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**Pregledni članak**
**Review**

# GENOME TYPING OF AUTOCHTHONOUS BREEDS OF DOMESTIC ANIMALS IN CROATIA

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## Summary

The national program for the protection of endangered breeds of domestic animals requests reliable evidence of originality of breeds. Besides good knowledge of the phenotypic features, it is necessary to know the genetic structure of breeds, their variability, similarity and distance in relation to cognate populations. The genetic characteristic of Croatia include autochthonous breeds of cattle, horses, donkeys, pigs, sheep, as well as turkeys, fish and bees. Genetic polymorphisms at the protein- (blood groups, blood and milk proteins) and DNA-level (microsatellites, mitochondrial DNA) can be successfully used for population analysis and for establishing relationships within and among species. On the basis of the research blood protein polymorphism and microsatellite markers the genetic distances among three cattle breeds (Istrian cattle, Slavonian-syrmian podolian cattle and Busha), three horse breeds (Posavina horse, Croatian Coldblood and Murinsulaner horse) and two donkey populations have been estimated. On the basis of mitochondrial DNA sequences, the genetic relationships of subpopulations of Lippizaner horse (Ergela and Field breeding), cold-blooded autochthonous breed of horses, type of donkeys and a few breeds of cattle have been determined. Rare breeds of sheep (Ruda sheep, Pag and Krk island sheep) have been typed using several methodological approaches: blood protein polymorphism, microsatellite markers and mitochondrial DNA.

In the Black Slavonian and Turopolje pig blood protein polymorphisms and eight microsatellite locis have been investigated. Endangered poultry populations (Zagorje turkey, Hen Hrvatica and Dravska goose) are now the object of research of phenotypic characteristics. The international joint project with neighbouring countries is trying to establish resistance to varoosis for the Mediterranean ecotype of Grey bee. Using RAPD method our aim is to determine genetic diversity in four lines of carp. The molecular research is

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carried out on endemic soft-muzzled trout using microsatellite markers and mitochondrial DNA. On the basis of the research microsatellite markers and mitochondrial DNA endemic soft-muzzled trout have been typed.

## *Introduction*

Autochthonous breeds of domestic animals are a component of total national and global animal genetic resources. The genetic richness can be seen in the existence of a considerable number of breeds and populations formed and adapted in some areas during past centuries. Genetic and paragenetic influences (climate, soil, food, illnesses, management) have profiled hundreds of breeds, types and subtypes adapted to specific ecological niches, with their own phenotypeic and genetic identity. The fact is that animal genetic resources have been decreasing around the world, which facilitated by the expressed trend of "monoculture" in cattle-breeding (small number of economically active breeds). Local breeds without genetic and economic value have frequently been pushed out. This is one of the main reasons for the sudden disappearance of many local breeds, populations and subpopulations genetically adapted to local conditions. Together with the disappearance of breeds, the biological variety within species is also disappearing, doing irreparable damage, since the variability is a precondition for survival and selection progress.

During the last decade, there was a great progress in Croatia in developing the awareness of the professional and general public of the need for protecting autochthonous breeds of domestic animals as a unique cultural heritage. During the eighties, esperts concentrated on the autochthonous cattle breeds, the Slavonian Syrmian Podolian and the Istrian cattle, which produced certain results. During the mid-nineties, these breeds already experienced certain revitalisation. Though they are still endangered, they have a solid base for survival. At the beginning of the nineties of the last century, the programmes were concentrated on the autochthonous breeds of horses, sheep, pigs, donkeys, turkeys, fish and bees. The activities that were under taken resulted in including genetically interesting breeds and populations in the system of state support for breeders (annual bonuses) and creating adequate programmes for preserving some uniqubreeds. Carlier information on autochthonous breeds was mostly based on historical records, previous research on exterior and production features. Aware of the deficiencies of the information, the attitude of the geueral public was to establish (confirm) the originality of autochthonous breeds, following the latest scientific achievements. The established information should, in a competent way, help in establishing justified and viable protection programmes, indicating the best way for preserving the genetic variability.

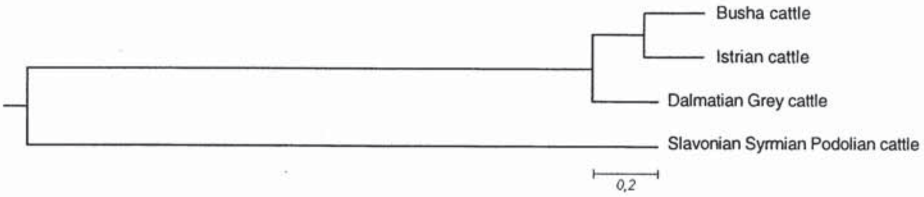
First studies on the polymorphisms of blood proteins of autochthonous cattle breeds were done fifteen years ago. New development of molecular genetics methods imposed the need for the DNA structure research, as the most reliable method for confirming originality, i.e. phylogenetic relationship. During several past years, there were numerous phylogenetic studies on several domestic animal species. A joint project for determining genetic structure of horses, cattle, sheep and pigs has been in progress. Some of the recent information have been included in the breeding programmes in order to protect the autochthonous breeds from extinction.

The latest molecular and genetic methods, of which a microsatellite typing and mtDNA typing are an obligatory phylogenetic tool, have been used to complete our information on autochthonous breeds of domestic animals. But, previously researched frequencies of polymorphic proteins, which are used for completing previously established information have not been left out from the phylogenetic studies. The following survey will deal with some of the information on the phylogenetic originality of the Croatian autochthonous breeds, based on various phylogenetic markers.

## *Cattle breeds*

The programme of preserving autochthonous breeds in Croatia has been initiated on the Slavonian-Syrmian Podolian and the Istrian cattle. At the same time, the idea of protecting Busha in Croatia was initiated by establishing a herd nucleus, but the then environment was not interested enough in such kind of protection. The interest of the professional and general public was concentrated on the Istrian cattle and the Slavonian-Syrmian Podolian cattle, while Busha was "forgotten". Today, when the protection of Busha has been initiated, it is obvious that only a few dozens of Busha have remained in the whole area, so an effort will be made to revitalise the whole population.

The research on blood and milk proteins polymorphism has been carried out for decades, followed by the genetic determination of the autochthonous cattle breeds. At the beginning of nineties, blood protein polymorphisms of the Slavonian-Syrmian Podolian cattle (Gašpert et al., 1990), the Istrian cattle (Caput et al., 1992), the Drey dalmatian cattle (Ivanković et al., 1997) and Busha (Ivanković et al., in press) were studied. The research results were used for the construction of the dendrogram showing phylogenetic relations.



Graph 1. - UPGMA TREE OF CATTLE BREEDS IN CROATIA, BASED ON BLOOD PROTEIN POLYMORPHISM (Ivanković *et al.*, in press; Gašpert *et al.*, 1990; Caput *et al.*, 1992, Ivanković and Caput, 1997).

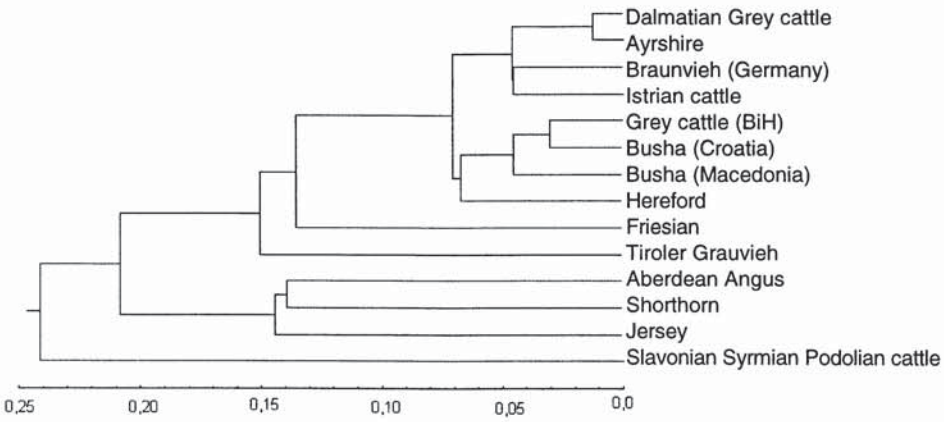
It can be noticed that Slavonian-Syrmian Podolian cattle is significantly philogenetically distanced in comparison to other breeds, which, regarding the assumption of its genesis, is expected. The Grey Dalmatian cattle is philogenetically related to Busha, since it emerged from merging Busha with the Austrian Tiroler Grauvieh.

Tabele 1. - NEI'S ORIGINAL MEASURES OF GENETIC IDENTITY (*ABOVE THE DIAGONAL*) AND GENETIC DISTANCE (*BELOW THE DIAGONAL*) (Ivanković *et al.*, in press)

| | Busha | Dalmatian Gray cattle | Istrian cattle | Slavonian Syrm. Pod. Cattle |
|---|---|---|---|---|
| Busha cattle | - | 0.9956 | 0.9962 | 0.9687 |
| Dalmatian Gray cattle | 0.0044 | - | 0.9962 | 0.9592 |
| Istrian Cattle | 0.0038 | 0.0038 | - | 0.9575 |
| Slavonian Syrm. Pod. cattle | 0.0318 | 0.0417 | 0.0434 | - |

Frequencies of other breed polymorphs were added to established values of frequencies of poylmorphic blood proteins of autochthonous Croatian cattle breeds in constructing dendrograms (Graph 1). The grouping of autochthonous Croatian cattle breeds with related and geographically closer breeds has been noticed. Thus, Busha shows the smallest genetic distance in comparison to the grey cattle from Bosnia and Herzegovina and slightly bigger in comparison to the Macedonian busha (Graph 2). The genetic distance of the Slavonian-Syrmian Podolian cattle regarding the polymorphic blood protein frequencies significantly deviates from other cattle breeds.

