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An insertion/deletion polymorphism in the 3'UTR encoding region of the porcine prolactin (*PRL*) gene*

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A novel mutation, insertion/deletion (InDel) was found in the porcine prolactin gene exon 5 coding for the 3'UTR. Polymorphism was detected and identified using PCR-MSSCP and DNA sequencing techniques, and then analysed with PCR-RFLP using *Hpy*CH4III endonuclease. Inserted/deleted was an 11-bp TGTTTCTTAAC motive that occurred once or twice at position 201-222 in the amplified DNA fragment. The investigations covered 150 gilts of the Puławska breed from the herds under the Preserve Breeding Project. The frequencies of the inserted and deleted PRL gene variants were 0.34 and 0.66, respectively. The estimated value of heterozygosity ($\hat{H}$) was 0.253 and the polymorphism information content (PIC) amounted to 0.348.

KEY WORDS: prolactin gene / polymorphisms / pigs / Puławska breed

Prolactin is a polypeptide hormone that is synthesized in and secreted from specialized cells of the anterior pituitary. In mammals it has a major role in the regulation of lactation and mammary growth and plays multiple homeostatic roles in the organism [Ben-Jonathan *et al*. 1996].

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The prolactin gene has been mapped to chromosome 6 in humans. It is formed by 5 exons and 4 introns, of total size 10 kbp. The comparative mapping studies have shown that there is conserved synteny between human chromosome 6 and pig (*Sus scrofa*) chromosomes 1 and 7, while the physical mapping revealed the porcine prolactin gene location on chromosome 7p.1.1-pl.2.

Conservation of heterozygosity at such a level so that population could be maintained in the breeding structure is addressed through the guidelines of the Pig Breeding Program [Walkiewicz *et al.* 2005]. Moreover, the assessment of the pig breeds genetic structure is compatible with a global tendency to determine and promote biodiversity of livestock [Martinez *et al.* 2000, Megens *et al.* 2008]. A key factor appears to be the selection of genetic markers of high polymorphicity that facilitates their further application, e.g. for the sows fertility. These needs are met by prolactin gene, which in pigs exhibits, among others, the luteotropic activity.

Material and methods

The investigations included a total of 150 gilts of the Puławska breed. The animals were kept in 9 farms under the Preserve Breeding Program of the Pulawska pig. The maintenance conditions and feeding regimen satisfied the requirements as well as animal welfare. Blood samples were taken from the external jugular vein (*v. jugularis externa*) from each gilt with disposable syringes (Monovette, Sarsted) using EDTA as an anticoagulant. DNA isolation from blood leukocytes was performed with the procedure of Kawasaki [1990], as modified by Coppieters *et al.* [1992], employing a commercial Dneasy* Blood & Tissue Kit (Qiagen). The PCR primers matching a part of the pig *PRL* gene exon 5 were designed basing on "Primer 3" program (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) and pig *PRL* gene sequence (GeneBank Ref. Seq - NM_213926.1): forward: 5' - GAATCAAGGAGAATGAGGTC 3'; reverse: 5' – TTAA-GAGGAGATGCGTTACA - 3'. The primer synthesis was performed at the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw.

The PCR samples of 20 µl volume contained 5 µl RedTaq™ReadyMix™PCR (SIGMA), 0.2 µl of each primer (0.2 µM), 3.6 µl water (SIGMA) and 2 µl DNA (20 mg/µl). The DNA amplification reactions were performed in the PTC-200 PELTIER THERMAL CYCLER at 95°C for 3 min, followed by 35 cycles of 94°C for 45 s, 58°C for 30 s, and 72°C for 2 min, followed by a final extension step at 72°C for 7 min, and completed by cooling for 10 min at 10°C. The identity of the PCR products was tested by electrophoresis in 4% agarose gels with ethidium bromide in 1 × TBE buffer. Gels were visualised and documented by the Molecular Imager System FX (BioRad, CA, USA).

