Use of Mitochondrial DNA Analyses in Verification of the Lipizzan Horse Pedigree

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Summary

The Lipizzan horse, established in 1580 in Lipica, is one of the most famous horse breed in the world. To verify the authenticity of maternal lineages in Lipizzan horses we analyzed maternal segregation of the nucleotide sequence of the mitochondrial control region (D-loop mtDNA) (nt 15476 – 15834) through the pedigree. The analyses were done for 258 Lipizzan horses sampled mainly in Croatia, State stud Lipik (40), State stud Đakovo (59) and private farms (129) as well as in Bosnia and Herzegovina, State stud Vučijak (19). Out of 24 analyzed mare lines we have found 23 different haplotypes. On a very long pedigree, tracing up to 36 generations, we found inconsistency (18.2%) in the maternal segregation. In several cases we were able to reconstruct the history and origin of the pedigree errors. Here, for populations with complex pedigree structure, we demonstrated the usefulness of the joint mtDNA and pedigree segregation analyses in tracing pedigree errors and maintenance of reliable herd book identification.

Key words

Lipizzan, mitochondrial DNA, pedigree error, maternal lineage

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Aim

Extensive and correct pedigree records are important part of a good stud books and provide valuable information in horse management and breeding. Unfortunately, maintaining correct pedigrees in a long time period, up to 30 generations or longer, is not an easy task. Polymorphic markers such as microsatellites are standard choice in the paternity testing of horses (Binns et al., 1995; Bowling et al., 1997; Lee and Cho, 2006). Simple organization, haplotype variability and the absence of recombination makes mitochondrial DNA (mtDNA) suitable molecular marker for testing correctness of the maternal segregation in a long and complex pedigrees. However, this possibility was exploited only in few studies (Bowling et al., 1998, 2000; Hill et al., 2002; Kavar et al., 2002).

The aim of this research was to test the suitability of mtDNA information as a tool in verification of pedigree correctness in a Lipizzan horse population with complex pedigree structure. Our analysis referred to horses from the state studs Lipik, Đakovo and Vučijak and from private farms in Croatia while pedigree information included whole Lipizzan population (upgraded with study of Kavar et al., 2002) and additional number of mtDNA haplotype sequences taken from the literature (Kavar et al., 2002).

Material and methods

The analysis was based on the pedigree of 7419 Lipizzan horse with pedigrees up to 36 generation long and with the average number of equivalent generations known (horses born from 1973 to 2008 as a referent population) equal to 14.88. The DNA was extracted from 258 Lipizzan horse blood samples (59 horses from the stud Đakovo, 40 horses from the stud Lipik, 129 horses from private breeding and 19 horses from the stud Vučijak) using Sigma blood kit (Sigma-Aldrich, Germany) according to manufacturer's recommendations. Sequencing was done following the procedure described in Aberle and Distl (2007). Thus, a 1260-bp fragment of the horse D-loop mtDNA, including parts of the tRNA-Thr, tRNA-Pro and tRNAPhe regions between nucleotides 15402 and 22, was amplified using the forward (5'-AACGTTTCCCAAGGACT-3') and reverse (5'-GCATTTTCAGTGCCTTGCTT-3') (Invitrogen) primers. The polymerase chain reaction (PCR) protocol was performed in a 20 µl reaction mix containing approximately 50 ng of total DNA, 0.2 µM of each forward and reverse primer and Master mix (Qiagen). The PCR was carried out in a iCycler (Biorad, USA) thermocycler and consisted of: an initial denaturation step at 95^{0}C for 15 min followed by 34 cycles at 94^{0}C, for 45 s, annealing at 62^{0}C for 45 s, and elongation at 72^{0}C for 80 s with a final elongation step of 10 min at 72^{0}C. PCR products were purified using ExoSAPIT (USB, Cleveland, OH) following the manufacturer's recommendations. DNA sequencing was preformed from the PCR product on an ABI 3130 DNA automated sequencer (Applied Biosystems, USA) using the ABI Prism Big Dye Terminator 3.1 Sequencing Kit (Applied Biosystems, USA). Obtained mtDNA sequences were aligned using the program CLUSTAL as implemented in the software MEGA 4.1 Beta 3 (Tamura et al., 2007; available at http://www.megasoftware.net/mega41.html). Aligned sequences were truncated to the length of 358 bp (nt 15476 – 15834, according to Xu and Arnason, 1994; accession number X79547) to enable comparison with results obtained in Kavar et al. (2002). To test the confidence of this sampling and molecular analysis we intentionally sampled 10 horses previously analysed by Kavar et al. (2002) and obtained fully concordant results.

