Polymorphism in BMP 15 Gene and its association with reproductive traits in sheep (*Ovis aries)*

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Abstract

The establishment of preferred prolific flocks through the selection of prolific ewes carrying fecundity genes associated with increased reproductive performance has indeed proven to be an effective strategy for enhancing prolificacy in sheep populations. Notably the *Fec B,* GDF 9 and BMP 15 genes are considered crucial in the regulation of fecundity. BMP 15 gene is an important gene in the regulation of fertility. By focusing on this gene, sheep breeders can significantly improve lambing rates which can lead to increased productivity and profitability in sheep farming. The aim of the current study aimed at screening for point mutations and detecting additional mutations of this specific gene in Corriedale and Local Kashmir Valley sheep. The methodology employed included PCR followed by SSCP analysis utilizing a 12% PAGE for effective separation of the SSCP genotypes. The results revealed two distinct SSCP genotypes: AA and AB, corresponding to the two labelled alleles, A and B. The phylogenetic analysis conducted on the designated alleles revealed significant insights into the genetic diversity of the investigated gene. The analysis indicated that the gene exhibits polymorphism within the studied populations with point mutations identified that result in alterations to the amino acid composition of the protein product. The overall frequencies of AA and AB genotypes were 0.48 and 0.52 and allelic frequencies of A and B alleles were 0.74 and 0.26, respectively. The genotypes AA showed highest average Age at First Lambing (AFL) (738.87±0.04 days) and AB showed lowest AFL (730.48±0.04 days). The genotype AA had average litter size of 1.37±0.07 and genotype AB had 1.34±0.07. Despite the observed polymorphism, the statistical evaluation of the influence of the BMP 15 gene alleles on important reproductive traits such as age at first lambing and litter size did not yield statistically significant results. This suggests that although variations in the BMP15 gene may exist, they do not appear to have a meaningful impact on these particular reproductive traits in the sheep populations studied.

Keywords: Prolificacy; BMP 15; SSCP; Phylogenetic Analysis; Genotypes; Litter Size; Age at First Lambing

Introduction

Sheep farming is source of livelihood for the marginal people in global south but the lack of scientific knowledge and input for breeding is hampering the profitability (Bhateshwar *et al.,* 2022; Djimon *et al.,* 2024). Prolific flocks of ewes with specific genes are a promising approach to improve the reproductive performance in sheep. This strategy can lead to higher lambing rates which is vital for enhancing productivity in sheep farming. By focusing on genetic traits that influence fertility and the number of lambs born, breeders can create flocks that consistently produce more offspring. This not only boosts the economic viability of sheep production but can also improve overall herd health and sustainability. These key genes commonly referred to as *Fec* genes have been identified in sheep, as reported by Davis (2005), have a major role in determining the reproductive outcomes in sheep. These include BMPR1B, GDF9, and BMP15 genes. All three of these fecundity genes are part of the transforming growth factor-beta (TGF-β) superfamily, as noted by Fabre *et al.* (2006). The intricate relationships between these genes and reproductive traits underscore their potential as targets for selection in breeding programs aimed at enhancing prolificacy. Bone morphogenetic proteins (BMPs) are a group of multifunctional proteins that play crucial roles in various biological processes including development and differentiation across multiple cell types. In mammals, BMPs are particularly significant in regulating fertility as they influence essential growth factors that govern the development of ovarian follicles and the ovulation process.

The BMP15 gene is especially important in ovarian function, actively contributing to the development of primary follicles. Research by Han *et al*. (2015) emphasizes the critical role of BMP15 in this context highlighting its influence on folliculogenesis and overall reproductive health. Several mutations within the BMP15 gene have been associated with variations in prolificacy among sheep. Specifically, five distinct mutations have been identified that impact reproductive outcomes. Notably heterozygous individuals carrying these mutations often exhibit significantly increased ovulation rates enhancing their reproductive performance. Conversely, homozygous individuals for certain mutations may experience severe reproductive challenges leading to primary ovarian breakdown and ultimately complete sterility. Understanding the genetic basis of these traits allows for more informed breeding strategies that can lead to improved fertility and productivity in sheep populations. By focusing on these genetic factors, sheep breeders can effectively improve lambing rates, contributing to the overall productivity and profitability of sheep farming operations.

