Genotyping of the Leptin Receptor Gene in Crna Slavonska Pig – Preliminary Results Suggests New Variants of the Promoter

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Summary

Researches on polymorphisms in the porcine *LEPR* gene and their association with economic traits were widely performed in the past. Manny polymorphisms in different part of the *LEPR* gene were described and majority of them was associated with economic traits such as growth and fatness. In present study, *LEPR* gene in 68 Crna Slavonska pigs was genotyped for *Hinf*I polymorphism in the 3.8kb part of *LEPR* promoter, for *Hpa*II and *Rsa*I polymorphisms in the intron 4 and for *Ape*KI polymorphism in the exon 14. Allelic and genotype frequencies on polymorphic sites were calculated. Restriction of the 3.8 kb of the promoter region with *Hinf*I revealed presence of two distinct restriction patterns, which haven't been described so far. Their exact location and also their potential role in *LEPR* expression, as well as their impact on important economic traits should be explored in the future. Allelic and genotypic frequencies for other three polymorphic sites studied were more or less comparable with previous findings in the literature.

Key words

Crna Slavonska pig, *LEPR* polymorphisms

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Introduction

The leptin receptor (LEPR) belongs to the type I cytokine receptor. At least 6 isoforms arising from alternative splicing are found in the LEPR family, including a long form, 4 short forms, which are distinct due to the length of the cytoplasmic region and a soluble circulating form (Tartaglia et al., 1995). In mammals, leptin receptor (*LEPR*) gene plays an important role in the control of feed intake, energy homeostasis, body weight regulation and fat mobilization. It modulates feed intake and GH secretion (Barb et al., 2006).

Due to its relevance on important economic traits such as growth and fatness, investigation on polymorphisms in the porcine *LEPR* gene and their association with economic traits were widely performed during the last decade.

Studies on Iberian x Landrace experimental cross reported significant effects of SNPs located in *LEPR* on pig productive traits (*Ovilo et al., 2005*). A significant effect on fatness and growth has been reported for *LEPR* c.1987 C>T polymorphism in this population. The effect of this SNP on growth and fatness has been confirmed also for other pig breed crosses (crossbred Iberian x Meishan, Duroc x Iberian and Duroc x Landrace/Large White pigs). Differential *LEPR*b (long form) expression connected to this SNP was found in hypothalamus (Ovilo et al., 2010). Moreover, Mackowski et al. (2005) identified a significant association between a *Tsp*509I RFLP *LEPR* genotype and backfat over shoulder. Hirose et al. (2014) genotyped the *LEPR* polymorphism c.2002C>T in exon14 (*Ape*KI restriction site) with an impact on average daily gain and backfat thickness. Biallelic polymorphisms in the intron 4 were found with *Hpa*II and *Rsa*I restriction enzymes by Stratil et al. (1998) and co-dominant inheritance of both polymorphisms was confirmed. *LEPR-Hpa*II polymorphism in Slovak Large White pigs showed significant impact on backfat thickness and lean meat percentage (Bauer et al., *2009*), which was also confirmed on Large White x Landrace crossbreeds (Trakovická et al, *2016*). The frequencies 0.214 and 0.786 were detected for *LEPRHpa*II alleles A and B in Slovak Large White pigs, while the frequency of the *LEPR-Rsa*I allele A was only 0.00357 (Bauer et al., *2009*). For the LEPR *Hinf*I polymorphism, alleles A and B were described (Vincent et al., *1997*). The frequencies for the A allele were lower than in Hampshire, Landrace, Duroc and Large White (from 0.9 to 0.18) and much higher than in Meishan (0.75).

Therefore, the aim of the present study was to genotype animals originating from Crna Slavonska pig at Leptin receptor (LEPR) gene and to estimate allele frequencies in investigated polymorphisms

Materials and methods

Genomic DNA was isolated from muscle samples of 68 Black Slavonian pigs using GeneJET™ Genomic DNA Purification Kit (Thermo Scientific™) following the manufacturer's instructions.

