

MOLECULAR CHARACTERIZATION OF BULGARIAN LIVESTOCK GENETIC RESOURCES. II. MICROSATELITE VARIATION WITHIN AND AMONG BULGARIAN CATTLE BREEDS**

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Abstract: This study was undertaken to determine the genetic structure and the diversity among 2 local cattle breeds from Bulgaria, the Rhodope Shorthorn and Grey cattle. A panel of 11 microsatellites was used for the evaluation. For these loci, allele frequencies, heterozygosity, HWE, genetic disequilibrium, genic differentiation were determined. Both populations displayed a relatively high level of genetic variation as estimated by allelic diversity and heterozygosity. Heterozygosities ranged from 0.5424 /SPS 115/ to 0.8983 /TGLA 227/ for the Rhodope population and 0.6333 /TGLA 53/ to 0.9333 /TGLA227/ for Grey cattle, with similar average values for the two groups – 0.7858 and 0.7757. These results clearly suggest that these breeds are suitable to preserve as a genetic resources.

Keywords: cattle breeds, linkage disequilibrium, microsatellites, genetic diversity

Introduction

One of the factors for improvement of livestock breeds in future is to develop new breeds with desirable traits based on cross-breeding between

traditional livestock breed breeds resistant to important diseases and commercial breeds. If done effectively, this would yield animals with productive characteristics of the commercial breed and the disease resistance of the traditional breed. However, when livestock breeds become extinct, their unique genes are lost forever. Loss of populations from local breeds contradicts to the principles for sustainable development of animal breeding and to the correct management of genetic resources. The old Bulgarian breeds, traditionally bred, have been adapted to the local conditions and they are resistant to diseases. Due to this they are usually preferred for organic raising of animals (which excludes the use of veterinary medicines and preparations). Utilising local breeds will be most effective in contributing to the achievement of local food security objectives. The necessity to extend, maintain and conserve genetic diversity has been outlined. In this context a number of molecular techniques have provided new DNA markers for the study of genetic variation (*Nijman et al.*, 1999; *Hansen et al.*, 2002).

During recent years, different studies of cattle breeds based on microsatellite markers have aimed at characterising the genetic variation, genetic relationships between the cattle breeds from Italy (*Ciampolini et al.*, 1995), Spain (*Martin-Burriel et al.*, 1998; *Canon et al.*, 2001), Belgium (*Mommens et al.*, 1999), Poland (*Radko and Duniec*, 2002), and the Czech Republic (*Czernekova et al.*, 2006).

The aim of this study was to assess the genetic diversity in two populations of Bulgarian local breeds and to evaluate possible the ranking of breeds for conservation purposes.

Material and methods

Bulgarian local cattle breeds

Two local cattle breeds are available in Bulgaria at present. The first one is described in our previous study (*Teneva et al.*, 2005). The other - Rhodope Shorthorn local cattle breed is dispersed in the Rhodope Mountains. Rhodope cattle are the last remnant of the prehistoric brachyceric cattle in Europe (*Danchev*, 1994). Small in size, they are capable of using any type of mountain pasture. Their average live weight is around 220 kg. Their average milk yield is between 1100-1200 liters with a maximum of 2623 liters. The fat content in the milk is on average 4.5%, with a maximum of 5.9%. Comparisons among all the local and improved breeds of cows in Bulgaria

show that the Rhodope Shorthorn breeds are second only to the Bulgarian Red Cows in production per 100 kg live weight. Moreover, they live 2-3 times longer than the highly-selected breeds and can produce offspring and milk into advanced age. They are practically immune to illness and have excellent adaptive capabilities. Forty years ago 52 956 cows were alive. Nowadays, despite their attractive traits, only a small population of the Rhodope Shorthorn breed remains in Bulgaria. After the Information of EAAP-Animal genetic data bank of the European Association for Animal Production /<http://www.tiho-hannover.de/einricht/zucht/eaap/> the status of endangerment of this breed is “critically endangered” (NFN =75). For the Grey cattle the status was described in our previous study (*Teneva et al.*, 2005).

Samples. Blood samples were collected from 89 animals belonging to the two breeds. The studied populations included 30 Grey cattle breed (G) and 59 Rhodope Shorthorn (R) from the regions of Burgas and Smoljan respectively.

