DISTRIBUTION OF ALLELE FREQUENCIES IMPORTANT TO MILK PRODUCTION TRAITS IN LITHUANIAN BLACK & WHITE AND LITHUANIAN RED CATTLE

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Summary. Lithuanian Black & White (LBW) and Lithuanian Red (LR) cattle belonging to the modern breeds form 99% of the total Lithuanian cattle population. These breeds have been intensively selected for milk production during the last 50 years. Associations were analysed between polymorphisms localized in six different polymorphic sites: four polymorphisms in the 5'-noncoding region of GHR gene: RFLP-*Alu*I, -*Acc*I and -*Fnu*4HI (located within the 1,206-bp LINE-1 element) and -*Sau*96I (located within the P1 promoter); one polymorphic site located in exon V of GH gene – RFLP-*Alu*I, and *Rsa*I polymorphic site located in exon 3 of the bovine PRL gene, including 52 LBW and 136 LR cattle. Genomes were identificated by the PCR-RFLP method.

The relative frequencies of alleles were determined for Lithuanian Black & White and Lithuanian Red cattle: $GHR^{(Alu+)}$ and $GHR^{(Alu-)}$ alleles – 0.45, 0.55 and 0.64, 0.36; $GHR^{(Acc+)}$ and $GHR^{(Acc-)}$ alleles – 0.62, 0.38 and 0.53, 0.47; $GHR^{(Fnu4H+)}$ and $GHR^{(Fnu4H-)}$ alleles – 0.87, 0.13 and 0.90, 0.10; $GHR^{(Sau96+)}$ allele – 1.00 and 1.00, $GHR^{(Sau96-)}$ allele were not found in both breeds; GH^{L} and GH^{V} alleles – 0.70, 0.30 and 0.77, 0.23; PRL^{A} and PRL^{B} alleles – 0.79, 0.21 and 0.87, 0.13, respectively. Except for GHR-*Fnu*4HI and GH-*Alu*I *loci*, significant differences between the allele frequencies of LBW and LR cattle were found of GHR-*Alu*I, -*Acc*I, and PRL-*Rsa*I genes.

Keywords: gene, polymorphism, polymerase chain reaction, GH, GHR, PRL.

ALELIŲ, SUSIJUSIŲ SU PIENO PRODUKTYVUMO POŽYMIAIS, DAŽNIŲ PASISKIRSTYMAS LIETUVOS JUODMARGIŲ IR LIETUVOS ŽALŲJŲ GALVIJŲ VEISLĖSE

Santrauka. Lietuvos juodmargiai (LJ) ir Lietuvos žalieji (LŽ) galvijai, priklausantys šiuolaikinėms veislėms, formuoja 99% visos Lietuvos pieninių galvijų populiacijos. Paskutiniuosius 50 metų šiose veislėse buvo vykdoma intensyvi selekcija pieno produkcijos kryptimi. (Malevičiūtė ir kt., 2003). Šiame darbe buvo ištirti 52 LJ ir 136 LŽ galvijai. Alelių ir genotipų dažniams nustatyti taikytas PCR-RFIP metodas. Tirtas ryšys tarp genų, veikiančių pieno produkciją, polimorfizmų, lokalizuotų šešiose skirtingose polimorfinėse srityse – keturių polimorfinių sričių 5'-nekoduojamame GHR geno regione: RFLP-*Alu*I, -*Acc*I ir -*Fnu*4HI (lokalizuoto 1,206-bp LINE-1 elemente) ir – *Sau*96I (lokalizuoto P1 promotoriuje); vienos polimorfinės srities, lokalizuotos GH geno V egzode, – RFLP-*Alu*I ir *Rsa*I polimorfinės srities, lokalizuotos galvijų PRL geno 3 egzone.

