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Full Length Research Paper

Polymorphism, sequencing and phylogenetic characterization of growth differentiation factor 9 (*GDF9*) gene in Assam Hill goat

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Assam hill goat (*Capra hircus*) is a prolific local goat in India. Growth differentiation factor (*GDF9*) gene was studied as a candidate gene for the prolificacy of goats. The objective of the present study was to detect the incidence of mutation in the exonic region of *GDF9* gene of Assam hill goat. Total number of 90 blood samples were collected randomly from different parts of Assam and genomic DNA were extracted using modified phenol-chloroform method. The quantity and quality of extracted DNA was examined using spectrophotometry and gel electrophoresis, respectively. A 294 bp fragment of GDF9 gene was amplified using polymerase chain reaction (PCR). The purified product was digested with *Dde*I, *Hha*I and *Alu*I restriction enzymes which produced single type of banding pattern 242 and 52 bp, 140 and 154 bp, 134 and 160bp, respectively. The present study revealed wild type alleles and all the samples showed AA genotype. Nucleotide sequencing revealed two new mutations 495 (C → A) and 387 (G → A). Phylogenetic analysis showed that the sequences of Assam Hill goat belong to a common cluster which differs from that of the other goat breeds. The analysis of polymorphism for *GDF9* in Assam Hill goat indicates that the genetic factor responsible for prolificacy or multiple kidding rate is not related to the reported mutated alleles of *GDF9 gene.* Therefore, attempts to be made to detect other single nucleotide polymorphism (SNPs) for *GDF9* gene or otherwise effort should be made towards other fecundity gene which might be responsible for the prolificacy of Assam Hill goat.

Key words: Assam Hill goat, GDF9, polymorphism, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

INTRODUCTION

Growth differentiation factor 9 (*GDF9*) belongs to the transforming growth factor-b superfamily. In female reproduction, growth and differentiation factor plays a critical role during early folliculogenesis in mammals (Elvin et al., 1999). The *GDF9* gene in sheep has been mapped to chromosome 5 between the BM7247 and BMS2258 markers (Sadighi et al., 2002). The expression of *GDF9* was oocyte-specific in ovine and bovine ovaries which begin at the primordial follicle stage (Bodensteiner et al., 1999). *GDF9* mRNA and protein were expressed in

Table 1. Primers (Forward and reverse) along with the properties for amplification of 294 bp of GDF9 gene.

Primer	Primer sequence (5'-3')	Molecular weight	Tm (°C)	Length (mer)	GC content (%)	Size of amplicon (bp)	Reference
Forward	GTTGGAATCTGAGGCTGAG	5923.9	54.18	19	52.6	294	Chu et al., 2011
Reverse	ATCTGCTCCTACACACCTG	5683.7	51.83	19	52.6		

follicles caprine ovary at all stages of their development, and additionally in luteal tissue (Silva et al., 2004). *GDF9* gene mutations in sheep may cause either an increased ovulation rate or infertility. In sheep, four different mutations of *GDF9* had been identified which affect fertility including FecGH (G8) mutation in Cambridge and Belclare sheep (Hanrahan et al., 2004), in Santa Ines sheep FecGE or FecGSI mutation (Melo et al., 2008; Silva et al., 2010), FecTT mutation in Thoka sheep (Nicol et al., 2009). In Moghani and Ghezel sheep (Barzegari et al., 2010) and in Garole sheep (Polley et al., 2010), G1 mutation was found.

Genetic mutation of *GDF9* identified in sheep which showed major effect on ovulation rate (Knight and Glister, 2003; Knight and Glister, 2006). In growing ovarian follicles *GDF9* growth factor is secreted and plays role in growth and differentiation of early ovarian follicles (McPherron and Lee, 1993). *GDF9* homozygous mutant animals are found to be anovulatory whereas animals heterozygous for *GDF9* have higher than normal ovulation rate (Chu et al., 2005). This shows that *GDF9* is essential for normal folliculogenesis in sheep. So, *GDF9* was an obvious candidate gene with a major effect on litter size in sheep (Davis, 2005), *GDF9* may be a potential major gene on litter size in goats. However, the literatures concerning *GDF9* gene and goat reproduction are relatively rare.

