



Association of c.974C>G Mutation in Growth Differentiation Factor 9 with Recovery Rate in Peranakan Ongole Cattle

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Abstract | Growth Differentiation Factor 9 (GDF9) is related to female reproductive characteristics, particularly folliculogenesis. The significant effect of GDF9 has been explored in previous investigations by determining the association between genetic diversity and phenotypic variations using c.589T>G, c.659T>G, c.974C>G, c.1105T>A, and c.1358G>A from Polymerase Chain Reaction (PCR) based on direct sequencing. This study aimed to analyze the association between GDF9 diversity and the superovulation response in Peranakan Ongole (PO), Belgian Blue (BB), and its crossbreeds (BB x PO). The experiment was conducted using 21 donors from Livestock Embryo Centre (LEC), which were selected for the superovulation based on oestrous synchronization. The normality distribution of data was evaluated using Kolmogorov-Smirnov, followed by non-parametric analysis. Furthermore, Single Nucleotide Polymorphism (SNP) was used to determine genotypes and their association was analyzed with the Kruskal-Wallis statistical test. In PO, SNP c.974C>G on GDF9 showed that GG genotypes had a higher recovery rate (82.83±5.83%) compared to CC genotypes (69.27±6.62%), with $p<0.05$. Based on c.589T>G, c.659T>G, c.974C>G, c.1105T>A, and c.1358G>A, mutant haplotype had a higher recovery rate (86.06±6.06%) compared to wild haplotype (69.06±6.14%), with $p<0.05$. The result showed that SNP at c.974C>G could be used as a marker for recovery rate in assisted selection and livestock breeding programs in the breeding centre location.

Keywords | GDF9, Mutation, Cattle, Recovery rate, Superovulation

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INTRODUCTION

Peranakan Ongole (PO) is among the Indonesian genetic diversity of cattle resources, widely distributed, and known to have the advantage of good adaptation. In 2017, the new taurine cattle breed, namely the Belgian Blue (BB),

was introduced by the Indonesian Government to improve the genetic resources of local cattle by crossing with PO (*Bos indicus*). Studies on the reproductive characteristics of PO, BB cattle, and the crosses kept in the tropics are crucial before being released as a new composite breed for commercial stocks to support their sustainability.

Genes present in the oocytes vary widely and one example majorly expressed is *GDF9*. The gene plays a crucial role in folliculogenesis, the process of follicle development in the ovary, which is a crucial aspect of female reproduction. Several studies reported that the *GDF9* in cattle is associated with oocyte number (Santos-Biase *et al.*, 2012), embryo quality (Tang *et al.*, 2013), multiple births (Marchitelli and Nardone, 2015), and calving rate (Rasheed *et al.*, 2021). *GDF9* is related to mammalian reproductive characteristics due to the role in the reciprocal communication of oocytes with the follicular environment, specifically theca and granulosa cells (Pfeffer *et al.*, 2007).

Currently, data on *GDF9* polymorphisms in cattle is still limited, specifically in Indonesian local cattle, and the crossbred in tropical areas by direct sequencing. Several studies only reported *GDF9* polymorphism in Bali cattle (Rahayu *et al.*, 2012), Peranakan Ongole (Arta and Rahayu, 2013), and Friesian Holstein (Inayah *et al.*, 2016). Various studies found the presence of *GDF9* in small ruminants such as goats and sheep. This study hypothesizes that PO (*Bos indicus*), BB (*Bos taurus*), and crossbred cattle (BB x PO) have different reproductive characteristics due to breed differences, specifically in the genetic aspect, namely the *GDF9*. The differences in reproductive variation are likely due to *GDF9* diversity occurring at the DNA level. This implies that the *GDF9* is likely associated with the level of recovery rate in the three breeds observed. Therefore, this study aimed to analyze the association of *GDF9* polymorphism found in PO, BB, crossbreeds (BB x PO), and the impact on the superovulation response. By examining *GDF9* as a candidate for reproductive characteristics, this study can significantly advance the current understanding of the genetic markers in cattle reproduction.

MATERIALS AND METHODS

ETHICAL STATEMENT

Ethical Committee, Universitas Padjadjaran, Bandung, West Java, Indonesia, has approved the research. The approval number is 132/UN6.KEP/EC/2022, Registration Number: 2201050051, dated February 11th, 2022.

