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A POLYMORPHISM ANALYSIS OF DYSFERLIN GENE LOCUS IN CHICKEN GENE POOL

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Abstract

Disferlin refers to proteins involved in the repair of the muscle membrane. It is assumed that some mononucleotide substitutions in the dysferlin gene (*DYSF*) are associated with the formation of muscle mass in poultry. In this work, for the first time in chickens of the Russian White breed, four mononucleotide substitutions have been identified that are in intron 32 on chromosome 4: rs317801013 (G/A) at position 90672849, rs16455118 (C/A) at 90672756, rs318045896 (A/G) at 90672862, and T/G at 90672805. Mononucleotide polymorphism T/G on chromosome 4 at position 90672805 was submitted for registration to the ENSEMBL database, since it was detected for the first time in the species *Gallus gallus*. In addition, for the first time, we assessed the frequency of occurrence of genotypes and the deviation of the observed genotype distribution from the expected Hardy-Weinberg equilibrium in the gene pool chickens of the Russian White breed. The aim of this work was to study single nucleotide polymorphisms (SNPs) of the dysferlin gene in chicken gene pools and to identify possible associations of *DYSF* gene polymorphisms with economically valuable traits. We studied meat (Cornish) chickens, laying hen (Russian White, Rhode Island, Aurora, Black-and-White Austrolorp, Leningrad Calico) and decorative breeds (Russian Crested, Light Brahma, Bare-Necked) from the gene pool population of the Genetic Collection of rare and endangered breeds of chickens (RRIFAGB, St. Petersburg—Pushkin). DNA was isolated from blood collected from the axillary vein by phenol extraction. Illumina Chicken 60K SNP iSelect BeadChip chip (Illumina, Inc., USA) was used to analyze the rs16455118 polymorphism. The observed and expected frequencies of genotypes *AA*, *AC*, *CC* and their deviations from the Hardy-Weiberg equilibrium were analyzed in laying hens based on the replacement of adenine for cytosine in the dysferlin gene (rs16455118). The reliability of the data obtained was assessed using the Pearson χ^2 test. Dysferlin gene polymorphism was analyzed by sequencing a 237 bp *DYSF* gene region on chromosome 4 in 76 Russian White hens. We analyzed the NCBI and ENSEMBL databases to identify the SNPs found. An analysis of the frequency of genotypes and alleles was carried out for four identified substitutions. Genotyping of 185 hens using Illumina Chicken 60K SNP iSelect BeadChip technology revealed a single nucleotide polymorphism SNP rs16455118. It was found that the allele frequencies shifted towards an increase in heterozygous genotypes of *AC* in decorative chickens while the *AA* genotype was present in the minority. In laying hen, the homozygous genotype *AA* had the highest frequency of occurrence, the *CC* genotype was small in number, and it was completely absent in the population of chickens of the Aurora breed. The Cornish beef breed had a more even distribution of genotypes as compared to decorative and laying hens. Sequencing of the 237 bp dysferlin gene region located on chromosome 4 in Russian White chickens identified mononucleotide substitutions in the intron 32. Single nucleotide substitutions G/A (rs317801013), C/A (rs16455118), A/G (rs318045896) corresponded to those in the publicly available chicken genome in the databases NCBI (https://www.ncbi.nlm.nih.gov/SNP) and ENSEMBL (https://www.ensembl.org/index.html). The single nucleotide polymorphism T/G at 90672805 has been detected for the first time. The shift in the genetic balance in the gene pool of Russian White hens indicates the effect of the founder or selection pressure on the region of the SNP rs16455118. The almost complete absence of heterozygotes in laying hen may indicate inbreeding or strong selection pressure. Our findings can be helpful in the future search for SNPs associated with productivity trait in chickens to create a system of molecular markers to accelerate breeding progress.

Keywords: dysferlin gene, SNP, single nucleotide polymorphism, poultry farming, allele, hens

Currently, traditional poultry breeding has reached a plateau, and progress in increasing productivity has declined significantly. The use of molecular genetic markers is becoming the most effective method for accelerating the selection process in farm animal production [1]. This approach is based on the search for single nucleotide polymorphisms (SNPs) associated with various traits in poultry, including using SNP chip panels. Based on several million SNP loci identified through years of scientific research by Illumina, Inc. (USA), chips of medium and high density were created for the main species of farm animals, including chickens, which allows obtaining data on the localization of areas and genes associated with traits. Sequencing makes it possible to study in more detail the regions of candidate genes to identify variants of genetic polymorphism associated with the traits of interest. For example, using the Illumina Chicken 60K SNP iSelect BeadChip chip technology, a reliable association of the 32nd intron region on chromosome 4 in the dysferlin gene (*DYSF*) with white fluff in Russian White chickens was found [1].

