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Single-Nucleotide Polymorphisms Identified within Exon 2 of Fertility-Associated Bone Morphogenetic Protein (BMP15) Gene in Three Romanian Sheep Breeds

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Abstract: The improvement of the reproductive traits of animals is of great interest for livestock production. Due to its positive impact on the sheep industry's profitability, prolificacy is one of the most economically significant biological traits, showing variation between and within breeds of domestic sheep (*Ovis aries*). Different mutations in *BMPR-1B*, *BMP15* and *GDF9* genes coding for the transforming growth factor-β (TGFβ) superfamily have been shown to influence the ovulation rate and litter size. Numerous single-nucleotide polymorphisms (SNPs) in the *bone morphogenetic protein 15* (*BMP15*) gene have been linked to ewe fecundity. Using targeted PCR amplification and Sanger sequencing, we were able to identify heterozygous SNPs in exon 2 of *BMP15* in three sheep breeds reared in Romania: Tsigai, Cluj Merino and Tsurcana. The sequence analysis revealed three previously documented mutations, namely the missense mutation c.755T>C (L252P), which is predicted to change the tertiary structure of the *BMP15* protein, and two silent mutations, c.747T>C (P249P) and c.1047G>A (V349V). In addition, we also identified one novel silent mutation, c.825G>A (S275S). Based on our findings and publicly available data, we indicate four putative mutational hotspots within exon 2 of *BMP15* that could be considered for improving the indigenous sheep breeds through targeted gene editing and SNP genotyping strategies.

Keywords: SNPs; BMP15; FecX; Tsigai; Cluj Merino; Tsurcana; litter size; fecundity

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1. Introduction

Sheep (*Ovis aries*) breeding plays a significant economic role and constitutes a major income stream, both for modern farming systems and small-scale farmers, due to its low resource requirements. In accordance with the advancement of ecological animal husbandry, the current development trend of sheep farming aims to reduce the number of adult individuals whilst increasing economic benefits. In order to achieve this goal, a key step is to improve the reproductive performance of ewes by producing more lambs. The majority of local Romanian sheep breeds, including Tsigai [1], Cluj Merino [2] and Tsurcana [3], have reduced litter sizes, an exception being the Prolific Palas composite sheep breed [4].

Tsurcana is the primary sheep breed in Romania and belongs to the Valachian (Zackel) broader group of sheep breeds, which is the leading phyletic group in central and south-eastern Europe [5]. Tsurcana and Tsigai breeds account for around 76.7% of the national Romanian ovine livestock [6]. Tsurcana was pivotal in creating the Cluj Merino breed, an endeavor that began in 1957, by crossing Transylvanian Merino rams with Tsurcana ewes, and this was successfully concluded in 1988 [7]. All three sheep breeds are well adapted

to Transylvania's climatic conditions, making them competitive in the context of modern sustainable agriculture, which aims to increase flock profitability.

Mutations in genes coding for the transforming growth factor-β (TGFβ) superfamily have been shown to influence ovulation rate and, in effect, litter size in sheep [8]. Known as *fecundity* (*Fec*) genes, these have the potential to significantly improve the reproductive performance of sheep flocks. Highly relevant examples include: *FecX* or *bone morphogenetic protein 15* (*BMP15*), an X-linked gene [9], *FecG* or *growth and differentiation factor 9* (*GDF9*), located on chromosome 5 [10], and *FecB* or *bone morphogenetic protein receptor type IB* (*BMPR1B*), or, alternatively, *activin-like kinase 6* (*ALK6*), which is mapped to chromosome 6 [11]. All these genes show documented hyperprolificacy-associated mutations that affect the bone morphogenetic signaling system in the ovaries [12].

Rams belonging to Romanian native breeds are suitable for assisted reproduction technologies (ARTs), as normal sperm quality is obtained using artificial collection methods [13]. Considering that sperm morphological features are maintained when artificial collection is performed and thus their genomic cargo is intact, specific traits controlled by fecundity genes can be effectively improved [13]. Moreover, ARTs hold the advantage of enhancing the transmission of beneficial genetic characteristics to offspring.

The BMP signaling pathway is critical for ewe fertility, as it contains the majority of genes associated with high fecundity (*BMP15*, *GDF9* and *BMPR1B*). BMP15 and GDF9 act as ligands in this biochemical pathway, whilst BMPR1B has a receptor function [14]. Together with growth and differentiation factors (GDF), activin/inhibin peptides, anti-Mullerian hormone (AMH), and the myostatin protein, the BMP system is an integrated component of the TGFβ superfamily [15]. TGFβ superfamily proteins are synthesized as precursors that are approximately three times larger than their mature form [16]. The BMP system is composed of approximately 30 ligands and preferential combinations of type I and type II receptors [17]. BMP proteins contribute to the regulation of gonadotrophin production in the anterior pituitary [18].