Through molecular techniques like Polymerase Chain Reaction (PCR), Single Strand Conformational Polymorphism (SSCP) and sequencing analysis, researchers can identify the specific single nucleotide polymorphisms (SNPs) and other potential mutations within the BMP15 gene. These findings could then be used for genetic selection strategies that focus on improving reproductive efficiency in these ovine breeds contributing to the development of more prolific and productive flocks.

Materials And Methods

Ewe Selection

The inclusion of 85 ewes with varying reproductive histories (twinning/triplets and single births) were selected for this study to provide valuable insights into the genetic factors influencing fecundity and reproductive success in sheep. This approach allows to investigate both the genetic predisposition to higher-order multiple births and the underlying factors contributing to single births. Incorporating Corriedale ewes from Mountain Research Centre for Sheep & Goat (MRCSG) of University of SKUAST Kashmir situated at Shuhama Campus further enhances the study's generalizability and applicability. The MRCSG is situated at 33°53′ N latitude and 74°37′ E longitude in district Srinagar at an altitude of 5300 feet above mean sea level. This breed serves as a widely used and standardized reference population enabling the identification of breed specific differences and similarities in reproductive traits. By comparing the genetic profiles of diverse populations, researchers can better understand the genetic architecture of fecundity and develop targeted breeding strategies.

Blood Collection and Isolation of Genomic DNA

Blood was drawn from the jugular vein of each ewe. Blood samples were stored at minus 20°C to prevent degradation until genomic DNA isolation was performed. The process described for isolating genomic DNA from the frozen blood samples using the phenol chloroform extraction method as outlined in the standard protocol by Sambrook and Russell (2001). The quality and concentration of the extracted genomic DNA was assessed using spectrophotometry (measuring absorbance at 260/280 nm) and gel electrophoresis. This well-established protocol provides high-quality genomic DNA suitable for downstream applications such as PCR, SSCP, sequencing and genotyping facilitating the genetic analysis of fecundity in the studied ewe populations.

Primer Design and PCR-SSCP analysis

In this study, Primer design for PCR amplification of *BMP15* gene of sheep was conducted using Fast PCR software. The target fragment encompassed the exonic region. The details of the primer sequence (5′-3′) are Forward GAGTGTTCAGAAGACCAAACCTC and Reverse TGGGGAGCAATGATCCAGTGATCC. A standardized PCR protocol to amplify specific DNA region of interest in local ewes and Corriedale ewes was

taken into consideration. For each 25 µl PCR reaction, a carefully optimized mix of reaction components was essential to achieve efficient amplification of the target amplicons.

After amplification, samples were prepared for SSCP analysis. The procedure involved taking 5 µl of the PCR product and mixing it with 15 µl of a denaturing formamide dye. This amplicon-dye mixture was then subjected to denaturation at 98°C for 5 minutes which is crucial for effectively separating the double stranded DNA into single strands. Following this step, the samples were snap cooled on ice for 15-20 minutes which helps to stabilize the single stranded conformations prior to electrophoresis. Gel electrophoresis process was optimized by running the polyacrylamide gels at a low temperature of 4°C to maintain the integrity and resolve the conformational variants. After the run, the SSCP gels were silver stained according to the protocol established by Bassam *et al.* (1991), which enhances the visibility of the DNA bands. The analysis revealed distinct banding patterns of the single-stranded amplicons, indicating the presence of polymorphisms within the studied genes. These variations in banding patterns are a hallmark of genetic diversity and can be attributed to differences in the nucleotide sequences that influence the secondary structures of the DNA. Thus, the SSCP analysis provided valuable insights into the genetic polymorphisms related to fecundity, which can be further explored for their implications in breeding programs aimed at improving reproductive traits in ewes.

