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PCR Analysis and Sequence Variation of Bone Morphogenetic Protein 15 (*BMP15/FecX*) Gene in Corriedale and Local Kashmir Valley Sheep

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Abstract

High prolificacy in sheep has been observed across different breeds and even within the same breed. Genetic conclusions have proven that in some situations ovulation rates and litter size can be genetically determined by the action of single gene(s) called *Fec* genes with major influences. Identification and exploitation of these genes by introgression in sheep can channel the current gap between demand and supply of meat to certain magnitude. Three major fecundity genes identified in sheep are FecB, GDF9, and BMP15. BMP15 gene is located on X chromosome and involves two exons. SNPs at diverse positions have been identified in this gene. To screen for these point mutations and detect any other mutation(s) in the mutational hotspot section of this gene, current study was carried out on Corriedale and Local Kashmir valley sheep. Polymerase Chain Reaction analysis was carried out in all the samples and two labelled alleles (A and B) were documented by the sequence analysis and there was no individual homozygous for A or B allele. The sequence similarity of nucleotide and predicted amino acid sequences of designated alleles was also achieved. These sequences were then subjected to multiple alignments with the reported sequences of different species. These point mutations lead to change in the amino acid sequences of the resulting protein, potentially impacting its structure and function and could affect the population at large. These also highlights the evolutionary implications as polymorphic genes may provide adaptive advantages to the population under study contributing its variability in traits that influence survival.

***Key words*:** BMP 15 Gene, Corriedale, Sequencing, SNP's, Alleles, Multiple alignment

The establishment of preferred prolific flocks, by selecting prolific ewes for the fecundity genes conferring prolificacy, has proven to be an applicable way to augment prolificacy. The fecundity genes, with differing magnitude of effect on ovulation rate and litter size, have become a new selection opportunity for a sheep farmer to increase lambing percentages significantly. By identifying and selectively breeding ewes that possess these genes, farmers can significantly improve lambing percentages, leading to higher productivity and profitability. The impact of fecundity genes varies; some genes have a stronger influence on ovulation rate and litter size than others. This genetic selection approach offers a sustainable and targeted method for increasing the number of lambs born per ewe, reducing the reliance on hormonal or nutritional interventions to boost fertility. Three types of fecundity genes have been reported in sheep [1]. These are Bone Morphogenetic Protein Receptor *IB* (*BMPRIB*) also known as Booroola fecundity gene (*FecB*) [2], growth differentiation factor 9 (*GDF9*) also known as *FecG* and bone morphogenetic protein 15 (*BMP15* or *GDF9B*) also known as *FecX* [3]. These three fecundity genes belong to the transforming growth factor-beta (*TGF-β*) superfamily [4]. BMP15 expression in ovary of prolific Jining Grey goat breeds was significantly higher than that of non-prolific Liaoning Cashmere goat breeds [5]. BMP15 gene, in both naturally mutated or knocked out animal models, is a key gene that affects ovulation rate or litter size in mammals [6]. Expression levels of *GDF9* gene in ovaries were higher in high prolific Small Tail Han sheep than that in low prolific specimens indicating that *GDF9* gene may play a positive regulator role in the lambing performance [7].

Moreover, the identification of specific fecundity genes, such as *BMPR1B* (Booroola gene), *GDF9* (Growth Differentiation Factor 9), and *BMP15* (Bone Morphogenetic Protein 15), has provided sheep breeders with precise genetic markers to guide their selection process. These genes regulate ovarian function and follicular development, making them valuable tools for improving reproductive performance in commercial and smallholder sheep farming systems. The implementation of a prolific flock strategy requires careful genetic screening, controlled breeding programs, and an understanding of how these genes interact with environmental and management factors. Farmers can leverage modern genetic testing technologies to identify carriers of desirable fecundity genes and incorporate them into breeding plans to maximize genetic gain over successive generations.

