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## Identification of 11 microsatellite markers in Bulgarian Rhodope cattle breed

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### ABSTRACT

The purpose of the present work was to study the genetic variation in 135 animals from the Bulgarian Rhodope cattle breed raised in the region of Smolyan, Bulgaria. For the purpose of the experiment, a panel of 11 microsatellite markers was used. A total of 113 alleles were identified and 5 of them were determined as population-specific. The allele number per locus varied from 7 (for SPS115) to 13 (for TGLA 227, TGLA 53, and INRA023 loci) with a mean number of 10.27. Allele frequency varied in different microsatellite loci. In locus TGLA227 the most frequent allele was with a length of 97 bp (0.59), and the rarest allele was with a length of 103 bp (0.025). The highest number of heterozygotes (125) was observed in locus INRA023 ( $n = 135$ ). The lowest number of heterozygotes (42) was detected in locus TGLA 122 ( $n = 135$ ). Microsatellite markers used in the current experiment showed PIC from 0.73 (loci SPS115 and BM2113) to 0.86 (loci INRA023 and TGLA227) with a mean value of 0.78. The observed heterozygosity ( $H_o$ ) varied from 0.828 (locus ETH3) to 0.959 (loci INRA23 and TGLA227). Expected heterozygosity (genetic diversity -  $H_e$ ) varied from 0.558 (loci ETH10 and BM1824) to 0.849 (locus INRA23). The mean heterozygosity for all investigated loci was  $H_o = 0.913$  and  $H_e = 0.734$ . Estimated values (PIC,  $H_o$ ,  $H_e$  и MNA) showed that all studied markers were polymorphic. All tested loci were with high polymorphic information content ( $>0.5$ ) and  $H_o > 0.6$ .

**Key words:** Bulgarian Rhodope cattle, Genetic diversity, SSR markers, Polymorphism

## Introduction

Conventional selection is mainly related to the evaluation of the breeding values of farm animals. This is a very slow and uncertain process. In recent decades, significant progress has been made in the understanding of gene behaviour and their application in the traditional selection, which led to a precise prediction and faster achievement of the desired results. Despite the hard work of scientists, it is still not entirely clear exactly how many genes are responsible for the expression of particular quantitative traits, where exactly they are located in the genome, and how exactly they interact with each other. That is why it is essential to continue searching for different candidate genes and molecular markers associated with economically important characteristics in farm animals (Shelyov *et al.*, 2017; Deb *et al.*, 2012).

Molecular markers are nucleotide variations at the DNA level which are specific for the different species and cause polymorphisms in the DNA sequence (Yadav *et al.*, 2017). Microsatellite markers (SSR – Single Sequence Repeats) are

repeated monomer sequences of the type  $(AT)_n$ ,  $(GC)_n$ ,  $(ATT)_n$ , etc. and the tandem repetition of the basis could be up to 60 times (Barker, 2002; Ellegren, 2004). In recent years, microsatellites have been the most popular and so called "marker of choice" in bovine genetic studies (Sharma *et al.*, 2020; Eusebi *et al.*, 2020).

Bulgarian Rhodope cattle breed was created on the basis of crossing the Rhodope Shorthorn cattle with Local Gray, Brown, and Jersey. The newly created breed was registered in 1981. Currently, the Rhodope cattle is below 2.0% in Bulgaria and it is raised mainly on private farms. The only larger and highly productive herd in the public sector is in the Experimental Station for Cattle Breeding in Smolyan. Bulgarian Rhodope cattle have the genetic potential for milk yield of about 2500 - 3500 kg, milk fat content – 5.12%, and protein content - 3.71% (Nikolov, 2012).

The purpose of the present work was to determine the genetic diversity by identification of 11 microsatellite markers in 135 animals from the Bulgarian Rhodope cattle breed raised in the region of Smolyan, Bulgaria.

## Materials and Methods

### Biological material

For the experimental object in the present study, there were randomly selected 135 animals from the Bulgarian Rhodope cattle breed, which were raised in Bulgaria. Blood samples were collected from each individual in vacuum tubes containing EDTA. The samples were stored at -20°C until the next step of experimental work.

