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ANALYSIS OF POLYMORPHISM IN THE MAJOR GENES FOR REPRODUCTIVE TRAITS IN SHEEP (*Ovis* spp.)

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Abstract

The reproductive traits significantly affect the cost of production of sheep products. The *BMP15, GDF9* and *BMPR1B* are the major genes for reproduction in sheep, mutations in which increase number of eggs per ovulation and litter size. The segregation of new mutations in an expanding breed diversity has been periodically reported. In this regard, the search for new SNPs in unexplored breeds is relevant to deepen knowledge about the genetic mechanisms underlying sheep prolificacy. In our work, for the first time, a comparative analysis of the complete nucleotide sequences of the *GDF9, BMP15* and *BMP15B* genes in the Romanov sheep was carried out in comparison with other breeds of domestic sheep (*Ovis aries* L.) and wild relatives. The most significantly varied SNPs were identified based on comparing the Romanov breed with autochthonous breeds of domestic sheep from Russia and the Persian Highlands, as well as wild *Ovis* species. Polymorphism in the major genes for reproduction in sheep was studied for argali (*O. ammon* L.) and mouflon (*O. orientalis* L.) for the first time. SNPs fixed in argali and domestic sheep were identified. The studies were conducted at Ernst Federal Research Center for Animal Husbandry in 2022-2023. We analyzed whole genome sequences of domestic sheep, Romanov (n = 9), other Russian breeds (n = 7), Iranian breeds (n = 6) and wild *Ovis* species, the Asian mouflon (*O. orientalis*, n = 16) and argali (*O. ammon*, n = 4). Alignment to the reference genome was performed using bwa-mem2 and SAMtools. The sequences of the *GDF9, BMP15,* and *BMP15B* genes were extracted from the whole genomes, in which the most different SNPs were searched based on the calculation of F_{ST} values for each SNP for each pair of groups. Gene sequences comparison of the Romanov breed with other breeds showed that the greatest differences were identified in the *BMPR1B* gene (F_{ST} = 0.562-0.749) when compared to the *BMP15* (F_{ST} = 0.051-0.374) and *GDF9* genes (F_{ST} = 0.037-0.660). Comparative analysis of gene sequences in the Romanov sheep and argali showed the presence of fixed SNPs (F_{ST} = 1), while one such SNP was identified in the *GDF9* gene. The highest F_{ST} values identified based on comparing Romanov breed sheep with mouflon were 0.702-0.780 (*BMPR1B* gene), 0.113-0.645 (*BMP15* gene) and 0.338-0.512 (*GDF9* gene). Thus, target SNPs were identified the effect of which on reproductive traits in the Romanov sheep should be studied in future work.

Keywords: SNP, candidate genes, *Ovis aries*, *Ovis ammon*, *Ovis orientalis*, domestic sheep, wild species, prolificacy

Prolificacy is an important economic trait that affects the cost of sheep

products. Mid- and high-latitude breeds still exhibit reproductive seasonality, with the reproductive season of ewes being shorter than that of rams, typically lasting from late summer to January [1]. To lengthen the reproductive season, the estrus cycle of sheep is synchronized, which is currently becoming an important element of reproduction programs [2]. However, breeders focus special attention on marker-assisted selection to fix desired alleles in target candidate genes associated with higher prolificacy in sheep. Many studies have reported a significant effect of the *BMP15*, *GDF9* and *BMPR1B* genes on reproductive traits in sheep. These genes are called the major candidate genes for sheep reproductive performance. The gene for bone morphogenetic protein 15 (BMP 15) *BMP15* and growth differentiation factor 9 (GDF9) *GDF9* are expressed in the ovaries and stimulate follicle growth [3, 4], promote the proliferation of granulosa cells [5, 6], influence cell viability signaling pathways [7, 8], and modulate other growth factors and hormones [9-11].

BMP15 is a key candidate gene controlling ovarian function. Several significant mutations have been identified in the gene, namely, $FecX^{I}$ (Fecundity Inverdale), $FecX^{H}$ (Fecundity Hanna) (12), $FecX^{B}$ (Fecundity Belclare), $FecX^{G}$ (Fecundity Galway) [13], $FecX^{L}$ (Fecundity Lacaune) [14], $FecX^{R}$ (Fecundity Rasa Aragonesa) [15, 16], and $FecX^{Bar}$ (Barbarine) [17]. The phenotypic expression of all of these mutations is generally uniform. A heterozygous genotype ensures higher prolificacy, and a homozygous genotype for the mutant allele leads to sterility. However, the French Grivette ($FecX^{Gr}$) and Polish Olkuska ($FecX^{O}$) sheep homozygous for the mutant allele have hyperprolificacy [18].

