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GENETIC CHARACTERISATION OF KRIVOVIRSKA PRAMENKA SHEEP

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Abstract: One of strategic goals in the field of improving our sheep breeding is to preserve sheep genofund. An important aspect in that context is genetic characterisation of our locally adapted breeds. The present research was conducted in summer season on 30 Krivovirska Pramenka sheep aged about 3 years on average and raised in 3 different localities. The blood of these animals was taken by venepuncture of *v.jugularis* into 5 ml test-tubes that were properly marked and samples stored at 4°C in a refrigerator and transported to a laboratory. Isolation of total genomic DNA was performed by a commercial kit for the isolation of DNA from blood for which 100 μl blood per animal was used. For genotyping a set of 12 nucleus microsatellites recommended by the International Society for Animal Genetics – ISAG was used. Molecular data obtained by amplifying nucleus microsatellites were processed by means of following standard bioinformatics packages: GenAlEx 6.5 (Peakall and Smouse 2012), HP-Rare 1.0 (Kalinowski 2005), Coancestry v.1.0.1.9 (Wang 2011) and STRUCTURE 2.3.4. (Pritchard et al. 2000). On the basis of the research conducted a relatively high level of genetic diversity was determined in studied population as well as a small number of animal pairs whose level of relatedness was higher than expected one. At the same time, a low and positive inbreeding coefficient not statistically significant was detected what indicates that examined population of Krivovirska Pramenka sheep is not burdened by inbreeding. The results obtained are informative and with an application of additional markers can serve for obtaining the guideliness for preserving and sustainable breeding of Krivovirska Pramenka sheep in Serbia.

Key words: Krivovirska Pramenka, genotyping, microsatellites, diversity

Introduction

Biodiversity (Greek *bios* - life, *diversio* - diversity) means genetic, species-wide ecosystem and molecular diversity (Campbell, 2003). Survival of humans is connected with a preservation of an overall biodiversity because an extinction of certain species may devastate our ecosystems, lead to their degradation and deterioration while an irreversible loss of biological diversity can permanently affect the survival of the other species and therefore humans as well. Due to its geographical position, climatic conditions, zones and orobiom Serbia is an important centre of biodiversity both at a national, European and global level. It is one of 153 centers of the world biodiversity and one of 6 centers of European diversity (Strategy of Biological Diversity of the Republic of Serbia, 2011-2018). On the other hand, it is expected that by 2050 human population rise to 9 billion people. Because of that an agricultural production will represent an unprecedented challenge in the next 30 years. About 40 species of domestic animals and poultry contribute to meeting the human population needs in providing meat, milk, eggs, animal skin and manure. Among these species about 8 800 breeds and strains make animal genetic resources (AnGR) that represent a key component of biological diversity and at the same time are crucially important for food and agriculture (FAO, 2021). A significant segment of social and economic structures of rural communities which contribute to satisfying the human population needs in food (Tempelman and Cardelino, 2007) are sheep genetic resources what is particularly noticeable in the conditions of climatic changes taking into account that autochthonous sheep breeds function better in an extreme conditions of environment compared to other ruminants (Al-Dawood, 2017). However, depopulation and deagrarianisation of rural regions have caused the endangerment of sheep genetic resources regarding their biological survival (Odjakova et al., 2023).

In order to preserve such sheep breeds the first necessary step would be to make out a sheep morphological characterisation followed closely by a genetic one taking into account that in a small autochthonous population endangered in its biological survival there may occur a phenomena of inbreeding, genetic drift and decreased flow of genes what may reduce a genetic variability and lead to genetic erosion resulting in a loss of potentially useful alleles in population genetic fund and in a decrease of heterozygosity in population individuals. In Serbia this could, among other things, be a consequence of prevailing morphological characterisation not followed by a genetic one although it is now deemed a common procedure both in Europe and the world at large since it can quantify negative genetic processes that represent a threat to survival of endangered populations.

