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MOLECULAR CHARACTERIZATION OF BULGARIAN LIVESTOCK GENETIC RESOURCES 1. GENETIC DIVERSITY IN BULGARIAN GREY CATTLE AS REVEALED BY MICROSATELLITE MARKERS[1]

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Abstract: Within–breed genetic diversity of a herd from indigenous Bulgarian Grey cattle breed was assessed for the first time with a microsatellite set (TGLA 227, BM 2113, TGLA 53, ETH 10, SPS 115, TGLA 126, TGLA 122, INRA 23, ETH 3, ETH 225, BM 1824) of the Internationally accepted panel for the cattle biodiversity study. Polymorphism in eleven SSR loci in a sample of 35 animals was evaluated and compared to other bovine populations.

In the 11 loci studied with an automated DNA sizing technology, 83 alleles were identified. Microsatellites were highly polymorphic with a mean number (MNA) of 7.6 alleles (ranging from 4 to 12 per locus). For each locus, allele frequencies, observed heterozygosity (Ho), gene diversity (He) and polymorphic information content (PIC) were computed. The average Ho (0.78), He (0.86) and PIC (0.72) displayed a high level of genetic variation. The present study confirmed the high polymorphism of the chosen set of microsatellite markers and its usefulness in determination of the population structure of bovine genetic resources from Bulgaria.

Key words: cattle, microsatellites, polymorphism, genetic resources

Introduction

In the last decades farm animal genetic diversity is rapidly eroding because of the strong emphasis on productivity. The increased specialization of breeds has favourited the prevalence of a few breeds. Consequently, concern about the reduction of genetic diversity has been expressed, primarily in terms of loss of breeds and strains. It`s only since the 1980 $_s$ that concerted conservation efforts have really been made to preserve the genetic diversity of cattle (*FAO, 1981*). A recent survey undertaken by the Food and Agriculture Organization of the United Nations, has determined that many breeds of livestock have become extinct, and that 35% of all remaining mammalian breeds and 63% of avian breeds included in the survey are currently at risk of extinction (*FAO, 2004*). However indigenous farm animals may appear to produce less than highly specialized exotic breeds, but they are highly productive in their use of local resources and more sustainable over than long term.

In consistence of the FAO Global Strategy for the Management of Farm Animal Genetic Resources and the Measurement of Domestic Animal Diversity *Hinkovski and Stoikov (2001)* pointed that one of the fundamental problems of Bulgarian `s livestock is the conservation of available indigenous breeds and creation of a molecular database. A programme for conservation of endangered breeds has recently started in Bulgaria (*Gelev, 2004*). Bulgarian Grey cattle breed is first one of affirmative indigenouse breeds, which biodiversity is protected at national level (*Gorinov, 2004*). There are 2 recognized forms of autochton breed Grey cattle in Bulgaria – the Local Grey and its offshoot the Iskar – Grey, selected mainly for milk productivity. The Grey is a result of crossbreeding between brachyceros type and a primigenius type. This crossbreeding has taken place with varying degree of intensity and continuity in different regions of Bulgaria. The Grey cows are exceptionally hardy and strong. They are used to provide tractive power – up to age of 15 to 20 years. In the recent past they numbered around 500 000 animals (40-42% of the cattle in the country) but nowadays they are on the brink of extinction. After criteria for similarities of breeds published by EAAP (*http://www.tiho-hannover.de/einricht/zucht/eapp/sim-grp.htm)* – origin, phenotypic characteristics, genetic

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polymorphisms (blood, protein and DNA) – the Bulgarian Grey cattle belongs to the main group – *Grey cattle group,* subgroup *Grey steppe/Podolian*. After EAAP and FAO survey (*www.fao.org/DAD-IS*) the status of endangerment of the breed is critically endangered (less than 1 000 individuals). The calculated number of females (NFN) is 280.

A first step in assessing genetic conservation needs is the development of baseline information for genetic diversity. There are many different sources of data relevant to genetic variation within and between breeds. Currently polymorphism in gene products such as enzymes, blood group systems, leukocyte antigens which have traditionally been used for population studies are being replaced by DNA markers. SSR markers are codominant nuclear sequences found at high density and randomly dispersed across all chromosomes. They are now the markers of choice for diversity studies, genome mapping, estimation of genetic variation, parentage determination, and population structure differentiation (*Fries, 1993; Mac-Hugh et al., 1994; 1997; Glowatzki-Mullius et al., 1995; Ciampolini et al., 1995; Arranz et al., 1996; Cavalli-Sforza, 1997; Glasko, 2001; Malevicinte et al., 2002; Viinalass et al., 2002; Moazami-Goudarzi et al., 1997; Luikart and England, 1999; Russel et al., 2000; Bruford at al., 2003; Gibson et al., 2004*).