Microsatellite analysis. The Genomic DNA extraction from whole blood, PCR amplifications and Genotyping were performed in multiplex reactions by methods, described in previous study (*Teneva et al.*, 2005). Eleven microsatellite loci recommended by ISAG /International Society of Animal Genetic/ for diversity studies in cattle - ETH225, INRA023, ETH10, ETH3, BM2113, BM1824, TGLA227, TGLA126, TGLA122, TGLA53, SPS115 were subjected to analysis.

Statistical analysis

Allele frequencies, the expected (H_e) and observed heterozygosity (H_o) were calculated using the computer program GENEPOP. Exact tests for deviations from Hardy-Weinberg equilibrium (HWE) and heterozygote deficiency were also performed using the GENEPOP package (*Raymond and Rousset*, 1995). The program performed a probability test using a Markov chain (dememorization 1 000, batches 100, iterations per batch 1000). Significance levels were calculated per locus, per population, and over all loci and populations combined. Fisher's method was used to combine the two populations and 11 microsatellite loci with 4n degrees of freedom, computed as in *Weir and Cockerham* (1984) and *Robertson and Hill* (1984). Genotypic linkage disequilibrium was estimated between all pairs of loci with GENEPOP (Markov chain using dememorization 1,000, batches 100, iterations per batch 100).

Results and discussions

A total of 178 alleles were identified in the 89 cattle at the 11 loci - 118 alleles in Rhodope (R) and 60 in Grey (G) cattle with a mean 16.2 alleles/locus.

The most polymorphic locus among the studied 11 microsatellite loci in both populations was TGLA 53, with each population having 13 alleles. High polymorphism was noted at loci TGLA 122, with 12 and 10 alleles respectively for R and G cattle, TGLA 227 (11 and 12 alleles) and ETH 3, with 10 and 9 alleles for R and G cattle. The lowest number of alleles in both breeds was observed at locus BM1824 (table 1).

The same variation of number of alleles were shown by *Zhou et al.* (2005) for 5 native Chinese cattle breeds - Luxi (LX), Nanyang (NY), Jinnan (JN), Qinchuan (QC) and Yanbian (YB). The number of alleles at locus TGLA 227 in present study is higher than observed by *Martin-Burriel et al.* (1998) and *Armstrong et al.* (2006) for Spanish native and Creole cattle breeds (7 alleles).

The Rhodope Shorthorn population had a greater mean number of alleles (9.0) than the Grey cattle (7.5), although this may have been due, in part, to the much larger sample size.

Allele frequency data for the microsatellites studied are shown in Figure 1. All loci were polymorphic in both breeds. The alleles detected at different loci varied with disparate frequency. At the most polymorphic locus TGLA 53 – 13 alleles were in range of 154 to 186 bp. Alleles of 160 bp and 170 bp were the most frequent alleles in both breeds at this locus. The allele 166 bp is present in Rhodope but absent in Grey cattle and allele 186 bp is present in Grey and absent in Rhodope Shorthorn cows.

The data for another high polymorphic locus – TGLA 122 showed the absence of alleles 138bp, 140 bp and 178 bp in the Grey cattle population. The most frequent allele in the studied populations was the 144 bp allele. The rarest alleles were 138bp and 152bp /0.008/for the Rhodope population and 156 bp (0.017) for the Greys.

ETH 3 amplified 10 and 9 alleles respectively for Rhodope and Grey cattle with the most common alleles being of 117 bp and 125 bp. In both breeds allele 121bp had the lowest frequency.

Four to five alleles were detected in locus BM1824. The most common allele was 184 bp. Allele 192 bp was present only in the Rhodope Shorthorn population.

The results concerning allele frequencies in Bulgarian local cattle are in

agreement with the data obtained from *Radko and Duniec* (2002). *Czernekova et al.* (2006) reported higher numbers of alleles in the same loci – from 7 at BM1824 to 14 at TGLA 227 for Czech cattle.

