Polymorphism of fecundity genes (*BMP15* and *GDF9*) and their association with litter size in Bangladeshi prolific Black Bengal goat

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27 Abstract

28 Identification of prolificacy associated genetic markers remains vital in goat breeding industry since an increase in litter size can
 29 generate significant profit. Black Bengal is a prolific goat breed in Bangladesh. There are no inland reports on polymorphisms
 30 associated with fertility of Black Bengal goats in Bangladesh. In this study, we investigated two major fecundity genes-bone
 31 morphogenetic protein 15 (*BMP15*) and growth differentiation factor 9 (*GDF9*) in order to detect any possible mutations in these two
 32 genes in Bangladeshi Black Bengal goats. We identified six single nucleotide polymorphisms (SNP), of which five (C735A, C743A,
 33 G754T, C781A, and C808G) in *BMP15* exon 2 and one (T1173A) in *GDF9* exon 2. We also studied their association with litter size.
 34 Association analysis results show that polymorphism at the 735, 754 and 781 nucleotide positions of *BMP15* exon 2 had significant
 35 association with litter size in Black Bengal goat. The effect of parity was also highly significant ($p < 0.001$) on litter size. This study
 36 explored, for the first time, SNP loci in fecundity genes in Bangladeshi prolific Black Bengal goats. Further studies with a high
 37 number of genetically unrelated animals for assessing the association of these loci and others in the fecundity genes with litter size
 38 may be useful.

39 **Keywords** Black Bengal goat, fecundity genes, polymorphisms, litter size

40 Introduction

41 Prolificacy (average number of kids born per kidding) is an essential economic trait in goats. This trait abides vital attention to the
 42 animal breeders since an increase in offspring size can yield substantial gains in profit. Prolificacy in domestic species genetically
 43 influenced by multiple genes called fecundity genes (Gootwine, 2020). *BMP15* and *GDF9* are two candidate fecundity genes among
 44 the several detected in goat so far (Ahlawat et al. 2015). Several studies reported the correlation between prolificacy and genetic
 45 polymorphisms in *BMP15* and *GDF9* genes in different goat breeds around the globe, including Black Bengal goat in India (Polley et
 46 al. 2009; Hadizadeh et al. 2014; Ahlawat et al. 2015; Ahlawat et al. 2016; Heikal and El Naby 2017; Jalbani et al. 2017; Mishra et al.
 47 2017; Wang et al. 2017; Ghoreishi et al. 2019; Wang et al. 2019; Yue et al. 2019).

48 In Bangladesh, with a census size of 25.7 million goat stands for the third largest livestock species. Most of the goats in Bangladesh
 49 are Black Bengal goat (>90%) (Husain et al., 1998), reared mainly by landless, small-scale farmers. Black Bengal goat is a dwarf goat
 50 breed and is well-known for its high prolificacy, meat quality and skin quality (Miah et al. 2016). The average litter size of this breed
 51 ranges from 1.50 to 2.13 (Choudhury et al. 2012; Paul et al. 2014; Miah et al. 2016). This breed also renders a significant source of red

meat (25%) production in Bangladesh (Amin et al. 2000). Considering its importance, increasing the numbers of black Bengal goat has invariably been the central breeding goal for selective goat breeding program in Bangladesh.

The use of molecular markers in animal breeding has added benefits over conventional breeding techniques. The advancement in molecular genetics has led to the detection of DNA markers with considerable effects on the traits of economic importance. To the best of our knowledge, no screening attempted to identify polymorphisms in fecundity genes in goats of Bangladesh, using DNA based technologies. Therefore, this study aimed at the detection of genetic variants in two fecundity genes (*BMP15* and *GDF9*) and their relationship with litter size in the only recognized native goat breed in Bangladesh, Black Bengal.