ANIMALS

This study was conducted with 21 donors, consisted five BB, ten PO, and six BB x PO crossbreed. The cattle used were in healthy reproduction condition, body weight ranged from 475–535 kg, age of 4–6 years, and Body Condition Score (BCS) range of 3.0–3.5 (scale 5).

The cattle were in normal oestrous and non-lactation status. The superovulation procedure is based on described by Irma *et al.* (2023). Sample size adjustment was based on the availability of donor at the breeding centre and used the

Slovin formula with a sampling error rate of 20% (0.2). The following equation denotes the Slovin formula:

$$n = \frac{N}{1 + N(e)^2}$$

Description:

n: sample size.

N: population size (total 54 donor population for PO, BB, and its crossbred raised in breeding centre location).

e: sampling error accuracy (e=0.2, ranges from 10% in large population and 20% in small population).

SINGLE NUCLEOTIDE POLYMORPHISM

Genetic diversity from the previous stages of study were used for the association study that occurred with superovulation response between individual. Five mutations (c.589T>G, c.659T>G, c.974C>G, c.1105T>A, and c.1358G>A) were found by PCR followed by Sanger sequencing. The protocol of PCR, primers used, annealing temperature, chromatogram, and alignment analysis to identify and validate these SNP referred to Irma *et al.* (2024).

VARIABLES

Superovulation response measured were: (1) response rate (%), (2) recovery rate (%), (3) fertilization rate (%), (4) degenerated embryo (%), (5) unfertilized oocytes (%), (6) and transferable embryos (%), as formulated by Jodiansyah *et al.* (2013). These variables were selected based on associated parameters observed with *GDF9* polymorphism in cattle, which has been published, such as referred in Marchitelli and Nardone (2015), Santos-Biase *et al.* (2012) and Tang *et al.* (2013).

IN-SILICO ANALYSIS

Prediction of mutation impact uses the HOPE webserver (<https://www3.cmbi.umcn.nl/hope/>), while protein domain prediction uses the NCBI conserved domain webserver (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>) (Wang *et al.*, 2023). Translation of codon changes uses the ExPASy webserver (<https://web.expasy.org/translate/>). Visualization of tertiary protein structure using Molstar (<https://molstar.org/>). Prediction of secondary protein structure using the Network Protein Sequence Analysis (NPS@) in SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html) (Deleage, 2017). Analysis of gene ontology using QuickGO (<https://www.ebi.ac.uk/QuickGO>). All in silico analyses used the Windows Operating System 64 Bit.

STATISTICAL ANALYSIS

The relationship between breeds in the same genotype with the average of the superovulation response was analyzed

non-parametrically using Kruskal-Wallis H-test analysis, as follows:

$$H = \frac{12}{N(N + 1)} \sum_{i=1}^k \frac{R_i^2}{n_i} - 3(N + 1)$$

Descriptions:

N: the number of sample sizes in the donor population.

k: the number of donors.

R_i: the number of ranks in the nth sample.

n_i: the nth sample size.

Dunn's post hoc test followed hypothesis testing with significant results (*p-value* < 0.05). Adjustment to the significance *p-value* for multiple testing correction using the Bonferroni error correction. All analyses used an alpha (significance level) of 0.05 and a confidential interval of 95%.

Differences in mean parameters in groups of two genotypes (mutant and wild) were analyzed using the Mann-Whitney test. All analysis uses SPSS (IBM Corp., Armonk, NY, USA). Data are presented in mean ± standard deviation. The use of non-parametric statistics is based on testing assumptions of normality and homogeneity that must be met in data, leading to non-normal distribution, heterogeneity, and limited sample size (less than 30 donors).

RESULTS AND DISCUSSION

SUPEROVULATION RESPONSES

A total of 21 donor were superstimulated with a total number of flushing were 74 procedures. The individual response in Belgian Blue (BB), PO and crossbred (BBxPO) cattle were presented in Table 1. The superovulation response rate showed a high percentage for Belgian Blue, PO, and crossbred respectively; 93.14%±9.60, 97.50%±8.33, and 94.44%±13.60. According to Supriatna (2018), the superovulation program is considered good when the donor responds rate above 70%. In this study, superovulation response rate showed a high percentage (>90%). Recovery rate in Belgian Blue, PO, and crossbred cattle were 73.67%±17.71, 82.69%±12.37, and 53.86%±39.40, respectively. According to Wiley (2017), the recovery rate value for the non-surgical flushing method is 49-92%. The mutant and wild cattle in this study showed a good recovery rate (>50%). In general, the performance of taurine and indicine donor raised in breeding location showed good superovulation response rate and recovery rate. Furthermore, performance of donor population in breeding location showed good response rate 76.32-80.82% and recovery rate 81.77-90.55% (LEC Annual Report, 2024).

