MINISTRY OF AGRICULTURE, FORESTRY AND WATER MANAGEMENT OF THE REPUBLIC OF SERBIA

INSTITUTE OF MOLECULAR GENETICS AND GENETIC ENGINEERING

University of Belgrade

CONSERVATION OF ANIMAL GENETIC RESOURCES USING MOLECULAR-GENETIC CHARACTERIZATION

- a model of Busha cattle in Serbia -

STUDY

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Preface

Genetic resources in the Republic of Serbia are extremely rich, and include, among others, around 30 autochthonous breeds of domestic animals, which, in addition to being of immediate importance to the animal husbandry and agriculture of our country, also represent our cultural and intangible heritage that needs to be preserved. However, in the implementation of policy in the field of conservation of animal genetic resources (AnGR), there are certain difficulties, which the state has recognized, and which it is trying to overcome. In this context, and having in mind the current situation in this field in the world, there is a need to employ science in a much greater extent at all levels of the process of conservation of AnGR in Serbia, i.e. from the improvement of legal frameworks and the system of information exchange, to the improvement of the management of AnGR by breeders.

In Serbia, the national breeding programs for breeding and selection of important species of domestic animals define strategic frameworks and implementation mechanisms that overall contribute to improving animal husbandry both quantitatively and qualitatively. Methods of assessing production characteristics, evaluating and selecting quality breeding animals, as well as a plan for their use in order to achieve breeding goals, are determined. However, in the case of autochthonous breeds of domestic animals, where the number of breeding herds and individuals are decreasing, unwanted genetic processes, such as inbreeding and genetic drift, must be taken into account. These processes, along with demographic instability, result in the loss of genetic diversity, i.e., genetic erosion, accounting for a decrease in the adaptive value and evolutionary potential of the breed. Namely, due to the loss of useful adaptive alleles in small-sized gene pools of the breed, there is a loss of adaptive potential of individuals, as well as inbreeding and outbreeding depression, and the expression of the effects of harmful alleles due to the loss of heterozygosity. The mentioned processes can have a negative impact on the reproductive properties, productivity and growth, and ultimately, they can lead to the extinction of the breed, especially if the number of individuals decreases below the critical level.

Quantification of the aforementioned negative and undesirable genetic processes that threaten not only the conservation but also the improvement and development of AnGR is not carried out, with the exception of the inbreeding level, which is determined based on the pedigree data available in the herd books. However, the pedigree data that are not accompanied with detailed and accurate characterization of individuals cannot be used for providing guidelines to breeders on the manipulation (management) of individuals in herds, such as, for example, recommendations for the introduction of selected genotypes (individuals) in herds in order to preserve the genetic integrity of the breed, as well as to increase the level of genetic diversity, i.e., to reduce inbreeding and homozygosity. The management of breeding herds today is, so to speak, left to the breeders themselves, and therefore, despite their desire to reduce the inbreeding in their herds, it can still be characterized as stochastic.

Bearing in mind the fact that uncontrolled mating is still sporadically present today, this approach can have serious consequences for the survival of autochthonous breeds of domestic animals in our country. Therefore, the definition of additional guidelines on how to manage AnGR in breeding programs is a priority area of the state aimed at improving the conservation of AnGR. This can be realized through implementation of a new methodology - molecular-genetic characterization of breeds, which can be applied along with standard morphological characterization, which is still one of the basic approaches when describing and defining breed standards, recognizing breeds of domestic animals, and formulating breeding programs. Molecular-genetic characterization analyzes the variability of the genetic material (DNA) of individuals belonging to one breed, and generates molecular data that are then used for population-genetic analyzes that provides the values of certain parameters, which can then be used to define management guidelines for conservation of AnGR as well as for the improvement of breeding programs. By applying this methodology, the state will be able to provide breeders not only incentives for the preservation of AnGR, but also further guidelines for their manipulation (management). This would represent a transition to genetically-based and planned management of AnGR, and will contribute to more efficient conservation, development and sustainable use of AnGR in Serbia. In this way, it will be possible to raise the entire system of conservation of AnGR in our country to a qualitatively completely new level, which is in line with modern trends that are applied today in Europe and the world.

In Serbia, the molecular-genetic characterization of AnGR is still rare and sporadic today, and is mostly limited to the analysis of a smaller number of selected individuals (up to 100 per breed) by researchers from different institutions. The main limiting factor for more extensive genetic research is funding. In addition, due to the selection of different molecular markers for the analyses of the same breed, comparisons between different studies are not possible, as well as the assessment of the genetic status of the breed, nor the formulation of guidelines for their management, development and sustainable use, and improvement of breeding programs.

Given that recommendations for the application of a unique methodology for the molecular-genetic characterization of breeds of domestic animals were formulated during the past decade by the Food and Agriculture Organization of the United Nations (FAO), as well as that in Serbia there are institutions with the state-of-the-art equipment for performing molecular-genetic and other analyses, such as the Institute for Molecular Genetics and Genetic Engineering (IMGGE), University of Belgrade, as well as experts in the field of population genetics who, in cooperation with experts in the field of animal husbandry and veterinary medicine, can successfully implement and perform all activities related to the conservation of AnGR by means of molecular-genetic characterization, it is possible to apply this methodology in Serbia, with the aim of more efficient conservation of genetic resources of indigenous breeds of domestic animals, and to improve both the institutional structure and the information system that will enable better functioning of all links in the process of conservation of AnGR in our country.

For the purposes of implementing this project, a multidisciplinary team of experts from IMGGI, the Institute of Animal Husbandry (IAH), the Faculty of Agriculture of the University of Belgrade (FA UB) and the Faculty of Veterinary Medicine of the University of Belgrade (FVM UB) was formed, which through mutual cooperation established and implemented a unique methodology for the conservation of AnGR using combined molecular-genetic and morphological analyses, which provides relevant information for the improvement of the process of conservation of AnGR to direct users, primarily the Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia (MAFWM RS), but also to end users - breeders. In this way, the basis for the implementation of genetically-based and planned management of the genetic resources of autochthonous breeds of domestic animals can be laid in Serbia. Given that the implementation of these activities requires both time and significant financial resources, within this project one autochthonous breed of domestic animals - Busha cattle, was examined. In this way, a model can be established for the improvement of the conservation of AnGR by means of molecular-genetic characterization. This model can then, depending on available resources, be applied to all other breeds of domestic animals, and in accordance with their specificities.

Therefore, this project represents a unique and exceptionally large positive step forward in the field of conservation of AnGR in Serbia, which significantly contributes to the improvement of animal husbandry and agriculture in our country in general.

Dr. Jelena M. Aleksic, PhD

Principal Research Felloe at the IMGGE

THE CONTENT

1. INTRODUCTION

Busha cattle is a common name for small short-horned cattle that in the past were widespread in the hilly and mountainous areas of the Balkan Peninsula and beyond, and which over time became highly adapted to the local, highly heterogeneous environmental conditions. Therefore, some authors believe that in the Balkans, depending on the geographical distribution, that is, the territory of breeding, there are 14 strains of Busha cattle [1]. In our country, Busha cattle is traditionally maintained in rural hilly, mountainous and rocky areas south of the rivers Sava and Danube, as well as in Kosovo and Metohia, and is also present in Croatia, Bosnia and Herzegovina, Montenegro, North Macedonia, Albania and Greece [1, 4, 43].

Phenotypically, the Busha cattle is characterized mainly by a rough body constitution that is small, and reaches a length of about 131 cm, a height of 105-120 cm, and a body weight of 150-320 kg (females) or 250-450 kg (males) [1- 5]. The body is often overbuilt (higher at rump than at withers). The head is small, light and narrow, the bones of the head are fine, the forehead is broad, sunken, with accentuated eye sockets. Horns are short and thin, semicircular. The neck is dry and weak. The back is narrow, short and straight, the croup is roofed (pointed) and turned down, the chest is narrow, but deep and spacious. The front and back parts of the body are equally developed, with slightly pronounced widths and depths [3 - 6]. The legs are relatively short, with well-articulated joints, with spacious and firm pigmented hooves. The front legs have the regular posture, while the hind legs have cow-hocked posture, which makes it easier to move on steep terrain. The udder is usually short, shallow and small, with a regular shape. The typical phenotype of the female and male of Busha cattle is given in Figure 1.

Figure 1. Phenotype of the female (left) and male (right) of Busha cattle.

The Busha cattle has specifically pigmented muzzle, called „roe snout“, with dark pigmentation of mucous membranes in the nose, and a wreath of white hair surrounding it. Also, a striped so-called „eel line“, which is always in contrast with the basic color of the coat, extends along the dorsal line. Coat color is usually monochromatic but very diverse: gray, brown, yellow, white, red. There are also rare examples of the so-called “tiger Busha” which typically have a gray coat with dense dark narrow stripes [2, 7]. In the past, strains with different coat colors were typically present in certain regions. For instance, the gray (gray-ruddy) polimska Busha was present in southwestern Serbia, and the red Metohia Busha in southern Serbia, in Kosovo and Metohia [3 - 6]. Variation in Busha cattle body coat color is given in Figure 2.

Figure 2. Variation in Busha cattle body coat color.

In addition to the usual coat colors, which are characteristic of certain strains, and which in the past were linked to certain growing territories, individuals with brown coat color are rather common today in Busha cattle herds in Serbia. However, the brown coat color has not been mentioned in the scientific and technical literature from previous periods. The reason for this phenomenon should be sought in the fact that in today's breeding herds, there are individuals with different coat colors; since it is not possible to perform mating between individuals of the same coat color (i.e., same strain), its has been assumed that the brown color emerged as a result of stirring strains of different coat colors, that has led to the occurance of new genetic combinations that resulted in new coat color variants [7]. The hooves are always dark, and the horns are light around the base of the horn, and dark at the top. The typical appearance of the horns and the head is given in Figure 3, and the undesired appearance of the horns in Figure 4.

Figure 3. Typical appearance of the horns and heads.

Figure 4. Undesired appearance of the horns and heads.

The Busha cattle is a late-maturity breed, which reaches sexual maturity with 16-17 months. Heifers enter breeding at the age of 18-26 months, and they finish growth with 5 years of age. The lifetime of the individuals is about 20 years (Figure 5), and they are used in breeding for 10-15 years. Cows give one calf annually, and are characterized by good fertility and pronounced maternal instincts [3]. They calve alone and easily, and the calves have a body weight of 15-20 kg at birth. The lactation takes about 8 months on average, during which cows give about 1,000 kg of milk. The quantity of milk can be doubled in terms of better management conditions. The milk from Busha cattle contains 4-6% milk fat.

Figure 5. The typical appearance of an old cow.

The Busha cattle is resistant to diseases, and adapted to maintenance in adverse and harsh environmental conditions. It has low requirements in terms of care, growing conditions and nutrition. It is kept outdoors most of the year, on pastures and fenced outlets, where typically feeds by grazing and eating leaves and twigs during the vegetation period. During the winter, the Busha cattle is kept in the barnd, and feed by straw and corn [3, 4]. Today, it is mostly maintained in the cow-calf system, in an extensive way. The Busha cattle is a low-productive breed with the three-direction of production (meat, milk, work), which today has maintained only the importance of growing for meat production in extensive conditions.

Historically, the Busha cattle was significant in Serbia until half of the twentieth century [44], when, due to its modest productivity, it is increasingly replaced by breeds intended for intensive production. By intensifying cattle production and introducing more productive breeds, the interest in growing Busha cattle has been lost. Moreover, in the earlier period, activities were carried out on Busha cattle breeding and replacement crossing, when new breeds, such as a Gatačko cattle and Domestic-colored breed, were developed [3, 8].

Some 30 years ago, the number of Busha cattle breeding herds/individuals dropped in Serbia to critical size, and therefore, the state initiated the conservation program of this breed, recognized as an important genetic resources of Serbia [44], in 1993. Activities on the conservation of Busha cattle are more intensely conducted since 2000, and refer to increasing the size of the active population [9]. *In situ* and *ex situ* conservation have been applied. The Busha cattle herds are nowadays present not only in areas where they were traditionally maintained (mountainous areas in rural parts of Serbia), but also in areas where this breed was not present in the past. This is evident from the data on locations of farms with Busha cattle in different districts of Serbia [4, 7, 9, 42]. Due to the absence of systematic work on improving this breed, the Busha kept poor production traits. However, in better diet and care conditions, the Busha cattle can achieve better milk production, which is certainly positively reflected in the greater body mass of calves in standby as well, and directly to meat production.

Today in Serbia, the most important area breeding of Busha cattle is at the Stara planina mountain, with the center in Dimitrovgrad. Other significant areas of breeding in Serbia are the Vlasina (Crna Trava), Kuršumlija, Prokuplje. On small family farms, Busha is also present in the area of Pešter (municipalities of Sjenica, Tutin and Novi Pazar), as well as on the territory of the municipalities of Priboj, Prijepolje and Vranje. In the other parts of Serbia is present in smaller or larger herds or sporadically, in almost all districts. The Busha cattle is also mainteined in Kosovo and Metohia and at the territory of Vojvodina, where several herds were established (municipalities of Vršac, Kovin and Bela Crkva) [10]. The herd book for Busha cattle was established in 2009 [4]. The total number of heads of breeding animals registered in herd book in Serbia is 847.

Trends of the changes of the number of breeding individuals from 1999 until 2020, taken from the FAO site Domestic Animal Diversity information System (DAD-IS, https://www.fao.org/dad-is/en/), is shown in Table 1. (Data for 2021 and 2022 were not used), and the trends of the changes of the estimated effective population size (Ne), which is calculated by formula Ne = 4x (NM x NF) / (NM + NF), (N-number of breeding individuals, M-breeding males, F-breeding females) in Graph 1. The positive growth trend is observed during the last 10 years, respectively, after the 1990s, when the effective population size of Busha cattle was very low (Ne = 7.73).

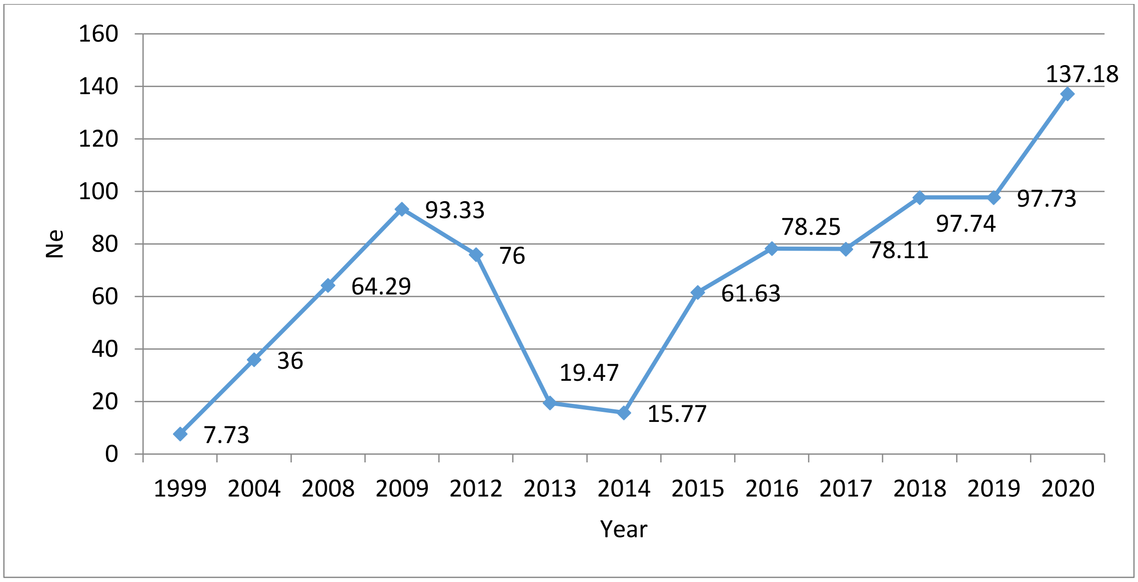
Table 1. Trends of the changes of the number of breeding animals of Busha cattle from 1999 until 2020.

	1999	**2004**	**2008**	**2009**	**2012**	**2013**	**2014**	**2015**	**2016**	**2017**	**2018**	**2019**	**2020**
Female*	57	90	150	350	380	185	270	416	896	827	1083	1077	1701
Male*	2	10	18	25	20	5	4	16	20	20	25	25	35
In total	**59**	**100**	**168**	**375**	**400**	**190**	**274**	**432**	**916**	**847**	**1108**	**1102**	**1736**

* breeding animals

Source: Domestic Animal Diversity Information System (DAD-IS, FAO).