### DNA extraction

Genomic DNA was extracted from whole blood using Exgene™ Tissue SV (plus) (GeneAll) purification kit according to the manufacturer's instructions. The concentration and quality of DNA were identified by spectrophotometer and agarose gel electrophoresis.

### PCR amplification

**Table 1.** Tested locus, chromosome localization, primers, allele length

№	Locus	Chromosome localization	Marker	Primer sequence (5' -> 3')	Length of alleles
1	ETH225 (D9S1)	9	M3	GAACACCTTGGCACTAATTCU ACATGACAGCCAGCTGCTACT	131-159
2	INRA023 (D3S10)	3	M9	GAGTAGAGCTACAAGATAAACTTC TAACTACAGGGTGTAGATGAACTC	195-225
3	ETH10(D5S3)	5	M10	GTTCAGGACTGGCCCTGCTAACA CCTCCAGCCACTTTCTCTTCTC	207-231
4	ETH3 (D19S2)	19	M14	GAACCTGCCTCTCCTGCATGG ACTCTGCCTGTGGCCAAGTAGG	103-133
5	BM2113(D2S26)	2	M15	GCTGCCTTCTACCAAATACCC CTTCCTGAGAGAAGCAACACC	122-156
6	BM1824(D1S34)	1	M16	GAGCAAGGTGTTTTCCAATC CATTCTCCAACCTGCTTCCTTG	176-197
7	TGLA227(D18S1)	18	M26	CGAATTCCAAATCTGTTAATTTGCT ACAGACAGAACTCAATGAAAGCA	75-105
8	TGLA126(D20S1)	20	M27	CTAATTTAGAATGAGAGAGGCTTCT TTGGTCTCTATTCTCTGAATATTCC	115-131
9	TGLA122(D21S6)	21	M28	CCCTCCTCCAGGTAAATCAGC AATCACATGGCAAATAAGTACATAC	136-184
10	TGLA53 (D16S3)	16	M29	GCTTTCAGAAATAGTTTGCATTCA ATCTTCACATGATATTACAGCAGA	143-191
11	SPS115(D15)	15	M30	AAAGTGACACAACAGCTTCTCCAG AACGAGTGCCTAGTTTGGCTGTG	234-258

A panel of 11 microsatellite markers recommended for cattle paternity testing by ISAG (Hoffman and Amos, 2004) was used (Table 1). Microsatellites were amplified using the "StockMarks for Cattle® Bovine Genotyping Kit" (Applied Biosystems Inc., Foster City, CA) in multiplex reactions according to the manufacturer's recommendations. PCR amplification was performed with thermal cycler EPPENDORF (PE, Applied Biosystems) under the following conditions: initial denaturation 95°C/15min, 31 cycles, denaturation 94°C/45s, annealing 55-65°C/45s, elongation 72°C/60s and final elongation 72°C/10min. The tested loci, primer sets, and allele range are presented in Table 1.

### Fragment analysis

The fluorescent labelled PCR products were submitted to fragment analysis by capillary electrophoresis, with an automated sequencer ABI PRISM 310 (Applied Biosystems), using the GeneScan-350 ROX® Size Standard (Applied Biosystems), according to the manufacturer's specifications. The information about fragment sizes was automatically estimated by the GENESCAN ANALYSIS v.3.1. Software.

### Statistical analysis

The genetic diversity of the tested animals was estimated based on allelic frequencies, the mean number of alleles (MNA), observed heterozygosity ( $H_o$ ), expected heterozygosity ( $H_e$ ), and the polymorphic information content (PIC) by Powerstat v.1.2 Software.

