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Institute of Genetics and Animal Breeding, Jastrzębiec, Poland

# Impact of LEP and LEPR gene polymorphisms on functional traits in Polish Holstein-Friesian cattle

**Jolanta Komisarek***

Department of Cattle Breeding and Milk Production,
Poznan University of Life Sciences,
Wojska Polskiego 71 A, 60-625 Poznań, Poland

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**Five single nucleotide polymorphisms in leptin and leptin receptor genes were analysed for their possible impact on estimating breeding values for somatic cell count score (SCS) in milk, longevity and reproductive traits. Used were 309 active Polish Holstein-Friesian bulls. The *LEP*-C(-963)T, *LEP*-Y7F, *LEP*-R25C, *LEP*-A80V, and *LEPR*-T945M genotypes were identified using the PCR-RFLP method. For linked leptin mutations, the additional haplotype analysis was performed. The results obtained suggest that three polymorphisms of bovine *LEP* gene may be associated with non-return rate in cows. The most significant effect was found for LEP-A80V. Moreover, the LEPR-T945M mutation seemed to be related to the age at first insemination.**

**KEY WORDS: cattle / functional traits / LEP / LEPR / polymorphism / reproduction**

During last decades, the strong genetic progress achieved in milk yield led to decreased reproductive performance in dairy cows. Declined fertility is believed to result from the negative energy balance (NEB), caused by high milk production combined with limited feed intake in early lactation. NEB was found to be associated with reduced plasma concentrations of several hormones, including insulin-like growth factor 1, insulin and leptin [Block *et al*. 2001, Reist *et al*. 2003]. These factors are thought to link changes in body energy reserves with changes in reproductive performance in cattle.

*Corresponding author: komisjol@jay.up.poznan.pl

Leptin is a proteinous hormone produced primarily by white adipose tissue and involved in regulation of feed intake, energy expenditure, growth and body composition, as well as immune system functions and several aspects of reproduction [Houseknecht *et al*. 1998]. In growing dairy heifers, leptin was shown to play a role in the initiation of puberty [Chelikani *et al*. 2009]. During early post-partum period, its concentrations were found to be positively correlated with pulsatile LH secretion and timing of the first ovulation after calving [Kadokawa *et al*. 2000, 2006].

Effects of leptin are exerted through six receptor isoforms, but only long form (LEPR-b) is fully functional and responsible for most hormone physiological functions [Tartaglia 1997]. A widespread expression of LEPR-b suggests that although most of leptin actions are mediated centrally, at the level of hypothalamus, it may also act in many peripheral tissues, including gonadal tissue [Silva *et al*. 2002]. In cattle and sheep, leptin was shown to affect directly the ovarian steroidogenesis [Kendall *et al*. 2004, Nicklin *et al*. 2007].

Genes encoding leptin (LEP) and leptin receptor (LEPR) are located in bovine chromosomes 4 and 3, respectively [Pomp *et al*. 1997, Pfister-Genskow *et al*. 1997]. Several polymorphisms have been found in *LEP* [Konfortov *et al*. 1999, Lagonigro *et al*. 2003, Liefers *et al*. 2005] and *LEPR* [Liefers *et al*. 2004, Guo *et al*. 2008]. Some of them may affect either activity or expression of leptin and its receptor. The aim of this study was to analyse the relationship between five single nucleotide polymorphisms (SNPs) in *LEP* and *LEPR* genes (listed in Table 1), and selected functional traits in cattle.

**Table 1**. Description of the single nucleotide polymorphisms considered in this study

| Gene | SNP code (and other codes used in literature) | Sequence poly-morphism | Gene region | Polypeptide region | Source |
|---|---|---|---|---|---|
| Leptin (*LEP*) | C(-963)T | C/T | promoter | - | Liefers *et al*. [2005] |
| Leptin (*LEP*) | Y7F (A252T) | A/T | exon 2 | signal sequence | Lagonigro *et al*. [2003] |
| Leptin (*LEP*) | R25C (C305T, R4C, C73T, Lep*Kpn2*I) | T/C | exon 2 | α-helix A | Konfortov *et al*. [1999] |
| Leptin (*LEP*) | A80V (Lep*Hph*I, A59V) | C/T | exon 3 | α-helix B | Konfortov *et al*. [1999] |
| Leptin receptor (*LEPR*) | T945M | T/C | exon 20 | intracellular domain of LEPR-b | Liefers *et al*. [2004] |

## Material and methods

The study included 309 Polish Holstein-Friesian bulls (Black-and-White type, with the average share of HF genes of 97%) from the active dairy population, born between 1992 and 2000.

