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Non-synonymous Single Nucleotide Polymorphisms (SNPs) on Growth Differentiation Factor 9 as candidate gene for reproduction in Indonesian local cattle

IRMA[1,♥], SITI DARODJAH RASAD[2], NENA HILMIA[2], CECE SUMANTRI[3]

[1]Graduate School of Animal Husbandry, Universitas Padjadjaran. Jl. Raya Bandung Sumedang Km. 21, Sumedang 45363, West Java, Indonesia. Tel.: +62-227-798241, ♥email: irma20006@mail.unpad.ac.id

[2]Department of Animal Production, Faculty of Animal Husbandry, Universitas Padjadjaran. Jl. Ir. Soekarno Km. 21 Jatinangor, Sumedang 45363, West Java, Indonesia

[3]Department of Animal Production and Technology, Institut Pertanian Bogor. Jl. Raya Dramaga Kampus IPB Dramaga, Bogor 16680, West Java, Indonesia

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Abstract. *Irma, Rasad SD, Hilmia N, Sumantri C. 2024. Non-synonymous Single Nucleotide Polymorphisms (SNPs) on Growth Differentiation Factor 9 as candidate gene for reproduction in Indonesian local cattle. Biodiversitas 25: 4011-4019.* Reproduction is a biological process that plays an essential role in livestock production. In mammals, the tendency to spontaneously conceive and maintain an embryo is a complex biological process affected by environmental and genetic factors, such as Growth Differentiation Factor 9 (GDF9). Therefore, this study aims to analyze the diversity of the GDF9 gene in the three breeds of cattle (*Bos taurus*), namely *Peranakan Ongole* (PO), Belgian Blue (BB), and its crossbreed (BB×PO) by molecular sequencing. A total of 20 blood samples were taken from cattle in the Livestock Embryo Centre, Bogor, Indonesia. DNA was extracted with genomic kit protocol followed by Polymerase Chain Reaction (PCR). Single nucleotide polymorphisms (SNPs) were then analyzed using Sanger sequencing. Sequence analysis was performed using Bio-Edit, Finch TV, and Molecular Evolutionary Genetic Analysis (MEGA) software. Variables analyses were allele and genotype frequency. The results showed that there were five non-synonymous mutations, namely c.589T>G (rs525937888), c.659T>G, c.974C>G, c.1105T>A, and c.1358G>A. These missense mutations altered the amino acid of GDF9 protein at position p.197Phe>Val, p.220Leu>Arg, p.325Ser>Cis, p.369Trp>Arg, and p.453Arg>His, respectively. The results also showed that the mutation prevalence was higher in *Peranakan Ongole* compared to Belgian Blue and its crossbreed.

Keywords: Crossbreds, GDF9, missense, mutations, sequencing

Abbreviations: GDF9: Growth Differentiation Factor 9; PCR: Polymerase Chain Reaction; SNP: Single Nucleotide Polymorphism; TGF-β: Transforming Growth Factor Beta; PO: *Peranakan Ongole*; LEC: Livestock Embryo Centre; EDTA: Ethylene Diamine Tetra Acetic acid; NCBI: National Centre for Biotechnology Information; DNA: Deoxyribo Nucleic Acid; RNA: Ribo Nucleic Acid; MEGA: Molecular Evolutionary Genetic Analysis; SIFT: Sorting Intolerant From Tolerant; BCS: Body Condition Score; UTR: Un Translated Region; BMP-15: Bone Morphogenetic Protein 15; BMPR-1B: Bone Morphogenetic Protein Receptor 1B

INTRODUCTION

GDF9 is a transforming growth factor-β superfamily member that primarily regulates female reproduction (Belli and Shimasaki 2018; Ajafar et al. 2022). During female reproduction, specifically folliculogenesis and ovulation, several factors and genes have been reported to play an essential role (Zheng and Dean 2007). For example, three related oocyte-derived members, GDF9, BMP-15, and BMPR1B, are associated with fertility and fecundity (Marchitelli and Nardone 2015). The role of GDF9 in folliculogenesis and ovulation has been extensively reported where it plays a role in facilitating both processes (Rasheed et al. 2021). Oocytes secrete GDF9 and BMP15 to form heterodimers to regulate cumulus cell differentiation, proliferation, and expansion (Turathum et al. 2021). Recent study of vaccination against GDF9 in deer showed high antibody titers, and sheep (*Ovis aries* Linnaeus 1758) were infertile (Eckery et al. 2014). Li et al. (2019) reported that GDF9 promotes follicle-stimulating hormone-induced progesterone production in chicken follicular granulosa cells.