## Results

In the studied 135 individuals of the breed Bulgarian Rhodope cattle, a total of 113 alleles were found in the analyzed 11 loci. The number of alleles per locus varied from 7 (for SPS115) to 13 (for TGLA 227, TGLA 53, and INRA023 loci) with a mean number/locus of 10.27. The study showed PIC values from 0.73 (loci SPS115 and BM2113) to 0.86 (loci INRA023 and TGLA227) with a mean

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**Table 2.** Allele frequency, homo- and heterozygous variants of 11 microsatellite loci in Bulgarian Rhodope cattle

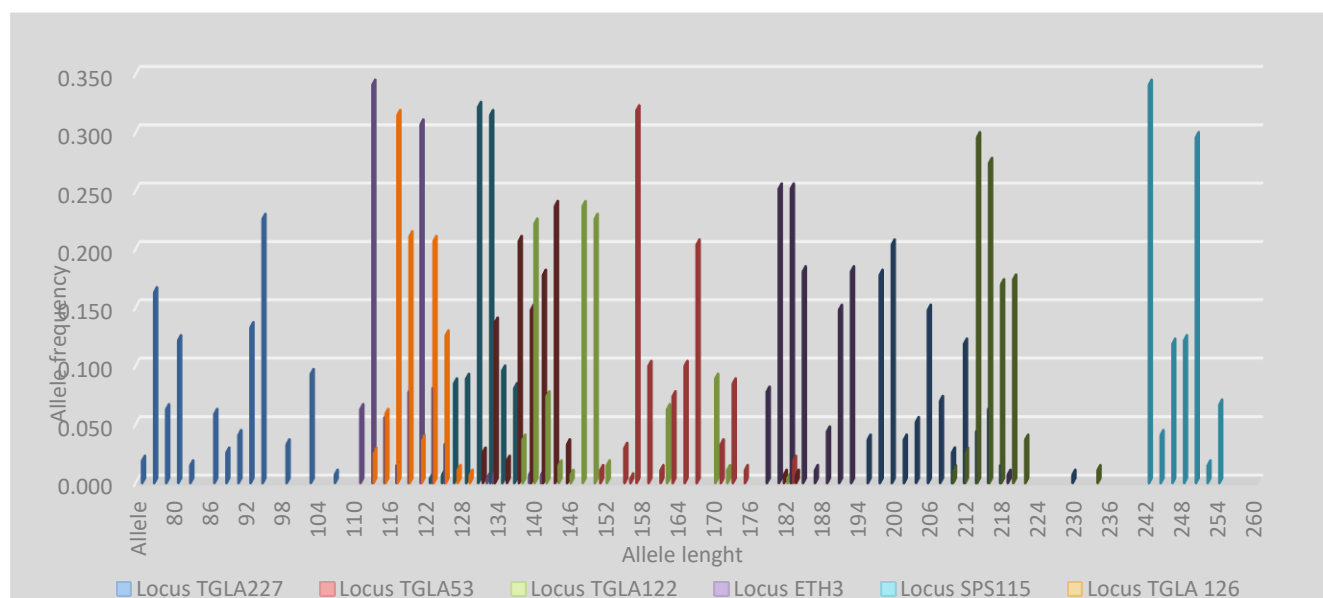
	TGLA 227 n=135	TGLA 53 n=135	TGLA 122 n=135	ETH3 n=135	BM2113 n=135	SPS115 n=135	TGLA 126 n=135	INRA023 n=135	ETH 225 n=135	ETH10 n=135	BM1824 n=135
Allele	75	151	138	111	122	242	113	195	131	209	178
Freq	0,019	0,011	0,037	0,063	0,004	0,341*	0,026	0,037	0,026	0,011	0,078
Allele	77	155	140	113	124	244	115	197	133	211	180
Freq	0,163	0,03	0,222	0,341*	0,007	0,041	0,059	0,178	0,137	0,026	0,252
Allele	79	156	142	115	126	246	117	199	135	213	182
Freq	0,063	0,004	0,074	0,056	0,085	0,119	0,315*	0,204	0,019	0,296	0,252
Allele	81	157	144	117	128	248	119	201	137	215	184
Freq	0,122	0,319*	0,015	0,015	0,089	0,122	0,211	0,037	0,207	0,274	0,181
Allele	83	159	146	119	130	250	121	203	139	217	186
Freq	0,015	0,1	0,007	0,078	0,322*	0,296	0,037	0,052	0,148	0,17	0,011
Allele	87	161	148	121	132	252	123	205	141	219	188
Freq	0,059	0,011	0,237	0,307	0,315	0,015	0,207	0,148	0,178	0,174	0,044
Allele	89	163	150	123	134	254	125	207	143	221	190
Freq	0,026	0,074	0,226	0,081	0,096	0,067	0,126	0,07	0,237	0,037	0,148
Allele	91	165	152	125	136	254	127	209	145	233	192
Freq	0,041	0,1	0,015	0,033	0,081	0,067	0,011	0,026	0,033	0,011	0,181
Allele	93	167	162	132	136	254	129	211	181	218	218
Freq	0,133	0,204	0,063	0,004	0,081	0,067	0,007	0,119	0,007	0,007	0,007
Allele	95	171	170	133	136	254	129	213	181	218	218
Freq	0,226	0,033	0,089	0,007	0,081	0,063	0,007	0,044	0,007	0,007	0,007
Allele	99	173	172	139	136	254	129	215	183	218	218
Freq	0,033	0,085	0,011	0,007	0,081	0,063	0,007	0,063	0,007	0,007	0,007
Allele	103	175	182	141	136	254	129	217	181	218	218
Freq	0,093	0,011	0,004	0,007	0,081	0,063	0,007	0,015	0,007	0,007	0,007
Allele	107	183	182	141	136	254	129	229	181	218	218
Freq	0,007	0,019	0,004	0,007	0,081	0,063	0,007	0,007	0,007	0,007	0,007
Homozygotes - n=11	11	13	9	12	11	26	14	10	16	30	30
Heterozygotes - n=124	124	122	42	123	124	109	121	125	119	105	105