The aim of this study is to characterize the genetic varibility in Bulgarian Grey cattle by 11 microsatellite markers.

Materials and Methods

Blood samples were collected from jugular vein of 35 Grey cattles from the herd, reared in the region of Sredetz, Bulgaria. Genomic DNA was extracted using GFX Genomic Blood Purification Kit (*Amersham Biosciences*) according to the manufacturer`s protocol.Eleven microsatellite markers corresponding to the loci currently used for diversity studies in cattle recommended by a joint ISAG/FAO working group were analyzed (*Hoffmann et al., 2004*) (table1).

The 11 microsatellites were amplified in multiplexes using Stock Marks® Cattle Paternity PCR Typing Kit (*Perkin Elmer*). The amplification was carried out in a thermocycler GeneAmp 9700, (*PE Applied Biosystems*) using the following conditions- an initial denaturation step at 95ºC for 15 min, pursued by 31 cycles of 94ºC for 45s, 61ºC for 45s, 72ºC for 60s, 72ºC for 60 min, and 25ºC for 2 hours. After the amplification microsatellites were mixed with respective to their size fluorescent dye group - FAM, JOE, and NED. The amplified products were separated in 5% long range gel on ABI PRISM 377 automated sequencer (*Applied Biosystems*), using a GeneScan - 350 internal size standard labeled with a ROX dye. The data of fragment sizes were analyzed automatically using the GENESCAN ANALYSIS v.3.1 software.

Table 1. List of primer sequences and chromosome location of microsatellites used in the study

N	Name of the locus	Chromosome location	Marker	Primer sequences (5' -> 3') Forward Reverse	Annealing temperature	Allele range
1	ETH225 (D9S1)	9	M3	GATCACCTTGCCACTATTTCCT ACATGACAGCCAGCTGCTACT	55-65°C	131-159
2	INRA023 (D3S10)	3	M9	GAGTAGAGCTACAAGATAAACTTC TAACTACAGGGTGTTAGATGAACTC	55°C	195-225
3	ETH10 (D5S3)	5	M10	GTTCAGGACTGGCCCTGCTAACA CCTCCAGCCCACTTTCTCTTCTC	55-65°C	207-231
4	ETH3 (D19S2)	19	M14	GAACCTGCCTCTCCTGCATTGG ACTCTGCCTGTGGCCAAGTAGG	55-65°C	103-133
5	BM2113 (D2S26)	2	M15	GCTGCCTTCTACCAAATACCC CTTCCTGAGAGAAGCAACACC	55-60°C	122-156
6	BM1824 (D1S34)	1	M16	GAGCAAGGTGTTTTTCCAATC CATTCTCCAACTGCTTCCTTG	55-60°C	176-197
7	TGLA227 (D18S1)	18	M26	CGAATTCCAAATCTGTTAATTTGCT ACAGACAGAAACTCAATGAAAGCA	55-56°C	75-105
8	TGLA126 (D20S1)	20	M27	CTAATTTAGAATGAGAGAGGCTTCT TTGGTCTCTATTCTCTGAATATTCC	55-58°C	115-131
9	TGLA122 (D21S6)	21	M28	CCCTCCTCCAGGTAAATCAGC AATCACATGGCAAATAAGTACATAC	55-58°C	136-184
10	TGLA53 (D16S3)	16	M29	GCTTTCAGAAATAGTTTGCATTCA ATCTTCACATGATATTACAGCAGA	55°C	143-191
11	SPS115 (D15)	15	M30	AAAGTGACACAACAGCTTCTCCAG AACGAGTGTCCTAGTTTGGCTGTG	55-60°C	234-258

Genetic diversity within studied herd was measured as allele frequencies, mean number of alleles (MNA), observed heterozygosity (H_o), gene diversity (H_e) and polymorphic information content (PIC) using the POWERSTAT software.

