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Mutation Analysis and Restriction Site Mapping of GDF9 in Indonesian Bligon Goat

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ABSTRACT

Bligon goat is one of the crossbred goats raised in Indonesia, which has a prolific nature, potentially increasing the economic benefits. The gene that particularly influences prolific traits is Growth Differentiation Factor 9 (GDF9). This study aims to identify the mutation analysis (SNPs and amino acid changes) and restriction enzymes map in the GDF9 of Bligon goats. Six pairs of primers were used to amplify target sequences of GDF9 by polymerase chain reaction method and continued by sequencing. The sequence products were analyzed to get information on the SNPs and restriction enzyme (RE) around the SNPs for genotyping by PCR-RFLP method. A total of 15 SNPs were found in position g.1956A>C, g.2248G>T, g.2470A>T, g.2172DelA (shift T, Heterozygote), g.2807C>T, g.2919C>T, g.2996C>T, g.3615T>C, g.3855A>C, g.3879A>G, g.3924A>G, g.3943G>T, g.3969G>A, g.3981G>A, and g.4314C>T. Eight out of fifteen SNPs are located at the exon. Thus, the amino acid shows one synonymous at Exon 1 (Leucine to Leucine) and seven non synonymous at exon 2 with varied amino acid alteration (Valine to Alanine, Glutamine to Proline, Lysine to Arginine, Lysine to Arginine, Glutamine to Histidine, Arginine to Lysine, and Serine to Asparagine, respectively). Two SNPs at position g.1956A>C in Exon 1 and g.3855A>C in exon 2 show the homozygote CC and heterozygote AC. The most sample at those two position 67% and 83% homozygote type, respectively. Recognitions of site restriction enzymes BsaI and BsmAI were found at g.1956A>C or g.667C>M (Exon I). SNP g.3855A>C or g.2566C>M was recognized by three restriction enzymes (MspI, HapII, and HpaII). Two SNPs were not recognized by the restriction enzyme, and two other SNPs have more than 12 fragmented sequences, and as a consequence, it is very difficult to analyze the genotype. In conclusion, fifteen site mutations were identified; however, only two potential genetic markers with transversion mutations in Exon 1 and Exon 2 were recognized by restriction enzymes (BsaI, HapII, and MspI). These enzymes were recommended as candidate markers for further genotype identification using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method.

Keywords: GDF9; restriction enzyme; single nucleotide polymorphism

INTRODUCTION

Bligon goat, produced from a cross between the Kacang and Ettawa Grade goats, is one of the livestock commodities bred in Indonesia. This crossbred goat is adaptable and has prolific traits and better productivity than Kacang goats. The prolificacy in goats is defined as the number of kids born per doe kidding (called litter size). This nature provides economic benefits and helps breeders select female goats with highly prolific traits. Natural selection takes a long time. Therefore, genetic marker technology has been developed using DNA that is identified at a certain location and expressed as phenotype, which can be inherited by the next generation based on inheritance laws. Based on previous studies, genetic markers can be used to select certain traits in living things. Genetic markers work specifically on certain individuals and genes; hence, they can be correlated with desired traits.

The prolific trait is influenced by several genes, such as Growth Differentiation Factor 9 (GDF9), which belongs to the superfamily of transforming growth factors. GDF9 plays an important role as a growth and differentiation factor during early follicle genesis in mammalian female reproduction (Elvin *et al.*, 1999) and is essential for normal follicle genesis in sheep (Hanrahan *et al.*, 2004). GDF9 gene had a role as oocyte-secreted paracrine factors and autocrine factors in granulosa cells (Silva *et al.*, 2004). Furthermore, the GDF9 strongly influences the increased ovulation rate, and polymor-

phism in the gene is associated with litter size (Wang *et al.,* 2021; Polley *et al.,* 2009). One point mutation of the GDF9 in Chinese goats is associated with ovulation rate (Du *et al.,* 2008). Several significant associations have also been reported between GDF9 polymorphism and ovulation rate, prolificacy, and fertility in sheep (Yuliana *et al.,* 2019). A genetic variant of the GDF9 gene is considered a strong gene marker for increasing fecundity in Egyptian Sheep and Goats (Aboelhassan *et al.,* 2021). A previous study stated that GDF9 shows some important enzymes of granulosa cells that are involved in the expansion and maintenance of the cumulus oophorous in the optimal microenvironment of the egg cell through the interaction and synergy, which is essential for normal ovulation, fertilization, and female reproduction (Chu *et al.,* 2011).

