Original Research

Molecular Variants of *FecB and BMP15* Fecundity Genes in Sheep (*Ovis aries*)

A. D. Asharani, M. M. Appannavar, H. M. Yathish*, M. S. Hussain, Vivek R. Kasaralikar[1] and M. D. Suranagi

Department of Animal Genetics and Breeding, Veterinary College, KVAFSU, Bidar – 585401, Karnataka, INDIA

[1]Department of Veterinary Medicine

***Corresponding author:** yathish.vety@gmail.com

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Abstract

The study was undertaken with an objective of detecting genetic variants of FecB and BMP15 genes using PCR-RFLP analysis. DNA of acceptable quality was isolated from blood samples of 30 each randomly chosen Kenguri and Kenguri x NARI Suwarna strain of sheep. Using gene specific primers, 190 nucleotides of FecB and 434 nucleotides of BMP15 gene was amplified. FecB/AvaII analysis in Kenguri x NARI Suwarna strain of sheep has revealed B+ and ++ genotypes with a respective frequency of 0.766 and 0.233. Similarly, BMP15/StuI analysis identified AB and AA genotypes with a frequency of 0.766 and 0.233, respectively. In Kenguri sheep, FecB/AvaII and BMP15/StuI polymorphism study revealed only ++ and AB genotypes, respectively. Analysis for population parameters revealed moderate PIC (0.3746 to 0.3576), 0.7407 to 1.000 heterozygosity, 0.4664 to 0.500 allelic diversity. The study revealed the introgression and presence of molecular variants of FecB and BMP15 genes in sheep.

Key words: Kenguri, NARI Suwarna, Nucleotide Variablities, Reproduction, Twinning

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Introduction

Sheep genetic resources stand out unique in diverse livestock species in India with shorter lambing interval, fast growth rate and no social taboos attached. Sheep contribute about 238.8 million kg meat and 40 million kg wool annually; and meat is the major produce. This is not sufficient to meet the demand of ever increasing human population for quality protein. To meet this demand from our sheep breeds seems unachievable due to their low productive and reproductive efficiency. Therefore, alternate strategy is to uplift the genetic potential of sheep breeds using appropriate selection methods for higher litter size or twinning.

The ovulation rate and litter size can be genetically regulated by a set of different genes. These multiple genes influencing fecundity/ ovulation rate in animals are collectively named as fecundity (Fec) genes (Davis *et al*., 1982) which can be targeted to identify DNA markers significantly influencing litter size in sheep. Some of the fecundity genes identified are *BMPR1B* (*FecB*), *BMP15* and *GDF9* genes. In *FecB*, one mutation and in *BMP15*, eight different mutations have been discovered with each producing the same phenotype. Therefore, these mutations in fecundity genes offers tool for selection of highly prolific animals. Kenguri is a medium sized sheep breed of Karnataka known to thrive well under scarcity condition and sparse vegetation and has higher meat production potential (Jain *et al*., 2004; Appannavar *et al*., 2010). However, the reproductive potential of this breed is poor. Therefore, to improve litter size/ fecundity in Kenguri sheep and evolve new strain of Kenguri sheep, introgressing *FecB* gene is substitute strategy. For this, NARI Suwarna strain developed at Nimbkar Agricultural research Institute (NARI), Phaltan, Pune, Maharashtra was considered as the choice of sheep suitable for this agro-climatic condition and has shown to yield 33% increase in productivity and income for shepherds. Keeping the potential of Kenguri sheep for mutton production and success of NARI in introducing *FecB* gene, the present investigation was designed to introgress *FecB* gene from NARI Suwarna into Kenguri without compromising on its physical attributes. In addition to *FecB* gene, Bone morphogenetic protein 15 (*BMP15*) was also investigated in Kenguri and Kenguri x NARI Suwarna strain of sheep. *BMP15* gene is also known as *FecX* (Galloway *et al*., 2000) and is an X linked gene (*FecX* locus) of sheep belonging to TGF-β family. The *BMP15* protein is a paracrine factor that stimulates follicle growth, granulosa cell proliferation and cell-survival signaling (Demars *et al*., 2013). *BMP15* is an oocyte-derived growth factor that is essential for follicular development in sheep (Hanrahan *et al*., 2004). The protein is expressed in primary stage of follicular development onwards and acts within the ovaries (Nicol *et al*., 2009). Till date, 8 mutations related to prolificacy have been identified in the sheep *BMP15* gene. Out of 8, six mutations ($FecX^{I}$, $FecX^{H}$, $FecX^{B}$, $FecX^{G}$, $FecX^{L}$, $FecX^{R}$) produce the same phenotype i.e. increased ovulation rate or litter size in heterozygous carriers and sterility in homozygous carriers. However, for $FecX^{Gr}$ and $FecX^{O}$ mutations, homozygous ewes are highly prolific. *BMP15* was found to be associated with prolificacy in Inverdale, Lacaune, Belclare and Small Tailed Han sheep. Therefore, *BMP15* gene was also targeted to identify DNA markers of higher prolificacy/ fecundity.

