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## ASSOCIATION OF GDF9 GENE POLYMORPHISMS WITH LITTER SIZE IN INDIGENOUS SHEEP OF BANGLADESH

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### ABSTRACT

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Sheep in Bangladesh are well known as efficient users of low quality roughages, well adapted to hot and humid agro-climatic conditions, capable of bi-annual lambing with multiple births and resistant to common prevalent diseases. They play an important role in the supply of animal protein. Present study aimed to investigate possible association of GDF9 gene polymorphisms with litter size. A total of 126 DNA samples extracted from the blood of indigenous sheep from 5 locations namely Tangail, Noakhali, Naogaon, Gaibandha and Satkhira with known litter size were used to study the association of GDF9 polymorphism by PCR based RFLP method. Two polymorphic regions of GDF9 (FecG1 and FecG8) were amplified by PCR, digested with respective restriction enzymes and 126 sheep were genotyped. Current study revealed that genotype and allele frequency for FecG1 varied among the sheep from different locations. The genotype (GG, AG, AA) frequency were 51.59%, 45.24% and 3.17% and the allele (G and A) frequencies in the overall population were 74.21% and 25.79%, respectively. There was a significant association of FecG1 of GDF9 gene polymorphism with litter size. The homozygous GG genotype had the lowest litter size ( $1.59 \pm 0.09$ ;  $n=65$ ) and homozygous AA genotype had the highest litter size ( $2.00 \pm 0.41$ ;  $n=4$ ). No genotypic variations were found for FecG8. Findings of this study specially the polymorphism of FecG1 together with genotyping of some sheep could be utilized in the selection program to increase the lamb production potentiality of indigenous sheep of Bangladesh.

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## INTRODUCTION

Agriculture is the main occupation of the people of Bangladesh employing about 47% of the labor force. This sector contributes about 14.2% of the gross domestic products (GDP) in the national economy (World Factbook, 2018) where, livestock sector plays a significant role. It contributes 1.54% and 13.62% of the total and agricultural Gross Domestic Product (GDP), respectively and provides livelihood for 20% directly and 45% partly of the population (DLS, 2017). There are 34.68 lakhs sheep population in Bangladesh (DLS, 2017) which stand third in number among the ruminant species in the country and are being used primarily for meat production. They are sparsely distributed throughout the country with a relatively higher concentration in three agro-ecological zones namely Coastal regions, Barind tracts and Jamuna river basin areas (Pervage et al., 2009). Most of the sheep of Bangladesh are nondescript indigenous with few crossbreds (Bhuiyan, 2006). Sheep in Bangladesh are well known as efficient users of low quality roughages, well adapted to hot and humid agro-climatic conditions, capable of bi-annual lambing with multiple births and resistant to common prevalent diseases (Hassan and Talukder, 2011). Litter size is one of the fundamental economic traits in farm animals especially in sheep. It is genetically influenced by multiple genes called fecundity genes. GDF9 (Growth differential factor 9) gene is crucially important among these fecundity genes. It is well known that growth differential factor 9 (GDF9) is one of the most promising genes for prolificacy and essential for normal folliculogenesis as well as increased ovulation rate in sheep. This gene encodes a member of the transforming growth factor-beta superfamily. Primordial follicle development is promoted by this factor and it stimulates granulosa cell proliferation. Eight different mutations (G1-G8) have been identified in GDF9 gene (Kolosov et al., 2015). Among the eight mutations of GDF9, G8 had additive effects on prolificacy and the G1 is associated with the increase in ovulation rate (Barzegari et al., 2010). For this reason, GDF9 gene is considered as a potential candidate gene for higher litter size in sheep.

Sheep is a small ruminant and it perhaps the most important species having a variety of functions with a unique ability to adopt and maintain themselves in a harsh environment in contrast with other ruminants. Sheep is being focused worldwide for their improvement and popularization of lamb/mutton to meet the increased demand of meat globally. Bangladesh is far away from developed countries to consume per capita meat per year. Therefore, sheep farming can be a potential option to manage the national meat demand as well as to alleviate poverty through generation of employment locally. So, genetic improvement of native sheep is a prime concern for sustainable sheep farming and move to demand of meat. Phenotypic and genetic characterization to improve the litter size could be an important way for increasing lamb production potentiality and overall productivity of native sheep. Considering the above facts and circumstance the present study was undertaken on native sheep from different agro-ecological zones of Bangladesh to identify the polymorphisms of FecG1 and FecG8 fragments of GDF9 gene in native sheep and to reveal the association of GDF9 gene polymorphisms with litter size.