Single Nucleotide Polymorphism (SNP) of GDF9 in goats has been studied to determine the type of mutation and amino acid changes at SNP sites (Ghoreishi *et al.,* 2019; Feng *et al.,* 2011; Das *et al.,* 2021). The difference sequence around the SNPs position can be developed as a marker associated with economic traits, such as growth and litter size in goats. The popular method is the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Restriction enzymes are used to recognize specific sequences and are also known as restriction endonucleases that cleave DNA. Site recognition consists of palindromic of 4–8 base pairs (Pingoud & Jelsch, 2001). This information was collected from several resources related to restriction enzyme mapping. Samples with nucleotides that differ in the same SNP sites will vary in the distance between the cleavage sites of certain restriction endonucleases that make the fragment length generated will be different. A restriction enzyme is one of the markers for genotyping using the PCR-RFLP method (Perdana & Hartatik, 2022). PCR-RFLP with BsaI (recognition site restriction: GGTCTCn'nnnn) has been used to investigate the polymorphism of exon I of GDF9 by Ghoreishi *et al.* (2019) The study found a mutation in Exon I of GDF9 which is associated with prolificacy of Markhoz goats. Three genotypes CC ($BsaI^+/BsaI^+$), CA ($BsaI^+/BsaI^-$), and AA ($BsaI^-/BsaI^-$), were detected for GDF9 with the size of fragment 234 bp/229 bp, 234 bp/229 bp/463 bp, and 463 bp/463 bp, respectively. Homozygote AA in GDF9 significantly increased litter size. Since GDF9 is one of the candidate genes which are associated with litter size and there is no study on the mapping of restriction enzymes of the GDF9 in Bligon goats, so it is interesting to explore the mutation analysis (SNPs and amino acid changes) and mapping of restriction enzymes in the full coding sequences of the GDF9 in Bligon goats.

MATERIALS AND METHODS

Samples and DNA Extraction

Genomic DNAs were obtained from the blood samples of 60 Bligon goats. The blood samples were collected from the jugular vein into a vacutainer K3EDTA (ethylene diamine tetra acetic acid) anticoagulant tubes and kept at 4 °C temperature before DNA isolation. Then the genomic DNA was extracted using SYNCTM DNA Extraction Kit (Geneaid, Taiwan) at Laboratory of Animal Breeding and Genetics, Department of Animal Breeding and Reproduction, Faculty of Animal Science, Universitas Gadjah Mada. The blood sampling was conducted according to the ethical clearance with no. 0103/ECFKH/Eks./2019.

DNA Amplification and Sequencing

Gene bank accession numbers EF446168, EU883989, and KY780296 were used as references for the alignment sequence of GDF9 and to design the primer of the target sequences with SNPs. The primer design in this study followed the online Primer3 program. Six pairs of primers GDF9-1, GDF9-2, GDF9-3, GDF9-4, GDF9-5, and GDF9-6 were used to amplify target sequences. The genomic DNA samples were amplified using the PCR method with six target sequences. The primer sequence, location, and product size are shown in Table 1, while the GDF9 primer location scheme based on the EF446168 GenBank reference is presented in Figure 1.

The polymorphism chain reaction was performed to amplify the target sequence. A total reaction of 25 µL, containing 12.5 µL of PCR Kit (KAPA BIOSYSTEMS, USA), 9.5 µL of DDW, 2 µL of DNA (100 ng/µL), and

Table 1. Primer information and sequence of target location of growth differentiation factor 9 (GDF9) in *Capra hircus* based on GenBank: EF446168.2

Primer name	Location	Primer sequence	Product (bp)	Annealing (°C)
GDF9-1	1652-2419	PF :5′- AGAAGTGAACCTAGCCCACC-3′	768	57
		PR : 5′-CTAACCTCCAGCAGCACTCT-3′		
GDF9-2	2924-3760	PF :5′- AACAAGCCTGGCAAGTGTCT-3′	837	58
		PR : 5′-AGGCTTCCTTTTAGGGTGGA-3′		
GDF9-3	3549-4004	PF : 5′-CTCCTCTTGAGCCTCTGGTG-3′	456	58
		PR : 5′-TCCAGTTGTCCCACTTCAGC-3′		
GDF9-4	1290-2057	PF:5′-CAGATCCAGGGAGAAGAGT-3′	768	53
		PR:5′-GCCTTATAGAGCCTCTTCATG-3′		
GDF9-5	2187-3176	PF: 5′-TGGTTAATGGGTGGAGGGAA-3′	990	58
		PR: 5′-TCAGGGTGGGATAGGGGTTA-3′		
GDF9-6	3826-4732	PF: 5′-TGTAAGATCGTCCCGTCACC-3′	907	56
		PR:5′- CACACTTCCTCTCCCTCTCA-3′		

0.5 µL (10 pmol) of both forward and reverse primers were used. The reactions were performed using a thermal cycle (PEQLAB Primus 25 advanced, Germany) with a pre-denaturation temperature at 94 °C for 5 minutes, followed by 35 cycles of reaction, denaturation at 94 °C for 30 seconds, annealing at a temperature depending on the recommendation of the primer for 40 seconds, as well as an extension at 72 °C for 30 seconds, then the last step was a final extension at 72 °C for 4 minutes (Table 2). The quality of the PCR product was determined using gel electrophoresis at 2%, 50 Volt, and for 30 minutes. The results were viewed using a UV illuminator, and the product size was adjusted with a DNA marker, as shown in Figure 2. Subsequently, the PCR product with good quality was selected for further sequencing using LPPT UGM facilities.