Therefore, to investigate the polymorphism at gene level is of massive importance since it would be advantageous to use molecular markers to recognize whether an animal has genetic potential to produce secure economically desirable

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characteristics even at younger age. The goal of present study was to explore the existence of any such polymorphism in local ewes of Kashmir valley and Corriedale.

MATERIALS AND METHODS

Studied animals

The animals explored for the experimental study encompassed ewes with the record of twinning and single births. In addition to it, ewes of Corriedale breed kept at organized farm of Sher-e-Kashmir University of Agricultural Sciences and Technology Kashmir at Shuhama Campus were also incorporated in this research study. All animals were in a good state of health and nutrition.

DNA preparation and primer sequences

Ten ml of blood was collected from jugular vein of every ewe in 15 ml sterile graduated polypropylene tubes containing EDTA. Blood samples were stored at -20° C till isolation of genomic DNA. Genomic DNA was isolated from frozen blood samples using the standard protocol of Sambrook and Russel [8] by phenol-chloroform extraction procedure. Primers for exon-2 DNA fragments of *BMP15* gene of sheep were designed utilizing "Fast PCR" software. The details of the primers are given in (Table 1).

Table 1 Primers along with the properties used for amplification of exon-2 fragment of *BMP15* gene

Primer	Primer sequence (5′-3′)	Molecular weight	Tm (°C)	Length (mer)	GC content (%)	Size of amplicon (bp)
(F)	GAGTGTTCAGAAGACCAAACCTC	7041.65	62.77	23	47.80	222
(R)	TGGGGAGCAATGATCCAGTGATCC	7417.89	66.28	24	54.20	

DNA amplification

The standardized combination of different reaction components for each 25 µl PCR reaction mix for amplification were 200 mM dNTP mixture, 30 ng each of forward and reverse primer, 1.5 mM $MgCl_2$, 2.5 mM buffer, 50-100 ng ovine genomic DNA and 1U Taq DNA polymerase. The optimized PCR programme used in the present study were as follows: Initial denaturation at 94°C for 2 minutes, cyclic denaturation at 94°C for 30 seconds, annealing at 60°C for 1 minute, extension at 72°C for 30 seconds and final extension at 72°C for 5 minutes. PCR products were separated by electrophoresis on 2% (w/v) agarose gel in 0.5x TBE, in parallel with 100bp DNA marker. The gels were stained with ethidium bromide before picturing them under UV light.

Sequencing and multiple alignment analysis

Amplified samples were sequenced using an ABI Genetic Analyzer. The sequences attained by forward and reverse primers were aligned and those showing mismatches were re-sequenced. The sequences achieved were also aligned with that of available sheep sequence existing in data bank of NCBI. Nucleotide sequences as well as their deduced amino acid sequences were subjected to multiple alignments with the reported sequences of different species using the Clustal W sub-programme of MegAlign Programme of DNASTAR Software (Lasergene, USA).

RESULTS AND DISCUSSION

Genomic DNA (Deoxyribonucleic acid) was isolated as per the standard protocol of Sambrook and Russel [8] with slight modifications. Modification in step of dissolution of DNA (Deoxyribonucleic acid) pellets in autoclaved triple distilled water rather than TE buffer (pH 8.0) was made to prevent likely chelation effect of Mg^{2+} ions by EDTA (existing in TE buffer). Chelation of Mg^{2+} ions hinder in amplification of DNA by interfering in DNA polymerase enzyme activity. To dissolve the DNA pellet completely in the autoclaved TDW, it was incubated at 60°C for 2 hours. This incubation was also favoured for its deactivating action on DNase present, if any, in the solution. PCR (Polymerase chain reaction) product of all samples was of 222 bp length (Fig 1).

