

Characterisation of Non-recognized Maternal Lines of the Croatian Lipizzan Horse Using Mitochondrial DNA

Mato ČAČIĆ^{1*}

Pavo CAPUT²

Ante IVANKOVIĆ²

SUMMARY

Under the assumption that some of the unrecognized Lipizzan lines originate from one of the classical female lines, the main goal of this studies was to prove that stud book data was not kept adequately. Since in the framework of LIF the conducted INCO Copernicus project „Biotechnical methods in the maintenance of genetic diversity of the Lipizzan horse breed“ comprised the research of the genetic structure of the entire studfarm Lipizzan horse population in European national stud farms, it was possible to compare its results to the results of this research and confirm or disprove set hypothesis. Analysed mares in the stud books of the Stud Farm of Đakovo are introduced as representatives of five families: Beba – Elza, Cura – Lela, Lenica – Lasta, Liza – Cica and Liza – Pliva. Characterising of mitochondrial genome has confirmed negligent record keeping of origin in private breeding. Some non-recognised families are descendants of recognised Lipizzan families. Haplotype Allegra is found in the family of Liza-Cica-Pliva, and haplotype Capriola in the family of Cura-Lela. Both haplotypes are very common in the Lipizzaner breed and inherent to classic Lipizzan families.

KEY WORDS

Lipizzan horse breed; private breeding; non-recognized female lines; mitochondrial DNA

¹ Croatian Livestock Center, Department for horse breeding and selection
Ilica 101, 10000 Zagreb, Croatia

*E-mail: mcacic@inet.hr

² University of Zagreb, Faculty of Agriculture
Svetošimunska cesta 25, 10000 Zagreb, Croatia

Received: August 23, 2005

ACKNOWLEDGMENTS

We are grateful to the private breeders of Lipizzan horses and the National Lipizzan State Stud Đakovo for the use of their horses. This original scientific paper is excerpt of the Master's thesis by Mato Čačić „Phenotypic and genetic characteristics of Lipizzan horses in territorial breeding of the Republic of Croatia“.

INTRODUCTION

Year 1580 is considered to be date of the Lipizzan breed foundation, when the Austrian Hungarian archduke Karlo II bought the Lipica estate intending to found a stud farm which would satisfy the court's needs for fine carriage horses (Dolenc, 1980). Particular ways of foundation, influence of a larger number of breeds in development, and different breeding goals of specific countries and regions have caused a large morphological diversity of the Lipizzan population. The wide origin of the breed has resulted in forming a larger number of pedigrees and families. The Lipizzan breed is the result of a long-term and systematic selection, breed has become and is considered as one of the best horse breeds in the world. This "baroque" horse is known for its noble and sturdy shape, resilience, modesty in housing and feeding, "easy" learning ability and will to work, as well as eagerness to tackle with the most difficult tasks.

Lipizzan horse breeding in Croatia has its long tradition in our country, exactly to these characteristics, especially in private breeding. The Lipizzan breed was considered the breed of the future, because it was not suited only for Slavonija, but also for Lika, Dalmacija, Istra and other Croatian regions (Ilančić, 1979). Slavonians accepted the Lipizzaner breed quickly not only because of its already mentioned characteristics which met the requirements of farmer enterprises, but because of spiritual satisfaction (Wichodif, 1899). Slavonian peasants have accepted Lipizzan breed and it took place in every segments of life – from working in field to cultural manifestation (Ljubešić, 1981; 1985). Lipizzan horses reflected prestige and the estate where they were bred, because of what horses the estate had, so much was it worth. Due to all that, the number of horses grew year-in and year-out. Lipizzan breeding in Slavonija became a part of tradition, what can be seen in today's folklore rallies, horse competitions and other events inevitably including Lipizzan horses.

Due to large cultivable areas, Slavonija had the largest needs for working horses, so that a more substantial development of Lipizzan horse breeding (and horse breeding in general) took place there in comparison to other regions (Steinhausz, 1924; 1943). Desirable characteristics of Lipizzan horses stipulated not only quality pure-blood breeding, but a large influence of Lipizzan horses to melioration horse breeding of this territory. It was often used as an ameliorator of other breeds and domestic horses in order to create a larger number of horses with desirable usage and breeding value. That is how the population of incomplete origin or B-book was found. To clear up, Lipizzan of incomplete origin (B-Book) are horses which didn't have a complete origin at the time when the Stud book was closed. While the Lipizzan horse has

become a part of cultural and national customs, horse experts in Croatia have decided that this population should be kept as a following population or B-book of Lipizzan breed despite of no recognition of Lipizzan International Federation - LIF.