Graph 1. Trends of the changes of the effective population size of Busha cattle in Serbia from 1999 to 2020.



Source: Domestic Animal Diversity Information System (DAD-IS, FAO).

According to the Serbian rulebook on conditions for recognition of new breeds, lines and hybrids of domestic animals [11], and based on the effective population size (Ne) as well as the total number of reproductive individuals (N), the Busha cattle is nowadays assigned to a group III, meaning that it represents a potentially endangered autochthonous breed. Therefore, the implementation of planned reproduction is very important in order to avoid mating among relatives. The Busha cattle represents a valuable cultural and intangible heritage of our state and beyond, and its conservation, development and improvement certainly represents one of the most important goals of the state.

The primary breeding goal for the Busha cattle in Serbia, defined in the breeding program for the period 2020-2024 [5], is an increase of the number of individuals, both in areas that represent species natural habitats (*in situ*) as well as in areas outside of its native habitats (*ex situ*). The aim is also to preserve the properties of health, resistance and longevity, which are typical for the Busha cattle, through maintaining the extensive breeding in conditions resembling its original habitats. That would maintain specific disease resistance as well as adaptation to modest maintaning conditions that evolved over the course of evolution. In such conditions, a coarse, i.e., rough body constitution, will be held, which is typical for this breed, and is essential for its adaptability and survival capacity. In addition, breeding objectives in terms of reproductive and production properties are also emphasized in the breeding program. However, it has been emphasized that as long as the population size does not increase and stabilize, the improvement of milk and meat yield cannot be considered as a primary breeding goal, due to the current threat to the breed survival associated with low population size.

As measures to preserve the genetic diversity of the Busha cattle population, and generally, biological diversity in animal husbandry in Serbia, procedures such as mating among unrelated individuals and monitoring of inbreeding levels and kinship have been put forward, as well as activities on providing genetic reserves. These measures need to be realized at all levels of the selection program, while respecting the provisions of the Livestock Low of the Republic of Serbia [12] and the Biological diversity strategy of the Republic of Serbia for the period 2011-2018 [13]. The maximum permitted degree of inbreeding is limited in certain countries to 5%, and the procedures for preventing inbreeding include planned mating of individuals who are not related to at least 4-6 generations. In the case of the Busha cattle in Serbia, due to the small size of the breeding herds the mating of individuals that are not related to at least 3-5 generations are allowed, as well as the maximum degree of inbreeding of 3.5%. However, since the uncontrolled mating is the most common way of mating in Busha cattle herds, it is recommended to perform regular replacement of breeding males in herds, at intervals of 3 years. Providing genetic reserves, on the other hand, is a strategic goal of each state, which is realized through *in situ* and *ex situ* conservation, as well as via the storage of genetic material (semen, oocytes, embryos, somatic cells, DNA and blood) in genebanks.

The breeding program for Busha cattle also emphasizes the obligation of phenotypic characterization, which determines the variability of relevant morphological and physiological characteristics, as well as the peculiarities of the production environment. According to the FAO guidelines from 2011 [14], the data that need to be provided for autochthonous breeds include: geographic distribution of the breed, size and structure of populations, phenotypic variability that includes the variability of morphological and physiological parameters, description of the exterior, information on economically profitable traits (e.g. growth, reproduction, productivity, yield-quality ratio), representative images of mature individuals of both sexes as well as herds in the natural environment, information on the origin and development of the breed, available research on phenotypic and genetic relations with other breeds inside or outside the country, data on natural habitats of the breed, information on the breed's response to stress factors in the external environment, such as diseases and parasites, extreme environmental conditions and nutrition, as well as relevant indigenous knowledge and traditional breeding management strategies used in local communities. Along with phenotypic characterization, the need for molecular-genetic characterization is highlighted.

Molecular-genetic characterization by means of selectively neutral molecular markers enables the determination of all relevant population-genetic parameters that describe genetic diversity, including the inbreeding level, then, the relatedness, genetic differentiation of breeding herds, number of genetic groups, gene flow, percentage of participation of gene pools of other breeds in the genetic structure of the breed of interest, etc. These data are of invaluable importance for the formulation of guidelines and strategies for the conservation and sustainable use of the breed, as well as for the improvement of breeding programs.

The most common type of molecular markers, which has been successfully used for more than 30 years in the genotyping of numerous animal and plant species are nuclear microsatelites (nuSSRs) [e.g., 15, 16], which are also called short tandem repeats (STRs), simple repetitive sequences (SSRs) or variable number of tandem repeats (VNTR). Microsatellites are highly specific, multiallelic, codominant and hypervariable molecular markers (mutation rate 10^{-2}-10^{-6} per locus per generation) [17, 18], and are very suitable for automation and parallel multiplication using a polymerase chain reaction (PCR) [19, 20, 21, 22, 23, 24, 25, 26, 27, 28]. Nuclear microsatellites are mainly found in non-coding part of the genome (so-called genomic microsatellites), and are considered not to be affected by the selection, so that the frequency and distribution of their alleles reflect exclusively mutation processes [21].

International Society for Animal Genetics (ISAG) in cooperation with FAO publishes regularly technical recommendations on usage of certain metholodologies for genotypization of domestic animals as well as for the use of highly informative and reliable molecular markers for application in certain species [14, 29]. The FAO/ISAG advisory body was founded in 1995, when the first recommendation for the usage of 30 nuclear microsatelites per species (cattle, poultry, sheep and pigs), was given [14, 29, 30]. The lists of molecular markers for each of the above mentioned species are periodically updated.

The application of nuclear microsatellites for the genotyping of Busha cattle in the Republic of Serbia represents a reliable and financially acceptable rapid screening of this breed, which provides insights into its genetic status and potential. After that, it is possible to apply the so-called genomic approach, which is based on assessing the variability of molecular markers of the second type, i.e., Single Nucleotide Polymorphisms (SNP), that allow: i) better resolution in terms of all the above-mentioned population genetics parameters and thus contribute to the improvements of the formulated breeding recommendations, ii) enable research on adaptive and other traits that are affected by selection, which are significant for productivity, reproduction, milkness, etc., iii) implementation of genomic selection for the desired production traits [31]. It is important to mention that numerous studies have demonstrated that the results obtained by means of selectively neutral SSRs and SNPs are concordant [32], and therefore, the results and recommendations resulting from application of nuSSRs for genotypization of Busha cattle are expected to be in line with those which are obtained by use of SNPs.

The aim of this project is to improve the process of conservation of AnGR in Serbia, through the establishment and implementation of a unique and modern methodology of molecular-genetic characterization by nuclear microsatellites, which will enable the improvement of breeding programs and transition to genetically-based and planned management of AnGR in our country. The Busha cattle was used as a model. To this end, we analyzed 287 individuals from breeding herds from 8 sites from 5 districts from Serbia, by means of 15 nuclear microsatellites recommended by FAO for use in cattle [14]. Based on the results of the project, the genetic status of the Busha cattle population in Serbia was assessed, as well as the genetic purity of individual herds, the genetic specificities of each herd, the degree of inbreeding; the guidelines were formulated for the management of Busha cattle in Serbia with the aim of maintaining the genetic integrity of the breed, increasing genetic diversity in herds and reducing of relatedness and inbreeding. In addition, the morphological characterization of the studied individuals was carried out, which enabled gaining insight into the compatibility of the studied individuals with the breed standard, body development and harmony of their structure, as well as the assessment of the development of the breed. This project represents the basis for genetically-based and planned management of Busha cattle in Serbia, both short-term and long-term.

2. MATERIAL AND METHODS

2.1. Selection of localities with herds/individuals for molecular-genetic and morphological characterization

The Ethical Permission for the use of animals for the project implementation was issued by the Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia, based on the request of the Ethical Commity of the Institute of Animal Husbandry. All rules related to animal welfare were followed.

Based on the data from the herd book, 13 farmers from 8 localities from 5 districts of Serbia maintaining Busha cattle are selected for participation in the project (Figure 6). The number of individuals used in the project ranged from three (Ub) to 90 (Dimitrovgrad). Sampled individuals included males, females and calves (Table 2). All individuals were used for molecular-genetic analyzes, and adults (males and females) for morphometric analyzes. Since population-genetic parameters for breeding herds comprising a few individuals cannot be performed, the individuals from the site Ub (farmer Branimir Živković), were grouped with individuals maintained by another farmer from this area, Nenad Velimirović, and were considered as a single breeding herd from Ub.

Table 2. Districts in Serbia with localities and Busha cattle breeding herds included in the project, and the number of individuals of different sexes and age used for molecular-genetic and morphometric analyzes.

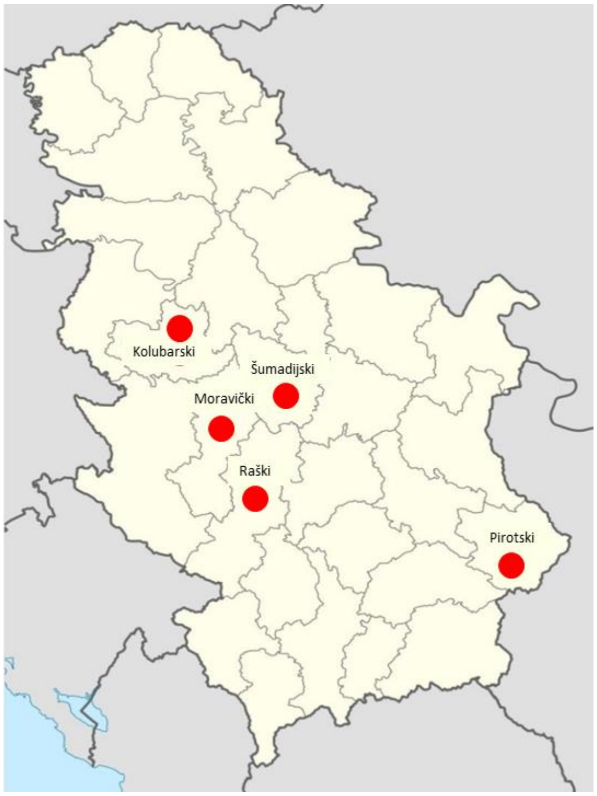
	District	**Locality (label) ***	**Breeder**	**Number of individuals**		
				males (m)**	females (z)	calves (t)
1	Kolubarski district	Osečina (R)	Igor Rabat	-/-	7/-	-/-
		Ub (B)	Nenad Velimirović	3/3***	24/25	30/-
			Branimir Živković	1/1	3/3	-/-
2	Moravički district	Ugrinovci (L)	Igor Dmitrović Slavica Jocović	1/1	17/17	10/-
3	Pirotski district	Dimitrovgrad (G)	Goran Jovanović, Goran Gogov, Milutin Nikolić and Jovanka Stevanović Ivanov	-/-	73/78	17/-
4	Raški district	Kamenjani-Ušće (Novi Pazar 1 - C)	Živorad Tošić	1/-	29/1	-/-
		Долово (Novi Pazar 2 - E)	Enver Ugljanin	2/1	14/15	3/-
		Rudnica (Novi Pazar 3 - I)	Ibrahim Ljuca	1/1	20/21	21/-
5	Šumadijski district	Gornja Sabanta (A)	Velimir Mijanović	1/-	9/-	-/-

* In parentheses are labels of locations used for presentation in charts, graphs and figures.

** m, z and t are labels for males, females and calves, respectively, in charts, graphs and figures.

*** Number of individuals used for molecular-genetic analyzes / number of individuals used for morphometric analyzes.

Figure 6. Districts where Busha cattle is maintained, which are included in the project (red circles).



Field work included the collection of blood samples (up to 1 ml per individual) for molecular-genetic analyzes, as well as assessment of exterior linear measures, relevant morphological properties and their frequency in the breeding herds and overall population. When working with animals, all animal welfare rules were respected. At all localities, a local veterinarian was present during field work, which took blood samples, as well as a representative of the local breeding organization.

2.2. Samples for molecular-genetic analyzes, molecular markers and laboratory work

Vacutainers with blood samples in sodium citrate are adequately marked, and kept in the refrigerator at +4 ° C during field work, and then, in the molecular laboratory, IMGGE, they were deposited in the freezer at -20 ° C prior to further manipulation.

The total genomic DNA was isolated from 100 µl of blood of each individual, using Zymo Research D3025 Quick-DNA miniprep kit (Zymo Research), according to the manufacturer's instructions. DNA yield and purity are determined spectrophotometrically, using the BioSpec-Nano (Shimadzu Europe). Molecular-genetic characterization was performed in the IMGGE laboratory, using 15 nuclear microsatelites recommended by FAO [14] for cattle genotyping: BM1818, BM1824, ETH10, ETH152, ETH225, ILST6, ILST29, INRA5, INRA23, INRA63, MAF70, MCM42, TGLA53, TGLA122 и TGLA126.

Direct primers for the amplification of nuclear microsatelites are labelled by one of four fluorescent colors from the dye set DS-33 (Applied Biosystems). Microsatellite loci are amplified in multiplex PCR reactions, using the Type-It microsellite PCR kit (Qiagen), which allows a parallel amplification of multiple loci in one reaction. Determination of sets of molecular markers that can be used together for parallel multiplication in PCR reactions was determined in preliminary analysis with 8 genotypes of Busha cattle.

Separation of PCR products of nuclear microsatellites were performed with capillary electrophoresis (fragment analysis) using automatic sequencer ABI 3500 Genetic Analyzer (Applied Biosystems).

Determining the length of PCR products was performed using program GeneMapper (Applied Biosystems), by comparing the length of PCR fragments with a ladder LIZ600 (ThermoFisher Scientific).

Molecular data obtained by parallel amplification of nuclear microsatelites via PCR reactions were used to determine the parameters of genetic diversity: number of alleles (A), effective number of alleles (Ae), allelic richness (Ar), number of private alleles (PA), obtained heterozygosity (H_O), expected heterozygosity (uH_E), inbreeding coefficient in populations (F_{ISpop}), effective population size (Ne), as well as to determine the coefficient of inbreeding of individuals (F_{ISind}), and the relatedness. They were also used to assess genetic differentiation of breeding herds (F_{ST}, G''st и Dest), gene flow (Nm), and to determine the number of genetic groups (К) and potential substructure of breeding herds. Assessments of all stated parameters were performed using standard, free bioinformatic packages Arlequin v3.5 [33], Coancestry v.1.0.1.9 [34], GenAlEx 6.5 [35], HP-Rare 1.0 [36], NeEstimator v2.1 [37], STRUCTURE 2.3.4. [38].

2.3. Morphological characterization

The number of individuals of Busha cattle that were used for morphometric analyzes was slightly lower than the number of individuals used for molecular-genetic analyzes, because it was not possible to assess morphological measures for certain number of individuals in accordance with animal welfare rules, and, in addition, measurements were not performed for calves. Therefore, 159 females and 6 males were included into morphological characterization (a total of 165 individuals), from six sites from four districts in Serbia (Table 2).

When measuring, the animals are individually directed via a corridor to a flat and stable site. After skillful and short-term restraint, body measurements were taken (Figure 7), in accordance with the methodology described in the literature [39]. The measurement was performed with Lydthin's rod and measuring tape, and the following exterior linear dimensions were measured:

1. Height at Withers (WtH) - vertical distance from the ground, behind the front hoof, to the highest point on the withers (area between the second and the fifth dorsal vertebrae).

2. Height at Rump (RuH) - vertical distance from the highest point of sacral bone (rump) to the ground.

3. Chest Circumference (ChC) - the circumference of the body immediately behind the shoulder blades in a vertical plane, perpendicular to the long axis of the body.

4. Chest Depth (ChD) - vertical distance from the chest floor to the highest point on the withers.

5. Chest Width (ChW) – the distance at the narrowest point behind the scapular blades (measured with a Lydtin’s rod from above).

6. Body Length (BL) - distance from the front edge of the shoulder-scapular joint (point of shoulder) to the rear point of the pin bone. In this study, the oblique length of the body was measured - during the measurement, the rod is held obliquely.