Table 1. Observed allele size range, heterozygosity and number of alleles in Bulgarian cattle breeds studied

Locus	Observed allele size range/	<i>He</i>	<i>Ho</i>	N
TGLA 227				
R	80-102	0.8983	0.8536	11
G	78-104	0.9333	0.8885	12
BM 2113				
R	125-143	0.8475	0.8141	8
G	125-141	0.8000	0.8172	7
TGLA 53				
R	117-127	0.7966	0.8383	13
G	109-123	0.6333	0.8517	13
ETH 10				
R	212-226	0.6610	0.6505	7
G	210-222	0.8333	0.7425	5
SPS 115				
R	248-260	0.5424	0.5143	7
G	248-258	0.7000	0.6488	6
TGLA 126				
R	117-127	0.6610	0.6531	6
G	109-123	0.6667	0.6149	5
TGLA 122				
R	138-174	0.8814	0.8369	12
G	142-176	0.6667	0.7523	10
INRA 23				
R	175-219	0.8814	0.8635	11
G	199-217	0.8333	0.7351	6
ETH 3				
R	109-131	0.8644	0.7702	10
G	103-127	0.8000	0.8351	9
ETH 225				
R	138-160	0.8814	0.8045	9
G	140-158	0.8333	0.7787	6
BM 1824				
R	180-192	0.7288	0.6749	5
G	180-190	0.8333	0.7402	4
Mean				
R		0.7858	0.7513	9.0
G		0.7757	0.7641	7.5

Both populations were described according to the expected (H_e) and observed (H_o) heterozygosity. The data in Table 1 shows a common trend in the two groups. Expected heterozygosity ranged from 0.5424 (SPS 115) to 0.8983 (TGLA 227) for the Rhodope population and 0.6333 (TGLA 53) to 0.9333 (TGLA 227) for Grey cattle. All the microsatellites showed expected heterozygosity greater than 0.500. The overall mean heterozygosity across all populations and all markers had similar average values for both groups – 0.7858 and 0.7757. In an analysis of six Spanish native breeds, Martín-Burriel *et al.* (1998) reported an average expected heterozygosity between 0.56 and 0.68. Additionally, *Rendo et al.* (2004) found an expected heterozygosity between 0.69 and 0.76 in four Western Pyrenees cattle breeds using 11 microsatellite markers, while in a study of 15 Portuguese cattle breeds *Mateus et al.* (2004) found an average expected heterozygosity between 0.63 and 0.74.

Similar results have been reported by *Zhou et al.* (2005) for 5 native Chinese populations which display a high heterozygosity – 0.51 to 0.86.

The obtained values of expected heterozygosity in our study are higher than those reported by *Citek and Rehout* (2001), *Czernekova et al.* (2006) for endangered populations – Czech Red, Czech Pied, Polish Red, and German Red cattle, which were in the range of 0.396 to 0.495 and 0.650 to 0.764 respectively

Observed heterozygosities were lower than the expected ones in the great of cases, however (Table 1). The difference between the expected (H_e) and observed (H_o) heterozygosity suggested a tendency towards heterozygote deficiency. This result could be due to local inbreeding or the presence of null alleles.

Our study showed the highest level of heterozygosity at locus TGLA 227 in both populations. This means that this marker could be included in subsequent genetic diversity studies of cattle populations.

The heterozygosity found in our samples of Bulgarian local cattle breeds was considerably higher in comparison to that found in studies on commercial breeds that used similar microsatellites. The studies of *Hansen et al.* (2002), *Maudet et al.* (2002) showed that highly selected commercial breeds are much less diverse and more inbred than local breeds, which reinforces the importance of local breeds as reserves of genetic diversity for sustainable agriculture.

Genetic equilibrium of the cattle breeds was calculated on the base of 11 studied loci. Analysis of the observed and expected genotype frequencies (table 2) showed excess of heterozygotes for all loci in the two populations

except TGLA 53 in Rhodope and BM 2113, ETH 3, TGLA 122 and TGLA 53 in Grey group.