Lietuvos juodmargiams ir Lietuvos žaliesiems galvijams nustatyti šie dažniai: $GHR^{(Alu+)}$ ir $GHR^{(Alu-)}$ alelėms – 0,45; 0,55 ir 0,64; 0,36; $GHR^{(Acc+)}$ ir $GHR^{(Acc-)}$ alelėms – 0,62; 0,38 ir 0,53; 0,47; $GHR^{(Fnu4H+)}$ ir $GHR^{(Fnu4H-)}$ alelėms – 0,87; 0,13 ir 0,90; 0,10; $GHR^{(Sau96+)}$ alelei – 1,00 ir 1,00, $GHR^{(Sau96-)}$ alelė nebuvo aptikta abiejose veislėse; GH^{L} ir GH^{V} alelėms – 0,70; 0,30 ir 0,77; 0,23; PRL^{A} ir PRL^{B} alelėms – 0,79; 0,21 ir 0,87; 0,13 atitinkamai. Reikšmingi skirtumai tarp LJ ir LŽ galvijų buvo rasti GHR-*Alu*I, -*Acc*I, ir PRL-*Rsa*I lokusų, išskyrus GHR-*Fnu*4HI ir GH-*Alu*I lokusus.

Raktažodžiai: genas, polimorfizmas, polimerazinė grandininė reakcija, GH, GHR, PRL.

Introduction. Growth hormone (GH) and prolactin (PRL) are polypeptide hormones, which have evolved from a common ancestral gene. Although PRL and GH are produced by cells of the anterior pituitary that have a common stem cell, there are clear and distinct functions of these two hormones (Bole-Feusot *et. al.* 1998). Both hormones have been shown to be important for control of mammary growth, lactogenesis and lactation (Kopečný *et. al.* 1998). Bovine growth hormone (bGH) plays a very important role in many physiological actions (Kratochvilová, 2000). It is made of 190 or 191 amino acids and alanine or phelynanine at the N-terminus (Wood *et. al.* 1989). The GH gene consists of 5 exons and 4 introns, is mapped on chromosome 19 in cattle (Hediger *et. al.* 1990). Growth hormone has wide physiological activities, which include the regulation of growth, lactation and mammary gland development, gluconeogenesis, the activation of lipolysis, and the enhancement of amino acid incorporation into muscle protein (Burton *et. al.* 1994). The gene encoding bPRL is located on chromosome 23. It is composed of five exons and four introns with an overall length of ~10 kb (Camper *et. al.* 1984). In the now classic reviews by Nicoll and Bern (1972), Nicoll (1974) different biological functions of PRL were subdivided into five broad categories: reproduction, osmoregulation, growth, integument, and synergism with steroids (Bole-Feusot *et. al.* 1998).

Growth hormone actions on target cells are accomplished through the GH receptor – GHR, (Burton *et. al.* 1994) located on the bovine chromosome 20. The

gene coding for GHR consist of 9 exons in the translated part and of 5'-noncoding region, that includes 9 untranslated exons: 1A, 1B, 1C, 1D, 1E, 1F, 1G, IH, 1I (Jiang *et. al.* 2001). Exons from the untranslated region are spliced alternatively and each of them has its own transcription start site. A LINE-1 element, about 1.2 Kbp-long, was found upstream from exon 1A (Lucy *et. al.* 1998).

Several polymorphisms at these three loci, representing genes important for milk production traits in cattle are presented in Table 2: growth hormone receptor (GHR) -*Alu*I, -*Acc*I reported by Aggrey *et. al.* (1999) and Klauzińska *et. al.* (2004), -*Fnu*4HI and -*Sau*96I - by Maj and Zwierzchowski (2002), Maj *et al.* (2004), growth hormone (GH) -*Alu*I by Lucy *et. al.* (1991) and prolactin (PRL) -*Rsa*I by Lewin *et. al.* (1992).

The present study was designed to test the distribution of the frequencies of alleles and genotypes at different polymorphic sites within GHR, GH and PRL genes of Lithuanian Red and Lithuanian Black & White cattle.

Material and methods. In total, were genotyped 136 individuals of Lithuanian Red cattle from 2 different cattle farms ("Bariūnai", "Atžalynas") and "Šiauliai" artificial insemination station; and 52 Lithuanian Black & White cattle from the Practical Training and Research Centre of Lithuanian Veterinary Academy, cattle farm "Bernatonys" and "Šiauliai" artificial insemination station.

