*Hinf*I Polymorphisms Characterization in the 1st intron of BMP15/FecX Gene in Turcana and Tsigai Sheep (*Ovis aries*)

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Abstract
BMP15-FecX is one of the major genes associated with prolificacy in different sheep breeds (*Ovis aries*), alongside BMPR15/FecB and GDF9/FecG. The majority of the polymorphisms, associated with ovulation rate in different breeds, are located in the exonic sequences of the gene. In the present study, a fragment of 356 bp, adjacent to the 2nd exon of the BMP-15 gene, was amplified by PCR in 50 individuals of Turcana and Tsigai breeds. The amplified products were subjected to digestion to identify the polymorphisms in the *Hinf*I restriction enzyme sites. The two putative enzyme sites presented in the fragment did not reveal mutations in neither breed, releasing the same electrophoretic profile.

Keywords: SNP, Polymorphism, BMP15 Gene, Restriction enzyme

1. Introduction

BMP-15 (Bone morphogenetic protein 15) gene, also known as GDF9B (Growth and Differentiation Factor 9B) belongs to the TGF β superfamily (Transforming Growth Factor β), which plays an important role in regulating ovarian follicular development [1,2]. The BMP15 gene is located on heterosome X and was discovered by Galloway et al. in 1999 [3] in a prolific Romney flock. An extensive range of mutations in the members of TGF β family have been identified in sheep and were associated with ovulation rate and embryo survival, the main component of litter size.

Genetics of theBMP15 locus:

The first genetic polymorphism associated with prolificacy was identified in Boorola Merino sheep, in the FecB gene (Fecundity gene Boorola) with autosomal location, on chromosome 6th [4-6]. The gene is also known as BMPR15, or as the BMP receptor type 1B. Another member of the TGF β superfamily is the GDF9 gene - Growth differentiation factor 9/FecG; [7] located on the 5th ovine chromosome [8]. The polymorphisms of all 3 genes have received special attention in the last couple of years in many breeds, as an important genetic tool to improve prolificacy in the flocks and for increasing economic efficiency of the farm. So far, at least 10 important mutations were identified in the BMP15/FecX gene and 3 mutations in the GDF9/FecG gene. The nomenclature of functional mutations identified in the BMP15 gene, associated with increased ovulation rates or infertility in different breeds, is as follows:

- $FecX^{I}$ (Inverdale/c.893T>A), results in valine substitution with aspartic acid in mature coding sequences [7], and $FecX^{H}$ (Hanna/ c.871C>7), which results in a premature codon stop; both mutations were identified in New Zeeland Romney ewe [3,9];
- $FecX^{G}$/BMP15c.718C>T, introduces a premature stop codon in the place of glutamic acid, at amino acid residue 239 of the unprocessed protein, which presumably results in complete loss of the gene's (BMP15) function; $FecX^{B}$/BMP15c1100G>T

