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Molecular genetics analysis of milk protein gene polymorphism of dairy cows and breeding bulls in Latvia

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Abstract. Milk protein is the most valuable component of milk from a dietary point of view. More than 95% of ruminants' milk proteins are coded by six structural genes: two whey proteins (α – lactalbumin and β – lactoglobulin) and four caseins (α_{S1} – and α_{S2} – caseins, β – casein, κ – casein). The object of the research was the genetic polymorphisms of milk protein genes in populations of cows and breeding bulls of milk producing breeds in Latvia. The aim was to promote cow breeding in Latvia by developing and testing molecular genetics analyses for future quantity and quality analysis of the dairy cows' population in Latvia, based on the research of genes encoding milk protein polymorphism. In methodology the molecular markers were chosen which would be suitable for characterization of polymorphism of five milk protein genes in the population of dairy cows reared in Latvia. As a genetic method chosen the Restriction Fragment Length Polymorphism (RFLP) method and most analysed alleles of milk proteins. Using data of 719 DNA samples of dairy cows, the analysis of Latvian cows' population was carried out through six SNP of five milk protein genes: C*SN1S1* c.–175A > G, *CSN2* – c.4451A > C, *CSN3* c.11625C > T and c.11661A > C, *LAA* c.15A > G and *LGB* c.3106T > C. The results of PCR-RFLP analysis showed, as it was expected, that all genotypes were found in the populations.

Key words: milk proteins, dairy cattle, polymorphisms, Latvian population.

INTRODUCTION

At the end of 2014 in Latvia agricultural holdings were breeding 422.0 thousand cattle (Central Statistical bureau of Latvia, 2015). But at the end of 2016, agricultural holdings were breeding 412.3 thousand cattle, the drop was due to the reduction in the number of dairy cows of 5.2% (Central Statistical bureau of Latvia, 2017).

The production of dairy cows or milk yield from the total milk, produced in the European Union, in year 2014 and 2015 was more than 96% (Eurostat, 2015; 2016). It is used not only in pure form (around 27% from all dairy products), but also in different dairy products. The amount of milk produced in Latvia (incl. goat milk) in 2014 comprised 971.8 thousand tons. The average milk yield from a dairy cow reached 5812 kg, (Central Statistical bureau of Latvia, 2015).

The primary genetic relationship with milk quality and quantity is sought in relation to milk proteins, which largely, till 95% depending from breed, (Artym, Zimecki, 2013; Tsabouri et al., 2014) consists of four caseins and two main whey proteins.

Caseins are the water insoluble fraction of milk proteins (Table 1): alfa$_{S1}$ – casein (gene *CSN1S1*), alfa$_{S2}$ – casein (*CSN1S2*), beta – casein (*CSN2*) and kappa – casein (*CSN3*), (Artym, Zimecki, 2013; Tsabouri et al., 2014). Cattle casein loci is located on chromosome 6, and closely interconnected in cluster (Caroli et al., 2009; Barłowska et al., 2012). The total casein locus varies from 250 kb to 370 kb for humans, but the gene sequence and genetic alignment are conservative (Vilotte et al., 2013).

The second groupe of milk proteins are whey or serum proteins – water soluble fraction. Two main whey proteins (Table 1) are α – lactalbumin (α – LA), which is encoded with *LAA (LALBA),* and β – lactoglobulin (β – LG; progestagen-associated endometrial protein) or *LGB (PAEP)* gene (Artym, Zimecki, 2013; Tsabouri et al., 2014). α – LA gene is localized on chromosome 5, but *LGB* gene is located on chromosome 11 (11q28; Barłowska et al., 2012).