BMP15 (also referred to as *GDF9B*) has two exons with a total coding sequence of 1179 nucleotides (nt), separated by a 5.4 kb intron. The gene encodes a prepropeptide of 393 amino acid residues, and the active mature peptide is composed of 125 amino acids [19]. Many functional studies aimed to characterize the *BMP15* gene expression in rams [20] and ewes [21,22], as well as its influence on reproduction efficiency. *BMP15* has been associated with numerous functions important for mammalian ovarian and follicular development, including promoting granulosa cell (GC) proliferation and steroidogenesis [17] and preventing premature luteinization [23]. *BMP15* expression increases progressively, starting with the primary follicle stage, after which it decreases in the antral follicles [24]. In addition, during the development of ovarian follicles grown both in vivo and in vitro settings, *BMP15* was expressed in the oocytes and cumulus cells and displayed stage-specific expression levels in sheep [21].

BMP15 protein induces granulosa cell mitosis and inhibits the actions of follicle-stimulating hormone (FSH) by suppressing the corresponding *FSH receptor* (*FSH-R*) gene expression [17]. In heterozygous individuals, a reduced *BMP15* level (50% normal level) causes a higher expression of *FSH-R* in granulosa cells, whilst luteinizing hormone receptors (LH-R) stimulate the production of more follicles [25]. Thus, in homozygous individuals with *BMP15* loss-of-function mutations, the lack of bioactive BMP15 protein, and its mitotic effects, leads to follicle development stalling at the primary follicle stage, which in turn causes infertility in affected females [19]. Although a lack of BMP15 protein inhibits follicular growth in homozygotes, the inactivation of only one copy of *BMP15* increases the ovulation rate [26]. When the BMP15 peptide level is half of the wild-type (wt) amount, delayed suppressive effects on plasma FSH concentrations are caused by a decreased granulosa cell mitosis and impaired inhibin or steroid levels produced by each follicle [26]. Thereby, this allows more than one follicle to be selected for ovulation, which leads to increased prolificacy [19]. However, unlike in monoovulatory species, such as humans and sheep, in polyovulatory mice, BMP15 protein is not necessary for folliculogenesis [27].

Regarding inter-species similarities, the sheep *BMP15* gene coding region is highly homologous with its corresponding gene in humans (82.9%), mice (78.8%) and rats (78.4%) [19]. A large number of mutations in the *BMP15* gene are particular to women with premature ovarian failure and to mothers of dizygotic twins [28].

In the last two decades, the links between high prolificacy in sheep and various mutations in *BMP15* have been thoroughly addressed [10,29–47]. To date, a number of sheep-prolificity-associated variants have been identified in the *BMP15* gene, especially in exon 2, as presented in Table 1: $FecX^R$ [47]; $FecX^B$ [10]; $FecX^I$, $FecX^H$ and $FecX^L$ [8]; $FecX^G$ [48]; $FecX^{Gr}$ and $FecX^O$ [49]; $FecX^{Bar}$ [38]; and c.755T>C [40]. Therefore, our study focused primarily on exon 2, where *BMP15* mutations are predominantly found.

Table 1. Known mutations in exon 2 of sheep *BMP15* gene; from top to bottom, the alleles are presented considering the timeline of their discovery.

Allele Symbol	DNA Change	Amino Acid Change	Reference
$FecX^R$	c.525_541del	p.A101Cfs * 113	[47]
$FecX^B$	c.1100G>T	p.S367I	[10]
$FecX^I$	c.896T>A	p.V299D	[8]
$FecX^H$	c.871C>T	p.Q291 *	
$FecX^L$	c.962G>A	p.C321Y	
$FecX^G$	c.718C>T	p.Q238 *	[48]
$FecX^{Gr}$	c.950C>T	p.T317I	[49]
$FecX^O$	c.1009A>C	p.N337H	
$FecX^{Bar}$	c.301_304delGCTAinsT	p.A101Cfs * 113	[38]
-	c.755T>C	p.L252P	[40]

The * symbol designates a Stop codon.

Prolific sheep are regarded as useful models for identifying the genes and mutations involved in the mechanisms regulating ovarian function. These findings are significant for various applications in animal farming, namely genetic selection for prolificacy, as well as possible exploitations in human medicine such as the treatment of female infertility or subfertility [12].

A decline in the BMP system's activity is associated with an increase in the ovulation rate. Considering this, as well as the role of BMP molecules during folliculogenesis, it was proposed that these increases in the ovulation rate can be explained by functional mutations in the BMP system [15]. This hypothesis is supported by a very recent study, which shows that intrafollicular injection (IFI) of recombinant human BMP15 protein inhibits ovulation in cattle [50]. Based on the available data, it is plausible that the analysis of *BMP15* mutant alleles could be an essential path for amelioration strategies that attempt to increase sheep prolificacy. Alternatively, *BMP15* alleles screening could be relevant for estimating the prolificacy of certain breeds or individuals.

The aim of this study was to sequence for the first time the exon 2 of *BMP15* alleles harbored by selected individuals pertaining to Tsigai, Cluj Merino and Tsurcana sheep breeds in order to identify specific variants that can impact its activity.