The first mutation ($FecB^{B}$ or Boorola allele) associated with fertility has been identified in the bone morphogenetic protein receptor 1B (*BMPR1B*) gene. This genetic variant, first identified in Booroola Merino, has an additive effect on the number of eggs at ovulation and a partially dominant effect on the number of lambs per lambing [19-21]. $FecB^{B}$ was then found in the Garole (or Bengal) sheep from India [22, 23], in the Javanese thin-tailed sheep in Indonesia [24], in the Hu, Small Tail Han, and Huyang, Cele, Duolang and Bayanbulak breeds from China [25-27], Bonpala from India [28] and Kalehkoohi from Iran [29].

Three mutations have been identified in the *GDF9* (*FecG*) gene, the $FecG^{H}$, $FecG^{T}$ and $FecG^{E}$. The $FecG^{H}$ (Fecundity High Fertility) [13] and $FecG^{T}$ (Fecundity Thoka) [30] mutations had a phenotypic inheritance pattern associated with the sterility of female carriers of homozygous mutations. The third mutation in the *GDF9* gene, $FecG^{E}$ (Fecundity Embrapa) associated with fecundity was identified in the Brazilian line of the Santa Inкs breed [31]. In this breed, a novel phenotype for the *GDF9* gene revealed for the first time was characterized not by sterility, but, on the contrary, by an increase in the number of eggs during ovulation in sheep homozygous for $FecG^{E}$ compared to non-mutant individuals, 2.22±0.12 vs. 1.22±0.11 with 1.78 lambs per lambing vs. 1.13 [31]. In 2014, another mutation in the protease cleavage site of *GDF9* ($FecG^{v}$, or Vacaria) was reported in the Île-de-France sheep [32]. In addition, several single nucleotide polymorphisms (SNPs) in the *GDF9* gene affect the number of lambs per lambing in the Chinese Hu local breed [33].

An analysis of scientific publications shows that in the global gene pool of sheep breeds, alleles have been identified that, both in hetero- and homozygous states are associated with increased fecundity, but the opposite effect has also been described when alleles are associated with sterility in homozygous females. Therefore, the identification of polymorphisms of the major candidate genes for reproductive qualities in sheep, especially in breeds that have not previously been studied in these aspects, is extremely important for practical breeding and a better understanding of the genetic mechanisms underlying the control of the reproductive

function of sheep.

In this work, we for the first time compared the complete nucleotide sequences of the *GDF9*, *BMP15* and *BMP15B* genes in highly prolific Romanov sheep, other domestic sheep breeds and their wild relatives. We have identified SNPs that differ most significantly between the Romanov sheep, autochthonous domestic sheep from Russia and the Persian Highlands, and wild species of the genus *Ovis*. Polymorphisms in the major candidate genes for reproductive qualities in argali (*O. ammon*) and mouflon (*O. orientalis*) are reported for the first time, and SNPs fixed in argali and domestic sheep are revealed.

The purpose of the work is to study polymorphism in the major genes of reproductive traits (*GDF9*, *BMP15* and *BMP15B*) in highly prolific Romanov breed sheep compared to low- prolific breeds and their wild relatives.

Materials and methods. The studied sample included domestic sheep (*Ovis aries* L.) of the Romanov breed (n = 9), Russian breeds Tushin (n = 3) and Karachaev (n = 4), and Iranian breeds Afshari, Ghezel, Gray Shiraz, Shal, Moghani, Karakul breed from a population bred in Iran (n = 6). Wild species were Asian mouflon (*Ovis orientalis* L.) (n = 16) and argali (*O. ammon* L.) (n = 4).

Ear tissue specimens of Tushin (n = 4), Karachaev (n = 4), Romanov (n = 9) sheep and argali (n = 4) were provided by the UNI Bank of Genetic Material of Domestic and Wild Species of Animals and Birds as part of the network bioresource collection of the sBRK SKhZh (Ernst Federal Research Center; agreement with the Ministry of Education and Science of Russia No. 075-15-2021-1037 of September 28, 2021).

