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Original article

SINGLE NUCLEOTIDE POLYMORPHISM OF THE B-LACTOGLOBULIN GENE IN SHEEP BREEDS REARED IN BULGARIA

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Summary

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In the present study, single nucleotide polymorphism in exon II of the β-lactoglobulin gene was investigated in four Bulgarian sheep breeds: Bulgarian Dairy Synthetic Population sheep (BDSP), Copper-red Shumen sheep (CRSH), Stara Zagora sheep (STZG) and Pleven Blackhead sheep (PLBH). Two genetic variants of *β-LG* gene (A and B) have been identified through PCR-RFLP assay. A 103 bp fragment of the polymorphic β-LG locus were amplified and digested with endonuclease enzyme RsaI. The obtained restriction fragments revealed three genotypes: AA, AB and BB, observed in 31%, 65.5% and 3.5% of the BDSP population and in 48%, 28% and 24% of CRSH sheep population, respectively, with departure from the Hardy-Weinberg equilibrium ($P<0.05$) in these groups. The allele frequencies demonstrated a prevalence of the A allele (0.638 and 0.620) over the B allele (0.362 and 0.380) in both populations. On the contrary, the distribution of allele frequencies in STZG and PLBH was 0.240 and 0.100 for allele A, respectively and 0.760 and 0.900 for allele B. Therefore, the homozygous BB genotype in these sheep populations was more frequently encountered (0.520 and 0.800) than the heterozygous AB genotype (0.480 and 0.200), with HWE correspondence ($P>0.1$). The homozygous genotype AA was absent in STZG and PLBH sheep populations. The greatest Nei's genetic distance calculated by UPGMA method was found between the populations BDSPand PLBH (0.5334), while the closest relationship (0.0006) was established between CRSH and BDSP. The results obtained from the present investigation confirmed the presence of the SNP polymorphism in exon II of the β-lactoglobulin gene. Therefore, the genetic variability established in this polymorphic locus could be applied in further association studies with milk production traits in sheep.

Key words: Bulgarian sheep breeds, β-lactoglobulin gene, PCR-RFLP analysis, single nucleotide polymorphism

INTRODUCTION

Milk protein polymorphisms have been intensively studied because of their effect on the yield and processing properties of milk and its products (Kemenes *et al.*, 1999). Beta-lactoglobulin (β-LG) is one of the most important proteins in

mammals'milk and plays a crucial role in milk quality (El-Shazly *et al.,* 2012). In sheep *β-LG* has been isolated and characterised by Ali & Clark (1988) based on genomes clones. This major whey protein consists of 162 amino acids and forms stable dimers in milk. Apart from its ability to bind and transport small hydrophobic molecules in milk, e.g. retinol and small fatty acids, its biological function is still unclear (Anton *et al.*, 1999a; Prinzenberg & Erhardt, 1999).

Taking into consideration that the *β-LG* gene in sheep breeds is a candidate gene for milk production, understanding the genetic diversity and variation in this locus is of particular relevance. One of the most extensively studied milk protein polymorphisms is the substitution of the amino acid Tyr with His in the β-lactoglobulin polypeptide resulting from a single base pair substitution in the *β-LG* gene, located on the ovine chromosome 3.

The genetic polymorphism of ovine *β-LG* is determined by 3 alleles: A, B and C. Alleles A and B are present in almost all the investigated breeds, whereas allele C is rather rare and confined to some specific breeds (Anton *et al.*, 1999b; Rozbicka-Wieczorek *et al.*, 2015). The polymorphisms are caused by a mutation changing the nucleotide sequence of the gene, which in turn affects the amino acid sequence of the protein. The genetic variant A differs from variant B in the amino acid sequence at position 20 (Tyr→His) (Moatsou *et al.*, 2005, Dario *et al.*, 2008, Selvaggi *et al.*, 2015). The *β-LG* C variant is a subtype of ovine *β-LG* A with a single exchange of Arg-Gln at position 148 (Erhardt *et al.*, 1989). The effect of *β-LG* polymorphism on milk quality was mostly described for dairy sheep from Mediterranean area, however not widely recognised in other sheep breeds focused on slaughter lambs production (Amigo *et al.*, 2000; Barillet *et al.*, 2005; Rozbicka-Wieczorek *et al.*, 2015). Genetic polymorphism of ovine *β-LG* and its influence on the milk yield, milk fatty acid composition technological properties of milk protein content, cheese-making ability and other economic traits were reviewed in different sheep breeds by Bolla *et al.* (1989); Recio *et al.* (1997); Pietrolà *et al.* (2000); Dario *et al.* (2008); Mele *et al.* (2007); El-Shazly *et al.* (2012).