Due to a comparatively simple isolation and organisation, maternal heritage, lack of recombinations and comparability of homologous nucleotide regions, mtDNA is a suitable, marker system in population and evolution biology. That is why it was included in our genetic studies of autochthonous breeds of domestic animals and D-loop mtDNA region of Busha, the Istrian cattle and the Grey Dalmatian cattle was sequenced. Sequences indicated the modest level of variability within the researched populations.



Graph 2. - UPGMA TREE (*EUCLIDEAN LINKAGE DISTANCE*) CONSTRUCTED ON THE BASIS OF FREQUENCIES OF POLYMORPHOUS BLOOD PROTEINS OF VARIOUS CATTLE BREEDS (Ivanković *et al.*, in press).

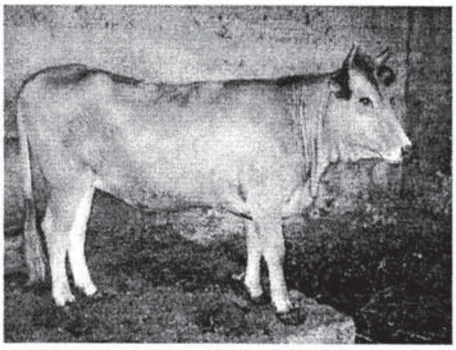
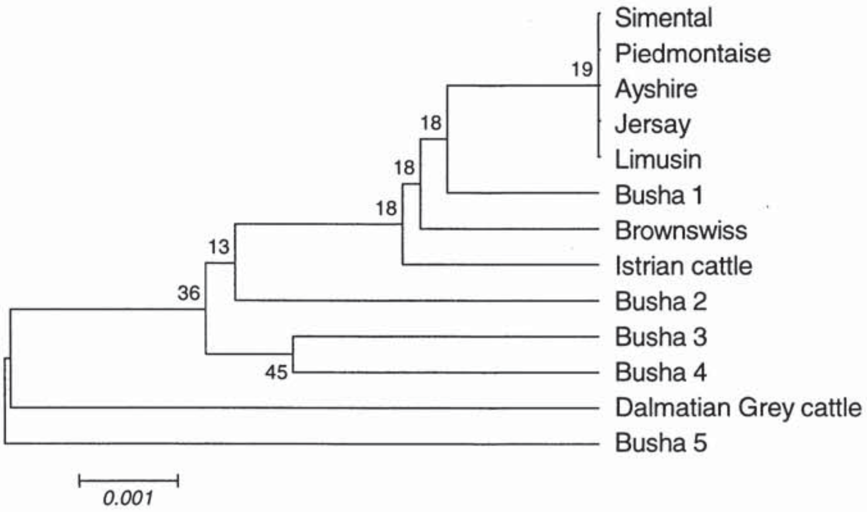


Photo 1. Busha


Photo 2. Istrian Cattle

The variability of mtDNA sequencies can be noticed on the constructed dendrogram in which, for comparison, sequences of some other cattle breeds, available in the NCBI database were included (AccNo. AF034442; AccNo. AF034439; AccNo. AF034446; AccNo. AF034443; AccNo. AF034440; AccNo. AF034438). The variability of Busha population is expected assuming its genesis based on available literature data. Significant spatial dispersion of collected samples contributed to this (Graph 3).



Graph 3. - UPGMA NEIGHBOR-JOINING TREE CONSTRUCTED ON THE BASIS OF D-LOOP MTDNA REGION SEQUENCES OF VARIOUS CATTLE BREEDS

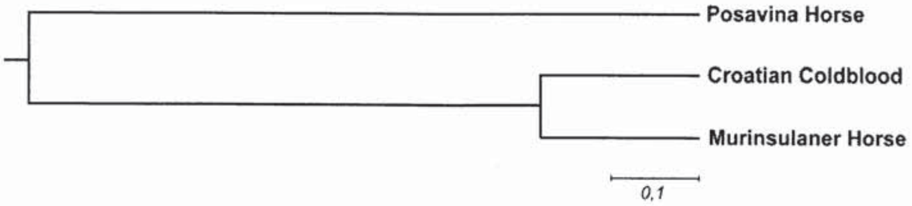
Available information will be completed by the use of other marker systems, primarily microsatellites and the established information will be included in the revision of the existing system of protection and in designing a programme for protection of these breeds. Information will be the basis for establishing adequate cooperation with institutions and services of neighbouring countries in order to avoid the inbreeding or other unwanted events met in the revitalisation of critically endangered domestic animal populations.

### *Horse breeds*

Autochthonous cold-blooded Croatian horse breeds have been dominantly represented, by their share, in the total horse population in Croatia. Their systematic breeding started in the 19th century in Međimurje and then it spread to Podravina, Posavina and parts of Slavonia. They were bred on the then horse population "Bushak" which, depending on the climate, was differently conformationally and genetically profiled. The breeding trends imposed the need for improving working and production features of these horses, thus during the 20th century, quality stallions of heavy European horse breeds were imported, primarily of the Noric (the Noriker) , the Percheron and the Belgian coldblood, which were used for "improving" the existing population. The differentiation of the base population and oriented selection approach conditioned the profiling of the population into breeds still existing in these areas. The Posavina horse and the Croatian Coldblood have remained in the marginal pasture areas in a small number which demands constant monitoring. The murinsulaner horse population has been reduced to forty heads which are carefully monitored, while its revitalisation is questionable. It requires the international cooperation of countries in the area of the river Mura.

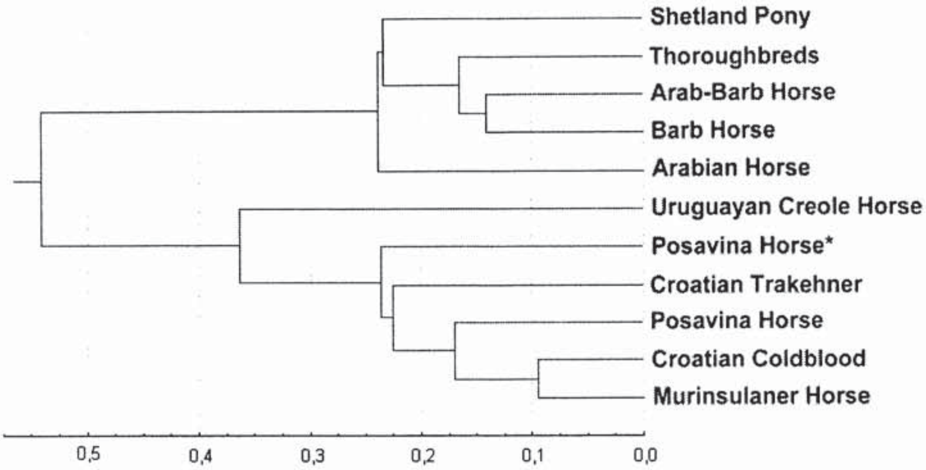
The state stimulates the preservation of autochthonous horse breeds by significant financial support. The exterior features have been investigated several times, but there is the need for their typing on the genetic level. The information on the genetic originality helps in giving the concrete answer to the need and way of preserving these breeds. Some research on the genetic structures of autochthonous horse breeds on the level of polymorphic proteins has already been carried out, while the research on the chosen genetic markers (microsatellites and mtDNA) is in progress.

The research on polymorphic blood proteins have shown the expectedly higher genetic closeness of the Croatian Coldblood and the Murinsulaner horse (Graph 4), while the genetic distance to the Posavina horse is significantly bigger.

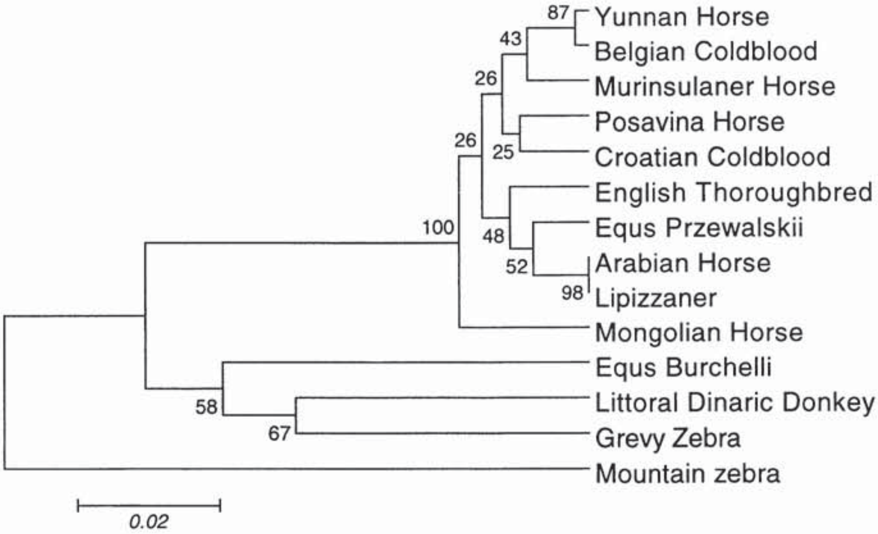


Graph 4. - UPGMA NEIGHBOR-JOINING DENDROGRAM CONSTRUCTED ON THE BASIS OF FREQUENCIES OF POLYMORPHIC BLOOD PROTEINS OF THE AUTOCHTHONOUS CROATIAN HORSE BREEDS (Ivanković *et al.*, 2004).

By including the phylogenetic relations of blood polymorph frequencies of some other breeds in the calculation, the grouping of autochthonous Croatian horse breeds can be noticed, in comparison to other breeds (Graph 5). These observations have to be taken with some restrictions, since the number of researched protein markers has been relatively small. The completion of existing information by including a higher number of microsatellite loci in the studies will give a more reliable picture of the phylogenetic relations of observed populations.



