RESEARCH ARTICLE

Identification of 11 microsatellite markers in Bulgarian Rhodope cattle breed

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Article info:
Received: 9 February 2022
Accepted: 29 March 2022

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^{0}C until the next step of experimental work.

DNA extraction

Genomic DNA was extracted from whole blood using ExgeneTM 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	GATCACCTTGCCACTATTTCCT ACATGACAGCCAGCTGCTACT	131-159
2	INRA023 (D3S10)	3	M9	GAGTAGAGCTACAAGATAAACTTC TAACTACAGGGTGTTAGATGAACTC	195-225
3	ETH10(D5S3)	5	M10	GTTCAGGACTGGCCCTGCTAACA CCTCCAGCCCACTTTCTCTTCTC	207-231
4	ETH3 (D19S2)	19	M14	GAACCTGCCTCTCCTGCATTGG ACTCTGCCTGTGGCCAAGTAGG	103-133
5	BM2113(D2S26)	2	M15	GCTGCCTTCTACCAAATACCC CTTCCTGAGAGAAGCAACACC	122-156
6	BM1824(D1S34)	1	M16	GAGCAAGGTGTTTTTCCAATC CATTCTCCAACTGCTTCCTTG	176-197
7	TGLA227(D18S1)	18	M26	CGAATTCCAAATCTGTTAATTTGCT ACAGACAGAAACTCAATGAAAGCA	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 AACGAGTGTCCTAGTTTGGCTGTG	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 95ºC/15min, 31 cycles, denaturation 94ºC/45s, annealing 55-65ºC/45s, elongation 72ºC/60s and final elongation 72ºC/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

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

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	**0.86**	**0.959**	0.833	**13**
BM 2113	122-136	**0.73**	0.865	0.833	8
TGLA 53	151-183	0.80	0.935	0.803	**13**
ETH 10	209-233	0.74	0.908	**0.558**	8
SPS 115	242-254	**0.73**	0.889	0.613	**7**
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	**0.86**	**0.959**	**0.849**	12
ETH 3	111-141	0.74	**0.828**	0.818	12
ETH 225	131-183	0.80	0.929	0.758	10
BM 1824	183-193	0.78	0.933	**0.558**	9
Total		8.6	10.043	8,08	113
Mean		0.78	0.913	0.734	10.27

[1]PIC – Polymorphic information content, [2]H_o – observed heterozygosity,[3]H_e – expected heterozygosity, [4]N – number of alleles

