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Research article

INSIGHT IN LEPTIN GENE POLYMORPHISM AND IMPACT ON MILK TRAITS IN AUTOCHTONOUS BUSHA CATTLE

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Leptin, a biomolecule secreted by adipose tissue, enchances productivity in cattle, especially affecting milk traits. The aim of this study was to detect leptin gene polymorphism on exon 3 (A59V locus) and intron 2 (SAU3AI locus) in the endangered population of autochtonous Busha cattle and associations with milk traits. The study included 46 cows: 36 Busha and 10 half-bred. Milk analyses comprised determination of somatic cell counts, fat, protein, lactose, total solids and solids-not-fat (SNF) concentrations and freezing point depression (FPD). Polymorphisms were determined by PCR-RFLP technique. A single A59V genotype (CC) was affirmed, and two SAU3AI genotypes, AA and AB, with frequencies of 78.26% and 21.74%, respectively. Comparing the obtained results for chemical characteristics of milk between cows with AA and AB, no significant differences were found, except for SNF content and FPD values. Cows with AA genotype had significantly lower ($p=0.021$) average SNF content (8.74%) in milk compared to the average SNF content (9.28%) in those with genotype AB, while cows with genotype AA (-0.54 °C) had significantly higher ($p=0.004$) average FPD values than those with AB genotype (-0.58 °C). The absence of BB genotype and significant differences in the investigated functional traits between two SAU3AI genotypes and the absence of A59V polymorphism (presence of only CC genotype) show that the Busha cattle breed, although being an autochtonous low-producing native breed used for meat and milk production, harbours polymorphism on gentic markers characteristic of high production dairy cows.

Key words: Busha cattle, leptin polymorphsm, milk traits, genetic markers

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INTRODUCTION

During past few decades, molecular markers proved to be an important instrument for selection and preservation of animal populations [1-4]. Association between DNA polymorphism and milk production traits have been studied for a number of genes, and among them, leptin (LEP) gene proved to be a powerful biomolecule that can increase the productivity of farm animals [5]. Leptin, a hormone secreted primarily by white adipose tissue, binds to receptors localized on neuropeptide Y-neurons [6,7]. Leptin influences immune and reproductive functions, lactogenesis, resistance to mastitis, feed intake, adipose tissue deposition and metabolism [5,7,8,]. Due to its roles, an insight in LEP gene polymorphism could help to predict the results of natural and artificial selection, as well as planning marker-assisted selection [9].

Located on chromosome 4, bovine LEP gene consists of three exons and two introns. Most investigated polymorphisms of LEP gene were single nucleotide polymorphisms (SNP) on A59V locus on exon 3 [6,8,10,11,13,14] and on SAU3AI locus on intron 2 [6,7,15,16,17]. The polymorphisms of LEP gene on these loci in most investigations involved dairy cattle breeds and are found to be highly associated with differenet milk traits [5]. However, according to available literature data, this methodology has not been applied in native cattle breeds.

Busha cattle (also known as Buša or Buscha) are autochthonous to the Balkan area, and are small, robust and long-living cattle, with relatively low production traits [18]. The aim of this investigation was to detect LEP gene polymorphism in the endangered population of autochtonous Busha cattle breed and its associations with milk traits.

MATERIAL AND METHODS

Sample collection and DNA extraction

The study included 46 cows, out of which 36 were of Busha breed and 10 were half-bred. Half-bred animals were born to Busha mothers and fathers of the Podolian cattle breed. The animals were 3-8 years old, housed in Tuzla Canton (Figure 1), Bosnia and Herzegovina.

Blood was sampled from v. coccigea media. DNA was extracted using KAPA Express Extract Kit (Kapa Biosystems, USA) following the manufacturer's protocol.