Graph 5. - UPGMA TREE (*EUCLIDEAN LINKAGE DISTANCE*) HAS BEEN CONSTRUCTED ON THE BASIS OF FREQUENCIES OF POLYMORPHIC BLOOD PROTEINS (Ivanković *et al.*, 2004).



Graph 6. UPGMA Neighbor-joining tree constructed on the basis of D-loop region sequences of various *Equidae* mtDNA (Przewalskii horse - AccNo. AF014409; Thoroughbred horse - AccNo. AF072990; Arabian horse - AccNo. AF132591; Belgian horse - AccNo. AF064632; Lipizzan horse - AccNo. AF168690; Cheju horse - AccNo. AF014412; Mongolian horse - AccNo. AF014414; Yunnan horse - AccNo. AF014417; *Equus burchelli* - AccNo. AF220923; Grevy zebra - AccNo. AF220930; Mountain zebra - AccNo. AF220925; Littoral Dinaric donkey –Croatia, Haplotype W) (Ivanković et al., 2004).

The research on mtDNA sequences has indicated a significant phylogenetic closeness of the Croatian autochthonous horse breeds, particularly the Croatian Coldblood and the Posavina horse (Graph 6). The Murinsulaner horse shows greater closeness to the Belgian Coldblood horse, which is expected due to its significant introduction to Croatian horse breeds and particularly the Murinsulaner horse.

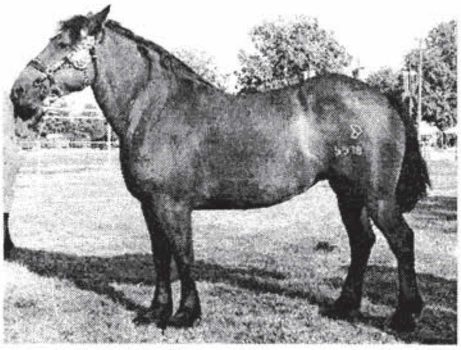


Photo 3. Posavina Horse

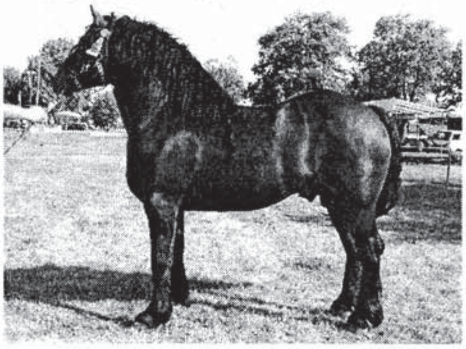
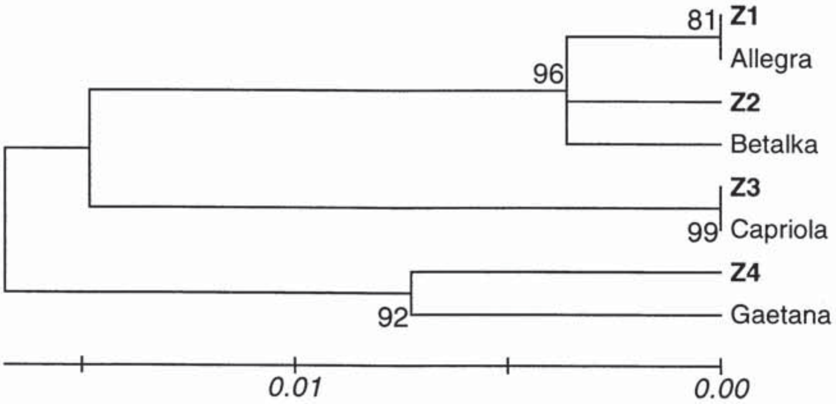


Photo 4. Croatian Coldblood

In Croatia, the Lippizaner population consists of the stud and country breeding. The genetic studies on the Lippizaner population in Croatia mostly included the stud breeding (COPERNICUS project), while the country population remained unresearched. Wanting to research the country population and to clarify some doubts about the reliability of keeping the pedigree in



Graph 7. UPGMA Neighbor-joining tree constructed on the basis of D-loop sequences of mtDNA regions of the part of Lippizaner genus (Čačić, 2003; Kavar et al., 1999)

country breeding, the research on D-loop sequences of mtDNA regions of several mare genera has been carried out. The mtDNA sequencing of unrecognised genera of the Lippizaner mares showed mistakes in keeping stud-books. It has been established that some mares do not belong to the genera as written in the stud-books. For the genus *Liza-Cica-Pliva* (haplotype Z1) it has been established that it is identical to the genus Allegra, and the genus *Cura-Lela* (haplotype Z3) to the genus Capriola (Graph 7).

Since the genus *Pliva* (Bosnia and Herzegovina) has been recognised by LIF, recognising the genus *Liza-Cica-Pliva* by LIF would complete already recognised genus of the Lippizaner.

### *Donkey breeds*

In Croatia, especially in its coastal part, donkeys have been living for centuries, helping a man to survive in savage environmental conditions. During several centuries of adaptation and natural selection in certain regions, donkey populations of special phenotype features have been profiled. The intensification of agrarian production and economic and social movements in rural areas have caused a significant decrease in the number of donkeys. The present information shows that in Croatia there are several thousands of donkeys, but some types are on the verge of extinction. In the nineties of the last century, the inevitable need for protection and preserving of donkeys in Croatia was realised.

In the first stage of the preparation of the donkey protection programme, several types within the total donkey population were noticed and defined exteriorly. In the later studies, these data were completed by research at the molecularly genetic level. The research of the genetic structure of the total donkey population carried out at the level on polymorphous proteins, microsatellites and mtDNA confirms the genetic differentiation of the total donkey population in three types. The research results of the polymorphous blood protein frequencies are shown in diagram 2.

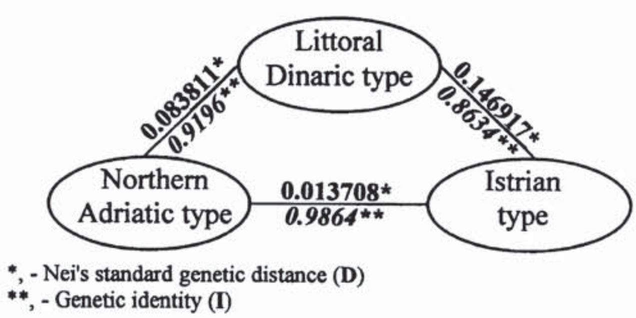


Diagram 2. - GENETIC DISTANCES AND GENETIC IDENTITY OF DONKEY TYPES IN CROATIA (Ivanković and Caput, 1997)

The DNA structure was completed by the analysis of microsatellite loci, chosen from the set recommended by ISAG for *Equidae* typing. The research results confirmed observations noted on the basis of blood polymorph analyses.

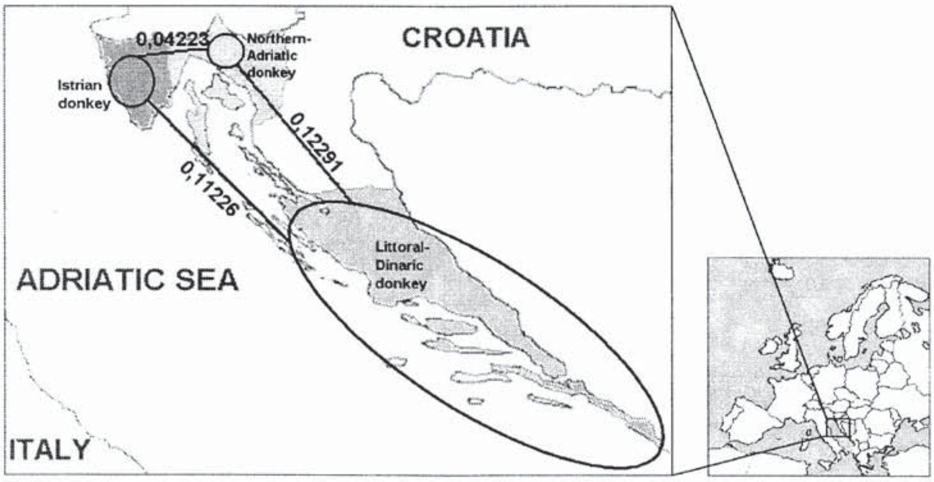
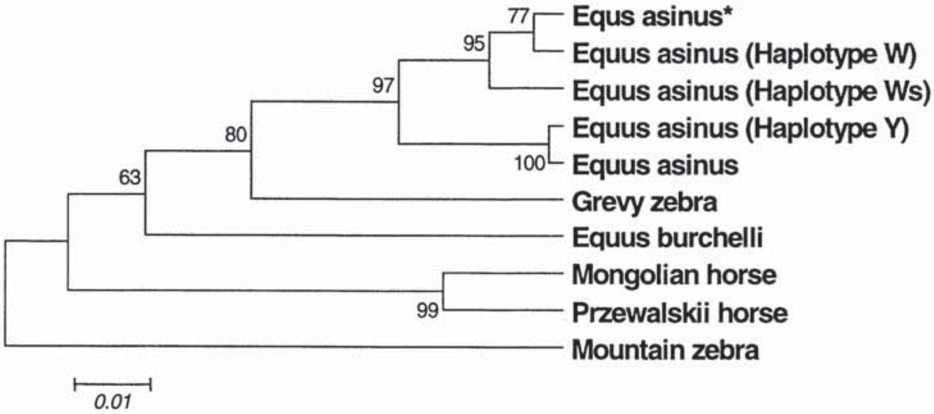


Diagram 3. - GENETIC DISTANCES OF DONKEY TYPES IN CROATIA ESTABLISHED ON THE BASIS OF MICROSATELLITE LOCI (Ivanković et al., 2003).