DNA was isolated using the DNA-Extran-2 kit (Syntol LLC, Russia). The DNA concentration (a Qubit® 4.0 fluorometer, Invitrogen/Life Technologies, USA) and quality ($OD_{260/280}$, a NanoDropTM 8000 spectrophotometer, ThermoFisher Scientific, Inc., USA) were assessed. The minimum amount of DNA to create sequencing libraries is 3 μg, so the threshold DNA concentration was 30 ng/μl with at least 100 μl volume. The optimal $OD_{260/280}$ was 1.8 and higher. At lower concentrations, DNA was re-isolated to increase the amount of starting material.

For whole-genome sequencing, NGS (next generation sequencing) method was used (NovaSeq 6000 sequencer, Illumina, Inc., USA). Sequencing libraries were prepared with TruSeq DNA Nano Library Prep kits (Illumina, Inc., USA) and Accel-NGS® 2S Plus DNA Library Kit (IDT) for Illumina® Platforms (Swift Biosciences, Inc., USA).

Whole genome sequences of Iranian sheep breeds were provided by Professor A. Esmailizadeh.

Whole genome sequences of mouflons (*O. orientalis*) were downloaded from the publicly available online NCBI database (project PRJNA624020, ID 624020) [34, 35]. Alignment to the reference genome Ovis_aries_rambouillet.Oar_rambouillet_v1.0.dna.toplevel.fa.gz (https://www.ens-embl.org/Ovis_aries_rambouillet/Info/Index/,?db=core) was performed using bwa-mem2 tools [36] and SAMtools [37]. From complete genomes, complete nucleotide sequences of candidate genes for sheep reproductive traits (*GDF9*, *BMP15* and *BMP15B*) were extracted and searched for genetic variants. The genes were found according to their coordinates in the reference genome Ovis_aries_rambouillet.Oar_ram-bouillet_v1.0.dna.toplevel.fa.gz. The coordinates included the chromosome number, the nucleotide position of the beginning of the gene, the nucleotide position of the end of the gene, and the indication of the DNA chain (1 means forward, –1 means reverse). The coordinates were X:56594565-56601245: 1 for the *BMP15* gene (Ensembl entry ENSOARG00020012408); 5:46544645-46547585: 1 for the *GDF9* gene (Ensembl entry ENSOARG00020021050); 6:33990928-34214488: 1

for the *BMP15B* gene (Ensembl entry ENSOARG00020020206.1). Direct extraction of complete sequences of the studied genes was carried out using the bwa-mem2 and SAMtools tools according to the author's scripts.

The comparison groups were Romanov breed vs. other Russian breeds, Romanov breed vs. Iranian breeds, Romanov breed vs. mouflon, and Romanov breed vs. argali.

The most divergent SNPs in the analyzed genes were detected by calculation of F_{ST} values for each SNP within each gene, using the R package StAMPP [38].

Results. Based on F_{ST} values, SNPs were identified within the *GDF9*, *BMP15* and *BMP15B* genes, which were the most different when comparing the nucleotide sequence of these genes in Romanov sheep and a group including sheep bred in Russia and Iran (Table 1).

1. SNPs in candidate genes for reproductive traits of *Ovis aries* the most different between Romanov sheep and breeds with low prolificacy (Ernst Federal Research Center for Animal Husbandry — VIZh, 2020-2023)

Comparison group	SNP	SNP position	F_{ST}
Gene *BMP15* located on X chromosome			
Romanov breed (n = 9) and Iranian breeds (n = 6)	rs400940002	56599692	0.374
	rs426251007	56600582	0.185
	rs1090246541	56597586	0.149
	X:56597710	56597710	0.146
	rs1086873546	56596059	0.051
Romanov breed (n = 9) and Russian breeds (n = 7)	rs55628000	56595188	0.126
	X:56597676	56597676	0.111
	X:56597286	56597286	0.109
	X:56599717	56599717	0.109
	X:56600871	56600871	0.065
Gene *GDF9* located on chromosome 5			
Romanov breed (n = 9) and Iranian breeds (n = 6)	rs160076413	46545932	0.580
	rs418388291	46546176	0.490
	rs421019907	46545415	0.416
	rs399579080	46545431	0.369
	rs594156088	46544743	0.037
Romanov breed (n = 9) and Russian breeds (n = 7)	rs160076413	46545932	0.660
	rs418388291	46546176	0.432
	rs421019907	46545415	0.309
	rs399579080	46545431	0.227
	rs160076408	46545688	0.114
Gene *BMPR1B* located on chromosome 6			
Romanov breed (n = 9) and Iranian breeds (n = 6)	rs409507123	34036290	0.749
	rs421851559	34036546	0.749
	rs425275620	34009200	0.703
	rs410857597	34053188	0.630
	rs426525353	34035758	0.609
Romanov breed (n = 9) and Russian breeds (n = 7)	rs400119613	34016579	0.701
	6:34091908	34091908	0.623
	6:34092013	34092013	0.623
	6:34113605	34113605	0.562
	rs417658413	34113624	0.562