Table 2 shows the relationship between genotype and superovulation response. *GDF9* mutations at point c.974C>G affect the recovery rate in PO cattle. PO with the GG genotype at point mutation c.974C>G had a higher recovery rate than the CC genotype (82.8±5.83 vs 69.27±6.62). The GG genotype in the c.974C>G mutation was found in three PO cattle with a frequency of 0.33% (3 heads cattle from 6 PO cows). Belgian Blue and crossbred cattle did not show significant differences between the GG (mutant) and CC (wild or control) genotypes at the c.974C>G mutation point.

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Table 1: Average performance of individual data per-flushing and per-breed donor (%).

	No Breed	Re- sponse Rate	Re- covery Rate	Fertili- zation Rate	Trans- ferable Embryos	Unfer- tilized Oocytes	Degen- erate Embryos
1	BB	100.00	83.67	82.93	53.66	17.07	29.27
2	BB	80.00	74.29	69.23	57.69	30.77	11.54
3	BB	100.00	84.21	18.75	18.75	81.25	0.00
4	BB	85.71	42.86	77.78	77.78	22.22	0.00
5	BB	100.00	83.33	73.33	73.33	26.67	0.00
6	PO	100.00	75.00	95.24	80.95	4.76	14.29
7	PO	100.00	90.00	76.67	57.78	23.33	18.89
8	PO	100.00	81.25	84.62	46.15	15.38	38.46
9	PO	100.00	85.37	40.00	25.71	60.00	14.29
10	PO	100.00	96.72	96.61	77.12	3.39	19.49
11	PO	75.00	60.00	75.00	25.00	25.00	50.00
12	PO	100.00	73.33	90.91	81.82	1818	9.09
13	PO	100.00	75.00	83.33	0.00	16.67	83.33
14	PO	100.00	95.79	57.14	24.18	42.86	32.97
15	PO	100.00	94.44	100.00	52.94	0.00	47.06
16	Cross- bred	100.00	82.35	89.29	75.00	10.71	14.29
17	Cross- bred	100.00	87.23	51.22	31.71	48.78	19.51
18	Cross- bred	100.00	88.89	68.75	62.50	31.25	6.25
19	Cross- bred	100.00	75.00	0.00	0.00	100.00	0.00
20	Cross- bred	100.00	0.00	0.00	0.00	0.00	0.00
21	Cross- bred	66.67	16.67	100.00	100.00	0.00	0.00

Notes: Data are presented in percentage (%); BB: (Belgian Blue); PO: (Peranakan Ongole), crossbred (BB x PO).

In this study, mutation c.589T>G, c.659T>G, c.1105T>A, and c.1358G>A did not affect the recovery rate (*p*>0.05). Variation originates from factors in the follicular dynamic conditions of each cow reproduction, which have not been observed in this study design, for example, deep ultrasono-

influenced by factors such as breed, age, parity, lactation, reproductive history, nutrition, stress, hormones, superovulation method, season, management, semen, and harvesting method (Mikkola *et al.*, 2019).

Table 2: Association between *GDF9* genotype and superovulation response (%).

