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New genetic polymorphisms in Indian goat BMPR1B gene

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ABSTRACT

Studies on the genetics of prolificacy in small ruminants have highlighted the importance of *BMPR1B* (bone morphogenetic protein receptor IB) gene in affecting ovulation rate and litter size. Most of the research on *BMPR1B* gene has revolved around screening sheep and goats for the presence of *FecB* mutation (A746G) that is associated with hyperprolific phenotype in many breeds around the world. In the present study, an attempt was made to detect polymorphism in the promoter and exonic regions (exon1, 2 and 6 to 9) of *BMPR1B* gene in 8 indigenous goat breeds (Barbari, Beetal, Black Bengal, Malabari, Jakhrana, Osmanabadi, Sirohi and Ganjam) varying in prolificacy and geographic distribution. Targeted DNA fragments (2612 bp) were obtained through PCR and DNA sequencing methods. *BMPR1B* promoter region was amplified in two fragments and a 1032 bp length contig was generated for the first time for goats. Two novel SNPs T(-242)C and G(–623)A were identified in the promoter region of caprine *BMPR1B* gene by sequence alignment. Hundred variations were observed between cattle and goat promoter sequence. The variations did not change or affect any transcription binding sites in promoter of *BMPR1B* gene. Sequence analysis revealed absence of any polymorphism including the *FecB* mutation in exonic regions of all the investigated breeds of Indian goats. This is the first report of identification of mutation in regulatory region of BMPR1B gene, which may be helpful for better understanding of the role played by this gene in reproduction of the goat.

Key words: *BMPR1B*, Indian goat, Polymorphism, Promoter, SNPs

Goats, integral component of farming system, support a large rural population of landless and marginal farmers in India. India ranks second in the goat population with more than 140 million goats, which constitute 25.6% of country's total livestock (Livestock census 2007). Goat diversity comprises 23 recognized breeds in India, a valuable gene pool for adaptive and economic traits providing diversified genetic pool that can help to meet future challenges. A moderate increase in litter size can lead to larger profit to the rural poor. Molecular genetics can overcome these limitations offering new opportunities to the improvement of reproductive traits, as it supplies tools to analyze genetic variability directly at the DNA level with the possibility of detecting the individual genes influencing the reproductive characteristics.

Genetic studies on sheep prolificacy across continents have indicated that litter size and ovulation rate is determined by the action of fecundity (*Fec*) genes, with major effects (Davis *et al.*1982). *FecB* is the first major gene for prolificacy identified in Australian Merino sheep. Single point mutation (A to G substitution) at base 746 (A746G) of the coding region of the *BMPR1B* gene caused an amino acid change (Q249R) that was associated with the hyper prolific phenotype of Booroola ewes (Souza *et al.* 2001). The genetic basis of caprine prolificacy remains to be explored completely. Recently reports have come related to interest in genetic basis of caprine prolificacy (Polley *et al.* 2009, Ahlawat *et al.* 2012a, 2012b). Knowledge of *FecB* gene prompted researchers to screen different goat breeds for mutation in *BMPR1B* to determine whether the gene is responsible for their high prolificacies. Recent studies on prolific goat breeds like Boer, Haimen, Huanghuai, Nubi, Matou and Jining Grey, suggested that higher prolificacy in goat is not like that of sheep (Hua *et al.* 2008). Based on the crucial role of *BMPR1B* gene in regulation of terminal folliculogenesis and the control of ovulation rate, *BMPR1B* gene was considered as a possible candidate gene for prolificacy in goats as the tendency of twining and triplets is inherited and possibly common in the two small ruminants (sheep and goat). The objectives of the present study were to

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identify SNPs (single nucleotide polymorphisms) of *BMPR1B* gene in 8 goat breeds with different prolificacy (high: Beetal, Barbari, Black-Bengal, Malabari; medium: Osmanabadi, Jakhrana; and low: Ganjam and Sirohi) and to investigate affect of polymorphism in promoter region on transcription factor binding sites.