Polymorphism was detected using the PCR-multitemperature single-strand conformation polymorphism (PCR-MSSCP) technique. The PCR product was mixed (1:1v/v) with loading buffer (95% formamide, 0.5M EDTA pH 8.0, and 0.05% bromophenol blue), denatured at 95°C for 5 min, chilled on ice, and loaded on 8%

polyacrylamide gels (acrylamide : bis-acrylamide - 29 : 1) containing 1 × TBE buffer. Gels (180 mm × 140 mm) were run at 300 V for about 3 h (1h at 30°C, 50 min. 18°C, 40 min. at 8°C) in 2 × TBE. Before the PCR-MSSCP procedure, a pre-running was applied (about 60 min., 300 V at constant temperature 30°C). The fragments were visualized by silver staining. The PCR products of different SSCP patterns were sequenced in ABI PRISM 310 Genetic Analyzer, according to procedure for BigDye Terminator Cycle Sequencing Kit designated by Applied Biosystems – Applera Corporation. The sequenced fragments of the exon 5 were analysed with Sequencer 4.2 (Gene Codes Corporation).

To perform the RFLP analyses the PCR-amplified DNA fragments were digested for 3 hours with 5 U of the *Hpy*CH4III endonuclease (FERMENTAS, Vilnius, Lithuania). Then, the 2% agarose gel separation of the *Hpy*CH4III digestion DNA fragments was made.

To assess the genetic variation within the studied population of Puławska pigs with the found InDel polymorphism, the following statistical indices were applied: (1) the frequency of alleles and genotypes was computed with the least square method; (2) the heterozygosity coefficient ($\hat{H}$) was estimated according to Ott [1992] and Weir [1990]; (3) the Polymorphic Information Content (PIC) was computed after the formula presented by Botstein *et al.* [1980]. Analyses were made with the software package SAS.

Results and discussion

PCR amplified was the fragment of the porcine gene exon 5 coding for the 3`UTR. Obtained were DNA fragments of different length – 287 or 298 bp – thus indicating on an insertion/deletion polymorphism in the *PRL* gene. The existence of such polymorphism was confirmed by using the PCR-multitemperature single-strand conformation polymorphism (PCR-MSSCP) technique (Fig. 1), and subsequently precisely identified by sequencing DNA samples showing different MSSCP patterns (Fig. 2). There was found an insertion/deletion of the 11-bp motive (TGTTTCTTAAC)

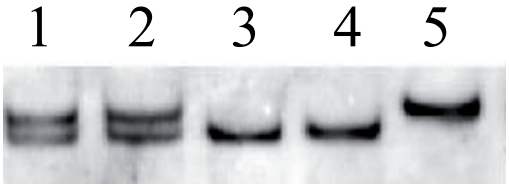

Fig. 1. PCR-MSSCP analysis of the In/Del polymorphism in the porcine *PRL* gene exon 5, coding for 3'UTR. Polyacrylamide gel electrophoresis of the amplified fragment of the porcine *PRL* gene. Lanes: 1, 2 show the heterozygous *In/Del* genotype (287/298-bp); lanes 3,4 – homozygous *Del/Del* genotype with one 11-bp TGTTTCTTAAC motive (287/287-bp), lane 5 – homozygous *Ins/Ins* genotype with two 11-bp motives (298/298-bp).

