

Preliminary result of a genetic polymorphism of β -lactoglobulin gene and the phylogenetic study of ten balkan and central european indigenous sheep breeds

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Genetic polymorphism at the β -lactoglobulin (β -LG) loci in indigenous sheep breeds (Tsigai, Racka, Pramenka) was determined. Altogether 904 sheep were genotyped for the presence of the A, B and C alleles of β -lactoglobulin by PCR-RFLP. The AB genotype was the most common and the β -lactoglobulin A was the most frequent in the Cokanski Tsigai (54%), while the B allele was the most common in the Rusty and the Zomborski Tsigai (59%, 60%). The C allele was found only in one individual from Serbian Cokanski flock. These results differ from those that refer to other native sheep breeds. In the Cokanski Tsigai, deviation from the Hardy-Weinberg equilibrium was detected. Genetic relationship based on β -lactoglobulin polymorphism was the closest between the Rusty and the Cokanski Tsigai among the studied populations and between sheep and goat among the other ruminants. Part of the promoter region (254 bp) of β -LG in studied sheep breeds were sequenced in order to identify polymorphisms, analyze haplotypes, and phylogenetic relationship among them. Sequencing analysis and alignment of the obtained sequences showed one haplotype. Analysis of more samples and longer parts of the promoter region of β -LG are needed to reconstruct a phylogenetic tree.

Key words: Tsigai group, Zackel sheep, β -LG, allele frequencies, genetic relationship, sequencing

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INTRODUCTION

Milk nutrients are essential to human consumption, especially during the early age of life. It is estimated that 3% of the infants born worldwide are sensitive to milk proteins, and this sensitivity declines to 1% by the time of adulthood. However, the milk protein fractions causing allergic reactions (α s1 casein, α s2 casein, β casein, and β lactoglobulin) consist of numerous variants and not all of the variants trigger such reactions. In order to provide the people manifesting sensitivity to certain milk protein fractions with the nutrients required, a selection method for dairy species should be developed so as to reduce or eliminate the triggering protein fractions causing milk allergies.

β -lactoglobulin is the major whey protein in the milk of ruminants and it also exists in the milk of some non-ruminants (pigs, horses, dogs, cats, dolphins, whales).

However, it is absent from the milk of rodents, lagomorphs, camels and humans (Hambling *et al.*, 1992; Kappeler, 1998). This protein was isolated about 60 years ago, but its clear biological function is still not described, in contrast to other main whey proteins, such as α -lactalbumin (Mercier & Vilotte, 1993). The ability of β -LG to bind retinol, fatty acids, and other hydrophobic substances has suggested that its biological function may be the participation in milk fat digestion and transportation of these substances to the newborn (Fogolari *et al.*, 2000). Ovine whey is characterized by a higher proportion of β -LG than caprine or bovine whey (Casper *et al.*, 1998).

Aschaffenburg & Drewry (1955) published the first genetic polymorphism data of β -LG. The β -LG encoding gene has been sequenced in sheep, cows and goats (Folch *et al.*, 1994). The β -LG gene is on chromosome 3 in sheep and chromosome 11 in goats and cows (Hayes & Petit, 1993). Allele frequencies of β -lactoglobulin have already been studied in several sheep breeds. The genetic variants A (tyrosine) and B (histidine) differ at the amino acid position 20 (Moioli *et al.*, 1998; 2007). The genetic variant C — a sub-type of variant A — was identified by Erhardt (1989). It is an arginine — glutamine substitution at position 148. Variants A and B were detected in all studied breeds, while variant C was found only in a few breeds. Frequencies of β -LG A were usually higher than that of β -LG B (Ivankovic & Dovc, 2004; Moioli *et al.*, 2007; Elmaci *et al.*, 2006; Dario *et al.*, 2008); however, in some breeds, the B allele was more frequent (Anton *et al.*, 1999; Di Stasio *et al.*, 1997; Pietrola *et al.*, 2000). Variant C was either absent in most of the studied breeds or its frequency was the lowest among β -LG alleles (Erhardt, 1989; Recio *et al.*, 1997; Meie *et al.*, 2006; Elmaci *et al.*, 2006).