Table 1. Distribution of SAU3AI locus polymorphisms in Busha cattle and their half-breed

Breed	Genotype			
	AA		AB	
	No.	%	No.	%
Busha	28	77.78	8	22.22
Half-breed	8	80.00	2	20.00
Total	36	78.26	10	21.74

Figure 1. Location of Busha cattle in the research

PCR amplification and RFLP

To analyse A59V polymorphism (C/T transition which results in substitution in the protein), exon 3 fragment (331 bp) was amplified by PCR using primers F 5'-GGGAAGGGCAGAAAGATAG-3', R 5'-TGGCAGACTGT TGAGGATC-3' (Haegeman et al., 2000); while SAU3AI polymorphism (C/T transition) was searched by amplifying intron 2 fragment (422 bp) was using primers F 5'-TGGAGTGGCTTGTTATTTTCTTCT-3' R 5'-GTCCCCGCTTCTGGCTACCTAACT-3' (Liefers et al., 2002). PCR reactions were performed using the MultiGene Gradient (Labnet International Inc., USA), following the manufacturer's (Kapa Biosystems, USA) recommendations.

The analyses of A59V and SAU3AI polymorphisms were done using RFLP method [20]. The PCR products were digested according to the manufacturer's recommendations (New England Biolabs, USA) using Hph I restriction enzyme for 331 bp product and Sau3 AI restriction enzyme for 422 bp product. The digestion products were separated by horizontal electrophoresis on 2% agarose gel, stained with ethidium bromide prior to visualization under UV light.

Milk analyses

Milk sampling began from 8 weeks of lactation, monthly, throughout the following 6 months. During milk sampling, efforts were made to avoid contamination and samples were immediately carried to the laboratory. Total somatic cell count (SCC) was determined using Fossomatic™ FC analyzer (Foss, Denmark), while chemical composition of milk (fat, protein, lactose, total solids and percent of solids-not-fat

(SNF) was determined using MilkoScan (Foss, Denmark). The evaluation of freezing point depression (FPD) was performed using Cryoscope (Advanced Instruments, USA).

Statistical analyses

The data recorded in this study were presented through mean, median and coefficient of variation (CV). Shapiro-Wilk's W test was used in order to assess the normality of the data distribution and was followed by Levene's test for determination of the homogeneity of variances. Variables with normally distributed data and homogeneous variances were compared between groups trough the t-test; otherwise, the Mann-Whitney U-test was used. Differences were considered significant for p values <0.05. The data obtained in this research were processed with statistical software Statistica 7 (StatSoft Inc., Tulsa, USA).

Informed consent has been obtained for client-owned animals included in this study.

RESULTS

Genotyping

Two SAU3AI genotypes were discovered; the first genotype, AA, recognizable by two digestion products (bands of 390 bp and 32 bp, the later not visible on gel) and the second one, AB, had two fragments of 390 bp and 303 bp, both visible on gel (Figure 2). The AA genotype was present in 36 cows (78.26%) and genotype AB in 10 cows (21.74%), while BB genotype was not detected. The frequency of genotype AA was significantly higher than the one of genotype AB ($\chi 2=14.696$; $p<0.001$), although this ratio did not differ significantly from the expected frequencies, no deviation from Hardy-Weinberg equilibrium was detected at this locus.

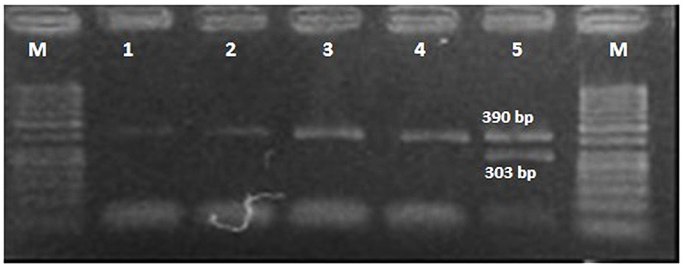

Figure 2. PCR-RFLP analysis of SAU3AI locus of the leptin gene in Busha cows (2% agarose gel, restriction enzyme – *Sau3AI*): genotype AA – samples 1-4; genotype AB – sample 5; M 50 bp ladder

Analysis of A59V polymorphisms revealed the existence of a single genotype – CC, with a corresponding product size of 331 bp (Figure 3).