In general, the greatest differences in nucleotide sequences are identified in the *BMPR1B* gene, F_{ST} from 0.609 to 0.749 for the Romanov breed vs. Iranian breeds and F_{ST} from 0.562 to 0.701 for the Romanov breed vs. other Russian breeds. In the *BMP15* genes with F_{ST} from 0.051 to 0.374 and from 0.065 to 0.126, respectively, and the *GDF9* genes with F_{ST} from 0.037 to 0.580 and from 0.114 to 0.660 the differences are significantly smaller.

Analysis of the nucleotide sequences of the studied genes in Romanov sheep and argali showed fixed SNPs (F_{ST} = 1), while only one such SNP was identified in the *GDF9* gene (5:46545406) (Table 2). The highest F_{ST} values for SNPs identified when comparing Romanov sheep with mouflon were 0.702-0.780 for the *BMPR1B* gene, 0.113-0.645 for the *BMP15* gene and 0.338-0.512 for the

GDF9 gene).

2. SNPs in candidate genes for reproductive traits the most different between Romanov sheep (*Ovis aries*) and wild relatives mouflon and argali (Ernst Federal Research Center for Animal Husbandry — VIZh, 2020-2023)

Comparison group	SNP	SNP position	FST
Gene *BMP15* located on X chromosome			
Romanov breed (n =9) and	rs400940002	56599692	0.645
mouflon (*O. orientalis*) (n = 16)	rs403715147	56597068	0.511
	X:56597710	56597710	0.260
	rs420350765	56599601	0.159
	X:56596503	56596503	0.113
Romanov breed (n =9)	rs422668280	56595720	1
and argali (*O. ammon*) (n = 4)	X:56595843	56595843	1
	X:56595928	56595928	1
	rs412479434	56597103	1
	X:56597429	56597429	1
	X:56598037	56598037	1
	X:56598317	56598317	1
	rs417053670	56599070	1
Gene *GDF9* located on chromosome 5			
Romanov breed (n =9) and	rs399579080	46545431	0.512
mouflon (*O. orientalis*) (n = 16)	rs421019907	46545415	0.481
	5:46546592	46546592	0.373
	5:46546650	46546650	0.338
Romanov breed (n =9)	5:46545406	46545406	1
and argali (*O. ammon*) (n = 4)	rs425601341	46546485	0.767
	5:46546592	46546592	0.767
	rs160076408	46545688	0.746
	rs427433335	46546966	0.680
Gene *BMPR1B* located on chromosome 6			
Romanov breed (n =9) and	rs424055720	34152408	0.780
mouflon (*O. orientalis*) (n = 16)	rs400936557	34121377	0.772
	rs400817842	34051068	0.744
	rs414227223	34059131	0.734
	rs400453556	34053288	0.705
	rs408680692	34058728	0.702
	rs403920069	34059147	0.702
Romanov breed (n = 9)	rs420236481	33995134	1
and argali (*O. ammon*) (n = 4)	6:34002894	34002894	1
	rs402116959	34003208	1
	rs401004280	34003416	1
	rs428477215	34005483	1

Some SNPs were identified in more than one comparative analysis. The SNPs rs400940002 and X:56597710 in the *BMP15* gene differed between the Romanov sheep and both Iranian breeds and mouflon.

We identified the largest number of SNP matches in the *GDF9* gene. SNPs rs160076413 and rs418388291 were among the most divergent when comparing the Romanov sheep with both Iranian and Russian breeds. SNPs rs421019907 and rs399579080 differed when comparing the Romanov breed with both domestic sheep and mouflon breeds. SNP 5:46546592 was identified when comparing the sequences of this gene in the Romanov breed with mouflon and argali, but the difference with argali was higher, F_{ST} = 0.767 vs. F_{ST} = 0.373. Interestingly, SNP rs160076408 coincided when compared with both Russian breeds and argali.

In the *BMPR1B* gene, all identified SNPs were unique for each of the compared groups.

Next, genotypes were identified in positions with F_{ST} = 1 (Table 3). In the studied domestic sheep and argali, exclusively opposite homozygous genotypes were found. Polymorphism in a number of analyzed SNPs was detected only in Asian mouflons. In other SNPs in which there was no polymorphism, the mouflon genotype corresponded to the genotype of domestic sheep.