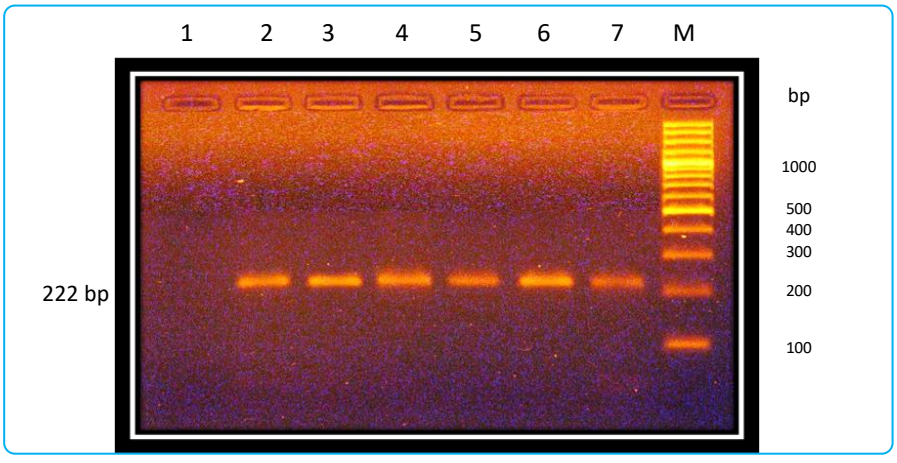

Fig 1 Amplification of fragment of exon-2 of *BMP15* gene of Corriedale and Kashmir valley sheep, Lane 1: Negative Control, Lane 2-4: Corriedale sheep, Lane 5-7: Kashmir valley sheep, Lane M: 100 bp DNA Ladder Marker

Two different allelic forms were confirmed by nucleotide sequencing and were labelled as A and B (Table 2). The nucleotide sequence of labeled A allele displayed nucleotide "G" at both the points of 171 and 173 in the present study. These two detected mutations were at variance to the published results in other sheep [3], Yunling goat [9], Cattle [10] and human [11], where "T" and "C" were present at these positions (Fig 2). By these variations, amino acid Phenylalanine (TTT) has been exchanged by Cysteine (TGT) at position 57 whereas Glutamine (CAA) has been switched by Glutamic acid (GAA) at position 58 (Fig 3, Table 2).

Other nucleotide variations of significance in designated B allele were "G" at position 106 like the published sequence of another sheep, however it was "T" in published sequence of cattle, goat, buffalo and human. At position 136 of the portion, "T" was observed in sheep under study, in another sequence of sheep, Boer goat, Jining grey goat and Yunling goat whereas at this locus in case of cattle, buffalo and human, "C" was detected (Fig 2). The mutations spotted here in are silent and did not change Serine and Aspartic acid amino acids at positions 35 and 45 of the corresponding amino acid sequences (Fig 3). The identified allelic variations in sheep, confirmed by nucleotide sequencing, reveal unique mutations compared to other species, with some causing amino acid substitutions while others remain silent, highlighting species-specific genetic diversity.

Table 2 Nucleotide and amino acid sequence variations between designated alleles of exon-2 of *BMP15* gene in Corriedale and Kashmir valley sheep

Gene	Fragment size (bp)	Allele	Nucleotide positions	Amino acid positions
			173	58
BMP15 (Exon2)	222 bp	A	Guanine (G)	Glutamic acid (E)
		B	Cytosine (C)	Glutamine (Q)

