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Analysis of coding DNA sequence of *GDF9* gene in Indian goats for prolificacy associated markers

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ABSTRACT

The present study was undertaken to characterize complete coding DNA sequence of one of fecundity genes: GDF9 (Growth differentiation factor 9), in a panel of 6 breeds of indigenous goats with different prolificacy. Nine nucleotide differences were identified between sheep (AF078545) and goats in exon 1 and flanking region and 9 transitions were seen in exon 2. Three non-synonymous SNPs (C818T, A959C and G1189A) were detected in exon 2 of indigenous goat breeds. One of the SNPs (A959C) identified in present study has been associated with high prolificacy in exotic goats. These SNPs were different from the 4 mutations ($FecG^H$, $FecG^E$, FecTT and G1) associated with fecundity in sheep. GDF9 coding DNA sequence of Indian goat was 99% similar to exotic goat as well as sheep, whereas it depicted 97% homology with *Bos taurus.* In silico translation of obtained coding DNA sequence of GDF9 gene in indigenous goats revealed a sequence of 454 amino acids with 1 change between Indian goat and exotic goat, 5 amino acid changes between Indian goat and sheep and 21 changes between Indian goat and cattle.

Key words: GDF9, Fecundity, Indian goat, Prolificacy

The population of goat in India is about 125.7 million (FAO STAT 2008). Goats contribute largely to the livelihoods of the livestock-keeping households of low- and medium - input farmers. Therefore, it is significant to improve the economic traits of goat such as reproductive trait. How precisely the litter size is controlled remains a critical and important question in reproductive biology. Transforming growth factor-beta (TGFβ) superfamily have crucial role in fecundity of sheep. Gene *GDF9* (growth differentiation factor 9) is associated with prolificacy in sheep. Ten single nucleotide polymorphisms (SNPs) have been identified so far in sheep *GDF9* gene. Out of these Hanrahan *et al*. (2004) reported 8 SNPs (labelled G1–G8). Four mutations in GDF9 were found associated with fertility in sheep, viz. $FecG^H$ (G8) mutation in Cambridge and Belclare sheep (Hanrahan *et al*. 2004), $FecG^E$ (also named as $FecG^{SI}$) mutation in Santa Ines sheep (Silva *et al.*2010), FecTT mutation in Thoka sheep (Nicol *et al*. 2009), and G1 mutation in Moghani and Ghezel sheep (Barzegari *et al*. 2010) and in Garole sheep (Polley *et al*. 2010). The G8 mutation, also indicated as $FecG^H$ (high fertility), causes increased ovulation rate in heterozygous ewes, while homozygous ewes are sterile. For the $FecG^E$ allele, homozygote ewes have shown an increase in their ovulation rate and prolificacy. Since *GDF9* is a gene with prominent effect on litter size in sheep it may be a potential gene affecting litter size in goats too.

Genetic mechanism of the goat prolificacy is to be explored because a moderate increase in litter size can lead to a large profit. Therefore, the objectives of the present study were to screen indigenous goat breeds for the mutations in GDF9 gene associated with prolificacy in sheep and to seek for the potential single nucleotide polymorphisms (SNPs) through sequence alignment in 6 goat breeds with different prolificacy.

MATERIALS AND METHODS

To explore the genetic variations within *GDF9* gene, blood samples from a panel of Indian goat breeds were used. The panel included samples from breeds, each based on phenotype (prolificacy), geographical distribution and genetic diversity (Table 1).

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 as described by Sambrook and Russell (2001). *GDF9* gene maps to chromosome 5 and contains 2 exons (Bodensteiner *et al.*1999) (Fig 1).

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

Phenotype	Breed	Geographical distribution	Twinning percentage%
High prolificacy	Beetal	Punjab	52.6
	Barbari	Uttar Pradesh	49.3
	Black-Bengal	West Bengal, Bihar, Jharkhand	54
	Malabari	Kerala	42.4
Medium prolificacy	Osmanabadi	Maharashtra	29
Low prolificacy	Ganjam	Orissa	1.6

Source: Acharya (1982).