Results and discussion

An example of the maternal pedigree concordance analysis is shown in Figure 1 for a maternal lineage pedigree of mare line Traviata with 44 members. From this maternal linage pedigree we considered 19 mares, 15 typed only in this study, three typed only in Kavar et al. (2002) and one mare typed in both studies. The concordance of the haplotype Dubovina through the segregation of mare line Traviata was confirmed for 12 mares. For one mare, 730 Trofetta XI Đ1992 (see Figure 1), the same haplotype was observed here and in Kavar et al. (2002). Non concordant haplotypes were observed three times for the haplotype Allegra (haplotype characteristic for mare lines Englanderia, Dama and Liza), two times for haplotype L (haplotype characteristic for mare line Wanda) and once for haplotype "MH2" first time observed in maternal lineages of the Lipizzan horses. Thus, calculating only error rate of typed horses we found 31.6% errors (six non concordant horses out of 19 considered horses). One might notice that this is very rigorous criteria as the error rate could have been calculated as a number of erroneous over total number of parent - offspring connections, here 9.5% (4/42). Overall pedigree concordance of maternal segregation for 23 specific mitochondrial haplotypes related to 258 Lipizzan horses is presented in Table 1. For 18.2% of typed horses there was disagreement with genealogical segregation. For 15.5% typed horses disagreement was caused by identity swap between different Lipizzan maternal lineages. Only for 2.7% typed horses we found pedigree inconsistency caused by the appearance of the new haplotypes previously not characterized for Lipizzan breed, due to non-regular introgression of non Lipizzan blood into the breeding. The most frequent pedigree inconsistencies were found in stud farm Vučijak (26.3%). As errors can be traced back to few remote ancestors, the high number of errors in Vučijak is not consequence of imprecise herd book management. More

Table 1. Pedigree concordance of maternal segregation for 23 specific mitochondrial haplotypes for 258 Lipizzan horses

Country	Typed horses	Pedigree concordance (%)	NCMS (NCMSnL)
Croatia	239	197 (82.4)	42 (5)
Bosnia and Herzegovina	19	14 (73.7)	5 (2)
Total	258	211 (81.8)	47 (7)

NCMS - The number of pedigree non-concordant maternal segregations. NCMSnL – The number of pedigree non-concordant maternal segregation caused by the appearance of new haplotypes previously not found in Lipizzan horse. Croatia is represented by Lipik stud, Đakovo stud and private breeding while Bosnia and Herzegovina is represented by Vučijak stud.