MATERIALS AND METHODS

Sample collection: To explore the genetic variations within *BMPR1B* gene, blood samples from a panel of Indian goat breeds were used. The panel included 8 breeds (Table 1) with 5 samples each based on phenotype (prolificacy), geographical distribution (Fig. 1) and genetic diversity.

The blood samples were collected from jugular vein into EDTA containing vacutainer tubes. Genomic DNA was isolated and purified from the blood cells using the standard phenol-chloroform-isoamyl alcohol extraction followed by ethanol precipitation (Sambrook and Russell 2001). The quality and quantity of isolated DNA was determined using agarose gel electrophoresis (0.8%) and Nanodrop spectrophotometer (GE Healthcare). Purified DNA ran as a single band on agarose gel and the OD 260/280 ratio for all the samples was in range of 1.8–2.0, indicating good quality of extracted DNA.

Table 1. Characteristics of Indian goat breeds employed in the study

Phenotype	Breed	Geographical distribution	Twinning percentage
High prolificacy	Beetal	Punjab	>50
	Barbari	Uttar Pradesh	
	Black-Bengal	West Bengal, Bihar, Jharkhand	
	Malabari	Kerala	
Medium prolificacy	Osmanabadi	Maharashtra	25–50
	Jakhrana	Rajasthan	
Low prolificacy	Ganjam	Odisha	<25
	Sirohi	Rajasthan	

Fig. 1. Geographical distribution of selected goat breeds.

Primer designing: To amplify exonic regions of *BMPR1B* gene, primers reported by Chu *et al.* (2010) were used. Two overlapping oligonucleotide primers were designed to amplify the promoter sequence of caprine *BMPR1B* gene from cattle promoter sequence (Accession number ENSBTAT00000002690) using PRIMERSELECT program of LASERGENE software. Characteristics of primers are listed in Table 2.

Table 2. Oligonucleotide primers for the promoter and coding DNA regions of caprine *BMPR1B* gene

Primer	Oligonucleotide sequence	Product size (bp)	Amplified region (location)	Annealing temperature (°C)
P1	F-5'-ATTTCTCAGGGTGACGATTTCTACAGTG-3' R-5'-CACGCAGCTAATAAGTCATGAATACAGG-3'	745	5'UTR[a](877-1621)	57
P2	F-5'-TCCCTTTTGCTTGTAAATACTGATGAGG-3' R-5'-GACCGAGTCTTCTGGACAATGGTG-3'	778	5'UTR[a](1364–2141)	57
P3	F-5'- AAGCAAACTTCCTTGATAACAT -3' R-5'- CTGCAAATATTGTTGACCGA -3'	163	Exon 1[b](137–299)	56.8
P4	F-5'- GCAGCACAGATGGATATTGTTT -3' R-5'- CGACACTGAAAATCTGAGCCT -3'	106	Exon 2[b](296–401)	57.1
P5	F-5'- GGTCCAGAGGACAATAGCAA-3' R-5'- GCCCAAGATGTTTTCATGC-3'	196	Exon 6[b](741–936)	59.8
P6	F-5'-GCTTCATTGCTGCAGATAT -3' R-5'CCTAATAAACTTAACAGCCAA-3'	299	Exon 7 [b](935–1233)	58.4
P7	F-5'-TATTAGTGACACGAATGAAGT-3' R-5'CTATACCTCCTGACACACA-3'	188	Exon 8 [b](1227-1414)	58.4
P8	F-5'-TCAGGAGGTATAGTGGAAGAATATC-3' R-5'-CGTCACTGCTCCACCGGTT-3'	137	Exon 9 [b](1401–1537)	61.1

[a]Corresponding to cattle sequence (Accession number ENSBTAT00000002690). [b]Corresponding to sheep sequence (Accession number AF357007.1).