GDF9 Sequence Alignment and Analysis

Single nucleotide polymorphism identification and comparison sequences were performed using BioEdit software. The sequences of partial GDF9 (GDF9-1 to GDF9-6) from the genomic DNA of Bligon goat were aligned using ClustalW on BioEdit ver. 7.2.5 to identify the SNPs representing the mutation type and determine the restriction enzyme mapping. To determine the longer sequences, the fragmentary sequences of each target gene (GDF9-1 to GDF9-6) were joined together by overlapping sequences.

GenBank Submission

Six individual representative sequences of full GDF9 were submitted to GenBank with BankIt tools with ID 2625695, submitted on 21 September 2022. The fasta format of each genomic DNA sequence and amino acid translation was prepared before the submission and followed the step of online submission (https://www.ncbi.nlm.nih.gov/WebSub/). The GenBank accession numbers OP494705, OP494706, OP494707, OP494708, OP494709, and OP4947010 were used for further analysis.

Identification of Restriction Enzyme and PCR-RFLP

The restriction enzyme sites in the full sequences of GDF9 were recognized by using Nebcutter V2 and or BioEdit ver. 7.2.5. The mappings were analyzed for selected enzymes that recognize the specific small number of sequences around the SNP. Different SNPs can lead to varying types of restriction enzymes, the frequency, and the position of the cutting site. A total number of 42 PCR products were digested with MspI restriction enzyme by following this procedure: A total volume of 12 µL mixture contains 7.7 µL of DDW, 1.2 µL of buffer, 3.0 µL of PCR product (DNA), 0.1 µL of MspI restriction enzyme were mixed into a 0.6 mL microtube, then incubated in a hotplate for 60 minutes at 60 °C, then inactivated with a hotplate for 20 minutes at 80 °C. The PCR-

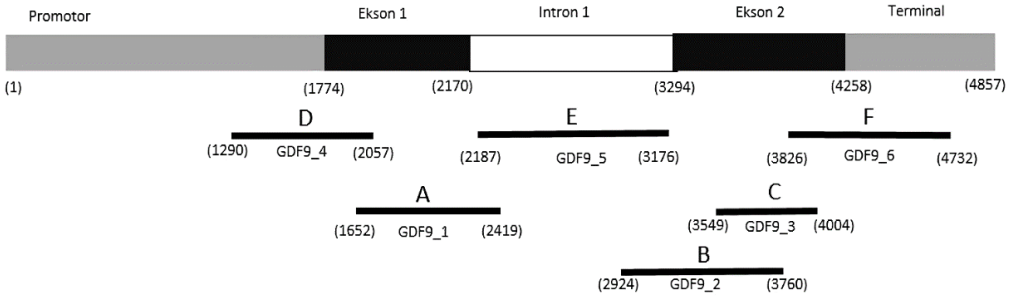

Figure 1. Gene structure of growth differentiation factor 9 (GDF9) in *Capra hircus* and six overlapping target positions based on GenBank EF446168. Note: A= GDF9-1 primer; B= GDF9-2 primer; C= GDF9-3 primer; D= GDF9-4 primer; E= GDF9-5 primer; F= GDF9-6 primer.

Table 2. Polymerase chain reaction-restriction (PCR) programs for amplification of sequence target of growth differentiation factor 9 (GDF9) in *Capra hircus*

PCR program	Sequence target					
	GDF9-1	GDF9-2	GDF9-3	GDF9-4	GDF9-5	GDF9-6
Initiation denaturation	94 °C	94 °C	94 °C	94 °C	94 °C	94 °C
	5 min.	5 min.	5 min.	5 min.	5 min.	5 min.
Denaturation	94 °C	94 °C	94 °C	94 °C	94 °C	94 °C
	30 sec.	30 sec.	30 sec.	30 sec.	30 sec.	30 sec.
Annealing	57 °C	58 °C	58 °C	53 °C	58 °C	56 °C
	40 sec.	40 sec.	40 sec.	40 sec.	40 sec.	40 sec.
Extension	72 °C	72 °C	72 °C	72 °C	72 °C	72 °C
	30 sec.	30 sec.	30 sec.	30 sec.	30 sec.	30 sec.
Final extension	72 °C	72 °C	72 °C	72 °C	72 °C	72 °C
	4 min.	4 min.	4 min.	4 min.	4 min.	4 min.