MtDNA sequencing confirmed the existence of several haplotypes (Y, W, Ws) within the total donkey population. The regional distribution of certain haplotypes has been noticed. The haplotype Y was represented in the Istrian donkey population, while W and Ws haplotypes were represented in the population of Littoral-Dinaric and Northern-Adriatic donkey (Graph 8).

Photo 5. Littoral-Dinaric Donkey

Photo 6. Istrian Donkey



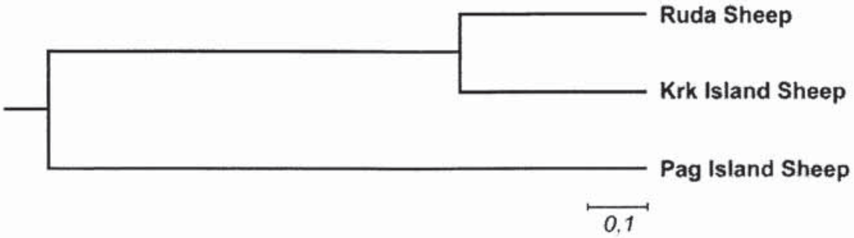
Graph 8. - UPGMA Neighbor-joining tree constructed on the basis of D-loop sequences of mtDNA regions of donkeys in Croatia (Przewalskii horse - AccNo. AF014409; Mongolian horse - AccNo. AF014414; *Equus burchelli* - AccNo. AF220923; Grevy zebra - AccNo. AF220930; Mountain zebra - AccNo. AF220925, *Equus asinus* - AccNo. X97337; *Equus asinus** - Ishida et al., 1995; Donkey in Croatia - Haplotype W, Ws and Y) (Ivanković et al., 2003).

Due to the applicability of established information in the breeding programme, trough the influence of certain restrictive endonucleases, it is possible in a relatively simple and fast way to determine the affiliation of individuals to a haplotype group. But, due to the abserve of an adequate breeding programme for donkey population, the information on the molecular and genetic features has had no practical influence on the profiling of breeding. The biggest problem in the revitalisation of donkey population in Croatia is a low level of animal fertility and insufficient motivation of relatively old breeders.

### *Sheep breeds*

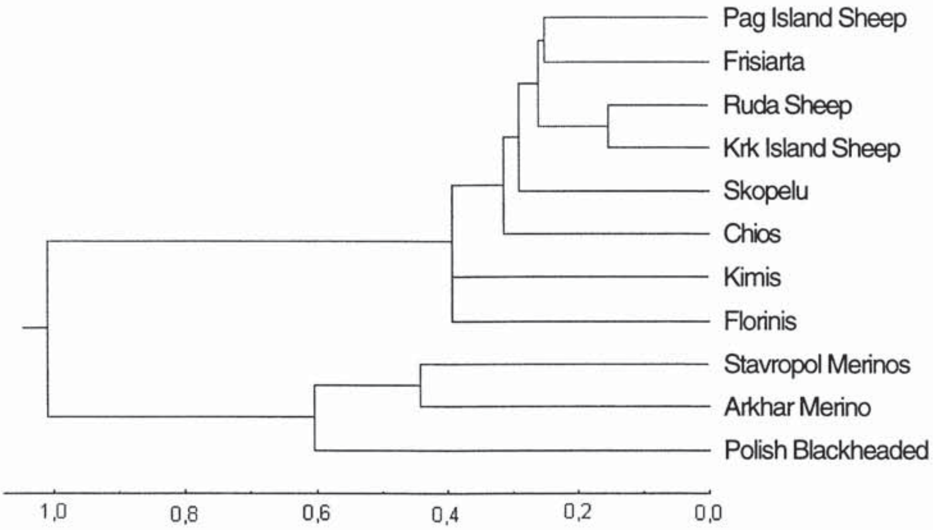
The sheep raising in Croatia has been based on traditional sheep breeds formed on Pramenka, merinized to a smaller or bigger extent, during last centuries. Regarding the phenotype, breeds have certain special features, but there have been atlempts to establish the real originality by studies on the DNA level. The research has included determining frequencies of polymorphc blood proteins, microsatellites and mtDNA sequences.

The research on polymorphic blood proteins indicates the special features of the Pag island sheep, while Ruda and the Krk island sheep are genetically closer (Graph 9).



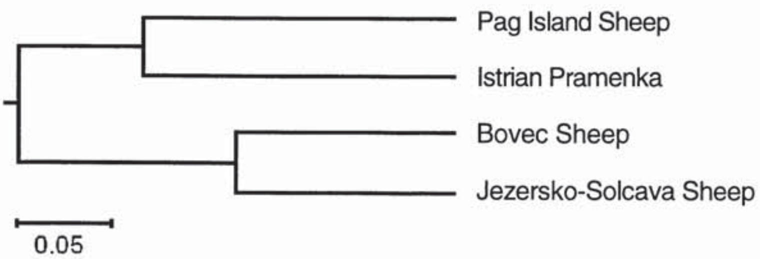
Graph 9. - UPGMA NEIGHBOR-JOINING TREE CONSTRUCTED ON THE BASIS OF BLOOD PROTEIN POLYMORPHISM OF SOME AUTOCHTHONOUS SHEEP BREEDS IN CROATIA (Mioč *et al.*, in press).

If frequencies of polymorphic blood proteins of other sheep breeds are taken into account in calculating genetic distances, the closeness, i.e. the specificity of autochthonous Croatian sheep breeds in comparison to other breeds can be noticed (Graph 10).



Graph 10. - UPGMA TREE (*EUCLIDEAN LINKAGE DISTANCE*) CONSTRUCTED ON THE BASIS OF FREQUENCIES OF POLYMORPHIC BLOOD PROTEINS OF SHEEP BREEDS (Mioč *et al.*, in press).

The analysis of microsatellite locus set of the Pag sheep is the first step in typing autochthonous sheep breeds at the DNA level. The research results were compared with the Slovenian autochthonous breeds (Graph 11). Nei's genetic distance (*D*) reveals a close relationship between two local Slovene breeds, Jezersko-Solcava sheep and Bovec sheep (0.2443), while the distance between the Istrian pramenka and the Pag island sheep was slightly greater (0.3606) (Ivanković *et al.*, 2004). Such a result has been expected, since phylogenetically, the Pag island sheep represents a merinized pramenka of the island of Pag.



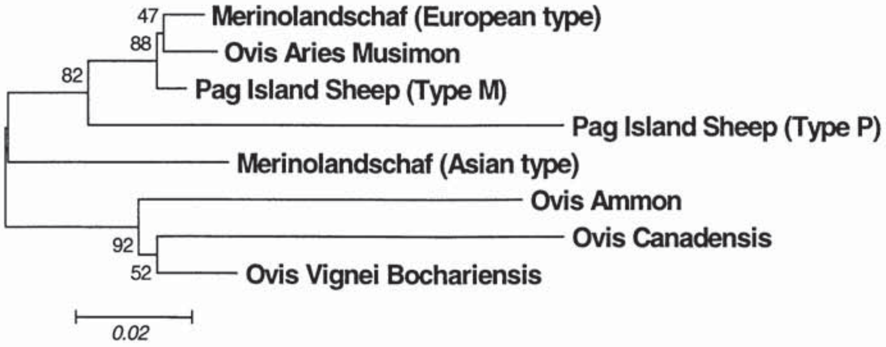
Graph 11. - UPGMA NEIGHBOR-JOINING TREE OF THE GENETIC RELATIONSHIPS AMONG THREE SLOVENIAN SHEEP BREEDS AND THE PAG ISLAND SHEEP (MEASURED ON SEVEN MICROSATELLITE LOCI) (Ivanković *et al.*, in press).

Photo 7. Ruda Sheep

Photo 8. Krk Island Sheep

The study of the Pag sheep genetic structure included the sequencing of the proximal part (nt 15816-16153) of the D-loop region at mtDNA. After clustering D-loop mtDNA sequences of the Pag island sheep, their grouping into two main haplotypes was proposed (type M and P). The difference of haplotype M in comparison to haplotype P (6.78 to 9.44 %) is higher than within haplotyes, which suggests their previous separation, i.e. longer genetic distance. The difference within P haplotypes shows longer independent existence and stratification of the original Pag island sheep haplotype diversified by mutations (Ivanković *et al.*, in press).

Since the Pag island sheep emerged by crossing the original island "Pramenka" and Merino, we assume that the determined P haplotype belongs to the original Pag sheep genotype, i.e. "the original island Pramenka". Similar or identical sequences were not found in available databases (NBCI, GenBank-Database, http://www.ncbi.nlm.nih.gov) and scientific papers. The low degree of sequence divergence of haplotype M from Merinolandschaf European type (2.58%) and *O. musimon* (3.16%) indicates that haplotype M entered the Pag sheep population by its merinisation (Ivanković *et al.*, in press).



Graph 12. - Neighbor-Joining tree of mtDNA D-loop sequence (nt 15816-16136) showing relations among the members of the subgenus *Ovinae*: Merinolandschaf European type (AccNo. AF039577); *O. aries musimon* (AccNo. AF039579); Pag island sheep (Haplotypes M and P); Merinolandschaf Asian type (AccNo. AF039578); *O. canadensis* (AccNo. AF076917); *O. vignei bochariensis* (AccNo. AF039580) and *Ovis ammon* (AccNo. AJ251327). (Ivanković *et al.*, in press)

### *Conclusion*

Previous informations will be completed by the use of other marker systems, primarily microsatellites and the established information will be included in the revision of the existing system of protection, state subsidies and designing a protection programme. Information will be the basis for establishing the adequate co-operation with institutions and services of neighbouring countries in order to avoid the inbreeding or other unwanted events which can occur in the revitalisation of the critically endangered population.