PCR amplification: Polymerase chain reaction (PCR) was carried out in a final reaction volume of 25 µl on i-cycler (BIO-RAD, USA). PCR cocktail consisted of 50 to 100 ng of genomic DNA, 200 µM of each dNTPs, 50 pM of each primer, 0.5 units of *Taq* DNA polymerase and *Taq* buffer having 1.5 mM $MgCl_2$ for each reaction. The PCR cycle was accomplished by denaturation for 1 min at 94°C; 30 cycles of denaturation at 94°C for 45 sec, annealing at specific temperature for 45 sec, extension step at 72°C for 45 sec with a final extension at 72°C for 5 min. The PCR products were visualized following electrophoresis through a 1.8% Ethidium bromide stained agarose gel.

Sequencing and analysis: The amplified region was sequenced using ABI 3100 automated DNA sequencer. Prior to sequencing, PCR products were purified by enzymatic method using exonuclease I and antarctic phosphatase. Raw sequence data were edited using Chromas (Ver. 1.45, http://www.technelysium.com. au=chromas.html). Multiple sequence alignments were performed with MegAlign program of LASERGENE software version 5.07 to identify polymorphisms (mutations or SNPs) in different regions of the *BMPR1B* gene in indigenous goats. Polymorphic sites identified by sequence comparison were further confirmed by manual inspection of chromatograms. The coding DNA sequences of different exonic regions were conceptually translated to amino acid sequences using Chromaspro software. The BLAST algorithm was used to search the NCBI Genbank (http://www.ncbi.nlm.nih.gov) databases for homologous sequences. The transcriptional factor binding sites were identified using MATCH (Kel *et al.* 2003) (http://www.cbil.upenn.edu/cgi-bin/pub/programs/match/bin/match.cgi). Phylogenetic analysis was performed with CLC Main Workbench version 5.0.2.

RESULTS AND DISCUSSIONS

DNA of 8 goat breeds was successfully amplified using 8 pairs of primers for *BMPR1B* gene. Amplified fragment sizes were consistent with the target ones. Sequences have been submitted to GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) vide accession numbers KC142197, KC142198 and JX315602. The polymorphic sites were explored in the eight different goat breeds such as Black Bengal, a meat-type Indian goat breed, which is famous for its quality lean meat production and well known for giving multiple births with a prolificacy of above 180% (Zeshmarani *et al.* 2007) and Ganjam goat a low prolificacy, late maturing, annual single kidding and slow growing goat (Acharya 1982).

Sequence analysis of exonic regions: Variations as compared to goat (DQ666418.1) as well as sheep (AF357007) were not found in exon1 and 2 of Indian goat BMPR1B gene. Four variations, one each in exon 6 (G674A), exon 7 (G1053A), exon 8 (C1253T) and exon 9 (C1367T) were observed in Indian goat as compared to Jining Grey goat of China (DQ666418.1). Whereas, a variation in exon 8 was observed in Indian goat coding regions as compared to sheep (Table 3). All the nucleotide variations were synonymous thus no change is expected in the amino acid sequence of corresponding protein in Indian goat, Jining Grey goat of China and sheep. Maximum number of nucleotide changes (19) was recorded in comparison to cattle (BC134547.1) of which 16 were transitions and three were transversion. Maximum variations were recorded in exon 7 (8) followed by exon 8 (5), exon 9 (3) and one each in exon 1, 2 and 6 (Table 3).

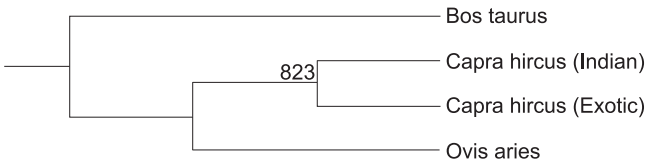

Fig. 2. Phylogenetic analysis of *BMPR1B* coding DNA sequence of different species following UPGMA algorithm (Branch number represent bootstrap value in 1000 replicates).