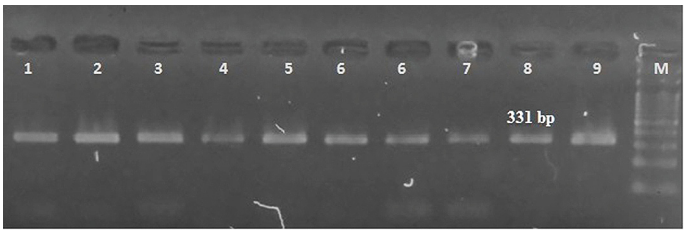

Figure 3. PCR-RFLP analysis of of A59V locus of the leptin gene in Busha cows (2% agarose gel, restriction enzyme – Hph I): genotype CC – samples 1-9; M 50 bp ladder

Milk yield and LEP gene polymorphism

Based on the determined SAU3AI polymorphism, the animals were divided in two groups (AA and AB). The values of milk yield in the two groups and overall (Table 2) displayed non-homogeneity (cv>30 %). According to Leven's test (F=0.164; p=0.688), variances of yield values for AA and AB group were homogeneous. Despite normal distribution of milk yield values, the heterogeneity of the data conditioned analysis of averge values with nonparametric tests. In spite of a slightly higher average milk yield in group AB comparing to AA (6.00 vs 5.94), Mann-Whitney U test (z=-0.202; p=0.840) showed that the difference in average milk yield between groups was not significant.

Table 2. Difference in milk yield (kg/day) between two *LEP* gene genotypes on SAU3AI locus

Genotype	n	$\bar{x}$	M_e	c_v (%)	MWU test
AA	36	5.94	5.5	**36.84**	
AB	10	6.00	6.0	**34.25**	
Total	46	5.96	6.0	**35.91**	p=0.840

$\bar{x}$ – Mean, M_e – median; c_v – coefficient of variation, MWU – Mann-Whitney U test; p – significant for P values <0.05.

Chemical characteristics of milk (fat, protein, lactose, total solids, SNF and FPD) and LEP gene polymorphism

The heterogenous (c_v>30 %) values of milk fat in both groups (AA and AB) and their non-normal data distribution (W_{AA}=0.874; p=0.001 i W_{AB}=0.974; p=0.924) dictated the use of median values and Mann-Whitney U-test for further analyses. Cows with AA genotype had average median values (4.19%) lower than cows with AB genotype (5.35%), but this difference was not significant (z=-1.465; p=0.143). Based on the obtained results (Table 3), the average milk protein concentrations did not differ significantly (t = -1,874, p = 0.068) between cows with the two genotypes.

Since the variance of the two groups was homogeneous for lactose content (Table 4), t-test (t = -1.105; p = 0.275) showed that the milk of these groups of cows did not differ significantly in lactose concentration.

Table 3. Difference in milk fat (%) and protein content (%) between two *LEP* gene genotypes on SAU3AI locus

		Milk fat				Protein			
Genotype	**n**	$\bar{x}$	M_e	c_v (%)	**MWU test**	$\bar{x}$	M_e	c_v (%)	**t- test**
AA	36	4.65	4.19	53.87		3.54	3.48	15.59	
AB	10	5.38	5.35	33.74		3.91	3.75	14.11	
Total	46	4.81	4.28	49.35	p=0.143	3.62	3.52	15.66	p=0.068

$\bar{x}$ – Mean, M_e – median; c_v – coefficient of variation, MWU – Mann-Whitney U test; p – significant for P values <0.05, % - g/100 g of milk

Table 4. Difference in lactose content (%) and SNF (%) between two *LEP* gene genotypes on SAU3AI locus

		Lactose				SNF			
Genotype	**n**	$\bar{x}$	M_e	c_v (%)	**t - test**	$\bar{x}$	M_e	c_v (%)	**t - test**
AA	36	4.45	4.48	8.14		8.74	8.80	7.37	
AB	10	4.59	4.55	5.24		9.28	9.27	5.99	
Total	46	4.48	4.52	7.63	p=0.143	8.86	8.83	7.45	**p=0.021**

$\bar{x}$ – mean, M_e – median; c_v – coefficient of variation, p – significant for P values <0.05, % - g/100 g of milk

SNF varied from 6.74% to 10.02% in cows with AA genotype, and in cows with AB genotype between 8.64% and 10.17%. Based on the results of the t-test (t = -1.397; p = 0.021), cows with AA genotype had significantly lower average SNF content (8.74%) in milk compared to the average SNF content (9.28%) in cows with AB genotype (Table 4).

The data on total solid (TS) showed homogeneous variances (Table 5) of data in both groups (AA and AB). However, Shapiro-Wilk's test rejected the hypothesis that the data were normally distributed in AA cows. Thus, the average TS concentrations in cows with AA and with AB genotype were compared by means of Mann-Whitney U-test. Results showed that average values for TS in milk did not differ significantly between the cow groups.

