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Genetic Variation Analysis of FSHB Gene Exon 3 in Goats (*Capra hircus*) Using In Silico Restriction Fragment Length Polymorphism (RFLP)

(Analisis Variasi Genetik Ekson 3 Gen FSHB pada Kambing (Capra hircus) Menggunakan Restriction Fragment Length Polymorphism (RFLP) Secara In Silico)

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ABSTRAK

Studi ini bertujuan untuk melakukan analisis in silico awal Restriction Fragment Length Polymorphism (RFLP) pada ekson 3 gen Hormon Perangsang

Folikel Beta (FSHB) pada kambing (Capra hircus) menggunakan enzim restriksi BspMAI dan PstI. Empat sekuens ekson 3 gen FSHB yang telah dipublikasikan, masing-masing sepanjang 310 bp, diperoleh dari basis data NCBI dan dianalisis dengan digesti restriksi virtual. Hasil analisis memprediksi dua pola restriksi pada sekuens yang dianalisis: pola tidak terpotong sepanjang 310 bp dan pola terpotong yang menghasilkan fragmen sepanjang 200 bp dan 110 bp. Pola tidak terpotong sepanjang 310 bp ditemukan pada tiga sekuens, sedangkan pola 200 bp dan 110 bp ditemukan pada satu sekuens. Hasil ini menunjukkan kemungkinan adanya perbedaan situs restriksi pada atau di sekitar situs pengenalan BspMAI/PstI pada ekson 3 gen FSHB kambing. Namun, karena penelitian ini terbatas pada analisis komputasional sekuens publik dan tidak melibatkan sampel biologis, amplifikasi PCR, pengurutan, pencernaan enzim restriksi langsung, atau validasi elektroforesis, temuan ini masih bersifat pendahuluan. Penelitian lebih lanjut menggunakan penyelarasan sekuens berganda, identifikasi SNP, dan validasi PCR-RFLP laboratorium pada populasi kambing sebenarnya diperlukan untuk mengkonfirmasi potensi penggunaan BspMAI/PstI dalam studi genetika kambing..
***Keywords** : BspMAI/PstI; FSHB gene; goat; restriction pattern; RFLP in silico*

INTRODUCTION

Follicle Stimulating Hormone beta subunit (FSHB) is a key gene that regulates the reproductive process in mammals, including goats (Capra hircus). Follicle-stimulating hormone (FSH) is a glycoprotein hormone expressed in the pituitary gland and is responsible for regulating reproduction in both male and female mammals (Das & Kumar, 2018; McDonald *et al.,* 2019). The FSH protein comprises alpha (α) and beta (β) subunits crucial in ovarian follicle development, spermatogenesis stimulation, and gonadal function regulation through interactions with specific receptors in target tissues (Andreas *et al.,* 2014; Santi *et al*., 2020). The beta subunit encoded by FSHB determines the biological specificity of the hormone, so genetic variation in this gene can directly impact reproductive performance. The beta subunit encoded by FSHB determines the biological specificity of the hormone. The functional specificity of FSH is determined by its β subunit, which ensures specific interaction of FSH with the FSH receptor (FSHR) (Haldar *et al*., 2022; Mao *et al.,* 2022). Therefore, genetic variation in the FSHB gene can potentially impact goats' reproductive performance directly.

Exploration of genetic variation in the FSHB gene, especially in the exon region, is of interest because this region is a coding sequence that can affect the structure and function of the protein. Exon 3 contains an important part of the receptor binding domain, so nucleotide changes in this region can potentially change the hormone affinity for the FSH receptor, affect the secretion of gonadotropic hormones, and ultimately affect fertility. Research conducted by