BLAST analysis was carried out to find the percentage identity in the coding region of Indian goat *BMPR1B* gene at the nucleotide level with other species. Coding DNA sequences (CDS) of *BMPR1B* gene revealed identity of 97% of Indian goat with *Bos taurus* (BC134547.1) and 99% with *Ovis aries* (AF357007.1) and *Capra hircus* (DQ666418.1). Homology of *BMPR1B* sequences among sheep and goat are especially higher, which coincide with the fact that these are evolutionary more related. Similar picture was depicted on

Table 3.Variations in coding DNA of Indian goat *BMPR1B* gene as compared to cattle, sheep and goat

Region	Cattle (BC134547.1)	Sheep (AF357007.1)	Jining grey goat (DQ666418.1)	Indian goat
Exon 1	C (108)	T (252)	T (105)	T
Exon 2	C (207)	T (351)	T (204)	T
Exon 6	G (677)	A (821)	G (674)	A
Exon 7	C (864)	T (1008)	T (861)	T
	T (903)	C (1047)	C (900)	C
	T (922)	C (1066)	C (919)	C
	T (934)	G (1078)	G (931)	G
	G (948)	C (1092)	C (945)	C
	T (975)	C (1119)	C (972)	C
	C (1029)	G (1173)	G (1026)	G
	G (1056)	A (1200)	G (1053)	A
Exon 8	C (1159)	T (1303)	T (1156)	T
	C (1164)	T (1308)	C (1161)	C
	C (1182)	T (1326)	T (1179)	T
	C (1218)	T (1362)	T (1215)	T
	C (1256)	T (1400)	C (1253)	T
	A (1257)	G (1401)	G (1254)	G
Exon 9	T (1323)	C (1467)	C (1320)	C
	C (1370)	T (1514)	C (1367)	T
	C (1389)	T (1533)	T (1386)	T

phylogenetic analysis of *BMPR1B* gene following UPGMA algorithm.

Exonic regions (Exon1-2, Exon 6–9) of *BMPR1B* gene were analyzed for the presence of polymorphic site. All the exons including exon 6 did not display polymorphism in the investigated Indian goats. One point mutation in *BMPR1B* gene at base 746 of the coding region (A746G) of exon 6 caused an amino acid change (Q249R) that was associated with the hyperprolific phenotype of Booroola ewes and has provided direct marker for the DNA mutation test for *FecB*. Based on the crucial role of *BMPR1B* gene in regulation of terminal folliculogenesis and the control of ovulation rate, *BMPR1B* gene was considered as a possible candidate gene for prolificacy of goats. However, *BMPR1B* gene polymorphism sites related to litter size trait were not detected in 10 goat breeds of China (Hua *et al.* 2008, Chu *et al.* 2010, Feng *et al.* 2010). Polley *et al.* (2009) reported that in Black Bengal goats of India the *BMPR1B* gene was polymorphic with the predominance of heterozygous genotype AG. However, the known point mutation of *BMPR1B* gene of sheep (A746G) was not detected in all the eight breeds of goat investigated in the present study. Our results are similar to those reported by Hua *et al.* (2008) who found that the point mutation of *BMPR1B* (*FecB*) is monomorphic in the prolific goat breeds of China such as Boer, Haimen, Huanghuai, Nubi, Matou. Other reports also demonstrate that Chinese goat breeds are monomorphic for this locus (He *et al.* 2010). Sheep *FecB* gene polymorphism was not found in the Thai multi-breed meat goat population too (Supakorn *et al*. 2010). Chu *et al*. (2010) reported absence of polymorphism in exon 6 of *BMPR1B* gene in high prolificacy as well as low prolificacy goat breeds of China. These findings indicate that there are possibly differences in prolificacy inheritance pattern of goat and sheep.

