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Research Article

PCR-SSCP ANALYSIS OF GROWTH DIFFERENTIATION FACTOR-9 GENE OF GAROLE SHEEP

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ABSTRACT: Growth differentiation factor-9 (GDF-9), one of the oocyte-derived members of the transforming growth factor - β super family has played an imperative role for follicular growth and ovulation. The present study was to investigate the polymorphism of exon-1 of GDF-9 gene in the most prolific Garole sheep breed. Blood samples were collected from 28 ewes and genomic DNA was extracted using the modified high salt method. The 462 bp amplified PCR product was analyzed for polymorphism by SSCP method. The results indicated two different banding patterns AA and AB for this fragment. The frequencies of AA and AB genotypes were 0.857 and 0.143. The allelic frequencies of A and B alleles were 0.928 and 0.072. The Calculated Chi-square value for exon-1 of GDF-9 gene (0.032) was found to be lesser than that of tabulated values at 5% and 1% level of significance indicating that the population under study was in Hardy-Weinberg Equilibrium. The 462 bp nucleotide sequence was subjected to BLAST analysis. Phylogenetic analysis revealed that nucleotide sequence of studied sample of Garole sheep is 100 percent similar with the published sequence of sheep and form a common cluster indicating their evolutionary closeness.

Key Words: GDF- 9, Polymorphism, Sheep, SSCP, Sequencing.

INTRODUCTION

Growth differentiation factor-9 (GDF-9) belongs to the transforming growth factor, ß super family. It plays a critical role in growth and differentiation during early folliculogenesis of mammalian female reproduction (Elvin *et al.*, 1999). GDF-9 was mapped to ovine chromosome 5 with a span of approximately 2.5 kb contains two exons and one intron (Sadighi *et al.*, 2002). The exon-1 spans 397 bp while exon- 2 spans 968 bp. The single intron spans 1,126 bp. The mutations in this gene cause increased ovulation rate and twin and triplet births in heterozygotes, and complete primary ovarian failure in homozygotes resulting in total infertility in some prolific breeds of sheep (Hanrahan *et al.*, 2004). So, characterization of fecundity gene GDF-9 in sheep could substantially improve the breeding programme in sheep breeds of the region. Keeping in view this aspect, the present study was envisaged to investigate the polymorphism in exon-1 of

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GDF-9 gene in most prolific Garole Sheep using PCR-SSCP technique.

MATERIALS AND METHODS

Collection of Blood Samples

A total of twenty eight ewes of Garole sheep were selected at random from the Sheep and Goat breeding Farm of West Bengal University of Animal and Fishery Sciences maintained at Mohanpur, Nadia,West Bengal. The blood samples (10 ml) were collected aseptically from jugular vein of each animal in a 15 ml disposable polypropylene centrifuge tube containing an amount of 300-400 µl of 10 percent EDTA as anticoagulant and the samples were stored at 4°C till further processing for the DNA isolation.

Genomic DNA Extraction

Genomic DNA samples of 28 Garole sheep were isolated from their blood samples using modified high-salt method (Miller *et al.*, 1988). The quality of the isolated DNA was checked on 0.8 percent agarose gel and almost all these samples showed a single genomic DNA band. From 10 ml of blood samples approximately 200-300 mg of genomic DNA was isolated and kept at -20^{0}C for further study.

PCR Amplification

Genomic DNA samples of 28 Garole sheep were adjusted to a concentration of 50 ng/µl and exactly 2.5 µl of the DNA samples were used as template for polymerase chain reaction (PCR). Amplification procedure for exon-1 of GDF-9 gene of Garole ewes has been standardized which yielded consistent and specific amplification. Genomic DNA was amplified using primer sequences (F: 5'-GAAGACTGGTATGGGGAAATG-3' and R: 5'- CCAATCTGCTCCTACACACCT-3') as described by Hanrahan *et al.* (2004). The amplification reaction conditions was carried out using 32 cycles at 94°C for 5 min., followed by 94°C for 45s, 56°C for 60s, 72°C for 60s, followed by 72°C for 10 min. The amplified products were consistent with the target fragments and had a good specificity on 2.5 percent agarose gel electrophoresis, which could be directly analyzed by SSCP.