Zhang *et al*., (2011) successfully detected a new mutation in exon 3 of the FSHB gene that causes a change in amino acids from glutamine (Gln) to arginine (Arg) at residue 115 and produces three genotypes, namely AA, AB, and BB, which are found in all tested goats. The results also showed that Boer and Matou goats with the AA genotype had the highest litter sizes ($P < 0.05$). In addition, Boer goats of AA genotype had the highest birth weight compared to other genotypes ($P < 0.05$). In contrast, after superovulation treatment, Matou goats of the AA genotype showed a higher number of ova, large follicles, and corpus luteum than the BB genotype ($P < 0.05$). Research conducted by Ishak et al., (2011) proved that FSHB gene polymorphisms affect the percentage of abnormal spermatozoa in Brahman, Friesian Holstein, Limousin, and Simmental cattle. Then, research conducted by Dai *et al*., (2009) showed that bulls with mutations in exon 3 of the FSH β subunit gene were found to have lower fresh semen concentration, lower acrosome integrity percentage in fresh and frozen semen, lower sperm motility in frozen semen, poor sperm quality, and resistance to freezing treatment, as well as lower fertility. Mokhtari *et al*. (2021) reported a synonymous SNP, g.59080365 C>T, in exon 3 of the ovine FSHβ gene. Although this mutation did not change the amino acid sequence, the SSCP patterns containing this SNP were significantly associated with litter size in Mehraban sheep.

In goat breeding, reproductive performance is one of the main economic parameters influencing the entire livestock industry (Dwatmadji *et al*., 2018; Ali *et al*., 2022). Identification of genetic markers associated with reproductive traits allows marker-assisted selection (MAS) to accelerate the improvement of superior traits. Although several studies have reported associations between FSHB polymorphisms and reproductive traits in livestock, studies on goats focusing on exon 3 genetic variation using an in silico RFLP approach remain limited. Previous studies have mostly used conventional or laboratory-based methods. Hence, the novelty of this study is the use of in silico RFLP analysis to identify genetic variation in exon 3 of the goat FSHB gene.

In silico RFLP analysis is a rapid, efficient, and low-cost initial approach for predicting restriction sites and DNA cleavage patterns based on available sequences. This approach can be used as an initial screening step prior to laboratory validation, but it cannot replace experimental analysis because it does not involve biological samples, PCR amplification, direct restriction enzyme digestion, or electrophoresis confirmation. Therefore, this study is an initial computational analysis of exon 3 of the FSHB gene in goats (*Capra hircus*) using sequences obtained from the NCBI database. This study aims to predict the

restriction pattern of exon 3 of the goat FSHB gene using the BspMAI/PstI restriction enzymes in silico. The results obtained are expected to provide preliminary information for selecting candidate restriction enzymes for further research, particularly for validating PCR-RFLP using biological samples from actual goat populations.

MATERIALS AND METHODS

DNA Sequence Data

The FSHB gene sequences used for in silico analysis were downloaded in FASTA format from the NCBI database (https://www.ncbi.nlm.nih.gov/), specifically from PopSet with ID 544207170. This dataset was submitted by Nikbin *et al*., (2013) in his study entitled "Association of FSHB gene polymorphisms with semen quality traits in Boer goats" (https://www.ncbi.nlm.nih.gov/nuccore?term=popset+representative+uid+544207170[word]). The PopSet includes four coding sequences of exon 3 of the FSHB gene in goats (Capra hircus), each 310 base pairs in size with accession numbers KF179313.1, KF179314.1, KF179315.1, and KF179316.1.

Restriction Enzyme Candidate Screening

The process of screening restriction enzymes to be used in in silico RFLP analysis can be done through the site http://insilico.ehu.es/restriction/. This site provides a "compare restriction pattern of many sequences" feature that allows users to simultaneously compare restriction patterns from several DNA sequences. Several gene sequences obtained in FASTA format are uploaded to the column provided on the site. After that, the system will display the alignment results of these sequences. If there are identical sequences, they will be removed to facilitate analysis. The next step is to select the option "only restriction enzymes with known bases (no N, R, Y...)" to obtain candidate restriction enzymes with definite recognition sites. Finally, click the "get the list of restriction enzymes" button to obtain the restriction enzymes to be used (Maretta *et al*., 2025).