Materials and Methods

Approximately 10ml of blood samples were collected aseptically from 30 each of Kenguri and Kenguri x NARI Suwarna strain of sheep maintained at the Instructional Livestock Farm Complex, Veterinary College, Bidar. Blood samples were collected, transported and stored according to standard procedure. These blood samples were used for genomic DNA (gDNA) extraction according to Phenol Chloroform method (Sambrook and Russel, 2001) with minor modifications. Isolated gDNA was subjected for analysis

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of purity and concentration by spectrophotometer reading and for quality assessment using 0.8% agarose gel electrophoresis. Working DNA was prepared from high purity stock DNA to contain 50ng of gDNA/µl.

Amplification of FecB and BMP15 Genes in *Ovis aries*

Using gene specific primers, 190bp region in exon2 of *FecB* and 434bp sequences in exon2 of *BMP15* genes were amplified in the gDNA samples of Kenguri and Kenguri x NARI Suwarna strain of sheep. For amplification of *FecB* gene primers (Forward- CCAGAGGACAATAGCAAAGCAAA and Reverse- CAAGATGTTTTCATGCCTCATCAACACGGTC) reported by Davis *et al.* (2002) were utilized. New primers were designed to amplify partial exon 2 region of *BMP15* gene (forward- AGAGCCACTGTGGTTTACCG and reverse- GATGCAATACTGCCTGCTTG). Primers were designed by referring the *BMP15* gene sequences of *Ovies aries* (NC–019484.2) available publicly at NCBI database with the help of primer 3 online softwares (Koressaar and Remm, 2007; Untergrasser *et al*., 2012). These primers were inspected for their properties using oligoanalyzer software (http://eu.idtdna.com/calc/analyzer) and for their specificities using primer BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/).

To ascertain ideal level of each components of PCR and thermal profile, different combinations of reaction mixture and thermal profiles were used for amplification and the acceptable concentration of reaction components and temperature profiles were used further for amplification of *Fec-B* and *BMP15* genes in gDNA samples (Table 1 and Table 2).

Table 1: The reaction composition for amplification of *FecB* and *BMP15* genes in Sheep

S. No.	Reaction Components	Stock Concentration	Working Concentration	Volume (µl) of each component to make 25 µl reaction mixture	
				***Fec-B* gene**	***BMP15* gene**
1.	*Taq* buffer	10x	1x	2.5	2.5
2.	dNTPs Mix	2.5mM	200µM	2.5	2.5
3.	$MgCl_2$	50mM	1.5/3mM	1.25	1.5
4.	Primer-F	100µM	10µM	1	1
5.	Primer-R	100µM	10µM	1	1
6.	*Taq* polymerase	1U/µl	1U	1	1
7.	NFW	-	-	13.75	13.5
8.	Genomic DNA	-	50ng	2	2

The reaction mixture was prepared according to standard procedure and amplification in final volume of 25µl was carried out in a thermocycler (Biorad iCycler, USA). Specific amplification of *Fec-B* and *BMP15* genes was confirmed by resolving the amplicons on 1.5% (w/v) agarose gel in a horizontal submarine agarose gel electrophoresis unit and checking under the UV Gel documentation system (Biorad, USA).

