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Genetic Variability of Indels in the Prolactin and Dopamine Receptor D2 Genes and Their Association with the Yield of Allanto-Amniotic Fluid in Russian White Laying Hens

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ABSTRACT
Currently, there is virtually no information on genetic factors affecting the yield of allanto-amniotic fluid, which is the raw material for the production of human and animal vaccines. Association studies including this trait are beneficial for increasing productivity of a biotechnological line of chickens used for the production of 'Clean Eggs'. We examined here a population of the Russian White breed for the effects of indels in the prolactin (*PRL*) and dopamine receptor D2 (*DRD2*) genes on the yield of extraembryonic fluid (YEF) and embryo weight at 12.5 days of development. A 24-bp insertion in the 5' flanking region of the *PRL* gene significantly (P<0.01) increases YEF in the embryos. The heterozygous embryos contained the highest YEF (9.6 mL) than that of the homozygous insertion (9.4 mL) and deletion embryos (8.4 mL). We also found a significant association (P<0.001) between the *PRL* genotypes and egg weight (EW). The results of the present study suggest a significant association between the *PRL* gene variation and quantitative traits in the Russian White chickens, contributing to a long-term programme on the effective use of the genetic potential of Russian gene pool breeds and populations of chickens.

Keywords: Chicken population; Biotechnological line; Candidate genes; Insertion-deletion polymorphism; Economically important traits

1. Introduction

Extraembryonic fluid obtained from chicken eggs is beneficial in the production of human and animal vaccines against viruses, and in pharmaceutical studies. Currently, a complex epizootic situation and the need for ever-increasing production of embryonic viral vaccines for humans, farm animals and poultry, using the developing chick embryos (DCEs), require search for new genetic approaches to create specialized poultry lines to produce eggs with increased volumes of extraembryonic fluid, which will not only reduce the number of embryos used, but also lower the cost of the vaccine itself.

One of the perspective directions in breeding is the marker-assisted selection that capitalises on studies of the polymorphisms of different target genes or genome regions and their relationship with production traits and is widely

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used in animal husbandry (Fulton 2008). Variant detection and studies of association between candidate genes or genetic markers and economically important traits have been broadly attempted in commercial poultry populations (Dunn et al 2004) and native breeds (Li et al 2008; Guo et al 2016). However, application of this approach in non-commercial, germplasm poultry populations has been complicated by their small size and the need to maintain their genetic diversity.

The prolactin (*PRL*) and dopamine receptor D2 (*DRD2*) genes have been suggested among the most promising candidate genes, allelic variants of which could be associated with the production traits in poultry. The biological activity of PRL manifests via interaction with membrane receptors found in numerous target tissues (Fleenor et al 2006). PRL is considered to be more associated with egg production traits, as it is believed that this hormone determines the broody instinct in the body of laying hens (Romanov 2001; Reddy et al 2002, 2007; Wang et al 2009; Wang et al 2011). Broodiness has a negative effect on egg production, which is crucial in egg industry. Genetic changes in the regulatory system comprising *PRL* and its receptor, and entailing inhibition of their activity may be used as genetic markers for breeding laying hens with the reduced instinct of broodiness, and hence, with increased egg production (Bagheri Sarvestani et al 2013). Sequencing of the promoter region of the chicken *PRL* gene resulted in detection of a 24-bp insertion located at nucleotide position 377 to 354 (GenBank Accession No. AB011438). In several studies, it was observed that the presence of the insertion at this site of the promoter region in the *PRL* gene was correlated positively with the intensity of lay in hens and negatively with incubation behaviour (Reddy et al 2002, 2007; Cui et al 2006; Jiang et al 2009; Wang et al 2009; Wang et al 2011; Kulibaba & Podstreshnyi 2012). It was also found that individuals with the heterozygous genotype for insertion and deletion (*In/Del*) had the greatest expression level of PRL mRNA (Cui et al 2006). This may suggest that this polymorphic locus could be associated with the chick hatchability characteristics through modulation of the *PRL* gene at the transcriptional level. Also, there has been an attempt to identify association between this mutation and egg production traits. There were reports of a significant correlation ($P<0.05$) found between the indel locus polymorphism and egg production (Cui et al 2006). On the other hand, an efficiency of using the *PRL* gene was also studied as a candidate gene and a marker in the selection programme of Silkie chicken, but no significant associations between genetic variation at this gene locus and performance traits were identified (Wada et al 2008; Rowshan et al 2012).