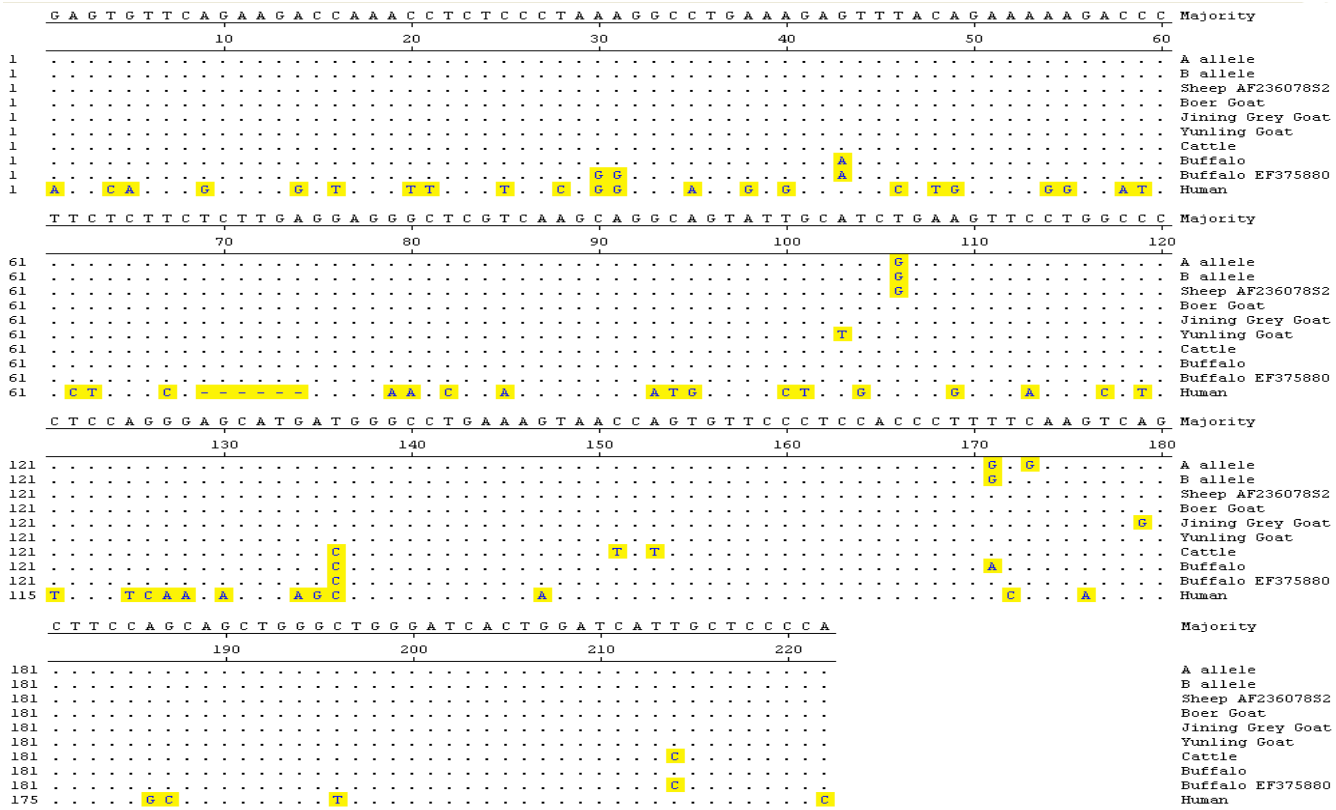

Fig 2 Multiple alignment of nucleotide sequences of fragment of exon-2 *BMP15* gene of sheep under study with that of other Sheep (Acc. No. AF236078S2), Boer Goat (Acc. No. EU847289), Jining Grey Goat (Acc. No. EU743938), Yunling Goat (Acc. No.EU847284), Cattle (Acc. No.AY304484), Buffalo (Acc.No.EF375880) and Human (Acc.No BC117264)

'.' indicates the nucleotides that match the consensus and yellow colour indicates the nucleotides that differ from the consensus

In designated B allele understudy, it showed "G" at position 171. This new mutation was in comparison to the sequence reported in other sheep [3], Boer goat [9], cattle [10] and human [11] (Fig 2). Cysteine (TGT) has replaced amino acid Phenylalanine (TTT) by this mutation (Fig 3). *FecXB* mutation by PCR-RFLP method was explored in Chios sheep. The investigated Chios breed was found monomorphic for *FecXB* mutation. All individuals were digested by DdeI restriction enzyme and exhibited wild-type genotype and did not carry *FecXB* mutation. In conclusion, it is supposed that the high-pitched prolificacy in Chios sheep may be based on additional region of BMP15 gene or different major gene [12]. Mutations in *FecB*, *FecX*^{G} and *FecX*^{I} genes were absent among the Egyptian prolific sheep breeds [13]. No mutation in *FecX* gene was detected in sheep homozygous with CC genotype regardless of their prolificacy [14-15]. Concurrent polymorphism at three important loci (*FecB, G1, and G4*) of two different fecundity genes (BMPR1B and GDF9) was found in Bonpala sheep [16].