Note: Each primer has a PCR program and different annealing temperatures.

RFLP product was electrophoresed at 3% agarose gel for 30 min, 100 Volt to separate the fragmented DNA.

RESULTS

Amplification GDF9 Sequence

The results of amplified DNA sequences are shown in Figure 2. The PCR process was carried out using six pairs of primers with overlapping at the ends of target sequences (Figure 1). The overlapping sequence length was varied from 193 bp to 406 bp. The PCR product of GDF9-1 was 768 bp. The overlapping sequences between GDF9-1 with GDF9-4 and GDF9-5 were 406 bp and 232 bp, respectively. Another example, the target sequence of GDF9-3 overlapped with GDF9-2 and GDF9-6 along 213 bp and 193 bp, respectively. The target sequences were coverage the full coding sequence of GDF9 in Bligon goats.

Single Nucleotide Polymorphisms of GDF9

The DNA sequencing was performed by direct primer forward GDF9-1 (10 sequence results), GDF9-2 (10 sequence results), GDF9-3 (10 sequence results), GDF9-4 (9 sequence results), GDF9-5 (10 sequence results), and GDF9-6 (6 sequence results), the sample alignment results are checked based on each target. Therefore, some SNP appear twice in overlapping target sequences depending on the position of the SNPs (Table 3). Based on the results, fifteen SNP positions were found in GDF9-1 (2 SNPs), GDF9-2 (2 SNPs), GDF9-3 (7 SNPs), GDF9-4 (1 SNP), GDF9-5 (6 SNPs), and GDF9-6 (7 SNPs). The first SNP was located at Exon 1, namely g.1956A>C at positions 1290 to 2057 along 768 bp, flanked by primer GDF9-4. GDF9-1 at position 1652-2419 along 768 bp had 2 SNPs, namely g.1956A>C (overlapped with GDF9-4) and g.2248G>T (overlapped with GDF9-5), while GDF9-5 at position 2187 to 3176 along

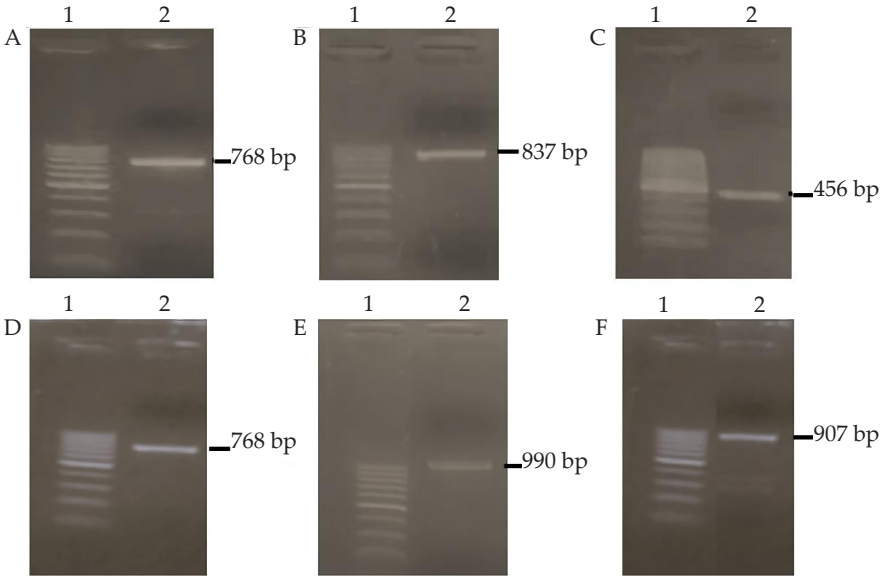

Figure 2. Product size of PCR using 6 pair of primers in growth differentiation factor 9 (GDF9) target gene of Bligon goat. Note: A= GDF9-1; B= GDF9-2; C= GDF9-3; D= GDF9-4; E= GDF9-5; F= GDF9-6; Lane 1= 100 bp DNA Marker; Lane 2= PCR Product; bp= base pair.