Single-Strand Conformation Polymorphism (SSCP)

The study of sequence variation in exon-1 region of GDF-9 gene was achieved by Single Strand Conformation Polymorphism as described by Orita *et al.* (1989) with minor modifications. About 4 µl PCR product and 12 µl SSCP loading buffer dye were taken in a 0.5 ml tube. Sample is denatured at 95ºC for 6 min. After denaturation it is kept immediately in ice for 6 minutes to make it single strand DNA for its conformation. The denaturated PCR products were run in a non-denaturing 8% poly-acrylamide gel for 6-8 hours at a constant volt (70 V) in room temperature. After electrophoresis, SSCP gels are fixed and stained in a solution containing 10% ethanol, 0.5% acetic acid, and 0.2% silver nitrate as per the method described by Bassam *et al.* (1991) with minor modifications to identify the DNA sequence variations. The silver stained gel was kept on trans-illuminator and SSCP variants were recorded for genotyping of the animals.

DNA Sequence Analysis

PCR products showing different banding patterns on SSCP gel were selected for sequencing. Primers for sequencing were the same as those used for PCR-SSCP

amplification. Same forward and reverse primers were used for sequencing to detect variations at the nucleotide level. Sequencing was done by automated sequencer (ABI prism) using Sanger's dideoxy chain termination method at GCC Biotech (India) Pvt. Ltd., Kolkata, West Bengal, India. The sequences obtained were screened first by BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST) to ascertain that sequences were of GDF-9 gene. The sequences were then aligned with that of the reported GDF-9 sequences of different species using Meg Align Programme of Lasergene Software (DNASTAR). Phylogenetic analysis with related species was also performed to determine the evolutionary relationship.

Statistical Analysis

Different genotypes were scored on the basis of PCR-SSCP banding in the gel. The following parameters were obtained: the genotype categories and the gene frequency of the alleles that corresponded to the exon-1 of GDF-9 gene. The frequency of exon-1 of GDF-9 allele and genotypes were estimated using standard procedure of Falconer and Mackay (1996).

RESULTS AND DISCUSSION

PCR Amplification

Genomic DNA samples of 28 Garole sheep were used as template for PCR. Amplification procedure for exon-1 of GDF-9 gene of Garole ewes were standardized which yielded consistent and specific amplicon. The amplification of exon-1 of GDF-9 gene resulted in a product size of 462 bp length on 2.5 percent agarose gel electrophoresis when visualized under the UV transilluminator in all the samples of Garole ewes (Fig. 1). The amplified fragment of exon-1 of GDF-9 gene of Garole sheep was assigned on the basis of the published reports of this gene in sheep.

The amplification of exon-1 of GDF-9 gene resulted in a product size of 462 bp in all the samples of Garole ewes of this University farm in the present study and was in good agreement with the result reported by Hanrahan *et al.* (2004) in Belclare and Cambridge sheep. Similar findings were also reported by Javanmard *et al.* (2011) in fat tailed Sheep breeds reared in Iran.

PCR-SSCP Analysis

PCR-SSCP technique was used to identify genetic variants of exon-1 of GDF-9 gene in a total of 28 Garole ewes. The results revealed two SSCP variants for PCR products in 8% poly acrilamide gel, which were arbitrarily assigned as AA and AB type of variants (Fig. 2). The AA pattern showed two bands whereas AB pattern shows four bands. Researchers earlier had already used the PCR-SSCP technique successfully to study the genetic variants of GDF-9 gene in Mehraban sheep (Abdoli *et al.*, 2013) and Guizhou White goat (Ran *et al.*, 2009).