7. Pelvic Length (PvL) – distance from the hip bone (tuber coxarum) to the pin bone (tuber ischium).

8. Pelvic Width between Hip Bones (PvW_{HB}) - distance between the outer points of the hip bones (tuber coxae).

9. Pelvic-Femoral Joints Width (PvW_{FJ}) - distance between the external edges of the pelvic-femoral joints.

10. Pelvic Width between Pin Bones (PvW_{PB}) - distance between the pins bones (tuber ischii).

11. Head Length (HdL) - Distance from the top of the front bone to the beginning of the muzzle.

12. Head Width (HdW) - distance between external edges of orbital arches.

13. Tibia circumference (TbC) - the circumference at the thinnest point on the tibia.

In addition, the authenticity of phenotypes, i.e., the compliance of morphological characteristics with the standard of breed defined in the breeding brogram [5] is assessed for each individual. For individuals which are not recognized as typical representatives of the breed, in cases where it was possible, the introgression of other breeds, such as Montafone and Tyrolean breeds, was recorded.

The color of the body coat, that is, the hair cover, was visually assessed for 121 adult individuals (females and males).

The obtained morphological data were used to calculate average, minimum and maximum values as well as a variation coefficient (descriptive statistics), using programs STATISTICA 13 (TIBCO, Palo Alto, CA, USA). They were also used to assess 11 conformation indices, determined as follows:

1. Length Index - LI: (BL / WtH) x 100
2. Thoracic Index - TI: (ChW / ChD) x 100
3. Relative Depth of Thorax Index - RDTI: (ChD / WtH) x 100
4. Relative Width of Thorax Index - RWTI: (ChW / BL) x 100
5. Thoracic Development Index - TDI: (ChW / WtH) x 100
6. Over Increase Index - OII: (RuH / WtH) x 100
7. Thoracic-Pelvic Index - TPI: (ChW / PvW_{FJ}) x 100
8. Pelvic Index - PSI: (PvW_{PB} / PvW_{HB}) x 100
9. Leg Length Index - LLI: ((WtH - ChD) / WtH) x 100
10. Cephalic Index - CI: (HdW / HdL) x 100
11. Relative Cannon Thickness Index - RCTI: (TbC / WtH) x 100

Translating absolute values into relative values - indices, allows for better insight into body harmonity, and allows comparisons between animals.

All calculations were performed specifically for females and males from all sites used in this survey, and then, female individuals from different sites were analyzed independently.

Figure 7. Measuring of body dimensions of Busha cattle individuals on the field.

2. RESULTS AND DISCUSSION

3.1. Molecular-genetic characterization

Genetic profiles have been successfully generated using 15 nuclear microsatelites in 287 Busha cattle individuals, namely, 196 females, 10 males and 81 calves, from all the examined sites. Since the number of Busha individuals available in herd book in 2022 is 847, the results of this project can be considered reliable, because they are based on analyzes of 33.88% of the entire Busha population in Serbia. In addition, the analyzes included individuals from the Dimitrovgrad site, with currently the largest Busha cattle breeding herd in Serbia, but also sites with a smaller number of individuals, found near Dimitrovgrad or distant from this site. Therefore, a sample used in this study can be considered representative. The inclusion of calves into analyses along with adult individuals enabled comparisons among generations.

Genetic diversity parameters: number of alleles (A), effective number of alleles (Ae), number of private alleles (PA), allelic richness rarefacted to 14 gene copies (Ar_{14}), obtained heterozygosity (H_O), expected heterozygosity (uH_E), inbreeding coefficient per populations (F_{ISpop}), and effective population size (Ne) of adult breeding herds are presented in Table 3, and these parameters of genetic diversity for calves herds are presented in Table 4. The values of these parameters were obtained using bioinformatics packages Arlequin v3.5 [33], GenAlEx 6.5 [35], HP-Rare 1.0 [36] и NeEstimator v2.1 [37].

Obtained values of parameters of genetic diversity in adult breeding herds from different sites, entire population of adults, as well as in calve herds and the entire calve population, reveal that all examined herds and entire Busha populations in Serbia are characterized by a relatively high level of genetic diversity, which is comparable to values obtained previously for this breed [45]. High levels of genetic diversity is a represents a prerequisite for the long-term survival of this breed. However, it is worth mentioning that the introgression of gene pools of other breeds (see later) may have affected, at least partially, the observed levels of genetic diversity in studued Busha cattle population in Serbia.

Table 3. Parameters of genetic diversity of adult Busha cattle breeding herds and overall sample of adult individuals.

Breeding herd	**N**	**A**	**Ae±SE**	**Ar_{14}**	**PA**	**H_O±SE**	**uH_E±SE**	**F_{ISpop}±SE**	**Ne**
Gornja Sabanta	10	75	3.27±0.23	4.49	-	0.783±0.04	0.709±0.02	-0.163±0.05	28.3 (12.5-1089.7)*
Ub	31	101	3.91±0.38	4.76	2	0.707±0.03	0.728±0.02	0.014±0.03	31.0 (24.8-39.9)
Ugrinovci	18	86	3.67±0.32	4.59	2	0.697±0.03	0.721±0.02	0.002±0.03	12.2 (9.3-16.5)
Oseččina	7	64	3.20±0.23	4.27	1	0.790±0.05	0.703±0.04	-0.213±0.05	8.6 (3.0-32.1)
Novi Pazar 1	30	84	3.10±0.32	4.18	1	0.569±0.03	0.638±0.04	**0.090±0.03**	5.5 (4.2-6.7)
Novi Pazar 2	15	62	2.92±0.19	3.62	-	0.666±0.02	0.658±0.03	-0.063±0.04	8.3 (5.2-13.2)
Novi Pazar 3	21	79	3.24±0.22	4.23	1	0.651±0.04	0.686±0.03	0.034±0.05	11.3 (8.7-14.8)
Dimitrovgrad	74	123	4.19±0.41	4.88	17	0.761±0.03	0.732±0.03	-0.049±0.02	69.7 (59.3-83.3)
Total/average	206	137	3.44±0.11	4.38	24	0.703±0.01	0.697±0.01	-0.044±0.02	55.2 (51.1-59.7)

N – number of individuals used in the project.

SE – Standard Error.

* 95% Confidence Intervals are presented in parentheses.

Table 4. Parameters of genetic diversity of herds of calves and overall calve sample.

Herd	N	A	Ae±SE	Ar$_{20}$	PA	H$_O$±SE	uH$_E$±SE	F$_{ISpop}$±SE	Ne
Ub	30	97	3.57±0.29	5.06	7	0.722±0.030	0.709±0.022	-0.036±0.032	24.1 (19.4-30.5)*
Ugrinovci	10	81	3.53±0.36	5.40	5	0.747±0.040	0.708±0.038	-0.130±0.053	21.0 (6.7-28.6)
Novi Pazar 2	3	44	2.49±0.18	-	-	0.733±0.067	0.676±0.045	-0.296±0.079	-
Novi Pazar 3	21	87	3.23±0.21	4.87	7	0.702±0.030	0.686±0.024	-0.057±0.042	30.6 (21.3-49.6)
Dimitrovgrad	17	85	3.30±0.27	4.98	1	0.708±0.043	0.688±0.029	-0.052±0.038	39.9 (23.5-100.0)
Total/average	81	119	3.22±0.13	5.08	20	0.722±0.019	0.693±0.014	-0.114±0.025	46.9 (41.6-53.2)

N – number of individuals used in the project.

* 95% Confidence Intervals are presented in parentheses.

SE – Standard Error.

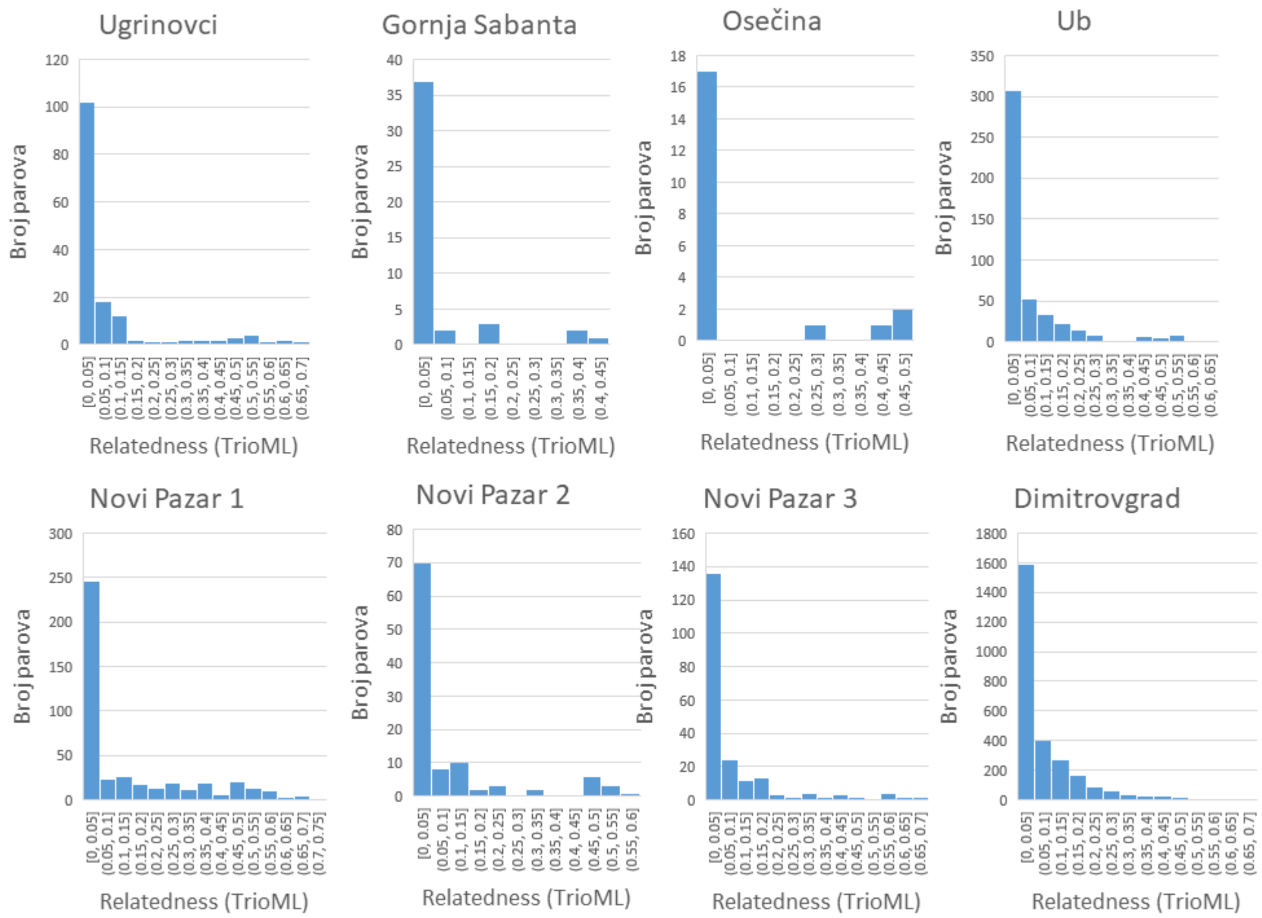
Statistically significant excess of homozygotes, expressed via inbreeding coefficients of populations, F_{ISpop}, was not observed in any adult and/or calve herd, with the exception of an adult breeding herd at the site Novi Pazar 1. However, since the substructure of this breeding herd was observed, that is, the presence of multiple genetic groups (see later), a positive and statistically significant F_{ISpop} parameter observed at this site does not reflect inbreeding, but is a result of Wahlund's effect [40], i.e., the mixing of different genetic groups with different allele frequencies. Given the fact that the herd substructure was observed at other localities as well (shown below), it is possible that the inbreeding coefficients at the herd level obtained in this project are slightly higher.

The effective population size (Ne) of the adult breeding herds ranged from 5.5 (4.2-6.7) in Novi Pazar 1 to 69.7 (59.3-83.3) in Dimitrovgrad, with an average value of 55.2 (51.1, 59.7). Given the variable size of the examined adult breeding herds, differences in Ne in breeding herds were expected. The effective population size of calve herds ranged from 21.0 (6.7-28.6) in Ugrinovci to 39.9 (23.5-100.0) in Dimitrovgrad, with an average value of 46.9 (41.6 -52.2). At sites with herds of smaller size, such as Ub and Novi Pazar 3, where the comparative number of adults and calves are analyzed, different trends in the effective population size are observed. Data for the calve herds in Dimitrovgrad, however, must be taken with cautions, because the number of analyzed calves is the half of the number of analyzed adults. In other words, increasing the number of analyzed calves would most likely contribute to a significant increase in Ne for the calve herds at this site.

Relatedness of pairs of adult individuals on each of the examined sites, estimated using the TrioML estimator implemented in the Bioinformatic package Coancestry v.1.0.1.9 [34], is shown on Chart 2.

Given that the relatedness of the two offsprings of the same parents estimated via TrioML estimator is ~ 0.5, as well as that lower values point to a lower level of relatedness, and higher values to a higher levels of relatedness, it is evident that the majority of adult individuals in all studied localites are not related to at least 3-5 generations. However, a higher relatedness of pairs of adult individuals was observed sporadically at several sites. This, however, reflects the presence of closely related groups at those site, i.e., grandmother-mother-daughter/son, and therefore, the occurance of such groups contributs towards somewhat higher estimates of relatedness (>0.5) at those sites.

Graph 2. Pairwise relatedness of adult individuals in the examined sites, estimated with the TrioML estimator.



Inbreeding coefficients for each adult individual, F_{ISind}, were also estimated with the TrioML estimator implemented in the Bioinformatic package Coancestry v.1.0.1.9 [34], and are presented in Chart 3.

Individual coefficients of the inbreeding of adult individuals at all examined sites were under 10% in most cases, and did not exceed 50%.

Genetic differentiation of adult breeding herds, expressed via F_{ST}, Hedrick's G''st parameter, and Jost's Dest parameter, are 0.066 ($p < 0.05$), 0.224 ($p < 0.05$) and 0.168 ($p < 0.05$), respectively, and genetic differentiation of calve herds, expressed via F_{ST}, Hedrick's G''st parameter, and Jost's Dest parameter, are 0.081 ($p < 0.05$), 0.270 ($p < 0.05$) and 0.203 ($p < 0.05$), respectively.

Genetic differentiation of pairs of adult breeding herds and pairs of calve herds, expressed via F_{ST} parameter, is given in Table 5.

Graph 3. Inbreeding coefficients of adult individuals, F_{ISind}, in the examined sites.

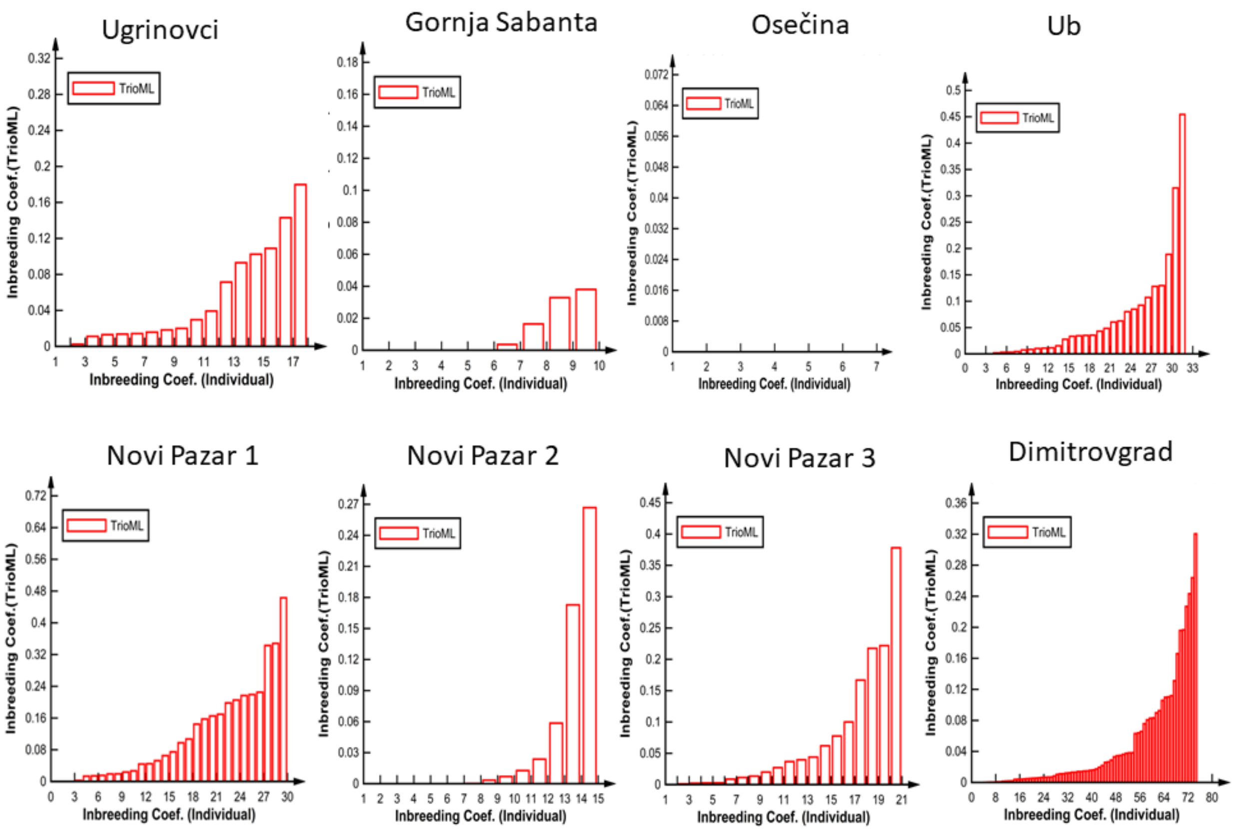


Table 5. Genetic differentiation of pairs of herds, expressed via F_{ST}. Below the diagonal are presented pairwise population F_{ST} values of adult breeding herds, and above the diagonal, pairwise population F_{ST} values of calve herds. All values are statistically significant ($p < 0.05$).