Over a considerable period of time, besides morphological characterisation of breeds, a genetic characterisation has also been performed globally by means of molecular markers (Baumung et al., 2004; Toro et al., 2009; FAO 2011; Lenstra et

al. 2012) serving not only as an extra addition to morphological characterisation but being also of a considerable use to solving doubts regarding differentation between one or more breeds when on the basis of relatively small differences in phenotype it cannot be clearly determined. This has recently been seen on the example of Pramenka sheep when its local populations from the regions of Croatia and Slovenia have been characterised as separate breeds primarily on the basis of their unique genetic profiles (Ćinkulov et al., 2008; Report, 2021; Programme of Rural Development, 2014).

The most common type of molecular markers having been more then 30 years successfully used in genotyping in numerous animal species are nucleus microsatellites (Ellegren, 2004; Selkoe and Toonen, 2006; Guichoux et al., 2011; Abdul-Muneer, 2014; Putman and Carbone, 2014; Ellegren and Galtier, 2016). For genotyping selectively neutral microsatellites are used. On the other hand, variability of control region (hypervariable region – HVS or D-loop) mitochondrial genome (mtDNA) inherited through mother is very successfully used in the research of evolutionary history both of animal and human species as well as in the process of domestication (Beja-Pereira et al., 2004; Pedrosa et al., 2005; Wang et al., 2007; Achilli et al., 2009; Lancioni et al., 2013). Keeping in mind a long tradition of sheep breeding not only in the region of Serbia but also in the region of a whole Balkan Peninsula (Ciani et al., 2020) as well as an important geographical position of this area where in the course of history many migrations happened it can be expected that local sheep populations therein contain such mitochondrial maternal lines that can contribute to better understanding of evolutive history of sheep in a given region as well as in a process of domestication such as was the case during the analysis of variability of mitochondrial genome in donkey (Stanišić et al., 2017).

A poor research interest for Pramenka sheep raised in the territory of Serbia during past decades has resulted in only a small number of scientific studies on its various populations including Krivovirska Pramenka sheep as one of the less studied genotypes among its examined phenotypes.

According to data of Domestic Animal Diversity Information System (DAD-IS) for 2025 in Serbia a 2661 female and 74 male animals of Krivovirska Pramenka sheep are being bred. An effective size of population is 315 animals and that designates it as a potentially endangered sheep breed. In the research conducted by Ružić Muslić et al. (2023) the results of morphometrical characterisation and the indices of physical development in Krivovirska Pramenka sheep were presented. This study focuses on further research on this issue, i.e. genetic characterisation of Krivovirska Pramenka sheep in order to both prevent its genetic erosion and contribute to its preservation, scientifically based sustainable utilisation and conservation as an essential activity in ANGr management planning at local, national and global level. In addition, preservation of its genetic integrity

is being closely connected with determination of an exact number of sheep breeds both in our region and in the world (FAO, 2000).

Material and Method

The research conducted in a summer season included 30 Krivovirska Pramenka individuals about 3 years old on average and raised in 3 different localities in Serbia: Valakonje, Podgorac and Boljevac.

Respecting all regulations regarding animal welfare and the consent granted by the Ethical Commission the animal blood samples were taken by venipuncture of *v.jugularis* into 5 ml test tubes properly marked and stored at 4°C in refrigerator and later transported to laboratory. The blood with EDTA in test tubes was frozen for further use. Isolation of total genomic DNA was performed by use of commercial kit for DNA extraction from sheep blood using 100 μl blood per animal. A set of 12 nucleus microsatellites recommended by the International Society for Animal Genetics – ISAG) and the Food and Agriculture Organisation of the United Nations – FAO for sheep genotyping was used for genotyping and 4 markers recommended by FAO 2011: INRA063, MAF65, MAF214 and OarFCB20 (FAO, 2011), and 8 markers from the updated ISAG list of markers recommended for sheep genotyping in 2017: CSRD247, ETH152/D5S2, INRA005, INRA006, INRA023, INRA172, MCM042 and MCM527.