Results and discussion

Eleven microsatellite markers distributed over 11 out of 30 pair of chromosomes in cattle were used for characterization of genetic variation and determination of population structure of 35 Grey cattle breed from Bulgaria.

Table 2. Allele frequency, homo- and heterozygote variants at 11 microsatellite loci in Bulgarian Grey cattle

TGLA 227 n=35		TGLA 53 n=35		TGLA 122 n=35		ETH3 n=35		BM2113 n=35	
Allele	Freq	Allele	Freq	Allele	Freq	Allele	Freq	Allele	Freq
78	0.014	154	0.086	142	0.100	103	0.071	125	0.157
80	0.043	160	0.329	144	0.343	109	0.029	127	0.029
82	0.114	162	0.043	148	0.029	115	0.071	133	0.300
84	0.200	164	0.043	152	0.028	117	0.229	135	0.200
86	0.114	168	0.171	154	0.371	119	0.143	137	0.114
90	0.072	170	0.029	156	0.029	121	0.029	139	0.129
92	0.172	172	0.029	164	0.043	123	0.100	141	0.071
94	0.071	174	0.029	172	0.028	125	0.286		
96	0.057	176	0.057	174	0.029	127	0.042		
98	0.014	180	0.086						
100	0.114	182	0.057						
104	0.015	184	0.041						
Homozygotes –n=2 Heterozygotes-n=33		Homozygotes–n=12 Heterozygotes-n=23		Homozygotes–n=10 Heterozygotes-n=25		Homozygotes –n=8 Heterozygotes-n=27		Homozygotes –n=6 Heterozygotes-n=29	

SPS115 n=35		TGLA 126 n=35		INRA 23 n=35		ETH 225 n=35		ETH10 n=35		BM1824 n=35	
Allele	Freq	Allele	Freq	Allele	Freq	Allele	Freq	Allele	Freq	Allele	Freq
248	0.571	109	0.014	199	0.186	140	0.371	210	0.071	180	0.157
250	0,014	117	0.529	207	0.115	144	0.143	216	0.057	182	0.200
252	0.029	119	0.300	209	0.129	146	0.129	218	0.286	184	0.386
254	0.157	121	0.014	211	0.057	148	0.157	220	0.329	190	0.257
256	0.086	123	0.043	215	0.471	150	0.157	222	0.257		
258	0.029	125	0.100	217	0.042	158	0.043				
260	0.114										
Homozygotes–n=11 Heterozygotes-n=24		Homozygotes–n=10 Heterozygotes-n=25		Homozygotes–n=6 Heterozygotes-=29		Homozygotes–n=5 Heterozygotes-n=30		Homozygotes –n=6 Heterozygotes-n=29		Homozygotes –n=6 Heterozygotes-n=29	

The efficiency of amplification and genotype determination was very high. PCR products were obtained in all samples at concentration of 30-50 ng/μl genomic DNA.

Within population variation (diversity) was measured by estimation of the level of heterozigosity and the number of alleles from microsatellite data.

A total of 83 alleles with an average 7.6 alleles per locus were detected across the studied 11 microsatellite loci in the population of 35 Bulgarian Grey cattle (table 3).

The large number of microsatellite alleles were determined at loci TGLA 227 and TGLA 53 (12 alleles). An equally high polymorphism was detected at loci ETH 3(9 alleles), BM2113 (7 alleles) and ETH 225 (6 alleles). The lowest number of alleles per locus was observed at locus BM2114 (4 alleles).

The alleles identified at each individual locus occurred with varying frequency in the studied population (table2). The most common alleles were allele with length 247 bp at the locus SPS 115 (0.571), allele 118 bp at locus (0.529) and allele 215 at locus INRA 23. Forty one alleles with low or very low in frequency (<0.10) were determined in this study.

The experimental results obtained showed an appearance of homo- and heterozygote variants as well as their different distribution over the 11 microsatellite loci. The highest number of heterozygotes was

observed in loci TGLA 227 (n=35) and ETH 225 (n=30) (Table 2). The lowest number of heterozygous individuals was obtained in the loci TGLA 53 and SPS 115 (n=23 and n=24 respectively).