The number of studies on genetic variation in whey proteins influencing sheep milk, particularly local sheep breeds is still limited in Bulgaria. Hence, the objective of this study was to assess SNP polymorphism in β-lactoglobulin gene using PCR-RFLP assay and to determine the genetic relationship between four sheep breeds reared in Bulgaria.

MATERIALS AND METHODS

Experimental animals and sample collection

The present investigation was carried out with a total of 104 animals (unrelated ewes) from one synthetic and three local sheep populations, reared in private farms in Bulgaria: Bulgarian dairy synthetic population sheep (BDSP, n=29), Copper-red Shumen (CRSH, n=25), Stara Zagora (STZG, n=25) and Pleven Blackhead (PLBH, n=25) sheep. Blood samples for analyses were obtained from *v. jugularis* of ewes in sterile EDTA vacuum tubes (3 mL) (Biosigma, Italy).

DNA extraction and PCR amplification

Genomic DNA was extracted from the whole sheep blood using Illustra Blood Genomic Prep DNA Purification Kit following the manufacturer protocol (GE

Healthcare, UK). The quantity of the obtained DNA (about 30–90 ng) was determined using NanoVue Plus Spectrophotometer (GE Healthcare) and samples were stored at –20 ºC until the assay. PCR amplifications were carried out in a total volume of 20 µL, containing 80 ng DNA template, 10 pM of each primer and 2× Red Taq DNA Polymerase Master mix (VWR, Belgium). Reactions were performed with primers designed by Feligini *et al.* (1998) in GeneAmp thermocycler (Applied Biosystems, USA) under the following conditions: an initial denaturation at 94 ºC for 5 min, followed by 30 cycles at 94 ºC for 30 s, primer annealing at 59 ºC for 45 s, extension at 72 ºC for 1 min and a final extension at 72 ºC for 10 min.

Genotyping

The genotypes of the analysed ewes with respect to the *β-LG* gene were established through RLFP analysis. The digestion reactions were carried out in 25 µL final volume, containing 10 µL PCR product, 10U/µL RsaI enzyme (Bioneer), 1× Buffer Tango (Fermentas), incubated at 37 ºC/7 h, followed by a process of enzyme deactivation at 80 ºC/20 min. The obtained PCR products and restriction fragments were separated on 2% agarose gel using GeneRuler™ Ladder, 50 bp (Fermentas) and visualised with Electrophoresis Gel Imaging Analysis System (Bio-Imaging Systems, Israel).

Statistical analysis

POPGENE software, version 1.31 (Yeh & Yong, 1999; Labate, 2000) was used to estimate the following parameters for the studied sheep populations: allele and genotype frequencies of *β-LG* gene and Nei-expected heterozygosity (H_e). The genetic distances (D_A) between sheep breeds were estimated according to the method of Nei (1978) using the UPGMA algorithm for phylogenetic tree construction. The same software was used to check whether the examined populations were in HWE, by the method of Guo & Thompson (1992), using the Markov Chain Monte Carlo algorithm.

RESULTS

As expected, a 103 bp fragment of the target polymorphic region (exon II) of the *β-LG* gene in sheep was successfully amplified in studied populations based on PCR technology. The T-C transition produced a RsaI restriction site which allows the genotyping of animals using RFLP method. After digestion, three different genotypes were identified in the studied sheep populations – AA, AB and BB regarding the studied exon II in *β-LG* locus (Fig. 1).

About 20% of the animals were homozygous AA and their RFLP profile on agarose gel showed three fragments with size 66, 37 and 17 bp. This genotype was detected in 12 CRSH ewes, in 9 BDSP ewes, and was absent in STZG and PLBH populations. The heterozygous AB genotype (about 40% and four fragments with size 103, 66, 37 and 17 bp) was found out in 19 BDSP, 12 STZG, 7 CRSH and 5 PLBH sheep. The homozygous animals with BB genotype were presented with a frequency of about 40% and they exhibited two fragments – 103 and 17 bp. This genotype was detected on the electrophoregram in 20 ewes of PLBH sheep population, in 13 STZG ewes, 6 CRSH ewes and in only one animal of BDSP sheep population.

