

*Research article***MOLECULAR CHARACTERIZATION OF AUTOCHTHONUS
SLAVONIAN SYRMIAN PODOLIAN CATTLE**

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The aim of the study is to increase the knowledge on the genetic structure status of the autochthonous Slavonian Syrmian Podolian cattle breed currently listed by the FAO as “critically endangered”. Blood samples obtained from a total of 20 randomly selected individua were included in genotyping upon microsatellite panel recommended by ISAG and FAO. The procedure enabled the identification of as many as 214 (96.34%) genotypes and, at the same time, 129 allelic variations were found with $\bar{x} = 11.73$ alleles per locus. The genotypes showed differences between the microsatellite loci and also significant differences ($p < 0.05$) in the frequency of both homozygosity and heterozygosity. The analyses of the allelic distribution within microsatellite loci showed marked variability. The distribution of genetic relationship was expressed by Wright’s F coefficients. A moderate level of genetic diversity was observed at the tested loci. The findings demonstrate the degree of breed relationship and breed purity but also indicate that gene pool of the tested population has been threatened. Therefore, urgent measures must be taken to support both the genetic consolidation and preservation of the remaining population of Slavonian Syrmian Podolian cattle in Croatia.

Key words: genetic diversity, genetic resource, microsatellite markers, Podolian cattle**INTRODUCTION**

Slavonian Syrmian Podolian is an autochthonous breed of cattle living in the Slavonia region in the northeastern part of Croatia and in the Serbian county Vojvodina. Due to their strength and endurance as well as other working abilities, the Podolian cattle used to be irreplaceable in farming activities. In the regions of Podravina, Slavonia and Syrmia, the Podolian cattle was the predominant breed in the first half of the early 20th century with as much as 90% of total bovine population. However, in the course of the 20th century and particularly in the second half, the demand for milk and meat products increased; whereas, due to technological advances in agriculture, the needs for working animals decreased. Therefore, in time, the highly productive Pinzgauer

and today the Simmental cattle replaced the Podolian breed. Complex breeding conditions and reduction of free grazing land significantly contributed to the decrease of Slavonian Sarmian Podolians so that some twenty years ago the breed was almost extinct. This was followed by studies of the remaining Podolian cattle in Croatia and in 1989 a Breed Registry was established for Slavonian Sarmian Podolians. Because of the efforts of enthusiastic breeders and some support from the government, the Slavonian Sarmian Podolians were saved to some extent and in 1998; they were included into the program of preservation and the system of protection [1,2].

Slavonian Sarmian Podolian cattle belong to the Podolian subspecies of the original cattle, originating from the steppe region of Russia and Ukraine, that is, from the Volhynian-Podolian hills, according to which the breed was named. In ancient times, Podolian cattle migrated into the Danube river region and Pannonia and spread into Central and Southern Europe; reaching Croatia from Romania and Hungary. It is assumed that the Podolian cattle migrated into these regions with Roman legions in the first centuries A.D. and in the fifth century, a great wave of cattle migration followed during the Tatar invasions of Attila the Hun to the West and the Great Migration. Over the centuries, various types of Podolian cattle emerged in various environmental conditions. Therefore, today's Slavonian Sarmian Podolian is genetically related to Podolian breeds in Ukraine, Romania and Hungary as well as to the Istrian cattle [3,4].

In the 1990s, Zlata Gašpert, Pavo Caput and collaborators tried to genetically determine the autochthonous breed of Slavonian Sarmian Podolian cattle. Zlata Gašpert and collaborators performed genetically important studies of nucleus herds which demonstrated the similarities in occurrence of the hemoglobin synthesis gene (Hb) with the variance established only in allelic distribution within transferrin locus (Tf). The differences in results can be explained by a significantly reduced number of animals [1,5,6].

Slavonian Sarmian Podolians are a late maturing breed the growth of which ends only in the sixth year of life and its general characteristics include longevity and adaptability to great climate changes. In its mature age, this breed is characterised by a solid skeleton with pronounced joints. The height of mature bulls is approximately between 135 and 145 cm and they weigh from 600 to 800 kg, whereas oxen can weigh up to a whole ton. The cows are slightly shorter, their height being approximately 128 cm but it can also range from 130 to 136 cm, and they can weigh approximately 470 (400 to 600) kg. The head is relatively small and narrow with a short neck and the back is sturdy and in some individuals it is mildly concave with well positioned shoulder blades and the heart girth ranges from 180 to 187 cm. The rump is mildly declined, 48 to 55 cm long and 39 to 49 cm wide; with moderate muscles sparsely covering bone protrusions. An important characteristic is the small udder in which, in non-lactating cows, only moderately long teats reaching above the hock are visible. The udder is often divided into fore udder and rear udder and the space between the teats is small, with visible pseudo-teats in certain cows. The skin and hair pigmentation is breed specific and monochromatic ranging from light-gray to dark-gray, often darkening to the dewlap.

