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

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Abstract

The primary objective of this study was to identify polymorphisms in two major fecundity genes (*BMP15* and *GDF9*) and their association with litter size in Black Bengal goat, a prolific goat breed in Bangladesh. Total 40 blood samples were collected from Black Bengal does with twining records in the first three parities. All sampled animals were genotyped for fragments of exon 2 of

BMP15 gene and exon 1 and 2 of *GDF9* gene using DNA sequencing. The results of DNA sequence analysis revealed six polymorphic loci (g.735G>A, g.743C>A, g.754G>T, g.781C>A, g.808C>G and g.1061C>T) in *BMP15* gene and three (g.118C>T, g.302_303insT and g.1173_1174insA) in *GDF9* gene. Association analysis for polymorphic loci showed litter size in Black Bengal goat significantly varied between genotypes at g.735G>A and g.781C>A loci of *BMP15* gene. Further studies with a high number of genetically unrelated animals for assessing the association of these loci and others in the fecundity genes with litter size may be useful.

Keywords: Black Bengal goat; fecundity genes, polymorphisms, litter size

Introduction

Prolificacy (increase in litter size) is an essential economic trait in farm animals. This trait abides vital attention to the animal breeders since a small increase in offspring size can yield substantial gains in profit. Prolificacy in domestic species genetically influenced by multiple genes called fecundity genes.[1] Studies on the genetics of prolificacy in goat detected several candidate fecundity genes.[2] Bone Morphogenetic Protein 15 (*BMP15*), and Growth Differentiation Factor 9 (*GDF9*) are two of them.[2] Several studies reported the correlation between prolificacy and genetic polymorphisms in *BMP15* and *GDF9* genes in different goat breeds around the globe. [2-11]

In Bangladesh, with a census size of 25.7 million goat stands for the third largest livestock species. Most of the goats in Bangladesh are Black Bengal goat (>90%), reared mainly by landless, small-scale farmers. [12] Black Bengal goat is a dwarf goat breed and is well-known for its high prolificacy, meat quality and skin quality.[13] The average litter size of this breed ranges from 1.93 to 2.33. [12,14,15] This breed also renders a significant source of red meat (25%) production in Bangladesh [16]. 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 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

Sample acquisition

In the present study, a total of forty (40) prolific Black Bengal does (with twining in the first three parities) were selected from two Upazilas (a sub-district unit) from Chattogram district, Bangladesh. We selected unrelated animals at random from their breeding tracts by picking up only two samples per smallholding and only five herds per village. Demographic data for all selected animal were collected using a predefined questionnaire.

Blood sample collection and DNA extraction

In the present study, a total of forty (40) prolific Black Bengal does (with twining in the first three parities) were selected from two Upazilas (a sub-district unit) from Chattogram district, Bangladesh. We selected unrelated animals at random from their breeding tracts by picking up

only two samples per smallholding and only five herds per village. Demographic data for all selected animal were collected using a predefined questionnaire. Blood samples 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. 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 quality of isolated DNA was investigated using agarose gel electrophoresis (0.8%).

Polymerase chain reaction (PCR) amplification

All selected goats were genotyped for segments of *BMP15* (Gene ID: 100861233) and *GDF9* (Gene ID: 100860859), respectively, associated with fecundity in sheep and goat [3,17-19]. To amplify exon 1 of *GDF9* gene and, exon 2 of *BMP15* gene, we used two sets primers reported in the literature (Table 1).

Polymerase chain reaction (PCR) was 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).

Sequencing and analysis

Amplified PCR products were bidirectionally sequenced in Macrogen Co., Korea. Nucleotide sequence data were edited and analysed by MEGA version 10.0.5.[20] The detected polymorphisms (SNPs) were compared to the *Capra hircus* nucleotide database in NCBI database using BLAST.[21] The sequences were submitted with the accession number of MN401415 for *BMP15* and MN401414 for *GDF9* gene, respectively.

Phylogenetic analysis

We performed a phylogenetic analysis of exon 2 of *BMP15* gene and exon 1 of *GDF9* gene with accessible published sequences of the same regions of genes in different goat breeds to identify the genetic diversity of Black Bengal goat. CLUSTALW[22] was used for multiple alignment and iTOL[23] to visualize the phylogenetic trees.

Statistical analysis

We used SHEsis online platform (http://analysis.bio-x.cn) [24] to calculate genotype frequencies, allele frequencies and $\chi 2$ values for Hardy–Weinberg equilibrium (HWE) test. The deviation from HWE for each polymorphism was tested using the Hardy–Weinberg law.[25,26] Heterozygosity (He), polymorphism information content (PIC) and effective allele number (Ne) for each polymorphism were estimated employing an online computing software (http://www.msrcall.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.