2. Materials and Methods

2.1. Ethics Standards

All the procedures that were carried out on animals were approved by the Research bioethics Commission of USAMV Cluj-Napoca, Romania (registration number of approval of application: 274/31.07.2021).

2.2. Animals and Sample Collection

Blood samples from thirty prolific sheep (2–3 years old), belonging to Cluj Merino (n = 10), Tsurcana (n = 10) and Tsigai Rusty variety (n = 10) breeds were collected from different livestock farms from Transylvania, Romania. The selected Tsigai, Cluj Merino and Tsurcana ewes had only twin (n = 27) or triplet (n = 3) lambing records in their whole reproductive history and were provided by the Agricultural Research and Development Station, Turda, Romania; Experimental Didactic Center, Cojocna, Romania and a commercial farm located in Dipsa, Bistrita-Nasaud county, Romania. The blood samples were collected from the jugular vein in 4 mL heparin vacutainers (Vacutest Kima, Arzergrande, Italy) and stored at 0–4 °C during transportation. Following DNA extraction, the remaining blood was placed at −20 °C for future use.

2.3. DNA Extraction

DNA was extracted from refrigerated blood approximately one hour after sampling using a commercially available DNA extraction kit as indicated by the manufacturer (Quick-DNA Miniprep D3025, ZymoResearch, Seattle, WA, USA). DNA concentration and purity were assessed using a NanoDrop ND1000 Spectrophotometer (Nanodrop Technologies, Wilmingtom, DE, USA), and DNA was stored at −20 °C before use. The genomic DNA had an average concentration of 40 ng/µL and a purity of 1.8 ($A_{260/280}$).

2.4. PCR Amplification

An extended genomic region comprising exon 2 (1008 bp) of the *BMP15* gene was amplified using ovine-specific primers as previously described [44] (Table 2). PCR amplifications were performed in a final volume of 50 µL containing 1 µL of each forward and reverse primers (10 µM each), 10 µL of 5X MyTaq Reaction Buffer (Bioline, London, UK), 10 U of MyTaq DNA polymerase (Bioline, London, UK), 4 µL DNA and 32 µL of PCR-grade H_2O. The PCR conditions were as follows: 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s and a final extension step at 72 °C for 8 min. From each PCR product, 6 µL was migrated in a 1.0% agarose gel to determine the PCR product quality.

Table 2. Primers used to amplify exon 2 of the *BMP15* gene.

Primer Sequence (5′→3′)	Position	Amplicon Size
F: CATCTCAAGGCTGCTTGTCA R: TTCGAATTTCTTGGGCAAAC	Exon 2	1008 bp

2.5. PCR Product Purification and Sequencing

The remaining 44 µL of PCR product was purified using a Bioline ISOLATE II PCR and Gel Kit (Bioline, London, UK). Briefly, 2 µL of Binding Buffer CB was added for each µL of PCR product solution. After loading the sample into the column, the silica membrane was washed twice with 700 µL Wash Buffer CW, and the DNA was eluted using 30 µL Elution Buffer C. The purified DNA samples corresponding to each sample were externalized for Sanger sequencing (Macrogen Europe, Amsterdam, The Netherlands).

2.6. Sequencing Data Analysis

The experimental DNA sequences were aligned against exon 2 of the *BMP15* gene using the BLAST software from Geneious Prime (version 2023.1) [51]. The reference exon sequence was in accordance with the chromosome X reference sequence (GenBank accession number NC_056080.1) from the NCBI ARS-UI Ramb v2.0 (GCF 016772045.1) genome assembly of *O. aries*. The Unipro UGENE software (version 45.1) was used for assessing the base calling quality of the experimental sequences [52]. Geneious Prime [51] was exploited in order to determine the potential effect of mutations on the amino acid sequence.

2.7. SNPs Analysis

SNPs analysis was performed by interrogating the available literature and by identifying the corresponding point mutations within dedicated databases, such as NCBI (https://www.ncbi.nlm.nih.gov/ accessed on 23 January 2023) and Ensembl (http://www.ensembl.org/ accessed on 23 January 2023). In addition, we considered the mutations identified in our study, as well as those previously reported, in order to arbitrary delimitate putative mutational hotspots. The mutations coordinates were based on the coding DNA sequence and according to the X chromosome reference sequence.

2.8. Protein Prediction and Modelling

The L252P amino acid substitution was evaluated for a possible effect on BMP15 biochemical properties by comparing it to the wt protein using web-available protein prediction tools such as CUPSAT [53], DynaMut [54], INPS-3D [55] and MAESTROweb [56]. Protein folding modelling was assessed using two dedicated tools, PyMOL [57] and ChimeraX version 1.5 [58], which ran AlphaFold simulations [59].

An overview of the procedures performed in the present study is presented in Figure 1.