HinfI polymorphism:

Polymerase Chain Reaction (PCR) was carried out in a final volume of 25 µl which contained 0.5 µM concentration of corresponding forward and reverse primers (Vincent et al., 1997), 12.5 µl of Maxima Hot Start Green PCR Master Mix (2X) (Thermo Scientific™), 50-100 ng of template DNA and nuclease free water to the final volume. Amplification conditions were 95°C for 7 min, followed by 35 cycles of 95°C (30s), 57.5°C (30s) and 72°C (3min 30s), with a final extension step of 10 min at 72°C. The 3.8kb PCR product was then digested overnight with *Hinf*I restriction endonuclease at 37°C and the products were checked on 3% agarose gel.

*Hpa*II and *Rsa*I polymorphisms.

The PCR reactions were performed in a volume of 25 µl containing 50 - 100 ng genomic DNA, standard PCR buffer, 1.5 mM MgCl2, 200 mM each dNTP, 5 pmol each primer (Stratil et al., *1998*) and 1,0 U Taq polymerase (Thermo Scientific™). Amplification conditions were 95°C for 7 min, followed by 35 cycles of 95°C (50s), 65°C (50s) and 72°C (2min), with a final extension step of 10 min at 72°C. PCR products were visualized on 2% agarose gel and the rest of products were divided into two restriction reactions, one containing *Hpa*II and the other *Rsa*I restriction endonucleases. Both reactions were incubated at 37°C and the products were visualized on 4% agarose gel.

*Ape*KI polymorphism

The PCR reactions were performed in a volume of 15 µl containing 50 ng genomic DNA, standard PCR buffer, 1.5 mM MgCl2, 200 mM each dNTP, 5 pmol each primer (Hirose et al., 2014) and 1,0 U Taq polymerase (Thermo Scientific™). Amplification conditions were 95°C for 7 min, followed by 35 cycles of 95°C (450s), 64°C (45s) and 72°C (45s), with a final extension step of 10 min at 72°C. PCR products were checked on 3% agarose gel. The restriction reaction with *Ape*KI was run overnight at 75°C. Products of restriction reaction were checked on 5% agarose gel.

Calculating genotypic and allelic frequencies

For all analyzed polymorphisms, except of *Hinf*I, as it was found to be monoallelic, genotypic and allelic frequencies were calculated. Genotypic frequencies were obtained by dividing the number of each genotype by the whole number of samples. Allelic frequencies were determined in compliance with Hardy-Weinberg basic formulas, $p2 + 2pq + q2 = 1$ and $p + q = 1$.

Results

Restriction of the 3.8 kb leptin receptor promoter with *Hinf*I revealed that majority of the genotyped samples possessed BB genotype (58 from 68 analyzed, i.e. 85.3%) with bands of approximate length of 2100, 700, 395, 240, 140, 110, 50 and 40 bp, previously described by Vincent et al. (1997). Among analyzed samples, we didn't find any of AA or AB genotype, as stated in above mentioned research. Nevertheless, two new genotypes were discovered. The first one, named N1, was found in 2 samples (3%) and is characterized by two additional bands of approximate lengths 300 and 160 bp. The second one, N2, was present in 8 samples (12%) and is characterized by two additional bands of approximate lengths 500 and 200 bp (figure 1).

Restrictions of 2kb part of the *LEPR* intron 4 with *Hpa*II and *Rsa*I revealed biallelic polymorphisms in both cases. Fragment lengths after restriction with *Hpa*II were for allele A 2kb (not cut) and for allele B 1450 and 550 bp. For *Rsa*I, fragment lengths of the A allele were 1kb, 349, 334 and 300bp, and for the B allele 750, 349, 334, 300 and 250bp. Genotypic and allelic frequencies

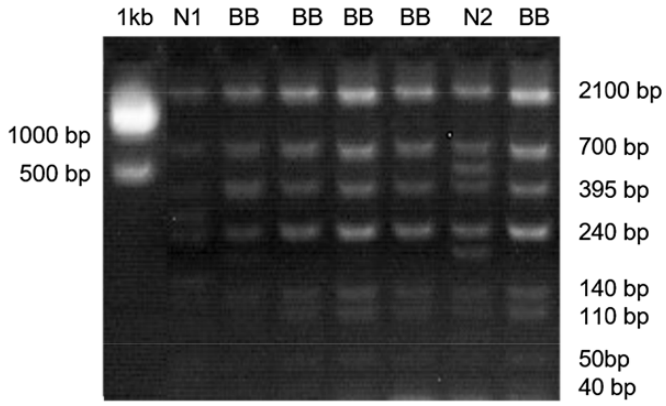

Figure 1. Restriction of the 3.8 kb leptin receptor promoter with *Hinf*I revealed two new genotypes, N1 and N2 in the picture