Materials and methods

Experimental animals and sample collection

In the present experimental study, a total 40 Black Bengal does with twinning record in first three parities were chosen from Hathazari Government Goat Farm, Chattogram district of Bangladesh. Selected Black Bengal does kidded in 2016–2017, there are data on litter size at the first, second and third parity. There were no selection on litter size or other fertility traits in the farm over previous years. The average age and litter size of the does were 38.9 months and 2.48, respectively. These animals were maintained under the standard conditions of management, health, and nutrition by the farm management.

Blood samples (approximately 10 ml) were collected aseptically from the jugular vein in a vacutainer tube containing ethylene diamine tetraacetic acid (EDTA) as the anticoagulant. All samples were delivered to the Poultry Research and Training Center (PRTC) laboratory at Chattogram Veterinary and Animal Sciences University using an icebox.

DNA extraction, amplification and DNA sequencing

Genomic DNA was extracted from the blood samples using GeneJET Genomic DNA Purification kit (Thermo Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instructions. The extracted DNA samples were stored at -20°C prior to being used in Polymerase chain reaction (PCR).

PCR was performed to amplify segments of *BMP15* exon 2 and *GDF9* exon 1 and exon 2. The sequences of primers and amplicon sizes of PCR products are presented in Table 1. PCR amplifications were performed in a final reaction volume of 25 µL on a Thermo-

cycler (2720 Thermal cycler, Applied Biosystems, USA). The PCR reaction was run under the following thermal condition: initial denaturation for 1 min at 94 °C; 30 cycles of denaturation at 94 °C for 45 s, annealing at 60°C for 45 s, extension step at 72 °C for 45 s with a final extension at 72 °C for 5 min. The amplified PCR product was electrophoresed by running 10 µl of through a 2% agarose gel stained with ethidium bromide (0.5µg/ml) (Sigma Aldrich, USA). The specific sizes of the fragments were distinguished by using 1kb plus DNA ladder (O,GeneRuler, 1 kb Plus, Thermo Scientific Fermentas) in the gel and visualized by a UV transilluminator gel-documentation system (BDA digital, Biometra GmbH, Germany).

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In order to detect any possible mutations in exons of *BMP15* and *GDF9* genes, amplified PCR products for 40 samples (one for each animal) were bidirectionally sequenced using BigDye Terminator v. 3.1 (ThermoFisher Scientific, Waltham, MA USA) cycle sequencing protocol by Macrogen Co., Korea.

87

88 Sequence analysis

Nucleotide sequence data were edited and analysed by MEGA version 10.0.5 (Kumar et al. 2018). The detected polymorphisms (SNPs) were compared to the *Capra hircus* nucleotide database in NCBI Genbank using BLAST (Altschul et al. 1990). We used Polyphen-2 (Adzhubei et al. 2013) to predict the possible impact of mutations (based on amino acid substitution) on the structure and function of the respective proteins.

93

94 Statistical analysis

We used SHEsis online platform (<http://analysis.bio-x.cn>) (Shi and He, 2005) to calculate genotype frequencies, allele frequencies and χ^2 values for Hardy–Weinberg equilibrium (HWE) test. The deviation from HWE for each polymorphism was tested using the Hardy–Weinberg law (Gorlov et al. 2018; Yue et al. 2019). Heterozygosity (He), polymorphism information content (PIC) and effective allele number (Ne) for each polymorphism were estimated employing an online computing software (<http://www.msncall.com/Gdicall.aspx>). For PIC, following classifications was used i) low polymorphism if PIC value <0.25, ii) moderate polymorphism if PIC value ≥ 0.25 to ≤ 0.50 and iii) high polymorphism if PIC 0.50.