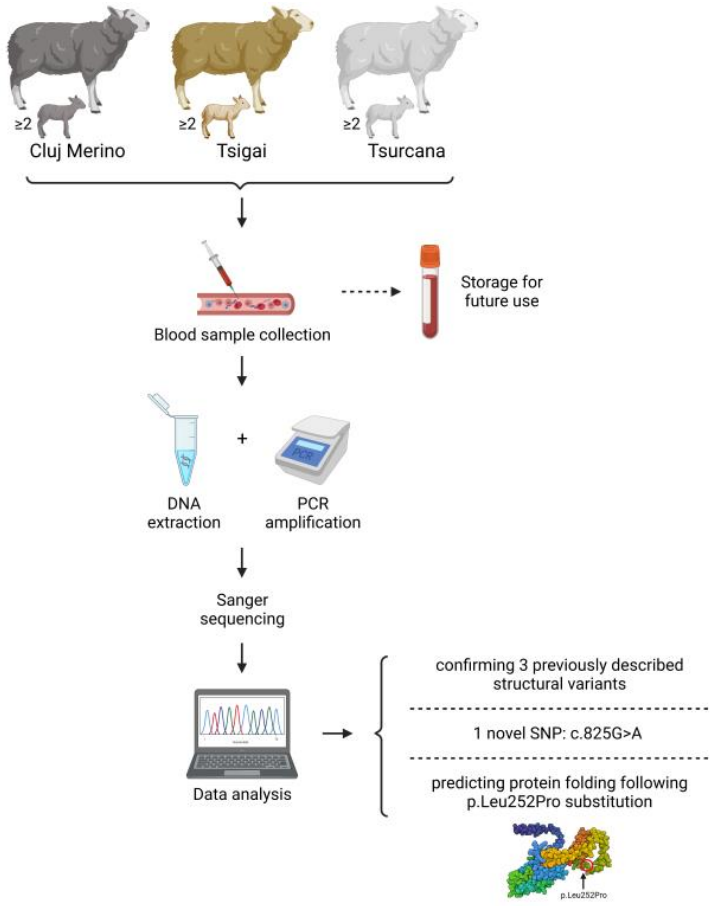

Figure 1. A synthetic workflow highlighting the procedures carried out in the present study, starting from sample collection and proceeding with sample processing, DNA extraction, targeted PCR amplification, Sanger sequencing and data analysis. Created with BioRender.com (accessed on 1 March 2023).

3. Results

PCR amplification was performed using ovine-specific primers flanking exon 2 of the *BMP15* gene, and agarose gel electrophoresis was used to verify the amplicon size (Figure 2). The target amplicons had an in silico estimated length of 1008 nt.

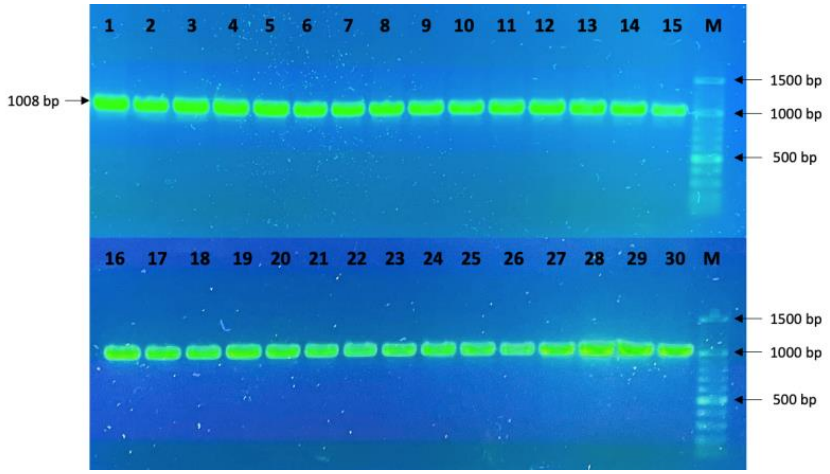

Figure 2. The electrophoregram of the target PCR products (1008 bp) belonging to Tsigai (lanes 1–10), Cluj Merino (lanes 11–20) and Tsurcana (lanes 21–30) breeds; M: 100 bp DNA ladder (O'RangeRuler 100 bp DNA ladder, Fermentas, Vilnius, Lithuania).

In order to identify possible structural variants associated with the prolificacy of the studied ewes, the target *BMP15* amplicons were sequenced. Consecutive to the sequencing data analysis, we identified four different mutations in exon 2 of *BMP15*, including a previously uncharacterized SNP.

3.1. Sequencing Data Analysis

Mapping the target amplicon sequences against the reference genomic sequence corresponding to *BMP15* revealed four distinct heterozygous SNPs: one previously documented missense mutation, c.755T>C (L252P), which was predicted to affect the tertiary structure of the BMP15 protein, two silent mutations, namely c.747T>C (P249P) and c.1047G>A (V349V), as well as a novel silent mutation (GenBank/NCBI accession number OQ593381), c.825G>A (S275S). All variants were confirmed by both forward and reverse sequencings. The sequencing electropherograms of the four structural variants identified in the ovine *BMP15* gene are presented in Figure 3.