The most urgent task in poultry farming is to increase egg and meat productivity. To work in this direction, the authors of this paper selected the gene *DYSF*, presumably affecting the formation of muscle mass and egg production in poultry [2-4].

Dysferlin (DYSF) is a type II transmembrane protein that is localized at the periphery of muscle fibers and serves as a regulator of vesicle fusion in the sarcolemma. Dysferlin plays an important role in vesicle movement, endocytosis, membrane receptor recirculation, muscle regeneration, and T-tubule formation [2]. It can perform additional functions in the vesicular transport of growth factor receptors that promote muscle growth and regeneration. It is assumed that the dysferlin-dependent transport of such signaling molecules modulates the expression of genes and the function of adult muscle stem (or satellite) cells responsible for the growth and regeneration of skeletal muscles in adults [5].

Mutations in the *DYSF* gene cause a number of muscle diseases with various clinical manifestations known as dysferlinopathies, including limb muscular dystrophy type 2B (LGMD2B) and Miyoshi myopathy [6–8]. Ferlins are proteins that affect Ca^{2+}-driven membrane dynamics and belong to the superfamily of proteins with multiple C2 domains (MC2D) that share functions in binding membrane-associated organelles and proteins on cell membranes. These proteins are often described as sensors of calcium ions (Ca^{2+}) for vesicular transport, capable of forming membranes [3, 9, 10]. In vertebrates, there are six ferlin genes; in humans, there are the dysferlin, otoferlin, myoferlin, *Fer1L5*, and *Fer1L6* genes, and a long gene that does not encode RNA, *Fer1L4* [11].

The most studied function of dysferlin is the restoration of damage in the surface membrane of striated muscle fibers — sarcolemma. The contraction of muscle fibers mechanically affects the sarcolemma, which leads to its microdestruction. The repair process is triggered by Ca^{2+} influx into the sarcoplasm, which depends on a number of proteins, including dysferlin as one of the key participants [12-14]. It probably promotes membrane aggregation and fusion during membrane repair through the interaction of Ca^{2+} with negatively charged phospholipids [15-17].

In the world scientific literature, there are no works devoted to the functions of dysferlin in chickens, including the association of dysferlin with poultry productivity has not been studied.

In the present study, four single nucleotide substitutions located in the 32nd intron on chromosome 4 were first identified in Russian White chickens:

rs317801013 (G/A) at 90672849, rs16455118 (C/A) at 90672756, rs318045896 (A/G) at 90672862, and SNP (T/G) at 90672805. Single nucleotide T/G polymorphism on chromosome 4 at position 90672805 was submitted for registration in the ENSEMBL database. Also, for the first time, an analysis of the frequency of occurrence of genotypes and the deviation of the observed distribution of genotypes from that expected at Hardy-Weinberg equilibrium in the gene pool of chickens of the Russian White breed was carried out for all the above substitutions in the dysferlin gene.

The aim of this work was to study the SNPs of the dysferlin gene in gene pool chicken breeds and to identify possible associations of *DYSF* gene polymorphisms with economically valuable characteristics.

Materials and methods. The studies were performed on chickens (*Gallus gallus*) from the gene pool population of the Genetic Collection of Rare and Endangered Chicken Breeds (All-Russian Research Institute of Genetics and Breeding of Agricultural Animals, St. Petersburg—Pushkin).

DNA was isolated by the phenol method from blood taken from the axillary vein into standard tubes with an anticoagulant (EDTA). To analyze the rs16455118 polymorphism, the research team used a database obtained as a result of genotyping using the Illumina Chicken 60K SNP iSelect BeadChip chip technology (Illumina, Inc., USA).

At the first stage, a total of 185 hens were used: meat (Cornish, $n = 39$), laying hen (Russian White, $n = 19$; Rhode Island, $n = 18$; Aurora, $n = 14$; Black-and-White Austrolorp, $n = 20$; Leningrad Calico, $n = 20$) and decorative (Russian Crested, $n = 20$; Light Brahma, $n = 18$; Bare-Necked, $n = 17$). The authors analyzed the frequency of *AA*, *AC*, and *CC* genotypes and the deviation of the observed genotype distribution from that expected under Hardy-Weinberg equilibrium in gene pool hens by the rs16455118 adenine to cytosine substitution in the dysferlin gene. The validity of the data obtained was assessed using Pearson's criterion χ^2.