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Table 2: Thermal profile for amplification of *FecB* and *BMP15* genes in Sheep

S. No.	Steps	*Fec-B*		*BMP15*	
1.	Initial denaturation	95°C for 5 min		95°C for 5 min	
2.	Cyclic denaturation	95°C for 60 Sec	35 cycles	95°C for 30 Sec	35 cycles
	Cyclic annealing	67°C for 60 Sec		61°C for 30 Sec	
	Cyclic extension	72°C for 30 Sec		71°C for 45 Sec	
3.	Final extension	72°C for 10 min		72°C for 10 min	
4.	Storage	4°C forever		4°C forever	

Restriction Fragment Length Polymorphism (RFLP) Analysis of Fec-B and BMP15 Genes

The specific 190bp and 434bp sized amplicons of *Fec-B* and *BMP15* genes were subjected for RFLP analysis using *Ava*II and *Stu*I restriction enzymes, respectively. The restriction site of *Stu*I within the 434bp amplicon of *BMP15* gene was identified using NEB-cutter V2.0 online tool (http://tools.neb.com/NEBcutter2/). This site had a reported SNP, which was identified using ensemble database (http://www.ensembl.org/index.html).

*FecB/Ava*II analysis in a total reaction mixture of 25µl containing 5µl of 10X assay buffer of RE, 10U of restriction enzyme and 19µl of amplicons. Similarly, *BMP15/Stu*I analysis was carried out in a total reaction mixture of 16µl containing 5µl of 10X assay buffer of RE, 10U restriction enzyme and 10µl of amplicons. These reaction mixtures were then incubated at 37°C for 15min and the enzymes were inactivated by heating at 80°C for 20 min. The digested products were resolved along with 100bp DNA ladder on 2.5 % agarose gel and the resultant fragments were analysed under Gel documentation system (Biorad, USA).

Statistical Analysis

The statistical analysis was done using SAS 9.3 package. Allelic and genotype frequencies were calculated using PROC ALLELE procedure. Also, the polymorphic parameters such as Polymorphic information content (PIC), heterozygosity, allelic diversity, and HWE were determined. The presence of variant/genotype in HWE was evaluated using χ^2 test.

Results and Discussion

Amplification of *FecB* and *BMP15* Genes in Sheep

Uniform and specific amplification of 190bp region of *Fec-B* gene was obtained in all the gDNA samples of Kenguri and Kenguri x NARI Suwarna strain of sheep. For specific amplification of *BMP15* gene, PCR reaction was optimised for annealing temperatures from 56-66˚C and for $MgCl_2$ concentration from 2.5mM to 3mM in reaction mixture. Though the 434bp region of *BMP15* gene was amplified between 59.0-62.5˚C but at 61˚C and at 3mM $MgCl_2$ specific and bright amplification was observed.

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RFLP Analysis of *Fec-B* and *BMP15* Genes in Kenguri Sheep

In Kenguri sheep, *Fec-B*/*Ava*II analysis revealed a single band of 190bp representing only ++ genotype (Fig. 1) and genotypes BB and B+ were not observed.