Mutation c.755T>C was identified in two ewes, one belonging to the Tsurcana breed and one to the Cluj Merino breed. The c.747T>C and c.1047G>A mutations were identified in the Tsurcana breed, with one individual carrying the c.747T>C mutation and another ewe carrying the other variant, c.1047G>A (V349V). The novel mutation, c.825G>A, was identified in a different individual from the Cluj Merino breed (Table 3).

Table 3. Structural variants identified in exon 2 of the *BMP15* gene. Three of the four substitutions, including the novel SNP presented in this study, were silent mutations (c.747T>C, c.825G>A and c.1047G>A). The last two columns present the mean litter size (LS) and the parity number of affected individuals.

HGVS (GenBank: NC_056080.1)	WT Allele	Mutant Allele	Amino Acid Change	Breed	LS	Parity
c.747T>C	T	C	p.Pro249=	Tsurcana	2	2
c.755T>C	T	C	p.Leu252Pro		2.66	3
c.1047G>A	G	A	p.Val349=		2.33	3
c.755T>C	T	C	p.Leu252Pro	Cluj Merino	2	2
c.825G>A	G	A	p.Ser275=		2	1

HGVS: Human Genome Variation Society.

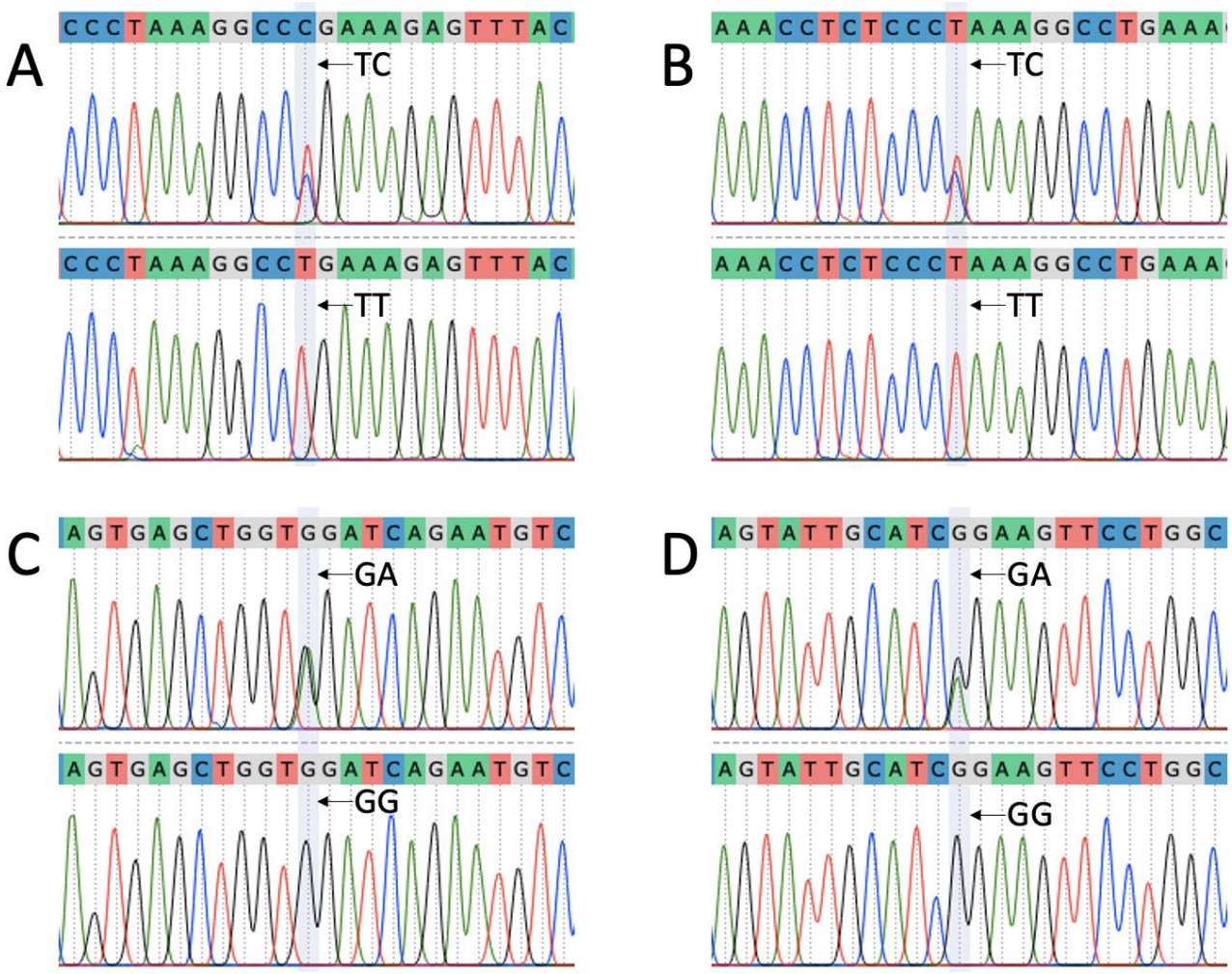

Figure 3. Sequencing electrophoregrams of the four structural variants found in ovine *BMP15* gene: (**A**) c.755T>C substitution (T/C genotype, **upper** panel) and T/T wt genotype (**lower** panel); (**B**) c.747T>C substitution (T/C genotype, **upper** panel) and T/T wt genotype (**lower** panel); (**C**) c.1047G>A substitution (G/A genotype, **upper** panel) and G/G wt genotype (**lower** panel); (**D**) c.825G>A novel substitution mutation (G/A genotype, **upper** panel) and G/G wt genotype (**lower** panel). Images were generated with Unipro UGENE software (version 45.1).

3.2. Putative Mutational Hotspots in Exon 2 of BMP15 Gene

Following the identification of the structural variants in the selected populations, we evaluated our results by consulting the literature and online databases. Out of the four identified SNPs, three were previously documented in other *O. aries* natural populations (c.747T>C, c.755T>C and c.1047G>A), and one was not previously reported (c.825G>A). Additionally, the NCBI database lists other structural variants within exon 2 of the *BMP15* gene. Based on the proximity and apparent grouping of several known exon 2 structural variants, we defined four arbitrary mutational hotspots (Table 4) containing SNPs or deletions. The hotspots confines were defined at ±5 nt relative to the coordinates of the outermost structural variants of each mutational hotspot.