	Gornja Sabanta	**Ub**	**Ugrinovci**	**Osečina**	**Novi Pazar 1**	**Novi Pazar 2**	**Novi Pazar 3**	**Dimitrovgrad**
Gornja Sabanta	0	-*	-	-	-	-	-	-
Ub	0.028	0	**0.041**	-	-	0.070	0.089	0.044
Ugrinovci	0.048	0.021	0	-	-	0.065	0.092	0.076
Osečina	0.049	0.037	0.034	0	-	-	-	-
Novi Pazar 1	0.095	0.044	0.077	0.107	0		-	-
Novi Pazar 2	0.069	0.067	0.073	0.097	0.111	0	0.076	**0.107**
Novi Pazar 3	0.099	0.082	0.112	**0.119**	0.118	0.086	0	0.137
Dimitrovgrad	0.057	**0.014**	0.037	0.047	0.073	0.082	0.107	0

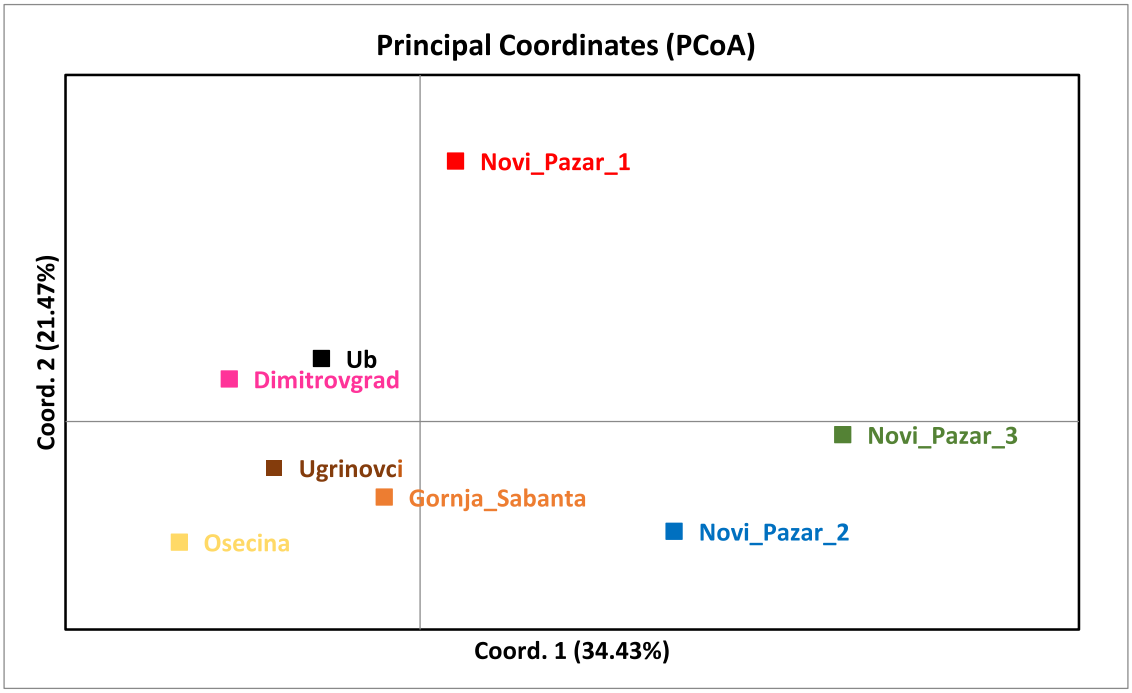
* The calves were not sampled at all localities, and therefore, it was not possible to determine the values for all pairs of calve herds.

Data in bold – minimal and maximal values.

Obtained pairwise population F_{ST} values among adult breeding herds reveal somewhat lower genetic differentiation between certain pairs of adult breeding herds, such as Dimitrovgrad and Ub ($F_{ST} = 0.014$), and also almost nine times higher genetic differentiation between certain pairs of adult breeding herds, such as Osečina and Novi Pazar 3 ($F_{ST} = 0.119$). This indicates that the gene flow, expressed via Nm, is not uniform among adult breeding herds, i.e., that the exchange of individuals between herds from different sites is differential, most likely due to the subjective preferences of farmers. The same holds for analyzed calve herds for which, however, somewhat higher minimal and somewhat lower maximal values of the pairwise population F_{ST} parameters were observed. The average gene flow among adult breeding herds is 2.85±0.23 migrants per generation. The average gene flow among calve herds is 2.38±0.22 migrants per generation.

The matrix of of genetic distances, F_{ST}, among breeding herds and/or individuals, were used for the Principal Coordinate Analysis (PCoA), and the results are presented in Charts 4 and 5 (adults with calves), Charts 6 and 7 (adults) and Charts 8 and 9 (calves).

Graph 4. Outcomes of the PCoA analysis on a matrix of pairwise population genetic distances (F_{ST}) of Busha herds (adults with calves).

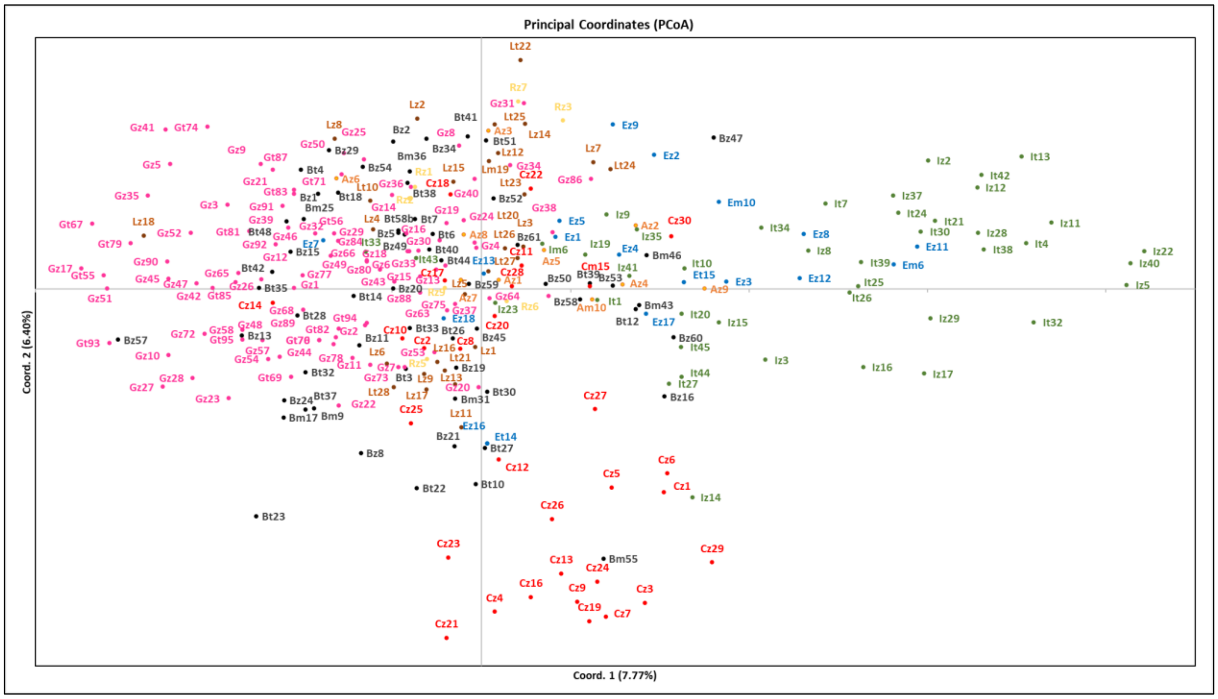


The first principal coordinate explains 34.43% of molecular variability, and separates herds at sites Novi Pazar 1, 2 and 3 from the herds from other examined sites, among which is locality Dimitrovgrad, with the largest number of individuals. The positioning of the scores of herds found at site Ub in the Chart 4 indicates its genetic affinity towards the herd from Dimitrovgrad, while herds at sites Ugrinovci, Gornja Sabanta and Osecina show somewhat lower genetic affinity towards herds at sites Dimitrovgrad/Ub.

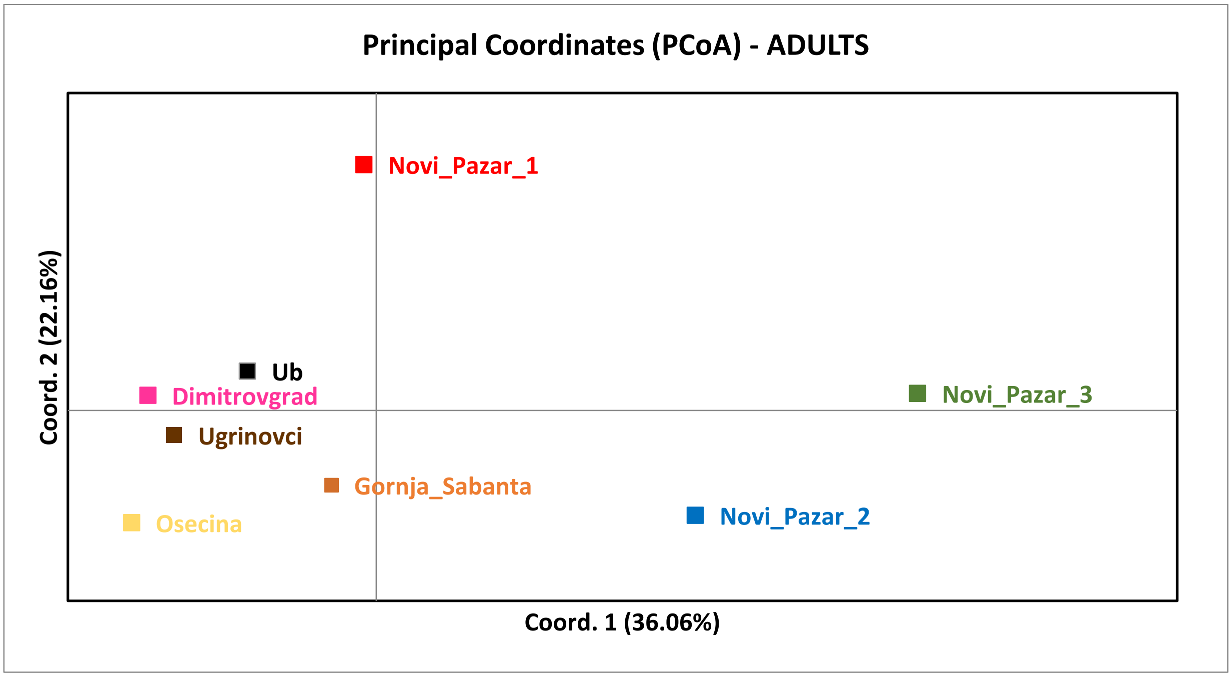
The second principal coordinate, which explains 21.47% of molecular variability, further separates herd at site Novi Pazar 1 from herds at sites Novi Pazar 2 and 3 sites, as well as herds from sites Dimitrovgrad/Ub from herds at sites Ugrinovci, Gornja Sabanta and Osecina.

The identical trend was observed when PCoA analysis was performed based on matrix of genetic distances (F_{ST}) between pairs of individuals (Chart 5), as well as when analyzes was performed with adult herds and individuals (Charts 6 and 7, respectively) and calves (Charts 8 and 9, respectively).

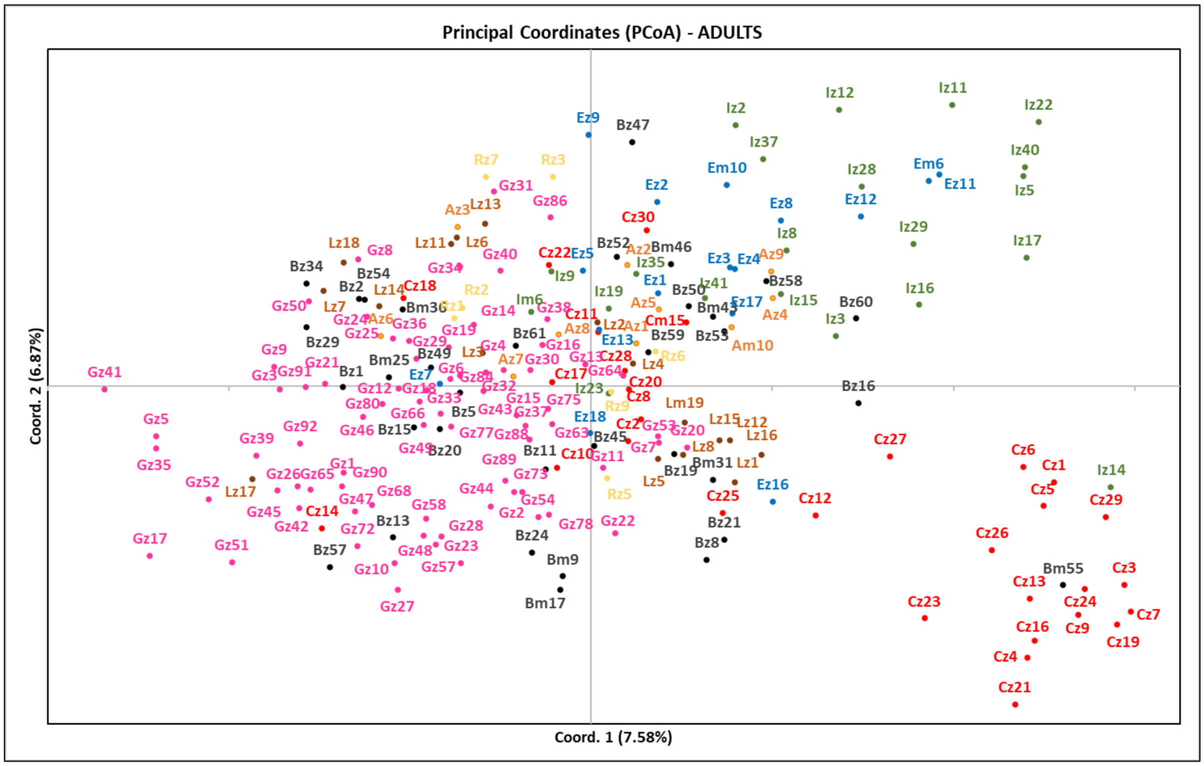
Graph 5. Outcomes of the PCoA analysis on a matrix of genetic distances, F_{ST}, of individuals in studied Busha cattle population (adults and calves).



