Polymorphism within the Intron Region of the Bovine Leptin Gene in Slovak Pinzgau Cattle

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Abstract
Leptin is 16 kDa protein synthesized by adipose tissue and involved energy balance by inhibiting food intake and stimulating energy expenditure, reproductive and immune functions. The physiological properties support leptin as a strong candidate gene for evaluating of genetic polymorphism, which has further been associated with growth, milk yield and other economical traits in livestock. The aim of this study was detection polymorphism in the intron region of the leptin gene on bovine chromosome 4 by using PCR-RFLP method. A total of 100 Slovak Pinzgau cows were used for SNP genotyping. A strategy employing PCR was used to amplify a 422 bp product from hair roots DNA samples. The genotype and allele frequencies for each breed were determined and tested to be in Hardy-Weinberg equilibrium. Digestion of PCR products with restriction enzyme *Sau*3AI revealed two alleles: allele A was 390, 32 fragments and allele B was 303, 88, 32. Three patterns were observed and frequencies were 0.4761, 0.4278 and 0.0961 for AA, AB and BB, respectively.

Keywords: cattle, leptin gene, PCR-RFLP, polymorphism.

1. Introduction

Leptin (LEP) gene is one of the potential genes that are involved intricately in the metabolism and growth of animals. Leptin biology in livestock is similar to human and plays important role in regulating productive performance of animals. Leptin as a hormonal product of the leptin gene expression has multiple physiological effects in the control of body growth, energy metabolism, feeding behaviour, reproduction and immune function. Leptin also plays a key role in the regulation of reproductive performance by stimulating GnRH, FSH and LH release [1] and appears to affect the central reproductive axis trough its own receptors and neurotransmitter, neuropeptide Y [2]. There is evidence that leptin may also modulate reproduction directly at gonadal sites [3]. In ruminants, leptin gene was shown to be expressed in adipose tissue, foetal tissue, mammary gland, rumen, abomasums, duodenum and pituitary gland [4] is one such candidate gene; his receptors are localized, among others, in the regions of the hypothalamus where somatostatin and growth hormone releasing factor – two primary regulators of growth hormone secretion – are produced [5]. The influence of leptin on the regulation of food intake and energy expenditure, as well as the location of his receptors, indicate its role in the growth processes [6].

Leptin is the 16 kDa molecular weight protein having 146 amino acids. LEP gene consists of three exons, which are separated by two introns. In mice, the first intron is more than 5 kb and second intron has a length of 1.6 kb [7]. The three exons of leptin gene cover approximately 15 kb of genomic DNA. The entire coding region is contained in exons 2 and 3, which are separated by a 2 kb intron. The gene structure, intron/exon boundaries and amino acid sequence are highly conserved in mammalian species [8]. The leptin gene itself is considered a potential QTL,

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influencing different production traits in cattle. The *SNP 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).
The aim of this study was to investigate the frequency of leptin gene polymorphism in population of Slovak Pinzgau cows.

2. Materials and methods

Sampling and DNA extraction
The total numbers of hair roots samples were taken from 100 samples of Slovak Pinzgau cows. Genomic DNA was extracted from samples with isolation kit Extract-N-Amp^{TM} Tissue PCR (Sigma-Aldrich) and according to Gábor [9]. DNA concentrations were calculated by spectrophotometer by taking the optical density at wave length of 260 nm.

PCR amplification
A 422 bp fragment of intron 2 in bovine LEP gene was amplified by PCR using forward and reverse primers according to Liefers et al. [10]. The polymerase chain reaction was performed in a 25 µl reaction mixtures, containing: 1 x PCR buffer $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 2 mM dNTPs, 8 pM primers (Generi-Biotech), 1 U Tag DNA polymerase (Fermentas), 6 µl BSA (Fermentas), 50 ng genomic DNA. PCR amplification was carried out in C1000^{TM} thermal cycler (Biorad). Thermal cycling conditions included: an initial denaturation step at 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec, 62°C for 20 sec, 72°C for 30 sec and a final extension at 72°C for 7 min.

Table 1. Primer sequence of LEP *Sau3AI* loci

Locus	Primer sequence
LEP *Sau3AI*[1]	F 5´ -TGG AGT GGC TTG TTA TTT TCT TCT- 3´
	R 5´-GTC CCC GCT TCT GGC TAC CTA ACT- 3´

Note: F= Forward, R= Reverse. [1] Liefers et al. [10]

Restriction reaction
Genotype analyses were performed using the polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) method. The PCR products of LEP gene were digested with 1 µl of FastDigest *Sau*3AI (Fermentas) restriction enzyme at 37°C in time 15 min. The digestion products were separated by horizontal electrophoresis in 3% agarose gels in 0.5 x TBE (130 V for 40 min) stained with GelRed (Biotium) prior to visualization under UV light.