Cattle SNP	Genotype	Recovery Rate	Fertilization	Degenerated
BB	c.659T>G	TT	46.66±10.03	44.64±11.31
		GG	5.21±3.63	12.00±12.00
	c.974C>G	CC	46.44±19.64	28.67±17.93
		GG	36.00±10.55	30.97±11.13
	c.1105T>A	TT	67.82±12.84	60.58±16.54
		TA	11.90±7.89	4.29±4.28
	c.1358G>A	TT	40.59±10.30	41.31±11.90
		TA	58.64±16.22	39.88±17.14
PO	c.659T>G	TT	73.22±5.33	70.44±6.55
		GG	32.06±6.74	18.12±7.85
	c.974C>G	CC	69.27±6.62 ^a	66.61±7.74
		GG	82.83±5.83 ^b	84.86±7.26
	c.1105T>A	TT	76.19±5.73	68.46±7.18
		TA	29.66±7.01	26.92±9.16
	c.1358G>A	GG	76.25±5.07	71.10±6.86
		GA	27.96±6.77	30.29±9.90
Cross-breeds	c.589T>G	TT	55.68±10.29	49.92±12.12
		GG	9.31±3.40	10.00±10.00
	c.659T>G	TT	65.83±22.04	57.01±23.80
		GG	10.00±10.00	5.00±5.00
	c.974C>G	CC	57.11±15.51	35.56±16.47
		GG	10.05±5.17	9.09±4.45
	c.1105T>A	TT	57.11±15.51	35.56±16.47
		TA	10.05±5.17	9.09±4.45

Notes: Data are presented in percentage (%) mean ($\mu \pm S.E.$). Superscripts on the same row: indicate differences (p -value < 0.05).

A similar thing occurs in allelic, where the haplotype affects the recovery rate in PO. The analysis results showed that the haplotype groups in PO cattle significantly differed in recovery rate (Table 3). One of the haplotype B groups, namely TKGWR, had the highest recovery rate (86.06±6.06%). This haplotype was found in one PO cattle with three heterozygous combinations at points c.659T>G, c.1105T>A, and c.1358G>A. Based on Table 1, PO cattle with ID Number 10 have an individual performance per flushing (4 times superovulation procedure), showing a high superovulation response (100%), high fertilization rate (96.61%±2.24), good transferable embryos (77.12%±11.93), and large grade 1 (excellent) embryos (70.75%±13.52).

FERTILIZATION RATE

Polymorphism both genotype and haplotype in PO, Bel-

gian Blue, and crossbreeds was not associated with fertilization rate (Tables 2). Table 1 showed that fertilization rate in Belgian Blue, PO, and crossbred were 56.24%±23.29, 79.95%±19.23, and 51.54%±43.32, respectively. This is presumably because fertilization is affected by multiple factors, which are influenced by genes from the female reproductive factors and others from the male reproductive factors. Fertilization is a fusion reaction between sperm and oocytes and influenced by many genes related to sperm quality. Furthermore, performance of donor in breeding location showed higher unfertilized oocytes in indicine (40.07%) than in taurine (25.21%) (LEC Annual Report, 2024).

Table 3: Association of *GDF9* haplotypes on recovery rates (%).

Cattle	Haplotype A (wild)	Haplotype B (mutant)
Peranakan Ongole (9/34)	69.06±6.14 (6/24) ^a	86.06±6.06 (3/10) ^b
Crossbred (6/18)	57.17±15.51 (3/7)	58.46±11.80 (3/11)
Belgian Blue (5/21)	44.28±10.00 (3/14)	63.56±12.93 (2/7)

Notes: Data are presented in percentage (%) mean ($\mu \pm S.E.$). The first number in brackets indicates the number of individuals, and the second number indicates the number of superovulation or flushing procedures. Superscripts on the same row indicate differences (p -value < 0.05).

Table 4: Association of *GDF9* haplotype on degenerated embryos (%).

Cattle	Haplotype A (Wild)	Haplotype B (Mutant)
Peranakan Ongole (9/34)	31.23±7.03 (6/24)	22.88±8.40 (3/10)
Crossbred (6/18)	10.05±5.17 (3/7)	9.09±4.45 (3/11)
Belgian Blue (5/21)	6.82±7.89 (2/7)	4.28±4.28 (3/14)

Notes: Data are presented in percentage (%) mean ($\mu \pm S.E.$). The first number in brackets indicates the number of individuals, and the second number indicates the number of superovulation or flushing procedures. Superscripts on the same row indicate differences (p -value < 0.05).

Ortega (2018) found that almost 26 genes are associated with male and female dairy cattle reproduction. Other genes related to fertility include BMP1B (FecB), BMP15 (FecX), and B4GALNT2 (FecL) which were not analyzed in this study. According to Zheng and Dean (2007), genetics factors involved in folliculogenesis, fertilization, and embryo development, are regulated by different genes. Genes related to fertilization were ZP1, ZP2, and ZP3, while ZARF1, NPM2, NALP5, DPPA3 regulate the embryogenesis. In the folliculogenesis, the genes involved include GDF9, FIGLA, NOBOX, SOH3H, DAZLA, YBX2, and CPEB1.