Table 3. Polymorphic information content, heterozygosity and number of alleles in Bulgarian Grey cattle

Locus	Observed allele size range	PIC	H_o	H_e	N
TGLA 227	78-104	0.86	0.943	0.957	12
BM 2113	125-141	0.79	0.829	0.944	7
TGLA 53	154-184	0.82	0.657	0.909	12
ETH 10	210-222	0.72	0.720	0.920	5
SPS 115	248-260	0.59	0.686	0.733	7
TGLA 126	109-125	0.56	0.714	0.742	6
TGLA 122	142-174	0.69	0.714	0.818	9
INRA 23	199-217	0.68	0.829	0.850	6
ETH 3	102-127	0.80	0.771	0.925	9
ETH 225	140-158	0.74	0.857	0.928	6
BM 1824	180-190	0.64	0.829	0.720	4
Total					83
Mean		0.72	0.78	0.86	7.6

The calculated values of genetic diversity parameters – PIC, H_o, H_e and MNA showed that all microsatellite markers are highly polymorphic (table3). All the examined loci were characterized by high (>0.5) polymorphic content (PIC) and observed and expected heterosygosity (H_o and H_e >0.6). PIC values ranged from 0.56 (locus TGLA 126) to 0.86 (locus 227) with a mean 0.72. Observed heterozygosity (H_o) and expected heterozygosity (gene diversity - H_e) varied from 0.66 (locus TGLA 126) to 0.94 (locus TGLA 227) and from 0.72 to (locus BM1824) to 0.96 (locus TGLA 227). For all 11 loci the average heterosygosity was respectively - H_o = 0.78 and H_e=0.86.

Recently the increased use of microsatellite markers in a wide array of species prouves that they are good tools for population genetic studies, identification of quantitative trait loci (QTL) and for parentage control (*Mac Hugh et al. 1994; 1997; Moazami –Goudarzi et al., 1997; Loftus et al., 1999; Edwards et al., 2000; Lubieniecka et al., 2001; Janic et al., 2003; Napolitano et al., 2003*). The utilization of PCR multiplex reaction and automated DNA sizing technology for microsatellite polymorphism have reduced the costs and duration of analyses and augmented their effectiveness and easiness.

All the 11 microsatellite loci in the Bulgarian Grey cattle herd were characterized by a high level of polymorphism, as pointed out by the number of alleles at each individual locus and the polymorphic information content. The following 5 loci can be ranked such as the most polymorphic: TGLA 227 (12 alleles, PIC=0.86), TGLA 53 (12 alleles, PIC=0.82), ETH3 (9 alleles, PIC=0.77), BM 2113 (7 alleles, PIC=0.79), and ETH 225 (6 alleles, PIC=0.74).

The majority of the loci used in this study have already been analyzed in many commercial and indigenouse breeds. The level of polymorphism here observed is similar to Piedmontese cattle in relation to locus TGLA 227 (12 alleles), TGLA 122 (9 alleles), and TGLA 126 (12 alleles) (*Lubieniecka et al., 1999*). The number of alleles in locus TGLA 227 is similar also to those of Brasilian Creole cattle (*Steigleder et al., 2004*).

The same results has been obtained for locus BM 2113 in Limousine cattle by *Janik et al. (2003*)and for loci ETH 3 and SPS 115 in Polish Red, Black-and White and Red – and White in Poland (*Lubieniecka et al., 2001*). Numerous studies also showed the high polyforphism of markers TGLA 53 (*Peelman et al., 1998; Schmid et al., 1999*), ETH 225 (*MacHugh et al., 1994; Ciampolini et al., 1995; Peelman et al., 1998; Steigleder et al., 2004)* and BM2113 (*Janik et al., 2003*). Usually the microsatellites display a high polymorphism, with a mean polymorphism information content (PIC) of 0.6 (*Vaiman et al., 1994*) but the value of this parameter registered in our investigation (0.72) was higher than those cited by these authors.

Estimates of heterozygosity (H_o), gene diversity (H_e) and mean number of alleles (MNA) are considered to be good indicators of within – breed genetic variability. The obtained values for Ho are higher than classical genetic markers, citated by *Nei (1987)*. After *Forbes et al.(1995)* in the case of microsatellite loci, the average heterozygosity seems generally to be between 0.5 and 0.8.

Results of calculated heterozygosity in individual loci and average heterozygosity in this study exceed 0.6. High level of genetic variation with H values more than of 0.6 at locus TGLA 227 were reported by *Peelman et al.(1998)* for Holstein-Friesian, Belgian Red Pied, East Flemish and Belgian Blue cattle breeds.