Both alleles – A and B – corresponding to the *β-LG* gene in the sheep population under investigation were

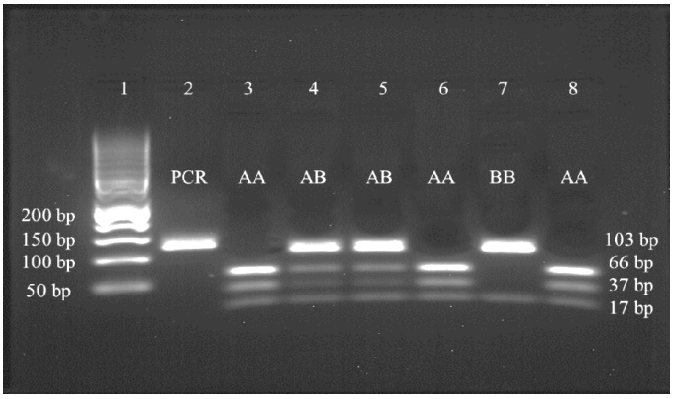

Fig. 1. Restriction fragments of amplified PCR products of the exon II in *β-LG* gene with Rsa I enzyme in sheep populations on 2% agarose gel electrophoresis. Lane 1: M – DNA ladder, 50 bp; lane 2 – PCR product, lanes 3–8: AA and BB – homozygous genotype, AB – heterozygous genotype.

Table 1. Distribution of the allele and genotype frequencies, expected heterozygosity (*He*) and chi-square test of HWE (χ^2) for the exon II of *β-LG* gene in the examined sheep populations

Breed	Population size	Allele frequencies		Genotype frequencies			He***	χ^2 (P)
		A	*B*	*AA*	*AB*	*BB*		
BDSP	29	0.638	0.362	0.310 (n=9)	0.655 (n=19)	0.035 (n=1)	0.462	4.686 (0.031)
CRSH	25	0.620	0.380	0.480 (n=12)	0.280 (n=7)	0.240 (n=6)	0.471	4.566 (0.033)
STZG	25	0.240	0.760	0.000 (–)	0.480 (n=12)	0.520 (n=13)	0.365	2.253 (0.134)
PLBH	25	0.100	0.900	0.00 (–)	0.200 (n=5)	0.800 (n=20)	0.180	0.242 (0.623)
Mean		0.399	0.601	0.198	0.404	0.398	0.367	

Legend: Bulgarian dairy synthetic population sheep (BDSP), Copper-red Shumen (CRSH), Stara Zagora (STZG); Pleven Blackhead (PLBH); He*** – expected heterozygosity calculated as per Nei (1978).

established based on PCR-RFLP analysis (Table 1). The mean frequency value of allele A was 0.399 and varied from 0.100 in PLBH to 0.638 in BDSP. For the allele B, the mean value of frequency was 0.601 and ranged from 0.362 in BDSP to 0.900 in PLBH.

All three genotypes were identified in BDSP sheep population with highest frequency (0.655) of the heterozygous genotype compared to homozygous AA and BB, where the values were 0.310 and 0.035, respectively. The opposite distribution of genotype frequencies was established in CRSH sheep population and thus the homozygous AA was found with a highest frequency of 0.480, whereas that of the heterozygous was 0.280. In contrast, the homozygous genotype AA was absent in STZG and PLBH populations.

The chi-square test for Hardy-Weinberg equilibrium (Table 1) at df=1 pointed a value of χ^2 with levels of probability P=0.134 and P=0.623, confirming the validity of the HWE for both sheep populations – STZG and PLBH. BDSP and CRSH sheep populations demonstrated deviation from HWE (P=0.031 and 0.033, respectively).

The genetic distances among the examined sheep breeds are shown in Table 2. The greatest (0.5334) Nei's genetic distance (D_A) was found between Bulgarian dairy synthetic population sheep and Pleven Blackhead sheep, while the closest relationship (0.0006) was established between Copper-red Shumen sheep and Bulgarian dairy synthetic population sheep. The genetic distances calculated by UPGMA method produced a phylogenetic tree which separates the investigated sheep breeds into two main clusters: one including Bulgarian dairy synthetic population sheep and Copper-red Shumen, and the other one consisting of the two remaining breeds – Stara Zagora and Pleven Blackhead sheep (Fig. 2).

DISCUSSION

The genetic polymorphisms of the milk proteins have been considered a potential tool for the selection of sheep breeds. In the present investigation, single nucleotide polymorphism in exon II of the β-lactoglobulin gene was investigated on a total of 104 ewes from four Bulgarian sheep breeds.

Among the four examined breeds in this study, BDSP and CRSH showed the highest frequency of allele A (0.638 and 0.620) versus allele B (0.362 and 0.380) which were similar to the allele frequency (0.630 for allele A and 0.370 for allele B) reported by Dario *et al.* (2008) in a total of 192 Leccese dairy ewes. Recently, in a comparatively research based on *β-LG* polymorphism, Kusza *et al.* (2015) have

Table 2. Nei's genetic distances (D_A) between the examined sheep breeds corresponding to the *β-LG* gene

Sheep breeds	SPBM	CRSH	STZG	PLBH
Bulgarian dairy synthetic population	0.000			
Copper-red Shumen	0.0006	0.000		
Stara Zagora	0.3112	0.2810	0.000	
Pleven Blackhead	0.5334	0.4885	0.0192	0.000