The blood samples (10 ml each) were collected to vacuum tubes with 19.5 mg EDTA (K_3) (Pharmacia Biotech, Sweden). Genomic DNA was isolated using phenol chloroform purification method according to Miller *et. al.* (1988). The methods of polymerase chain reaction (PCR) and restriction length polymorphism (RFLP) were used (Sakai *et al*., 1988). All PCR reactions were performed using the MJ Research PTC-225 Thermal Cycler.

The PCR-based detection of restriction length polymorphism (RFLP) was carried out using primers and restriction endonucleases given in Table 1 (according to Klauzińska *et. al.* (2004) and techniques and procedures used at the Institute of Genetics and Animal Breeding, PAN). The primer sequences and polymerase chain reaction conditions are summarized in the same table. The PCR was performed in a reaction volume of 10 µl, using 5.2 µl ddH_2O, 1.00 µl 10xPCR buffer, 0.8 µl (2.5 mM) dNTPs, 0.50 µl (0.25 µM) primers, 1.5 µl (approx.100 ng) genomic DNA and 0.5 µl (1 unit) of *Taq* polymerase. The amplified DNA was digested for 3 hours at 37°C with 5-10 U of appropriate restriction endonuclese (Biolabs, New England, USA).

Table 1. **Polymorphic sites and used techniques in this study.**

Locus, polymorphic site	Position of mutation	Primer	Amplified fragment (bp)	DNA restriction fragments obtained after digestion	PCR conditions
GHR-*Acc*I	5'-noncoding region -887, C/T	F: 5'-TGCGTGCACAGCAGCTCAACC-3' R: 5'-GGCAAACAGTGCGGGGTTGGA-3'	1934	+/+ 422, 289, 265 +/- 958, 422, 289, 265 -/- 958, 265	95°-2 min. (95°-45 s, 58°-50s, 72°-2 min., 72°-10 min.) x 39
GHR-*Alu*I, GHR-*Fnu*4HI	5'-noncoding region -1177, A/T, -1104, C/T	F: 5'-TGCGTGCACAGCAGCTCAACC-3' R: 5'-AGCAACCCCACTG-CTGGGCAT-3'	836	+/+ 836, 75 bp +/- 836, 602, 75, 15 bp -/- 602, 75 bp +/+ 433 bp +/- 433, 315, 75, 13 bp -/- 315 bp	95°-2 min. (95°-30 s, 69°-45s, 72°-2 min.) x 39, 72°-6 min.)
GHR-*Sau* 96I	P1 promoter 262, C/T	F: 5'-CTGGCGTATGGTCTTTGTCA-3' R: 5'-TGGTCTTGCTGCTTTC-CTAT-3'	318	-/- 318 bp +/- 318, 262, 56 bp +/+ 262, 56 bp	95°-2 min. (95°-20 s, 58°-30s, 72°-40s, 72°-4 min.) x 38
GH-*Alu*I	exon 5, 2241, C/G	F: 5'-CCGTGTCTATGAGAAGC-3' R: 5'-GTTCTTGAGCAGCGCGT-3'	428	LL 265, 96, 51, 16 bp LV 265, 96, 51, 16 bp VV 265, 96 bp	(94°-1 min., 60°-1 min., 72°-1 min.) x 44, 94°-1 min., 60°-1 min., 72°-10 min.
PRLP-*Rsa*I	exon 3, 103, A/G	F: 5'-CGAGTCCTTATGAGCTTGATTCTT-3' R: 5'-GCCTTCCAGAAGTCGTTTGTTTTC-3'	156	AA 156 bp AB 156, 82, 74 bp BB 82, 74 bp	(94°-1 min., 60°-1 min., 72°-1 min.) x 31

The digested DNA fragments were then separated by electrophoresis in 2 % agarose (Gibco, BRL, England) in 1 x TBE buffer (0.09 M Tris-boric acid, 0.002 M EDTA) with 0.5 mg/ml ethidium bromide (Et-Br) added to the gels, visualized under UV light and scanned in FX Molecular Imager apparatus (Bio-Rad).