Phylogenetic Tree Analysis

In the study, amplified samples of the BMP15 gene exhibiting distinct banding patterns in Polyacrylamide Gel Electrophoresis (PAGE) were subjected to further analysis through automated sequencing using the ABI Genetic Analyzer. This method allowed for the precise determination of nucleotide sequences which is crucial for understanding genetic variation within and between populations of ewes. The subsequent phylogenetic analysis aimed to assess the evolutionary relationships among the obtained BMP15 sequences and those from other related species. By comparing these sequences, phylogenetic tree was created that illustrates the genetic relationships, revealing how closely related the local Kashmir Valley ewes and Corriedale ewes are to each other and to other sheep and other breeds.

In addition to constructing the phylogenetic tree, the study investigated homology which involves comparing the nucleotide sequences to identify regions of similarity and divergence. This comparison can provide insights into the functional importance of specific genetic variations related to fecundity. Relative distance calculations between the nucleotide sequences help to quantify genetic differences. Smaller distances indicate closer genetic relationships while larger distances suggest greater divergence potentially associated with differences in reproductive traits.

Association Study

In conducting an association study to analyse the relationship between age at first lambing and litter size in ewes, the least squares maximum likelihood method as described in Harvey (1990) was employed to evaluate the data. This analytical approach allows for the examination of the effects of various factors on the traits of interest while controlling for potential confounding variables.

Statistical Analysis

Different patterns of the fragment were designated as different and genotypes were counted differently. The frequency of different genotypes and alleles were calculated using the standard procedure given by Falconer and Makay (1996).

Results and Discussion

Genomic DNA was isolated following the standard protocol of Sambrook and Russell (2001) with some modifications. Notably, during the dissolution of DNA pellets, autoclaved triple-distilled water (TDW) was used instead of TE buffer (pH 8.0) to avoid the potential chelation of Mg^{2+} ions by EDTA present in the buffer. The chelation of Mg^{2+} can hinder DNA amplification by interfering with the activity of DNA polymerase. To ensure complete dissolution of the DNA pellet in the autoclaved TDW, an incubation step at 60°C for 2 hours was implemented. This temperature not only aids in dissolving the DNA but also serves to inactivate any DNase that may be present in the solution.

PCR-SSCP Analysis

The aim of this study was to analyse polymorphisms within the BMP15 gene in sheep by focusing on 222 bp fragment encompassing exon 2. The findings from the single-strand conformational polymorphism (SSCP) analysis indicate a genetic diversity within the targeted gene in the assessed sheep population. The observation of two distinct banding patterns, labelled as AA and AB, suggests the presence of at least two genotypes corresponding to variations in the amplicons derived from the gene of interest. The identification of different banding patterns signifies genetic polymorphisms within the sheep population. This kind of variation can be crucial for breeding programmes, disease resistance studies, and population genetics analyses. By designating the two patterns as AA and AB, it effectively categorizes the sheep into at least two distinct genetic types based on their SSCP profiles (Fig 1). The observed polymorphism indicates that there is variation in the genetic material which could be linked to heterozygosity or specific traits within the sheep. Understanding this polymorphism can help in selecting animals for breeding with desirable traits. This study highlights the importance of using molecular

techniques like SSCP to unveil genetic diversity, which is valuable for both research and applied breeding strategies in livestock management.