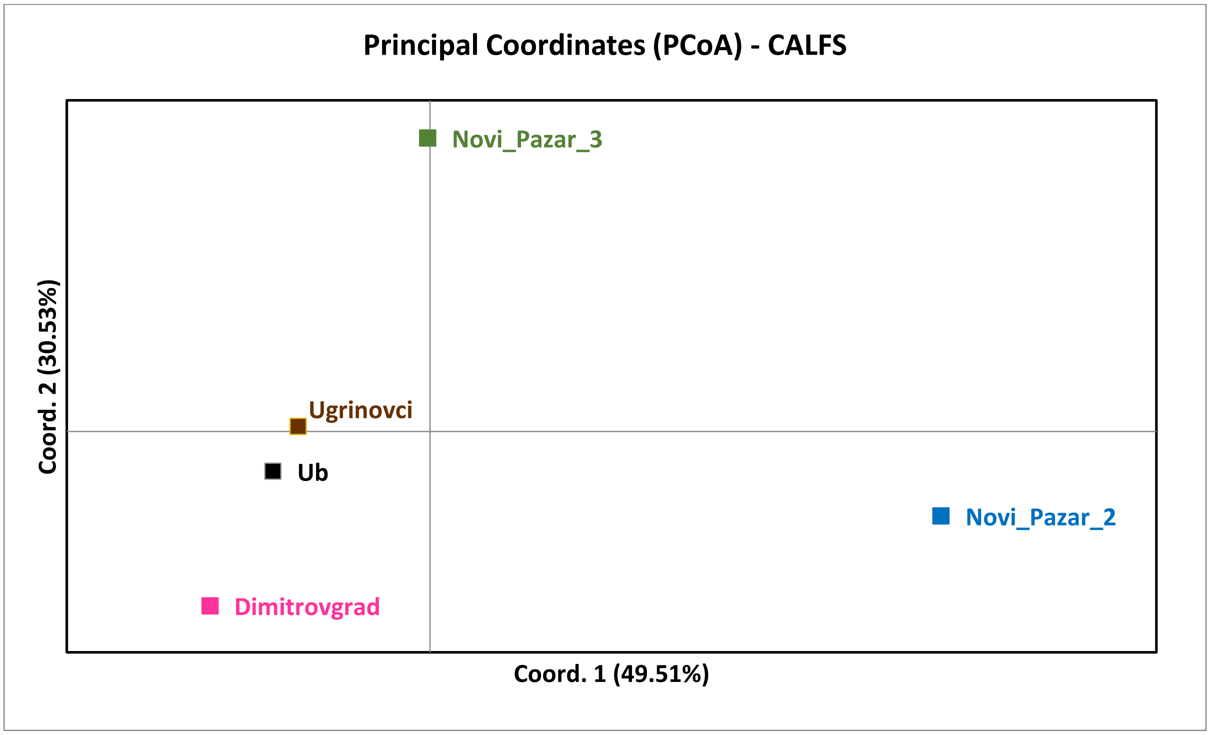
Graph 6. Outcomes of the PCoA analysis on a matrix of genetic distances, F_{ST}, of adult Busha cattle breeding herds.



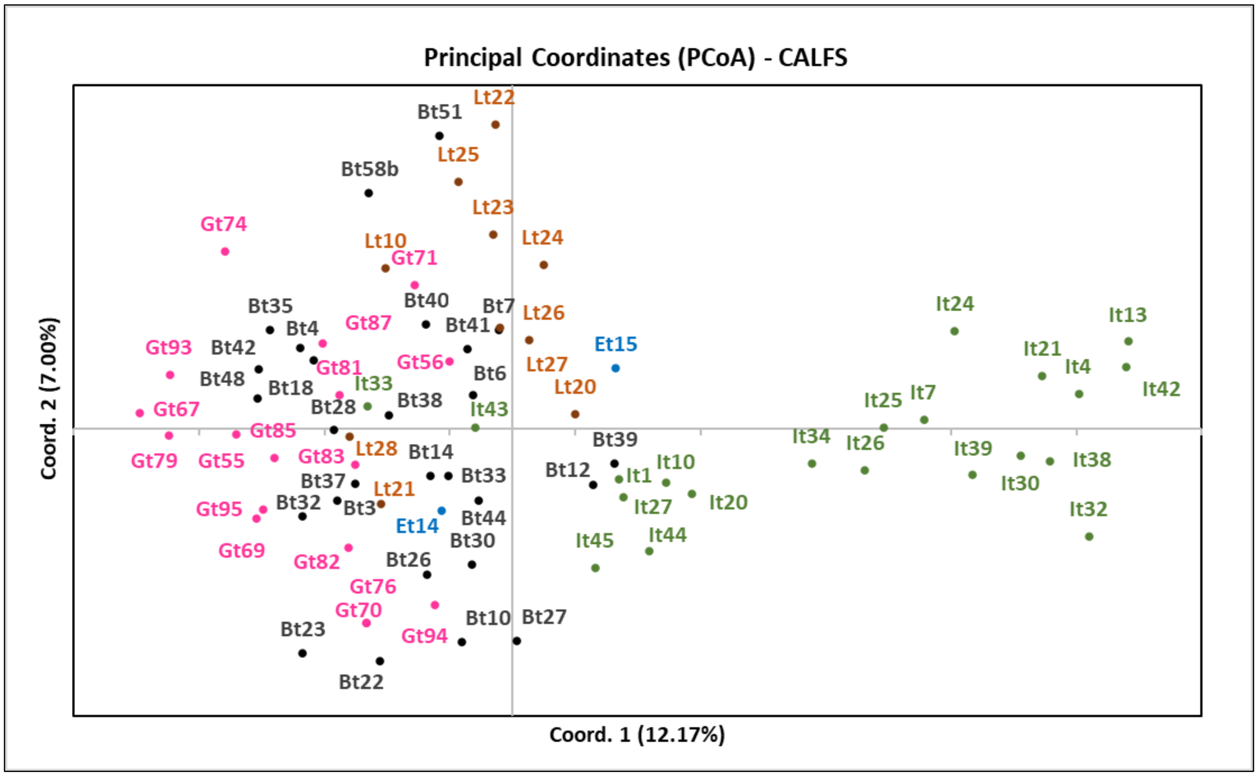
Graph 7. Outcomes of the PCoA analysis on a matrix of genetic distances, F_{ST}, of adult Busha cattle individuals.



Graph 8. Outcomes of the PCoA analysis on a matrix of genetic distances, F_{ST}, of calve herds.

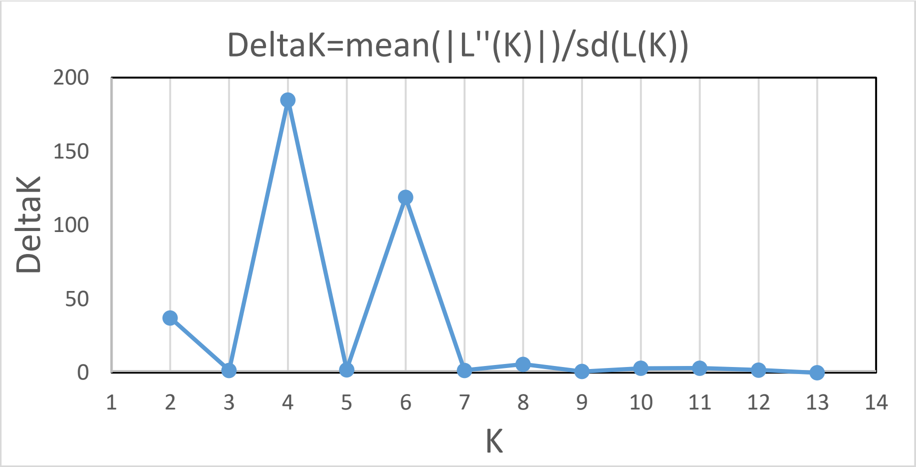


Graph 9. Outcomes of the PCoA analysis on a matrix of genetic distances, F_{ST}, of calve individuals.



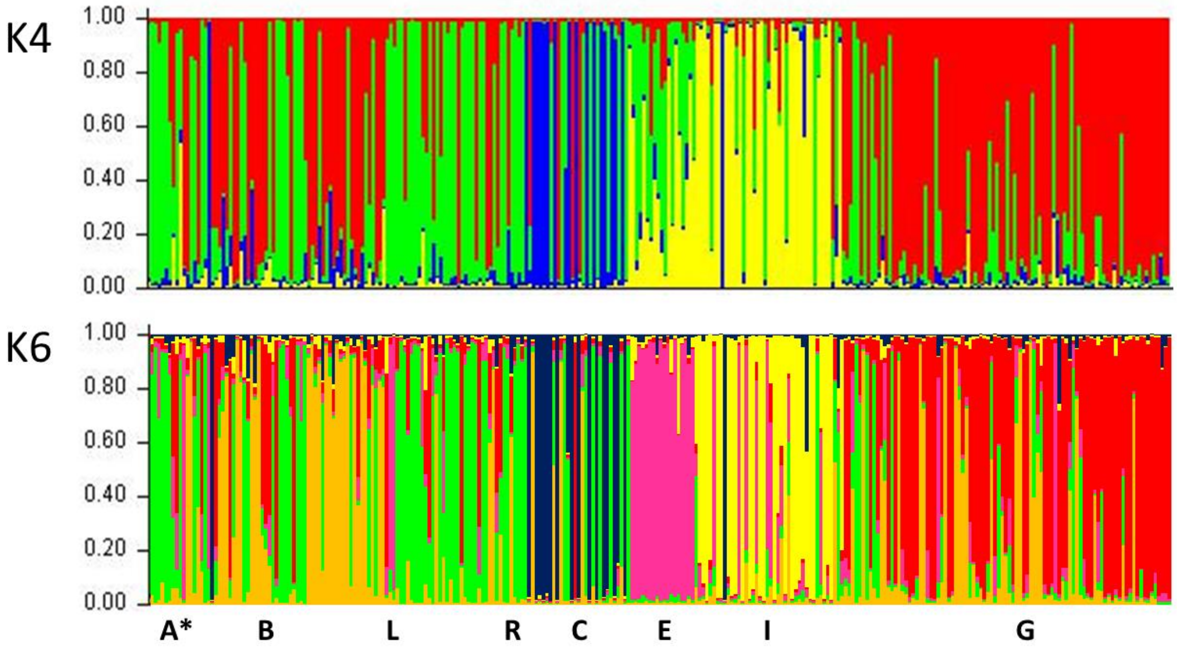
The number of genetic groups in the entire sample, K, as well as substructure of herds, are assessed using Bayesian method implemented in the Bioinformatic package STRUCTURE 2.3.4. [38]. The default parameter settings of the admixture model with correlated allele frequencies and no LOCPRIOR were used, with K varying from 1 to 14. Each run consisted of 500,000 burn-in iterations and 1,000,000 data collection iterations, and ten runs for each K were performed. The number of clusters (K) in which individuals can be divided, assessed using the empirical statistic ΔK, i.e., Evanno's model [41], was four. However, a higher level of genetic structure, with K=6, was observed as well (Chart 10).

Chart 10. The optimal number of genetic groups, K, in the studied population of Busha cattle in Serbia, determined by application ΔK Evanno's model.



Results of STRUCTURE analyzes for the optimal number of genetic groups К=4 and К=6 for the entire studied Busha cattle population (males and females, adults and calves) are presented in Figure 8.

Figure 8. Results of STRUCTURE analyzes for the optimal number of genetic groups К=4 and К=6 for the entire studied Busha cattle population (males and females, adults and calves).



К – Optimal number of genetic groups.

* Sites: A – Gornja Sabanta, B – Ub, L – Ugrinovci, R – Osecina, C – Novi Pazar 1, E – Novi Pazar 2, I – Novi Pazar 3, and G – Dimitrovgrad.

Four genetic groups, obtained in STRUCTURE analysis for К=4, and which, due to the simpler interpretation of results, are named according to the colors, are found at sites:

1) Dimitrovgrad, the so-called "red genetic group" or gene pool (individuals strongly assigned to this gene pool, however, are also present at the sites Gornja Sabanta, Ub, Ugrinovci and Osecina, indicating the substructure of herds at those sites),

2) Novi Pazar 1, "blue genetic group",

3) Novi Pazar 2 and 3, "yellow genetic group", and

4) Gornja Sabanta, UB, Ugrinovci and Osecina, which comprise individuals strongly assigned to the "green genetic group", and also individuals belonging to the red genetic group characteristic of Dimitrovgrad (so-called mixed groups/herds).

Four genetic groups determined based on Bayseian method coincide with genetic groups that are determined by applying a different methodology, that is, PCoA Analysis, and correspond to the separation of the herds by the first and the second coordinates in all samples (adults and calves, Chart 4 and Chart 5).

When the higher level of the hierarchical structure, with six genetic groups (K=6), shown in Figure 8, is considered, the following separation of herds/sites was observed:

1) Separation of the herd at site Novi Pazar 2 from the herd at site Novi Pazar 3, which now represent separate genetic groups, i.e., "pink and yellow gene pools", respectively, indicates the existence of genetic differences between indivuduals from these two sites.

It is worth mentioning that during morphological characterization, when the authenticity of phenotypes, i.e., compliance with morphological characteristics with a breed standard [5] as well as the genetic impact of other breeds, such as Montafone and Tyrolean breed (explained later), was evaluated, it has been observed that the morphological appearance of eight out of the 16 individuals from the site Novi Pazar 2 display the morphological features indicating the introgression of the Montafone breed, while five out of the 22 individuals from the site Novi Pazar 3 display the morphological feautures indicating the introgression of the Tyrolean breed. Therefore, we may assume that, although these two sites are grouped together in STRUCTURE analysis for K=4, which certainly indicates their genetic similarity, the separation of these sites into two separate genetic groups in STRUCTURE analysis for K=6 may most likely be explained by the differential introgression of gene pools of different breeds in these localities.

2) Separation of the new, "orange genetic group", within Dimitrovgrad, as well as at the localities Gornja Sabanta, Ub, Ugrinovci and Osecina, was observed. At this point, the reasons for the occurrence of individuals belonging to the orange gene pool cannot be inferred. This gene pool may represent individuals introgressed by gene pools of other breeds not used in this project, or, alternatively, the organge gene pool may have emerged via admixture of different Busha strains (see later).

It is important to note that in one individual from the Ub site, as well as in six out of 18 individuals from the Ugrinovci site, during morphological characterization, we also observed the introgression of the Montafone breed. We observed that most of individuals from the Ugrinovci site belongs to the green gene pool, while those from the site Novi Pazar 2, also assumed to have had been exposed to the introgression of the Montafone breed, belong to the yellow gene pool. This observation could be explained by the introgression of the Montafone breed into the different genetic background present in Busha cattle individuals at sites Ugrinovci/Ub and Novi Pazar 2.

Six genetic groups detected by Baysian methods are also implied in PCoA analyses, which are based on genetic distances, i.e., on a different methodology (adults and calves - Chart 4 and Chart 5, respectively).

As already mentioned, sites Novi Pazar 2 and 3 are rather specific from both genetic and morphological aspects. Individuals from these sites differ genetically from individuals from all other studied sites, as well among themselves. At the morphological level, individuals from these sites display some features typical for the Montafone and Tyrolean breed, respectively (explained later). In addition, these individuals are slightly larger in relation to those from the area of Pirot district. This indicates, on the one hand, that the phenotype of individuals currently found at sites Novi Pazar 2 and 3 most likely reflects the genetic input of larger breeds, and, on the other hand, that the individuals from the Pirot district (Dimitrovgrad), with smaller body size, are more similar to those typical for the original, small-sized Busha cattle phenotype [3].

Given that in this project we did not analyze at the molecular-genetic level individuals belonging to other breeds, such as Montafone and Tyrolean breeds, it is not possible to determine the percentage of contribution of their gene pools to the Busha gene pool at sites Novi Pazar 2 and 3. However, it is worth mentioning that in the STRUCTURE analyzis at K=4, almost all individuals at the site Novi Pazar 2 are admixed. This would indicate that the introgression of an exotic gene pool at this site is relatively recent. In STRUCTURE analysis at K=6, these individuals form a separate, pink gene pool, found also at other sites (e.g., Ub, Ugrinovci, Novi Pazar 3, Dimitrovgrad). Therefore, it could be assumed that the pink gene pool may represent individuals introgressed by the Montafone breed. To support this assumption, as well as for confirmation of the assumption that the yellow gene pool typical of Novi Pazar 3 could indicate the introgression of the Tyrolean breed, however, additional research is needed, which would also include the individuals of Montafone breed and Tyrolian breeds.