The shading being more prominent in bulls and young animals, with the dark aureoles encircling the eyes. The pigmentation of the muzzle is dark, while the hoofs are black and partly open. The calf are born with brownish-red hair which starts turning into gray after a few weeks. A particular characteristic of the Slavonian Sarmian Podolian, as well as of other Podolians, are the horns which are about 50 cm long, jutting to the side, and spanning up to a hundred centimeters, resembling a lyre instrument. The horns are often set more perpendicularly with the tips bent backwards so they resemble a pitchfork. The upper parts of the horns and the tips are always of darker pigmentation.

The temperament of the breed is vivacious but of unreliable nature and shy in contact with people, and, sometimes, very nervous. The cows are known to calve easily but their ability to lactate is unfortunately low and is hardly sufficient to feed the calf. A recent research indicates to the fact that the cows in lactation produce from 800 to 1000 and even up to 1100 liters of milk which is rich in milk fat (4.2 to 6.5%). The fattening ability of the Slavonian Sarmian Podolian is average and with average dietary requirements [1-3].

Slavonian Sarmian Podolians were included into the program of systematic protection only fifteen years ago because the breed was classified according to FAO as “bordering critical endangerment” with $N_e = 33$. According to official data by Croatian Agricultural Agency (CAA) published in the yearly report for 2013, there were 13 bulls, and 171 cows with 126 specimens of female offspring out of which 56 were under a year old and 70 over a year old. They are owned by 19 breeders [7,2].

The aim of the study was to investigate the allelic polymorphism occurring within 11 microsatellite loci in the remaining herds of Slavonian Sarmian Podolians. The allelic data findings in the present population study would contribute to the knowledge of the present status of the genetic structure of the breed.

MATERIALS AND METHODS

The twenty randomly selected animals (19 cows and 1 bull), 4 - 10 years old in good health, breeding status and condition were included in the sample of the population. Blood samples were obtained by jugular vein puncture, collected in BD Vacutainer test tubes with anticoagulant KBE 15% 0.005 mL (Systems Pharmaceutical Solution, Plymouth, PL5, 7BP, UK). The pure genomic DNA was extracted from separated leucocytes upon the procedure using NucleoSpin Blood kit (Macherey - Nagel, Düren, Germany) according to the protocol recommended by the manufacturer. The 11 microsatellite panel: TGLA227, BM2113, TGLA53, ETH10, SPS115, TGLA126, TGLA122, INRA23, ETH3, ETH225, BM1824 selected from the set which was recommended by ISAG and FAO in the Mo DAD program [8,9] was evaluated in the obtained samples of genomic DNA.

The microsatellite loci investigated were amplified through the multiplex PCR (Polymerase Chain Reaction) with the use of a commercial Stock Marks® Cattle Bovine Paternity PCR Typing Kit (Applied Biosystems, Foster City, Ca, USA), and it was carried out according to the protocol in the Gene Amp® PCR System 9700 device (Applied Biosystems). The gene analysis of PCR products was made by capillary electrophoresis in the ABI Prism 310 Genetic Analyzer device (Applied Biosystems) with the use of marked primers and linear GS STR Pop 4 polymer, as well as the DNA control according to the manufacturer's guidelines.

The statistical analysis of microsatellite occurrence was made by the ABI Prism Genemapper Software (Applied Biosystems). The statistical significance of allelic peak-ratio values was checked by the Mann–Whitney non-parametric test. The statistical significance of the differences of homozygosity and heterozygosity between and within the observed populations as well as the assessment of the conformity of the results with HWE were checked by the standard Chi square (\bar{X}) – test and Fischer's exact–test. Genetic variety, i.e., the values of observed heterozygosity (H_o) and the expected heterozygosity (H_e) as well as Wright's F coefficients were determined by the GENETIX 4,05 program [10,11].

RESULTS

A total of 214 (96.34%) genotypes were established in 20 animals showing the value of blood samples as an exceptional source of genomic DNA. A 100% incidence was recorded in 8 microsatellite loci and only slightly expressed in SPS115 (3/20), BM2113 (2/20) and TGLA53 (1/20) loci. The success of genotyping simultaneously demonstrated the appropriateness of the selected microsatellite loci and the fact that DNA sequences, to which the primers were bound, were evolutionarily preserved, which was a basic requirement for including certain microsatellites into the research.