Phylogenetic Tree Analysis

The insights gained from the sequencing and phylogenetic analysis of the BMP 15 gene contribute significantly to our understanding of the genetic basis of fecundity in sheep. This knowledge not only aids in making informed breeding decisions but also enhances the genetic management of sheep populations, promoting the sustainability of livestock farming in the region. The phylogenetic evaluation based on nucleotide sequences of exon-2 revealed that both designated A and B alleles are closely related from the evolutionary opinion of interpretation. Buffalo and cattle were diverged before formation of sheep and goat sequences. Human showed a discrete separation from the point of origin compared to animals (Fig 2). Based on derived amino acid sequences, designated A and B alleles form a common cluster from the evolutionary point of view as expected. Jining grey goat and Bhadawari buffalo were close to these two designated alleles whereas human cluster in another group located away from the animals (Fig 3). Polymorphism of bone morphogenetic protein 15 gene shared a likeness in sequence matched to 9 accession numbers of Ovis aries found in GenBank (Fiky *et al.,*2017). The results specify that 5 accession numbers of Ovis aries are closely related with Ossimi and Saidi female that yield single or twins lamb in UPGMA investigation. In supplement, PCR-RFLP method using Pst1 and Msp1 restriction enzymes was used to mask polymorphisms of partial exon-2 0f BMP 15 gene (Fiky *et al.,*2017). Increased prolificacy of 0.52 ± 0.05, 0.42 ± 0.05 and 0.32 ± 0.01 were found when comparing $FecX^{GR}$, $FecX^{RA}$ and $FecX^{R}$ heterozygous ewes to wild type homozygous ones. These effects are of the same order of magnitude as the effect of most of other known major genes for prolificacy (Calvo *et al.,*2020).

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.

Association Study

The influence of none of the alleles of *BMP15* gene of sheep on age at first lambing (AFL) was found to be statistically significant. The genotype AA showed the highest average of AFL 738.87±0.04 days and genotype AB showed the lowest AFL 730.48±0.04 days (Table 1). The genotype AA of exon-2 of *BMP15* gene of sheep under study had average litter size of 1.37±0.07 and genotype AB had average litter size of 1.34±0.07 (Table 2).

The effect of polymorphism of the fragment of *BMP15* gene of sheep under study on litter size was found to be statistically non-significant ($p \geq 0.05$). BMP15 c.31_33CTTdel mutation and the *GDF 9* mutations (G2, G3, G4) were associated with an increased tendency in litter size. However, no significant difference was observed (Zhang *et al*., 2025). Litter size did not differ significantly between sheep breeds regardless of the presence/absence of c.31_33del. Results suggested that c.31_33del might be a genetic marker for improving fecundity in some New Zealand sheep (Najafabadi *et al.,* 2021). The relevant alleles and environmental variables did not have a statistically significant effect on the twinning phenotype and the genes associated with multiple births in Kangal Akkaraman breed may have different variants specific to the breed (Aksel *et al*., 2024). SNP loci in *GDF 9* gene were low polymorphic, while loci in *BMP 15 gene* were moderately polymorphic. The genotypes at the G1330T locus had a significant difference in litter size in Jamunapari goats but no significant difference was observed for all genotypes at other loci (Shaha *et al*., 2022). *BMPR1B*, *GDF9* and *BMP15* are the major genes that influence prolificacy in Beetal and Teddy goats and polymorphism in such genes can be used as molecular markers to select the prolific animals (Islam *et al*., 2019). *BMP15* gene is an essential gene affecting the reproductively in animals. Reduction in *BMP* system activity in the ovaries of sheep has been implicated with higher ovulation rate (Montgomery *et al*., 1992; Montgomery *et al*., 2001). To explore the effects of various identified mutations in the present study, an association of genotypes and age at first lambing was studied. None of the effect of labelled alleles of the fragments of *BMP15* gene on age at first lambing (AFL) was significant ($P \leq 0.05$). The overall least squares mean of AFL was 734.68±0.03 days. The genotype AA showed 738.87±0.04 days and genotype AB showed 730.48±0.04 days average of AFL. This study specifies that the spotted mutations are not affecting the age at first lambing in sheep. As clarified above those mutations in *BMP15* gene influence the prolificacy and consequently litter size is increased in sheep. No significant difference was observed in expression characteristics of the GDF9 gene in ovarian tissue from the multiparous and uniparous goat breeds (Pan *et al*., 2015). To explore the impact of mutations observed in the present study on litter size, the presented data was analysed and subsequent inferences were made. The overall least squares mean of litter size was 1.36±0.06. The genotype AA of *BMP15* gene of sheep under study had average litter size of 1.37±0.07 and genotype AB had average of 1.34±0.07. G40G was found in two local Chinese goat breeds (Lezhi black goats and Tibetan goats) with opposite fecundity (Yang *et al*., 2012). The association analyses showed that the genotypic structure did not significantly affect litter size in the Akkaraman ewes. However, ewes with one copy of the G1 mutation produced 0.13 more lambs than those