Several studies (Tang et al. 2013; Marchitelli and Nardone 2015) reported that GDF9 *Bos taurus* Linnaeus 1758 gene is in the fifth bovine autosomal chromosome. The length of approximately 2,754 bp and contains two exons separated by a single intron. Exon 1 is 397 bp from the ATG until CAG codon, while exon 2 is 1,360 bp from GAA until CTG codon (Access GenBank: GQ922451.1) (Marchitelli and Nardone 2015). According to UniProt Database (ID: Q9GK68), the bovine GDF9 encodes 453 amino acids which have glycosylation sites at Asparagine residues in positions 106, 163, 236, 255, 269, and 337. Other post-transitional modification was found in the disulfide bond between cysteine residue (352-418, 381-450, and 385-452). The result of GDF9 transcription is mRNA with a length of 1,816 bp (Access GenBank: NM_174681). The inactivation of GDF9 in pigs (*Sus domesticus* Erxleben 1777) showed blocked folliculogenesis (Chen et al. 2023a). This occurs because GDF9 is involved

in cumulus expansion maintenance of the microooocyte environment and is synergistic with BMP15 for regular ovulation. The mechanism by which GDF9 influences reproduction is explained by its ability to stimulate the production of proteins that promote follicular development, thereby regulating granulosa and theca cells during steroidogenesis.

Due to the site of GDF9 expression in ovarian cells, numerous studies have referenced its role in ovulation rate. In addition, it was investigated for the effect on reproduction in humans (*Homo sapiens* Linnaeus 1758), mice (*Mus musculus* Linnaeus 1758), sheep (*Ovis aries* Linnaeus 1758), cattle, goats (*Capra hircus* Linnaeus 1758), chickens (*Gallus gallus* Linnaeus 1758), and pigs. In human, a number of GDF9 mutations have been identified in the mother dizygotic twin (Belli and Shimasaki 2018). In mice, GDF9 significantly improve oocyte developmental competence (Akin et al. 2022). GDF9 polymorphism in sheep was reported to be associated with ovulation rate (Chen et al. 2023b). In chicken, mutation of GDF9 was associated with high egg production and egg weight (Qin et al. 2015). Another study in pigs reported only SNPs without trait association (Zhang et al. 2008). Intensive studies in GDF9 were conducted in small ruminants as animal models. At the same time, there is limited information on the genetic diversity of this gene in large animals such as cattle and buffalo (*Bubalus bubalis* Linnaeus 1758).

There are limited studies on GDF9 diversity in local cattle and its crossbred with taurine cattle in tropical areas by direct sequencing. Certain studies reported GDF9 polymorphism in Friesian Holstein by direct sequencing (Inayah et al. 2016). Santos-Biase et al. (2012) found a relationship between polymorphism and the number of oocytes. Another study revealed that GDF9 polymorphism was associated with the number of transferable embryos (Tang et al. 2013), prolificacy, twinning rate (Marchitelli and Nardone 2015), and calving rate (Rasheed et al. 2021). The use of markers assisted selection has been shown to have the potential to overcome the problems encountered in conventional breeding. This biotechnology has led to the widespread use of molecular markers in partial and total genome approaches (Singh et al. 2014). Therefore, this study aims to investigate SNPs in the bovine GDF9 that can be used as molecular marker of embryo production. Belgian Blue, *Peranakan Ongole*, and its crossbreed. The results are expected to provide potential candidate genes to improve reproduction in cattle.

MATERIALS AND METHODS

Ethical statement

The study procedures were approved by the Ethical Committee, Universitas Padjadjaran, Indonesia, with approval number 132/UN6.KEP/EC/2022, Registration Number: 2201050051, dated February 11, 2022.

Animals

This study was conducted with a sample of 20 donor cattle, including Belgian Blue (BB, 5 heifers), *Peranakan Ongole* (PO, 9 cows), and Belgian Blue x *Peranakan Ongole* (BB x PO crossbreed, 6 heifers). Sample size justification was based on the availability of donor at the breeding center and used the Slovin formula with a sampling error rate of 20% (0.2). The following equation denotes the Slovin formula: The cattle used during the procedures were healthy, with a body weight of 475-535 kg, a range age of 4-6 years, and with Body Condition Score range of 3.0-3.5 on a scale of 5 BCS. The cattle were in normal estrous condition and non-lactation status. The procedures were conducted at Livestock Embryo Centre (LEC), Cipelang Bogor, West Java, Indonesia, and the Animal Molecular Genetics Laboratory, Institut Pertanian Bogor, Indonesia, from February 2022 to February 2023.

Blood samples and DNA extraction

All blood sample procedures were performed according to the standard protocols approved by the Livestock Embryos Center. The procedures followed the principles of animal welfare. Blood samples were collected from Belgian Blue (BB) heifers, *Peranakan Ongole* (PO) cows, and Belgian Blue×*Peranakan Ongole* crossbred (BB×PO) heifers. Blood samples were taken from the jugular vein using a 10 mL syringe. The blood samples were immediately transferred into a vacutainer containing anticoagulant EDTA and stored at -20°C. Additionally, genomic DNA was extracted using the Geneaid® DNA extraction kit following the manufacturer's instructions.