However, during the optimisation of protocol for PCR amplification it was observed that amplification of locuses ETH152/D5S2, INRA005 and INRA023 was not successful in neither of the individuals even after repeated reactions of PCR amplifications. Besides, PCR amplification of locuses MAF214, INRA172 and MCM527 was only partly successful in studied sample. Thus, only 6 of 12 molecular markers selected for genotyping (CSRD247, INRA006, INRA063, MAF65, MCM042 and OarFCB20) provided reliable molecular and genetic data while molecular markers ETH152/D5S2, INRA005, INRA023, INRA172, MAF214 and MCM527 were determined as not suitable to be used in further analyses.

Taking into account that reliability of the results of molecular and genetic analyses depends on the number of used locuses it was decided that the analyses should be directed to an additional set of 12 nucleus microsatelites such as follows: BM1818, BM2113, ETH225, ILST29, ILST87, INRABERN185, MAF70, OarFCB48, SPS113, SRCRSP8, TCRVB6 and TGLA53, among which 8 were successfully amplified: ETH225, ILST87, MAF70, OarFCB48, SPS113, SRCRSP8, TCRVB6 and TGLA53. Therefore, genotyping of 30 Krivovirska sheep was done by using not 12 but 14 highly informative and reliable nucleus microsatelites. The list of markers is shown in Table 1.

Table 1. The list of nucleus microsatellites used in genotyping of Krivovirska Pramenka sheep population

	Marker
1	CSRD247
2	INRA005
3	INRA006
4	INRA23
5	INRA063
6	INRA172
7	MAF065
8	McM527
9	ETH152
10	MAF214
11	McM042
12	OarFCB20

An amplification of control region of mitochondrial genome about 1300 based pairs long being multiplied in all animals was done by means of the 15346for, 157rev and 15393 for primers (Lancioni et al., 2013) and F_BDG (Pedrosa et al., 2005).

Direct primers for multiplying nucleus microsatellites were marked by one of 4 fluorescence colours from DS-33 set of colours (Applied Biosystems). Nucleus loci were multiplied by a polymerase chain reaction (*Polymerase Chain Reaction* - PCR) using Type-it microsatellite kit (Qiagen) which enables a parallel amplification of more loci in one reaction. The amplification of control region of mitochondrial genome was done by standard PCR reactions. Separating the products of PCR amplification of nucleus microsatellites was done by a capillary electrophoresis (fragment analysis) using ABI 3130 Genetic Analyzer automatic sequencer (Applied Biosystems). Determination of the length of PCR amplification of nucleus microsatellites products was performed using a GeneMapper programme (Applied Biosystems) by comparing the length of the fragments of PCR amplification with a LIZ500 scale (ThermoFisher Scientific). Determination of primer sequence of nucleotids of control region of mitochondrial genome was performed by use of a commercial Sanger sequencing by a Macrogen Europe company (https://dna.macrogen-europe.com/eng/). Molecular data obtained by amplifying nucleus microsatellites in Krivovirska Pramenka sheep were processed by means of following standard bioinformatics packages: GenAlEx 6.5 (Peakall and Smouse, 2012), HP-Rare 1.0 (Kalinowski, 2005), Coancestry v.1.0.1.9 (Wang, 2011) and STRUCTURE 2.3.4. (Pritchard et al., 2000). Following standard parameters of genetic diversity: number of alleles (A), number of private alleles (PA), average number of alleles (Na), effective number of alleles (Ne), obtained heterozygosity (HO), expected heterozygosity (He) and coefficient of inbreeding

(F) were determined by using a GenAlEx 6.5 programme package. The abundance of alleles was determined by means of a HP-Rare 1.0 programme package. The values of parameters relatedness in animals (relatedness - r) and inbreeding coefficient of individual animals (F) were determined by use of TrioML estimator implemented in Coancestry v.1.0.1.9 programme package. An optimal number of genetic groups in mentioned population was determined by STRUCTURE 2.3.4 programme package.