It was found that out of 28 studied Garole ewes, 24 animals had AA genotypes and only 4 animals had AB genotypes. The research finding of this present investigation revealed that 85.71 percent of studied animals had AA genotype while only 14.29 percent animals had AB genotype. The present finding revealed higher frequency of AA genotype followed by AB genotype for exon-1 of GDF-9 gene in Garole ewes. However, among the animals examined, BB genotype could not be detected. This finding was in agreement with those of Ghaderi *et al.*, (2010), who also reported rare

occurrence of homozygote BB in Kordi sheep and Arabic sheep. Two genotypes (GG and GA) of Garole sheep for exon-1 of GDF-9 gene was also reported by Polley *et al*. (2009). However, Roy *et al*. (2011), Moradband *et al*. (2011) and Aboli *et al*. (2013) reported three distinct genotypes in Bonpala, Baluchi and Mehraban ewes respectively.

Genetic Structure

The present findings revealed a higher frequency of wild type AA genotype followed by AB genotype for exon-1 of GDF-9 gene in this studied population of Garole sheep. The allelic frequencies of A and B alleles were 0.928 and 0.072, respectively. This finding was in conformity with earlier observation of Abdoli *et al*. (2013) in Mehraban sheep breed. This finding was also in accordance with those of Hanrahan *et al*. (2004) in Cambridge and Belclare sheep breeds. These results were in conformity with those of Polley *et al*. (2010) and Roy *et al*. (2011) who also reported the highest frequency of wild type GG genotype in Indian sheep. Likewise, wild type GG genotype was the most frequent for Iranian sheep (Abdoli *et al.*, 2013) and also among Chinese sheep (Shi *et al.*, 2010).

However, there were reports, where GDF-9 variant A showed a higher frequency over variant G in Belclare and Cambridge sheep (Hanrahan *et al.*, 2004). Because of the effects of genetic variants of GDF-9 gene on adaptation, selection of animals with the favourable GDF-9 G allele is considerable for proper adaptation in the farm. However, among the animals examined, mutant genotype BB could not be detected. This finding was in agreement with those of Polley *et al*. (2010), who also reported a rare occurrence of homozygote mutant BB genotype in Garole sheep breed. The absence of mutant BB genotype in the population studied, however, might be due to limited sample size. Chi square test was used and indicated that the population followed Hardy-Weinberg equilibrium (χ^2 =0.032), so low genetic diversity in this population was observed. Hence the population under study was found to be in Hardy-Weinberg Equilibrium with respect to Exon-1 of GDF 9 gene. Similar findings were reported also by Ghaderi *et al*. (2010) in Arabic sheep breed and Kordi sheep breed. In studies of genetic

Table 1: Blast analysis of Garole sheep with other closely related species.

Accession No.	Species	Identity
FJ529501.2	*Bubalus bubalis*	96%
GQ922451.1	*Bos taurus*	97%
HN462265.1	*Capra hircus* (Barbari)	99%
JN680860.1	*Capra hircus* (Sirohi)	99%
HM462256.1	*Capra hircus* (Jakhrana)	99%
HM462267.1	*Capra hircus* (Osmanabadi)	99%
JN601040.1	*Capra hircus* (Black Bengal)	99%
HM462268.1	*Capra hircus* (Jamunapari)	99%
JN601041.1	*Capra hircus* (Ganjam)	99%
HE866499.1	Norwegian white sheep	100%
AF078545.2	Reference Sheep	100%