## MATERIALS AND METHODS

### Phenotypic data and sample collection

Data were collected from five distinctly located sheep populations from five regions and a total of 310 farmers were interviewed from the selected locations for collecting data on the litter size of individual sheep. A total of 126 blood samples from ewes with known litter size were collected from a foresaid five locations and analyzed for genotyping. Blood was taken from the jugular vein using venoject tubes (Addbio manufacturing company, South Korea) coated with Di Sodium Ethylene Di Amine Tetra Acetate (EDTA) as anticoagulant. The collected blood sample in the venoject tube was kept at 4-6°C in icebox and carried as soon as possible to Animal Genomics and Breeding Laboratory, Department of Animal Breeding and Genetics, Bangladesh Agricultural University, Mymensingh and preserved at 4-6°C until DNA extraction.

### DNA extraction

DNA was extracted following the standard protocol (Hanrahan et al., 2004) from the whole blood using PrimePrep™ genomic DNA extraction kit, (GeNet Bio, South Korea) according to the manufacturer's instruction manual. In this method, RBC lysis buffer was used prior to the extraction in order to breakdown of RBC and concentrating WBC. Extracted DNA was stored in -20°C until use.

### DNA quantification and detection

After extraction, the concentration of DNA was measured by Nanodrop 1000 spectrophotometer (Model ND1000) according to the instruction of (Hanrahan *et al.*, 2004). Agarose gel electrophoresis for visualizing/detecting the isolated DNA was performed. The Purity of extracted DNA was assessed by calculating the OD260/OD280 nm ratio using NanoDrop (Model ND1000) spectrophotometer. The range of concentration and the purity determined by OD 260/280 ratio from Nanodrop 1000.

### PCR amplification

DNA samples were amplified in Biometra gradient thermocycler using standard PCR protocol (Polley et al., 2010). All samples were subjected to polymerase chain reaction. PCR was carried out in 16 µl reaction mixture containing 8.00 µl PCR master mix, 0.7µl primer forward, 0.7µl primer reverse, 4.2 µl deionized water and with 2.4 µl of DNA sample. A no-template negative control was used every time. The thermocycler program used for the amplification of two target sequences is noted in Table 1 and Table 2.

**Table 1.** PCR program used for amplification of G1 variant of GDF9 gene

Activity	Temperature	Duration	Cycles
Initialization	95°C	10 min	1
Denaturation	95°C	30s	37
Annealing	62°C	30s	
Extension	72°C	45s	
Final extension	72°C	10 min	1
Final hold	12°C	ours	1

**Table 2.** PCR program used for amplification of G8 variant of GDF9 gene

Activity	Temperature	Duration	Cycles
Initialization	95°C	10 min	1
Denaturation	95°C	30s	37
Annealing	55°C	30s	
Extension	72°C	45s	
Final extension	72°C	10 min	1
Final hold	12°C	ours	1

### Agarose gel electrophoresis of PCR products

Agarose gel electrophoresis was performed for checking the amplification of target FecG1 and FecG8 sequence of GDF9 followed by PCR. A volume of 5 µl of each PCR products was used for the agarose gel electrophoresis. 220 volt and 2% agarose used for 30 minutes electrophoresis.

### Digestion of PCR products with endonuclease enzymes:

After confirmation of PCR amplification of target DNA sequence by electrophoresis, digestion of the PCR products was done by *HhaI* and *DdeI* restriction enzymes (Hanrahan *et al.*, 2004) using a digital incubator. A 15 µl of reaction volume for each amplicon containing a mixture of 10 µl of PCR product, 0.35 µl of *HhaI* (for FecG1) and *DdeI* (for FecG8) enzyme, 1.6 µl of buffer and 3.05 µl of deionized water were kept into incubator for 30 minutes at 37°C. After completion of enzymatic digestion, the digested products were checked in agarose gel electrophoresis. In this case, 2.5% agarose gel was used. Agarose gel with electrophoresed digested products was examined in the gel documentation system (GDS-200, Sunil-Bio., I N K, Made in South Korea) and the particular images were captured for further analysis.

### GDF9 gene amplification by PCR:

Primers used for the amplification of FecG1 (462bp) and FecG8 (324bp) by PCR are presented in Table 3. In a 16 µl of reaction volume, 0.7 µl of each primer was used in single PCR and 2.4 µl of genomic DNA mixed with 8 µl PCR master mix. After amplification, the PCR product was used in agarose gel (2.0%) electrophoresis (30 minutes) and then visualized for the acquisition of images in the digital Gel Documentation System (GDS). The results showed and confirmed the expected amplification of the DNA fragments of interest.

