

Genetic polymorphisms of leptin and leptin receptor genes in relation with production and reproduction traits in cattle*

Anna Trakovická[✉], Nina Moravčíková and Radovan Kasarda

Department of Animal Genetics and Breeding Biology, Slovak University of Agriculture in Nitra, Nitra, The Slovak Republic

Leptin and leptin receptor genes are considered as production traits markers in dairy or beef cattle. The aim of this study was to verify the associations of polymorphisms in bovine *LEP* and *LEPR* genes with production and reproduction traits in Slovak Spotted and Pinzgau cows. Long-life production was evaluated: milk, protein, and fat yield and reproduction traits: age at first calving, calving interval, days open, and insemination interval. In total, 296 blood samples of Slovak Spotted and 85 hair roots samples of Pinzgau cows were analyzed. In order to detect *LEP/Sau3AI* (BTA 4, intron 2) and *LEPR/T945M* (BTA 3, exon 20) genotypes PCR-RFLP method was used. In Slovak Spotted and Pinzgau cows allele frequencies were 0.838/0.162 and 0.694/0.306 for A and B *LEP* variants, and 0.954/0.046 and 0.912/0.088 for C and T *LEPR* variants, respectively. For testing the associations between SNPs *LEP/Sau3AI* and *LEPR/T945M* and evaluated traits, the General Linear Model procedure in SAS Software was used. Statistical analysis showed that SNP *LEP/Sau3AI* significantly affected milk, protein and fat yield ($P < 0.05$), and age at first calving ($P < 0.01$) in analyzed population of cows. Statistically, SNP *LEPR/T945M* affected significantly calving interval ($P < 0.01$) only. Results of our study suggest that especially leptin is a candidate gene, which influences mainly milk production traits and might be implemented in breeding strategies to improve the production performance of both analyzed cattle breeds.

Key words: cattle; leptin; leptin receptor; milk production; polymorphism; reproduction

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INTRODUCTION

The importance of reproduction traits in dairy cattle breeding programs has dramatically increased recently. Achievement and profitability of modern cattle production determined stable and efficient reproduction. Milk yield in dairy cattle is governed both by genetic and environmental factors. Animals are subject to constant improvement for this quantitative trait, which is based on phenotype observations of cows and evaluation of the progeny and kins. Most associations between the polymorphism at a given locus and the phenotypic variability are virtually meaningless, or appear in local populations only. Thus, the research in pursuit of a functional marker, which would be of importance for selection, focuses mainly on protein-coding genes involved in the processes of lactation, as well as the genes that regulate these complex processes.

Leptin is a polypeptide hormone synthesized and secreted primarily in the adipose cells (Forhead & Fowden, 2009). Leptin binds to a receptor mainly localized on Neuropeptid — Y — neurons, which in hypothalamus appear also to play a key role in the integration of feeding behaviour with internal signals of body energy status (Wayne *et al.*, 1995). As leptin concentrations are strongly influenced by adipose tissue mass, leptin is thought to be a key signalling molecule linking nutritional status to reproductive function. An adequate amount of circulating leptin appears to be necessary for the attainment of puberty (Cunningham *et al.*, 1999). Leptin may help regulate ovarian development and steroidogenesis, and serve as either a primary signal initiating puberty, or as a permissive regulator of sexual maturation (Lindersom *et al.*, 1998). In cattle, *LEP* gene is located on chromosome 4. It consists of three exons and two introns of which only 2 exons are translated into protein. The coding region of *LEP* gene (501 nucleotide length) is contained in exon 2 and 3 (Liefers *et al.*, 2002). SNP *LEP/Sau3AI* is situated in the second intron and results in amino acid change at position 2059 of the protein chain (cytosine, C to thymine, T).