Two genotypes (GG and AG) with a new point mutation at position 121bp of the studied fragment, deducing an amino acid exchange in the codified amino acid sequence were identified in the Mehraban and Lori sheep ewes [17]. Polymorphism was recognized in GDF9 gene by PCR-RFLP method at points G1 (G260A) and G4 (G721A) in Salskaya and Romanov sheep breeds. AG and GG genotypes were noticed at points G1 and AA and AG genotypes were detected at points G4. In Romanov breed AG and GG genotypes were spotted at points G1 and AA and AG genotypes were detected at points G4 [18]. PCR-RFLP was used to identify polymorphisms of BMP-15 gene in Awassi ewes. Gene which encloses the *Hinf1* endonuclease restriction enzyme illustrates two genotypes (AA and BB). The frequencies of the A and B alleles not differed significantly in the sample (0.48 and 0.52 respectively). The results showed the absence of heterozygous (AB). Litter size was affected significantly (P<0.01) by BMP-15 genotype, the highest litter size was in BB group (1.26) while the least litter size was in AA group (1.07) [19]. A *FecX* mutated allele called

FecX^{R} in the BMP15 gene has been described in Rasa aragonesa sheep, *FecX*^{R} responsible for increased prolificacy when heterozygous and sterility when homozygous in ewes [20]. Polymorphism in exon1 of BMP15 gene (*FecX* ^{Bar}) is associated with increased prolificacy and sterility, homozygous females carrying *FecX* ^{Bar} exhibited streaky ovaries with a blockade at the primary stage of folliculogenesis [21]. PCR (Polymerase chain reaction) products digested with restriction enzymes exhibited a substitution in the G7 locus of the GDF9 gene in the Ossimi and Rahmani sheep breeds, and a substitution in the FecGH locus of the GDF9 gene in all sheep breeds [22]. Sequencing result of *BMP15* gene of sheep breeds presented segregation of T755C mutation and ensuing non-conservative substitution of L252P in BMP15 pro-peptide. Association exploration revealed that this mutation was significantly linked to the ewe's litter size, ewes with CT genotype had 0.24 and 0.30 more lambs than those with CC and TT genotypes, respectively [23]. Point mutation (G → A) was found at position 57 of the amplified 312bp fragment of BMP15 gene exon 2. Frequencies of AG and GG genotypes of BMP 15 gene stood 69.4 and 30.6% in Mehraban and 44.7 and 55.3% in Lori ewes, respectively [24].

