



Polymorphism Analysis of BMP15 and GDF9 Genes in Dorper Sheep Using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) Technique

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Abstract. This study examined BMP15 and GDF9 gene polymorphisms in Dorper sheep and their potential link to prolificacy traits. Blood samples from 17 Dorper ewes with recorded litter sizes were analyzed using PCR-RFLP with HAEIII and HhaI restriction enzymes for BMP15 and GDF9 genes, respectively. Despite observed litter size variations (70.59% single births, 29.41% twin births), PCR-RFLP analysis revealed monomorphic patterns for both genes across all samples. All individuals exhibited wild-type genotypes, with no genetic variation detected at the examined restriction sites. Genotype and allele frequencies were 1 for both genes, resulting in zero heterozygosity and polymorphic information content (PIC) values. These findings suggest that the PCR-RFLP method with the selected restriction enzymes may not effectively detect prolificacy-related genetic variations in Dorper sheep. The discrepancy between genetic homogeneity and phenotypic variability in litter size indicates that other genes or molecular mechanisms may influence prolificacy traits in this breed. Further research using larger sample sizes, alternative genetic markers, and more sensitive techniques is recommended to elucidate the genetic basis of prolificacy in Dorper sheep.

Keywords: Dorper Sheep, BMP15 Gene, GDF9 Gene, Litter Size, PCR-RFLP

1 Introduction

The population growth and increased public awareness of the importance of animal protein have led to a rising demand for meat in Indonesia. To meet this demand, the livestock sector needs to optimize production from various types of livestock. Sheep play a crucial role in fulfilling the national animal protein requirements. According to data from the Central Bureau of Statistics in 2023 [1], the sheep population in Java Island (West Java, Central Java, and East Java), which serves as the center of sheep farming in Indonesia, has shown a decline over the past three years. The sheep population reached 15.7 million head in 2020, then decreased to 13.8 million head in 2021, and further declined to 13.7 million head in 2022. The main factors contributing to this

population decline are uncontrolled sales due to high market demand and the relatively low productivity of local sheep compared to superior foreign breeds. Consequently, effective breeding strategies are necessary to increase the population and improve genetic performance.

Improving livestock production efficiency is one of the primary focuses in the global livestock industry. One approach involves introducing Dorper sheep and crossbreeding them with local breeds. According to Noor and Hidayat [2], Dorper sheep are a cross between Dorset Horn (from south-west England) and Blackhead Persian (from Persia). This breed possesses excellent qualifications as meat sheep, including the ability to reach a weight of 36 kg at 3.5–4 months of age. Dorper sheep demonstrate good adaptability, strong physical characteristics, high reproductive and growth rates, and good mothering ability. These characteristics make Dorper a promising breed for the development and improvement of sheep production in various regions.

In addition to meeting domestic meat demand, another target is to increase the sheep population. According to Sholikhah et al. [3], twin birth types (prolific) can significantly increase the population, albeit with less optimal pre-weaning weight gain. The prolific trait or twin litter size, as stated by Hamdani et al. [4], results in a high maternal productivity index. The average litter size of Dorper sheep ranges from 1.28 to 1.87, with results varying considerably depending on the birth season [5]. An increase in the litter size of Dorper sheep is expected to aid in the provision of breeding stock. To date, the selection of potential rams or ewes in sheep breeding has been conducted conventionally, focusing on measurable traits such as morphometric assessments. Conventional selection is considered less effective as it requires a large number of animals, extended time, and comprehensive individual records. Currently, more rapid selection methods using molecular biotechnology have been developed.

Candidate genes affecting prolificacy in sheep are regulated by three major genes: Bone morphogenetic protein receptor 1B (BMPR-1B), Bone morphogenetic protein 15 (BMP15), and Growth differentiation factor 9 (GDF9) [6]. The BMP15 gene, also known as the FecX gene, is found to be specifically expressed in developing oocytes in the ovaries of several mammalian species, including rodents [7], ruminants [8], and primates (including humans) [9]. This gene is associated with prolificacy traits in various sheep breeds. The GDF9 gene is a member of the transforming growth factor β superfamily and plays a crucial role in ovarian follicle development and ovulation rate [10]. Mamutse et al. [11] explained that the GDF9 protein is one of the fecundity genes that plays a vital role during early folliculogenesis as a growth and differentiation factor secreted by oocytes in mammals. According to Rahayu [12], the GDF9 gene is associated with increased ovulation rates and litter size in livestock.