The population of goat in the world was approximately 861.9 million (FAOSTAT, 2008) and India holding the second position with 125.7 millions goat. In India, mainly the landless farmers and small size holders maintain goats, which provide a dependable source of income to most of the rural population who are below the poverty line. Improvement of reproductive traits in livestock species has become of increasing interest, where small increases in litter size can equal large gains in profit. Genetic improvement of reproductive traits has traditionally been restricted to use of quantitative genetic methods but gain has been limited when using these methods. Provided that the major genes associated with reproduction are identified, they can be utilized in breeding through marker-assisted selection (MAS). Reproductive traits are often suggested as prime targets for MAS for their low heritability and the fact that the trait can be measured only in one sex (Ghaffari et al., 2009).

The Assam Hill goat which is known for its high prolificacy has kidding records varying from single to quadruplet. Their average litter size at birth was reported to be 1.41 (Gogoi, 1987). They are found mostly in the hilly regions of Assam and its adjoining areas. Even though the Assam Hill goat is considered as one of the most prolific goat in India, so far no attempts have been made to carry out research work on the fecundity gene, at molecular level, which may be responsible for prolificacy in Assam Hill goat. Therefore, the present investigation was designed to study the polymorphism of GDF9 as important fecundity gene in Assam hill goats.

MATERIALS AND METHODS

Blood collection and DNA extraction

Total of 92 samples Assam Hill goats were collected based on their history of litter size. Goats were obtained from different parts of state Assam, India. Out of this, 50 samples were twins, 10 singlet, 20 triplet and 12 quadruplets. 5 (five) ml of blood sample was collected aseptically from jugular vein of each goat using 0.5 ml of EDTA (ethylene diamine tetra acetate, 0.5 M, pH = 8) as an anticoagulant. The samples were brought to the laboratory in double walled ice-boxes containing icepacks and stored at -20°C until the genomic DNA was extracted. Genomic DNA of goat was extracted by phenol-chloroform extraction procedure (Sambrook and Russel, 2001).

Polymerase chain reaction (PCR) amplification and gel electrophoresis

A 294 bp region of GDF9 gene was amplified by using a set of forward (5′-GTTGGAATCTGAGGCTGAG-3′) and reverse (5′-ATCTGCTCCTACACACCTG-3′) primers (Table 1). PCR reaction was performed in thermocycler (Applied Biosystem, USA) in 25 µl reaction volume containing 40 ng of each primers, 200 µM dNTP mix, 1.5 nM $MgCl_2$, 2.5 mM of 10X buffer, 100 ng DNA template and 1U Taq DNA polymerase (MBI, Fermentas). The PCR cycling parameters were optimized as follows: Initial denaturation at 94°C for 5 min, followed by 94°C for 30 s, 46°C for 30 s, 72°C for 30 s for 35 cycles and final extension at 72°C for 5 min. The PCRproducts were separated by horizontal submarine agarose gel (2%, free from DNAse and RNAse) electrophoresis in 0.5X TBE buffer at 110

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Abbreviations: *GDF9*; Growth differentiation factor 9; **MAS,** marker-assisted selection; **PCR,** polymerase chain reaction; **RFLP,** restriction fragment length polymorphism; **SSCP,** single strand conformation polymorphism.