Traits analysed were estimated breeding values (EBVs) obtained from the officially published assessments in January 2009 (http://wycena.izoo.krakow.pl) for non-return rate to 56 days after the first insemination in heifers (NRH56, %), non-return rate to 56 days after the first insemination in cows (NRC56, %), calving-to-first insemination interval after first calving (CFI, days), calving-to-conception interval after first calving (CCI, days), age at first insemination (AFI, days), somatic cell count score of milk (SCS, ln), and length of productive life (LPL, days). The mean reliability of EBVs was 0.77, ranging from 0.52 to 0.99. The higher breeding values for LPL indicate the increased longevity of daughters, the higher breeding values for NRH56 and NRC56 indicate the increased per cent of daughters which have become pregnant after first insemination, whereas the higher breeding values for CFI, CCI, AFI and SCS may be interpreted as indicative of shorter intervals from first calving-to-first insemination and from first calving-to-conception in daughters, decreased age at first insemination of daughters, and lower somatic cell count in milk of daughters, respectively.

Genomic DNA for molecular analyses was isolated from semen using the standard phenol method. Genotypes were determined with the PCR-RFLP technique as described by Szyda and Komisarek [2007]. The Genomatix MatInspector (ver. 8.0) programme (http://www.genomatix.de) was used to search for potential transcriptional factors (TF) binding domains in the leptin gene promoter region around the C(-963)T polymorphism site. For three linked SNPs in the leptin gene (C(-963)T, R25C and A80V), the haplotype assignment was performed. Haplotypes were inferred from homozygotes and single-site heterozygotes, whereas double and triple heterozygotes were excluded from the analysis.

Impact of LEP and LEPR gene polymorphisms on bulls' breeding values was analysed by one-way ANOVA followed by the post-hoc Duncan's test. Additionally, allele and haplotype substitution effects were estimated by regressing EBVs on the number of copies of each allele or haplotype carried by each individual. In both models, EBVs were weighted by their corresponding reliabilities. Statistical analyses were carried out using STATISTICA (ver. 8) software.

All investigations were performed according to the rules accepted by the Local Commission for Ethics in Animal Experimentation Investigation on Animals.

**Results and discussion**

Allele frequencies of five SNPs analysed in this study for 309 Polish Holstein-Friesian bulls (Tab. 2) did not differ notably from those reported for other HF cattle populations [Buchanan *et al*. 2003, Liefers *et al*. 2004, 2005, Madeja *et al*. 2004]. Very low frequencies of the minor alleles revealed for polymorphisms Y7F and T945M (0.02 and 0.08, respectively), resulted in lack of animals with the Y7F-TT genotype and only two with T945M-TT genotype in the population studied. The T945M-TT genotype class, as too small for reliable statistical estimations, was excluded from

**Table 2**. Genotype and allele frequencies

| SNP | Genotype frequencies | | | Allele frequencies | |
|---|---|---|---|---|---|
| LEP-C(-963)T | CC – 0.28 | CT – 0.55 | TT – 0.17 | C – 0.56 | T – 0.44 |
| LEP-Y7F | AA – 0.97 | AT – 0.03 | TT – 0.00 | A – 0.98 | T – 0.02 |
| LEP-R25C | TT – 0.19 | TC – 0.52 | CC – 0.29 | T – 0.45 | C - 0.55 |
| LEP-A80V | CC – 0.50 | CT – 0.43 | TT – 0.07 | C – 0.72 | T – 0.28 |
| LEPR-T945M | TT – 0.01 | TC – 0.15 | CC – 0.84 | T – 0.08 | C – 0.92 |

the association analysis. Distributions of all genotypes followed the expected values obtained according to the Hardy-Weinberg law.

The computer analysis of promoter of bovine leptin gene with the use of MatInspector software showed that C(-963)T mutation might co-localize with a binding site for several transcriptional factors (Fig. 1). Positive matches were found only for the T allele sequence, whereas they were absent in the C variant. No sequence with 100% identity to TF-binding sites was identified. A highest sequence similarity (99%) was found for the hematopoietically expressed homeobox, known also as the proline-rich homeodomain protein (HEX/PRH) binding site. The HEX/PRH protein is a critical controller of vertebrate development, regulating cell proliferation and differentiation, and required for the formation of many vital organs. In adults, its expression was detected e.g. in thyroid, lung, liver, oocytes and hematopoietic cells [Soufi and Jayaraman 2008]. However, presence of HEX/PRH in adipose tissue, the primary leptin secretion site, has not been reported, so far.