We mapped the novel SNP c.825G>A variant within the third mutational hotspot, along with the two previously reported structural variants (Figure 4).

Table 4. Putative mutational hotspots within exon 2 of *BMP15* gene in *O. aries*. The structural variants also identified in our study are highlighted in bold characters, including the novel c.825G>A substitution.

	Size (nt)	CDS Region	Variants	CDS Change	Variant Effect	Variant Type
Hotspot 1	79	c.455–534	rs1091219782 rs421419167	c.529C>G c.460_476del	p.Pro177Ala p.Trp153AsnfsTer55	missense frameshift deletion
Hotspot 2	48	c.713–760	**rs55628000** **rs1089512607**	**c.755T>C** **c.747T>C**	**p.Leu252Pro** **p.Pro249=**	**missense** **silent**
			rs425019156	c.718C>T	p.Gln240Ter	nonsense
Hotspot 3	82	c.820–901	rs398521635 rs413916697 **Novel SNP**	c.896T>A c.871C>T **c.825G>A**	p.Val299Asp p.Gln291Ter **p.Ser275=**	missense nonsense **silent**
Hotspot 4	21	c.1032–1052	**rs587958054** rs402413016	**c.1047G>A** c.1037G>A	**p.Val349=** p.Ser346Asn	**silent** missense

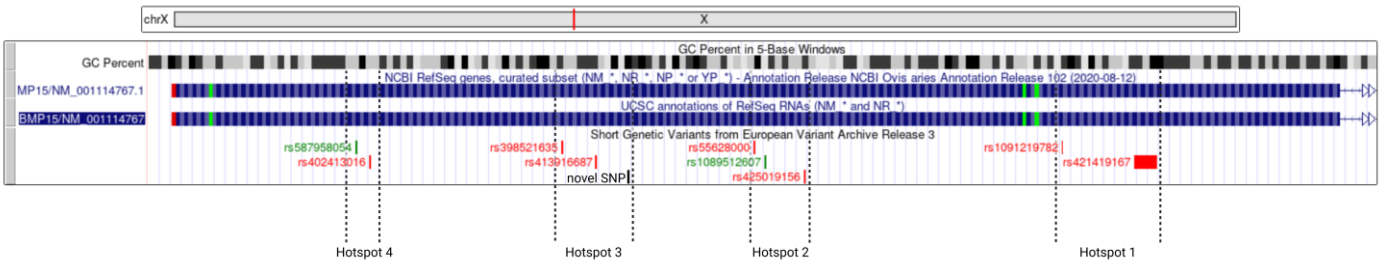

Figure 4. Structural variants and putative mutational hotspot (hotspot 1 to hotspot 4) sites within exon 2 of *BMP15* gene. Screenshot from UCSC database edited in BioRender (accessed on 6 March 2023). Data presented according to reference strand of the genome.

3.3. Predicting the Functional Impact of the Mutations Affecting BMP15 Protein

We estimated the potential effect of the mutations identified in this study on amino acid coding using the Geneious Prime software (version 2023.0.1). The three silent SNPs were not expected to have a serious impact on the BMP15 protein function and on its interactions within the BMP signaling pathway.

The missense mutation c.755T>C determines an amino acid substitution, p.L252P. Therefore, we investigated its possible effect on protein structure and biochemical properties. The secondary structure analysis revealed that the p.L252 amino acid was located in the near vicinity of an α-helix and a glycosylation site at position p.N237. The PAM250 matrix predicted a score of −3 for the substitution of L with P, a mutation that may decrease the protein's hydrophobicity, which in turn might influence its normal folding, according to dedicated protein tertiary structure analysis tools, such as CUPSAT [53], DynaMut [54], INPS-3D [55] and MAESTROweb [56].