In addition, the authors analyzed in more detail the polymorphism of the dysferlin gene in 76 chickens of the Russian White egg breed by sequencing a 237-bp section of the *DYSF* gene on chromosome 4. Amplification primers were designed based on the NCBI database (https://www.ncbi.nlm.nih.gov/) using the PRIMER_3 computer program (https://bioinfo.ut.ee/primer3-0.4.0/). The primer sequences were used: Fw — 5′-GGATGCCATAAGGACGTTGC-3′, Rv — 5′-TCCCCACAGCATCCCCTATAC-3′. PCR was performed in 10 rl of reaction mixture containing 67 mM Tris-HCl (pH 8.6), 2.5 mM $MgCl_2$, 16.6 mM NHOH, 0.125 mM dNTP, 0.5 rM primer, 50-100 ng of genomic DNA, and 2.5 units. Taq polymerases (Sibenzyme, Novosibirsk) on a C 1000 Touch amplifier (Bio-Rad, USA). Amplification mode: 5 min at 95 °C (denaturation); 20 s at 95 °C, 20 s at 62 °C, 20 s at 72 °C (40 cycles); 4 min at 72 °C (final elongation). The analysis of PCR products was carried out in a 2% agarose gel.

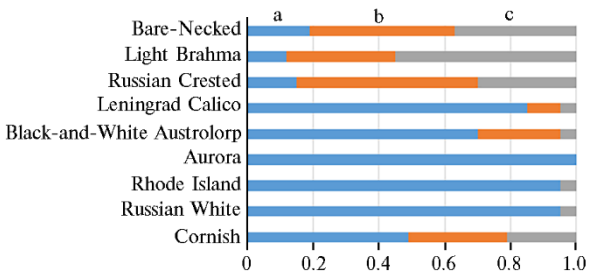
PCR products were purified using a commercial kit ExoSAP-IT Express (Affimetrix, USA) according to the manufacturer's protocol. Sanger sequencing was performed on an Applied Biosystems 3500 genetic analyzer (Thermo Fisher Scientific, Inc., USA) using a commercial BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Inc., USA). Alignment and processing of sequences were performed using the MEGA 6 software (https://www.megasoftware.net/web_help_10/index.htm#t=Citing_MEGA_In_Publications.htm).

To identify the identified substitutions, an analysis of the international genetic databases NCBI (https://www.ncbi.nlm.nih.gov/SNP) and ENSEMBL (https://www.ensembl.org/index.html) was carried out. For four substitutions, the

analysis of the frequency of genotypes and alleles was carried out. The deviation of the observed heterozygosity from the expected heterozygosity in Russian White hens was statistically processed and the validity of the obtained results was calculated using Pearson's criterion χ^2.

Results. Based on the results of genotyping of 185 hens using Illumina Chicken 60K SNP iSelect BeadChip chip technology by the rs16455118 (C/A) replacement, a shift towards an increase in the frequency of the *C* allele in decorative birds was observed. In egg chickens, the homozygous *AA* genotype had the highest frequency, the *CC* genotype was the least frequent, and was completely absent in the Aurora population. The Cornish meat breed had a more even distribution of genotypes in comparison with decorative and laying breeds (Fig.).

Database analysis (https://www.ensembl.org/Gallus_gallus/Variation/Population?db=core;r=4:90672256-90673256;v=rs16455118;vdb=variation;vf=6811490) showed equal distribution of *A* and *C* alleles (C: 0.500; A: 0.500) in red jungle hen, white Plimutrock, white leghorn, and silk hen (native Chinese breed).



Frequency of *AA* (a), *AC* (b), *CC* (c) genotypes by single nucleotide substitution A/C (rs16455118) at position 90672756 on chromosome 4 in the *DYSF* gene in gene pool populations from the Genetic Collection of Rare and Endangered Chicken Breeds (All-Russian Research Institute of Genetics and Breeding of Farm Animals, St. Petersburg—Pushkin).