Restriction Fragment Length Polymorphism (RFLP) in Silico

Restriction Fragment Length Polymorphism (RFLP) is a method of analyzing nucleotide sequence variations based on the DNA cutting pattern by certain restriction enzymes, resulting in polymorphisms in the form of differences in the length of DNA fragments. This RFLP analysis is performed in silico or virtually using the platform https://www.benchling.com/. The analysis begins by importing the DNA sequence obtained from NCBI into the Benchling platform. Next, click the scissors sign in the right corner. Then, the "find enzyme" tool is selected, and the names of the restriction enzymes previously

determined during screening are typed in the column. Next, click the "run digest" menu for restrictions. With the "virtual digest" tool, you can view the electrophoregram image (Afionita *et al*., 2025).

RESULTS AND DISCUSSION

Restriction Enzyme Candidate Screening

Based on the output obtained from the screening of restriction enzyme candidates on the site (http://insilico.ehu.es/restriction/), there are many sides of restriction enzyme recognition, then one of them was chosen, namely the BspMAI/PstI enzyme. This enzyme was chosen because it has a fairly high restriction fragment polymorphism, codominance, and consistent results between laboratories. DNA cutting analysis using the BspMAI/PstI restriction enzyme which has a C_TGCA'G sequence recognition site showed a specific cut at position 200 bp in the 3rd gene. No cutting sites were found for genes 1, 2, and 4. This pattern indicates that the enzyme recognizes and cuts only certain DNA segments that have the target sequence.

Restriction enzyme	Cleaves defined at	Cleaves gene3 at	Cleaves gene4 at	Cleaves gene2 at	Cleaves gene1 at
AatI,Eco147I,PceI,SseBI,StuI AGG'CCT		240	240	240	240
Bsp120I,PspOMI G'GGCC_C		245	245	245	245
Bsp1407I,BsrGI,BstAUI,SspBI T'GTAC_A		60 165	60 165	60 165	60 165
BspMAI,PstI C_TGCA'G		200			
Csp6I,CviQI,RsaNI G'TA_C		61 99 118 166 172	61 99 118 166 172	61 99 118 166 172	61 99 118 166 172
CviAII,FaeI,Hin1II,Hsp92II,NlaIII _CATG'		99 155	99 155	99 155	99 155
FatI 'CATG_		95 151	95 151	95 151	95 151
HpyCH4IV,HpySE526I,MaeII A'CG_T		170	170	170	170

Figure 1. In silico restriction enzyme screening

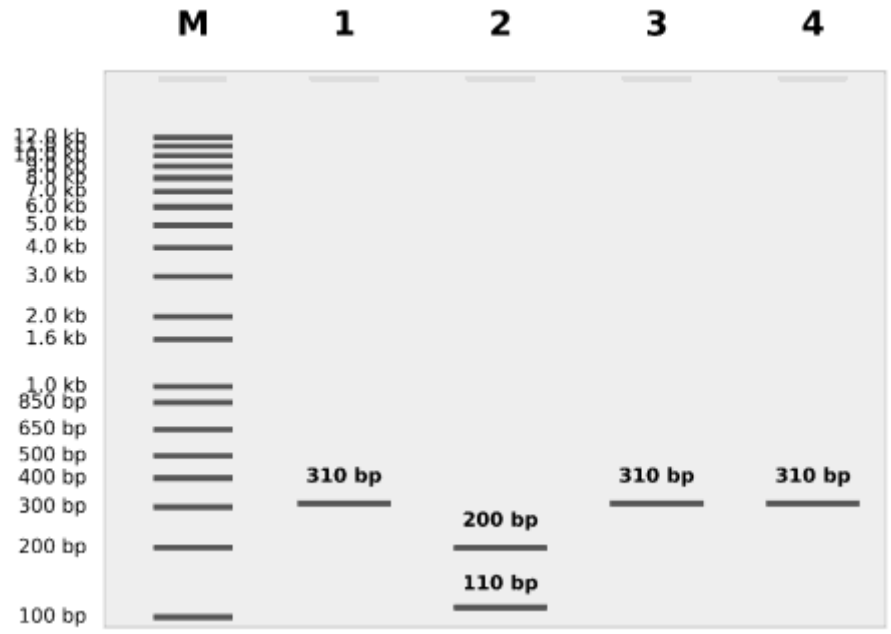
These results confirm that BspMAI/PstI has a high level of specificity for the recognition site corresponding to the nucleotide motif C_TGCA'G, cutting only at position 200 bp in the third gene. This limited