**Table 3.** Primers used in PCR-RFLP, designed by (Hanrahan *et al.*, 2004)

Gene fragment	Primer	Sequence of primer	Amplicon Size (bp)
<i>GDF9, G1</i>	FecG1-F	5'-GAAGACTGGTATGGGGAAATG-3'	462
	FecG1-R	5'-CCAATCTGCTCCTACACACCT-3'	
<i>GDF9, G8</i>	FecG8-F	5'-CCATGACTTTAGACTTAGC-3'	324
	FecG8-R	5'-TGGTTTTACTTGACAGGAG-3'	

### Sequencing of GDF9 (FecG1 & G8) gene and bioinformatics analysis

According to the standard procedure (Hanrahan *et al.*, 2004), PCR products were purified and subsequently sequencing of the amplicons has been performed by Genetic Analyzer to confirm the amplification of candidate gene fragments. The sequencing service was performed by commercial company (National Institute of Biotechnology, Bangladesh and aAddbio manufacturing company, South Korea). The generated raw sequences were checked thoroughly and aligned using software package Chomas and Clustalw.

### Digestion of PCR amplified GDF9 fragments by endonuclease

After amplification of two fragments namely FecG1 and FecG8 of GDF9 gene by PCR, restriction enzyme digestion of the two amplicons was performed. For enzymatic digestion in 15 µl reaction volume, 0.35µl of *HhaI* (for FecG1) and *DdeI* (for FecG8) restriction enzyme was used for each reaction consisting of 1.6 µl of buffer, 3.05 µl of deionized water and 10 µl of PCR products. Enzyme digestion was performed in an incubator at the temperature of 37°C for 30 minutes. After completion of enzymatic digestion, agarose gel electrophoresis was performed for the products and gel images were analyzed in the Digital Gel Documentation system (GDS) (Figure 5 and 6).