100

A linear mixed model was employed to analyse the association of polymorphisms in BMP15 and GDF9 gene with litter size using SPSS 25 statistical software, SPSS Inc., Chicago, IL, USA. The mixed model included fixed effects genotypes and parity (1, 2 and 3). Litter size data from consecutive parities in the same individual were considered as repeated measures.

$$Y_{ijk} = \mu + p_i + g_j + d_{ki} + e_{ijk}$$

where, Y_{ijk} is the phenotypic value of litter size, μ is the overall population mean, p_i is the effect of parity, g_j is the effect of genotype, d_{ki} is a normally distributed random variable with mean zero and variance σ_d^2 corresponding to doe k in parity i and e_{ijk} is the random error. The interactions between fixed effects were not significant hence excluded from the model. Mean separation procedures were performed by using the least significant difference test.

Results

Sequence results and SNP detection

The sequences were submitted to NCBI Genbank with the accession number of MN401415 and MN401414 for *BMP15* and *GDF9*, respectively. In a comparison of coding sequence sequences of caprine *BMP15* and *GDF9* genes that were recorded in NCBI GenBank (*BMP15*, NM_001285588.1; *GDF9*, NM_001285708.1), we identified five SNPs (G735A, C743A, G754T, C781A, and C808G) in the *BMP15* exon 2 and one SNP (T1173A) in *GDF9* exon 2 (Fig.1). Five of these polymorphisms leading to amino acid substitutions (Table 2). The predicted possible effect of identified polymorphisms on the structure and function of the functional protein revealed G754T and T1173A SNP loci have a significant impact on the coding BMP15 and GDF9 peptide, respectively (Fig.2). The C781CA mutation deduced to cause a moderate change while remaining two mutations did not show significant effects on the functional BMP15 protein.

Genetic parameters for the detected SNP loci

The genotypic and allelic frequencies, PIC, HE, Ne and χ^2 values for detected polymorphisms in two fecundity genes are presented in Table 2. The G735A of *BMP15* showed all the three possible genotypic combinations while remaining SNPs recorded with two genotypes. All tested goats expressed heterozygous genotype at C808G of *BMP15* gene. Genotype frequencies for most of the detected polymorphisms were significantly different from the expectations of Hardy-Weinberg equilibrium in Black goat population in this study ($P < 0.01$), except G735A and G754T in *BMP15* gene. Three polymorphisms in *BMP15* genes (C743A, C781A and

C808G) and one in *GDF9* gene (T1173A) were found to moderate polymorphic according to the classification of PIC. The remainder of the detected polymorphisms was found to be low polymorphic.

Association between SNP loci and litter size

The least squares means and standard error for litter size of different genotypes at the various loci of *BMP15* and *GDF9* genes are presented in Table 4. The effect of genotype was significant only for locus G735A ($p < 0.01$), G754T ($p < 0.05$) and C781A ($p < 0.001$) in *BMP15* gene. Parity had a highly significant effect on litter size in tested Black Bengal goat ($p < 0.001$). However, the interaction effect of parity and genotype had no significant effect on litter size. We were unable to test the C808G SNP in *BMP15* gene for association analysis since that had only one genotype in the study population.

Discussion

We herein reported nine polymorphic sites in two well-studied fecundity genes namely *BMP15* and *GDF9* in Black Bengal goat, the only prolific goat breed in Bangladesh. The genetic parameters of the detected loci and their association with the litter size in the Black Bengal goat were also analysed. The G735A locus of *BMP15* gene recorded with three possible combinations of genotypes. However, for the remaining five SNP loci, we did not observed any homozygous mutant genotype in the study population. One plausible reason is that these mutations are inactivating mutations in homozygote form, therefore does carrying two copies of mutated alleles are sterile and are expected to remove from the reproductive flock. Till date, reports on infertility among Black Bengal goat are scarce. Therefore, absence of homozygote mutant does for these five loci in the Black Bengal goat, similar to the situation that was observed for Small-Tail Han sheep, Tunisian-Barbarine sheep and Markhoz goat (Chu et al. 2007; Lassoued et al. 2017; Ghoreishi et al. 2019).