Dopamine functionally interacts with PRL, which determines its indirect effect on the instinct of incubation. In some studies (Xu et al 2010), there was a suggestion that mutations in the chicken *DRD2* gene might also affect the broody instinct. Involvement of dopamine has been demonstrated in the processes of stimulation and inhibition of the PRL secretion in the brain. In addition, the inhibitory effect of dopamine on the PRL secretion was mediated by DRD2 in the pituitary (Youngren et al 1995; Al Kahtane et al 2003). In chickens, which were treated with antagonists or blockers of dopamine receptors, termination of incubation behaviour was identified due to the inhibition of PRL secretion (Hall & Chadwick 1984). An indel mutation was found in the 5'-flanking region of the *DRD2* gene, and effect of the *DRD2* indel polymorphisms on body weight was examined in Chinese and Japanese chicken breeds (Zhou et al 2010). Yet, the influence of variability in the genes of dopamine and its receptors on performance of chickens remains insufficiently clarified.

The Russian White breed was produced in the Soviet period (in 1929 to 1953) by crossing different White Leghorn populations with local chickens of Russian Central regions (Paronyan & Yurchenko 1989). More recently, a population under the name of 'Russian Snow White' was developed at the Russian Research Institute of Farm Animal Genetics and Breeding (RRIFAGB) Branch 'Genofond' (Tyshchenko 2002). The population is unique and characterized by tolerance of chicks to cold growing conditions, and is of particular interest for use in genetic research. It was created by selection for tolerance to low temperatures in the first days of life and for high egg production. Chicks of this breed can safely bear temperature regime of 8-10 degrees below normal and are also resistant to certain avian leukosis sarcoma complex diseases. Furthermore, possibly due to the recessive gene *sw* (Hutt 1951), 25% day old chicks in the population have completely snow-white (instead of yellow) down. This breed was also selected for increased resistance to leukemia, Marek's disease and carcinomas of the internal organs.

On the basis of this population, a specialized biotechnological line is bred at the RRIFAGB for the yield of extraembryonic fluid (YEF) to be used in the production of 'Clean Eggs' and vaccines in the bioindustry. For this purpose, we assessed 30-35-week females for extraembryonic fluid volume in 12.5-day DCEs (Lapa et al 2015).

The present study has been undertaken to test indel polymorphisms in the *PRL* and *DRD2* genes in the biotechnological line of Russian White chickens and search for their associations with YEF and egg laying traits as a part of a broader survey on genetic features of the Russian gene pool chicken breeds.

2. Material and Methods

2.1. Experimental population and traits

A population of the egg-type Russian White chickens was chosen for the study. It represents a biotechnological line of chickens used for the production of 'Clean Eggs'. The experimental group consisted of 160 females and 24 males. All selected roosters met the sperm quality requirements for artificial insemination.

To examine YEF, three to six consecutively laid eggs were collected for evaluation from each chicken at 34 weeks of age, weighed and labeled with the mother's pedigree number, egg weight, and date of lay. Then, the eggs were placed in an incubator. The age of embryos was chosen to be 12.5 days to assess the genetic potential of chickens in terms of YEF of their descendants, as found in the previous study (Lapa et al 2015). To determine YEF at 12.5 days of development, the eggs were taken out of the incubator and placed for two days in a refrigerator, and after that the volume of extraembryonic fluid was measured. To do this, the egg was weighed (to determine shrinkage), and the shell over the air pocket was broken and removed. Puncturing the exposed membrane and pushing the embryo aside with tweezers, the allantoic fluid was collected with a pipette and the volume of the allantoic fluid was measured. A total of 451 eggs were evaluated for YEF in 12.5-day-old embryos.

A number of performance traits were also recorded during the experiment time. In particular, chickens were assessed for egg production (by months for the entire laying period), body weight, age at first egg and egg weight at 30 weeks.

2.2. DNA sampling and genotyping

One hundred to 500 μL of whole blood per sample was collected as a DNA source from the wing vein into a tube containing anticoagulant (30 μL 200 mM EDTA). The whole procedure for collection of the blood samples of all animals was carried out in strict accordance following the standard protocols approved by the RRIFAGB. Samples were frozen and stored at -20 ºC.