Effects of leptin are exerted through six isoforms of leptin receptor. The leptin receptor is a glycoprotein with a single transmembrane-spanning region. The long, fully active isoform (*LEPR-b*) is expressed mainly in hypothalamus, where it takes part in energy homeostasis and in regulation of the activity of the secretory organs, it may also act in many peripheral tissues, including gonadal tissues (Matteis *et al.*, 2012). Leptin receptors are present in the ovary, where leptin can modulate steroidogenesis and improve the ability of the oocyte to sustain subsequent embryonic development (Boelhaue *et al.*, 2005). *LEPR* gene is located on bovine chromosome 3. The leptin receptor gene consists of 20 exons divided over 1.75 Mb. Inside *LEPR* gene, Liefers *et al.* (2004) described a missense mutation *T945M*. It is a cytosine to thymine base substitution at position 115 in exon 20, which results in a substitution of the amino acid (threonine, T to methionine, M) at residue 945 of leptin. SNP *T945M* may

[✉] e-mail: nina.moravcikova1@gmail.com

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Abbreviations: BTA, bovine autosome; H_e , heterozygosity; H_o , homozygosity; *LEP*, leptin; *LEPR*, leptin receptor; *LEP/Sau3AI*, name of single nucleotide polymorphism in leptin gene; *LEPR/T945M*, name of single nucleotide polymorphism in leptin receptor gene; N_e , effective allele number; PIC, polymorphic information content; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; SNP, single nucleotide polymorphism; *Sau3AI*, *BseGI*, restriction enzymes; TBE, electrophoresis buffer

Table 1. Sequence of the primers, size and region of the amplified fragments in PCR and observed genotypes with size of the digestion patterns

Gene	Primer sequences	Size (bp)	Amplified region	Genotype/Digestion patterns (bp)	Reference
LEP	F 5' -TGG AGT GGC TTG TTA TTT TCT TCT- 3' R 5' -GTC CCC GCT TCT GGC TAC CTA ACT- 3'	422	intron 2	AA 390, 32 AB 390, 303, 88, 32 BB 303, 88, 32	Liefers <i>et al.</i> (2002)
LEPR	F 5' -ACTACAGATGCTCTACTTTGG-3' R 5' -TGCTCCTCCTCAGTTT-3'	197	exon 20	CC 130, 67 CT 130, 93, 67, 37 TT 93, 67, 37	Almeida <i>et al.</i> (2008)

have induced a structural change in the intracellular domain of *LEPR*.

The aim of this study was to establish *LEP* and *LEPR* genes allele and genotypes frequencies, and significance of associations between these polymorphisms and long-life milk production, and reproduction traits in Slovak Spotted and Pinzgau cows.

MATERIAL AND METHODS

Animals and DNA extraction method. The study covered a total of 296 Slovak spotted and 85 Pinzgau cows. Evaluated cattle breeds belong to the dual purpose production type. Genomic DNA for animal's genotyping was extracted from blood samples and hair roots. DNA concentrations were calculated by spectrophotometer by taking the optical density at wave length of 260 nm.

Analyses of polymorphisms. The analyzed polymorphic sites are situated within the second intron in the bovine *LEP* gene (C/T transition results in amino acid change arginine by cysteine at position 2059 in the secreted protein) and the exon 20 in the bovine *LEPR* gene (C/T transition at a position 115, which results in a substitution of the amino acid threonine by methionine at residue 945 of leptin). A 422 bp fragment of *LEP* gene was amplified by PCR using forward and reverse primers according to Liefers *et al.* (2002). The polymerase chain reaction was performed in a 25 µl reaction mixtures, containing: 1 × PCR buffer (NH₄)₂SO₄, 1.5 mM MgCl₂, 2 mM dNTPs, 8 pM primers, 1 U Tag DNA polymerase and 50 ng genomic DNA. Thermal cycling conditions included: an initial denaturation step at 95°C/5 min, followed by 30 cycles of 95°C/30 sec, 55°C/20 sec, 72°C/30 sec and a final extension at 72°C/7 min. Amplification of a 197 bp fragment of *LEPR* gene was performed with appropriate primer pairs proposed by Almeida *et al.* (2008). DNA was amplified in a total volume of 25 µl containing: 1 × PCR buffer (NH₄)₂SO₄, 2 mM MgCl₂, 2 mM dNTPs, 6 pM primers, 1 U Tag DNA polymerase and 50 ng genomic DNA. PCR conditions were at 94°C/3 min, followed by 35 cycles of 94°C/30 s, 51°C/50 s and 72°C/30 s. After 35 cycles, reactions were completed by an extension at 72°C/5 min. PCR products of *LEP* and *LEPR* genes were digested at 37°C in time 10 min with 1 µl of Fast-Digest restriction enzymes *Sau3AI* and *BseGI*, respectively. The digestion products were separated by horizontal electrophoresis in 3% agarose gels in 0.5 × TBE (130 V for 50 min) stained with GelRed prior to visualization under UV light.