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changes the serine residue at amino acid 99 of the mature active protein in an isoleucine, in Cambridge and Belclare sheep [7,10,11]; - $FecX^{L}$/BMP15c.962G>A identified in French Lacaune [12]; - $FecX^{R}$/BMP15c.455_471del;$FecX^{RA}$/c.1172C>T, and $FecX^{GR}$ in Spanish Rasa Aragonesa [13,14];
- Fec^{Bar}, a composite polymorphism associating a single nucleotide substitution (c.301G > T), a 3 bp deletion (c.302_304delCTA) and a C insertion (c.310insC) in the ovine BMP15 cDNA, leading to a frame shift at protein position 101, in Tunisian Barbarine sheep [15], and
- $FecX^{GR}$/ BMP15c.950C>T and $FecX^{O}$/BMP15c.1009C>T in French Grivette and Olkuska sheep [16] in which homozygous ewe are more prolific than their heterozygous counterparts. As a X-linked gene (BMP15), the homozygous or heterozygous combinations of alleles in females produced different phenotypes, from sterile to prolific. The interactions between loci, regarding genes BMPR15, GDF9 and BMP15, were first reviewed by Davis in 2001, where several research groups have reported the multi-marker combination effects on litter size in sheep. In previous association studies, the mutations in $FecX^{I}$, $FecX^{H}$, $FecX^{G}$, $FecX^{B}$ and $FecX^{R}$ genes exhibit one or two additional ovulations or increased prolificacy in heterozygous females compared to noncarriers, while homozygotes are sterile due to a blockade of follicular development at the primary stage [2,5,7,12,13,16]. Even though the validity of these associations holds for the majority of breeds, the homozygous ewes $FecX^{GR}$ and FecXO, are not sterile in other breeds, but on the contrary, are hyper-prolific [14,17]. In some hyper-prolific sheep (such as the Garole), the polymorphisms associated with this trait were detected only in the BMPR15 and GDF9 genes and not in the BMP15 gene (Polley et al, 2010). In other breeds, such as the Suffolk, Dorset, Charolais, or hybrids Suffolk × China Merino, Dorset × China Merino, the polymorphisms in the BMPR15 gene or in other major genes was absent, even though the sterile phenotype exists [18]. Few studies in literature investigate the intronic region for mutations found in Corriedale, Kashmir valley sheep and Friesian x Corriedale hybrids, in which only one new SNP mutation (A>G) was identified [19,20]. Even though the two breeds taken in this study are not known to be hyper-prolific (Turcana with litter size - 118.94 ± 0.051%, and Tsigai - 1.211 ± 0.398), management and feeding conditions can be used to improve prolificacy [21-23]. The study of presence of possible association between alleles with this genetic trait, even at low frequencies, can be used to enhance the offspring production in the flocks. On the other hand, some specific mutations in the coding or intronic region can be in linkage disequilibrium with loci from the regulatory region of the genes, responsible for prolificacy. To characterise the structure of these genes in Romanian sheep breeds - Turcana and Tsigai, we start the study of polymorphisms of the first intron of the BMP15 gene, which is part of a large study investigating all polymorphisms in the BMPR15, GDF9 and BMP15 genes.

2. Materials and methods

2.1 Biologic material:

All blood samples, from which DNA was extracted, come from animals with registered data of origin, sex, and age. 22 mature ewes (1.5 – 5 years of age) and 3 males from each breed (Turcana and Tsigai) were selected according to the history of lambing: single lamb (14), twin lambs (3) or sterile females (5).

2.2 Genomic DNA extraction from blood

200 µl of blood samples, collected on K_2EDTA, from each animal were subjected to extraction with Quick DNA Microprep Plus Kit following the manufacturer's instructions (BioZyme). The DNA samples were analysed on Spectrophotometer NanoDrop ND1000 for quantity and quality of the DNA. All samples analysed had optimum purity, ranging between 1.8 and 2 and the quantity ranging between 69-150 ng DNA/µl.

2.3 Sequence identification and region selection

The primary approach used was to identify the position of intronic DNA sequence of Bone Morphogenetic Protein 15 – BMP-15 gene, in GeneBank/NCBI (NC_040278.1). BMP15 full length sequence of the gene is of 6485 nucleotides and encodes a pre-proprotein of 393 amino-acid residues, while the active mature peptide is of 125 amino acids long [3]. The entire intronic nucleotide sequence lies between positions 323 – 5629 and the length of intronic genomic sequence amplified by PCR, stretches between positions <5259 – 5615>, in the total length of 6485

nucleotides of the gene. The primers used to amplify the 356 bp fragment of the intronic sequence had the following structure (Table 1).