What also depicts the development and tradition of horse breeding in Slavonija, are records of many private and state-run stud farms, stallion farms and associations active in the territory, which contributed through their work to horse breeding development in the region in general (Čačić, 1998). Only one Lipizzan stud farm is active in Croatia today – the State Lipizzan Stud Farm in Đakovo, which is together with, one of the oldest stud farms in Europe. The stud farm in Đakovo as the mother stud farm of the Lipizzan breed is the Lipizzan breeding centre in Croatia, and it continually directly and indirectly influences the rise of quality in territorial breeding through its work. The largest part of the territorial subpopulation is connected to the stud farm through horse breeding associations.

Prior to the Homeland War the Lipizzan horse was bred at the stud farm of Lipik as well, but now there are only ruins because the stud farm was destroyed in the first months of the aggression (Stephen et al., 1993). The consequences of the Homeland War aggression left a deep mark in Croatian horse breeding, and this is one of the causes of the decline in the number of horses. The other reason, still present, is low breeding profitability. Considering the share in Croatian horse breeding, the Lipizzan horse is in the third place.

The Stud Farm Đakovo and Lipizzan horse breeders in the Republic of Croatia have been full members of the Lipizzan International Federation – LIF since December 1995 (Mandić and Rastija, 1997).

Within INCO-Copernicus Project: "Biotechnical methods in the maintenance of genetic diversity of the Lipizzan horse breed", maintained from 1996 to 2000, morphology (Zechner et al. 2001), pedigree structure (Zechner et al. 2002) as well as microsatellite (Achmann et al. 2004) and mtDNA (Kavar et al. 1999; Kavar et al. 2002) diversity have been thoroughly studied. The research comprised Lipizzan breed subpopulations bred in 9 traditional European stud farms (Lipica, Szilvásvár, Đakovo, Topolcianky, Beclean, Monterotondo, Piber and Vienna (Spanish Riding School)).

The Lipizzaner breed population in Croatia is divided into two subpopulations; a subpopulation consisting of horses from National stud Đakovo and the other subpopulation consisting of horses kept in private breeding.

Under the assumption that some of the unrecognized Lipizzan lines originate from one of the classical female lines, the main goal of this studies was to

prove that stud book data was not kept adequately. Since in the framework of LIF the conducted INCO Copernicus project “Biotechnical methods in the maintenance of genetic diversity of the Lipizzan horse breed” have comprised the research of the genetic structure of the entire Lipizzan horse population held in European national stud farms, it was possible to compare its results to the results of this research and confirm or disprove set hypothesis.

MATERIAL AND METHODS

The research comprises four unrecognized Lippizan female lines: Liza-Cica-Pliva, Beba-Elza, Cura-Lela and Lenica-Lasta. The mitochondrial DNA (mtDNA) was isolated from blood samples. The blood was taken from the jugular vein 7 ml test-tubes (BD Vacutainer system™) filled with 0,084 ml of preanalytic solution (K3E 15%) and stored at -20 °C. The isolation of the mtDNA was conducted according to the White and Densmore (1992). Testing of isolation efficiency was done on 1% agarose gel in 1 x TBE puffer system (20 min; 5v/cm) and visualisation was done by Macruve UV transilluminator. For the PCR multiplication of the D-loop region we used the first two oligonucleotides, P28 i HF, in central CSB (Conserved Sequence Block). The obtained PCR products were split by teh restrictive endonuclease AluI which splits the products at one site in the recognizable sequence ag/ct. The cutting was conducted using the Webcutter 2.0 (Heisman, 1997). The electrophoresis was conducted on an 8% PAA gel (37,5 : 1 = acrylamide : bisamilacryd) during 14 hours. The Silver Stain Plus Kit, Bioad was used to colour the gel. The PCR reactions with the nucleotides (nt) P28 and HF were conducted in a microprocessed wather thermostat MJ Research PTC – 100 with the following temperature programme after 5 minutes of initial denaturation at 95 °C, folowed 32 cycles; denaturation 94 °C/1 min, fitting 52 °C/30 s, extension 72 °C/1 min and final synthesis 2 min at 72 °C. The analysis was done on an Abi Prism™ 310 Genetic Analyzer laser sequencer in the laboratory of the Department for Zootehnology of the Biotechnical Faculty in Domžale, Slovenija. The obtained sequences were analyzed using the MEGA

2.1. programme package (Kumar et al., 2001) on a segment of 280 nucleotides. The obtained haplotypes were compared to the referential sequences and to the haplotypes of the recognized Lipizzan lines, with NCBI GenBank used as data base.