Using various web tools [60] for predicting protein stability changes, based on a similar approach described in a recent study, we found inconsistent results with regard to the ΔΔG (kcal/mole) value. Two tools predicted a positive change in energy, i.e., ΔΔG = 1.01 kcal/mole, and an unfavorable modification in torsion at the substitution site (CUPSAT) with ΔΔG = 1.016 kcal/mole (MAESTROweb). In contrast, the other two tools predicted a negative change in energy, namely ΔΔG = −0.153 kcal/mole (DynaMut) and ΔΔG = −0.544 kcal/mole (INSP-3D).

Upon computing the protein folding models on the dedicated software packages ChimeraX and PyMOL, the results showed no modification in the protein's tertiary structure as a result of the p.L252P substitution compared to the wt BMP15 protein. A computational model of the BMP15 protein highlighting the localization of the amino acids specified by the affected codons found in the analyzed *O. aries* populations is presented in Figure 5.

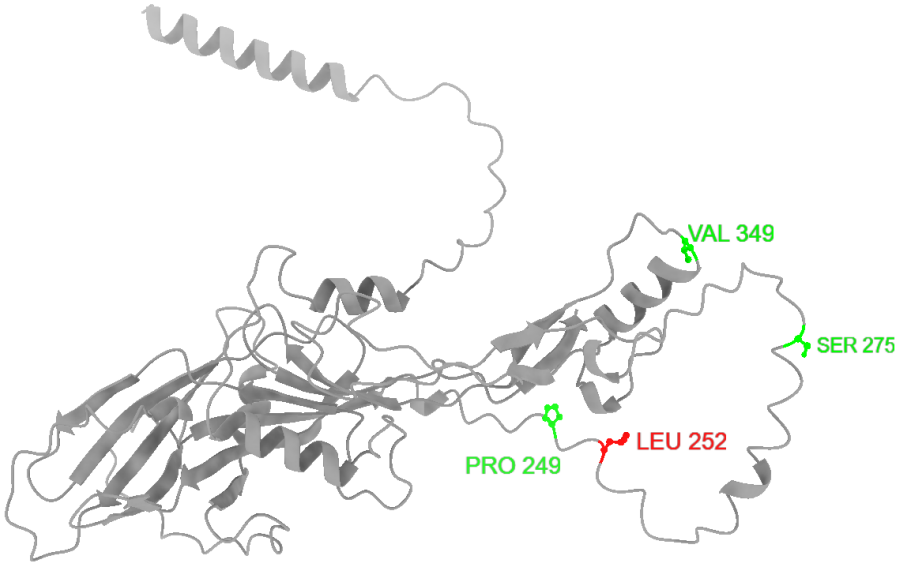

Figure 5. Predicted model of the BMP15 protein. The highlighted amino acids are specified by the codons encompassing the mutations identified in this study. The amino acids resulting from silent mutations are highlighted in green, and the amino acid changed by the missense mutations is highlighted in red. Image generated with ChimeraX software version 1.5.

4. Discussion

Our results showed that in the selected ewes from the Tsurcana and Cluj Merino local breeds, the heterozygous missense c.755T>C mutation was identified in 10% (2/20) of the individuals. One possible explanation for the low incidence of the c.755T>C mutation is that, traditionally, in Romania, farmers do not keep replacements from twin or triplet lambings, and thus, a detailed breeding plan between mutant rams and ewes is lacking, which in turn causes relatively low litter size in the native sheep breeds.

Since the BMP signaling pathway plays a crucial role in ewe fecundity, assessing the mutations affecting the *BMP15* gene is an essential feat in order to improve reproductive performance. The mutations identified in the various genes pertaining to the BMP pathway led to specific alleles, herein symbolized as B. Numerous sheep breeds around the world have been identified to possess various mutations in *BMP15*, many of them leading to an improvement in the litter size of heterozygous mutants (B/+) and infertility in homozygous ewes (B/B). An example is represented by Romney ewes that carry the Inverdale (I) mutation ($FecX^I$) located on the X chromosome. Davis et al. (1992) [61] showed that ewes heterozygous for this mutation (I/+) have ovulation rates about 1.0 units higher than non-carriers (+/+). They also demonstrated that I/I homozygous sheep have "streak", nonfunctional ovaries, which are about 1/8 the size of normal ovaries and do not exhibit any signs of follicular activity [61].

The mutations identified in our study were in the heterozygous state (B/+). We identified the missense mutation c.755T>C, which results in a replacement of the leucine at p.252 with proline. This mutation was firstly described in Iranian Shal, Ghezel, Afshari and Lori-Bakhtiari sheep breeds [40] and was associated with high fertility. Furthermore, heterozygous ewes (c.755T>C) had a higher prolificacy than the mutant homozygous ones. Homozygous c.755T>C mutation does not lead to sterility, in contrast to the majority of the known *BMP15* mutations. Therefore, this mutation could be pivotal in designing a breeding program aiming to improve the reproductive performance of *O. aries* breeds.