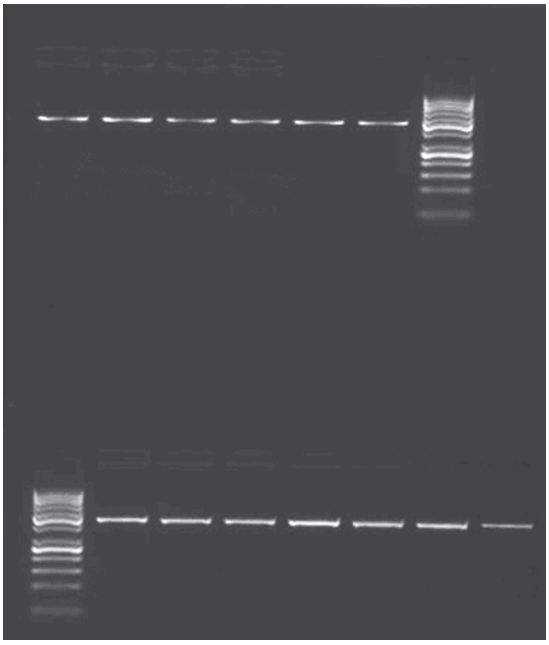

Fig. 1: Resolution of Exon 1, PCR Product on 2.5% agarose gel.

characterization of Garole sheep breeds, it was found that the wild type A of exon-1 of GDF-9 gene occurred at higher frequencies in breeds originating from Asian countries than in those of breeds originated in European countries origin.

Sequence Analysis

PCR products corresponding to each of the different PCR-SSCP alleles/patterns were selected and sequenced using the forward and reverse primer to detect variations at the nucleotide level. The sequence obtained was subjected to BLAST www.ncbi.nlm.nih.gov/BLAST) analysis to ascertain that sequences were of GDF-9 gene. Single stranded DNA (ssDNA) usually yielded two bands for homozygote AA and four for heterozygote AB genotype of Garole sheep ewes of this

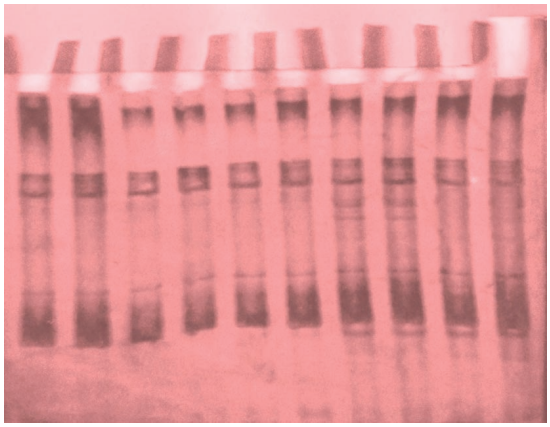

Fig. 2: Different SSCP Patterns (AA and AB Genotypes) of exon 1of GDF-9 gene.

University farm. Nucleotide sequences of these two groups of ewes revealed that 462 bp and 464 bp (Fig. 3) respectively.

Multiple Sequence Alignment

Sequencing of exon-1 and partial portion of intron of 462 and 464 bases for these two SSCP variants revealed that nucleotide sequence variations at different places as indicated in the MegAlign Clustal W Multiple Sequence Alignment. A close look at Clustal W Multiple Sequence Alignment results showed that there were two differences in AB SSCP variants (Fig. 3). Two insertion mutation of nucleotides were observed at position 38(R=A/G) and 420(C) of nucleotide sequence of B allele although such was remain absence in reference nucleotide sequence (AF078545.2). Hence, further extensive study based on a large number of observations may be taken up in future for validity of this present finding.

Basic Local Alignment Search Tool (BLAST) Analysis

The nucleotide sequences obtained for exon-

Fig. 3: Multiple Sequence Analysis of A Allele and B Allele of Exon 1 of GDF9 Gene of Garole sheep

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GAAGACTGGTATGGGGAAATGTGTTCCTTGCTAATTC-TTCCAAGCCATG Majority
          10        20        30        40        50
1 GAAGACTGGTATGGGGAAATGTGTTCCTTGCTAATTC-TTCCAAGCCATG A Allele
1 GAAGACTGGTATGGGGAAATGTGTTCCTTGCTAATTCRTTCCAAGCCATG B Allele
1 GAAGACTGGTATGGGGAAATGTGTTCCTTGCTAATTC-TTCCAAGCCATG Black Bengal Goat
1 -------------------GTGTTCCTTGCTAATTC-TTCCAAGCCATG Bos taurus
1 GAAGACTGGTATGGGGAAATCTGTTCCTTGCTAATTC-TTCCAAGCCATG Bubalus bubalis
1 GAAGACTGGTATGGGGAAATGTGTTCCTTGCTAATTC-TTCCAAGCCATG Jamnapari Goat
1 ----------------------------CTAATTC-TTCCAAGCCATG Norwegian white sheep
1 GAAGACTGGTATGGGGAAATGTGTTCCTTGCTAATTC-TTCCAAGCCATG Reference Sheep
1 ----------------------------------------------ATG Bos grunniens