Statistical analysis
The allele and genotype frequencies of *Sau3AI* polymorphism were examined for deviation from Hardy – Weinberg equilibrium using χ^2 test.

3. Results and discussion

Inside the second intron of the bovine LEP gene using digestion of PCR fragment with restriction enzyme *Sau*3AI was detected restriction fragment length polymorphism. There were two *Sau3AI* sites in 422 bp fragments. The digested AA PCR product exhibited two fragments of 390 and 32 bp. For the BB genotype exhibited 303, 88 and 32 bp. Figure 1 shows PCR product size and the restriction patterns of the tree genotypes AA, AB and BB and they confirmed GAC»GAT, (C to T) transversion.

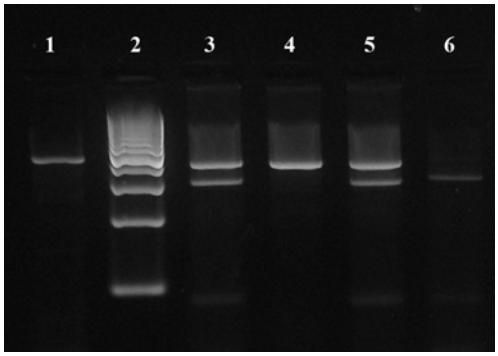

Figure 1. Representative result of PCR-RFLP analysis LEP *Sau3AI* loci on 3% agarose gel

Line 1 is PCR product (422 bp), line 2 is a marker of molecular weight (Fermentas, 100 bp), line 3 is AB genotype (390, 303, 88 and 32 bp), line 4 is AA genotype (390 and 32 bp), line 5 is AB genotype (390, 303, 88 and 32 bp) and line 5 is BB genotype (303, 88 and 32 bp)

On the basis of the Hardy-Weinberg formulas, the expected frequencies of A and B alleles were 0.69 and 0.31, respectively. The expected frequencies of the three genotypes were 47.61% (AA), 42.78% (AB) and 9.61% (BB). The observed numbers of genotypes were 44% (AA), 50% (AB) and 6% (BB). The most frequent genotype for LEP *Sau3AI* loci in observed population was AB with 50 individuals. Table 2 shows frequencies of three detected genotypes AA, AB and BB. The calculated χ^2 test value was 2.848, indicating Hardy-Weinberg genetic equilibrium in the population.

Table 2. Allele and genotype frequencies of LEP *Sau3AI* loci

Cows (n=100)	LEP *Sau3AI* loci					χ^2
	Genotype			Allele		
	AA	AB	BB	A	B	2.848-
Number	44	50	6	0.69	0.31	
Frequency	0.4761	0.4278	0.0961			

P > 0.05

Pomp et al. [7] verified that the frequency of a restriction fragment length polymorphism (*Sau3AI*) in bovine LEP gene was different between *Bos Taurus* and *Bos indicus* cattle breeds, being possible that genotype differences in leptin could explain some of the phenotypic variation observed between breeds of cattle. It is generally accepted that leptin may be a strong candidate gene for economically import production traits such as meet quality, feed intake and reproduction function. Passos et al. [11] described effect of leptin polymorphism on its expression on adipose tissues in beef cattle, when frequency of A allele was 0.91 and B allele 0.09. A higher frequency of A allele (0.95) found Javanmard et al. [12], they verified associations between *Sau3AI* polymorphisms and breeding value of milk traits in Holstein cows. These findings were similar previously reported for Holstein-Friesian cows [10], Black-and-White cows [13] and Holstein bulls [12]. Almeida et al. [14] found significant effect of *Sau3AI* polymorphism on calving interval and weight at first calving, when allele A seem to increase calving interval. According Passos et al. [11] leptin controls feed intake and therefore, animals with higher leptin gene expression will probably have lower daily weight gain than others with similar forage offer and nutritional condition and probably will also have longer calving interval. Kulig et al. [15] showed, than polymorphism in leptin gene is associated with growth traits in Limousine cattle. Moreover was this polymorphism associated with energy balance and fertility [16, 10], milk yield [13, 10] and somatic cell count in milk [15].

4. Conclusions

Genetic polymorphisms in the bovine leptin gene have been detected by using PCR-RFLP method. The PCR-RFLP method employed to screening the LEP *Sau3AI* SNP permitted the identification of both alleles. In the studied population of 100 Slovak Pinzgau cows were detected all three AA, AB and BB. The frequent allele in studied population was allele A with observed frequency of 0.69. The B allele was present at a lower frequency (0.31). The most frequent genotype was AB with observed frequency 0.5.

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