Significant results of the association analysis are presented in Table 3. The LEPR-T945M polymorphism was found

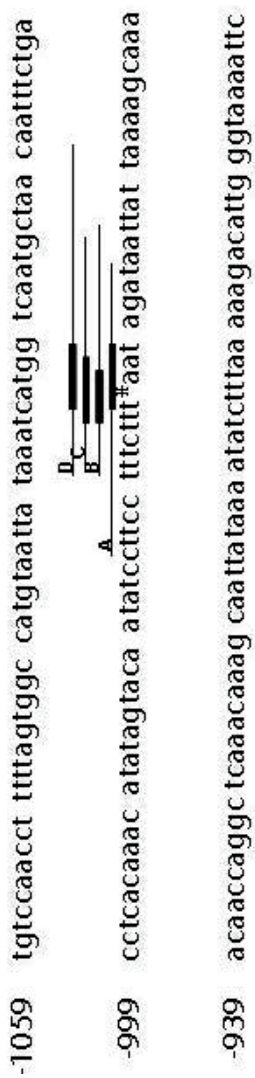


Fig. 1. The 180 bp sequence (from -1059 to -880 bp upstream the transcription start site at position 2998 according to GenBank, accession No. AB070368) of bovine leptin gene promoter region. Positions of the putative binding regions (with more than 90% sequence similarity) for transcriptional factors (A – hematopoietically expressed homeobox/proline-rich homeodomain protein, B – homeodomain transcription factor HOXC13, C – NK6 homeobox 1, D – Phox2a and Phox2b) in the vicinity of the C/T polymorphism site at position -936 (marked with an asterisk) are shown with thin lines, while the TF core-binding sequences (100% identity) are shown with bold lines.

**Table 3**. Least squares means (LSM) and standard errors (SE) of estimated breeding values (EBVs) for functional traits in bulls with different LEP and LEPR genotypes, and regression coefficients with standard errors (SE) for the number of copies of the LEP-C(-963)$T^T$, LEP-R25$C^C$, LEP-A80$V^T$ and LEPR-T945$M^C$ alleles representing half of the allele substitution effects

| SNP | Effect | | Trait | | |
|---|---|---|---|---|---|
| | | | NRC56 | AFI | SCS |
| LEP-C(-963)T | Genotype | CC (N=88) | 98.8 (10.9) | 102 (8) | 101 (10) |
| | | CT (N=169) | 97.0 (9.6) | 102 (9) | 100 (11) |
| | | TT (N=52) | 95.3 (9.6) | 103 (9) | 99 (10) |
| | | Overall P | 0.124 | 0.605 | 0.399 |
| | Allele substitution | α/2 | -1.7 (0.9) | 0.4 (0.7) | -1.1 (0.9) |
| | | P | **0.049** | 0.541 | 0.207 |
| LEP-R25C | Genotype | TT (N=58) | 95.6 (10.4) | 103 (9) | 99 (10) |
| | | TC (N=162) | 96.7 (9.8) | 102 (8) | 100 (11) |
| | | CC (N=89) | 98.8 (10.9) | 102 (7) | 100 (10) |
| | | Overall P | 0.052 | 0.341 | 0.950 |
| | Allele substitution | α/2 | 1.6 (0.7) | -0.6 (0.6) | 0.2 (0.7) |
| | | P | **0.021** | 0.267 | 0.797 |
| LEP-A80V | Genotype | CC (N=155) | 96.0[a] (10.5) | 102 (8) | 99 (19) |
| | | CT (N=131) | 97.8 (9.6) | 103 (9) | 101 (11) |
| | | TT (N=22) | 100.4[a] (11.6) | 102 (6) | 101 (11) |
| | | Overall P | **0.037** | 0.394 | 0.113 |
| | Allele substitution | α/2 | 1.9 (0.8) | 0.7 (0.6) | 1.6 (0.8) |
| | | P | **0.012** | 0.303 | 0.055 |
| LEPR-T945M | Genotype | TC (N=47) | 96.7 (9.7) | 105[a] (9) | 100 (10) |
| | | CC (N=260) | 97.3 (10.1) | 103[a] (8) | 100 (10) |
| | | Overall P | 0.717 | **0.014** | 0.839 |
| | Allele substitution | α/2 | 0.6 (1.6) | -3.3 (1.3) | -0.3 (1.7) |
| | | P | 0.717 | **0.014** | 0.839 |