In this study, three SNPs in *BMP15* gene viz. C743A, G754T and C781A were found to be novel based on an extensive literature search. Remaining two SNPs identified by us in *BMP15* gene viz. G735A and C808G have also been reported in Black Bengal goats in the neighbouring country India (Ahlawat et al. 2013; Ahlawat et al. 2015; Maitra et al. 2016). The SNP in *GDF9* gene (T1173A) detected by us also not reported so far in goat. However, several studies reported polymorphisms in the flanking sequences of *GDF9* gene (Ahlawat et al. 2015; Shokrollahi and Morammazi 2018; Yue et al. 2019). Polley et al. (2009) who screened Black Bengal goats of India for the presence of mutations in *BMP15* and *GDF9* genes already associated with prolificacy in sheep and reported the wild

type genotype only. Similar findings have also been reported in five Indian goat breeds in addition to Black Bengal (Ahlawat et al. 2013). These findings differ with the findings of the present study since we screened for goat specific markers. The five non-synonymous (C743A, G754T, C781A, C808G and T1173A) goat specific mutations we detected in the present study might despite the necessity of tested in larger population be valuable for future studies on the molecular and functional effect of mutations on differential prolificacy in Bangladeshi goat breeds. The molecular and functional effect of a mutation can be neutral, harmful or beneficial depending on their context or location (Williams, 2016). In our study, two of the identified polymorphisms (G754T and T1173A) predicted to have a significant effect on resulting protein sequences.

Results of association study showed genotypes at three loci in *BMP15* gene had a significant effect on litter size in tested Black Bengal goat ($p = <0.05$). Significant effect of genotypes on litter size have been reported in Liaoning cashmere goats and Markhoz goat (Ghoreishi et al. 2019; Yue et al. 2019). In contrast, Ahlawat et al. (2015) and Ahlawat et al. (2016) reported a non-significant effect of genotypes on litter size in Indian Black Bengal goat. Heterogeneity among studies investigating effects on mutations on prolificacy in sheep has also been shown by Gootwine (2020). Presence of more than one segregating mutation in a major gene (*BMP15*) for high prolificacy in the present study is in line with those observed in some prolific sheep breed. For example, both FecXB and FecXG mutations in *BMP15* segregate in Beclatare sheep in Ireland (Hanrahan et al. 2004). Similarly, Tunisian-Barbarine sheep carries FecXBar and B5 mutation in *BMP15* gene (Vacca et al. 2010; Lassoued et al. 2017). None of the loci in *GDF9* gene we detected significantly associated with litter size in Black Bengal goat. Gootwine (2020) also showed that estimates of the effects of major genes vary among studies. Ahlawat et al. (2015) reported a highly significant ($p < 0.01$) effect of parity on litter size in Black Bengal goat of India. In the present study, we also observed a highly significant ($p < 0.001$) effect of parity on litter size. Faruque et al. (2010) reported a significantly ($p < 0.001$) lower litter size in first parity than in the seventh parity in Black Bengal goat. Halder et al. (2014) also confirmed that larger litter size in Black Bengal goat is highly influenced by higher parity and higher litter size in previous parity.

Variations of a quantitative trait are controlled by several genes, genetic variants and their interactions. Hence, the detection of polymorphisms that are underlying the differences in a quantitative trait, for instance, litter size remains as a challenge in modern genetics. However, in some prolific sheep breeds, prolificacy inherited as a qualitative trait, rather than quantitative due to presence of major genes with large effects on litter size (Gootwine, 2020). *BMP15*, *GDF9* and *BMPIB* are three well-documented candidate genes for litter size in sheep. Till date, no association of *BMPIB* gene with litter size in goat has been established. However,

