PCR-RFLP assay for genotyping of three different regions of the BMP15 gene in sheep from the Bulgarian dairy synthetic population

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Abstract

Bone morphogenetic protein 15 (BMP15/FecX) gene is one of the candidate genes for the reproduction in farm animals, especially sheep. The present study aimed to detect the genetic polymorphism in three different regions of BMP15 gene in sheep from the Bulgarian Dairy Synthetic Population (BDSP) using PCR-RFLP technique. For the purpose of the experiment were used 163 ewes from the herd of the Agricultural Institute-Shumen with records of the number of lambs born from a minimum of two consecutive lambing. The average number lambing of ewe is 4.07. DNA was extracted from blood samples of all ewes, subjected to PCR amplification. The PCR products of 141 bp ($FecX^G$), 153 bp ($FecX^B$), and 240 bp ($FecX^H$) were cut with Hin*f*I, DdeI, and SpeI restriction enzymes, respectively. In $FecX^G$ region were visualized two fragments with length 112 bp and 29 bp (wild genotype ++) in all tested ewes. For $FecX^B$ in all animals were obtained two fragments of 122 bp and 31 bp which corresponded to homozygous wild genotype *AA*. For $FecX^H$ all animals were homozygous with *CC* genotype and only one determined fragment of 240 bp. In conclusion, the findings in present study did not detect any polymorphisms in BMP-15 gene in sheep, regardless their good prolificacy.

Key words: Sheep; genetic polymorphism; PCR-RFLP; BMP15 gene; Bulgarian Dairy Synthetic Population

Introduction

FecX (BMP-15) is a member of the transforming growth factor beta (TGFβ) superfamily that is expressed specifically in oocyte of the developing follicle. Sheep Bone morphogenetic protein 15 (BMP-15, FecX) has been mapped on the X chromosome of *Ovis Aries L.* genome. The full coding sequence length is 1179 bp and it is consisted of two exons separated by one intron. BMP-15 is a protein that blocks FSH receptor expression in the ovaries. According this fact heterozygous individuals have multiple ovulations and therefor increased ovulation rate. The mutation in only one copy of BMP-15 leads to increased ovulation rate (Kumar et al., 2016; Nagdy et al., 2018).

Eight different mutations have been identified in this locus – Inverdale-FecX^{I}, Hanna-FecX^{H}, Belclare-FecX^{B}, Galway-FecX^{G}, Lacaune-FecX^{L}, RasaAragonesa-FecX^{R}, Grivette-FecX^{Gr}, Olkuska-FecX^{O} (Galloway et al., 2004; Davis, 2005; Bodin et al., 2007; Demars et al., 2013; Monteagudo et al., 2019). FecX^{B} locus covers a sequence containing the missense mutation 1100 G < T(S99I) in exon 2 causing a non-synonymous substitution of serine with isoleucine. The nonsense mutation C < T in FecX^{G} causes the substitution of glutamine with a stop codon (Q239*) resulting in premature termination of translation. The point mutation C < T in the FecX^{H} locus causes the substitution of glutamine with a stop codon (Mohamed et al., 2020).

Twinning in sheep is a crucial economic factor. The slow and uncertain process of improving reproduction traits by traditional selection is due to the low heritability rate. Hence, the application of marker-assisted selection (MAS) is essential in enhancing fecundity in sheep. Therefore, the development of different fecundity genes and the study of their genetic diversity would lead to a rapid and efficient improvement of fertility in sheep (Rahman et al., 2021).

Dairy sheep constitute 75% of the sheep population in Bulgaria, most numerous being the representatives of the Bulgarian Dairy Synthetic Population (BDSP), registered in 2005. The applied methods of creation, with the participation of the East Friesian (EF) and Awassi (Aw) breeds (Hinkovski et al., 1984, 2008; Stancheva, 2003; Stancheva et al., 2014[ab], 2016), classify sheep from the Synthetic population as a composite commercial breed for milk, according to international standards (Rasali et al., 2006). At the same time, sheep from the Bulgarian Dairy Synthetic Population have good adaptability and potential for good fecundity – 150 lambs from 100 ewes (Stancheva et al., 2014[b]).

The available literature on the genetic diversity of genes related to fecundity in Bulgarian sheep breeds is very limited. For the BMP 15 gene, only two studies have been performed (Bozhilova-Sakova and Dimitrova, 2021; Bozhilova-Sakova and Stoykova-Grigorova, 2022).