There are no significant differences in degenerated and retarded embryo between both in genotype and haplotype

in BB, PO, and BB x PO (Table 2 and Table 4). Based on Table 1, degenerated embryo in PO were 32.79%±23.25, while in Belgian Blue and crossbred were 8.16%±12.81 and 6.67%±8.44, respectively. However, donor performance at breeding centre showed no difference degenerated embryo between taurine and indicine, 23.50 and 20.54, respectively (LEC Annual Report, 2024). Degenerated or retarded embryo might be occurred due to the cessation of division during the embryo genome activation (EGA) period. The EGA period occurs in bovine embryos, namely the fourth or fifth cell cycle transition period, around 8-16 cells in the early morula stage (Graf *et al.*, 2014).

TRANSFERABLE EMBRYO

In this study, *GDF9* polymorphism in all donor breeds did not affect transferable embryos (Table 5). According to Supriatna (2018), around ±30-40% of ova recovered from superstimulated ovary in cattle cannot be transferred. Practically the number of embryos that can be transferred is around ±60-70%. The transferable embryo in this study was still lower in Belgian Blue, PO, and crossbred, 56.24%±23.29, 47.17%±26.98, and 44.87%±41.12, respectively. This lower numbers due to high unfertilized oocyte and degenerated or retarded.

Table 5: Association of *GDF9* haplotype on the transferable embryo (%).

Cattle	Haplotype A (wild)	Haplotype B (Mutant)
Peranakan Ongole(9/34)	52.54±7.90(6/24)	60.95±11.34(3/10)
Crossbred (6/18)	49.55±16.63(3/7)	52.55±12.10(3/11)
Belgian Blue (5/21)	54.21±11.10(2/7)	48.67±16.34(3/14)

Notes: Data are presented in percentage (%) mean (μ±S.E). The first number in brackets indicates the number of individuals, and the second number indicates the number of superovulation or flushing procedures. Superscripts on the same row indicate differences (*p*-value<0.05).

Furthermore, performance of donor in the breeding center showed that taurine tends to show a higher of transferable embryos (51.28%) than indicine (39.39%) (LEC Annual Report, 2024). Genetic factors influencing female reproduction are relatively small, showed by low heritability (Cohran *et al.*, 2013). Some heritabilities include the number of oocytes 0.31 (Cornelissen *et al.*, 2017), the number of embryos 0.14-0.21 (Cornelissen *et al.*, 2017; Jatón *et al.*, 2016), the fertilization rate 0.21 (Parker *et al.*, 2016) and embryo quality 0.04 (Asada and Terawaki, 2002). The genetic aspect of the preimplantation embryo, apart from having a small genetic portion, is also determined by many gene loci (Pfeffer *et al.*, 2007) and related to many transduction signals (Li *et al.*, 2012).

According to Asada and Terawaki (2002), non-genetic

factors in the form of physical factors (estrous condition, hormones used) and environmental factors (breeding conditions, climate, and nutrition) are more dominant in influencing the success of superovulation. This mechanism is because the production and quality of oocytes depend on cross-communication between the oocyte and the surrounding cell environment since preantral during folliculogenesis (Figueiredo *et al.*, 2018). The stage-appropriate development of follicles provides an environment for oocytes to develop and ovulate (Scaramuzzi *et al.*, 2011). There are factors outside the design of this study not captured in the design, making it more challenging to observe the causes of varying superovulation responses between individual.

MUTATION ON BIOLOGICAL FUNCTION

Non-synonymous mutation at the c.974C>G alters the amino acid Serine to Cysteine (p.325Ser>Cys) with the same structure and properties but different molecular weight and charge characteristics. Cysteine has a higher molecular weight, which is polar, compared to Serine, lighter and non-polar. Prediction of the conserved domain showed *GDF9 Bos taurus* located at amino acids from 348 to 453 (Figure 2). The conserved region is rigid, hence, geometric errors in this part can lead to changes in structure and function (Lukitaningsih *et al.*, 2015). The p.325Ser>Cys mutation occurs outside the primary protein structure (helical chains and sheets), such as in the loop region usually composed of coils and turns, which have high flexibility during protein folding. Changes in this region have little impact and do not contribute directly to the structure and function of the protein (Lukitaningsih *et al.*, 2015).