A generalized linear model was used to analyse the association of polymorphisms in *BMP15* and *GDF9* gene with litter size by applying the least-squares method in SPSS 25 statistical software, SPSS Inc., Chicago, IL, USA.

$$Yi = \mu + Pi + eij$$

where, *Yi* is the phenotypic value of litter size; μ, is the overall population mean; *Pi*, is the genotype and *eij*, is the random error.

Results

Sequence analysis of the BMP15 gene exon 2

In a comparison of caprine *BMP15* gene sequence, we identified six single nucleotide polymorphisms (SNPs): g.735G>A, g.743C>A, g.754G>T, g.781C>A, g.808C>G (Fig.1) and g.1061C>T in the exon 2 of *BMP15* gene (Fig.1 and Supplementary Fig. S1). Five of these polymorphisms leading to amino substitutions (Table 2). The predicted possible effect of identified polymorphism on the structure and function of the functional protein revealed two mutations: g.754G>T and g.1061C>T have a significant impact on the coding BMP15 peptide (Fig. 2) indicating a substantial phenotypic effect. The g.781C>A mutation deduced to cause a moderate change while remaining two mutations did not show significant effects on the functional BMP15 protein.

Sequence analysis of the GDF9 gene exons 1 and 2

The sequence of the exon 1 of *GDF9* gene showed a SNP g.118C>T and an insertion mutation g.302_303insT. The g.302_303insT mutation causes a frameshift in the reading frame of the *GDF9* gene which leads to a premature stop codon at third codon downstream of the mutated

codon (Supplementary Fig. S2). We identified a one bp insertion in between nucleotide 1173 and 1174 in the exon 2 of *GDF9* gene, which deduced a premature stop codon at 16th codon downstream of the mutated codon (Supplementary Fig. S3).

Genetic parameters for the detected polymorphisms in BMP15 and GDF9 gene

The genotypic and allelic frequencies, PIC, HE, Ne and $\chi 2$ values for detected polymorphisms in two fecundity genes are presented in Table 3. The g.735G>A of *BMP15* showed all the three possible genotypic combinations while remaining SNPs recorded with two genotypes. All 40 tested individuals had only homozygous mutant genotypes for g.1061C>T of *BMP15* and g.118C>T of *GDF9* gene. All tested goats expressed heterozygous genotype at g.808C>G and homozygous mutant genotype at g.1061C>T 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 g.735G>A and g.754G>T in *BMP15* gene. Three polymorphisms in *BMP15* genes (g.743C>A, g.781C>A and g.808C>G) and two in *GDF9* gene (g.302_303insT and g.1173_1174insA) were found to moderate polymorphic according to the classification of PIC. The remainder of the detected polymorphisms was found to be low polymorphic.

Phylogenetic analysis

Results of phylogenetic analysis show that the sequences of the exon 2 of *BMP15* gene in this study clustered in a shared cluster with previously published sequences for Black Bengal goat, however, diverges from that of other goat breeds (Fig. 4). On the other hand, sequences of the exon 1 of *GDF9* gene did not form a breed-specific cluster (Fig. 5).

Association between SNPs of BMP15 and GDF9 and litter size trait

The litter size of AA genotype individuals at G735A locus of *BMP15* was significantly higher than that of GG genotype ($p<.05$), however, there is no significant difference between individuals with GA and GG genotypes at this locus. Individuals with heterozygous CA genotype at C781A locus of *BMP15* had a significantly higher ($p<.01$) litter size than those of CC genotype. Individuals with different genotypes at other loci in both *BMP15* and *GDF9* gene did not show any significant difference for litter size. In this study, the mean litter size was highest (3.04±0.45) in individuals with heterozygous CA genotype at C781A locus of *BMP15* (Table 4).