The aim of present experiment was to study the genetic diversity of three different regions in BMP 15 gene in 163 ewes from the Bulgarian Dairy Synthetic population from the flock of the Agricultural Institute – Shumen.

Materials and methods

Animals and blood sampling

A total of 163 ewes with records of the number of lambs born from a minimum of two consecutive lambing's were tested in this study. The average number lambing of ewe is 4.07. The ewes were part of the nucleus flock of the Bulgarian Dairy Synthetic Population, raised at the Agricultural Institute-Shumen. (Figure 1). Blood

Fig. 1. Ewes from the flock of the Bulgarian Dairy Synthetic population, raised in Agricultural Institute-Shumen, Bulgaria

samples (5 mL) were collected from each animal through *vena jugularis* into EDTA containing tubes and were stored at -20 °C until DNA extraction. All animals were kept under the same nutritional and environmental condition.

DNA extraction

DNA was extracted from whole blood using DNA preparation kit QIAamp DNA Blood Mini Kit (Qiagen), the concentration and purity of extracted genomic DNA were determined by spectrophotometer and agarose electrophoresis on 1% agarose gel.

DNA amplification by polymerase chain reaction

The DNA samples were amplified through polymerase chain reaction technique developed by Mullis et al. (1986) in thermal cycler B-96 (Quanta Biotech). The following components were used for preparation of one reaction mixture with 20 μL final volume: 40 ng DNA template, 20 pM of each primer and 2 × (1.5 mM MgCl2) Red Taq DNA Polymerase Master mix (VWR, Int., Belgium). The primers sequences and annealing T was presented in Table 1. The reactions were amplified at 94 °C for 1 min (initial denaturation), then 30 cycles of denaturation at 94 °C for 45 s, annealing, extension at 72 °C for 45 s, and a final extension at 72 °C for 5 min. PCR products were subjected to electrophoresis in 2.5% agarose gel stained by Red Gel Nucleic Acid Stain (Biotium), 1 × TBE buffer with, at 90 V for 30–40 min. The bands were visualized under ultraviolet trans illumination and photographed in Hi-UVTM Duo Capture (HIMEDIA).

Restriction fragment length polymorphism (RFLP)

The PCR products of 141 bp ($FecX^G$), 153 bp ($FecX^B$), and 240 bp ($FecX^H$) were digested with *HinfI* (G/ACT), *DdeI* (C/TTAG), and *SpeI* (A/CTAGT) restriction enzymes (Thermo Fisher Scientific, UK), respectively. All reactions were prepared in a total volume of 10 μL (6 μL PCR product, 1 μL enzyme buffers, 0,5 μL digestion enzymes and 2,5 μL water) and incubated at 37 °C for 1 h. After digestion, the results were visualized by electrophoresis at a concentration of 3% agarose gel.

Results

After DNA extraction were received 163 samples with concentration approximately 15–20 ng/μl. A 141 bp product of $FecX^G$ gene was amplified using PCR-RFLP technique in all sheep under the study. The PCR products were cut with *HinfI* restriction enzyme into two digested fragments of 29 bp and 112 bp in all sheep, which determined the homozygous wild genotype ++ (Figure 2).

After RFLP analysis of $FecX^H$ gene with restriction enzyme *SpeI* all PCR products of 240 bp remained uncut, which revealed the homozygous wild genotype *CC* (Figure 3).

The PCR products of $FecX^B$ with length of 153 bp were cut with restriction enzyme *DdeI*

Table 1. Primer sequences, length of PCR products, and annealing temperature of the three investigated regions of BMP-15 gene

Region	Primer sequence	Length of PCR product	Annealing T	References
$FecX^G$	F F:5'-CACTGTCTTCTTGTTACTGTATTTCAATGAGAC-3 R: 5'GATGCAATACTGCCTGCTTG-3'	141 bp	60 °C	Hanrahan et al. (2004)
$FecX^B$	F: 5'GCCTTCCTGTGTCCCTTATAAGTATGTTCCCCTTA-3'R: 5'-TTCTTGGGAAACCTGAGCTAGC -3'	153 bp	60 °C	Barakat et al. (2017)
$FecX^H$	F: TATTTCAATGACACTCAGAG R: GAGCAATGATCCAAGTGATCCCA	240 bp	55 °C	Hua et al., (2008)