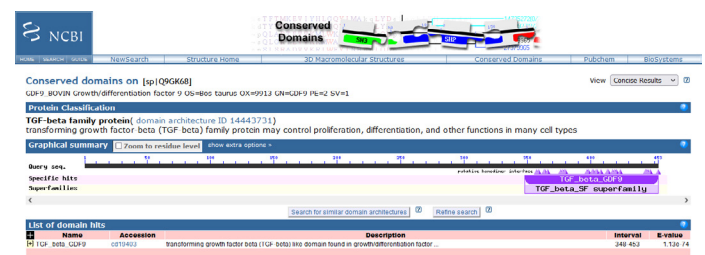


Figure 2: Conserved region protein *GDF9 Bos taurus*; **Sources:** <https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi> (Wang *et al.*, 2023).

Based on in silico analysis, the c.974C>G mutation changes the 325th amino acid, which converts serine to cysteine. The amino acid serine was identified in the random coil structure, and the change to cysteine remained in the random coil condition. However, SOPMA analysis shows that there is a change in the percentage of secondary constituent components of *GDF9*, for example an increase in the percentage of alpha-helix structure from 71 unit (15.67%) to 79 unit (17.44%), a decrease in extended strand from 68 unit (15.01%) to 67 unit (14.79%), as well as a decrease

random coil structure from 314 unit (69.32%) to 307 unit (67.77%). Other in silico analysis showed that this point mutation has its specific size, charge, and hydrophobicity. The original wild and mutant often differ in these properties. The mutant residue is more hydrophobic than the wild type.

The c.1105T>A mutation impacts changes in the amino acid p.369Trp>Arg, while the c.1358G>A mutation impacts changes in the amino acid p.453Arg>His. A combination of both points occurs in mutant haplotypes, which showed a high recovery rate, potentially impacting changes in the energy affinity of proteins associated with folliculogenesis. According to Inayah *et al.* (2016), the substitution of arginine to histidine (p.453Arg>His) reduces the interaction affinity of GDF9 with BMP-15 (Bone Morphogenetic Protein 15), resulting in lower change energy. Based on molecular docking, this point mutation is located in the GDF9 heterodimer binding domain, the interaction site with BMP15 (Figure 1). The interaction energy shows that variations change the affinity strength and patterns of GDF9 and BMP15 (Inayah *et al.*, 2016). However, Inayah *et al.* (2016) did not associate these results with certain traits related to reproduction in cows with mutant genotype conditions.

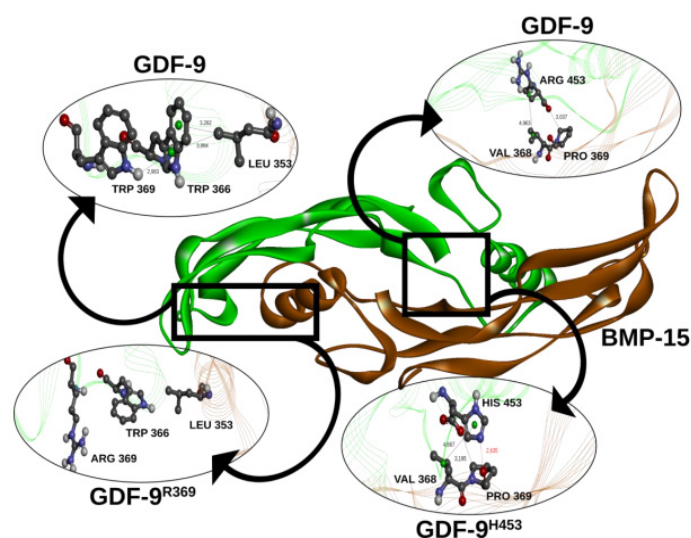


Figure 1: Visualization of mutation point at p. 369 Trp>Arg and p.453His>Arg in GDF9 *Bos taurus* cattle. GDF9 and BMP15 interaction in both point mutations (p.369Trp>Arg and p.453Arg>His). (Caption: green color GDF9 protein, brown color BMP15, top circle: control GDF9, bottom circle: GDF9 mutant, black box: interaction area).