Table 3. Mutation type at SNPs position of growth differentiation factor 9 (GDF9) in Bligon goat with GenBank EF446168

No.	Position	Mutation	Primer	Location
1*	g.1956A>C	Transversion	GDF9-4 and GDF9-1	Exon1
2	g.2248G>T	Transversion	GDF9-1 and GDF9-5	Intron
3	g.2470 A>T	Transversion	GDF9-5	Intron
4*	g.2712IndelA	Deletion	GDF9-5	Intron
5	g.2807C>T	Transition	GDF9-5	Intron
6	g.2919C>T	Transition	GDF9-5	Intron
7	g.2996 C>T	Transition	GDF9-5 and GDF9-2	Intron
8	g.3615T>C	Transition	GDF9-2 and GDF9-3	Exon2
9*	g.3855A>C	Transversion	GDF9-3 and GDF9-6	Exon2
10*	g.3879A>G	Transition	GDF9-3 and GDF9-6	Exon2
11	g.3924A>G	Transition	GDF9-3 and GDF9-6	Exon2
12*	g.3943G>T	Transversion	GDF9-3 and GDF9-6	Exon2
13*	g.3969G>A	Transition	GDF9-3 and GDF9-6	Exon2
14	g.3981G>A	Transition	GDF9-3 and GDF9-6	Exon2
15*	g.4314C>T	Transition	GDF9-6	3′-UTR

990 bp had 6 SNPs (Table 3). One deletion was found at position 2712 bp, which indicates the heterozygous sample. No homozygous deleted sequence was found in this study. The sample numbers K46.1 & K48.1 show a heterozygous pattern on the electropherogram sequence with one deletion A nucleotide and shift next sequence T (Figure 3). The frequency of genotype with deletion allele and insertion allele were 0.2 and 0.8, respectively. The position of GDF9-2 at 2924 to 3760, along with 837 bp, had 2 SNPs, namely g.2996C>T (overlapped with GDF9-5 at position 2186 to 3176) and g.3615T>C (overlapped with the GDF9-3 at position 3549 to 3760). The position of GDF9-3 at 3549 to 4004 along 456 bp had 7 SNPs in exon 2 containing one SNP g.3615T>C, which overlapped with the GDF9-2 target sequence, and 6 SNPs which overlapped with the GDF9-6 target sequence at position 3820 to 4004. The last SNP g.4314C>T flanked by GDF9-6 primers was located at 3'-UTR (Table 3). Seven out of fifteen SNPs in Table 3 indicated the variation of Bligon goat (marked with star "*").

The scheme of GDF9 in this study based on the EF446168 GenBank reference is presented in Figure 1. The alignment sequences represented the position of SNPs. Subsequently, SNPs were also detected using an electropherogram, showing homozygous and heterozygous sequences. Homozygous SNPs are indicated by a single peak, while heterozygous SNPs are indicated by double peaks, as shown in Figure 3.

Mutation Analysis and Amino Acid Changes

Eight out of fifteen SNPs are located at Exon (Table 4). Thus, the amino acid shows one synonymous (silent mutation) at Exon 1 and seven non synonymous (missense mutation) at exon 2. Indel variation was located at intron (g.2712IndelA) and one SNP at 3'UTR. Two SNPs at position g.1956A>C in Exon 1 and g.3855A>C in exon 2 showed the homozygote CC and heterozygote AC. The most sample at those two positions had 67% and 83% homozygote type, respectively. SNP at position g.4314C>T in 3'UTR also had homozygote TT (50%) and heterozygote CT (50%).

GenBank GDF9 of Bligon Goat and SNPs Analysis

Six representative GDF9 sequences of Bligon goat were OP494705, OP494706, OP494707, OP494708, OP494709, and OP4947010. The length of GDF9 se-

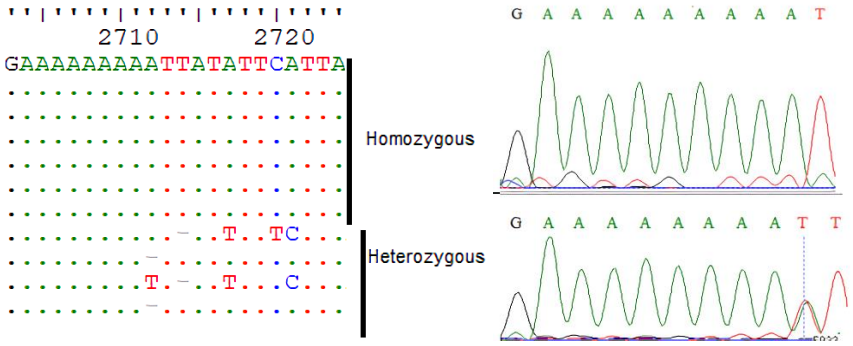

Figure 3. Sequence alignment and chromatogram that represented the deletion, homozygous (single peak A), and heterozygous (double peaks T and A, as a result of deletion of A and shift of one T) in growth differentiation factor 9 (GDF9) target gene of Bligon goat.