Results and Discussion

Table 2 presents the results obtained for genetic characterisation of Krivovirska Pramenka sheep implying the parameters of genetic diversity. In the population of 30 Krivovirska sheep individuals a total of 98 alleles was detected on 14 studied loci. Number of alleles per locus (Na) ranged from 3 (ETH225) to 11 (ILST87 and MAF70) with an average count of 7 alleles per locus. An average effective number of alleles (Ne) was 4.24 while the values of this parameter per individual loci ranged from 2.36 (MCM042) to 6.55 (MAF70). The results obtained correspond with the research conducted by Odjakova et al. (2023), on 3 autochthonous sheep breeds (Rhodopean Tsigai, RT; Middle Rhodopean sheep, MRS; and Karakachan sheep, KS) in which they determined 4.72 effective alleles on average.

The average value of the abundance of alleles parameter performed according to the method of rarefaction was 7.00 for 60 copies of genes.

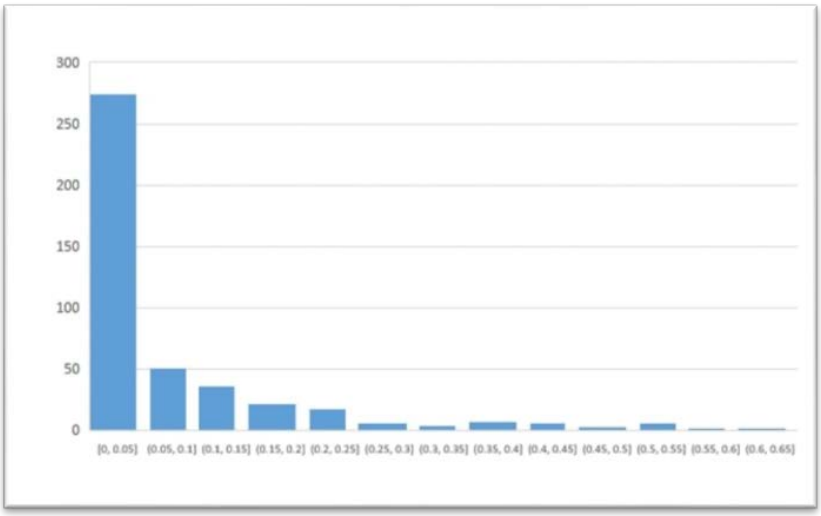
The heterozygosity obtained (Ho) per individual loci ranged from 0.433 (SPS113) to 0.900 (TCRVB6). The average obtained heterozygosity was 0.717 (SE=0.033). The expected heterozygosity (He) per individual loci ranged from 0.576 (MCM042) to 0.847 (MAF70). The mean expected heterozygosity was 0.737 (SE=0.024). The expected heterozygosity (He) that is being considered as a significant indicator of genetic diversity in Krivovirska pramenka population is somewhat lower than 0.76, 0.77, 0.77 values obtained in the studies by Odjakova et al. (2023), Georgieva et al. (2013), as well as Hristova et al. (2017), respectively. In the examined population a low and positive inbreeding coefficient of FIS=0.027 (SE=0.034) was detected but it was not statistically significant.

Table 2. Parameters of genetic diversity in examined population of Krivovirska sheep

Locus	N	A	Ne	Ar_{60}	Ho	He	F
CSRD247	30	7	5.33	7.00	0.767	0.812	0.056
ETH225	30	3	2.60	3.00	0.533	0.616	0.134
ILST87	30	11	6.23	11.00	0.800	0.839	0.047
INRA006	30	5	3.79	5.00	0.700	0.736	0.049
INRA063	30	6	2.85	6.00	0.667	0.649	-0.027
MCM042	30	5	2.36	5.00	0.667	0.576	-0.157
MAF65	30	7	3.16	7.00	0.733	0.683	-0.073
MAF70	30	11	6.55	11.00	0.833	0.847	0.016
OarFCB20	30	8	5.73	8.00	0.767	0.826	0.071
OarFCB48	30	8	5.83	8.00	0.833	0.828	-0.006
SPS113	30	5	3.03	5.00	0.433	0.670	0.353
SRCRSP8	30	8	3.94	8.00	0.667	0.746	0.106
TCRVB6	30	7	4.81	7.00	0.900	0.792	-0.136
TGLA53	30	7	3.23	7.00	0.733	0.690	-0.063
TOTAL/AVERAGE	30	98	4.24	7.00	0.717 (SE=0.033)	0.737 (SE=0.024)	0.027 (SE=0.034)

N – size of population; A – number of alleles; Ne – effective number of alleles; Ar_{60} – abundance of alleles per method of rarefaction for 60 gene copies; Ho – obtained heterozygosity; He – expected heterozygosity; F – inbreeding coefficient.