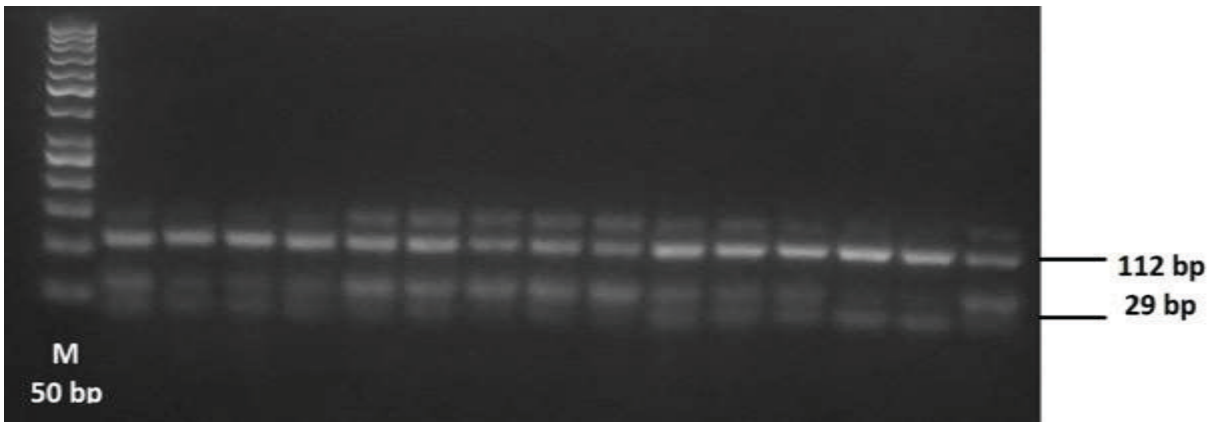

Fig. 2. Restriction fragments after digestion with *HinfI*

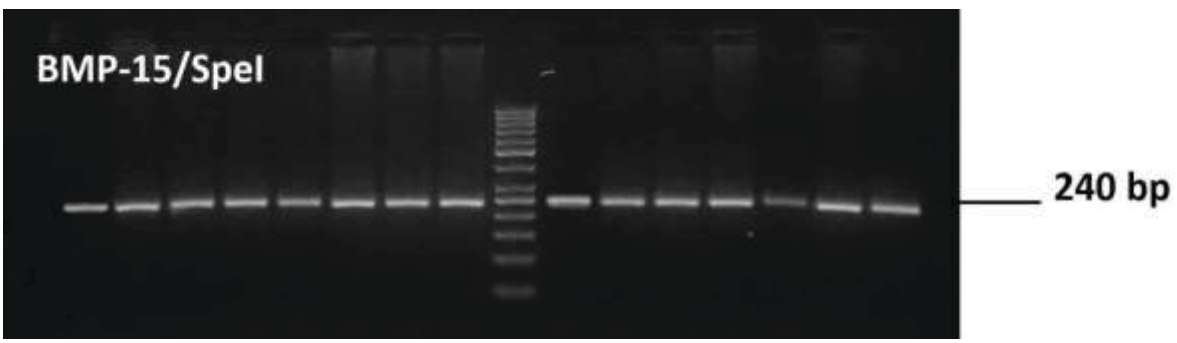

Fig. 3. Restriction profile of $FecX^H$ in sheep from the Bulgarian Dairy Synthetic population