GDF9, as a growth factor, plays a significant regulatory role during gonadotropin-independence (Pramod *et al.*, 2013). In the context of reproductive biology, GDF9 acts as a growth factor that stimulates mitogenic activity in local tissues and modulates the responsiveness of target cells to FSH and LH. A study by Figueiredo *et al.* (2018)

showed that GDF9 influences reproduction through a signaling pathway of SMAD proteins. Activation of SMADs leads to translocation from the cytoplasm into the nucleus, where transcription is activated or repressed together with transcription factors to regulate target gene expression (Attisano and Lee-Hoeflich, 2001). A signaling pathway related to the mechanism by which GDF9 affect ovulation shows the involvement of other genes presented in Figure 3.

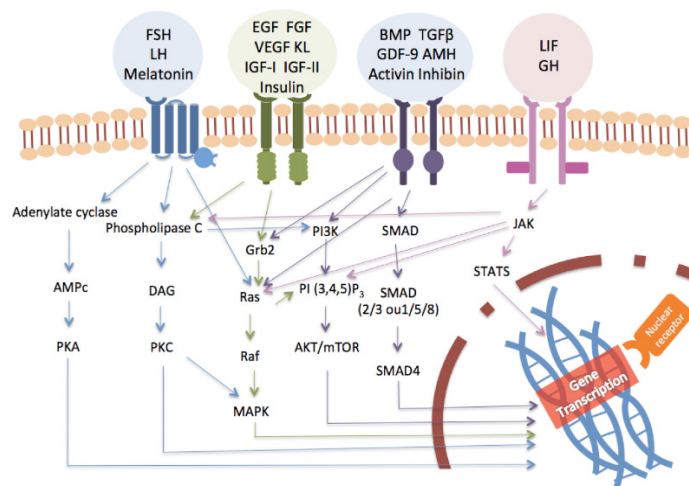


Figure 3: Ligand intra- and extraovarian signaling and molecular pathways in follicle development (Figueiredo *et al.*, 2018).

Based on Gene Ontology, GDF9 activity is related to the function as a receptor ligand activity in the signaling transduction pathway. This process is part of the cellular response to stimulus, regulation of cellular processes, cell communication, and signaling. GDF9 also functions in signaling receptor binding and signaling receptor activator activity. These two functions are critical activities for signaling receptor regulator and molecular function activator activity (Figure 4).

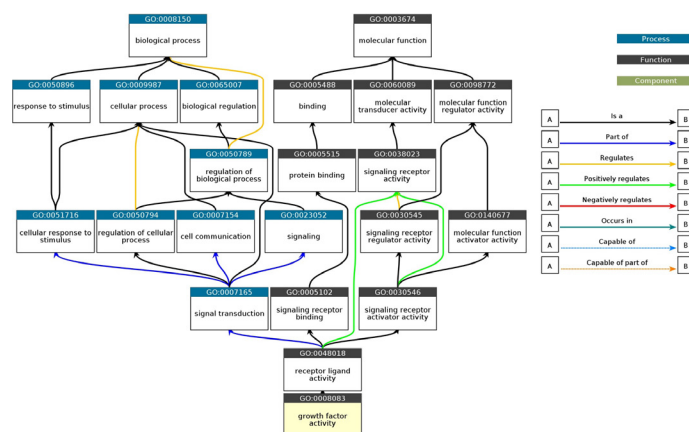


Figure 4: Gene ontology of growth factor activity in biological processes.

ASSOCIATION STUDY OF GDF9

Several studies reported that GDF9 polymorphism in

Maremmmana cattle was associated with prolificacy and multiple births (Marchitelli and Nardone, 2015). The g.231T>C mutation in exon 1 of *GDF9* changes the amino acid p.66Leu>Ser, affecting prolificacy and multiple births such as twins. The mutation occurs in conserved/pro-region proteins required in post-translational processing for the structure of the mature *GDF9* (Shimasaki *et al.*, 2004).

GDF9 mutation in Nellore cattle was reported by Santos-Biase *et al.* (2012), associated with the number of oocytes. The AA and CC genotypes produced the highest number of oocytes (20.80±0.44; 19.99±0.01) compared to the CA genotype (14.00±0.02). This mutation has significant implications for the reproductive potential of Nellore cattle, affecting the number of oocytes produced, which in turn influences the fertility of the cattle. In this study, the mutation point was not found even in the same exon 2 location. Differences in cattle breeds may impact the incidence of *GDF9* mutations.