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 g.735G>A locus of *BMP15* recorded with three possible combinations of genotypes while the remaining loci expressed either two or one genotypes in the study population. The frequency distribution of genotype in a population is the simplest way to describe mendelian variation[27], which might not be the fact in this study since we sequenced only 40 goats for each locus. However, other factors such as such matting pattern, random genetic drift, individual survival, reproductive success, and migration also generate genetic variation in a population.[28]

In this study, four SNPs in *BMP15* gene viz. g.743C>A, 754G>T, g.781C>A and g.1061C>T were found to be novel based on an extensive literature search. Remaining two SNPs identified by us in *BMP15* gene viz g.735G>A and g.808C>G have also been reported in Black

174 Bengal goats in the neighbouring country Indian.[2,4,18] None of the polymorphisms in *GDF9* gene
175 detected by us was reported so far in goat. However, several studies reported polymorphisms in
176 the flanking sequences of *GDF9* gene. [2,25,29]

177 The diversity of the impact on molecular function determines the effect of a mutation.
178 Mutational effects can be neutral, harmful or beneficial depending on their context or location[30].
179 In our study, two of the identified polymorphisms in *BMP15* gene predicted to have a significant
180 effect on resulting protein sequence, hence might contribute to phenotypic change. An
181 advantageous phenotypic effect of two insertions detected by us in *GDF9* gene might be
182 excluded since frameshifting generally assume to cause a loss of function resulting from
183 degrading mutant mRNA by nonsense-mediated or non-stop-mediated mRNA decay[31-33].
184 However, any harmful or neutral effect[34] of these frameshift mutations on GDF9 protein function
185 cannot be ruled out.

186 Phylogenetic analysis of *BMP15* gene revealed Black Bengal goat including the present
187 study assorted in a common cluster which differs from that of other goat breeds. The *GDF9* gene
188 sequences show more genetic diversity in different goat breeds. A similar trend of genetic
189 divergence in the coding region of *BMP15 and GDF9* gene has reported by Xue-qin et al.[35]

190 Results of association study showed that goat with AA genotype at G735A of *BMP15*
191 had significantly higher litter size than that of GG genotypic individuals ($p = <0.05$). The effect
192 of G735A in *BMP15* gene on litter size also reported in Indian Black Bengal goat[2,4]. Besides, the
193 phenotypic effects of this synonymous mutation could not be ruled out since there are many
194 mechanisms exist by which a synonymous mutation can affect a phenotype.[36] In this study,
195 Black Bengal goat with genotype CA at C781A of *BMP15* gene had significantly higher litter
196 size than those with CC at the same locus. Ahlawat et al[2,4] and Feng et al[37] have reported a

197 significant effect of allelic variants in *BMP15* gene on the litter size in different goat breeds
198 including Black Bengal.

199 Variations of a quantitative trait are controlled by several genes, genetic variants and their
200 interactions. Hence, detection of polymorphisms that are underlying the differences in a
201 quantitative trait, for instance, litter size remains as a challenge in modern genetics. *BMP15*,
202 *GDF9* and *BMPR1B* are three well-documented candidate genes for litter size in sheep and goat.
203 Till date, no association of *BMPR1B* gene with litter size in goat has been established. However,
204 researchers have explored the association between litter size in goat and polymorphisms in
205 *BMP15* and *GDF9* genes. These two genes are part of the ovary-derived transforming growth
206 factor β (TGF β) that have an integral role as growth factors and receptors in follicular
207 development in the ovaries.[38] Both bone morphogenetic proteins (BMPs) and growth
208 differentiation factors (GDFs) have critical role in follicle growth and cell-survival signalling
209 hence causal mechanism underlying the high prolificacy or fertility in female animal.[39]
210 Considering the biological importance of *BMP15* and *GDF9* genes, this study investigated
211 polymorphisms in these genes and their association with the litter size in Bangladeshi prolific
212 Black Bengal goat.

213 **Conclusion**

214 This is the first study exploring polymorphisms in *BMP15* and *GDF9* genes and investigating
215 their association with litter size in Bangladeshi goat. The findings of the study reveal that
216 different genotypes at two loci in *BMP15* gene had significant ($p \leq 0.05$) effect on litter size in
217 the prolific Black Bengal breed of Bangladesh. Hence, there is a need for further research with a
218 substantially large number of animals across a wide range of geographically divergent
219 populations of this breed. Our results enrich the repository of molecular markers database of

caprine fecundity genes which pave the way for association studies with fecundity trait, hence contribute to molecular breeding in goat.

Data availability

The assembled and annotated sequences for *BMP15* and *GDF9* genes were deposited to GenBank database under accession numbers MN401414 and MN401415, respectively.

Conflict of Interest

The authors declare that they have no competing financial interests.

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Author contributions

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

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

Gene	Region	Oligonucleotide sequence (5'-3')	Amplicon size (bp)
BMP15	Exon2	F: CACTGTCTTCTTGTTACTGTATTTCAATGAC	141
		R: GATGCAATACTGCCTGCTTG	
		F: TCCCTAAAGGCCTGAAAGAGT	575
		R: GCTGAAGGCAAGGAATAGAATC	
GDF9	Exon1	F: GAAGACTGGTATGGGGAAATG	462
		R: CCAATCTGCTCCTACACACCT	
	Exon2	F: CCACACAAATACAACCCTCGATAC	183
		R: AGGCTCGATGGCCAAAACACT	

337

338 **Table 2.** Identified polymorphisms in *GDF9* and *BMP15* genes in Bangladeshi Black Bengal goat breed.