GCGCTTCCCAACAAATTCTTCCTTTGGTTTTGCTGCTTTGCCTGGCTCTG Majority
          60        70        80        90       100
50 GCGCTTCCCAACAAATTCTTCCTTTGGTTTTGCTGCTTTGCCTGGCTCTG A Allele
51 GCGCTTCCCAACAAATTCTTCCTTTGGTTTTGCTGCTTTGCCTGGCTCTG B Allele
50 GCGCTTCCCAACAAATTCTTCCTTTGGTTTTGCTGCTTTGCCTGGCTCTG Black Bengal Goat
30 GCGCTTCCCAACAAATTCTTCCTTTGGTTTTGCTGCTTTGCCTGGCTCTG Bos taurus
50 GCGCTTCCCAACAAATTCTTCCTTTGGTTTTGCTGCTTTGCCTGGCTCTG Bubalus bubalis
50 GCGCTTCCCAACAAATTCTTCCTTTGGTTTTGCTGCTTTGCCTGGCTCTG Jamnapari Goat
20 GCGCTTCCCAACAAATTCTTCCTTTGGTTTTGCTGCTTTGCCTGGCTCTG Norwegian white sheep
50 GCGCTTCCCAACAAATTCTTCCTTTGGTTTTGCTGCTTTGCCTGGCTCTG Reference Sheep
4 GCGCTTCCCAACAAATTCTTCCTTTGGTTTTGCTGCTTTGCCTGGCTCTG Bos grunniens

TTTTCCTATTAGCCTTGATTCTCTGCCTTCTAGGGGAGAAGCTCAGATTG Majority
          110       120       130       140       150
100 TTTTCCTATTAGCCTTGATTCTCTGCCTTCTAGGGGAGAAGCTCAGATTG A Allele
101 TTTTCCTATTAGCCTTGATTCTCTGCCTTCTAGGGGAGAAGCTCAGATTG B Allele
100 TTTTCCTATTAGCCTTGATTCTCTGCCTTCTAGGGGAGAAGCTCAGATTG Black Bengal Goat
80 TTTTCCTATTAGCCTTGATTCTCAGCCTTCTAGGGGAGAAGCTCAGATTG Bos taurus
100 TTTTCCTATTAGCCTTGATTCTCAGCCTTCTAGGGGAGAAGCTCAGATTG Bubalus bubalis
100 TTTTCCTATTAGCCTTGATTCTCTGCCTTCTAGGGGAGAAGCTCAGATTG Jamnapari Goat
70 TTTTCCTATTAGCCTTGATTCTCTGCCTTCTAGGGGAGAAGCTCAGATTG Norwegian white sheep
100 TTTTCCTATTAGCCTTGATTCTCTGCCTTCTAGGGGAGAAGCTCAGATTG Reference Sheep
54 TTTTCCTATTAGCCTTGATTCTCAGCCTTCTAGGGGAGAAGCTCAGATTG Bos grunniens