Given the importance of these two genes in sheep reproduction, analyzing the polymorphisms of BMP15 and GDF9 in Dorper sheep can provide valuable insights into their genetic potential related to reproductive traits. This information can be used to develop more effective breeding strategies, which in turn can enhance productivity and economic efficiency in Dorper sheep husbandry. Saiki et al. [13] demonstrated that the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) technique has proven to be an effective and affordable method for analyzing these gene polymorphisms. PCR-RFLP involves two main steps: first, the amplification of specific

DNA segments using PCR, and second, the digestion of PCR products using specific restriction enzymes. The fragment patterns resulting from this digestion can reveal the presence of polymorphisms at the studied loci [14].

The advantages of the PCR-RFLP technique lie in its high sensitivity, ability to detect point mutations, and relative ease of implementation using standard laboratory equipment. Furthermore, this method allows for the simultaneous analysis of a large number of samples, making it ideal for population studies [15]. This research aims to analyze the polymorphisms of BMP15 and GDF9 genes in Dorper sheep using the PCR-RFLP technique. The information obtained from this study can serve as a foundation for further research, such as association analyses between the discovered genetic variations and reproductive phenotypic data. Additionally, the results of this study may contribute to the development of genetic markers for molecular-based selection, potentially enhancing the efficiency and accuracy of future Dorper sheep breeding programs.

2 Materials and Methods

2.1 Sample Collection

The objects of the research were 17 ewe Dorper sheep that had given birth and had their litter sizes recorded. The sheep's peripheral blood was used as biological material. Blood samples were collected in EDTA vacuum tubes via the jugular vein. Subsequently, the blood samples were delivered to the Laboratory of Biotechnology, Faculty of Animal Science, Brawijaya University in containers with refrigerant and stored in a freezer (at -25°C) until they were used for DNA analysis.

2.2 DNA Extraction and Quality Assessment

DNA isolation was performed using the Gysinc Geneaid DNA kit (for DNA extraction) according to the manufacturer's protocol. The determination of quantitative indicators was carried out on the nanodrop spectrophotometer while the concentration of DNA. DNA qualitative characteristics were checked through agarose gel electrophoresis. Purified DNA was stored in a freezer at -25°C before being used for PCR-RFLP (Polymerase Chain Reaction–Restriction Fragment Length Polymorphism) analysis.

2.3 PCR-RFLP Analysis

The PCR reaction process was carried out with a total volume for each sample, consisting of $0.4\ \mu\text{l}$ each of forward and reverse primers, $6.2\ \mu\text{l}$ of Nuclease-Free Water (NFW), $7\ \mu\text{l}$ of GoTaq Green, and $1\ \mu\text{l}$ of DNA sample. This mixture was incubated in a PCR Thermocycler-Biorad machine with an initial DNA denaturation stage at 95°C for 5 minutes. The second stage consisted of 35 cycles, each comprising a denaturation process at 95°C for 10 seconds, primer annealing at 60°C for 20 seconds, and DNA extension at 72°C for 30 seconds. The final stage was primer extension (final extension) at 72°C for 5 minutes, ending with a cooling down process at 12°C for 2 minutes. The

DNA amplification results were visualized using 1.5% agarose gel through electrophoresis.

All PCR products from the BMP15 gene and GDF9 gene were each digested using restriction enzymes. 5 μ l of PCR product was used, along with a master mix consisting of 0.9 μ l of NFW/DW solution, 0.7 μ l of Tango buffer, and 0.4 μ l of each restriction enzyme (Table 1). A 100-bp molecular marker was used to determine the DNA sequence size. The digested fragments were examined using 2% agarose gel and visualized with Gel Documentation Blue Light (Gite 965 GW). Primers for the BMP15 Gene with genbank accession number (ENSOARG00000009372) and the GDF9 Gene with accession number (ENSOARG00000013229) were designed by ourself using the Ensemble genbank website (<https://asia.ensembl.org/>).