RESULTS AND DISCUSSION

The result of the comparison of obtained haplotypes to the referential sequence Acc.n:X79547 (GenBank NCBI, 2002) is 14 polymorphic sites. All mutation positions are of transitional type (A↔G; C↔T). Based on polymorphic positions, the samples are arranged into four haplotypes – Z1, Z2, Z3 and Z4 (Table 1). In individual comparison of gained haplotypes with the relative sequence, the number of polymorphic positions was from 4 to 8, and the genetic distance (Tamura Nei - Gamma) from 0.016 to 0.032. The identity of nucleotide sequence is from 97.14% to 98.57%.

The number of nucleotide substitutions between determined haplotypes of the B book mares was from two to 10, and the genetic distance in the range from 0.008 to 0.043. The identity between nucleotide sequence is in the interval from 96.43% to 99.29%. The smallest difference was established between haplotypes Z1 and Z2, and the largest between Z3 and Z4.

The number of nucleotide switches between the established haplotypes of unrecognized lines ranged from 2 (Z1 i Z2) to 10 (Z3 i Z4) and the genetic distance ranged from 0.008 to 0.043. The identity of the nucleotide order is in the interval from 96.43% to 99.29%.

The haplotype of the unrecognized lines were compared to 40 haplotypes found in recognized Lippizan female lines (Kavar et al. 2002). The haplotypes Z1 and Z3 are identical to two haplotypes found in classical lines.

The haplotyp Z1 characteristic for the line Liza-Cica-Pliva is identical to the haplotype Allegra which is typical of the classical line Englanderia and a younger one, Dama. The same haplotype is found in the lines Deflorata, Bradamanta, Fistula and in the Romanian

Table 1. The polymorphic sequence sites of D-loop regions of the unrecognized Lipizzan maternal line and the referential sequence X79547, direction 5'-3', according to the nucleotide ordinal number from 15528 nt to 15807 nt.

X79547	Nucleotide ordinal number														The number of switches
	1553	1558	1559	1560	1560	1560	1564	1565	1566	1570	1572	1574	1577	1577	
Haplotype Z1 - Line Liza-Cica-Pliva	C	G	A	C	T	G	A	A	G	T	G	A	C	A	6
Haplotype Z2 - Line Beba-Elza	T			T	C		G				A		T		8
Haplotype Z3 - Line Cura-Lela		A						G	A		A				4
Haplotype Z4 - Line Lenica-Lasta			G	T		A				C	A	G	T	G	8

line with the ancestress 89 SH XVII. The progenitress of the line Liza-Cica-Pliva is the mare Liza, foaled in 1914. in Široko Polje. From the beginning, the genealogy has been correct from the point of view of the principle of breeding in pure blood. One of the mares from this line is the progenitress of the recognized Lipizzan female line Pliva in Bosnian Lipizzan studfarm in Prnjavor.

The haplotype Z3 found in the line Cura-Lela is identical to the haplotype Capriola characteristic for a great number of classical lines and is also the prevalent haplotype in the lipizzan breed. Except in the Capriola line it was also found in classical lines Sardinia, Deflorata, Bradamanta, Fistula, Argentina, Contesina and Sistiana. The ancestress of the line is the mare Cura, genealogy is very short (some 50 years). But breeding in pure blood was not respected. The father of the third known mare in the lineage is nonius stailon.

The other two haplotypes have not been established in Lipizzan lines. The haplotype Z2 is the most similar to the haplotype Monteaura found in the classical line Spadiglia, the difference is in only one nucleotide switch (15604). The haplotype Z4 shows the greatest similarity to the haplotypes I and Z. The haplotype I is characteristic for the mares of Hungarian origin found at Szilvasvarad stud farm, and the haplotype Z in mares of Hungarian origin. Between haplotypes Z4 and I the switch is in the nucleotide spot 15597, and in reference to the haplotype Z in 15595. The genetic distance between Z2 and Z4 and the named recognized haplotypes (Monteaura, I and Z) is $0,004 \pm 0,004$ and the identity of the nucleotide order is 99,64%.