DNA amplification

The GDF9 primer pair (Table 1) was designed based on GenBank (Accession: GQ922451.1) using Primer- Basic Local Alignment Search Tool (BLAST) (https:// www.ncbi.nlm.nih.gov/tools/primer-blast/). Amplification of the target gene by Polymerase Chain Reaction (PCR) used a total media volume of 28 µL, including 12.5 µM PCR Master Mix 2x, 0.3 µM each primer, and 100 ng DNA. The PCR thermocycler was set for 95°C denaturation for 1 minute for one cycle, 35 cycles of denaturation (95°C for 15 seconds), annealing (56°C and 60°C for 45 seconds), initial extension (60°C for 15 seconds), and final extension (72^0C for 10 seconds). PCR uses a Master Cycler Gradient. Each reaction in a final volume of 24 µL had one µL of DNA sample, 10.9 µL of nuclease-free water, 0.3 µL of forward primer, 0.3 µL of reverse primer, and 12.5 µL of Mix. PCR products were visualized under ultraviolet light on a 1.5% agarose gel containing FlouroSafe stain.

DNA sequencing and Single Nucleotide Polymorphism identification

Amplicons were purified and sequenced by Macrogen (Singapore) according to the Sanger sequencing. The sequence of the GDF9 was analyzed using Finch-TV and Bio-Edit software. The sequence alignment followed the procedure of the Clustal W, while SNPs were determined using Molecular Evolutionary Genetics Analysis (MEGA) version 6 (Tamura et al. 2021). Amino acid sequence estimation was performed by aligning the DNA sequence with the GDF9 cDNA sequence in GenBank (Accession: GQ922451.1). The sequences of each sample were aligned

to GDF-9 *B. taurus* (Accession GQ922451.1). Nucleotide positions refer to access sequences AC_000164 (Marchitelli and Nardone et al. 2015), GQ922451.1, NM_174681.2 (Inayah et al. 2016), 19537 with NCBI reference sequence: NC_007305.3 (Santos-Biase et al. 2012) and GenBank Accession No. 282574 (Tang et al. 2013). The nucleotide sequences (coding regions) of the GDF9 were translated into amino acids using the ExPASy program (https://web.expasy.org/translate/). The identification of mutation found was compared with SNPs database in Ensembl Genome Browser (Yates et al. 2020).

Data analysis

Analysis of the GDF9 loci in the cattle population using molecular data, including allele and genotype frequencies.

Allele frequencies

Allele frequencies were calculated based on the Nei and Kumar (2000) formula:

$$X_i = \frac{2n_{ii} + \sum n_{ij}}{2N}$$

Where:

X_i : frequency of the i^{th} allele
n_{ii} : genotyped ii number
N_{ij} : genotyped ij numbers
N : total sample

Genotype frequency

Genotype frequencies were calculated based on the formula Nei and Kumar (2000):

$$X_{ii} = \frac{n_{ii}}{N}$$

Where:

X_{ii} : genotype ii frequency
n_{ii} : number of genotypes ii
N : total number of samples observed

RESULTS AND DISCUSSION

The PCR products were successfully obtained, resulting in a targeted region with a sequence length of exons 2 is 902 bp from the GAA codon to the TGA stop codon (Figure 1). PCR obtained two amplicons measuring 418 bp and 484 bp. A total of 5 SNPs were identified in GDF9 exon 2 (Table 2) which four mutations are transversions and one mutation is transition. The distribution of alleles and frequency of genotypes in each cattle breed was presented in Tables 3 and 4.

Table 1. Pairs of primer forward and reverse gen GDF9

Primers	Strand	Primer sequence	Annealing temperature (°C)	Amplicon size (bp)
GDF9-2A	Forward	5'CTGCTGTTTAACCTGGATCG'3	56	418 bp
	Reverse	3'ACCTGTGAAAAGCCTGAGCA'5		
GDF9-2B	Forward	5'AGACCAGGAGAGTGTCAGCT'3	60	484 bp
	Reverse	3'TCCCAAAGGCATAGACAGGTG'5		

Table 2. Non-synonymous mutation GDF9 in donor cattle

Point (snps)	Mutation types	Codon changes	Residu changes	Polarity changes	Hydrophobicity changes
c.589T>G	Transversion	TTT>GTT	p.197 Phe > Val	Non-Polar	Hydrophobic > Hydrophobic
c.659T>G	Transversion	CTT>CGT	p.220 Leu > Arg	Non-Polar > Polar	Hydrophobic > Hydrophilic
c.974C>G	Transversion	TCT>TGT	p.325 Ser > Cys	Polar > Non-Polar	Hydrophilic > Hydrophilic
c.1105T>A	Transversion	TGG>AGG	p.369 Trp > Arg	Non-Polar > Polar	Hydrophobic > Hydrophilic
c.1358G>A	Transition	CGT>CAT	p.453 Arg > His	Polar > Polar	Hydrophilic > Hydrophilic

Note: point of mutation based on coding sequence order. Phe (phenylalanine), Val (valine), Leu (leucine), Arg (arginine), Ser (serine), Cys (cysteine), Trp (tryptophan)