Table 4. Amino acid change and mutation type of growth differentiation factor 9 (GDF9) in Bligon goat compared to GenBank reference EF446168

No.	SNP position	Codon	Amino acid	Mutation type	
				Amino Acid	Nucleotide
1	g.1956A>M	CUA	Leucine	Silent mutation	Transversion
		CUC	Leucine		
2	g.3615T>C	GUG	Valine	Missense mutation	Transition
		GCG	Alanine		
3	g.3855A>M	CAG	Glutamine	Missense mutation	Transversion
		CCG	Proline		
4	g.3879A>G	AAG	Lysine	Missense mutation	Transition
		AGG	Arginine		
5	g.3924A>G	AAA	Lysine	Missense mutation	Transition
		AGA	Arginine		
6	g.3943G>T	CAG	Glutamine	Missense mutation	Transversion
		CAU	Histidine		
7	g.3969G>A	AGA	Arginine	Missense mutation	Transition
		AAA	Lysine		
8	g.3981G>A	AGU	Serine	Missense mutation	Transition
		AAU	Asparagin		

quences with one deletion A was 3442 bp, and the others were 3443 bp. Analysis mutation according to the GDF9 GenBank data of Bligon goat was presented in Table 5. There are seven SNPs along the regions of Exon 1, Intron, Exon 2, and 3'UTR.

Restriction Enzyme Mapping

The restriction enzymes found around the SNPs sequence at a position in g.1956A>C or g.667C>M (Exon I) were BsaI (three sites for allele A and four sites for allele C) and BsmAI (six sites for allele A and seven sites for allele C) restriction enzymes which recognized the sequence GGTCTCn'nnnn. This site identifies two genotypes CC and AC, of the Bligon goat. The genotype CC has product sizes of 305 bp and 463 bp, while genotype AC has product sizes of 768 bp, 463 bp, and 305 bp. Restriction enzymes HapII, HpaII, and MspI in SNP g.3855A>C or g.2566C>M show the unique site restriction that only one restriction site and can be used for genotyping with three forms of genotypes CC, AA, and AC (Table 5). Genotype AA has one fragment with a 456 bp product size, genotype AC has three fragments with 456 bp, 307 bp, and 149 bp product sizes, and genotype CC has two fragments with 307 bp and 149 bp product sizes. The restriction mapping results based on Bioedit software are shown in Table 6, that only four out of six SNPs were recognized by the restriction enzyme. However, only two marker SNPs were recommended as markers using restriction enzymes (BsaI at exon 1 and MspI at exon 2). Total samples of 42 with single (21 samples) and twin (21 samples) litter size has been digested with MspI; however, all samples show homozygote AA (456 bp).

DISCUSSION

The PCR product is very clear, with product sizes of 456 bp to 990 bp. According to Garibyan & Avashia (2013), there are two main ways to visualize PCR products, i.e., (1) stain the amplified DNA product with a chemical dye such as ethidium bromide that is inserted between the two double-stranded strands, or (2) label it with PCR primers or nucleotides. PCR is the starting point for all analyses for PCR-RFLP and sequence analysis. The result of mutation analysis in GDF9 of Bligon goat contains 6 SNP substitutions at Exon 1, Exon 2, and 3'UTR. One insertion/deletion was found in the intron. Restriction enzyme sites of BsaI, HapII, HhaI, HpaII, Hpy188I, and MspI were found along the sequence in GDF9 of Bligon goat. Restriction enzyme BsaI located at Exon I. Restriction enzyme BsaI digestion at the SNP of exon 1 GDF9 gene in Markhosz goats resulted in three genotypes: AA, AC, and CC (Ghoreishi *et al.*, 2019), but only two genotypes (CC and AC) were found in Bligon goat with the low frequency

Table 5. GenBank accession number of Bligon goat (OP494705- OP4947010) and SNPs location of growth differentiation factor 9 (GDF9)

Type	GenBank Acc. No.	Sequence size	Location/SNPs of GDF9						
			1	2	3	4	5	6	7
			Exon 1 g.667C>M	Intron 1423IndelA	Exon 2 g.2566C>M	Exon 2 g.2590G>A	Exon 2 g.2654G>T	Exon 2 g.2680A>G	3'-UTR g.3025C>Y
1	OP494705	3442 bp	C	Deletion	C	G	G	A	Y
2	OP494706	3443 bp	C	Insertion	A	A	T	A	Y
3	OP494707	3443 bp	M	Insertion	C	G	G	G	T
4	OP494708	3443 bp	M	Insertion	M	G	T	A	T
5	OP494709	3443 bp	C	Insertion	C	G	G	A	T
6	OP4947010	3442 bp	C	Deletion	A	A	T	A	Y

Note: A= adenine, C= cytosine, G= guanine, T= thymine, M= C or A , Y= C or T.