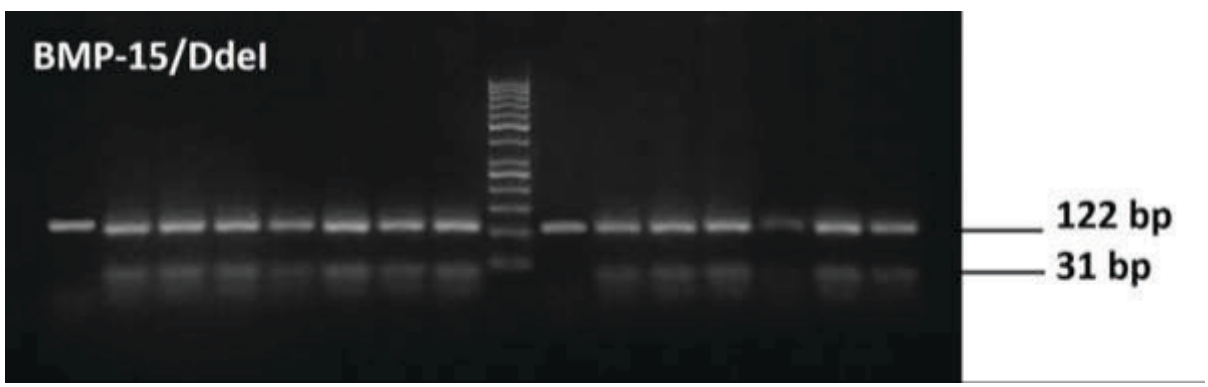

Fig. 4. Restriction profile of $FecX^B$ in sheep from the Bulgarian Dairy Synthetic population

into two digested fragments of 31 bp and 122 bp, which identified the homozygous wild genotype *AA* in all tested animals (Figure 4).

The mean values and results of the analysis of variance for the litter size of the animals studied are reflected in Table 2. Ewes have a good fecundity potential (1.65 lambs born per ewe), despite the fact that no polymorphisms were detected in the three regions of the BMP 15 gene. Litter size was significantly affected by parity ($P \leq 0.01$) and ranged from 1.52 number of lambs born at 2nd lambing to 1.77 number of lambs born at 4th

Table 2. Overall mean and analysis of variance for litter size by ewes parity

Variable	Number	Average	C.V. %	P-value
Total litter size	163	1.65	39.48	
Liter size by parity				
1-st lambing	163	1.61	41.14%	0.021615
2-nd lambing	163	1.52	32.52%	
3-rd lambing	143	1.70	40.89%	
4-th lambing	103	1.77	47.46%	
5-th lambing	62	1.74	32.58%	
6-th lambing	29	1.66	37.68%	

$^{*}P \leq 0.01$

lambing. Possibly, the good fecundity of ewes from the studied flock may be based on a different region of the BMP15 gene or a different major gene.

Discussion

According to many authors, these three regions are highly conservative in various sheep breeds worldwide.

In different study in Bulgaria were tested 50 ewes of Ile de France sheep breed and the genetic status of $FecX^{B}$ was determined by means of PCR-RFLP method. Similar to the results in this experiment it was found the presence only of homozygous wild genotype *AA* (Bozhilova-Sakova and Stoykova-Grigorova, 2022).

In a study of Ali et al. (2020) were investigated $FecX^{H}$ and $FecX^{G}$ mutation in Karadi sheep. After the PCR amplification, the research team received products of 240 bp and 141 bp for $FecX^{H}$ and $FecX^{G}$ gene, respectively. When PCR products were digested with *SpeI* and H*infI* restriction enzymes, was revealed the absence of restriction sites for both genes in all tested animals.

Mohamed et al. (2020) investigated the same three regions of BMP-15 gene in 156 Watish ewes of 2–6 years of age. Their results confirm the results obtained in the present experiment. The BMP15 ($FecX^{B}$) locus was analyzed, targeting a 153 bp PCR fragment. The PCR reaction was carried out with the restriction endonuclease enzyme *DdeI*. It resulted in one type of restriction pattern that produced two fragments in all animals under study and was assigned as homozygous genotype (wild type) (122-31 bp). The PCR product of $FecX^{G}$ was 141 bp and it was digested by *Hinf1I* endonuclease enzyme, generating two types of restriction patterns (112 bp + 29 bp) and (141 bp + 112 bp + 29 bp) representing ++ and *G* + genotypes, respectively. The wild type (++) was the more frequent type in the population, but none of the animals carried the homozygous mutant genotype *GG* (141 bp), which was not sensitive to the enzyme at the site of cutting. The point mutation polymorphism C < T in the causes the substitution of glutamine with a stop codon. The PCR products (240 bp) of $FecX^{H}$ gene were digested by *SpeI* endonuclease enzyme. The uncut pattern (240 bp) resulted in one type of restriction fragment pattern, which was assigned as wild type genotype ++ in all animals under study.

The BMP15 genotypes of 77 fertile Chios sheep were investigated by PCR-RFLP method. Monomorphism was established in all studied individuals who showed a wild type genotype and did not carry the $FecX^{B}$ mutation. In conclusion, it is believed that the high fertility of Chios sheep may be based on a different region of the BMP15 gene or a different major gene (Dinçel et al., 2018). Their results correspond with those in the present study.