# TIPIZACIJA GENOMA AUTOHTONIH PASMINA DOMAĆIH ŽIVOTINJA U HRVATSKOJ

## *Uvod*

Autohtone pasmine domaćih životinja čine sastavnicu ukupnih nacionalnih i globalnih životinjskih genetskih resursa. Genetsko bogatstvo se ogleda u postojanju većeg broja pasmina i populacija formiranih i adaptiranih tijekom minulih stoljeća na određenim područjima. Genetski i paragenetski utjecaji (klima, tlo, hrana, bolesti, management) profilirali su stotine pasmina, tipova i podtipova prilagođenih specifičnim ekološkim nišama, s vlastitom fenotipskom i genetskom prepoznatljivošću. Činjenica je da se životinjski genetski resursi diljem svijeta smanjuju, čemu je svakako pogodovao u stočarstvu izražen trend "monokulture" (malog broja gospodarsko aktivnih pasmina). Lokalne su se pasmine često bez genetskog i gospodarskog vrednovanja istiskivale. To je jedan od glavnih razloga naglog nestanka mnogih lokalnih pasmina, populacija i subpopulacija genetski adaptiranih na lokalne uvjete. Nestankom pasmina nepobitno nestaje i biološka raznolikost unutar vrsta, što je nenadoknadiva šteta, budući da je varijabilnost preduvjet opstanka i selekcijskog napretka.

Tijekom proteklog desetljeća u Hrvatskoj su načinjeni veliki pomaci u razvijanju svijesti stručne i šire javnosti o potrebi očuvanja autohtonih pasmina domaćih životinja kao jedinstvenog kulturnog naslijeđa. Stručna javnost se tijekom osamdesetih godina usredotočila na autohtone pasmine goveda, slavonsko-srijemski podolac i istarsko govedo, što je postiglo i određene rezultate. Ove su pasmine već sredinom devedesetih godina doživjele stanovitu revitalizaciju. Premda su još ugrožene, imaju solidnu osnovu za opstanak. Početkom devedesetih godina prošlog stoljeća programi se usredotočuju na autohtone pasmine konja, ovaca, svinja, magaraca, purana, riba i pčela. Poduzimane aktivnosti rezultirale su uvrštavanjem genetski interesantnih pasmina i populacija u sustav državne potpore uzgajivačima (godišnje premije) a za dio pasmina osmišljeni su adekvatni programi očuvanja njihove jedinstvenosti. Dotadašnje spoznaje o autohtonim pasminama temeljile su se uglavnom na povijesnim zapisima, ranijim istraživanja eksterijera i proizvodnih odlika. Svjesna manjkavosti ovih spoznaja, stručna javnost zauzela je stav da treba, prateći najnovija znanstvena dostignuća, utvrditi (potvrditi) originalnost autohtonih pasmina. Utvrđene spoznaje trebale bi na meritoran način pomoći uspostavi opravdanih i održivih programa zaštite, ukazujući na najbolji put očuvanja genetske varijabilnosti.

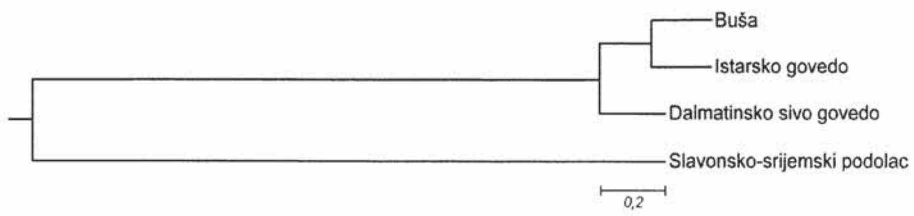
Prije petnaestak godina učinjena su prva proučavanja studije polimorfnosti krvnih proteina autohtonih pasmina goveda. Noviji razvoj metoda molekularne genetike nametnuo je potrebu istraživanja strukture DNA, kao najmjerodavnije metode potvrde originalnosti odnosno filogenetske srodnosti. Tijekom nekoliko minulih godina učinjen je niz filogenetskih pručavanja više vrsta domaćih životinja. U tijeku je kolaborativni projekt determinacije genetske strukture konja, goveda, ovaca i svinja. Dio novijih spoznaja ugrađen je u uzgojne programe kojima se nastoje autohtone pasmine očuvati od izumiranja.

Nastojeći upotpuniti naše spoznaje o autohtonim pasminama domaćih životinja koriste se najnovije molekularno genetske metode, od kojih je mikrosatelitska tipizacija i tipizacija mtDNA neizostavan filogenetski alat. No u filogenetskim proučavanjima ne izostavljaju se ni ranije istraživane frekvencije polimorfnih proteina, kojima upotpunjujemo ranije postignute spoznaje. U narednom prikazu dotaknut ćemo neke spoznaje o filogentskoj originalnosti hrvatskih autohtonih pasmina, bazirane na različitim filogenetskim markerima.

### *Pasmine goveda*

Program očuvanja autohtonih pasmina u Hrvatskoj započeo na slavonsko srijemskom podolcu i istarskom govedu. Istodobno je pokrenuta ideja zaštite buše u Hrvatskoj zasnivanjem nukleus stada, no okruženje toga doba još nije imalo dovoljno sluha za takav oblik zaštite. Interes stručne i šire javnosti ustredotočio se na istarsko govedo i slavonsko srijemskog podolca, a buša je "zaboravljena". Danas, kada je započela zaštita buše, spoznalo se da je preostalo samo nekoliko desetaka buša na cijelom području, na kojima će se pokušati revitalizirati cijela populacija.

Istraživanja polimorfizma proteina krvi i mlijeka provode se desetljećima, od čega se krenulo i u genetskoj determinaciji autohtonih pasmina goveda. Početkom devedesetih istraženi su polimorfizmi proteina krvi slavonsko srijemskog podolca (Gašpert i sur., 1990), istarskog goveda (Caput i sur., 1992), sivog dalmatinskog goveda (Ivanković i sur., 1997) i buše (Ivanković i sur., u tisku). Rezultati istraživanja su poslužili za konstrukciju dendograma koji prikazuje filogenetske odnose.



Graf 1. - UPGMA DENDROGRAM PASMINA GOVEDA U HRVATSKOJ, ZASNOVAN NA POLIMORFIZMU PROTEINA KRVI (Ivanković i sur., u tisku; Gašpert i sur., 1990; Caput i sur., 1992, Ivanković i Caput, 1997).

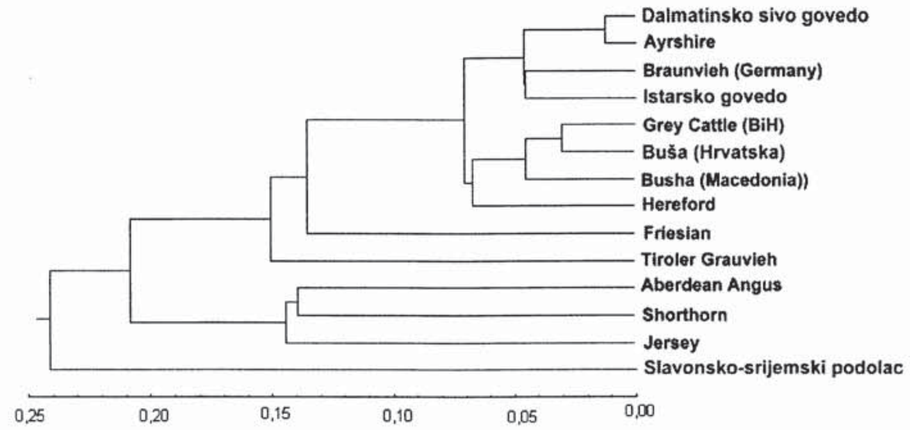
Uočljivo je da je slavonsko-srijemski podolac značajno filogenetski udaljen naspram ostalih pasmina, što je s obzirom na pretpostavke o njegovoj genezi i očekivano. Sivo dalmatinsko govedo je filogentski srodno buši, budući da je nastalo pretapanjem buše austrijskim sivim govedom.

Tablica 1. - NEI-EVA MJERA GENETSKE IDENTIČNOSTI (*IZNAD DIJAGONALE*) I GENETSKE DISTANCE (*ISPOD DIJAGONALE*)

| | Buša | Dalmatinsko sivo govedo | Istarsko govedo | Slavonsko-srijemski podolac |
|---|---|---|---|---|
| Buša | - | 0,9956 | 0,9962 | 0,9687 |
| Dalmatinsko sivo govedo | 0,0044 | - | 0,9962 | 0,9592 |
| Istarsko govedo | 0,0038 | 0,0038 | - | 0,9575 |
| Slavonsko-srijemski podolac | 0,0318 | 0,0417 | 0,0434 | - |

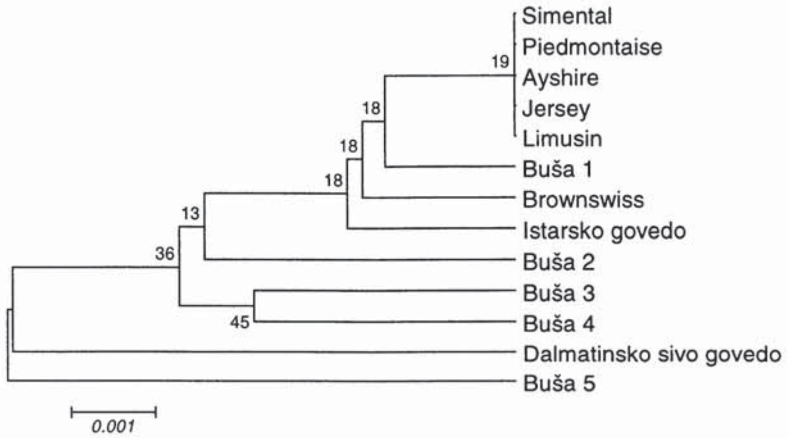
Utvrđenim vrijednostima frekvencija polimorfnih krvnih proteina autohtonih hrvatskih pasmina goveda pridodane su frekvencije polimorfa drugih pasmina pri konstrukciji dendrograma (Graf 2). Uočljivo je grupiranje autohtonih hrvatskih pasmina goveda sa srodnijim i zemljopisno bližim pasminama. Buša pokazuje najmanju genetsku distancu naspram sivog goveda iz BiH, a nešto veću spram makedonske buše. Genetska distanca slavonsko-srijemskog podolca s obzirom na frekvencije polimorfnih krvnih proteina čini značajan otklon naspram drugih pasmina goveda.