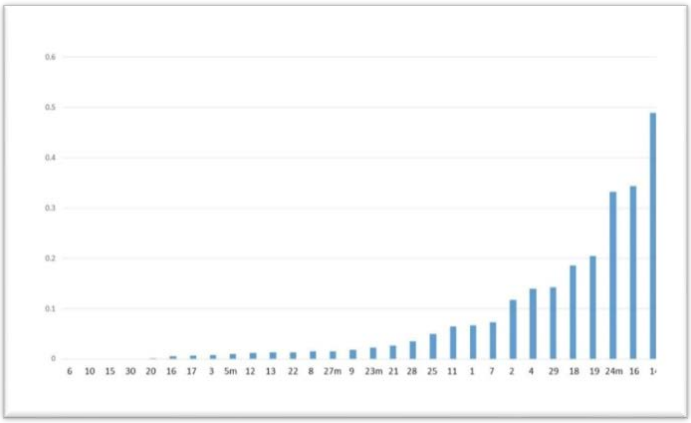
The values of the animal pairs relatedness parameter (relatedness - r) that can give an insight into a pedigree of studied animals and the inbreeding coefficient of individual animals determined by use of TrioML estimator are shown in graphs 1 and 2, respectively.



Graph 1. The values of relatedness–r parameters in animal pairs determined by TrioML estimators

Relatedness of the pairs of animals in a studied population of Krivovirska sheep ranged from 0.00 to 0.622 in the highest number of animal pairs the relatedness ranging from 0.0 to 0.05 was observed implying that unrelated animals are in question. However, the pairs whose value of r - parameter was ≥0.50 (KV14/KV25, KV3/KV11, KV11/KV17, KV13/KV29, KV9/KV25, KV16/KV23m, KV2/KV16, KV12/KV27m, KV14/KV26 and KV23m/KV28) and the two pairs of animals whose value of the same parameter was ≥0.60 (KV19/KV27m and KV25/KV26) were detected. Taking into account that ~0.5 value of r-parameter indicate the first-degree relatives it can be concluded that in the studied population the pairs of animals being in a higher degree of relatedness than expected are present as well. For the purpose of determining the pedigree of studied animals it is however necessary to perform a calibration of the value of r parametre using the animal with known pedigree (Wang, 2011).

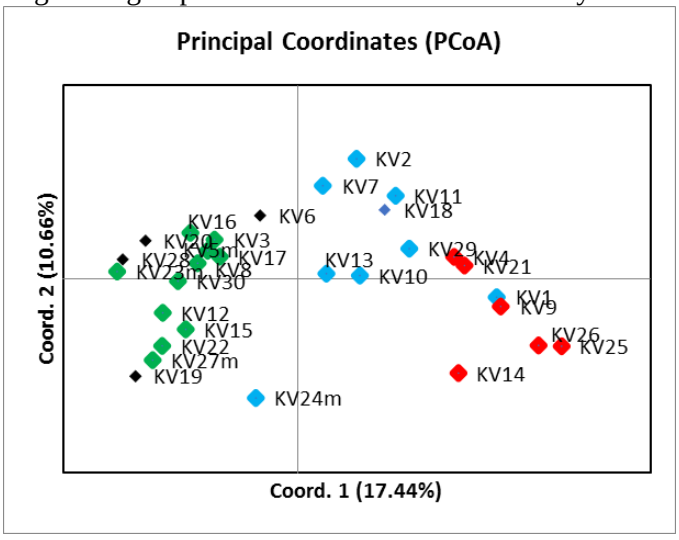
The inbreeding coefficient in individual animals (Graph 2) ranged from 0.00 (KV_6, KV_10, KV_15 and KV_30) to 0.49 (KV_14) not exceeding the value of 0.25 in majority of animals suggesting that the inbreeding is not present in a high degree in majority of animals.