Gene	Region	Mutation	Amino acid substitution	Type of mutation
BMP15	Exon2	g.735G>A	-	Synonymous
		g.743C>A	p.Pro248His	Non-synonymous
		g.754G>T	p. Gly252Cys	Non-synonymous
		g.781C>A	p. Pro261Thr	Non-synonymous
		g.808C>G	p.Gln270Glu	Non-synonymous
		g.1061C>T	p.Ala354Val	Non-synonymous
GDF9	Exon1	g.118C>T	-	Synonymous
		g.302_303insT	p.Gly101Glyfs2X	Frameshift
	Exon2	g.1173_1174insA	p.Arg392Glnfs16X	Frameshift

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Table 3. Genotypic and allelic frequencies and population parameters for mutations detected in *GDF9* and *BMP15* genes in Bangladeshi Black Bengal Goat breed (N=40).

Gene	Mutation	Genotypic frequencies			Allelic frequencies		PIC	He	Ne	H-W test ($\chi 2$)	p value
		PP	Pq	qq	P	q					
BMP15	g.735G>A	0.625	0.275	0.100	0.762	0.237	0.237	0.361	1.567	2.317	0.127
	g.743C>A	0.300	0.700	0	0.650	0.350	0.351	0.455	1.834	11.597	0.000
	g.754G>T	0.700	0.300	0	0.85	0.15	0.222	0.255	1.342	1.245	0.264
	g.781C>A	0.450	0.550	0	0.725	0.275	0.319	0.398	1.663	5.755	0.016
	g.808C>G	0	1.000	0	0.500	0.500	0.375	0.500	2.000	40.000	0.000
	g.1061C>T	0	0	1.000	0	1.000	0	0	1.000	-	-
GDF9	g.118C>T	0	0	1.000	0	1.000	0	0	1.000	-	-
	g.302_303insT	0.200	0.800	0	0.600	0.400	0.364	0.480	1.923	17.778	0.000
	g.1173_1174insA	0.300	-	0.700	0.300	0.700	0.331	0.420	1.724	39.999	0.000

PP, homozygous reference allele, Pq, heterozygous mutant allele; qq, homozygous mutant allele; *PIC*, polymorphism information content; *He*, effective number of heterozygosities; *Ne*, effective number of alleles.

Table 4. Genotype wise least squares mean ± standard error of litter size in Bangladeshi Black Bengal goat.

Gene	Locus	Genotype	N	Litter size	Significance
BMP15	G735A	GG	25	3.00^{a}±0.33	*
		GA	11	2.52^{ab}±0.13	
		AA	4	2.27^{b}±0.20	
	C743A	CC	12	2.42±0.20	NS
		CA	28	2.53+0.13	
		AA	0	-	
	G754T	GG	28	2.39±0.14	NS
		GT	12	2.75±0.13	
		TT	0	-	
	C781A	CC	18	1.83^{a}±0.09	***
		CA	22	3.04^{b}±0.45	
GDF9	302_303insT	GG	8	2.47±0.12	NS
		GGT	32	2.63±0.24	
	1173_1174insA	TT	7	2.86±0.69	NS
		TTA	28	2.42±0.66	

*, p value <0.05 ***, p value <0.01; rows with different superscripts differed significantly.

359

360 **Figure Legends**

361 **Figure 1.** Sequencing chromatograms of the detected single nucleotide polymorphisms (SNPs) in exon 2 of the *BMP15* gene in Black Bengal
362 goat breed. a. g.735G>A (heterozygous); b. g.743C>A (heterozygous); c. g.754G>T (heterozygous); d. g.781C>A (heterozygous); d. g.808C>G
363 (heterozygous) and f. g.1061C>T (homozygous TT). Positions of the mutations are based on the full sequences of the *BMP15* gene (Gene ID:
364 100861233).

365

366 **Figure 2.** Sequencing chromatograms of the detected polymorphisms in the *GDF9* gene in Black Bengal goat breed. a. g.118C>T (homologous
367 TT) and b. 302_303insT in the exon 1 of *GDF9* gene; c. g.1173_1174insA in the exon 2 of *GDF9* gene. Positions of the mutations are based on
368 the full sequences of the *GDF9* gene (Gene ID: 100861233).