Differ from the results in this study, in a previous study of $FecX^{B}$ gene in Bulgaria, it was investigated the genetic diversity of 60 ewes from

the Northeast Bulgarian Merino sheep breed. It was revealed the presence of heterozygous genotype G+ with frequency 0.12 (Bozhilova-Sakova and Dimitrova, 2021).

Conclusions

As a result, in the present investigation it could be concluded that the three investigated sites from the FecX gene were highly conservative in all 163 ewes from the Bulgarian Dairy Synthetic population. In $FecX^G$ was produced only allele + and only genotype ++, respectively. After RFLP analysis of $FecX^H$ was determined only allele *C* and only genotype *CC*, respectively. In FecXB was identified only the homozygous wild genotype *AA* in all tested animals. However, worldwide (including in Bulgaria) there were different breeds and studies with genetic diversity of FecX gene. The good fecundity of ewes from the Bulgarian Dairy Synthetic population may be based on a different region of the BMP15 gene or a different major gene. Thus the effect of other major genes or regions should be investigated in future studies.

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References

Ali, M. K., Khan, K. M. H., & Abdullah, M. M. (2020). Identification of FecxH and FecxG Mutations in Karadi Sheep Breed Using RFLP-PCR Technique in Sulaimani Province. *Journal of Animal and Poultry Production*, *11*(2), 27-29.

Barakat, I. A., Salem, L. M., Daoud, N. M., Khalil, W. K., & Mahrous, K. F. (2017). Genetic polymorphism of candidate genes for fecundity traits in Egyptian sheep breeds. *Biomed Res*, *28*(2), 851-857.

Bodin, L., Di Pasquale, E., Fabre, S., Bontoux, M., Monget, P., Persani, L., & Mulsant, P. (2007). A novel mutation in the bone morphogenetic protein 15 gene causing defective protein secretion is associated with both increased ovulation rate and sterility in Lacaune sheep. *Endocrinology*, *148*(1), 393-400.

Bozhilova-Sakova, M., & Dimitrova, I. (2021). Application of PCR-RFLP method to determine polymorphism in BMP-15 and GDF9 fecundity genes in Northeast Bulgarian Merino sheep breed. *Journal of BioScience and Biotechnology*, *10*(2), 107-111.

Bozhilova-Sakova, M., & Stoykova-Grigorova, R. (2022). Genetic study on BMP-15 as candidate gene of prolificacy in Ile de France sheep breed. *Zhivotnovadni Nauki*, *59*(1), 32-35.

Davis, G. H. (2005). Major genes affecting ovulation rate in sheep. *Genetics Selection Evolution*, *37*(Suppl. 1), S11-S23.

Demars, J., Fabre, S., Sarry, J., Rossetti, R., Gilbert, H., Persani, L., Tosser-Klopp, G., Mulsant, P., Nowak, Z., Drobik, W., Martyniuk, E., & Bodin, L. (2013). Genome-wide association studies identify two novel BMP15 mutations responsible for an atypical hyperprolificacy phenotype in sheep. *PLoS Genetics*, *9*(4), e1003482.

Dinçel, D., Ardiçli, S., Şamli, H., & Balci, F. (2018). Genotype frequency of FecXB (Belclare) mutation of BMP15 gene in Chios (Sakiz) sheep. *Uludağ Üniversitesi Veteriner Fakültesi Dergisi*, *37*(2), 87-91.

Galloway, S. M., McNatty, K. P., Cambridge, L. M., Laitinen, M. P., Juengel, J. L., Jokiranta, T. S., McLaren, R. J., Luiro, K., Dodds. K. G., Montgomery, G. W, Beattie,A. E., Davis, G. H. & Ritvos, O. (2000). Mutations in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner. *Nature genetics*, *25*(3), 279-283.

Hanrahan, J. P., Gregan, S. M., Mulsant, P., Mullen, M., Davis, G. H., Powell, R., & Galloway, S. M. (2004). Mutations in the genes for oocyte-derived growth factors GDF9 and BMP15 are associated with both increased ovulation rate and sterility in Cambridge and Belclare sheep (Ovis aries). *Biology of reproduction*, *70*(4), 900-909.