The research resulted in finding 129 alleles with the median value $\bar{X} = 11.73$, while the number of alleles per each locus ranged from 7 at TGLA53 and ETH10 to 19 at ETH3 loci. Simultaneously, significant differences of homozygosity and heterozygosity were recorded for TGLA227, ETH3, SPS115, TGLA126 and BM1824 alleles. Peak-ratio values of separate alleles' electropherograms were different in certain microsatellite loci and the check by Mann-Whitney test showed statistically significant differences in both peak and ratio values in TGLA227 alleles (Table 1). Main Number of Alleles (MNA) and median heterozygosity of all microsatellite loci are valid indicators of population genetic variability. However, high values of heterozygosity can indicate on genetic variability caused by changes in the environment [12,13]. Locus TGLA227 had the greatest allelic variability ranging from 67 to 108 bp, but another five loci also stood out with high variability (TGLA53, TGLA122, INRA23, ETH225, BM 1824).

The analysis of individual occurrence of microsatellite loci and allelic variability (Table 2) showed more pronounced differences of H_o and H_e median values, which are

a valid indicator of variability within breeds [13]. In the studied population the H_e was relatively high and the highest variability was established within TGLA227 (16 different alleles, $H_o=0.947$, $H_e=0.901$) and BM2113 (15 different alleles, $H_o=0.777$, $H_e=0.905$) loci. Loci ETH3 (8 different alleles, $H_o=0.95$, $H_e=0.803$) and ETH225 (8 different alleles, $H_o=0.789$, $H_e=0.732$) loci showed the least variability.

Table 1. Number of alleles, homozygous/heterozygous frequency and allelic peaks in the tested loci

Locus				Peaks of alleles
	Non-expressed	homozygotes	heterozygotes	
TGLA227		2	18	67.108
BM2113	2	4	14	111.141
TGLA53	1	12	7	150.185
ETH10		13	7	209.221
SPS115	3	13	4	246.261
TGLA126		3	17	100.127
TGLA122		10	10	139.172
INRA23		8	12	196.232
ETH3		1	19	113.123
ETH225		6	14	132.136
BM1824		2	18	177.192

Table 2. Allelic variability of the observed and expected heterozygosity in the studied population

Locus	H_o	H_e	Allelic variability
TGLA227	0.947	0.901	16
BM2113	0.777	0.905	15
TGLA53	0.368	0.694	12
ETH10	0.35	0.692	10
SPS115	0.588	0.847	12
TGLA126	0.9	0.853	12
TGLA122	0.57	0.757	11
INRA23	0.6	0.858	14
ETH3	0.95	0.803	8
ETH225	0.789	0.732	8
BM1824	0.9	0.797	11
Total no – without alleles			129
\bar{X} (SE)			11.73 (0.77)

H_o – observed heterozygosity; H_e – expected heterozygosity; \bar{X} – median value; SE – standard error; Allelic variability – number of alleles per locus

The distribution of genetic relations between the entire researched group and within the group was expressed by Wright's F coefficients [14,15]. The value of inbreeding coefficient (F_{IS} index) which shows the deviation of individuals from HWE, was positive in most loci tested with $F_{IS} = 0.26514$, but negative values were observed in two loci: TGLA227 and ETH3. These findings with relatively high H_e values point to the potential influence of other breeds, that is, mating of individuals of different genotypes, more often than expected in random mating (Table 3). The index of population kinship (F_{IT}) measures the total deviation of the entire population from HWE and for the established values were expected for almost all tested microsatellite loci ($F_{IT}=0.31905$), except at TGLA227 ($F_{IT}= -0.00959$) which was negative thus indicating frequent matings of genetically unrelated individuals. The fixation index (F_{ST}) measures genetic differences between animals in their population groups, and in the studied group it had a positive value for all loci with $F_{ST} = 0.07336$, which points to the fact that the average degree of genetic variability is not statistically significant.

Table 3. Interbreeding coefficient (F_{IS}) in the studied population

Locus	F_{IS} index
TGLA227	-0.0237
BM2113	0.16928
TGLA53	0.4750
ETH10	0.51371
SPS115	0.33333
TGLA126	0.02857
TGLA122	0.36242
INRA23	0.32444
ETH3	-0.15705
ETH225	0.05058
BM1824	0.10323
All loci	
HO (SD)	0.6974 (0.2264)
HE (SD)	0.8024 (0.0799)
P(0.95)	1.00
P(0.99)	1.00

P – Probability criteria

DISCUSSION

The Slavonian Syrmian Podolian cattle is a fragment of the formerly Podolian population which is well adapted to the Panonian and undoubtedly have a cultural value as a historical witness of local traditions. At the same time this livestock possesses an economic value in the tourist industry, which is an important part of the Croatian economic system [16].

The presented results of our molecular genetics research confirmed the statistical findings by Ivanković *et al.* regarding the increased level of kinship between the remaining Slavonian Syrmian Podolians. At the time of that genetic research, the effective size of the population was $N_e = 15.06$, but with the necessary correction, it was significantly smaller ($N_e = 6.40$), whereas the level of kinship inbreeding was $\Delta F = 0.0186$. The period of systematic protection allowed a small improvement of the population status but the breed is still endangered [2,16].

