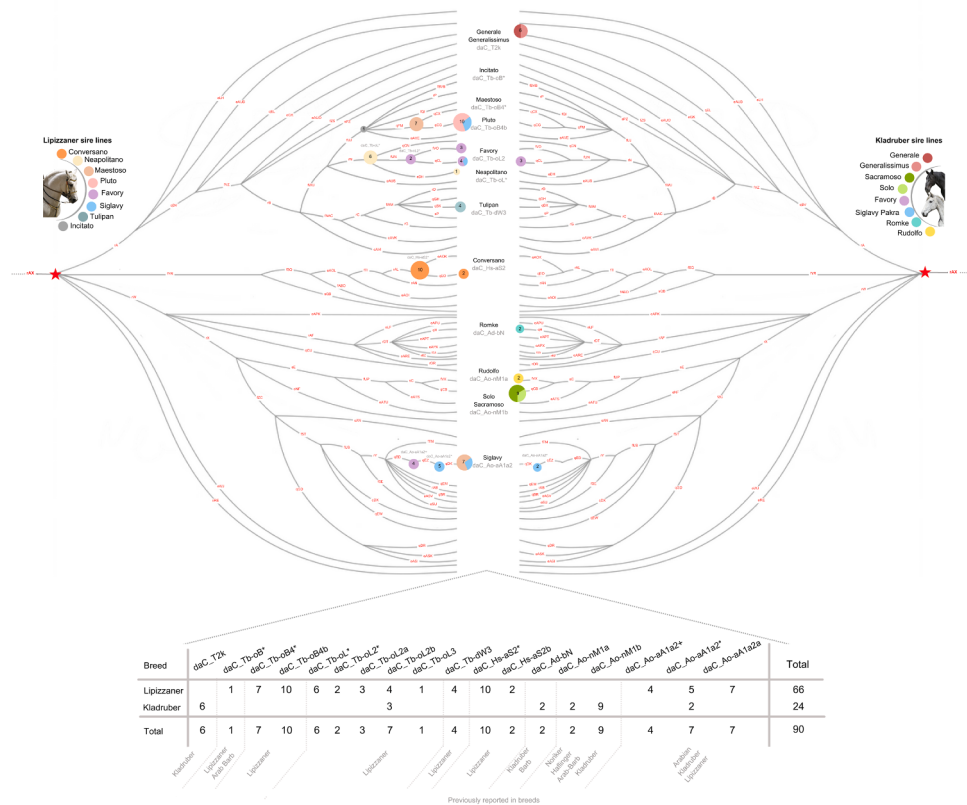


Figure 2. MSY haplotypes in Lipizzaner and Kladruber horses. HT frequency plot based on the MSY tree after Bozjak et al. [3]. HT determining variants used for genotyping are denoted on branches in red. Clustering of MSY HT in 90 horses (66 Lipizzaner and 24 Kladruber horses) based on genotyping is illustrated as pies. Pie radiuses are scaled to the number of individuals and colors correspond to foundation sires. The major HTs found in each sire line is given in the center. Red stars indicate the common ancestor (root) of the “Crown” (daC) HG. A summary of all detected HTs with corresponding number of individuals in each breed is given in the bottom panel. Details on individuals are listed in Supplementary Table S1 and information on screened variants can be found in Supplementary Table S2.

Our findings also highlighted the influence of Iberian stallions in both Baroque breeds. In Lipizzaner, an Iberian ancestry is evident in the Conversano line, where all males carry HT daC_Hs-aS2*. This HG has been previously reported in breeds of Iberian origin [3] as well as North African horses [6], which have historical ties to the Iberian Peninsula. In Kladruber, the presence of the HT daC_Ao-nM1a in Rudolfo's sire line further corroborates Iberian ancestry, consistent with its occurrence in Lusitanos [3].

As shown in Table 1, the foundation sires in Lipizzaner originate from different European regions, and the source breeds were mostly Iberian/Neapolitan horses. Remarkably, individuals from six sire lines carry HTs in the daC_Tb, a frequent HG in Thoroughbreds, Akhal Teke, Arabian, Turkoman and many breeds from the Middle East [3,4,6,9,10]. This clustering could indicate that MSY HTs of West Asian origin permeated and got established in the early phase of Lipizzaner breed consolidation in the early 19th century [10].

Numerous migrations and war conquests were sweeping through Europe at that time and severely affected the herds. For instance, in 1796, all Lipizzaner horses needed to be relocated from the Lipica stud to Hungary, where they stayed until 1798. From 1805 to 1807, the Lipizzaner stock from Lipica was housed in Đakovo and Karád; shortly after, in 1809, the horses were moved near the Mezöhegyes stud farm in Hungary, where they remained for six years [37]. In the course of these and many other relocations [19] it is possible that unrecorded horses of Turkish origin have influenced the breed. Nevertheless, grouping of the

Lipizzaner sire lines – a classical Baroque type breed – into the MSY HG daC_Tb was sudden and needs further characterization.