During the last decades, genetic polymorphism studies of milk protein genes in different species gained the interest of the animal breeding and dairy industries (Elmaci *et al.*, 2006). One prominent reason for this interest is the increase in the number of people who are suffering from allergies which are mainly caused by the proteins in milk. It has been known for years that α s1-casein, α -lactalbumin and β -LG are cow's milk allergens. Therefore, manufacturers want to prove the presence or absence of these proteins (Sieber, 2000). Since β -LG is absent from

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Abbreviations: β -LG, β -lactoglobulin; RFLP, restriction fragment length polymorphism; ng, nanogram; HWE, Hardy-Weinberg equilibrium

the milk of humans and camels, it is supposed to be the most responsible for milk allergies related to the consumption of cow milk (Kappeler, 1998). However, there is still no clear opinion on whether a product made from cow milk could be substituted by the one that contains the milk of other animal species. Since in camel milk there is a similar amount of essential amino acid content to the levels found in cow milk, camel milk could be a new protein source for children who are allergic to cow milk (Kappeler, 1998; El-Agamy *et al.*, 1997). Buergin-Wolff *et al.* (1980) and Taylor (1986) have not found any significant difference in the allergenic effect of β -LG and casein of cow milk. Milk allergies cannot be cured while taking medicines at the same time, however they are enduring, conditioned for the affected person to maintain a strict diet. Given that the different variants/alleles can be detected by the use of DNA tests, the use of genomic selection in dairy species represents a viable solution for producing allergen free milk.

Zackel and Tsigai strains are the two most widespread indigenous sheep breed groups found in Eastern and Southern Europe, currently being reared in 14 countries from this regions (Draganescu, 2007). There are historical evidences for the presence of similar types of sheep in the ancient Egypt, from where the migrations at different periods of time took these breeds to the Middle East and Europe. The exact origins of breeds are still unknown.

To the best of our knowledge, no other study concerning the β -lactoglobulin genetic polymorphism in Tsigai and Zackel breed groups exists up to this moment. However, the importance of indigenous breeds as gene reservoirs is recognized worldwide following the large scale crossbreeding's with exotic dairy or meat specialized breeds during the last decades.

In this study, our aim was to determine the allele and genotype frequencies of the β -LG gene in ten different Balkan and Central European indigenous sheep breeds and to study their genetic differences based on their β -LG polymorphism and promoter region.

MATERIALS AND METHODS

Materials. The Tsigai, Racka, Pramenka breeds are considered as indigenous sheep breeds in Balkan and Carpathian basin. In this work, altogether 904 blood samples were used. Unrelated animals with no shared common ancestor for at least two generations were sampled. Individuals were between ages of 3 to 6 year, sampling was made from different number of flocks across the countries. Hungarian Tsigai (gene reserve type) were collected from five (n=20, n=25, n=15, n=20, n=17), Hungarian Tsigai (milking type) from four (n=30, n=25, n=20, n=20), Racka from eight (n=35, n=30, n=27, n=30, n=30, n=35, n=30, n=20), Gyimesi Racka from three (n=30, n=35, n=33), Romanian Rusty Tsigai from two (n=20, n=33), Slovakian Tsigai from three (n=15, n=20, n=15), Croatian Tsigai from two (n=20, n=23), Bulgarian Tsigai from four (n=30, n=25, n=20, n=19), Serbian Tsigai (Cokanski) from three (n=35, n=30, n=34) and Bosnian Pramenka from three (n=34, n=29, n=35) flocks (Table 1).

Methods. Genomic DNA was extracted from total blood samples using standard protocol (Zsolnai & Orbán, 1999). PCR amplification was carried out in a 25 μ l reaction mixture containing 100–150 ng DNA. β -LG A and B alleles were determined, as previously reported by Feligini *et al.* (1998). β -LG C allele was characterized