Statistical analysis. The allele and genotype frequencies of *LEP* and *LEPR* genes were estimated by direct counting, and the differences of the observed and expected frequencies of genotypes were tested using Chi-square (χ²) test in order to verify if the population was in

Hardy-Weinberg equilibrium. Genetic indices of population, including observed and expected genes heterozygosity (H_j), homozygosity (H_o) and effective allele numbers (N_e) were performed by Popgene32 software version 1.3 (Yeh *et al.*, 2000). Moreover, polymorphism information content (PIC) was calculated according to Botstein *et al.* (1980). Associations of the animal genotypes with long-life milk production and reproduction traits were determined by analyses of quantitative traits. Statistical analysis was performed using SAS Enterprise Guide 4.2 software (SAS Institute Inc., 2009), and significance of differences based on genotypes effect of production and reproduction traits were tested by following general linear models:

$$Y_{ijklmn}^{1,2,3} = G_i + BT_j + S_k + A_l + CI_m + L_n + e_{ijklmn}$$

$$Y_{ijk}^4 = G_i + BT_j + S_k + e_{ijk}$$

$$Y_{ijklmn}^5 = G_i + BT_j + S_k + DO_l + MY_m + L_n + e_{ijklmn}$$

$$Y_{ijklm}^6 = G_i + BT_j + S_k + MY_l + I_m + e_{ijklm}$$

$$Y_{ijklm}^7 = G_i + BT_j + S_k + MY_l + I_m + e_{ijklm}$$

where: $Y_{ijklmn}^{1,2,3}$ – milk, protein and fat yield, Y_{ijk}^4 – age at first calving, Y_{ijklmn}^5 – calving interval, Y_{ijklm}^6 – days open, Y_{ijklm}^7 – insemination interval, G_i – effect of genotype, BT_j – effect of breed type, S_k – effect of sire, A_l – effect of age at first calving, DO_l – effect of days open, CI_m – effect of calving interval, L_n , L_m – effect of number of lactation, MY_m , MY_l – effect of milk yield, I_m – effect of insemination interval, e_{ijk} , e_{ijklm} , e_{ijklmn} – random error.

RESULTS

Three genotypes for *LEP/Sau3AI* (AA, AB, and BB) were identified. For *LEPR* gene only two genotypes (CC and CT) were detected, whereas TT genotype was not observed. Frequencies of individual alleles and genotypes in the analyzed populations of Slovak Spotted and Pinzgau cows are presented in Table 2. The highest frequencies of SNP *LEP/Sau3AI* were observed for homozygous AA genotype in Slovak Spotted cows and heterozygous AB genotypes in Pinzgau cows, and lowest for homozygous BB genotype in both analyzed populations. For SNP *LEPR/T945M* was predominant in both cows populations CC homozygous genotype. Slovak Spotted and Pinzgau cows populations were in Hardy-Weinberg equilibrium ($p > 0.05$). The heterozygosity, effective allele numbers and polymorphism information content of SNPs *LEP/Sau3AI* and *LEPR/T945M* in two analyzed populations are presented in Table 2. The decrease in heterozygosity can be caused as result of eventual populations inbreeding. The low heterozygosity values indicate, that inbreeding probably may be a potential prob-

Table 2. Distribution of SNPs *LEP/Sau3AI* and *LEPR/T945M* alleles and genotypes in Slovak Spotted and Pinzgau cows.