Table 6. Identification restriction enzyme around the SNPs in growth differentiation factor 9 (GDF9) target gene of Bligon goat

Type	GenBank Acc. No.	Location/SNPs					
		Exon 1 g.667C>M	Exon 2 g.2566C>M	Exon 2 g.2590G>A	Exon 2 g.2654G>T	Exon 2 g.2680A>G	3'-UTR g.3025C>Y
		Sequence around SNP GGTCT**C**n'nnnn_	C'CG_G	-	TCNGA	-	T'TA_A
		Restriction enzyme BsaI/BsmAI	MspI/HapII/HpaII	No Enzyme	Hpy188I	No Enzym	MseI
1	OP494705	+/+	+/+	-	-/-	-	+/+
2	OP494706	+/+	-/-	-	+/-	-	+/+
3	OP494707	+/-	+/+	-	+/-	-	+/+
4	OP494708	+/-	+/-	-	+/-	-	+/+
5	OP494709	+/+	+/+	-	+/-	-	+/-
6	OP4947010	+/+	-/-	-	-/-	-	+/-

Note: +/+ (homozygote, cut by restriction enzyme), -/- (homozygote, uncut by restriction enzyme), +/- (heterozygote).

of heterozygote AC. Genotype AA was lost from the population of Bligon goats. However, according to the result from Markhosz goats, genotype AA showed the largest litter size of the other genotypes. Four mutations in exon 2 of the GDF9 were detected extensively in several goat breeds (Wouobeng *et al*., 2020). Other results were presented by Feng *et al*. (2011) in the study of the Jining Gray goat. Four SNPs at GDF9 in Jining Gray goat nucleotide changes were identified based on AF078545. Our results showed that six SNPs at exon 2 of GDF9 in the Bligon goat were identified based on EF446168. One of those SNPs is located at Exon 2 and recognized by MspI restriction enzyme. PCR-RFLP with MspI restriction enzyme at the locus of 959 GDF9 in Jining Gray goats (g.2566C>M in Bligon goat) expressed three genotypes: AA (MspI-/MspI-), AC (MspI-/MspI+), and CC (MspI+/MspI+). The genotypes were determined by different fragment sizes: 150 bp for AA, 108/42 bp for CC, and 150/108/42 bp for AC at partial Exon 2 of GDF9 in Jining Grey goats, while fragment sizes of genotype at partial Exon 2 of GDF9 in Bligon goats were 456 bp for AA, 309/147 bp for CC, and 456/309/147 bp for AC. The litter size of Jining Gray goats with genotype AA, AC, and CC were 2.00±0.14, 2.63±0.12, and 2.81±0.17, respectively. Litter size in Jining Grey goats was significantly influenced by sire, kidding season, and parity. For the locus of 959, the Jining Grey goat does with genotype CC or AC had 0.81 or 0.63 kids more than those with genotype AA, respectively. No significant difference was found in litter size between CC and AC genotypes in Jining Grey goats (Feng *et al*., 2011). Even though the restriction mapping of MspI was recognized at SNP g.2566C>M and resulted in three genotypes (Tabel 6) of Bligon goats, further analysis with PCR-RFLP method showed homozygote AA. The major genotype AA at Bligon goat probably was caused by negative selection and or a limited population in certain locations which influenced the mating process. The mating process in a small population also influenced the allele frequencies in a population over generations. An evolutionary mechanism that produces random (rather than selection-driven) changes in allele frequencies in a population over time was called genetic drift.

Bi *et al*. (2020) reported that three SNPs of the GDF9 (p.proline27alanine (P27R), p.leucine61leucine (L61L), and p.alanine85glycine (A85G)) were identified in Shaanbei white cashmere (SBWC) goats. Two SNPs are missense mutations (P27R and A85G) among the three SNPs. SNP P27R, where TGC (proline) transformed to TGG (alanine), and SNP A85G locus, where CCT (alanine) transformed to CCG (glycine). They contribute to amino acid type change during encoding of the goat GDF9 and significantly correlate with litter size. This information provides insights for exploiting different markers to be associated with beneficial traits. The discovery of SNPs can be used as genetic markers to improve traits that have economic value in goat breeding (Zhang *et al*., 2019). Wang *et al*. (2019) used NCBI data to find effective molecular markers for the genetic breeding of goats and accelerate repair. There are 45 SNPs classified into four types, as well as compared and analyzed the effects of similar and related effects of different SNPs on reproductive traits in different breeds of goats. Although there were several SNPs in the goat GDF9, only 15 have been identified in more than 30 goat breeds worldwide, and they show distinct effects on litter size. Therefore, this study primarily selected these 15 SNPs and discussed their relationships to goat productivity. The results showed that three non-synonymous SNPs, namely A240V, Q320P, V397I, and three identical L61L, N121N, and L141L, play an "actual" role in litter size traits, but the regulatory mechanism still needs further investigation. These results provide an effective tool for further studies to develop goat molecular breeding strategies and enhance goat reproductive traits. Similar to the study by Das *et al.* (2021) using 40 Black Bengal goats, the study found that one SNP in GDF9 had a significant on litter size.