NRC56 – non-return rate in cows, AFI – age at first insemination, SCS – somatic cell score in milk.
Means within columns bearing the same superscript differ at P≤0.05.
P values significant at P≤0.05 are shown in bold; P values significant at P≤0.10 are underlined.

to affect significantly the bulls' EBVs for AFI (age at first insemination). Daughters of bulls with the TC genotype were characterized by a lower age at first insemination compared to daughters of CC homozygotes. These findings should be treated with caution, because the considerable discrepancy in T945M allele frequencies reduced the power of the association test. They suggest, however, the possible effect of LEPR gene polymorphism on maturation time in cattle. Earlier, Liefers *et al*. [2004] revealed that the T945M was correlated with the leptin plasma concentrations and might influence the signal transduction pathway of the hormone.

C(-963)T, R25C and A80V polymorphisms influenced EBVs for non-return rate in cows (NRC56). As these three SNPs are positioned close to each other in bovine leptin gene, they may be in linkage disequilibrium, and the revealed effects might be ascribed to only one of them. On the other hand, it can not be excluded, that the

underlying mutation is the other closely linked polymorphism, not tested in this study. The most significant genotype and allele substitution effects were found for A80V, and the T allele appeared to be the most favourable variant for NRC56. Additionally, this variant tended to positively affect the EBV for somatic cell score in milk (SCS). The LEP-A80V is a conservative substitution, as both alanine and valine belong to the same group of non-polar amino acids. This SNP should not, therefore, undererlie the variation of phenotype. Moreover, the known binding sites of leptin protein to its receptor [Peelman *et al*. 2004] do not include the α-helix B, that contains the Ala to Val substitution site. On the other hand, the LEP-A80V is located at the between-species conserved region of leptin, that may be important for maintaining the structural integrity of the protein [Zhang *et al*. 1997].

Results of the haplotype analysis also indicate that A80V is more probable causative mutation affecting non-return rate variation than C(-963)T and R25C. Inference of haplotypes was possible for 140 bulls. The C(-963)T, R25C and A80V SNPs occurred in six haplotypes: TTC (38.2%), CCT (30.7%), CCC (29.3%), CTC (0.7%), CTT (0.7%) and TCC (0.4%). Due to the sample size, their impact on EBVs for functional traits was tested only for TTC, CCT and CCC. The association analysis showed that only CCT haplotype (including T allele at A80V *locus*) significantly affected bulls' breeding values for NRC56 (non-return rate to 56 days after first insemination in cows) (Tab. 4).

**Table 4**. Regression coefficients (and their standard errors) for the number of copies of the TTC, CCT and CCC haplotypes representing half of the haplotype substitution effects

| Haplotype | C(-963)T-R25C-A80V | Trait | |
|---|---|---|---|
| | | NRH56 | NRC56 |
| TTC | α/2 | -0.90 (0.86) | -1.37 (0.92) |
| | P | 0.298 | 0.113 |
| CCT | α/2 | 2.23 (1.80) | 2.40 (1.11) |
| | P | 0.095* | 0.033** |
| CCC | α/2 | -0.67 (1.16) | 1.25 (0.69) |
| | P | 0.565 | 0.491 |

NRH56 – non-return rate in heifers, NRC56 – non-return rate in cows.
*Significant at P≤0.10. **Significant at P≤0.05.

There are very few published reports on the relationships between SNPs analysed in this study and reproductive traits in cattle [Liefers *et al*. 2002, 2005, Almeida *et al*. 2003, Komisarek and Antkowiak 2007], and none of them included the non-return rate. In Holstein-Friesians, the LEP-C(-963)T polymorphism was shown to affect the first observed oestrus [Liefers *et al*. 2005]. In Jerseys, Komisarek and Antkowiak [2007] found effect of neither LEP-C(-963)T nor R25C polymorphisms on cows'

fertility. However, they revealed the positive impact of TT genotype at the LEP-A80V polymorphism on number of inseminations per conception as well as on days open and calving interval. The association with the latter trait was confirmed neither in Holstein-Friesians (this study) nor in beef cattle [Almeida *et al*. 2003]. Moreover, Liefers *et al*. [2002] showed no correlation between LEP-A80V and commencement of luteal activity.