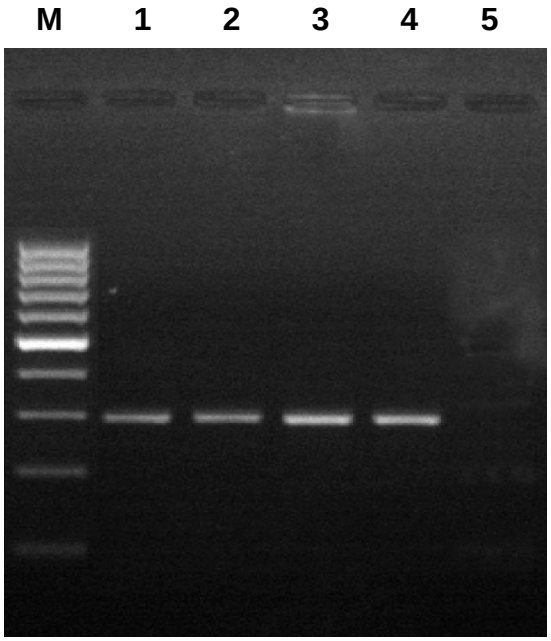

Figure 1. PCR amplicon of *GDF9* gene (294 bp). L1-L4, PCR amplicons of *GDF9* gene of Assam Hill goat; L-5, negative control; M, marker 100 bp.

V and visualised using a gel documentation system (Gel Logic 100, KODAK).

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis

PCR products of *GDF*9 gene were used for restriction enzyme digestion by *Dde*I, *Hha*I and *Alu*I at 37°C for 3 h. The restriction enzyme digested products were resolved in 2.5% (w/v) agarose gel in 0.5X TBE buffer. 10 µl of RE digested product along with 5 µ of 6 x gel loading dye, after mixing was loaded in well. A 50 and 100 bp marker ladder was also run alongside the samples to ascertain the size of the amplified products. Electrophoresis was carried out at 110 V for 1.15 h. The digested products were visualized under UV light on a trans-illuminator and the gels were recorded in a gel documentation system.

Sequencing and phylogenetic analysis

PCR amplicon of *GDF9* gene were sequenced at South Campus, Department of Biochemistry, University of Delhi by automated DNA sequencer (ABI Genetic Analyser) following Sanger's dideoxy chain termination method (Sanger et al., 1977). The sequences were analysed by using Clustal W method of DNASTAR Software (Lasergene, USA) and MEGA 5 Software to generate sequence alignment reports, sequence distance, residue substitution and phylogenetic analysis.

RESULTS

Concentration of extracted DNA and purity

After quantification of each DNA sample, a uniform final concentration of 100 ng/µl was prepared by dilution of the entire sample in 0.5M Tris EDTA (TE) buffer.

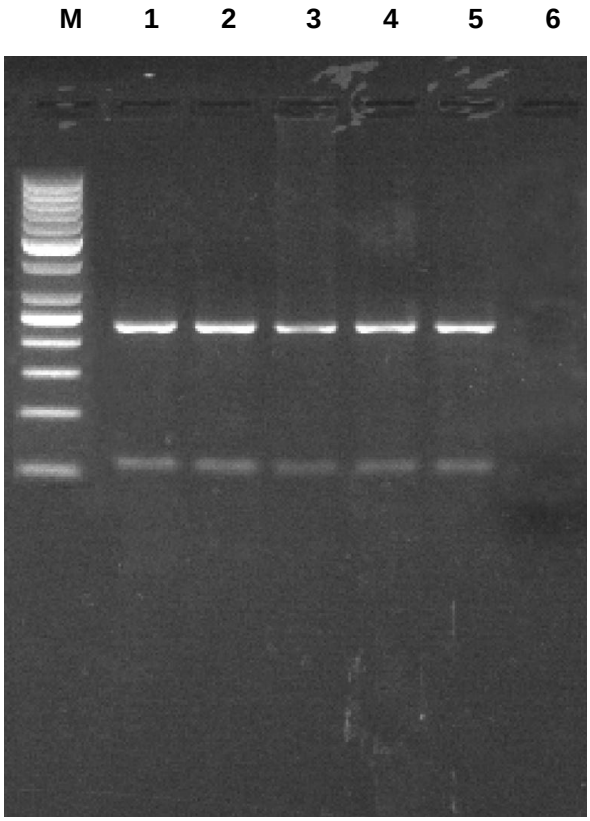

Figure 2. PCR-RFLP of *GDF9* gene using *Dde*I (242 and 52 bp). L1- L5, digested products (252 and 52 bp); M, Marker 50 bp.