Breed	Genotype frequency			Allele frequency		χ^2 test	H_e	PIC	N_e
	<i>LEP/Sau3AI</i> ^{AA}	<i>LEP/Sau3AI</i> ^{AB}	<i>LEP/Sau3AI</i> ^{BB}	<i>LEP/Sau3AI</i> ^A	<i>LEP/Sau3AI</i> ^B				
SS	0.70	0.27	0.03	0.839	0.161	0.18	0.28	0.25	1.39
P	0.45	0.49	0.06	0.694	0.306	2.23	0.42	0.34	1.74
	<i>LEPR/T945M</i> ^{CC}	<i>LEPR/T945M</i> ^{CT}	<i>LEPR/T945M</i> ^{TT}	<i>LEPR/T945M</i> ^C	<i>LEPR/T945M</i> ^T				
SS	0.92	0.08	-	0.958	0.043	0.20	0.08	0.08	1.09
P	0.82	0.18	-	0.911	0.088	1.26	0.16	0.15	1.19

$P > 0.05$, SS — Slovak spotted, P — Pinzgau, H_e — observed heterozygosity, H_o — observed homozygosity, PIC — polymorphic information content, N_e — effective allele number

Table 3. Basic statistical variation measurements of long-life milk production and reproduction traits

	Milk production traits								
	Slovak Spotted cows			Pinzgau cows			Total		
	n	mean	SD	n	mean	SD	n	mean	SD
MY (kg)	248	20668.45	8310.80	83	17316.98	11300.86	331	19828.05	9249.22
PY (kg)	248	703.05	274.12	83	659.31	421.22	331	678.55	306.79
FY (kg)	248	829.87	336.59	83	605.35	381.06	331	787.09	366.56
	Reproduction traits								
AFC (d)	248	896.23	117.98	83	1054.70	122.61	331	936.01	137.42
CI (d)	240	404.04	64.62	66	386.18	56.93	306	404.58	70.58
DO (d)	224	117.64	64.25	75	166.64	137.09	299	129.93	90.60
II	224	73.72	31.52	75	88.44	79.64	299	77.41	48.58

n — number of individuals, mean — average value, S.D. — standard deviation, MY — milk yield, PY — protein yield, FY — fat yield, AFC — age at first calving, CI — calving interval, DO — days open, II — insemination interval

lem at the population level, therefore should be included pedigree information in further population's evaluation. According to the classification of PIC (low polymorphism if PIC value < 0.25, median polymorphism if $0.25 < \text{PIC value} < 0.5$, and high polymorphism if PIC value > 0.5) (Botstein *et al.*, 1980), the analyses show low (*LEPR/T945M*) or median (*LEP/Sau3AI*) levels of polymorphic information content of analyzed SNPs across populations based on the expected heterozygosity. The effectiveness of loci allele impact in populations has been expressed by effective allele numbers. Providing that in biallelic system both alleles result in the genotypes creation, threshold value is 2,000. Comparison of N_e showed higher effective allele numbers across populations for SNP *LEP/Sau3AI* and indicated good level of genetic variability in analyzed cows population at the considered locus.

Table 3 and 4 shows average values of analyzed production and reproduction traits in relation to specific genotype in analyzed cows populations. Significance of observed differences in association analyses between polymorphisms and analyzed traits was affected by number of data available for particular trait. Only animals with relevant data of production and reproduction parameters were used in subsequent analyses.

The study results of SNP *LEP/Sau3AI* show significant differences between the long-life milk production traits ($P < 0.05$) and age at first calving ($P < 0.01$). Cows of homozygous *LEP/Sau3AI*^{AA} genotype were characterized by highest milk, protein and fat yield and lowest age at first calving. The opposite relationships between milk production and *LEP/Sau3AI* genotypes were found in the group of cows of *LEP/Sau3AI*^{BB} genotype. The highest age at first calving was determined in heterozy-

gous *LEP/Sau3AI*^{AB} cows. Differences between other analyzed traits were very low and non-significant.

The statistical analysis of *LEPR* genotype effect on long-life milk production and reproduction traits shows significant differences ($P < 0.01$) only for length of calving interval. The shorter calving interval was characterized for group of cows with heterozygous *LEPR/T945M*^{CT} genotype. This genotype was also favourable for days open and insemination interval, but only non-significant. Significant results of association analyses between *LEPR/T945M* genotypes and analyzed production and reproduction traits were affected by number of identified heterozygous individuals and missing homozygous TT genotype.