but specific cutting pattern can potentially produce restriction fragment polymorphisms (RFLPs) that can be used as molecular markers. This is consistent with Volkandari *et al*., (2019) statement that the BspMAI/PstI restriction enzyme cuts the CTGCA^G site in PCR products. The advantages of BspMAI/PstI in this study align with the recommended criteria for selecting restriction enzymes: its ability to detect a high degree of sequence variation (polymorphism), its codominance, allowing the detection of both alleles in heterozygous individuals, and its consistent results across laboratories. This consistency is crucial for data standardization in comparative studies and meta-analyses.

Furthermore, the limited number of identified cutting sites within the gene can be advantageous in genetic mapping analysis, as it reduces the complexity of fragment patterns and simplifies the interpretation of electrophoresis results (Brown, 2015; Hathaway *et al*., 2007). These findings support the potential use of BspMAI/PstI in genetic diversity studies, variety identification, and phylogenetic analysis, especially in species with medium to high levels of genetic variation.

Restriction Fragment Length Polymorphism (RFLP) in Silico

Deoxyribonucleic acid (DNA) fragment restriction analysis using the BspMAI/PstI enzymes on the goat *FSHB* gene showed a specific cutting pattern corresponding to the C_TGCA'G recognition site. Agarose gel electrophoresis results (Figure 1) showed the presence of two alleles, namely A1 (uncut) and A2 (cut into two fragments).



Notes : M = DNA Ladder
1 = KF179313.1

2 = KF179313.1
3 = KF179315.1
4 = KF179316.1

Figure 2. In silico gel electrophoresis simulation of restriction digestion using the BspMAI/PstI enzymes. M: DNA ladder; lane 1: KF179313.1 produces a 310-bp fragment; lane 2: KF179314.1 produces 200 bp and 110 bp fragments; lane 3: KF179315.1 produces a 310 bp fragment; lane 4: KF179316.1 produces a 310 bp fragment.

Deoxyribonucleic acid (DNA) fragments with a size of 310 bp (allele A1) were visible in lanes 1, 3, and 4, while fragments of 200 bp and 110 bp (allele A2) were detected in lane 2. Based on the four samples analyzed, three samples (75%) showed a single band pattern measuring 310 bp, and one sample (25%) showed two bands with 200 bp and 110 bp sizes. Thus, the frequency of allele A1 was 0.75, while the frequency of allele A2 was 0.25 (Table 1).

Table 1. Allele frequency of *FSHB* gene using BspMAI/PstI enzyme

Restriction Enzymes	**Restriction Recognition Site**	**Fragment size (bp)**	**Allele**	**Number of fragments present (N = 4)**	**Percentage of fragment presence (%)**	**Allele Frequency**
BspMAI/PstI	C_TGCA'G	310	A1	3	75	0,75
		200 and 110	A2	1	25	0,25

The analysis using the BspMAI/PstI restriction enzymes successfully detected genetic variation in the FSHB gene through thein silico RFLP analysis. A single band pattern of 310 bp indicates that the A1 allele does not have a cutting site for the BspMAI/PstI enzyme, while the pattern of two bands (200 bp and 110 bp) in the A2 allele indicates the presence of a restriction site that produces a truncated DNA fragment. The allele frequency distribution shows the dominance of the A1 allele in this sample population, with a frequency of 0.75. This indicates that the A1 allele is more common in the analyzed samples and is potentially associated with favorable phenotypic traits in this population. However, further association studies are needed to confirm this relationship. Meanwhile, the presence of the A2 allele at a frequency of 0.25 indicates

the presence of genetic variation, although in a lower proportion.