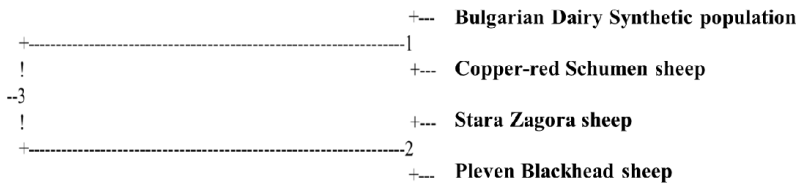

Fig. 2. UPGMA dendrogram generated from Nei's genetic distances of the examined sheep breeds based on *β-LG* genotypes.

established genetic differences in a total of 904 individuals of ten different Balkan and Central European indigenous sheep breeds. The β-lactoglobulin A has been the most common in the Cokanski Tsigai (54%), while the B allele has been the most frequent in the Rusty and the Zomborski Tsigai (59% and 60%). In the four flocks representing Bulgarian Tsigai breed, the authors reported frequencies similar to those from the present study – 0.680 for allele A and 0.320 for allele B. On the contrary, Arora *et al.* (2010) established a mean value of 0.370 for allele A and 0.630 for allele B in 15 native Indian sheep breeds. The distribution of allele frequency in other sheep breeds – STZG and PLBH demonstrated a higher preponderance of allele B (0.760 and 0.900) compared to allele A (0.240 and 0.100). Similar results were established by Georgescu *et al.* (2006) for 57.5% of animals from Racka sheep breed, as well as by Elmaci *et al.* (2006) in three sheep breeds (Kivircik, Gökceada and Sakiz), with allele B frequencies of 0.776, 0.763 and 0.976, respectively.

Although the distribution of allele frequencies in BDSP sheep population was 0.638 (allele A) and 0.362 (allele B), the heterozygous AB genotype was more frequently (0.655) encountered than both homozygous AA and BB (0.310 and 0.035). Similar results were recorded by Mroczkowski *et al.* (2004) in Polish Merino breed, where the frequency of heterozygous AB was 0.493 versus those of both homozygous AA (0.251) and BB (0.256), and by Kučinskiene *et al.* (2005) in Lithuanian blackface and native coarse-wooled sheep, with AB genotype frequencies 0.670 and 0.460, respectively. Celik & Özdemir (2006) also confirmed the largest share of individuals with heterozygous AB genotype in Awassi and Morkaraman sheep. In this study, the mean genotype frequency for homozygous BB was 0.398 varying from 0.035 in BDSP to 0.800 in PLBH. The mean frequency for homozygous AA genotype was low (0.198), due to the absence of homozygous AA individuals in STZG and PLBH sheep populations –. Kevorkian *et al.* (2008) established no animals with the homozygous BB genotype in Karakul sheep breed.

The observed heterozygosity value (0.655) in SPBM sheep population was higher compared to the expected one (0.462) resulting in a deviation from HWE (P=0.031). The same was observed for CRSH sheep population (P=0.033). This could be attributed to the fact that observed heterozygosity value in SPBM sheep was substantially higher than theoretically expected one, similarly to the reported observed/expected heterozygosity ratio (0.540/0.499) by Nassiry *et al.* (2007) in their study on the genetic variability of *LGB* gene in Iranian Kurdi sheep.

Studies on livestock diversity by molecular genetic distances were in the focus of the researchers during the last two decades. In the first cluster on the presented dendrogram (Fig. 2), Bulgarian dairy synthetic population sheep diverged from Stara Zagora and Pleven Blackhead sheep. Thus, the investigated *β–LG* polymorphism did not provide the expected separation of the breeds. The BDSP breed being synthetic population was expected to be closer to STZG and PLBH sheep breeds. Bulgarian dairy synthetic population sheep was established by crossing ewes of fine-wool and local dairy breeds with rams of the following dairy breeds: Pleven Blackhead, East-Friesian, Awassi and Local Stara Zagora sheep. Partial crossing with sheep

of Chios and Lacaune breeds has been applied in the last years (EASAB, 2011).

CONCLUSIONS

The obtained experimental results from the present PCR-RFLP study confirmed the presence of single nucleotide polymorphism in the relevant polymorphic region (exon II) of the β-lactoglobulin gene in all studied sheep populations. The distribution of allele frequencies in the *β-LG* gene suggested that the observed genetic polymorphism could be a useful marker in future research on association analysis for ovine milk traits. Therefore, additional investigations are planned to estimate the favourable *β-LG* genotypes that would allow accurate sheep selection. This will give an opportunity to define the genetic structure of sheep breeds in order to facilitate and plan their sustainable development, utilisation and conservation.

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