Another intriguing speculation drawn from MSY haplotype topology regarding the origin and relatedness of Lipizzaner foundation sires is highlighted by the clustering of the Favory stallions (daC_Tb-oL2*/a/b) downstream of Neapolitanos (daC_Tb-oL*), and Plutos (daC_Tb-oB4b) downstream of Maestosos (daC_Tb-oB4*). It can be speculated that in the 19th century, the Maestoso line may have integrated into the Pluto line, while the Neapolitano line could have merged with the Favory line, though both events remained unrecorded. However, we detected an unambiguous Thoroughbred influence in Lipizzaner breed. The daC_Tb-dW HG, found in the Tulipan sire line, has previously been attributed to descendants of the famous English Thoroughbred 'Eclipse, 1876' [4].

Here, we delineated the Kladruber sire lines for the first time through Y-chromosomal analysis, which is particularly interesting given the breed's turbulent history [20]. Previously, strong differences between the Kladruber sire lines were reported based on microsatellite data, specifically regarding color variations [38]. While the Favory, Siglavy, and Romke sire lines are present in other breeds, the Generale/Generalissimus and Sacramoso/Solo lines are private for the Kladruber breed, along with their associated MSY HTs. Specifically, the HTs daC_T2k and daC_Ao-nM1b have not previously been reported in any other breed, and Kladruber's private clustering highlights their uniqueness [13].

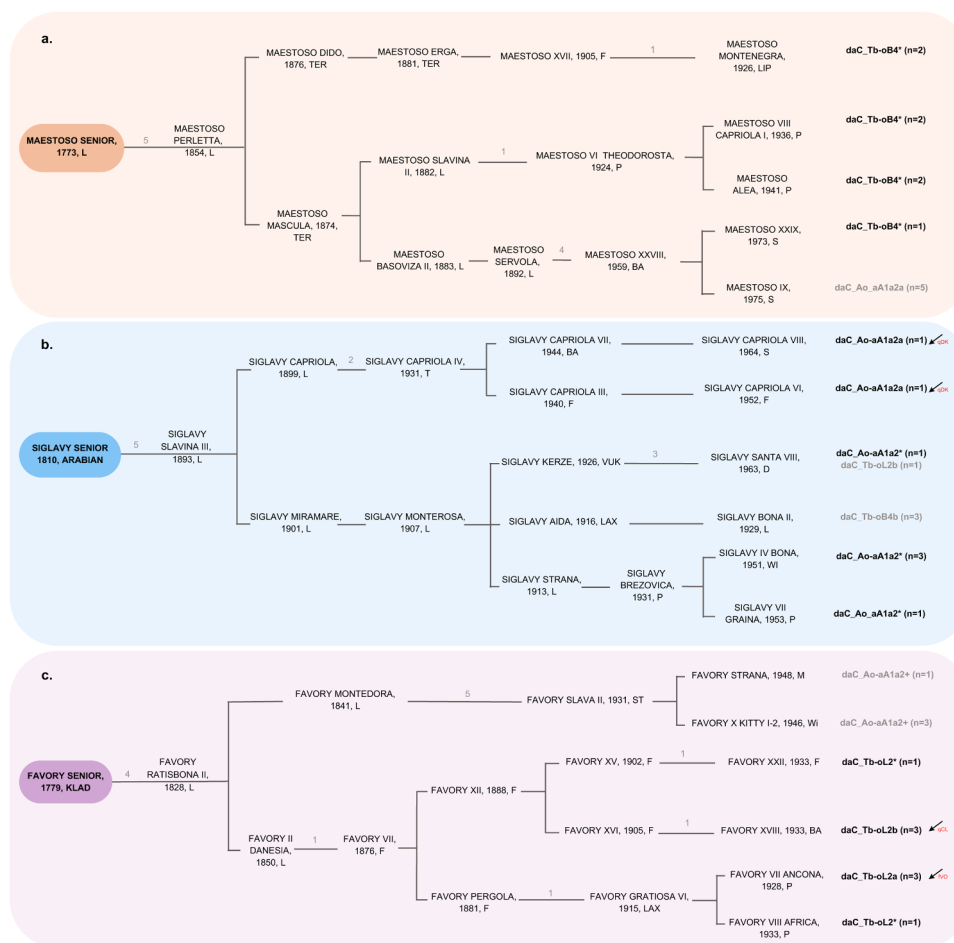


Figure 3. Incongruences between MSY HT and paternal lineage documentation. a) Paternal genealogies of twelve males from the Maestoso stallion line. b) Paternal genealogies of eleven males from the Siglavy stallion line. c) Paternal genealogies of twelve males from the Favory stallion line. For each stallion the name and year of birth is listed, with numbers on the lines indicating the number of omitted generations. The extended name also indicates the stud: Babolna (Ba), Đakovo (D), Vukovar (Vuk), Fagaras (F), Kladruber (Klad), Laxenburg (Lax), Lipica (L), Lipik (Lip), Monte Rotondo (M), Piber (P), Terezovac (Ter), Topolcianky (T), Stančić (St), Szilvasvarad (S), Wimsbach (Wi). MSY HTs are indicated on the right side of the panel, with mismatched HTs in red. *De novo* mutations are marked with arrows and displayed beside them in red. The full pedigree is given in Supplementary Table S1.