The differences in the predicted restriction patterns suggest the possibility of sequence variations at or around the BspMAI/PstI recognition sites in exon 3 of the goat FSHB gene. The 310-bp uncut fragment indicates the absence of the BspMAI/PstI restriction site, whereas the 200-bp and 110-bp fragments indicate the presence of this restriction site in one of the analyzed sequences. This finding is relevant because exon 3 of the goat FSHB gene has previously been reported to contain polymorphisms associated with reproductive traits, including litter size and superovulation response (Zhang *et al*., 2011). Additionally, the NCBI PopSet sequences used in this study were derived from a previous study on the association of FSHB gene polymorphisms with semen quality traits in Boer goats (Nikbin *et al*., 2013). Therefore, the restriction patterns predicted in this study can provide preliminary information for selecting BspMAI/PstI as candidate enzymes for validating FSHB gene polymorphisms in goats in future studies.

The biological relevance of the FSHB gene is supported by its role in encoding the beta subunit of follicle-stimulating hormone (FSH), which determines the biological specificity of FSH and contributes to reproductive processes such as follicular development, ovulation, and spermatogenesis (Wang *et al*., 2021; Janjic *et al*., 2019). Previous studies in livestock have also reported that variation in the FSHB gene may be associated with reproductive performance, semen quality, and fertility-related traits (Dai *et al*., 2009; Zhang *et al*., 2011). Thus, the predicted restriction pattern obtained in the present study may serve as an initial reference for further molecular validation. However, this result should not be interpreted as experimentally confirmed functional genetic variation or as direct evidence of association with reproductive traits.

This study has several limitations because the analysis was limited to in silico restriction mapping of four exon 3 sequences of the goat FSHB gene obtained from the NCBI database. This study did not include more in-depth molecular genetic analyses, such as multiple sequence alignment, SNP identification, nucleotide substitution analysis, or interpretation of potential amino acid changes. Therefore, the differences in restriction patterns observed in this study can only be interpreted as sequence-based predictions of cleavage patterns, not as genetic variations or functionally confirmed mutations. Furthermore, this study did not utilize biological samples, PCR amplification, sequencing, direct restriction enzyme digestion, or electrophoresis validation. Thus, further research using a larger number of sequences, alignment-based SNP analysis, amino acid change prediction, and laboratory PCR-RFLP

validation in real goat populations is needed to confirm the potential use of BspMAI/PstI as candidate molecular markers in the goat FSHB gene.

CONCLUSION

This study is an initial in silico analysis of exon 3 of the FSHB gene in goats (Capra hircus) using the restriction enzymes BspMAI and PstI. The virtual restriction digestion results predicted two restriction patterns in the four NCBI sequences analyzed, namely an uncut pattern of 310 bp and a cut pattern yielding fragments of 200 bp and 110 bp. The 310-bp pattern was found in three sequences, while the 200-bp and 110-bp patterns were found in one sequence. These results indicate that BspMAI/PstI has the potential to be used to detect differences in restriction sites in exon 3 of the goat FSHB gene. However, since this study used only a limited number of publicly available sequences and did not involve biological samples, PCR amplification, sequencing, direct restriction enzyme digestion, or electrophoresis validation, these findings are still preliminary. Further research using a larger number of sequences, multiple sequence alignment, SNP identification, and laboratory-based PCR-RFLP validation in actual goat populations is needed to confirm the potential use of BspMAI/PstI in goat genetic studies.

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