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**Table 3.** Polymorphic information content (PIC), heterozygosity and number of alleles in the studied microsatellite loci of Bulgarian Rhodope cattle

Locus	Allele length /bp/	PIC	Ho	He	N
TGLA 227	75-107	<b>0.86</b>	<b>0.959</b>	0.833	<b>13</b>
BM 2113	122-136	<b>0.73</b>	0.865	0.833	8
TGLA 53	151-183	0.80	0.935	0.803	<b>13</b>
ETH 10	209-233	0.74	0.908	<b>0.558</b>	8
SPS 115	242-254	<b>0.73</b>	0.889	0.613	<b>7</b>
TGLA 126	113-129	0.76	0.904	0.788	9
TGLA 122	138-182	0.80	0.934	0.669	12
INRA 23	195-229	<b>0.86</b>	<b>0.959</b>	<b>0.849</b>	12
ETH 3	111-141	0.74	<b>0.828</b>	0.818	12
ETH 225	131-183	0.80	0.929	0.758	10
BM 1824	183-193	0.78	0.933	<b>0.558</b>	9
Total		8.6	10.043	8,08	113
Mean		0.78	0.913	0.734	10.27

<sup>1</sup>PIC – Polymorphic information content, <sup>2</sup>H<sub>o</sub> – observed heterozygosity, <sup>3</sup>H<sub>e</sub> – expected heterozygosity, <sup>4</sup>N – number of alleles

**Figure 1.** Allele frequency and allele length of Bulgarian Rhodope cattle in 11 microsatellite loci

value of 0.78 (Table 3). Genetic diversity in the population was determined based on the level of heterozygosity and the number of alleles in microsatellite loci. The observed heterozygosity ( $H_o$ ) in this study ranged from 0.828 (locus ETH3) to 0.959 (loci INRA23 and TGLA227). The expected heterozygosity (genetic diversity -  $H_e$ ) varied from 0.558 (loci ETH10 and BM1824) to 0.849 (locus INRA23). For all

11 loci, the mean observed and expected heterozygosity were  $H_o = 0.913$  and  $H_e = 0.734$ , respectively. The effective number of alleles (N) was between 12 (loci ETH3, INRA23, and TGLA122) and 13 (loci TGLA53 and TGLA227). The calculated values of the parameters of genetic diversity - PIC,  $H_o$ ,  $H_e$ , and MNA showed that all microsatellite markers were polymorphic. All studied markers were characterized with

high polymorphic content (PIC) > 0.5 and observed heterozygosity  $H_o > 0.6$ .