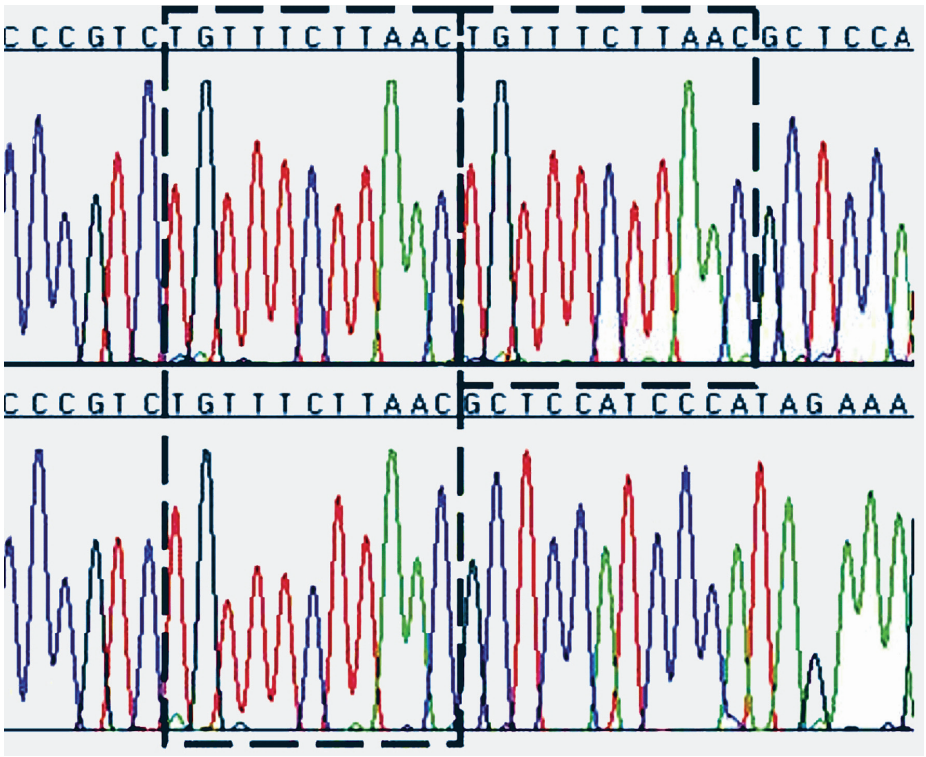

Fig. 2. Partial sequence of the amplified *PRL* gene fragment comprising 3'UTR coding region within 5-th exon. The upper panel – PRL gene variant "Ins", with two 11-bp TGTTTCTTAAC motives; the bottom panel – variant "Del", with one 11-bp motive (both in the dotted-line frames).

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5′GAATCAAGGA GAATGAGGTC TACTCTGTGT GGTCCGGACT TCCCTCCCTG   50

  CAGATGGCTG ATGAAGACAC TCGCCTTTTT GCTTTTTATA ACCTGCTCCA 100

  CTGCCTACGC AGGGATTCAC ATAAGATTGA CAATTATCTC AAGCTTCTCA 150

  AGTGCCGAAT CATCTACGAC AGCAACTGCT AAGCCCACGT CCATCCCGTC 200

  TGTTTCTTAAC TGTTTCTTAAC GCTCCATC CCATAGAAAG ATTCTTTTAG 250

  TTTTATAGCT TTTTAATGCA TGCTTGGGTG TAACGCATCT CCTCTTAA 3′ 298
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Fig. 3. The sequenced fragment of the porcine prolactin gene exon 5 as analysed with the Sequencer 4.2 (Gene Codes Corporation) program. The repeated **TGTTTCTTAAC** motive is shown in bold. Underlined is the sequence encoding 3'UTR in the PRL mRNA.

at position 200-222 in the amplified DNA fragment. In the "Del" variant of the *PRL* gene the single TGTTTCTTAAC motive was found while in the "Ins" variant this motive was repeated twice (Fig. 2 and 3). The sequence obtained for PRL gene was deposited in GenBank (acc. no. EU825774).

Separation of the *PRL* gene variants (differing by 11-bp) carried out on 4% agarose gel yielded some identification difficulties. The restriction analysis with the computer package Annhyb has shown that the examined site is recognized by the restriction enzyme *Hpy*CH4III, therefore the enzyme RFLP-*Hpy*CH4III technique was employed to genotype a cohort of 150 Puławska gilts. The enzyme cleaved the 298-bp PCR product of the Ins allele to produce two restriction fragments – 86 and 212 bp, whereas the 287-bp amplicone of the *Del* allele (with the 11-bp deletion) remained uncut (Fig. 4).