369

370 **Figure 3.** Predicted effects (using Ployphen2) of five single nucleotide polymorphisms (SNPs). a, b, c, d and e represent the of effect of
371 g.743C>A, g.754G>T, g.781C>A, g.808C>G and g.1061C>T on the functional BMP15 protein, respectively.

372

373 **Figure 4.** Phylogenetic tree of *BMP15* gene.

Figure 5. Phylogenetic tree of *GDF9* gene.

Supplementary Figure S1. Multiple alignment for g.1061C>T of *BMP15* gene (indicated by box arrow). *BMP15* gene sequences from this study aligned with BMP15 mRNA sequence from the NCBI database using MEGA version 10.0.5.[20]

Supplementary Figure S2. Multiple alignment for 302_303insT of *GDF9* gene (Condon changes are indicated by box arrow). *GDF9* gene sequences from this study aligned with GDF9 mRNA sequence from the NCBI database using MEGA version 10.0.5.[20]

Supplementary Figure S2. Multiple alignment for 302_303insT of *GDF9* gene (Condon changes are indicated by box arrow). *GDF9* gene sequences from this study aligned with GDF9 mRNA sequence from the NCBI database using MEGA version 10.0.5.[20]

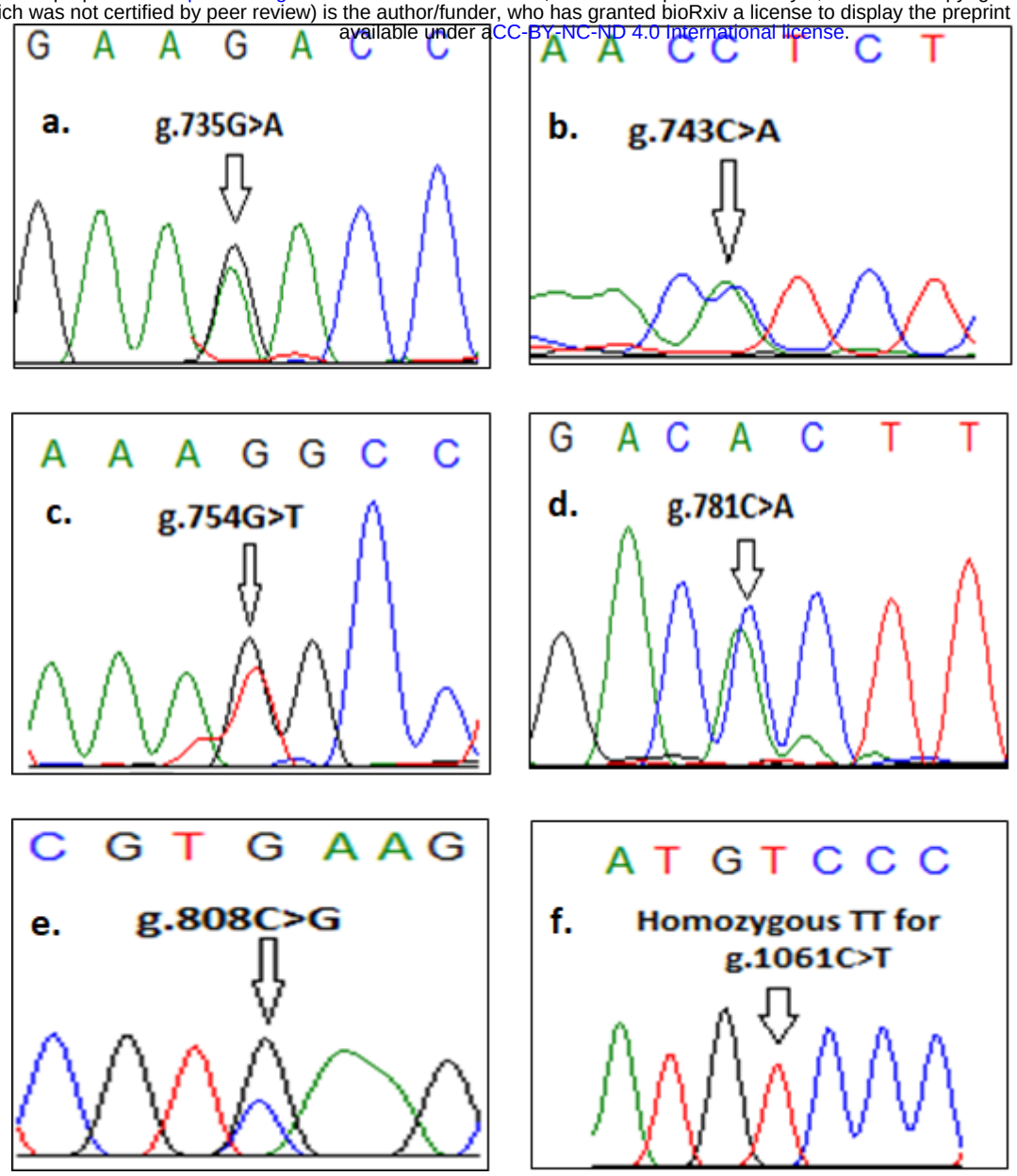

Figure 1. Sequencing chromatograms of the detected single nucleotide polymorphisms (SNPs) in exon 2 of the *BMP15* gene in Black Bengal goat breed. a. g.735G>A (heterozygous); b. g.743C>A (heterozygous); c. g.754G>T (heterozygous); d. g.781C>A (heterozygous); d. g.808C>G (heterozygous) and f. g.1061C>T (homozygous TT). Positions of the mutations are based on the full sequences of the *BMP15* gene (Gene ID: 100861233).

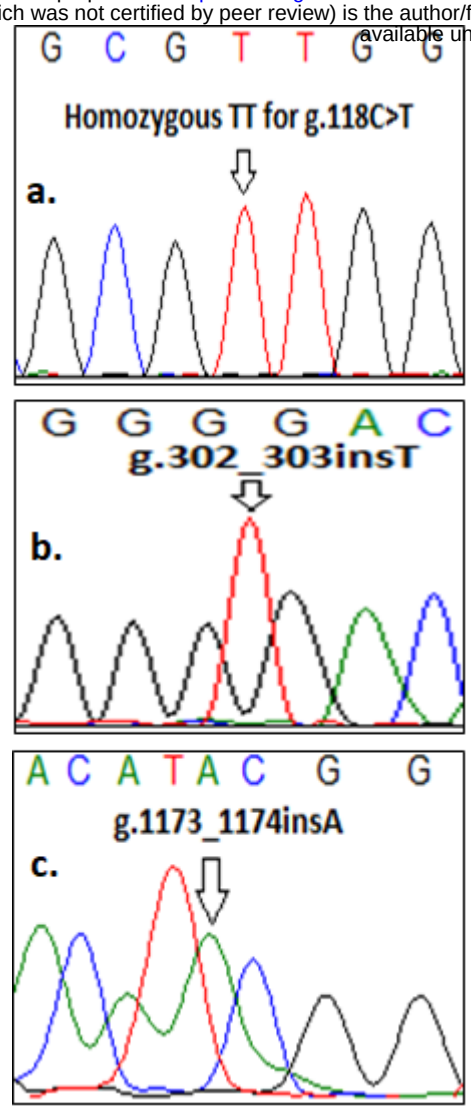

Figure 2. Sequencing chromatograms of the detected polymorphisms in the *GDF9* gene in Black Bengal goat breed. a. g.118C>T (homologous TT) and b. 302_303insT in the exon 1 of *GDF9* gene; c. g.1173_1174insA in the exon 2 of *GDF9* gene. Positions of the mutations are based on the full sequences of the *GDF9* gene (Gene ID: 100861233).