Table 2. Observed and expected homo- and heterozygosity for the Rhodope /n=59/ and Grey /n=30/ populations

Locus	HomExp	HomObs	HetExp	HetObs	Fis /W&C/	Fis /R&H/
TGLA 227						
Rhodope	8.6154	6	50.3846	53	-0.0524	-0.0460
Grey	3.3220	2	26.6780	28	-0.0505	-0.0259
BM 2113						
Rhodope	10.9487	9	48.0513	50	-0.0409	-0.0234
Grey	5.4915	6	24.5085	24	+0.0211	+0.0390
TGLA 53						
Rhodope	9.5641	12	49.4359	47	+0.0497	+0.0520
Grey	4.5593	11	25.4407	19	+0.2564	+0.1265
ETH 10						
Rhodope	20.6154	20	38.3846	39	-0.0162	-0.0179
Grey	7.6780	5	22.3220	25	-0.1223	-0.0937
SPS 115						
Rhodope	28.6410	27	30.3590	32	-0.0545	-0.0474
Grey	10.5085	9	19.4915	21	-0.0788	-0.0506
TGLA 126						
Rhodope	20.4615	20	38.5385	39	-0.0121	+0.0993
Grey	11.5254	10	18.4746	20	-0.0841	-0.0564
TGLA 122						
Rhodope	9.5983	7	49.4017	52	-0.0531	-0.0392
Grey	7.4746	10	22.5254	20	+0.1138	+0.0388
INRA 23						
Rhodope	8.0427	7	50.9573	52	-0.0206	+0.0997
Grey	7.8983	5	22.1017	25	-0.1337	-0.0712
ETH 3						
Rhodope	13.5128	8	45.4872	51	-0.1224	-0.0468
Grey	4.9661	6	25.0339	24	+0.0420	+0.1004
ETH 225						
Rhodope	11.4957	7	47.5043	52	-0.0955	-0.0577
Grey	6.6102	5	23.3898	25	-0.0701	-0.0702
BM 1824						
Rhodope	19.1538	16	39.8461	43	-0.0799	-0.0388
Grey	7.7458	5	22.2542	25	-0.1258	-0.1341

Tests of genotypic disequilibrium across populations resulted in 110 comparisons. Significant linkage disequilibrium ($P < 0.05$) was found between locus pairs BM2113 and TGLA53 on one hand and ETH 3 in the

other hand in Rhodope Shorthorn breed. In the second local Bulgarian breed was observed a genetic disequilibrium between the following loci ($P < 0.05$): BM2113 and ETH3 and ETH 225, BM1824 and ETH10. Gametic disequilibrium can be due to a different causes, including epistatic selection, physical linkage, genetic hitchhiking. Because these markers have been mapped to different chromosomes (respectively BM1824 on chromosome 1, BM2113 on chromosome 2, ETH 3 on chromosome 9, ETH 225 on chromosome 10 and TGLA 53 on chromosome 16) one can exclude physical linkage. The most probable reason may be gametic selection.

The main objective of conservation genetics is to preserve variability within populations. As the present investigation has used neutral markers, the possible reason for the high degree of genetic diversity in these two populations may be that intensive breeding programmes have not been undertaken.