DISCUSSION

Improvement of production traits and increase of genetic and economic gain are the aim of all cattle breeders. This gain can be achieved with traditional selection programs or breeding programs using genetic markers. Marker assisted selection or genomic selection in livestock uses quantitative trait loci and candidate genes, which directly or indirectly affect phenotype of individuals. Analyses of increase genetic gain using marker assisted selection can put genetic evaluation more exactly and decrease time, which is needed for realization in cattle breeding. It is generally accepted that leptin plays a key role during pregnancy, in feed intake, energy expenditure and growth, or reproduction functions of dairy cattle, and leptin gene might be a strong candidate for economically important production traits. For leptin hormone functioning leptin receptor is necessary. This receptor is

Table 4. Average values of analyzed production and reproduction traits in cows of different LEP/Sau3AI and LPR/T945M genotype variants

	LEP/Sau3AI ^{AA}			LEP/Sau3AI ^{AB}			LEP/Sau3AI ^{BB}			LEPR/T945M ^{CC}			LEPR/T945M ^{CC}		
	n	mean	S.D.	n	mean	S.D.	n	mean	S.D.	n	mean	S.D.	n	mean	S.D.
MY (kg)	211	20813.94**	8649.63	108	18442.19**	10177.32	12	14965.58**	8204.19	292	20283.51	9248.25	39	16417.92	8625.96
PY (kg)	211	710.48*	286.57	108	632.77*	334.78	12	529.14*	317.82	292	693.10	306.71	39	569.63	288.33
FY (kg)	211	825.39*	339.87	108	730.79*	405.03	12	620.57*	374.81	292	805.56	367.27	39	648.87	334.18
AFC (d)	211	921.82**	139.71	108	963.26**	131.05	12	940.08**	124.95	292	933.52	136.90	39	954.61	141.62
CI (d)	201	408.33	68.93	95	397.52	75.03	10	396.04	59.08	272	407.82**	73.11	34	378.60**	36.88
DO (d)	189	124.19	75.10	98	141.60	115.8	12	125.00	76.85	265	131.82	88.36	34	115.20	106.85
II	189	75.25	40.67	98	82.93	62.69	12	66.25	22.33	265	79.13	50.21	34	64.00	30.45

* $P < 0.05$, ** $P < 0.01$, n — number of individuals, mean — average value, S.D. — standard deviation, MY — milk yield, PY — protein yield, FY — fat yield, AFC — age at first calving, CI — calving interval, DO — days open, II — insemination interval

involved also in regulation of secretory organs activity. Among potential genetic markers analyzed in this study, LEP/Sau3AI^A allele can play a role in determination of level of milk, protein and fat yield, and age at first calving and LEPR/T945M^C allele in determination of length of calving interval. The Effect of leptin and leptin receptor genes were evaluated in many studies on different cattle breeds. The SNP LEP/Sau3AI have been significantly associated with somatic cells count (Kulig *et al.*, 2010), milk production traits (Anton *et al.*, 2012; Liefers *et al.*, 2002), reproduction traits (Chebel & Santos, 2011; Almeida *et al.*, 2003) and growth traits (Kulig & Kmiec, 2009; Othamn *et al.*, 2011). For SNP LEPR/T945M effect on milk production traits (Glantz *et al.*, 2012), reproduction traits (Clempton *et al.*, 2011; Glantz *et al.*, 2011; Komisarek, 2010) and growth traits (Almeida *et al.*, 2008; Silva *et al.*, 2012) was confirmed. Selection of animals with LEP/Sau3AI or LEPR/T945M genotype could have beneficial results in production and reproduction traits in cows.

In conclusion, genes encoding hormones, growth factors, and other regulatory proteins associated with milk production and reproduction can be evaluated as a genetic markers candidate for quantitative traits in farm animals. We confirmed that LEP/Sau3AI genotypes may cause differences in milk production phenotypes, and also SNP affected the reproduction of analyzed individuals. Statistical analyses of associations between LEPR/T945M genotypes and analyzed traits were significant only for length of calving interval. The leptin and leptin receptor genes could contribute to the progress in selection of farm animals, whereas for application in breeding practice confirmation of their effect is needed by further associations analyses. The confirmation of our results can increase economic effectiveness of animal production resulting from selection of individuals with preferred genotype.

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