Table 3. Allele frequency analysis of GDF9 mutations per cattle breed

Cattle	c.589 T>G		c.659 T>G		c.974 C>G		c.1105 T>A		c.1358 G>A	
	T	G	T	G	C	G	T	A	G	A
Belgian Blue (5)	1.00 (5)	0.00 (0)	0.90 (4)	0.10 (1)	0.40 (2)	0.60 (3)	0.80 (3)	0.20 (2)	1.00 (5)	0.00 (0)
PO (9)	1.00 (9)	0.00 (0)	0.89 (7)	0.11 (2)	0.67 (6)	0.33 (3)	0.83 (6)	0.17 (3)	0.78 (5)	0.22 (4)
BB x PO (6)	0.83 (4)	0.17 (2)	0.75 (3)	0.25 (3)	0.50 (3)	0.50 (3)	0.75 (3)	0.25 (3)	1.00 (6)	0.00 (0)

Note: The first number in parentheses shows the number of allele frequencies, while the second number shows the number of individuals

Table 4. Genotype frequency analysis of GDF9 mutations per cattle breed

Cattle	c.589 T>G		c.659 T>G		c.974 C>G		c.1105 T>A		c.1358 G>A	
	TT	TG	TT	TG	CC	GG	TT	TA	GG	GA
Belgian Blue (5)	1.00 (5)	0.00 (0)	0.80 (4)	0.20 (1)	0.40 (2)	0.60 (3)	0.60 (3)	0.40 (2)	1.00 (5)	0.00 (0)
PO (9)	1.00 (9)	0.00 (0)	0.78 (7)	0.22 (2)	0.67 (6)	0.33 (3)	0.67 (6)	0.33 (3)	0.56 (5)	0.44 (4)
Belgian Blue x PO (6)	0.67 (4)	0.33 (2)	0.50 (3)	0.50 (3)	0.50 (3)	0.50 (3)	0.50 (3)	0.50 (3)	1.00 (6)	0.00 (0)

Note: The first number in parentheses showed several genotype frequencies, while the second number showed the number of individuals

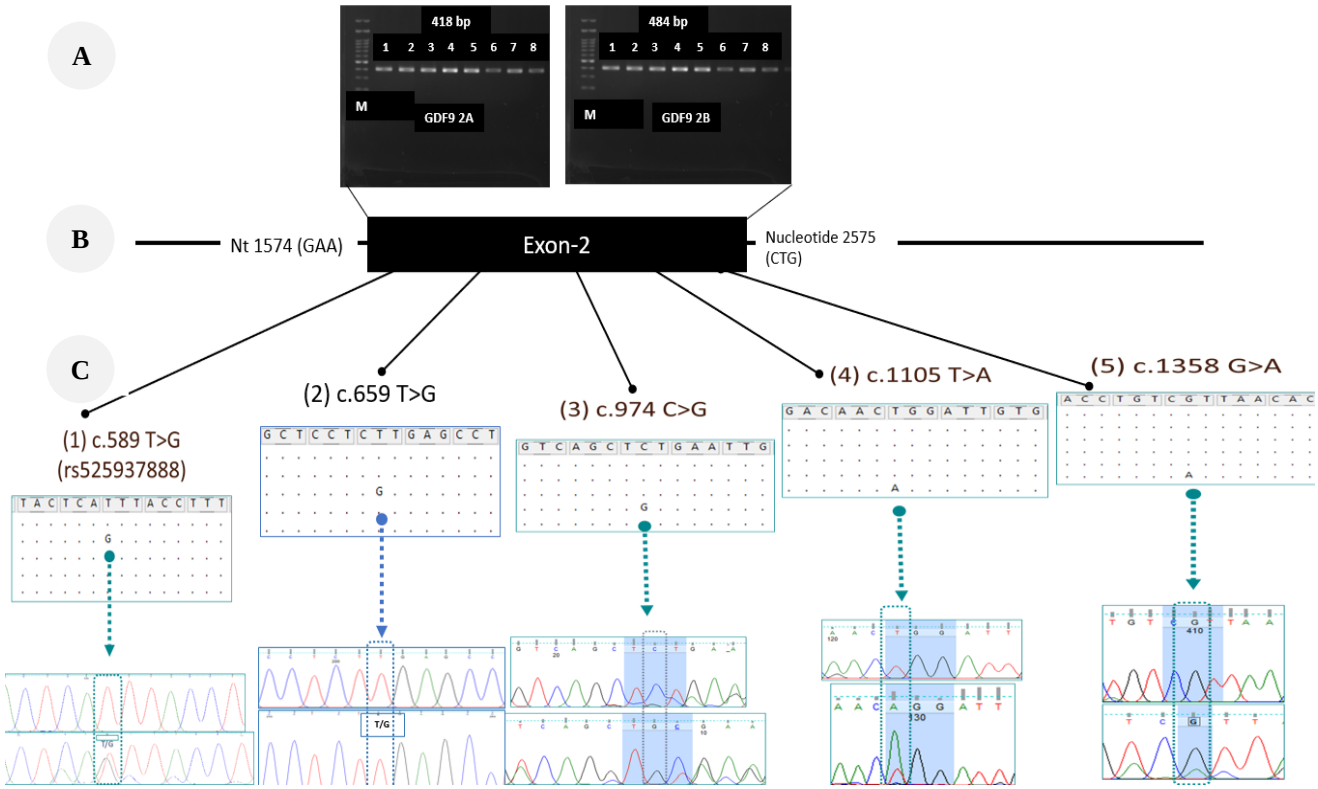

Figure 1. Profiling of the GDF9 in Belgian Blue, *Peranakan Ongole*, and crossbred. A. Electrophoresis analysis of the PCR product (Marker: 100 bp DNA ladder, Lines 1-8 were samples); B. Structure and SNPs position of the GDF9; C. GDF9 genotyping (TG, TG, CG, TA, and GA)

