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Search for SNP mutations of chosen genes of the hypothalamic-pituitary axis in laying hens

Poszukiwanie mutacji SNP wybranych genów osi podwzgórzowo-
-przysadkowej kur nieśnych

Summary. The analyses performed aimed at the detection of possible point mutations in genes from the multi-level neuro-hormonal system, whose products are expressed in various organs of the bird reproductive system. In future, they could be a basis in the search for relations between polymorphism and the level of hatchability traits. The study involved two lines: Rhode Island White (200 individuals) and Rhode Island Red (100 individuals) laying hens. Molecular analyses consisted in preliminary verification of the SNP mutation in selected gene fragments: Insulin-like growth factor 1 (*IGF1*, exon 3, GeneID: 418090, amplicon length 479 bp) and Progesterone receptor (*PGR*, intron 6, GeneID: 396198, amplicon length 362 bp). The PCR-RFLP reaction was the second step of the investigations. A point mutation in the 38965 gene nucleotide within exon 3 was detected as a result of the *IGFI* gene sequencing reaction. The sense mutation involved an adenine-to-guanine nucleotide change. In both hen lines, allele A and a mutated allele B with a 50% frequency were identified using the PCR-RFLP method for further analyses. The *PGR* sequencing reaction revealed a mutation in nucleotide position 34216 in intron 6 of the gene. Here, transition of the adenine to guanine nucleotide occurred. Allele A was identified, after 155 bp and 207 bp fragments had been obtained in the restriction analysis, as well as a mutated 362 bp allele B. Heterozygosity in the study *locus* was 0.38 for the RIW line and 0.23 for RIR.

Key words: *IGFI* gene, *PGR* gene, SNP, laying hens

INTRODUCTION

Estimation of bird breeding merits in terms of reproduction is difficult due to the specificity of these traits. Research is being conducted with the aim of improving the reliability of the BLUP estimation of hatchability traits in laying hens [Rozempolska-

-Rucińska *et al.* 2009, 2010, Rozempolska-Rucińska 2010], for which selection decisions are made only on the basis of the relationship matrix rather than upon consideration of the usability traits of individuals. Investigations have been initiated to determine candidate genes for markers of reproduction traits in hens, which could possibly facilitate BLUP estimation and selection of birds for the breeding flock. Their use is particularly justified in the case of low heritability of traits, which is an objective hindrance in effective selection. This group of genes includes the *Insulin-like growth factor 1* and *Progesterone receptor* genes; they are part of the multi-level neuro-hormonal system and their products are expressed in various organs of the reproductive system and may determine the level of hatchability-related reproduction traits. The *IGFI* gene enhances expression of FSH receptors, and together with FSH and LH sustains survival of the granular layer cells, stimulates the process of their proliferation and differentiation, controls the activity of ovaries and exerts an effect on estradiol and progesterone secretion. The *Insulin-like growth factor 1 (IGF1, somatomedin C*) gene has been localised in the short arm of chromosome 1, near the centromere [Klein *et al.* 1996]. Polymorphism of *IGF1* and its relations with usability traits in poultry has been extensively described in literature [Nagaraja *et al.* 2000, Amills *et al.* 2003, Kim *et al.* 2004, Zhou *et al.* 2005, Bian *et al.* 2008, Tang *et al.* 2010].

Similarly to estradiol, progesterone participates in regulation of ovulation, differentiation of gonads and is responsible for the sexual and nesting behaviour in birds [Johnson *et al.* 1985, Gahr 2001]. Moreover, literature data indicate its considerable role in various physiological and pathological processes. Progesterone displays its activity through binding to specific intracellular receptors (PGR), through which it regulates numerous physiological and morphological processes [Camacho-Arroyo *et al.* 2007]. It is currently well-known that there are two alternative isoforms of the progesterone receptor. Camacho-Arroyo *et al.* [2007] reported occurrence of two progesterone receptor isoforms, which are functionally distinct in terms of their capacity of gene activation and regulation of physiological processes of chicks (full-length PR-B and short PR-A isoforms). The isoforms were detected in the brain, ovaries and oviducts of both adult hens and chick embryos. The hen *Progesterone receptor* (*PGR*, *NR3C3*) gene was identified in chromosome 1 [Toye *et al.* 1997]. Despite extensive data about the structure and function of the progesterone receptor, the accessible literature fails to provide information about mutations occurring within the *PGR* gene sequences in hens. Identification thereof would be essential due to the great range of functions that progesterone receptors have in numerous physiological processes in hens.

The analyses performed were a preliminary step of investigations aiming to determine candidate genes for markers of hatchability traits in laying hens. We have attempted at selection of possible point mutations of the genes in question that might serve as the basis for finding relationships between polymorphism and the level of bird hatchability traits.