Examples of Busha cattle individuals introgressed by the Montafone, Tyrolean and Jersey breed, taken from the archive of Prof. dr P. Perišić, which was created before this project, are presented in Figure 9.

Figure 9. Examples of Busha cattle individuals introgressed by the Montafone breed (upper right picture), a Jersey breed (lower right) and Tyrolean breed (upper and lower left).

A very intriguing site from the genetic aspect is Novi Pazar 1, which stands out in all analyzes as genetically completely different, blue genetic group. On this site, 16 of the 30 analyzed individuals are characterized by a very high proportion of membership (q_i) to the blue gene pool, not only in STRUCTURE analysis at

K=4 but also in the analysis at K=6, indicating very high genetic integrity of these individuals (i.e., blue genetic pool), which is clearly separated from all other genetic groups detected in this project. The reason for this may be due to the fact that the blue gene represents genetically pure Busha cattle individuals, or alternatively, that individuals found at this site were introgressed previously by unknown breed(s), i.e., by a breed whose impact on phenotype of individuals was not poosible to assess during the morphological characterisation. Given the results of morphological characterization, in which the individuals from the site Novi Pazar 1 are characterized by larger bodies in relation to the original, small-sized Busha cattle individuals [3], the second assumption is more likely. Since we did not analyze at the molecular-genetic level individuals belonging to other breeds, we were not able to determine the breed which served as a source for the introgression at the site Novi Pazar 1 at this point.

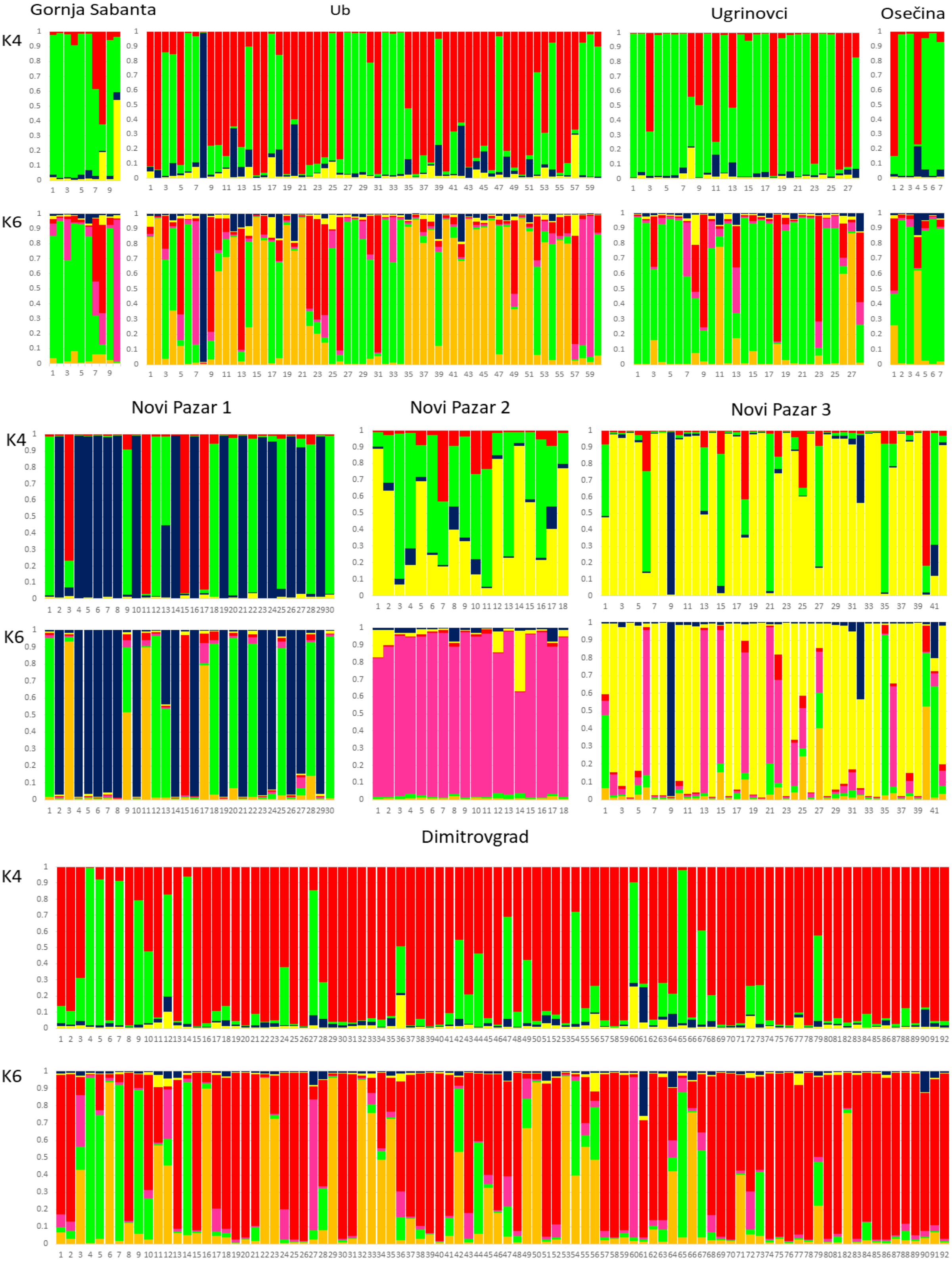
Further research, in which the Busha cattle individuals will be analyzed at the molecular-genetic level along with individuals belonging to other breeds, i.e., those known and/or assumed to have had introgressed Busha cattle in the past, will enable distinction of typical Busha cattle genotypes from introgressed ones. Given a very complex genetic structure of Busha cattle population from Serbia observed in this project, it is also necessary to increase the number of molecular markers in order to achieve better resolution. Nonetheless, it is possible to say that our molecular data support the presence of distinct genetic groups in Busha cattle population from Serbia, while morphological data, at least partially, provide evidence regarding the breeds which served as sources for the introgression. That is, morphological data support the assumption that the observed genetic differences may be explained, at least partly, by the differential introgression of other breeds, such as Montaphone and Tyrolean breed (explained later). Also, given the lack of morphological evidence on introgression of other breeds at one site, Novi Pazar 1, and clear separation of individuals from this site from those at other studied sites according to the molecular data, we inferred an introgression of currently unknown breed at this site.

It is worth mentioning that we additionally included into the analyses two individuals that are F1 crosses of Jersey x Busha cattle, found at the site Ugrinovci. These individuals, however, were not positioned as outliers in PCoA analyses, but rather contrary, were grouped along with several other Busha cattle individuals. This would suggest that the introgression of the Jersey breed may be larger than expected. Expectedly, these individuals displayed admixed genetic profiles in STRUCTURE analysis, indicative of their hybrid origin.

Our results also indicate that in the area of Raška district, the exchange of individuals among farmers is relatively low, while in the area of Dimitrovgrad and adjacent sites Gornja Sabanta, Ub, Ugrinovci and Osecina, the exchange of individuals between farmers is more frequent. Also, the management practice at all sites is introduction of genetically distinct individuals, males and females, into herds, which, contribute to the emergence of new gene pools over time.

Herd substructure, that is, the presence of individuals strongly assigned to different gene pools, is observed at all sites, as shown in Figure 10. The observed substructure of herds is a consequence of the introduction of individuals belonging to one gene pool into the herd in which individuals belonging to another gene pool are prevalent. This is in line with the known fact that breeders exchange Busha cattle individuals, as well as with the fact that individuals from a new sources are frequently introduced in their flocks. Over time, genetically distinct immigrants mate with individuals belonging to the gene pool prevalent at one site, and that results in the occurrence of new genetic profiles.

Figure 10. Herd substructure as assessed in STRUCTURE analyzes at K=4 and K=6 (individulas of both sexes and different ages).



3.2. Morphological characterization

Morphological data obtained for females (159 individuals) were used to calculate average, minimum and maximum values as well as the variation coefficient (descriptive statistics) shown in Table 6. Data for male individuals (a total of 6) are presented in the table 7.

Table 6. Descriptive statistics of morphometric parameters of female individuals (N=159).

Parameters	WtH	RuH	ChC	ChD	ChW	BL	PvL	PvW_{HB}	PvW_{FJ}	PvW_{PB}	HdL	HdW	TbC
Average (cm)	110.52	113.40	151.72	55.22	30.57	129.60	39.79	38.39	34.43	15.41	42.11	18.96	15.69
SD	6.81	7.04	12.56	4.51	4.96	10.17	3.86	4.22	4.15	2.40	2.86	1.88	1.45
Min (cm)	94.00	97.00	125.00	43.00	17.00	93.00	28.00	26.00	16.00	9.00	35.00	13.00	13.00
Max (cm)	130.00	132.00	183.00	68.00	43.00	154.00	51.00	49.00	44.00	22.00	52.00	24.00	20.00
CV	6.16	6.21	8.28	8.17	16.23	7.85	9.70	10.99	12.04	15.59	6.80	9.90	9.25

Parameters: WtH - Height at Withers; RuH - Height at Rump; ChC - Chest Circumference; ChD - Chest Depth; ChW - Chest Width; BL - Body Length; PvL - Pelvic Length; PvW_{HB} - Pelvic Width between Hip Bones; PvW_{FJ} - Pelvic-Femoral Joints Width; PvW_{PB} - Pelvic Width between Pin Bones; HdL - Head Length; HdW - Head Width; TbC - Tibia circumference.
SD - Standard Deviation.
Min - minimum value.
Max - maximum value.
CV - Coefficient of Variation.

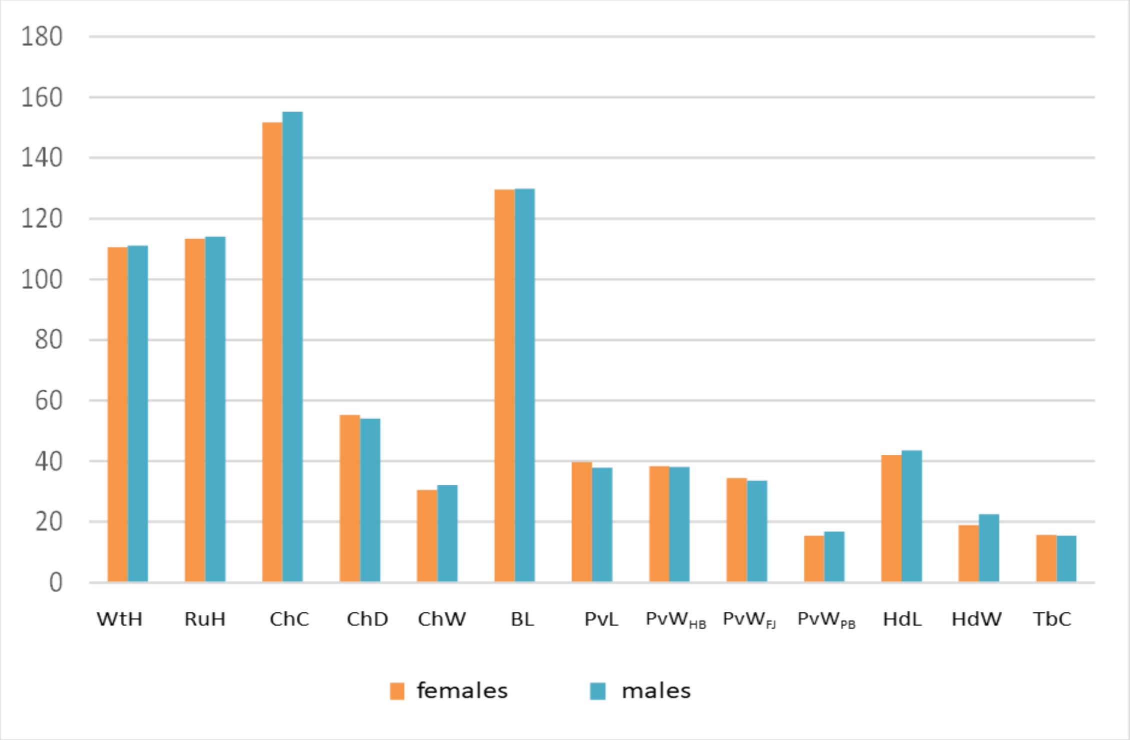
Table 7. Descriptive statistics of morphometric parameters of male individuals (N=6).

Parameters	WtH	RuH	ChC	ChD	ChW	BL	PvL	PvW_{HB}	PvW_{FJ}	PvW_{PB}	HdL	HdW	TbC
Average (cm)	111.17	114.17	155.25	54.00	32.17	129.83	37.83	38.17	33.67	16.83	43.50	22.50	15.50
SD	3.06	5.42	7.89	3.52	4.96	8.75	4.45	1.94	4.27	1.60	3.11	1.73	2.38
Min (cm)	109.00	109.00	146.00	49.00	24.00	117.00	29.00	36.00	27.00	16.00	40.00	21.00	12.00
Max (cm)	116.00	123.00	164.00	59.00	39.00	144.00	41.00	41.00	39.00	20.00	47.00	25.00	17.00
CV	2.75	4.75	5.08	6.52	15.41	6.74	11.75	5.09	12.69	9.52	7.15	7.70	15.36

Parameters: WtH - Height at Withers; RuH - Height at Rump; ChC - Chest Circumference; ChD - Chest Depth; ChW - Chest Width; BL - Body Length; PvL - Pelvic Length; PvW_{HB} - Pelvic Width between Hip Bones; PvW_{FJ} - Pelvic-Femoral Joints Width; PvW_{PB} - Pelvic Width between Pin Bones; HdL - Head Length; HdW - Head Width; TbC - Tibia circumference.
SD - Standard Deviation.
Min - minimum value.
Max - maximum value.
CV - Coefficient of Variation.

The comparative representation of the average values of morphometric parameters of female and male individuals is shown in Graph 11.

Graph 11. A comparative representation of the average values of morphometric parameters of female and male individuals.



Morphometric parameters were used for calculation of 11 body conformation indices for females (Table 8) and males (Table 9). Their comparative presentation is given in Chart 12.

Table 8. Body conformation indices for female individuals in the studied population (N=159).

Parameters	Length Index	Thoracic Index	Relative Depth of Thorax Index	Relative Width of Thorax Index	Thoracic Development Index	Over Increase Index	Thoracic-Pelvic Index	Pelvic Index	Leg Length Index	Cephalic Index	Relative Cannon Thickness Index
Average (cm)	117.30	55.52	49.99	117.44	137.38	102.62	89.03	44.91	50.01	45.09	14.21
SD	6.63	7.74	3.09	8.36	7.70	2.03	11.29	5.30	3.09	4.11	1.06
Min (cm)	90.29	30.91	41.80	95.95	118.33	98.17	56.67	33.33	40.87	30.95	11.61
Max (cm)	134.04	85.42	59.13	145.19	166.32	107.27	125.00	65.52	58.20	56.76	17.02
CV	5.65	13.95	6.19	7.12	5.60	1.98	12.69	11.80	6.19	9.11	7.48

SD - Standard Deviation.

Min - minimum value.

Max - maximum value.