Discussion

In this study, 5 SNPs were identified: c.589T>G, c.659T>G, c.974C>G, c.1105T>A, and c.1358G>A in exon 2 (Table 2). A total of 4 mutations are transversions that change pyrimidine bases (C or T base) to purines (A or G base) or vice versa. A mutation c.1358G>A was transitional (base transition between pyrimidines or between purines). Chromatogram analysis shows that most mutations were heterozygous. This study found three novel mutations: c.659T>G, c.974 C>G, c.1105 T>A. The number of cattle in the five mutations occurred randomly and was spread across various samples. Mutations c.659T>G, c.974C>G, and c.1105T>A were found in Belgian Blue (taurine cattle), *Peranakan Ongole* (indicine cattle) and its crossbred (BB x PO).

Santos-Biase et al. (2012) found differences in the amino acid properties in the GDF9 between *B. taurus* and *Bos indicus* Linnaeus 1758, which were caused by a point mutation at the Chr7:44093437 C>T location. *B. indicus* with homozygous AA and CC genotypes showed higher oocyte recovery (20.80±0.44, 19.99±0.01, respectively) than heterozygous genotypes CA (14.00±0.02). Oocyte recovery in embryo transfer is essential for enhancing fertility in shorting generation intervals, increasing selection intensity, improving selection accuracy, and genetic improvement (Jaton et al. 2016). Another SNP GDF9 was also found in *B. indicus* based on the *B. taurus* reference sequence of exon 2 (3'UTR) (Chr7: 44.090.844 T>G) and intron 1 (Chr7: 44.092.967 C>T) but did not alter the amino acid composition (Santos-Biase et al. 2012). There were some differences in GDF9 sequences between *Bos taurus* and *Bos indicus*. All *Bos taurus* showed allele T, while *B. indicus* showed allele G in SNP in exon 2 (3'UTR). Other SNPs were founded in intron 1; all *Bos taurus* showed allele C, while all *B. indicus* showed allele T (Santos-Biase et al. 2012).

Mutation c.1358G>A occurred only in PO cattle, while mutation c.589T>G occurred only in crossbred cattle with frequencies 0.44 and 0.33, respectively. In Belgian Blue and PO cattle, the c.589T>G mutation was not found and

did not correlate with mutation in the crossbred because Belgian Blue and PO purebreds were not the parents of the crossbred (different generation). There were fewer mutations in Belgian Blue, while four mutations were found in PO and crossbred. The c.1358G>A mutation was reported by Inayah et al. (2016) and altered Arginine to Histidine (p.453Arg>His). This missense mutation predicted an altered side chain of GDF9 and changed its interaction with BMP15. Mutation c.1358G>A, which was found in this study, reduces the interaction affinity of GDF9 with BMP-15 with lower change energy (Irma 2024-unpublished data). A similar decrease in energy at the mutation point was also reported by Inayah et al. (2016). The p.453Arg>His mutation is located in the GDF9 heterodimer binding domain, the interaction site with BMP15 (Figure 2). The interaction energy shows that variations in p.453Arg>His change the affinity strength and interaction patterns of GDF9 and BMP15. The p.453Arg>His mutation in this study was only found in PO cattle in heterozygote condition. According to Monestier et al. (2014), high variations of BMP15 and GDF9 in polyovulating species change the equilibrium between homodimers and heterodimers, modifying the biological activity and thus allowing polyovulation.

According to Christoforou et al. (2019), the ratio of GDF9 and BMP15 determines litter size in mammals. GDF9 played a significant role in early ovarian development and folliculogenesis in mammal reproduction. GDF9 stimulated protein in the development process of primordial and primary follicles to regulate granulosa and theca cells. The process of follicle formation in the ovaries involves GDF9, a local factor that functions in granulosa cell proliferation, increasing LH receptors in granulosa cells, oocyte cumulus cell expansion, hyaluronic acid production, and oocyte maturation. Another effect is an increase in follicular fluid protein secretion, especially transport traffic and the binding of amino acids into protein. GDF9 in the ovaries also functions in DNA synthesis in granulosa cell membranes, reducing cAMP activity so that the meiosis process occurs.