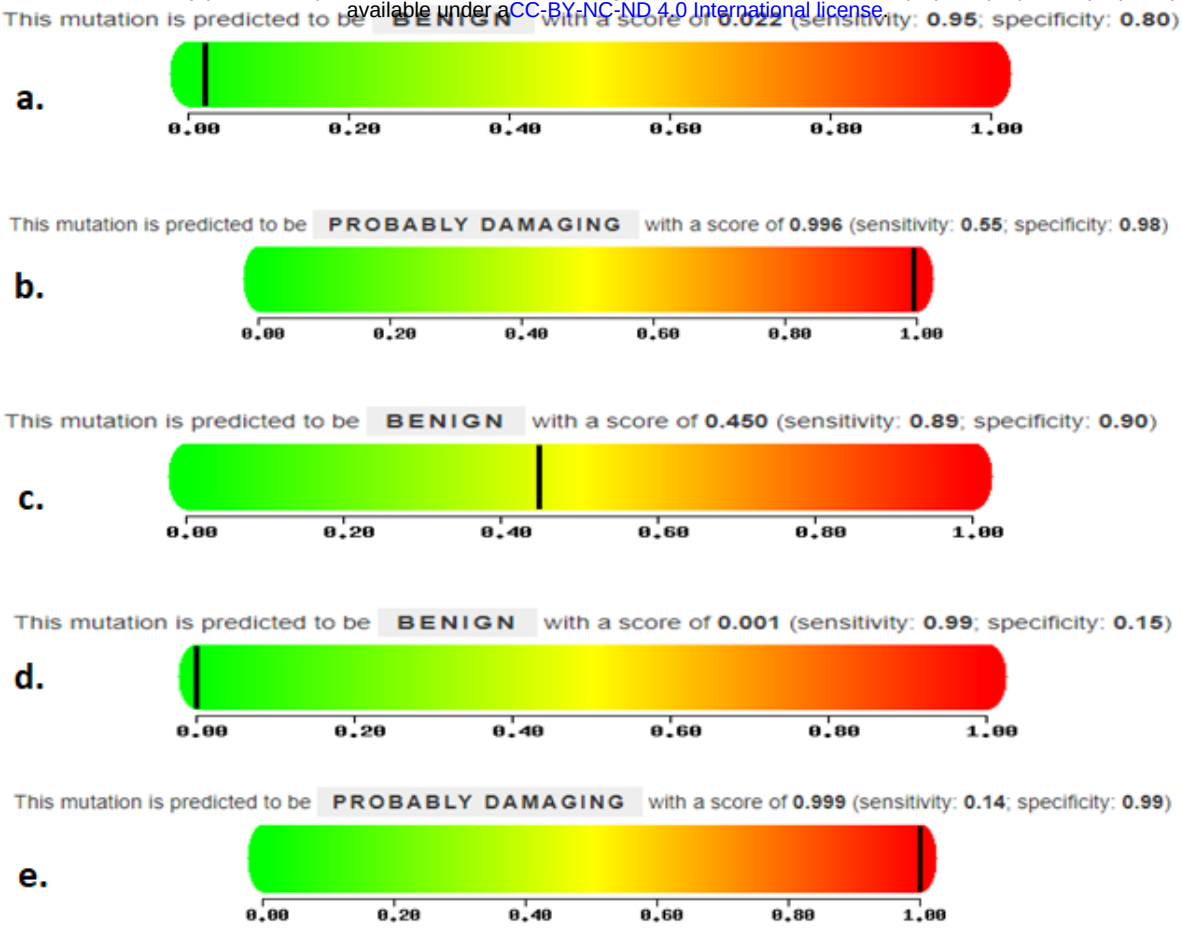

Figure 3. Predicted effects (using Ployphen2) of five single nucleotide polymorphisms (SNPs). a, b, c, d and e represent the of effect of g.743C>A, g.754G>T, g.781C>A, g.808C>G and g.1061C>T on the functional BMP15 protein, respectively.