Amplification of the first exon of *GDF9* was performed by using the primers described by Hanrahan *et al*. (2004). For the second exon, 2 pairs of overlapping primers were designed using PRIMERSELECT program of LASERGENE software. Characteristics of primers are listed in Table 2.

Polymerase chain reaction (PCR) was carried out in a final reaction volume of 25 µl on i-cycler. PCR cocktail consisted of 50 to 100ng of genomic DNA, 200µM of each dNTPs, 50pM of each primer, 0.5units of Taq DNA polymerase and Taq buffer having 1.5 mM $MgCl_2$ for each reaction. The PCR reaction cycle was accomplished by denaturation for 1 min at 94°C; 30 cycles of 94°C for 45 sec, annealing at specific temperature for 45 sec, 72°C for 45 sec with a final extension at 72°C for 5 min. The PCR products were visualized following electrophoresis through a 2% ethidium bromide stained agarose gel.

The amplified region was sequenced using 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 to identify polymorphisms in the exon 1 and exon 2 of *GDF9* gene in indigenous goats and phylogenetic analysis was performed with CLC Main Workbench version 5.0.2. The coding sequences of different exonic regions were conceptually translated to amino acid sequences using Chromaspro software.

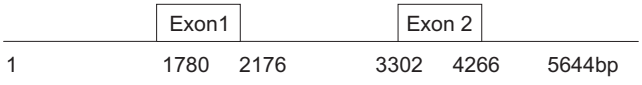

Fig 1. Sheep *GDF9* gene.

RESULTS AND DISCUSSION

Genomic DNA of 6 indigenous goat breeds was successfully amplified by the primers designed for both the exons of *GDF9* gene. There were 44 nucleotide changes in the amplified regions (exon 1 along with the boundary regions and exon 2) of caprine *GDF9* gene as compared to *Bos taurus* (GQ922451.1) out of which 63.5% were transitions, 32% were transversions and 4.5% were deletions. On comparing the sequence of indigenous goats with sheep (AF078545), 3 transitions, 2 transversions and 4 consequetive deletions were observed in exon 1 and flanking region and 9 transitions were seen in exon 2 (Table 3). When the GDF9 sequence of Indian goats was compared with exotic goat (Jining grey goat, EF446168), 2 transversions (A1962C, G2254T) were seen in exon 1 and flanking region whereas 3 transitions (T3623C, C3722T and G4093A) and 1 transversion (A3863C) were observed in exon 2.

Interesting observation in our study was that two variations (G477A and G721A) in exon 2 of indigenous goats as compared to sheep were also the site of reported SNPs (G3 and G4 mutations) in Cambridge and Belclare sheep (Hanrahan *et al.* 2004). However, allele A was fixed at loci 477 and 721 in the investigated goat breeds.

Exon 1 and exon 2 were screened for SNPs by direct sequencing of PCR amplicons. None of the ten SNPs identified so far in sheep *GDF9* gene including four mutations ($FecG^H$, $FecG^E$, FecTT and G1) associated with fecundity (Table 4) could be identified in indigenous goats by sequence alignment.

Similar results were also reported by Polley *et al.* (2009) in Black Bengal goats, where 5 mutations, viz. G1, G4, G6, G7 and G8 were analysed by tetra-primer based ARMS-PCR. All the goats tested had only the wild type genotype and same is true for all these loci with animals of 6 breeds tested by us.