Table 5. Difference in total solids (%) and freezing point depression (°C) between two *LEP* gene genotypes on SAU3AI locus

		TS				FPD			
Genotype	**n**	$\bar{x}$	M_e	c_v (%)	**MWU test**	$\bar{x}$	M_e	c_v (%)	**t - test**
AA	36	13.47	12.98	17.61		-0.54	-0.54	7.00	
AB	10	14.59	14.18	14.61		-0.58	-0.57	8.69	
Total	46	13.71	13.26	17.11	p=0.129	-0.55	-0.54	8.09	**p=0.004**

$\bar{x}$ – mean, M_e – median; c_v – coefficient of variation, MWU – Mann-Whitney U test; p – significant for P values <0.05, % - g/100 g of milk.

In cows with AA genotype, minimum and maximum values for FPD of milk were -0.63°C and -0.46 °C, respectively, while in cows with AB genotype the corresponding FPD values for milk were -0.68 °C and -0.54 °C. The use of t-test was justified based on average FPD values in milk (Table 5), cows with genotype AA had significantly higher ($t = -3.082$; $p = 0.004$) average FPD values than those with AB genotype.

SCC and LEP gene polymorphism

In all examined cows, the SCC counts were below 2 x 10^6 cell/mL (data not shown here) of milk, indicating and confirming that the individuals did not suffer from any form of mastitis. The SCC values did not differ significantly between the two SAU3AI genotypes ($p > 0.05$).

DISCUSSION

In this study, polymorphism of A59V locus (transition C/T) and SAU3AI locus was investigated in the autochtonous Busha cattle breed and the half-breds.

Using the PCR-RFLP method, single A59V genotype (CC) was detected. The remaining two genotypes, CT (331, 311 and 20 bp) and TT (311 and 20 bp), were not found. The obtained data on A59V polymorphism in Busha breed are similar to the polymorphism found in dairy cattle with high milk yield. Clempson et al. (2011) detected all three A59V genotypes in 509 Holstein Friesian heifers from 19 UK dairy farms, in which CC genotype was most frequent (0.66). Investigations conducted on Jersey cows [15] also pointed to the prevalence of CC genotype (CC 52 %, CT 40 % i TT 8 %), as in other investigations on Holstein breed [6,16]. However, in a study on Nellore cattle [17], CC genotype was not found and the highest frequency was detected for the T allele (0.85). In addition, Öztabak et al. (2010) studied A59V polymorphism in 120 cattle of 3 beef breeds from Turkey (South Anatolian Red, East Anatolian Red and Turkish Grey) and found a higher frequency for the T allele. The results of our investigation of A59V polymorphism in the Busha cattle population showed that this autochtonous breed has more similarity to dairy cattle breeds with higher frequency of C allele, while studies of other native beef and dual-purpose breeds showed higher frequency for the T allele.

Analyses of Busha cattle investigated in this research, revealed two SAU3AI genotypes, AA and AB, in the 78.26% to 21.74% ratio, while the BB genotype was not detected. Javanmard et al. (2010) had similar results in 66 Iranian Holstein cattle, where two genotypes were found and their frequencies were 0.90 (AA) and 0.10 (AB), while BB was not observed. In more extensive research on 613 Holstein cows, Liefers et al. (2002) also detected high prevalence of AA genotype (0.813), and only one cow with BB genotype. By contrast, a higher proportion of heterozygous cows for SAU3AI locus was observed in Slovak Pinzgau cattle [13], where AA (0.47) and AB (0.43) frequencies were almost equal. In addition, Kulig et al. (2010) also found more

heterozygous animals in the population of Jersey cows. The observed results of SAU3AI polymorphism in Busha cattle population with prevalence of AA genotype and absence of BB genotype were in compliance with most of the previous results.