When analyzing the frequency distribution of genotypes for the replacement rs16455118 among the gene pool breeds, a strong shift of genotypes towards homozygosity was revealed. The deviation of the observed heterozygosity (H_o) from the expected (H_e) turned out to be statistically insignificant for the bare-necked breeds ($\chi^2 = 0.28$, $p = 0.9$), Light Brahma ($\chi^2 = 0.28$, $p = 0.5$), Russian Crested ($\chi^2 = 0.68$, $p = 0.71$), Black-and-White Austrolorp ($\chi^2 = 0.009$, $p = 1$) in accordance with the Hardy-Weinberg law. In other words, the actual frequencies were in good agreement with the theoretically expected ones. The deviation of the observed distribution of genotypes from that expected at Hardy-Weinberg equilibrium was statistically significant for the populations of Cornish ($\chi^2 = 7.38$, $p = 0.025$), Rhode Island ($\chi^2 = 5.18$, $p = 0.05$), Leningrad Calico ($\chi^2 = 7.9$, $p = 0.019$), and Russian White ($\chi^2 = 40$, $p = 0.00001$). The obtained value of criterion χ^2 was greater than the critical one (3.84 with the number of degrees of freedom 1); therefore, the shift in genetic equilibrium in the analyzed populations indicated the founder's effect or selection pressure on the region of the rs16455118 single nucleotide substitution, presumably associated with egg production.

Frequency of occurrence of genotypes and alleles for four SNPs in the dysferlin gene *DYSF* in gene pool chickens of the Russian White breed from the Genetic Collection of Rare and Endangered Chicken Breeds (All-Russian Research Institute of Genetics and Breeding of Farm Animals, St. Petersburg—Pushkin).

SNP	Allele, genotype	Frequency	χ^2	p	H_o	H_e
rs318045896	*A*	0,593	6.004	0.014	13	20.75
	G	0,401				
	AA	0,442				
	AG	0,302				
	GG	0,256				

Continued Table

rs16455118	*A*	0,623	45.1	1.026187e–10	4	28.6557
	C	0,377				
	AA	0,590				
	AC	0,066				
	CC	0,344				
rs317801013	*A*	0.280	4.31	0.037	18	24.52
	G	0.720				
	AA	0.131				
	AG	0.295				
	GG	0.573				
Отсутсвует в базе ENSEMBL	*T*	0.180	2.68	0.1	15	18.8
	G	0.820				
	TT	0.063				
	TG	0.234				
	GG	0.703				

N o t e. H_o — observed heterozygosity, H_e — expected ожидаемая heterozygosity.

The almost complete absence of *AC* heterozygotes in egg chicken breeds and a few *CC* homozygotes also indicated the association of the homozygous *AA* genotype with egg productivity and strong selection pressure on these populations, which resulted in an increase in the degree of inbreeding. Apparently, selection for increasing egg production using closely related crosses led to the founder effect, as a result of which the proportion of heterozygotes and individuals with the *CC* genotype decreased, which, in turn, led to a shift in the distribution of genotypes according to the Hardy-Weinberg law (Table).

Sequencing of a 237 bp region of the dysferlin gene, located on the 4th chromosome in Russian White chickens ($n = 76$), revealed four single nucleotide substitutions in the 31st intron in the following positions: G/A in position 90672849 (rs317801013), C/A in position 90672756 (rs16455118), A/G in position 90672862 (rs318045896), T/G in position 90672805. Single nucleotide substitutions rs317801013, rs16455118, and rs318045896 coincided with known substitutions in the chicken genome (NCBI and ENSEMBL databases). Mononucleotide polymorphism T/G at position 90672805 was detected for the first time and was submitted by the authors for deposition in the ENSEMBL database.

Despite the increase in the sample of egg chickens to 76 individuals, when genotyping using the Illumina Chicken 60K SNP iSelect BeadChip chip technology to replace rs16455118, a deviation of the actual distribution of genotypes from that expected at Hardy-Weinberg equilibrium was also observed ($\chi^2 = 45.1$, $p = 1.026187e{-}10$), the tendency towards homozygosity remained, the *AA* genotype remained the most numerous, but the number of individuals with the *CC* genotype increased.

In a previous study [4], no significant relationship was found between alternative genotypes *AA*, *AC*, *CC* according to the dysferlin gene with live weight, age of first egg-laying, and egg weight in Russian White chickens; however, a relationship was established with egg production. Chickens with the *CC* genotype laid in 180 days on average 10 eggs less (134.06 ± 5.96) than those with the *AA* (145.00 ± 2.35) and *AC* (143.94 ± 2.31) genotypes ($p \leq 0.05$). This work partly confirms the hypothesis about the influence of the *AA* genotype on the egg production of chickens, since the *AA* genotype prevailed in laying breeds. Therefore, the A/C rs16455118 substitution in the dysferlin gene can be considered associated with egg production and used as a molecular marker after a more detailed study in the panel of marker polymorphisms.