Table 1. Description of polymorphisms of five cow milk proteins

Gene		Polymorphisms of analyse			Protein alleles	
Abr.	Chr.	ID nr.	SNP^	Inr/Ex	Short	Full
CSN1S1	6q31	rs109817504	g.4646092A > G	5'UTR	B > C	A,**B**,D,F,G,H > **C**,E,I
CSN2	6q31	rs43703011	c.4451A > C	Ex 7	A1 > A2	**A1**,B,C,F,G > **A2**,A3,D,E,H1,H2,I,J
CSN3	6q31	rs43703015	c.11625C > T	Ex 4	A > B	**A**,A1,D,E,F2,G1,H,I > **B**,B2,C,J
		rs43703016	c.11661A > C	Ex 4		
LAA	5q21	rs209045823	c.15A > G	5'UTR	A > B	-
LGB	11q28	rs109625649	c.3106T > C	Ex 4	A > B	**A**,H > **B**,C,D,Dr,E,F,G,I,J,W

^ – Full name of polymorphisms taking in account position in gene (DNA) sequence.

In different studies in last 80 years have found out that all six major cows' milk proteins are variably not only in genome level (polymorphisms) but mainly after amino acid sequences of proteins. So far, various protein synthesis summaries (Hristov et al., 2014; Martin et al., 2013) have shown that for six proteins a total variation of alleles are 53: from 3 till 16 depending on gene/protein (Table 1), despite the fact that there are more polymorphisms in each gene. In the last five years, research on the dairy cow population in several of these genes has also been launched in Latvia (Petrovska et al., 2017a; 2017b; 2017c).

By 2008, more than 1200 breeds of dairy cows have been registered in the world (Utsunomiya et al., 2015), but in Latvia are registered 16 breeds in Agricultural Data Centre of Republic of Latvia. The number of dairy cows at 2014 were more than 125 thousands. More often farmed breed in Latvia is Holstein Black and White (HB; frequency around 33% in 2014, but in 2017 – around 47%), which is known in more than 128 countries (Utsunomiya et al., 2015). Dairy cows' milk yield per one cow of HB breed is greater than of Latvian local or historical breeds: Latvian Brown and Latvian Blue (Cielava et al., 2015; 2017), what's mean that in case of HB breed is a need more urgent to carry out a rebuilding of the bulk or additional costs of buying or growing new cows.

The aim of all research is to promote cow breeding in Latvia by developing and testing molecular genetics analyses for future quantity and quality analysis of the dairy cows' population in Latvia, based on the research of genes encoding milk protein

polymorphism. But the task of this study is to make the primary characterization of selected polymorphisms of bovine milk protein genes in the population of dairy cows reared in Latvia.

MATERIALS AND METHODS

Research was elaborated in the period of April 2009 to June 2014.

Description of the study object

Research group of breeds of dairy cows raised in Latvia was formed from 719 cattle biological material, including blood of 625 cows and sperm samples of 94 breeding bulls from Breeding and artificial insemination stations (BAIS) of seven breeds (Latvian Brown (LB) and Latvian Blue (LZ), Holstein Black and White (HB), Holstein Red and White (HR), Sweden Red and White (SR), Danish Red (DR) and Angeln (AN)) of dairy cows raised in Latvia (Table 2).

Table 2. Description of cows' group and the number of animals with positive genotyping in each locus

Breed of dairy cow		Sample	Freq., %	Positive genotyping (number of cows)				
				CSN1S1	*CSN2*	*CSN3*	*LAA*	*LGB*
Latvian Brown	LB	367	51,04	352	365	299	292	342
Latvian Blue	LZ	179	24,90	154	178	65	142	138
Holstein Black and White	HB	79	10,99	79	79	52	79	79
Holstein Red and White	HR	15	2,09	15	15	14	15	15
Sweden Red and White	SR	4	0,56	4	4	3	4	4
Danish Red	DR	55	7,65	55	55	41	55	55
Angeln	AN	4	0,56	4	4	4	4	4
Breeds crossing	XX	16	2,23	12	16	9	12	12
Sum		719		675	716	487	603	649
Including breeding bulls of BAIS		94	13,07	86	94	74	94	86

Overall, biological material was obtained from 81 herds of dairy cows in different regions of Latvia. In research were used also samples of cows of Latvian Brown and Latvian Blue breeds from the Depository of Latvian animal genetic resources.

DNA extraction

DNA extraction was done from 625 cows' blood and 94 bulls' sperm samples. In work were used two kits of genomic DNA extraction: Genomic DNA Purification Kit #K512 (Fermentas, Vilnius, Lithuania) and PUREGENE® DNA Isolation Kit (QIAgene, USA). Methods of both kits were adjusted for work with different blood and sperm quantity than in protocol.