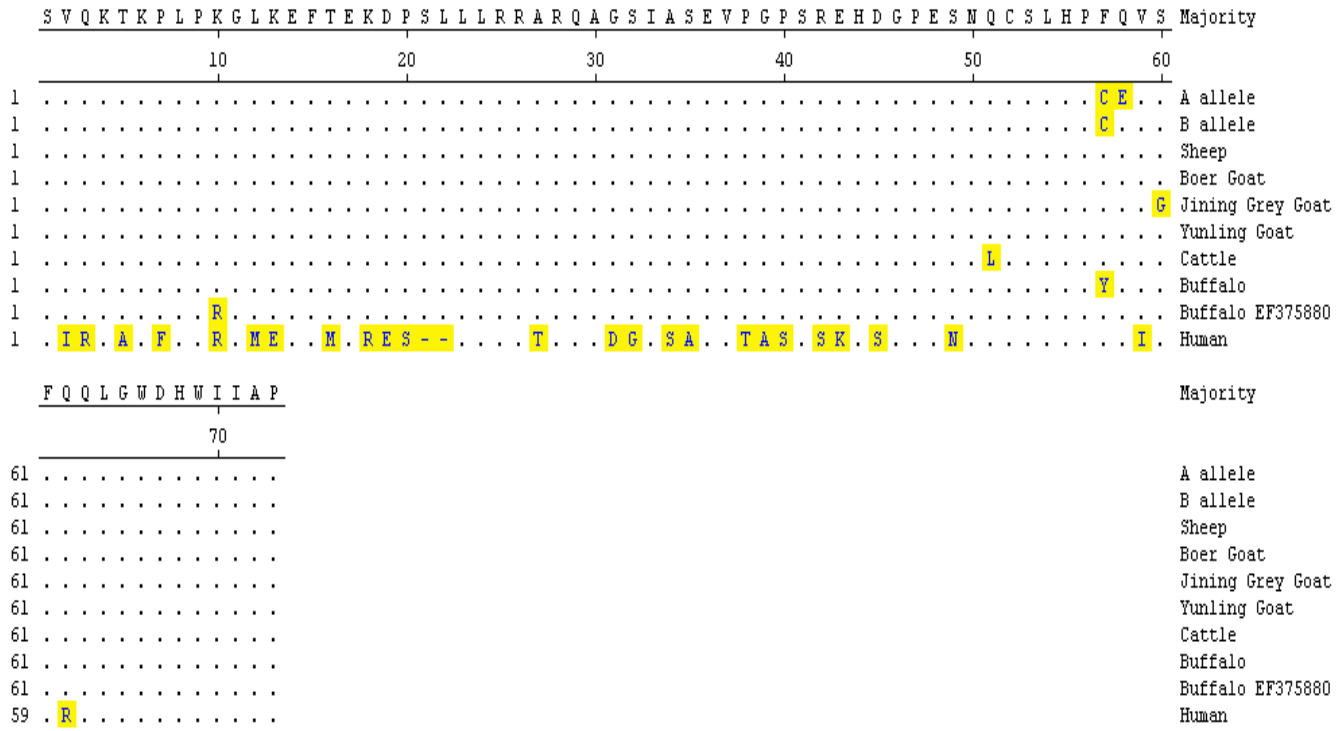

Fig 3 Multiple alignment of inferred amino acid sequences of fragment of exon-2 *BMP15* gene of sheep under study with that of other Sheep (Acc. No. AF236078S2), Boer Goat (Acc. No. EU847289), Jining Grey Goat (Acc. No. EU743938), Yunling Goat (Acc. No. EU847284), Cattle (Acc. No. AY304484), Buffalo (Acc. No. EF375880) and Human (Acc. No. BC1172)

'.' indicates the amino acid that match the consensus and yellow colour indicates the amino acids that differ from the consensus

The overall frequencies of AA and AB genotypes of sheep under study were 0.48 and 0.52, respectively and no animal was observed to have BB genotype whereas the frequencies of both the genotypes were 0.50 in Corriedale sheep. With respect to Kashmir valley sheep, the genotype frequencies of AA and AB genotypes were 0.47 and 0.53, respectively. The overall allelic frequencies of A and B alleles of exon-2 fragment of *BMP15* gene were 0.74 and 0.26 in the sheep under study. Breed wise allelic frequencies of A and B alleles in Corriedale ewes were 0.75 and 0.25. The allelic frequencies of A and B alleles of Kashmir valley sheep were 0.73 and 0.27, respectively (Table 3).

Table 3 Genotypic and allelic frequencies of *BMP15* gene of sheep under study

Overall				Corriedale				Kashmir Valley Sheep			
Genotypic		Allelic		Genotypic		Allelic		Genotypic		Allelic	
AA	AB	A	B	AA	AB	A	B	AA	AB	A	B
0.48	0.52	0.74	0.26	0.50	0.50	0.75	0.25	0.47	0.53	0.73	0.27

CONCLUSION

The previously reported mutations at nucleotide positions 152 and 177 conferring the fecundity to sheep were not observed in any of the twin producing sheep under study. Instead of that, two unreported mutations at positions 171 and 173 were detected. The consequences of such mutations may extend to the population at large influencing traits crucial for reproduction and fertility. This variability can also enhance the species resilience contributing to increased survival rates under selective pressures such as diseases, climate change and resource availability.

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