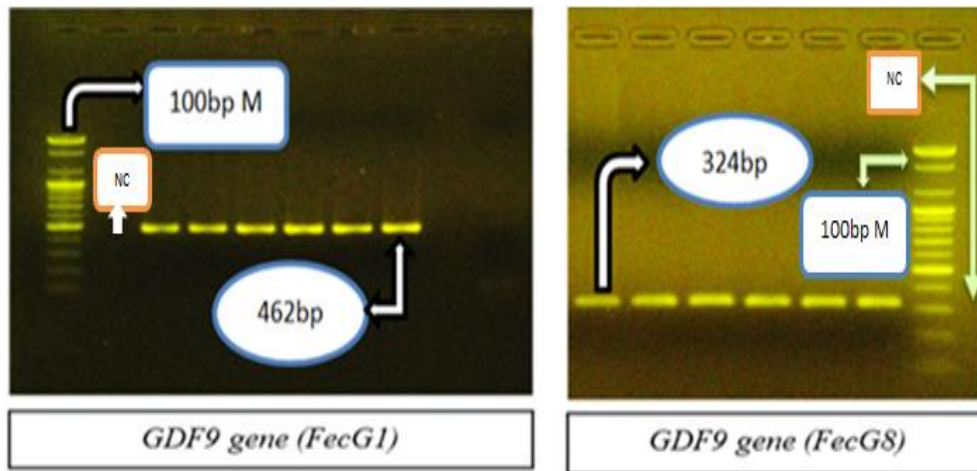


Figure 2. PCR amplification of FecG1 and FecG8 fragments of GDF9 gene

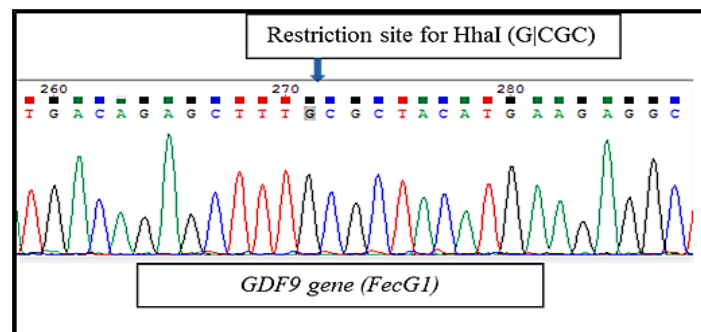


Figure 3. Chromatogram of FecG1 of GDF9 gene along with restriction site

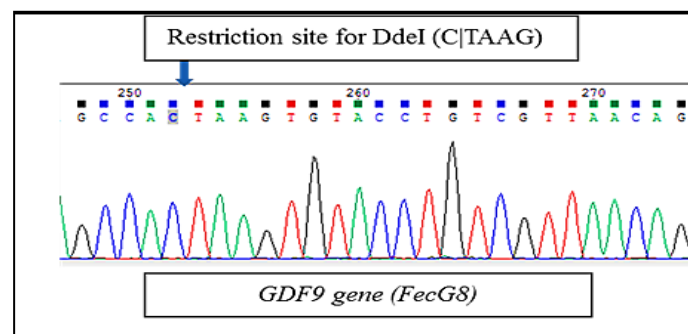
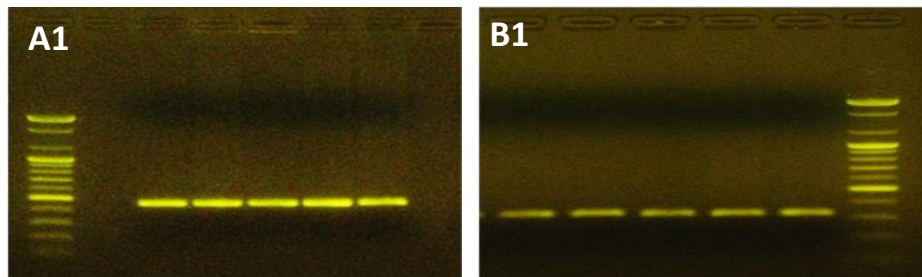
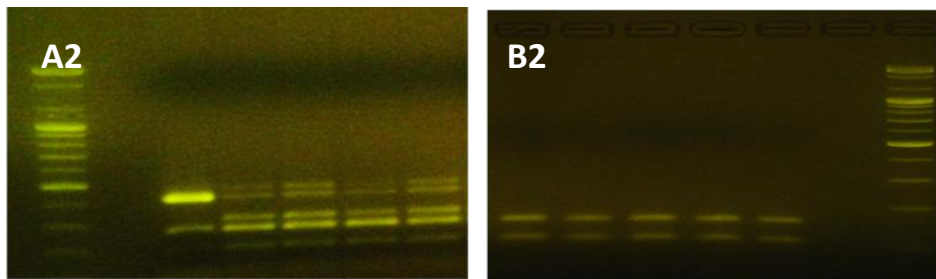


Figure 4. Chromatogram of FecG8 of GDF9 gene along with restriction site



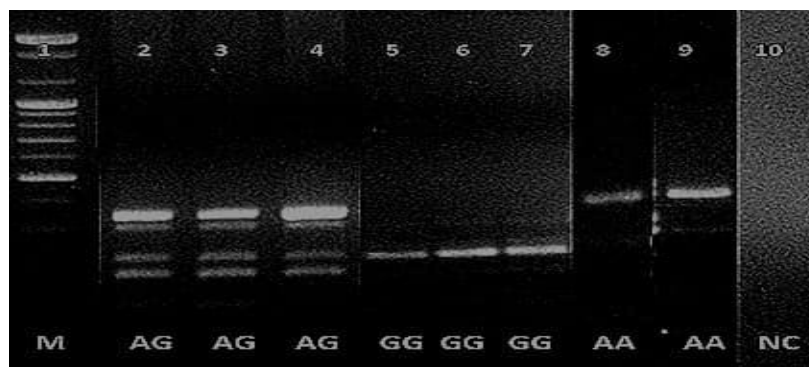
**Figure 5.** PCR amplified product of FecG1 (A1) and FecG8 fragments (B1) of *GDF9*



**Figure 6.** PCR product of FecG1 (A2) and FecG8 (B2) after enzymatic digestion of *GDF9* gene

**Gel image analysis and genotyping:** Gel images were captured by Digital Gel Documentation system and marked for identification of each individual sample. The images were then analyzed and subsequently genotyped following PCR-RFLP genotyping system.

**PCR-RFLP Genotyping:** The genotyping of each polymorphism was carried out by PCR-RFLP method. According to the gel image and the sequence of the gene, genotyping was done as shown in Figure 4.