MATERIAL AND METHODS

Blood sampled from the wing-vein of the laying hens and kept in test tubes containing the K_2EDTA anti-coagulant was the material for the genetic investigations. The study was conducted on a poultry farm and involved a pedigree population of two lines

of laying hens: Rhode Island White and Rhode Island Red, which were under individual control regarding the usability traits.

The first step of the investigations consisted in identification of single nucleotide polymorphism (SNP) in the particular gene fragments: *Insulin-like growth factor 1* (*IGF-1*, exon 3, GeneID: 418090, amplicon length 479 bp) and *Progesterone receptor* (*PGR*, intron 6, GeneID: 396198, amplicon length 362 bp). The choice of fragments designated for the analyses was made taking into considerations the presence of protein encoding sequences in a particular fragment; however, designing optimal primer sequences for the protein encoding regions (*PGR*) was not always possible. The analyses were carried out on 10 individuals from the RIR line and 10 from the RIW line. The birds were selected on the basis of the BLUP estimations of the reproduction traits (the breeding merit is determined to meet the needs of a commercial pedigree farm). The group selected was characterised by high variability of hatchability traits. Simultaneously, the relations between the bird lineages were analysed in order to select individuals with the least possible relatedness.

DNA was isolated from whole peripheral blood with the use of the QIAamp DNA Blood Mini Kit (Qiagen). The DNA solution was analysed with the quantitative-qualitative assay (Biophotometer, Eppendorf). PCR amplification was performed with the use of primer sequences designed by Primer 3 [http://frodo.wi.mit.edu/primer3/] software on the basis of the hen genome sequences (*PGR* – forward primer (5'–gccatcttctttcagcac-3'), reverse primer (5'–gagttagccaccacacctt-3'); *IGF-1* – forward primer (5'-gcaaccactcatagacagagg-3'), reverse primer (5'-tagcaggcagaacacatcag-3'). The PCR reaction and the composition of the reaction mixture were optimised. The amplification products were visualised on 2% agarose gel with respect to the size marker (Gene Ruler 50bp DNA Ladder, ready-to-use). The time-temperature profile of the PCR reaction consisted in: preliminary denaturation (95°C, 10 min), 35 cycles of: denaturation (95°C, 1 min), binding (60°C, 1 min for both gene fragments), elongation of the primers (72°C, 1 min), and final elongation of the primers (72°C, 7 min). The reaction mixture (sample volume – 27.5 μl) contained: 100–200 ng DNA; 1.1 μl 360 GC enhancer, 2.2 μl dNTP mix (2 mM/ml), 110 pM of each starter, 55 mM $MgCl_2$, 0.7 U AmpliTaq Gold polymerase.

The amplicons were purified (QIAquick PCR Purification Kit, QIAGEN) and sequenced (BigDye® Terminator v3.1 Cycle Sequencing Kits, Applied Biosystems) in both directions with the use of 3100 Avant Genetic Analyzer, Applied Biosystems. In the subsequent stage, the sequences obtained were analysed and point mutations were identified with the use of the DNASTAR programme.

Once SNP was observed, the PCR-RFLP reaction was performed in the chosen gene regions. The analyses involved 200 unrelated RIW and 100 RIR individuals. In the case of the *IGF-1* gene, the *Bsm* I restrictive enzyme was used, while for the *PGR* gene – *Dra* III. The results were statistically elaborated by determination of gene frequencies and genotypes in the population of the study hens. Heterozygosity was estimated in accordance to the formula provided by Ott [1985].

RESULTS AND DISCUSSION

As a result of IGF-1 gene sequencing, a point mutation was discovered in position 38965 of the gene nucleotide in exon 3. The sense mutation involved an adenine-to-guanine nucleotide change. The PCR-RFLP method employed in further analyses revealed allele A and a mutated allele B, the frequency of which exceeded 50% in both hen lines (Tab. 1). Three genotypes were identified: AA – 263 bp and 216 bp fragments, AB – 479 bp, 263 bp and 216 bp, and BB – a 479 bp fragment. Heterozygosity in the locus of the study gene was relatively high, reaching 0.45 in RIW and 0.46 in RIR. Heterozygotic individuals predominated in both lines.