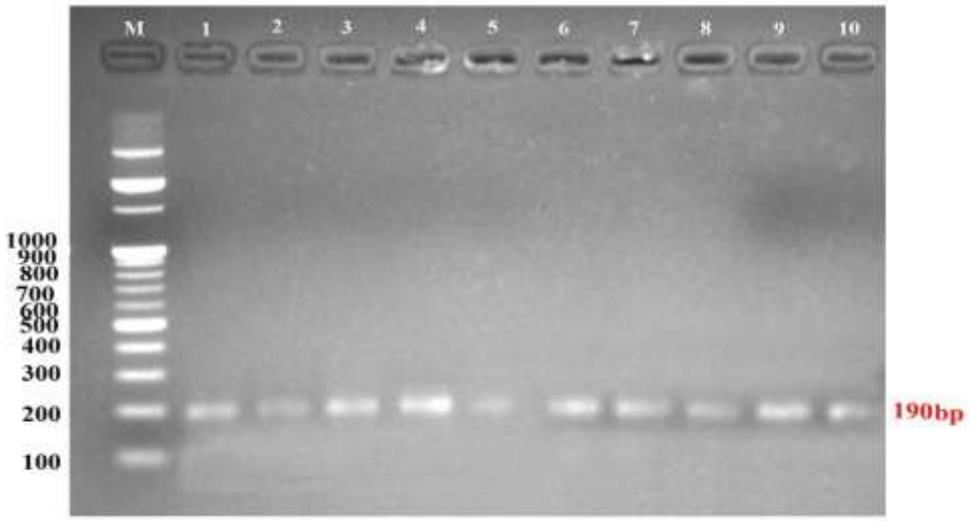

Fig. 1: PCR-RFLP band patterns of ovine *Fec-B* gene revealed by *Ava*II restriction enzyme in Kenguri sheep. Lane M: 100bp molecular marker; Lane 1-10: Restriction enzyme digested amplicons (190bp) of *FecB* gene in Kenguri sheep.

The frequency of ++ genotype was 1.00 and the frequency of '+' allele was 1.00. Similarly, *BMP15*/*Stu*II analysis has revealed single pattern consisting of 434bp, 362bp and 72bp bands representing AB genotype (Fig. 2) and AA and BB genotype were not observed. The frequency of AB genotype was 1 and the frequency of each A and B alleles was 0.5. The polymorphic parameter such as polymorphic information content (PIC), heterozygosity, allelic diversity and test for analysis of Hardy-Weinberg equilibrium (HWE) for *Fec-B* and *BMP15* genes in Kenguri sheep is given in Table 3. The analysis revealed moderate PIC values and departure of Kenguri population from HWE.

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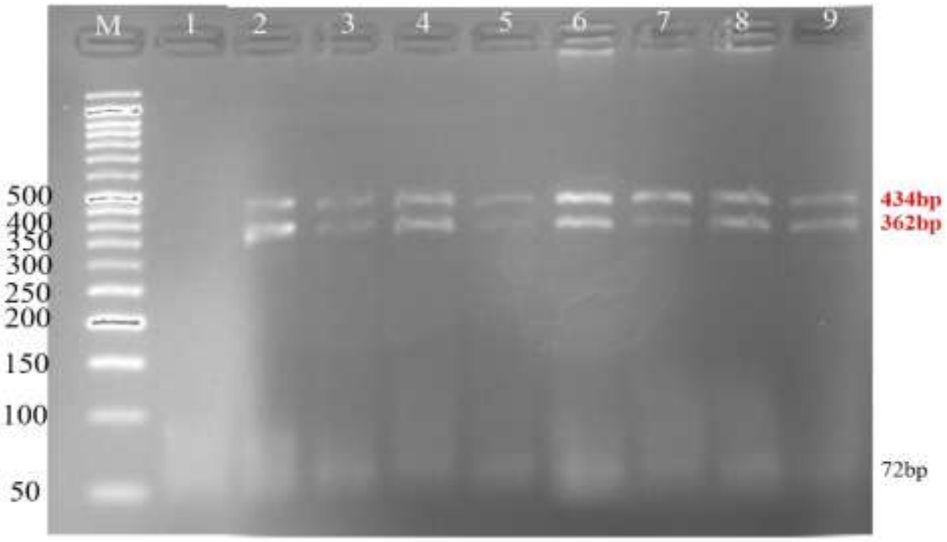

Fig. 2: PCR-RFLP band patterns of ovine *BMP15* gene revealed by *Stu*I restriction enzyme in Kenguri sheep. Lane M: 50bp molecular marker; Lane 2-9: Restriction enzyme digested amplicons (434bp, 362bp and 72bp) of *BMP15* gene in Kenguri sheep.