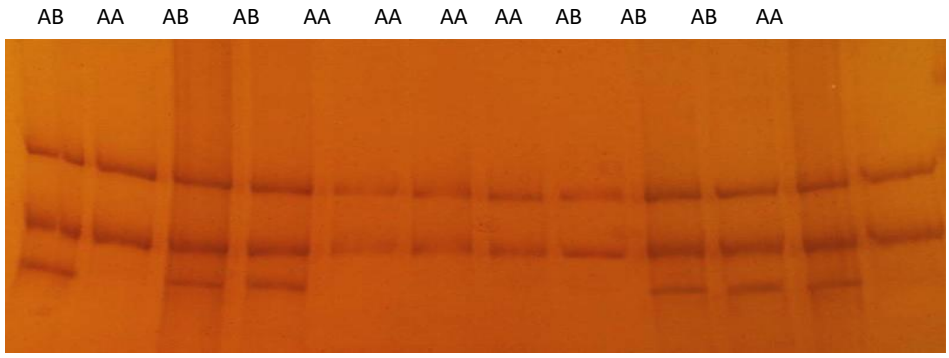

Fig.1 SSCP genotypes of 222 bp fragment of exon-2 *BMP15* gene of Corriedale and Kashmir valley sheep

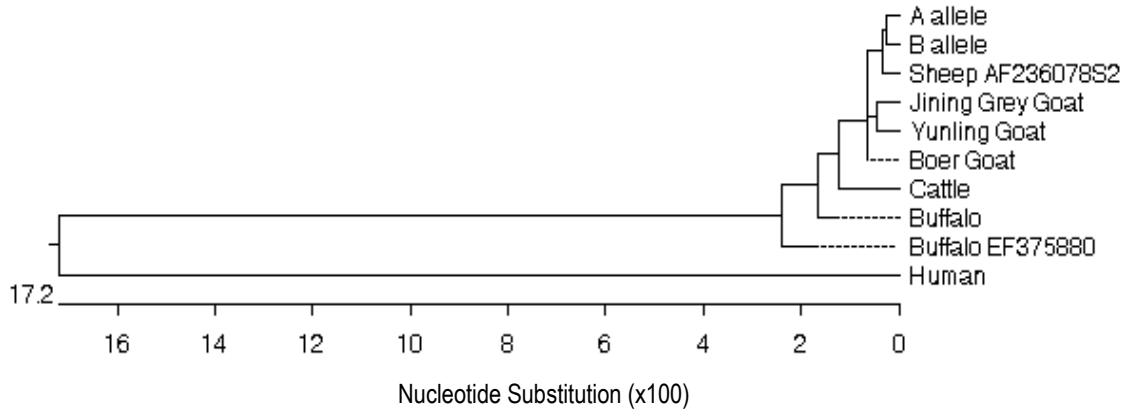

Fig. 2 Diagram showing the phylogenetic analysis on the basis of nucleotide sequences of exon-2 *BMP15* gene of sheep under study with that of other Sheep (Acc. No. AF236078S2), Jining Grey Goat (Acc. No. EU743938), Yunling Goat (Acc. No. EU847284), Boer Goat (Acc. No. EU847289), Cattle (Acc. No. AY304484), Buffalo (Acc. No. EF375880) and Human (Acc. No. BC117264)