Ajafar et al. (2021) review that there was an association of GDF9 with litter size in livestock. GDF9 regulates fertility and litter size in most livestock species. Ovarian follicles release GDF9 that are involved in the maturation of primary follicles into the basal form, proliferation of granulosa and theca cells, steroidogenesis, ovulation, and corpus luteum formation. Mutations of GDF9 in intron were reported to affect the superovulatory response in cattle (Tang et al. 2013). Association analysis showed that SNP g.485A>T and c.625A>T significantly affected the number of transferable embryos. Cattle with genotype g.485TT showed more viable embryos (5.26±1.0) than cattle with genotype g.485AA and g.485AT (3.77±0.23, 3.70±0.27). Cattle with genotype g.625AA showed a higher number of ova (8.28±0.69) compared to cows with genotype g.625AT and g.625TT (6.73±0.29, 7.50±0.54, respectively). Several studies (Santos-Biase et al. 2012; Tang et al. 2013; Marchitelli and Nardone 2015; Inayah et al. 2016; Rasheed et al. 2021) showed that GDF9 is a candidate gene related to reproductive performance in various cattle, including Ongole or Nellore cattle (Table 5**).**

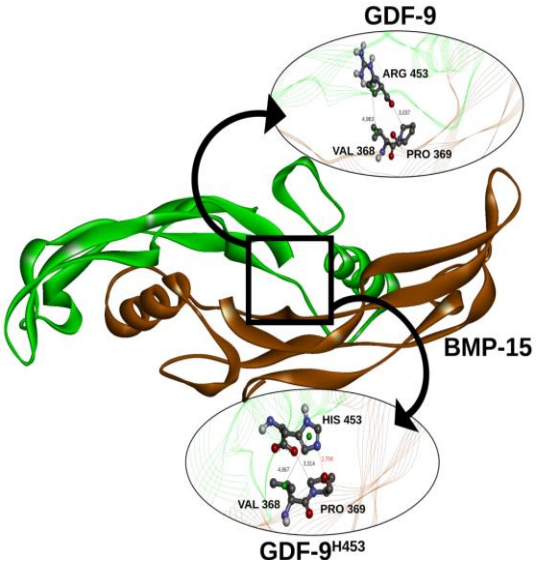

Figure 2. Interaction of GDF9 and BMP15 at the c.1358G>A alters p.453Arg>His point (Caption: green color GDF9 protein, brown color BMP15, top circle: control GDF9, bottom circle: GDF9 mutant, interaction area indicated with black box) (Irma 2024, unpublished)

Table 5. Mutation of GDF9 in various cattle

Region	Mutation	Amino acid change	Trait associated	Cattle	References
3' UTR	2630 C>T			Maremma	Marchitelli and Nardone (2015)
Exon 1	231 T>C		Prolific, twinning	Maremma	Marchitelli and Nardone (2015)
Intron 1	485 A>T		Embryo quality	Holstein	Tang et al. (2013)
Intron 1	625 A>T		Embryo quality	Holstein	Tang et al. (2013)
Exon 1	Chr7: 44093437C>A	Ala > Glu	Oocyte number	Nellore (Ongole)	Santos-Biase et al. (2012)
Intron 1	Chr7: 44092967C>T			Nellore (Ongole)	Santos-Biase et al. (2012)
Exon 2	Chr7: 44090844T>G			Nellore (Ongole)	Santos-Biase et al. (2012)
Exon 2	1109 A>T		Calving rate	Holstein	Rasheed et al. (2021)
Exon 2	1133 G>A		Calving rate	Holstein	Rasheed et al. (2021)
Exon 2	1358 G>A	Arg > His		Holstein	Inayah et al. (2016)
Exon 2	2087 T>C			Maremma	Marchitelli and Nardone (2015)
Exon 2	2456 T>C			Maremma	Marchitelli and Nardone (2015)

In this study, mutation of GDF9 exon 2 at point 1109A>T and 1133G>A (Rasheed et al. 2021) and Chr7: 44090844T>G (Santos-Biase et al. 2012) were not found. Based on the Ensemble Genome Browser, approximately 518 mutations were found in bovine GDF9 and distributed in various locations. However, out of the 518 mutations, 494 were single base mutations, and the rest were insertions (13 variants) and deletions (11 variants). These mutations were located in 3'untranslated region or UTR (56 variants), downstream (125 variants), intron (56 variants), exons either in the form of synonymous mutations (14 variants) as well as missense (40 variants) and upstream (227 variants). Some missense SNP in exon 2 of GDF9 in *Bos taurus* are presented in Table 6. The c.589T>G (rs525937888) mutation was identified in this research with a tolerated effect indicated by SIFT score (>0.05).

Another study reported exon 2 GDF9 mutations in two populations of cattle (dizygotic twin birth/DZTB and single birth/SB) associated with twin births and calving rate (Rasheed et al. 2021). However, in this study, that mutation was not found. Two SNPs were revealed in exon 2 of Holstein Friesians (Rasheed et al. 2021). These SNPs, 1109A>T and 1133G>A, were compared with Sequence XM_027546514.1 and ensemble ENSBTAT00000012476.2. In the population of non-identical, the 1109A>T mutation with the TT genotype showed a higher frequency of twinning and calving rate (66.6% and 1.83, respectively) compared to the AA genotype (33.3% and 1.27, respectively). However, in the single-birth population, the AA genotype was dominant with single birth and a lower calving rate (86.6% and 1.27). The missense SNP of 1109A>T has altered the amino acid from Isoleucine into Asparagine (p.370Ile>Asn), which give transversion effect. Mutation of 1133G>A with the AA genotype showed a higher frequency of twinning and calving rate (60% and 1.81) compared to the GG genotype (40% and 1.31) in the DZTB population. However, the GG genotype was dominant with single birth, with a lower calving rate (86.6% and 1.31) in the SB population. This mutation also alters the amino acid from Aspartic acid into Asparagine (p.376Asp>Asn), which is predicted to give a transition effect.