Table 3: PIC, Heterozygosity, Allelic diversity and test for HWE in sheep

Breed/ Strain of sheep	Gene	No. of Individuals	PIC	Heterozygosity	Allelic Diversity	Test for HWE (Pr>Chi)
Kenguri	*FecB*	31	0	0	0	0
	BMP15	33	0.375	1	0.5	<0.0001
Kenguri x NARI Suwarna	*FecB*	27	0.3576	0.7407	0.4664	0.0002
	BMP15	24	0.3746	0.9583	0.4991	<0.0001

RFLP Analysis of *Fec-B* and *BMP15* Genes in Kenguri x NARI Suwarna Strain of Sheep

In Kenguri × NARI Suwarna strain of sheep, *Fec-B*/*Ava*II analysis identified two band patterns- one pattern with 190bp and 160bp representing B+ genotype and another band pattern with single band of 190bp representing ++ genotype (Fig. 3). 30bp band in B+ genotype was not visible on gels due to their fast resolution during electrophoresis. The genotype BB was not observed in the investigated population. The frequency of genotype B+ and ++ was 0.766 and 0.233, respectively and the respective frequency of 'B' and '+' allele was 0.383 and 0.616. Similarly, *BMP15*/*Stu*I analysis has produced two band patterns; one pattern with 362bp and 72bp bands representing AA genotype and another pattern with 434bp, 362bp and 72bp bands representing AB genotype (Fig. 4). The frequency of AA and AB genotypes was 0.033 and 0.967, respectively. The respective frequency of A and B allele was 0.516 and 0.484.

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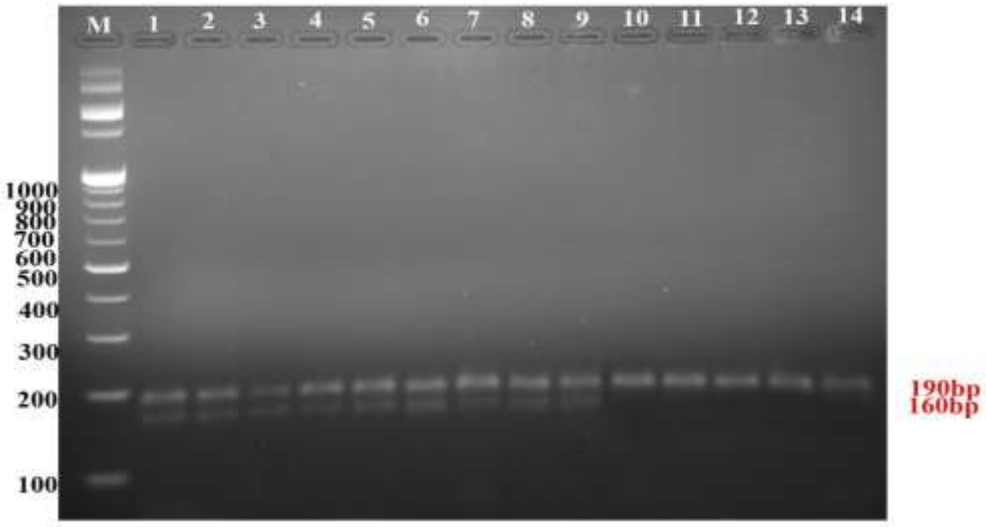

Fig. 3: PCR-RFLP band patterns of ovine *FecB* gene revealed by *AvaII* restriction enzyme in Kenguri x NARI suwarna sheep. Lane M: 100bp molecular marker; Lane 1- 9: Restriction enzyme digested amplicons (190bp and 160bp) representing B+ genotype of *FecB* gene; Lane 10-14: Restriction enzyme digested amplicons (190bp) representing ++ genotype of *FecB* gene.