Table 1 -The 5'-3' primer's structure for amplification of 356 bp fragment of the 1th intron of BMP-15 gene (according to Shabir and Ganai, 2012 [20])

	Primer structure	Ta
Forward	5' TTCTCCGTCTAGGGGTATGAG 3'	60°C
Reverse	5' AGGGAACAAGAGCAAAGCGTTAGC 3'	

2.4 PCR amplification and RFLP analysis

The fragment of 356 bp was amplified in 25 selected ewes and rams of each breed by conventional PCR, in 25µl final volume, using 5µl - 5xFirePol Master Mix (BioZyme); 0.6 µl of each (forward and reverse) primer (from 10pmol/µl solution), DNA template - 2µl and H_2O -16.8 µl. After 35 cycles of amplification in an MasterCycler thermocycler (Eppendorf, Germany) (94°C – (30 s); 58°C (45 s); 72°C (45 s) and a final extension at 72°C for 8 min) the PCR products were analysed on 1% agarose gel. After electrophoresis 15 µl of each sample were submitted to restriction with 1 µl of *Hinf*I enzyme, 2.5 µl buffer and 6.5 µlH_2O, at 37°C for 2 hours. The restriction products were separated in 2.5% agarose gel (Figure 1).

3. Results and discussion

Genotyping the possible polymorphisms G/ACTC, G/ATTC (positions X:50971977; X:50972094) in the fragment amplified by PCR was realised by enzymatic restriction with *Hinf*I. The primer pairs used, amplified a 356 bp fragment from intron 1 of BMP15, in samples and the *Hinf*I enzyme recognized the two putative restriction sites in all samples, resulting in three fragments of characteristic length: 70 bp, 117 bp and 169 bp. All samples presented the same profile, with three expected fragments, with no modifications in the enzyme's sites (Figure 1).

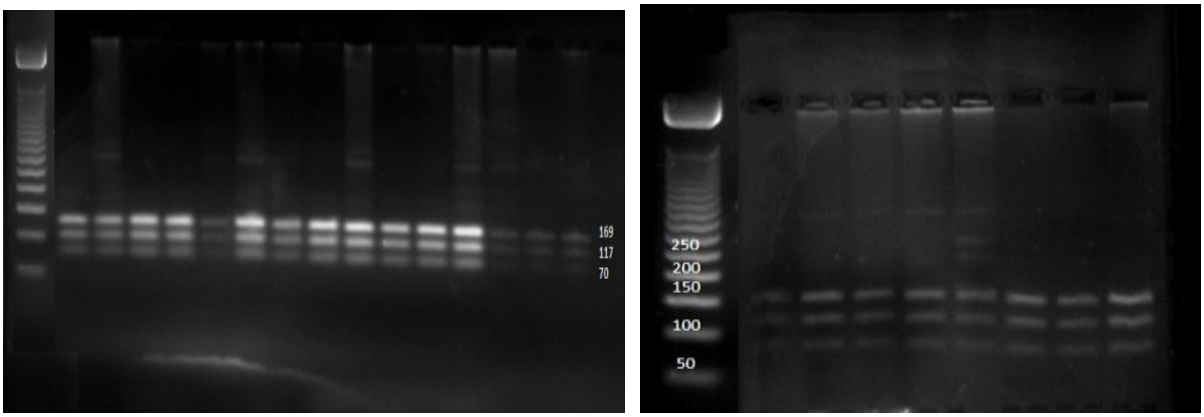

Figure 1 Electrophoretic profile of 356 bp fragment of BMP15 gene, restricted with *Hinf*I enzyme (line1 – 50 bp DNA ladder; individual profiles from Turcana (left) and Tsigai (right) breeds).

4. Conclusions

Short term immunisation with a GDF9 or BMP15 peptide-protein conjugate has been shown to enhance ovulation rate and lamb production. Recent studies of genetic mutations in sheep highlight the importance of oocyte-secreted factors in regulating ovulation rate, and these discoveries may help to explain why some mammals have a predisposition to produce two or more offspring rather than one [24]. The study of possible specific mutations in genes associated with prolificacy is necessary and important for characterizing a particular phenotype in various local breeds. Research undertaken in the past 3 years for characterizing the structure of the loci has revealed two new alleles in BMP15 gene in different breeds which are associated with prolificacy. The only new mutation, identified in 204th bp position of this fragment of BMP15 gene, in Corriedale and Kashmir Valley sheep indicates this type of study is often useful for a full characterisation of a breed

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