The Bulgarian Rhodope cattle was characterized with equal value PIC for TGLA227 and INRA23 (–PIC 0.86), which was the highest in these loci. The highest  $H_o = 0.959$  was also observed in both loci. TGLA227 and TGLA53 showed the largest number of alleles - 13. Alleles with the highest frequency in Bulgarian Rhodope cattle were observed in loci ETH3, SPS115, BM2113, and TGLA126, and their frequency varied between 0.315 and 0.341 (Table 3).

From all 113 alleles identified for this breed, 5 (4%) were identified as population-specific (Table 2), based on the relatively higher allele frequencies at the following microsatellite loci: TGLA 53 - 1 allele (allele 157 with frequency 0.319), TGLA126 - 1 allele (allele 117 with frequency 0.315), ETH 3 - 1 allele (allele 113 with frequency 0.341), SPS115– 1 allele (allele 242 with frequency 0.341), BM2113 - 1 allele (allele132 with frequency 0.315).

## Discussion

In Bulgaria, similar study was conducted by Dalvit *et al.* (2009). The research team tested a total of 195 samples taken from three cattle breeds - Rhodope Shorthorn ( $n=73$ ), Iskar ( $n=82$ ), and Bulgarian Rhodope cattle ( $n=40$ ). They applied a panel of 19 microsatellite markers in order to study the genetic diversity in selected breeds - ILSTS008, BM1818, TGLA57, ETH3, RM12, INRA006, MM12, TGLA126, INRA016, TGLA122, CSSM14, TGLA53, INRA64, ETH152, BM203, ETH10, ETH185, BL42 and SPS115. Identical to SSRs markers in the present study were ETH3, TGLA126, TGLA122, TGLA53, ETH10, and SPS115. The obtained results by Dalvit *et al.* (2009) for the Bulgarian Rhodope cattle were  $0.619 \pm 0.177$  for the expected heterozygosity ( $H_e$ ),  $0.576 \pm 0.228$  for the observed heterozygosity ( $H_o$ ) 0.069 (0.001 – 0.105) for the inbreeding coefficient ( $F_{is}$ ) and 6.3 of allelic richness (AR). In the current study of the Bulgarian Rhodope cattle, the number of tested animals was higher ( $n=135$ ) and the observed heterozygosity ( $H_o$ ) was 0.913, while the expected heterozygosity was 0.734.

Worldwide have been conducted many studies on different cattle breeds and with different numbers of microsatellite markers.

Kramarenko *et al.* (2018) studied the genetic diversity of the Red Steppe cattle based on the same 11 microsatellite markers as in the present experiment - BM1818, BM1824, BM2113, ETH3, ETH10, INRA023, TGLA53, TGLA122, TGLA126, TGLA227, and SPS115. The research team tested 39 animals and 71 alleles were detected. The lowest observed heterozygosity ( $H_o$ ) was determined in the locus TGLA53 with the value of 0.185 and the highest  $H_o$  was detected in

locus TGLA227 – 0.872. While for the Bulgarian Rhodope cattle in the present study the lowest  $H_o$  was for locus BM2113 – 0.865 and the highest  $H_o$  for loci TGLA 227 and INRA 23 – 0.945. The lowest expected heterozygosity ( $H_e$ ) for Red Steppe was observed in locus INRA023 with the value of 0.459. The highest  $H_e$  was 0.830 in the same locus. In the Bulgarian Rhodope cattle in this study the lowest  $H_e$  was for loci BM1824 and ETH10 with the value of 0.558 and the highest  $H_e$  was for locus INRA 23 – 0.849.