Conclusions

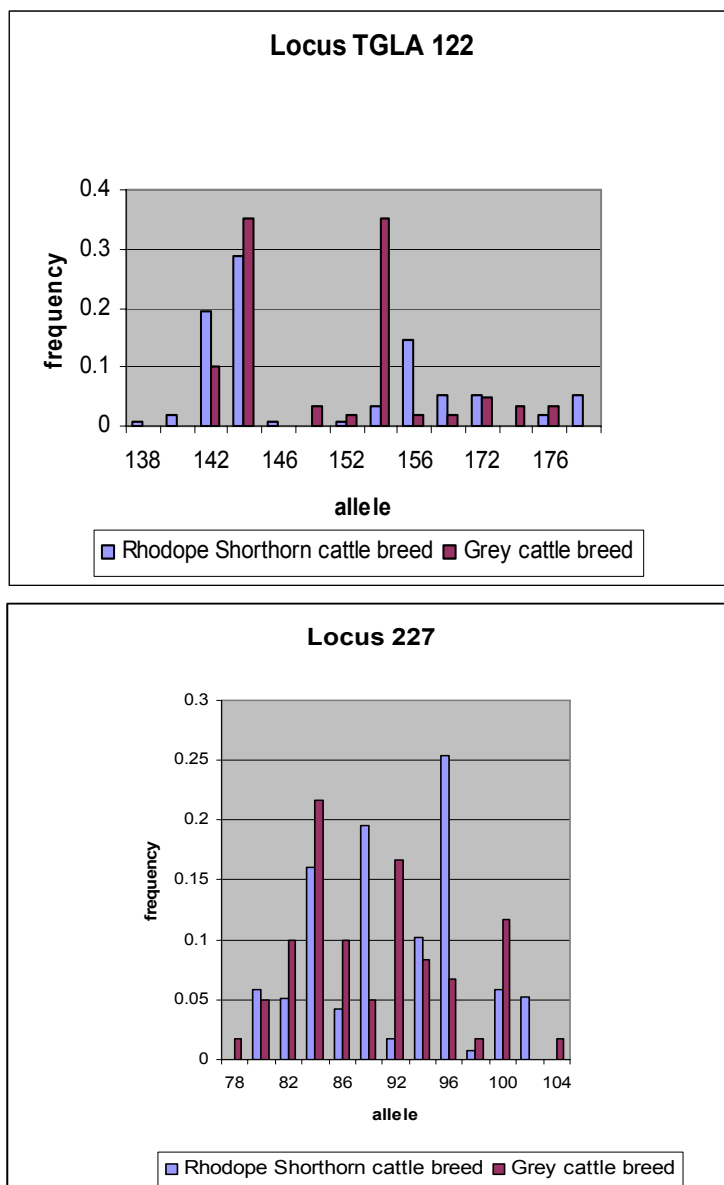
This study contributes to the knowledge of the genetic diversity, genetic structure and to the molecular characterization of small populations on the brink of extinction.

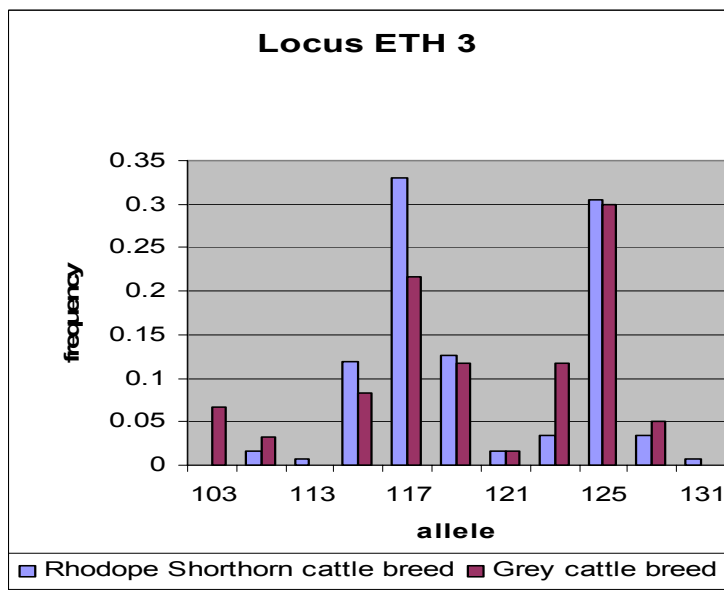
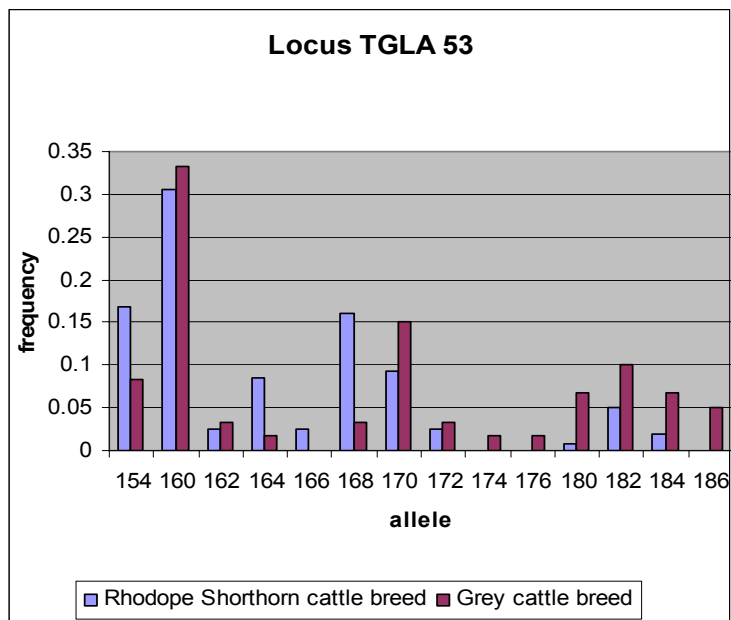
For the first time in Bulgaria the Rhodope Shorthorn breed was genetically characterized by using DNA markers. All loci were polymorphic and this indicates that the microsatellite markers used are suitable for genetic diversity study. The comparison between the two local breeds shows that they display a remarkable high variability. This clearly suggests that these breeds have potential value to be preserved as genetic resources.