In the Cele black sheep breed, a recent study showed that ewes heterozygous for the c.755T>C mutation had larger litter sizes than wt ewes, presumably due to a change in the structure and function of the BMP15 protein [45]. Our in silico modelling did not provide conclusive results regarding the ΔΔG changes, but other parameters, such as hydrophobicity, can decisively influence the protein properties. Heterozygous females

exhibited higher litter size than wt individuals, but no homozygous mutant ewes were identified [45]. Another study carried out on Mongolia and Ujimqin breeds showed that the c.755T>C mutation was significantly associated with an increased litter size [46]. Sheep that had a c.755T>C heterozygous genotype were significantly more prolific than wt ewes. Additionally, the same study described the c.1047G>A mutation for the first time, which we also identified in one Tsurcana ewe.

The c.747T>C silent mutation found in one Tsurcana ewe was firstly identified in the Cambridge and Belclare sheep breeds [10] and later in the Noire de Thibar [33] and Grivette [35] breeds, but it was never associated with prolificacy.

The arbitrarily defined hotspots highlighted exon regions that could be considered for targeted genome editing strategies, since several *BMP15* mutations have a positive impact on fertility. Moreover, although relatively cost-efficient, sequencing could be difficult to consider and/or to perform in real farm settings if fast and efficient sheep genotyping is required. Such mutational hotspots could be relevant for various PCR applications, such as the amplification refractory mutation system PCR that was previously employed for identifying SNP markers in sheep [62].

There are growing concerns regarding the effect of livestock production on the environment, specifically in terms of greenhouse gas emissions. In this context, fertility is extremely important, because the number of offspring lambed per mated ewe is one of the determining factors regarding the reduction in the carbon footprint (CF) per kg of lamb [63]. In addition, due to their ability to adapt to local conditions, local breeds represent valuable genetic resources. Nevertheless, in order to identify other candidate genes and variants that could be associated with increased prolificacy, sequencing, genome-wide association studies and/or gene expression (microarray/ARN-seq) strategies should be performed on highly prolific individuals from local sheep populations.

5. Conclusions

In this study, a total of four mutations were identified in exon 2 of the *BMP15* gene, including one novel variant (c.825G>A) that could be used as possible candidate in order to enhance the reproductive efficiency of these sheep breeds. With the exception of c.825G>A, all of the other SNPs have been previously documented in sheep breeds worldwide.

Considering the necessity to enhance the reproductive traits of local sheep breeds in order to increase farmers' profitability and sustainability, our study could be of interest for future managerial decisions regarding the improvement of fecundity traits in Tsigai, Tsurcana and Cluj Merino breeds. Out of the four identified SNPs, the c.755T>C (L252P) mutation had a documented effectiveness in boosting sheep fertility. Moreover, the p.L252P polymorphism does not induce sterility in homozygous condition [40], unlike most of the *BMP15* variants associated with prolificacy. Although identified in a few individuals, it is of interest that the c.755T>C mutation is effectively present in the Tsurcana and Cluj Merino genetic backgrounds. This feature could be of pivotal relevance for future breed improvement projects, since the dissemination of this mutation in the analyzed local populations would not affect their specific allelic background. If c.755T>C-centered strategies are to be considered, mattings involving individuals from other local populations are to be avoided since they would bring, along with the intended mutation, non-specific gene alleles with unforeseeable effects.

We could not identify any mutation in the Tsigai breed, which means that additional studies focused on the high prolificacy associated *GDF9* gene or other candidate genes that can lead to an improvement in fecundity are required.

Furthermore, no definitive relationship was determined between the novel c.825G>A mutation and litter size. Future research, eventually evaluating its impact on mRNA structure and processing, could reveal if there is any association between this SNP and litter size in larger Cluj Merino populations.

Our study is the first to describe the *BMP15* gene alleles in three Romanian native sheep breeds. Further studies could aim to determine the genetic penetrance of the identified

variants, their association with litter size using a vast sample size and to investigate the genotype of these breeds with respect to the prolificacy influences exerted by the TGFβ superfamily of genes.

In a future where food products will be labeled according to their CF levels, which is reported per unit of food product, an increase in a flock's litter size could drastically reduce CFs [63]. Subsequently, achieving this goal could improve the competitiveness of lamb products and could positively influence consumers' purchasing decisions when buying meat-based products. Considering that the main source of income of sheep breeders in Romania comes from lamb marketing, intended both for domestic consumption and for export, the main goal in this industry is to improve the reproductive performance of native sheep breeds.

Romania has the second largest livestock population of sheep in the European Union (16.7% of the EU total) [64], and accounting for future challenges in this branch of agriculture, we can strongly state that more studies are required in order to improve the fertility and prolificacy of native sheep breeds through breeding programs based on modern molecular biology methods and genetic selection.

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