By using 20 microsatellite markers, Demir and Balcioglu (2019) genotyped 120 animals belonging to 4 breeds - Turkish Grey Steppe, Holstein Friesian, Eastern Anatolian Red, and Anatolian Black. Three of the studied loci (SPS115, TGLA227, and ETH3) were used in this study. The highest registered alleles were: 10 for Turkish Grey Steppe in locus SPS115, 9 for Anatolian Black, 8 – Holstein Friesian, and 7 – Eastern Anatolian Red. The lowest number of established alleles were in locus TGLA227 – 4 for Turkish Grey Steppe, Holstein Friesian, Eastern Anatolian Red, and 5 for Anatolian Black. For the locus ETH3 – 8 alleles were detected for Eastern Anatolian and Red Anatolian Black, 6 for Turkish Grey Steppe, and 4 for Holstein Friesian. The highest registered PIC was in locus SPS115 with the value of 0.82 detected in Turkish Grey Steppe and the lowest for ETH3 – 0.51 also for the Turkish Grey Steppe. The highest observed heterozygosity ( $H_o$ ) was estimated in Turkish Grey Steppe, Eastern Anatolian, and Red Anatolian Black with the value of 1.00 and 0.90 for Holstein Friesian in locus SPS115 and the lowest in locus ETH3 – 0.37 detected in Anatolian Black and Holstein Friesian. The highest expected heterozygosity ( $H_e$ ) was registered in locus SPS115 with the value of 0.85 in the Turkish Grey Steppe and the lowest in locus ETH3 – 0.55 also for the Turkish Grey Steppe.

Heryani *et al.* (2019) sampled 18 animals from the Taro white cattle using 4 microsatellite markers - BM1824, BM2113, INRA23, and ETH225 to characterize their genetic status for improving breeding programs. This was a unique group with a very small number of animals kept in the Taro forest at Tegallalang. There were only 33 individuals in the area. According to the authors, this cattle breed played an important role in the local culture (Heryani *et al.*, 2019). A total of 13 alleles were successfully observed in the tested population. The reported highest PIC was in locus BM2113 with a value of 0.627 and the highest number of the observed alleles (4). The lowest reported was in locus BM1824 – 0.448 with 3 alleles. In the Bulgarian Rhodope Cattle for the locus BM2113 the registered PIC was 0.730 and for BM1824 was 0.780. Nine alleles were observed for locus BM1824 and 8 alleles in locus BM2113 in the Bulgarian Rhodope cattle. The observed heterozygosity ( $H_o$ ) in Taro White cattle for locus BM2113 was 0.444 and for locus BM1824 – 0.111. The expected heterozygosity ( $H_e$ ) was 0.703 for locus BM2113

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and for locus BM1824 – 0.565. In the Bulgarian Rhodope cattle the observed heterozygosity ( $H_o$ ) for locus BM2113 was 0.865 and expected heterozygosity ( $H_e$ ) – 0.833, and for the locus BM1824 the observed heterozygosity ( $H_o$ ) was 0.933 and the expected heterozygosity ( $H_e$ ) was 0.558.

Analyzing the genetic structure of a population of Lebedyn cattle, Ladyka *et al.* (2019) sampled 30 individuals from the farm "Komyschans'ke" in Sumy region by using 10 microsatellite loci: ETH225, BM2113, ETH3, BM1818, BM1824, ILSTS006, INRA023, TGLA053, TGLA122, and ETH10. They detected 43 alleles in 8 of the used loci. For the same eight loci in the present study was detected a total of 84 alleles. It also should be considered the higher number of the samples. The highest observed registered PIC was 0.617. The most polymorphic loci were TGLA053 (8 alleles), BM2113 (6), and ETH3 (6). The highest value of  $H_e$  (0.811) and  $H_o$  (0.833) was in locus BM2113.

From the results obtained in the present study, and the results of other scientists as well, it is clear that microsatellite markers are a suitable tool for studying the genetic status of various cattle breeds.

## Conclusions

According to the results in the present study in the Bulgarian Rhodope cattle breed, it can be concluded that all identified 11 SSRs markers were polymorphic. A total of 113 alleles were found. Alleles with the highest frequency in Bulgarian Rhodope cattle were reported in loci ETH3, SPS115, BM2113, and TGLA126, and their frequency varied between 0.315 and 0.341. The integration of the analyzed results would give an opportunity for additional information about genetic structure and diversity at intra- and inter-population levels, therefore it would improve the management of the genetic resources in cattle breeding.

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