Table 1. Primer sequences and PCR-RFLP analysis conditions for GDF9 and BMP15 genotyping.

Gene	Primer Sequences	Frage Length	Annealing Temperatur	Enzyme
BMP15	F: GCATAGCTTCTCTGAGCTTC R: GGTCTTCTGAACACTCTGAG	665bp (Exon 2)	60°C	HaeIII (GG/CC)
GDF9	F: GGAGAAGCTCAGATTGTAGC R: GACAAGATGCTAACCTCCAG	568bp (Exon 1)	60°C	HhaI (GCG/C)

2.4 Data Analysis

Allele and Genotype Frequency Values. The formulas for calculating allele and genotype frequencies Nei and Kumar [16] are as follows:

$$x_{ii} = \frac{n_{ii}}{N} \quad x_i = \frac{(2n_{ii} + \sum n_{ij})}{2N} \quad (1)$$

Where :

X_{ii} = Phenotype of genotype ii

X_i = Frequency of allele i

n_{ii} = Number of individuals with genotype ii

n_{ij} = Number of individuals with genotype ij

N = Total number of individuals

Degree of Heterozygosity. The calculation of the degree of heterozygosity based on allele frequencies at each fecundity gene locus according to Nei and Kumar [16] is as follows:

$$H_0 = \sum_{i \neq j} \frac{n_{ij}}{N} \quad H_e = 1 - \sum_{i=1}^q x_i^2 \quad (2)$$

Where :

H_0 = Observed heterozygosity (population)

n_{ij} = Number of heterozygous individuals

- N = Number of individuals analyzed/observed
- H_e = Expected heterozygosity value
- X_i = Frequency of homozygous allele
- q = Number of alleles

Polymorphic Informative Content (PIC) Value. The degree of polymorphism in BMP15 and GDF9 genes will be calculated using the Polymorphic Information Content (PIC) mathematical model, with the following formula [17]:

$$PIC_i = 1 - \sum p_{ij}^2 \tag{3}$$

- Where :
- PIC_i = PIC for locus i
- p_{ij} = Frequency of allele j at locus i

Hardy-Weinberg Equilibrium (H-W). The equilibrium of a population can be determined by testing the Hardy-Weinberg equilibrium (H-W) using chi-square (χ²) with the following formula [18]:

$$\chi^2 = \sum \frac{(O - E)^2}{E} \tag{4}$$

- Where:
- χ² = Hardy-Weinberg (H-W) test
- O = Observed genotype value/number of observations
- E = Expected genotype value

Statistical Analysis. The statistical data on litter size percentage and coefficient of variation were analyzed using SPSS version 26.0. The coefficient of variance (CV) percentage was calculated according to the formula as follows:

$$CV = \frac{\sigma}{\mu} \times 100\% \tag{5}$$

- Where:
- CV = Coefficient of Variation
- σ = Standard Deviation
- μ = Mean

3 Results and Discussion

3.1 Reproduction Traits

Analysis of birth patterns in a sample of 17 Dorper ewes showed significant variation in the number of lambs per birth. The majority of births (70.59%) were single births, while the remaining 29.41% were twin births. The coefficient of variation (CV) of

36.41% indicates a relatively high variability in the number of lambs per birth (Table 2).

Table 2. Coefficient of Variation Percentage for Litter Size of Dorper Ewes

Traits	Producing Single Lamb	Producing Twin Lamb	CV (%)
Litter size	0.29	0.71	35.53%

This coefficient of variation value reflects heterogeneity in the population regarding reproductive capacity, which may be influenced by genetic, nutritional, or management factors. The prevalence of single births indicates that, although twin births occur in nearly a third of the population, most ewes in this sample tend to give birth to a single lamb. These findings have important implications for farm management and breeding programs, as the number of lambs per birth is a key factor in the productivity and economic efficiency of sheep farming.