According to Kirkpatrick and Morris (2015), GDF9 is a significant candidate gene for folliculogenesis and ovulation in sheep and goats. Numerous studies have reported the polymorphism of GDF9 in small ruminants, such as reported by El Fiky et al. (2017), which showed that genetic polymorphism of GDF9 is related to fecundity in Egyptian sheep. GDF9 in sheep, known as *FecG*, is an autosomal gene on the seventh chromosome. GDF9 mutations in Barki sheep (c.25C>T and c.260G>A) were associated with multiple births (Ibrahim 2021). In goats, GDF9 mutations also occur in Indonesian local goat in the form of c.836G>A and 1019 G>C polymorphisms (Batubara et al. 2014). The p.320Glu>Pro and p.397Val>Ile amino acid mutations are significantly associated with the first litter size in goats (Wang et al. 2019). However, According to Ahlawat et al. (2015), the c.818C>T, c.959A>C, and c.1189 G>A mutations in Black Bengal goats were reported as not associated with litter size. According to Hartatik et al. (2023), six SNPs and one deletion were identified in GDF9 of the Bligon goat. Other GDF9 SNPs in goat reported by Song et al. (2023), which reported four polymorphisms 719C>T, 1189G>A, 732G>A, and 1189G>A in Tibetan cashmere goats.

Table 6. Missense mutations in the GDF9 in exon 2 *Bos taurus*

Variant ID	Locations	Allele	Alleles	Amino acids	Effect	SIFT
rs439231021	7:44.443.020	A	T/A	p.398His>Leu	Deleterious	0
rs457691414	7:44.443.023	T	A/T	p.397Val>Asp	Deleterious	0
rs443916243	7:44.443.027	A	G/A	p.396Pro>Ser	Deleterious	0
rs462372270	7:44.443.196	T	A/T	p.339Ser>Arg	Deleterious	0.04
rs480835503	7:44.443.252	G	C/G	p.321Glu>Gln	Tolerated	0.23
rs459843964	7:44.443.305	G	A/G	p.303Met>Thr	Tolerated	0.93
rs445206700	7:44.443.312	C	A/C	p.301Cys>Gly	Tolerated	0.37
rs437875448	7:44.443.318	G	A/G	p.299Ser>Pro	Tolerated	0.31
rs468388984	7:44.443.324	T	C/T	p.297Gly>Arg	Tolerated	0.07
rs519920027	7:44.443.413	C	T/C	p.267Tyr>Cys	Deleterious	0
rs472341708	7:44.443.476	G	T/G	p.246His>Pro	Tolerated	0.23
rs451343180	7:44.443.570	A	C/A	p.215Asp>Tyr	Deleterious	0
rs525937888	7:44.443.624	C	A/C	p.197Phe>Val	Tolerated	0.52
rs476182598	7:44.443.701	T	G/T	p.171Pro>His	Deleterious	0.02
rs443977774	7:44.443.719	A	G/A	p.165Ser>Phe	Deleterious	0
rs462441123	7:44.443.764	C	A/C	p.150Val>Gly	Deleterious	0.05
rs474457932	7:44.443.767	C	A/C	p.149Val>Gly	Deleterious	0.01
rs441404240	7:44.443.771	A	T/A	p.148Thr>Ser	Tolerated	0.11
rs459853659	7:44.443.773	C	A/C	p.147Val>Gly	Deleterious	0
rs445269918	7:44.443.777	A	G/A	p.146Arg>Cys	Tolerated	0.07
rs463611810	7:44.443.779	C	T/C	p.145Asp>Gly	Tolerated	0.06
rs482016725	7:44.443.786	A	T/A	p.140Asn>Tyr	Deleterious	0
rs468503023	7:44.443.794	G	A/G	p.140Leu>Pro	Deleterious	0
rs435441979	7:44.443.797	C	T/C	p.139Asp>Gly	Deleterious	0.03

Source: https://asia.ensembl.org, Accessed February 12th 2023 based on ENSBTAG000000094781

The heterozygote genotypes were associated with high egg production and egg weight in chicken (Qin et al. 2015). Mutations were found at points c.238A>G and c.1609G>T. The c.1609G>T exon two polymorphism produces a TC genotype with higher egg production and egg weight (Qin et al. 2015). Other research in pigs reported SNPs (c.359G>C, c.1801C>T, c.1806T>C) as not associated with litter size (Zhang et al. 2008). In pigs, polymorphisms were found at c.359G>C, c.1801C>T, c.1806T>C, and 314 bp indels. The c.359G>C point mutation was only found in indigenous Chinese pigs, Erhualian and Dahuabai, while the 314 bp indel was only found in Duroc and Landrace pigs. The c.1801C>T and c.1806T>C mutations were found in Erhualian, Dahuabai, and Landrace (Zhang et al. 2008). According to Lin et al. (2013), GDF9 in porcine stimulates maternal genes such as CDC2 [regulatory subunits of maturation/M-phase-promoting factor (MPF)] and *C-MOS* [a regulatory subunit of mitogen-activated protein kinase (MAPK)] activities. The presence of GDF9 was identified in pre-ovulatory follicle in sow (Stankiewicz and Błaszczyk 2016).