178 researchers have explored the association between litter size in goat and polymorphisms in *BMP15* and *GDF9* genes. These two genes
179 are part of the ovary-derived transforming growth factor β (TGF β) that have an integral role as growth factors and receptors in
180 follicular development in the ovaries (Davis, 2005). Both bone morphogenetic proteins (BMPs) and growth differentiation factors
181 (GDFs) have critical role in follicle growth and cell-survival signalling hence causal mechanism underlying the high prolificacy or
182 fertility in female animal (Otsuka et al., 2011). Considering the biological importance of *BMP15* and *GDF9* genes, this study
183 investigated polymorphisms in these genes and their association with the litter size in Bangladeshi prolific Black Bengal goat.
184
185 The frequency distribution of genotype in a population is the simplest way to describe Mendelian variation (Mather and Jinks 1977).
186 Other factors such as such matting pattern, random genetic drift, individual survival, reproductive success, and migration also generate
187 genetic variation in a population (Chen et al. 2019). In the current study we sequenced only 40 goats, it would have an effect on the
188 genotypic and allelic frequencies we observed; hence, large population-scale research is required to confirm the genotypic and allelic
189 frequencies in Black Bengal goat of Bangladesh.

190

191 **Conclusion**

192 This is the first study exploring polymorphisms in *BMP15* and *GDF9* genes and investigating their association with litter size in
193 Bangladeshi goat. The findings of the study reveal that different genotypes at three loci in *BMP15* gene had significant ($p \leq 0.05$)
194 effect on litter size in the prolific Black Bengal breed of Bangladesh. Hence, there is a need for further research with a substantially
195 large number of animals across a wide range of geographically divergent populations of this breed. Our results enrich the repository of
196 molecular markers database of caprine fecundity genes which pave the way for association studies with fecundity trait, hence
197 contribute to molecular breeding in goat.

198

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202 Bengal goat”.

203 **Data availability**

204 The data that support the findings of this study are available from the authors upon reasonable request.

205 **Author contributions**

206 A. Das conceived this project. M. Shaha performed sample collection. M. Shaha and A. Dutta extracted DNA and performed PCR. A.
207 Das and M. Das Gupta performed data curation and formal data analysis. A. Das and O. F. Miazhi involved in funding acquisition,
208 project administration and supervision. A. Das drafted the original manuscript. All authors read and approved the final manuscript.

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210 **Statement of animal rights**

211 The manuscript does not contain clinical studies or patient data.

212

213 **Competing Interests**

214 The authors declare that they have no competing interests.

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312 Tables

313

314 **Table 1** List of primer used to amplify specific segment of *BMP15* and *GDF9* gene.

Gene	Region	Oligonucleotide sequence (5'-3')	Amplicon size (bp)	Reference
<i>BMP15</i>	Exon2	F: CACTGTCTTCTTGTTACTGTATTTC AATGAC	141	(Javanmard et al., 2011)
		R: GATGCAATACTGCCTGCTTG		
		F: TCCCTAAAGGCCTGAAAGAGT	575	(Maitra et al., 2016)
		R: GCTGAAGGCAAGGAATAGAATC		
<i>GDF9</i>	Exon1	F: GAAGACTGGTATGGGGAAATG	462	(Abdoli et al., 2018)
		R: CCAATCTGCTCCTACACACCT		
	Exon2	F: CCACACAAATACAACCCTCGATAC	183	(Ahlawat et al., 2016)
		R: AGGCTCGATGGCCAAAACACT		

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318 **Table 2** The identified mutations in *BMP15* and *GDF9* within the Bangladeshi Black Bengal Goat breed

Gene	Mutation	Coding amino acid residue	Amino acid change
<i>BMP15</i>	G735A	245	Unchanged Lys(K)
	C743A	248	Pro(P) to His(H)
	G754T	252	Gly(G) to Cys(C)
	C781A	261	Pro(P) to Thr(T)
	C808G	270	Gln(Q) to Glu(E)
<i>GDF9</i>	T1173A	391	His(H) to Gln(Q)

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Table 3 Genotype and allele frequencies of six SNP loci in two fecundity genes in Bangladeshi Black Bengal Goat (N=40).