DNA was isolated by standard phenol procedure using proteinase K (SibEnzyme, Russia) and diluted in TE buffer. The concentration and purity of the samples was determined using a NanoDrop 2000 instrument.

Polymerase chain reaction (PCR) was performed using Thermal Cycler T1000 (Bio-Rad, USA) as follows: initial denaturation for 5 minutes at 95 ºC, followed by 40 cycles of denaturation for 30 seconds at 95 ºC, annealing for 30 seconds at 60 ºC (for *PRL* gene) or 63 ºC (for *DRD2* gene) and extension for 30 seconds at 72 ºC, and final elongation for 5 minutes at 72 ºC.

The *PRL* and *DRD2* genes were tested for the presence of indel polymorphisms. The following primers were used for the PCR (Rahman 2014): *PRL* gene, forward primer: 5'-GGTGGGTGAAGAGACAAGGA-3'; reverse primer: 5'-TGCTGAGTATGGCTGGATGT-3'; *DRD2* gene, forward primer: 5'-TGCACTTCAATCCTTCCCAGCTT-3'; reverse primer: 5'-TTGCGCTGCCCATTGACCA-3'. Amplification products obtained for the *PRL* and *DRD2* genes were separated on 2% agarose gel. In case of *PRL* gene, the amplified fragment size was 130 and 154 bp depending on the 24-bp indel. For the *DRD2* gene, size of the PCR fragments was 165 and 187 bp due to the 22-bp indel (Figure 1 and 2).

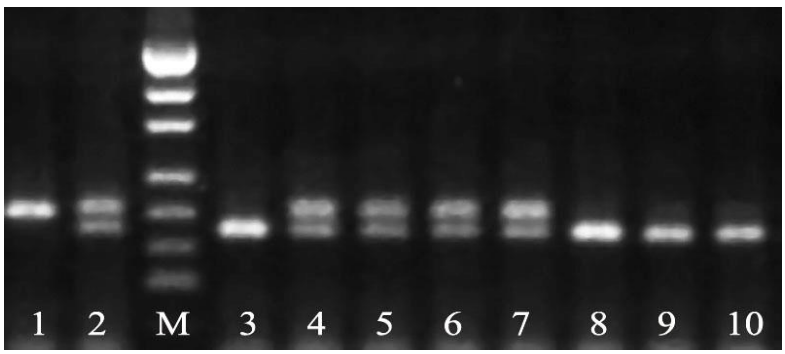

Figure 1- Electrophoregramme of the amplified fragments of the chicken *PRL* gene for genotypes *In/In* (154 bp, lane 1), *Del/Del* (130 bp, lanes 3, 8, 9 and 10), and *In/Del* (lanes 2, 4, 5, 6 and 7). Lane M= DNA size marker pUC19 DNA *Hae*III Digest (Sigma)

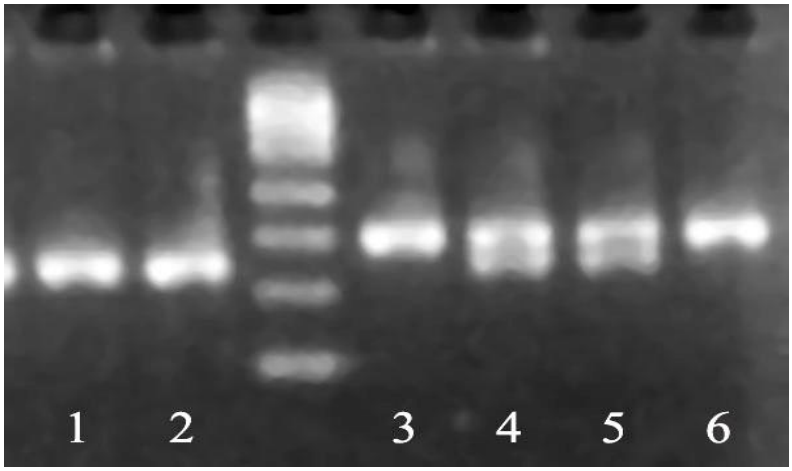

Figure 2- Electrophoregramme of the amplified fragments of the chicken *DRD2* gene for genotypes *In/In* (187 bp, lanes 3 and 6), *Del/Del* (165 bp, lanes 1 and 2), and *In/Del* (lanes 4 and 5). Lane M= DNA size marker pUC19 DNA *Hae*III Digest (Sigma)

2.3. Statistical analysis

Frequencies of genotypes and alleles were calculated based on the PCR genotyping data obtained. Deviation of frequencies from Hardy-Weinberg equilibrium was evaluated using the $\chi 2$ test.