Cows' milk protein polymorphism analysis

Six SNPs of five cows' milk protein genes (Table 3) were analysed with one of the oldest DNA fingerprinting method (Restriction Fragment Length Polymorphism (RFLP)), where each restriction endonuclease targets different nucleotide sequences in a DNA strand and cuts at different sites. Respectively, in casein protein genes: C*SN1S1* c.–175A > G, which leading to the protein variation change B to C; *CSN2* – c.4451A > C

(Pro$_{67(82)}$His; A1 and A2 variations); for *CSN3* were selected two SNPs: c.11625C > T and c.11661A > C, which leading to Thr$_{136(157)}$Ile and Asp$_{148(169)}$Ala or A variation change to B variation. For whey proteins, respectively, for *LAA* was selected SNP located in no-translated region or c.15A > G, in which case it is considered that changes A variations of the B, but for *LGB* c.3106T > C, which leading to the amino acid change Val$_{118(134)}$Ala or protein variation change A to B.

Table 3. Description of PCR – RFLP for study of polymorphisms of milk protein genes at cows' population reared in Latvia

Gene	Polymorphism[#]	PCR		RFLP	
		sequence of primers	product, bp	**enzyme,** sequence	fragments, bp and protein variations
CSN1S1	c.–175A > G - B > C	F 5' – TGC ATG TTC TCA TAA TAA CC – 3' R 5' – GAA GAA GCA GCA AGC TGG – 3'	310	***MaeIII*** ↓GTNAC	214/96 – B 310 – C
CSN2	c.4451A > C Pro$_{67(82)}$His A1 > A2	F 5' – CCT TCT TTC CAG GAT GAA CTC CAG – 3' R 5' – GAG TAA GAG GAG GGA TGT TTT GTG GGA GGC TCT – 3'	121	***DdeI*** C↓TNAG	121 – A1 86/35 – A2
CSN3	c.11625T > C and c.11661A > C Thr$_{136(157)}$Ile and Asp$_{148(169)}$Ala A > B	F 5'– TAT CAT TTA TGG CCA TTG GAC CA – 3' R 5'– CTT CTT TGA TGT CTC CTT AGA GTT – 3'	228	***HinfI*** G↓ANTC ***HindIII*** A↓AGCTT	133/93 – A 135/95 – B
LAA (LALBA)	c.15A > G - A > B	F 5' – CTC TTC CTG GAT GTA AGG CTT– 3' R 5' – AGC CTG GGT GGC ATG GAA TA– 3'	166	***MnlI*** CCTCN$_7$↓	78/52/36 – A 114/52 – B
LGB (PAEP)	c.3106T > C Val$_{118(134)}$Ala A > B	F 5' – TGT GCT GGA CAC CGA CTA CAA AAA G – 3' R 5' – GCT CCC GGT ATA TGA CCA CCC TCT – 3'	247	***HaeIII*** GG↓CC	148/99 – A 74(x2)/99 – B

[#] – Nucleotide replacement and, if so, the replacement amino acids in activated protein (inactive protein), and protein variations.

Data analysis

Numbers and frequencies of alleles and genotypes for entire population were estimated by direct counting, but for group of each breed were calculated by dividing samples.

Expected heterozygote indexes were estimated for both alleles at the locus or polymorphism. Population heterozygote was calculated as the ratio of the number of heterozygous individuals vs. the total number of subjects. Deviations from the Hardy – Weinberg equilibrium were tested by the *chi square (χ^2) test*.

Also for entire population was analysed association between non-parametrical indicators (number of alleles and genotypes for different sex or breeds of cows). For that calculation was used crosstab method with χ^2 *tes*t or *Pirson* χ^2 *test* at the confidence $p < 0.05$.

For all analyzes was used statistically programs: IBM SPSS Statistics version 22.0 and PAST (PAlaentological Statistics, ver. 1.63).