Restriction fragment length polymorphism analysis

In order to determine the polymorphism, if any in amplified fragment (Figure 1) of *GDF9* gene, restriction enzyme *Dde*I, *Hha*I and *Alu*I having recognition site of (C↓TNAG), (C↓CGG) and (AG↓CT) were used to digest 294 bp fragment. In the present study, a total of 92 Assam hill goats were genotyped with PCR-RFLP approach. All 92 Assam hill goats were screened for *Dde*I, *Hha*I and *Alu*I enzyme digestion followed by agarose gel electrophoresis revealed single type of restriction pattern consisting two fragments. *Dde*I digestion produced two fragments approximately 242 and 52 bp (Figure 2). Similarly, *Hha*I gave two fragments of 140 and 154 bp (Figure 3) and *Alu*I cut the amplicon of 294 bp into two fragments measuring approximately 134 and 160 bp (Figure 4), respectively. The genotype was deduced to be homozygous normal wild type in all the animals and suggest that in spite of totally different breeds of European and Indian origin, no new site was created for the enzymes in *GDF9* gene.

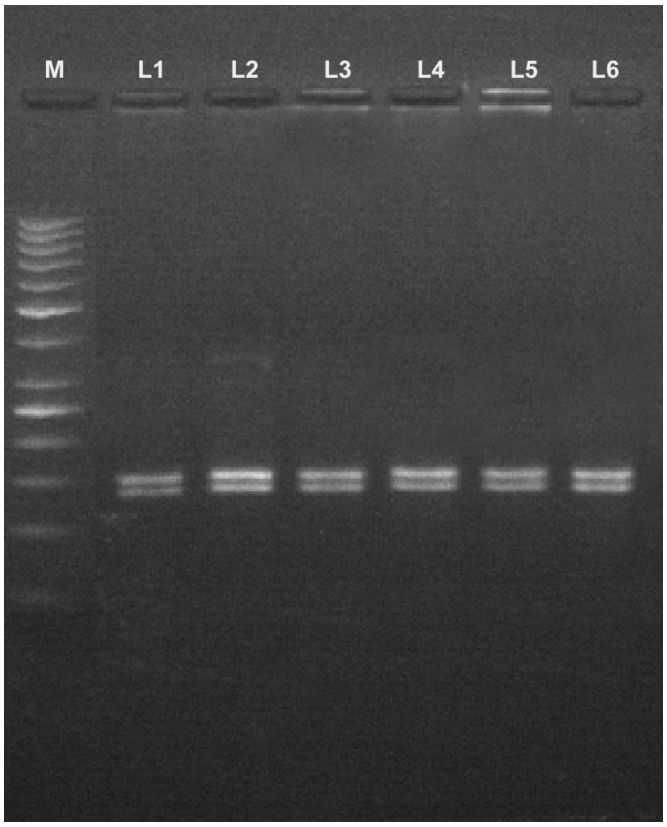

Figure 3. PCR-RFLP of *GDF9* gene using *Hha*I (154 and 140 bp). L1-L6, Digested products (154 and 140 bp); M, marker 50 bp.