The *Chi*-square test was used to evaluate associations and significance level of allele frequencies for LBW and LR cattle breeds. The Hardy-Weinberg equilibrium (HWE) was performed to evaluate the population differentiation (http://www.kursus.kvl.dk). Observed number of alleles (N), observed heterozygosity (H_o) for each locus, average heterozygosity over all loci was used to assess the genetic variability of populations studied.

Results and discussion. Number of animals of different genotypes and allele frequencies in the Lithuanian Black & White and Lithuanian Red cattle are shown in Table 2. Extremely significant associations were found of allele frequency between two studied populations at GHR-*Alu*I, -*Acc*I, and PRL-*Rsa*I genes. The estimated frequency of the $GHR^{(Alu+)}$ allele in LBW was 0.45, with highly significant difference (P≤0.0003) from LR cattle (0.64); $GHR^{(Acc+)}$ allele in LBW was 0.62 with highly significant difference (P≤0.0008) from LR cattle (0.53); PRL^{A} in LBW was 0.79 with highly significant difference (P≤0.0007) from LR cattle (0.87). The frequency of $GHR^{(Fnu4H)}$ and GH^{L} alleles were not significantly different between estimated breeds. Rare $GHR^{(Sau96-)}$ allele was found neither in LBW nor in LR cattle.

Frequencies of GH-*Acc*I, -*Sau* 96I, GH-*Alu*I and PRL-*Rsa*I alleles obtained for Lithuanian Red and Lithuanian Black & White cattle in this study remained within the range of the Polish Red (PR) and Polish Black & White (PBW) cattle reported previously by Dybus. *et. al.* (2002), Klauzińska *et. al.* (2004). The higher frequency of $GHR^{(Acc+)}$ allele (0.78) in Holstein cattle was identified by Aggrey *et. al.* (1999). The rare V allele appeared at frequency of 0.30 and 0.23 for LBW and LR, respectively do not differing much from frequencies: (0.28) - detected by Chrenek *et. al.* (1998) and (0.30) – by Čitek *et. al.* (1998). Løvendahl *et. al.* (1997) have shown frequency of (0.93) of GH^{L} allele in Danish Holstein and (0.83) in Danish Red. Frequency of GH^{L} (0.78) was observed in Polish Black and White cattle by Zwierzchowski *et. al.* (2001) and the same frequency detected in Bohemian Red by Kopečný *et. al.* (1998).

Table 2. **Alleles and genotypes frequencies in the Lithuanian Black & White and Lithuanian Red cattle.**

Polymorphism	Genotype	Number of animals (genotype frequency)		Allele frequency		*Chi*-square test
		Lithuanian Black & White	Lithuanian Red	Lithuanian Black & White	Lithuanian Red	
GHR RFLP-*Alu*I	+/+ +/- -/-	8 (0.15) 31 (0.59) 13 (0.25) total: 52	58 (0.43) 58 (0.43) 20 (0.14) total: 136	(+) 0.45 (-) 0.55	(+) 0.64 (-) 0.36	***
GHR RFLP-*Acc*I	+/+ +/- -/-	15 (0.29) 35 (0.67) 2 (0.04) total: 52	41 (0.30) 62 (0.46) 33 (0.24) total: 136	(+) 0.62 (-) 0.38	(+) 0.53 (-) 0.47	***
GHR RFLP-*Fnu*4HI	+/+ +/- -/-	38 (0.73) 14 (0.27) 0 (0.00) total: 52	111 (0.82) 24 (0.18) 1 (0.07) total: 136	(+) 0.87 (-) 0.13	(+) 0.90 (-) 0.10	n. s.
GHR RFLP-*Sau* 96I	+/+ +/- -/-	52 (1.00) 0 (0.00) 0 (0.00) total: 52	136 (1.00) 0 (0.00) 0 (0.00) total: 136	(+) 1.00 (-) 0.00	(+) 1.0 (-) 0.0	n. e.
GH RFLP-*Alu*I	LL LV VV	26 (0.50) 21 (0.40) 5 (0.10) total: 52	87 (0.64) 36 (0.26) 13 (0.10) total: 136	(L) (0.70) (V) (0.30)	(L) (0.77) (V) (0.23)	n. s.
PRL RFLP-*Rsa*I	AA AB BB	34 (0.65) 14 (0.27) 4 (0.08) total: 52	102 (0.75) 32 (0.24) 2 (0.15) total: 136	(A) (0.79) (B) (0.21)	(A) (0.87) (B) (0.13)	***