In conclusion, the results obtained suggest that three SNPs in bovine leptin gene may be associated with EBVs for non-return rate in cows, the most significant being the effect found for LEP-A80V. Additionally, the LEPR-T945M mutation seemed to be related to age at first insemination. These initial association between leptin and its receptor gene polymorphisms and reproductive traits require, however, to be validated in other cattle populations.

**REFERENCES**

1. ALMEIDA S.E.M., ALMEIDA E.A., MORAES J.C.F., WEIMER T.A., 2003 – Molecular markers in the LEP gene and reproductive performance of beef cattle. ***Journal of Animal Breeding and Genetics*** 120, 106-113.
2. BLOCK S.S., BUTLER W.R., EHRHARDT R.A., BELL A.W., VAN AMBURGH M.E., BOISCLAIR Y.R., 2001 – Decreased concentration of plasma leptin in periparturient dairy cows is caused by negative energy balance. ***Journal of Endocrinology*** 171, 339-348.
3. BUCHANAN F.C., VAN KESSEL A.G., WALDNER C., CHRISTENSEN D.A., LAARVELD B., SCHMUTZ S.M., 2003 – An association between a leptin single nucleotide polymorphism and milk and protein yield. ***Journal of Dairy Science*** 86, 3164-3166.
4. CHELIKANI P.K., AMBROSE D.J., KEISLER D.H., KENNELLY J.J., 2009 – Effects of dietary energy and protein density on plasma concentrations of leptin and metabolic hormones in dairy heifers. ***Journal of Dairy Science*** 92, 1430-1441.
5. GUO Y., CHEN H., LAN X., ZHANG B., PAN C., ZHANG L., ZHANG C., ZHAO M., 2008 – Novel SNPs of the bovine LEPR gene and their association with growth traits. ***Biochemical Genetics*** 46, 828-834.
6. HOUSEKNECHT K.L., BAILE C.A., MATTERI R.L., SPURLOCK M.E., 1998 – The biology of leptin: a review. ***Journal of Animal Science*** 76, 1405-1420.
7. KADOKAWA H., BLACHE D., MARTIN G.B., 2006 – Plasma leptin concentrations correlate with luteinizing hormone secretion in early postpartum Holstein cows. ***Journal of Dairy Science*** 89, 3020-3027.
8. KADOKAWA H., BLACHE D., YAMADA Y., MARTIN G.B., 2000 – Relationships between changes in plasma concentrations of leptin before and after parturition and the timing of first post-partum ovulation in high-producing Holstein dairy cows. ***Reproduction, Fertility and Development*** 12, 405-411.
9. KENDALL N.R., GUTIERREZ C.G., SCARAMUZZI R.J., BAIRD D.T., WEBB R., CAMPBELL B.K., 2004 – Direct in vivo effects of leptin on ovarian steroidogenesis in sheep. ***Reproduction*** 128, 757-765.
10. KOMISAREK J., ANTKOWIAK I., 2007 – The relationship between leptin gene polymorphisms and reproductive traits in Jersey cows. ***Polish Journal of Veterinary Sciences*** 10, 193-197.
11. KONFORTOV B.A., LICENCE V.E., MILLER J.R., 1999 – Re-sequencing of DNA from a diverse panel of cattle reveals a high level of polymorphism in both intron and exon. ***Mammalian Genome*** 10, 1142-1145.