Hossain *et al*. (2020) reported two polymorphic regions of GDF9 (FecG1 and FecG8) digested by HhaI and DdeI restriction enzymes. There are three genotypes (GG, AG, AA) for FecG1, with the genotype frequency continuously 51.59%, 45.24%, and 3.17%, and the allele (G and A) frequencies in the overall population were 74.21% and 25.79%. There was a significant association of FecG1 of GDF9 gene polymorphism with litter size. The homozygous GG genotype had the smallest litter size, and the homozygous AA genotype had the highest (Hossain *et al*., 2020). Hanrahan *et al.* (2004) reported the G to A nucleotide change in GDF9 exon 1 disrupts a Hha I restriction enzyme cleavage site (GCGC to GCAC) at nucleotide 260 of the 462-bp PCR fragment product. Restriction enzyme digestion of the PCR product from wild-type animals with HhaI resulted in cleavage of

Table 7. Recognition site of restriction enzymes in growth differentiation factor 9 (GDF9) target gene of Bligon goat

Enzyme	Recognition	Frequency	Recommendation	References
BsaI	GGTCTCn'nnnn_	3	+	This study
BsmAI	GTCTCn'nnnn_	6	-	This study
DdeI	C'TnA_G	16	-	This study, El-Maaty *et al.* (2022)
HapII	C'CG_G	1	+	This study
HhaI	G_CG'C	5	-	This study, Hanrahan *et al.* (2004)
HpaII	C'CG_G	1	+	This study
Hpy188I	TC_n'GA	12	-	This study
MseI	T'TA_A	33	-	This study
MspI	C'CG_G	1	+	This study, Feng *et al.* (2021)

Note: + (recommended for PCR-RFLP), - (not recommended for PCR-RFLP).

the 462-bp product (at two internal HhaI sites) into fragments of 52 bp, 156 bp, and 254 bp. However, DNA fragments containing the A nucleotide yield only two fragments (52 bp and 410 bp). Animals heterozygous for the mutation have fragments of all four sizes (52 bp, 156 bp, 254 bp, and 410 bp). HhaI restriction enzyme in our study was identified at 5 positions (491, 745, 2355, 2391, and 3022), but all of the sites do not match the GDF9 SNPs position in Bligon goats. Thus, HhaI restriction enzymes in GDF9 are not recommended for the genetic marker of litter size in Bligon goats.

Exon 1 showed the SNP g. 667C>M and this SNP position GGTCT**C**n'nnnn_was recognized by BsaI and BsmAI, which cut at 3 and 6 sites, respectively. Restriction enzyme DdeI found 16 sites along 3443 bp in GDF9 sequence of Bligon goat. In a study to explore the diet restriction and the polymorphism in the bone morphogenetic protein-15 (BMP15) and growth differentiation factor-9 (GDF9) on ovulation and reproductive hormones in three sheep breeds, by El-Maaty *et al.* (2022), reported that the result of PCR-RFLP with Ddel endonucleases in FecGH locus (exon-1) of the GDF9 showed mutation. Regarding GDF9 (FecGH), the allele (B) frequency is higher than that of (A), and the homozygote (BB) frequency is markedly higher than heterozygote (AB) in both control and treated ewes. The fertility rate in these breeds is related to the GDF9 (FecG7 and FecGH). Restriction enzyme DdeI recognized the sequence C'TnA_G. There are 16 site frequencies in the Bligon goat study. Therefore, DdeI restriction enzyme and other restriction enzymes with more than 5 restriction sites were not included as marker for Bligon goats (Table 7). Less than 5 fragments with site restriction ranging from one to 4 sites can be used as a reference to make the right decision to choose a restriction enzyme. BsaI and MspI restriction enzymes are the most appropriate choices as a marker for Bligon goats. Different populations have specific conditions and genetic diversity, so studying SNPs and restriction enzyme mapping are important as alternative methods for genotyping in large-scale samples.

CONCLUSION

Six novel SNPs and one deletion were identified along 3443 bp of GDF9 sequence in Bligon goat. There are two SNPs that were potential for the genetic markers: g.667C>M (Exon I) and g.2566C>M (Exon 2) with transversion mutation. Three restriction enzymes (BsaI, HapII, and MspI) were recommended as candidate markers for further research. The implication of the research about mutation analysis and restriction enzyme map is expected to provide accurate information, quicker and cheaper for genotyping livestock using the PCR-RFLP method. Since GDF9 has a potential role in follicles development and affects the litter size, the mutations at g.667C>M (Exon I) and g.2566C>M (Exon 2) in GDF9 of Bligon goat can be proven as genetic markers for the female. Further research with a larger sample size is needed to adjust mating patterns to increase the number of minor genotypes.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest with any organization or third party regarding the material discussed in this research.

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