TAGCTAGGACTGCGTTGGAATCTGAGGCTGAGACTTGGTCCTTGCTGAAC Majority
          160       170       180       190       200
150 TAGCTAGGACTGCGTTGGAATCTGAGGCTGAGACTTGGTCCTTGCTGAAC A Allele
151 TAGCTAGGACTGCGTTGGAATCTGAGGCTGAGACTTGGTCCTTGCTGAAC B Allele
150 TAGCTAGGACCGCGTTGGAATCTGAGGCTGAGACTTGGTCCTTGCTGAAC Black Bengal Goat
130 TAGCTAGGACTGCGTTGGAATCTGAGGCTGAGACTTGGTCCTTGCTGAAG Bos taurus
150 TAGCTAGGACTGCGTTGGAATCTGAGGCTGAGACTTGGTCCTTCCTGAAG Bubalus bubalis
150 TAGCTAGGACCGCGTTGGAATCTGAGGCTGAGACTTGGTCCTTGCTGAAC Jamnapari Goat
120 TAGCTAGGACTGCGTTGGAATCTGAGGCTGAGACTTGGTCCTTGCTGAAC Norwegian white sheep
150 TAGCTAGGACTGCGTTGGAATCTGAGGCTGAGACTTGGTCCTTGCTGAAC Reference Sheep
104 TAGCTAGGACTGCGTTGGAATCTGAGGCTGAGACTTGGTCCTTGCTGAAG Bos grunniens

CATTTAGGTGGGAGACACAGACCTGGTCTCCTTTCCCCTCTCTTAAAGGT Majority
          210       220       230       240       250
200 CATTTAGGTGGGAGACACAGACCTGGTCTCCTTTCCCCTCTCTTAGAGGT A allele
201 CATTTAGGTGGGAGACACAGACCTGGTCTCCTTTCCCCTCTCTTAGAGGT B allele
200 CATTTAGGTGGGAGACACAGACCTGGTCTCCTTTCCCCTCTCTTAAAGGT Black Bengal Goat
180 CATCTAGATGGGAGACACAGACCTGGTCTCCTTTCCCCTCTCTTAAACGT Bos taurus
200 CATCTAGATGGGAGACACAGACCTGGTCTCCTTTCCCCTCTCTTAAAGGT Bubalus bubalis
200 CATTTAGGTGGGAGACACAGACCTGGTCTCCTTTCCCCTCTCTTAAAGGT Jamnapari Goat
170 CATTTAGGTGGGAGACACAGACCTGGTCTCCTTTCCCCTCTCTTAGAGGT Norwegian white sheep
200 CATTTAGGTGGGAGACACAGACCTGGTCTCCTTTCCCCTCTCTTAGAGGT Reference Sheep
154 CATCTAGATGGGAGACACAGACCTGGTCTCCTTTCCCCTCTCTTAAACGT Bos grunniens
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Fig. 3: Multiple Sequence Analysis of A Allele and B Allele of Exon 1 of GDF9 Gene of Garole sheep (Contd).

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TCTGTATGATGGGCACGGGGAACCCCCCAGGCTGCAGCCAGATGACAGAG Majority
                                260       270       280       290       300
250 TCTGTATGATGGGCACGGGGAACCCCCCAGGCTGCAGCCAGATGACAGAG A Allele
251 TCTGTATGATGGGCACGGGGAACCCCCCAGGCTGCAGCCAGATGACAGAG B Allele
250 TCTGTATGATGGGCACGGGGAACCCCCCAGGCTGCAGCCAGATGACAGAG Black Bengal Goat
230 TCTGTATGATGGGCACAGGGAACCCCCCAGGCTTCAGCCAGATGACAGAG Bos taurus
250 TCTGTATGATGGGCACAGGGAACCCTCCAGGCTTCAGCCAGATGACAAAG Bubalus bubalis
250 TCTGTATGATGGGCACGGGGAACCCCCCAGGCTGCAGCCAGATGACAGAG Jamnapari Goat
220 TCTGTATGATGGGCACGGGGAACCCCCCAGGCTGCAGCCAGATGACAGAG Norwegian white sheep
250 TCTGTATGATGGGCACGGGGAACCCCCCAGGCTGCAGCCAGATGACAGAG Reference Sheep
204 TCTGTATGATGGGCACAGGGAACCCCCCAGGCTTCAGCCAGATGACAGAG Bos grunniens