Study by Gamal *et al.* (2024) found that methylation of the *GDF9* in promoter region occurs in the epigenetic regulation of *GDF9* expression in the ovaries. According to Pan *et al.* (2018), *GDF9* expression is high in the ovaries due to methylation in the promoter, at the mC-4 (methyl-cysteine) site in the CpG region. Study by Pan *et al.* (2018) contributes to understanding the mechanism by which *GDF9* regulates reproduction epigenetically without DNA changes at the genome level.

Polymorphism of *GDF9* in Holstein was associated with the number of transferable embryos and recovery rate (Tang *et al.*, 2013). The 485A>T and 625A>T mutations in intron 1 impact the number and quality of transferable embryos. The g.485TT genotype cattle had more transferable embryos (5.26±1.0) than g.485AA and g.485AT (3.77±0.23, 3.70±0.27, respectively). Mutation at 625A>T showed that the g.625AA genotype had higher number of ova (8.28±0.69) than the g.625AT and g.625TT (6.73±0.29, 7.50±0.54, respectively). Even though the intron part of *GDF9* does not code for amino acids, mutations in the intron part may impact the DNA splicing process. In our study, the intron that separates exons is not flanked by primers designed.

Rasheed *et al.* (2021) found two polymorphisms in Holstein at c.1109A>T and c.1133G>A. The TT and AA genotypes show twin births and a high calving rate. The g.1109TT genotype showed a higher frequency of twin births and calving rate than the AA genotype. Cattle with AA genotype at 1133G>A shows a higher frequency of twin births than those with the GG genotype. Both mutations occurred in exon 2 in Holstein, but we were not found in this study either in *Bos taurus* (Belgian Blue) cattle or *Bos indicus* (PO) cattle and their crosses (BB x PO).

Study by Gunawan *et al.* (2011) showed non-genetic factors such as parity and age affect reproduction traits in cattle. This finding has implications for improving reproduction; apart from being improved through genetic factors, it can also be improved through improving management and utilizing reproductive techniques appropriate to reproductive characteristics. A review of the maintenance of donor cattle at the breeding location showed that donor cattle were maintained with adequate management.

LIMITATION, FURTHER STUDY, AND IMPLICATION

The limitations of this study are not supported by the large sample size due to the limited number of purebred donors kept in the breeding center, the condition of the follicular wave, which was not observed, and the limited number of donor cows that can be used as controls for natural mating. All of the limitations in this study are a factor might be contributed to the variations between individuals in the same breed and between individuals in different breeds.

The results need to be supported by further studies, such as multi-omics, which considers multidisciplinary science to determine the crystal structure of *GDF9* and transcriptomics in cattle. Currently, *GDF9* structure available in protein databases for in-silico studies is still limited to predictions from Alpha-Fold (AF-Q9GK68-F1). Protein-protein interaction studies can also be carried out to determine how *GDF9* interacts with other proteins to influence molecular, cellular, and biological properties based on gene ontology. Furthermore, molecular docking studies should be performed to predict whether the location of mutations occurring in the active site of *GDF9* is related to affinity with other proteins that influence folliculogenesis.

CONCLUSION AND RECOMMENDATIONS

In conclusion, the recovery rate is associated with the *GDF9* polymorphism at point mutation c.974C>G in the PO cattle population at the breeding center location. The main result is that point mutation of c.974C>G of *GDF9* in PO cattle can be a marker for recovery rates. This can assist in livestock breeding programs in the donor population at the breeding center, specifically in the Livestock Embryo Centre. Donor cattle with specific genotypes should be selected for higher recovery rates early to support the livestock breeding program.

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NOVELTY STATEMENTS

This study presents the discovery of new point mutations associated with recovery rate in PO cattle at the Indonesian Breeding Center, with a comprehensive discussion of genomics and predictions at the proteomic level.

AUTHOR'S CONTRIBUTIONS

Irma: Conceptualization, Formal Analysis, Writing Original Draft, and Editing.

Nena Hilmi: Conceptualization, Methodology, Supervision, Validation.

Siti Darodjah Rasad: Conceptualization, Methodology, Supervision, Validation.

Cece Sumantri: Conceptualization, Methodology, Supervision, Validation.

CONFLICT OF INTEREST

The authors do not have any conflict of interest.

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