Since the A/C mutation is located in the intron and does not lead to an amino acid substitution, let us consider how one can explain its effect on the phenotype. In many eukaryotes, including mammals, plants, insects, and yeast, introns can upregulate gene expression without functioning as a binding site for

transcription factors. This phenomenon is called intron-mediated amplification [18]. Introns can increase the number of transcripts, affecting the rate of transcription, nuclear export, and stability of transcripts, as well as increase the efficiency of mRNA translation [19–21]. Introns proximal to promoters are capable of enhancing transcription in mammalian and plant cells [22–24]. Chromatin immunoprecipitation (ChIP) analysis showed that the number of binding sites for RNA polymerase II (Pol II) on a reporter construct containing an intron was 4 times higher compared to the construct without an intron [25].

Intron-mediated enhancement of transcription correlates with the formation of a loop conformation of genes, which unites their promoter and terminator regions, possibly facilitating recycling and re-initiation of Pol II [26]. According to the results of genome-wide analysis, mRNA stability positively correlated with the number of introns in mice and humans [27–30]. In addition to increasing the mRNA content, the presence of introns increases the efficiency of mRNA translation in yeast, plants, mammals, and other animals [31, 32].

Another hypothesis about the mechanisms of influence of single nucleotide substitution in the intron is to change the pre-mRNA splicing required for the corresponding translation of the protein, which depends on the presence of consensus cis sequences that define the exon-intron boundaries and regulatory sequences [33]. Point mutations in these consensus sequences can cause misrecognition of exons and introns and result in an aberrant transcript of the mutated gene. Typically, such mutations cause errors in the splicing process, lead to improper removal of an intron, and thus cause changes in the open reading frame. Recent studies have highlighted the significant number and importance of splicing mutations in the etiology of inherited diseases.

In the paper by Chinese scientists [34], a reliable association of single nucleotide substitutions in the introns of the *MAGI-1* gene (encodes membrane-associated guanylate kinase 1) and the *ACSF2* gene (encodes acetyl-coA synthetase, an enzyme of the mitochondrial matrix) with egg production in geese was found. Bai *et al.* [35] also showed that polymorphism (A412G) in intron 1 of the *PRL* prolactin gene was significantly associated with egg production in two populations of Chinese domestic ducks. Arango *et al.* [36] showed an association between polymorphism in intron 3 of the bovine growth hormone *BGH* and body weight during the first estrus and first calving.

Therefore, despite the fact that the rs16455118 (A/C) mutation in the dysferlin gene does not change the amino acid sequence, it possibly affects the gene expression and the stability of its transcription, which leads to a change in the DYSF protein content [18–21]. The rs16455118 A/C mutation in the *DYSF* gene may be associated with economically significant traits by affecting mRNA splicing or stability, as well as with linkage disequilibrium with an unidentified missense mutation associated with the trait. The influence of the dysferlin gene on egg production is presumably related to the ability of the ferlins superfamily to trigger the influx of Ca^{2+}, an important macronutrient for oviposition, into the reproductive tract of the laying hen, since these proteins are described as sensors of calcium ions (Ca^{2+}) and key participants in a number of physiological processes [11–16].

The authors plan to continue studying the possible associations of single nucleotide substitutions rs317801013, rs318045896, and the T/G substitution at position 4:90672805 presented in the ENSEMBL database with signs of productivity. The identified associations can be further used in the selection of highly productive lines of domestic chicken breeds.

Thus, the study of SNPs rs16455118 of the dysferlin gene in chickens from

the gene pool of the Genetic Collection of Rare and Endangered Breeds of Chickens (All-Russian Research Institute of Genetics and Breeding of Farm Animals) showed that the homozygous genotype *AA* had the highest frequency of occurrence in poultry of the egg direction, the lowest the *CC* genotype, and the latter was completely absent in the aurora population. The Cornish meat breed was characterized by a more even distribution of genotypes in comparison with decorative and laying breeds. As a result of the sequencing of the dysferlin gene region located on chromosome 4, four single nucleotide substitutions in intron 32 were identified in Russian White chickens: rs317801013 (G/A) in position 90672849, rs16455118 (C/A) in position 90672756, rs318045896 (A/G) in position 90672862, and mononucleotide polymorphism (T/G) in position 90672805. Analysis of the frequency of occurrence of genotypes and the deviation of their observed distribution from the expected by Hardy-Weinberg showed a shift in the genetic equilibrium for all substitutions in the dysferlin gene found in gene pool chickens. The substitution of rs16455118 in the *DYSF* gene is associated with increased egg production and may be a consequence of selection pressure during breed improvement.

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