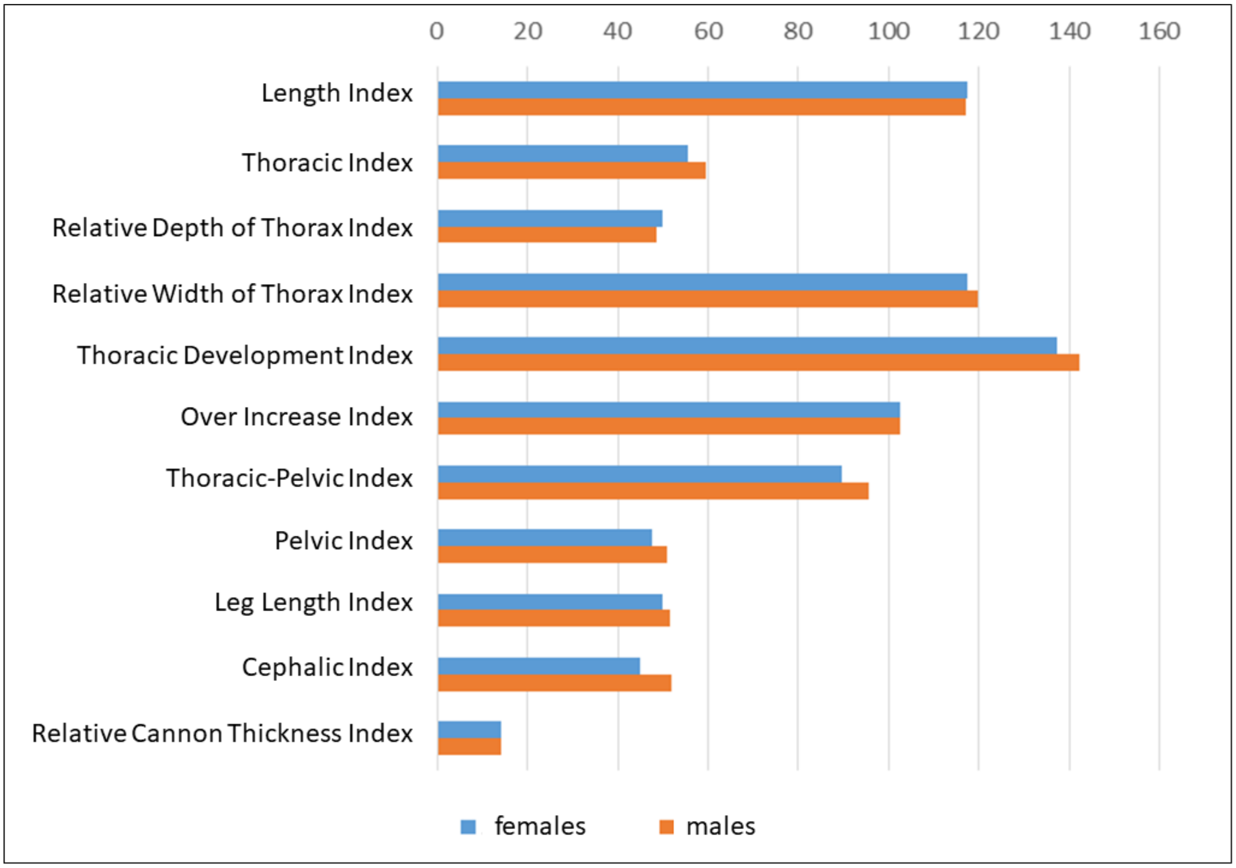
CV - Coefficient of Variation.

Table 9. Body conformation indices for male individuals in the studied population (N=6).

Parameters	Length Index	Thoracic Index	Relative Depth of Thorax Index	Relative Width of Thorax Index	Thoracic Development Index	Over Increase Index	Thoracic-Pelvic Index	Pelvic Index	Leg Length Index	Cephalic Index	Relative Cannon Thickness Index
Average (cm)	116.91	59.49	48.57	119.78	142.09	102.66	95.40	50.98	51.43	51.81	14.20
SD	9.33	7.55	2.71	16.29	6.75	2.48	6.59	10.17	2.71	3.64	2.23
Min (cm)	106.36	45.28	44.95	101.39	133.94	100.00	86.11	41.03	47.71	46.81	10.91
Max (cm)	132.11	66.10	52.29	140.17	149.09	106.03	103.23	64.52	55.05	55.56	15.60
CV	7.98	12.69	5.58	13.60	4.75	2.41	6.90	19.96	5.27	7.03	15.73

SD - Standard Deviation.
Min - minimum value.
Max - maximum value.
CV - Coefficient of Variation.

Graph 12. Comparative representation of values of body conformation indices for females and males.



The average height at withers and height at rump in females are 110.52±6.81 cm and 113.40±7.04 cm, respectively, and in males 111.17±3.06 cm and 114.17±5.42 cm, respectively. Thus, femals are slightly smaller than males, and individuals of both sexes are generally characterized by lower heights, which is expected in Busha cattle, as well as overbuilt constitution, indicated by the over increase index of 102.62±2.03 (females) and 102.66±2.48 (males). Such body constitution corresponds to that typical for the brachicern type of cattle. Also, the values of other morphological characters related to the length, width and depth point to the primitive and rough constitution of studied individuals.

The narrow thorax in studied individuals is indicated by the values of the chest width and chest depth, which are 30.57±4.96 cm and 55.22±4.51 cm, respectively, in females, and 32.17±4.96 cm and 54.003.52 cm, respectively, in males. However, given the values of the thoracic index of approximately 57% (average in females and males) and relative depth of thorax index of approximately 50% (average in females and males), the thorax is sufficiently deep and spacious to provide satisfactory support to the respiratory tract. This is also supported by the values of the relative width of thorax index (females; 117.44±8.36, males: 119.78±16.29) and thoracic development index (females; 137.38±7.70, males: 142.09±6.75).

The values of morphological parameters related to the pelvic in females: pelvic length, pelvic width between hip bones, pelvic-femoral joints width, and pelvic width between pin bones, which are 39.79±3.86 cm, 38.39±4.22 cm, 34.43±4.15 cm and 15.41±2.40 cm, respectively, together with the pelvic index of 44.91±5.30, indicate a less spacious pelvic in Busha cattle in comparison to that typical for noble breeds. Nonetheless, given the relatively small size of female individulas as well as small body weight of calves at birth (15-20 kg), relatively small pelvic does not represent an obstacle for the calving, which is typically rather easy. Furthermore, Busha is usually characterized by good reproductive abilities, as well as the pronounced maternal instincts.

Regarding the morphological parameters related to the length and width of the head, the differences among sexes are more pronounced in case of the head width (18.96±1.88 cm in females, 22.50±1.73 cm in males) than in case of the head length (42.11±2.86 cm in females, 43.50±3.11 cm in males). Expectedly, males are characterized by higher values of the relative width of thorax index (119.78±16.29) than females (117.44±8.36), and thoracic development index (142.09±6.75 vs 137.38±7.70).

Given the minimal and maximal values of studied morphological parameters as well as the corresponding coefficients of variation, it is evident that the variability of all parameters is rather high in Busha cattle population in Serbia. This can be explained by variable genetic and environmental factors which alone and in interaction typically account for the phenotype of individuals. Alternatively, relatively high variability of morphological parameters may also result from differential introgression of larger breeds, such as Montafone, Tyrolean and Jersey, which have been observed as several localities, such as Novi Pazar 1, 2 and 3. To further elaborate our observations, we analyzed 159 females in relation to the locality at which they are maintained. The values of all studied morphological parameters for females according to the locality are given in Table 10. Comparative presentation of values of important morphological parameters describing the body size of females: height at withers, height at rump, body length, chest depth, chest circumference and pelvic-femoral joints width, from different localities is given in Chart 13.

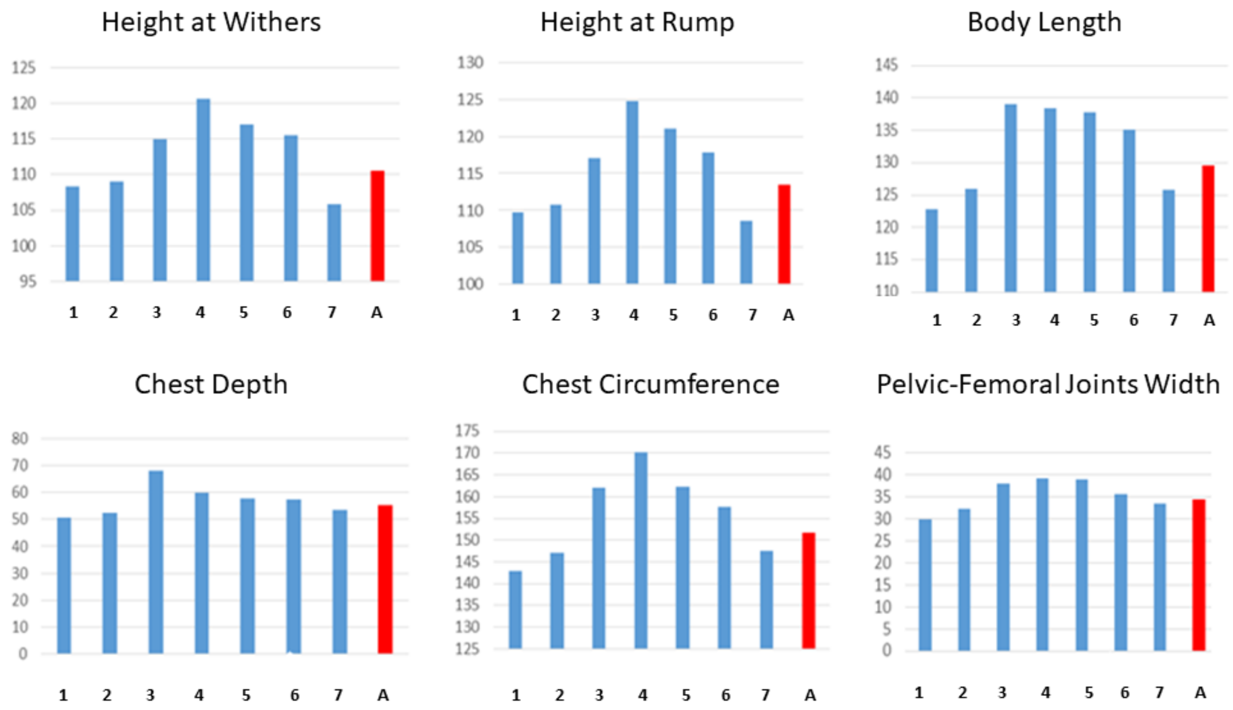
Overall, according to the morphological analyses of female individuals from different localities, we infer that those from the Pirot district (municipality Dimitrovgrad) are smaller than those from the Raška district (Novi Pazar Municipality), and thus, more similar to the original size typical for the Busha cattle breed [3].

Table 10. Average values of morphological parameters for females by localities.

Locality	N	WtH	RuH	ChC	ChD	ChW	BL	PvL	PvW$_{HB}$	PvW$_{FJ}$	PvW$_{PB}$	HdL	HdW	TbC
Ugrinovci	17	115.53	117.82	157.65	57.53	31.76	135.12	43.24	39.71	35.65	16.88	44.88	19.65	16.24
Ub - Cvena Jabuka	14	108.36	109.71	143.00	50.50	28.21	122.86	38.31	35.36	29.86	12.86	43.00	19.14	14.23
Ub - Zvizdar	14	109.07	110.71	147.00	52.50	27.86	125.93	37.86	37.64	32.36	15.07	42.93	18.14	15.64
Dimitrovgrad	78	105.78	108.56	147.61	53.39	29.83	125.72	39.08	36.47	33.44	22.39	41.17	18.47	15.32
Novi Pazar 2 - Dolovo	14	120.64	124.71	170.17	59.71	38.50	138.36	43.57	42.86	39.21	17.64	41.86	20.07	16.92
Novi Pazar 3 - Rudnica	21	117.00	121.10	162.38	57.81	33.19	137.71	42.19	42.14	39.00	16.81	42.24	20.67	16.90
AVERAGE	159	110.52	113.40	151.72	55.22	30.57	129.60	39.79	38.39	34.43	15.41	42.11	18.96	15.69

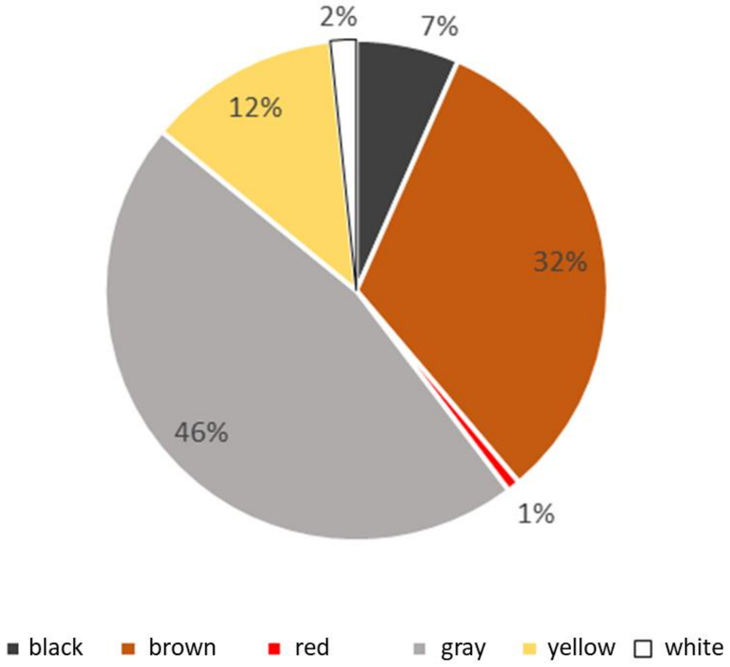
Parameters: WtH - Height at Withers; RuH - Height at Rump; ChC - Chest Circumference; ChD - Chest Depth; ChW - Chest Width; BL - Body Length; PvL - Pelvic Length; PvWHB - Pelvic Width between Hip Bones; PvWFJ - Pelvic-Femoral Joints Width; PvWPB - Pelvic Width between Pin Bones; HdL - Head Length; HdW - Head Width; TbC - Tibia circumference.

Graph 13. Comparative presentation of important morphological parameters describing the body size of females from different localities (1 – Ub, Crvena jabuka, 2 – Ub, Zvizdar, 3 – Novi Pazar 1, 4 – Novi Pazar 2, 5 – Novi Pazar 3, 6 – Ugrinovci, 7 – Dimitrovgrad, A – average).



The color of the coat was assessed for 121 individuals (females and males). Six basic colors of the coat color were observed: black, brown, red, gray, yellow and white, which are differentially represented, as shown on the Chart 14.

Graph 14. Abundance of coat colors in a sample of 121 Busha individuals (females and males).



The coat colors described previously in Busha and commonly associated with different strains maintained at different territories [2, 3] are differentialy represented in the studied population: gray (46%), yellow (12%), black (7), white (2%) and red (1%). The brown coat color, however, has not been described previously in scientific and technical literature [3, 6, 7]. This coat color most likely emerged due to the mixture of strains of different colors, that has resulted in the new gene combinations that resulted in the occurrence of new coat color variants [7]. According to newer data, brown coat color is becoming increasingly frequent in the populations of Busha cattle in Serbia [2], and in our sample, it is rather abundant – it is present in 32% of studied individuals.

Although a monochromatic coat color was observed in the largest number of studied individuals, a relatively common presence of partial or complete shading of coat color, i.e., presence of different shades of basic color, such as light, dark and cream, was observed. Also, the presence of other colors (gray, brown, yellow, black, gray-ruddy, white and gray) was noted on the basic coat color. The tiger pattern was noticed not only in individuals with the gray basic coat color, but also in individuals with different basic coat color, such as brown, yellow and white.

The largest number of individuals with the tiger pattern was observed in Pirot district (8 out of 10 individuals with the tiger stripes recorded in the studied sample), while two individuals with the tiger stripes were observed in the Kolubara district. In total, the tiger pattern was observed in 8.3% of studied individuals. Thus, it could be stated that the tiger pattern is no longer so rare as before. Namely, according to the data collected for the purposes of the "Bushalive" project (Save Foundation 2014), which was performed 7-8 years ago, the largest number of Busha individuals with the tiger stripes was recorded in

Croatia (5/30), only one individual was present in Bosnia and Herzegovina, and individuals with the tiger stripes were not recorded in Serbia. Nevertheless, it is necessary to emphasize that although a large number of countries from the Balkans was included into the project, the total number of Busha cattle individuals analyzed in this project was relatively small (227).

During the morphological characterization, a number of individuals were not characterized as typical representatives of the breed. For those individuals, the impact of other breeds, such as Montafone and Tyrolean breed, was observed whenever possible (Figure 9).

All of the above observations were used to establish a descriptive-analytical model that best describes the diversity in coat color in the studied Busha cattle population, which is presented in Table 11. The presence of specific characteristics, such as shades, tiger stripes, black eel lines, presence of other coat colors along with the basic coat color, is marked with a symbol + for those characteristics which occur in parallel with the basic coat color.

Table 11. Description of basic coat color and specific characteristics of the coat color in the studied Busha cattle population.

Basic coat colors (1-6) and other characteristics (7-9)		**N**	**Shades**			**Tiger stripes**	**Black eel lines**	**Presence of other coat colors**	**Impact of other breeds on phenotype**
			light	**dark**	**cream**				
1	Black	8						Grey, Brown	Montaphone
2	Brown	39	+	+		+		Yellow	Montaphone
3	Red	1							
4	Gray	56	+	+	+	+	+	Black, Ruddy,	Montaphone Tyrolean
5	Yellow	15	+	+	+	+		White	
6	White	2				+		Gray	
7	Tiger	10							
8	Montafone	10							
9	Tyrolean	2				+			
	Total (1-6):	**121**							

N – number of individuals.