Zbog relativno jednostavne izolacije i organizacije, maternalnog nasljeđivanja, odsutnosti rekombinacija i usporedivosti homolognih nukleotidnih regija, mtDNA pogodan je markerski sustav u populacijskoj i evolucijskoj biologiji. Stoga smo ga uključili u genetsko pročavanje autohtonih pasmina domaćih životinja. Radi navedenoga sekvencirana je D-loop regije mtDNA buše, istarskog i dalmatinskog sivog goveda. Sekvence su ukazale na umjerenu razinu varijabilnosti unutar istraživanih populacija.



Graf 2. - UPGMA DENDROGRAM SASTAVLJEN NA TEMELJU FREKVENCIJA POLIMORFNIH KRVNIH PROTEINA RAZLIČITIH PASMINA GOVEDA (*EUCLIDEAN LINKAGE DISTANCE*) (Ivanković i sur., u tisku)

Varijabilnost sekvenci mtDNA uočljiva je na dendrogramu u koji smo radi usporedbe uključili sekvence nekih drugih pasmina goveda, dostupnih u NCBI bazi podataka (AccNo. AF034442; AccNo. AF034439; AccNo. AF034446; AccNo. AF034443; AccNo. AF034440; AccNo. AF034438). Varijabilnost populacije buše je očekivana pretpostavljajući njenu genezu na osnovi dostupnih podataka u literaturi. Ovome je doprinijela i znatna prostorna disperzija prikupljenih uzoraka (Graf 3).



Graf 3. - UPGMA NEIGHBOR-JOINING DENDROGRAM SASTAVLJEN NA TEMELJU SEKVENCI D-LOOP REGIJA MTDNA RAZLIČITIH PASMINA GOVEDA

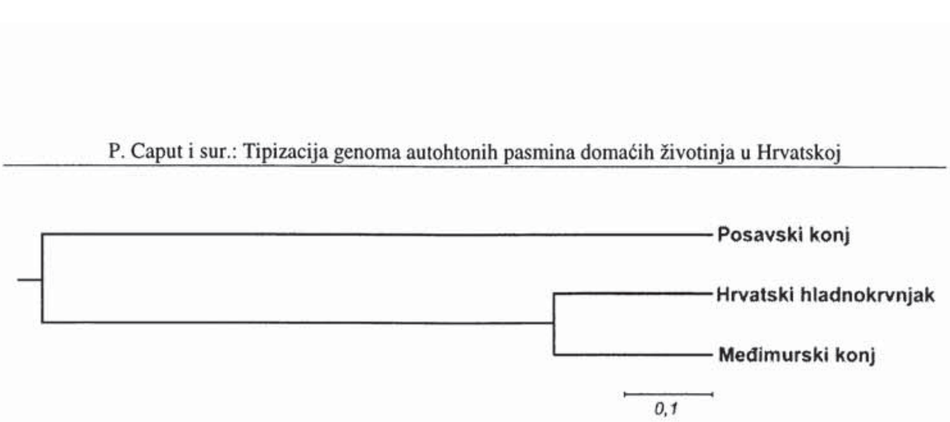
Dosadašnje spoznaje upotpunit će se uporabom drugih markerskih sustava, prvenstveno mikrosatelita, a utvrđene spoznaje uključit će se u reviziju postojećeg sustava zaštite i dizajniranje programa očuvanja ovih pasmina. Spoznaje će biti osnova za uspostavljanje primjerene suradnje s institucijama i službama susjednih zemalja kako bi se izbjegao uzgoj u srodstvu ili druge neželjene pojave na koje se nailazi u revitalizaciji kritično ugroženih populacija domaćih životinja.

### *Pasmine konja*

Autohtone hladnokrvne hrvatske pasmine konja udjelom su dominantno zastupljene u ukupnoj populaciji konja u Hrvatskoj. Njihov sustavni uzgoj započeo je početkom devetnaestog stoljeća na području Međimurja a zatim se proširio na područja Podravine, Posavine i Slavonije. Izgrađene su na populaciji konja "bušaka", koji je, ovisno o podneblju, bio različito konformacijski i genetski profiliran. Uzgojni trendovi nametnuli su potrebu popravljanja radnih i proizvodnih značajki ovih konja te su tijekom dvadesetog stoljeća uvažani rasplodnjaci teških europskih pasmina primarno noričke, pešeronske i belgijske krvi, kojima se "oplemenjivala" postojeća populacija. Diferenciranost bazne populacije te usmjeren selekcijski pristup uvjetovali su profiliranje ondašnje izvorne populacije u autohtone pasmine koje se danas nalaze na ovim područjima. Posavski konj i hrvatski hladnokrvnjak zadržali su se na marginalnim pašnjačkim područjima u nevelikom broju koji iziskuje konstantan monitoring. Populacija međimurskog konja svedena je na četrdesetak grla koja se pomno prate, a njegova revitalizacija je upitna. Ona zahtijeva međudržavnu suradnju zemalja u području rijeke Mure.

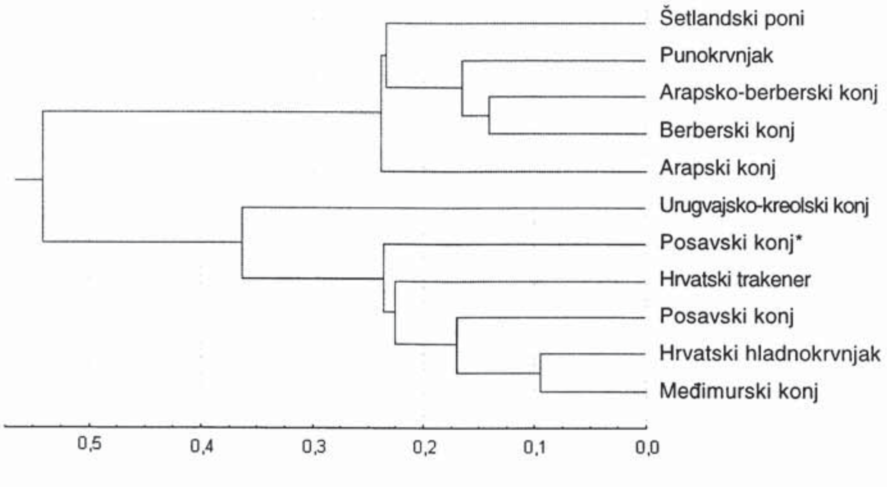
Očuvanje autohtonih pasmina konja država potiče znatnim novčanim subvencijama. Njihove eksterijerne odlike su već u više navrata istražene no nametnula se potreba njihovog tipiziranja na genetskoj razini. Spoznaje o genetskoj originalnosti pomažu u davanju konkretnog odgovora o potrebi i načinu očuvanja ovih pasmina. Već je provedeno nekoliko istraživanja genetske strukture autohtonih pasmina konja na razini polimorfnih proteina a u tijeku su istraživanja izabranih genetskih markera (mikrosateliti i mtDNA).

Istraživanja polimorfnih krvnih proteina potvrdila su očekivano veću genetsku bliskost hrvatskog hladnokrvnjaka i međimurskog konja (Graf 4), dok je genetska distanca naspram posavskog konja znatno veća.



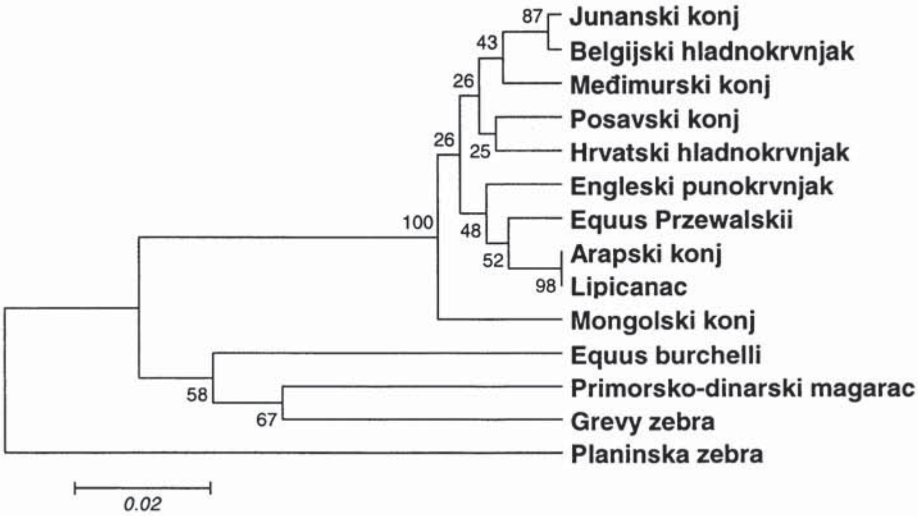
Graf 4. - UPGMA NEIGHBOR-JOINING DENDROGRAM SASTAVLJEN NA TEMELJU FREKVENCIJA POLIMORFNIH KRVNIH PROTEINA AUTOHTONIH HRVATSKIH PASMINA KONJA (Ivanković i sur., 2004)

Uvrštavanjem u izračun filogenetskih odnosa frekvencija krvnih polimorfa i nekih drugih pasmina uočava se grupiranje autohtonih hrvatskih pasmina konja naspram drugih pasmina (Graf 5). Ova zapažanja treba uzeti s rezervom, budući da je istraženi broj proteinskih markera relativno mali. Nadopuna postojećih spoznaja uključivanjem većeg broja mikrosatelitskih lokusa proučavanja dat će pouzdaniju sliku o filogenetskim odnosima promatranih populacija.