Sequence analysis of promoter region: Since information is not available for the goat *BMPR1B* promoter region, the present investigation was directed to sequence the 5´ upstream region from start codon of *BMPR1B* gene in Indian goat. *BMPR1B* promoter region was amplified in two fragments and a 1032 bp length contig was generated. Four putative binding sites for the transcription factors were observed within the promoter region of Indian goat sequence (Fig. 3). These included COMP1 (cooperates with myogenic proteins 1), BR-C Z1 (belongs to family of zinc-finger transcription factors encoded by *Broad-Complex* (*BR-C*) gene), HNF-1(Hepatocyte nuclear factor-1) and FOX J-2 (Forkhead box J-2). Binding of these factors to their respective sites is known to be associated with growth and differentiation. Most important among these seems to be FOXJ-2 as FOX (Forkhead box) proteins belong to a family of transcription factors that play important roles in regulating the expression of genes which are involved in cell growth, proliferation, differentiation, and longevity and many FOX proteins are important to embryonic development too.

Table 4. Variations in promoter of Indian goat as compared to cattle (ENSBTAT00000002690) and sheep (GU117619)

Position as per cattle	Cattle	Indian Goat	Sheep	Position as per cattle	Cattle	Indian Goat	Sheep
Between –39 and –40		Insertion of TAAAT	Insertion of TAAAT	–598	C	-	
–50	T	G	G	–599	T	-	
–60	C	-	C	–600	A	-	
–65	T	C	C	–601	T	-	
–93	T	G	T	–602	A	-	
–113	A	C	C	–603	T	-	
–117	G	A	A	–604	A	-	
–118	T	C	C	–605	A	-	
–120	C	T	C	–606	A	-	
–132	A	A	G	–607	G	-	
–136	T	T	G	–608	T	-	
–138	G	C	C	–609	T	-	
–142	C	T	T	–610	T	-	
–148	T	C	T	–611	T	-	
–159	G	G	C	–612	T	-	
–167	T	C	C	–613	T	-	
–210	C	T	C	–614	A	-	
–237	A	G		–615	T	-	
–303	A	G		–616	T	-	
–316	A	G		–636	T	A	
–324	G	T		–717	G	C	
–395	C	A		–721	G	A	
–480	G	A		–741	T	C	
–491	T	A		–743	A	G	
–528	C	T		–750	G	A	
–574	A	-		–752	G	A	
–575	T	-		–772	G	T	
–576	A	-		–825	T	C	
–577	T	-		–845	A	G	
–578	A	-		–846	C	T	
–579	A	-		–847	A	-	
–580	C	-		–848	G	-	
–581	T	-		–849	T	-	
–582	A	-		–850	A	-	
–583	T	-		–851	C	-	
–584	A	-		–852	A	-	
–585	A	-		–864	C	T	
–586	C	-		–870	C	T	
–587	T	-		–871	C	G	
–588	A	-		–888	A	G	
–589	T	-		–896	C	T	
–590	T	-		–912	G	A	
–591	A	-		–913	T	C	
–592	A	-		–942	G	A	
–593	T	-		–984	G	T	
–594	A	-		–1005	A	G	
–595	G	-		–1041	G	C	
–596	T	-		–1050	C	T	
–597	T	-		–1054	C	T	
–598	C	-		–1071	A	G	

The name of your search is: BMPR1B_promoter_sequence
Search for sites by WeightMatrix library: matrixTFP60.lib
Sequence file: default.seq
Matrix groups: all
Cut-offs: to minimize false positive matches

Scanning sequence ID: default_0
View a graphic of the following search results

matrix identifier	position (strand)	core match	matrix match	sequence (always the (+)-strand is shown)	factor name
V$COMP1_01	20 (-)	1.000	0.832	acttaataggtgtCAATCcatgct	COMP1
I$BRCZ1_01	320 (+)	0.950	0.930	agctgataaACAATaaag	BR-C_Z1
V$HNF1_C	628 (-)	0.942	0.855	ttcatgacttaTTAGCt	HNF-1
V$FOXJ2_02	697 (+)	1.000	0.939	atgATAATatttac	FOXJ2

Total sequences length=1032
Total number of sites found=4
Frequency of sites per nucleotide=0.003876

Fig. 3. Transcription factor binding sites (TFBS) in promoter of *BMPR1B* gene of Indian goat.