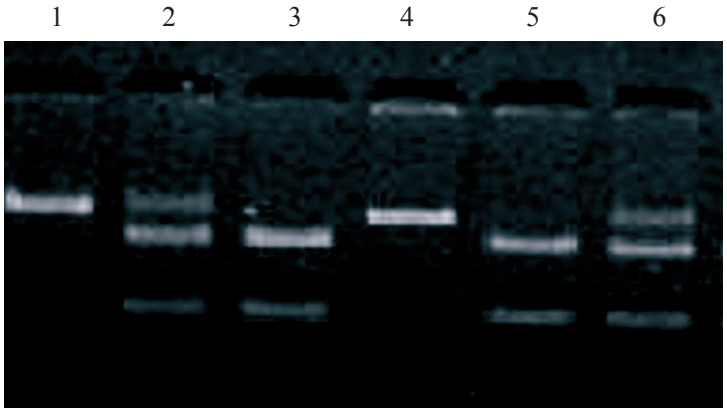

Fig.4. The In/Del polymorphism in the porcine *PRL* gene as recognized by the *Hpy*CH4III endonuclease digestion. Allele Ins – the 298 PCR product cleaved into 212-bp and 86-bp restriction fragments; allele *Del* – 287-bp PCR product not cleaved with the enzyme. Genotype *Ins/Ins* – paths 1 and 4; genotype *Del/Del* – paths 3 and 5; genotype *Ins/Del* – paths 2 and 6.

Table 1. Genetic variation coefficients of the Puławska gilts population under study as referred to the In/Del polymorphism in the *PRL* gene exon 5

PIC	Ĥ	Frequency of	
		alleles	genotypes
0,348	0,253	*Ins* – 0.34	*Ins/Ins* – 0.21
		Del – 0.66	*Ins/Del* – 0.25
			Del/Del – 0.54

In the studied population of Puławska pigs the frequency of prevailed the *Del/Del* genotype was 0.54, and the frequency of genotypes *Ins/Del* and *Ins/Ins* amounted to 0.25 and 0.21, respectively (Tab. 1). The frequency of allele *Ins* was 0.34 and that of allele *Del* – 0.66. The estimated value of heterozygosity ($\hat{H}$) was 0.253 and the Polymorphism Information Content (PIC) amounted to 0.348 (Tab. 1).

Only a few polymorphisms were detected so far in the porcine prolactin gene. Two polymorphic sites in intron 2, both recognized as RFLP-*BstU*I, were reported by Vincent *et al*. [1998]. Searching for putative molecular marker for reproductive traits in pigs resulted in finding a SNP, the *C*499*T* transition in the 5'UTR coding region of the porcine *PRL* gene [Korwin-Kossakowska *et al*. 2006].

In the present study, a novel mutation of the InDel type was identified in exon 5 encoding the 3'UTR PRL region. The 11-bp deletion/insertion was found at position 212-222 using PCR-MSSCP and DNA sequencing techniques. For convenience, this polymorphism was further identified with RFLP-*Hpy*CH4III. In the studied population of 150 Puławska gilts, included into the conservation breeding project, the frequency of alleles Ins and Del was 0.34 and 0.66, respectively. Such distribution of values was reflected in a number of genotype variants of the Puławska gilts, *i.e*. homozygotes and heterozygotes. The obtained heterozygosity coefficient (0.253) proved to be markedly lower than those recorded for the Puławska breed with other genetic markers [Babicz *et al.* 2003]. The preliminary studies of Puławska breed indicate the presence of genetic variation between different herds of Puławska pigs (not shown).

The present results indicate the potential employment of the novel polymorphism in the *PRL* gene for the genetic variation determination, especially that such deletion/insertion could influence the stability of the PRL transcript. Moreover, genetic variation in the investigated PRL *locus* can be used in future studies of the effect of the *PRL* gene on reproduction performance of Puławska pigs.

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Polimorfizm typu insercja/delecja w rejonie kodującym 3'UTR genu prolaktyny (*PRL*) świni

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

Wykryto nowy polimorfizm w genie prolaktyny (*PRL*) świni. Mutacja typu insercja/delecja (InDel) została zidentyfikowana w 5 eksonie kodującym fragment 5'UTR. Polimorfizm wykryto i zidentyfikowano metodami PCR-MSSCP i sekwencjonowania DNA, a następnie analizowano metodą PCR-RFLP, za pomocą endonukleazy restrykcyjnej *Hpy*CH4III. Allele *Ins* i *Del* genu *PRL* różniły się obecnością jednego z dwóch 11-nukleotydowych motywów TGTTTCTTAAC w pozycji 212-222 w amplifikowanym fragmencie DNA. Zgenotypowano 150 loszek rasy puławskiej, pochodzących ze stad objętych hodowlą zachowawczą. Frekwencja alleli Ins i Del wyniosła odpowiednio 0.34 i 0.66. Wartość wskaźnika heterozygotyczności ($\hat{H}$) wyniosła 0,253, a współczynnika informatywności polimorfizmu (PIC) – 0,348.