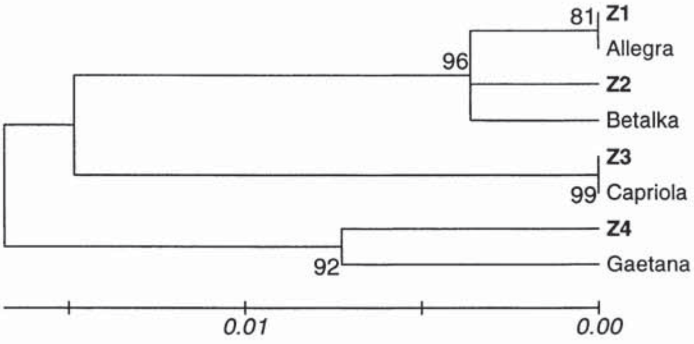
Graf 5. - UPGMA DENDROGRAM SASTAVLJEN NA TEMELJU FREKVENCIJA POLIMORFNIH KRVNIH PROTEINA (*EUCLIDEAN LINKAGE DISTANCE*) (Ivanković i sur., 2004)

Istraživanje sekvenci mtDNA ukazalo je na znatnu filogenetsku bliskost hrvatskih autohtonih pasmina konja, osobito hrvatskog hladnokrvnjaka i posavskog konja (Graf 6). Međimurski konj pokazuje veću bliskost s belgijskim hladnokrvnim konjem, što je s obzirom na njegovu znatnu introdukciju u hrvatske pasmine konja, osobito međimurskog konja, očekivano.



Graf 6. UPGMA Neighbor-joining dendrogram sastavljen na temelju sekvenci D-loop regija mtDNA različitih *Equida* (Przewalskii horse - AccNo. AF014409; Engleski punokrvnjak - AccNo. AF072990; Arapski konj - AccNo. AF132591; Belgijski hladnokrvnjak - AccNo. AF064632; Lipicanac - AccNo. AF168690; Cheju konj - AccNo. AF014412; Mongolski konj - AccNo. AF014414; Yunnan horse - AccNo. AF014417; *Equus burchelli* - AccNo. AF220923; Grevy zebra - AccNo. AF220930; Mountain zebra - AccNo. AF220925; Primorsko-dinarski magarac – haplotip W*)* (Ivanković i sur., 2004).

Populaciju lipicanca u Hrvatskoj osim ergelskog čini i zemaljski dio uzgoja. Genetska proučavanja populacije lipicanca u Hrvatskoj uglavnom su



Graf 7. - UPGMA NEIGHBOR-JOINING DENDROGRAM KONSTRUIRAN NA TEMELJU SEKVENCI D-LOOP REGIJA MTDNA DIJELA RODOVA LIPICANCA (Čačić, 2003; Kavar i sur., 1999)

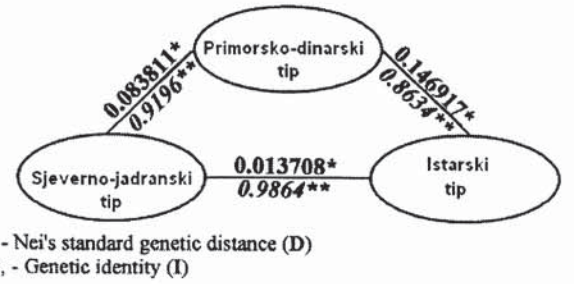
obuhvatila ergelski dio uzgoja (COPERNICUS projekt), dok je zemaljski dio populacije ostao neistražen. Želeći istražiti zemaljski dio populacije te pojasniti neke dvojbe o vjerodostojnosti vođenja rodoslovlja u zemaljskom uzgoju provedeno je istraživanje sekvenci D-loop regije mtDNA više rodova kobila. Sekvenciranje mtDNA nepriznatih rodova lipicanskih kobila ukazalo je na greške u vođenju matičnih knjiga. Za neke kobile je utvrđeno da ne pripadaju rodovima kojima po rodovniku pripadaju. Za rod *Liza-Cica-Pliva* (haplotip Z1) utvrđeno je da je identičan rodu Allegra, a rod *Cura-Lela* (haplotip Z3) rodu Capriola (Graf 7).

Budući da je rod *Pliva* (BiH) priznat u LIF-u, priznavanjem roda *Liza-Cica-Pliva* od strane LIF-a upotpunio bi se već priznati rod lipicanca.

### *Pasmine magaraca*

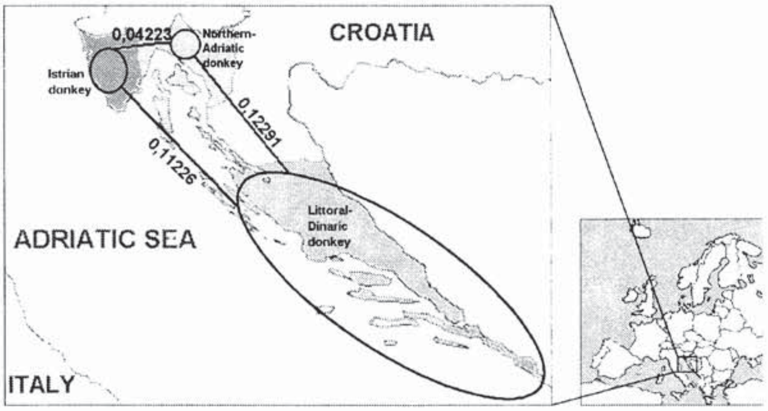
Na području Hrvatske, osobito njenog priobalnog dijela, magarci tisućlje-ćima preživljavaju u surovim okolišnim uvjetima. Tijekom višestoljetnog razdoblja adaptacije i prirodne selekcije u pojedinim regijama profilirale su se populacije osobitih fenotipskih značajki. Intenziviranje agrarne proizvodnje te gospodarsko-socijalna kretanja u ruralnim područjima uvjetovali su znatan pad broja magaraca. Aktualne spoznaje ukazuju da na području Hrvatske obitava nekoliko tisuća magaraca s tim da su neki tipovi na rubu nestanka. Devedesetih godina minulog stoljeća uvidjela se neodgodiva potreba zaštite i očuvanja magaraca u Hrvatskoj.

U prvoj fazi pripreme programa zaštite magaraca eksterijerno je uočeno i definirano nekoliko tipova unutar ukupne populacije. Ove su spoznaje u kasnijim studijama upotpunjene istraživanjima na molekularno-genetskoj razini. Provedena istraživanja genetske strukture ukupne populacije magaraca na razini polimorfnih proteina, mikrosatelita i mtDNA potvrđuju genetsku diferenciranost ukupne populacije magaraca na tri tipa. Rezultati istraživanja frekvencija polimorfnih krvnih proteina prikazani su na prikazu 2.



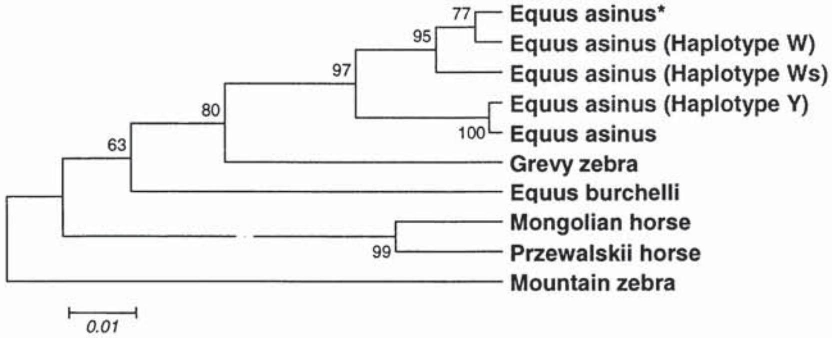
Prikaz 2. - GENETSKE DISTANCE I GENETSKI IDENTITET TIPOVA MAGARACA U HRVATSKOJ UTVRĐENI NA OSNOVI FREKVENCIJA POLIMORFNIH KRVNIH PROTEINA (Ivanković i Caput, 1997)

Proučavanje DNA strukture upotpunjeno je analizom mikrosatelitskih lokusa, izabranih iz seta preporučenog od strane ISAG-a za tipiziranje *Equida*. Rezultati istraživanja su potvrdili zapažanja uočena na bazi analize krvnih polimorfa.



Prikaz 3. - GENETSKE DISTANCE TIPOVA MAGARACA U HRVATSKOJ UTVRĐENE NA OSNOVI MIKROSATELITSKIH LOKUSA (Ivanković i sur., 2002)

Sekvenciranje mtDNA potvrdilo je postojanje nekoliko haplotipova (Y, W, Ws) unutar ukupne populacije magaraca. Uočena je regionalna distribucija pojedinih haplotipova. Haplotip Y zastupljen je u populaciji istarskog magarca, dočim su haplotipovi W i Ws zastupljeni u populacijama primorsko dinarskog i sjeverno jadranskog magarca (Graf 8).



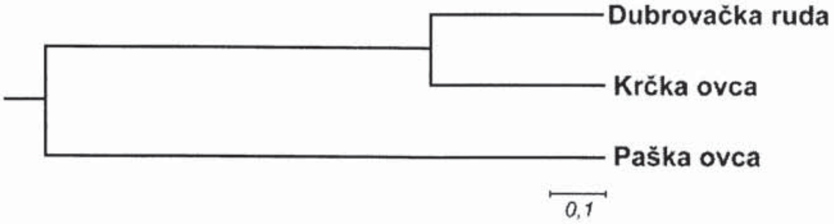
Graf 8. - UPGMA Neighbor-joining dendrogram sastavljen na temelju sekvenci D-loop regije mtDNA magaraca u Hrvatskoj (Przewalskii konj - AccNo. AF014409; Mongolski konj - AccNo. AF014414; *Equus burchelli* - AccNo. AF220923; Grevy zebra - AccNo. AF220930; Brdska zebra - AccNo. AF220925, *Equus asinus* - AccNo. X97337; *Equus asinus** - Ishida i sur., 1995; magarci u Hrvatskoj - haplotipovi W, Ws i Y) (Ivanković i sur., 2002).