In accordance with the obtained results, we examined the association only between SAU3AI polymorphism and milk yield and its chemical characteristics since A59V polymorphism was not detected in Busha cattle. However, the A59V polymorphism (CC genotype) detected in Busha cattle showed a positive influence on milk yield, protein and fat composition in other studies. Clempson et al. (2011) proved that cows with CC – A59V polymorphism had significant associations with both a daily and a 305-day milk yield, since the TT substitution of A59V significantly decreased milk output by 3.3 kg per day and 1.365 kg after 305 days of lactation. In addition, Kulig et al. (2009) found significant differences in milk yield, protein and fat yield in Jersey cows with different A59V leptin genotypes: TT cows produced significantly less milk with significantly lower protein and fat content than CC and CT cows did.

The current research on the influence of SAU3AI polymorphism on functional traits (milk yield, fat, protein, and lactose, TS, SNF, FPD and SCC) was conducted on Busha cattle. The majority of cows had AA genotype (78.26%). Between cows with AA and AB genotype, significant defferences were found only for SNF and FPD. Similar results were found for 117 Polish Black and White bulls [12], which did not prove any associations between the Kpn2I and SAU3AI polymorphism, and the production traits. By contrast, Liefers et al. (2002) tested 613 Holstein cows and found significant differences between genotypes AA and AB in milk production, protein and lactose concentrations, and pointed out that SAU3AI can be a possible marker for milk and protein yield.

The polymorphic sites (SAU3AI and A59V) on LEP gene in investigated Busha cattle exhibited reduced levels of observed heterozygosity, revealing a loss of genetic variability. This could be due to artificial selection that occurred in the environment of the animals or/and due to possible losses and fixation of alleles, which might occur as a result of the selection method used. The absecnce of BB genotype, insignificant differneces in functional traits between two SAU3AI polymorphisms and the presence of only one A59V genotype (CC), showed that the Busha, although an autochtonous, low-producing native breed used for meat and milk production, have polymorphism on genetic markers characteristic of high-producing dairy cows. These results may be very important for the conservation programmes aimed at the preservation of this autochtonous breed.

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Authors' contributions

MM, PN and SLj carried out the molecular genetic studies, participated in the sequence alignment and drafted the manuscipt. PN and LM participated in experimental design, sampling and helped to draft the manuscript. AH and VD participated in the design of the study and performed the statistical analysis. SZ conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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UVID U POLIMORFIZAM LEPTIN GENA I NJEGOV UTICAJ NA KARAKTERISTIKE MLEKA KOD AUTOHTONE RASE GOVEDA BUŠA

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Leptin je biomolekul koji sekretuje masno tkivo i utiče na povećanje proizvodnih karakteristika kod goveda. Cilj ovog istraživanja je detekcija polimorfizama LEP gena na egzonu 3 (lokus A59V) i intronu 2 (lokus SAU3AI) kod ugrožene populacije autohtone buša rase goveda i njihova povezanost sa karakteristikama mleka. Obuhvaćeno

je 46 krava od kojih je 36 krava rase buša a preostalih 10 su melezi buše i podolskog govečeta. Analize mleka obuhvatale su sledeće parametre: broj somatskih ćelija; procenat masti, proteina, laktoze, suve materije, suve materije bez masti i tačka mržnjenja. Za utvrđivanje polimorfizama korišćena je PCR-RFLP tehnika. Utvrđen je samo jedan A59V genotip (CC) i dva SAU3AI genotipa, AA i AB, sa učestalošću 78,26% i 21,74%, redom. Upoređujući dobijene rezultate za hemijske karakteristike mleka između krava sa AA i AB genotipom, nađena je statistički značajna razlika za dva parametra: procenat suve materije bez masti i tačka mržnjenja. Krave sa AA genotipom su imale statistički niže ($p=0,021$) prosečne vrednosti procenta suve materije bez masti (8,74%) u mleku kada se uporede sa kravama AB genotipa (9,28%), dok su krave sa AA genotipom (-0,54 ^{o}C) imale statistički veće vrednosti ($p=0,004$) tačke mržnjenja nego krave sa AB genotipom (-0,58 ^{o}C). Odsustvo BB genotipa i značajnih razlika u ispitivanim karakteristikama mleka poreklom od krava AA i AB, kao i odsustvo A59V polimorfizma (prisustvo samo CC genotipa), pokazuju da buša rasa goveda, čak i ako je autohtona niskoproduktivna rasa koja se koristi za proizvodnju mesa i mleka, poseduje polimorfizme na gentičkim markerima karakteristične za visokoproduktivne mlečne rase krava.