CTTTGCGCTACATGAAGAGGCTCTATAAGGCATACGCTACCAAGGAGGGG Majority
          310       320       330       340       350
300 CTTTGCGCTACATGAAGAGGCTCTATAAGGCATACGCTACCAAGGAGGGG A Allele
301 CTTTGCGCTACATGAAGAGGCTCTATAAGGCATACGCTACCAAGGAGGGG B Allele
300 CTTTGCGCTACATGAAGAGGCTCTATAAGGCATACGCTACCAAGGAGGGG Black Bengal Goat
280 CTTTGAGCTACATGAAGAGGCTCTATAAAGCATATGCTACCAAGGAGGGG Bos taurus
300 CTTTGAGCTACATGAAGAGGCTCTATAAAGCATACGCTACCAAGGAGGGG Bubalus bubalis
300 CTTTGCGCTACATGAAGAGGCTCTATAAGGCATACGCTACCAAGGAGGGG Jamnapari Goat
270 CTTTGCGCTACATGAAGAGGCTCTATAAGGCATACGCTACCAAGGAGGGG Norwegian white sheep
300 CTTTGCGCTACATGAAGAGGCTCTATAAGGCATACGCTACCAAGGAGGGG Reference Sheep
254 CTTTGAGCTACATGAAGAGGCTCTATAAAGCATATGCTACCAAGGAGGGG Bos grunniens

ACCCCTAAATCCAACAGACGCCACCTCTACAACACTGTTCGGCTCTTCAC Majority
          360       370       380       390       400
350 ACCCCTAAATCCAACAGACGCCACCTCTACAACACTGTTCGGCTCTTCAC A Allele
351 ACCCCTAAATCCAACAGACGCCACCTCTACAACACTGTTCGGCTCTTCAC B Allele
350 ACCCCTAAATCCAACAGACGCCACCTCTACAACACTGTTCGGCTCTTCAC Black Bengal Goat
330 ACCCCTAAATCCAACAGAAGCCACCTCTACAACACTGTTCGGCTCTTCAC Bos taurus
350 ACCCCTAAATCCAACAGAAGCCACCTCTACAACACTGTTCGACTCTTCAC Bubalus bubalis
350 ACCCCTAAATCCAACAGACGCCACCTCTACAACACTGTTCGGCTCTTCAC Jamnapari Goat
320 ACCCCTAAATCCAACAGACGCCACCTCTACAACACTGTTCGGCTCTTCAC Norwegian white sheep
350 ACCCCTAAATCCAACAGACGCCACCTCTACAACACTGTTCGGCTCTTCAC Reference Sheep
304 ACCCCTAAATCCAACAGAAGCCACCTCTACAACACTGTTCGGCTCTTCAC Bos grunniens

CCCCTGTGCTCAGCACAAGC-AGGCTCCTGGGGACCTGGCGGCAGGTGTG Majority
          410       420       430       440       450
400 CCCCTGTGCTCAGCACAAGC-AGGCTCCTGGGGACCTGGCGGCAGGTGTG A Allele
401 CCCCTGTGCTCAGCACAAGCCAGGCTCCTGGGGACCTGGCGGCAGGTGTG B Allele
400 CCCCTGTGCTCAGCACAAGC-AGGCTCCTGGGGACCTGGCGGCAGGTGTG Black Bengal Goat
380 CCCCTGTGCTCAGCACAAGC-AGGCTCCTGGGGACCAGGCAGCAGGTGTG Bos taurus
400 CCCCTGTGCTCAGCACAAGC-AAGCTCCTGGGGACCAGGCTGCAGG      Bubalus bubalis
400 CCCCTGTGCTCAGCACAAGC-AGGCTCCTGGGGACCTGGCGGCAGGTGTG Jamnapari Goat
370 CCCCTGTGCTCAGCACAAGC-AGGCTCCTGGGGACCTGGCGGCAGGTGTG Norwegian white sheep
400 CCCCTGTGCTCAGCACAAGC-AGGCTCCTGGGGACCTGGCGGCAGGTGTG Reference Sheep
354 CCCCTGTGCTCAGCACAAGC-AGGCTCCTGGGGACCAGGCAGCAGG      Bos grunniens