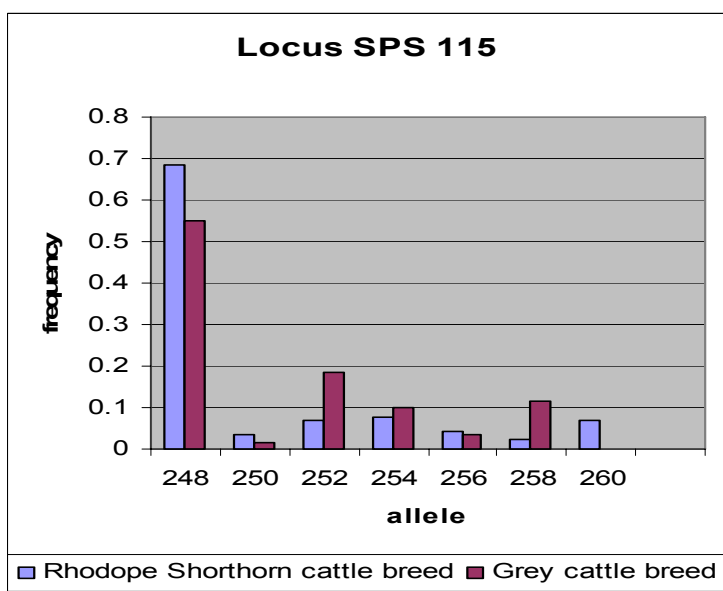
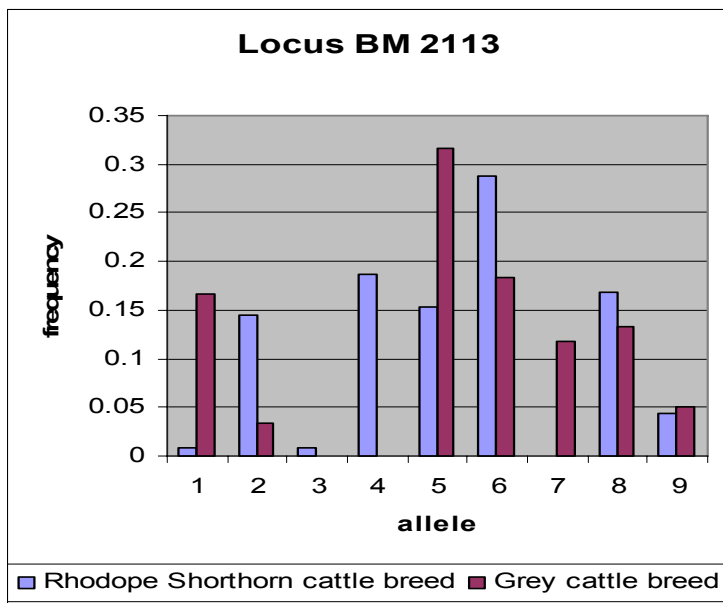
Since the cows studied represent the female parents of future generations, it should be possible to maintain an adequate level of genetic diversity in the reserve for the next years. Further analysis of other categories and of the reserve as a whole will reveal more information about the diversity and genetic structure of the population and will yield the necessary data for achieving characterization and conservation goals.