All the transcription binding sites presented high core match and matrix match similarity. Minimum value of 0.83 and 0.94 was observed for matrix match and core match, respectively. The maximum core similarity of 1.0 was observed for COMP1 and FOXJ-2 and is reached when the highest conserved bases of a matrix match exactly in the sequence.

Variations in promoter region: A total of hundred variations were observed between cattle (Accession number ENSBTAT00000002690) and goat (Table 4) which include 32 transitions, 13 transversions, 50 deletions and 5 insertions. Thirteen variations (4 transition, 3 transversion, 1 deletion and 5 nucleotide insertion) were recorded as compared to 5´UTR sequence of sheep (210 bp, GU117619). Insertion of five nucleotides was observed in goat sequence between 39th and 40th nucleotide of cattle sequence.

Comparison of promoter region in 40 samples of Indian goat from eight breeds resulted in identification of two SNPs, T(–242)C and G(–623)A (Fig. 4). The numbering of SNPs is with respect to transcriptional start site. Both the SNPs are found to be novel as these have not been reported in any other goat breeds. Both these polymorphisms did not change or affect any of four transcription binding sites.

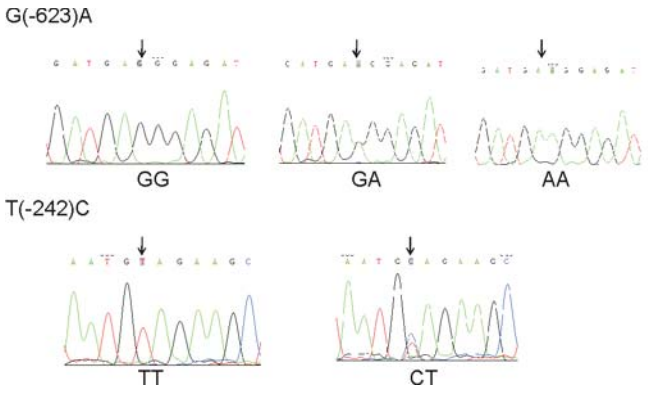

Fig. 4. Sequence comparison of different genotypes at –623 and –242 locus of the goat *BMPR1B* gene (the arrow pointed to the mutation site).

Table 5. SNPs in *BMPR1B* promoter and their genotype frequencies in Indian goats

Locus	Cattle (ENSBTAT00000002690)	Overall (number of animals)	Genotype frequency		
			High prolific	Medium prolific	Low prolific
–242	TT	TT = 0.95 (38)	TT=0.9	TT=1.0	TT=1.0
		TC = 0.05 (2)	TC=0.1		
		CC = 0.00 (0)			
–623	GG	GG = 0.693 (27)	GG=0.79	GG=0.11	GG=1.0
		GA = 0.282 (11)	GA=0.21	GA=0.78	
		AA = 0. 025 (1)		AA=0.11	

At –623 locus, G to A transition expressed 3 genotypes: GG, GA and AA in investigated goat population whereas, transition (A to C) at –242 locus expressed 2 genotypes: TT and TC (Fig 4). For G(–623)A polymorphism, all the 3 genotypes (Table 5) were detected in Osmanabadi goats of medium prolific goat group (Osmanabadi, Jakhrana), 2 genotypes (GG, GA) were observed in all the breeds of high prolificacy groups (Beetal, Barbari, Black-Bengal, Malabari) whereas, low prolificacy group (Ganjam and Sirohi) presented single genotype (GG). For T(–242)C, 2 genotypes, TT and TC were detected only in high prolific group (Malabari and Black-Bengal) and medium prolificacyand low prolific group presented single genotype (TT).

Presence of C allele in the high prolific group only, that too in Black-Bengal the most prolific goat breed of India (Zeshmarani *et al.* 2007), makes this mutation an interesting site for further exploration with reference to prolificacy associated markers. There is a need to undertake a further research on a substantially larger number of the population to reach to some logical conclusion. The results of the present study provide, for the first time, partial sequence as well as two polymorphisms of 5´UTR region of BMPR1B gene in Indian goat. Further studies are necessary to validate the association and/or physiological significance of these novel SNPs.

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