3.2 PCR-RFLP Analysis

PCR-RFLP analysis of the BMP15 (exon 2) and GDF9 (exon 1) genes, utilizing HAEIII and HhaI restriction endonucleases in Dorper sheep, revealed that all samples exhibited wild-type or non-prolific genotypes and were monomorphic. The PCR-RFLP results for the BMP15 gene demonstrated a single DNA band, indicating the absence of genetic variation or polymorphism at the restriction sites of the examined samples (Figure 1). Concurrently, the analysis of the GDF9 gene yielded two distinct DNA bands following enzymatic digestion (Figure 2), this digestion pattern further corroborates the absence of genetic variation, suggesting that the investigated locus is monomorphic.



Fig. 1. PCR-RFLP Visualization Results of the BMP15 Gene with HAEIII Restriction Enzyme.



Fig. 2. PCR-RFLP Visualization Results of the GDF9 Gene with HhaI Restriction Enzyme.

The PCR-RFLP analysis of the BMP15 gene's exon 2 using the HaeIII restriction enzyme revealed a monomorphic DNA band pattern across all Dorper sheep ewes. A single DNA band of 665 base pairs (bp) was observed. The restriction enzyme failed to recognize the existing DNA sequence due to the HaeIII cutting site being GG/CC. All samples were genotyped as CC, while the Ensemble gene bank indicated a base change from C to G, representing a missense mutation. This finding demonstrates the absence of genotypic variation at the BMP15|HaeIII locus.

The PCR-RFLP analysis of the GDF9 gene's exon 1 using the HhaI restriction enzyme also showed a monomorphic DNA band pattern in all Dorper sheep ewes. The DNA bands comprised two to three fragments, specifically 173 bp and 395 bp. The restriction enzyme successfully cleaved the DNA sequence, generating a single DNA band with the GG genotype. HhaI has a recognition cutting site of GCG/C, and the Ensemble gene bank revealed a base change from G to C, which represents a missense mutation. This result indicates the presence of genotypic variation at the GDF9|HhaI locus.

This finding contradicts field observations indicating that the studied Dorper sheep samples exhibited litter sizes exceeding one. This discrepancy suggests that the PCR-RFLP method employing the selected restriction enzymes may not serve as a reliable detection method for prolificacy traits in Dorper sheep. Nevertheless, previous investigations on other ovine lineages have demonstrated associations between BMP15 and GDF9 gene polymorphisms and prolificacy traits. Chu et al. [19] identified polymorphisms in exon 2 of the BMP15 gene correlated with increased litter size in Small Tail Han sheep. Similarly, Hanrahan et al. [20] discovered mutations in both genes linked to elevated ovulation rates in Belclare and Cambridge sheep breeds.

The discrepancy between this study and previous findings can be explained by several factors. First, genetic variation among sheep lineages may influence the expression and function of BMP15 and GDF9 genes. This is supported by a meta-analysis conducted by Abdoli et al. [21], which showed that the effects of mutations in these genes vary among lineages. Second, the detection method employed may not be sufficiently sensitive to identify genetic variations present in Dorper sheep. Wang et al. [22] used sequencing methods to identify SNPs in exon 2 of the BMP15 gene correlated with increased litter size in Hu sheep, which might not be detectable using PCR-RFLP methods.

Furthermore, research by Mullen et al. [23] on Dorset sheep, which are related to Dorper, showed that although there were no significant mutations in the BMP15 and GDF9 genes, variations in other genes influenced prolificacy traits. This raises the possibility that prolificacy traits in Dorper sheep may be influenced by other genes or different molecular mechanisms. However, the determination of prolificacy traits in this study was based on the number of offspring born. As the BMP15 gene functions in granulosa cells [24,25], there is a possibility that Dorper sheep have more than one ovulated oocyte.