Graph 2. The values of parameters of inbreeding coefficients in individual animals of Krivovirska Pramenka sheep

PCoA analysis performed on the basis of genetic distances of the animal pairs is shown in Graph 3.

In PCoA analysis the first and second coordinates explain 17.44% and 10.66% of a molecular variability, respectively. The first and second coordinates cummulatively explain 40.90% molecular variability. Distribution of the scores of animals in a two-dimensional space defined by the first and second coordinates most match the genetic groups detected in STRUCTURE analysis for K=3.

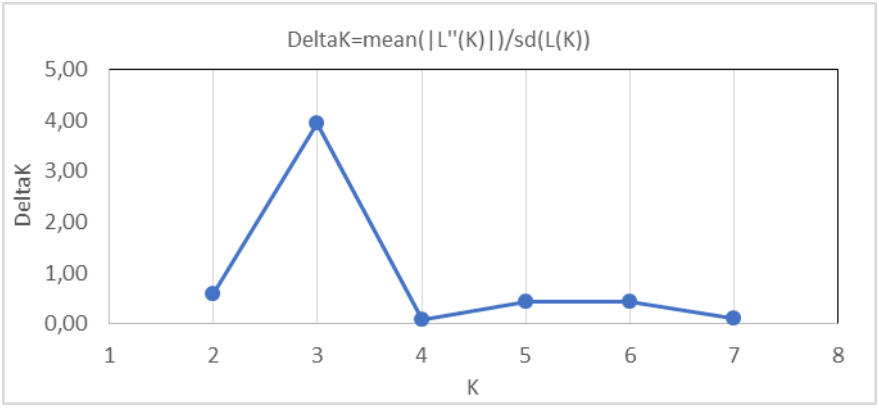


Graph 3. PCoA analysis performed on the basis of genetic distances of the animal pairs (animals characterised with a high degree of relatedness distributed either in red, blue or green gene pool in STRUCTURE analysis are marked by a rhomb of a corresponding colour while the other animals are marked by a black rhomb).

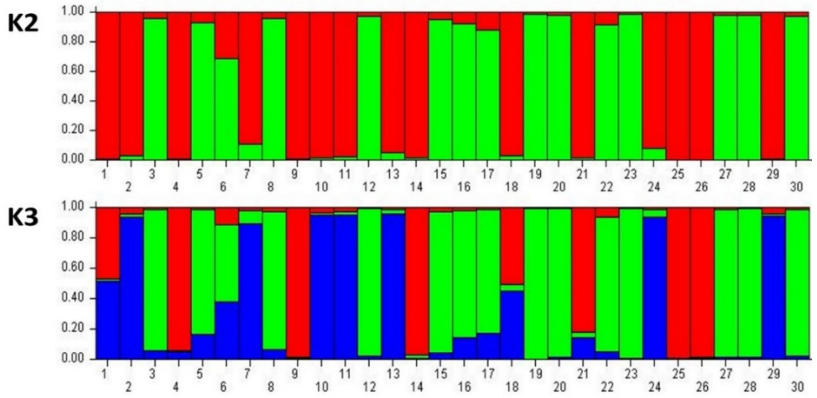
A substructure of studied population including 30 individuals of Krivovirska strain was determined by Bayesian method implemented in a STRUCTURE 2.3.4. bioinformatics package incorporating already given parameters: MCMC pre-analysis of 20 000 iterations, MCMC analysis of 20 000 iterations, the correlated allele frequency model and the relatedness of individuals per admixture model. The number of assumed genetic groups was K=1-8 while the number of analysis repetitions for each group was 10.