Three SNPs (C818T, A959C and G1189A) were identified in exon 2 of indigenous goat breeds which correspond to

Table 2. Oligonucleotide primers designed to amplify the coding DNA sequence of caprine *GDF9* gene

Region	Oligonucleotide sequence	Product size (bp)	Annealing temperature (°C)
Exon 1	F: 5′ - GAATTGAACCTAGCCCACCCAC-3′ R: 5′ -AGCCTACATCAACCCATGAGGC-3′	704	60.5
Exon 2	F 5′ -GGGAGAAAAGGGACAGAAGC-3′ R 5′ -TTCAGAGCTGGCACTCTCCT-3′	677	56.7
	F 5′ -AGATTGATGTGACGGCTCCT-3′ R 5′ -CTCCCAAAGGCATAGACAGG-3′	799	55.6

Table 3. Variations in coding DNA sequence (CDS) of Caprine GDF9 gene

Region	Position	Sheep AF078545	Exotic goat EF446168	Indian goat	Region	Position	Sheep	Exotic goat	Indian goat
Exon 1	1723	G	A	A	Exon 2	3381	G	A	A
and flanking	1893	T	C	C		3623	C	T	C
region	1962	C	A	C		3625	G	A	A
	1978	G	A	A		3722	C	C	C/T
	2254	G	G	T		3788	A	G	G
	2327	T	-	-		3794	G	A	A
	2328	A	-	-		3810	C	T	T
	2329	C	-	-		3863	A	A	A/C
	2330	T	-	-		3872	C	T	T
	2345	G	C	C		4071	C	T	T
						4093	G	G	G/A
						4146	A	G	G

Table 4. Polymorphic sequence variations in GDF9 in sheep

Gene	Allele	Base change	Coding Base (bp)	Amino acid change	Accession number	Reference
GDF9	G1	G-A	260	Arg(R)87 his(H)	AF078545	Hanrahan *et al*. (2004)
	G2	C-T	471	Unchanged val(V)		
	G3	G-A	477	Unchanged leu(L)		
	G4	G-A	721	Glu(E)241 lys(K)		
	G5	A-G	978	Unchanged glu(E)		
	G6	G-A	994	Val(V)332 ile(I)		
	G7	G-A	1111	Val(V)371 met(M)		
	G8/FecG^{H}	C-T	1184	Ser(S)395 phe(F)		
	FecTT	A-C	1279	Ser(S)109 arg(R)		Nicol *et al*. (2009)
	FecG^{E}	T-G	1034	Phe(F)345 cys(C)	FJ429111	Silva *et al.* (2010)

nucleotide positions 3722, 3863 and 4093, respectively. All the 3 mutations identified in our study are non-synonymous in nature leading to change in amino acid in the corresponding protein. The position of these SNPs, their genotype frequencies and position of amino acid change are presented in Table 5.

Two amino acid changes are conservative as amino acid with nonpolar groups are substituted by the amino acids of same category, viz. C818T and G1189A change alanine to valine and valine (conserved across most TGFβ superfamily members) to isoleucine at position 273 and 397, respectively, in the corresponding protein. However, A959C is a non-conservative change as it corresponds to glutamine to proline change which replaces an uncharged polar group with a nonpolar group at amino acid residue 320. The change may be affecting the functioning of mature protein as this is the only SNP among the 3 SNPs identified in present study on indigenous goat that has been associated with prolificacy in exotic goat (Feng *et al.* 2010).

Four SNPs (G3288A in intron 1, G423A, A959C and G1189A in exon 2) including the 2 reported by us (A959C and G1189A) were detected in 4 goat breeds of China with

Table 5. SNPs in exon 2 of caprine GDF9 gene

Region	Coding base (bp)	Base change	Genotype frequency	Amino acid change
Exon 2	818	C-T	CC=0.86CT=0.083 TT=0.055	A273V
	959	A-C	AA=0.83AC=0.16	Q320P
	1189	G-A	GG=0.86GA=0.13	V397I