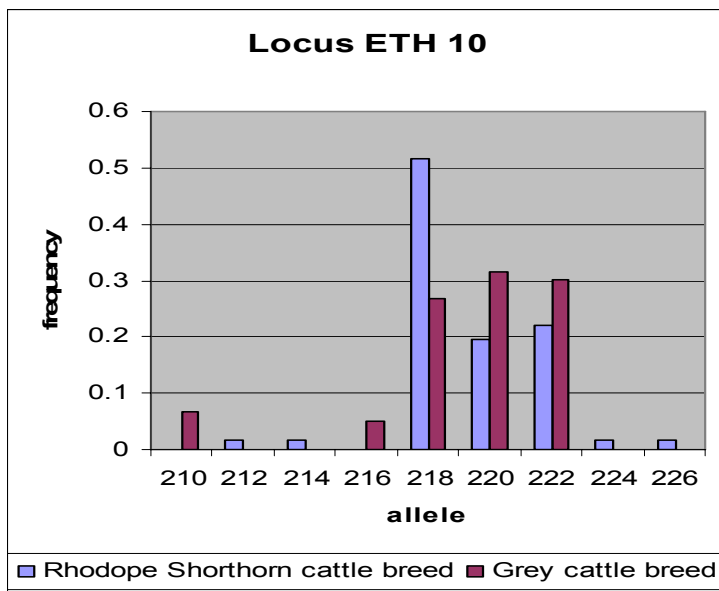
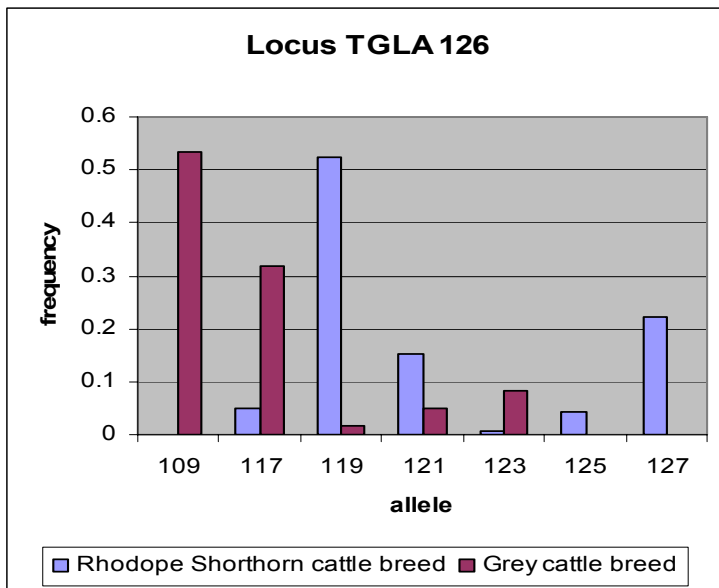
Additional information on productive, morphological, and fitness-related traits of these breeds is needed, however, as these factors should also be taken into account when ranking breeds for preservation purposes. Strategies based on neutral markers can be efficient in maximising the retention of the highest number of neutral alleles in small populations.

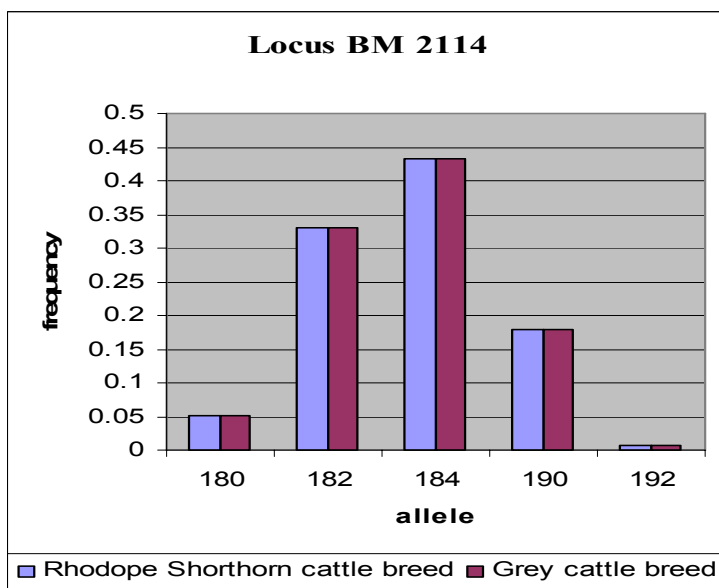
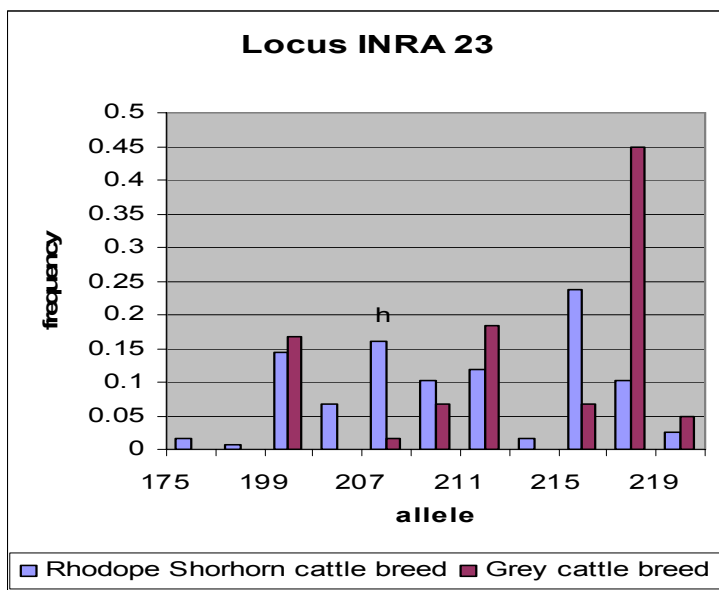
Fig 1. Allelic frequencies at 11 microsatellite loci in Bulgarian local cattle breeds