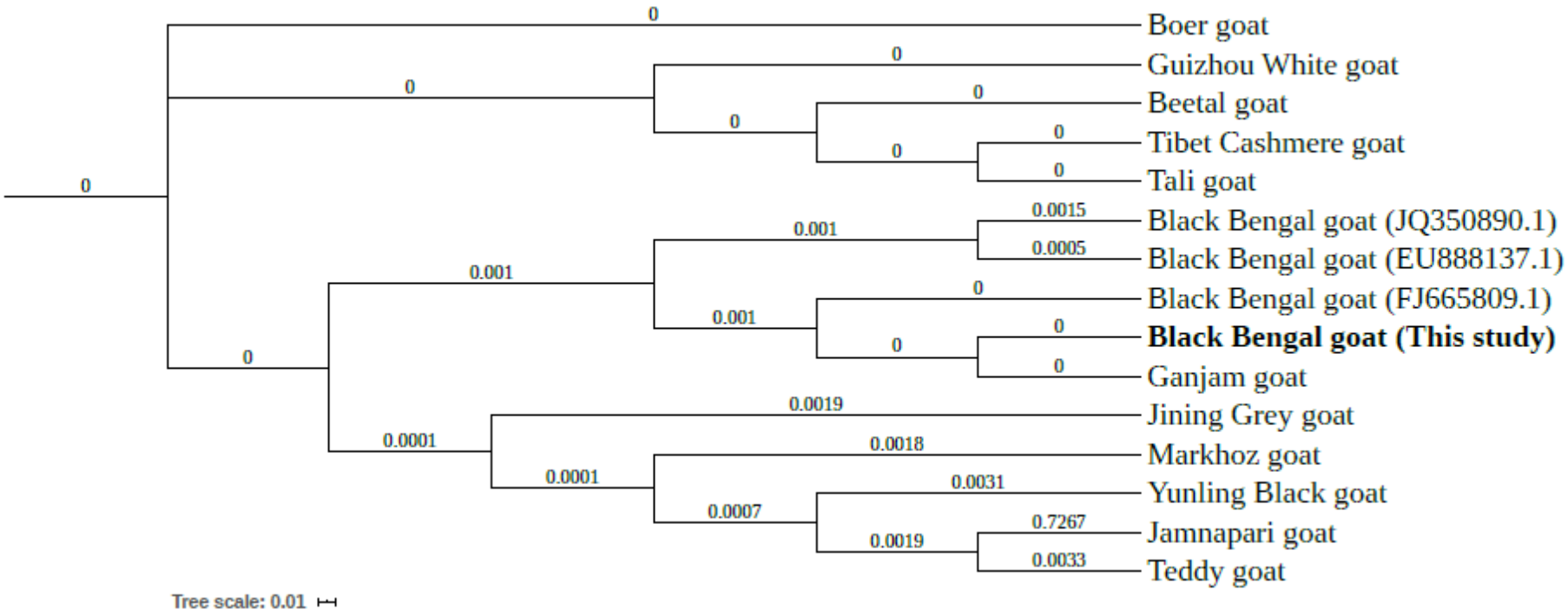

Figure 4. Phylogenetic tree of *BMP15* gene.

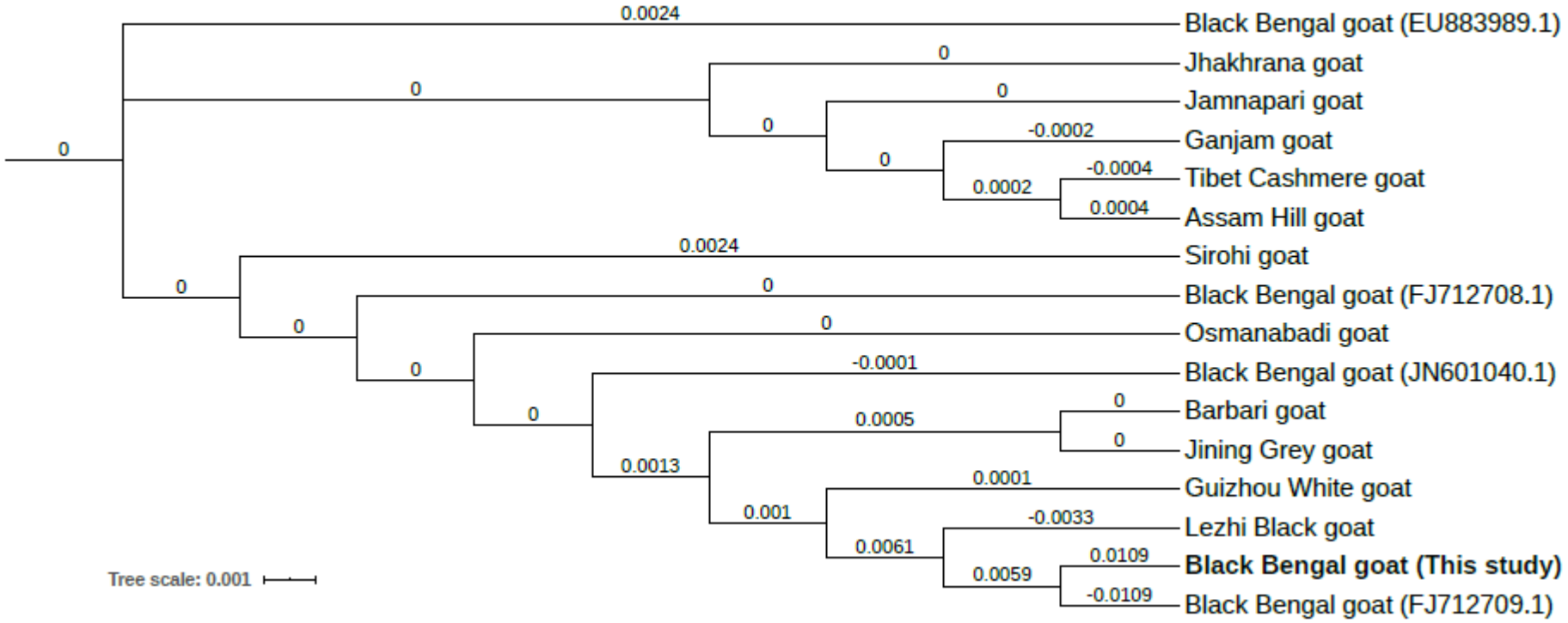

Figure 5. Phylogenetic tree of *GDF9* gene.