*** - p≤0.001, n. s. – non-significant; n. e. – not estimated.

The most frequent genotypes in both Lithuanian cattle populations were: (0.73), (0.82) for $GHR^{(Fnu4H+/+)}$ and (0.65), (0.75) for PRL^{AA} for LBW and LR cattle, respectively. In LBW and LR observed frequencies of $GHR^{(Fnu4H+)}$ alleles, i.e. 0.87 and 0.90, was higher than frequency previously reported by Maj *et. al.* (2004) for Polish Red and Polish Black & White cattle breeds: 0.78 and 0.87, respectively,. Higher frequency of PRL^{A} allele (0.95) was found in Holstein cattle by Chrenek *et. al.* (1998). However the $GHR^{(Fnu4H+/+)}$ genotype was absent in LBW and carried only by one individual in LR cattle; four PRL^{BB} genotypes were found in LBW and two in LR.

Determined average heterozygosity did not differ much between breeds and was 0.325 and 0.285 for LBW and LR cattle, respectively. The estimated heterozygosity (H_o) at GH locus (RFLP-*Sau* 96I) was equal to 0.00 for both breeds, as $GHR^{(Sau96I-)}$ allele was absent in both breeds. Low heterozygosity 0.23, 0.17 was found for GHR locus (RFLP-*Fnu*4HI) (Table 3). Lithuanian Black & White departured from the Hardy-Weinberg genetic equilibrium for GHR-*Acc*I, Lithuanian Red – for GH-*Alu*I *loci*.

Table 3. **Hardy-Weinberg equilibrium test* and Heterozygosity estimated in Lithuanian Black & White, Lithuanian Red cattle.**

Locus	Lithuanian Black & White		Lithuanian Red	
	HWE p-value	Heterozygosity (H_o)	HWE p-value	Heterozygosity (H_o)
GHR RFLP-*Alu*I	n.s.	0.50	n.s.	0.46
GHR RFLP-*Acc*I	0.0072	0.47	n.s.	0.50
GHR RFLP-*Fnu*4HI	n.s.	0.23	n.s.	0.17
GHR RFLP-*Sau* 96I	n.e.	0.00	n.e.	0.00
GH RFLP-*Alu*I	n.s.	0.42	0.0153	0.35
PRL RFLP-*Rsa*I	n.s.	0.33	n.s.	0.23
Average	-	0.325	-	0.285

*Numbers of animals are given in table 1.

n. s. – non-significant; n. e. – not estimated

Conclusions. The differences in allele frequency exist between the two studied cattle breeds. Highly significant differences were found in the frequency of alleles at the *loci:* GHR-*Alu*I, -*Acc*I, and PRL-*Rsa*I. The $GHR^{(Fnu4H+/+)}$ and PRL^{AA} genotypes were observed at the highest frequency in both Lithuanian cattle populations: (0.73), (0.82) and (0.65), (0.75) for LBW and LR cattle, respectively. Rare $GHR^{(Sau96-)}$ allele typically existing in *Bos Indicus* was absent in LBW and LR cattle. Non divergence average heterozygosity and observed low heterozygosity (H_o) in *GHR*(RFLP-*Fnu*4HI) *locus* reflect low genetic variability, between studied breeds. These differences in allele frequency might be raised due to different history of selection for milk yield appeared in both populations. However, the HWE analysis showed that selection for high milk yield did not affected GHR-*Acc*I and GH-*Alu*I *locus* of studied cattle breeds.

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