different prolificacy (Feng *et al.* 2010). PCR-RFLP of these loci was done using restriction endonucleases SspI, PvuII, MspI and MvaI, respectively. At 1189 locus, G to A transition expressed 3 genotypes: GG, GA and AA. Transversion (A to C) at 959 locus expressed 3 genotypes: AA, AC and CC and allele C showed significant correlation with high litter size in Jining grey goats. Proportion of animals with favourable C allele (A959C) was highest in the prolific Black Bengal goat in our results. Thus A959C SNP should be explored as the potential marker associated with fecundity in Indian goats. Wu *et al.* (2006) found 2 single nucleotide polymorphisms in exon 2 of GDF9 gene in Jining Grey, Boer, Wendeng dairy, Liaoning Cashmere and Beijing native goats by PCR-SSCP,

which were G423A and G1189A, and the latter was also detected in Guizhou White goats by Du *et al.* (2008) and in Yangtse River Delta White, Huanghuai and Boer goats by Zhang *et al*. (2008). Furthermore, in the results of Zhang *et al*. (2008) mutation A959C was detected in Yangtse River Delta White, Huanghuai and Boer goats, mutation C818T was detected in Yangtse River Delta White goat, and G1189A was detected in Yangtse River Delta White and Boer goats. Ran *et al.* 2009 found 4 polymorphic sites from GDF9 mature peptide (V18A, P78Q, V79I and T81A) in Guizhou White goats. Thus all the 3 SNPs identified in the indigenous goats have also been reported in a number of goat breeds in the world.

BLAST analysis was carried out to find the % homology in the DNA sequence of Indian goats with other species. Coding DNA sequences (CDS) of *GDF9* gene revealed homology of 97% of goat sequence with *Bos taurus,* 99% with *Ovis aries* and *Capra hircus*. Homology of *GDF9* sequences among cattle, buffalo, sheep and goat are especially higher than other mammals, which coincide with the fact that 4 species belong to domestic ruminants and are evolutionary more related. Phylogenetic analysis of *GDF9* gene following UPGMA algorithm revealed that domestic animals and murines form distinct clusters (Fig. 2) and small ruminants (sheep and goat) were closer to large ruminants (cattle and buffalo) as compared to pig, horse, human and cat. Chicken and zebra fish were found to be distinct from domestic animals. In silico translation of coding DNA sequence of *GDF9* gene in caprines revealed a sequence of 454 amino acids. Five amino acid changes were observed between Indian goat and sheep while 21 such changes were found between Indian goat and cattle. As expected, the similarity between Indian goat and exotic goat was 99.8% with only one amino acid different between them.

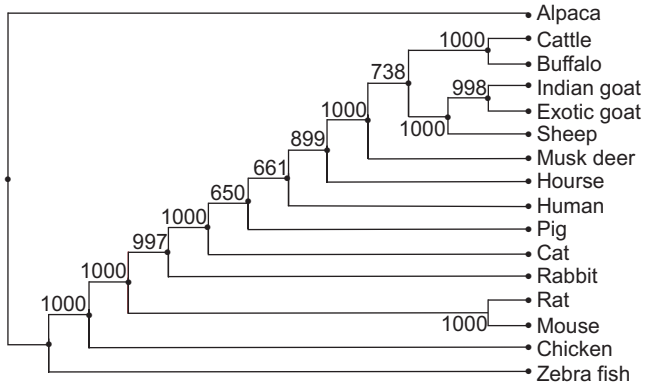

Fig 2. Phylogenetic analysis of *GDF9* coding DNA sequence of different species following UPGMA algorithm

A panel constituting 6 breeds of indigenous goats differing in prolificacy and geographic distribution was utilized for PCR-direct DNA sequencing analysis of *GDF9* gene. The present study provided sequence of two exons of the caprine *GDF9* gene, as well as polymorphisms of *GDF9* in six Indian goat breeds. The polymorphism scanning of *GDF9* gene uncovered 3 potentially meaningful SNPs in the investigated goat breeds have also been reported in several other goat breeds around the world. Since one of the SNP that we identified (A959C) has already been associated with high litter size in Jining grey goats, further studies are necessary for function validation of these polymorphisms with the fecundity traits in Indian goats.

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