TAGGAGCAGATTGG                                        Majority
          460
449 TAGGAGCAGATTGG                                    A Allele
451 TAGGAGCAGATTGG                                    B Allele
449 TAGGAGCAGATTGG                                    Black Bengal Goat
429 TGGGAGCAGATTGG                                    Bos taurus
444                                                   Bubalus bubalis
449 TAGGAGCAGATTGG                                    Jamnapari Goat
419 TAGGA                                             Norwegian white sheep
449 TAGGAGCAGATTGG                                    Reference Sheep
398                                                   Bos grunniens
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1 of GDF-9 gene in Garole ewes were arranged to represent the coding region and were compared with other exon-1 sequences of GDF-9 gene of the related species available in NCBI Gene Bank. BLAST analysis was performed by using the online BLAST analysis software available at http://www.ncbi.nlm.nih.gov/BLAST. The BLAST analysis was used to find the extent of homology of the nucleotide sequences that has obtained in Garole sheep and a comparison was made with those of other closely related species.

BLAST analysis of obtained nucleotide sequence of A allele revealed that 462 bp of this sequence was 100 percent identical from nucleotide position 1734 to 2195 of the reference sequence of sheep (AF078545.2), which covered the entire exon-1 and partial portion of intron of GDF-9 gene (Table 1). Although BLAST analysis of obtained nucleotide sequence of B allele revealed that 464 bp of this sequence was 99 percent identical from nucleotide position 1734 to 2195 of the reference sequence of sheep (AF078545.2), which also covered the entire exon-1 and partial intron of GDF-9 gene. Insertion of two nucleotides at position 38 and 420 of the nucleotide sequence of A allele reduced identity one percent less compared to earlier one. Although Hanrahan *et al*. (2004) identified one SNP (G→A) at position 306 in exon-1 of GDF-9 gene in Belclare and Cambridge sheep. Kasiriyan *et al.* (2011) also reported G→A mutation at position 306 in Sangsari Sheep breed. Yosefabad *et al.* (2011) found a mutation at exon-1 in base No. 233 with C¦A type in Markhoz breed of goat. Singh *et al.* (2013) reported Two SNPs in exon-1 at the position 1893C→T and 1962C→A (heterozygous) in Gaddi goat.

Phylogenetic Analysis

The phylogeny of those species was studied to reveal the genetic distance due to the process of evolution. The sequences of exon-1 fragments of GDF-9 gene were aligned with available homologous sequences of the other species. Phylogenetic analysis of the sequence information of exon1 of GDF-9 gene of Garole sheep was compared with published sequences of this gene in other sheep breeds (NCBI accession no. AF078545.2 and HE 866499.1) and Black Bengal goat (NCBI accession no. JN601040.1). The sequences of exon-1 fragments were also aligned with available homologous sequences of the other species. The present study showed 96-99 percent homology between species for the obtained sequences of Garole in the exon-1 of GDF-9. The observations demonstrated conservation (100 percent) among the ovis species, however less conserved among other mammalian species (Table 1). BLAST analysis of exon 1 of Garole sheep revealed homology of 99 percent with all Indian goats (*Capra hircus*), 97 percent with *Bos taurus* and 96 percent with *Bubalus bubalis*. Phylogenetic analysis on the basis of nucleotide sequences revealed Garole sheep sequence as well as published sequence of sheep (accession no. AF078545.2 and HE 866499.1) form a common cluster indicating that they are most closely related from the evolutionary point of view as expected.

CONCLUSION

In the present study, all the tested animals of prolific Garole sheep breed carried wild type homozygous genotype for exon-1 of GDF 1 gene. The prolificacy genotypes at exon 1 of GDF 1 gene mutation found so far in the

genomes of many prolific breeds throughout the world were absent in the selected small population of Garole sheep breed reared in this herd. Hence, further investigation on large diversified population is required to confirm this finding.

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