**Figure 7.** Determination of different genotype for the polymorphism of FecG1 fragment of *GDF9* gene. (M=100bp marker, NC= Negative Control)

### Statistical analysis

The data were analyzed using Generalized Linear Model (GLM) procedure of Statistical Analysis System (SAS) (SAS Institute Inc., 2009) computer package, version 9.1.3

To find out the genotype and location effect on litter size the following model was used:

$$Y_{ij} = \mu + L_i + G_j + e_{ij}$$

Where,  $Y_{ij}$  = the dependent variable (litter size of the individual animal)

$\mu$  = the overall mean

$L_i$  = the fixed effect of  $i^{\text{th}}$  location

$G_j$  = the effect of  $j^{\text{th}}$  genotype

$e_{ij}$  = the residual error

## RESULTS AND DISCUSSION

### Quantification of DNA concentration and purity:

The highest concentration of extracted DNA was 244.9 ng/ $\mu$ l and the lowest was 26.1 ng/ $\mu$ l and the maximum purity was 2.06 and minimum purity was 1.35 (Table 4)

**Table 4.** Summary of concentration and purity value of extracted DNA

	Minimum	Maximum	Average
Concentration of Extracted DNA (ng/ $\mu$ l)	26.1	244.9	79.70
Purity of Extracted DNA (260/280)	1.35	2.06	1.86

### Genotype and allele frequency for the polymorphism of FecG1

Analysis of the genotype showed the frequency of GG genotype in sheep of Noakhali, Satkhira, Naogaon, Tangail and Gaibandha (Table 5). The maximum frequency of GG, AG and AA genotype were found in Satkhira, Tangail, and Naogaon, respectively.

The maximum frequency of allele G and A were found in Satkhira and Tangail, respectively (Table 5).

**Table 5.** Genotype and allele frequency for FecG1 polymorphism of GDF9 gene in indigenous sheep of Bangladesh

Parameter	Noakhali n=45	Satkhira n=34	Naogaon n=25	Tangail n=15	Gaibandha n=07	Overall n=126
Frequency of GG genotype	44.44%	82.35%	44.00%	6.67%	71.43%	51.59% <sup>a</sup>
Frequency of AG genotype	53.33%	14.71%	48.00%	93.33%	28.57%	45.24% <sup>a</sup>
Frequency of AA genotype	2.22%	2.94%	8.00%	0.00%	0.00%	3.17% <sup>b</sup>
Frequency of G allele	71.11%	89.71%	68.00%	53.34%	85.72%	74.21% <sup>a</sup>
Frequency of A allele	28.89%	10.29%	32.00%	46.66%	14.28%	25.79% <sup>a</sup>

(n=number of observations)

The study explains that the highest frequency was found in GG genotype and there were no significant differences between GG and AG genotype in the indigenous sheep of Bangladesh but the genotype AA significantly varied with GG and AG genotype. The results also show that the G allele has the highest frequency in the study population. A previous experiment on Indian Garole sheep population has shown that both mutant (A) and wild-type (G) alleles of GDF9 (FecG1) gene were detected with frequencies of 0.18 and 0.82, respectively. The wild type (GG) was found to be the predominant genotype in tested Indian Garole sheep population and the frequencies of GG and AG genotypes were 0.64 and 0.36, respectively (Polley *et*

al., 2010). Another study has detected three genotypes (AA, AB and BB) in GDF9 gene in Small Tail Han sheep with the frequencies of 0.30, 0.60 and 0.10 respectively (Chu et al., 2010).

#### Association of GDF9 (FecG1) gene polymorphisms with litter size

Association analysis of genotypes found in sheep from selected five locations with their litter size revealed that there is an association of FecG1 polymorphism of GDF9 gene with litter size (Table 6).

**Table 6.** Effect of genotypes for FecG1 polymorphism of GDF9 gene and location on litter size

Source	df	SS	MS	F value	Pr> F	R-square
Genotype	2	1.79	0.89	3.91	0.02	0.55
Location	4	26.94	0.74	29.30	< .0001	
Error	102	23.45	0.23			

\*\*Dependent variable: Litter size

\*\*Independent variable: Genotype and Location

Results suggest that the litter size has significant (F value= 3.91) effect on genotype for FecG1 polymorphism of GDF9 gene. The study also showed that the homozygous GG genotype had the lowest litter size ( $1.59 \pm 0.09$ ) and homozygous AA genotype had the highest litter size ( $2.00 \pm 0.41$ ). The mean litter size found to be varied among genotypes. Several studies (Polley et al., 2010), (Vacca et al., 2010) and (Moradband et al., 2011) reported more or less similar result.