The optimal number of genetic groups, determined by an application of ΔK Evanno model (Evanno et al., 2005) was 3 (Graph 4). For K=3 a certain number of animals having a high share of relatedness (qi) distributed in each of 3 gene pools named by colour for easier tracking as red, green and blue gene pools was observed.

A display of substructure of 30 Krivovirska sheep studied population based on the results of STRUCTURE analysis for the number of K2 and K3 genetic groups is presented in Picture 1. Apart from the animals with a high share of relatedness (qi) distributed in one of the three gene pools the animals in which the admixture of gene pools was observed were also detected.



Graph 4. The optimal number of genetic groups in studied sample



Picture 1. Substructure of studied population of 30 Krivovirska sheep individuals based on the results of STRUCTURE analysis for the number of K2 and K3 genetic groups.

Three genetic groups detected in STRUCTURE analysis are matching the separation of animals obtained in PCoA analysis (Graph 3). Namely, per first PCoA coordinate the animals distributed in the so-called red and blue gene pool are separated from the animals distributed in the so-called green gene pool while per second PCoA coordinate the animals distributed in red gene pool are mostly separated from the animals distributed in a blue gene pool.

In spite of a small number of genotyped animals the results obtained in present research can offer useful information regarding both the structure of Krivovirska Pramenka sheep population and the estimation of genetic diversity serving as a starting point in designing effective breeding programmes.

Conclusion

Based on the research conducted it can be concluded that a relatively high level of genetic diversity was determined in the examined population of 30 Krivovirska sheep from the region of Serbia as well as a small number of animal pairs with the relatedness level higher than expected.

In the analysed population a low and positive inbreeding coefficient not statistically significant was detected indicating that the examined population of Krivovirska sheep is not burdened by inbreeding.

The obtained genetic data can be deemed both reliable and informative and together with morphological and other data and analysis on more populations as well as the application of additional markers in analyses can help formulate the guidelines and strategies for planned conservation of Krivovirska sheep in Serbia.

Genetska karakterizacija krivovirske pramenke

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Rezime

Jedan od strateških ciljeva na polju unapređenja ovčarstva je očuvanje genofonda ovaca. Važan aspekt u tom kontekstu je genetska karakterizacija naših lokalno adaptiranih rasa. Istraživanje je sprovedeno na 30 grla krivovirske pramenke, prosečne starosti oko 3 godine, u letnjoj sezoni, gajenih na 3 različita lokaliteta. Od navedenih životinja, uzeta je krv venepunkcijom *v.jugularis*, u epruvete od 5 ml, koje su uredno obeležene i uzorci su uskladišteni na 4°C u hladnjaku, nakon čega su transportovani u laboratoriju. Izolacija totalne genomske DNK je obavljena primenom komercijalnog kita za izolaciju DNK iz krvi za šta je korišćeno 100 μl krvi po grlu. Za genotipizaciju je korišćen set od 12 jedarnih mikrosatelita koji su preporučeni od strane Međunarodne organizacija za animalnu genetiku (*International Society for Animal Genetics* – ISAG). Molekularni podaci dobijeni umnožavanjem jedarnih mikrosatelita su obrađeni primenom standardnih bioinformatičkih paketa: GenAlEx 6.5, HP-Rare 1.0, Coancestry v.1.0.1.9 i STRUCTURE 2.3.4. Na osnovu obavljenih istraživanja, utvrđen je relativno visok nivo genetičkog diverziteta u ispitivanoj populaciji kao i mali broj parova grla kod kojih je stepen srodstva viši od očekivanog. Istovremeno, detektovan je nizak i pozitivan koeficijent inbridinga koji nije bio statistički značajan što ukazuje na to da ispitivana populacija krivovirske ovce nije opterećena inbridingom. Dobijeni podaci su informativni i uz primenu dodatnih markera mogu poslužiti za dobijanje smernica za očuvanje i održivo gajenje krivovirske rase ovaca u Srbiji.

Ključne reči: Krivovirska pramenka, genotipizacija, mikrosateliti, diverzitet

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Conflict of interest
The authors declare that they have no conflict of interest

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