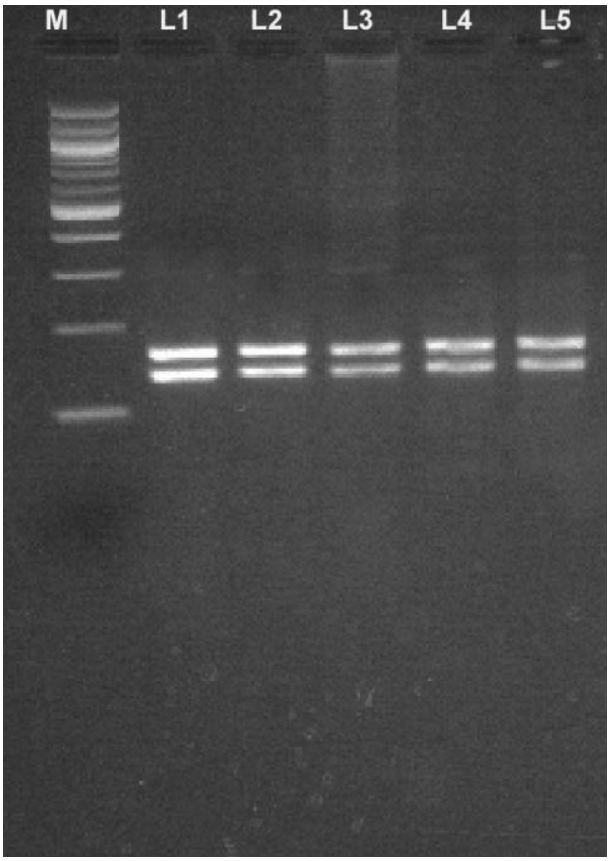

Figure 4. PCR-RFLP of *GDF9* gene using *Alu*I (160 and 134 bp). L1-L5, Digested product of (160 and 134 bp); M, marker 100 bp.

Nucleotide sequence comparison

The sequence of same region was analyzed. The nucleotide sequences (NCBI accession no. JX483872) of our experiment were aligned and compared using DNAstar software (USA). Eight published sequences of *GDF9* gene of other breeds of goat viz. Barbari (NCBI accession no. HM462265), Black Bengal (NCBI accession no. FJ665810), Ganjam (NCBI accession no.JN601041), Jaminapari (NCBI accession no.HM462268), Lezhi Black (NCBI accession no.JF824149), Osmanabadi (NCBI accession no.HM462267), Sirohi (NCBI accession no.JN680860) and Tibetan (NCBI accession no. JN100108) *GDF9* genes were obtained. All the nucleotides of *GDF9* genes obtained in the present study showed 97-100% similarity with the other goats. Similarity study of *GDF9* gene of Assam Hill goat showed that it has similarity of 97.0, 97.4, 97.4, 97.4, 95.3, 97.4, 97.0 and 97.0% with Barbari, Black Bengal, Ganjam, Jaminapari, Lezhi Black, Osmanabadi, Sirohi and Tibetan goats, respectively (Figure 5).

The partial sequences of *GDF9* gene of Assam Hill goat were aligned to the position of 260 to 495 bp of the complete *GDF9* gene sequence of Sirohi goat (NCBI accession no. JN680860). At position 495 (C > A) and at 387 (G > A), nucleotide substitution were found in all the sequences of Assam Hill goat, which was found to be unique in comparison to the other sequences. In one sequence of Assam Hill goat, an insertion of nucleotide ‘A’ was found at position 268, which might be an individual variation. The Assam Hill goat having insertion of nucleotide “A” at position 268 showed remarkable change in amino acid sequence. None of the ten SNPs identified so far in sheep *GDF9* gene including four mutations (FecGH, FecGE, FecTT and G1) associated with fecundity (Table 2) could be identified in Assam Hill goats.

Phylogenetic analysis

The nucleotide sequenced of GDF9 gene of Assam hill goat used for construction of phylogenetic tree along with published sequences of this gene. Phylogenetic analysis revealed that the sequences Assam Hill goat belongs to a commons cluster which differ with that of the other goat