According to genotype, the maximum, minimum and mean value of litter size is presented in Table 7. The study also found that there is a strong effect of genotype and location on litter size which is presented in Table 6.

Therefore, it can be assumed that there is a strong association of GDF9 gene polymorphism with litter size in indigenous sheep of Bangladesh and the sheep that have AA genotype for FecG1 polymorphism of GDF9 gene is the best for higher litter size (Table 7).

**Table 7.** Statistics of litter size (LS) of different genotype for FecG1 polymorphism of GDF9 gene in indigenous sheep of Bangladesh

	LS of GG genotype	LS of AG genotype	LS of AA genotype
Max	1	4	3
Min	1	1	1
Mean	1.59	1.83	2
Std. Error	0.09	0.10	0.41

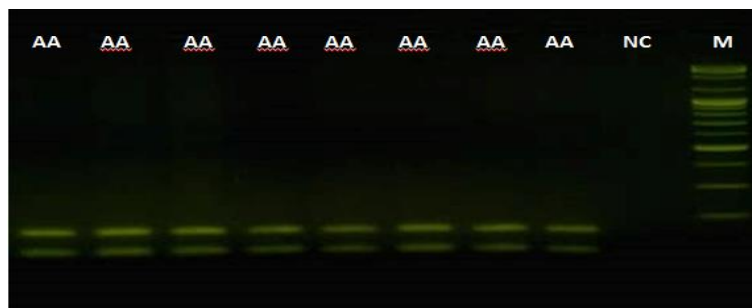
Max=Maximum, Min= Minimum, Std. Error= Standard Error

Several studies (Vacca et al., 2010), (Chu et al., 2011) and (Moradband et al., 2011) described GDF9 gene as polymorphic gene and associates with litter size.

#### Polymorphisms of FecG8 of GDF9 gene in indigenous sheep of Bangladesh

In case of GDF9 (FecG8), a total of 126 individuals from the indigenous sheep were genotyped with the forced PCR-RFLP approach. The electrophoretic profiles of RFLP analysis from GDF9 (FecG8) gene are shown in Figure 2 and 3. The results show that no polymorphism in exon 2 of GDF9 (FecG8) gene exists in the typed samples and all individuals had the wild type (AA) genotype (Figure 5). That means there was no genetic variation for FecG8 within the GDF9 gene among the studied animal population.





**Figure 8.** *DdeI* PRC-RFLP analysis of *GDF9* (FecG8) gene in indigenous sheep.  
(NC: Negative control, M: 100bp DNA ladder)

Until now, among Iranian sheep breeds, G8 mutation of *GDF9* was not detected. Our samples were randomly taken from the flock and one would expect to find all possible genotypes of *GDF9* if it really segregates in our indigenous population. Moreover, G8 mutation of *GDF9* was not detected in small tail Han, Hu, Dorset, Texel, German Mutton Merino sheep, Suffolk, Dorset, Charollais, Romney Hills, Chinese Merino sheep (Guan et al., 2005), Cele and Duolang sheep in China (Bai et al., 2007), Shal sheep in Iran (Ghaffari et al., 2009), Barbarine, Noire dehibar, Sicilo-Sarde and D'man sheep in the North Africa (Vacca et al., 2010), that is in agreement with our study. However, rare mutation rate (0.645%) in FecG8 of *GDF9* gene has been reported one study in Hu sheep through PCR-RFLP method (Guan et al., 2005).

## CONCLUSION

PCR-RFLP based genotyping analysis revealed that the genotype and allele frequency for FecG1 polymorphism of *GDF9* gene in the sheep populations varies between locations. Current study showed that there was an association of FecG1 polymorphism of *GDF9* gene with litter size in indigenous sheep of Bangladesh. In addition, it was observed that the litter size had significant ( $P < 0.02$ ) effect on genotype. The homozygous GG genotype showed lowest litter size ( $1.59 \pm 0.09$ ) and homozygous AA genotype represented the highest litter size ( $2.00 \pm 0.41$ ). On the other hand, there were no genotypic differences for the polymorphism of FecG8 fragment of *GDF9* gene in any of the sheep population in this study. Therefore, it can be concluded that there is an association of polymorphism of FecG1 fragment of *GDF9* gene with litter size in indigenous sheep of Bangladesh and the sheep with AA genotype which might be the suitable candidate as breeding animal in a selection program for improving prolificacy of indigenous sheep of Bangladesh.

## CONFLICT OF INTEREST

There is no conflict of research interest.

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