3.3 Genotype and Allele Frequency, PIC Value and Hardy-Weinberg Equilibrium (H-W)

Genotype and allele frequencies are essential quantitative parameters for evaluating genetic variability within a population. Intrapopulation genetic diversity can be analyzed through the quantification of genotype and allele frequencies. The primary objective of determining genotype and allele frequencies is to detect the presence of selective pressures operating within the population. Empirical data regarding genotype and allele frequencies for the BMP15 and GDF9 loci in the Dorper Ewes population are comprehensively presented in Table 3 and Table 4.

Table 3. Genotype and Allele Frequency of the BMP15 ene in Dorper Ewes

Gene	n	Genotype Frequency	Allele Frequency
		CC	C
BMP15	17	1	1

Table 4. Genotype and Allele Frequency of the GDF9 Gene in Dorper Ewes

Gene	n	Genotype Frequency	Allele Frequency
		GG	G
GDF9	17	1	1

Analysis of BMP15 and GDF9 genes in samples from 17 female Dorper sheep revealed a monomorphic population. All individuals exhibited the same genotype, resulting in an allele frequency of 1 for each gene. Consequently, this population deviates from Hardy-Weinberg equilibrium due to the absence of observable genetic variation at these loci. According to Riyanto [26], polymorphism values are closely correlated with the number of alleles produced at each locus. The greater the number of alleles, the higher the polymorphism value. Genetic polymorphism is crucial for mapping quantitative trait loci associated with productivity and enhancing breeding strategies [27]. Polymorphic loci are characterized by the presence of heterozygous individuals within a population. Yuniarsih [28] further elaborates that variations in allele and genotype frequencies are influenced by several key factors (natural selection, gene mutation, population mixing, inbreeding, and outbreeding).

Both the expected (H_e) and observed (H_o) heterozygosity values were 0 (Table 5), further confirming the lack of genetic variation at these loci. These results indicate that the BMP15 and GDF9 genes are highly conserved within the studied sheep population.

Table 5. Hardy-Weinberg Equilibrium (H-W) and PIC Value

Gene	n	H_e	H_o	(X^2)	PIC
BMP15	17	0	0	0	0
GDF9	17	0	0	0	0

The Polymorphic Information Content (PIC) value was calculated as 0, indicating that the BMP15 and GDF9 gene loci were not informative for genetic diversity studies in this population. These genetic markers could not be used to differentiate between individuals in this population because all individuals possessed the same genotype. The number of samples used in this study was very small and still highly homogeneous, consistent with the findings of Lassoued et al. [29], who reported low polymorphism levels in the BMP15 gene for Barbarine sheep. In that study, only one SNP in the BMP15 gene was identified from 65 samples. Low genetic variation in the BMP15 and GDF9 genes was also reported by Abdoli et al. [21] in Iranian Shal sheep, with only one polymorphism found in the BMP15 gene and no polymorphisms in the GDF9 gene. Similarly, Våge et al. [30] found limited genetic variation in the GDF9 gene in Norwegian White Sheep, with only one mutation related to fertility.

However, these results contrast with the findings of Demars et al. [31] in Lacaune sheep, where significant genetic variation was found in the BMP15 gene, with several mutations identified that were associated with ovulation rate. Similarly, Chu et al. [19] reported high levels of polymorphism in the GDF9 gene in Small Tail Han sheep, with several SNPs related to prolificacy traits. These differences in results suggest that the polymorphism levels of BMP15 and GDF9 genes can vary significantly among sheep breeds, possibly due to differences in selection history and breeding management. Therefore, although these genes are monomorphic in the studied population, they may still be important in sheep breeding programs in other populations.

4 Conclusion

This study on Dorper sheep revealed monomorphic BMP15 and GDF9 genes through PCR-RFLP analysis, despite observed variations in litter size. The PCR-RFLP method employing HaeIII and HhaI restriction enzymes proved ineffective in detecting genetic variations associated with prolificacy in this population. The discrepancy between genetic homogeneity and phenotypic variability suggests that prolificacy traits in Dorper sheep may be influenced by other genes or molecular mechanisms not captured by this analysis. Future research should explore a larger sample size, alternative genetic markers, and more sensitive techniques to elucidate the genetic basis of prolificacy in this breed. These findings have significant implications for Dorper sheep breeding programs aimed at improving reproductive performance.

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