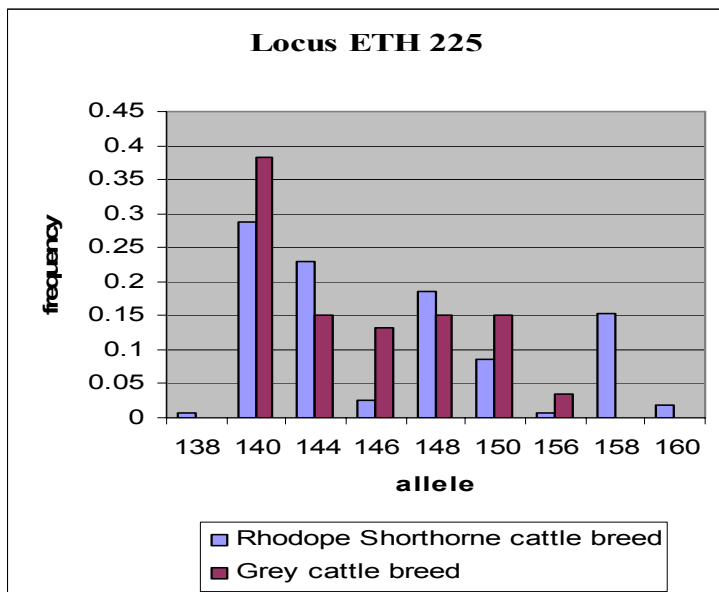












MOLEKULARNA KARAKTERIZACIJA GENETSKIH RESURSA BUGARSKIH DOMAĆIH ŽIVOTINJA II. VARIJACIJE MIKROSATELITA UNUTAR I IZMEĐU BUGARSKIH RASA GOVEDA

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Rezime

Istraživanje je obavljeno kako bi se odredila genetska struktura i različitost dve lokalne rase goveda iz Bugarske, rodopsko goveče i sivo goveče. Za ispitivanje je korišćen panel od 11 mikrosatelita za koje su određivani lokusi, frekvencije alela, heterozigotnost, HWE, genetska neravnoteža, genska diferencijacija. Obe populacije su pokazale relativno visok nivo genetskih varijacija kako je ocenjeno analizom diverzitetom alela i heterozigotnosti. Heterozigotnost je bila u opsegu od 0.5424 /SPS 115/ do

0.8983 /TGLA 227/ za rodopsku populaciju i 0.6333 /TGLA 53/ do 0.9333 /TGLA227/ za svou populaciju goveda, sa sličnim prosečnim vrednostima za dve grupe – 0.7858 i 0.7757. Ovi rezultati jasno ukazuju da su ove rase pogodne za očuvanje kao genetski resurs.

Ključne reči: rase goveda, disekvilbrijum veza, genetski diverzitet

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