Certain coat color-related specifics are recorded in individuals which were characterized as atypical representatives of the breed, i.e., in which the impact of other breeds was observed. For instance, in individuals for which the impact of the Tyrolean breed was observed, the presence of monochromatic black or gray coat cover, the “roe snout”, as well as the short horns, curved upwards and forward, were recorded.

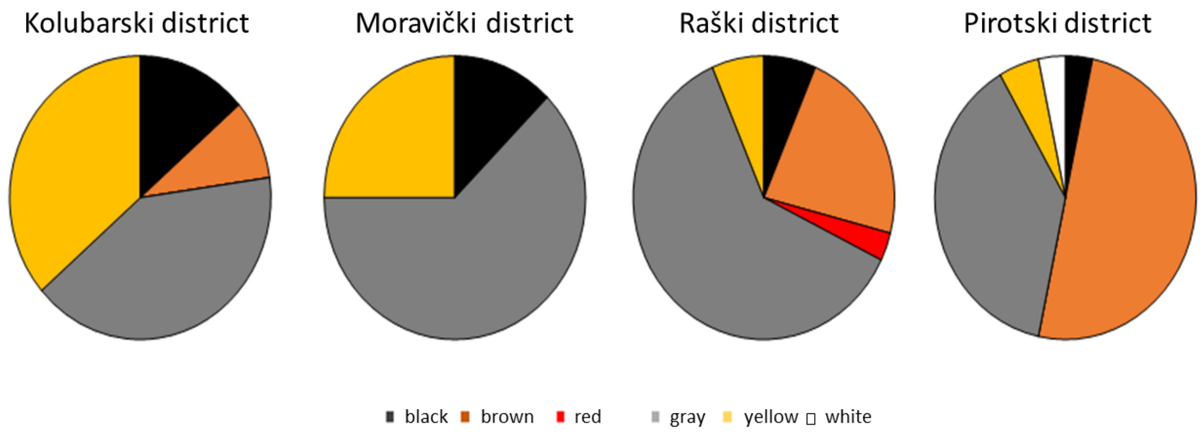
The majority of atypical individuals impacted by the Montafone breed was detected in Raška and in the Kolubara district, while the individuals with the impact of the Tyrolean breed were observed only in the Raška district.

Peculiarities of the herds found at different sites, as well as the abundance of different coat colors in different districts, are shown in Table 12, and in Chart 15.

Table 12. Features and structure of herds of Busha cattle (females and males) according to the coat color in four districts in Serbia.

Basic coat colors (1-6) and other characteristics (7-9)		**N in all districts**	**Number of individuals with a given characteristic**			
			Kolubarski district	**Moravicki district**	**Raski district**	**Pirotski district**
1	**Black**	8	3	1	2	2
2	**Brown**	39	2	0	7	30
3	**Red**	1	0	0	1	0
4	**Gray**	56	9	5	19	23
5	**Yellow**	15	8	2	2	3
6	**White**	2	0	0	0	2
7	Tiger	10	2	0	0	8
8	Montaphone	15	1	6	8	0
9	Tyrolean	6	0	0	6	0
Total (1-6)		121	22	8	31	60

Graph 15. Structure of Busha cattle population~~s~~ (females and males) according to the coat color in four districts in Serbia.



Morphological characterization is still one of the basic approaches when describing and defining the breed standard, recognizing the breed, and formulating breeding programs. In Serbia, morphological data are a component of production sheets of individuals. Continuous monitoring of morphological parameters in Busha cattle individuals registered in the herd book enables insights into the variability of morphological characteristics in the population, body development, constitution harmony, compliance with the breed standards, as well as the impact of maintaining conditions on body constitution and the impact of the bulls on the offspring.

Breed standard is defined based on the values of morphological parameters and indices, which, among other things, are used for the comparisons between animals of the same or different breeds. Positive and/or negative deviations from the breed standard are used for the classification of individuals into certain categories or classes. Also, morphological data are important from both individual and population aspects, and are necessary to monitor the growth rate and development from birth to the complete development. In breeding & selection work, they are essential for selecting individuals to be parents of the next generations.

In this context, molecular-genetic characterization does not represent a replacement but rather an invaluable addition to morphological characterization, which must certainly be carried out in Serbia in the future. The molecular-genetic characterization allows us to generate additional and qualitatively new data, which are of great importance for conservation of genetic resources, the improvement of the Busha cattle breeding program, as well as for the enhancement of animal husbandry and agriculture in Serbia.

By applying a molecular-genetic characterization, a basis for genetically-based and planned management of genetic resources of Busha cattle in our contry was founded. This contributes towards raising the system of conservation of AnGR in Serbia to a qualitatively brand new level, which is in line with modern trends in this field in Europe and the world today. Also, the results of our project are the basis for further research of adaptive and other traits related to productivity, reproduction, resistance to diseases, milk production, as well as on better understanding evolutionary history of this breed [46], by applying modern molecular genetic/genomic approaches and techniques.

4. CONCLUSION AND RECOMMENDATIONS

1. The sample of Busha cattle from Serbia analyzed at the molecular level within this project is representative, because it covers 33.88% of the entire Busha cattle population in Serbia. Studied herds are maintained by 13 farmers from 8 localities from 5 districts in Serbia. We analyzed individuals from the locality Dimitovgrad, where the largest breeding herd of Busha cattle in Serbia is currently found, as well as those from the sites in close vicinity or further away from the Dimitrivgrad. Also, we analyzed both adult individuals and calves, and thus, it was possible to compare genetic trends in two successive generations.

2. Since a high level of genetic diversity is considered as a prerequisite for the long-term survival of the breed, our findings on the relatively high levels of genetic diversity in both adult and offspring Busha cattle herds in Serbia indicates that the joint efforts of the state, which provides subsidies to breeders for the conservation of Busha cattle genetic resources, as well as the breeders themselves, which manage their herds in an adequate manner, were fruitful. Therefore, we infer that the Busha cattle population in Serbia today has good prospects for long-term survival, if it is properly managed from now on. Further research, which will include other breeds, will show the extent to which the introgression of their gene pools contributed to genetic diversity in the studied Busha cattle population and affected the breed purity.

3. Adult individuals not related to at least 3-5 generations are currently present in studied herds, which also comprise rare adult individuals that are more closely related than expected and allowed. The latter, however, most likely reflects the fact that closely related groups, such as grandmother-mother-daughter/son, are present at several sites. It is recommended that breeders continue to follow such closely related individuals, and to avoid mating within such groups.

4. Inbreeding coeficints at the herd level in the studied adult and offspring herds from the Serbia are negligible. Statistically significant excess of homozygotes detected at one site reflects herd substructure, i.e., Wahlund's effect.

5. Inbreeding coefficients of individuals are in most cases below 10%, and do not exceed 50%. The recommendation is that the identified individuals with a higher inbreeding coefficient are not used for mating.

6. The effective population size of the studied adult Busha cattle herds found at different sites ranges from 5.5 (4.2-6.7) to 69.7 (59.3-83.3), with an average value of 55.2 (51.1-59.7). It is recommended to increase the size of unrelated breeding individuals at sites with smaller Ne. However, it is very important that individuals introduced into herds belong to the same genetic group determined in this project, in order to maintain the genetic integrity of detected groups.

7. The effective population size of the studied calve herds found at different sites ranges from 21.0 (6.7-28.6) to 39.9 (23.5-100.0), with an average value of 46.9 (41.6-53.2). Although smaller number of calves are analyzed in some localities in relation to the number of studied adult individuals, the effective population size of the calve Busha cattle herds is maintained at a lower but comparable level in the offspring generation in relation to the adult breeding herds. An increase in the number of calves

analyzed at the studied sites would most likely contribute to the increase in the values of Ne for calve herds.

8. Genetic differentiation of adult breeding herds, expressed via F_{ST}, Hedrick's G''st parameter, and Jost's Dest parameter, is low but statistically significant [0.066 ($p < 0.05$), 0.224 ($p < 0.05$) and 0.168 ($p < 0.05$), respectively], indicating the existence of a genetic structure, i.e., genetic differences between the herds of Busha cattle in Serbia. An identical trend was also observed in the case of studied calve herds: F_{ST}, Hedrick' s G''st parameter, and Jost's Dest parameter values were 0.081 ($p < 0.05$), 0.270 ($p < 0.05$) and 0.203 ($p < 0.05$), respectively. It is recommended to take into account the observed genetic structure in the studied Busha cattle population in Serbia when formulating guidelines for the management of the herds.

9. Pairwise adult population F_{ST} values ranged from 1.4% (Dimitrovgrad and Ub) to 11.9% (Osecina and Novi Pazar 3). That supports farmer's statements that they have certain preferences in terms of origin of individuals that they introduce into their flocks, and reflects the differential exchange of individuals from different localities, i.e., differential gene flow between different localities, which is on average 2.85±0.23 migrants per generation. In the calve herds, somewhat higher minimal as well as a slightly lower maximal values of pairwise population F_{ST} parameter were observed. Average gene flow among studied calve herds was estimated to 2.38±0.22 migrants per generation. The observed pattern suggests that the process of equalization of genetic structure in offspring population is in progress.

10. The optimal number of genetic groups in the studied Busha cattle population from Serbia is four, but a higher level of hierarchical structure, i.e., six genetic groups, was observed, according to both the Bayesian analyses and the PCoA analysis based on genetic distances. The first and the largest genetic group consists of individuals predominantly found in the Pirot district, the Dimitrovgrad site, but also at the sites Osecina, Ugrinovci and Gornja Sabanta. Such specific distribution of individuals belonging to this gene pool, which are present at different localities, indicates an intensive exchange of individuals between the mentioned sites, as indicated also by the farmers themselves. The gene pool present at these locations is heterogeneous, and within it, two genetically different groups, which contribute to higher levels of hierarchical genetic structure, were detected by further analyzes. The heterogeneity of this gene pool is a consequence of the introduction of individuals belonging to one genetic group into a herd characterized by different genetic background, or alternatively, by the genetic impact of other breeds. The second gene pool comprise individuals from the two sites from the Raška district, Novi Pazar 2 and 3. This gene pool is also heterogeneous, and a higher level of the hierarchical genetic structure, with two genetic groups, was observed in this gene pool. The first genetic group consists of individuals from the site Novi Pazar 2, and the second of individuals from the site Novi Pazar 3. The genetic group currently present at the site Novi Pazar 2, most likely reflects the genetic impact of the Montafone breed, while the genetic group present at the site Novi Pazar 3 most likely reflects the genetic impact of the Tyrolean breed, as indicated by the morphological data. The third gene pool found in the Busha cattle population from Serbia comprise individuals from the site Novi Pazar 1, which stands out with a unique genetic profile, in relation to all other examined sites. For this gene pool, the genetic impact of currently unknown breed was postulated, and this certainly deserves futher research. The fourth gene pool in the overall sample comprise individuals predominantly found at the sites Ugrinovci and Gornja Sabanta, which do not belong to the gene pool typical for Dimitrovgrad. The individuals strongly assigned to this gene pool are also present at

Dimitrovgrad site. The existence of a pronounced genetic structure of Busha cattle population from Serbia, as well as detected admixture of individuals, indicate the need for molecular-genetic research to become a necessary aspect when selecting individulas that will be introduced into herds, in order to preserve the genetic integrity of genetic groups observed in this project, as well as to prevent the occurrence of inbreeding and outbriding depression.

11. The observed substructure of herds found at almost all examined sites, that is, the presence of individuals that are strongly assigned to different gene pools at the same site, has severe and long-term consequences for the maintenance of the genetic groups (possible strains) of Busha cattle in Serbia. Namely, the consequence of mixing and mating of individuals belonging to different genetic groups leads to the loss of genetic specificities of different genetic groups, which may represent different strains. This way of managing Busha cattle genetic resources may be justified in the case when the population is small and critically endangered, but it is not justified in cases when the size of the population is above the critical level. Therefore, it is recommended that from now on, the introduction of individuals into the herd should be based on its genetic profile, and its assignment to a specific genetic group.

12. Further research, with increased number of molecular markers, which will also include the remaining individuals of Busha cattle from Serbia and also individuals of different breeds that are known or for which there are indications that have had introgressed Busha cattle in the past, will enable the distinction of genetically pure groups from introgressed ones. Along with the introgression of the Montafone and Tyrolean breeds, the introgression of the Jersey breed has been postulated, as well the introgression of a currently unknown breed.

13. Based on the outcomes of the analyses of morphological characteristics of studied individuals, we infer that the Busha cattle population in Serbia generally retained morphological features typical for the original Busha cattle breed. This primarily refers to the body dimensions according to which a small body size of individuals and their coarse constitution has been maintened. However, taking into account the values of coeficients of variations, as well as the range of minimal and maximum values, it is evident that studied morphological characteristics of Busha cattle individuals in Serbia are rather variable. The comparison of morphological data between different regions has shown that the individuals found in Raška district (municipality Novi Pazar) have larger body size, while those from the Pirot district (municipality Dimitrovgrad) have smaller body size. Therefore, the body measures of individuals from the herd from the Pirot district are more similar to those typical for the original Busha cattle breed.

14. Morphological observations that refer to the coat color of studied Busha cattle individuals reveal that this feature greatly evolved in the recent past, most likely as a results of admixture of different strains that are characterized by different coat colors. The studied Busha cattle population in Serbia is dominated by gray coat color (46%), as well as brown (32%), the latter being considered to be of a more recent origin. In addition, a large aray of different coat colors was observed, as well as shading of the basic coat colors, and the presence of multiple colors in otherwise monochromatic coats typical for the Busha cattle breed. The tiger Busha cattle, with specific tiger stripes present at the monochromatic coat, is no longer so rare, as it has been observed in 8.3% of the studied individuals.

Furthermore, tiger stripes were noticed not only in individuals with the gray basic coat color, but also in individuals with different basic coat color, such as brown, yellow and white.

15. For individuals identified as atypical representaives of the breed at the phenotypic level resulting from the introgression of different breeds such as Montafone and Tyrolean breed, some specificities in the coat color have been observed. That is, in individuals introgressed by the Tyrolean breed, the presence of monochromatic black or gray coat color, the "roe snout", as well as the short horns, curved upwards and forward, have been observed. The majority of atypical individuals in the type of Montafone breed was recorded in Raška district and in the Kolubara district, while the individuals in the type of the Tyrolean breed were observed only in the Raška district.

16. Overall, the outcomes of our study demonstrate that despite the relatively small number of remnant Busha cattle individuals in Serbia (847 individuals recorded in the herd book in 2022), the current population of Busha cattle in Serbia has a relatively high level of genetic diversity, which indicates good prospects for long-term survival of this breed, if it is properly managed from now on. In addition, inbreeding and the relatedness of studied individuals are in line with recommendations given in the breeding program for Busha cattle. In this study, for the first time, four distinct genetic groups have been detected in Busha cattle population in Serbia, which may represent different strains, but the support for this hypothesis requires further investigations. Furthermore, a higher level of hierarchical genetic structure, with six genetic groups, has been observed as well, suggesting rather complex genetic structure in extant Busha cattle population in Serbia. The occurrence of novel genetic groups as well as novel coat colors most likely resuts from the specific management, i.e., the mating of individuals belonging to different genetic groups and/or breeds. Our findings support our hypothesis that molecular-genetic characterisation, which will enable assignment of each individual to a specific genetic group as well as the assessment of inbreeding coefficients of individuals and their relatedness, must become an essential component for the management of Busha cattle genetic resources in Serbia. In this way, genetically-based and planned management of genetic resources of Busha cattle in Serbia will be established, and the entire system of conservation of AnGR in our country will be raised to a qualitatively brand new level, which is in line with modern trends in this field present in Europe and the world. Also, the results of our study represent the basis for further research of adaptive and other traits related to productivity, reproduction, resistance to diseases, milk quality, etc., which may be accomplished by applying modern molecular genetic/genomic approaches and techniques. In addition, further activities on molecular-genetic characterization of Busha cattle in Serbia, and their comparative analysis with other breeds, are also necessary.

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6. LITERATURE

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Some photos have been taken from prof. Dr. Predrag Perišić archives, created before this project.

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