Table 1. Genotypic and allelic frequencies of analyzed *LEPR* polymorphisms with indicated location in the *LEPR* gene

Polymorphism	Location	Genotypic frequencies			Allelic frequencies	
		AA	AB	BB	A	B
*Hpa*II	intron 4	0.04	0.16	0.80	0.12	0.88
*Rsa*I	intron 4	0	0.09	0.91	0.04	0.96
*Ape*KI	exon 14	0.12	0.54	0.34	0.38	0.62

of both polymorphisms are shown in the table 1. For both polymorphisms, frequencies of the A allele were low, 0.125 for the *Hpa*II and 0.04 for the *Rsa*I, in which genotype AA was completely absent.

Digestion of the 133bp part of *LEPR* exon 14 with *Ape*KI also revealed a biallelic polymorphism with uncut allele A (133bp) and cut allele B (107 and 26 bp). Genotypic and allelic frequencies are shown in the table 1. Allelic frequencies were 0.38 for allele A and 0.62 for allele B and all three genotypes (AA, AB and BB) were found.

Discussion

Discovered new restriction patterns of the *LEPR* promoter region suggest presence of new SNPs in that region in Crna Slavonska pigs. It is well known that polymorphisms in the promoter region can affect transcription factors binding sites, which in turn influences transcription and expression of genes (*van 't Hooft et al., 1999*). Also, promoters have role in splicing of introns and some polymorphisms in the promoter region can lead to alternative splicing (Cramer et al., 1999). So polymorphisms in *LEPR* promoter could impact not only quantity of LEPR in different tissues, but also its form, especially when taking into account that at least six different splicing forms of LEPR exist (Tartaglia et al., *1995*). The quantity of *LEPR* expression could have an impact on different performance (average daily gain) and meat quality traits (backfat thickness, intramuscular fat). Considering above mentioned, new polymorphisms in the promoter region certainly deserve further attention.

Regarding allelic frequencies for *Hpa*II and *Rsa*I polymorphisms in the intron 4, allelic frequency of the A allele on *LEPR-Hpa*II locus was 0.12, while in literature different values for different breeds were described: 0.214 for Slovak Large White and 0.0484 for Landrace pigs (Bauer et al., 2009), while *Stratil et al.* (1998) observed frequencies ranging from 0.07 in Meishan, between 0.17 and 0.29 for Landrace, Czech Meat Pig, Pietrain and Black Pied Přestice, to 0.50 for Large white and 0.75 for Hampshire. However, small number of animals per breed were included in the research, ranging from 6 to 15. Allelic frequencies for the *LEPR- Rsa*I locus A allele, reported by *Bauer et al.* (2009), were 0.00357 in Slovak large white and 0.008 for Landrace pigs, which is comparable with our results, where the A allele frequency was 0.04. In Crna Slavonska pig, genotypic frequencies for *Hpa*II polymorphism were between values, reported by *Bauer et al.* (2009) for the Landrace and Slovak large white. Also, on *LEPR- Rsa*I locus the AA genotype was completely absent in both breeds and the frequency of AB genotype was low, which coincides with our results of the present study.

Allelic and genotypic frequencies on *LEPR- Ape*KI locus in Crna Slavonska pigs were similar with that reported by Hirose et al. (2014) for Duroc pig breed.

Conclusions

Genotyping four polymorphic sites in different part of the *LEPR* gene revealed two new restriction patterns of the *LEPR* promoter region, suggesting a presence of new SNPs in that region in Crna Slavonska pigs. Their exact location and also their potential role in *LEPR* expression, as well as their impact on important economic traits should be explored in the future. Allelic and genotypic frequencies for other three polymorphic sites included in our study were more or less comparable with previous findings in the literature. Nevertheless, correlation of all investigated *LEPR* polymorphic sites with important performance and meat quality traits in Crna Slavonska pigs is planned for the future.

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