12. LAGONIGRO R., WIENER P., PILLA F., WOOLLIAMS J.A., WILLIAMS J.L., 2003 – A new mutation in the coding region of the bovine leptin gene associated with feed intake. ***Animal Genetics*** 34, 371-374.
13. LIEFERS S.C., TE PAS M.F.W., VEERKAMP R.F., VAN DER LENDE T. 2002 – Associations between leptin gene polymorphisms and production, live weight, energy balance, feed intake, and fertility in Holstein heifers. ***Journal of Dairy Science*** 85, 1633-1638.
14. LIEFERS S.C., VEERKAMP R.F., TE PAS M.F.W., DELAVAUD C., CHILLIARD Y., PLATJE M., VAN DER LENDE T., 2005 - Leptin promoter mutation affects leptin levels and performance traits in dairy cows. ***Animal Genetics*** 36, 111-118.
15. LIEFERS S.C., VEERKAMP R.F., TE PAS M.F., DELAVAUD C., CHILLIARD Y., VAN DER LENDE T., 2004 – A missense mutation in the bovine leptin receptor gene is associated with leptin concentrations during late pregnancy. ***Animal Genetics*** 35, 138-141.
16. MADEJA Z., ADAMOWICZ T., CHMURZYŃSKA A., JANKOWSKI T., MELONEK J., ŚWITOŃSKI M., STRABEL T., 2004 - Effect of leptin gene polymorphisms on breeding value for milk production traits. ***Journal of Dairy Science*** 87, 3925-3927.
17. NICKLIN L.T., ROBINSON R.S., MARSTERS P., CAMPBELL B.K., MANN G.E., HUNTER M.G., 2007 – Leptin in the bovine corpus luteum: receptor expression and effects on progesterone production. ***Molecular Reproduction and Development*** 74, 724-729.
18. PEELMAN F., VAN BENEDEN K., ZABEAU L., ISERENTANT H., ULRICHTS P., DEFEAU D., VERHEE A., CATTEEUW D., ELEWAUT D., TAVERNIER J., 2004 – Mapping of the leptin binding sites and design of a leptin antagonist. ***Journal of Biological Chemistry*** 279, 41038-41046.
19. PFISTER-GENSKOW M., HAYES H., EGGEN A., BISHOP M.D., 1997 – The leptin receptor (LEPR) gene maps to bovine chromosome 3q33. ***Mammalian Genome*** 8, 227.
20. POMP D., ZOU T., CLUTTER A.C., BARENDSE W., 1997 – Mapping of leptin to bovine chromosome 4 by linkage analysis of a PCR-based polymorphism. ***Journal of Animal Science*** 75, 1427.
21. REIST M., ERDIN D., VON EUW D., TSCHUEMPERLIN K., LEUENBERGER H., DELAVAUD C., CHILLIARD Y., HAMMON H.M., KUENZI N., BLUM J.W., 2003 – Concentrate feeding strategy in lactating dairy cows: metabolic and endocrine changes with emphasis on leptin. ***Journal of Dairy Science*** 86, 1690-1706.
22. SILVA L.F.P., VANDEHAAR M.J., WEBER NIELSEN M.S., SMITH G.W., 2002 – Evidence for a local effect of leptin in bovine mammary gland. ***Journal of Dairy Science*** 85, 3277-3286.
23. SOUFI A., JAYARAMAN P.S., 2008 – PRH/Hex: an oligomeric transcription factor and multifunctional regulator of cell fate. ***Biochemical Journal*** 412, 399-413.
24. SZYDA J., KOMISAREK J., 2007 – Statistical modeling of candidate gene effects on milk production traits in dairy cattle. ***Journal of Dairy Science*** 90, 2971-2979.
25. TARTAGLIA L.A., 1997 – The leptin receptor. ***Journal of Biological Chemistry*** 272, 6093-6096.
26. ZHANG F., BASINSKI M.B., BEALS J.M., BRIGGS S.L., CHURGAY L.M., CLAWSON D.K., DIMARCHI R.D., FURMAN T.C., HALE J.E., HSIUNG H.M., SCHONER B.E., SMITH D.P., ZHANG X.Y., WERY J.P., SCHEVITZ R.W., 1997 – Crystal structure of the obese protein leptin-E100. ***Nature*** 387, 206-209.

Jolanta Komisarek

## Wpływ polimorfizmu w genach LEP i LEPR na cechy funkcjonalne bydła rasy polskiej holsztyńsko-fryzyjskiej

S t r e s z c z e n i e

Analizowano pięć polimorfizmów w genach leptyny i receptora leptyny pod względem ich wpływu na szacowaną wartość hodowlaną dla zawartości komórek somatycznych w mleku, długowieczności oraz cech reprodukcyjnych. Badania prowadzono w grupie 309 buhajów hodowlanych rasy polskiej holsztyńsko-fryzyjskiej. Genotypy w *loci* LEP-C(-963)T, LEP-Y7F, LEP-R25C, LEP-A80V i LEPR-T945M identyfikowano metodą PCR-RFLP. Dla sprzężonych mutacji w genie leptyny przeprowadzono dodatkową analizę haplotypów. Uzyskane wyniki wskazują, że trzy polimorfizmy w genie LEP mogą być związane ze wskaźnikiem niepowtarzalności rui u krów, przy czym najistotniejszy efekt wykryto dla LEP-A80V. Ponadto, mutacja LEPR-T945M może mieć wpływ na wiek pierwszego unasienienia.