Percent Identity

	1	2	3	4	5	6	7	8	9	10	11	
1		97.9	97.0	97.0	98.0	99.8	99.9	99.8	99.6	99.9	97.0	1
2	2.2		97.4	96.6	99.9	98.4	99.8	98.3	97.5	99.8	97.4	2
3	2.6	2.2		99.6	97.4	97.4	95.3	97.4	97.0	95.3	100.0	3
4	2.6	2.2	0.0		96.6	96.6	94.5	96.6	96.2	94.5	99.6	4
5	2.1	0.1	2.2	2.2		98.5	99.9	98.4	97.6	99.9	97.4	5
6	0.1	2.1	2.2	2.2	2.0		99.9	100.0	99.8	99.9	97.4	6
7	0.1	0.2	0.0	0.0	0.1	0.1		99.9	99.9	99.9	95.3	7
8	0.1	2.1	2.2	2.2	2.0	0.0	0.1		99.8	99.9	97.4	8
9	0.1	2.2	2.6	2.6	2.0	0.1	0.1	0.1		99.9	97.0	9
10	0.1	0.2	0.0	0.0	0.1	0.1	0.1	0.1	0.1		95.3	10
11	2.6	2.2	0.0	0.0	2.2	2.2	0.0	2.2	2.6	0.0		11
	1	2	3	4	5	6	7	8	9	10	11	

Divergence

1 gdf9 Barbari Goat
2 gdf9 Black Bengal
3 gdf9 g1 Assam Hill Goat
4 gdf9 g2 Assam Hill Goat
5 gdf9 Ganjam Goat
6 gdf9 Jamnapari Goat
7 gdf9 Lezhi Black Goat
8 gdf9 Osmanabadi goat
9 gdf9 Sirohi Goat
10 gdf9 Tibetan Goat
11 gdf9 G3 Assam Hill Goat

Figure 5. Percent identity and divergence of *GDF9* gene.

Table 2. Major mutations of *GDF9* gene identified in sheep.

	Base change	Coding base (bp)	Coding residue (amino acid)	Mature peptide residue (amino acid)	Amino acid change	Reference
G1	G→A	260	87	-	Arg→His	Hanrahan et al. (2004)
G2	C→T	471	157	-	Unchanged Val	-
G3	G→A	477	159	-	Unchanged Leu	-
G4	G→A	721	241	-	Glu→Lys	-
G5	A→G	978	326	8	Unchanged Glu	-
G6	G→A	994	332	14	Val→Ile	-
G7	G→A	1111	371	53	Val→Met	-
G8	C→T	1184	395	77	Ser→Phe	-
FecGSI	T→G	1034	345	27	Phe→Cys	Melo et al. (2008)
FecTT	A→C	1279	427	109	Ser→Arg	Nicol et al. (2009)
A152G	A→G	152	51	-	Asn→Asp	Li et al. (2003)
T692C	T→C	692	231	-	Leu→Thr	Gao (2007)

breeds when clustering was done at 1.2 x 100 nucleotide substitution. The close proximity of location of GDF9 gene in the same clade of tree indicated the descendent of the genes in those groups from a common ancestor (Figure 6).

DISCUSSION

Nucleotide substitution from G to A at position 387 show that the predicted change in amino acid is observed to be glycine (G) to aspartic acid (D) at position 129 in GDF9 protein. Li et al. (2003) identified one single nucleotide mutation (A152G) of GDF9 gene in Hu, Dorset and Suffolk sheep by PCR-single strand conformation polymorphism (SSCP), which resulted in an amino acid change Asn51Asp. Hanrahan et al. (2004) reported eight DNA variants in *GDF9* of Cambridge and Belclare sheep including G1 to G8. Out of these eight polymorphisms, three nucleotide changes did not alter amino acids (G2, G3 and G5). Four G >A mutations of the eight SNPs resulted in amino acid changes (G1, G4, G6 and G7)