Hinkovski, T., Stoyanov, A., Donchev, P., & Boikovski, S. (1984). Methodical instructions for creation of synthetic sheep population and technologies of raisin. *Agric. Academy, Sofia*.

Hinkovski, T., Rajcheva, E., & Metodiev, N. (2008). Estimation of the productivity of ewes from the Bulgarian Dairy Synthetic Population. *Journal of Animal Science (Bulgaria)*, *3,* 35-41.

Hua, G. H., Chen, S. L., Ai, J. T., & Yang, L. G. (2008). None of polymorphism of ovine fecundity major genes FecB and FecX was tested in goat. *Animal Reproduction Science*, *108*(3-4), 279-286.

Kumar, R., Taraphder, S., Sahoo, A., Senpati, P., Paul, R., Roy, M., Sarkar, U., Raja, D., Samanta, I., & Baidya, S. (2016). Polymorphism and nucleotide sequencing of BMP-15 gene in an organised herd of Garole sheep (Ovis aries). *Indian J. Anim. Hlth*, *55*(1), 77-86.

Mohamed, S. E. I., Ahmed, R. M., Jawasreh, K. I., Salih, M. A. M., Abdelhalim, D. M., Abdelgadir, A. W., Obeidat, M. T., Musa, L. M. & Ahmed, M. K. A. (2020). Genetic polymorphisms of fecundity genes in Watish Sudanese desert sheep. *Veterinary world*, *13*(4), 614-621.

Monteagudo, L. V., Ponz, R., Tejedor, M. T., Lavina, A., & Sierra, I. (2009). A 17 bp deletion in the Bone Morphogenetic Protein 15 (BMP15) gene is associated to increased prolificacy in the Rasa Aragonesa sheep breed. *Animal reproduction science*, *110*(1-2), 139-146.

Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G., & Erlich, H. (1986). Spesific enzymatic amplification of DNA in vitro: The polymerase chain reaction. Cold spring Harbor Symposium on quantitative biology, vol. LI, 0-87969-052-6/86.

Nagdy, H., Mahmoud, K. G. M., Kandiel, M. M., Helmy, N. A., Ibrahim, S. S., Nawito, M. F., & Othman, O. E. (2018). PCR-RFLP of bone morphogenetic protein 15 (BMP15/FecX) gene as a candidate for prolificacy in sheep. *International journal of veterinary science and medicine*, *6*(sup1), S68-S72.

Al Qasimi, R. H., Jaffar, A. A., & ALKhauzai, A. L. (2021). Relationship GDF9B (BMP-15) Gene Polymorphism with litter size in Iraqi Awassi Sheep. *Euphrates Journal of Agriculture Science*, *13*(4).

Rahman, H. H., Al Qasimi, Azhar, A., Jaffar Allawi, L. D., & Al Khauzai. (2021). Relationship GDF9B (BMP-15) Gene Polymorphism with litter size in Iraqi Awassi Sheep, Euphrates *Journal of Agriculture Science, 13* (4):187-194.

Rasali, D. P., Shrestha, J. N. B., & Crow, G. H. (2006). Development of composite sheep breeds in the world: A review. *Canadian Journal of Animal Science*, *86*(1), 1-24. 10.4141/A05-073

Stancheva, N. (2003). *Phenotypic and Genotypic Parameters of Selection Indices in the Newly Created Milk Sheep Population in Bulgaria.* Ph D Thesis, Sofia, 188 pp. (Bg).

Stancheva, N., Dimitrova, I., & Georgieva, S. (2014). Biological fertility and milk yield in Bulgarian Dairy Synthetic Population sheep according to breeding line. *Agricultural Science & Technology (1313-8820)*, *6*(1), Pp, 17–20.

Stancheva, N., Raicheva, E., Laleva, S., Ivanova, T., Iliev, M., & Kalaydhziev, G. (2014). Present status, problems and development of the Synthetic Population Bulgarian Milk sheep from the herds of Agricultural Academy. *Journal of Animal Science*, *6*, 3-11.

Stancheva, N., Krastanov, J., Angelova, T., Kalaydhziev, G., Yordanova, D., & Laleva, S. (2016). Genetic structure of the sheep from the Bulgarian Dairy Synthetic Population on the Experimental Farm of the Agricultural Institute in Shumen. *Macedonian Journal of Animal Science*, *6*(1), pp 17–24.