A dendrogram was constructed based on gained results (Figure 1).

It was mentioned in the text that Liza – Cica and Liza – Pliva families were joined in a unique genealogy under the new name of Liza – Cica – Pliva. Results gained by genome typifying of non-recognised families have stipulated the need for a more detailed review of mare family. The family genealogy is shown in Figure 2, and the line represents the joining point. Joining of families was enabled by mtDNA typifying and a spoken statement by the still living mare breeder of the family about the origin of Pliva family existence. As the family has been bred in his family through generations, the mentioned breeder claims that the living mares of that family are the descendants of mares known under the name of Alka in the stud farm of Đakovo from the time of Bishop Strossmayer. The oldest available origin record is about the founder of bay horse family, mare Liza, foaled in 1914 in Široko Polje near Đakovo, from father 517 Favory.

The origin of the Pliva (Lipizzan International Federation: Establishing acts. 1995; 2001) family is

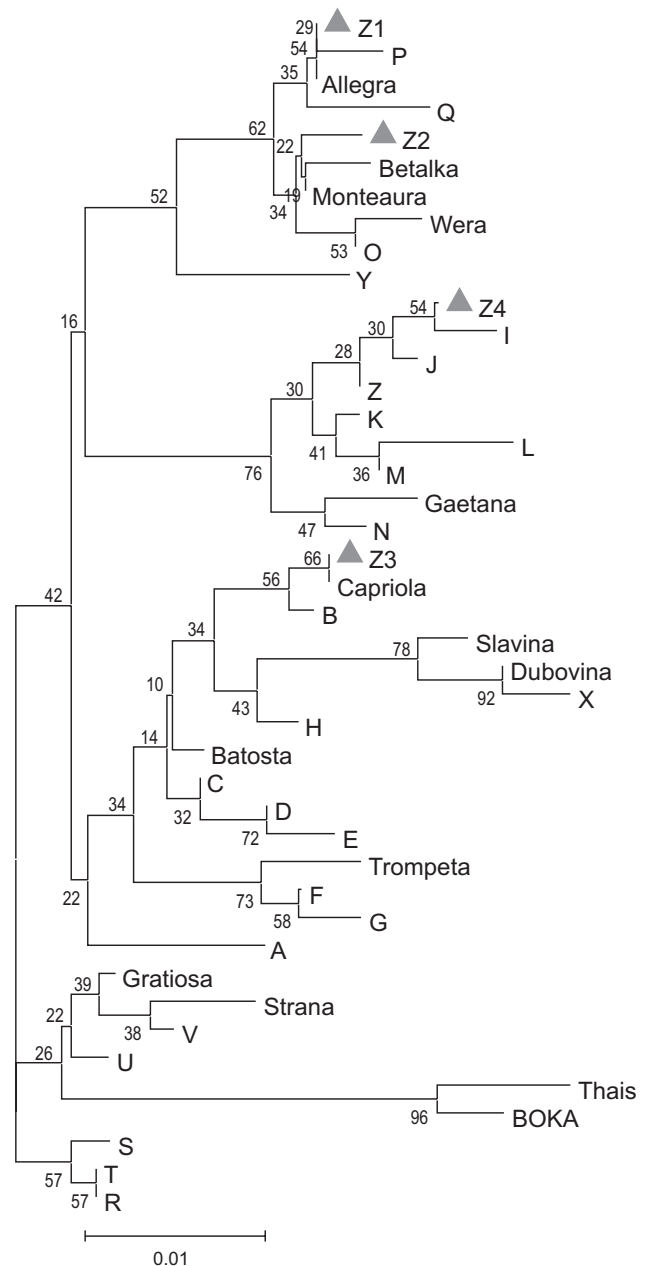
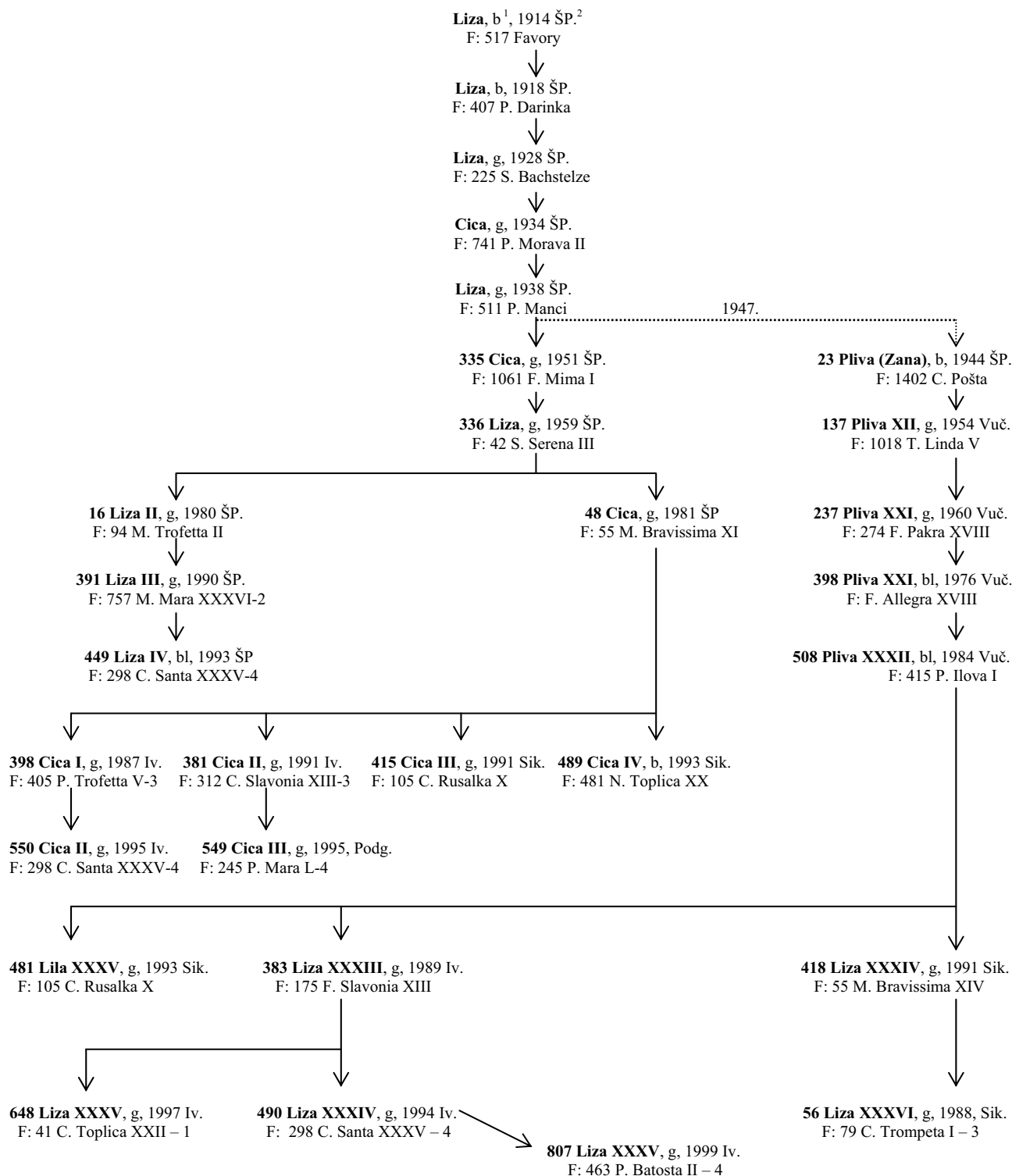