Table 1. Distribution of β -lactoglobulin genotype and allele frequencies

Breed	Country	Total number	β -lactoglobulin genotype			Gene frequency		λ^2
			AA*	AB	BB	A*	B	
Hungarian Tsigai (gene reserve type)	Hungary	97	31	56	10	0.61	0.39	4.33 ^a
			35.94	46.25	14.88			
Hungarian Tsigai (milking type)	Hungary	95	23	50	22	0.51	0.49	0.26 ^a
			24.25	47.50	23.25			
Racka	Hungary	237	94	114	29	0.64	0.36	0.38 ^a
			96.21	109.59	31.21			
Gyimesi Racka	Hungary	98	17	57	24	0.46	0.54	2.81 ^a
			21.13	48.75	28.13			
Rusty Tsigai	Romania	53	7	31	15	0.42	0.58	2.06 ^a
			9.55	25.90	17.55			
Slovakian Tsigai	Slovakia	50	11	32	7	0.54	0.46	4.15 ^a
			14.58	24.84	10.58			
Croatian Tsigai	Croatia	43	11	28	4	0.58	0.42	4.91 ^a
			14.53	20.93	7.53			
Bulgarian Tsigai	Bulgaria	34	13	20	1	0.68	0.32	4.02 ^a
			15.56	14.88	3.56			
Serbian Tsigai (Cokanski)	Serbia	99	18	72	9	0.55	0.45	21.56 ^b
			29.45	49.10	20.45			
Bosnian Pramenka	Bosnia	98	14	64	20	0.47	0.53	9.48 ^c
			21.60	48.82	27.59			

*A+C alleles together. ^aTest of Hardy-Weinberg equilibrium; NS, not significant. ^bTest of Hardy-Weinberg equilibrium; $p < 0.001$. ^cTest of Hardy-Weinberg equilibrium; $p < 0.01$. ^dUpper row: observed value; Lower row: expected value.

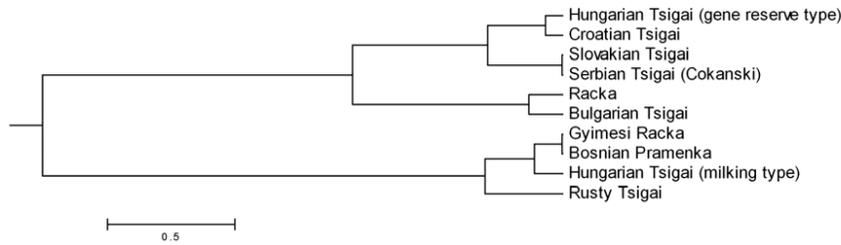


Figure 1. Genetic distance between the studied indigenous breeds based on β -LG genotypes

from β -LG A, as described by Anton *et al.* (1999). Promoter region of β -LG gene of five animals per breed was sequenced by Mastrangelo *et al.* (2012) procedure.

Genotype and allele frequencies of β -LG were calculated by Popgene vers.1.31. (Yeh & Yong, 1999). The chi-square test was used for Hardy-Weinberg equilibrium (HWE). To determine genetic relationships, neighbor-joining trees were constructed with MEGA 4.0.2 (Molecular Evolutionary Genetics Analysis) software using the bootstrap test in 1000 replications (Edgar, 2004; Tamura *et al.*, 2007). Number of haplotypes (h) were estimated with the DnaSP v5.10.01 software (Librado & Rozas, 2009).