3. Genotypes for SNPs in the studied samples of domestic sheep (*Ovis aries*), mouflon (*O. orientalis*) and argali (*O. ammon*) (Ernst Federal Research Center for Animal Husbandry — VIZh, 2020-2023)

SNP	Identified genotypes		
	O. aries	*O. ammon*	*O. orientalis*
Gene *BMP15* located on X chromosome			
rs422668280	GG	AA	GG
X:56595843	CC	TT	TT, CC
X:56595928	TT	CC	TT
rs412479434	AA	TT	AA
X:56597429	GG	AA	GG
X:56598037	CC	TT	CC
X:56598317	AA	GG	AA
rs417053670	GG	AA	GG
Gene *GDF9* located on chromosome 5			
5:46545406	AA	CC	AA
Gene *BMPR1B* located on chromosome 6			
rs420236481	AA	CC	AA, CA
6:34002894	GG	AA	GG, AG
rs402116959	CC	GG	GG, CC, GC
rs401004280	AA	TT	AT, TT, AA
rs428477215	GG	AA	AG, GG

Reproductive traits, ultimately expressed in the number of lambs per lambing, significantly influence the profitability of sheep farming. Because most ewes produce one lamb, identifying the genes responsible for specific fertility traits is of great scientific and economic interest. The genes *SPOCK1* for age of first estrus, *GPR173* for mediator of ovarian cyclicity, *HB-EGF* for signals about successful onset of pregnancy, *SMARCAL1* and *HMGN3a* which regulate gene expression during embryogenesis [39], *B4GALNT2* for follicle development, the $FecL^L$ mutation has been described in the Lacaune breed [40], are considered as potential candidates influencing reproductive traits. In addition, some genes are not discussed as likely candidates for marker selection, but they may influence reproductive traits to varying degrees, in particular the genes *ESR1* [41], *FSHR* [42], *FTF* or *NR5A2* [43].

Nevertheless, it is the *BMP15*, *GDF9* and *BMPR1B* genes that continue to attract the greatest interest. For example, attempts have been made to link known mutations in these genes, especially the *GDF9* gene, in sheep bred in Russia. The frequencies of alleles of the *GDF9* gene (polymorphism c.260G>A) were studied in the Altai Mountain [44], Dagestan Mountain [45] breeds, and in the Manych Merino [46].

In the Romanov breed, genetic screening for the main mutations in the *BMP15* gene, the $FecX^G$, $FecX^H$, $FecX^I$, $FecX^L$, $FecX^B$, and in the *GDF9* gene revealed the absence of these mutant alleles in all animals in the sample [47]. Continuing screening in an expanded sample also did not bring a success [48]. However, it should be noted that in general these results are consistent with our data, since none of the identified SNPs coincided with previously known substitutions. It is likely that unique reproductive traits may be associated with genetic variants in other genomic regions.

Other researchers [44-48] have studied polymorphisms at established positions that have a known effect in certain breeds, which does not always guarantee positive results in other breeds. Therefore, we chose a different methodology based on the complete sequencing the *BMP15*, *GDF9* and *BMPR1B* genes to study polymorphisms in each SNP in these genes and identify SNPs. most significantly different between highly- and low prolific sheep breeds. We plan further research to determine whether these substitutions affect reproductive traits in Romanov sheep.

In addition, our work was the first to analyze the complete sequences of

the *BMP15*, *GDF9* and *BMPR1B* genes in argali (*O. ammon*) and mouflon (*O. orientalis*). It should be noted that the specific distribution of genotypes by fixed SNPs in mouflon, that is, common allelic variants with argali, could be due to introgression events that occurred before or after domestication. This hypothesis is consistent with complete genome sequencing of wild species, which revealed adaptive introgression from argali into the genomes of European and Asian mouflon [49].

Thus, here, we compared polymorphisms in the candidate genes *GDF9*, *BMP15* and *BMP15B* for reproductive traits of highly prolific Romanov sheep, low prolific breeds and wild species of the genus *Ovis*, the argali and mouflon. SNPs have been identified that differ most significantly between the Romanov breed, Russian and Iranian breeds, and wild species. Polymorphisms in the main candidate genes for reproductive traits in argali (*O. ammon*) and mouflon (*O. orientalis*) were examined for the first time, and SNPs fixed in argali and domestic sheep were identified.

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