Radi primjene utvrđenih spoznaja u uzgojnom programu, djelovanjem određenih restrikcijskih endonukleaza moguće je na relativno jednostavan i brz način utvrditi pripadnost jedinki haplotipskoj skupini. No radi nepostojanja primjerenog uzgojnog programa za populaciju magaraca, spoznaje o molekularno-genetskim značajkama nisu imale praktičnog odraza na profiliranje uzgoja. Najveći problem revitalizacije populacije magaraca u Hrvatskoj je niska razina plodnosti životinja i nedovoljna motiviranost relativno starih uzgajivača.

### *Pasmine ovaca*

Ovčarstvo Hrvatske dominantno se temelji na tradicionalnim pasminama ovaca koje su oblikovane na pramenci, meriniziranoj u manjoj ili većoj mjeri tijekom minulih stoljeća. Pasmine s obzirom na fenotip imaju određene osobitosti, no stvarna se originalnost nastoji utvrditi proučavanjima na razini DNA. Istraživanja su uključila određivanje frekvencija polimorfnih krvnih proteina, mikrosatelita i sekvenci mtDNA.

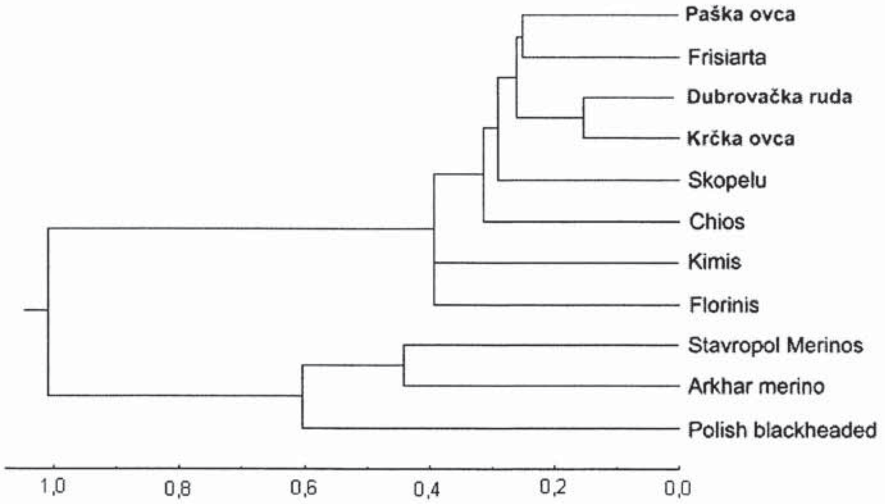
Istraživanja polimorfnih krvnih proteina ukazuje na osobitost paške ovce, dok su ruda i krčka ovca genetski bliskije (Graf 9).



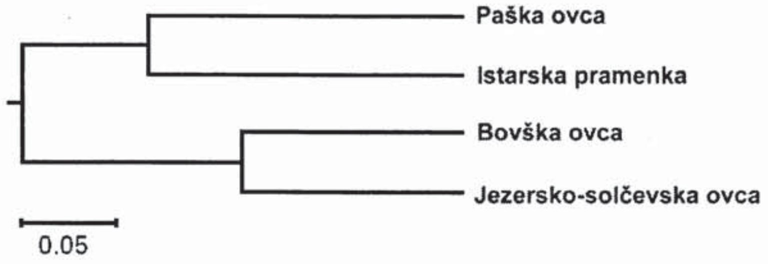
Graf 9. - UPGMA NEIGHBOR-JOINING DENDROGRAM SASTAVLJEN NA TEMELJU POLIMORFIZMA KRVNIH PROTEINA NEKIH AUTOHTONIH PASMINA OVACA U HRVATSKOJ (Mioč i sur., u tisku)

Uzmu li se u obzir prilikom izračuna genetskih distanci frekvencije polimorfnih krvnih proteina drugih pasmina ovaca, uočava se bliskost, odnosno specifičnost autohtonih hrvatskih pasmina ovaca naspram drugih pasmina (Graf 10).

Analizom seta mikrosatelitskih lokusa paške ovce učinjen je prvi korak u tipiziranju autohtonih pasmina ovaca na DNA razini. Rezultati istraživanja uspoređeni su sa slovenskim autohtonim pasminama (Graf 11). Nei-eva genetska distanca (*D*) između dvije lokalne slovenske pasmine, jezersko-solčavske i bovške ovce je nešto manja (0,2443), nego distanca između istarske pramenke i paške ovce (0,3606). Ovakav rezultat je očekivan budući da filogenetski paška ovca predstavlja meriniziranu pramenku otoka Paga.



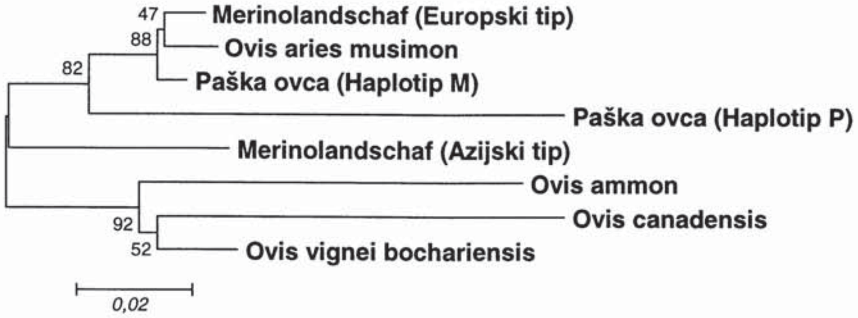
Graf 10. - UPGMA DENDROGRAM SASTAVLJEN NA TEMELJU FREKVENCIJA POLIMORFNIH KRVNIH PROTEINA PASMINA OVACA (*EUCLIDEAN LINKAGE DISTANCE*) (Mioč i sur., u tisku)



Graf 11. - UPGMA DENDROGRAM GENETSKIH ODNOSA IZMEĐU TRI SLOVENSKE PASMINE OVACA I PAŠKE OVCE (UTVRĐENO NA OSNOVI SEDAM MIKROSATELITSKIH LOKUSA) (Ivanković i sur., u tisku).

U proučavanje genetske strukture paške ovce uključeno je sekvenciranje proksimalnog dijela (nt 15816-16153) D-loop regije mtDNA. Nakon grupiranja sekvenci paške ovce, uočena su dva osnovna haplotipa (haplotip M i P). Različitost haplotypa M u odnosu na haplotip P (6,78 do 9,44 %) veća je nego između haplotipova spomenutih u ranijim studijama.

Uvažavajući činjenicu da je paška ovca nastala i merinizacijom originalne otočke pramenke, pretpostavljamo da uočeni P haplotip pripada originalnom genotipu izvorne paške ovce (originalnoj otočkoj pramenci). Slične ili identične sekvence nismo pronašli u dostupnim bazama sekvenci (NBCI, GenBank-Database, http://www.ncbi.nlm.nih.gov) i znanstvenim publikacijama. Mali stupanj sekvenčne različitosti haplotipa M naspram europskog tipa merina (Merinolandschaf - europski tim) (2,58%) i *O. musimon* (3,16%) ukazuje da je haplotip M introduciran u populaciju paške ovce merinizacijom (Ivanković i sur., u tisku).



Graf 12. Neighbor-joining dendrogram D-loop sekvence mtDNA (nt 15816-16136) pokazuje odnose članica subgenusa *Ovinae*: Merinolandschaf – europski tip (AccNo. AF039577); *O. aries musimon* (AccNo. AF039579); paška ovca (haplotipovi M i P); Merinolandschaf - azijski tip (AccNo. AF039578); *O. canadensis* (AccNo. AF076917); *O. vignei bochariensis* (AccNo. AF039580) i *Ovis ammon* (AccNo. AJ251327) (Ivanković i sur., u tisku).

## *Zaključak*

Prethodne informacije bit će upotpunjene korištenjem i drugih sistema markiranja, primarno mikrosatelita. Provjereni rezultati poslužit će reviziji postojećeg sustava zaštite, državnoj potpori i dizajniranju programa.

Rezultati istraživanja bit će baza za definiranje adekvatne suradnje s institucijama i stručnim službama susjednih zemalja u namjeri sprečavanja inbridinga i drugih nepoželjnih pojava do kojih može doći u revitalizaciji malih, kritično ugroženih populacija.

Dvosmjerno pisani zapisi zapisani su na više pisama, tj. na pismima koja se pišu s lijeva na desno i pismima koja se pišu s desna na lijevo.

Svaka promjena pisma započinje u novom redu opisa na odgovarajućoj margini, ali samo kad se smjer pisma ne mjenja unutar elementa. Propisana interpunkcija navodi se na početku novog elementa na način kako to pismo zahtjeva, osim propisane točke, zareza ili točke-zareza, koji se navode na kraju prethodnog elementa na pismom propisan način (vidi 0.4). Tako se, na primjer, točka, iz simbola točaka, razmak, crta, razmak (.-) bilježi na kraju prethodnog elementa, a sljedeći element počinje crtom u novom retku uz odgovarajuću marginu.

Npr. Kuwait business statistics [Elektronička građa]
------------------------------------------------=
Kuwait City: Dar al Nasher, cop. 1996.
(Najprije su navedeni engleski podaci)
=Kuwait business statictics.
-Kuwait Ciy: Dar al Nasher, cop. 1996.
(Najprije su navedeni arapski podaci)

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