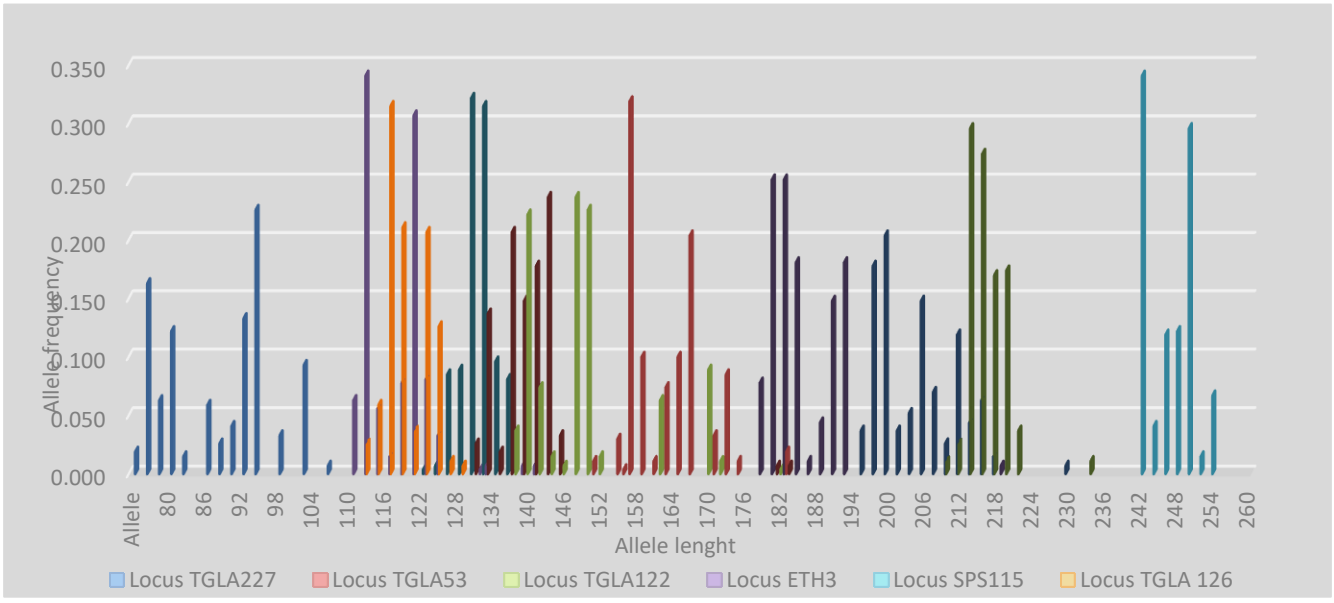

Figure 1. *Allele frequency and allele length of Bulgarian Rhodope cattle in 11 microsatellite loci*

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 ± 0.177 for the expected heterozygosity (H_e), 0.576 ± 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

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 "Komyshans'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.

References

Kramarenko A, Gladyr E, Kramarenko S, Pidpala T, Strikha L, Zinovieva N. 2018. Genetic diversity and bottleneck analysis of the Red Steppe cattle based on microsatellite markers. Ukrainian Journal of Ecology, 8(2), 12-17.

Barker G C. 2002. Microsatellite DNA: a tool for population genetic analysis. Transactions of The Royal Society of Tropical Medicine and Hygiene, 96 (Supplement), 21 - 24 (0035-9203).

Dalvit C, Krastanov J, Maretto F, Oblakov N, Angelova T , Cassandro M. 2009. Monitoring genetic variability of Bulgarian cattle biodiversity. Ital. J. Anim. Sci. vol. 8 (3): 89-91.

Deb R, Chakraborty S, Singh U. 2021. Molecular Markers and Their Application in Livestock Genomic Research, Journal Veterinary Science Technology, 3: 106-120.

Ellegren H. 2004. Microsatellites: simple sequences with complex evolution, Nature Reviews Genetics, 5: 435–445.

Eusebi P G, Martinez A, Cortes O. 2020. Genomic Tools for Effective Conservation of Livestock Breed Diversity, 12 (1): 8-24.

Demir E and Balcioglu E. 2019. Genetic diversity and population structure of four cattle breeds raised in Turkey using microsatellite markers. Czech Journal of Animal Science, 64 (10): 411–419.

Heryani L, Wandia N, Suarna W, Puja K, Susari N, Agustina K. 2019. Molecular characteristic of taro white cattle based on DNA microsatellite markers. Biodiversitas Journal of Biological diversity, 20 (3): 671-675.

Sharma R, Ahlawat S, Sharma H, Bhagat R, Singh P, Tantia M. 2020. Identification of a new potential native Indian cattle breed by population differentiation based on microsatellite markers, Mol Biol Rep, 47: 6429–6434.

Shelyov A V, Kopylov K, Kramarenko S, Kramarenko O. 2017. Analysis of population-genetic processes in different cattle breeds by microsatellite loci of dna. Agricultural Science and Practice. 4: 74-78.

Ladyka V, Khmelnychyi L, Lyashenko Y, Kulibaba R. 2019. Analysis of the genetic structure of a population of Lebedyn cattle by microsatellite markers. Regul. Mech. Biosyst., 2019, 10(1), 45–49.

Yadav A, Tomar S, Jha A, Singh J. 2017. Importance of Molecular Markers in Livestock Improvement: A Review, International Journal of Agriculture Innovations and Research, 5 (4): 2319-1473.

Nikoilov V. Rhodope Brachycern cattle, Monography, Taftprint, Plovdiv 2012.

Hoffman J and Amos W. 2004 Microsatellite genotyping errors: detection approaches, common sources and consequences for paternal exclusion. Mol. Ecol., 14(2): 599-612.