The HT daC_T2k detected in Generale/Generalissimus forms a solitary, early separating branch (Fig. 2). This topology does not allow any conclusion of the origin of the foundation sire. In contrast, the HT daC_Ao-nM1b in Sacromoso/Solo clustered closely to daC_Ao-nM2 (a HT previously noted in another Baroque breed - the Fredriksborg horse [3] and Ao-nM1a (a HT previously associated with heavier horses like the Noriker draft horse [3,4,10] corroborates the Baroque/Iberian signature in this Kladruber line.

Fine-scaled MSY haplotyping is ideal for re-evaluating paternal genealogies documented in the pedigree [4,9]. Here, we detected inconsistencies in Lipizzaner horses that may have arisen from errors in record-keeping or misidentification of horses several generations ago (Fig. 3). Similar investigations of Lipizzaner matrilineal lines with mtDNA analysis uncovered more severe pedigree errors within dam lines [39].

However, the importance of sires in breeding programs resulted in more thorough documentation of their contributions, as evidenced by the higher pedigree completeness of stallions [16]. Thus, the higher accuracy of records of paternal lines is expected, making the noted inconsistencies relatively minor. Nonetheless, our results underline the importance and need of verifying parentage via genetic markers to ensure the accuracy of modern horse pedigrees [40].

It is noteworthy that the Kladruber horses in our dataset showed absolute consistency between the MSY signals and pedigree records. This is surprising, considering that the Kladruber pedigrees were lost in a fire at the Kladruber nad Labem stud farm in 1945, which destroyed vital historical records [20]. Thus, the consistency in Kladruber horse data highlights the reliability and robustness of MSY analysis in preserving accurate breed information despite historical disruptions.

Overall, we enlightened the ancestry of Lipizzaner and Kladruber sire lines, paving the way for further exploration of other Baroque type breeds with complex breeding histories. Incorporating historical samples [41] will help validate our hypotheses and further address unresolved questions regarding the presumed or unrecorded ancestry of these breeds. Future research should focus on a broader screening of individuals to support our findings and leverage the exemplified strengths of the Y chromosome for patrilineal tracing and pedigree validation.

5. Conclusion

We highlight the Y chromosome's strength as a genetic marker in Lipizzaner and Kladruber horses, demonstrating that Y-chromosomal variation offers valuable insights into unique heritage of these breeds. Beyond pedigree validation, our findings can enhance breeding programs by supporting the careful selection of breeding stallions to maximize genetic diversity. More broadly, the Y-chromosomal patterns deepen our understanding of equine genetics.

CCRediT authorship contribution statement

L. Radovic: Writing – original draft, Formal analysis, Data curation, Conceptualization. **V. Remer:** Resources, Data curation. **D. Rigler:** Writing – review & editing, Validation, Data curation. **S. Felkel:** Writing – review & editing, Investigation, Formal analysis, Data curation. **G. Brem:** Resources, Funding acquisition. **B. Wallner:** Writing – review & editing, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Declaration of generative AI in scientific writing

Not applicable.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.jevs.2024.105252](https://doi.org/10.1016/j.jevs.2024.105252).

Supplementary Table S1. Detailed information on analyzed samples. Sample IDs of 90 analyzed Lipizzaner and Kladruber horse samples are given in the first column, followed by information on breed, biological source of DNA (biosample), region and country of origin and coat color of the horse, if available. Following information provides sire line and detail pedigree, given as string for each individual from generation 16 until the last known founder. Last, summary of genotyping is given in columns X-AA containing information on most terminal derived variant and HT. If the sample was used in previous study, the information is given in the last column.

Supplementary Table S2. Variant information. Gives information on 95 variants genotyped in the dataset, including HT identifier and corresponding HT determining variant ID and type of variant. For each variant, coordinates on LipY764 reference [4] are indicated along with the reference, alternative, ancestral states, and flanking sequence. Additional notes on KASP genotyping and allelic signals of female controls are given in last column. Column J specifies the study where variant was first published.

Supplementary Figure S1. Genotyping backbone. Downscaled MSY HT phylogeny based on 95 variants ascertained by [3,9]. Selected HT determining variants used for genotyping are denoted on each branch in red and listed in Supplementary Table S2. HT names are given in proximity of the corresponding node. The star indicates the most recent common ancestor (MRCA) of the Crown HG. Triangles mark HTs previously reported in Lipizzaner (blue) and Kladruber (orange) stallions [3,9]. The red X indicates an intermediate HT defined within this manuscript, corresponding to MSY patterns detected in a subline of the Lipizzaner line.

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