Gene	Loci	Genotype frequency	Allele frequency	PIC	He	Ne	H-W test (χ^2)	p value
<i>BMP15</i>	G735A	GG-0.625	G-0.762	0.237	0.361	1.567	2.317	0.127
		GA-0.275	A-0.237					
		AA-0.100						
	C743A	CC-0.300	C-0.650	0.351	0.455	1.834	11.597	0.000
		CA-0.700	A-0.350					
		AA-0.0						
	G754T	GG-0.700	G-0.85	0.222	0.255	1.342	1.245	0.264
		GT-0.300	T-0.15					
		TT-0.0						
	C781A	CC-0.450	C-0.725	0.319	0.398	1.663	5.755	0.016
		CA-0.550	A-0.275					
		AA-0.0						
	C808G	CC-0.0	C-0.500	0.375	0.500	2.000	40.000	0.000
		CG-1.000	G-0.500					
		GG-0.0						
<i>GDF9</i>	T1173A	TT-0.300	T-0.300	0.331	0.420	1.724	39.999	0.000
		TA-0.700	A-0.700					
		AA-0.0						

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PIC, polymorphism information content; *He*, effective number of heterozygosities; *Ne*, effective number of alleles.

Table 4 Parity wise least squares of litter size for different genotypes at different loci of *BMP15* and *GDF9* genes.

Gene	Loci	Genotype	N	Litter size (Mean±SE)			Effect of genotype
				Parity 1	Parity 2	Parity 3	
<i>BMP15</i>	G735A	GG	25	1.92±0.11	2.64±0.11	2.88±0.11	**
		GA	11	1.82±0.16	2.36±0.16	2.55±0.16	
		AA	4	1.75±0.27	2.00±0.27	2.25±0.27	
	C743A	CC	12	2.00±0.16	2.58±0.16	2.75±0.16	NS
		CA	28	1.82±0.11	2.46±0.11	2.71±0.11	
	G754T	GG	28	1.82±0.10	2.39±0.10	2.64±0.10	*
		GT	12	2.00±0.16	2.75±0.16	2.91±0.16	
	C781A	CC	18	1.67±0.12	2.39±0.12	2.39±0.12	***
		CA	22	2.04±0.11	2.59±0.11	3.00±0.11	
<i>GDF9</i>	T1173A	TT	12	1.75±0.16	2.42±0.16	2.75±0.16	NS
		TA	28	1.89±0.10	2.57±0.10	2.71±0.10	

N, number of observations; *, P<0.05; **, P<0.01; ***, P<0.001; NS, Not significant.

Figures

Fig.1. Sequencing chromatograms of the detected SNPs in *BMP15* and *GDF9* genes in Black Bengal goat. **A, B, C, D, and E** represent the SNP loci G735A, G743A, G754T, C781A, and C808G in exon 2 of the *BMP15* gene, respectively. **F**, represents the SNP T1173A in exon 2 of the *GDF9* gene. Positions of the mutations are based on the full sequences of the *BMP15* and *GDF9* gene (Gene IDs: 100861233 and 100861233, respectively).

Fig.2. Predicted effects (using Ployphen2) of five SNP loci. **A**, **B**, **C**, and **D** represent the effect of C743A, G754T, C781A, and C808G on the functional BMP15 protein, respectively. **E**, represents the effect of T1173A on the functional GDF9 protein.

This mutation is predicted to be **DESTRUCTIVE** with a score of 0.933 (positivity: 0.93, specificity: 0.93)

A

This mutation is predicted to be **FUNCTIONALLY BENEFICIAL** with a score of 0.000 (positivity: 0.00, specificity: 0.00)

B

This mutation is predicted to be **DESTRUCTIVE** with a score of 0.444 (positivity: 0.44, specificity: 0.44)

C

This mutation is predicted to be **DESTRUCTIVE** with a score of 0.444 (positivity: 0.44, specificity: 0.44)

D

This mutation is predicted to be **FUNCTIONALLY BENEFICIAL** with a score of 0.000 (positivity: 0.00, specificity: 0.00)

E