RESULTS AND DISCUSSION

DNA samples of 719 dairy cows raised in Latvia, including 94 breeding bulls, was analysed on polymorphisms of five milk proteins, by studding alleles and genotypes of each SNPs of each gene. Therefore we get a view about Latvian cows' population (Table 4).

Table 4. Distribution of polymorphisms of alleles and genotypes of the milk protein genes in population of dairy cows' in Latvia

Gene	Allele/	Frequency of allele/genotype									Statistic
	Genotype	All	LB	LZ	HB	HR	SR*	DR	AN*	XX	p_x
CSN1S1	B	0.96	0.97	0.95	0.97	0.97	1.00	0.97	0.88	0.92	0.42
	C	0.04	0.03	0.05	0.03	0.03	-	0.03	0.13	0.08	
	BB	0.93	0.93	0.90	0.94	0.93	1.00	0.95	0.75	0.83	
	BC	0.07	0.07	0.10	0.06	0.07	-	0.05	0.25	0.17	0.76
	CC	-	-	-	-	-	-	-	-	-	
CSN2	A1	0.67	0.69	0.76	0.43	0.60	0.38	0.63	0.50	0.53	1.25
	A2	0.33	0.31	0.24	0.57	0.40	0.63	0.37	0.50	0.47	$x10^{-11}$
	A1A1	0.42	0.44	0.56	0.16	0.20	0.25	0.38	-	0.19	
	A1A2	0.49	0.50	0.42	0.53	0.80	0.25	0.49	1.00	0.69	1.57
	A2A2	0.09	0.06	0.03	0.30	-	0.50	0.13	-	0.13	$x10^{-9}$
CSN3	A	0.92	0.93	0.72	1.00	1.00	1.00	1.00	1.00	1.00	2.32
	B	0.08	0.07	0.28	-	-	-	-	-	-	$x10^{-17}$
	AA	0.86	0.88	0.54	1.00	1.00	1.00	1.00	1.00	1.00	
	AB	0.11	0.10	0.35	-	-	-	-	-	-	2.66
	BB	0.03	0.02	0.11	-	-	-	-	-	-	$x10^{-13}$
LAA	A	0.06	0.03	0.08	0.18	0.10	0.13	0.02	-	-	8.04
	B	0.94	0.97	0.92	0.82	0.90	0.88	0.98	1.00	1.00	$x10^{-9}$
	AA	-	-	-	0.01	-	-	-	-	-	
	AB	0.12	0.06	0.15	0.34	0.20	0.25	0.04	-	-	2.70
	BB	0.88	0.94	0.85	0.65	0.80	0.75	0.96	1.00	1.00	$x10^{-3}$
LGB	A	0.22	0.17	0.20	0.42	0.33	0.38	0.25	0.50	0.04	7.16
	B	0.78	0.83	0.80	0.58	0.67	0.63	0.75	0.50	0.96	$x10^{-11}$
	AA	0.03	0.01	0.01	0.14	0.13	0.25	0.05	0.25	-	
	AB	0.37	0.33	0.37	0.57	0.40	0.25	0.40	0.50	0.08	3.25
	BB	0.60	0.67	0.62	0.29	0.47	0.50	0.55	0.25	0.92	$x10^{-5}$

* – sample only from bulls.

The analysis of polymorphism of milk protein genes

For **CSN1S1** gene the most frequently occurring allele is B (in all population 0.96) and genotype – BB (0.93) in Latvia (Table 4). There isn't difference of distribution of alleles or genotypes between dairy cow breeds.

Analysing frequencies of alleles depending from sex, we have recognized, that frequency of allele B of breeding bulls' is 0.07 lower than in cows (0.90 vs. 0.97; $p = 2.,07 \times 10^{-7}$). Looking at frequencies of genotypes, we can make conclusion, that samples with heterozygote are more common between breeding bulls than cows (20.93% for bulls and 5.43% for cows; $p = 4.99 \times 10^{-7}$ for all genotypes). So, it might imply, that breeding bulls are responsible about C allele in different stoking, because we didn't found among 675 samples any with homozygote genotype CC.