RESULTS AND DISCUSSION

At the β -LG locus, 3 alleles were characterized. The 904 sheep, belonging to three breeds (Zomborski, Rusty, Cokanski), were genotyped for the presence of the A, B and C alleles. The method of Feligini *et al.* (1998) was used to differentiate between A+C and B alleles, while since C is a subtype of allele A, a second genotyping was needed to differentiate between A and C alleles using the method of Anton *et al.* (1999). Genotype and allele frequency data of the β -LG obtained by PCR-RFLP of all three studied breeds are presented in Table 1. Three genotypes (AA, AB, BB) and two alleles (A, B) were presented because C allele was detected only in one individual (Cokanski Tsigai) in heterozygote form. The most frequent genotype was AB in all three breeds, just like in Pag ewes, Polish Merino and crosses derived from Finn, Romanov and Booroola rams (Cubrik-Curik *et al.*, 2002; Piwczynski *et al.*, 2002). BB genotype was in the Russian Karakul breed, while AB genotype in Indian Jalauni sheep was not detected (Nassiry *et al.*, 2002; Arora *et al.*, 2010). Frequency of genotypes of AA, AB, BB were observed at 18%, 72%, 9% in the Cokanski, at 11%, 59%, 30% in the Rusty Tsigai and at 13%, 54%, 33% in the Zomborski Tsigai, respectively. Except for the Cokanski Tsigai, the other breeds were in Hardy-Weinberg equilibrium at the β -LG locus (Table 1). The frequency of A, B and C β -LG alleles were estimated to be 0.54, 0.46 and 0.005 in the Cokanski, 0.41, 0.59 and 0.00 in the Rusty, as well as 0.40, 0.60 and 0.00 in the Zomborski Tsigai. Results revealed that the β -LG A allele was more frequent than the B allele only in the Cokanski breed from among the studied breeds; however, based on other publications, β -LG A seems to be more frequent than the B allele in the Iranian Karakul, the Finnish Landrace, the Russian Karakul, the Ghezel, the Hungarian Racka, the Pag island sheep, the Manchega and the Hyfer Border Leicester breeds (Mohammadi *et al.*, 2006; Thomas *et al.*, 1989; Rampilli *et al.*, 1997; Lopez-Galvez *et al.*, 1994; Ivankovic & Dovc, 2004; Kerekes *et al.*, 2008; Amigo *et al.*, 2000). The β -LG B allele was found to be more fre-

quent than A in the Romanov (Macha & Novackova, 1974), Tajik (Aliiev & Koloteva, 1975), Sarda (Bolla *et al.*, 1989), Lacha (Recio *et al.*, 1997), and Chios (Macha & Novackova, 1974) breeds. Our results showed that the frequency of β -LG B allele in the studied breeds was higher than in the breeds studied previously (Mohammadi *et al.*, 2006; Amigo *et al.*, 2000). A relatively low incidence of the C allele was found in the studied breeds, as previously

reported in the Merino, Massese, Merinoland, Kivircik, Gokceada and Sakiz breeds by Recio *et al.* (1997), Erhardt (1989), Meie *et al.* (2006), Elmaci *et al.* (2006). Amigo *et al.* (2000) stated that β -LG A could be the original form of ovine β -LG.

Genetic relationships between the studied breeds based on β -LG polymorphism are presented in Fig. 1. The closest relationship was found between the Rusty and the Cokanski Tsigai, while the Zomborski Tsigai was clustered into another group. Since there is a similarity between caprine, ovine and bovine milk proteins, genetic relationship between ovine, bovine and caprine was also studied based on the β -LG nucleotide sequence (Fig. 2). The smallest genetic distance was observed between ovine and caprine.

After alignment and reduction (254 bp) of the sequences of promoter region of β -LG only one haplotype was detected.

Regrettably, within the sampling farms, no available data existed regarding the milk chemical composition for the studied populations. As a result, the comparison between milk content and individual genotypes, even though it could had given interesting results, was not feasible in the current study.

Kawecka & Radko (2011) have not found statistical differences between β -lactoglobulin genotype and milk yield composition in the Polish Merino. Conversely to results published by Mroczkowski *et al.* (2004) for the same breed. Yousefi *et al.* (2013), Ramos *et al.* (2009) and Dario *et al.* (2008) revealed significant associations between AB genotype of β -LG and high percentages of fat and milk lactose in different dairy breeds, respectively.

For the future, we would like to characterize all known alleles of ovine milk protein genes in the studied breeds, sequence the whole promoter region of β -LG and determine genetic variability within breeds. Our plan is also to extend the study by examining the association between milk composition and β -LG polymorphism.

Hereby, we took the first steps forward towards allergy-free products based on genotyped animals from indigenous sheep breeds reared in Central-, Eastern- and Southern-Europe.

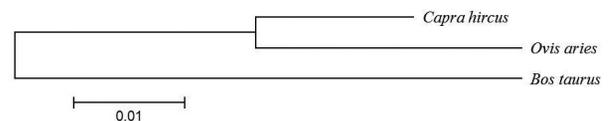


Figure 2. Genetic relationship between goat, sheep and bovine based on β -LG gene sequences. Sequence references are as follows: *C. hircus* (Genebank: Z33881), *O. aries* (Genebank: X12817), *B. taurus* (Genebank: Z48305).

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