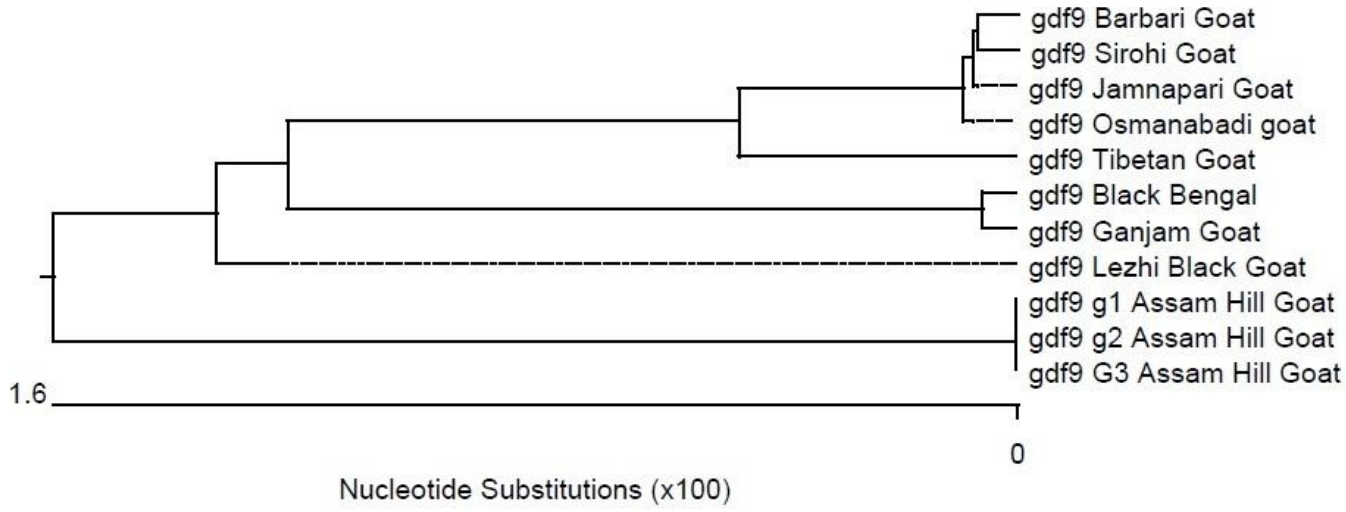

Figure 6. Phylogenetic tree of *GDF9* gene.

which occurred at position before the furin processing site or unprocessed protein and were unlikely to affect the mature active coding region. However, G8 variant also known as FecGH caused serine to phenylalanine at residue 395 which replaced an uncharged polar amino acid with a non polar one at residue 77 of the mature coding region and may change the function GDF9 in sheep. Recently, Melo et al. (2008) and Silva et al. (2010) detected FecGE mutation (c.1024T>G resulting in p.F345C) in *GDF9* gene of Brazilian Santa Ines sheep. Another new mutation (c.1279A>C, named as FecTT) was found in *GDF9* gene of Icelandic Thoka sheep resulting in a non-conservative p.S109R in the C-terminus of the mature *GDF9* protein (Nicol et al., 2009). Three mutations (c.423G>A, c.959A>C [p.Q320P] and c.1189G>A [p.V397I]) in exon 2 of *GDF9* gene had been detected extensively in several goat breeds, in which mutation c.423G>A was detected in Jining Grey, Liaoning Cashmere and Boer goats (Wu et al., 2006; Feng et al., 2010), Wendeng Dairy and Beijing native goats (Wu et al., 2006) and Guizhou White goats (Feng et al., 2010); mutation c.959A>C was found in Yangtse River Delta White and Huanghuai goats (Zhang et al., 2008), Jining Grey, Liaoning Cashmere and Guizhou White goats (Feng et al., 2010) and Boer goats (Zhang et al., 2008; Feng et al., 2010); mutation c.1189G>A was identified in Jining Grey, Liaoning Cashmere and Boer goats (Wu et al., 2006; Feng et al., 2010), Guizhou White goats (Du et al., 2008; Feng et al., 2010), Wendeng Dairy and Beijing native goats (Wu et al., 2006; Feng et al., 2010).

Conclusion

In the present study, the results showed monomorphic banding pattern in all the studied samples of Assam Hill goat with respect to *GDF9* gene. Regarding the records of high prolificacy in Assam Hill goat, it is concluded that probably the genetic factor controlling twinning and triplet is not related to the mutation which is reported in the *GDF9* gene. It may be concluded that probably the litter size in Assam Hill is either not affected by major genes or it is possible that other SNP in the *GDF9* gene or some other major genes may be controlling the prolificacy in Assam Hill goat.

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