Figure 1. Neighbour joining dendrogram constructed on the basis of Tamura Nei (Gamma) genetic distances (B-book haplotypes are pointed out by a grey triangular marker)

connected to the foundation of the Lipizzan stud farm of Vučjak (Prnjavor) in Bosnia in 1947. The mares which made the initial mother herd came from a number of stud farms, and a part of the mares was shipped from territorial breeding. Mandić and Rastija (2001) claimed that the B-book mares of private breeding were also a part of the initial herd of the stud farm of Vučjak. In the same way, the mare foaled under the name of Zana (1994, m: Liza, 1938, f: 1402 Conversano Pošta, 1931) in Široko Polje in the family enterprise of the breeder mentioned before were leaving to the stud farm of Vučjak as a three-year old, and as families are renamed in this stud farm, it gets a number and a new name: 23 Pliva. Based on the



¹ horse colours: b – bay, g – grey, bl – black:

² place of birth: Iv.-Ivanovac, Podg.-Podgorač, Sik.-Sikirevci, ŠP.-Široko Polje, Vuč.-Vučjak (Prnjavor)

Figure 2. Genealogy of female line Liza – Cica – Pliva

results of research, it can be concluded that mare 23 Pliva (Zana) is not a founder of a new family, but of a Bosnian family branch by the name of Liza – Cica – Pliva, which is used in the study. Based on the Stud Book of Lipizzan horses in the Stud Farm of Đakovo (Mandić and Rastija, 1997), it can be said that the existence of the Liza – Cica – Pliva family is relatively

long in comparison to the recognised Croatian Lipizzan families. For instance, the recognised family of Mima (Nana – Wanda) originates in 1898, the family of Mara (Margit) around 1900, the family of Trofetta (Traviata) in 1913, the family of Toplica (Siglavý) in 1913 and the family of Munja (Famosa) in 1905. In the tables of the Establishing acts of the

Lipizzan International Federation from 1995. and 2001. (Lipizzan International Federation; Establishing Acts, 1995 and 2001), which show recognised Lipizzan families of Croatian origin, there is the family of Alka (1898; father Maestoso, Đakovo), which is confirmed by the Stud Book of the Stud Farm of Đakovo as well. The importance of the statement in the Establishing Acts is that the mares of this family are bred in the Bosnian stud farm of Vučjak under the name of Pliva as a recognised Lipizzaner family, which brings us back to the breeder's spoken statement. Both statutes that the family of Alka is probably a continuation of the original family of Engländeria, and it was verified by mitochondrial genome typifying in this research. Haplotype Allegra, which is established for the mares of this family, is inherent exactly to the classic family of Engländeria. According to records, mare Alka is the descendant of one of seven mares bought by Bishop Strossmayer in Lipica in December 1854 (Stipić, 1975). Only mare Alda (father Neapolitano Valdemora, mother Alda) was of the family of Engländeria. Stallion Neapolitano Valdemora (born 1839 in Lipica) was mating in his native stud farm, and its significance for breeding is evident from the record of its entry into the Neapolitano pedigree genealogy. Out of mentioned seven mares bought in 1854, three mares are this stallion's daughters. The temporal distance of 16 years between the foundation of the family of Alka and the family of Liza – Cica – Pliva is a very short period of documented origin cessation. As it can be concluded from the statements of LIF's Establishing Acts and from research results of this thesis, a half of the family, i.e. the Bosnian branch of Pliva, is an internationally recognised Lipizzan family, while the mares of the family's original part in private breeding of the Republic of Croatia are classified under non-recognised families, i.e. the so-called B-book of Lipizzan horses.

The founder of family line Beba - Elza is mare Beba. In a relatively short existence of the family (approximately 45 years) there was introduction of inadmissible breeds in Lipizzan breeding. The father of the third known mare in the genealogy is a stallion of the Nonius breed of the stud farm of Lipik. One of the family characteristics is frequent occurrence of dark colour, especially bay. The family is mostly bred in the vicinity of Županja. Family mares are used in everyday work in former Agricultural Livestock Enterprise "Stočar" in Županja. After abolishment of this organisation the mares were transferred into private breeding.

The founder of family Cura - Lela is mare Cura. This family shows some resemblance to the family of Beba – Elza. The origin of the families is known for about the same number of years, and in both genealogies there is a Nonius stallion from Lipik. Although haplotype Capriola, which is found in classic Lipizzan breeds, is established in mares of this

family by mitochondrial genome typifying, mentioned consolidation failures do not justify more detailed consideration of this family.

The family originator of family Lenica - Lasta is mare Lenica, foaled in 1921 in Slavonski Kobaš. The family origin is known for a number of years. A family characteristic is that it did not expand geographically to a significant extent, and that it is bred today in the same territory where it originated. All known stallions who are fathers of the mares in the genealogy are of Lipizzan origin.

CONCLUSION

Mitochondrial genome typifying has confirmed negligent record keeping of origin in territorial breeding. Some non-recognised families are descendants of recognised Lipizzan families. Haplotype Allegra is found in the family of Liza-Cica-Pliva, and haplotype Capriola in the family of Cura-Lela. Both haplotypes are very common in the Lipizzan breed and inherent to classic Lipizzan families.

The family of Liza – Cica – Pliva should be given more attention, based on the research on documented origin, existence length and sequence analysis results for the family's D-loop region. Established results should be presented to the LIF for the purpose of family recognition. As the Bosnian branch of this family is recognised as a separate family of Pliva within the framework of the LIF, it is not about the recognition of a new Lipizzan family, but about the recognition of the entire family.