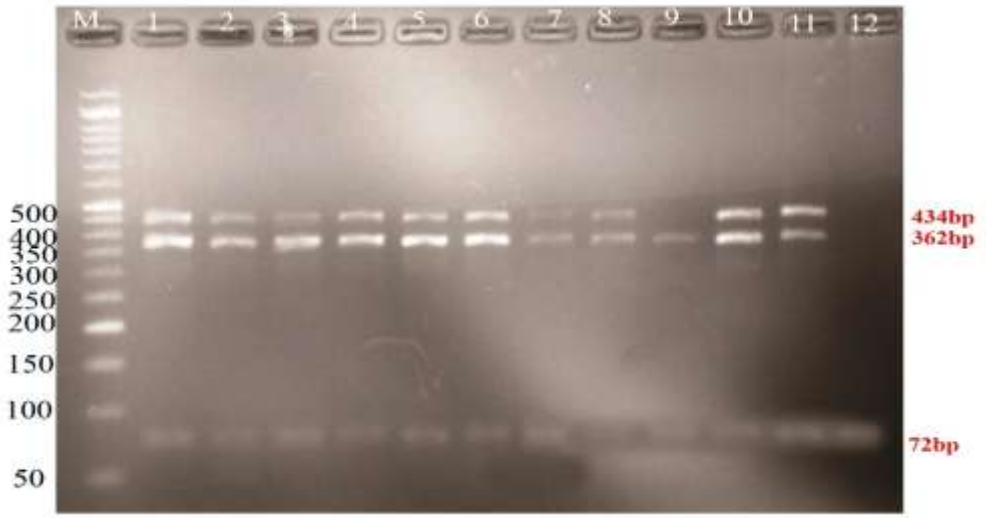

Fig. 4: PCR-RFLP band patterns of ovine BMP15 gene revealed by StuI restriction enzyme in Kenguri x NARI suwarna sheep. Lane M: 50bp molecular marker; Lane 1-8 and 10-11: Restriction enzyme digested amplicons (434bp, 362bp and 72bp) representing AB genotype of BMP15 gene; Lane 9: Restriction enzyme digested amplicons (362bp and 72bp) representing AA genotype of BMP15 gene.

The polymorphic parameter such as polymorphic information content (PIC), heterozygosity, allelic diversity and test for analysis of Hardy-Weinberg equilibrium (HWE) for *Fec-B* and *BMP15* genes in Kenguri x NARI Suwarna strain of sheep is given in Table 3. The analysis revealed moderate PIC values and departure of Kenguri x NARI Suwarna strain of sheep from HWE.

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PCR-RFLP analysis of *Fec-B* gene has revealed the presence of only ++ genotype in Kenguri sheep and B+ and ++ genotypes in Kenguri x NARI Suwarna strain of sheep. Similar to our results in Kenguri sheep, absence of *Fec-B* mutation was reported in Macheri (Sudhakar *et al*., 2014), in Sangsiri sheep of Iran (Jamshidi *et al*., 2013) and in 5 Egyptian sheep breeds *viz*. Rahmani (R), Ossami (O), Awassi (A), Barki (B) and Awassi×Barki (A×B) (Amr *et al*., 2009). On the contrary to our results for *Fec-B* gene in Kenguri, B+ and ++ genotypes were reported with a frequency of 0.77 and 0.23, respectively, in Garole sheep (Polley *et al*., 2010) and a frequency of 0.39 and 0.61 for B and + alleles. Likewise, PCR-RFLP analysis of *Fec-B* gene in Garole sheep revealed BB, B+ and ++ genotypes with a frequency of 0.6, 0.31 and 0.09 and the same in Shahabadi were 0.20, 0.70 and 0.15 (Banerji *et al*., 2002). In Muzaffarnagari sheep, BB, B+ and ++ genotypes were identified with a frequency of 0.03, 0.41 and 0.54 and allelic frequency of 0.235 and 0.745 for B and + alleles, respectively (Singh *et al*., 2008). In Nilgiri sheep, Sudhakar *et al*. (2013) have reported BB, B+ and ++ genotypes of *Fec-B* with a respective frequency of 0.006, 0.262 and 0.731 and frequency of 0.131 and 0.862 for B and + alleles. Similar to our indigenous breeds, in Hu sheep of China BB, B+ and ++ genotypes of *Fec-B* gene with a respective frequency of 0.33, 0.58 and 0.09. In Garole×Malpura sheep, RFLP analysis of *Fec-B* sheep revealed three genotypes namely BB, B+ and ++ with respective frequency of 0.06, 0.73 and 0.20 and the respective frequency of B and + alleles was 0.37 and 0.41 (Kolte *et al*., 2005).