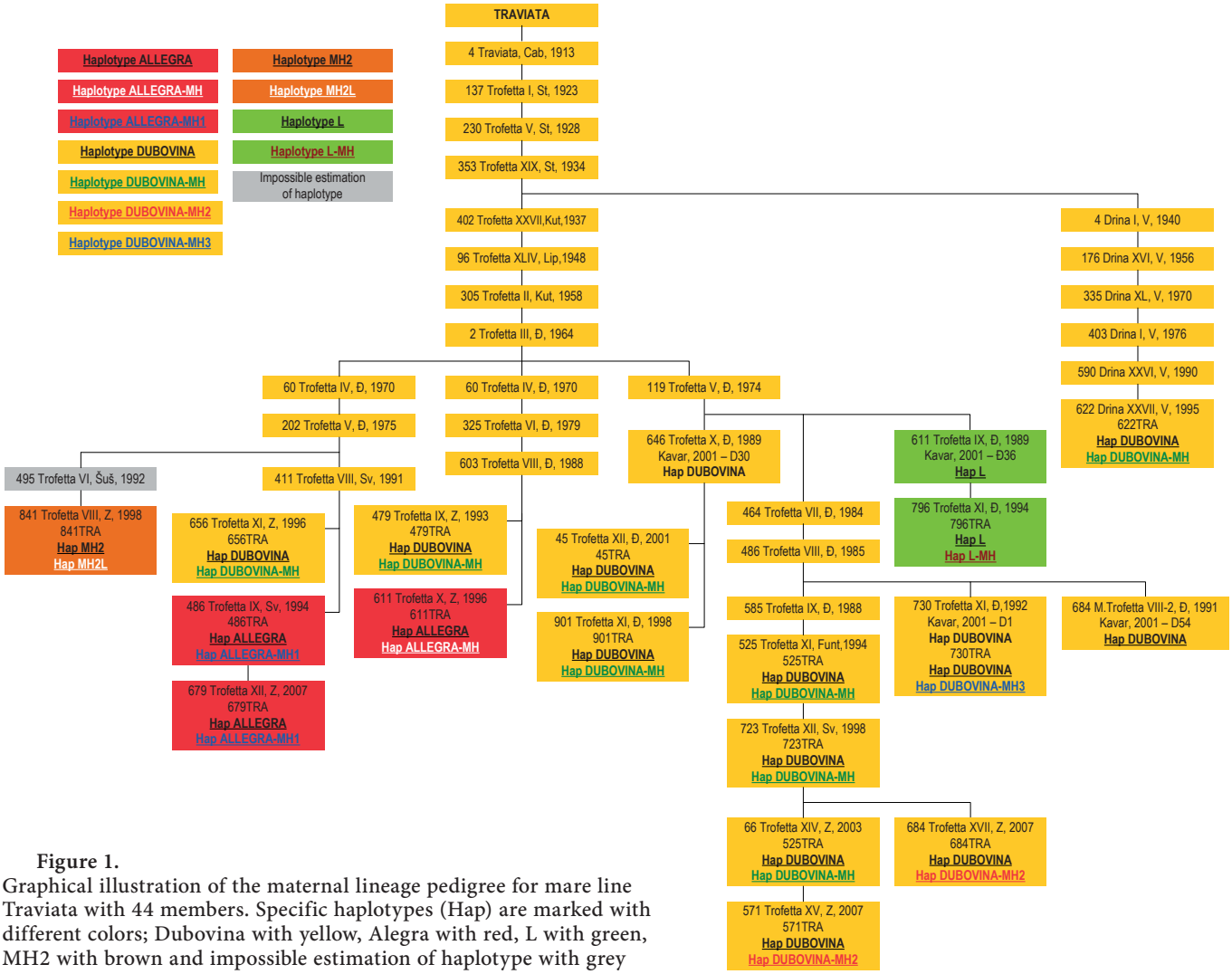

Figure 1.
Graphical illustration of the maternal lineage pedigree for mare line Traviata with 44 members. Specific haplotypes (Hap) are marked with different colors; Dubovina with yellow, Alegra with red, L with green, MH2 with brown and impossible estimation of haplotype with grey

precisely, replacements in mare lines Pliva (mare line Liza) and Neretva (mare line Gidrana) because of pedigree inconsistencies accumulated over the time, is the main reason of inconsistency in stud farm Vučijak. Somewhat less frequent number of errors was found in Croatian breeding (17.6%). There were no pedigree inconsistencies in Lipik. On the other side 18.4% and 23.0% of typed horses had inconsistent maternal lineage in Đakovo and in private breeding, respectively. Unfortunately, inconsistencies were found in important maternal lineages such as Gidrana, Wanda, Munja, Traviata, Almerina, Africa, Siglavy and Margit. No errors were found for Englanderia and Liza, mare lines that have the same haplotype as mare line Allegra. Kavar et al. (2002) have found 25 typed horses with inconsistent maternal segregation (11.79 %) in the sample of 212 Lipizzan horses. The lower error rate might result from the better maintenance of the herd books and/or just by chance i.e. large random maternal contribution of few mares with inconsistent pedigree. In this study, we were able to trace all erroneous segregation up to 17 mares where we suspect replacement (swaps), eight related to mares from Đakovo and nine to private breeding. Those 17 mares left female and male descendants in first, second, third, fourth and fifth generation as follows: 30 : 25, 52 : 42, 46 : 32, 15 : 15 and 6 : 3. Hill et al. (2002), in Thoroughbred horses, also reported large vertical transmission of pedigree errors.

We would like to point that some mare lines, for example Capriola, Allegra, etc…, share the same specific haplotype. For those mare lines we were not able to detect pedigree errors, although they could have been present. By increasing the length of the mtDNA sequence analyzed, additional number of pedigree errors could have been detected. On the other hand, it is also possible that some mare lines would still have the same haplotypes because they share the same maternal origin not recorded by pedigree. The power of finding pedigree errors also depends on pedigree complexity as, for example, in one-lined genealogical topologies the possibility to find inconsistency is limited to chronological sampling (Kavar, 2001).

Conclusions

In a long time period it is very difficult to maintain herd book without making any pedigree mistake. Furthermore, in small and closed populations genetic contribution of certain founders can be very high, so, any pedigree error related to such founders can increase overall number of pedigree errors. Here, we have shown that in populations with complex and long pedigrees their correctness can be supported by analyzing pedigree consistency through maternal segregation of specific mtDNA haplotypes. In such situations described analyses (procedure) can be a useful tool assisting for maintaining the pedigree errors at a very low level.

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