Mutation of GDF9 in rabbit (*Oryctolagus cuniculus* Linnaeus 1758) was reported by Sun et al. (2017). Three SNPs (C.539C>T, C.562 G>C and C.718C>G) in exon 2 of the GDF9 were found, and altered amino acids (p.183Thr>Met, p.188Glu>Gly and p.240Leu>Val, respectively). A recent study by Chen et al. (2022) reported that loss of GDF9 causes an arrest of early folliculogenesis in zebrafish (*Danio rerio* Hamilton 1822). Stefaniuk-Szmukier et al. (2018) found sequence tagged site (STS) in equine GDF9 in intronic region (g.42750467GT[7]GA [9], g.42750467GT[8]GA[11], g.42750467GT[8]GA[12]) and were not correlated with ovulation rate. Belly and Shimasaki (2018) review GDF9 in women's reproduction. Most of the mutations in the GDF9 identified in mothers of dizygotic twins are located in the pro region of the proprotein, as the proregion is necessary for the dimerization of the mature protein. Mutations in the pro region may cause the impaired processing of the proproteins by forming misfolded proprotein dimers, thereby negatively impacting the production of functional mature protein dimers. Misfolded proteins are degraded in the endoplasmic reticulum by the ER-associated degradation process. This is necessary for protein quality control in the eukaryotic secretory pathway to ensure that only correctly folded proteins transit through cellular organelles. A recent study reported two biallelic of GDF9 in infertile women contributed to the defect in antral follicle development (Duan et al. 2024).

The mechanism of how GDF9 affected reproduction was explained by its function, as summarized by Kaivo-Oja (2007). The function of GDF9 in various locations such as preantral follicle, antral and pre-ovulated follicle, and cumulus cell. In the preantral follicle, GDF9 function is related to the mitosis of granulosa cells, primary follicle development, suppresses kit ligand expression, and prevents granulosa cell apoptosis. In the antral and pre-ovulated follicles, GDF9 function is related to the mitosis of granulosa cells, modulation of steroidogenesis, suppresses LHR expression, and induces the expression of pentaxin 3 and gremlin. GDF9 in cumulus cells has the function of induction of cumulus cell expansion, Induces the expression of HAS2, COX2, StAR, PGE2, and EP, and Suppresses uPA and LHR (Kaivo-Oja 2007). In granulosa cells, GDF9 regulation increases the activity of the HAS2, COX2, StAR, Pentraxin 3, TNFIP6, Gremlin, Inhibin β Subunit, Peroxiredoxin 6, and PGE2, while in theca cells, GDF9 increases the activity of the *CYP17* (Kaivo-Oja 2007). GDF9 plays critical functions in ovarian architecture and fertility with a primary task in follicle growth and development (formation, growth, and functionalization of granulosa and theca). Granulosa cells, as target cells for regulation and modulation of GDF9, are responsible for oocyte maturation and maintaining oocyte survival.

The TGF-β signaling system activates target cells through the SMAD2/3 or SMAD1/5/8 pathway, so biological processes such as proliferation, differentiation, apoptosis, and embryo development occur. The TGF-B superfamily group can potentially be a genetic marker for fertility due to the function of GDF9 in theca cell proliferation, differentiation, apoptosis, and embryo development. The GDF9 factor is also involved in the interconnection communication between the cumulus cell and the oocyte by regulating amino acid absorption, glycolysis, and cholesterol biosynthesis in cumulus cells. Swinerd et al. (2023) review the difference in GDF9 in various litter-size animals. In pig, sheep, and deer species, oocytes secreted contained both premature and maturity of GDF9 in similar levels between species. The predominant forms of GDF9 found within and secreted from the oocyte across the species were the promature and cleaved mature forms of GDF9 (Al Hussini 2016).

In conclusion, there were genetic polymorphisms in the GDF9 gene in Belgian Blue, *Peranakan Ongole*, and its crossbred. There were five non-synonymous mutations, namely c.589T>G, c.659T>G, c.974C>G, c.1105T>A, and c.1358G>A which altered the amino acid of GDF9 protein at position p.197Phe>Val, p.220Leu>Arg, p.325Ser>Cis, p.369Trp>Arg, and p.453Arg>His, respectively. The mutant-type allele frequency is lower than the wild-type allele. The non-synonymous mutation may alter the characteristics and properties of the amino acid of the GDF9 protein and the interaction of other oocyte-specific proteins in folliculogenesis related to the female reproduction system. This study revealed that PO cattle showed a high mutation rate in the GDF9. Moreover, these findings are different from the previously reported. The high level of diversity had implications for further study regarding the characteristics of folliculogenesis in mutant and wild-type cattle related to regulating these genes in the female reproductive process in ovulating oocytes for embryo production and quality marker-assisted selection.

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