The lowest level of heterozygosity in the present study was detected at loci SPS 115 and TGLA 126 in which the H values estimated were 0.73 and 0.74. Also low values for heterozygosity were obtained by *Lubieniecka et al.(2001)* in Black – and White and Red-and White in Poland and *Peelman et al.(1998)* in cattle in Belgium. The heterozygosity level of both microsatellites BM2113 and TGLA 126 observed in this investigation were similar to those of Limousine cattle in Poland (*Janik et al., 2003*).

More recently, *Napolitano et al. (2002)* and Moioli at al. (2004*)* when analyzing three native Italian cattle breeds – Piedmontese, Maremmana and Podolica at 17 microsatellites showed the highest mean gene diversity (MGD) for the Podolica breed (0.72) which is lower than those estimated for Bulgarian Grey cattle (0.86). Also H_e and mean number of alleles (MNA=7.6) computed here were higher than those obtained in endangered German Pustertaler Sprinzen (0.69 and 5.3), Pinzgauer (0.71and 6.0), Vosges (0.68 and 5.4) and Simmental (0.58 and 5.2) (*Edwards et al. 2000*). The MNA detected in our survey is considerably higher than Jersey (4.5), N`Dama(4.4), Ongole (5.2), Hungarian Grey (5.2), Charolais (5.6), reported by *Loftus et al. (1999)*. Low values of this parameter were determined in cattle of Pustertaler breed (5.3), Pinzgauer (6.0), Vosges (5.4) and Simmental breeds (5.2) (*Edwards et al. 2000*) and in 7 autochtonous Italian breeds (5.5) (*Del bo et al. 2001).*

The studied Bulgarian cattle population displays higher average observed and expected heterozygosity than the populations from British Isles, African Zebu, European and Azian cattle which values varied from 0.47 to 0.65 and 0.43 to 0.66 (*MacHugh et al. 1997*). *Moazami-Goudarzi et al. (1997*) analyzed 17 microsatellite markers in 10 cattle breeds. They have shown an average heterozygosity ranged from 0.53 in the Jersey breed to 0.65 in the Parthenais breed which values were also lower than these for Bulgarian Grey cattle breed. The values of MNA received by the same authors for Monbeliard (7.2) and Normandy (7.5) are in consistence with those obtained in this study.

Conclusion

The present study is the first one where microsatellite markers are used for characterization of genetic variation and determination of the population structure of indigenous cattle breed in Bulgaria by application of SSR markers. The investigation confirmed the high polymorphism of the selected set of microsatellite markers and its usefulness in determination of genetic structure of cattle populations.

From the calculated genetic diversity parameters and the comparison with the data of some native and commercial breeds it appears that the studied herd of Bulgarian Grey cattle breed exhibits a high level of genetic variability. On the base of the results received here it can be suggested the importance of the preservation of this breed and its exploitation as a source of genetic diversity in future breeding programmes.

Further studies involving more animals from this cattle breed will be required to broaden the knowledge on their genetic structure, based on microsatellite loci. The searching for candidate genes of economically important quantitative traits will provide new arguments for their protection.

MOLEKULARNA KARACTERIZACIJA BUGARSKIH GENETSKIH RESURSA. 1. GENETSKI DIVERZITET KOD BUGARSKOG SIVOG GOVEČETA PREKO MIKROSATELITSKIH MARKERA

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Rezime

Polimorfizam kod jedanaest SSR lokusa u uzorku od 35 životinja je ocenjivan korišćenjem automatske DNK tehnologije i upoređen sa drugim populacijama goveda. Genetski diverzitet unutar rase u okviru zapata autohtone rase bugarsko sivo goveče je ocenjivan po prvi put korišćenjem mikrosatelitskog seta.

Ispitivanje je potvrdilo visoki polimorfizam odabranog seta mikrosaltelitskih markera i njegovu korisnost u određivanju genetske strukture populacije goveda. Izračunati parametri genetskog diverziteta - Ho (0.78), He (0.86) i PIC (0.72) su pokazali visok nivo genetske varijacije u ispitivanom zapatu bugarskog sivog govečeta. Na bazi dobijenih rezultata može se sugerisati značaj očuvanja ove rase I njene dalje eksploatacije kao izvora genetskog diverziteta.

Ključne reči: goveda, mikrosateliti, polimorfizam, genetski resursi

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