Differences between genotypes and production traits were explored in SigmaPlot software package (version 12.0.5) using analysis of variance correction for multiple comparisons (All Pairwise Multiple Comparison Procedures).

3. Results and Discussion

To study genetic variability for indel polymorphisms in the *PRL* and *DRD2* genes, we genotyped respectively 155 and 140 laying hens of the Russian White breed. At the *PRL* locus, predominance of individuals homozygous for the 24-bp insertion (*In/In*) was observed. In the case of the *DRD2* gene, heterozygotes for the 22-bp indel (*In/Del*) prevailed (Table 1). Such a genotype distribution appears to be characteristic for egg-type breeds with a decent egg production, as also confirmed by other authors (Cui et al 2006; Kulibaba & Podstreshnyi 2012).

Table 1- The frequency of alleles and genotypes at the *PRL* and *DRD2* loci in the chicken population of the Russian White breed

Genes	*No. of observations*	*Alleles*		*Genotypes*			*H*	*PIC*	χ^2
		Del	*In*	*Del/Del*	*In/Del*	*In/In*			
PRL	152	0.26	0.74	0.07	0.37	0.56	0.37	0.298	0.081
DRD2	152	0.56	0.44	0.33	0.46	0.21	0.46	0.371	0.239

The analysis of the observed and theoretical distribution of genotypes for the *PRL* and *DRD2* gene did not reveal any deviations from Hardy-Weinberg equilibrium (with χ^2 being equal to 0.0808 and 0.1411, respectively; Table 1).

To examine potential associations and improve further the selection efficiency, we studied the effect of indel polymorphisms on basic performance traits in the Russian White chicken population. In particular, we looked for associations between polymorphic variants of the two genes and the main selected traits such as body weight at 52-week age, age at first egg, egg production for 360 days of life, and egg weight at 30-week age. The performance data was collected in 117 individuals genotyped for the *PRL* gene and in 104 birds genotyped for the *DRD2* gene.

According to the ANOVA results (Table 2), a significant difference ($P<0.001$) was found between the *PRL* indel genotypes relative to egg weight. Hens heterozygous for the insertion in the *PRL* gene promoter demonstrated an earlier

sexual maturity as well as a tendency for a greater egg production and egg weight. As for the *DRD2* gene, there were no significant associations between its indel genotypes and the characteristics studied including age at fist egg, egg production for 360 days of life and egg weight at 30-week age (Table 3).

Table 2- Genotype variants at the *PRL* locus as associated with economically important traits

Trait	*Genotype*			*P*
	Del/Del (N= 11)	*In/Del (N= 56)*	*In/In (N= 85)*	
Age at fist egg, days	168.3±2.0	166.4±0.6	167.4±0.5	0.336
Egg production for 360 days of life	136.7±7.9	145.2±2.2	141.7±2.4	0.391
Egg weight at 30 weeks of age, g	48.2±0.7	50.7±0.5	50.0±0.3	0.066
Body weight, g	1685±75	1677±25	1693±25	0.910

Del, deletion; In, insertion; P, significance value

Table 3- Genotype variants at the *DRD2* locus as tested for association with economically important traits

Trait	*Genotype*			*P*
	Del/Del (N=50)	*In/Del (N=70)*	*In/In (N=32)*	
Age at fist egg, days	166.9±0.8	167.5±0.5	166.5±0.9	0.610
Egg production for 360 days of life	143.9±3.1	141.49±2.4	143.3±3.8	0.804
Body weight, g	1667±32	1719±25	1662±39	0.326

Del, deletion; In, insertion; P, significance value

Using ANOVA, we analysed main quantitative estimates of extraembryonic fluids in the groups of polymorphic variants in the *PRL* and *DRD2* genes. Table 4 summarises the ANOVA results concerning effect of the hens' genotype on embryo weight and YEF in the 12.5-day-old embryos. The insertion in the *PRL* gene in the dams was associated with a YEF increase by 1.3 mL in the embryos produced from the heterozygous hens (*In/Del*) and by 1.1 mL from the homozygotes (*In/In*) as compared with the homozygotes having the deletion (*Del/Del*) in the *PRL* gene (P<0.01).