PCR-RFLP analysis of *BMP15* gene has revealed the presence of only AB genotype in Kenguri sheep and AA and AB genotypes in Kenguri x NARI Suwarna strain of sheep. Similar to our results, in Lleyn (Cambridge and Belclare) sheep breeds AA and BB genotype with a frequency of 0.5 and 0.5, respectively were reported (Mullen *et al*., 2003). Likewise, in Small Tailed Han sheep, AB and BB genotypes with a frequency of 0.60 and 0.40, respectively were reported (Chu *et al*., 2007). Contrast to our results, *BMP15* mutation was reported absent in Garole, Malpura, Kendrapada sheep (Kumar *et al*., 2008), Nilgiri sheep (Sithi, 2009), Bonpala sheep (Roy *et al*., 2011), Marwari goat (Aditya *et al*., 2011), Little tailed Han sheep (Liu *et al*., 2003) and Iranian Sangsari sheep (Kasiriyan *et al*., 2009). Interestingly, BMP15 mutation was reported by many researchers. In Black Bengal goat, AA, AB and BB genotypes were reported with a respective frequency of 0.44, 0.59 and 0.17 (Ahlawat *et al*., 2015). Also, Wang *et al*. (2011) have identified three genotypes (AA, BB and AB) in Fumin White goats and two genotypes (AA, AB and BB) in Tailang black goats using PCR–SSCP and DNA–Sequencing of exon 2 of *BMP15* gene. Feng *et al*. (2014) have identified a mutation in *BMP15* gene in Chinese goats.

Although, polymorphism of *BMP15* gene was identified in goat, it was not observed in sheep. However, we are foremost in reporting the polymorphism of *BMP15* gene in sheep. For this new primers were designed to amplify partial exon2 of *BMP15* gene (434bp) and restriction endonuclease enzymes within this region was identified using NEB cutter online program (http://nc2.neb.com/NEBcutter2/). Among

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these REs, we selected RE site that contained reported SNPs. One such RE was *Stu*I, which was useful for PCR – RFLP genotyping of *BMP15* gene in Kenguri and Kenguri x NARI Suwarna strain of sheep. This report of *BMP15/StuI* polymorphism in sheep breeds/ strains of India is foremost. The χ^2-square test to know whether *Fec-B* and *BMP15* genotype frequencies are in Hardy-Weinberg equilibrium revealed significant difference between observed frequencies and expected frequencies that indicate the departure of both Kenguri and Kenguri x NARI Suwarna strain of sheep population for both *Fec-B* and *BMP15* genes from HWE. Similar to these findings, Polley *et al.* (2010) in Garole, Shahabadi sheep and Ahlawat *et al.* (2015) in Black Bengal goat had also shown that the populations were not in HWE equilibrium for *FecB* and *BMP15* genotypes.

Conclusion

Polymorphism of *FecB* gene was not observed in Kenguri but in Kenguri x NARI Suwarna strain of sheep. Likewise, polymorphism of *BMP15* gene was not detected in Kenguri but in Kenguri x NARI Suwarna strain of sheep. Investigating the polymorphism of *FecB* and *BMP15* genes in larger population along with other candidate genes of fecundity *viz*. *GDF9, FSHR* and *GnRHR* is suggested. The polymorphism in these genes if identified may be used to associate with fecundity rate/ litter size in sheep. The genetic markers significantly associated with fecundity rate/ litter size will certainly assist the breeders to select sheep with better reproductive performance and make sheep rearing a profitable enterprise.

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