Investigating results of other researchers of different countries, we found out that result of research of Estonia (EE) is similar to ours. For Estonian native breed cows' (n = 118) α_{s1} – CN allele B is with frequency 0.92, but allele C – 0.08 (Lien et al., 1999). Also Lithuanian (LT) researchers have similar results: for dairy cows' population of LT (n = 427) allele B is with frequency 0.95 (Pečiulaitiene, 2005). In others countries of Europa, the results of similar studies are a little different. For example, for Bulgarian Grey breed (Neov et al., 2013) allele B is with frequency 0.43, but allele C – 0.57. As well as in this research, contrary to our, was found all three genotypes with frequencies: BB = 0.18, BC = 0.79, CC = 0.03. These results can be explained with this historical occurrence of breed (*Bos taurus brachiceros* x *Bos taurus primigenius*; Neov et al., 2013). In contrast, the Czech scientists' results for 440 Czech Fleckvieh breed cows' by alleles are closer to our results (B = 0.89, C = 0.11), but by genotypes – to Bulgarian' results with all three genotypes, respectively, BB = 0.80, BC = 0.18, CC = 0.16 (Kučerova et al., 2006).

In case of **CSN2** the common allele is A1 with frequency 0.67 and genotypes A1A1 (0.42), and A1A2 (0.49), despite the fact that in literature as preferable for cows' populations and as common allele more often is allele A2 (Cardak, 2005; Cieslinska et al., 2007; Pečiulaitiene et al., 2007). Looking at the population balance or deflection from Hardy – Weinberg at a given locus, there is statistically significant different ($p = 2.72 \times 10^{-3}$) between expected and obtained heterozygote frequencies. Similar results are for half of breeds (LB, LZ, HR and AN). For all investigated dairy breeds of Latvia the frequency of genotype A2A2 is very low. In addition, it appears that for HB breed, which has higher milk yield than other breeds, also observed higher frequency of A2A2 and equilibrium by Hardy – Weinberg equation.

Frequency of preferable allele A2 of CSN2 in Estonian native breed cows (Värv et al., 2009) is significantly higher (0.60) than in LB breed cows (0.31) or Latvian Blue (LZ) breed cows (0.24). We can conclude that in Estonian native breed frequency of benevolent allele A2 of CSN2 is two times higher than in Latvian native breeds.

In study included 94 breeding bulls are with small tendency to dominance of CSN2 allele A1 (0.54) over CSN2 allele A2 (0.46), but 622 dairy cows are with bigger tendency to CSN2 allele A1 dominance (0.69 vs. 0.31; between sex $p = 4.22 \times 10^{-5}$). Breeding bulls has higher frequency level of heterozygote and homozygote of CSN2 allele A2 genotypes ($p = 3.75 \times 10^{-3}$). So, there is a better background for CSN2 genotype A2A2 formation.

In Czech is the better situation for selection of CSN2 allele A2 than in Latvia. In their study was found that CSN2 alleles A1, A2, A3 and B are with frequencies 0.18, 0.80, 0.01 and 0.01, respectively (Kučerova et al., 2006).

In study of third casein protein gene **CSN3** we found, that in our population of dairy cows the common allele is A with frequency 0.92 and common homozygote genotype AA – 0.86. From literature is known that cows with genotype BB of CSN3 distinguished by better dairy technological features, the higher cheese outcome (Martin et al., 2002), but in our population this genotype and, respectively, CSN3 allele B are very rear (BB = 0.03 and B = 0.08). There is pronounced predominance of CSN3 allele A, as demonstrated by analyse of Hardy – Weinberg equation ($p = 1.19 \times 10^{-3}$). It should be noted that only in historical dairy cattle breeds of Latvia (LB and LZ) was found all three genotypes, but in other breeds – only genotype AA. The results differ from our colleges work (Petrovska et al., 2017 a, b, c), but the reason could be in fact, that in their collection have only genetic resources animals (bloodiness > 50%) and therefore groups of breeds are smaller.