The determined specific polymorphisms of the loci tested showed the level of kinship and purity of Slavonian Syrmian Podolian breed as the well as the level of endangerment. The median of expected heterozygosity of Slavonian Podolians was fairly high but with higher occurrence of identical alleles due to shared data that is, mating between related individuals. At the same time, an inappropriate frequency of mating with genetically unrelated individuals was observed.

In a previous comparative study, specific polymorphisms of loci were determined and they showed related genetic characteristics between Slavonian Syrmian Podolians and the Croatian autochthonous breed of Istrian cattle. A similar variability within TGLA227 and ETH225 loci was determined with the established negligible differences of allelic variability, together with approximated similarity of allelic polymorphism at ETH10, ETH3, TGLA53, TGLA126 and BM1824 loci, whereas the difference was revealed at BM2113, SPS115, TGLA132 and INRA23 loci [9,17]. By analyzing the results of our study and comparing them with the results obtained by similar studies from other countries, a great number of different alleles at the same locus were observed. For example, in a study of 35 Bulgarian Grey cattle the same loci were involved and 83 alleles were determined in total with an average 7.6 alleles per locus. The large number of alleles were determined at loci TGLA227 and TGLA53 (12 alleles), and lowest number at locus BM1824 (4 alleles) with equally high polymorphism detected at loci ETH3, BM2113 and ETH225, [18]. A similar study in Romania determined 125 different alleles in 5 different cattle breeds. Loci TGLA53 and TGLA122 showed the most variability with 16 alleles each and the smallest number, that is 7 alleles, was found in locus ETH225 [19]. The comparison of these results shows that some of the alleles identified in our study do not appear in Bulgarian and Romanian cattle breeds. It should also be pointed out that our study results show the variability in the INRA23, TGLA122 and in ETH225 loci. Significant is that simultaneously in Serbia on Vojvodina county (Fruška Gora, Deliblatska Peščara) exists a population with about 500 Podolian cattle [8]. Therefore, it may be very valuable to compare the genetic structure status of both parentage breeds from Croatia and Serbia.

The results of our research can be applied in the protection and breeding programs of preservation of autochthonous Croatian Podolian breeds. In order to preserve the remaining purebred animals urgent measures are needed, and apart from creating special systems of breeding, it is necessary to solve spatial limitations by moving the animals and, if necessary, to obtain some unrelated high-quality breeding animals from other breeders [2]. In addition, the recent researches [20,21] point at the usefulness

of one complement study about effects of certain gene polymorphisms on the improvement of fat deposition and milk yield traits. Any possible following projects on crossbreeding, in order to increase beef and milk production, primarily require breeding of 'purebred' animals and the data collected in this study can undoubtedly contribute to this.

In summary, the conducted study of molecular characterization of the autochthonous Slavonian Sirmian Podolian breed points to the need of urgent genetic consolidation of the remaining population of our multi-valid Podolians. The preservation of the remaining population of 'purebred' animals is possible by following the appropriate patterns of selection and mating as well as by introducing 'fresh' genetic material from high-quality breeding animals including the related breeds (for example Istrian cattle, Podolian cattle from Serbia or Hungarian Grey cattle). Data from this study can certainly help in creating a valid selection program for further breeding. That is the only way to protect and save our autochthonous cattle, and use them in future breeding.

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MOLEKULARNA KARAKTERIZACIJA HRVATSKOG AUTOHTONOGA SLAVONSKO SREMSKOG PODOLSKOG GOVEČETA

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Cilj istraživanja bio je unaprediti znanje o stanju genetskih struktura autohtone rase slavonsko sremskog podolskog govečeta, trenutno proglašene od FAO kao “kritično ugrožena“. U istraživanje je uključeno dvadeset nasumično odabranih goveda, te je iz uzoraka krvi obavljena genotipizacija pomoću mikrosatelitskih markera preporučenih od ISAG i FAO-a. Pri tome je identifikovano čak 214 (96,34 %) genotipova i utvrđeno je 129 alelskih varijacija s prosečno $\bar{X} = 11,73$ alela po lokusu. Genotipovi su pokazali razlike u mikrosatelitskih lokusa i također značajne razlike ($p < 0,05$) u učestalosti oba homozigotnosti i heterozigotnosti. Analiza pojavnosti mikrosatelitskih lokusa takođe pokazuje znatnu alelnu varijabilnost. Raspodela genetskog odnosa izražena je Wright

F koeficijentima i zabeležena je umeren nivo genetske raznolikosti u pojedinim lokusa. Rezultati pokazuju stepen rasnog odnosa i rasne čistoće, ali i ugroženost nekih delova genoma proučavane populacije . Stoga je neophodno preduzeti hitne mere za potporu genetske konsolidacije i očuvanje preostale populacije čistokrvnog slavonsko srem-skog podolskog govečeta.