Table 1. Table of contingency of alleles and genotypes of the *IGF1* gene in exon 3 in the population of laying hens
Tabela 1. Tabela kontyngencji alleli i genotypów genu *IGF1* eksonu 3 w populacji kur nieśnych

IGFI gene *IGFI* gen		Frequency – Frekwencja	
		Rhode Island White	Rhode Island Red
Alleles Allele	A	0.39	0.43
	B	0.61	0.57
Genotypes Genotypy	AA	0.16	0.20
	AB	0.46	0.46
	BB	0.38	0.34

Table 2. Table of contingency of alleles and genotypes of the *PGR* gene in intron 6 in the population of laying hens
Tabela 2. Tabela kontyngencji alleli i genotypów genu PGR intronu 6 w populacji kur nieśnych

PGR gene *PGR* gen		Frequency – frekwencja	
		Rhode Island White	Rhode Island Red
Allele Allele	A	0.79	0.80
	B	0.21	0.20
Genotypes Genotypy	AA	0.60	0.69
	AB	0.38	0.23
	BB	0.02	0.08

Studies carried out by other authors indicate a type A→C mutation at position 570 of this gene [Amills *et al.* 2003, Zhou *et al.* 2005]. Up to date, it has been found that polymorphism of the *IGF1* gene is associated with growth improvement, increased breast muscle weight, reduction of fat deposits in the abdominal cavity and increased skeletal system congruity, or egg weight [Nagaraja *et al*. 2000, Zhou *et al.* 2005]. Also, correlation between the *IGF1* polymorphism and body weight of cocks has been confirmed, thus the *IGF1* was determined to be the candidate gene for this trait [Bian *et al.* 2008]. The *IGF1* gene has been assumed to be the main gene for body weight and reproduction traits in hens [Tang *et al.* 2010].

Search for correlations between the mutation discovered in exon 3 and hen reproduction traits will be the next step of the study. Currently, it may be suggested on the basis of the frequency of the genotypes in the *IGF1 locus* that heterozygotic individuals are desirable in selection. This may result from possible relationships between a particular genotype and the level of traits or a trait taken into account in the selection criterion in the hen population investigated.

The sequencing reaction of the *PGR* gene revealed a mutation in position 34216 of intron 6. Here, transition of the adenine nucleotide to the guanine nucleotide took place.

The mutation resulted in loss of the *Dra III* endonuclease restriction site. Allele A with a sequence consistent with the reference sequence (155 bp and 207 bp fragments were obtained upon the restriction analysis) and a mutated allele B (no cleavage by restrictive endonuclease – 362 bp) were identified. Heterozygosity in the study *locus* was 0.38 for the RIW line and 0.23 for RIR. Although the frequency of the mutated allele was not low, reaching approximately 0.2, it is worth mentioning that there was a very low percentage of birds with a homozygotic genotype (Tab. 2). This may imply that unintentional selection against genotype BB takes place while choosing individuals for the breeding flock.

In conclusion, it may be claimed that the particular point mutations in the chosen fragments of the *PGR* and *IGF-1* genes are a basis for further analyses aimed at selection of candidate genes for markers of the usability traits in laying hens.

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Streszczenie. Przeprowadzone analizy miały na celu wytypowanie ewentualnych mutacji punktowych w genach należących do wielopoziomowego systemu neurohormonalnego, których produkty wykazują ekspresję w różnych organach układu rozrodczego ptaków. W przyszłości mogłyby one stanowić podstawę do poszukiwania powiązania pomiędzy polimorfizmem a poziomem cech wylęgowych. W badaniach wykorzystano dwa rody kur nieśnych Rhode Island White (200 osobników) i Rhode Island Red (100 osobników). Analizy molekularne obejmowały wstępną weryfikację mutacji typu SNP w wybranych fragmentach genów: Insulin-like growth factor 1 (*IGF1*, ekson 3, GeneID: 418090, długość amplikonu 479 pz) oraz Progesterone receptor (*PGR*, intron 6, GeneID: 396198, długość amplikonu 362 pz). Drugim etapem badań była przeprowadzona reakcja PCR-RFLP. W wyniku reakcji sekwencjonowania genu *IGFI* wykryto mutację punktową, w 38965 nukleotydu genu, mieszczącą się w obrębie eksonu 3. Mutacja typu sensownego dotyczyła podstawienia nukleotydu adeninowego na guaninowy. Wykorzystując w dalszych analizach metodę PCR-RFLP, stwierdzono występowanie w obu rasach kur allelu A i zmutowanego allelu B, którego frekwencja przekraczała ponad 50%. Przeprowadzając reakcje sekwencjonowania genu *PGR,* stwierdzono występowanie mutacji na pozycji 34216 nukleotydu, w intronie 6 genu. W tym przypadku nastąpiła tranzycja nukleotydu adeninowego na guaninowy. Zidentyfikowano allel A, uzyskując po analizie restrykcyjnej fragmenty o długości 155 pz i 207 pz oraz zmutowany allel B o długości 362 pz. Heterozygotyczność w badanym *locus* wynosiła 0,38 dla rasy RIW i 0,23 dla rasy RIR.

Słowa kluczowe: gen *IGFI*, gen *PGR*, SNP, kury nieśne