Our data about very high occurrence of allele A of CSN3 in dairy cows of Latvian population comport with the research data in Lithuania and Estonia (Pečiulaitiene et al., 2007; Värv et al., 2009). Estonian researchers found higher frequency of CSN3 allele B in Estonian native breed cows (0.24) and in Estonian Red breed cows (0.37). Despite the low level of CSN3 allele B, Estonian scientists found that all parameters of milk coagulation were better to cows with genotype BB of CSN3 (Kübarsepp et al., 2005). In Lithuanian population of dairy cows' frequency of CSN3 allele A is 0.74 and of CSN3 allele B – 0.22 (Pečiulaitiene et al., 2007).

Second group is whey proteins, where for both **LAA** and **LGB** common allele is B (0.94 and 0.78, respectively) and common genotype is BB (0.88 and 0.60, respectively).

In *LAA* case analysed locus is in balance after Hardi – Weinberg equation across the population. In our all six breeds are observed predominance of LAA allele B (from 0.82 till 1.00). With the result can be recognized, that in result of genetic drift of LAA allele A is forced out of the population and the B allele frequency increases. Furthermore, LAA allele A is only in heterozygote genotypes, except in HB breed, where is one breeding bull with homozygote genotype after allele A. In this breed also frequency of LAA allele A is highest, which, compares to Latvian Brown breed, have six times higher (0.18 vs. 0.03). Looking to apportionment of rear LAA allele A in cows and breeding bulls, can be seen that for breeding bulls this allele is two times common than for cows (0.12 vs. 0.05; $p = 5.38 \times 10^{-4}$). Prevalence of LAA allele A resulting as double prevalence of frequency of heterozygote genotype (0.21 vs. 0.10; $p = 6.00 \times 10^{-4}$).

From data of different researches (Bell et al., 1981; Formaggioni et al., 1999) we can conclude that LAA variation B is typical or ancestral for *Bos taurus, Bos indicus, Bos (Poephagus) grunniens*, therefore LAA variation A isn't widespread in *Bos taurus*. Hypothetically, we can conclude that our data confirm the researchers' indicated the results about low frequency of variation A in different breeds and origins.

In our data, the highest frequency of LAA allele A is for dairy cows of HB breed (0.18) in Latvia. Comparing our results to the data of other researches, we conclude, that in other countries (Voelker et al., 1998; Bojarojc-Nosowicz et al., 2005) frequency of LAA allele A is higher (up to 0.77 or 76.60%) than in our studded breeds in Latvia.

In case of second largest whey protein or **LGB** in historical Latvian dairy cows' breeds frequency of LGB allele B is relative higher: in Latvian Brown 0.83, in Latvian Blue 0.80, but in Holstein Black and White (HB) only 0.58. Analysing variations of LGB genotypes of Latvian dairy cows' population, we found out that in tested HR, SR and AN breeds homozygote form of LGB rear allele A is markedly higher than in other breeds of Latvian population and average frequency of all population (0.03). Difference of distribution of LBG alleles and of genotypes between breeds is statistically significant ($p = 7.16 \times 10^{-11}$ and $p = 3.25 \times 10^{-5}$, respectively) in Latvia.

Comparing our results with other researches' publicised data (Pečiulaitiene, 2005; Kübarsepp et al., 2005; Zaton-Dobrowolska et al., 2006), we conclude that they show a slightly lower frequency of allele B, researching polymorphisms of whey milk protein LGB in different cows' breeds at national and/or special herds. Only one breed with higher frequency of allele B comparing to Latvian population is Lithuanian Red with frequency 0.92 (Pečiulaitiene, 2005).

CONCLUSIONS

The results of population analyse provide an opportunity to analyse B and C variations of CSN1S1, of A1 and A2 variations of CNS2 and of A and B variations of CSN3, LAA and LGB in association analyses with milk productivity indicators and with breeding bulls' estimated breeding values and yield index.

These loci can be used in future, after association analyses, as potential for gene, more precisely for marker assisted selection (MAS) in Latvian milk cattle breeding. Compared to the traditional breeding, MAS can be significantly more effective than traditional dairy cattle breeding. Judging by the data from scientific literature of different countries, it can be significant for increasing the productivity of animals, improving the quality and safety of milk products, by reducing the risks of milk and/or milk product related diseases and by promoting biological and food safety.

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