REFERENCES

- Achmann R, Curik I, Dovc P, Kavari T, Bodo I, Habe F, Marti E, Sölkner J, Brem G. (2004): Microsatellite diversity, population subdivision and gene flow in the Lipizzan horse. *Animal Genetics*, 35: 285-292
- Čačić M. (1998): Državna ergela lipicanaca u Đakovu. Agronomski fakultet Sveučilišta u Zagrebu, Diplomski rad
- Čačić M. (2003): Fenotipske i genetske odlike lipicanaca u zemaljskom uzgoju Republike Hrvatske. Agronomski fakultet Sveučilišta u Zagrebu. Magistarski rad
- Dolenc M. (1980): Lipica. Mladinska knjiga, Ljubljana
- GenBank. National Center for Biotechnology Information. <http://www.ncbi.nlm.nih.gov>, 18.12.2002.
- Heisman, M. (1997): Webcutter 2.0. <http://rna.lundberg.gu.se/cutter2>, 01.11.2002.
- Ilančić D. (1979): Osnivanje i djelovanje pastuharnice u Dalmaciji. *Stočarstvo*, 33: 43-49
- Kavari T., Habe F., Brem G., Dovc P. (1999): Mitochondrial D – loop sequence variation among the 16 maternal lines of the Lipizzan horse breed. *Animal Genetics*, 30: 423-430

- Kavar T., Brem G., Habe F., Sölkner J., Dovč P. (2002): History of lipizzan horse maternal lines as revealed by mtDNA analysis. *Genetics Selection Evolution*, 34: 1-14.
- Kumar S., Tamura K., Jakobsen I., Nei M. (2001): Molecular Evolutionary Genetics Analysis, Version 2.1. <http://www.megasoftware.net>, 13.11.2001.
- Lipizzan International Federation (1995): Establishing acts., Rebecq. Belgium
- Lipizzan International Federation (2001): Establishing acts., Rebecq. Belgium
- Ljubešić J. (1981): Prilog poznavanju utjecaja lipicanaca na tip konja u Brodskoj Posavini. Doktorska disertacija, Poljoprivredni fakultet u Sarajevu
- Ljubešić J. (1985a): Lipicanac u zemaljskom uzgoju u SR Hrvatskoj s osvrtom na uzgoj u Slavoniji. *Veterinarski glasnik*, 39: 1095-1103
- Mandić I., Rastija T. (1997): Matična knjiga lipicanskih konja Ergele Đakovo: 1855.-1997. Poljoprivredni fakultet Osijek i PIK Đakovo, Osijek
- Steinhausz M. (1924): Lipicanac. Postanak i gojidbena izgradnja pasmine. Hrvatski štamparski zavod, Zagreb
- Steinhausz M. (1943): Linije pastuha i rodova kobila hrvatskog lipicanca. Gospodarska knjižnica, Zagreb
- Stephen G. M., Carding N., James J., Franetič A., Hrastnik Z., Grabarević Ž., Salamon D. (1993): Izvještaj međunarodne komisije nezavisnih promatrača i veterinarskih inspektora o uništavanju lipicanaca u Lipiku. Stradanja životinja u hrvatskom domovinskom ratu 1990.-1992., Veterinarski fakultet sveučilišta u Zagrebu, Zagreb, 83-107
- Stipić L. (1975): Bijeli lipicanci Đakova. Centar za selekciju konja Đakovo, GIP Tipografija Đakovo
- White P.S., Densmore L.D. (1992): Mitochondrial DNA isolation: Molecular genetic analysis of populations. A practical approach. Oxford, Oxford University Press
- Wichodif G. A. (1899): Die Pferdezucht in Kroatien und Slavonien. Sonderabdruck aus der "Wiener Landwithfchaftlichen Zeitung", Wien
- Zechner P., Zohman F., Sölkner J., Bodo I., Habe F., Marti E., Brem G. (2001): Morphological description of the Lipizzan horse population. *Livestock Production Science*, 69: 163-177
- Zechner P., Sölkner J., Bodo I., Druml T., Baumung R., Achmann R., Marti E., Habe F., Brem, G. (2002): Analysis of diversity and population structure in the Lipizzan horse breed based on pedigree information. *Livestock Production Science*, 77: 137-146

acs70_17