Table 4: Genotype variants at the *PRL* and *DRD2* loci as associated with yield of extraembryonic fluids (YEF) and embryo weight

Trait	*Genotype*			*P*
	Del/Del	*In/Del*	*In/In*	
PRL				
No. of eggs	32	173	246	
Egg weight, g	47.94±0.48[a]	50.52±0.31[b]	49.78±0.22	0.003
YEF, mL	8.391±0.327[c]	9.622±0.165[d]	9.423±0.121[e]	0.006[*]
Embryo weight, g	9.769±0.161	9.302±0.009	9.473±0.331	0.795
DRD2				
No. of eggs	149	205	97	
Egg weight , g	50.01±0.29	50.19±0.26	49.50±0.41	0.333
YEF, mL	9.19±0.17	9.64±0.150	9.42±0.19	0.116
Embryo weight, g	9.41±0.08	9.17±0.08	9.19±0.12	0.109

Del, deletion; In, insertion; P, significance value; YEF, yield of extraembryonic fluids; Significant difference; [a, b], P<0.001; [c, d], P<0.001; [c, e], P<0.01; [*], significant association, P<0.01

A variety of current poultry breeds can be divided into two groups, old breeds and novel lines and populations. Certain new populations have been or are being created on the base of old breeds and contemporary commercial lines. Breeding purpose in such new populations is often to solve problems of commercial strains including disease resistance, and to handle insistence to growing conditions and specific production technologies. Within the current breeding programme that focuses on the Russian White chicken breed kept at the RRIFAGB, one of the selection objectives is to create a population with high biotechnological qualities of eggs. Such a population is an ideal material for conducting effective selection and creating poultry lines that can be used in the production of embryonic viral vaccines.

At present, efforts are undertaken to develop a procedure of breeding chicken lines with an increased volume of allantoic/amniotic fluid for producing further a biotechnological virus-containing material (Lapa et al 2015). VALO Biomedia GmbH (http://www.valobiomedia.com/) is the world's largest supplier of specific pathogen-free (SPF) and 'Clean Eggs' used for vaccine manufacture. In Russia, there are no specialized poultry populations for vaccine production, with eggs from commercial crosses kept at large poultry farms being utilised for that purpose. The main feature of the SPF eggs is the absence of any vaccinations of hens, and that of 'Clean Eggs' is a gentle vaccination against diseases. There are also no enterprises for the production of 'Clean' and SPF eggs in Russia, and SPF eggs have to be imported from other countries, while 'Clean Eggs' are substituted for chicken eggs of commercial crosses exposed to a rigid schedule of vaccination, which significantly reduces the quality of the vaccines. There are no reports about genetic factors that may influence the amount of allantoic/amniotic fluid, which is the raw material for the production of vaccines, and the titer of the vaccine virus in it. Therefore, search for genomic associations with the increased amount of amniotic fluid is one of the areas in our research. Accordingly, we evaluated 30-35-week parents in terms of extraembryonic fluid from 12.5-day-old DCEs and genotypes in the *PRL* and *DRD2* genes and observed a significant association with the *PRL* indel variation.

4. Conclusions

In summary, our search for possible candidate genes including the *PRL* and *DRD2* genes associated with the volume of extraembryonic fluid in 12.5-day-old embryos has suggested that YEF may be affected by the genes that control the growth and development of the embryo. As found in the current study, one of such candidate genes is the *PRL* gene showing the indel polymorphism in its promoter region associated with YEF and egg weight in the Russian White chicken population. The results can be used within a long-term programme on the effective use of the genetic potential of Russian chicken breeds and populations, and specifically for breeding poultry lines with performance characteristics of interest (YEF, egg and meat production, egg quality, etc.) and producing progeny with desirable genotypes.

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Abbreviations and Symbols	
PRL	Prolactin
DRD2	Dopamine receptor D2
DCEs	Developing chick embryos
YEF	Yield of extraembryonic fluids
RRIFAGB	Russian research institute of farm animal genetics and breeding
SPF	Specific pathogen-free

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