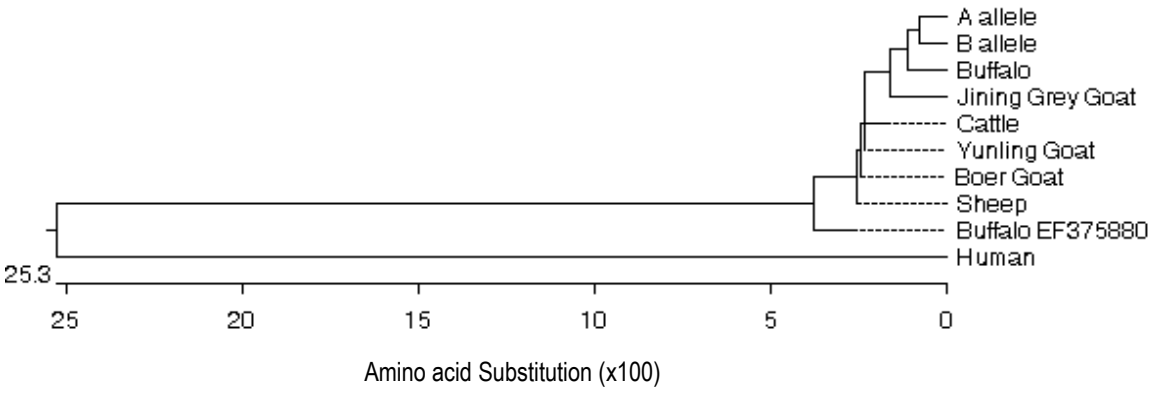

Fig. 3 Diagram showing the phylogenetic analysis on the basis of amino acid sequences of exon-2 *BMP15* gene of sheep under study with that of other Sheep (Acc. No. AF236078S2), Jining Grey Goat (Acc. No. EU743938), Cattle (Acc. No. AY304484), Yunling Goat (Acc. No. EU847284), Boer Goat (Acc. No. EU847289), other sheep (Acc. No. AF236078S2), Buffalo (Acc. No. EF375880) and Human (Acc. No. BC117264)

without the mutation, suggesting that heterozygous ewes have an inclination to increase litter size (Kirikci K. 2023).

Table 1 Genotype wise least squares mean with standard error for age at first lambing (days)

Fragment	Genotype	Age at First Lambing
BMP-15 Exon-2	AA	738.87±0.04 (39)
	AB	730.48±0.04 (46)

Figures in parentheses indicate number of observations

Table 2 Genotype wise least squares mean with standard error for litter size

Fragment	Genotype	Litter Size
BMP-15 Exon-2	AA	1.37±0.07 (41)
	AB	1.34±0.07 (44)

Figures in parentheses indicate number of observations

Point mutation (G→A) was found at position 57 of the amplified fragment of BMP15 gene exon 2. Average litter size in the AG genotype (1.56) was significantly (P<0.01) higher than the GG ewes (1.08). The results of the present study indicated the potential of the observed SNP in exon 2 of BMP15 for further exploitation to improve reproduction efficiency of Mehraban and Lori sheep (Zamani *et. al*., 2015). Mutations in GDF9 exon I and in BMP15 exon II significantly increased litter size in heterozygote form for BMP15 and homozygote form for GDF9 in Markhoz goat breed. Homozygote females for the BMP15 mutation were not identified which is like the situation found in Belclare sheep, small-tailed Han sheep, and Jining Grey goats (Ghoreishi *et al*., 2019).

Conclusion

The finding here concludes that the influence of alleles of the BMP 15 gene in Corriedale and local Kashmir valley ewes on age at first lambing and litter size were found to be statistically non-significant indicates that variations in this gene do not play a substantial role in influencing these reproductive traits in the sheep population studied. Further research in this domain may yield significant insights into the genetic mechanisms underlying fecundity and reproductive success in sheep.

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Conflict of interest

The authors declare that they have no conflict of interest.

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